EFFECT OF ENZYMATIC HYDROLYSIS ON BRAN MICROFLORA

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Abstract
The present study was undertaken to estimate enzymatically hydrolysed and non–hydrolysed wheat (*Triticum aestivum*) and rye *(Secale cereale)* bran microflora. Enzymatic hydrolysis was accomplished by α – amylase from *Bacillus amyloliquefaciens* and by Viscozyme L which contain a wide range of enzymes responsible for the breakdown of carbohydrates into simple sugars. Wheat and rye bran samples were collected from native mills, namely Stock Company (SC) 'Rigas dzirnavnieks' wheat bran with large particle size (WLSR), SC 'Jelgavas dzirnavas' rye bran with small particle size (RSSJ), SC 'Dobeles dzirnavnieks' wheat bran with small particle size (WSSD) and wheat bran with large particle size (WLSD). Gained results indicate that before enzymatic hydrolysis all of the bran samples showed similar microbiological contamination with total plate count (TPC), yeasts and lactic acid bacteria. Enzymatic hydrolysis of bran give the possibility to partially eliminate the microbiological contamination with TPC, yeasts and lactic acid bacteria. The amount of microorganisms after enzymatic hydrolysis (before storage) were decreased and ranged from 5.26 ± 0.04 to 5.45 ± 0.01 log CFU g^{-1}, from 4.81 ± 0.01 to 5.60 ± 0.05 log CFU g^{-1}, and from 4.09 ± 0.01 to 5.10 ± 0.05 log CFU g^{-1}, respectively. After eight weeks of storage (temperature – 20 ± 1 °C, relative humidity – 40 ± 1%) enumeration of microorganisms showed significant decrease of colony–forming units in all bran samples. The amount of TPC, yeasts and lactic acid bacteria in the control bran samples fluctuated in a range from 4.84 ± 0.04 to 5.49 ± 0.05 log CFU g^{-1}, from 4.86 ± 0.03 to 5.25 ± 0.03 log CFU g^{-1}, 3.53 ± 0.03 to 4.21 ± 0.02 log CFU g^{-1} respectively.
Key words: microbiological contamination, enzymatic hydrolysis, Viscozyme L, depolymerisation.

Introduction

Wheat (*Triticum aestivum*) bran is the coarse outer layer of the wheat kernel that is separated from the cleaned and scoured kernel. It consists mainly of the large pieces of bran remaining after the flour has been extracted from the wheat (Radenkovs and Klava, 2012). Wheat bran is a composite material formed from different histological layers, and three different strips can be obtained from the soaked outer layers. The outer strip corresponds to outer pericarp (epidermis and hypodermis), the inner one corresponds to the aleurone layers, and the intermediate one remains a composite of several tissues (inner pericarp, testa, and nuclear tissue (Hemery et al., 2010).

Rye *(Secale cereale)* bran like wheat bran is by-product of the rye milling. Likewise wheat bran rye bran composed from different histological layers such as: fruit coat (pericarp), seed coat (testa), aleurona layer.

Different types of bran have different chemical compositions, it depends on grain genetics, agricultural background and milling process (Harris et al., 2005).

The chemical composition of the wheat as well as rye bran depends on certain factors associated with the grain chemical composition or with milling processes. Wheat and rye bran has a significant amount of carbohydrates, proteins, minerals (magnesium, potassium, phosphorus, iron, manganese, and zinc), bioactive compounds (tocopherols and tocotrienols, phenolic compounds, alkylresorcinols), and other growth factors, which support growth of microorganisms, including the fastidious lactic acid bacteria (Hemery et al., 2010).

Due to its nutritional value, low cost, and potential use in human nutrition, many studies have been conducted to evaluate its use in food.

Microbiological conditions during harvesting together with outer part residues and starch contamination during dehulling and polishing processes limit its direct use as food.

