Universidade Estadual de Maringá
Programa de Pós-Graduação em Ciências Biológicas
Área de Concentração: Biologia Celular e Molecular

FABIANE CRISTINA DOS SANTOS

PROSPECÇÃO DE BACTÉRIAS PRODUTORAS DE ENZIMAS HIDROLÍTICAS. PRODUÇÃO RECOMBINANTE, PURIFICAÇÃO E CARACTERIZAÇÃO DE UMA CICLODEXTRINASE DE *Massilia timonae*

Maringá
2018

FABIANE CRISTINA DOS SANTOS

PROSPECÇÃO DE BACTÉRIAS PRODUTORAS DE ENZIMAS HIDROLÍTICAS. PRODUÇÃO RECOMBINANTE, PURIFICAÇÃO E CARACTERIZAÇÃO DE UMA CICLODEXTRINASE DE *Massilia timonae*

Tese apresentada ao Programa de Pós-Graduação em Ciências Biológicas (Área de Concentração – Biologia Celular e Molecular) da Universidade Estadual de Maringá, como parte dos requisitos exigidos para obtenção do Título de Doutor em Ciências Biológicas.

Orientadora: Prof[a]. Dr[a]. Ione Parra Barbosa-Tessmann

Maringá
2018

Dados Internacionais de Catalogação na Publicação (CIP)
(Biblioteca Central - UEM, Maringá, PR, Brasil)

S237p

Santos, Fabiane Cristina dos
Prospecção de bactérias rodutoras de enzimas hidrolíticas. Produção recombinante, purificação e caracterização de uma Ciclodextrinase de *Massilia timonae* / Fabiane Cristina dos Santos. -- Maringá, 2018.

Orientador: Prof. Dr. Ione Parra Barbosa-Tessmann.
Tese (doutorado) - Universidade Estadual de Maringá, Programa de Pós-Graduação em Ciências Biológicas, 2018.

1. Bactéria. 2. Milho. 3. Prospecção. 4. Enzimas hidrolíticas. 5. Ciclodextrinase - Caracterização. 6. Purificação. 7. *Massilia timonae*. 8. Amilase. I. Barbosa-Tessmann, Ione Parra, orient. II. Universidade Estadual de Maringá. Programa de Pós-Graduação em Ciências Biológicas. III. Título.

CDD 21.ed. 572.7

Mariza Nogami - CRB 9/1569
CC-003949

FABIANE CRISTINA DOS SANTOS

PROSPECÇÃO DE BACTÉRIAS PRODUTORAS DE ENZIMAS HIDROLÍTICAS. PRODUÇÃO RECOMBINANTE, PURIFICAÇÃO E CARACTERIZAÇÃO DE UMA CICLODEXTRINASE DE *Massilia timonae*

Tese apresentada ao Programa de Pós-Graduação em Ciências Biológicas da Universidade Estadual de Maringá como parte dos requisitos para obtenção do título de Doutor em Ciências Biológicas – Biologia Celular e Molecular.

Aprovado em 29 de junho de 2018.

BANCA EXAMINADORA

Profa. Dra. DAYANE ALBERTON
Universidade Federal do Paraná - UFPR - Curitiba

Profa. Dra. MARCIA PORTILHO
Universidade Estadual de Maringá - UEM - Sede

Profa. Dra. CRISTINA GIATTI MARQUES DE SOUZA
Universidade Estadual de Maringá - UEM - Sede

Profa. Dra. ROSANE MARINA PERALTA
Universidade Estadual de Maringá - UEM – Sede

Profa. Dra. IONE PARRA BARBOSA TESSMANN
Universidade Estadual de Maringá - UEM - Sede

BIOGRAFIA

Fabiane Cristina dos Santos nasceu em Campo Mourão, Paraná, em 19/09/1989. Possui graduação em Biomedicina pela Universidade Paranaense (UNIPAR) no Campus de Cascavel (2011) e mestrado em Ciências Biológicas – Biologia Celular e Molecular pela Universidade Estadual de Maringá, Paraná (2014). Em março de 2014 iniciou o curso de Doutorado em Ciências Biológicas - Biologia Celular e Molecular pela mesma instituição. Tem experiência na área de Bioquímica e Biologia Molecular de Microrganismos.

DEDICATÓRIA

Aos meus pais, Adélio Ricardo dos Santos e Célia Yoshiko dos Santos pela paciência, pelo amor, incentivo e principalmente, pela educação que me deram.

AGRADECIMENTOS

Primeiramente a Deus, em sua infinita bondade, por permitir seguir adiante com sabedoria todos os dias.

À minha família, pelo apoio e amor incondicional durante essa caminhada.

À Universidade Estadual de Maringá e ao Programa de Pós-Graduação em Ciências Biológicas, pela oportunidade.

À Professora Doutora Ione Parra Barbosa Tessmann, pela incrível orientação, confiança, dedicação e principalmente pelos conhecimentos adquiridos em mais essa etapa.

À Capes pela concessão da bolsa.

Ao CNPq pelo auxílio financeiro concedido.

Aos docentes do Programa de Pós-Graduação em Ciências Biológicas, que ao longo do doutorado nos ajudaram com parcerias e troca de conhecimento.

Aos colegas que estão e aos que passaram pelo laboratório. É difícil nomear todos, visto que passei seis anos no mesmo laboratório. Fica difícil lembrar quem passou pelo laboratório no período do doutorado. Mas, agradeço particularmente ao Fausto e à Damaris, pela troca, pelo apoio e pela amizade.

Aos servidores técnicos do da secretaria e do laboratório de ensino do Departamento de Bioquímica, pelo apoio técnico.

Aos alunos dos minicursos dos eventos científicos e aos alunos das disciplinas de graduação do estágio à docência, fica o meu agradecimento pelo companheirismo e pela permuta de conhecimentos. Desejo a todos um sucesso enorme e espero ter colaborado em algo na aquisição de conhecimento de vocês.

Aos colegas do programa de pós-graduação, obrigada pela torcida e apoio. Vocês mais do que ninguém entendem as flores e as dores de um doutorado do início ao fim.

Aos colegas de fora do programa de pós-graduação, de Maringá e de Cascavel, por serem tão incríveis e compreensíveis, mesmo quando a situação não era das melhores.

À Coordenação do Programa de Pós-Graduação em Ciências Biológicas – Área de concentração Biologia Celular e Molecular e à todas as pessoas que, de maneira direta ou indireta, fizeram parte deste processo.

“Pra começar, cada coisa em seu lugar, e nada como um dia após o outro. Pra que apressar? Se nem sabe aonde chegar. Correr em vão se o caminho é longo...

“... O novo virá, pra re-harmonizar, a terra, o ar, a água e o fogo. E sem se queixar, as peças vão voltar pra mesma caixa no final do jogo...

“... Pode esperar, o tempo nos dirá, e nada como um dia após o outro...

Um dia após o outro
Tiago Iorc

APRESENTAÇÃO

Esta tese é composta por dois artigos científicos. O primeiro concerne à prospecção de bactérias de grãos de milho apresentando sinais de podridão produtoras de enzimas hidrolíticas. O segundo artigo concerne sobre a expressão recombinante, purificação e caracterização de uma ciclodextrinase da bactéria *Massilia timonae*.

Santos, F.C. dos; Castro, F.F.; Apolonio, T.M.; Yoshida, L.; Martim, D.B.; Tessmann, D.J; Barbosa-Tessmann, I.P. Screening of hydrolytic enzymes production by bacteria isolated from maize (*Zea mays* L.) grains. Submetido ao periódico **Biocatalysis and Agricultural Biotechnology**.

Santos, F.C. dos; Barbosa-Tessmann, I.P. Recombinant expression, purification, and characterization of a cyclodextrinase from *Massilia timonae*. **Protein Expression and Purification**. In Press, Accepted manuscript. doi.org/10.1016/j.pep.2018.08.013.

SUMÁRIO

LISTA DE TABELAS

CAPÍTULO I

CAPÍTULO II

LISTA DE FIGURAS

CAPÍTULO I

CAPÍTULO II

LISTA DE ABREVIATURAS

µg – Microgram(s)

µL – Microliter(s)

AN – GenBank Acession Number

bp – Base pairs

CTAB – Cetyltrimethylammonium bromide

CTI – Irrigation Tecnology Center

DNA – Deoxyribonucleic Acid

EDTA – Ethylenediaminetetraacetic acid

EMB – Eosin Methylene Blue medium

FEI – Igatemi Experimental Farm

g – Gram(s)

g – Gravitacional force

g/L – gramas por litro

h – Hour(s)

kb - Kilobases

K_M – Michaelis-Menten Constant

M – Molar

M.M. – Molecular markers

mg – Miligram(s)

min – Minute(s)

mL – Mililiter(s)

mM – Milimolar

ng – Nanogram(s)

MR – Methyl Red

PCR – Polymerase Chain Reaction

PMSF – Phenylmethylsulfonyl fluoride

RNase – Ribonuclease.

s – Second(s).

SD – Standard Deviation.

SDS – Sodium Dodecyl Sulphate.

SDS-PAGE – Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis.

SIM – Sugar Indole Motility medium

TAE – Tris-Acetate-EDTA Buffer.

TE – Tris-EDTA Buffer.

TSI – Triplice Sugar Iron Medium

U – International Units.

UEM – Maringá State University

USA – United States of America.

UV – Ultraviolet.

v/v – volume/volume

V_{max} – Maximal Reaction Velocity.

VP – Voges Proskauer

w/v – Weight/volume.

°C – Celsius degree.

RESUMO GERAL

Introdução: Os processos industriais catalisados por enzimas são considerados importantes uma vez que atuam em condições brandas de temperatura e pressão, além de serem processos simples e apresentam menor impacto ambiental. A tecnologia de hidrólise enzimática movimenta uma soma considerável de recursos e vem apresentando grande destaque no mercado industrial. No mercado industrial as enzimas hidrolíticas são as mais empregadas e incluem amilases, celulases, lipases e proteases, entre outras. Elas são utilizadas em diversos ramos da indústria como na de alimentos, bebidas, papel, têxtil, produtos de limpeza, farmacêutica e até no tratamento de efluentes. Nestas indústrias a atual preferência é por enzimas de origem microbiana por causa de sua consistência, produção constante, estabilidade e grande capacidade catalítica. Embora as enzimas possam ser obtidas de diferentes microrganismos, ainda existe um desafio em se obter isolados que produzam enzimas hidrolíticas em grandes quantidades. Nesse sentido, a biologia molecular tem papel importante na produção e caracterização de enzimas recombinantes com potencial industrial. **Objetivos:** Devido à demanda comercial por biocatalisadores, a prospecção de novos microrganismos produtores de enzimas hidrolíticas, juntamente com o desenvolvimento da biotecnologia enzimática, auxilia o meio científico-industrial na busca e no melhoramento de enzimas com características comerciais. Sendo assim, os objetivos desse trabalho foram o isolamento e a caracterização bioquímica de bactérias em grãos de milho apresentando sinais de podridão. Além da identificação molecular e avaliação de produção de enzimas hidrolíticas pelos isolados, técnicas de bioquímica e biologia molecular foram utilizadas para a clonagem, expressão recombinante, purificação e caracterização de uma ciclodextrinase de *Massilia timonae*. **Materiais e Métodos**: A bioprospecção foi realizada com grãos de milho usando a técnica de diluição seriada em água peptonada. A produção das enzimas amilase, celulase, lipase e protease foi verificada de modo qualitativo em meios seletivos. Isolados de bactérias obtidas em ágar nutriente foram transferidos para meios seletivos contendo amido, celulose microcristalina, óleo de oliva e leite desnatado. A formação de halo hidrolítico evidenciou os isolados produtores de enzimas e aqueles que produziram os maiores halos foram armazenados. Para a caracterização bioquímica, os isolados foram analisados com a coloração de Gram e cultura nos meio sólidos EMB (Eosina Methylene Blue), TSI (Triplice Sugar Iron), Agar Citrato de Simmons e SIM (Sulfide, Indole, Motility). Alguns isolados foram melhor analisados pelos testes de Vermelho de metila (VM) e Voges-Proskauer (VP), além de fermentação individual de açúcares em meio líquido e cultura em meio sólido contendo ureia. A identificação molecular de 55 isolados foi feita pela amplificação parcial do rDNA 16S com iniciadores universais, seguido de sequenciamento e comparação com sequências depositadas em bancos de dados. Como há um número menor de espécies de bactérias descritas na literatura como produtoras de amilases, lipases e celulases, foi feito um teste de produção em meio líquido para alguns dos isolados produtores identificados. Um isolado de *Pantoea dispersa* e um de *Massilia* sp. foram identificados como produtores de lipase/esterase e amilase, respectivamente, o que é um dado novo para a literatura. Todo o gene 16S do isolado de *Massilia* sp. foi amplificado e sequenciado e uma análise de DNA *barcoding* revelou que era a espécie *Massilia timonae*. Uma pesquisa no genoma desta bactéria revelou a presença de cinco genes para amilases. Iniciadores foram desenhados para estes genes. Entretanto, apenas um deles foi amplificado por PCR com sucesso. Este gene que codifica uma ciclodextrinase (EC 3.2.1.54) foi clonado e sequenciado. Programas de bioinformática auxiliaram na análise *in silico* da estrutura da enzima. A expressão recombinante da proteína codificada foi otimizada em *E. coli*. A proteína expressa continha uma cauda de histidinas e foi purificada por afinidade em coluna de níquel. Após a purificação, SDS-PAGE e

cromatografia por filtração em gel foram usados para determinar a massa molecular da enzima produzida. Em adição, a enzima recombinante foi caracterizada cineticamente e uma cromatografia em papel descendente foi realizada para avaliar os produtos de hidrólise. **Resultados e Discussão:** Na prospecção de novos isolados produtores de enzimas hidrolíticas foram armazenados 137 isolados, com predominância de bacilos Gram negativos. A maior parte destes isolados eram produtores de celulase, seguidos de produtores de protease, lipase e amilase. Muitos apresentaram mais de uma atividade hidrolítica em meio sólido. Os isolados foram agrupados segundo suas características bioquímicas e isolados com características bioquímicas únicas, diferenciados dos demais, e isolados representantes dos diferentes grupos bioquímicos obtidos foram selecionados para a identificação molecular. Cinquenta e cinco isolados produtores de enzimas foram identificados molecularmente. Os produtores de proteases apresentaram maior diversidade de gênero: *Klebsiella*, *Pseudomonas*, *Ochrobactrum*, *Pantoea*, *Stenotrophomonas*, *Staphylococcus*, *Burkholderia*, *Sphingomonas*, *Isoptericola*, *Massilia* e *Bacillus*. Isolados dos seguintes gêneros produziram amilases: *Klebsiella* e *Massilia*. Produtores de lipases tanto em meio sólido como líquido pertenciam aos gêneros: *Stenotrophomonas*, *Burkholderia*, *Klebsiella e Pantoea.* Isolados dos gêneros *Klebsiella*, *Enterobacter* e *Modestobacter* produziram celulases. A análise de fermentação submersa confirmou a produção de lipases e esterases por alguns isolados, entre eles o de *P. dispersa*, e de amilases por todos os isolados produtores, entre eles o de *Massilia* sp., mas não foi evidenciada a produção de celulase pelos isolados testados. Com os resultados encontrados no primeiro trabalho, foi feita uma busca por sequências codificadoras de amilases no genoma da *M. timonae*. Um gene de uma ciclodextrinase (EC 3.2.1.54) foi amplificado, clonado e sequenciado. Ciclodextrinases são enzimas que participam do metabolismo do amido pelas bactérias hidrolisando ciclodextrinas. O gene clonado codificava uma proteína que continha todos os domínios estruturais de uma glicosil hidrolase da família 13 (GH13). O meio ZYM-5052 de autoindução foi considerado o melhor para a expressão da ciclodextrinase em *Escherichia coli*, comparado com o meio LB. Uma análise estrutural *in silico* da proteína expressa identificou sítios de ligação do íon cálcio, corroborando com os dados da literatura e os dados laboratoriais obtidos do papel essencial do cálcio para a ativação da enzima após purificação e para a estabilidade térmica da enzima purificada (T_{50} = 48.45 °C). A enzima purificada apresentou pH ótimo de 7.0 e temperatura ótima de 40 °C, permanecendo estável por 30 min de incubação a 45 °C. A análise de massa molecular por SDS-PAGE e cromatografia de exclusão em gel mostrou que a proteína purificada é um tetrâmero de 260 kDa com monômeros de 65 kDa. A enzima purificada foi fortemente inibida por íons metálicos como cobre, zinco, ferro e magnésio, e por EDTA. Os detergentes CTAB e SDS, catiônico e aniônico, respectivamente, inibiram completamente a enzima purificada, mas o detergente neutro Tween 80 não foi um forte inibidor. A enzima purificada atuou preferencialmente sobre a β-ciclodextrina e em bem menor proporção sobre o amido e a maltodextrina. O K_M para a β-ciclodextrina foi de 2,1 mM, a $V_{máx}$ da enzima purificada foi 0,084 μmol/min, o k_{cat} foi de 8326 min^{-1}, e k_{cat}/K_M foi de 4,1 × 10^6 $M^{-1}min^{-1}$. A ciclodextrinase de *M. timonae* produziu glicose e maltose como produtos finais da hidrólise de β-ciclodextrina, maltotetraose e maltoheptaose, mas não hidrolisou a maltose. **Conclusões:** Vários isolados de bactérias obtidas dos grãos de milho foram identificados como produtores de enzimas hidrolíticas. Algumas das espécies encontradas não tinham sido ainda descritas como produtoras ou suas enzimas ainda não foram estudadas. O gene de uma nova ciclodextrinase de *M. timonae* foi clonado e expresso em *E. coli*. A enzima recombinante foi expressa em um alto nível e apresentou características enzimáticas que podem ser interessantes em aplicações industriais, como na hidrólise de oligossacarídeos lineares.

GENERAL ABSTRACT

Introduction: Industrial processes catalyzed by enzymes are considered important since they run under mild conditions of temperature and pressure, besides being simple and having lower environmental impact. Enzymatic hydrolysis technology has attracted a considerable amount of resources and has shown great prominence in the industrial market. In the industrial market the hydrolytic enzymes are the most employed and include amylases, cellulases, lipases, and proteases, among others. They are used in various industries such as food, beverage, pulp juice, paper, textiles, cleaning, pharmaceutical, and even effluent treatment. In these industries, the current preference is for enzymes of microbial origin because of its consistency, regular supply, stability, and greater catalytic activity. Although enzymes can be obtained from different microorganisms, there is still a challenge in obtaining isolates that produce hydrolytic enzymes in large amounts. In this sense, molecular biology plays an important role in the production and characterization of recombinant enzymes with industrial potential. **Objectives:** Due to the commercial demand for biocatalysts, the prospection of new microorganisms producing hydrolytic enzymes, together with the enzymatic biotechnology development, assists the scientific-industrial environment in the search and improvement of enzymes with commercial characteristics. Thus, the aims of this work were the isolation and biochemical characterization of bacteria from maize grains presenting rotten symptoms. In addition to the molecular identification and evaluation of the hydrolytic enzymes production by the isolates, biochemical and molecular biology techniques were used for the cloning, recombinant expression, purification, and biochemical characterization of a *Massilia timonae* cyclodextrinase. **Materials and Methods:** Bioprospecting was carried out with the maize grains, using the serial dilution technique in peptone water. The amylase, cellulase, lipase, and protease production was qualitatively verified in selective media. Bacteria isolates obtained in nutrient agar were transferred to selective media containing starch, microcrystalline cellulose, olive oil, and skimmed milk. The hydrolytic halo formation showed the enzyme-producing isolates and the ones with the largest halo were stored. For the biochemical characterization of these isolates, they were treated with Gram staining and culture in the solid media EMB (Eosin Methylene Blue), TSI (Triplice Sugar Iron), Simmons Agar Citrate, and SIM (Sulfide, Indole, Motility). Some isolates were better analyzed with the tests Methyl Red (RM) and Voges-Proskauer (VP), besides fermentation of single sugars in liquid medium as well as culture in solid medium containing urea. Molecular identification of 55 isolates was done by partial amplification of the 16S rDNA with universal primers, followed by sequencing and comparison with sequences deposited in databases. As there is a smaller number of species of bacteria described in the literature as producers of amylases, lipases, and cellulases, a test of production in liquid medium of these enzymes by some of the identified producing isolates was performed. An isolate of *Pantoea dispersa* and another of *Massilia* sp. were identified as lipase/esterase and amylase producers, respectively, what is a new data for the literature. The entire 16S gene from the *Massilia* sp. isolate was amplified and sequenced and a DNA barcoding analysis revealed that it was the species *Massilia timonae*. A survey on the genome of this bacterium revealed five genes coding for amylases. Primers were designed for these genes. However, only one of them has been successfully amplified by PCR. The gene that encoded a cyclodextrinase (EC 3.2.1.54) was cloned and sequenced. Bioinformatics programs aided in the *in silico* analysis of the enzyme structure. The recombinant expression of the encoded protein was optimized in *E. coli*. The expressed protein contained a histidine tag and was purified by affinity on a nickel column. After purification, SDS-PAGE and gel filtration chromatography were used to find the molecular mass of the produced enzyme. In addition, the recombinant enzyme was kinetically characterized and paper chromatography was

performed to check the hydrolysis products. **Results and Discussion:** In the prospection of new isolates producing hydrolytic enzymes, 137 isolates were stored, with Gram-negative bacilli predominance. Most of these isolates were cellulase producers, followed by protease, lipase, and amylase producers. Many had more than one hydrolytic activity on a solid medium. The isolates were grouped according to their biochemical characteristics and bacteria isolates with unique biochemical characteristics, differentiated from the others, and isolated representatives of the different biochemical groups obtained were selected for molecular identification. Fifty-five enzyme-producing isolates were identified molecularly. Protease producers had the greater genera diversity: *Klebsiella*, *Pseudomonas*, *Ochrobactrum*, *Pantoea*, *Stenotrophomonas*, *Staphylococcus*, *Burkholderia*, *Sphingomonas*, *Isoptericola*, *Massilia*, and *Bacillus*. Isolates from the following genera produced amylases: *Klebsiella* and *Massilia*. Producers of lipases, identified in solid and liquid media, belonged to the genera: *Stenotrophomonas*, *Burkholderia*, *Klebsiella*, and *Pantoea*. Isolates of the genera *Klebsiella*, *Enterobacter*, and *Modestobacter*, have produced cellulases. The submersed fermentation analysis confirmed the lipases and esterases production by some isolates, including *P. dispersa*, and amylases by all the producer isolates, among them the *Massilia* sp. isolate, but cellulases were not produced. With the results found in the first work, a search was made for amylases coding sequences in the *M. timonae* genome. A cyclodextrinase gene (EC 3.2.1.54) was amplified, cloned and sequenced. Cyclodextrinases are enzymes that take part in the metabolism of starch by the bacteria hydrolyzing cyclodextrins. The cloned gene encoded a protein which contained all the structural domains of a family 13 glycosyl hydrolase (GH13). The auto induction ZYM-5052 medium was considered the best for the cyclodextrinase expression in *Escherichia coli*, compared to the LB medium. An *in silico* structural analysis of the expressed protein identified calcium ion binding sites, corroborating with the literature and the obtained laboratory data of the essential role of calcium for activation of the enzyme after purification and for thermal stability of the purified enzyme (T_{50} = 48.45 °C). The purified enzyme presented optimum pH of 7.0 and optimum temperature of 40 °C, remaining stable for 30 min of incubation at 45 °C. The molecular weight analysis by SDS-PAGE and gel exclusion chromatography showed that the purified protein is a 260 kDa tetramer with 65 kDa monomers. The purified enzyme was strongly inhibited by metals ions such as copper, zinc, iron, and magnesium, and by EDTA. CTAB and SDS, cationic and anionic detergents, respectively, completely inhibited the purified enzyme, but the neutral detergent Tween 80 was not a strong inhibitor. The purified enzyme acted preferentially on β-cyclodextrin and in a smaller proportion on starch and maltodextrin. The K_M for the β-cyclodextrin was of 2.1 mM, the V_{max} of the purified enzyme was 0.084 μmol/min, the k_{cat} was 8326 min^{-1}, and k_{cat}/K_M was 4.1 × 10^6 $M^{-1}min^{-1}$. The *M. timonae* cyclodextrinase released glucose and maltose as final products of the hydrolysis of β-cyclodextrin, maltotetraose, and maltoheptaose, but did not hydrolyze maltose. **Conclusions:** Several isolates of bacteria obtained from the maize grains were identified as producers of hydrolytic enzymes. Some of the species found have not yet been described as producers or their enzymes have not yet been studied. The gene of a novel cyclodextrinase of *M. timonae* was cloned and was expressed in *E. coli*. The recombinant enzyme was expressed at a high level and presented enzymatic characteristics that may be interesting in industrial applications, as in the hydrolysis of linear oligosaccharides.

