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Use of the cotton husk for the production of a Basidiomycete fungus in submerged culture

Abstract

The agro-industrial waste has become the point of research for many researches due to their potential to get different products of high added value. Due to its low cost and the need to dispose them to safe guard the environment, they have become the most profitable raw materials. Mushroom production is one of the strategies for the use of these residues due their ability to degrade complex compounds such lignin, which together with cellulose and hemicellulose are the most abundant in this type of waste. Therefore developing a strategy that combines the use of agro-industrial waste with the advantages of biotechnological culture for mushrooms production is the aim of this research. In this case, cotton seed hulls 30g/L were employed as media culture to flask scale for mushrooms production. The non-conventional substrate supplemented was evaluated with glucose for finding the best result in terms of degradability of substrate.

Keywords: agro-industrial waste, biotechnological culture, mushroom, cottonseed hulls

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Introduction

At present, agro-industrial waste is becoming a source of profitable raw materials for obtaining biotech-based value-added products. Its applications range from chemical and thermal use, to obtaining essential oils, pectin, and its use as a substrate for obtaining basidiomycete fungi,[1] which have a promising market due to the multiple properties that are conferred to them.[2] Basidiomycete fungi, also called white rot fungi, are known for their ability to degrade residues rich in complex compounds such as lignin and cellulose, since they have the enzymatic machinery to metabolize them and obtain their source of carbon and energy. Bioremediation,[3] enzyme production[4,5] and functional foods for humans[6,7] and animals[8] are some of the most relevant applications of this type of fungi.

Biotechnological cultivation has multiple benefits when the main objective is to obtain biomass, secondary metabolites and non-fruiting bodies. Products of commercial interest such as enzymes or polysaccharides produced by fungi are mostly extracellular, they can be separated and extracted easily and they can also easily induce their production by modifying operational variables such as temperature, agitation, aeration and pH.[9] The submerged culture is presented as a cost-effective solution due to the improvement in time of production, increasing the productivity of the process, in addition to provide more availability and homogeneity of the substrate and a better control of the conditions under which the fermentation takes place.

Most of the reports in which submerged culture has been studied use simple substrates such as glucose.[10,11] However, there are reports where these types of fungi are used in other more complex substrates, such as *Schisophyllum commune*, was grown in goat cheese.[12] In the case of complex substrates such as lignin, studies focus on solid-state culture for the production of fruiting bodies.[13,14] In order to study the fungi growth in submerged culture with complex substrate, we evaluated the feasibility of obtaining fungi biomass by biotechnology culture of basidiomycete fungi using cotton husks residues as a substrate. This approach aims to evaluate degradation capacity and the morphological behavior of these microorganisms and the potential use of lignocellusic residue for production of fungi biomass and metabolites of interest. The cotton husk is a waste from the textile industry which on average generates 39,000 tons per year of waste in our country.[15] Due to contents of carbon and some trace elements such as copper and manganese, fundamental cofactors for the activation of specific enzymes in metabolic pathways, cotton husk can be used as a substrate for the growth of a fungus.[4]

Materials and methods

Conditioning and pre-treatment of the raw material

The substrate concentration for fermentation processes was established using a wettability test, which consisted of evaluating the proportion of cotton hulk and water to obtain a complete submerged culture. The amount of substrate reported for the submerged culture of basidiomycete fungi in complex substrates[16,17] was used as a substrate concentration of reference with the materials. The wettability tests were made with cotton hulk concentration of 5/L, 15g/L, 30g/L and 50g/L. They were left at 100 rpm and 27°C for 48 hours. The tests were done in triplicate. The treatment with the largest amount of material that could be kept in suspension was selected. The cotton hulk was pre-treatment in a knife mill to have a homogeneous size particle. The substrate was divided in two fractions: Cotton Fraction (FA), characterized by mainly containing cotton fibres and Lignocellulosic Fraction (FL), a fraction with the smallest particle material. The cotton hulk without pre-treatment was called complete material (MC). To avoid the action of a different organism on the raw material, it was kept in a cool and dry place.

Microorganism and maintenance means

To obtain the inoculums, a commercial mushroom seed of *Grifola frondosa* was used. It was cultivated for conservation, in solid medium composed of (g/L): barley flour 30, yeast extract 3, sucrose 5, 3 and

agar-agar 8. The pH was adjusted to 5.5±0.1 and left in incubation until the fungus invaded the entire box. Then the strain was brought to liquid culture, in a medium composed of (g/L): barley flour 35 and yeast extract 1; the pH was adjusted to5.5±0.1. This culture was stirred at 100 rpm for 10 days.[16] This culture was used as inoculum for the experimentation.

