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PREPARATION OF FUNGAL INOCULUM FOR LEAF LITTER COMPOSTING FROM SELECTED FUNGI

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ABSTRACT

Fungi are the major degraders of organic matter in natural environments, and almost every naturally occurring organic compound can be degraded by one fungus or another. An enormous range of organic compounds can be utilized by fungi, especially the major organic compounds such as cellulose, xylan and lignin. The aim of the research was to provide fungal inoculum of selected fungi as a Composting Starter Kit (CSK) to increase decomposition process of leaf litter. The fungi were isolated from decomposing leaf litter and selected based on qualitative measurement of cellulase, xylanase and ligninase activity. The result showed that there were 5 cellulolytic, 6 xylanolytic, and 2 ligninolytic isolates potentially used as inocula for leaf litter composting. Based on a halo test indicated that isolates *Shj*, *Lht*, and *Lpt1* had the highest ratio for cellulase, xylanase and ligninase activity respectively. Inoculum of those three isolates were developed on sorghum seeds and were able to produce spores of *Shj* = 1.5 x 10^9, *Lht* = 9.3 x 10^7 and *Lpt1* = 4.2 x 10^9 spores/ml. The viability test for inoculum showed the number of colonies of *Shj* = 9.6 x 10^9, *Lht* = 5.6 x 10^8 and *Lpt1* = 8.2 x 10^6 CFUs. In conclusion, development of fungal inoculum is possibly prepared using sorghum grains inoculated with selected fungi.

Keywords: leaf litter composting, inoculum, cellulase, xylanase, ligninase.

1. INTRODUCTION

Various studies on composting is constantly evolving with reviewing various aspects, such as the type of substrate, the physico-chemical parameters, microbial populations, additional elements of compost, technology, inoculum, enzymatic and others (Zhang *et al*., 2011). The addition of inoculum on the composting process is one of the topics widely studied by scientists. Therefore, construction of inoculum composition of fungal decomposer able to initiate and enhance the composting process is very interesting to study and this research was dealt with this matter. The major substrate for leaf litter composting is lignocelluloses difficult to decompose naturally and it has led to find a proper inoculum. It has been recognized that the decomposition process is complex process and involves many microorganisms, but attention is focused on decomposer fungi. The aim of the study was to select fungal decomposer (cellulolytic, xylanolytic and ligninolytic isolates) based on the activity of cellulase, xylanase and ligninase qualitatively to develop as inoculum for leaf litter composting. The best isolates were subjected to inoculum development and fungi were selected as inoculum due to some decomposition properties relating to their role in nature.

2. LITERATURE REVIEW

In general, fungi are able to absorb only small soluble nutrients such as monosaccharide and amino acids, or peptides composed of two or three amino acids. If nutrients are available in the form of the disaccharide must first be degraded into monosaccharide before they can be absorbed by most fungi. So the availability of nutrients for fungi is highly dependent on the release of degradation enzymes (Deacon, 1997). The level of complexity of substrates that can be degraded fungi in nature is so diverse that fungi need to release extracellular enzymes to obtain their carbon sources. Extracellular enzymes are released through cell walls in the form of stored materials in vesicles that are sent from the Golgi body to the tip of hyphae and then released into the environment by exocytosis (Wessels, 1990). However this imposes constraints because enzymes are large molecules, about 20.000 - 60.000 Da (in the case of fungal cellulases), so they do not diffuse far from the hyphal surface. As a result, fungi create localized zones of erosion of insoluble substrates (such as cellulose) (Beguin, 1990).

Organic compounds in a broad category can be used by fungi as a source of nutrition. Most fungi are able to use glucose, a monosaccharide and disaccharide. A small group of fungi are capable of using alcohol and even methane. However, polymers which are most widely available as a nutritional for fungi in nature are cellulose. Forty percent of the plant cell wall material is cellulose. Cellulose has a simple chemical structure comprising a straight chain of glucose residues 3000-10000 bound by ties ß-1, 4. In order to break this chain it needs cellulose enzymes which are a complex enzymes consist of selobiohidrolase, endoglukanase and ß-glukosidase (Deacon, 1997). Cellobiohydrolases are able to break disaccharide units (Cellobiose) from the end of the chain, endoglucanase attack the middle of the chain randomly and ß-glucosidases split cellobioses into glucoses.

