NARDI FUNDULEA, ROMANIA
www.incda-fundulea.ro

ROMANIAN AGRICULTURAL RESEARCH, NO. 29, 2012
Print ISSN 1222-4227; Online ISSN 2067-5720

THE INFLUENCE OF A CELLULASE BEARING ENZYME COMPLEX FROM ANAEROBIC FUNGI ON BREAD STALING

Seyhun Yurdugul[1*], Natalija-Atanasova Pancevska[2], Gülgez Gökçe Yildiz[1] and Faruk Bozoglu[3]

[1]Abant Izzet Baysal University, Faculty of Arts and Sciences, Department of Biology, 14280, Bolu, Turkey.

[2]Institute of Biology, Faculty of Natural Sciences and Mathematics, St. Cyril and Methodius University, Gazi Baba bb, Skopje, Macedonıa.

[3]Middle East Technical University, Department of Food Engineering, 06531, Ankara, Turkey.

*Corresponding author. E-mail: yurdugul_s @ibu.edu.tr

ABSTRACT

The digestive system of the ruminants possesses anaerobic fungi, which are responsible for the degradation of cellulose and cell wall structures by their enzyme systems. An enzyme complex, bearing cellulase activity from *Neocallimastix* spp. which belongs to anaerobic rumen fungi, was partially isolated and its effect at various concentrations on bread quality was tested. The addition of enzyme complex into bread dough resulted in a decrease of hardness, gumminess and chewiness, providing a softer crumb, indicating the retardation of the staling in bread, mostly due to the degradation of cell wall components. A professional sensory analysis, carried out by trained panellists indicated that 0.3 mL cellulase containing enzyme solution added was preferred.

Key words: anaerobic rumen fungi, enzyme complex, bread, bread texture.

INTRODUCTION

Bread is the main staple food item in many developing countries, and in the Turkish diet, its importance has not changed for centuries. However; the short shelf-life of bread, due to staling, causes a major problem and this may be costly to producer, distributor, consumer and the country in general. When a loaf of bread is removed from the oven after baking, series of changes starts that eventually leads to deterioration of quality (Sahlström and Brathen, 1996). Changes in flavour and texture taking place during storage are commonly called staling (Bollaín et al., 2005), and are explained in two categories: crust staling and crumb staling. The major cause of crust staling is believed to be moisture redistribution. Water migrates to the crust from the crumb or becomes adsorbed from the air, causing hydration of crust components (Primo-Martin et al., 2006), resulting in a soft, leathery texture and is generally less offensive than the crumb staling. Crumb staling is more complex, more important, and less understood.

The firmness of bread varies with position within a loaf, with maximum firmness occurring in the central portion of the crumb (Gray and Bemiller, 2003), therefore starch retrogradation is accepted as the main cause of bread staling (Xie et al., 2004); emulsifiers, sugars and enzymes are also reported to affect crumb softness of fresh bread as well as shelf-life (Katina et al., 2006).

During transformation of dough to bread in bread making process, the increased temperature causes gelatinisation of starch content of bread at the microscopic level. At the macroscopic level, baking includes the solidification of dough and change from a foam type system with gas cells to an open pore system. On cooling and ageing of bread, rearrangements in starch structure cause a series of changes which are referred to as crystallization and gelation. This transformation is generally called as the starch retrogradation and this is accepted as the major cause of bread crumb firming which is referred as bread staling (Hug-Iten et al., 1999). The production of bread using limited

Received 19 May 2011; accepted 15 February 2012

amount of chemicals like ethanol, potassium sorbate and calcium propionate (Katsinis et al., 2008) sodium 9 stearoyl-2-lactylate (SSL), monoglycerides (MG), hydroxypropyl methyl cellulose gum (HPMC), high-fructose corn syrup (HFCS) (Abu-Ghoush et al., 2008a), fumaric acid and sodium propionate (Abu-Ghoush et al., 2008b) which is regarded as natural, can be improved by using enzymes which are isolated from natural sources. Microbial enzymes can be of great help to improve the consumers' acceptance to whole wheat bread with improved body, texture, flavour and other desirable properties, and can be added as processing aids/additives. The added enzymes can be easily inactivated by the baking time-temperature treatment (Shah et al., 2006). Thus, using microbial enzymes with no side effects can be favourable in baking industry. The source of microbial enzymes can change depending on the enzyme type and the effect of enzyme on baked product. Microbial fermentation of ingested plant materials in the rumen is the key process to the digestion and nutrition of ruminants. As a result of extensive studies over decades, the rumen microbiota has been shown to comprise a dense population of microbes consisting predominantly the anaerobic bacteria, the protozoa, and large populations of obligate anaerobic fungi (Bauchop, 1989), all responsible for hydrolysis of plant biomass in the rumen (Theodorou et al., 1991).

