UNIVERSIDADE ESTADUAL DE CAMPINAS
FACULDADE DE ENGENHARIA DE ALIMENTOS

RICARDO RODRIGUES DE MELO

STUDY OF BACTERIAL GLYCOSIDE HYDROLASES FOR BIOTECHNOLOGICAL APPLICATIONS: BIOPROSPECTION, PRODUCTION AND IMMOBILIZATION

ESTUDO DE HIDROLASES GLICOSÍDICAS BACTERIANAS PARA APLICAÇÕES BIOTECNOLÓGICAS: BIOPROSPECÇÃO, PRODUÇÃO E IMOBILIZAÇÃO

CAMPINAS - SP
2017

RICARDO RODRIGUES DE MELO

STUDY OF BACTERIAL GLYCOSIDE HYDROLASES FOR BIOTECHNOLOGICAL APPLICATIONS: BIOPROSPECTION, PRODUCTION AND IMMOBILIZATION

ESTUDO DE HIDROLASES GLICOSÍDICAS BACTERIANAS PARA APLICAÇÕES BIOTECNOLÓGICAS: BIOPROSPECÇÃO, PRODUÇÃO E IMOBILIZAÇÃO

Thesis presented to the Faculty of Food Engineering of the University of Campinas for partial fulfillment of the requirements for the degree of Doctor in FOOD SCIENCE

Tese apresentada à Faculdade de Engenharia de Alimentos da Universidade Estadual de Campinas como parte dos requisitos exigidos para a obtenção do título de Doutor em CIÊNCIA DE ALIMENTOS

Supervisora/Orientadora: Dra. Hélia Harumi Sato

Co-supervisor/Co-orientador: Dr. Roberto Ruller

ESTE EXEMPLAR CORRESPONDE À VERSÃO FINAL DA TESE DEFENDIDA PELO ALUNO RICARDO RODRIGUES DE MELO E ORIENTADO PELA Dra. HÉLIA HARUMI SATO.

CAMPINAS - SP

2017

Agência(s) de fomento e nº(s) de processo(s): CNPq, 140610/2014-6

Ficha catalográfica
Universidade Estadual de Campinas
Biblioteca da Faculdade de Engenharia de Alimentos
Claudia Aparecida Romano - CRB 8/5816

M491s

Melo, Ricardo Rodrigues de, 1985-
Study of bacterial glycoside hydrolases for biotechnological applications: Bioprospection, production and immobilization / Ricardo Rodrigues de Melo. – Campinas, SP : [s.n.], 2017.

Orientador: Hélia Harumi Sato.
Coorientador: Roberto Ruller.
Tese (doutorado) – Universidade Estadual de Campinas, Faculdade de Engenharia de Alimentos.

1. Hidrolases glicosídicas. 2. Streptomyces. 3. Genômica. 4. Proteômica. 5. Enzimas imobilizadas. I. Sato, Hélia Harumi. II. Ruller, Roberto. III. Universidade Estadual de Campinas. Faculdade de Engenharia de Alimentos. IV. Título.

Informações para Biblioteca Digital

Título em outro idioma: Estudo de hidrolases glicosídicas bacterianas para aplicações biotecnológicas: Bioprospecção, produção e imobilização
Palavras-chave em inglês:
Glycoside hydrolases
Streptomyces
Genomics
Proteomics
Immobilized enzymes
Área de concentração: Ciência de Alimentos
Titulação: Doutor em Ciência de Alimentos
Banca examinadora:
Hélia Harumi Sato [Orientador]
Letícia Maria Zanphorlin Murakami
Luciana Ferracini dos Santos
Luciana Francisco Fleuri
Marcela Pavan Bagagli
Data de defesa: 30-10-2017
Programa de Pós-Graduação: Ciência de Alimentos

BANCA EXAMINADORA

Dra. Hélia Harumi Sato
DCA-FEA-UNICAMP - Orientador(a)

Dra. Letícia Maria Zanphorlin Murakami
CTBE-CNPEM - Membro Titular

Dra. Luciana Ferracini dos Santos
UNIARARAS - Membro Titular

Dra. Luciana Francisco Fleuri
IBB - UNESP - Membro Titular

Dra. Marcela Pavan Bagagli
IFSP-Avaré - Membro Titular

A ata de defesa com as respectivas assinaturas dos membros encontra-se no processo de vida acadêmica do aluno.

AGRADECIMENTOS

A DEUS por me dar vida, saúde e vigor para enfrentar os desafios e porque, sem Ele, não teria chegado até aqui.

Aos meus pais pelo amor, constante apoio, e por não medirem esforços para que eu alcançasse meus objetivos.

À Profa. Dra. Hélia Harumi Sato pela oportunidade desde o mestrado, pela orientação, incentivo e por acreditar no meu trabalho.

Ao Dr. Roberto Ruller pela oportunidade única de desenvolver o trabalho no CTBE, pela co-orientação ao longo de todo o trabalho, pela coordenação das pesquisas no CTBE, e também por confiar no meu trabalho.

Aos membros da banca por avaliarem e darem sugestões preciosas para o trabalho: Profa. Dra. Marcela Pavan Bagagli, Profa. Dra. Rosana Goldbeck, Profa. Dra. Luciana Francisco Fleuri, Profa. Dra. Luciana Ferracini dos Santos, Dra. Samantha Christine Santos, Dr. Clelton Aparecido dos Santos e Dra. Letícia Maria Zanphorlin Murakami.

Aos amigos do CTBE/CNPEM pela amizade e tornarem o ambiente de trabalho mais agradável. Agradecimento em especial à ilha 2, pela grande amizade e troca de experiências.

Aos amigos do laboratório de Bioquímica de Alimentos - DCA/FEA pela amizade e boa convivência.

Aos funcionários da Pós-graduação pela paciência e por estarem sempre prontos a nos atender e tirar nossas dúvidas.

À Universidade Federal de Viçosa pela excelente formação ao longo de cinco anos de graduação.

À Universidade Estadual de Campinas pela oportunidade de cursar a pós-graduação em um centro de excelência em pesquisa, tanto no mestrado como agora no doutorado.

Ao CNPq pelos recursos destinados à realização das pesquisas.

Ao CNPq pela concessão da bolsa de doutorado.

À todos que de alguma forma, direta ou indiretamente, contribuíram para a realização deste trabalho.

À todos vocês, meu mais sincero, Muito Obrigado!

RESUMO

A biomassa lignocelulósica é um importante recurso renovável que está prontamente disponível, sendo uma fonte de polissacarídeos com alto potencial biotecnológico. Os polissacarídeos complexos que compõem a lignocelulose podem ser convertidos em monossacarídeos fermentescíveis, com grande aplicabilidade em diversos bioprocessos industriais. A degradação dos materiais lignocelulósicos pode ser realizada por uma diversidade de vias enzimáticas complexas, onde é requerido um número considerável de enzimas ativas sobre carboidratos. Entre elas, as famílias das celulases e hemicelulases, além de atuarem na hidrólise dos materiais lignocelulósicos, possuem um uso versátil em setores industriais, tais como, nas áreas alimentícia, bebidas e de biocombustíveis. A tese teve como principais objetivos o delineamento de estratégias para a produção de enzimas e coquetéis enzimáticos eficientes para o uso na hidrólise da biomassa vegetal e, a aplicação de técnicas de imobilização para ampliar a utilização de enzimas em escala comercial. Inicialmente, a bioprospecção de novos micro-organismos secretores de enzimas atuantes na biomassa lignocelulósica foi realizada, e dentre as oitenta linhagens de *Streptomyces* testadas, duas linhagens (F1 e F7) se destacaram por apresentarem elevadas atividades celulolíticas e hemicelulolíticas. Uma abordagem genômica dessas linhagens possibilitou a identificação de 85 hidrolases glicosídicas (GHs) distribuídas em 33 famílias diferentes na linhagem F1, e 100 GHs dispostas em 44 famílias na linhagem F7. Além disso, os dados genômicos das linhagens F1 e F7 também indicaram a presença de genes relacionados à degradação da lignina. Ferramentas estatísticas também foram aplicadas e possibilitaram a ampliação da produção de GHs pela linhagem F1. Com a otimização, elevadas concentrações de GHs foram alcançadas com meio nutriente adicionado de 16,4 g L^{-1} de farelo de trigo e 10,0 g L^{-1} de caseína, onde se obteve 9,27 U mL^{-1} de xilanase e 0,22 U mL^{-1} de celulase. Para confirmar a diversidade de GHs expressas pela linhagem F1, uma análise por espectrometria de massa foi realizada e observou-se que quanto maior a complexidade da fonte de carbono utilizada, maior foi a gama de proteínas expressas, incluindo vários tipos de celulases e hemicelulases. A eficiência do extrato enzimático produzido pela linhagem F1 foi estudada para a sacarificação da biomassa vegetal e possibilitou um aumento significativo na liberação de açúcares quando adicionado ao extrato celulolítico comercial, indicando que as enzimas secretadas pela *Streptomyces* sp. F1 podem ser aplicadas para o melhoramento dos atuais coquetéis comerciais. Foi estudada também a criação de métodos de imobilização de enzimas em condições neutras de pH. Os novos suportes produzidos com a agarose foram utilizados para a imobilização de enzimas

monoméricas e multiméricas de grande importância biotecnológica. Um estudo mais detalhado explorando os novos suportes e o uso de técnicas de pós-imobilização foi também proposto. O processo desenvolvido com a aplicação do polímero polietilenimina (PEI) possibilitou a formação de um excelente sistema para imobilizar e estabilizar a β-glicosidase obtida de *Exiguobacterium antarcticum*. A β-glicosidase imobilizada apresentou uma melhora em suas características, incluindo estabilidade térmica e de armazenamento. Além disso, a β-glicosidase manteve sua atividade elevada mesmo após vários ciclos de hidrólise com celobiose como substrato.

Palavras chave: Hidrolases glicosídicas; *Streptomyces*; Genômica; Proteômica; Imobilização de enzimas.

ABSTRACT

Lignocellulosic biomass is an important renewable resource that is readily available, being a source of polysaccharides with high biotechnological potential. The complex polysaccharides that compose lignocellulose can be converted into fermentable monosaccharides, with great applicability in several industrial bioprocesses. The degradation of lignocellulosic materials can be accomplished by a variety of complex enzymatic pathways, where a considerable number of carbohydrate active enzymes are required. Among them, the families of cellulases and hemicellulases, besides acting in the hydrolysis of lignocellulosic materials, have a versatile use in industrial sectors, such as in food, beverages and biofuels. The main aims of this thesis were the design of efficient strategies for the production of enzymes and enzymatic cocktails for use in plant biomass hydrolysis and the application of immobilization techniques to increase the use of enzymes in a commercial scale. Initially, bioprospection of new enzyme secreting microorganisms active in lignocellulosic biomass was performed, and among the eighty *Streptomyces* strains tested, two strains (F1 and F7) were distinguished by their high cellulolytic and hemicellulolytic activities. A genomic approach of these strains allowed the identification of 85 glycoside hydrolases (GHs) distributed in 33 different families in the strain F1, and 100 GHs arranged in 44 families in the strain F7. In addition, the genomic data from strains F1 and F7 also indicated the presence of genes related to lignin degradation. Statistical tools were also applied and allowed the increase in GH production by strain F1. With the optimization, high concentrations of GHs were achieved with a nutrient medium containing 16.4 g L^{-1} of wheat bran and 10.0 g L^{-1} of casein, where 9.27 U mL^{-1} of xylanase and 0.22 U mL^{-1} of cellulase were obtained. To confirm the diversity of GHs expressed by the strain F1, an analysis using mass spectrometry technique was performed and it was observed that the greater the complexity of the carbon source used, the greater the range of proteins secreted, including several types of cellulases and hemicellulases. The efficiency of the enzymatic extract produced by strain F1 was studied for the saccharification of plant biomass and allowed a significant increase in sugar release when added to the commercial cellulolytic extract, indicating that the enzymes expressed by *Streptomyces* sp. F1 can be applied for the improvement of the current commercial cocktails. The creation of enzyme immobilization methods under neutral conditions of pH was also study. The new agarose supports were used for the immobilization of monomeric and multimeric enzymes of great industrial and biotechnological importance. A more detailed study exploring the new supports and the use of

post-immobilization techniques with polymers and small molecules was also proposed. The process developed with the application of the polymer polyethyleneimine (PEI) enabled the formation of an excellent system to stabilize the glucose-tolerant tetrameric β-glucosidase obtained from *Exiguobacterium antarcticum*. The immobilized β-glucosidase showed an improvement in its characteristics, with an increased activity, including thermal and storage stability. In addition, the β-glucosidase maintained a high activity even after several cycles of hydrolysis applying cellobiose as substrate.

Keywords: Glycoside hydrolases; *Streptomyces*; Genomics; Proteomics; Enzyme immobilization.

SUMÁRIO

INTRODUÇÃO GERAL 14
CAPÍTULO 1 17
Revisão bibliográfica 17
1. FUNDAMENTAÇÃO CIENTÍFICA 18
1.1. Biomassa lignocelulósica e seu potencial biotecnológico 18
1.2. Enzimas e proteínas ativas na desconstrução da biomassa lignocelulósica 20
1.3. Micro-organismos produtores de CAZymes e o potencial biotecnológico do gênero Streptomyces 24
1.4. Imobilização de enzimas 26
1.5. Aplicações das hidrolases glicosídicas em biorrefinarias e outras indústrias 29
2. REFERÊNCIAS 31
CAPÍTULO 2 43
Unraveling the cellulolytic and hemicellulolytic potential of two novel *Streptomyces* strains 43
RESUMO 44
ABSTRACT 45
1. INTRODUCTION 46
2. MATERIAL AND METHODS 47
2.1. Streptomyces strains isolation 47
2.2. Qualitative and quantitative screening 47
2.3. Cultivation and enzymatic production 48
2.4. Enzymatic assays 48
2.5. Identification of Streptomyces strains 48
2.6. Genome sequencing, assembly and annotation 49
2.7. Phylogenetic analysis 50
2.8. Nucleotide sequence accession number 51
3. RESULTS AND DISCUSSION 51
3.1. Strain screening and enzymatic activity evaluation 51
3.2. General features and phylogenetic analysis 55
3.3. CAZYme profiles 58
3.4. Aromatic compounds degradation pathways 60
4. CONCLUDING REMARKS 63

5. REFERENCES 63
CAPÍTULO 3 69
Enhancement in the production of hydrolytic enzymes from *Streptomyces* sp. strain F1 for optimized lignocellulosic biomass degradation 69
RESUMO 70
ABSTRACT 71
1. Introduction 72
2. Material and Methods 73
2.1. Streptomyces strain 73
2.2. Plant biomass feedstocks 73
2.3. Growth and analysis of culture conditions using statistical tools 74
2.4. Enzymatic assays 75
2.5. Protein analysis and SDS-PAGE 75
2.6. Preparation of secretomes 75
2.7. Secretome peptide mapping by liquid chromatography coupled in-line to mass spectrometry (LC–MS/MS) 76
2.8. Biochemical assays 76
2.9. Biomass saccharification 77
3. Results and Discussion 77
3.1. Culture medium analysis: Effects of carbon and nitrogen sources 77
3.2. Statistical evaluation as a tool to increase the production of biomass-degrading enzymes 79
3.3. Secretome analysis 82
3.4. Partial characterization 87
3.5. Hydrolytic potential of the biomass-degrading enzymes system from strain F1 88
4. Conclusions 90
5. References 90
CAPÍTULO 4 94
New heterofunctional supports based on glutaraldehyde-activation: A tool for enzyme immobilization at neutral pH 94
RESUMO 95
ABSTRACT 96
1. Introduction 97
2. Results and Discussion 100

2.1. Construction and Analysis of New Heterofunctional Supports 100
2.2. Immobilization of Different Biocatalysts on the New Heterofunctional Supports ... 104
2.3. Thermal Stability 108
3. Materials and Methods 111
3.1. Materials 111
3.2. Standard Determination of Enzymatic Activities 111
3.3. Supports for Immobilization 112
3.3.1. Activation of Agarose-Based Beads 112
3.3.2. Modification of the Epoxy-Activated Agarose with Different Reactive Groups 113
Cationic Supports 113
Support Activated with the Glutaraldehyde Group 113
Anionic Supports Activated with the Glutaraldehyde Group and Metal Chelate .. 113
Hydrophobic Supports Activated with the Glutaraldehyde Group 114
3.4. Immobilization of Enzymes 114
3.5. Protein Assays and SDS-PAGE 115
3.6. Fourier Transform Infrared (FTIR) 115
3.7. Desorption Process Analysis 115
3.8. Thermal Stability 116
4. Conclusions 116
5. References 117
CAPÍTULO 5 122
Hydrolysis of cellobiose using an improved immobilized-stabilized glucose-tolerant β-glucosidase from *Exiguobacterium antarcticum* B7 122
RESUMO 123
ABSTRACT 124
1. Introduction 125
2. Material and Methods 127
2.1. Material 127
2.2. Expression and purification of recEaBglA 127
2.3. Determination of proteins concentration and SDS-PAGE 128
2.4. β-glucosidase activity assays 128
2.5. Construction of supports for recEaBglA immobilization 128
2.5.1.Activation of agarose-based beads 128
2.5.2.Modification of epoxy-activated agarose beads 129

2.6. The recEaBglA immobilization 130
2.7. Biochemical assays 131
2.8. Operational stability of the immobilized recEaBglA and storage 131
3. Results and Discussion 132
3.1. Study of recEaBglA immobilization 132
3.2. Use of different polymers or small molecules and increased stability against temperature 132
3.3. Effects of the immobilization on the catalytic properties of recEaBglA 134
3.4. Operational stability of the immobilized recEaBglA and storage 138
4. Conclusions 139
5. References 140
DISCUSSÃO GERAL 144
CONCLUSÃO GERAL 147
PERSPECTIVAS PARA TRABALHOS FUTUROS 148
REFERÊNCIAS BIBLIOGRÁFICAS 149
ANEXO 1 175
CAPÍTULO 1 175
CAPÍTULO 2 176
CAPÍTULO 3 184
CAPÍTULO 4 185
CAPÍTULO 5 186
ANEXO 2 187
DOCUMENTOS CIENTÍFICOS PUBLICADOS NO PERÍODO DE DESENVOLVIMENTO DA TESE 187
FORMULÁRIO DE APROVAÇÃO DA COMISSÃO INTERNA DE BIOSSEGURANÇA (CIBIO). 196

INTRODUÇÃO GERAL

A parede celular vegetal constitui uma importante barreira física contra patógenos e estresse químico e físico para as plantas e é formada essencialmente por polissacarídeos de grande importância biológica e industrial. Os biopolímeros constituintes da biomassa vegetal, em especial a fração lignocelulósica, são considerados matérias-primas renováveis, ilimitadas, e biodegradáveis, sendo uma eficiente fonte de carboidratos e outros compostos que podem ser aplicados como blocos construtores de uma imensa gama de moléculas de grande valor econômico (Castro; Pereira Jr, 2010; Howard et al., 2003). Os açúcares obtidos a partir da despolimerização da lignocelulose (principalmente, xilose/C5 e glicose/C6) podem ser aplicados na produção de adoçantes (tais como, xilitol), e do etanol de segunda geração, bem como, alguns oligossacarídeos oriundos da degradação em potenciais nutracêuticos (como os celo- e xilo-oligossacarídeos) (Brienzo; Carvalho; Milagres, 2010; Jiao et al., 2014; Kuhad; Gupta; Singh, 2011; Mussatto; Roberto, 2004).

Entretanto, o desafio de desmontar a parede celular vegetal e posteriormente despolimerizar estes polissacarídeos em açúcares simples (monossacarídeos) ainda não é um processo totalmente consolidado e economicamente viável para diversas aplicações industriais. Assim, desenvolver e descobrir novas estratégias e processos bioquímicos mais baratos e eficientes para degradação e modificação dos polissacarídeos da parede celular vegetal são de grande interesse econômico para o crescimento do conceito de biorrefinarias lignocelulósicas.

Um dos obstáculos à ascensão desta tecnologia e a viabilização comercial dos produtos obtidos a partir da biomassa vegetal se encontra na relação custo-eficácia da produção e obtenção das enzimas hidrolíticas imprescindíveis para a desconstrução dos materiais lignocelulósicos. Estima-se que o custo com enzimas e/ou coquetéis enzimáticos, mesmo considerando-se os diferentes cenários, representa cerca de 30% do custo total (Macrelli; Galbe; Wallberg, 2014), sendo ainda um grande desafio tecnológico. Portanto, a busca por novas tecnologias que visem maior sustentabilidade na produção dos coquetéis enzimáticos e de novas enzimas que atuem de forma mais eficiente no processo de hidrólise, certamente é um dos caminhos para a redução dos custos e aumento dos rendimentos dos atuais processos.

Em relação à bioprospecção de enzimas que atuam na lignocelulose e suas fontes naturais, as bactérias são consideradas como uma eficiente plataforma de genes envolvidos na desconstrução da biomassa vegetal. As bactérias, especialmente o gênero *Streptomyces* são

descritas habitando diversos nichos, desempenhando um papel importante na reciclagem de carbono e na biodegradação dos polissacarídeos da biomassa vegetal (Anné et al., 2014). Outra estratégia viável para a diminuição de custos se encontra no uso dos subprodutos lignocelulolíticos como fonte de nutrientes de baixo custo em meios de cultivo para a obtenção dos coquetéis enzimáticos. Além disso, o uso de ferramentas estatísticas, como a metodologia de superfície de resposta (RSM), pode contribuir de forma randômica na análise de meios de cultivo, maximizando a produção e viabilizando o processo (Latha; Sivaranjani; Dhanasekaran, 2017).

Além da possibilidade de se reduzir custos com a bioprospecção e etapas de otimização da produção, a aplicação de métodos de imobilização enzimática pode contribuir como uma ferramenta eficiente para minimizar os gastos durante o processamento (Mateo et al., 2007). Embora as enzimas da família das celulases e hemicelulases sejam apresentadas como excelentes propriedades catalíticas, contendo alta especificidade e seletividade, algumas desvantagens biotecnológicas e importantes ainda são observadas. Características tais como baixa termoestabilidade, faixa de temperatura e pH de atuação restritas e inibição pelo produto e ou componentes do meio são descritas como sendo prejudiciais a aplicação destas hidrolases glicosídicas em alguns processos industriais. De acordo com Mateo et al. (2007), muitas destas adversidades podem ser superadas com a implementação de técnicas de imobilização de biocatalisadores que não são estáveis. Além disso, o uso de métodos de imobilização é um importante aliado na busca pelo aumento de produtividade e redução de custos em diversos bioprocessos, principalmente pela possibilidade de reuso do biocatalisador enzimático (Mateo et al., 2007).

Por isso, a maior motivação desta tese de doutorado foi justamente ampliar o conhecimento e delinear estratégias alternativas que facilitem o acesso a enzimas e coquetéis enzimáticos eficientes que atendam a demanda nas etapas de hidrólise dos complexos polissacarídeos da biomassa vegetal. Além disso, a aplicação de técnicas de imobilização enzimática como uma ferramenta valiosa poderá refletir em novas perspectivas para viabilizar a aplicação em escala industrial destes importantes biocatalisadores. Para tanto, o trabalho desenvolvido foi dividido em capítulos, visando dar maior ênfase aos objetivos de cada uma das etapas propostas.

O **CAPÍTULO 1** desta tese descreve uma breve revisão da literatura, onde é apresentada informações que contextualizam o leitor sobre a composição estrutural e o potencial biotecnológico da biomassa lignocelulósica, além de tópicos abordando a

sacarificação enzimática, estratégias para a redução e otimização de custos de produção, e a aplicação das enzimas lignocelulolíticas em diferentes segmentos industriais.

No **CAPÍTULO 2** uma expansão do atual conhecimento da diversidade de GHs contidas no gênero *Streptomyces* foi apresentada como uma nova solução para aplicações biotecnológicas onde o uso das enzimas ativas em carboidratos tem um papel fundamental. Neste capítulo foi abordada uma análise genômica dos genes codificantes de enzimas que atuam na biomassa vegetal de duas linhagens do gênero *Streptomyces* (linhagens F1 e F7) bioprospectadas no estudo a partir de amostras de solo.

No **CAPÍTULO 3** é apresentado um estudo de sustentabilidade na produção de um coquetel enzimático a partir da linhagem *Streptomyces* sp. F1. A escolha da linhagem se baseou em análises prévias de expressão de enzimas, onde foi observada uma elevada produção de GHs em um menor tempo de fermentação. Neste capítulo, se encontram descritos o uso de ferramentas estatísticas para otimizar o meio cultura com intuito de elevar a expressão de um coquetel enzimático (rico em enzimas celulolíticas e hemicelulolíticas), análises do secretoma por espectrometria de massas, e a avaliação do extrato enzimático obtido na suplementação de uma preparação enzimática comercial (Celluclast 1.5 L).

Nos **CAPÍTULOS 4 e 5** são demonstrados dados sobre a aplicação de técnicas de imobilização enzimática como uma importante e eficiente ferramenta na busca pelo aumento de produtividade e redução de custo, principalmente pela possibilidade de reuso do biocatalisador. Os resultados obtidos nestes capítulos se referem a uma colaboração com o Institute of Catalysis and Petrochemistry, Spanish National Research Council, Madrid-Spain (programa Ciências Sem Fronteiras). O **CAPÍTULO 4** descreve o desenvolvimento de novos suportes ativados com o agente glutaraldeído aplicáveis a imobilização de enzimas em condições de pH neutro, e no **CAPÍTULO 5** é apresentado uma análise de diferentes polímeros e pequenas moléculas na estabilização de uma β-glicosidase tetramérica tolerante à glicose obtida de *Exiguobacterium antarctic*um B7, imobilizada no novo suporte de agarose desenvolvido.

CAPÍTULO 1

Revisão bibliográfica

1. FUNDAMENTAÇÃO CIENTÍFICA

1.1.Biomassa lignocelulósica e seu potencial biotecnológico

A biomassa lignocelulósica é o recurso biológico renovável mais abundante no planeta e o maior componente estrutural das plantas. Estruturalmente, a biomassa é considerada uma importante fonte de carboidratos renováveis (denominados de açúcares de segunda geração) e outros compostos que podem ser utilizados como matérias-primas fundamentais para a obtenção de uma imensa gama de produtos de valor agregado, como o etanol, ácidos orgânicos, prebióticos, biopolímeros, entre outros. Desta forma, o conceito de biorrefinaria lignocelulósica se enquadra a essa tecnologia, visando o reaproveitamento total dos resíduos agroindustriais gerados em uma determinada cadeia produtiva, de modo a agregar valor à mesma (Castro; Pereira Jr, 2010; Howard et al., 2003; Pandey et al., 2000; Petruccioli et al., 2011).

A maior perspectiva sobre a biomassa lignocelulósica se deve a sua ampla disponibilidade. Estima-se que a produção mundial seja de 2×10^{11} milhões de toneladas por ano, sendo que 8 a 20×10^{9} milhões são descritas como potencialmente acessíveis para serem processadas e convertidas em produtos de valor agregado (Kricka; Fitzpatrick; Bond, 2014). Os materiais lignocelulósicos, em geral, são majoritariamente formados por polissacarídeos complexos como celulose e hemicelulose, os quais se encontram complexados a um heteropolímero fenólico denominado lignina. Em geral, a proporção de cada um destes componentes pode variar dependendo do tipo de biomassa vegetal analisada, mas em média é 33-55% de celulose, 13-33% de hemicelulose e 13-32% de lignina (Brethauer; Studer, 2015; Climent; Corma; Iborra, 2014; Kricka; Fitzpatrick; Bond, 2014; Volynets; Ein-Mozaffari; Dahman, 2017) (**Figura 1**). Compostos, tais como pectina, cinzas, lipídeos, proteínas, açúcares solúveis e minerais estão presentes junto à estrutura lignocelulósica em diferentes graus e de acordo com a fonte da biomassa estudada (Pauly; Keegstra, 2008; Sánchez, 2009).

A celulose é o polissacarídeo mais abundante e o maior constituinte da parede celular das plantas. As fibras de celulose correspondem a homopolissacarídeos lineares (não ramificados), constituídos por unidades de D-glicose conectadas por ligações covalentes do tipo β-D-1,4. Estes biopolímeros possuem como principal característica regiões de alta cristalinidade, formadas por ligações de hidrogênio entre as cadeias de celulose adjacentes, conferindo assim à fibra alta resistência a hidrólise e insolubilidade em água, e por regiões

amorfas (não-cristalinas) que são estruturas não organizadas mais suscetíveis aos processos de hidrólise (Carvalho et al., 2009; Fujita et al., 2004; Rubin, 2008).

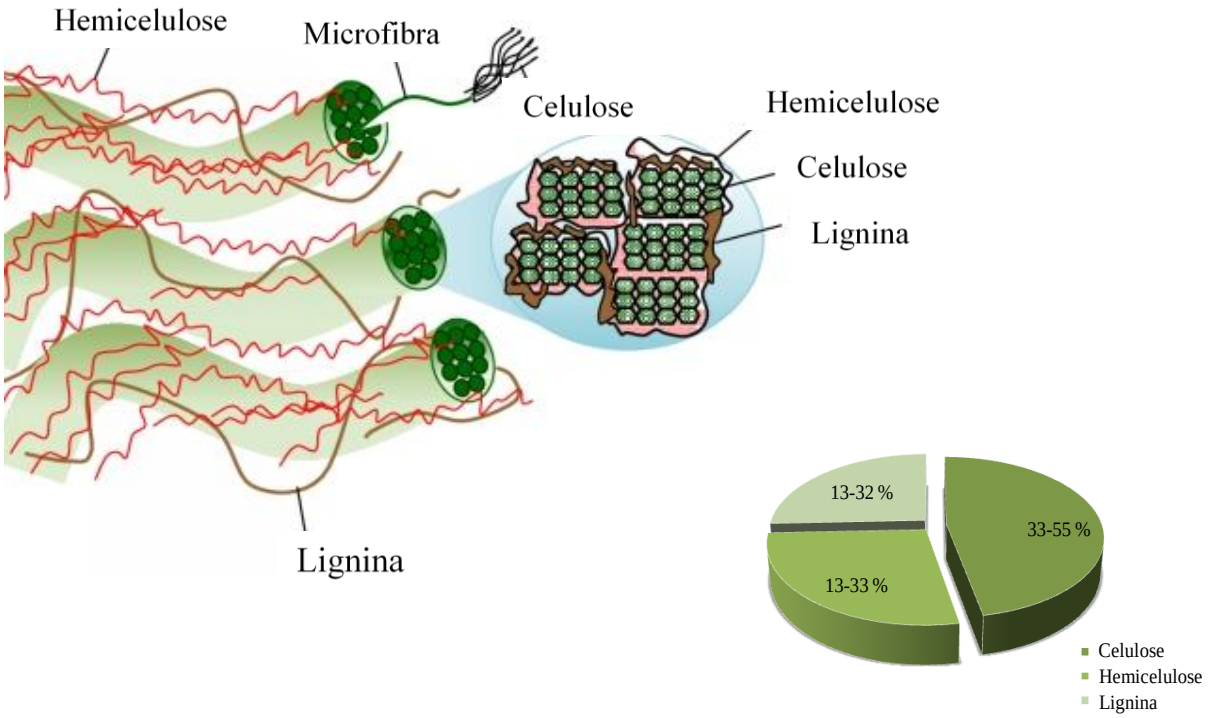


Figura 1 - Modelo simplificado da arquitetura da lignocelulose encontrada na parede celular vegetal (celulose, hemicelulose e lignina) e, sua composição estrutural (Lee; Hamid; Zain, 2014; Volynets; Ein-Mozaffari; Dahman, 2017).

A fração hemicelulósica, em contraste, é representada por um grupo diversificado de heteropolímeros complexos, compostos tanto por moléculas de pentoses (β-D-xilose, α-L-arabinose), hexoses (β-D-manose, β-D-glicose, α-D-galactose), bem como alguns outros monossacarídeos (ramnose e frutose) em menores quantidades (Gírio et al., 2010; Kqczkowski, 2003). As cadeias hemicelulósicas se encontram distribuídas na biomassa lignocelulósica aderindo-se às microfibrilas de celulose, ligando-se firmemente a região hidrofílica de sua superfície. O componente majoritário na hemicelulose, a xilana, é formada por uma cadeia principal de resíduos de β-1,4-D-xilopiranose podendo conter substituintes nas ramificações como L-arabinofuranose, ácido glucurônico, ácido 4-O-metilglucurônico, e cadeias laterais de acetil, os quais podem ser encontrados em diferentes frequências dependendo da origem da biomassa vegetal. O xiloglucano tem como cadeia principal resíduos de glicose com ligações do tipo β-1,4 com uma alta porcentagem desses resíduos substituídos por xilose com ligações do tipo α-1,6. Dependendo do tecido e da espécie

vegetal, estes resíduos de xilose podem estar unidos com resíduos de galactose ou arabinose por ligações do tipo β-1,2 ou α-1,2, respectivamente. Além disso, uma parte dos resíduos de galactose pode ser encontrada ligada com resíduos de fucose por meio de ligações do tipo α-1,2 (Gírio et al., 2010). A cadeia principal de manana é composta inteiramente por resíduos de manose, como em mananas e galactomananas, ou por resíduos de manose e glicose, como em glicomananas e galactoglicomananas (Adsul et al., 2011; Brink; van den; Vries, 2011; Horn et al., 2012; Ogeda et al., 2010; Vries et al., 2001).

A lignina, o terceiro componente principal da estrutura lignocelulósica, é um heteropolímero essencial presente na biomassa, uma vez que proporciona suporte estrutural, resistência contra o ataque microbiano e impermeabilidade à água. Estruturalmente, a lignina é um polímero altamente complexo sintetizado a partir de três precursores monoméricos: álcool p-cumaril, álcool coniferílico e álcool sinapílico. Estas unidades monoméricas quando incorporadas aos polímeros de lignina são conhecidas como p-hidroxifenil (H), guaiacil (G) e siringil (S), e estão conectadas através de ligações do tipo éter ou éster e, por resistentes ligações carbono-carbono. Assim, a biomassa lignocelulósica encontra-se organizada tendo suas fibras de celulose circundadas por hemicelulose e lignina, as quais estão associadas entre si por meio de ligações covalentes e interações fracas, formando as estruturas que conferem estabilidade à parede celular vegetal (Carvalho et al., 2009; Munk et al., 2015). Portanto, a organização da biomassa lignocelulósica torna sua estrutura altamente resistente à hidrólise química e biológica (Ogeda et al., 2010).

1.2. Enzimas e proteínas ativas na desconstrução da biomassa lignocelulósica

A heterogeneidade e complexidade dos polissacarídeos da biomassa lignocelulósica faz com que interações sinérgicas entre uma grande diversidade de enzimas e/ou proteínas sejam necessárias para uma eficiente quebra de todos os seus constituintes estruturais. A completa desconstrução da biomassa lignocelulósica em açúcares monossacarídicos é realizada por um vasto grupo de enzimas e/ou proteínas, denominadas de Enzimas Ativas em Carboidratos (*CAZymes*, do inglês *Carbohydrate-Active enZymes*). As classes de enzimas ativas em carboidratos são descritas como possuindo um enorme potencial biotecnológico industrial, sendo responsáveis pela síntese, degradação, reorganização e modificação de todo tipo de carboidrato disponível no planeta (Yin et al., 2012).

Atualmente, as enzimas atuantes na modificação de carboidratos e glicoconjugados se encontram distribuídas em famílias, sendo organizadas de acordo com suas similaridades de

sequência de aminoácidos, aspectos estruturais e mecanismos enzimáticos. As *CAZymes* estão classificadas no banco de dados CAZy (do inglês, ***C**arbohydrate **A**ctive En**z**ymes*, http://www.cazy.org/), sendo divididas entre classes funcionais, cuja função biológica pode se basear na síntese (Glicosil Transferases, GTs) e, na degradação e/ou rearranjo de complexos de carboidratos (Hidrolases Glicosídicas - GHs; Polissacarídeo Liases - PLs; Carboidrato Esterases - CEs; Enzimas Auxiliares - AAs) (Henrissat; Vegetales; Grenoble, 1991; Levasseur et al., 2013). Essas enzimas ainda podem se apresentar associadas à módulos de ligação a carboidratos (CBMs), que são domínios auxiliares proteicos sem função catalítica, que aderem especificamente à cadeia dos polissacarídeos, permitindo a proximidade do biocatalisador ao substrato (Guillén; Sánchez, 2010).

Na natureza, a desconstrução estrutural da celulose por via enzimática é realizada por múltiplas enzimas com diferentes módulos e mecanismos de atuação (Berlemont e Martiny, 2015). A desconstrução da celulose em monossacarídeos pode ser realizada por meio de grupo de hidrolases glicosídicas denominadas genericamente de celulases. As celulases são enzimas que clivam as ligações β-1,4 presentes na estrutura da celulose, e são subdivididas em três principais grupos como endoglucanases (EGLs), celobiohidrolases I e II (CBHs) e β-glicosidases (BGL) **(Figura 2)** (Volynets; Ein-mozaffari; Dahman, 2017).

As endoglucanases (EGLs, EC 3.2.1.4) são hidrolases glicosídicas essenciais ao processo de desconstrução da celulose, sendo capazes de atuar de forma aleatória, hidrolisando randomicamente as ligações do tipo β-1,4 contidas nas regiões internas da estrutura amorfa da celulose liberando oligossacarídeos de vários tamanhos (Kumar; Singh; Singh, 2008; Lynd et al., 2002). A atuação das EGLs sobre a estrutura da celulose promove a diminuição do grau de polimerização das cadeias celulolíticas e, consequentemente, a viscosidade do substrato, com isso expondo novas extremidades na cadeia principal da celulose para a atuação dos outros grupos de enzimas (Dimarogona; Topakas; Christakopoulos, 2012; Percival Zhang; Himmel; Mielenz, 2006). De acordo com o banco de dados CAZy, as principais famílias de GHs que contêm endoglucanases são: GHs 5-10, 12, 26, 44, 45, 48, 51, 74 e 124. As celobiohidrolases são também enzimas que desempenham um papel crucial na decomposição da celulose, atuando junto as extremidades redutoras (CBH I, EC 3.2.1.176, GHs 7, 9 e 48) e não redutoras (CBH II, EC 3.2.1.91, GHs 5, 6, e 9) da cadeia de celulose, liberando unidades celobiose como principal produto da hidrólise **(Figura 2)** (Lynd et al., 2002). A grande maioria das enzimas estudadas e caracterizadas como fazendo parte do grupo das celobiohidrolases (CBH I e CBH II), se encontram presentes em fungos

filamentosos pertencentes aos filos *Ascomycetes* e *Basidiomycetes* (Cantarel et al., 2009; Levasseur et al., 2013).

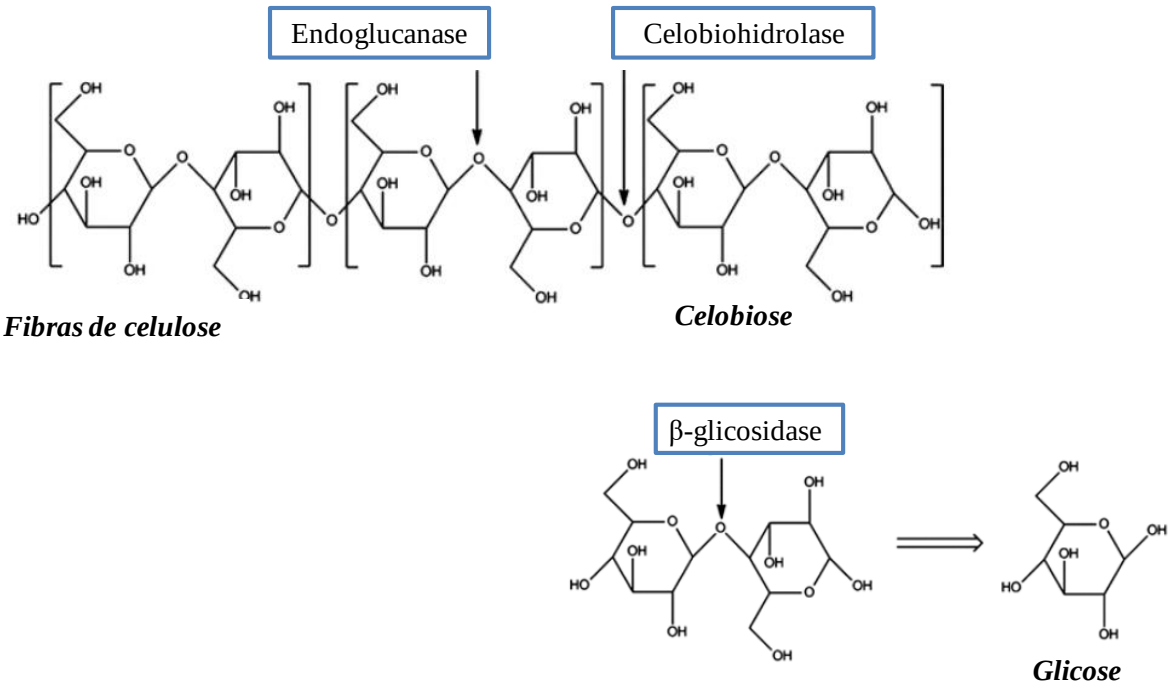


Figura 2 – Principais enzimas celulolíticas atuantes na despolimerização das fibras de celulose (Volynets; Ein-Mozaffari; Dahman, 2017).

O processo final de hidrólise dos polissacarídeos constituintes da celulose é realizado pelas β-glicosidases (BGs, EC 3.2.1.21, GHs 1, 2, 3, 5, 9, 30, 39 e 116), as quais se distinguem das outras duas enzimas celulolíticas pela sua capacidade em hidrolisar unidades solúveis de celobiose (dissacarídeo de glicose) ou oligossacarídeos (até celohexose-C6) gerados a partir das atividades catalíticas das endoglucanases e celobiohidrolases em monômeros de glicose (Ogeda et al., 2010). A atuação das β-glicosidases dentro da cascata de despolimerização dos substratos celulósicos é de extrema importância, porque estas enzimas diminuem o efeito inibitório da celobiose sobre as atividades enzimáticas das endoglucanases e das celobiohidrolases. Além das hidrolases mencionadas, proteínas acessórias podem auxiliar a degradação da celulose pelo enfraquecimento das ligações de hidrogênio presentes entre suas cadeias, facilitando assim o acesso das enzimas celulolíticas (Lee; Choi; Kende, 2001; Obeng et al., 2017).

Já a organização estrutural da hemicelulose, faz com que para a sua completa despolimerização seja necessário um conjunto maior de enzimas as quais atuam em uma diversidade de polissacarídeos complexos **(Figura 3)**. A hidrólise da cadeia de xilana, por

exemplo, ocorre por meio da ação sinérgica das endo β-1,4 xilanases (EC 3.2.1.8, GHs 3, 5, 8-12, 16, 26, 30, 43, 44, 51, 62 e 98) que catalisam a hidrólise aleatória das ligações β-1,4-xilosídicas na cadeia da xilana produzindo xilo-oligossacarídeos, e pelas exo β-1,4 xilosidases (EC 3.2.1.37, GHs 1, 3, 5, 30, 39, 43, 52, 52, 54, 116 e 120) que clivam os xilo-oligossacarídeos e xilobiose produzindo xilose. Entretanto, a biodegradação da hemicelulose ainda requer a ação de outras enzimas adicionais, tais como: endo β-1,4-mananase (EC 3.2.1.78, GHs 5, 9, 26, 44, 113 e 134) e β-1,4-manosidase (EC 3.2.1.25, GHs 1, 2 e 5) para a cadeia galactomanano (Brink; van den; Vries, 2011; Vries et al., 2001); endo α-1,5-L-arabinanases (EC 3.2.1.99, GH 43) e α-L-arabinofuranosidases (EC 3.2.1.55, GHs 2, 3, 10, 43, 51, 54 e 63) para a remoção dos resíduos de arabinose; α-galactosidase (EC 3.2.1.22, GHs 4, 27, 31, 36, 57, 97 e 110) que libera a galactose; α-glucuronidases (EC 3.2.1.139, GHs 4 e 67) para a remoção de ácido glucurônico ligada à xilose; e a acetil xilana esterase (EC 3.1.1.72, CEs 5 e 11), acetil manano esterase (EC 3.1.1.6, CE1), feruloil esterases e p-cumaroil esterases (EC 3.1.1.73, CE1), entre outras (Himmel et al., 2010; Kumar; Singh; Singh, 2008; Lynd et al., 2002; Pérez et al., 2002).

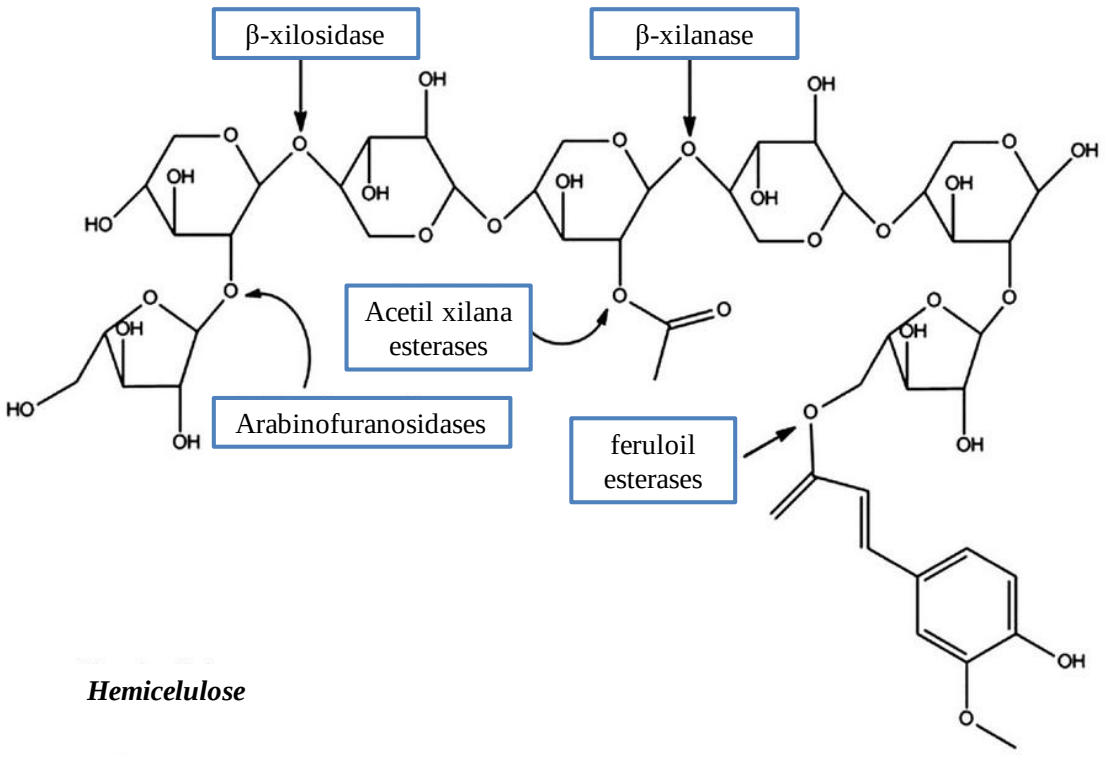


Figura 3 – Enzimas na despolimerização da hemicelulose (Volynets; Ein-Mozaffari; Dahman, 2017).

Ao contrário do sistema hidrolítico mencionado, existem enzimas específicas voltadas à degradação e modificação da lignina. As enzimas ligninolíticas atuam sobre os polímeros de

lignina gerando radicais livres altamente reativos que atacam as ligações carbono e éter, preferencialmente as ligações envolvendo C4 e C6. As principais enzimas que atuam na estrutura da lignina são a manganês peroxidase (MnP, EC 1.11.1.13, AA2) e lignina peroxidase (LiP, EC 1.11.1.14, AA2), que catalisam uma variedade de reações oxidativas dependentes de H_2O_2, e por último, as lacases (EC 1.10.3.2, AA1) que são enzimas oxidoredutoras envolvidas na degradação do polifenol, principal componente recalcitrante na lignocelulose. As lacases são enzimas induzíveis extracelulares que empregam oxigênio como agente oxidante. A baixa especificidade ao substrato das lacases permite que degradem uma grande variedade de compostos (Hammel; Cullen, 2008; Pérez et al., 2002; Sánchez, 2009; Sweeney; Xu, 2012).

1.3. Micro-organismos produtores de CAZymes e o potencial biotecnológico do gênero Streptomyces

Notoriamente, muitos sistemas biológicos naturais evoluíram para superar o desafio de degradar os complexos carboidratos da biomassa lignocelulósica e assim utilizá-lós de forma eficiente devido às suas capacidades para a produção de enzimas específicas capazes de clivar as cadeias de celulose, hemicelulose e lignina (Lynd et al., 2002; Xie et al., 2014).

Atualmente, as enzimas ativas em carboidratos (CAZymes) têm sido exploradas e, obtidas por uma grande variedade de organismos como fungos (*Aspergillus niger*, *Trichoderma reesei, Trichoderma harzianum, Phanerochaete chrysosporium, Penicilium* sp.) (Alsheikh-Hussain; Altenaiji; Yousef, 2014; Dashtban; Schraft; Qin, 2009; Delabona, et al., 2012), bactérias anaeróbicas (*Clostridium thermocellum, Clostridium cellulolyticum*, *Acetivibrio cellulolyticus*) (Bélaich et al., 1997; Dassa et al., 2012), bactérias aeróbicas (*Xanthomonas*, *Streptomyces* sp., *Bacillus* sp., *Thermobifida fusca, Flavobacterium* sp., *Pseudomonas* sp.) (Hazlewood; Gilbert, 1998; Lee et al., 2006; Rastogi et al., 2010; Santos et al., 2014; Zhang et al., 2010) e, as extraídas no trato digestivo de cupins, baratas e entre outros (Franco Cairo et al., 2016; Tramontina et al., 2017). Dentre os organismos descritos, em nível comercial, os fungos filamentosos são os maiores produtores, uma vez que evoluíram e se desenvolveram como sendo eficientes secretores de um conjunto de enzimas hidrolíticas e oxidativas capazes de clivar de forma eficiente os complexos polissacarídeos da parede celular vegetal. Os fungos filamentosos mais descritos como produtores de enzimas ativas em carboidratos são os pertencentes aos gêneros *Aspergillus*, *Trichoderma*, *Penicillium*,

Humicola e *Fusarium* (Castro; Pereira Jr, 2010; Kumar; Singh; Singh, 2008; Polizeli et al., 2005).

Embora as enzimas fúngicas possuam um maior enfoque comercial, as CAZymes de origem bacterianas têm sido analisadas e descritas como eficientes catalisadores (Maki; Leung; Qin, 2009; Pradeep et al., 2013; Sadhu; Maiti, 2013). Com relação aos gêneros bacterianos explorados para a obtenção das CAZymes, as bactérias tais como, os *Clostridium*, *Streptomyces*, *Bacillus*, *Pseudomonas* e *Cellulomonas* são as mais estudadas (Hamann et al., 2015; Heck; Hertz; Ayub, 2002; Shabeb et al., 2010). No entanto, o gênero *Streptomyces* tem sido consideravelmente analisado devido às suas enzimas possuírem características especialmente úteis em bioprocessos em nível industrial (Beg et al., 2000; Jang; Chen, 2003; Kiddinamoorthy et al., 2008).

O gênero *Streptomyces* é um grupo de bactérias ecologicamente importante, especialmente nos ambientes de solo, onde atuam como eficientes decompositores da biomassa vegetal (Book et al., 2014; Takasuka et al., 2013). Assim, as linhagens de *Streptomyces* são relatadas como sendo capazes de secretar uma grande variedade de enzimas relacionadas à despolimerização da biomassa lignocelulósica. Por exemplo, a análise do genoma de *S. coelicolor* possibilitou a identificação de 819 proteínas responsáveis por codificar genes para diferentes CAZymes, como hidrolases, quitinases, celulases/endoglucanasas, amilases e pectato liases (Bentley et al., 2002). Além disso, várias enzimas secretadas pelas linhagens de *Streptomyces* são economicamente valiosas, tais como, a glicose isomerase obtida por *S. olivaceus*, *S. olivochromogenes*, *S. rubigenosus* e *S. murinus*, a qual é utilizada na produção de xarope de frutose, e a enzima transglutaminase secretada por *S. mobaraensis* utilizada na indústria para melhorar a qualidade de carnes processadas e produtos à base de peixe. Dentro do tema de energias bio-sustentáveis, as linhagens de *Streptomyces* possuem um interessante papel devido à sua capacidade em secretar diversas enzimas celulolíticas e xilanolíticas. Por exemplo, *S. lividans* (Hurtubise et al., 1995), e *S. thermoviolaceus* (Kittur et al., 2003) são produtores de eficientes xilanases.

Além da possibilidade de se obter eficientes GHs a partir da bioprospecção, um dos obstáculos que ainda inviabiliza a ampla disseminação destas enzimas em níveis industriais está na relação custo-eficácia da produção e obtenção. Estima-se que o custo com enzimas e/ou coquetéis enzimáticos atuantes na despolimerização da biomassa vegetal, mesmo considerando-se os diferentes cenários, represente cerca de 30% do custo total (Macrelli; Galbe; Wallberg, 2014). Portanto, a busca por novas tecnologias que visem uma maior

sustentabilidade na produção de coquetéis enzimáticos ou de enzimas é certamente ainda um grande desafio tecnológico.

Atualmente, vários trabalhos têm relatado o uso de materiais lignocelulósicos como fontes de nutrientes para a obtenção de uma diversidade de enzimas de interesse industrial, tais como as hidrolases glicosídicas (Delabona, et al., 2012; Garcia et al., 2015; Heck; Hertz; Ayub, 2002; Mander et al., 2014). Em parte, o maior interesse sobre estes materiais lignocelulósicos se deve ao fato de ser uma fonte de nutrientes sustentável e economicamente viável, sendo gerados anualmente em grandes quantidades por diversos setores, tais como, industriais e agrícolas (Delabona, et al., 2012; Kumar; Satyanarayana, 2012, 2014). Além disso, o uso de ferramentas estatísticas, como a metodologia de superfície de resposta (RSM), pode contribuir de forma randômica na análise de meios de cultivo, maximizando a produção e viabilizando o processo (Latha; Sivaranjani; Dhanasekaran, 2017).

Finalmente, tendo em consideração o contexto de viabilidade econômica, outro importante ponto que pode ser explorado é a possibilidade de produção simultânea de várias enzimas. Segundo Kaur et al. 2010, a produção simultânea de diversas enzimas em um mesmo meio de cultivo por um único micro-organismo, faz com que o processo de obtenção de diferentes biocatalisadores se torne mais atraente e viável para o uso em processos industriais em comparação com a obtenção destas enzimas separadamente (Kaur et al., 2010). Neste contexto, a utilização dos subprodutos lignocelulósicos em conjunto com a possibilidade de obtenção de um coquetel enzimático pode aumentar a viabilidade de se produzir diversas GHs de interesse comercial.

1.4. Imobilização de enzimas

O uso de enzimas como eficientes catalisadores tem sido descrito em uma gama de processos industriais devido à sua especificidade, rapidez e economia de materiais, energia e água, comparados com outros processos convencionais (Jegannathan; Nielsen, 2013). No entanto, um dos principais desafios que envolvem a utilização de enzimas em escala industrial é a competição econômica com os processos químicos, que estão bem estabelecidos e foram aperfeiçoados por décadas. As enzimas encontradas na natureza muitas vezes precisam ser melhoradas quanto à estabilidade, atividade e eficiência catalítica para suportar o processo industrial (Mateo et al., 2007). Além disso, a atuação enzimática requer na maioria das vezes meio aquoso, o que não permite a recuperação e reciclo do biocatalisador, tornando os custos do processamento mais elevados (Mateo et al., 2007).

Tabela 1 - Estudos de recuperação e reuso de hidrolases glicosídicas imobilizadas na desconstrução de materiais lignocelulósicos e outros substratos.

	Enzima/Preparação comercial	***Suporte***	***Substrato***	***Reciclo ou reuso***	***Referências***
Celulase					
	Cellic CTec2	Partículas magnéticas de sílica	Palha de trigo pré-tratada	80% da conversão inicial no segundo ciclo	(John; Alftre, 2014)
	Celulase de *T. reesei*	Partículas magnéticas	Carboximetilcelulose (CMC)	30% da conversão inicial após 10 ciclos	(Abraham et al., 2014)
	Cellulase NS50013	Partículas de sílica com polímero de ácido poliacrílico	Papel de filtro e celulose Solka-Floc	95% da atividade recuperada após hidrólise	(Samaratunga et al., 2015)
	Celulase de *T. reesei*	CLEA com sílica	Fibra de óleo de palma pré-tratada	81% da conversão inicial após 5 ciclos	(Sutarlie; Yang, 2013)
β-glicosidase					
	β-glicosidase Megazyme e Novozym 188	Partículas magnéticas de sílica	Biomassa de pinheiro pré-tratada	52% da conversão inicial após quatro ciclos	(Alftrén; Hobley, 2013)
	Novozym 188	Eupergit C	Madeira pré-tratada	90% da conversão inicial após 6 ciclos	
	β-glicosidase de *Agaricus arvensis*	Nanopartículas de óxido de silício funcionalizadas	Celobiose	95% da conversão inicial após 25 ciclos	(Singh et al., 2011)
Xilanase					
	Xilanase de *Thermomyces lanuginosus*	Partículas magnéticas de sílica	Xilana de madeira de faia	65% da conversão inicial após 9 ciclos	(Soozanipour, Taheri-kafrani; Landarani, 2015)
	Xilanase de *Thermomyces lanuginosus*	Partículas de hidróxido de alumínio	Xilana de madeira de faia	71% da conversão inicial após 5 ciclos	(Jiang; Wu; Li, 2016)

Para um maior favorecimento dos processos enzimáticos para fins industriais, diferentes estratégias têm sido desenvolvidas, incluindo a imobilização de enzimas. O uso de métodos de imobilização é um importante aliado na busca pelo aumento de produtividade e redução de custos em diversos bioprocessos, principalmente pela possibilidade de reuso do catalisador enzimático aplicado. Além da possibilidade de reutilização, as enzimas imobilizadas podem apresentar vantagens, tais como, a melhoria da estabilidade operacional frente à diferentes condições operacionais (temperatura e pH), redução da inibição e/ou redução da agregação. A imobilização traz também como benefício o desenvolvimento de tecnologias limpas proporcionando a obtenção de produtos livres do biocatalisador (Datta, Christena; Rajaram, 2013; Mateo; Grazu, et al., 2007; Rodrigues et al., 2013; Spahn; Minteer, 2008).

Para o desenvolvimento de estratégias de imobilização eficientes, uma avaliação inicial de três principais pontos, como a estrutura da enzima, o suporte (matriz) e o modo de fixação da enzima ao suporte, é de extrema importância para a viabilidade do processo (Brenda; Batista-Virra, 2006). As técnicas de imobilização exploram o fato de que as enzimas são compostas por aminoácidos com diferentes características, (Brenda; Batista-Virra, 2006; Sheldon, 2007) e que estes constituintes podem ser utilizados na interação com o suporte por meio de vários tipos de ligações e interações. As interações mais comumente aplicadas entre as enzimas e os diferentes suportes ocorrem através de interações do tipo reversíveis, como a adsorção física, e aprisionamento ou encapsulamento, ou por técnicas de *cross-linking* e ligação covalente das proteínas ao suporte, as quais se utilizam de interações do tipo irreversíveis (Brenda; Batista-Virra, 2006).

