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Study on the reduction of acrylamide in mixed rye bread by fermentation with bacteriocin-like inhibitory substances producing lactic acid bacteria in combination with *Aspergillus niger* glucoamylase

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ABSTRACT

This study mainly focuses on a strategy for reducing of acrylamide in cereal products, predominantly in bakery products. The effect of mesophilic lactic acid bacteria (LAB) strains as well as a novel fermentation media on the bases of extruded rye wholemeal on the acrylamide formation in mixed rye bread of different weight of loaf was studied. The LC–MS/MS method for acrylamide determination in bread crumb has been applied. Addition of 15% low pH (pH 3.4–4.3) fermented product to bread dough, which were produced by using commercial strain *Lactobacillus casei* and bacteriocin-like inhibitory substances (BLIS) producing strains *Lactobacillus sakei KTU05-6*, *Pediococcus acidilactici KTU05-7* and *Pediococcus pentosaceus KTU05-8* separately, caused significant reduction of acrylamide in mixed rye bread. All bread loafs of 1000 g contained less by 27% acrylamide concentrations versus loafs of 500 g. Acrylamide formation was affected ($r^2 = 0.7193$) by total reducing sugar content in bread and slightly correlated ($r^2 = 0.5587$) with reducing sugar content in sourdough. The treatment of extruded rye wholemeal with *Aspergillus niger* glucoamylase as compared to the control sample was found to have a positive effect on the acidification process lowering the acrylamide formation on an average by 59.4% and 40% in 500 g and 1000 g loafs of bread, respectively. This study demonstrates that acrylamide content could be reduced by using LAB excreting lower amylolytic activity in the medium, while the higher proteolytic activity is preferred.

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1. Introduction

Acrylamide is formed in foods *via* the Maillard reaction with free asparagine and reducing sugars as the precursors, with the former generally being limiting in cereal-based products (Mottram, Wedzicha, & Dodson, 2002; Stadler et al., 2002; Zyzak et al., 2003). Moderate levels of acrylamide (5–50 μg/kg) in heated protein-rich foods and higher levels (150–4000 μg/kg) have been found in carbohydrate-rich foods, including potato, and also in certain heated commercial potato products and crisp bread (Claus, Carle, & Schieber, 2008; Dybing & Sanner, 2003; Surdyk, Rosén, Andersson, & Åman, 2004). Acrylamide occurs in many foods common to diets globally. It is formed during the heating of carbohydrate-rich foods containing the reducing sugars glucose and fructose and the amino acid asparagine, which are common to the plant ingredients used in the preparation of many foods (Lineback & Jones, 2011).

Acrylamide concentration in processed food products has become a very serious health issue. The possibility to remove acrylamide from foods by exploiting its chemical and physical properties was studied (Amrein, Schonbachler, Escher, & Amado, 2004; Anese, Suman, & Nicoli, 2010; Blom, Baardseth, Sundt, & Slinde, 2009). Lower protein (asparagines) content of bread is associated with lower acrylamide. In dough-based applications, addition of asparaginase resulted in reduction of acrylamide content in the final products of 34–92% (Hendriksen, Kornbrust, Østergaard, & Stringer, 2009). Also it is known that acrylamide content could be reduced by fermentation processes (Fink,

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Andersson, Rosen, & Aman, 2006; Fredriksson, Tallving, Rosen, & Aman, 2004) due to a consumption of the limiting precursor asparagine by yeast. Therefore, one of the possibilities to reduce the levels of acrylamide and herewith to ensure the safety of bakery products (Digaitiene, Hansen, Juodeikiene, Eidukonyte, & Josephsen, 2012; Juodeikiene et al., 2012) the use of bacteriocin-like inhibitory substances (BLIS) producing lactic acid bacteria (LAB) with high proteolytic activity (Elder, Fulcher, Leung, & Topor, 2007; Wehrle, Crowe, van Boeijen, & Arendt, 1999) should be envisaged.

