José Max Barbosa de Oliveira Junior
(Organizador)

Análise Crítica das Ciências Biológicas e da Natureza 3

Atena Editora
Ano 2019

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APRESENTAÇÃO

A obra *"Análise Crítica das Ciências Biológicas e da Natureza"* consiste de uma série de livros de publicação da Atena Editora. Com 96 capítulos apresenta uma visão holística e integrada da grande área das Ciências Biológicas e da Natureza, com produção de conhecimento que permeiam as mais distintas temáticas dessas grandes áreas.

Os 96 capítulos do livro trazem conhecimentos relevantes para toda comunidade acadêmico-científica e sociedade civil, auxiliando no entendimento do meio ambiente em geral (físico, biológico e antrópico), suprindo lacunas que possam hoje existir e contribuindo para que os profissionais tenham uma visão holística e possam atuar em diferentes regiões do Brasil e do mundo. As estudos que integram a *"Análise Crítica das Ciências Biológicas e da Natureza"* demonstram que tanto as Ciências Biológicas como da Natureza (principalmente química, física e biologia) e suas tecnologias são fundamentais para promoção do desenvolvimento de saberes, competências e habilidades para a investigação, observação, interpretação e divulgação/interação social no ensino de ciências (biológicas e da natureza) sob pilares do desenvolvimento social e da sustentabilidade, na perspectiva de saberes multi e interdisciplinares.

Em suma, convidamos todos os leitores a aproveitarem as relevantes informações que o livro traz, e que, o mesmo possa atuar como um veículo adequado para difundir e ampliar o conhecimento em Ciências Biológicas e da Natureza, com base nos resultados aqui dispostos.

Excelente leitura!

José Max Barbosa de Oliveira Junior

SUMÁRIO

CAPÍTULO 1 1

INIBIÇÃO DA PEÇONHA DE *Bothrops alternatus* (URUTU) 'IN VIVO' PELO PRINCIPIO ATIVO ISOLADO VEGETAL LUPEOL

Benedito Matheus dos Santos
Klaus Casaro Saturnino
Vanderlúcia Fonseca de Paula
Mirian Machado Mendes

DOI 10.22533/at.ed.5901927051

CAPÍTULO 2 7

INVESTIGAÇÃO DAS ATIVIDADES TÓXICA, ANTIDIARREICA E ANTIESPASMÓDICA DAS PARTES AÉREAS DE *SIDA RHOMBIFOLIA* L. (MALVACEAE)

Rafael Lima Marinho Paiva
Antônio Raphael Lima de Farias Cavalcanti
Rayane Fernandes Pessoa
Indyra Alencar Duarte Figueiredo
Sarah Rebeca Dantas Ferreira
Otemberg Souza Chaves
Micaelly da Silva Oliveira
Maria de Fátima Vanderlei de Souza
Fabiana de Andrade Cavalcante

DOI 10.22533/at.ed.5901927052

CAPÍTULO 3 22

INVESTIGAÇÃO DE LECTINA E INIBIDOR DE TRIPSINA EM TUBÉRCULOS DE INHAME (*Dioscorea alata*) CULTIVADO NO NORDESTE DO BRASIL

Julia Mariano Caju de Oliveira
Edilza Silva do Nascimento
Tatiane Santi Gadelha
Carlos Alberto de Almeida Gadelha

DOI 10.22533/at.ed.5901927053

CAPÍTULO 4 38

ISOLAMENTO E IDENTIFICAÇÃO DE FUNGOS FILAMENTOSOS ALERGÊNICOS ENCONTRADOS EM PEÇAS ANATÔMICAS HUMANAS CONSERVADAS EM SOLUÇÃO DE FORMALDEÍDO

Hércules Gonçalves de Almeida Medeiros
Adna Cristina Barbosa de Sousa

DOI 10.22533/at.ed.5901927054

CAPÍTULO 5 50

MEIO AMBIENTE GENÉTICO E EMBRIÕES EXCEDENTÁRIOS

Odair Bufolo
Daiane Silva Berdusco Freire
Andréia de Fátima Selvati Bredariol

DOI 10.22533/at.ed.5901927055

CAPÍTULO 6 62

PRODUÇÃO DE ÁCIDOS PROPANOICO E ACÉTICO POR PROPIONIBACTERIUM ACIDIPROPIONICI ADSORVIDA EM MONTMORILONITA K-10

Taciani do Santos Bella de Jesus
Lucidio Cristovão Fardelone
Gustavo Paim Valença
José Roberto Nunhez
José Augusto Rosário Rodrigues
Paulo José Samenho Moran

