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CHARACTERISTICS OF SELECTED FEATURES OF BREWING YEASTS IN ENVIRONMENTS CONTAINING T-2 TOXIN

Barbara Foszczyńska[1], Ewelina Dziuba[1], Joanna Kawa-Rygielska[1], Joanna Chmielewska[1], Maria Wojtatowicz[2]
[1] *Department of Food Storage and Technology, Agricultural University of Wrocław, Poland*
[2] *Department of Biotechnology and Food Microbiology, Agricultural University of Wrocław, Poland*

ABSTRACT

The influence of various concentrations of T-2 toxin on the growth ability and fermentation activity of brewing yeasts was examined. Four cultures of top and bottom fermenting yeast strains were maintained in YEPG medium containing T-2 toxin at the concentration of 2,5; 5; 10; 15; 20 i 50 μg·ml^{-1}. The maximum specific growth rate (μ_{max}) and biomass yield were determined. Concentrations of 5, 10 and 15 μg T-2·ml^{-1} were selected for the fermentation of malt wort. During top (20-22°C) and bottom fermentation (12-14°C), the following were examined: physiological condition of yeasts, fermentation dynamics and the degree of utilisation of amino acid nitrogen and the extract. It was observed that the presence of T-2 toxin in the medium resulted in lowered specific growth rate and biomass yield. T-2 toxin had an unfavourable influence on the physiological condition of yeasts and disrupted the mechanism of the intake of extract components, including amino acid nitrogen, which affected the dynamics of fermentation. Top fermenting yeasts, esp. *S. cervisiae* 46 strain, were more susceptible to T-2 toxin than bottom fermenting yeasts.

Key words: T-2 toxin, brewing yeasts, yeast growth, fermentation.

INTRODUCTION

The grain of brewing barley is contaminated with filamentous fungi, among which the most dangerous group is composed of toxin-producing "field" fungi (e.g. *Fusarium graminearum, F. culmorum, F. sporotrichoides*) and "storage" fungi (eg. *Aspergillus ochraceus, A. flavus, A. parasiticus, Penicillium sp.*) [3,6,7,10,12]. These microorganisms produce various toxins, such as: deoxynivalenol (DON) and its acetyl derivative (Ac-DON), diacetoxyscirpenol (DAS), T-2 toxin, zearalenone (ZEA) and its derivatives, aflatoxins, ochratoxins, patulin and cytochlasin [4,5,10].

Numerous mycotoxins are characterised by high thermostability, thanks to which they penetrate into food products, e.g. malt and beer, and pose threat to human health [4,8,14,15,17]. As mycotoxins may accumulate in the organs, the symptoms of poisoning may appear even a few years after the infection. T-2 toxin is especially dangerous because it

attacks human bone marrow and may cause death [10]. Although the concentration of toxins in grain or beer is generally low, it may be increased depending on the weather conditions during vegetation season or as a result of improper conditions of grain storage.

Contamination of brewing raw materials with mycotoxins may lead to disruptions in the process of malt and beer production. The results of experiment malting indicated that T-2 toxin inhibited germination, delayed the synthesis of proteins and weakened the synthesis of alpha-amylase [16]. DAS was characterised by similar effect, whereas contamination with DON was less disrupting. The presence of mycotoxins in malt wort may influence the activity of brewing yeasts. Boiera *et al.* [1, 2] examined the influence of deoxynivalenol (DON) and fumonisin B_1 (FB_1) on the growth of brewing yeasts. Top fermenting yeasts were more sensitive to DON toxin than bottom fermenting yeasts and their growth was inhibited within the first 6 hours of incubation. The presence of FB_1 in growth medium resulted in growth inhibition of bottom fermenting yeasts.

The aim of the study was to examine the influence of various concentrations of T-2 toxin on the growth ability and fermentation activity of top and bottom fermenting brewing yeasts.

MATERIAL AND METHODS

T-2 toxin (Sigma Aldrich) and the following yeast strains:

- *Saccharomyces cerevisiae I-S.c./46*
- *Saccharomyces cerevisiae I-S.c./57*
- *Saccharomyces carlsbergensis I-S.ca./13*
- *Saccharomyces cerevisiae (lager*) 23

were used as an experiment material.