CAPÍTULO I
SCREENING OF HYDROLYTIC ENZYMES PRODUCTION BY BACTERIA ISOLATED FROM MAIZE (*ZEA MAYS* L.) GRAINS

SANTOS, F.C. dos[1]; CASTRO, F.F. de[1]; APOLONIO, T.M. [1]; YOSHIDA, L. [1]; MARTIM, D.B.; TESSMANN[1], D.J.[2]; BARBOSA-TESSMANN, I.P.[1]

ABSTRACT

Microbial hydrolytic enzymes are industrially preferred due to their consistency, regular supply, stability, and greater catalytic activity. A bioprospection of hydrolytic enzymes production by bacteria isolated from maize grains with rot symptoms was performed in this work. Harvest leftover maize ears were collected from three different locations in Southern Brazil. The ears (samples) were threshed and the grains were treated with insecticide and stored at room temperature until analysis. Grains of seven samples were incubated in peptone water and aliquots of this were diluted and inoculated in nutrient agar. Individualized colonies were transferred to selective media containing starch, microcrystalline cellulose, skimmed milk, or triolein. The hydrolytic activities production was evaluated by the development of halos around the colonies. Isolates (137) presenting hydrolytic activity were stored and their biochemical profile was analyzed. The molecular identification of 55 isolates of higher interest was done by DNA barcoding. A phylogenetic analysis showed that the big majority of the obtained enzyme producers belonged to the phylum Proteobacteria and just a few of them belonged to the phyla Actinobacteria and Firmicutes. Some of the isolated species have already their enzymes well described. However, new producers were found. For instance, an amylase producer isolate of *Massilia* sp. and a lipase and esterase producer isolate of *Pantoea dispersa*, both confirmed to produce these enzymes in submerse fermentation.

Key words: Bacteria, Hydrolases, Bioprospection, Maize.

[1] Maringá State University, Department of Biochemistry, Av. Colombo, 5790, 87020-900 Maringá, PR, Brazil.
[2] Maringá State University, Department of Agronomy, Av. Colombo, 5790, 87020-900 Maringá, PR, Brazil.

1. INTRODUCTION

With the enzyme technology development, microbial enzymes are currently acquiring a great deal of attention. The global market for industrial enzymes is expected to reach nearly 6.2 billion dollars by 2020 (Singh et al., 2016). Almost 75% of all industrial enzymes are hydrolytic enzymes and proteases, amylase, lipases, and cellulases account for more than 70% of all enzyme sales (Li et al., 2012; Gurung et al., 2013). Microbial enzymes are preferred due to their consistency, stability, greater catalytic activity, and regular supply because of the seasonal fluctuations absence (Gurung et al., 2013). Microorganisms grow easily on simple and low-cost substrates and offer easy culture conditions optimization and genetic alteration to increase enzyme production (Gurung et al., 2013). Furthermore, with the use of recombinant DNA technology, a microbe can be manipulated to produce large quantities or an engineered protein (Singh et al., 2016). Currently, about 200 microbial enzymes are used commercially, but only about 20 enzymes are produced on an industrial scale (Liet al., 2012). Near 150 industrial processes use enzymes or whole microbial cell catalysts and over 500 industrial products are made using enzyme technology (Adrio and Demain, 2014).

Proteases represent over 60% of the enzymes global market (Adrio and Demain, 2014). The largest commercial proteases segment is held by detergent alkaline proteases (Kumar and Takagi, 1999). Most commercial alkaline proteases are produced by bacteria belonging to the genus *Bacillus*, mainly by the species *Bacillus licheniformis*, *Bacillus subtilis*, *Bacillus amyloliquifaciens*, and *Bacillus mojavensis*, but the proteases production is reported on several other bacteria genera (Rao et al. 1998; Kumar and Takagi, 1999; Kirk et al., 2002; Jisha et al., 2013). Besides their use in detergents, proteases are also employed in the food industry, leather treatment, bioremediation, and pharmaceutical industry (Rao et al., 1998; Adrio and Demain, 2014).

Amylases embody 25% of the worldwide enzyme commercialization (Gurung et al., 2013). They are used in the following industries: textile, brewery, bakery, chemical, pharmaceutical, and distillery for bio-ethanol production (Pandey et al., 2000; Sun et al., 2010). Amylases can be derived from several bacteria, yeasts, and fungi, but the bacterial enzymes are generally preferred due to their thermal stability (Gupta et al., 2003). Strains of *B. amyloliquefaciens* and *B. licheniformis* are employed for the commercial production of α-amylase, an endoamylase. β-amylase, an exoamylase, is generally of plant origin, but bacterial strains belonging to the genera *Bacillus*, *Pseudomonas*, and *Clostridium* are reported to produce it (Pandey et al., 2000). Filamentous fungi characterize the major source of glucoamylases, another exoamylase (Gupta et al., 2003).

After proteases and amylases, lipolytic enzymes, i.e. lipases and esterases, are the most important enzyme group in terms of sales volume (Gurung et al., 2013). Esterases hydrolyze short chain fatty acids esters, whereas lipases hydrolyze long chain fatty acids esters, mainly triacylglycerol (Jaeger and Eggert, 2002). Lipases are used in the detergent, paper, and food industries, and in effluent treatment, while esterases can be employed in the synthesis of optically pure compounds (Bornscheuer, 2002; Jaeger and Eggert, 2002). In organic interfaces, lipases and esterases also accomplish esters synthesis. Because of this they can be applied for biodiesel synthesis, which is a mixture of esters of fatty acids with ethanol or methanol (Fjerbaek et al., 2009). Lipolytic enzymes can be obtained from microorganisms, plants, and animals (Pandey et al., 1999; Sayali et al., 2013). Several bacteria genera are reported to produce lipases, and those from the genera *Burkholderia*, *Chromobacterium*, and *Pseudomonas* are commercially available (Jaeger and Eggert, 2002; Sharma et al., 2001). The main bacterial esterase producers are from the genera *Bacillus* and *Pseudomonas* (Bornscheuer, 2002; Sayalli et al., 2013).

Cellulases are sold in great volumes and are applied in the following industries: food, fruit juices, paper, brewery, textile, laundry, and distillery for bio-ethanol production (Bhat, 2000; Lynd et al., 2002, Dashtban et al., 2009). Cellulases are synthesized by a large diversity of microorganisms including fungi and bacteria. Structurally, fungal cellulases are non-complexed systems, whereas bacterial cellulases can either be non-complexed or complexed systems, the so-called cellulosomes (Bhat, 2000; Lynd et al., 2002, Dashtban et al., 2009). The best-studied non-complexed cellulase bacterial producers are species of the aerobic genera *Cellulomonas* and *Thermobifida* (Lynd et al., 2002; Kuhad et al., 2011). Cellulosomes from different species of the anaerobic genera *Clostridium* and *Ruminococcus* have also been studied in detail (Lynd et al., 2002).

Bioprospection can be carried out by using environmental or culture collection samples. Maize (*Zea mays* L.) is originated from Central America and is extensively cultivated in Brazil. The economic importance of maize is characterized by its high nutritional value, due to its richness in starch, protein, triacylglycerol, and fibers. Because of this, it was hypothesized that maize grains presenting rotten symptoms might carry bacteria that could produce hydrolases for degrading the maize grain components. Moreover, these bacteria isolates could belong to genera or species before undescribed as hydrolytic enzymes producers.

2. MATERIALS AND METHODS

2.1 Maize grain samples

Harvest leftover maize ears containing grains presenting rotten signs were collected from three different locations: the Iguatemi Experimental Farm (FEI) and Irrigation

Technology Center (CTI) of the Universidade Estadual de Maringá, and from a private property in the Marialva (MVA) municipality, Pr, Brazil. The ears (samples) were threshed and the grains were treated with insecticide and stored at room temperature until analysis. Each ear was called a sample and seven grain samples were collected: FEI1, FEI2, FEI3, FEI4, FEI5, CTI, MVA.

2.2 Bacteria isolates

Grains (2.5 g) from each sample were incubated in 250 mL flasks containing 47.5 mL of sterile 0.1% (w/v) peptone water for one hour at 37 °C, with orbital shaking at 100 rpm. Aliquots (100 μL) of this suspension, pure or diluted 2 and 4 times, were spread on the surface of a 10 cm diameter Petri dish (triplicate) containing nutrient agar [3.0 g/L (w/v) yeast extract, 5.0 g/L (w/v) peptone, pH 8.0, 15 g/L (w/v) agar,] and malachite green (2.5 μg/mL of medium) to inhibit fast growing fungi. The inoculated dishes were incubated for 16 h at 37 °C.

2.3 Detection of enzymatic activity in solid media

The obtained bacterial isolates in the nutrient agar culture (about 160 isolates from each grain sample) were transferred, with a sterile wooden toothpick, to each specific substrate medium. The dishes were inoculated with about 40 colonies, which were placed 1 cm equidistant from each other. These dishes were incubated at 37 °C for 24-72 h. After this, the results were evaluated by measuring a clear or fluorescent halo around the colonies, which indicated the tested enzyme production (Ceska, 1971).

Protease producers were identified by casein hydrolysis in skimmed milk-agar [300 mL/L of non-fat milk and 20 g/L agar] (Sarath, 1989). As the milk agar is opaque, the enzyme

activity was revealed by a degradation transparent halo around the colonies after 24-48 h of culture.

The bacterial isolates ability to degrade starch was used as a criterion to check for amylases production, using nutrient agar [3 g/L yeast extract, 5 g/L peptone, 15 g/L agar] containing 2 g/L of soluble starch (Hankin and Anagnostakis, 1975). After growth of the bacteria for 48 h at 37 °C, the dishes were treated with 5 mL of the iodine reagent [2% (w/v) KI; 0.2% (w/v) I_2] for the remaining substrate detection. The amylase activity was discovered by a clear yellow halo around the isolate colony (Hankin and Anagnostakis, 1975).

The lipolytic activity was evaluated in Rhodamine B medium [2.0% (v/v) olive oil; 1.0% (v/v) Tween 80; 2 mg/L Rhodamine B; 0.5 g/% (w/v) yeast extract; 0.3 g/% (w/v) peptone; 0.125 g/% (w/v) tryptone; 4 g/L NaCl; pH 7.0; 15 g/L agar. Rhodamine B was prepared at 2 mg/mL in sterile water:ethanol 100% (1:1) and added into the medium after sterilization in autoclave and cooling to 60 °C in the proportion of 1 μL/mL of medium (Jaeger and Kouker, 1987). After an incubation for 72 h at 37 °C, lipase production was detected by a pink fluorescent halo around the colonies visualized under UV light (312 nm) in a transilluminator.

A medium containing microcrystalline cellulose was used to determine cellulase activity production [7.0 g/L KH_2PO_4; 2.0 g/L K_2HPO_4; 0.1 g/L $MgSO_4 \cdot 7H_2O$; 1.0 g/L $(NH4)_2SO_4$; 0.6 g/L yeast extract; and 10 g/L microcrystalline cellulose (Sigmacell, Sigma-Aldrich, St Louis, MO, USA); pH 5.0; and 15 g/L agar] (ABE et al., 2015). After bacteria growth for 48-72 h, as described above, the dishes were incubated for 16 h at 50 °C, considering the cellulases optimum temperatures that are in this range (Montenecourt and Eveleigh, 1977). After this period and to visualize the hydrolytic halo, the dishes were revealed with 5 mL of the iodine reagent and distained by several washes with distilled water (Kasana et al, 2008).

2.4 Maintenance of the isolates

Bacteria isolates that demonstrated the ability to produce at least one hydrolytic activity and that presented the largest halos (larger than 1.0 cm) were inoculated in nutrient agar slants were and incubated for 24-48 h at 37 °C. After growth, the cultures were stored at 4 °C and at room temperature. The same bacteria isolates were also inoculated in Luria Bertani medium [10 g/L tryptone; 5 g/L NaCl; 5 g/L yeast extract] and after 14 h of incubation at 37 °C, under orbital shaking (100 rpm), the resulting cultures were added to sterile glycerol to a final concentration of 50% and were stored at -20 °C.

2.5 Biochemical characterization of the isolates

All stored isolates were stained by Gram and their morphology was evaluated under optical microscopy. Gram negative and positive isolates were confirmed by culture at 37 °C, for 24 h, in EMB agar (Eosin Methylene Blue) [10 g/L peptone; 10 g/L lactose; 2 g/L K_2HPO_4; 0.4 g/L eosin Y; 0.065 g/L methylene blue; 15 g/L agar]. EMB culture also indicated lactose fermentation. The isolates capacity to use glucose, lactose, or sucrose as a carbon source and to produce H_2S and gas was verified by culture in Triple Sugar Iron slants (TSI) [0.3% (w/v) meat peptone; 0.3% (w/v) yeast extract; 2% (w/v) casein peptone; 0.5% (w/v) NaCl; 1% (w/v) lactose; 1% (w/v) sucrose; 0.1% (w/v) glucose; 0.03% (w/v) ammonium iron(III) citrate; 0.03% (w/v) sodium thiosulphate ($Na_2S_2O_3$); 0.0024% (w/v) phenol red; 1.2% (w/v) agar]. The inoculated medium was incubated at 37 °C for 18 to 24 h.

The isolated bacteria were also cultured in Simmons citrate agar [0.02% (w/v) $MgSO_4 \cdot 7H_2O$; 0.02% (w/v) $NH_4H_2PO_4$; 0.08% (w/v) sodium ammonium phosphate; 0.2% (w/v) tribasic sodium citrate; 0.5% (w/v) NaCl; 0.008% (w/v) bromothymol blue; 1.5% (w/v) agar] to verify their capacity to use citrate as main carbon and energy source. The slants tubes were incubated for 24-48 h at 37 °C.

The Sulfide, Indole, and Motility (SIM) medium [2% (w/v) tryptone; 0.61% (w/v) peptone; 0.02% (w/v) ammonium iron(II) sulfate hexahydrate ($(NH_4)_2Fe(SO_4)_2 \cdot 6H_2O$); 0.02% (w/v) sodium thiosulphate, 0.35% (w/v) agar] was used to detect H_2S and indole production, in addition to test for motility. The tubes were incubated for 24-48 h at 37 °C. The indole production was evaluated by adding drops of the Kovacs Reagent [0.6 g/% (w/v) *p*-dimethylaminobenzaldehyde and 3.2% (v/v) hydrochloric acid in ethyl alcohol] to the medium surface.

Isolates of highest interest, considering their enzyme profile, were further characterized to differentiate among them. For the Methyl Red (MR) and Voges-Proskauer (VP) tests, the isolates were inoculated in 2 mL of glucose broth [7 g/L peptone; 5g/L KH_2PO_4; 5 g/L glucose; pH 7.0] for 24 h, without agitation, at 37 °C. One mL of each culture was examined by the MR test by adding 4-5 drops of the methyl red solution [0.1 g methyl red, 800 mL of ethanol 35%]. The MR test determines sufficient acid production during glucose fermentation so an old culture still has an acidic pH. The other one mL of the culture was inspected by the VP test by addition of 600 μL of 5% (w/v) alpha naphthol in absolute ethanol and 200 μL of 40% (w/v) KOH. The VP test detects acetyl methyl carbinol production. To test the use of urea by the selected isolates, they were inoculated and cultured for 18 h at 37 °C in agar urea [1g/L glucose; 1 g/L peptone; 2 g/L KH_2PO_4; 5 g/L NaCl; 0.012 g/L phenol red; 20 g/L agar; pH 6.8; urea was added after autoclaving and cooling to 60 °C to a final concentration of 0.4 % (w/v)]. The selected isolates were also subjected to fermentation tests with single sugars. Samples were inoculated in specific sugar broth [5 g/L peptone; 5 g/L NaCl; 0.018 g/L phenol red; 10% (w/v) of one of the following sugars: glucose, lactose, raffinose, sucrose, and mannitol; pH 7.4]. The culture was performed in tubes with 10 mL of the liquid medium with an inverted Durham tube inside, for 18-24 h, at 37 °C.

The general and specific biochemical results were analyzed by ABIS online program (http://www.tgw1916.net/bacteria_abis.html) and classification tables (Holt, 1994).

2.6 Molecular identification of the selected isolates

The isolates were grouped based on the biochemical tests results. One or two isolates from each group and out-group isolates were chosen for DNA barcoding identification. The strains were chosen to meet the largest number of species. For the DNA extraction, the isolates were inoculated in 5 mL of Luria Bertani (LB) medium [10 g/L (w/v) tryptone; 5 g/L (w/v) NaCl; 5 g/L (w/v) yeast extract] and incubated for 18 h, at 37 °C under orbital stirring at 100 rpm. Cells from 1.5 mL of this culture were harvested by centrifugation and washed 3 times with 500 μL TE buffer (10 mM Tris; 1 mM Na_2EDTA; pH 8.0). Then, cells were resuspended in 200 μL of TE buffer and were boiled for 10 minutes The obtained lysate was centrifuged for 1 min (10,000×*g*) and the obtained supernatant was the source of DNA and was stored at -20 °C. Bacterial isolates that did not grow in LB liquid medium were inoculated in solid medium (LB or nutrient agar) and cultured at 30 or 37 °C for 24-72 h. The obtained colonies were suspended in 1.0 mL of nutrient broth. The cells were collected by centrifugation (9,000×*g*, 2 min) and treated as above to extract the DNA.

PCR reactions were carried out for the 16S rRNA partial gene amplification, using the universal primers 91E (5´-GGAATTCAAAKGAATTGACGGGGGC) and 13B (5´-CGGGATCCCAGGCCCGGGAACGTATTCAC) (Relman, 1993), which amplify a 440 bp fragment at positions 930 to 1370 of the *Escherichia coli* 16S rDNA.

The PCR reaction was performed with 1 U of Platinum® *Taq* DNA Polymerase (Thermo Fisher Scientific, USA), 1 X enzyme buffer, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 25 pmol of each primer, and 2 μL of the DNA solution, in a total volume of 50 μL. The PCR consisted of an initial incubation of 3 min at 95 ºC and of 25 cycles of: 1 min a 94 ºC, 1 min

at 55 ºC, and 2 min at 72 ºC. Then, the samples were incubated at 72 ºC for 10 min for complete extension of the fragments. The amplicons were cleaned using the ExoSAP-IT PCR Clean-up Kit (GE Healthcare, USA), following the manufacturer´s protocol. Alternatively, the PCR reaction was run in a 1.0% agarose gel and the PCR product was cleaned from the gel with the Wizard® SV Gel and PCR Clean-Up system (Promega, USA) via the vendor´s protocol. The obtained amplicons were sequenced in the Centro de Estudos do Genoma Humano (CEGH) at the Universidade de São Paulo (USP), Brazil. Each amplicon was sequenced once using the 13B primer in the sequencing reaction. After trimming at 5′ and 3′ extremities, the resulting sequences were compared with sequences deposited in data Banks. All obtained 16S rRNA partial gene sequences were deposited in GenBank and the accession numbers are listed in Table 2. The identification was considered only at the genera level when the identity was too high or lower than 98% for several species.

