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Morphological Characteristics of Some Microfungal Species Isolated From Spontaneously Fermented *Jatropha curcas* Seed Meal

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ABSTRACT

Jatropha curcas seed meal (JCSM) is known as a good substrate for enzyme production via solid state fermentation. The aim of this research was to study the mycology of spontaneously fermented JCSM. JCSM was spontaneously fermented for six days. Mycelia of fungus were cultured and incubated at 25°C on potato dextrose agar, sabouraud for six days. Microslide culture was carried out to obtain greater detail of the resulting morphological features. Five fungal species (*Aspergillus flavus, Aspergillus niger, Rhizopus stolonifer, Penicillium chrysogenum and Fusarium* species) were isolated. This is the first report of fungal species associated with spontaneous fermentation of defatted *Jatropha curcas* seed meal.

Introduction

Microbial fermentation of foods or substrates has been a wonder tool in biotechnological innovations. Fermentation technology has served as a tool in many research works in producing novel products. This technology has been employed in the detoxification of defatted *Jatropha curcas* seed or kernel meal. Since the industrial method via chemical means of detoxification of *J. curcas* seed meal is expensive and poses risk of residual chemical effect (Aregheore *et al*., 2003), as a result, fermentation technology has served as an alternate means of detoxifying of *Jatropha curcas* seed/kernel meal.

The seed meal of *J. curcas* is the by-product obtained after oil extraction from *J. curcas* seeds. The use of *J. curcas* seed or kernel meal as substitute for feed has received global attention in growing areas. This is because, *J. curcas* seed meal (JCSM) or kernel meal is noted for being a good substrate for enzyme production via solid state fermentation (Mahanta *et al.*, 2007). And also, the *J. curcas* kernel and seed meal serve as a good protein source for animal feeds, due to the rich protein content with high concentrations of essential amino acids (Makkar *et al*., 1998a; Tjakradidjaja *et al*., 2009).

However, the *J. curcas* meal is toxic due to the presence of anti-nutritional factors such as phorbol ester, the main principal toxic agents; curcin, trypsin inhibitors, lectin and tannins (Makkar *et al*., 1998b).

Detoxification approaches to eliminate phorbol esters and other anti-nutrient in *J. curcas* seed and kernel meal using microbes have been reported. Solid state fermentation of *J. curcas* seed meal using specific *Trichoderma* spp and endophytic fungi were able to reduce phorbol esters by 96.9% - 99.7% and 88.9% - 92.2% respectively (Najjar *et al.*, 2014). Fungi (*Aspergillus niger, Penicillium chrysogenum, Rhizopus oligosporus, Rhizopus nigricans and Trichoderma longibrachitum*) used in the fermentation of *J. curcas* kernel cake for 7 days had significant increase in the crude protein content of all the fungi treated samples (Belewu and Sam 2010). Instances where spontaneous solid state fermentation was employed in detoxification of defatted *Jatropha curcas* kernel meal, the results showed significant increases ($p < 0.05$) in dry matter, crude protein and ash content of all fermented meals, crude phorbol ester (CPE) and phytic acid concentrations in the kernel meals decreased significantly ($p < 0.05$) (Chikpah and Demuyakor 2012). Though such nutritional benefits accrue from spontaneously fermentted *J. curcas* seed/kernel meal (Chikpah and Demuyakor 2012), the mycology of the fermented substrate was identified.

The present study aims to isolate, identify and characterize the mycoflora of spontaneously fermented *Jatropha curcas.*

Materials and Methods

Study area

Dried *J. curcas* seeds for the study were harvested from Nyankpala (A Guinea savanna zone, Location Lat. 09° 25‘N, Long. 00° 58‘ W; average daily temperature 28.3 °C; annual rainfall of 1043 mm; average humidity of 58 %) and Bole (located on the coordinates: Lat. 9° 2’ N and Long. 2° 29’ W) all in the Northern Region of Ghana. The study was conducted at the Spanish grant laboratory of the Department of Biotechnology, University for Development Studies and the Department of Clinical Pathology, Noguchi Memorial Institute for Medical Research, College of Health Sciences, University of Ghana.

