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REDUCTION OF DEOXYNIVALENOL (DON) USING XYLANOLYTIC ENZYMES DURING ALCOHOLIC FERMENTATION OF *FUSARIUM* CONTAMINATED WHEAT

SMANJENJE DEOKSINIVALENOLA UPOTREBOM KSILANOLITIČKIH ENZIMA TOKOM ALKOHOLNE FERMENTACIJE PŠENICE ZARAŽENE FUZARIJUMOM

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SUMMARY

*Deoxynivalenol (DON) in food and feed is considered as important safety and economic issue of growing concern. The cereal grains contaminated by Fusarium fungi may also be referred to as biological inhibitors. In recent years, the interest in biological detoxification of mycotoxins has increased due to the potential application of different strains of yeast and bacteria. This study was dedicated to evaluate the influence of new biotechnological means: xylanolytic enzymes in combination with traditional amylolytic enzymes on the efficiency of the alcoholic fermentation process and DON detoxification during fermentation of Fusarium contaminated wheat with high concentration of DON (*3950 μg/kg*). The results show that Fusarium contaminated wheat has a negative influence on alcoholic fermentation: the quantity of alcohol was 13.5% lower then in the case of wholesome grain fermentation and 73% of DON came into DDGS (Dried Distillers Grains with Solubles), usually used for feed. The application of a new combination of amylolytic and xylanolytic enzymes allowed to increase the concentration of alcohol in the broth by 35.3% and in the same way increased the efficiency of the fermentation process. By using this enzyme combination for cereal saccharafication, the highest degree (51.5%) of partial detoxification of DON was achieved during the fermentation process. However the residual DON concentration in DDGS was still too high despite its reduction as to use it in feed. Therefore, cereal material must be properly investigated before bioethanol production to avoid that higher amounts of mycotoxins come in the DDGS and onwards in feed.*

Key words: *deoxynivalenol (DON), Fusarium contaminated wheat, xylanolytic enzymes, alcoholic fermentation, Dried Distillers Grains with Solubles (DDGS).*

INTRODUCTION

Fusarium species are a common problem in cereal grains worldwide. These pre-harvest fungi not only degrade cereal quality and yield, but also contaminate the harvested grain with several mycotoxins such as trichothecenes (T-2, deoxynivalenol (DON) and others) [1]. They attract worldwide attention because of the significant economic losses associated with their impact on human health, animal productivity and trade [2]. *Fusarium* fungi on cereals are hard to master because their occurrence is mainly the result of weather conditions. The combination of moisture, temperature and relative humidity at time of flowering plays an important role for the occurrence of head blight or scab on e.g. wheat. The most ubiquitous of these fungal toxins is the trichothecene deoxynivalenol (DON), which occurs in wheat, barley and corn infected by *Fusarium* species such as *F. graminearum* and *F. culmorum*. DON inhibits DNA, RNA, and protein synthesis can cause haematic and anorexic syndromes in mammals as well as neurotoxic and immunotoxic effects. Although DON is not as toxic as T-2, HT-2 toxin and other type A trichothecenes, its occurrence and exposure is much bigger and not seldom. Surveys on the occurrence of DON showed that it is a common contaminant of wheat world wide. It was recently reported by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) [3] that out of 11444 tested samples of common and durum wheat 57 % was contaminated up till maximum levels of 30.000 μg/kg. Also the data collection on the occurrence of *Fusarium* toxins in food in the EU, performed within an ad hoc SCOOP project, showed a similar incidence of contamination among the over six thousand samples of wheat (common and durum) included in the report [4]. Therefore, regulatory limits for DON have been established in many countries worldwide, also in the EU [5]. The natural occurrence of DON in cereals and the influence of cereal processing too cereal products and the different decontamination possibilities of DON have therefore a great social and economical impact together with appropriate regulations to protect the health of the consumer. Cereal processing, that may involve physical, chemical or microbiological decontamination, can often be effective in destroying or redistributing *Fusarium* mycotoxins [6, 7]. Several authors have studied detoxification procedures for *Fusarium* mycotoxins [8-15]. Physical processing of grains can reduce the levels of mycotoxins and their contaminating metabolites. Peeling and wet grinding stand out among these processes, but their results vary depending on the procedure [9, 10]. During the fermentation process used in bread making from wheat contaminated with DON, levels of mycotoxin were shown to be reduced [12]. According to Garda *et al.* [13], alcoholic fermentation with *Saccharomyces cerevisiae* can be considered as a promising method of detoxification of different levels of DON and T-2 toxin. It was found that the fermentation process of malt contaminated with DON and T-2 caused a decontamination of 53% for these mycotoxins, taking into account both the wort and the filtered sample. On the contrary, the results mentioned by Bennet and Richard showed that DON was not completely destroyed by the alcoholic fermentation. High levels in both the solid residue and the fermented liquid [14] could be detected, and Scott mentioned, that DON was stable during the wort fermentation process [15]. These results suggest that other procedures should be carried out on *Fusarium* contaminated grains used as raw materials for fer-

mentation and that also studies of the effect of fermentation on the decontamination process should be better evaluated.

