STUDIES ON SHELF-LIFE EXTENSION OF BREAD BY A CELLULASE BEARING ENZYME COMPLEX FROM ANAEROBIC FUNGI

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ABSTRACT

STUDIES ON SHELF-LIFE EXTENSION OF BREAD BY A CELLULASE BEARING ENZYME COMPLEX FROM ANAEROBIC FUNGI

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Anaerobic fungi, intensively present in the digestive system of the ruminants has distinctive properties in helping the degradation of cellulose and cell wall structures due to their enzyme systems. An enzyme complex, from *Neocallimastix* spp. which belongs to anaerobic rumen fungi, was partially isolated and its effect at various concentrations on bread quality was tested by using an additive during mixing of flour. The addition of enzyme complex into bread dough resulted in the decrease of hardness, gumminess and chewiness, thus provided a softer crumb, as the means of retardation in the staling of bread.

Keywords: anaerobic rumen fungi, enzyme complex, bread, bread texture

ÖZET

ANAEROBiK MANTARDAN ELDE EDiLEN SELÜLOZ iÇEREN ENZiM KOMPLEKSiNiN EKMEK RAF ÖMRÜNÜN ARTTIRILMASI ÜZERiNE ÇALIŞMALAR

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Anaerobik mantarlar, geviş getiren hayvanların sindirim sistemlerinde yoğun olarak bulunurlar, enzim sistemleri ile selüloz ve diğer hücre duvarı yapılarını parçalayarak sindirime yardımcı olurlar. Anaerobik işkembe mantarı olan *Neocallimastix* cinsine ait enzim kompleksi kısmi olarak izole edilerek, çesitli konsantrasyonları katkı maddesi olarak un ile karıştırılarak ekmeğin niteliğine etkisi test edilmiştir. Enzim kompleksinin, ekmek hamuruna katılması, ekmek sertliği, ezilmesi ve çiğnenmesinde bir azalmaya neden olup, daha yumuşak ekmek kırıntısı oluşmasını sağlamış ve bayatlamasında gecikmeye neden olduğu gözlemlenmiştir.

Anahtar Kelimeler: anaerobik işkembe mantarları, enzim kompleksi, ekmek, ekmek yapısı

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

1. INTRODUCTION

Bread is the main staple food item in many developing countries and the Turkish diet. Its consumption varies among one country to another and individuals in the same country but its importance does not change. 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.

In today's modern society, the consumer's food demand as well as the preference has changed substantially. In order to have more wholesome and fiber-rich foods, the baking industry is shifting towards producing whole wheat bread with limited use of chemicals. However, production of such type of bread poses certain technological difficulties. In order to improve the consumers' acceptance to whole wheat bread with improved body, texture, flavour and other desirable properties, development of the appropriate technology is required. Microbial enzymes can be of great help in overcoming these problems and they 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 become favorable in baking industry. The source of microbial enzymes can change depending on the enzyme type and the effect of enzyme on baked product.

Consequently, production of bread using limited amount of chemicals, which is regarded as natural, can be improved by using enzymes which are isolated from natural sources.

1.1. Anaerobic Rumen Fungi

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 and the protozoa. In recent years, large populations of fungi, possessing the exceptional property of being obligate anaerobes, have been found to constitute an integral part of the rumen microbiota (Bauchop, 1989).

Prior to discovering the rumen fungi, it was assumed that the cellulolytic bacteria and, perhaps to a lesser extent, the protozoa were responsible for hydrolysis of plant biomass in the rumen (Theodorou et al., 1991). Evidence has accumulated since the mid 1970s, which leaves little doubt as to the involvement of obligate anaerobic ruminal celluloysis. These fungi colonize and grow on plant fragments in the rumen and are able to degrade cellulose and other plant structural polysaccharides, functions at the heart of herbivore fermentative digestion.

This major oversight in failure to detect these fungi may be explained by the fact that microorganisms associated with the rumen plant fragments (solids)

have been little studied. This solid, which have been discarded during the studies, is now known to contain the thalli of rumen anaerobic fungi (Bauchop, 1989). Also until that time fungi were generally considered as being the aerobic organisms, which are not expected to exist in absolutely anaerobic ecosystems (Chen et al., 1995).

In the past, common saprophytic fungi had been isolated from rumen contents but they were not regarded as indigenous rumen microbes (Clarke, 1977, in Bauchop, 1989). These fungi were considered to be merely transients, present in low numbers, and unable to grow under the anaerobic conditions of the rumen environment. However the first fungus which was isolated from sheep rumen by Orpin in 1975, belongs in an entirely different category; unlike all other known fungi they are obligate anaerobes (Chen et al., 1995).

1.1.1. Classification and life cycle of anaerobic rumen fungi

The taxonomy of the anaerobic fungi is still based on the traditional classification methods used for the chytrid fungi which rely on studies of morphology and ultrastructure, particularly of the zoospore, and the chemistry of cell walls and the metabolic characteristics of the fungi (Gordon and Phillips, 1998).

The classification of fungi is based principally upon the morphological characteristics of the thallus (Ushida et al., 1997). Thallus may be classified as: i) monocentric and endogeneous; ii) monocentric, exogenous and uni- or

multisporangiate; iii) mycelial-polycentric (Ho and Barr, 1995). In a monocentric thallus, either the encysted zoospore retains the nucleus and enlarges into a sporangium or prosporangium (called endogeneous sporangial development regarded as the most primitive type of thallus development), or the nucleus migrates out of the zoospore, and the sporangium is formed in the germ tube or rhizomycelium (called exogenous sporangial development) (Theodorou et al., 1991). In polycentric thallus, the nucleus migrates out of the zoospore cyst and grows into the germ tube (Ho and Barr, 1995). The germ tube usually develops into a vegetative system which varies from a fine, unbranched filament or a lobate holdfast to a richly branched and extensive rhizomycelium. The germ tube may arise from the base of zoosporangium, or at several points on the periphery of the zoosporangium, or the rhizoids may be centered on an apophysis (Theodorou et al., 1991).

The traditional physiological tests, such as sugar utilization or end product formation for classification, do not seem to be efficient for identifying species, because little difference is usually observed in sugar utilization and end-product formation among genera (Phillips and Gordon, 1988; Gordon and Phillips, 1989, in Ushida et al., 1997).

The anaerobic rumen fungi were first classified as *Chytridiomycetes*, in the order *Spizellomycetales*, by Heath et al. 1983 (Özköse et al., 2001).

However, from recent rRNA analyses, Li and Heath (1992) and Li et al. (1993) have proposed to elevate the family *Neocallimastigaceae* to the new

order *Neocallimasticales* in the *Chytridiomycota.* To date, five genera have been described; on the basis of monocentric and polycentric thallus, filamentous and bulbous rhizoid, and monoflagellated and polyflagellated zoospores (Thedorou et al., 1996). These five genera can be classified as; *Neocallimastix, Piromyces* (formerly *Piromanas*), *Caecomyces* (formerly *Sphaerpmanas*), *Orpinomyces, Anaeromyces* (syn *Ruminomyces*) and 17 species are known (Table 1) (Ushida et al., 1997). Of the five genera of rumen fungi, three develop monocentric thalli, and latter two produce polycentric thalli (Özköse et al., 2001). Among the rumen Chytridiomycetes, species belonging to genera *Neocallimastix* and *Orpinmyces* produce multiflagellated zoospores, and the remaining species produce monoflagellated zoospores (Ushida et al, 1997).

Table 1. Classification of anaerobic chytridiomycete fungi (Ushida et al., 1997)

Genera and characteristics	Species	Source	Reference
Neocallimastix Monocentric, polyflagellate zoospore extensive, filamentous rhizomycelium	*N. frontalis*	sheep	Heath et al., 1983
	N. patriciarum	sheep	Orpin & Munn, 1986
	N. hurleyensis	sheep	Webb & Theodorou, 1991
	N .variabilis	sheep	Ho et al., 1993b
Piromyces Monocentric, monoflagellate zoospore filamentous rhizomycelium	*P. communi*	sheep	Gold et al., 1988
	P. mae	horse	Li & Heath, 1990
	P. dumbonicus	elephant	Li & Heath, 1990
	P. rhizinflatus	ass	Breton et al.,1991
	P. minutus	deer	Ho et al., 1993c
	*P. spirali*s	goat	Ho et al., 1993d
	P. citronii	horse	Gaillard-Martine et al., 1995
Orpinomyces Polycentric, polyflagellate zoospore filamentous rhizomycelium	*O. joyonii*	sheep	Li et al., 1991
	O. intercalaris	cattle	Ho et al., 1993
Caecomyces Polycentric or monocentric, monoflagellate zoospore, spherical holdfasts	*C. commun*is	sheep	Gold et al., 1988
	C. equi	horse	Gold et al., 1988
Anaeromyces Polycentric, monoflagellate zoospore, filamentous rhizomycelium	*A. muconatus*	cow	Breton et al., 1991
	A. elegans	sheep	Ho et al., 1993a

The biology of anaerobic fungi is characterized by a complex life cycle similar to the aerobic chytrid fungi (Heath et al. 1983 in Gordon and Phillips,

1998). The most commonly observed life cycle of anaerobic rumen fungi is defined by Orpin (1994), which consists of an alteration between a motile flagellate zoospore stage free-living in the liquid phase of the digesture, and a nonmotile vegetative reproductive one, saprophytic on digestive fragments in the alimentary tract of the animal (Chen et al., 1995). A third stage, the survival, occurs in the lower gut and faeces (Davies et al., 1993; Nielsen et al., 1995, in Thedorou et al., 1996).

Depending on the fungal genus, the zoospores, which have either a single flagellum or a bundle of flagella; these zoospores are attracted to pieces of freshly-ingested plant biomass (Gordon and Phillips, 1998). This mechanism is a result of chemotactic response to carbohydrates (Orpin and Joblin, 1988). After attaching to feed particle, they encyst and germinate to produce a fungal thallus consisting of a rhizoidal system and one (monocentric) or more (polycentric) zoosporangia (Thedorou et al., 1996).

