Izolacija i karakterizacija proteolitičkih sojeva bakterija i kvasaca iz ovčjeg i kozjeg sira

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ISOLATION AND CHARACTERIZATION OF PROTEOLYTIC BACTERIA AND YEAST STRAINS FROM SHEEP AND GOAT CHEESES

This work was done under supervision of dr. sc. Monika Kovacs at Department of Microbiology and Biotechnology, on the Faculty of Food science at Szent Istvan University, Budapest, and prof. dr. sc. Vladimir Mrša on the Faculty of Food Technology and Biotechnology, University of Zagreb.

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IZOLACIJA I KARAKTERIZACIJA PROTEOLITIČKIH SOJEVA BAKTERIJA I KVASACA IZ OVČJEG I KOZJEG SIRA

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Sažetak: Cilj ovog rada bila je izolacija i karakterizacija mikroorganizama iz ovčjeg i kozjeg feta sira. Provedena je selektivna izolacija i prebrojavanje kvasaca i bakterija te izolacija pojedinačnih kolonija i njihova karakterizacija na WL hranjivoj podlozi. Ukupno je izolirano 17 kolonija, od toga 4 kvasca s površine ovčjeg sira i 13 bakterija s površine i unutarnjih dijelova obje vrste sira. Provedena je karakterizacija izoliranih spojeva promatranjem morfologije stanica i kolonija, rasta na različitim temperaturama, bojanja po Gramu i provedbom katalaza, oksidaza i ureaza testa. Proteolitička aktivnost nije detektirana u slučaju izoliranih sojeva kvasca, dok su bakterijski izolati pokazali značajnu proteolitičku aktivnosti detektiranu na SM agar podlozi. Nadalje, testiranjem proteolitičke aktivnosti u različitim medijima, pokazalo se da određene komponente hranjivog medija, poput peptona i natrijevog klorida mogu utjecati na proteolitičku aktivnost izoliranih sojeva. Spektrofotometrijskim mjerenjem aktivnosti sojeva pri različitim temperaturama, pH vrijednostima i načinima uzgoja, soj G-2b je pokazao najveću aktivnost pri pH 9, 25°C i uzgojem na tresilici.

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ISOLATION AND CHARACTERIZATION OF PROTEOLYTIC BACTERIA AND YEAST STRAINS FROM SHEEP AND GOAT CHEESES

Dragana Zadro, 642/PI

Abstract: Aim of this work was to isolate and characterize microorganisms from two types of Feta cheese made from sheep and goat milk. Selective isolation and enumeration of yeasts and bacteria were performed, which was followed by obtaining and characterizing single colonies on WL nutrient medium. Altogether 17 colonies were isolated, 4 yeasts from surface of sheep cheese, and 13 bacteria from surface and inside parts of both cheeses. Isolated strains were characterized by observing their cell and colony morphology, growth at different temperatures, Gram staining, and catalase, oxidase and urease tests. Proteolytic activity was not detected in case of yeast strains, while bacterial isolates showed considerable proteolytic activity detected on SM agar. Furthermore, testing the proteolytic activity in different media, it has been shown that certain components of growth media, like peptone and sodium chloride, could affect ability of bacterial strains to produce proteolytic enzymes. Quantifying the proteolytic activity with spectrophotometric measurements at different temperatures, pH values and way of culturing, strain G-2b showed the highest activity at pH 9 and 25°C in agitating cultivation conditions.

Keywords: Proteases, isolation, bacteria, yeasts, characterization, proteolytic activity
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Table of Contents

1. INTRODUCTION 1

2. LITERATURE REVIEW 2

2.1. Sources of proteases 2

2.1.1. Plant Proteases 2

2.1.2. Animal Proteases 2

2.1.3. Microbial Proteases 3

2.1.3.1. Bacterial proteases 3

2.1.3.2. Fungal proteases 4

2.2. Classification of proteases 4

2.2.1. Endopeptidases (proteinases) (E.C. 3.4.21-99) 5

2.2.1.1. Serine Proteases (E.C. 3.4.21) 6

2.2.1.2. Cysteine/Thiol Proteases (E.C. 3.4.22) 6

2.2.1.3. Aspartic Proteases (Acidic Proteases) (E.C. 3.4.23) 7

2.2.1.4. Metalloproteases (E.C. 3.4.24) 7

2.2.2. Exopeptidases (peptidases) (E.C. 3.4.11-19) 7

2.3. Industrial Applications of Proteases 8

2.3.1. Detergent Industry 9

2.3.2. Food Industry 10

2.3.2.1. Dairy industry 10

2.3.2.2. Baking industry 10

2.3.3. Other applications 10

2.4. Bacteria and yeasts in cheese 10

3. MATERIALS AND METHODS 13

3.1.1. Cheese samples for isolation 13

3.1.2. Growth media and solutions 13

3.1.2.1. RBC (Rose Bengal Chloramphenicol) agar 13

3.1.2.2. Milk PC (Milk Plate Count) agar 14

3.1.2.3. WL Nutrient Agar 15

3.1.2.4. YEPD (Yeast Extract Peptone Dextrose) and TGE (Tryptone Glucose Extract) agar 15

3.1.2.5. SM (Skim Milk) agar 16

3.1.2.6. MM (Minimal Media) 17

3.1.2.7. Urea R Broth (Urea Rapid Broth) 17
3.1.2.8. Urea broth base 18
3.1.3. Solutions used for serial dilution and sample processing 19
3.1.4. Chemicals and components for growth media and solutions 19
3.1.5. Equipment and apparatus 19
3.1.5.1. Densitometer 19
3.1.5.2. Laboratory centrifuge 19
3.1.5.3. Spectrophotometer and Multiskan machine 19
3.1.6. Other equipment and apparatus 19
3.2. Methods 20
3.2.1. Processing of cheese samples 20
3.2.2. Preparation of pure cultures and characterization of isolates 21
3.2.3. Strain maintenance 21
3.2.4. Microscopic investigation of isolates 21
3.2.4.1. Preparation of native dissection in case of yeast isolates 21
3.2.4.2. Gram-staining of bacterial isolates 21
3.2.5. KOH test 22
3.2.6. Testing the growth of isolates at different temperatures 22
3.2.7. Testing the proteolytic activity of isolates on SM Agar 22
3.2.8. Extracellular proteolytic activity of strains in different media 22
3.2.9. Multi scanning analysis 22
3.2.10. Influence of NaCl on proteolytic activity of S-5b strain 23
3.2.11. Urea R Broth test 23
3.2.12. Urea Broth Base 23
3.2.13. Catalase test and Oxidase test 23
3.2.14. Quantification of produced extracellular enzyme with spectrophotometric measurement 24
3.2.14.1. Shaked and static bacterial cultures 24
3.2.14.2. Different temperatures and pH values on enzyme activity 25
4. RESULTS AND DISCUSSION 26
4.1. Enumeration of microbes in different cheeses 26
4.2. Preparation of pure cultures and investigation of colony morphology on WL Nutrient Agar 28
4.3. Cell morphology, Gram properties, oxidase and catalase test of isolates 30

4.3.1. Cell morphology 30
4.3.2. Gram properties, oxidase and catalase test of bacterial isolates 32
4.3.3. Urease test of yeasts isolates 35
4.4. Growth of isolates at different temperatures 36
4.5. Proteolytic activity of isolated strains 38
4.5.1. Testing the proteolytic activity of isolates on SM Agar 38
4.5.2. Extracellular proteolytic activity of strains in different media 40
4.6. Influence of NaCl on growth and proteolytic enzyme production 43
4.6.1. Influence on growth 43
4.6.2. Influence on proteolytic activity 44
4.7. Quantification of produced extracellular enzyme with spectorphotometric measurement 47
4.7.1. Spectrophotometric measurements of shaked and static bacterial cultures 47
4.7.2. Effect of temperature and pH on enzyme activity 49
5. CONCLUSIONS 51
6. LITERATURE 52

1. INTRODUCTION

Proteolytic enzymes, also called proteases, proteinases, or peptidases, are group of enzymes, whose catalytic function is to hydrolyze peptide bonds of proteins and break them down into polypeptides or free amino acids. They possess catalytic activity in wide temperature (5-100°C) and pH (0-14) range. Predominantly, they are extracellular and secreted into the fermentation medium. There are thousands of different protease molecules that have been isolated and characterized, and also several hundred proteases that have commercial relevance.

Proteolytic enzymes can be classified by source organism **(**animal, plant, bacterial, fungal), proteolytic mechanism **(**serine, threonine, cysteine, aspartic, metalloproteases and glutamic acid proteases), active pH range (acid, neutral, alkaline and high-alkaline), and peptide bond specificity (endopeptidases and exopeptidases). The protease enzymes, from the hydrolytic group of enzyems, are the most important groups in the industrial enzymes with wide applications in several industrial sectors, particularly in the food, pharmaceutical, chemicals, detergent and leather processing industries.

Two thirds of the industrially produced proteases are from microbial sources. In order to obtain commercial quantities, microbial strains that produce the desired enzyme are being isolated and characterized. Microorganisms are an essential component of all natural cheese varieties, what makes cheese desirable source of potential microbial proteases.

The aim of this work is to isolate different microorganisms from two types of Feta cheese made from sheep and goat milk, characterize isolated strains by obtaining cell and colony morphology, and to characterize various characteristic of isolated strains. Furthermore, to test and quantify proteolytic activity at different conditions.

2. LITERATURE REVIEW

2.1. Sources of proteases

For living organisms proteolytic enzymes are physiologically necessary and have degradative and synthetic functions. Proteolytic enzymes can be found in a wide diversity of sources, from prokaryotes, eukaryotes to viruses.

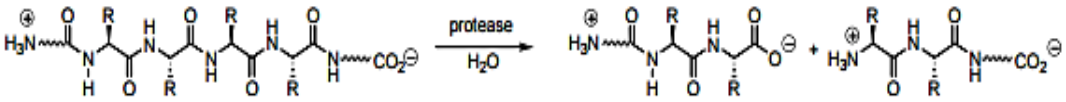

Fig. 1. The mechanism of protease hydrolysis (Source: Güracar, 2011)

2.1.1. Plant Proteases

Production of proteases from plants is limited by climatic conditions and the availability of land for cultivation, what makes production a time-consuming process. Also, processing of large amounts of plant material is necessary since the plant tissue contains low concentration of enzyme (Veloorvalappil et al., 2013). Papain, bromelain, keratinases, and ficin represent some of the well-known proteases of plant origin. The performance of the enzyme depends on the plant source, the climatic conditions for growth, and the methods used for its extraction and purification (Pandey et al., 2006). Plant proteases contain sulfhydryl groups in the active site which are responsible for the catalytic activity and used as meat tenderizers and in other food applications (Uhlig, 1998).

2.1.2. Animal Proteases

The main characteristic of animal proteases is their ability to occur as inactive precursor in producing organs until they are secreted into the digestive tract where they activate to the active form. The most familiar proteases of animal origin are pancreatic trypsin, chymotrypsin, pepsin, and rennin. However, availability of livestock for slaughter determinate their production (Pandey et al., 2006). They are used in the manufacture of protein hydrolysates, processing meat and fish

residues, in medicine as part of digestive medical aid preparations and also in leather manufacturing industry (Uhlig, 1998).

2.1.3. Microbial Proteases

Microorganisms are the most common sources of commercial enzymes due to their physiological and biochemical properties, wide biochemical diversity, ability of genetic manipulation, the rapid growth of the microorganisms and limited space required for cell cultivation. Among microbial enzymes, proteases are the most important for the industry, and constitute approximately 60% of the total industrial enzyme market (Rodarte et al., 2011).

Proteolytic enzymes not only play a critical role in cellular metabolic processes but have also gained considerable attention in the industrial community which leads to increasing interests in the study of proteolytic enzymes. In order to obtain commercial quantities, microbial strains that produce the desired enzyme are being isolated and characterized by using optimal conditions for growth. Proteases can be secreted inside or outside of cell. Intracellular proteases playing an important role in different cells and metabolism processes, while extracellular hydrolyze proteins in fermented media and enable the cell to absorb and utilize hydrolytic products (Veloorvalappil et al., 2013).

2.1.3.1. Bacterial proteases

Mostly, they are produced by genus *Bacillus* due to their vital importance in the industry (Priest, 1977). Bacterial proteases are mainly neutral and alkaline. The bacterial neutral proteases have low thermotolerance what represents advantageous in controlling their reactivity during the production of food hydrolysates with a low degree of hydrolysis. They are active in pH range 5-8, not sensitive to the natural plant proteinases and are, therefore, useful in the brewing industry. One of the main characteristics is their high affinity for hydrophobic amino acids. Bacterial alkaline proteases have wide substrate specificity, activity at alkaline pH (e.g. pH 10) and optimal temperature around 60°C what makes them suitable for use in the detergent industry (Mala et. al., 1998).

2.1.3.2. Fungal proteases

One of the many advantages of fungal proteases is the ability of fungi to grow on low cost materials and secret enzymes into the culture medium, making them easily recoverable (Anitha and Palanivelu, 2013). They are active over a wide pH range (pH 4 to 11), but have lower heat tolerance than the bacterial enzymes what makes them useful in the cheese-making industry. Fungal proteases are recognized as GRAS (generally regarded as safe) and are, therefore, safer for usage than bacteria (Germano et al., 2003).

2.2. Classification of proteases

Proteases are divided into three groups, intracellular, periplasmic, and extracellular proteases (Kohlmann et al., 1991). Proteases are of relevant importance in cellular, metabolic and regulatory processes (intracellular) and in the hydrolysis of proteins in cell-free environments (extracellular). Many factors influence production of intracellular or extracellular enzymes, such as pH, temperature, nitrogen and carbon sources, inorganic salts, and dissolved oxygen concentration (Gupta and Beg, 2002).

