

## Effect of Media and Temperature on Growth and Preliminary Detection of Ligninolytic and Cellulolytic Activity of *Trametes* spp.

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### ABSTRACT

Effects of media and temperatures on growths of *Trametes cervina* (Schwein.) Bres., *T. ljubarski* Pilat, *T. orientalis* (Yasuda) Imazeki and *T. velutina* (Pers.) G. Cunn were studied. There was significant different of effect of media on growth of all the *Trametes* isolates. All of the four *Trametes* isolates were able to grow on Malt Extract Agar (MEA), Potato Dextrose Agar (PDA), Yeast Malt Agar (YMA) and Corn Meal Agar (CMA), however, no growth of *T. cervina* was seen on Czapek Dox Agar (CDA). Colony growth rates varied depending on the media and isolate of the fungi. *T. orientalis* showed the fastest growth while *T. cervina* showed the slowest growth on all the tested media. Although, *T. ljubarski*, *T. orientalis* and *T. velutina* can grow on CDA, the formation of mycelia was sparsely. There were significant differences of effect of temperature on growth of the *Trametes* isolates. *T. orientalis* and *T. velutina*, both, can grow at temperature up to 40°C, *T. ljubarski* can growth at temperature up to 35°C while *T. cervina* at temperature up to 30°C only. The optimum growth temperature for, both, *T. orientalis* and *T. velutina* was at 30°C, while for isolate *T. ljubarski* at 35°C and *T. cervina* at 25°C. All of the four *Trametes* isolates showed excellent cellulolytic activity, indicated by the formation of clearing zone in the test media. The diameters of the degraded area formed by all of the *Trametes* isolates were more than 8 cm. *T. orientalis* and *T. ljubarski* degraded lignin better than *T. cervina* or *T. velutina*

Keywords: *Trametes* species, *Trametes ljubarski*, *Trametes velutina*, cellulolytic fungi, fungal growth

### INTRODUCTION

Most of Polyporacea live in nature utilizing components of wood cell walls as their source of energy resulting into decay through secretion of various intercellular enzymes (Akhtar *et al.* 1997; Kirk & Cullen 1998; Pataký 1999). Species of *Trametes* is well known as wood decaying fungi and as the cause of white rot decay on wide range of tree species. Species of this genus are able to degrade the different components of wood cell wall which included cellulose, hemicelluloses, and lignin (Obst *et al.* 1994; Akhtar *et al.* 1997; Burdsall Jr. 1998). Aust & Benson (1993), Deacon (1997) and Gonzales *et al.* (2003) reported the ability of certain *Trametes* species to degrade lignin completely, then

Akhtar *et al.* (1997) suggested this genus as a potential resource for biopulping and degradation process.

Although the abilities of certain *Trametes* species have been identified for commercialization in wide range of economic importance, only a few species are successfully explored. The difficulty of certain potential species to be isolated and cultured on commercial media is always become a big challenge in industry. Types of media used not only determine the growth development of fungi outside their natural habitat but also affecting the colony morphology and colour as well as the formation of particular structure (Smith & Onions 1994). Generally, fungi are able to utilize various types of natural and artificial substrates to ensure that they can survive in various host types. On artificial substrate, most fungi can reach the best growing

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phase on the media which is formulated with natural materials from which they are isolated (Smith & Onions 1994). However, several fungi species are host selective and specific media are required for their growth.

Besides media, the growth of fungi isolates on culture is also affected by the temperature (Cooke & Whipps 1993; Smith & Onions 1994). The physiological temperature where the fungi are able to grow is usually in a range between 0°C to 40°C (Jennings & Lysek 1996) and it could be up to 50°C (Cooke & Whipps 1993) and up to 60°C to 62°C for thermophilic species (Maheshwari *et al.* 2000). However, to achieved optimum growth, the temperature requirement may be either same or differ among species even the species are in the same genus. For example, *Trametes pocas* was reported to have optimum growth rate at the temperature of 30°C to 35°C, whereas *T. versicolor* at 30°C, *T. cingulata* at 37 °C and *T. elegans* at 25°C (Tekere *et al.* 2001a).

Realizing the important to determine the media and temperature requirement for growth of fungi isolates, this study was conducted. The aim was mainly to determine the growth performance of different species of *Trametes* cultured on different media and at different temperature. As several species of the genus was well known as the wood decaying fungus, the ability of the isolates used in this study in producing both cellulose and ligninase was also assayed.