To evaluate the identified bacteria phylogenetic distances, the 16S- rDNA (388 to 393 bp) obtained sequences were aligned with ClustalW and the alignment was used to build a phylogenetic tree using the neighbor-joining method (Saitou and Nei, 1987) in the MEGA 7.0 program (Kumar et al., 2016). Bootstrap analyses were conducted to assess the confidence limits of the branching with 1000 heuristic replicates (Felsenstein, 1985). Values higher than 70% in the bootstrap test of phylogenetic accuracy have indicated reliable grouping among bacterial isolates. Pairwise deletion was used to remove gaps because a complete removal of the gaps could cut a large part of phylogenetically meaningful sites. The evolutionary distances were computed using the maximum composite likelihood method (Tamura et al., 2004) and as number of base substitutions per site. The grouping was also performed using other methods, such as maximum parsimony, minimum evolution and UPGMA with similar results.

2.7 Submerse culture for hydrolytic enzymes production and enzyme assays

Since there are fewer reported bacterial genera and species to produce amylases, lipolytic enzymes, and cellulases, compared with the greater number of bacterial genera and species described to produce proteases, those enzymes production was evaluated in liquid medium culture to refine the search for new producers and to better determine their levels.

Identified isolates producing amylase were cultured in a starch liquid medium (6 g/L peptone; 0.5 g/L $MgSO_4 \cdot 7H_2O$; 0.5 g/L KCl; 1 g/L starch; pH 7.0). Identified isolates producing lipase were cultured in a triolein liquid medium (2.7 g/L KNO_3; 2.0 g/L Na_2HPO_4; 0.37 g/L $MgSO_4 \cdot 7H_2O$; 0.02 g/L $CaCl_2$; 1% olive oil; 0.1 g/L KCl; 0,014 g/l ammonium ferric citrate; pH 7.5). Some of the identified isolates (CTI 14, CTI 38, CTI 49, FEI5 124, FEI5 153) producing cellulase were cultured in a microcrystalline cellulose medium [7.0 g/L KH_2PO_4; 2.0 g/L K_2HPO_4; 0.1 g/L $MgSO_4 \cdot 7H_2O$; 1.0 g/L $(NH_4)_2SO_4$; 0.6 g/L yeast extract; and 10 g/L microcrystalline cellulose (Sigmacell); pH 5.0]. The inoculum for those media was prepared as follows. The isolates were first streaked on nutrient agar medium in 10 cm diameter plates and were incubated overnight at 37 °C. Afterwards, the obtained colonies were suspended in 5 mL of nutrient broth or LB medium and 1 mL of this solution was transferred to each 125 mL flasks with 25 mL of the liquid medium. The inoculated flasks were incubated for 36 h, 100 rpm, at 37 °C, in duplicate. Cells were collected by centrifugation (3 min, 10,000×*g*) and the supernatant was used in the enzyme assay.

Amylase activity was evaluated with the method of the starch-iodine complex discoloration (Palanivelu, 2001). Five hundred µL of the supernatant were added of 0.5 mL of 0.1% (w/v) soluble starch and of 1 mL of 100 mM sodium phosphate buffer (pH 7.0) and incubated for 10 minutes at 50 °C. The control for assessing the initial starch concentration was prepared by adding 0.5 mL 0.1% (w/v) soluble starch to 1.5 mL of 100 mM sodium phosphate buffer, pH 7.0. The reaction was stopped by addition of 0.5 mL of 0.1 M HCl. The

remaining starch was stained by the addition of 0.5 mL of the iodine solution [2.0% (w/v) KI; 0.2% (w/v) I_2]. The absorbance was read at 690 nm. One unit of enzyme activity was defined as the amount of enzyme causing a 20%/min·mL reduction in the absorbance. In addition, amylase activity was also evaluated by reducing sugar release from starch, using the dinitrosalicylic acid (DNS) method (Miller, 1959). The reaction consisted of 500 μL of the culture supernatant with 1.5 mL of 0.5% (w/v) soluble starch in 50 mM sodium phosphate buffer, pH 7.0, and incubation for 30 min at 50 °C. The reaction was stopped by addition of 0.5 mL of the DNS solution (1 g/% (w/v) DNS, 30 g/% (w/v) sodium potassium tartrate, 1.6 g/% (w/v) NaOH). After boiling for 5 min, 2.5 mL of deionized water were added and the absorbance was read in at 540 nm. One unit of enzyme activity was considered as the amount of enzyme that produced 1.0 μmol/min·mL of reducing sugar, under the assay conditions. Glucose was used as a standard in concentrations ranging from 0.25 to 2.5 μmol.

Lipase and esterase activities were assayed by hydrolysis of *p*-nitrophenyl palmitate (*p*-NPP) and *p*-nitrophenyl butyrate (*p*-NPB), respectively, after the method described by Winkler and Stuckmann (1979), with modifications. For the lipase assay, 100 μL of the culture supernatant were added to 100 μL of 0.5 M phosphate/citrate buffer, pH 5.0, and to 900 μL of the substrate solution. The substrate solution was prepared with 10 mL of isopropanol containing 30 mg of *p*-NPP mixed with 90 mL of 0.1% (w/v) arabic gum and 0.4% (v/v) Triton X-100, resulting in a final concentration of 0.8 mM of *p*-NPP. The reaction was carried out at 35 °C, for 30 min. For the esterase assay, 100 μl of the culture supernatant were added of 100 μl of 100 mM phosphate buffer, pH 7.0, and of 900 μl of substrate solution (10 mL of isopropanol containing 2 μl of *p*-NPB mixed with 90 mL of 0.1% (w/v) arabic gum and 0.4% (v/v) Triton X-100) containing 113 μM of *p*-NPB. The reaction was carried out at 35 °C, for 30 min. In both reactions the released *p*-nitrophenol (*p*-NP) was read at 410 nm. One lipase and esterase unit was defined as the amount of enzyme that liberated 1

μmol/mL.min of *p*-NP. A blank without enzyme was used to subtract the natural hydrolysis of the *p*-NP derivative.

Cellulase was assayed by reducing sugar release from carboxymethylcellulose (CMC), using the DNS method (Miller, 1959). The reaction consisted of 500 μL of the culture supernatant with 1.5 mL of 1.0% (w/v) CMC in 50 mM acetate buffer, pH 4.8, and incubation for 30 min at 50 °C. Next, the DNS method was performed as described above.

3. RESULTS

3.1 Maize samples and bacterial isolates

Maize grains samples FEI-1 and FEI-2 did not yield bacterial colonies. The used culture medium, the chemical added to control rapid fungal growth, or the used culture conditions were probably not adequate for the bacterial flora of those samples to develop. Samples FEI-3, FEI-4, FEI-5, CTI, and MVA rendered innumerable colonies in nutrient agar in all tested dilutions, with the highest dilution being the best for obtaining isolated colonies. One hundred and thirty seven (137) isolates that produced the largest hidrolytic halos in the selective media were stored (Table 1).

3.2 Detection of enzymatic activities and identification of the isolates

Examples of the obtained hydrolytic halos are shown in Figure 1. Some of the stored isolates produced more than one hydrolytic activity (Table 1). The DNA barcoding identified isolates are shown in Table 2. It was evidenced 13 different genera occurrence among the 55 identified isolates, of which nine were Gram negative bacilli, one was Gram positive cocci, and three were Gram positive bacilli. The generated phylogenetic tree (Figure 2) shows these

species separation. All the identified Gram negative isolates belonged to the phylum Proteobacteria, with predominance of the γ-proteobacteria class (genera: *Klebsiella*, *Enterobacte*r, *Stenotrophomonas*, *Pantoea*, and *Pseudomonas*), followed by β-proteobacteria class (genera: *Burkholderia* and *Massilia*), and by α-proteobacteria class (genera: *Ochrobactrum* and *Sphingomonas*). The Gram positive isolates belong to the phyla Firmicutes (genera: *Bacillus* and *Staphylococcus*) and Actinobacteria (genera: *Isoptericola* and *Modestobacter*).

3.3 Enzyme activities

The DNA barcoding identified isolates and their hydrolytic enzymes production are shown in Table 2. In total, 26 (47.2%) isolates presented protease activity, 18 (32.7%) exhibited amylase enzyme activity, 16 (29.1%) showed lipase activity, and 25 (45.4%) displayed cellulase activity (Table 2).

3.4 Submerse culture for amylases, lipolytic enzymes, and cellulases production

When the chosen isolates were grown in liquid medium containing the specific substrate as a carbon source to activate enzyme production, there were production of amylases, lipases and esterases, but no production of cellulases. The obtained results for amylases, lipases and esterases production are shown in Figure 3.

4.DISCUSSION

Maize originates from southern Mexico and is a domestication product of wild teosinte (Matsuoka et al., 2002). Its seeds are reported to have a set of associated bacteria, despite

9,000 years of culture, selection, and genetic improvement by primitive and modern breeders (Johnston-Monje and Raizada, 2011). As in our methodology no grain disinfection was performed, it can be conclude that our isolates are epiphytic as well as endophytic. All the identified Gram negative isolates belonged to the phylum Proteobacteria, with predominance of the γ-proteobacteria class. Isolates from β-proteobacteria and α-proteobacteria classes, as well as from the Phyla Firmicutes and Actinobacteria were also obtained in this work, representing a total of 13 genera. According to our results, Johnston-Monje and Raizada (2011) have described the massive presence of bacteria from the γ- Proteobacteria class as maize seed endophytes and also has observed the presence of bacteria from the β-proteobacteria and α-proteobacteria classes, and from the Firmicutes and Actinobacteria Phyla. In addition, those authors have described the presence of 26 genera as endophytic bacteria, but they have used a dual approach of culture and cloning and DNA fingerprinting using terminal restriction fragment length polymorphism. Regarding maize roots bacteria diversity, Chelius and Triplett (2001) have found predominance of Actinobacteria using culture technique and of α-proteobacteria within a constructed clone library.

From the 13 different bacteria genera found in maize grains in this work, the vast majority was already describe to occur in this substrate, such as the genera *Klebsiella*, *Sphingomonas*, *Bacillus*, *Pantoea*, *Staphylococcus*, *Stenotrophomonas*, *Enterobacter*, *Burkholderia,* and *Pseudomonas* (Chelius and Triplett, 2001; Johnston-Monje and Raizada, 2011). The occurrence of the genera *Massilia* and *Ochrobactrum* have been reported in maize roots and rizosphere (Niu et al., 2017; Silva et al., 2017). The occurrence of *Modestobacter* and *Isoptericola* genera isolates have apparently not been yet described in maize grains. However, the occurrence of *Modestobacter* sp. in rice roots have been described (Hernandez et al., 2015) as well as the occurrence of *Isoptericola* sp. in rhizosphere of tomato and cucumber and soil around of a mangrove plants root (Kämpfer et al., 2016; Antoniou et al.,

2017, Bibi et al., 2017). As our maize samples were in soil contact when collected, it may have been contaminated with this soil related bacteria.

This study was an attempt to isolate new bacterial strains producers of hydrolytic enzymes. We used a culture dependent approach to gain the isolates and studied amylase, lipase, cellulase and protease production in solid media. The 26 identified isolates of bacteria that were protease producers belonged to the following genera or species: *K. variicola*, *K. oxytoca*, *P. putida*, *Ochrobactrum* sp., *P. dispersa*, *S. maltophilia*, *S. hominis*, *Burkholderia* sp. *Sphingomonas* sp., *Isoptericola* sp., *Massilia* sp., and *Bacillus* sp. The *Bacillus* genus is vastly reported in the literature as a significant protease producer and, indeed, most of the commercial neutral and alkaline proteases are obtained from it (Jisha et al., 2013). However, there are many other genera described as proteases producers (Rao et al., 1998). The proteases of the species *S. maltophilia*, *P. putida*, *K. oxytoca*, and *P. dispersa* have already been well characterized (Tondo et al., 2004; Nicodème et al., 2005; Gohelab et al., 2007; Ribitsch et al., 2012). For the genera *Burkholderia*, *Staphylococcus*, and *Ochrobactrum*, the proteases of the species *Burkholderia pseudomallei*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, and *Ochrobactrum anthropi* were also already characterized (Bompard-Gilles et al., 2000; Shaw et al., 2004; Chin et al., 2007; Martínez-García et al., 2018). The proteases production by the genus *Staphylococcus* is reported to be involved with its human pathogenesis virulence. Despite the proteases production by the genera *Isoptericola*, *Massilia*, and *Sphingomonas* being also described in the literature, none has been studied (Lindquist et al., 2003; Willsey and Wargo, 2015, Bibi et al., 2017).

Bacteria can use starch as an energy source, however, amylase production by bacteria varies across genera and species. Among the 18 identified amylase producer isolates were the following species or genera: *K. variicola*, *K. oxytoca*, and *Massilia* sp. In fact, fewer genera have been shown to produce amylases, such as the genera *Bacillus* and *Clostridium* (Gupta et

al., 2003), which explains, at least in part, the lower number of producing species, in comparison with the other enzymes. The species *K. variicola* is described as a plant endophyte and is much related to the human isolates of *K. pneumoniae*, with some strains sharing more than 95% of genome identity (Rosenblueth et al., 2004; Chem et al., 2016). No amylase production by *K. variicola* has hitherto been described, despite its similar species, *K. pneumoniae*, being known as a producer and having a well characterized enzyme (Sun et al., 2010). *K. oxytoca* has been reported to produce an extracellular cyclodextrin glucanotransferase, which can degrade starch and synthesize α-cyclodextrin (Lee et al., 1992). Moreover, both *K. oxytoca* and *K. variicola* ae reported to exhibit pullulanase production (Francetić and Pugsley, 2005; Chen et al., 2013). Both cyclodextrin glucanotransferase and pullulanase belong to the α-amylase family of enzymes. The performed submerse fermentation studies have shown that all tested isolates produced amylase activity using the reducing sugar releasing method, but only isolates of *K. oxytoca* and *Massilia* sp. produced amylase activity using the starch-iodine complex color reduction method (Figure 3). Even though no amylase from species of the genus *Massilia* has been characterized so far, they were shown to use starch to produce polyhydroxyalkanoates, which are biodegradable and biocompatible with hydroxycarboxylic acid polyester (Cerrone et al., 2011). In fact, the isolate of *Massilia* sp. CTI 57 has been further identified as *Massilia timonae* and a cyclodextrinase gene was amplified from its DNA (Santos and Barbosa-Tessman, in press). The amplified gene was cloned and expressed in *E. coli* and the expressed protein was fully characterized (Santos and Barbosa-Tessman, in press).

Among the 55 identified isolates, there were 16 producers of lipases, identified in agar Rhodamine B, which belong to the species or genera: *S. maltophilia, Burkholderia* sp., *Ochrobactrum* sp., *P. dispersa, S. hominis, Sphingomonas* sp., and *K. variicola*. When evaluated for lipase and esterase production in liquid medium, the major producers of both

enzymes were the isolates of *Burkholderia* sp., *S. maltophilia*, *P. dispersa*, and *K. variicola* (Figure 3), whereas the isolates of *Ochrobactrum* sp. and *S. hominis* have produced insignificant amounts of both enzymes (Figure 3). Few species of bacteria are described as good producers of lipolytic enzymes, but the *Burkholderia cepacia* lipase, for instance, is commercially available (Sharma et al., 2001). In addition, lipases production by *S. maltophilia* is well documented and one enzyme has been fully characterized (Li et al., 2016). Although isolates of the genus *Klebsiella* have been shown to produce lipase in bioprospection studies, none has been studied (Mazzucotellii et al., 2013). Nonetheless, no lipase or esterase from *P. dispersa* has been studied yet and this is the first time, to the best of our knowledge, which this species is shown to synthesize lipolytic enzymes.

As reviewed by Kuhad et al. (2011), there are many cellulase-producing genera of aerobic and anaerobic bacteria widely distributed in nature. The species or genera among the 25 identified cellulase producer identified were: *K. oxytoca*, *K. variicola*, *E. cloacae*, and *Modestobacter* sp. In this work, most of the identified isolates of *K. variicola* and *K. oxytoca* were found to produce both amylase and cellulase, indicating their involvement with glycan polymers breakdown. Cellulase production by *Klebsiella* genus isolates and *E. cloacae* is well described in the literature and some of them have latterly been characterized (Li et al., 2015; Attigani et al., 2016; Akintola et al., 2017). The cellulase production by the Actinobacteria *Modestobacter* has not been reported yet. Members of the Actinobacteria are tolerant to several environments, and so are their enzymes (Mohammadipanah and Wink, 2015). However, the submerse fermentation analysis, using CMC as substrate, did not evidence cellulase production by the *Modestobacter* sp. isolate and by any other tested isolates. A new culture in microcrystalline cellulose agar did not confirm cellulase production by *Modestobacter* sp. and by several other cellulase producing isolates (results not shown). In fact, it seems that the cellulase production was evidenced soon after the bacteria isolation

from maize grains, but their ability to produce this enzyme seems to be repressed under laboratory storage.

CONCLUSIONS

Fifty-five strains of bacteria isolated from maize grains found to produce hydrolytic enzymes have been identified either to the genus or species level in this work. In total, the isolates belonged to 13 different genera. Some of these strains belonged to well-documented species, whose enzymes have been already purified and characterized. Yet, new species have been described as producers for the first time. For example, *Massilia* sp. as an amylase producer and *P. dispersa* as a lipase and esterase producer. Further studies on the characterization of these newly identified enzymes may enhance the biotechnological importance of these species.

ACKNOWLEDGEMENTS

The authors are thankful to CNPQ (Conselho Nacional de Desenvolvimento Científico e Tecnológico, Ministry of Science and Technology, Brazil) and to CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, Ministry of Education, Brazil), for the student scholarships.

REFERENCES

Abe, C.A.L, Faria, C.B., Castro, F.F., Souza, S.R., Santos, F.C., Silva, C.N., Tessmann, D.J., Barbosa-Tessmann, I.P., 2015. Fungi isolated from maize (*Zea mays* L.) grains and production of associated enzyme activities. Int. J. Mol. Sci. 16, 15328-15346.

Adrio, J.L., Demain, A.L., 2014. Microbial enzymes: Tools for biotechnological processes. biomolecules 4, 117-139.

Akintola, A.I., Oyedeji, O., Bakare, M.K., Adewale, I.O., 2017. Purification and characterization of thermostable cellulase from *Enterobacter cloacae* IP8 isolated from decayed plant leaf litter. Biocatal. Biotransfor. 35(5), 79-387.

Antoniou1, A., Tsolakidou, M.D., Stringlis, I.A., Pantelides, I.S., 2017. Rhizosphere microbiome recruited from a suppressive compost improves plant fitness and increases protection against vascular wilt pathogens of tomato. Front. Plant Sci. 8, Article 2022, https://doi.org/10.3389/fpls.2017.02022.

Attigani, A., Sun, L., Wang, Q., Liu, Y., Bai, D., Li, S., Huanga, X., 2016. The crystal structure of the endoglucanase Cel10, a family 8 glycosyl hydrolase from *Klebsiella pneumoniae*. Acta Crystallogr. F 72(Pt 12), 870–876.

Bhat, M.K., 2000. Cellulases and related enzymes in biotechnology. Biotechnol. Adv. 18, 355-383.

Bibi, F., Ullah, I., Alvi, S.A, Bakhsh, S.A., Yasir, M., Al-Ghamdi, A.A.K., Azhar, E.I., 2017. Isolation, diversity, and biotechnological potential of rhizo- and endophytic bacteria associated with mangrove plants from Saudi Arabia. Genet. Mol. Res. 16 (2), gmr16029657, https://doi.org/10.4238/gmr16029657.

Bompard-Gilles, C., Remauta, H., Villereta, V., Prangé, T., Fanuel, L., Delmarcelle, M., Joris, B., Frèrec, J.M., Beeumena, J.V., 2000. Crystal structure of a D-aminopeptidase from *Ochrobactrum anthropi*, a new member of the 'penicillin-recognizing enzyme' family. Structure 8(9), 971-980.

Bornscheuer, U.T., 2002. Microbial carboxyl esterases: Classification, properties and application in biocatalysis. FEMS Microbiol. Rev. 26, 73–81.

Ceska, M., 1971. Enzyme catalysis of solidified media. Eur. J. Biochem. 22, 186-192.

Chelius, M.K., Triplett, E.W., 2001. The diversity of Archaea and bacteria in association with the roots of *Zea mays* L. Microb. Ecol. 41, 252–263.

Chen, M., Li, Y., Li, S.; Tang, L.; Zheng, J., An, Q., 2016. Genomic identification of nitrogen-fixing *Klebsiella variicola, K. pneumoniae* and *K. quasipneumoniae*. J. Basic Microbiol. 56, 78–84.

Chen, W.B., Nie, Y., Xu, Y., 2013. Signal peptide-independent secretory expression and characterization of pullulanase from a newly isolated *Klebsiella variicola* SHN-1 in *Escherichia coli*. Appl. Biochem. Biotech. 169(1), 41-54.

Chin, C.-Y., Othman, R., Nathan, S., 2007. The *Burkholderia pseudomallei* serine protease MprA is autoproteolytically activated to produce a highly stable enzyme. Enzyme Microb. Tech. 40(2), 370-377.

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

Felsenstein, J., 1985. Confidence limits on phylogenies: An approach using the bootstrap. Evolution 39, 783-791.

Fjerbaek, L., Christensen, K.V., Norddahl, B., 2009. A review of the current state of biodiesel production using enzymatic transesterification. Biotechnol. Bioeng. 102, 1297-1315.

Francetić, O., Pugsley, A.P., 2005. Towards the identification of type II secretion signals in a nonacylated variant of pullulanase from *Klebsiella oxytoca*. J. bacterial. 187(20), 7045-7055.

Gohelab, V., Maisuriaa, V., Chhatpara, H.S., 2007. Utilization of various chitinous sources for production of mycolytic enzymes by *Pantoea dispersa* in bench-top fermenter. Enzyme Microb. Tech. 40(6), 1608-1614.

Gupta, R., Gigras, P., Mohapatra, H., Goswami, V.K., Chauhan, B., 2003. Microbial α-amylases: Biotechnological perspective. Process Biochem. 38, 1-18.

Gurung, N., Ray, S., Bose, S., Rai, V., 2013. A broader view: Microbial enzymes and their relevance in industries, medicine, and beyond. BioMed Res. Int. 2013, 329121, https://doi.org/10.1155/2013/329121.