Cultivation in cotton husks

To evaluate the growth of the fungus in the cotton husk, fermentations were performed using the three fractions described above. 1000mL flasks with an effective volume of 500mL composed of 30g/L of complete material (MC) or the concentration corresponding to the percentage of each fraction after separation were used: FA 50%±5 (%P/V) and FL 40%±7 (% P/V). Each treatment was completed with 500mL of water, sterilized under standard conditions: 121°C, 15psi for 20 minutes and was inoculated, as reported by different authors,[18] with 25mL (5%) of a liquid culture of *G. frondosa* of 9 days of fermentation. The operational conditions in submerged culture were selected according to those reported in the literature. The best results for biomass production[16] were: pH 5.5; 120rpm; 25°C. The experimentation was done in triplicate for each substrate and the samples for the analysis were taken in 0, 15 and 30 days. In order to eliminate the uncertainty about whether the changes suffered by the material are due to the action of water and agitation, a control treatment was prepared, which consisted of 30g/L of Complete Material (CM) without inoculation. This control treatment was incubated at the same conditions of the culture. The biomass growth was establish qualitatively with a visual tracking.

Supplementation of the cotton husk

To assess the effect of supplementation on material degradation, fermentations were performed using the three fractions described above. The MC treatment was supplemented with 15.5g/L of anhydrous glucose and the FA and FL treatment with the equivalent amount of anhydrous glucose to the percentage of each fraction. Each treatment was completed with 500mL of water and they were sterilized under standard conditions. The fermentation process was performed in the same conditions.

Substrate consumption

To determine if there was an action of the fungus on the material, it was proposed to perform degradability analysis using the Van Soest method.[19] This method consists in the determination and classification of the forages according to the fibre content and their solubility in neutral and acidic detergents. The less digestible walls of the material due to its high content of cellulose, hemicellulose and lignin are separated from accessible nutritional constituents such as sugars, pectin, proteins and lipids, soluble in a neutral detergent (Na-LurilSulphate, EDTA). The remainder of this digestion is called Neutral Detergent Fibre (NDF) and consists of cellulose, hemicellulose and lignin, the main components of the cell walls of agro industrial materials. The NDF is then treated with an acid detergent (cetyltrimethyl ammonium bromide) in which lignin and cellulose are separated from the hemicellulose, in addition to a protein fraction that is attached to the cell walls. This remainder consisting of cellulose and lignin is called Acid Detergent Fibre (FDA).Finally, a treatment with H_2SO_4 to the FDA allows to determine the amount of lignin present, thus obtaining the percentage of lignin (% Lig). It is possible to determine the percentage of cellulose and hemicellulose contained in the sample with these three values, based on two simple relationships:

Hemicellulose = FDN - FDA

Cellulose = Lignin- Hemicellulose

An important aspect to consider is the fraction called organic matter (MO), also given in percentage. This measure refers to all the material that is removed in the first step of the Van Soest method and in which cellular debris such as carbohydrates, proteins and pectin, mainly. This value is important because it is an indirect measure of the cellular material produced, and can be calculated as:

% MO= 100- (% Lignine + % Cellulose + % Hemicellulose)

Determination of sugars by HPLC

This analysis was performed in order to monitor the consumption of added glucose to supplement the medium. Each sample was previously filtered on a 0.20µm filter. For the determination, readings were made using an Agilent Technologies© 1200 series HPLC type chromatographic device with an HPR-87H Aminex column from BioRad, with a mobile phase of acidified water at 0.008N with H_2SO_4 at a flow of 0.6mL/minute and a temperature of 50°C, using a standard curve of Glucose and Xylose.

Results and discussion

The results obtained for the conditioning and pre-treatment of the raw material and to estimate the concentration of material for the culture, allowed establish that using a concentrations of 5g/L (Figure 1a) and 15g/L (Figure 1b) were below the capacity of the system to be suspended. In the case of the concentration of 50g/L (Figure1d), the opposite effect can be seen, in crops supersaturated with material which behaves more like a solid state culture.

However, for the concentration of 30g/L (Figure 1c) it was found that the material remained in suspension when stirred and retained its submerged culture characteristics. This substrate concentration was consistent with the selection criteria. When the complete material (CM) (Figure 2a) was passed through the knife mill to obtain a homogeneous size particle, it found that a whirlwind was generated inside the equipment, causing the less dense fraction, composed of cotton (FA) to go to the walls, while a heavier portion, composed of small timber particles (FL), remained close to the blades; in this way the material was separated. In the case of AF (Figure 2b), it found a bulky material after separation, which comprised about 50% of the initial material and which is mostly made of cotton fibres, which according to Van Soest's analysis, presents more than 70% cellulose and only 12% Lignin. On the other hand, FL (Figure 2c) is mainly composed of small timber particles, which comprise about 40% of the initial material and that have a lignin content of more than 30% and cellulose of 20%, according to Van's analysis Soest. Thus the MC has the physical and chemical characteristics of both fractions, presenting a lignin and cellulose content around 20% and 60%, respectively.

