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Enzymatic Hydrolysis of Lignocellulosic Biomass Using an Optimized Enzymatic Cocktail Prepared From Secretomes of Filamentous Fungi Isolated From Amazonian Biodiversity

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Enzymatic hydrolysis of lignocellulosic biomass using an optimized enzymatic cocktail prepared from secretomes of filamentous fungi isolated from Amazonian biodiversity

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Abstract

The use of lignocellulosic biomass (LCB) has emerged as one of the main strategies for generating renewable biofuels. For the efficient use of such feedstock, pretreatments are essential. The hydrolysis of cellulose – major component of LCB - demands enzymatic cocktails with improved efficiency to generate fermentable sugars. In this scenario, lignocellulolytic fungi have enormous potential for the development of efficient enzyme platforms. In this study, two enzymatic cocktails were developed for hydrolysis of two lignocellulosic biomasses: industrial cellulose pulp and cassava peel. The solid biomass ratio in relation to the protein content of the enzyme cocktail were performed by experimental design. The optimized cocktail for the hydrolysis of cellulose pulp (AMZ 1) was composed, in protein base, by 43% of *Aspergillus sp* LMI03 enzyme extract and 57% of *T. reesei* QM9414, while the optimal enzyme cocktail for cassava peel hydrolysis (AMZ 2) was composed by 50% of *Aspergillus sp* LMI03 enzyme extract, 25% of the extract of *P. citrinum* LMI01 and 25% of *T. reesei*. The ratio between solids and protein loading for AMZ 1 cocktail performance was 52 g/L solids and 30mg protein/g solids, resulting in a hydrolytic efficiency of 93%. For the AMZ 2 cocktail, the hydrolytic efficiency was 78% for an optimized ratio of 78g/L solids and 19mg protein/g solids. These results indicate that cocktails formulated with enzymatic extracts of *P. citrinum* LMI01, *Aspergillus sp* LMI03 and *T. reesei* QM9414 are excellent alternatives for efficient hydrolysis of plant biomass and for other processes that depend on biocatalysis.

Keywords lignocellulolytic fungi, enzymatic cocktail, lignocellulosic biomass, hydrolysis.

Introduction

The issues related to the step of lingnocellulosic biomass saccharification remain one of the main factors that determine the performance and cost of cellulosic ethanol, the so called second generation (2G) ethanol. Therefore, much research has been devoted to increasing the enzymatic saccharification efficiency seeking a cheaper production of bioethanol [1] [2]. Researches with strains of *Trichoderma reesei* were pioneered in the design of enzymatic cellulose saccharification by a synergistic combination of different cellulase activities [3]. Of the species of *Trichoderma sp* isolated during World War II and subsequently analyzed by the researchers Mary Mandels and Ewyn Reese, the QM6 strain (sixth of the 6 fungus cultures of the genus Trichoderma) from the Quartemaster collection (US Army Quartermaster Research and Development Center), presented an excellent cellulose degradation capacity. This strain is considered a reference of *Trichoderma reesei* and from which all mutant strains that are used today in the industry were derived [4], as *T. ressei* QM9414 and Rut 30; the latter produces cellulases 2 to 4 times more than the original strain QM6a [5]. The formulations of commercial enzymes widely known and used industrially are produced with *T. reesei* enzymes. According to Bischof et al. (5), the filamentous fungus *T.reesei* still represents great importance in the research, it has been that on average more than 100 articles related to this fungus are published annually[4].

The hydrolysis of biomass to fermentable sugars by biological pathway, requires the action of multiple enzymes with different specificities to deconstruct the lignocellulosic structure of plant biomass [1][6]. The classic model of enzymatic degradation of cellulose is described by a set of enzymes belonging to the group of glycosil hydrolases (GH) that act synergistically to hydrolyze the cellulose fiber. However, it is currently known that other proteins that do not belong to the GH family, act in a way to cooperate with the catalytic power of cellulases, thus increasing the efficiency of hydrolyzing lignocellulosic materials and generating fermentable sugars [7][8]. These proteins called accessory proteins were classified within the AA (Auxiliary activity) family and the main ones contributed to the degradation of lignocellulose are: polysaccharide lytic monoxygenases (LPMOs) and swollenins [9].

The action of LPMO´s is influenced by the presence of cellobiose dehydrogenases (CDH´s), CDHs are fungal hemoflavoenzymes that belong to the oxidoreductase superfamily, and catalyze the oxidation of two cellobiose electrons [10]. It has been described that CDHs are secreted by filamentous fungi in conditions of cellulose degradation, and act in conjunction with LPMOs, increasing the conversion of cellulose to glucose [8]. LPMOs act in the crystalline region of cellulose, generating oxidized and non-oxidized chain ends and require an external electron donor to induce its activity [9] . While, swollenins have disruptive activity on various cellulosic substrates, such as filter paper, cotton and plant cell walls, without, however, releasing detectable amounts of sugars [11]. Enzymes that degrade lignin may not act on carbohydrates, but, because lignin is invariably and closely associated with

carbohydrates in the cell wall of plants, lignolytic enzymes cooperate with classic polysaccharides enzymes [9].

The challenge for the production of ethanol and other chemicals using lignocellulosic biomass is due to the recalcitrant nature of biomass and technical difficulties of the process, such as: type of pre-treatment, balance of enzymatic cocktails, microorganisms used, conditions of the fermentative process and type of bioreactor configurations [6]. Thus, obtaining high levels of fermentable sugars from lignocellulosic biomass depends on the efficient production of enzymes and well-balanced enzymatic cocktails containing: cellulases, hemicellulases, and other accessory proteins that act synergistically [12]. The optimization of enzyme mixtures to improve hydrolysis is among the factors that will allow further advances in the area of second generation technologies, adding to ~~a~~ greater understanding of the interactions between enzymes and lignocellulosic substrates and the development of enzyme engineering [13]. In this sense, the studies of proteins involved in the degradation of lignocellulosic material have allowed a broader knowledge of the main proteins of the hydrolytic complex that can be applied in processes inserted in the context of biorefinery. [14] [15] [2].

The synergistic action between hydrolases, especially cellulases and hemicellulases, and between lytic polysaccharide monooxygenases, swolenins and other auxiliary proteins in the pre-treated material, is extremely necessary for the effective deconstruction of biomass [12]. Strains of filamentous fungi of the species *T. reesei* have an effective cellulase production system (endoglucanases and exoglucanases), but with a very low production of β-glucosidases, while the *Aspergillus* genus are described for producing high levels of β-glucosidase, key-enzymes in glucose production [16] [17]. The prepared commercial of cellulases, Multifect®, was complemented with *Penicillium funiculosum* and *Trichoderma harzianum* enzymatic complexes, displaying greater efficiency than when used alone; in this case, the higher hydrolytic efficiency was correlated with an adequate level of β-glucosidase and xylanase activities [18].

Other studies have highlighted the need to complement enzymatic preparations with enzymes from different sources, as well as added accessory proteins that take part in the cellulose amorphogenesis [14][8]. Thus, the objective of this work was to produce enzymatic cocktails of high catalytic performance using fungal platforms isolated from the Amazon region: *Penicillium citrinum* LMI01 and *Aspergillus sp* LMI03 together with the hydrolytic complex of a strain of *Trichoderma reesei* QM9414.

Materials and Methods

Microorganisms

For the production of enzymatic extracts used to formulate the cocktails composition, the filamentous fungi were used: *Trichoderma reesei* QM9414

obtained from the Tropical Culture Collection of the André Tosselo Foundation (Campinas, São Paulo, Brazil); *Penicillium citrinum* strain LMI01 (NCBI access code: NoKU686951), isolated from decaying plant material [19], and *Aspergillus sp* strain LMI03 (NCBI access code: No. MT989354) isolated from recycled cellulose pulp (unpublished data). For all experiments, a pre-inoculum for mycelial growth of each fungus was prepared from cultures reactivated in BDA (Potato Dextrose agar) with a maximum of 10 days of growth. Spore suspensions for the pre-inoculum were made in sterilized distilled water, followed by spore counting in a Neubauer chamber.

Enzymatic production

The enzymatic extracts of the filamentous fungi *P. citrinum* LMI01, *Aspergillus sp*. LMI03 and *T. reesei* QM9414 were obtained by submerged fermentation that was conducted using industrial cellulose pulp as a carbon source. For production of cellulases by *T. reesei* QM9414, Mandels medium [20] was used composed of: KH_2PO_4 (2.0g/L); $CaCl_2 2H_2O$ (0.4g/L); $MgSO_4 7H_2O$ (0.3g/L); $(NH_4)_2SO_4$ (1.4g/L); urea (0.3g/L); peptone (1.0g/L); yeast extract (0.25g/L); $FeSO_4 7H_2O$ (5mg/L); $MnSO_4 7H_2O$ (1.6mg/L); $ZnSO_4 7H_2O$ (1.4mg/L) and $CoCl_2$ $6H_2O$ (2mg/L), added 15g/L of cellulosic carbon source. For the production of enzymes by *Penicillium citrinum* LMI01, a medium previously optimized for this strain was used, composed of: $MgSO_4$: 0.10g/L, KH_2PO_4: 12g/L; Urea: 0.25 g/L and peptone 1.32 g/L, cellulosic carbon source: 15g/L, and for enzyme production by *Aspergillus sp* LMI03 the medium optimized for this strain was composed of: cellulosic carbon source: 15g/L, $CaCl_2$: 0.15g/L, $(NH_4)2SO_4$: 2.80g/L, Urea: 0.39g/L and peptone: 1.30g/L.