Hankin, L., Anagnostakis, S.L., 1975. The use of solid media for detection of enzyme production by fungi. Mycologia 67, 597-607.

Hasan, F., Shah, A.A., Hameed, A. 2006. Industrial applications of microbial lipases. Enzyme Microb. Tech. 39, 235-251.

Hernández, M., Dumont, M.G., Yuan, Q., Conrad, R., 2015. Different bacterial populations associated with the roots and rhizosphere of rice incorporate plant-derived carbon. Appl. Environ. Microbiol. 81(6), 2244-2253.

Holt, J.G., 1994. Bergey's manual of determinative bacteriology. 9th edition. Lippincott Williams & Wilkins, Philadelphia.

Jaeger, K.E., Eggert, T., 2002. Lipases for biotechnology. Curr. Opin. Biotech. 13, 390-397.

Jaeger, K.E., Kouker, G., 1987. Specific and sensitive plate assay for bacterial lipases. Appl. Environ. Microbiol. 53, 211-213.

Jisha, V.N., Smitha, R.B., Pradeep, S., Sreedevi, S., Unni, K.N., Sajith, S., Priji, P., Josh, M.S., Benjamin, S., 2013. Versatility of microbial proteases. Adv. Enzyme Res. 1(3), 39-51.

Johnston-Monje, D., Raizada, M.N., 2011. Conservation and diversity of seed associated endophytes in *Zea* across boundaries of evolution, ethnography and ecology. PLoS ONE 6(6), e20396.

Kämpfer, P., Glaeser, S.P., Kloepper, J.W., Hu, C.-H., McInroy, J.A., Martin, K., Busse, H.J., 2016. *Isoptericola cucumis* sp. nov., isolated from the root tissue of cucumber (*Cucumis sativus*). Int. J. Syst. Evol. Microbiol. 66, 2784–2788.

Kasana, R.C., Salwan, R., Dhar, H., Dutt, S., Gulati, A., 2008. A rapid and easy method for the detection of microbial cellulases on agar plates using Gram's iodine. Curr. Microbiol. 57, 503-507.

Kirk, O., Borchert, T.V., Fuglsang, C.C., 2002. Industrial enzyme applications. Curr. Opin. Biotech. 13(4), 345‑351.

Kuhad, R.C., Gupta, R., Singh, A., 2011. Microbial cellulases and their industrial applications. Enzyme Res. 2011, ID 280696, https://doi.org/10.4061/2011/280696.

Kumar, C.G., Takagi H., 1999. Microbial alkaline proteases: From a bioindustrial viewpoint. Biotechnol. Adv. 17, 561–594.

Kumar, S., Stecher, G., Tamura, K., 2016. MEGA7: Molecular evolutionary genetics Analysis version 7.0 for bigger datasets. Mol. Biol. Evol. 33, 1870-1874.

Lee, J.-H., Choi, K.-H., Choi, J.Y., Lee, Y.-S., Kwon, I.-B., Yu, J.H., 1992. Enzymatic production of α-cyclodextrin with the cyclomaltodextrin glucanotransferase of *Klebsiella oxytoca*. Enzyme Microb. Tech. 14(12), 1017-1020.

Li Q., Hong X., Chen X., Chen D., 2015. Expression and characterization of recombinant cellulases from an isolated *Klebsiella*. Afr. J Microbiol. Res. 9(38), 2057-2066.

Li, M., Yang, L.R., Xu, G., Wu, J.P., 2016. Cloning and characterization of a novel lipase from *Stenotrophomonas maltophilia* GS11: The first member of a new bacterial lipase family XVI. J. Biotechnol. 228, 30-36.

Lindquist, D., Murrill, D., Burran, W.P., Winans, G., Janda, M.J., 2003. Characteristics of *Massilia timonae* and *Massilia timonae*-like isolates from human patients, with an emended description of the species. J. Clin. Microbiol. 41, 192-196.

Lynd, L.R., Weimer, P.J., van Zyl, W.H., Pretorius, I.S., 2002. Microbial cellulose utilization: Fundamentals and biotechnology. Microbiol. Mol. Biol. R. 66, 506-577.

Martínez-García, S., Rodríguez-Martínez, S., Cancino-Diaz, M.E., Cancino-Diaz, J.C., 2018. Extracellular proteases of *Staphylococcus epidermidis*: Roles as virulence factors and their participation in biofilm. APMIS 126(3), 177-185.

Matsuoka, Y., Vigouroux, Y., Goodman, M.M., Sanchez, G.J., Buckler, E., Doebley, J., 2002. A single domestication for maize shown by multilocus microsatellite genotyping. Proc. Natl. Acad. Sci. USA 99, 6080-6084.

Mazzucotellii, C.A, Ponce, A.G., Kotlar, C.E., Moreira, M.delR., 2013. Isolation and characterization of bacterial strains with a hydrolytic profile with potential use in bioconversion of agroindustial by-products and waste. Food Sci. Technol. 33(2), 295-303.

Miller, G.L., 1959. Use of dinitrosalicylic acid reagent for determination of deducing sugar. Anal. Chem. 31(3), 426–428.

Mohammadipanah, F., Wink, J., 2015. Actinobacteria from arid and desert habitats: Diversity and biological activity. Front. Microbiol. 6, article1541, https://doi.org/10.3389/fmicb.2015.01541.

Montenecourt B.S., Eveleigh D.E., 1977. Semiquantitative plate assay for determination of cellulase production by *Trichoderma viride*. Appl. Environ. Microbiol. 33, 178-183.

Nicodème, M., Grill, J.-P., Humbert, G., Gaillard, J.-L., 2005 Extracellular protease activity of different *Pseudomonas* strains: Dependence of proteolytic activity on culture conditions. J. Appl. Microbiol. 99, 641-648.

Niu, B., Paulson, J.N., Zheng, X., Kolter, R., 2017. Simplified and representative bacterial community of maize roots. Proc. Natl. Acad. Sci. USA 114(12), E2450-E2459, https://doi.org/10.1073/pnas.1616148114.

Palanivelu, P., 2001. Analytical Biochemistry and separation techniques. Kalamani Printers, Madurai, India.

Pandey, A., Nigam, P., Soccol, C.R., Soccol, V.T., Singh, D., Mohan, R., 2000. Advances in microbial amylases. Biotechnol. Appl. Bioc. 31, 135-152.

Rao, M.B., Tanksale, A.M., Ghatge, M.S., Deshpande, V.V., 1998. Molecular and biotechnological aspects of microbial proteases. Microbiol. Mol. Biol. R., 62, 597-35.

Relman, D.A., 1993. Universal bacterial 16S rDNA amplification and sequencing. In Persing, D.H., Smith, T.F., Tenover, F.C., White T.J. (Eds.), Diagnostic molecular mcrobiology, principles and applications. ASM, Washington DC, pp. 489–495.

Ribitsch, D., Heumann, S., Karl, W., Gerlach, J., Leber, R., Birner-Gruenberger, R., Gruber, K., Eiteljoer, G.I., Remler, P., Siegert, P., Lange, J., Maurer, K.H., Berg, G., Guebitz, G.M., Schwab, H., 2012. Extracellular serine proteases from *Stenotrophomonas maltophilia*: Screening, isolation and heterologous expression in *E. coli*. J. Biotechnol. 157(1), 140-7.

Rosenblueth, M., Martínez, L., Silva, J., Martínez-Romero, E., 2004. *Klebsiella variicola*, a novel species with clinical and plant-associated isolates. Syst. Appl. Microbiol. 27, 27-35.

Saitou, N., Nei, M., 1987. The neighbor-joining method: A new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4, 406-425.

Santos, F.C., Barbosa-Tessmann, I.P., 2018. Recombinant expression, purification, and characterization of a cyclodextrinase from *Massilia timonae*. Protein Expression and Purification. In Press, https://doi.org/10.1016/j.pep.2018.08.013.

Sarath, G., de La Motte, R.S., Wagner, F.W., 1989. Protease assay methods. In: Beynon, R.J., Bonde, J.S. (Eds.), Proteolytics Enzymes: An practical approach. Oxford University Press, Oxford, p. 25-54.

Sayali, K., Sadichha, P., Surekha, S., 2013. Microbial esterases: An overview. IJCMAS 2, 135-146.

Sharma, R., Chistib, Y., Banerjeea, U.C., 2001. Production, purification, characterization, and applications of lipases. Biotechnol. Adv. 19, 627-662.

Shaw, L., Golonka, E., Potempa, J., Foster, S.J., 2004. The role and regulation of the extracellular proteases of *Staphylococcus aureus*. Microbiology 150, 217-228.

Silva, U.C., Medeiros, J.D., Leite, L.R., Morais, D. K., Cuadros-Orellana, S., Oliveira, C.A., Lana, U.G.P., Gomes, E.A., Santos, V.L., 2017. Long-term rock phosphate fertilization impacts the microbial communities of maize rhizosphere. Front. Microbiol. 8, Article 1266, https://doi.org/10.3389/fmicb.2017.01266.

Singh, R., Kumar, M., Mittal, A., Mehta, P.K., 2016. Microbial enzymes: Industrial progress in 21st century. 3 Biotech 6(2), 174, https://doi.org/10.1007/s13205-016-0485-8.

Sun, H., Zhao, P., Ge, X., Xia, Y., Hao, Z., Liu, J., Peng, M., 2010. Recent advances in microbial raw starch degrading enzymes. Appl. Biochem. Biotech. 160, 988-1003.

Synnes, M., 2007. Bioprospecting of organisms from the deep sea: Scientific and environmental aspects. Clean Technol. Envir. 9, 53-59.

Tamura, K., Nei, M., Kumar, S., 2004. Prospects for inferring very large phylogenies by using the neighbor-joining method. Proc. Natl. Acad. Sci. USA 101, 11030-11035.

Tondo, E.C., Lakus, F.R., Oliveira, F.A., Brandelli, A., 2004. Identification of heat stable protease of *Klebsiella oxytoca* isolated from raw milk. Lett. Appl.Microbiol. 38, 146–150.

Vandecandelaere, I., Depuydt, P., Nelis, H.J., Coenye, T., 2014. Protease production by *Staphylococcus epidermidis* and its effect on *Staphylococcus aureus* biofilms. Pathog. Dis., 70(3), 321-331.

Willsey, G.G, Wargo, M.J., 2015. Extracellular lipase and protease production from a model drinking water bacterial community is functionally robust to absence of individual members. PLoS One 10(11), e0143617, https://doi.org/10.1371/journal.pone.0143617.

Winkler, U.K., Stuckmann, M., 1979. Glycogen, hyaluronate, and some other polysaccharides greatly enhance the formation of exolipase by *Serratia marcescens*. J. Bacteriol. 138, 663-70.

Table 1. Bacterial isolates identified as producers of hydrolytic activities.

Maize samples	**Protease**	**Amylase**	**Lipase**	**Cellulase**	**Total**
FEI3	4, 19, 33, 40, 62, 63, 71, 73, 79, 81		53, 63, 81	11, 12, 28, 32, 55, 57, 58, 89, 91	20
FEI4	6, 7, 10, 13, 16, 17, 18, 20, 31, 40, 49, 50, 52, 55, 56, 57, 62, 65, 66, 80, 90, 92, 104, 106, 107, 108, 109, 116, 119		6, 7, 10, 13, 31, 49, 50, 52, 55, 56, 57, 62, 65, 66	3, 43, 79, 61, 114, 120	35
FEI5	137, 138, 139, 140, 141, 145, 146, 151, 152, 155, 157, 159	87, 104, 117	8, 26, 63, 137, 138, 139, 140, 141, 145, 146, 151, 152, 155, 157, 159	19, 23, 30, 50, 64, 79, 81, 89, 97, 117, 122, 124, 126, 127, 130, 131, 132, 144, 153	36
CTI	13, 20, 39, 56, 57, 61, 70, 76, 99, 100	1, 14, 15, 16, 20, 37, 39, 40, 47, 57, 60, 63, 64, 83, 90, 92, 94, 96	15	1, 13, 14, 15, 16, 20, 31, 37, 38, 39, 40, 49, 51, 60, 64, 68, 79, 83, 84, 90, 92, 93, 94	36
MAR				20, 26, 31, 35, 40, 51, 55, 57, 75, 100	10
Total	61	21	33	67	**137** / **182**

FEI – Iguatemi Experimental Farm, Maringá, PR. CTI – Irrigation Technology Center, Maringá, PR. MAR – Marialva, PR. In red are the isolates that produced more than one hydrolytic activity. Underlined are the isolates that were molecularly identified.

Table 2. DNA barcoding identified bacterial isolates and hydrolytic enzymes production.

Isolate	Genera and species	GenBank AN	GenBank AN of similar sequences (% of identity)	Protease	Amylase	Lipase	Cellulase
FEI3 11	*Klebsiella variicola*	MH819801	CP012426.1 (100%); MF179616.1 (100%)				X
FEI3 33	*Pseudomonas putida*	MH819802	MF079266.1 (100%); KY849572.1 (100%)	X			
FEI3 40	*Pseudomonas putida*	MH819803	MF079266.1 (100%); KY849572.1 (100%)	X			
FEI3 53	*Klebsiella variicola*	MH819804	MH111590.1 (100%); KY856926.1 (100%)			X	
FEI3 62	*Stenotrophomonas maltophilia*	MH819805	MH179323.1 (100%); KY933291.1 (100%)	X			
FEI4 3	*Klebsiella variicola*	MH819806	MH111590.1 (100%); KY856926.1 (100%)				X
FEI4 6	*Stenotrophomonas maltophilia*	MH819807	MH179323.1 (100%); KY933291.1 (100%)	X		X	
FEI4 13	*Stenotrophomonas maltophilia*	MH819808	MH179323.1 (100%); KY933291.1 (100%)	X		X	
FEI4 16	*Ochrobactrum* sp.	MH819809	KX822680.1 (100%); FJ603030.1 (100%)	X			
FEI4 49	*Stenotrophomonas maltophilia*	MH819810	MH179323.1 (100%); KY933291.1 (100%)	X		X	
FEI4 65	*Pantoea dispersa*	MH819811	MG923542.1 (100%); LT969731.1 (100%)	X		X	
FEI4 92	*Stenotrophomonas maltophilia*	MH819812	MH179323.1 (100%); KY933291.1 (100%)	X			
FEI4 106	*Bacillus* sp.	MH819813	MH337993.1 (100%); KY860723.1 (100%)	X			
FEI4 107	*Stenotrophomonas maltophilia*	MH819814	MH179323.1 (100%); KY933291.1 (100%)	X			
FEI4 108	*Stenotrophomonas maltophilia*	MH819815	MH179323.1 (100%); KY933291.1 (100%)	X			
FEI4 119	*Isoptericola* sp.	MH819816	KX034605.1 (100%); KY939565.1 (98%)	X			
FEI5 8	*Klebsiella variicola*	MH819817	MH111590.1 (100%); KY856926.1 (100%)			X	
FEI5 63	*Klebsiella variicola*	MH819818	MH111590.1 (100%); KY856926.1 (100%)			X	
FEI5 87	*Klebsiella oxytoca*	MH819819	KY614353.1 (100%); MF289236.1 (100%)	X	X		
FEI5 89	*Klebsiella variicola*	MH819820	MH111590.1 (100%); KY856926.1 (100%)				X
FEI5 117	*Klebsiella oxytoca*	MH819821	KY614353.1 (100%); MF289236.1 (100%)		X		X
FEI5 124	*Enterobacter cloacae*	MH819822	MF471480.1 (100%); KY495207.1 (100%)				X
FEI5 127	*Klebsiella variicola*	MH819823	MH111590.1 (100%); KY856926.1 (100%)				X
FEI5 139	*Sphingomona*s sp.	MH819824	FR750304.1 (99%); DQ145741.1 (99%)	X		X	
FEI5 141	*Staphylococcus hominis*	MH819825	MH201347.1 (100%); KY992547.1 (100%)	X		X	
FEI5 145	*Klebsiella variicola*	MH819826	MH111590.1 (100%); KY856926.1 (100%)	X		X	
FEI5 146	*Ochrobactrum* sp.	MH819827	KX822680.1 (100%); FJ603030.1 (100%)	X		X	
FEI5 151	*Staphylococcus hominis*	MH819828	MH201347.1 (100%); KY992547.1 (100%)	X		X	
FEI5 152	*Stenotrophomonas maltophilia*	MH819829	MH179323.1 (100%); KY933291.1 (100%)	X		X	
FEI5 153	*Modestobacter* sp.	MH819830	KY510681.1 (98%); FM995613.1 (98%)				X
FEI5 157	*Burkholderia* sp.	MH819831	LC385678.1 (99%); MF464547.1 (99%)	X		X	
FEI5 159	*Stenotrophomonas maltophilia*	MH819832	MH179323.1 (100%); KY933291.1 (100%)	X		X	
CTI 13	*Klebsiella variicola*	MH819833	CP013985.1 (100%); CP001891.1 (100%)	X			X
CTI 14	*Klebsiella variicola*	MH819834	MH111590.1 (100%); KY856926.1 (100%)		X		X
CTI 15	*Klebsiella oxytoca*	MH819835	KY614353.1 (100%); MF289236.1 (100%)		X	X	X

Table 2. (Continuation).

Isolate	Genera and species	GenBank AN	GenBank AN of similar sequences (% of identity)	Protease	Amylase	Lipase	Cellulase
CTI 16	*Klebsiella oxytoca*	MH819836	KY614353.1 (100%); MF289236.1 (100%)		X		X
CTI 20	*Klebsiella oxytoca*	MH819837	KY614353.1 (100%); MF289236.1 (100%)	X	X		X
CTI 37	*Klebsiella oxytoca*	MH819838	KY614353.1 (100%); MF289236.1 (100%)		X		X
CTI 38	*Enterobacter cloacae*	MH819839	MF471480.1 (100%); KY495207.1 (100%)				X
CTI 39	*Klebsiella oxytoca*	MH819840	KY614353.1 (100%); MF289236.1 (100%)	X	X		X
CTI 40	*Klebsiella variicola*	MH819841	MH111590.1 (100%); KY856926.1 (100%)		X		X
CTI 47	*Klebsiella oxytoca*	MH819842	KY614353.1 (100%); MF289236.1 (100%)		X		
CTI 49	*Klebsiella variicola*	MH819843	CP012426.1 (100%); MF179616.1 (100%)				X
CTI 57	*Massilia* sp.	MH819844	KM187207.1 (99%); JX566630.1 (99%)	X	X		
CTI 60	*Klebsiella variicola*	MH819845	CP012426.1 (100%); MF179616.1 (100%)		X		X
CTI 63	*Klebsiella oxytoca*	MH819846	KY614353.1 (100%); MF289236.1 (100%)		X		
CTI 64	*Klebsiella oxytoca*	MH819847	KY614353.1 (100%); MF289236.1 (100%)		X		X
CTI 70	*Bacillus* sp.	MH819848	MF418036.1 (100%); KY860716.1 (100%)	X			
CTI 79	*Klebsiella variicola*	MH819849	CP012426.1 (100%); MF179616.1 (100%)				X
CTI 83	*Klebsiella oxytoca*	MH819850	KY614353.1 (100%); MF289236.1 (100%)		X		X
CTI 92	*Klebsiella oxytoca*	MH819851	KY614353.1 (100%); MF289236.1 (100%)		X		X
CTI 94	*Klebsiella variicola*	MH819852	CP012426.1 (100%); MF179616.1 (100%)	X		X	
CTI 96	*Klebsiella oxytoca*	MH819853	KY614353.1 (100%); MF289236.1 (100%)	X			
MVA 26	*Klebsiella variicola*	MH819854	CP012426.1 (100%); MF179616.1 (100%)			X	
MVA 35	*Klebsiella variicola*	MH819855	CP012426.1 (100%); MF179616.1 (100%)			X	

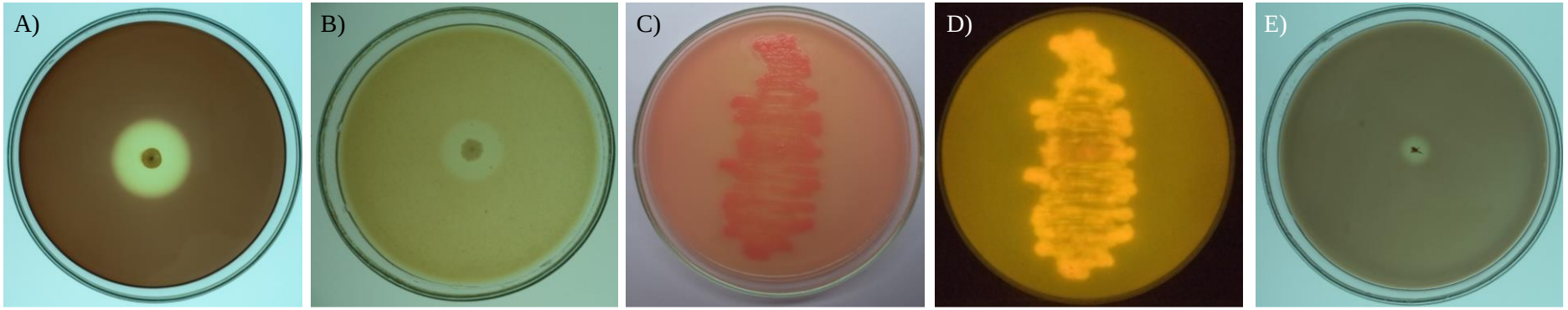

Figure 1. Petri dishes with distinct substrate media for the enzyme activities detection. A) Amylolytic activity. Nutrient agar containing soluble starch. *K. oxytoca* CTI 63; B) Proteolytic activity. Medium containing skimmed milk casein. *S. maltophilia* FEI4 49. C) Lipolytic activity. Medium containing triolein and rhodamine B. *S. maltophilia* FEI4 6. D) The reverse of the dish shown in C) photographed under UV light. E) Cellulolytic activity. Medium containing microcrystalline cellulose. *K. oxytoca* CTI 15.

