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# Process Parameters Affecting the Synthesis of Natural Flavors by Shiitake (*Lentinula edodes*) during the Production of a Non-Alcoholic Beverage

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**Abstract:** A novel alcohol-free beverage with a fruity, slightly sour, sweetish, fresh, and plum-like flavor was produced by incorporating the edible mushroom shiitake (*Lentinula edodes*) into the fermentation process. Shiitake pellets were used as a biocatalyst to promote the synthesis of the fruity esters methyl 2-methylbutanoate and 2-phenylethanol from amino acids and an organic acid present in the wort. We investigated the impact of two critical process parameters (volumetric power input and inoculum concentration) on the morphology of, and flavor production by, the shiitake pellets in a 1 L stirred bioreactor. Increasing the volumetric power input and biomass concentration influenced the morphology of the pellets and promoted the production of the most important flavor compound methyl 2-methylbutanoate in the beverage. Furthermore the worty off-flavor methional was degraded during the cultivation in stirred bioreactor by shiitake pellets. These findings provide useful information to facilitate the scale-up of the biotransformation and fermentation process in bioreactors.

**Keywords:** shiitake; volumetric power input; inoculum concentration; natural flavor; stirred bioreactor; non-alcoholic beverage; methyl 2-methylbutanoate

## 1. Introduction

The submerged cultivation of basidiomycetes, such as the shiitake mushroom (*Lentinula edodes*), allows the production of valuable secondary metabolites, including pharmaceuticals, enzymes, and natural flavor compounds [1–3]. The increasing demand for alcohol-free beverages with natural flavors has forced breweries to look for new beverages to broaden their product portfolios. Preliminary research has shown that it is possible to produce non-alcoholic beverages based on malt by incorporating basidiomycetes that produce diverse natural flavors during the fermentation [4,5]. However, the complex morphology of these organisms makes the cultivation process challenging, because the submerged fungi can grow as viscous mycelia, or as dense pellets depending on process

parameters. There is little information concerning the impact of these parameters on the fermentation of mushrooms in submerged cultures for flavor production.

The volumetric power input is one of the critical process parameters in the submerged cultivation of shear-sensitive organisms such as basidiomycetes. This process parameter can be regulated by agitation and aeration intensity and by stirrer geometry. Agitation is required for mixing and to achieve adequate oxygen transfer, but excessive agitation subjects the mycelia to mechanical stress and this can influence the production of target metabolites. It is, therefore, important to achieve the optimal balance between adequate oxygen supply and minimal mechanical stress [6,7]. Therefore, the used impeller type is another aspect, which should be considered in the cultivation of shear-sensitive basidiomycetes. A pitched blade impeller is used mainly for cell culture applications and achieves higher oxygen supply and less shear forces than a Rushton impeller [8]. The amount of inoculum is another critical process parameter that can influence the efficiency of fermentation. Many researchers have shown that the inoculum concentration affects the morphology, mycelia biomass yields, and metabolite production by basidiomycetes. It is important to determine the optimum biomass concentration that achieves effective biotransformation for the production of natural flavor compounds [6,7].

Odor-active compounds such as methyl 2-methylbutanoate and 2-phenylethanol generated by shiitake pellets contribute to the overall flavor profile of beverages. In addition, the flavor compounds of wort (β-damascenone and methional) are degraded during the biotransformation [5]. Methional is a typical worty off-flavor and has an odor impression of "potato-like" [9]. In contrast, β-damascenone has a fruity odor impression and smells like pear [4,5]. We used the ability of shiitake to produce natural flavors in order to develop a fermentation process for novel beverages. Due to the loss of volatile flavor compounds through the aeration of medium with air, the oxygen supply of the basidiomycete is achieved with the regulation of agitation rates. In addition to create less mechanical forces in comparison to a cultivation with a Rushton impeller, a 3 × 45° pitched blade impeller ($d$ = 45 mm, downward pumping, Applikon, Delft, The Netherlands) is used for the cultivation of shiitake. We investigated the impact of inoculum size, and volumetric power input under different agitation rates, on the morphology of shiitake pellets in the wort, and the quantities of key flavor compounds produced during cultivation. We compared different volumetric power inputs and maximal shear rates in a stirred tank bioreactor to provide information that will facilitate the scale-up of the fermentation process.

## 2. Materials and Methods

### *2.1. Microorganism and Media*

Shiitake (*Lentinula edodes*) was kindly donated by the Institute of Food Chemistry and Food Biotechnology, Justus-Liebig-University, Giessen, Germany. The fungus was subcultured every month on standard nutritional medium with agar (see below) in Petri dishes and was incubated at 24 °C until half of the agar was overgrown with mycelia (approximately 14 days). The plates were stored at 4 °C.

The standard nutritional medium (SNLH) comprised 30 g/L glucose, 4.5 g/L L-asparagine, 1.5 g/L $KH_2PO_4$, 1.0 g/L $MgSO_4 \cdot 7H_2O$, 3.0 g/L yeast extract and 1 mL trace element solution (plus 15 g/L agar-agar for the preparation of agar medium) in distilled water. The trace element solution comprised 5 mg/L $CuSO_4 \cdot 5H_2O$, 80 mg/L $FeCl_3 \cdot 6H_2O$, 90 mg/L $MnSO_4 \cdot H_2O$, and 400 mg/L Titriplex III (EDTA). The medium was adjusted to pH 6.0 with 1 M NaOH and autoclaved at 121 °C for 20 min.

