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Article

Bagasse of *Agave tequilana*, *A. angustifolia* and *A. salmiana* for cultivation of the fungus *Pleurotus ostreatus*

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Abstract

The residues of tequila and mezcal are a problem since they are not properly managed; for this reason, bagasse from three species of agave (*A. tequilana*, *A. angustifolia*, and *A. salmiana*) was used as a substrate for the cultivation of the fungus *P. ostreatus*, as proposal for adding value to bagasse and to avoid clandestine landfills and contamination. The research was carried out at the University Center of Los Lagos, in Lagos de Moreno, Jalisco in 2019. Mixtures of each of the substrates were made with cattle manure or corn stubble, both in proportions of 20 and 30%; in addition to these mixtures, another substrate was made, 50% *A. angustifolia* plus 50% *A. salmiana*; in total, 16 experimental models and a control (100% corn stubble), all in quadruplicate, in order to know which mixture can generate better biological efficiency (BE), production rate, and yield. The efficacy was obtained by comparing the morphological measurements of the fruiting body: cap length and width, stem length and diameter, and the percentage of degradation capacity of the substrates by measuring the biodegradation rate. An improvement in biological efficiency, production rate, and yield was observed for *A. salmiana* and *A. angustifolia*, and *A. tequilana* did not improve when mixing with corn stubble or manure compared to the use of *A. tequilana* alone. Therefore, the use of bagasse of *Agave* spp. is suitable for the cultivation of *P. ostreatus* since a medium biological efficiency was obtained, which can be improved by adding nitrogen; this allowed the bagasse to be used and its accumulation in polluting landfills to be avoided.

Palabras clave:

Pleurotus spp., *Agave* spp., productive parameters, substrates.

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Introduction

Agro-industrial waste should be considered raw material for other processes. This research focuses on the bagasse of *Agave* spp. that is used for the manufacture of bricks, paper, livestock feed, mattress filling, obtaining alcohols and organic acids, but it is not enough for the large amount of bagasse generated in the production of tequila and mezcal (Iñiguez *et al*., 2005; González *et al*., 2005). Agave bagasse has been applied to sow *P. ostreatus* (mushroom); cultivating it is economical and simple since it is a viable alternative; it is known that it has been used for the manufacture of bricks, paper, livestock feed, as well as mattress filling.

The fungus is lignocellulosic; it rapidly and effectively degrades cellulose, hemicellulose and lignin, the main compounds in bagasse (Heredia *et al*., 2014); in addition to the fact that it has a large amount of nutrients and protein, growing this mushroom is inexpensive and easy; Mexico ranks first in production within Latin America, generating around 80.8% of the total production of the region, ranking 13th worldwide.

Some of its advantages are the ease of its cultivation, the investment required is minimal, it does not require a large infrastructure, its price is accessible to the consumer and it has a good flavor; in addition, it is an ecological conversion crop so it can be a profitable alternative for agave bagasse (Heredia *et al*., 2014; Romero *et al*., 2018). The area of denomination of origin of tequila and mezcal has constant growth in production and therefore greater amount of bagasse, according to the Tequila Regulatory Council (CRT), for its acronym in Spanish, in 2019, 351.7 million liters of tequila and a byproduct of 537.04 thousand tons of bagasse were produced (CRT, 2019).

For this reason, a mixture of agave bagasse, corn stubble and cattle manure were created to obtain a substrate of high biological efficiency (BE) to grow *P. ostreatus*. The purpose of this research was to create mixtures based on agave bagasse together with corn stubble and cattle manure in order to obtain a substrate of high biological efficiency (BE) for the cultivation of *P. ostreatus*.

Materials and method

The work was conducted in a greenhouse of the University Center of Los Lagos, University of Guadalajara. Av. Enrique Díaz de León No. 1144, Col. Paseos de la Montaña, Lagos de Moreno, Jalisco, Mexico. The bagasse of *A. salmiana* and *A. angustifolia* was obtained in San Felipe, Guanajuato, donated by the Villasuso and DYPICURIAN mezcal factories (Figures 1 and 2); the bagasse of *A. tequilana* was donated by Tequila Centinela in Arandas, Jalisco; the cattle manure and corn stubble were obtained from farms in the region and a commercial strain (seed) of the Prodiset brand variety BPR-246 was used.