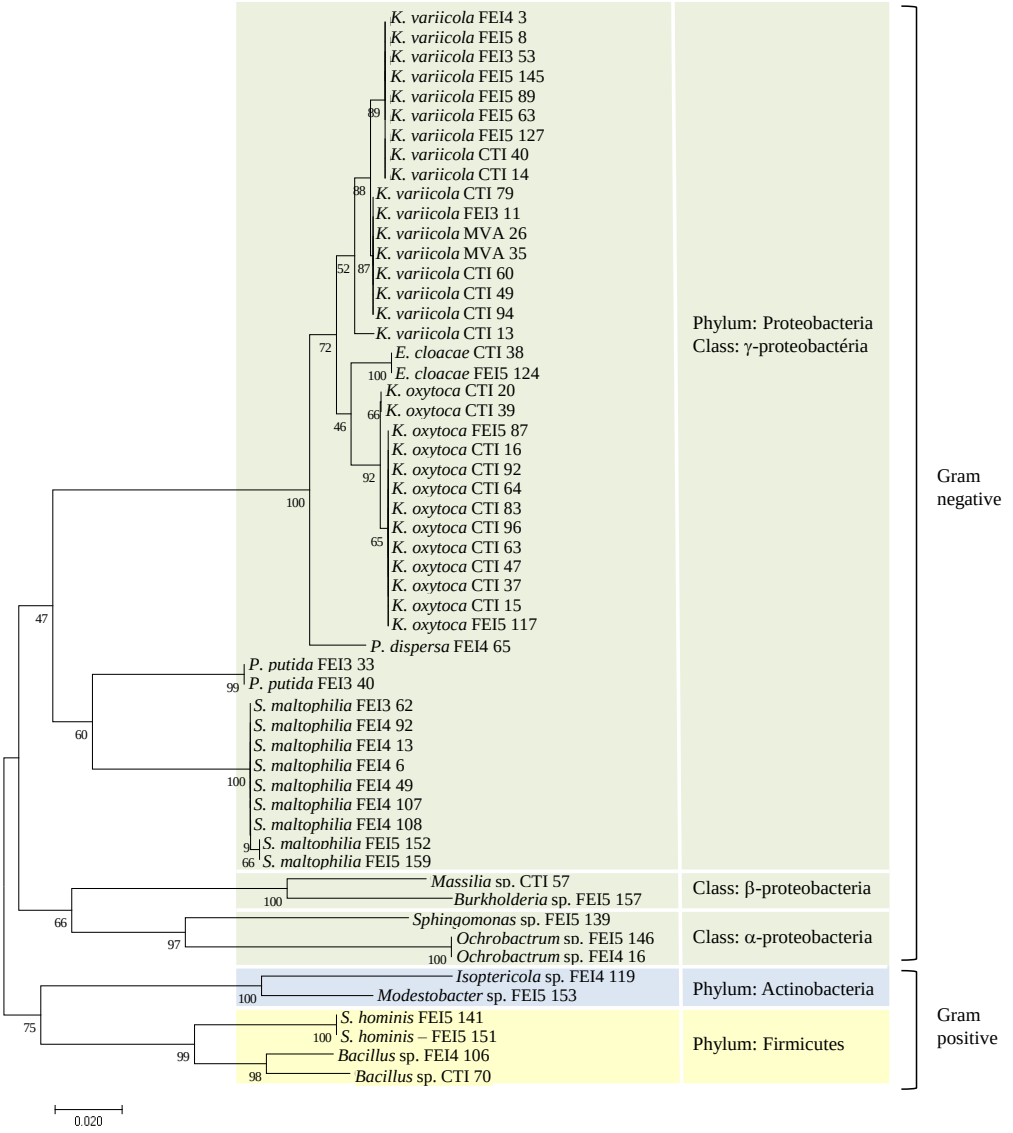

Figure 2. The evolutionary history of the 16S rRNA partial gene sequences. The optimal tree with the sum of branch length = 0.94393273 is shown. The percentages of replica trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (FELSENSTEIN, 1985). The tree is drawn to scale, with branch lengths in number of base substitutions per site. The analysis involved 55 nucleotide sequences for rDNA. All ambiguous positions were removed for each sequence pair. There were a total of 398 positions in the final dataset. Evolutionary analyses were conducted in MEGA 7.0 program (KUMAR et al., 2016).

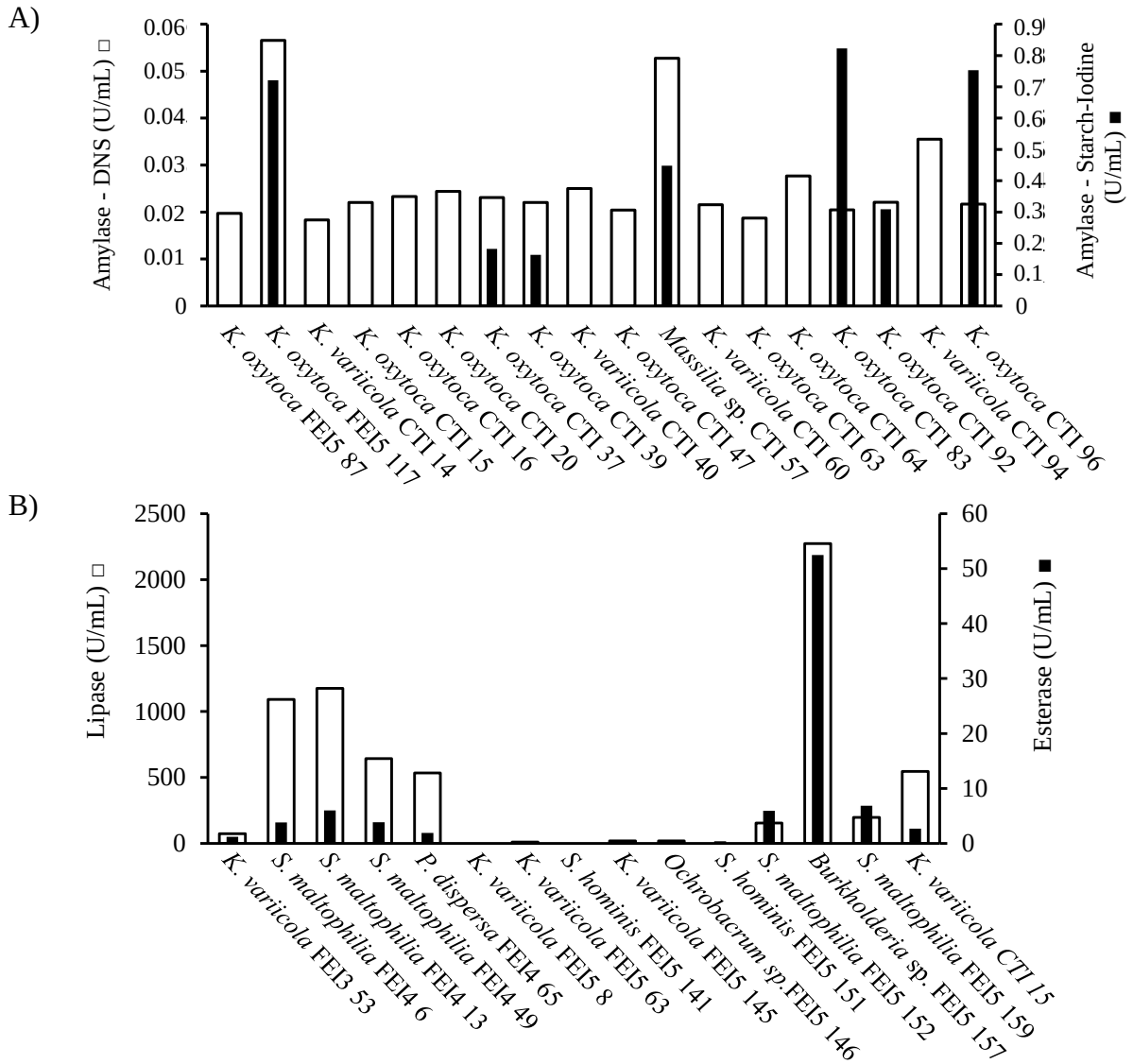

Figure 3. Enzyme activity analysis in submerse fermentation. The data are the average obtained in two repetitions. Enzyme activity was expressed as U/mL. A) Amylase activity analysis with the DNS and starch-iodine methods. B) Lipase and esterase activity. The enzyme activities were evaluated by release of *p*-NP from *p*-NPP and *p*-NPB, respectively.

CAPÍTULO II
RECOMBINANT EXPRESSION, PURIFICATION, AND CHARACTERIZATION OF A CYCLODEXTRINASE FROM *MASSILIA TIMONAE*

SANTOS, F.C. dos[1]; BARBOSA-TESSMANN, I.P.[3]

ABSTRACT

Some microorganisms can produce cyclodextrin glycosyltransferases, which degrades starch by catalyzing cyclization and giving rise to cyclodextrin. Thus, to fully degrade starch, microorganisms can also synthesize cyclodextrinases, which hydrolyze cyclodextrins. In this work, a truncated gene, without the signal peptide sequence, encoding a cyclodextrinase from *Massilia timonae* was PCR amplified, cloned, and expressed in *E. coli*. The histidine-tagged recombinant enzyme was purified by immobilized metal ion affinity chromatography. The purified protein was found to be a tetramer of about 260 kDa, with monomers of about 65 kDa, as estimated by gel filtration and SDS-PAGE, respectively. The enzyme presented an optimum temperature of 40 °C, optimum pH of 7.0, and remained stable after 30 min of incubation at 45 °C, with a T_{50} of 48.45 °C. The enzyme showed a higher activity toward β-cyclodextrin compared to that for maltodextrin and starch. K_M for β-cyclodextrin was 2.1 mM, V_{max} was 0.084μmol/min, k_{cat} was 8326 min^{-1}, and k_{cat}/K_M was 4.1×10^6 $M^{-1}min^{-1}$. Calcium acted as an activator and SDS, CTAB, several cations, and EDTA acted as strong inhibitors. The purified cyclodextrinase produced glucose and maltose as final products by hydrolysis of β-cyclodextrin, maltotetraose, and maltoheptaose. This novel cyclodextrinase could be a promising alternative for the enzymatic hydrolysis of starch.

Keywords: Cyclodextrinase, β-cyclodextrin, *Massilia timonae*, Expression, Starch.

[3] Department of Biochemistry, Universidade Estadual de Maringá, Maringá, Paraná, Brazil

1. INTRODUCTION

Cyclodextrins are cyclic carbohydrates formed during starch degradation by enzymes called cyclodextrin glucantransferases (EC 2.4.1.19), which belong to the class of transferases [34]. The prevalent α-, β-, and γ-cyclodextrins are composed of six, seven, and eight d-glucopyranosyl units linked via α(1,4)-glycosidic bonds [9]. The hydrophobic environment of their cavity enables them to form inclusion complexes with water-insoluble compounds, which are useful in pharmaceutical and chemical industries [9]. The growing application of cyclodextrins has increased the interest in their degradation mechanisms, mainly because they are resistant to hydrolysis by most α-amylases. Cyclodextrins can be degraded by cyclomaltodextrinases (EC 3.2.1.54), maltogenic amylases (EC 3.2.1.133), and neopullulanases (EC 3.2.1.135). These three enzymes are almost equal in their catalytic properties and three-dimensional structures; therefore, they have been placed in a common subfamily, the glycoside hydrolase family 13 (GH13) [24]. They have also been reported to be capable to hydrolyze all or two of the following three types of substrates: cyclodextrins, pullulan, and starch. Cyclomaltodextrinases (cyclodextrinases, cyclomaltodextrin dextrinhydrolases) and maltogenic amylases (maltogenases) catalyze the hydrolysis of cyclodextrins faster than pullulan or starch and neopullulanases hydrolyzes pullulan more efficiently than starch [33].

Microbial amylases generally meet high industrial demand [16, 32]. The industrial applications of cyclodextrinases include starch structure change to get amylose-free or low-amylose food/bread products, what influences their taste and make them suitable for freezing [2].

Cyclodextrinases are mostly restricted to bacteria but have also been reported from Archaea [33]. In addition to their hydrolytic activity, cyclodextrinases have significant transglycosylation capacity and can form α-1,4- and α-1,6-glycosidic linkages [33]. Regarding cyclodextrin metabolism in bacteria, it has been reported that they are formed outside the cell during starch metabolism and are transported into the cytoplasm to be hydrolyzed by intracellular cyclodextrinases to form molecules that are used as energy source [33]. Alternatively, when in high intracellular concentration, they are subjected to transglycosylation to form branched oligosaccharide that modulates the bacterial osmotic pressure and cytoplasm viscosity [33]. Otherwise, it has also been reported that some cyclodextrinases have secretion signal peptides and could be secreted to play a role in extracellular cyclodextrins hydrolysis or transglycosilation [31, 39].

Recently, a bioprospection on maize grains presenting rotten symptoms was conducted in our laboratory to find hydrolases producing bacteria, using solid media with a specific substrate [36]. An isolate producing amylase activity was identified as *Massilia timonae* by the partial amplification and sequencing of its 16S rRNA gene [36]. The members of the genus *Massilia* were first isolated from clinical samples and later isolated from environmental samples of many sources [10, 13, 21, 27, 30]. Isolates of the genus *Massilia* have already been suggested to produce amylase because they can use starch as a carbon source to produce polyhydroxyalkanoates, which are biodegradable and biocompatible to hydroxycarboxylic acids polyesters [7].

A search in the *M. timonae* CCUG 45783 sequenced genome revealed five amylase genes. Primers were designed to PCR amplify those genes. A gene coding for a cyclodextrinase was amplified, cloned, and expressed in *Escherichia coli*. The recombinant enzyme was purified and characterized.

2. MATERIALS AND METHODS

2.1 Bacterial strains

E. coli DH5α™ was used for cloning and *E. coli* BL21 Star™(DE3) was used as an expression host. The isolate of *Massilia* sp. was obtained in a nutrient agar plate inoculated with an aliquot of sterile 0.1% (w/v) peptone water that was incubated with maize grains presenting rotten symptoms [36]. The grains were collected from the soil as harvest leftovers, during August 2015, at the Irrigation Technical Center (CTI), at the main campus of the Universidade Estadual de Maringá, Paraná, Brazil. In order to search for amylase producers, the colonies were transferred with a sterile toothpick to nutrient agar containing starch (5g/L meat peptone; 3 g/L yeast extract; 2g/L starch; 15 g/L agar). After culture for three days at 37 °C, the dishes were stained with iodine reagent (2% (w/v) KI; 0.2% (w/v) I_2) and a hydrolytic halo formation was considered positive for amylase production [17]. Classical biochemical tests were done to characterize the *Massilia* sp. isolate.

2.2 Genomic DNA extraction and molecular identification of the isolate

In order to get genomic DNA, a bacterial colony obtained from a fresh five-day-old culture in nutrient agar, grown at 30 °C, was transferred to 5 mL of liquid nutrient medium containing starch and grown for 24 h at 30 °C (100rpm). The cells from 1.5 mL of this culture were harvested by centrifugation (12,000 × *g*, 1min) and washed twice with 500 μL of TE buffer (10 mM Tris; 1mM Na_2EDTA; pH 8.0). The last pellet was suspended in 200 μL of TE buffer before being boiled for 10 min and centrifuged for 1 min (10,000 × *g*). The obtained supernatant was the source of DNA and was stored at −20 °C.

In order to better classify at the species level, the entire 16S rRNA gene of the *Massilia* sp. isolate was PCR amplified from the bacterial genomic DNA with the primers

27F and 13B [23, 35]. The amplified DNA fragment was treated with ExoSAP-IT™ PCR Product Cleanup Reagent (Thermo Fisher Scientific, USA), using the manufacturer's protocol, and sequenced at the CEGH (Centro de Estudos do Genoma Humano) at the University of São Paulo, Brazil, with the same primers and with the internal primers 534R and 91E [23, 35]. A contig was generated in the BioEdit program and the 1381 bp sequence was deposited in GenBank with the accession number MF800962. Comparison of the obtained sequence with sequences available in data banks identified the isolate as *Massilia timonae*.

2.3 PCR amplification, cloning, and subcloning of the cyclodextrinase gene in the expression vector

Five amylolytic enzymes were found in the *M. timonae* CCUG 45783 sequenced and annotated genome (GenBank AN: EKU82996.1, EKU83004.1, EKU82989.1, EKU82293.1, EKU83291.1). Primer pairs were designed for the five genes and if a signal peptide was present in the protein, primers were designed to amplify a partial gene coding for the mature form of the protein, i.e., without the signal peptide. When these primers were used in PCR reactions, only one pair was able to amplify a DNA fragment, indicating some variations between our isolate and the sequenced one. The amplified DNA corresponded to a putative cyclodextrinase gene (GenBank EKU82989.1) coding for the hypothetical protein HMPREF9710_01718.

The forward primer (5′-TTCATATGCAGACCTCCGACATCGCCCG) had a restriction site for the enzyme *Nde*I (underlined) that provided the first ATG to the partial gene. The reverse primer (5′-TTAAGCTTTCGCGGCGCGTCCAGCC) had a restriction site for the enzyme *Hind*III (underlined) and had the stop codon deleted. The PCR reaction was performed with 1 U of Platinum® Taq DNA Polymerase High Fidelity (Thermo Fisher Scientific, USA), 1X enzyme buffer, 2 mM $MgSO_4$, 0.2 mM of each dNTP, 25 pmol of each

primer, and 2 μL of the DNA obtained supernatant, in a total volume of 25 μL. The PCR consisted of an initial incubation of 5 min at 94 ºC and of 25 cycles of 1minat 94 °C, 1 min at 60 °C, and 2 min at 68 °C. After that, the sample was incubated at 72 °C for 10 min for complete extension of the fragments.

The amplified DNA fragment was cloned into the pCR2.1® plasmid from the TA Cloning kit (Thermo Fisher Scientific, USA), via the vendor's instructions. The insert of the obtained recombinant plasmid was sequenced at the CEGH at the University of São Paulo, using internal primers targeting the cyclodextrinase gene from *M. timonae* CCUG 45783 and primers targeting to the plasmid regions flanking the insert. The contig was generated in the BioEdit program and the 1797 bp fragment sequence was deposited in GenBank with the accession number MG386881.

To express a C-terminally His-tagged cyclodextrinase, the insert was subcloned from the TA vector to the pET21a(+) plasmid by restriction/ligation, using *Nde*I and *Hind*III restriction enzymes.

2.4 Bioinformatics analysis

BLASTp was used to search for proteins in data banks. Protein sequences were aligned using CLUSTAL Omega. Phyre2 was used to predict the protein structure. PyMol was used to analyze the enzyme structure model and active site. SignalP 4.0 server was used to search for secretion signal peptide. MEGA 7.0 was used to build a protein phylogenetic tree. CDD was used to search for conserved domains. Phobius and TMHMM were used to predict transmembrane segments. Phobius was also used for subcellular localization prediction. Translate was used to derive the amino acid sequence from the cloned gene. Chromas Lite 2.01 was used for viewing the sequencing files. Compute pI/Mw was used to calculate the molecular weight and pI value of the recombinant protein.

2.5 Expression optimization

The empty pET21a(+) vector (60 ng), as a control, and the recombinant vector pET21a(+)-cyclodextrinase (60 ng) were used to transform *E. co*li BL21 Star™(DE3), as described by Chung et al. [8]. The transformed cells were inoculated in 10 mL of Luria-Bertani (LB) medium containing ampicillin (10 g/L tryptone; 5 g/L yeast extract; 10 g/L NaCl; 50 μg/mL ampicillin) and cultured overnight at 37 °C with agitation at 100 rpm. Of this culture, 300 μL was used to inoculate 30 mL, in Erlenmeyer of 125 mL, of LB-ampicillin medium or of slight modified ZYM-5052-ampicillin medium [1% (w/v) tryptone; 0.5% (w/v) yeast extract; 0.5% (v/v) glycerol; 0.05% (w/v) glucose; 0.2% (w/v) lactose monohydrate; 25 mM Na_2HPO_4; 25 mM KH_2PO_4; 50 mM NH_4Cl; 5 mM Na_2SO_4; 2 mM $MgSO_4.7H_2O$; 10 μM $MnSO_4.H_2O$; 2 μM $CuSO_4.5H_2O$; 10 μM $ZnSO_4.7H_2O$; 25 mM Sodium succinate dibasic hexahydrate; 20 μM $CaCl_2.2H_2O$; 50 μM $FeCl_3$; 50 μg/mL ampicillin] [37]. After incubation for 4 h at 37 °C under agitation (100 rpm), when an optical density of 0.6–0.8 at 620 nm was achieved, isopropyl β-D-1-thiogalactopyranoside (IPTG) was added in the concentrations of 0.5 mM and 1 mM in both media, to induce protein expression, and the flasks were incubated at 20 °C. Each treatment was prepared in triplicate. Three flasks of the ZYM-5052 medium were left without IPTG. Aliquots (1 mL) were taken at 0, 2, 4, and 18h and were centrifuged at 9000 × *g* for 2 min.

The obtained cell pellets were suspended in 400 μL of a lysis buffer (50 mM Tris, 50 mM NaCl, pH 7.5) containing 1 mM phenylmethylsulfonyl fluoride (PMSF) and the cells were disrupted by sonication in a Fisher Scientific (USA) sonicator using 32 cycles of 15 s (5 s on and 10 s off), with an amplitude of 40%. The sonicated samples were centrifuged at 12000 × *g* for 2 min and the pellet was separated from the supernatant. The supernatants were used in the enzyme assay to assess enzyme expression. The results were expressed as average and standard deviation of the triplicate. Averages and standard deviations of the 18 h time

point were submitted to ANOVA and compared using the Tukey test ($\alpha = 0.01$), in the SASM–Agri program [6].

The soluble (supernatant) and insoluble (pellets) proteins in the samples of the LB and ZYM-5052 media cultures induced with 1.0 mM IPTG and in the ZYM-5052 medium without addition of IPTG were analyzed by SDS-PAGE.