### *2.2. Wort Production*

Wort (Kölsch type, 12–13 °Brix) was produced at the University of Applied Sciences (Giessen, Germany) using a KB50 pilot-scale brewing device (Alfred Gruber GmbH, Eugendorf, Austria). A mixture of Pilsner (80% $w/w$), Munich (13% $w/w$) ,and wheat (7% $w/w$) malt types were used to produce the wort, which was passed through a 3-µm filter and a 0.25-µm sterile filter, filled into

aluminum bottles and tyndallized twice at 80 °C for 1 h before storing at −20 °C. Before fermentation, the wort was thawed and pasteurized at 80 °C for 1 h and then cooled to room temperature.

### *2.3. Pre-Culture Cultivation*

The basidiomycete *Lentinula edodes* was initially grown on SNLH-Agar (see Section 2.1) in Petri dishes for 14 days and then stored in refrigerator at 4 °C. An agar plug with an edge length of about 1 cm with growing mycelia from the periphery of a Petri dish was removed with a sterilized spatula and transferred to a 500 mL shake flask filled with 200 mL standard nutritional medium. The culture was homogenized at 9500 rpm for 15 s using an Ultra Turrax T25 homogenizer (Janke and Kunkel, IKA Labortechnik, Staufen, Germany). The inoculated medium was then incubated on a rotary shaker (InforsHT, Multitron Standard, Bottmingen; Switzerland) at 24 °C and 150 rpm for 12 days in the dark.

### *2.4. Biotransformation*

Fermentations were carried out in a 1 L stirred glass bioreactor with a 0.8 L working volume (Applikon, Delft, The Netherlands) and a 3 × 45° pitched blade impeller ($d$ = 45 mm, downward pumping, Applikon, Delft, The Netherlands). The standard fermentation was set up with the following parameter: 800 mL wort, 35 mg/L pre-culture, 150 rpm and 24 °C. Parallel fermentations with different agitation rates (100 and 250 rpm) and inoculum sizes (21 and 68 mg/L) were carried out for 72 h to determine the effect of these process parameters on the production of natural flavors. The agitation rate, pH, and temperature were controlled using BioXpert (Applikon, Delft, The Netherlands). Dissolved oxygen in the fermentation process was measured in-line using an oxygen probe (Presens GmbH, Regensburg, Germany). Samples were taken every 12 or 24 h for further analysis. Each sample for flavor analysis was analyzed in duplicate. Samples were also tested for sterility on lysogeny broth agar medium (5 g/L yeast extract, 10 g/L tryptone, 10 g/L NaCl, and 15 g/L agar).

The volumetric power input was calculated using the following equations:

$$P = \rho \times \left(\frac{N}{60}\right)^3 \times D^5 \times Np \tag{1}$$

$$Volumetric\ power\ input\ \left(\frac{W}{m^3}\right) = \frac{P}{V} \tag{2}$$

where $P$ = power (W), $\rho$ = the density of the medium (kg/m$^3$), $N$ = stirrer speed (s$^{-1}$), $D$ = impeller diameter (m), and $Np$ = the power number of the impeller type (1.5 for the Applikon pitched blade impeller).

The maximal shear rate $(\gamma_{max})$ in the stirred bioreactor was calculated to characterize the mechanical stress according to the following equation [10]:

$$\gamma_{max} = 3.3N^{1.5}D\left(\frac{\rho_L}{\mu_L}\right)^{0.5} \tag{3}$$

where $N$ = agitation speed (s$^{-1}$), $D$ = impeller diameter (m), $\rho_L$= the density of the medium (kg/m$^3$), and $\mu_L$= viscosity of the fluid (Pa·s).

### *2.5. Measurement of Cell Dry Weight*

The dry weight of mycelial biomass was determined after recovery with a sieve (Rotilabo®, mesh size: 0.5 mm, Carl Roth, Karlsruhe, Germany), washing with distilled water, and drying at 80 °C for 24 h in aluminum cups.

### 2.6. Characterization of Fungal Morphology

Mycelial pellets gathered from the pre-culture (inoculum) and fermentation were separated using a round sieve (Rotilabo®, mesh size: 0.5 mm, Carl Roth, Karlsruhe, Germany), suspended in a water-filled centrifuge tube and stained with 3 mL methylene blue solution (0.3 g methylene blue (Merck, Darmstadt, Germany), 30 mL 95% ethanol, and 100 mL water) for 5 min. After staining, the pellets were separated using a sieve (Rotilabo®, mesh size: 0.5 mm, Carl Roth, Karlsruhe, Germany) and washed in distilled water. The stained pellets (approximately 100–200 pellets per Petri dish) were placed in a Petri dish filled with water and scanned (CanoScan 9000F, Canon Deutschland, Krefeld, Germany). The pellets were also carefully separated from each other with a help of tweezers to obtain a good image for the analysis. The images were then analyzed using ImageJ v1.47. The morphology of the pellets was characterized by their equivalent diameter and the D10, D50 and D90 value of the particle size distribution. The equivalent diameter ($d_{eq}$) was calculated according to the following equation:

$$d_{eq} = \sqrt{\frac{4A}{\pi}} \tag{4}$$

where $A$ = projected area ($cm^2$).