Studies in vivo and in vitro showed that the life cycle lasts about 24-32 hours, although zoosporogenesis of young sporangia may occur under appropriate conditions as early as 8 hours after encystment (Orpin and Joblin, 1988). Also the fungi in feces and the post-ruminal organs remain viable when dried in air at ambient temperature. By these findings, the generally accepted life cycle of the gut fungi was amended by inclusion of an additional survival cycle (Thedorou et al., 1996).

1.1.2. Distribution of anaerobic rumen fungi

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) (Gordon and Phillips, 1998). Also obligate anaerobic fungi have been isolated from the rumen and other parts of the ruminant digestive tract, the horse caecum, the fore-stomach of kangaroo, and the feces of numerous other herbivorous mammals (Table 2) (Theodorou et al., 1991).

Owing to this wide range of host animal species, anaerobic fungi have been found in all geographical regions where they have been sought (Gordon and Phillips, 1998). All present studies indicate that fungal colonization in herbivores requires a high-fiber diet and capacious organ of fermentative digestion. Anaerobic fungi were not detected in fresh feces of rabbits, possum, or man (Bauchop, 1989).

Table 2. Distribution of Anaerobic Fungi in Herbivores (Theodorou et al., 1991)

Common Name	Specific Name	Pregastric(P), Hindgut(H) fermenter	Anaerobic fungi isolated (or observed)	Source reference
African elephant	*Loxodonta africana*	H	feces	Bauchop 1979b
Asian elephant	*Elephas maximus*	H	feces	Milne et al., 1989
Arabian Oryx	*Oryx leucoryx*	P	feces	Milne et al., 1989
Bactrian camel	*Camelus bactrianus*	P	feces	Milne et al., 1989
Bongo	*Taurotrapus eurycerus*	P	feces	Milne et al., 1989
Common zebra	*Equus burchelli*	H	feces	Milne et al., 1989
Domestic cattle	*Bos* spp.	P	rumen, feces	Various, and Milne et al., 1989
Domestic goat	*Capra* spp.	P	rumen, feces	Orpin & Joblin (1988)
Domestic sheep	*Ovis* spp.	P	esophagus, rumen, feces	Milne et al., 1989
Gaur	*Bos gaurus*	P	feces	Milne et al., 1989
Greater kudu	*Tragelaphus strepsicero*	P	feces	Milne et al., 1989
Grey kangaroo	*Macropus giganticus*	P	fore stomach	Bauchop, 1983
Horse	*Equus caballus*	H	feces, caecum	Bauchop, 1979b; Orpin, 1981
Impala	*Aepyceros melampus*	P	rumen	Bauchop, 1979b
Lama species	*Lama glama*	P	feces	Milne et al., 1989
	Lama guanicoe	P	feces	Milne et al., 1989
	Lama pacos	P	feces	Milne et al., 1989
Musk ox	*Ovibos moscharus*	P	not specified	Orpin & Joblin (1988)
Red deer	*Cervus elaphus*	P	rumen	Bauchop, 1979*b*
Redneck wallaby	*Macropus rufogriseus*	P	fore stomach	Bauchop, 1983
Reindeer	*Rangifer tarandus*	P	rumen	Orpin et al.,1985
Black rhinoceros	*Diceros bicornis*	H	not specified	Orpin & Joblin (1988)
Roan antelope	*Hippotragus equinis*	P	feces	Milne et al., 1989
Swamp wallaby	*Wallabia bicolor*	P	fore stomach	Bauchop, 1983
Vicuna	*Vicugna vicugna*	P	feces	Milne et al., 1989
Wallaroo	*Macropus robustus*	P	ore stomach	Bauchop, 1983
Water buffalo	*Bubalus babalis*	P	rumen	Ho et al.,1988; Foong et al., 1987

1.1.3. Transfer of fungi between individual animals

The transfer of fungi between animals can be considered as occurring in two phases: the initial acquisition of a fungal population, which is followed by the addition to replace this population later in life (Gordon and Phillips, 1998).

In newborn ruminant, the anaerobic fungi are not present but develop even before these animals start to eat solid food (Milne et al., 1989). The possible routes of transfer of microorganisms from adult to young ruminants include saliva and feces. Rumination brings large number of organisms into the mouth and this is considered to be one of the most important routes as direct contact with the saliva is thought to be the method of transfer of these anaerobic fungi (Lowe et al., 1987), and the aerosols have also been indicated as possible means of dissemination between animals (Gordon and Phillips, 1998).

1.1.4. Enzyme systems of anaerobic rumen fungi

Plant biomass consists of predominantly of cellulose (280-500g/kg), hemi cellulose (200-300g/kg) and lignin (180-300g/kg) (Ljungdahl and Eriksson, 1984, in Thedorou et al., 1996), with pectin contributing up to 100 g/kg dry weight of some forage. To facilitate the primary colonization of plant cell wall material, the gut fungi produce a range of cell wall degrading enzymes. These enzymes are present on the fungal surface, localized on the rhizoids or rhizomycelia, with a larger proportion being secreted into the extracellular environment (Lowe et al., 1987*b, c* in Thedorou et al., 1996). Also the growth conditions greatly influence

the enzyme production (Gordon and Phillips, 1998). The rumen fungi produce polysaccharidases (endo-glucanase, exo-glucanase, xylanase, cellodextrinase, amylase), glycosidases (α- and β- glycosidase, β-fructosidase, β-xylosidase) and esterase (acetylxylan esterase,feruroyl esterase) (Ushida et al., 1997). In general it can be said that rumen fungi produce high levels of cellulases and hemicellulases and are particularly proficient in producing xylanases (Akin and Borneman, 1990). The activities of these enzymes were observed in pure cultures and co-cultures, the physical association with the lignocellulosic tissues of plant fragments, and the ability to penetrate and weaken the plant tissue *in vivo* (Chen et al., 1995).

1.1.4.1. Cellulose, cellulases and glucosidases

All forms of cellulose can be described as β-1,4 polyglucosidase (Pomeranz and Meloan 2000). Cellulose consists of β-glucopyranosyl residues joined by 1 -4 linkages (Belitz et al., 2004). The cellulose molecule is elongated and rigid, even when in solution. The hydroxyl groups that protrude the chain may readily form hydrogen bonds, resulting in a certain amount of crystallinity; and the areas of crystallinity are denser and more resistant to enzymes and other chemicals than the noncrystalline areas (deMan, 1999) (Figure 1).

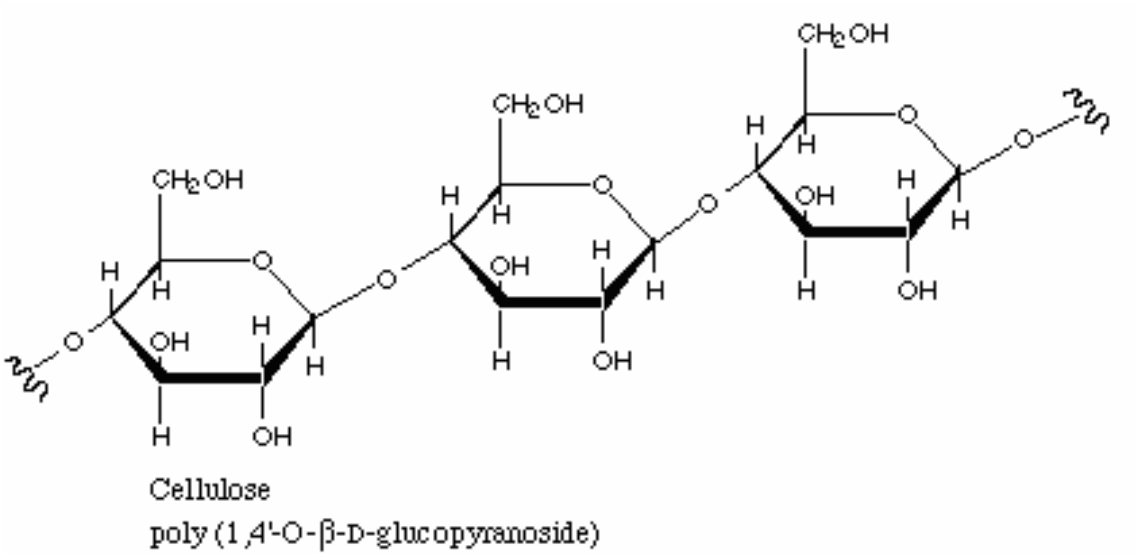

Figure 1. Section of a cellulose molecule (URL 1)

The hydrolysis of crystalline cellulose to glucose has been studied extensively with aerobic fungi, notably using enzymes from *Trichoderma* and *Phanerochaete* species (Eriksson et al., 1990 in Chen et al., 1995). The anaerobic rumen bacteria and the rumen fungi have an identifiable extracellular macromolecular complex of cellobiohydrolases called a cellulosome, whereas the cellulase components of aerobic fungi can not aggregate (Thedorou et al., 1996). The hydrolysis of crystalline cellulose requires several enzymes acting synergistically; they have been characterized as endo-1,4-β-D-glucanases (carboxymethylcellulases, CMCases, EC 3.2.1.4), exo-1,4-β-D-glucanases (cellobiohydrolases, EC 3.2.1.91) and β-glucosidases (cellobiases, EC 3.2.1.21) (Chen et al., 1995).

The most widely accepted hypothesis to incorporate observed endo-exo synergism is that endoglucanases initiate the attack by cleaving cellulose chains

at random, creating exposed chain ends from which exocellobiohydrolases remove successive cellobiose units, however the ordered structure of cellulose is also maintained by interchain hydrogen bonds, so that interchain glycolytic bonds cleaved by endoglucanases acting alone will rapidly reform unless exocellobiohydrolases are immediately on hand; also, without the latter acting to solubilize the fragment outermost chains on the microfibril surface, the endoglucanases are denied access to the internal cellulose chains (Sharrock, 1988). (Figure 2).