Anaerobic rumen fungi colonize in the alimentary tract of herbivorous animals that consume a fibrous diet and have a digestive retention time sufficient for a complete fungal life cycle; thus, anaerobic fungi are present in the more important species of domesticated ruminants (sheep, goats, cattle and water buffalo). To facilitate the primary colonization of plant cell wall material, the gut fungi produce a range of cell wall degrading enzymes. Also the growth conditions greatly influence the enzyme production (Gordon and Phillips, 1998). The rumen fungi produce poly-saccharidases (endo-glucanase, exo4 gluca-nase, xylanase, cellodextrinase, amylase), glycosidases (α- and ß-glycosidase, ß-fructosidase, ß-xylosidase) and esterase (acetylxylan esterase, feruroyl esterase) (Ushida et al., 1997).

The control of bread staling can be done in two ways: during the bread making process by the addition of enzymes which may change the molecular mechanism of bread, or after baking by avoiding the loss of water by the use of good packaging and holding microbial parameters under control. Different types of enzymes are used in baking industry such as amylases, xylanases, lipases, proteases. In bread making industry amylases have been used for improving dough-handling properties and enhancing bread quality. Thus the addition of α-amylase not only shows a substantial anti-staling effect but also improves the elasticity of bread crumb (Kim et al., 2006). In general fungal α-amylases are used in baking industry, because of their thermostability (Keskin, 2003). The general point of adding α-amylases to flour was to generate fermentable compounds, mainly maltose in the dough.

On the other hand, some other changes, such as increased bread volume, improved crumb grain, crust colour and flavour may also be obtained (Sahlström and Brathen, 1996). The influence of α-amylase on the staling of bakery products was originally thought to result from a modification to the structure of the starch. Since α-amylase is an endoenzyme that hydrolyses the α-1,4-glucosidic bonds in a random fashion along the chain, it hydrolyses amylopectin to oligosaccharides that contain two to six glucose units. This action, therefore leads to rapid decrease in viscosity, but little monosaccharide formation. Xylanases as non-starch polysaccharide degrading enzymes are widely used as additives in baking industry to improve dough flexibility, machinability and stability, and a larger volume as well as an improved crumb structure (Collins et al., 2006), to hydrolyse water non-extractable arabinoxylans (Shah et al., 2005), to reduce the staling rate (Harada et al., 2000). The proteases break down the gluten protein which makes the dough softer and extensible and to improve flavour profiles, flow characteristics, machining properties, gas retention, and mixing time; lipases result in more uniform

crumb structure by producing mono and diglycerides from lipids and thus an improvement in crumb softness during storage (Gray and Bemiller, 2003).

Cellulose is also present in the bread structure and believed to have a role in staling. The enzymes required for the hydrolysis of cellulose include endoglucanases, exoglucanases and ß-glucosidases. While cellulase is an endoglucanase that hydrolyses cellulose randomly, producing oligosaccharides, cellobiose and glucose, exoglucanases hydrolyze ß-1,4-D-glycosidic linkages in cellulose releasing cellobiose from the non-reducing end (Haki and Rakshit, 2003). Cellulases/hemicellulases cleave non-starch polysaccharides contained in flour. This affects the water retention and water binding capacity, viscosity, and proofing (rising) capacity of the dough as well as the texture, aroma, taste and freshness of the bread. The use of cellulases/hemicellulases gives an improved oven spring to the dough and an improved bread volume, grain structure and anti-staling properties to the finished bakery product.

The main objective of this study was to extend the shelf-life and quality of bread by using cellulolytic activity bearing enzyme complex. This complex produced by the anaerobic rumen fungi, classified as *Neocallimastix s*pp. was studied for its activity. Following the partial isolation of the enzyme complex including cellulase, hemicellulase and xylanases, the effect on bread quality and shelf-life was examined.

