BIOCHEMICAL AND MOLECULAR CHARACTERIZATION OF EXTRACELLULAR ENZYME PRODUCING STAPHYLOCOCCI ISOLATED FROM DIFFERENT ORIGINS

A Thesis Submitted to
the Graduate School of Engineering and Sciences of
İzmir Institute of Technology
in Partial Fulfillment of the Requirements for the Degree of

MASTER OF SCIENCE

in
Molecular Biology and Genetics

by
Sıla APPAK

December 2006
İZMİR

We approve the thesis of **Sıla APPAK**

Date of Signature

…….. **6 December 2006**
Prof. Dr. Hatice GÜNEŞ
Supervisor
Department of Biology
Muğla University

…….. **6 December 2006**
Assist. Prof. Dr. Ahmet KOÇ
Department of Biology
İzmir Institute of Technology

…….. **6 December 2006**
Assist. Prof. Dr. Belgin TAŞKIN
Department of Biology
Mugla University

…….. **6 December 2006**
Assist. Prof. Dr. Ayten NALBANT
Head of Department
İzmir Institute of Technology

……
Assoc. Prof. Dr. Barış ÖZERDEM
Head of the Graduate School

ACKNOWLEDGEMENTS

I would firstly thank my thesis advisor Prof. Dr. Hatice GÜNEŞ for providing me the oppurtinity to study in this project. I would also want to thank instructor Dr. İlhan DOĞAN for his sympathy and understanding. I would like to thank Assist. Prof. Dr. Ahmet KOÇ for his kind support and help offers. I would also like to thank our departement chair Assist. Prof. Dr. Ayten NALBANT for her patience and understanding. I would like to thank my friend Mert SUDAĞIDAN for the isolates, for the self sacrifices he made, his guidance and for his beneficial suggestions.

I would thank Assist. Prof. Dr. Adnan AYHANCI for always being there whenever I need, for his friendship and his guidance in all the troubles I met not only in the thesis but also in the real life.

I would like to thank my friends Zeynep YEĞİN, Çelenk MOLVA, Özgür APAYDIN, Burcu OKUKLU, Sinem ÇELİK, Oylum ERKUŞ, Hatice YAVUZDURMAZ, Layka ABBASİ ASBAGH, Burcu ÜNSAL, Ersin AKINCI, Ercan Selçuk ÜNLÜ, Deniz GÖL, Göksenin DURSUN and our bachelor degree student Geylani CAN for their kind help offers and for the moral supports they gave to me. I would also like to thank our department secretary Selda BOZKURT KIZILIŞIK for her sympathy, understanding and friendship whenever I need.

I do not know which words could describe my gratitude towards my family for the moral and financial support they gave to me both during this thesis and in my whole life. I would like to thank my mother Esma APPAK, my father Bedri APPAK, my brother Hasan APPAK and my little brother Yunus APPAK for their endless love.

ABSTRACT

BIOCHEMICAL AND MOLECULAR CHARACTERIZATION OF EXTRACELLULAR ENZYME PRODUCING STAPHYLOCOCCI ISOLATED FROM DIFFERENT ORIGINS

Staphylococci are pathogenic bacteria known to cause diseases among diferrent organisms including human. The two species *Staphylococcus aureus* and *Staphylococcus epidermidis* are well defined in human diseases although their exact mechanism of pathogenesis is still not fully understood. These pathogenic bacteria could be isolated from soil, water, air, as well as from the living organisms and they are both pathogenic and saprophytic.

Extracellular enzymes of the organisms are used for the industrial purposes. The isolation and characterization of these enzymes are crucial steps in biotechnology. The extracellular enzymes derived from the bacteria serve for many purposes in the industry.

In this project 128 *Staphylococcus sp*. were used. Of these 128 bacteria, 12 were isolated from patients, 40 were isolated from the foodhandler's hygiene detections, 27 were isolated from pygeons and 49 of them were reference strains. They were searched for the presence of some of the industrially important extracellular enzymes: protease, lipase, cellulase, xylanase, amylase, laccase, urease, DNase and pectinase with biochemical tests. They were also searched for the presence of the lipase, protease and thermonuclease amplifications by PCR. The bacteria apart from the refence strains were also tried to be identified by 16S-ITS-rRNA RFLP analysis.

The results would indicate the extracellular enzyme production among these pathogenic bacteria and would also be used as a guide in further studies to correlate between Staphylococcal pathogenity and enzyme production.

ÖZET

FARKLI KAYNAKLARDAN İZOLE EDİLEN EKSTRA SELÜLER ENZİM ÜRETEN STAFİLOKOKLARIN BİYOKİMYASAL VE MOLEKÜLER KARAKTERİZASYONU

Stafilokoklar insanlar da dahil olmak üzere birçok organizmada hastalık yapan bakterilerdir. İki türü *Staphylococcus aureus* and *Staphylococcus epidermidis* insan hastalıklarında oldukça iyi bilinmelerine karşın hastalık yapma mekanizmaları tam olarak anlaşılamamıştır. Bu bakteriler topraktan, sudan, havadan ve canlılardan izole edilebilen hem patojenik hem de saprofitik bakterilerdir. Organizmalardan elde edilen ekstrasellüler enzimler endüstriyel amaçlarla kullanılmaktadır. Bioteknolojide bu enzimlerin izolasyon ve karakterizasyonları önemli basamaklardır. Bakterilerden elde edilen ekstrasellüler enzimler endüstride birçok amaç için kullanılmaktadır. Bu projede 128 *Staphylococcus sp*. kullanılmıştır. Bu 128 bakteriden, 12 tanesi hastalardan, 40 tanesi gıda personeli hijyen denetimlerinden ve 27 tanesi güvercinlerden izole edilmiştir. Aynı zamanda 49 referans suş kullanılmıştır. Bu bakterilerde endüstriyel açıdan önemli olan proteaz, lipaz, sellülaz, amilaz, ksinalaz, lakkaz, üreaz, DNAz ve pektinaz gibi enzimlerin varlığı biyokimyasal testlerle araştırılmıştır. Aynı zamanda PCR ile lipaz, proteaz ve termonükleaz gen amplifikasyonları incelenmiştir. Referans suşlarının haricindeki bakteriler 16S-ITS-rRNA RFLP tekniği kullanılarak tür düzeyinde tanmlanmaya çalışılmıştır. Sonuçlar bu patojenik bakterilerdeki ekstrasellüler enzim üretimini gösterip aynı zamanda ileri dönemde Staphylococcal patojenite ve ekstrasellüler enzim üretimi arasındaki ilişkiyi belirlemek için yapılacak çalışmalara da ışık tutacaktır.

TABLE OF CONTENTS

LIST OF FIGURES ix
LIST OF TABLES xi

CHAPTER 1. INTRODUCTION 1
1.1. Staphylococcus 1
1.1.1. *Staphylococcus aureus* 1
1.1.2. *Staphylococcus epidermidis* 3
1.1.3. Extracelullar enzymes of *Staphylococcus sp.* 3
1.2. Industrially Important Extracellular Enzymes 6
1.2.1. Lipases 6
1.2.2. Proteases 6
1.2.3. Amylases 7
1.2.4. Cellulases 8
1.2.5. Xylanases 8
1.2.6. Laccases 9
1.2.7. Pectinases 9
1.3. Identification Methods for Bacteria 10
1.3.1. Phenotypic Methods 10
1.3.2. Genotyping Methods 11
1.4. Aim of the Project 12

CHAPTER 2. MATERIALS AND METHODS 14
2.1. Materials 14
2.1.1. Reference strains 14
2.2. Methods 15
2.2.1. Bacteriological Techniques 15
2.2.1.1. Isolation of Staphylococci 15
2.2.1.2. Preservation of the Isolates 15
2.2.1.3. Gram Reaction and Morphology 15
2.2.1.4. Catalase Test 16
2.2.1.5. DNase Activity 16

2.2.2. Staphylococcus characterization assays 16
2.2.2.1. Coagulase Test 16
2.2.2.2. Mannitol Salt Phenol Red Agar 16
2.2.2.3. Blood Agar 17
2.2.3. Screening for Extracellular Enzymes 17
2.2.3.1. Screening for Protease Activity 17
2.2.3.2. Screening for Amylase Activity 17
2.2.3.3. Screening for Lipase Activity 17
2.2.3.4. Screening for Urease Activity 17
2.2.3.5. Screening for Xylanase Activity 18
2.2.3.6. Screening for Cellulase Activity 18
2.2.3.7. Screening for Pectinase Activity 18
2.2.3.8. Screening for Laccase Activity 18
2.2.4. Genomic DNA Isolation 19
2.2.5. Amplification of the 16S rDNA-ITS region 19
2.2.5.1. Electrophoresis of the Amplified 16S-ITS region 20
2.2.5.2. Na-Acetate Precipitation of the PCR Products 20
2.2.5.3. Restriction Fragment Length Polymorphism (RFLP) 21
2.2.5.4. Electrophoresis of the Restriction Fragments 21
2.2.6. PCR Analysis of the Extracellular Enzyme Encoding Genes 22
2.2.6.1. Polymerase Chain Reaction of *sspA* 22
2.2.6.2. Electrophoresis of the Amplified Products of *sspA* 22
2.2.6.3. Polymerase Chain Reaction of *geh* 22
2.2.6.4. Electrophoresis of the Amplified Products of *geh* 23
2.2.6.5. Polymerase Chain Reaction of *sspB* 23
2.2.6.6. Electrophoresis of the Amplified Products of *sspB* 23
2.2.6.7. Polymerase Chain Reaction of *aur* 24
2.2.6.8. Electrophoresis of the Amplified Products of *aur* 24
2.2.6.9. Polymerase Chain Reaction of *prt* 24
2.2.6.10. Electrophoresis of the Amplified Products of *prt* 25
2.2.6.11. Polymerase Chain Reaction of *nuc* 25
2.2.6.12. Electrophoresis of the Amplified Products of *nuc* 25

CHAPTER 3. RESULTS 26
3.1. Isolation of Staphylococci 26
3.2. Phenotypic Characterizations 26
3.2.1. Gram Reaction and Morphology 26
3.2.2. Catalase Test 27
3.2.3. DNase Test 27
3.3. Staphylococcus Characterization Assays 27
3.3.1. Coagulase Test 27
3.3.2. Mannitol Salt Phenol Red Agar 27
3.3.3. Blood Agar 28
3.4. Extracellular Enzyme Profiles of Staphylococci 29
3.5. Genotypic Characterization of Staphylococci 40
3.5.1. PCR Analysis of the Genes for the Extracellular Enzyme Production 40
3.5.2. 16S rRNA- ITS (Internally Transcribed Spacer) RFLP (Restricition Fragment Length Polymorphism) Analysis of the Bacteria 47
3.5.2.1. Amplification of Bacteria by 16S rRNA –ITS 47
3.5.2.2. 16S rRNA-RFLP Analysis of Isolates and Reference Strains 47

CHAPTER 4. DISCUSSION 51

REFERENCES 53

APPENDICES

APPENDIX A. CHEMICALS USED 61
APPENDIX B. MEDIA 63
APPENDIX C. PCR-RFLP RECIPIES 70
APPENDIX D. STAINS AND INDICATORS 72
APPENDIX E. BUFFERS AND STOCK SOLUTIONS 73
APPENDIX F. PCR RESULTS OF THE ISOLATES 76

LIST OF FIGURES

Figure **Page**

Figure 1.1. Pathogenesis of *Staphylococcus aureus* 2
Figure 3.1. Extracellular enzyme production among staphylococci 29
Figure 3.2. Extracellular enzyme production of staphylococci 30
Figure 3.3. Percentage of extracellular enzyme production among staphylococci 31
Figure 3.4. The extracellular enzyme production among the isolates 35
Figure 3.5. The percentage of the lipase activity in four different media among all the lipase positive cultures 36
Figure 3.6. The percentage of DNase positive bacteria in the four lipase media 37
Figure 3.7. The percentage of DNase positive bacteria in the two proteinase media 38
Figure 3.8. Protease, lipase and thermonuclease amplifications 41
Figure 3.9. The percentage of the *sspA* expressing bacteria in the two protease media 41
Figure 3.10. The percentage of *sspB* expressing bacteria in the solid protease media 42
Figure 3.11. The percentage of *prt* expressing bacteria in the two protease media 43
Figure 3.12. The percentages of the aur expressing bacteria in the proteinase media 43
Figure 3.13. The perentage of the *geh* expressing bacteria in the four lipase media 44
Figure 3.14. The percentage of the thermonuclease (*nuc*) positive bacteria in DNase agar 45
Figure 3.15. The PCR products of *nuc*, *sspA* and *geh*. 45
Figure 3.16. PCR products of *sspB* 46
Figure 3.17. PCR products of *aur* 46
Figure 3.18. PCR products of *prt*. 46

Figure 3.19. 16S- ITS amplification of some isolates 47
Figure 3.20. *Taq 1* digested 16S-ITS-rRNA RFLP profiles of the isolates..... 48
Figure 3.21. Dendogram of isolates and reference strains 50

LIST OF TABLES

Table **Page**

Table 2.1. The reference strains 14
Table 3.1. Sample, name and numbers of the isolates 26
Table 3.2. Growth in Mannitol Salt Agar 28
Table 3.3. The results of the each extracellular enzyme assay for the isolates..... 32
Table 3.4. Lipase enzyme groups for the DNase positive bacteria..... 37
Table 3.5. Enzyme groups, the names and the codes of the bacteria..... 39

CHAPTER 1

INTRODUCTION

1.1. Staphylococcus

Taxonomically, the genus Staphylococcus is in the bacterial family Staphylococcacea. Staphylococci are non-motile Gram positive cocci being approximately 1µm in diameter. If these microorganisms are observed under microscope it would clearly be noticed that these spherical bacterial cells are arranged in irregular clusters. These clusters resemble a bunch of grapes. In Greek the term stapyle means a bunch of grapes. So these bacteria are named Staphylococcus.