The residues may carry a high level of microbiological impurities, such as yeast, fungi, the spores of which are resistant to heat and are able to produce mycotoxins. The most common mycotoxins contamination in cereals is *Aspergillus spp.*, *Penicillium spp.*, *Fusarium* spp. and *Claviceps* spp. (Finnegan, 2010). Mycotoxins are formed during cereal growth or in post–harvest storage during the wet season; sun drying practised by most farmers may not adequately reduce the moisture content in grains. As a result, grains with moisture content higher than permissible level enter the storage system.

Fermentation may be a useful strategy for reducing microbiological contamination. Enzymatic or microbial fermentation is a process of bioconversion of organic substances by microorganisms and/or enzymes of microbial, plant or animal origin. It is one of the oldest forms of food preservation which is applied globally. Indigenous fermented foods such as bread, cheese and wine, have been prepared and consumed for thousands of years and are strongly linked to culture and tradition, especially in rural households and village communities. It is estimated that fermented foods contribute to about one–third of the diet worldwide (Food and Agriculture Organization, 2004).

Microbial fermentation leads to a decrease in the level of carbohydrates, as well as some non–digestible poly and oligosaccharides. Certain amino acids may be synthesised and the availability of B group vitamins may be improved (Nout and Ngoddy, 1997). During the fermentation of cereals by lactic acid bacteria the content of free amino acids was increased. Several studies imply that the fermentation gained the positive effect on cereal nutritional value, on the content of essential amino acids, particularly lysine, methionine and tryptophan (Adams, 1990). During the microbial fermentation the optimal pH is necessary for enzymatic degradation of cereal substances e.g. cell walls, protein/starch matrix. It was reported in the literature that the more appropriate condition of enzymatic pre–treatment with Viscozyme L is pH 4.6 (Guan and Yao, 2007), while for most of microorganisms this pH is critical for growing and developing. Temperature, pH, the control of water activity, and use of antimicrobial agents are the available methods to prevent the growth of organisms or production of microbial toxins in food. Reducing pH below 4.0 – 4.5 by fermentation of acidification with acid foods can similarly inhibit proliferation and the availability of water to microorganisms by adding salt or sugar, or by freezing (Brown et al., 1998).

The aim of this work was to estimate enzymatically hydrolysed and non–hydrolysed wheat and rye bran microflora.

Materials and Methods

The experiments were performed at the Faculty of Food Technology of Latvia University of Agriculture in collaboration with the Latvia State Institute of Fruit–Growing. All analyses were conducted with threefold repetition.

Bran samples

Summer wheat (*Triticum aestivum*) and rye *(Secale cereale)* bran samples were collected from industrial mills of Latvia:

1) SC ‘Dobeles dzirnavnieks’– wheat bran with large particle size (~441 μm) **(WLSD);**
2) SC ‘Dobeles dzirnavnieks’– wheat bran with small particle size (~215.8 μm) (**WSSD);**
3) SC ‘Rigas dzirnavnieks’– wheat bran with large particle size (~600.0 μm) (**WLSR);**
4) SC ‘Jelgavas dzirnavas’– rye bran small particle size (~276.0 μm) **(RSSJ).**

Two different methods were used for pre–treatment of bran samples: enzymatic hydrolysis (by using enzymes, heating, pH adjustment);

the control treated bran samples (by using heating, excluding adding of enzyme and citric acid). This type of pre–treatment is needed to decide whether the temperature (100 ± 1 °C) has any influence on the colony–forming units or not;

the control samples – samples that were not treated but used like raw material.

Enzymes

Industrial enzyme preparations were produced by ‘Novozyme Corporation’ (Bagsvaerd, Denmark) and purchased from Sigma – Aldrich. Two commercial preparations of enzymes: α – amylase from *Bacillus amyloliquefaciens* (EC 232 – 560 – 9) and Viscozyme L from *Aspergillus spp*., (EC 263 – 462 – 4) were used to hydrolyse carbohydrates. The α – amylase has a declared activity ≥250 units g^{-1}, optimum conditions of enzymatic pre–treatment is pH 5.0 – 8.0, temperature 55 ± 1 °C and incubation time 0.5h (Demirkan et al., 2004) form Viscozyme L declared activity is 100 fungal beta glucanase (FBG) g^{-1}, optimum conditions are pH 4.6, temperature 44 ± 1 °C and incubation time 3.2 h (Guan and Yao, 2007).

