Hydrolysis of cellulose and oil palm empty fruit bunches by using consortia of fungi isolated from the soil of Colombian high andean forest

Hidrólisis de celulosa y tusa de palma de aceite usando consorcios de hongos aislados del suelo del bosque alto andino colombiano

Luz Aida Moya A.[1] and Esperanza Torres R.[2]

ABSTRACT

Hydrolytic activity was evaluated in a mixture of supernatants produced by filamentous fungi grown individually on microcrystalline cellulose and empty fruit bunches. The strains that were used correspond to two types of isolates; the first was made from soil samples from a transect of a high andean forest of Colombia, in the Parque Natural Nacional de Los Nevados, where, based on previous studies, we selected the strains B7, B11, B11M and B19. The second isolate was obtained from a pool of oil palm empty fruit bunches (*Eleaeis guinensis* Jacq.) in different states of decomposition on the Unipalma plantation located in the eastern plains; strains TA1 and TA2. To perform the hydrolysis of cellulose and empty fruit bunches, the previously obtained supernatants from each of the selected strains were cultivated for 300 hours in cellulose and characterized individually by endoglucanase, exoglucanase, and β-glucosidase activity. Individual supernatants were mixed at a 1:1 ratio to form consortia; and hydrolytic activity was evaluated in the substrates at two hours. The glucose concentration was determined by the 3,5-dinitrosalicylic acid (DNS) method. The results show that hydrolysis of empty fruit bunch to glucose was favored by three pools of supernatants, with increases greater than 400% in comparison with the hydrolysis obtained by individual supernatants, demonstrating the potential to decompose palm empty fruit bunches; thereby contributing to the reduction of decay time of empty fruit bunches and the decrease of environmental and health problems.

Key words: consortia, enzymatic hydrolysis, empty fruit bunches, cellulases, filamentous fungi, biomass.

RESUMEN

Se evaluó la actividad hidrolítica a partir de la mezcla de sobrenadantes producidas por hongos filamentosos cultivados individualmente sobre celulosa microcristalina y tusa. Las cepas utilizadas corresponden a dos tipos de aislamientos, el primero se realizó a partir de muestras de suelo de un transepto del bosque altoandino colombiano del Parque Natural Nacional de Los Nevados, en donde con base en estudios previos, se seleccionaron las cepas B7, B11, B11M y B19. El segundo aislamiento se obtuvo a partir de un pool de tusas de palma de aceite (*Eleaeis guinensis* Jacq.) en diferentes estados de descomposición obtenidos de la plantación UNIPALMA ubicada en los Llanos Orientales, cepas TA1 y TA2. Para realizar la hidrólisis de tusa y celulosa, previamente se obtuvieron los sobrenadantes de cada una de las cepas seleccionadas a las 300 horas de cultivo en celulosa y se caracterizaron individualmente por su actividad endoglucanasa, exoglucanasa y β-glucosidasa. Se mezclaron los sobrenadantes individuales en una proporción 1:1 para formar los consorcios y se evaluó la actividad hidrolítica sobre los sustratos a las dos horas. La concentración de la glucosa se determinó por el método ácido 3,5-dinitrosalicílico DNS. Los resultados muestran que la hidrólisis de tusa a glucosa fue favorecida por tres consorcios de sobrenadantes, con aumentos mayores al 400% en comparación con la hidrólisis obtenida por los sobrenadantes individuales, lo cual demuestra su potencial para degradar tusas de palma. De esta forma se pretende contribuir a la reducción en el tiempo de descomposición de las tusas y disminuir la fuente de problemas ambientales y sanitarios.

Palabras clave: consorcios, hidrólisis enzimática, tusa, celulasas, hongos filamentosos, biomasa.

Introduction

The biological conversion of cellulose, the insoluble, linear homopolymer constituting more than 40% of the ground biomass, occurs through hydrolysis, chemical or biological, by breaking β-1,4-glucosidic bonds. Chemical methods involve the use of strong acids or bases in large quantities, which when released cause an environmental hazard (Singh *et al.*, 2010). In contrast, biological methods provide an alternative with the use of microorganisms or enzymes produced by same, which hydrolyze cellulose to obtain simpler molecules such as glucose.