Depending on optimum pH, proteases are divided in acidic proteases, neutral proteases, alkaline proteases and high-alkaline proteases. Acidic proteases require alkaline conditions for their production and because of their catalytic nature they have wide range of application. They are found in animal cells, moulds, yeasts and rarely bacteria (Asokan, 2010). Neutral proteases comprise cystein proteases (papain, bromelain, ficin) isolated from botanical origin and the metalloproteases. They require neutral and low thermal conditions (Rao et al., 1998; Siddalingeshwara et. al., 2010).

Depending on their site of action, proteases are divided into two groups: exopeptidases or endopeptidases, according to the Enzyme Commission (EC) classification (García-Carreńo, 1993). If the peptide bond is cleaved closer to the amino or carboxy terminus of the substrate, enzymes are classified as exopeptidases. If the enzyme cleaves peptide bonds in the inner regions of peptide chains, distant from the termini, they are classified as endopeptidases (also known as proteinases) (Güracar, 2011).

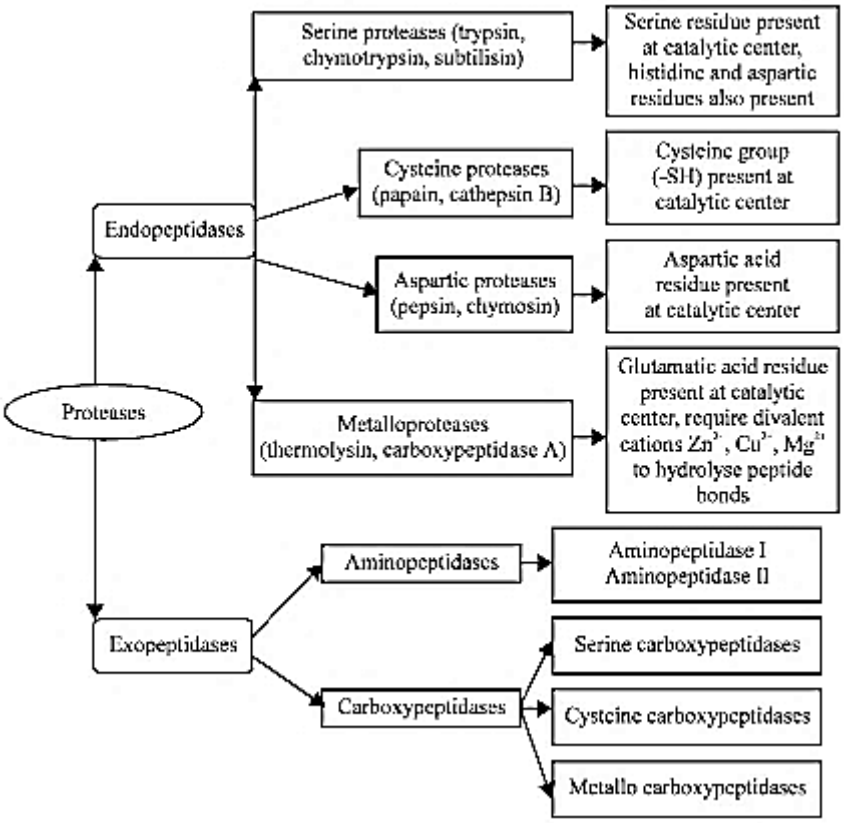

Fig. 2. The classification of proteases (Source: Kumar et al., 2008)

2.2.1. Endopeptidases (proteinases) (E.C. 3.4.21-99)

Endopeptidases hydrolyze peptide bonds in the inner regions of polypeptide chains, away from the N or C terminus (Polaina and MacCabe, 2007). They don't require presence of an unsubstituted N- or C-terminus in the substrate (Sterchi and Stöcker, 1999), on the contrary, the presence of the free amino or carboxyl group has a negative influence on enzyme activity (Wu, 2010). Based on their catalytic mechanism, the endopeptidases are divided on aspartic, serine, cysteine, threonine and metallo-endopeptidases (Rawlings and Barrett, 1993). The catalytic nucleophile in serine, threonine and cysteine peptidases is reactive group in the side chain of an amino acid, either a hydroxyl group in case of serine and threonine, or a sulfhydryl group in case of cysteine. The aspartic and metallopeptidases have the common characteristic that the nucleophile in catalysis is an activated water molecule. While the water molecule is directly bound by the side chains of aspartic residues, in metallopeptidases, one or two divalent metal (most commonly zinc) ions hold the water molecule in place (Polaina and MacCabe, 2007). Some endopeptidases act only on substrates smaller than proteins, and these are termed as

oligopeptidases. An example of an oligopeptidase is thimet oligopeptidase (Rawlings and Barrett, 1993). Endopeptidases initiate the digestion of food proteins, generating new N- and C-termini that are substrates for the exopeptidases that complete the process (Polaina and MacCabe, 2007).

2.2.1.1. Serine Proteases (E.C. 3.4.21)

Serine proteases are the most researched and understood group of proteolytic enzymes characterized by the essential serine residue that acts as a nucleophile in the active site. They are widely distributed and spread among viruses, bacteria and eukaryotes and can be found among exopeptidases, endopeptidases, oligopeptidases and omega peptidases (Güracar, 2011). Based on structure, they can be divided into a series of subfamilies. In comparison with other microorganisms, bacteria are the best known producers of serine proteases, where bacterial subtilisins have wide application as detergent additives (Maheshwari et al., 2000). The production of serine proteases usually occurs at neutral (pH 7) and alkaline (pH 11) conditions, while their isoelectric points rang between pH 4 and 6. They generally have low molecular weight between 18,5-35 kDa, and can be recognized by irreversible inhibition by di-isopropyl fluorophosphates (DFP) and phenyl methyl sulfonyl fluoride (PMSF) (Ellaiah et al., 2002).

2.2.1.2. Cysteine/Thiol Proteases (E.C. 3.4.22)

Cysteine proteases are characterized by the presence of a cysteine (SH-) and histidine residues at the active site (Garcia-Carreno, 1992) and produced by prokaryotes and eukaryotes where they occur in only a few fungi (Kalisz, 1988). They are divided into twenty families depending on the differences in the order of cysteine and histidine residues (Barett, 1994), and in four groups based on their side chain specificity: papain-like, trypsin-like, glutamic acid-like and others (Güracar, 2011). Cysteine proteases have neutral pH-optima and they are active only in the presence of reducing agents such as potassium cyanide or cysteine, EDTA or dithiothreitol (Maheshwari et al., 2000). They have molecular masses between 30 and 40 kDa, isoelectric points from pH 4,9 to pH 8,4 (Rao et al., 1998), and are inhibited by thiol reagents (heavy metals, alkylating-oxidizing agents), as well as by sulphydryl reagents (chloromercuribenzoate and iodoacetamide) (Zeigler, 2001).

2.2.1.3. Aspartic Proteases (Acidic Proteases) (E.C. 3.4.23)

Aspartic proteases are a group of proteases which contain an aspartic acid residue in their active sites. Acidic conditions are required for their production and they show maximum activity at acidic pH, between 3 and 4. Mostly, they are found in fungi, rarely in bacteria (Ellaiah et al., 2002). They can be divided into three groups, pepsin, retropepsin and enzymes from pararetroviruses (Güracar, 2011). Their molecular masses range between 30 and 40 kDa, while isoelectric points are between pH 3,4 and 4,6. Aspartic proteases are not sensitive to inhibitors of other three groups of enzymes, but are sensitive to diazo-ketone and epoxy compounds in the presence of copper cations, such as diazoacetyl norleucine methyl ester (DAN), and 1, 2-epoxypnitrophenoxy propane (EPNP) (Rao et al., 1998; Zeigler, 2001).

2.2.1.4. Metalloproteases (E.C. 3.4.24)

Metalloproteases require divalent metal ions (catalytic zinc, manganese, cobalt, nickel or copper) for biological activity. The metal ion is complexed by three conserved amino acid residues that can be glutamic acid (Glu), aspartic acid (Asp), histidin (His) or lysin (Lys) in their active sites. They are widespread and the most diverse of the catalytic types of proteases (Barett, 1995). Zinc is essential for their activity (one atom of zinc per molecule of enzyme), while calcium is important for structure stability. According to the amino acid sequences and the relation between the amino acids and the metal binding sites, they are divided into thirty families. According to the catalytic action they are neutral, alkaline, Myxobacter I and Myxobacter II (Ellaiah et al., 2002). Metalloproteases have maximum activity at neutral to alkaline pH, in the range of 5-9, with molecular masses between 19 and 37 kDa They are sensitive to metal-chelating reagents, for example EDTA (Ziegler, 2001).

2.2.2. Exopeptidases (peptidases) (E.C. 3.4.11-19)

Exopeptidases are the enzymes which cleave peptide bonds in the terminal amino end or carboxylic end of the substrate (Garcia-Carreno, 1992). They remove single amino acid, dipeptide or tripeptide from the N- or C-terminus. The exopeptidases that act at a free N-terminus are named aminopeptidases. They are generally intracellular enzymes, widespread among microbial species. Exopeptidases acting at a free C-terminus are called carboxypeptidases. Based on the nature of the amino acid residues at the active site of the proteases, they are divided into serine, metal and cistein carboxypeptidases (Mekashwari et al., 2010).

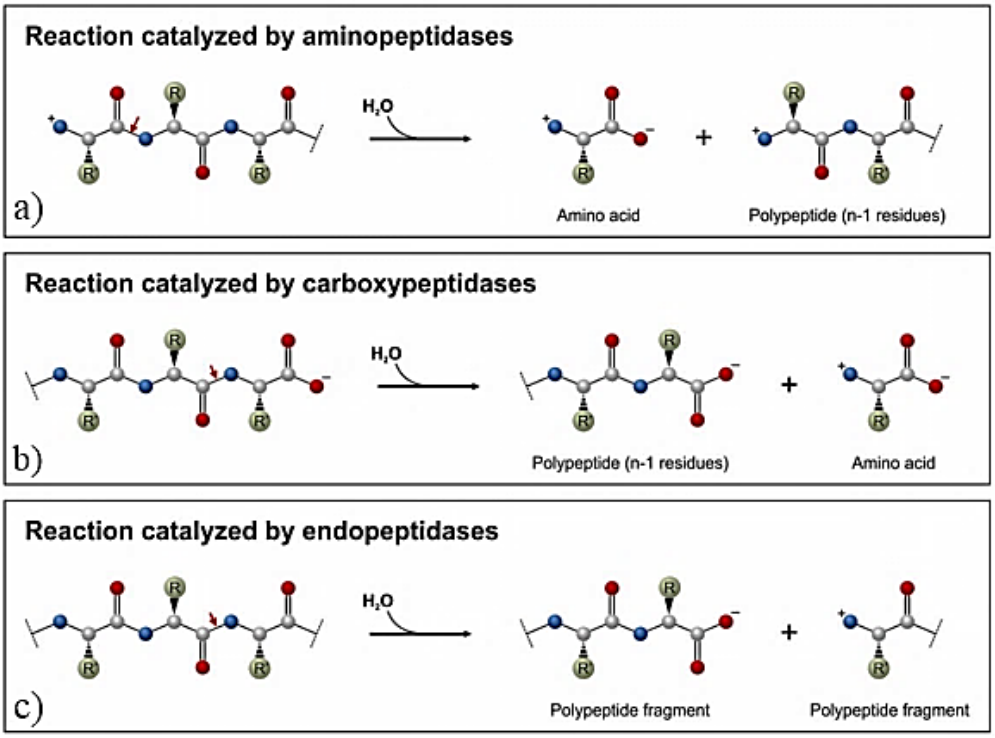

Fig. 3. Action of aminopeptidases and carboxypeptidases removing the terminal amino acid residues as well as endopeptidases on a polypeptide substrate: (**a**) reaction catalyzed by aminopeptidases; (**b**) reaction catalyzed by carboxypeptidases; (**c**) reaction catalyzed by endopeptidases (Source: Mótyán et al., 2013)

2.3. Industrial Applications of Proteases

Proteases are commercial enzymes and have a large variety of applications playing irreplaceable role in industrial biotechnology, especially in detergent and food industries. 75% of the commercial enzymes are hydrolase enzymes. In the industrial enzyme, the protease enzymes from the hydrolytic enzymes are the most important groups, accounting for nearly 60% of the total enzymes in the world (Rao et al., 1998).

Table 1. Application of proteases in industry (Source: Kumar and Savitri, 2008)

Industry	Protease	Application
Baking	Neutral protease	Dough conditioner
Beverage	Papain	Chill proofing, removal of haze in beverages
Dairy	Fungal proteases, chymosin, other proteases	Replacement of calf rennet, whey protein processing, production of enzyme modified cheese (EMC)
Detergent	Alkaline protease, subtilisin	Laundry detergents for protein stain removal
Food processing	Several proteases	Modification of protein rich material i.e., soy protein or wheat gluten
Leather	Trypsin, other proteases	Bathing of leather, dehairing of skin
Meat and fish	Papain, other proteases	Meat tenderization, recovery of protein from bones and fish waste
Medicine	Trypsin	Dead tissue removal, blood clot dissolution
Photography	Several proteases	Recovery of silver from used X-ray and photographic films
Sweetener	Thermolysin	Reverse hydrolysis in aspartame synthesis

2.3.1. Detergent Industry

Proteases are one of the standard ingredients of all kinds of detergents. The use of proteases in laundry detergents accounts for approximately 25% of the total worldwide sales of enzymes. The cleaning mechanism is based on forming complex structure with detergents and the hydrolysis of the proteins in stains (Anwar and Saleemuddin, 1998; Beg and Gupta, 2003). In the detergent industry, activity and stability at high pH and temperature and compatibility with other chelating and oxidizing agents is necessary for their usage. Proteases are most suitable for this application when the pI of the enzyme is similar to the pH of the detergent (Rao et al., 1998). The serine proteases produced by *Bacillus* strains *subtilis* are generally used in the laundry detergent technology (Fogarty et al., 1974).