Uma revisão de literatura mostra que a imobilização de enzimas possibilita a obtenção de catalisadores mais estáveis e compatíveis a processos envolvendo fins industriais **(Tabela 1)**. Singh *et al.* (2011) estudaram a imobilização de uma β-glicosidase produzida por *Agaricus arvensis* covalentemente ligada à nanopartículas de óxido de silício funcionalizadas, e observaram que a técnica de imobilização proporcionou o reuso da β-glicosidase em 25 ciclos, tendo a enzima ainda apresentado 95% da sua atividade inicial. O uso desta técnica também proporcionou uma maior estabilidade da enzima a qual apresentou um tempo de meia-vida aumentado 288 vezes quando submetida à temperatura de 65^{o}C, em comparação com a enzima livre (Singh et al., 2011). O uso de agarose-MANAE e derivados de DEAE-celulose na imobilização de uma β-glicosidase promoveu um aumento na estabilidade de cerca de 120 e 75 vezes comparado com a enzima livre, respectivamente (Silva, Da et al., 2014). Vieira et al. (2011) constataram que uma β-glicosidase da Novozymes, covalentemente

imobilizada sobre agarose amina-epoxi foi 200 vezes mais estável quando aplicada a 65^{o}C e pH 4,8 em comparação com a forma livre (Vieira et al., 2011).

Além de estudos utilizando as β-glicosidases, diversos outros trabalhos demonstram que a imobilização das enzimas celulolíticas e hemicelulases resulta em uma melhoria de suas estabilidades e características em diferentes condições de processamento. Driss et al. (2014) mostraram que a xilanase imobilizada através da ligação covalente em nanopartículas magnéticas Fe_3O_4 revestidas com quitosano exibiu uma elevada estabilidade quando aplicada em diferentes valores de pH e temperatura (Driss et al., 2014). Benassi et al. (2013) mostraram que a β-xilosidase imobilizada em PEI-Sepharose foi mais termoestável do que a enzima livre (Benassi et al., 2013). Assim, as técnicas de imobilização podem ser úteis e ferramentas eficazes para estabilizar e proporcionar o reuso de diversos biocatalisadores, estimulando uma maior aplicabilidade de algumas enzimas em processos industriais.

No entanto, apesar da imobilização apresentar diversas vantagens, tornando seus usos uma rota atraente e promissora para a melhoria da estabilidade frente a diferentes condições operacionais e reciclagem de enzimas, novos estudos ainda são necessários para se encontrar condições ideais para a imobilização. Isto é importante, devido aos passos realizados durante a imobilização frequentemente não promoverem a recuperação de total da atividade enzimática inicialmente aplicada, ocorrendo assim perdas do biocatalisador. Uma explicação para a baixa eficiência dos processos de imobilização pode estar relacionado com interações inespecíficas entre o suporte e os aminoácidos presentes no sítio ativo do biocatalisador, os quais são necessários para a ligação com o substrato (Eijsink et al., 2004; Závodszky et al., 1998). Portanto, o desenvolvimento de novos métodos que permitam o aumento das taxas de aprisionamento da enzima ao suporte e maiores taxas de eficiências dos biocatalisadores imobilizados é atualmente requisitado.

1.5.Aplicações das hidrolases glicosídicas em biorrefinarias e outras indústrias

A hidrólise enzimática tem sido aplicada como uma ferramenta poderosa para extrair os principais compostos ou açúcares monoméricos fermentescíveis presentes na biomassa lignocelulósica, com o objetivo de transformá-los em produtos com maior valor agregado (Wong; Saddler, 1992). Os materiais lignocelulósicos (por exemplo, celulose e hemicelulose) estão entre os mais viáveis e abundantes recursos renováveis, o que os torna excelentes substratos, com aplicações em diversos setores industriais alimentícios e, de biocombustíveis (Peng et al., 2011).

Atualmente, entre os processos biotecnológicos aplicados para a utilização da biomassa lignocelulósica, o mais amplamente pesquisado é a produção de etanol lignocelulósico ou de segunda geração. O maior interesse se deve a possibilidade de esgotamento das reservas de combustíveis fósseis, junto com problemas associados ao aumento da emissão de gás carbônico (CO_2), o que ampliou o interesse por fontes renováveis de energia (Cerqueira Leite et al., 2009; Clark; Luque; Matharu, 2012; Santos, et al., 2014). O processo de produção de etanol a partir da biomassa lignocelulósica consiste de um pré-tratamento inicial (podendo ser mecânico, químico e/ou biológico), necessário para a desconstrução da recalcitrância da biomassa lignocelulósica tornando os seus constituintes mais acessíveis à hidrólise, seguido pela etapa de sacarificação, onde são aplicadas as hidrolases glicosídicas, que desconstroem os polissacarídeos em açúcares monoméricos (pentoses e hexoses). Assim, os açúcares obtidos podem ser fermentados para bioetanol utilizando-se micro-organismos específicos (Kuhad; Gupta; Singh, 2011; Sánchez; Cardona, 2008).

Além da obtenção dos biocombustíveis, os açúcares oriundos da biomassa podem ser utilizados na produção de vários ácidos orgânicos, incluindo os ácidos lático e acético. Estes ácidos podem ser obtidos a partir dos materiais lignocelulósicos utilizando-se alguns passos sequenciais envolvendo o processamento da biomassa (a fim de tornar a celulose mais acessível para as enzimas), seguido da sacarificação empregando as enzimas celulolíticas (obtenção de hidrolisado rico em açúcares) e, finalmente, este último pode ser fermentado por micro-organismos específicos para a produção dos ácidos orgânicos (Mussatto et al., 2008; Ravinder et al., 2001).

Na indústria alimentícia, as enzimas celulolíticas e hemicelulases têm sido utilizadas em diversos processos para a produção de uma ampla gama de compostos. Atualmente, uma das aplicações de grande destaque se encontra no uso das hidrolases glicosídicas para a obtenção dos prebióticos. Os prebióticos são ingredientes alimentares não digeríveis que estimulam o crescimento seletivo de organismos probióticos no trato gastrointestinal, tais como *Bifidobacterium* e *Lactobacillus*. As principais vantagens do consumo dos prebióticos, além de poderem ser aplicados como um adoçante de baixa caloria se encontra na possibilidade destes compostos poderem atuar na redução da glicose e colesterol no sangue, ou na manutenção da flora gastrointestinal, aumentando a absorção de minerais e a imuno estimulação (Gibson; Roberfroid, 1995; Samanta et al., 2015; Sousa; Santos; Sgarbieri, 2011).

Importantes prebióticos podem ser obtidos a partir da hidrólise da biomassa lignocelulósica utilizando as hidrolases glicosídicas. As xilanases hidrolisam os materiais hemicelulósicos produzindo xilo-oligossacarídeos (xilobiose, xilotriose e oligossacarídeos

com um maior grau de polimerização), os quais possuem propriedades prebióticas importantes. De forma similar, outro tipo de oligossacarídeo funcional (celo-oligossacarídeos) pode ser produzido pela hidrólise enzimática da celulose utilizando-se enzimas celulolíticas específicas (Hasunuma et al., 2011; Otsuka et al., 2004).

As hidrolases glicosídicas, especificamente as celulases e as hemicelulases, também possuem aplicações potenciais em outros setores alimentícios. Na produção de sucos de frutas, são necessários métodos aperfeiçoados para a extração, clarificação e estabilização dos diferentes sucos. Neste caso, as celulases e xilanases têm sido utilizadas como uma parte importante do complexo enzimático aplicado no processo de maceração utilizado durante a extração e na etapa de clarificação dos sucos de frutas para aumentar o rendimento (Carvalho; Castro; Silva, 2008; Minussi; Pastore; Durán, 2002).

As celulases microbianas, especificamente glucanases, desempenham um papel importante nos processos fermentativos utilizados na produção de bebidas alcoólicas, incluindo cervejas e vinhos (Bamforth, 2009; Sukumaran; Singhania; Pandey, 2005). Estas enzimas podem ser aplicadas na melhora da qualidade e do rendimento dos produtos fermentados (Bamforth, 2009; Canales et al., 1988). Na produção de vinhos, as enzimas glucanases e hemicelulases desempenham um importante papel, os quais incluem; o melhoramento da extração de pigmentos, facilitação do processo de clarificação e filtração e, finalmente a melhora na estabilidade e da qualidade do vinho (Kuhad; Gupta; Singh, 2011). No processo de fabricação de cerveja, as celulases podem ser utilizadas na fase de maceração, a fim de hidrolisar as cadeias de β-glucanos presentes em excesso, reduzindo assim a viscosidade do mosto e melhorando a capacidade de filtração (Bamforth, 2009).

Outro produto com considerável valor de mercado que pode ser obtido pela conversão dos açúcares da biomassa lignocelulósica, são os adoçantes. O uso de adoçantes tem recebido maior atenção como uma forma viável de se evitar problemas de riscos à saúde associados com os açúcares calóricos (Chattopadhyay; Raychaudhuri; Chakraborty, 2014; Pinheiro; Oliveira, 2005). Neste contexto, a maior busca por adoçantes (por exemplo, xilitol, sorbitol e manitol) tem estimulado o crescimento do mercado para esses açúcares-alcoóis alternativos, o que pode facilitar a produção de adoçantes a partir de açúcares obtidos da hidrólise enzimática da biomassa.

2. REFERÊNCIAS

ABRAHAM, R. E.; VERMA, M. L.; BARROW, C. J.; PURI, M. Suitability of magnetic nanoparticle immobilised cellulases in enhancing enzymatic saccharification of pretreated

hemp biomass. **Biotechnology for Biofuels**, v. 7, n. 1, p. 1–12, 2014.

ADSUL, M. G.; SINGHVI, M. S.; GAIKAIWARI, S. A.; GOKHALE, D. V. Development of biocatalysts for production of commodity chemicals from lignocellulosic biomass. **Bioresource Technology**, v. 102, n. 6, p. 4304–4312, 2011.

ALFTRÉN, J.; HOBLEY, T. J. Covalent immobilization of β-glucosidase on magnetic particles for lignocellulose hydrolysis. **Applied Biochemistry and Biotechnology,** p. 2076–2087, 2013.

ALSHEIKH-HUSSAIN, A.; ALTENAIJI, E. M.; YOUSEF, L. F. Fungal cellulases from mangrove forests - A short review. **Journal of Biochemical Technology**, v. 5, n. 169, p. 765–774, 2014.

ANNÉ, J.; VRANCKEN, K.; MELLAERT, L. VAN; IMPE, J. VAN; BERNAERTS, K. Protein secretion biotechnology in Gram-positive bacteria with special emphasis on *Streptomyces lividans*. **BBA - Molecular Cell Research**, v. 1843, n. 8, p. 1750–1761, 2014.

BAMFORTH, C. W. Current perspectives on the role of enzymes in brewing. **Journal of Cereal Science**, v. 50, n. 3, p. 353–357, 2009.

BEG, Q. K.; BHUSHAN, B.; KAPOOR, M.; HOONDAL, G. S. Production and characterization of thermostable xylanase and pectinase from *Streptomyces* sp. QG-11-3. **Journal of Industrial Microbiology and Biotechnology**, v. 24, p. 396–402, 2000.

BÉLAICH, J. P.; TARDIF, C.; BÉLAICH, A.; GAUDIN, C. The cellulolytic system of *Clostridium cellulolyticum*. **Journal of Biotechnology**, v. 57, n. 1, p. 3–14, 1997.

BENTLEY, S. D.; CHATER, K. F.; CERDEÑO-TÁRRAGA, A.-M.; CHALLIS, G. L.; THOMSON, N. R.; JAMES, K. D.; HARRIS, D. E.; QUAIL, M. A.; KIESER, H.; HARPER, D.; BATEMAN, A.; BROWN, S.; CHANDRA, G.; CHEN, C. W.; COLLINS, M.; CRONIN, A.; FRASER, A.; GOBLE, A.; HIDALGO, J.; HORNSBY, T.; HOWARTH, S.; HUANG, C.-H.; KIESER, T.; LARKE, L.; MURPHY, L.; OLIVER, K.; O'NEIL, S.; RABBINOWITSCH, E.; RAJANDREAM, M.-A.; RUTHERFORD, K.; RUTTER, S.; SEEGER, K.; SAUNDERS, D.; SHARP, S.; SQUARES, R.; SQUARES, S.; TAYLOR, K.; WARREN, T.; WIETZORREK, A.; WOODWARD, J.; BARRELL, B. G.; PARKHILL, J.; HOPWOOD, D. A. Complete genome sequence of the model actinomycete *Streptomyces*. **Nature**, v. 417, n. 2, p. 141–147, 2002.

BOOK, A. J.; LEWIN, G. R.; MCDONALD, B. R.; TAKASUKA, T. E.; DOERING, D. T.; ADAMS, A. S.; BLODGETT, J. A. V; CLARDY, J.; RAFFA, K. F.; FOX, B. G.; CURRIE, C. R. Cellulolytic *Streptomyces* strains associated with herbivorous insects share

a phylogenetically linked capacity to degrade lignocellulose. **Applied and Environmental Microbiology**, v. 80, n. 15, p. 4692–4701, 2014.

BENASSI, V. M.; SILVA, T. M. DA; PESSELA, B. C.; GUISAN, J. M.; MATEO, C.; LIMA, M. S.; JORGE, J. A.; POLIZELI, M. D. L. T. M. Immobilization and biochemical properties of a β-xylosidase activated by glucose/xylose from *Aspergillus niger* USP-67 with transxylosylation activity. **Journal of Molecular Catalysis B: Enzymatic**, v. 89, p. 93–101, 2013.

BERLEMONT, R.; MARTINY, C. Genomic potential for polysaccharide deconstruction in bacteria. **Applied and Environmental Microbiology**, v. 81, n. 4, p. 1513–1519, 2015.

BRENDA, B.; BATISTA-VIRRA, F. Immobilization of enzymes. *In*: GUISAN, J. (Ed.). **Immobilization of enzymes and cells**. 2. ed. [s.l.] New Jersey (NJ): Humana Press Inc., v. 22p. 123–124, 2006.

BRETHAUER, S.; STUDER, M. H. Biochemical conversion processes of lignocellulosic biomass to fuels and chemicals – A review. **The Swiss Competence Center for Energy Research on Bioenergyv**. 69, n. 10, p. 572–581, 2015.

BRINK, J. VAN DEN; VRIES, R. P. DE. Fungal enzyme sets for plant polysaccharide degradation. **Applied Microbiology and Biotechnology**, v. 91, n. 6, p. 1477–92, 2011.

CANALES, A. M.; GARZA, R.; SIERRA, J. A.; ARNOLD, R. The application of beta-glucanase with additional side activities in brewing. **MBAA Technical Quarterly**, v. 25, p. 27–31, 1988.

CANTAREL, B. I. L.; COUTINHO, P. M.; RANCUREL, C.; BERNARD, T.; LOMBARD, V.; HENRISSAT, B. The Carbohydrate-Active EnZymes database (CAZy): an expert resource for glycogenomics. **Nucleic Acids Research**, v. 37, p. D233–D238, 2009.

CARVALHO, L. M. J.; CASTRO, I. M.; SILVA, C. A. B. A study of retention of sugars in the process of clarification of pineapple juice (*Ananas comosus*, L. Merril) by micro- and ultra-filtration. **Journal of Food Engineering**, v. 87, n. 4, p. 447–454, 2008.

CARVALHO, W.; CARRILHA, L.; FERRAZ, A.; MILAGRES, A. M. F. Uma visão sobre a estrutura, composição e biodegradação da madeira. **Química Nova**, v. 32, n. 8, p. 2191–2195, 2009.

CASTRO, A. M. DE; PEREIRA JR, N. Produção, propriedades e aplicação de celulases na hidrólise de resíduos agroindustriais. **Química Nova**, v. 33, n. 1, p. 181–188, 2010.

CERQUEIRA LEITE, R. C. DE; VERDE LEAL, M. R. L.; BARBOSA CORTEZ, L. A.; GRIFFIN, W. M.; GAYA SCANDIFFIO, M. I. Can Brazil replace 5% of the 2025 gasoline world demand with ethanol? **Energy**, v. 34, n. 5, p. 655–661, 2009.

CHATTOPADHYAY, S.; RAYCHAUDHURI, U.; CHAKRABORTY, R. Artificial sweeteners - A review. **Journal of Food Science and Technology**, v. 51, n. 4, p. 611–621, 2014.

CLARK, J. H.; LUQUE, R.; MATHARU, A. S. Green chemistry, biofuels, and biorefinery. **Annual Review of Chemical and Biomolecular Engineering**, v. 3, n. 1, p. 183–207, 2012.

CLIMENT, M. J.; CORMA, A.; IBORRA, S. Conversion of biomass platform molecules into fuel additives and liquid hydrocarbon fuels. **Green Chemistry**, v. 16, n. 2, p. 516, 2014.

DASHTBAN, M.; SCHRAFT, H.; QIN, W. Fungal bioconversion of lignocellulosic residues; Opportunities & perspectives. **International Journal of Biological Sciences**, v. 5, n. 6, p. 578–595, 2009.

DASSA, B.; BOROVOK, I.; LAMED, R.; HENRISSAT, B.; COUTINHO, P.; HEMME, C. L.; HUANG, Y.; ZHOU, J.; BAYER, E. A. Genome-wide analysis of *Acetivibrio cellulolyticus* provides a blueprint of an elaborate cellulosome system. **BMC Genomics**, v. 13, n. 1, p. 210, 2012.

DATTA, S.; CHRISTENA, L. R.; RAJARAM, Y. R. S. Enzyme immobilization: An overview on techniques and support materials. **3 Biotech**, p. 1–9, 2013.

DELABONA, P. DA S.; FARINAS, C. S.; SILVA, M. R. DA; AZZONI, S. F.; PRADELLA, J. G. DA C. Use of a new *Trichoderma harzianum* strain isolated from the Amazon rainforest with pretreated sugar cane bagasse for on-site cellulase production. **Bioresource Technology**, v. 107, p. 517–521, 2012.

DELABONA, P. DA S.; PIROTA, R. D. P. B.; CODIMA, C. A.; TREMACOLDI, C. R.; RODRIGUES, A.; FARINAS, C. S. Using Amazon forest fungi and agricultural residues as a strategy to produce cellulolytic enzymes. **Biomass and Bioenergy**, v. 37, p. 243–250, 2012.

DIMAROGONA, M.; TOPAKAS, E.; CHRISTAKOPOULOS, P. Cellulose degradation by oxidative enzymes. **Computational and Structural Biotechnology Journal**, v. 2, n. 3, p. e201209015, 2012.

DRISS, D.; ZOUARI-ELLOUZI, S.; CHAARI, F.; KALLEL, F.; GHAZALA, I.; BOUAZIZ, F.; GHORBEL, R.; CHAABOUNI, S. E. Production and in vitro evaluation of xylooligosaccharides generated from corncobs using immobilized *Penicillium occitanis* xylanase. **Journal of Molecular Catalysis B: Enzymatic**, v. 102, p. 146–153, 2014.

EIJSINK, V. G. H.; BJØRK, A.; GÅSEIDNES, S.; SIREVÅG, R.; SYNSTAD, B.; BURG, B. VAN DEN; VRIEND, G. Rational engineering of enzyme stability. **Journal of**

Biotechnology, v. 113, n. 1–3, p. 105–120, 2004.

FRANCO CAIRO, J. P. L.; CARAZZOLLE, M. F.; LEONARDO, F. C.; MOFATTO, L. S.; BRENELLI, L. B.; GONÇALVES, T. A.; UCHIMA, C. A.; DOMINGUES, R. R.; ALVAREZ, T. M.; TRAMONTINA, R.; VIDAL, R. O.; COSTA, F. F.; COSTA-LEONARDO, A. M.; PAES LEME, A. F.; PEREIRA, G. A. G.; SQUINA, F. M. Expanding the knowledge on lignocellulolytic and redox enzymes of worker and soldier castes from the lower termite *Coptotermes gestroi*. **Frontiers in Microbiology**, v. 7, 2016.

FUJITA, Y.; ITO, J.; UEDA, M.; FUKUDA, H.; KONDO, A. Synergistic saccharification, and direct fermentation to ethanol, of amorphous cellulose by use of an engineered yeast strain codisplaying three types of cellulolytic enzyme. **Applied and Environmental Microbiology**, v. 70, n. 2, p. 1207–1212, 2004.

GARCIA, N. F. L.; SILVA SANTOS, F. R. DA; GONÇALVES, F. A.; PAZ, M. F. DA; FONSECA, G. G.; LEITE, R. S. R. Production of β-glucosidase on solid-state fermentation by *Lichtheimia ramosa* in agroindustrial residues: Characterization and catalytic properties of the enzymatic extract. **Electronic Journal of Biotechnology**, v. 18, n. 4, p. 314–319, 2015.

GIBSON, G. R.; ROBERFROID, M. B. Dietary modulation of the human colonic microbiota: Introducing the concept of prebiotics. **The Journal of Nutrition**, v. 125, n. 6, p. 1401–1412, 1995.

GÍRIO, F. M.; FONSECA, C.; CARVALHEIRO, F.; DUARTE, L. C.; MARQUES, S. Hemicelluloses for fuel ethanol : A review. **Bioresource Technology**, v. 101, n. 13, p. 4775–4800, 2010.

GUILLÉN, D.; SÁNCHEZ, S. Carbohydrate-binding domains : Multiplicity of biological roles. A**pplied Microbiology and Biotechnology,** p. 1241–1249, 2010.

HAMANN, P. R. V.; SERPA, D. L.; BARRETO DA CUNHA, A. S.; CAMARGO, B. R. DE; OSIRO, K. O.; VALLE DE SOUSA, M.; FELIX, C. R.; MILLER, R. N. G.; NORONHA, E. F. Evaluation of plant cell wall degrading enzyme production by *Clostridium thermocellum* B8 in the presence of raw agricultural wastes. **International Biodeterioration & Biodegradation**, v. 105, p. 97–105, 2015.

HAMMEL, K. E.; CULLEN, D. Role of fungal peroxidases in biological ligninolysis. **Current Opinion in Plant Biology**, v. 11, n. 3, p. 349–355, 2008.

HASUNUMA, T.; KAWASHIMA, K.; NAKAYAMA, H.; MURAKAMI, T.; KANAGAWA, H.; ISHII, T.; AKIYAMA, K.; YASUDA, K.; TERADA, F.; KUSHIBIKI, S. Effect of cellooligosaccharide or synbiotic feeding on growth performance, fecal condition and

hormone concentrations in *Holstein calves*. **Animal Science Journal**, v. 82, n. 4, p. 543–548, 2011.

HAZLEWOOD, G. P.; GILBERT, H. J. Structure and function analysis of *Pseudomonas* plant cell wall hydrolases, **Biochemical Society Transactions,** p. 185–190, 1998.

HECK, J. X.; HERTZ, P. F.; AYUB, M. A Z. Cellulase and xylanase production by isolated amazon *Bacillus strains* using soybean industrial residue based solid-state cultivation. **Brazilian Journal of Microbiology**, v. 33, n. 3, p. 213–218, 2002.

HENRISSAT, B.; VEGETALES, M.; GRENOBLE, F.-. A classification of glycosyl hydrolases based sequence similarities amino acid. **Biochemical Journal**, v. 280, p. 309–316, 1991.

HURTUBISE, Y.; SHARECK, F.; KLUEPFEL, D.; MOROSOLI, R. A cellulase / xylanase-negative mutant of *Streptomyces fividans* 1326 defective in cellobiose and xylobiose uptake is mutated in a gene encoding a protein homologous to ATP-binding proteins. **Molecular Microbiology**, v. 17, n. 2, p. 367–377, 1995.

HIMMEL, M. E.; XU, Q.; LUO, Y.; DING, S.-Y.; LAMED, R.; BAYER, E. A. Microbial enzyme systems for biomass conversion: Emerging paradigms. **Biofuels**, v. 1, n. 2, p. 323–341, 2010.

HORN, S. J.; VAAJE-KOLSTAD, G.; WESTERENG, B.; EIJSINK, V. G. Novel enzymes for the degradation of cellulose. **Biotechnology for Biofuels**, v. 5, n. 1, p. 45, 2012.

HOWARD, R. L.; ABOTSI, E.; JANSEN VAN RENSBURG, E. L.; HOWARD, S. Lignocellulose biotechnology: Issues of bioconversion and enzyme production. **African Journal of Biotechnology**, v. 2, n. 12, p. 602–619, 2003.

JANG, H. D.; CHEN, K. S. Production and characterization of thermostable cellulases from *Streptomyces* transformant T3-1. **World Journal of Microbiology and Biotechnology**, v. 19, n. 3, p. 263–268, 2003.

JEGANNATHAN, K. R.; NIELSEN, P. H. Environmental assessment of enzyme use in industrial production-A literature review. **Journal of Cleaner Production**, v. 42, p. 228–240, 2013.

JIANG, Y.; WU, Y.; LI, H. Immobilization of *Thermomyces lanuginosus* xylanase on aluminum hydroxide particles through adsorption: characterization of immobilized enzyme. **Journal of Microbiology and Biotechnology,** v. 25, n. 2015, p. 2016–2023, 2016.

JOHN, T.; ALFTRE, J. Immobilization of cellulase mixtures on magnetic particles for hydrolysis of lignocellulose and ease of recycling. **Biomass and Bioenergy,** v. 5, p. 4–10,

2014.

KAUR, A.; MAHAJAN, R.; SINGH, A.; GARG, G.; SHARMA, J. Application of cellulase-free xylano-pectinolytic enzymes from the same bacterial isolate in biobleaching of kraft pulp. **Bioresource Technology**, v. 101, n. 23, p. 9150–9155, 2010.

KIDDINAMOORTHY, J.; ANCENO, A. J.; HAKI, G. D.; RAKSHIT, S. K. Production, purification and characterization of *Bacillus* sp. GRE7 xylanase and its application in eucalyptus Kraft pulp biobleaching. **World Journal of Microbiology and Biotechnology**, v. 24, n. 5, p. 605–612, 2008.

KITTUR, F. S.; MANGALA, S. L.; ABU, A.; KITAOKA, M.; TSUJIBO, H.; HAYASHI, K. Fusion of family 2b carbohydrate-binding module increases the catalytic activity of a xylanase from *Thermotoga maritima* to soluble xylan. **FEBS Journal**, v. 549, p. 147–151, 2003.

KQCZKOWSKI, J. Structure , function and metabolism of plant cell wall and their functions. **Acta Physiologiae Plantarum**, v. 25, n. 3, p. 287–305, 2003.

KRICKA, W.; FITZPATRICK, J.; BOND, U. Metabolic engineering of yeasts by heterologous enzyme production for degradation of cellulose and hemicellulose from biomass: a perspective. **Frontiers in Microbiology**, v. 5, p. 1–11, 2014.

KUHAD, R. C.; GUPTA, R.; SINGH, A. Microbial cellulases and their industrial applications. **Enzyme Research**, v. 2011, p. 1–10, 2011.

KUMAR, R.; SINGH, S.; SINGH, O. V. Bioconversion of lignocellulosic biomass: Biochemical and molecular perspectives. **Journal of Industrial Microbiology & Biotechnology**, v. 35, n. 5, p. 377–91, 2008.

KUMAR, V.; SATYANARAYANA, T. Thermo-alkali-stable xylanase of a novel polyextremophilic *Bacillus halodurans* TSEV1 and its application in biobleaching. **International Biodeterioration & Biodegradation**, v. 75, p. 138–145, 2012.

KUMAR, V.; SATYANARAYANA, T. Production of thermo-alkali-stable xylanase by a novel polyextremophilic *Bacillus halodurans* TSEV1 in cane molasses medium and its applicability in making whole wheat bread. **Bioprocess and Biosystems Engineering**, v. 37, n. 6, p. 1043–1053, 2014.

LADEIRA, S. A.; CRUZ, E.; DELATORRE, A. B.; BARBOSA, J. B.; MARTINS, M. L. L. Cellulase production by thermophilic *Bacillus* sp. SMIA-2 and its detergent compatibility. **Electronic Journal of Biotechnology**, v. 18, n. 2, p. 110–115, 2015.

LATHA, S.; SIVARANJANI, G.; DHANASEKARAN, D. Response surface methodology : A non- conventional statistical tool to maximize the throughput of *Streptomyces* species

biomass and their bioactive metabolites. **Critical Reviews in Microbiology**, v. 7828, 2017.

LEE, C. C.; SMITH, M.; KIBBLEWHITE-ACCINELLI, R. E.; WILLIAMS, T. G.; WAGSCHAL, K.; ROBERTSON, G. H.; WONG, D. W. S. Isolation and characterization of a cold-active xylanase enzyme from *Flavobacterium* sp. **Current Microbiology**, v. 52, n. 2, p. 112–116, 2006.

LEE, H. V; HAMID, S. B. A.; ZAIN, S. K. Conversion of lignocellulosic biomass to nanocellulose: structure and chemical process. **The Scientific World Journal**, v. 2014, p. 1–22, 2014.

LEE, Y.; CHOI, D.; KENDE, H. Expansins: Ever-expanding numbers and functions. **Current Opinion in Plant Biology**, v. 4, n. 6, p. 527–532, 2001.

LEVASSEUR, A.; DRULA, E.; LOMBARD, V.; COUTINHO, P. M.; HENRISSAT, B. Expansion of the enzymatic repertoire of the CAZy database to integrate auxiliary redox enzymes. **Biotechnology for Biofuels**, v. 6, n. 1, p. 41, 2013.

LYND, L.; WEIMER, P.; WH, VAN Z.; IS., P. Microbial cellulose utilization: fundamentals and biotechnology. **Microbiology and Molecular Biology Reviews**, v. 66, n. 3, p. 506–577, 2002.

MACRELLI, S.; GALBE, M.; WALLBERG, O. Effects of production and market factors on ethanol profitability for an integrated first and second generation ethanol plant using the whole sugarcane as feedstock. **Biotechnology for Biofuels**, v. 7, n. 1, p. 26, 2014.

MAKI, M.; LEUNG, K. T.; QIN, W. The prospects of cellulase-producing bacteria for the bioconversion of lignocellulosic biomass. **International Journal of Biological Sciences**, v. 5, n. 5, p. 500–516, 2009.

MANDER, P.; CHOI, Y. H.; G.C., P.; CHOI, Y. S.; HONG, J. H.; CHO, S. S.; YOO, J. C. Biochemical characterization of xylanase produced from *Streptomyces* sp. CS624 using an agro residue substrate. **Process Biochemistry**, v. 49, n. 3, p. 451–456, 2014.

MATEO, C.; PALOMO, J. M.; FERNANDEZ-LORENTE, G.; GUISAN, J. M.; FERNANDEZ-LAFUENTE, R. Improvement of enzyme activity, stability and selectivity via immobilization techniques. **Enzyme and Microbial Technology**, v. 40, n. 6, p. 1451–1463, 2007.

MINUSSI, R. C.; PASTORE, G. M.; DURÁN, N. Potential applications of laccase in the food industry. **Trends in Food Science & Technology**, v. 13, n. 6–7, p. 205–216, 2002.

MUNK, L.; SITARZ, A. K.; KALYANI, D. C.; MIKKELSEN, J. D.; MEYER, A. S. Can laccases catalyze bond cleavage in lignin? **Biotechnology Advances**, v. 33, n. 1, p. 13–24,

2015.

MUSSATTO, S. I.; FERNANDES, M.; MANCILHA, I. M.; ROBERTO, I. C. Effects of medium supplementation and pH control on lactic acid production from brewer's spent grain. **Biochemical Engineering Journal**, v. 40, n. 3, p. 437–444, 2008.

OBENG, E. M.; NURUL, S.; ADAM, N.; BUDIMAN, C.; ONGKUDON, C. M.; MAAS, R.; JOSE, J. Lignocellulases : a review of emerging and developing enzymes , systems , and practices. **Bioresources and Bioprocessing**, 2017.

OGEDA, T. L.; PETRI, D. F. S. Hidrólise enzimática de biomassa. **Química Nova**, v. 33, n. 7, p. 1549–1558, 2010.

OTSUKA, M.; ISHIDA, A.; NAKAYAMA, Y.; SAITO, M.; YAMAZAKI, M.; MURAKAMI, H.; NAKAMURA, Y.; MATSUMOTO, M.; MAMOTO, K.; TAKADA, R. Dietary supplementation with cellooligosaccharide improves growth performance in weanling pigs. **Animal Science Journal**, v. 75, p. 225–229, 2004.

PANDEY, A.; SOCCOL, C. R.; NIGAM, P.; SOCCOL, V. T. Biotechnological potential of agro-industrial residues. I: Sugarcane bagasse. **Bioresource Technology**, v. 74, n. 1, p. 69–80, 2000.

PAULY, M.; KEEGSTRA, K. Cell-wall carbohydrates and their modification as a resource for biofuels. **The Plant Journal**, v. 54, n. 4, p. 559–568, 2008.

PENG, F.; REN, J. L.; XU, F.; SUN, R. Chemicals from hemicellulose: A review. **Sustainable Production of Fuels, Chemicals, and Brans from Forest Biomass**, p. 219–259, 2011.

PERCIVAL ZHANG, Y. H.; HIMMEL, M. E.; MIELENZ, J. R. Outlook for cellulase improvement: Screening and selection strategies. **Biotechnology Advances**, v. 24, n. 5, p. 452–481, 2006.

PÉREZ, J.; MUÑOZ-DORADO, J.; LA RUBIA, T. DE; MARTÍNEZ, J. Biodegradation and biological treatments of cellulose, hemicellulose and lignin: an overview. **International Microbiology**, v. 5, n. 2, p. 53–63, 2002.

PETRUCCIOLI, M.; RAVIV, M.; SILVESTRO, R. DI; DINELLI, G. **Agriculture and Agro-Industrial Wastes, Byproducts, and Wastewaters**. Second Edi ed. [s.l.] Elsevier, v. 1, 2011

PINHEIRO, M.; OLIVEIRA, M. The effect of different sweeteners in low calorie yogurts - a review. **International Journal of Dairy Technology**, v. 58, n. 4, p. 193–199, 2005.

POLIZELI, M. L. T. M.; RIZZATTI, A. C. S.; MONTI, R.; TERENZI, H. F.; JORGE, J. A.; AMORIM, D. S. Xylanases from fungi: Properties and industrial applications. **Applied**

Microbiology and Biotechnology, v. 67, n. 5, p. 577–591, 2005.

PRADEEP, G. C.; CHOI, Y. H.; CHOI, Y. S.; SEONG, C. N.; CHO, S. S.; LEE, H. J.; YOO, J. C. A novel thermostable cellulase free xylanase stable in broad range of pH from *Streptomyces* sp. CS428. **Process Biochemistry**, v. 48, n. 8, p. 1188–1196, 2013.

RASTOGI, G.; BHALLA, A.; ADHIKARI, A.; BISCHOFF, K. M.; HUGHES, S. R.; CHRISTOPHER, L. P.; SANI, R. K. Characterization of thermostable cellulases produced by *Bacillus* and *Geobacillus* strains. **Bioresource Technology**, v. 101, n. 22, p. 8798–8806, 2010.

RAVINDER, T.; SWAMY, M. V.; SEENAYYA, G.; REDDY, G. *Clostridium lentocellum* SG6 - A potential organism for fermentation of cellulose to acetic acid. **Bioresource Technology**, v. 80, p. 171–177, 2001.

RODRIGUES, R. C.; ORTIZ, C.; BERENGUER-MURCIA, Á.; TORRES, R.; FERNÁNDEZ-LAFUENTE, R. Modifying enzyme activity and selectivity by immobilization. **Chemical Society Reviews**, v. 42, n. 15, p. 6290–6307, 2013.

RUBIN, E. M. Genomics of cellulosic biofuels. **Nature**, v. 454, n. 7206, p. 841–845, 2008.

SADHU, S.; MAITI, T. K. Cellulase production by bacteria : A review. **British Microbiology Research Journal**, v. 3, n. 3, p. 235–258, 2013.

SAMANTA, A. K.; JAYAPAL, N.; JAYARAM, C.; ROY, S.; KOLTE, A. P.; SENANI, S.; SRIDHAR, M. Xylooligosaccharides as prebiotics from agricultural by-products: Production and applications. **Bioactive Carbohydrates and Dietary Fibre**, v. 5, n. 1, p. 62–71, 2015.

SAMARATUNGA, A.; KUDINA, O.; NAHAR, N.; PRYOR, S. W. Impact of enzyme loading on the efficacy and recovery of cellulolytic enzymes immobilized on enzymogel nanoparticles. A**pplied Microbiology and Biotechnology,** p. 2872–2882, 2015.

SÁNCHEZ, C. Lignocellulosic residues: Biodegradation and bioconversion by fungi. **Biotechnology Advances**, v. 27, n. 2, p. 185–194, 2009.

SÁNCHEZ, Ó. J.; CARDONA, C. A. Trends in biotechnological production of fuel ethanol from different feedstocks. **Bioresource Technology**, v. 99, n. 13, p. 5270–5295, 2008.

SANTOS, C. R.; HOFFMAM, Z. B.; MATOS MARTINS, V. P. DE; ZANPHORLIN, L. M.; PAULA ASSIS, L. H. DE; HONORATO, R. V.; OLIVEIRA, P. S. L. DE; RULLER, R.; MURAKAMI, M. T. Molecular mechanisms associated with xylan degradation by *Xanthomonas* plant pathogens. **Journal of Biological Chemistry**, v. 289, n. 46, p. 32186–32200, 2014.

SANTOS CASTRO, L. DOS; PEDERSOLI, W. R.; ANTONIÊTO, A. C. C.; STEINDORFF,

A. S.; SILVA-ROCHA, R.; MARTINEZ-ROSSI, N. M.; ROSSI, A.; BROWN, N. A.; GOLDMAN, G. H.; FAÇA, V. M.; PERSINOTI, G. F.; SILVA, R. N. Comparative metabolism of cellulose, sophorose and glucose in *Trichoderma reesei* using high-throughput genomic and proteomic analyses. **Biotechnology for Biofuels**, v. 7, n. 1, p. 41, 2014.

SHABEB, M. S. A.; YOUNIS, M. A. M.; HEZAYEN, F. F.; NOUR-ELDEIN, M. A. Production of cellulase in low-cost by *Bacillus subtilis* KO strain**. World Applied Sciences Journal**, 2010.

SHELDON, R. A. Cross-linked enzyme aggregates (CLEAs): Stable and recyclable biocatalysts. **Biochemical Society Transactions**, v. 35, n. 6, p. 1583–1587, 2007.

SILVA, T. M. DA; PESSELA, B. C.; SILVA, J. C. R. DA; LIMA, M. S.; JORGE, J. A.; GUISAN, J. M.; POLIZELI, M. D. L. T. M. Immobilization and high stability of an extracellular β-glucosidase from *Aspergillus japonicus* by ionic interactions. **Journal of Molecular Catalysis B: Enzymatic**, v. 104, p. 95–100, 2014.

SINGH, R. K.; ZHANG, Y.-W.; NGUYEN, N.-P.-T.; JEYA, M.; LEE, J.-K. Covalent immobilization of β-1,4-glucosidase from *Agaricus arvensis* onto functionalized silicon oxide nanoparticles. **Applied Microbiology and Biotechnology**, v. 89, n. 2, p. 337–344, 2011.

SOOZANIPOUR, A.; TAHERI-KAFRANI, A.; LANDARANI, A. Covalent attachment of xylanase on functionalized magnetic nanoparticles and determination of its activity and stability. **Chemical Engineering Journal**, v. 270, p. 235–243, 2015.

SOUSA, V. M. C. DE; SANTOS, E. F. DOS; SGARBIERI, V. C. The importance of prebiotics in functional foods and clinical practice. **Food and Nutrition Sciences**, v. 2, n. 2, p. 133–144, 2011.

SPAHN, C.; MINTEER, S. D. Enzyme immobilization in biotechnology. **Recent Patents on Engineering**, v. 2, n. 3, p. 195–200, 2008.

SUKUMARAN, R. K.; SINGHANIA, R. R.; PANDEY, A. Microbial cellulases - Production, applications and challenges. **Journal of Scientific and Industrial Research**, v. 64, n. 11, p. 832–844, 2005.

SUTARLIE, L.; YANG, K. Hybrid cellulase aggregate with a silica core for hydrolysis of cellulose and biomass. **Journal of Colloid and Interface Science**, v. 411, p. 76–81, 2013.

SWEENEY, M. D.; XU, F. Biomass converting enzymes as industrial biocatalysts for fuels and chemicals: recent developments. **Catalysts**, v. 2, n. 2, p. 244–263, 2012.

TAKASUKA, T. E.; BOOK, A. J.; LEWIN, G. R.; CURRIE, C. R.; FOX, B. G. Aerobic

deconstruction of cellulosic biomass by an insect-associated *Streptomyces*. **Scientific reports**, v. 3, p. 1030, 2013.

TRAMONTINA, R.; FRANCO CAIRO, J. P. L.; LIBERATO, M. V.; MANDELLI, F.; SOUSA, A.; SANTOS, S.; RABELO, S. C.; CAMPOS, B.; IENCZAK, J.; RULLER, R.; DAMÁSIO, A. R. L.; SQUINA, F. M. The *Coptotermes gestroi* aldo–keto reductase: a multipurpose enzyme for biorefinery applications. **Biotechnology for Biofuels**, v. 10, n. 1, p. 4, 2017.

VIEIRA, M. F.; VIEIRA, A. M. S.; ZANIN, G. M.; TARDIOLI, P. W.; MATEO, C.; GUISÁN, J. M. β-Glucosidase immobilized and stabilized on agarose matrix functionalized with distinct reactive groups. **Journal of Molecular Catalysis B: Enzymatic**, v. 69, n. 1–2, p. 47–53, 2011.

VOLYNETS, B.; EIN-MOZAFFARI, F.; DAHMAN, Y. Biomass processing into ethanol : pretreatment , enzymatic hydrolysis , fermentation , rheology , and mixing. p. 1–22, 2017.

VRIES, R. P. DE; VISSER, J.; RONALD, P.; VRIES, R., DE; P. *Aspergillus* enzymes involved in degradation of plant cell wall polysaccharides. **Microbiology and Molecular Biology Reviews**, v. 65, n. 4, p. 497–522, 2001.

WONG, K. K. Y.; SADDLER, J. N. *Trichoderma* xyIanases, their properties and application . **Critical Reviews in Biotechonology**, v. 12, n. 5/6, p. 413–435, 1992.

XIE, S.; SYRENNE, R.; SUN, S.; YUAN, J. S. Exploration of Natural Biomass Utilization Systems (NBUS) for advanced biofuel-from systems biology to synthetic design. **Current Opinion in Biotechnology**, v. 27, p. 195–203, 2014.

YIN, Y.; MAO, X.; YANG, J.; CHEN, X.; MAO, F.; XU, Y. dbCAN: a web resource for automated carbohydrate-active enzyme annotation. **Nucleic Acids Research**, v. 40, p. W445-51, 2012.

ZÁVODSZKY, P.; KARDOS, J.; SVINGOR; PETSKO, G. A. Adjustment of conformational flexibility is a key event in the thermal adaptation of proteins. **Proceedings of the National Academy of Sciences of the United States of America**, v. 95, n. 13, p. 7406–7411, 1998.

ZHANG, Y.; CHEN, S.; XU, M.; CAVOCO-PAULO, A. P.; WU, J.; CHEN, J. Characterization of *Thermobifida fusca* cutinase-carbohydrate-binding module fusion proteins and their potential application in bioscouring. **Applied and Environmental Microbiology**, v. 76, n. 20, p. 6870–6876, 2010.

CAPÍTULO 2

Unraveling the cellulolytic and hemicellulolytic potential of two novel *Streptomyces* strains

Ricardo Rodrigues de Melo, Geizecler Tomazetto, Gabriela Felix Persinoti, Hélia Harumi Sato, Roberto Ruller, Fabio Marcio Squina

Submetido para publicação na International Journal of Genomics

RESUMO

*Streptomyce*s spp. são notórios decompositores da biomassa vegetal em ambientes de solo, mas apenas poucas linhagens foram bioquimicamente e geneticamente caracterizadas. Para acelerar a descoberta de enzimas, uma seleção funcional de oitenta linhagens de *Streptomyces* foi realizada, resultando em duas linhagens (F1 e F7) com elevadas atividades celulolíticas e xilanolíticas que foram selecionadas para sequenciamento genômico e análises bioquímicas. Após o cultivo em meios à base de bagaço de cana-de-açúcar, as linhagens F1 e F7 exibiram atividades enzimáticas contra arabinana, arabinoxilano de centeio, beta-glucano, amido, CMC, xilano e quitina. Além destas atividades, a linhagem F7 foi capaz de degradar pectina liquenana e manana. A análise genômica de ambas as linhagens revelou uma diversidade de enzimas ativas em carboidratos. Os genomas de F1 e F7 codificam 33 e 44 tipos diferentes famílias de hidrolases glicosídicas, respectivamente. Além disso, a análise genômica também revelou genes relacionados à degradação de compostos aromáticos derivados da lignina. Coletivamente, o estudo revelou duas novas linhagens de *Streptomyce*s e informações sobre a capacidade de degradação de bactérias lignocelulolíticas, das quais podem surgir várias tecnologias, como a conversão de biomassa vegetal em bioprodutos.

Palavras-chave: *Streptomyces*, bagaço de cana-de-açúcar, genômica, ensaios enzimáticos, bioconversão.

ABSTRACT

The *Streptomyces* spp. are notorious plant biomass decomposers in soil environments, but only few strains were biochemically and genetically characterized. To accelerate enzyme discovery, functional screening of eighty *Streptomyces* strains was performed, resulting on two strains (F1 and F7) of superior cellulolytic and xylanolytic activities that were selected for biochemical and genomic sequencing. After cultivation on sugarcane bagasse based media, F1 and F7 exhibited enzymatic activity against arabinan, rye arabinoxylan, β-glucan, starch, CMC, xylan and chitin. Furthermore, strain F7 was able to degrade pectin, lichenan and mannan. The genomic analysis of both strains revealed a diversity of carbohydrates-active enzymes. The F1 and F7 genomes encode 33 and 44 different types of glycosyl hydrolases families, respectively. Moreover, the genomic analysis also revealed genes related to degradation of lignin-derived aromatic compounds. Collectively, the study revealed two novel *Streptomyces* strains and further insights on the degradation capability of lignocellulolytic bacteria, from which a number of technologies can arise, such as plant biomass conversion into bioproducts.

Keywords: *Streptomyces*, sugarcane bagasse, genomics, enzymatic assays, bioconversion.

1. INTRODUCTION

The depletion of fossil fuels and environmental concerns have driven global politics towards the development of renewable energy resources. Lignocellulosic biomass represents the largest reservoir for renewable energy and value-added chemical production [1,2,3]. The term lignocellulosic biomass refers to nonfood materials available from plants, including rice straw, cotton straw, corn stover, sugarcane bagasse, wood, grass, and others [1,3,4].

The transformation of the lignocellulosic biomass into fermentable sugars and biofuel production is a challenging process. Generally, lignocellulosic biomasses are composed variable contents of cellulose, hemicellulose and lignin. These polymers are highly organized and interlinked among themselves into recalcitrant structure [1,2]. However, in nature ecosystems, lignocellulosic biomass is constantly recycled by microorganisms, which efficiently degrade lignocellulosic material [2,5,6]. The biomass-degrading microorganisms are found in various environments, including hot spring pool [7,8], cow rumen [9], biogas reactor [10,11], and soil [12,13,14]. These environments represent resource for isolation of novel microorganisms and enzymes involved on plant biomass conversion. For instance, *Streptomyces* have been reported to play an important role in the carbon cycle and plant biomass deconstruction in soils [15,16,17,18]. Indeed, *Streptomyces* species genomes have revealed several Carbohydrates-Active Enzymes (CAZy), including cellulases, hemicellulases, lytic polysaccharide monooxygenases [18,19,20]. Moreover, a recent study described three *Streptomyces* strains (pl6, pl88 and pr55) isolated from soil capable to metabolize polysaccharides and poplar lignin [17].

Although several *Streptomyces* strains have been described as plant biomass decomposer, the molecular and biochemical understanding on how these microorganisms degrade lignocellulosic biomass remain rather fragmented and restricted to a few strains. In this sense, eighty *Streptomyces* strains were isolated from soil and their cellulolytic and hemicellulolytic capacity evaluated. The biochemical assays demonstrated enzymatic capability of the strain in the degradation of plant-based polysaccharides carbohydrates. To further investigate the mechanisms of lignocellulosic biomass degradation, the genomes of two strains were sequenced and analyzed regarding their CAZy profiles. Their genomes encode several CAZymes confirming enzymatic profiles. In addition, genomic analysis indicated that both strains may perform lignin degradation.

2. MATERIAL AND METHODS

2.1. Streptomyces strains isolation

Eighty *Streptomyces* spp. strains were isolated from soil samples collected from different places at Brazil (São Paulo, Brazil: 22°49´8.861"S, 47°3´39.085"W; Minas Gerais, Brazil: 19°57'08.1"S, 44°12'55.7"W; Minas Gerais, Brazil: 20°36´29.624"S, 46°2´30.739"W). Briefly, soil samples corresponding to the upper 10 cm were collected and transported to the laboratory into sterile bags. These samples were mixed with calcium carbonate (1:1), ground with a pestle and air-dried [21]. For each sample collected, 4 g of air-dried soil were mixed with 40 mL sterilized water and stirred vigorously. Several dilutions (10^{-1} to 10^{-4}) were spread on isolation medium ISP 4 agar plates [22] and incubated at 30 °C for 3 days. Single colonies were transferred to fresh medium ISP 2 [22] and grown at 30 °C for 6-8 days. The procedure was repeated until obtaining pure cultures. The isolated strains were stored at -80 °C as mixtures of mycelial fragments in 20 % (*v/v*) glycerol or in medium ISP 2.

2.2. Qualitative and quantitative screening

To screen cellulolytic and hemicellulolytic microorganisms among the isolated ones, they were cultivated on Bushnell Haas Broth (BHB, Sigma Aldrich®) mineral salts medium agar plates (g/L: K_2HPO_4 1, KH_2PO_4 1, $CaCl_2 \cdot 2H_2O$ 0.02, NH_4NO_3 1, $FeCl_3$ 0.05, $MgSO_4 \cdot 7H_2O$ 0.2, and agar 15, pH 7.0) supplemented with 0.5 % (*w/v*) of beechwood xylan or carboxymetilcellulose (CMC). After incubation at 30 °C for 48-72 h, agar plates were stained with Congo red and distained with 1 M NaCl [23]. Colonies showing clear halos were selected as indicative for the CMC and xylan degradation. Of the 80 strains screened, 48 shown a visual enzymatic activity (**Table 1**). The 14 best candidates were selected for further analysis described as follows.

The strains were initially grown on medium ISP 2 agar plates at 30 °C for 7 days. From each culture, were inoculated 10^6 spores mL^{-1} into 25 mL of BHB medium and supplemented with beechwood xylan or CMC (0.5 %, *w/v*) and incubated at 30 °C for 6 days in a shaker (New Brunswick Scientific, New Jersey, USA) at 180 rpm. Samples were taken daily to monitor the corresponding enzymatic activity (**Table 2**). All experiments were done in biological triplicates.

2.3. Cultivation and enzymatic production

Based on CMCase and xylanase activity results, the two best candidates, namely F1 and F7, were cultivated on sugarcane bagasse (SB) as sole carbon source for enzyme production. SB was pretreated by steam explosion at 200 °C for 15 min, as described by Rocha et al. [24]. The strains were grown on medium ISP 2 agar plates at 30 °C for 7 days, and then 10^6 spores mL^{-1} were inoculated into 80 mL of BHB medium supplemented with 1.0 % (*w*/*v*) pretreated SB. The flasks were incubated at 30 °C for 10 days in a rotary shaker at 180 rpm. Samples were taken in order to evaluate the set of enzymes produced. All experiments were done in biological triplicates.

2.4. Enzymatic assays

The enzymatic activity (International Units, U) was performed using different substrates. All the polysaccharides were purchased from Sigma-Aldrich or Megazyme [beechwood xylan, rye arabinoxylan, β-glucan (barley), sugar beet arabinan, debranched arabinan, carboxymetilcellulose, tamarind xyloglucan, starch, icelandic moss lichenan, chitin from shrimp shells, arabinogalactan, mannan (ivory nut), and citrus pectin]. The enzymatic reactions were carried out in a miniaturized fashion by mixing 20 µL of crude extract preparation, 50 µL of the distinct substrates (0.5 %, *w/v*), and 30 µL of sodium phosphate buffer (0.1 M) at pH 6. Reactions were incubated at 50 °C in a Thermostat® (Eppendorf, Hamburg, Germany) for 30 min or 18 h, and stopped with the addition of 100 µL of DNS following of immediately by boiled for 5 min at 99 °C [25]. The solutions were analyzed at 540 nm using the Infinite M200®spectrophotometer (Tecan, Switzerland) to measure the release of reducing sugars. One unit (1 U) of enzymatic activity corresponded to the formation of 1 µmol of reducing sugar equivalent per minute under the assay conditions. Total protein was measured using microtiter plates with Bio-Rad protein assay reagent (Bio-Rad Laboratories, USA), employing a procedure based on Bradford's method [26]. Bovine serum albumin was used as standard.

2.5. Identification of Streptomyces strains

The strains F1 and F7 were grown in ISP 2 broth at 30 °C in flasks agitated at 180 rpm for 3 days, and their cells harvested by centrifugation at 10,000 rpm for 10 min. The cell pellets were washed twice with sterile water. Genomic DNA was isolated using FastDNA SPIN Kit for soil (MP Biomedicals, Irvine, CA) according to the manufacturer's instructions. The quality of the total DNA was assessed by electrophoresis in a 0.8 % agarose gel stained with ethidium bromide and visualized using UV transilluminator. The DNA concentration was measured by fluorimetry (Qubit® 2.0 Fluorometer-Life Technologies. Carlsbad, California, EUA) using the BR Qubit® dsDNA Assay (Life Technologies, Carlsbad, California, EUA).

To determine the relatedness of strains with their closest described relative *Streptomyces*, 16S rRNA gene sequences were amplified using polymerase chain reaction (PCR) with primers 27f (5' AGAGTTTGATCMTGGCTCAG 3') and 1492r (5' TACCTTGTTACGACTT 3'). All PCR reactions were performed in 50 µL containing 50 ng of individual genomic DNA, 1 mM dNTP, 10 pmoL each primer, 2 mM $MgCl_2$, and 1 U of Taq DNA polymerase (Thermo Fischer Scientific, Waltham, USA). The PCR conditions consisted as follows: an initial denaturation at 94 °C for 3 min, followed by 30 cycles of 94 °C for 60 s, 55 °C for 60 s, and 72 °C for 2 min and a final extension at 72 °C for 4 min. PCR products were purified using Illustra GFX PCR DNA and Gel Band Purification kit (GE Healthcare Bio-Sciences, Pittsburgh, USA) and sequenced using a DNA ABI PRISM 377 Genetic Analyzer system (Applied Biosystems, USA). BLASTn search analysis revealed that strains F1 and F7 shared more than 97 % 16S rRNA gene sequences identity with members of the genus *Streptomyces*.

2.6. Genome sequencing, assembly and annotation

Genomic DNA of both strains was used for libraries construction using Nextera® DNA Library Preparation Kit (Illumina, San Diego, CA), according to the manufacturer's protocol. The genomic libraries were sequenced on Illumina HiSeq sequencing platform at NGS sequencing facility at CTBE, generating approximately 8 and 6 million 2 x 100 bp reads, respectively.

Raw fastq files were quality checked using FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/), and processed with Trimmomatic version 0.32 [27] to quality trimming, adaptor removal and minimum length filtering. High-quality reads were further analyzed using ProDeGe [28] to remove possible

contaminants and then assembled using Spades version 3.6.2 [29], applying several kmers (k = 21, 27, 33, 55, 77) and careful mode option as parameters.

The drafts genome sequences of strains F1 and F7 were uploaded into the IMG/ER annotation pipeline [30] for automatic prediction of genes using Prodigal [31]. Functional annotation of predicted proteins were obtained using Prokka automatic pipeline [32]. Briefly, it uses hierarchical searches against different databases such as bacterial proteins from RefSeq genomes, UniProt, Pfam and TIGRFAM models. The Aragorn tool [33] was used to predicted tRNA genes whereas rRNA genes were identified using RNAmmer 1.2. Signal peptides and transmembrane helices were predicted by SignalP and TMHMM, respectively. To determine the Carbohydrates-Active Enzymes (CAZYmes) profiles for strains F1 and F7, putative proteins sequences were annotated using dbCAN database version 4 [34] using HMMER3.1b package. Results obtained were manually assessed based on their functional annotation prediction and conserved protein domains to identify the corresponding best matching CAZymes.

2.7. Phylogenetic analysis

The taxonomic assignment of strains F1 and F7 within *Bacteria* domain was determined based on 16S rRNA sequences analysis and multilocus phylogenetic analysis. Phylogenetic tree was reconstructed based on 16S rRNA gene sequences of strains isolated and closest related species with validly published name. All sequences were aligned using the ClustalX tool [35] and manually refined. From those aligned, pairwise distances were calculated using the Maximum Composite Likelihood approach and phylogenetic tree was constructed applying the neighbour-joining method, as implemented in MEGA version 6 [36]. Bootstrap tests with 1,000 replications/iterations were calculated to assess the nodes confidence level.

Multilocus phylogenetic analysis was done as previous described [19]. Briefly, TIGRFAM models were used to search for single copy genes conserved among 172 *Streptomyces* genomes sequences available at RefSeq database, and the genome of *Kitasatospora setae* was considered as an out group. The sequences recovered from each genome were aligned using Mafft v7.299 [37] and the resulting alignment was concatenated using FASconCAT-G v1.02 [38]. The phylogenetic tree was generated using FastTree version 2.1.8 [39] under WAG as substitution model and the final tree was visualized using iTOL version 3 [40].

2.8. Nucleotide sequence accession number

The draft genome sequences of strains F1 and F7 are available at the NCBI with the accession numbers FKJI03000000 and FKJH01000000, respectively.

3. RESULTS AND DISCUSSION

3.1. Strain screening and enzymatic activity evaluation

Of the 80 strains isolated from soil samples in study, 48 exhibited degradation halo in CMC or xylan-agar plates (**Table 1**). Among them, the 14 best candidates were selected for further analysis using submerged fermentation.

The cellulolytic and hemicellulolytic activity of 14 bacterial isolates cultivated on liquid media containing cellulose and xylan as carbon source was studied (**Table 2**). All supernatant showed CMCase activity varying between 0.18 to 0.40 U mL^{-1} and xylanase 1.97 to 10.78 U mL^{-1} using xylan as sole carbon source, and CMCase activity between 0.10 to 0.17 U mL^{-1} and xylanase 0.12 to 0.28 U mL^{-1} using CMC as carbon source. Considering the enzymatic activities, two strains (F1 and F7) exhibited superior cellulolytic and xylanolytic activities and they were selected for enzymatic studies and genomic analysis.

The enzymatic assays using culture supernatants revealed that strains F1 and F7 after grown on SB were able to secrete enzymes involved in the hydrolysis of arabinan, rye arabinoxylan, β-glucan, starch, CMC, xylan and chitin (**Fig. 1**). The strain F7 was also able to degrade pectin, lichenan and mannan. Moreover, the strain F7 supernatant was more efficient in the degradation of plant-based polysaccharides than strain F1. No activity against arabinan sugar beet, xyloglucan and arabinogalactan was detected for both strains.

