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Utilization of Chlorine dioxide to Reduce *Fusarium* sp Growth on Malt

One of the long term issues the malt producers and brewers deal with is contamination of barley and malt with Fusarium mould. Fusarium sp. contributes to beer gushing, formation of red grains, production of several carcinogenic toxins and other negative phenomenons. Different methods are used to reduce Fusarium sp. growth but none of them is fully effective. This presentation describes a concept with Chlorine dioxide (ClO_2) added during steeping and germination of barley. Laboratory, semi industrial as well as industrial trial proved functionality of ClO_2 application on malt during steeping and germination to push back Fusarium sp. Particularly, use of chlorine dioxide during germination was found to have most impact on controlling Fusarium growth. This application has also positive impact on gushing, red grains and it does not influence quality of the produced malt. No residues of ClO_2 and its by-products were found in the malt and there were no quality changes in the wort produced from malt treated with ClO_2.

Descriptors: *Fusarium*, chlorine dioxide, barley, malt, gushing

1 Introduction

The *Fusarium* moulds (*Fusarium graminarum, Fusarium culmorum* etc.) are considered to be the most important malt contaminants [10]. *Fusarium* sp. can attack grains already on the field and their further growth can be boosted during (incorrect) warehousing, steeping and germination [32]. The frequency of contamination is gradually increasing and it is very much dependent on wheather conditions [20]. Also the previous crop and agronomic practices (ploughing or non-ploughing after the harvest) play an important role [9], as do the variety of barley and application of fungicides. This knowledge enables development of tools for predicting the risk of early gushing from harvest [2].

Fusarium moulds cause failures during the germination process because they take away nutrition from the germ and furthermore, mycotoxins produced by *Fusarium* sp. can be harmful to the grain itself. These moulds are known to produce enzymes, causing undesired changes in the grain and thus having a negative impact on its quality. The contamination of barley by *Fusarium* sp. is accompanied by formation of so called red grains [7, 8] which are considered to be one of the key parameters in malt quality assesment. *Fusarium* moulds are toxic for brewer's yeast and by influencing fermentation, deviations in taste and colour have been noted. Some of the toxins produced by *Fusarium* sp. can be not only toxic, but are also mutagenous. Moulds like *Alternaria*, *Stemphylium*, *Fusarium*, *Aspergillus*, *Penicillium* and *Nigrospora* contribute to gushing [1, 11, 12], on barley and on wheat malts [5, 3]. The chemical composition of substances produced by moulds and the reason for gushing is not yet fully clarified, but most of them are protein type. A detailed study was performed on a substance produced by *Nigrospora* and marked as Nigrospora gushing faktor (NGF). This protein factor with molecular weight 16 500 and isoelectric point 4,0 was hydrophobic, it was active after 2 hours of boiling and even proteolytical enzymes did not reduce its activity [15]. Fungispumins are proteins with surfactant properties produced for example by *Fusarium culmorum.* They don't induce gushing directly, but contribute to its intensification. Furthermore, the gushing potential seems to be related to the content of fungi metabolites and ergosterol [28]. Moulds known to contribute to gushing also have ability to produce pathogenic compounds and substances causing premature yeast floculation (PYF) and supression of yeast growth [17, 24].

As stated above *Fusarium* moulds are also a source of toxins. The level of barley contamination by mycotoxins can be influenced by treatment of the grains after harvest as well as during storage. *Fusarium* sp. growth can be reduced by application of fungicides, but these can also put stress on moulds resulting in hyperproduction of mycotoxins. Therefore, new types of more effective fungicides have been developed [14]. Some strains of *Fusarium* sp. produce highly toxic mycotoxins, as for example deoxynivalenol (DON), nivalenol (NIV), HT-2, T-2 toxin and zearalenon (ZON) [20]. Also the role of deoxynivalenol-3-glycoside (DON-3-Glc) has been investigated. *Fusarium* mycotoxins are relatively stable and usually are not degradated during technological operations, therefore can easily be passed to the final malt product. *Fusarium* sp. is able to grow and produce mycotoxines during barley storing, steeping, germination, and even during kilning [32]. Part of the mycotoxins are removed with steeping water, but further growth can occur during germination [16]. The content of mycotoxins is reduced, but not fully eliminated during kilning [19, 25, 31]. Vegetative moulds including spores and eventually being present on contaminated malt are killed during process of mashing, but some mycotoxins can even arrive to beer, thanks to their thermal resistance [32].