2.3.2. Food Industry

The food industries are the major protease using industries. They have been routinely used for various purposes such as cheese making, baking, preparation of soya hydrolysates, and meat tenderization (Cheong et al., 1993).

2.3.2.1. Dairy industry

The major application of proteases in the dairy industry is in the manufacture of cheese. The proteases are used as milk-coagulation agents for cheese production. Advantages of using proteases in dairy industry is ability of staying at higher temperatures in the pasteurization and obtaining high quality of milk in the dairy industry (Meer et al., 1991).

2.3.2.2. Baking industry

Endo- and exoproteinases from *Aspergillus oryzae* have been used to modify wheat gluten by limited proteolysis. Enzymatic treatment of the dough improves handling and machining and permits the production of a wider range of products. The addition of proteases reduces the mixing time, improve the extensibility and strength of the dough (Argos, 1987).

2.3.3. Other applications

Proteases from the *Bacillus* species are generally used in brewing industry in order to cleave peptide bonds in proteins. In the leather industry they are used instead of hazardous chemicals (sodium sulfide) to prevent the pollution problems, obtain high quality, easy control, speed up dehairing and reduce the waste materials. The usage of these enzymes is also common in developing therapeutic agents, preparation of medicines, medical diagnosis, biopharmaceutical products (contact-lens enzyme cleaners, enzymatic debriders) and cosmetics (skin care ointments) (Anwar and Saleemuddin, 2000).

2.4. Bacteria and yeasts in cheese

Microorganisms are an essential component of all natural cheese varieties as the major contributor to cheese flavor, aroma, texture, and appearance (Beresford et al., 2001; Irlinger and Mounier, 2009; Ogier et al., 2002). The variety and number of specific types of organisms present in cheese depend on the microbial quality of the milk, heat treatment of the milk, manufacturing

conditions, temperature and humidity during ripening, amount of salting, and exposure of the cheese to exogenous microorganisms during and after manufacture (Torkar and Teger, 2006).

In cheese making, two types of cultures are used. The primary cultures include all the starter lactic acid bacteria responsible for acid production during manufacture and in cheese ripening. The secondary cultures do not contribute to acid production during manufacture, but generally play a significant role during ripening. The secondary culture is composed of non-starter lactic acid bacteria which grow internally in most cheese varieties and other bacteria, yeasts and molds, which grow internally or externally and are usually unique to specific cheese types. During cheese ripening, the starter culture, along with the secondary flora promotes a complex series of biochemical reactions which are vital for proper development of flavor and texture (Beresford et al., 2001).

During ripening, proteolysis in cheese is catalyzed by enzymes from coagulant (e.g., chymosin, pepsin, microbial or plant acid proteinases), milk (plasmin), enzymes from the starter, nonstarter, or secondary cultures and exogenous proteinases or peptidases (fig 4). Proteolysis contributes to the taste of cheese by the production of peptides and free amino acids. Large peptides do not contribute directly to cheese flavor, but are important for the development of the correct texture. Engels and Visser (1994) analyzed water-soluble fractions from Cheddar, Edam, Gouda, Gruyere, Maasdam, Parmesan and Proosdij cheeses and suggested that low-molecular compounds (small peptides, amino acids, free fatty acids or their breakdown products) were responsible for the basic taste of cheese.

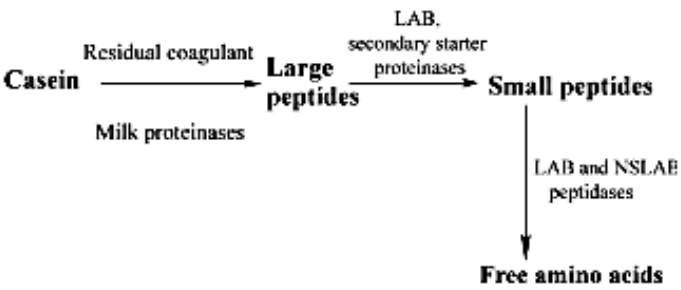

Fig. 4. Proteolytic agents in cheese during ripening (Source: Sousa et al., 2001)

The starter is not only responsible for acid metabolism but also for metabolic activities in sensory properties of cheese. They are often used in cheese because of their proteolytic characteristics which contributes flavor development. However, compared to yeasts and molds, starter bacteria are very weakly proteolytic (Donnelly, 2014). The starter cultures commonly used in cheese manufacture include mesophilic *Lactococcus* and *Leuconostoc* species, thermophilic *Lactobacillus* species and *Streptococcus thermophilus*. Although lactic acid bacteria (LAB) are weakly proteolytic, they possess proteinase/peptidase system capable of hydrolyzing oligopeptides to small peptides and amino acids (Pederson and Steele, 1999).

The secondary and adjunct cultures include yeasts (e.g., *Geotrichum candidum*), moulds (e.g., *Penicilium camemberti*) and bacteria (e.g. *Micrococcus*), and grow mainly on the cheese surface. Yeasts are used mainly in mould and bacterial surface-ripened cheeses because they promote the growth of other microorganisms. They colonise numerous cheeses, particulary their surface and show a large diversity in proteolytic activity between species and strains of the same species. They have caseinolytic, aminopeptidase and carboxypeptidase activities (Fox et al., 2004).

Bacteria cause fermentation of milk and are therefore considered to be of major importance during cheese making (Cousin, 1982). Yeasts, however, possess the ability to grow under conditions unfavorable to many bacteria and play an important role in the spoilage of dairy products (Fleet and Mian, 1987; Seiler and Busse, 1990). Role of yeasts as spoilage organisms is related to their nutritional requirements, growth at low temperatures, low pH values, low water activities and high salt concentration. However, in some cases yeasts may contribute positively to the fermentation and maturation process of cheeses by inhibiting undesired microorganisms, supporting the function of the starter culture by proteolytic activity, lipolytic activity and metabolizing lactic acid leading to an increase in pH (Welthagen and Viljoen, 1998).

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. Cheese samples for isolation

For the purpose of this work, two types of Feta cheese made from sheep and goat milk were used. They were originated from a Újpest Marketplace in Budapest, Hungary. Cheeses were refrigerated at 4ºC.

3.1.2. Growth media and solutions

Different media and solutions were sterilized at 121 °C for 15 minutes (if otherwise not stated). Media with agar were cooled down to 40-50 °C in water bath and were poured in sterile Petri dishes (20-25 ml into each dish). Media were left to solidify on flat surface and after solidification they were turned upside down to prevent condense water forming. Isolated strains were maintained on agar slops and stored at 4 ºC.

Main components of growth media are **peptone** (provides nitrogen, vitamins, minerals and amino acids essential for growth), **glucose** (fermentable carbohydrate providing carbon and energy), **yeast extract** (source of vitamins, particularly of the B-group; minerals) and **bacteriological agar** (solidifying agent).

3.1.2.1. RBC (Rose Bengal Chloramphenicol) agar

RBC agar was used for selective isolation and enumeration of yeasts. Chloramphenicol is used as the selective agent to suppress most bacteria. Pre-mixed formula (table 2) containing 5 g L^{-1} peptone, 10 g L^{-1} glucose, 1 g L^{-1} dipotassium phosphate, 0,5 g L^{-1} magnesium sulphate, 0,05 g L^{-1} rose-bengal, 15,5 g L^{-1} agar was used where 32,2 grams of the powder has to be dissolved in one liter of double distilled water.

Table 2. Composition of RBC agar for selective isolation and enumeration of yeasts

Ingredients	Concentration [g L^{-1}]
Mycological peptone	5,0
Glucose	10,0
Dipotassium phosphate	1,0
Magnesium sulphate	0,5
Rose-Bengal	0,05
Chloramphenicol	0,1
Agar	15,5
pH 7,2 ± 0,2 (at 25°C)	

3.1.2.2. Milk PC (Milk Plate Count) agar

Milk PC agar was used for selective isolation and enumeration of bacteria in milk and dairy products. The nutrients supplied by antibiotic free skim milk and peptone, vitamins from yeast extract and glucose used as energy source, favor the growth of most bacteria. Pre-mixed formula (table 3) containing 5 g L^{-1} peptone, 2,5 g L^{-1} yeast extract, 1 g L^{-1} glucose, 1 g L^{-1} antibiotic free skim milk, 10 g L^{-1} agar was used where 19,95 grams of the powder has to be dissolved in one liter of double distilled water.

Table 3. Composition of Milk PC agar used for selective isolation and enumeration of bacteria

Ingredients	Concentration [g L^{-1}]
Peptone	5,0
Yeast extract	2,5
Glucose	1,0
Antibiotic free skim milk	1,0
Agar	10,0
pH 6,9 ± 0,1 (at 25°C)	

3.1.2.3. WL Nutrient Agar

WL Nutrient agar contains a variety of substances to promote the growth and selection of bacteria and yeast. Monopotassium phosphate is the buffer. Potassium, calcium and ferric chlorides all provide essential ions for the osmotic balance. Magnesium and manganese sulfates are sources of divalent cations. Bromocresol green is the pH indicator. Ingredients (table 4) have to be dissolved in one liter of double distilled water.

Table 4. Composition of WL Nutrient Agar for preparation of pure cultures

Ingredients	**Concentration [g L^{-1}]**
Yeast extract	4,0
Peptone	5,0
Glucose	50,0
Potassium dihydrogen phosphate	0,55
Potassium chloride	0,425
Calcium chloride	0,125
Ferric chloride	0,0025
Magnesium sulphate	0,125
Manganese sulphate	0,0025
Bromocresol green	0,022
Agar	15,0
pH 5,5 ± 0,2 (at 25°C)	

3.1.2.4. YEPD (Yeast Extract Peptone Dextrose) and TGE (Tryptone Glucose Extract) agar

YEPD agar was used for the maintenance, detecting and propagation of yeasts, while TGE agar for bacteria in milk and other dairy product. Ingredients (table 5) have to be dissolved in one liter of double distilled water.

Table 5. Composition of YEPD and TGE agar for yeast and bacteria growth

Ingredients	Media	Concentration [g L^{-1}]
Yeast extract	YEPD/TGE	5,0
Peptone	YEPD	5,0
	TGE	2,5
Glucose	YEPD	10,0
	TGE	1,0
Agar	YEPD/TGE	15,0
pH 6,2 ± 0,2 (at 25°C)		

3.1.2.5. SM (Skim Milk) agar

Skim Milk agar is used for cultivation and differentiation of microorganism based on proteolysis of casein. Addition of skim milk in the medium makes the conditions optimal for microorganisms encountered in milk. Sodium chloride is added in medium in case of testing two strains. Ingredients (table 6) have to be dissolved in one liter of double distilled water.

Table 6. Composition of SM (Skim Milk) agar for testing proteolytic activity

Ingredients	Concentration [g L^{-1}]
Yeast extract	2,5
Peptone	5,0
Glucose	1,0
Agar	15,0
Skim milk powder	10,0
*Sodium chloride	0,95
pH 7,0 ± 0,2 (at 25°C)	

3.1.2.6. MM (Minimal Media)

Minimal Media contains the essentials for bacterial species to grow. For testing bacteria proteolytic activity in different growth media, beside Minimal Media, other modified Minimal Media were used. All ingredients are specified in Table 7 and dissolved in one liter of double distilled water.

Table 7. Composition of different Minimal Media for testing proteolytic activity of bacteria

Media	**Ingredients**	**Concentration [g L^{-1}]**
MM	Ammonium sulfate	5,0
MM/Modified MM1/Modified MM2	Monopotassium phosphate	1,0
	Magnesium Sulphate Heptahydrate	0,5
	Glucose	1,0
	Vitamin	1 mL
MM1/MM2	Sodium caseinate	0,1
MM2	Sodium chloride	20
pH 7,40 ± 0,2 (at 25°C)		

3.1.2.7. Urea R Broth (Urea Rapid Broth)

Urea R Broth is a highly sensitive medium used for the rapid detection of urease activity by microorganisms. Monopotassium phosphate and disodium phosphate are used in very low concentrations in order to detect the smallest amount of alkali, yeast extract acts as a source of nutrients, carbon, and nitrogen for those organisms incapable of using ammonia as a nitrogen source. Phenol red is used as the pH indicator, changing from an orange-red to a bright pink.

Table 8. Composition of Urea R Broth (Urea Rapid Broth) media for the detection of urease activity

Ingredients	Concentration [g L^{-1}]
Urea	20
Yeast extract	0,1
Disodium Phosphate	0,095
Monopotassium Phosphate	0,091
Phenol red	0,01
pH 6,9 ± 0,2 (at 25°C)	

3.1.2.8. Urea broth base

Urea Broth Base used for the detection of urease producing microorganisms. The peptone provides the required nitrogen, carbon and vitamins. Glucose is a fermentable carbohydrate. The disodium phosphate and potassium dihydrogen phosphate are buffers, and sodium chloride maintains osmotic balance. Phenol red is a pH indicator.

Table 9. Composition of Urea broth base for testing the urease activity

Ingredients	Concentration [g L^{-1}]
Peptone	1,0
Glucose	1,0
Sodium chloride	5,0
Disodium phosphate	1,2
Potassium dihydrogen phosphate	0,8
Phenol red	0,012
∗Urea solution (40% w/v)	50,0 mL
pH 6,8 ± 0,2 (at 25 °C)	

3.1.3. Solutions used for serial dilution and sample processing

Solution used for serial dilution and sample processing contained 1,0 g L^{-1} casein peptone and 8,5 g L^{-1} NaCl. For serial dilution 9 ml of prepared solution was distributed into tubes before sterilization.

3.1.4. Chemicals and components for growth media and solutions

All used chemicals were of the laboratory grade of purity.

