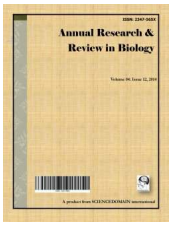

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The Use of Ozone-air Mixture for Reduction of Microbial Contamination in Grain Brewing Raw Material

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Authors' contributions

This work was carried out in collaboration between all authors. Authors LT, MR and AT designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors RZ, LP and AA managed the analyses of the study. Authors OL and EO managed the literature searches. Authors TZ and LT involved in constant monitoring of the experiment, data analysis. All authors read and approved the final manuscript.

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ABSTRACT

Considering the fact that different types of microbiological impurities of grain brewing raw materials (barley and malt) significantly influence the organoleptic properties of beer and leading to expressed consumer rejection, the efficient ways of preventing mycotoxins from entering the wort, and eventually the finished beer are offered. The influence of various ways of sterilization of grain

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brewing raw materials has been experimentally studied. The effectiveness of the processing method is estimated on the change of the standardized microbiological indicators of microbial population of the surface and deep layers (endosperms) of barley and malt. The Influence of ozone-air mixture to internal (sub epidermal) and surface (epiphytic) micro flora of brewing barley and malt is investigated. The technological scheme of sterilization and periodic drying of malt in a fluid bed of grain raw materials and the function chart of automation of the process are offered. The recommendations for granaries of the brewing enterprises equipped with installations of drying and sterilization are provided. The optimum concentration of ozone -air necessary for sterilization of grain and technological capacities of beer production line is also recommended.

Keywords: Grain disease; microbiology quality; sterilization and drying unit; ozonation; sterilization in "boiling bed".

1. INTRODUCTION

The effect of grain brewing raw material (barley and malt) is the determining factor of quality and safety of beer. Due to the high risk of infection and contamination of barley and malt, the traditional processes of brewing cannot provide the required quality and safety for beer. It was determined that the microbial biota of various sort of barley and other grain varieties have a little difference [1]. These grains commonly contain particular counts of bacterial species, filamentous fungi, yeasts and mold as concluded by the extensive research carried out from 1980-1990. Thus, Clarke J.H., Hill S.T. [2] and Flannigan B. [3] determined that fresh barley, gathered from the field, contained predominantly *Erwinia caratovora* and *Xanthomonas campestris* bacteria, but dried barley used for malting, contained Pseudomonadaceae, *Bacillus spp*. and micrococcaceae. Yeast content occupies the second place of all viable microorganism count in barley grain. The effect of microorganisms in the grain has a negative impact on the quality of malt, wort, and beer. For example, after 24 hours of malt mashing the count of microorganisms increases significantly. These microorganisms reproduced and grew not only in the malt but on the equipment walls and packing materials. Moreover, it caused bad smell and storage flavor, contaminated with toxic products (aflatoxins, ochratoxin A, tentoxin, deoxynivalenol and others with a total concentration of 130 mg/g) [4].

The existing classic brewing technology does not provide required safety and quality of the finished product above all due to the high risk of infection and contamination of brewing grain. The investigations of [5,6] revealed that the micro biota of different varieties of barley and other cereals is largely similar and usually prevails with a limited number of bacteria, filamentous fungi, yeasts, and molds. The most prominent effect of the impact of barley and malt micro biota to the beer is the gushing effect, which is caused by the presence of *Fusarium* (*F. Graminearum* and *F. moniliforme*) in the cereal and leads to an increase in the total nitrogen content in the wort and Formalin nitrogen in wort and beer [7] and is capable of producing mycotoxins DON (deoxynivalenol) [8]. Gushing effect in beer is also stimulated by the microorganisms producing ZEA (zearalenone). Studies of Czech brewers [9] showed that aflatoxin content in light and dark Czech beer depends on the level of microbial contamination of grain. As is well known [10,11, 12,13], aflatoxins prevailing in barley during the storage, lead not only to severe toxicosis in humans but have been found to be carcinogenic.