The cultivation of each filamentous fungus was carried out in three replicates, by submerged fermentation in 500mL Erlenmeyer flasks containing 200mL of medium. The flasks were inoculated with 10% (v/v) culture containing cells previously grown (pre-inoculum) and incubated at 30 °C, 200rmp, for 72h. After fermentation, the mycelial mass was removed by vacuum filtration on glass wool. Then, mycelium-free culture supernatants were subjected to a tangential filtration system (QuixStand® -GE), using Hollow-fiber cartridges.

A volume of 4 liters of each supernatant was applied in cut-off cartridge 0.1µm (microfiltration) for removal of remaining cells and clarification, and then, was applied in cut-off cartridge 3kDa (ultrafiltration), the working conditions were: cartridge inlet pressure up to 10 and 15 Psi, respectively. The volume permeated in the ultrafiltration was neglected and the volume withheld (40mL) was recovered. The enzymatic activities and the total protein content in the permeate and in the withheld were monitored during the process.

Electrophoretic profile of proteins and enzymatic activity in gel SDS-PAGE

The electrophoretic profile of the proteins present in the concentrated enzymatic extracts of the fungi *P. citrinum* LMI01, *Aspergillus sp* LMI03 and *T. reesei* were analyzed on SDS/PAGE gel (12% polyacrylamide) prepared as

described by Laemmli [21]. At the same time, a zymogram for cellulases was performed on SDS-PAGE gel copolymerized with 0.2% carboxymethyl Cellulose (CMC). The samples were prepared in denaturing buffer (0.2 M Tris-HCl pH 6.8; 4% (v / v) SDS; 20% (v / v) glycerol; 0.1% (p / v) bromophenol blue) without β-mercaptoethanol. Electrophoresis was performed in 50 mA, 200W Tris-glycine buffer for approximately 1h30min.

After electrophoresis, the SDS-PAGE gel was stained with Coomassie blue R-250 overnight and discolored by incubating the gel in a bleach solution containing 50% methanol and 12% acetic acid, until visualization of the protein. While the zymogram gel was incubated in 1% (v/v) Triton X-100 buffer under slight agitation for 1 hour to remove the SDS and renaturate the proteins. Then, the gel was washed in distilled water and incubated in reaction buffer (sodium citrate pH 4.8 50 mM) for 1 hour. In the sequence, the gel was incubated in Congo Red 0.1% solution with shaking for 20 minutes, and then, decolored in 1M NaCl solution, until the appearance of clear bands against the red background indicating the degradation of CMC, for a better visualization of the degradation halos was added 100uL of glacial acetic acid [22].

Enzymatic hydrolysis

Industrial cellulose pulp, provided by the company Benaion Indústria de Papel e Celulose S.A., and cassava peel (*Manhiot esculenta* Crantz), obtained in the municipal market of Manaus-AM-Brazil were used for the enzymatic hydrolysis assays. First, the cassava peel was crushed and passed through a decontamination process (washing, drying in an oven at 100 °C, autoclaved at 120 °C for 60min, drying at 100 °C 24 hours) to remove possible contaminants. These biomasses were quantified in terms of cellulose, hemicellulose, lignin and ash content, using the methodology described by the National Renewable Energy Laboratory-NREL (33) and Ververis et al. (34).
The hydrolysis experiments were carried out according to the methodologies described by Barcelos et al. [23], with modifications. The hydrolysis was carried out using industrial cellulose pulp and cassava peel as solid substrates, in separate experiments. The proportion of solid waste was 25g/L, in a reaction volume of 1mL, in 2mL *eppendorf* tubes. The volumes of the concentrated enzymatic extracts were added to the tubes according to the protein concentration of each one, so that it had the protein load required by the experiments. The final volume of the hydrolysis reaction was completed with 50mM pH5.0 citrate buffer. The reaction was carried out at 50°C for 48 hours, and after that period, glucose determination in solution was performed by the glucose-oxidase method [24] [25] using the Enzyme Glucose Kit (*In Vitro*®).

Formulation of enzyme cocktail for hydrolysis of biomass

To obtain an ideal mixture from the concentrated enzymatic extracts of the fungi LMI01, LMI03 and *T. reesei*, experimental design of the simplex-centroid mixture was used, which generated a matrix with 10 experiments. The independent factors were the protein percentages of each concentrated

enzyme extract, where the value of 15mg of protein/g of solid waste (cellulose pulp or cassava peel) was considered 100%. The response variable was the glucose concentration (g/L) after 48h of hydrolysis at 50°C. The formulation of the cocktails was conducted using the values of total proteins (mg/mL) of each enzymatic extract to be added in the matrix experiments.

With the results obtained, statistical analysis was performed using the 95% confidence interval ($p \leq 0.05$), to decide which model was adequate to explain the data, the following tools were used: analysis of variance with *F* test ($p \leq 0.5$), lack of fit and R^2. After the statistical analysis and the obtention of the model, the validation of the optimal mixture was performed with 3 experimental repetitions.

Optimization of solid and protein loading

The proportion of solid residue (g/L) and protein concentration (mg/g) for an efficient hydrolysis, was optimized through the Central Composite Rotational Design (CCRD), followed by validation. Table 1 shows the levels investigated. The experimental matrix was composed of 11 tests, with 3 repetitions at the central point. The enzymatic cocktails were incubated together with the solid residue (cellulose pulp or cassava peel) within the proportions indicated by the matrix. Glucose production g/L and hydrolytic efficiency (HE%) were considered the response variables.

Table 1 CCRD factors and levels for optimization of solid and protein loading

Factors	Levels				
	- 1.44	- 1	0	+ 1	+ 1.44
Solid concentration (g/L)	6.75	25	52	89	97.25
Protein (mg/g)	9	15	30	45	51

For the calculation of hydrolytic efficiency, the equation below was used, where: *HE*: Efficiency of enzymatic hydrolysis (%); *Solid waste* (g/L): concentration of solid waste used in hydrolysis; *Cellulose* (g/g): cellulose concentration in the solids; 1.11: Conversion factor, related to the addition of a water molecule for the release of a glucose molecule, after the rupture of each covalent bond during hydrolysis.

$$HE(\%) = \frac{Glucose\ (g/L)}{Solid\ waste\ \ (g/L) \times Cellulose\ (g/g) \times 1{,}11} \times 100$$

Enzymatic assays and total protein quantification

The enzymatic activities of endoglucanase (CMCase), β-glucosidase and xylanase were determined using 2% carboxymethylcellulose, 2% cellobiose and 2% xylan birchwood as substrate, respectively, in 50mM citrate buffer, pH 5.0. The enzymatic assays were conducted according to the methodologies described by Ghose [24] and Eveleigh *et al*. [25]. Enzymatic activities were

determined in IU (International Units), where one unit of activity corresponds to 1µmol of product released per minute. The total protein concentration was determined by the Bradford method [26].

Results and discussion

Production of the enzymatic extracts, protein profile in SDS-Page and Zimogram

The levels of enzymatic activities and total protein concentration observed in the extracts after 72h of submerged fermentation, were different among *Penicillium citrinum* LMI01, *Aspergillus sp* LMI03 and *T.reesei*, as shown in Fig. 1 According to the enzymatic activity profiles of the *P.citrinum LMI01* and *Aspergillus sp* LMI03, the enzymatic preparations of these fungi can contribute to offer synegisms to enzymatic preparations of *T. reesei*, particularly with regard to beta-glucosidase activity, a key enzyme in glucose production [27] [17] . Likewise, the high levels of xylanases from these fungi can contribute strongly to synergisms for disruption of solid substrates rich in hemicellulose [27] [28]

Table 2 exhibits the levels of enzymatic activities and the concentration of total proteins (mg/mL) in the enzyme extracts crude, whose volumes were reduced 100 times. FPase activity was higher in the enzymatic extract of *T.reesei* (250 U/L), while LMI03 showed a higher level of β-glucosidase activity (2240 U/L) and considerable xylanase activity (29020 U/L), and the total protein content in the crude extract of *T. reesei* was also higher than that observed in strain LMI01 and LMI03, after 72h of submerged fermentation. These enzymatic activities increased after extracts enzymatics volume concentration, *T. reesei*´s fpase activity increased to 1,388.5 U/L, *Aspergillus sp* LMI03´s β-glucosidase activity and xylanase activity rise for 105,447.2 U/L and 234,103.2 U/L, respectively.

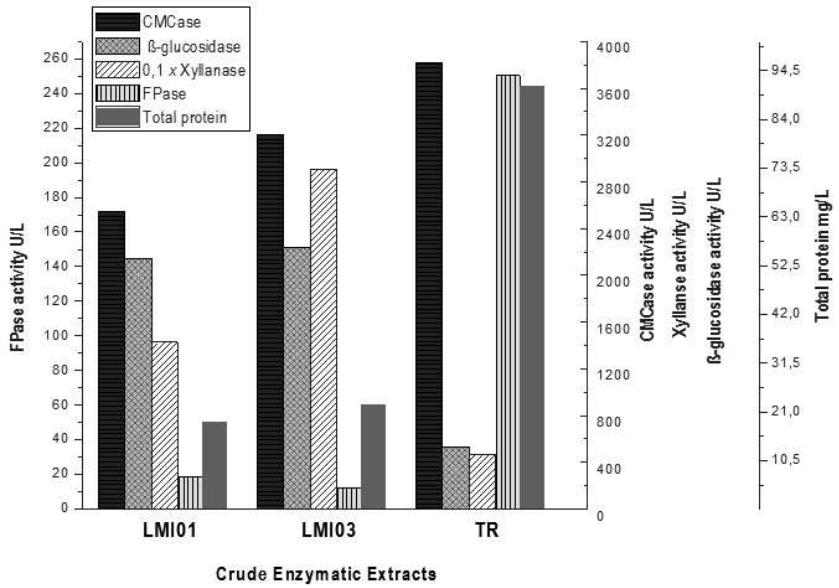


Fig. 1 Enzymatic activities and total protein concentration in crude enzymatic extracts, after 72 hours of submerged fermentation, with industrial cellulose pulp as a carbon source