DOI 10.22533/at.ed.5901927056

CAPÍTULO 7 72

PRODUÇÃO DE B-GLUCANASES E AVALIAÇÃO DA ATIVIDADE ANTIMICROBIANA E REDUÇÃO DE BIOFILME DE *Candida albicans*

Glaucia Hollaender Braun
Henrique Pereira Ramos
Maria Laura Lucas Natal
Rosemeire Cristina Linhari Rodrigues Pietro

DOI 10.22533/at.ed.5901927057

CAPÍTULO 8 80

PRODUCTION AND STABILITY OF LIPASE AND PECTINASE PRESENT IN AGROINDUSTRIAL RESIDUES

Millena Cristiane de Medeiros Bezerra Jácome
Carlos Eduardo de Araújo Padilha
Murilo Ricardo do Nascimento Arrais
Maria Cecília Bezerra Caldas
Everaldo Silvino dos Santos

DOI 10.22533/at.ed.5901927058

CAPÍTULO 9 84

PROPRIEDADES FÍSICAS E MECÂNICAS DE UM CIMENTO DE IONÔMERO DE VIDRO APÓS ADIÇÃO DE NANOPARTÍCULAS DE TIO2

Luis Eduardo Genaro
Luana Mafra Marti
Ana Carolina Bosco Mendes
Rafael Amorim Martins
Angela Cristina Cilense Zuanon

DOI 10.22533/at.ed.5901927059

CAPÍTULO 10 91

PURIFICATION OF A XYLANASE FROM *Penicillium crustosum* AND ITS POTENTIAL USE IN CLARIFYING FRUIT JUICE

Jaina Caroline Lunkes
Vanessa Cristina Arfelli
Jorge William Fischdick Bittencourt
Rafael Andrade Menolli
Alexandre Maller
Jose Luís da Conceição Silva
Rita de Cássia Garcia Simão
Marina Kimiko Kadowaki

DOI 10.22533/at.ed.59019270510

CAPÍTULO 11 101

SENSIBILIDADE CELULAR E DE BIOFILME DE *Enterococcus* sp. AOS DESINFETANTES DE USO INDUSTRIAL

Luciana Furlaneto Maia
Naieli Mücke
Márcia Regina Terra
Danielle Karine Ohashi
Talita Butzske Bússolo
Márcia Cristina Furlaneto

DOI 10.22533/at.ed.59019270511

CAPÍTULO 12 115

SIMULAÇÃO NUMÉRICA DA PROPAGAÇÃO DE ONDAS CISALHANTES EM ROCHAS SEDIMENTARES A PARTIR DE IMAGENS MICROTOMOGRÁFICAS DE RAIOS X

Túlio Medeiros
José Agnelo Soares
Ronildo Otávio de Oliveira Neto
Juliana Targino Batista

DOI 10.22533/at.ed.59019270512

CAPÍTULO 13 127

STABILITY OF PECTINASE OF ASPERGILLUS NIGER IOC 4003 IN DIFFERENT SALTS FOR PURIFICATION IN BIPHASIC AQUEOUS SYSTEM

Millena Cristiane de Medeiros Bezerra Jácome
Murilo Ricardo do Nascimento Arrais
Carlos Eduardo de Araújo Padilha
Everaldo Silvino dos Santos

DOI 10.22533/at.ed.59019270513

CAPÍTULO 14 131

TÉCNICA DE FISH APLICADA NA IDENTIFICAÇÃO DA MICROBIOTA DE REATOR DE LODO ATIVADO UTILIZADO NA DEGRADAÇÃO DE BLENDAS

Lívia Cordi
Nelson Durán

DOI 10.22533/at.ed.59019270514

CAPÍTULO 15 142

TEMPERATURE AND pH EFFECTS ON THE ACTIVITY AND STABILITY OF THR XYLANASES PRODUCED BY THE THERMOPHILIC FUNGUS *Rasamsonia emersonii* S10

Jéssica de Araujo Zanoni
Eleni Gomes
Gustavo O. Bonilla-Rodriguez

DOI 10.22533/at.ed.59019270515

CAPÍTULO 16 147

TRIAGEM DE TRATAMENTO DE *Luffa cylindrica* PARA IMOBILIZAÇÃO DE Saccharomyces cerevisiae VISANDO A PRODUÇÃO DE INVERTASE

Beatriz Paes Silva
Brenda Kischkel
Nicolle Ramos dos Santos
André Álvares Monge Neto