In the biotechnological culture made with each fraction, it found morphological differences for the treatment. In Figures 3, 4 and 5 it can be seen how the fungus grows in the form of a pellet for the three fractions, also presenting an enveloping growth for the MC (Figure 3) and FA (Figure 4) due to the bulky characteristics of the fibrous material; while for FL there is only pellet formation until the end of the fermentation, this due to the size of the substrate particle in this medium.

It is important to clarify that no quantitative data on biomass production is reported, since due to the characteristics of the substrate and the filamentous growth it is not possible to separate the fungus

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from the substrate for the determination of biomass production. However, the change in the medium and the presence of structures such as pellets corroborate that the material can be used as a substrate for the fungus. Another way to demonstrate the effect of the fungus on the material is the change it undergoes in its structure (Figure 6b, 6c & 6d). After 30 days of fermentation, each culture was homogenized with a knife mill, where the transformation that the material underwent due to the growth of the fungus became evident.

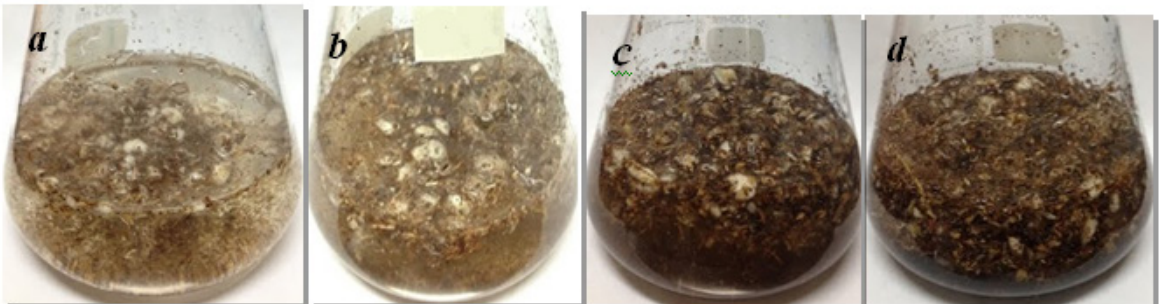

Figure 1 Wettability tests. Suspended substrate with water in Erlenmeyer of 500mL for the determination of the substrate concentration used in the fermentations: a) 5g/L; b) 15g/L; c) 30g/L and d) 50g/L.

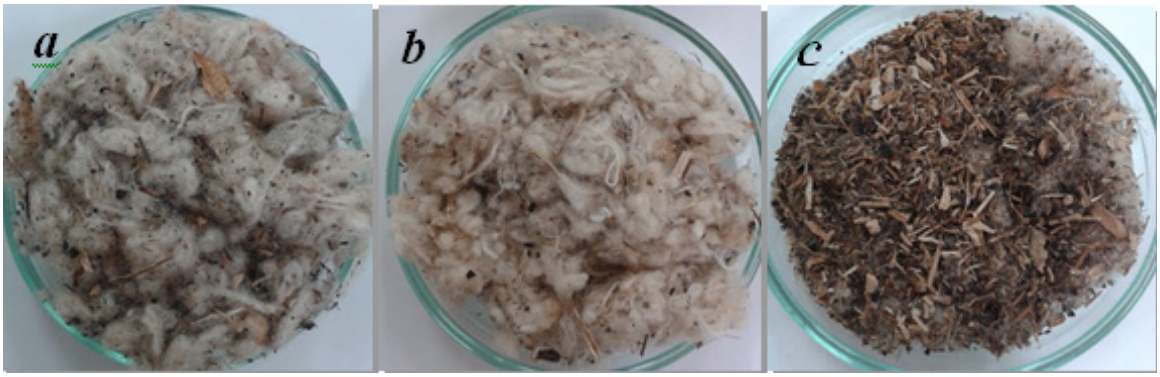

Figure 2 Appearance of dry cotton husk: a) Complete Material (MC), b) Cotton Fraction (FA) and c) Lignocellulosic Fraction (FL).

Figure 3 Complete material (MC): a) 1 day of fermentation, b) 7 days of fermentation, c) 15 days of fermentation.

Figure 4 Cotton fraction (FA): a) 1 day of fermentation, b) 7 days of fermentation, c) 15 days of fermentation.