Our findings corroborated with previous studies and confirmed the lignocellulolytic potential of *Streptomyces* spp. [15,16,19,20]. In a recently study using comparative genomic, transcriptomic and biochemical analysis [18], 29 *Streptomyces* strains have shown a relatively high rate of cellulose degrading activity. Pinheiro et al. [20] reported that *Streptomyces* spp. are able to secrete a set of enzymes involved in degradation of different natural carbohydrates, including cellulose, xylan, mannan, starch, chitin, pectin, and β-glucan.

Table 1. Growth of soil strains in agar plates supplemented with carboxymetilcellulose or beechwood xylan.

Source	*Strain*	*beechwood xylan*	*carboxymetilcellulose*
		E.A.I. (cm)	*E.A.I. (cm)*
Soil collected from Minas Gerais, Brazil: (20°36´29.624"S, 46°2´30.739"W)	A1	2.00	2.30
	A2	2.75	1.64
	A3	2.00	2.00
	A4	N.D.	N.D.
	A11	2.00	3.67
	A13	2.00	1.70
	A14	N.D.	N.D.
	A19	2.00	1.77
	A24	**5.00**	**3.67**
	A25	**3.50**	**2.00**
	A26	**3.60**	**1.47**
	A27	2.22	1.47
	A29	2.53	1.67
	A31	**3.00**	**1.69**
	A33	2.67	1.70
	A34	N.D.	N.D.
	A35	N.D.	N.D.
	A36	2.00	1.82
	A37	N.D.	N.D.
	A38	N.D.	N.D.
	A39	N.D.	N.D.
	A42	N.D.	N.D.
	A44	2.33	1.50
	A45	1.75	1.87
Soil collected from São Paulo, Brazil: (22°49´8.861"S, 47°3´39.085"W)	B5	2.22	1.56
	B6	N.D.	N.D.
	B9	N.D.	N.D.
	B14	**3.00**	**2.20**
	B15	2.00	1.67
	C2	N.D.	N.D.
	C3	1.75	1.53
	C4	2.33	1.53
	C6	3.00	1.54
	C8	2.67	2.33
	C9	N.D.	N.D.
	C10	2.80	1.53
	C11	**3.00**	**2.25**
	C12	**5.33**	**1.83**
	E1	2.50	1.56
	E3	2.10	1.63
Soil collected from São Paulo, Brazil: (22°49´8.861"S, 47°3´39.085"W)	**E5**	**2.69**	**2.67**
	E8	2.50	1.77
	F1	**3.70**	**2.67**
	F2	2.67	1.53
	F3	2.18	1.32
	F4	2.82	1.80
	F6	**3.80**	**1.67**
	F7	**3.30**	**2.38**
	F8	**3.10**	**2.20**
	F9	**3.70**	**1.69**
	F10	2.70	1.67
	F11	**3.00**	**2.30**
	F12	2.89	2.33
Soil collected from Minas Gerais, Brazil: (19°57'08.1"S, 44°12'55.7"W)	G1	N.D.	N.D.
	G2	N.D.	N.D.
	G3	N.D.	N.D.
	G4	2.54	2.71
	G5	3.00	2.00
	G6	N.D.	N.D.
	G7	2.30	2.00
	G8	3.50	1.80
	G9	2.50	1.56
	G10	2.50	1.67
	G11	N.D.	N.D.
	G12	N.D.	N.D.
	G13	N.D.	N.D.
	G14	N.D.	N.D.
	G15	N.D.	N.D.
	G16	N.D.	N.D.
	G17	N.D.	N.D.
	G18	N.D.	N.D.
	G19	N.D.	N.D.
	G20	N.D.	N.D.
	G21	N.D.	N.D.
	G22	N.D.	N.D.
	G23	N.D.	N.D.
	G24	N.D.	N.D.
	G25	N.D.	N.D.
	G26	N.D.	N.D.
	G27	2.80	1.82

E.A.I. - Enzymatic activity index (ratio between the halo diameter and the colony diameter).

N.D. – none detected. The strains of higher enzymatic activity are highlighted in bold.

Table 2. Quantitative assay of the selected isolates using submerged fermentation system.

Strain		***Carbon source (beechwood xylan)***		***Carbon source (carboxymetilcellulose)***	
		Xylanase	*Cellulase*	*Xylanase*	*Cellulase*
A24	Enzymatic activity (U mL^{-1}) *	3.68 ± 0.77 ^{120h}	0.27 ± 0.04 ^{48h}	0.24 ± 0.02 ^{144h}	0.12 ± 0.01 ^{120h}
	Total protein (µg mL^{-1})	22.50 ± 0.90	25.20 ± 0.80	13.50 ± 1.10	16.20 ± 2.80
	Specific activity (U mg^{-1})	163.58	10.71	18.20	7.22
A25	Enzymatic activity (U mL^{-1}) *	3.17 ± 0.15 ^{120h}	0.35 ± 0.01 ^{24h}	0.13 ± 0.01 ^{120h}	0.11 ± 0.01 ^{120h}
	Total protein (µg mL^{-1})	34.00 ± 0.10	12.40 ± 3.60	11.80 ± 3.90	11.80 ± 3.90
	Specific activity (U mg^{-1})	93.19	28.39	11.15	9.55
A26	Enzymatic activity (U mL^{-1}) *	3.93 ± 0.17 ^{72h}	0.36 ± 0.02 ^{24h}	0.25 ± 0.01 ^{144h}	0.12 ± 0.01 ^{120h}
	Total protein (µg mL^{-1})	18.70 ± 4.40	19.60 ± 0.40	8.10 ± 1.30	8.80 ± 1.20
	Specific activity (U mg^{-1})	210.18	18.24	31.53	14.21
A31	Enzymatic activity (U mL^{-1}) *	4.45 ± 0.64 ^{144h}	0.37 ± 0.01 ^{24h}	0.12 ± 0.01 ^{48h}	0.10 ± 0.01 ^{120h}
	Total protein (µg mL^{-1})	34.50 ± 8.10	15.30 ± 0.30	2.70 ± 0.50	4.50 ± 0.40
	Specific activity (U mg^{-1})	129.18	24.48	44.55	22.88
B14	Enzymatic activity (U mL^{-1}) *	1.97 ± 0.07 ^{96h}	0.35 ± 0.07 ^{24h}	0.18 ± 0.01 ^{120h}	0.11 ± 0.01 ^{120h}
	Total protein (µg mL^{-1})	22.50 ± 0.20	12.50 ± 1.60	5.20 ± 0.90	5.20 ± 0.90
	Specific activity (U mg^{-1})	87.61	28.14	34.54	22.33
C11	Enzymatic activity (U mL^{-1}) *	6.20 ± 0.50 ^{120h}	0.31 ± 0.01 ^{24h}	0.21 ± 0.01 ^{120h}	0.12 ± 0.01 ^{144h}
	Total protein (µg mL^{-1})	24.20 ± 1.20	18.40 ± 0.50	11.80 ± 1.00	12.20 ± 3.30
	Specific activity (U mg^{-1})	256.20	17.15	17.69	9.80
C12	Enzymatic activity (U mL^{-1}) *	2.30 ± 0.10 ^{72h}	0.18 ± 0.01 ^{96h}	0.28 ± 0.02 ^{120h}	0.17 ± 0.06 ^{96h}
	Total protein (µg mL^{-1})	11.20 ± 1.80	14.40 ± 0.80	15.80 ± 1.90	13.30 ± 0.30
	Specific activity (U mg^{-1})	205.53	12.81	18.01	12.80
E5	Enzymatic activity (U mL^{-1}) *	7.30 ± 0.16 ^{120h}	0.24 ± 0.01 ^{24h}	0.21 ± 0.02 ^{120h}	0.1189 ± 0.0602 ^{144h}
	Total protein (µg mL^{-1})	56.00 ± 2.80	11.40 ± 5.30	13.70 ± 2.10	14.80 ± 3.80
	Specific activity (U mg^{-1})	130.39	21.00	15.77	8.03
F1	Enzymatic activity (U mL^{-1}) *	9.58 ± 0.24 ^{72h}	0.24 ± 0.01 ^{24h}	0.14 ± 0.01 ^{120h}	0.12 ± 0.01 ^{144h}
	Total protein (µg mL^{-1})	29.00 ± 0.40	16.30 ± 1.00	12.20 ± 2.40	9.10 ± 1.70
	Specific activity (U mg^{-1})	330.42	14.76	11.70	13.17

Strain		***Carbon source (beechwood xylan)***		***Carbon source (carboxymetilcellulose)***	
		Xylanase	*Cellulase*	*Xylanase*	*Cellulase*
F6	Enzymatic activity (U mL^{-1}) *	7.43 ± 0.86 ^{120h}	0.19 ± 0.01 ^{24h}	0.23 ± 0.04 ^{120h}	0.13 ± 0.01 ^{96h}
	Total protein (µg mL^{-1})	45.40 ± 1.70	14.20 ± 0.80	8.80 ± 0.20	26.90 ± 1.20
	Specific activity (U mg^{-1})	163.74	14.05	25.97	4.81
F7	Enzymatic activity (U mL^{-1}) *	10.78 ± 0.01 ^{96h}	0.34 ± 0.02 ^{24h}	0.16 ± 0.02 ^{120h}	0.14 ± 0.01 ^{120h}
	Total protein (µg mL^{-1})	20.10 ± 0.30	2.30 ± 0.10	6.00 ± 1.40	6.00 ± 1.40
	Specific activity (U mg^{-1})	535.04	149.69	27.7	23.08
F8	Enzymatic activity (U mL^{-1}) *	6.11 ± 0.01 ^{120h}	0.41 ± 0.07 ^{24h}	0.26 ± 0.01 ^{96h}	0.11 ± 0.01 ^{120h}
	Total protein (µg mL^{-1})	82.60 ± 0.10	17.00 ± 2.40	7.30 ± 0.50	4.30 ± 0.20
	Specific activity (U mg^{-1})	73.99	24.00	35.68	25.28
F9	Enzymatic activity (U mL^{-1}) *	5.75 ± 0.68^{120h}	0.28 ± 0.06 ^{24h}	0.19 ± 0.01 ^{120h}	0.11 ± 0.01 ^{120h}
	Total protein (µg mL^{-1})	31.10 ± 0.10	3.00 ± 0.80	6.40 ± 0.70	6.40 ± 0.70
	Specific activity (U mg^{-1})	184.88	95.20	30.75	17.53
F11	Enzymatic activity (U mL^{-1}) *	7.89 ± 1.12 ^{72h}	0.27 ± 0.02 ^{24h}	0.17 ± 0.01 ^{120h}	0.15 ± 0.01 ^{144h}
	Total protein (µg mL^{-1})	39.30 ± 0.90	17.10 ± 2.60	13.10 ± 2.20	8.30 ± 0.20
	Specific activity (U mg^{-1})	200.88	15.81	13.30	17.65

* Time of highest enzymatic activity. The strains of higher enzymatic activity are highlighted in bold.

Results are expressed as the average of triplicate assays ± the standard error of the mean.

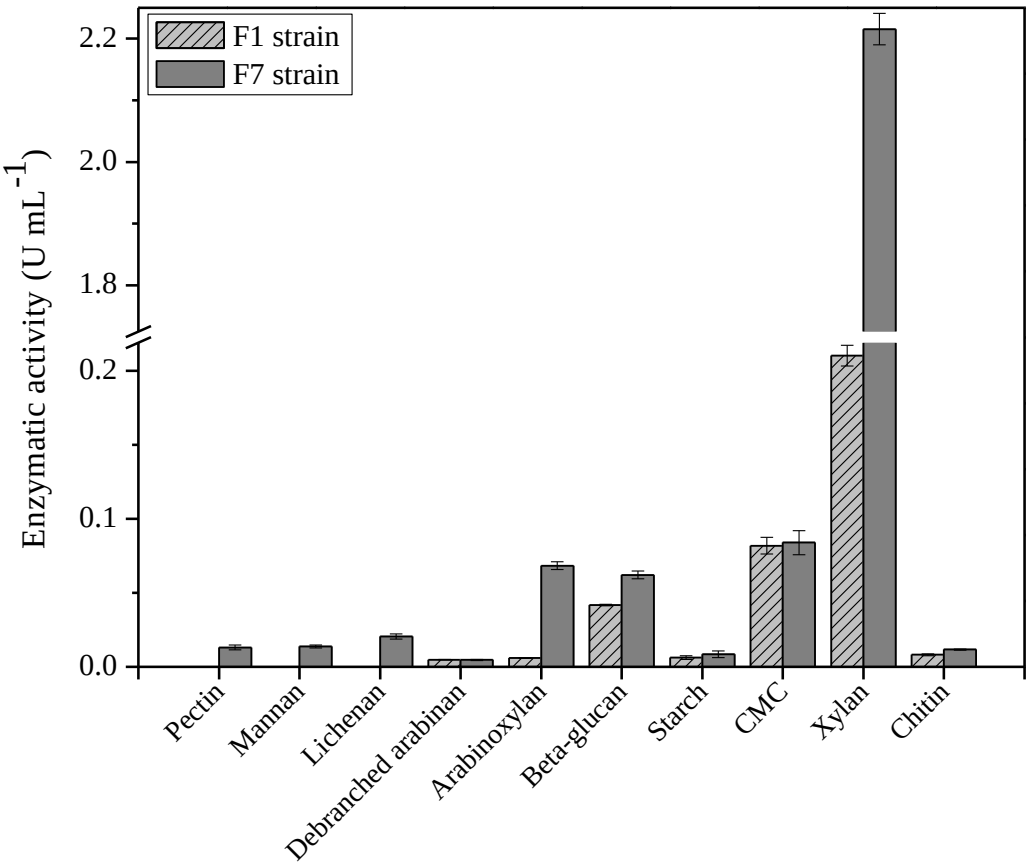


Fig. 1. Enzymatic activities obtained from crude extracts of strains F1 and F7 grown on sugarcane bagasse pretreated with steam explosion (SB). Results are expressed as the average of triplicate assays ± the standard error of the mean. Abbreviations: CMC - Carboxymetilcellulose. The maximum activity obtained for each substrate was used to construct the graph.

3.2. General features and phylogenetic analysis

Based on cellulolytic and hemicellulolytic profiles of strains F1 and F7, we decided to genetically characterize both strains to uncover their metabolic pathways dedicated to biomass degradation. The genomes of both strains were sequenced on the Illumina HiSeq system using the 2x100 pb sequencing strategy. The genomes of strains F1 and F7 resulted in 69 and 66 contigs, respectively, ranging from 10,212 to 760,841 bases (**Table 3**). Strain F1 consists of an 8.1 megabase pair (Mb) chromosome coding 7,262 protein coding sequences (CDS). The strain F7 genome is 7.33 Mb in size and contains 6,463 CDSs. The genomes display a similar GC content of 72 %.

The 16S rRNA gene sequences analyses placed strains F1 and F7 within the genera *Streptomyces*. Pairwise analysis revealed that strain F1 was closely related to *S. misionensis* NRRL B-3230^T sharing 99 % 16S rRNA gene sequence identity. Whereas, strain F7 showed

100 % 16S rRNA gene sequence identity with *S. viridodiastaticus* IFO 13106^{T} (**Fig. 2**). It is important to underline that the percentage of identity obtained from 16S rRNA gene sequences alignment were calculated disregarding a gap (26 and 31 nucleotides) that are present only in strains F1 and F7.

Table 3. Genome statistics for strains F1 and F7.

Attribute	Strain	
	F1	**F7**
Genome size (bp)	8,142,296	7,327,391
DNA coding (bp)	7,100,494	6,492,599
DNA G + C (bp)	5,915,378	5,326,280
GC content (%)	72.65	72.69
DNA contigs	69	66
Total genes	7,355	6,548
Protein coding genes	7,262	6,463
RNA genes	93	86
Genes with function prediction	4093	3478
Genes with Pfam domains	5,526	5,172
Genes with signal peptides	447	439
Genes with transmembrane helices	1,641	1,577
CRISPR repeats	2	2

Previous study reported that 16S rRNA gene sequences analysis is not suitable to distinguish closely related *Streptomyces* species [41] since several *Streptomyces* type strains share completely identical 16S rRNA gene sequences. Therefore, to refine the phylogenetic relationship of the strains isolated within *Streptomyces* genus, a multilocus phylogenetic tree was constructed based on 288 single copy conserved genes found across strains F1, F7 and 172 *Streptomyces* genomes available at RefSeq database (**Fig. 3**). Indeed, multilocus analysis indicated a phylogenetic relationship different from the analysis based on 16S rRNA gene sequences. While, strain F1 forms a monophyletic clade with *S. griseofuscus* NRRL B 5429^{T}, which is a type species of this genus, strain F7 is closely related to species with currently not validly published names.

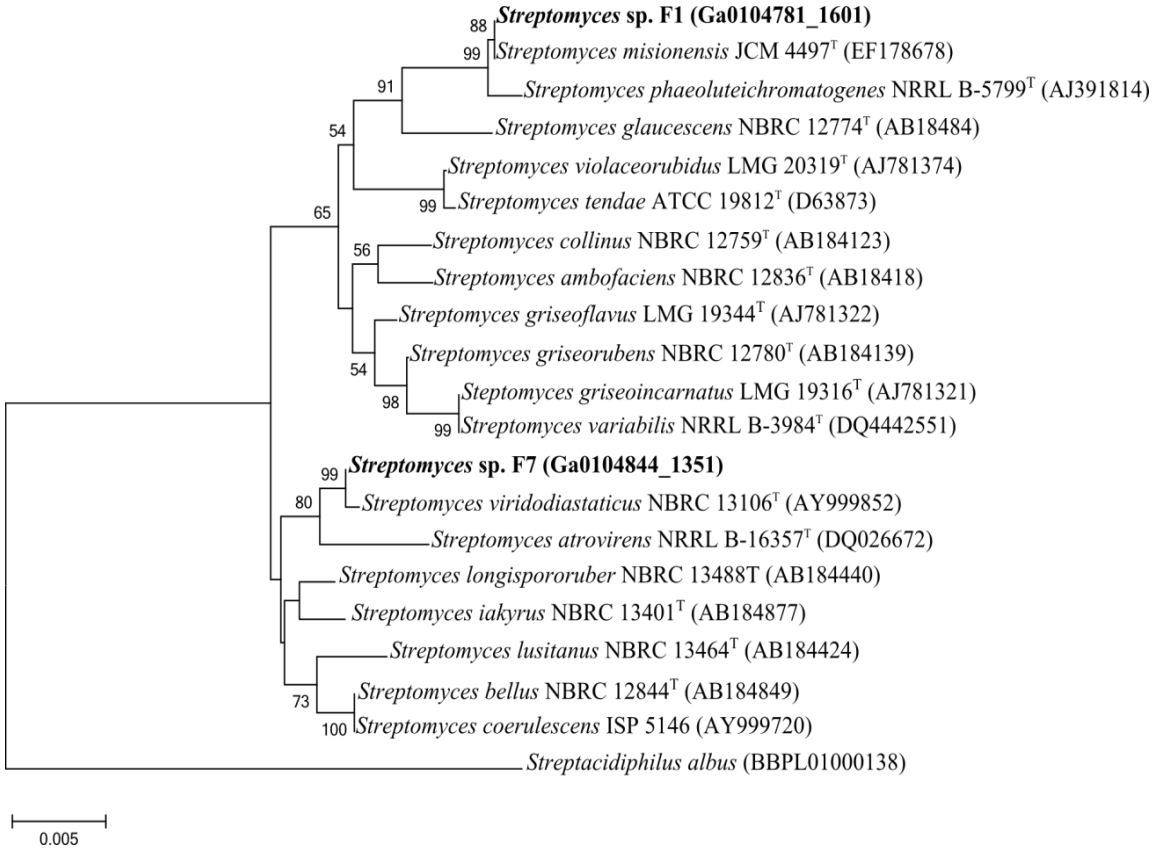


Fig 2. Phylogenetic analysis of 16S rRNA gene sequences of strain F1 and F7 (printed in bold) relative to the most closely related strains of the genus *Streptomyces*. The phylogenetic tree was constructed using neighbor-joining (NJ) algorithm. Bootstrap values higher than 60 % are shown (1,000 replications for bootstrapping were done). Genbank accession numbers are shown in parentheses and type strain with superscript “T”. The sequence of *Streptacidiphilus albus* was used as an out group.

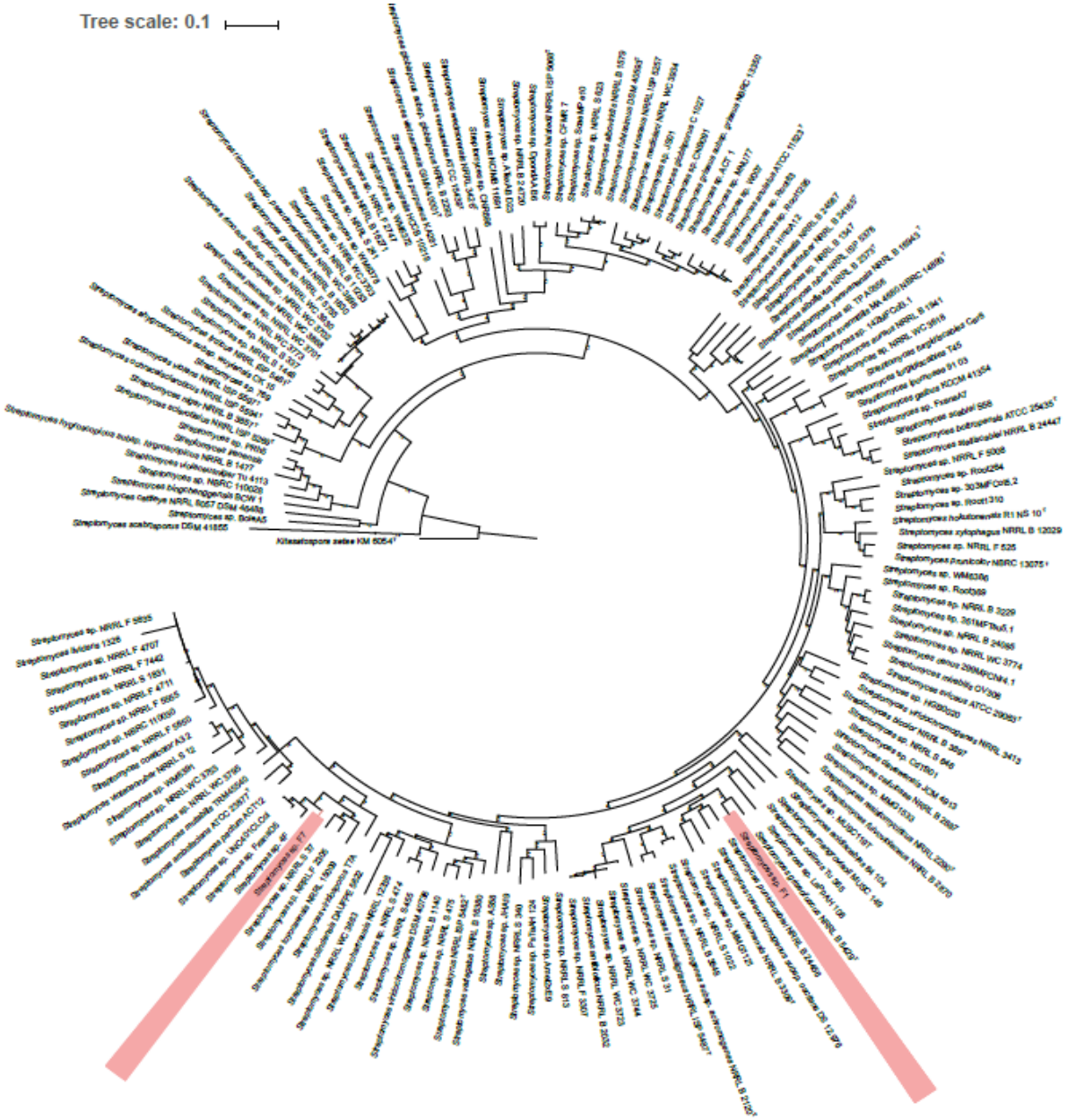


Fig 3. Multilocus phylogenetic analysis of *Streptomyces* genus. The multilocus phylogenetic tree was generated from a concatenated alignment of 288 conserved genes across all genomes of *Streptomyces*. Bootstrap values (expressed as percentages of 1,000 replicates) higher than 0.8 are shown at branch nodes.

3.3. CAZYme profiles

The CAZymes [42] present in *Streptomyces* strains F1 and F7 were identified using dbCAN web resource [34]. Both strains have similar profile of CAZy genes in their genomes (**Table 4**). Strain F1 genome encodes 85 glycoside hydrolyses (GH), 31 glycosyltransferases (GT), 18 carbohydrate esterases (CE), one polysaccharide lyases (PL), 31 carbohydrate

binding motifs (CBM), and nine classified as auxiliary activities (AA), which are CAZymes with redox activities (Table 3). Whereas, strain F7 genome possesses 100 GHs, 22 GTs, 26 CEs, 5 PLs, 26 CBMs and 9 AAs. A detailed CAZy gene prediction indicated that strains F1 and F7 genomes encode 33 and 44 different types of GH families, respectively, which are potentially involved in degradation of starch, chitin, hemicellulose and cellulose (**Table 5** and **Table S3 – ANEXO 1**). However, only strain F7 genome encodes pectin-, arabinogalactan-, lichenan-, and mannan-degradation from families PL1, PL3, GH35, GH64, GH5 and GH2, respectively.

Table 4. Comparison of CAZy genomic profile between strains F1 and F7.

	F1	**F7**
Genome size (Mb)	8.14	7.32
Protein coding genes	7,262	6,463
No. of CAZymes proteins	175	193
% CAZymes proteins [a]	2.36	2.92
Total GH [b]	85	100
Total PL [c]	1	5
Total GT [d]	31	22
Total CE [e]	18	26
Total AA [f]	9	9
Total CBM [g]	31	31

[a] Carbohydrate-Active Enzymes

[b] Glycosyl Hydrolases

[c] Polysaccharide Lyases

[d] Glycosyltransferase

[e] Carbohydrate Esterase

[f] Auxiliary Activity

[g] Carbohydrate-Binding Modules

As expected, *Streptomyces* strains F1 and F7 do not encode cellulosomes, which is the enzymatic complex composed of cohesins and dockerins modules and several lignocellulolytic enzymes with CBMs domains [43]. However, both strains possess GHs connected to CBMs, which is not surprising since lignocellulolytic enzymes and CBMs are broadly distributed in *Bacteria* domain [11,14, 20]. The F7 genome encodes different CAZymes associated with CBMs domains, including endoglucanases, chitinase, xylanases, α-

amylase (**Table S1 – ANEXO 1**). Whereas, strain F1 genome, it was found to encode CBMs associated with only chitinase and endoglucanase. CBMs domains bind to plant cell wall polysaccharides and facilitating GHs activity [44]. In addition, both strains contain genes encoding lytic polysaccharide monooxygenases (LPMOs) belonging to family AA10 (**Table S1 – ANEXO 1**). Chitinolytic and cellulolytic AA10 genes are frequently found in *Streptomyces* genomes [18]. Nevertheless, strains F1 and F7 genomes have only AA10 genes related to chitin degradation.

3.4. Aromatic compounds degradation pathways

Several bacteria have been reported to metabolize lignin, releasing aromatics compounds that are imported into the cell for catabolism [45,46]. Among the lignin-degrading bacteria, few *Streptomyces* strains were described to degrade lignin or lignin-derived compounds, such as catechol, protocatechuate, and gentisate [17,47,48,49]. In this context, the corresponding metabolic pathways in strains F1 and F7 were manually reconstructed from their genomes sequence data.

The genomic analyses of strains F1 and F7 revealed several genes involved on protocatechuate and gentisate catabolism (**Fig. 4 and Table S2 – ANEXO 1**). For gentisate catabolism, an identical gene clusters consisting of four genes were found in both strains genomes. Sequence analysis predicted four encoding genes, including DNA-binding transcriptional regulator (IclR family), gentisate 1,2 dioxygenase, fumarylacetoacetate hydrolase, and maleylpyruvate isomerase. For protocatechuate catabolism, all genetic determinants for this aromatic degradation were found only in strain F1. These seven genes are organized in a cluster and flaked by transcriptional regulators. It is important to mention the presence of transcriptional regulator in all these clusters. For instance, the protocatechuate cluster in *Streptomyces* F7 genome consists of four genes involved on aromatic degradation preceded by a transcriptional regulator. Members of IclR family frequently control genes whose products are involved degradation of aromatics [50]. These findings suggest the clusters could be expressed and allow the strains to use these aromatic compounds as carbon sources.

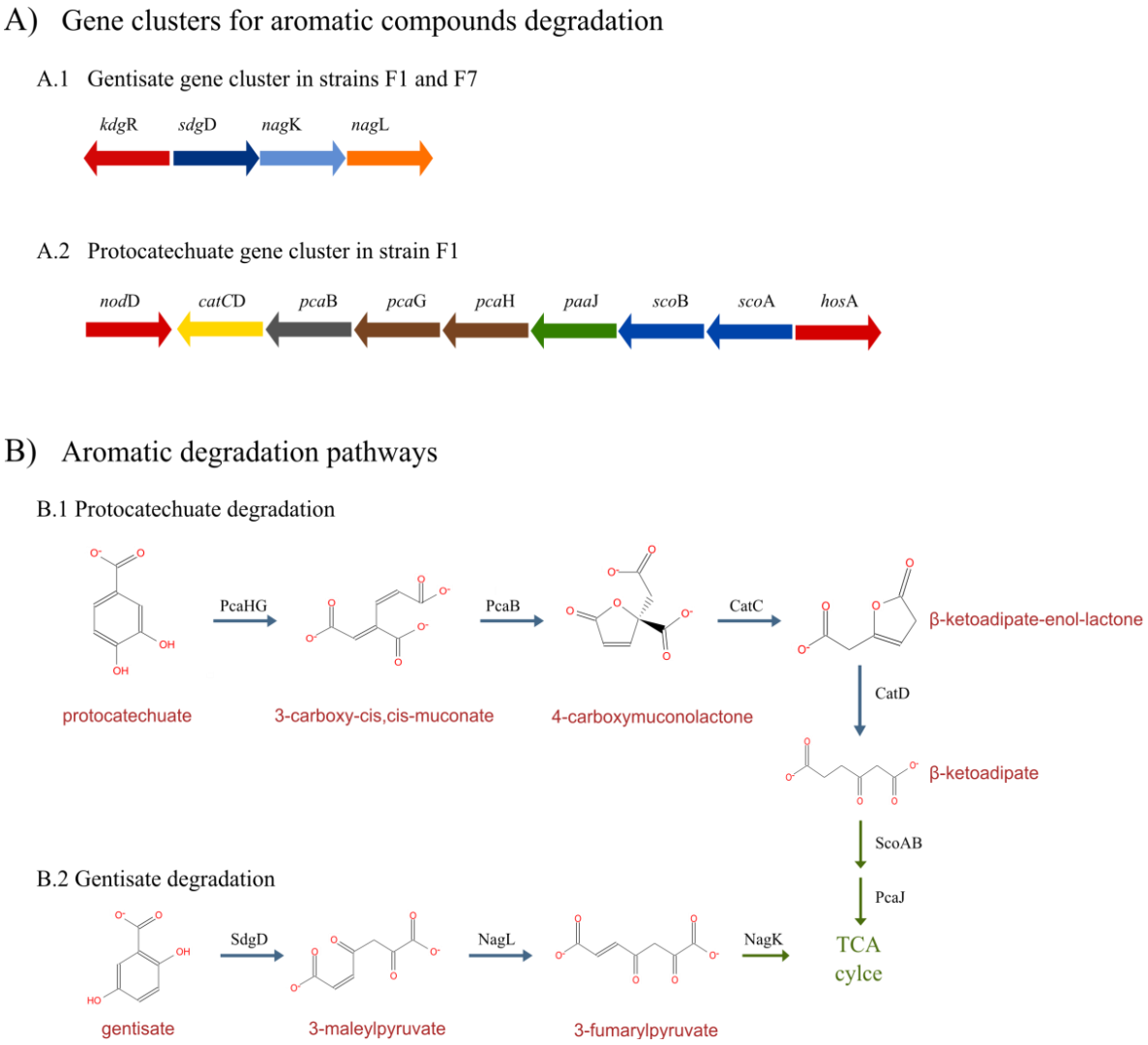


Fig 4. Schematic representation of the gene clusters encoding the gentisate and protocatechuate degradation and their corresponding metabolism found in F1 and F7 genome. **A.1** and **A.2**, Organization of the gentisate and protocatechuate clusters, respectively. The putative functions of the gene products are as follows: *kdg*R, transcriptional regulator KdgR (MarR family transcriptional regulator); *sdg*D, gentisate 1,2-dioxygenase; *nag*K, fumarylacetoacetate hydrolase family; *nag*L, maleylpyruvate isomerase; *nod*D, nodulation protein D (MarR Family); *cat*D, 3-oxoadipate enol-lactonase; *pca*B, β-carboxymuconatecycloisomerase; *pca*B,3-carboxy-cis,cis-muconate cycloisomerase; *pca*GH, Protocatechuate 3,4-dioxygenase, α and β-subunits;*paa*J, 3-oxoadipyl-CoA thiolase**;***sco*AB, 3-oxoacid CoA-transferase subunit A and B; *hos*A, transcriptional regulator (MarR family). **B**. Metabolic pathways of the protocatechuate and gentisate degradation. The enzyme names are shown above the arrows. Abbreviation: TCA cycle, tricarboxylic acid cycle.

Table 5. Summarizes of CAZyme profile encoded by strains F1 and F7.

Substrate	Main known activity	CAZYme family	EC number[1]	Number of genes predicted	
				F1	F7
Pectin	Pectinesterase		3.1.1.11	0	1
	Pectate lyase	PL1	4.2.2.2	0	2
	Pectate lyase	PL3	4.2.2.2	0	1
	Alpha-L-rhamnosidase	AA10	3.2.1.40	0	1
Starch	Alpha-amylase	GH13	3.2.1.1	5	4
	Alpha-glucosidase	GH13	3.2.1.20	3	4
	Alpha-glucosidase	GH31	3.2.1.20	1	1
	4-alpha-glucanotransferase	GH77	2.4.1.25	0	1
	Starch phosphorylase	GT35	2.4.1.1	1	1
Chitin	Chitinase	GH18	3.2.1.14	3	4
	Chitinase	GH19	3.2.1.14	0	1
	Beta-N-acetylhexosaminidase	GH3	3.2.1.52	2	2
	Hexosaminidase	GH20	3.2.1.52	3	3
	N-acetylglucosamine 6-phosphate deacetylase	CE9	3.5.1.25	0	1
Arabinogalactan	Endo-beta-1,4-galactanase	GH35	3.2.1.89	0	1
Lichenan	Endo-1,3-beta-glucanase	GH64	3.2.1.39	1	2
Xyloglucan (hemicellulose)	Alpha-D-xylosidase	GH31	3.2.1.177	1	1
Arabinan (hemicellulose)	Alpha-L-arabinofuranosidase	GH62	3.2.1.55	1	0
	Alpha-L-arabinofuranosidase	GH51	3.2.1.55	1	1
Xylan (hemicellulose)	Endo-1,4-beta-xylanase	GH10	3.2.1.8	4	5
	Endo-1,4-beta-xylanase	GH11	3.2.1.8	1	2
	Xylan 1,4-beta-xylosidase	GH39	3.2.1.37	0	1
	Beta-xylosidase	GH43	3.2.1.37	1	2
	Alpha-N-arabinofuranosidase	GH51	3.2.1.55	1	1
	Alpha-L-arabinofuranosidase	GH54	3.2.1.55	4	0
	Alpha-glucuronidase	GH67	3.2.1.139	0	1
Mannan (hemicellulose)	Endo-1,4-beta-mannosidase	GH5	3.2.1.78	0	1
	Beta-mannosidase	GH2	3.2.1.25	0	1
Cellulose	Beta-glucosidase	GH1	3.2.1.21	4	5
	Beta-glucosidase	GH3	3.2.1.21	1	3
	Endoglucanase	GH5	3.2.1.4	1	1
	Endoglucanase	GH6	3.2.1.4	2	3
	Cellulose 1,4-beta-cellobiosidase	GH6	3.2.1.91	0	1
	Endoglucanase	GH9	3.2.1.4	0	1
	Cellulose 1,4-beta-cellobiosidase	GH12	3.2.1.91	1	0

[1] – Enzyme Commission number

4. CONCLUDING REMARKS

This work disclose two novel *Streptomyces* strains isolated from soil able to grown on CMC, xylan and sugarcane bagasse as sole carbon sources and secrete a range of hydrolytic enzymes. The enzymatic assays indicated that strain F7 was more efficient in the degradation of natural carbohydrates than strain F1. This observation was confirmed by genomic analysis: the genome of strain F1 encodes 85 glycoside hydrolases (GHs) which comprise 33 different types of GH families, and strain F7 harbors 100 GHs representing 44 GH families. Finally, the identification of gene clusters encoding enzymes dedicate to aromatic compounds degradation suggests that the strains could also perform lignin degradation.

ANEXO I, CAPÍTULO 2 - Supplementary material (Page 176)

Table S1. The genes associated to Carbohydrate active enzymes (CAZy) encoded in the strain F1 and F7.

Table S2. Gene clusters encoding the gentisate and protocatechuate degradation patways found in F1 and F7 genome.

Competing interests

The authors declare that they do not have any competing interests.

Acknowledgement

This work was financially supported by grants from National Counsel of Technological and Scientific Development - CNPq (442333/2014-5 and 310186/2014-5) and São Paulo Research Foundation - FAPESP (14/50371-8). R.R.M was supported by CNPq fellowship (140610/2014-6) and G.T. was supported by FAPESP fellowship (2015/23279-6).

5. REFERENCES

[1] L.D. Gomez, C.G. Steele-king, S.J. Mcqueen-mason, Sustainable liquid biofuels from biomass : The writing`s on the walls, (2008) 473-485.

[2] M.E. Himmel, Q. Xu, Y. Luo, S.-Y. Ding, R. Lamed, E.A. Bayer, Microbial enzyme systems for biomass conversion: Emerging paradigms, Biofuels. 1 (2010) 323-341.

[3] J.S. Van Dyk, B.I. Pletschke, A review of lignocellulose bioconversion using enzymatic hydrolysis and synergistic cooperation between enzymes-factors affecting enzymes, conversion and synergy, Biotechnol. Adv. 30 (2012) 1458-1480.

[4] K. Sanderson, A chewy problem, Nature. 474 (2011) S12-14.

[5] K.L. McGuire, K.K. Treseder, Microbial communities and their relevance for ecosystem models: Decomposition as a case study, Soil Biol. Biochem. 42 (2010) 529-535.

[6] D.E. Koeck, A. Pechtl, V.V. Zverlov, W.H. Schwarz, Genomics of cellulolytic bacteria., Curr. Opin. Biotechnol. 29C (2014) 171-183.

[7] S.D. Hamilton-Brehm, J.J. Mosher, T. Vishnivetskaya, M. Podar, S. Carroll, S. Allman, T.J. Phelps, M. Keller, J.G. Elkins, *Caldicellulosiruptor obsidiansis* sp. nov., an anaerobic, extremely thermophilic, cellulolytic bacterium isolated from obsidian pool, Yellowstone National Park, Appl. Environ. Microbiol. 76 (2010) 1014-1020.

[8] P. Brumm, M.L. Land, L.J. Hauser, C.D. Jeffries, Y.J. Chang, D.A. Mead, Complete genome sequence of *Geobacillus* strain Y4.1MC1, a novel CO-utilizing *Geobacillus thermoglucosidasius* strain isolated from bath hot spring in Yellowstone National Park, Bioenergy Res. 8 (2015) 1039-1045.

[9] M. Hess, A. Sczyrba, R. Egan, T.-W. Kim, H. Chokhawala, G. Schroth, S. Luo, D.S. Clark, F. Chen, T. Zhang, R.I. Mackie, L.A. Pennacchio, S.G. Tringe, A. Visel, T. Woyke, Z. Wang, E.M. Rubin, Metagenomic discovery of biomass-degrading genes and genomes from cow rumen., Science. 331 (2011) 463-467.

[10] A. Hanreich, U. Schimpf, M. Zakrzewski, A. Schlüter, D. Benndorf, R. Heyer, E. Rapp, A. Pühler, U. Reichl, M. Klocke, Metagenome and metaproteome analyses of microbial communities in mesophilic biogas-producing anaerobic batch fermentations indicate concerted plant carbohydrate degradation, Syst. Appl. Microbiol. 36 (2013) 330-338.

[11] G. Tomazetto, S. Hahnke, D.E. Koeck, D. Wibberg, I. Maus, A. Pühler, M. Klocke, A. Schlüter, Complete genome analysis of *Clostridium bornimense* strain M2/40T: A new acidogenic *Clostridium* species isolated from a mesophilic two-phase laboratory-scale biogas reactor, J. Biotechnol. 232 (2015) 38-49.

[12] Y. Zhou, P.B. Pope, S. Li, B. Wen, F. Tan, S. Cheng, J. Chen, J. Yang, F. Liu, X. Lei, Q. Su, C. Zhou, J. Zhao, X. Dong, T. Jin, X. Zhou, S. Yang, G. Zhang, H. Yang, J. Wang, R. Yang, V.G.H. Eijsink, J. Wang, Omics-based interpretation of synergism in a soil-derived cellulose-degrading microbial community, Sci. Rep. 4 (2014) 1-6.

[13] D.J. Jiménez, M.J. de Lima Brossi, J. Schückel, S.K. Kračun, W.G.T. Willats, J.D. Van Elsas, Characterization of three plant biomass-degrading microbial consortia by metagenomics- and metasecretomics-based approaches, Appl. Microbiol. Biotechnol. (2016) 1-15.

[14] R. López-Mondéjar, D. Zühlke, T. Větrovský, D. Becher, K. Riedel, P. Baldrian, Decoding the complete arsenal for cellulose and hemicellulose deconstruction in the highly efficient cellulose decomposer *Paenibacillus* O199, Biotechnol. Biofuels. 9 (2016) 104.

[15] K.F. Chater, S. Biró, K.J. Lee, T. Palmer, H. Schrempf, The complex extracellular biology of *Streptomyces*, FEMS Microbiol. Rev. 34 (2010) 171-198.

[16] C. Bontemps, M. Toussaint, P.V. Revol, L. Hotel, M. Jeanbille, S. Uroz, M.P. Turpault, D. Blaudez, P. Leblond, Taxonomic and functional diversity of *Streptomyces* in a forest soil, FEMS Microbiol. Lett. 342 (2013) 157-167.

[17] T. Větrovský, K.T. Steffen, P. Baldrian, Potential of cometabolic transformation of polysaccharides and lignin in lignocellulose by soil Actinobacteria, PLoS One. 9 (2014). doi:10.1371/journal.pone.0089108.

[18] A.J. Book, G.R. Lewin, B.R. McDonald, T.E. Takasuka, E. Wendt-Pienkowski, D.T. Doering, S. Suh, K.F. Raffa, B.G. Fox, C.R. Currie, Evolution of high cellulolytic activity in symbiotic *Streptomyces* through selection of expanded gene Content and coordinated gene expression, PLoS Biol. 14 (2016) 1-21.

[19] A.J. Book, G.R. Lewin, B.R. McDonald, T.E. Takasuka, D.T. Doering, A.S. Adams, J.A. V Blodgett, J. Clardy, K.F. Raffa, B.G. Fox, C.R. Currie, Cellulolytic *Streptomyces* strains associated with herbivorous insects share a phylogenetically linked capacity to degrade lignocellulose, Appl. Environ. Microbiol. 80 (2014) 4692-4701.

[20] G.L. Pinheiro, A.C. de Azevedo-Martins, R.M. Albano, W. de Souza, S. Frases, Comprehensive analysis of the cellulolytic system reveals its potential for deconstruction of lignocellulosic biomass in a novel *Streptomyces* sp., Appl. Microbiol. Biotechnol. 101 (2016) 301-319.

[21] M.A. El-Nakeeb, H.A. Lechevalier, Selective isolation of aerobic Actinomycetes, Appl. Microbiol. 11 (1963) 75-77.

[22] E.B. Shirling, D. Gottlieb, Methods for caracterization of *Streptomyces* species, Int. J. Syst. Bacteriol. 16 (1966) 313-340.

[23] R.M. Teather, P.J. Wood, Use of congo red-polysaccharide interactions in enumeration and characterization of cellulolytic bacteria from the bovine rumen, Appl. Environ.

Microbiol. 43 (1982) 777-780.

[24] G.J.M. Rocha, A.R. Gonçalves, B.R. Oliveira, E.G. Olivares, C.E. V Rossell, Steam explosion pretreatment reproduction and alkaline delignification reactions performed on a pilot scale with sugarcane bagasse for bioethanol production, Ind. Crops Prod. 35 (2012) 274-279.

[25] G.L. Miller, Use of dinitrosalicylic acid reagent for determination of reducing sugars, Anal. Chem. (1959) 426-429.

[26] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72 (1976) 248-254.

[27] A.M. Bolger, M. Lohse, B. Usadel, Trimmomatic: A flexible trimmer for illumina sequence data, Bioinforma. Adv. 30 (2014) 2114.

[28] K. Tennessen, E. Andersen, S. Clingenpeel, C. Rinke, D.S. Lundberg, J. Han, J.L. Dangl, N. Ivanova, T. Woyke, N. Kyrpides, A. Pati, ProDeGe: A computational protocol for fully automated decontamination of genomes, ISME J. 10 (2015) 1-4.

[29] A. Bankevich, S. Nurk, D. Antipov, A.A. Gurevich, M. Dvorkin, A.S. Kulikov, V.M. Lesin, S.I. Nikolenko, S. Pham, A.D. Prjibelski, A. V. Pyshkin, A.V. Sirotkin, N. Vyahhi, G. Tesler, M.A. Alekseyev, P.A. Pevzner, SPAdes: A new genome assembly algorithm and its applications to single-cell sequencing, J. Comput. Biol. 19 (2012) 455-477.

[30] V.M. Markowitz, K. Mavromatis, N.N. Ivanova, I.M.A. Chen, K. Chu, N.C. Kyrpides, IMG ER: A system for microbial genome annotation expert review and curation, Bioinformatics. 25 (2009) 2271–2278.

[31] D. Hyatt, G.-L. Chen, P.F. Locascio, M.L. Land, F.W. Larimer, L.J. Hauser, Prodigal: Prokaryotic gene recognition and translation initiation site identification, BMC Bioinformatics. 11 (2010) 119.

[32] T. Seemann, Prokka: Rapid prokaryotic genome annotation, Bioinformatics. 30 (2014) 2068-2069.

[33] D. Laslett, B. Canback, ARAGORN, a program to detect tRNA genes and tmRNA genes in nucleotide sequences, Nucleic Acids Res. 32 (2004) 11-16.

[34] Y. Yin, X. Mao, J. Yang, X. Chen, F. Mao, Y. Xu, dbCAN: A web resource for automated carbohydrate-active enzyme annotation., Nucleic Acids Res. 40 (2012) W445-51.

[35] M.A. Larkin, G. Blackshields, N.P. Brown, R. Chenna, P.A. Mcgettigan, H.

McWilliam, F. Valentin, I.M. Wallace, A. Wilm, R. Lopez, J.D. Thompson, T.J. Gibson, D.G. Higgins, Clustal W and Clustal X version 2.0, Bioinformatics. 23 (2007) 2947-2948.

[36] K. Tamura, G. Stecher, D. Peterson, A. Filipski, S. Kumar, MEGA6: Molecular evolutionary genetics analysis version 6.0, Mol. Biol. Evol. 30 (2013) 2725-2729.

[37] K. Katoh, D.M. Standley, MAFFT multiple sequence alignment software version 7: Improvements in performance and usability, Mol. Biol. Evol. 30 (2013) 772-780.

[38] P. Kück, G.C. Longo, FASconCAT-G: Extensive functions for multiple sequence alignment preparations concerning phylogenetic studies, Front. Zool. 11 (2014) 81.

[39] M.N. Price, P.S. Dehal, A.P. Arkin, FastTree 2 - Approximately maximum-likelihood trees for large alignments, PLoS One. 5 (2010) e9490.

[40] I. Letunic, P. Bork, Interactive Tree Of Life (iTOL): An online tool for phylogenetic tree display and annotation, Bioinformatics. 23 (2007) 127-128.

[41] Y.P. Guo, W. Zheng, X.Y. Rong, Y. Huang, A multilocus phylogeny of the *Streptomyces griseus* 16S rRNA gene clade: Use of multilocus sequence analysis for Streptomycete systematics, Int. J. Syst. Evol. Microbiol. 58 (2008) 149-159.

[42] B.I.L. Cantarel, P.M. Coutinho, C. Rancurel, T. Bernard, V. Lombard, B. Henrissat, The Carbohydrate-Active EnZymes database (CAZy): An expert resource for glycogenomics, Nucleic Acids Res. 37 (2009) D233-D238.

[43] S.P. Smith, E.A. Bayer, Insights into cellulosome assembly and dynamics: From dissection to reconstruction of the supramolecular enzyme complex., Curr. Opin. Struct. Biol. 23 (2013) 686-94.

[44] B.M. Campos, M.V. Liberato, T.M. Alvarez, L.M. Zanphorlin, G.C. Ematsu, H. Barud, I. Polikarpov, R. Ruller, H.J. Gilbert, A.C. De Mattos Zeri, F.M. Squina, A novel carbohydrate-binding module from sugar cane soil metagenome featuring unique structural and carbohydrate affinity properties, J. Biol. Chem. 291 (2016) 23734-23743.

[45] M.E. Brown, M.C.Y. Chang, Exploring bacterial lignin degradation, Curr. Opin. Chem. Biol. 19 (2014) 1-7.

[46] T.D.H. Bugg, M. Ahmad, E.M. Hardiman, R. Rahmanpour, Pathways for degradation of lignin in bacteria and fungi. Nat. Prod. Rep. 28 (2011) 1883-96.

[47] S. Wa, D. Ishiyama, D. Vujaklija, J. Davies, Novel pathway of salicylate degradation by *Streptomyces* sp . strain WA46, Appl. Environ. Microbiol. 70 (2004) 1297-1306.

[48] L.M.F. Gottschalk, E.P.S. Bon, R. Nobrega, Lignin peroxidase from *Streptomyces viridosporus* T7A: Enzyme concentration using ultrafiltration, Appl. Biochem.

Biotechnol. 147 (2008) 23-32.

[49] J.R. Davis, L. Goodwin, H. Teshima, C. Detter, R. Tapia, C. Han, M. Huntemann, C.-L. Wei, J. Han, A. Chen, N. Kyrpides, K. Mavrommatis, E. Szeto, V. Markowitz, N. Ivanova, N. Mikhailova, G. Ovchinnikova, I. Pagani, A. Pati, T. Woyke, S. Pitluck, L. Peters, M. Nolan, M. Land, J.K. Sello, Genome sequence of *Streptomyces viridosporus* strain T7A ATCC 39115, a lignin-degrading Actinomycete, Genome Announc. 1 (2013) e00416-13.

[50] A.J. Molina-Henares, T. Krell, M. Eugenia Guazzaroni, A. Segura, J.L. Ramos, Members of the IclR family of bacterial transcriptional regulators function as activatorsand/or repressors, FEMS Microbiol. Rev. 30 (2006) 157-186.

CAPÍTULO 3

Enhancement in the production of hydrolytic enzymes from *Streptomyces* sp. strain F1 for optimized lignocellulosic biomass degradation

Ricardo Rodrigues de Melo, Thiago Augusto Gonçalves, Elaine Crespim, Roberto Ruller, Hélia Harumi Sato

Trabalho em preparação para submissão na revista Bioresource Technology

RESUMO

O gênero *Streptomyces* inclui bactérias que naturalmente degradam a biomassa lignocelulósica, secretando importantes enzimas ativas em carboidratos (CAZymes) que caracterizam seu estilo de vida saprofítico. Neste estudo, nosso objetivo foi desenvolver um bioprocesso para a produção de um extrato rico em celulase e xilanase a partir da bactéria *Streptomyces* sp. F1 para melhorar a sacarificação do bagaço de cana por via enzimática. Utilizando ferramentas estatísticas, a secreção máxima de celulases (0,22 U mL^{-1}) e xilanases (9,27 U mL^{-1}) foi alcançada com a suplementação do meio nutriente com 16,4 g L^{-1} de farelo de trigo e 10,0 g L^{-1} de caseína. As análises dos secretomas da linhagem F1 cultivada em quatro fontes de carbono diferentes: carboximetilcelulose (CMC), xilano de madeira de faia (Xyl), farelo de trigo (WF) e glicose (Glu) revelaram um total de 61 proteínas, com 24, 30, 43 e 18 proteínas para os substratos CMC, Xyl, WF e Glu, respectivamente. As CAZymes corresponderam a mais de 15 % do total identificado nos secretomas analisados. O extrato enzimático bruto otimizado da linhagem F1 demonstrou valores ótimos de atividade enzimática a pH 5,5 e 45 ^{o}C com o substrato CMC e pH 5,5 e 55 ^{o}C com substrato xilano. A hidrólise enzimática do bagaço de cana pré-tratado com alcali e antraquinona (ABaq) utilizando o extrato comercial Celluclast 1,5 L suplementado com enzimas degradantes da biomassa da linhagem F1 gerou 2,5 vezes mais açúcares fermentescíveis. A estratégia proposta pode contribuir para aumentar a hidrólise enzimática de materiais lignocelulósicos.

Palavras-chave: Bagaço de cana-de-açúcar; Sacarificação da biomassa; *Streptomyces*; Bactérias do solo; Meio de cultivo.

ABSTRACT

The genus *Streptomyces* includes bacteria that naturally degrade the lignocellulosic biomass, secreting important carbohydrate-active enzymes (CAZymes) that characterize their saprophytic lifestyle. In this study, our aim was to develop a bioprocess for the production of an extract rich in cellulase and xylanase from the bacterium *Streptomyces* sp. F1 to improve the saccharification of sugarcane bagasse *via* the enzymatic route. Using statistical tools, the maximum secretion of cellulases (0.22 U mL^{-1}) and xylanases (9.27 U mL^{-1}) was achieved at the highest concentrations of wheat bran (16.4 g L^{-1}) and casein (10.0 g L^{-1}) near the center point value. Secretome analyses of strain F1 grown on four different carbon sources; carboxymethylcellulose (CMC), beechwood xylan (Xyl), wheat bran (WF) and glucose (Glu) revealed a total of 61 proteins, with 24, 30, 43 and 18 proteins in the substrates CMC, Xyl, WF and Glu, respectively. CAZymes corresponded to more than 15 % of the total identified in the secretomes analyzed. The optimized crude extract of strain F1 showed optimum values of enzymatic activity at pH 5.5 for CMC and xylan as substrates, with an optimum temperature of 45 ^{o}C for CMCases and 55 ^{o}C for xylanases. The enzymatic hydrolysis of sugarcane bagasse treated with alkali and anthraquinone (ABaq) using the commercial cocktail Celluclast 1.5 L loaded with biomass-degrading enzymes of strain F1 yielded ~ 2.5 more times fermentable sugars than without the extract from this stain. The strategy proposed may contribute to increase the enzymatic hydrolysis of lignocellulosic materials.

Keywords: Sugarcane bagasse; Biomass saccharification; *Streptomyces*; Soil bacteria; Culture medium.

1. Introduction

Cellulose and hemicellulose, the major constituents of plant cell wall, are the most abundant renewable polysaccharides in the biosphere (Book et al., 2014; Nagieb et al., 2014). These polysaccharides and the monomers that compose them (especially cellulose and xylan) have been widely employed for diverse industrial applications, such as in food and feed (Erick et al., 2014; Patel et al., 2015), paper and pulp (Das et al., 2015; Pathak et al., 2014) and biofuel (Anwar et al., 2014; Srivastava et al., 2015) industries. Cellulosic ethanol production has received increasing attention in the last decade, as a way to improve bioethanol yield by the use of plant lignocellulosic material (Farinas, 2015).

In the context of enzymatic hydrolysis for lignocellulosic ethanol production, fungal enzymes (especially those from *Trichoderma* spp. and *Aspergillus* spp.) have been traditionally used, both as commercial cocktails or cloned and expressed in laboratory, since fungi produce many types of glycoside hydrolases (GHs) and in large amounts, besides the fact that they use secretory pathways, thus enabling their easy recovery and downstream processing (Hasunuma et al., 2012; Lambertz et al., 2014). On the other hand, bacterial enzymes have been receiving an increasing attention. Bacteria have the advantage of a faster growth in relation to fungi, which results in a faster production of the desired enzymes. Furthermore, since many bacteria can strive in hostile environments, with extreme temperatures, pH or salinity, they can be a source for the production of enzymes that are more tolerant to the harsh conditions found in industrial environments (Dashtban et al., 2009; Maki et al., 2009). Bacterial enzymes are usually more complex than their fungal counterparts and might act in synergy with other enzymes from their own metabolism, as observed in cellulosome-producing bacteria (Doi and Kosugi, 2004). In this sense, Gram-positive bacteria have an additional advantage of releasing the enzymes into the medium in an active conformation, similarly to what is observed for fungi (Anné et al., 2014; Lambertz et al., 2014).

Some actinomycetes, including bacteria from the Genus *Streptomyces*, are able to produce and secrete cellulases and hemicellulases (Mander et al., 2014; Nagieb et al., 2014). They are Gram-positive bacteria, usually part of the soil microbiota, which are renowned for the production of secondary metabolites of industrial interest (Book et al., 2014; Pinheiro et al., 2016). In terms of enzyme yield improvement, bacterial cultivation in media containing cellulose as the sole carbon source can induce the expression of relevant genes for cellulase production, therefore increasing its expression. Nevertheless, the carbon source is not the only

factor that can contribute to a high protein yield (Lambertz et al., 2014). Reports in literature show that nitrogen and phosphorous sources can also influence homologous cellulase expression. For instance, Lin and Wilson (1987) verified, for an actinomycete strain and its mutants, that endocellulase synthesis can be upregulated by an inducer, such as cellulose, and repressed by high growth rates, which are directly proportional to the amount of the nitrogen, and to a lesser extent, phosphorous, available in the medium.

In a previous work (Melo *et al*., unpublished), eighty *Streptomyces* strains were screened for cellulolytic and xylanolytic activities, and two strains showing a great potential for GH production and application for enzymatic hydrolysis of plant biomasses were selected for genomic analyses. *Streptomyces* sp. strain F1 was selected for the present study, which aimed the optimization of conditions for cellulase and xylanase production. A final secretome analysis was performed to investigate the influence of different carbon sources on the secretion of cellulases and hemicellulases in this strain. The synergistic effect with the use of the enzymatic extract from strain F1 for the supplementation of the commercial cocktail Celluclast in the hydrolysis of treated bagasse was tested.

2. Material and Methods

2.1. Streptomyces strain

Streptomyces sp. strain F1 was previously isolated from soil samples in an exploratory study of new *Streptomyce*s strains capable of hydrolyzing different polysaccharides from plant biomass (Melo *et al*., unpublished). Strain F1 was stored at -80 °C as a mixture of mycelial fragments in 20 % (v/v) glycerol or in ISP 2 agar plates at 4 ^{o}C (El-Nakeeb and Lechevalier, 1963).