DOI 10.22533/at.ed.59019270516

CAPÍTULO 17 159

AÇÃO FIBRINOLÍTICA DE PROTEASES PRODUZIDAS POR BACTÉRIAS ISOLADAS DE AMBIENTES AMAZÔNICOS

Thayana Cruz de Souza
Anni Kelle Serrão de Lima
Michele Silva de Jesus
Raimundo Felipe da Cruz Filho
Wim Maurits Sylvain Degrave
Leila de Mendonça Lima
Ormezinda Celeste Cristo Fernandes

DOI 10.22533/at.ed.59019270517

CAPÍTULO 18 164

ÁCIDO CÍTRICO: UM ENFOQUE MOLECULAR

Letícia Fernanda Bossa
Daniele Sartori

DOI 10.22533/at.ed.59019270518

CAPÍTULO 19 174

ACTINOBACTÉRIAS ISOLADAS DE MANGUEZAL E SEU POTENCIAL BIOTECNOLÓGICO

Gabriela Xavier Schneider
Jean Carlos Ramos de Almeida
Kassiely Zamarchi
Débora Santos
Danyelle Stringari
Renata Rodrigues Gomes

DOI 10.22533/at.ed.59019270519

CAPÍTULO 20 188

IDENTIFICAÇÃO DE BACTÉRIAS COM A CAPACIDADE DE BIODEGRADAÇÃO DO HERBICIDA ÁCIDO 2,4-DICLOROFENOXIACÉTICO

Juliana Barbosa Succar
Andressa Sbano da Silva
Lidiane Coelho Berbert
Vinícius Ribeiro Flores
João Victor Rego Ferreira
Alexander Machado Cardoso
Ida Carolina Neves Direito

DOI 10.22533/at.ed.59019270520

CAPÍTULO 21 199

REABILITAÇÃO DE AREAS DEGRADADAS PELA MINERAÇÃO DE QUARTZITO COM INSTALÇAO DE USINA SUSTENTAVEL

Gabriel Silva Gomes

DOI 10.22533/at.ed.59019270521

CAPÍTULO 22 218

COMPOSIÇÃO FITOQUÍMICA E TOXICIDADE DAS FOLHAS DE *Nectandra megapotamica* (Spreng.) Mez (LAURACEAE)

Viviane Mallmann
Lucas Wagner Ribeiro Aragão
Edineia Messias Martins Bartieres
Valdeci José Pestana
Shaline Séfara Lopes Fernandes
Rogério César de Lara da Silva
Tauane Catilza Lopes Fernandes
Ana Francisca Gomes da Silva

DOI 10.22533/at.ed.59019270522

CAPÍTULO 23 223

CRESCIMENTO DE MUDAS DE *Dipteryx odorata (Aubl.) Willd. (Fabaceae*) EM SUBSTRATOS ORGÂNICOS COMPOSTOS COM RESÍDUOS DE CASTANHA-DO-BRASIL

Givanildo Sousa Gonçalves
Lúcia Filgueiras Braga
Letícia Queiroz de Souza Cunha

DOI 10.22533/at.ed.59019270523

CAPÍTULO 24 236

SUBSTRATOS ORGÂNICOS NO CRESCIMENTO DE MUDAS DE *Dipteryx odorata (Aubl.) Willd. (Fabaceae*)

Givanildo Sousa Gonçalves
Lúcia Filgueiras Braga
Letícia Queiroz de Souza Cunha

DOI 10.22533/at.ed.59019270524

SOBRE O ORGANIZADOR 253

CAPÍTULO 10

PURIFICATION OF A XYLANASE FROM *Penicillium crustosum* AND ITS POTENTIAL USE IN CLARIFYING FRUIT JUICE

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ABSTRACT: Xylan-degrading enzymes have been used in several industrial sectors, such as in pulp and paper processing, brewing, baking, animal feed, starch, textiles and wine production. This study aimed to purify an extracellular xylanase produced by *Penicillium crustosum* and investigate its potential for fruit juice clarification. The enzyme was purified after three chromatography columns (DEAE, CM-Sephadex, and Sephadex G-75), and it exhibited 23.4 kDa of molecular mass. The purified xylanase-I hydrolyzed the beechwood xylan and yield mainly xylobiose and xylotriose as end products. Furthermore, xylanase-I showed no cytotoxic effects and exhibited a significant increase in the clarification of mango, orange, strawberry, peach, apple, and papaya juices. This is also first report on the potential of a xylanase produced from *Penicillium crustosum* isolated from the Atlantic Forest that showed promising characteristics for biotechnological application.