## MATERIALS & METHOD

### Organism

Isolates of four species of *Trametes* namely *T. ljubarski* Pilat, *T. cervina* (Schwein.) Bres., *T. orientalis* (Yasuda) Imazeki and *T. velutina* (Pers.) G. Cunn were used in this study. Samples of *T. ljubarski* and *T. orientalis* were collected in forest of Kubah National Park whereas *T. cervina* and *T. velutina* were collected in forest of Matang Wildlife Center. Pure cultures of all the isolates were obtained by tissue culture technique using the inner tissue of fruiting body and Malt Extract Agar (MEA) as the growth media. All of the cultures were maintained on MEA throughout the study

period. New cultures were prepared for each experiment.

### Effect of media on the growth rate

The growth rates of the *Trametes* species on five different types of media were tested. The tested media were MEA, Potato dextrose Agar (PDA), Yeast Malt Agar (YMA), Czapek Dox Agar (CDA) and Corn Meal Agar (CMA). Plug (0.5 cm x 0.5 cm) containing young mycelia from seven days old pure culture of the tested fungi was inoculated onto the tested media in 9 cm sterilized plastic Petri dishes. Three replicates were prepared for each isolates for each media used. All the inoculated Petri dishes were incubated in light condition at room temperature (25-27°C). The radial extension of the fungal colony size was recorded every day, then the average colony diameter and growth rate were determined using the method described by Whiting *et al.* 2001. Changes in cultural characteristic were also observed and recorded.

### Effect of temperature on the growth rate

Plug (0.5 cm x 0.5 cm) containing young mycelia cut from seven days old pure culture was inoculated onto MEA in 9 cm sterilized plastic Petri dishes, then incubated in dark condition at the temperature; 15°C, 20°C, 25°C, 30°C, 35°C and 40°C. Three replicates were prepared for each isolates for each temperature. The radial extension of the colony size was recorded every day. The average colony diameter and growth rate was determined as the method mentioned above.

### Plate assay for cellulose degradation

Cellulase activity of the samples were assayed using Carboxymethylcellulose sodium salt (CMC) media, based on the method described by Hankin & Anagnostakis (1977) and Kasana *et al.* (2008). The test medium was comprised of yeast extracts 2g/l, Carboxymethylcellulose sodium salt (CMC) 6 g/l,

MgSO<sub>4</sub> 6 g/l, KH<sub>2</sub>PO<sub>4</sub> 1 g/l and agar 20 g/l. Plug (0.5 cm x 0.5 cm) containing young mycelia of seven days old pure culture was inoculated onto the tested media in a 9 cm sterilized plastic Petri dishes and then incubated in dark at room temperature (25-27°C) for seven days. After seven days, the mycelia were scraped out from the media plate and then poured with 0.1% of Congo red solution for 15 minutes before washed with 1 M NaCl. The diameter of clear zone which indicated the degradation zone of cellulose area was then measured.

#### Plate assay for lignin degradation

The ability of the fungi to degrade lignin was tested using alkaline lignin (Sigma Aldrich) as the lignin source, using the same method as described by Tekere *et al.* (2001b). The test medium were comprised of 5 g/l glucose, 5 g/l Ammonium tartrate, 1g/l malt extract, 0.5 g/l MgSO<sub>4</sub>.H<sub>2</sub>O, 0.01 g/l CaCl<sub>2</sub>.2H<sub>2</sub>O, 0.1 g/l NaCl, 0.01 g/l FeCl<sub>3</sub>, 1 mg/l thiamine and 20 g/l agar. The 0.25 % (w/v) of alkali lignin was added in the medium before autoclaved. Plug (0.5 cm x 0.5 cm) containing young mycelia from seven days old pure culture was inoculated onto tested media in a 9 cm sterilized plastic Petri dishes and then incubated in dark at room temperature (25-27°C) for seven days. After seven days, the mycelia were scraped out from the media plate and then poured with 1% of FeCl<sub>3</sub> and K<sub>3</sub>[Fe(CN)<sub>6</sub>] aqueous solution. The diameter of clear zone which indicated the degradation zone of lignin was measured.

