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Comparison of the impact of *Lactobacillus casei* and *Lactobacillus rhamnosus* on acrylamide reduction in flat and bulk bread

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RESEARCH ARTICLE

Abstract

The effect of yeast and lactic acid bacteria (LAB), *Lactobacillus casei* and *Lactobacillus rhamnosus* fermentation, on reducing acrylamide and physicochemical properties of the Iranian flat bread named, Sangak and bulk bread were studied. Bread containing whole grain such as Sangak bread contains a high level of enzymes and nutrients related to the bran and outer layers of the grain. In this study, the activity of amylase in whole wheat flour was 19.8% higher than to white wheat flour, based on falling number analysis. Results showed after 24 h of fermentation, in similar temperature, the pH values of the sourdoughs made from whole wheat flour were higher, compared to sourdoughs prepared from white wheat flour. Acrylamide formation was affected (r=0.69) by pH of sourdough. In addition, moisture and water activity of bread reversely influenced (r=-0.807 and -0.588, respectively) the formation of acrylamide in bread. As an important result, acrylamide content of Sangak bread in all cases was lower than in the bulk bread. According to a sensory analysis, Sangak and bulk bread leavened with LAB starters had the most acceptable to yeast starter. The addition of sourdough starters decreased pH of bread, which causes of enhancing the texture and sensory properties, as well as reducing of contaminants like acrylamide.

Keywords: cereals quality, contaminants, food safety, wheat

1. Introduction

Acrylamide can be a heat-induced contaminant naturally occurring in home cooking and industrial process of the many foods consumed daily around the world. It comes primarily from the reaction between reducing sugars (carbonyl moiety) and the amino group of free L-asparagine. French-fried potatoes, potato chips, bread, biscuits, and coffee all make contributions to the human dietary exposure. International bodies concerned with food safety and industrial sectors are active in implementing ways of minimising acrylamide formation during preparation, baking, frying and toasting (Arribas-Lorenzo and Morales, 2012; Bemiller and Huber, 2008). The European Chemicals Agency has added acrylamide to the list of substances of high concern (EC, 2006). As acrylamide has been classified as a probable carcinogenic substance for humans (IARC, 1994) and is thought to be a neurotoxin (WHO, 2002), national and international regulatory agencies have focused their attention on the detection of acrylamide in food items.

Among the food mentioned above, bread is a staple food in the worldwide. It is a good source of energy, protein, dietary fibre, minerals, vitamins and bioactive compounds. Moreover, several choices and tools for reducing acrylamide in cereal products are reported. The results obtained in several studies have clearly showed that raw materials, formulation and product composition, additive formulations, processing methods and baking technology have effects on the acrylamide contents of bakery product (Claus *et al.*, 2008).

Since 1982, biodegradation of acrylamide has been explored extensively in the non-food industry. Biodegradation of acrylamide occurs via hydrolysation of the amide group of acrylamide to acrylic acid and ammonia by amidase. Microorganisms capable of using acrylamide and

intermediate metabolites in this pathway as an energy source, as well as, many microorganisms can produce amidase (Charoenpanich, 2013).

It is well-known that the acrylamide content in bakery products can be reduced by fermentation processes (Fink *et al.*, 2006; Fredriksson *et al.*, 2004). It was demonstrated that acrylamide of yeast fermented bread with long fermentation is 87% lower than bread with short fermentation because of the consumption of the limiting precursor asparagine by *Saccharomyces cervisiea* (bakers' yeast) (Fredriksson *et al.*, 2004).

Therefore, one of the options for cutting back the amount of acrylamide and to ensure the safety of bakery products the utilisation of lactic acid bacteria (LAB) ought to be explored (Digaitiene *et al.*, 2012; Elder *et al.*, 2007). The use of sourdough by LAB has increased recently, because of consumers demand for food consumption without added chemical preservatives. Some of the advantages of using sourdough are as follows: improvement of sensorial characteristics via proteolysis and lipolysis activity which produce aromatic compounds, improvement of volume, texture, and shelf life of bread by producing exopolysaccharides, improvement in the availability of proteins and amino acids, increasing divalent cations (zinc, iron, and calcium) bioavailability through degradation of phytic acid (De Vuyst and Neysens, 2005; Gamel *et al.*, 2015; Katina *et al.*, 2005; Torkamani *et al.*, 2015).