MATERIAL AND METHODS

Organisms and cultivation

The rumen anaerobic fungi, which was previously classified as *Neocallimastix* spp, was isolated from deer feces and obtained from the laboratory of Prof. Dr. Dzoko Kungulovski, Institute of Biology, Faculty of Natural Sciences and Mathematics, Ss. Cyril and Methodius University, Skopje, Macedonia. The cultures were maintained anaerobically at 39°C, in serum bottles containing M10 medium, with CM-cellulose and subcultured every week.

The growth medium (M10) ingredients (for 1 litre) were prepared according to Caldwell and Bryant (1966). The pH of the medium was adjusted to 6.7-6.9, which was optimal for the growth of rumen anaerobic fungi. Carbondioxide gas was provided before closing the serum vials to maintain the anaerobic property of the growth media.

Determination of cellulolytic activity

The cellulolytic activity of anaerobic rumen fungi was determined by the dinitrosalicylic acid (DNS) method. The isolates were incubated at 39°C for four days in M10 medium containing carboxymethylcellulase (CMC) at an incubator (Nüve EN 400, Turkey). After the incubation period, 1 mL of medium was centrifuged at 4000 g/15 minutes and 0.5 mL of the supernatant was used as enzyme complex containing solution and placed into a glassware test tube. The enzyme solution was mixed with 1.8 mL 50 mM citrate-phosphate buffer (SIGMA-ALDRICH) (pH=6.8); and 10 mg AVICEL (MERCK) and the reaction was stopped by adding 3 mL DNS to enzyme and buffer solution; incubation was done in water bath (Nüve NB20, Turkey) at 50 °C for 30 minutes. The absorbance was determined by a spectrophotometer (CADAS 50S Dr. Bruno Lange GmbH-Berlin) at O.D. 550 nm.

The "blank" for spectrophotometric determination of enzymatic activity of anaerobic rumen fungi, contained 0.5 mL dH_2O instead of 0.5 mL supernatant of the enzyme solution.

Determination of glucose

Glucose determination was carried out by using dinitrosalicylic acid (DNS) method (Miller, 1959). The stock solution of glucose (D-glucose) (MERCK) was adjusted to a concentration of 1,099 g/1000 mL. After preparing glucose standards, 1 mL sample from each dilution was taken to test tubes and 3 mL DNS was added; than these samples were incubated in boiling water bath for 10 minutes. The absorbance was determined by using a spectrophotometer at O.D.550 nm. A linear standard curve for glucose was

constructed using the absolute amounts of glucose plotted against spectrophotometric values to determine the amount of glucose released from each sample.

The effects of temperature and pH on enzyme activity

The effects of temperature and pH on enzymatic activity were determined by the same procedure (DNS method).

The effect of temperature

In order to determine the effect of temperature on enzyme activity, the isolates were incubated at 39°C for four days in M10 Medium with CMC at an incubator (Nüve EN 400, Turkey). 1 mL of medium was centrifuged at 4000 g/15 minutes. After the incubation 0.5 mL supernatant was used as enzyme solution and placed into a glassware test tube. The enzyme solution was mixed with 1.8 mL 50 mM citrate-phosphate buffer (SIGMA-ALDRICH) (pH=6.8) and 10 mg AVICEL (MERCK). The reaction was stopped by adding 3 mL DNS to enzyme and buffer solution. The test tubes, containing the same solutions were prepared as above; and kept at different temperature-time combinations.

For all of the tests, the absorbance was determined by a spectrophotometer at O.D. 550 nm.

The effect of pH

The effect of pH on enzyme activity was determined by incubating the anaerobic rumen fungi at 39°C for four days in M10 Medium with CMC at an incubator. After the incubation period, 1 mL of medium was centrifuged at 4000 g/15 minutes and 0.5 mL supernatant was used as enzyme solution and placed into several glassware test tubes. The pH of the test tubes was adjusted with 1.8 mL 50 mM citrate-phosphate buffer and pH 6.8 was used as control.

Following the pH adjustment 10 mg AVICEL (MERCK) was added and the reaction was stopped by adding 3 mL DNS; incubation was done in water bath at 50°C for 30 minutes. The absorbance was determined at O.D. 550 nm.

Partial purification of the cellulolytic enzymatic complex

After growing *Neocallimastix* spp the growth media was first centrifuged at 18,000 rpm for 10 minutes, the supernatant of the centrifugate was carefully removed by using needle and passed through 0.45 μm pore size filters and used in the assays.

Bread making process

The breads used in this study were made in the laboratories of Pakmaya A.Ş. Cumayeri Production Plant, Düzce, TURKEY and the experiments were repeated six times. All the bread ingredients were supplied from the same laboratory.

