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Research Article
Solid State Fermentation of *Lentinula edodes* on Solid Olive Substrate: Evaluation of Growth Factors

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Abstract

Background: Culture of shiitake attracts attention of several researchers for its proved medicinal properties and antimicrobial effect and biodegradation abilities of phenolic compounds by producing large spectrum of enzymes. However, the growth kinetics of the mycelium is related to nutritional and environmental factors. A statistical approach according to the factorial design has been employed for identifying significant variables influencing biomass growth of *Lentinula edodes* Le119 strain and polyphenol removal from olive mill wastewater (OMW). **Materials and Methods:** The optimal formulation of substrates, including olive twigs and leaves (OTL), olive cake (OC) and OMW which acts as the wetting/moistening agent, for *L. edodes* culture was determined. Then, the growth factors including, initial pH, aeration rate, inoculums age, inoculums size, particle size and mineral solution were evaluated following the experimental screening design methodology using $2^{5}5^{1}//16$ experiments. The fungal biomass was estimated by ergosterol technique and total phenol was determined according to the Folin-Ciocalteu assay. **Results:** The optimal formulation of substrate for *L. edodes* solid culture was OTL (20%)+OC (30%)+OMW (50%) allowing highest mycelial biomass production of 16.24 mg g^{-1} of substrate dry weight. Among the variables screened, the nitrogen added (through ammonium sulfate and urea) and spawn size had significant effects on mycelial biomass production as well as polyphenol removal from OMW. The optimized conditions of Le119 culture on solid olive substrate (SOS) with 20% of spawn and without nitrogen input led to mycelial biomass production of 48.72 mg g^{-1} of substrate dry weight and 82% drop in total phenols. **Conclusion:** The obtained results support the potential effective and profitable utilization of undiluted OMW in mixture with OTL and OC for *L. edodes* cultivation. However, farther experiments investigating the impact of these substrates on *L. edodes* fruiting yield and quality have to be conducted.

Key words: Solid olive wastes, olive mill wastewater, solid state fermentation, *Lentinula edodes*, experimental design methodology

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

The importance of olive oil mill industry in Mediterranean countries is well known, as well as the serious problems that olive mill factories have in disposing their by-products[1,2]. Commonly, olive mills produce residual solids, the olive cake which make up a fibrous lignocellulosic waste and generally used as fuel[3]. However, the olive mill wastewater is a heavy pollutant mainly due to its high phenolic content and organic load[4]. In addition, it has been estimated that each olive tree could produces 25 kg leaves and twigs per year[5].

Several investigations were carried out to select microorganisms (notably fungi) capable of eliminating the inhibitory effects of those substances and converting the initial waste either directly into a useful end-product or by making it susceptible to further physicochemical and biological treatments[6,7]. Among basidiomycotina in particular, white-rot fungi belonging to the genus of *Pleurotus* and *Lentinula* were selectively tested for their ability to decompose the lignin and are among the most efficient producers of lignocellulosic enzymes[8,9]. Moreover, they can be cultivated on a large lignocellulosic substrates transforming them into food and feed[10].

Lentinula edodes (Berk.) Pegler (shiitake) is the second most popular edible mushroom in the world because of its flavor, taste and quality[11] as well as the best characterized mushrooms used for medicinal purposes[12-14]. Owing to shiitake characteristic, different substrate formulations have been developed in different countries, depending on their readily available raw material, agricultural wastes such as oak, cereal straw, corn cobs, sugarcane bagasse are used alone or in combination with other wastes in shiitake cultivation[9,15,16]. However, the use of mixture of olive cake, olive twigs and leaves supplemented with OMW is reported for the first time in this study.

To determine the parameters affecting biomass production of *L. edodes* as well as polyphenol removal from OMW, with the intention to optimize the conditions of culture, the set of factors influencing the growth of *L. edodes* on olives residues was studied. Factorial matrix was established. Processing the results with the Nemrodw software (New Efficient Methodology for Research using Optimal Design) enabled us to quantify the direct effects of the various factors on the responses chosen and to identify those that will require fine-tuning during the optimization phase.

