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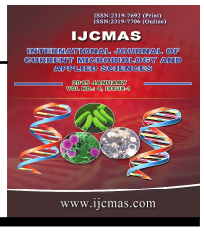


**Original Research Article**

# Preculture of *Pleurotus ostreatus* Increases the Yield of Yeast Biomass

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**A B S T R A C T**

The effect of pre-culturing of *Pleurotus ostreatus* in wheat bran extract after consequent heat treatment (5 min – 100°C) and without it on the growth rate of the yeast *Rhodosporidium diobovatum* and *Xanthophyllomyces dendrorhous* was investigated. It was found that the pre-cultivation of *Pleurotus ostreatus* from 2 to 12 days thereafter depressed the yeast growth rate as compared to the base culture medium. In that case, if *Pleurotus ostreatus* was precultured in the base medium, and then treated by heat, *Rhodosporidium diobovatum* and *Xanthophyllomyces dendrorhous* growth rate increased by 60-80% as compared with the base medium cultivation. It is shown that the composition of the base medium after culturing of *Pleurotus ostreatus* and consequent heat treatment changed greatly, in particular glucose content increased. It has been shown that the presence of lipid components in natural nutrient media inhibits yeast growth rate.

**Keywords**

Yeast biomass, Sequential culturing, Lipid components in cultivation media

## Introduction

The yeast biomass is used in animal feeding, it is a source of biologically active compounds, and it is used in the pharmaceutical and food industries. Growth rate and composition of the yeast biomass depends on culturing conditions (Frengova and Beshkova, 2009), in particular on the composition of the growth media (Somashekar and Joseph, 2000; Aksu and Eren, 2005; Libkind and van Brook, 2006), as well as the genetic characteristics of species and yeasts strains (Marova *et al.*, 2011).

Since different types of yeasts are adapted to different natural habitats, it is necessary to select culturing conditions for each species. Representatives of the *Rhodosporidium* genus are of great interest for industrial culturing, as they relate to the carotene-synthesizing yeasts species (Yurkov, 2008; Guo *et al.*, 2014). They can be cultured in a liquid fraction of vinasse and other organic wastes. However, the intensity of their growth in industrial conditions is low. This may be due to several reasons. Natural growth media are poor in polysaccharides

and rich in monosaccharides that are poorly metabolized by the yeast. Furthermore, such media may lack of growth factors necessary for the yeast growth. Isolation, purification and application of growth factors to the yeast culturing medium are unprofitable in industrial environments.

To meet the challenges of the yeast growth intensification, we suggested the feasibility of biological modifying the natural growth media which can enrich the growth media in monosaccharides and, possibly, in growth factors, and ensure the growth of yeast.

The essence of biological modification of natural growth media was reduced to a sequence in the culturing yeast on fungi bran extract, followed by yeast culturing. We assumed that the short-term culturing of *Pleurotus ostreatus* fungi can provide the cleavage of bran extract polysaccharides to monosaccharides and enrich the medium with exometabolites, which may affect the intensity of the yeast growth.

It is known that wheat bran on 53% consists of fibre components which are insoluble in water. The chemical composition of these fibers is a complex, which includes cellulose and pentosans, i.e. xylose and arabinose polymers associated with proteins. Proteins make about 16% of the total dry weight of the bran, minerals – about 7.2% (Šramková *et al.,* 2009). As we know, fungi are able to excrete in the medium the various hydrolytic enzymes, which split the polymers (Safari Sinegani *et al.,* 2005; García-Soto *et al.,* 2006).

Great interest in solving this problem may be *Pleurotus ostreatus* basidiomycetes at deep culturing, as their biomass has high food and forage value, they do not excrete toxic substances in the medium and excrete hydrolases (Flores *et al.,* 2010).

In the present study, we examined the growth rate of the *Rhodosporidium diobovatum* and *Xanthophyllomyces dendrorhous* yeasts grown in the medium after consecutive culturing of the *Pleurotus ostreatus* with subsequent heat treatment, and without it; and also the carbohydrate grows media composition; the effect of lipid components in natural nutrient media on yeast growth rate.