Biological hydrolysis is generated by enzymes, cellulases, produced by highly diverse microorganisms, including, the most reported due to their ability to produce extracellular

[1] Faculty of biological sciences, Universidad Militar Nueva Granada; Centro de Genómica y Bioinformática-GeBiX. Bogotá (Colombia). etorresr@unal.edu.co

[2] Department of Agronomy, Faculty of Agronomy, Universidad Nacional de Colombia; Centro de Genómica y Bioinformática-GeBiX. Bogotá (Colombia).

enzymes, the aerobic filamentous fungi *Trichoderma reesei* and *Aspergillus niger*, which play an important role in the recycling of this polymer in the biosphere (Yang *et al*., 2011) and are used industrially in the production of cellulases for different uses.

Cellulases are complex enzyme systems consisting of three classes of enzymes: endoglucanases, exoglucanases and β-glucosidases, which are complementary and act synergistically during the degradation process (Lynd *et al*., 2002). Endoglucanase or CMCase (1,4-β-D-glucan-glucanohydrolase, EC 3.2.1.4) breaks non-covalent interactions between linear cellulose chains and links of internal β-1,4-glycosidic leaving more free short cellulose chains, alters the crystalline structure of cellulose and exposes the polysaccharide chains of individual cellulose. Exoglucanase (1,4-β-D-glucan-cellobiohydrolase, EC 3.2.1.91) forms from two or four glucose units from the ends of the exposed chains produced by endoglucanase and releases celotetrosa or cellobiose from non-crystalline cellulose. Finally, β-glucosidase or cellobiase (β-D-glucoside glucohydrolase, EC 3.2.1.21) hydrolyses cellobiose units and short oligosaccharide, obtaining individual monosaccharide glucose (Martins *et al*., 2008; Lynd *et al*., 2002).

The action of cellulases may be used for the transformation of certain substrates of agroindustrial interest, considering the high content of cellulose, as in the case of oil palm empty fruit bunches (*Eleaeis guinensis* Jacq.) (Alam *et al*., 2009; Umikalsom *et al*., 1997). This substrate is the main solid byproduct of the oil production process, which is of great importance within agribusiness in Colombia, Malaysia, Indonesia, Thailand and Nigeria (García-Núñez *et al*., 2008). The empty fruit bunch correspond to 23% of the weight of fresh fruit bunches processed into oil. Just one oil production plant with a capacity of 60 t of fruit bunches per hour produces more than 54,000 t of empty fruit bunches per year, with reported cellulose content between 37 and 62% (Chew and Bhatia, 2008). In Colombia, contents of cellulose and hemicellulose in empty fruit bunches have been reported at 47 and 21% respectively, with 41% C, 0.87% N and 0.09% S, and although some empty fruit bunches are used in boilers for steam production, a plant typically has 30% of the total biomass available for other uses (García, 1993). Due to the heat sterilization process and the temperature to which the clusters are subjected for oil extraction, microorganisms such as enzymes involved in the natural decomposition process of empty fruit bunches are denatured, increasing decay time in the field, causing severe environmental and health problems (Atlas, 2002; Prasertsan and Prasertsan, 1996). Additionally, in many producing areas, environmental authorities increasingly restrict the direct disposal of empty fruit bunches in the field.

Cellulytic activity has been reported on *in vitro* for empty fruit bunches of *A. niger* (Prasertsan and Prasertsan, 1996; Wen *et al*., 2005), *Trichoderma reesei* (Rodríguez and Piñeros, 2007; Wen *et al*., 2005) and *Chaetomium* sp. (Umikalsom *et al*., 1997), among others. However, none of the strains, including the best mutant, are capable of producing high concentrations of the three types of enzymes at the same time (Zhou *et al*., 2008). For example, *T. reesei* produced CBH and EGs in high quantities, but β-glucosidase activity was low in contrast to *A. niger* which produces more β-glucosidase but is limited for EG (Dashtban *et al*., 2009) An alternative to supplement this deficiency is the use of enzymes for hydrolysis of vegetable biomass, either purified or in a culture of supernatants of cellulolytic fungi species, with an exogenous addition which depends on the purpose of the transformation and may be enhanced by glucosidases supplementation and commercial xylanases (Kovacs *et al*., 2009).