Lactic acid bacteria are generally fastidious on artificial media, but they grow readily in most food substrates and lower the pH rapidly to a point where competing organisms are no longer able to grow. The formation of lactic acid is obtained by acidification below pH 4.2 which is a safety factor, and also gives a nice taste to whey products (Manea & Buruleanu, 2010). The leavening by LAB in association with yeast forming a softer and more palatable bread crumb, modifying flour components, e.g. swelling and partial hydrolysing proteins and polysaccharides (Salovaara, 2004). The highly promising results of these studies underline the important role that functional, bacteriocins of certain LAB strains play in the food industry as starter cultures, co-cultures, or bioprotective cultures, to improve food quality and safety (Juodeikiene et al., 2012).

The use of the lactic acid sourdough is a way to enhance dough properties, to improve flavour, texture and microbiological shelf-life of bread (De Vuyst & Vancanneyt, 2007). In the Baltic States, a traditional scald process is applied for the production of bread. In compare with rye bread making processes used in other parts of Europe, the major difference is a scalding step (15–20% of rye flour brewing up with hot water and saccharification during 3 h prior to fermentation) that contributes to the excellent quality and long shelf life of the bread. However, scalded bread preparation is a long, complicated and economically inefficient process. The alternative technological means for the intensification of sourdough production, higher stability of sourdough and improved bread quality are still being studied. Recently, considerable interest has arisen in the application of a new fermentation media composed of extruded products possessing specific physical properties (Juodeikiene et al., 2011). Extrusion cooking causes gelatinization of starch among the other physicochemical and functionality changes the grain components undergo; moreover, extrusion enhances the amount of dietary fibre and eliminates the bacterial contamination of the cereal material.

However, there is a lack of literature on the effect of scald saccharification process using enzymatic treatment of extruded rye wholemeal as well as of lactic acid fermentation on acrylamide formation in rye mixed bread.

The aim of this study was to investigate the effect of *Lactobacillus* and *Pediococcus* strains in combination with *Aspergillus niger* glucoamylase used for saccharification of extruded rye wholemeal on acrylamide formation in the most popular mixed rye bread of different loaf weight.

2. Materials and methods

2.1. Flours and microorganisms

Rye flour (type 700, moisture content 14.4%, falling number 130 s, ash 0.74%) and wheat flour (type 550D, moisture content 14.5%, falling number 350 s, gluten 27%, ash 0.68%) obtained from Kauno Grudai Ltd. mill (Kaunas, Lithuania) were used for mixed rye bread production. Extruded rye wholemeal (moisture content 8.6%) produced by a single-screw extruder (Ustukiu malunas Ltd, Lithuania) was tested as the target medium.

Lactobacillus sakei KU05-6, *Pediococcus acidilactici* KTU05-7 and *Pediococcus pentosaceus* KTU05-8 able to produce BLIS were isolated from rye sourdoughs (Digaitiene et al., 2012). Strains were stored at −70 °C in a Microbank system (PRO-LAB DIAGNOSTICS) and were propagated in MRS broth (CM 0359, Oxoid Ltd, Hampshire, UK) at 30 °C (KTU05-6) or 35 °C (KTU05-7 and KTU05-8) for 48 h with the addition of 40 mM fructose and 20 mM maltose prior to be used. Lyophilized bacteria of *Lactobacillus casei* (optimal temperature 35 °C) from the commercial product Lactobacterin (ImBio, Nizhny Novgorod, Russia) was used for comparison. The bacteria were diluted with a physiological saline to a concentration of 10^8 cfu/mL before the experiment. Fermented products (sourdoughs) for mixed rye bread baking were made using one single culture.

Spirizyme Plus FG (*A. niger* glucoamylase, Novozymes, Denmark) with specific activity 400 GU/g (GU is the amount of enzyme which hydrolyses 1 μmol of maltose per minute under specified conditions) was used for scald saccharification.