Barley microbiota composition in a large part determines the quality of malt and beer [14,15]. More detailed description of barley microbiota and its impact on the quality of malt, semi products, and finished beer is shown in [16,17, 18].

Codex Alimentarius Commission adopted "CODEX GENERAL STANDARD FOR CONTAMINANTS AND TOXINS IN FOOD AND FEED" (CODEX STAN 193-1995), as well as EEC Regulation №1881 / 2006 setting maximum levels of certain contaminants in foodstuffs, including grain and beer. Mycotoxins monitoring in barley represents Hazard Analysis Critical Control Point, as currently, it is essentially the last test before grain drying and sending it to the silo for storage.

Currently a worldwide practice, grain contamination is defined by ergosterol content with a maximum allowable level not more than 14 mg/kg (CAC/RCP 51-2003) [19].

Since the problem of grain protection from mycotoxins is an international one, there is an urgent need to develop rapid methods for their

detection and neutralization. For example, the Charm Company (US) developed the method ROSA-test for determination of mycotoxins [20].

This method has several advantages compared to the enzyme-linked immunosorbent assay analysis which is widely used in national analytical practice. It should be noted that the rapid detection methods of mycotoxins are relevant to other countries. There was a relationship of aflatoxin accumulated in the malt, with their quantity in the beer [21].

Special investigations of the author [22] found that the microbiological contamination of black brewing raw materials significantly affects the organoleptic properties of beer and leads to a pronounced consumer rejection.

Attempts to sterilize grain by chemical methods of soil microbiota accumulated on the surface of the grain glume showed the failure of antibiotic treatment of grain before storing in a silo. In particular, in Denmark, antifungal fungicide treatment of fresh barley showed the opposite effect - an increase of *Fusarium* fungi [23].

It is known that microbial "field" contamination of grain brew in grain stores is enhanced by the products of birds and rodents lifestyle, which leads to the development of "grain storage diseases." Moreover, the microbiota produces toxic elements, is accumulated not only on glume but also in the endosperm [24].

Especially important is the fact of elevators "contamination" with a large number of different types of "storage fungi" which present, both in the air and in the dust and contaminate the newly incoming grain [24]. Excess grain moisture enhances the activity of microbiota contamination.

The technological way of reducing the increased grain moisture, which is provoking irreversible changes in the finished beer quality, is using extremely energy-intensive column grain dryers. However, the sustained movement of huge masses of grain allows removing the dust and the waste of insects and rodents, and only temporarily reduces the moisture of grain from critical to optimal [25].

It can be stated that thermal drying does not help to remove pathogenic micro flora of grain which will inevitably lead to infection of the wort and a reduced beer quality. In addition, it is known that it provokes rapid microbiota growth during the malting and poses difficulties for brewers in ensuring the required quality of beer [26].

Thus, an effective sterilization technology of the grain, which must be used at the stage of mash preparation, is coming to the forefront of the problems among the quality and safety assurance of the final product.

Data analysis of the methods of reducing microbial contamination of barley and malt showed the inefficiency of the main processing methods which include:

- Sulphureting of the malt at drying stage, used in particular in the American and British breweries;
- Blocking of the micro flora reproduction opportunities in the malt by drastically reducing its wetness below the level required for the reproduction of most common microorganisms in the malt, It is concluded [26], that storing the grain at low temperature does not inhibit the growth of the field fungi (including, *Fusarium*, *Cladosporium*). Moreover, the most common among the "storage fungi" *Aspergillus* fungus is adapted for reproduction on the grain surface at low moisture. In practice, it is difficult to achieve optimal conditions for the main grain storage - low temperature and reduced humidity;
- Creating a controlled atmosphere for grain storage, impeding the possibility of microorganisms reproducing by saturation with nitrogen (95±1%). This method is more effective compared to the refrigerated room [27], but non universal, as much of the malt microbiota are anaerobes and are able to reproduce without oxygen;
- Quartz treatment of the grain storage area (mercury-quartz lamps). The method leaves open the possibility of an occurrence of tolerant forms of microorganisms and may cause overheating of the upper layers of the grain.

