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Cultivation of *Trametes versicolor* on supplemented agro-forestry wastes: yield, antioxidant activity, and first report from Iran

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Abstract

Trametes versicolor (turkey tail) is one of the wild medicinal mushrooms, and the most important of all medicinal mushrooms. To date, there are no reports of commercial cultivation of this species and this is the first report of successful cultivation in Iran. *Trametes versicolor* was collected from the Hyrcanian forest area in the north of Iran and identified by analyzing of the *rpb2* and ITS sequences. Sawdust 90% and wheat bran 10% substrate formulation revealed the longest duration for spawn running, primordial initiation, total cultivation period, and days to the first harvest, recording values of 21.7 ± 1, 24.3 ± 1, 39.7 ± 2, and 29.7 ± 4 days, respectively. However, better yield performance and biological efficiency were recorded in the sawdust 70% + wheat bran 30% substrate formulation (S10; C/N ratio = 40), with values of 159 ± 4 g/kg and 45.5%, respectively. The substrate moisture content was maintained at 65%, while the relative humidity and temperature in the fruiting room were maintained at 80–90% and 24 ± 1 °C, respectively. Among fruiting bodies harvested from 13 various substrate formulations, the highest values of total phenolic content (TPC) and total flavonoid content (TFC) were recorded in S10, with values of 78 mg GAE/g DW extract and 14.76 ± 2 mg QE/g DW extract, respectively. Subsequently, the highest DPPH free radical scavenging activity (antioxidant activity) was recorded in the same medium, with an IC50 value of 24.28 µg/mL. These findings demonstrate the significant potential for using local agricultural waste for the cultivation of medicinal mushrooms. Furthermore, *T. versicolor* extracts show promise as a natural source of antioxidant(s) and/or radical scavengers, which could eventually be used as medicinal compounds or functional food supplements in the treatment of diseases.

Keywords – Antioxidant activity – Phylogeny – Promising agent – *Trametes versicolor* – Waste management

Introduction

For many centuries, edible mushrooms have been considered a delicious food component due to their sensory properties and culinary uses (Kamiyama et al. 2013). In general, edible mushroom cultivation is widespread worldwide due to its low resource requirements and ease of cultivation (Luo et al. 2014). Recently, edible fungi have become more noteworthy as functional foods due to their significant impact on human health (Ito et al. 2020). The majority of the edible mushroom species are grown, consumed, or commercially cultivated from their wild vegetation (Luo et al. 2014).

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Mushrooms have been used as medicine and food even before their function was carefully examined and confirmed (Rašeta et al. 2020). In recent years, common mushroom species such as *Pleurotus* spp. (shell mushroom), *Lentinus* spp. (Panus giant), *Hericium* spp. (lion's mane mushroom), *Ganoderma* spp. (hemlock lacquer shelf), and *Agaricus* spp. (button mushroom) have been commercially cultivated (Anusiya et al. 2021). In this context, *Trametes versicolor* (turkey tail), a member of the Polyporaceae family, is one of the best medicinal mushrooms studied in recent years (Justo & Hibbett 2011). “Turkey tail” is the most common name in the Western world. Its medicinal importance in traditional Chinese medicine dates back at least 2000 years, where it has been associated with general health-promoting effects, including improved longevity, enhanced stamina, immunomodulatory activity and anti-cancer effects (Cheng & Leung 2008). Furthermore, *Trametes versicolor* extracts contain the number of polysaccharide fractions, including d-glucose polymers composed of glucuronic acids, ß-glucans, mannose, arabinose, fucose, galactose, and xylose, and these polysaccharides are responsible for several biological activities (Thatoi et al. 2018). Morphologically, *T. versicolor* has a thin, solid, ovoid pileus and is known as an annual fungus commonly grown on old conifers or broadleaf trees (Wahab 2021). According to reports on the first successful cultivation of *T. versicolor* in Vietnam, Vietnamese industrial *T. versicolor* strains has been identified with high performance potential. Searching for wild strains before they become extinct is a helpful strategy to improve the economic efficiency of *T. versicolor* cultivation in Mexico, china, and Japan (Gonzalez et al. 2013, Le 2021).

The ratio of carbon to nitrogen (C/N) ratio directly affects the behavior of mushroom cultivation. The types and sources of nitrogen and carbon affect polysaccharide production and fungal mycelial growth. Basidiomycetes readily absorb organic nitrogen sources and benefit the production of maximum mycelial biomass and polysaccharide formation in culture (Elisashvili et al. 2009).

To our knowledge, no study has been conducted in Iran to date to evaluate the potential of wild strains of *T. versicolor* for commercial cultivation. Cultivation conditions and cultured strains are two main factors to improve mushroom product yield (Le 2021). However, the optimal culture conditions for mycelial growth and fruiting body development of *T. versicolor* have been insufficiently studied (Jo et al. 2010). Until now, sawdust was the only appropriate substrate for *T. versicolor* cultivation (Gonzalez et al. 2013). Substrate for mushroom cultivation is generally selected based on the lignocellulosic waste available on-site. Thus, further studies are needed to discover suitable substrate types.

This study was conducted to determine the optimal cultivation conditions for a native wild strain of *T. versicolor*. Using industrial and agricultural waste to produce mushrooms is an effective way to reduce production costs (Jahedi et al. 2024, Osma et al. 2011). Agriculture is one of the most important economic activities in Iran. Therefore, there is extensive production of agricultural and industrial products, along with concerns about their disposal (Gonzalez et al. 2013). In this study, locally available agro-forestry wastes, alder sawdust, wheat straw, and bagasse, were evaluated as the base substrates, along with wheat bran, for the production of *T. versicolor* under solid-state culture conditions. In general, this study aimed to assess the potential cultivation of the native wild *T. versicolor* strain by optimizing cultivation conditions. In this study, under artificial cultivation conditions, the first successful cultivation report of *T. versicolor* in Iran and their ability to produce fruiting bodies were highlighted.