2.2. Preparation of fermented products

The fermented product (65% moisture content) for mixed rye bread has been prepared using 300 g of extruded rye wholemeal flour and 450 mL of water (50 °C). Saccharification was made with or without addition of a selected amount of *A. niger* glucoamylase. After 30 min LAB cell suspension (5 mL) containing about 9.2 cfu/mL was added, followed by fermentation for 24 h at temperatures optimal for the strains. The sourdough for control bread was made following the same formulation using rye flour and boiling water (95 °C), saccharified at 30 °C temperature for 3 h, and fermented under the same conditions as the test sourdough without addition of starter cultures (spontaneously). The pH value of sourdough was measured and recorded by a pH electrode. Prepared fermented product (750 g) was used for bread production.

2.3. Bread production

Experimental bread-making was done according to the traditional procedure used for sourdough bread making in Lithuania and Latvia. Breads, containing 15% flour basis of *L. casei*, *P. acidilactici*, *L. sakei* or *P. pentosaceus* fermented product, were made from 1 kg of wheat and 700 g of rye flours, 750 g of fermented product and 700 mL of tap water. The recipe of the control bread without addition of fermented product was based on 2 kg of flour weight (1 kg wheat flour and 1 kg of rye flour): salt 1.7%, fresh compressed yeast 2.5% and tap water 56%. The dough was then mixed at a low regime in a mixer (Diosna SP25, Osnabrück, Germany) for 10 min, fermented at 27 °C and 75% RH for 30 min. Then the dough was shaped and proofed at 35 °C and 80% RH for 40 min. Dough loafs of 1000 and 500 g were formed and baked in a deck oven (MIWE Michael Wenz GmbH, Germany) at 240 °C for 50 and 35 min, respectively. Breads were cooled at room temperature and bread crumb was subjected to acrylamide analysis.

2.4. Determination of amylase activity

The amylase levels excreted by single LAB were determined by the starch-iodine method described by Nguyen, Rezessy-Szabó, Claeyssens, Stals, and Hoschke (2002). The dough sample (5 g) was homogenized with 50 mL of distilled water and centrifuged at 5000× *g* for 10 min. Reaction mixture containing 1 mL of 1% (w/v) soluble starch as substrate in 1/15 M phosphate buffer (pH 6) and sample extract (0.5 mL) was incubated for 10 min at 30 °C. The reaction was stopped and the colour was developed by addition of 1.5 mL of diluted iodine reagent [2 mL of iodine (0.25% w/v) with KJ

(2.5% w/v) solution diluted with 0.5 M HCl to 100 mL]. Absorbance was measured at 670 nm using a Genesys 10 spectrophotometer (Thermo Fisher Scientific Inc., Langensenbold, Germany). One unit of α-amylase activity (1 AU) was defined as an amount of enzyme that catalyzes 1 g soluble starch hydrolysis to dextrins in 10 min at 30 °C temperature.

2.5. Determination of protease activity

The mode of action of the LAB protease was determined by Sigma–Aldrich non-specific protease assay (Cupp-Enyard, 2008). Dough sample (5 g) was dissolved in 50 mL of 10 mM sodium acetate buffer (pH 7.5) with 5 mM calcium acetate. For each sample 5 mL of 65% casein (w/v) as substrate and 1 mL of sample extract were incubated at 37 °C for 10 min. The reaction was stopped by the addition 5 mL of 110 mM trichloroacetic acid and was kept at 37 °C for 30 min. After centrifugation at 5000× *g* for 10 min the supernatant (2 mL) was taken and added to 5 mL of 0.5 M sodium carbonate and 1 mL of Folin–Ciocalteus reagent. The protease activity was detected spectrophotometrically since the released tyrosine developed a blue colouration. Each sample was read in a Genesys 10 spectrophotometer (Thermo Fisher Scientific Inc., Langensenbold, Germany) at 660 nm. One protease unit was defined as the amount of casein hydrolysed to produce a colour equivalent to 1.0 μmol (181 μg) of tyrosine per minute at 37 °C and pH 7.5.