MATERIALS AND METHODS

Microorganism and spawn preparation: The strain of *L. edodes* (Le119), selected for its high ability in polyphenol removal from OMW was maintained at 4°C on potato dextrose agar (PDA, Sigma, France) supplemented with 10% of OMW[7]. Wheat grains (Ebly®, Casino, France) were used as substrate and growth support for spawn preparation. The grains were cooked for 5 min in a water solution containing 10% (v/v) of OMW at a ratio of 1:1 (Ebly®: Solution; w:v). Once cooked, the wheat grains were packaged (250 g of wheat grains in jar of 500 mL) and sterilized in an autoclave at 121°C for 30 min. The grains were then inoculated with 5 agar disks of 5 mm diameter containing mycelium and incubated at 25±2°C in the absence of light for 10 and 25 days.

Preparation of substrates: The substrates were composed of different parts; OC, olive twigs and leaves (OTL) and OMW as wetting agent. The OC and OMW were obtained from an olive oil plant after three-phase extraction. The OMW dilutions were performed in distillate water. The OTL were ground and sieved to desirable particle size. The individual ingredients of the substrate were mixed thoroughly by mechanical means to achieve a homogenous mixture.

Formulation of substrate for shiitake culture: In order to determine the optimal formulation of substrates which allow high mycelial biomass, different mixtures of OC and OTL was soaked into OMW to reach final humidity of 50% (Table 1). The mixtures were sterilized at 121°C for 30 min and inoculated with spawn at rate of 10%. The Erlenmeyer were then incubated at 25±2°C for 1 month. Three Erlenmeyers per treatment were evaluated.

Experimental methodology

Asymmetrical screening design: Inspiration was drawn from previous works[17,18]. In this study, a $2^5 5^1//16$ experimental design was processed using Nemrodw software[19]. It allowed the investigation of six factors (A-F) in 16 experiments; 5 factors A-E each at two levels and one factor F at five levels. Table 2 and 3 listed the values given to each factor. The choice

Table 1: Mixing ratio of olive twigs and leaves (OTL), olive cake (OC) and olive mill wastewater (OMW) used as substrate for *Lentinula edodes* growth

	Ingredient		
Treatment No.	Olive cake (%)	OTL (%)	OMW (%)
1	50	0	50
2	30	20	50
3	20	30	50
4	0	50	50

was based on our preliminary experiments. Table 4 showed the $2^5 5^1$//16 experimental design. The results treatment was processed using Nemrodw® software.

From the 16 runs, we can compute the "Weight" of each factor level using the least square method[17,18]. For each factor, the weight of each level is related to the upper level weight, which becomes the "Reference state" among each factor[20]. The weight describes the factor effects on the response when changing factor levels with respect to the reference state. The obtained results are generally presented as histograms which graphically illustrate the variable differential weights[21].

Culture of *L. edodes*: The series of fermentations (Table 4) were carried out in glass columns so-called "Raimbault columns[22]". Sterilization of the mixtures was achieved in an autoclave at 121°C for 30 min. After inoculation, the columns were incubated during 2 days without forced aeration. Then, they were placed in a water bath at 25±2°C and the air supply from compressor was saturated in moisture and regulated following the experiments conditions. Two replicates were performed for each experiment.

Methods of analysis

Standard chemical analysis: The pH value of the sample of substrates was assayed following dilution of 1 g in 10 mL of distilled water and by using a digital pH meter. Mycelial biomass was measured by estimation of ergosterol as reported by Yuan *et al.*[23]. The total phenol was determined as reported by Lakhtar *et al.*[7]. Five replicates per treatment were used.

Table 2: Particle size and composition of mineral solution used in experimental conditions

Particle size	Sieve (mm)	
	0.8<Ø<2	Ø<0.8
T1	80	20
T2	70	30

	g L^{-1}		
Mineral solution	Urea	$(NH_4)_2SO_4$	KH_2PO_4
SO	0.00	0.00	0.75
MS1	0.25	0.25	0.75
MS2	0.25	1.00	0.75
MS3	1.00	0.25	0.75
MS4	1.00	1.00	0.75

Elemental analysis: The instrument used for carbon and nitrogen concentrations was a Flash EA 1212 Elemental Analyzer (France). About 1 mg (0.8-1.2 mg) of the sample in a capsule made of silver (assay of C and N) was burned at 920-1000°C. The quantity of each element is expressed in percent mass. Each determination was performed in duplicate. The instrument was calibrated with aspartic acid standard.