3.1.5. Equipment and apparatus

3.1.5.1. Densitometer

McFarland Densitometer DEN-1B (bioSan, Latvia) (suspension turbidity meter) was used for measuring turbidity of cell suspensions. It is calibrated to measure turbidity in the range of 0,5 to 4,0 McFarland units (0,5 McFarland Standard for bacteria and 2,5 McFarland Standard for yeasts) with a small standard deviation.

3.1.5.2. Laboratory centrifuge

Laboratory centrifuge Jouan BR4i (Thermo Scientific Labor, Hungary) was used for preparing extracellular cells samples for testing proteolytic activity of strains in different media and for spectrophotometric measurements.

3.1.5.3. Spectrophotometer and Multiskan machine

Using UV/Vis Spectrophotometer SPECORD 200 PLUS (Analytik Jena, Germany) apsorbance at 280 and 366 nm were determined for measuring concentration of proteins. Thermo Multiskan Ascent is a microplate photometer (Labexchange, Germany) used for measuring concentrations of proteins in different growth media chapter.

3.1.6. Other equipment and apparatus

Table 10 shows the list of equipment and apparatus used in this research. Also, other equipment was used, including basic laboratory equipment, Erlenmeyer flask, Eppendorf tubes,

inoculation loop, test tubes, microscope slides, Petri dishes, Stomacher bag, filter papers, burners, fridge for storage of chemicals and strains.

Table 10. Other equipment and apparatus

Equipment	Company
BagMixer	INTERSCIENCE, France
Autoclave	Horizontal Benchtop, UniEquip, Germany
pH Meter	Orion Star A111, Thermo Fisher Scientific Inc, USA
Light microscope	Olympus BH2-RFCA, Hungary
Olympus DP-10 Microscope Digital Camera	Olympus America Inc., USA
Vortex	IKA Vortex 1, China
Analytical balance	SCALTEC, Germany
Technical balance	Kern EW, Sigma-Aldrich, Germany

3.2. Methods

3.2.1. Processing of cheese samples

10 grams of cheese samples (sheep cheese, goat cheese) and 90 ml of solution were measured into a Stomacher bag. Bags were homogenized for 120 seconds using BagMixer. From homogenized samples a decimal dilution was prepared and 0,1 ml from dilutions was spread on the surface of different agar plates. RBC agar was used for selective isolation and enumeration of yeasts while Milk PC was used for bacteria. RBC and Milk PC plates were incubated for 48 hours at 25 °C and 30 °C respectively.

3.2.2. Preparation of pure cultures and characterization of isolates

Single colonies from RBC and Milk PC agar were streaked using a loop on WL nutrient media to obtain single colonies. Plates were incubated for 3 days at 25 °C.

3.2.3. Strain maintenance

Yeast and bacterial isolates were inoculated on YEPD and TGE slopes (5 ml of agar was distributed into tubes before sterilization). After autoclaving, agar in tubes was left to solidify slanting the tubes to obtain agar slopes, respectively and incubated at 25 °C. After growth, slopes were stored at 4 °C.

3.2.4. Microscopic investigation of isolates

3.2.4.1. Preparation of native dissection in case of yeast isolates

Yeast suspensions were prepared from one day old yeast cultures (one loopful of cells was transferred into 0,5 ml sterile double distilled water) from which 10 µl were pipetted onto microscope slides. Cover-slides were laid on the smears and investigated under light-microscope.

3.2.4.2. Gram-staining of bacterial isolates

Smears were prepared on microscope slides using 10 µl of sterile double distilled water and a small amount of one day old bacteria colonies. Smears were air dried and heat fixed before staining. Cells were stained with crystal violet dye for two minutes and rinsed with distilled water. Lugol solution (iodine and potassium iodide) was added as mordant. After one minute slides were rinsed with distilled water. Ethanol was dropped onto tilted slides for a few seconds to decolorize Gram-negative bacteria. After differentiation slides were rinsed with distilled water immediately. Safranin was used for counterstaining. Cells were stained for two minutes and rinsed with distilled water. Slides were air dried and investigated by light-microscope.

3.2.5. KOH test

One drop of 3% KOH was pipetted onto a microscope slide. A loopful of one day old bacteria culture was transferred into the KOH drop and stirred. Microbiological loop was raised up and down to detect mucoid string formed by Gram-negative strains.

3.2.6. Testing the growth of isolates at different temperatures

Cell suspensions were prepared from one day old yeast and bacteria cultures (one loopful of cells were transferred into 0,5 ml sterile double distilled water) from which 10 µl was pipetted onto YEPD and TGE agar plates, respectively. Plates were incubated for three days at 10 ºC, 15 º C, 20 ºC, 25 ºC, 30 ºC and 37 ºC.

3.2.7. Testing the proteolytic activity of isolates on SM Agar

Cell suspensions with adjusted density (0,5 McFarland Standard for bacteria and 2,5 McFarland Standard for yeasts) were prepared from one day old yeast and bacteria cultures. 10 µl of each strain sample was inoculated on SM Agar plates. Plates were incubated for 3 days on 25 ºC.

3.2.8. Extracellular proteolytic activity of strains in different media

Strains who showed proteolytic activity were inoculated in different growth media to test their proteolytic activity after 24, 48 and 72 h of incubation at 25 ºC in rotator. Media used for this experiment were YEPD, YEPD+2% NaCl, MM, MM+2% NaCl, mMM1 and mMM2. 100 µl of one day old cell suspension with adjusted density was incubated in 3 ml of each media. First, second and third day 500 µl from each tube was mixed with 5 µl gentamicin and centrifuged at 14000 rpm, 5 min. 100 µl of supernatant was distributed in drilled circle holes in SM agar and incubated at 25 ºC 72 h.

3.2.9. Multi scanning analysis

Cell suspensions of S-5a and S-5b bacteria strains with adjusted density were prepared from one day old bacteria cultures and inoculated in TGE media with different concentration of NaCl (0%, 0,5%, 1%, 1,5%, 2%) to test effect of NaCl on bacteria growth ability. 100 µl of bacteria, 400 µl double distilled water and 500 µl TGE media with different concentration of

NaCl were prepared in Eppendorf tubes from which 300 µl was pipetted in kits and putted on multi scanning analysis for 48 h.

3.2.10. Influence of NaCl on proteolytic activity of S-5b strain

One day old cell suspension of S-5b with adjusted density was inoculated in five tubes with TGE media containing different concentration of NaCl (0%, 0,5%, 1%, 1,5%, 2%) and incubated 24 h in rotator at 25º. 700 µl from each tube was distributed in Eppendorf tubes and centrifuged at 14 000 rpm for 5 min after adding 7 µl of gentamicin. 100 µl of supernatant was distributed in drilled circle holes in SM agar plates containing different concentration of NaCl (0%, 0,5%, 1%, 1,5%, 2%). SM agar plates were incubated 48 h at 25 ºC.

3.2.11. Urea R Broth test

Components were added to distilled water and mixed thoroughly. Media was filtered and distributed to Eppendorf tubes. One loop of one day old yeasts cells were aseptically distributed into tubes. After incubation at 35 ºC, change of color in inoculated media was checked after 6 to 8 and 12 to 18 hours.

3.2.12. Urea Broth Base

Components were suspended in distilled water and sterilized by autoclaving for 15 min at 121°C. 40% sterile urea solution was added to media and mixed. 2 mL of prepared media was distributed in tubes and inoculated with one loop of one day old yeasts cells while one tube was left as control sample (without yeast cells). Tubes were incubated 24 h at 25°C in rotator. The release of ammonia during urea hydrolysis will change the pH of the medium to alkaline, converting the light-orange phenol red indicator to pink.

3.2.13. Catalase test and Oxidase test

Catalase test demonstrates the presence of catalase, an enzyme that catalyzes the release of oxygen from hydrogen peroxide (H_2O_2). Small amount of one day old bacteria culture was transferred with toothpick to 20 µl of 3% H_2O_2 on the surface of glass slide and mixed. The presence of the enzyme in a bacterial isolate is evident when a small inoculum is introduced into

hydrogen peroxide, and the rapid elaboration of oxygen bubbles occurs. The lack of catalase is evident by a lack or weak bubble production.

The oxidase test is used to determine if an organism possesses the cytochrome c oxidase enzyme. Small amount of one day old bacteria culture was rubbed with toothpick on 10 µl of Kovacs Oxidase Reagent (5% tetra-methyl-p-phenylenediamine dihydrochloride) dropped on filter paper. Organisms which contain cytochrome c as part of their respiratory chain are oxidase-positive and turn the reagent blue/purple appearing within 5-10 seconds. When the enzyme is not present, the reagent remains reduced and is colorless.

3.2.14. Quantification of produced extracellular enzyme with spectrophotometric measurement

3.2.14.1. Shaked and static bacterial cultures

Absorbance was measured with two different methods. Six bacteria strains (G-1, G-2b, G-3, G-4, S-5b, S-7) were inoculated in 6 Erlenmeyer flask containing YEPD medium. Flasks were incubated at 25 °C with and without slow rotation. After incubation, 1 mL from each culture was centrifuged at 14000 rpm for 5 min and supernatant was used for measuring in spectrophotometer. Absorbance was measured after 24, 48 and 72 h.

- *Method 1 – Sodium caseinat*

1% casein sodium salt was prepared with 50mM Sorensen's phosphate buffer (pH 7), 250µl of each supernatant was added in 500µl of casein solution and incubated for two hours at 30°C. Reaction was stopped with 375µl TCA (20% trichloroacetic acid). The tubes were placed in an ice bath for 30 minutes and then centrifuged at 6000 rpm for 15 minutes at 4 °C. Blank samples were prepared for each strain using 500µl of casein solution, 250µl of supernatant and 375µl TCA without previous incubation. Blank samples were placed in an ice bath for 30 minutes. Absorbance was measured at 280nm.

- *Method 2 – Azocasein*

0,5% azocasein was dissolved in 0,1mol citrate buffer (pH 6) by boiling and then filtered. 100µl of culture supernatant was added to 500µl azocasein dissolvent and 500µl of citrate buffer.

After incubation at 30 ºC for 30 min, the reaction was stopped with 1,1 ml 10% TCA and left on ice for 15 min. The samples were centrifuged at 6000 rpm 15 min and the absorbance of the supernatant was measured at 366 nm in a spectrophotometer.

3.2.14.2. Different temperatures and pH values on enzyme activity

- For testing effect of different temperatures, bacterial cultures were incubated at 25, 30 and 37 ºC for 2 h.
- For testing effect of pH value on enzyme activity, three types of buffers were used:

pH 5 ⇒ pH 3,0-6,2, pk_a=6,40

Stocks solutions:

A: 0,1 M solution of citric acid; B: 0,1 M solution of sodium citrate

x mL of A + y mL of B, diluted to a total of 100 mL

Table 11. Composition of citrate buffer

0,1 M citric acid [mL]	0,1 M sodium citrate [mL]	pH
20,5	29,5	**5,0**

pH 7 ⇒ Sørensen's phosphate buffer; pH 5,8–8,0, pk_a = 7,20

Stocks solutions:

A: 0,2 M Monosodium phosphate; B: 0,2 M Disodium phosphate

Table 12. Composition of Sørensen's phosphate buffer

0,2 M monosodium phosphate [mL]	0,2 M disodium phosphate [mL]	pH
39,0	61,0	**7,0**

pH 9 ⇒ Tris-HCl buffer; pH 7,0- 9,2; pk_a = 8,0

Stocks solutions: 0,2 M Tris (hydroxymethyl) aminomethane (Tris base) and HCl for adjusting pH value

4. RESULTS AND DISCUSSION

4.1. Enumeration of microbes in different cheeses

RBC and Milk PC agar were used for selective isolation and enumeration of yeast and bacteria in milk and dairy products. Enumeration was performed on RBC and Milk PC agar plates (fig 5), respectively. From sheep cheese two samples (cheese crust, cheese inside), while from goat cheese three samples (cheese crust, cheese inside, yellow coloured cheese side) were prepared. Yeasts could be counted only from sheep cheese crust sample, while bacteria from all samples including samples from RBC media in case of chloramphenicol resistant bacteria. Table 13 shows counted colonies expressed as cfu/g (Colony-Forming-Unit per gram). To compare amount of counted bacterial colonies, considering different parts of cheese samples, more isolated bacterial colonies were counted on sheep crust part ($7,7x10^7$ CFU/g), while in goat cheese in inside part ($1,53x10^9$ cfu/g).