Enzymatic Hydrolysis

For starch hydrolysis, wheat bran (10g) was mixed with 90 mL of distilled water in 1000 – mL Reagent bottle with screw cap with dilutions 1:9, and then 500μL of α – amylase was added. Hydrolysis was carried out in a water bath at temperature 55 ± 1 °C, incubation time 0.5h and shaking intensity 60 rpm. After starch hydrolysis bran mash was homogenized for 3 minutes, the pH of the suspension was adjusted to pH 4.6 with 0.2 mL of 50% citric acid in each dilutes and Viscozyme L 400μL was added. Incubation time is 3.2h, temperature 44 ± 1 °C, and shaking intensity 60rpm. After enzymatic hydrolysis and enzymes inactivation (10 min at temperature 100 ± 1 °C), bran mash was cooled to room temperature (20 ± 1 °C) and then freeze–dried (temperature (– 50 °C, vacuum – 0.5 mbar, drying time – 72 ± 5h), and then stored at room temperature (20 ± 1 °C).

Microbiological analysis

Microbiological evaluation of bran was performed according to the standard ‘Microbiology of food and animal feeding stuffs’ LVS EN ISO 7218:2007. All microbiological evaluations were conducted with threefold repetition.

Enumeration of aerobic colony count (ACC) was performed according to the standard LVS EN 4833:2003, colony–count technique at 30 °C (Fig. 1).

Microbiology of food and animal feeding stuffs – Horizontal method was used for the enumeration of yeasts and moulds – LVS ISO 21527 – 2:2008, and colony count technique in products with water activity lower than or equal to 0.95.

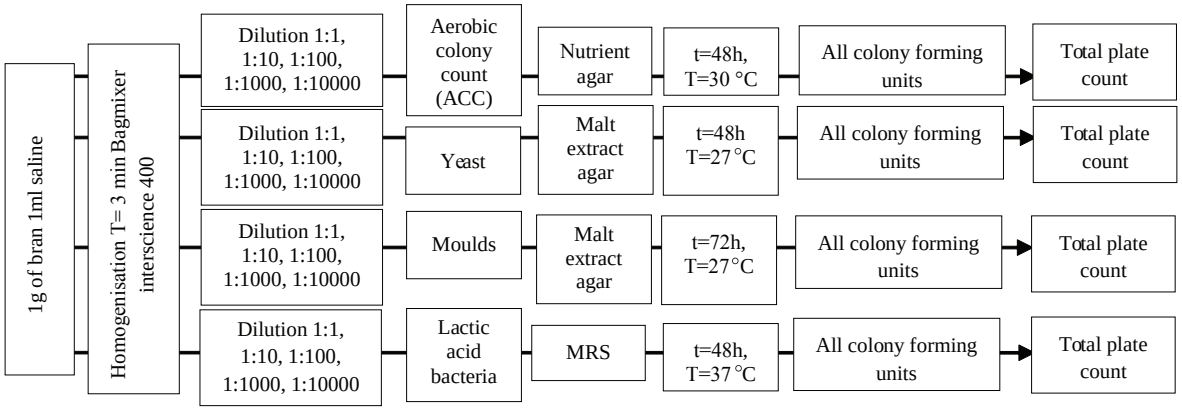

Figure 1. Scheme of microbiological testing of enzymatically treated and non–treated wheat and rye bran.

Enumeration of mesophilic lactic acid bacteria was performed according to standard LVS ISO 15214:1998.