In 1884, Rosenbach described staphylococci according to the colony types they had and proposed the appropriate nomenclature: *Staphylococcus aureus* (yellow) and *Staphylococcus albus* (white). The latter species is now named *Staphylococcus epidermidis. Staphylococcus aureus* forms yellowish large colony and *Staphylococcus epidermidis* forms relatively small white colony. There are many other Staphyloccus species but these two are well defined in human diseases.

Staphylococci are facultative anaerobes grow by aerobic respiration or by fermentation resulting in lactic acid production. They are oxidase negative and catalase positive. Their cell wall contains teichoic acid. Teichoic acid composition is also different among the Staphylococcal species. The cell wall contains ribitol teichoic acid (polysaccharide A) in *Staphylococcus aureus*, glycerol teichoic acid (polysaccharide B) in *S. epidermidis* .

Staphylococci are common in skin, nasal cavity, oropharynx, gastrointestinal tract and in genitourinary tract flora.

1.1.1. *Staphylococcus aureus*

Staphylococcus aureus is always considered to be potentially pathogenic bacteria. It is responsible for many of the human diseases. The clinical syndromes caused by this bacteria can be grouped as: coutaneous infections which includes folliculitis, impetigo, wound infections, toxin-mediated infections that includes toxic

shock syndrome, foodpoisoning, scalded skin syndrome which is seen in children under the age of four, other diseases such as pneumonia, bacteremia, endocarditis, osteomyelitis and septic arthritis (Figure 1. 1).

This bacteria forms large yellow colonies and it is often hemolytic in blood agar. Nearly all strains of *Staphylococcus aureus* produce the enzyme coagulase, being coagulase positive is an important feature of this bacteria. It has both bound and free coagulase while the other staphyloccocci are coagulase negative. The surface of this bacteria is coated with Protein A and Protein A is not found on the surface of coagulase negative staphylococci . It ferments mannitol and most other staphylococci are mannitol negative. *S. aureus* can grow at a temperature range of 15 to 45 degrees and at NaCl concentrations as high as 15 percent. Salt is useful as a selective constituent in isolation media.

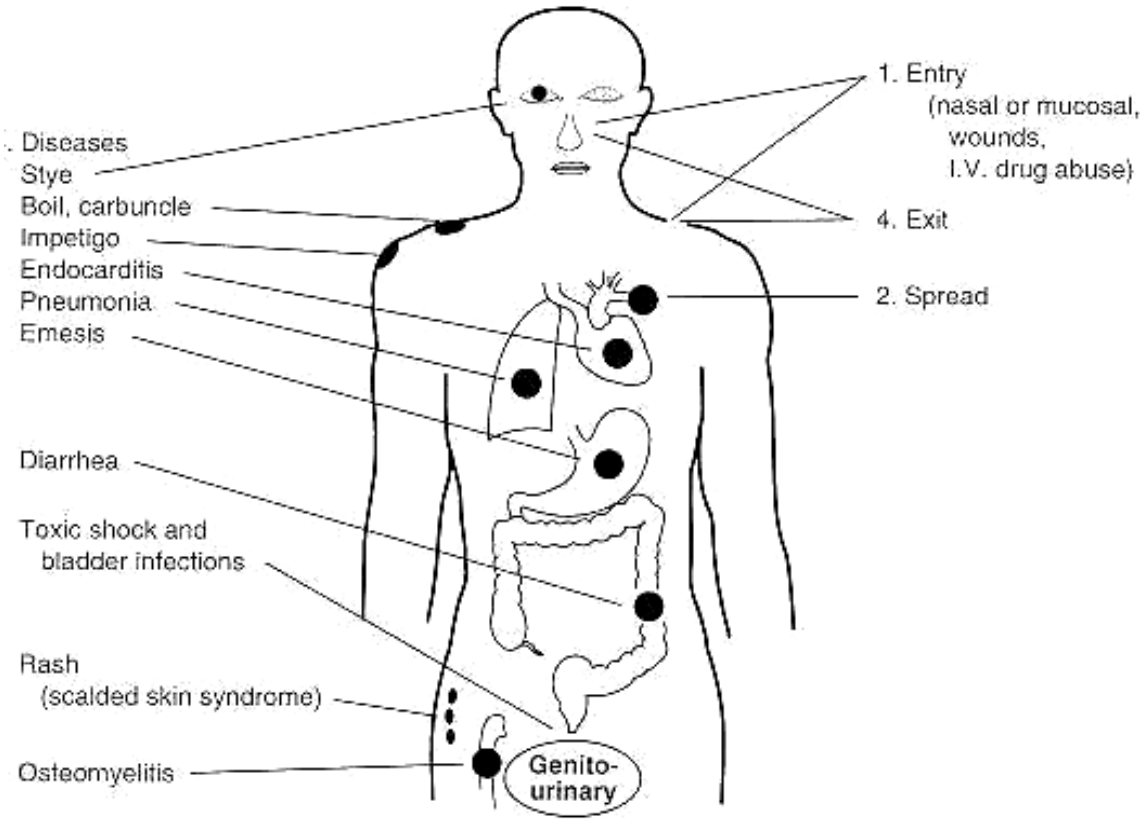

Figure 1.1. Pathogenesis of *Staphylococcus aureus*

1.1.2. *Staphylococcus epidermidis*

Staphylococcus epidermidis is a coagulase negative, catalase positive bacteria usually resident in skin flora, in gut and in the respiratory tract. These coagulase negative staphylococci are known as the leading cause of the nosocomial sepsis. These bacteria are also known to cause medical device derived infections.

Biofilm production is an important feature among staphylococci it may also be the reason why *Staphylococcus epidermidis* once thought to be non-pathogenic is cosidered to be pathogenic these days (Costerton et al. 1995). Biofilms are the highly organized multicellular complexes occuring in two main steps: attachment of bacteria on solid surface and proliferation of cells in multilayers leading in the formation of the enclosed bacterial community in a polymeric matrix (Cucarella et al. 2002).

1.1.3. Extracelullar Enzymes of *Staphylococcus sp.*

Staphylococcus sp. produce some of the industrially important extracellular enzymes. Lipase is the most abundant enzyme produced by this bacteria. The lypotic activity of staphylococci was originally observed in 1901 (Ejkman, 1901). The lypotic activity is now known to be the result of the enzymes lipase and esterase which act against water-soluble, water-insoluble glycerol esters and on water-soluble Tween polyoxyethlene esters. So staphylococci split variety of lipid substrates with lipase acting on fat-soluble glycerides and with esterase acting on water-soluble esters (Vadehra 1974). It is known that staphylococci tend to remain in lipoid secretions in the cutaneous habitat of the host organism (Saggers and Stewart 1965). Lipids are found ubiquitously on the surface of human skin, and are largely composed of sebum-derived triacylglycerides (Nicolaides 1974). When the natural host defence is weakened the opportunistic pathogens invade the host. One of these microorganisms is *Staphylococcus epidermidis* known as the human cutaneous commensal that lives on the skin of its host which is also able to become an opportunistic pathogen. It is thought that during the infection process, two secreted lipases support the colonisation and growth of the bacteria by the cleavage of the triacylglycerols derived from the sebum of the skin (Longshaw 2000). The clinical studies have proven that *Staphylococcus aureus* that were isolated from the deep wound infections produced higher amounts of lipase than

those isolated from the more superficial ones (Rollof 1987) suggesting that lipase activity might be important for nutrition or dissemination of the bacteria. The strongest hint that was ever found out about the correlation between lipase activity and pathogenity of staphylococci is the detection of anti-lipase IgG antibodies in patients with the *Staphylococcus aureus* infections which showed the pathogenetic potential of the extracellular lipase (Ryding 1992). However the contribution of this enzyme alone to virulence is not clearly understood but it is suggested that lipases may be important for the colonisation and persistence of the bacteria on the skin possibly in terms of nutrition or by the release of free fatty acids which may promote adherence (Gribbon et al. 1993). Staphylococcal lipase is encoded by *geh* which stands for glycerol eser hydrolyse that was identified from *Staphylococcus epidermidis* strain 9 in the studies aiming to identify extracellular colonization factors important for the persistence of cutaneous bacteria on skin (Farrell et al. 1993).

Staphylococci mainly the species *Staphylococcus aureus* are known to produce several extracellular proteases serine, cycteine and metalloenzymes. These proteases are important in the pathogenity processes of the bacteria (Maeda 1996). The proteases of staphylococci are insensitive to the plasma protease inhibitors of the human furthermore the proteases can also inactivate some of these plasma protease inhibitors. By the proteolytic inactivation, staphylococcal proteases could deregulate internally derived proteolytic activity which results in the destruction of the host tissues allowing the dissemination of bacteria as well as the acquisition of nutrients (Potempa et al. 1986, Maeda and Yamamoto 1996). Alterations in the coordinated expression of the proteases diminished the virulence.

The first purified and characterized staphylococcal protease was serine glutamylendopeptidase (V8 protease) (Drapeau et al. 1972). As in the case of some other staphylococcal extracellular proteins, V8 protease is synthesized as a preproenzyme. V8 protease coding *ssp*A is encoded in one operon with *ssp*B coding for an extracellular cysteine protease. This operon is up-regulated by *agr* (Rice et al. 2001) and repressed by *sar*A. In vitro the enzyme V8 protease of *Staphylococcus aureus* has shown to interfere with the host defence mechanisms (Arvidson 2000) by inactivating the α1-Proteinase inhibitor the neutrophil elastase inhibitor of the human plasma (Protempa et al 1986). V8 protease also generates kinin from kininogen which is thought to have a role in the pain and edema accompying the staphylococcal infections. Recently this kinin activity has shown to result in the aid to the bacteria in transfer into

the systemic circulation that leads to septicemia (Maeda and Yamamoto 1996). In vitro V8 protease cleaves the heavy chains of all human immunoglobulins and impairs the host defense mechanism (Arvidson 2000).

Aureolysin is a metalloprotease encoded by the *aur*. Aureolysin has shown to inactivate in vitro human protease inhibitors like V8 protease but it is not as efficient as V8 protease. Both proteases may act synergically in deregulating neutrophil derived proteolytic activity (Potempa et al. 1986). In addition to V8 protease aureolysin can modify the cell surface proteins of *Staphylococcus aureus* .

The proteases of Staphylococci have shown to have effects in the virulence of these bacteria. The enzymes have shown to interact in vitro with the host's defence mechanisms causing deleterious effects on host and increasing their chances of survival. However, in the animal models of the staphylococcal infections only epidermolytic toxins were shown to have a direct linkage for pathogenity (Prevost et al. 1991). It was assumed that the orchestrated expression and interaction of variety of proteases all together played an important role in the pathogenity in vivo, rather than any particular gene product or an extracellular enzyme. Neverthless proteases appereantly play an important role in this complex process of pathogenity.

It was seen that during harsh heat conditions staphylococci especially *Staphylococcus aureus* remained alive. This is due to the production of thermonucleases encoded by *nuc*. Staphylococcal thermonuclease has been shown to be stable at high temperatures (Kulkarni and Barve 1983) and as well as to low temperatures (Jaspal 1999) and in chemical preservatives. Thermonucleases along with the enterotoxins serve for the pathogenity of *Staphylococcus aureus,* their activities have proven to be the cause of the food poisoning (Niskanen and Koiranen 1977) indeed thermonuclease tests were developed for the identification of *Staphylococcus aureus* .

1.2. Industrially Important Extracellular Enzymes

Extracellular enzymes of the organisms are used for the industrial purposes. The isolation and characterization of these enzymes are crucial steps in biotechnology. The extracellular enzymes derived from the bacteria serve for many purposes in the industry**.** To date, enzymologists have turned their attention to bacteria as a source of the enzymes as the bacterial origins of enzymes are cheaper and less time consuming than the animal sources. The estimated world-wide sales volume for industrial enzymes in 1995 was 1 billion U.S dollars and this volume has doubled in recent years (Godfrey 1996). 90% of these enzymes are produced from microorganisms by fermentation. The first group of these enzymes are proteases, second is charbohydrases and third group is the lipases based on the total sales volume.

1.2.1. Lipases

Lipases (acylglycerol hydrolyses, EC 3.1.1.3) catalyse both the hydrolysis and the synthesis of esters formed from glycerol and long-chain fatty acids. The microbial lipases are important in the biotechnological industry as they are stable in organic solvents, they do not require cofactors and they have a broad substrate specifity.

In the industry the hydrolytic lipases are added to the detergents which are used in household, industrial laundry and in household dishwashers, in the pharmaceuticals microbial lypases are used to enrich polyunsaturated fatty acids from the animal or plant lipids therefore free polyunsaturated fatty acids and their mono and diglycerids are used to produce anticholesterolemics, anti-inflammatories and thrombolytics. In the paper industry the hydrophobic components of the wood are removed by the lipases.

1.2.2. Proteases

Proteases account for about the 60% of the total worldwide sale of enzymes. Microbial proteases account for the approximately 40% of the total worldwide enzyme sales (Rao et al. 1998). Proteases are the enzymes cleaving proteins in peptide chains or further in free amino acids. They are divided into two subroups: exopeptidases that cleave the peptide bond near to the amino or carboxy termini of the substrate,

endopeptidases that cleave the peptide bonds distant from the termini of the substrate. They are further divided into four groups based on the functional groups they have: serine proteases, aspartic proteases, cycteine proteases and metalloproteases (Rao et al. 1998).