The morphological shape parameter roundness was used to characterize the particles. Particles with a roundness smaller than 0.5 were not considered as pellets, but rather as irregular particles such as mycelium filaments and clumps. The shape parameter roundness was calculated by using ImageJ v1.47 according to the following equation:

$$Roundness = 4 \times \frac{[Area]}{\pi \times [Major\ axis]^2} \tag{5}$$

### 2.7. Flavor Analysis

Ten milliliters of the wort or fermented wort was transferred to a 20 mL headspace vial. Then 100 µL thymol (TCI, Eschborn, Germany) or ethyl butyrate (Acros Organics) was added as an internal standard and the volatile components were extracted by headspace solid phase micro-extraction (HS-SPME) using a carboxen/polydimethylsiloxane (CAR/PDMS) fiber (Supelco, Steinheim, Germany). The extract was then analyzed by gas chromatography tandem mass spectrometry/olfactometry (GC-MS/MS-O) as previously described [5]. The key odor-active compounds were detected by olfactometry, and changes in the levels of methyl 2-methylbutanoate, 2-phenylethanol, β-damascenone and methional during fermentation were expressed as relative peak areas. The odor activity values (OAVs) for the main flavor compounds were also calculated as previously described [5].

### 2.8. Measurement of the Oxygen Gradient

The upper part of a cuvette was removed and fixed to a Petri dish, and a hole was introduced in the middle of the cuvette using a hot needle. The Petri dish was then filled with water. Different size of pellets from pre-culture was carefully placed onto the hole to provide stability during measurement (see Figure 1). Before measurement, the oxygen sensor was prepared by two-point calibration in oxygen-free and air-saturated water. The oxygen profile in the shiitake pellets was measured using a needle-type oxygen sensor (OXR50, tip diameter 50 µm; Pyro Science, Aachen, Germany) which was driven into the pellet with a depth of 100 µm with an automatic manipulator. Measurements were recorded using Profix software (Pyro Science GmbH, Aachen, Germany).

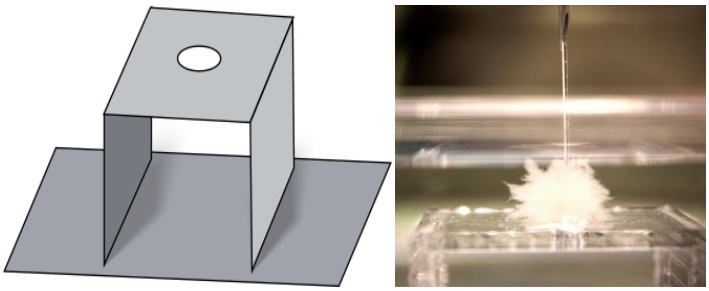

**Figure 1.** Holder for the measurement of the oxygen gradient in shiitake pellets.

### *2.9. Statistical Analysis*

Analysis of variance (one-way ANOVA) was used to test the significance of the factors volumetric power input and inoculum concentration on the flavor production at 72 h. A significance level of 5% was used to run the analysis of variance. Error bars are standard errors of the mean for two measurements with two samples.

## 3. Results and Discussion

### *3.1. Microprofiling*

The oxygen gradient in shiitake pellets was measured using a microelectrode oxygen sensor and it was found that the fungal cells in the center of the pellets were subjected to oxygen starvation (Figure 2). Oxygen penetrated to a depth of approximately 0.10–0.15 cm at 84%–100% air saturation. Therefore, oxygen can reach deeper into pellets, which have a diameter less than 0.20 cm. The D50 value of the pre-culture used in the fermentations under different agitation rates and inoculum concentration was 0.22 and 0.10 cm, respectively. According to the microprofiling measurement it is obviously clear that the cells in the center of pellets can become oxygen-limited during the fermentation process. A negative effect on the flavor production was not observed during the fermentation of the beverage.

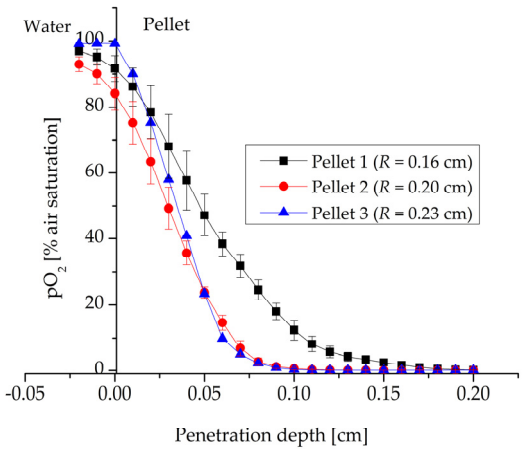


**Figure 2.** Measurement of oxygen profile in shiitake pellets with different particle sizes (*R* = Radius of the pellet).

Oxygen can penetrate fully into small pellets and all the cells remain active, whereas in larger pellets the cells in the center are starved of oxygen. The reported critical pellet radius for filamentous microorganisms in the literature is about 200–400 µm [11–13]. It has been shown that the particle size plays an important role in the production of metabolites. For example, microprofiling in *Phanerochaete chrysosporium* also revealed that the oxygen concentration in the pellet declined with

penetration depth. The oxygen limitation in the *P. chrysosporium* pellets reduced the production of lignin-degrading enzymes [14]. For the production of the ligninolytic enzyme manganese peroxidase (MnP) by *Pleurotus ostreatus*, mycelial pellets of 0.1–0.2 cm in diameter were necessary. However, the cultivation in shake flask with larger pellets (0.5–1.0 cm) resulted in a lower MnP production by *P. ostreatus* [15]. Leisola and Fiechter also reported that *P. chrysosporium* pellets with an average diameter of 0.1–0.2 cm produced the maximal lignin peroxidase activity (LiP) [16]. On the other hand there are investigations with basidiomycetes which state that the oxygen limitation in pellets can promote the secondary metabolite production [17–19].