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Diverse countermeasure can be taken to push back gushing at different stages of malt and beer production processes [4]:

- Raw material selection with respect to mould contamination level
- Mold supression during malting (hot water, hydrogene peroxide, formaldehyde, ozon)
- Reduction of oxalates by adding calcium salts
- Wort and hopped wort filtration
- Shorter mashing and wort boiling times
- Maximum 80 °C mashing temperature (by infusion process)
- Prolonged lautering time
- Degrading gushing inductors by proteolytical enzymes
- Adding absorbents to beer (active carbon, caoline, nylon PVPP)
- Stripping with CO_2

Other meassures used by malt producers are:

- Sieves with bigger eyes for elimination of small contaminated grains – this messure may have a negative impact on the overall economical results)
- Proper removal of barley tailings during steeping
- Competitive fungi (Geotrichum candidum) during steeping

Also lactic acid bacteria can contribute to gushing reduction if added during the malting process [13, 18, 21, 26, 27].

Non of the above described measures however are completly effective. Therefore, Sealed Air's Diversey and the Research Institute for Brewing and Malting investigated Chlorine dioxide for reduction of *Fusarium* sp. growth during the malting process.

Chlorine dioxide (ClO_2) has a number of advantages over hypochlorite and other chlorine-based disinfectants. It doesn't form trihalogenmethanes (THM), adsorbable organic halogens (AOX) or chlorophenols, it acts against a wide range of microorganisms including funghi and spores, it doesn't leave residues, it is cost effective and it is easy to measure . This is why ClO_2 is commonly used as terminal disinfectant in the Food and Beverage industry as well in potable water treatment.

Chlorine dioxide is under normal conditions a gas which is usually prepared by mutual reaction of hydrochloric acid and sodium chlorite:

$$4\ HCl + 5\ NaClO_2 \rightarrow 4\ ClO_2 + 5\ NaCl + H_2O$$

Due to difficulte storage it is usually generated on-site and diluted from a stock solution (5–20 g/L).

2 Material and methods

2.1 Laboratory tests

Initial laboratory tests were performed in parallel at the Diversey R&D center in Mannheim (GER) and at the Research Institute for Brewing and Malting in Prague (CZ). Key target of the tests were:

- Verification of ClO_2 action against Fusarium
- Setting of minimum effective concentration
- Checking presence of ClO_2 residues in the treated grains

The performance of chlorine dioxide to reduce initial *Fusarium* on barley was investigated in suspension testing. Prior to treatment with chlorine dioxide, barley samples were artificially contaminated with cells of *Fusarium* by spraying and drying. A fresh stock solution of Chlorine dioxide was generated (approximately 60 mg ClO_2/L). Concentrations of chlorine dioxide in stock and use solutions were determined with a Dr. Lange test-kit (LCK 310/343) and photometric DR 2800 device. Chlorine dioxide use solutions were prepared by diluting the stock solution with aid of CEN hard water diluent (300 mg/kg $CaCO_3$). The neutralizing solution was based on sodium thiosulfate (5 g/l in ¼ strength of Ringer-solution). Experiments were carried out at ambient temperature and no soil on the basis of EN 1276. Chlorine dioxide use solutions for testing were determined to be 1 ppm and 10 ppm. Test runs were conducted as follows. In a test tube, chlorine dioxide solution (10 ml) was added to barley (1 g) and the start time of the experiment was recorded. Barley and chlorine dioxide were mixed by whirling for 10 seconds and then allowed to react for one hour. After treating the barley sample with chlorine dioxide solution for one hour, the solution was decanted. Shortly thereafter, a subsequent treatment for either 60 or 300 min with a fresh solution containing either 1 ppm ClO_2 or 10 ppm ClO_2 was performed. After completion of the second treatment, a portion of the mixture (1 ml) was pipetted into 9 ml of neutralizer and mixed. After 5 minutes reaction time, 1 ml of the neutralized solution was added to 9 ml Ringer diluent. A dilution series was prepared up to 1:10,000. From each dilution step, 1 ml (in duplicate) was plated on potato dextrose agar. Plates were incubated at 30 °C for 3–5 days. Grown colonies on plates were counted and expressed at CFU per gram barley. A control experiment using Ringer solution instead of chlorine dioxide was performed following the same procedure.