Table 2 Enzymatic activities and total protein concentration in the concentrated extracts*

	P. citrinum LMI01	*Aspergillus sp.* LMI03	*T.reesei*
FPase U/L	216.3 ± 0.3	227.4 ± 30.8	1,388.5 ± 18.8
CMCase U/L	121,448.5 ± 9323.6	179,005.1 ± 878.3	457,057.2 ± 6148.7
β-glucosidase U/L	101,793.1 ± 6630.5	105,447.2 ± 9,766.0	13,050.4 ± 904.2
Xylanase U/L	105,720.4 ± 1184.0	234,103.2 ± 5,800.8	277,920.8 ± 966.8
Total protein mg/L	333.2 ± 13.5	1,045.1 ± 20.2	1,524.4 ± 24.4

*Volume enzymatic extract crude concentrated up to 100 times by tangential filtration in 3kDa cutoff hollow-fiber membrane

(Fig. 2a) shows the electrophoretic profile of the crude enzymatic extracts of the fungi platforms. In the extract of *P. citrinum* LMI01 it was observed the presence of 8 protein bands with an approximate molecular weight of: 20 kDa, 38 kDa, 35kDa, 29 kDa, 30 kDa, 50 kDa, 76kDa and 140 kDa. In the *Aspergillus sp* LMI03 extract, it was identified at least 16 defined bands with molecular weight of approximately: 13kDa, 15 kDa, 17 kDa, 19kDa, 22kDa, 31kDa, 35kDa, 38kDa, 40kDa, 50kDa, 52kDa, 60kDa, 68kDa, 76kDa, 80kDa and 130kDa. And, in the extract of *T. reesei* QM9414, 5 protein bands with high intensity were revealed, with molecular weight of approximately: 80kDa, 52kDa, 40kDa, 35kDa and 19kDa, and 5 bands with less intensity: 102kDa, 30kDa, 28kDa, 24kDa and 26kDa.

In the zymogram for endoglucanases (Fig. 2b), the crude extract of *T. reesei* QM9414 showed areas of CMC degradation at the height of protein bands with a molecular weight between 40 kDa to 100 kDa, and three other bands of approximately: 35 kDa, 30 kDa and 19 kDa. Several studies have shown that Trichoderma genus are efficient in secreting cellulolytic enzymes [16] [29] [30]. A similar result to the obtained in this work was reported for *T. harzianum*, an effective strain species in degrading sugarcane bagasse cellulignin, which produces 6 different proteins with cellulosic activity with molecular weight of

approximately: 48 kDa, 55 kDa, 59 kDa, 62 kDa, 69 kDa and 78 kDa [31], and for *Trichoderma viride* VKF3 which presented proteins with cellulolytic activities of molecular weight between 20.1- 29.0 kDa and 14.3 KDa [32]. While other Trichoderma strains such as *T. harzianum* L04 showed two cellulolytic activities with distinct molecular weight (50 kDa and 20 kDa) [33], and two other strains of *T. reesei* and *T. viride* that presented proteins with cellulolytic activity of molecular weight between 23 - 42 KDa [34].

P. citrinum LMI01 showed a CMC degradation region in the zymogram corresponding to proteins with an approximate molecular weight between 50 kDa to 100kDa, and a halo of degradation in the height of proteins with molecular weight ≅ 35kDa. Unlike what was observed for another species of the same genus, such as *P. verruculosum* BS3 isolated from the wood-yards on Kallai river belts, which in the zymogram showed a low molecular weight cellulase estimated at 17 kDa [35]. Other species of *Penicillium sp* have been described presenting proteins with cellulase activities close to those observed for *P.* citrinum LMI03, such as *P. ochrochloron* with a cellulase of 55kDa [36], *P. echinulatum* in which two bands of apparent molecular weight of approximately 80 kDa and 250 kDa were observed [37] and *P. digitatum*, which presented CMCase activity with an estimated molecular weight of 74 kDa [38].

In *Aspergillus sp* LMI03 extract, it was observed an area of CMC degradation corresponding to proteins of approximately 52 kDa, 80 kDa, and 2 bands with a degradation area at the height of ≅ 37kDa and ≅ 29kDa. Other fungi of *Aspergillus* genus produce multiple cellulases with molecular weight similar to those found in this work, as observed in the zymogram of *A. tubingensis*, which presented protein bands with cellulose activity in the range of 30kDa - 50kDa [39]. *A. fumigatus* Z5 zymogram analysis showed eight types of CMCase with molecular weight between 20kDa - 50kDa secreted by this fungus [40], and *A. fumigatus* FBSPE-05 in which six bands of cellulase activity were observed with estimated molecular weight of 94.9, 82.2, 73.4, 56.4, 35.3 and 27.3 kDa [41]. While, some species are described producing only one type of cellulose (presenting only 1 protein band), as for example, *A. glaucus*, which presented a cellulolytic activity protein band with molecular weight 31 kDa [42], *A. niger* IMMIS1 whose electrophoretic profile of its secreted proteins revealed a 71 KDa molecular weight cellulase [43]; Another strain, *A. fumigatus* ABK9 that presented a protein with cellulolytic activity of a size of 56.3 kDa (Jana et al., 2013) and *A. terreus* with cellulase activity protein of 29.1 kDa (Narra et al., 2014).

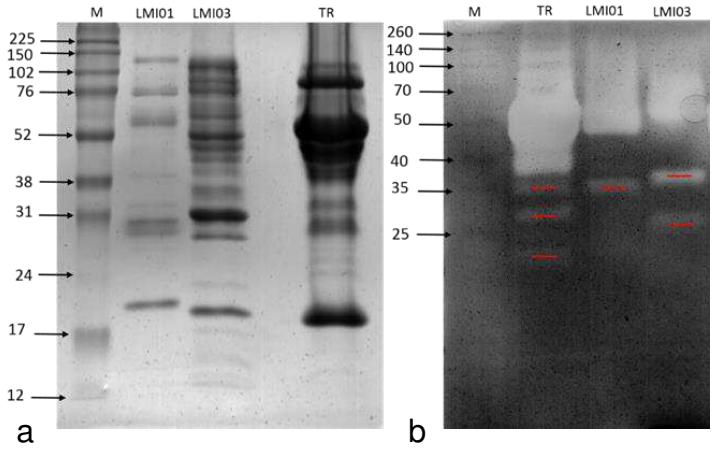


Fig. 2 Electrophoretic protein profile and zymogram for cellulases of the crude extract of fungi. **a** SDS-PAGE polyacrylamide gel stained with Coomassie blue R-250; M: Molecular weight marker (225kDa to 12kDa). LMI01: *P. citrinum* (6 µg); LMI03: *Aspergillus sp* (9 µg); TR: *T. reesei* QM9414 (13.6 µg). **b** SDS-PAGE gel zymogram (0.2% CMC) stained with 1% Congo red. M: molecular weight marker (260kDa to 10kDa), samples with a concentration of 5 µg of total proteins

According to these results, the proteins secreted by the fungi *P. citrinum LMI01*, *Aspergillus sp LMI03* and *T. reesei* QM9414 are very representative of enzymes that degrade cellulose. In addition, it is observed that proteins with cellulolytic activity of different molecular weight occur among the extracts of these fungi. As for example, in the *Aspergillus sp* LMI03 extract there is a protein band with cellulolytic activity of ≅ 29kDa, which was not seen in the crude extract of *T. reesei* or in the extract of *P. citrinum* LMI01. These conditions are quite interesting, since the synergistic action between different enzymes can be advantageous for increasing the catalytic efficiency in enzymatic hydrolysis of polysaccharides.

Besides, proteins from different fungal sources are described as responsible by increasing the performance of commercials enzyme cocktails [14]. For example, the saccharification level of pre-treated sugarcane bagasse, produced by commercial enzymatic cocktails, was increased by the addition of extracellular proteins from wood-decaying fungi, and this fact was attributed to different proteins identified in the extracellular extract of these fungi, mainly proteins belonging to the families GH5 and GH45-endoglucanases, GH3-betaglucosidases and GH10-xylanases [8].

Composition of different lignocellulosic biomass used in the enzymatic hydrolysis

Industrial cellulose pulp can be an excellent alternative carbon source for the production of enzymes by filamentous fungi that are capable of being induced to produce lignocellulolytic enzymes by the presence of this carbon source rich in polysaccharides. In addition, this biomass from the cellulose industry can also be used in process conceptions of biorefineries for the production of second generation ethanol and other molecules [44]. Cassava peel is a common agro-industrial solid residue in Brazil, which can be also used in the

production of cellulosic ethanol, in addition of being a source with great potential for the production of sugar syrups to obtain other molecules of industrial interest, due to its high fiber content. Current studies have demonstrated the potential of this residual biomass for obtaining biofuels [45] [46]. Table 3 shows the percentage composition of main components present in the cellulose pulp and cassava peel, used for enzymatic hydrolysis in this work.

Table 3 Composition of lignocellulosic biomass used in the enzymatic hydrolysis

Biomass	Cellulose %	Hemicellulose %	Lignin %	Ashes %	Others %
Cellulose pulp	83.3	7.7	1.8	3.2	4.0
Cassava peel	55.7	11.5	15.0	5.0	12.0

Experimental Design for optimizing the composition of enzyme cocktails

The matrix of experiments and results obtained in the design of Simplex-centroid mixture for the hydrolysis of cellulose pulp and cassava peel are described in table 4. The quadratic model provided a best fit to the data with a correlation coefficient *(R2)* = 99%, for hydrolysis of cellulose pulp. This formulation was called cocktail AMZ1, and the model of the ideal cocktail formulation is represented by the equation 1. For the hydrolysis of cassava peel, the ideal mixture was called the AMZ2 cocktail. The quadratic model fitted best the experimental data, with R2 = 88%, and the equation 2 represent this model.