Bran samples were packaged in plastic bags and stored at room temperature (temperature – 20 ± 1 °C, relative humidity – 40 ± 1%). Microbiological evaluation was conducted before storage (initial microflora), after the third, the fifth (the data are not included) and eighth week of storage. Particularly the eighth week of storage showed significant (p=0.001) decreasing of microorganisms comparing with initial microflora.

Moisture, pH value and water activity (a_w)

Moisture content was analysed using 'Determination of the Moisture Content of Cereals and Cereal Products method'– ICC Standard No, 110/1, by drying for 2 h at 150 °C. Procedure was carried out in triplicates.

pH was measured using 'Hydrogen–Ion Activity (pH)Electrometric method' – AACC 02 – 52.01, using JENWAY 3520 (Barloworld Scientific Ltd., ESSEX, UK) pH–meter. The pH electrode was dipped into a mixture of homogenized sample and distilled water. For a more precise measurement the calibration of pH meter has been done using 'Two–Point Calibration Procedure'. Measuring procedure was carried out in triplicates.

Water activity was measured using 'LABSwift a_w measurement device'. Before the measurement of water activity in the samples, calibration was done by using re–usable saturated salt calibration standard. Measuring procedure was carried out in triplicates.

Statistical analysis

Data was processed by SPSS software version 17.0. Data was analysed using descriptive statistics and processed by one–way analysis of variance ANOVA (one way ANOVA), as well as for comparing all bran samples depending on pre – treatment ways two-way ANOVA were used. Microsoft office software version 2007 was used to determine significant differences between the samples.

Results and Discussion

Moisture content, pH value and water activity of analysed bran.

Wheat and rye bran is a by–product of the milling process of flour, and is a composite material formed from different histological layers and three different strips. The outer strip corresponds to outer pericarp (epidermis and hypodermis), the inner one corresponds to the aleurone layers, and the intermediate one remains a composite of several tissues (inner pericarp, testa, and nuclear tissue) (Hemery et al., 2010).

One of the most important factors for microbial contamination, as well as for microorganism development is grain harvesting and storage conditions. Influence of temperature is closely associated with grain moisture (Dimic et al., 2009). Fungal infection, as well as insect invasion is of particular concern with wheat and rye stored at moisture content, time or length of storage and storage temperature (Karunakaran et al., 2001). It was reported that fungi produce mycotoxins, such as ochratoxin A, which was produced by *Aspergillus* and *Penicillium* spp., as a result it presents a health risk. Several studies imply that synthesis is highest when the product humidity is above 13% and temperature is between 24 ± 1 and 37 ± 1 °C. That is why, warm and wet geographic regions are the most favourable environments for mycotoxins (Dimic et al., 2009). For elimination of fungal contamination it is strongly recommended to control the moisture content of grain before harvesting, as well as during storage.

One of the factors which can affect bran moisture content is grain milling and drying technology. In the previous experiments it was ascertained that the

increase of moisture content might be caused by the increase of particle sizes in bran that leads to intensive growth and reproduction of fungi spore (Radenkovs et al., 2013).

In total, twelve samples including four controls were treated without enzymatic modification and four enzymatically treated bran samples were analysed. The results obtained from the determination of moisture content are summarized in Figure 2. The obtained results give a possibility to conclude that the highest moisture content was detected in the control bran samples without enzymatic modification. The content of moisture fluctuated in a range from 7.66 ± 0.07 to 15.38 ± 0.05%, while the lowest content was recorded in the control samples which were treated similarly to enzymatically hydrolysed bran, provided they are not enzymatically modified. The amount of moisture content in the tested bran samples ranged from 4.60 ± 0.09 to 6.1 ± 0.04%. Analysing data of moisture obtained after enzymatic modification suggest that the moisture content was significantly ($p<0.05$) decreased comparing with the control bran samples, for WSSD from 13.69 ± 0.05 to 10.21 ± 0.05% ($p=0.0001$) and for WLSD from 15.38 ± 0.05 to 11.73 ± 015% ($p=0.0001$). An exception was detected for WLSR, as well as for RSSJ bran samples, the content of moisture of which has increased from 7.66 ± 0.07 to 7.92 ± 0.14% ($p=0.009$) and from 12.05 ± 0.08 to 13.20 ± 0.11% ($p=0.002$), respectively. Differences between the samples can be explained with the fact that during the freeze-drying the uneven moisture elimination has occurred. It is because the water holding capacity in brans with small particle size is weaker, compared to the brans with large particles.