Studying the bactericidal effect of quartz treatment (ozone emission from the air under the influence of radiation exposure) led us to the conclusion that ozonation as a method of disinfecting the grain is a perspective one.

2. MATERIALS AND METHODS

The objective of this work was experimentally studying the effect of different methods of sterilization of grain brewing (barley and malt) and developing the devices and processing methods that ensure the reduction of concentrations of toxic trace product - microbiota producers in the finished beer.

The modern technology of patent search and design simulation was used. The efficiency of processing methods was estimated by a variation of the standardized microbiological indicators of surface and deep layers (endosperm) contamination of barley and malt. According to the Sanitary Rules SanPiN 2.3.2.1078 (TR CU 021/2011), the microbiological indicators such as CFU of yeast and molds were determined by State Standard GOST 10444.12-88; and total viable count QMAFAnM (TBC) - by GOST 10444.15-94.

The total viable count was determined by a swab test method. A petri dish is used to provide a growth medium using a mix of agar. The suspension is either spread onto the surface of agar plates, or is mixed with molten agar, poured into plates, and allowed to solidify (pour plate method). The plates are then incubated under conditions that permit microbial reproduction so that colonies develop that can be seen without the aid of a microscope. It is assumed that each bacterial colony arises from an individual cell that has undergone cell division. Therefore, by counting the number of colonies and accounting for the dilution factor, the number of bacteria in the original sample can be determined.

3. RESULTS AND DISCUSSION

According to the conducted study, the effectiveness of the micronizing sterilizing treatment is visible in the reduction of the surface (epiphytic) colonization from 10 to 2000 times in the investigated samples. Microbial population in land (sub epidermal) layers decreased from 1000 to 20 times CFU 10^6/g, and also it is found that outsider (wild) yeast micro flora in micronized grain is completely destroyed.

The most stable were molds (*Alternaria*, *Aspergillus*, and *Penicillium*): after a 50-second exposure, their single colonies still presented. The number of CFU mycelial micro flora on the barley surface has decreased from $1{,}0\cdot10^3$ to $1{,}0\cdot10^1$, and inside the grain from $4{,}0\cdot10^3$ to $1{,}0\cdot10^1$ CFU/g. The corresponding data for malt are: in control samples epiphytic flora decreased from 1, $8\cdot10^3$ to 1, $0\cdot10^1$ and sub epidermal flora - from $2{,}0\cdot10^2$ to $1{,}0\cdot10^1$ CFU/g (Figs. 1,2). Our obtained data on barley and malt contamination were significantly below what was published in [16,17].

It is known that during the malting process the contamination of grain increases several times, and even after drying and sorting malt the molds count is increased four times.

On modern malt house, the dynamics of progressive infection is critical and even more threatening. The number of heterotrophic bacteria in malt within two days dramatically increases for 2000-times (to $1{,}3\cdot10^9$ CFU/g); most increased is the number of lactic acid bacteria (40 CFU/g before soaking up to $1\cdot10^8$ CFU/g, - after soaking); the number of Pseudomonas increases in 29000 times (up to $3{,}6\cdot10^8$ CFU/g); Enterobacteriaceae amount increases only 23 times ($3{,}0\cdot10^7$ CFU/g); yeast on dry barley increased 1300 times; the number of filamentous fungi increases in the finished malt 8 times (relative to the original amount of $2{,}0\cdot10^2$ CFU/g – in dry grain). These facts highlight the need for more effective microbiota control measures to prevent the contamination of the entire process line of beer production.

3.1 Methods of Elimination of Microbial Contamination of Grain and Malt

The above mentioned facts show the ineffectiveness of antimicrobial treatment of barley and malt stored in the silo. As we believe, the best way to prevent contamination of commercial beer by nitrosamines and mycotoxins is to prevent microbial growth during the grain storage and to sterilize just before cooking the mash.