Defattening of *Jatropha curcas* whole seed

Dried *J. curcas* seeds were milled into powder using an Elbee blender (model: LB-1323). Defattening of *J. curcas* seed was done using cold maceration method with petroleum ether (at 40-60 ^{o}C). The ratio of milled *Jatropha curcas* and petroleum ether was 1:3 by weight. The defatted samples was air-dried, kept in sterile polyester plastic containers and refrigerated at 4 °C for later use and analysis.

Fermentation setup

J. curcas seed meal (JCSM) was divided into four portions; sterilized portion (A) was the control, while the second (B), third

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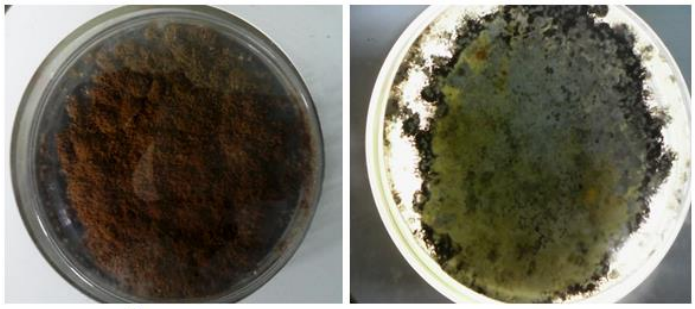
(C), and fourth (D) portions were not autoclave. Each treatment contained 10 g of defatted *J. curcas* seed and placed in sterile Petri dish. Approximately 5 ml of sterile distilled water was added to each sample is to it moist. Each treatment was replicated thrice and allowed to ferment spontaneously for 6 days.



A B

Figure 1. Plate (A) front view of spontaneously fermented JCSM on a petri dish; Plate (B) reverse view of spontaneously fermented JCSM on a petri dish.

Media preparation

Thermo Scientific Oxoid Sabouraud dextrose agar media and potato dextrose agar (PDA) media **(**Sigma-Aldrich) were used in the identification of fungi. Each media was prepared according to manufacturer's instructions. Corn meal agar (2g of corn meal with 15g of agar; Suspend 17g in 1 litre of distilled water. Bring to the boil to dissolve completely and sterilise by autoclaving at 121°C for 15 minutes) was used to induce sporulation of *Fusarium* species.

Isolation of fungus from spontaneously fermented JCSM

In order to isolate fungi from the fermented JCSM into pure culture; sterile inoculating loop was used to transfer mycelia from each unique fungus in the mixed colony onto sterile Potato Dextrose Agar (PDA) and Sabouraud agar. The cultures were incubated at (28 °C ± 2 °C) for seven days to facilitate morphological studies and identification

Morphology and microscopic characterization

Morphological characteristics of isolates were based on colour and growth pattern on PDA and Sabouraud agar media. Microscopic characterization of fungal isolates was carried out by preparing a micro-slide to obtain greater detail of morphological features and using a Leica DMLS2 light microscope (Microsystem 11020518016).

Growth studies of fungus

In measuring the growth of fungus, sterilized 5 mm cork-borer was used in cutting circular disk of fungal colony at the edges or margins of growing mycelium. The circular disk was sub- cultured on both PDA and Sabouraud agar media. The average diameter of the pure colony was measured at intervals of 6 h for 72 h using a calibrated transparent ruler.

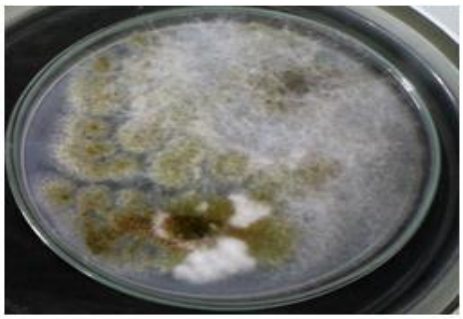
Data analysis

Data collected were presented in tables and figures using Microsoft Word and Excel 2010 edition.