Materials & Methods

Sampling and isolation

Trametes versicolor was sampled from Hyrcanian forests unprotected areas (36°35′40″N and 53°20′36″E) in northern Iran and transferred to the Tarbiat Modares University laboratory. Pure mycelium culture was isolated from the collected fruiting body by the tissue culture method and cultivated in PDA (potato dextrose agar) medium at 25 °C. The purified isolate was stored in PDA at 4 °C for the next step. The experiment was conducted at the Agricultural Faculty of the University of Tarbiat Modares, Tehran.

DNA extraction, PCR amplification, and sequencing

Total genomic DNA was prepared from a fungal colony (Wu et al. 2015). The regions of ITS were amplified with primers ITS5 and ITS4 (White et al. 1990), and the *rpb2* with primers b7.1R and b6F (Table 1) (Binder & Hibbett 2003). The PCR reaction (25 µl) contained 1.5 µl DNA, 1 µl reverse primer, 1 µl forward primer, 9 µl deionized water, and 12.5 µl Mastermix. The PCR for ITS was performed as follows: an initial denaturation at 95 °C for 3 min, followed by 35 cycles of 1 min at 72 °C, 45 s at 58 °C, and 40 s at 94 °C, and a final extension for 10 min at 72 °C. In addition, the PCR process for *rpb2* was done as follows: an initial denaturation at 95 °C for 5 min, followed by 35 cycles of 90 s at 72 °C, 90 s at 57 °C, and 1 min at 94 °C, and a final extension for 5 min at 72 °C. The product of PCR was studied with GreenView Ultra on a 1% agarose gel stained. The PCR products were directly sequenced and purified using similar primers from Biomagic Company, China, and manually aligned with Chromas software.

Table 1 Primer sequences were utilized for PCR analysis.

Regions	Primer names	Primer sequences	References
ITS	ITS4	TCCTCCGCTTATTGATATGC	White et al. (1990)
	ITS4	GGA AGT AAA AGT CGT AAAAG G	
rpb2	bRPB2-6F	TGG GGY ATG GTN TGY CCY GC	Binder and Hibbett (2003)
	bRPB2-7.1R	CCC ATR GCY TGY TTM CCC ATD GC	

Sequencing and phylogenetic analyses

The quality of the sequences received from the Biomagic Company was first evaluated using Chromas software (Biomagic Company, China). Re-sequencing was done for samples that failed to sequence. In the next step, suitable and valid sequences were tested for similarity (up to 98%) and compared with sequences of all species of the desired genus, along with an outgroup sample, using the latest published, valid articles for phylogenetic analysis and tree drawing (Table 2). Both the alignment of the obtained sequences with the desired sequence online in the MAFFT program (Katoh & Standley 2013) and using the Clustalx2 program (Clustal W) were first converted to FASTA format and then to NEXUS format. Also, in MAFFT alignment, the Q-INS-i strategy was used, whereas in Clustal W, alignment was performed according to the software's predefined parameters.

To perform phylogenetic analyses, alignment editing was conducted using Gblocks Server in the Castresana Lab online program (Nylander 2004) and the output was saved in NEXUS format. To construct the phylogeny tree, the Bayesian Inference (BI) approach was used. During this research, the MrModeltest2 software and the MrModelblock file containing 24 evolutionary models, mostly GTR+G+I, were used according to the AIC (Akaike Information Criterion) (Nylander 2004). It was applied to select the evolutionary model according to aligned sequences, in conjunction with PAUP. After choosing the evolutionary model, MrBayes was used to run the Bayes test and draw the resulting tree. The MrBayes output file was viewed with Dendroscope V.3.2.8.

Spawn preparation

Spawns were prepared using wheat grains. The wheat seeds were soaked overnight in water and then transferred into clear glass bottles. The bottles were sterilized in an autoclave at 20 psi for 50 minutes (Jahedi et al. 2024). The next day, the inoculum was prepared under sterile conditions pure mycelium and incubated at 26 °C. This was done to cover the wheat grains with white mycelium.

Preparation of cultivation substrate

The main research phase of this project aims to identify the optimal combination of cultivation substrates from local agricultural waste and hardwood sawdust. The substrate formula used for fruiting body production was prepared from sugarcane bagasse waste, wheat straw (an average particle size of about 8-10 x 2-3 mm), sieved alder tree sawdust, and wheat bran, as shown in Table 3.

Table 2 List of strains and Taxa and accession numbers of Genbank for the *rpb2* and ITS study (New sequences created for this research are in bold).

Species name	Species number	Origin	Genbank Accession Numbers	
			ITS	*rpb2*
T. betulina	CBS 695.94	Austria	JN645081	JN645126
T. aff. meyenii	BRFM 1361	French Guiana	JN645083	JN645144
T. gibbosa	BRFM 1115	France	JN645064	JN645110
T. hirsuta	BRFM 994	France	JN645100	JN645142
T. junipericola	–	Italy	AY684171	–
T. maxima	BRFM 1367	Guadeloupe – FWI	JN645084	JN645146
T. meyenii	CBS 453.7	India	JN645067	JN645112
T. ochracea	BRFM 632	France	JN645092	JN645133
T. polyzona	CBS 319.36	–	JN645078	JN645123
T. pubescens	CBS 696.94	Austria	JN645080	JN645125
T. socotrana	BRFM 1293	Zimbabwe	JN645073	JN645118
T. suaveolens	BRFM 578	France	JN645090	JN645131
T. versicolor	BRFM 1219	France	JN645058	JN645113
T. versicolor	FP135156	USA	JN164919	JN164850
T. versicolor	**Jahedi1400**	**Iran**	**OR461755**	**OR471649**
T. villosa	CBS 334.49	Argentina	JN645079	JN645124
Trametes elegans	–	Florida	JV021237	–
Daedaleopsis tricolor	BRFM 954	France	JN645096	JN645138

Table 3 Prepared formulations based on four agro-forest wastes.