(1):

$$Glucose\ (g/L) = 0.72A + 1.9B + 7.9C + 6.2AB + 42.1AC + 54.4BC + 0.28\varepsilon$$

(2):

$$Glucose\ (g/L) = 10.5\mathrm{A} + 13.1\mathrm{B} + 9.7\mathrm{C} + 12.1\mathrm{AB} + 16.2\mathrm{AC} + 10.4\mathrm{BC} + 0.65\varepsilon$$

Where, *A: P. citrinum* LMI01; *B: Aspergillus sp* LMI03; *C: T. reesei* QM9414; *ε*: *Error*

Table 4 Matrix of experiments simplex-centroid mixture design and results

	Percentage of enzymatic extract[a]			Glucose release (g/L)	
Run	*LMI01*	*LMI03*	*QM9414*	cellulose pulp[b]	cassava peel [c]
1	1.00	0.00	0.00	0.93	9.97
2	0.00	1.00	0.00	1.87	10.65
3	0.00	0.00	1.00	8.13	7.85
4	0.50	0.50	0.00	2.50	11.98
5	0.50	0.00	0.50	15.31	12.36
6	0.00	0.50	0.50	18.75	12.17

7	0.33	0.33	0.33	15.00	11.60
8	0.67	0.17	0.17	9.37	13.02
9	0.17	0.67	0.17	11.87	13.02
10	0.17	0.17	0.67	15.62	11.74
11	1.00	0.00	0.00	0.62	7.42
12	0.00	1.00	0.00	1.56	11.27
13	0.00	0.00	1.00	8.12	7.47
14	0.50	0.50	0.00	2.81	13.31
15	0.50	0.00	0.50	14.68	11.27
16	0.00	0.50	0.50	18.12	11.60
17	0.33	0.33	0.33	15.03	12.69
18	0.67	0.17	0.17	8.43	12.45
19	0.17	0.67	0.17	10.62	12.45
20	0.17	0.17	0.67	16.87	12.36

*[a]: Percentage of enzymatic extract used in hydrolysis, according with total proteins concentration (mg). [b; c]: Solid waste used in the proportion of 25g/L

In tables 5 and 6 are shown the Mathematical models coefficients from DOE mix to hydrolysis of cellulose pulp and cassava peel, respectively. It is observed that for the release of glucose from the cellulose pulp, the enzymatic extract of *P. citrinum* LMI01 had no statistically significant effect ($p \geq 0.05$), whereas, the enzymatic extracts of *Aspergillus sp* LMI03 and *T. reesei* QM9414 were statistically significant ($p < 0.05$). However, for the hydrolysis of cassava peel, the enzymatic extracts of the three fungi LMI03, LMI01 and *T. reesei* QM9414 were statistically significant ($p < 0.05$).

In triangular contour surfaces generated by the models (Fig. 3), it is possible to observe that, for the hydrolysis of the cellulose pulp, the cocktail formulation around 50% of enzymatic extract of *Aspergillus sp* LMI03 and 50% of *T.reesei* QM9414, in terms of mg of protein, provides an improvement in response. While, for cassava peel hydrolysis, as higher the relative proportion of the *Aspergillus sp* LMI03 enzymatic extract, the greater is the glucose release.

Table 5 Mathematical model coefficients from DOE mix to cellulose pulp hydrolysis

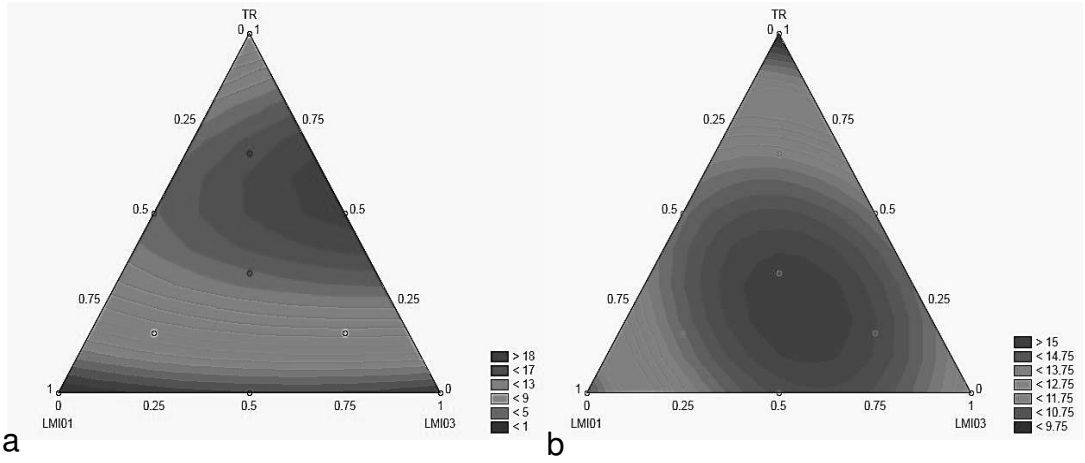
Factor	Coeff.	Std.Err.	*t*	*p*
(A) *P. citrinum* LMI01	0.72	0.36	2.00	0.070
(B) *Aspergillus sp.* LMI03	1.90	0.36	5.26	< 0.05
(C) *T.reesei* QM9414	7.97	0.36	22.04	< 0.05
AB	6.21	1.67	3.73	< 0.05
AC	42.09	1.67	25.25	< 0.05
BC	54.45	1.67	32.67	< 0.05

p

Table 6 Mathematical model coefficients from DOE mix to cassava peel hydrolysis

Factor	Coeff.	Std.Err.	*t*	*p*
(A) *P. citrinum* LMI01	8.88	0.57	15.70	< 0.05
(B) *Aspergillus sp*. LMI03	10.93	0.57	19.33	< 0.05

(C) *T.reesei* QM9414	7.81	0.57	13.82	< 0.05
AB	10.72	2.61	4.11	< 0.05
AC	14.37	2.61	5.51	< 0.05
BC	9.71	2.61	3.72	< 0.05



a b

Fig. 3 Contour surfaces for quadratic models generated in the mix design. **a** AMZ1 cocktail for cellulose pulp hydrolysis; **b** AMZ2 cocktail for cassava peel hydrolysis

The validations of the optimized mixtures were made by the mathematical function *Desirability*, obtained in the software Statistica®10.0. The *Desirability value* shows the percentage that the function meets the maximum that can be obtained in the answer. In table 7 shows the validation of the results and the ideal cocktail composition for cellulose pulp and cassava peel hydrolysis. The results experimentally obtained were close to those predicted by the function, indicating that the conditions predicted by the models and the *Desirability function* were validated.

Table 7 Validation of the optimized cocktails

Cocktail	Validation condition	Desirability value	Glucose *g/L*		Confidence limits	
			Observed	Predicted	-95.%	+95.%
AMZ 1	43%LMI03+57%TR	0.96	19.1	18.7	18.0	19.4
AMZ 2	50%LMI03+ 25%LMI01+25%TR	0.97	12.7	13.1	12.5	13.7

The individual enzymatic extracts of the fungi and the optimized cocktails AMZ 1 and AMZ 2 were evaluated in the hydrolysis of industrial cellulose pulp and cassava peel, using a protein loading of 15mg/g and a solid loading (25g/L). In Fig. 4, the hydrolytic efficiency (%) of the individual enzymatic fungal platforms

and of the optimized cocktails are demonstrated, and it is possible to observe the superiority of the combined cocktails in relation to the individual enzymatic extracts. The individual enzymatic extracts of *P. citrinum* LMI01, *Aspergillus sp* LMI03 and *T. reesei* QM9414 presented hydrolysis efficiency of 4.0%; 8.1% and 35.3% respectively, while hydrolytic efficiency AMZ1 cocktail was 82.9%. For hydrolysis of the cassava peel, the AMZ2 cocktail showed 81.7% hydrolytic efficiency much higher than the individual enzymatic extracts *P. citrinum*, *Aspergillus sp* and *T. reesei*, which values were as follows: 55.9%; 68.5%; 50.5%, respectively.

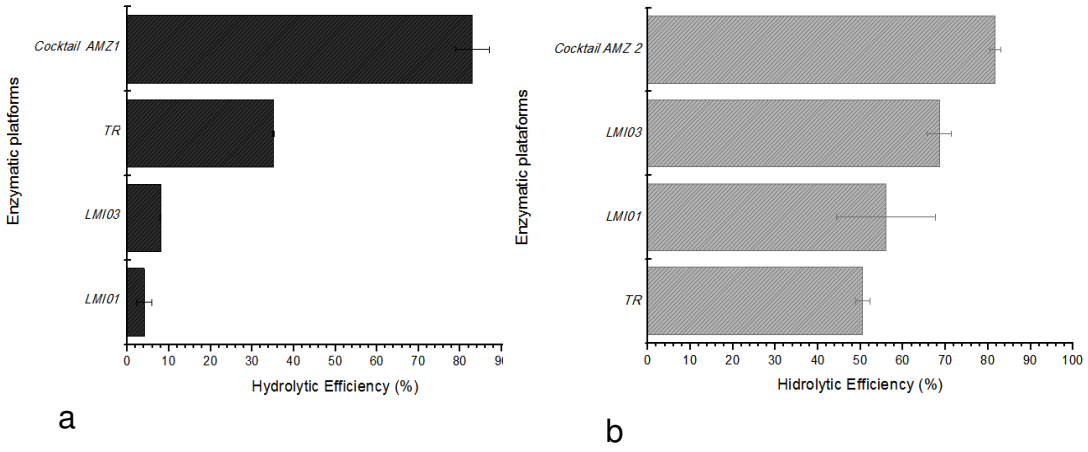


Fig. 4 Hydrolytic efficiency of the individual enzyme platforms and of the optimized cocktails, using 15mg of protein/g of cellulose and 25 g/L of solid residue. **a** hydrolysis of industrial cellulose pulp; **b** hydrolysis of cassava peel. LMI01: *P. citrinum*; LMI03: *Aspergillus sp*; TR: *T. reesei* QM9414; Cocktail AMZ1: 47% LMI03 + 53% TR; Cocktail AMZ2: 50% LMI03 + 25% TR + 25% LMI01

The synergistic performance of the *Aspergillus sp* LMI03 enzymatic complex in combination with that of *T. reesei* QM9414 in cellulose pulp hydrolysis can be ascribed with the level of β-glucosidase production by LMI03, which is much higher than that of *T. reesei* QM9414, what contributed for an increase release of glucose and consequently led to an increase hydrolytic capacity of *T. reesei* enzymatic extract, since β-glucosidase prevents the inhibition of exoglucanases, by increasing the rate of cellobiose degradation that is known to be an strong inhibitor of these enzymes [17].