In Iran, a traditional formulation by sourdough is applied for the production of a sort of bread, named Sangak, which contributes to the superb quality and long shelf life of the bread. Sangak bread is created by using whole-grain wheat flour.

The increased intake of dietary fibre has been linked with a reduced risk of obesity, cardiovascular disease, diabetes, certain cancers and constipation. Bran or fibre in bread slows sugar absorption. The utilisation of refined flour in bread decreases its dietary fibre content and associated bioactive compounds. Thus, in many types of bread around the world, whole-meal flour, composite flours, bran, fructan/fructo-oligosaccharides and fibre concentrates are added to bread to complement it with the dietary fibre (Gamel *et al.*, 2015). However, these ingredients will modify dough properties and also the bread, therefore the bread-making method must be custom-made to realise desirable product (Rakha *et al.*, 2013). In addition to dietary fibre and phenolic compounds, bran is considered to be a source of minerals and phytic acid, which is the major anti-nutritional factor for micronutrient absorption. It has been widely discussed within the literature that in sourdough fermentation, the activity of endogenous enzymes beside the microorganism phytase activity, leads to an improvement of mineral bioavailability (Coda *et al.*, 2015).

Therefore, the aim of this study was to evaluate the impact of LAB, yeast, and also the type of bread in the fermentation process, on the reduction of acrylamide in bread. For this purpose, totally different formulations of Sangak and bulk bread were made and acrylamide was determined after baking. The impact of flour specifications were also evaluated.

2. Material and methods

Chemicals

All chemicals and solvents utilised in the tests were of analytical grade and for high pressure liquid chromatography were purchased from Merck (Darmstadt, Germany) and Sigma-Aldrich (Saint Louis, MO, USA).

Ingredients

Two types of wheat flour obtained from a local mill (Karaj, Iran) were used for bread preparation. The specifications of the flours are shown in Table 1. Dried instant yeast (Iran Maye Co., Karaj, Iran), salt and different ingredients were purchased domestically.

LAB strains and culture conditions

The LAB strains were *Lactobacillus casei* (DSM 20011) and *Lactobacillus rhamnosus* (LGGID 100271), that were obtained from the culture collection of the Food Microbiology Laboratory of the faculty of Agricultural Engineering and Technology, Tehran University (Tehran, Iran). The LAB strains were previously selected based on their capability for improving crumb firmness, flavour and overall acceptability. The strains were cultivated and propagated by doubling in De Man, Rogosa and Sharpe

Table 1. Flour specifications.[1]

Specifications	Whole wheat flour	White wheat flour
Moisture (%)	10.4±0.1	11.2±0.1
Ash (%)	1.63±0.01	0.70±0.01
Protein (%)	15.5±0.5	11.3±0.3
Falling number(s)	479±1	574±2
Damaged starch (UCD)	21.5±0.3	18.1±0.1
Gluten (%)	27±0.1	29.6±0.1
Gluten index	92±0.1	72±0.3
pH	6.2±0.3	5.6±0.2
Fibre (%)	8.2±0.1	1.4±0.1
Total reducing sugars (mg/g)	26.2±0.1	12.9±0.1

[1] Results are mean ± standard deviation of three determinations.

broth medium (Merck) for 24 h at 37 °C up to 10^9 cfu/g, before the experiment.

Sourdough preparation

Stationary phase cells of the LAB strains, as described above, were harvested by centrifugation at 8,000×*g*, for 10 min, at 4 °C. Then they were washed twice with sterile 0.85% saline solution, suspended in the milk, and incubated at 37 °C for 24 h. The cell suspension was added to the substrate (wheat flour and water) to obtain 10^9 cfu/g in the dough.

Bread making

Bulk bread

A commercial bakery (Nanavaran Company, Tehran, Iran) made the bread in their laboratory. The dough of bulk bread was prepared according to the approved AACC methodology 10.10B (AACC, 2000). Ingredients based on flour weight (100 g) were, instant dry yeast (1.7 g), sourdough (13.0 g), bread improver (16.0 g), and vegetable oil (5.0 g).