Substrate number	Substrate code	Formulation (%)	C/N
1	S1	Wheat Straw 100	71
2	S2	Sawdust 100	80
3	S3	Bagasse 100	41
4	S4	Wheat Bran 100	18
5	S5	Wheat Straw 90 + Wheat Bran 10	53
6	S6	Wheat Straw 80 + Wheat Bran 20	50
7	S7	Wheat Straw 70 + Wheat Bran 30	45
8	S8	Sawdust 90 + Wheat Bran 10	46
9	S9	Sawdust 80 + Wheat Bran 20	42
10	S10	Sawdust 70 + Wheat Bran 30	40
11	S11	Bagasse 90 + Wheat Bran 10	40
12	S12	Bagasse 80 + Wheat Bran 20	35
13	S13	Bagasse 70 + Wheat Bran 30	30

The supplements of the above culture substrates with the mentioned percentages were thoroughly mixed. A moisture content of 65% was maintained, and 5% calcium carbonate was added to adjust the pH to 7. Subsequently, 1000 grams of the compound were transferred to sterilizable propylene bags measuring 20 × 40 cm. And their tips were closed by PVC rings and cotton caps. The bags were autoclaved at 121 °C and 1.5 atmospheres for 2 hours to be sterilized. After cooling, the substrates, under the hood under sterile conditions, 10 grams of spawn were inoculated into each bag.

C/N analysis

To measure the ratio of C/N (carbon to nitrogen), it was done according to the Valkli-Black and Kjeldahl method (Hossain et al. 2023) and the following formula:

The ratio of C/N of an n-component composition: (C/N ratio of component 1 × component 1's contribution in the composition) + ... + (C/N ratio of component n × component n's contribution in the composition).

Mycelial growth on different substrates

The bags were moved to a special room maintained at 25 ± 1 °C and with a 2:22 light:dark cycle. This environment was designed to complete the mycelial growth phase and to be filled with mushroom mycelium.

Fruiting body formation

At this stage, when the bags were filled with mycelium, to induce the primordia to enter the fruiting phase, they were transferred to the mushroom cultivation room at 23 ± 1 °C and 200 lux light to promote pin emergence and fruiting body formation. Periodically, approximately four incisions were made with a sterile scalpel in the four directions of the bags. The humidity level was maintained at 80–90% using the mist sprayer, which was turned on every 2.5 hours to provide moisture to the floor. In general, the wall was equipped with a constant relative humidity system with tips installed around the hall, and holes were drilled at 1 meter intervals that dripped (Jahedi et al. 2024).

Harvesting mushrooms and recording the parameters of the substrates

Evaluation of harvested mushroom yield performance

The following relation was utilized to obtain the yield of the whole harvested mushroom (including flushes 1, 2, and 3):

The total yield of harvested mushrooms (Y) = The total weight of freshly harvested mushrooms divided by one kilogram of the substrate wet weight was used (Thawthong et al. 2014).

Evaluation of biological efficiency (BE%)

This formula is widely used to measure a mushroom strain's ability to convert substrate into fruit, and it reflects the mushroom's function. BE is defined as the ratio of the freshly harvested mushroom weight to the dry weight of the used substrate and is considered as a percentage (Liang et al. 2019).

Biological efficiency (BE%) = (the weight of freshly removed mushroom divided by the substrate dry weight) × 100

Determination of the antioxidant activity, total phenol, and total flavonoid

TPC (Total polyphenol content) was specified by the method of Folin-Ciocalteu (Slinkard & Singleton 1977). Ground powder extracts were prepared at a 0.01 g/mL in methanol. Also, 7% Na2CO3 dissolved in distilled water was prepared separately. Then, 20 µl of the extract, 2 ml of distilled water, and 100 µl of Folin were added to the tube. After a 30-minute interval, 300 µl of Na_2CO_3 was added. Then, the solution was placed in a shaker incubator (for 2 hours), and finally, the measurement was performed by a spectrophotometer at a wavelength of 765 nm. All treatments were prepared in triplicate. Using a linear equation, the TPC obtained from the standard calibration curve of gallic acid (sigma) was expressed in mg of gallic acid equivalent per gram of dry weight extract (mg GAE/g DW ext.). All materials were run in triplicate. The findings were expressed as mean ± standard deviation.

The flavonoid content (TFC) was determined using the aluminum chloride method reported by Slinkard & Singleton (1977). Briefly, 1 mL of each mushroom extract was added to 0.3 mL AlCl3 10%, 0.3 mL NaNO2 5%, and 2 mL NaOH solution (1 M). The final mixture volume was adjusted to 10 mL with distilled water. The maximum absorbance was calculated at 510 nm after incubation for 15 min. A Jasco v530 spectrophotometer did the analysis. TFC was reported as milligrams of quercetin equivalent per dry matter gram of mushroom extract (mg QE/g DW ext.) according to the quercetin calibration curve (Sigma). All the materials were run in triplicate. Findings were reported as mean values ± standard deviations.

The antioxidant activity of the *Trametes versicolor* methanol extract was specified using the method of Sadh et al. (2018). Shortly thereafter, 200 µl of extract was mixed with 2 ml of 0.1 mM DPPH solution, and the mixture was kept in the dark for 30 min. An alteration in DPPH color from

purple to pale yellow was reported, and absorbance was calculated at 517 nm by a UV–visible spectrophotometer. The following relation determined the activity of free radical scavenging percentage:

$$\text{Inhibition (\%)} = (A - B) \div A \times 100$$

Where A is the DPPH absorbance, and B is the treatment absorbance. Ascorbic acid was utilized as the positive control. Ascorbic acid was used as a positive control. IC50 (the concentration of the extract required to inhibit 50% of free radicals) was also used to compare the extracts' activities. Finally, the results obtained were analyzed using SPSS software and one-way analysis of variance, and the comparison of data means was performed using Duncan's multiple range test at the 1% level.

