

Original Research Article

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The Effect of Lipid Components of Corn Vinasse on the Growth Intensity of Yeast *Rhodospiridium diobovatum* IMB Y-5023

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ABSTRACT

It was studied the effect of lipid components of corn vinasse on the growth intensity of yeast *Rhodospiridium diobovatum* IMB Y – 5023. It was established that when cultured on the primary corn vinasse the yeast growth intensity was quite low, and in such form it cannot be used for industrial production of yeast biomass. For use of corn vinasse in industrial production of yeast biomass it must be preliminary prepared, using flotation or separation for dividing into fractions and, respectively, for removing of mechanical particles and some lipids, inhibiting the growth of *Rhodospiridium diobovatum* IMB Y – 5023, pH of corn vinasse must be 5.0 – 6.0. After such vinasse modification, the yield of *Rh. diobovatum* IMB Y-5023 dry biomass was increased by 44% compared to primary medium, and at the same time *Rh. diobovatum* IMB Y – 5023 actively assimilated the residual lipid components of corn vinasse, which provided quite intensive growth of *Rh. diobovatum* IMB Y – 5023 culture. For optimal growth and high yield of *Rhodospiridium diobovatum* IMB Y – 5023 biomass on corn vinasse the amount of its lipids must be about 300 mg/ml, and at the concentration of 400 mg/ml their growth is inhibited. Also, it was found that when the media contained lipids with concentrations from 50 to 400 mg/ml and without mechanical particles, yeast cells formed aggregates.

Keywords

Yeast, lipids, cornvinasse, *Rhodospiridium diobovatum* IMBY – 5023, growth intensity, biomass.

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Introduction

Yeast *Rhodospiridium diobovatum* IMB Y – 5023 refer to the carotene-accumulating species and they can accumulate up to 25 mg carotenoids per 1 g dry biomass. Our investigations demonstrated, that the most of *Rh. diobovatum* IMB Y – 5023 carotenoids are represented by lycopene, β -carotene and astaxanthin (Ielchishcheva and Goltvianskiy, 2015). The literature evidence that some species of *Rh. diobovatum* also synthesize

torulin, torulorodin and γ -carotene (Buzzini *et al.*, 2007; Yurkov *et al.*, 2008). Together with carotenoids they contain protein, amino acids, vitamins, lipids, the amount of which can vary in biomass, depending on the cultivation conditions, organic acids (Munch *et al.*, 2015), ubiquinone (Yurkov *et al.*, 2008),

It was shown that under the optimal cultivation conditions *Rh. diobovatum* IMB

Y – 5023 can accumulate from 3 to 7-8 g/l dry biomass (Goltvianskiy *et al.*, 2015; Ielchishcheva and Goltvianskiy, 2015).

Therefore, *Rh. diobovatum* IMB Y – 5023 is the prospective object for industrial cultivation. One of the nutrient media, rich in sugars, can be wheat vinasse - waste of distilleries. Quite often the raw materials of distilleries can contain corn together with wheat. It is known, that corn is rich in fats (Belyea *et al.*, 2004), which fall into the liquid fraction of vinasse. The effect of lipid components of corn vinasse on the growth intensity of *Rh. diobovatum* IMB Y – 5023 is still has not been investigated.

It is known, that yeast *Rh. diobovatum* also refer to microorganisms, who can accumulate more than 20% lipids in their biomass, depending on the medium and cultivation conditions (Munch *et al.*, 2015). Such microorganisms, who can accumulate significant amount of lipids, and who are of great interest as a source of oil for the synthesis of biodiesel (Sitepu *et al.* 2014), are *Rhodospiridium toruloides* (Xu *et al.*, 2012), *Rhodospiridium babjevae* (Munch *et al.*, 2015), *Rhodotorula glutinis* (Chi *et al.*, 2011) and others. In the work of Munch *et al.*, 2015 it was studied the effect of pour glycerine and glycerine, obtained from the waste of biodiesel production, as a single source of carbon, on accumulation of biomass and lipids by *Rh. diobovatum* 08 – 225. It had been shown that when culturing on such media, therein limiting the amount of nitrogen, *Rh. diobovatum* 08 – 225 had accumulated up to 14 g/l biomass with lipid content up to 50% of the total cell mass.

