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The use of spent brewery grains for *Pleurotus ostreatus* cultivation and enzyme production

Research Paper

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In the brewing industry, spent brewery grains (SBGs) are byproducts with a low economic value. The potential use of this leftover as a substrate ingredient for *Pleurotus ostreatus* fruiting body cultivation and enzyme production was evaluated. The best substrate mixture for *P. ostreatus* mycelium growth comprised 30% wheat bran (WB), 68% beech sawdust (BS) and 2% $CaCO_3$. On the substrates containing SBG, the fastest mycelium growth was observed on the substrate composed of 10% SBG, 20% WB, 68% BS and 2% $CaCO_3$. The highest biological efficiency (51%) of fruiting bodies was determined on the mixtures containing 20% WB, 10% SBG and 2% $CaCO_3$. The SBGs with the addition of WB were also shown to be suitable as a substrate for enzyme production. However, the supplementation levels designate which enzymes are produced and in what amounts.

Introduction

Spent brewery grains (SBGs) are byproducts of the brewing industry containing about 17% cellulose, 28% noncellulosic polysaccharides, mostly arabinoxylans and 28% lignin, and they have very little or no economically interesting further uses [1]. Their water content is approximately 80% when produced; therefore, they cannot be stored for a long period. In Slovenia they are mostly used as animal feed but must be dried to achieve long storage times and smaller transportation costs. Drying large amounts of SBG requires considerable energy and represents a great financial burden to the brewing industry. SBG are available in large quantities (80–100 tons daily at the brewery factory in Ljubljana, Slovenia) throughout the year. Owing to their high protein and fiber content (approximately 20 and 70% dry basis, respectively) they can also present an attractive adjunct in the human diet and biotechnological processes, such as cultivation of mushrooms and actinobacteria [1]. It has been proposed that SBG favor the growth of mushrooms not only due to their high protein content [2], but also to high moisture content and physical properties such as particle size, volume weight, specific density, porosity and water-holding capacity [3]. In a study by Wang *et al*. [3], nonpretreated SBG were successfully used as a basic substrate material for the cultivation of *Pleurotus ostreatus*, while SBG alone and mixed with maguey tequila bagasse were fruitfully used for *P. ostreatus* and *Pleurotus pulmonarius* cultivation [4]. In the laboratory phase, a higher growth rate and biomass production resulted with the SBG agar than with malt extract agar. In the field phase, the mixture of SBG and bagasse produced earlier fruiting bodies formation [4]. SBG were also reported to be used for the cultivation of *Agrocybe* sp., *Lentinus* sp. [5] and *Hericium* sp. [6]. *P. ostreatus* and other *Pleurotus* species are able to utilize ligninocellulosic waste materials for the production of industrially important lignolytic and cellulolytic enzymes. However, one must be aware that the substrate composition as well as the fungal strain used determines enzyme type and activity [7,8].

In this study, substrates containing various proportions of fresh SBG, wheat bran (WB), beech sawdust (BS) and $CaCO_3$ were used to determine *P. ostreatus* mycelium growth rate, enzyme activity and

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TABLE 1

Composition of the substrates used for *P. ostreatus* cultivation and their carbon/nitrogen ratio

Wheat bran (%)	Spent brewery grains (%)	Beech sawdust (%)	$CaCO_3$ (%)	Carbon/ nitrogen ratio
20	0	78	2	54
20	10	68	2	32
20	20	58	2	24
20	30	48	2	19
20	40	38	2	16
30	0	68	2	38
30	10	58	2	26
30	20	48	2	21
30	30	38	2	17
30	40	28	2	15

All substrates contained 2% $CaCO_3$.

the biological efficiency (BE) of acquired fruiting bodies to find the optimal substrate composition.

Materials and methods

C/N determination

Total and organic carbon (C) and total nitrogen (N) content in SBG, WB and BS were determined after dry combustion and incineration at 900°C in a carbon, nitrogen and sulphur analyzer (Vario MAX CNS, Elementar Corp., Germany). For each type of substrate mixture the carbon/nitrogen (C/N) ratio was calculated (Table 1).

Substrate preparation

SBG were obtained from the Pivovarna Union Brewery Company, Ljubljana, Slovenia and stored at −20°C. Fresh BS with 46% water content was obtained from a local saw mill (Ljubljana, Slovenia) and stored outside in a 20 m^3 pile covered with plastic covering. WB was bought at Mlin Katić mill, Leskovec pri Krškem, Slovenia. $CaCO_3$ used was Kalcivit, a commercial product of Unichem Company, Vrhnika, Slovenia.

Substrates with 65% water content containing different proportions of SBG, WB, BS and 2% $CaCO_3$ were prepared (Table 1). 400 g of substrate was filled into a 720 ml glass jar with the packing density of 62 g substrate per 100 ml volume. Jars were covered with punctured lids (9 mm hole closed with cotton plug), and sterilized for three hours at 121°C. Three replicates were conducted for each substrate.