## Materials and Methods

### Microorganisms and culture conditions

We obtained the IMB Y-5023 *Rhodosporidium diobovatum* basidiomycetic yeast strain from the depository of microorganisms of the Institute of Microbiology and Virology, National Academy of Sciences of Ukraine, and *Xanthophyllomyces dendrorhous* DSM 5626 (*P. rhodozyma* ATCC 24202 and CBS 5905) yeast from the Deutche Sammlung von Mikroorganismen and Zellkulturen.

We maintained yeast stock cultures on agar medium (10% of carrot extract, 1% of glucose, 0.1% of peptone, 0.1% of NaCl, and 2% of agar; pH = 5.0). Before culturing on liquid media investigated, the inoculum was prepared for further culturing. For doing that, the yeast culture from agar stocks was transferred to liquid media studied (1 stock washed with 10 ml of fresh medium and the resultant suspension was inoculated into 50 ml of liquid medium in 300 ml volume flasks).

The following media were used in this study:

1. The BY medium was 3% of wheat bran extract, 1% of yeast extract, which was a basic medium. For sterilization, the

resulting extract was autoclaved at 1.5 atmospheres, for 1.5 hours.

2. The BYP medium (culture medium of the *Pleurotus ostreatus* filamentous fungus after 2 and 12 days of mycelium growth in the BY base medium, and after removing the mycelium by filtration).
3. The BYP-T medium **(**BYP medium after thermal treatment at a T=100°C, for 5 minutes).
4. Distillery corn vinasse was obtained from Karavan alcohol factory, Kharkiv Region, Ukraine.

**Preparation of BYP *Pleurotus ostreatus* filamentous fungus cultural medium**

The *Pleurotus ostreatus* mycelium of НК-35 strain (6.5 g of inoculum was added to 300 ml of the medium) were subcultured on the basic medium (the BY medium) and cultured for 2 and 12 days at a temperature of 22°C without shaking. The mycelium was removed by filtration after 2 or 12days of growth, respectively.

**Conditions for yeast culturing**

Five-day yeast culture was seeded in the flasks (50 ml medium in a 250 ml flask), was cultured for 5 days at 22°C on an orbital shaker at 150 rev/min.

**Determination of the dynamics of growth and yield of the yeast biomass**

The number cells of *Rhodosporidium diobovatum* yeast was determined on the $1^{st}$ -$5^{th}$ day of growth by counting the cells in Goryaev chamber on all of the media studied. The growth rate of the culture was expressed by the million/ml of cells. On the $5^{th}$ day of growth the output of dry yeast biomass *Rhodosporidium diobovatum* and *Xanthophyllomyces dendrorhous* was determined on all the studied media. To this biomass was dried at a temperature of 105ºC to constant weight and biomass output was expressed as g/l of dry weight.

**Determination of the base medium and BYP-T modified medium content**

We measured ash, crude fat, total nitrogen, total protein, nitrogen-free extractives, calcium, and phosphorus content in the base (BY) and modified (BYP-T) grows medium at a specialized laboratory using classical methods.

**Sugar contents in tested media**

To determine the content of monosaccharides and oligosaccharides in the base (BY), and modified (BYP-T) grows medium; we centrifuged 10 ml of the medium (15 minutes, 3500 g). We selected the supernatant liquid and added methanol (1:4) and left it overnight in the refrigerator. We then filtered it through the membrane filters Millipore filter 0.45 μμ from Merck Millipore (USA, MA) for separation of large molecules and cell aggregates. In the obtained samples, we determined content of simple carbohydrates (monosaccharides and oligosaccharides) using an ultra-high performance liquid chromatography conjugated with the mass spectrometry (LC-MS).

We made separation of carbohydrates on the apparatus of (UHPLC) UltiMate 3000 (Dionex Softron GmbH part of Thermo Fischer Scientific Inc., Germany), on the column of RCM-Monosaccharide $Ca^{+2}$ (8%) (phenomenex) at a temperature of 80º C, (right pump of 0.4 ml/min $H_2O$, left pump of 0.1 ml/min 0.5% $NH_4$ in ACN (Acetonitrile) at a flow-rate of 0.400 ml/min. The further determination was continued with the maXis impact mass spectrometry apparatus (Bruker Daltonik GmbH, Germany).