As part of the activities the Colombian Center for Genomics and Bioinformatics of Extreme Environments (GeBix), the collection and characterization of filamentous fungi in different ecosystems of the high andean forest of the Parque Nacional Natural de los Nevados was carried out (PNNN) (Avellaneda *et al*., 2009) in order to determine the feasibility of their use in biotechnological processes, in which isolates were obtained *in vitro* with high cellulolytic capacity, determined by activity curves for endoglucanase, exoglucanase, and β-glucosidase. The most promising strains, designated B7, B11, B11M and B19, along with two isolates obtained from palm empty fruit bunch decay (Moya-Álvarez, 2012), denominated TA1 and TA2, were characterized by activity curves for endoglucanase, glucosidase and β-exoglucanase, in a Mandel's medium with cellulose and empty fruit bunches as carbon sources. The most promising isolate corresponds to the strain B19 (*Penicillium* sp.), having an endoglucanase activity of 277.88 *nkat* mL^{-1} and exoglucanase activity of 88.18 *nkat* mL^{-1} at 300 h of incubation in cellulose. Likewise, it was found that the strain B7 has a potential β-glucosidase activity of 25.5 and 17.17 *nkat* mL^{-1}, obtained in cellulose and empty fruit bunches, respectively (Moya-Álvarez, 2012).

As a consequence of the information obtained from the isolates, we conducted the present study, which aimed to determine the ability of hydrolysis of empty fruit bunches and microcrystalline cellulose and the presence of an enzymatic synergy *in vitro* of the enzyme consortia obtained from the mixture of supernatants individually produced

from promising fungi of the PNNN soil and decaying palm empty fruit bunches of the eastern plains of Colombia.

Materials and methods

Fungal strain

The study employed four isolates of filamentous fungi from high-andean forests of the PNNN with a high activity of total cellulase, endoglucanase, exoglucanase and β-glucosidase, in accordance with a previous characterization performed at the Agro-biotechnology lab, Universidad Nacional, called B-7, B-11, B-11M and B-19, corresponding to *Penicillium* sp. (Avellaneda *et al.*, 2009); and two isolates from decaying palm empty fruit bunches called TA1 and TA2 and identified as *Emericella nidulans* strain EN-KSU-09 and *Aspergillus fumigatus*, respectively (Moya-Álvarez, 2012). The initial inoculum was obtained from agar plates with CMC as a carbon source, incubated until the appearance of conidia at 25°C, suspended in water with 1% (v/v) Tween® 80, and homogenized at a concentration of $2 \cdot 10^6$ spores/mL.

Culture medium

A modified Mandel's medium was used, with the following composition: 0.004 g L^{-1} NH_4Cl, 2.1 g L^{-1} $(NH_4)_2SO_4$, 2.0 g L^{-1} KH_2PO_4, 0.3 g L^{-1} $CaCl_2$, 0.3 g L^{-1} $MgSO_4$ $7H_2O$, 0.00156 g L^{-1} $MnSO_4$ $5H_2O$, 0.0014 g L^{-1} $ZnSO_4$ $7H_2O$, 0.00266 g L^{-1} $CoCl_2$ $6H_2O$, 0.25 g L^{-1} yeast extract, 0.01 g L^{-1} succinic acid, 10 g L^{-1} microcrystalline cellulose powder (20 μ) (St. Louis, MO) or empty fruit bunches pretreated with 0.5 M NaOH and 0.5% (v/v) H_2O_2, 29.94 g L^{-1} with a content of 55.5% (w/v) cellulose, 20% (w/v) hemicellulose, and 18.2% (w/v) lignin (Moya- Álvarez, 2012). The pH was adjusted to 5.0 before sterilization (Kumar, 2001; Umikalsom *et al.*, 1997). Supernatants with cellulase activity used for hydrolysis were obtained in 15 mL tubes with 5 mL of culture medium and incubated at 25°C for 300 h with orbital shaking at 130 rpm. The consortia were prepared with a 1:1 mixture of the individual supernatants.