The same substrates were filled into racing tubes for mycelium growth measurements. 40 g of substrate per racing tube (25 mm diameter, 175 mm length) was used and sealed with cotton plugs on both sides of the tube. Racing tubes were steam sterilized at 121°C for three hours. Four replicates were made for each substrate.

Experiments were performed at the mycological laboratory of Institute of Natural Sciences, Ljubljana, Slovenia.

Fungal inoculum preparation

P. ostreatus strain Pl.o4 was obtained from the fungal culture collection at the Department of Wood Science and Technology, Biotechnical Faculty, University of Ljubljana, Slovenia. Cultures were transferred to potato dextrose agar (PDA) and maintained in dark at 24°C. PDA overgrown with mycelium was cut with a selfdesigned cutter to acquire 9-mm diameter discs of mycelium culture, which were used for inoculation.

Substrate inoculation

After cooling, a 9-mm diameter disc was used for inoculation of each filled glass jar and centered to the leveled substrate surface. The inoculation procedure was the same for racing tubes. Inoculated substrates were incubated at 24 ± 1°C.

Mycelium growth measurements

Mycelium growth on substrates filled in racing tubes was measured and the average growth was calculated from the fastest and slowest mycelium growth front point and averaged for all four replicates.

Mushroom cultivation

When the mycelium reached the bottom of filled glass jars, they were transferred to the mushroom cultivation room with 16 ± 1°C, 90 ± 5% relative humidity, ten hours light cycle and approximately 1300 ppm CO_2. The mushrooms were harvested before caps started to invert. Acquired fruiting bodies were cleaned of excess substrate and their weight was determined. BE, defined as weight of fresh fruiting bodies divided by initial weight of dry substrate multiplied by 100, was calculated for each substrate. The experiment was conducted twice and the average BE for each substrate was determined.

Enzyme activity determination

After 33 days of growth in the racing tubes the extracellular enzymes were extracted from 5 g of overgrown substrate with 10 ml of the extraction buffer composed of 0.1 M sodium phosphate (pH 6.5) with 5% (v/v) Tween-80 and screened for manganese-dependent peroxidase (MnP), manganese-independent peroxidase (MiP), lignin peroxidase (Lip) and laccase (Lac) activity. Lac activities were determined as described in [9]. MnP, MiP and Lip activities were measured according to [10]. The enzyme activity experiments were performed at least in triplicates.

Results

Mycelium growth

Out of all tested substrates the highest mycelium growth was measured on that containing 30% WB, 68% BS and 2% $CaCO_3$. Out of the substrates containing SBGs the fastest mycelium growth was observed on the one containing 10% SBG, 20% WB, 68% BS and 2% $CaCO_3$. Visually examined mycelium growth density increased with the rising proportions of WB and SBG, but it was noticed that mycelium expansion fell with increasing WB and SBG proportions in the mixtures containing 20%, as well as 30% WB (Figs 1,2).

Biological efficiency of fruiting bodies

The highest BE of fruiting bodies (51%) was determined on the substrate containing 20% WB, 10% SBG and 2% $CaCO_3$. With the substrates containing 30% WB the highest BE was measured when the substrate composed of sawdust and $CaCO_3$ contained 20% SBG. On the 30% WB-containing substrates with 30% or 40% SBG

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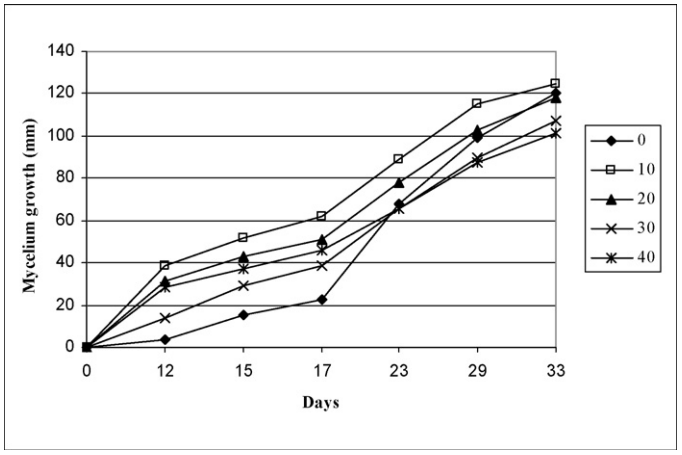

FIGURE 1

Growth of *P. ostreatus* mycelium on the substrates composed of 20% wheat bran and different proportions (%) of spent brewery grains.

and substrate-containing 20% WB with 40% SBG, primordial formation was noticed but fruiting bodies failed to develop. All of these substrates had a C/N ratio below 17 (Fig. 3).