2.2. Plant biomass feedstocks

All materials applied in the study were extensively washed after the pretreatment, dried at room temperature, milled, sieved and stocked at room temperature until used. Hydrothermal sugarcane bagasse (HB) was obtained by pretreatment of the sugarcane bagasse for 12 min with water at 190 °C (Santucci et al., 2015). Steam-exploded sugarcane bagasse (SB) was pretreated by steam explosion at 200 °C, for 15 min. SB was delignified (DSB) conducted using the concentration of 100 g L^{-1} pulp, 10 g L^{-1} NaOH, at 30 °C for 2 h (Rocha

et al., 2012). Sugarcane bagasse treated with alkali and anthraquinone (ABaq) was obtained according to optimized conditions described by Nascimento et al (2016), where 12 kg of dry sugarcane bagasse were processed at 130 °C with NaOH solution (1.5 % *w/v*) with the addition of 0.15 % (*w/w*) of anthraquinone. The chemical compositions of pretreated bagasses were determined according to the method of the National Renewable Energy Laboratory (NREL) (Sluiter et al., 2006). The sugarcane bagasse compositions were, respectively (*w/w* % dry basis): HB (cellulose 74.0 ± 1.3 %; hemicellulose 6.0 ± 0.5 %; lignin 26 ± 0.1 %; and ash 0.2 ± 0.1 %), SB (cellulose 57.3 ± 0.2 %; hemicellulose 10.9 ± 0.1 %; lignin 27.6 ± 0.8 %; and ash 4.6 ± 0.0 %), DSB (cellulose 74.0 ± 0.1 %; hemicellulose 6.8 ± 0.1 %; lignin 16.2 ± 0.0 %; and ash 4.9 ± 0.1 %), and ABaq (cellulose 60.5 ± 1.1 %; hemicellulose 24.1 ± 0.5 %; lignin 9.4 ± 0.1 %; and ash 6.3 ± 2.8 %).

Wheat bran (WF) was obtained from a local vendor (Yoki Alimentos Ltda., São Bernardo do Campo, SP, Brazil). Wheat bran (WF) contains approximately (*w/w*), 22.2 % of starch and 58.5 % of total bran (comprising mainly cellulose, hemicellulose, and lignin).

2.3. Growth and analysis of culture conditions using statistical tools

Strain F1 was initially recovered from the glycerol stock and grown on agar plates with the medium ISP 2 at 30 °C. After 7 days, 10^6 spores mL^{-1} were inoculated in Erlenmeyer flasks containing 50 mL of Bushnell Haas Broth (BHB, Sigma Aldrich®) mineral salts medium (g L^{-1}: K_2HPO_4, 1; KH_2PO_4, 1; $CaCl_2 \cdot 2H_2O$, 0.02; NH_4NO_3, 1; $FeCl_3$, 0.05; $MgSO_4 \cdot 7H_2O$, 0.2 and pH 7.0) with the addition of 1.0 % (*w/v*) of HB, SB, DSB, ABaq or WF as carbon sources. The flasks were incubated at 30 °C for 7 days in a rotary shaker (New Brunswick Scientific, New Jersey, USA) at 180 rpm. Samples were taken to evaluate the set of enzymes produced. With the choice of the best carbon source, five different nitrogen sources (casein, yeast extract, ammonium sulfate, peptone and ammonium chloride) were tested at a final concentration of 1.0 % (*w/v*). All experiments were conducted in biological triplicates.

To study the best carbon and nitrogen concentration to support the optimum cellulase and xylanase production, a central composite rotatable design (CCRD) was performed with data from shake flask experiments. The design was conducted considering 12 trials.

The mathematical relationship connecting the factors and the response was analyzed by the following quadratic polynomial Eq. 1:

$$Y = \beta_0 + \sum \beta_i x_i + \sum \beta_{ii} x_i^2 \quad \text{(Eq. 1)}$$

where Y is the response (enzyme yield), β_0 is the intercept, $X_i s$ are the levels of independent variables (factors) under study, β_i is the *i*th linear coefficient and β_{ii} is the *i*th quadratic coefficient. Statistica™ 8.0 software from Statsoft Inc. (Tulsa, Oklahoma, USA) was employed for the experimental design, data analysis and model building.

2.4. Enzymatic assays

All enzymatic assays were carried out in sodium phosphate buffer, 100 mM, pH 6.0, at 50 °C in triplicate and the mean values calculated. Xylanase and cellulase activities were determined with the commercial substrates (0.5 %, *w/v*), beechwood xylan and carboxymetilcellulose, respectively. For the reactions, 50 µL of substrates, 30 µL of sodium phosphate buffer, and 20 µL of the crude enzymatic extract were used. After 20 min of incubation at 50 °C, the reactions were stopped by the addition of 100 µL of dinitrosalicylic acid (DNS) (Miller, 1959), and the measurement was made at 540 nm using an Infinite M200® spectrophotometer (Tecan, Männedorf, Switzerland). One enzyme activity unit (U) was defined as the amount of enzyme which released a 1 µmol of the product (equivalent glucose or xylose) per minute under assay conditions used for all activities.

2.5. Protein analysis and SDS-PAGE

Total protein content was measured in micro plates using the Bio-Rad assay reagent (Bio-Rad Laboratories, Hercules, USA), employing a procedure based on the method described by Bradford (Bradford, 1976) with bovine serum albumin as standard. For electrophoresis (SDS-PAGE) in denatured gels, samples were mixed with loading buffer (containing β-mercaptoethanol and SDS), and heated at 99 ^{o}C for 5 min. After electrophoresis, the gels were stained with Coomassie blue (Laemmli, 1970). A mixture of high molecular weight proteins was used as a molecular standard.

2.6. Preparation of secretomes

Streptomyces sp. strain F1 was grown in Erlenmeyer flasks using Bushnell Haas Broth mineral salts medium, 1.0 % of casein and supplemented with different polysaccharides or plant biomass (1.0 % *w/v*: carboxymethylcellulose, beechwood xylan or wheat bran). Glucose, at a final concentration of 1.0 % (*w/v),* was used as an internal control. The proteins secreted were collected after a culture period of 48 h at 30 °C, 180 rpm. The supernatants were cleared by centrifugation (5000 × *g*), concentrated by ultra-filtration (10,000 MWCO, PES membrane, Vivaspin, Littleton USA), rinsed twice with MiliQ water and the proteins were separated in a 12 % SDS-PAGE gel.

2.7. Secretome peptide mapping by liquid chromatography coupled in-line to mass spectrometry (LC–MS/MS)

For secretome peptide mapping experiments, four independent cultures and three protein separations by SDS-PAGE were performed. In the LC-MS/MS analysis 30 μg of total secretome proteins were loaded onto an SDS-PAGE gel. The SDS-PAGE was run, stained with Coomassie blue and the entire protein banding profile was excised and processed for LC-MS/MS (Shevchenko et al., 1996). The gel bands isolated were reduced with Tris (2-carboxyethyl) phosphine (DTT), alkylated with 2-Iodoacetamide (IAA), digested for 16 h with 8 μg mL^{-1} trypsin (Promega™, USA) using ammonium bicarbonate buffer and analyzed by LC-MS/MS using LTQ-Orbitrap XL hybrid mass spectrometer (Thermo Scientific, Waltham, MA, USA). For this analysis, an Eksigent LC pump was used to separate peptide populations on analytical C18 nanocolumns, with the column effluent being sprayed directly into a New Objective Picoview ion source. Using a "TopThree" MS/MS method, the Orbitrap analyzer collected accurate (5 ppm) scans of intact peptides for one second; simultaneously, the LTQ ion trap performed MS/MS fragmentation analysis of each of the three most abundant peptides, eluted in that 1 s chromatographic fraction (0.8 Da mass accuracy).

The LC-MS/MS raw files were used for database Mascot (version 2.2.04, Matrix Science, London UK) searches run on a *Streptomyces* sp. F1 specific subset at NCBI. The searches were validated using Scaffold (version 4.0.7, Proteome Software Inc. Portland, OR) with a protein threshold of 5 % FDR and a peptide threshold of 99 %.

2.8. Biochemical assays

To evaluate the optimum pH, assays with the enzymatic extract produced by strain F1 were conducted ranging the pH from 2.0 to 10.0 using McIlvaine glycine-supplemented buffer at 50 mM. The optimum temperature was evaluated in a range from 15 to 70 °C. The initial activities were regarded as 100 %, and relative activities were expressed as a percentage of the initial activity. All assays were performed in triplicate.

2.9. Biomass saccharification

Bioconversion assays were conducted in 1 mL (2 mL Eppendorf tubes) final volumes, containing sodium citrate buffer (50 mM at pH 4.8), 5 % (*w/v*) of biomass (ABaq) and a commercially available cellulolytic preparation (Celluclast 1.5 L, Novozymes®) at 0.4 $FPUg^{-1}$ of bagasse supplemented with the optimized extract from strain F1 at different concentrations (see Figure 6). Eppendorf tubes were incubated in a ThermoMixer (Eppendorf, Germany) operated at an agitation speed of 1000 rpm at 50 ^{o}C. Samples were collected at different time intervals (0, 24 and 48 h), cooled on ice, centrifuged at 10 000 × *g* for 30 min at 4 °C and the amount of sugars released was quantified in the recovered supernatants using the DNS method. The saccharification yields, that are the means of three replicates, were expressed as percentages related to the sugar content of the pretreated material before the hydrolysis.

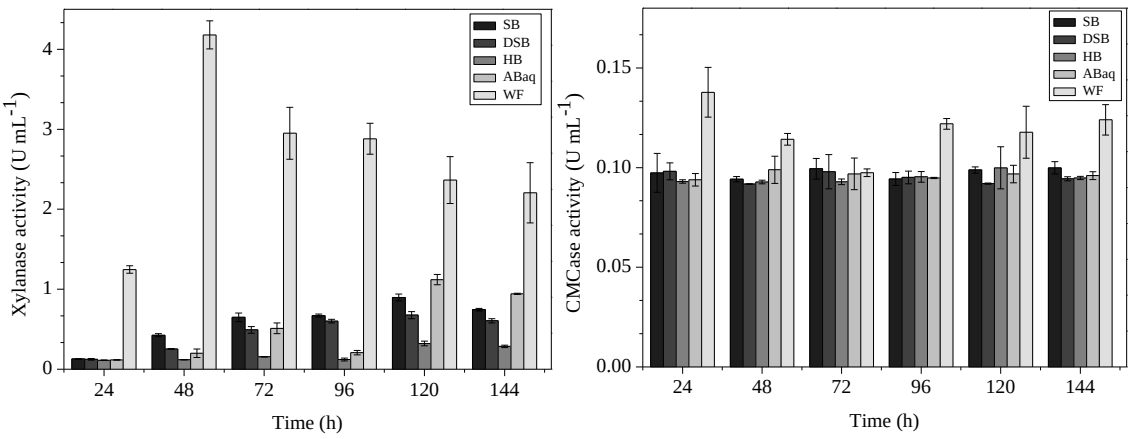
3. Results and Discussion

3.1. Culture medium analysis: Effects of carbon and nitrogen sources

Nutrient medium supplementation with the agro-industrial wastes/by-products as carbon sources was initially explored to accelerate biomass-degrading enzymes secretion by the *Streptomyces* sp. strain F1. The application of agro-industrial wastes/by-products as primary carbon sources in enzyme production reduces the operation cost and helps in solving problems with their disposal (Anwar et al., 2014; Chandel et al., 2012). An expressive impact on xylanase secretion by strain F1 was obtained with the use of wheat bran (4.3 U mL^{-1} at 48 h, Figure 1a), while the other lignocellulosic materials tested, i.e., HB, SB, DSB and ABaq did not exhibit a great influence on xylanase accumulation (Figure 1a). Besides, it was observed that xylanase production by HB, SB, DSB and ABaq only occurred at high levels after 120 hours of fermentation (Figure 1a), a much longer time than required for enzyme production with WF. Similarly, high cellulase secretion was obtained using wheat bran as

carbon source (0.14 U mL^{-1} at 24 h, Figure 1a). Regarding the other carbon sources, no significant increase in cellulase release was detected and their enzymatic secretion capacities remained very close throughout the growth process of strain F1 (ranging from 0.09 to 0.10 U mL^{-1}, Figure 1a). Again, the employment of sugarcane bagasses with different pretreatments used as carbon sources and inducers were not efficient to improve enzymatic secretion.

(a)



(b)

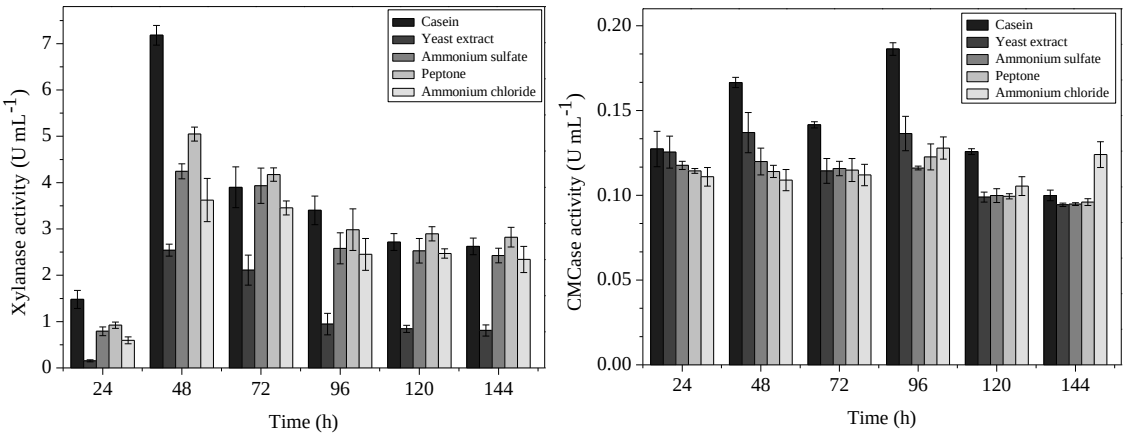


Figure 1 - Influence of (a) carbon sources and, (b) nitrogen sources on biomass-degrading enzymes secretion by *Streptomyces* sp. strain F1. Steam-exploded sugarcane bagasse (SB), Steam-exploded sugarcane bagasse delignified (DSB), Hydrothermal sugarcane bagasse (HB), Sugarcane bagasse treated with alkali and anthraquinone (ABaq) and Wheat bran (WF). Each value represents mean ± SD (n = 3).

By adding the various nitrogen sources to the nutrient medium, xylanase secretion by strain F1 was observed to be slightly higher in the presence of casein (7.2 U mL^{-1} at 48 h), followed by peptone (5.1 U mL^{-1} at 48 h), ammonium sulfate (4.3 U mL^{-1} at 48 h) and

ammonium chloride (3.6 U mL^{-1} at 48 h) (Figure 1b). The use of yeast extract as a nitrogen source to the nutrient medium was not an efficient inducer for the increase of xylanase secretion (Figure 1b). Likewise, cellulase secretion was more stimulated by casein as a nitrogen source than the other sources tested (Figure 1b). Therefore, wheat bran was selected as the carbon source and casein as the nitrogen source for the next optimization step.

3.2. Statistical evaluation as a tool to increase the production of biomass-degrading enzymes

To increase the production of biomass-degrading enzymes, a study using the experimental design of the CCRD type was proposed. Here, the CCDR will help in understanding the pattern in which the dependent variables (cellulase and xylanase production) are affected by the corresponding changes in the independent variables (wheat bran and casein concentration), predicting the interactive effects, to select the best conditions where the maximum secretion of biomass-degrading enzymes can be obtained.

The independent variables (wheat bran - X_1 and casein - X_2) used in the CCRD, the values observed and the values predicted by the mathematical model for the improvement of cellulase and xylanase production by *Streptomyces* sp. strain F1 are illustrated in Table 1. The CCRD results indicate that xylanase secretion by strain F1 was affected by the concentration of carbon and nitrogen source in the culture medium. As shown in Figure 2, the highest wheat bran and casein concentrations near the center point value increased xylanase production, which ranged from 0.62 to 9.27 U mL^{-1}, a 15-fold difference (Table 1). The best condition for xylanase production corresponded to 16.4 g L^{-1} wheat bran and 10.0 g L^{-1} casein (Figure 2a). In addition, cellulase secretion by strain F1 was explored, and a 3.7-fold difference is reported, depending on the wheat bran and casein concentration applied (ranging from 0.06 to 0.22 U mL^{-1}, Table 1). Using CCDR, the maximum cellulase secretion was achieved close to the values obtained for xylanase, where the maximum yield was obtained at the highest wheat bran and casein concentration near the center point value (16.4 g L^{-1} wheat bran and 10.0 g L^{-1} casein Figure 2b).

The suitability of models was further corroborated by ANOVA tests (Tables 2 and 3). The coefficients of determination ($R^2 = 0.92$ and 0.89) indicates that there is a good correlation between the predicted and experimentally observed values, implying that 92 and 89 % of the variability in the response can be explained by the models (Tables 2 and 3). The closer the value of R^2 to 1, the better is the correlation between the observed and predicted

values (Singhania et al., 2007). The validity of fitted models (Eqs. 2 and 3) was also controlled by F-*tests* (*Ft*). The model F_t-values of 33.4 and 25.5 implied that the models are considerable. Values of 'F_{tab}' lower than 7.67 for xylanase ($Ft = 33.4 > F_{\text{tab } 0.05;\ 3;\ 7} = 4.35$) and 5.87 for cellulase ($Ft = 25.5 > F_{\text{tab } 0.05;\ 3;\ 7} = 4.35$) indicate that model terms are significant (Tables 2 and 3).

The multiple regression analysis of the data obtained generated the following quadratic models for xylanase yield, U mL^{-1} (Y1, Eq. 2) and cellulase yield, U mL^{-1} (Y2, Eq. 3), respectively:

$$\boldsymbol{Y1} = 7.5618 + 2.1910\, x_1 - 0.9903\, x_1^2 + 1.9356\, x_2 - 1.7694\, x_2^2 \qquad \text{(Eq. 2)}$$

$$\boldsymbol{Y2} = 0.1818 + 0.0369\, x_1 - 0.0196\, x_1^2 + 0.0253\, x_2 - 0.0380\, x_2^2 \qquad \text{(Eq. 3)}$$

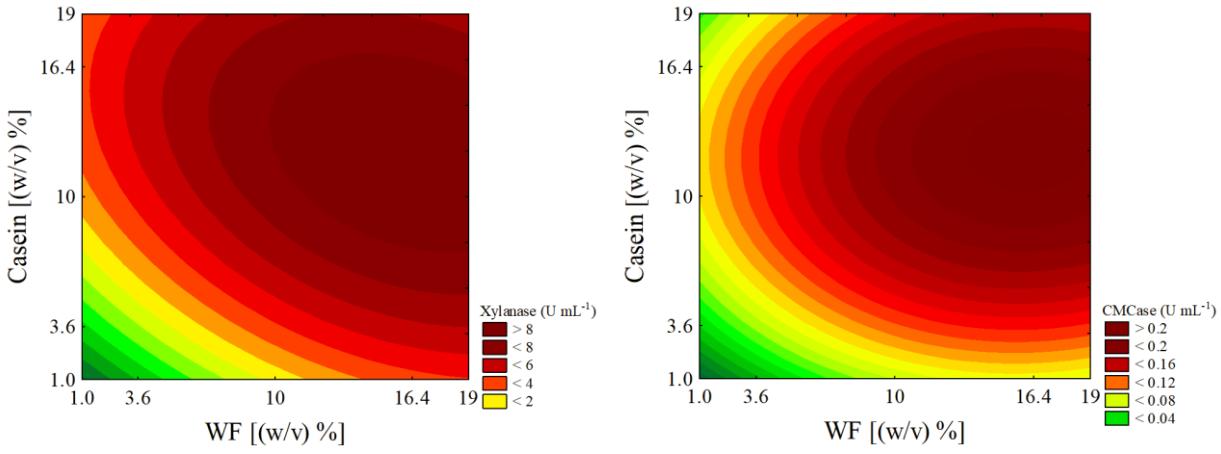


Figure 2 - Contour plots for the production of biomass-degrading enzymes from strain F1 in relation to the combined effect of variables - WF (wheat bran) and casein. (a) xylanase production and (b) cellulase production.

Table 1 - Central composite rotatable design (CCDR) with coded values of variables and responses for the production of biomass-degrading enzymes from strain F1.

Variables		**Levels**				
		-1.41	-1	0	1	1.41
Wheat bran [(w/v) %]	*x1*	1.0	3.6	10	16.4	19
Casein [(w/v) %]	*x2*	1.0	3.6	10	16.4	19

Runs	***x1***	***x2***	**Activity (U mL^{-1})**			
			Experimental value		**Predicted value**	
			Xylanase	***CMCase***	***Xylanase***	***CMCase***
1	-1	-1	0.62	0.06	0.68	0.06
2	1	-1	6.00	0.12	5.06	0.14
3	-1	1	5.44	0.11	4.55	0.11
4	1	1	7.81	0.18	8.93	0.19
5	-1.41	0	1.56	0.09	2.50	0.09
6	1.41	0	9.27	0.22	8.68	0.19
7	0	-1.41	1.13	0.09	1.31	0.07
8	0	1.41	6.59	0.14	6.77	0.14
9 (C)	0	0	7.60	0.18	7.56	0.18
10 (C)	0	0	7.61	0.18	7.56	0.18
11 (C)	0	0	7.54	0.18	7.56	0.18
12 (C)	0	0	7.50	0.18	7.56	0.18

Table 2 - Analysis of variance (ANOVA) and regression analyses for the response of the central composite rotatable design of xylanase production.

Source of variation	Sum of squares	Degrees of freedom	Mean squares	*F*-test	*p*-value
Regression	91.1238	4	22.7809	33.36	0.0024
Residual	4.7797	7	0.6828		
Lack of fit	4.7724	4			
Pure error	0.0073	3			
Total	95.8962	11			

Coefficient of determination: $R^2 = 0.922$; $F_{t\ 0.05;\ 3;\ 7} = 4.35$

Table 3 - Analysis of variance (ANOVA) and regression analyses for the response of the central composite rotatable design of cellulase production.

Source of variation	Sum of squares	Degrees of freedom	Mean squares	*F*-test	*p*-value
Regression	0.026257	4	0.006564	25.54	0.000001
Residual	0.001801	7	0.000257		
Lack of fit	0.001798	4			
Pure error	0.000003	3			
Total	0.028058	11			

Coefficient of determination: $R^2 = 0.899$; $F_{t\ 0.05;\ 3;\ 7} = 4.35$

3.3. Secretome analysis

The secretome (all extracellular non-anchored proteins) produced by *Streptomyces* sp. strain F1 cultures grown on glucose (control), pure polysaccharides (beechwood xylan and carboxymethylcellulose), and complex polysaccharides (wheat bran - WF) were analyzed by LC-MS/MS (Table 4; Figure 3). In the study, the glucose was used as a promoter of catabolite repression of proteins, while the substrates CMC and xylan represented the main components of plant cell wall, cellulose and hemicellulose. The use of wheat bran (WF) was tested as a heterogeneous carbon source containing complex lignocellulosic material, and as the best source chosen in the optimization tests. The proteins analyses were sorted into a descending rank according to spectral counts. Figure 3 shows 1-D SDS-PAGE protein profiles secreted to the medium as a response to the different carbon sources after 48 h of culture. The analysis of *Streptomyces* sp. strain F1 secretomes by 1-D SDS-PAGE (Figure 3) reveals remarkable

differences in terms of protein profile. A more intense number of proteins were observed with the medium formulated by WF than with the other sources tested, which clearly indicates that the secretion of proteins has changed according to the carbon source.

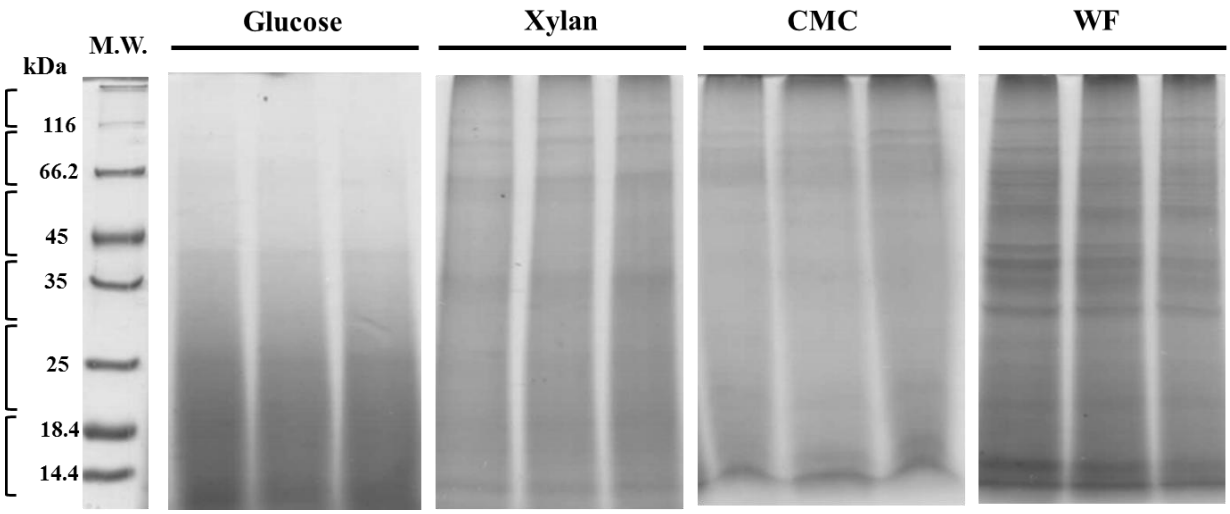


Figure 3 - *Streptomyces* sp. strain F1 secretome composition. Strain F1 secretomes developed on a variety of carbon sources: glucose, beechwood xylan, carboxymethylcellulose (CMC), and wheat bran (WF) were analyzed by LC-MS/MS from SDS-PAGE separated proteins.

Streptomyces sp. strain F1 secretome showed a diverse set of proteins with predicted molecular weights in the range of 11-126 kDa (Table 4). In the secretomic analyses of strain F1, 61 proteins were found. However, using bioinformatics tools to predict secreted proteins, it has been found that 47 of identified proteins (77 %) are predicted to contain signal peptide and/or are secreted by nonclassical routes. The growth on glucose resulted in the secretion of 18 proteins of which 7 were exclusively for this carbon source (Figure 4). Using glucose, 5.5 % of the total spectral counts to secretome were identified as having a CAZy annotation. In the xylan secretome, 30 proteins were detected, being 3 exclusive; however, 17 % were identified as having a CAZy annotation. In secretome coming from media supplemented with CMC, 24 proteins were identified, and 4 were exclusively secreted in this condition (Figure 5). Besides the putative proteins, 5 CAZymes were secreted with CMC substrate.

Table 4 - Identified proteins involved in plant cell wall modifications and spectrum counts in different carbon sources by *Streptomyces* sp. strain F1 at 48 h during submerged cultivation.

Accession number	Identified proteins	dbCAN	Signal peptide	Secretion[b]	MW (kDa)	Spectrum count[a]			
						Glu	Xyl	CMC	WF
STEPF1_03546	Immune inhibitor A		Yes	Yes	85	23	16	23	0
STEPF1_01735	Subtilisin inhibitor-like protein 4		Yes	Yes	15	1	18	17	15
STEPF1_01439	hypothetical protein		Yes	Yes	76	9	10	0	18
STEPF1_04878	putative D,D-dipeptide-binding periplasmic		Yes	Yes	64	9	9	0	6
STEPF1_06845	hypothetical protein		Yes	Yes	22	13	11	0	11
STEPF1_05766	Major membrane protein I		No	No	52	1	10	0	16
STEPF1_05643	hypothetical protein		No	No	65	4	4	0	8
STEPF1_01813	hypothetical protein		Yes	Yes	21	10	12	0	5
STEPF1_03259	Superoxide dismutase [Fe-Zn] 1		No	Yes	24	1	13	22	10
STEPF1_03436	hypothetical protein		Yes	Yes	45	0	5	11	1
STEPF1_03288	hypothetical protein	CBM13	Yes	Yes	38	4	7	2	9
STEPF1_01584	Serine/threonine-protein kinase PknD		Yes	Yes	60	2	6	2	4
STEPF1_05783	hypothetical protein		Yes	Yes	31	3	10	0	8
STEPF1_00210	hypothetical protein		Yes	Yes	22	0	0	13	0
STEPF1_02633	Isocitrate dehydrogenase [NADP]		No	No	80	0	0	0	6
STEPF1_06531	Transglutaminase-activating metalloprotease		Yes	Yes	57	0	3	5	4
STEPF1_04128	Malate dehydrogenase		No	No	35	1	1	6	2
STEPF1_01473	Endoglucanase E1	GH5/CBM3	Yes	Yes	69	19	0	0	0
STEPF1_03754	Dihydrolipoyl dehydrogenase		No	Yes	49	0	0	0	8
STEPF1_01440	hypothetical protein		Yes	Yes	19	10	2	0	3
STEPF1_00302	hypothetical protein		Yes	Yes	32	23	2	0	0
STEPF1_02894	hypothetical protein		Yes	Yes	23	3	6	0	3

Accession number	Identified proteins	dbCAN	Signal peptide	Secretion[b]	MW (kDa)	Spectrum count[a]			
						Glu	Xyl	CMC	WF
STEPF1_02122	Aconitate hydratase A		No	No	98	0	0	2	2
STEPF1_01250	Glycerophosphoryl diester phosphodiesterase		Yes	Yes	43	0	11	0	2
STEPF1_05821	Subtilisin BL		Yes	Yes	52	10	0	0	2
STEPF1_04692	hypothetical protein		Yes	Yes	23	0	0	14	0
STEPF1_03704	Citrate synthase 1		No	No	49	0	0	0	7
STEPF1_03778	Cytochrome c oxidase subunit 2		Yes	Yes	34	0	2	0	3
STEPF1_03077	Endo-1,4-beta-xylanase B	GH11/CBM2	Yes	Yes	36	0	6	0	1
STEPF1_00819	hypothetical protein		No	Yes	11	0	0	8	0
STEPF1_05102	Serine protease AprX		Yes	Yes	126	17	0	0	0
STEPF1_05569	Aminopeptidase N		Yes	Yes	54	0	1	0	2
STEPF1_03784	Menaquinol-cytochrome c reductase		Yes	Yes	28	0	0	8	0
STEPF1_03989	Phosphoglycerate kinase		No	No	42	0	0	0	3
STEPF1_06302	1-pyrroline-5-carboxylate dehydrogenase		No	No	59	0	0	7	0
STEPF1_01441	Aminodeoxyfutalosine deaminase		Yes	Yes	58	0	0	0	1
STEPF1_04348	Endoglucanase A	GH6	Yes	Yes	42	6	0	0	3
STEPF1_07199	Endo-1,4-beta-xylanase B	GH11/CBM2	Yes	Yes	35	0	4	0	1
STEPF1_05913	hypothetical protein		Yes	Yes	29	1	0	0	2
STEPF1_07200	Bifunctional xylanase/deacetylase	CE4/CBM2	Yes	Yes	33	0	2	0	0
STEPF1_05085	hypothetical protein		Yes	Yes	14	17	0	0	0
STEPF1_01393	hypothetical protein		Yes	Yes	44	0	2	30	0
STEPF1_02545	hypothetical protein	GH115	Yes	Yes	116	0	12	0	0
STEPF1_04825	Streptogrisin-B		Yes	Yes	30	2	0	0	1
STEPF1_03864	Phospholipase D		Yes	Yes	60	0	1	0	1
STEPF1_01368	General stress protein 16U		No	No	20	0	0	0	1

Accession number	Identified proteins	dbCAN	Signal peptide	Secretion[b]	MW (kDa)	Spectrum count[a]			
						Glu	Xyl	CMC	WF
STEPF1_06701	hypothetical protein		Yes	Yes	65	0	0	3	0
STEPF1_04283	Tripeptidyl aminopeptidase		Yes	Yes	57	1	0	0	0
STEPF1_00136	Chaperone protein DnaK		No	No	66	0	0	0	1
STEPF1_02431	Elongation factor Ts		No	No	32	0	0	0	2
STEPF1_04141	hypothetical protein		Yes	Yes	50	0	1	6	0
STEPF1_03539	4-hydroxyphenylpyruvate dioxygenase		No	No	42	0	0	0	2
STEPF1_05677	hypothetical protein	CBM13	Yes	Yes	60	0	0	0	1
STEPF1_00818	hypothetical protein		No	Yes	12	0	0	3	0
STEPF1_01121	hypothetical protein		Yes	Yes	49	0	2	0	1
STEPF1_03676	hypothetical protein		Yes	Yes	36	0	1	0	0
STEPF1_01583	hypothetical protein		No	Yes	17	0	0	0	1
STEPF1_02995	hypothetical protein	GH54/CBM42	Yes	Yes	53	0	0	0	1
STEPF1_00157	Fructose-bisphosphate aldolase		No	No	37	0	0	0	1
STEPF1_06670	Polyribonucleotide nucleotidyltransferase		No	No	79	0	0	0	1
STEPF1_02195	hypothetical protein		Yes	Yes	21	0	0	0	1

[a] Secretomic analysis based on spectral counting.

[b] The secretion of each protein was verified by the softwares SignalP, SecretomeP and YLoc. When at least two of three softwares give a positive result the protein was considered secreted.

Glucose (Glu); beechwood xylan (Xly); carboxymethylcellulose (CMC); and wheat bran (WF).

The secretome isolated from cells grown on wheat bran (WF), contained the highly abundant secreted proteins identified in experiments with cellulose and xylan and some additional proteins. These additional proteins likely reflect cellular response to the more complex composition of polysaccharides present in WF (Figure 4). In total, wheat bran secretome contained 43 proteins of which 15 were unique to this carbon substrate, and 6 proteins (~14 %) identified as belonging to the CAZymes group.

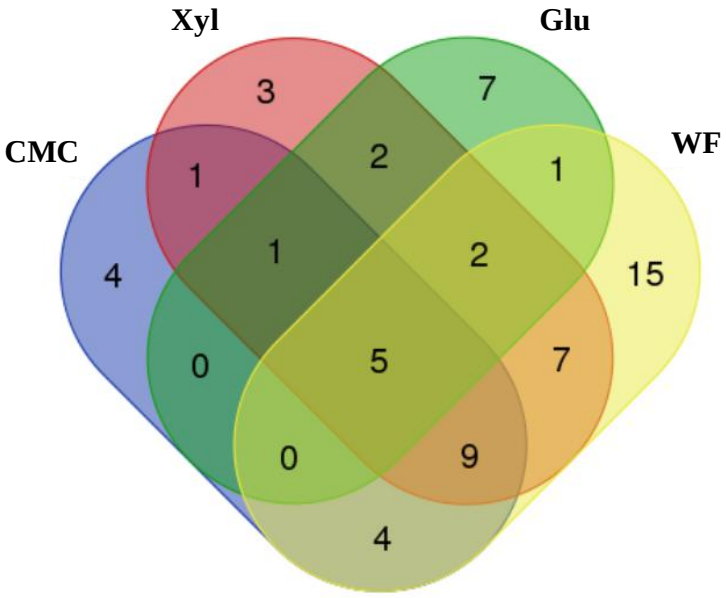
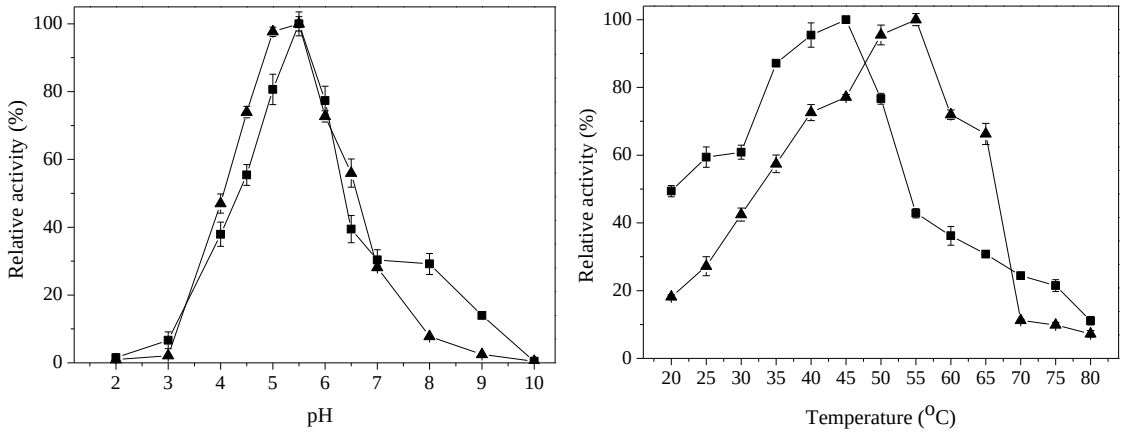


Figure 4 - Venn diagram representing unique and common protein identified in each secretome of *Streptomyces* sp. strain F1. A total of 61 nonredundant protein groups were identified in the secretomes resulting from growth in media with the addition of *GLU* glucose, *Xly* beechwood xylan, *CMC* carboxymethylcellulose, and *WF* wheat bran by means of the gel-free LC–MS/MS strategy. The venn diagram was prepared by means of the tool, Venny (http://bioinfogp.cnb.csic.es/tools/venny/index.html).

3.4. Partial characterization

Effects of pH and temperature on the system of biomass-degrading enzymes expressed by *Streptomyces* sp. strain F1 were evaluated using commercial polysaccharides as substrates. Using the McIlvaine glycine-supplemented buffers at 50 mM, the optimized crude extract produced by the *Streptomyces* strain exhibited an optimum activity at the same pH value (pH 5.5) in both cellulase and xylanase analyses (Figure 5a). CMCase and xylanase activities decreased as pH increased towards alkalinity. This result indicated an acidophilic characteristic of CMCase and xylanase enzymes expressed by strain F1, which is a close

behavior in comparison to other cellulases and xylanases derived from *Streptomyces* strains (Bajaj and Singh, 2010; Pinheiro et al., 2016).



(a) (b)

Figure 5 - Effects of (a) pH and (b) temperature on the biomass-degrading enzymes system expressed by *Streptomyces* sp. strain F1. Regarding (*square*) cellulase activity; and (*triangle*) xylanase activity.

When the temperature profile of the optimized crude extract was studied, it was found that (Figure 5b) the incubation temperature of 45 ^{o}C favored the maximum CMCase activity and, 55 ^{o}C the maximum xylanase activity. A temperature increment of up to 80 °C leads to a decline in enzymatic activities. Thus, the optimum pH and temperature observed for the crude extract of strain F1 indicates that the biomass-degrading enzymes system may be applied in the supplementation and act in synergy with the current extract cellulolytic commercial extract i.e., Celluclast 1.5 L (Gama et al., 2015) used in biomass saccharification.

3.5. Hydrolytic potential of the biomass-degrading enzymes system from strain F1

To explore the hydrolytic potential of the crude extract expressed by *Streptomyces* sp. strain F1, enzymatic hydrolysis using sugarcane bagasse treated with alkali and anthraquinone (ABaq) was performed. The strategy used in this study was the supplementation of Celluclast 1.5 L (Novozymes®), a benchmark commercial cocktail for hydrolyzing raw lignocellulosic materials. It is known that the Celluclast 1.5 L cocktail contains mainly an extract rich in cellulolytic enzymes (Gama et al., 2015) and a previous study showed that the addition of xylanase to its composition has improved the hydrolysis efficiency (Hu et al., 2011). Enzymatic crude extract from strain F1 was produced in an optimized liquid medium, concentrated by ultra-filtration and adopted to perform saccharification experiments. The

extract from strain F1 showed a higher prevalence of xylanolytic enzymes than the cellulolytic enzymes. This information is interesting because the absence or lack of xylanolytic enzymes limits the action of cellulases in cellulose bran hydrolysis, because the hemicellulases act synergistically with cellulases in the hydrolysis of lignocellulosic materials (Hu et al., 2011; Li et al., 2014).

The supplementation of the cellulolytic commercial extract with the biomass-degrading enzymes system from strain F1 allowed sugar yield to reach, after 72 h, almost 2.5-fold (Mix, Cell + F1 50 %) that obtained only by the commercial counterpart after the same incubation time (6.14 g L^{-1}, Figure 6). Moreover, considering our experiment, we could observe that an increase in commercial extract loading with enzymes from strain F1 (cellulases and xylanases above 0.8 U or 13.7 U, respectively) did not cause additional benefits for biomass conversion (Figure 6). It has previously been suggested that loading with high enzyme concentrations may promote a decrease in the relative numbers of enzyme binding sites and the enzymes in the cocktail start competing for the same binding sites, leading to a reduction in the overall rate of activity (Banerjee et al., 2010).

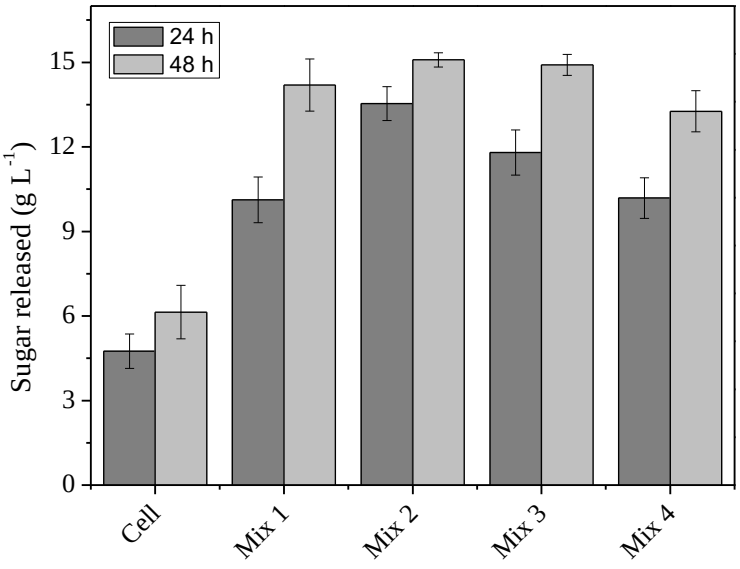


Figure 6 - Saccharification of the sugarcane bagasse treated with alkali and anthraquinone (ABaq) by using commercial enzymatic cocktail Celluclast 1.5 L (Novozymes®) supplemented with different proportions of crude enzymatic cocktail expressed by *Streptomyces* sp. strain F1. ***Cell***: Celluclast 1.5 L (0.4 FPU g^{-1} of bagasse); ***Mix 1***: Celluclast 1.5 L (0.4 FPU g^{-1} of bagasse) with cellulase (0.4 U) and xylanase (6.8 U) from strain F1; ***Mix 2***: Celluclast 1.5 L (0.4 FPU g^{-1} of bagasse) with cellulase (0.8 U) and xylanase (13.7 U) from strain F1; ***Mix 3***: Celluclast 1.5 L (0.4 FPU g^{-1} of bagasse) with cellulase (1.2 U) and xylanase (20.5 U) from strain F1; ***Mix 4***: Celluclast 1.5 L (0.4 FPU g^{-1} of bagasse) with cellulase (1.6 U) and xylanase (27.4 U) from strain F1.

Therefore, the results of hydrolysis experiments indicated that the enzymatic extract produced by strain F1 showed an interesting ability to produce significantly more reducing sugars in a shorter time, compared to the commercial enzymatic extract (Celluclast 1.5 L, Novozymes®) as the only tool. Thus, enzymatic extract from *Streptomyces* sp. strain F1 could potentially be used as a source of enzymes to improve the commercial enzymatic formulations intended for biomass deconstruction.

4. Conclusions

The extent of enzyme accumulation by *Streptomyces* sp. strain F1 was strongly dependent on the type and concentration of carbon and nitrogen sources. The best condition for xylanase and CMCase production corresponded to supplementation of nutrient medium with 16.4 $g\,L^{-1}$ wheat bran and 10.0 $g\,L^{-1}$ casein. Proteomic analysis of the secretome of *Streptomyces* sp. F1 identified 61 proteins, with approximately 15 % of their proteins with CAZy function. Enzymatic hydrolysis of the pretreated ABaq yielded a higher (~ 2.5 times) amount of fermentable sugars. These findings allow us to confirm the potential that the enzymes of *Streptomyces* sp. strain F1 have for lignocellulosic biomass degradation.

Acknowledgements

The authors thank National Counsel of Technological and Scientific Development - CNPq, and the National Laboratory of Science and Technology of Bioethanol (CTBE-CNPEM) for technical assistance. R.R.M was supported by CNPq fellowship (140610/2014-6). We gratefully acknowledge the CNPEM facility MAS at LNBio-CNPEM.

5. References

Anné, J., Vrancken, K., Mellaert, L. Van, Impe, J. Van, Bernaerts, K., 2014. Protein secretion biotechnology in gram-positive bacteria with special emphasis on *Streptomyces lividan*s. BBA - Mol. Cell Res. 1843, 1750–1761.

Anwar, Z., Gulfraz, M., Irshad, M., 2014. Agro-industrial lignocellulosic biomass a key to unlock the future bio-energy : A brief review. J. Radiat. Res. Appl. Sci. 7, 163–173.

Bajaj, B.K., Singh, N.P., 2010. Production of xylanase from an alkalitolerant *Streptomyces* sp. 7b under solid-state fermentation, its purification, and characterization. Appl. Biochem.

Biotechnol. 162, 1804–1818.

Banerjee, G., Car, S., Scott-craig, J.S., Borrusch, M.S., Bongers, M., Walton, J.D., 2010. Synthetic multi-component enzyme mixtures for deconstruction of lignocellulosic biomass. Bioresour. Technol. 101, 9097–9105.

Book, A.J., Lewin, G.R., McDonald, B.R., Takasuka, T.E., Doering, D.T., Adams, A.S., Blodgett, J.A. V, Clardy, J., Raffa, K.F., Fox, B.G., Currie, C.R., 2014. Cellulolytic *Streptomyces* strains associated with herbivorous insects share a phylogenetically linked capacity to degrade lignocellulose. Appl. Environ. Microbiol. 80, 4692–4701.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Chandel, A.K., Silva, S., Singh, O. V, 2012. Sugarcane bagasse and leaves : foreseeable biomass of biofuel and bio-products. Fungal Genet Biol 87, 11–20.

Das, A., Paul, T., Ghosh, P., Halder, S.K., Das Mohapatra, P.K., Pati, B.R., Mondal, K.C., 2015. Kinetic study of a glucose tolerant β-glucosidase from *Aspergillus fumigatus* ABK9 entrapped into alginate beads. Waste and Biomass Valorization 6, 53–61.

Dashtban, M., Schraft, H., Qin, W., 2009. Fungal bioconversion of lignocellulosic residues; Opportunities & perspectives. Int. J. Biol. Sci. 5, 578–595.

Doi, R.H., Kosugi, A., 2004. Cellulosomes : Plant-cell-wall-degrading enzyme complexes. Nat. Rev. 2, 541–551.

El-Nakeeb, M.A., Lechevalier, H.A., 1963. Selective isolation of aerobic actinomycetes. Appl. Microbiol. 11, 75–77.

Erick, J., Gomes, G., Camila, T., Nascimento, S., Emília, A., Queiroz, S.D.F., Ivaldo, J., Silva, D.S., Souza-, C.M. De, Medeiros, E.V. De, Moreira, K.A., 2014. Production, characterization and evaluation of in vitro digestion of phytases, xylanases and cellulases for feed industry. African J. Microbiol. Res. Full 8, 551–558.

Farinas, C.S., 2015. Developments in solid-state fermentation for the production of biomass-degrading enzymes for the bioenergy sector. Renew. Sustain. Energy Rev. 52, 179–188.

Gama, R., Dyk, J.S. Van, Pletschke, B.I., 2015. Optimisation of enzymatic hydrolysis of apple pomace for production of biofuel and biorefinery chemicals using commercial enzymes. 3 Biotech 5, 1075–1087.

Hasunuma, T., Okazaki, F., Okai, N., Hara, K.Y., Ishii, J., Kondo, A., 2012. A review of enzymes and microbes for lignocellulosic biorefinery and the possibility of their application to consolidated bioprocessing technology. Bioresour. Technol. 1–9.

Hu, J., Arantes, V., Saddler, J.N., 2011. The enhancement of enzymatic hydrolysis of lignocellulosic substrates by the addition of accessory enzymes such as xylanase : is it an additive or synergistic effect ? Biotechnol. Biofuels 4, 36.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685.

Lambertz, C., Garvey, M., Klinger, J., Heesel, D., Klose, H., Fischer, R., Commandeur, U., 2014. Challenges and advances in the heterologous expression of cellulolytic enzymes: A review. Biotechnol. Biofuels 7, 135–150.

Li, J., Zhou, P., Liu, H., Xiong, C., Lin, J., Xiao, W., Gong, Y., Liu, Z., 2014. Synergism of cellulase, xylanase, and pectinase on hydrolyzing sugarcane bagasse resulting from different pretreatment technologies. Bioresour. Technol. 155, 258–265.

Lin, E., Wilson, D.B., 1987. Regulation of 3-1, 4-endoglucanase synthesis in *Thermomonospora fusca*. Appl. Environ. Microbiol. 53, 1352–1357.

Maki, M., Leung, K.T., Qin, W., 2009. The prospects of cellulase-producing bacteria for the bioconversion of lignocellulosic biomass. Int. J. Biol. Sci. 5, 500–516.

Mander, P., Hee, Y., Pradeep, G.C., Seok, Y., Hee, J., Sik, S., Cheol, J., 2014. Biochemical characterization of xylanase produced from *Streptomyces* sp. CS624 using an agro residue substrate. Process Biochem. 49, 451–456.

Miller, G.L., 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugars. Anal. Chem. 426–429.

Nagieb, Z.A., Milegy, M.G. El, Faat, H.M.R., Isis, K.B., 2014. Effect of EDTA on production of xylanase by *Streptomyces* species and their bleaching effect on papyrus and cotton stalk pulp. Int. J. For. Wood Sci. 1, 2–9.

Patel, A.K., Singhania, R.R., Pandey, A., 2015. Novel enzymatic processes applied to the food industry. Curr. Opin. Food Sci.1-27.

Pathak, P., Centre, A., Roorkee, T., 2014. Production of crude cellulase and xylanase from *Trichoderma harzianum* PPDDN10 NFCCI-2925 and its application in photocopier waste paper recycling. Appl. Biochem. Biotechnol. 172, 3776–3797.

Pinheiro, G.L., de Azevedo-Martins, A.C., Albano, R.M., de Souza, W., Frases, S., 2016. Comprehensive analysis of the cellulolytic system reveals its potential for deconstruction of lignocellulosic biomass in a novel *Streptomyces* sp. Appl. Microbiol. Biotechnol. 101 (1), 301-319.

Rocha, G.J.M., Gonçalves, A.R., Oliveira, B.R., Olivares, E.G., Rossell, C.E. V, 2012. Steam explosion pretreatment reproduction and alkaline delignification reactions performed on

a pilot scale with sugarcane bagasse for bioethanol production. Ind. Crops Prod. 35, 274–279.

Santucci, B.S., Maziero, P., Rabelo, S.C., Curvelo, A.A.S., Pimenta, M.T.B., 2015. Autohydrolysis of hemicelluloses from sugarcane bagasse during hydrothermal pretreatment: A kinetic assessment. BioEnergy Res 8, 1778–1787.

Shevchenko, A., Wilm, M., Vorm, O., Mann, M., 1996. Mass spectrometric sequencing of proteins from silver-stained polyacrylamide gels. Anal. Chem. 68, 850–858.

Singhania, R.R., Sukumaran, R.K., Pandey, A., 2007. Improved cellulase production by *Trichoderma reesei* RUT C30 under SSF through process optimization. Appl Biochem Biotechnol 142, 60–70.

Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D., 2006. Determination of sugars, byproducts, and degradation products in liquid fraction process samples. Laboratory Analytical Procedure (LAP). Golden, Natl. Renew. Energy Lab.1-11.

Srivastava, N., Srivastava, M., Mishra, P.K., Singh, P., 2015. Application of cellulases in biofuels industries : An overview. J. Biofuels Bioenergy 1, 55–63.

CAPÍTULO 4

New heterofunctional supports based on glutaraldehyde-activation: A tool for enzyme immobilization at neutral pH

Ricardo Rodrigues de Melo, Robson Carlos Alnoch, Adriana Ferreira Lopes Vilela, Emanuel Maltempi de Souza, Nadia Krieger, Roberto Ruller, Hélia Harumi Sato, Cesar Mateo

Publicado na revista Molecules 2017, 22 (7), 1088; (Open Access) doi:10.3390/molecules22071088

RESUMO

A imobilização é uma promissora alternativa para melhorar a estabilidade de processos enzimáticos. No entanto, parte das estratégias covalentes aplicadas para a imobilização utilizam condições específicas, geralmente pH alcalino, onde algumas enzimas não são estáveis. Aqui, uma nova geração de suportes heterofuncionais com aplicações em condições neutras de pH foi proposto. Os novos suportes foram desenvolvidos com diferentes grupos bifuncionais (*isto é*, hidrofóbicos ou carboxílicos/metálicos) capazes de adsorver os biocatalisadores por diferentes regiões (regiões ricas em grupos hidrofóbicos ou histidina), juntamente com um grupo glutaraldeído capaz de promover uma imobilização irreversível em condições neutras. Para testar estes suportes, um sistema modelo multi-proteico (extrato de *E. coli*) e quatro enzimas (lipase de *Candida rugosa*, lipase metagenômica, β-galactosidase e β-glicosidase) foram utilizadas. O mecanismo de imobilização foi testado e indicou que força iônica moderada deverá ser aplicada para evitar possíveis adsorções inespecíficas. O uso de diferentes suportes permitiu a imobilização da maioria das proteínas contidas no extrato bruto de proteínas. Além disso, os diferentes suportes produziram catalisadores com diferentes propriedades catalíticas. Em pH neutro, os novos suportes foram capazes de adsorver e imobilizar covalentemente as quatro enzimas testadas com diferentes valores de atividade recuperadas. Notavelmente, o uso desses suportes provou ser uma ferramenta alternativa eficiente para a imobilização enzimática em neutras condições de pH.

Palavras-chave: imobilização enzimática; suportes heterofuncionais; glutaraldeído; estabilidade térmica; lipase de *Candida rugosa*; lipase metagenômica; β-glucosidase; β-galactosidase.

ABSTRACT

Immobilization is an exciting alternative to improve the stability of enzymatic processes. However, part of the applied covalent strategies for immobilization uses specific conditions, generally alkaline pH, where some enzymes are not stable. Here, a new generation of heterofunctional supports with application at neutral pH conditions was proposed. New supports were developed with different bifunctional groups (*i.e.*, hydrophobic or carboxylic/metal) capable of adsorbing biocatalysts at different regions (hydrophobic or histidine richest place), together with a glutaraldehyde group that promotes an irreversible immobilization at neutral conditions. To verify these supports, a multi-protein model system (*E. coli* extract) and four enzymes (*Candida rugosa* lipase, metagenomic lipase, β-galactosidase and β-glucosidase) were used. The immobilization mechanism was tested and indicated that moderate ionic strength should be applied to avoid possible unspecific adsorption. The use of different supports allowed the immobilization most of proteins contained in a crude protein extract. In addition, different supports yielded catalysts of the tested enzymes with different catalytic properties. At neutral pH, the new supports were able to adsorb and covalently immobilize the four enzymes tested with different recovered activity values. Notably, the use of these supports proved to be an efficient alternative tool for enzyme immobilization at neutral pH.

Keywords: enzyme immobilization; heterofunctional supports; glutaraldehyde; thermal stability; *Candida rugosa* lipase; metagenomic lipase; *β*-glucosidase; *β*-galactosidase.

1. Introduction

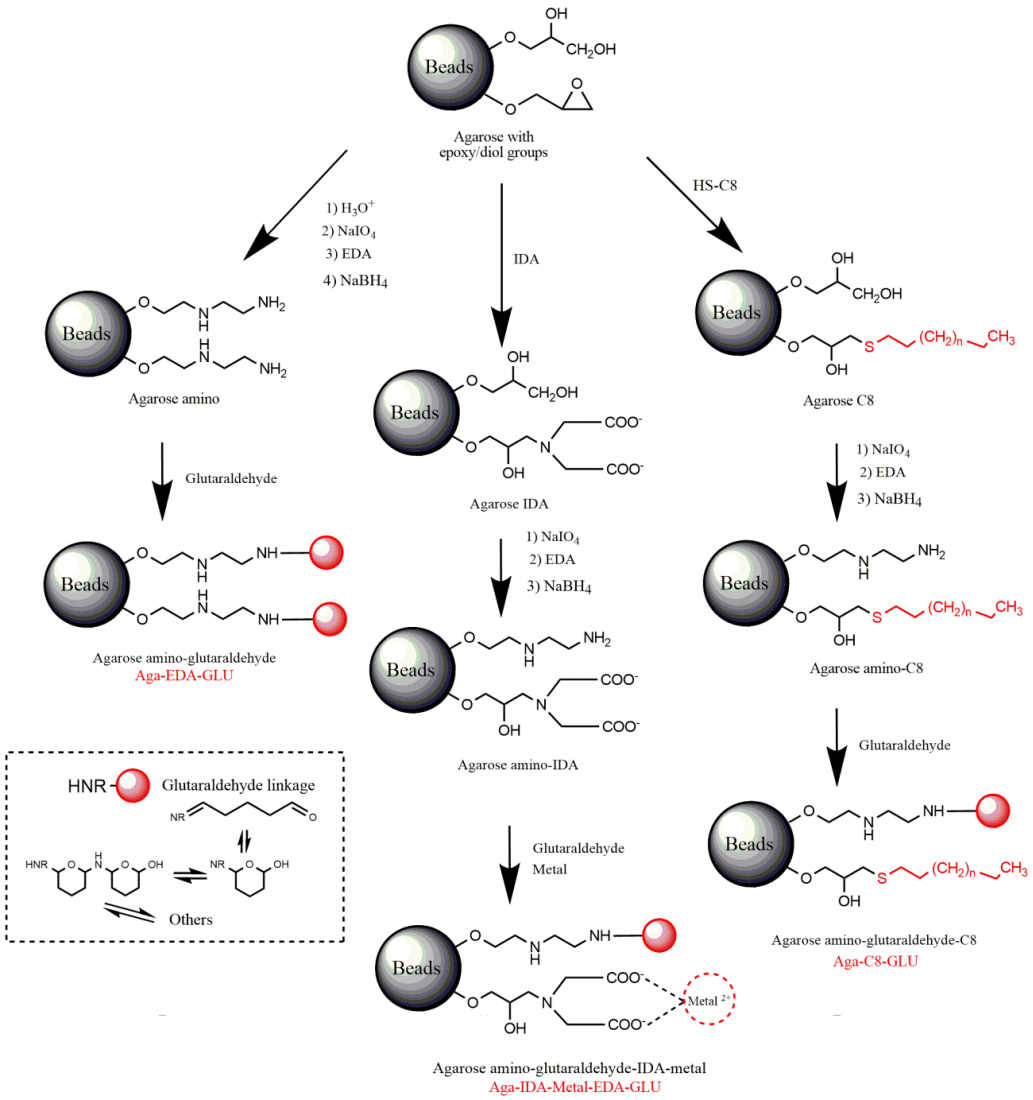
A simple, cheap and efficient immobilization strategy represents one of the main bottlenecks in the implementation of many industrial-scale enzymatic processes. Immobilization technology, as a very powerful tool, has been intensively applied to prepare various high-performance and economically-feasible biocatalysts improving activity, stability and selectivity [1-4]. By careful adjusting of the immobilization strategies, heterogeneous biocatalysts can work in broader pH and temperature ranges, and show greater thermal stability than their native free form [5,6].