T. reesei QM9414 is known to produce high levels of cellullases, mainly endoglucanases and exoglucanases [47] [30], and this justifies the fact that its enzymatic extract was more efficient in the hydrolysis of cellulose pulp, considering that this biomass has a high content of available cellulose fibers, due to the process by which it is produced. In the process of producing cellulose pulp, the wood chips are subjected to several stages: cooking, purification and washing, delignification, washing again and storage, leaving this material partially delignified [48].

In contrast, the enzymatic extract of the fungi *Aspergillus sp* LMI03 was more influential in the cocktail during the hydrolysis of the cassava peel, although a percentage of 25% *T. reesei* and 25% *P. citrinum* was required. This is quite interesting, since this biomass has a higher percentage of lignin and hemicellulose (12% hemicellulose and 15% lignin) when compared to cellulose pulp (8% hemicellulose and 2% lignin). Thus, these results indicate that *Aspergillus sp* LMI03, most likely, have an efficient disruptive lignocellulolytic apparatus, making its enzymatic extract very interesting from the point of view of developing new cocktails for degradation of other lignocellulosic substrates with biotechnological potential [49].

This synergistic effect was similar to that observed in other works reported in literature. An enzymatic mixture prepared with commercial enzyme reinforced with enzymes of *P. funiculosum* and *T. harzianum* resulted in high sugar content released from the enzymatic hydrolysis of the pre-treated sugarcane bagasse [18]. Yet, in a blend prepared from enzymatic extracts of *P. janthinellum*, *A. tubingenesis* and *T. reesei* Rut C-30 resulted in an increase in Avicel hydrolysis [50]. In the hydrolysis of pre-treated sugar cane bagasse by an optimized mixture composed of 15% *T. harzianum*, 50% *P. funiculosum* and 35% *A. niger* in protein basis, a hydrolysis yield of 91% was reached [51].

Optimization of the solid waste and protein loading for biomass hydrolysis.

In the process of biomass saccharification, the efficiency of hydrolysis is as important as the production of glucose itself, since high loads of enzymes entail a high cost in the process of deconstructing plant biomass aiming at the production of second generation ethanol and other chemicals [1][23]. Thus, the cocktails AMZ1 and AMZ2 were used to optimize the loading of solid waste (g/L) and protein (mg/g), in hydrolysis of industrial cellulose pulp and cassava peel, by means of CCRD. Aiming at to obtain an ideal proportion of developed cocktails, the effect of the different concentrations of solids and protein content required were evaluated, having as response the glucose release (g/L) and the hydrolysis efficiency (%). The results obtained in the CCRD are shown in table 8. The highest values of glucose release were obtained at the midpoints of the experimental matrix, which for hydrolysis of the cellulose pulp it was around 45.7 g/L, and for hydrolysis of the cassava peel it was 29.4 g/L. Similarly, hydrolytic efficiency showed higher percentage values around the midpoint, in both solids used.

Table 8 CCRD results for optimization solid and protein loading

Run	Solid load [a] g/L	Protein load mg/g	Enzymatic cocktail			
			AMZ1		AMZ2	
			Glucose g/L	HE %	Glucose g/L	HE %
1	25	15	3.1	13.4	11.4	73.2
2	25	45	9.1	39.5	12.3	79.0
3	89	15	41.1	50.1	44.1	79.7
4	89	45	26.6	32.4	27.0	48.9
5	7	30	1.9	30.0	0.8	20.5

6	97	30	36.1	40.3	34.7	57.5
7	52	9	24.6	51.3	20.2	62.5
8	52	51	18.6	38.8	20.2	62.5
9 (C)	52	30	47.1	98.3	27.4	84.8
10 (C)	52	30	45.6	95.2	30.5	94.5
11 (C)	52	30	44.6	93.0	30.1	93.3

[a] Solid residues used in hydrolysis with AMZ1 and AMZ2 cocktails: Industrial cellulose pulp and cassava peel respectively. Results obtained after 48h of hydrolysis at 50°C

The CCRD analysis of variance to AMZ1 cocktail showed that the model was adequate to explain the data, providing high values of the parameter R for glucose g/L (R^2 = 98%) and for hydrolytic efficiency (R^2 = 97%), and in addition, the lack of fit was not significant ($p > 0.05$) for both responses. As seen in the response surfaces (Fig. 6), the solid and protein loads around the average level studied (52g/L of solid waste and 30mg of protein/g) allowed for higher glucose production and maximum hydrolytic efficiency. The following equations describe the model as a function of solid/protein loading, using the AMZ 1 cocktail, where: *S: solid loading*; *P*: *protein loading*, *ε*: *pure error*.

Equation 3:

$$Glucose\ \ g/L = 45.76 + 12.97S - 13.45S^2 - 2.12P - 12.16P^2 - 5.12SP + 1.56\varepsilon$$

Equation 4:

$$Hydrolytic\ efficiency\ \% = 95.52 + 5.51S - 31.74S^2 - 1.16P - 26.78P^2 - 10.93SP + 6.89\varepsilon$$

The analysis of variance of the CCRD to optimize the solid and protein loadings using the AMZ2 cocktail, the explained variation percentages (R2) were 94% for glucose g/L production and 70% for hydrolytic efficiency, and for both, the lack adjustment was not significant ($p>0.05$). Analyzing the response surfaces for glucose production, it is observed that the increase in the loading of solids caused an improvement in the response, whereas the hydrolytic efficiency (%) was higher in the central points (Fig. 5c and 5d).

To obtain an ideal proportion of the loading solid and protein in the cocktails, that would allow an optimal response for the production of glucose g/L and for hydrolytic efficiency, the validation of the models obtained in the CCRD, was done by the *Desirability function* (*value = 0.91*). According to the mathematical model of this function (*Desirability value = 0.79*) for an optimal AMZ1 cocktail performance in the hydrolysis of cellulose pulp, 52g/L of solids should be used in combination with 30mg of protein in cocktail per *g* of solids. Yet for the AMZ2 cocktail, the solid and protein loadings should be 78g/L and 19mg of protein per/g of waste. The results obtained in the validation after 48 hours of hydrolysis were satisfactory, within the established confidence limits (95%).

In order to compare the performance of the cocktails developed in this work, with commercially available formulations, the commercial enzymes Multifect® and Cellic®CTec2 were incubated for hydrolysis of cellulose pulp and cassava peel, under the same conditions as AMZ1 and AMZ2. In cellulose pulp

hydrolysis, it was found that the AMZ1 cocktail had higher levels of glucose production (g/L) and hydrolytic efficiency (%), than that observed for the commercial blend Multifect®, and presented a performance similar to that obtained with Cellic®CTec2 (Fig. 6). While in the hydrolysis of cassava peel, the cocktail AMZ 2, presented a slightly higher performance (38 g/L glucose and 78% HE) than that obtained with Multifect (34g/L glucose; 70% HE). Similar results to these were obtained by Agrawal et al. [52], who reported a value of 75% for the enzymatic hydrolysis, using an enzyme mix developed with *Trichoderma* enzymes, and critical levels of β-glucosidase and xylanase, compared to individual commercial preparations, in the hydrolysis of pre-treated wheat straw by steam explosion.

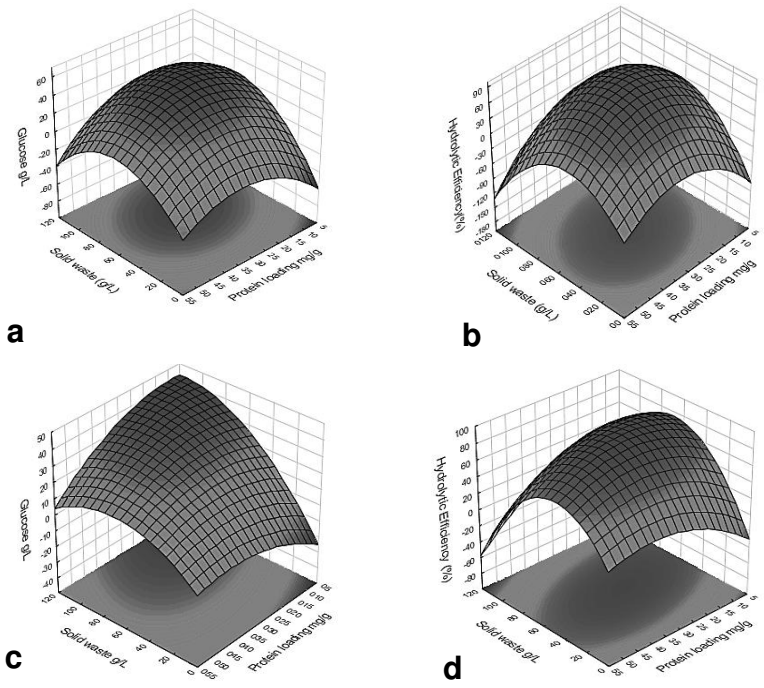


Fig. 5 Response surfaces for glucose released and hydrolytic efficiency as a function of loading solid and protein. **a** glucose (g/L), **b** Hydrolytic efficiency (%) to AMZ1 cocktail. **c** and **d** glucose (g/L) and hydrolytic efficiency (%), to AMZ2 cocktail

Efficiency is linked not only to the enzymatic cocktail's ability to convert cellulose and hemicellulose into fermentable sugar monomers, but to how much of this material a low protein loading of enzymes can hydrolyze [1][53]. Accordingly, modern biorefinery require high efficiency enzyme cocktails for the generation of fermentable sugars from lignocellulosic biomass. In this sense, fungi are the microorganisms that have shown the greatest potential in terms of enzyme productivity and efficiency in enzyme production [14][29].