The fact of ozone utilization in the food industry is not disputed [28]. It is known that ozone at concentrations in the air of 0.08-0.2 mg/l kills bacteria that cause decay of products and prevents the formation of mold and mucous deposition [29,30,31,32]. However, the existing methods do not provide "shoveling" of bulk material that is strictly necessary for disinfecting volume weight of grain products. The unit, developed by our team [33] (Fig. 3) consists of 4 functionally interconnected blocks: the block of air preparation, the block of ozone-air mixture preparation (OMP) and the block of drying and sterilization of malt and process control panel.

The unit operates in the following ways. Compressor 5 inhausts the outer air through the air intake apparatus 1 and the air handling block (including filter 2, drier 3 and cooler 4) and injects it into the receiver 6; with the possibility of air recirculation for the optimum properties: temperature not exceeding 10°C at a relative humidity of 10%.

The ozone air mixture (OMP) preparation line switches on directly during the treatment of malt by the commands from a central control panel, initiated by temperature and humidity sensors (-3 and -3) of silo 12. Thus ozone generator 7 feeds ozone directly into the air supply line from receiver 6 to receiver 9 before the ozone concentration in OAM receiver 9 reaches 10.0-15.0 mg/m3 on the testimony of the sensor and the set mixture pressure value (P2 probe). Immediate drying and disinfecting of malt in silo 12 is performed by compressor 11 O.V.S. from the receiver 9 to the system of the nozzles 16 (1+n), located under the bottom 12 of the silo. Alternation nozzles 16 (1+n) switching based on the principle of "traveling wave" causes spiraling displacement of grain malt by compressed OAM stream, creating the effect of "fluidized bed." Action time of each nozzle does not exceed 3-5 minutes. Exhaust air, taking away excessive moisture and dust particles, actively aspirated by exhaust vent line 13 equipped with a fan and a dust collector 14. «Stop» command is supplied by the control processor according to the readings of temperature sensor T3 and humidity sensor 3, which is installed over the surface of the malt of silo 12 indicates the normalization of storage options of grain products.

From the world grain storage practices [34] it is known that fungi, which is potentially capable of producing mycotoxins, are no longer reproduced on the grain with a moisture content lower than 14.5%, which is why the correct moisture of whole grain reaches below 14,5% (For malt = 12%) at drying. This necessitates the re-sterilization of malt. The criterion, which we propose to consider, are the parameters of humidity and temperature of grain and increasing it is necessary in order to control in automatic mode, instead of retrospective analyzes of the presence of mold contaminants.