S. cerevisiae 46 and *57* and *S. carlsbergensis 13* yeasts were obtained from the collection of microorganism cultures of the Institute of Agricultural and Food Biotechnology in Warsaw. The strain *S. cerevisiae (lager) 23* was isolated at the Department of Fermentation Technology, Agricultural University of Wroclaw, from a 48 h culture of dried brewing yeast Saflager S-23 (Lesaffre Bio-Corporation Ltd.).

The following were performed in the study:

- Assessment of the yeasts' growth ability in the presence of T-2 toxin at the concentrations of 2.5, 5, 10, 15, 20 and 50 $\mu g \cdot ml^{-1}$ YEPG medium. T-2 toxin was introduced to the medium as ethanol dilution. Yeast culture in a YEPG medium without the toxin was used as control sample. The culture was conducted in a Bioscreen C turbidimeter (Labsystem) at 25°C;
- Assessment of the process and results of fermentation of 12% malt wort with 5, 10 and 15 μg T-2 $toxin \cdot ml^{-1}$. Top fermentation was conducted for 5 days at 20 – 22°C, whereas bottom fermentation was conducted for 10 days at 12 – 14°C.

The following were determined in the study:

- Maximum specific growth rate (μ_{max}) of the yeast during logarithmic growth phase in YEPG medium;
- Maximum biomass yield ΔOD_{max};
- The physiological condition of yeasts during wort fermentation, expressed as budding index I_p, i.e. the count of budding cells in active cells;
- The diameter of yeast cells during fermentation using a laser particle size analyser Mastersizer 2000. Weighted mean value of the diameter of cells seen by the analyser as spherical objects was calculated for selected phases of the process;
- Fermentation dynamics (the percentage of CO_2 produced during the phases of fermentation compared to its total amount were calculated);
- Extract content (refractometer method). The degree of extract consumption (primary extract – final extract) / (final extract) · 100% was calculated for selected phases of fermentation;
- The content of alpha-amine nitrogen (FAN). The degree of nitrogen compounds consumption (primary FAN content – final FAN content) / (primary FAN content) · 100% was calculated for selected phases of fermentation.

After fermentation, ethanol content, real extract content and real attenuation were determined (Electronic Beer Analyser DSA 48, courtesy of Namysłów Brewery laboratory).

RESULTS AND DISCUSSION

Results of earlier studies, both unpublished [11] and published [9], were used for the selection of brewing yeasts for the present study. Strains with different fermentation activity and other features were selected: two top fermentation strains: *S. cerevisiae 46* and *57* and two bottom fermentation strains: *S. carslsgergensis 13* and *S. cerevisiae (lager)23.*

The assessment of the influence of T-2 toxin on the cell metabolism was began with a culture of brewing yeasts in model media (YEPG without the toxin and at toxin concentration of 2.5, 5, 10, 15, 20 and 50 $\mu g \cdot ml^{-1}$) where the specific growth rate and maximum biomass yield were determined.

The strains used in the study differed significantly in the rate and effects of growth in a medium contaminated with T-2 toxin. At a growing amount of toxin, the growth rate and biomass yield were lower (Table 1). The strain *S. cerevisiae 46* was the most sensitive and at 15 $\mu g \cdot ml^{-1}$ of toxin, due to low growth rate, it had the biomass yield almost 50% lower than in the control sample. The strain *S. cerevisiae (lager) 23* was the least sensitive. At T-2 toxin concentrations from 5 to 20 $\mu g \cdot ml^{-1}$ it was characterised by the highest specific growth rate and relatively lower decrease of biomass yield. The results show that T-2 toxin inhibits the growth of *Saccharomyces* yeasts, similarly to another toxin from the same group, i.e. diacetoxiscirpenol (DAS) [18]. An addition of DAS to the growth medium at 5 $\mu g \cdot ml^{-1}$ resulted in 55% reduction of cell count. At the concentration of 10 µg DAS$\cdot ml^{-1}$ the reduction was 62% and the vitality of cells was reduced as well. Deoxynivalenol (DON) significantly inhibited the yeast growth only when its concentration was 50 $\mu g \cdot ml^{-1}$.