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Figure 1. Stone mill in Villasuso factory in San Felipe, Guanajuato, to obtain bagasse from *A. salmiana* and *A. angustifolia*.

Figure 2. Bagasse of *A. salmiana* and *A. angustifolia* of DYPICURIAN in San Felipe, Guanajuato.

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Substrate preparation

Substrate formulation (dry weight percentages): carbon source (agro-industrial waste) 78%, nitrogen source (cattle manure, corn stubble) 20%, sugar 1% and lime 1% (it raises the pH of the substrate), water (moisture) 65-75% (Zamora, 1998; López-Rodríguez *et al*., 2008).

Substrate pretreatment

Prior to pasteurization, the agro-residue of *A. salmiana*, coming from an artisanal mezcal company, had dimensions of 15-20 cm, and as it was necessary to have smaller particles for moisture retention, cuts were made with a machete (Figure 3) (Gaitán *et al*., 2012). All bagasse was exposed to the sun (Figure 4) to dry; when subjected to manual pressure, they broke (López-Rodríguez *et al*., 2008). In order to be able to make the dry weight relationship with the mushroom obtained at the time of measuring the BE.

Figure 3. Cuts of *A. salmiana* with a machete.

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Figure 4. Sun exposure of *A. angustifolia* to remove moisture.

The sun exposure of the substrates was: *A. salmiana* three days, the fibers were bound together and retained moisture; *A. angustifolia* two days, its fibers were spread and separated and *A. tequilana* four days, since it was donated immediately after juice extraction and contained greater moisture.

Greenhouse temperature

The mycelium of *P. ostreatus* grows in temperatures between 0 and 35 °C, the optimal growth temperature is 30 °C. On the other hand, to stimulate fruiting, a temperature between 18 and 25 °C is needed (Garzón and Cuervo, 2008; Martínez, 2014); constant measurements were made with a digital hygro-thermometer (Steren 0.0) To control the parameter.

Relative humidity of the greenhouse

The optimal humidity should be 50-80% (Garzón and Cuervo, 2008; Martínez, 2014); measurements were made with a digital hygro-thermometer (Steren 0.0); humidity was regulated with irrigation (micro-sprinkler) between 11:00 am and 12:00 pm and 4:00 and 5:00 pm by wetting walls and the ground, as well as directly to the substrates, containers with water were also placed. Temperature and humidity measurements were taken before and after irrigation to keep track of the parameters and standardize the process. For the fruiting phase, it was necessary to increase the humidity, between 85 and 90%, so the irrigation times were extended (Garzón and Cuervo, 2008; Martínez, 2014).

Greenhouse aeration

The space was completely covered with black polyethylene plastic to avoid air currents, a condition that was maintained during the 20 days of the incubation phase. Once the phase was finished, two sections of plastic were removed: back and front walls, and shade mesh was left (Figure 5) to increase the ventilation and oxygenation required for the fruiting phase, which allowed air to enter and decreased CO2 (Martínez, 2014).

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Figure 5. Opening of the nursery for ventilation and light entry.

pH in the growing medium

The growth of *P. ostreatus* has an optimal pH range between 5.5 and 6.5; it was measured and controlled by adding calcium carbonate of 20-30 g kg^{-1} of wet substrate (Garzón and Cuervo, 2008).

Inoculation

Because the strain had remained refrigerated, it was necessary to expose it to room temperature for 40 h prior to cultivation and reactivate its metabolism (Martínez, 2014). In a closed and clean area, the substrate was inoculated in maximum asepsis with the use of gloves and face masks (Escobedo, 2008). Bags 1-16 and 21-28 were inoculated on April 23, 29-64 on the 24th and 17-20 and 65-72 on the 25th. Each replication was arranged in different sectors of the greenhouse, upper and middle level, except for pure manure, pure stubble and *A. salmiana* with *A. angustifolia*, which were placed on the lower level (Table 1).

Table 1. Number of bags corresponding to each of the mixtures to be used.