After combining the dry ingredients in a stand mixer, 50 g tap water was gradually added, with mixing at low speed for 3 min, followed by 5 min at medium speed until the dough was formed.

The dough was divided into 50 g portions and formed as a round roll. Thereafter the dough was fermented and proofed at 29±0.5 °C for 90 min, following by baking at 190 °C for 20 min in a convection oven. The oven was provided with two centred fans that effectively facilitate heat transfer within the oven. All of bread was cooled at room temperature until packaging and transfer to the laboratory. Control bread was prepared exactly as described above, without sourdough.

Sangak bread

Experimental Sangak bread was prepared according to the traditional procedure used for this bread making in Iran, and a bakery was selected locally in Karaj, Iran.

The Sangak bread formula consisted of 100 g whole wheat flour, 90 g tap water, 1.2 g salt, 1 g instant dry yeast, 13 g sourdough. The ingredients were blended and the dough mixed at a low speed in a mixer for 3 min followed by 7 min at medium speed until the dough was formed. This dough was fermented at 29±0.5 °C for 90 min, and then was flattened on the little stones within the traditional furnace. Baking was performed at the beginning at 330 °C for 2 min, followed by 300 °C for 1 min. The control bread was prepared precisely as defined above, without sourdough.

Analytical techniques

Preparation of test samples

Sangak bread and Bulk breads were sliced and then dried in an oven at 30-40 °C. The drying temperature was set relatively low to avoid acrylamide formation throughout the drying process. The bread samples were ground and homogenised by employing a mixer (model Mj-176NR; National Co., Osaka, Japan).

Acrylamide analysis

Acrylamide determination was performed according to a sensitive technique of Lim and Shin (2013) and Ghasemzadeh-Mohammadi *et al.* (2012) with modifications. Gas chromatography with mass spectrometry (GC-MS; a 7890A GC system from Agilent Technologies, Palo Alto, CA, USA, with a triple-axis detector coupled with a 5975C inert MSD network mass selective detector) was applied to determine acrylamide in bread. For the first time, the planned technique very sensitively determines ultra-trace levels of acrylamide concentration in bread after derivation with xanthydrol and dispersive liquid-liquid micro extraction (DLLME).

Briefly, in order to achieve acrylamide primary extraction from the matrix, a mixing solution containing potassium hydroxide, and ethyl alcohol was added to the sample. Microwaving (type MW 602; Delonghi, Appliances S.r.l, Treviso, Italy) at 500 MHz for 2 min was employed to hydrolyse the sample. After cooling, the compounds were centrifuged at 4,000×*g* for 5 min (Rotorfix 32A; Hettich, Kirchlengern, Germany). Then the pH of the aqueous phase was decreased to 6.5 by adding hydrochloric acid. Finally, to precipitate the proteins and carbohydrates, Carrez solutions 1 and 2 were added to the vessel, which was then centrifuged again at 4,000×*g* for 5 min. The filtered sample was shaken for 30 min at 300 rpm using a mechanical shaker after adding xanthydrol solution, HCl, and acrylamide-d3 (1.0 mg/l in methanol). The derivation reaction was conducted at ambient temperature for 30 min in the dark, and then the solution was neutralised with KOH. Then the DLLME procedure was applied to extract the xanthyl-acrylamide. A solution consisting of tetrachloroethylene as the extracting solvent and acetone as the disperser solvent was injected rapidly into the xanthyl-acrylamide solution. The mixture was gently shaken and centrifuged at 4,000×*g* for 10 min. The dispersed fine particles of the extraction phase formed as a sediment at the bottom of the vessel. The upper aqueous phase was separated with a syringe and about 1.5 µl of the sediment phase was injected directly into the GC-MS using a micro syringe.

