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Full Length Research Paper

Development of compatible fungal mixed culture for composting process of oil palm industrial waste

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Six filamentous fungal strains/isolates such as *Aspergillus niger* (A), *Trichoderma viride* (Tv), *Trichoderma reesei* (Tr), *Penicillium* sp. (P), Basidiomycete M1 (M1) and *Panus tigrinus* M609RQY (IMI 398363)(M6) were tested to find their mutual growth in the laboratory. Potato dextrose agar (PDA) as a media was used for their fifteen combinations and two different fungi were grown 4 cm apart in every combination. The results of this present study showed that the combinations of *T. viride* and *Penicillium* sp. (Tv/P), *T. viride* and Basidiomycete M1 (Tv/M1), *T. reesei* and *P. tigrinus* M609RQY (Tr/M6) may interact as compatible, while *A. niger and T. viride* (A/Tv), *A. niger* and *T. reesei* (A/Tr), *T. viride* and *T. reesei* (Tv/Tr) and *Penicillium* sp. And *P. tigrinus* M609RQY (P/M6) were partially compatible and the other combinations were incompatible or inhibited by each other. Furthermore, the cellulolytic fungus *T. viride* was the dominant in all its combinations, and its growth rate and hyphal expansion showed the highest responses as compared to all combinations. These compatible filamentous fungi would be useful for effective composting process in further study.

Key words: Fungal compatibility, mixed culture, filamentous fungi, *in vitro* interactions, composting.

INTRODUCTION

Mixed culture fermentation is that in which the inoculum consists of two or more organisms (Gutierrez-Correa et al., 1999). Oriental food fermentations are good examples of this type of fermentation. Other examples include mushroom cultivation, composting, anaerobic digestion of organic matter, dairy fermentation and ensiling (Wood, 1984; Hogan et al., 1989; Fogarty and Tuovinen, 1991). Product or process- specific mixed culture fermentations have been used for bio delignification and enzyme production (Arora, 1995; Asiegbu et al., 1996; Gutierrez-Correa and Tengerdy, 1998).The mixed culturing of fungi may lead to better substrate utilization, increased productivity, and it also can strengthen and accelerate the bioconversion process on encouraging compatibility results reported in solid-state bioconversions than for liquid state fermentations (Alam et al., 2003; Molla et al., 2001). Strain compatibility is a critical factor in mixed culturing, and the most important observation was that synergistic interactions between compatible partners may overcome nutritional limitations in poor agricultural residues (Gutierrez-Correa et al., 1999).

The purpose of compatible mixed culturing is to let fungi work together mutually and degrade substrates faster. Many studies showed that evaluation of fungal possible compatible interactions was first studied in two agar media: potato dextrose agar (PDA) and malt extract agar (MEA) (Skidmore and Dickinson, 1976; Webber and Hedger, 1986). *Trichoderma reesei* LM-UC4, the parent strain and its hypercellulolytic mutant LM-UC4E1 were co-cultured with *Aspergillus niger* ATCC 10864 in solid substrate fermentation by Gutierrez-Correa et al. (1999). Bagasse was *Phanerochaete chrysosporium* supplemented with either soymeal or ammonium sulfate and urea, and the mutant strain was more responsive to mixed culturing than the parent strain when *A. niger* was the cooperating partner. Molla et al. (2001) observed the

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Table 1. Modes of interaction between filamentous fungi.

S/N	Interaction	Definition
1	Mutual intermingling	The growth where both fungi grow into one another without any macroscopic sign of interconnections.
2	Partial intermingling	The growth where the fungus being observed is growing into the opposed fungus either above/below or touching each other without making any inhibition zone.
3	Invasion/replacement	One mycelium grows into the other and begins to consume another one, it may finally replace it.
4	Inhibition at touching point	The fungi approached each other until almost in contact and a narrow demarcation line, 1 to 2 mm, between the two colonies was clearly visible.
5	Inhibition at distance	Inhibition at a distance of >2 mm

compatibility test where six strains such as, *Aspergillus versicolour, Mucor hiemalis*, Basidiomycete RWPI 512 and two strains of *Trichoderma harzianum* used and two combinations of *P. chrysosporium* and *T. harzianum* and *T. harzianum* and *M. hiemalis* were shown as compatible mixed culture for composting of domestic waste sludge in solid state bioconversion process. Another study was done by Alam et al. (2003) where four filamentous fungal strains such as *Penicillium corylophilum* (P), *A. niger* (A), *T. harzianum* (T) and *P. chrysosporium* (PC) were selected for compatible/incompatible mixed cultures for effective degradation of sewage sludge. This study showed that the combinations of P/A, P/PC and A/PC were compatible among the six combinations.