In addition, recent research has shown high levels of saccharification of lignocellulosic biomass and hydrolytic efficiency using enzymatic cocktails produced by filamentous fungi that contain proteins with auxiliary activities of cellulases, in the same way, extracellular proteins produced by fungi from

rotting wood when added to commercial enzymatic preparations, enhanced their catalytic power [8][13]. Furthermore, Zerva et al [28] established the synergistic action between an active LPMO in xylan and a new fungal xylanase on cellulosic substrates, and observed a strong synergistic interaction in the degradation of the recalcitrant part of xylan, and also found that LPMO increased the cellobiose release by cellobiohydrolases during the hydrolysis of pretreated lignocellulosic substrates and microcrystalline cellulose.

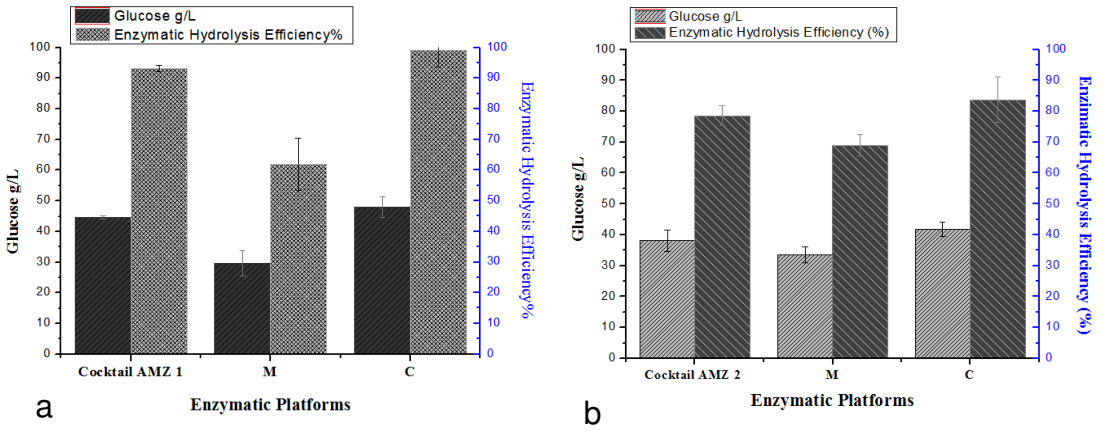


Fig. 6 Glucose released (g/L) and hydrolytic efficiency (%) after 48h of hydrolysis at 50°C. **a** hydrolysis of industrial cellulose pulp (52g/L) with 30mg of protein. **b** hydrolysis of cassava peel (78g/L) with 19mg of protein. M and C: commercial enzyme Multifect® and Cellic®CTec2 respectively

The increase in hydrolytic efficiency when combining extracellular proteins from the three different fungal platforms used in this work corroborate with the results observed in other works, in which higher levels of hydrolysis efficiency were obtained in balanced mixtures with different protein apparatus [18][50] [51][52]. According to this findings and compared with the results of the current literature, in fact it is evident that the efficiency of hydrolysis obtained in formulations with different fungi is of great impact when compared to the performance of enzymatic complexes obtained from only one species of fungi.

And, to strengthen this issue, other studies have confirmed that cellulolytic fungi such as *A. niger* and *T. reesei* RUT-C30 present a difference in genes related to the decomposition content of lignocellulose and also in its expression profile [15]. However, it is necessary to increase knowledge about the interactions between these proteins and between their substrates in terms of the complexity of the recalcitrant lignocellulosic matter. Once mixing enzymes from different sources is a sustainable approach that can increase the hydrolysis performance of cellulose, reducing the enzymatic deconstruction costs of lignocellulosic biomass [50][49]..

Conclusions

Extracellular proteins from *Penicillium citrinum* LMI01 and *Aspergillus sp* LMI03 isolated from the Amazon biome, showed high levels of xylanase and

beta-glucosidase activity. In addition, the extracellular protein complexes of these fungi are able to act synergistically with the proteins secreted by the cellulolytic *T. reesei* QM9414, strain equivalent to QM 9414, which is of great importance, since the QM9414 strain is still one of the main bases of enzymatic formulations for hydrolysis of lignocellulosic biomass, due to its protein apparatus rich in endoglucanases, exoglucnases and carbohydrate binding modules (CBMs). The optimization of the ideal mixture for hydrolysis of cellulose pulp and cassava peel showed that the LMI03 protein complex was the most required to achieve a higher performance of hydrolytic efficiency, being required in critical values such as 47% and 53% of *Aspergillus sp* LMI03 in its protein composition. This fact shows the disruptive catalytic power of the enzymatic extract produced by this strain.

The results reported herein also show that the enzymatic cocktails formulated with the enzymatic apparatus produced by the fungi *Aspergillus sp* LMI03 and *P. citrinum* LMI01 are able to perform as well as commercial cocktails of high performance, and with satisfactory hydrolytic efficiency, since resulting in an hydrolysis efficiency above 80% in the hydrolysis of cellulose pulp (cocktail AMZ1) and cassava peel (cocktail AMZ2). In this way, the enzymatic cocktails obtained in this work present an efficient alternative for hydrolysis of pre-treated or untreated vegetable biomass. In addition to providing investigative bases for future performance evaluations of these preparations in the deconstruction of other lignocellulosic biomass.

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Declarations

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Conflicts of interest/Competing interests

We wish to confirm that there are no known conflicts of interest associated with this publication. We confirm that all of us have approved the order of authors listed in the manuscript, and we confirm further that there are no other persons who satisfied the criteria for authorship but are not listed.

We understand that the Corresponding Author is the sole contact for the Editorial process (including Editorial Manager and direct communications with the office). He is responsible for communicating with the other authors about progress, submissions of revisions and final approval of proofs. We confirm that we have provided a current, correct email address which is accessible by the Corresponding Author and which has been configured to accept email from pamellasuely@gmail.com

Availability of data and material

All datasets presented in this study are included in the article/Supplementary Material

Code availability

Not applicable

Authors' contributions

PSSR, NPJ, and SAF conceived the study. PSSR and NPJ planned the experiments. PSSR and JBO carried out the experiments. PSSR, NPJ, and SAF contributed to the interpretation of the results. NPJ and SAF supervised the work. PSSR wrote the manuscript and NPJ and SAF contributed to the text and revised it. All authors read and approved the final version of the manuscript

Ethics approval

The activity of access to Genetic patrimony was registered in SisGen (National System of Management of Genetic Patrimony and Associated Traditional Knowledge) with the number A63F955, in compliance with the provisions of Law No. 13,123 / 2015 and its regulations of the Ministry of the Environment – Brazil.

Consent to participate

Not applicable

Consent for publication

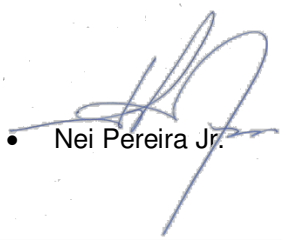
Not applicable

we confirm that the manuscript has been read and approved by all named authors Signed by all authors as follows:

- Pamella S. Santa-Rosa

- Jessica Batista de Oliveira

- Spartaco Astolfi-Filho



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Figures

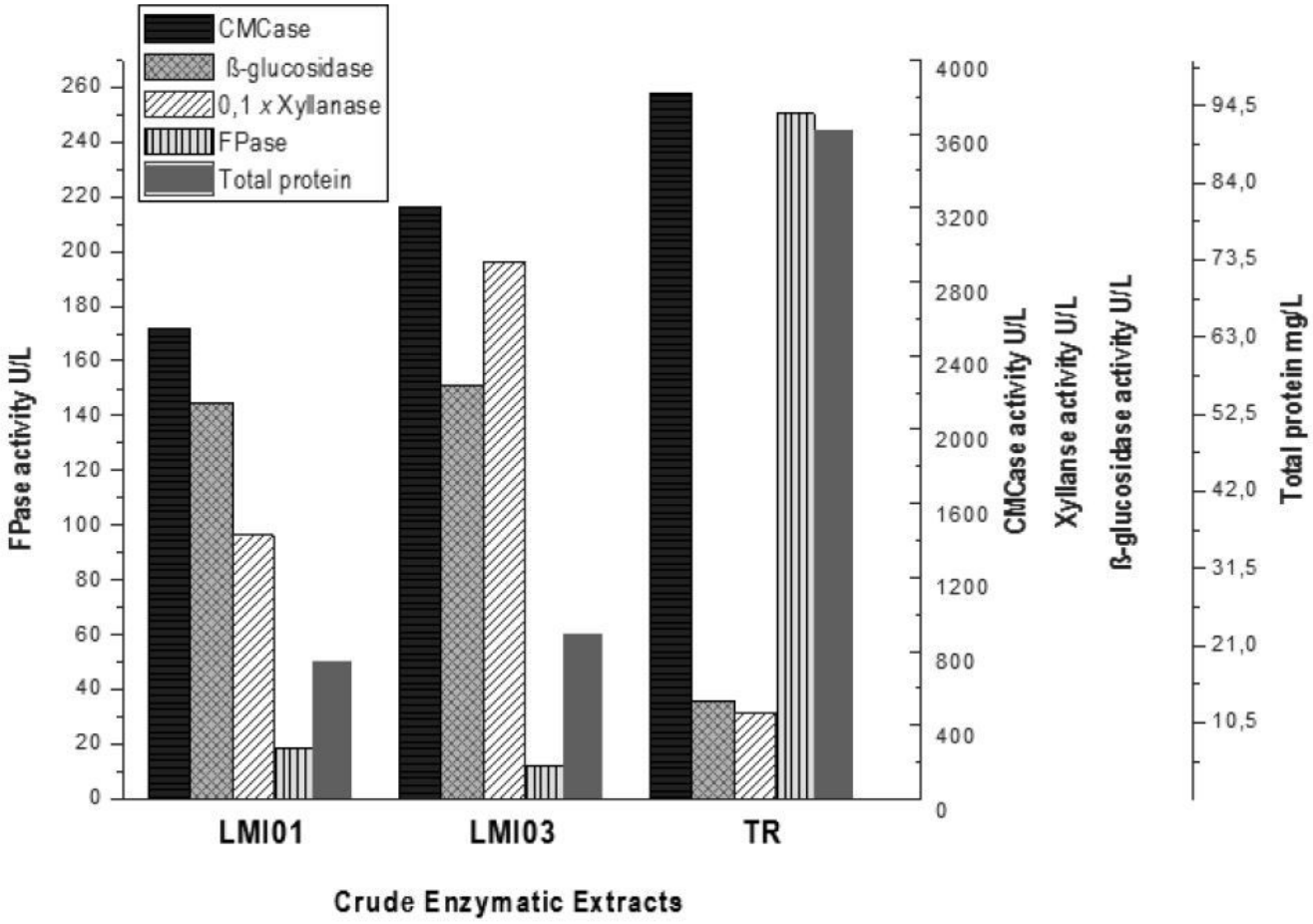

Figure 1

Enzymatic activities and total protein concentration in crude enzymatic extracts, after 72 hours of submerged fermentation, with industrial cellulose pulp as a carbon source

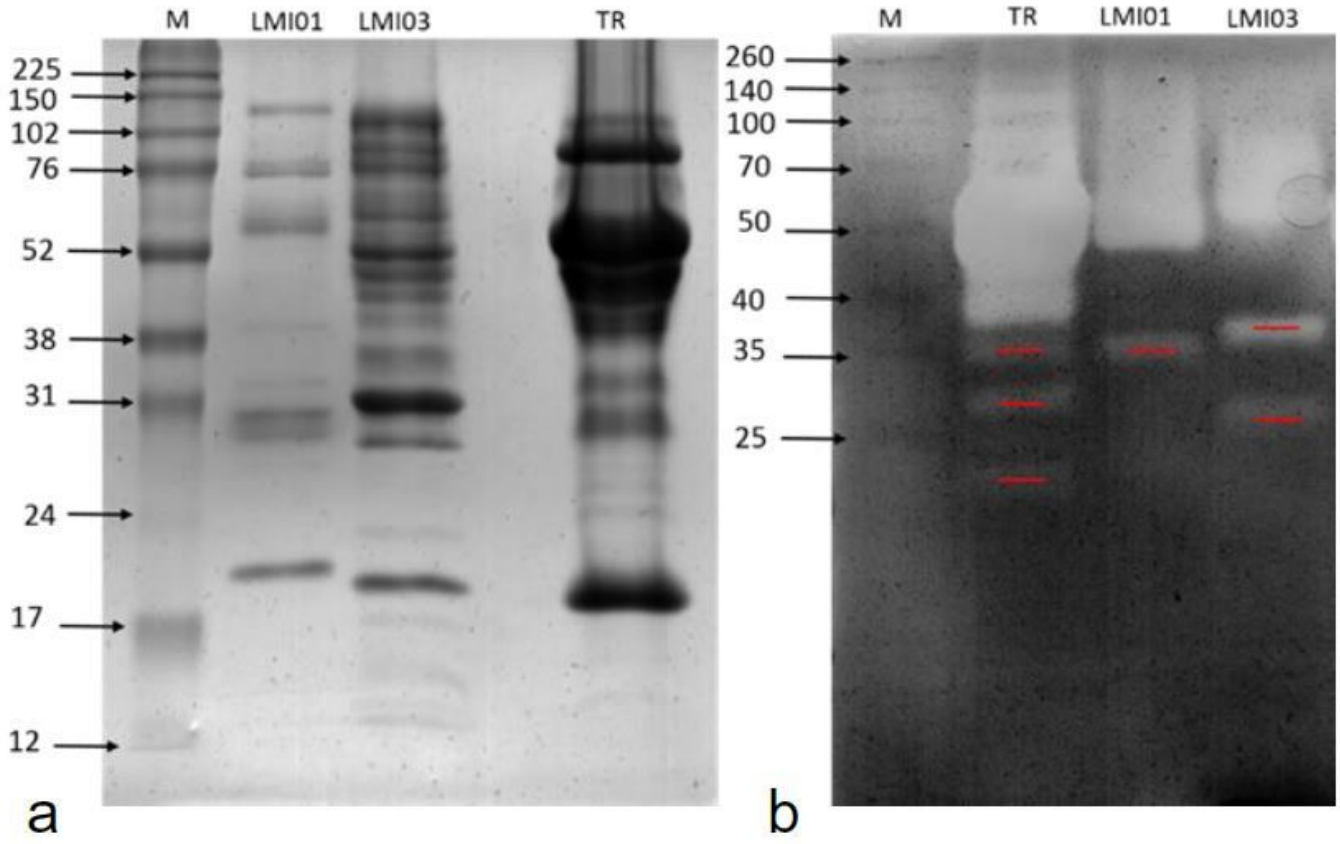

Figure 2

Electrophoretic protein profile and zymogram for cellulases of the crude extract of fungi. a SDS-PAGE polyacrylamide gel stained with Coomassie blue R-250; M: Molecular weight marker (225kDa to 12kDa). LMI01: P. citrinum (6 µg); LMI03: Aspergillus sp (9 µg); TR: T. reesei QM9414 (13.6 µg). b SDS-PAGE gel zymogram (0.2% CMC) stained with 1% Congo red. M: molecular weight marker (260kDa to 10kDa), samples with a concentration of 5 µg of total proteins

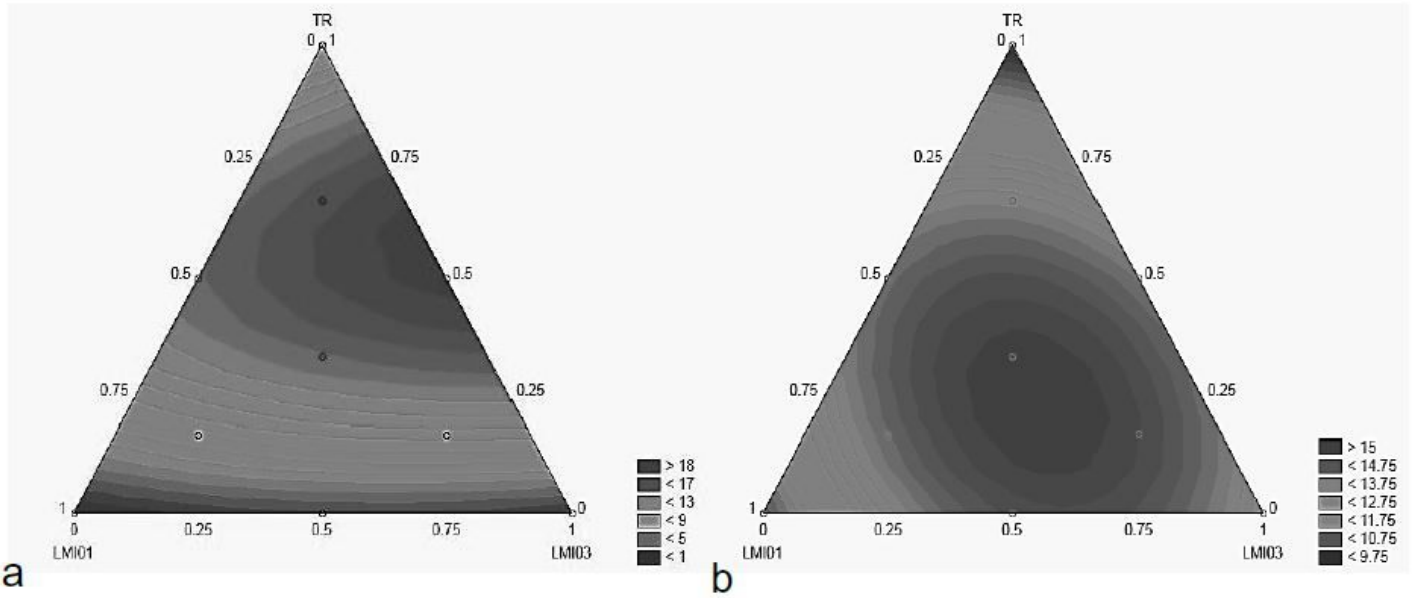

Figure 3

Contour surfaces for quadratic models generated in the mix design. a AMZ1 cocktail for cellulose pulp hydrolysis; b AMZ2 cocktail for cassava peel hydrolysis