Chemical determinations

Water activity (A_w) was determined using a thermo constanter (RS 232; Novasina AG, Lachen, Switzerland) at 25 °C. The moisture of flour and bread samples were determined according to the ISO method 712 (ISO, 2009). The ash contents of flours were analysed according to the ISO method 2171 (ISO, 2007). Wet gluten and gluten index of flours were determined by the mechanical method according to the ISO method 21415-2 (ISO, 2006). The hydrogen ion activity (pH) of flour, sourdough and bread was measured by making a 10% solution in water. The measurement was carried out employing a pH meter (model 632; Metrohm AG, Herisau, Switzerland), based on AOAC method 943.02 (AOAC, 1995). Titratable acidity or acid content was measured as the amount of 0.1 N NaOH (ml) required to neutralise solution (method 947.05; AOAC, 1995). Protein and fibre of flours were determined based on AACC methods (method 46-12 and 32-05; AACC, 2000).

Total reducing sugar was considered as the sum of glucose, fructose and maltose content and quantitated by ultra-high pressure liquid chromatography (platin blue; Knauer, Berlin, Germany) equipped by a refractive index detector (smart line, 2300; Knauer) after aqueous extraction from the sample matrix based on AACC method 80-04 (AACC, 2000).

Sensory analysis

A panel of ten specialists was used to assess the staleness of bread (method 74-30; AACC, 2000), based on sensory analysis. They were asked to judge the overall acceptance of every sample regarding general properties. The ranking scale ranged from 1 (unacceptable and very stale) to 6 (ideal and very fresh). They judged the sample by feel with the fingers, odour, flavour, mouth-feel, and in any manner that they commonly judge the freshness of bread.

Statistical analysis

A two-way ANOVA was applied to bread samples with the SPSS software package (version 22.0; IBM, Armonk, NY, USA). When the difference between the samples in ANOVA was statistically significant, pairwise comparisons of those samples were analysed with Duncan's test (significance of variations at $P<0.05$).

3. Results and discussion

Flour characteristics

Table 1 shows the characteristics of the 2 flours in this study. The whole wheat flour had a higher content of ash, protein, fibre and damaged starch than the white wheat flour. Falling number is a methodology based on the power of alpha-amylase to liquefy a starch gel. The activity of the enzyme is measured by falling number. So, the activity of alpha-amylase in whole wheat flour was higher than that of the white flour.

Bread containing whole grain such as Sangak bread has a high level of enzymes such as proteases, amylases, decarboxylases and transaminases due to the bran and outer layers of wheat. During this study, the activity of the α-amylase enzyme in whole wheat flour was 19.8% higher than the white wheat flour based on falling number analysis. In addition, damaged starch which is denatured by amylolytic enzymes in the whole wheat flour was 15.8% more.

When the enzyme activity is high in the flour, the breakdown of proteins and starch to amino acids and simple sugars can easily happen and the nutrients available for microorganisms increase in the dough. This is often caused by an excessive amount of acid production and the accompanying reduction of pH and this was determined by the present study. The variations in terms of falling number will affect the final total titratable acidity (TTA) of the sourdough (Katina *et al.*, 2005). Other researchers have shown that after 20 h of fermentation, at identical temperature, the TTA values of the sourdoughs made with whole rye flour were higher compared to sourdoughs prepared from dark flour (De Vuyst and Neysens, 2005). This may be because of the variations in nutrient content (vitamins and minerals), and in addition, in buffering capability, related to the phytic acid from the aleuronic layer of the cereals. These two factors will stimulate the growth and biochemical activity of the sourdough microflora, increasing the acids and flavour compounds synthesis (Brummer and Lorenz, 1991; De Vuyst and Neysens, 2005).

Figure 1 shows the content of acrylamide in two kinds of bread. This figure shows well that the acrylamide content in Sangak bread, in all cases, is less than in the bulk bread.

Mulla *et al.* (2010) investigated the influence of damaged starch in whole wheat flour on the creation of acrylamide in an Indian flat bread, Chapatti, and showed that the total reducing sugars of whole wheat flour were raised with an increase in the damaged starch content. These conclusions are in agreement with our results in that the total reducing sugars in whole wheat flour were 50.8% more than the white wheat flour, with an increase in damaged starch (15.8%) in whole wheat flour. However, Mulla *et al.* (2010) also showed that the damaged starch content in Chapatti bread, had a strong positive correlation with acrylamide formation that is in contrast to our results, probably due to the lack of fermentation in Chapatti making.