Table 13. Yeast and bacteria count in different cheeses

Sample	Yeast count on RBC media [CFU/g]	Bacteria count on Milk PC agar [CFU/g]
Sheep cheese crust	$7,2x10^4$	$7,1x10^7$
Sheep cheese, inside part	no yeasts	$4,9x10^6$
Goat cheese crust	no yeasts, but chloramphenicol resistant bacteria ($5,56x10^7$)	$4,1x10^8$
Goat cheese, inside part	no yeasts, but chloramphenicol resistant bacteria ($8,2x10^6$)	$1,53x10^9$
Goat cheese, yellow coloured part	no yeasts, but chloramphenicol resistant bacteria ($1,15x10^7$)	$7,7x10^8$

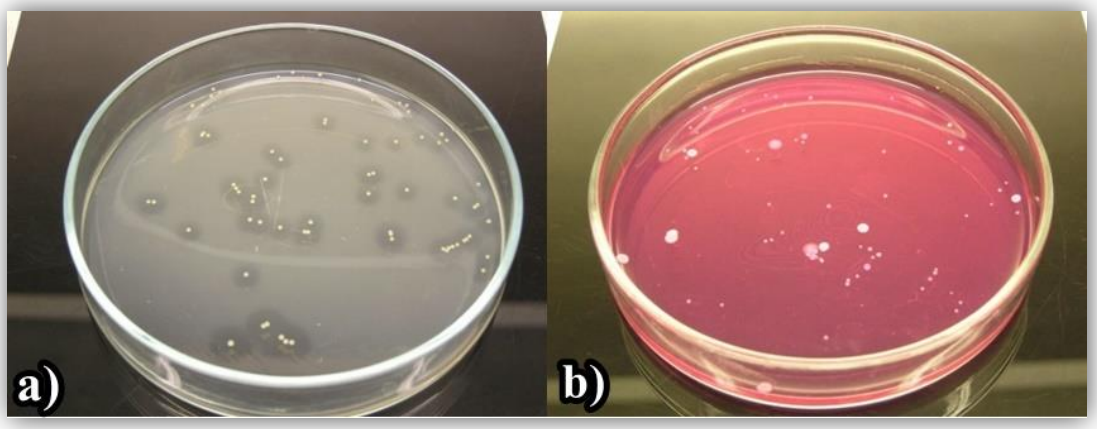

Fig. 5. Grown bacteria and yeast colonies: (**a**) Milk PC agar: Sheep outside part; (**b**) RBC agar: Sheep outside part

Yeasts colonise numerous cheeses, particularly their surfaces and they can grow during early stages of cheese making. Generally, their number in the interior of the cheese is 100 or 1000 times lower. The yeasts encountered on the surface of cheese show varied abilities to metabolise sugars, lactate and citrate (Eliskases-Lechner and Ginzinger, 1995). Yeasts can make contributions to the flavor, taste and appearance; however, some species can benefit one type of cheese while spoiling another. Particular yeasts on the surface of the cheese can cause spoilage or generate undesirable aromas, flavors, or other metabolic products that reduce the quality of cheese (Atanassova et al., 2003). It is not unusual to find yeast counts of approximately 10^5–10^6 cfu/g and, in some varieties, counts as high as 10^7–10^8 cfu/g were reported (Fleet, 1990). Yeasts also show a large diversity in proteolytic activity between species and strains of the same species. Banjara *et al.* (2015) were investigating diversity of yeast and mold species from a variety of cheese types. Eight different yeasts were isolated from 39 types of cheese. *Debaryomyces hansenii* was the most abundant species and was present in 79 % of all cheeses. The second most frequently isolated yeast species was *Galactomyces candidus*, present in 13 % of all cheeses.

Starter bacteria are either added deliberately at the beginning of manufacture or may be natural contaminants of the milk, as is the case in many traditional handmade cheese varieties made from raw milk. They grow during manufacture and typically reach densities of 10^8 cfu/g within hours of the beginning of manufacture; approximately 10^8 –10^9 cfu/g in most cheese varieties but levels decrease during ripening due to the hydrolytic activity of their own autolysin

enzymes (Thomas and Batt, 1969). The primary function of starter bacteria in cheese is to produce acid during the fermentation process; however, they also contribute to cheese ripening while their enzymes are involved in proteolysis and conversion of amino acids into flavor compounds (Fox and Wallace, 1997).

The predominant surface microflora of fresh feta cheese is composed of LAB, yeasts, and salt-tolerant microbes. *Leuconostoc*, *Lactococcus lactis*, and *Lactobacillus plantarum* constitue the predominant LAB microflora develop in the beginning of ripening, while at the end the LAB microflora is composed of lactobacilli, with *L. plantarum* being the predominant species (Fang and O'Toole, 2009). The predominant yeasts species in the beginning of ripening are *Saccharomyces cerevisiae* and *Debaryomyces hansenii*, while at the end of ripening at room temperature, *Saccharomyces cerevisiae* predominate (Raes and Bork, 2008). The halotolerant surface microflora is composed of staphylococci, micrococci, enterococci, and coryneform bacteria. *Staphylococcus saprohyticus* predominates the halotolerant bacteria content on the surface of cheese in the beginning, while coryneforms in further stages of ripening. As indicated by their enzyme systems, lactobacilli of the cheese surface may affect proteolysis, yeasts participate in both proteolysis and lipolysis, and staphylococci contribute to cheese lipolysis. Lactococci are the most frequently group among the NSLAB found in the fresh cheese, with *L. Lactis* subsp. *lactis* predominating. Lactobacilli are also isolated more frequently than enterococii, and leuconostocs are equal to lactobacilli (Bassler, 2002).

4.2. Preparation of pure cultures and investigation of colony morphology on WL Nutrient Agar

Colonies showing different colony morphology on RBC and Skim PC were streak-plated on WL Nutrient agar to separate cells so that discrete colonies could be isolated. Altogether 17 colonies (4 yeasts and 13 bacteria) were isolated. Yeasts could be isolated only from sheep cheese crust. The observed colony morphology and the strain codes used later are listed in Table 14. Out of 12 bacteria two could not grow on WL Nutrient agar and another two showed weak growth, therefore these isolates were streak-plated on TGE agar. Colony morphology including colour, shape and surface is described in Table 14.

Table 14. Colony morphology of isolates on WL Nutrient and/or TGE agar and strain codes

Sample	Used media for isolation	Appearance of colonies on WL Nutrient agar	Shape	Surface	Colony morphology on TGE agar	Strain code	
		Colour					
Sheep cheese crust	RBC	white	round	lined	-	**S-1**	**YEASTS**
		light yellow; brighter middle	irregular	rough	-	**S-2**	
		light greenish	rounded	shiny	-	**S-2**	
		white to light greenish	rounded	shiny and smooth	-	**S-4**	
	Milk PC	light yellow	rounded	shiny	yellow	**S-5a**	**BACTERIA**
		yellowish	rounded	smooth	yellow	**S-5b**	
Sheep cheese, inside part	Milk PC	-	rounded	rough	yellowish	**S-6**	
		-	rounded	shiny and smooth	white to light yellow	**S-7**	
		light green	rounded	shiny	-	**S-8**	
		green to bluish (green edge)	rounded	rough	-	**S-9**	
Goat cheese crust	Milk PC	white to light bluish	rounded	shiny and smooth	-	**G-1**	
		light blue to greenish (with darker circle in the middle)	rounded	rough	-	**G-2a**	
		light blue to greenish with lighter middle	rounded	rough	-	**G-3**	
Goat cheese, inside part	Milk PC	light blue to greenish (with darker circle in the middle)	irregular	shiny	-	**G-2b**	
		light blue colonies (with darker greenish middle)	rounded	smooth and shiny	-	**G-4**	
Goat cheese, yellow coloured part	Milk PC	light blue	rounded	smooth and shiny surface	-	**G-5**	
		greenish (with darker greenish middle)	rounded	smooth and shiny	-	**G-6**	

Different yeast and bacteria population can be identified by unique colony morphology with WL nutrient agar what validates the effectiveness of using WL nutrient agar as classification and identification culture medium. All isolated colonies were rounded, mostly yellow, blue or greenish with smooth, shiny or rough surface (fig 6).

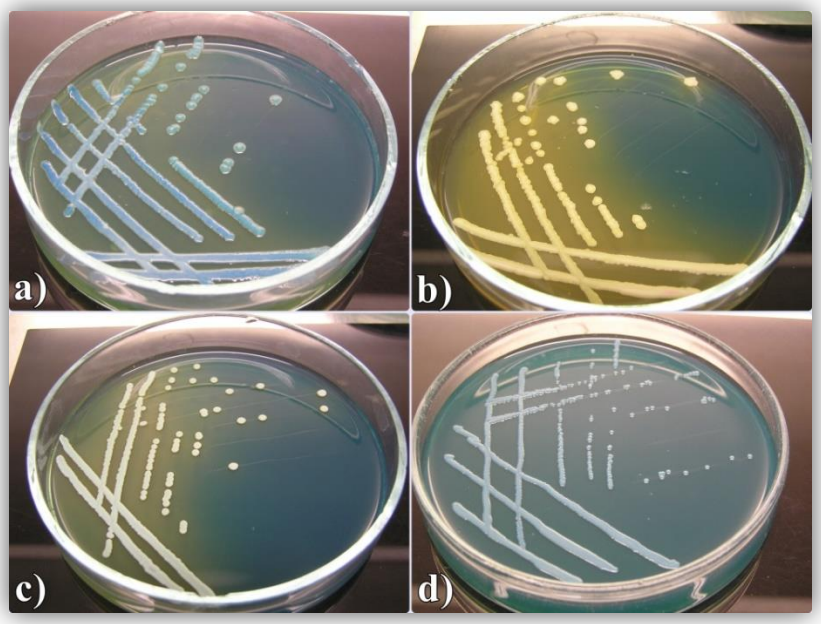

Fig. 6. Colonies on WL Nutrient agar: (**a**) Goat cheese crust (Milk PC; G-2a); (**b**) Sheep cheese crust (RBC; S-2); (**c**) Sheep cheese crust (RBC; S-3); (**d**) Goat cheese, yellow coloured part (Milk PC; G-5)

WL nutrient agar contains bromocresol green pH indicator. Colonies of different yeasts and bacteria not only differ in size and shape, but laso in pH and amount of dye take up; therefore there is a wide range of yellow-gree-blue colony colors (Priest, 2003).

4.3. Cell morphology, Gram properties, oxidase and catalase test of isolates

4.3.1. Cell morphology

Bacteria are classified into 5 groups according to their basic shapes: spherical (cocci), rod (bacilli), spiral (spirilla), comma (vibrios) or corkscrew (spirochaetes), but there are two basic shapes of starters: rods (bacillus) and cocci (fig 7b,c). Colony morphology of isolated yeast and bacteria are shown in Table 15. Rods are longer than they are wide and cocci are round or oval.

They occur as single cells or pairs, but most commonly, they are connected end to end as short chains. Bacteria isolated from sheep chesse were all cocci, except strain S-8 who was rods. In case of isolates from goat cheese, all were rod rods, except coccus strains G-2a and G-2b.

The size and shape of cells within a strain are the same when the yeast is propagated under identical conditions; they may change with the nutrient and the environment. Some strains produce one cell form, while others propagate in several shapes (dimorphism- two shapes predominate; polymorphism- more than two cell forms occur). According to cell morphology observed under light microscope, all isolated yeast cells were oval shaped (fig 7a).

Table 15. Colony morphology of yeast and bacteria isolates

Strains	Cell morphology
S-1, S-2, S-3, S-4	oval
S-5a, S-5b, S-7, S-9	coccus; tetrad
S-6	coccus
S-8	bacillus
G-1, G-3, G-4, G-5, G-6	bacillus
G-2a, G-2b	coccus

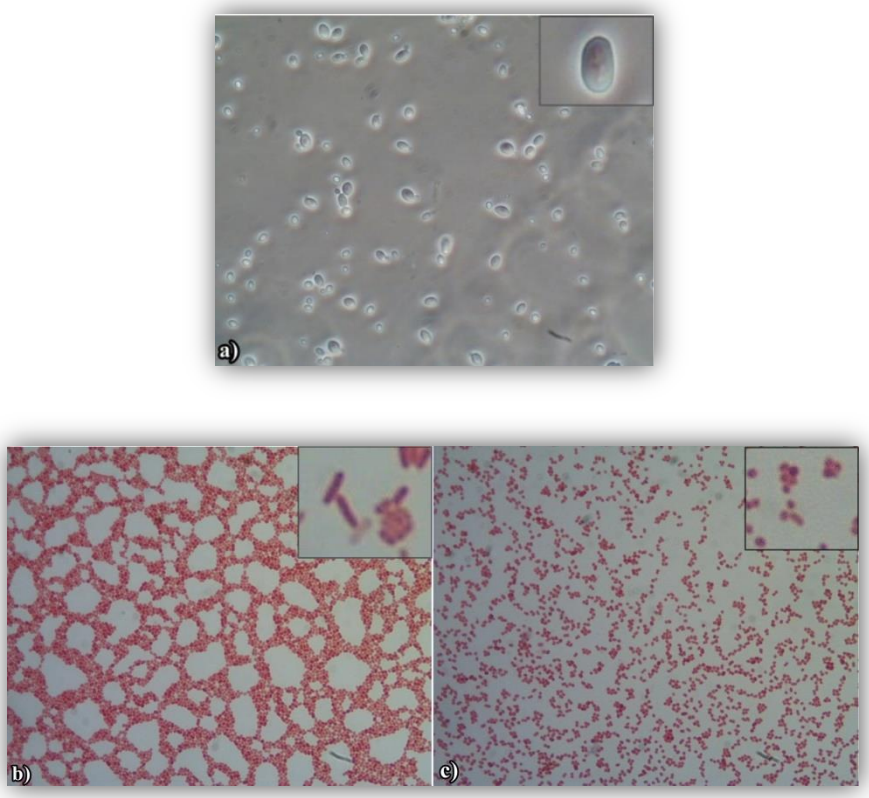

Fig. 7. Microscope photo of isolated cells: (**a**) yeast isolate-oval cell; (**b**) bacteria isolate-rods; (**c**) bacteria isolate-cocci

Classifying any unknown culture typically starts with a phenotypic analysis, usually Gram staining and microscope analysis. This is especially useful for dairy cultures as they are composed of both cocci- and rod-shaped bacteria. Of the five genera of LAB generally associated with cheese, three are cocci (*Streptococcus*, *Enterococcus* and *Leuconostoc*), one is coccoid (*Lactoccous*), and one (*Lactobacillus*) is rod-shaped. Growth temperature can further subdivide the four cocci genera, as *S.thermophilus*, a thermophilic culture (Kirschner et al., 2001).

4.3.2. Gram properties, oxidase and catalase test of bacterial isolates

Table 16 shows results of catalase, oxidase, Gram-staining and KOH test, used for bacterial classification. All 13 bacteria isolates were catalase positive what indicates they possess a catalase enzyme which is generally produced by microorganisms that live in oxygenated environments to neutralize the bactericidal effects of hydrogen peroxide. Bacteria isolated from

sheep cheese (strains S5a-S9) were all oxidase negative, while from goat cheese all bacteria isolates (G-1, G-3, G-4, G-5, G-6) except G-2a and G-2b strains were oxidase positive (table 16).