Results and Discussion

Mycoflora of spontaneously fermented *Jatropha curcas* seed meal

The study employed spontaneous fermentation on defatted *Jatropha curcas* seed meal. During the six days of spontaneous fermentation, four different fungal cultures were isolated. This included *Aspergillus* species (*A. niger* and *A. flavus*), *Fusarium* species, *Rhizopus stolonifer* and *Penicillium chrysogenum* (Table 1). The morphology and microscopic features of each fungus are described in table 1. The manifestation of these fungi resulted from spores associated with the seeds prior to fermentation which were noticed during preliminary studies as shown in figure 2. Sterile defatted *Jatropha curcas* seed meal had no fungi colony growth under the same environmental condition which implies that spores in the defatted JCSM were destroyed (Table 2). The growth of the various fungi within six days suggests that the Jatropha seed meal (substrate) provided the required nutrient for their growth and development. These fungi have been reported to be associated with *Jatropha curcas* seed. Srivastava *et al*., (2011) isolated *Aspergillus niger*, *Aspergillus flavus*, *Rhizopus* species and *Fusarium* species among nine storage fungi from *Jatropha curcas* seed stored over a period of time.



C

Figure 2. Plate (C) Jatropha seeds with mixed culture of fungi on PDA after 3 days of incubation

Morphological and microscopic characteristics of the isolated fungi

Mycologists have adapted different concepts in defining fungal diversity. One classical approach is based on morphological study (characteristics or features) and differentiating among them (Tiwari *et al.*, 2011). In recent times, molecular, biochemical and physiological methods are significant tools in identifying some fungi species; however morphological properties are commonly employed in identification of fungi species (Asan, 2004).

Isolated *Aspergillus niger* colonies from *J. curcas* substrate exhibited characteristic morphological features indicated in Table 1 and Figure 3. De *et al.* (2014) also characterized *A. niger* morphologically and found it to possess a characteristic growth which begins initially as a yellow colony that soon develops a black, dotted surface as conidia. As the culture becomes old, the colony becomes jet black and powdery (Figure 3C). The reverse side remains buff with cream colour; this occurs on any culture medium. On Sabouraud media, exhibited a whitish morphology which turned black as colonies became old (Fig. 3, plate B). Microscopically, the fungus revealed septated hyphea which were hyaline (Fig. 3, plate D). Conidiophores were formed and the elongations of conidiosphores were halted as a result of the formation of a swollen vesicle which were spherical or round (Fig. 3, plate E). The microscopic characteristics of *A. niger* were similar to reports of Forbes *et al*. (2007). Thus, *A. niger* has a characteristic darkly pigmented roughened spores with the hyaline septate hyphae. Similar features were revealed by De *et al.* (2014) in microscopic characterization of *A. niger*.

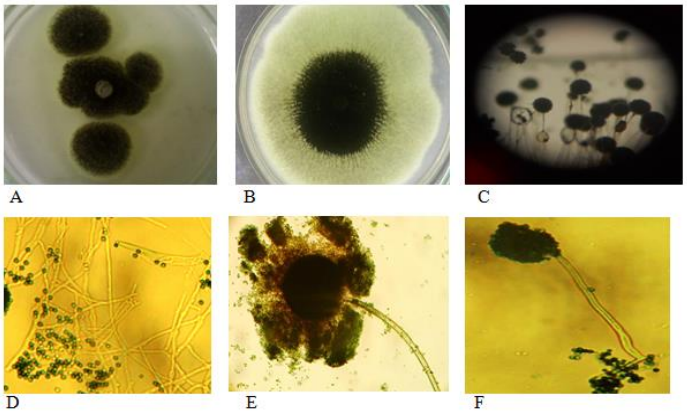


Figure 3. Plate A). *A. niger* on PDA; B) *A. niger* on Sabouraud; C) Microscopic view of *A. niger* colony 10X; D) Septated mycelium of *A. niger* 20X; E. *A. niger* head on a conidiophores. Conidiophores terminating in swollen vesicle and conidiophores releasing spore; F) Microscopic image of sporulating structure. Magnification 20X