In addition to cellulose, xylan is the next compound which is also found in the cell walls of gramineae and some monocots. Besides xylan also a major component of hemicelluloses as much as 15-30% which is found in hardwood tree species, 7-10% in softwood and some up to 30% in perennial plants (Collins *et al*., 2002). Hemicelluloses are compounds that are normally degraded in the young plants shown with the degradation of the

middle lamella (Worrall *et al*., 1997). Xylan is a homopolisaccharides bonded in the form of ß-1, 4-D connecting residues xylosa (Lehninger, 1975). Hydrolysis of xylan involves the enzyme ß-1, 4 xylanases and ß xylosidases and these enzymes were able to be isolated. In general, xylanases attack internal part of xylosidic linkage (Cho *et al*., 1996).

Lignin is also an important part of the constituent elements of plants including the plant litters. Lignin term is generally used to indicate that a significant fraction tested as acid-insoluble residue or Klason lignin which is also based on proximate analysis, such as sulfuric acid hydrolysis. Lignin fraction contains not only true lignin but also cutin and tannin (Ko gel-Knabner *et al*., 1989). Lignin is a three-dimensional polymer composed of various phenolic acids such as dihydroxy benzoic acid, coumaril alcohol, and ferulic acid as a monomer.

Due to their complex chemical structure, it is very difficult to degrade lignin and only certain types of microbes are able to degrade these compounds. Wittmann *et al*. (2004) and Saiya-Cork *et al.* (2002) reported that bacteria and saprotrophic basidiomycetes fungi degrading litter can produce non-specific extracellular enzymes, which are able to depolymerase detritus land (including the celluloses, hemicelluloses, and sulfur-containing compounds) into oligomers and monomers which then become a source of nutrients for microbes and plants.

3. MATERIALS AND METHODS

The isolation and selection of the isolates producing cellulase, xylanase and ligninase, was performed using a selective medium. Samples were small pieces of leaf litter collected from piles of decomposed leaves on the floor of Biological Garden (Arboretum) UGM (Gadjah Mada University). Leaf litter composition was *ketepeng* (*Terminalia catappa*), *kenari* (*Canarium vulgare* Leenh), *keben* (*Barringtonia asiatica*), *jati belanda* (*Guazuma ulmifolia* Lamk.), *Nagasari* (*Mesua ferrea*), *kantil* (*Michelia alba*) and *puring* (*Codiaeum variegatum*).

3.1. Isolation and screening of cellulose-degrading fungi

Isolation of cellulase-producing fungi was carried by dilution and direct plating methods (Malloch, 1981). Selection of cellulolytic fungi isolates was done by modification of Congo-Red method of Teathre and Wood (1982). Isolates obtained were cultured in the media Cellulose Agar (cellulose 5.0, $NaNO_3$ 1.0, K_2HPO_4 1.8, $MgSO_4.7H_2O$ 0.9, KCl 0.5, 0.5 yeast extract, casein hydrolysat 0.5, agar 20 and distilled water 1L). Confirmation of cellulose-degrading ability of fungal isolates was performed by streaking it on cellulose agar media. Media were 2 layer media (bilayer) with the bottom layer was a PDA of 1/5 recipes, agar 1.5, and distilled water 100 ml.

The top layer was Carboxymethyl Cellulose (CMC) 1-2%, agar 1.5 and distilled water 100 ml. Once inoculated with fungi in the middle of the test media, the cultures were then incubated for 4 days. The media were added with 0.1% Congo-Red and allowed to stand for 20 minutes at room temperature. Media was washed with 1 M NaCl (left overnight if necessary). Isolates producing cellulase formed halo (clear zone) around the colony. The use of Congo-Red as an indicator for cellulose degradation in an agar medium provides the basis for a rapid and sensitive screening test for cellulolytic fungi. Colonies showing discolorization of Congo-Red were taken as positive cellulose-degrading fungal colonies (Lu, *et al*., 2004), and only these were taken for further study. Cellulose-degrading potential of the positive isolates were also qualitatively estimated by calculating hydrolysis capacity (HC), that is the ratio of diameter of clearing zone and colony (Hendricks *et al*., 1995).

3.2. Isolation and screening of xylan-degrading fungi

Isolation and selection method of isolates used a modified method of Teathre and Wood (1982). Isolates obtained were cultured in medium for fungi xylanolitik (xylan from beechwood 1 g, peptone 5 g, yeast extract 5 g, K_2HPO_4 0.2 g, agar 20 g, and distilled water 1000 ml). Confirmation of xylan-degrading ability of fungal isolates was performed by streaking on xylan enriched media. Media were 2 layer media (bilayer) with the bottom layer was a PDA of 1/5 recipes, agar 1.5, and distilled water 100 ml. The top layer consisted of xylan from beechwood 1%, agar 1.5 and distilled water 100 ml.