Overall, the interaction effects among the strains are very important in a bioconversion program by mixed cultures. Therefore, this study was undertaken to ascertain the compatibility among unexpected failure due to non-compatible strain combination for solid-state bioconversion of oil palm empty fruit bunch (EFB) with palm oil mill effluent (POME) for composting. The objective of this study was to find compatibility among fifteen fungal mixed cultures for effective composting process. An experiment was carried out with fifteen fungal mixed cultures (combinations) of six individual strains of filamentous fungi such as *A. niger, Trichoderma viride, T. reesei, Penicillium* sp.*,* Basidiomycete M1 and *Panus tigrinus* M609RQY obtained from local relevant sources to optimize the compatible mixed culture.

MATERIALS AND METHODS

In vitro interactions

The interactions were studied by dual direct opposition mycelial cultures. Interactions between the opposition colonies were visually assessed by various researchers (Skidmore and Dickinson, 1976; Stahl and Christensen, 1992; Molla et al., 2001). There are 5 recognized separate modes of interactions which can be assumed and are shown in Table 1. Their graphical representations are shown in Figure 1.

Fungal strains

Six strains of filamentous fungi such as *A. niger*, *T. viride*, *T. reesei*, *Penicillium* sp., Basidiomycete M1 and *P. tigrinus* M609RQY (IMI 398363) were used in this study. *A. niger* and *T. viride*, the two important cellulolytic fugi, were collected from the laboratory stock, University Putra Malaysia (UPM). The rest, *T. reesei* and *Penicillium* sp., another two cellulolytic fungi Basidiomycete M1 *and P. tigrinus* M609RQY (IMI 398363) and two lignin decomposers, were taken from Environmental Biotechnology laboratory stock of International Islamic University Malaysia.

Preparation of inoculum

The fungal strains/isolates used were broadly classified into two groups: slow- and fast- growing fungi. The fungi covered the full plate within 3 days and those needed after 3 days were considered as slow-growing and fast-growing fungi, respectively. The strains *T. reesei,* Basidiomycete M1 and *P. tigrinus* M609RQY were designated as slow-growing and the rest were fast-growing after evaluation of their growth rates in Petri dishes. All fungal strains/isolates were cultured on potato dextrose agar (PDA) (Oxoid Ltd., England, 3.9%, pH 5.5) as inocula sources. After 3 days for the fast growers and 7 days for the slow growers, the fungal cultures were used for inoculation of the interactions study. The agar discs containing the inocula were transferred with a 5 mm diameter paper pieces and used for inoculation from the young peripheral edges of cultures.

Experimental design for compatibility test

Evaluation of fungal possible interactions was studied in PDA media. Fifteen milliliters of each medium PDA (3.9%, pH 5.5, Oxoid) was taken in plastic Petri dishes (9 cm diameter). Two discs of different fungal strains/isolates were cultured 4.0 cm apart from each other in the same Petri dish to examine the consequences of different fungal interactions. Controls were single and dual cultured

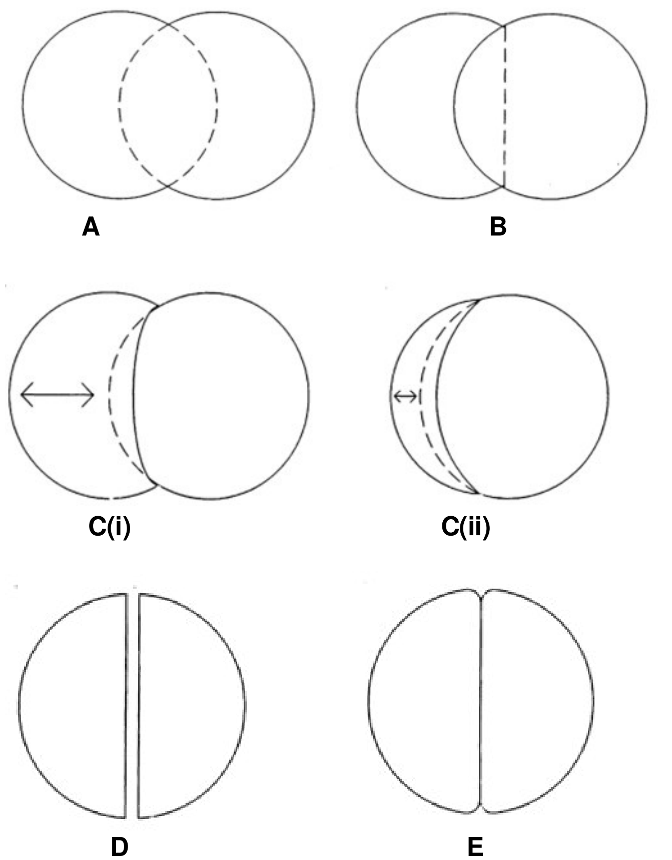

Figure 1. Graphical representation of possible outcomes of interacting fungi. (A) Mutual intermingling (compatible); (B) partial mutual intermingling (partial compatible); (C)(i) invasion/ replacement (early stage), (ii) invasion/replacement (final stage); (D) inhibition/deadlock (at touching point); (E). inhibition/deadlock (at distance).

by the same fungus, which was inoculated at the centre as well as at 4 cm apart, respectively.