Table 1. The recipe of dough

Ingredients	Amount
Flour	280 g
Water	170 mL
Instant active dry yeast	1.7 g
Salt	4 g

The composition of the dough containing no enzyme (control group) was on flour basis, as shown in Table 1. The dough was prepared by using straight dough method in which all of the dry ingredients are mixed, subsequently, following the addition of water. After the removal of dough from the mixer and placing into baker's pans, and after fermentation, the loaf was baked at 250ºC for 30 minutes in an oven (Heraeus ST5060, Germany), was cooled for 3 hours at room temperature (25ºC), was sealed in plastic bag and stored at room temperature.

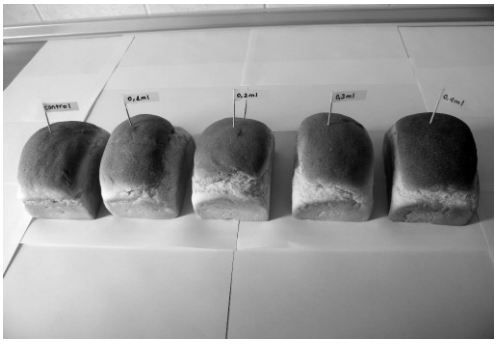

Figure 1. Baked bread samples including enzyme complex

The enzyme complex from anaerobic rumen fungi was added to dough at

concentrations varying from 0.1 mL to 0.4 mL. The enzyme complexes were added to flour while mixing with water.

The microbial counts of bread samples

The microbial activity of loaves was studied by using serial dilution technique. Potato Dextrose Agar (MERCK), Yeast Extract Agar (MERCK) and Nutrient Agar (MERCK) were used as cultivation media for the mold, yeast and the total mesophilic anaerobic bacteria counts respectively.

Each bread sample was tested for its microbial counts at the zero, fourth and eighth days of the storage of bread samples. One gram of bread sample was weighed and placed into a test tube containing 9 mL sterile dH_2O as diluent and the same procedure was repeated up to 10^{-5} dilution. 0.1 mL of sample was taken from each dilution and placed on the Petri dishes, separately containing Potato Dextrose Agar (PDA) (MERCK) and Yeast Extract Agar (YEA) (MERCK) incubated at an incubator (Nüve EN 400, Turkey) at 30 ºC for 48 hours. For the total mesophilic aerobic bacterial counts, the plates were incubated at 37ºC for 48 hours.

Crumb firmness measurements

The firmness of bread was measured by a universal testing machine (Lloyd Instruments LR 30K, UK) in the laboratories of the Department of Food Engineering, Middle East Technical University, Ankara, Turkey.

Bread samples were sliced, from the middle part of the loaf, to 1 mm thickness and compressed for 25% at a speed of 55 mm/minutes with 2.5 probe diameter and the load cell was adjusted to 50 N. Firmness measurements were done in the first 24 hours after baking bread samples and two slices were used for each group.

Taste panel

A taste panel, composed of 20 professionally trained panellists was performed in order to evaluate the bread according to the criteria of saltiness, sourness, sweetness, bitterness, hardness, colour, texture and overall perspective.

RESULTS AND DISCUSSION

The cellulolytic activity of enzyme complex produced by anaerobic rumen fungi, *Neocallimastix* spp. was determined by DNS method where Avicel (microcrystalline cellulose) was used as carbon source. This study indicated the degradation of 10 mg cellulose by the extracellular enzyme complex of *Neocallimastix* spp. for different samples in each replicate.

The anaerobic rumen fungi have already been studied many times by scientists since their discovery in 1988 by Orpin (Orpin and Joblin, 1988). The production of extracellular enzymatic complexes has also been described for the anaerobic rumen fungi. In general the extracellular enzyme complexes of anaerobic rumen fungi species are intensively studied due to their ability for degradation of the cell wall components of the plant tissues where they generally colonize. The production of a wide range of hydrolytic enzymes provides utilization of carbon sources ranging from simple sugars to complex polymers; thus it was explained by the fact that cellulolytic, xylanolytic, glycolytic, amylolytic and proteolytic enzyme activities were observed against substrates (Orpin and Joblin, 1988). In this study, Avicel was used as a substrate for determination of the cellulolytic activity of our isolates. The activity of the enzyme complex was measured by using DNS method, based on the colour change, while testing the presence of free carbonyl group (C=O), the so-called reducing sugars. This involves the oxidation of the aldehyde functional group present in glucose. As dissolved oxygen can interfere with glucose oxidation, sulphite, which itself is not necessary for the colour reaction, is added in the reagent to absorb the dissolved oxygen.

