Agronomy Research **13**(4), 992–1001, 2015

Yeast Physiological State Influence on Beer Turbidity

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Abstract. The physiological state of yeast affects the intensity of propagation and biosynthesis of secondary metabolites and determines the sensory profile of beer. Besides that, yeast with low physiological activity increases the number of colloidal particles in beer, which consist of proteins and polysaccharides. The purpose of this study was to select a method for assessing the physiological state of yeast and to study the influence of yeast physiological state on the adsorption of compounds that determine the colloidal stability of beer. As a result of comparative analysis of different methods for determining the non-viable and weakened cells with dyes a certain correlation between them was found. The highest correlation (R^2 = 0.84) was set between parameters obtained by staining yeast cells by methylene blue with Safranin O, which stains both dead and weakened cells and dihydrorhodamine 123, which detects only physiologically least active yeast. Sufficiently high correlation (R^2 = 0.83) was observed when cells were stained with methylene blue, which identifies only dead cells, and with methylene blue with Safranin O. The worse the physiological condition of yeast, the less they adsorb turbidity-inducing compounds, so more substances remain in beer, reducing its stability. Thus, the increase in the proportion of damaged and non-viable cells from 24.2 to 32.2% leads to increased beer turbidity from 1.5 to 3.3%. Z-potential of yeast cell walls determines their sorption properties. Physiologically active yeast cells are able to adsorb positively charged colloids due to the negative charge of their surface. Activation of the yeast's surface potential occurs in the presence of oxygen.

Key words: yeast, Z-potential, colloids, staining methods.

INTRODUCTION

Beer represents a complex colloidal system with particles from 0.01 to 3 microns in size. Bad conditions of beer storage cause integration of colloidal particles by their condensation and polymerization. Formed deposit cause colloidal instability of beer and problems with its realization. Chemical, physical, fermentative and mechanical ways of impact on colloidal system of beverages are widely used in modern brewing production for the purpose of product stability increase. The choice direction is defined by the Specific objectives facing the brewer define the choice direction. Some chemical ways reduce oxidizing processes speed in beer. For this purpose, brew masters use antioxidants, interacting with oxygen from air and preventing oxidation of phenolic beer compounds. Physical and chemical ways are making it possible to remove colloids of

various natures by means of adsorbents. In particular, silica gels remove the haze forming proteins and polyvinyl piralidone decreases the concentration of phenolic compounds. Besides, such technological operations as separation and filtration could increase colloidal stability of beer (Meledina, 2003). Antioxidants interact with oxygen from air and prevent oxidation of phenolic substences. Adsorbtion is physical and chemical way directed on removal of colloids of various nature. For extraction colloids from beer, filtration materials are used. The most popular filtering material is diatomaceous earth of various brands having wide range of particles size (Meledina, 2003). Besides at this stage various adsorbents of colloidal particles are used (Nikolashkin, et al., 2004; Rinqwaer, et al., 2010). In particular, silica gels remove proteins and polypeptides (Rehmandji, et al 2000; Evans, 2001). Brew masters use polyvinilpirrolidon (PVPP) for long storage beer production (up to 12 months) (Mcmurrough, et al 1997; Siebert, 1999; Mc Keown, et al., 2003). PVPP represents polyamide and contains NH-groups, interacting with phenolic substances due to formation of hydrogen bonds. As a result, part of phenolic substances removal, Protein and tannic compoundes form fewer complexes during beer storage because of part of phenolic substances removal. Besides, there are less condensation and copolymerization, leading to tannoid formation. In particular, silica gels remove haze-forming proteins and PVPP decreases the concentration of phenolic substances. Such technological operations as a separation and a filtration increase beer stability (Burrell, to et al., 1994; Meledina, 2003; Dedegkaev, 2011).

All listed processing methods of beer physical and chemical stability increase are rather well studied. However, now the role of a physiological condition of yeast in this process is not well known. Contents of reserved carbohydrates (glycogen and trehalose) which are the source of endogenous glucose used during the period log growth phase (the adaptation period), sterol and non-saturated fatty acids biosynthesis which are the most important components of cellular membranes or the speed of carbon dioxide release and oxygen consumption can determine physiological activity of yeast cells.