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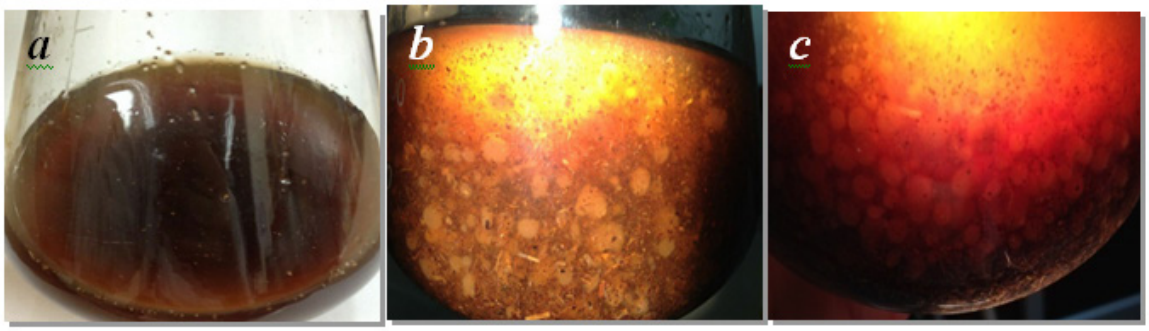


Figure 5 Lignocellulosic Fraction (FL): a) 1 day of fermentation, b) 7 days of fermentation, c) 15 days of fermentation.

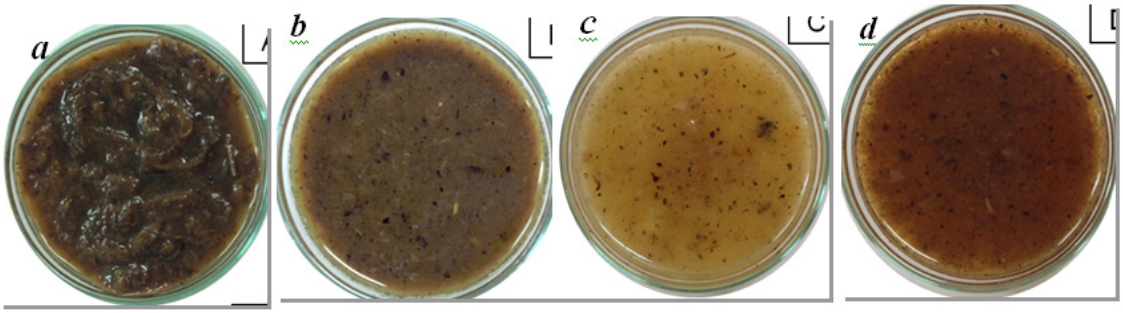


Figure 6 Homogenized material in knife mill after 30 days of fermentation. It is observed how in a) Control there was no complete homogenization of the material. The change in fibrous structures of: b) Complete Material is evident, c) Cotton Fraction, d) Lignocellulosic Fraction.

-While the treatments (MC, FA and FL) were easily homogenized, the control treatment was not homogenized and conserved the rigid structures of the material (Figure 6a), which indicates that the changes observed are due exclusively to the action of the fungus on the substrate. The transformation of this material rich in cellulose and lignin could take place by the enzymatic machinery of the fungus to degrade this type of substrates: Lignin-Peroxidase (LiP), Manganese-Peroxidase (MgP) and Lacasa (Lac);[20] these enzymes are secreted to the medium where hydrolysis of the material occurs, so the fungus can absorb free glucose and the resulting intermediates to complete other metabolic processes. This machinery is highly nonspecific so it can act on many substrates.[14,20] The effect of these enzymes on lignin and cellulose could cause the weakening of the fibres, making them more sensitive to the physical breakdown of the mill. For this reason, one of the most recognized applications of white rot fungi is the production of these enzymes.

With respect to the morphology observed in the different fractions, it is sought that there is pellet formation, since this morphology is associated with high yields in the production of biomass and polysaccharides,[21,22] in addition to the separation and extraction processes of metabolites are facilitated by preventing the enveloping growth of the fungus on the substrate. This characteristic growth of the most voluminous treatments such as MC and AF can present a lower degradation rate since the catabolic processes of lignin and cellulose are aerobic, and in these cases the diffusion of oxygen decreases by compacting the material.[21,22]

The division of the material is an important input to investigate more about the morphology of the fungus and its relation to the physical characteristics of the material since according to these results the formation of pellets or the envelope growth depends on the type of material with which it is worked: bulky or particulate. In this way, a strategy can be established to favour the production of some metabolite, directing the morphology of the fungus through the physical characteristics of the substrate. Based on the presence of spherical structures (pellets), the compaction by the enveloping growth of the fungus and the change of the rigid structure of the cotton husk, it is inferred that this material can be used as a substrate for the submerged culture production of a basidiomycete fungus. With respect to the substrate consumption or degradability analyses, the Van Soest's analyzes corresponding to the percentages of lignin, cellulose and hemicellulose for each treatment: MC (Graph1), FL (Graph 2) and FA (Graph 3) in the times represented by bars: 0, 15 and 30 days.