Once inoculated with fungi in the middle of the test media, the cultures were then incubated for 4 days. The media were added with 0.1% Congo-Red and allowed to stand for 20 minutes at room temperature. Media was washed with 1 M NaCl (left overnight if necessary). Isolates producing xylanase formed halo (clear zone) around the colony. The use of Congo-Red as an indicator for xylan degradation in an agar medium provides the basis for a rapid and sensitive screening test for xylanolytic fungi. Xylan-degrading potential of the positive isolates were also qualitatively estimated by calculating hydrolysis capacity (HC), that is the ratio of diameter of clearing zone and colony (Hendricks *et al*., 1995).

3.3. Isolation and screening of lignin-degrading fungi

Qualitatif assay for Lignin Degrading Enzymes (LDE's) were done by Okino *et al*. (2000) method. Fungal isolates were isolated on B and K medium (glucose 10 g, peptone 2 g, yeast extract 1 g, agar 18 g, aquadest 1 L) (Atalla *et al*., 2010). The seven day old isolates were then cut in diameter of 10 mm from the middle of cultures and transferred to qualitative screening media (Guaiacol-supplemented agar: contained glucose 10 g, peptone 2 g, yeast extract 1 g, agar 18 g and 4 mM guaiacol in 1 L water) (D'Souza *et al*., 2006). The inoculated plates were incubated at 25^0C in dark for 7 days. The production of intense brown color under and around the fungal colony was considered as a positive reaction resulting from guaiacol oxidation (Okino *et al*., 2000).

3.4. Inoculum development

Inoculum development was made using modification of Gaind *et al*. (2009) method. Sorghum grains were used as substitute for fungal strain growth. The sorghum grains were finely ground and sifted before it was mixed with paddy rice bran in the ratio of 2 : 1 (v / v), 4% calcium sulphate, and 2% calcium carbonate (in 1 L distilled water). A loopful of individual culture potential of cellulose, xylan and lignin degrading fungi was inoculated in each 100 g grains (sorghum and bran) added with 25 ml of solutions (sterilized at 15 lb pressure for 1 h) and incubated at 25^0C for 15 days. The whole growth of each strain including mycelium, spores, and the grains were used as the inoculum. The inoculum was counted for the number of spores and viability by calculating CFUs.

4. RESULTS AND DISCUSSIONS

The isolation of cellulolytic, xylanolytic, and ligninoytic fungi from mixed leaf litter are shown in Table-1. These data indicate that obtaining such fungi isolates from mixed litter substrate is quite potential since lignocellulose is a main constituent of plant cell. Isolates of cellulolytic, xylanolytic and ligninolytic fungi were then selected based on enzymatic activity and the best of them was used to make a consortium of fungal inoculum expected to accelerate the decomposition of mixed leaf litter.

Table-1. Number of fungal colonies from leaf litter substrates.

No.	Isolates	CFU/ml
1	Cellulolytic	2. 7 x 10^5
2	Xylanolytic	5. 3 x 10^4
3	Ligninoytic	2. 5 x 10^5

4.1. Cellulolytic potential of fungal isolates

A total of 5 cellulolytic fungi (Htm1, Htm2, Pth1, Pth2, and Shj) were isolated from decomposing leaf litters in Biological Garden (Arboretum) UGM (Gadjah Mada University) (Table-2). The results of the clearing zone method revealed that all isolates were able to hydrolyze cellulose exhibited clearing zones around their colonies (Figure-1). Carboxymethyl cellulose CMC was a more favorable carbon source for screening the cellulolytic fungi (Shahriarinour *et al*., 2011). The data showed that isolates *Shj* had the greatest ratio which was 3.978 and this means that isolate *Shj* (Figure-1(C)) has a relative greater cellulolytic enzyme activity than other isolates. Screening of cellulolytic fungi is based on the size of the diameter of the clear zone indicating the ability of isolates to hydrolyze cellulose (Shahriarinour *et al*., 2011).