No. bags	Mixture of substrate	No. bags	Mixture of substrate	No. bags	Mixture of substrate
1-4	St	29-32	A+St20%	57-60	S+St30%
5-8	T	33-36	A+M20%	61-64	S+M30%
9-12	T+St20%	37-40	A+St30%	65-68	S+A
13-16	T+M20%	41-44	A+M30%	69-72	M
17-20	T+St30%	45-48	S	73-76	St
21-24	T+M30%	49-52	S+St20%	57-60	S+St30%

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No. bags	Mixture of substrate	No. bags	Mixture of substrate	No. bags	Mixture of substrate
25-28	A	53-56	S+M20%		

St= stubble; M= manure; T= *A. tequilana*; S= *A. salmiana*; A= *A. angustifolia*.

The substrate inside the bag was slightly compressed. The added mycelium was equivalent to 5% of the total weight of the wet substrate; it was then closed without air to prevent death from poisoning and labeled (date of inoculation, bag number, and mixture code) (Varnero *et al*., 2010; Martínez, 2014).

The bags were perforated the day after sowing with a disinfected cutter to promote a condition of semi-anaerobiosis (incubation phase); the luminosity was low (less than 100 lux), which was verified with an Amprobe LM-120 luxmeter (Gaitán *et al*., 2006; Varnero *et al*., 2010).

Fruiting

In this phase, part of the plastic was removed to provide ventilation and fresh air entry, reduce CO_2, and increase the luminosity to 200 lux, optimal light for fruiting. Since this phase is aerobic, the holes in the plastic bags were enlarged and they were later removed (Martínez, 2014) to make way for the primordia.

Harvesting, weighing, and measuring carpophores

The primordia emerged in one week, they are shown in Figure 6; they were harvested when the cap was flat, maximum moment of growth (Figure 7); a cut was made at the base of the stem to prevent the tissue from being susceptible to rot and contaminate the fruiting area. On average, two to four harvests are produced, but only three were considered so that the quality of the mushroom does not decrease (Gaitán *et al*., 2006).

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Figure 6. Primordium in bag no. 35 A+M20%.

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Figure 7. First fruiting of sample no. 3.

The weight of the carpophores was calculated after cutting with a digital scale (Precisa BJ4100D), they were separated from the bunches and the stem and cap were left; the length and width of the cap and stem were obtained with a Vernier (Karlen, accuracy ±0.2 mm), this procedure was performed during the three harvests (Lopez *et al*., 2008).

BE, PR and Yi variables

Biological efficiency (BE) consists of the bioconversion of energy and the biodegradation of the substrate, thus expressing the yield of fresh fruiting bodies (Heredia-Solis *et al*., 2014). The BE basically depends on the composition of the substrate and these can have a value ranging from 20% using leaves as substrate to approximately 160% using coffee pulp as substrate (Heredia-Solis *et al*., 2014).

The production rate (PR) is the relationship between biological efficiency and the time in days from inoculation to harvest (Martínez *et al*., 2014). Yield (Yi) is defined as the ratio in percentage of the weight of fresh mushrooms to the wet weight of the substrate (Garzón and Cuervo *et al*., 2008).

The biodegradation rate (BR) is the variable that measures the percentage of degradation carried out by a biological agent and is determined by the difference between the dry weight of the initial substrate and the dry weight of the final substrate divided by the dry weight of the initial substrate multiplied by 100 (Romero *et al*., 2018). An Anova statistical analysis (Tukey, *p*# 0.05) was performed for each variable with the Sas program, version 9.4.

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Results

Temperature, relative humidity, and light

The experiment was carried out in spring-summer 2019, with temperatures of 20.5-38 °C and humidity of 50-80%. A visual measurement of mycelium growth was performed 15 days prior to inoculation, graded from 1-5, where 1 is minimal growth and 5 when mycelium is found throughout the substrate (Table 2).

Table 2. Mycelium growth in the substrates used after 15 days of sowing.

Substrate	Growth	Substrate	Growth	Substrate	Growth
St	5	A	2.25	S+St20%	1.5
T	4.75	A+St20%	4.25	S+St30%	2.25
T+St20%	3.5	A+St30%	5	S+M20%	2.25
T+St30%	4.25	A+M20%	3.25	S+M30%	1.75
T+M20%	4.5	A+M30%	3.75	S+A	1.25
T+M30%	4.5	S	1.25		

St= stubble; M= manure; T= *A. tequilana*; S= *A. salmiana*; A= *A. angustifolia*.