Gündüza and Cengiza (2015) studied the levels of acrylamide in different bread samples taken from a Turkish market (all bread fermented by yeast) and they detected

the highest level of acrylamide in the whole wheat bread (479±325 µg/kg). They concluded that the results related to the use of the outer bran layers of wheat. Also, Springer *et al.* (2003) showed higher levels of acrylamide in baked products from flour with high extraction and Capuano *et al.* (2009) demonstrated that in a whole wheat model system more acrylamide was produced than with white. These findings are in contrast to the results of our research which showed a lower level of acrylamide in Sangak bread from whole wheat flour than bulk bread.

In addition, the proteolytic activity of wheat flour attributed mostly to aspartic proteinases and carboxypeptidases and both protease groups are active in acidic conditions. The level of these enzymes in the outer layers of endosperm and germ in whole wheat flour are higher than elsewhere (Ganzle *et al.*, 2008). In the present study the level of protein and, therefore, amino acids, in whole wheat flour was 27% more than the white flour.

Several studies have shown different protein or amino acids, especially sulphur-containing amino acids and peptides such as glycine, methionine, cysteine, and glutathione, were effectively used for acrylamide reduction in bakery products (Claus *et al.*, 2008; Keramat *et al.,* 2011).

Therefore, the results obtained have clearly shown that flour characteristics have a significant effect on reducing acrylamide. In many cases, as has been explained, our results differed from the others.

The effect of pH and acidity on the acrylamide formation in bread

Our results showed pH values of sourdoughs (with whole wheat flour) fermented with *L. casei*, *L. rhamnosus* in Sangak bread were 2.90 and 3.00, and sourdoughs (with white wheat flour) in bulk bread were 3.59 and 3.68, respectively, at the end of the fermentation (Table 2). Moreover, the data in Figure 1 show a tendency for reduction of acrylamide contents in bread with the decreased pH values. A significant reduction of acrylamide in Sangak bread was obtained with a pH of sourdough below three. Although the pH of bulk bread was less than the Sangak bread, due to the bulk bread dough being chemically acidified by a bread improver, this did have an influence on acrylamide reduction.

These results are in agreement with other authors that low pH values (pH<5) achived through microorganism sources, can be one of the solutions for preventing Maillard reaction and decreasing the acrylamide content in bread. The primary step in acrylamide formation in Maillard

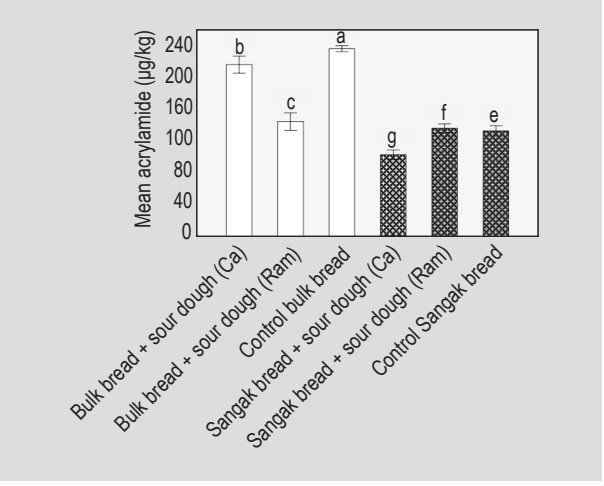

Figure 1. Comparison of mean acrylamide levels in different types of bread (Ca = *Lactobacillus casei*; Ram = *Lactobacillus rhamnosus*; error bars: standard deviation).

Table 2. Specifications of bread.[1]