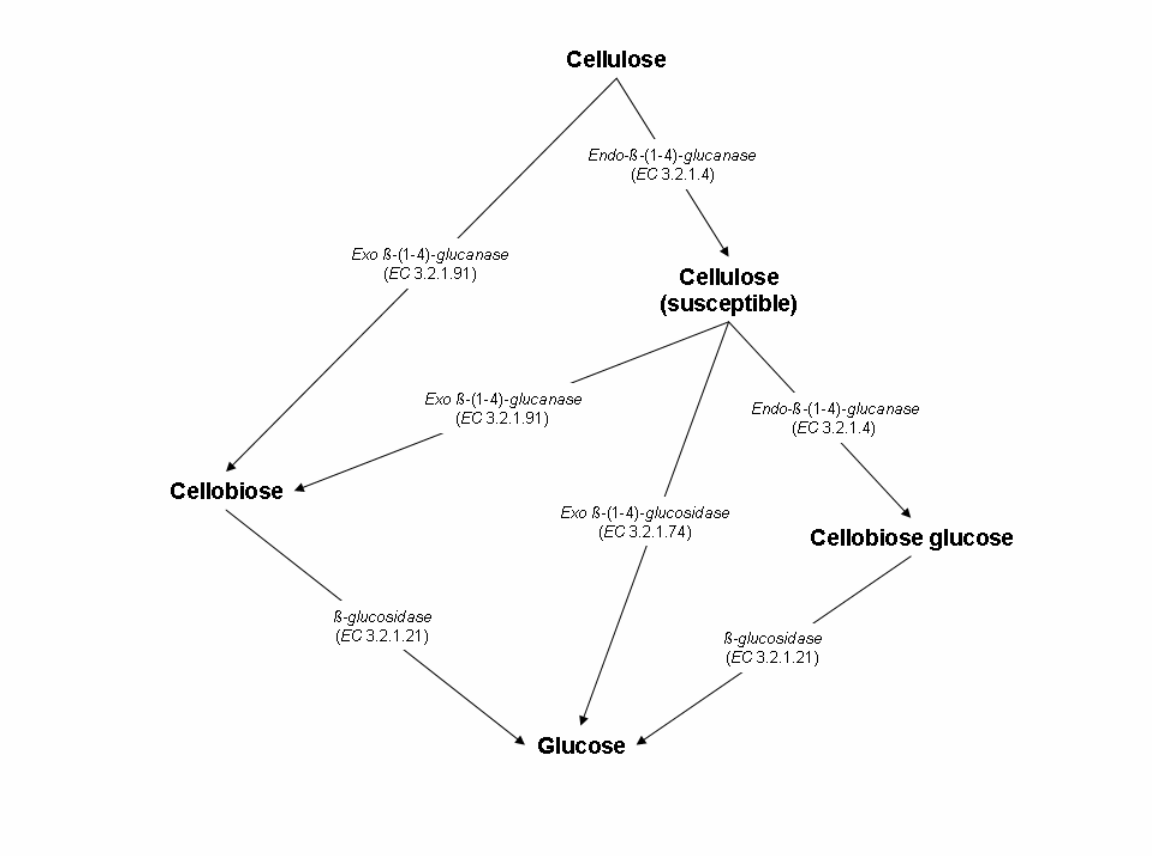

Figure 2. Mode of action of fungal cellulase on cellulose (Sharrock, 1988).

Cellulases from the anaerobic rumen fungi have a pH and temperature optima of 5.0- 6.0 and 45-55°C, respectively and the activities described include carboxymethylcellulase, FPase (filter paper activity), cellobiase (β-glucosidase) and Avicelase (Theodorou et al., 1991). Avicel is often used as substrate and the combined action of the enzymes on crystalline cellulose is usually referred as Avicelase activity (Chen et al., 1995).

In general total cellulolytic ability depends on the enzyme composition of the cellulolytic complex, the arrangement of the cellulolytic complex, specific activities of individual enzymes, enzyme location, enzyme inducibility and enzyme affinity to the insoluble substrate (Hodrova et al., 1998).

1.1.4.2. Hemicelluloses, Pentosans and Xylanases (Hemicellulases)

Hemicellulose refers to the water-insoluble, nonstarchy polysaccharides; pentosan refers to water-soluble, non starchy polysaccharides (D' Appolonia et al., in deMan,1999). Hemicelluloses are classified according to the sugars present. Xylans are very common hemicellulose and they are polymers of xylose, mannose and galactans of galactose. Xylan is composed of β-1,4-linked xylose units as a xylan backbone connected with side chains; these side chains can include one or more of α-L-arabinose, D-glucuronic acid, 4-o-methyl-D-glucuronic acid, acetyl, feruloyl, and β-coumaroyl groups. Xylan plays a major role in cell wall cohesion, being the major interface between lignin and other carbohydrates in secondary plant cell- walls (Chen et al., 1995).

Extensive breakdown of xylans requires the synergistic action of a variety of hydrolytic enzymes, which include the endo-1,4-β-D-xylanase and β-xylosidase, and the those that liberate side chain substituents (Chen et al., 1995).

Xylanases which are produced by anaerobic rumen fungi can be found associated with a high molecular weight complex containing both endo- and exo-acting enzymes that have been identified and characterized (Gordon and Phillips, 1998). Xylanase and xylosidase are produced by both mono- and polycentric anaerobic fungi (Chen et al., 1995). In general xylanase activities have a pH and temperature optima of 5.5-6.0 and 50°C, respectively and are partially cell associated or mainly extracellular. The production of xylanases by anaerobic fungi is reported to be constant but further production is inducible in the presence of xylan (Theodorou et al., 1991).

1.2. Bread and Bread Staling Mechanism

1.2.1. Bread

Bread is ‘‘soft’’ and, like many other foodstuff, is comprised, at a macroscopic level, of two phases- a fluid (air) and a solid (cell wall material) which gives its unstable, elastic, solid foam structure (Scanlon and Zghal, 2001). The solid part contains a continuous phase composed in part of an elastic network of cross-linked gluten molecules and in part of the leached starch polymer molecules, primarily amylose, both uncomplexed and complexed with

polar lipid molecules, and a discontinuous phase of entrapped, gelatinized, swollen and deformed (wheat) starch granules (Gray and Bemiller, 2003).

1.2.2. Bread Making

The basic procedure of bread-making includes all ingredients of bread, mixed into developed dough. The operations are carried out in such a way that the dough can possess the appropriate mechanical properties which will permit it to retain gas and thus produce a well-expanded loaf of bread with an even crumb structure (Bloksma and Bushuk, 1988; Gan et al., 1990; Kokelaar and Prins, 1995 in Scanlon and Zghal, 2001).

The processing of bread can be divided into three basic parts which are, mixing and development of dough, fermentation (resting and proofing) and baking. After resting, the dough is divided into loaf sized pieces, rounded, moulded, placed on a baking tray, proofed and baked (Sahlström & Brăthen, 1996).

In the process of bread making, mixing does more than just dispersing the ingredients homogeneously that fulfills different functions. In bread making the large amount of water is added to the flour which has to be absorbed by the polymers of the flour, the water added to make up the dough is absorbed by the hydrophilic groups of the protein molecules and also up to an extent, the amount of water uptake by starch is observed. However, some of the flour proteins are water-insoluble which are the focal point of studies aimed at elucidating how

mechanical work input converts flour and water into a cohesive dough mass (Weegels, Hamer, & Schofield, 1996 in Scanlon and Zghal, 2001). When the dough is optimally developed by the mixer, the proteins (which appear to have complexed with flour lipids and some carbohydrate components) form a coherent viscoelastic mass encapsulating the air, the starch granules and other filler materials. Optimal gluten development by the mixing process is vital to the development of structure in the crumb (Scanlon and Zghal, 2001). It can be said that the development of the gluten into elongated fibers has to occur, so that it can form a structure capable of retaining dispersed gas to give a light loaf of bread (Matz, 1960).

In fermentation stage of bread making, carbondioxide and alcohol generated by the leavening agent diffuse in solution to the nuclei due to a concentration gradient so that the nuclei expand into gas cells and the density of the dough is reduced (Scanlon and Zghal, 2001). As fermentation continues, more gas is produced and the gas cells in the dough become larger and larger however the 60% of total gas produced is lost during fermentation, punching, molding and proofing of the dough. Yeast cells do not have mobility in dough so punching brings the yeast cells and fermentables together. After punching, the dough is molded by sheeting followed curling and rolling. The dough must be sheeted in different directions to have strong dough in all directions (Hoseney, 1986).

The final stage of bread making takes place in the oven, in which the final bread crumb structure is set by the conversion of the liquid phase to a solid one. This occurs because, at the temperatures and moisture contents typical for bread baking, thermal transitions occur (Scanlon and Zghal, 2001). One class of polymers within the gluten proteins are known to undergo aggregation; rigidity is conferred to the structure when the polymers are more effectively cross-linked but association of gluten polymer chains with numerous other components is also expected. The other major class of polymers in dough cell walls is also affected by the heat–moisture system of the baking process. Partial melting of the hydrated starch granules is induced, but not their complete homogenization, so that individual granules are readily recognisable in the bread (Scanlon and Zghal, 2001). The reactions that take place during baking are the physical changes and the chemical / biochemical changes. These reactions must take place in order, at the specified temperature, in the correct time and in the proper atmosphere.

1.2.3. Bread Staling

When a loaf of bread is removed from the oven after baking, series of changes starts that eventually leads to deterioration of quality (Sahlström & Brăthen, 1996). Changes in flavor and texture taking place during storage are commonly called staling (Bollaín et al., 2005).