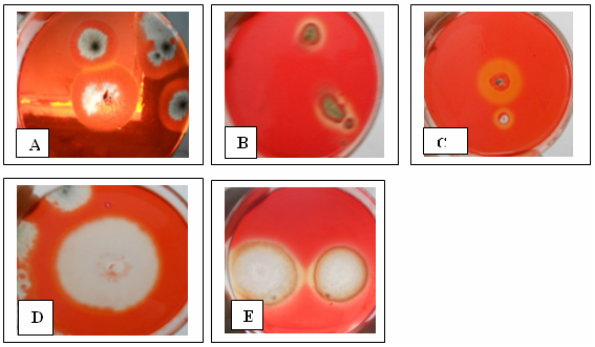

Figure-1. Plate screening of CMCase in the surrounding of colonies and Congo red dye staining for displaying clearing zone (halo). The isolates are *Htm1* A), *Htm2* (B), *Shj* (C), *Pth1* (D) and *Pth2* (E).

Table-2. Selection of cellulolytic fungi according to clear zone (halo) ratio.

No.	Fungal isolate	Diameter of colony (cm)	Diameter of halo (cm)	Colony area (cm^2)	Halo area (cm^2)	Ratio of halo/colony	Ratio average
1	*Htm 1*	1. 4 1. 3	2. 5 1. 4	1. 539 1. 327	4. 906 1. 539	3. 189 1. 160	2. 174
2	*Htm 2*	3. 0 2. 7	3. 4 3. 3	7. 065 5. 723	9. 075 8. 549	1. 285 1. 494	1. 390
3	*Pth 1*	3. 4 3. 5	3. 9 0	9. 075 9. 616	11. 940 0	1. 316 0	0. 658
4	*Pth 2*	4. 7 2. 8	5. 3 3. 2	17. 431 6. 154	22. 051 8. 034	1. 272 1. 305	1. 289
5	*Shj*	2. 0 1. 2	3. 4 2. 7	3. 140 1. 130	9. 075 5. 723	2. 890 5. 065	3. 978

Shj expressed the high cellulolytic potential for decomposition of cellulose. These results indicated that the isolates *Shj* having cellulolytic enzyme activity relatively higher than other isolates, and this isolate were selected for the preparation of inoculum in subsequent studies. The ability of this isolate in producing a clear zone was an evidence to his ability in degrading CMC (cellulose source) by cellulase actively released by colonies of fungi (Shahriarinour, *et al*., 2011). Cellulases are complex enzymes consisted of cellobiohydrolase, endoglucanase and β-glucosidase acting synergistically to degrade natural cellulose (Taylor and Marsh, 1963).

Decomposition of cellulose requires the activity of three enzymes simultaneously, namely: 1) cellobiohydrolase, an exo-acting enzyme cutting successive disaccharide units (cellobiose) from the non-

reducing end of the chain, 2) endoglucanase, an endo-acting enzymes working at the center and randomly breaking down molecule chains into smaller parts and 3) β-glucosidase which break down cellobiose into glucose (Deacon, 1997; Perez *et al*., 2002). Therefore this selected isolate (*Shj*) was developed to be a potential inoculum for CSK.

4.2. Xylanolytic potential of fungal isolates

There were 6 isolates indicating xylanolytic isolates (*Lht, Lhj, Lpt1, Lpt2, Lpt3* and *Xabu*). The xylanase production ability of fungi assessed by estimating zone around the colony formed due to ability of fungal isolates to hydrolyze xylan (Figure-2). The sizes of clearing zone diameter for each isolate are shown in Table-3. Results showed that isolate *Lht* has highest enzyme activity among total isolates which is 3.591 (Figure-2(A)). Screening of xylanolytic fungi is based on the size of the diameter of the clear zone indicating the ability of isolates to hydrolyze xylan (Sridevi and Charya, 2011). Solid screening medium containing xylan as the sole carbon source developed for this purpose was employed by Flannigan and Gilmour (1980). Xylanase producing organisms were first identified on the basis of the clearing zone formed around the colonies.