Type of bread	Added strain in sourdough	Water activity	Moisture (%)	pH of sourdough	pH of bread	Total reducing sugar (mg/g)	Scores of sensory analysis
Sangak	*Lactobacillus casei*	0.91±0.003^{a}	35.71±0.01^{a}	2.90±0.02^{b}	5.68±0.02^{a}	19.28±0.10^{e}	5±0.0^{a}
Sangak	*Lactobacillus rhamnosus*	0.79±0.005^{c}	33.19±0.01^{c}	3.00±0.04^{b}	5.69±0.03^{b}	15.14±0.11^{d}	5±0.5^{a}
Control Sangak	–	0.86±0.005^{b}	33.51±0.01^{b}	–	5.91±0.01^{b}	12.35±0.05^{f}	4±0.0^{b}
Bulk	*L. casei*	0.79±0.001^{c}	21.90±0.05^{d}	3.59±0.1^{a}	5.02±0.01^{d}	51.06±0.20^{b}	5±0.3^{a}
Bulk	*L. rhamnosus*	0.75±0.001^{d}	21.90±0.05^{d}	3.69±0.1^{a}	5.00±0.01^{d}	54.42±0.78^{c}	5±0.0^{a}
Control bulk	–	0.79±0.001^{c}	21.80±0.005^{e}	–	5.19±0.01^{c}	67.35±0.10^{a}	3±0.5^{c}

[1] Results are mean ± standard deviation of three determinations. Different letters in the same column indicate significant differences between the values (P<0.05) by Duncan's multiple-comparison test.

reaction is that the formation of a Schiff base which will change to form 3-aminopropionamide, a potent precursor of acrylamide (Bemiller and Huber, 2008).

Low pH in the dough inhibits the formation of the Schiff base by protonation of the amine group of asparagine, leading to aspartic acid production, which led to a strong decrease of the key intermediate Maillard reaction compounds and inhibited acrylamide formation. The addition of food grade acids (lactic, tartaric, citric acids) would be a very simple method for minimising acrylamide in bakery products, but the subsequent acidic taste may not be acceptable and must be assessed for each product (Amrein *et al.*, 2004; Claus *et al.*, 2008). Furthermore, acidic flavours in bakery products are just accepted in the cases of sourdough, which might contribute to improvement of bread quality and prevention of acrylamide formation, as confirmed by the other researchers (Bartkiene *et al.*, 2013; Fredriksson *et al.*, 2004). Fast acid production is a most popular property of LAB as a starter culture for the fermented product processes.

The effect of LAB and total reducing sugars on the acrylamide formation in bread

The impact of a single LAB strain used, in whole wheat flour and white flour fermentation on the acrylamide formation in Sangak bread and bulk bread was analysed. In order to know the role of the fermented product on acrylamide formation, the specifications of bread like, A_w, moisture, pH and acidity and specifications of flour were taken into account.

Acrylamide concentrations in bread samples ranged from 104.30 till 239.12. The results showed that the amount of acrylamide in bread made with LAB starters in two kinds of bread was less than in the control sample, except Sangak bread fermented by *L. rhamnosus* with a 2.5% increase. Moreover, the acrylamide content of Sangak bread in all cases was lower than in the bulk bread, the data are presented in Table 3 and Figure 1.

A reduction in the effect of lactofermentation on the acrylamide formation in bread samples depended on the activity of the LAB strain used in sourdough and compatibility or competition with yeast in the utilisation of nutrients. The fermentation with commercial strain *L. casei* was found to have a higher effect on acrylamide reduction in Sangak bread samples. Lower (by 22.8%) concentrations of acrylamide in sourdough bread versus control bread (Table 3) may be achieved due to utilisation of reduced sugars by *L. casei* and yeast.

The results show that total reducing sugars of breads influenced the formation of acrylamide in bread and this correlation is significant (Figure 2). This is a consequence of a consumption of the precursors by microorganisms in the fermentation process. Sugar looks to be the most important in fermentation process for acrylamide formation in bakery products because free asparagine is relatively low in wheat flour. This result is confirmed by other studies (Keramat *et al.*, 2011). Although asparagine is the limiting factor in

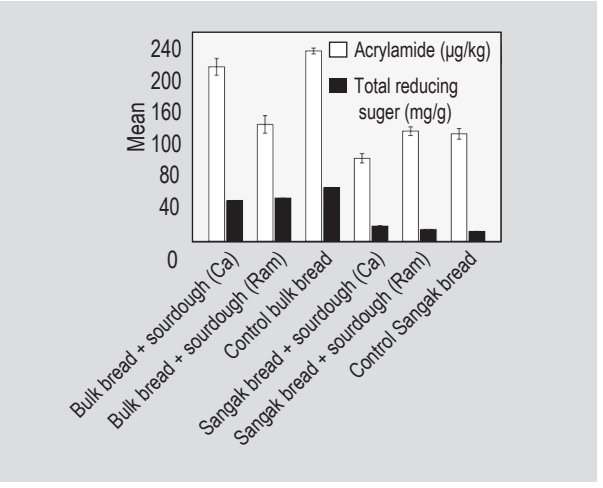

Figure 2. Comparison of means of acrylamide and total reducing sugar levels in bread (Ca = *Lactobacillus casei*; Ram = *Lactobacillus rhamnosus*; error bars: standard deviation).