2.2 Semi-industrial trials

Initial laboratory tests proved good efficacy of ClO_2 against *Fusarium* at ca. 10 ppm in steeping water. Based on this finding we continued with a semi-industrial trial performed at a micro malting facility of the Research Institute for Brewing and Malting in Brno, Czechia (Fig. 1).

A comparison test with a batch of barley treated with chlorine dioxide in second steeping versus a control using tap water only was carried out. Harvested barley from a high load *Fusarium* batch was provided by Czech Malt and Brew Institute. Four perforated baskets each containing 0,5 kg of contaminated barley were steeped in pure water and four other baskets in water containing ClO_2.

A starting probe (parallel) was taken to determine initial *Fusarium* counts on barley. Barley (1 g) was whirled in Ringer-solution (10 ml) to detach cells. After thorough whirling ten-fold dilutions steps in sterile Ringer-solution were prepared. 1 ml sample in parallel from dilution steps 10^{-4}, 10^{-5} and 10^{-6} were poured with sterile potato agar medium. Chlorine dioxide treated barley was prior to dilution series once washed with Ringer-solution to remove residual Chlorine dioxide. Plates were transferred into a temperature-controlled (30 °C) incubation box and grown for 3–5 days until colony formation was observed. Colonies were

Fig.1 Steeping of contaminated barley in ClO_2 solution

Fig. 2 ClO_2 application in spray water during germination

counted and calculated to CFU/g. Baskets filled with barley were placed into the steeping/germination box (Fig. 1). The chamber was filled with town tap water to remove dust and dirt (first steeping). The first steeping was performed for 5 hours at about 14.5 °C. During this time the water content in exposed barley increased up to 31.8 %. After the first steeping, residual water was drained and parallel barley sample were taken to determine *Fusarium* counts. The steeped barley was then exposed to air until next morning (approximately 16 hours), and another (parallel) barley sample was taken to determine *Fusarium*. The second steeping was performed using water only (blank), or an aqueous solution of chlorine dioxide. The chlorine dioxide steeping solution was prepared from stock by diluting with town tap water. The chlorine dioxide concentration of the steeping solution was determined to be 11 ppm. Four boxes with 0.5 kg barley each were placed into a plastic container, which then was filled with 11 L of above mentioned chlorine dioxide steeping solution. The plastic container was transferred into the steeping/germination chamber and left for another 4 hour steeping period. The chlorine dioxide concentration was periodically monitored. It dropped within 4 hours steeping by approximately 27 % (data not shown). Barley samples were taken to determine residual cell load of *Fusarium* after the chlorine dioxide steeping. After second steeping with chlorine dioxide, the boxes were exposed to air in the closed chamber for another 20 hour period. The water level was determined to 38.1% and then adjusted to final 45.5 % after a third steeping with tap water for a period of 2.5 hours following standard process used by the Institute to compare different varieties of barley. A final parallel barley sample was taken to determine residual cell load with *Fusarium* prior to germination.

2.3 Industrial trials

For the industrial trials a malting plant equipped with stainless steel steeping tanks and germination aparatus was selected. A ProMinent CDVC generator with output 2000 g/h was installed for generation of Chlorine dioxide.