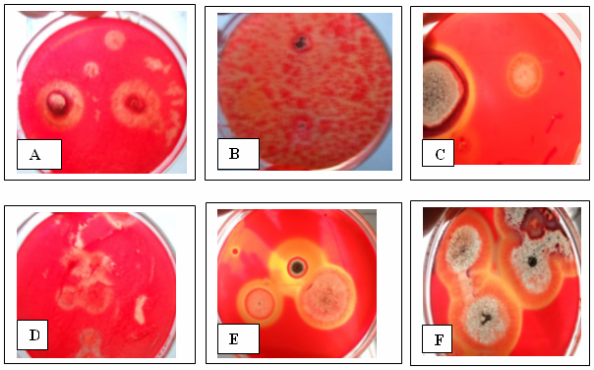

Figure-2. Plate screening of xylanase in the surrounding of colonies and Congo red dye staining for displaying clearing zone (halo). The isolates are *Lht* (A), *Lhj* (B), *Lpt1* (C), *Lpt2* (D), *Lpt3* (E) and *Xabu* (F).

Table-3. Selection of xylanolytic fungi according to clear zone (halo) ratio.

No.	Fungal isolate	Diameter of colony (cm)	Diameter of halo (cm)	Colony area (cm^2)	Halo area (cm^2)	Ratio of halo/colony	Ratio average
1	*Lht*	1. 3 1. 5	2. 5 2. 8	1. 327 1. 766	4. 906 6. 154	3. 697 3. 485	3. 591
2	*Lhj*	1. 5 1. 5	2. 2 1. 8	1. 766 1. 766	3. 799 2 543	2. 151 1 440	1. 796
3	*Lpt 1*	1. 6 2. 7	2. 3 3. 1	2. 010 5. 723	4. 153 7. 544	2. 066 1. 318	1.692
4	*Lpt 2*	1. 4 1. 5	2. 2 2. 5	1. 539 1. 766	3. 799 4. 906	2. 468 2. 808	2. 638
5	*Lpt 3*	1. 8 0. 9	2. 6 1. 7	2. 543 0. 636	5. 306 2. 269	2. 086 3. 567	2. 826
6	*Xabu*	3. 8 2. 6	4. 3 3. 0	11. 335 5. 307	14. 515 7. 065	1. 280 1. 331	1. 306

Xylanolytic enzymes catalyze the hydrolysis of xylan, the major constituent of hemicellulose, which is the second abundant molecule in plant cell wall (Coughlan and Hazlewood, 1993). Xylan is a branched heteropolysaccharide constituting a backbone of β-1, 4 linked xylopyranosyl units substituted with arabinosyl, glucuronyl and acetyl residues (Shallom and Shoham, 2003). Biodegradation of xylan is a complex process that requires the coordination of several xylanolytic enzymes which hydrolyze xylan and arabinoxylan polymers. This enzyme group includes endo-β 1, 4-xylanase (1, 4-β-D xylan xylanohydrolase, EC 3.2.1.8), which attack the main chain of xylans and β-D-xylosidase (1, 4-β-xylan xylanohydrolase, EC 3.2.1.37), which hydrolyze xylooligosaccharides into D-xylose, in addition to a variety of debranching enzymes that is, α–L-arabinofuranosidases, α-glucuronidases and acetyl esterases (Collins *et al*., 2005).

4.3. Ligninolytic potential of fungal isolates

There were 2 ligninolytic isolates (*Lpt1* and *Lpt3*) among 6 isolates observed (*Lht, Lhj, Lpt1, Lpt2, Lpt3*, and *Xabu*) (Table-4). Fungi ligninolytic were indicated by the appearance of brown color on the bottom and around the colonies (Figure-3). The qualitative assessment of oxidation scales of color was made based on brown color intensity. The darker of the color indicates that the oxidation of lignin was stronger. *Lpt 1* showed the value of the intensity and ratio of zone wich was 4 and 6.25, respectively.

Table-4. Qualitatif assay for lignin degrading enzymes.

No.	Fungal Isolat	Oxidation color	Color diameter (mm)	Colony diameter (mm)	Zone ratio	Oxidation scale
1	*Lht*	-	-	-	-	0
2	*Lhj*	-	-	-	-	0
3	*Lpt 1*	Dark brown	25	10	6.25	4
4	*Lpt 2*	-	-	-	-	0
5	*Lpt 3*	Light brown	35	30	1.36	2
6	*Xabu*	-	-	-	-	0

Oxidation scales measured on the 7th day of cultivation on B and K medium containing 4 mM guaiacol:: 4=dark brown c; 3= brown; 2= light brown, 1= smear color and 0= no color zone

Screening of ligninolytic fungi was observed on the presence of oxidation color of guaiacol surrounding a colony (Atalla *et al*., 2010). Guaiacol oxidation is one of the most convenient qualitative assay of Lignin Modifying Enzymes (LMEs) production among fungi. Guaiacol is a polymer that is widely used to study the biodegradation of lignin because it has a lot of character as synthetic lignin and has been shown to be recognized by the ligninolytic systems in microorganisms (Crawford *et al*., 1981).