Table 3. Acrylamide concentrations of bread and reduction or increase compared to control.[1]

Type of bread	Added strain in sourdough	Acrylamide (µg/Kg)	% reduction	% increase
Sangak	*Lactobacillus casei*	104.31±4.7^{f}	22.8%	–
Sangak	*Lactobacillus rhamnosus*	138.44±5.1^{d}	–	2.5%
Control Sangak	–	135.06±5.2^{e}	–	–
Bulk	*L. casei*	219.11±8.2^{b}	8.37%	–
Bulk	*L. rhamnosus*	146.78±6.5^{c}	38.61%	–
Control bulk	–	239.12±8.6^{a}	–	–

[1] Results are mean ± standard deviation of three determinations. Different letters in the same column indicate significant differences between the values (P<0.05) by Duncan's multiple-comparison test.

bakery products, sugars play a critical role and necessary for converting of asparagine into acrylamide (Claus *et al.*, 2008).

Reduction in the effect of lactofermentation on the acrylamide formation in Sangak bread depended on the specifications of flour and production process. In Bulk bread after fermentation and baking, the increase in total reducing sugar concentrations was noted (Table 2). In fermented products, maltose and glucose are increased during the fermentation through starch hydrolysis by flour amylolytic enzymes and then utilised by the lactobacilli (Korakli *et al.*, 2001).

L. casei and *L. rhamnosus* could well reduce (8.37 and 38.61%, respectively) the acrylamide content of bulk bread compared to control bread. *L. rhamnosus* and yeast had better competition and coexistence in bulk bread and they could reduce the acrylamide effectively. As regards the high remaining reducing sugar in bulk bread, less activity or consumption of the other nutrients such as amino acids by microorganisms to supply energy will be expected.

Baardseth *et al.* (2004) reported that after lactofermentation (NCIMB 40450) process in crispbread acrylamide was reduced by 75%. This result is because of the reduced pH (3.7 compared to 6.0 in the control) instead of consumption of asparagine by the LAB. These findings are also in accordance with our results that reduced pH of sourdough in Sangak bread influenced reduced acrylamide in this kind of bread. In other studies it was reported that a reduction of acrylamide could also be as a result of decreasing level of sugars during the fermentation rather than the reduction of free asparagine (Baardseth *et al.*, 2006).

In contrast to our findings, Fredriksson *et al.* (2004) showed that the free asparagine content of fermented bread by spontaneous sourdough did not decrease as efficiently but had a strong negative influence on asparagine consumption by the yeast. The authors suspected that this type of fermentation may result in a bread with a higher acrylamide content than bread fermented with yeast only (Fredriksson *et al.*, 2004). In this study, the role of reducing sugars and type of LAB has been ignored.

Bartkiene *et al.* (2013) showed that fermentation with a commercial strain *L. casei* has a greater impact on acrylamide reduction in bread samples with totally different loaf weights (500 and 1000 g) by 20.2 and 29.4%, respectively compared to *Lactobacillus sakei*, *Pediococcus acidilactici* and *Pediococcus pentosaceus* strains. Although in the survey mentioned above, the sourdough bread was prepared with mixed rye and wheat flour, but the effect of *L. casei* was similar to our results. However, only very few or almost no studies have been reported regarding the influence of fermentation by LAB and yeast together on the acrylamide level in flat bread.