## RESULTS

#### Effect of different media types on growth rate

Statistical analysis shows that there are significant differences of growth rate of all the *Trametes* isolates on the different types of media (Table 1). All the four *Trametes* isolates were able to grow on all the tested media except for *T. cervina* which was unable to grow on CDA. The highest growth rate of *T. ljubarski* was on MEA with 1.52 cm/day. Growth rate of *T. velutina* on PDA, MEA and YMA showed no significant different, which were faster than its growth rate on CDA and on CMA. Growth rate of *T. orientalis* and *T. cervina* showed no significant

difference on, both, MEA and YMA. Both of these fungi showed the fastest growth on these media compared with the other media. *T. velutina* was recorded had the lowest growth rate on CDA with only 0.55 cm/day. Statistical analysis to compare the effect of each tested media on the growth rate of the different isolates also shows significant different. Isolate *T. orientalis* was recorded had the highest growth rate whereas *T. cervina* showed the slowest growth rate on all the tested media compared to the other isolates. Observation on the culture characteristics of each isolates on each tested media revealed that, the mycelia formation for *T. ljubarski*, *T. orientalis* and *T. velutina* were more abundant on PDA, YMA and MEA whereas sparsely mycelia formation was observed on CDA. Strong vanillin-like odour can be smell when *T. velutina* was cultured on MEA media but the odour was less on PDA while no smell was produced on CDA and CMA. Further study should be carried out to better quantify the level of odour formation on the different media.

#### Effect of temperature on growth rate

Statistical analysis shows that there are significant different of growth rate of the *Trametes* isolates at the different temperature (Figure 1). *T. orientalis* was recorded had the highest growth rate at all of the tested temperatures compared to other isolates, while *T. cervina* had the slowest growth rate. The growth rates of *T. cervina* at all the tested temperature were between 0.00 cm/day to 0.42 cm/day. This species was unable to grow at temperature  $\geq 30^\circ\text{C}$ . All of the isolates were able to grow at 15°C. The temperature range for the isolates to grow was also varied. *T. orientalis* and *T. velutina* showed having a wider temperature range to grow compared with the other two isolates. Both of these isolates can grow at temperature 15°C to 40°C. *T. ljubarski* can grow at temperature between 15°C to 35°C while *T. cervina* at 15°C to 30°C only. The optimum growth temperature of *T. orientalis* and *T. velutina* was at 30°C with 2.66 cm/day and 1.42 cm/day of growth rate respectively, while isolate of *T. ljubarski* at 35°C with 1.21 cm/day and *T. cervina* at 25°C with 0.47 cm/day of growth rate.

#### Cellulase plate assay and ligninase plate assay

All of the isolates demonstrated having excellent cellulosic activity since they degraded almost all the

**Table 1.** The average growth rates of the *Trametes* isolates on different types of media incubated at temperature 25±2°C. Mean ± s.d for each media, values in row followed by the same letter indicate no significant different of growth rate at  $\alpha=0.05$  level by the Turkey's test.

Isolate	Average Growth Rate (cm/day)				
	MEA	PDA	YMA	CDA	CMA
<i>T. velutina</i>	1.03 ± 0.03a	1.05 ± 0.03a	1.04 ± 0.12a	0.55 ± 0.00c	0.93 ± 0.07b
<i>T. cervina</i>	0.42 ± 0.01a	0.34 ± 0.02c	0.42 ± 0.00a	0.00 ± 0.00d	0.38 ± 0.03b
<i>T. ljubarski</i>	1.52 ± 0.13a	0.92 ± 0.03d	0.70 ± 0.02e	1.08 ± 0.06c	1.39 ± 0.04b
<i>T. orientalis</i>	1.63 ± 0.02a	1.27 ± 0.01c	1.65 ± 0.02a	1.16 ± 0.08c	1.47 ± 0.08b