The study revealed the association of *Aspergillus flavus* with spontaneous fermented Jatropha meal. It was observed that *A. flavus* grew in almost all the spontaneous fermented samples as shown in Table 2. The occurrence of *A. flavus* in stored *J. curcas* seed have been reported by Sahab *et al.* (2011). The fungus had yellowish green colony on PDA (Fig. 4, plate A). On sabouraud media the fungus had a concentric morphology with colour varying from dark brown region at the centre of the colony due to its aging, followed by brown circle, deep yellow circle and whitish mycelia around the colony (Fig. 4, plate 4B). On both media, the fungus displayed powdery surface. The characteristics exhibited by *A. flavus* in the study correspond with the observation of Forbes *et al.,* (2007). The microscopic characteristics of the fungus were all similar to the features described by Forbes *et al.,* (2007), which include globule vesicles and phialides that are produced directly from the vesicle surface (uniserate). The phialides give rise to short chains of spherical conidia.

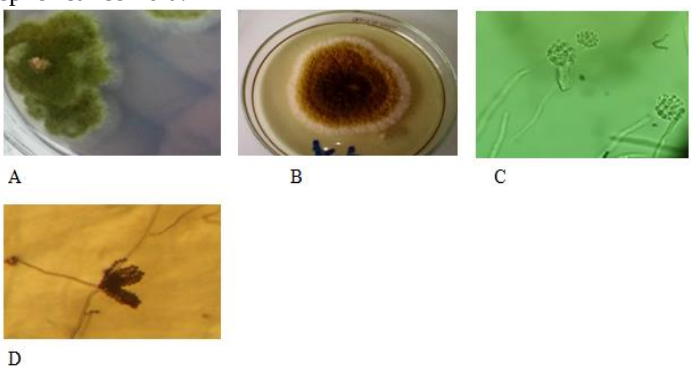


Figure 4. Plate (A). *A. flavus* on PDA; (B) *A. flavus* on Sabouraud; (C) Conidiophores terminating in swollen vesicles. Vesicles are globose and phialides are produced directly from the vesicle surface; (D) Microscopic view of *Aspergillus flavus*. Magnification 20X

Rhizopus stolonifer possessed copious cottony and aerial mycelia growth on PDA with black globules in it (Fig. 5 Plate A). Similar growth pattern was observed on sabouraud agar media (Fig. 5, plate B). *R. stolonifer* had high growth rate on both potato dextrose and sabouraud agar. This indicates that both media were appropriate for *R. stolonifer* culturing. These morphological characteristics corroborate with the description of *R. stolonifer* by Hernández-Lauzardo *et al.,* (2006). The identification of this mould was based mainly on the appearance of a root-like structure and the connection of sporangiosphores at a point by septated hyphae known as stolons (Fig. 5 plate C and D). The *stolonifer* species is characterized by the formation of well-defined complex rhizoids according to Hernández-Lauzardo *et al.* (2006), which gives rise to unbranched sporangiophores which are terminated by sporangium (Fig. 5, plate D)

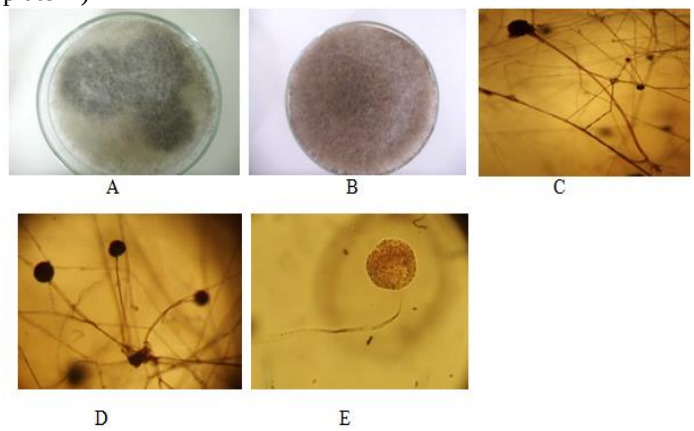


Figure 5. Plate (A) *Rhizopus stolonifer* on PDA; (B) *Rhizopus stolonifer* on sabouraud; (C) Microscopic view of colonY; (D) Sporangiophores are connected to one another by septate hyphae; (E) Large sac-like sporangia that contain sporangiospores on long sporangiophore. Magnification 20X.