Bread staling is an extremely complex phenomenon and is difficult to define in uncomplicated terms. In general, bread staling refers to all changes

that occur in bread after baking. Bread staling falls into 2 categories: crust staling and crumb staling.

The major cause of crust staling is believed to be due to 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 is 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).

The bread crumb is a complex solid matrix composed mainly of gluten, starch, and water (Bollaín et al., 2005). Thus bread stales largely as a result of physical changes that occur in the starch-protein matrix of bread crumb. Retrogradation is the process by which starch amylopectin reverts to more ordered state after gelatinization (Katina et al, 2006), therefore starch retrogradation is accepted as the main cause of bread staling (Xie et al., 2004); also proteins of wheat flour may effect the staling rate (Katina et al., 2006). Other components of dough like pentosans, lipids (D' Appolonia and Morad, 1981); emulsifiers, sugars and enzymes also affect crumb softness of fresh bread and shelf-life (Katina et al., 2006).

Changes occur in both the crumb and the crust of the bread. The increase in crumb firmness has probably been used to the largest extent by the

investigators following bread staling. Other changes, loss of flavor, decrease in water absorption capacity, amount of soluble starch crystallinity and opacity, and changes in x-ray diffraction patterns have also been listed (D' Appolonia and Morad, 1981).

1.2.3.1. Starch in bread staling

Starch is a polymer of glucose linked to one another through the C1 oxygen, known as the glycosidic bond and this glycosidic bond is stable at high pH but hydrolyzes at low pH. Two types of glucose polymers are present in starch: i) amylose and ii) amylopectin. The organization of starch granules are designed as amorphous, and crystalline. While amylopectin is soluble in water amylose and starch granule are insoluble in cold water. When water and starch solution is heated, the granules start to a swelling process which is irreversible and referred as gelatinization. During this process, amylose leaches out the granule and causes an increase in the viscosity of the slurry. The continued increase in temperature leads to maximum swelling finally granules break apart and resulting in a complete viscodous colloidal dispersion (van der Maarel et al., 2002).

During transformation of dough to bread in bread making process, the increased temperature causes gelatinization of starch content of bread at microscopic level. At 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).

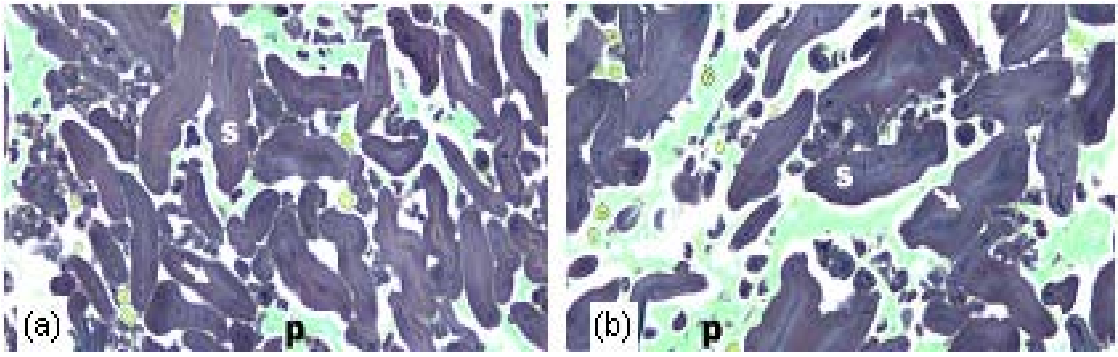

Figure 3. Micrographs of bread crumb stained with Light Green and iodine to visualize protein (p) and starch (s), respectively: (a) bread, fresh and (b) 6 days old (Katina et al., 2006).

During retrogradation, the starch substance undergoes a change from dissolved and dissociated state to associated stage and retrogradation is caused by the amylose content of the starch rather than amylopectin content which has highly branched organization that is prone to less retrogradation (Van Der Maarel et al, 2002).

1.2.3.2. Proteins in bread staling

It has been suggested that protein content is an important factor in the rate of bread staling, that protein (gluten) reduces the firming rate of bread during staling, has no effect on the firming rate, and is required for firming, meaning, that staling is depend on starch–gluten interactions (Gray and Bemiller, 2003). Gluten is a combination of proteins that forms a large network

during dough formation. This network holds the gas in microstructures during dough proofing and baking. The strength of this gluten network is therefore extremely important for the quality of all bread raised using yeast (URL 2). On heating, the gluten transforms from a gel to a coagel by polymerization, which means that the gel looses its cohesiveness. The resulting network, combined with partially gelatinized starch granules, is most certainly responsible for the semirigid structure of baked bread (Hug-Iten et al, 1999). During aging of the bread, amylopectin recrystallizes resulting in increased rigidity of the starch granule and decreased flexibility of the gluten matrix. All these changes seem to contribute to crumb firming (Valjakka et al., 1994; Zobel and Kulp, 1996, in Keskin, 2003).

1.2.3.3. Non-starch polysaccharides in bread staling

Arabinoxylans and arabinogalactans (arabinogalactan-proteins) are the “pentosans” (more properly pentoglycans) of wheat flour (Gray and Bemiller, 2003); which are polysaccharide materials, are a minor component of wheat flour present at the 2-3% level (D’ Appolonia and Morad, 1981). They are supposed to play important role in both the preparation and the shelf-life of bakery products and it was shown that water-soluble and -insoluble pentosans have been reported both to slow down staling (Gray and Bemiller, 2003); by reducing the amount of starch components available for crystallization, and the effect exerted by the water insoluble pentosans was more pronounced than that exerted by water soluble pentosans (Kim and D’Appolina 1977).

Both water-soluble and water-insoluble pentosans of wheat flours are important functional ingredients in dough and bread systems because of their ability to bind large quantities of water (Michniewicz et al., 1992 in Gobetti et al., 1999).

Pentosans have been estimated to absorb about one-third of the water of the total water in normal dough. Water soluble pentosans dissolve in water to give extremely viscous solutions, whereas water-insoluble pentosans become highly hydrated without truly dissolving. Thus, pentosans could remarkably affect rheological properties of dough and, thereby, the quality of bread (Krishnarau and Hoseney, 1994).

1.2.4. Control of Bread Staling

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 and the other way of controlling the staling is done after baking bread. The control of staling after baking includes the avoidance of loss of water by the use of good packaging and holding microbial parameters under control.

As the first parameter, moisture migration into and around the bread must be avoided. This moisture control starts during baking. A minimal but sufficient baking time gives a longer freshness to the bread. Cooling under conditioned parameters can diminish the water evaporation. Plastic packaging makes the

barrier for water migration during long shelf-life. There is also water migration between the dry crust and the humid crumb. Decrusting the bread can eliminate this crumb–crust migration. If there is a filling with a lower water activity than the crumb, the latter will dry out. If the filling has a higher water activity than the crumb, moulds can start to grow. This may affect the microbial parameters also storage temperature may affect microbial activity. Cold temperatures are speeding up the staling of the bread. Better conditions are 15 °C or higher for a minimal staling rate (Decock and Cappelle, 2005).

1.2.4.1. Enzymes in Bread Staling

The use of enzymes in baking industry is mainly concerned with bread staling and extending bread sensory quality throughout its shelf life, by acting on major functional flour components (Gimenez et al., 2005). Consequently different types of enzymes are used in baking industry such as, amylases, xylanases, lipases, proteases.

1.2.4.1.1. α-amylases

In bread making industry amylases have been used for improving dough-handling properties and enhancing bread quality thus the addition of α-amylase which has not only a substantial anti-staling effect but also improves the elasticity of bread crumb (Kim et al., 2006).

The general point of adding α-amylases to flour was to generate fermentable compounds, mainly maltose in the dough. In the other hand some

other changes, such as increased bread volume, improved crumb grain, crust colour and flavour may also obtained (Sahlström and Brăthen, 1997). The influence of α-amylase on the staling 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. Thus a mixture of amylase and amylopectin will be hydrolyzed into a mixture of dextrins, maltose, glucose and oligosaccharides (deMan, 1999). Depending on the suggestions in literature dextrin production by α-amylases persist in the baked bread which may give a different dextrin distribution that in control of loaf volumes.

Commercial α-amylases aimed at the baking industry are obtained from cereals, fungi and bacteria. The main difference between these enzymes lie in their temperature optima, the fungal enzymes having the lowest (50-60°C) and bacterial the highest (70-80 °C) (Hamer, 1992 in Sahlström and Brăthen, 1997). In general fungal α-amylases are used in baking industry, because of their thermostability and lower risk for overdose. In fact bacterial α-amylases should be carefully dosed for the reason that overproduction of dextrins may observed. This would resulted with a sticky bread crumb (Zobel and Kulp, 1996; Poutanen, 1997 in Keskin 2003).

1.2.4.1.2. Xylanases, Hemicellulases and Cellulases

Xylanases as non-starch polysaccharide degrading enzymes are widely used as additives in baking industry. The usage of xylanases in bread making have been shown to effect enhancements in dough and bread quality leading to improved dough flexibility, machinability and stability and a larger volume as well as an improved crumb structure (Collins et al., 2006).

The non-starch polysaccharide in the endosperm cell walls of wheat constitute up to 75% of the cell wall dry material weight. There are 3-4% (w/w) pentosans in normal wheat flour, partially soluble and partially insoluble. Arabinoxylan occur as minor components of wheat flour (2-3% dry basis) and can be divided as water extractable and water unextractable. Water unextractable arabinoxylans, which make up 70% of wheat endosperm cell walls, can be hydrolyzed by xylanases (Shah et al., 2005). Thus, xylanases lead to moderate release of water initially absorbed by water-insoluble pentosans, which causes a redistribution of water that becomes available for gluten leading to optimal development. Solubilization of arabinoxylan also results in higher viscosity that is likely to play a positive role in dough rheology and gas retention (Rouau et al., 1994 in Jimenez and Martínez-Anaya, 2001).