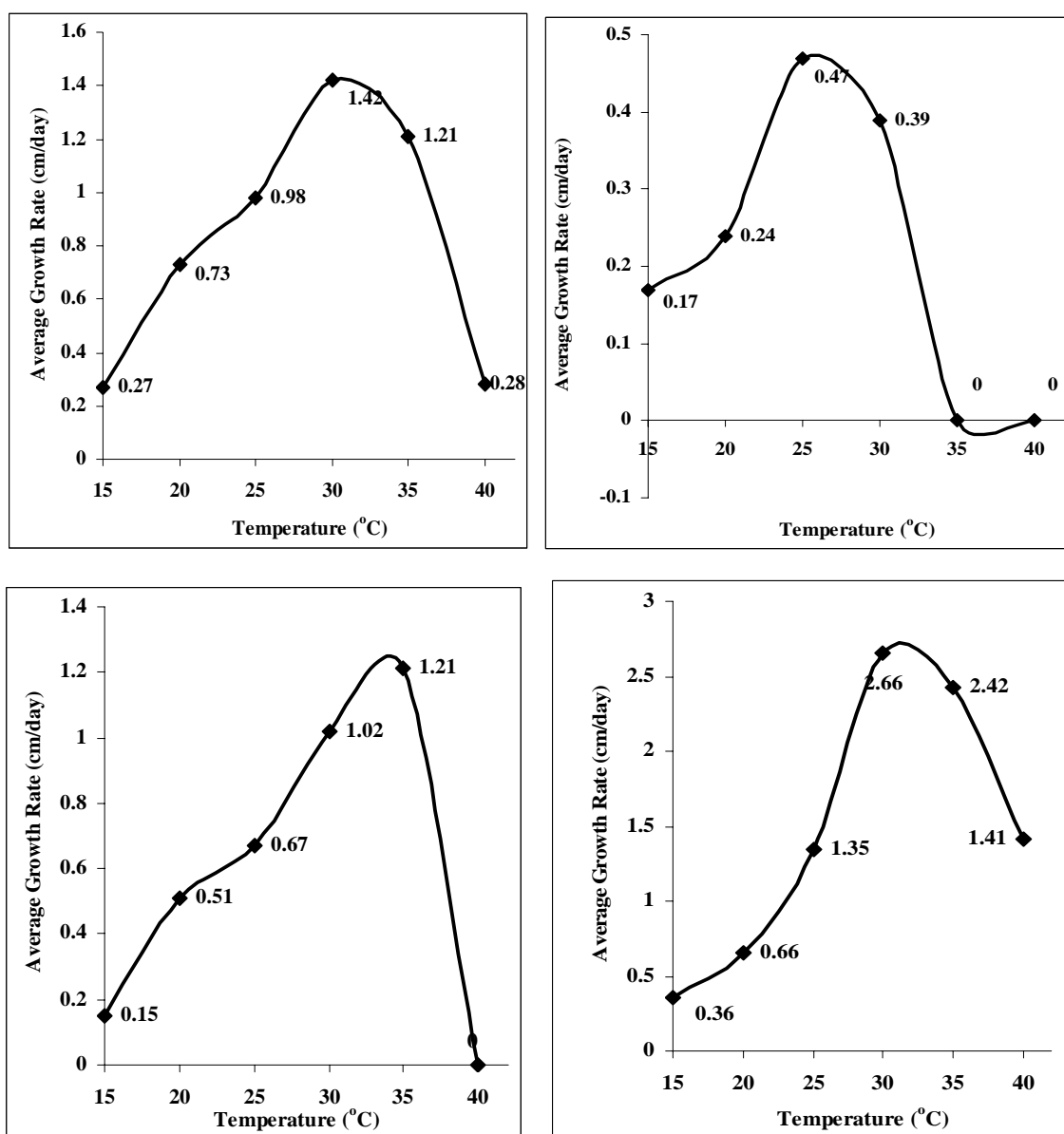
**Table 2.** Rate of lignin and cellulose degradation after seven days inoculation.

Isolate	Rate (magnitude) of degradation	
	Lignin	Cellulose
<i>T. velutina</i>	+	+++++
<i>T. cervina</i>	++	+++
<i>T. ljubarski</i>	++++	+++++
<i>T. orientalis</i>	++++	+++++

Symbol of (+) = clear zone ≥ 1 cm, (++) = clear zone ≥ 2 cm, (+++) = clear zone ≥ 3 cm, (++++) = clear zone ≥ 4 cm, (+++++) = clear zone ≥ 6 cm

cellulose that had been provided in the media, which were indicated by the formation of clearing zone. Based on the degradation rate scaling as applied by Tekere *et al.* (2001b) in their study, all the *Trametes* isolates except *T. cervina* were placed on the highest scale of cellulose degradation rate. *T. velutina*, *T. ljubarski* and *T. orientalis* were placed under the scale of six and above with exact diameter of clearing zone present by them were 8.17 cm, 8.60 cm and 8.72 cm respectively. In ligninase assay,

isolates *T. orientalis* and *T. ljubarski* showed a good ligninolytic activity while *T. cervina* showed the lowest activity. After seven days of inoculation, *T. orientalis* and *T. ljubarski* formed degraded zone of diameter about 5.83 cm and 5.03 cm, respectively. *T. cervina* formed degraded zone of diameter 2.22 cm while *T. velutina* was only 1.80 cm. The magnitude of both ligninolysis and cellulolysis activities based on the degradation rate scaling applied by Tekere *et al.* (2001b) are shown in Table 2.