**Determination of total lipids in corn vinasse**

Total lipid content was determined in the initial corn vinasse and after centrifugation (15 minutes, 10000 g) and removing the top of the lipid phase. To measure the total lipids, we added successively the mixtures of organic solvents of chloroform - methanol (1:2, v/v) and chloroform - methanol - water (1:2:0.8, v/v) were added sequentially to the prepared samples. The extract was evaporated to dryness and mineralized at 120°C for 20 minutes in a 98% sulfuric acid. The samples were then diluted with water and the extinction was determined at 400 nm on a spectrophotometer SF-46 (LOMO, Russia). Contents of lipid fractions was calculated from the calibration curve and expressed in µg/ml of the sample.

**Statistical analysis**

All experiments were done in triplex. We assessed the mean value and the statistical error of mean. The probability of the difference between control and experiment was assessed by one-way analysis of variance (ANOVA) method.

## Result and Discussion

**The growth rate of *Rhodosporidium diobovatum* in the media after culturing of *Pleurotus ostreatus***

In control (base culture medium) the *Rhodosporidium diobovatum* culture reached the stationary phase at the 4th day. At this time the number of cells increased by 110 times as compared to the initial concentration (Figure 1).

In the case if *Pleurotus ostreatus* was cultured on the base medium for 2 days, and then *Rhodosporidium diobovatum* was cultured, the intensity of yeast growth was 2 times less at the time of entering the stationary phase (Figure 1A).

In the case if *Pleurotus ostreatus* was cultured in the base medium for 12 days, the intensity of growth of *Rhodosporidium diobovatum* was even lower and the number of cells at the time of entering the stationary phase increased only by 69 times (110 times in control) (Figure 1A).

Consequently, pre-culturing of *Pleurotus ostreatus* in the base medium inhibits the growth of *Rhodosporidium diobovatum.* The degree of the growth rate inhibition depends on the culturing time of *Pleurotus ostreatus.*

We can assume that *Pleurotus ostreatus* excretes components having inhibitory activity to *Rhodosporidium diobovatum*. If these components are temperature sensitive, they may be inactivated by the heat treatment.

It was found that heat treatment of the base medium was accompanied by a decrease of intensity of yeast growth by 33% in comparison to the medium without the heat treatment. Thus, if the number of cells in the base medium without the heat treatment on the 4th day was increased by 110 times in comparison to the initial concentration, after the thermal treatment of medium it increased only by 83 times (Figure 1B).

In the case where *Pleurotus ostreatus* was cultured in the base medium for 2 days, and then *Rhodosporidium diobovatum* was cultured, the growth rate of the culture to the 4th day 3-fold compared to the same medium, untreated (Figure 1). We should note that practically the same effect of stimulating the growth of *Rhodosporidium diobovatum* was also observed after 12 days

of the culturing period of *Pleurotus ostreatus* (Figure 1).

If we compare the number of yeast cells in culture at the fourth day of cultivation after the heat treatment of the medium, it should be noted that the initial amount of cells in the basic medium increased by 110 times, but after the previous culturing of *Pleurotus ostreatus* it increased by 184 times.

Consequently, the short-term heat treatment of the base medium decreased the growth rate, but heat treatment of the base medium after 2 to 12 days of culturing of *Pleurotus ostreatus* significantly increased the growth rate of *Rhodosporidium diobovatum.* These results may indicate inactivation of components excreted by *Pleurotus ostreatus.*

To confirm this, we determined the yield of the dry biomass of *Rhodosporidium diobovatum* at 5th day cultured in the base medium without heat treatment and after pre-culturing of *Pleurotus ostreatus* with heat pretreatment of the medium. The yield ofdry biomass of *Rhodosporidium diobovatum* in the base medium on the 5th day of growth was 4.2 g/litre, but after the sequential culturing it was 6.9 g/litre, i.e. it was greater by 64% (Figure 2).

We can assume that this stimulatory effect on yeast cultured in the medium after 2 days of culturing of mycelium of *Pleurotus ostreatus* (after heat treatment) is linked to two possible mechanisms. The heat treatment provides an increase in the content of sugars in the medium after culturing of *Pleurotus ostreatus* and/or inactivates inhibitors of growth of *Rhodosporidium diobovatum,* which may occur in the medium after growth of *Pleurotus ostreatus.* However, regardless of the mechanism we can argue that the thermal treatment modifies the medium after the growth of *Pleurotus ostreatus.* It is of practical interest for determining "specificity" of such a modification of the culturing medium.