Xylanases also have been reported to extend shelf life of bread by reducing the staling rate. As a result of xylanase activity, hydrolyzed cell wall polysaccharides may affect the water balance; the released water can reduce dough viscosity resulting in softer dough (Harada et al., 2000). Also products of

xylanase activity may interfere with other wheat flour components that may affect the staling rate of bread (Haros et al., 2002 in Keskin 2003). Beside this, Kim and D'Appolonia (1977) suggested that pentosans decreased the bread staling rate, and the effect exerted by the water-insoluble pentosans was more pronounced than that exerted by the water-soluble pentosans.

Cellulose consists of glucose units linked by β-1, 4-glycosidic bonds in a linear fashion. The difference in the type of bond and the highly ordered crystalline form of the compound between starch and cellulose make cellulose more resistant to digestion and hydrolysis. The enzymes required for the hydrolysis of cellulose include endoglucanases, exoglucanases and β-glucosidases. While cellulase is an endoglucanase that hydrolyses cellulose randomly, producing oligosaccharides, cellobiose and glucose, exoglucanase hydrolyze β-1, 4-D-glycosidic linkages in cellulose releasing cellobiose from the nonreducing 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. Generally speaking; 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.

1.2.4.1.3. Proteases

In general proteases break down the gluten protein which makes the dough softer and extensible. The purpose of adding proteases to bread is to improve flavor profiles, flow characteristics, machining properties, gas retention, and mixing time (Gray and Bemiller, 2003). Also, protein has significant effect on bread staling mechanism; the activity of proteases may affect this mechanism via some modifications in gluten network. These modifications can be explained as the cleavage of peptide bonds by releasing water which has been bound to gluten; thus viscosity of dough is reduced (URL 1).

g1.2.4.1.4. Lipases

Depending on the type of flour and the formula, addition of some lipases resulted 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). As the action of lipase, monoglycerides are also formed which are reported to have antistaling characteristics (Gray and Bemiller, 2003). The most accepted theory about the mechanism of antistaling action of monoglycerides is based on the ability of monoglycerides to form complexes with amylose and amylopectin. Additionally, the antifirming effect of emulsifiers was attributed to mainly the weakened cohesion between the swollen starch granules, which are rich in amylopectin (Zobel and Kulp, 1996, in Keskin, 2003).

2. THE AIM AND SCOPE OF THE STUDY

The main objective of this study is 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* spp 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.

3. MATERIALS AND METHOD

3.1. Organisms and Cultivation

The rumen anaerobic fungi, which was previously classified as *Neocallimastix* spp, was isolated from deer feces and obtained from Prof. 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 liter) were prepared according to Caldwell and Bryant, 1966 and showed in Table 3.

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.

Table 3. The ingredients of M 10 medium.

Ingredients	Formula	Amount
Yeast Extract	(OXOID)	0.5 g
Peptone From Casein	(FLUKA)	2.0 g
Mineral Solution I	6g K_2PO_4/1L or 7.8 g $K_2HPO_4.3H_2O$/1L	38 mL
Mineral Solution II	12 g NaCl, 12 g $(NH_4)_2SO_4$, 6 g KH_2PO_4, 1.2 g $CaCl_2$, 2.5 g $MgSO_4.7H_2O$ (per liter)	38 mL
Hemin (ALDRICH)	50 mg hemin, 1mL1NaOH	10 mL
Volatile Fatty Acid (VFA) Mix	10 mL methylbutric acid (ALDRICH), 170 mL acetic acid (PANREAC), 60 mL propionic acid (PANREAC), 40 mL butryric acid (FLUKA), 10 mL valeric acid (FLUKA), 10 mL isovaleric acid (FLUKA), 10 mL isobutyric acid (FLUKA), 100 mg Phenylacetic acid , 100 mg phenylpropionic acid (ALDRICH))	3.1 mL
Resazurin (0.1%)	(ALDRICH)	0.5 mL
Vitamins	200 mg pyridoxin B6, 200 mg riboflavin B2, 200 mg nicotinamide, 200 mg thiamine –HCl B1, 200 mg Ca-D- panthothenate B3, 1mg p-aminobenzoic acid, 0.5 mg biotin, 0.5 mg cobalamine, 5 mg acidumfolicum (per 100 mL)	2 mL
Microelements	253 mg $NiCl_2$, 333 mg H_3PO_4, 500 mg $Na_2MoO_4.2H_2O$, 11 mg $FeSO_4.7H_2O$, 500 mg $MnSO_4.4H_2O$, 500 mg $ZnSO_4.7H_2O$, 253 mg $CuSO_4.5H_2O$, 253 mg $CoCl_2.6H_2O$, 100 g chelaton, 100 mg $KAl(SO_4)_2.12H_2O$, 253 ml 5N NaOH	0.5 mL
Rumen Fluid		200 mL
Water (distilled)		809 mL
Substrate	(CM cellulose)	4 g
Antibiotic Solution	2.000.000 1U penicillin, 1 g streptomycin, 1 g chloramphenicol.	100 mL

3.2. Determination of Cellulolytic Activity

The cellulolytic activity of anaerobic rumen fungi was determined by the dinitrosalicylic acid (DNS).

The isolates were incubated at 39 °C four days in M10 Medium containing carboxymethycellulase (CMC) at an incubator (Nüve EN 400, Turkey). After the incubation period, 1 mL of medium was centrifuged at 4000g / 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 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, was contained 0.5 mL dH_2O instead of 0.5 mL supernatant of the enzyme solution.

3.3. Glucose Determination

Glucose determination was carried out by using DNS method according to Miller, 1959.

The stock solution of glucose (D- Glucose) (MERCK) was adjusted to a concentration of 1, 099 g/ 1000 mL and several concentrations of glucose from stock solution were prepared in the following manner:

- ✓ 55 ppm = 5 mL + 95 mL dH_2O
- ✓ 110 ppm = 20 mL + 80 mL dH_2O
- ✓ 165 ppm = 15 mL+ 85 mL dH_2O
- ✓ 220 ppm = 20 mL + 80 mL dH_2O

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. A linear standard curve for glucose was constructed using the absolute amounts of glucose plotted against spectrophotometric values. Finally, the amount of glucose released from each sample was determined.

3.4. 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).

3.4.1 The Effect of Temperature

For determining 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 4000g / 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. Several test tubes having the same solutions as above were prepared as follows; and kept at the given temperature and time below:

- ✓ 4 °C for 10 minutes, 20 minutes, 30 minutes, 40 minutes and 60 minutes.
- ✓ 25 °C for 10 minutes, 20 minutes, 30 minutes, 40 minutes and 60 minutes.
- ✓ 50 °C for 10 minutes, 20 minutes, 30 minutes, 40 minutes and 60 minutes.
- ✓ 100 °C for 10 minutes, 20 minutes and 30 minutes.

For all test tubes the absorbance was determined at O. D. 550 nm by spectrophotometer.

3.4.2. 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 (Nüve EN 400, Turkey). After the incubation period, 1 mL of medium was centrifuged at 4000g / 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 were adjusted with 1.8 mL 50 mM Citrate-Phosphate buffer to the following pH values; 2, 3, 4, 5, 6, 7,8, 9, 10; 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 also, incubation was done in water bath (Nüve NB20, Turkey) at 50 °C for 30 minutes. The absorbance was determined at O. D. 550 nm by a spectrophotometer.

3.5. Partial Isolation of Enzymatic Complex

An enzyme containing solution complex from *Neocallimastix* spp. were obtained by using 0.45 µm pore sized, Sartorius- Minisart filters.

By incubating the isolates at 39 °C, 1 mL of medium, supposed to contain extracellular enzyme complex of anaerobic rumen fungi, was removed to sterile Eppendorf tubes; and 1 mL of medium was centrifuged at 18,000 rpm for 10 minutes. The supernatant of the sample was carefully removed by using sterile needle and passed through 0.45 µm pore sized filters. The final solution was collected into sterile Eppendorf tubes and used as an enzyme complex.

3.6. Bread Making

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 for six times. All the bread ingredients were supplied from the same laboratory.

The composition of the dough containing no enzyme (control group) was on flour basis is shown in the table below.

Table 4. The recipe of dough

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

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, the fermentation step starts composed of two parts. The samples are placed into an oven and then baked.

The bread baking process was initially started with the adjustment of the surrounding temperature of the laboratory to 25 °C by using a heater. 280 g commercial standard white flour, 1.7 g instant yeast, 4 g salt were weighed for each loaf and mixed in the Farinograph® (BRABENDER® Farinograph Resistograph, OGH Duisburg, Germany) for 1 minute. The optimal amount of water for dough preparation was determined by recording farinographs at 500 BU. Thus, 170 mL water was added during mixing for 5 minutes while keeping the temperature of the mixer constant at 27.8 ºC. After that dough was placed into baker's pan (13 cm X 9 cm) for proofing for 30 minutes and covered with moisten cloth for fermentation. At the end of this period, the dough was kneaded for a while and placed again into baker's pan and then placed into

incubator (Heraeus B5060E, Germany) for 45 minutes at 45 ºC for the second step of fermentation. The proofing was accomplished after 45 minutes. The loaf was baked at 250 ºC for 30 minutes in an oven (Heraeus ST5060, Germany), and was cooled for 3 hours at room temperature (25 ºC) and was sealed in plastic bag and stored at room temperature.