The morphological and microscopic characteristics of *Penicillium chrysogenum* is described in table 1. Tiwari *et al.* (2011) described *P. chrysogenum* as green in colour with the edges of colonies being white with yellow back side on potato dextrose agar (PDA). Similar morphological features were observed in this study (Table 1 and Fig. 6, plate A). The morphological characteristics of *P. chryosgenum* were unique when compared to eleven *Penicillium* species isolated during morphological and molecular differentiation of different *Penicillium* species by Tiwari *et al.* (2011). The colony grew faster on saboraud than on PDA. It was beige in appearance and strongly adhered to the sabouraud media. Microscopically, *Penicillium chrysogenum* was observed to have septate hyphae and hyaline (Fig. 6, plate C). The conidiospore elongation was halted by a brush-like structure (Fig. 6, Plate D). The brush-like structure was formed due to the formation of branching swollen vesicles on which long chains of conidia were formed (Fig. 6, plate D). The microscopic features were similar to the description by Forbes *et al.* (2007). The hyphae were hyaline and brush-like which served as a classical morphological characterization for *Penicillium* species (Forbes *et al.,* 2007).

Fusarium species isolated in the study grew rapidly on PDA. It was unable to sporulate on PDA and saboouraud media. When cultured on corn meal agar, *Fusarium* sp. sporulated with cylindrical macroconidia (Fig. 7, plate D) five days after culturing. Moreover, the fungi growth on corn meal agar was not conspicuous as compared to the growth on PDA and sabouraud agar. *Fusarium* sp colony was initially white on PDA but changed to pink as the culture became old (Fig. 7, plate A), similar observation were made on Sabouraud media (Fig. 7, plate B).

Table 1. Mycoflora isolated from spontaneously fermented defatted *Jatropha curcas* by agar plate methods

Fungi	Morphology on PDA	Morphology on sabouraud	Microscopy of fungi	Suspected fungi
U	Black colony with yellow edges or hallow region. Aerial growth (Plate 3A). Surface of colony were powdery. An erect structures (like a stand) supporting a globule body (a head) initially transparent and becomes black finally (Plate 1C)	Colony exhibited white mycelium growth initially with black centre which spread or increases with time. The margins of the fungi were whitish. The colony had The reverse face of the plate was yellowish. As shown in Plate 3B.	Hyphae are septate (Plate 3D). Conidiophores formed terminate in a swollen vesicle (Plate 3E).	*Aspergillus niger*
V	Yellow-green colony with whitish powdery edge (Plate 4A).	Yellow-brown and powdery with whitish mycelium at the edges (Plate 4B).	Vesicles are globule and phialides are produced directly from the vesicle surface. Conidiophores terminating in swollen vesicles (Plate 4C).	*Aspergillus flavus*
W	Initially white and cotton like but turns gray after three days with black globule structure present all over (sporangia). Aerial mycelium growth on media. The reverse plate has a black colour. Fast growth rate. Covers the entire petri dish (90 mm diameter) in 3 days (Plate 5A).	It produces a fluffy, whitish hyphal but turns brown after three days. Presence of dotted brown sporangia. Aerial mycelium growth on media. Reverse plate is yellowish. Fast growth rate. Covers the entire petri dish (90 mm diameter) in 3 days (Plate 5B).	Large saclike that contain sporangiophores are connected to one another by septate hyphae (Stolons) (Plate 5C and D). Large saclike sporangia that contain sporangiospores on long sporangiophore (Plate 5E).	*Rhizopus stolonifer*
X	Greenish with white edges. Powdery surface (Plate 6A). Slow growth rate.	Whitish green colour with margins within the colony (Plate 6B).	The presence of hyphae which are hyaline and septate (Plate 6C). Conidiophores produces a brush like structure (Plate 6D)	*Penicillium chrysogenum*
Y	Snow white colony initially. Aerial mycelium growth. Colony turns pink as it ages (Plate 7A).	White colony like cotton but less aerial mycelium growth as compared to PDA (Plate7B).	Septated hyphae (Plate 7C). Production of macroconidia on corn meal agar. Rod like and slightly bent (Plate 7D).	*Fusarium* species

