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Full Length Research Paper

Growth conditions of mycelium medicinal mushroom *Lentinula edodes* (Berk.) Pegl. in the substrate colonization phase

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Apart from the status of nutrients within the substrate, the level of inoculum and virulence, major factors relevant for the infection occurrence are temperature and pH necessary for wood colonization process. The impact of these factors on the growth and production of mycelial mass of *Lentinus edodes* was investigated under laboratory conditions. The aim of the investigation was to determine optimal conditions for the development of the fungus *L. edodes* for the purpose of their comparison with the conditions where other rival microorganisms develop. The results showed that the optimal temperature for the development of *L. edodes* was 23°C, whereas the highest weight of the mycelial dry mass was formed at the substrate pH values between 3.00 and 3.63, which means that this fungus can easily perform spontaneous infection.

Key words: Colonization, growth conditions, shiitake*, Lentinus edodes*.

INTRODUCTION

There is a growing interest in cultivation of the fungus Shiitake (*Lentinula edodes* (Berk.) Pegl. (Syn. *Lentinus edodes* (Berk.) Sing.)) due to its high nutritive value and therapeutic properties acknowledged by the civilizations of the Orient, especially China and Japan (Chang and Buswell, 1996; Wasser, 2005). Shiitake significantly increases the strength and vitality of the organism and empowers the body to ward off a large number of organic disorders. Shiitake contains immunostimulants, components effective against tumours and viruses which lower the cholesterol level, prevent clogging of blood vessels, regulate the blood pressure, improve the circulation, balance the blood sugar level, regulate digestion, improve the respiratory system, have anti-rheumatic and anti-allergenic effect, stimulate or soothe the central nervous system in an innocuous way, boost physical strength and stamina and slow down the aging process. Japanese and American researchers have demonstrated that this type of fungus contains a certain number of biologically active compounds that inhibit cell degeneration. The fungus contains medicinal polysaccharides - lentinan and LEM are compounds that have shown anti-fatigue, anti-virus, anti-bacterial and hepatoprotective effects (Hobbs, 2000; Maeda et al., 1998; Mizuno, 1996; Yap, 2003; Ngai, 2003), its production does not entail use of chemicals and pollution of the environment and, finally, the production itself requires minimal investment and pays off before long (Markovic, 2003; Stamets, 1993; Tsivileva et al., 2008). For these reasons, this paper investigated the basic conditions allowing successful colonization of the substrate. The basic conditions that enable the fungus to perform infection are the temperature, the substrate pH, the status of the nutrients within the substrate, the level of inoculum, virulence and the competition on the part of other microorganisms (Cartwright and Findlay, 1946). For the fungus to carry out successful colonization of the

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substrate, to counteract the competitive species and perform maximum fructification, certain basic environmental conditions must be met depending on the type and strain of the fungus and the phase of the wood decay. This paper examines the basic environmental conditions during the first phase of the development of Shiitake – the phase of substrate colonization.

For the fungus to carry out successful colonization of the substrate to counteract the competitive species and perform maximum fructification, certain basic environmental conditions must be met depending on the type and strain of the fungus and the phase of the wood decay. This paper examines the basic environmental conditions during the first phase of the development of Shiitake – the phase of substrate colonization. The mycelial growth was investigated at different temperatures so as to determine the temperature range within which the fungus formed mycelial mass to the extent where it was capable of performing the adequate physiological activity. Apart from food, concentration of hydrogen ions is one of the major factors that define the dynamics of the substrate-based fungal development (Grosser, 1985; Li, 1992). Substrate acidity may affect the stimulation or inhibition of the growth of lignicolous fungi, while changes in pH value significantly influence the speed of nutrient consumption and substrate decomposition. Environment acidity also affects the enzyme system of the fungi which satisfies the vital organisms' needs for food. Decomposing wood (by oxidation and hydrolysis of the wood constituents), epixylous fungi increase wood acidity by means of oxalic acid obtained as a product of these processes. Edible fungus *L. edodes* belongs to this group of fungi. In this way, the order and succession of nutritive substrate colonization by microorganisms are arranged under natural conditions.