When compared with the blank, after the reaction period, most samples indicated a certain difference of glucose concentration. This was mostly due to the enzymatic activity of the complex, which can be assayed by glucose calibration curve. The percentage values of samples indicated the activity of enzyme complex, as the degradation of

substrate, varying from 9.5% to 36.5%. The most active sample was Sample 1, which had 36.5% degradation value (Figure 2). From the same figure, it was observed that the enzymatic activity declined after each subcultivation.

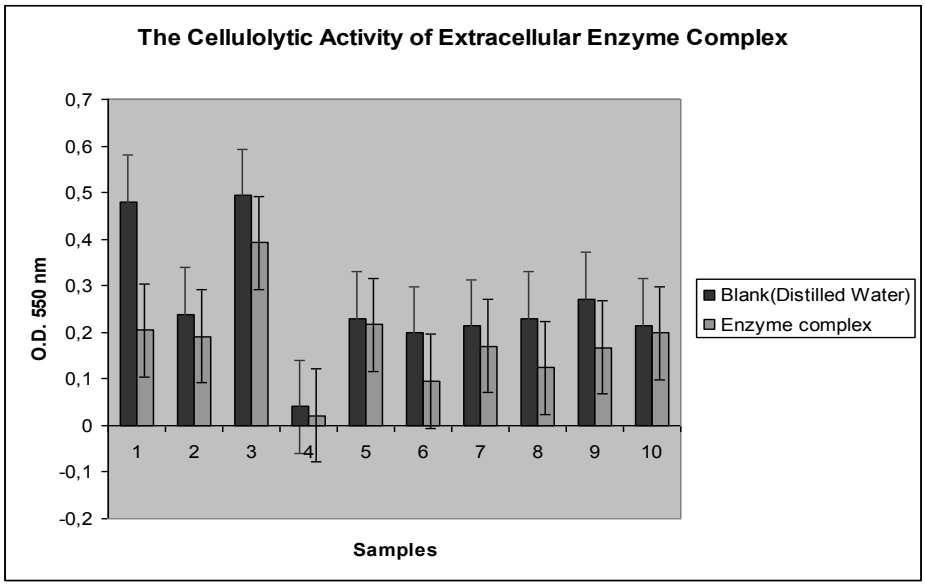

Figure 2. Difference of cellulolytic activity (μmol/mg/min^{-1}) of extracellular enzyme complex of *Neocallimastix* spp with reference to the blank containing no enzyme solution

The glucose determination was carried out for the calculation of absolute degradation of substrate (Avicel) by cellulolytic enzymatic complex of anaerobic rumen fungi *Neocallimastix* spp. By a standard calibration curve, the amounts of glucose produced by the enzymatic action of Neocallimastix spp., and the percentages of degradation of substrate by samples were calculated.

When the effect of temperature on the cellulolytic enzyme complex was studied, it was observed that the enzymatic activity is more or less the same during incubation at 4°C, 25°C and 50°C (Figure 3). At high temperatures like 100°C, the enzymatic activity is observed by verification of spectroscopic findings correlated with the standard curve of glucose. This may allow the enzyme complex to be used in such cases in which high-temperature processes are commonly used in certain food production technology, like baking industry.