Currently, a variety of methodologies for the immobilization of enzymes have been described. Among them, covalent immobilization on solid supports has been shown to be able to stabilize a variety of enzymes. Thus, supports activated with different functional groups (epoxy, aldehyde, glutaraldehyde, and others) have been developed and applied. However, the use of these supports only permit the random immobilization or the immobilization through only one region (*e.g.*, richest in lysine region for aldehyde supports). This makes it so that only one kind of catalyst can be obtained using a functional covalent group; the product of the rigidification through one region or the average of the properties of the different enzyme molecules by random immobilization [7-10]. Because of these properties, several studies have shown the efficiency of immobilizing different enzymes in heterofunctionalized supports using various adsorbing groups (octyl, primary or secondary amino groups, ionic exchangers, metallic chelates and others) and reactive moieties (glyoxyl, epoxy, aldehyde, divinyl sulfone, amino, or glutamic groups), which are capable of adsorbing the protein physically and subsequently establishing covalent or ionic linkages with nucleophilic groups on the adsorbed protein [6,11-16]. One of the main advantages of use this type of supports is that they allows the orientation of the enzyme immobilization in different zones in its surface, being an alternative to the monofunctional supports. Heterofunctional supports are currently being studied as a useful tool in the immobilization of lipases (lipases from *Pseudomonas stutzeri*, *Rhizomucor miehei*, *Thermomyces lanuginosus*, and *Candida antarctica*), for instance, immobilizing silica or agarose supports containing distinct hydrophobic adsorbing groups (different alkyl groups) and reactive moieties [12,13,15-17]. In addition to the lipases, β-galactosidases (e.g., *Aspergillus oryzae* and *Thermus* sp.) and other enzymes have been immobilized on heterofunctional supports, obtaining good results with respect to their use [18-21]. For most strategies involving the application of heterofunctional supports, the immobilization process needs to be carried out in at least two steps: first the enzyme is

adsorbed into the different adsorbent groups under mild conditions (*i.e.*, low ionic strength and neutral pH), followed by elevation to alkaline conditions (around pH 10) for the occurrence of multiple interactions between nucleophilic groups on the enzyme (typically amino, hydroxyl, and/or thiol moieties) and reactive groups on the surface of the support. In some cases an additional step is carried out involving the reduction process necessary to establish multiple covalent linkages between the support and the enzyme [9]. However, the reduction step can cause some deleterious effects on the protein structure, cleaving disulfide and peptide linkages or reducing some groups essential for catalysis [22,23].

Although these immobilization methods are very useful, their uses have several limitations. One disadvantage of its use is the need to apply alkaline pH conditions (around pH 10) to enhance the reactivity of the nucleophilic residues found on the protein surface. Thus enzymes that are intrinsically unstable at alkaline pH values (*e.g.*, the lipase from *Candida rugosa*) can be inactivated by the immobilization process [9,24,25]. An alternative process is the use of the reagent glutaraldehyde. The chemical covalent attachment process using glutaraldehyde is particularly attractive, since it provides a carefully regulated connection with specific groups found on the proteins under mild pH conditions [26]. Furthermore, the chemical reactivity shown by glutaraldehyde means that the reduction process with sodium borohydride is not strictly necessary [9].

Standard immobilization techniques using glutaraldehyde as the coupling agent are quite simple, efficient and probably the most used technique to carry out enzyme immobilization. Glutaraldehyde can react with different portions of the enzyme, mainly involving the primary amino groups of proteins, although it may eventually react with other groups (thiols, phenols and imidazoles) [9,26]. Supports functionalized with glutaraldehyde groups are described as being readily constructed from different supports containing primary amino groups (*i.e.*, ethylenediamine-activated supports). However, the chemistry of glutaraldehyde in aqueous solution as well as the structures related to protein crosslinking or to enzyme immobilization are not yet fully understood. Some structures have been proposed for aqueous solutions of glutaraldehyde, which reported that in the pH from 3.0 to 8.0 and under dilute conditions, the glutaraldehyde reagent may exist as monomers (free aldehyde form or pre-dominantly cyclic hemiacetal), whereas in concentrations above 25 % or under acidic conditions, oligomeric hemiacetals are formed. The equilibrium between the linear and cyclic monomeric forms is described as being linearly oriented as the temperature increases. Under basic conditions, oligomeric aldehydes may be formed by way of intermolecular aldol condensations [9,26,27].



Scheme 1. Schematic representation of the chemical modification in the surface of the agarose. The structures of the supports were idealized based on previous reviews concerning glutaraldehyde chemistry [26,27]. IDA (iminodiacetic acid); EDA (ethylenediamine); $NaBH_4$ (sodium borohydride); $NaIO_4$ (sodium periodate); GLU (glutaraldehyde); and HS-C8 (1-octanothiol).

Apart from the structural complexity presented by glutaraldehyde, its configuration already characterizes it as having distinct heterofunctionalities. Depending on the conditions applied during the process of immobilization with glutaraldehyde-activated supports, the proteins can be immobilized by three different mechanisms. On using very high ionic strength, the protein may first be immobilized by way of hydrophobic adsorption before forming the covalent bonds, whereas when using low ionic strength, primary immobilization will be by way of anion exchange. If the ionic strength applied were moderate, protein immobilization is described as being mainly due to covalent bonds [9,26,28-30].

In this work, a new generation of heterofunctional supports was proposed using different groups (hydrophobic or metal-chelate groups) capable of adsorbing proteins via different regions, followed by activation with the glutaraldehyde group, which can react covalently with the adsorbed proteins under neutral pH conditions (**Scheme 1**). Therefore, this work proposed the use of glutaraldehyde for the activation and formation of covalent linkages as a viable alternative to the alkaline mechanics currently applied. After the construction, a study was carried out with a multi-protein model system (*E. coli* BL21 crude extract) to define the conditions under which a pure immobilization mechanism occurred by way of adsorbed groups, and to define the conditions under which a connection can occur via only covalent bonds using the reagent glutaraldehyde. Structural analyses of the supports were carried out using Fourier Transformed Infrared (FTIR) spectroscopy with the aim of demonstrating the connection between the amine groups present in the support and the glutaraldehyde groups. Moreover studies using four different enzymes (*Candida rugosa* lipase-CRL, metagenomic lipase-LipC12, β-galactosidase-*Kl*Bgal and β-glucosidase-*Ea*BglA) were evaluated to verify the ability of the new supports to adsorb and form covalent linkages under neutral pH conditions. The properties of the immobilized enzymes, such as their activity and thermal stability, were also studied.

2. Results and Discussion

2.1. Construction and Analysis of New Heterofunctional Supports

Agarose-based beads were chosen as a base matrix for the construction of different heterofunctional supports. Agarose is a strongly hydrophilic, lyophilic and inert colloid that can reversibly form stable and firm gels. Its suitability as an excellent support is confirmed by its high area rich in hydroxyl groups [31,32]. Briefly, agarose was activated with epichlorohydrin, a bifunctional reagent used to obtain epoxy groups [33]. The reaction was performed in alkaline conditions where most of the primary hydroxyl groups of the support were deprotonated. The primary reaction formed epoxy groups but as the reaction occurred in an alkaline environment, some of the epoxy groups created were hydrolyzed and yielded diol groups **(Scheme 1)**. The total amount of activated primary hydroxyl groups obtained was around 65 ± 0.3 μmoL g^{-1}, with epoxy groups accounting for 23 ± 0.4 μmoL g^{-1} and diol groups accounting for 42 ± 0.4 μmoL g^{-1}. By obtaining a basic structure containing epoxy/diol groups, the new heterofunctional supports were prepared using the following

strategy: (*i*) the epoxy groups formed were functionalized with different bifunctional reagents (*i.e.*, hydrophobic or carboxylic/metal groups); and (*ii*) the diol groups were firstly oxidized with sodium periodate ($NaIO_4$), and then activated with amino groups using ethylenediamine (EDA) **(Scheme 1)**. The amino groups were applied to future activation with glutaraldehyde molecules. The new supports were constructed using very stable bifunctional groups and inert amino groups that allow storage for long periods prior to activation with the glutaraldehyde molecules.

The new heterofunctional supports are expected to perform an initial protein adsorption only by the inserted heterofunctional groups (*i.e.*, hydrophobic groups or metal chelate linked to the carboxylic groups), and only later to establish a covalent interaction with the glutaraldehyde group. However, reactive groups used in their construction, such as amino (from ethylenediamine, EDA), may interfere in a pure immobilization process. A conjugation chemistry that introduces a charged functional group into the support can cause nonspecific binding by promoting ion-exchange effects. However, in some cases, as in the traditional glutaraldehyde activated supports, a pure immobilization process is expected. Therefore, a linkage that alters the flow and binding characteristics of the support, *e.g.*, EDA, is not of interest for the work. Thus, a possible unspecific adsorption *via* ion-exchange by the amino groups was studied. A multi-protein model system (*E. coli* BL21 crude extract, 5 mg g^{-1} of support) was offered to a pure amino support (activated only with ethylenediamine/Aga-EDA), and then, the adsorption process at different ionic strengths was analyzed **(Figure 1a)**. The *E. coli* extract was applied because it contains proteins with different properties and characteristics, therefore providing different affinities for the adsorption [30]. At pH 7 and low ionic strength (25 mM sodium phosphate buffer), 50 % of proteins were quickly adsorbed in 30 min **(Figure 1a)**. An increase in the ionic strength promoted a decrease in the adsorption by amino groups until a negligible adsorption of proteins was found in 500 mM sodium phosphate buffer (even at 200 mM sodium phosphate buffer, the adsorption was almost negligible during the first hour). The results show that the new supports could not be used under low ionic strength due to protein interaction being conducted *via* a double mechanism, which would be promoted by amino groups (from ethylenediamine), and by the heterofunctional groups inserted in the new supports.

Similarly, a possible adsorption by the glutaraldehyde structure was studied. Depending on the conditions applied, the immobilization process with glutaraldehyde groups can occur by a double step: in a first step, there is the adsorption of proteins on the support and then, the covalent immobilization [26,30]. Thus, a study using a support activated only by

glutaraldehyde groups but reduced with sodium borohydride (Aga-EDA-GLU-Control) was done. The reduction step with sodium borohydride was used to transform the Schiff's bases (–N=C– double bond) into stable primary amino bonds [34]. A negligible adsorption was observed using either 200 mM or 500 mM sodium phosphate buffer **(Figure 1b)**. On the other hand, when low ionic strengths were applied, a significant protein adsorption was found (21 % in 100 mM). Therefore, the results indicate that the process of the immobilization on the new heterofunctional supports has to be carried out at a moderate ionic strength (200-500 mM) to prevent unspecific interaction, such as ion-exchange. Furthermore, it has been described that protein immobilization using glutaraldehyde groups with moderate ionic strength occur by direct covalent attachment [9,26,35], which is of interest in the present study.

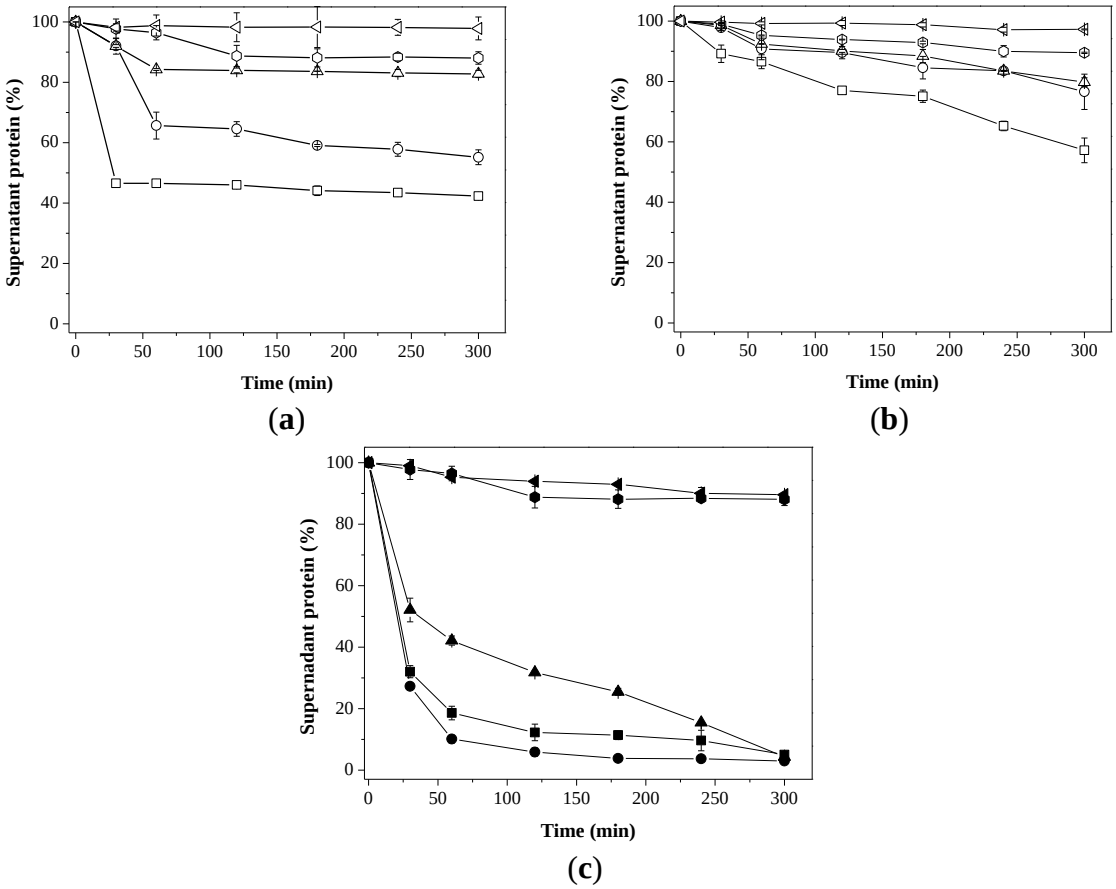
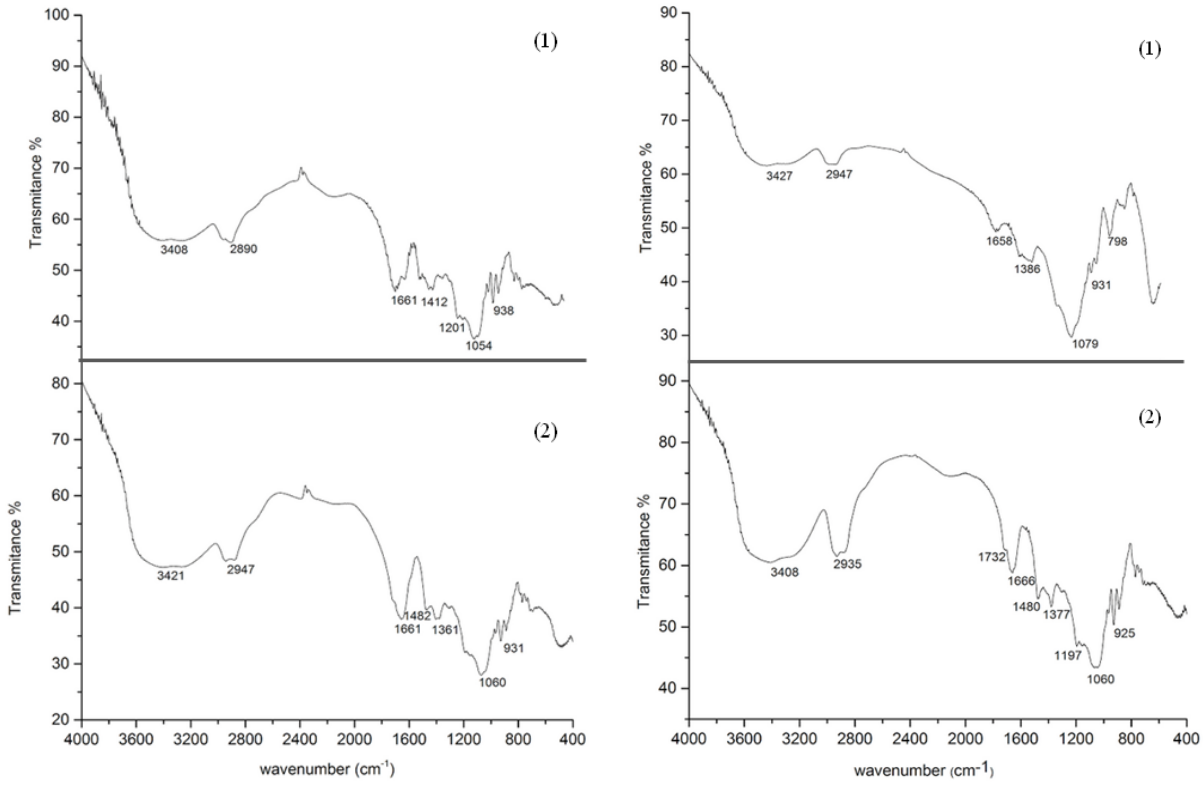


Figure 1. Immobilization of proteins present in the crude extract of *E. coli* BL21. **(a)** Immobilization on Aga-EDA support (Control). **(b)** Immobilization on Aga-EDA-GLU-Control support after the reduction process (Control).-□- 25 mM;-○- 50 mM;-△- 100 mM;-◌- 200 mM; and -◁- 500 mM sodium phosphate buffer at pH 7. **(c)** Immobilization on different supports using 200 mM sodium phosphate buffer at pH 7.-■- Aga-C8-GLU; -●- Aga-IDA-Ni^{2+}-EDA-GLU; -▲- Aga-EDA-GLU; -●-Aga-EDA (Control); and -◀- Aga-EDA-GLU-Control (Control). The results are expressed as the average of triplicate assays ± the standard error of the mean.

Considering these data, the *E. coli* BL21 crude extract was offered to new supports using moderate ionic strength (200 mM sodium phosphate buffer at pH 7) to prevent unspecific absorptions, and to promote a direct covalent and irreversible immobilization by glutaraldehyde groups. Using the new supports (Aga-C8-GLU and Aga-IDA-Metal-EDA-GLU), more than 95 % of all proteins from *E. coli* BL21 crude extract were immobilized after 5 h **(Figure 1c)**. To compare the immobilization efficiency of the new supports, the crude extract was offered to the traditional support activated using glutaraldehyde (Aga-EDA-GLU), and a similar result was obtained after 5 h **(Figure 1c)**. The controls (Aga-EDA and Aga-EDA-GLU-Control) were repeated and no protein adsorption was found **(Figure 1c)**. The findings suggest that the proteins can be immobilized on new glutaraldehyde-activated supports at moderate ionic strength, where unspecific interactions are not found.



(**a**) (**b**)

Figure 2. Fourier Transformed Infrared (FTIR) spectra for agarose supports: **(a)** Aga-IDA-EDA support (1) before modification, and (2) after chemical modifications with the agent glutaraldehyde; and **(b)** Aga-C8-EDA support, (1) before modification, and (2) after chemical modifications with the agent glutaraldehyde. Aga (agarose beads); IDA (iminodiacetic acid); EDA (ethylenediamine); and C8 (1-octanothiol).

FTIR spectra analysis was used to characterize the activation with the glutaraldehyde group, reflecting the effectiveness of the developed procedure in producing different glutaraldehyde-activated supports. The FTIR spectra were taken from untreated and treated with the glutaraldehyde group, using EDA-functionalized supports to determine any changes in chemical bonding states **(Figure 2)**. The new band around 1660 cm^{-1} (as shown in **Figure 2a (2) and 2b (2)**) was ascribed to the presence of imine (–N=C–) in the support as the result of the reaction between glutaraldehyde and the amino groups of the EDA found on the supports. Previous studies indicate that the reaction of the agent glutaraldehyde with primary amine groups in the treated support is well accepted [27,36].

2.2. Immobilization of Different Biocatalysts on the New Heterofunctional Supports

The new supports were used for the covalent immobilization of different enzymes in mild conditions (neutral pH). For the immobilization assays on these supports, four enzymes of biotechnological interest were used, namely: *Candida rugosa* lipase (CRL), metagenomic lipase (LipC12), *β*-galactosidase (*Kl*Bgal) and *β*-glucosidase (*Ea*BglA).

To prevent non-specific adsorptions, the immobilization tests were carried out using a moderate ionic strength (200 mM at pH 7). In all cases, the non-specific adsorption was negligible. **Table 1** shows the tests with the different glutaraldehyde-activated supports using the lipase from *Candida rugosa* (CRL). Lipase CRL is an enzyme used in several biocatalytic processes; however, it shows great structural instability especially at alkaline pH [25,37]. At pH 7, immobilization tests with lipase CRL on the new glutaraldehyde-activated supports were done, and the data showed that the final recovered activity was strongly dependent on the support used. The recovered activity (R) values were from 210 % (new hydrophobic group-glutaraldehyde support, Aga-C8-GLU) to 54 % (new metal group-glutaraldehyde support, Aga-IDA-Metal-EDA-GLU). Traditional glutaraldehyde support (Aga-EDA-GLU) showed almost unaltered recovered activity (R = 95 %) compared to the initial activity of the free enzyme. For further studies, lipase CRL was also immobilized on a commercial support activated only with hydrophobic groups (Octyl-Sepharose). The commercial hydrophobic support showed a recovered activity of R = 225 %, similar to that found by the new hydrophobic group-glutaraldehyde support (Aga-C8-GLU, **Table 1**). Both hydrophobic supports have shown a hyperactivation described as being related to the interfacial activation of the enzyme, whereby the catalyst may be adsorbed in its open form, compared to the soluble form in the standard conformational equilibrium [38]. Thus, the presence of the agent

glutaraldehyde on Aga-C8-GLU did not significantly alter lipase adsorption by the hydrophobic group. The results for lipase CRL immobilized on the Aga-C8-GLU were in general agreement with a previous study with octyl activated with divinyl sulfone (OCDVS, more than 3 fold recovered activity) [14].

Table 1. *Candida rugosa* lipase (CRL) and metagenomic lipase (LipC12) immobilization on glutaraldehyde-activated supports.

Enzyme	Support	Immobilization Efficiency [a] (%), IE	Recovered Activity after Immobilization at pH 7 [b] (%), R
CRL	Aga-C8-GLU	100	210
	Aga-IDA-Ni^{2+}-EDA-GLU	95	54
	Aga-EDA-GLU	100	95
	Octyl-Sepharose	100	225
LipC12	Aga-C8-GLU	100	269
	Aga- IDA-Ni^{2+}-EDA-GLU	100	100
	Aga-EDA-GLU	100	42

C8 (1-octanothiol); Ni^{2+} (nickel); EDA (ethylenediamine); IDA (iminodiacetic acid); Aga (agarose beads); and GLU (glutaraldehyde). [a] Calculated as the difference between the initial and final activities in the supernatant after 1 h of immobilization. [b] Recovered activity (%), measured as the ratio between the real activity (U g^{-1} of support) of immobilized enzymes (CRL or LipC12) and theoretical activity of the immobilized enzymes (CRL or LipC12) (U g^{-1} of support).

To analyze if CRL was covalently attached to the new supports and not only by adsorption, the different CRL preparations were boiled in Laemmli's disruption buffer (which contains mercaptoethanol and SDS) and analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE, **Figure 3**). CRL was used as a model because it is a monomeric enzyme. The glutaraldehyde is known to establish covalent bonds at pH 7 [9,39]. According to SDS-PAGE results, the new glutaraldehyde-activated supports did not allow the release of the immobilized CRL, proving that the new supports can promote the adsorption and subsequent covalent immobilization by the glutaraldehyde group at pH 7. CRL immobilized on Octyl-Sepharose produced a band in SDS-PAGE **(Figure 3)**, which indicates only the adsorption to the hydrophobic groups.

Further experiments of lipase CRL desorption from the different supports were done by the addition of Triton X100, NaCl and imidazol **(Table 2)**. The tests were used to prove that lipase CRL was not hydrophobic or ion-bound to the new supports. In the analysis, the activities of supernatants and suspensions were measured to verify the desorption of the immobilized CRL. The use of the non-ionic detergent Triton X100 produced a significant

desorption of the immobilized CRL on Octyl-Sepharose (81 % initial activity), while a negligible activity was obtained in the supernatant for the new hydrophobic group-glutaraldehyde support/Aga-C8-GLU **(Table 2)**. Higher concentrations of Triton X100 were not able to remove CRL from the new glutaraldehyde-activated support (data not shown). Analysis using NaCl and imidazol showed a negligible activity found in the supernatant for the immobilized CRL on Aga-EDA-GLU and Aga-IDA-Metal-EDA-GLU supports. These results confirm the covalent immobilization of lipase CRL by the glutaraldehyde group at pH7.

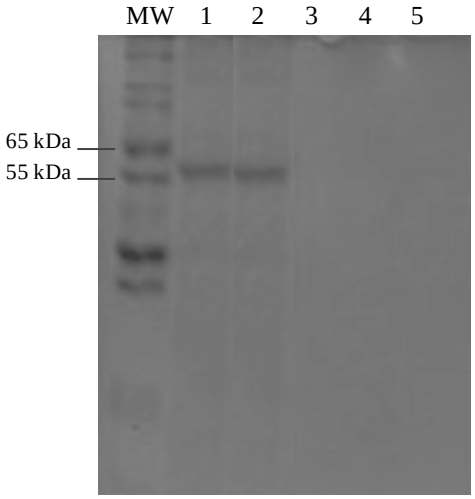


Figure 3. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of CRL desorption assays from different supports. The lanes were loaded as follows: **lane MW**, protein molecular weight markers; **lane 1**, Free CRL lipase; **lane 2**, eluted fraction from the commercial Octyl-Sepharose; **lane 3**, eluted fraction from Aga-EDA-GLU at pH 7; **lane 4**, eluted fraction from Aga-C8-GLU at pH 7; **lane 5**, eluted fraction from Aga-IDA-Ni^{2+}-EDA-GLU at pH 7. The proteins were stained with Coomassie Brilliant Blue R-250.

Another monomeric lipase obtained from metagenomics (lipase LipC12, [40]) was also studied **(Table 1)**. All the assayed supports allowed the complete immobilization of lipase LipC12. The recovered activities were different depending on the support used in the immobilization **(Table 1)**. Upon immobilization on all supports, a high-recovered activity (R = 269 %) was obtained with the new hydrophobic group-glutaraldehyde support (Aga-C8-GLU) showing a hyperactivation of lipase LipC12 **(Table 1)**. Moreover, LipC12 immobilized on Aga-IDA-Metal-EDA-GLU and Aga-EDA-GLU (traditional support) showed recovered activity of 100 % and 42 %, respectively. The traditional glutaraldehyde-activated support used as control system showed a low recovered activity of LipC12 in relation to the new

supports. A similar study of covalent attachment by the glutaraldehyde group was accomplished, and no desorption was found for LipC12 immobilized on the new supports (data not shown).

Table 2. Desorption assays of different CRL preparations.

Support	Immobilization Efficiency [a] (%), IE	Recovered Activity after Incubation [b] (%), R	Activity in the Supernatant after Incubation (%)
Aga-EDA-GLU *	100	97	0
Aga-C8-GLU **	100	90	0
Octyl-Sepharose **	100	12	81
Aga-IDA-Ni^{2+}-EDA-GLU	100	98	0

C8 (1-octanothiol); Ni^{2+} (nickel); EDA (ethylenediamine); IDA (iminodiacetic acid); Aga (agarose beads); and GLU (glutaraldehyde). [a] Calculated as the difference between the initial and final activities in the supernatant after 1 h of immobilization. [b] Recovered activity (%), measured as the ratio between the real activity (U g^{-1} of support) of immobilized lipase CRL and theoretical activity of the immobilized lipase CRL (U g^{-1} of support).
* Preparation incubated in 1 M NaCl; ** Preparation incubated in 0.5 % of Triton X100; *** Preparation incubated in 0.5 M imidazol.

Table 3. *β*-galactosidase (*Kl*gal) and *β*-glucosidase (*Ea*glA) immobilization on glutaraldehyde-activated supports.

Enzyme	Support	Immobilization Efficiency [a] (%), IE	Recovered Activity after Immobilization at pH 7 [b] (%),
*Kl*Bgal	Aga-C8-GLU	100	81
	Aga-IDA-Co^{2+}-EDA-	>94	78
	Ag-EDA-GLU	100	70
*Ea*BglA	Aga-C8-GLU	100	0
	Aga-IDA-Co^{2+}-EDA-	100	64
	Aga-EDA-GLU	100	50

C8 (1-octanothiol); Co^{2+} (cobalt); EDA (ethylenediamine); IDA (iminodiacetic acid); Aga (agarose beads); and GLU (glutaraldehyde). [a] Calculated as the difference between the initial and final activities in the supernatant after 1 h of immobilization. [b] Recovered activity (%), measured as the ratio between the real activity (U g^{-1} of support) of immobilized enzymes (*Kl*Bgal or *Ea*BglA) and theoretical activity of the immobilized enzymes (*Kl*Bgal or *Ea*BglA) (U g^{-1} of support).

Tests analyzing the possibility of using the new glutaraldehyde-activated supports on the immobilization of two complex multimeric enzymes were studied. *β*-galactosidase (*Kl*Bgal) and *β*-glucosidase (*Ea*BglA) are described as tetrameric enzymes [41,42]. The structural complexity of these catalysts represents an especially complex problem for

immobilization because small disturbances in their quaternary-structures may alter the precise distribution of the native state ensemble [39,43], with dissociation of subunits producing enzymatic inactivation. Therefore, these enzymes are fragile even in mild conditions. **Table 3** shows the results for *Kl*Bgal and *Ea*BglA with a final recovered activity ranging from 81 to 70 % for the *β*-galactosidase and from 64 to 0 % for the *β*-glucosidase. *Kl*Bgal immobilized on the new supports showed recovered activity values of 81 % (Aga-C8-GLU) and 78 % (Aga-IDA-Co^{2+}-EDA-GLU). The traditional GLU supports (Aga-EDA-GLU) had an R 70 %. The traditional glutaraldehyde-activated support (control system) showed a low recovered activity of *Kl*Bgal in relation to the new supports. A similar result has been reported for the covalent immobilization of *Kluyveromyces lactis* β-galactosidase in glyoxyl-Sepharose support [20], retaining approximately 82 % of its initial activity after immobilization. In the case of *Ea*BglA, Aga-IDA-Co^{2+}-EDA-GLU showed the highest R (64 %), following the traditional Aga-EDA-GLU with R 50 %. The new hydrophobic-glutaraldehyde support yielded an inactive derivative after the immobilization process. Zanphorlin et al. [42] describe that the selective pressure at low temperatures favored mutations that redesigned the *Ea*BglA protein surface, reducing the number of salt bridges, and exposing more the hydrophobic regions to the solvent. Its can thus be concluded that one reason for the inactivation of *Ea*BglA when immobilized in the hydrophobic support may be due to the interaction between high number of hydrophobic groups in the protein surface and the hydrophobic group of the support. As in the other cases, no activity was detected after desorption of the different preparations after incubation in Triton X100, NaCl and imidazol.

2.3. Thermal Stability

Enzymatic stabilization has a great importance due to the increasing number of applications of enzymes in almost all areas and it is one of the main aims when the immobilization of enzymes is performed [6]. Therefore, the thermal stabilities of different immobilized preparations and free forms were studied in 100 mM phosphate buffers, pH 7 using distinct temperatures. **Figure 4a** shows the tests using lipase CRL preparations. The stability of the adsorbed preparation (Octyl-Sepharose) was considerably similar to the free form with around 3.6-fold stabilization. The maximal stability was obtained with the traditional glutaraldehyde support (Aga-EDA-GLU) with a half-life of around 1560 min after incubation at 50 °C **(Table 4)**. Considering that the half-life of the free CRL was 21 min, the

stabilization factor obtained was around 74.3-fold. CRL immobilized on Aga-C8-GLU and Aga-IDA-Ni^{2+}-EDA-GLU was 5.7 and 2.8-fold more stable than the free form.

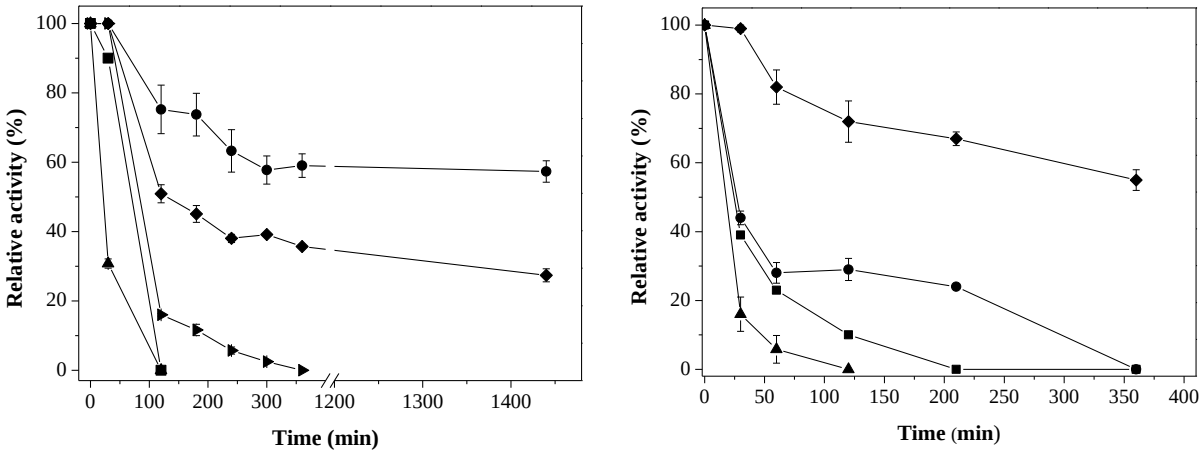


Figure 4. Thermal stability. **(a)** Lipase CRL at pH 7, 50 °C. (■) Aga-IDA-Metal-EDA-GLU; (◆) Aga-C8-GLU; (●) Aga-EDA-GLU; (►) Octyl-Sepharose; and (▲) free enzyme. **(b)** Lipase LipC12 at pH 7, 70 °C. (■) Aga-IDA-Metal-EDA-GLU; (◆) Aga-C8-GLU; (●) Aga-EDA-GLU; and (▲) free enzyme. The results are expressed as the average of triplicate assays ± the standard error of the mean.

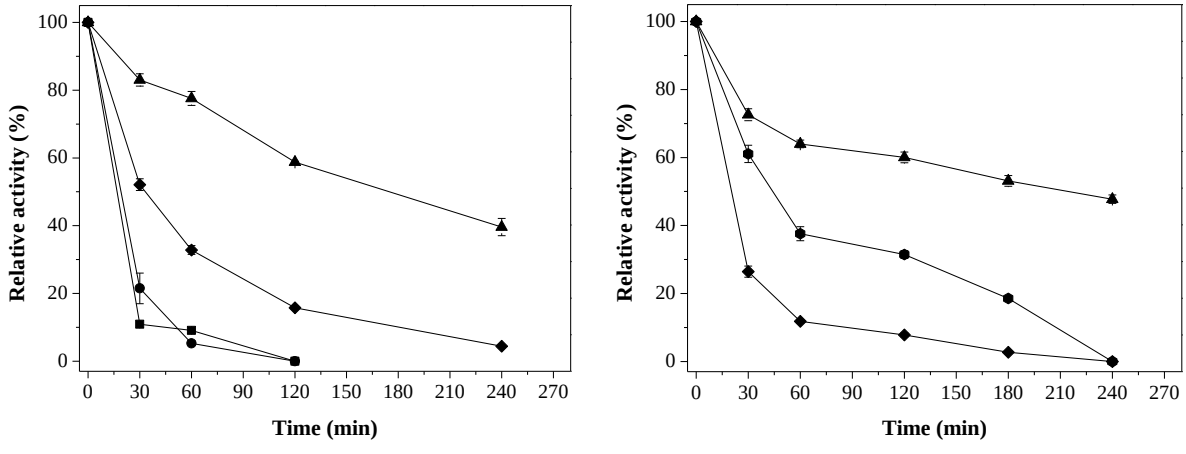


Figure 5. Thermal stability. **(a)** *β*-galactosidase (*Kl*gal) at pH 7, 40 °C. (■) Aga-C8-GLU; (●) Ag-EDA-GLU; (▲) Aga-IDA-Metal-EDA-GLU, and (◆) free enzyme. **(b)** *β*-glucosidase (*Ea*glA) at pH 7, 40 °C. (●) Ag-EDA-GLU; (▲) Aga-IDA-Metal-EDA-GLU, and (◆) free enzyme. The results are expressed as the average of triplicate assays ± the standard error of the mean.

The thermal stability of lipase LipC12 preparations was assessed by incubation at 70 °C **(Figure 4b, Table 4)**. At this temperature, the native free LipC12 had a half-life time of 13 min. The most stable preparation was found using the new hydrophobic group-glutaraldehyde support (Aga-C8-GLU) with a stabilization factor around 32.3-fold compared to the free form. The stability of LipC12 immobilized on the traditional amino (Ag-EDA-GLU) and new metal group-glutaraldehyde support (Aga-IDA-Ni^{2+}-EDA-GLU) was, respectively, 5.8 and 1.8-fold higher than the free LipC12. The high improvement of lipase LipC12 after immobilization (Aga-C8-GLU/LipC12) is important because it permits the transformation of a mesophilic enzyme into an enzyme with properties that are similar to those of thermophilic organisms, such as *Bacillus thermocatenolatus* lipase (BTL) and *Thermus thermophilus* lipase (TTL) [44,45].

Table 4. Half-lifes (in minutes) of different immobilized preparations.

Enzyme	Support	Half-Life ($T_{1/2}$, Minutes)	Stability Factor
CRL[a]	Free form	21	
	Aga-C8-GLU	120	5.7
	Aga-IDA-Ni^{2+}-EDA-GLU	60	2.8
	Aga-EDA-GLU	1560	74.3
	Octyl-Sepharose	76	3.6
LipC12[b]	Free form	13	
	Aga-C8-GLU	420	32.3
	Aga- IDA-Ni^{2+}-EDA-GLU	23	1.8
	Aga-EDA-GLU	76	5.8
*Kl*Bgal[c]	Free form	33	
	Aga-C8-GLU	17	0.52
	Aga- IDA-Co^{2+}-EDA-GLU	160	4.8
	Aga-EDA-GLU	12	0.36
*Ea*BglA[c]	Free form	21	
	Aga-C8-GLU	-	-
	Aga- IDA-Co^{2+}-EDA-GLU	230	10.9
	Aga-EDA-GLU	39	1.8

C8 (1-octanothiol); Ni^{2+} (nickel); Co^{2+} (cobalt); EDA (ethylenediamine); IDA (iminodiacetic acid); Aga (Agarose beads); and GLU (glutaraldehyde). [a] Thermal stability of lipase CRL preparations was checked by incubation at 50 °C. [b] Thermal stability of lipase LipC12 preparations was checked by incubation at 70 °C. [c] Thermal stability of *Kl*gal and *Ea*glA preparations was checked by incubation at 40 °C. Aliquots were periodically withdrawn for quantification of the residual enzymatic activity to estimate the half-life according to Henley and Sadana [46]. The stability factors were done in relation to the free form.

Figure 5 shows the thermal stability of the immobilized *Kl*Bgal and *Ea*BglA at 40 °C. For *Kl*Bgal preparations, only Aga-IDA-Co^{2+}-EDA-GLU promoted a significant

stabilization (4.8-fold factor) compared to the free form (half-life of 33 min at 40 °C, **Figure 5a** and **Table 4**). All other preparations showed a lower stabilization than the free form (Aga-C8-GLU 0.52 and Aga-EDA-GLU 0.36-fold factor, **Table 4**). For studies with the immobilized *Ea*BglA, it was observed that the Aga-IDA-Co^{2+}-EDA-GLU (half-life time of 230 min) showed the best result, which was more stable (5.9-fold factor) than the traditional GLU preparation (Aga-EDA-GLU), and 10.9-times more stable than free enzyme (half-life time of 21 min at 40 °C, **Figure 5b** and **Table 4**). The Aga-EDA-GLU preparation also presents a half-life of 39 min (1.8-fold factor more stable than free form). In all cases, the stability of different preparations was strongly dependent on the support used in the immobilization process.

3. Materials and Methods

3.1. Materials

Agarose 4 BCL was purchased from Agarose Bead Technologies (Madrid, Spain). Epichlorohydrin, iminodiacetic acid, triethylamine, sodium borohydride, sodium periodate, 1-octanothiol, bovine serum albumin, *o*-nitro-phenyl-*β*-D-galactopyranoside (*o*-NPG), *p*-nitrophenyl proprionate (*p*-NPP), 4-nitrophenyl-*β*-D-glucopyranoside (*p*-NPG), and high molecular weight protein (Sigma Marker™) were purchased from Sigma (Sigma-Aldrich®, St Louis, MO, USA). Glutaraldehyde solution (25 %, *v/v*) and ethylenediamine were purchased from Alfa Aesar (Thermo Fisher Scientific®). Octyl-Sepharose CL-4B was purchased from GE Healthcare Bio-Sciences (Uppsala, Sweden). The *β*-galactosidase from *Kluyveromyces lactis* (Lactozym pure 6500L) (*Kl*Bgal) was kindly supplied by Novozymes A/S (Denmark). The lipase from *Candida rugosa* (CRL) was purchased from Sigma (Sigma-Aldrich®, St Louis, MO, USA). Overexpression and purification of lipase (LipC12) and *β*-glucosidase (*Ea*BglA) were performed as previously described by Glogauer et al. [40] and Crespim et al. [47], respectively. All other chemicals used were of analytical grade.

3.2. Standard Determination of Enzymatic Activities

β-glucosidase assays. The activity was determined using 4-nitrophenyl *β*-D-glucopyranoside (*p*-NPG) as substrate. The free or immobilized enzyme was added to a mixture solution (5 mM *p*-NPG in 50 mM sodium phosphate buffer at pH 7) and the increase

in absorbance was monitored at 410 nm (pH 7.0, $\varepsilon_{410\ nm}$= 7320 M^{-1} cm^{-1}) [47]. One unit (U) of enzyme was defined as the amount of enzyme required to release1 μmoL of *p*-nitrophenol per min and the specific activity was defined as the number of units per mg of protein.

Lipase assays. The activities were determined using *p*-nitrophenyl propionate (*p*-NPP) as substrate. The free or immobilized enzymes were added to a mixture solution (0.4 mM *p*-NPP in 50 mM sodium phosphate buffer at pH 7) and the increase in absorbance was monitored at 348 nm (pH 7.0, $\varepsilon_{348\ nm}$= 5150 M^{-1} cm^{-1}) [29]. One unit (U) of enzyme was defined as the amount of enzyme required to release 1 μmoL of *p*-nitrophenol per min and the specific activity was defined as the number of units per mg of protein.

β-galactosidase assays. The activity was determined using *o*-nitro-phenyl-*β*-D-galactopyranoside (*o*-NPG) as substrate. The free or immobilized enzyme was added to a mixture solution (5 mM *o*-NPG in 50 mM potassium phosphate buffer at pH 7 with the addition of 2 mM $MgCl_2$) and the increase in absorbance was monitored at 410 nm (pH 7.0, $\varepsilon_{410\ nm}$= 3500 M^{-1} cm^{-1}) [48]. One unit (U) of enzyme was defined as the amount of enzyme required to release 1 μmoL of *o*-NP per min and the specific activity was defined as the number of units per mg of protein.

3.3. Supports for Immobilization

3.3.1. Activation of Agarose-Based Beads

Epoxy-activated agarose was prepared by the reaction of the hydroxyl groups on the agarose beads with epichlorohydrin, as described by Mateo et al. [24]. Under gentle agitation and in an ice bath, 50 g of agarose 4 BCL, previously washed with distilled water and vacuum dried, was mixed with 220 mL of distilled water, 16.4 g of NaOH, 1.0 g of sodium borohydride ($NaBH_4$), 80 mL of acetone, and 55 mL of epichlorohydrin. The suspension was incubated for 16 h at 25 °C, and then washed with the excess of distilled water, vacuum dried, and stored at 4 °C.

The number of epoxy/ligand groups was calculated from the difference in periodate consumption between the hydrolyzed support and the initial epoxy support. Periodate consumption was quantified using potassium iodide, as previously described [49].

3.3.2. Modification of the Epoxy-Activated Agarose with Different Reactive Groups

Cationic Supports

Epoxy-activated agarose (10 g) was modified using 1 M triethylamine solution (acetone/water, 50:50 *v/v*) for 18 h at 25 °C [24]. After this, the support was oxidized with 100 mL of 100 mM sodium periodate ($NaIO_4$) for 2 h at 25 °C, washed with distilled water, vacuum dried and stored at 4 °C.

Support Activated with the Glutaraldehyde Group

The support activated using the glutaraldehyde group was prepared as previously described by Betancor et al. [35]. Briefly, the epoxy-activated agarose (10 g) was hydrolyzed with 100 mL of 1 M H_2SO_4 for 2 h at 25 °C. Afterwards, the support was washed with distilled water and oxidized using 100 mL of 100 mM $NaIO_4$. Then, the support was treated with 100 mL of 2 M ethylenediamine (EDA) at pH 10, and kept under gentle stirring at 25 ^{0}C. After 2 h, sodium borohydride (10 mg mL^{-1}) was added and stirred for further 2 h at 25 °C. The particles were successively washed with distilled water and 11 mL of glutaraldehyde solution (25 %, *v/v*) was added together with 17 mL of sodium phosphate buffer (200 mM at pH 7). The system was kept under gentle stirring for 18 h at 25 °C. Finally, the activated support was washed with distilled water and vacuum dried.

Anionic Supports Activated with the Glutaraldehyde Group and Metal Chelate

Agarose support (10 g) activated with the glutaraldehyde group and metal chelate was obtained by treatment of the epoxy-activated agarose with 100 mL of 500 mM iminodiacetic acid (IDA) at pH 11 for 18 h at 25 °C [24]. Then, the support was washed with distilled water and oxidized using 100 mL of sodium periodate ($NaIO_4$) at a final concentration of 100 mM. After 2 h of gentle agitation at 25 °C, the oxidized support was washed with distilled water and treated with 100 mL of 2 M ethylenediamine (EDA) pH 10 for 2 h at 25 °C. Sodium borohydride (10 mg mL^{-1}) was added, and stirred for further 2 h at 25 °C. The activated agarose was successively washed with distilled water. Glutaraldehyde solution (25 %, *v/v*) and sodium phosphate buffer (200 mM at pH 7) were added, and the system was kept under gentle stirring for 18 h at 25 °C. The agarose activated with glutaraldehyde was added to the

metal chelate solutions (30 mg mL^{-1}, $CoCl_2$ or $NiSO_4$) at pH 7 for 30 minutes at 25 °C. The activated support was washed with distilled water and vacuum dried.

Hydrophobic Supports Activated with the Glutaraldehyde Group

Agarose activated with the hydrophobic group and glutaraldehyde was prepared by treatment of the epoxy-activated agarose with 100 mL of 100 mM 1-octanothiol in $NaHCO_3$ (25 mM) at pH 10 for 24 h at 25 °C. Thereafter, the support was oxidized with 100 mL of $NaIO_4$ (100 mM), washed, and filtered using a glass filter. The hydrophobic support was then treated with 100 mL of 2 M ethylenediamine (EDA), pH 10 for 2 h at 25 °C, following the addition of sodium borohydride (10 mg mL^{-1}) for 2 h at 25 °C. After it was washed and dried, the glutaraldehyde solution (25 %, *v/v*) and sodium phosphate buffer (200 mM at pH 7.0) were added. The activated support was washed with distilled water and vacuum dried.

3.4. Immobilization of Enzymes

For all immobilization experiments, *β*-glucosidase (*Ea*BglA) and lipases (CRL and LipC12) were solubilized in 200 mM sodium phosphate buffer at pH 7, and *β*-galactosidase (*Kl*Bgal) was solubilized in 200 mM potassium phosphate buffer at pH 7 with the addition of 2 mM $MgCl_2$. Then, different immobilization supports were suspended in an enzyme solution (1 g support: 10 mL of enzyme solution in the immobilization buffer, 1 mg g^{-1} of support). The immobilization suspensions were gently stirred at 25 °C. Periodically, samples of supernatants were withdrawn, and their enzymatic activities were analyzed. The immobilization was considered complete when there was no activity in the supernatant.

After immobilization, the enzymes were washed with phosphate buffers. The immobilization efficiency (IE, %) was calculated as (Equation 1):

$$IE = \frac{A_i - A_f}{A_i}\ x\ 100\% \quad (1)$$

where A*i* is the amount activity (U) of the enzyme solution before immobilization and A_f is the amount activity (U) remaining in the supernatant at the end of the immobilization procedure.

The recovered activity (R, %) was calculated as (Equation 2):

$$R = \frac{A_o}{A_T} x\ 100\% \quad (2)$$

where A_0 is the as the ratio between the real specific activity (U g^{-1} of support) of immobilized preparation and A_T is the theoretical specific activity of the immobilized preparation (U g^{-1} of support).

Escherichia coli BL21 crude extract was used as a multi-protein model system during the immobilization tests with the different supports. One gram of activated supports was suspended in 10 mL of *E. coli* BL21 crude cell extract (5 mg g^{-1} of support). The adsorption of proteins was monitored using the Bradford method.

3.5. Protein Assays and SDS-PAGE

Protein content was determined by the Bradford method [50], using a Coomassie Protein Assay Kit (Pierce Biotechnology, Rockford, IL, USA) with bovine serum albumin as the standard. Electrophoresis of protein samples was done with 12 % (*w/v*) SDS-PAGE [51]. The gel was stained with Coomassie Brilliant Blue R-250 and distained with methanol/acetic-acid/water (5/1/4 *v/v/v*). A mixture of high molecular weight proteins (Sigma Marker™, Sigma-Aldrich®) was used as the molecular weight standard.

3.6. Fourier Transform Infrared (FTIR)

Identification of the chemical groups on the untreated and treated support was performed using the FTIR spectroscopy Vertex 70 Bruker. The spectra were obtained with a wave number range from 4000 to 400 cm^{-1} at a resolution of 4 cm^{-1} over 32 cumulative scans. FTIR spectra were not used as quantitative relationship, but only as a qualitative reference.

3.7. Desorption Process Analysis

Samples of 1 g of immobilized enzymes were suspended in 3 mL of phosphate buffer (5 mM, at pH 7). Then, Triton X100, NaCl and imidazol were added to a final concentration of 0.5 % (*v/v*), 1.0 M and 0.5 M, respectively. The different preparations were gently stirred at 25 °C and periodically, samples from supernatants were withdrawn and their enzymatic activities were analyzed.

3.8. Thermal Stability

Thermal stabilities were assessed by incubation in sodium phosphate buffer (100 mM, pH 7 at 40 °C) for *β*-glucosidase, potassium phosphate buffer containing 2 mM $MgCl_2$ (100 mM, pH 7 at 40 °C) for *β*-galactosidase, and sodium phosphate buffer (100 mM, pH 7 at 50 °C or 70 °C) for lipases (CRL and LipC12). Aliquots were periodically withdrawn for the quantification of the residual enzymatic activity to estimate the half-life time according to Henley and Sadana [46]. The relative activities of free and immobilized forms without incubation were defined as control and attributed a relative activity of 100 %. Inactivation parameters were determined from the best-fit model of the experimental data which was the one based on a two-stage series inactivation mechanism with residual activity. Half-lifes (time in which the residual enzyme activity is half of its initial value) were used to compare the stability of the different preparations, being determined by the interpolation from the respective models described in [52].

4. Conclusions

The new generation of supports proposed in this study overcomes some of problems associated with the immobilization of enzymes intrinsically unstable at alkaline pH. The new supports were capable of immobilizing distinct enzymes with significant recovered activity values. Thus, this strategy allowed obtaining heterogeneous catalysts with different catalytic properties (*i.e.*, activity and stability) under mild conditions (neutral pH).

The strategy of this study can be applied to other supports activated in similar ways, such as cellulose, permitting the improvement of the materials or the use of supports with distinct morphologies or properties.

Acknowledgments: The authors gratefully acknowledge the Ramón Areces Foundation for financial support and the Brazilian National Council for Scientific and Technological Development (CNPq) for fellowships (Grant No: 201757/2015-0 and 201688/2015-8). FAPESP is also recognized (FAPESP 2013/01710-1 and FAPESP fellowships 2016/15037-5). We thank Novozymes A/S for the donation of the β-galactosidase used in this research.

Author Contributions: R.R.M. and R.C.A. performed most of the experiments and contributed with manuscript writing. A.F.L.V. performed some of experiments. E.M.S, N.K.,

R.R. and H.H.S. contributed in the analysis of the data and in writing the paper. C.M. conceived and designed the experiments and contributed with the writing of the paper.

Conflicts of Interest: The authors declare no conflict of interest.

5. References

1. Roy, J. J.; Abraham, T. E. Strategies in making cross-linked enzyme crystals. *Chem. Rev.* **2004**, *104*, 3705–3721.

2. Cao, L. Immobilised enzymes: Science or art? *Curr. Opin. Chem. Biol.* **2005**, *9*, 217–226.

3. Eş, I.; Vieira, J. D. G.; Amaral, A. C. Principles, techniques, and applications of biocatalyst immobilization for industrial application. *Appl. Microbiol. Biotechnol.* **2015**, *99*, 2065–2082.

4. Sheldon, R. A.; Van Pelt, S. Enzyme immobilisation in biocatalysis: Why, what and how. *Chem. Soc. Rev.* **2013**, *42*, 6223–6235.

5. Rodrigues, R. C.; Ortiz, C.; Berenguer-Murcia, Á.; Torres, R.; Fernández-Lafuente, R. Modifying enzyme activity and selectivity by immobilization. *Chem. Soc. Rev.* **2013**, *42*, 6290–6307.

6. Mateo, C.; Palomo, J. M.; Fernandez-Lorente, G.; Guisan, J. M.; Fernandez-Lafuente, R. Improvement of enzyme activity, stability and selectivity via immobilization techniques. *Enzyme Microb. Technol.* **2007**, *40*, 1451–1463.

7. Mateo, C.; Abian, O.; Bernedo, M.; Cuenca, E.; Fuentes, M.; Fernandez-Lorente, G.; Palomo, J. M.; Grazu, V.; Pessela, B. C. C.; Giacomini, C.; Irazoqui, G.; Villarino, A.; Ovsejevi, K.; Batista-Viera, F.; Fernandez-Lafuente, R.; Guisán, J. M. Some special features of glyoxyl supports to immobilize proteins. *Enzyme Microb. Technol.* **2005**, *37*, 456–462.

8. Mateo, C.; Palomo, J. M.; Fuentes, M.; Betancor, L.; Grazu, V.; López-Gallego, F.; Pessela, B. C. C.; Hidalgo, A.; Fernández-Lorente, G.; Fernández-Lafuente, R.; Guisán, J. M. Glyoxyl agarose: A fully inert and hydrophilic support for immobilization and high stabilization of proteins. *Enzyme Microb. Technol.* **2006**, *39*, 274–280.

9. Santos, J. C. S. Dos; Barbosa, O.; Ortiz, C.; Berenguer-Murcia, A.; Rodrigues, R. C.; Fernandez-Lafuente, R. Importance of the support properties for immobilization or purification of enzymes. *ChemCatChem* **2015**, *7*, 2413–2432.

10. Tural, B.; Tarhan, T.; Tural, S. Covalent immobilization of benzoylformate decarboxylase from *Pseudomonas putida* on magnetic epoxy support and its carboligation reactivity. *J. Mol. Catal. B Enzym.* **2014**, *102*, 188–194.

11. Alnoch, R. C.; Melo, R. R. De; Palomo, J. M.; Souza, E. M. De; Krieger, N.; Mateo, C.

New tailor-made alkyl-aldehyde bifunctional supports for lipase immobilization. *Catalyst* **2016**, *6*, 191.

12. Garmroodi, M.; Mohammadi, M.; Ramazani, A.; Ashjari, M.; Mohammadi, J.; Sabour, B.; Yousefi, M. Covalent binding of hyper-activated *Rhizomucor miehei* lipase (RML) on hetero-functionalized siliceous supports. *Int. J. Biol. Macromol.* **2016**, *86*, 208–215.

13. Rueda, N.; Albuquerque, T.; Bartolome-Cabrero, R.; Fernandez-Lopez, L.; Torres, R.; Ortiz, C.; dos Santos, J.; Barbosa, O.; Fernandez-Lafuente, R. Reversible immobilization of lipases on heterofunctional octyl-amino agarose beads prevents enzyme desorption. *Molecules* **2016**, *21*, 646.

14. Albuquerque, T. L. de; Rueda, N.; dos Santos, J. C. S.; Barbosa, O.; Ortiz, C.; Binay, B.; Ozdemir, E.; Gonçalves, L. R. B.; Fernandez-Lafuente, R. Easy stabilization of interfacially activated lipases using heterofunctional divinyl sulfone activated-octyl agarose beads. Modulation of the immobilized enzymes by altering their nanoenvironment. *Process Biochem.* **2016**, *51*, 865–874.

15. Guajardo, N.; Bernal, C.; Wilson, L.; Cabrera, Z. Selectivity of R-α-monobenzoate glycerol synthesis catalyzed by *Candida antarctica* lipase B immobilized on heterofunctional supports. *Process Biochem.* **2015**, *50*, 1870–1877.

16. Hirata, D. B.; Albuquerque, T. L.; Rueda, N.; Virgen-ortíz, J. J.; Tacias-pascacio, V. G.; Fernandez-lafuente, R. Enzymatic evaluation of different immobilized lipases in transesterification reactions using tributyrin: Advantages of the heterofunctional octyl agarose beads. *J. Mol. Catal. B, Enzym.* **2016**, *133*, 117–123.

17. Rueda, N.; Cleiton, S.; Daniela, M.; Albuquerque, T. L.; Barbosa, O.; Torres, R.; Ortiz, C.; Fernandez-lafuente, R. Enzymatic reversible immobilization of lipases on octyl-glutamic agarose beads: A mixed adsorption that reinforces enzyme immobilization. *J. Mol. Catal. B, Enzym.* **2016**, *128*, 10–18.

18. Pessela, B. C. C.; Mateo, C.; Fuentes, M.; Vian, A.; Carrascosa, A. V; Guisa, M. Stabilization of a multimeric β-galactosidase from *Thermu*s sp . strain T2 by immobilization on novel heterofunctional epoxy supports plus aldehyde-dextran cross-linking. *Biotechnol. Prog.* **2004**, 388–392.

19. Guerrero, C.; Vera, C.; Serna, N.; Illanes, A. Immobilization of *Aspergillus oryzae* β-galactosidase in an agarose matrix functionalized by four different methods and application to the synthesis of lactulose. *Bioresour. Technol.* **2017**, *232*, 53–63.

20. Bernal, C.; Marciello, M.; Mesa, M.; Sierra, L.; Fernandez-lorente, G.; Mateo, C.; Guisan, J. M. Immobilisation and stabilisation of β-galactosidase from *Kluyveromyces lactis* using a

glyoxyl support. *Int. Dairy J.* **2013**, *28*, 76–82.

21. Mateo, C.; Grazu, V.; Palomo, J. M.; Lopez-gallego, F.; Fernandez-lafuente, R. Immobilization of enzymes on heterofunctional epoxy supports. *Nature.* **2007**, *5*, 133.

22. Blanco, R. M.; Guistin, J. M. Stabilization of enzymes by multipoint covalent attachment to agarose-aldehyde gels . Borohydride reduction of trypsin-agarose derivatives. *Enzym. Microb. Technol.* **1989**, *11*, 360–366.

23. Tardioli, P. W.; Fernandez-Lafuente, R.; Guisan, J. M.; Giordano, R. L. C. Design of new immobilized-stabilized carboxypeptidase a derivative for production of aromatic free hydrolysates of proteins. *Biotechnol. Prog.* **2003**, *19*, 565–574.