Study of the effect of lipids on the growth intensity is of great interest not only from the viewpoint of preparation of industrial nutrient media, but also for clarifying the regulation mechanisms of yeast cells proliferation.

In the present work it was determined the effect of lipid components of corn vinasse on the growth intensity of *Rh. diobovatum* IMB Y – 5023.

Materials and Methods

Microorganisms and culture conditions

The strain of basidiomycete yeast *Rhodospiridium diobovatum* IMB Y – 5023 was obtained from the Microorganisms Depository of Institute of Microbiology and Virology, National Academy of Sciences of Ukraine.

The stock culture was supported on the agar medium (26% of wort, 2% of agar). Before cultivation in the test liquid media it was obtained the inoculum for further cultivation. For this purpose the yeast culture was transferred from the agar slant into a liquid Ryder nutrient medium: NH_4NO_3 – 2; $\text{FeSO}_4 \times 7\text{H}_2\text{O}$ – 0.1; MgSO_4 – 0.75; KCl – 0.4; K_2HPO_4 – 0.1; KH_2PO_4 (g/l); 2% of glucose as a carbon source (1 tube was rinsed with 10 ml fresh medium and the obtained suspension was added to 50 ml of liquid medium in 300 ml flasks).

As experimental media were used:

1. Ryder (g/l): NH_4NO_3 – 2; $\text{FeSO}_4 \times 7\text{H}_2\text{O}$ – 0.1; MgSO_4 – 0.75; KCl – 0.4; K_2HPO_4 – 0.1; KH_2PO_4 – 1; glucose – 20.
2. BY: wheat bran extract 3%, yeast extract 1%. The extract was obtained due to thermal treatment at $T = 132^\circ\text{C}$ under the pressure 1.5 atm. for 1.5 hours.
3. CV: distillery corn vinasse, obtained at Karavanovskiy distillery of Kharkiv region, Ukraine, $\text{pH} = 3.4$.

4. CV with different value of pH (range from 3.4 to 7.0).
5. CV after dividing into 3 fractions with the help of sedimentation (for 3 hours with preliminary bringing of pH up to 6.5 1N NaOH. In this way the primary corn vinasse was divided into 3 fractions - lower fraction (LF), containing large mechanical flakes-like particles, middle fraction (MF), containing some small mechanical particles; and top fraction (TF), which did not contain any mechanical particles).
6. BY with addition of corn oil with concentrations of 50, 100, 200, 300 and 400 mg/ml of medium.

Conditions for yeast cultivation

The flasks were inoculated with three-day yeast culture (5 ml of inoculum was added to 50 ml of medium in 250 ml flask), cultured for 3-4 days at 22°C on orbital shaker at 150 rpm, round the clock.

Determination of growth dynamics and biomass yield of yeast

The number of yeast cells was determined on 1-4 days of growth by direct counting of cells in the Gorjaev chamber on all test media. The growth intensity of the culture was expressed by the quantity of cells million/ml. On the 3d day of the culture growth the yield of yeast dry biomass on the test media was determined. For this purpose the biomass was dried at the temperature of 105°C up to constant weight and expressed in g/l of dry mass.

Determination of the total lipids in the corn vinasse

The total lipids content was determined in primary corn vinasse after centrifugation

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

Statistical analysis

All experiments were performed in three replicates. All results were statistically processed using STATISTICA 10 program. The statistical error and significant difference between the control and experiment were determined using non-parametric method of analysis.

Results and Discussion

The growth intensity of *Rh. diobovatum* IMB Y-5023 on the test media

In the first set of experiments *Rh. diobovatum* IMB Y-5023 was cultured on the classical Ryder medium with 2% glucose as a carbon source. During 24 hours of growth the number of *Rh. diobovatum* IMB Y - 5023 cells had increased 3.7 times compared to the primary amount, during 48 hours - 15.2 times, and after 72 hours - 22 times (Table 1).