Proteases are one of the standard ingredients of all kinds of detergents ranging from household detergents to reagents used for contact lenses. In the leather industry proteases are used to eliminate the noncollagenous constituents of the skin and for the removal of nonfibrillar proteins such as albumins and globulins. In the baking industry bacterial proteases are used to improve the extensibility and the strength of the dough. There are many applications of proteases in the pharmaceutical industry: as a digestive aid to correct certain lytic enzyme deficiency syndromes, as an agent to be used with the broad spectrum antibiotics in the treatment of burns and wounds. Asparginase is used to eliminate aspargine from the bloodstream of the lymphocytic leukemia patients (Anvar and Saleemuddin 1998).

1.2.3. Amylases

It is the group of the extracellular enzymes which degrades starch. Amylase hydrolalyzes the bonds between the adjacent glucose units in the starch molecule. Within the family of microbial amylases α-amylase, β-amylase and amyloglucosidase have found wide scale industrial application. α-amylase is an endo-acting enzyme cleaving the linkages in the interior of the starch molecule which results in the formation of the linear and branced oligosaccharides. On the other hand β-amylase and amyloglucosidase along with the α-glucosidase are the exo-acting enzymes that attack the substrate from the non-reducing end resulting in the production of oligo and/or monosaccharides. Amylases have approximately 25% of the worldwide enzyme sales. Amylases are used in food industry especially for baking, in dairy industry, fermentation, paper and detergent industries. Microbial sources of amylases are used in biological hydrolysis of starch so that no longer the chemical catalysts are used.

1.2.4. Cellulases

Cellulose is a linear insoluble glucan biopolymer composed of the repeating structural unit cellobiose (Brown 1996). The association of the glucan chains lead to the formation of a microfibril in a regular crystalline arrangement. Amorphous and crystalline regions alternate within the microfibril. In nature, cellulose is essentially synthesized by plants, algae, bacteria and in animals. But cellulose degradation in the environment is still considered as a microbiological process. Microorganisms have developed strategies to digest cellulose, some produce lipases secreted into the extracellular medium and some regroup the cellulases into an extracellular enzymatic complex called cellulosome. The concept of cellulosome was first introduced with thermophilic, cellulolytic, anaerobic bacterium *Clostridium thermocellum* (Lamed et al. 1983). Cellulases are used in the textile industry, detergent industry, in animal feed, in the paper industry for the deinking of the recycled paper.

1.2.5. Xylanases

Xylan is a complex polysaccharide comprising a backbone of xylose residues linked by β-1,4-glycosidic bonds. The main chain of xylan is composed of β-xylopyranose residues. Xylan is the most common hemicellulosic polysaccharide in cell walls of land plants representing up to 30%-35% of the total dry weight. The xylan layer with its covalent linkage to lignin and its non-covalent interaction with cellulose may be important in maintaining the integrity of the cellulose and in helping to protect the fibers against degredation to cellulases. Several microorganisms have several multifunctional enzyme complexes on their surfaces, these complexes are named xylanosomes and they play an important role in the degradation of hemicelluloses. Xylanases are widely used in industrial applications to:

- Prebleach the pulps for paper manufacture,
- Improve the nutritional qualities of wheat,
- Clarify the juices and wines,
- Extract juices, oils, spices and pigments,

- Modify cereal flours in order to enhance the texture and volume of the bread (Gilbert and Hazlewood 1993).

1.2.6. Laccases

Laccases (benzendial: oxygen oxidoreductases, EC1.10.3.2) are diverse group of multi-copper proteins that catalyse the oxidation of a variety of aromatic and non-aromatic compounds. Phylogenetically, laccases are members of the multi-copper protein family including ascorbate oxidase, ceruloplasmin and bilirubin oxidase (Alexandre and Zhulin 2000). Laccases are involved in the metabolic turnover of complex organic substances such as lignin. Until recently laccases were only found in eukaryotes (fungi, higher plants, insects) the first convincing data for the laccase activity in prokaryotes were presented for *Azospirillum lipoferum* (Givaudan et al. 1993). The spore coat protein cotA of *Bacillus subtilis* has been identified as a laccase (Martins et al. 2002) and the crystal structure has been presented. Mutants in the gene encoding CotA lost the ability to produce a brownish spore pigment which aids to protect the spores against UV radiation. Since they have a high non-specific oxidation capacity, laccases are useful biocatalysts for the biotechnical applications.

1.2.7. Pectinases

Pectin and pectic substances are complex plant polysaccharides. They contribute to the plant tissue as a part of primary cell wall and as middle lamella component (Naidu and Panda 1998). The pectin is made up mostly of D-galacturonic acid in the form of macromolecule that is based on α-1,4 glycosidic linkages. Pectins are mainly synthesized by plants and microorganisms. In the industrial applications *Aspergillus niger* which synthesizes polygalacturonases, polymethyl galacturonases, pectin lysases and pectin esterases is mostly used. Pectins are used since 1930 in the preparation of wines and fruit juices commercially. They are also used in textile processing, coffee and tea fermentation, in paper and pulp industry, oil extraction.

1.3. Identification Methods for Bacteria

As it would be the case with all organisms, microbial organisms are identified with two methods known as the phenotypic and genetoypic methods. Accurate identification of the bacterial isolates is an essential task in microbiology.

1.3.1. Phenotypic Methods

Organisms genetic structure could not always be seen in the phenotype neverthless phenotypic methods are important for the microorganisms' classification because these methods allow us to place a microorganism in a group according to the phenotypic characteristics they posess. In the phenotypic determination of the bacterial morphology determination is the first step. The bacteria could be rod shaped, coccus, vibria, helical or they could have some other shapes. Nutritional classification would be of second importance. Because some bacteria are ototrophic, they could be photosynthetic, chemosynthetic or they could be heterotrophs. The metabolism is important in the identification process as some bacteria are anaerobic and fermentative, some have an aerobic metabolism and some could be facultative anaerobes being fermentative in case of the absence of oxygen. Gram reaction is one of the phenotypic characterization methods and it depends on the structure of the cell wall structure of the bacteria. Growth in different habitats, pH requirements, pathogenity, antibiotic sensitivity and immunological characteristics are the other phenotypic characterization methods (Madigan et al. 1997).

In recent years with the aid of the technology phenotypic characterization methods have improved and became more sensitive. These tests include antibiogram, serotyping, biotyping, phage typing, immunoblotting and enzyme electrophoresis (Busch and Nitschko 1999). Antibiogram is the test in which bacteria's growth in the presence of various antibiotics are measured. But antibiotic resistance is not always a good selective agent in phenotyping since the resistance genes could be transferred horizontally from one bacteria to another by plasmids. Bacteria's antigen antibody reactions are the basis of serotyping as microorganisms are characterised by using special antibodies. In biotyping, microorganisms are grouped according to their reactions against various biochemical reagents. Immunoblotting is a method that

characterizes bacteria by the seperation of bacterial proteins with polyacrylamide gel electrophoresis (Busch and Nitschko 1999).

The phenotypic test results alone are not sufficient in the identification of the bacteria and they must be matched with the genotypic tests as well. Since the improvements in DNA technology some phenotypic results alone have proven to be not satisfactory in the characterization assays of bacteria.

1.3.2. Genotyping Methods

The phenotypic methods' interperations are sometimes subjective so there are commercial systems offering computer-assisted identification of various bacteria but they are again insufficient as they are based on the phenotypic identification such as gas-liquid chramatography system measuring the cellular fatty acid profile but they still rely on phenotypic identification.

Genotypic identification has emerged as an alternative or complement to the established phenotypic methods. Typically these methods include the DNA based analysis of chromosomal and extrachromosomal genetic material. Molecular methods have proven to have more discriminatory power than the phenotypic methods which means that molecular results could distinguish the differences between the two closely related species (Busch and Nitschko 1999). Molecular characterization methods include the amplification of a single part of a gene by polymerase chain reaction, 16S rRNA ITS (Internally Transcribed Spacer) region RFLP (Restriction Fragment Length Polymorphism), Pulsed Field Gel Electrophoresis (PFGE), plasmid typing and DNA sequencing.

PCR (Polymerase Chain Reaction) allows the DNA from a selected region of the genome to be amplified by selectively purifying this DNA part away from the remainder of the genome. PCR is a relatively simple technique by which a DNA or cDNA template is amplified many thousand or million-fold quickly and reliably. The steps of the polymerase chain reaction are the denaturation, annealing, elongation and extension. Denaturation by heat (usually >90ºC) separates double-stranded DNA into two single strands. Since the hydrogen bonds linking the bases to one another are weak, they break at high temperatures, whereas the bonds between deoxyribose and phosphates, which are stronger covalent bonds, remain intact. The whole DNA is not

aimed to be replicated but a target sequence is intended to be replicated. This sequence is marked by the primers which are two in number. One for each of the complementary single DNA strands that was produced during denaturation. The beginning of the DNA target sequence of interest is marked by the primers that bind to the complementary sequence. Since the aim is to amplify the target sequence there is a unique temperature at which the primers anneal to the target sequence. After the annealing the temperature rises about 72ºC and a recombinant thermostable *Taq* DNA polymerase begins the synthesis marked by the primers so there is the synthesis of the new double stranded DNA. This amplified region could well be used for further analysis.

In the prokaryotes, the ribosomal subunits hold the ribosomal RNA genes in the chromosome (16S-23S-5S) and this part is transcribed as a single polycistronic RNA. Between the 16S-23S region genes there are conserved and highly variable sequence motifs such as antiterminators and this region is known as intergenic or internal transcribed spacer (ITS) or intergenic spacer region (ISR). These regions have been found to differ even different operons within a single cell (Garcia-Martinez et al. 1999) so these regions can differentiate between different species. This region is amplified as a single amplicon and then digested with the restriction enzymes, the restriction fragments would then be separated with agarose gel electrophoresis and would specify the bacteria at species level. The 16S internal transcribed spacer (16S-ITS) rRNA gene RFLP method has already been used for the identification of bacteria of diverse origin: lactic acid bacteria (Bulut et al. 2005), lactobacilli (Yavuz et al. 2004a) thermophilic bacilli (Yavuz et al. 2004b) alkalophilic bacilli (Akbalik et al. 2004) and in staphylococcus (Sudagidan et al. 2005).

Probably the most powerful discriminating way of identification or genotyuping between the individual microorganisms would be pulse field gel electrophoresis which allows large molecules of DNA to be resolved as well as which has an identification power at subspecies level.

1.4. Aim of the Study

A substantial evidence in the literature indicated that both coagulase positive and coagulase negative staphylococci are pathogenic. Especially coagulase-negative

staphylococci has emerged as a major source of biomaterial-related nasocomial infections.

In a previous study of our laboratory, staphylococcal strains were isolated from clinically used polymeric biomaterial surfaces and characterized on the basis of their slime production and biofilm forming abilities. In addition, 16S-ITS rRNA RFLP method was shown to have discriminatory power on type strains of 16 *Staphylococcus sp*. at distinct RFLP haplotypes. Because staphylococci are pathogenic bacteria and known to cause diseases in human, it is important to isolate and characterize staphylococci from different origins. Therefore, the purpose of this present study is to characterize previously isolated staphylococci: 12 isolates from patients; 27 isolates from pygeons and 40 isolates from foodhandler's hygiene detections at both phenotypic and genotypic levels in the presence of the reference and type strains. Especially, the identification of extracellular enzyme profiles of staphylococci may be helpfull for understanding the mechanism of their pathogenity in the future studies.

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

A detailed list of commonly used chemicals, solutions and their suppliers are presented in Appendix A and the media used for bacteriological studies are presented in Appendix B.

2.1.1. Reference strains

The reference and type strains used in this study are outlined in Table 2.1. The references were the isolates of 24 different species of staphylococci.

Table 2.1. The reference strains

S.aureus	ATCC 25923	*S.saprophyticus*	128.76- A, 128.76 -B
S.epidermidis	NRRL B-4268, AA-O1,YK-02, RSKK 01015	*S.cohnii spp. cohnii*	NRRL B-14756, cohnii 77, YK-05
S.capitis	YK-06 (OM2), NRRL B-14752	*S.pettenkoferi*	B-3117
S.caprae	JSCC 2/22, NRRL B-14757	*S.condimenti*	DSM 11674
S.simulans	NRRL B-14753	*S.felis*	K-1247
S.lentus	17, 293, 296-3	*S.lugdunensis*	NRRL B-14774
S.sciuri	8-1, 2, 3-2, 13-1, 10-1, SK1	*S.equorum*	NRRL B-14765
S.warnerii	316, warneri 93	*S.chromogens*	NRRL B-14759
S.intermedius	CCM 5739	*S.gallinarum*	NRRL B-14763
S.vitulinus	294-1, 295-1	*S.carnosus*	NRRL B-14760
S.hemolyticus	SH 621	*S.auricularis*	NRRL B-14762
S.xylosus	DSM 20266	*S.arlettae*	NRRL B-14764

2.2. Methods

2.2.1. Bacteriological Techniques

2.2.1.1. Isolation of Staphylococci

The bacteria were isolated from the patients' used shringes, from the mouth of the pygeons and from the foodhandler's. Sterile swabs were used during the procedure. Streak plate method was used with the swabs and single colonies were aimed to get. Blood agar (Appendix B) was used. The petri dishes were incubated overnight at 37ºC and further characterization assays were carried out.