### *3.2. Effect of Volumetric Power Input and Maximum Shear Rate*

#### 3.2.1. Morphology and Biomass Growth

There is a well-known relationship between fungal morphology and the production of target metabolites [6,19]. It was found that increasing the volumetric power input and maximum shear rate influenced the morphology of the shiitake pellets. Pellet growth and erosion of pellets were observed during the fermentation process, which led to the formation of a heterogeneous culture. As seen in Figures 3 and 4 the pellet hairs were shaved-off by mechanical forces in the bioreactor system. This led to the formation of mycelium filaments and clumps. With increasing volumetric power input, the percentage of mycelium filaments and clumps in the culture were also increased. Especially the fermentation with the highest volumetric power input of 26.2 W/m$^3$ led to the formation of smaller pellets. On the other hand the increase of volumetric power input up to a value of 5.7 W/m$^3$ promoted the enlargement of the pellets, which may reflect the enhanced oxygen transfer (Figure 3).

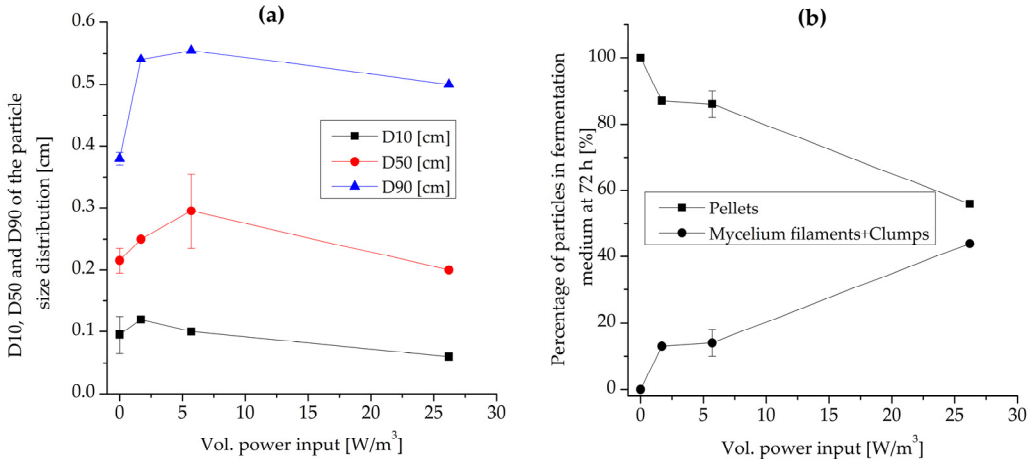


**Figure 3.** D10, D50, and D90 value of particle size distribution of pre-culture and fermentations at different volumetric power inputs at 72 h (**a**); Percentage of pellets, mycelium filaments and clumps of pre-culture and fermentations at different volumetric power inputs at 72 h (**b**), the points at 0 W/m$^3$ volumetric power input indicate the parameter values of the pre-culture.

Due to the heterogeneous distribution of pellets, the D10, D50 (median), and D90 value of the particle size distribution was used to characterize the particle size distribution of the fermentations. With increasing volumetric power input, the D50 and D90 values increased first, which indicates the growth of pellets. However at the highest volumetric power input of 26.2 W/m$^3$, a decrease of the values D50 and D90 was observed. Furthermore it was found that the D10 value decreased with increasing volumetric power input (Figure 3). This result shows that the pellets were damaged by the mechanical forces created in stirred tank bioreactor. It is generally observed that the pellet size decreases with increasing agitation intensity, which is related to mechanical shear forces [20,21]. It was

reported that higher shear forces created in stirred tank bioreactor can destroy the hairiness of the fungal pellets [22].

The increase of volumetric power input through agitation rate was used in the fermentation process to increase the oxygen supply of the pellets and to avoid the loss of volatile flavor compounds by stripping via exhaust air. The increase in volumetric power input within the range we analyzed promoted the biomass productivity and the production of biomass, which may reflect better oxygen transfer to the pellets, mycelium filaments and clumps (Table 1). The biomass concentration of *Pleurotus ostreatus* was also improved with increasing agitation and aeration rates in a stirred tank bioreactor [23]. On the other hand it was found that the pellet size of *P. ostreatus* was decreased when the agitation rate was increased from 200 rpm to a value of 400 rpm in a stirred tank bioreactor. It was speculated that the shear forces were sufficient to decrease the pellet stability which led to the restriction of cell growth [24].

**Table 1.** Kinetic parameters of *L. edodes* fermentation at different agitation rates in a 1 L stirred tank bioreactor. Data were part of a design of experiment (DoE) with four repetitions of the center point experiment. The corresponding experimental standard deviation was below 28%.

| Agitation Rate (rpm) | Max. Shear Rate (1/s) | P/V [1] ($W/m^3$) | $X_{start}$ (mg/L) | $X_{final}$ [2] (mg/L) | Biomass Productivity (mg/L h) |
|---|---|---|---|---|---|
| 100 | 247 | 1.7 | 35 | 112 | 1.08 |
| 150 | 453 | 5.7 | 35 | 167 | 1.84 |
| 250 | 974 | 26.2 | 35 | 231 | 2.72 |

[1] P/V: Volumetric power input, [2] $X_{final}$: biomass dry weight at 72 h.