It is necessary to mention the test of ‘the acidulation force’ (Patino et al., 1993) and the modified Davydenko's method (Davydenko, 2012) among efficient practical methods of yeast physiological condition assessment. Besides, various dyes staining methods can estimate physiological activity of microorganisms (Selivanov, 2003; Smart et al 1999; Mailloux et al. 2008). Visible light or fluorescent microscopy determines the particular staining methods (Van Zandycke et al., 2003). The method of dead cells identification by means of the methylene blue is widely used. Living yeast cells reductases restored the dye to non-coloured substances; dead cells are blue (Smart, 1999). Staining by methylene blue, tannin and safranin gives fuller information on physiological condition of cells. Safranin O and some other dyes are also efficient for this purpose. The histone forming chromatin leaves the DNA surface and phosphoric groups of DNA molecules and reacts with safranin and other dyes during an active transcription of a certain site of DNA. Thus, active process of a transcription and active metabolism correlate. The methylene blue stains cytoplasm proteins (Selivanov, 2003).

Magnesium salt 1-anilino-8-naftalen of sulfonic acid (Mg-ANS) is widely used for yeast cells viability. Getting into the yeast cell, Mg-ANS forms fluorescent complexes with cytoplasm proteins (Jenkins et al., 2003; Van Zandycke et al., 2003). Dead cells have fluorescent luminescence.

It is possible to reveal the existence of the active oxygen forms in yeast cells by means of digidrorhodamine 123 (Fluka). Digidrorhodamine oxidizes to the fluorescing rhodamine (Henderson et al., 1993). Accumulation of free radicals (ROS, reactive oxygen species) in the yeast cell reflects to its aging, or stress conditions. In both cases, the cells are physiologically poorly active. The digidrorhodamine 123 method allows ROS status estimation by quantity fluorescing (red or green) cells. Unlike methylene blue, which stains already dead cells, rhodamine stains cells which are in poor physiological condition.

The purpose of this work was to choose yeast physiological condition assessment method by cells staining and microscopy and to study the yeast strains genetically based features on yeast physiology in normal and stress conditions and its influence on beer haze indicators.

MATERIALS AND METHODS

Research objects were lager yeast strains 34/70 from Hefebank Weihenstephan collection, strain Y-3194 selected in the research center of 'Baltika Breweries'–Part of Carlsberg Group St Petersburg (Davydenko et al., 2008, Davydenko, et al., 2010) and the strain which is widely used in the European brewing which we designated as X.

Beer production was carried out by Mini Brewery plant system (Beraplan Harter) with cylindro conical tanks (CCTs) containing 120 l of 12° beer wort at 14°C with 100% barley malt according to classical beer production technology, including mashing, boiling, wort filtration, fermentation, yeast cropping, beer stabilization and filtration (*Нарµисс*). Wort (12°P), contained 160 mg l^{-1} of free nitrogen and 8.6% of sugars.

Beer turbidity was measured by Haffmans Hazemeter at red light (650 ± 30 nm) with formazin calibration standard. Particles smaller than 1µm (such as proteins, polysaccharides and polyphenols) were measured at 90Âº. Particles larger than 1µm (yeast cells, diatomaceous earth etc.) were measured at 25Âº.

Particle size of particles in beer was estimated by Nanotrac U2313ES (Microtrac Inc.).

Microscopy of yeast cultures was performed using an Axioskpo MOT Microscope (Zeiss); preparations were photographed using an Axiocam video camera.

Zeta potential measurements were performed with a Brookhaven Instruments NanoBrook ZetaPALS. For enhanced sensitivity, the motion of the charged protein is probed with so-called phase analysis light scattering (PALS). In traditional Laser Doppler electrophoresis, the frequency shift in the scattered light that arises due to the motion of the scatters determines the motion of the proteins.

Centrifugation was executed by Sigma 2-5 centrifuge.

Methods of cell's staining

In order to choose the most informative method of yeast physiological conditions assessment and existence of dead cells in yeast population, the results of cell's from different generations of strain 34/70 staining by both lifetime and fixed staining methods, were compared.

Techniques of cells staining by methylene blue

Methylene blue was prepared as follows: 0,01g methylene blue and 2.0 g dihydric citrate were dissolved in 10 ml of the distilled water, filtered and volume was adjusted to 100 ml. For definition of quantity of dead cells, one drop of yeast suspension and solution of a methylene blue (0.01%) was subjected on glass slide and in 2 min the calculation of total number of cells and quantity of the blue dead cells was carried out. We counted yeast cells in five vision fields, total counted of cell number not less than 500. Yeast cells were counted in five vision fields; total count of cell number was no less than 500. The percent of dead cells was calculated accordingly to Smart et al (1999).