The light was 2-20 lux. The primordia emerged 19 days after inoculation in St, T+St and T+M substrates, as they appeared, the holes were enlarged until the bags were completely removed; a back and a front section were removed on day 21 to give oxygenation and light (80-170 lux).

BE, PR and Yi variables

The growth of the fruiting body was satisfactory. BE outperformed the control substrate (Table 3). The PR is the product-time ratio and is obtained by dividing the BE by the number of days it took to produce the three crops since their inoculation.

Table 3. Comparison of BE of each species. 1) *A. tequilana*; 2) *A. angustifolia*; 3) *A. salmiana*; and 4) *A. salmiana* + *A. angustifolia*.

[1] Substrate	BE (%)	[2] Substrate	BE (%)	[3] Substrate	BE (%)	[4] Substrate	BE (%)
T	55.41^{A}	A	31.435^{C}	S	27.15^{B}	A	31.435^{AB}
T+St20%	26.445^{B}	A+St20%	54.83^{AB}	S+St20%	50.725^{A}	S	27.15^{B}
T+M20%	48.52^{A}	A+M20%	41.075^{BC}	S+M20%	52.04^{A}	S+A	36.89^{A}
T+St30%	54.735^{A}	A+St30%	67.72^{A}	S+St30%	45.305^{A}		
T+M30%	54.015^{A}	A+M30%	49.825^{B}	S+M30%	55.935^{A}		

Values with the same letter, in each table, are statistically equal to each other (Tukey, $p \leq 0.05$). St= stubble; M= manure; T= *A. tequilana*; S= *A. salmiana*; A= *A. angustifolia*.

The (Table 4) shows that the control substrate obtained the best production rate despite having taken more days in its cycle (59.25 days) compared to A+M30% (50.5 days); this difference is determined by the BE, which is much higher for the St substrate.

Table 4. Production rate obtained by each substrate.

Substrate	PR (%)	Substrate	PR (%)	Substrate	PR (%)
St	1.997775^{A}	A	0.4373^{FG}	S+St20%	0.596375^{EF}
T	0.9603^{BCD}	A+St20%	0.987325^{BC}	T+M20%	0.885275^{CDE}

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Substrate	PR (%)	Substrate	PR (%)	Substrate	PR (%)
T+St20%	0.467225^{FG}	A+M20%	0.713975^{CDEF}	S+St30%	0.68605^{DEF}
T+M20%	0.885275^{CDE}	A+St30%	1.214575^{B}	S+M30%	0.88035^{CDE}
T+St30%	0.96885^{BCD}	A+M30%	0.982625^{BC}	S+A	0.45395^{FG}
T+M30%	1.003025^{BC}	A+M20%	0.713975^{CDEF}		

Values with the same letter are statistically equal to each other (Tukey, $p \leq 0.05$). St= stubble; M= manure; T= *A. tequilana*; S= *A. salmiana*; A= *A. angustifolia*.

The Yi allows us to know if the substrate is adequate; it is obtained by dividing the fresh weight of the fungi by the fresh weight of the substrate; a viable medium must obtain at least 10% yield (Bermúdez *et al*., 2007) (Table 5), the unsuitable substrates were those of T+St20% and S, the best result was St with 27.68% and A+St30% with 20.7%. BR is measured by the degradation carried out by a biological decomposer agent, it is obtained by the difference between the dry weight of the initial substrate minus the dry weight of the final substrate divided by the initial dry weight (Romero *et al*., 2018) (Table 6).

Table 5. Yield for each substrate.

Substrate	Yi (%)	Substrate	Yi (%)	Substrate	Yi (%)
St	27.6795A	A	10.59075EFG	S+St20%	16.2883BCDE
T	17.947125BCD	A+St20%	17.565825BCD	S+M20%	18.1818BCD
T+St20%	7.93335G	A+M20%	14.314825CDEF	S+St30%	13.87425DEFG
T+M20%	15.746075BCDE	A+St30%	20.700625B	S+M30%	19.3721BC
T+St30%	15.8634BCDE	A+M30%	17.220225BCD	S+A	13.098275DEFG
T+M30%	17.5807BCD	S	9.654375FG		

Values with the same letter are statistically equal to each other (Tukey, $p \leq 0.05$). St= stubble; M= manure; T= *A. tequilana*; S= *A. salmiana*; A= *A. angustifolia*.