Results

Phylogenetic analysis

By analyzing the *rpb2* region and ITS1-5.8S-ITS2 sequences, the strain of the current study was specified as *T. versicolor* (Fig. 1). The partial *rpb2* gene and ITS rDNA sequences obtained from the current research strain Jahedi1400 were maintained in GenBank (NCBI) under OR461755 and OR471649 accession numbers, respectively (Table 2).

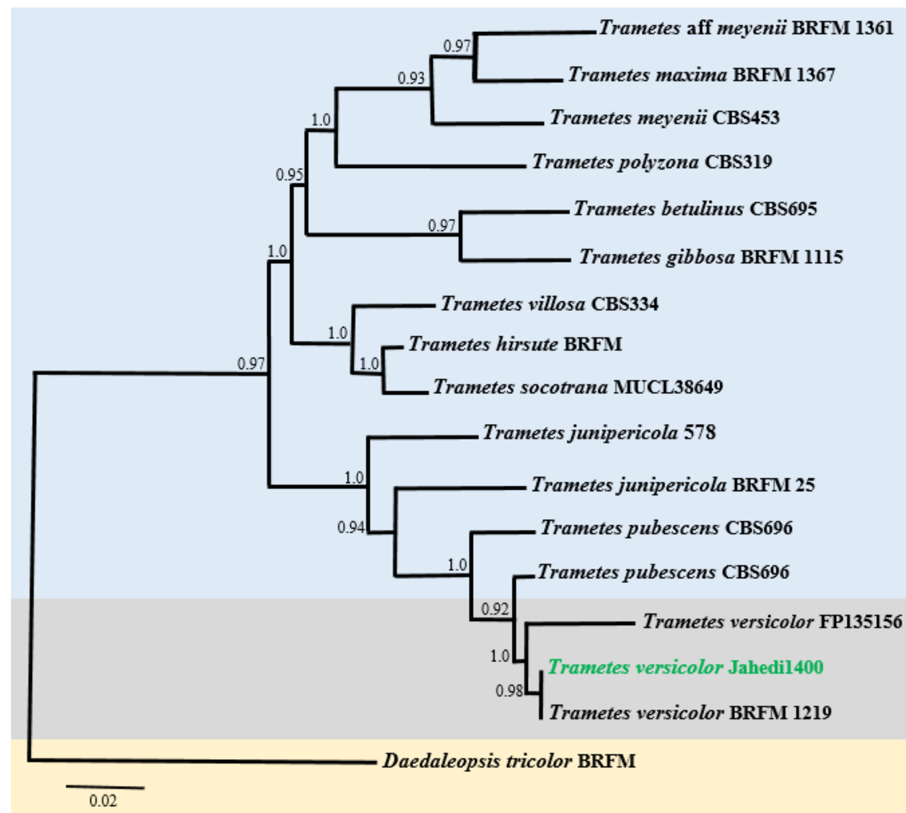

Fig. 1 – Bayesian tree inferred under the model of GTR + G + I from the ITS sequences of the entire Trametes genus, by *Daedaleopsis tricolor* BRFM 1327. New sequences are shown in green font and were considered as strong support to 0.98 Bayesian posterior probabilities.

Determination of substrate composition during the spawn running

The growth of *T. versicolor* on bag media made of 13 substrates is presented in Table 4. It showed better outcomes in the studied parameters when the substrate was supplemented with wheat bran. The shortest spawn running period was observed on wheat straw, followed by rice straw, and the longest on a sawdust substrate, with a spawning time of 37–44 days.

Pinhead formation period

The research found that the fruiting body started to form a needle head as soon as the mycelium fully colonized the substrate. This finding could be because of the compounds in the material composition. The 13 substrates used in the current study revealed a primordia formation period of 24.3–46 days; indeed, the sawdust 90% + wheat bran 10% substrate (24.3 days) showed a faster initial formation period than the wheat bran 100% substrate (46 days).

Fruiting body harvesting time

According to these results, the average time to fruit body harvest ranged from 29.7 to 51.7 days. The shortest mushroom fruiting body harvesting time is on substrate 8, with an average of 29.7 days, followed by substrates 5, 10, and 12, with values of 32.3, 32.3, and 32.7 days, respectively, without significant differences. The longest fruiting body harvest time in this study was recorded by substrate 4 (51.7 days) (Fig. 2).

Fig. 2 – Fructification of native *T. versicolor* (domestication at TMU, Tehran, Iran).

Evaluation of the total yield and biological efficiency of the harvested fruiting body

Table 4 shows that the yield performance of *T. vesicolor* varies between several substrates. In general, according to the data in Table 5, it can be seen that the highest yield of mushrooms in substrate 10 (sawdust 70% + wheat bran 30%) with 159 grams per bag and biological efficiency 45.5% and subsequently in substrate No. 9 (sawdust 70% + wheat bran 30%) with a value of 148 and a biological efficiency of 42.3% was observed. The lowest fruit body yield was observed in substrate 4 (wheat bran 100%) with 29 grams per bag and a biological efficiency of 8.29%.

Table 4 Impacts of the various substrates prepared from various portion levels (%) of waste of agricultur, sawdust, wheat straw, and bagasse enriched with wheat bran, on PI (primordial initiation), SRP (spawn running period), DFFH (days for the first harvest), and TCP (total cultivation period) of *T. versicolor.*

Sub. No	Sub. Code	C/N	Substrate formula (%)	SRP (day)	PI (day)	DFFH	TCP(day)
1	S1	71	Wheat Straw 100	35.7 ± 2^{b}	40.3 ± 1^{b}	43.7 ± 1^{b}	54.3 ± 2^{b}
2	S2	80	Sawdust 100	29 ± 1^{cd}	33 ± 1^{de}	37 ± 0^{de}	47.7 ± 1^{de}
3	S3	41	Bagasse 100	32.7 ± 1^{bc}	37 ± 1^{bc}	41.3 ± 3^{bc}	51.3 ± 2^{bc}
4	S4	18	Wheat Bran 100	40 ± 1^{a}	46 ± 1^{a}	51.7 ± 2^{a}	60 ± 2^{a}
5	S5	53	Wheat Straw 90 + Wheat Bran 10	22 ± 2^{f}	27 ± 0^{hi}	32.3 ± 1^{gh}	40.7 ± 1^{hi}
6	S6	50	Wheat Straw 80 + Wheat Bran 20	26.7 ± 2^{de}	32 ± 0^{def}	36.7 ± 1^{de}	47 ± 2^{def}

Table 4 Continued.