2.6 Enzyme assay

The cyclodextrinase activity was determined by measuring the released reducing sugars, using the dinitrosalicylic acid (DNS) method [28]. A sample of 500 μL was added to 1.5 mL of 0.5% (w/v) β-cyclodextrin in 50 mM Tris buffer, pH 7.5, and the reaction was incubated for 30 min at 40 °C. The reaction was terminated with addition of 0.5 mL of the DNS solution [1% (w/v) DNS; 0.4 M NaOH; 1 M potassium sodium tartrate]. After boiling for 5 min and the addition of 2.5 mL of deionized water, the absorbance was estimated in a spectrophotometer at 540 nm. The obtained results with different concentrations of glucose (0.5–4 μmol) were used to draw a calibration curve. One unit of enzyme activity was defined as the amount of enzyme that releases 1 μmol of reducing sugar per minute per mL under the assay conditions.

2.7 SDS-PAGE

An aliquot of the purified protein (15 μg) was added to the same volume of sample dilution buffer [20% (v/v) glycerol, 1% (w/v) SDS, 0.03 mg/mL bromophenol blue, 125 mM Tris, pH 6.8, 0.72 M β-mercaptoethanol] and loaded on a discontinuous denaturing 7.5% polyacrylamide gel (SDS-PAGE), pH 8.9, with a 4.5% stacking polyacrylamide gel, pH 6.8, and was run in the Tris-glycine buffer system [22]. The proteins (20–40 μg) from the pellets and supernatant obtained after centrifugation of the cell homogenate were treated in the same

way. Unless otherwise stated, all samples were boiled for 10 min before being applied in the gel. Broad range molecular markers (Thermo Fisher Scientific, USA, or BioRad, USA) were used to determine the proteins approximate size. Protein concentration was determined using the Bradford method using albumin as a standard [3].

2.8 Expression and purification of the recombinant cyclodextrinase

Sixty ng of the recombinant vector pET21a(+)-cyclodextrinase were used to transform E. coli BL21 Star™(DE3), as described by Chung et al. [8]. The transformed cells were inoculated in 10 mL of LB medium containing 50 μg/mL ampicillin and grown overnight at 37 °C with agitation at 100 rpm. Of this culture, 500 μL were used to inoculate 50 mL of ZYM-5052-ampicillin medium. After incubation for 4 h, at 37 °C, under agitation (100 rpm), although unnecessary, IPTG was added to a final concentration of 1.0 mM and the culture was incubated for 18 h at 20 °C with agitation of 100rpm. After this incubation, the cells were harvested by centrifugation (2000 × *g*, 10 min, 4 °C) and were suspended in 10 mL of the lysis buffer containing 1 mM PMSF (described above).

For purification of the expressed protein, the cells were disrupted by sonication as described above. The sonicated sample was centrifuged (10,000 × *g*, 5 min, 4 °C) and the supernatant was applied on a HisTrap™ HP column (GE Healthcare Life Sciences, USA) charged with Ni^{2+} and equilibrated with a buffer containing 50 mM Tris, pH 7.5, 50 mM NaCl, and 20 mM imidazole. Afterward, the column was washed with the same equilibrium buffer and the recombinant protein was eluted with the elution buffer (50 mM Tris, pH 7.5, 50 mM NaCl, 450 mM imidazole). Protein elution was followed by absorbance at 280 nm. Fractions containing the enzyme were combined and dialyzed to remove the excess of imidazole against 50 mM Tris buffer, pH 7.5, containing 50 mM NaCl and 2 mM $CaCl_2$.

After dialysis, the enzyme was supplemented with glycerol to a final concentration of 50% (v/v) and kept frozen at −20 °C until use.

2.9 Determination of the purified protein molecular weight

Gel filtration chromatography was performed on a Sepharose 6B (Sigma-Aldrich, USA) column (83 cm×1.6 cm) to determine the protein molecular weight in its oligomeric form. The column was packaged and equilibrated with 50 mM Tris, pH 7.5, containing 100 mM KCl. A flow rate of 0.8 mL/min was used and fractions of 2.0 mL were collected. The void volume (V_o) was determined by loading 2.0 mL of a Blue Dextran solution (2 mg/mL) prepared in the equilibration buffer containing 5% (v/v) glycerol, and following its elution by absorbance reading at 280 nm. The used molecular weight standards were: bovine thyroglobulin (669 kDa), horse spleen apoferritin (443 kDa), yeast alcohol dehydrogenase (150 kDa), and bovine serum albumin (66 kDa) (Sigma-Aldrich, USA). Albumin and apoferritin were prepared at 10 mg/mL. Thyroglobulin and alcohol dehydrogenase were prepared at 8 mg/mL and 5 mg/mL, respectively. The standards were prepared freshly with the equilibration buffer and were supplemented with 5% (v/v) glycerol. Two mL of each standard solution were individually loaded in the column and the elution volume (V_e) was determined by absorbance reading at 280 nm. Finally, 2 mL (0.58 mg) of the purified cyclodextrinase in the dialysis buffer was supplemented with 5% (v/v) glycerol and loaded in the column. The elution profile was also recorded by the absorbance reading at 280nm.

A 7.5% SDS-PAGE was used to determine the monomeric protein molecular weight. The same procedure was used to determine the native protein molecular weight, where the sample was not boiled and β-mercaptoethanol was not added.

2.10 Enzyme characterization

To find the effect of putative inhibitors and activators on the purified enzyme, the sample was first dialyzed in 50 mM Tris buffer, pH 7.5, to remove NaCl, $CaCl_2$, and glycerol, which were used in the purification and storage processes. The effect of metal ions and detergents in the enzyme activity was evaluated by adding them at 5 or 10 mM (for solids) and 0.5 or 1% (v/v) (for liquids) in the enzyme assay. The relative activity was defined as the percentage of activity related to the activity at standard conditions.

The optimum temperature was determined by examining the cyclodextrinase activity against β-cyclodextrin in 50 mM Tris buffer, pH 7.5, with temperatures ranging from 25 °C to 55°C, in increments of 5°C. To discover the thermal stability, the purified enzyme was diluted in 50 mM Tris buffer, pH 7.5, containing 200 μM or 2 mM $CaCl_2 \cdot 2H_2O$, and incubated for 30minat 25, 30, 35, 40, 45, 50, and 55 °C, and then the β-cyclodextrin enzyme assay was accomplished at 40 °C, in 50 mM Tris buffer, pH 7.5. The temperature at which the enzyme lost 50% of its activity (T_{50}) was calculated from the obtained second order polynomial regression curve equation. A 24-h thermal stability time course was performed at 0, 25, and 40 °C in 50 mM Tris buffer, pH 7.5, containing 2mM $CaCl_2 \cdot 2H_2O$. The cyclodextrinase activity pH influence was determined using the substrate prepared in 50 mM Tris buffer adjusted to have its pH varying from 6.0 to 9.0, in an increment of 0.5. A 24-h pH stability time course was performed at pHs 6.5, 7.0, and 8.0, at 25 °C, in the presence of 2 mM $CaCl_2 \cdot 2H_2O$.

The initial velocities of the enzymatic reactions were examined by varying the β-cyclodextrin concentration from 0.88 to 8.8 mM, in 50 mM Tris buffer, pH 7.0, at 40 °C. A Lineweaver-Burk plot of initial velocity against β-cyclodextrin concentration was drawn and the kinetic constants K_M and V_{max} were then determined. k_{cat} was calculated using the

obtained V_{max} divided by the used enzyme concentration and the obtained value was used to calculate k_{cat}/K_M.

To evaluate the substrate specificity of the purified cyclodextrinase, the enzyme reaction was performed with 0.5% (w/v) β-cyclodextrin and also with 0.5% (w/v) starch or 0.5% (w/v) maltodextrin (dextrose equivalent 4.0–7.0, Aldrich 419672, USA).

2.11 Analysis of the enzymatic products

Two hundred and fifty μL (0.12 U) of the enzyme were incubated with 750 μL of 1.0% (w/v) β-cyclodextrin or 750 μL of 0.1M maltose, maltotetraose, and maltoheptaose, in 50 mM Tris buffer, pH 7.5, for 16 h at 40 °C. The obtained hydrolysis solutions (50 μL) were spotted on a Whatman paper chromatogram n°. 1, along with 20 μL of the 0.1 M standards: glucose, maltose, maltotetraose, and maltoheptaose. The used solvent system in the descending chromatography was butanol/pyridine/acetic acid/water in a 12:6:1:4 proportion. The chromatogram was developed with the ammoniacal silver nitrate method [40]. The chromatogram was sprayed with a saturated solution of $AgNO_3$ in acetone (1.5 g of $AgNO_3$ in 0.5 mL of distilled water added to 12 mL of 60% (v/v) acetone) followed by a spray with NaOH in ethanol (0.5 mL of 10 M NaOH added to 12 mL de ethanol). After drying, the chromatogram was exposed to water vapor for 5 min and was dipped in a 10% (w/v) sodium thiosulfate bath. The chromatogram was then dried and photographed.

3. RESULTS

3.1 *Massilia timonae* isolate characterization

The isolate was found to be a bacilliform Gram-negative bacterium that could not use glucose, sucrose, lactose, mannitol, and raffinose as a carbon source and did not produce any gas or H_2S. It could not grow on Simon's citrate medium and could not use urea as a nitrogen source. In addition, the strain had a faster growth rate at 30 °C. Fig. 1 shows a plate containing nutrient agar-starch with *M. timonae* CTI–57, which was revealed with iodine solution.

3.2 Sequence analysis of the recombinant cyclodextrinase

The cloned 1797 bp length cyclodextrinase partial gene, in which a 57 bp sequence coding for a 19 amino acids *N*-terminal signal peptide was missing, codes for a mature protein of 598 amino acids residues. The expressed protein had an extra 13 amino acids sequence at *C*-terminus given by the plasmid, which includes a 6-His tag. The predicted molecular mass of the expressed protein was 69.4 kDa and the putative isoelectric point was 6.04. The obtained protein sequence was 96% identical to the *M. timonae* cyclodextrinase HMPREF9710_01718 and 67.7% identical to the cyclodextrinase from *Flavobacterium* sp. (Fig. 2), a well-described enzyme [4, 12]. Two aspartic acids and one glutamic acid residues (D310, E339, D417) in the active site are well conserved in the alpha-amylase domain of the expressed protein (Fig. 2).

In silico analysis revealed that the protein derived from the cloned partial gene has an *N*-terminal (R7-E93) cyclodextrinase domain (PF09087) PF09087) with an E value of $8.7\times e^{-28}$, followed by a central (G144-S458) alpha-amylase domain (PF00128; CL0058) from the hydrolase family 13 (GH13), with an E value of $4.8\times e^{-51}$, and a *C*-terminal (M518-E593)

cyclodextrinase domain (PF10438; CL0369), with an E value of $8.8\times e^{-25}$ (Fig. 2). Additionally, the Domain B, characteristically present in cyclodextrinases [12, 42] and involved in substrate binding, is also present in the studied enzyme (Figs. 2 and 3A). The four calcium binding sites of the *Flavobacterium* sp. enzyme aligned well with the *M. timonae* enzyme and may play a role in calcium binding in this enzyme as well (Fig. 2).

The structural model of *M. timonae* cyclodextrinase build by the comparison with the *Flavobacterium* sp. cyclodextrinase structure (PDB 3EDE) [4] is shown in Fig. 3A and details of the active site are shown in Fig. 3B.

Based on the similarities of amino acid sequences, a phylogenetic tree was constructed (Fig. 4) to find the relationship between the *M. timonae* CTI–57 cyclodextrinase with other bacterial cyclodextrinases retrieved from GenBank.

3.3 Expression optimization and purification of the cyclodextrinase

Although the enzyme production was higher in both LB and ZYM-5052 media supplemented with IPTG in the first hours of culture, the highest cyclodextrinase expression, statistically significant ($\alpha = 0.01$), was observed after 18 h of culture in IPTG-free ZYM-5052 medium. However, the expression in this medium, in this time-point, was also statistically similar to the expression obtained by the culture in ZYM-5052 medium supplemented with 0.5 mM and 1.0 mM IPTG and in LB medium induced with 0.5 mM IPTG (Fig. 5A). The soluble (supernatant) and insoluble (pellet) proteins obtained after cell lysis were analyzed by SDS-PAGE (Fig. 5B). None of the analyzed culture conditions was able to decrease the insoluble cyclodextrinase level.

The obtained yield after dialysis of the Ni-column purified protein, from 50 mL culture in the ZYM-5052 medium supplemented with 1.0 mM of IPTG, was 1.58 mg of protein, corresponding to 26.8 U of the enzyme with a specific activity of 17 U/mg of protein.

Considering the calcium fully activated protein, the yield was 134.8 U, with a specific activity of 85 U/mg of protein.

3.4 Molecular mass determination

Fig. 6A shows the gel filtration chromatography results. The calculated enzyme molecular weight, using the obtained line regression equation, is 260 kDa. The SDS-PAGE analysis results in complete and partial denaturing conditions are shown in Fig. 6B. In complete denaturing conditions the purified protein runs as a monomer of 65 kDa and in partial denaturing conditions it runs as a tetramer of 260 kDa.

3.5 Effects of various reagents on enzyme activity

The addition of Ca^{2+} at 5 and 10 mM activated the *M. timonae* cyclodextrinase, increasing its activity by more than 200% (Table 1). The cations Fe^{3+}, Cu^{2+}, Zn^{2+}, Mg^{2+}, and Mn^{2+} were inhibitors of the enzyme. NaCl and KCl addition to the enzyme reaction slightly inhibited the enzyme. Regarding detergents, SDS and CTAB were strong inhibitors in both the concentrations, but Tween 80, at 0.5% and 1.0% (v/v), was not able to fully inhibit the enzyme.

3.6 Effect of temperature and pH on the cyclodextrinase activity

The highest catalytic activity for the β-cyclodextrin hydrolysis could be achieved at 40 °C (Fig. 7A). The cyclodextrinase was able to remain relatively stable after 30 min at 30 °C and 40 °C, but lost all its activity when incubated for 30 min at 50 °C (Fig. 7B). Addition of 2 mM calcium did not improve the enzyme thermal stability but did the enzyme activity and performance at lower temperatures (Fig. 7B). The temperature at which the enzyme lost 50% of its activity (T_{50}) with 2 mM calcium was 48.45 °C (Fig. 7B). The enzyme remained 60%

active after 24 h of incubation at 25 or 40 °C but was only 20% active at 0 °C (Fig. 7C). The optimum pH for the cyclodextrinase assayed with 50 mM Tris bufferwas 7.0 (Fig. 7D). The enzyme remained 100% active when incubated at pHs 6.5 and 8.0 after 24 h of incubation, but was only 60% active when incubated at pH 7.0 (Fig. 7E).

The highest catalytic activity for the hydrolysis of β-cyclodextrin could be achieved at 40 °C (Figure 7A). The cyclodextrinase was able to remain relatively stable after 30 min at 30 °C and 40 °C, but lost all its activity when incubated for 30 min at 50 °C (Figure 7B). The addition of 2 mM calcium did not improve the enzyme thermal stability but did the enzyme activity and performance at lower temperatures (Figure 7B). The temperature at which the enzyme lost 50% of its activity (T_{50}) in the presence of 2 mM calcium was 48.45 °C (Figure 7B). The enzyme remained 60% active after 24 h of incubation at 25 or 40 °C but was only 20% active at 0 °C (Figure 7C). The optimal pH for the cyclodextrinase assayed with 50 mM Tris buffer was 7.0 (Figure 7D). The enzyme remained 100% active when incubated at pHs 6.5 and 8.0 after 24 h of incubation, but was only 60% active when incubated at pH 7.0 (Figure 7E).

3.7 Kinetic parameters and substrate specificity

The Lineweaver-Burk plot of the recombinant cyclodextrinase with the different concentrations of β-cyclodextrin is shown in Fig. 7F. K_M for β-cyclodextrin was 2.1 mM, V_{max} was 0.084 μmol/min, k_{cat} was 8326 min^{-1}, and k_{cat}/K_M was 4.1×10^6 $M^{-1}min^{-1}$.

The purified enzyme presented the highest specificity for β-cyclodextrin (relative activity 100%) followed by maltodextrin (relative activity 62%), and by starch (relative activity 8%).

3.8 Mode of action of the recombinant cyclodextrinase

The recombinant cyclodextrinase action on β-cyclodextrin, as well as on malto-oligosaccharides like maltotetraose and maltoheptaose, is shown in Fig. 8. The reactions produced predominantly glucose and maltose.

DISCUSSION

Considering the sequence similarity of the studied protein with other cyclodextrinases, it can be inferred that the expressed enzyme is a member of the glycoside hydrolase family 13 (GH13) (EC 3.2.1.54). The virtual structure of the protein derived from the cloned partial gene has a distinct N-domain as well as a central and much larger $(\beta/\alpha)_8$-barrel domain, characteristic of alpha-amylases, and another small C-domain. Unlike the central $(\beta/\alpha)_8$-barrel domain, the *N*-domain and *C*-domain are composed exclusively of β-strands. The extra *N*-terminal domain presence contrasts with most GH13 family members but agrees with other cyclodextrinases [12, 24]. This domain is related to dimer and other oligomers formation in other cyclodextrinases, where the *N*-terminal domain of one subunit contacts the active site of the other subunit, constricting it and affecting the ligation of large molecules, such as starch [24]. The C-terminal domain is involved with protein solubility and supramolecular structures formation [24]. These *N*- and *C*-terminal domains may be involved with the tetrameric structure of the purified protein.

When the purified protein sequence was matched with other sequences in data banks, several cyclodextrinase sequences with more than 50% of identity were retrieved, which were used to build a phylogenetic tree. The retrieved sequences were from the phyla Proteobacteria (Classis: Betaproteobacteria and Gammaproteobacteria), Bacteroidetes (Classis:

Flavobacteriia, Cytophagia, Bacteroidia, and Chitinophagia), and Balneolaeota (Classis: Balneolia). These three phyla belong to Gram-negative bacteria and are evolutionarily related. The outgroup sequence from *Bacillus* sp. [20] belongs to the phylum Firmicutes, a more phylogenetically distant phylum.

In the protein expression optimization analysis, the cyclodextrinase expression was higher in LB medium induced with 0.5 mM IPTG in the first hours of induction, but was higher in ZYM-5052-IPTG free medium after 18 h of expression (Fig. 5A). One explanation for these different expressions is that non expressing cells grow better than expressing cells, and hence express more enzymatic activity per mL after induction. In addition, ZYM-5052 has lactose that can be used by cells as a carbon source and this could explain why, even in IPTG presence, it allows a stronger expression slope than LB. Besides, the auto-induction ZYM-5052 medium can vigorously enhance *E. coli* growth as it has several ions which improve the bacterial metabolism and increase enzyme expression. Another difference between LB and ZYM-5052 is that the latter is buffered, which better maintains the culture pH around 7.0. The induction in the ZYM-5052 medium occurs when glucose is depleted and lactose starts being metabolized generating allolactose, which activates T7 regulated gene expression because it deactivates the lac repressor. Nevertheless, the lactose is slowly metabolized what ends further induction. The different level of cyclodextrinase expression from *Anoxybacillus xavithermus* in *E. coli* with different concentrations of IPTG was also observed by Turner et al. [41]. Regarding protein solubility, at 20 °C, both soluble and insoluble proteins accumulate in the same proportion in both media at 2, 4, and 18 h after induction (Fig. 5B).

Although the highest obtained production in the optimization experiment occurred in ZYM-5052 medium without addition of IPTG, the enzyme production for the purification procedure was performed in ZYM-5052 medium induced with 1.0 mM IPTG, at 20 °C, for 18

h. The purified enzyme yield was 26.8 U and 1.28 mg of protein per 50 mL of culture, with a specific activity of 17 U/mg of protein. Fritzsche et al. [12] have obtained a yield of 8 mg/L of culture in LB medium induced with 0.1 mM IPTG (equal to 0.4 mg/50 mL of medium) of the *Flavobacterium* sp. cyclodextrinase expressed in *E. coli* and purified in a cation-exchange column. Turner et al. [41] have obtained a specific activity of 3.5 U/mg and a total of 11 U of a Ni-column purified cyclodextrinase from *A. flavithermus* expressed in *E. coli*, which was obtained from 5 mL out of a 13 mL cell extract from a 2 L culture in LB medium induced with IPTG. Kim et al. [20] have obtained a yield of 10368 U per 5 L culture (corresponding to 103 U/50 mL of culture), with a specific activity of 1629 U/mg of protein, of the *Bacillus* sp. cyclodextrinase expressed in *E. coli* and purified with a series of chromatographic steps.

The gel filtration chromatography analysis indicated that the recombinant enzyme has a molecular mass of about 260 kDa (Fig. 6A). This result was confirmed by SDS-PAGE, where the purified enzyme migrated as a sole band of 65 kDa in total denaturing condition and as a 260 kDa protein band in partial denaturing condition, when the boiling step was not performed and β-mercaptoethanol was omitted (Fig. 6B). Thus, it can be concluded that the studied cyclodextrinase is a tetramer. The molecular mass of other characterized Bacteria or Archaea cyclodextrinases is in the same size range, as the one from *Bacillus* sp. of 64.8 kDa and 75 kDa, and from *Thermococcus kodakarensis* of 76.4 kDa [20, 31, 38]. Likewise, the cyclodextrinase from *Flavobacterium* sp. also is a tetramer [12]. In addition, bacterial cyclodextrinases with more complex structures have also been described, as the protein from *Bacillus* sp. that is a dodecamer of equal subunits [25].

The gel filtration analysis was conducted using Sepharose 6B equilibrated with a buffer containing 100 mM KCl. Kim et al. [19] have demonstrated that the *Thermus* maltogenic amylase was predominantly a dimer in KCl absence and a monomer in 0.8–1.0 M KCL presence. Ferrer et al. [11] have incubated a metagenomic cyclodextrinase with 200 mM

NaCl or KCl before performing gel filtration and they observed no difference in the tetramer state and relative activity. Considering that the *M. timonae* cyclodextrinase tetramer was confirmed by SDS-PAGE, it is possible that the used 100 mM KCl in the column could not convert a higher size to a lower size enzyme polymer.