For each interaction, the fast-growing fungi were inoculated 3 day later once the slow-growing fungi stated to grow. Otherwise, with simultaneous inoculation, the fast-growing fungus will quickly suppress and overlap the slow-growing partner without allowing its growth and expansion (Molla et al., 2002; Alam et al., 2004).

The interacting fungi were incubated at room temperature (32 ± 2°C) until they were matured enough and they were observed every day to know their responses. The experiments were done with three replicas.

RESULTS AND DISCUSSION

Observations on compatible/incompatible mixed culture

Among the 15 combinations (mixed culture), the results shown in Table 2 indicated that 7 combinations of *A. niger* and *T. viride (A/Tv)*, *A. niger* and *T. reesei (A/Tr)*, *T. viride* and *T. reesei (Tv/Tr), Penicillium* sp. and *P. tigrinus* M609RQY (P/M6), *T. viride* and *Penicillium* sp. *(Tv/P), T. viride* and Basidiomycete M1 (Tv/M1), and *T. reesei* and *P. tigrinus* M609RQY (Tr/M6) were found to be compatible or partially compatible mixed cultures (Figures 2 and 3) and the other 8 combinations of *A. niger* and *Penicillium* sp. *(A/P), A. niger* and Basidiomycete M1 (A/M1), *A. niger* and *P. tigrinus* M609RQY (A/M6), *T. viride* and *P. tigrinus* M609RQY (Tv/M6), *T. reesei* and *Penicillium sp* (Tr/P), *T. reesei* and Basidiomycete M1 (Tr/M1), *T. reesei* and *P. tigrinus* M609RQY (Tr/M6), *Penicillium* sp. and Basidiomycete M1 (P/M1), and Basidiomycete M1 and *P. tigrinus* M609RQY (M1/M6) were observed to be incompatible (Figure 4). Their growth patterns and mycelia's movement characterized their compatibility and inhibition for each other (Table 2). The combinations of Tv/P, Tv/M1 and Tr/M6 may interact as compatible (Figure 2), while the combinations of A/Tv; A/Tr; Tv/Tr and P/M6 were partially compatible (Figure 3). The combinations of A/P, A/P, Tr/P, Tr/M1 and P/M1 were inhibited at a distance (Figure 4a and b), but the combinations of M1/M6 and Tv/M6 were inhibited at their touching point, appearing yellow in color (Figure 4c) and *P. tigrinus* M609RQY replaced *A. niger* in the combination of A/M6 (Figure 4d).

In vitro interactions with each other and mycelium expansion were mingled mutually in the combination of Tv/P, Tv/M1 and Tr/M6 as shown in the experiments (Figure 2). Their mycellial growth expanded into each other and overlapped mutually. *A. niger* and *T. reesei* with *T. viride* were cultured and observed for 18 days to characterize their compatible natures (Figure 3a and b). This study shows that hyphal growth of *T. viride* interacted mutually at the touching points with its partner but it did not precede long distance to its partner. Furthermore, its partners, *A. niger* and *T. viride* also did not penetrate into it as much as mutual compatible cultures. This partial intermingling can be called partial compatible (Figure 3). Similar results were also observed in the combinations of *A. niger* with *T. reesei* and *Penicillium* sp. with *P. tigrinus* M609RQY. However, the combination of *A. niger* with *Penicillium* sp. showed compatible interaction in the study of Alam et al. (2003).

Figure 4a shows the combination of slow and fast growing fungi where *A. niger* was grown followed by slow growth fungus, Basidomycete M1. After certain period, it was seen that the growth of *A. niger* was inhibited by its partner at a certain distance (they were not compatible at all). Almost same pattern was observed but inhibition distance was less as compared to the combination of A/M1 (Figure 4b). In Figure 4d, the observations in incompatible cultures revealed that one of the single cultures was almost replaced by another and its growth was dominated by the other. So it can be assumed that *P. tigrinus* M609RQY is stronger than *A. niger*. Similarly, results were also recorded continuously until 21 days for Basidomycete M1 and *P. tigrinus* M609RQY where both were inhibited at the touching point (Figure 4c). A strong inhibition line showed that they were not mutually inter-

Table 2. Effects of compatible mixed culture on their growth.