2.2.1.2. Preservation of the Isolates

All of the isolates were enriched in the nutrient broth by incubating at 37ºC overnight in orbital shaker. Then 0.5 ml of each culture was transferred into cryotubes then 20% 500 µl glycerol was added. Tubes were mixed gently and stored at -80ºC.

2.2.1.3. Gram Reaction and Morphology

Isolates grown on tryptic soy agar overnight were used for Gram staining. A few drops of water were placed onto the microscope slides. A loopfull of the overnight culture was mixed with water drops and spread onto the microscope slides until a thin film was formed. The slides were then kept for drying. After drying, the slides were fixed with flame for 1–3 seconds. Then the fixed smear of the culture was first stained with crystal violet for 1 minute. It was then rinsed undertap water. The second chemical used was iodine. The slides were kept in the iodine solution for a minute and after this step the slides were washed by 95% alcohol for 10–15 seconds. They were then transferred into the safranin solution and kept there for 30 seconds. The slides were then washed with tap water and they were left for drying on paper towel. The dry slides were examined under the microscope. They were examined for their both colour and morphology. Gram positive cells appeared purple while the negative ones appeared pink. For this study Gram positive cells with spherical shape (coccus) were looked for.

2.2.1.4. Catalase Test

Isolates were grown on nutrient agar for 24 hours at 37ºC . 3% hydrogen peroxide solution was poured onto the colonies. The formation of the air bubbles indicated the catalase activity.

2.2.1.5. DNase Activity

DNase Test Agar was used for the determination of DNase activity and was prepeared according to the manufacturer's description. The cultures were inoculated to the test agar and were grown for a day at 37ºC. 37 % HCl solution was poured onto the colonies and after 5 minutes passed the colonies the HCl solution was removed. The clear zones around the colonies indicated DNase activity.

2.2.2. Staphylococcus characterization assays

2.2.2.1. Coagulase Test

For the coagulase test of the bacteria, Dry Spot Staphytect Plus (Oxoid) was used. The manufacturar's description was followed. Briefly an overnight culture is prepeared on agar plates. Then a few saline drops are put on the test rea tion area. The saline drops and the culture were well mixed on the test area. The test worked by detecting the clumping factor Protein A and certain polysaccharides found in methicillin resistant *S.aureus*. The agglutination formed indicated the strain was *Staphylococcus aureus* .

2.2.2.2.Mannitol Salt Phenol Red Agar

The solid media used was listed in Appendix B. The culture was inoculated on the mannitol salt agar and was incubated for three days at 37ºC. The pH changes directed the change in colour of the indicator phenol red and the yellow colour was seen as a positive result.

2.2.2.3. Blood Agar

The media prepared was listed in Appendix B. The culture was inoculated on the blood agar and incubated for three days at 37ºC. The hemolytic zones around the colonies indicated the hemolysis was whether α, β or if there was no zone then it was γ.

2.2.3. Screening for Extracellular Enzymes

2.2.3.1. Screening for Protease Activity

The solid media used for the protease screening are listed in Appendix B (Priest et al. 1988) . Cultures were inoculated in the media and incubated for 36 hours at 37ºC. Clear zones around the colonies indicated the presence of the protease activity.

2.2.3.2. Screening for Amylase Activity

The solid media used was listed in Appendix B. The cultures were inoculated and incubated for 1–2 days at 37ºC. Iodine solution (I_2 =1 g, KI= 2g/ 300 ml) was poured on the plates. The iodine dyed the plates with dark blue. The positive result was seen as whiteness appeared around the colonies aginst the dark blue colour of the iodine dyed petri dishes. So this indicated the positiveness of the colonies for the amylase activity (Bragger et al. 1989).

2.2.3.3. Screening for Lipase Activity

The solid media used in detection of the lipase activity were listed in Appendix B. The cultures were inoculated in the media and incubated for three days at 37ºC. The presence of the lipase activity of the colonies was detected as opaque halos occured around the colonies positive for the lipase activity (Haba et al. 2000).

2.2.3.4. Screening for Urease Activity

The cultures were inoculated in the solid media (Appendix B) and incubated overnight at 37ºC. The yellow colour of the phenol red in the agar changed into pink red

colour due to the degradation of urea so that the pH increase changed the colour of the phenol red.

2.2.3.5. Screening for Xylanase Activity

After the inoculation of the cultures in the solid media (Appendix B) and the incubation period of 3-4 days at 37ºC. 0,1 % Congo Red solution was poured onto the plates. The plates were then incubated for 30 minutes at room temperature. Then the plates were washed with 1 M NaCl solution. Clear zones around the colonies on the red background dyed with the Congo Red solution indicated the positive results for the xylanase activity (Bragger et al. 1989) .

2.2.3.6. Screening for Cellulase Activity

The solid media used for the detection of the cellulase activity was listed in Appendix B. The cultures were inoculated in the media and incubated for 3-4 days at 37ºC. 1 % Congo red solution was poured onto the plates, the plates were incubated for 30 minutes at room temperature. The positiveness for the cellulase activity was seen as there were clear colonies around the colonies against the red background of the Congo Red solution (Bragger et al. 1989) .

2.2.3.7. Screening for Pectinase Activity

The cultures were inoculated in the solid media (Appendix B) and incubated for 3-4 days at 37ºC . 1% cetyltrimethylammoniumbromide solution was poured onto the plates and the plates were incubated for ten minutes at room temperature. The clear zones around the colonies indicated the pectinase activity (Kobayashi et al. 1999) .

2.2.3.8. Screening for Laccase Activity

The media used for the detection of the laccase activity was listed in Appendix B. The cultures were inoculated in the media and incubated for 2-3 days at 37ºC.

Whiteness occured around the colonies aginst the blue background of the solid media which included Comassie Brillant Blue.

2.2.4. Genomic DNA Isolation

Genomic DNA was isolated as described by Arciola *et. al* (2001). Fresh tryptic soy broth was used (Appendix B). The bacterial cultures were inoculated into the broth in the tubes and allowed for growth at 37ºC overnight in orbital shaker. The tubes were then vortexed. 100 µl of the cultures were taken into ependorf tubes. They were centrifuged at 10000 rpm for five minutes and the soup was removed. The pellets were resuspended in 45µl d H_2O and 15 µl lysostaphin (100 µg/ µl). They were incubated at 37ºC for an hour in the water bath. After this step 15 µl Proteinase K (100 µg/ µl) and 150 µl TrisHCl were added and incubated at 37ºC for an hour in the water bath. The samples were then boiled at 100ºC for five minutes. Genomic DNAs were ready and stored at -20ºC .

2.2.5. Amplification of the 16S rDNA-ITS region

The 16S rDNA –ITS region was amplified by polymerase chain reaction. The results were used for the verificaiton of the genomic DNA isolation and used for the RFLP (restriction fragment length polymorfism) analysis.

Two µl of the genomic DNA samples were transferred into 0.2 ml PCR tubes. The PCR mixture was prepared as indicated in Appendix C. 48 µl of the PCR mix was transferred to each of the tubes. All the steps were carried on ice. Amplifications were performed in a Mini Cycler System. The PCR conditions used for amplificaitons were:

94ºC for 5 minutes	→	initial denaturation	
94ºC for 1 minute	→	denaturation	40 cycles
42ºC for 1 minute	→	annealing	
72ºC for 1 minute	→	elongation	
72ºC for 10 minutes	→	final extension	

The forward primer used in the experiment L1 was complementary to the upstream of 16S rDNA region and the reverse primer EGE1 was complementary to the downstream sequences of ITS region.

The base orders of the primers were as follows:
L1 (forward primer): 5' - AGAGTTTGATCCTGGCTCAG- 3' (Mora et al. 1998)
EGE1 (reverse primer): 5' - CAAGGCATCCACCGT- 3' (Jensen et al. 1993).

2.2.5.1. Electrophoresis of the Amplified 16S-ITS region

Agorose gels of 0.8 % were prepared for the seperation of the amplified fragments. 0.8 gr agarose was dissolved in 100 ml of 1x TAE by boiling. When the agarose solution was cooled to 40ºC 7,5 µl of ethidium bromide solution was added . The combs were placed on the gel casting stand and the agarose solution was poured . After the gel was cooled and became solid the combs were taken out. The gel was placed in the tank with its casting stand. The tank was filled with 1x TAE until the buffer covered the gel. 5 µl of PCR products were taken from the PCR tubes and were mixed with 1 µl of the 6x gel loading buffer (Appendix C). The samples were then loaded into the wells of the agarose gel. DNA ladder (molecular weight marker) was loaded into the first well of the agarose gel. The electrophoresis was performed for appraoxiamately two hours at 80 V. The PCR products were visualised on an UV illuminator.

2.2.5.2. Na-Acetate Precipitation of the PCR Products

The volume of the PCR products were adjusted to 100 µl by adding 1x TE . Two volumes of chloroform isoamyl alcohol solution (24 chloroform:1 isoamyl alcohol) were added and vortexed for 15-20 seconds. They were then centrifuged for two minutes at 10.000 rpm in microcentrifuge. The bottom phase containing chloroform was removed and two volumes of chloroform isoamyl alcohol solution was added, vortexed and centrifuged for two minutes at 10.000 rpm. The aqueous phase containing the sample was transferred into a new eppendorf tube. 10 µl 3 M sodium acetate (pH 5.2) was added. After mixing well, two and a half volume of 99% ethanol was added and

vortexed for 15-20 seconds. The tubes were centrifuged for 15 minutes at 10.000 rpm. The liquid phase was removed and the pellet was washed with 300 µl 70 % ethanol, vortexed for 15-20 seconds and centrifuged for five minutes at maximum speed of the microcentrifuge. The ethanol was then removed without disturbing the pellet. The eppendorf tubes were then incubated for 15-20 minutes at 37ºC in order to let the ethanol dried out. The pellets were then dissolved in 20 µl 1xTE and stored at -20ºC for later use.

2.2.5.3. Restriction Fragment Length Polymorphism (RFLP)

The endonuclease *Taq*I (T'CGA) was used. The amplification products were digested with 5 units of the enzyme in a final volume of 50 µl, ten µl of the products were mixed with 40 µl enzyme mixture (Appendix C). Two drops of the mineral oil were added and the tubes were incubated overnight at 65ºC in a water bath. After this step in order to reattain the DNA the procedure described in 2. 2. 5. 2 was followed again.

2.2.5.4. Electrophoresis of the Restriction Fragments

Restriction fragments were separated in 2 % agarose gel. 2 gr agarose was dissolved in 100 ml of 1x TAE buffer by boiling. 20 µl of ethidium bromide solution was added after the agarose solution was cooled to 40ºC . The coombs were placed and the agarose solution was poured. After the gel was cooled the combs were taken out. The casting tray with the gel was placed into the tank. 1x TAE buffer was added until the buffer covered the gel. 8 µl of the restriction fragments were taken, mixed with 2µl of 6x loading dye and were loaded into the wells of the gel. 4 µl of DNA molecular weight marker was loaded into the first well of the gel.

Electrophoresis was performed for one hour at 40 mA and for three hours at 60 mA and the gel was visualised and analysed in a gel documentation system (Vilber Lourmat, France).

Restriction patterns were analysed using Bio-1D++ software (Vilber Lourmat) with a 13 % homology coefficient.

2.2.6. PCR Analysis of the Extracellular Enzyme Encoding Genes

2.2.6.1. Polymerase Chain Reaction of *sspA*

0,5µl of template was put in 0,2 ml PCR tubes. The PCR mixture content was prepared as described in Appendix C. 45µl of the PCR mix was distrubuted to each of the PCR tubes. The PCR conditions were .

95ºC for 5 minutes	→	initial denaturation	
95ºC for 30 seconds	→	denaturation	40 cycles
47ºC for 1 minute	→	annealing	
72ºC for 1 minute	→	elongation	
72ºC for 10 minutes	→	final extension	

The base orders of the forward and reverse primers are:
Forward 5'- GACAACAGCGACACTTGTGA - 3'
Reverse 5'- AGTATCTTTACCTACAACTACA - 3'

2.2.6.2. Electrophoresis of the Amplified Products of *sspA*

1,5 % gel was prepared as described in 2. 2. 5. 1, the gel was run for about an hour and a half and visualised

2.2.6.3. Polymerase Chain Reaction of *geh*

0,5µl of template was put in 0,2 ml PCR tubes. The PCR mixture content was prepared as described in Appendix C. 45µl of the PCR mix was distrubuted to each of the PCR tubes. The PCR conditions were .

95ºC for 5 minutes	→	initial denaturation	
95ºC for 30 seconds	→	denaturation	35 cycles
55ºC for 1 minute	→	annealing	
72ºC for 1 minute	→	elongation	
72ºC for 10 minutes	→	final extension	

The base orders of the forward and reverse primers are:

Forward 5' - GCACAAGCCTCGG - 3'

Reverse 5' - GACGGGGGTGTAG - 3'

2.2.6.4. Electrophoresis of the Amplified Products of *geh*

The gel was prepared, the gel was run and visualised as described in 2. 2. 5. 1.

2.2.6.5. Polymerase Chain Reaction of *sspB*

0,5µl of template was put in 0,2 ml PCR tubes. The PCR mixture content was prepared as described in Appendix C.

The PCR conditions were .