KEYWORDS: endoxylanase, fruit juice, filamentous fungi, *Penicillium.*

1 I INTRODUCTION

Xylan is a heterogeneous polysaccharide that consists of a backbone of xylose residues linked by β-1,4 bonds with short lateral branches (BAJAJ AND MANHAS, 2012). The

most common substituents that comprise the side branches are arabinosyl, mannosyl, galactosyl, acetyl, and glucuronosyl, which are found in polymers such as arabinoxylans, glucomannans, arabinogalactans, glucuronoxylans, galactoglucomannans, and arabinoglucuronoxylans (HEINEN, BETINI and POLIZELI, 2017). Xylan is a major component of hemicellulose and the second-most abundant polysaccharide in nature (COLLINS, GERDAY and FELLER, 2005). However, the complexity of the structure of xylan requires the action of a group of enzymes for complete hydrolysis (BEG et al., 2001). These enzymes are known as endoxylanase (EC 3.2.1.8), β-xylosidase (EC 3.2.1.37), acetylxylan esterase (EC 3.1.1.72), α-L-arabinofuranosidase (EC 3.2.1.55), α-D-glucuronidase (EC 3.2.1.139), feruloyl esterase (EC 3.1.1.73), and p-coumaroyl esterase (EC 3.1.1.B10) (CHÁVEZ, BULL & EYZAGUIRRE, 2006). Endoxylanases are responsible for cleavage of the β-1,4 glycosidic linkages of the xylan backbone, releasing xyloligoosaccharides (KOCABAS et al., 2015).

Several organisms found in nature, including bacteria, protozoa, algae, and fungi, are producers of xylanase (COLLINS, GERDAY and FELLER, 2005). Among the microorganisms, fungi present as potential producers of large-scale biocatalysts because of their ability to secrete enzymes (HEINEN, BETINI and POLIZELI, 2017; KOCABAS et al., 2015). In recent decades, interest in microbial xylanases has increased because of their potential in biotechnology, and they have been used in various industrial sectors, including the following: supplementation of feed for monogastric animals, pre-bleaching of pulp and paper, saccharification of lignocellulosic biomass, textiles, and baking (HEINEN et al., 2018). Furthermore, xylan-degrading enzymes, together with cellulase, amylase, and pectinase, have been used in the stabilization of fruit pulp; recovery of flavors, essential oils, vitamins, minerals, pigments, and edible coloring; reduction in viscosity; and liquefaction of fruits and vegetables (HEINEN, BETINI and POLIZELI, 2017; DOBREV et al., 2007). Studies have shown that the addition of enzymes in fruit juice and wine provides improvement in quality and characteristics by clarifying these products (BAJAJ & MANHAS, 2012). Xylanases have been used to improve filtration and to reduce the turbidity of the final product (DOBREV et al., 2007). Thus, this study aimed to purify an extracellular xylanase produced by *Penicillium crustosum,* isolated from the Atlantic forest biome of Brazil, and to analyze the performance of xylanase in the clarification of fruit juice.

2 I MATERIALS AND METHODS

2.1 Fungus and Culture Conditions

The fungus *P. crustosum* was previously isolated from the Atlantic forest in Paraná-Brazil. The sequence of ITS1 - ITS4 of the fungus can be found in the database at the National Center for Biotechnical Information (NCBI) by GenBank accession number **KM065878** (http://www.ncbi.nlm.nih.gov/). The fungus strain was cultivated in potato-

dextrose-agar (PDA) at 28°C for seven days, and after its growth, maintained at 4°C. The liquid cultures for xylanase production were optimized previously as reported by Silva et al., 2016. The cultures were incubated at 28 ºC under static condition for six days, and the crude extract was obtained by vacuum-filtered. The filtrate was centrifuged at 1200 x *g* for 10 min, and the supernatant was used to determine enzymatic activity and purification.

2.2 Enzymatic Assay and Protein Quantification

Xylanase was determined based on the amount of reducing sugar released after incubation in adequately diluted enzyme solution containing 1% (w/v) beechwood xylan, in a 50 mM acetate buffer (pH 5.0) at 50ºC for 10 min. The amount of reducing sugar was determined using the dinitrosalicylic acid method (MILLER, 1959). One unit of enzymatic activity was defined as the amount of enzyme capable of releasing 1 *µ*mol of reducing sugar per minute under the experimental conditions. All assays were performed in triplicate.

The amount of protein was estimated by Bradford (1976) method using bovine serum albumin as standard, and an absorbance of 280 nm was used for monitoring the protein in the column eluates.