Table 6. Comparison of substrate Yi for each species of Agave. 1) *A. tequilana*; 2) *A. angustifolia*; 3) *A. salmiana*; and 4) *A. salmiana* + *A. angustifolia*.

[1] Substrate	Yi (%)	[2] Substrate	Yi (%)	[3] Substrate	Yi (%)	[4] Substrate	Yi (%)
T	17.947125A	A	10.59075C	S	9.6543B	A	10.5907AB
T+St20%	7.93335B	A+St20%	17.565AB	S+St20%	16.288A	S	9.65437B
T+M20%	15.746075A	A+M20%	14.314BC	S+M20%	18.181A		
T+St30%	15.8634A	A+St30%	20.7006A	S+St30%	13.87B		
T+M30%	17.5807A	A+M30%	17.220AB	S+M30%	19.372A		

Values with the same letter are statistically equal to each other (Tukey, $p \leq 0.05$). St= stubble; M= manure; T= *A. tequilana*; S= *A. salmiana*; A = *A. angustifolia*.

Discussion

Temperature, relative humidity and light

The optimal conditions of temperature should be 30 °C and humidity of 50-80% (Garzón and Cuervo, 2008; Martínez, 2014), as indicated in the supplier's recommendation sheet (Prodiset). The following has been reported: temperatures of 25-27 °C (Heredia *et al*., 2014), 12-32 °C and 18.4-25 °C (Bermúdez *et al*., 2007) and humidity of 60-65% (Heredia *et al*., 2014), 75-80% and 70-75% (Bermúdez *et al*., 2007), similar results were obtained in this work. The BPR-246 strain of

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Prodiset was adequate and its growth was stable. In the fruiting bodies, there was no alteration of organoleptic properties: consistency and coloration (UNAM, 2012).

The development of the mycelium was stable, the substrates had a continuous sprouting from day 20 of incubation, except for those based on *A. salmiana* bagasse with longer cycles. The cycle was similar for each substrate, 55-60 days, except for those containing S since their cycle lasted 63-90 days, consistent with what was cited by Bermúdez *et al*. (2007); Varnero *et al*. (2010), with cycles of 54-104 and 45-63 days.

BE, PR and Yi variables

The BE of the control substrate was positive, presenting an average of 118%, higher than the 64.3% reported by Romero *et al*. (2018) and within the range of 20-154% achieved in different species of *Pleurotus* (Piña *et al*., 2015). The result obtained could be to the particle size of the corn stubble as it allowed gas and nutrient exchange, since the agronomist who donated the stubble commented that it had a lot of corn grain; according to Rivera-Omen *et al*. (2013), *Pleurotus* has an enzymatic capacity to degrade large polymers (lignin and cellulose); Bermúdez *et al*. (2007) mention that the substrates with the best colonization are those with the highest content of structural carbohydrates (lignin, cellulose, and hemicellulose), found in corn, coinciding with Amador and Boschini (2000), who point out that the ear of corn contains 31% lignin and cellulose.

The BE of *A. tequilana* varied between 48 and 55%, with the exception of substrate to which 20% of stubble was added, since a BE of 26.4% was obtained. These results contrast with those mentioned by Lara *et al*. (2002), who indicate a BE of 69.1%. The BE of *A. angustifolia* was 31.4% with substrate A, and 67.7% with A+St30%, which showed a significant difference, this can be attributed to the addition of corn stubble to the bagasse of *A. angustifolia*; these results are higher than those obtained by Heredia *et al*. (2016), with 10% in *A. angustifolia* bagasse, 33.2% in a mixture of 65% *A. angustifolia*, 35% in walnut shavings, and 5% in wheat bran.