Succession is one of the key factors of the wood colonization process in addition to the vitality of the host plant, degree of parasitism and the manner fungi consume food (Schmidt, 1994), which in turn regulates connexion or succession of the species on the same substrate.

MATERIALS AND METHODS

Dycariotic mycelia of two strains of the edible fungus *L. edodes* – AV (originally from Italy) and T 72 (originally from the USA) were examined under laboratory conditions. These strains are highly valued in the market as they form regular large fruiting bodies, rich in medicinal polysaccharide lentinan. The base of malt and agar with or without adequate solutions of phosphates added was used as the substrate.

The influence of temperature on the *L. edodes* mycelial growth investigation method

The investigation of the speed of mycelial growth was carried out by standard methods in plastic Petri dishes with malt and agar bases (5 Bé sugar and 2% agar) at pH 6.0 and in complete darkness. The inoculums were set by the rim of a Petri dish and the mycelial growth was followed at 16 different temperatures in the range of 4 to 31°C in five replications each. The mycelial growth was marked every 24 h in three directions - in the direction of the dish radius as well as to the left and right at 22.5° angles, and noted as a mean value in millimetre per day.

The influence of the substrate pH value on the mycelial growth and mass production investigation method

For the purpose of investigating the influence of the constant substrate pH values on the *L. edodes* mycelial growth and mass production, a buffered nutritive base was prepared according to the Wolpert method (Rehm and Reed, 2007). By mixing different volumes of 0.3 molar solutions of phosphates (H_3PO_4, KH_2PO_4 and K_2HPO_4), five series of phosphates with different pH values were obtained, yet they all had equal quantities of nutrients in certain parts of the buffer so that different quantities would not influence the results (Table 1). A portion of double-concentrated malt and agar base (10 Bé sugar and 4% agar) was prepared separately and autoclaved independently of 0.3 M solutions of phosphates. Upon pH value check, the base was mixed with the phosphates under aseptic conditions. In this way, a base of standard concentration was obtained with the physiologically equal percentage of buffer (0.15 M). Thus prepared buffered base was poured into plastic Petri dishes. Five replications were used for each fungus strain and each pH value examined. The experiments were set in a poly-thermostat at the temperature of 21°C. The mycelial growth was marked every 24 h and measured in the direction of the dish radius as well as both sides at angles of 22.5°. In order to check the stability of the buffered system, a test series was simultaneously set on a liquid base where dry mycelial mass was measured at the end of the experiment.

The influence of the mycelium on the substrate pH change investigation method

For the study of the influence of the fungus on the change of nutritive substrate pH, the unbuffered (liquid) medium was prepared according to Schmidt and Liese method (Schmidt and Liese, 1987). A double-concentrated malt base was prepared with distilled water. According to the recipe (Table 2), 1M-solutions of HCl or NaOH were added thus providing five series of liquid base of standard sugar concentration (5 Bé). Base acidity for each series (five replications each) was measured before the sterilization. The base pH values were also measured after the sterilization and treated as initial values. Upon inoculation of the substrate with strains of the fungus *L. edodes*, the changes in pH value of the substrate were measured every 7 days. 22 days after the commencement of the experiment, the mycelium was extracted with a water vacuum pump, air-dried in a laminar flow chamber and dry mycelial mass was measured.

RESULTS

The influence of temperature on the *L. edodes* mycelial growth

Table 3 shows the effects of different temperature values on the average daily mycelial growth of the fungus *L. edodes* strains AV and T 72. At 4°C both strains exhibited very weak growth whereas the temperature of 31°C was

Table 1. Recipe for preparation of buffered substrates.

