

## RESEARCH ARTICLE

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## Alternative substrates for higher mushrooms mycelia cultivation

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Cultivation of 29 species of higher mushroom mycelia on alternative substrates – wastes of Ukrainian oil-fat industry, has been investigated. The amount of mushroom mycelia obtaining on 12 investigated substrates varied significantly, from 1.0 g/L to 22.9 g/L on the 14th day of cultivation. The superficial cultivation adopted in this study allows for easy to choose appropriate medium (substrate) for mycelia production. Alternative substrates (compared to glucose-peptone-yeast medium) were selected for all studied species, from soybean cake – most suitable for the mycelial growth of 24 species, to walnut cake – suitable only for 2 species. The utilization of substrates has been evaluated by biological efficiency. The best index of biological efficiency varied from 19.0% to 41.6% depending on the mushroom species. It was established high biological efficiency of mycelia cultivation on substrates: wheat seed cake – *Pleurotus djamor*, *Lyophyllum shimeji*, *Crinipellis schevczenkovi*, *Phellinus igniarius*, *Spongipellis litschaueri*; oat seed cake – *Ganoderma applanatum* and *G. lucidum*; soybean cake – *Hohenbuehelia myxotricha*, *Trametes versicolor*, *Morchella esculenta*, *Cordyceps sinensis*, *C. militaris*, and *Agrocybe aegerita*; rape seed cake – *Auriporia aurea*; camelina seed cake – *Fomes fomentarius*. The cultivation of these species are perspective as a biotechnological process of agricultural wastes converted into mycelia, which could be used in different forms of products with therapeutic action: powder or tablets nutraceuticals or ingredients for functional foods.

**Key words:** higher mushrooms mycelia, superficial cultivation, oil-fat industry wastes, biological efficiency

### ABSTRACT

#### Introduction

In the basis of the priority directions of modern biotechnology is the study of the possibility of wastes bioconversion for effective bioresources usage by waste-free processing, first of all – agricultural wastes. Global trends of oilseeds consumption for food, cosmetic, health and non-food (biodiesel production) usage demonstrate dynamic growth with a corresponding increase in the amount of oil-fat industry wastes. The main methods of vegetable oils producing: extraction of pre-peeled and crushed oilseeds by organic solvents and liquid or supercritical carbon dioxide; cold pressed oils from seeds; combined method. Sunflower seed, linseed, soybean, rapeseed, cottonseed oil cakes are the

main wastes of oil-fat industry. The growth of the demand for nontraditional oils with preventive medical appointment have promoted of expanding the range of oil cakes such as: rosehip, amaranth seeds, pumpkin seeds, milk thistle, wheat germs, oats, rapeseed, mustard seeds, walnuts, sunflower seeds, pine nuts, peanut etc. Usually, oil and fat industry wastes are used for the production of feed for animals and birds. These by-products are high in protein (35–50%), low in fat (about 1% in oil cake after extraction and up to 10% of fat by cold pressed). Oil cakes contain a certain amount of micro- and macro elements and vitamins. The ability of dry oil cakes for long storage without sacrificing of quality (provided of proper storage conditions – humidity, temperature) is also important. Therefore, recycling of such

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solid substrates by direct bioconversion using organisms with powerful enzyme systems capable of acting on biopolymer materials is more appropriate. From the point of view of effectiveness of the process of waste utilisation, mushrooms can be good tools for bioconversion of oil cakes due to their ability to produce different enzymes. Mushrooms cultivation on cheap substrates is becoming a topic of great interest since this process converted wastes to a food with high nutritional value and therapeutic potential. Usually, agrowastes used for production of fruit bodies on solid state cultivation. A number of different types of agricultural, industrial wastes and by-products as substrates or as additives for growing of 46 mushroom species are summarized by Poppe (2000). At the same time, there is an increasing interest in use of mushroom biomass (mycelia) in different forms of powders, tablets as dietary supplements or as components of functional foods. Despite the large number of publications, only some of them are devoted to the use of different wastes to produce biomass and biologically active substances in liquid nutrient media. There are few reports which describe natural mono substrates for mycelia production (Reyes et al., 2009; Barakat et al., 2011; Krupodorova & Barshteyn, 2012; Ivanova et al., 2014). Some studies have been done in liquid medium with addition of various types of plants, generally, in the form of extracts from plant wastes (Nieto-López & Sánchez-Vázquez, 1997; Curvetto et al., 2002; Mshandete & Mgonja, 2009; Taskin et al., 2011; Salvador et al., 2012; Yang et al., 2012; Zhang et al., 2012; Petre & Petre, 2013).

The purpose of this work was to study the possibility of utilization of oil-fat industry wastes (as basis of liquid substrate) through mushrooms cultivation in static condition.