In general, it is observed that there is no significant difference between the percentages of degraded cellulose, hemicellulose and lignin for the materials worked (Graphs 1, 2, 3).This may be due to the lack of a simple nutrient source that allows the fungus to adapt to the complex substrate and activate the enzymatic machinery for its transformation. However, the remaining fraction (% MO) shows a significant increase when working with the CM (Graph 1).

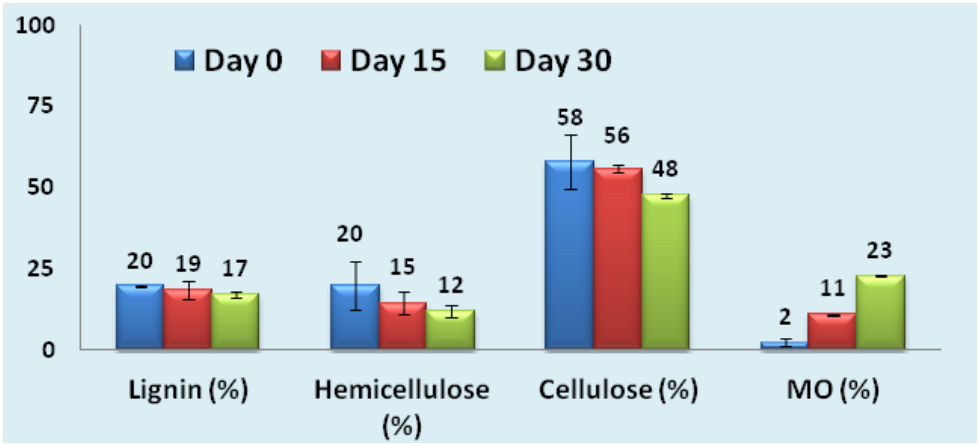
The presence of simple sugars (residual glucose) and polysaccharides from the inoculum that is added and that can act as a carbon source for the fungus, validating the increase in the MO fraction (Graph 1) without showing a decrease in lignocellulosic material.

With respect to the degradability analyses, the Van Soest analysis corresponding to the percentages of lignin, cellulose and hemicellulose for each treatment are presented in the graphs: MC (Graph4), FL (Graph 5) and FA (Graph 6) in the time represented by bars: 0, 15 and 30 days.

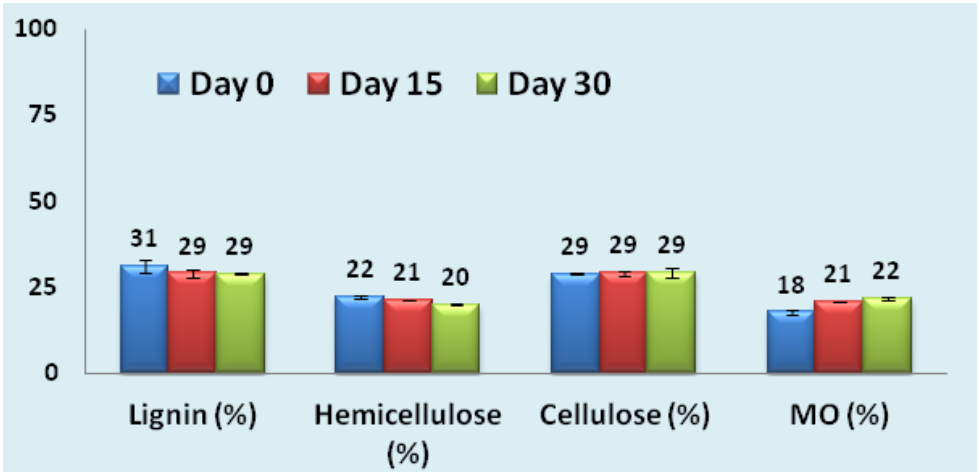
In the case of the MC, the culture presented the same physical characteristics observed in Figure 3: Envelope growth and presence of

some pellets. As the fermentation progressed, the fungus compacted the substrate, so that the homogenization of the culture was affected.