Therefore, the addition of glucose up to 2% to the Ryder medium had provided relatively good culture growth.

When *Rh. diobovatum* IMB Y - 5023 cultured on the corn vinasse, it was appeared that after 24 hours of growth the number of

yeast cells increased 7 – fold more than on Ryder medium, however, then, the intensity of growth increased with the less rate (by 11.7 and 19 times) after 48 and 72 hours, compared to the primary number of cells, respectively (Table 1).

The present results evidence that the growth-determining components, contained in CV, were actively assimilated by yeasts, but the medium was exhausted more quickly, then the Ryder medium.

The comparison of the growth intensity of *Rh. diobovatum* IMB Y – 5023 (Table 1) with the other media allows to conclude, that the growth intensity on these media was relatively low.

For experimental verification of this fact *Rh. diobovatum* IMB Y – 5023 was cultured on water extract of wheat bran BY, prepared as it was described in “Methods”. It was found that on BY during 24 hours of growth the number of cells increased by 25 times, and after 48 and 72 hours – by 51 and 102 times, compared with the primary number, respectively (Table 1).

Therefore, the growth intensity of *Rh. diobovatum* IMB Y – 5023 on CV, which is the waste of alcohol industry, is lower compared to BY, and in such form it cannot be used for yeast production.

Low growth intensity of *Rh. diobovatum* IMB Y-5023 on CV, compared with BY, can be explained by the low primary pH value of this medium, it was 3.4. Thereby, in the further set of experiments it was determined the yield of *Rh. diobovatum* IMB Y-5023 dry biomass when cultured on the corn vinasse with various pH values. It was established that under pH 5.0 – 6.5 the growth intensity of yeast was 34% more compared to the primary medium, and under

pH 7.0 their growth intensity decreased again by 28% compared to pH 5.0 (Fig. 1).

Consequently, bringing pH of CV up to 5.0 – 6.5 provide intensification of their growth, however, they grew significantly slower, compared with BY medium. Lower growth intensity of *Rh. diobovatum* IMB Y – 5023 on CV, compared with BY medium, may be connected with some more factors: lack of sugars and other nutrients, poor assimilation of corn vinasse nutrients, presence of factors, inhibiting yeast growth. Such factors can include lipids, since it is known that the corn vinasse contains more lipids (Belyea *et al.*, 2004), compared with the wheat bran extract (Goltvianskiy *et al.*, 2015).

Dividing of the corn vinasse into three fractions and the growth intensity of *Rh. diobovatum* IMB Y-5023 on these fractions

Since the corn vinasse has heterogeneous composition, it was divided into 3 constituent fractions. After bringing pH up to 6.0 – 6.5 and settling for an hour with a liquid column height of 15 cm, it was well divided into 3 fractions.

The top fraction (TF) did not contain mechanical particles, the middle fraction (MF) contained many small mechanical residues - corn seeds and the lower fraction (LF) contained a lot of large mechanical particles. Yeast growth intensity on the 3 fractions was different.

Thus, the number of yeast cells during 2 days of cultivation on LF was increased by 2 times, compared to the primary amount, and further it remained the same (Fig. 2A)

The yeast grew much better on MF. After 3 days the number of cells on this fraction increased by 17.2 times (Fig. 2A).

However, the best growth of *Rh. diobovatum* IMB Y – 5023 was noticed on TF. The number of cells already after 1 day of growth was by 16 times more than the primary number, and after 2 and 3 days of growth it was increased by 63 and 77 times, respectively, as it was on BY (Fig. 2A).

The various rate of growth of *Rh. diobovatum* IMB Y – 5023 on the different fractions of CV is evidenced by the yield of yeast dry biomass on the 3 days of cultivation (Fig. 2B).