Series no.	Parts of 0.3 M solution (ml)			Parts of solution of double-concentrated base of malt (ml)
	H_3PO_4	KH_2PO_4	K_2HPO_4	
1	13.0	62.0	-	75
2	4.6	70.4	-	75
3	-	74.7	0.3	75
4	-	62.5	12.5	75
5	-	16.8	58.2	75

lethal for both strains which was confirmed when the inoculum was transferred onto a fresh base and exposed to the optimal temperature 25 days after the commencement of the experiment. The mycellial growth in the AV strain was somewhat more aggressive at all the tested temperatures (between 0.1 and 0.6 mm/day), with the greatest difference at the temperatures close to the optimum at 23°C the difference was 0.5, while at 24°C it came up to 0.6 mm/day. The temperature value was determined at which both strains of the fungus *L. edodes* used the nutrients from the substrate best: 23°C.

The influence of the substrate pH value on the *L. edodes* mycelial growth and mass production

The mycelia of both strains of the fungus *L. edodes* developed in all the series with acidic reaction (initial pH 3.0 to 4.9) as shown in Table 4. For the most part, the AV strain had a more aggressive growth than the T 72 strain while both strains had the fastest growth and formed the greatest mycelial mass in series 2 (at the initial pH 3.6). Both strains had the smallest mycelial mass and the lowest growth in series 3 (initial pH 4.9), whereas in series 5 (at the initial pH 7.3) there was no mycelial growth in either strain. Series 5 was not lethal for the fungus which was confirmed when fragments were transferred onto the substrate at optimal pH value 3.5. Graph in Figure 1 was formed on the basis of data provided in Table 4. Figure 1 show the average daily mycelial growth presented in millimetre per day. The graph (Figure 3) demonstrates that the most favourable initial pH value is 3.6 as the mycelial growth is approximately 4.25 mm/day in both strains of the fungus, while for all other initial pH values (3.0, 4.6 and 4.9), the growth amounts to 3.5 to 3 mm/day. At the initial pH value of 7.3 there was no mycelial growth at all.

The influence of *L. edodes* mycelium on the change of pH of the nutritive substrate

Table 5 shows changes in the pH values of the malt substrate of the standard concentration under the influence of the *L. edodes* mycelium. After three weeks of the activity of the fungus, the pH of the base was reduced to the range of 3.52 to 5.15 for AV strain and 3.63 to 5.05 for T 72 strain in all the series investigated which means that those pH values were most favourable for the mycelia of both strains of the fungus *L. edodes* in this phase of growth. The greatest change of the substrate pH value through the effect of both strains of the fungi *L. edodes* was found in series 5 at the initial pH value 6.1 while the smallest one occurred in series 1 – initial pH 3.9. In three series (no. 3, 4 and 5), the T 72 strain caused greater total change of the substrate pH than the AV strain. Graphs (Figures 2 and 3) were formed on the basis of Table 5. The graph showing the change of base pH in the function of measuring once every 7 days (Figure 2, curve T72_3.9) demonstrates that for the initial pH value of 3.9 (strain T72) base pH slightly decreases until the seventh day when it reaches a constant value. This means that this pH value is optimal for the fungus *L. edodes* and that the fungus strives towards it or in other words that these values of the pH substrate should be aimed for during the production of fungus in the substrate colonization phase. The same occurs in the AV strain (Figure 2, curve AV_3.9); at the initial substrate pH value of 3.9, the base pH decreases mildly until day 14 when it reaches its constant value. It is characteristic for the initial pH values of 5.4, 5.5 and 5.7 that both strains of the fungus (AV and T72) reach stagnation of the base pH value by the 14th day after which the base pH value suddenly drops (Figure 2). At the initial pH value of 6.1, the base pH value abruptly decreases since day 1 (Figure 2) in both strains of the fungus. The Figure 2 served as basis for creating a model of prognosis of *L. edodes* growth per days which in turn can be used to generate data on changes of the pH value for each day along with the explanation on the manner of its application. In order to use the graph in Figure 2, a specific day should be selected (between 1 and 21) and for that day a vertical line should be drawn on the horizontal axis until it crosses the curve for a particular strain of the fungus and a particular initial base pH. Starting from that intersection, a horizontal line should be drawn on the left-hand side until it crosses the vertical axis so that the change of the base pH value can be determined.