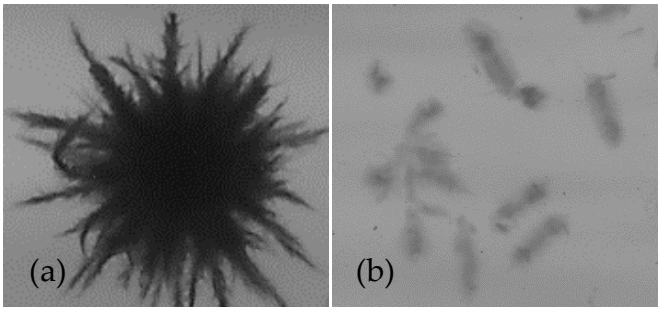


**Figure 4.** Shiitake pellet stained with methylene blue solution (**a**); hyphel fragments observed in fermentations (**b**).

### 3.2.2. Flavor Production

Odor-active flavor compounds have been identified in wort and fermented wort with shiitake [4]. However, in this study key odor-active compounds in wort and fermented wort were regarded. Flavor compounds with an odor activity value (OAV) > 1 such as methyl 2-methylbutanoate (OAV = 30), 2-phenylethanol (OAV = 1.3), β-damascenone (OAV = 1.1), and methional (OAV = 10), were chosen for the characterization of the beverage [5]. It was found that methyl 2-methylbutanoate and 2-phenylethanol were produced during the fermentation whereas methional was degraded (Figure 5). Almost 80% of the methional was degraded during the fermentation of the beverage (Figure 5d). However, ß-damascenone was not degraded significantly at all of the fermentations with different volumetric power inputs (Figure 5c) ($p$-value > 0.05). Zhang et al. showed that both of the flavor compounds were degraded by *L. edodes* pellets in shake flask fermentation [5].

Shiitake pellets utilize the precursor 2-methylbutanoic acid and L-isoleucine to produce the fruity ester methyl 2-methylbutanoate. Methyl 2-methylbutanoate has the highest odor activity value with

a fruity impression and is therefore regarded as a key quality control parameter [5]. The increase in volumetric power input up to a value of 5.7 W/m$^3$, which was regulated with the agitation rate, enhanced the production of this fruity aroma significantly ($p$-value: 0.04), perhaps reflecting the more efficient oxygen supply to the pellets, promoting biomass, and pellet growth (Figure 5a). A further increase of volumetric power input did not enhance the production of methyl 2-methylbutanoate significantly ($p$-value: 0.08). The higher shear force might restrict the further production of fruity ester, which was also reflected in the pellet morphology. A similar finding was observed in the cultivation of shiitake mycelia for eritadenine production. Increasing the agitation rate from 50 to 250 rpm in a 1 L bioreactor also promoted biomass growth and eritadenine production by shiitake. The enhanced eritadenine production was related to the formation of dispersed mycelial filaments. However, the type of mixer used in the cultivation of shiitake was not mentioned which can influence the morphology [25]. It was shown that agitation rate affects the morphology of shiitake pellets in shake flasks. The pellet size was increased with decreasing agitation rates when vegetative homogenized mycelium was used as inoculum. Furthermore it was stated that the pellet formation was faster at the lower agitation rates of 50 and 100 rpm. Although the biomass growth was promoted with increasing agitation rate, the enhanced production of ergothioneine was related to the compact and larger pellets produced at the lower agitation rate of 50 rpm in shake flasks. It was assumed that the oxygen limitation in larger pellets favored the production of ergothioneine [17].

Basidiomycetes can synthesize 2-phenylethanol de novo or by the biotransformation of precursors such as asparagine or L-phenylalanine [26]. In shiitake, 2-phenylethanol can be produced from L-phenylalanine in the wort [5]. Figure 5b shows the production of 2-phenylethanol, which has a rose-like odor. The result shows that the production of 2-phenylethanol was not significantly influenced by increasing volumetric power input ($p$-value: 0.86).

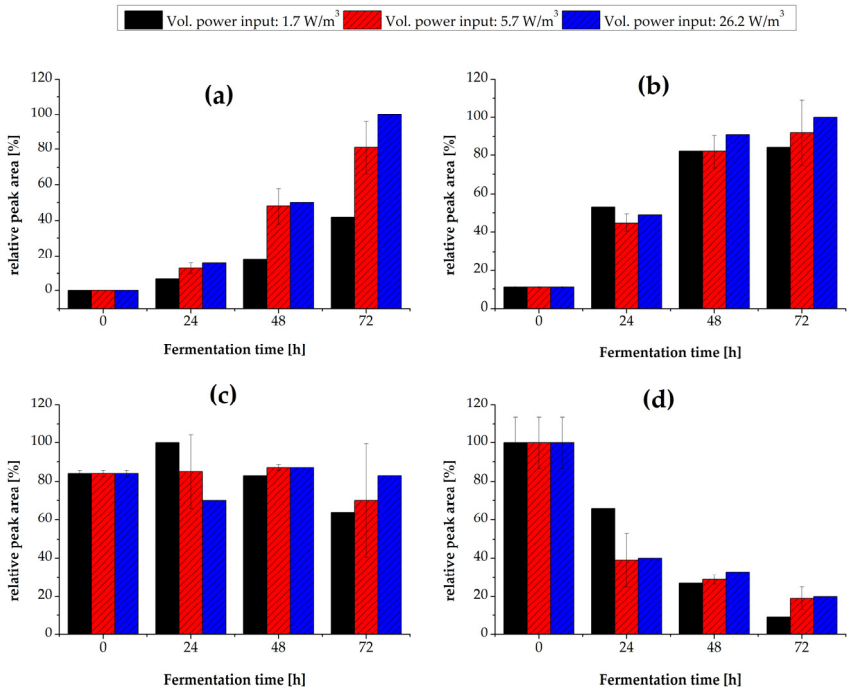


**Figure 5.** Production of (**a**) methyl-2-methylbutanoate, (**b**) 2-phenylethanol, and degradation of (**c**) β-damascenone, (**d**) methional by shiitake under different volumetric power inputs. Vol.: Volumetric. Error bars are standard errors of the mean for two samples.