It must be noticed, that the yield of yeast dry biomass, when cultured on the corn vinasse on TF after removing of mechanical particles and bringing pH up to 5.0 – 6.5 was almost the same, as on BY, and it was 4.5 – 5.0 g/l dry yeast biomass during 3 days of growth.

The total lipids determination in 3 fractions of the corn vinasse

As it was noted, corn is rich in lipids and their amount can remain unchanged in the vinasse. Most lipids were detected in TF and their content was 334 mg/ml, and in the middle and lower fractions it was by 3 times less (Fig. 3A).

It should be noted, that there was no direct relationship between the lipid content in the vinasse and the growth intensity in MF and LF. Thus, MF contained 100.3 mg/ml lipids, and LF 149.9 mg/ml, in this case the growth intensity of *Rh. diobovatum* IMB Y – 5023 in MF was higher, than in LF.

During the next set of experiments the content of lipids in TF before and after 3 days of yeast cultivation was determined. It was found that after 3 days of yeast growth, the content of lipids in TF was decreased by

2.5 times, compared to their primary amount (Fig. 3B).

Hence, *Rh. diobovatum* IMB Y – 5023 yeast actively assimilated the lipid components of corn vinasse.

Additionally, it was found, that namely in TF of corn vinasse, contained more than 300 mg/ml lipids and where the yeast culture grew intensively, the cells of *Rh. diobovatum* IMB Y – 5023 formed aggregates, which were absent in the control culture (CV) and in the samples, growing on MF and LF.

Therefore, removing of mechanical particles in CV and its high lipid content provided intensive growth of yeasts, that didn't differ from the growth of culture on BY. It may be assumed, that presence of such amount of lipids in the vinasse stimulated the growth of *Rh. diobovatum* IMB Y – 5023 culture.

To verify this fact, on the next stage of the work it was determined the growth intensity of *Rh. diobovatum* IMB Y – 5023 on BY, adding different amounts of corn lipids. When selecting the concentrations of lipids we based on the principle that the fraction of corn vinasse (TF), which had the highest yield of yeast biomass, contained about 300 mg/ml lipids.

The lipid suspensions were prepared on BY with concentrations of 50,100, 200, 300 and 400 mg/ml lipids.

Growth intensity of *Rh. diobovatum* IMB Y-5023 culture on BY, adding of 50 mg/ml corn lipids, lightly increased only in the beginning of cultivation: by 30 – 32% after 24 and 48 hours, compared to the control (Fig. 4).

The rise of corn lipid content in the medium up to 100 mg/ml increased the growth intensity by 60, 41, 12 and 18 % after 24, 48, 72 and 96 hours, respectively (Fig. 4).

The greatest impact on stimulation of culture growth was obtained after addition of 300 mg/ml corn lipids to the culture medium. Thus, the number of cells was increased by 71, 65, 26, 36 % compared to the control after 24, 48, 72 and 96 hours, respectively (Fig. 4). The rise of corn lipid content in the medium up to 400 mg/ml further slightly slowed down the culture growth, compared to the concentration of 300 mg/ml (Fig. 4).

It must be noticed, that when *Rh. diobovatum* IMB Y – 5023 cultured on these media with addition of corn lipids with concentrations from 50 to 400 mg/ml, cells of *Rh. diobovatum* IMB Y – 5023 formed aggregates (Fig. 5), as in TF.

Hence, corn lipids stimulate growth of *Rh. diobovatum* IMB Y-5023, the greatest impact of stimulation was demonstrated during the first 24 – 48 hours of cultivation. The optimal concentration of lipids for the yeast growth is 300 mg/ml.

It is known, that the growth of microorganisms as well as the other organisms is a complex integrative process. Under the integrative processes of growth we mean the creation of "single" functional dynamic system between the organism's metabolism and the components of environment, and more precisely, the organism's microenvironment.