2.3 Purification of Xylanase

The crude extract from *P. crustosum* was loaded to the ion exchange column DEAE-Sephadex (2 x 20 cm), pre-equilibrated with Tris-HCl buffer (0.02 M, pH 7.5). A 5 mL fraction in a flow of 1.3 mL/min using the same buffer for elution was collected. The fractions of the first peak with xylanolytic activity unbound in the DEAE-Sephadex column were pooled, dialyzed, and loaded to a second ion exchange column of CM-Sephadex (2 x 20 cm), pre-equilibrated with 0.02 M of sodium acetate buffer, at pH 5.0. Elution was performed with the same buffer added of linear NaCl gradient from 0.05 to 1.0 M. Fractions with enzyme activity were pooled, dialyzed and concentrated by lyophilization, and loaded to the Sephadex G-75 column (2 x 70 cm) pre- equilibrated with a 0.02 M sodium acetate buffer, at pH 5.0. Fractions with enzyme activity were pooled, dialyzed, and concentrated by lyophilization for further analysis of the properties of the enzyme.

2.4 SDS-PAGE and Zymogram

The purity of the enzyme was verified by both electrophoresis native-PAGE and denaturing conditions (SDS-PAGE) at a concentration of 10% acrylamide, according to Laemmli (1970). The gels were silver-stained according to Blum, Beier and Gros (1988), and the molecular weight ladder was as follows: phosphorylase B (97 kDa), albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (21.1 kDa), and α-lactalbumin (14.1 kDa). Native-PAGE (10%) of the sample was carried out to obtain the zymogram using the methodology described by Reisfeld,

Lewis and Williams (1962). After electrophoresis, the gel was incubated for 30 min in 0.5% Triton X-100. After being washed with distilled water, the gel was incubated with 1% beechwood xylan (w/v) in 0.05 M sodium acetate buffer, at pH 5.0, for 30 min in a water bath at 50 °C and then stained with Congo Red (0.2%) for 10 min, and washed with 1 M NaCl to remove excess dye.

2.5 Thin-Layer Chromatography (TLC) Analysis of the Hydrolytic Products

The enzymatic hydrolysis of beechwood xylan was performed by incubating the beechwood xylan 1% with purified enzyme under the standard assay condition. Aliquots were collected at different time points (30, 60, 120, and 160 min), and products were analyzed by thin-layer chromatography (TLC). Thin-layer chromatography was developed using n-butanol:pyridine:water (7:3:1, v/v/v) as the mobile phase, and xylose, xylobiose, and xylotriose as standards. The hydrolysis products were detected by spraying with a 1:1 (v/v) mixture of 0.4% (w/v) orcinol in sulfuric acid/methanol (1:9, v/v) followed by heating at 100 °C.

2.6 Macrophage Isolation and Measurement of Cell Viability

Albino Swiss mice (eight weeks old) were used as peritoneal macrophage donors. All legal recommendations of Brazilian legislation (Law No. 6.638, Nov. 5, 1979) for animal-handling procedures for scientific research were approved by the Animal Ethics Committee. Mouse peritoneal macrophages were collected by infusing the donors' peritoneal cavities with 10 mL of chilled phosphate-buffered saline (PBS). The cells were plated in culture medium (RPMI-1640, 5% fetal bovine serum, and antibiotic) in 96-well plates (2 x 10^5 cells/well). After 2 h of incubation at 37 °C under 5% CO_2 in a humidified incubator, non-adherent cells were removed by washed twice with PBS at 37°C (NOLETO et al., 2002).

Adherent macrophages were incubated for 48 h in the standard medium in the absence (control) or presence of purified xylanase (5 U mL^{-1} or 12.38 μg of protein) or crude extract (274.5 μg of protein) from *P. crustosum*. Cytotoxicity was evaluated using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium reduction assay at 550 nm in a spectrophotometer, as described by Reilly et al., (1998). The significance of the differences between the means was calculated by one-way ANOVA with Dunnet's multiple comparison tests using GraphPad Prism 5.0 at a value of $p<0.05$.

2.7 Effect of Xylanase-I on Clarification of Fruit Juice

Two hundred grams of fruits (apple, kiwi, mango, melon, orange, papaya, peach, pear, plum, and strawberry) previously washed with distilled water and cut into cubes were ground in a blender with 200 mL of distilled water; then the juice was filtered in sterile gauze. The mixture of 3 mL of each juice and *P. crustosum* xylanase (5 U

mL^{-1}) was incubated at 50°C according to the modified method of Bajaj and Manhas (2012). After 10 min, the reaction was stopped by boiling at 100°C for 1 min followed by centrifugation at 3000 x *g* for 10 min. The turbidity in the supernatant of fruit juice was determined by a spectrophotometer at 600 nm. The control was conducted under the same conditions, except that the enzyme was replaced by deionized H_2O. Results are presented as means ± mean standard error (SEM), and the significance of the differences between the means was calculated by two-way ANOVA with Bonferroni multiple comparison tests using GraphPad Prism 5.0, in which $p<0.01$ was considered to represent significance differences.