S/N	Interacting species	Observation
1	*A. niger* *T. viride*	The two-partner strains stopped their growth at a contact point without inhibition.
2	*A. niger* *T. reesei*	Growth patterns were like that of *A. niger* and *T. viride.*
3	*A. niger* *Penicillium sp*	None could proceed towards each other and distance 2 to 3mm was observed between them.
4	*A. niger* Basidiomycete M1	Clear deadlock from a certain distance.
5	*A. niger* *P. tigrinus* M609RQY	M6 mycelium grows into *A. niger* and starts to consume it.
6	*T. viride* *T. reesei*	They are partially mutual to each other and stopped at the touching point.
7	*T. viride* *Penicillium sp*	These two strains grew more or less mutually and overlapped each other.
8	*T. viride* Basidiomycete M1	Mutual like *T. viride* and *Penicillium* sp.
9	*T. viride* *P. tigrinus* M609RQY	Both were stopped at touching point and made a clear inhibition zone.
10	*T. reesei* *Penicillium sp.*	Inhibition at certain distance.
11	*T. reesei* Basidiomycete M1	Inhibition at certain distance like before
12	*T. reesei* *P. tigrinus* M609RQY	More or less mutual and mycelia propagate towards the partner.
13	*Penicillium sp* Basidiomycete M1	Inhibition at certain distance.
14	*Penicillium sp* *P. tigrinus* M609RQY	They are partially mutual to each other.
15	Basidiomycete M1 *P. tigrinus* M609RQY	The two fungal strains met each other but did not allow further growth after contact point, making inhibition line at their territories.

mingled. Perhaps they synthesized some strong fungal metabolites around their territories which restricted the growth of the others (Molla et al., 2001).

Hyphal interactions and their growth were observed and recorded accordingly. The microscopic interactions of two different fungi grown adjacently were also studied

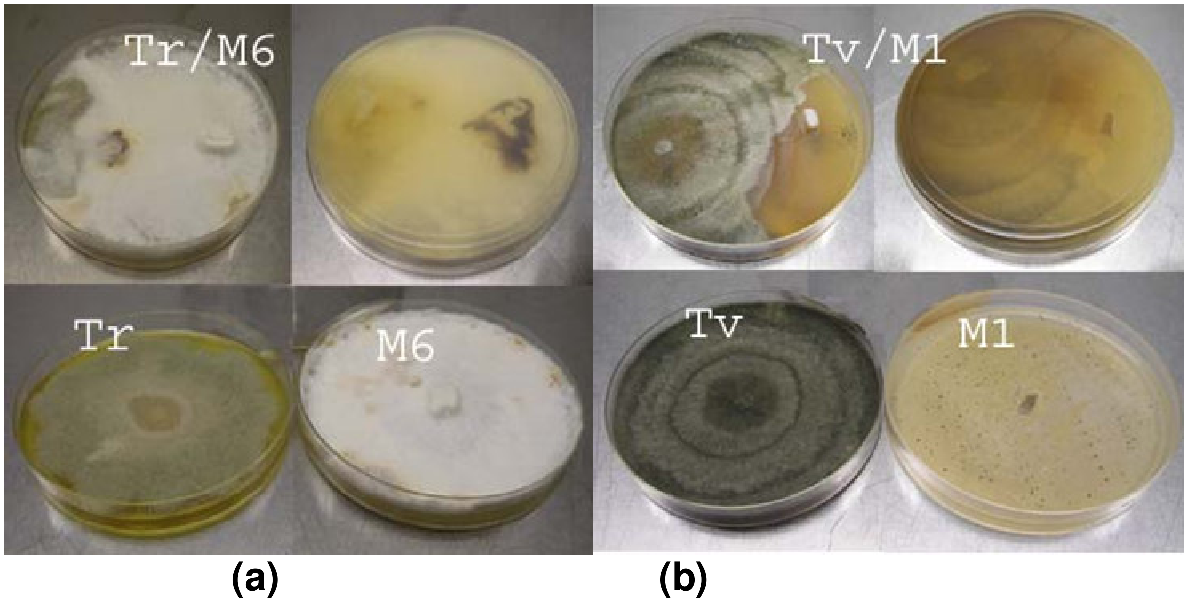

Figure 2. Compatible mixed cultures. (a) *T. viride* and Basidomycete M1 and (b) *T. reesei* and *P. tigrinus* M609RQY.