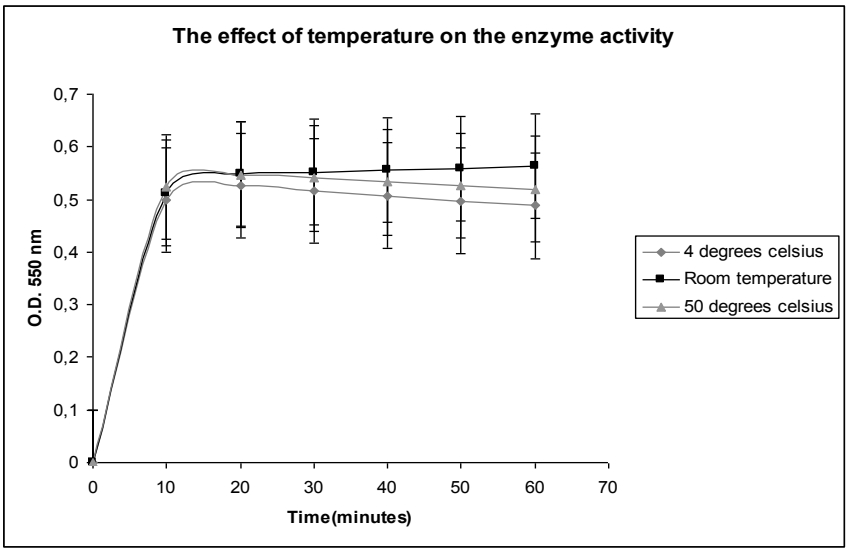

Figure 3. The effect of temperature on the enzyme activity complex of anaerobic rumen fungi
The activity of the enzyme is expressed as cellulolytic activity (μmol/mg/min^{-1})

The pH studies indicated that the enzyme shows its activity above pH=5. The enzyme complex seems to be inactivated below pH=4 (Figure 4).

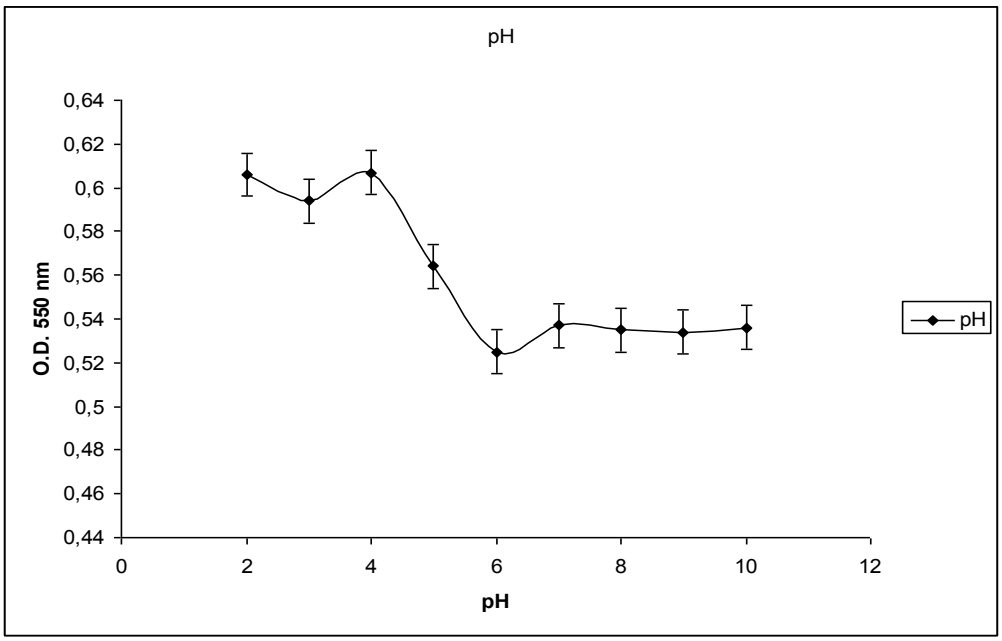

Figure 4. The effect of pH on the activity of enzyme complex of anaerobic rumen fungi
The activity of the enzyme is expressed as cellulolytic activity (μmol/mg/min^{-1})

Depending on the food texture definition, the various constituents and structural elements of food are arranged and combined into a micro- and macrostructure and the external manifestations of this structure was provided in terms of flow and deformation (de Man, 1999). By measuring the mechanical textural characteristic of bread, a texture analyser was used in this study. The main parameters as the hardness, the cohesiveness (includes brittleness, chewiness and gumminess) and the adhesiveness were determined.

Hardness is often used as a measure of bread staling; a standard method for bread staling based on force-deformation for firmness in the static compression mode is available (Bollaín et al., 2005). The hardness of bread samples decreased when the cellulolytic enzyme complex of *Neocallimastix* spp. was added.

0.3 mL enzyme complex added bread sample showed the lowest value for hardness as compared with the control sample. However, as seen from the data, 0.4 mL enzyme complex containing bread sample showed higher value for hardness than 0.3 mL sample; this may be caused from the irregularities in size and shape of the samples. During sample preparation, samples were not sliced with an automatic knife; they were sliced with a sharp knife which may be the probable cause of the irregularities. Also, the optimum level of enzyme preparations was another factor that may affect the crumb firmness, thus the optimum level of enzyme complex of *Neocallimastix* spp. could be determined by using this data. Therefore, it can be said that the enzyme complex of anaerobic rumen fungi *Neocallimastix* spp. may affect the hardness of bread.