Table 1. Selected features of the growth of brewing yeast in the medium contaminated with T-2 toxin

Parameter	Yeast strain	Concentration of T-2 toxin [µg·ml^{-1}]						
		0	2.5	5	10	15	20	50
Specific growth rate μ_{max} [h^{-1}]	46	0.19	0.02	0.04	-[1)]	-[1)]	0.03	-[1)]
	57	0.12	0.11	0.08	0.06	0.05	0.05	0.04
	13	0.22	0.16	0.08	0.05	0.02	-[2)]	-[2)]
	23	0.12	0.10	0.10	0.08	0.07	0.07	0.05
Biomass yield ΔOD_{max}	46	1.18	1.0	1.0	0.58	0.63	0.87	0.26
	57	1.26	1.25	1.21	1.17	0.96	1.11	0.59
	13	1.54	1.53	1.51	1.41	1.20	-[2)]	-[2)]
	23	1.23	1.17	1.10	1.01	1.01	1.05	0.94

[1)] no specific growth rate of *S. cerevisiae 46* was calculated because of very weak growth of this strain
[2)] no culture for *S. carlsbergensis 13* at the concentration of 20 i 50 µg T-2·ml^{-1} was conducted because of very low growth rate in the medium with 15 µg T-2·ml^{-1}

The results of the cultures of brewing yeast strains used in the study were used for the preparation of fermentation samples. Concentrations of T-2 toxin: 5, 10 and 15 $\mu g \cdot ml^{-1}$ were used for the contamination of malt wort. The lowest concentration (2.5 $\mu g \cdot ml^{-1}$) did not have a significant influence and the highest concentrations (20 and 50 $\mu g \cdot ml^{-1}$) brought about changes in yeasts metabolism during growth in a model medium which were too big.

During fermentation, the physiological state of yeasts was examined. In the present study it is presented as a ratio between budding and active cells (budding index I_p), as this fraction is mostly responsible for the rate and effectiveness of fermentation.

The inocula of the brewing yeasts used in the study, which were introduced to the wort, had various budding indices I_p. The population of *S. cerevisiae (lager) 23* yeast was characterised by the highest I_p index value (2.34). *S. cerevisiae 57* strain had the lowest I_p index (1.02). The culture of the control sample (no toxin in the wort) made it possible to examine the budding ability of used strains during fermentation. Based on the data (Table 2), it may be assumed that the cells of *S. carlsbergensis 13*, *S. cerevisiae 57*, and especially *S. cerevisiae 46*, budded intensively at the beginning of fermentation. The inoculum of *S. cerevisiae (lager) 23* introduced to the fermentation had lots of daughter cells that had already come off the mother cells. In the next stages of fermentation, the budding was less intensive as there was less dissolved oxygen. Similar observations were made in case of worts with the toxin, but at its lowest concentration the differences in relation to control group were small. The increased concentration of T-2 toxin in the wort resulted in decreased budding because the presence of the toxin, most probably, limited the intake of oxygen by the cells [13].

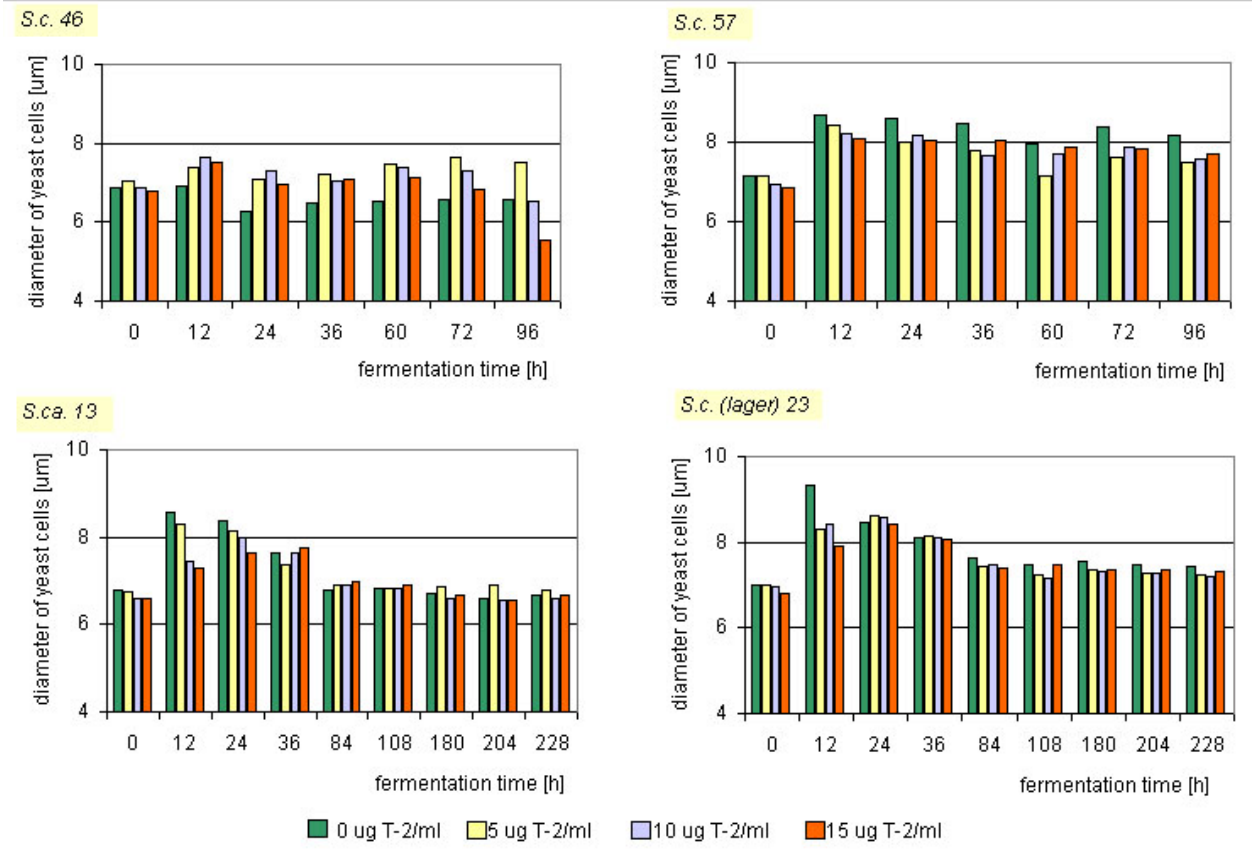
Table 2. Physiological state of yeasts (I_p index – ratio between budding and active cells) during fermentation of wort contaminated with T-2 toxin