Figure 3 shows that the percentage of decrease of the

Table 2. Recipe for preparation of unbuffered (liquid) substrates.

Series no.	Parts of M solution (ml)		Distilled water (ml)	Parts of solution of double-concentrated base of malt (ml)	pH of base	
	HCl	N_aOH			Before sterilization	After sterilization (initial pH)
1	4.80	-	200	204	3.8	3.9
2	0.28	-	204	204	5.2	5.4
3	-	0.40	204	204	5.8	5.5
4	-	1.62	202.8	204	5.9	5.7
5	-	12.80	194.8	204	6.7	6.1

Table 3. Total average daily mycelial growth of the fungus *L. edodes* (strain AV and T 72) at different temperatures (mm).

Strains *L. edodes*	Temperature (°C)															
	4	6	8	10	12	14	16	18	20	22	23	24	25	26	29	31
AV	0.4	0.9	1.7	2.1	2.5	2.7	3.2	3.5	4.1	4.5	4.7	4.6	4.0	3.5	1.9	0.0
T 72	0.3	0.8	1.6	1.9	2.4	2.6	2.9	3.2	3.8	4.1	4.2	4.0	3.6	3.1	2.2	0.0

Table 4. Average daily increment (mm/day) and weight of dry mycelial mass of the fungus *L. edodes* (AV and T 72) (g) on buffered nutritive substrates.

Series no.	Fungus strain	pH of base			Average daily increment of mycelium (mm/day)	Weight of dry mycelial mass (g)
		pH of buffer	Initial pH	pH at the end of experiment		
1	AV	3.0	3.0	3.1	3.48	0.195
	T 72			3.2	3.37	0.180
2	AV	3.5	3.6	3.4	4.23	0.252
	T 72			3.3	4.37	0.271
3	AV	4.6	4.9	4.7	3.01	0.148
	T 72			4.5	3.05	0.155
4	AV	4.7	4.6	4.3	3.25	0.160
	T 72			4.2	3.07	0.161
5	AV	7.5	7.3	7.0	0.0	0.0
	T 72			7.1	0.0	0.0