RESULTS

Physico-chemical characterization of substrates: The physical and chemical characteristics of the three substrates are presented in Table 5. The pH of all the three substrates was between 5.45 and 4.9 for OTL and OMW, respectively. While nitrogen content was the same for all substrates (0.82±0.1%), the C/N ratio was different for all substrate and OTL presented the lowest value (54.07±2.12) followed by OC and OMW (60.11±3.24 and 63.2±0.65, respectively). Statistical analysis ($p = 0.05$) showed no significant difference in carbohydrate and lignin content were observed for OMW and OC but significant difference for OTL which presented the lowest carbohydrate content (46.64±2.68%) and highest lignin content (26.87±1.86%).

Formulation of substrate: The effect of various olive substrate mixtures on mycelial biomass of LE119 strain is shown in Table 6. It can be seen that the mycelial biomass produced is dependent on the ingredients rate. The high production was recorded for treatments 2 and 3 which had mycelial biomass of 16.24 and 14.58 mg g^{-1} of substrate dry weight (dw), respectively. However hyphal growth on treatments 3 was less profuse than treatments 2. By contrast, the substrates containing only OC or OTL moistened with addition of OMW led to low mycelia production (9.91 and 5.98 mg g^{-1} of substrate dry weight, respectively and high mould contamination.

On the basis of mycelial production, the substrate composed from OC (30%), OTL (20%) and OMW (50%) was selected for further optimization.

Table 3: Experimental conditions of the asymmetrical screening design with 6 factors (A-F)

	Factors					
Levels	(A) Initial pH of substrate	(B) Aeration (mL min^{-1})	(C) Inoculum age (day)	(D) Inoculum size (%)	(E) Particle size (mm)	(F) Mineral solution
1	5	20	10	10	T1	SO
2	6	30	25	20	T2	MS1
3	-	-		-	-	MS2
4	-	-	-	-	-	MS3

Table 4: Experimental conditions of the screening design with 6 factors (A-F)

Run No.	A	B	C	D	E	F
1	5	20	10	10	T1	SO
2	6	30	25	20	T2	SO
3	5	20	10	10	T2	MS1
4	6	30	25	20	T1	MS1
5	5	20	25	20	T1	MS2
6	6	30	10	10	T2	MS2
7	5	20	25	20	T2	MS3
8	6	30	10	10	T1	MS3
9	5	30	10	20	T1	MS4
10	6	20	25	10	T2	MS4
11	5	30	10	20	T2	MS2
12	6	20	25	10	T1	MS2
13	5	30	25	10	T1	MS1
14	6	20	10	20	T2	MS1
15	5	30	25	10	T2	SO
16	6	20	10	20	T1	SO

Table 5: Chemical composition of olive twigs and leaves (OTL), olive cake (OC) and olive mill wastewater (OMW) used as substrate in different culture media for *Lentinula edodes* growth

	Substrate		
Physicochemical properties	OTL	OC	OMW (dry matter)
Moisture content (%)	10.00±0.41	10.0±0.350	90.00±1.20
pH	5.45±0.15	5.10±0.20	4.90±0.40
C (%)	42.72±2.01	51.12±0.18	41.60±1.50
N (%)	0.65±0.15	0.85±0.09	0.80±0.01
C/N	65.72±2.12	60.11±3.24	63.20±0.65
Carbohydrate (%)	42.64±2.68	52.00±2.32	49.72±1.62
Lignin (%)	26.87±1.86	25.81±1.31	22.25±3.65
Carbohydrate:lignin ratio	1.58±0.02	2.01±0.01	2.23±0.35
Fat (%)	-	10.00±1.20	10.00±2.21

Table 6: Mycelial biomass of *Lentinula edodes* (Le119 strain) on different mixtures of substrate

Treatment	Ingredient			
No.	Olive cake (%)	OTL (%)	OMW (%)	Mycelial biomass (mg g^{-1})*
1	50	0	50	9.91^{c}**
2	30	20	50	16.24^{a}
3	20	30	50	14.58^{b}
4	0	50	50	5.98^{d}

*Biomass produced after 1 month of incubation, **Means followed by the different letter are significantly different (p = 0.05 after LSD)

Screening design: In the set of experiments arranged according to the factorial design, the maximum production of biomass (48.72±1.47 mg g^{-1}) were obtained in run 1 followed by run 16 with value of 44.66±1.27 mg g^{-1}. The both runs were inoculated with rate of 20% of spawn. On the other hand, the minimum biomass production of 12.18±0.34 mg g^{-1} was recorded in treatments 10 with inoculums size of 10%. In relation to the effect of mycelial culture on OMW polyphenols, the maximum reduction was recorded in the treatment 1 (82±1.3%) followed by treatments 16 (80±1.2%) with spawn size of 20% and without mineral supplements. While, the lowest phenol removal is shown by treatment 10 (15.48±1.8%) with 10% of spawn.