How had this unity been created? On the one hand, due to the presence of alternative metabolic pathways, metabolism forms such metabolic pattern, which provides its self-regulation in the particular environment, i.e. the organism utilizes that components for

growth and reproduction, which surround it at the moment. On the other hand, the organism as the open system, excreting metabolites in the environment, dynamically "adjust" its microenvironment under the selected metabolic strategy of functioning. As a result, the metabolic system of organism and the components of microenvironment form a single dynamic system – "SDS". The faster the SDS will be formed, the shorter will be the lag-period, and the more effectively SDS will be formed, the higher will be the rate of culture reproduction. In that case, if SDS will not be formed because of "incompatibility" between the possibilities of metabolism and components of microenvironment, the culture will die.

The effectiveness of SDS forming depends on many factors and conditions. Conceptually, it may be highlighted only some of them: the characteristics of genotype, i.e. structural and dynamic organization of the genetic system. The most important characteristic of SDS is the presence of genes that provide alternative pathways, i.e., metabolism of sugars, lipids etc. The second feature of the genetic system is its instability or dynamic adaptive conversion. As it is known, the instability of microbial genome is provided by plasmids (Bibb *et al.*, 1978; Lichtetal, 2001), transposons (Nagyand Chandler, 2004) and high mutability as well as horizontal gene transfer (Craigetal, 2002).

Epigenetic mechanisms play an equally important role in the "organism - environment" "unity". Change of culture medium was shown to influence on the characteristics and the extent of DNA methylation (Nicholson and Low, 2000), and other epigenetic mechanisms, that are inadequate studied in microorganisms.

The next important factor is the characteristics of metabolic regulation, and more precisely, the amount of alternative metabolic pathways in a single organism. It is known that yeast can simultaneously assimilate various substrates (Marova et al., 2011). This is also evidenced by the fact, that the same species or strain can grow with the same rate on different substrates (Buzzini and Martini, 1999). At the same time they accumulate different amount of metabolites. Thus, *Rh. diobovatum* IMB Y – 5023 can accumulate a similar biomass per time unit on BY medium and corn vinasse (Fig.2B). Previously, it had been shown that *Rh. diobovatum* IMB Y – 5023 accumulate

different amount of carotenoids and other metabolites on different media (Yurkov et al., 2008; Ielchishcheva and Goltvianskiy, 2015). The effective use of alternative metabolic pathways by yeast *Rh. diobovatum* IMB Y-5023 is evidenced by the studies of Munch et al. (Munch et al., 2015). These authors had demonstrated that *Rh. diobovatum* 08 – 225 can be cultivated on pure glycerol as a single source of carbon. In such case, its growth intensity was high - they accumulated up to 14 g/l biomass with lipid content up to 50%. This yeast can be a promising source of biodiesel (Chietal, 2011; Xu et al., 2012; Sitpuetal, 2014).

Table.1 The number of the *Rh. diobovatum* IMB Y-5023 cells when cultured during 72 hours on different media. The average values from 3 independent experiments are presented

Culture media	Cultivation time, hours			
	0	24	48	72
Ryder	5.4±0.27	20.4±1.02	82.3±4.11	120.9±6.04
CV	5.4±0.27	38.1±1.91	63.2±3.16	103.7±5.18
BY	5.4±0.27	134.5±6.72	279.6±13.98	555±27.75

Fig.1 The yield of *Rh. diobovatum* IMB Y-5023 dry biomass when cultured on the corn vinasse (CV) with pH from 3.4 to 7.0.

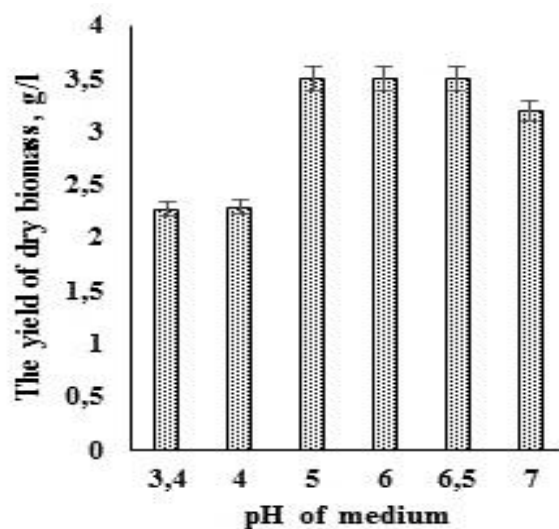


Fig.2 The intensity of growth of *Rh. Diobovatum* IMB Y-5023 on the different fractions (LF, MF, TF) of the corn vinasse (A); The yield of *Rh. diobovatum* IMB Y-5023 dry biomass when cultured on the primary medium (CV), on different fractions of the corn vinasse and on the bran extract (BY) on the 3d day of growth (B)