We observed no difference in the degradation of β-damascenone and methional among the different volumetric power inputs ($p$-value > 0.05), (Figure 5c,d). The degradation of methional

improves the taste attributes of the beverage because methional is responsible for the strong potato-like taste. Therefore, the fermented beverage has a milder and more pleasant taste than the wort. Shiitake can secrete many different catabolic enzymes [2,27], one of which may degrade methional. The milder taste of the fermented beverage was confirmed by a tasting. Fermentation of the wort by the shiitake mushroom produced a fruity, slightly sour, sweetish, fresh, and plum-like taste.

The agitation rate has also been shown to boost the synthesis of fungal metabolites due to the efficient mixing and improved oxygen transfer, e.g., a higher agitation rate (400 rpm) promoted mycelial growth and EPS production by *Paecilomyces japonica* in a 5-L stirred tank bioreactor [28]. The production of EPS by *Tricholoma matsutake* was also enhanced at a higher agitation rate although cell growth was less efficient [29]. Furthermore, exopolysaccharide (EPS) production by *Pycnoporus sanguines* and *Grifola frondosa* was more efficient in a stirred tank bioreactor than an air-lift bioreactor [22,30]. The degradation of cyanide by *Ganoderma lucidum*, *Polyporus arcularius* and *Schizophyllum commune* in shake flasks was enhanced by increasing the agitation rate to 100 rpm, whereas degradation remained stable at higher rates [31]. On the other hand mycelial growth and EPS production by *G. frondosa* was more efficient at the lowest agitation rate in shake flask cultures [32].

In contrast to the studies listed above, there are many others showing that mechanical stress damages cells and inhibits product formation. For example, *Ganoderma lucidum* is a shear-sensitive basidiomycet and 300 rpm was reported as a critical impeller speed in a 10 L stirred tank bioreactor mixed by three Rushton turbines [33]. It was also shown that the cell growth and the intracellular polysaccharide production of this basidiomycet in a stirred tank bioreactor were inhibited by higher shear stress [34]. The morphology of *Cordyceps militaris* was significantly affected by agitation intensity, and mild agitation (150 rpm) promoted EPS production in a 5 L stirred tank bioreactor. Smaller and less compact pellets were formed at the highest agitation rate of 300 rpm [20]. Mild agitation rates are preferable for laccase production by *Dichomitus squalens* and *Pleurotus ostreatus* in stirred tank bioreactors [21,23]. The laccase production by *Panus tigrinus* on olive mill wastewater-based media was influenced negatively when the stirrer speed was increased [35]. The production of ligninolytic enzymes by *D. squalens* in a 3 L stirred tank bioreactor enhanced when the agitation intensity increased from 175 rpm (P/V: 20 W/m$^3$) to 250 rpm (P/V: 80 W/m$^3$). However, a further increase to an agitation intensity of 350 rpm (P/V: 180 W/m$^3$) led to a decrease in enzyme production [21]. Therefore, bioreactor systems with lower shear environment are sometimes preferable for the fungal metabolites. The production of a manganese-dependent peroxidase (MnP) by *D. squalens* was promoted when the fungus was cultivated in a bubble column reactor, which has lower shear effects than a stirred tank bioreactor [21]. Phenolic compounds in olive mill wastewater were also degraded more rapidly by *Panus tigrinus* in a bubble column bioreactor reflecting the production of larger amounts of the enzymes laccase and Mn (II) peroxidase [36]. The production of biomass and EPS by *Tremella fuciformis* was also enhanced in an air-lift bioreactor compared to a stirred tank bioreactor [37]. An alternative disposable bag bioreactor system for the submerged cultivation of shear-sensitive basidiomycetes was shown to promote mycelial growth and enzyme activity [38].

### *3.3. Effect of Inoculum Concentration*

#### 3.3.1. Morphology and Biomass Growth

To increase the number of pellets and cell density in the medium, biotransformation was carried out in a glass bioreactor with increasing amounts of inoculum. Oxygen consumption, which is a good indicator of cell activity, increased as the inoculum concentration increased (see Figure 6b). The values of D10, D50, and D90 show that there is an increase in the pellet diameter compared to pre-culture, which indicates pellet growth. The D50 value increased from 0.10 to 0.24 cm in both biotransformation reactions (21 mg/L and 35 mg/L inoculum) and decreased to 0.18 cm in the fermentation with the highest initial inoculum concentration (Figure 6a). Compared to the fermentations with an inoculum concentration of 21 mg/L and 35 mg/L smaller pellets were obtained in the fermentation (at 72 h) with

an inoculum concentration of 68 mg/L (Figure 6a). When the inoculum concentration was increased to 68 mg/L, the dissolved oxygen concentration in the medium decreased to fewer than 5% air saturation after 12 h, which also reduced the biomass productivity and the final biomass concentration (Figure 6b and Table 2).