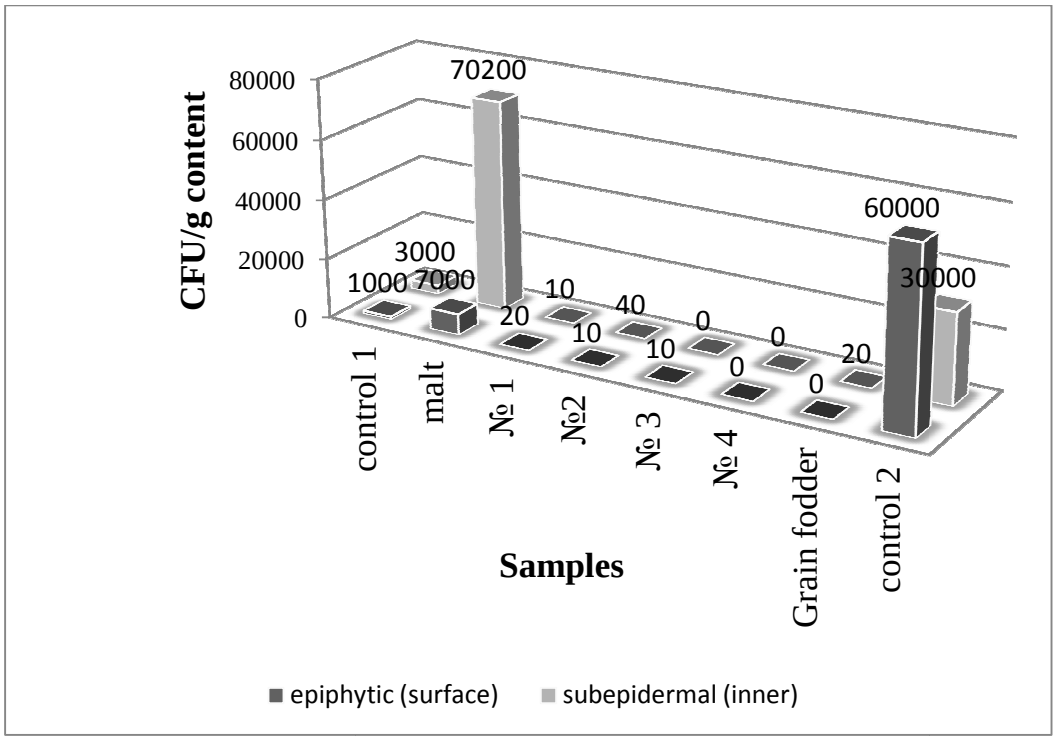

Fig. 1. Influence of micronization to the microbial population of barley and malt
Control 1– control for malt barley; Control 2– control for feeding grain

We recommended while the humidity of grain is of higher critical value (higher of 12%) and the temperature of stored grain is more than 20ºC we recommend to impact by providing to the malt the properties of "fluidized bed" for no more than 20 min, but without moving the whole grain mass from one silo to another. The concentration of ozone in OAM cannot exceed 1.0 mg per cubic meter of silo capacity (storage), and the exhaust air with residual ozone must be discharged to the environment through the filter-dust collector.

Another advantage of our developed method is the disinfection of product pipelines and tanks of brewing industry. However, the ozone concentration must be set up to 20 mg per cubic meter of disinfected storage capacity on the memory of control processor board on exposure for at least 10 min. When developing the program, we took into account the results of specialized studies (Table. 1) [30,31].

Latest global technologies show that effective disinfection of beer tanks is achieved at ozone concentrations of 40 mg / m3 and 7.5 minutes of exposure.

In order to prevent the negative effect of ozonation on staff, we have decided to reduce its current concentration to 20 mg per cubic meter of capacity with a double increase of exposure up to 20 minutes, which will provide natural ozone decontamination during its contact with the surface of the container. The unit allows automated control of malt condition and sends the alarm to the control board for implementation of corrective actions.