2.6. Reducing sugar content

Reducing sugars in dough and bread were determined by a Waters HPLC system (Waters Associates, Milford, USA) consisting of a photodiode array detector and a Waters Micromass ZQ-2000 mass detector. The sourdough (5 g) or bread samples homogenized in 50 mL of the distilled water were shaken for 20 min and heated for 10 min at 90 °C to denature the enzymes. After centrifugation 15 min at 4500× *g*, the water extracts were filtered through the 0.45 μm syringe filters and aliquots of the filtrates were subjected for HPLC analysis.

The sugar separation was performed using a 250 × 4.6 mm i.d; 5 μm silica-based, aminopropyl-modified phase packing Supelcosil™LC-NH_2 column (Sigma–Aldrich, Steinheim, Germany) and a 2 cm × 4.0 mm; 5 μm NH_2 guard column (Sigma–Aldrich, Steinheim, Germany) within 20 min with an isocratic elution of acetonitrile/water (75/25 v/v%). The eluent was delivered at a 1.0 mL/min flow rate and the injection volume of 20 μL was used. The detection wavelength was set to 191 nm. Compounds were quantified using a calibration curve of the corresponding standards ranging between 50 and 500 μg/mL. Sucrose, glucose, D-fructose and D-maltose standards were obtained from Sigma–Aldrich (Steinheim, Germany) and of an analytical grade.

2.7. Determination of acrylamide by LC–MS/MS

Acetonitrile, methanol, hexane and acrylamide (>99.5% purity) were purchased from Merck (Darmstadt, Germany). The d_3-acrylamide was obtained from Fluka (Buchs SG, Switzerland) and was used as internal standard. Primary and secondary amines (PSA-sorbent) were purchased from Acros Organics (Geel, Belgium).

Two grams of sample, the internal standard (100 ng/g) and 5 mL of hexane were added into a 50 mL centrifuge tube, then the tube was vortexed. Distilled water (10 mL) and acetonitrile (10 mL) were added followed by the the QuEChERS extraction salt mixture (4.0 g anhydrous $MgSO_4$ and 0.5 g NaCl). The sample tube was shaken for 1 min vigorously and centrifuged at 4500× *g* for 5 min. The hexane layer was discarded and 1 mL of the acetonitrile extract was transferred to the tube containing 50 mg of PSA-sorbent and 150 mg of anhydrous $MgSO_4$. The tubes were vortexed for 30 s and then analysed by LC–MS/MS.

The quantitative analysis of acrylamide was based on the pretreatment of selected final products and performed by liquid chromatography–tandem mass spectrometry (LC–MS/MS) using a Waters Alliance HPLC system 2695 coupled to a Micromass Premier XE mass spectrometer (Micromass, Watford, UK). The separation of acrylamide was achieved with Luna 3um HILIC dC_{18} column (50 × 2.00 mm i.d., 3 μm; Phenomenex, Macclesfield, UK). Methanol (6%) in acidified acetonitrile (0.1% acetic acid) was used as a mobile phase (flow rate 0.25 mL/min, column temp. 40 °C, injection volume 10 μL). The conditions for detection by MS/MS were as follows: ionization was performed using electrospray in the positive mode (source temp. 120 °C, desolvation temperature 400 °C; cone gas flow 25 L/h, desolvation gas flow 600 L/h). The acrylamide levels in selective samples were quantified by the internal standard ($^{13}C_3$-acrylamide) method. The limit of quantification (LOQ) for acrylamide was 10 μg/kg.

2.8. Statistical analysis

All analytical experiments were carried out in triplicate. Data were subjected to the analysis of variance (ANOVA) using statistical package SPSS for Windows (Ver.15.0, SPSS Inc., Chicago, IL, USA, 2006). Calculated mean values were compared using Duncan's multiple range test with significance defined at $p < 0.05$.

3. Results and discussion

3.1. The effect of LAB on the acrylamide formation in mixed rye bread

The effect of a single LAB strain used for extruded rye wholemeal fermentation on the acrylamide formation in mixed rye breads of different loaf weight (500 g and 1000 g) was analysed (Fig. 1). In order to understand the role of fermented product on acrylamide formation and to prevent the end product contamination, the acidification kinetics of fermentation media, enzymatic profiles of LAB and reducing sugar formation have been taken into consideration.