**Figure 1.** Average growth rate (cm/day) at different temperature (°C) of (a) *T. velutina* (b) *T. cervina* (c) *T. ljubarski* (d) *T. orientalis*

## DISCUSSION

The requirement of fungi to grow in artificial media may vary among strain even though culture from same species and genera were tending to grow in the same media (Smith & Onions 1994). Generally each species of fungi had their own suitable substrate where they can grow in optima level. Fungi were found to grow best in a media which is formulated from natural materials of their natural environment (Smith & Onions 1994). For white rot fungi such as species of *Trametes*, several studies reported that they were successfully cultured on 2% or 3% of Malt Extract Agar (Tekere *et al.* 2001a; Tomsovsky, 2008). Other study reported that *T. versicolor* can easily being cultured on malt base media with addition of trace element (Koller *et al.* 2000). In this study, it was found that all isolates of the *Trametes* were able to grow in all the commercial media used except for *T. cervina* which was unable to grow on CDA. According to Smith & Onions (1994), in certain conditions, some species were able to grow in poor nutrient media but other species might need a rich nutrient source of media. This situation was revealed by *Mucorales* sp. that it could grow well on Malt Agar but unable to grow on Czapek Agar.

The type of media used was not only affecting the growth rate of isolates but also the colony morphology (Smith & Onions 1994). In the present study, the mycelia formations were abundant when cultured on media with rich nutrient content (MEA, PDA, and YMA). While, media with poor nutrient content such as CDA (Smith & Onions 1994) caused very thin culture even the growth rate was quite high.

Temperature is identified as one of the factor that affected the growth of fungi. Fungi from the same genera may differ in a range of temperature in which they were able to live or grow by producing mycelia and reproduction structure. According to Jennings & Lysek (1996), the temperature range for fungal growth was usually between 0°C to 40°C. Of the four isolates used in this study, only *T. ljubarski* and *T. velutina* were able to grow at 40°C whereas *T. cervina* was only able to grow up to the temperature of 30°C. Although all of the tested isolates were able to grow at 15°C, their growth rates were slow. According to Roy & De (1996), most wood decaying fungi of genera Polyporacea are mesophiles where they have an optimum growth

development at the range of temperature 16°C to 32°C. In some cases, isolates which come from the same macro-environment may differ in their temperature requirement as to achieve optima growth (Tekere *et al.* 2001a). They reported that the optimum growth temperature of *T. pocas* was at temperature range between 30°C to 35°C, while *T. versicolor* at 30°C, *T. cingulata* at 37°C and *T. elegans* at 25°C. In the present study, the optimum temperature of *T. ljubarski* was at 35°C whereas for *T. orientalis* was at the range of 30°C to 35°C. The optimum temperature of growth for *T. cervina* and *T. velutina* was at 25°C and 30°C, respectively.

All isolates of the *Trametes* spp. used in the present study were able to degrade both cellulose and lignin. However, the magnitudes of the degradation activities were depending on the isolates. Species which have good cellulase activity may also have good ligninase activity as showed by *T. ljubarski* and *T. orientalis*. In contrast, although *T. cervina* and *T. velutina* had good cellulase activity, they showed poor ligninase activity. According to Tomsovsky (2008), the production of ligninolytic enzyme of *T. cervina* is lower if compared to the other *Trametes* isolates. The differences in enzymatic characteristic of other *Trametes* isolates have also been reported by Mswaka and Magan, 1998. They found that, *T. modesta* and *T. pocas* had high cellulase activity on all types of cellulose sources and able to degrade lignin sources. However, *T. menzei* that able to express high cellulase activity was found unable to degrade lignin.

## CONCLUSION

Types of growth media and incubation temperature were significantly influenced the growth rates and characteristics of culture of the four tested *Trametes* isolates. The most suitable media for these *Trametes* spp. were MEA and YMA whereas temperature range between 25°C to 35°C was required to obtain the optimum growth rate. The optimum growth temperature for *T. cervina* and *T. ljubarski* was at 25°C and at 35°C, respectively, while for *T. velutina* and *T. orientalis* was at 30°C. All of these *Trametes* spp. were also able to degrade, both, cellulose and lignin, thus they had a great potential as the sources of natural enzymes for degradation of various environment pollutant.

In cellulose degradation tests, *T. velutina*, *T. ljubarski* and *T. orientalis* showed relatively great cellulase activities, while in lignin degradation studies only *T. ljubarski* and *T. orientalis* showed a good activity of ligninases.

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