Determination of endoglucanase, exoglucansa and β-glucosidase activity

Before determining the hydrolysis of the supernatants, cellulolytic activity was determined. For endoglucanase activity, the study employed a 50 μL substrate of carboxymethyl cellulose (CMC), (Sigma, St. Louis, MO) 0.8% (w/v) in a sodium acetate buffer pH 5.0 with 50 μL of supernatant from each fungus, according to Ghose (1987). For the exoglucanase determination, a substrate was used of 15 μL of *p*-nitrophenyl-β-D-cellobioside (pNPC) (Sigma, St. Louis, MO) at 1% (w/v) as a substrate under the same conditions

and 100 μL of supernatant. Both reactions were incubated at 40°C for 1 h and 4°C for 10 min. Then, 50 μL of the solution was mixed with 50 μL of a DNS solution (Ghose 1987), 1% (w/v) NaOH and 16% (w/v) sodium potassium tartrate, 43.8% (v/v) distilled water, for determination of released reducing sugars. The mixture was incubated for 5 min at 90°C and then cooled 10 min at 4°C. Then, 50 μL of the above solution were added with 250 μL of distilled water. Absorbance readings were made at 540 nm in a microplate reader model BioRad 680XR (Bio- Rad Laboratories, Hercules, CA) from the Biotechnology Laboratory, Faculty of Agronomy, Universidad Nacional de Colombia. Calibration curves were made with glucose (0.1, 0.7, 1.5, 2.5 and 3 g L^{-1}) for endo-and exoglucanases taking into account that one unit of enzymatic activity corresponds to 1 μmol min^{-1} of glucose released during hydrolysis.

Determination of β-glucosidase activity

We used 180 μL of *ρ*-nitrophenyl-β-D-glicopiranósido (PNPG) (Sigma, St. Louis, MO) as a substrate and 50 μL of supernatant. Following incubation with the substrate, 20 μL of this solution were added with 80 μL of 2% sodium carbonate and 180 μL water. We measured the release of *p*-nitrophenol as the increase in absorbance at 405 nm and used pNitrofenol (10, 20, 30 and 50 μg mL^{-1}) for the calibration curve of β-glucosidases (Sadana and Patil, 1988). In all cases, the tests were performed in triplicate.

Determination of hydrolysis of supernatant consortia

The fungal cellulases for the study were obtained from 15 mL Falcon tubes with 4 mL of modified Mandel's medium with cellulose (Umikalsom *et al.*, 1997) as the carbon source, incubated under stirring at 130 rpm and 25°C for 300 h. Each culture was centrifuged at 4.500 g_n for 15 min. The supernatant corresponded to the crude enzyme extract. Enzymatic hydrolysis was performed in triplicate in 1.5 mL tubes with 100 μL of acetate buffer and 100 μL of cell-free supernatants containing the enzyme, with 1% (w/v) microcrystalline cellulose and empty fruit bunches 5% (w/v) as the substrate at 45°C and 175 rpm on an orbital shaker MaxQ 4000 (Barnstead/Labline, Melrose Park, IL) for 2 h. The total volume of enzyme extract was 100 μL, and was divided according to the number of strains in each mixture. Thus, for the mixture of supernatants obtained from two strains each volume was 50 μL and for mixtures of four strains, it was 25 μL. A control with acetate buffer pH 5.0 was used. The glucose obtained was determined at 2 h of incubation with the DNS method (Ghose, 1987). A factorial model was used with carbon source as the main factor and strain as a secondary factor

Determination of synergy between the enzymes of the consortium

The determination of the synergy between the enzymes when acting in consortium was performed as reported by Andersen *et al*. (2008). Wherein, a measure of synergy can be calculated as the ratio of the activity exhibited by a mixture of components divided by the sum of activities of the components separately and which may be obtained based on the product formation, or the extent of substrate conversion. This measure is called the degree of synergy (DS). When the DS is greater than 1, there is said to be synergy between the components of the consortium, and competition when it is less than one.

Statistical analysis

Statistical analysis of the data was performed using a factorial model with carbon source, as the main factor, at two levels: cellulose or empty fruit bunches called "tusa". The secondary factor was strain with different levels according to the strains or consortia supernatants used. The evaluated variables correspond to protein biomass, endoglucanase, exoglucansa and β-glucosidase activity. Then Tukey and Sheff method with a significance level of $P \leq 0.05$ and 0.01 were used to detect statistically significant differences among of variable used. Each experiment was done in triplicate and the analysis of variances was conducted using SAS (v. 9.1) statistical software.