Enzyme activities

When comparing enzyme activities, *P. ostreatus* exhibited a markedly higher peroxidase-type activity compared with Lac-type enzyme activity on all substrates. Overall, the tendency was that enzyme activities decreased as the addition of SBG increased or the C/N ratio fell. It was also noticeable that enzyme activities were higher on substrates where more WB was added. Lip activity was not found in any experiment. The enzyme activities as well as the C/N ratio are presented in Fig. 4 for the substrates with 20% WB addition and in Fig. 5 for the substrates containing 30% WB.

Discussion

Substrate with addition of SBGs could be an appropriate medium for *P. ostreatus* cultivation. Although in previous research BE is determined as dry fruiting bodies weight divided by initial weight of dry substrate multiplied by 100 [3], we were unable to compare

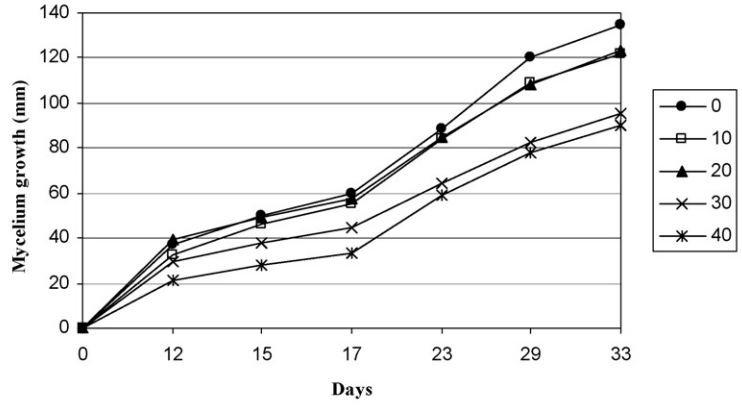

FIGURE 2

Growth of *P. ostreatus* mycelium on the substrates composed of 30% wheat bran and different proportions (%) of spent brewery grains.

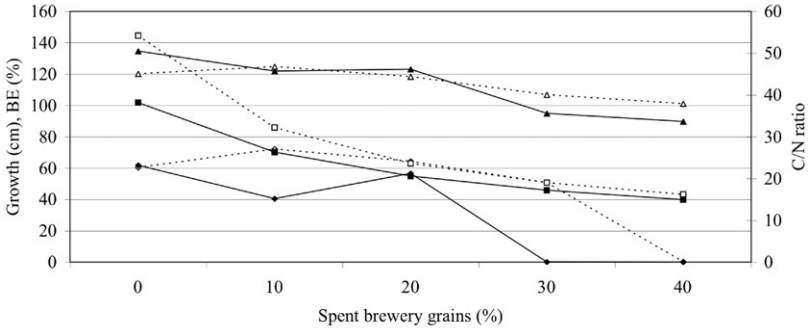

FIGURE 3

Biological efficiency – BE (◆ and ◇) of *P. ostreatus* fruiting bodies, mycelium growth (▲ and △) and carbon/nitrogen ratio – C/N (■ and □) on substrates with different proportions of spent brewery grains. Dashed lines and empty symbols represent substrates containing 20% wheat bran while full lines and symbols represent substrates containing 30% wheat bran.

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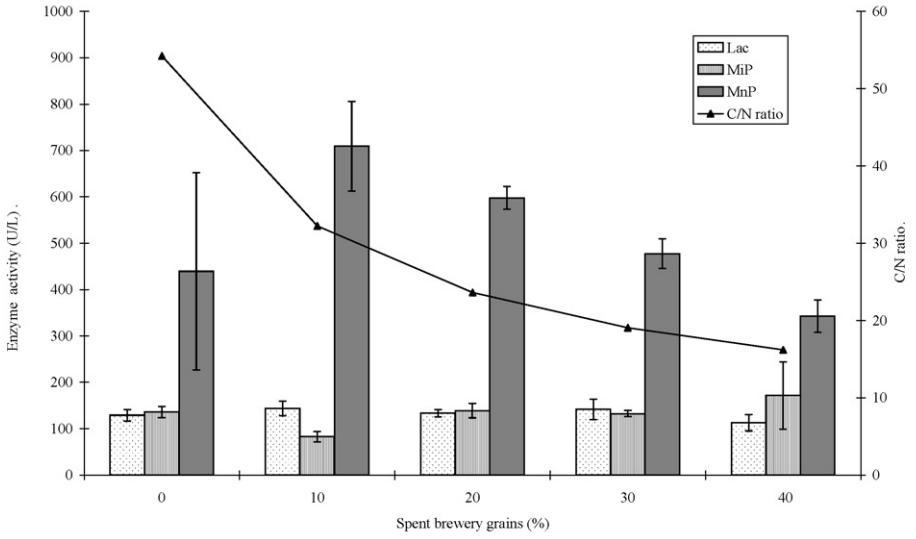