## Materials and Methods

### Mushrooms cultures

The mushroom species *Agrocybe aegerita* 1853 (V. Bring.) Singer, *Auriporia aurea* 5048 (Peck) Ryvarden, *Coprinus comatus* 137 (O.F. Müll.) Pers., *Cordyceps militaris* 1862 (L.) Fr., *C. sinensis* 1928 (Berk.) Sacc., *Crinipellis schevchenkovi* 31 Bukhalo, *Flammulina velutipes* 1878 (Curtis) Singer, *Fomes fomentarius* 355 (L.) Fr., *Fomitopsis pinicola* 1523 (Sw.) P. Karst., *Ganoderma applanatum* 1701 (Pers.) Pat, *G. lucidum* 1900 (Curtis) P. Karst., *Grifola frondosa* 976 (Dicks.) Gray, *Hericium erinaceus* 970 (Bull.) Pers., *Hohenbuehelia myxotricha* 1599 (Lév.) Singer, *Hypsizygus marmoreus* 2006 (Peck) H.E. Bigelow, *Inonotus obliquus* 1877 (Ach. ex Pers.) Pilát,

*Laetiporus sulphureus* 352 (Bull.) Murrill, *Lentinus edodes* 502 (Berk.) Singer, *Lepista luscina* 64 (Fr.) Singer, *Lyophyllum shimeji* 1662 (Kawam.) Hongo, *Morchella esculenta* 1843 (L.) Pers., *Oxyporus obducens* 5085 (Pers.) Donk, *Phellinus igniarius* 1589 (L.) Quel., *Piptoporus betulinus* 327 (Bull.) P. Karst., *Pleurotus djamor* 1526 (Rumph. ex Fr.) Boedijn, *P. eryngii* 2015 (DC.) Quél., *Schizophyllum commune* 1768 Fr., *Spongipellis litschaueri* 5312 Lohweg, and *Trametes versicolor* 353 (L.) Lloyd were kindly supplied by the Culture Collection of Mushrooms (IBK) of the M.G. Kholodny Institute of Botany of the National Academy of Sciences of Ukraine (Buchalo et al., 2011).

### Cultivation medium basis

The basis of cultivation medium was oil-fat wastes – cakes of: rosehip fruit, linseed (flaxseed), pumpkin seed, milk thistle seed, wheat germ, oat seed, walnut, soyabean, camelina seed, rapeseed (canola seed), mustard seed and sunflower seed (composition – in accordance with the regulations of the manufacturer).

Cakes: rosehip fruit, linseed, pumpkin seed, milk thistle seed, wheat germ, oat seed, walnut were obtained from Scientific and Production LLC «Zhitomirbioprodukt» (Ukraine), cakes: rapeseed, soybean, sunflower seed – from PSC «Nizhynsky Zhyrcombinat» (Ukraine), mustard seed cake – from PSC «Factoria» (Ukraine), camelina seed cake – from M.M. Gryshko National Botanical Garden (Ukraine).

### Growth conditions

Mycelial cultures were initially grown in Petri dishes (90 mm in diameter) on culture medium with pH 6.0, composed of (g/L): glucose – 25.0, yeast extract – 3.0, peptone – 2.0, K<sub>2</sub>HPO<sub>4</sub> – 1.0, KH<sub>2</sub>PO<sub>4</sub> – 1.0, MgSO<sub>4</sub>·7H<sub>2</sub>O – 0.25, agar – 20. This GPY medium (glucose-peptone-yeast) without agar was selected as a control for evaluation of mushrooms growth.

Then the mycelium of each mushroom species (three agar disks, diameter 8 mm) was transferred to sterilized liquid cultivation medium (autoclaving for 20 min at 121°C) with appropriate waste previously crushed in powder consistence in amount 60 g on 1 L of distilled water.

Mycelium was grown at static cultures in 250-mL flasks with 50-mL liquid medium for 14 days at 26±2°C. Mycelium was separated from the medium by filtration through Whatman's filter paper N 4, washed with distilled water and dried at a temperature of 105°C to constant weight. The

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growth of mushrooms was evaluated by absolutely dry weight (a.d.w.) of mycelium.

The utilization of substrates evaluated on biological efficiency. Biological efficiency (%) was calculated using the following equation (Jwanny et al., 1995):

$$\text{Biological efficiency} = (A/B) \times 100,$$

where A is weight of dry mycelium, B is weight of the dry substrate.

### Statistical analysis

All samples were carried out in triplicate. The data were analyzed by Excel statistical functions using Microsoft Office XP software the Statistical Package for Social Sciences, Program 11.5 Version (SPSS, Inc., 2002). Values are presented as means  $\pm$  standard error of the mean (SEM). Differences at  $P \leq 0.05$  were considered to be significant.

