

# Breadcrumb as a New Substrate for *Trametes versicolor* and *Schizophyllum commune* Submerged Cultivation

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A new substrate, breadcrumbs, was investigated for biomass accumulation, the pH of the cultural broth, the formation of primary metabolites such as the proteins and endopolysaccharides of *Schizophyllum commune* 1768 and *Trametes versicolor* 353, as well as its bioconversion efficiency. The results showed that *S. commune* gives more mycelial mass ( $23.96 \pm 0.8$  g/l) and in a shorter period (4 days) than *T. versicolor* ( $15.76 \pm 0.5$  g/l in 5 days). The pH values changed from the initial 6.1 to 3.6 in *S. commune* cultural broth and to 4.4 in *T. versicolor* cultural broth. Maximal endopolysaccharide content in the mycelia of *S. commune* and *T. versicolor* were 7.13% and 6.42%, correspondingly. Crude protein content in *S. commune* mycelium was 18.83 % on the 4th day of cultivation, and 20.03%, in the mycelium of *T. versicolor*, on the 6th day of cultivation. Kinetic parameters for the quantitative estimation of cultivation efficiency were calculated for biomass, endopolysaccharide, and crude protein concentrations.

**Keywords:** *Trametes versicolor*, *Schizophyllum commune*, breadcrumb, crude protein, endopolysaccharides, submerged cultivation

## Introduction

Basidiomycetes are high prized for dietary and medicinal properties, which makes them a promising raw material for creation of functional foods and dietary supplements. Mushrooms have high amounts of protein with a sufficient content of the essential amino acids, low fat which consists predominantly of unsaturated fatty acids, polysaccharides with wide ranges of medicinal activities; vitamins and mineral elements complement well-balanced composition of mushrooms [14].

*Trametes versicolor* (L.: Fr.) Quel. and *Schizophyllum commune* Fr.: Fr. are well-known medicinal Basidiomycetes, which can be cultivated artificially, and cause white rot of trees in the nature. In terms of high medicinal

value of these mushrooms, dietary supplements with variety of therapeutic activity were developed in the USA, Korea, China, Belarus, Ukraine, and some other countries. These products are mostly based on the fruiting bodies. At the same time, substances of commercial value were isolated not only from fruiting bodies, but also from mycelia (for example, polysaccharide-protein complexes Krestin and PSP from *T. versicolor*) and cultural broth (e.g.,  $\beta$ -glucan schizophyllan from *S. commune*) [20]. Submerged cultivation takes less time and gives more predictable content of active substances in comparison with cultivation of fruiting bodies.

It was shown [11, 13, 23] that bioconversion efficiency, accumulation rate and amounts of biologically active substances in mycelia and cultural broth depended on substrate for cultivation. Fungi have an ability to utilize wide range of substrates, but synthetic easily accessible media can suppress generation of some valuable secondary metabolites [18]. Nowadays wastes of agriculture, food, paper, and

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other industries are considered to be the most perspective substrates for cultivation of mushrooms because of low cost (or lack thereof) and necessity to recycle refuses. Thus, researchers applied beet molasses, milk whey, beer wort [13], detoxified hydrolysate of rice hull [19] for *S. commune* submerged cultivation. In turn, *T. versicolor* submerged cultivation was conducted on milk whey [4, 11], beer wort [11], tomato pomace [7], barley husk, oak and hornbeam sawdust [22].

In our previous work [10], we performed screening of medicinal Basidiomycetes and Ascomycetes fungi biomass accumulation on liquid media containing wastes of food industry of Ukraine, such as aspiration wastes of barley from brewery, the 3-d category solid wastes from wheat and rye mill, and waste of bread production – breadcrumb. To the best of our knowledge, such wastes were never used before for the purpose of fungal cultivation on liquid media. The investigation has shown that the highest biomass concentrations were achieved during *S. commune* and *T. versicolor* cultivation on liquid medium with breadcrumb.

The purpose of the present work was to investigate the process of *T. versicolor* and *S. commune* submerged cultivation on the new substrate – breadcrumb: the dynamics of biomass accumulation, primary metabolite content of mycelia (polysaccharides and protein), pH of cultural broth, and bioconversion efficiency.