Acrylamide concentrations in bread samples ranged from 57.84 ± 0.88 to 128.00 ± 1.64 μg/100 g. The results showed that the levels of acrylamide in breads made with LAB starters in all cases were lower than in the control sample. A reduction effect of lactofermentation on the acrylamide formation in mixed rye bread

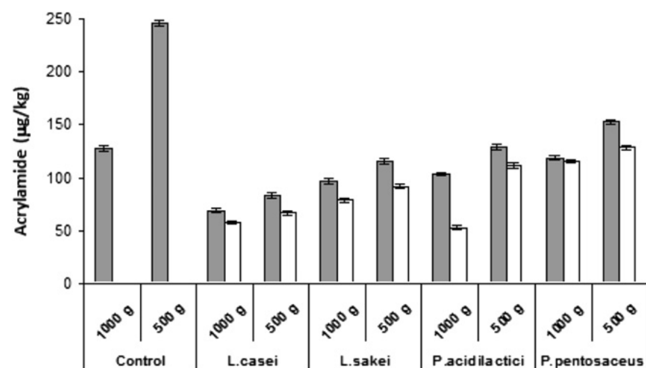

Fig. 1. Acrylamide formation in mixed rye breads (500 g and 1000 g loaf weight) made with different LAB and with (white) or without (grey) addition of *A. niger* glucoamylase for scald saccharification.

depended on the LAB strain used. The fermentation with commercial strain *L. casei* was found to have a higher effect on acrylamide reduction in bread samples of 500 g and 1000 g loaf weight on an average by 20.2% and 29.4%, respectively compared to *L. sakei*, *P. acidilactici* and *P. pentosaceus* strains (Fig. 1).

The difference in acrylamide reduction between tested LAB strains also was noticed. Acrylamide content in breads made with *L. sakei* fermented product was lower by 52.8 and 22.9%, followed by *P. acidilactici* – 47.6 and 19.2% and *P. pentosaceus* – 37.8 and 7.8% in 500 g and 1000 g loafs of bread, respectively compared to the control samples.

Less (by 27%) concentrations of acrylamide in 1000 g loafs of mixed rye bread versus 500 g loafs (Fig. 1) may were achieved due to prolonged heat treatment and higher moisture contents (Claus, Mongili, Weisz, Schieber, & Carle, 2008). The acrylamide content was shown as to be influenced by the product thickness and change of temperature in different locations of the bread (Açar & Gökmen, 2009). Acrylamide is predominantly generated in the outer crust layer where more than 99% can be found, while only trace amounts are detectable in the crumb (Fredriksson et al., 2004). This can be ascribed to the lower temperatures of only 100 °C in the inner parts of bakery products. The higher acrylamide levels in 500 g breads most probably resulted from the forced loss of humidity, leading to a faster and more intense drying of the bread crust. Since low moisture contents enhance acrylamide formation during the Maillard reaction, these effects may, at least partially, be avoided by applying a higher relative humidity during baking (FEDIL, 2011).

In the present study pH values of sourdoughs fermented with *L. casei*, *L. sakei*, *P. acidilactici* and *P. pentosaceus* ranged between 3.46 and 4.32 at the end of the fermentation, though on the initial stage pH was measured within the value of 5.24 (Fig. 2). The highest acidity was measured of dough fermented with *L. casei* strain (pH 3.46). *L. sakei* and *P. acidilactici* reduced the pH on an average by 31% after 24 h of fermentation, while the *P. pentosaceus* was found to be a weaker acidifier decreasing the pH by 17.5% compared to control sample (Fig. 2).

Furthermore, the tendency of the reduction of acrylamide contents in bread with the decreased pH values of the fermented products was found (Fig. 2). A significant reduction of acrylamide in all bread samples was obtained by pH of sourdough below 3.7. Saccharification of extruded rye wholemeal with glucoamylase as compared to the control sample was found to have a positive effect on acidification process by lowering the pH values of fermented products on average by 8.6% (Fig. 2). Herewith, fermentation with *P. acidilactici* in combination with glucoamylase enabled the decrease of pH from 3.65 to 3.35 (Fig. 2) indicating the reduction of acrylamide in the bread therefore by 48.9% compared to the corresponding bread without enzyme (Fig. 1).