## Results and Discussion

One of important trend in development of modern mycology and fungotherapy is the mycelia obtaining not only as the stage of subsequent fruiting bodies production, but also in finding in mycelia as well as in mushroom fruit bodies, spores, liquid culture new biologically active compounds with numerous therapeutic properties: antiviral, antibacterial, antiparasitic, antitumor, antihypertensive, antiatherosclerotic, hepatoprotective, antidiabetic, anti-inflammatory, and immune modulating (Turło, 2014). This entails increasing interest to the cultivation of many new species of mushroom in addition to the most traditional species from genus *Agaricus*, *Agrocybe*, *Pleurotus*, *Lentinus*, *Flammulina*, *Trametes*, *Ganoderma*, *Grifola*, *Hericium*, *Schizophyllum*.

The cost of substrates for mushrooms growth is one of the important parts of the final cost of the biomass. In recent decades the most popular substrates for mushroom cultivation became agricultural wastes characteristic for this or that region of the world. Agriculture is one of the leading sectors of the economy of Ukraine, one of the world leaders of oil and fat production. So, its dynamic and effective development leads to the appearance of different wastes such as oil seed cakes.

Cultivation of 29 species of higher mushroom mycelia on alternative substrates – wastes of Ukrainian oil-fat industry has been investigated in this study. The superficial cultivation adopted in this study allows for easy to choose appropriate medium (substrate) for mycelia production. It was necessary

to get answers on two fundamental questions: is it possible to cultivate the mushrooms on each of the studied substrates and whether it is effective (compared with the biomass growth on the control culture medium).

The influence of investigated substrates on mushrooms growth varied in terms of mycelium quantity from 1.0 g/L to 22.9 g/L (Figure 1–3). It is obvious that the investigated substrates affected differently on mycelial growth, depending on the content of those or other nutrients in substrates and individual requirements of studied species of fungi in nutrients. Principal ingredients of media for mushroom nutrition are: carbon sources (sugars), nitrogen sources ( $\text{NH}_4^+$  salts, urea, amino acids), vitamins (first of all – B vitamins), minerals (P, K, Mg, S), trace elements (Fe, Zn, Mn, Ca, Cu) and other components (Walker & White, 2011). However, the ratio between these components is different for the growth of each type of fungi. Many factors (temperature, pH of the nutrient medium, aeration) may influence of the biomass production and metabolite formation, but the most important are a sufficient amount of carbohydrates and nitrogen in substrates.

The soybean cake turned to be the most suitable for the mycelial growth of 24 species (Figure 1, 4). Among the studied substrates it is particular rich in protein (42 %), contains carbohydrates (7%), fat (8%), B vitamins, micro- and macro elements and supported very good mycelial growth of most mushroom species (Figure 1). Two studied *Cordyceps* species (*C. sinensis* and *C. militaris*) which are endoparasitoids, parasitic mainly on insects and other arthropods, found only one substrate – soybean cake, by which showed an increase in biomass higher compared to the control (more than 17 g/l).

The potential of conversion of various types of soybean components such as soybean straw, soybean hulls, defatted soybean (soy cake powder), soybean whey, soybean meal or flour into fruit bodies of mushrooms have been described in earlier studies (Vijay & Gupta, 1992; Bano et al., 1993; Upadhyay et al., 2002; Dehariya et al., 2011; Zied et al., 2011; Jape et al., 2014). Zied et al. (2011) summarized investigations in which soy-containing components have used as organic nitrogen supplementation for mushrooms cultivation. However influence of soybean components has been studied mainly on fructification of commercially cultivated mushrooms – *Agaricus spp.* (Vijay & Gupta, 1992; Zied et al., 2011), *Agrocybe spp.*, *Pleurotus spp.* (Bano et al., 1993; Upadhyay et al., 2002; Dehariya et al., 2011; Zied et al., 2011; Jape et al., 2014), *Lentinula spp.* (Zied et al., 2011)

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and seldom on mycelial mass production or useful metabolites (Falanghe, 1964; Hsieh et al., 2008; Hu et al., 2009; Barakat et al. 2011; Chimilovski et al., 2011; Maftoun et al., 2013). Production of mycelial mass on soybean cake

was higher when compared to submerged cultivation in natural mono substrates as soybean whey (Falanghe, 1964), lower than that obtained on soybean oil (Hu et al., 2009) and soybean curd residue (okara) (Barakat et al., 2011).