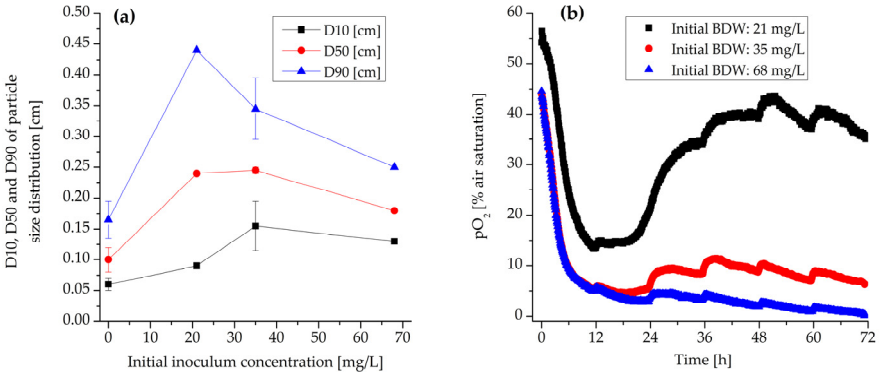


**Figure 6.** (**a**) D10, D50, and D90 value of particle size distribution of pre-culture and fermentations at different initial inoculum concentration at 72 h (volumetric power input: 1.7 W/m$^3$, temperature: 24 °C, the points at 0 W/m$^3$ volumetric power inputs indicates the pre-culture); (**b**) oxygen profile during the fermentation at the initial inoculum concentration of 21, 35, and 68 mg/L.

**Table 2.** Kinetic parameters of *L. edodes* fermentation at different initial inoculum concentrations in a 1 L stirred tank bioreactor. Data were part of a DoE with four repetitions of the center point experiment. The corresponding experimental standard deviation was below 14%.

| Inoculum Volume (mL) | Initial BDW [1] (mg/L) | $X_{final}$ [2] (mg/L) | Biomass Productivity (mg/L h) |
|---|---|---|---|
| 40 | 21 | 175 | 2.14 |
| 80 | 35 | 225 | 2.63 |
| 160 | 68 | 205 | 1.90 |

[1] BDW: biomass dry weight, [2] $X_{final}$: biomass dry weight at 72 h.

### 3.3.2. Flavor Production

The increase in inoculum concentration accelerated and enhanced the production of the fruity flavor compound methyl 2-methylbutanoate significantly (*p*-value: 0.02) (Figure 7a). This reflected the increasing number of active cells which contributed to the biotransformation. Although the highest inoculum concentration resulted in the lowest biomass productivity (Table 2), it also resulted in the highest concentration of the fruity ester perhaps reflecting the combined effect of the smaller pellet size and the larger number of pellets. At the end of the fermentation with the highest inoculum concentration 4331 pellets were counted, whereas only 705 and 1570 pellets were counted for the fermentations with an initial biomass concentration of 21 and 35 mg/L, respectively. The results showed that the shiitake pellets can even produce the fruity ester at a low dissolved oxygen concentration (<5% air saturation) after 12 h (Figures 6b and 7a). Therefore, it is crucial to elucidate the biochemical pathway of the production of methyl 2-methylbutanoate by *Lentinula edodes* to understand this observed effect. With an isotopic labelling experiment it was confirmed that methyl 2-methylbutanoate can be produced by biotransformation of 2-methylbutanoic acid and L-isoleucine by *L. edodes*, and 2-methylbutanoic acid was assumed to be an intermediate in the biochemical pathway of methyl 2-methylbutanoate [5]. A similar pathway in the production of this fruity ester was observed in strawberry and apple, when they were fed with the amino acid L-isoleucine [39,40] It was also reported that methyl branched fruity esters in apple can be produced from the precursor ethyl tiglate [41],

but there is no information about the effect of oxygen concentration on the fruity flavor production by *L. edodes* in the literature. It can be speculated that the metabolism of *L. edodes* may be shifted more to metabolite production.

The accumulation of 2-phenylethanol was also influenced significantly by the increase in inoculum concentration ($p$-value: 0.001), but did not increase further in the biotransformation inoculated with 68 mg/L shiitake pellets (Figure 7b). The degradation of methional and β-damascenone was not influenced significantly by the inoculum concentration ($p$-value > 0.05). However, the results showed that methional and ß-damascenone were degraded significantly during the fermentation ($p$-value < 0.05) (Figure 7c,d). The same findings were observed during the fermentation of this beverage by shiitake pellets in shake flasks [5].