Results and discussion

Determining the hydrolyzing ability of the enzyme consortia

The supernatants used for forming consortia were individually characterized by endoglucanase, exoglucanase, and β-glucosidase activity. The cellulase activity of the supernatants used for hydrolysis is seen in Fig. 1. The results obtained from hydrolysis with the supernatants obtained from the individual strains and their mixtures (consortia) are presented in Fig. 2. B19 was observed with the greatest hydrolysis compared with supernatants produced by the individual strains. Given this fact, the hydrolysis of B19 was used as a comparison with that produced by the consortia and assumed to produce 100% hydrolysis.

Highly significant differences were observed in the microcrystalline cellulose hydrolysis due to the interaction consortium x substrate ($P \leq 0.01$). In the consortia that showed significant differences, most hydrolysis occurred in empty fruit bunches with less crystallinity than microcrystalline cellulose and possibly a lower degree of polymerization. In this regard, it has been reported that the microcrystalline cellulose is degraded more slowly to cellobiose and glucose than amorphous cellulose, which is more accessible to enzymatic action (Yang *et al*., 2011).

In empty fruit bunches, the consortia of B19B11, B19B11M and B19TA1 produced higher hydrolysis as compared to that of B19 individually at 86, 110 and 111%, respectively, as presented in Tab. 1. There may be a synergistic effect between the supernatants mentioned. The hydrolysis of the more efficient consortia was close to that shown by the supernatant of B19 with 3.5 times greater enzymatic activity and was obtained from the individual supernatant of B19 at 300 h of growth, according to preliminary studies (Moya-Álvarez, 2012). A similar effect was reported by Jørgensen *et al*. (2005) who, with increased β-glucosidase activity, obtained a saccharification of the substrate similar to that obtained with a lower activity.
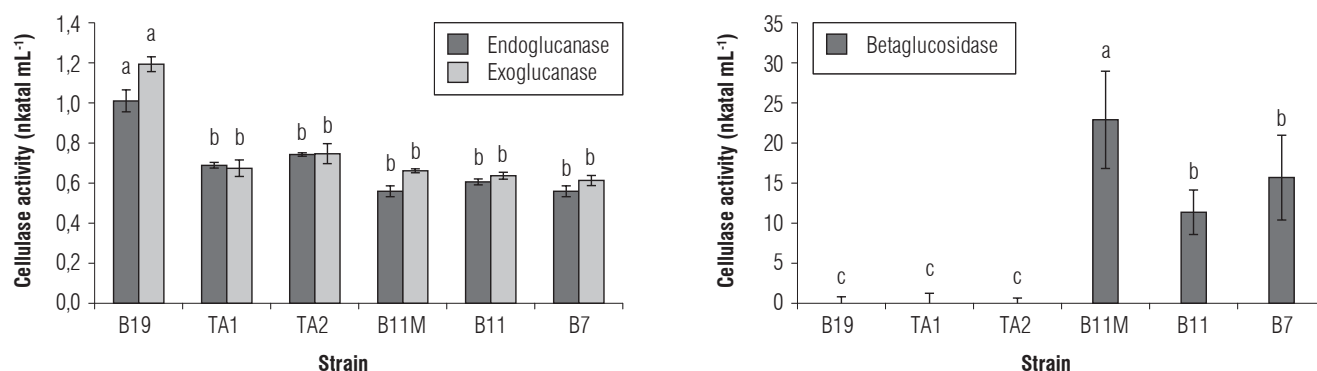