The initial focus of the trial was on treatment of steeping water. Same like during the micro malting test, barley was mixed with water treated with 10 ppm ClO_2. However, the ratios between barley and water were very different. In the industrial steeping tank it was 1:1 (56 m^3 treated water: 56 MT barley) while in the micro malting conditions it was 5,5:1 (11 L treated water : 2 kg barley). In spite of this the *Fusarium* sp. growth was partly reduced after the steeping (log 1–2), but then further growth was observed during germination, most likely due to activation of spores. As a consequence it was decided to increase total amount of available ClO_2 and to apply it during germination for elimination of *Fusarium* sp. growth at end of malting process. Chlorine dioxide concentration in the air was measured with GasAlert Extreme GAXT-V-DL (BW Technologies) and safety rules were set for operators working in germination area during ClO_2 application. The in-use concentration was increased to 20 ppm and instead of steeping water, water for barley spraying during germination was treated (Fig. 2).

As a side effect cost of treatment was reduced due to significantly smaller volume of water needed in germination.

During 2012 in total 3 trials were run with different malt varieties. Each trial included 2 batches treated with ClO_2 and 2 batches of the same variety sprayed with water without ClO_2. For those counts on Fusarium, malt quality parameters, number of red grains, and gushing potential were analysed according to methods described before. Residual ClO_2 was measured on-site by means of photometric test kit (Prominent Dulcotest DT1) in the green malt after last spraying and a quick drop of ClO_2 after application as well no increased residual values in the ready product was observed (data not shown). In order to analyse for known by-products of chlorine dioxide like chlorite, chlorate, and chloride, samples of green malt and malt after kilning 12 samples from two different lots (blank and ClO_2 treated) were selected. 30 g aliquot of a sample was put in a beaker and 100 g of deionized water were added. Samples were stirred for 10 min with help of a magnetic stirrer at room temperature. The solution was filtered via funnel and paper filter to remove dirt and particles and again filtered with help of a 0.45 µm pre-syringe filter to give a clear solution. For determination of Chlorite, Chlorate and Chloride Ion chromatography was carried out using a Methrohm IC with supp 7 Anion chromatography column and 3.6 mmol/L sodium carbonate as eluent. Chlorine dioxide was determined by UV spectroscopy at 360 nm (Chlorine dioxide handbook, publis-

hed by American Water Works Association in 1998, author Don Gates).

3 Results and discussion

Results from laboratory trials (Bojos variety) are presented in tables 1 and 2.

The treatment with chlorine dioxide compared to a control without chlorine dioxide demonstrated a clear reduction of *Fusarium* counts on barley. The efficiency of killing performance depends on the strength of chlorine dioxide and contact time. A first treatment with 10 ppm chlorine dioxide followed by 1 ppm chlorine dioxide in a second step resulted in less reduction in *Fusarium* counts than a subsequent second step with 10 ppm chlorine dioxide (Tab. 1). A treatment with 10 ppm chlorine dioxide at contact times of about 2–6 hours gave the highest reduction of *Fusarium* on barley (Tab. 2).

A comparison test with a batch of barley (Bojos variety) treated with chlorine dioxide in second steeping versus a control using tap water only was carried out in a pilot chamber. Results on cell counts of Fusarium are shown in table 3.

As shown in table 3, initial cell counts on dry barley increased during first steeping and subsequent aeration. Fungal spores were activated by the water to start germination and growing. Due to the presence of chlorine dioxide within the second steeping step

Table 1 Treatment of barley with Ringer solution (control) compared to chlorine dioxide solutions at varying chlorine dioxide concentration. First treatment for 60 minutes was followed by a second treatment with fresh solutions at two different contact times

Batch (ClO_2 as ppm)	Contact Time (min)	CFU/g after treatment	R (log batch control – log batch ClO_2)
Control (no ClO_2)	60 min	3.8×10^7	–
10 ppm + 1 ppm	60 min + 60 min	3.3×10^6	1.06
10 ppm+ 1 ppm	60 min + 300 min	1.3×10^7	0.47

R = reduction in viability

Table 2 Treatment of barley with Ringer solution (control) compared to chlorine dioxide solution at varying chlorine dioxide concentration. First treatment for 60 minutes was followed by a second treatment with fresh solutions at two different contact times

Batch ClO_2 Concentration (ppm)	Contact time (min)	CFU/g after treatment	R (log batch control – log batch ClO_2)
Control (0 ppm)	60 min	3.8 x 107	–
10 ppm + 10 ppm (2nd steep)	60 min + 60 min	1.1 x 104	3.58
10 ppm+ 10 ppm (2nd steep)	60 min + 300 min	5 x 103	3.88