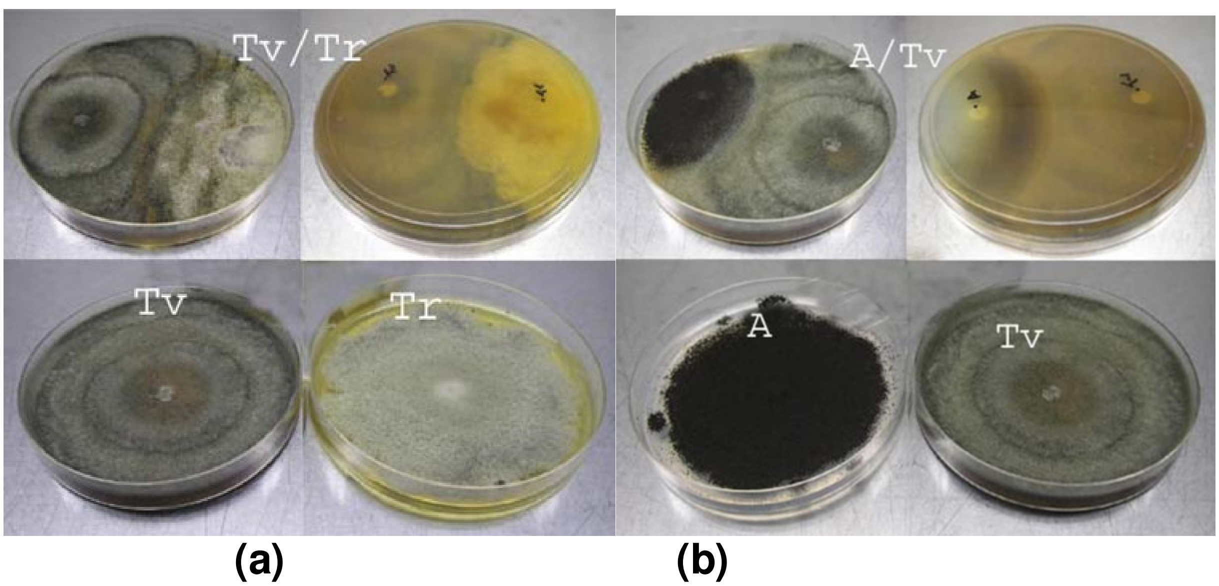

Figure 3. Partial compatible mixed cultures. (a) *A. niger* and *T. viride*, (b) *T. viride* and *T. reesei*.

to confirm the actual outcomes of the interactions (Figure 5). Mycelium growth and synergetic or antagonistic characteristics were categorized based on literature review (Porter, 1924; Skidmore and Dickinson, 1976; Stahl and Christensen, 1992; Molla et al., 2001; Alam et al., 2003) and whole scenario is stated in Table 2. Six fungal strains were selected to evaluate their successful adaptation and growth to domestic wastewater sludge (Molla et al., 2001). The interaction of *T. hazianum* Rifai with *P. chrysosporium* 2094 was identified as mutual intermingling. The strain *T. hazianums* with *M. hiemalis* Wehmer showed partial compatibility but *A. versicolor* Vuill acted as a strong repellent and all interactions exhibited deadlock/inhibition at a certain distance. Another study by Alam et al. (2003) was done to evaluate the fungal performance as compatible/incompatible mixed culture for the treatment of municipal wastewater sludge in a bioconversion process. The filamentous fungal strains such as *P. corylophilum, A. niger, T. harzianum* and *P. chrysosporium* isolated from relevant sources (waste-