This study aims to evaluate the influence of a combined treatment with a mixture of xylanolytic and amylolytic enzymes on the decontamination of DON present in a high concentration in *Fusarium* contaminated wheat and at the same time look at the efficiency of the alcohol fermentation process.

MATERIAL AND METHOD

Wheat grains. Fusarium contaminated wheat (*Triticum aestivum L.*) stored with a moisture content between 11.5-12%, was obtained from the Lelystad branch of the Plant Research Institute (PRI) in Wageningen, The Netherlands. An initial DON determination by Enzyme-Linked Immuno Sorbent Assay (ELISA) on the wheat, indicated a level of 3950 µg/kg. Wholesome wheat (without DON contamination) used for parallel studies was obtained from a Lithuanian milling company "Kauno grudai".

Enzymes and yeast. The enzyme preparations Vilzim SKA and Vilzim SKG used for starch hydrolysis and saccharification, and Vilzim SKK used for the non-starch polysaccharides degradation were supplied by SC "Biosinteze" (Lithuania). Vilzim SKA is a liquid α-amylase preparation (from *Bacillus subtilis*) with an activity of 2350 AU/ml (amylase activity units). This enzyme preparation also contains neutral protease and β-glucanase activities. The most important components of Vilzim SKG (from *Aspergillus awamori*), used for saccharification of raw materials, are glucoamylase (activity 2500 GAU/ml), α-amylase and β-glucanase. Vilzim SKK (from *Trichoderma reesei*), contains β-xylanase (activity 2540 XU/ml), cellulase and β-glucanase activities. Baker's yeast of *Saccharomyces cerevisiae* was donated by SC "SEMA" (Lithuania).

Fermentation. A low-temperature technological process was used for the ethanol production under the laboratory conditions. The raw material was treated by a two-step enzymatic hydrolysis procedure consisting of a liquefaction and saccharification step. Wheat was ground in a laboratory mill WZ-1 (ZBPP, Bydgoszcz, Poland). 100 g of the ground grain was mixed with water previously heated to 90^{o}C at ratio 1:5 and kept at the same temperature in a water bath for 30 minutes to reach partial degradation of polysaccharides and to decrease microbial activity. Liquefaction of starch was carried out for 90 minutes at 65^{o}C temperature and a pH between 6.0-6.5 by adding a selected amount of Vilzim SKA (150 AU/100 g wheat). A simultaneous enzymatic liquefaction and saccharification step was performed in 120 minutes by a temperature between 55-60^{o}C and pH 5.0-6.0. An initial supplementation of a selected amount of Vilzim SKG (300 GU/100 g) and an appropriate amount of Vilzim SKK (100, 150, 200, 250 XU/100 g) for the degradation of non-starch polysaccharides was carried out. The assays were carried out in stirred glass vessels. 300 ml of the wort was fermented at 30-33^{o}C temperature for 72 h using baker's yeast in a 500 ml glass flask, sealed with a rubber stopper. A tube was inserted into the stopper to allow carbon dioxide to escape.

All fermentations were performed in duplicates. Wort and fermented broth were analyzed for acidity and soluble dry matter. The fermented broth was filtered and the filtrate was subjected to quantitative analysis of the ethanol**.** The residue was dried in an oven at 80 °C with aeration for 6 h and was used for DON analysis in Dried Distillers Grains with Solubles (DDGS).

Analysis of efficiency of the fermentation process. The amount of soluble dry matter was determined according to the AACC Method 68-62 [16]. The acidity analysis was performed by titration with 0.1 N NaOH. One degree (1^{o}) of acidity corresponds to 1 ml of 1 N NaOH required to neutralize the acids present in 20 ml of filtrate. The ethanol concentration was determined by using direct distillation and pycnometry [17]. The analyses were carried out 3 times.

Determination of DON. DON concentration was determined by using the Enzyme-Linked Immuno Sorbent Assay (ELISA) technique for both the untreated wheat samples and for wheat samples being processed (DDGS). Samples were analyzed for DON using a Veratox®DON 5/5 Quantitative Test Kit (Neogen Corporation, USA) according to the manufacturer's instructions. This test system is a Competitive Direct Enzyme-Linked Immuno Sorbent Assay (CD-ELISA) which allows obtaining a DON concentration in mg/kg (ppm). Free DON in the samples and controls is allowed to compete with enzyme-labeled DON (conjugate) for the antibody binding sites. After a wash step, K-Blue Substrate reacts with the bound conjugate to produce a blue color. The optical densities of the controls form the standard curve, and the sample optical densities are plotted against the curve to calculate the concentration of DON. Limits of detection and quantitation for this method were reported as 0.1 and 0.25 mg/kg, respectively. This test kit does not differentiate between DON and 3-acetyl DON.