To this end, the growth rate of *Xanthophyllomyces dendrorhous* in the basic medium, and the medium modified after culturing other yeast species – *Pleurotus ostreatus,* – was determined. It was found that when culturing *Xanthophyllomyces dendrorhous* in the base medium, the dry biomass yield on the 5th day was only 1.2 g, while the output in the modified medium was 6 times larger and amounted to more than 7 g/litre, that even exceeded the yield of *Rhodosporidium diobovatum* (Figure 2).

Therefore, a two-day's culturing of *Pleurotus ostreatus* and the heat treatment, most likely, modified and enriched the medium with monosaccharides and other components necessary for the yeast growth, and it was not related to the species characteristics of the yeast.

**A characteristic of the basic and modified grows media**

The basic grows medium changed significantly after a two-day's culturing of *Pleurotus ostreatus.* Thus, the ash content has been reduced by 26%, and the fat content has been reduced by 2.4 times compared to the original medium (Table 1). A two-day's culturing of *Pleurotus ostreatus* was accompanied by a reduction of total nitrogen and protein by 23% (Table 1). Contents of calcium and phosphorus were not significantly changed. With that, the content of nitrogen-free extractives (NFE) has been increased by 23% compared to the base medium (Table 1). As we know, NFE include mono-, di- and trisaccharides, starch, glycogen, part of pectins and

hemicelluloses, gum, and organic acids (Iskanderova and Ibadulayeva, 2013).

Measuring of the total content of mono- and oligosaccharides showed that their amount increased by 3.5 times after the culturing of *Pleurotus ostreatus* (Table 2). Analysis of the individual components revealed that a two-day's culturing of *Pleurotus ostreatus* in the base medium induces a significant increase, primarily, in glucose – by 278.5 times, and pentasaccharides – by 13.7 times, whereas fructose content was reduced by 1.7 times compared to its content in the base medium (Table 2).

Consequently, pre-culturing of *Pleurotus ostreatus* in the base medium provided the modification of the medium, which manifested itself in a significant increase in glucose and other low molecular weight sugars and reducing fat and protein.

However, such a large increase in glucose might contribute to greater effect of stimulation of the *Rhodosporidium diobovatum* growth. This situation suggests that the culturing medium contains components that partly "retard" the yeast growth. We can assume that lipids may have these properties in the composition of the medium.

We know that when using corn as the additional raw material for distilleries, the corn vinasse, which is rich in lipids, is formed after production of alcohol. Measuring of total lipids in corn vinasse alcohol showed that it contained of 582 µg per ml of them. Removal of 40% of the lipids from corn vinasse increased the yeast growth rate by 34% on the 1st day of growth, and by 26% on the 3rd day of growth (Figure 3).

Consequently, the presence of a large amount of lipid components in the culturing medium of *Rhodosporidium diobovatum* can inhibit, or retard, the yeast growth.

Pre-culturing of *Pleurotus ostreatus* basidiomycete in the base medium modifies the medium, and this is evident in the yeast growth inhibition on such a medium. Since this inhibitory effect was well expressed and manifested itself after only 2 days after the growth of *Pleurotus ostreatus,* it is unlikely that the fungi had time to assimilate the medium carbohydrates during this time. We can assume that *Pleurotus ostreatus* excreted during this time exometabolites into the medium, inhibiting the yeast growth.

Furthermore, fungi excrete various hydrolytic enzymes during the growth, which actively hydrolyse cellulose and hemicellulose (Elisashvili, 2008; Téllez-Téllez *et al.*, 2013). This may in turn lead to further enrichment of such a medium in mono- and oligosaccharides. This is supported by evidence of the data presented in Table 2.

However, the stimulatory effect of the medium, modified by fungi, appeared only after further 5-minute heat treatment. These results suggest that the inhibitory effect of the *Pleurotus ostreatus* pre-culturing is associated with excretion of exometabolites constraining the growth of *Rhodosporidium diobovatum.*

This is supported by the results of slowing down the growth of yeast after heat treatment of the base medium. Furthermore, these results indicate that such processing may not provide additional hydrolysis of polysaccharides.