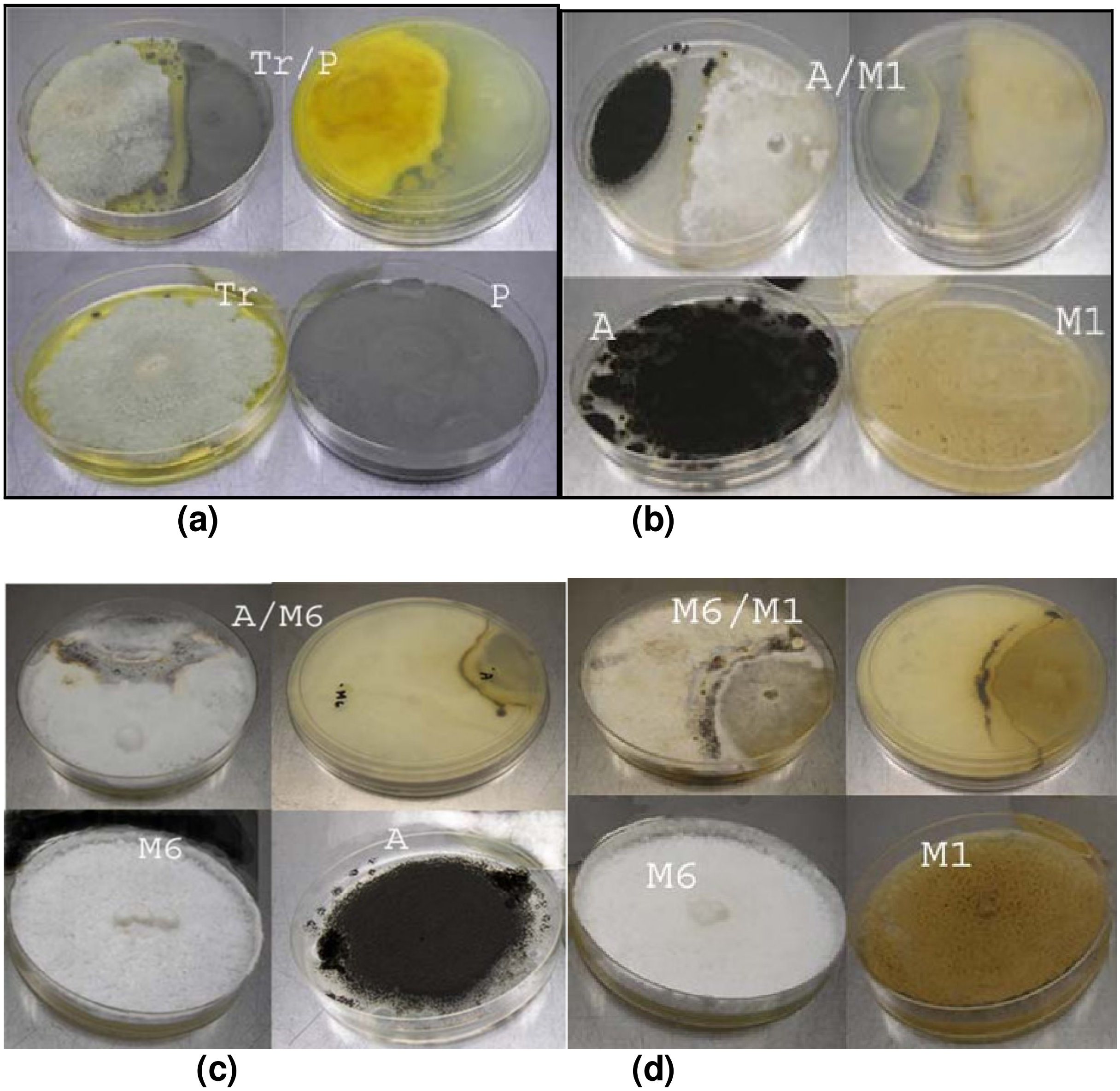

Figure 4. Incompatible mixed cultures. (a) *A. niger* and Basidomycete M1, (b) *T. reesei* and *Penicillium* sp., (c) Basidomycete M1 and *P. tigrinus* M609RQY and (d) *A, niger* and Basidomycete M1.

water, sewage sludge and sludge cake) were selected for compatible/incompatible mixed cultures. This study shows that the combinations of *P. corylophilum* and *A. niger, P. corylophilum* and *P. chrysosporium* and *A. niger* and *P. chrysosporium* had compatible growth among six combinations.

Observation of mycelium growth rates

Six filamentous fungi were split into two groups based on their growth rates. *A. niger, T. viride* and *Penicillium* sp. were estimated as fast growing whereas, *T. reesei,* Basidomycete M1 and *P. tigrinus* M609RQY were considered as slow growing. Observations were compared in three different combinations. Every day observations until matured stage (2 weeks for fast-growing and 3 weeks for slow-growing) are shown in Tables 3 to 5. These observations were compiled and growth rates were calculated after 3 days, that is, at 2nd day and at matured stage. Hyphal expansion towards or away from partner were also measured properly as well.

The *T. viride* had the maximum growth rate (4.5 cm) amongst the fast growing fungi (Table 3). This growth rate decreased slightly after one week and it remained the same until its matured stage at day 15. However, the