Of all the different classification systems, the Gram staining remains an important and useful technique to this day. Gram positive bacteria have a large peptidoglycan structure and some of them are able to form spores in stressful environment conditions. Gram negative bacteria have a small peptidoglycan layer but have an additional cytoplasmic membrane. All isolated bacteria from goat cheese samples were Gram-negative. On the other hand, in case of isolates from sheep cheese, only strains S-6 and S-8 were Gram-negative (table 16). Gram property was tested both with Gram-staining and KOH test.

Table 16. Catalase, Oxidase, Gram staining and KOH test of isolated bacterial strains

Strain	Catalase test (3% H_2O_2)	Oxidase test	Gram property	
			Gram-staining	KOH test
S-5a	+	-	Gram-positive	Gram-positive
S-5b	+	-	Gram-positive	Gram-positive
S-6	+	-	Gram-negative	Gram-negative
S-7	+	-	Gram-positive	Gram-positive
S-8	+	-	Gram-negative	Gram-negative
S-9	+	-	Gram-positive	Gram-positive
G-1	+	+	Gram-negative	Gram-negative
G-2a	+	-	Gram-negative	Gram-negative
G-2b	+	-	Gram-negative	Gram-negative
G-3	+	+	Gram-negative	Gram-negative
G-4	+	+	Gram-negative	Gram-negative
G-5	+	+	Gram-negative	Gram-negative
G-6	+	+	Gram-negative	Gram-negative

Organisms that produce an intracellular oxidase enzyme that catalyze the oxidation of cytochrome c, are oxidase-positive and turn the reagent blue/purple appearing within 5-10 seconds. When the enzyme is not present, the reagent remains reduced and colorless (fig 8a).The presence of the catalase enzyme in a bacterial isolate is evident when a small inoculum is introduced into hydrogen peroxide, and the rapid elaboration of oxygen bubbles occurs. On the other hand, lack of catalase is evident by a lack or weak bubble production (fig 8b).

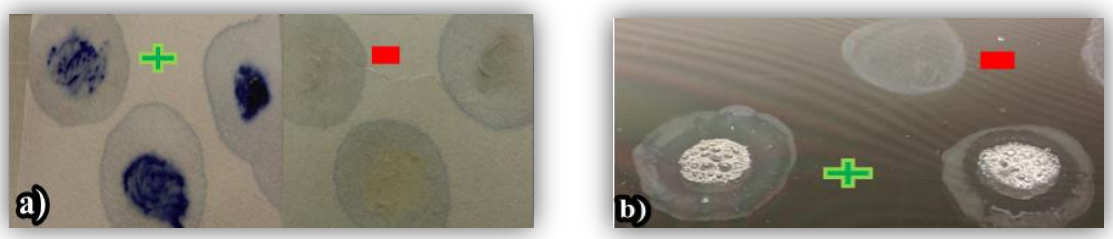

Fig. 8. (**a**) Oxidase test: (+) positive; (-) negative; (**b**) Catalase test: (+) positive; (-) negative

The catalase and oxidase test are one of the main tests used by microbiologists to identify species of bacteria. Catalase-positive bacteria include strict aerobes as well as facultative anaerobes. They all have the ability to respire using oxygen as a terminal electron acceptor. Anaerobes generally lack the catalase enzyme, or they may be facultative anaerobes that only ferment and do not respire using oxygen as a terminal electron acceptor. The enzyme catalase mediates the breakdown of hydrogen peroxide into oxygen and water. Bacteria use catalase enzyme as a self-protection from the lethal effect of hydrogen peroxide which is accumulated as an end product of aerobic carbohydrate metabolism.

The cytochrome system is usually only present in aerobic organisms which are capable of using oxygen as the final hydrogen receptor, but this doesn't mean they are strict aerobes. The end product of this metabolism is either water or hydrogen peroxide (broken down by catalase). Oxidase-negative bacteria are anaerobic, aerobic, or facultative. The oxidase negative means that they don't have the cytochrome c oxidase that oxidizes the test reagent. However, they may respire using other oxidases in electron transport.

Generally, all starter bacteria and bacteria found in ripening cheese are Gram-positive (Fox et al., 2000). The bacteria used in the manufacture of fermented dairy products are generally lactic acid bacteria (LAB) including *Enterococci*, *Streptococci*, *Leuconostoc*, *Lactococcus*, and *Lactobacilli*, who are facultative anaerobic and catalase and oxidase negative (Baluja et al., 2011).

4.3.3. Urease test of yeasts isolates

The principle of the urease test is to determine if organism has the ability to hydrolyze urea by the enzyme urease which results in increased alkalinity of the medium. This increase in alkalinity is detected by the use of phenol red as the pH indicator, changing from an orange-red to a bright pink.

Urea R Broth is rapid and sensitive for the detection of urease using weak pH buffers monopotassium phosphate and disodium phosphate in very low concentrations in order to detect the smallest amount of alkali. In this test change of color was not detected; none of the 4 yeast strains showed any urease activity. On the other hand, using Urea Broth Base, a change of color was detected with strain S-1. According to this result, the S-1 strain was urease positive (fig 9b) what means it belongs to *Basidiomycota*, while other 3 strains were urease negative, and therefore belongs to *Ascomycota*.

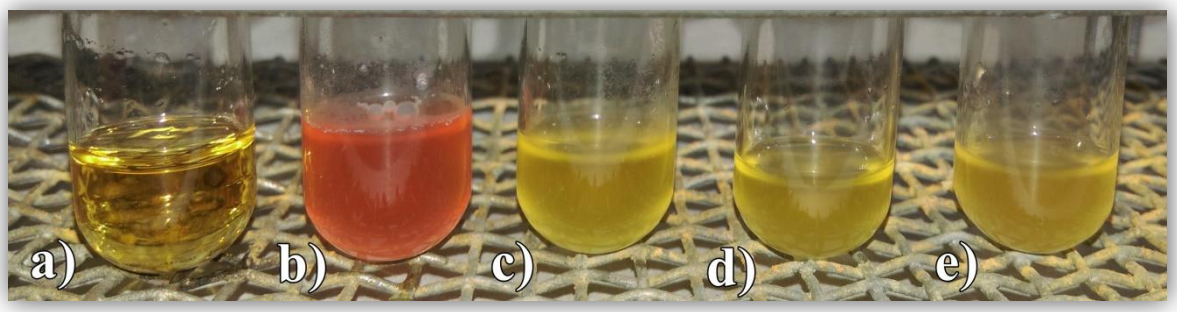

Fig. 9. Urea Broth Base: (**a**) control; (**b**) S-1 strain, urease +; (**c**) S-2 strain; urease -; (**d**) S-3 strain, urease -; (**e**) S-4 strain, urease –

Practically all yeasts can utilize urea in low concentrations as sole source of nitrogen provided that adequate amounts of vitamins are supplied. Nevertheless, yeasts differ in their ability to hydrolyse high concentrations of urea to ammonia in complete media containing an organic nitrogen source such as peptone. Urease activity is generally lacking in the ascogenous species, whereas it is particulary marked in the basidiomycetous genera *Cryptococcus* and *Rhodotorula* (Hagler and Ahearn, 1981).

4.4. Growth of isolates at different temperatures

Yeast strains had best growth at 25/30 °C but no growth at 10 °C and 37 °C. In case of bacteria strains optimal growth was at 30 °C and no growth has been detected at 10 °C. Four isolates could grow well at 37 °C (all were isolated from sheep cheese) indicates that they were mesophile. However, bacteria and yeast showed the highest growth ability in a temperature range 20-30 ºC what places them on the boarder between of psychrophiles and mesophiles.

Table 17. Growth of yeast strains at different temperatures during 3 days of incubation

Strains	10 ºC			15 ºC			20 ºC			25 ºC			30 ºC			37 ºC		
	1d	2d	3d	1d	2d	3d	1d	2d	3d	1d	2d	3d	1d	2d	3d	1d	2d	3d
S-1	-	-	(+)	(+)	+	+++	+	++	+++	+++	+++	+++	++	+++	+++	-	-	-
S-2	-	-	-	-	(+)	++	(+)	++	+++	+++	+++	+++	++	+++	+++	-	-	-
S-3	-	-	-	-	(+)	+	(+)	+	+++	+	++	+++	++	+++	+++	-	-	-
S-4	-	-	-	-	(+)	+	(+)	+	+++	+	++	+++	+	+	+++	-	-	-

Table 18. Growth of bacteria strains at different temperatures after 3 days of incubation

Strains	10 ºC			15 ºC			20 ºC			25 ºC			30 ºC			37 ºC		
	1 d	2 d	3 d	1 d	2 d	3 d	1 d	2 d	3 d	1 d	2 d	3 d	1 d	2 d	3 d	1 d	2 d	3 d
S-5a	-	-	-	-	-	-	(+)	(+)	++	+	++	+++	+++	+++	+++	++	++	+++
S-5b	-	-	-	-	-	-	(+)	(+)	++	+	+	+++	+++	+++	+++	++	++	+++
S-6	-	-	(+)	(+)	++	+++	+	+++	+++	++	+++	+++	+++	+++	+++	-	-	-
S-7	-	-	-	-	-	-	(+)	+	++	+	++	+++	++	+++	+++	+++	+++	+++
S-8	-	-	-	-	-	-	-	(+)	++	(+)	+	+++	+	+	+++	(+)	(+)	+

Table 18. Growth of bacteria strains at different temperatures after 3 days of incubation (continue)

S-9	-	-	-	-	-	(+)	(+)	+	++	+	++	+++	++	+++	+++	++	++	+++
G-1	-	-	(+)	(+)	++	+++	+	+++	+++	++	+++	+++	++	+++	+++	-	-	-
G-2a	-	-	-	(+)	++	+++	+	+++	+++	++	+++	+++	+++	+++	+++	(+)	+	+
G-2b	-	-	-	(+)	++	+++	+	+++	+++	++	+++	+++	+++	+++	+++	(+)	+	+
G-3	-	-	(+)	(+)	++	+++	+	+++	+++	++	+++	+++	+++	+++	+++	-	-	-
G-4	-	-	(+)	(+)	++	+++	+	+++	+++	++	+++	+++	++	+++	+++	-	-	-
G-5	-	-	(+)	(+)	+	+++	+	++	+++	++	++	+++	+++	+++	+++	-	-	-
G-6	-	-	(+)	(+)	++	+++	+	+++	+++	++	+++	+++	++	+++	+++	-	-	-

* no growth; (+) visible growth; + growth

Traditional microbiologists use the following terms to indicate the general optimum temperature for growth of bacteria: pyschrophiles (15 to 20°C), mesophiles (30 to 37°C), thermophiles (50 to 60°C) and extreme thermophiles (up to 122°C). However, there is a big difference between optimum growth temperature and the lowest temperatures at which bacteria can grow. Psychrotropic bacteria can grow at temperatures as low as 0 to 15°C but they may be mesophilic when it comes to their optimum growth temperature. Yeast are common psychrotrophic microorganisms that can cause spoilage of fermented dairy products because of their ability to grow at refrigeration temperature (4 to 8°C) and under the acidic conditions of these products.

Several starters are used for Feta cheese, which are mostly combinations of mesophilic cocci, thermophilic cocci and thermophilic rods (Vafopoulou et al., 1989; Litopoulou-Tzanetaki et al., 1993; Katsiari et al., 1997). Their counts increase rapidly during the first days, remain relatively high during ripening in the warm room and, later on, decline significantly, especially those of mesophilic cocci. Mesophilic lactic acid bacteria constitue the main flora of Feta cheese

(Tzanetakis and Litopoulou, 1992; Sarantinopoulos, 2002). Their number significantly increases during the first 15 days in the warm room and remains high throughout the ripening time. This explains the high proteolytic activity observed at the beginning of the ripening period. Proteolytic activity of the extracellular proteinases from psychrotrophic bacteria in raw milk reduces cheese yield and increase the levels of nitrogenous compounds in whey. The loss in cheese yield directly relates to the storage time of the raw milk. The proteolytic activity in cheese by heat stable proteinases can cause flavour quality and higher texture problems (Erkmen and Bozoglu, 2016)

4.5. Proteolytic activity of isolated strains

4.5.1. Testing the proteolytic activity of isolates on SM Agar

A clear zone of skim hydrolysis gave an indication of protease producing organisms. The proteolytic activity was assayed using SM agar and expressed as a radius of clear zone in mm (table 19; fig 10a,b). Strain G-2b exhibited the highest proteolytic activity during all three days of incubation, with a clear zone radius of 12,5 mm measured on third day of incubation. G-2b strain is followed by strains S-5b, G-1 and G-6 with clear zone of 11 mm . Strains S-8 and G-2a didn't show proteolytic activity, while S-6 had low, not measurable activity. The surface of the cheese has a relatively high salt content and therefore the microorganisms that grow on it are salt tolerant and can require salt for growth and activity. Strains isolated from sheep cheese crust part, S-5a and S-5b were not growing, and S-1, S-2, S-3 and S-4 didn't show any activity. Therefore, they have been tested on 0,85% NaCl containing SM agar. Depending on the existing zone of clearance, strains S-5a, S-5b, S-7, S-9, G-1, G-2b, G-3, G-4, G-5, and G-6 were selected for further experimental studies as isolated proteolytic bacterial strains.