## Materials and Methods

### Fungal strains and cultivation

*S. commune* 1768 and *T. versicolor* 353 were kindly supplied by the Culture Collection of Mushrooms from the M.G. Kholodny Institute of Botany of the National Academy of Sciences of Ukraine (IBK) [2]. The stock cultures were maintained on wort agar slants at 4°C. The cultures were inoculated in Petri dishes containing glucose-peptone medium, g/l: 25 glucose, 3 peptone, 2 yeast extract, 1 KH<sub>2</sub>PO<sub>4</sub>, 1 K<sub>2</sub>HPO<sub>4</sub>, 0.25 MgSO<sub>4</sub> × 7 H<sub>2</sub>O, and 20 agar.

The substrate for submerged cultivation (breadcrumb) was donated by Bread Plant No 12 of the Open Joint-Stock Company “Kyivkhlіb”, Kyiv, Ukraine. The liquid media with breadcrumb were autoclaved for 40 min, with 1 atmosphere.

Fully colonized Petri dish with glucose-peptone medium was homogenized and used for 10% (v/v) inoculation of 250 ml flasks containing 50 ml liquid medium with bread-

crumb. The flasks were incubated at 28 ± 2°C in a rotary shaker (120 rpm).

To select the optimal substrate concentrations the following ratios of breadcrumb concentrations (g/l) were chosen: 25, 30, 40, 50, and 60. After incubation for 7 days, the fungal mycelia were harvested by filtration, washed three times with water, and desiccated at 105°C to a constant weight. Bioconversion efficiency parameter was used to evaluate the optimal concentration of the substrate. Bioconversion efficiency (*E*, %) was calculated as follows:

$$E = \frac{M_n}{M_w} \times 100\% \quad (1) [23]$$

where *M<sub>n</sub>*: biomass concentration, dry weight;

*M<sub>w</sub>*: initial concentration of breadcrumb, dry weight.

To study the cultivation parameters of investigated fungi, mycelia in active phase of growth (with average diameter of pellets about 0.5 mm) grown on breadcrumb in selected concentrations for each strain were used for 10% (v/v) inoculation of media with the same concentrations of the substrate for elimination of the lag phase.

### Chemical composition of breadcrumb

The chemical composition of breadcrumb, including moisture, ash, total carbohydrates, crude fat, and crude protein, was determined according to AOAC methods [1]. To obtain moisture content, samples of the substrate were dried at 105°C until constant weight. The ash content was determined by incineration at 600°C until constant weight.

Nitrogen content was defined by Kjeldahl's method. For the calculation of crude protein in breadcrumb, the Nitrogen content was multiplied by a factor of 5.7 [16]. In present work we determined crude fat content by extracting a known weight of powdered sample with hexane as a solvent, using a Soxhlet apparatus. The amount of total carbohydrates was calculated by difference:

$$\text{Total carbohydrates} = 100 - (\text{g moisture} + \text{g crude protein} + \text{g crude fat} + \text{g ash}) \quad (2)$$

The method of vitamin B<sub>1</sub> (thiamine) evaluation was based on oxidation of thiamine to thiochrome, extraction thiochrome into organic solvent, and measurement of fluorescence [15]. Vitamin B<sub>2</sub> (riboflavin) content was determined using riboflavin binding apoprotein from chicken eggs [12]. The method of vitamin B<sub>3</sub> (PP) estimation was

based on hydrolysis, quantitative obtaining of colored glutamic aldehyde derivate, and further colorimetric determination [21]. Vitamin B<sub>9</sub> (folic acid) content was analyzed by the change in fluorescence intensity before and after oxidation of folates previously purifying them from interfering compounds [7].

### Endopolysaccharide and crude protein assay of fungal mycelia

The mycelia were filtered, washed with water, and desiccated at 105°C to a constant weight. Biomass concentration was determined gravimetrically, and further used for nitrogen determination by Kjeldal's method [1]. Crude protein conversion factor was  $N \times 4.38$  [14]. Filtered and washed mycelia were dried at 60°C for obtaining endopolysaccharides; endopolysaccharide content was measured by gravimetric method [3].