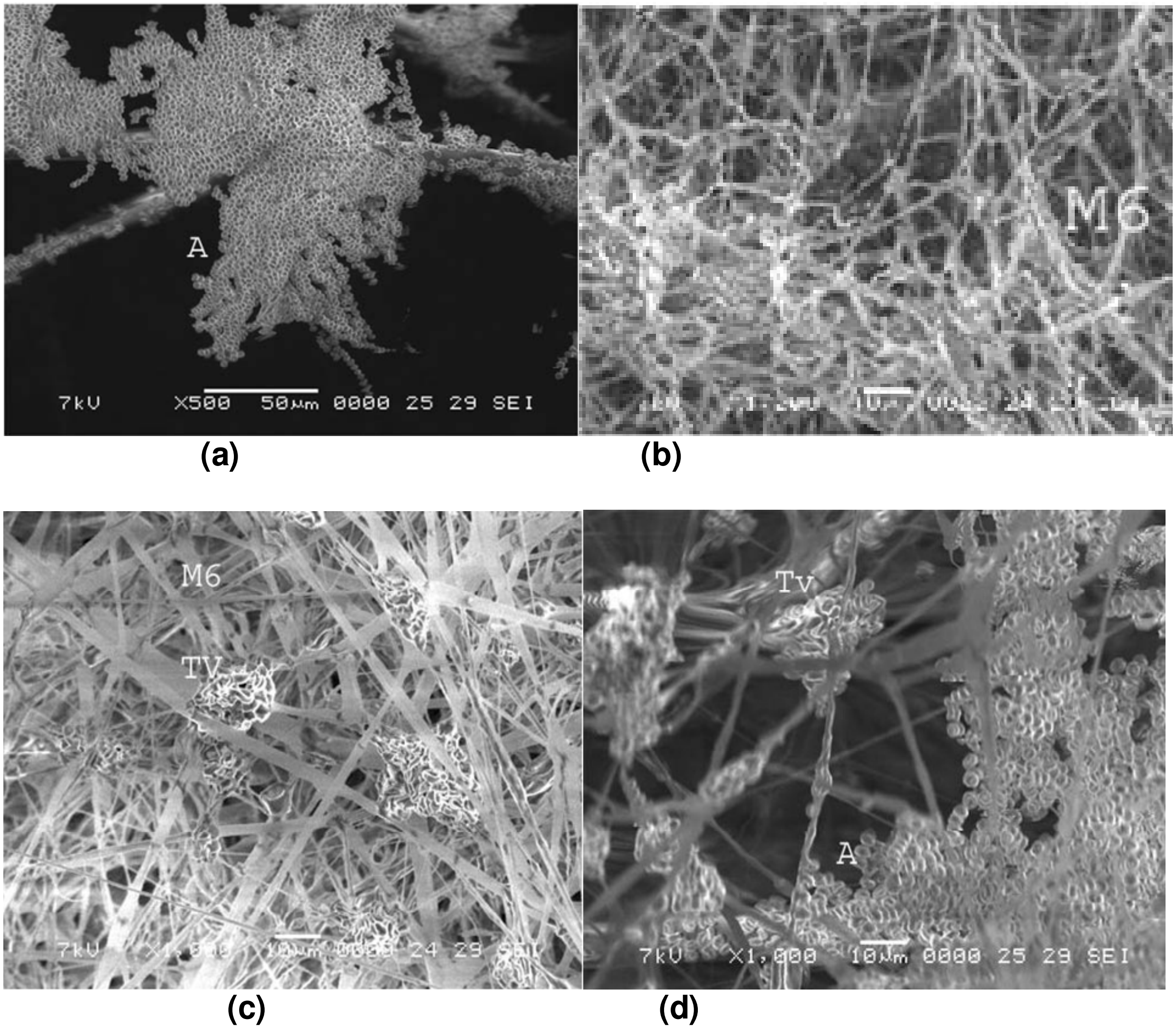

Figure 5. Scanning electron microscopy (SEM) pictures of filamentous fungi. (a) *A. niger* (b) *P. tigrinus* M609RQY (c) combination of *A. niger* and *T. viride* and (d) combination of *P. tigrinus* M609RQY and *T. viride*.

growth rate of *Penicillium* sp. remained the same at 4 cm and the growth rate of A. *niger* was different between 2nd and 15th day. The maximum colony diameter as well as mycelial growth away and towards the partners was also highest in *T. viride* as compared to other species. The growth rates were about 7.5 and 9 cm at 2nd and 15th day where the growth rates were quite lower, less than 7 cm for both *A. niger* and *Penicillium* sp. Furthermore, *T. reesei*, the slow growing fungus showed the highest growth rate and hyphal expansion, whereas, *P. tigrinus* M609RQY and Basidomycete M1 were 2nd and last, accordingly in terms of their growth and expansion towards and against their partners (Table 4). In Table 5, interaction responses for combined slow-fast growing fungi are shown where *T. viride* dominated over its partners among other combinations. The interactions between two colonies of *A. niger* and Basidomycete M1 appeared less responsive where maximum colony diameters were 5.9 and 2.2 cm, respectively and the least combined radial growth (8.1 cm) is shown in Table 5. Molla et al. (2001) showed the highest growth response of *T. harzianum* and *P. chrysosporium* with RW-PI 512 among their 15 combinations. The growth rate of *Trichoderma* was more than 7.6 cm and the growth rate of *P. chrysosporium* was almost 7.5 cm.

Conclusion

It is shown that the strain compatibility is a determining factor for successful mixed culture fermentations. The fifteen combinations of six different fungi were observed

Table 3. Interaction responses of filamentous fungi for or against their partners (fast growing fungi).