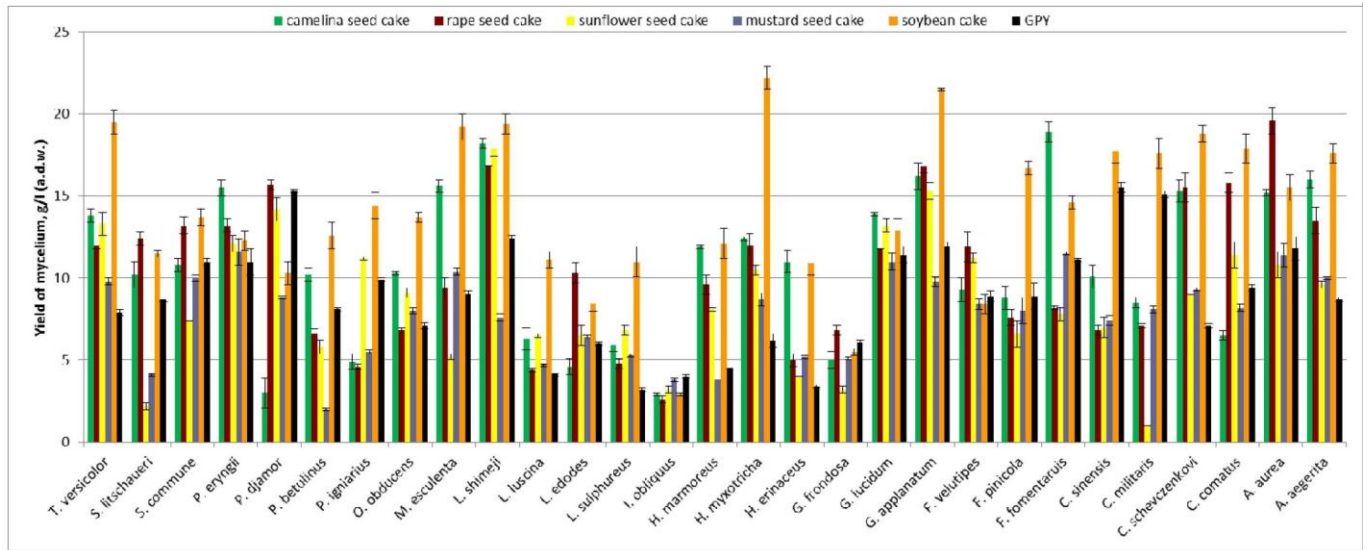


Figure 1. Growth of mushrooms on different media.