As another parameter of texture analysis, cohesiveness was measured. The cohesiveness expresses how well the product withstands a second deformation relative to the behaviour under the first deformation. It was measured as the area of work during the second compression, divided by the area of work during first compression (URL 1). It can also be defined as the strength of the internal bond making up the body of the product, and is usually tested in the terms of the second parameters of texture analysis (brittleness, chewiness and gumminess). While comparing these parameters they showed strong correlation with each other. The energy

required to masticate and disintegrate the bread to a state ready for swallowing was decreased by the addition of enzyme complex. Overall, this may cause an increase in volume, more uniform grain structure and slower aging during storage.

In agreement with the studies of Harada et al. (2000) our data demonstrated that texture profile, crumb firmness values, hardness and cohesiveness were significantly affected by the addition of cellulolytic enzyme complex. The anti-staling effect of enzyme complex could be due to the degradation of cell wall components, leading to altered water distribution between starch-protein matrixes, the connection between the swollen starch granules and the continuous protein network responsible for crumb firming in the bread during aging.

A professional sensory analysis, by trained panellists, was also carried out to determine flavour of the baked bread and whether the other quality parameters were acceptable or not. The breads preferred by the panel belonged to the group with 0.3 mL cellulase containing complex added (Table 2).

Table 2. The sensory analysis of bread
(The letters 'a' to 'e' indicated the preference: a – the most acceptable; b – quite acceptable; c – acceptable; D – unacceptable; e – the most unacceptable)

Type of bread	Hardness	Sweetness	Sourness	Bitterness	Saltiness	Color	Texture	Overall
Control	e	e	d	e	e	e	e	e
0.1 mL	d	e	d	e	e	d	d	d
0.2 mL	b	e	d	e	e	c	c	d
0.3 mL	a	e	d	e	e	c	b	c
0.4 mL	a	e	d	e	e	b	b	c

CONCLUSIONS

The enzymatic complex of anaerobic rumen fungi produced by *Neocallimastix* spp., was partially isolated and the effects of addition of this complex on bread texture and shelf life were studied in this study. We found that the usage of this enzyme in bread baking may increase the consumer's acceptability, being a natural additive, which prolonged shelf life. The cellulolytic activity was present in the partially isolated enzyme complex, as demonstrated by the DNS method.

The addition of this enzyme complex provided an improvement of the sensory and texture characteristics of bread (hardness, gumminess and chewiness) and an increase of overall acceptability.

The impact of side activities of the enzyme complex of *Neocallimastix* spp. could have influenced the results, because the complex was not highly purified. If this can be done, the exact effect of cellulase and other constituents of enzyme complex on bread texture could be studied. Moreover, combination of enzymes, because of their synergetic effects, may be tested for the extension of shelf life of bread.

Also the effects of this enzyme complex of anaerobic rumen fungi on the quality of different varieties of bread, pastry and cakes could be studied.

Acknowledgements

This project was supported by Turkish Scientific and Technical Research Council (TUBITAK)-TBAG projects (U/73 103 T 084) in cooperation with The Ministry of Science and Education of Macedonia.

REFERENCES

Abu-Ghoush, M., Herald, T. J., Dowell, F., Xie, F., Aramouni, F.M., Walker, C., 2008a. *Effect of antimicrobial agents and dough conditioners on the shelf-life extension and quality of flat bread, as determined by near-infrared spectroscopy.* International Journal of Food Science & Technology, 43: 365-372.

Abu-Ghoush, M., Herald, T.J, Dowell, F., Xie, F., Aramouni, F.M., Madl, R., 2008b. *Effect of preservatives addition on the shelf-life extensions and quality of flat bread as determined by near-infrared spectroscopy and texture analysis*. International Journal of Food Science & Technology, 43.

Bauchop, T., 1989. *Biology of gut anaerobic fungi.* Biosystems. 23: 53-64.

Bollaín, C., Angioloni, A., Collar, C., 2005. *Bread staling assessment of enzyme-supplemented pan breads by dynamic and static deformation measurements*. European Food Research and Technology, 220: 83-89.

Caldwell, D.R. and Bryant, M.P., 1966. *Medium without rumen fluid for nonselective enumeration and isolation of rumen bacteria*. Applied Microbiology, 14: 794-801.