The *M. timonae* recombinant cyclodextrinase activity was inhibited by cations as Fe^{3+}, Cu^{2+}, Zn^{2+}, Mn^{2+}, and Mg^{2+}. The same results were obtained for the enzymes from *Paenibacillus* sp., *Bacillus* sp., and *Thermococcus* sp. [18, 20, 26]. This may indicates the ionization state importance of the negatively charged aspartate and glutamate residues present in the active site or of other negatively charged amino acids residues that are important for the protein structure or stabilization. In line with this, the anionic and cationic detergents SDS and CTAB, respectively, completely inhibited the enzyme activity but the neutral detergent Tween 80 showed only a mild decrease in the enzyme activity, even at a higher concentration. Another positive point for this hypothesis is that weak cations, such as Na^{+} and K^{+}, were not potent inhibitors of the enzyme.

Calcium was observed to be an effective activator of the studied recombinant cyclodextrinase, even though it is also a cation. The calcium role in cyclodextrinases structure maintenance and catalytic activity is well described and this involves the interaction of this ion with negatively charged amino acids residues [12, 29, 33]. However, the cyclodextrinases from *Paenibacillus* sp., *Bacillus* sp., and *Thermococcus* sp. were not or were only barely activated by calcium [18, 20, 26]. EDTA completely inhibited the *M. timonae* cyclodextrinase, which corroborates the hypothesis that a bivalent cation is required for the enzyme activity. In general, EDTA, which chelates stabilizing Ca^{2+} ions, inhibits amylases [16]. Quite the opposite, EDTA was an activator of the recombinant cyclodextrinase from *Bacillus* sp. [20] and had no effect on the enzyme from *Paenibacillus* sp. [18].

The optimum temperature for the greatest catalytic activity of the purified cyclodextrinase was 40 °C (Fig. 7A). Generally, bacterial cyclodextrinases have optimal activities at temperatures below 50 °C [33]. For instance, the cyclodextrinases from *Bacillus sphaericus* and *Bacillus* sp. have an optimum temperature of 40 °C and 50 °C, respectively, and the enzyme from *Paenibacillus* sp. has an optimum temperature of 40 °C [14, 18, 43]. Some exceptions are reported for cyclodextrinases from hyperthermophilic Archaea species, such the one from *T. kodakarensis*, with the optimum temperature at 65 °C and the one from *Thermococcus* sp. at 85 °C [26, 38].

Many factors can affect the thermal stability of amylases, such as preparation purity, type of substrate, calcium presence, and other stabilizers presence [32]. The *M. timonae* cyclodextrinase remained relatively stable in temperatures ranging from 30 °C to 40 °C, after 30min of incubation, with 200 μM $CaCl_2 \cdot 2H_2O$ (Fig. 7B). Calcium increase to 2 mM during the enzyme incubation at different temperatures did not improve its thermal stability but had a profound effect on its stability at lower temperatures. Actually, when the enzyme was frozen at −20 °C without calcium or glycerol it was completely denatured after thawing (data not shown). The thermal time course also showed the negative effect of low temperature on the enzyme stability (Fig. 7C). In contrast, Ca^{2+} ions have been reported to increase the *Bacillus* sp. cyclodextrinase thermal stability by 6.8-fold at 70 °C [20]. The Ca^{2+} ions salting out effect for different hydrophobic residues can make proteins to adopt a more compact structure, which explains its stabilizing effects [15]. A further explanation of the Ca^{2+} ions role is the active site cleft stabilizationby an ionic bridge formation between the two domains around it [5]. In the *Flavobacterium* sp. cyclodextrinase three-dimensional structure there are two calcium-binding sites, which are involved in conformational stabilization and substrate binding [12]. The same sites are conserved in the recombinant *M. timonae* cyclodextrinase (Fig. 2) and this observation affirms an important role of calcium in its stabilization.

An enzymatic activity is markedly affected by pH since the substrate binding to the enzyme catalytic site is often dependent on charge distribution. The greatest β-cyclodextrin hydrolysis activity of the purified cyclodextrinase was found at pH 7.0 (Fig. 7D). The same result was found for the *Paenibacillus* sp. cyclodextrinase [18]. The optimal reaction pH for the *T. kodakarensis* and *Bacillus* sp. recombinant cyclodextrinases was 7.5 and for the *Thermococcus* sp. recombinant cyclodextrinase was pH 5.0 [2, 26, 38]. The pH stability time course indicated the pH importance on the enzyme activity (Fig. 7E). The enzyme was more stable at pHs above or below pH 7.0 indicating that the enzyme structure depends on its residues ionization state.

The K_M for β-cyclodextrin and V_{max} found for the *M. timonae* purified enzyme was 2.1 mM and 0.084 μmol/min, respectively (Fig. 7F). In agreement with our data, a K_M for β-cyclodextrin of 2.47 mM has been reported for the recombinant enzyme from *Paenibacillus* sp. [18] and of 3.9 mM for the enzyme from *B. sphaericus* [14]. However, lower K_M values for other cyclodextrinases such as, 0.42 mM for β-cyclodextrin, was found for the *Bacillus stearothermophilus* enzyme [1]. The obtained values for the catalytic rate constant, k_{cat}, and catalytic efficiency constant, k_{cat}/K_M, were observed to be 8326 min^{-1} and 4.1×10^6 $M^{-1}min^{-1}$, respectively. Comparable values of k_{cat} and k_{cat}/K_M of 2044 min^{-1} and 8.28×10^5 $M^{-1}min^{-}1$ were observed for the *Paenibacillus* sp. recombinant cyclodextrinase [18].

The *M. timonae* recombinant cyclodextrinase hydrolyzed cyclodextrins faster than maltodextrin and starch. These results conform to the earlier studies on substrate specificity of the recombinant cyclodextrinases, which prefer cyclodextrins than malto-oligosaccharides or soluble starch, e.g., the enzymes from *T. kodakarensis*, *Thermococcus* sp., and *Enterococcus faecium* [26, 38, 42]. In the soluble cyclodextrinases dimer or tetramer forms, the active site is a narrow and deep groove and large substrates like starch are not accessible, while small and compact substrates as cyclodextrins and malto-oligosaccharides fit well [33]. The studied

enzyme substrate specificity indicates that it may form oligomers in solution, what was confirmed by gel filtration.

As shown in Fig. 8, the purified enzyme can hydrolyse not only β-cyclodextrin but also maltotetraose and maltoheptaose, releasing glucose and maltose as products, which is compatible with most cyclodextrinases reaction mode [33]. However, the enzyme could not use maltose as a substrate. The latter observation, together with data published by other authors [39], reinforces the suggestion that maltose is too small to bind at the active site. In line with this reasoning and together with the fact that starch is not the preferred substrate, it is possible to draw the conclusion that there is probably a substrate size limitation for the enzyme catalysis. The variable domain B present in cyclodextrinases participates in substrate binding and is considered to play a role in determining the enzyme specificity [12]. This domain is present in the *M. timonae* recombinant enzyme (Figs. 2 and 3) and may also be involved in the substrate specificity.

CONCLUSIONS

An *E. coli* expressed cyclodextrinase from *M. timonae* was purified and characterized. The recombinant enzyme preferred β-cyclodextrin as a substrate but also reacted with maltodextrin, starch, and malto-oligosaccharides. The purified enzyme was more active at 40 °C, pH 7.0, and was relatively stable at temperatures below 40 °C. The enzyme was calcium activated and was completely inhibited by several cations and detergents as SDS and CTAB, and by EDTA. Indeed, this study offers a method for expression and purification of a new enzymewith interesting characteristics for future industrial applications. In addition, this study

proposes an interesting approach to investigate and find novel enzymes from annotated genomes.

ACKNOWLEDGMENT

The authors are thankful to CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior), from the Education Ministry, for the student scholarship.

REFERENCES

[1] J.-I. Abe, O. Nakazono, S. Hizukuri, Characterization of cyclodextrinase from *Bacillus stearothermophilus* K-12481, J. Appl. Glycosci. 43 (2) (1996) 155–159.

[2] J.-H. Auh, H.Y. Chae, Y.-R. Kim, K.-H. Shim, S.-H. Yoo, K.-H. Park, Modification of rice starch by selective degradation of amylose using alkalophilic *Bacillus* cyclomaltodextrinase, J. Agric. Food Chem. 54 (2006) 2314–2319.

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

[4] S. Buedenbender, G.E. Schulz, Structural base for enzymatic cyclodextrin hydrolysis, J. Mol. Biol. 385 (2009) 606–617.

[5] G. Buisson, E. Duee, R. Haser, F. Payan, Three dimensional structure of porcine pancreatic α-amylase at 2.9-Å resolution. Role of calcium in structure and activity, EMBO J. 6 (13) (1987) 3909–3916.

[6] M.G. Canteri, R.A. Althaus, J.S. Virgens Filho, E.A. Giglioti, SASM-AGRI – system for analysis and mean separation in agricultural assays using Scott-Knott, Tukey and Duncan methods, Braz. J. Agrocomp. 1 (2001) 18–24.

[7] F. Cerrone, M. Del Mar Sánchez-Peinado, M. Rodríguez-Díaz, J. González-López, C. Pozo, PHAs production by strains belonging to *Massilia* genus from starch, Starch Staerke 63 (2011) 236–240.

[8] C.T. Chung, S.L. Niemela, R.H. Miller, One-step Preparation of competent *Escherichia coli*: Transformation and Storage of Bacterial Cells in the Same Solution. Proc. Natl. Acad. Sci. USA, 86)1989) 2172-2175.

[9] E.M.M. Del Valle, Cyclodextrins and their uses: a review, Process Biochem. 39 (9) (2004) 1033–1046.

[10] C. Fahlgren, G. Bratbak, R.A. Sandaa, R. Thyrhaug, U.L. Zweifel, Diversity of airborne bacteria in samples collected using different devices for aerosol collection, Aerobiologia 27 (2011) 107–120.

[11] M. Ferrer, A. Beloqui, O.V. Golyshina, F.J. Plou, A. Neef, T.N. Chernikova, L. Fernández-Arrojo, I. Ghazi, A. Ballesteros, K. Elborough, K.N. Timmis, P.N. Golyshin, Biochemical and structural features of a novel cyclodextrinase from cow rumen metagenome, Biotechnol. J. 2 (2007) 207–213.

[12] H.B. Fritzsche, T. Schwede, G.E. Schulz, Covalent and three-dimensional structure of the cyclodextrinase from *Flavobacterium* sp. no. 92, Eur. J. Biochem. 270 (2003) 2332–2341.

[13] V. Gallego, C. Sánchez-Porro, M.T. García, A. Ventosa, *Massilia aurea* sp. nov., isolated from drinking water, Int. J. Syst. Evol. Microbiol. 56 (2006) 2449–2453.

[14] N.M. Galvin, C.T. Kelly, W.M. Fogarty, Purification and properties of the cyclodextrinase of *Bacillus sphaericus* ATCC7055, Appl. Microbiol. Biotechnol. 42 (1994) 46–50.

[15] N. Goyal, J.K. Gupta, S.K. Soni, A novel raw starch digesting thermostable a-amylase from *Bacillus* sp. I-3 and its use in the direct hydrolysis of raw potato starch, Enzym. Microb. Technol. 37 (2005) 723–734.

[16] R. Gupta, P. Gigras, H. Mohapatra, V.K. Goswami, B. Chauhan, Microbial α-amylases: biotechnological perspective, Process Biochem. 38 (11) (2003) 1–18.

[17] L. Hankin, S.L. Anagnostakis, The use of solid media for detection of enzyme production by fungi. Mycologia 67 (1975) 597–607.

[18] J. Kaulpiboona, P. Pongsawasdib, Purification and characterization of cyclodextrinase from *Paenibacillus* sp. A11, Enzym. Microb. Technol. 36 (2005) 168–175.

[19] T.J. Kim, V.D. Nguyen, H.-S. Lee, M.-J. Kim, H.-Y. Cho, Y.-W. Kim, T.-W. Moon, C.S. Park, J.-W. Kim, B.-H. Oh, S.-B. Lee, B. Svensson, K.-H. Park, Modulation of the multisubstrate specificity of *Thermus* maltogenic amylase by truncation of the *N*-Terminal domain and by a salt-induced shift of the monomer/dimer equilibrium, Biochemistry 40 (2001) 14182–14190.

[20] T.-J. Kim, J.-H. Shin, J.-H. Oh, M.-J. Kim, S.-B. Lee, S. Ryu, K. Kwon, J.-W. Kim, E.-H. Choi, J.F. Robyt, K.-H. Park, Analysis of the gene encoding cyclomaltodextrinase from alkalophilic *Bacillus* sp. I-5 and characterization of enzymatic properties, Arch. Biochem. Biophys. 353 (2) (1998) 221–227.

[21] B. La Scola, R.J. Birtles, M.N. Mallet, D. Raoult, *Massilia timonae* gen. nov., sp. nov., Isolated from blood of an immunocompromised patient with cerebellar lesions, J. Clin. Microbiol. 36 (1998) 2847–2852.

[22] U.K. Laemmli, Cleavage of structural proteins during the assembly of the 21 head of bacteriophage T4, Nature 227 (1970) 680–685.

[23] D.J. Lane, 16S/23S rRNA sequencing, in: E. Stackebrandt, M. Goodfellow (Eds.), Nucleic Acid Techniques in Bacterial Systematics, John Wiley and Sons, New York, NY, 1991, pp. 115–175.

[24] H.S. Lee, M.S. Kim, H.S. Cho, J.I. Kim, T.J. Kim, J.H. Choi, Cyclomaltodextrinase, neopullulanase, and maltogenic amylase are nearly indistinguishable from each other, J. Biol. Chem. 277 (2002) 21891–21897.

[25] H.-S. Lee, J.-S. Kim, K.-H. Shim, Jung-Wan Kim, C.-S. Park, K.-H. Park, Quaternary structure and enzymatic properties of cyclomaltodextrinase from alkalophilic *Bacillus* sp. I-5, Biologia 60 (16) (2005) 73–77.

[26] J.-E. Lee, I.-H. Kim, J.-H. Jung, D.-H. Seo, S.-G. Kang, J.F. Holden, J. Cha, C.-S. Park, Molecular cloning and enzymatic characterization of cyclomaltodextrinase from hyperthermophilic archaeon *Thermococcus* sp. CL1, J. Microbiol. Biotechnol. 23 (8) (2013) 1060–1069.

[27] D. Lindquist, D. Murrill, W.P. Burran, G. Winans, M.J. Janda, Characteristics of *Massilia timonae* and *Massilia timonae*-like isolates from human patients, with an emended description of the species, J. Clin. Microbiol. 41 (2003) 192–196.

[28] G.L. Miller, Use of dinitrosalicylic acid reagent for determination of reducing sugar, Anal. Chem. 31 (3) (1959) 426–428.

[29] G. Muralikrishna, M. Nirmala, Cereal alpha-amylases—an overview, Carbohydr. Polym. 60 (2) (2005) 163–173.

[30] M.L. Nagy, A. Pérez, F. Garcia-Pichel, The prokaryotic diversity of biological soil crusts in the Sonoran desert (Organ Pipe Cactus National Monument, AZ), FEMS Microbiol. Ecol. 54 (2005) 233–245.

[31] R.M. Ong, K.M. Goh, N.M. Mahadi, O. Hassan, R.N.Z.R.A. Rahman, R. Md Illias, Cloning, extracellular expression and characterization of a predominant beta-CGTase from *Bacillus* sp. G1 in E. coli, J. Ind. Microbiol. Biotechnol. 35 (2008) 1705–1714.

[32] A. Pandey, P. Nigam, C.R. Soccol, V.T. Soccol, D. Singh, R. Mohan, Advances in microbial amylases, Biotechnol. Appl. Biochem. 31 (2) (2000) 135–152.

[33] K.-H. Park, T.-J. Kim, T.-K. Cheong, J.-W. Kim, B.-H. Oh, B. Svensson, Structure, specificity and function of cyclomaltodextrinase, a multispecific enzyme of the α-amylase family, Biochim. Biophys. Acta 1478 (2000) 165–185.

[34] Q. Qi, W. Zimmermann, Cyclodextrin glucanotransferase: from gene to applications, Appl. Microbiol. Biotechnol. 66 (2005) 475–485.

[35] D.A. Relman, Universal Bacterial 16S rDNA amplification and sequencing, in: D.H. Persing, T.F. Smith, F.C. Tenover, T.J. White (Eds.), Diagnostic Molecular Microbiology, Principles and Applications, ASM, Washington DC, 1993, pp. 489–495.

[36] F.C. Santos, F.F. Castro, T.M. Apolonio, L. Yoshida, D.B. Martim, D.J. Tessmann I.P. Barbosa-Tessmann, Screening of hydrolytic enzymes production by bacteria isolated from maize (*Zea Mays* L.) grains. (Manuscript in preparation).

[37] F.W. Studier, Protein production by auto-induction in high-density shaking cultures, Protein Expr. Purif. 41 (2005) 207–234.

[38] Y. Sun, X. Lv, Z. Li, J. Wang, B. Jia, J. Liu, Recombinant cyclodextrinase from *Thermococcus kodakarensis* KOD1: expression, purification, and enzymatic characterization, Archaea (2015) https://doi.org/10.1155/2015/397924, Article ID 397924, 8 pages, 2015.

[39] K. Tang, T. Utairungsee, P. Kanokratana, R. Sriprang, V. Champreda, L. Eurwilaichitr, S. Tanapongpipat, Characterization of a novel cyclomaltodextrinase expressed from environmental DNA isolated from Bor Khleung hot spring in Thailand, FEMS Microbiol. Lett. 260 (2006) 91–99.

[40] W.E. Trevelyan, D.P. Procter, J.S. Harrison, Detection of sugars on paper chromatograms, Nature 166 (1950) 444–445.

[41] P. Turner, O. Holst, E.N. Karlsson, Optimized expression of soluble cyclomaltodextrinase of thermophilic origin in *Escherichia coli* by using a soluble fusion-tag and by tuning of inducer concentration, Protein Expr. Purif. 39 (2005) 54–60.

[42] K. Unban, A. Kanpiengjai, S. Lumyong, T.-H. Nguyend, D. Haltrichd, C. Khanongnuch, Molecular structure of cyclomaltodextrinase derived from amylolytic lactic acid bacterium *Enterococcus faecium* K-1 and properties of recombinant enzymes expressed in Escherichia coli and *Lactobacillus plantarum*, Int. J. Biol. Macromol. 107 (2018) 898–905.

[43] A. Yoshida, Y. Iwasaki, T. Akiba, K. Horikoshi, Purification and properties of cyclomaltodextrinase from alkalophilic *Bacillus* sp, J. Ferment. Bioeng. 71 (1991) 226–229.

Table 1. Effect of metal ions and detergents on the cyclodextrinase activity.

Substance	**Concentration**	**Relative activity (%)**	**Concentration**	**Relative activity (%)**
Control	5 mM	100.0	10 mM	100.0
$MnSO_4 \cdot H_2O$	5 mM	76.2	10 mM	0.0
$CuSO_4 \cdot 5H_2O$	5 mM	3.6	10 mM	13.2
$CaCl_2 \cdot 2H_2O$	5 mM	276.8	10 mM	264.6
$MgSO_4 \cdot 7H_2O$	5 mM	60.7	10 mM	0.0
KCl	5 mM	88.9	10 mM	78.0
$FeCl_3$	5 mM	13.9	10 mM	44.0
$ZnSO_4 \cdot 7H_2O$	5 mM	0.0	10 mM	0.0
NaCl	5 mM	91.5	10 mM	65.9
SDS	5 mM	0.0	10 mM	0.0
CTAB	5 mM	0.0	10 mM	0.0
EDTA	5 mM	0.0	10 mM	0.0
Tween 80	0.5% (v/v)	68.4	1.0% (v/v)	62.1

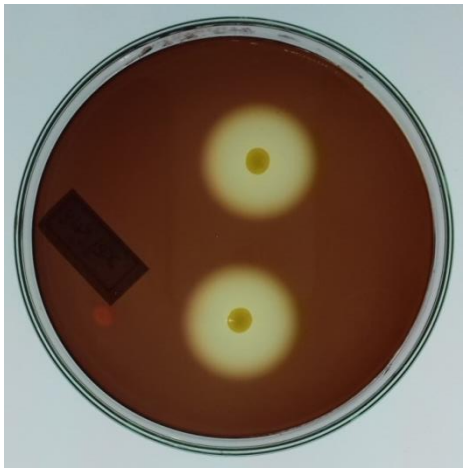

Figure 1. The analysis in a specific medium for amylase production by *M. timonae* CTI–57. The photo represents a dish with nutrient agar-starch with two *M. timonae* CTI–57 colonies. The dish was inoculated with a toothpick with an inoculum of a fresh culture in nutrient agar and then was incubated at 30 °C for five days. Finally, the dish was exposed to an iodine solution.

Figure 2. The sequence alignment of *M. timonae* CTI–57 cyclodextrinase with other cyclodextrinases. Clustal Omega alignment of the *M. timonae* CTI–57 cyclodextrinase (GenBank MG386881) sequence from this work with the cyclodextrinase HMPREF9710_01718 sequence from *M. timonae* CCUG 45783 (GenBank EKU82989.1), and with the cyclodextrinase sequence from *Flavobacterium* sp. (GenBank CAD32957.1). The active site residues are in bold and have a diamond on the top. The four segments of the calcium binding site in the *Flavobacterium* sp. cyclodextrinase sequence [12] are boxed and the domain B in the same sequence is underlined. The *N*-terminal and *C*-terminal domains, the alpha-amylase domain, and the domain B in the *M. timonae* CTI–57 cyclodextrinase sequences, are in magenta, yellow, blue, and green, respectively.