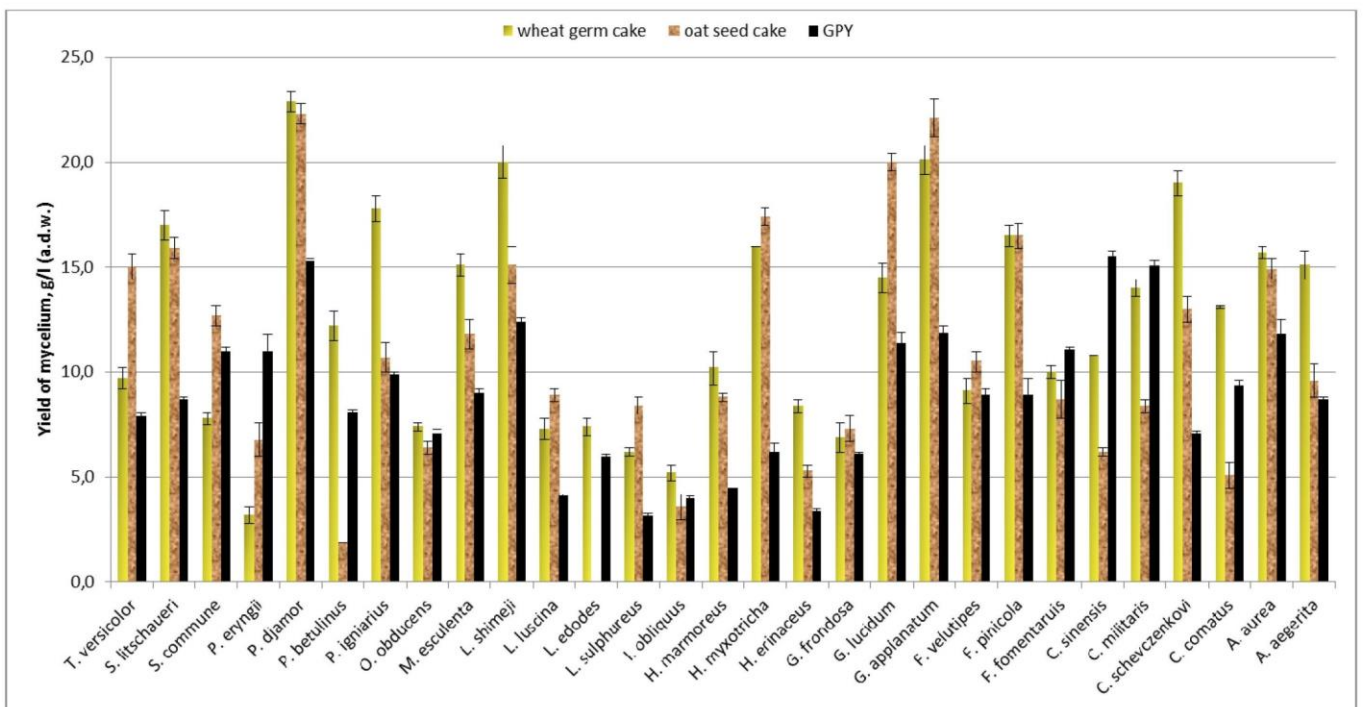
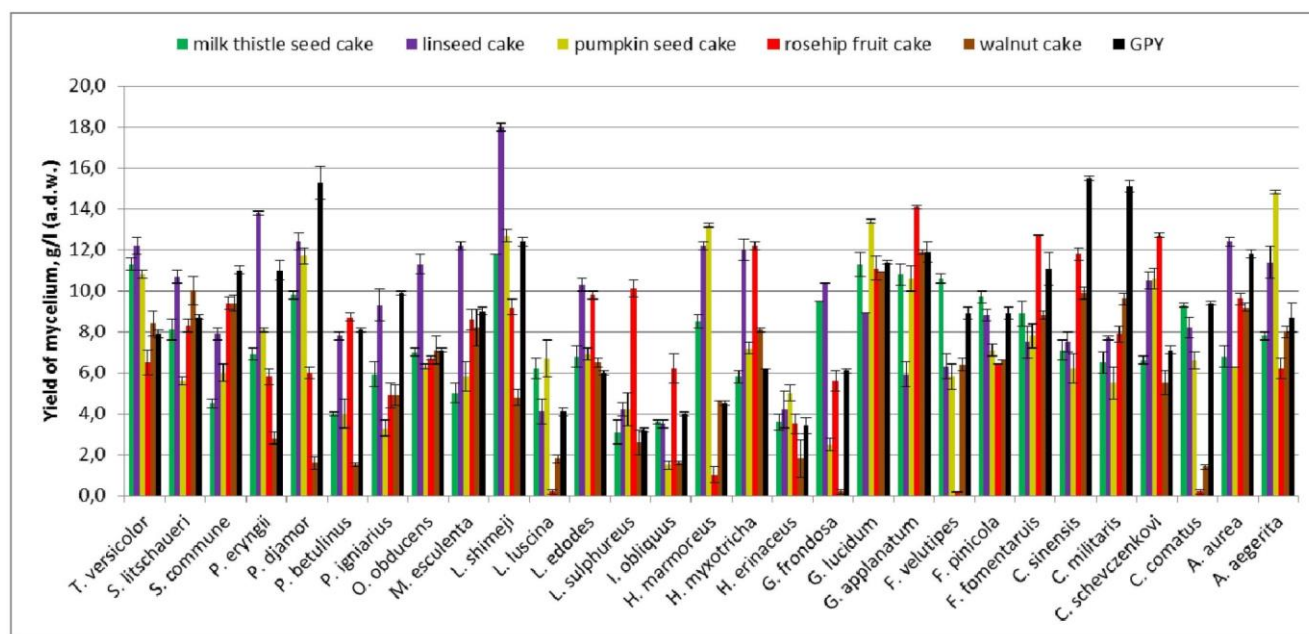


Figure 2. Growth of mushrooms on different media.

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**Figure 3.** Growth of mushrooms on different media.

Camelina seed cake allowed to get the mycelium yield over than 10 g/l for 18 species of fungi (*C. sinensis* and *S. commune* – less than on control) and rape seed cake – for 17 species (about and over than 10 g/l) (Figure 1). Camelina seed cake consists of approximately 31% of protein, 14% – carbohydrates, 10% – residual oil, 5% – minerals, and 10% – phytochemical constituents such as glucosinolates, flavonols, lignans, phenolic acids, B vitamins as well as nucleic acids. The rapeseed cake comprises a similar amount of basic components: protein – 37%, carbohydrates – 16%, fat – 9%, phosphorus – 1.5%, calcium – 1%.

Some mushrooms (12 species) cultivated easily on sunflower seed cake (protein – 28%, carbohydrates – 26%, fat – 10%) (Figure 1). Only *A. aurea* produced less biomass on this cake than on control. The published previously results suggest that replacing of peptone on an equivalent quantity of sunflower seed cake, have contributed to strong growth of *G. lucidum* mycelium and lipids synthesis, and slightly increased the biomass accumulation of *L. sulphureus* mycelium and carotenoid pigments (Babitskaya et al., 2012).