Statistical analysis. Data are presented as arithmetic means plus standard errors. Data were obtained by using a Microsoft Excel spreadsheet and a program Origin 50 (Origin Lab Corporation) was used for comparison of the means by one-way analysis of variance. The significance of the results from the data analysis was considered by $P< 0.05$.

RESULTS AND DISCUSSION

The influence of xylanase on the fermentation of with Fusarium contaminated wheat. It was found that enzymatic hydrolysis of with *Fusarium* contaminated wheat (FW) caused the formation of the lower contents of soluble dry matter (SDM) in the wort in comparison with wholesome wheat (WW). After fermentation of FW the ethanol concentration determined in the broth was found to be 13.5 % lower (Fig. 1) and SDM content – 20% higher than that of WW (Fig. 2). The analysis of the acidity in the medium shows the formation of a 40% higher amount of organic acids during the fermentation of FW (Fig. 3).

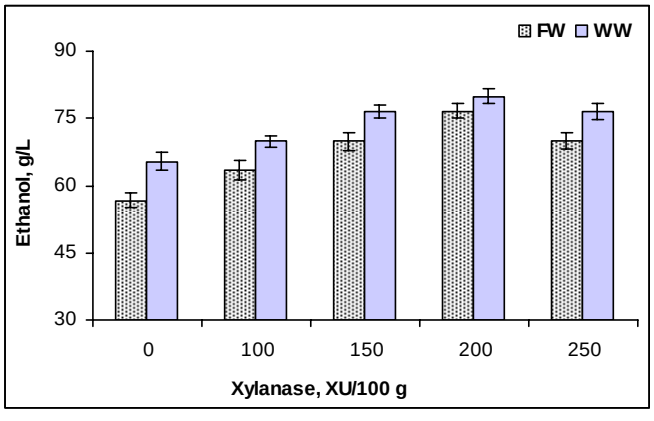

Fig. 1. Ethanol concentration in the fermented broth produced from wholesome wheat (WW) and with Fusarium contaminated wheat (FW); 0 – sample prepared by using only amylolytic enzymes

Sl. 1. Koncentracija etanola u fermentisanom kljuku od zdrave pšenice i pšenice zaražne fuzarijumom; 0 - uzorci pripremani korišćenjem samo amilolitičkih enzima

In this study the influence of xylanase in combination with traditional ethanol production used amylolytic enzymes on the efficiency of the hydrolysis of FW was investigated. It was determined that the xylanase addition (100-250 XU/100 g grains) resulted in a 3.7-35.2% higher SDM concentration in the wort. The results showed that by application of xylanase**,** it is possible to increase the ethanol concentration on average by 12-35.3% and 6.9-22.2%, respectively for FW and WW, in comparison with the reference sample without xylanase. The maximum ethanol concentrations were achieved by a xylanase activity of 200 XU/100 g. In summary, the addition of xylanase played a positive role on the enzymatic hydrolysis of with *Fusarium* contaminated wheat grains. The increase of the efficiency of alcohol fermentation could be caused by the synergetic action of the xylanase/cellulase complex of Vilzim SKK, which renders the solubilization and depolymerization of non-starch polysaccharides to their monomeric constituent sugars and the higher concentration of hexoses in the medium.

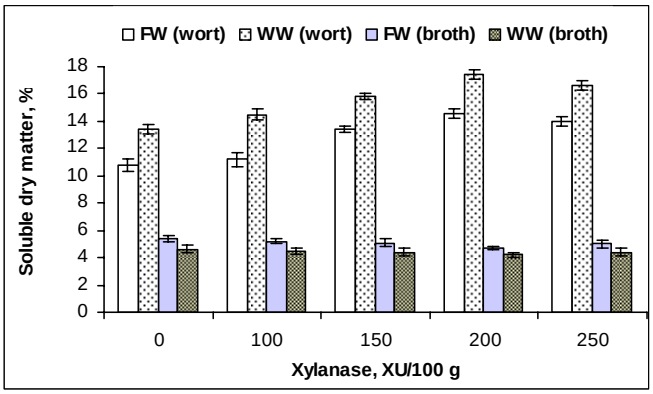

Fig. 2. Effect of xylanase on soluble dry matter (SDM) content in the wort and fermented broth produced from wholesome wheat (WW) and with Fusarium contaminated wheat (FW); 0 – sample prepared by using only amylolytic enzymes