FIGURE 1. Enzymatic activity of the supernatants used for hydrolysis. The substrate used for determining the activity of each enzyme is indicated in the materials and methods section, carboxymethyl cellulose (CMC) for endoglucanase activity, ρ-nitrophenyl-β-D-cellobioside (pNPC) and ρ-nitrophenyl-β-D -glicopiranósido (PNPG) for β-glucosidase and exoglucanases respectively. Values are mean of three determinations and error bars are standard deviations. Andean forest strains: B19, B7, B11 and B11M. Empty fruit bunch isolates: TA1 and TA2. Different letters indicate significant differences in enzyme activity in evaluated substrates. ($P < 0.01$).
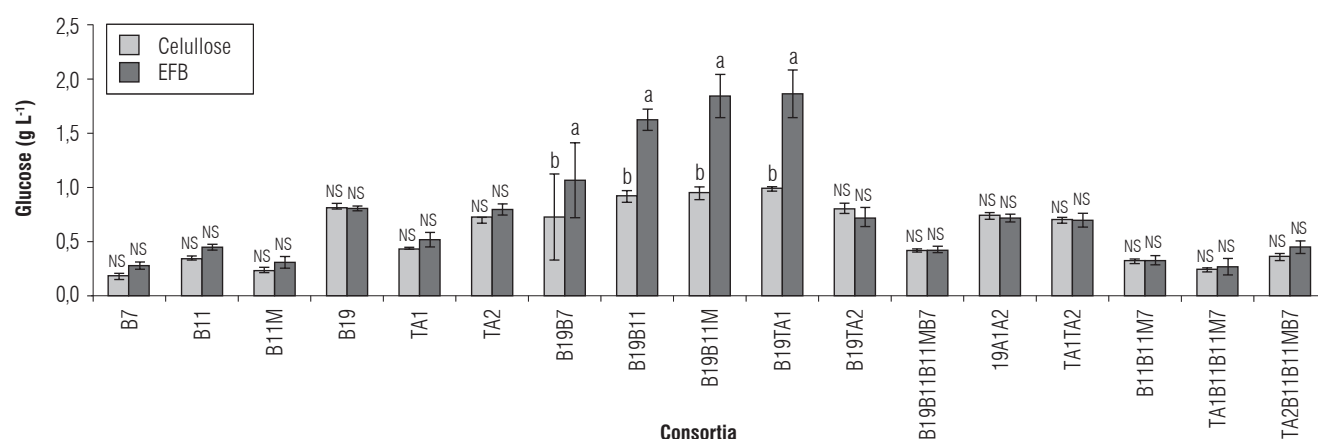
FIGURE 2. Empty fruit bunch (EFB) and cellulose hydrolysis produced by consortia supernatants of homogeneous enzyme activity. Andean forest strains: B19, B7, B11 and B11M. Empty fruit bunch isolates: TA1 and TA2. Different letters indicate significant differences ($P \leq 0.01$). NS: no significant differences.

TABLE 1. Comparison of the percentage of hydrolysis of cellulose and empty fruit bunches of the B19 supernatant, with respect to the supernatant consortia. Hydrolysis for 2 h in acetate buffer at 45°C with stirring at 175 rpm. Each value is the average of three replicates.

Strain consortium	Hydrolysis with respect to B19 B19 (%)	
	Cellulose	Empty fruit bunch
B19		
B19	100.00	100.00
B11	50.08	62.27
B7	32.68	43.58
B11M	38.76	47.37
TA1	59.54	69.24
TA2	86.48	98.70
B19B7	89.86	127.13
B19B11	108.94	186.73
B19B11M	112.41	210.07
B19TA1	116.29	211.79
TA1 TA2	86.73	87.93
B11B11MB7	46.95	49.09
B19TA1TA2	90.28	90.78
B19B11 B11MB7	57.76	59.94
TA1B11B11MB7	38.51	42.97
TA2 11 B11MB7	51.34	61.84

According to the results, consortia culture supernatants produced individually could reduce empty fruit bunch hydrolysis costs considering that supernatants are obtained with lower enzyme activity, requiring less time of incubation with the consequent reduction in consumption energy.

When comparing the hydrolysis of each individual supernatant with that obtained when in consortium with B19 (Tab. 2), it was observed in empty fruit bunches, that the consortium hydrolysis of the B19 supernatant with TA1 was 306% greater than that obtained individually for TA1.

For B11M, hydrolysis with B19 was 441% higher compared to B11M individually. In cellulose, although in all cases hydrolysis increased, the rate of increase was smaller; only in the TA2 consortium was hydrolysis higher in cellulose than empty fruit bunches.