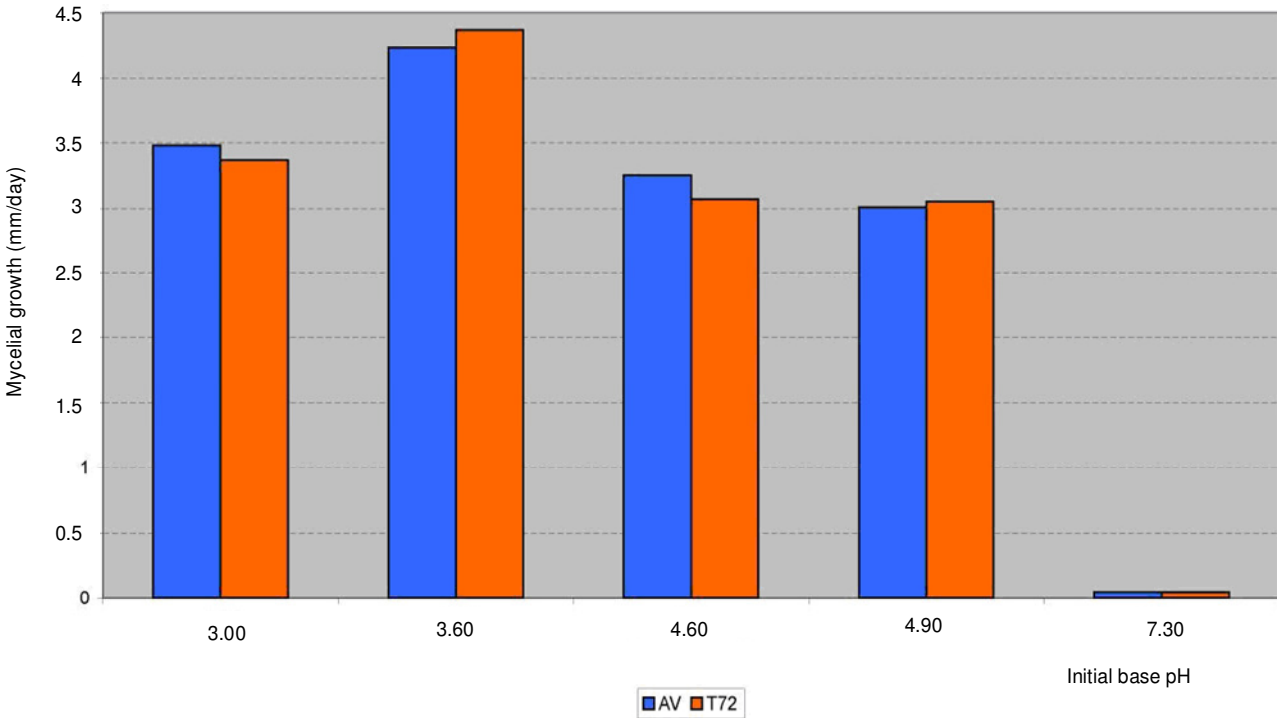

Figure 1. Average daily mycelial growth of the strains AV and T72 of the fungus *L. edodes* (mm/day).

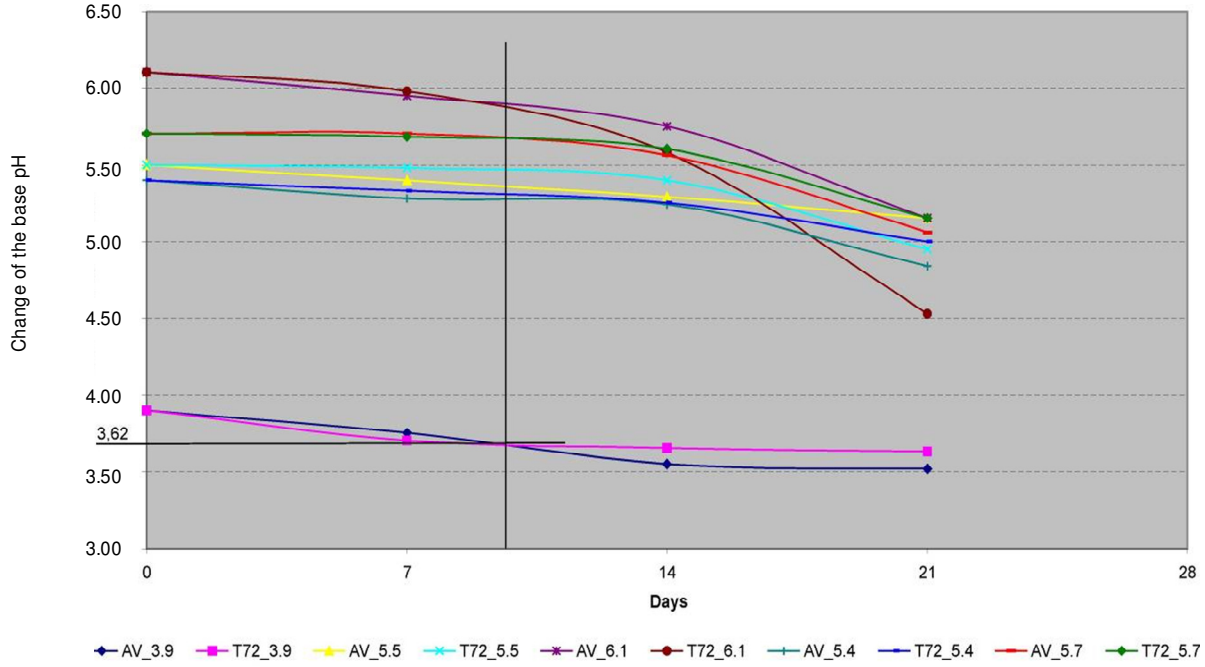

Figure 2. Determining the change of the base pH through action of the strains AV and T72 of the fungus *L. edodes* per days.