It is important to know not only the moisture content of bran, - another important marker is water activity (a_w) which can influence the development of microorganisms. Bacteria need higher water activity than yeasts and moulds, consequently foods with low water ability may be contaminated mainly with yeasts and moulds (King, 2009). Our obtained results indicate that the highest water activity of bran was recorded in the control samples (Figure 3). Water activity fluctuated in a range from 0.30 ± 0.01 to 0.71 ± 0.01. After enzymatic hydrolysis as well as bran treatment without enzymes gave positive results in decreasing the water activity. Water activity was decreased particularly 2 times and ranged from 0.38 ± 0.01 to 0.39 ± 0.01 for control samples treated without enzymes and from 0.35 ± 0.01 to 0.37 ± 0.01 for enzymatically hydrolysed bran samples.

The results obtained from the determination of pH value of the samples are summarized in Figure 4.

The highest pH value was found in the control bran samples, as well as in bran samples which

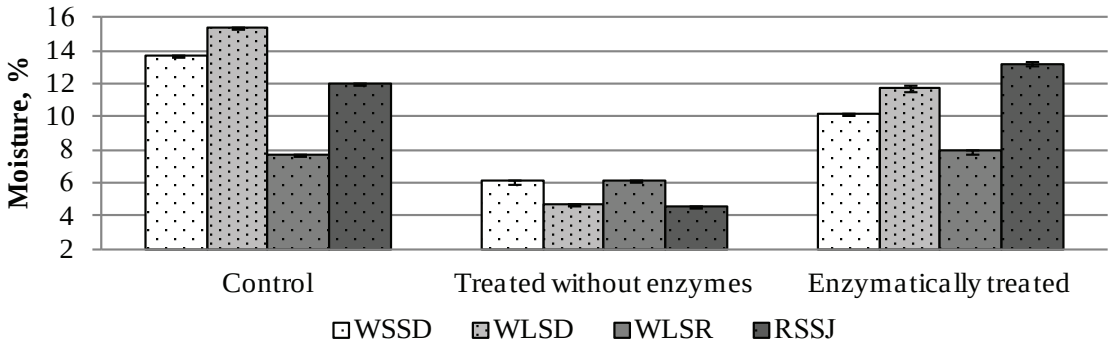

Figure 2. Moisture content of different bran samples, %.

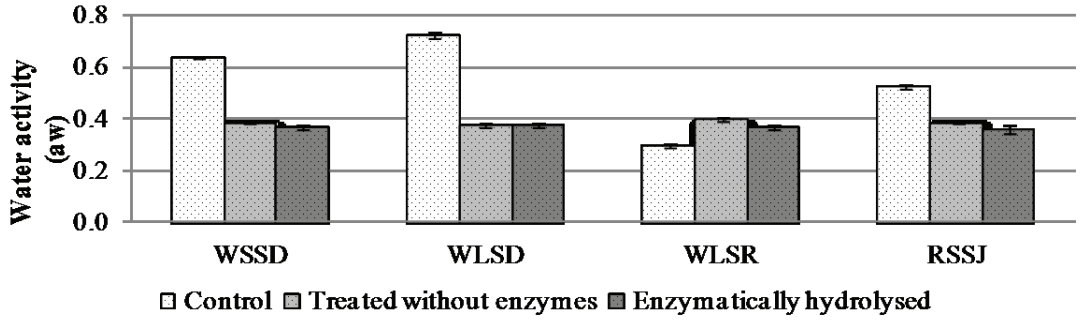

Figure 3. Water activity of different bran samples, %.