Such hydrolysis is realized only after culturing of *Pleurotus ostreatus*, i.e. after excretion of the hydrolytic enzymes with it.

We should note that such an inhibitory effect of pre-culturing of *Pleurotus ostreatus* on the yeast should not be considered as presence of toxic compounds among exometabolites. Most likely, this mechanism is implemented by the allelopathic substances action type. The allelopathy phenomenon was discovered in 1937 by Hans Molisch (Willis and Rick, 2007).

We know that in response to stress factors, vegetable objects enhance the activity of excretory system and they may significantly change the composition of the substances released into the medium (Lazzeri and Manici, 2001). In particular, we have shown that in response to exposure of the external extreme factors, plants excrete a large amount of phenolic compounds in the medium (Bais *et al.*, 2003).

Since the transfer of *Pleurotus ostreatus* in the submerged culture is their stress factor, we can assume that fungi can excrete in such circumstances the thermolabile substances having an inhibitory effect on *Rhodosporidium diobovatum* growth.

## Conclusion

A preliminary short-term (2 days) culturing of *Pleurotus ostreatus* on wheat bran extract is accompanied by a change of the medium composition. This change in the composition of the medium was accompanied by inhibition of growth of *Rhodosporidium diobovatum* yeast on this medium. A short-term (5 minutes) heating to 100º C of the medium modified by *Pleurotus ostreatus,* provided significant growth of the yeast on the medium to increase the yield to 64%. The medium, modified by *Pleurotus ostreatus,* is characterized by increase in glucose and other low molecular weight sugars content. The presence of the lipid components in the culturing medium of *Rhodosporidium diobovatum* inhibited their growth. In industrial production the corn vinasse can be used as a substrate for *Rhodosporidium diobovatum* only after removal of lipids.

**Table.1** The content of the grows media – the base (BY) and the medium after 2 days of the *Pleurotus ostreatus* culturing on the base medium, and after 5 minutes of heat treatment (BYP-T), per cent (%)

| Medium | Ash | Crude fat | Total nitrogen | Total protein | NFE | Calcium | Phosphorus |
|---|---|---|---|---|---|---|---|
| BY | 15.5 ±0.8 | 1.6 ±0.08 | 5.9 ±0.3 | 36.9 ±1.8 | 45.9 ±2.1 | 0.27 ±0.01 | 2.6 ±0.1 |
| BYP-T | 11.4 ±0.5 | 0.7 ±0.03 | 4.6 ±0.2 | 28.6 ±1.4 | 59.4 ±2.7 | 0.24 ±0.01 | 2.3 ±0.1 |

**Table.2** The content of the low molecular weight sugars (μg/ml) in the base medium (BY) and in the medium after 2 days of culturing of *Pleurotus ostreatus* on the base medium, and after 5 minutes of heat treatment (BYP-T)

| Sugar content | Tested media | |
|---|---|---|
| | BY | BYP-T |
| DP2 | 160,74±5,7 | 163,88±6,0 |
| DP3 | 2870,95±49,2 | 9249,89±67,3 |
| DP4 | 134,29±4,3 | 1325,81±21,8 |
| DP5 | 6,04±0,3 | 82,74±3,5 |
| DP6 | 0,91±0,04 | 4,46±0,2 |
| rabinose | 0 | 0 |
| fructose | 1,87±0,09 | 1,04±0,05 |
| galactose | 0 | 0 |
| glucose | 1,19±0,05 | 331,38±7,6 |
| ribose | 0 | 0 |
| xylose | 0 | 0 |
| total | 3175,98±51,1 | 11159,19±97,6 |

* DP2 - disaccharides; DP3 - trisaccharides; DP4 - tetrasaccharides; DP5, DP6 – oligosaccharides consisting of 5 and 6 monomers, respectively.