FIGURE 4

Enzyme activities ± standard errors in the substrates containing 20% wheat bran with different proportions of spent brewery grains. See text for abbreviation definitions.

precisely the results without knowing the water content in mushrooms which these authors cultivated. The results of our experiments showing the maximum BE of fruiting bodies at 20% WB, 10% SBG and 2% $CaCO_3$ are in contrast with the results of Wang *et al*. [3] who obtained highest yields on substrates composed of 45% WB and 55% SBG. In our work, no fruiting bodies were obtained on substrates with even lower SBG and WB proportions were used. Lara *et al*. [4] reported the lowest BE for *P. ostreatus* and *P. pulmonarius* (26–29%) on substrate composed equal proportions of SBG and maguey tequila bagasse. At lower proportions of SBG they reported a higher BE of fruiting bodies. The pronounced differences in results obtained by ourselves and [4] compared to those reported by [3] could be explained by different beer manufacturing procedures influencing the chemical composition of SBG or by differences in fungal strains used.

Pleurotus ostreatus exhibited a higher peroxidase-type activity compared with Lac-type enzyme activity with almost all substrates tested here. With substrates containing 20% WB, the Lac and MiP activities were present in all SBG formulations. However, MnP activities tend to decrease when the SBG addition was increased.

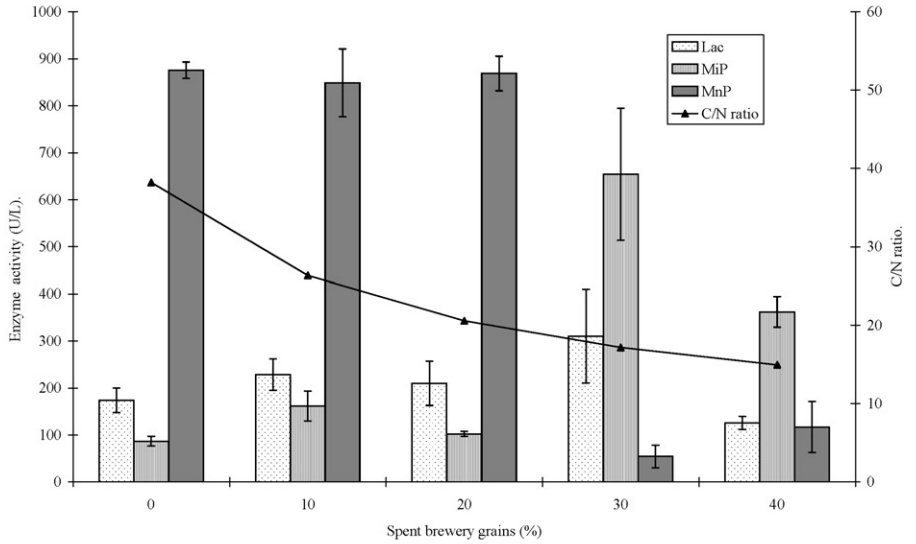

FIGURE 5

Enzyme activities ± standard errors in the substrates containing 30% wheat bran with different proportions of spent brewery grains. See text for abbreviation definitions.

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The substrates with 30% WB have a tendency to yield higher enzyme activities. MiP and Lac activities predominate when the addition of SBG is higher than 30%, while MnP activity diminishes almost completely. This coincides well with the fact that the carbon and nitrogen source influences enzyme production [8]. In our case, WB tends to be a better substrate than SBG in regard to enzyme activity; however, further experiments are needed to evaluate enzyme expression over time. Our method for Lip determination is based on veratryl aldehyde detection produced by veratryl alcohol oxidase. The fact that we did not detect any Lip activity is in agreement with [11], even though Lips in *P. ostreatus* have been discovered and characterized [12,13].

Conclusions

According to these results, substrates with the addition of SPBs could be an appropriate substrate for *Pleurotus ostreatus* cultivation. It can be concluded that the substrate-containing 20% WB, 10% SBG and 2% $CaCO_3$ with 65% water content, resulting in 51% BE, is the most appropriate one for cultivating strain Pl.o4.

The substrate-containing 10% SBG, 20% WB, 68% BS and 2% $CaCO_3$ overgrown with *P. ostreatus* mycelium could be used for other applications, for example as animal feed or as mycoremediation media, because of its excellent mycelium growth promoting properties. In further experiments different strains should be evaluated to determine the one most appropriate for cultivation on the substrates containing SBG. Adaptation of different fungal strains for the growth on the SBG-containing substrates should be done as well to potentially increase BE and the quality of fruiting bodies.

This work shows that formulations composed of SBG and WB can also be successfully used for enzyme production. Nevertheless, we noted that a higher WB addition tends to yield higher enzyme activities, for different enzyme types at different SBG supplementation rates.

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