24. Mateo, C.; Bolivar, J. M.; Godoy, C. A.; Rocha-Martin, J.; Pessela, B. C.; Curiel, J. A.; Munoz, R.; Guisan, J. M.; Fernández-Lorente, G. Improvement of enzyme properties with a two-step immobilizaton process on novel heterofunctional supports. *Biomacromolecules* **2010**, *11*, 3112–3117.

25. Rivero, C. W.; Palomo, J. M. Covalent immobilization of *Candida rugosa* lipase at alkaline pH and their application in the regioselective deprotection of Per-O-acetylated thymidine. *Catalyst* **2016**, *6*, 115.

26. Barbosa, O.; Ortiz, C.; Berenguer-Murcia, Á.; Torres, R.; Rodrigues, R. C.; Fernandez-Lafuente, R. Glutaraldehyde in bio-catalysts design: A useful crosslinker and a versatile tool in enzyme immobilization. *RSC Adv.* **2014**, *4*, 1583.

27. Migneault, I.; Dartiguenave, C.; Bertrand, M. J.; Waldron, K. C. Glutaraldehyde: Behavior in aqueous solution, reaction with proteins, and application to enzyme crosslinking. *Biotechniques* **2004**, *37*, 790–802.

28. Barbosa, O.; Torres, R.; Ortiz, C.; Fernandez-lafuente, R. Versatility of glutaraldehyde to immobilize lipases: Effect of the immobilization protocol on the properties of lipase B from *Candida antarctica*. *Process Biochem.* **2012**, *47*, 1220–1227.

29. Fernández-Lorente, G.; Palomo, J. M.; Mateo, C.; Munilla, R.; Ortiz, C.; Cabrera, Z.; Guisán, J. M.; Fernández-Lafuente, R. Glutaraldehyde cross-linking of lipases adsorbed on aminated supports in the presence of detergents leads to improved performance. *Biomacromolecules* **2006**, *7*, 2610–2615.

30. Bezbradica, D. I.; Mateo, C.; Guisan, J. M. Novel support for enzyme immobilization prepared by chemical activation with cysteine and glutaraldehyde. *J. Mol. Catal. B Enzym.* **2014**, *102*, 218–224.

31. Trivedi, T. J.; Srivastava, D. N.; Rogers, R. D.; Kumar, A. Agarose processing in protic and mixed protic–aprotic ionic liquids: Dissolution, regeneration and high conductivity, high

strength ionogels. *Green Chem.* **2012**, *14*, 2831.

32. Zucca, P.; Fernandez-Lafuente, R.; Sanjust, E. Agarose and its derivatives as supports for enzyme immobilization. *Molecules* **2016**, *21*, 1–25.

33. Mateo, C.; Abian, O.; Fernandez-Lafuente, R.; Guisan, J. M. Increase in conformational stability of enzymes immobilized on epoxy-activated supports by favoring additional multipoint covalent attachment. *Enzyme Microb. Technol.* **2000**, *26*, 509–515.

34. Rodrigues, D. S.; Mendes, A. A.; Adriano, W. S.; Gonçalves, L. R. B.; Giordano, R. L. C. Multipoint covalent immobilization of microbial lipase on chitosan and agarose activated by different methods. *J. Mol. Catal. B Enzym.* **2008**, *51*, 100–109.

35. Betancor, L.; López-Gallego, F.; Hidalgo, A.; Alonso-Morales, N.; Dellamora-Ortiz, G.; Mateo, C.; Fernández-Lafuente, R.; Guisán, J. M. Different mechanisms of protein immobilization on glutaraldehyde activated supports: Effect of support activation and immobilization conditions. *Enzyme Microb. Technol.* **2006**, *39*, 877–882.

36. Soltani, N.; Reza, F.; Babak, P. Direct introduction of amine groups into cellulosic paper for covalent immobilization of tyrosinase: Support characterization and enzyme properties. *Cellulose* **2017**, *24*, 1407–1416.

37. Suescun, A.; Rueda, N.; Dos Santos, J. C. S.; Castillo, J. J.; Ortiz, C.; Torres, R.; Barbosa, O.; Fernandez-Lafuente, R. Immobilization of lipases on glyoxyl-octyl supports: Improved stability and reactivation strategies. *Process Biochem.* **2015**, *50*, 1211–1217.

38. Palomo, J. M.; Muoz, G.; Fernández-Lorente, G.; Mateo, C.; Fernández-Lafuente, R.; Guisán, J. M. Interfacial adsorption of lipases on very hydrophobic support (octadecyl-Sepabeads): Immobilization, hyperactivation and stabilization of the open form of lipases. *J. Mol. Catal. B Enzym.* **2002**, *19–20*, 279–286.

39. Balcão, V. M.; Vila, M. M. D. C. Structural and functional stabilization of protein entities: State-of-the-art. *Adv. Drug Deliv. Rev.* **2015**, *93*, 25–41.

40. Glogauer, A.; Martini, V. P.; Faoro, H.; Couto, G. H.; Müller-Santos, M.; Monteiro, R. A.; Mitchell, D. A.; de Souza, E. M.; Pedrosa, F. O.; Krieger, N. Identification and characterization of a new true lipase isolated through metagenomic approach. *Microb. Cell Fact.* **2011**, *10*, 54.

41. Pereira-Rodríguez, Á.; Fernández-Leiro, R.; González-Siso, M. I.; Cerdán, M. E.; Becerra, M.; Sanz-Aparicio, J. Structural basis of specificity in tetrameric *Kluyveromyces lactis* β-galactosidase. *J. Struct. Biol.* **2012**, *177*, 392–401.

42. Zanphorlin, L. M.; de Giuseppe, P. O.; Honorato, R. V.; Tonoli, C. C. C.; Fattori, J.; Crespim, E.; de Oliveira, P. S. L.; Ruller, R.; Murakami, M. T. Oligomerization as a strategy

for cold adaptation: Structure and dynamics of the GH1 β-glucosidase from *Exiguobacterium antarcticum* B7. *Sci. Rep.* **2016**, *6*, 23776.

43. Fernandez-Lafuente, R. Stabilization of multimeric enzymes: Strategies to prevent subunit dissociation. *Enzyme Microb. Technol.* **2009**, *45*, 405–418.

44. Palomo, J. M.; Fuentes, M.; Fernández-Lorente, G.; Mateo, C.; Guisan, J. M.; Fernández-Lafuente, R. General trend of lipase to self-assemble giving bimolecular aggregates greatly modifies the enzyme functionality. *Biomacromolecules* **2003**, *4*, 1–6.

45. Palomo, J. M.; Segura, R. L.; Mateo, C.; Fernandez-Lafuente, R.; Guisan, J. M. Improving the activity of lipases from thermophilic organisms at mesophilic temperatures for biotechnology applications. *Biomacromolecules* **2004**, *5*, 249–254.

46. Henley, J. P.; Sadana, A. Deactivation theory. *Biotechnol. Bioeng.* **1986**, *28*, 1277–85.

47. Crespim, E.; Zanphorlin, L. M.; de Souza, F. H. M.; Diogo, J. A.; Gazolla, A. C.; Machado, C. B.; Figueiredo, F.; Sousa, A. S.; Nóbrega, F.; Pellizari, V. H.; Murakami, M. T.; Ruller, R. A novel cold-adapted and glucose-tolerant GH1 β-glucosidase from *Exiguobacterium antarcticum* B7. *Int. J. Biol. Macromol.* **2016**, *82*, 375–380.

48. Pessela, B. C. C.; Vian, A.; Mateo, C.; Fernandez-Lafuente, R.; Garcia, J. L.; Guisán, J. M.; Carrascosa, A. V. Overproduction of *Thermus* sp. strain T2 β-galactosidase in *Escherichia coli* and preparation by using tailor-made metal chelate supports. *Appl. Environ. Microbiol.* **2003**, *69*, 1967–1972.

49. Whistler, R. L.; Wolfrom, M. L.; BeMiller, J. . *Methods in Carbohydrate Chemistry*; 2nd ed.; Academic Press: New York, NY, USA; London, UK, 1963.

50. Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248–254.

51. Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **1970**, *227*, 680–685.

52. Addorisio, V.; Sannino, F.; Mateo, C.; Guisan, J. M. Oxidation of phenyl compounds using strongly stable immobilized-stabilized laccase from *Trametes versicolor*. *Process Biochem.* **2013**, *48*, 1174–1180.

CAPÍTULO 5

Hydrolysis of cellobiose using an improved immobilized-stabilized glucose-tolerant β-glucosidase from *Exiguobacterium antarcticum* B7

Ricardo Rodrigues de Melo; Robson Carlos Alnoch; Roberto Ruller; Hélia Harumi Sato; Cesar Mateo

Trabalho em preparação para submissão na revista Process Biochemistry

RESUMO

As β-glucosidases (BGL) são enzimas ubíquas, bem caracterizadas, e biologicamente importantes com consideráveis aplicações industriais. Neste trabalho, uma β-glicosidase tetramérica tolerante à glicose de *Exiguobacterium antarcticum* B7 (*recEa*BglA) foi imobilizada, seguida de pós-imobilização com polímeros e/ou pequenas moléculas e caracterizada bioquímicamente. A *recEa*BglA foi eficientemente imobilizada em Agarose GLU-IDA-Co^{2+} revestido com polietilenimina (PEI), e sua eficiência catalítica foi avaliada com base na estabilidade térmica (46 vezes mais estável que a forma livre). A *recEa*BglA imobilizada mostrou elevada atividade catalítica na faixa de pH 3 a 9, enquanto que a *recEa*BglA livre apresentou elevada atividade catalítica em uma faixa mais estreita de pH (> 80% a pH 6.0-8.0), e pH ótimo em 7.0. A *recEa*BglA apresentou temperatura ótima alterada de 30 °C para 50 °C com o processo de imobilização. A *recEa*BglA imobilizada mostrou uma expressiva adaptação ao pH e estabilidade de armazenamento maior do que a sua forma livre. Dados de armazenamento a 4 °C revelaram que a BGL imobilizada reteve 67% de sua atividade inicial após 60 dias. A *recEa*BglA imobilizada proporcionou uma bem sucedida hidrólise de 100% da celobiose mesmo após 9 ciclos sem reduzir a sua atividade inicial. Assim, o presente estudo descreve uma estratégia eficiente para a imobilização e estabilização de uma β-glucosidase tetramérica com grande potencial de biotecnológico.

Palavras-chave: imobilização da β-glucosidase; enzima tetramérica; glutaraldeído; estabilidade térmica; hidrólise de celobiose.

ABSTRACT

β-glucosidases (BGLs) are ubiquitous, well-characterized, and biologically important enzymes with considerable industrial applications. Here, an important multimeric glucose-tolerant β-glucosidase from *Exiguobacterium antarcticum* B7 (*recEa*BglA) was immobilized, followed by post-immobilization with polymers or small molecules and its biochemical characterization. The *recEa*BglA was successfully immobilized onto Agarose GLU-IDA-Co^{2+} coated with polyethyleneimine (PEI), and its catalytic efficiency was assessed based on thermal stability (46-fold more stable than free form). The immobilized *recEa*BglA showed a optimum pH at acid region and a retention with high catalytic activity in the pH range 3 to 9, while free *recEa*BglA showed high catalytic activity in a narrow range of pH (>80 % at pH 6.0-8.0) and optimum pH at 7.0. The *recEa*BglA has the optimum temperature changed from 30 °C to 50 °C with the immobilization process. The immobilized *recEa*BglA showed an expressive adaptation to pH and storage stability than its free form. Storage data at 4 °C revealed that the immobilized BGL retained 67 % of its initial activity after 60 days. The immobilized *recEa*BglA provided successful hydrolysis of 100 % cellobiose even after 9 cycles without reducing its initial activity. Hence, the current study describes an efficient strategy for the immobilization and stabilization of a tetrameric β-glucosidase with great potential biotechnological.

Keywords: β-glucosidase immobilization; tetrameric enzyme; glutaraldehyde; thermal stability; cellobiose hydrolysis.

1. Introduction

Cellulose, the most abundant biopolymer on Earth, plays an important role in the recycling of photosynthetically fixed carbon [1]. Cellulosic biomass has a great industrial relevance, mainly due to its saccharified form, i.e., glucose units useful for use by yeast and other organisms to produce biofuel or other bioproducts [2,3]. The most important enzymes involved in the biodeconstruction of cellulose brans, cellulases, cleave the β-1,4-glycosidic bonds of cellulose to glucose molecules. In a classical view, cellulases are catalogued according to their mechanisms of action as endoglucanases (EGLs, EC 3.2.1.4) that cleaving internal bonds in cellulose brans, creating more free short cellulose chains; cellobiohydrolases (CBH I, EC 3.2.1.176 and CBH II, EC 3.2.1.91) acting on the reducing or non-reducing ends of cellulose polymer; and lastly, β-glucosidases (BGLs, EC 3.2.1.21) hydrolyze short-oligosaccharides and cellobiose to produce glucose molecules [4,5]. BGLs are key hydrolases in the conversion process due its reaction is considered to be the rate limiting step in the enzymatic hydrolysis of cellulose polymers. Thus, the function of BGLs is not only to convert cellobiose to glucose, but also to reduce the cellobiose inhibition, resulting in efficient functioning of other enzymes (EGLs and CBHs) [6].

β-glucosidases are enzymes found in most living of kingdoms, from bacteria to higher eukaryotes [3]. BGLs are enzymes involved in a variety of natural processes, and being extensively explored by different biotechnological procedures in industrial level. Recently, β-glucosidases have drawn intensive attention because of its critical role in the biological conversion of cellulose brans to glucose [2]. Besides, the reactions with BGLs can be particularly used in industrial processes in the pharmaceutical field (e.g., production of bioactive agents, and antimicrobial compounds for use in cosmetics) and in food areas (e.g., processing of wines, teas or fruit juices, and aroma enhancement) [2,7–10]. Thus, the β-glucosidases appear as the important enzymes in a variety of biotechnological applications, obtaining more active and stable BGLs under a large conditions range makes highly interesting.

Recently, a novel GH1 β-glucosidase from *Exiguobacterium antarcticum* B7 (*recEa*BglA) with higher k_{cat} over mesophilic counterparts at 30 °C was identified and characterized [11]. The *recEa*BglA shown a cold-active mechanism, and also a high glucose tolerant capacity (up to 1 M of glucose); desirable feature in bioprocesses with high production of glucose, such as biomass saccharification [12]. Structural analyses, revealed that *recEa*BglA have a tetrameric arrangement, being the first kind to be reported within the

GH1 family [13]. Although, *recEa*BglA has interesting features as biocatalyst, still are necessary carry out studies to economically viable application.

A notorious alternative to develop more cost-effective enzymatic process has been the use of immobilization methods [14,15]. The benefits of enzyme immobilization, such as longer shelf life, reusability and stability against temperature and pH variations have been extensively studied in numerous research works [16]. However, although the immobilization techniques have been applied as efficient tools, increases in demand for new enzymatic processes and the structural diversity found in the new biocatalysts, means that the materials and/or methodologies used in the immobilization also have to be updated constantly.

Multimeric enzymes are among the most interesting enzymes with potential industrial applications [17–22]. However, a characteristic behavior of many multimeric enzymes is their lack of thermal stability outside the cell owing to facile multimer dissociation with concomitant loss of activity [23]. Thus, the increase of its structural and functional stabilities assumes special relevance, and the use of a sequential combination of immobilization techniques and post-immobilization has been shown to be a viable strategy [24–27]. Modification in the microenvironment of biocatalysts via covalent attachment onto a solid support followed by post-immobilization with polymers or small molecules are some of the (latest) strategies discussed aiming at full structural and functional stabilization of said multimeric biomolecules [23,28]. Currently, there are several studies proving in an extensive and specific form that the stabilization of multimeric enzymes, in particular with regard to strategies to prevent dissociation of the subunit, may be achieved with the use of post-immobilization techniques applying polymers and small molecules [23–27]. López-Gallego et al. [29] reported on the structural and functional stabilization of several different multimeric alcohol oxidases *via* covalent immobilization onto glyoxyl-agarose followed by post-immobilization with aldehyde-dextran to prevent subunit dissociation.

In this work, a sequential combination of multi-subunit immobilization followed by post-immobilization with polymers and small molecules as a general strategy to stabilize a multimeric glucose-tolerante GH1 β-glucosidase from *Exiguobacterium antarcticum* B7 (*recEa*BglA) was studied. To immobilize the *recEa*BglA, agarose supports based on glutaraldehyde-activation were applied as useful tools. Then, different post-immobilization treatments using multifunctional polymers (i.e., DEAE-Dextran, polygalacturonic acid and polyethylenimine), distinct bifunctional small molecules (i.e., aspartic acid and glycine), and/or mixture of both were evaluated. To access the modifications on the catalytic efficiency of immobilized *recEa*BglA, studies of the thermal stability were performed. Subsequently,

optimum pH, temperature, pH-tolerance, reusability assay and storage were also investigated with the *recEa*BglA.

2. Material and Methods

2.1. Material

Agarose 4 BCL was purchased from Agarose Bead Technologies (Madrid, Spain). Epichlorhydrin, iminodiacetic acid, sodium borohydride, sodium periodate, bovine serum albumin, polyethylenenimine (PEI - 25000 Da), isopropyl-β-D-thiogalactopyranoside (IPTG), 4-nitrophenyl β-D-glucopyranoside (*p*NPG) were purchased from Sigma (Sigma-Aldrich®, St Louis, MO, USA). Glutaraldehyde and ethylenediamine were purchased from Alfa Aesar (Thermo Fisher Scientific® Waltham, Massachusetts, EUA). The protein assay kit (Bio-Rad protein dye reagent concentrate) was sourced from Bio-Rad. Superdex-200 column and His-Trap Ni^{2+}-chelating affinity were purchased from GE Healthcare. The silica gel TLC plates (DC Kieselgel 60 F_{254}) was purchased from Merck®. Expression vector pET28a(+) was purchased from Novagen® (Thermo Fisher Scientific® Waltham, Massachusetts, EUA). *Escherichia coli* strains DH5α and pRARE2 were used as cloning and expression hosts, respectively. All other reagents and solvents are of analytical grade and commercially available.

2.2. Expression and purification of recEaBglA

Escherichia coli pRARE2 competent cells were transformed with construct pET28a(+) containing the β-glucosidase (*recEa*BglA) gene, and recombinant protein expression was performed with LB medium containing the antibiotic kanamycin (40 μg mL^{-1}) at 37 ^{o}C followed by addition of IPTG to a final concentration of 0.5 mM. For enzyme purification, the cells were collected by centrifugation, disrupted using sonication, and the soluble fraction was loaded onto a His-Trap Ni^{2+}-chelating affinity column. A non-linear imidazole gradient (5-500 mM) was used for elution of proteins, and subsequently the selected fractions were pooled and submitted to a size-exclusion chromatography (Hiload Superdex 200 16/60) [11]. The purity of the final *recEa*BglA was verified by 12 % SDS-PAGE. The concentration of β-glucosidase was determined by absorbance at 280 nm. The

molar extinction coefficient ($\varepsilon_{280\,nm}$) was determined using the ProtParam tool (http://web.expasy.org/protparam/).

2.3. Determination of proteins concentration and SDS-PAGE

Protein content was determined by the Bradford method [30], using a Protein Assay Kit (Bio-Rad protein dye reagent concentrate) with bovine serum albumin as the standard. Electrophoresis of protein samples was done with 12 % SDS-PAGE [31]. The gel was stained with Coomassie Brilliant Blue R-250 and distained with methanol/acetic-acid/water (5/1/4 v/v/v). A mixture of high molecular weight proteins (Sigma Marker™, Sigma-Aldrich) was used as the molecular weight standard.

2.4. β-glucosidase activity assays

The enzyme assay for free and immobilized *recEa*BglA was carried out by measuring the increase in absorbance at 410 nm produced by the release of *p*-nitrophenol (*p*NP) in the hydrolysis of 0.5 mM 4-nitrophenyl β-D-glucopyranoside (*p*NPG) in sodium phosphate buffer (50 mM, pH 7.0) ($\varepsilon_{410\,nm,\ pH\ 7} = 7320\ M^{-1}\ cm^{-1}$). One unit (U) of enzyme was defined as the amount of enzyme required to release 1 μmoL *p*NP per min and the specific activity was defined as the number of units per mg of protein [11].

2.5.Construction of supports for recEaBglA immobilization

2.5.1. Activation of agarose-based beads

Epoxy-activated agarose was prepared by the reaction of hydroxyl groups on the agarose beads with epichlorohydrin, as described by Mateo et al [32]. For this, agarose gel 4 BCL (50 g) was washed with excess distilled water, and then treated with 220 mL of NaOH 1.84 M under agitation and in an ice bath. Subsequently, 1.0 g of sodium borohydride ($NaBH_4$), 80 mL of acetone, and 55 mL of epichlorhydrin were added and the suspension formed was gently stirred at 25 ^{o}C for 16 h. Agarose support was then washed five times with distilled water, and filtered under vacuum. After the last washing, the support was thoroughly sucked dry to remove the interstitial humidity and stored at 4 ^{o}C.

The number of epoxy/ligand groups was characterized from the difference in the periodate consumption between the hydrolyzed support and the initial epoxy support. Periodate consumption was quantified using potassium iodide, as previously described [33].

2.5.2. Modification of epoxy-activated agarose beads

2.5.2.1. Support activated with glutaraldehyde group

The support activated using the glutaraldehyde group was prepared as previously described by Betancor et al [34]. Briefly, the epoxy agarose beads (10 g) were hydrolyzed with 100 mL of 1 M H_2SO_4 for 2 h at 25 °C. Afterwards, the support was washed with distilled water and oxidized using 100 mL of $NaIO_4$ (100 mM). Then, the support was treated with 100 mL of 2 M ethylenediamine (EDA) at pH 10, and kept under gentle stirring at 25 ^{o}C. After 2 h, sodium borohydride (10 mg mL^{-1}) was added and stirred for further 2 h at 25 °C. The particles were successively washed with distilled water and 11 mL of glutaraldehyde solution (25 %, v/v) was added together with 17 mL of sodium phosphate buffer (200 mM at pH 7.0). The system was kept under gentle stirring for 18 h at 25 °C. Finally, the activated support was washed with distilled water and vacuum dried.

2.5.2.2. Anionic support activated with glutaraldehyde group and metal chelate

Agarose support (10 g) activated with the glutaraldehyde group and metal chelate was obtained by treatment of epoxy agarose beads with 100 mL of 0.5 M iminodiacetic acid (IDA) at pH 11 for 18 h at 25 °C. Then, the support was washed with distilled water and oxidized using 100 mL of sodium periodate ($NaIO_4$) at a final concentration of 100 mM. After 2 h of gentle agitation at 25 °C, the oxidized support was washed with distilled water and treated with 100 mL of 2 M ethylenediamine (EDA) pH 10 for 2 h at 25 °C. Sodium borohydride (10 mg mL^{-1}) was added, and stirred for further 2 h at 25 °C. The activated agarose was successively washed with distilled water. Glutaraldehyde solution (25 %, v/v) and sodium phosphate buffer (200 mM at pH 7.0) were added, and the system was kept under gentle stirring for 18 h at 25 °C. The agarose activated with glutaraldehyde was added to the metal chelate solutions (30 mg mL^{-1}, $CoCl_2$) at pH 7 for 30 minutes at 25 °C [35]. The activated support was washed with distilled water and vacuum dried.

2.6. The recEaBglA immobilization

The immobilization course of recombinant β-glucosidase (*recEa*BglA) was monitored measuring the enzyme activity in the supernatant and in the whole suspension at different time intervals. Additionally, controls with free *recEa*BglA were used to determine a possible inactivating effect (pH, temperature or dilution) on the enzyme during the immobilization process. In all cases, β-glucosidase was diluted in sodium phosphate buffer (200 mM at pH 7.0), and the suspension was gently stirred during 10 min. Then, different immobilization supports were suspended in an enzyme solution (1 g agarose beads: 10 mL enzyme solution in immobilization buffer), and gently stirred at 25 ^{o}C at different times. The immobilization was considered complete when there was not activity in the supernatant.

After *recEa*BglA immobilization, several polymers or small molecules as glycine (200 mM), aspartic acid (200 mM), DEAE-Dextran (200 mM), polygalacturonic acid(20 mg mL^{-1}), polyethylenimine (20 mg mL^{-1}), a mixture of glycine (200 mM) with polygalacturonic acid (20 mg mL^{-1}), and glycine (200 mM) with polyethylenimine (20 mg mL^{-1}) were evaluated in a post-immobilization to stabilize the *recEa*BglA. The reactions were maintained under agitation for 1 h (glycine reaction), and 30 min (all the others) using sodium phosphate buffer (200 mM at pH 7.0).

The immobilization efficiency (IE, %) was calculated as (Equation 1):

$$\text{IE} = \frac{A_i - A_f}{A_i} \text{ x } 100\% \quad (1)$$

where *Ai* is the hydrolytic activity (U) of the enzymatic solution before immobilization and A_f is the hydrolytic activity (U) remaining in the supernatant at the end of the immobilization procedure.

The recovered activity (R, %) was calculated as (Equation 2):

$$R = \frac{A_o}{A_T} x\ 100\% \quad (2)$$

where A_0 is the as the ratio between the real activity (U g^{-1} of support) of the immobilized preparation and A_T is the theoretical activity (U g^{-1} of support) of the immobilized preparation.

2.7. Biochemical assays

The optimum pH of *recEa*BglA activity was evaluated onto pH range from 3.0 to 10.0 using McIlvaine glycine-supplemented buffer at 50 mM. The optimum temperature of enzyme activity was determined in assays ranging from 15 to 70 °C. Thermal stability was evaluated by pre-incubating the free and immobilized *recEa*BglA in sodium phosphate buffer (50 mM at pH 7.0) at 40 ^{o}C. For the pH tolerance tests, free and immobilized *recEa*BglA were evaluated in the pH range from 3.0 to 10.0 using McIlvaine glycine-supplemented buffer at 50 mM and 30 ^{o}C. Periodically, samples of these suspensions were withdrawn, and the remaining activities assayed. The initial activities are regarded as 100 %, and relative activities were expressed as a percentage of initial activity. All assays were performed in triplicate.

For analysis of activity inhibition by glucose concentration (final concentrations: 0 to 1 M) was investigated using immobilized *recEa*BglA and compare against the free form as control.

2.8. Operational stability of the immobilized recEaBglA and storage

The reusability of the immobilized *recEa*BglA preparation was assessed at 25 ^{o}C by carrying out reaction that consisted of 1 mL cellobiose (10 %, w/v) diluted in sodium phosphate buffer (50 mM at pH 7.0), and 100 mg immobilized *recEa*BglA (167 U g^{-1} of support). After each cycle, the immobilized enzyme was washed three times with assay buffer, performed its activity, and added to a novel cellobiose solution. Analysis of cellobiose hydrolysis using immobilized *recEa*BglA was performed by ascending chromatography on a thin layer of silica (DC Kieselgel 60 F_{254}). Aliquots were sampled and applied to a silica plate. The run was carried out with a mixture of *n*-butanol: acetic acid: distilled water (2:1:1 v/v). The revelation of sugars was performed by dipping the plate in 5 % sulfuric acid in ethanol and heating.

Storage stability was tested using free and immobilized BGL in sodium phosphate buffer (50 mM at pH 7.0) for 60 days at 4 °C. The activity was measured with the *p*NPG assay.

3. Results and Discussion

3.1. Study of recEaBglA immobilization

To obtain the recombinant β-glucosidase (*recEa*BglA) and proceed with the immobilization process, the coding region of *recEa*BglA was sub-cloned into pET28a(+) vector, produced in *E. coli* pRARE2 cells and purified to homogeneity [11] (**Figure S1**, Supplementary data). The pure *recEa*BglA used in this study is described as tetrameric enzyme [13]. Thus, the structural complexity shown by *recEa*BglA makes its functional stabilization a difficult strategy.

Agarose beads functionalized with different reactive groups has been described as a suitable support to immobilize both monomeric and multimeric enzymes, allowing its full structural stabilization and considerable yields [14, 25, 27, 34, 36, 37]. Due to the multimeric nature of *recEa*BglA, the preservation of the enzyme quaternary structure is critical to avoid the dissociation of its subunits and retain its catalytic activity. Thus, these multimeric biomolecules require special immobilization methods that maintain their unperturbed conformation and correct orientation during the immobilization process. To achieve this goal, the use of a recent bifunctional metal glutaraldehyde-activated support was used in the study. This bifunctional support was previously described with the ability to covalently immobilize two tetrameric enzymes, even under moderate pH conditions, and it is not necessary to raise the pH, which is common to some supports to establish covalent interactions [35].

Using the novel bifunctional agarose supports (Agarose GLU-IDA-Co^{2+}), in less than 1 h, applying a ratio of 1 g of support to 10 mL of the enzyme suspension (~ 7.0 U g^{-1} of support), the *recEa*BglA was immobilized and showed a efficiency more than 100 % (**Table 1**). To compare the immobilization efficiency obtained by the novel platform, the *recEa*BglA was offered to the traditional support activated using glutaraldehyde molecules (Agarose GLU), and a similar result was found (IE = 100 %, **Table 1**).

3.2. Use of different polymers or small molecules and increased stability against temperature

As illustrated in **Table 1**, a sequential combination of multi-subunit immobilization followed by post-immobilization of the tetrameric *recEa*BglA with different polymers and

small molecules was evaluated as a tool to increase its structural stability. In this step, treatments using multifunctional polymers (i.e., DEAE-Dextran, polygalacturonic acid and polyethylenimine-PEI), distinct bifunctional small molecules (i.e., aspartic acid and glycine), and/or mixture of both were studied. Through the use of polymers and small molecules, full stabilization of any protein entity (monomeric or multimeric) may be possible from the point of view of its structure (rigidification) or of its functionality (contributing to the maintenance of the quaternary assembly), allowing a tremendous increase of its structural stability [23–27, 29]. To Agarose GLU supports, the post-immobilization methods showed recovered activities from 19 to 56 %, while in Agarose GLU-IDA-Co^{2+} recovered activities from 8 to 68 % were found (**Table 1**). The control preparation (supports without treatments) showed recovered activity of 54 % in Agarose GLU and 64 % in Agarose GLU-IDA-Co^{2+}.

To explore the efficiency of immobilization on solid supports and the post-immobilization with different compounds on increase in structural stability of *recEa*BglA, the thermal stability at 40 ^{o}C were performed (**Table 2**). As shown in the **Table 2**, using the immobilized *recEa*BglA on the traditional glutaraldehyde support (Agarose GLU), it was observed that the *recEa*BglA coated with polyethylenimine and glycine (2.4 and 2.3-fold more stable than free form, respectively) showed the best results. To visualize the increase in the structural stability of BGL promoted by the treatments, the *recEa*BglA immobilization on Agarose GLU without addition of compounds was performed, and demonstrated to be 1.8-fold more stable than free form. In addition, immobilized *recEa*BglA on Agarose GLU showed a slight improvement in thermal stability than free *recEa*BglA when the treatments with aspartic acid, DEAE-dextran, polygalacturonic acid and a mixture of glycine with polygalacturonic acid were applied. At the same conditions, the treatment with the mixture glycine and polyethylenimine showed not significant improve in the thermal stability in relation the native free *recEa*BglA (half-life time of 21 minutes at 40 °C).

For studies with the immobilized *recEa*BglA on the Agarose GLU-IDA-Co^{2+}, the best result was observed by the treatment with polyethylenimine (half-life time of 960 min), which was more stable than the preparation without addition of compounds (4.2-fold factor than control support), and 46-times more stable than free *recEa*BglA (half-life time of 21 min at 40 °C, **Table 2**). All other treatments showed a slight improvement in thermal stability than free *recEa*BglA, but no better than control preparation (*recEa*BglA- GLU-IDA-Co^{2+} without treatment, **Table 2**).

As mentioned above, *recEa*BglA immobilized in both supports coated with polyethylenimine exhibited a greater heat resistance than free *recEa*BglA. Polyethyleneimine

is advantageous because due to its cationic polymeric nature, it is likely to interact with areas on the protein surface located in different enzyme subunits, and is therefore desirable in the case of multimeric enzymes [24]. Thus, polyethyleneimine is highly suitable for use in the post-immobilization stabilization of the quaternary structure of multimeric enzymes that are very sensitive to covalent rigidifications [24,27]. Therefore, due to the excellent result found in the Agarose GLU-IDA-Co^{2+} support coated with PEI to attach and stabilize the tetrameric structure of *recEa*BglA, it was selected for the studies of biochemical studies, storage and operational stability.

3.3. Effects of the immobilization on the catalytic properties of recEaBglA

The biochemical assays of the free (*recEa*BglA) and immobilized *recEa*BglA (*recEa*BglA-GLU-IDA-Co^{2+}-PEI) were investigated using the general artificial substrate for β-glucosidase activity, 4-nitrophenyl β-D-glucopyranoside (*p*NPG). The effects of different pH values (3.0-10) on the activity of free and immobilized *recEa*BglA were compared at 30 °C, and the results were displayed in **Figure 1A**. The pH-dependent enzyme activity showed that the free *recEa*BglA retains its highest relative activity (>80 %) between pH 6.0 and 8.0, with a catalytic optimum at 7.0, while immobilized *recEa*BglA had its catalytic optimum in a broader range of pH values (>80 % at pH 3.0-9.0) (**Figure 1A**). The optimum pH for the *recEa*BglA-GLU-IDA-Co^{2+}-PEI showed a small displacement being found in acid pH values (pH 6.0, 100 %). A similar pH stability observation has been reported while conducting immobilization of cellulase, where it shown that the enzyme preparation was quite stable in a wide pH range (pH 1.5 to 12.0), but the activity of free cellulase was found to be reduced for pH 6.0 [39].

Table 1 - Immobilization of *recEa*BglA on glutaraldehyde-activated supports and the analysis of the addition of different polymers and small molecules.

Support	Treatment	Immobilization efficiency [a] (%), EI	Recovered activity after treatments [b] (%), R
Agarose GLU	Control (without treatment)	100	50
	Glycine 200 mM, pH 7.0	100	34
	Aspartic acid 200 mM, pH 7.0	100	47
	DEAE-Dextran 20 mg/mL, pH 7.0	100	51
	Polygalacturonic acid 20 mg mL^{-1}, pH 7.0	100	54
	Polyethylenimine 20 mg mL^{-1}, pH 7.0	100	32
	Glycine 200 mM and polygalacturonic acid 20 mg mL^{-1}, pH 7.0	100	56
	Glycine 200 mM and polyethylenimine 20 mg mL^{-1}, pH 7.0	100	19
Agarose GLU-IDA-Co^{2+}	Control (without treatment)	100	64
	Glycine 200 mM, pH 7.0	100	63
	Aspartic acid 200 mM, pH 7.0	100	68
	DEAE-Dextran 20 mg/mL, pH 7.0	100	8
	Polygalacturonic acid 20 mg mL^{-1}, pH 7.0	100	64
	Polyethylenimine 20 mg mL^{-1}, pH 7.0	100	55
	Glycine 200 mM and polygalacturonic acid 20 mg mL^{-1}, pH 7.0	100	63
	Glycine 200 mM and polyethylenimine 20 mg mL^{-1}, pH 7.0	100	10

Co^{2+} (cobalt); IDA (iminodiacetic acid); Aga (agarose beads); and GLU (glutaraldehyde).

[a] Calculated as the difference between the initial and final activities in the supernatant after 1 h of immobilization.

[b] Recovered activity (%), measured as the ratio between the real activity (U g^{-1} of support) of the immobilized *recEa*BglA and theoretical activity of the immobilized *recEa*BglA (U g^{-1} of support).

Table 2 - Time course of thermal stability of different immobilized *recEa*BglA derivates.

Support	Treatment	Half life ($T_{1/2}$, min 40 °C)	Stability factor
*recEa*BglA free		21	
Agarose GLU	Control (without treatment)	39	1.8
	Glycine 200 mM, pH 7.0	49	2.3
	Aspartic acid 200 mM, pH 7.0	26	1.2
	DEAE-Dextran 20 mg/mL, pH 7.0	25	1.2
	Polygalacturonic acid 20 mg mL^{-1}, pH 7.0	30	1.4
	Polyethylenimine 20 mg mL^{-1}, pH 7.0	50	2.4
	Glycine 200 mM and polygalacturonic acid 20 mg mL^{-1}, pH	30	1.4
	Glycine 200 mM and polyethylenimine 20 mg mL^{-1}, pH 7.0	22	1.0
Agarose GLU-IDA-Co^{2+}	Control (without treatment)	230	11
	Glycine 200 mM, pH 7.0	40	1.9
	Aspartic acid 200 mM, pH 7.0	23	1.1
	DEAE-Dextran 20 mg/mL, pH 7.0	28	1.3
	Polygalacturonic acid 20 mg mL^{-1}, pH 7.0	60	2.9
	Polyethylenimine 20 mg mL^{-1}, pH 7.0	960	46
	Glycine 200 mM and polygalacturonic acid 20 mg mL^{-1}, pH	55	2.6
	Glycine 200 mM and polyethylenimine 20 mg mL^{-1}, pH 7.0	28	1.3

Co^{2+} (cobalt); IDA (iminodiacetic acid); Aga (agarose beads); and GLU (glutaraldehyde).
Thermal stability of *recEa*BglA preparations was checked by incubation at 40 °C.
Aliquots were periodically withdrawn for quantification of the residual enzymatic activity to estimate the half-life according to Henley and Sadana [38]. The stability factors were done in relation to the free form.

The catalytic activity of free and immobilized BGL was investigated at different temperatures ranging from 15 to 70 °C, in an attempt to understand the effects of temperature on the catalytic activity of *recEa*BglA following immobilization. The optimum reactive temperature for free *recEa*BglA was observed at 30 °C; however, in assays performed with *recEa*BglA-GLU-IDA-Co^{2+}-PEI the highest catalytic activity was presented at 50 ^{o}C (**Figure 1B**). The use of the immobilization technique allowed the *recEa*BglA to increase its catalytic performance at higher temperatures, relative activity R >50 % between 30-50 ^{o}C. The results for pH-stability using *recEa*BglA-GLU-IDA-Co^{2+}-PEI were evaluated at different pH values ranging from of 3 to 10 and the results were illustrated in **Figure 1**. After 72 h, the *recEa*BglA-GLU-IDA-Co^{2+}-PEI showed to be stable over a relatively broad pH range, i.e. between pH 5-9, as it retained 50-100 % activity (**Figure 1C**). Differently, free *recEa*BglA had a low activity after 24 h at all pH values tested (**Figure 1D**).

To investigate the suitability of *recEa*BglA-GLU-IDA-Co^{2+}-PEI catalyzed processes in industrial green reactions, the effects of glucose tolerance were analyzed. β-glucosidase from *Exiguobacterium antarcticum* B7 was reported previously as having a high tolerance to glucose inhibition (retaining 50 % relative activity at 1 M glucose) [11]. The properties showed by *recEa*BglA are extremely desirable for biocatalysts involved with the breakdown of polysaccharides such as cellulose or cellobiose into glucose [4]. Glucose inhibition in immobilized *recEa*BglA was not significant affected by glucose up to 100 mM when *p*NPG was used as a substrate (103 % relative activity). The *recEa*BglA-GLU-IDA-Co^{2+}-PEI retained 55 % relative activity in the presence of 600 mM glucose and when 1 M glucose was applied, immobilized β-glucosidase retained around 27 % of its initial activity (data not shown). The significant tolerance of *recEa*BglA-GLU-IDA-Co^{2+}-PEI to high concentrations of glucose may facilitate the adoption this BGL as a viable biocatalyst in sustainable biotechnological processes.

Exploring this natural variability through immobilization technique approach, the heterogeneous biocatalyst developed could now be expand its use in mesophilic reactions, *i.e.*, commercial cocktails used to saccharification of lignocellulosic biomass [3, 40, 41], or even act in industrial preparations where the products involved are heat-sensitive [42].

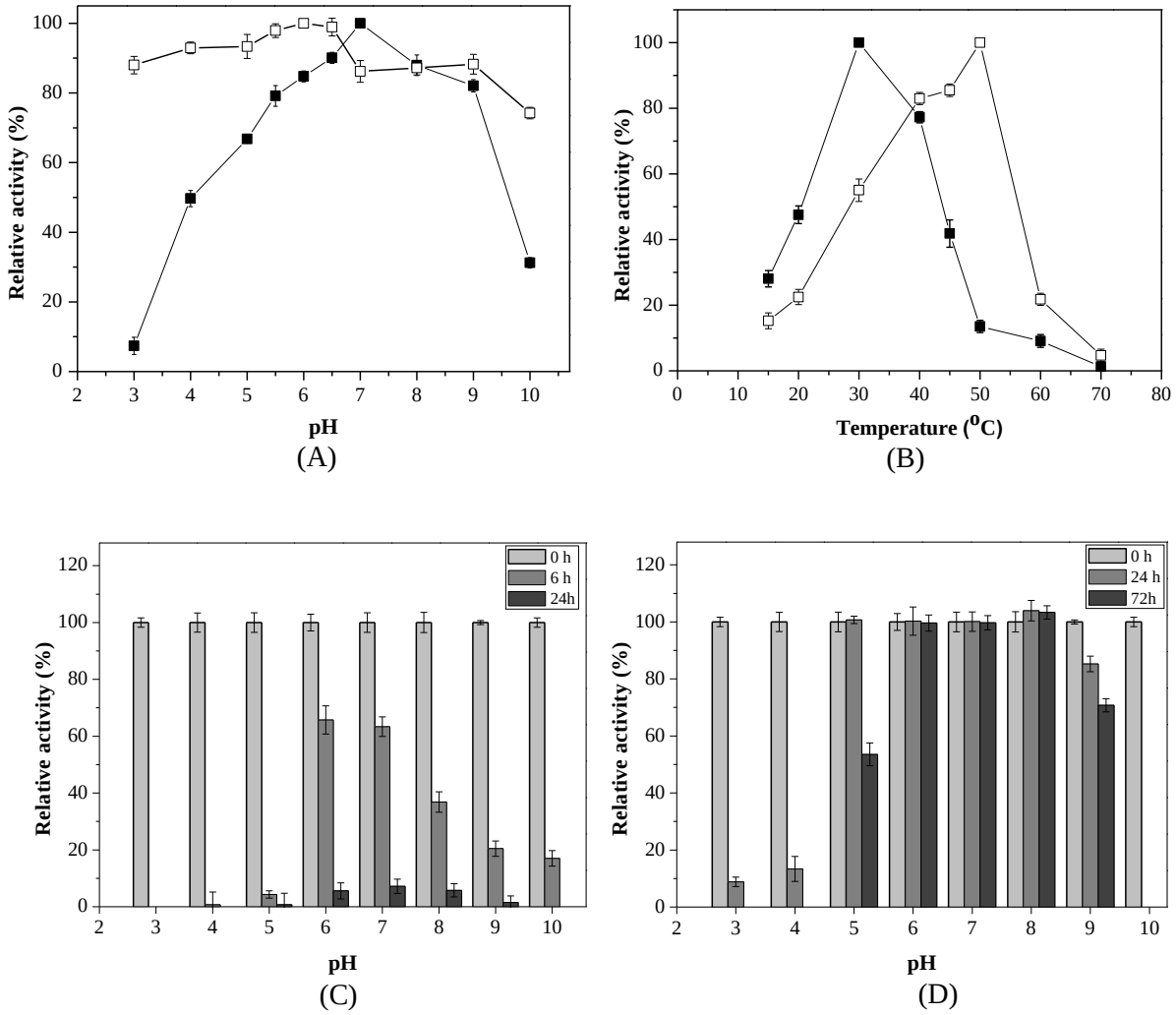


Figure 1 - Biochemical analysis of free (filled square) and immobilized *recEa*BglA (empty square); (A) Effect of pH and (B) temperature on the enzymatic activity. pH-stability under different pH values at 30 °C; (C) free and (D) immobilized *recEa*BglA.

3.4. Operational stability of the immobilized recEaBglA and storage

Immobilization of *recEa*BglA can facilitate enzyme recycling in a sequential batch-wise process, thereby lowering the enzyme cost. To assess the operational stability, immobilized *recEa*BglA was applied in successive reactions using cellobiose as substrate. After each reaction cycle (30 min), the biocatalyst was thoroughly washed several times with sodium phosphate buffer (50 mM at pH 7.0) to remove residual substrate and/or product formed. As illustrated in **Figure 2**, after 9 cycles, *recEa*BglA-GLU-IDA-Co^{2+}-PEI retained approximately 100 % of its initial activity with a yield of 100 %. Therefore, immobilized *recEa*BglA can be suitable for its repeated use, reducing processing costs in practical applications.

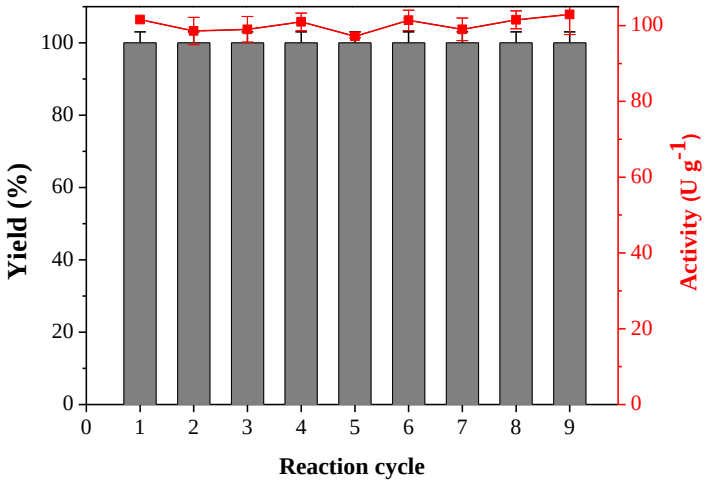


Figure 2 - Reusability assay of *recEa*BglA-GLU-IDA-Co^{2+}-PEI using cellobiose as substrate. Line (activity U g^{-1} of support), and bars (yield %).

Good storage stability is another essential property for an economically-feasible biocatalyst. Both free and immobilized *recEa*BglA were stored in sodium phosphate buffer (50 mM at pH 7.0) at 4 ^{o}C. The immobilized *recEa*BglA kept 67 % of its original activity, while the free enzyme retained 16 % of its activity at 4 °C (data not shown). It suggests that the mechanical coordination formed by the Agarose- GLU-IDA-Co^{2+} and by flexible cationic polymer (PEI) on the enzyme structure could hold *recEa*BglA in a stable state in comparison to the free enzyme.

4. Conclusions

Motivated by the need to obtain a better and more cost-efficient enzyme preparation, the use of immobilization techniques as viable tools was applied. The immobilization of *recEa*BglA onto a heterofunctional glutaraldehyde-activated support (Agarose GLU-IDA-Co^{2+}) coated with polyethylenimine (PEI) proved to be an excellent system to attach and stabilize the *recEa*BglA. The *recEa*BglA-GLU-IDA-Co^{2+}-PEI showed an expressive improved pH and temperature adaption, higher activity and stronger stability including thermal and storage stability. Also, it retained more than 100 % of its initial activity after 9 cycles of cellobiose hydrolysis. Therefore, the technique of the present work contributed to the production of a new *recEa*BglA derivate more stable, which can expand its application, having a good perspective in areas as biofuels, food and pharmaceutical.

Acknowledgment

The authors gratefully acknowledge the Ramón Areces Foundation for financial support and the Brazilian National Council for Scientific and Technological Development (CNPq) for fellowships (Grant No: 201757/2015-0 and 140610/2014-6). We also thank the CNPEM-CTBE for the use of facilities LAM (Laboratório de Análises de Macromoléculas).

ANEXO I, CAPÍTULO 5 - Supplementary data (Page 186)

Figure S1 - SDS-PAGE of the purified *recEa*BglA - M, molecular marker (kDa); 1 - *E. coli* BL21 crude extract with *recEa*Bgl; 2 - *recEa*BglA after purification by affinity column and molecular exclusion chromatography

5. References

[1] S. Wang, G. Dai, H. Yang, Z. Luo, Lignocellulosic biomass pyrolysis mechanism : A state-of-the-art review, Prog. Energy Combust. Sci. 62 (2017) 33–86.

[2] Y. Bhatia, S. Mishra, V.S. Bisaria, Microbial β-glucosidases: Cloning, properties, and applications, Crit. Rev. Biotechnol. 22 (2002) 375–407.

[3] A. Sørensen, M. Lübeck, P.S. Lübeck, B.K. Ahring, Fungal beta-glucosidases: A bottleneck in industrial use of lignocellulosic materials., Biomolecules. 3 (2013) 612–31.

[4] L.R. Lynd, P.J. Weimer, W.H. Van Zyl, S. Isak, Microbial cellulose utilization : Fundamentals and biotechnology microbial cellulose utilization : Fundamentals and Biotechnology. Indian Institute of Technology Madras, 66 (2002) 506–577.

[5] V. Lombard, H. Golaconda Ramulu, E. Drula, P.M. Coutinho, B. Henrissat, The carbohydrate-active enzymes database (CAZy) in 2013, Nucleic Acids Res. 42 (2014) 490–495.

[6] R.R. Singhania, A.K. Patel, R.K. Sukumaran, C. Larroche, A. Pandey, Role and significance of beta-glucosidases in the hydrolysis of cellulose for bioethanol production, Bioresour. Technol. 127 (2013) 500–507.

[7] M. Chen, Y. Qin, Z. Liu, K. Liu, F. Wang, Y. Qu, Isolation and characterization of a β-glucosidase from *Penicillium decumbens* and improving hydrolysis of corncob residue by using it as cellulase supplementation, Enzyme Microb. Technol. 46 (2010) 444–449.

[8] G. Li, Y. Jiang, X.J. Fan, Y.H. Liu, Molecular cloning and characterization of a novel

β-glucosidase with high hydrolyzing ability for soybean isoflavone glycosides and glucose-tolerance from soil metagenomic library, Bioresour. Technol. 123 (2012) 15–22.

[9] P. González-Pombo, L. Fariña, F. Carrau, F. Batista-Viera, B.M. Brena, A novel extracellular β-glucosidase from *Issatchenkia terricola*: Isolation, immobilization and application for aroma enhancement of white Muscat wine, Process Biochem. 46 (2011) 385–389.

[10] G. Singh, A.K. Verma, V. Kumar, Catalytic properties, functional attributes and industrial applications of β-glucosidases, 3 Biotech. 6 (2016) 3.

[11] E. Crespim, L.M. Zanphorlin, F.H.M. de Souza, J.A. Diogo, A.C. Gazolla, C.B. Machado, F. Figueiredo, A.S. Sousa, F. Nóbrega, V.H. Pellizari, M.T. Murakami, R. Ruller, A novel cold-adapted and glucose-tolerant GH1 β-glucosidase from *Exiguobacterium antarcticum* B7, Int. J. Biol. Macromol. 82 (2016) 375–380.

[12] U.S. Aswathy, R.K. Sukumaran, G.L. Devi, K.P. Rajasree, R.R. Singhania, A. Pandey, Bio-ethanol from water hyacinth biomass: An evaluation of enzymatic saccharification strategy, Bioresour. Technol. 101 (2010) 925–930.

[13] L.M. Zanphorlin, P.O. de Giuseppe, R.V. Honorato, C.C.C. Tonoli, J. Fattori, E. Crespim, P.S.L. de Oliveira, R. Ruller, M.T. Murakami, Oligomerization as a strategy for cold adaptation: Structure and dynamics of the GH1 β-glucosidase from *Exiguobacterium antarcticum* B7., Sci. Rep. 6 (2016) 23776.

[14] C. Mateo, J.M. Palomo, G. Fernandez-Lorente, J.M. Guisan, R. Fernandez-Lafuente, Improvement of enzyme activity, stability and selectivity via immobilization techniques, Enzyme Microb. Technol. 40 (2007) 1451–1463.

[15] L. Cao, Immobilised enzymes: Science or art?, Curr. Opin. Chem. Biol. 9 (2005) 217–226.

[16] A.A. Homaei, R. Sariri, F. Vianello, R. Stevanato, Enzyme immobilization:An update, J. Chem. Biol. 6 (2013) 185–205.

[17] A. Barzegar, A.A. Moosavi-movahedi, J.Z. Pedersen, M. Miroliaei, Comparative thermostability of mesophilic and thermophilic alcohol dehydrogenases : Stability-determining roles of proline residues and loop conformations, Enzyme Microb. Technol. 45 (2009) 73–79.

[18] V. Carbonea, A. Harab, O. El-Kabbania, Structural and functional features of dimeric dihydrodiol dehydrogenase, Cell. Mol. Life Sci. 65 (2008) 1464–1474.

[19] N.K. Lokanath, Y. Nodake, M. Sugahara, S. Kuramitsu, M. Miyano, Structure of

aldolase from *Thermus thermophilus* HB8 showing the contribution of oligomeric state to thermostability research papers, Acta Crystallogr. Sect. D. 60 (2004) 1816–1823.

[20] A.K. Kumar, P. Goswami, Dissociation and reconstitution studies of a broad substrate specific multimeric alcohol oxidase protein produced by *Aspergillus terreus*, J. Biochem. 145 (2009) 259–265.

[21] P. Ozimek, M. Veenhuis, I.J. Van Der Klei, Alcohol oxidase : A complex peroxisomal, oligomeric flavoprotein, FEMS Yeast Res. 5 (2005) 975–983.

[22] B.W. Matthews, The structure of *E . coli* β -galactosidase, C. R. Biol. 328 (2005) 549–556.

[23] R. Fernandez-Lafuente, Stabilization of multimeric enzymes: Strategies to prevent subunit dissociation, Enzyme Microb. Technol. 45 (2009) 405–418.

[24] I. Serra, C. Daniela, S. Rocchietti, D. Ubiali, M. Terreni, Stabilization of thymidine phosphorylase from *Escherichia coli* by immobilization and post immobilization techniques, Enzyme Microb. Technol. 49 (2011) 52–58.

[25] V.M. Balca, C. Mateo, R. Ferna, F.X. Malcata, Structural and functional stabilization of L-asparaginase via multisubunit immobilization onto highly activated supports, Biotechnol. Prog. 17 (2001) 537–542.

[26] V.M. Balca, C. Mateo, R. Ferna, M. Guisa, Coimmobilization of L-asparaginase and glutamate dehydrogenase onto highly activated supports, Enzyme Microb. Technol. 28 (2001) 696–704.

[27] J.M. Bolivar, J. Rocha-martín, C. Mateo, J.M. Guisan, Stabilization of a highly active but unstable alcohol dehydrogenase from yeast using immobilization and post-immobilization techniques, Process Biochem. 47 (2012) 679–686.

[28] J.C.S. Dos Santos, O. Barbosa, C. Ortiz, A. Berenguer-Murcia, R.C. Rodrigues, R. Fernandez-Lafuente, Importance of the support properties for immobilization or purification of enzymes, ChemCatChem. 7 (2015) 2413–2432.

[29] F. Lopez-gallego, L. Betancor, A. Hidalgo, G. Dellamora-ortiz, C. Mateo, R. Fern, J.M. Guis, Stabilization of different alcohol oxidases via immobilization and post immobilization techniques, Enzyme Microb. Technol. 40 (2007) 278–284.

[30] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding., Anal. Biochem. 72 (1976) 248–254.

[31] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4., Nature. 227 (1970) 680–685.

[32] C. Mateo, J.M. Bolivar, C.A. Godoy, J. Rocha-Martin, B.C. Pessela, J.A. Curiel, R. Munoz, J.M. Guisan, G. Fernández-Lorente, Improvement of enzyme properties with a two-step immobilizaton process on novel heterofunctional supports, Biomacromolecules. 11 (2010) 3112–3117.

[33] R.L. Whistler, M.L. Wolfrom, J.. BeMiller, Methods in carbohydrate chemistry, 2nd ed., Academic Press: New York, NY, USA; London, UK, 1963.

[34] L. Betancor, F. López-Gallego, A. Hidalgo, N. Alonso-Morales, G. Dellamora-Ortiz, C. Mateo, R. Fernández-Lafuente, J.M. Guisán, Different mechanisms of protein immobilization on glutaraldehyde activated supports: Effect of support activation and immobilization conditions, Enzyme Microb. Technol. 39 (2006) 877–882.

[35] R.R. De Melo, R.C. Alnoch, A. Ferreira, L. Vilela, E.M. De Souza, N. Krieger, R. Ruller, H. Sato, C. Mateo, New heterofunctional supports based on glutaraldehyde-activation : A tool for enzyme immobilization at neutral pH, Molecules. 22 (2017) 1088–1106.

[36] C. Bernal, L. Sierra, M. Mesa, Improvement of thermal stability of beta-galactosidase from *Bacillus circulans* by multipoint covalent immobilization in hierarchical macro-mesoporous silica, J Mol. Catal. B, Enzym. 84 (2012) 166–172.

[37] G.F. Rocha, F. Kise, A.M. Rosso, M.G. Parisi, Potential antioxidant peptides produced from whey hydrolysis with an immobilized aspartic protease from *Salpichroa origanifolia* fruits, Food Chem. 237 (2017) 350–355.

[38] J.P. Henley, A. Sadana, Deactivation theory., Biotechnol. Bioeng. 28 (1986) 1277–85.

[39] I. Hegedüs, J. Hancsók, E. Nagy, Stabilization of the cellulase enzyme complex as enzyme nanoparticle, Appl. Biochem. Biotechnol. 168 (2012) 1372–1383.

[40] L. Olsson, Fermentation of lignocellulosic hydrolysates for ethanol production, Enzyme Microb. Technol. 229 (1996) 312–331.

[41] C.A. Cardona, Fuel ethanol production : Process design trends and integration opportunities, Bioresour Technol. 98 (2007) 2415–2457.

[42] S. Brethauer, C.E. Wyman, Continuous hydrolysis and fermentation for cellulosic ethanol production, Bioresour. Technol. 101 (2010) 4862–4874.

DISCUSSÃO GERAL

O **CAPÍTULO 1**, como revisão bibliográfica, traça um panorama geral sobre a composição estrutural e o potencial biotecnológico da biomassa lignocelulósica. Este capítulo ainda traz informações sobre tópicos abordando a sacarificação enzimática, estratégias para a redução e otimização de custos de produção, e a aplicação das hidrolases glicosídicas em diferentes segmentos industriais.

No **CAPÍTULO 2** uma expansão do atual conhecimento e diversidade de GHs contidas no gênero *Streptomyces* foi apresentada. Neste trabalho, uma seleção funcional com oitenta linhagens de *Streptomyces* foi realizada, resultando em duas linhagens (F1 e F7) com elevadas atividades celulolíticas e xilanolíticas que foram selecionadas para sequenciamento genômico e análises bioquímicas. Após o cultivo em meio à base de bagaço de cana-de-açúcar, as linhagens F1 e F7 exibiram uma gama de atividades enzimáticas contra arabinana, arabinoxilano de centeio, beta-glucano, amido, CMC, xilano e quitina. Além destas atividades, a linhagem F7 foi capaz de degradar pectina e manana. A análise genômica de ambas as linhagens revelou uma diversidade de enzimas ativas em carboidratos. Os genomas codificaram 85 hidrolases glicosídicas (GHs) distribuídas em 33 famílias diferentes na linhagem F1, e 100 GHs em 44 famílias na linhagem F7. Além disso, a análise genômica também revelou genes relacionados à degradação de compostos aromáticos derivados da lignina. Coletivamente, o estudo revelou duas novas linhagens de *Streptomyce*s e informações sobre a capacidade de degradação de bactérias lignocelulolíticas, que podem a vir contribuir para o melhoramento dos processos de conversão da biomassa vegetal em bioprodutos.