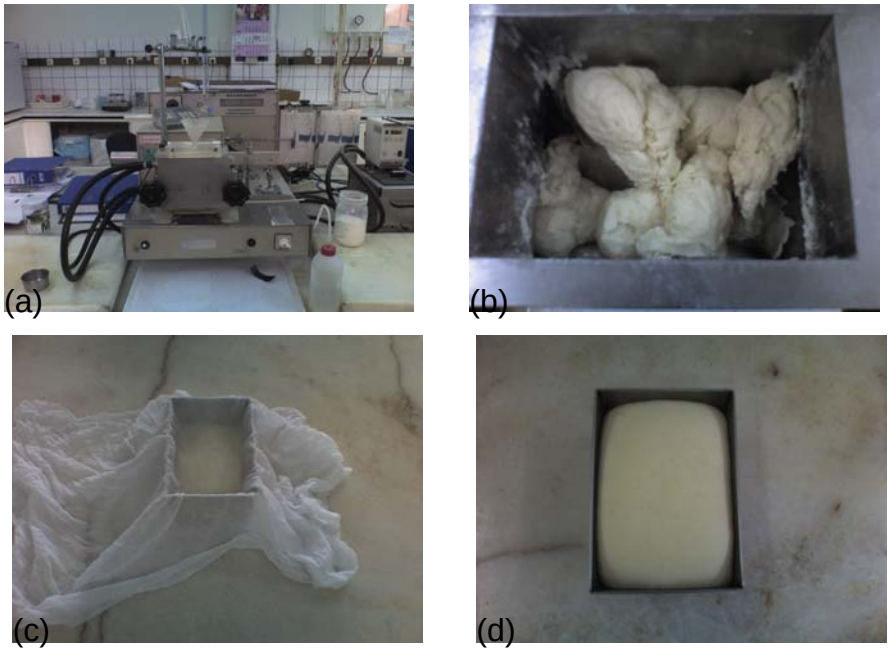

Figure 4. (a) (b) Mixing of dough at Brabender farinograph. (c) (d) Fermentation stages.

The enzyme complex which has been partially isolated from anaerobic rumen fungi was added to dough at different concentrations varying from 0.1 mL to 0.4 mL. Thus five loaves were made at each replicate, including the control, 0.1 mL, 0.2 mL, 0.3 mL and 0.4 mL enzyme added loaves. The enzyme complexes were added to flour while mixing with water.

Figure 5. Baked bread samples including enzyme complex.

3.7. The Microbial Counts of Bread Samples

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

Each bread samples were tested for its microbial counts at the first, third and seventh days of the storage of bread samples. Bread sample (1 g) was weighed and placed into a test tube containing 9 mL sterile dH_2O as diluent and shaken well. 1 mL from the sample was added to another test tube containing 9 mL sterile dH_2O 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 37 ºC for 48 hours.

3.8. Crumb Firmness Measurements

The firmness of breads was measured by a universal testing machine (Lloyd Instruments LR 30K, UK) in the laboratories of department of Food Engineering, Middle East Technical University, Ankara, Turkey. The principle of Texture Profile Analysis (TPA) is summarized schematically in Figure 7 and Figure 8 in Appendix section.

Bread samples were tried to be sliced, from the middle portion 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 50N. Firmness measurements were done in the first 24 hours after baking bread samples and two slices were used for each group.

4. RESULTS AND DISCUSSION

4.1. Determination of Cellulolytic Activity

The cellulolytic activity of enzyme complex which has been produced by anaerobic rumen fungi, *Neocallimastix* spp. was determined by DNS method Avicel (microcrystalline cellulose) was used as carbon source. These experiments indicated the degradation of 10 mg cellulose by extracellular enzyme complex of *Neocallimastix* spp. for different samples.

The anaerobic rumen fungi have already been studied many by scientists since its discovery in 1988 by Orpin, also the production of extracellular enzymatic complexes has been described. In general the extracellular enzyme complexes of anaerobic rumen fungi species are studied because of their ability for degrading the cell wall components of the plant tissues where they generally colonize. The production of wide range of hydrolytic enzymes provides utilization of carbon sources ranging from simple sugars to complex polymers, thus it was explained by Theodorou et. al, in 1991 that cellulolytic, xylanolytic, glycolytic, amylolytic and proteolytic enzyme activities were observed against substrates. In this study the 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 that is simply based on the color 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. Because dissolved oxygen can interfere with glucose oxidation, sulfite, which itself is not necessary for the color 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 the glucose calibration curve (Figure 3). Percentage values of samples indicated the activity of enzyme complex, as the degradation of substrate, varying from 9.5% to 36.5%; and the most active sample was Sample 1, which has 36.5% degradation value. Also as seen from the figure 3 the enzymatic activity declined after each subcultivation. However, after the isolation of new anaerobic rumen fungi from feces, they had high values of enzymatic activity. Thus, fresh isolates and the samples which have high enzymatic activity were used in further studies.

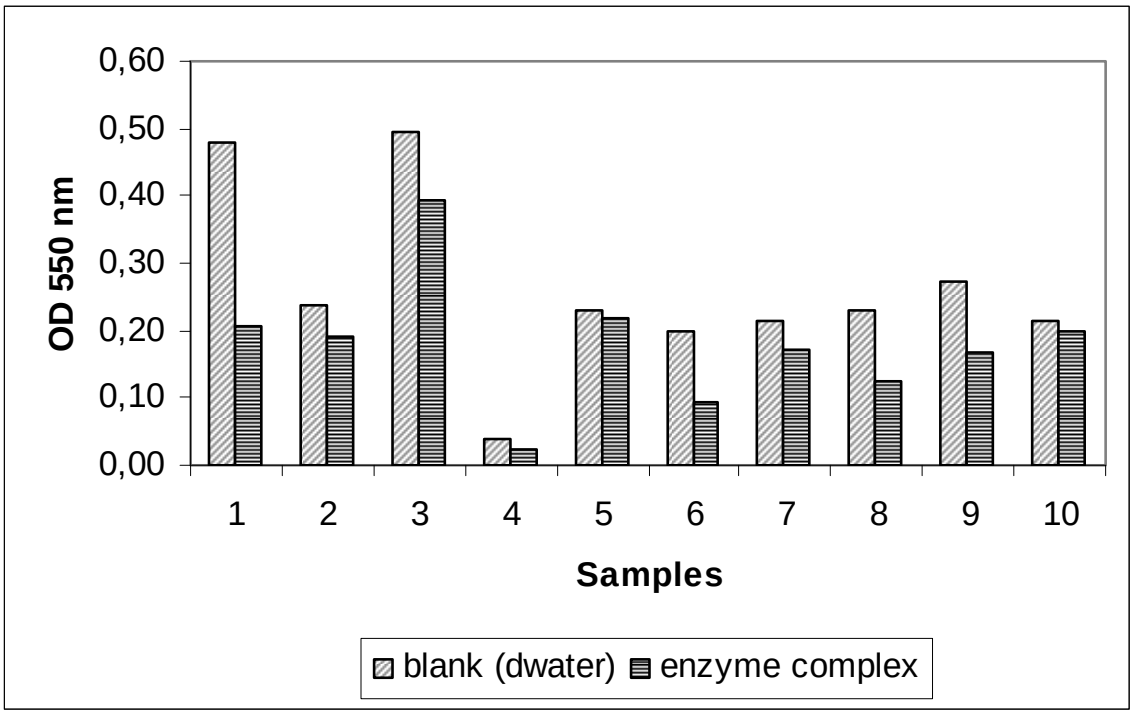

Figure 6. Results and difference of cellulolytic activity of extracellular enzyme complex of *Neocallimastix* spp.

4.2. Glucose Determination

The glucose determination was done 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 amount of glucose produced by the enzymatic action of *Neocallimastix* spp., and the percentages of degradation of substrate by samples were calculated (Table 7 in Appendix Section).

4.3. Effects of Temperature and pH on Enzyme Activity

4.3.1. Effects of temperature on enzyme activity

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 as the time goes along for 4 °C, 25 °C and 50 °C (Figure 4). At high temperatures like 100 °C, the enzymatic activity is observed by verification of spectroscopical 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.

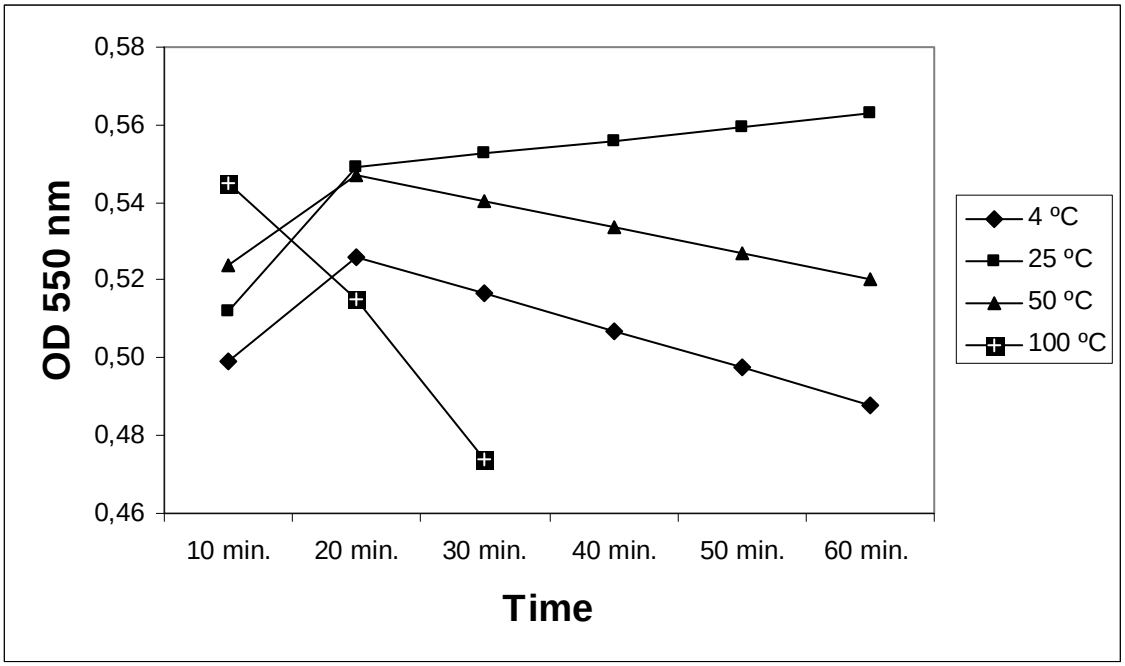

Figure 7. The temperature effect on enzyme complex of anaerobic rumen fungi.