As previously mentioned, in the whole wheat flour, due to the high activity of enzymes and damaged starch, carbohydrates and proteins are easily broken. The first result is the availability of nutrients for microorganisms increases. As well the protein content of whole wheat flour was 27% more than white flour, therefore in the fermented Sangak dough a greater amount of amino acids and reducing sugars could be synthesised and less reducing sugars remain in the final product. Decreasing the amount of reducing sugars in Sangak bread supported this claim. Also, because of the low level of asparagine in wheat flour, its combination with reducing sugars will be much lower and so in this kind of bread would have lower acrylamide.

The impact of moisture and A_w on the acrylamide formation in bread

The lower content of acrylamide in Sangak bread versus Bulk bread could also be achieved due to short heat treatment and higher moisture contents (Figure 3), as suggested by the other studies (Claus *et al.*, 2008). Fermented Sangak bread with *L. casei* had the lowest arylamide and the highest moisture of all samples. Açar and Gökmen (2009) showed that the acrylamide concentration was influenced by the product depth and the temperature in different locations of the bread.

Since low moisture contents enhance acrylamide formation through the Maillard reaction, these effects might at least partially, be avoided by using a higher of relative humidity throughout baking (FoodDrinkEurope, 2011). During the baking of dough, the water content on the surface of the loaf quickly decreases providing optimum conditions for the formation of Maillard reaction products (MRPs) and an intense brown colour. Inside the dough, the temperature

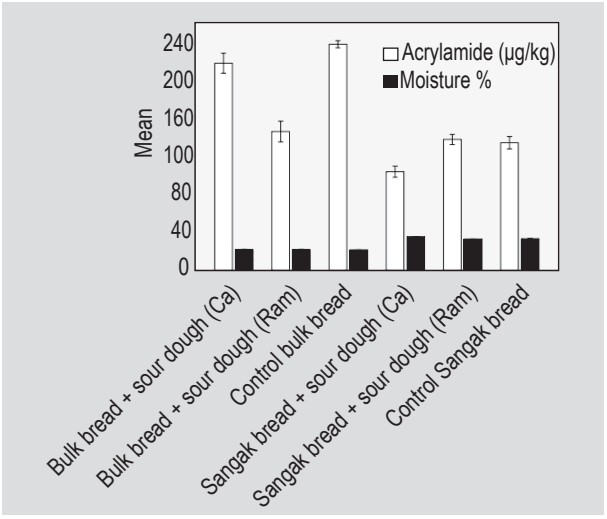

Figure 3. Comparison of mean levels of acrylamide and moisture of bread (Ca = *Lactobacillus casei*; Ram = *Lactobacillus rhamnosus*; error bars: standard deviation).

is lower and the water activity remains relatively high. Therefore, the crumb is just weakly coloured with a low concentration of MRPs (Capuano *et al.*, 2008).

Bassama *et al.* (2011) suggested that Schiff base formation is reversible, it is likely that with higher A_w the elimination of water molecules during Schiff base formation is more difficult than at lower A_w. Therefore, the moisture content or water activity could act in changing the balance between Schiff base and the reactants (Bassama *et al.*, 2011).

Sadd and Hamlet (2005) showed that flat bread such as pizza bases and naan had amazingly low levels of acrylamide, despite their larger proportion of crust to the crumb. This could be as a result of only a small proportion of the crust surface is deeply coloured by baking.

Low moisture content could be a more important promoter of acrylamide than temperature, thus crust moisture could be a key factor in controlling acrylamide levels, and this accounts for a lot of the variation seen in different kinds of bakery products (Sadd and Hamlet, 2005).

In our study, the results showed that moisture and A_w of bread samples negatively influenced (r=-0.807 and -0.588, respectively) the formation of acrylamide in bread. The bread made with high moisture and A_w had lower acrylamide concentrations.

4. Conclusions

The results reported in this research showed that the type of flour and fermentation process are both factors can strongly influence the acrylamide formation in bread. In summary, fermentation by microorganisms could inhibit or decrease acrylamide formation in bakery products in three ways. First, by lowering the pH through increased microbial and bran endogenous enzymatic activities, which may attribute to protonation of the amine group of asparagine, so decreasing the key intermediate Maillard reaction. Secondly, by reducing acrylamide precursors as a result of consumption by microorganisms. And thirdly, by biodegradation and destruction of acrylamide through deamination or utilisation of acrylamide by microorganisms.

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