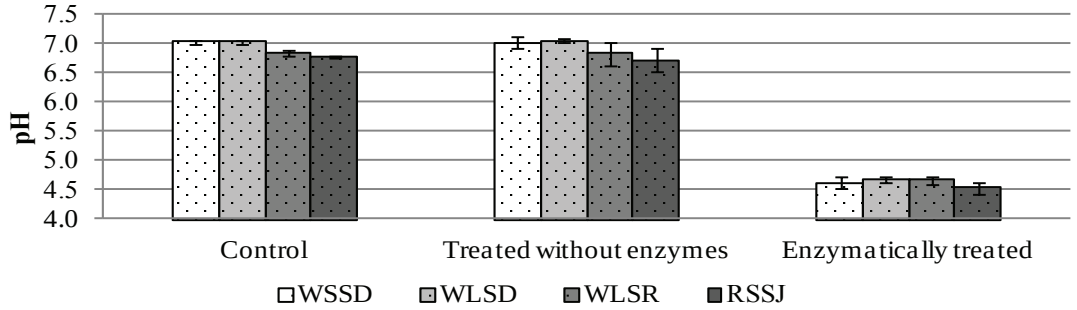

Figure 4. Comparison of pH value in wheat bran samples.

were treated without enzyme addition. The pH value for these bran samples ranged from 6.79 ± 0.02 to 7.04 ± 0.01 and from 6.72 ± 0.20 to 7.06 ± 0.04, respectively. Our previous studies show similar results in pH value among bran samples (Radenkovs and Klava, 2012). Analysing data of pH value obtained after enzymatic modification suggest that pH was significantly ($p<0.05$) decreased, pH value fluctuated in a range from 4.54 ± 0.10 to 4.69 ± 0.05. The decrease of pH may be explained with the fact that during enzymatic hydrolysis pH was adjusted to 4.6 with addition of citric acid. The pH 4.6 is necessary for enzymatic degradation of cell walls with Viscozyme L.

Microbiological contamination

The microbiological contamination of cereals as well as cereal products is diverse and includes a wide range of microorganisms, basically moulds, bacteria, yeasts, lactic acid bacteria, rope–forming bacteria (*Bacillus spp.*), pathogens, enterococci and coliforms. The microbiological contamination occurs mostly during growth, harvest and storage time, and is dominated by the moulds. The most important genera of the storage fungi are *Penicillium* and *Aspergillus*, although species of *Fusarium* may also be involved in spoilage when grain is stored under moist conditions (Adams and Moss, 2000). In our results obtained from four bran samples and by two different pre–treatments of bran we did not find any mould impurities, while our previous study indicated that moulds were detected in all bran samples with the genus *Penicillium* being the most frequent. Fungal counts ranged from 5 to 8 log CFU g^{-1} (Radenkovs et al., 2013). Differences between the samples may be explained by the fact that obviously the microbiological contamination has occurred during transporting, milling or storage of cereals.

Contamination with lactic acid bacteria

The results obtained from four bran samples and by two different methods of pre–treatment of bran suggest that the highest contamination with lactic acid bacteria was in the control bran samples, particularly in WLSD bran samples (5.75 ± 0.04 log CFU g^{-1}) (Figure 5). It was detected that during the storage of wheat, as well as rye bran samples, the colony–forming units have significantly decreased ($p<0.05$) comparing with starting point (1 week). The obtained results suggest that after eight weeks of storage in treated without enzymes bran samples lactic acid bacteria were completely inactivated in the WLSR bran sample, but in other bran samples significantly ($p<0.05$) decreased. In enzymatically hydrolysed bran samples the concentration of colonies significantly ($p<0.05$) decreased, and this amount was equal to 4.19 ± 0.07 log CFU g^{-1} in WSSD, 3.04 ± 0.03 log CFU g^{-1} in WLSD, 3.68 ± 0.06 log CFU g^{-1} in WLSR and 3.28 ± 0.05 log CFU g^{-1} in RSSJ.