Good yield of mycelial mass can be obtained from mediums with oat seed cake and wheat germ cake (Figure 2). These substrates supported growth of all tested mushroom species, except *L. edodes* in case of application the medium with oat seed cake. Twenty species of mushrooms showed the mycelium yield about and over than 10 g/l on wheat germ

cake (*C. sinensis*, *C. militaris* and *F. fomentarius* – less than on control). The absolutely best result (all mushrooms on all substrates) has been demonstrated by *P. djamor* – 22.9 g/l. Defatted wheat germ, the main by-product in the wheat germ oil extraction process, has relatively high protein content (about 35%) and contains many other nutritional ingredients, such as carbohydrates (about 35%), pigments, minerals, and B vitamins. Fifteen species (Figure 2) produced the mycelium yield about and over than 10 g/l on oat seed cake, which is rich in carbohydrates (34%) and proteins (32%). The use of agricultural wastes – straw of various type of cereal as substrates is accepted worldwide. Regarding wheat and oats, they are used as an additive in combined substrates for mycelia growing (for subsequent fruiting bodies production) and mushrooms, but not used as mono substrate for mycelium growth (as the commercial product), as in our study.

Our result reveals that linseed cake supported good mycelial growth (over 10 g/l) of 13 species (Figure 3). Only *P. djamor* produced less biomass on linseed cake than on control. The defatted linseed cake consists of 4% of protein, 18% of carbohydrates and 17% of fat. It was also found that supplementation linseed meal has stimulated effect on mycelial growth and fructification of *Pleurotus spp.* cultivated on soybean straw (Jape et al., 2014).

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Thus, cakes from major typical oil cultures supported relatively good mycelial growth due to their chemical composition rich in protein, fibre, energy contents, nitrogenous substances and residues of oil (Ramachandran et al., 2007; Sivaramakrishnan & Gangadharan, 2009) necessary for growth. The positive effect of slight fat content in cakes for mushrooms biomass and their metabolites production is also in line with other studies (Yang et al., 2000; Hsieh et al., 2008; Hu et al., 2009; Malinowska et al., 2009; Halim et al., 2012), which confirmed a stimulating influence of some plant oils as good adjuvants.

The worst results were obtained with pumpkin seed cake, rosehip fruit cake, mustard seed cake, milk thistle seed cake and walnut cake (Figure 3). Slight growth of mushroom species was generally obtained on these five substrates and probably due to the limitation of essential components or availability of hard-to-reach components. Pumpkin seed cake (proteins – 16%; carbohydrates – 10%; fats – 18%), defatted rosehip fruit (proteins – 3%; carbohydrates – 9%, fats – 0.1%), mustard seed (protein – 37%, carbohydrates – 6%, fat – 11%), milk thistle (proteins – 7%; carbohydrates – 15%; fats – 12%), and walnut cakes (protein – 12%, carbohydrates – 8%, fat – 18%) contain medium and low amount of protein (except mustard seed) and high amount of fat (except rosehip

fruit). Probably, the high fat content in cakes does not contribute the growth of mushrooms. Low results obtained on the rosehip fruit cakes can be also explained by the low acidity of the medium (pH 4.0).

The use of oil cakes as mono substrates for the mushrooms mycelia production has shown promising results. However it is necessary to take into account composition changes of waste depending on the variety of oilseed plants, geographic and climatic growing conditions, technology of oil production and its waste obtaining (Shahidi & Miraliakbari, 2006; Concha et al., 2006; Zhu et al., 2006; Pashchenko et al., 2007; Gutiérrez et al., 2010; Leming & Lember, 2010; Al-Jasass & Al-Jasser, 2012; Berhow et al., 2014; Steiner-Asiedu et al., 2014; Mahmoud et al., 2015).

Results of screening allowed to find alternative substrates (compared to GPY medium) for the mycelia production of all studied mushroom species. Thus our investigation has expanded the knowledge of the possibility of studied mushrooms to growth on different substrates. The preferred substrates for mushrooms growth are shown in Figure 4 and the ability of mushrooms to assimilate different substrates – in Figure 5.