Technique of cells staining by methylene blue and safranine O

Yeast was centrifuged for 5 min at 4000 rpm, washed by physiological solution and centrifuged again. Further yeast suspension concentration of 10^7cells ml^{-1} was then prepared. The drop of yeast suspension was dried at the room temperature on the fat-free glass. After that, it was fixed in flame and filled with methylene blue solution and maintained 4 min at the room temperature. Then dye was washed away by warm water and filled in by freshly prepared 5% tannin solution for 2 min. Dye was washed away under a water stream and felled with 1% solution of safranin and maintain for 16 min and then the dye was washed away. Microscopy was carried out at 400 multiplication, non-fluorescent oil was used (Selivanov, 2003).

Technique of yeast cells staining by dihydrorhodamine

2 mg of a dihyidrorhodamine 123 (Fluka) were dissolved in 1.0 ml 0.1M Tris buffer (2 amino-2-(hydroxy-methyl)-1,3-propanedinol) pH 8.0. Yeast suspension was centrifuged within 5 min at 4000 rpm and then yeast suspension of 10^7 cells ml^{-1} was prepared (Henderson et al., 1993).

Technique of yeast cells staining by magnesium salt 1-anilino-8-naftalen of sulfonic acid (Mg-ANS)

300 mg of Mg-ANS (Sigma) were dissolved in 2 ml of ethyl alcohol and added to 98 ml of sterile water. Solution can be stored at 4°C in glass bottles (dark glass) within 6 months (Jenkins et al., 2003). 0.5 ml of 0.3% Mg-ANS solution was added to 0.5 ml of yeast suspension and incubated for 5 min at 25°C. Then dead (yellow-green fluorescence) and living cells were calculated.

RESULTS AND DISCUSSION

In current research, the investigation of various yeast strains and generations and determination of correlation between different staining methods by means of which it is possible to estimate a physiological condition of cells, was carried out. Yeast was stained

by methylene blue, methylene blue together with safranin, and digidrorhodamine. Partially weakened cells were detected using digidrorhodamine and methylene blue with safranin; dead yeast stained by methylene blue, Mg-ANS and methylene blue with safranin. As a result of comparison of different methods the highest coefficients of determination were found between methylene blue with safranin and digidrorhodamine and between methylene blue, and methylene blue with safranin O, $R^2 = 0.84$ (Fig. 1) and $R^2 = 0.83$ (Fig. 2) respectively. R^2 shows reliability of linear dependence between the studied indicators.

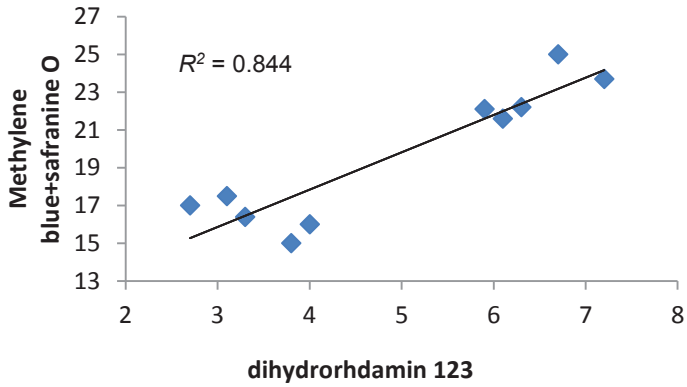

Figure 1. Linear relationship between Methylene blue + safranine O and digidrorodamin 123 staining methods.

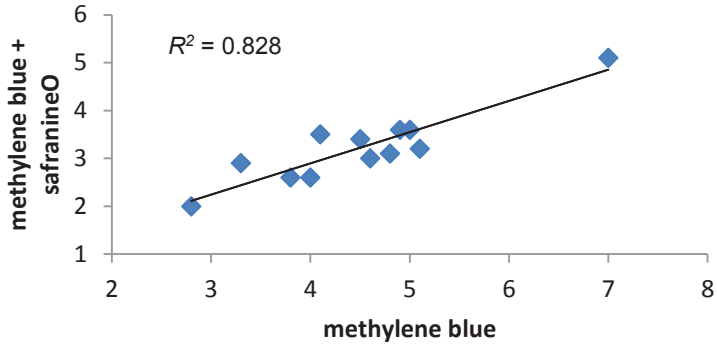

Figure 2. Linear relationship between methylene blue + safranine O and methylene blue methods of staining.

Coefficient of determination for Mg-ANS and methylene blue with safranin O was $R^2 = 0.63$ (Fig. 3). Lowest coefficients of determination was between methylene blue and Mg-ANS, $R^2 = 0.43$ (Fig. 4). Further, methods of staining cells by methylene blue and methylene blue with safranin O were used for yeast physiological condition assessment.