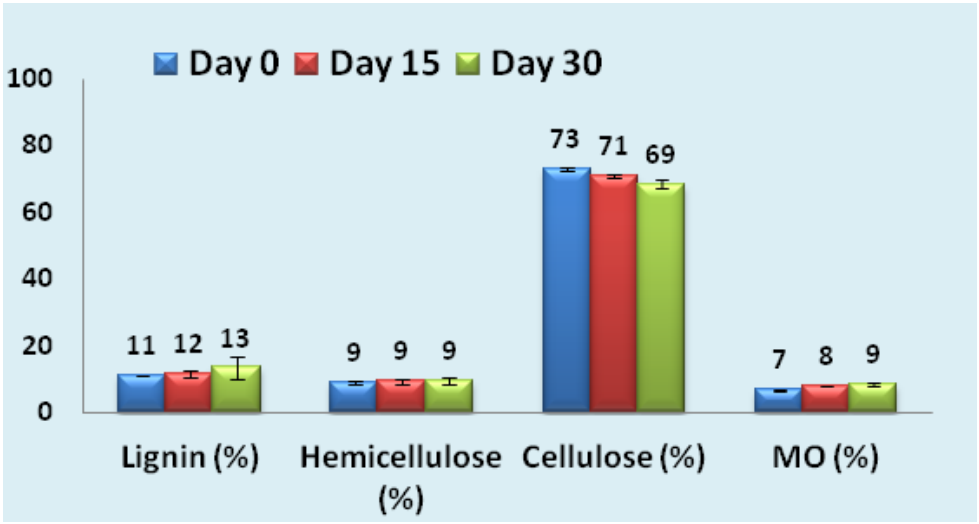
Regarding the percentages of lignin, cellulose and hemicellulose, there were no significant changes during the 30 days of fermentation (Graph 4). However, with respect to glucose consumption, there is a significant change, which for this treatment was 9.31g/L equivalent to 67% of the available sugar (Graph 7). We found that for MC at 15 days of fermentation the consumption was 5.03g/L, which represents 54.02% of the total sugar consumed during the first 15 days.



Graph 1 Material degradability, Full Material (MC).



Graph 2 Degradability of the material, Lignocellulosic Fraction (FL).



Graph 3 Degradability of the material, Lignocellulosic Fraction (FA).