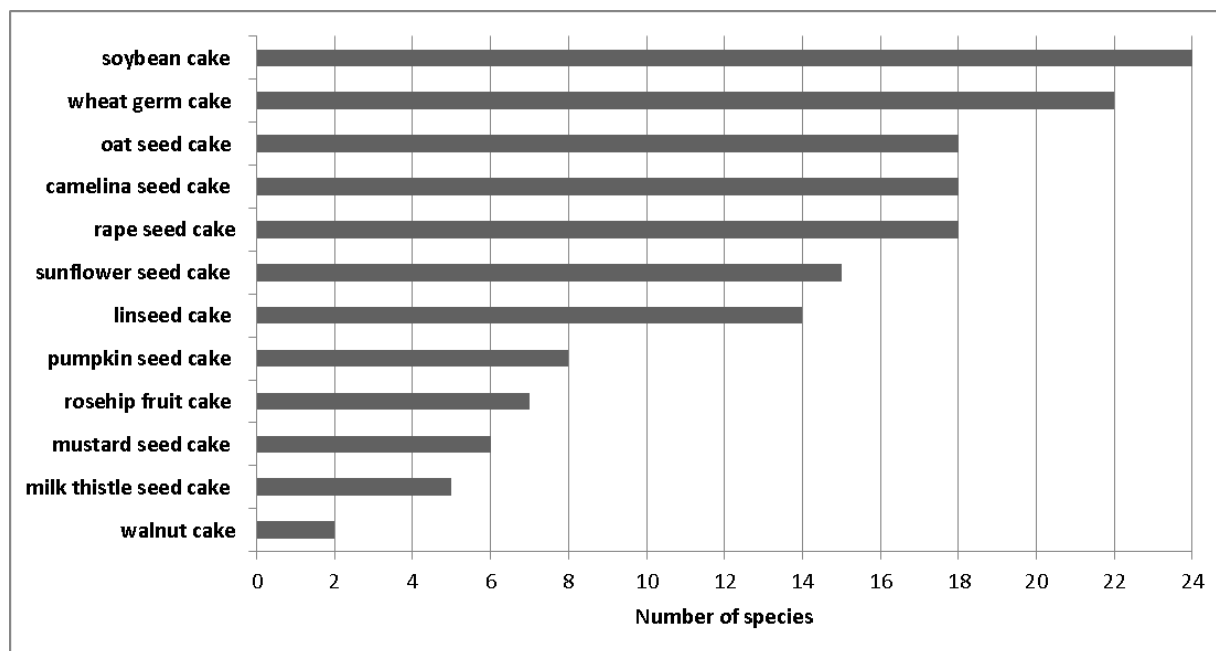


Figure 4. The preferred substrates for mushrooms growth.

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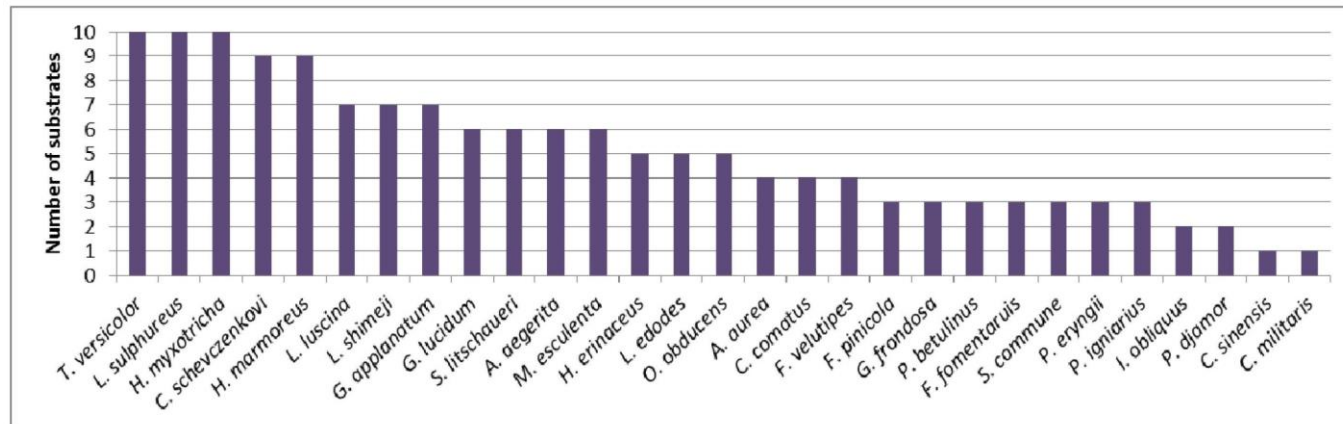


Figure 5. The ability of mushrooms to assimilate different substrates.