Time [h]	Concentration of T-2 toxin [µg·ml^{-1}]															
	0				5				10				15			
	Yeast strain															
	46	57	13	23	46	57	13	23	46	57	13	23	46	57	13	23
0	1.51	1.02	1.54	2.34	1.51	1.02	1.54	2.34	1.51	1.02	1.54	2.34	1.51	1.02	1.54	2.34
12	3.79	1.59	2.0	0	3.23	1.19	2.03	0	0.81	0.66	0.58	1.01	0.57	0.53	0.40	0.25
36	1.24	0.96	0.79	0.85	1.02	0.89	0.83	0.85	0.62	0.77	0.45	0.66	0.68	0.69	0.70	0.37
60	0.70	1.60	1.30	0.82	0.72	1.81	1.07	0.57	0.36	0.35	0.77	0.47	0.30	0.29	0.62	0.37
84	0.25	0.25	0.93	0.58	0.78	0.04	0.77	0.29	0.12	0.25	0.35	0.25	0.11	0	0.45	0.17
108	0.14	0.20	0.46	0.20	0.11	0	0.27	0.17	0.08	0	0.17	0.11	0.04	0	0.39	0.28
180	-	-	0.07	0.16	-	-	0.04	0.22	-	-	0.04	0.10	-	-	0.09	0.14
204	-	-	0.06	0.12	-	-	0.08	0.08	-	-	0.05	0.08	-	-	0.06	0.11

During the fermentation process the diameter of yeast cells were measured because the size of a cell ready to bud, or of a cell already budding is increasing at this moment.

The changes of cell size during fermentation in control groups (without the toxin), except *S. cerevisiae 46* strain, were typical (Fig. 1). Higher diameters at the beginning of fermentation were related to intensive budding and coming off of the buds from mother cells. The cells of *S. cerevisiae 46* strain budded intensively during the first 12 hours of the process, probably due to better oxygen metabolism, and young cells in wort with some amount of ethanol and limited oxygen content in further stages of the process were unable to grow and bud. This is confirmed by weak growth of the cells of this strain in model medium. The diameter of the cells of the other strains used in the study was decreasing and was similar until the end of turbulent fermentation, which was consistent with low budding index.

Fig. 1. Changes of yeast cell' diameter during fermentation of wort contaminated with T-2 toxin



As in the budding process, with an increased concentration of the toxin in the wort, the cells were smaller and smaller. The strain *S. cerevisiae 46*, characterised by stable size in the control sample, increased its size after the toxin was added and it was similar to the size of the other strain of top fermenting yeast strain, i.e. *S. cerevisiae 57*.