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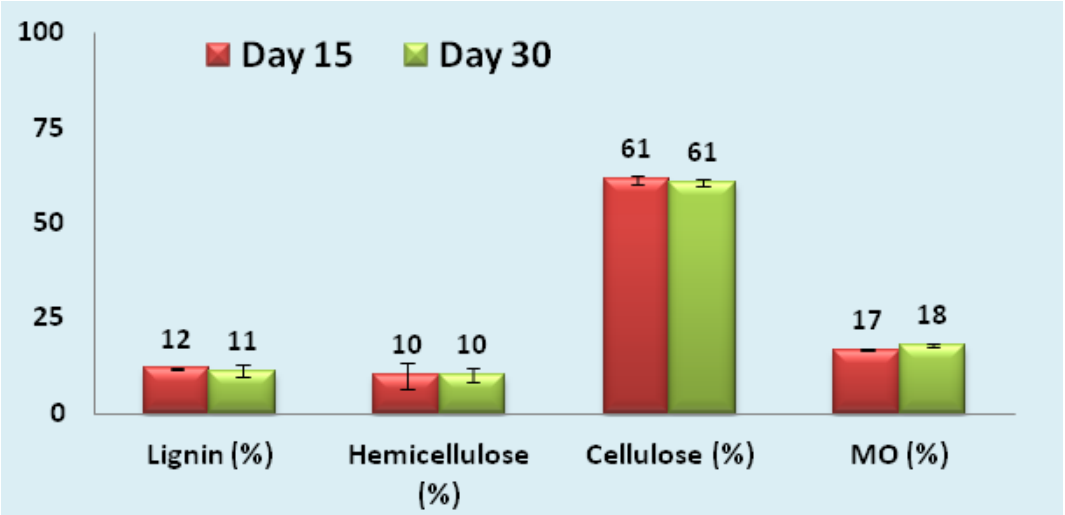
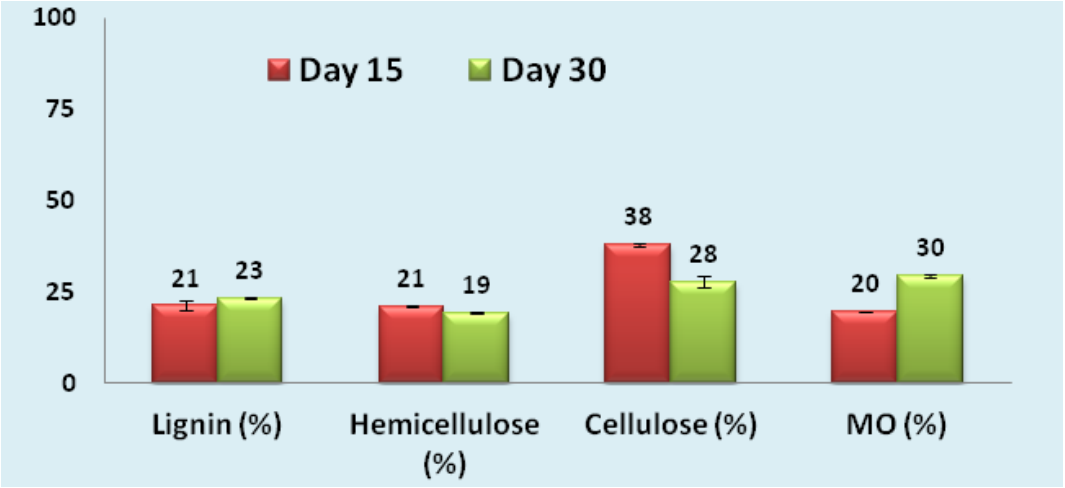
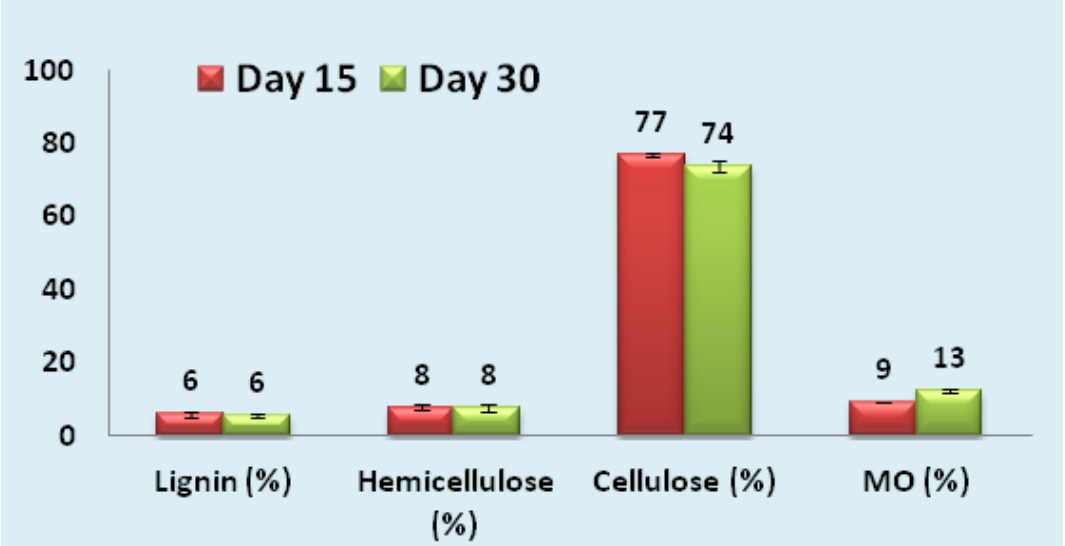


Graph 4 Degradability of the supplemented material, Full Material (MC).



Graph 5 Degradability of the supplemented material, Lignocellulosic Fraction (FL).



Graph 6 Degradability of the supplemented material, Cotton fraction (FA).

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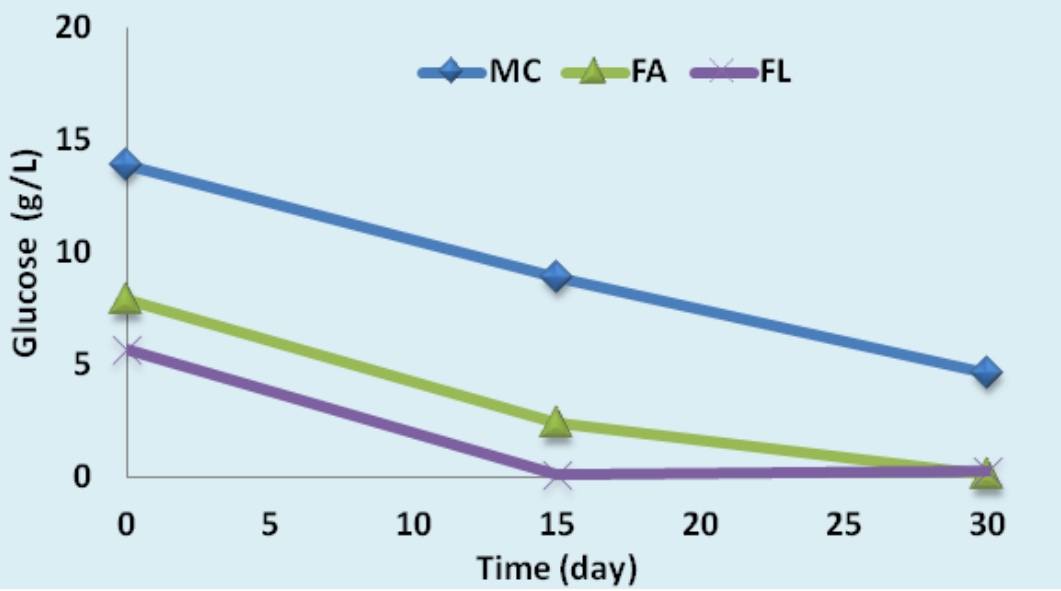

Graph 7 Glucose consumption for the three materials.