### Kinetic parameters for quantitative estimation of cultivation efficiency

Growth rate ( $X$ , g/l per day), endopolysaccharide production rate ( $Y$ , g/l per day), productivity of endopolysaccharide biosynthesis ( $P$ , mg/g per day), protein production rate ( $Z$ , g/l per day), and productivity of protein biosynthesis ( $C$ , mg/g per day) were calculated as follows [2]:

$$X = \frac{M_n - M_0}{\Delta t} \quad (3)$$

$$Y = \frac{M'_n}{\Delta t} \quad (4)$$

$$P = \frac{M'_n}{M_n \times \Delta t} \quad (5)$$

$$Z = \frac{M''_n}{\Delta t} \quad (6)$$

$$C = \frac{M''_n}{M_n \times \Delta t} \quad (7)$$

where  $M_n$  : biomass concentration, dry weight;

$M_0$  : dry weight of inoculum;

$M'_n$  : endopolysaccharide content;

$M''_n$  : crude protein content;

$\Delta t$  : time of cultivation;

### Statistical analysis

Values are mean of three independent experiments done in triplicate and are expressed as mean  $\pm$  errors. Data were statistically analyzed by  $t$  test using OriginPro 8.5.1, Origin-Lab Corporation, USA. Differences between means at 5% ( $p < 0.05$ ) level were considered to be significant.

## Results and Discussion

In Ukraine bread is made of wheat and rye flour. Unsold and off-test bread undergoes inspection for visible mold, bacterial infection, contaminations, followed by crumbling, desiccation, and this is the way how breadcrumb forms. The composition of breadcrumb is present in Table 1. As shown in the table, the most part of the substrate ( $77.45 \pm 0.63\%$ ) is carbohydrates. This data is corresponding to literature data [5] which report that cereals contain 50-80% of carbohydrates, the main component of which is starch. Breadcrumb contains vitamins of B group (thiamine, riboflavin, niacin, and folic acid).

According to the maximal bioconversion efficiency rates, the optimal concentrations of breadcrumb appear to be

**Table 1. Composition of dried crust crumbs as percent of dry matter.**

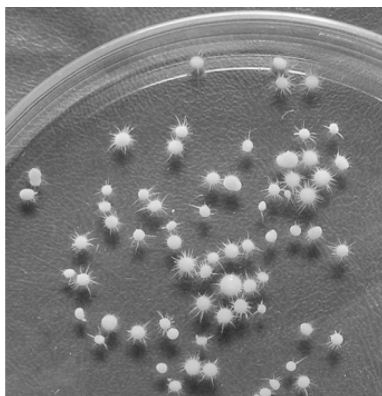
Moisture (%)	Ash (%)	Crude protein (%)	Crude fat (%)	Total carbohydrates (%)	Vitamins (mg%)			
					B1	B2	B3	B9
$9.38 \pm 0.03$	$1.75 \pm 0.04$	$10.94 \pm 0.55$	$0.48 \pm 0.01$	$77.45 \pm 0.63$	$0.105 \pm 0.006$	$0.042 \pm 0.003$	$1.25 \pm 0.08$	$0.0130 \pm 0.0002$

**Table 2. Bioconversion efficiency of breadcrumb<sup>a</sup> (%).**

Mushroom species	Concentrations of breadcrumb (g/l)				
	25	30	40	50	60
<i>T. versicolor</i>	42.06a	46.48a	53.16b	57.17c	49.23d
<i>S. commune</i>	55.68a	56.17a	54.11b	54.95b	58.32c

<sup>a</sup>Means with the same letter within the same row are not significantly different ( $p < 0.05$ )

The amount of inoculum for *T. versicolor* was  $11.2 \pm 0.3$  g/l and for *S. commune* -  $5.1 \pm 0.7$  g/l



**Fig. 1.** *T. versicolor* pellets on the 5-th day of submerged cultivation on breadcrumb (in Petri dish with diameter 9 cm).

50 g/l for *T. versicolor* and 60 g/l for *S. commune* (Table 2) and were used for further experiments. In such concentrations of the substrate bioconversion efficiency reached the highest levels of 57.17 and 58.32% correspondingly. Higher than 60 g/l concentrations of breadcrumb led to the growth of mycelia on the top of the substrate, did not result in increase in bioconversion efficiency rates and were eliminated. Bioconversion efficiency of *T. versicolor* grown on breadcrumb in all investigated concentrations was higher than values obtained by other researches [11] during submerged cultivation on milk whey (39%) or beer wort (36%). Bioconversion efficiency of *S. commune* grown on breadcrumb was more than two times higher than on rice hull's detoxified hydrolysate (24%) [19].