The relations between the intensity of budding and the size of cells observed in the process indicate the difficulty of cells to grow in fermentation environment. During fermentation, with an increasing level of the toxin in wort, the cells were becoming smaller and were losing their ability to bud.

The physiological condition of yeast in a given environment determines the ability of cells to bud and grow. However, in case of industrial strains, the process, dynamics and final results of fermentation are equally important. Good fermentation and proper organoleptic characteristics of beer depend on the use of amino acids and the components of wort extract by the yeasts. The process of fermentation in control groups was characterised by typical changes of the compounds, no matter what yeast strain was used. Contamination of the wort with T-2 toxin weakened the brewing yeast's ability to uptake of amino acid nitrogen and other extract compounds. Significant differences between the sensitivity of the yeast strains used in the study to T-2 toxin were observed.

The T-2 toxin inhibited the use of amino acid nitrogen at a concentration as low as 5 $\mu g \cdot ml^{-1}$ (Table 3). The top and bottom fermenting yeasts decreased the use of free nitrogen compounds in the wort when the concentration of toxin was increased. At the begining of fermentation of contaminated worts, the degree of the use of amino acid nitrogen by top fermenting yeasts was from 1% to 6% (except strain *S. cerevisiae 57* at 15 $\mu g \cdot ml^{-1}$ toxin), and it was ca. 40% in the control group. Such lowered ability to use nitrogen resulted in significant amounts of amino acid nitrogen remaining in the fermented wort, which may negatively influence the organoleptic features and stability of beer. The strain *S. cerevisiae 46* was the most sensitive to T-2 toxin. The other strain of top fermenting yeasts, *S. cerevisiae 57*, was characterised by relevantly lower sensitivity and at the toxin concentration of 10 and 15 $\mu g \cdot ml^{-1}$ it was characterised by the best utilisation of nitrogen among all yeast strains used in the study.

Table 3. The use of amino acid nitrogen [%] by brewing yeast strains during fermentation of wort contaminated with T-2 toxin

Time [h]	Concentration of T-2 toxin [$\mu g \cdot ml^{-1}$]															
	0				5				10				15			
	Yeast strain															
	46	57	13	23	46	57	13	23	46	57	13	23	46	57	13	23
12	40	35	8	4	1	4	5	2	2	6	2	2	4	22	1	2
60	69	70	53	52	28	49	43	36	17	49	31	30	9	42	21	28
84	71	73	72	58	36	53	56	36	25	51	40	35	19	44	28	28
end	72	74	75	65	39	54	68	39	27	53	53	37	21	46	45	29

The yeasts differed in their utilisation of extract components. The T-2 toxin weakened the yeasts' ability to utilise the components of wort extract (Table 4). The utilisation of extract was lower with increased concentration of T-2 toxin. It was from 7% to 19% at the beginning of fermentation in the control samples and 0% in the wort with the highest content of T-2 toxin. During fermentation, the yeasts partially regained their ability to utilise extract (except strain *S. cerevisiae 46*), but after fermentation the utilisation of extract in worts with T-2 toxin was lower than in the control group. Strain *S. cerevisiae 46* was the most sensitive to T-2 toxin and at toxin concentration as low as 5 $\mu g \cdot ml^{-1}$ utilised only 45% of the extract, whereas the utilisation in the control wort was at the level of 56%.