Sl. 2. Uticaj ksilanaze na sadržaj čvrste suve materije u nefermentisanoj podlozi i prevreloj komini dobijenom od zdrave pšenice i pšenice zaražene fuzarijumom; 0 - uzorci pripremani korišćenjem samo amilolitičkih enzima

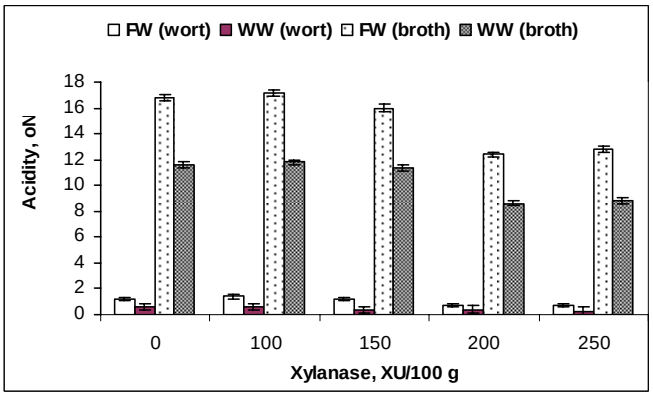

Fig. 3. Effect of xylanase on the acidity of the wort and fermented broth produced from wholesome wheat (WW) and with Fusarium contaminated wheat (FW); 0 – sample prepared by using only amylolytic enzymes

Sl. 3. Uticaj ksilanaze na na kiselost podloge i fermentisanom podlozi dobijenom od zdrave pšenice i pšenice zaražene fuzarijumom; 0 - uzorci pripremani korišćenjem samo amilolitičkih enzima

The influence of alcoholic fermentation on DON levels in the DDGS. This part of work includes the investigation of the effect of enzymatic hydrolysis and alcoholic fermentation by *S. cerevisiae* on DON levels in the processing of grains. The results showed (Fig. 4) that by using amylolytic enzymes a decrease in DON concentration by 26.6 % of the initial contamination was observed. It can be indicated that by using the complex of amylolytic enzymes and xylanase, it was possible to decrease DON levels in the draff by 51.5% and 31.5%, respectively, in comparison with the initial contamination and the sample with amylolytic enzymes.

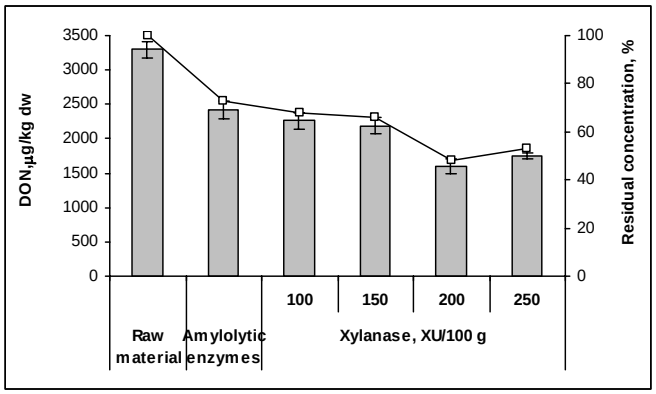

Fig. 4. DON concentration in wheat and DDGS after alcoholic fermentation by using different combinations of enzymes

Sl. 4. Koncentracija deoksinivalenola u pšenici i suvoj džibri posle alkoholne fermentacije upotrebom različitih kombinacija enzima

The decrease in DON levels may have happened due to its solubility in the aqueous medium or the ability of yeast to absorb and modify the mycotoxin by forming the various compounds. With reference to our investigations, the most part of them was formed by using xylanase supplementation for saccharification of contaminated grains. Modified yeast cell wall mannanoligosaccharide (MOS) has been reported to effectively bind aflatoxins and the fusariotoxins to a lesser degree. Glucomannans extracted from the external part of cell walls of the yeast S. *cerevisae* are able to bind certain mycotoxins. The results of the study showed that alcoholic fermentation by *S. cerevisiae* and xylanolytic enzymes reduce DON levels in DDGS.

CONCLUSION

The *Fusarium* contaminated wheat grains which contain DON negatively affect the fermentation process and reduce the ethanol concentration by 13.5% in the fermented broth; after alcoholic fermentation 73 % of the initial DON level could be detected in DDGS.

The created complex of enzyme preparation containing xylanase from *Trichoderma reesei* (200 XU/100 g grains) increased the efficiency of the fermentation process of *Fusarium* contaminated wheat: the ethanol concentration increased by 35.3%.

The reduction of the levels of DON up to 51.5 % of the initial degree of contamination was achieved during the alcoholic fermentation process of *Fusarium* contaminated wheat grain with a high concentration of DON (3950 µg/kg).

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