Table 3 Fusarium counts on barley (average from duplicates) during steeping treatment with tap water (blank) or chlorine dioxide

Step	Blank (log CFU/g)	ClO_2 (log CFU/g)	Log reduction vs. Blank	Reduction vs. Blank (%)
start (prior to 1 steeping)	4.88	–	–	–
after 1st steeping (16 hours)	5.99	–	–	–
after 2nd steeping (4 hours)	4.84	3.66	1.18	93.4
after 2nd aeration (20 hours)	4.80	3.64	1.16	93.1
after 3rd steeping	4.62	3.50	1.12	92.6

Fusarium numbers on barley were reduced by more than 1 log (> 90 %) compared to the blank and stayed lower till end of steeping. Samples of malted barley produced from this batch were free from *Fusarium* (not shown).

Residual ClO_2 in the grains after steeping was measured spectrophotometrically and no increased values were indicated by spectrophotometer (data not shown). Treated barley, processed according to a quality parameter protocol of the Malting Institute (results shown in table 4). There were no deviations in malt and wort quality by samples treated with ClO_2 compared to samples steeped in pure water and the concentration of *Fusarium* – related toxins was partly pushed back as well.

During 2012 in total 3 trials were run in an industrial malting site and all of them gave similar results, proving positive effect of ClO_2 treatment on Fusarium contamination. The most significant improvement was achieved by strongly contaminated barley (Batyk variety) batch processed in October 2012. On batches treated with ClO_2 a significant drop in Fusarium counts were achieved compared to blank controls (Fig. 3).

Another focus parameter was number of red grains in ready malt (Tab. 5). The processed barley was free of red grains and ClO_2 significantly reduced their formation during steeping and germination.

Gushing was significantly reduced (by 62 %) as shown in table 6.

Malt and wort produced from barley treated with ClO_2 did not show any deviations compare to pieces sprayed with pure water (Tab. 7).

Residual ClO_2 was measured on-site by means of photometric test kit (Prominent Dulcotest DT1) in the green malt after last spraying and a quick drop of ClO_2 after application as well no increased residual values in the ready product was observed (data not shown). In order to analyse for known by-products of chlorine dioxide like chlorite, chlorate, and chloride, samples of green malt and malt after kilning 12 samples from two different lots (blank and ClO_2 treated) were selected and analyzed by means of IC and UV-photopectroscopy. There was no difference on chromatogrammes (and peak heights) for chlorine dioxide, chlorate and chloride of blank control samples compared to those from ClO_2 treated batches (data not shown), indicating no difference between ClO_2 treated and blank control batches.

Table 4 **Comparison of malt and wort quality by sample treated with ClO_2 and with pure water [6, 22, 23, 29]**

	Moisture content of barley (%)	Protein content of barley (%)	Moisture content of malt (%)	Extract of malt (%)	Mash method according to Hartong and Kretschmer VZ 45 °C (%)	Kolbach index (%)	Diastatic power (WK)	Final attenuation of laboratory wort from malt (%)	Friability (%)	β - glucan content of malt (mg/l)	Protein content of malt (%)	Total nitrogen of malt (%)	Soluble nitrogen of malt (mg/l)	Soluble nitrogen of malt (%)	Saccharide extract of malt (%)	DON (µg/kg)	DON 3 Glc (µg/kg)	ZON (µg/kg)
References	EBC 3.2	EBC 3.3.2	EBC 4.2	EBC 4.6	MEBAK 3.1.4.11	EBC 4.3.2	EBC 4.12	Mühlbauer, J.	EBC 4.15	Trojanowicz, M.	EBC 4.3.2	EBC 4.3.2	EBC 8.9.2	EBC 8.9.2		ČSN EN 12955*	ČSN EN 12955	ČSN EN 12955
Malt with ClO_2	x	x	4.6	82.0	39.9	47.1	350	78.6	84	103	11.7	1.865	878	0.878	76.5	450.4	751	12.1
Malt without ClO_2	x	x	4.6	81.6	39.9	48.2	341	78.7	86	99	11.6	1.853	894	0.894	76.0	505.6	853.4	19.1
Barley	12.6	12.2	X	X	x	x	x	x	x	x	x	x	x	x	x	1438.3	505.3	6.0