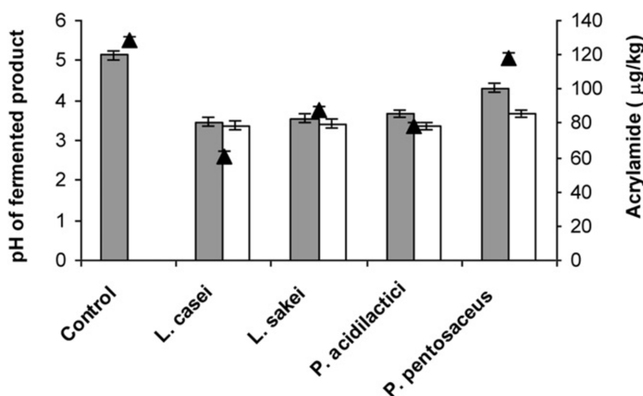

Fig. 2. Effect of pH of fermented products made with different LAB with (white) or without (grey) treatment with *A. niger* glucoamylase on acrylamide formation (average values) in 1000 g breads (triangles).

These results are in agreement with other authors that low pH values (pH < 5) can be one of the solutions to inhibit the Maillard reaction, thereby decreasing the acrylamide content in bread. The first step in acrylamide formation reaction (Maillard) is the formation of a Schiff base that can hydrolyse to form 3-aminopropionamide, a potent precursor of acrylamide (Granvogl, Jezussek, Koehler, & Schieberle, 2004). The reduction of the pH in the system inhibits the formation of the Schiff base by favouring protonation of the amine group of asparagine. This effect is due to the reduced pH to 3.7 rather than to assimilation of asparagine by the lactic acid bacteria (Fredriksson et al., 2004).

Hereby, the addition of consumable acids would be a very simple but efficient method to minimise acrylamide in bakery products (Amrein et al., 2004). As reported by Baardseth et al. (2004) and Blom et al. (2009), similar effects could be observed by lacto-fermentation. Rapid acid production is a preferred property of lactic acid bacteria as a starter culture for the fermented product processes (Simsek, Con, & Tulumoglu, 2006). Furthermore, an acidic flavour of bakery products is only accepted in the case of sourdough, which according to this study could attribute to improvement of mixed rye bread quality by prevention of acrylamide formation.

3.2. *Impact of the A. niger glucoamylase on saccharification of fermentation media and acrylamide formation in bread*

Although amino acid such as asparagine is the limiting factor of acrylamide formation in bakery products, sugars in fermentation media also could play an important role (Amrein et al., 2004). The effect of *A. niger* glucoamylase on the saccharification process was analysed by qualitative and quantitative composition of reducing sugars at different stages of bread preparation: saccharified scald, fermented product and bread (Table 1).

The results of the quantitative sugar analysis confirmed the saccharification degree (qualified as the total amount of saccharides) of the extruded rye wholemeal to be lower by 71.5% as compared to the rye flour scald (control) prepared under prolonged saccharification process (Table 1). The increase by 84.8% in a total amount of reducing sugars of scald as measured with the addition of glucoamylase preparation compared to the samples of extruded material (ERF) with no addition of glucoamylase was noticed (Table 1).

According to the obtained results, a significant reduce in terms of saccharification processes of extruded flour can be achieved with the addition of glucoamylase. In the case of extruded rye wholemeal, the highest amount of sugars was measured after 30 min saccharification, meanwhile in the control sample after 2.5 h.

In the perspective of the sourdough samples after fermentation the increase in fructose concentrations, and decrease in maltose and glucose concentrations was noticed (Table 1). The higher maltose amount in bread (Table 1) might be explained by maltose formation due to the breakdown of starch achieved by flour amylolytic enzymes. In fermented products, maltose and glucose are formed during the fermentation through the starch hydrolysis by cereal amylases and then utilized by the lactobacilli (Korakli, Rossman, Gänzle, & Vogel, 2001).