With the information obtained from the hydrolysis of the individual strains and consortia, the degree of synergy was calculated for the consortia based on the glucose concentration obtained as a product of the hydrolysis of cellulose and empty fruit bunches by supernatants corresponding to a mixture of the enzymes endoglucanase, exoglucanase, and β-glucosidase produced by each strain (Tab. 3). It was determined that consortium synergy occurs only in empty fruit bunches in the consortia B19B11, B19B11M and B19TA1 with DS values greater than 1. In other consortia, there was competition, both for empty fruit bunches and cellulose, as the DS was less than 1. Interestingly, there was synergy between B19, the strain with the most endoglucanase and exoglucanase activity, and B11M and B11, the strains with the most β-glucosidase activity, as compared with TA1 and TA2. In these consortia, endoglucanase and exoglucanase initiated hydrolysis and β-glucosidase hydrolyzed cellobiose produced by the two primaries to produce glucose, as described in the literature as one of the possible action mechanisms of the cellulase enzyme complex. This effect has been observed in enzyme consortia of *A. niger* with *T. reesei,* the latter deficient in β-glucosidase, essential to convert cellobiose to glucose (Alam *et al.*, 2005). The synergy of endoglucanases and exoglucanases with β-glucosidase has been described in the enzyme interaction of *P. pinophilum* and *T. koningii* (Wood and McCrae, 1986).

TABLE 2. Evaluation of B19 potentiation over the hydrolytic action of supernatants of five strains of fungi *vs*. the endoglucanase activity and hydrolytic activity ratio, individual and consortia.

Strain	Endoglu-canasa activity ($nkat$ mL^{-1})	Exoglu-canasa activity ($nkat$ mL^{-1})	β-glucosidasa activity ($nkat$ mL^{-1})	Hydrolysis of the individual strain (mg glucose/mL)		Consortium hydrolysis with B19 (mg glucose/mL)		Percentage of consortium hydrolysis with B19 (%)	
				Cellulose	Empty fruit bunch	Cellulose	Empty fruit bunch	Cellulose	Empty fruit bunch
B19	1.0168	1.217	Non detectable	0.721	0.707	-	-	-	-
TA1	0.683	0.683	Non detectable	0.429	0.489	0.839	1.498	195.57	306.34
TA2	0.751	0.750	Non detectable	0.624	0.698	0.708	0.646	113.461	92.550
C 11	0.600	0.650	11.352	0.361	0.44	0.786	1.32	217.72	300
C 7	0.567	0.617	15.670	0.235	0.308	0.64	0.89	272.34	288.96
B11M	0.567	0.667	22.888	0.279	0.335	0.81	1.48	290.32	441.79

TABLE 3. Degree of synergy of the evaluated consortia, corresponding to the activity of the mix/sum of observed individual activity. Each measurement is the average of three replicates.

Consortium	Degree of synergy (DS)	
	Cellulose	Empty fruit bunch
B19B7	0.670	0.877
B19C11	0.726	1.151
B19B11M	0.810	1.420
B19TA1	0.729	1.252
B19TA2	0.526	0.460
B19TA1TA2	0.367	0.339
B19B11B11MB7	0.261	0.237
TA1TA2	0.594	0.523
B11B11MB7	0.386	0.320
TA1 B11 B11M 7	0.213	0.193
TA2 B11 B11M 7	0.2467	0.245

The results obtained in this study do not agree with the claim of Andersen (2008) that as a rule the highest DS occurs in highly crystalline substrates (such as crystalline cellulose and cotton) and decreases with the decrease of the crystallinity of the substrate. In this case, the higher obtained DSs were in empty fruit bunch consortia, a substrate with crystallinity lower than microcrystalline cellulose, where DSs were lower.

The hydrolysis produced by B19, 0.721 and 0.707 g L^{-1}, respectively for microcrystalline cellulose and empty fruit bunches, with supernatants with 5.001 $nkat$ mL-1 of endoglucanase activity and $nkat$ mL-1 of exoglucanase activity was similar to that obtained by Ariffin *et al*. (2008) with 0.73 g L^{-1} of reducing sugars in untreated empty fruit bunches using a pure enzyme that contained 941.85 $nkat$ mL^{-1} total cellulase, 2458.825 $nkat$ mL^{-1} endoglucanase, and 280.05 $nkat$ mL^{-1} β-glucosidase. Considering the difference in the activity of enzymes used in this study, one can conclude that the B19 supernatants used in this study are highly efficient. The subsequent evaluation of pretreatments is also important, increasing availability of cellulose since the synergy could be modified with actions over the availability of cellulose in empty fruit bunches with pretreatments that

reduce the concentration of lignin and hemicellulose as has been previously demonstrated (Simarani *et al*., 2009).