Sub. No	Sub. Code	C/N	Substrate formula (%)	SRP (day)	PI (day)	DFFH	TCP(day)
7	S7	45	Wheat Straw 70 + Wheat Bran 30	25 ± 1^{ef}	29.7 ± 1^{fgh}	33.7 ± 2^{fg}	44.3 ± 2^{fg}
8	S8	46	Sawdust 90 + Wheat Bran 10	21.7 ± 1^{f}	24.3 ± 1^{i}	29.7 ± 4^{h}	39.7 ± 2^{i}
9	S9	42	Sawdust 80 + Wheat Bran 20	31.3 ± 2^{c}	34.3 ± 1^{cd}	38.7 ± 2^{cd}	48.7 ± 2^{cd}
10	S10	40	Sawdust 70 + Wheat Bran 30	24 ± 1^{ef}	28.3 ± 1^{gh}	32.3 ± 1^{gh}	43 ± 0^{gh}
11	S11	40	Bagasse 90 + Wheat Bran 10	26.7 ± 3^{de}	30.7 ± 1^{defg}	35 ± 1^{def}	44.7 ± 2^{efg}
12	S12	35	Bagasse 80 + Wheat Bran 20	24 ± 3^{ef}	29 ± 4^{gh}	32.7 ± 2^{gh}	40.7 ± 1^{hi}
13	S13	30	Bagasse 70 + Wheat Bran 30	23.3 ± 1^{f}	29 ± 0^{gh}	33.3 ± 1^{fg}	43.3 ± 1^{ghi}

Values are expressed as means ± SD. Values shown in different lowercase letters in each column differ at a significant level (P = 0.01).

Table 5 Impacts of the various substrates prepared from various portion levels (%) of waste of agricultur, sawdust, wheat straw, and bagasse enriched with wheat bran, on yield and biological efficiency (BE%) of *T. versicolor.*

Sub. No	Sub. Code	C/N	Substrate formula (%)	Y(g/Kg)	BE (%)
1	S1	71	Wheat Straw 100	67.8 ± 3^{i}	19.4 ± 1^{i}
2	S2	80	Sawdust 100	96.8 ± 6^{gh}	27.7 ± 2^{gh}
3	S3	41	Bagasse 100	91.1 ± 6^{h}	26 ± 2^{h}
4	S4	18	Wheat Bran 100	29 ± 4^{j}	8.29 ± 1^{j}
5	S5	53	Wheat Straw 90 + Wheat Bran 10	106 ± 4^{fg}	30.3 ± 1^{fg}
6	S6	50	Wheat Straw 80 + Wheat Bran 20	122 ± 2^{e}	34.8 ± 0^{e}
7	S7	45	Wheat Straw 70 + Wheat Bran 30	118 ± 3^{ef}	33.6 ± 1^{ef}
8	S8	46	Sawdust 90 + Wheat Bran 10	148 ± 2^{abc}	42.3 ± 1^{abc}
9	S9	42	Sawdust 80 + Wheat Bran 20	152 ± 3^{ab}	43.5 ± 1^{ab}
10	S10	40	Sawdust 70 + Wheat Bran 30	159 ± 4^{a}	45.5 ± 1^{a}
11	S11	40	Bagasse 90 + Wheat Bran 10	135 ± 4^{d}	38.7 ± 1^{d}
12	S12	35	Bagasse 80 + Wheat Bran 20	141 ± 10^{bcd}	40.2 ± 3^{bcd}
13	S13	30	Bagasse 70 + Wheat Bran 30	144 ± 4^{cd}	41.3 ± 1^{cd}

Values are expressed as means ± SD. Values shown in different lowercase letters in each column differ at a significant level (P = 0.01).

Total cultivation period

The shortest cultivation period was recorded at 39.7 and 40.7 days in the substrates sawdust 90% + wheat bran 10% and sugarcane bagasse 80% + wheat bran 20%. The longest cultivation period was observed in the 100% wheat bran substrate, at 60.2 days.

C/N ratio analysis

Since C/N is a key parameter in mushroom cultivation, the current research found that low or high ratios of C/N are not appropriate for cultivation (Table 4). Supplemented substrates (numbers 5 to 13) with a balanced C/N ratio (30 to 45) showed high performance. However, substrates with a high ratio of C/N (substrates no. 1–3) or a low C/N ratio (substrate no. 4) had a low performance.

Total phenolic and flavonoid contents

TFC and TPC of the extracts are presented in Table 6. The S10 exhibited the highest TPC with 78 ± 1.41 mg GAE/ g DW extract, followed by S4 with 72 ± 1.41 mg GAE/ g DW extract.

Table 6 TFC (mg QE/g DW ext.), TPC (mg GAE/g DW ext.), and antioxidant activity (IC50 µg/mL) of fruiting body extracts of *T. versicolor*.