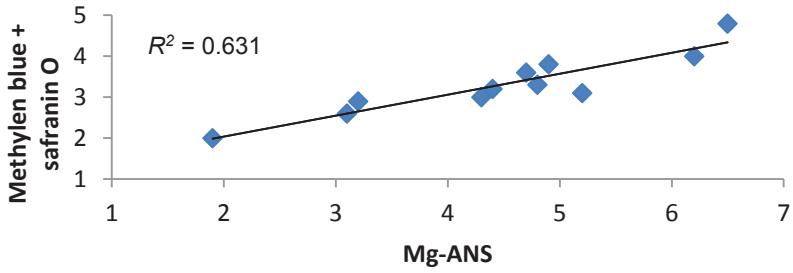

Figure 3. Linear relationship between methylene blue + safranine O and Mg-ANS methods of staining.

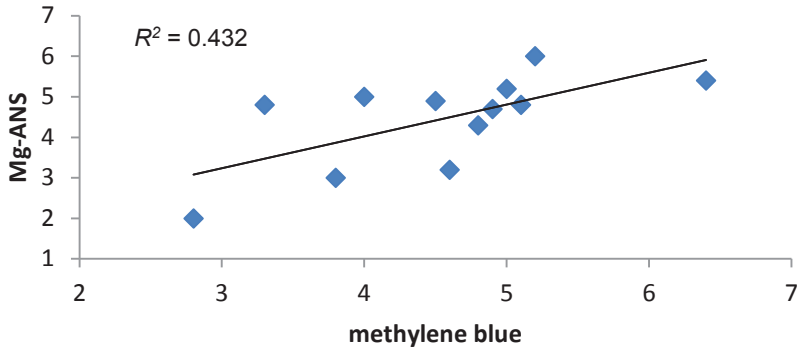

Figure 4. Linear relationship between Mg-ANS and methylene blue methods of staining.

Adsorption of the colloidal particles on yeast surface and their sedimentation with flocculating cells makes the main changes of fermenting wart colloidal system during fermentation are connected with. Zeta-potential of yeast cellular wall defines sorption properties of yeast. Physiologically active yeast due to negative charge of their surface is capable to adsorb positively charged colloids, such as proteins and phenolic substances (Table 1).

Table 1. Change of quantity of the colloidal particles of a different chemical composition during beer fermentation

Duration of fermentation process, days	Quantity of particles, % of total of particles		
	proteins (B)	phenolic substances (FS)	dextrins (D)
0	55 ± 4	25 ± 1	20 ± 4
3	33 ± 5	32 ± 3	35 ± 2
10	36 ± 4	22 ± 3	42 ± 3
14 (ready beer)	29 ± 3	14 ± 5	57 ± 6

The reduction of their concentration in the fermenting wort in parallel increases a share of particles partly formed by dextrins. As a result, in the course of fermentation zeta-potential value of cells falls from 46 mV to 23 mV (Table 2), thus concentration of 0.01–1.0 μm colloidal particles (Fig. 5), i.e. those particles, which influence beer turbidity, decreases. In general, the number of small and big colloidal particles decreases during fermentation.

In parallel, cells zeta-potential decreases and the superficial potential of colloidal particles increases. Thus, the potential of 0.01–0.10 μm particles increased from 37 to 49 mV and of 0.1–1.0 μm particles potential changed from 33 to 41 mV.

When using yeast with low physiological activity adsorption of the proteins and phenolic substances, which are the reason of low colloidal stability of beer decreases.

Table 2. Superficial potential of yeast (strain 34/70) and colloidal particles change in the course of wort fermentation

Fermentation time, days	Superficial potential of particles, mV		
		Range of the colloidal particles, microns	
		0.01–0.1	0.1–1.0
0	46 ± 2.3	37 ± 1.8	33 ± 1.0
2	37 ± 1.5	37 ± 1.5	33 ± 1.2
4	31 ± 0.3	39 ± 1.5	35 ± 0.8
6	29 ± 0.5	41 ± 1.8	38 ± 1.3
8	26 ± 0.02	43 ± 2.0	41 ± 0.9
10	24 ± 1.2	45 ± 1.5	41 ± 1.0
12	23 ± 0.5	47 ± 2.3	42 ± 2.2
14	23 ± 1.0	49 ± 2.3	41 ± 1.9

The physiological condition of cells decrease, if yeast has any stresses: osmotic and hydrostatic pressure, sharp fluctuations of temperature etc. It is possible to judge it based on trehalose segregation from cells (Attfield et al., 1992; Eleutherio et al., 1993), glycerol content in beer (Dedegkaev et al., 2005) and concentration increase of dead and weakened cells. Data showing decrease of the number of colloidal particles during wort fermentation (Table 3).