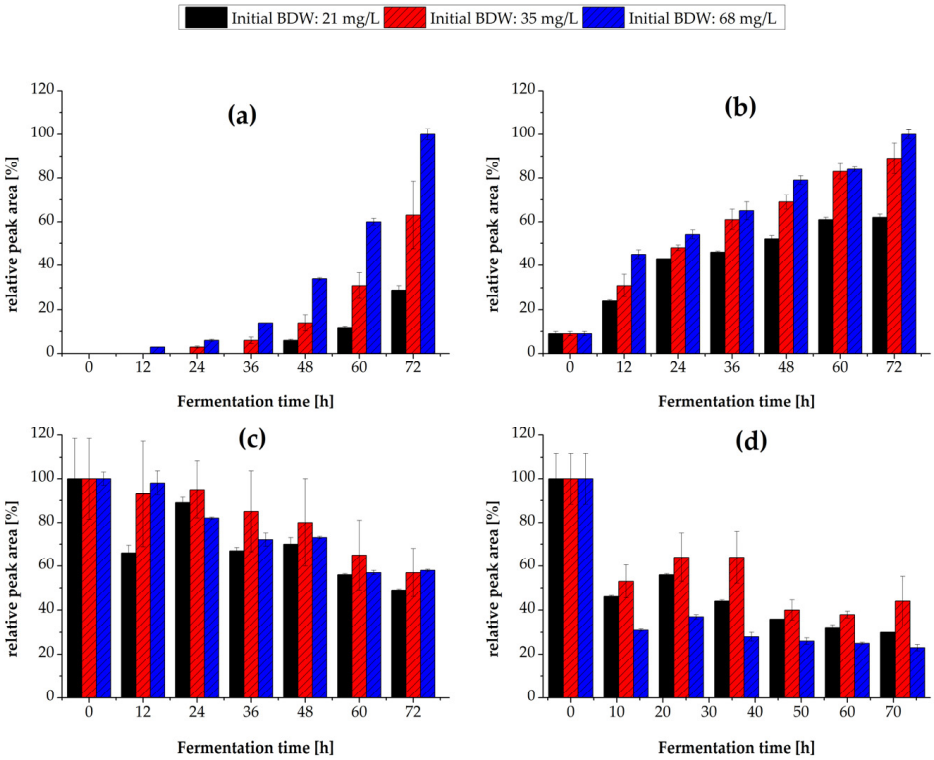


**Figure 7.** Production of (**a**) methyl-2-methylbutanoate, (**b**) 2-phenylethanol, and degradation of (**c**) ß-damascenone and (**d**) methional by shiitake under different inoculum concentrations. BDW: biomass dry weight. Error bars are standard errors of the mean for two samples.

Homogenized vegetative mycelia are generally used to test the impact of inoculum size on cell morphology and the impact of this parameter on metabolite production. Berovic et al. showed that the inoculum concentration affected the biomass growth of *Ganoderma lucidum* in a 10 L stirred tank bioreactor [33]. Vegetative inoculum of *G. lucidum* (six-day old shake flask culture) in concentrations of 14%, 17%, and 20% (wet weight) was used for the investigation. The maximal biomass concentration was obtained in the cultivation with an inoculum concentration of 17%. However, the highest inoculum concentration (20%) led to the decrease of biomass growth probably due to the substrate limitation in the culture. In addition, the inoculum concentration greatly affected the pellet size and the production of polysaccharide and ganoderic acid in shake flasks by *Ganoderma lucidum*. The maximal cell concentration of *G. lucidum* was promoted when the inoculum concentration was increased from 70 to 330 mg dry weight/L. A further increase in inoculum concentration (670 mg dry weight/L) led to the decrease of the maximal cell concentration. At the lowest inoculum concentration 91% of the pellets in the culture had a diameter larger than 0.16 cm. On the contrary, most of the pellets were smaller than 0.12 cm when the cultivation was carried out with the highest inoculum concentration. Polysaccharide

production was enhanced by high concentrations of inoculum, which led to the formation of small pellets, whereas ganoderic acid production was promoted by lower inoculum concentrations which led to the formation of large pellets. The limitation of nutrients in large pellets, thus, appears to favor ganoderic acid production [19]. As reported by Yang and Liau, an increase in inoculum concentration promoted the mycelium yield but decreased the pellet size of *G. lucidum* [42]. Lin and Yang also stated that a linear correlation exists between inoculum concentration and mycelia yield. However, the mycelia growth of *Agaricus blazei* Murrill was not significantly influenced when the cultivation was done at an inoculum concentration of higher than 40 mg/L [43]. Laccase production by *P. ostreatus* is promoted by a biomass concentration of up to 1.38 g/L (wet weight of mycelia) immobilized on polyurethane foam cubes, but a further increase in biomass concentration reduces the yield of this enzyme [44]. Laccase production by *P. ostreatus* was also enhanced by the inoculum size when increased numbers of fungal disks were used as the inoculums [45]. The biomass concentration of *G. frondosa* was significantly higher at an inoculum ratio of 4% than the inoculum ratios of 2% and 6%. A higher biomass concentration did not increase the secretion of EPS by *G. frondosa* in shake flask cultures, but the smaller and hairier pellets formed at an inoculation rate of 2% favored EPS production, suggesting the outer layer of the pellet regulates the synthesis of EPS [32]. Other investigations with basidiomycetes also showed that a too low and a too high inoculum concentration decreased the mycelia growth and metabolite production [30,46,47].

## 4. Conclusions

We investigated volumetric power input and inoculum concentration as critical process parameters affecting the production of natural flavor compounds by shiitake pellets. The production of methyl 2-methylbutanoate, which has the highest odor activity value and generates the fruity flavor of the beverage, was promoted by the better mixing and growth conditions. Furthermore, the results showed that the production of the fruity ester can be enhanced by the regulation of inoculum concentration. The findings highlight the importance of process parameters in the cultivation of basidiomycetes in a stirred tank bioreactor for the production of natural flavors. Our future work will focus on the scale-up of the fermentation process and its effect on the metabolic pathways leading to methyl 2-methylbutanoate and 2-phenylethanol.

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**Author Contributions:** Sibel Özdemir did the experimental work, research, and wrote the paper. Doreen Heerd critically reviewed the paper and helped in preparing the manuscript. Hendrich Quitmann gave advices in the experimental design. Yanyan Zhang and Marco Alexander Fraatz conducted the flavor analysis of the samples and analyzed the flavor data. Holger Zorn and Peter Czermak reviewed the paper and gave advice in preparing the manuscript. Peter Czermak gave advice in the experimental design and is the head of the research program.

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