Sub. No	Sub. code	Formulation (%)	TPC	TFC	Antioxidant activity (IC50)
1	S1	Wheat Straw 100	70 ± 2.44abc	12.66 ± 2 .49c	46.88 ± 0.9b
2	S2	Sawdust 100	70 ± 3.74abc	10 ± 1.63c	26.62 ± 0.86de
3	S3	Bagasse 100	65 ± 1.41bcd	10.65 ± 2.49c	57.7 ± 0.9a
4	S4	Wheat Bran 100	72 ± 1.41ab	12.21 ± 2.21c	34.16 ± 1.11c
5	S5	Wheat Straw 90 + Wheat Bran 10	71 ± 3.74ab	12.73 ± 2.33c	26.62 ± 0.86de
6	S6	Wheat Straw 80 + Wheat Bran 20	69 ± 2.44abc	10.83 ± 2.02c	49.49 ± 0.29b
7	S7	Wheat Straw 70 + Wheat Bran 30	70 ± 3.74abc	12 ± 1.63c	49.49 ± 0.29b
8	S8	Sawdust 90 + Wheat Bran 10	60 ± 0cde	8.67 ± 2.46c	29.88 ± 0.29d
9	S9	Sawdust 80 + Wheat Bran 20	69 ± 4.89abc	7.33 ± 0.94b	29.88 ± 0.29d
10	S10	Sawdust 70 + Wheat Bran 30	78 ± 1.41a	14.76 ± 2.32a	24.28 ± 1.7e
11	S11	Bagasse 90 + Wheat Bran 10	63 ± 2.44cd	10.9 ± 2.04c	49.06 ± 1.92b
12	S12	Bagasse 80 + Wheat Bran 20	61 ± 3.74de	8.25 ± 2.08c	49.06 ± 1.92b
13	S13	Bagasse 70 + Wheat Bran 30	53 ± 1.41def	10.88 ± 1.08c	49.06 ± 1.92b
-	AS	-	-	-	7 ± 1.25f

Values are expressed as means ± SD. Values shown in different lowercase letters in each column differ at a significant level (P = 0.01).

The DPPH free radical scavenging activity

In general, the findings of this research showed that DPPH free radical inhibition was enhanced with increasing concentration. At a 100 µg/ml concentration, the highest DPPH inhibition was 86.94, and 85.07 at 200 µg/l for substrates 10, 5, and 2, respectively. Subsequently, at a 10 µg/ml concentration, the lowest inhibition occurred: 66.79% for substrate 3. At a 50 µg/ml concentration, the inhibition was similar to that recorded at the final concentration. Ascorbic acid is considered a standard antioxidant (positive control) (Fig. 3). Although the synthetic antioxidant ascorbic acid is regarded as a standard antioxidant, the fruiting body of *T. versicolor* also showed relatively higher inhibition activity.

As shown in Table 6, the comparison of the antioxidant activity of different fungi of investigated substrates showed that the highest amount of antioxidant activity is related to S10 (Sawdust 70% + Wheat bran 30%) and S2 (Sawdust 100%) with IC50 (the test substance concentration that inhibits 50 % of free radicals) to 24.28 and 26.62 µg/mL. The lowest activity was observed with S3 (Bagasse 100%), with an IC50 of 57.7 µg/mL.

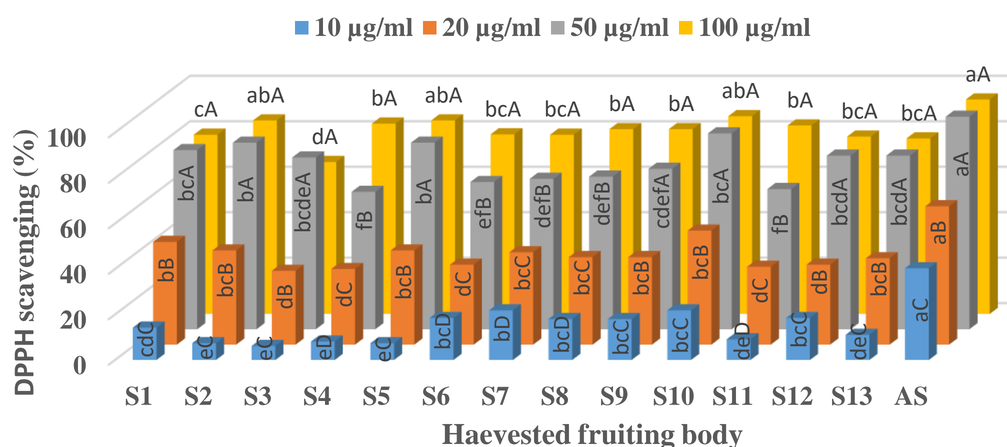

Fig. 3 – Antioxidant activity of different fruiting body extracts using a DPPH free radical scavenging assay; A–D: Values bearing the different superscripts within the same substrate are remarkably different at the 1% level (P<0.01); a–b: Values bearing the different superscripts within the different

substrates are significantly different at the 5% level (P<0.01). S1 = wheat straw 100%; S2 = sawdust 100%; S3 = bagasse 100%; S4 = wheat bran 100%; S5 = wheat straw 90% + wheat bran 10%; S6 = wheat straw 80% + wheat bran 20%; S7 = wheat straw 70% + wheat bran 30%; S8 = sawdust 90% + wheat bran 10%; S9 = sawdust 80% + wheat bran 20%; S10 = sawdust 70% + wheat bran 30%; S11 = bagasse 90% + wheat bran 10%; S12 = bagasse 80% + wheat bran 20%; S13 = bagasse 70% + wheat bran 30%; AS = ascorbic acid.

Pearson correlation between the measured parameters of substrates and the harvested fruiting body

Analysis of Pearson correlation between the substrate parameters and mushrooms harvested on various media substrates is given in Table 7.

Table 7 Pearson Correlation between different cultivation substrate parameters, harvested fruiting body, and mycochemical content.

	SRP	PI	DFFH	TCP	Yield	BE	TPC	TFC	IC50
SRP	1								
PI	0.958***	1							
DFFH	0.935***	0.956***	1						
TCP	0.93***	0.943***	0.938***	1					
Yield	-0.777***	-0.819***	-0.826***	- 0.799***	1				
BE	-0.777***	-0.819***	-0.826***	-0.799***	1***	1			
TPC	0.432	0.521	-0.642	-0.444	0.444	0.321	1		
TFC	0.232	-0.532	0.111	0.341	0.332	0.232	0.989***	1	
IC50	0.132	0.223	0.434	0.201	0.435	0.4	-0.837***	-0.915**	1

**Significance at the level of .01, ***Significance at the level of .001. SRP (spawn running period), PI (primordial initiation), DFFH (days for the first harvest), TCP (total cultivation period), BE (biological efficiency), TPC (Total polyphenol content), TFC (total flavonoid content)

There was a high positive correlation between spawn running and pinhead formation (r = 0.958), spawn running and first harvest (r = 0.935), and spawn running and total cultivation period (r = 0.930). Also, positive correlations were found between pinhead formation and first harvest (r = 0.956), and pinhead formation and the period of entire cultivation (r = 0.943), and first harvest and the period of entire cultivation (r = 0.938) at the level of 0.001.