pH value ranges between 6 and 11% for the initial pH values of 3.9, 5.4, 5.5 and 5.7 in both strains of the fungus (AV and T72), whereas at the initial pH value of 6.1 the decrease of the acidity is far greater and amounts

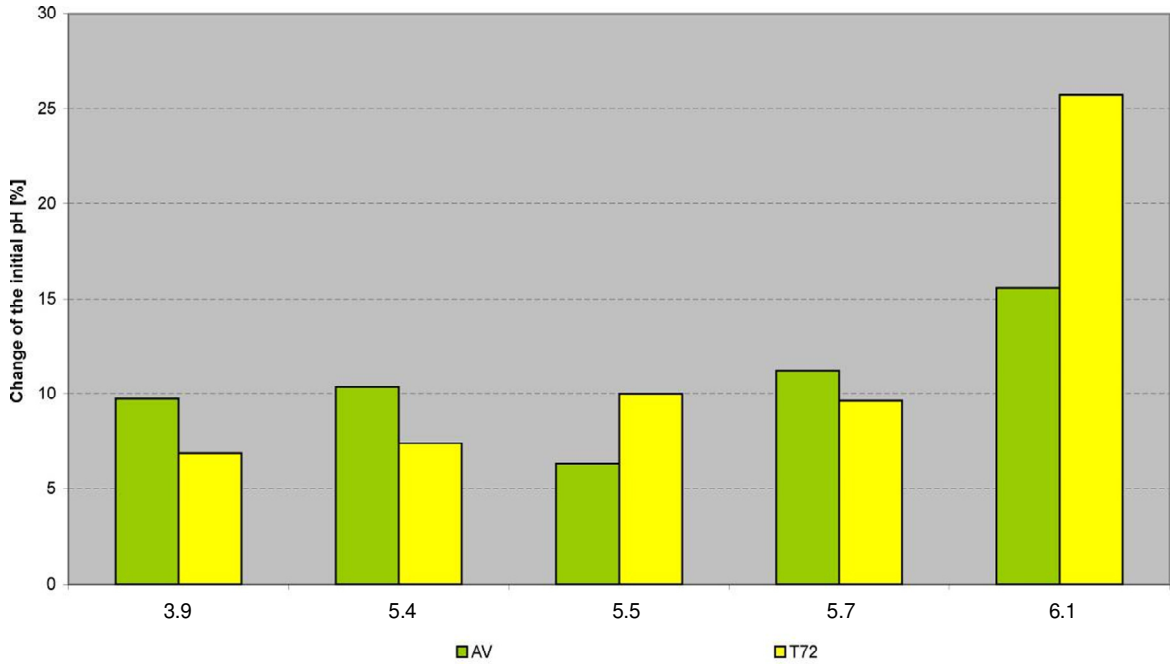

Figure 3. Change of the initial base pH through action of the strains AV and T72 of the fungus *L. edodes* (%).

to approximately 16% for the AV strain and 26% for the T72 strain.