Table 19. Proteolytic activity of strains on SM agar during 3 days of incubation

Strains	25 ºC					
	1d		2d		3d	
	growth	activity	growth	activity	growth	activity
S-1(SM Agar + NaCl)	+	-	+	-	+	-
S-2(SM Agar + NaCl)	+	-	+	-	+	-
S-3(SM Agar + NaCl)	+	-	+	-	+	-
S-4(SM Agar + NaCl)	+	-	+	-	+	-
S-5a (SM Agar + NaCl)	+	-	+	**4 mm**	+	**9 mm**
S-5b (SM Agar + NaCl)	+	-	+	**6 mm**	+	**11 mm**
S-6	+	+	+	+	+	-
S-7	-	-	+	**2 mm**	+	**7,5 mm**
S-8	+	-	+	-	+	-
S-9	+	**4 mm**	+	**6 mm**	+	**6 mm**
G-1	+	**3 mm**	+	**8 mm**	+	**11 mm**
G-2a	-	-	-	-	-	-
G-2b	+	**4,5 mm**	+	**9 mm**	+	**12,5 mm**
G-3	+	**2 mm**	+	**6,5 mm**	+	**10,5 mm**
G-4	+	**3 mm**	+	**8 mm**	+	**10,5 mm**
G-5	+	**3 mm**	+	**7 mm**	+	**10 mm**
G-6	+	**3 mm**	+	**7,5 mm**	+	**11 mm**

In order to grow on cheese, one of conditions is the ability of organisms to breakdown casein, the major protein in milk. Initial screening methods for protease detection and characterization of microbial proteases are of major importance considering microbial proteases one of the most important groups of industrially and commercially produced enzymes. Rapid and sensitive techniques are desirable and according to that, enzyme substrates have been incorporated in solid culture agar media for protease screening (Vijayaraghavan, 2013).

Degradation of casein contributes to the texture of the cheese and about 50% of the flavour components in cheese are amino acids, short peptides and products of their metabolism (Kieronczyk, 2006). Amino acids and short peptides can typically be bitter, but di-, tri- and short peptides can be bioactive contributing to cheese as a functional food (Korhonen and Pihlanto, 2003).

4.5.2. Extracellular proteolytic activity of strains in different media

Two nutrient media were used to test effect of growth media compositiont on proteolytic activity of isolated bacteria strains: YEPD and Minimal Media. Both media provides all required components for bacteria growth, but results indicated that bacteria for proteolytic activity require certain components that were not included in growth media. Sodium caseinate is the major protein source for the proteolytic organism while casein enzymic hydrolysates and, also yeast extract, provide nitrogenous nutrients to the proteolytic organisms. Nitrogen source in Minimal Media was replaced with sodium caseinate as a new source, and NaCl is added to test his influence on activity. To test extracellular proteolytic activity of isolated bacterial strains, after cultivation in different media, samples were treated with antibiotic gentamicin and centrifuged to get extracellular proteolytic enzymes in supernatant. Well diffusion method on SM agar plates was used for determination of protease activity. After incubation in SM agar, strains S-5b, S-7, G-1, G-2b, G-3, and G-4 showed proteolytic activity detected as the clear zone around drilled holes. Proteolytic activity increased during three days of incubation, showing the biggest radius of clearing zone in case of strains S-7 (11mm) and G-2b (10,5mm) (table 20). Strains S-5a, S-9, G-5, and G-6 didn’t show extracellular proteolytic activity.

Table 20. Extracellular proteolytic activity of strains in different media

Strains	Media	1d	2d	3d
S-5b	YEPD + NaCl	**2mm**	**5,5mm**	**9,5 mm**
	modified MM + NaCl	-	-	-
S-7	YEPD	-	**5mm**	**11 mm**
	modified MM	-	-	-
G-1	YEPD	-	**2mm**	**6 mm**
	modified MM	-	-	**7,5mm**
G-2b	YEPD	**4mm**	**5mm**	**10,5mm**
	modified MM	-	-	-
G-3	YEPD	-	**1mm**	**3mm**
	modified MM	-	-	-
G-4	YEPD	-	-	**8mm**
	modified MM	-	-	-

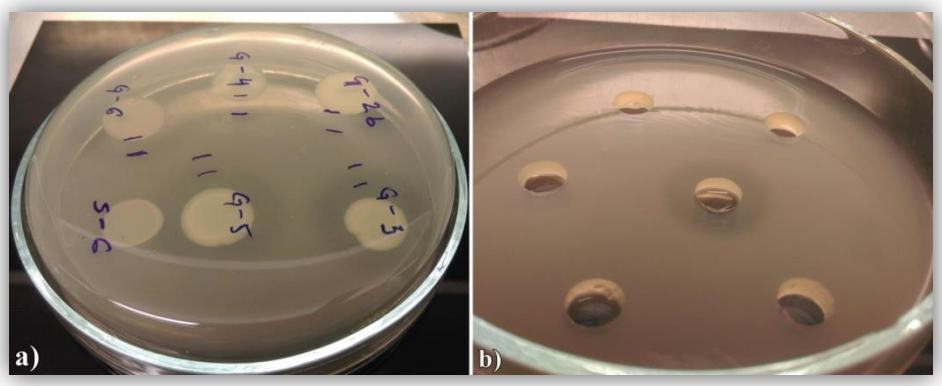

Fig. 10. Proteolytic activity of isolated bacterial strains on SM agar: (**a**) Proteolytic activity of isolated bacteria strains (**b**) Extracellular proteolytic activity of isolated bacterial strains cultivated in different growth media

Proteases can be localized inside or outside of the cell. Intracellular proteases play an important role in different cells and metabolic processes, they are highly specific and involved in several biological processes such as removal of signal peptides from newly synthesized proteins, activation of inactive precursors, inactivation of regulatory proteins, and the breakdown of abnormal or foreign proteins. A wide range of bacteria also secrete proteases into the extracellular medium. Some of these proteases are toxins or factors involved in virulence, while others exhibit low specificity and degrade proteins to produce small peptides and amino acids which can be transported and utilized (Wandersman, 1989).

In this experiment, the growth of isolated bacterial strains and ability to produce protease in liquid media was examined in the presence of two different nitrogen sources, ammonium sulfate in Minimal Media, and sodium caseinate in Modified Minimal Media. Both nitrogen sources tested support bacterial growth, but protease activity was not detected after testing on SM agar. On the other hand, YEPD showed as a good media for enzyme production due to peptone as a basic component of media, and principal source of organic nitrogen for the growing bacteria.

Most of proteolytic bacteria showed a variation in their requirement for nitrogen and carbon sources for growth as well as for the enzyme production (Ibrahim et al., 2006). Examinating of growth and ability to produce protease in liquid media containing various nitrogen and carbon sources, it has been shown that almost all of organic nitrogen source support bacterial growth, but protease activity was limited by certain compounds, like peptone and casein (Rahman et al., 2003).

Nurkasanah and Widodo were (2015) investigating the effect of different media content on protease activity of two *Bacillus subtilis* isolates in NB (Nutrient Broth) and TSB (Tryptic Soy Broth). Protease activities from both isolates of *B.* were higher on TSB production media than on NB production media. It showed that the content of substrate influenced the bacterial protease activity. TSB production media contained tryptone, soy peptone, dextrose, NaCl, and K2HPO4 while NB production media containing peptone and beef extract. The content of TSB production media was more complex than the one of NB production media. Content of nutrients in the media

was one of the variables that affected enzymes production. Also, the higher concentration of substrate also affected the activity of the enzyme.

4.6. Influence of NaCl on growth and proteolytic enzyme production

4.6.1. Influence on growth

NaCl in growth media has effect on strains growth while on previous experiment (chapter 4.5) during investigation of proteolytic activity of isolated bacterial strains on SM agar, bacterial strains S-5a and S-5b required NaCl for growth. Using Multi scanning analysis, growth of these two strains was tested during 48 h of incubation in TGE media with different concentration of NaCl (0%, 0,5%, 1%, 1,5%, 2%). Results in Charts showed that are higher concentration of NaCl increases growth in case of these two strains, however salt concentration above 1,5% is not favourable.

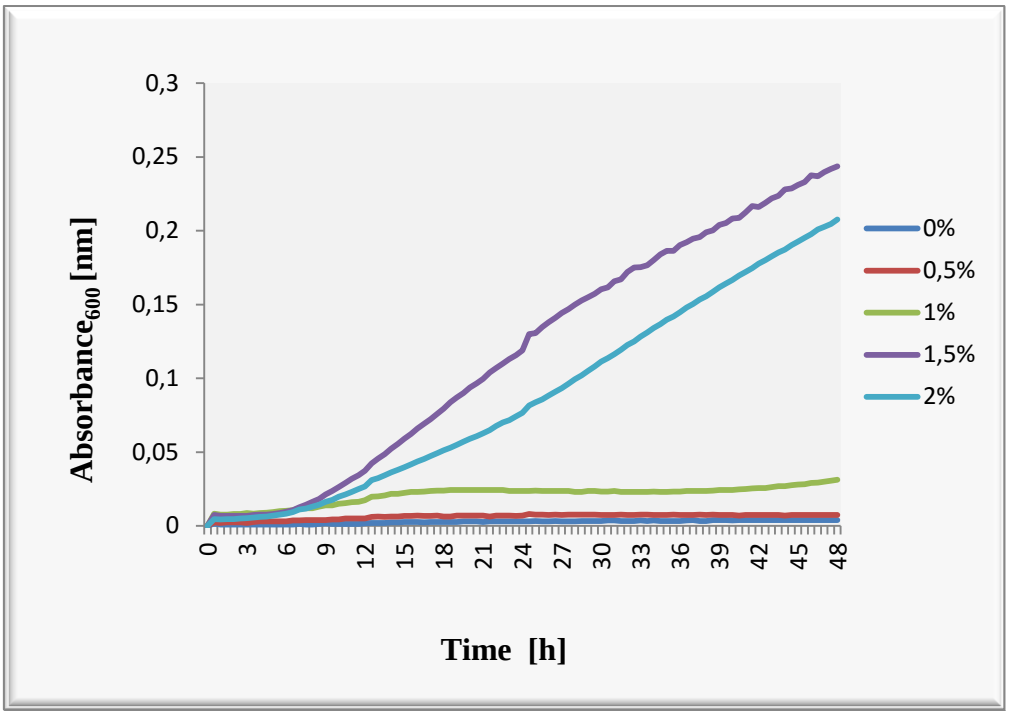

Fig. 11. S-5a strain growth after 48 h

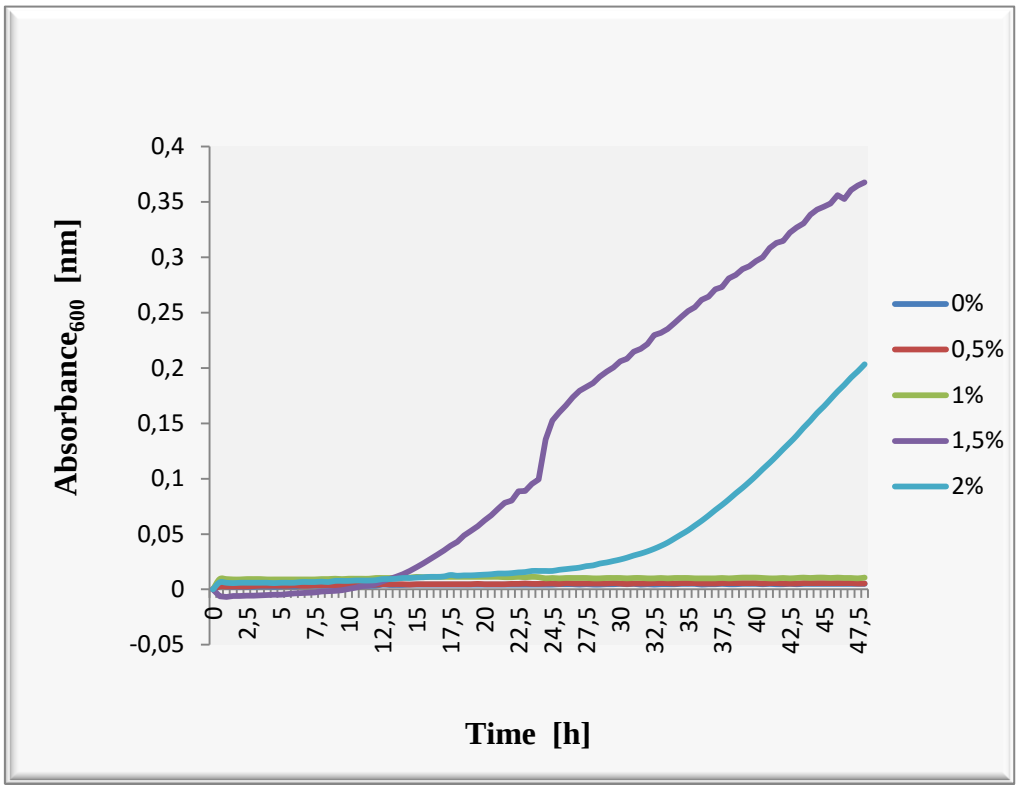

Fig. 12. S-5b strain growth after 48 h

4.6.2. Influence on proteolytic activity

Concentrations 0%, 0,5%, 1% , 1,5%, and 2% of NaCl were added to media for bacterial growth and media for proteolytic activity assay. Difference between influence on growth, and influence on proteolytic activity is existed in case of NaCl. Biggest proteolytic activity was measured on SM agar containing no salt, while on SM agar with 1,5% and 2% of salt, activity was lacking (table 21). Adding NaCl to growth media has increasing positive effect on bacteria growth and it is require for strains S-5a and S-5b. However, higher concentrations of NaCl can have inhibitory influence on proteolytic activity.