In *A. salmiana*, the BE with a nitrogen source was 27.1% by means of *A. salmiana* bagasse and 45.3-55.9% for the other mixtures, in agreement with Ruilova and Monzón (2014); nevertheless, no significant difference was observed between the proportions of 20 and 30%, they were encouraging results when compared to the BE of 12-18.9% reported by Heredia *et al*. (2016). The S+A mixture has an improvement in BE compared to S; however, there is no significant difference against A.

The PR achieved for the control substrate was much higher than the other substrates, with a high BE. The PR for *A. angustifolia*-based substrates showed the same result as the BE, A +St30% was the most outstanding mixture. The PR of the A substrate was lower since it presented a longer cycle than the substrates added with a nitrogen source, a behavior similar to that reported by Heredia *et al*. (2016).

A. salmiana-based substrates added with a nitrogen source shortened the fungus cycle, obtaining a PR of 0.6-0.88%, a significant difference against 0.29% of the substrate with *A. salmiana* alone. The S+A substrate is not significantly different from A in PR. The PRs of this project are correct for the production of *P. ostreatus* and similar to the results of other studies, such as 0.68% in *A. tequilana* bagasse, 0.68% with brewer's spent grain (25%)/*A. tequilana* bagasse (75%), and 48.5% in brewer's spent grain (50%)/*A. tequilana* bagasse (50%), described by Lara *et al*. (2002), and wheat straw, barley straw, corn stubble, and alfalfa, with results of 1.06, 0.84, 0.53, and 0.29%, obtained by Romero *et al*. (2018).

The substrates of *A. salmiana* showed an improvement when adding a nitrogen source; nonetheless, no significant difference was obtained between S and S+St30%; however, the BE and PR of S+St30% were higher than S; the Yi was affected because it contained 30% stubble and this has greater water retention, so it's wet weight was high. The S+A substrate did not make a significant difference when compared to A.

Authors such as Bermúdez *et al*. (2007) reported a Yi of 58.7% in coffee pulp substrate; 23.6% in cedar shavings, and 22.4% with a 1:1 mixture of such substrates, results with a better Yi compared to those of this experiment; however, Bermúdez *et al*. (2007) report that, in an acceptable

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production, the Yi should be greater than 10%, the results obtained in this project, except for S, exceeded that 10%.

No substrate exceeded the results of the control; it was observed that the addition of the nitrogen source helps to increase the BE, as mentioned by Gil *et al*. (2012); BR enhanced biodegradation in *A. angustifolia* substrates when the nitrogen source was added.

Morphological variables

Although when using *A. salmiana* there is a tendency to increase the size of the fruiting bodies, a significant difference cannot be stipulated since the number of bodies studied are not equivalent; this scenario occurs in all substrates. Forero *et al*. (2008) obtained diameters of 4.2 cm and 3.8 cm in King Grass and chili pepper residues, each with 5% wheat bran and 2% calcium sulfate. Bermúdez *et al*. (2007) report diameters of 9.26 cm, 4 cm, and 5.15 cm for substrates of coffee pulp, cedar shavings, and 50% pulp + 50% shavings, respectively.

In most substrates, the diameter of the caps was approximately 5 cm, similar to the previous results. The size and quantity of the fruiting bodies decreased as they were harvested since the fungus depletes the substrate; the substrates that promptly produced their three harvests generated fruiting bodies on two more occasions; nevertheless, they were very small and scarce and were not considered, as indicated by Fracchia *et al*. (2009) (only the first three harvests should be taken for statistical purposes) (Table 2).

Conclusions

The conditions and mycelium were suitable for the experiment. The addition of cattle manure or corn stubble as a nitrogen supplement to the bagasse of *A. angustifolia* and *A. salmiana* improves BE, PR, and Yi. The S+A mixture did not show improvement when compared with *A. angustifolia* bagasse, except for the BR variable. The addition of supplements to the substrate did not result in an improvement for BR.

The use of *P. ostreatus* for agave bagasse decreasing is a great option as the substrate waste is easier to compost. Morphological measures are not affected by adding supplements. The S substrate was larger in the measurements; nonetheless, it was due to the low production of fruiting bodies. Growing *P. ostreatus* in agave bagasse is recommended, giving added value to the agro-residue, and at least three harvests can be obtained.

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