3 I RESULTS AND DISCUSSION

3.1 Purification of Xylanase-I from *P. crustosum*

The crude extract was loaded onto the ion-exchange DEAE-Sephadex, and two enzymatic activity peaks were obtained (Figure 1A). The first peak with xylanase-I unbounded to resin was loaded onto the CM-Sephadex column and eluted with a NaCl gradient of 100 mM and 275 mM (Figure 1B). The presence of more than one xylanase produced by *Penicillium* fungi has also been described by Chávez, Bull and Eyzaguirre (2006). After enzyme eluting from the size exclusion column Sephadex G-75 (Figure 1C), xylanase-I was purified, with recovery of 0.06% and a 22.18 purification fold (Table 1). Liao and colleagues (2014) used three steps of purification of xylanase from *Penicillium oxalicum* GZ-2; however, recovery was 6.9% with a 6.4 purification fold. Knob and colleagues (2013) reported recovery of 76.94% and a 5.10-fold with purified xylanase from *Penicillium glabrum*. The homogeneity of the xylanase purified by column chromatography was confirmed by a single band found in SDS-PAGE, and the molecular mass was 23.4 kDa (Figure 1D). The molecular mass of xylanase-I *P. crustosum* was near those of *Penicillium* genera xylanases, such as the xylanase from *P. oxalicum*, with molecular mass of 21.3 kDa (LIAO et al., 2014).

Steps	Total protein (mg)	Total activity (U)	Specific activity (U mg^{-1})	Purification (fold)	Yield (%)
Crude culture filtrate	420	7,650	18.21	1	100
DEAE-Sephadex	19.02	1,104	58.04	3.18	4.52
CM-Sephadex	2.25	818	391.55	21.50	0.53
Sephadex-G75	0.255	103	403.92	22.18	0.06