**Figure.1** The number of cells of *Rhodosporidium diobovatum* during culturing in the base medium (BY), on the same medium after culturing of *Pleurotus ostreatus* (BYP) for 2 days (BYP-2), and on the same medium after culturing of *Pleurotus ostreatus* for 12 days (BYP-12) without additional heat treatment of the fluids (A) and after 5 minutes of heat treatment of the medium (B)

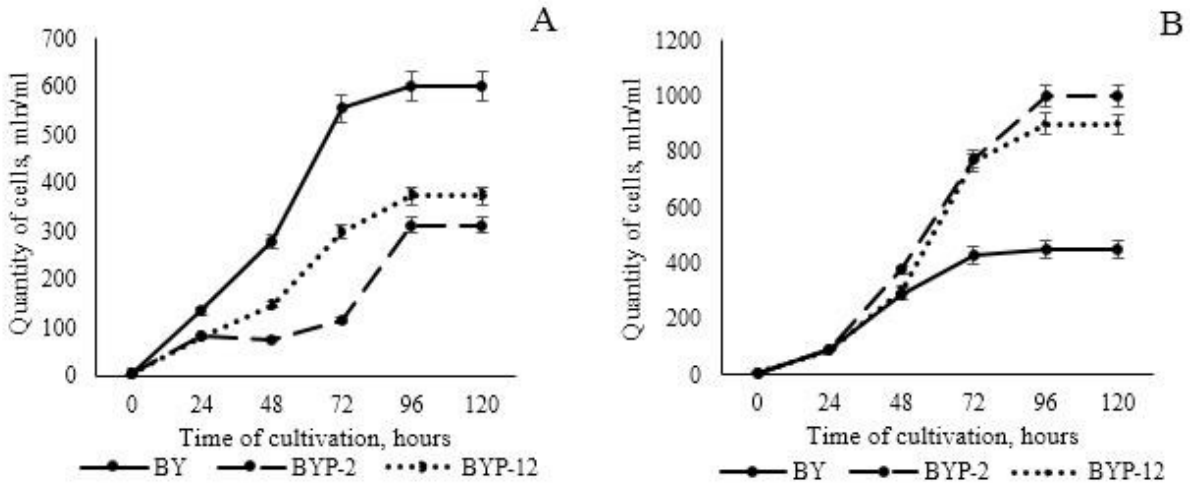


**Figure.2** The dry biomass yield of the *Rhodosporidium diobovatum* and *Xanthophyllomyces dendrorhous* yeasts on the BY and BYP-T media on the 5th day of the culturing

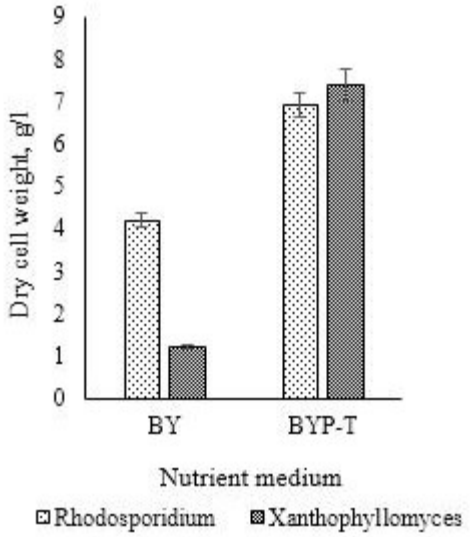
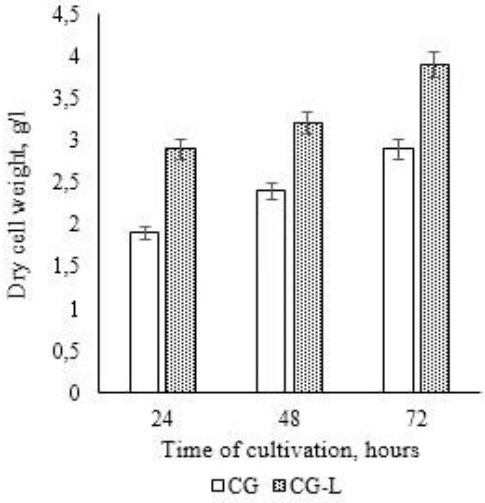



**Figure.3** The dry biomass yield of *Rhodosporidium diobovatum* yeast when culturing on corn vinasse (CV) and on corn vinasse after partial removal of lipids (CV-L), for 3 days

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