Chen, H., Li, X.L., Ljungdahl, L.G., 1995. *Biomass degrading enzymes from anaerobic rumen fungi.* Saas Bulletin Biochemistry and Biotechnology: 1-6.

Collins, T., Hoyoux, A., Dutron, A., Georis, J., Genot, B., Dauvrin, T., Arnaut, F., Gerday, C., Feller, G., 2006. *Use of glycosidase hydrolase family 8 xylanases in baking*. Journal of Cereal Science, 43:79-84.

De Man, J.M., 1999. *Principles of Food Chemistry*. Third Edition. Aspen Publishers U.S.A.: 191-193; 403.

Gordon, G.L.R. and Phillips, M.W., 1998. *The role of anaerobic fungi in ruminants*. Nutrition Research Reviews, 11: 133-168.

Gray, J.A. and Bemiller, J.N., 2003. *Bread Staling: Molecular basis and control*. Comprehensive Reviews in Food Science and Food Safety, 2: 1-21.

Haki, G.D., Rakshit, S.K., 2003. *Developments in industrially important thermostable enzymes: a review.* Bioresource Technology, 89: 17-34.

Harada, O., Lysenko, E. D. and Preston, K.R., 2000. *Effects of commercial hydrolytic enzyme additives on Canadian short process bread properties and processing characteristics.* Cereal Chemistry, 77: 70-76.

Hug-Iten, S., Handschin, S., Conde-Petit, B., Escher, F., 1999. *Changes in Starch Microstructure on Baking and Staling Wheat Bread.* Lebensmittel-Wissenschaft und Technologie: 255-260.

Katina, K., Salmenkallio-Marttila, M., Partanen, R., Forssell, P., Autio, K., 2006. *Effects of sourdough and enzymes on staling of high-fibre wheat bread*. Swiss Society of Food Science and Technology, 39: 479-491.

Katsinis, G., Rigas, F., Doulia, D., 2008. *Synergistic effect of chemical preservatives with ethanol on the microbial shelf life of bread by factorial design.* International Journal of Food Science & Technology, 43: 208-215.

Keskin, S.Ö., 2003. *Effects of different ovens and enzymes on quality parameters of bread.* Master thesis, The Graduate School of Natural And Applied Sciences of the Middle East Technical University, Ankara,Turkey.

Kim, J.H., Maeda, T., Morita, N., 2006*, Effect of fungal α-amylase on the dough properties and bread quality of wheat flour substituted with polished flours*. Food Research International, 39: 117-126.

Miller, G.L., 1959. *Use of dinitrosalicylic acid reagent for determination of reducing sugar.* Analytical Chemistry, 31: 426-428.

Orpin, C.G., Joblin, K.N., 1988. *The Rumen Anaerobic Fungi*. The Rumen Microbial Ecosystem. Elsevier Applied Science. London: 129-150.

Primo-Martin, C., van de Pijpekamp, A., van Vliet, T., de Jongh, H.H.J., Plijter, J.J., Hamer, R.J., 2006. *The role of the gluten network in the crispness of bread crust*. Journal of Cereal Science, 43: 342-352.

Sahlström, S., Brăthen, E., 1996. *Effects of enzyme preparations for baking, mixing time and resting time on bread quality and bread staling*. Food Chemistry, 58: 75-80.

Shah, A.R., Shah, R.K., Madamwar, D., 2006. *Improvement of the quality of whole wheat bread by supplementation of xylanase from Aspergillus foetidus*. Bioresource Technology, 97: 2047-2053.

Theodorou, M.K., Lowe, S.E. & Trinci, A.P.J., 1991. *Anaerobic Fungi and the Rumen Ecosystem. In The Fungal Community*: 43-71. Edited by G.C. Carroll & D.T. Wicklow. New York: Marcel Dekker Inc.

Ushida, K., Matsui, H., Fujino, Y., Ha, J.K., 1997. *Role and Potential of Ruminal Fungi in Fiber Digestion*. Asian-Australasian Journal of Animal Sciences, 10 (6): 541-550.

Xie, F., Dowell, F.E., Sun, X.S., 2004. *Using visible and near-infrared reflectance spectroscopy and differential scanning calorimetry to study starch, protein and temperature effects on bread staling*. Cereal Chemistry, 81(2): 249-254. (URL1) http://www.food.upm.edu.my/kuliah/lectures/foodsci/fst4109h/Solids%20Rheology.pdf (Accessed on 25.10.2005).