95ºC for 5 minutes	→	initial denaturation	
95ºC for 30 seconds	→	denaturation	40 cycles
54ºC for 1 minute	→	annealing	
72ºC for 1 minute	→	elongation	
72ºC for 10 minutes	→	final extension	

The base orders of the forward and reverse primers are:

Forward 5'- TGAAGAAGATGGCAAAGTTAG - 3'

Reverse 5'- TTGAGATACACTTTGTGCAAG - 3'

2.2.6.6. Electrophoresis of the Amplified Products of *sspB*

An agarose gel of 1.5 % was prepared and cooled, ethidium bromide solution was added and the gel was poured as described in 2. 2. 5. 1. The products were loaded and run then the gel was visualized under U. V.

2.2.6.7. Polymerase Chain Reaction of *aur*

The PCR conditions were .

95ºC for 5 minutes	→	initial denaturation	
95ºC for 30 seconds	→	denaturation	35 cycles
58ºC for 1 minute	→	annealing	
72ºC for 1 minute	→	elongation	
72ºC for 10 minutes	→	final extension	

The base orders of the forward and reverse primers are:

Forward 5'- TAGTAGCACACGAATTAACACACG - 3'

Reverse 5'- TTCCCTATTGCTTGAATCACG - 3'

2.2.6.8. Electrophoresis of the Amplified Products of *aur*

The gel was prepared the PCR products were loaaded and the gel was run for about an hour and a half at 75 V. Then the gel was visualized.

2.2.6.9. Polymerase Chain Reaction of *prt*

The PCR conditions were .

95ºC for 5 minutes	→	initial denaturation	
95ºC for 30 seconds	→	denaturation	40 cycles
57ºC for 1 minute	→	annealing	
72ºC for 1 minute	→	elongation	
72ºC for 10 minutes	→	final extension	

The base orders of the forward and reverse primers are:

Forward 5' -CAAGTTGAAGCACCTACTGG - 3'

Reverse 5' - TAGAGTGTGAATCGGCTTTGG - 3'

2.2.6.10. Electrophoresis of the Amplified Products of *prt*

The gel was prepared as described in 2. 2. 5. 1. The samples were loaded and run for about an hour and visualized.

2.2.6.11. Polymerase Chain Reaction of *nuc*

0,5µl of template was put in 0,2 ml PCR tubes. The PCR mixture content was prepared as described in Appendix C.

The PCR conditions were :

95ºC for 5 minutes	→	initial denaturation	
95ºC for 30 seconds	→	denaturation	35 cycles
45ºC for 1 minute	→	annealing	
72ºC for 1 minute	→	elongation	
72ºC for 10 minutes	→	final extension	

The base orders of the forward and reverse primers are:

Forward 5'- GGCAATTGTTTCAATATTAC- 3'

Reverse 5'- TTTTATTTGCATTTTCTACC - 3'

2.2.6.12. Electrophoresis of the Amplified Products of *nuc*

1,5 % gel was prepared as described in 2. 2. 5. 1, the gel was run for about an hour and a half and visualised.

CHAPTER 3

RESULTS

3.1. Isolation of Staphylococci

The isolation procedure was described in 2. 2. 1. The isolates were selected according to their colony morphologies at first then further characterized. 12 isolates were isolated from the patients', 40 of them were isolated from the foodhandler's hygiene detections and 27 of the isolates were isolated from pygeons (Table 3.1)

Table 3.1. Sample, name and numbers of the isolates

Sample	No.	Isolate
Patients'	12	YT04, YT26, YT39, YT70, YT72, YT58, YT81, YT83, YT165, YT170, YT75, YT49
Foodhandlers'	40	Per1, 2, 3A, 4, 5, 7, 8, 9, 10A, 10B, 13A, 14, 16B, 17, 19A1, 20A, 20B, 21B23, 24, 25A, 25B, 26B, 27, 28, 29, 30, 32, 33, 34, 35A, 36, 37A, 38, 39A, 39B1, Per40, Hüs1#2, EY-1, EY-2
Pygeons'	27	GS6, 11, 16, 18, 21, 27, 35, 43, 48, 55, 57, 59, 62, 63, 67, 87, 90, 98, 99, 104, P8#1, P10#4, P20#1, P10#2, bid1#1, P12#1, H3#2

3.2. Phenotypic Characterizations

3.2.1. Gram Reaction and Morphology

The isolates were Gram stained as described in 2. 2. 1. 3 examined under the microscope. Purple-violet cells in coccus morphology forming clusters were accepted as staphylococci.

3.2.2. Catalase Test

As one of the main features of staphylococci was being catalase positive, all isolates were tested and the positive ones were selected.

3.2.3. DNase Test

All of the isolates including the reference strains were examined for the presence of the DNase activity. The positive result was seen as there was the opaque degradation zone around the DNase positive colonies. DNase positive isolates were suspected to be *Staphylococcus aureus.*

3.3. Staphylococcus Characterization Assays

3.3.1. Coagulase Test

There are many commercially available *Staphylococcus aureus* agglutination assays, in this study Oxoid-Dry Spot Assay was used according to the manufacturer's description 2. 2. 2. 1. *Staphylococcus aureus* was used as a reference. DNase positive isolates were selected in order to be further characterized. The results revealed that four of the 25 DNase positive isolates were *Staphylococcus aureus.* Since some other species of staphylococci *S.hyicus*, *S. intermedius* (Phillips et al. 1981*)*, *S.lugdunensis* (Freney et al. 1988), *S.xylosus*, *S.schleiferi* (Jean-Pieree et al. 1989) and *S.haemolyticus* could possess Protein A, the agglutination would not give the exact results if all the samples were tested including the DNase negative ones.

3.3.2. Mannitol Salt Phenol Red Agar

This phenotypic test is aimed to characterize staphylococci able to grow in extreme salt contitions. All of the isolates were tested. Mannitol Salt Agar also contains the sugar mannitol and the pH indicator phenol red (Appendix B). If an organism can ferment mannitol, an acidic byproduct is formed that will cause the phenol red in the

agar to turn yellow. Most pathogenic staphylococci, such as *Staphylococcus aureus*, will ferment mannitol. Most non-pathogenic staphylococci will not ferment mannitol.

The strains having the other features of *Staphylococcus aureus* expected to be positive as the salt tolerence as well as mannitol fermentation were the characteristics of this species so a reference of *Staphylococcus aureus* included as a positive control.

The results showed that 28 of the isolates were positive whereas there was a change in colour into orange in 5 of the isolates, 13 of the isolates were negative as there was no sign of change in colour and 7 of the isolates were not able to grow in the mannitol salt agar (Table 3.2).

Table 3.2. Growth in Mannitol Salt Agar (the isolates written in bold indicate DNase positive staphylococci that were also positive in coagulase test)

Result	**Isolate**
Positive	Per32, 27, 1, 20A, 29, 25A, 3A, 24, 34, 28, 14, 4, **19A1**, 35A, 23, 39B1, 20B, 25B, **21B**, 7, 38, GS55, 43, 99, **59**, 87, **62**, 104, **St.02(reference)**
Negative	Per8, 9, 10A, 10B, 17, 40, GS6, 11, 48, 90
Orange colour	Per16B, Per26B, GS18, GS35, GS67
No growth	Per2, 5, 13A, 16, 30, 37A, GS16, 98

3.3.3. Blood Agar

The aim of the test is to examine the bacteria according to their ability to breakdown the red blood cells and hemoglobin. Beta-hemolysin breaks down the red blood cells and hemoglobin completely. This leaves a clear zone around the bacterial growth. Such results are referred to as β-hemolysis. Alpha-hemolysin partially breaks down the red blood cells and leaves a greenish colour behind. This is referred to as α-hemolysis. The greenish color is caused by the presence of biliverdin, which is a by-product of the breakdown of hemoglobin. Hemolysins are not produced and the red blood cells are not broken down, no clearing will occur. This is called γ-hemolysis. Some strains of *Staphylococcus aueus* are known to produce β-hemolysin

so all of the isolates and reference strains were examined for their hemolysin activity in order to be further grouped. The resuluts showed that 31 of the cultures had a greenish colour around the colonies and reffered to as α-hemolysis 13 of the cultures had clear zones around the colonies and referred to as β-hemolysis, the cultures that had no hemolysin activity were to have γ-hemolysis.

3.4. Extracellular Enzyme Profiles of Staphylococci

All of the cultures including the references and the isolates were screened for the presence of the activities of the enzymes lipase, proteinase, amylase, cellulase, xylanase, urease, pectinase and laccase by adding their substrates to the media (AppendixB). Briefly the substrates were Tween 20, Tween 80, lipid emulsion and tributyrin for lipase, casein and skim milk for proteinase, starch for amylase, carboxymethylcellulose sodium salt for cellulase, birchwood xylan for xylanase, uric acid for urease, polygalacturonic acid, pectin from citrus peel and pectin from citrus fruits for pectinase and potato dextrose agar for the laccase activity. There was no laccase nor pectinase activity detected among 128 bacteria. The enzyme screening was done twice for each of the cultures (Figure 3.1).

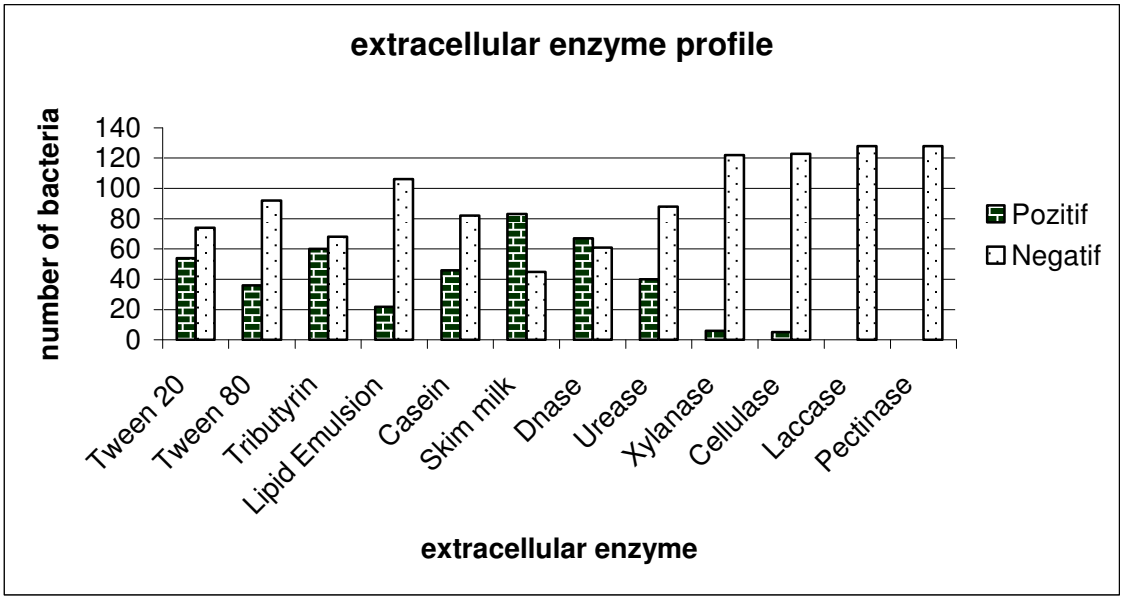

Figure 3.1. Extracellular enzyme production among staphylococci

All of the bacteria had been searched for the presence of the extracellular enzyme production biochemically. All the positive results for each of the biochemical assay is shown on Figure 3. 2

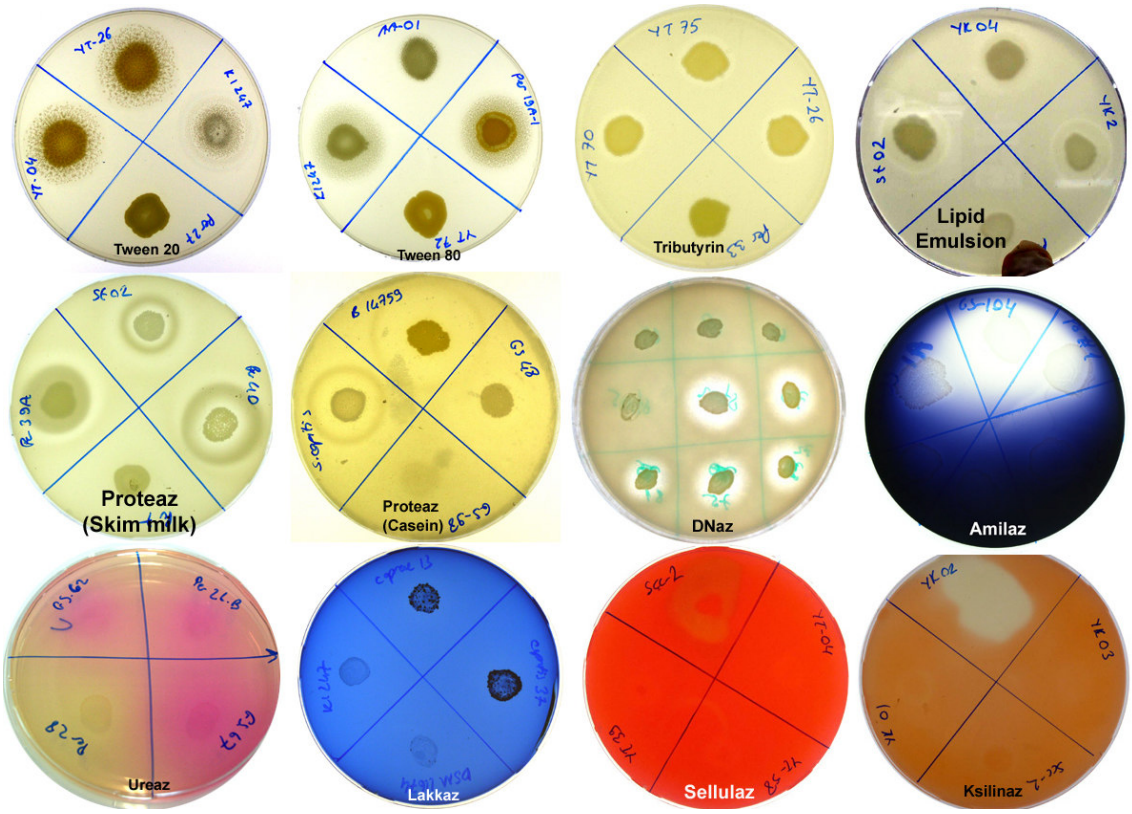

Figure 3.2. Extracellular enzyme production of staphylococci

The amylase enzyme data was omitted. Although amylase enzyme profile was searched with three different media (Appendix B) the positive results were obtained not immediately when the iodine was poured on the plates but after there was a long time passed by. So that was why amylase data of these bacteria was found unreliable. An amylase producing bacteria *Bacillus thrungiensis* was used as a positive control and the amylase degradation was visualized immediately after the iodine was poured on the plate.