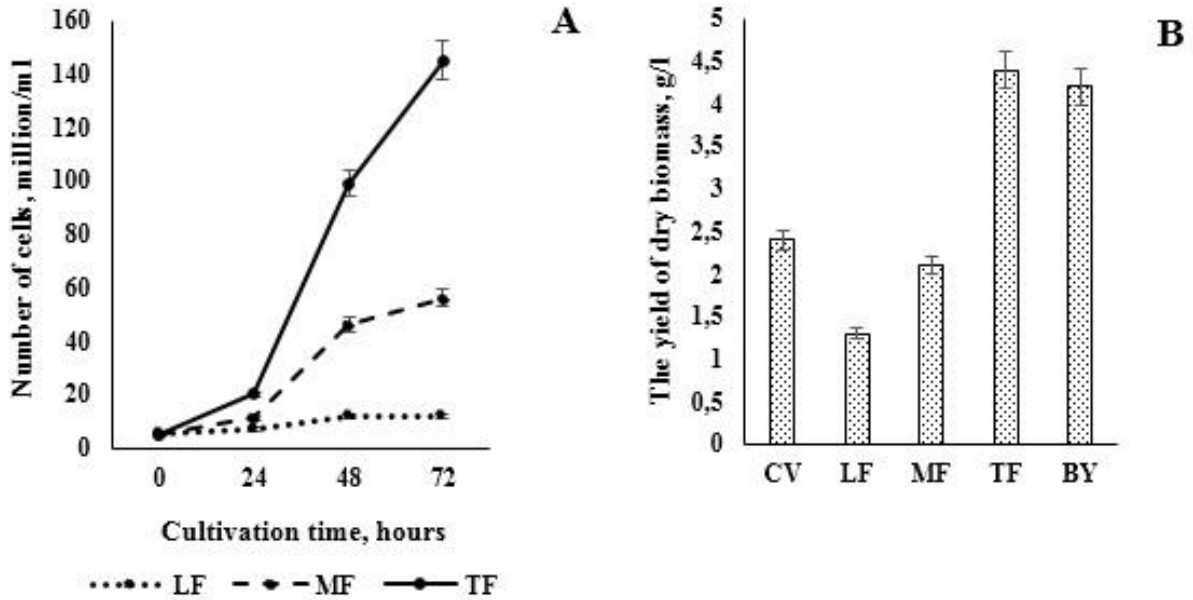


Fig.3 The content of total lipids in the different fractions of corn vinasse (CV) (A) and the content of total lipids before and after cultivation of *Rh. diobovatum* IMB Y-5023 in the top fraction (TF) (B)