Interacting fungi	Day 2				Day 15			
	Growth rate (day^{-1})	Maximum colony diameter	Mycelial growth towards partner	Mycelial growth away from partner	Growth rate (day)	Maximum colony diameter	Mycelial growth towards partner	Mycelial growth away from partner
A. niger	4.0	6.5	2.0	2.0	4.0	8.5	2.4	2.5
T. viride	4.5	7.8	2.4	2.4	4.0	9.0	2.5	2.5
A. niger	3.9	6.0	1.5	2.0	4.0	8.6	1.8	2.5
Penicillium sp.	4.0	6.5	1.8	2.2	4.0	8.2	2.0	2.5
T. viride	4.5	7.5	2.5	2.5	4.0	9.0	2.5	2.5
Penicillium sp.	4.3	6.9	2.4	2.4	4.3	8.5	2.5	2.5

All values are in cm.

Table 4. Interaction responses of filamentous fungi for or against their partners (slow growing fungi).

Interacting fungi	Day 2				Day 21			
	Growth rate (day^{-1})	Maximum colony diameter	Mycelial growth towards partner	Mycelial growth away from partner	Growth rate (day^{-1})	Maximum colony diameter	Mycelial growth towards partner	Mycelial growth away from partner
T. reesei	0.6	2.1	1.0	1.0	1.0	8.5	2.0	2.5
Basidiomycete M1	0.4	2.0	0.8	0.8	0.8	9.0	1.8	2.5
T. reesei	0.4	2.0	0.9	0.9	2.5	8.6	2.0	2.5
P. tigrinus M609RQY	0.5	2.5	1.2	1.2	2.9	9.0	2.3	2.5
Basidiomycete M1	0.3	2.0	0.9	0.9	1.1	9.0	2.0	2.5
P. tigrinus M609RQY	0.5	2.1	1.2	1.2	1.5	9.0	2.0	2.5

All values are in cm.

Table 5. Interaction responses of filamentous fungi for or against their partners (combined slow-fast growing fungi).

Interacting fungi	Day 2				Day 18			
	Growth rate (day^{-1})	Maximum colony diameter	Mycelial growth towards partner	Mycelial growth away from partner	Growth rate (day)	Maximum colony diameter	Mycelial growth towards partner	Mycelial growth away from partner
A. niger	3.9	6.0	1.5	1.5	4.0	8.6	2.0	2.5
T. reesei	0.4	2.0	1.0	1.0	1.0	8.5	2.1	2.5
A. niger	3.8	6.0	1.4	1.4	3.8	8.5	1.8	2.5
Basidiomycete M1	0.4	2.0	0.8	0.8	0.8	9.0	2.0	2.5
A. niger	3.8	5.9	1.4	1.4	3.8	8.5	1.5	2.5
P. tigrinus M609RQY	0.5	2.2	1.3	1.3	1.8	9.0	2.6	2.5
T. viride	4.5	7.5	2.5	2.5	4.5	9.0	2.2	2.5
T. reesei	0.4	2.0	1.0	1.0	1.0	8.5	2.2	2.5
T. viride	4.6	7.8	2.6	2.6	4.6	9.0	4.2	2.5
Basidiomycete M1	0.4	2.1	0.9	0.9	0.9	9.0	2.1	2.5
T. viride	4.5	7.6	2.5	2.5	4.5	9.0	2.0	2.5
P. tigrinus M609RQY	0.5	2.2	1.4	1.4	1.9	9.0	2.0	2.5
T. reesei	0.4	7.4	1.0	1.0	4.4	8.7	2.2	2.5
Penicillium sp	4.2	6.8	1.8	1.8	4.3	8.5	1.6	2.5
Penicillium sp	4.3	6.7	1.8	1.8	4.4	9.0	1.9	2.5
Basidiomycete M1	0.5	2.1	0.8	0.9	1.0	9.0	1.9	2.5
Penicillium sp	4.4	6.9	1.8	1.8	4.4	9.0	2.0	2.5
P. tigrinus M609RQY	0.5	2.5	1.5	1.5	2.0	9.0	3.9	2.5

All values are in cm.

to find their mutual responses. There were only four combinations of *T. viride* and *Penicillium* sp. (Tv/P), *T. viride* and Basidiomycete M1 (Tv/M1), *T. reesei* and *P. tigrinus* M609RQY (Tr/M6) that appeared as compatible interactions. Furthermore, *A. niger and T. viride* (A/Tv), *A. niger* and *T. reesei* (A/Tr), *T. viride* and *T. reesei* (Tv/Tr), and *Penicillium* sp. and *P.* M609RQY (P/M6) showed partial compatible combinations and the other combinations were incompatible or inhibited by each other. Fungal growth rates and hyphal expansions were also measured accordingly based on their interacting responses. The overall growth rate of *T. viride* with its partners was the highest (4.5 to 4.5 cm) where that of *T. reesei* and *P. tigrinus* M609RQY was almost less than 0.5 cm, which was the least growth response in the fifteen observations. This study would contribute to develop an effective and faster composting process using compatible fungal mixed culture for further scale up production of compost.

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