O **CAPÍTULO 3** relata um estudo da produção de um extrato rico em celulase e xilanase a partir da bactéria *Streptomyces* sp. F1 para melhorar o processo de sacarificação da biomassa lignocelulósica por *via* enzimática. A seleção da linhagem se baseou em análises prévias onde foi observada uma elevada expressão de GHs em um menor tempo de fermentação. Inicialmente cinco substratos de origem agroindustrial foram utilizados como fonte de carbono para a elevação da secreção de GHs pela *Streptomyces* sp. F1. Dentre os substratos analisados, a maior produção foi alcançada com a linhagem F1 cultivada em farelo de trigo como substrato. Em seguida, cinco diferentes fontes de nitrogênio foram aplicadas e observou-se que o uso de caseína foi um ótimo indutor para elevar a secreção das enzimas no estudo. Após a seleção das melhores fontes indutoras, um delineamento composto central rotacional (DCCR) foi aplicado e a secreção máxima de celulases (0,22 U mL^{-1}) e xilanases (9,27 U mL^{-1}) foi alcançada com a suplementação do meio cultivo com 16,4 g L^{-1} de farelo de

trigo e 10,0 g L^{-1} de caseína. No **CAPÍTULO 3** foi também realizada uma análise dos secretomas da linhagem F1 quando cultivada em quatro diferentes fontes de carbono: carboximetilcelulose (CMC), xilano de madeira de faia (Xyl), farelo de trigo (WF) e glicose (Glu), onde se revelou um total de 61 proteínas, sendo 24, 30, 43 e 18 proteínas para os substratos CMC, Xyl, WF e Glu, respectivamente. As CAZymes corresponderam a mais de 15 % do total identificado nos secretomas analisados. O extrato enzimático bruto otimizado da linhagem F1 demonstrou valores ótimos de atividade enzimática a pH 5,5 e 45 ^{o}C com o substrato CMC e pH 5,5 e 55 ^{o}C com substrato xilano. A hidrólise enzimática do bagaço de cana pré-tratado com alcali e antraquinona (ABaq) utilizando o extrato comercial Celluclast 1,5 L suplementado com enzimas degradantes da biomassa obtidos da linhagem F1 gerou 2,5 vezes mais açúcares fermentescíveis.

Os **CAPÍTULOS 4 e 5** relatam dados sobre a aplicação de técnicas de imobilização enzimática como uma importante e eficiente ferramenta, principalmente pela possibilidade de reuso do biocatalisador aplicado. No **CAPÍTULO 4** foi estudado a criação de métodos de imobilização de enzimas em condições neutras de pH. Os novos suportes de agarose foram desenvolvidos com diferentes grupos bifuncionais (isto é, hidrofóbicos ou carboxílicos/metálicos) capazes de adsorver os biocatalisadores por diferentes regiões (regiões ricas em grupos hidrofóbicos ou histidina), juntamente com um grupo glutaraldeído capaz de promover uma irreversível imobilização em condições neutras. Para testar estes novos suportes, um sistema modelo multi-proteico (extrato de *E. coli*) e quatro enzimas (lipase de *Candida rugosa*, lipase metagenômica, β-galactosidase e β-glicosidase) foram utilizadas. O uso de diferentes suportes permitiu a imobilização da maioria das proteínas contidas no extrato bruto de proteínas. Além disso, os diferentes suportes produziram catalisadores com diferentes propriedades catalíticas. Em pH neutro, os novos suportes foram capazes de adsorver e imobilizar covalentemente as quatro enzimas testadas com diferentes valores de atividade recuperadas. No **CAPÍTULO 5** um estudo mais detalhado explorando os novos suportes e o uso de técnicas de pós-imobilização foi proposto. Neste trabalho, uma β-glicosidase tetramérica tolerante à glicose de *Exiguobacterium antarcticum* B7 (*recEa*BglA) foi imobilizada, seguida de pós-imobilização com polímeros e/ou pequenas moléculas e caracterizada bioquímicamente. A *recEa*BglA foi eficientemente imobilizada em Agarose GLU-IDA-Co^{2+} revestido com polietilenimina (PEI), e sua eficiência catalítica foi avaliada com base na estabilidade térmica (46 vezes mais estável que a forma livre). A *recEa*BglA imobilizada mostrou elevada atividade catalítica na faixa de pH 3 a 9, enquanto que a *recEa*BglA livre apresentou elevada atividade catalítica em uma faixa mais estreita de pH (>

80% a pH 6.0-8.0), e pH ótimo em 7.0. A *recEa*BglA apresentou temperatura ótima alterada de 30°C para 50°C com o processo de imobilização. A *recEa*BglA imobilizada mostrou uma expressiva adaptação ao pH e estabilidade de armazenamento maior do que a sua forma livre. Dados de armazenamento a 4 °C revelaram que a BGL imobilizada reteve 67% de sua atividade inicial após 60 dias. A *recEa*BglA imobilizada proporcionou uma bem sucedida hidrólise de 100% da celobiose mesmo após 9 ciclos sem reduzir a sua atividade inicial.

CONCLUSÃO GERAL

Entre as linhagens de *Streptomyces* sp. isoladas do solo e testadas no trabalho, duas linhagens (F1 e F7) foram selecionadas como sendo capazes de produzir quantidades consideráveis de hidrolases glicosídicas (GHs) (**CAPÍTULO 2**). A análise genômica de ambas as linhagens F1 e F7 revelou uma diversidade de hidrolases glicosídicas (GHs) distribuídas em 33 e 44 diferentes famílias, respectivamente (**CAPÍTULO 2**). Na fermentação da linhagem *Streptomyces* sp. F1 em meio otimizado composto de sais mínimos suplementado com 16,4 g L^{-1} de farelo de trigo e 10,0 g L^{-1} de caseína foi obtido elevadas concentrações de GHs expressas (9,27 U mL^{-1} de xilanase e 0,22 U mL^{-1} de celulase). O aumento da diversidade de GHs foi confirmado por espectrometria de massas, na qual se visualizou uma expansão no número de GHs com o aumento da complexidade da fonte de carbono aplicada. O extrato enzimático otimizado obtido da linhagem F1 foi utilizado na suplementação da preparação comercial (Celluclast 1,5 L) e possibilitou um aumento significativo na liberação de açúcares, indicando que as GHs secretadas pela *Streptomyces* sp. F1 podem ser aplicadas para o melhoramento dos atuais coquetéis comerciais (**CAPÍTULO 3**).

Os novos suportes construídos com diferentes grupos bifuncionais (hidrofóbicos ou carboxílicos/metálicos), juntamente com o agente glutaraldeído demonstraram ser eficientes na imobilização de enzimas monoméricas e multiméricas de grande interesse biotecnológico (**CAPÍTULO 4**). A imobilização da β-glicosidase tetramérica glicose-tolerante de *Exiguobacterium antarcticum* expressa em *Escherichia coli* pRARE2 no suporte de agarose ativado com glutaraldeído e revestido com um polímero catiônico (polietilenimina-PEI) provou ser um excelente sistema para fixar e estabilizar a enzima. A β-glicosidase imobilizada apresentou atividade na faixa de pH 3 a 9 e pH ótimo em 6, enquanto que a enzima livre mostrou atividade em uma faixa mais estreita de pH (> 80% em pH 6-8) e pH ótimo em 7. A β-glicosidase imobilizada foi capaz de hidrolisar 100% da celobiose após 9 ciclos sem perda de atividade e reteve 67% da atividade inicial após 60 dias de armazenamento a 4^{o}C (**CAPÍTULO 5**).

PERSPECTIVAS PARA TRABALHOS FUTUROS

A continuidade deste assunto em obras futuras deve ser feita e pode produzir informações relevantes para as áreas onde o uso de GHs tem um papel fundamental.

- Novos estudos de produção de GHs pelas linhagens de *Streptomyces* sp. isoladas podem ser realizados para prospecção de novas enzimas.
- As plataformas gênicas obtidas das linhagens F1 e F7 revelaram uma diversidade de enzimas ativas em carboidratos que podem ser estudadas em trabalhos futuros.
- O coquetel enzimático expresso pela linhagem *Streptomyces* sp. F1 descrito no **CAPÍTULO 3,** poderia ser aplicado na suplementação de outros extratos enzimáticos comerciais para visualizar o aumento na sacarificação da biomassa lignocelulósica.
- Os novos suportes criados em conjuto com as técnicas de pós-imobilização com polímeros e pequenas moleculas podem ser úteis para testes de imobilização explorando outras enzimas, tais como as secretadas pelas linhagens de *Streptomyces* prospectadas.

REFERÊNCIAS BIBLIOGRÁFICAS

ABRAHAM, R. E.; VERMA, M. L.; BARROW, C. J.; PURI, M. Suitability of magnetic nanoparticle immobilised cellulases in enhancing enzymatic saccharification of pretreated hemp biomass. **Biotechnology for Biofuels**, v. 7, n. 1, p. 1–12, 2014.

ADDORISIO, V.; SANNINO, F.; MATEO, C.; GUISAN, J. M. Oxidation of phenyl compounds using strongly stable immobilized-stabilized laccase from *Trametes versicolo*r. **Process Biochemistry**, v. 48, n. 8, p. 1174–1180, 2013.

ADSUL, M. G.; SINGHVI, M. S.; GAIKAIWARI, S. A.; GOKHALE, D. V. Development of biocatalysts for production of commodity chemicals from lignocellulosic biomass. **Bioresource Technology**, v. 102, n. 6, p. 4304–4312, 2011.

ALBUQUERQUE, T. L.; RUEDA, N.; SANTOS, J. C. S.; BARBOSA, O.; ORTIZ, C.; BINAY, B.; OZDEMIR, E.; GONÇALVES, L. R. B.; FERNANDEZ-LAFUENTE, R. Easy stabilization of interfacially activated lipases using heterofunctional divinyl sulfone activated-octyl agarose beads. Modulation of the immobilized enzymes by altering their nanoenvironment. **Process Biochemistry**, v. 51, p. 865–874, 2016.

ALFTRÉN, J.; HOBLEY, T. J. Covalent immobilization of β-glucosidase on magnetic particles for lignocellulose hydrolysis. **Applied Biochemistry and Biotechnology**, p. 2076–2087, 2013.

ALNOCH, R. C.; MELO, R. R.; PALOMO, J. M.; SOUZA, E. M.; KRIEGER, N.; MATEO, C. New tailor-made alkyl-aldehyde bifunctional supports for lipase immobilization. **Catalysts**, v. 6, p. 191, 2016.

ALSHEIKH-HUSSAIN, A.; ALTENAIJI, E. M.; YOUSEF, L. F. Fungal cellulases from mangrove forests - A short review. **Journal of Biochemical Technology**, v. 5, n. 169, p. 765–774, 2014.

ANNÉ, J.; VRANCKEN, K.; MELLAERT, L. V.; IMPE, J. V.; BERNAERTS, K. Protein secretion biotechnology in Gram-positive bacteria with special emphasis on *Streptomyces lividans*. **BBA - Molecular Cell Research**, v. 1843, n. 8, p. 1750–1761, 2014.

ANWAR, Z.; GULFRAZ, M.; IRSHAD, M. Agro-industrial lignocellulosic biomass a key to unlock the future bio-energy : A brief review. **Journal of Radiation Research and Applied Sciences**, v. 7, n. 2, p. 163–173, 2014.

ASWATHY, U. S.; SUKUMARAN, R. K.; DEVI, G. L.; RAJASREE, K. P.; SINGHANIA, R. R.; PANDEY, A. Bio-ethanol from water hyacinth biomass: An evaluation of

enzymatic saccharification strategy. **Bioresource Technology**, v. 101, n. 3, p. 925–930, 2010.

BAJAJ, B. K.; SINGH, N. P. Production of xylanase from an alkali tolerant *Streptomyces* sp. 7b under solid-state fermentation, its purification, and characterization. **Applied biochemistry and biotechnology**, v. 162, p. 1804–1818, 2010.

BALCA, V. M.; MATEO, C.; FERNA, R.; GUISA, M. Coimmobilization of L-asparaginase and glutamate dehydrogenase onto highly activated supports. **Enzyme and Microbial Technology**, v. 28, p. 696–704, 2001.

BALCA, V. M.; MATEO, C.; FERNA, R.; MALCATA, F. X. Structural and functional stabilization of L-asparaginase via multisubunit immobilization onto highly activated supports. **Biotechnology Progress**, v. 17, n. 3, p. 537–542, 2001.

BALCÃO, V. M.; VILA, M. M. D. C. Structural and functional stabilization of protein entities: State-of-the-art. **Advanced Drug Delivery Reviews**, v. 93, p. 25–41, 2015.

BAMFORTH, C. W. Current perspectives on the role of enzymes in brewing. **Journal of Cereal Science**, v. 50, n. 3, p. 353–357, 2009.

BANERJEE, G.; CAR, S.; SCOTT-CRAIG, J. S.; BORRUSCH, M. S.; BONGERS, M.; WALTON, J. D. Synthetic multi-component enzyme mixtures for deconstruction of lignocellulosic biomass. **Bioresource Technology**, v. 101, n. 23, p. 9097–9105, 2010.

BANKEVICH, A.; NURK, S.; ANTIPOV, D.; GUREVICH, A. A.; DVORKIN, M.; KULIKOV, A. S.; LESIN, V. M.; NIKOLENKO, S. I.; PHAM, S.; PRJIBELSKI, A. D.; PYSHKIN, A. V.; SIROTKIN, A. V.; VYAHHI, N.; TESLER, G.; ALEKSEYEV, M. A.; PEVZNER, P. A. SPAdes: A new genome assembly algorithm and its applications to single-cell sequencing. **Journal of Computational Biology**, v. 19, n. 5, p. 455–477, 2012.

BARBOSA, O.; ORTIZ, C.; BERENGUER-MURCIA, Á.; TORRES, R.; RODRIGUES, R. C.; FERNANDEZ-LAFUENTE, R. Glutaraldehyde in bio-catalysts design: A useful crosslinker and a versatile tool in enzyme immobilization. **RSC Advances**, v. 4, n. 207890, p. 1583, 2014.

BARZEGAR, A.; MOOSAVI-MOVAHEDI, A. A.; PEDERSEN, J. Z.; MIROLIAEI, M. Comparative thermostability of mesophilic and thermophilic alcohol dehydrogenases: Stability-determining roles of proline residues and loop conformations. **Enzyme and Microbial Technology,** v. 45, p. 73–79, 2009.

BEG, Q. K.; BHUSHAN, B.; KAPOOR, M.; HOONDAL, G. S. Production and characterization of thermostable xylanase and pectinase from *Streptomyces* sp. QG-11-3. **Journal of Industrial Microbiology and Biotechnology**, v. 24, p. 396–402, 2000.

BÉLAICH, J. P.; TARDIF, C.; BÉLAICH, A.; GAUDIN, C. The cellulolytic system of *Clostridium cellulolyticum*. **Journal of Biotechnology**, v. 57, n. 1, p. 3–14, 1997.

BENASSI, V. M.; SILVA, T. M.; PESSELA, B. C.; GUISAN, J. M.; MATEO, C.; LIMA, M. S.; JORGE, J. A.; POLIZELI, M. D. L. T. M. Immobilization and biochemical properties of a β-xylosidase activated by glucose/xylose from *Aspergillus niger* USP-67 with transxylosylation activity. **Journal of Molecular Catalysis B: Enzymati**c, v. 89, p. 93–101, 2013.

BENTLEY, S. D.; CHATER, K. F.; CERDEÑO-TÁRRAGA, A.-M.; CHALLIS, G. L.; THOMSON, N. R.; JAMES, K. D.; HARRIS, D. E.; QUAIL, M. A.; KIESER, H.; HARPER, D.; BATEMAN, A.; BROWN, S.; CHANDRA, G.; CHEN, C. W.; COLLINS, M.; CRONIN, A.; FRASER, A.; GOBLE, A.; HIDALGO, J.; HORNSBY, T.; HOWARTH, S.; HUANG, C.-H.; KIESER, T.; LARKE, L.; MURPHY, L.; OLIVER, K.; O'NEIL, S.; RABBINOWITSCH, E.; RAJANDREAM, M.-A.; RUTHERFORD, K.; RUTTER, S.; SEEGER, K.; SAUNDERS, D.; SHARP, S.; SQUARES, R.; SQUARES, S.; TAYLOR, K.; WARREN, T.; WIETZORREK, A.; WOODWARD, J.; BARRELL, B. G.; PARKHILL, J.; HOPWOOD, D. A. Complete genome sequence of the model actinomycete *Streptomyces*. **Nature**, v. 417, n. 2, p. 141–147, 2002.

BERLEMONT, R.; MARTINY, C. Genomic potential for polysaccharide deconstruction in bacteria. **Applied and Environmental Microbiology**, v. 81, n. 4, p. 1513–1519, 2015.

BERNAL, C.; MARCIELLO, M.; MESA, M.; SIERRA, L.; FERNANDEZ-LORENTE, G.; MATEO, C.; GUISAN, J. M. Immobilisation and stabilisation of beta-galactosidase from *Kluyveromyces lactis* using a glyoxyl support. **International Dairy Journal**, v. 28, n. 2, p. 76–82, 2013.

BERNAL, C.; SIERRA, L.; MESA, M. Improvement of thermal stability of β-galactosidase from *Bacillus circulans* by multipoint covalent immobilization in hierarchical macro-mesoporous silica. **Journal of Molecular Catalysis. B, Enzymatic**, v. 84, p. 166–172, 2012.

BETANCOR, L.; LÓPEZ-GALLEGO, F.; HIDALGO, A.; ALONSO-MORALES, N.; DELLAMORA-ORTIZ, G.; MATEO, C.; FERNÁNDEZ-LAFUENTE, R.; GUISÁN, J. M. Different mechanisms of protein immobilization on glutaraldehyde activated supports:

Effect of support activation and immobilization conditions. **Enzyme and Microbial Technology**, v. 39, n. 4, p. 877–882, 2006.

BEZBRADICA, D. I.; MATEO, C.; GUISAN, J. M. Novel support for enzyme immobilization prepared by chemical activation with cysteine and glutaraldehyde. **Journal of Molecular Catalysis B: Enzymatic**, v. 102, p. 218–224, 2014.

BHATIA, Y.; MISHRA, S.; BISARIA, V. S. Microbial β-glucosidases: Cloning, properties, and applications. **Critical Reviews in Biotechnology**, v. 22, n. 4, p. 375–407, 2002.

BOLGER, A. M.; LOHSE, M.; USADEL, B. Trimmomatic: A flexible trimmer for illumina sequence data. **Bioinformatics Advance**, v. 30, p. 2114, 2014.

BOLIVAR, J. M.; ROCHA-MARTÍN, J.; MATEO, C.; GUISAN, J. M. Stabilization of a highly active but unstable alcohol dehydrogenase from yeast using immobilization and post-immobilization techniques. **Process Biochemistry**, v. 47, n. 5, p. 679–686, 2012.

BONTEMPS, C.; TOUSSAINT, M.; REVOL, P. V.; HOTEL, L.; JEANBILLE, M.; UROZ, S.; TURPAULT, M. P.; BLAUDEZ, D.; LEBLOND, P. Taxonomic and functional diversity of *Streptomyces* in a forest soil. **FEMS Microbiology Letters**, v. 342, n. 2, p. 157–167, 2013.

BOOK, A. J.; LEWIN, G. R.; MCDONALD, B. R.; TAKASUKA, T. E.; DOERING, D. T.; ADAMS, A. S.; BLODGETT, J. A. V; CLARDY, J.; RAFFA, K. F.; FOX, B. G.; CURRIE, C. R. Cellulolytic *Streptomyces* strains associated with herbivorous insects share a phylogenetically linked capacity to degrade lignocellulose. **Applied and Environmental Microbiology**, v. 80, n. 15, p. 4692–4701, 2014.

BOOK, A. J.; LEWIN, G. R.; MCDONALD, B. R.; TAKASUKA, T. E.; WENDT-PIENKOWSKI, E.; DOERING, D. T.; SUH, S.; RAFFA, K. F.; FOX, B. G.; CURRIE, C. R. Evolution of high cellulolytic activity in symbiotic *Streptomyces* through selection of expanded gene content and coordinated gene expression. **PLoS Biology**, v. 14, n. 6, p. 1–21, 2016.

BRADFORD, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. **Analytical Biochemistry**, v. 72, n. 1–2, p. 248–254, 1976.

BRENDA, B.; BATISTA-VIRRA, F. Immobilization of enzymes. In: GUISAN, J. (Ed.). Immobilization of enzymes and cells. 2. ed. [s.l.] New Jersey (NJ): Humana Press Inc., v. 22p. 123–124, 2006.

BRETHAUER, S.; STUDER, M. H. Biochemical conversion processes of lignocellulosic biomass to fuels and chemicals - A review. **The Swiss Competence Center for Energy Research on Bioenergyv**. 69, n. 10, p. 572–581, 2015.

BRETHAUER, S.; WYMAN, C. E. Review : Continuous hydrolysis and fermentation for cellulosic ethanol production. **Bioresource Technology**, v. 101, n. 13, p. 4862–4874, 2010.

BRINK, J. VAN DEN; VRIES, R. P. DE. Fungal enzyme sets for plant polysaccharide degradation. **Applied Microbiology and Biotechnology**, v. 91, n. 6, p. 1477–92, 2011.

BROWN, M. E.; CHANG, M. C. Y. Exploring bacterial lignin degradation. **Current Opinion in Chemical Biology**, v. 19, n. 1, p. 1–7, 2014.

BRUMM, P.; LAND, M. L.; HAUSER, L. J.; JEFFRIES, C. D.; CHANG, Y. J.; MEAD, D. A. complete genome sequence of *Geobacillus* strain Y4.1MC1, a novel CO-utilizing *Geobacillus thermoglucosidasius* strain isolated from bath hot spring in yellow stone national park. **Bioenergy Research**, v. 8, n. 3, p. 1039–1045, 2015.

BUGG, T. D. H.; AHMAD, M.; HARDIMAN, E. M.; RAHMANPOUR, R. Pathways for degradation of lignin in bacteria and fungi. **Natural Product Reports**, v. 28, n. 12, p. 1883–96, 2011.

CAMPOS, B. M.; LIBERATO, M. V.; ALVAREZ, T. M.; ZANPHORLIN, L. M.; EMATSU, G. C.; BARUD, H.; POLIKARPOV, I.; RULLER, R.; GILBERT, H. J.; MATTOS ZERI, A. C. DE; SQUINA, F. M. A novel carbohydrate-binding module from sugar cane soil metagenome featuring unique structural and carbohydrate affinity properties. **Journal of Biological Chemistry**, v. 291, n. 45, p. 23734–23743, 2016.

CANALES, A. M.; GARZA, R.; SIERRA, J. A.; ARNOLD, R. The application of beta-glucanase with additional side activities in brewing. **MBAA Technical Quarterly**, v. 25, p. 27–31, 1988.

CANTAREL, B. I. L.; COUTINHO, P. M.; RANCUREL, C.; BERNARD, T.; LOMBARD, V.; HENRISSAT, B. The Carbohydrate-Active EnZymes database (CAZy): An expert resource for glycogenomics. **Nucleic Acids Research**, v. 37, p. D233–D238, 2009.

CAO, L. Immobilised enzymes: Science or art? **Current Opinion in Chemical Biology**, v. 9, n. 2, p. 217–226, 2005.

CARBONEA, V.; HARAB, A.; EL-KABBANIA, O. Review Structural and functional features of dimeric dihydrodiol dehydrogenase. **Cellular and Molecular Life Sciences**, v. 65, p. 1464–1474, 2008.

CARDONA, C. A. Fuel ethanol production : Process design trends and integration opportunities. **Bioresource Technology**, v. 98, p. 2415–2457, 2007.

CARVALHO, L. M. J.; CASTRO, I. M.; SILVA, C. A. B. A study of retention of sugars in the process of clarification of pineapple juice (*Ananas comosus*, L. Merril) by micro- and ultra-filtration. **Journal of Food Engineering**, v. 87, n. 4, p. 447–454, 2008.

CARVALHO, W.; CARRILHA, L.; FERRAZ, A.; MILAGRES, A. M. F. Uma visão sobre a estrutura, composição e biodegradação da madeira. **Química Nova**, v. 32, n. 8, p. 2191–2195, 2009.

CASTRO, A. M. DE; PEREIRA JR, N. Produção, propriedades e aplicação de celulases na hidrólise de resíduos agroindustriais. **Química Nova**, v. 33, n. 1, p. 181–188, 2010.

CERQUEIRA LEITE, R. C. DE; VERDE LEAL, M. R. L.; BARBOSA CORTEZ, L. A.; GRIFFIN, W. M.; GAYA SCANDIFFIO, M. I. Can Brazil replace 5% of the 2025 gasoline world demand with ethanol? **Energy**, v. 34, n. 5, p. 655–661, 2009.

CHANDEL, A. K.; SILVA, S.; SINGH, O. V. Sugarcane bagasse and leaves: foreseeable biomass of biofuel and bio-products. **Fungal Genetics and Biology**, v. 87, p. 11–20, 2012.

CHATER, K. F.; BIRÓ, S.; LEE, K. J.; PALMER, T.; SCHREMPF, H. The complex extracellular biology of *Streptomyces*: Review article. **FEMS Microbiology Reviews**, v. 34, n. 2, p. 171–198, 2010.

CHATTOPADHYAY, S.; RAYCHAUDHURI, U.; CHAKRABORTY, R. Artificial sweeteners - A review. **Journal of Food Science and Technology**, v. 51, n. 4, p. 611–621, 2014.

CHEN, M.; QIN, Y.; LIU, Z.; LIU, K.; WANG, F.; QU, Y. Isolation and characterization of a β-glucosidase from *Penicillium decumbens* and improving hydrolysis of corncob residue by using it as cellulase supplementation. **Enzyme and Microbial Technology**, v. 46, n. 6, p. 444–449, 2010.

CLARK, J. H.; LUQUE, R.; MATHARU, A. S. Green chemistry, biofuels, and biorefinery. **Annual Review of Chemical and Biomolecular Engineering**, v. 3, n. 1, p. 183–207, 2012.

CLIMENT, M. J.; CORMA, A.; IBORRA, S. Conversion of biomass platform molecules into fuel additives and liquid hydrocarbon fuels. **Green Chemistry**, v. 16, n. 2, p. 516, 2014.

CRESPIM, E.; ZANPHORLIN, L. M.; SOUZA, F. H. M. DE; DIOGO, J. A.; GAZOLLA, A. C.; MACHADO, C. B.; FIGUEIREDO, F.; SOUSA, A. S.; NÓBREGA, F.; PELLIZARI, V. H.; MURAKAMI, M. T.; RULLER, R. A novel cold-adapted and glucose-tolerant

GH1 β-glucosidase from *Exiguobacterium antarcticum* B7. **International Journal of Biological Macromolecules**, v. 82, p. 375–380, 2016.

DAS, A.; PAUL, T.; GHOSH, P.; HALDER, S. K.; MOHAPATRA, P. K. DAS; PATI, B. R.; MONDAL, K. C. Kinetic study of a glucose tolerant β-glucosidase from *Aspergillus fumigatus* ABK9 entrapped into alginate beads. **Waste and Biomass Valorization**, v. 6, n. 1, p. 53–61, 2015.

DASHTBAN, M.; SCHRAFT, H.; QIN, W. Fungal bioconversion of lignocellulosic residues; Opportunities & perspectives. **International Journal of Biological Sciences**, v. 5, n. 6, p. 578–595, 2009.

DASSA, B.; BOROVOK, I.; LAMED, R.; HENRISSAT, B.; COUTINHO, P.; HEMME, C. L.; HUANG, Y.; ZHOU, J.; BAYER, E. A. Genome-wide analysis of *Acetivibrio cellulolyticus* provides a blueprint of an elaborate cellulosome system. **BMC Genomics**, v. 13, n. 1, p. 210, 2012.

DATTA, S.; CHRISTENA, L. R.; RAJARAM, Y. R. S. Enzyme immobilization: An overview on techniques and support materials. **3 Biotech**, p. 1–9, 2013.

DAVIS, J. R.; GOODWIN, L.; TESHIMA, H.; DETTER, C.; TAPIA, R.; HAN, C.; HUNTEMANN, M.; WEI, C.-L.; HAN, J.; CHEN, A.; KYRPIDES, N.; MAVROMMATIS, K.; SZETO, E.; MARKOWITZ, V.; IVANOVA, N.; MIKHAILOVA, N.; OVCHINNIKOVA, G.; PAGANI, I.; PATI, A.; WOYKE, T.; PITLUCK, S.; PETERS, L.; NOLAN, M.; LAND, M.; SELLO, J. K. Genome sequence of *Streptomyces viridosporus* strain T7A ATCC 39115, a lignin-degrading actinomycete. **Genome Announcements**, v. 1, n. 4, p. e00416--13, 2013.

DELABONA, P. DA S.; FARINAS, C. S.; SILVA, M. R. DA; AZZONI, S. F.; PRADELLA, J. G. DA C. Use of a new *Trichoderma harzianum* strain isolated from the Amazon rainforest with pretreated sugar cane bagasse for on-site cellulase production. **Bioresource Technology**, v. 107, p. 517–521, 2012.

DELABONA, P. DA S.; PIROTA, R. D. P. B.; CODIMA, C. A.; TREMACOLDI, C. R.; RODRIGUES, A.; FARINAS, C. S. Using Amazon forest fungi and agricultural residues as a strategy to produce cellulolytic enzymes. **Biomass and Bioenergy**, v. 37, p. 243–250, 2012.

DIMAROGONA, M.; TOPAKAS, E.; CHRISTAKOPOULOS, P. Cellulose degradation by oxidative enzymes. **Computational and Structural Biotechnology Journal**, v. 2, n. 3, p. e201209015, 2012.

DOI, R. H.; KOSUGI, A. Cellulosomes: Plant-cell-wall- degrading enzyme complexes. **Nature Reviews**, v. 2, p. 541–551, 2004.

DRISS, D.; ZOUARI-ELLOUZI, S.; CHAARI, F.; KALLEL, F.; GHAZALA, I.; BOUAZIZ, F.; GHORBEL, R.; CHAABOUNI, S. E. Production and in vitro evaluation of xylooligosaccharides generated from corncobs using immobilized *Penicillium occitanis* xylanase. **Journal of Molecular Catalysis B: Enzymatic**, v. 102, p. 146–153, 2014.

DYK, J. S. VAN; PLETSCHKE, B. I. A review of lignocellulose bioconversion using enzymatic hydrolysis and synergistic cooperation between enzymes - Factors affecting enzymes, conversion and synergy. **Biotechnology Advances**, v. 30, n. 6, p. 1458–1480, 2012.

EIJSINK, V. G. H.; BJØRK, A.; GÅSEIDNES, S.; SIREVÅG, R.; SYNSTAD, B.; BURG, B. VAN DEN; VRIEND, G. Rational engineering of enzyme stability. **Journal of Biotechnology**, v. 113, n. 1–3, p. 105–120, 2004.

EL-NAKEEB, M. A.; LECHEVALIER, H. A. selective isolation of aerobic Actinomycetes. **Applied Microbiology**, v. 11, n. July, p. 75–77, 1963.

ERICK, J.; GOMES, G.; CAMILA, T.; NASCIMENTO, S.; EMÍLIA, A.; QUEIROZ, S. D. F.; IVALDO, J.; SILVA, D. S.; SOUZA-, C. M. DE; MEDEIROS, E. V. DE; MOREIRA, K. A. Production , characterization and evaluation of in vitro digestion of phytases, xylanases and cellulases for feed industry. **African Journal of Microbiology**, v. 8, n. 6, p. 551–558, 2014.

EŞ, I.; VIEIRA, J. D. G.; AMARAL, A. C. Principles, techniques, and applications of biocatalyst immobilization for industrial application. **Applied Microbiology and Biotechnology**, v. 99, n. 5, p. 2065–2082, 2015.

ESTRUCH, I.; TAGLIANI, A. R.; GUIS, M.; FERN, R.; TOMA, L.; TERRENI, M. Immobilization of the acylase from *Escherichia coli* on glyoxyl-agarose gives efficient catalyst for the synthesis of cephalosporins. **Enzyme and Microbial Technology**, v. 42, p. 121–129, 2008.

FARINAS, C. S. Developments in solid-state fermentation for the production of biomass-degrading enzymes for the bioenergy sector. **Renewable and Sustainable Energy Reviews**, v. 52, p. 179–188, 2015.

FERNANDEZ-LAFUENTE, R. Stabilization of multimeric enzymes: Strategies to prevent subunit dissociation. **Enzyme and Microbial Technology**, v. 45, n. 6–7, p. 405–418, 2009.

FERNANDEZ-LAFUENTE, R. Stabilization of multimeric enzymes: Strategies to prevent subunit dissociation. **Enzyme and Microbial Technology**, v. 45, n. 6–7, p. 405–418, 2009.

FERNÁNDEZ-LORENTE, G.; PALOMO, J. M.; MATEO, C.; MUNILLA, R.; ORTIZ, C.; CABRERA, Z.; GUISÁN, J. M.; FERNÁNDEZ-LAFUENTE, R. Glutaraldehyde cross-linking of lipases adsorbed on aminated supports in the presence of detergents leads to improved performance. **Biomacromolecules**, v. 7, n. 9, p. 2610–2615, 2006.

FRANCO CAIRO, J. P. L.; CARAZZOLLE, M. F.; LEONARDO, F. C.; MOFATTO, L. S.; BRENELLI, L. B.; GONÇALVES, T. A.; UCHIMA, C. A.; DOMINGUES, R. R.; ALVAREZ, T. M.; TRAMONTINA, R.; VIDAL, R. O.; COSTA, F. F.; COSTA-LEONARDO, A. M.; PAES LEME, A. F.; PEREIRA, G. A. G.; SQUINA, F. M. Expanding the knowledge on lignocellulolytic and redox enzymes of worker and soldier castes from the lower termite *Coptotermes gestroi*. **Frontiers in Microbiology**, v. 7, 2016.

FUJITA, Y.; ITO, J.; UEDA, M.; FUKUDA, H.; KONDO, A. Synergistic saccharification, and direct fermentation to ethanol, of amorphous cellulose by use of an engineered yeast strain codisplaying three types of cellulolytic enzyme. **Applied and Environmental Microbiology**, v. 70, n. 2, p. 1207–1212, 2004.

GAMA, R.; DYK, J. S. VAN; PLETSCHKE, B. I. Optimisation of enzymatic hydrolysis of apple pomace for production of biofuel and biorefinery chemicals using commercial enzymes. **3 Biotech**, v. 5, n. 6, p. 1075–1087, 2015.

GARCIA, N. F. L.; SILVA SANTOS, F. R. DA; GONÇALVES, F. A.; PAZ, M. F. DA; FONSECA, G. G.; LEITE, R. S. R. Production of β-glucosidase on solid-state fermentation by *Lichtheimia ramosa* in agroindustrial residues: Characterization and catalytic properties of the enzymatic extract. **Electronic Journal of Biotechnology**, v. 18, n. 4, p. 314–319, 2015.

GIBSON, G. R.; ROBERFROID, M. B. Dietary modulation of the human colonic microbiota: Introducing the concept of prebiotics. **The Journal of Nutrition**, v. 125, n. 6, p. 1401–1412, 1995.

GÍRIO, F. M.; FONSECA, C.; CARVALHEIRO, F.; DUARTE, L. C.; MARQUES, S. Hemicelluloses for fuel ethanol : A review. **Bioresource Technology**, v. 101, n. 13, p. 4775–4800, 2010.

GLOGAUER, A.; MARTINI, V. P.; FAORO, H.; COUTO, G. H.; MÜLLER-SANTOS, M.; MONTEIRO, R. A.; MITCHELL, D. A.; SOUZA, E. M. DE; PEDROSA, F. O.;

KRIEGER, N. Identification and characterization of a new true lipase isolated through metagenomic approach. **Microbial cell factories**, v. 10, p. 54, 2011.

GOMEZ, L. D.; STEELE-KING, C. G.; MCQUEEN-MASON, S. J. Sustainable liquid biofuels from biomass : the writing´s on the walls. **New Phytologist**. 473–485, 2008.

GONZÁLEZ-POMBO, P.; FARIÑA, L.; CARRAU, F.; BATISTA-VIERA, F.; BRENA, B. M. A novel extracellular β-glucosidase from *Issatchenkia terricola*: Isolation, immobilization and application for aroma enhancement of white Muscat wine. **Process Biochemistry**, v. 46, n. 1, p. 385–389, 2011.

GOTTSCHALK, L. M. F.; BON, E. P. S.; NOBREGA, R. Lignin peroxidase from *Streptomyces viridosporus* T7A: Enzyme concentration using ultrafiltration. **Applied Biochemistry and Biotechnology**, v. 147, n. 1–3, p. 23–32, 2008.

GUILLÉN, D.; SÁNCHEZ, S. Carbohydrate-binding domains: Multiplicity of biological roles. **Applied Microbiology and Biotechnology**, p. 1241–1249, 2010.

GUO, Y. P.; ZHENG, W.; RONG, X. Y.; HUANG, Y. A multilocus phylogeny of the *Streptomyces griseus* 16S rRNA gene clade: Use of multilocus sequence analysis for streptomycete systematics. **International Journal of Systematic and Evolutionary Microbiology**, v. 58, n. 1, p. 149–159, 2008.

HAMANN, P. R. V.; SERPA, D. L.; BARRETO DA CUNHA, A. S.; CAMARGO, B. R. DE; OSIRO, K. O.; VALLE DE SOUSA, M.; FELIX, C. R.; MILLER, R. N. G.; NORONHA, E. F. Evaluation of plant cell wall degrading enzyme production by *Clostridium thermocellum* B8 in the presence of raw agricultural wastes. **International Biodeterioration & Biodegradation**, v. 105, p. 97–105, 2015.

HAMILTON-BREHM, S. D.; MOSHER, J. J.; VISHNIVETSKAYA, T.; PODAR, M.; CARROLL, S.; ALLMAN, S.; PHELPS, T. J.; KELLER, M.; ELKINS, J. G. *Caldicellulosiruptor obsidiansis* sp. nov., an anaerobic, extremely thermophilic, cellulolytic bacterium isolated from obsidian pool, yellowstone National Park. **Applied and Environmental Microbiology**, v. 76, n. 4, p. 1014–1020, 2010.

HAMMEL, K. E.; CULLEN, D. Role of fungal peroxidases in biological ligninolysis. **Current Opinion in Plant Biology**, v. 11, n. 3, p. 349–355, 2008.

HANREICH, A.; SCHIMPF, U.; ZAKRZEWSKI, M.; SCHLÜTER, A.; BENNDORF, D.; HEYER, R.; RAPP, E.; PÜHLER, A.; REICHL, U.; KLOCKE, M. Metagenome and metaproteome analyses of microbial communities in mesophilic biogas-producing anaerobic batch fermentations indicate concerted plant carbohydrate degradation. **Systematic and Applied Microbiology**, v. 36, n. 5, p. 330–338, 2013.

HASUNUMA, T.; KAWASHIMA, K.; NAKAYAMA, H.; MURAKAMI, T.; KANAGAWA, H.; ISHII, T.; AKIYAMA, K.; YASUDA, K.; TERADA, F.; KUSHIBIKI, S. Effect of cellooligosaccharide or synbiotic feeding on growth performance, fecal condition and hormone concentrations in Holstein calves. **Animal Science Journal**, v. 82, n. 4, p. 543–548, 2011.

HASUNUMA, T.; OKAZAKI, F.; OKAI, N.; HARA, K. Y.; ISHII, J.; KONDO, A. A review of enzymes and microbes for lignocellulosic biorefinery and the possibility of their application to consolidated bioprocessing technology. **Bioresource Technology**, p. 1–9, 2012.

HAZLEWOOD, G. P.; GILBERT, H. J. Structure and function analysis of *Pseudomonas* plant cell wall hydrolases. **Biochemical Society Transactions**, p. 185–190, 1998.

HECK, J. X.; HERTZ, P. F.; AYUB, M. A Z. Cellulase and xylanase production by isolated amazon *Bacillus* strains using soybean industrial residue based solid-state cultivation. **Brazilian Journal of Microbiology**, v. 33, n. 3, p. 213–218, 2002.

HEGEDÜS, I.; HANCSÓK, J.; NAGY, E. stabilization of the cellulase enzyme complex as enzyme nanoparticle. **Applied Biochemistry and Biotechnology**, v. 168, p. 1372–1383, 2012.

HENLEY, J. P.; SADANA, A. Deactivation theory. **Biotechnology and bioengineering**, v. 28, n. 8, p. 1277–85, 1986.

HENRISSAT, B.; VEGETALES, M.; GRENOBLE, F.-. A classification of glycosyl hydrolases based sequence similarities amino acid. **Biochemical Journal**, v. 280, p. 309–316, 1991.

HESS, M.; SCZYRBA, A.; EGAN, R.; KIM, T.-W.; CHOKHAWALA, H.; SCHROTH, G.; LUO, S.; CLARK, D. S.; CHEN, F.; ZHANG, T.; MACKIE, R. I.; PENNACCHIO, L. A; TRINGE, S. G.; VISEL, A.; WOYKE, T.; WANG, Z.; RUBIN, E. M. Metagenomic discovery of biomass-degrading genes and genomes from cow rumen. **Science**, v. 331, n. 6016, p. 463–467, 2011.

HIMMEL, M. E.; XU, Q.; LUO, Y.; DING, S.-Y.; LAMED, R.; BAYER, E. A. Microbial enzyme systems for biomass conversion: Emerging paradigms. **Biofuels**, v. 1, n. 2, p. 323–341, 2010.

HIRATA, D. B.; ALBUQUERQUE, T. L.; RUEDA, N.; VIRGEN-ORTÍZ, J. J.; TACIAS-PASCACIO, V. G.; FERNANDEZ-LAFUENTE, R. Evaluation of different immobilized lipases in transesterification reactions using tributyrin: Advantages of the

heterofunctional octyl agarose beads. **Journal of Molecular Catalysis. B, Enzymatic**, v. 133, p. 117–123, 2016.

HOMAEI, A. A.; SARIRI, R.; VIANELLO, F.; STEVANATO, R. Enzyme immobilization: An update. **Journal of Chemical Biology**, v. 6, n. 4, p. 185–205, 2013.

HORN, S. J.; VAAJE-KOLSTAD, G.; WESTERENG, B.; EIJSINK, V. G. Novel enzymes for the degradation of cellulose. **Biotechnology for Biofuels**, v. 5, n. 1, p. 45, 2012.

HOWARD, R. L.; ABOTSI, E.; JANSEN VAN RENSBURG, E. L.; HOWARD, S. Lignocellulose biotechnology: Issues of bioconversion and enzyme production. **African Journal of Biotechnology**, v. 2, n. 12, p. 602–619, 2003.

HU, J.; ARANTES, V.; SADDLER, J. N. The enhancement of enzymatic hydrolysis of lignocellulosic substrates by the addition of accessory enzymes such as xylanase: is it an additive or synergistic effect ? **Biotechnology for Biofuels**, v. 4, n. 1, p. 36, 2011.

HURTUBISE, Y.; SHARECK, F.; KLUEPFEL, D.; MOROSOLI, R. A cellulase/xylanase-negative mutant of *Streptomyces fividans* 1326 defective in cellobiose and xylobiose uptake is mutated in a gene encoding a protein homologous to ATP-binding proteins. **Molecular Microbiology**, v. 17, n. 2, p. 367–377, 1995.

HYATT, D.; CHEN, G.-L.; LOCASCIO, P. F.; LAND, M. L.; LARIMER, F. W.; HAUSER, L. J. Prodigal: Prokaryotic gene recognition and translation initiation site identification. **BMC bioinformatics**, v. 11, p. 119, 2010.

JANG, H. D.; CHEN, K. S. Production and characterization of thermostable cellulases from *Streptomyces transformant* T3-1. **World Journal of Microbiology and Biotechnology**, v. 19, n. 3, p. 263–268, 2003.

JEGANNATHAN, K. R.; NIELSEN, P. H. Environmental assessment of enzyme use in industrial production - A literature review. **Journal of Cleaner Production**, v. 42, p. 228–240, 2013.

JIANG, Y.; WU, Y.; LI, H. Immobilization of *Thermomyces lanuginosus* xylanase on aluminum hydroxide particles through adsorption: characterization of immobilized enzyme. **Journal of Microbiology and Biotechnology**, v. 25, n. 2015, p. 2016–2023, 2016.

JIMÉNEZ, D. J.; LIMA BROSSI, M. J. DE; SCHÜCKEL, J.; KRAČUN, S. K.; WILLATS, W. G. T.; ELSAS, J. D. VAN. Characterization of three plant biomass-degrading microbial consortia by metagenomics- and metasecretomics-based approaches. **Applied Microbiology and Biotechnology**, p. 1–15, 2016.

JOHN, T.; ALFTRE, J. Immobilization of cellulase mixtures on magnetic particles for hydrolysis of lignocellulose and ease of recycling. **Biomass and Bioenergy**, v. 5, p. 4–10, 2014.

KATOH, K.; STANDLEY, D. M. MAFFT multiple sequence alignment software version 7: Improvements in performance and usability. **Molecular Biology and Evolution**, v. 30, n. 4, p. 772–780, 2013.

KAUR, A.; MAHAJAN, R.; SINGH, A.; GARG, G.; SHARMA, J. Application of cellulase-free xylano-pectinolytic enzymes from the same bacterial isolate in biobleaching of kraft pulp. **Bioresource Technology**, v. 101, n. 23, p. 9150–9155, 2010.

KIDDINAMOORTHY, J.; ANCENO, A. J.; HAKI, G. D.; RAKSHIT, S. K. Production, purification and characterization of *Bacillus* sp. GRE7 xylanase and its application in eucalyptus Kraft pulp biobleaching. **World Journal of Microbiology and Biotechnology**, v. 24, n. 5, p. 605–612, 2008.

KITTUR, F. S.; MANGALA, S. L.; ABU, A.; KITAOKA, M.; TSUJIBO, H.; HAYASHI, K. Fusion of family 2b carbohydrate-binding module increases the catalytic activity of a xylanase from *Thermotoga maritima* to soluble xylan. **FEBS Journal**, v. 549, p. 147–151, 2003.

KOECK, D. E.; PECHTL, A.; ZVERLOV, V. V; SCHWARZ, W. H. Genomics of cellulolytic bacteria. **Current opinion in biotechnology**, v. 29C, p. 171–183, 2014.

KQCZKOWSKI, J. Structure, function and metabolism of plant cell wall and their functions. **Acta Physiologiae Plantarum**, v. 25, n. 3, p. 287–305, 2003.

KRICKA, W.; FITZPATRICK, J.; BOND, U. Metabolic engineering of yeasts by heterologous enzyme production for degradation of cellulose and hemicellulose from biomass: A perspective. **Frontiers in Microbiology**, v. 5, p. 1–11, 2014.

KÜCK, P.; LONGO, G. C. FASconCAT-G: Extensive functions for multiple sequence alignment preparations concerning phylogenetic studies. **Frontiers in Zoology**, v. 11, n. 1, p. 81, 2014.

KUHAD, R. C.; GUPTA, R.; SINGH, A. Microbial cellulases and their industrial applications. **Enzyme Research**, v. 2011, p. 1–10, 2011.

KUMAR, A. K.; GOSWAMI, P. dissociation and reconstitution studies of a broad substrate specific multimeric alcohol oxidase protein produced by *Aspergillus terreus*. **The Journal of Biochemistry**, v. 145, n. 2, p. 259–265, 2009.

KUMAR, R.; SINGH, S.; SINGH, O. V. Bioconversion of lignocellulosic biomass: Biochemical and molecular perspectives. **Journal of industrial microbiology & biotechnology**, v. 35, n. 5, p. 377–91, 2008.

KUMAR, V.; SATYANARAYANA, T. Production of thermo-alkali-stable xylanase by a novel polyextremophilic *Bacillus halodurans* TSEV1 in cane molasses medium and its applicability in making whole wheat bread. **Bioprocess and Biosystems Engineering**, v. 37, n. 6, p. 1043–1053, 2014.

KUMAR, V.; SATYANARAYANA, T. Thermo-alkali-stable xylanase of a novel polyextremophilic *Bacillus halodurans* TSEV1 and its application in biobleaching. **International Biodeterioration & Biodegradation**, v. 75, p. 138–145, 2012.

LADEIRA, S. A.; CRUZ, E.; DELATORRE, A. B.; BARBOSA, J. B.; MARTINS, M. L. L. Cellulase production by thermophilic *Bacillus* sp. SMIA-2 and its detergent compatibility. **Electronic Journal of Biotechnology**, v. 18, n. 2, p. 110–115, 2015.

LAEMMLI, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. **Nature**, v. 227, n. 5259, p. 680–685, 1970.

LAMBERTZ, C.; GARVEY, M.; KLINGER, J.; HEESEL, D.; KLOSE, H.; FISCHER, R.; COMMANDEUR, U. Challenges and advances in the heterologous expression of cellulolytic enzymes : A review. **Biotechnology for Biofuels**, v. 7, p. 135–150, 2014.

LARKIN, M. A.; BLACKSHIELDS, G.; BROWN, N. P.; CHENNA, R.; MCGETTIGAN, P. A.; MCWILLIAM, H.; VALENTIN, F.; WALLACE, I. M.; WILM, A.; LOPEZ, R.; THOMPSON, J. D.; GIBSON, T. J.; HIGGINS, D. G. Clustal W and Clustal X version 2.0. **Bioinformatics**, v. 23, n. 21, p. 2947–2948, 2007.

LASLETT, D.; CANBACK, B. ARAGORN, a program to detect tRNA genes and tmRNA genes in nucleotide sequences. **Nucleic Acids Research**, v. 32, n. 1, p. 11–16, 2004.

LATHA, S.; SIVARANJANI, G.; DHANASEKARAN, D. Response surface methodology : A non- conventional statistical tool to maximize the throughput of Streptomyces species biomass and their bioactive metabolites. **Critical Reviews in Microbiology**, v. 7828, 2017.

LEE, C. C.; SMITH, M.; KIBBLEWHITE-ACCINELLI, R. E.; WILLIAMS, T. G.; WAGSCHAL, K.; ROBERTSON, G. H.; WONG, D. W. S. Isolation and characterization of a cold-active xylanase enzyme from *Flavobacterium* sp. **Current Microbiology**, v. 52, n. 2, p. 112–116, 2006.

LEE, H. V; HAMID, S. B. A.; ZAIN, S. K. Conversion of lignocellulosic biomass to nanocellulose: Structure and chemical process. **The Scientific World Journal**, v. 2014, p. 1–22, 2014.

LEE, Y.; CHOI, D.; KENDE, H. Expansins: Ever-expanding numbers and functions. **Current Opinion in Plant Biology**, v. 4, n. 6, p. 527–532, 2001.

LETUNIC, I.; BORK, P. Interactive Tree Of Life (iTOL): An online tool for phylogenetic tree display and annotation. **Bioinformatics**, v. 23, n. 1, p. 127–128, 2007.

LEVASSEUR, A.; DRULA, E.; LOMBARD, V.; COUTINHO, P. M.; HENRISSAT, B. Expansion of the enzymatic repertoire of the CAZy database to integrate auxiliary redox enzymes. **Biotechnology for Biofuels**, v. 6, n. 1, p. 41, 2013.

LI, G.; JIANG, Y.; FAN, X. J.; LIU, Y. H. Molecular cloning and characterization of a novel β-glucosidase with high hydrolyzing ability for soybean isoflavone glycosides and glucose-tolerance from soil metagenomic library. **Bioresource Technology**, v. 123, p. 15–22, 2012.

LI, J.; ZHOU, P.; LIU, H.; XIONG, C.; LIN, J.; XIAO, W.; GONG, Y.; LIU, Z. Synergism of cellulase, xylanase, and pectinase on hydrolyzing sugarcane bagasse resulting from different pretreatment technologies. **Bioresource Technology**, v. 155, p. 258–265, 2014.

LIN, E.; WILSON, D. B. Regulation of 3-1,4-endoglucanase synthesis in *Thermomonospora fusca*. **Applied and Environmental Microbiology**, v. 53, n. 6, p. 1352–1357, 1987.

LOKANATH, N. K.; NODAKE, Y.; SUGAHARA, M.; KURAMITSU, S.; MIYANO, M. Structure of aldolase from *Thermus thermophilus* HB8 showing the contribution of oligomeric state to thermostability research papers. **Acta Crystallographica Section D**, v. 60, p. 1816–1823, 2004.

LOMBARD, V.; GOLACONDA RAMULU, H.; DRULA, E.; COUTINHO, P. M.; HENRISSAT, B. The carbohydrate-active enzymes database (CAZy) in 2013. **Nucleic Acids Research**, v. 42, n. D1, p. 490–495, 2014.

LOPEZ-GALLEGO, F.; BETANCOR, L.; HIDALGO, A.; DELLAMORA-ORTIZ, G.; MATEO, C.; FERN, R.; GUIS, J. M. Stabilization of different alcohol oxidases via immobilization and post immobilization techniques. **Enzyme and Microbial Technology**, v. 40, p. 278–284, 2007.

LÓPEZ-GALLEGO, F.; BETANCOR, L.; MATEO, C.; HIDALGO, A.; ALONSO-MORALES, N.; DELLAMORA-ORTIZ, G.; GUISÁN, J. M.; FERNÁNDEZ-LAFUENTE, R. Enzyme stabilization by glutaraldehyde crosslinking of adsorbed proteins on aminated supports. **Journal of Biotechnology**, v. 119, n. 1, p. 70–75, 2005.

LÓPEZ-MONDÉJAR, R.; ZÜHLKE, D.; VĚTROVSKÝ, T.; BECHER, D.; RIEDEL, K.; BALDRIAN, P. Decoding the complete arsenal for cellulose and hemicellulose deconstruction in the highly efficient cellulose decomposer *Paenibacillus* O199. **Biotechnology for Biofuels**, v. 9, n. 1, p. 104, 2016.

LYND, L.; WEIMER, P.J.; VAN, Z. W. H.; PRETORIUS, I. S. Microbial cellulose utilization: Fundamentals and biotechnology. **Microbiology and Molecular Biology Reviews**, v. 66, n. 3, p. 506–577, 2002.

MACRELLI, S.; GALBE, M.; WALLBERG, O. Effects of production and market factors on ethanol profitability for an integrated first and second generation ethanol plant using the whole sugarcane as feedstock. **Biotechnology for Biofuels**, v. 7, n. 1, p. 26, 2014.

MAKI, M.; LEUNG, K. T.; QIN, W. The prospects of cellulase-producing bacteria for the bioconversion of lignocellulosic biomass. **International Journal of Biological Sciences**, v. 5, n. 5, p. 500–516, 2009.

MANDER, P.; CHOI, Y. H.; G.C., P.; CHOI, Y. S.; HONG, J. H.; CHO, S. S.; YOO, J. C. Biochemical characterization of xylanase produced from *Streptomyces* sp. CS624 using an agro residue substrate. **Process Biochemistry**, v. 49, n. 3, p. 451–456, 2014.

MANRICH, A.; KOMESU, A.; ADRIANO, W. S.; TARDIOLI, P. W. Immobilization and stabilization of xylanase by multipoint covalent attachment on agarose and on chitosan supports. **Applied Biochemistry and Biotechnology**, v. 161, p. 455–467, 2010.

MARKOWITZ, V. M.; MAVROMATIS, K.; IVANOVA, N. N.; CHEN, I. M. A.; CHU, K.; KYRPIDES, N. C. IMG ER: A system for microbial genome annotation expert review and curation. **Bioinformatics**, v. 25, n. 17, p. 2271–2278, 2009.

MATEO, C.; ABIAN, O.; BERNEDO, M.; CUENCA, E.; FUENTES, M.; FERNANDEZ-LORENTE, G.; PALOMO, J. M.; GRAZU, V.; PESSELA, B. C. C.; GIACOMINI, C.; IRAZOQUI, G.; VILLARINO, A.; OVSEJEVI, K.; BATISTA-VIERA, F.; FERNANDEZ-LAFUENTE, R.; GUISÁN, J. M. Some special features of glyoxyl supports to immobilize proteins. **Enzyme and Microbial Technology**, v. 37, n. 4, p. 456–462, 2005.

MATEO, C.; ABIAN, O.; FERNANDEZ-LAFUENTE, R.; GUISAN, J. M. Increase in conformational stability of enzymes immobilized on epoxy-activated supports by favoring additional multipoint covalent attachment. **Enzyme and Microbial Technology**, v. 26, n. 7, p. 509–515, 2000.

MATEO, C.; BOLIVAR, J. M.; GODOY, C. A.; ROCHA-MARTIN, J.; PESSELA, B. C.; CURIEL, J. A.; MUNOZ, R.; GUISAN, J. M.; FERNÁNDEZ-LORENTE, G.

Improvement of enzyme properties with a two-step immobilizaton process on novel heterofunctional supports. **Biomacromolecule**s, v. 11, n. 11, p. 3112–3117, 2010.

MATEO, C.; PALOMO, J. M.; FERNANDEZ-LORENTE, G.; GUISAN, J. M.; FERNANDEZ-LAFUENTE, R. Improvement of enzyme activity, stability and selectivity via immobilization techniques. **Enzyme and Microbial Technology**, v. 40, n. 6, p. 1451–1463, 2007.

MATEO, C.; PALOMO, J. M.; FUENTES, M.; BETANCOR, L.; GRAZU, V.; LÓPEZ-GALLEGO, F.; PESSELA, B. C. C.; HIDALGO, A.; FERNÁNDEZ-LORENTE, G.; FERNÁNDEZ-LAFUENTE, R.; GUISÁN, J. M. Glyoxyl agarose: A fully inert and hydrophilic support for immobilization and high stabilization of proteins. **Enzyme and Microbial Technology**, v. 39, n. 2, p. 274–280, 2006.

MATTHEWS, B. W. The structure of *E. coli* β-galactosidase. **Comptes Rendus Biologies**, v. 328, p. 549–556, 2005.

MCGUIRE, K. L.; TRESEDER, K. K. Microbial communities and their relevance for ecosystem models: Decomposition as a case study. **Soil Biology and Biochemistry**, v. 42, n. 4, p. 529–535, 2010.

MELO, R. R. DE; ALNOCH, R. C.; FERREIRA, A.; VILELA, L.; SOUZA, E. M. DE; KRIEGER, N.; RULLER, R.; SATO, H.; MATEO, C. New heterofunctional supports based on glutaraldehyde-activation: A tool for enzyme immobilization at neutral pH. **Molecules**, v. 22, p. 1088–1106, 2017.

MIGNEAULT, I.; DARTIGUENAVE, C.; BERTRAND, M. J.; WALDRON, K. C. Glutaraldehyde: Behavior in aqueous solution, reaction with proteins, and application to enzyme crosslinking. **BioTechniques**, v. 37, n. 5, p. 790–802, 2004.

MILLER, G. L. Use of dinitrosalicylic acid reagent for determination of reducing sugars. **Analytical Chemistry**, n. 31, p. 426–429, 1959.

MINUSSI, R. C.; PASTORE, G. M.; DURÁN, N. Potential applications of laccase in the food industry. **Trends in Food Science & Technology**, v. 13, n. 6–7, p. 205–216, 2002.

MOLINA-HENARES, A. J.; KRELL, T.; EUGENIA GUAZZARONI, M.; SEGURA, A.; RAMOS, J. L. Members of the IclR family of bacterial transcriptional regulators function as activators and/or repressors. **FEMS Microbiology Reviews**, v. 30, n. 2, p. 157–186, 2006.