The number of the positive bacteria for an enzyme was also further correlated with the total number of bacteria for each of the enzymes and figured on Figure 3. 3

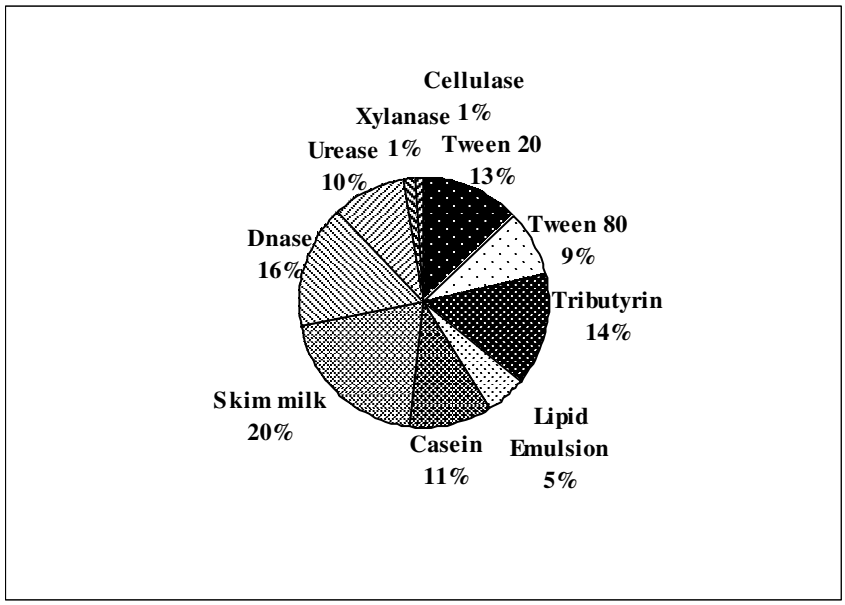

Figure 3.3. Percentage of extracellular enzyme production among staphylococci

The highest percentage of the production was seen in the protease activity within the skim milk agar followed by the DNase activity and the lipase activity. In 128 samples, 54 of them were positive in the Tween 20 media that accounts for the 13 % of the bacteria, 36 of the bacteria were positive in the Tween 80 media that accounts for the 9 % of the bacteria, the lipase activity of 60 bacteria was detected in the Tributryin media that accounts for the 14 %, the lipase activity of 22 bacteria was detected in the lipid emulsion media that accounted for the 5 % of all the bacteria. DNase activity was detected 67 of the cultures and the percentage is 16 %. The protease activity was detected within two different media and 83 of the bacteria was positive in skim milk agar that accounted for the 20 % of the bacteria, in the casein agar 46 of the bacteria had protease activity which accounted for the 11 % of the bacteria. There were 40 bacteria being urease positive and the percentage of the urease activity is 10 %. 6 of the bacteria had xylanase activity that accounted for the 1 % of all the bacteria and 5 of the bacteria had cellulase activity having 1 % percentage within all the bacteria. The results of the each of the extracellular enzyme assay for the isolates were shown on the table 3.3.

Table 3.3 The results of the each extracellular enzyme assay for the isolates.

Isolates	Urease	DNase	Tw20	Tw80	Lipid Emulsion	Tributryin	Casein	SkimMilk Milk	Amylase	Pectinase	Cellulase	Xylanase	Laccase
Per1	-	-	+	-	-	-	-	+	-	-	-	-	-
Per2	-	-	-	-	-	-	-	+	-	-	-	-	-
Per3A	+	-	-	-	-	-	-	+	-	-	+	-	-
Per4	+	-	-	-	-	-	-	-	-	-	-	-	-
Per5	-	-	-	-	-	-	-	-	-	-	-	-	-
Per7	-	-	+	-	-	-	-	-	-	-	-	+	-
Per8	+	-	-	-	-	-	-	-	-	-	-	-	-
Per9	-	-	-	-	-	+	-	+	-	-	-	-	-
Per10A	+	-	+	+	+	+	-	+	-	-	-	-	-
Per10B	-	+	+	-	-	-	-	+	-	-	-	+	-
Per13A	-	-	-	-	-	-	-	+	-	-	-	-	-
Per14	-	-	-	-	-	+	-	+	-	-	-	-	-
Per16B	-	-	+	+	-	+	-	+	-	-	-	-	-
Per17	+	-	+	-	+	+	+	+	-	-	-	-	-
Per19A	-	+	+	+	+	+	-	-	-	-	-	-	-
Per20A	-	-	-	-	-	+	-	+	-	-	-	-	-
Per20B	-	-	-	-	-	+	-	+	-	-	-	-	-
Per21B	+	-	-	-	-	-	-	+	-	-	-	-	-
Per23	-	-	-	-	-	-	-	-	-	-	-	-	-
Per24	-	-	-	-	-	+	-	-	-	-	-	-	-
Per25A	-	-	+	+	+	+	-	-	-	-	-	-	-
Per25B	-	-	-	-	-	+	-	-	-	-	-	-	-
Per26B	-	-	-	-	-	+	-	-	-	-	-	-	-
Per27	+	-	-	-	-	-	-	-	-	-	-	+	-
Per28	-	-	-	-	-	-	+	+	-	-	-	-	-
Per29	-	-	-	-	-	-	+	+	-	-	-	-	-
Per30	-	-	-	-	-	-	-	-	-	-	-	-	-
Per32	-	-	+	-	-	+	-	-	-	-	-	-	-

Table 3.3 The results of the each extracellular enzyme assay for the isolates. (continued)

Isolates	Urease	DNase	Tw20	Tw80	Lipid Emulsion	Tributryin	Casein	SkimMilk Milk	Amylase	Pectinase	Cellulase	Xylanase	Laccase
Per33	-	-	-	-	-	-	-	+	-	-	-	-	-
Per34	+	-	-	-	-	+	-	+	-	-	-	-	-
Per35A	-	-	-	-	-	-	+	+	-	-	-	-	-
Per36	-	-	-	-	-	+	-	+	-	-	-	-	-
Per37A	-	-	+	+	-	+	-	+	-	-	-	-	-
Per38	-	-	+	+	-	+	-	+	-	-	-	-	-
Per39A	+	-	+	+	+	+	+	+	-	-	-	-	-
Per39B	-	-	+	-	+	+	-	-	-	-	-	-	-
Per40	-	-	+	+	+	+	+	+	-	-	-	-	-
Hüs1.2	-	-	-	-	-	-	+	-	-	-	-	-	-
P12.1	-	+	-	-	-	-	+	+	-	-	-	-	-
GS6	-	+	-	-	-	-	+	+	-	-	-	-	-
GS11	-	+	-	-	-	-	+	-	-	-	-	-	-
GS16	-	-	-	-	-	-	-	-	-	-	-	-	-
GS18	-	-	+	+	-	+	-	+	-	-	-	-	-
GS21	-	+	-	-	-	-	+	+	-	-	-	-	-
GS27	-	-	-	-	-	-	-	-	-	-	-	-	-
GS35	-	+	+	+	-	-	-	+	-	-	-	-	-
GS43	-	-	-	-	-	-	+	+	-	-	-	-	-
GS48	-	+	+	-	-	-	+	+	-	-	-	-	-
GS55	-	+	-	-	-	-	+	+	-	-	-	-	-
GS57	-	+	-	-	-	-	+	+	-	-	-	-	-
GS59	-	+	+	+	-	-	-	+	-	-	-	-	-
GS62	-	+	+	+	-	-	-	+	-	-	-	-	-
GS63	-	-	+	+	-	-	-	+	-	-	-	-	-
GS67	-	-	+	-	-	-	-	+	-	-	+	-	-
GS87	-	+	+	-	-	-	+	+	-	-	-	-	-
GS90	-	+	-	-	-	-	+	+	-	-	-	-	-

Table 3.3 The results of the each extracellular enzyme assay for the isolates. (continued)

Isolates	Urease	DNase	Tw20	Tw80	Lipid Emulsion	Tributryin	Casein	SkimMilk Milk	Amylase	Pectinase	Cellulase	Xylanase	Laccase
GS98	-	-	-	+	-	-	-	-	-	-	+	-	-
GS99	-	+	-	-	-	-	+	+	-	-	-	-	-
GS104	-	+	+	-	-	-	-	+	-	-	-	-	-
GS106	-	-	-	-	-	-	-	+	-	-	+		-
P8.1	-	-	-	+	-	-	+	+	-	-	-	-	-
P10.4	-	-	-	-	-	+	-	-	-	-	-	-	-
P20.1	-	+	+	-	-	+	-	+	-	-	-	-	-
BÇ-1	-	-	+	-	-	-	-	+	-	-	-	-	-
P10.2	-	-	-	-	-	-	-	-	-	-	-	-	-
Bid 1.1	-	-	-	-	-	-	-	-	-	-	-	-	-
Hüs1.1	+	-	-	-	-	-	-	-	-	-	-	-	-
EY1	-	+	+	-	-	-	-	-	-	-	-	-	-
EY2	-	-	+	-	-	-	-	-	-	-	-	-	-
H3.2	-	+	-	-	-	-	+	+	-	-	-	-	-

The extracellular enzyme production of the isolates were examined separately (Figure 3. 4).

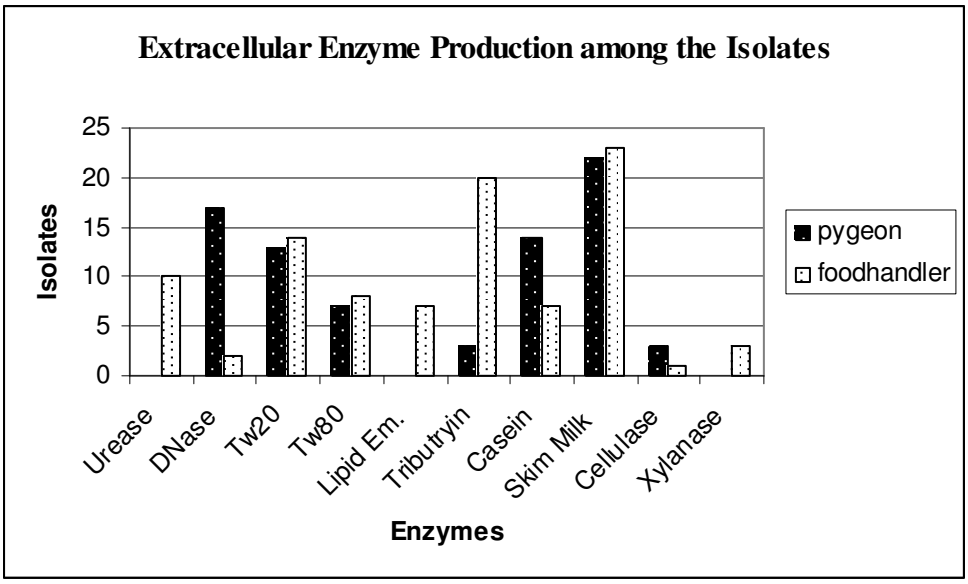

Figure 3.4. The extracellular enzyme production among the isolates.

The results show that urease activity was seen in the foodhandlers' isolates whereas DNase activity were high among the pygeon isolates. In the case of the lipase activity, the two isolates had nearly the same number of positiveness in Tw20 and Tw80 media, in the lipid emulsion media only the foodhandlers' isolates had activity. In another lipid media tributryin foodhandlers' isolates had more lipase activity than the pygeon isolates. When the activity in the two proteinase media were compared, pygeon isolates had more activity in the casein media whereas the foodhandlers'isolates had more activity in the skimmilk media. Pygeon isolates had more cellulase activityand the xylanase activity was only detected among the foodhandlers' isolates.

Since there were four different media were used for the screening of the lipase activity, lipase positive cultures were further characterized and the percentage of the positiveness in each media to all of the lipase positive samples were examined (Figure 3.5).