This result indicates that the fungus used glucose added to the medium but did not take advantage of transforming the cellulosic and hemi-cellulosic fractions of the material. In addition, in these fermentations there is no change in the M.O. of the material, which is evidence that when the medium is supplemented with glucose, the fungus has a preference for this substrate, this result is significantly different from that obtained in fermentation without supplementation, where fungus growth and significant variation were observed in the MO fraction of the material.

Another reason is that lignin degradation mechanism depends on a high concentration of oxygen,[23] a factor that was affected by the envelope growth of the fungus, making the colonization of the substrate only superficial, in this case it is difficult to transfer oxygen to the areas where there was agglomeration due to the growth of the fungus; For this reason, biomass production was evidenced, but not material degradation. For FL (Graph 5) there were significant changes in cellulose transformation, going from 37.99% +0.65 to 27.83% +1.58, which represents a 27% decrease in the amount of cellulose present in the medium. This change can be seen in the physical characteristics that the FL presents, since the fungus can act better on the surface of the material to be this particulate. In addition to presenting a greater amount of lignin, this can act as an elicitor for the enzymatic expression of the fungus.

Biomass production was another significant aspect in this treatment, since the% MO fraction went from 19.69% + 0.08 to 29.51% + 0.57 between day 15 and day 30. This corroborates the statement in the MC with respect to the transfer phenomena, since in this case the material, being particulate, allowed a homogenization of the substrate, facilitating the transfer of oxygen necessary for the degradation of the lignocellulosic complex.[23] Glucose consumption is another evidence of metabolic activity, since for this treatment it was consumed completely during the first 15 days of fermentation (Graph 7).

In contrast to the results obtained for this treatment in the first objective, it is inferred that the increase in the ability of the fungus to degrade the material lies in the presence of a simple carbon source that helps the fungus to adapt to the material during the phase latency, allowing an initial growth and adaptation to the environment by activating the machinery, which in turn allows other important nutrients to be obtained from the substrate used, such as cofactors and intermediate metabolites.[23]

In the case of AF (Graph 6) there were no significant changes in the degradation of lignin, cellulose and hemicellulose; however, fungus growth and glucose consumption were evidenced (Graph 7). This behaviour is similar to that observed in the CM, since the same characteristics are presented in terms of the growth of the fungus envelope, making transfer phenomena difficult, which can be a strong indicator that the use of bulky materials that induce this type morphology significantly affects the degradation of the material by making it difficult to transfer oxygen in the environment.[24–47]

In morphological terms, the development of the first two objectives allowed to establish to what fraction of the material the growth of the fungus is attributed in enveloping form or in the form of pellet and the effect that it has on the phenomena of transfer and the degradation of the substrate. If transfer phenomena are facilitated, the fungus response to degrade the complex material may be better. It is advisable to develop a new approach using the complete material but in specialized equipment such a stirred tank reactor, in order to treat the entire substrate and not just a fraction, but improving operational conditions to ensure homogeneity in terms of oxygen transfer, which is only achieved in bioreactor.

Conclusions

The cotton husk is an agro-industrial waste that can be degraded and used as a substrate for the growth of *Grifola frondosa* with potential production of metabolites of interest, such as enzymes. Depending on the fraction of material used in submerged culture, the morphology of *G. frondosa* is different. The Lignocellulosic Fraction (FL), which is particulate, favours the formation of pellets, while in the bulky fractions, Complete Material (MC) and Cotton Fraction

(FA), an enveloping morphology is favoured on the substrate. The supplementation of the cotton husk with glucose presents advantages for the production of biomass with respect to the medium without supplementing, since a simple source of glucose is provided to the fungus during the first days of fermentation. For the control of fungus morphology and increased degradation of complex substrate like cotton hulks it is recommended to evaluate the fermentation in specialized equipment as a stir tank bioreactor. In this equipment it is possible a better control of transport phenomena, oxygen diffusion and pellet size.

Acknowledgments

None.

Conflicts of interest

Authors declare that there is no conflict of interest.

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