MUNK, L.; SITARZ, A. K.; KALYANI, D. C.; MIKKELSEN, J. D.; MEYER, A. S. Can laccases catalyze bond cleavage in lignin? **Biotechnology Advances**, v. 33, n. 1, p. 13–24, 2015.

MUSSATTO, S. I.; FERNANDES, M.; MANCILHA, I. M.; ROBERTO, I. C. Effects of medium supplementation and pH control on lactic acid production from brewer's spent grain. **Biochemical Engineering Journal**, v. 40, n. 3, p. 437–444, 2008.

NAGIEB, Z. A.; MILEGY, M. G. EL; FAAT, H. M. R.; ISIS, K. B. Effect of EDTA on Production of xylanase by *Streptomyces* species and their bleaching effect on papyrus and cotton stalk pulp. **International Journal of Forestry and Wood Science**, v. 1, n. 1, p. 2–9, 2014.

OBENG, E. M.; NURUL, S.; ADAM, N.; BUDIMAN, C.; ONGKUDON, C. M.; MAAS, R.; JOSE, J. Lignocellulases: A review of emerging and developing enzymes, systems, and practices. **Bioresources and Bioprocessing**, v.4, n.16, 2017.

OGEDA, T. L.; PETRI, D. F. S. Hidrólise enzimática de biomassa. **Química Nova**, v. 33, n. 7, p. 1549–1558, 2010.

OLSSON, L. Fermentation of lignocellulosic hydrolysates for ethanol production. **Enzyme and Microbial Technology**, v. 229, n. 95, p. 312–331, 1996.

OTSUKA, M.; ISHIDA, A.; NAKAYAMA, Y.; SAITO, M.; YAMAZAKI, M.; MURAKAMI, H.; NAKAMURA, Y.; MATSUMOTO, M.; MAMOTO, K.; TAKADA, R. Dietary supplementation with cellooligosaccharide improves growth performance in weanling pigs. **Animal Science Journal**, v. 75, p. 225–229, 2004.

OZIMEK, P.; VEENHUIS, M.; KLEI, I. J. VAN DER. Alcohol oxidase : A complex peroxisomal, oligomeric flavoprotein**. FEMS Yeast Research**, v. 5, p. 975–983, 2005.

PALOMO, J. M.; FUENTES, M.; FERNÁNDEZ-LORENTE, G.; MATEO, C.; GUISAN, J. M.; FERNÁNDEZ-LAFUENTE, R. General trend of lipase to self-assemble giving bimolecular aggregates greatly modifies the enzyme functionality. **Biomacromolecules**, v. 4, p. 1–6, 2003.

PALOMO, J. M.; MUOZ, G.; FERNÁNDEZ-LORENTE, G.; MATEO, C.; FERNÁNDEZ-LAFUENTE, R.; GUISÁN, J. M. Interfacial adsorption of lipases on very hydrophobic support (Octadecyl-Sepabeads): Immobilization, hyperactivation and stabilization of the open form of lipases. **Journal of Molecular Catalysis B: Enzymatic**, v. 19, n. 20, p. 279–286, 2002.

PALOMO, J. M.; SEGURA, R. L.; MATEO, C.; FERNANDEZ-LAFUENTE, R.; GUISAN, J. M. Improving the activity of lipases from thermophilic organisms at mesophilic temperatures for biotechnology applications. **Biomacromolecules**, v. 5, p. 249–254., 2004.

PANDEY, A.; SOCCOL, C. R.; NIGAM, P.; SOCCOL, V. T. Biotechnological potential of agro-industrial residues: Sugarcane bagasse. **Bioresource Technology**, v. 74, n. 1, p. 69–80, 2000.

PATEL, A. K.; SINGHANIA, R. R.; PANDEY, A. Novel enzymatic processes applied to the food industry. **Current Opinion in Food Science**, 2015.

PATHAK, P.; CENTRE, A.; ROORKEE, T. Production of crude cellulase and xylanase from *Trichoderma harzianum* PPDDN10 NFCCI-2925 and its application in photocopier waste paper recycling. **Applied Biochemistry and Biotechnology**, v. 172, p. 3776–3797, 2014.

PAULY, M.; KEEGSTRA, K. Cell-wall carbohydrates and their modification as a resource for biofuels. **The Plant Journal**, v. 54, n. 4, p. 559–568, 2008.

PENG, F.; REN, J. L.; XU, F.; SUN, R. Chemicals from hemicellulose: A review. **Sustainable Production of Fuels, Chemicals, and Fibers from Forest Biomass**, p. 219–259, 2011.

PERCIVAL, Z. Y. H.; HIMMEL, M. E.; MIELENZ, J. R. Outlook for cellulase improvement: Screening and selection strategies. **Biotechnology Advances**, v. 24, n. 5, p. 452–481, 2006.

PEREIRA-RODRÍGUEZ, Á.; FERNÁNDEZ-LEIRO, R.; GONZÁLEZ-SISO, M. I.; CERDÁN, M. E.; BECERRA, M.; SANZ-APARICIO, J. Structural basis of specificity in tetrameric *Kluyveromyces lacti*s β-galactosidase**. Journal of Structural Biology**, v. 177, n. 2, p. 392–401, 2012.

PÉREZ, J.; MUÑOZ-DORADO, J.; LA RUBIA, T. DE; MARTÍNEZ, J. Biodegradation and biological treatments of cellulose, hemicellulose and lignin: an overview. **International Microbiology**, v. 5, n. 2, p. 53–63, 2002.

PESSELA, B. C. C.; VIAN, A.; MATEO, C.; FERNANDEZ-LAFUENTE, R.; GARCIA, J. L.; GUISÁN, J. M.; CARRASCOSA, A. V. Overproduction of *Thermus* sp. strain T2 β-galactosidase in *Escherichia coli* and preparation by using tailor-made metal chelate supports. **Applied and Environmental Microbiology**, v. 69, n. 4, p. 1967–1972, 2003.

PETRUCCIOLI, M.; RAVIV, M.; SILVESTRO, R. DI; DINELLI, G. Agriculture and agro-industrial wastes, byproducts, and wastewaters. Second Edi ed. [s.l.] Elsevier, v. 1, 2011

PINHEIRO, G. L.; AZEVEDO-MARTINS, A. C. DE; ALBANO, R. M.; SOUZA, W. DE; FRASES, S. Comprehensive analysis of the cellulolytic system reveals its potential for deconstruction of lignocellulosic biomass in a novel *Streptomyces* sp. **Applied Microbiology and Biotechnology**, v. 101, n.1, p. 301-319, 2016.

PINHEIRO, M.; OLIVEIRA, M. The effect of different sweeteners in low calorie yogurts - A review. **International Journal of Dairy Technology**, v. 58, n. 4, p. 193–199, 2005.

POLIZELI, M. L. T. M.; RIZZATTI, A. C. S.; MONTI, R.; TERENZI, H. F.; JORGE, J. A.; AMORIM, D. S. Xylanases from fungi: Properties and industrial applications. **Applied Microbiology and Biotechnology**, v. 67, n. 5, p. 577–591, 2005.

PRADEEP, G. C.; CHOI, Y. H.; CHOI, Y. S.; SEONG, C. N.; CHO, S. S.; LEE, H. J.; YOO, J. C. A novel thermostable cellulase free xylanase stable in broad range of pH from *Streptomyces* sp. CS428. **Process Biochemistry**, v. 48, n. 8, p. 1188–1196, 2013.

PRICE, M. N.; DEHAL, P. S.; ARKIN, A. P. FastTree 2 - Approximately maximum-likelihood trees for large alignments. **PLoS ONE**, v. 5, n. 3, p. e9490, 2010.

RASTOGI, G.; BHALLA, A.; ADHIKARI, A.; BISCHOFF, K. M.; HUGHES, S. R.; CHRISTOPHER, L. P.; SANI, R. K. Characterization of thermostable cellulases produced by *Bacillus* and *Geobacillus* strains. **Bioresource Technology**, v. 101, n. 22, p. 8798–8806, 2010.

RAVINDER, T.; SWAMY, M. V.; SEENAYYA, G.; REDDY, G. *Clostridium lentocellum* SG6 - A potential organism for fermentation of cellulose to acetic acid. **Bioresource Technology**, v. 80, p. 171–177, 2001.

RIVERO, C. W.; PALOMO, J. M. Covalent immobilization of *Candida rugosa* lipase at alkaline pH and their application in the regioselective deprotection of Per-O-acetylated thymidine. **Catalyst**, v. 6, p. 115, 2016.

ROCHA, G. F.; KISE, F.; ROSSO, A. M.; PARISI, M. G. Potential antioxidant peptides produced from whey hydrolysis with an immobilized aspartic protease from *Salpichroa origanifolia* fruits**. Food Chemistry**, v. 237, p. 350–355, 2017.

ROCHA, G. J. M.; GONÇALVES, A. R.; OLIVEIRA, B. R.; OLIVARES, E. G.; ROSSELL, C. E. V. Steam explosion pretreatment reproduction and alkaline delignification reactions performed on a pilot scale with sugarcane bagasse for bioethanol production. **Industrial Crops and Products**, v. 35, n. 1, p. 274–279, 2012.

RODRIGUES, D. S.; MENDES, A. A.; ADRIANO, W. S.; GONÇALVES, L. R. B.; GIORDANO, R. L. C. Multipoint covalent immobilization of microbial lipase on chitosan and agarose activated by different methods. **Journal of Molecular Catalysis B: Enzymatic**, v. 51, n. 3–4, p. 100–109, 2008.

RODRIGUES, R. C.; ORTIZ, C.; BERENGUER-MURCIA, Á.; TORRES, R.; FERNÁNDEZ-LAFUENTE, R. Modifying enzyme activity and selectivity by immobilization. **Chemical Society Reviews**, v. 42, n. 15, p. 6290–6307, 2013.

ROY, J. J.; ABRAHAM, T. E. Strategies in making cross-linked enzyme crystals. **Chemical Society Reviews**, v. 104, n. 9, p. 3705–3721, 2004.

RUBIN, E. M. Genomics of cellulosic biofuels. **Nature**, v. 454, n. 7206, p. 841–845, 2008.

RUEDA, N.; ALBUQUERQUE, T.; BARTOLOME-CABRERO, R.; FERNANDEZ-LOPEZ, L.; TORRES, R.; ORTIZ, C.; SANTOS, J. DOS; BARBOSA, O.; FERNANDEZ-LAFUENTE, R. reversible immobilization of lipases on heterofunctional octyl-amino agarose beads prevents enzyme desorption. **Molecules**, v. 21, n. 5, p. 646, 2016.

SADHU, S.; MAITI, T. K. Cellulase production by bacteria : A review. **British Microbiology Research Journal**, v. 3, n. 3, p. 235–258, 2013.

SAMANTA, A. K.; JAYAPAL, N.; JAYARAM, C.; ROY, S.; KOLTE, A. P.; SENANI, S.; SRIDHAR, M. Xylooligosaccharides as prebiotics from agricultural by-products: Production and applications. **Bioactive Carbohydrates and Dietary Fibre**, v. 5, n. 1, p. 62–71, 2015.

SAMARATUNGA, A.; KUDINA, O.; NAHAR, N.; PRYOR, S. W. Impact of enzyme loading on the efficacy and recovery of cellulolytic enzymes immobilized on enzymogel nanoparticles. **Applied Microbiology and Biotechnology**, p. 2872–2882, 2015.

SÁNCHEZ, C. Lignocellulosic residues: Biodegradation and bioconversion by fungi. **Biotechnology Advances**, v. 27, n. 2, p. 185–194, 2009.

SÁNCHEZ, Ó. J.; CARDONA, C. A. Trends in biotechnological production of fuel ethanol from different feedstocks. **Bioresource Technology**, v. 99, n. 13, p. 5270–5295, 2008.

SANDERSON, K. A chewy problem. **Nature**, v. 474, p. S12-S14, 2011.

SANTOS CASTRO, L. DOS; PEDERSOLI, W. R.; ANTONIÊTO, A. C. C.; STEINDORFF, A. S.; SILVA-ROCHA, R.; MARTINEZ-ROSSI, N. M.; ROSSI, A.; BROWN, N. A.; GOLDMAN, G. H.; FAÇA, V. M.; PERSINOTI, G. F.; SILVA, R. N. Comparative metabolism of cellulose, sophorose and glucose in *Trichoderma reesei* using high-throughput genomic and proteomic analyses. **Biotechnology for Biofuels**, v. 7, n. 1, p. 41, 2014.

SANTOS, C. R.; HOFFMAM, Z. B.; MATOS MARTINS, V. P. DE; ZANPHORLIN, L. M.; PAULA ASSIS, L. H. DE; HONORATO, R. V.; OLIVEIRA, P. S. L. DE; RULLER, R.; MURAKAMI, M. T. Molecular mechanisms associated with xylan degradation by *Xanthomonas* plant pathogens. **Journal of Biological Chemistry**, v. 289, n. 46, p. 32186–32200, 2014.

SANTOS, J. C. S. DOS; BARBOSA, O.; ORTIZ, C.; BERENGUER-MURCIA, A.; RODRIGUES, R. C.; FERNANDEZ-LAFUENTE, R. Importance of the support

properties for immobilization or purification of enzymes. **ChemCatChem**, v. 7, n. 16, p. 2413–2432, 2015.

SANTUCCI, B. S.; MAZIERO, P.; RABELO, S. C.; CURVELO, A. A. S.; PIMENTA, M. T. B. Autohydrolysis of hemicelluloses from sugarcane bagasse during hydrothermal pretreatment : A kinetic assessment. **BioEnergy Research**, v. 8, p. 1778–1787, 2015.

SEEMANN, T. Prokka: Rapid prokaryotic genome annotation. **Bioinformatics**, v. 30, n. 14, p. 2068–2069, 2014.

SERRA, I.; DANIELA, C.; ROCCHIETTI, S.; UBIALI, D.; TERRENI, M. Stabilization of thymidine phosphorylase from *Escherichia coli* by immobilization and post immobilization techniques. **Enzyme and Microbial Technology**, v. 49, n. 1, p. 52–58, 2011.

SHABEB, M. S. A.; YOUNIS, M. A. M.; HEZAYEN, F. F.; NOUR-ELDEIN, M. A. Production of cellulase in low-cost by *Bacillus subtilis* KO strain. **World Applied Sciences Journal,** v.8, n.1, p. 35-42, 2010.

SHELDON, R. A. Cross-linked enzyme aggregates (CLEAs): Stable and recyclable biocatalysts. **Biochemical Society Transactions**, v. 35, n. 6, p. 1583–1587, 2007.

SHELDON, R. A.; PELT, S. VAN. Enzyme immobilisation in biocatalysis: why, what and how. **Chemical Society Reviews**, v. 42, n. 42, p. 6223–6235, 2013.

SHEVCHENKO, A.; WILM, M.; VORM, O.; MANN, M. Mass Spectrometric sequencing of proteins from silver-stained polyacrylamide gels. **Analytical Chemistry**, v. 68, n. 5, p. 850–858, 1996.

SHIRLING, E. B.; GOTTLIEB, D. Methods for characterization of *Streptomyces* species. **International Journal of Systematic Bacteriology**, v. 16, n. 3, p. 313–340, 1966.

SILVA, T. M. DA; PESSELA, B. C.; SILVA, J. C. R. DA; LIMA, M. S.; JORGE, J. A.; GUISAN, J. M.; POLIZELI, M. D. L. T. M. Immobilization and high stability of an extracellular β-glucosidase from *Aspergillus japonicus* by ionic interactions. **Journal of Molecular Catalysis B: Enzymatic**, v. 104, p. 95–100, 2014.

SINGH, G.; VERMA, A. K.; KUMAR, V. Catalytic properties, functional attributes and industrial applications of β-glucosidases. **3 Biotech**, v. 6, n. 1, p. 3, 2016.

SINGH, R. K.; ZHANG, Y.-W.; NGUYEN, N.-P.-T.; JEYA, M.; LEE, J.-K. Covalent immobilization of β-1,4-glucosidase from *Agaricus arvensis* onto functionalized silicon oxide nanoparticles. **Applied Microbiology and Biotechnology**, v. 89, n. 2, p. 337–344, 2011.

SINGHANIA, R. R.; PATEL, A. K.; SUKUMARAN, R. K.; LARROCHE, C.; PANDEY, A. Role and significance of beta-glucosidases in the hydrolysis of cellulose for bioethanol production. **Bioresource Technology**, v. 127, p. 500–507, 2013.

SINGHANIA, R. R.; SUKUMARAN, R. K.; PANDEY, A. Improved cellulase production by *Trichoderma reesei* RUT C30 under SSF through process optimization. **Applied Microbiology and Biotechnology**, v. 142, p. 60–70, 2007.

SLUITER, A.; HAMES, B.; RUIZ, R.; SCARLATA, C.; SLUITER, J.; TEMPLETON, D. Determination of Sugars, Byproducts, and Degradation Products in Liquid Fraction Process Samples. Laboratory Analytical Procedure (LAP), Determination of Sugars, Byproducts, and Degradation Products in Liquid Fraction Proce. Golden, National Renewable Energy Laboratory, n. January, 2006.

SMITH, S. P.; BAYER, E. A. Insights into cellulosome assembly and dynamics: From dissection to reconstruction of the supramolecular enzyme complex. **Current Opinion in Structural Biology**, v. 23, n. 5, p. 686–94, 2013.

SOLTANI, N.; REZA, F.; BABAK, P. Direct introduction of amine groups into cellulosic paper for covalent immobilization of tyrosinase: Support characterization and enzyme properties. **Cellulose**, v. 24, n. 3, p. 1407–1416, 2017.

SOOZANIPOUR, A.; TAHERI-KAFRANI, A.; LANDARANI, A. Covalent attachment of xylanase on functionalized magnetic nanoparticles and determination of its activity and stability. **Chemical Engineering Journal**, v. 270, p. 235–243, 2015.

SØRENSEN, A.; LÜBECK, M.; LÜBECK, P. S.; AHRING, B. K. Fungal beta-glucosidases: A bottleneck in industrial use of lignocellulosic materials. **Biomolecules**, v. 3, n. 3, p. 612–31, 2013.

SOUSA, V. M. C. DE; SANTOS, E. F. DOS; SGARBIERI, V. C. The importance of prebiotics in functional foods and clinical practice. **Food and Nutrition Sciences**, v. 2, n. 2, p. 133–144, 2011.

SPAHN, C.; MINTEER, S. D. Enzyme immobilization in biotechnology. **Recent Patents on Engineering**, v. 2, n. 3, p. 195–200, 2008.

SRIVASTAVA, N.; SRIVASTAVA, M.; MISHRA, P. K.; SINGH, P. Application of cellulases in biofuels industries: An overview. **Journal of Biofuels and Bioenergy**, v. 1, n. 1, p. 55–63, 2015.

SUESCUN, A.; RUEDA, N.; SANTOS, J. C. S. DOS; CASTILLO, J. J.; ORTIZ, C.; TORRES, R.; BARBOSA, O.; FERNANDEZ-LAFUENTE, R. Immobilization of lipases

on glyoxyl-octyl supports: Improved stability and reactivation strategies. **Process Biochemistry**, v. 50, n. 8, p. 1211–1217, 2015.

SUKUMARAN, R. K.; SINGHANIA, R. R.; PANDEY, A. Microbial cellulases - Production, applications and challenges. **Journal of Scientific and Industrial Research**, v. 64, n. 11, p. 832–844, 2005.

SUTARLIE, L.; YANG, K. Hybrid cellulase aggregate with a silica core for hydrolysis of cellulose and biomass. **Journal of Colloid and Interface Science**, v. 411, p. 76–81, 2013.

SWEENEY, M. D.; XU, F. Biomass converting enzymes as industrial biocatalysts for fuels and chemicals: Recent developments. **Catalysts**, v. 2, n. 2, p. 244–263, 2012.

TAKASUKA, T. E.; BOOK, A. J.; LEWIN, G. R.; CURRIE, C. R.; FOX, B. G. Aerobic deconstruction of cellulosic biomass by an insect-associated *Streptomyces*. **Scientific Reports**, v. 3, p. 1030, 2013.

TAMURA, K.; STECHER, G.; PETERSON, D.; FILIPSKI, A.; KUMAR, S. MEGA6: Molecular evolutionary genetics analysis version 6.0. **Molecular Biology and Evolution,** v. 30, n. 12, p. 2725–2729, 2013.

TEATHER, R. M.; WOOD, P. J. Use of Congo red-polysaccharide interactions in enumeration and characterization of cellulolytic bacteria from the bovine rumen. **Applied and Environmental Microbiology**, v. 43, n. 4, p. 777–780, 1982.

TENNESSEN, K.; ANDERSEN, E.; CLINGENPEEL, S.; RINKE, C.; LUNDBERG, D. S.; HAN, J.; DANGL, J. L.; IVANOVA, N.; WOYKE, T.; KYRPIDES, N.; PATI, A. ProDeGe: A computational protocol for fully automated decontamination of genomes. **The ISME Journal**, v. 10, n. 1, p. 1–4, 2015.

TOMAZETTO, G.; HAHNKE, S.; KOECK, D. E.; WIBBERG, D.; MAUS, I.; PÜHLER, A.; KLOCKE, M.; SCHLÜTER, A. Complete genome analysis of *Clostridium bornimense* strain M2/40T: A new acidogenic *Clostridium* species isolated from a mesophilic two-phase laboratory-scale biogas reactor. **Journal of Biotechnolog**y, v. 232, p. 38-49, 2016.

TRAMONTINA, R.; FRANCO CAIRO, J. P. L.; LIBERATO, M. V.; MANDELLI, F.; SOUSA, A.; SANTOS, S.; RABELO, S. C.; CAMPOS, B.; IENCZAK, J.; RULLER, R.; DAMÁSIO, A. R. L.; SQUINA, F. M. The *Coptotermes gestroi* aldo-keto reductase: A multipurpose enzyme for biorefinery applications. **Biotechnology for Biofuels,** v. 10, n. 1, p. 4, 2017.

TRIVEDI, T. J.; SRIVASTAVA, D. N.; ROGERS, R. D.; KUMAR, A. Agarose processing in protic and mixed protic–aprotic ionic liquids: Dissolution, regeneration and high conductivity, high strength ionogels. **Green Chemistry**, v. 14, n. 10, p. 2831, 2012.

TURAL, B.; TARHAN, T.; TURAL, S. Covalent immobilization of benzoylformate decarboxylase from *Pseudomonas putida* on magnetic epoxy support and its carboligation reactivity. **Journal of Molecular Catalysis B: Enzymatic**, v. 102, p. 188–194, 2014.

VĚTROVSKÝ, T.; STEFFEN, K. T.; BALDRIAN, P. Potential of cometabolic transformation of polysaccharides and lignin in lignocellulose by soil Actinobacteria. **PLoS ONE**, v. 9, n. 2, p. e89108, 2014.

VIEIRA, M. F.; VIEIRA, A. M. S.; ZANIN, G. M.; TARDIOLI, P. W.; MATEO, C.; GUISÁN, J. M. β-glucosidase immobilized and stabilized on agarose matrix functionalized with distinct reactive groups. **Journal of Molecular Catalysis B: Enzymatic**, v. 69, n. 1–2, p. 47–53, 2011.

VOLYNETS, B.; EIN-MOZAFFARI, F.; DAHMAN, Y. Biomass processing into ethanol: Pretreatment, enzymatic hydrolysis, fermentation, rheology, and mixing. **Green Processing and Synthesis**, v.6, n.1, p. 1–22, 2017.

VRIES, R. P. DE; VISSER, J.; RONALD, P.; VRIES, R., DE; P. *Aspergillus* enzymes involved in degradation of plant cell wall polysaccharides. **Microbiology and Molecular Biology Review**s, v. 65, n. 4, p. 497–522, 2001.

WA, S.; ISHIYAMA, D.; VUJAKLIJA, D.; DAVIES, J. Novel pathway of salicylate degradation by novel pathway of salicylate degradation by *Streptomyces* sp. strain WA46. **Applied and Environmental Microbiology**, v. 70, n. 3, p. 1297–1306, 2004.

WANG, S.; DAI, G.; YANG, H.; LUO, Z. Lignocellulosic biomass pyrolysis mechanism : A state-of-the-art review. **Progress in Energy and Combustion Science**, v. 62, p. 33–86, 2017.

WHISTLER, R. L.; WOLFROM, M. L.; BEMILLER, J.. Methods in carbohydrate chemistry. 2. ed. [s.l.] Academic Press: New York, NY, USA; London, UK, 1963.

WONG, K. K. Y.; SADDLER, J. N. *Trichoderma* xylanases, their properties and application. **Critical Reviews in Biotechonology**, v. 12, n. 5/6, p. 413–435, 1992.

XIE, S.; SYRENNE, R.; SUN, S.; YUAN, J. S. Exploration of natural biomass utilization systems (NBUS) for advanced biofuel-from systems biology to synthetic design. **Current Opinion in Biotechnology**, v. 27, p. 195–203, 2014.

YIN, Y.; MAO, X.; YANG, J.; CHEN, X.; MAO, F.; XU, Y. dbCAN: A web resource for automated carbohydrate-active enzyme annotation. **Nucleic Acids Research**, v. 40, p. W445-51, 2012.

ZANPHORLIN, L. M.; GIUSEPPE, P. O. DE; HONORATO, R. V.; TONOLI, C. C. C.; FATTORI, J.; CRESPIM, E.; OLIVEIRA, P. S. L. DE; RULLER, R.; MURAKAMI, M. T. Oligomerization as a strategy for cold adaptation: Structure and dynamics of the GH1 β-glucosidase from *Exiguobacterium antarcticum* B7. Scientific reports, v. 6, p. 23776, 2016.

ZÁVODSZKY, P.; KARDOS, J.; SVINGOR; PETSKO, G. A. Adjustment of conformational flexibility is a key event in the thermal adaptation of proteins. **Proceedings of the National Academy of Sciences of the United States of America**, v. 95, n. 13, p. 7406–7411, 1998.

ZHANG, Y.; CHEN, S.; XU, M.; CAVOCO-PAULO, A. P.; WU, J.; CHEN, J. Characterization of *Thermobifida fusca* cutinase-carbohydrate-binding module fusion proteins and their potential application in bioscouring. **Applied and Environmental Microbiology**, v. 76, n. 20, p. 6870–6876, 2010.

ZHOU, Y.; POPE, P. B.; LI, S.; WEN, B.; TAN, F.; CHENG, S.; CHEN, J.; YANG, J.; LIU, F.; LEI, X.; SU, Q.; ZHOU, C.; ZHAO, J.; DONG, X.; JIN, T.; ZHOU, X.; YANG, S.; ZHANG, G.; YANG, H.; WANG, J.; YANG, R.; EIJSINK, V. G. H.; WANG, J. Omics-based interpretation of synergism in a soil-derived cellulose-degrading microbial community. **Scientific Reports**, v. 4, p. 1–6, 2014.

ZUCCA, P.; FERNANDEZ-LAFUENTE, R.; SANJUST, E. Agarose and its derivatives as supports for enzyme immobilization. **Molecules**, v. 21, n. 11, p. 1–25, 2016.

ANEXO 1

CAPÍTULO 1

Não possui material Suplementar

CAPÍTULO 2

Table S1. The genes associated to Carbohydrate Active Enzymes (CAZy) encoded in the strain F1 and F7.

Polysaccharide	***Enzymatic Activity***	***EC number***	***CAZy***	***Locus Tag***	
				Strain F1	***Strain F7***
Pectin	Pectinesterase	3.1.1.11	PL1	Not predicted	STEPF7_03951
	Pectate lyase	4.2.2.2	PL3	Not predicted	STEPF7_00221
	Pectate lyase	4.2.2.2	PL1	Not predicted	STEPF7_01521
	Pectate lyase.	4.2.2.40	AA10	Not predicted	STEPF7_03950
Starch	Alpha-amylase	3.2.1.1	GH13	STEPF1_06988;STEPF1_00433	STEPF7_00207;STEPF7_01788;STEPF7_04080;STEPF7_02288
	Alpha-glucosidase	3.2.1.20	GH13	STEPF1_01608;STEPF1_06989;STEPF1_00074;STEPF1_01081	STEPF7_05747;STEPF7_01788;STEPF7_06469
	Alpha-glucosidase	3.2.1.20	GH31	Not predicted	STEPF7_03439
	4-alpha-glucanotransferase	2.4.1.25	GH77	STEPF1_03274	STEPF7_05216
	Starch phosphorylase	2.4.1.1	GT35	STEPF1_06534	STEPF7_04556
Chitin	Chitinase	3.2.1.14	GH18	STEPF1_06614;STEPF1_01119;STEPF1_01872	STEPF7_03476;STEPF7_01123;STEPF7_04655;STEPF7_02361
	Beta-N-acetylhexosaminidase	3.2.1.52	GH3	STEPF1_02104;STEPF1_03679	STEPF7_04385;STEPF7_03200
	Hexosaminidase	3.2.1.52	GH20	STEPF1_03527;STEPF1_04159;STEPF1_03649	STEPF7_03806;STEPF7_01245;STEPF7_04415
	Chitinase	3.2.1.14	GH19	STEPF1_05163	STEPF7_04714
	N-acetylglucosamine 6-phosphate deacetylase	3.5.1.25	CE9	STEPF1_00260	STEPF7_02740
Chitosan	Chitosanase	3.2.1.132	GH46	STEPF1_01445	STEPF7_00018
	Exo-1,4-Beta-D-glucosaminidase.	3.2.1.165	GH2	STEPF1_00768	Not predicted

Polysaccharide	***Enzymatic Activity***	***EC number***	***CAZy***	***Locus Tag***	
				Strain F1	***Strain F7***
Arabinogalactan	Arabinogalactan endo-Beta-1,4-galactanase.	3.2.1.89	GH53	Not predicted	STEPF7_06008
Xyloglucan (Hemicellulose)	Alpha-D-xyloside xylohydrolase	3.2.1.177	GH31	STEPF1_03025;STEPF1_01873	STEPF7_00499
Arabinan (Hemicellulose)	Alpha-L-arabinofuranosidase	3.2.1.55	GH62	STEPF1_01633;STEPF1_01633;STEPF1_02514	Not predicted
	Alpha-L-arabinofuranosidase	3.2.1.55	GH51	STEPF1_01638	STEPF7_03647
Xylan (Hemicellulose)	Endo-1,4-Beta-xylanase (glycosyl hydrolase family 10)	3.2.1.8	GH10	STEPF1_02515;STEPF1_03077;STEPF1_04518;STEPF1_07199	STEPF7_05844;STEPF7_00325;STEPF7_03021;STEPF7_05844;STEPF7_00325
	Endo-1,4-Beta-xylanase	3.2.1.8	GH11	STEPF1_07199	STEPF7_03152;STEPF7_05182
	Xylan 1,4-Beta-xylosidase	3.2.1.37	GH39	Not predicted	STEPF7_04179
	Beta-xylosidase	3.2.1.37	GH43	STEPF1_01633	STEPF7_00906;STEPF7_04137
	Alpha-N-arabinofuranosidase	3.2.1.55	GH51	STEPF1_01638	STEPF7_03647
	Alpha-glucuronidase	3.2.1.139	GH67	Not predicted	STEPF7_04177
	Alpha-L-arabinofuranosidase	3.2.1.55	GH54	STEPF1_02995;STEPF1_01633;STEPF1_02515;STEPF1_05660	Not predicted
Mannan (Hemicellulose)	Mannan endo-1,4-Beta-mannosidase	3.2.1.78	GH5	Not predicted	STEPF7_01472
	Beta-mannosidase	3.2.1.25	GH2	Not predicted	STEPF7_04152

Polysaccharide	*Enzymatic Activity*	*EC number*	*CAZy*	*Locus Tag*	
				Strain F1	*Strain F7*
Cellulase	Beta-glucosidase	3.2.1.21	GH1	STEPF1_03634;STEPF1_03639;STEPF1_03160;STEPF1_07040	STEPF7_00458;STEPF7_04428;STEPF7_05964;STEPF7_04263;STEPF7_00235
	Beta-glucosidase	3.2.1.21	GH1	Not predicted	STEPF7_00235
	Beta-glucosidase	3.2.1.21	GH3	STEPF1_06315	STEPF7_06178;STEPF7_06410
	Endoglucanase	3.2.1.4	GH5	STEPF1_01473	STEPF7_00502
	Endoglucanase	3.2.1.4	GH6	STEPF1_04348;STEPF1_01473	STEPF7_00904;STEPF7_03895;STEPF7_03496
	Cellulose 1,4-Beta-cellobiosidase	3.2.1.91	GH6	Not predicted	STEPF7_01028
	Endoglucanase	3.2.1.4	GH9	Not predicted	STEPF7_00645
Lichenan	Endo-1,3-Beta-glucanase	3.2.1.39	GH64	STEPF1_03028	STEPF7_00491;STEPF7_00586
Auxiliary Activities	Catalase-peroxidase		AA2	STEPF1_00667;STEPF1_02865	STEPF7_02188
	Choline dehydrogenase		AA3	STEPF1_06102;STEPF1_04130;STEPF1_03021	STEPF7_06087;STEPF7_01283
	Cholesterol oxidase		AA3	STEPF1_07010;STEPF1_04093	STEPF7_01326
	Chitin-binding protein		AA10	STEPF1_01045;STEPF1_07264	STEPF7_05046;STEPF7_00485;STEPF7_02156;STEPF7_02236
Carbohydrate Esterases	Esterase, PHB depolymerase family		CE1	Not predicted	STEPF7_03778
	Pimeloyl-ACP methyl ester carboxylesterase		CE1	Not predicted	STEPF7_03705;STEPF7_02209
	S-formylglutathione hydrolase FrmB		CE1	Not predicted	STEPF7_05976
	Carboxymethylenebutenolidase		CE1	Not predicted	STEPF7_02272
	Polysaccharide deacetylase		CE4	STEPF1_07200;STEPF1_02173	STEPF7_05183

Polysaccharide	*Enzymatic Activity*	*EC number*	*CAZy*	*Locus Tag*	
				Strain F1	*Strain F7*
Carbohydrate Esterases	Polysacc_deac_1		CE4		STEPF7_01355;STEPF7_01356;STEPF7_06146;STEPF7_01763
	Peptidoglycan/xylan/chitin deacetylase, PgdA/CDA1 family		CE4		STEPF7_06035;STEPF7_00786;STEPF7_04123;STEPF7_04342
	N-acetylglucosamine-6-phosphate deacetylase.		CE9	STEPF1_00260;STEPF1_05136	STEPF7_02740
	Para-nitrobenzyl esterase		CE10	Not predicted	STEPF7_00282
	Acetyl esterase		CE10	Not predicted	STEPF7_02116
	N-acetyl-1-D-myo-inositol-2-amino-2-deoxy-Alpha-D-glucopyranoside deacetylase.		CE14	Not predicted	STEPF7_02457
	N-acetylglucosaminyl deacetylase, LmbE family		CE14	STEPF1_00619	STEPF7_03169;STEPF7_00675
	Carboxymethylenebutenolidase.		CE7	Not predicted	STEPF7_02272
	Dienelactone hydrolase		CE7	Not predicted	STEPF7_02331;STEPF7_03952
Polysaccharide Lyases	Xanthan lyase		PL8	STEPF1_01801	Not predicted
	Pectate lyase			Not predicted	STEPF7_05746;STEPF7_02199
	Pectate lyase			Not predicted	STEPF7_03950;STEPF7_01521;STEPF7_00221
Others CAZymes	GH2			Not predicted	STEPF7_00945
	GH3			STEPF1_06095	Not predicted
	GH12			Not predicted	STEPF7_00484
	GH13			STEPF1_06987	Not predicted
	GH18			STEPF1_05904;STEPF1_02474;STEPF1_05163	Not predicted
	GH25			STEPF1_05800	Not predicted

Polysaccharide	*Enzymatic Activity*	*EC number*	*CAZy*	*Locus Tag*	
				Strain F1	***Strain F7***
Others CAZymes	GH30			Not predicted	STEPF7_00512
	GH35			STEPF1_05092	STEPF7_03646
	GH43			Not predicted	STEPF7_03947
	GH48			STEPF1_01473	STEPF7_01027
	GH59			STEPF1_02513	Not predicted
	GH62			Not predicted	STEPF7_05845
	GH64			STEPF1_05270	Not predicted
	GH75			STEPF1_06380;STEPF1_00828	STEPF7_04672
	GH81			Not predicted	STEPF7_06106;STEPF7_00230
	GH85			STEPF1_04249	Not predicted
	GH92				Not predicted
	GH127			STEPF1_05673	STEPF7_00482
CBM	CBM_2			STEPF1_06480;STEPF1_06122;STEPF1_01045;STEPF1_03077;STEPF1_02251;STEPF1_02314;STEPF1_06614;STEPF1_02510;STEPF1_07200;STEPF1_07199	STEPF7_01472;STEPF7_01028;STEPF7_01027;STEPF7_01026;STEPF7_00759;STEPF7_05182;STEPF7_00325;STEPF7_01123;STEPF7_00485;STEPF7_00484
	CBM_3			STEPF1_0147	Not predicted
	CBM_4_9			STEPF1_02474;STEPF1_06240;STEPF1_05904	STEPF7_04655;STEPF7_02361
	CBM_5_12			STEPF1_01872	Not predicted
	CBM_6_35_36			Not predicted	STEPF7_06008
	CBM_11			STEPF1_06315	Not predicted
	CBM_35			STEPF1_00258	STEPF7_02134
	CBM_41			STEPF1_06987	STEPF7_00205
	CBM_48			STEPF1_02213;STEPF1_00711;STEPF1_06826;STEPF1_02213;STEPF1_06517	STEPF7_00205;STEPF7_04543;STEPF7_01069;STEPF7_04783;STEPF7_02288

Polysaccharide	*Enzymatic Activity*	*EC number*	*CAZy*	*Locus Tag*	
				Strain F1	*Strain F7*
Glycosyltransferases	N-glycosyltransferase		GT1	STEPF1_06947	Not predicted
	glycosyltransferase, MGT family		GT1	STEPF1_02843	Not predicted
	Glycosyltransferase, GT2 family		GT2	STEPF1_06482	Not predicted
	Glycos_transf_2		GT2	STEPF1_01114	Not predicted
	Dolichyl-phosphate Beta-D-mannosyltransferase.		GT2	Not predicted	STEPF7_03470
	D-inositol-3-phosphate glycosyltransferase.		GT4	Not predicted	STEPF7_02687
	Alpha,Alpha-trehalose-phosphate synthase (UDP-forming).		GT20	Not predicted	STEPF7_02745
	Glycogen phosphorylase.		GT35	Not predicted	STEPF7_04556
	Dolichyl-phosphate-mannose-protein mannosyltransferase		GT39	Not predicted	STEPF7_00594
	Dolichyl-phosphate-mannose-protein mannosyltransferase		GT76	STEPF1_06521;STEPF1_04836	STEPF7_05057;STEPF7_04688
	Mannosyltransferase (PIG-V)		GT76	STEPF1_00149;STEPF1_00707	
	Glucosyl-3-phosphoglycerate synthase		GT81	Not predicted	STEPF7_02746
	mannosyltransferase		GT83	STEPF1_03499	Not predicted
	Dolichyl-phosphate-mannose-protein mannosyltransferase		GT83	STEPF1_01707	Not predicted

Polysaccharide	*Enzymatic Activity*	*EC number*	*CAZy*	*Locus Tag*	
				Strain F1	*Strain F7*
	4-amino-4-deoxy-L-arabinose transferase		GT83	STEPF1_04659	STEPF7_01998
	Alpha-1,2-mannosyltransferase		GT87	STEPF1_00846	Not predicted
	arabinofuranan 3-O-arabinosyltransferase		GT87	STEPF7_01385	Not predicted

Table S2. Gene clusters encoding the gentisate and protocatechuate degradation patways found in F1 and F7 genome.

Aromatic compound	*Enzymatic activity*	*EC number*	*Locus Tag*	
			strain F1	*strain F7*
Protocatechuate	DNA-binding transcriptional regulator, LysR family	-----	STEPF1_04370	STEPF7_05728
	4-carboxymuconolactone decarboxylase.	4.1.1.44	STEPF1_04371	-----
	3-oxoadipate enol-lactonase.	3.1.1.24	STEPF1_04371	STEPF7_05732
	3-carboxy-cis,cis-muconate cycloisomerase.	5.5.1.2	STEPF1_04372	STEPF7_05731
	Protocatechuate 3,4-dioxygenase.	1.13.11.3	STEPF1_04373	STEPF7_05729
	Protocatechuate 3,4-dioxygenase.	1.13.11.3	STEPF1_04374	STEPF7_05730
	3-oxoadipyl-CoA/3-oxo-5,6-dehydrosuberyl-CoA thiolase	2.3.1.174	STEPF1_04375	-----
	3-oxoacid CoA-transferase subunit A	2.8.3.6	STEPF1_04376	-----
	3-oxoacid CoA-transferase subunit B	2.8.3.6	STEPF1_04377	-----
	Transcriptional regulator	-----	STEPF1_04378	-----
Gentisate	DNA-binding transcriptional regulator, IclR family	-----	STEPF1_01998	STEPF7_02132
	Gentisate 1,2-dioxygenase	1.13.11.4	STEPF1_01999	STEPF7_02131
	Fumarylacetoacetate (FAA) hydrolase family	3.7.1.5	STEPF1_02000	STEPF7_02130
	Maleylpyruvate isomerase domain	5.2.1.4	STEPF1_02001	STEPF7_02129

CAPÍTULO 3

Não possui material Suplementar

CAPÍTULO 4

Não possui material Suplementar

CAPÍTULO 5

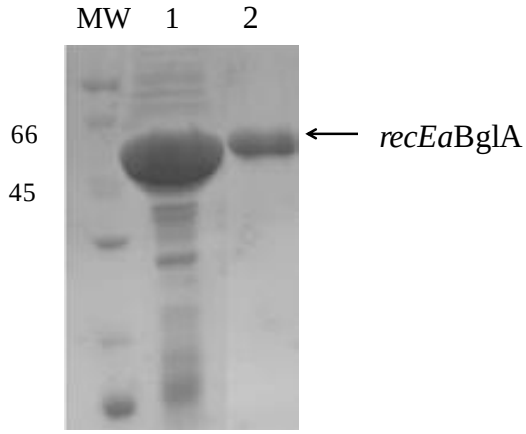


Figure S1 - SDS-PAGE of the purified *recEa*BglA - M, molecular marker (kDa); 1 - *E. coli* BL21 crude extract with *recEa*Bgl; 2 - *recEa*BglA after purification by affinity column and molecular exclusion chromatography

ANEXO 2

DOCUMENTOS CIENTÍFICOS PUBLICADOS NO PERÍODO DE DESENVOLVIMENTO DA TESE.

Publicação 1

Brazilian Journal of Microbiology

Available online 10 February 2017

open access

In Press, Corrected Proof

Genome Announcement

Draft genome sequence of *Streptomyces* sp. strain F1, a potential source for glycoside hydrolases isolated from Brazilian soil

Ricardo Rodrigues de Melo [a, b, 1], Gabriela Felix Persinoti [a, 1], Douglas Antonio Alvaredo Paixão [a], Fábio Márcio Squina [a], Roberto Ruller [a] ✉, Helia Harumi Sato [b] ✉

Show more

https://doi.org/10.1016/j.bjm.2016.11.010

Abstract

Here, we show the draft genome sequence of *Streptomyces* sp. F1, a strain isolated from soil with great potential for secretion of hydrolytic enzymes used to deconstruct cellulosic biomass. The draft genome assembly of *Streptomyces* sp. strain F1 has 69 contigs with a total genome size of 8,142,296 bp and G + C 72.65%. Preliminary genome analysis identified 175 proteins as Carbohydrate-Active Enzymes, being 85 glycoside hydrolases organized in 33 distinct families. This draft genome information provides new insights on the key genes encoding hydrolytic enzymes involved in biomass deconstruction employed by soil bacteria.

Publicação 2

CRITICAL REVIEWS IN BIOTECHNOLOGY, 2017
https://doi.org/10.1080/07388551.2017.1354354

REVIEW ARTICLE

An overview of *Bacillus* proteases: from production to application

Fabiano Jares Contesini, Ricardo Rodrigues de Melo and Hélia Harumi Sato

Laboratory of Food Biochemistry, Department of Food Science, College of Food Engineering, State University of Campinas (UNICAMP), Campinas, SP, Brazil

ABSTRACT

Proteases have a broad range of applications in industrial processes and products and are representative of most worldwide enzyme sales. The genus *Bacillus* is probably the most important bacterial source of proteases and is capable of producing high yields of neutral and alkaline proteolytic enzymes with remarkable properties, such as high stability towards extreme temperatures, pH, organic solvents, detergents and oxidizing compounds. Therefore, several strategies have been developed for the cost-effective production of *Bacillus* proteases, including optimization of the fermentation parameters. Moreover, there are many studies on the use of low-cost substrates for submerged and solid state fermentation. Other alternatives include genetic tools such as protein engineering in order to obtain more active and stable proteases and strain engineering to better secrete recombinant proteases from *Bacillus* through homologous and heterologous protein expression. There has been extensive research on proteases because of the broad number of applications for these enzymes, such as in detergent formulations for the removal of blood stains from fabrics, production of bioactive peptides, food processing, enantioselective reactions, and dehairing of skins. Moreover, many commercial proteases have been characterized and purified from different *Bacillus* species. Therefore, this review highlights the production, purification, characterization, and application of proteases from a number of *Bacillus* species.

ARTICLE HISTORY
Received 20 April 2015
Revised 5 June 2017
Accepted 18 June 2017

KEYWORDS
Bioactive peptides; detergent; protease; *Bacillus*; genetic engineering of proteases; food industry

Publicação 3

ACADEMIC PRESS

Biotechnology of Microbial Enzymes

Production, Biocatalysis and Industrial Applications

2017, Pages 475–511

Chapter 18 – Agro-Industrial Residues and Microbial Enzymes: An Overview on the Eco-Friendly Bioconversion into High Value-Added Products

Jose Valdo Madeira Jr[1], Fabiano Jares Contesini[2], Felipe Calzado[2], Marcelo Ventura Rubio[2], Mariane Paludetti Zubieta[2], Danielle Branta Lopes[1], Ricardo Rodrigues de Melo[1]

Show more

https://doi.org/10.1016/B978-0-12-803725-6.00018-2

Abstract

Bioconversion of renewable lignocellulosic biomass to biofuels and high value-added products is nowadays a field of much attention and promise. Furthermore, lignin can be used for the smooth generation of polymers using laccase or a laccase-mediator system. Food industries provide other important uses of residues, such as underutilized fish, and by-products from the fishing industries for the production of bioactive peptides using microbial proteases. On the other hand, fat wastes, such as waste from cooking oil, are very interesting substrates for the production of industrially relevant compounds, mainly biodiesel, using lipases from different microorganisms. Again, phenolic compounds are very important because of their biological activities, presenting impressive antioxidant activity. More interestingly, they can be obtained using enzymes from different microorganisms, which are capable of producing antioxidative phenolics from different wastes. Although microbial enzymes are highly effective tools for modifying agro-industrial residues in generating high value-added products, the use of native enzymes are frequently infeasible in large scales. Therefore, different techniques of molecular biology are necessary to surpass these limitations. These techniques include the use of expression models that are more feasible for the industrial production of enzymes, and genetic and protein engineering focusing on the overexpression of the enzymes to have the desired enzymes with improved characteristics, such as better enzymatic activity, stability, and selectivity.

Keywords

Microbial enzymes; genetic engineering; agro-industrial residues; biomass; value-added products

Publicação 4

Catalysts **2016**, *6*(12), 191; doi:10.3390/catal6120191 Open Access Feature Paper Article

New Tailor-Made Alkyl-Aldehyde Bifunctional Supports for Lipase Immobilization

Robson Carlos Alnoch [1,2], Ricardo Rodrigues de Melo [1,3], Jose M. Palomo [1], Emanuel Maltempi de Souza [2], Nadia Krieger [4] and Cesar Mateo [1,*]

[1] Departamento de Biocatálisis, Instituto de Catálisis y Petroleoquímica (CSIC), Marie Curie 2. Cantoblanco, Campus UAM, 28049 Madrid, Spain

[2] Departamento de Bioquímica e Biologia Molecular, Universidade Federal do Paraná, Cx. P. 19081 Centro Politécnico, 81531-980 Curitiba, Paraná, Brazil

[3] Departamento de Ciência de Alimentos, Faculdade de Engenharia de Alimentos (FEA), Universidade Estadual de Campinas, 13083-862 Campinas, São Paulo, Brazil

[4] Departamento de Química, Universidade Federal do Paraná, Cx. P. 19081 Centro Politécnico, 81531-980 Curitiba, Paraná, Brazil

* Author to whom correspondence should be addressed.

Academic Editor: David D. Boehr

Received: 28 October 2016 / Revised: 24 November 2016 / Accepted: 27 November 2016 / Published: 30 November 2016

(This article belongs to the Special Issue Asymmetric and Selective Biocatalysis)

Abstract

Immobilized and stabilized lipases are important biocatalytic tools. In this paper, different tailor-made bifunctional supports were prepared for the immobilization of a new metagenomic lipase (LipC12). The new supports contained hydrophobic groups (different alkyl groups) to promote interfacial adsorption of the lipase and aldehyde groups to react covalently with the amino groups of side chains of the adsorbed lipase. The best catalyst was 3.5-fold more active and 5000-fold more stable than the soluble enzyme. It was successfully used in the regioselective deacetylation of peracetylated D-glucal. The PEGylated immobilized lipase showed high regioselectivity, producing high yields of the C-3 monodeacetylated product at pH 5.0 and 4 °C.

Keywords: regioselective hydrolysis; biocatalysis; lipase; interfacial activation; covalent immobilization; tailor-made supports; enzyme stabilization

Publicação 5

Living Reference Work Entry
Fungal Metabolites
Part of the series Reference Series in Phytochemistry pp 1-28

Date: 11 May 2016 Latest Version

Aspergillus Lipases: Biotechnological and Industrial Application

Fabiano Jares Contesini , Felipe Calzado, Jose Valdo Madeira Jr, Marcelo Ventura Rubio, Mariane Paludetti Zubieta, Ricardo Rodrigues de Melo, Thiago Augusto Gonçalves

Get Access

Abstract

Lipases are enzymes with remarkable properties and catalytic versatility. These proteins are capable of catalyzing hydrolytic and synthetic reactions, allowing the production of different compounds. *Aspergillus* are important producers of lipases, since they are able to secrete large amounts of these proteins to the extracellular media. Several studies have reported the importance of fermentation parameters as well as genetic engineering of *Aspergillus* strains in order to improve lipase production. Different *Aspergillus* species secrete lipases with interesting characteristics such as thermostability, stability in a wide pH range, stability in organic solvents, and enantioselectivity toward the substrate. The obtainment of lipases with highlighted characteristics for use in industry is the main focus of several studies. Such lipases can be obtained with screening of *Aspergillus* strains, protein engineering, and immobilization of lipases that can frequently improve thermostability and enantioselectivity. Among the applications of lipases from *Aspergillus*, there are studies on the improvement of sensorial properties of different products in the food industry, compatibility with detergents for removal of fat stains from fabrics, and the obtainment of enantiopure pharmaceuticals.

Keywords

Lipases – *Aspergillus* – Lipase properties – Biotechnological applications – Biotechnology – Immobilization – Lipase production

Publicação 6

Vegetable Oil: Properties, Uses and Benefits **$190.00**

Click to enlarge

Editors: Brittany Holt

Book Description:
Vegetable oils are hydrophobic compounds, such as tryacylglicerols and essential oils extracted from plants that have been used by humans for centuries in many different areas. The use of isolated enzymes and microorganisms applied to vegetable oils has been shown to be very interesting from an industrial viewpoint, due to the broad variety of products that may be obtained from it. This book provides current research on the properties, uses and benefits of vegetable oils. (Imprint: Nova)

Table of Contents:
Preface

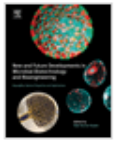
Chapter 1
An Overview on Vegetable Oils and Biocatalysis
(Ricardo Rodrigues de Melo, Gustavo Pagotto Borin, Guilherme Keppe Zanini, Antonio Adalberto Kaupert Neto, Bruna Soares Fernandes and Fabiano Jares Contesini, Brazilian Bioethanol Science and Technology Laboratory (CTBE), Brazilian Centre of Research in Energy and Materials (CNPEM), Campinas, São Paulo, Brazil, and others)

Publicação 7

New and Future Developments in Microbial Biotechnology and Bioengineering

Aspergillus System Properties and Applications

2016, Pages 155–169



Chapter 11 – β-Glucosidase From *Aspergillus*

G. Molina[1,2], F.J. Contesini[1], R.R. de Melo[1], H.H. Sato[1], G.M. Pastore[1]

Show more

https://doi.org/10.1016/B978-0-444-63505-1.00011-7

Abstract

Different species from the genus *Aspergillus*, such as *A. niger* and *A. oryzae*, are able to produce a wide range of carbohydrate-active enzymes (CAZymes), including β-glucosidase, that present several industrial applications. This glycosyl hydrolase hydrolyzes β-linked *O*-glycosyl bond of various oligomeric saccharides or glycosides from cellulose. Hence, it is a promising biocatalyst for the synthesis of stereo- and regiospecific glycosides or oligosaccharides, molecules potentially useful as functional materials, nutraceuticals, or pharmaceuticals. In addition, this enzyme can be used in combination with other enzymes in the degradation of lignocellulosic materials for the obtainment of second-generation ethanol. In this perspective, this chapter highlights the production, purification, characterization, applications, and immobilization of β-glucosidases from different *Aspergillus* species.

Publicação 8

Pectin: Chemical Properties, Uses and Health Benefits
July 01, 2014, Pages 49-63

Pectin: An efficient matrix for cell and enzyme immobilization

(Book Chapter)

Contesini, F.J., de Melo, R.R., Lopes, D.B., Junior, J.V.M., Kawaguti, H.Y., Ceresino, E.B.

Laboratory of Biochemistry, Department of Food Science, State University of Campinas, Campinas, SP, Brazil

Abstract

View references (64)

Pectins are polysaccharides containing D-galacturonic acid and galacturonic acid with methyl ester residues that can be acetylated to some degree. This biopolymer has been used as a gelling agent for the last two centuries and is extensively applied in food and pharmaceutical industries. In this case, pectins with a methylation degree lower than 50%, called low-methoxyl pectin (LMP), form gel in the presence of calcium ions, and hence, may be used as a gelling agent in numerous types of products such as: low-calorie jams and jellies, confectionery jelly products, and other food applications. However, one highlighted use of LMP is for the entrapment, encapsulation or immobilization of enzymes and cells for biotechnological applications. The encapsulation of a lipase in pectin gels cross-linked with calcium ions brought three to four times more enzymatic activity in water miscible organic co-solvents compared with aqueous systems. In another study, a-amylase and glucoamylase enzymes were immobilized to pectin by covalent binding showing greater thermal and pH stability over the free enzyme system with the complete retention of original activities. The immobilized enzymes showed the highest release of glucose compared with free enzymes when applied in starch hydrolysis. Another important use of LMP is in the entrapment of microbial cells for biocatalytic/ bio-transformation and fermentation uses. When the cells of the Nocardia tartaricans bacterial strain were immobilized in pectate gel to obtain L-tartrate, higher cisepoxysuccinate hydrolase activity was observed compared with the free cells. An additional study reports the immobilization of Saccharomyces cerevisiae cells in pectin gel for ethanol produc-tion, indicating that no significant changes occurred. Cells maintained their growth capacity, and the beads could be reutilized several times in successive batch fermentations, which is one of the major advantages of cell immobilization. The uses of pectin will be reviewed in this chapter since different highadded- value-compounds can be obtained showing the remarkable relevance of this matrix for biocatalysts immobilization.

Publicação 9

Oleic Acid: Production, Uses and Potential Health Effects
April 01, 2014, Pages 55-78

Oleic acid and microbial lipases: An efficient combination

(Book Chapter)

Contesini, F.J., Lopes, D.B., Ceresino, E.B., Madeira Junior, J.V., Speranza, P., Barros, F.F.C., de Melo, R.R.

Laboratory of Biochemistry, Department of Food Science, State University of Campinas, Campinas - SP, Brazil

Abstract

View references (111)

Oleic acid is a monounsaturated fatty acid found in high concentrations in vegetable oils, presenting a broad number of applications in many industrial areas, such as food, pharmaceutical, cosmetic, oleochemical and biodiesel industries. Due to the lipophilicity, unsaturation and acidic characteristics that this compound presents, oleic acid can be effectively used in esterification and acidolysis, among other reactions. Recent studies have used oleic acid as an efficient substrate for synthesis of trimethylolpropane esters by esterification using lipase from Candida Antarctica, since this polyol ester is widely applied in hydraulic fluids with several applications. Other studies used C. antarctica lipase for improving the lipophilicity of bioactive molecules, such as ferulic acid and L-ascorbic acid by esterification with oleic acid, which is very interesting, taking into account that it increases the solubility of these molecules in hydrophobic environments, resulting in higher biological activities. On the other hand, some studies showed that lipases can be used to convert oleic acid into epoxies, which are useful intermediates in organic synthesis due to the high reactivity they present. They are used to produce plasticizers that increase flexibility, workability or distensibility of plastics, hence rendering them suitable for several applications. One study reported biodiesel production by esterification of oleic acid with aliphatic alcohols using immobilized Candida antarctica lipase, showing high yields of biodiesel (above 90%) in less than 24 h with ethanol, n- propanol and n-butanol; whereas with methanol, the enzyme was inactive after ten cycles of reaction. In addition to the various reactions involving oleic acid as a promising substrate for various reactions, oleic acid can also be used to induce microbial lipase production, as seen in a study using the fungal strain Rhizopus arrhizus. Therefore, different high- added-value compounds can be obtained using oleic acid as a cheap and efficient substrate for microbial lipases, which can be considered as environmentally friendly alternatives for chemical catalysts. Within this context, this chapter reviews some studies and trends on the use of oleic acid as an efficient substrate for microbial lipases.

ANEXO 3

FORMULÁRIO DE APROVAÇÃO DA COMISSÃO INTERNA DE BIOSSEGURANÇA (CIBIO).

CNPEM

Campinas, 04 de outubro de 2017.

Para Programa de pós-graduação em Ciência de Alimentos

Assunto aprovação de projeto na CIBio - CNPEM

Prezado coordenador, declaramos para os devidos fins que Ricardo Rodrigues de Melo, teve seu projeto analisado e aprovado pela CIBio do CNPEM em 09/02/2015, protocolo 2015-2. O Título originalmente submetido foi "Estudos da produção, clonagem e imobilização de enzimas hemicelulolíticas e lignolíticas dos gêneros *Streptomyces* e *Bacillus* visando potenciais aplicações industriais", sob supervisão do Roberto Ruller, PhD. Este projeto rendeu resultados que foram utilizados para o desenvolvimento da tese, intitulada "Estudos de hidrolases glicosídicas bacterianas para aplicações biotecnológicas: Bioprospecção, produção e imobilização". Outrossim, informamos que os trabalhos contidos na sua tese estão contemplados no projeto aprovado pela CIBio.

Atenciosamente,

Ricardo Rodrigues de Melo
Doutorando em Ciência de Alimentos
FEA – UNICAMP

Roberto Ruller, PhD
Pesquisador do Laboratório Nacional de Ciência e Tecnologia do Bioetanol
CTBE-CNPEM

Marcio Chaim Bajgelman, PhD
Presidente da CIBio - CNPEM