4.3.2. Effects of pH on enzyme activity

The pH values indicated that the enzyme shows its activity mainly between 5 to 7 pH interval, which can be stated as very closer to neutral pH values. This may allow the use of enzymatic complex in neutral food items, although the strongest acid treatment indicated less degradation of cellulose when compared with the neutral case, which might be due to probable inactivation of this enzymatic complex (Figure 5).

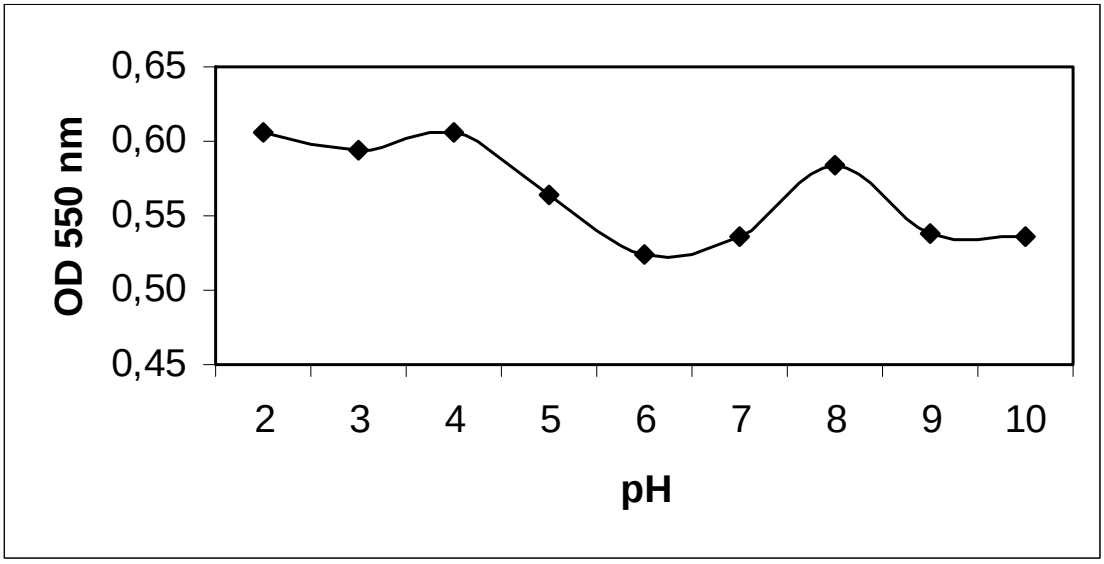

Figure 8. The effect of pH on enzyme complex of anaerobic rumen fungi

4.4. The Microbial Counts of Bread Samples

The microbial analysis was performed as initial (zero-day), fourth and eighth days of storage of bread at room temperature. In the initial and fourth days of storage the microbial activity was low on bread, but when the eighth day counts were considered, high mold counts were achieved on the plates

containing potato dextrose agar. The storage time could be prolonged by using an effective packaging technique, supporting the positive effect of enzyme and if adequate cooling of the bread samples were maintained for longer time after baking, mold appearance would be reduced.

4.5. Crumb Firmness Measurements

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 (deMan, 1999). By measuring the mechanical textural characteristic of bread, a texture analyzer was used in this study. The primary parameters, the hardness, the cohesiveness (includes brittleness, chewiness and gumminess) and the adhesiveness were determined (Table 10 and 11).

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; and it can be said that the enzyme complex of anaerobic rumen fungi *Neocallimastix* spp*.* may affect the hardness of bread.

As another primary parameter of texture analysis, cohesiveness was measured. The cohesiveness can be explained as, 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 3). Also it can be defined as the strength of the internal bond making up the body of the product; and was 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 increased volume, more uniform grain structure and slower aging during storage.

In general, depending on the studies of Harada et al. (2000) and the data of Texture Profile Analysis, crumb firmness values, hardness and cohesiveness

were significantly affected by the addition of cellulolytic enzyme complex. Also the anti-staling effect of enzyme complex could be explained 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.

5. CONCLUSIONS

The enzymatic complex of anaerobic rumen fungi which is produced by *Neocallimastix* spp., was partially isolated and the effect of addition of this complex on bread texture and shelf life were studied in this study. It was found that the usage of this enzyme in bread baking may increase the consumer's acceptability because of its natural state with prolonged shelf life. The cellulolytic activity was present in the partially isolated enzyme complex supported by the DNS method.

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

The impact of side activities of the enzyme complex of *Neocallimastix* spp. could influence the results; as because it was not highly purified. If this can be done, the exact effect of cellulase and other constituents of enzyme complex on bread texture can be studied. Moreover, combination enzymes, because of their synergetic effects, may be tried in the extention of shelf life of bread. Also the effects of enzyme complex of anaerobic rumen fungi on the quality of different varieties of bread, pastry and cakes can be studied.

A professional sensory analysis, by trained panellists, is also recommended to determine flavor of the baked bread and whether the other quality parameters were brought to an acceptable point or not.

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URL3: http://www.food.upm.edu.my/kuliah/lectures/foodsci/fst4109h/Solids%20Rheology.pdf

APPENDIX

Table 5. O. D. 550 (nm) values for cellulolytic activity of extracellular enzyme complex of *Neocallimastix* spp.

Sample no	Date	O. D. $_{550}$ blank	O. D. $_{550}$ sample
1	06.06.2004	0,480	0,205
2	29.06.2004	0,239	0,191
3	03.07.2004	0,494	0,392
4	15.07.2004	0,040	0,022
5	18.11.2004	0,229	0,217
6	12.12.2004	0,198	0,095
7	25.01.2005	0,214	0,170
8	08.06.2005	0,229	0,124
9	29.06.2005	0,272	0,168
10	11.07.2005	0,215	0,199

Table 6. O. D. values for glucose determination.

Amount of Glucose (mg / L)	O. D. 550 nm
50	0,001
100	0,153
150	0,299
200	0,446
250	0,589

Table 7. Amount of glucose produced by the result of enzymatic activity of *Neocallimastix* spp.

Sample No	O. D. $_{550}$ Blank	Glucose Content	O.D $_{550}$ Sample	Glucose Content	Degradation %
1	0,480	186,387	0,205	118,528	36,5
2	0,239	129,893	0,191	113,848	12,4
3	0,494	215,135	0,392	181,038	15,8
4	0,040	63,371	0,022	57,354	9,5
5	0,229	126,551	0,217	122,539	3,1
6	0,198	116,188	0,095	81,757	30,6
7	0,214	121,536	0,170	106,828	12,0
8	0,229	126,551	0,124	91,451	27,0
9	0,272	140,925	0,168	106,159	24,0
10	0,215	121,871	0,199	116,522	4,0

Table 8. O. D. 550 (nm) values for optimum temperature of enzymatic complex of anaerobic rumen fungi

TIME	O.D $_{550}$ at 4°C	O.D $_{550}$ at 25°C	O.D $_{550}$ at 50°C	O.D $_{550}$ at 100°C
10 minutes	0,499	0,512	0,524	0,545
20 minutes	0,526	0,549	0,547	0,515
30 minutes	0,516	0,552	0,540	0,474
40 minutes	0,507	0,556	0,533	
50 minutes	0,497	0,559	0,526	
60 minutes	0,488	0,563	0,520	

Table 9. O. D. 550 (nm) values for optimum pH value for enzymatic complex of anaerobic rumen fungi

pH	O.D $_{550}$
2	0,606
3	0,594
4	0,607
5	0,564
6	0,525
7	0,537
8	0,535
9	0,534
10	0,536

Table 10. The results of bread analysis by texture analyzer

Reference	Sample Height (mm)	Hardness 1 (kgf)	Hardness 2 (kgf)	Area 1 (kgf.mm)	Area 2 (kgf.mm)	Cohesiveness	Springiness (mm)	Springiness Index	Gumminess (kgf)	Chewiness (kgf.mm)	Fracture Force (kgf)	Adhesive Force (kgf)	Adhesiveness (kgf.mm)	Stiffness (kgf/mm)
control-1	12,20	0,6384	0,5261	0,3073	0,1609	0,6234	2,8091	0,8150	0,3288	0,9236	0,0057	0,0013	-0,0006	0,2832
control-2	12,66	0,6282	0,5954	0,2281	0,1399	0,6132	2,7137	0,8517	0,3301	0,8958	0,5197	0,0003	-0,0009	0,2653
0,1 ml-1	12,64	0,5541	0,4914	0,2241	0,1336	0,5958	2,6580	0,7994	0,3301	0,8775	0,0056	0,0001	-0,0010	0,2986
0,1 ml-2	12,87	0,6341	0,5708	0,3805	0,2128	0,5594	2,8713	0,8512	0,3547	1,0185	0,0065	0,0003	-0,0004	0,2455
0,2 ml-1	13,42	0,5962	0,5040	0,3978	0,1980	0,4977	2,9108	0,8091	0,2941	0,8561	0,0058	0,0001	-0,0007	0,2442
0,2 ml-2	13,45	0,5909	0,4354	0,2655	0,1367	0,5147	2,2654	0,7824	0,2502	0,5669	0,0063	-0,0003	-0,0008	0,2370
0,3 ml-1	12,45	0,4861	0,3317	0,2133	0,1095	0,5135	2,5394	0,7950	0,1968	0,4998	0,0060	0,0005	-0,0008	0,2626
0,3 ml-2	12,00	0,3833	0,5181	0,4148	0,2066	0,4980	2,5673	0,8122	0,2967	0,7618	0,0062	0,0005	-0,0006	0,2537
0,4 ml-1	11,90	0,5958	0,3478	0,1826	0,0888	0,4862	2,2814	0,8061	0,2022	0,4613	0,0067	0,0002	-0,0008	0,3623
0,4 ml-2	10,50	0,4159	0,5261	0,3990	0,2312	0,5794	2,9357	0,8269	0,3454	1,0141	0,0063	0,0001	-0,0007	0,2119