TABLE 1: Summary of purification steps of xylanase-I from *P. crustosum*

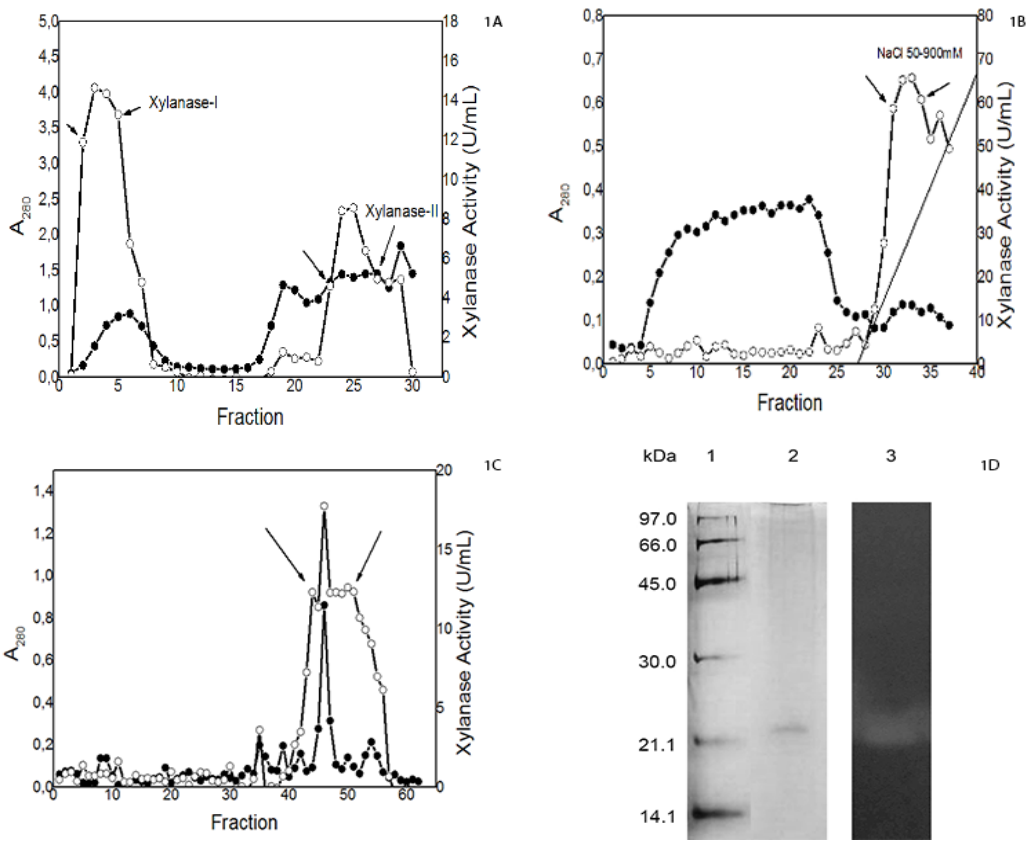

FIGURE 1: Chromatographic profile of purification of xylanase-I from *P. crustosum* by DEAE-Sephadex (A), CM-Sephadex (B), and gel filtration Sephadex G-75 (C); Xylanase activity (o), protein absorbance at 280 nm (●), and NaCl (straight line). The collected fractions are indicated by arrows (à); SDS-PAGE gel electrophoresis (D). Lane 1: molecular weight ladder with phosphorylase b (97 *kDa*), albumin (66 *kDa*), ovoalbumin (45 *kDa*), carbonic anhydrase (30 *kDa*), trypsin inhibitor (20.1 *kDa*), and α-lactalbumin (14.4 *kDa*); Lane 2: purified xylanase from *P. crustosum*; Lane 3: zymogram by native PAGE.

3.2 Hydrolysis of Xylan

The mode of action of the purified xylanase-I was shown by TLC analysis of hydrolysates of beechwood xylan. After 160 min incubation, no xylose was produced, but xylobiose and xylotriose were the main hydrolysis products of beechwood, indicating that it is a typical endo-acting xylanase (Figure 2).

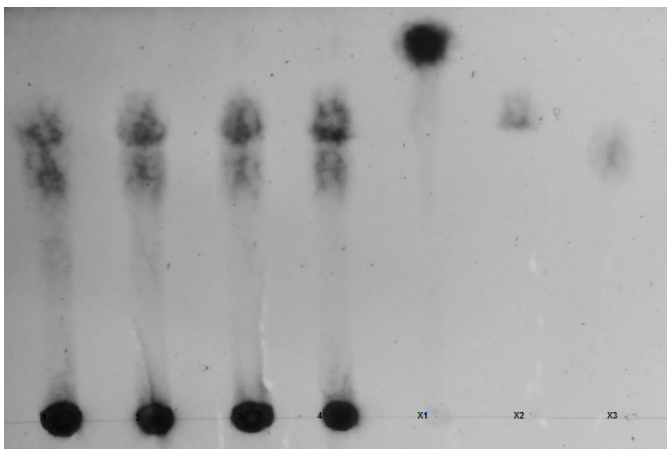

FIGURE 2: TLC analysis of the products after hydrolysis of beechwood xylan by xylanase-I from *P. crustosum*. The numbers 1, 2, 3, and 4 represent aliquots that were removed at times 30, 60,

120, and 160 min, respectively. X1 (xylose), X2 (xylobiose), and X3 (xylotriose) were used as standards.

3.3 Cytotoxicity Analysis of the Crude Extract and Purified Xylanase-I from *P. crustosum*

Considering the application of the enzyme in different sectors of the food industry, the cytotoxic effects of both crude extract and purified xylanase-I from *P. crustosum* were analyzed using the cell viability of mouse macrophage. Both crude extract and purified xylanase-I from *P. crustosum* showed no cytotoxic effects, that is, they did not cause cell death of mice macrophages at the concentration tested (5 U mL^{-1} or 12.38 μg of protein). The statistical analysis also showed that there is no significant difference between the control and the samples (Figure 3), suggesting that crude extract and purified xylanase-I enzyme from *P. crustosum* are safe and can be added as a supplement to food products.

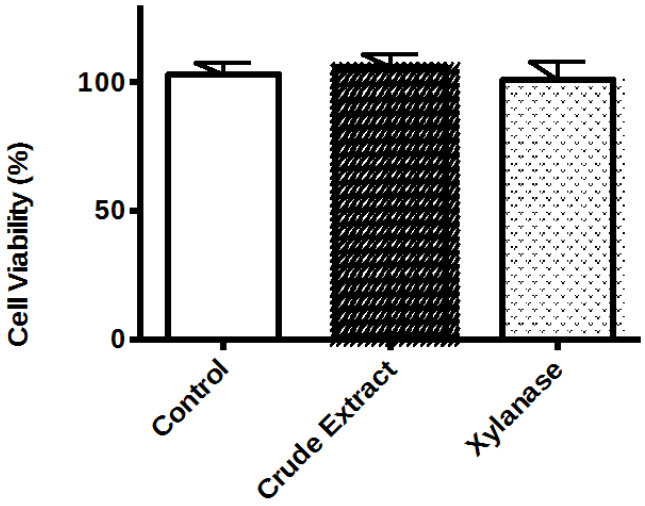

FIGURE 3: Effect of crude extract and purified xylanase-I from *P. crustosum* on cell viability. Differences were considered significant at a value of $p<0.05$ compared with control.