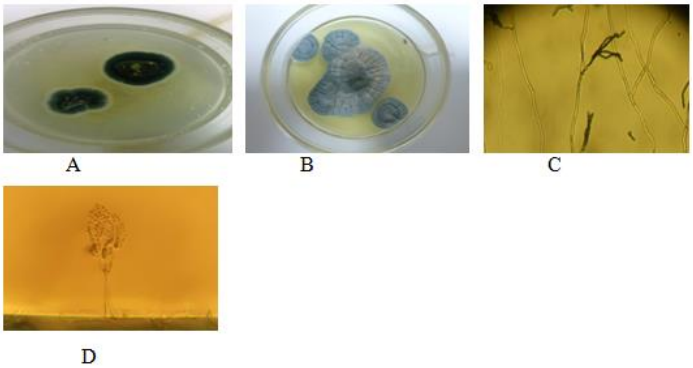


Figure 6. Plate (A) *P. chrysogenum* on PDA; (B) *P. chrysogenum* on sabouraud; (C) The presence of hyphae which are hyaline and septate. (D) Conidiophores produce a brush like structure Magnification 20X.

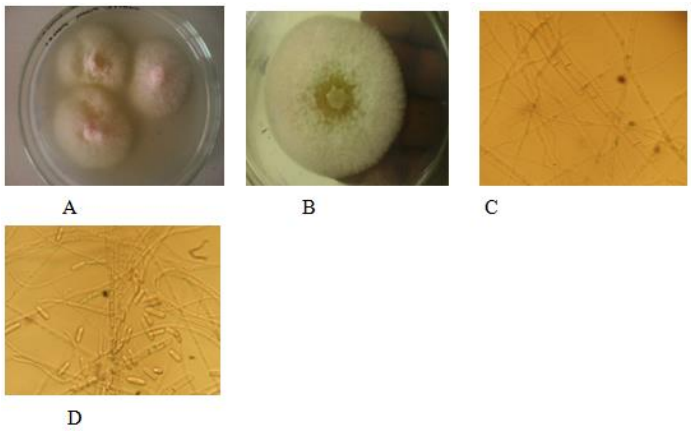


Figure 7. Plate (A) *Fusarium* species on PDA; (B) *Fusarium* species on sabouraud; (C) Mycelium septated (D) Macroconidia of *Fusarium* species on corn meal agar. Magnification 20X.

Fungi occurrence in spontaneously fermented defattened *Jatropha curcas* seed

From the study conducted *Aspergillus flavus* and *A. niger* occurrence were dominant in the spontaneously fermented samples (table 2). *Aspergillus flavus* dominance in the defatted JCSM was similar to observation made by Chikpah and Demuyakor (2012). *Fusarium* species was the fungus which had the least colonies on defatted JCSM (Table 2).

Table 2. Mycoflora isolated from spontaneous fermented JCSM

		Samples							
Fungus		A		B		C		D	
	Rep	Bole	Ny	Bole	Ny	Bole	Ny	Bole	Ny
	1	-	-	+	+	-	+	+	-
# *Aspergillus*	2	-	-	+	-	-	-	-	-
niger	3	-	-	+	-	+	+	-	+
	1	-	-	-	-	+	+	+	+
# *Aspergillus*	2	-	-	+	+	-	-	+	-
flavus	3	-	-	-	+	+	+	-	+
	1	-	-	-	+	+	-	-	-
Penicillium	2	-	-	-	-	-	+	+	+
chrysogenum	3	-	-	+	-	-	+	-	-
	1	-	-		-	+	-	-	-
Rhizopus	2	-	-	+	+	-	-	+	+
stolonifer	3	-	-	-	-	-	+	-	-
	1	-	-	+	+	+	-	-	-
Fusarium spp	2	-	-	-	-	-	+	-	-
	3	-	-	+	-	-	-	-	+