Table 11. Average values of texture analysis

Reference	Sample Height (mm)	Hardness 1 (kgf)	Hardness 2 (kgf)	Area 1 (kgf.mm)	Area 2 (kgf.mm)	Cohesiveness	Springiness (mm)	Springiness Index	Gumminess (kgf)	Chewiness (kgf.mm)	Fracture Force (kgf)	Adhesive Force (kgf)	Adhesiveness (kgf.mm)	Stiffness (kgf/mm)
control	12,43	0,6333	0,5608	0,2677	0,1504	0,6183	2,7614	0,8333	0,3294	0,9097	0,2627	0,0008	-0,0007	0,2742
0,1 mL	12,76	0,5941	0,5311	0,3023	0,1732	0,5776	2,7647	0,8253	0,3424	0,9480	0,0060	0,0002	-0,0007	0,2720
0,2 mL	13,44	0,5936	0,4697	0,3317	0,1673	0,5062	2,5881	0,7958	0,2722	0,7115	0,0061	-0,0001	-0,0007	0,2406
0,3 mL	12,23	0,4347	0,4249	0,3141	0,1581	0,5058	2,5533	0,8036	0,2468	0,6308	0,0061	0,0005	-0,0007	0,2581
0,4 mL	11,20	0,5058	0,4370	0,2908	0,1600	0,5328	2,6086	0,8165	0,2738	0,7377	0,0065	0,0001	-0,0007	0,2871

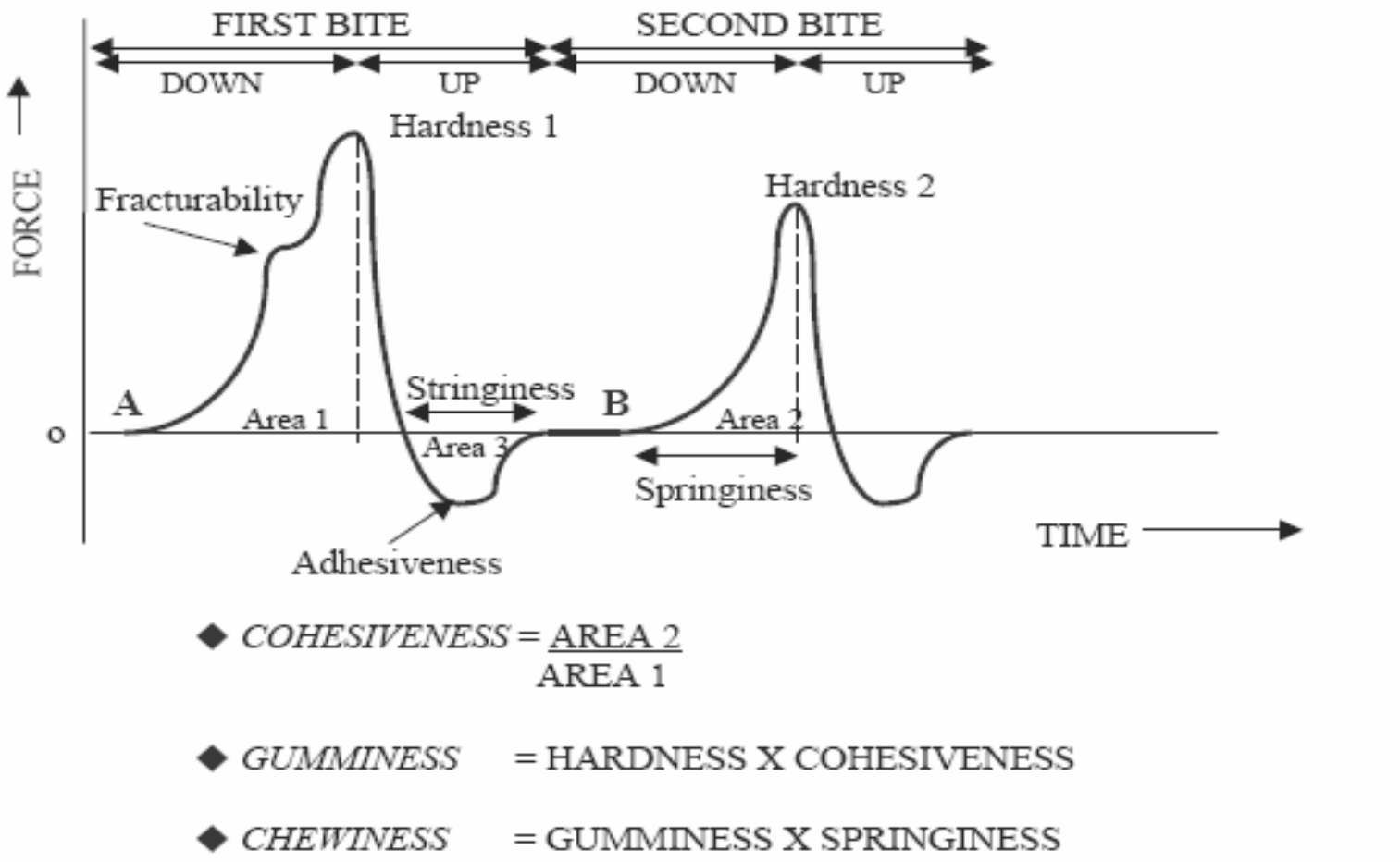

Figure 9. Summary of Texture Profile Analysis (URL 3)

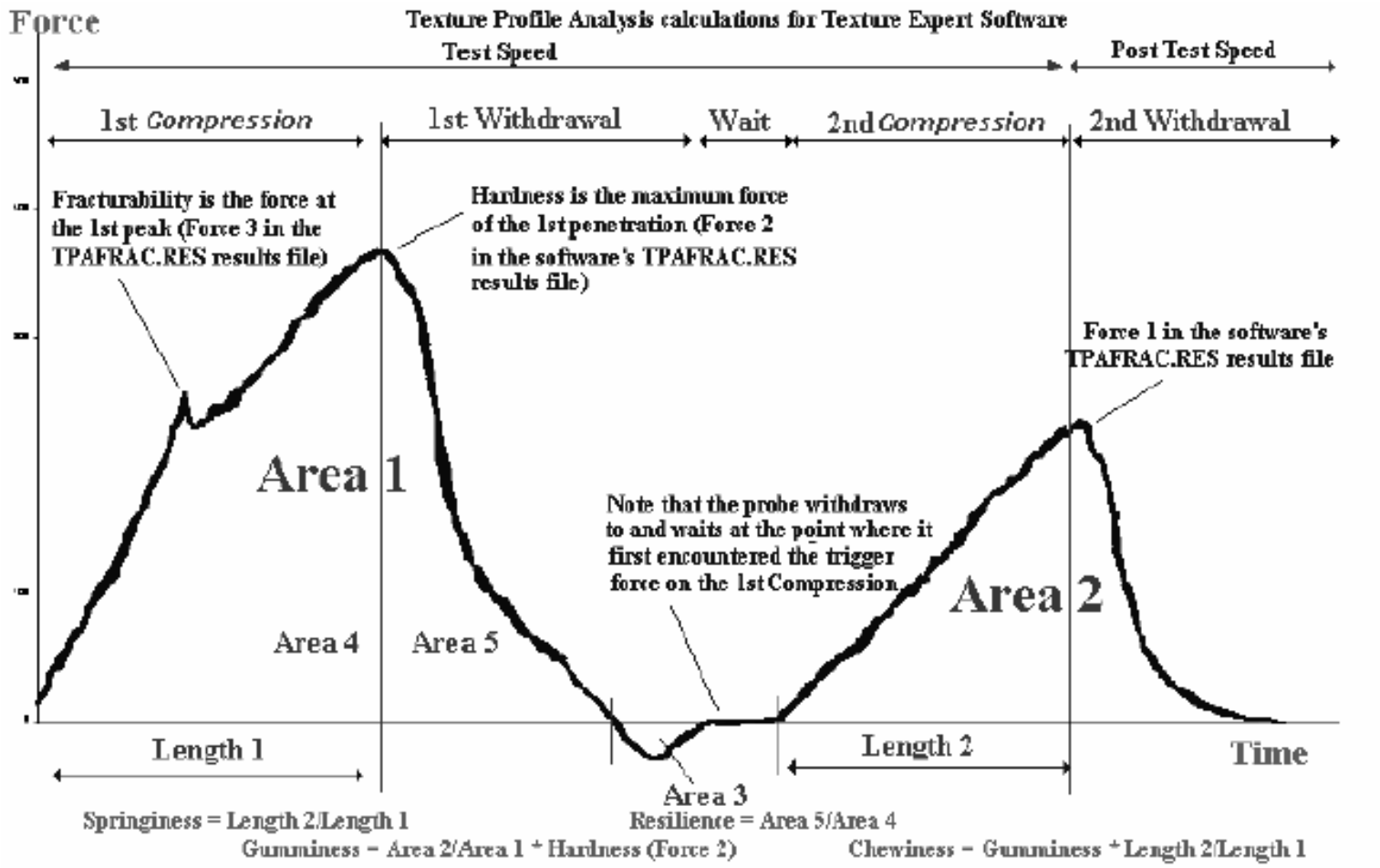

Figure 10. Summary of Texture Profile Analysis (URL 3)