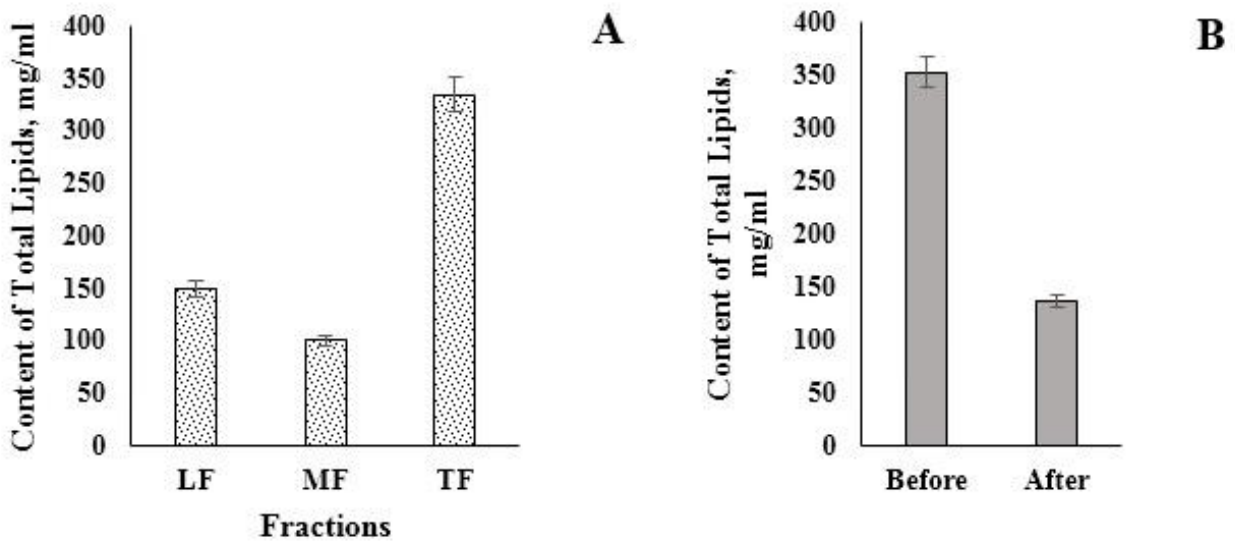


Fig.4 The growth intensity of *Rh. diobovatum* IMB Y-5023 culture on the bran extract (BY), adding corn lipids with concentrations of 50, 100, 200, 300 and 400 mg/ml

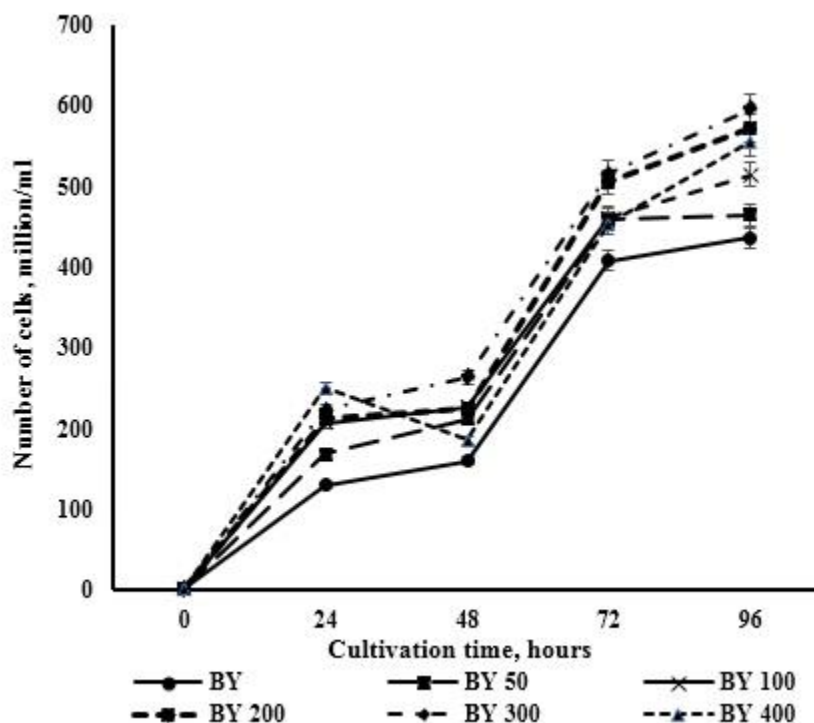
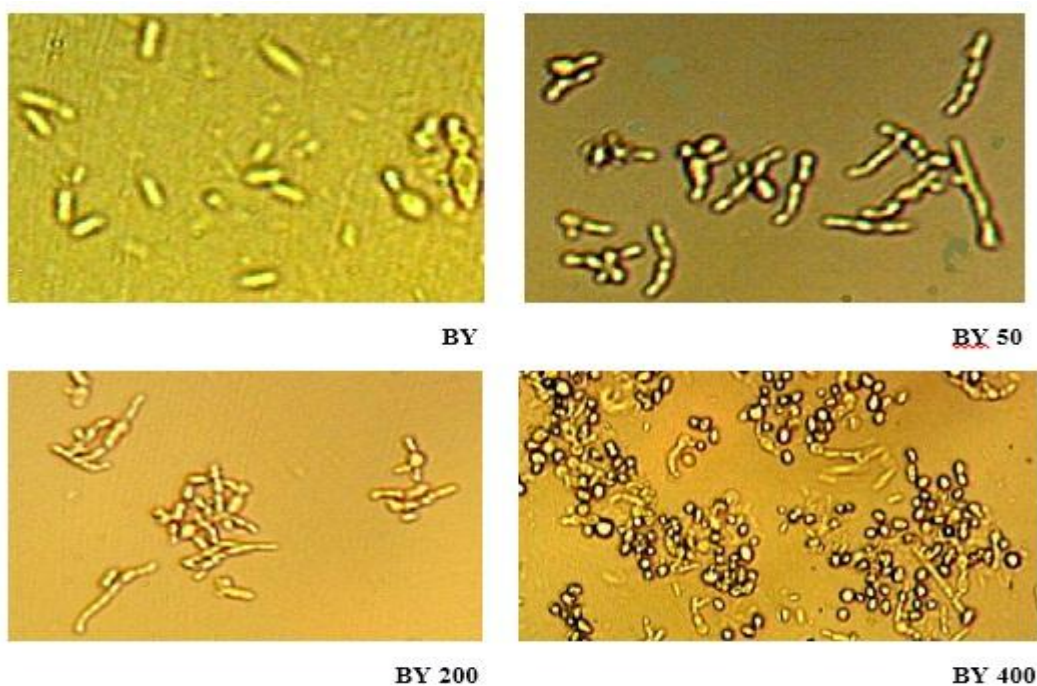