*ČSN = Czech technical standards

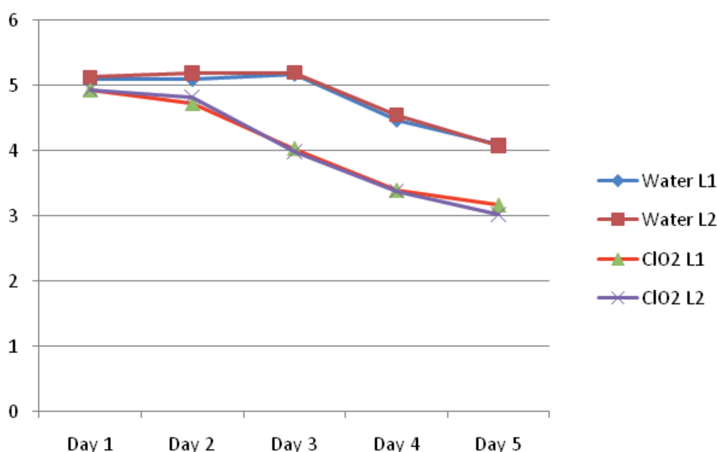

Fig. 3 **Comparison of Fusarium sp. counts with and without ClO_2 on lines L1 and L2 (y-axis is showing log cfu/g barley). Counts are averages of 5 individual samples from different place in germination boxes (standard variation was less than 5 %).**

Table 5 **Number of red grains in the samples of ready barley (200 g)**

Batch No.	ClO_2 treatment	Number of red grains
C12 L1 211012	No	35
C12 L2 211012	No	36
C12 L1 221012	Yes	16
C12 L2 221012	Yes	11

4 Conclusion

Laboratory, semi industrial as well as industrial trials proved functionality of ClO_2 application on malt during steeping and germination to push back *Fusarium* sp. Particularly, use of chlorine dioxide during germination was found to have most impact on controlling

Table 6 **Comparison of gushing potential by pieces sprayed with and without ClO_2 [*30*]**

Batch No.	ClO_2 treatement	Gushing (ml)
C12 L1 211012	No	24
C12 L2 211012	No	26
C12 L1 221012	Yes	11
C12 L2 221012	Yes	5

Table 7 **Malt and wort analysis [6, 22, 23, 29]**

	Sample No.			
Sample code	**C12L1 2 11012**	**C12L2 211012**	**C12L1 221012**	**C12L2 221012**
Type of water spraying	No	No	Yes	Yes
Moisture content of malt (%)	3.6	4.5	4.2	4.1
Extract of malt (%)	79.9	79.8	80.1	80.0
Mash method according to Hartong and Kretschmer VZ 45 °C (%)	47.1	45.7	47.5	48.2
Kolbach index (%)	40.8	40.9	41.7	41.2
Diastatic power (jWK)	294	368	377	381
Final attenuation of laboratory wort from malt (%)	79.2	79.5	79.6	79.8
Friability (%)	100	98	99	98
ß–Glucan content of malt (mg/l)	25	30	31	21
Protein content of malt (%)	12.6	12.8	12.8	12.9
Total nitrogen of malt (%)	2.01	2.04	2.05	2.07
Soluable nitrogen of malt (mg/l)	819	835	857	852
Soluable nitrogen of malt (%)	0.82	0.83	0.86	0.85
Saccharide extract of malt (%)	74.8	74.6	74.7	74.7

Fusarium growth. This application has also positive impact on gushing, red grains and it does not influence quality of the produced malt. No residues of ClO_2 and its by-products were found in the malt and there were no quality changes in the wort produced from malt treated with ClO_2. In the meantime use of chlorine dioxide as processing aid in this application has been approved by Czech Health Ministry under EU 1333/2008. National approval for other EU and non – EU countries has to be applied for using the existing approval by Czech Authority as a reference.

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