Table 7: Experimental conditions of the screening design with 6 factors (A-F) and the corresponding experimental responses

Run No.	Biomass yield (mg g^{-1})	Total phenol removal (%)
1	29.30	82.35
2	48.72	51.28
3	22.24	42.00
4	34.50	61.65
5	33.50	51.91
6	12.18	24.12
7	28.56	37.75
8	19.44	26.23
9	22.32	29.49
10	12.18	15.48
11	28.42	47.20
12	14.20	31.51
13	18.26	40.71
14	34.50	55.45
15	30.44	57.98
16	44.66	80.68

Measurements of pH of different experiments after the colonization process were different. A steady decrease of pH in all media without supplement was recorded; the initial pH values (5.0-6.0) fell at the end of incubation to 4.39 and 4.66 for experiment 1 and 16, respectively. On the other hand, the supplemented media showed a slow increase of pH after incubation (from 5-5.2 for experiment 9) thanks to the potassium phosphate addition. However, good correlation was found between biomass yield and total phenol removal ($r^2 = 0.8$) for all treatments.

Variable effects on mycelial biomass and total phenol removal responses: Coefficient values (the weight associated to each factor level) and statistical analyses, using t-test are reported in Table 7. These results are illustrated by two kinds of histograms as shown in Fig. 1 and 2. The Pareto diagrams (Fig. 1a, 2a) represent the weight of each factor taking its highest level as reference. While Fig. 1b and 2b represent the differential effects of each factor when considering two different levels taken two by two[19,21].

It was found that the significant parameter effects were inoculum size (D) and mineral solution (F). For the others, there were no significant effects. The addition of high nitrogen content exhibited a negative effect on biomass yield (Fig. 1) as well as drop in polypehnols (Fig. 2). The use of OMW without supplements nitrogen showed an interesting positive effect on both responses. On the other hand, inoculum size exhibited positive effect on both responses. Among the two spawn size (10 and 20%) tested, the 20% size resulted in both significantly higher mycelial biomass and rapid development than the other size. Indeed, the mycelial biomass and drop in polyphenol at the 10% size was 58 and 47%, respectively lower than at the 20% size for the substrate without supplement. Moreover, the mycelial growth at the 10% size