During the research of literature about enzymatic hydrolysis and their impact on cereal microflora no explanation was found for colony count decrease; the limitation of literature review does not allow the possibility to completely describe the obtained

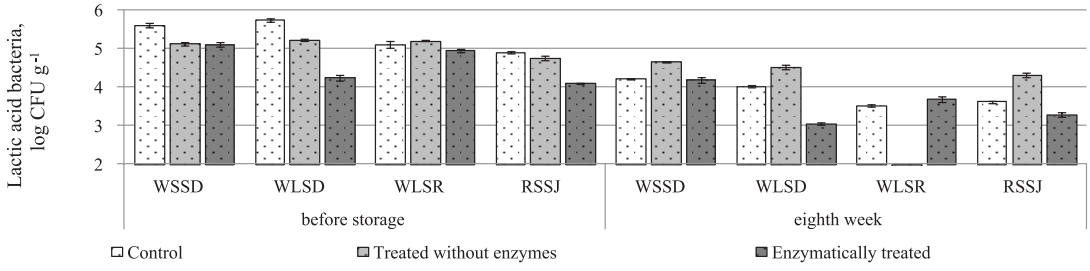

Figure 5. Colony – forming units of particular microbiological contamination with lactic acid bacteria.

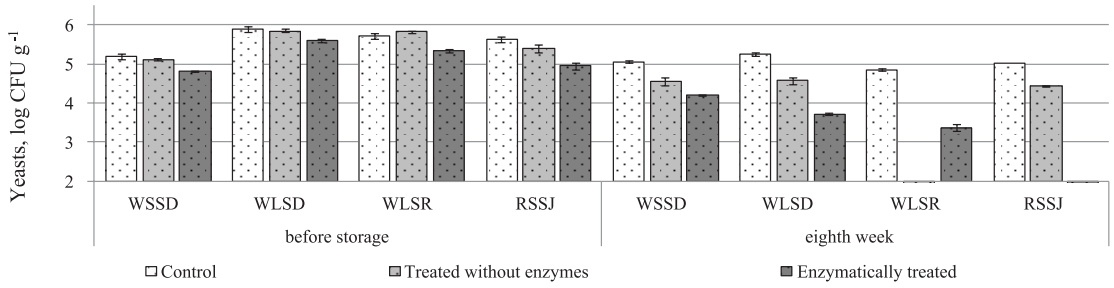

Figure 6. Colony – forming units of particular microbiological contamination with yeasts.

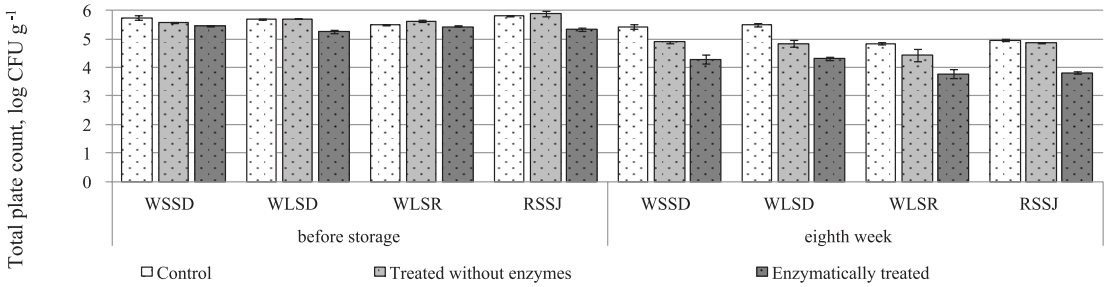

Figure 7. Colony–forming units of particular microbiological contamination with total plate count.

results. But we can assume that during the enzymatic modification of bran, water activity (a_w), moisture, nutrient accessibility (carbohydrate hydrolysis), as well as the pH value were changed, which can significantly alter the implementation of the development of microorganisms (Rushing et al., 2004).