3.4 Effect of Xylanase-I on Clarification of Fruit Juice

The clarification of ten different types of fruit juices (apple, kiwi, mango, melon, orange, papaya, peach, pear, plum, and strawberry) by adding 5 U mL^{-1} xylanase-I from *P. crustosum* for 10 min was analyzed (Figure 4). Six types of juices showed a significant increase in clarification, in the following order: mango (36.68%), orange (29.89%), strawberry (28.89%), and peach (28.29%), with lower values for apple (17.77%) and papaya (18.45%). The other types of juice (plum, kiwi, melon, and pear) did not exhibit significant clarification with xylanase-I. Fruit juice treated with xylanase was clarified because of the hydrolysis of xylan that is present in these juices. According to Bajaj & Manhas (2012), the turbidity and viscosity of the raw juice are mainly because of the polysaccharides such as pectin, starch, and hemicellulosic components. However, treatment with enzymes that degrade polymers such as hemicellulose, pectin, and

cellulose improve the extraction of some compounds like sugars, and further it clarifies the juice (DHIMAN et al., 2011; SHARMA & KUMAR, 2013). The enzymes used by industry for the clarification of juice, beer, and wine are primarily composed of enzyme cocktails, including hemicellulases to improve juice clarification (YANG et al., 2011). Similar reports of improvement in the clarification of citrus juice have been described for xylanases from *Bacillus stearothermophilus* (DHIMAN et al., 2011) and for *Bacillus licheniformis* xylanase added to pineapple, mousambi, and apple juices (BAJAN & MANHAS, 2012).

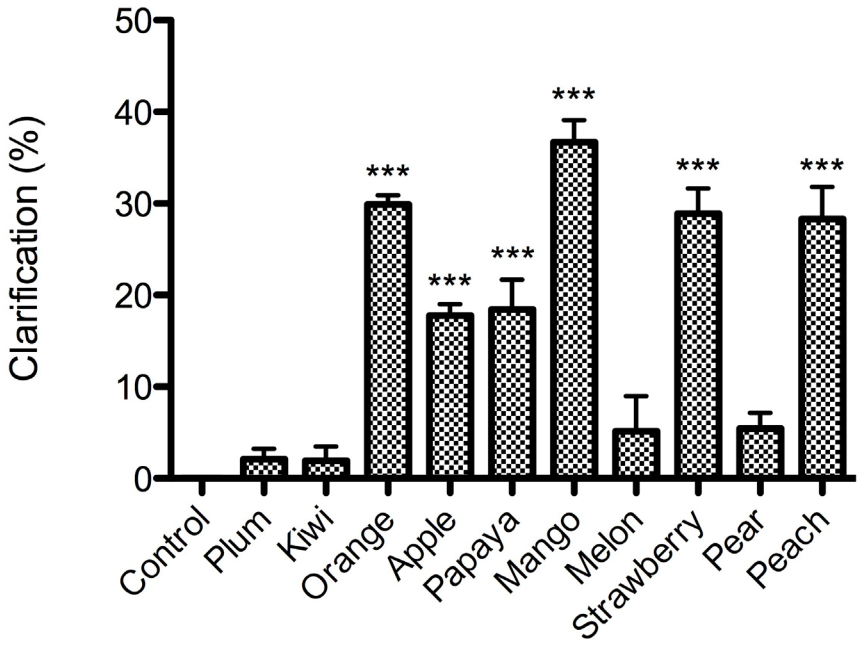

FIGURE 4: Effect of xylanase-I on clarification of fruit juice. The bars represent means ± SEM and ***$p < 0.01$ denotes significant differences compared with control.

4 I CONCLUSION

The purified endoxylanase from P. crustosum showed promising and desirable characteristics for biotechnological applications. The clarification of mango, orange, strawberry, peach, apple, and papaya juices was significantly improved with the addition of xylanase-I from P. crustosum. To our knowledge, this is the first report on the potential application of a xylanase produced by Penicillium crustosum fungus.

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