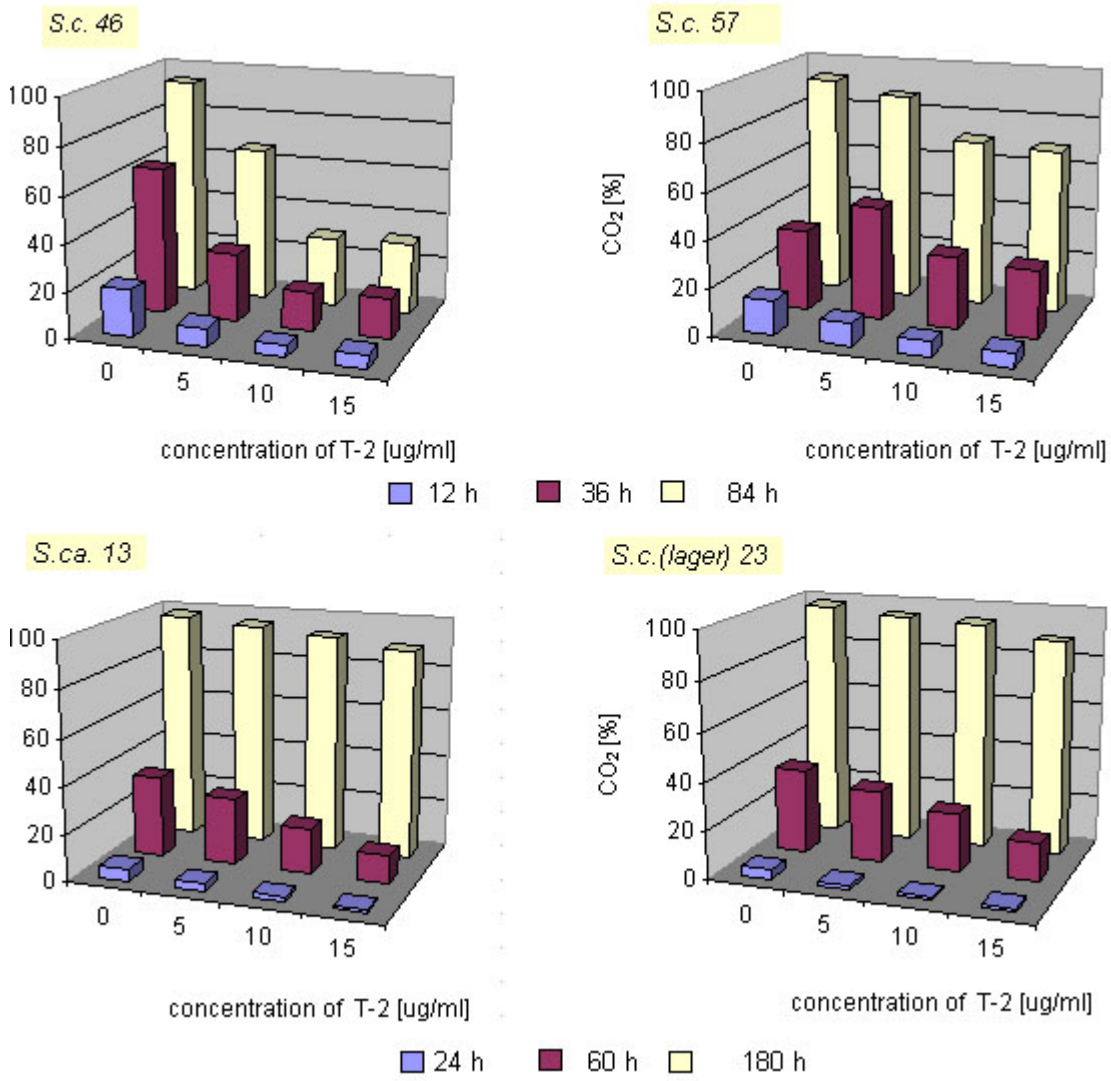
Table 4. The use of extract [%] by brewing yeast strains during fermentation of wort contaminated with T-2 toxin

Time [h]	Concentration of T-2 toxin [$\mu g \cdot ml^{-1}$]															
	0				5				10				15			
	Yeast strain															
	46	57	13	23	46	57	13	23	46	57	13	23	46	57	13	23
12	19	16	7	7	6	11	3	3	2	2	1	0	0	0	0	0
60	52	55	40	42	35	51	34	40	14	46	27	40	2	2	19	38
84	55	58	56	56	44	52	54	53	23	48	43	46	14	47	34	41
end	56	57	66	66	45	55	65	65	35	51	63	63	26	49	63	61

The dynamics of fermentation during the process determines the ability of cells to adapt to the environment. The T-2 toxin had various influence on the fermentation activity of bottom and top fermenting yeasts (Fig. 2). The toxin

concentration increasing from 5 to 15 μg·ml^{-1} resulted in slower fermentation, although in top fermenting yeasts the rate was back to normal at the end of fermentation. The amount of CO_2 produced was similar to that in the control group and was from 89% to 98%. The top fermenting yeasts were characterised by lower dynamics of fermentation of contaminated worts at the end of the process. It was mainly the strain *S. cervisiae 46* which at 10 and 15 μg·ml^{-1} toxin was characterised by weak dynamics during all the process of fermentation.