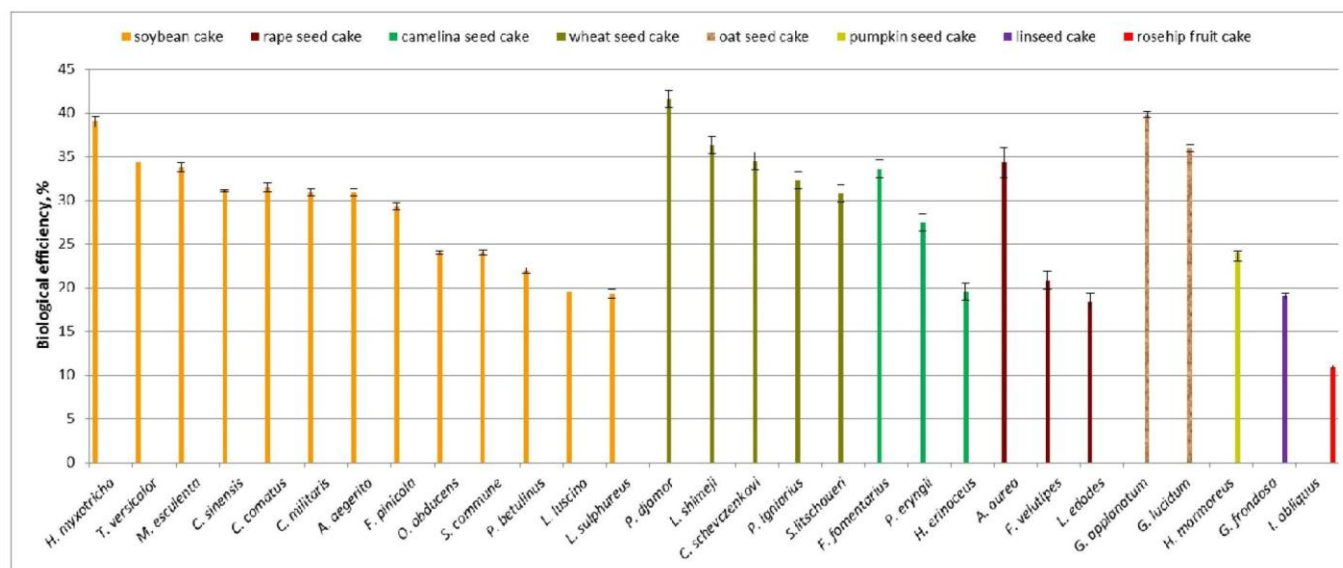


Figure 6. Maximal level of biological efficiency of the substrates for each studied mushroom species.

The best ability for wastes recycling has been found for wood-decay species: *T. versicolor*, *L. sulphureus*, *H. myxotricha*, followed by *C. schevczenkovi*, and *H. marmoreus*. These species can be perspective for commercial production. It should be noted unique soil saprotrophic mushroom – *L. shimeji* as the most active producer of mycelia (over 18 g/l) on greatly differ substrates: linseed cake, camelina seed cake, sunflower seed cake, soybean cake, wheat germ cake.

Biological efficiency is one of significant factors for understanding of expediency of the use of substrate for mushrooms growing. A significant variation of biological efficiency levels was observed in our study. The best indexes

of biological efficiency for every mushroom were varied from 19.0% to 41.6% (Figure 6). Taking into account that we studied the process of vegetative mycelium cultivation, but not fruit bodies, our results showed relatively high biological efficiency of mycelium cultivation on substrates: wheat seed cake – *P. djamor*, *L. shimeji*, *C. schevczenkovi*, *P. igniarius*, *S. litschaueri*; oat seed cake – *G. applanatum* and *G. lucidum*; soybean cake – *H. myxotricha*, *T. versicolor*, *M. esculenta*, *C. sinensis*, *C. militaris*, and *A. aegerita*; rape seed cake – *A. aurea*; camelina seed cake – *F. fomentarius*. These species are perspective for a biotechnological process since they are converted wastes (substrates) into mycelium (rich in protein, vitamins, micro- and macro elements), which could

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be used in different forms of products with therapeutic action: powder or tablets nutraceuticals or ingredients for functional foods.

## Conclusion

Bioconversion of processing industry wastes using Macromycetes is a perspective direction of biotechnology. Thus two problems are solved: effective use of bioresources by waste-free processing and production of nutraceuticals or ingredients for functional foods (rich in protein, vitamins, micro- and macro elements) on the basis of mushroom mycelia. The investigation has expanded the knowledge of the possibility of studied mushrooms to utilize oil-fat wastes. The preferred substrates for mushrooms growth and the ability of mushrooms to assimilate different substrates have been revealed. Alternative substrates (compared to glucose-peptone-yeast medium) were selected for all 29 studied species, from soybean cake – most suitable for the mycelial growth of 24 species to walnut cake – suitable only for 2 species. The preferred substrates can be successfully used to develop a protocol for cheap mycelial growth and also can be easily applied technically for the production of liquid spawn (for subsequent production of fruiting bodies) in a mushroom farm as mono substrates or as supplements. Production of various kinds of plant oils is an essential component of the global food industry and accordingly our results give reason to recommend investigated in present study substrates and others like by-products of oil-fat industry for further studies for mycelia production in culture elsewhere in the world.

The success of the mushroom cultivation on different substrates – a multifactorial process that depends on the presence in the substrate carbon, nitrogen, vitamins, fats and their ratio, pH of the substrate, and these factors are unique for each species of fungi. Subsequent researches must be devoted to the correction of substrate chemical composition: pH change, the addition of those or other biologically active substances, combinations of substrates; utilization of selected substrates in submerged culture.

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