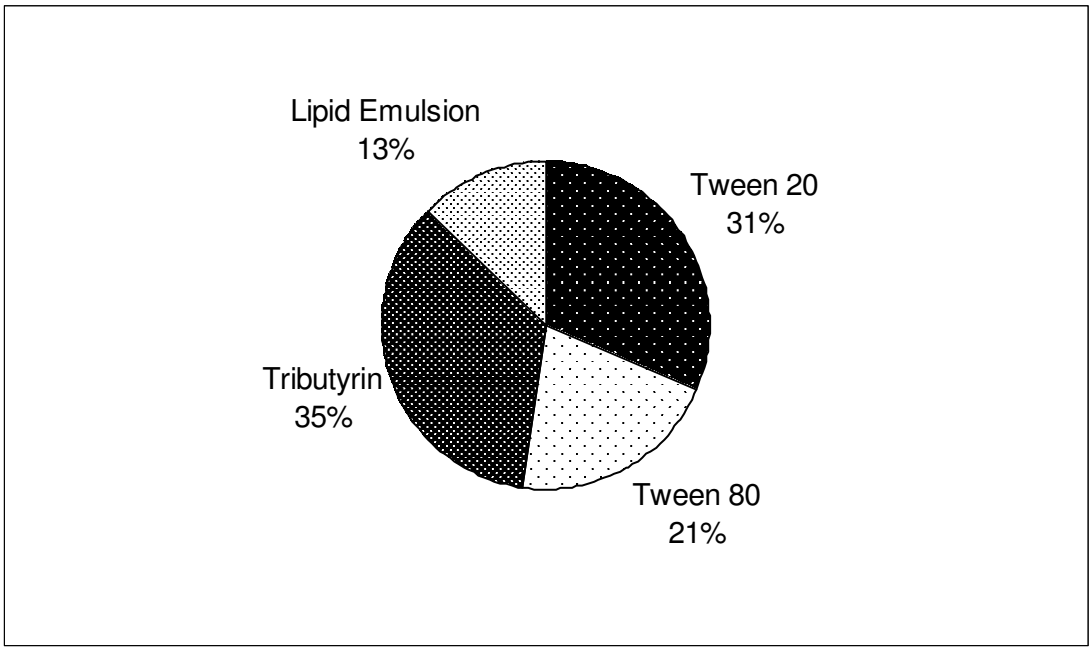

Figure 3. 5 The percentage of the lipase activity in four different media among all the lipase positive cultures.

The highest percentage among the lipase media was the Tributyrin degradation followed by Tween 20 and Tween 80, the lowest positiveness for the lipase activity was seen in the lipid emulsion media.

If the positive result for the lipase activity is the ability to degragade lipid molecules in any of the media rich in lipid (Tween 20, Tween 80, Tributryin and Lipid Emulsion) 82 of the bacteria are positive for the lipase activity with %29 percentage.

The protease screening was carried out with two different media in skim milk and casein, therefore if the positive result was due to the positiveness in any of the protease media then 85 of the bacteria was positive that accounts for the %29 percentage.

Extracellular enzyme producing bacteria tried to be further characterized by grouping the bacteria according to their enyme production together. Firstly the DNase positive cultures were further characterized in correlation with their activities in four different lipase media (Figure 3.6)

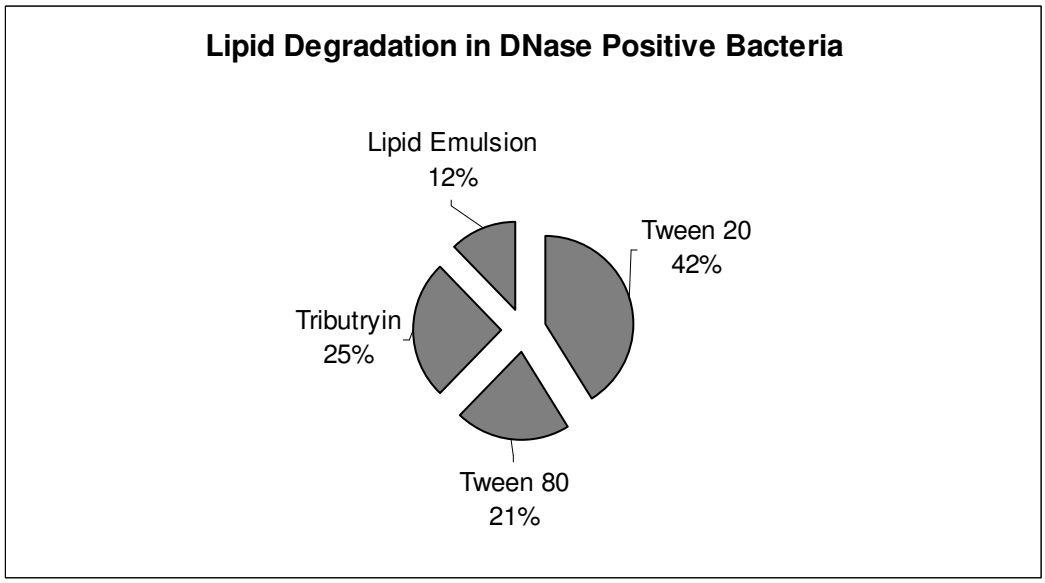

Figure 3. 6 The percentage of DNase positive bacteria in the four lipase media

Of all the DNase positive bacteria 47 of them had lipase activity in Tween 20 with the percentage of 42%, 29 of them had lipase activity in Tributryin with the percentage of 25%, 24 of them were positive in Tween 80 media with a percentage of 21% and 14 of them with the percentage of 12% were positive in the lipid emulsion media.. It was seen that some of the DNase positive bacteria were positive for two or more of the four lipid rich media. These are listed in table 3.4.

Table 3.4. Lipase enzyme groups for the DNase positive bacteria

DNase positive bacteria also lipase positive in two or more media	Number of bacteria	The code of the bacteria
DNase, Tw20, Tw80, Lipid Emulsion, Tributryin	6	Per19A–1, K1247, St.02, YT04, YT83, YK06
DNase, Tw20, Lipid Emulsion, Tributryin	1	EY–1
DNase, Tw20, Tw80, Tributryin	10	YT04, YT70, YT72, YT58, YT81, YT165, YT170, YT75, YT49
DNase, Tw20, Tw80, Lipid Emulsion	1	B14752
DNase, Lipid emulsion, Tributryin	1	Caprae13
DNase, Tw80, Tw20	7	GS35, GS48, GS59, GS87, GS104, P20#1, YT39

DNase positive bacteria also correlated with the protease activity in the two protease media (Figure 3.7)

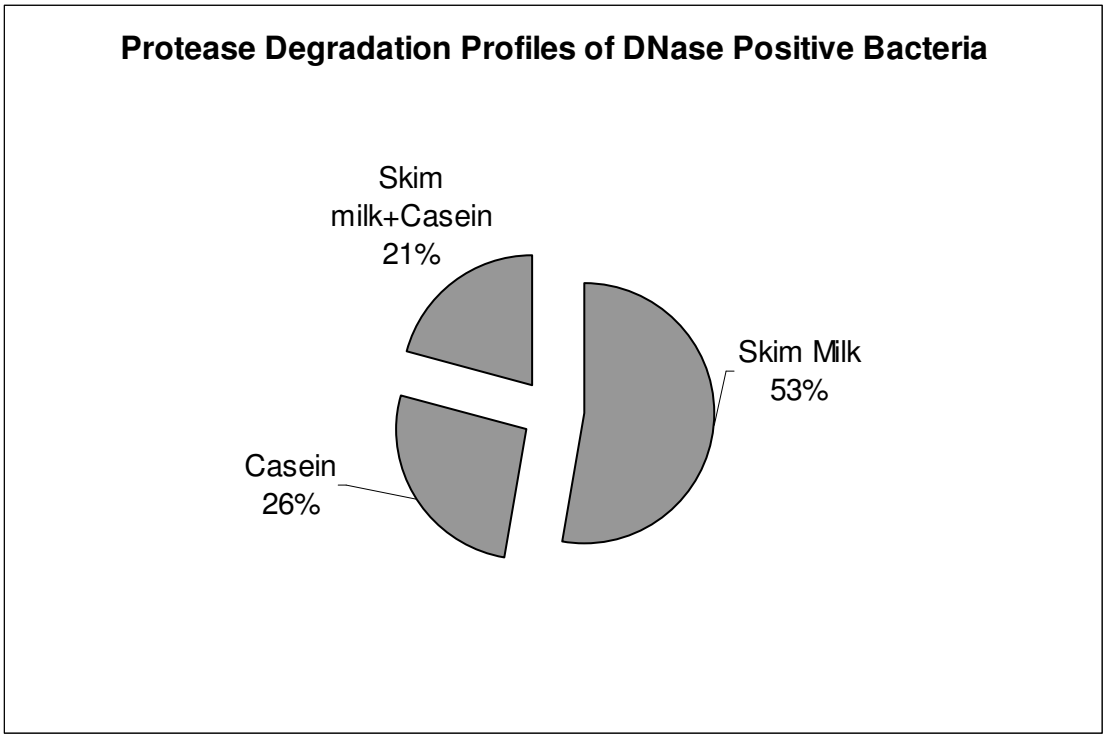

Figure 3.7. The percentage of DNase positive bacteria in the two proteinase media

38% of the DNase positive bacteria had a degradation zone in skim milk media that accounted for 67% and 19 of them had proteolytic zones in casein media with a percentage of 33%. It was also seen that 15 of the bacteria had proteolytic zones within the two media with a percentage of 21%.

Within these three data of the enzymes DNase, protease and lipase, the isolate Per19A-1 was found to be positive in the four lipase media and had a DNase activity but it had no protease degrading ability. On the other hand, the references K1247 (*S. felis*), YK-6 (*S. capitis*) and St.02 (*S. aureus*) had DNase activity as well as lipase activity in the four lipid rich media and protease activity in the two media.

Within the whole 128 bacteria 40 of them had both lipase and DNase activity, these bacteria were grouped together. The second group was consisted of the DNase positive and protease positive bacteria which were 38 in number. The protease, lipase and DNase positive bacteria were grouped together and this group was consisted of 21 bacteria. Another group was lipase and protease positive bacteria that were 55 in

number. Urease and protease positive bacteria were also grouped together and the result showed that 20 bacteria had both urease and protease activity. Urease and lipase producing bacteria were grouped together and 23 bacteria were found to be both urease and lipase positive. Urease, lipase, protease producing bacteria were grouped together, the group consisted of 16 bacteria capable of both urea, protein and lipid degradation biochemically in the media rich in their substrates. There were five cellulase and six xylanase producing bacteria and these were grouped separetely (Table 3. 4).

Table 3.5. Enzyme groups, the names and the codes of the bacteria

Enzyme Groups	Number of the bacteria	Codes of the bacteria
Lipase+DNase	40	Per19A1, Per25A, GS18, GS35, GS48, GS59, GS62, GS63, GS67, GS87, GS104, P2014757, B14760, capitis37, 104.20, Caprae13, 128.76A, St.02, YT–04, YT–26, YT–39, YT–70, YT–72, YT-81, YT-83, YT-165, YT-170, YT-75, YT-49
Protease+DNase	38	Per10B, P12#1, GS6, GS18, GS21, GS35, GS48, GS55, GS57, GS59, GS62, GS63, GS67, GS87, GS90, GS99, GS104, P8#1, P20#1, H3#2, SS-8, K1247, DSM11674, YK3, YK4, Y5, CCM5739, YK6, B14757, B14759, B14760, B14774, S.capitis37, 104-20, caprae13, 128.76.A, St.02, YT81
Lipase+Protease+ DNase	21	Per10B, Per19A-1, GS35, GGS48, GS59, GS62, GS87, GS90, GS99, GS104, P8#1, P20#1, EY1, K1247, DSM11674, YK-4, CCM5739, YK-06, B14752, B14757, caprae13, St.02, YT-81
Lipase+Protease	55	Per1, Per10A, Per10B, Per14, Per16B, Per17, Per20A, Per24, Per20B, Per25A, Per36, Per37A, Per38, Per39A, Per40, GS11, GS18, GS21, GS35, GS46, GS59, GS62, GS63, GS67, GS87, GS90, GS99, GS104, P8#1, P20#1, BÇ-1, EY1, AA01, K1247, DSM20266, DSM11674, YK1, YK2, YK4, CCM5739, 128.76.B, YK-6, YK-7, B14752, B4268, RSKK01015, B14757, warneri93, B14753, B14756, 104.20, caprae13, 128.76A, YT81

Table 3.5. Enzyme groups, the names and the codes of the bacteria (continued)

Enzyme Groups	Number of the bacteria	Codes of the bacteria
Protease+Urease	20	Per3A, Per20A, Per17, Per21B, Per27, Hüs1#1, AA-01, K1247, DSM20266, DSM11674, YK-4, CCM5739, 128.76-B, YK-7, B-14763, B-14753, S.capitis37, 104.20, caprae13, 128.76-A
Lipase+Urease	23	Per10A, Per17, Per27, Per34, Per39B1, AA01, K1247, DSM20266, DSM11674, YK-4, CCM5839, 128.76B, YK-7, warneri93, B14753, 104.20, EGE40, caprae13, 128.76A, YT39, YT70, YT72, YT49
Lipase+Protease+ Urease	16	Per10A, Per17, AA-01, K1247, DSM20266, DSM11674, YK-4, CCM5839, 128.76-B, YK-7, B14753, 104-20, caprae13, 128.76A, YT-70, YT-72
Cellulase	5	Per3A, GS67, GS97, GS104, GS98
Xylanase	6	Per7, Per10B, Per17, Scc–2, B14760, YT165

3.5. Genotypic Characterization of Staphylococci

3.5.1. PCR Analysis of the Genes for the Extracellular Enzyme Production

There were 114 isolated genomic DNA used for the polymerase chain reactions.The PCR conditions and procedures for the amplification of the genes were described in 2. 2. 6. The PCR reactions were done twice for each of the samples. The PCR results forthe isolates were shown on a table in Appendix F.