As it is clear from Table 7, there is a strong negative correlation at the level of 0.001 between the parameters of yield and biological efficiency with the traits of spawn running time, pinhead formation, first harvest, and the period of total cultivation, respectively, with coefficients of 0.777, 0.819, 0.826, and 0.799, respectively. There was a perfect correlation between yield and biological yield (r = 1). There was a high positive correlation between TFC and TPC (r = 0.958) at the 0.001 level, a strong negative correlation at the 0.0001 level between IC50 and TPC (r = 0.837), and a 0.001 level correlation between TFC and IC50 (r = 0.915).

Discussion

According to the obtained results (Table 4), data analysis shows that *T. versicolor* mushroom strain grows faster than mycelium in the substrate produced from sawdust 90% + wheat bran 10%, wheat straw 90% + wheat bran 10% and sugarcane bagasse 70% + wheat bran 30% with values of 21.7, 22.2, and 23.3 days, respectively. Still, the slowest spawn run time was recorded in the substrate made from 100% wheat bran after 40 days. Atila et al. (2018) and Jahedi et al. (2024) obtained the maximum mycelium growth rate or the shortest spawning time of *H. erinaceus* on oak sawdust substrate with wheat bran supplementation, with a spawning time of 27 days, and wheat as the most essential supplement in reducing the spawning time (Table 4). In addition, the minimum spawn running time of *H. erinaceus* on a substrate of wheat bran was determined (Chang & Roh 1999). In this research, it was found that a mixture of sawdust, wheat straw, and sugarcane bagasse, with the

addition of wheat bran, reduced the spawn running time and is recommended for the production of fruiting bodies. The study's findings are inconsistent with those of Hassan (2007). In the current research, among all the wastes, sawdust mixed with food supplements showed better outcomes than the other two cases. The study's results are inconsistent with those of Stamets (1993), who reported mycelial growth rates over 10–14 days. The findings were also inconsistent with those of Oei (2003), who stated a spaw running time of 6 weeks at 20–30 °C.

It can be seen that in certain sections of the substrate surface, primordia began to appear, forming small, round masses that were grouped. According to Yang et al. (2013), mycelial expansion in the substrate depends directly on the fungus's performance. In addition, the statistical analysis demonstrated a significant difference among the 13 substrates in the formation of *T. versicolor* mushroom pinheads. In general, the results of the present research did not agree with those reported by Bunroj et al. (2017), who found the period of pinhead formation ranged from 57.1 to 149.67 days in rubber sawdust containing brewer's yeast, dolomite, gypsum, rice bran, and Lucana.

In general, there are multiple essential parameters for the performance of mushroom yield, including the optimal nutrient levels in the energy available for the spawn run, the substrate, and the production of mushroom fruiting bodies. Based on the studies of Yang et al. (2013), the increase in water absorption capacity depends on the physical structure of the substrate. The results of the present research did not agree with those reported by Bunroj et al. (2017), who found that the yield of *H. erinaceus* mushroom strains ranged from 84.77 g/kg to 211.42 g/kg on rubber para bar sawdust. Nguyen showed that compared to sawdust alone, 62% sawdust + 30% rice husk + 7% wheat bran + 1% CaCO 3 exhibited higher BE and thus, should be used as the optimal substrate mixture to cultivate *T. versicolor* (Nguyen 2021). A combination of diverse substrates could enhance the yield performance of mushrooms because of a variation in the capability of such substrates to provide nutritional and environmental requirements and the difference in cellulose, hemicellulose, and lignin contents (Owaid et al. 2015). Our results are consistent with those of Bunroj et al. (2017), who reported the highest yield in enriched sawdust.

According to the data above, one of the most critical factors for successful cultivation is the cultivation period, which maximizes nutrient use from the substrate in the shortest time and produces high-quality fruit with high yield and efficiency. In this research, the reinforced substrates provided favorable conditions for carbon, nitrogen, minerals, and other compounds in the structural composition of the materials, especially nutrients, resulting in a favorable outcome. Needless to say, it is not always true that a substrate that has high carbon and nitrogen content or vitamins and minerals will give a favorable outcome, but this is due to the quality and simplicity of the chemical complexity of the composition that fungal enzymes can convert into absorbable structures, and can be used to convert mushroom mycelium. The findings of Uhart et al. (2008) and Jahedi et al. (2023, 2024, 2025) showed that wheat bran contains higher-quality nutrients that increase mycelial growth rate, findings similar to those of this research. Shashirekha et al. (2005) reported that the activities of amino acid transaminases and proteases in wheat bran will improve mycelial performance.

Jin et al. (2018) stated that a high ratio of C/N could be related to the *P. ostreatus* slow growth. The optimal ratio of C/N provides the materials required to make an arbitrary compost. If the ratio of C/N is high, it slows the activity of the culture media, and if it is low, nitrogen is released by the mass as ammonia, creating an disagreeable smell (Zhu 2007). Based on the results of Chang & Roh (1999), the *Auricularia fungus* growth in a substrate with a carbon-to-nitrogen ratio of 35:1 is appropriate, and an increase or decrease of this ratio causes a reduction in mycelium growth. It is known that lignocellulosic materials generally have low protein content and are insufficient for mushroom cultivation, which requires potassium, phosphate, and nitrogen. Nitrogen supplementation is a key factor to consider in mushroom cultivation, as the ratio of C/N plays a vital role in spore germination and fruiting body growth.