The study showed that total reducing sugar content of sourdough slightly ($r^2 = 0.5587$) correlated with acrylamide formation in bread, and was strongly affected by reducing sugar content measured in bread ($r^2 = 0.7193$). A tendency was noticed that the type of LAB strains used was determining for the course of the sourdough fermentation. In summary, *P. pentosaceus* and

Table 1
Contents of sugars (mg/100 g d.w.) in saccharified scald, fermented product and bread.

Samples	Sacharose	Fructose	Glucose	Maltose	Total sugars
RF scald:					
without glucoamylase	297.2 ± 28[c]	58.0 ± 7[c]	520.8 ± 38[c]	607.6 ± 42[c]	1483.7[c]
with glucoamylase	502.2 ± 36[b]	145.2 ± 23[b]	1286.3 ± 82[a]	2030.4 ± 88[a]	3964.1[a]
ERF scald:					
without glucoamylase	91.5 ± 5[d]	41.0 ± 2[d]	182.3 ± 8[d]	108.5 ± 6[d]	423.3[d]
with glucoamylase	1417.1 ± 69[a]	169.8 ± 13[a]	406.4 ± 44[b]	792.4 ± 62[b]	2785.8[b]
Fermented product (sourdough):					
Control	–	724.1 ± 23[a]	388.3 ± 13[a]	828.8 ± 38[a]	1941.2[a]
L. casei	–	355.2 ± 10[c]	92.2 ± 4[d]	487.6 ± 16[b]	935.0[c]
L. sakei	–	386.7 ± 12[c]	88.3 ± 5[d]	528.8 ± 14[b]	1003.8[c]
P. acidilactici	–	480.5 ± 16[b]	246.5 ± 10[b]	402.1 ± 12[c]	1129.1[b]
P. pentosaceus	–	513.7 ± 15[b]	121.2 ± 6[c]	512.7 ± 11[b]	1147.6[b]
Bread:					
Control	–	1125.9 ± 37[a]	920.4 ± 32[a]	3804.4 ± 62[a]	5850.8[a]
L. casei	–	82.5 ± 3[bc]	166.4 ± 5[cd]	1819.3 ± 49[c]	2068.2[d]
L. sakei	–	93.5 ± 5[b]	182.3 ± 6[c]	1975.2 ± 43[c]	2251.0[d]
P. acidilactici	–	75.6 ± 4[c]	330.2 ± 11[b]	3021.9 ± 56[b]	3427.7[b]
P. pentosaceus	–	88.6 ± 5[b]	161.9 ± 6[d]	2960.5 ± 42[b]	3211.0[b]

[a–d]represent significant ($p < 0.05$) differences in the same column.
d.w. – dry weight.
RF – rye flour, ERF – extruded rye flour.

P. acidilactici were the key strains for the formation of reducing sugars in fermented product and bread as well as acrylamide concentrations in breads, while the lower contents were measured with the *L. sakei* and *L. casei* strains (Table 1).

The experimental results showed that the addition of glucoamylase at an amount of 500 GU/kg for scald saccharification lowered the formation of acrylamide up to 16% in mixed rye bread compared to appropriate samples without enzyme (Fig. 1). Such observations indicate that the reduction of acrylamide may be due to decreasing level of sugars during the fermentation stage rather than reduction of free asparagine (Baardseth et al., 2006). In practice carbohydrates are necessary to affect the conversion of asparagine into acrylamide (Yaylayan, Wnorowski, & Locas Perez, 2003). It has been demonstrated that α-hydroxy carbonyl compounds, such as fructose or glucose, are much more efficient than others (Yaylayan & Stadler, 2005). Enzyme containing bakery improvers, especially amylases and amyloglucosidases are widely used in bread production and might have an impact on acrylamide. However as has been demonstrated by Claus, Mongili, et al. (2008), acrylamide remained unchanged by the use of enzyme-containing bakery improvers. This is in agreement with the obtained results that the contents of glucose and maltose declined greatly during fermentation.