Contamination with yeasts

Similar results were obtained for presence of yeasts in bran (Figure 6), - their development has occurred most intensively in the control bran samples. Among four bran samples the highest amount was in WLSR (5.89 ± 0.08 log CFU g^{-1}), while the lowest amount was found in WSSD bran samples (5.19 ± 0.07 log CFU g^{-1}). Our previous studies suggest that the control wheat and rye bran samples had the highest contamination with yeasts. The highest colony forming units were recorded in WLSD, and corresponding log 10 CFU g^{-1}, while the lowest contamination was in RSSJ, which corresponds to log 7 CFU g^{-1} (Radenkovs et al., 2013).

A positive fact was it that after eight weeks of storage enzymatically hydrolysed bran the presence of yeasts in RSSJ bran sample was not detected. This can be explained by the fact, that rye has a higher tolerance to disease, because the grain–filling assimilates are photosynthesized mainly in the stalk and head, comparing with wheat. It is probable that during the enzymatic hydrolysis the liberating of bound bioactive compound has occurred, and it has influenced the colony counts of yeasts. In the other bran samples after eight weeks of storage the colony forming units had significantly ($p<0.05$) decreased. The amount of colony forming units in WSDD comparing with initial microflora decreased from 4.81 ± 0.01 to 4.21 ± 0.00, from 5.60 ± 0.05 to 3.72 ± 0.07 log CFU g^{-1} (WLSD), from 5.35 ± 0.04 to 3.37 ± 0.09 log CFU g^{-1} (WLSR).

The results (Figure 7) showed contamination with total plate count. The initial study showed that the highest contamination with microorganisms was in the control bran samples. Among four control samples the amount of colony ranged from 5.50 ± 0.02 log CFU g^{-1} to 5.82 ± 0.04 log CFU g^{-1}.

Similar to the previous case, the lowest colony count was found after eight weeks of storage in enzymatically hydrolysed bran samples, the amount of TPC ranging from 3.79 ± 0.16 to 4.31 ± 0.05 log CFU g^{-1}.

Conclusions

1. This study asserts that no mould presence was detected in any of the bran samples, which allows to assume, that the moisture content and water activity of samples during storage, as well as after enzymatic hydrolysis were not appropriate for fungal growing and developing.
2. Analysing data of bran initial contamination with TPC, yeasts and lactic acid bacteria, suggest that all bran samples contain contamination of these microorganisms. Partial reduction of microbiological impurities was gained after enzymatic hydrolysis.

3. In all bran samples TPC, yeasts and lactic acid bacteria were detected in a range from 5.50 ± 0.02 to 5.82 ± 0.04 log CFU g^{-1}, from 5.19 ± 0.07 to 5.89 ± 0.08 log CFU g^{-1}, and from 5.11 ± 0.09 to 5.75 ± 0.04 log CFU g^{-1}, respectively.
4. After enzymatic hydrolysis the concentration of colony forming units decreased significantly ($p<0.05$) with yeasts ($p=0.002$) and lactic acid bacteria ($p=0.001$) which corresponds 4.81 ± 0.01 to 5.60 ± 0.05 log CFU g^{-1} and from 4.09 ± 0.01 to 5.10 ± 0.05 log CFU g^{-1}, respectively.
5. Analysing the data obtained after eight weeks of storage indicate significant decrease of TPC, yeasts and lactic acid bacteria. The amount of TPC fluctuated in a range from 3.79 ± 0.16 to 4.310.05 log CFU g^{-1}, yeasts from 3.37 ± 0.09 to 4.21 ± 0.08 log CFU g^{-1} and lactic acid bacteria 3.04 ± 0.03 to 4.19 ± 0.04 log CFU g^{-1}.

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