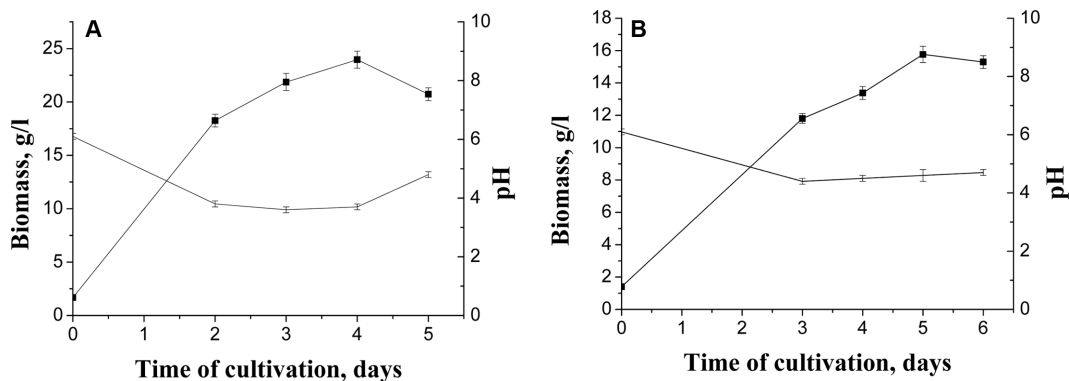
When grown in submerged culture, fungi exhibit different morphological forms, ranging from dispersed mycelia filaments to densely interwoven mycelial mass referred to as pellets (fluffy or smooth, with hollow or compact center)

[17]. Mycelia of *S. commune* and *T. versicolor* grew in the form of fluffy and smooth pellets with size 0.5-3 mm during submerged cultivation on breadcrumb (Fig. 1). We observed hollows in pellets, which had diameter about 3 mm. Appearance of hollows can be explained by autolysis due to oxygen and nutrition deficiency in the center of the colony [17]. The growth in form of pellets has its preferences such as easier separation of biomass and lower viscosity of cultured broth which cause better nutrition and oxygen transfer as compared to dispersed form.

*S. commune* produced significant amounts of biomass ( $23.96 \pm 0.8$  g/l) in 4 days of cultivation on investigated substrate (Fig. 2A) compared to others results: 7.29 g/l on detoxified hydrolysate of rice hull [19], 8.8 g/l on beet molasses, 6.7 g/l on corn extract, 1,8-2,7 g/l (depending on the strain) on beer wort [13]. *T. versicolor* accumulated  $15.76 \pm 0.5$  g/l biomass on the 5-th day (Fig. 2B), which was similar to results of Klechak *et al.* [11] on milk whey but twice more than on beer wort. Other investigators obtained 8.9-10.6 g/l (depending on the strain) of *T. versicolor* biomass on complex medium with addition of milk whey [4].

Values of pH altered more significantly during active growth of cultures: pH descended from the initial ( $6.1 \pm 0.1$ ) to  $3.6 \pm 0.1$  in *S. commune* cultural broth and to  $4.4 \pm 0.1$  in *T. versicolor* cultural broth on the 3-d day of cultivation, after that pH ascended to 4.7-4.8 in both cases on the end of cultivation (Fig. 2). The same general view of pH curve was observed in submerged cultivation of *G. lucidum* on breadcrumb [8].

We evaluated cultivation efficiency by calculations of kinetic parameters (Table 3, 4). The highest growth rate for *S. commune* and *T. versicolor* occurred until the 2-d and 3-d days of cultivation, respectively. After consuming most



**Fig. 2.** Time courses of biomass accumulation (-■-) and pH of cultural broth (-) in submerged cultivation on the breadcrumb: (A) *S. commune*, (B) *T. versicolor*.