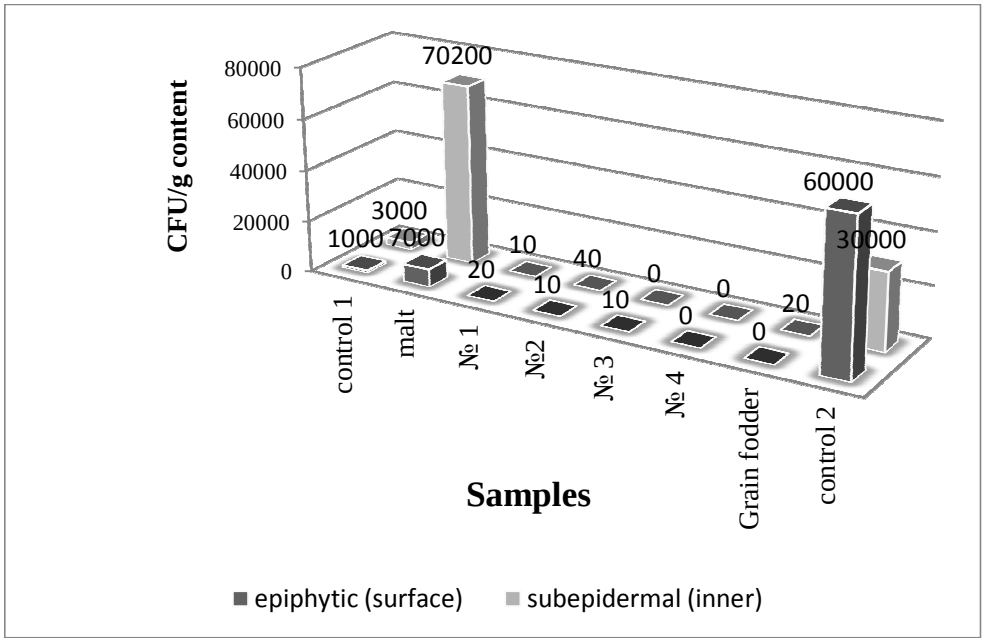

Fig 2. Microflora composition of samples (yeast, fungus)

Table 1. Effect of testing culture and treatment time to the ozone concentration

Testing-culture	Ozone concentration, mg/m^3			
	15 min	25 min	35 min	45 min
Coliform bacillus E.coli	30	10	10	5
Dysentery Sh.sonnei	20	10	7.5	7.5
Salmonella S.java	20	20	10	10
Anthracoid B.cereus	30	20	15	10
Staphylococcus St.aureus	30	20	7.5	7.5
Disease yeasts, *Saccharomyces, Candida, lactic-acid and acetic acid bacterium Lactobacillus, Sarcinia Micrococcus,*	30	20	10	7,5