Our studies were consistent with the results of a previous research by Tripathy et al. (2009), which found that mushroom yield performance depends on factors beyond the C/N ratio, including the type and physical and chemical structures of compounds, vitamins, minerals, and other nutrients. The findings were consistent with those of Pop et al. (2018): 46.22 ± 0.89 and 15.40 ± 0.81 mg

GAE/g DW extract for total phenols in water and methanol extracts of *T. versicolor,* respectively. According to previous articles, bioactive compounds from mushroom species, such as phenolics (flavonoids and phenolic acids), polysaccharides and proteins, or polysaccharide-protein complexes (Hybelbauerova et al. 2008), contribute significantly to the antioxidant potential of these species. The results of the current study for DPPH, TF, and TP are shown in Table 6. Antioxidant activity is closely associated with flavonoids, anthocyanins, total phenol content, and vitamins. However, based on the many researchers, antioxidant activity is more affected by total phenol (Wang & Lin 2000). Therefore, the high antioxidant capacity of the mushrooms produced in substrates containing sawdust, sugar beet waste, and walnut and tea hard skins can be attributed to the high levels of total phenols in these substrates. It has been shown that phenolic compounds are potential antioxidants and free radical inhibitors, suggesting a relation between the amounts of phenolic and flavonoid compounds and antioxidant activity (Kumar et al. 2008). As expected, the addition of wheat bran of up to 7% to the substrates showed an improvement in the yield as well as BE in all treatments, whereas the 9% wheat bran-supplemented basal substrate did not enhance the yield performance of *T. versicolor* (Dzaka 2017). Based on the literature review, higher and lower values have been reported, for example, in the studies of Knežević, values of 24.8 µg/mg dry ext. were obtained (Knežević et al. 2018) . In general, basal substrates are known to be poor in nutrients. Thus, supplements are used as co-substrates to improve the nitrogen content for cellular protein and enzyme synthesis (Nguyen 2021).

The DPPH radical-scavenging activity strongly depends on the concentration of the sample. In general, DPPH radical scavenging activity increases with increasing the concentration of the sample. According to Johnsy & Kaviyarasan (2011), extract yield is influenced by the solvent type, extraction time, temperature, and the sample's chemical nature. The solvent used and the chemical characteristics of the samples are two essential factors. Also, the extraction method used for antioxidant activity is important. In the experiment conducted by Johnsy & Kaviyarasan (2011), free radical scavenging increased with increasing concentration, and the synthetic antioxidant revealed greater free radical scavenging activity than the mushroom, consistent with the findings of this study.

According to research conducted by Kalbarczyk & Jamroz (1989), the rate of mycelium growth and accumulated biomass depends on the activity of lignin-cellulosic enzymes (cellulose, hemicellulose, and lignin). On the other hand, the development of mycelium depends on the nutrient content of the substrate and its degree of availability. Considering that sawdust contains carbon, cellulose and lignin (Joshua et al. 2016) and on the other hand, research has shown that it contains compounds such as protein, fat, carbohydrates, fiber, water, iron, potassium, calcium, manganese, copper, sodium, zinc (Dambalkar et al. 2015). Phenolic compounds, such as flavonoids, phenolic acids, and tannins, are considered the essential factors in a mushroom's antioxidant capacity. These antioxidants also exhibit various biological activities, including anticancer, anti-inflammatory, and anti-atherosclerotic activities.

Despite numerous studies on the beneficial effects of *T. versicolor* and other plant extracts in cancer treatment, controversy persists over their clinical use. Thus, the effectiveness of the *T. versicolor* extract is most likely due to its known spectrum and yet-to-be-specified bioactive compounds (Hsieh & Wu 2001). According to Philippoussis (2009), adding supplements to the substrate increases nutrient content, accelerates growth and pinhead formation, and ultimately reduces the cultivation period. Previous research results show that the growth parameters and amount of phenolic compounds is affected by factors such as species, substrate composition, cultivar, environmental conditions, cultivation operations, harvesting stage, and drying and storage conditions. Among the environmental factors affecting the accumulation of phenolic compounds are light intensity and temperature (Toor & Savage 2005). Fabros et al. (2023) study showed *T. versicolor* with a mycelial growth rate of 8.36 mm/day.

According to this research, Shashirekha et al. (2005) reported that the activities of transaminases, amino acids, and proteinases in wheat bran improve yield performance and, subsequently, biological efficiency in mycelium and fruiting bodies. These results are consistent with those of Ferreira et al. (2009), who reported a strong relation between phenolic compounds and

antioxidant activity. Antioxidant activity depends on phenolic compounds. According to previous studies, there is a direct relationship between total phenol and total flavonoid content (Mihaylova & Lante 2019).

Conclusions

In the current study, solid-state cultivation of the medicinal mushroom native to Iran, *T. versicolor,* was carried out. All the examined fruiting body extracts revealed a significant antioxidant activity. The results indicate that the highest values of TPC and TFC among fruiting bodies harvested from 13 different substrates were reported in the Sawdust 70% + Wheat bran 30% substrate, with amounts of 78 ± 1 and 14.76 ± 2 mg QE/g DW extract, respectively. Subsequently, the highest antioxidant activity was recorded in the same medium, with an IC50 of 24.28 ± 1 µg/mL. These findings have shown significant potential for using local agricultural wastes for the production of medicinal mushrooms, as well as for *T. versicolor* extracts as antioxidant(s) natural sources, and/or radical scavengers, which could eventually be applied as food supplements or medicinal compounds in the treatment of diseases. Evidently, our study elaborates on the development of a bioconversion concept to valorize waste to added-value products with potential bioactive attributes.This is the first report of the successful cultivation of *T. versicolor* native to Iran. The successful cultivation of this mushroom can play a vital role in Iran's mushroom industry by enabling optimum use of two valuable but abandoned members of the environment: agro-waste valorization and the production of a medicinal mushroom with high antioxidant activity. Finally, we suggest that the use of substrates in industrial scale be used for final evaluation and production of commercial substrates for this species in the future.

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Accessibility of data

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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