Fig. 2. Dynamics of fermentation of wort contaminated with T-2 toxin with the use of brewing yeasts



The physiological changes of yeasts, resulting from the contact with wort containing T-2 toxin, influenced partly the final results of fermentation (Table 5). Irrespective of the toxin concentration in the medium, top fermenting yeasts utilised less extract than bottom fermenting yeasts and left more of it in the wort, which resulted in lower attenuation and lower ethanol content. This may have been influenced by various conditions of fermentation depending on the strain of yeasts. Fermentation was conducted at temperature typical for top and bottom fermenting yeasts, 20-22°C and 12-14°C, respectively. As a result, the metabolism was fast in top fermenting strains and slow in bottom fermenting strains. Bottom fermenting strains retained the ability to assimilate the components of wort better than top fermenting strains. Most probably, longer period of contact with wort contaminated with the T-2 toxin enabled them to adapt to the environment through a mechanism of detoxication or degradation of the toxin into less harmful compounds [1,2,17]. As a result, even at the highest T-2 toxin concentration used in the study (15 μg·ml^{-1}), the degree of fermentation and ethanol content were only slightly different than those in the groups without the toxin. More detailed research on the composition of environment during fermentation is necessary to understand these problems.

Table 5. The effects of fermentation of wort contaminated with T-2 toxin by used brewing yeasts

Feature	Yeast strain	Concentration of T-2 toxin [μg·ml^{-1}]			
		0	5	10	15
Ethanol content [% v/v]	46	5.55	5.32	5.18	-[1)]
	57	5.43	4.83	5.20	-[1)]
	13	5.63	5.69	5.81	5.81
	23	5.60	5.68	5.70	5.70
Real extract content [%]	46	4.59	4.00	4.61	-[1)]
	57	3.61	5.21	4.61	-[1)]
	13	2.57	2.87	2.90	3.10
	23	2.77	2.92	3.13	3.41
Real attenuation [%]	46	64.6	66.9	63.0	-[1)]
	57	69.6	58.4	63.1	-[1)]
	13	77.1	75.2	75.4	74.1
	23	75.6	74.9	73.6	71.8

[1)]no value were obtained because of inncorect read of beer analyser

CONCLUSIONS

It needs to be indicated in conclusion that the changes observed proved a negative influence of T-2 toxin on the morphological and physiological features of the yeasts. The sensitivity to the toxin was an individual feature of the yeasts. The presence of T-2 toxin in the growth and fermentation medium resulted in:

- lower specific growth rate and lower biomass yield during the growth;
- worsened physiological state of the population of yeasts, i.e. significantly lower count of budding cells;
- morphological changes due to which the cells had problems in regulating their size depending on the conditions of environment.

The changes should be attributed to T-2 toxin which negatively influenced the mechanism of the intake of extract components, especially of amino acid nitrogen. This worsened the fermentation dynamics although, in most strains used in the study, the dynamics by the end of fermentation was similar to that observed in samples without T-2 toxin. The strain *S. cerevisiae 46* was especially sensitive to the presence of T-2 toxin in the medium, even if its concentrations were low.

Thus, the presence of T-2 toxin may be dangerous for the proper process of fermentation of brewing wort. It should be remembered that disruption of yeasts' metabolism results the changes of organoleptic features of beer. Apart from ethanol and CO_2, yeasts produce many other compounds which are commonly referred to as by-products of fermentation. Because obtaining harmonised taste and smell of beer depends on the accessibility of cells to amino acid nitrogen and carbohydrates, the presence of T-2 toxin in wort may bring about worse taste of beer.

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Barbara Foszczyńska
Department of Food Storage and Technology,
Agricultural University of Wrocław, Poland
Norwida 25, 50-375 Wrocław, Poland
phone: (+ 48 71) 32-05-237
fax: (+ 48 71) 32-05-273
email: bfoszcz@wnoz.ar.wroc.pl

Ewelina Dziuba
Department of Food Storage and Technology,
Agricultural University of Wrocław, Poland
Norwida 25, 50-375 Wrocław, Poland

Joanna Kawa-Rygielska
Department of Food Storage and Technology,
Agricultural University of Wrocław, Poland
Norwida 25, 50-375 Wrocław, Poland

Joanna Chmielewska
Department of Food Storage and Technology,
Agricultural University of Wrocław, Poland
Norwida 25, 50-375 Wrocław, Poland

Maria Wojtatowicz
Department of Biotechnology and Food Microbiology,
Agricultural University of Wrocław, Poland
C.K. Norwida 25, 50-375 Wrocław, Poland
Fax. 4871- 3284124
Phone: 48-71-3205117
email: mwojt@wnoz.ar.wroc.pl