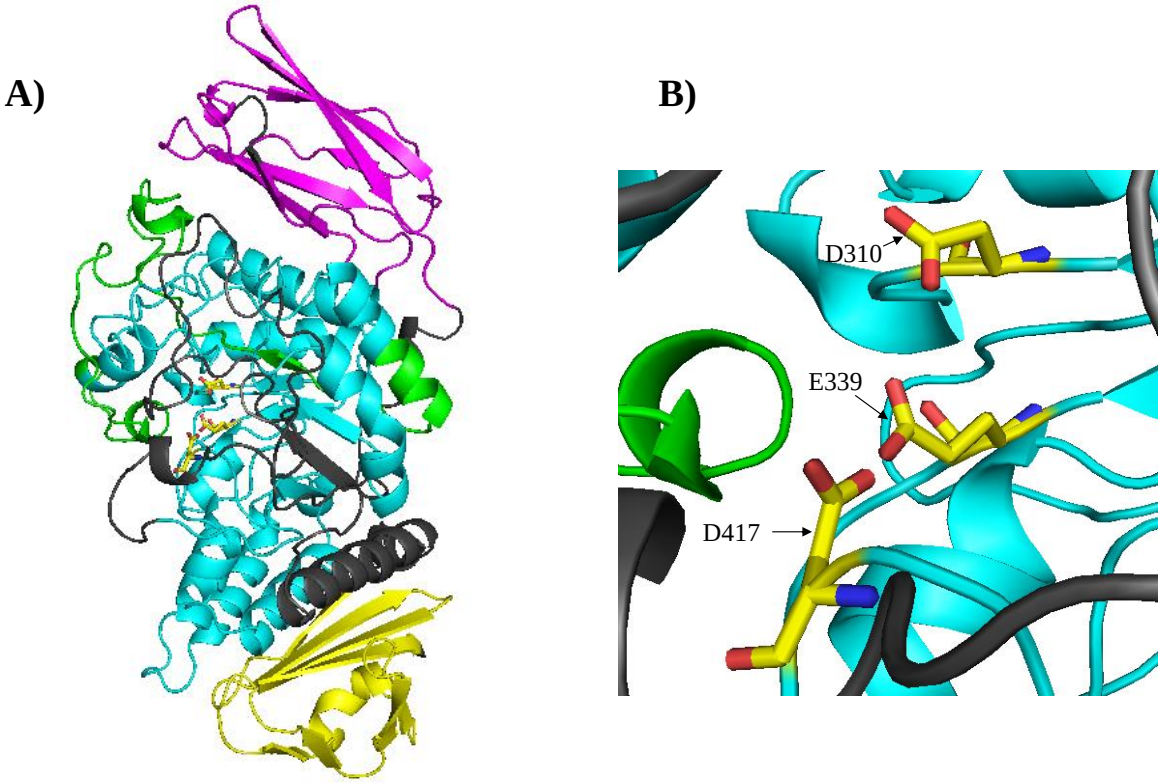

Figure 3. *M. timonae* CTI–57 cyclodextrinase structure. A) The structural model of the cyclodextrinase modelled in the Phyre2 platform by comparison with the *Flavobacterium* sp. protein structure (PDB 3EDE) [4]. The *N*-domain is shown in magenta, the *C*-domain in yellow, the alpha-amylase domain in blue, and the assumed domain B in green. B) Details of the predicted active site.

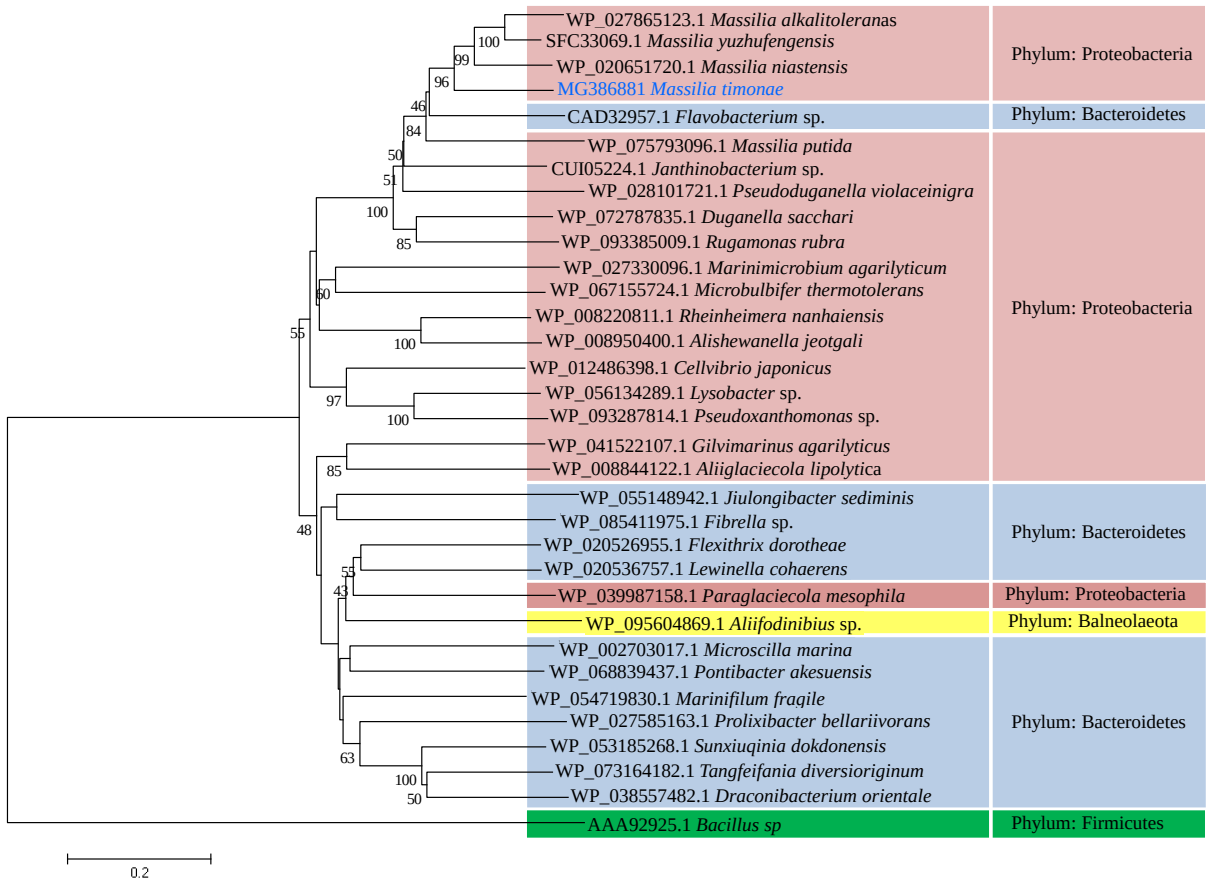

Figure 4. The phylogenetic tree of cyclodextrinases. The tree was inferred using the Neighbor-Joining method using MEGA 7.0. The percentage in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches but values lower than 30% were omitted. The distances were computed using the Poisson method and are in number of amino acids substitutions per site. Ambiguous positions were removed for each sequence pair. *M. timonae* CTI–57 cyclodextrinase sequence is in blue. All sequences have more than 50% of identity, except the outgroup sequence from *Bacillus* sp. [20].

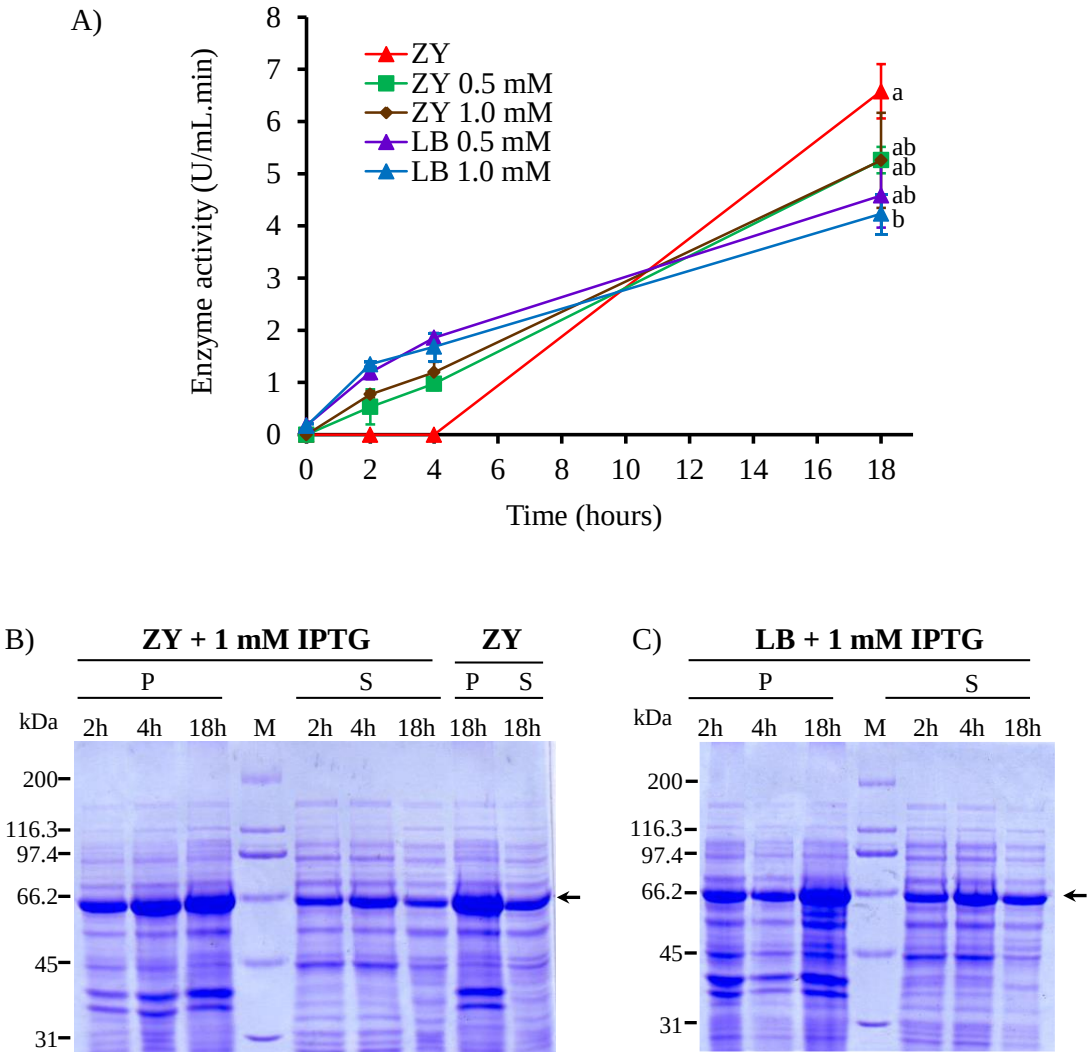

Figure 5. Expression analysis of the cyclodextrinase from pET21a(+) in *E. coli* BL21 Star™(DE3). A) The time-scale indicates the production activity analysis. Time zero is the induction time. The data represent the average and the standard deviation of the results obtained in three culture flasks. Averages at 18 h of culture followed by different letters are different (α = 0.01) in the Tukey test. B) A 7.5% SDS–PAGE containing the proteins of the cyclodextrinase expression analysis in ZYM-5052 medium, at 20 °C. C) A 7.5% SDS–PAGE containing the proteins of the cyclodextrinase expression analysis in LB medium, at 20 °C. P – Pellet. S – Supernatant. M− Broad Range SDS-PAGE molecular marker from BioRad (USA). The gels were stained with Coomassie Blue. The 65k Da expressed protein is indicated with an arrow.

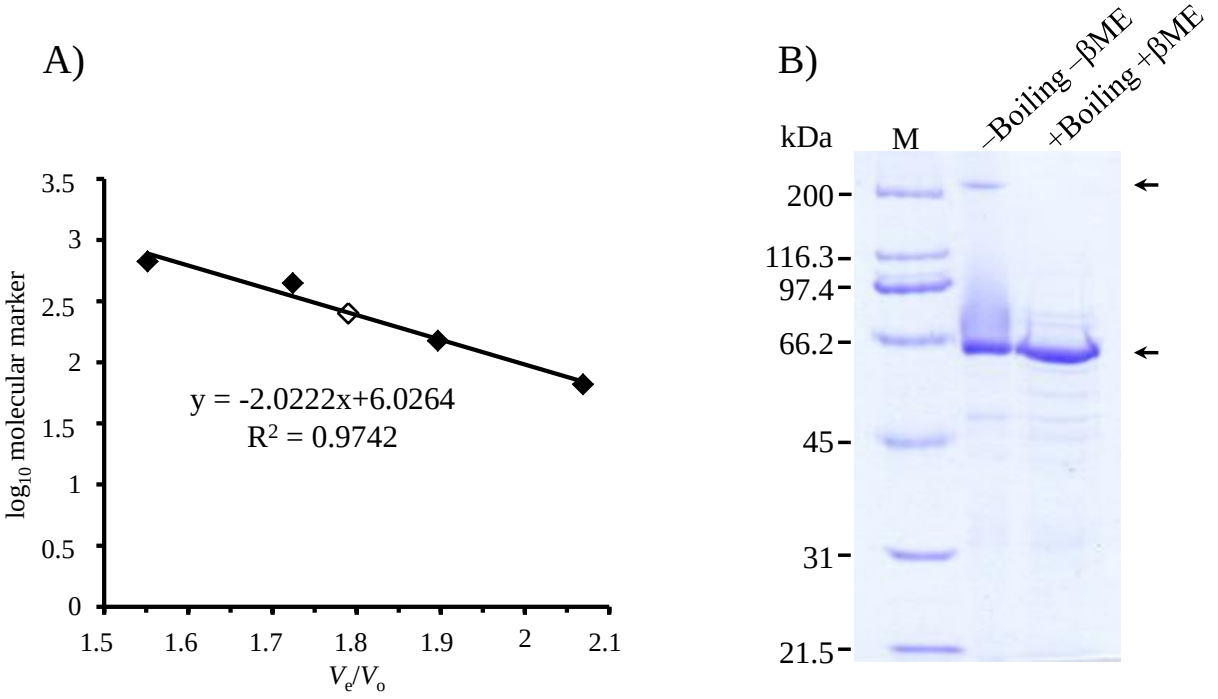

Figure 6. Molecular weight determination. A) Gel filtration chromatography analysis using the standards: bovine thyroglobulin (669 kDa), horse spleen apoferritin (443 kDa), yeast alcohol dehydrogenase (150 kDa), and bovine serum albumin (66 kDa). The black diamonds represent the standards and the white diamond represents the cyclodextrinase. V_e/V_o is the elution volume divided by the void volume. B) An SDS-PAGE gel showing the purified protein that was boiled or not in the presence or absence of β-mercaptoethanol, respectively, before loading on the gel. The arrows indicate the enzyme in its monomeric and oligomeric forms.

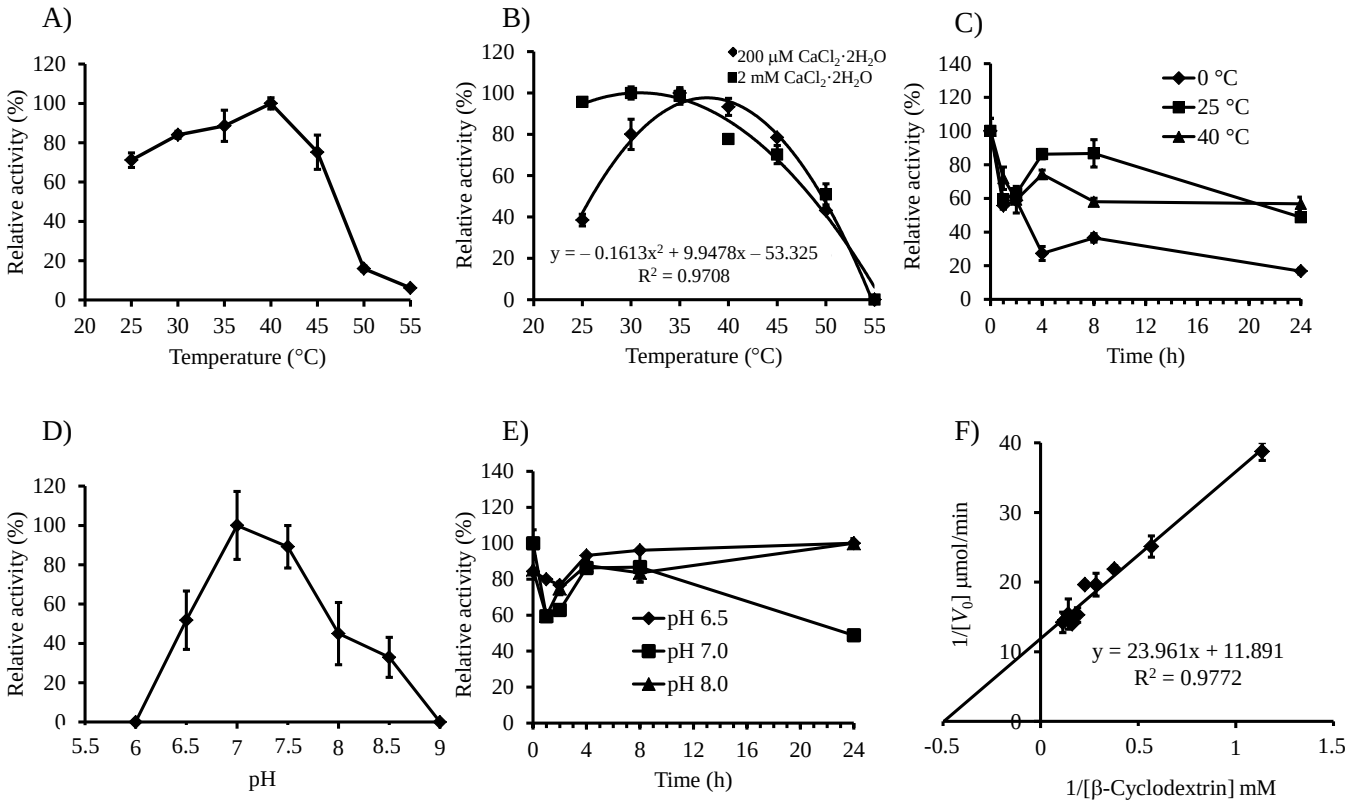

Figure 7. Biochemical characterization of the purified recombinant cyclodextrinase from *M. timonae*. A) Optimum temperature. B) Thermal stability. C) Thermal stability time course with 2 mM $CaCl_2 \cdot 2H_2O$. D) Optimum pH. E) pH stability time course with 2 mM $CaCl_2 \cdot 2H_2O$. F) Lineweaver-Burk plot. The data represent the average and the standard deviation of three experimental sets.

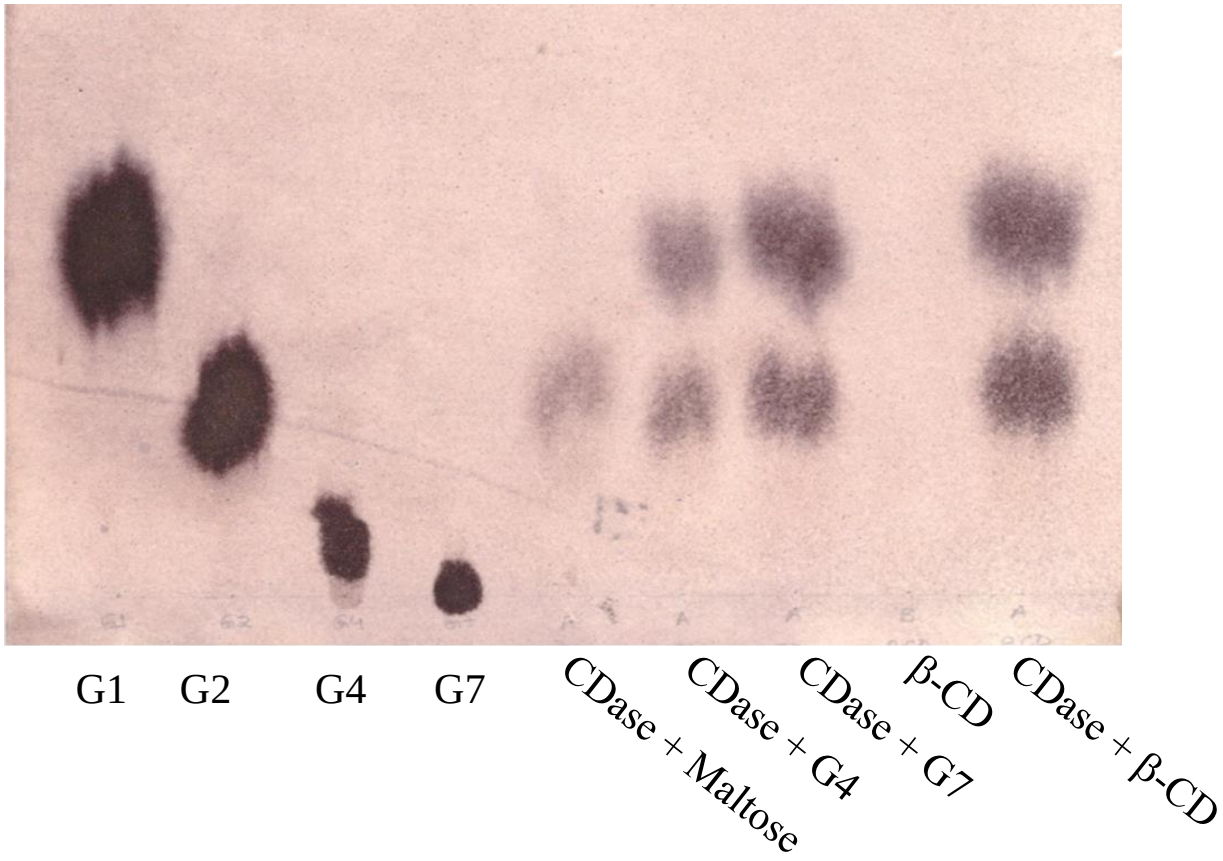

Figure 8. Mode of action for *M. timonae* recombinant cyclodextrinase. A descending chromatogram with the hydrolysis products from different substrates incubated with the recombinant cyclodextrinase at 40 °C, for 16 h. Standards: G1-glucose, G2 - maltose, G4 - maltotetraose, and G7-maltoheptaose. CDase - Cyclodextrinase. β-CD - β-cyclodextrin.