In this study, it was determined that the consortia B19B11, B19B11M and B19TA1 obtained 1.5 g of reducing sugars per g empty fruit bunches 5% (W/V) and 0.8 g g^{-1} of cellulose 5% (W/V) with supernatants for B19 with 1.02, 0.061, and 1.22 $nkat$ mL^{-1} of endoglucanase and exoglucanase activity, respectively, 0.04 and 0.67 $nkat$ mL^{-1} for the other strains (Tab. 2). This result agrees with those previously obtained by Umikalsom *et al*. (1997) who obtained 0.7 g reducing sugars per g of delignified empty fruit bunches (78% cellulose and 5.5% lignin) with enzymes of *Chaetomium globosum* with 250 $nkat$ mL^{-1} PFasa, 7134.7 $nkat$ mL^{-1}, CMCase (endoglucanase) and 1500 $nkat$ mL^{-1} β-glucosidase. The supernatant of B19, acting individually, produced 0.75 g of reducing sugars for both empty fruit bunches and cellulose. Other authors reported that an endophytic fungus isolated from the paramo of Cruz Verde (Cundinamarca) was able to produce more than 25 g L^{-1} of reducing sugars in 6 d with chemically pretreated empty fruit bunches as the carbon source, possibly due to the action of endo and exoglucanases (González *et al.,* 2008). These data, however, are not comparable with those obtained by the strains under the present study; the evaluation was performed at 2 h of incubation and cannot be extrapolated to 6 d since the linearity of the reaction is not known. In general, one could say that the time of empty fruit bunch digestibility is enhanced by a reduction in crystallinity index of cellulose and an increase in surface area (Park *et al.,* 2010). Likewise, the evaluation would be needed over time, where factors of stability of the supernatant enzyme mixture would be related. It is possible that the consortia that effectively increased empty fruit bunch digestion contributed to the reduction of surface roughness of the crystalline cellulose with the increased traffic of cellulases, flattening the surface, removing obstacles and increasing the number of inputs and outputs of enzymes which reduce congestion,

improve mobility of cellulases and increase the efficiency of the hydrolysis (Igarashi *et al.*, 2011).

Conclusions

The study allowed us to determine the ability of empty fruit bunch and cellulose hydrolysis, *in vitro*, by consortia of enzymes obtained from the mixture of supernatants produced by fungi from a high andean forest transect of the Parque Natural Nacional de Los Nevados and decaying empty fruit bunches of individually cultivated oil palms. The efficiency of the hydrolysis of empty fruit bunches by supernatants of consortia of B11, B11M and TA1 with B19 is greater than that obtained by the supernatants produced by each strain individually, with hydrolysis capacity increases up to 400%. The action of such consortia could be complemented by the timely addition of supernatants of B7 due to the β-glucosidase activity. The dilution of the activity of the supernatant of B19, 5.001 to 1.002 *nkat* mL^{-1} of endoglucanase activity, does not reduce the capacity of hydrolysis in consortia.

Promising consortia here show great potential and the action can be optimized with the study of the characteristics of the enzymes involved and their interactions. Thus, it would be possible to obtain a balanced synergy that takes into account: suitable combinations of enzymes, which maximize hydrolysis, achieve higher performance and reduced glucose production costs. It is necessary to take into account aspects such as the proportion of supernatants of each strain of the consortium, time in which they are added, pH, agitation, reaction temperature and reaction medium. It is also important to note that to completely disassemble heterogeneous structures of plant cell walls, reactions also require synergy with other enzymes such as hemicellulases and ancillary enzymes and modifying lignin (Yang *et al.*, 2011).

The use of consortia of supernatants from cultivations of the studied fungi has great potential for the initial treatment of empty fruit bunches after oil extraction, considering that the temperature after the sterilization process in the processing plant may be optimal for the action of cellulases. Thus, hydrolysis to glucose could be initiated more rapidly in empty fruit bunches, which could allow a faster colonization by decomposing microorganisms with an easily employed increase in substrate concentration and could therefore reduce decomposition time, which should be further tested. On the other hand, subsequent studies could be directed towards optimizing hydrolysis to glucose with different purposes.

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