Table 21. Proteolytic activity of S-5b strain in SM agar with different NaCl concentrations after 48 h of incubation at 25 ºC

	Salt concentration of culturing YEPD broth				
Salt concentration in SM agar	0 %	0,5 %	1 %	1,5 %	2 %
0 %	-	-	**3 mm**	**2 mm**	**3 mm**
0,5 %	-	-	**1,5 mm**	-	**1 mm**
1 %	-	-	**2,5 mm**	-	-
1,5 %	-	-	-	-	-
2 %	-	-	-	-	-

The salt content of cheese depends on variety and ranges from 0,5% to over 5% in the moisture. Salt play an important role in cheeses for flavor, texture and shelf life. It has influence to the flavor of cheese in several ways, by controling of microbial growth and having impact on enzyme and water activity. Salt in cheese helps regulate pH of cheese and eventually ripening and texture through the control of microbial growth (Mistry and Kasperson, 1998). When inhibition of lactic acid bacteria occurs due to salt, salt-tolerant non-starter lactic acid bacteria are likely to continue the metabolism of lactose. Proteolytic activity is inhibited at higher levels of salt and stimulated at lower levels (Fox and Guinee, 2000). On the other hand, lower levels of salt inhibit the proteolysis of β-casein through conformational changes. For example, Thomas and Pearce (1981) reported that more than 50% of the β–casen is degraded after 4 weeks of ripening with 4% salt-in-moisture but only 10% is degraded with 8% salt-in-moisture. Cheese with little or no salt tends to develop excessive acidity and bitternes during ripening because of restrained lactid acid starter bacteria growth and enzyme activity. In Feta cheese, which has relatively high salt content, biogenic amine content is low because of unfavorable conditions for amino acid decarboxylation due to low pH and high salt concentration (Valsamaki et al., 2000).

Sheibani and Ayyash (2015) investigated the effects of salt reduction on characteristics of a hard cheese prepared using high proteolytic starter cultures. Cheeses were made with 1, 1,5, 2 and 2,5% salt and analyzed for composition, starter bacteria count, and proteolysis before and after storage for 8 weeks. Results showed that salt reduction significantly increased proteolysis and there was no differences in the sensory attributes after storage for 8 weeks in cheeses made with 1,5% and 2% salt compared to the control (2,5% salt). Furthermore, salt reduction has a significant effect on starter culture cell growth while L. *helveticus* and *S. thermophilus* growths in cheese samples containing the lowest salt content were significantly higher than in the other cheeses. This is due to higher water activity, which is a key requirement for microbiological activity (Guinee and Fox, 2004). Aly (1995) and Schroeder *et al.* (1988) reported an increase in proteolysis of low-salt Feta and Cheddar cheeses, respectively, during storage. Further, the increase in proteolysis during storage may be attributed to the residual rennet and starte10r culture activities in cheeses (Sousa et al., 2001).

Nonstarter lactic acid bacteria in five samples of Feta cheese were studied throughout a 90 days ripening period by Tzanetakis and Litopoulou-Tzanetakis (1992). Low pH of the cheese favored the growth of lactobacilli, which predominated over enterococci and pediococci throughout ripening. Combined effect of pH and NaCI-in-moisture appeared to have a selective effect on lactic acid bacteria that dominated in the cheese. *Lactobacillus plantarum*, which was the dominant species, is NaCl-resistant (Rasic, 1962) while Enterococci do not appear to be an important group in Feta cheese, in contrast with previous results on similar cheeses. Predominant NaCl-resistant groups and species of lactic acid bacteria in Feta cheese may play an important role in cheese ripening (Tzanetakis and Litopoulou-Tzanetakis, 1992).

4.7. Quantification of produced extracellular enzyme with spectorphotometric measurement

4.7.1. Spectrophotometric measurements of shaked and static bacterial cultures

The effect of agitation on enzyme production was investigated in six bacterial strains who possess extracellular proteolytic activity, by incubating bacteria strains in YEPD medium with and without agitating conditions. Supernatants from centrifuged cultures were used for absorbance measuring with two different methods including sodium caseinate and azocaseine as substrate. According to results in Table 22, the biggest difference in activity is measured in case of strain G-2b, where first day of incubation measured activity was 10,42 U/mL/h in case of shaking condititons , and 0 U/mL/h in case of non shaking. On the other hand, higher enzyme production in standing culture was detected in case of all other strains. According to these results, agitation was used only for strain G-2b for further experiments.

Table 22. Spectrophotometric measurements of shake bacterial cultures

Strain	1 day of incubation		2 days of incubation				3 days of incubation	
	method 1		method 1		method 2		method 1	
	A_{280}[nm]	[U/ml/h]	A_{280}[nm]	[U/ml/h]	A_{366}[nm]	[U/ml/h]	A_{280}[nm]	[U/ml/h]
G-1	0	0	0	0	0,096	**1,92**	0	0
G-2b	0,521	**10,42**	0,132	**2,64**	0,130	**2,60**	0,224	**4,48**
G-3	0	0	0	0	0,051	**1,02**	0	0
G-4	0	0	0,063	**1,26**	0,023	**0,46**	0,110	**2,22**
S-5b	0	0	0,104	**2,08**	0,046	**0,92**	0	0
S-7	0	0	0,085	**1,70**	0,041	**0,82**	0	0

Table 23. Spectrophotometric measurements of static bacterial cultures

Strain	1 day of incubation		2 days of incubation		3 days of incubation	
	method 1		method 1		method 1	
	A_{280}[nm]	[U/ml/h]]	A_{280}[nm]	[U/ml/h]	A_{280}[nm]	[U/ml/h]
G-1	0,079	**1,58**	0,078	**1,56**	0,098	**1,96**
G-2b	0	0	0,211	**4,22**	0,339	**6,78**
G-3	0	0	0	0	0,101	**2,02**
G-4	0	0	0,078	**1,56**	0,033	**0,66**
S-5b	0	0	0	0	0,168	**3,36**
S-7	0	0	0	0	0,221	**4,42**

Microorganisms vary in their oxygen requirement. The enzyme production in aerobic microorganisms also depends on the availability of oxygen in the fermentation medium. The variation in the agitation speed has been found to influence the nutrient availability for oxygen (Nascimento and Martins, 2004). Agitation rates have been shown to affect protease in various strains of bacteria (Pourrat et al., 1998; Mabrouk et al., 1999). For example, Sepahy and Jabalameli (2011) were doing research how agitating conditions effect on production of an extracellular protease by *Bacillus* sp. isolated from soil sample. Isolated *Bacillus* sp. strain CR-179 showed maximum protease activity at 150 rpm agitation speed after 24 h incubation, while at lower or higher agitation speed, proteolytic activity was reduced probably due to denaturations of enzyme caused by high agitation speed or the inability of bacteria to grow efficiently in case of low agitation speed.

4.7.2. Effect of temperature and pH on enzyme activity

The protease activities were assayed at different temperatures ranging from 25°C to 37°C at a constant pH 7 using 1% casein sodium salt as substrate to measure enzyme activity of proteolytic bacterial strains. From the figure, it can be seen that different strains have different optimum temperature for enzyme activity. Strains S-7, G-2b and G-4 have optimum temperature for enzyme production at 25ºC, strain G-1 at 30ºC, and strain G-3 at 37ºC.

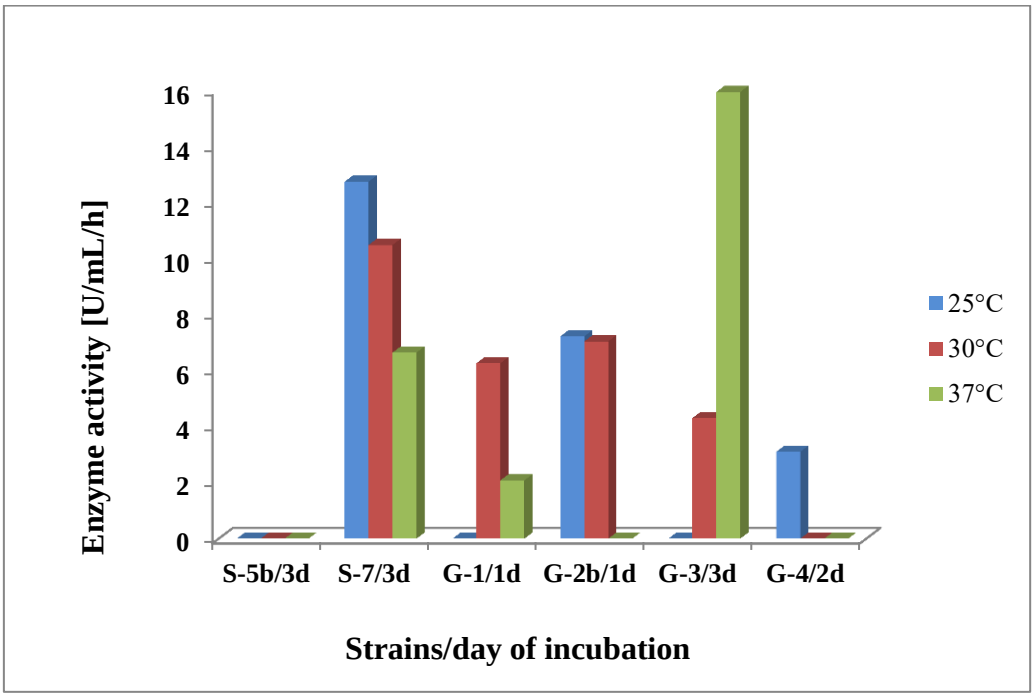

Fig. 13. Effect of temperature on enzyme activity of bacteria strains

The optimum pH for enzyme activity was determined with 1% casein sodium salt as substrate dissolved in different buffers (Citrate buffer , pH 5; Sørensen's phosphate buffer, pH 7; Tris-HCl, pH 9). Strain G-2b was incubated 24 h in shaking conditions in YEPD, while agitating showed in previous experiment as a better condition for enzyme producing than non shaking incubation. These strains had biggest activity in case pH 9, and lowest at pH 7.

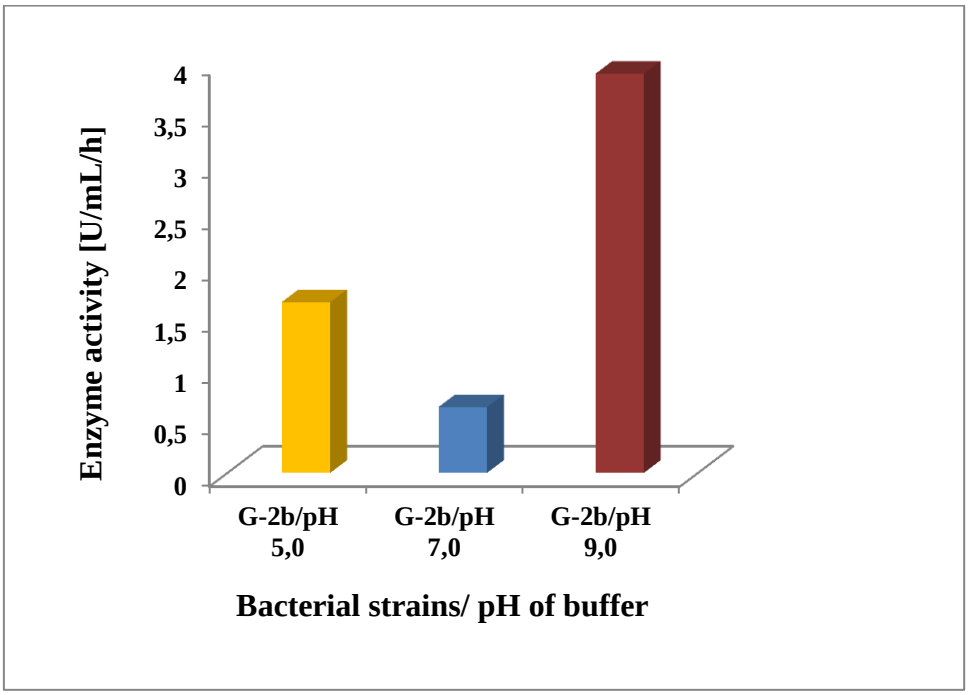

Fig. 14. Effect of different pH on enzyme activity

From an industrial prospective, the protease must exhibit considerable activity at high pH and temperature. Sepahy and Jabalameli (2011) were testing effect of culture conditions on the production of an extracellular protease by *Bacillus* sp. isolated from soil sample. A pH range between 6,5 and 11,0 was used to study the effect of pH on protease activity. The crude protease had a relatively wide pH range between 8,0 to 10,5, with maximum enzyme activity at pH 9,0. Proteases that have pH optima in the range of 8,0 − 11,0 are grouped under the category of alkaline proteases (Hameed and Evans, 1996; Tang et al., 2004). Maximum activity at pH 9,0 indicates the enzyme to be alkaline protease. In a similar study Ibrahim *et al.* (2007) found an alkaline protease which had maximum activity at pH 10,0. The proteolytic activity of these microorganisms varies according to the bacterial species and environmental conditions (Exterkate, 1976; Milla and Thomas, 1978).

De Giori *et al.* (1985) researched how pH and temperature affect proteolytic activity of lactic acid bacteria. Lactobacilli and thermophilic streptococci showed highest proteolysis at 15

and 45°C, and mesophilic streptococci showed only a slight proteolysis at all temperatures, except for *S. cremoris* strains at 50°C and *S. lactis* at 45°C. Variations in pH also had a strong influence on casein hydrolysis; this effect was more evident in streptococci than in lactobacilli.

5. CONCLUSIONS

1. Temperature needed for growth was not always the same as the temperature optimal for developing enzymatic activity.
2. One of the factors that influenced the proteolytic activity of bacteria was the composition of the media used as nutritional sources. Different nitrogen sources, ammonium sulfate, sodium caseinate, and peptone, support bacterial growth, but protease activity on SM agar was not detected when growth media did not contain peptone.
3. YEPD media showed good properties for enzyme production, while modified MM was not appropiate due to the abscence of peptone as a basic component.
4. Sodium chloride has a positive effect on bacteria growth but inhibitory influence on proteolytic activity.
5. The growth and production rates could be correlated to each other, but good growth did not necessarily mean a high enzyme production.
6. Skimmed milk agar plate assays allows qualitative determination of protease activity and provides a sensitive, convenient, and inexpensive method for screening protease production.

6. LITERATURE

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