Table 3. Change of concentration of 0.01 to 1.0 μm colloidal particles during wort fermentation of yeast strain 34/70 $|p| \leq 0.15$.

Fermentation time, days	Number of particles	
	Range of the colloidal particles, microns	
	0.01–0.10	0.10–1.0
0	24500	137
2	24300	120
4	23000	105
6	22500	87
8	22000	85
10	21700	80
12	21200	74
14	20000	73

In order to study the influence of yeast cells physiological condition on adsorption of colloids defining beer turbidity, 7 samples of the yeast were received after beer fermentation. In these samples by means of staining cells by methylene blue and safranin the maintenance of partially weakened and dead cells was estimated (Table 4). Further, subsequent to centrifugation yeast was washed 3 times with water. After each centrifugation, turbidity of washing water was measured by Haffmans Hazemeter. Then total relative turbidity was counted in terms of 1 g of biomass with a mass fraction of solids 22% (damp yeast), for this purpose, we summarized values of a turbidity of all three washouts and divided into amount of the washed-out yeast.

Table 4. Influence of a physiological condition of yeast on a turbidity of beer and adsorption of colloids

Sample	Beer turbidity, EBC ($\|p\| \leq 0.10$)	Relative turbidity of washing waters, biomass EBC g^{-1} ($\|p\| \leq 0.10$)	Results of cells staining by methylene blue and safranin, % ($\|p\| \leq 0.20$)		
			good shape	partially weakened	dead
1	1.5	42.0	75.8	20.5	3.7
2	3.3	11.9	67.8	19.6	12.6
3	2.0	17.5	73.1	18.6	8.3
4	1.9	51.0	75.1	20.7	4.3
6	1.91	47.6	75.5	21.0	3.5
7	2.32	22.7	74.5	19.0	7.5

The less normal physiological conditions of yeast, the less they adsorb the haze coursing substances and as a result, more substances remain in beer that raises its turbidity. Least of all cells in a good physiological shape in the second sample (67.8%) and this beer has maximum turbidity – 3.3 units of EBC. However, not only a physiological condition of yeast, but also features of various strains in certain conditions can influence adsorption of colloids. Influence of colloidal particles concentration in beer depending on particles size was investigated in case of two yeast strains. Y-3194 and X strains were used for 12% wort fermentation with 15 ± 2 million cells ml^{-1}.

Table 5. The characteristic of colloidal particles in beer, using different yeast strains ($|p| \leq 0.10$)

Strain	Hydrostatic stress	Average size of particles, μm	Interval of fluctuation	Concentration of particles, million ml^{-1}
Y-3194	no	5.6	4–9	0.24
X	no	4.7	4–10	0.22
Y-3194	yes	8.2	4–15	1.10
X	yes	8.3	3–13	2.30

There was no difference in number and in size of colloidal particles in beer fermented by two strains (Table 5). However, increase of pressure during fermentation up to 2 bars affected adsorption of colloids by various strains differently. As a result, the quantity of the colloid particles in beer when using strain X was twice higher, than in the beer made by Y-3194 yeast. It testifies that yeast X, is less resistant against hydrostatic stress. Under the influence of various factors hydrophilic colloids that are present in beer, coagulate. Large colloidal particles start reflecting light, causing opalescence. Then particles integrate with each other, so that become visible, and beer becomes turbid.

Different substances form haze in beer – proteins, polysaccharides, polyphenols, etc., as different sorbents – absorb colloidal complexes from beer.

CONCLUSIONS

It is possible to draw a conclusion that all methods of staining have rather exact correlation among themselves, and if necessary, they can substitute each other or be used in total to obtain accurate results. During beer production, it is possible to use only methylene blue method to determine yeast cell's physiological activity. However, staining with methylene blue with safranin gives an opportunity to divide cells on physiological activeness–weakened and dead. This is important from practical point of view, since it is possible to activate the weakened cells, for example, by addition of wart. When using only methylene blue one estimates both weakened and not viable cells as dead.

Falling of zeta-potential of cells causes, on one hand the reduction of colloidal particles concentration during fermentation and on the other hand–increase in their charge. Decrease of physiological status of cells leads to colloids adsorption reduction and increase of beer turbidity.

In the absence of stress (during fermentation), the concentration of colloidal particles depends on the strain used in beer production. However, in stressful conditions, for example at elevated pressure, different strains are capable to adsorb various colloidal particles in different quantity. Thus, yeast stress resistance influence the turbidity of beer.

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