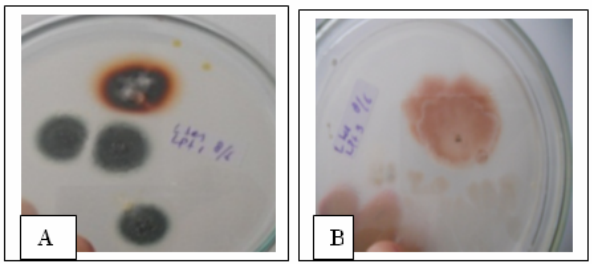

Figure-3. Positive guaiacol oxidation of *Lpt1* (A) dan *Lpt3* (B).

The use of clear zone as an indicator of enzyme activity gave different responses on different enzyme activity (cellulase, xylanase and ligninase). Shahriarinour *et al*. (2011) showed that on cellulolytic scrrening of *Aspergillus* sp indicated that the ratio of clear zone also related to the CMCase activity, filter paper (FP) and β-glucosidase. The data showing highest ratio of clearing zone also has the highest enzyme activity. Meanwhile Sridevi and Charya, (2011) stated that there is no correlation between the zone of the clearing and the enzyme units (xylanases) produced. They stated that zone of inhibition is not a perfect marker to decide the xylanase highest producer however, might indicate about the selection of isolates (Teather and Wood, 1982; Tseng *et al*., 2000). On the other hand, Atalla *et al*. (2010) concluded that in screening for lignin degrading enzymes on *Trematosphaeria mangrovei* exhibited fast and large oxidation of guaiacol on agar plates. Thus the clear zone or oxidation area could at least give an idea of speed and coverage area of the enzyme released and we believe that this parameters are still be possible to assign as a character of certain isolates. The use of clear zone as an indicator of enzyme activity gave different responses on different enzyme activity (cellulase, xylanase and ligninase).

4.4. Fungal inoculum development

Fungal inoculum on sorghum seeds with the three selected isolates (*Shj*, *Lht* and *Lpt1*) after 12 days of incubation (Figure-4) were able to produce spores and CFU as shown in Table-5. This result demonstrates the potential of inoculum is very possible to be applied as Composting Starter Kit. CFU values of the inoculum (Table-5) was higher comparing to CFU of spores from decomposed leaf litter substrate in first isolation (Table-1). Viability of spores is very critical for CSK usage. The successful use of inoculum is largely determined by the level of spore viability, whereby if the spores have high viability it will produce a large fungal biomass. This will certainly give significant effect if this inoculum is applied for composting of organic material.

Figure-4. Fungal inoculum of selected isolates on sorghum seeds.

Table-5. Production and viability of spores from 12 day old inocula.

No.	Isolate	Fungi	spores/ml	**Viability** (CFU/ml)
1	*Shj*	Cellulolytic	1.5×10^9	9.6×10^9
2	*Lht*	Xylanolytic	9.3×10^7	5.6×10^8
3	*Lpt1*	Ligninolitic	4.2×10^9	8.2×10^6

The number of propagules from sorghum was relatively high and has a good prospect as an inoculum compared to a previous study by Beary *et al.* (2002) which used fungi *Ceriporiopsis subvermispora* (cellulolytic) with bacteria *Cellulomonas* sp., and *Azospirillum brasilense* as a consortium for enhancing sugarcane waste decomposition, which produced 8.4×10^5 propagules / ml. Production and viability of spores produced on sorghum media is also relatively higher than the results of previous research by Elzein, *et al.* (2004) which made inoculum of microconidia, mycelia and chlamydospores of *Fusarium oxysporum* entrapped in pesta granules (32 g semolina, a coarse durum wheatflour, 6 g kaolin and 2 g sucrose) with the highest spores of 1.6×10^9 and viability of 4.6×10^7 CFU/g.

5. CONCLUSIONS

Selected fungi of cellulolytic (*Shj*), xylanolytic (*Lht*) and ligninolytic (*Lpt1*) isolates were very potential to develop as inoculum for Composting Starter Kit. Sorghum seeds are able to use as a substrate for manufacturing fungal inoculum due to the high spore's production and the number and spores viability. This indicates that the development of fungal inoculum was quite successful.

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