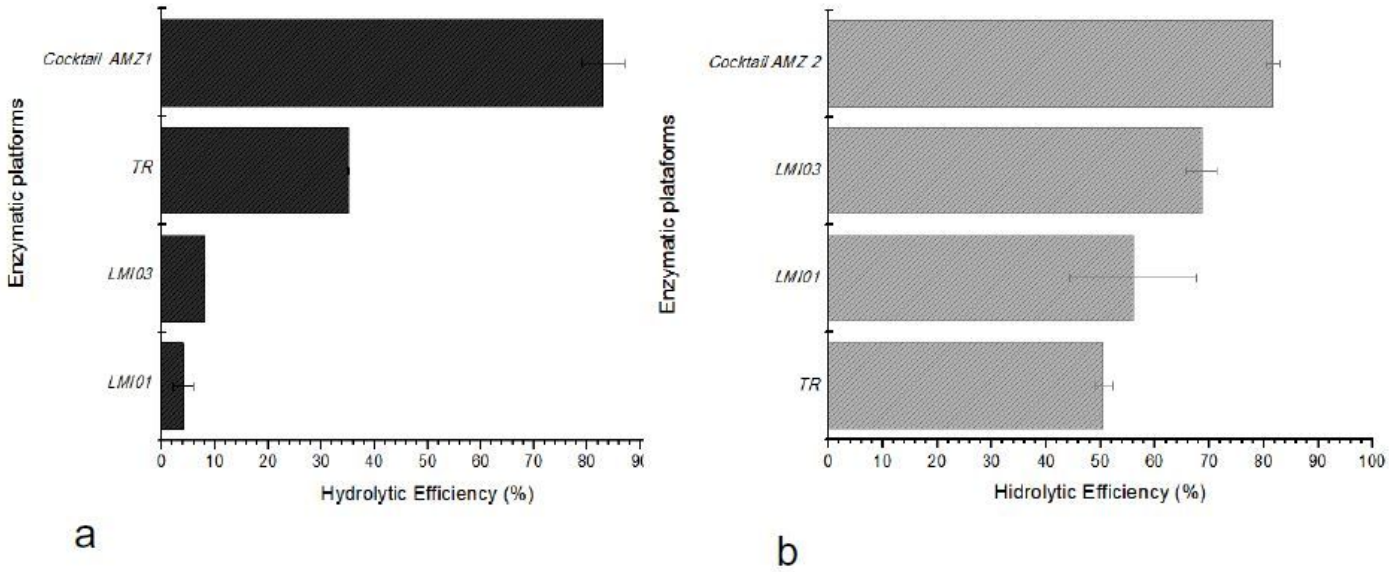

Figure 4

Hydrolytic efficiency of the individual enzyme platforms and of the optimized cocktails, using 15mg of protein/g of cellulose and 25 g/L of solid residue. a hydrolysis of industrial cellulose pulp; b hydrolysis of

cassava peel. LMI01: P. citrinum; LMI03: Aspergillus sp; TR: T. reesei QM9414; Cocktail AMZ1: 47% LMI03 + 53% TR; Cocktail AMZ2: 50% LMI03 + 25% TR + 25% LMI01

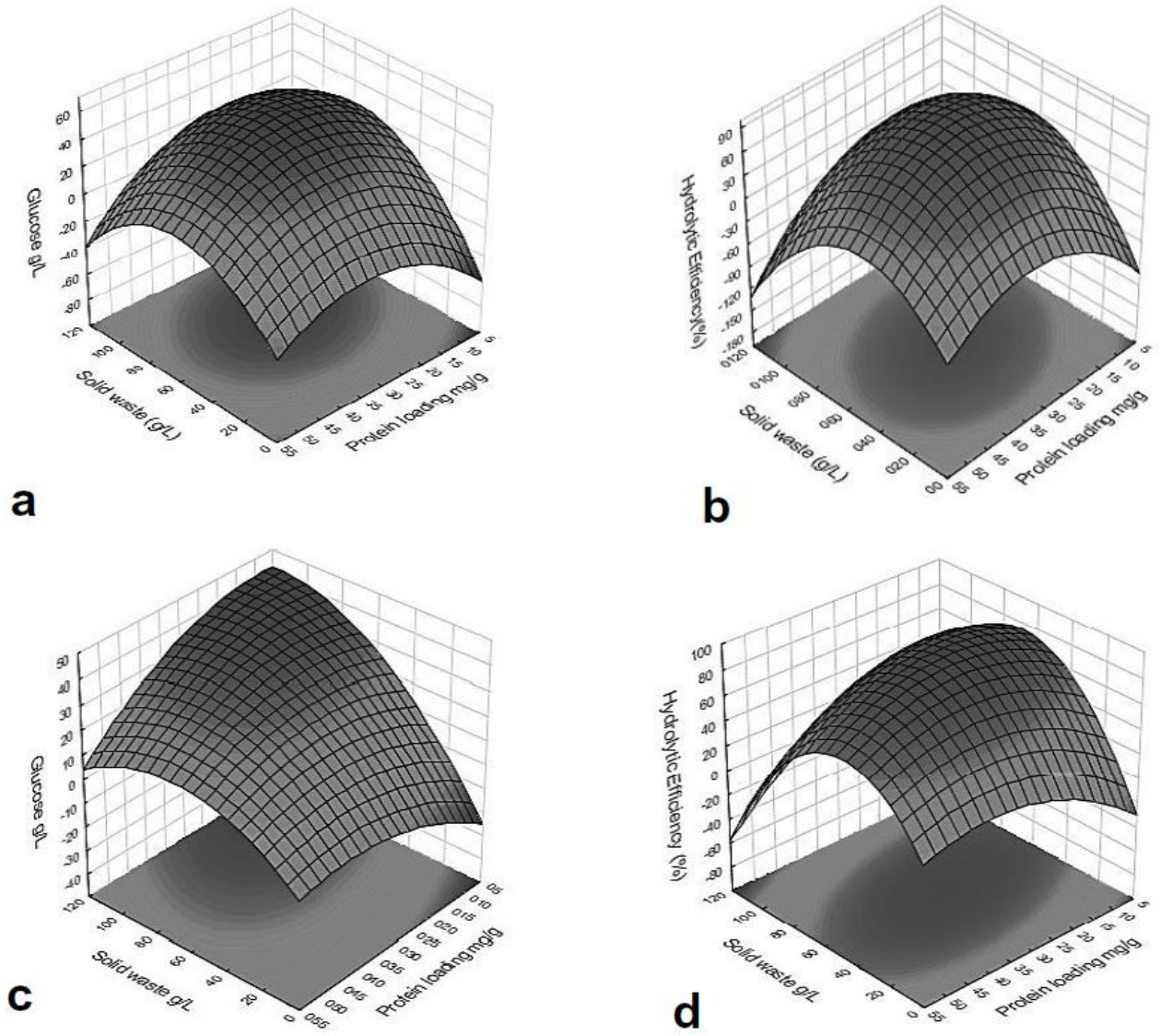

Figure 5

Response surfaces for glucose released and hydrolytic efficiency as a function of loading solid and protein. a glucose (g/L), b Hydrolytic efficiency (%) to AMZ1 cocktail. c and d glucose (g/L) and hydrolytic efficiency (%), to AMZ2 cocktail

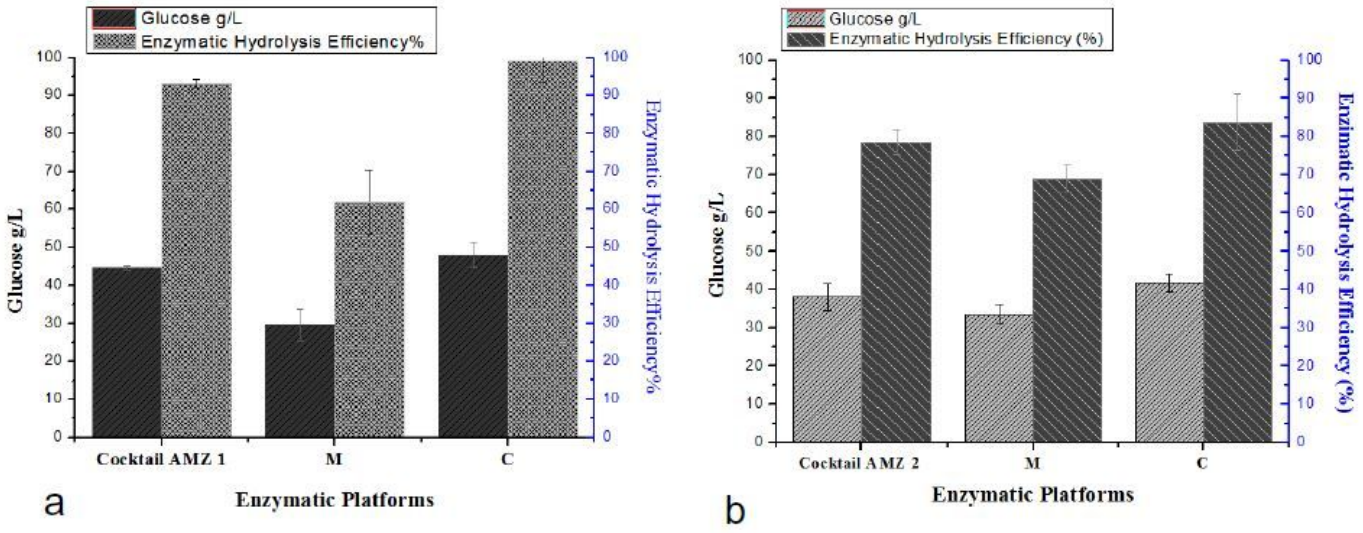

Figure 6

Glucose released (g/L) and hydrolytic efficiency (%) after 48h of hydrolysis at 50°C. a hydrolysis of industrial cellulose pulp (52g/L) with 30mg of protein. b hydrolysis of cassava peel (78g/L) with 19mg of protein. M and C: commercial enzyme Multifect® and Cellic®CTec2 respectively