Additional investigations of LAB enzymatic activities in fermentation media have revealed that obtained significant difference in the excreted protease as well as in amylase profiles could be related to the acrylamide formation in the bread making process (Fig. 3).

The highest protease activity after 24 h of fermentation in extruded rye flour media was found to be excreted by *L. casei* (189.01 PU/g), followed by the *L. sakei* (165.05 PU/g). *P. pentosaceus* and *P. acidilactici* strains were able to excrete protease levels of 28.06 and 20.29 PU/g, respectively. Results showed that proteolytic activity of LAB negatively influenced ($r^2 = 0.6605$) the formation of acrylamide in bread. The breads made with high proteolytic *L. casei* and *L. sakei* contained lower acrylamide concentrations (64.23 and 96.69 μg/kg, respectively), while breads prepared with low proteolytic strains *P. acidilactici* and *P. pentosaceus* contained higher acrylamide concentrations (103.48 and 121.47 μg/kg, respectively) (Fig. 3).

Acrylamide formation in bread was found to be slightly affected by LAB amylase activity excreted in extruded rye material ($r^2 = 0.57$). *P. pentosaceus* and *P. acidilactici* strains were found to have a higher amylase activity (172.57 and 120.24 AU/g, respectively) than *L. sakei* and *L. casei* (86.48 and 91.52 AU/g, respectively) (Fig. 3).

Despite the fact that increased protease activity of LAB may result in an enhanced release of amino acids from proteins, several factors (e.g. pH, fermentation time and activities of indigenous cereal enzymes) are responsible for the proteolytic events during sourdough fermentation. A cell envelope associated proteinase hydrolyses proteins to small peptides and amino acids, especially proline, glutamic and aspartic acids, increase in sourdoughs during fermentation and are important for rapid microbial growth (Zotta, Piraino, Ricciardi, McSweeney, & Parente, 2006; Zotta, Ricciardi, & Parente, 2007). Di Cagno et al. (2002) reported that the addition of one of a selected group of amino acids to the recipe for the food inhibited the formation of acrylamide during the thermal processing. The efficient amylolytic activity of LAB is expected to increase the availability of energy sources for yeasts to contribute to a rapid pH decrease (Sanni, Morlon-Guyot, & Guyot, 2002). The acrylamide content could be lowered by using lactic acid bacteria with lower amylolytic activity. Thus, the significant effect of LAB protease activity on the fermentation process as well as on acrylamide formation in bread can be related. *A. niger* glucoamylase in

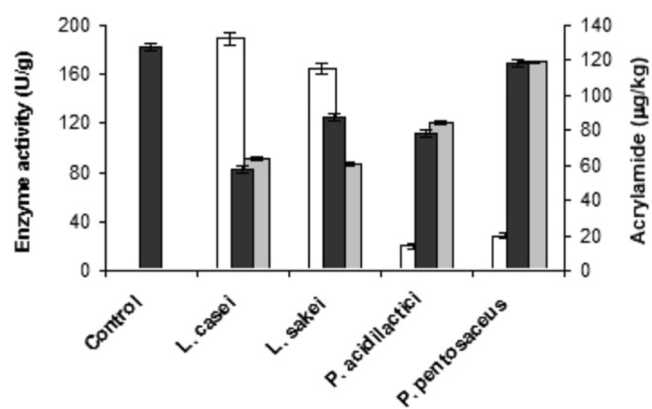

Fig. 3. Acrylamide content (black) in bread and activities of amylase (grey) and protease (white) excreted by LAB in extruded rye wholemeal media after 24 h of fermentation.

conjunction with an appropriate LAB strain could be used as one of the factors regulating acidification in dough, herewith decreasing the acrylamide content in bread. Above all, manufacturers need to keep in mind consumer expectations regarding flavour, colour, and other sensory properties in order to ensure their products remain marketable.

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