Fig.5 Formation of *Rh. diobovatum* IMB Y-5023 cell aggregates when cultured on the bran extract (BY) with addition of corn lipids with concentrations from 50 to 400 mg/ml



The ability of yeast *Rh. diobovatum* IMB Y-5023 to assimilate lipids is evidenced by the results of the present study. It should be noted, that there is a well-defined dose dependence between the increase of culture growth intensity and the lipid content in the culture medium (Fig. 4).

The presence of "zone" of saturation by lipid components may indicate the presence of genetic and metabolic species limitations.

Together with the genetic and metabolic characteristics of SDS formation, the same role, and, probably, defining role belong to characteristics of microenvironment. It should be noted, that the most important feature of the microenvironment is 1 - high rate of parameters changing - its physical and chemical characteristics (pH, viscosity, osmolality, temperature, etc.), 2 - formation of gradients around the functionally active cells. These are the gradients of medium components, which are formed due to the opposite direction of flows of exometabolites excretion and nutrients absorption, temperature gradients, cell heat generation, pH gradients, etc. and finally, the presence of growth inhibitors and promoters in medium for particular representative of microorganisms.

The results of the present work allow to assume that together with necessary nutrients for *Rh. diobovatum* IMB Y-5023 corn vinasse contains the inhibitors of yeast growth. Thus, after its dividing into fractions, the components of heavy fraction of medium did not "provide" the effective formation of SDS, i.e. the intensity of biomass accumulation. Thus, despite the slight differences in the lipid content in fraction and heavy fraction, the growth intensity of *Rh. diobovatum* IMB Y-5023 differed strongly (Fig. 2,3).

In conclusion, therefore, yeast *Rh. diobovatum* IMB Y-5023 can grow on media rich in lipids, and corn vinasse after preliminary preparation (dividing by flotation or separation and bringing of pH up to 5 - 6.5) can be used for industrial production of yeast biomass rich in carotenoids and other biologically active substances.

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