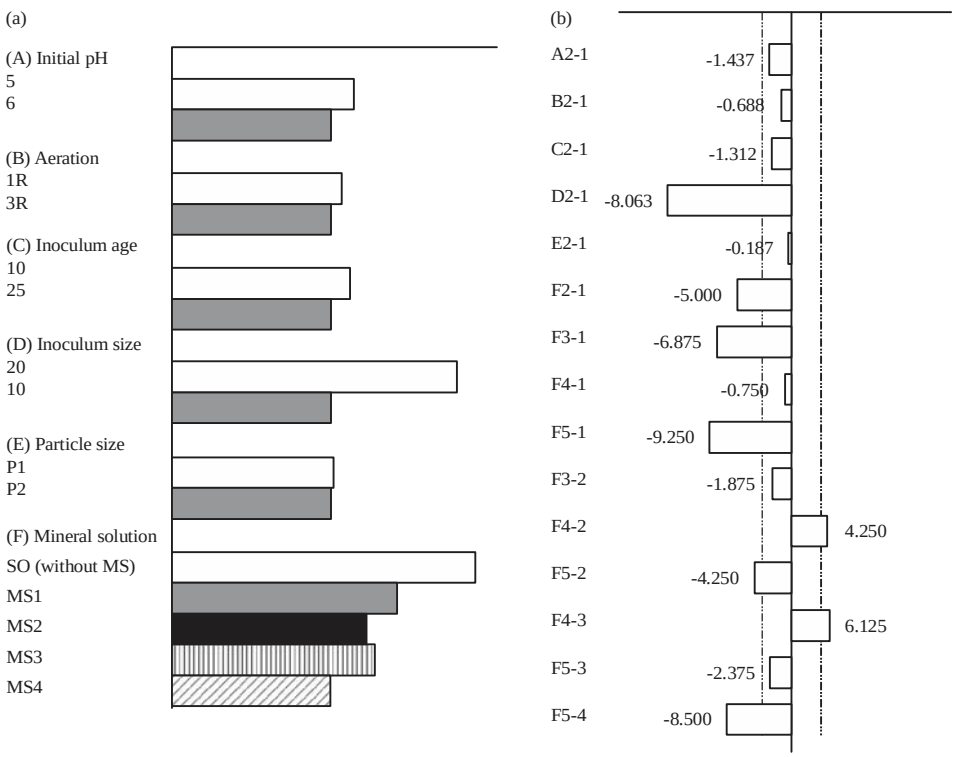

Fig. 1(a-b): Graphical study of the effects of the factors A-F on the mycelial biomass response, (a) Graphical study of the total effects and (b) Differences of the weights of the different levels

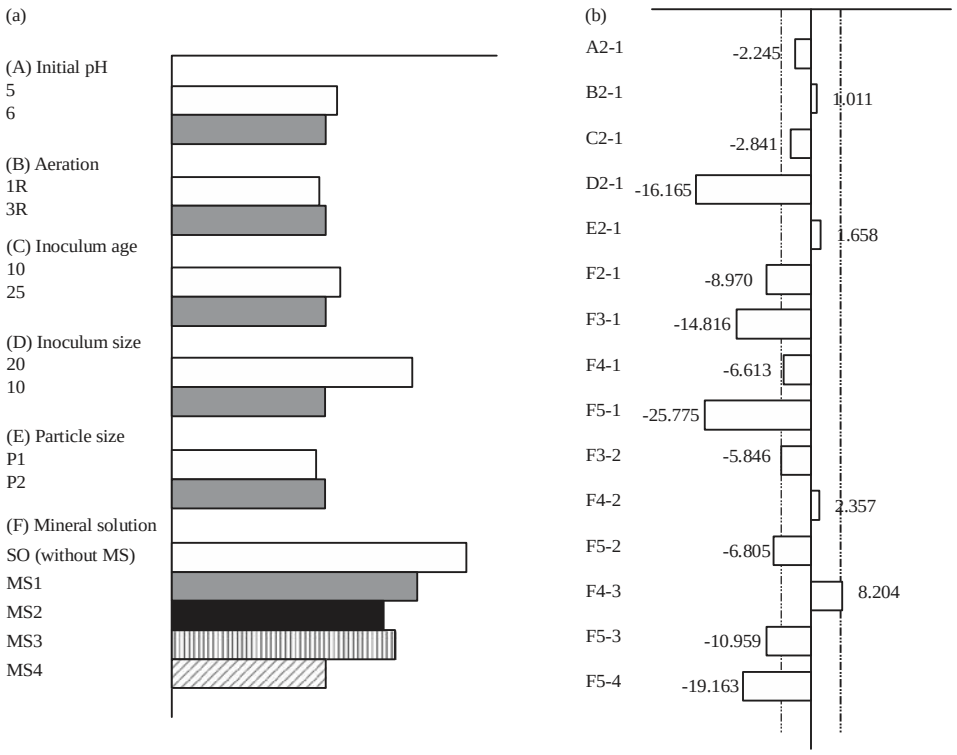

Fig. 2(a-b): Graphical study of the effects of the factors A-F on total phenol removal, (a) Graphical study of the total effects and (b) Differences of the weights of the different levels

showed contamination by green and black moulds during cultivation of the *L. edodes* Le119 strain.

Regarding to contamination of the culture during SSF, the combination of moisture content, which was maintained at 50% (v/v), as well as the incubation of substrate in oven for two days without forced aeration, allowed reducing the contamination of shiitake culture with filamentous fungi which are known as major antagonistic agent in mushrooms culture[15,24,25]. While, the supply of culture with humidified air allowed to increase the substrate moisture content slowly and keeping the substrate with suitable moisture content.

DISCUSSION

The present study aimed at screening 6 factors of shiitake growth on optimized mixture of OTL and OC supplemented with OMW. One strain of shiitake, which was previously selected for its capability to remove phenolic compounds from OMW[7] was employed to convert the substrate into mycelial biomass and remediate the phytotoxic effects of OWW through the solid state fermentation of shiitake mycelium.