JCSM means *Jatropha curcas* seed meal; +: present; -: absent and #: dominant; Rep: replicates

Growth studies

Rhizopus stolonifer grew rapidly on PDA, filling the entire 85 mm petri-dish within 36 hours, with growth rate of 2.36 mm/h, (Figure 8) which was similar to the findings of Hernández-Lauzardo (2005). On sabouraud dextrose agar

(SDA), *R. stolonifer* growth rate was 1.18 mm/h and filled the petri-dish in 48 h (Figure 9). *Fusarium* species had a lower growth rate on PDA and SDA (Figures 8 and 9). At 72 hours of incubation the growth rates of *Fusarium* species on PDA and SDA were 0.62 and 0.57 mm/h, respectively. The growth rate of *A. niger* on PDA (0.46 mm/h) was higher than on SDA (0.41 mm/h). On PDA and SDA, the growth rate of *A. flavus* was 0.47 mm/h and 0.43 mm/h, respectively. The growth rate of *A. niger* and *A. flavus* on PDA and SDA were similar (Figure 8 and 9).

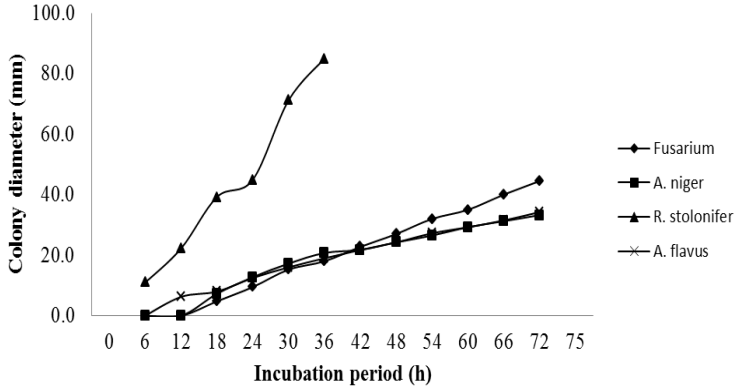


Figure 8. Colony diameter of fungal isolates incubated at room temperature on PDA

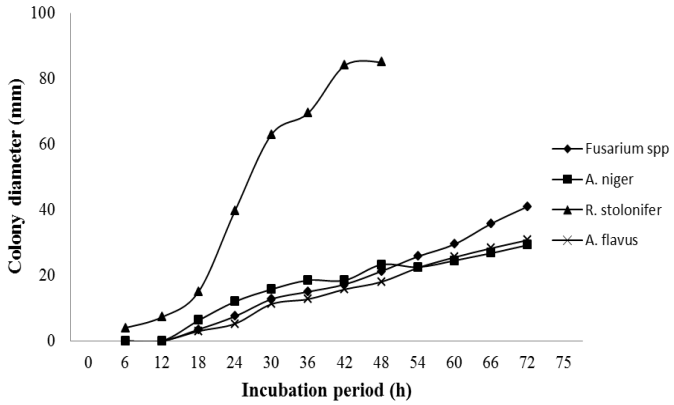


Figure 9. Colony diameter of fungal isolates incubated at room temperature on Sabouraud agar media

Conclusion

Our study has revealed that spontaneous fermentation of *Jatropha curcas* was influenced by some fungal load on the seeds. However it was noticed that mycological load of spontaneously fermented *Jatropha curcas* seed meal varied with each treatment. *A. niger* and *A. flavus* were isolated in most of the fermented samples whiles *Rhisopus stolonifer*, *Penicillium chrysogenum* and *Fusarium* species was less abundant.

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