DISCUSSION

Generally, the AV strain displayed a faster mycelial growth than the T 72 strain at almost all temperatures examined. The temperature value was determined at which both strains of the fungus *L. edodes* used the nutrients from the substrate best: 23°C. This value is within the temperature range optimal for the development of most rot fungi that infect oak wood. According to the findings of other authors, the optimal temperatures where maximal mycelial growth of the fungus *L. edodes* was recorded range from 20 to 25°C (Miric, 1993; Lee et al., 2008). During the experiment (22 days) it was determined that both strains of the fungus changed the initial pH values of the substrate shifting them towards the optimal values. As this shifting was within the range of tolerance, all buffered systems were considered stable. Both strains of the fungus *L. edodes* had the fastest growth and formed the greatest mycelial mass at the initial pH 3.6. According to the research results of other authors, the optimum pH for *L. edodes* mycelial growth was 3.0 to 3.5 (Hassegawa et al., 2005). It is well known that all fungi need different pH values depending on the phase of development – germination of spores, mycelial growth, formation of fruiting bodies, sporulation etc. Hence the fungi may prefer more acidic substrate for germination of spores, whereas this changes in the phase of fruiting body formation and quite often less acidic or almost neutral reaction in the substrate is more suitable. This phenomenon has particular importance in production of the fruiting bodies of the fungi for commercial purposes as is the case with fungus *L. edodes*.

Practice has shown that formation of fruiting bodies for this species is the best on substrates with acidic reaction. This should certainly be taken into consideration for the pH value of the substrate is the key factor for the infection process, substrate colonization or rot progress and dynamics.

Conclusions

The temperature value of 23ºC at which both examined strains of the fungus *L. edodes* colonized the substrate the fastest is pertinent to Serbia's climate. However, at the same time it is the optimum for development of many other species of rot fungi, so one has to take into account that there are a large number of rivals in both food and environment which also develop under these favourable temperature conditions. The changes in pH of the substrate where the examined strains of the fungus *L. edodes* grew shifted toward acidic reaction (5.15 to 3.52) which suggests that such acidity favours the development of the fungus investigated. The graph (Figure 2, using graph in Figure 1) shows the stagnation of base pH value at the pH value of about 3.5 which indicates that these are the most favourable conditions for development of both strains of the fungus *L. edodes* in the substrate

Table 5. Changes of pH values of substrate provoked by fungus *L. edodes* (AV and T 72) after 7, 14 and 21 days.

Series no.	Initial pH	Fungus strain	Change in pH of the base				pH at the end of experiment	Weight of dry mycelial mass (g)
			After 7 days	After 14 days	After21 days	Total change of pH		
1	3.9	AV	- 0.15	- 0.20	-0.03	- 0.38	3.52	0.081
		T 72	- 0.20	- 0.05	- 0.02	- 0.27	3.63	0.085
2	5.4	AV	- 0.12	-0.04	- 0.40	- 0.54	4.86	0.062
		T 72	- 0.07	- 0.08	- 0.25	- 0.40	5.00	0.059
3	5.5	AV	- 0.10	- 0.11	- 0.14	- 0.35	5.15	0.051
		T 72	0.02	- 0.08	- 0.45	- 0.55	4.95	0.045
4	5.7	AV	0.00	- 0.14	- 0.50	- 0.64	5.06	0.040
		T 72	0.10	- 0.15	- 0.40	- 0.65	5.05	0.042
5	6.1	AV	- 0.15	- 0.20	- 0.60	- 0.95	5.15	0.033
		T 72	- 0.12	- 0.40	- 1.05	- 1.57	4.53	0.038

colonization phase (base pH of 3.6 being the most favourable for mycelial growth). The assumptions from the reference literature (Hassegawa et al., 2005) were thus confirmed by using the graph methods. On mildly acidic bases, the fungus *L. edodes* developed a colony of hyphae which means that lower pH values resulting from the development of a rival species under natural conditions would not hinder the development of *L. edodes.* Alkaline reaction, however, does not suit this fungus and its development stops even at pH 7.3. If the pH is reduced again the fungus continues to develop, in other words, in mildly alkaline medium it will not lose its vitality.

The competition of microorganisms on the same substrate, inhibition or growth, or the occurrence of antagonism represent a phenomenon that may be the consequence of the metabolism of rival fungal species, excretion of mycotoxins or antibiotics from the growing front of the mycelia and sensitivity, that is the reaction of the rival species thereafter. This phenomenon has a direct impact on the speed, course and consequences of decomposition of wood as a substrate and food source.

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