Mycelial biomass revealed that the substrate formulation exercised a considerable influence on the mycelium production and therefore on its morphologic aspect . The substrate composed entirely of OC supplemented with OMW caused the oxygen (O_2) depletion which could reduce mycelial biomass development in substrates[26]. Indeed, small air spaces present in OC moistened with OMW may slow gas exchange from deep within the interior of the substrate to the surface[27]. Addition of OTL to mixture formulation could improve the oxygen transfer production but reduce the bioavailable carbohydrate existing in OC. The trend of this ingredient was a decreasing in mycelial biomass when its ratio increased. Between ratio of 20 and 30%, OTL can be added to the growing medium with satisfactory results.

However, the use of OTL as unique solid support may be detrimental to the mycelial growth because of it limited bioavailability of carbohydrate which was reported as an important factor at the beginning of cultivation. Philippoussis *et al.*[9] have demonstrated that the growth of *L. edodes* is closely associated to hemicellulose content of substrate. Indeed, hemicellulose and other water-soluble sugars are mainly utilized during the active growth phase, prior to the breakdown of lignin and cellulose. Likewise, lignin could act as a barrier for the breakdown of cellulose and delays the colonization of the substrate[9].

The treatments 1 and 16, where fungal biomass exhibited its maximum at 30 days of incubation were lower than found for the same strain cultivated on wheat straw and reed grass substrates[9]. This reduction is attributed to the presence of OMW without any dilution or pre-treatment. Raw olive mill wastes in mixtures with wheat straw in various ratios hindered the growth of several white-rot fungi, including *L. edodes* and the ratio exceeding 60% was the worst performing medium noted on mushroom yields as reported by Zervakis *et al.*[28]. Meanwhile, It was found that the pre-treatment of olive mill waste through aerobic thermophilic composting greatly enhance their ability to support growth of the mushroom strains[28,29].

The influence of the composition of the substrate on mushroom production characters has been emphasized in several studies[9,28]. In the framework of the present study, the mineral solution with high nitrogen content added to the OMW-based substrates was the main parameter affecting negatively the growth of shiitake (Fig. 1a, 2a). This can be attributed to the nitrogen imported through urea and ammonium sulfate, which did not favor the growth of the mycelium as reported by Kalberer[30], Feng *et al.*[31] and Pedri *et al.*[32]. Mostly, the basidiomycetous fungi grow best in the presence of aminoacids[31] differently in organic nitrogen.

For the cultivation of *L. edodes* a source of nitrogen, such as rice bran or soybean flower, is favorable as observed by Philippoussis *et al.*[9,33]. However, in *L. edodes* cultivation on synthetic composite media (OTL+OC+OMW), supplementary nutritive solution should not be used. Otherwise, when the nutritive solution is added, we observed the risk of contamination of the growing media increased and this fact was already reported by Ozcelik and Peksen[34].

Similarly, the treatments, were fungal biomass was hindered, showed a low polyphenols reduction. This result is attributed to low production of lignolytic enzymes, which is strictly related to the fungal biomass production. In our previous study[7], laccase production was the main lignolytic activity expressed by the strain cultivated in OMW-based media. It has also been reported that LiP and MnP are capable of decolorizing and removing phenolics from OMW[35,36]. On the other hand, nitrogen content could also repress the lignolytic activity as reported by Philippoussis *et al.*[9] and Contreras-Dominguez *et al.*[37].

Compared to the straw, spawn is a relatively expensive item. Using the lowest inoculation size possible without sacrificing the mushroom yield would give the best economics. In the present sutdy, the 20% size resulted in significantly highest mycelial biomass and polyphenol reduction. However, in literature, several rates of inoculation (from 3-18%) were used for mushroom production[28,38], but the optimum spawn size depends on the type of substrate,

mushroom species, spawn quality and the cultivation conditions. When the substrate-based media contained the recalcitrant compound (e.g., polyphenols), the inoculation rate recommended was high in order to enhance the mycelial growth and reduce the incubation period as concluded from different studies[39-41].

CONCLUSION

Therefore, based on these results, it would be recommended that at least 20% spawn level be used to achieve a colonization of the substrate by *L. edodes* without any mould contamination. Further studies are recommended for determining the optimum spawn inoculation levels for large scale cultivation.

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