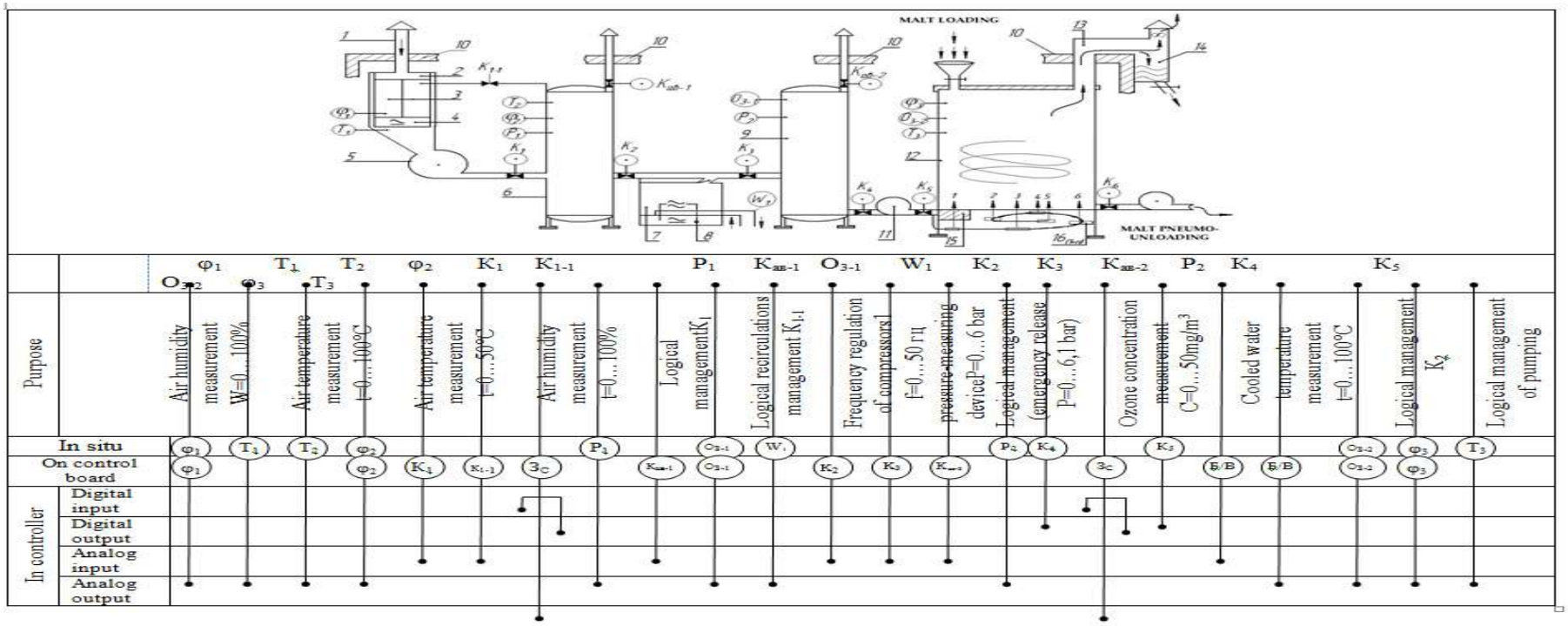

Fig. 3. Unit scheme of drying and sterilization of grain products and functional scheme of process automation

Symbol identification:
1 - air-intake device; 2 – filter;
3 – air drying container; 4 – air freezing device;
5 – compressor; 6 – receiver; 7 – ozone generator; 8 – water-cooled discharged electrode; 9 – OAM receiver; 10 - roof of the building; 11 - OAM to silo compressor; 12 – malt silo; 13 - exhaust ventilation system; 14 - dust pan; 15 - sequential reclosing unit of nozzles, transporting OAM to silo; 16 – (1+n) –OAM to the silo 12 transporting nozzles.
Sensors: 1,2,3 – air humidity; - air temperature; - air pressure in receiver; -1,2 – ozone concentration sensor, mg/m^3; – temperature sensor of cooling water electrode; K-1,2,3,4,5,6 – electromagnetic remote controlled taps; – emergency valves on the lines of releasing of pressure in receivers

The proposed scheme of drying and sterilization of grain products provides only a temporary effect since it is known that the existence of "grain storage warehouse" microbiota inevitably re-infects malt, as soon as the relative humidity exceeds the minimum critical level (for malt 12%) and the malt must be inevitably re-sterilized. The criterions for the periodic (re) sterilization are the humidity and temperature of grains, which are controlled in automatic mode. We offer this criterion instead of control of mold contaminants presence, which is currently practiced. It must be acknowledged that the criterion is retrospective.

During the practical application of this unit for disinfecting malt, it is highlighted that the problem of catching and recycling of malty hulling bran which accumulates up to 10% of processed malt in dusters. However, as the main byproducts during purging of barley are small trash, husks, and dietary fiber, then purging of the silo with brewing malt, the malty hulling bran is accumulated in the dust collector that is not a waste collector. Malty hulling bran is an important source of vitamins and minerals, so it is proposed for use in the preparation of the mash.

4. CONCLUSION

The analysis of microbiological contamination factors of grain that significantly affect the quality of beer showed the following:

- To prevent the microbial contamination of brewing raw materials on the process line is almost impossible, and the extreme diversity of microorganisms are associated with the microbiota of the field (grain) pollution, storage microorganisms, with the most dangerous of silo microbiota;
- The diversity of contaminants of barley and malt makes it economically impractical for a microbiological inspection of incoming raw materials in the breweries;
- Reducing the relative humidity of the grain up to 5% as a method of preventing microbial contamination is not economically justified and technically impracticable. The optimal storage of malting grain is under the humidity of not more than 12%, and microbiota growth is recommended to neutralize the antimicrobial effect of ozonation, which is not as effective as micronization, but less energy consuming;
- The ability to reproduce microbiota in wet (over 12%) malt during storage requires periodic sterilization of grain brewing raw materials, as well as sterilization directly before cooking the mash;
- Equipping each brewery company with ozone sterilization unit will reduce the total toxicity of finished beer by preventing the entry of mycotoxins to the wort, and then to the finished beer.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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