**Table 3. Kinetic parameters for quantitative estimation of *S. commune* cultivation efficiency<sup>1</sup>.**

Parameters	Time of cultivation (days)			
	2	3	4	5
Average growth rate (g/l per day)	8.30a	6.73b	5.57c	– <sup>2</sup>
Endopolysaccharide content (%)	6.06a	6.53b	6.79b	7.13b
Endopolysaccharide production rate (g/l per day)	0.56a	0.48b	0.41c	0.30d
Productivity of endopolysaccharide biosynthesis (mg/g per day)	30.39a	21.80b	17.01c	14.28d
Crude protein content (%)	17.39a	17.48a	18.83b	18.35b
Protein production rate (g/l per day)	1.59a	1.34b	1.13c	0.80d
Productivity of protein biosynthesis (mg/g per day)	87.08a	61.12b	47.06c	38.69d

<sup>1</sup>Means with the same letter within the same row are not significantly different ( $p < 0.05$ )

<sup>2</sup>The biomass amount is decreased

**Table 4. Kinetic parameters for quantitative estimation of *T. versicolor* cultivation efficiency<sup>1</sup>.**

Parameters	Time of cultivation (days)			
	3	4	5	6
Average growth rate (g/l per day)	3.47a	3.00b	2.87b	– <sup>2</sup>
Endopolysaccharide content (%)	5.36a	5.98b	6.10b	6.42b
Endopolysaccharide production rate (g/l per day)	0.21a	0.20a	0.19a	0.16b
Productivity of endopolysaccharide biosynthesis (mg/g per day)	17.80a	14.95b	12.18c	10.68d
Crude protein content (%)	18.92a	19.22b	19.85c	20.03c
Protein production rate (g/l per day)	0.74a	0.64b	0.61b	0.51c
Productivity of protein biosynthesis (mg/g per day)	62.99a	48.02b	38.58c	33.36d

<sup>1</sup>Means with the same letter within the same row are not significantly different ( $p < 0.05$ )

<sup>2</sup>The biomass amount is decreased

part of nutrients from medium fungal cultures moved to stationary phase and growth rate slowed down until fungi reached maximal biomass concentration. On the last investigated days of cultivation biomass concentrations decreased and the phase of culture senescence became.

Mushrooms are considered to be a good source of important biologically active substances such as polysaccharides and protein. In our investigations the content of endopolysaccharides in mycelia of both species increased insignificantly (Table 3 and 4), but for *T. versicolor* it was ten times higher compared to others results [6] on the complex medium with addition of milk. Maximal crude protein content was 18.83% in mycelium of *S. commune* and 20.03% in mycelium of *T. versicolor*. The curve of biomass accumulation did not correlate with protein and endopolysaccharide content for both mushroom species. Mycelia of fungi accumulated maximal amounts of aforementioned primary metabolites before reaching maximal biomass (Fig. 2, Tables 3 and 4). Production rates of primary metabolites indicate how much desired product can be obtained in unit

of medium volume per unit of time, and productivity of biosynthesis shows how much desired product can be generated in unit of biomass per unit of time. The obtained results of active processes of primary metabolites accumulation occur at the active phase of growth which is corresponding with literature data [2].

The results of our work showed that the new substrate breadcrumb is promising for submerged cultivation of *S. commune* 1768 and *T. versicolor* 353. Significant bioconversion efficiency of breadcrumb by *S. commune* and *T. versicolor* was established at concentrations of the substrate 60 and 50 g/l respectively. We gained higher biomass concentrations than those in most of researches for cultivation of these species on other substrates. The maximal biomass concentrations were obtained on the 4-th for *S. commune* (23.96 g/l) and on the 5-th days for *T. versicolor* (15.76 g/l), and such periods of submerged cultivation on breadcrumb are proposed for further employment. Maximal endopolysaccharide contents in mycelia of *S. commune* and *T. versicolor* were 7.13% and 6.42%, correspondingly.

Crude protein content in *S. commune* biomass was 18.83% on the 4-th day of cultivation, and 20.03% in biomass of *T. versicolor* on the 6-th day of cultivation.

Breadcrumb can be assumed as a safe, cheap, and widely available substrate for cultivation of medicinal mushrooms. The use of breadcrumb for medicinal mushroom cultivation needs subsequent investigations in order to evaluate detailed content and medicinal properties of primary and secondary metabolites. It is also important to perform optimization of culture conditions to obtain increased production of mycelial growth and metabolites.

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