The amplification of four protease genes (*sspA*, *sspB*, *aur* and *prt*) revealed that 32 bacteria were positive for the *sspA* amplification whereas the number was 12 for the *sspB*, 18 for the *aur* and 28 for the *prt* .

13 bacteria expressed *geh* (glycerol ester hydrolyse) and *nuc* (thermonuclease) was expressed by 37 bacteria (Figure 3. 8).

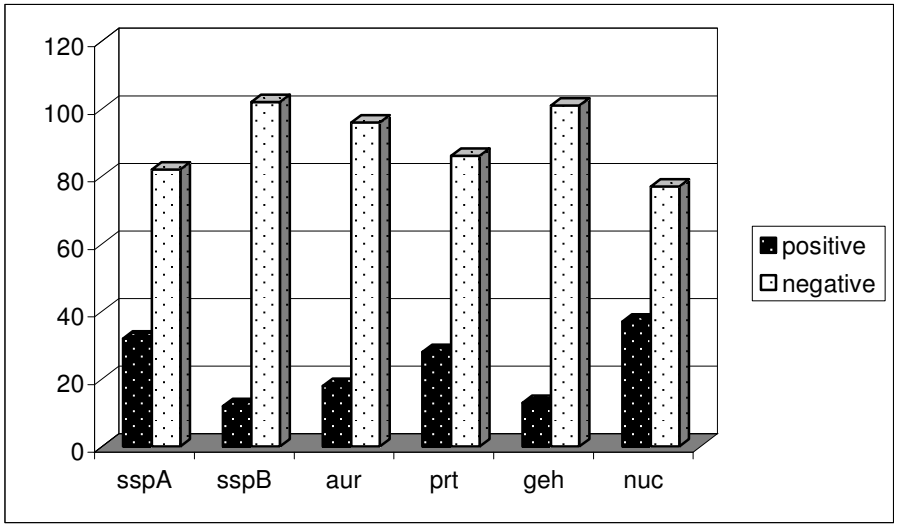

Figure 3. 8 Protease, lipase and thermonuclease amplifications.

SspA (serine protease) amplification resulted in the production of 292 bp. product . 32 bacteria were found to express this gene .

The gene results were correlated with the solid agar protease tests . The results showed that within these 32 bacteria 12 of them had protease activity only in skim milk agar, one of them had protease activity only in casein agar, 4 of them had protease activity within the two media and 15 of them showed no protein degradation ability within the two test media. The percantage of the bacteria were shown on (Figure 3. 9) .

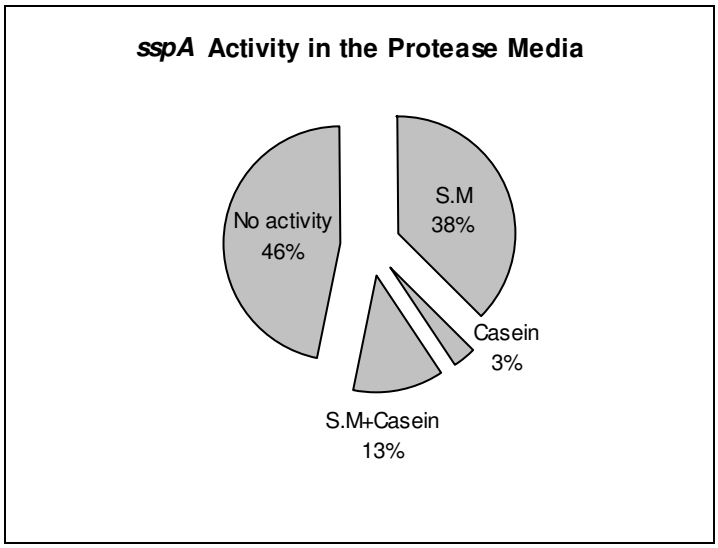

Figure 3. 9 The percentage of the *sspA* expressing bacteria in the two protease media

The percentages showed that within the whole *sspA* expressing bacteria 38% had activity in only skim milk agar whereas 3% had activity only in casein agar. 13% of the *sspA* expressing bacteria were able to degrade proteins in both skim milk and casein agar and 46% of the bacteria had no activity in the two media.

sspB (cycsteine protease) yielded a product of 500 bp. The amplification was studied for the bacteria and 12 bacteria were found to express this gene. Within these 12 bacteria 3 of them were found to hydrolyse protein in skim milk agar, 2 of them in both skim milk and casein agar, none of had a degradation zone only in casein agar and 7 of them had no activity neither in casein nor skim milk agar. The percentage of the *sspB* expressing bacteria in the two media is shown on the Figure 3. 10.

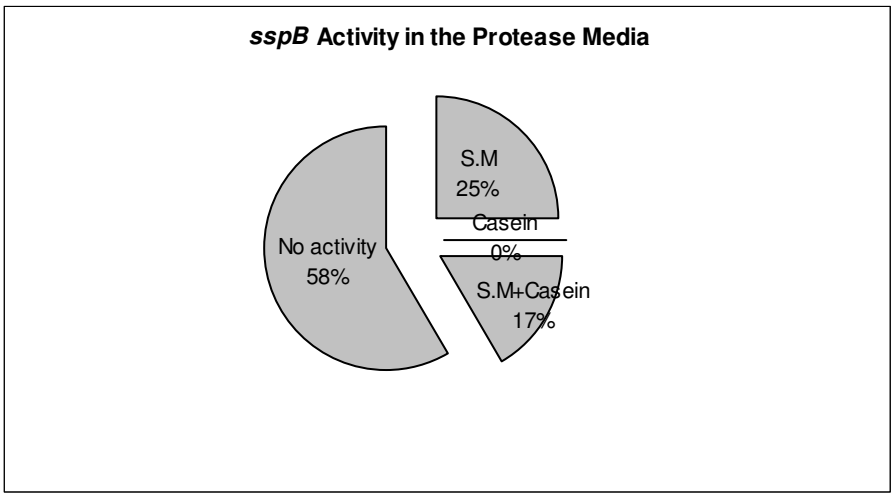

Figure 3. 10 The percentage of *sspB* expressing bacteria in the solid protease media

Of all *sspB* expressing bacteria, 25% had proteolytic ability only in skim milk agar, there was no activity detected in the casein agar and 58% of them had no hydrolysing ability in skim milk and casein agar.

The amplification of the *prt* (serine protease) revealed a product of 740 bp. There were 28 bacteria found to be positive for this gene amplification. When further correlated with the solid media results, it was seen that 7 bacteria had proteolytic ability only in the skim milk agar, 3 bacteria could only hydrolyze casein, 7 bacteria were able to hydrolyze both skim milk and casein and 11 bacteria had no proteolytic activity detected within these two media. The percentage of the prt expressing bacteria having the ability to hydrolyze skim milk and casein is presented on the Figure 3. 11.

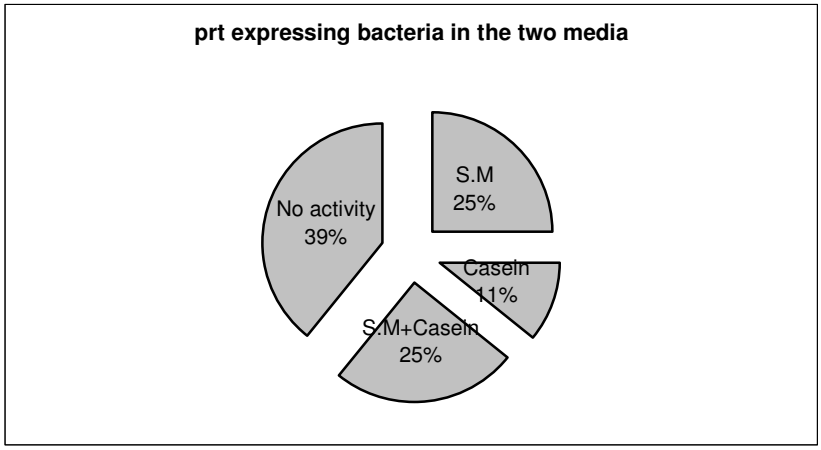

Figure 3. 11 The percentage of *prt* expressing bacteria in the two protease media

Of all the *prt* expressing bacteria, 25% had protease activity in the skim milk agar, 11% had this ability only in the casein agar, 25% had proteolytic ability both in the skim milk and casein agar and 39% showed no degradation neither in skim milk nor in casein agar.

aur (aurolysin) amplification resulted in the production of 340 bp product. 18 bacteria were found to express this gene. The soid media skim milk and casein agar used for the protease activity were also correlated with this gene. The results indicated that five of them were positive for the proteolysis within the skim milk agar, one of them was only positive for the casein agar, one of them was positive in the two media and finally 11 of them showed no proteolytic ability. The percentages were presented in figure 3. 12.

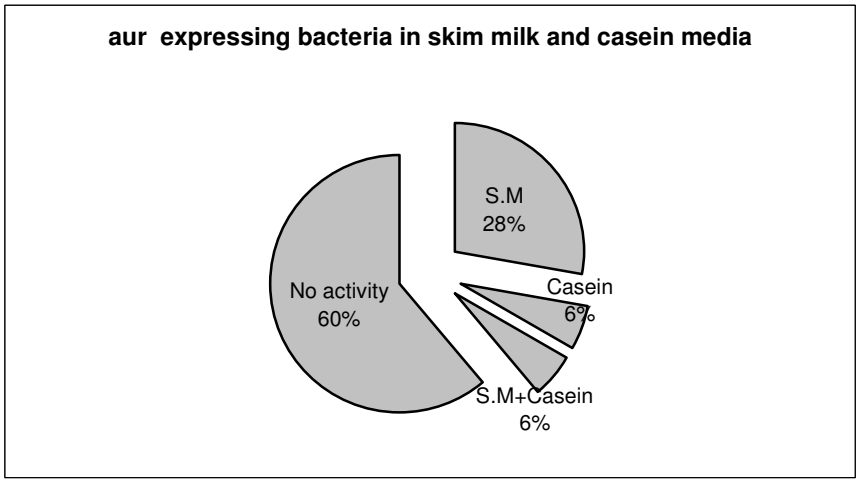

Figure 3. 12 The percentages of the aur expressing bacteria in the proteinase media.

The percentages revealed that 28% of the *aur* expressing bacteria had hydrolyzing ability only in skim milk media, 6% of them were able to hyrolyze only casein, 6% of them had proteolytic ability both in skim milk and casein media whereas 60% of them showed no hydrolyzing ability.

geh (glycerol ester hydrolyze) yields a product of 473 bp gene when amplified. PCR analysis was also done for the presence of this gene. Since the lipase activity of the bacteria was studied with fourdifferent lipase media, the results of *geh* amplification was correlated with these four solid media.

The results showed 13 bacteria expressed this gene when correlated with the solid media, 1 bacteria (Per10A) had lipid degradation ability in the Tween 20, Tween 80, lipid emulsion and tributryin. 2 bacteria had clear zones of degradation in Tween 20, Tween 80 and tributryin and 4 of the bacteria were positive for lipase activity only in tributryin media. 6 of the *geh* expressing bacteria had no lipase activity in the solid media. The percentages of the *geh* expressing bacteria in the four solid media are presented inthe Figure 3. 13.

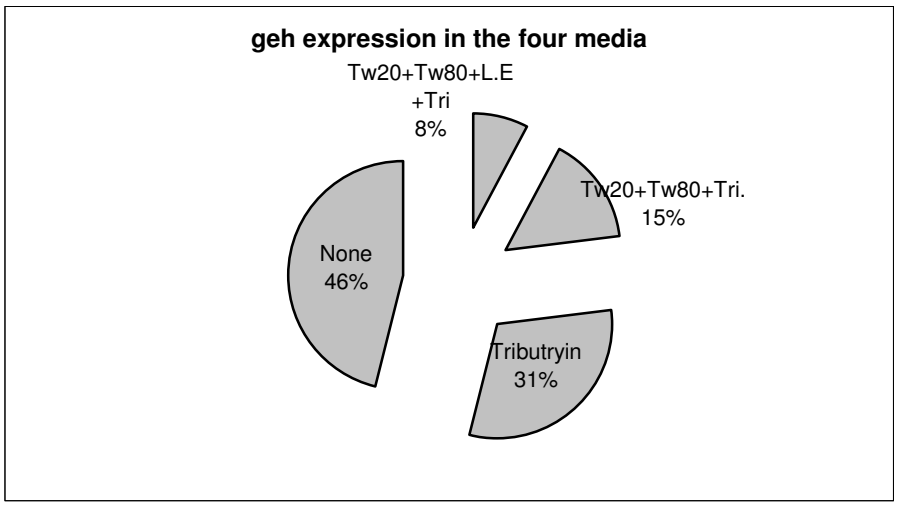

Figure 3. 13 The percentage of the *geh* expressing bacteria in the four lipase media.

46% of the bacteria had no positive result within the four lipase media, 31% had activation onlyin the tributryin media whereas 8% of the bacteria were positive in the four bacteria and finally 15% of the bacteria had lipid degrading ability in Tween 20, Tween 80 and tributryin media.

nuc (thermostablenuclease) when amplified yields a product of 417 bp. 37 bacteria were found to express this gene. Since thermonuclease gene *nuc* encodes for an

enzyme which degrades DNA and RNA, the *nuc* expression profiles were correlated with the results of the solid DNase media. It was seen that of all *nuc* expressing bacteria, the ones which also had DNase activity in the solid media were 20 in number. The percentage is shown on Figure 3. 14 .

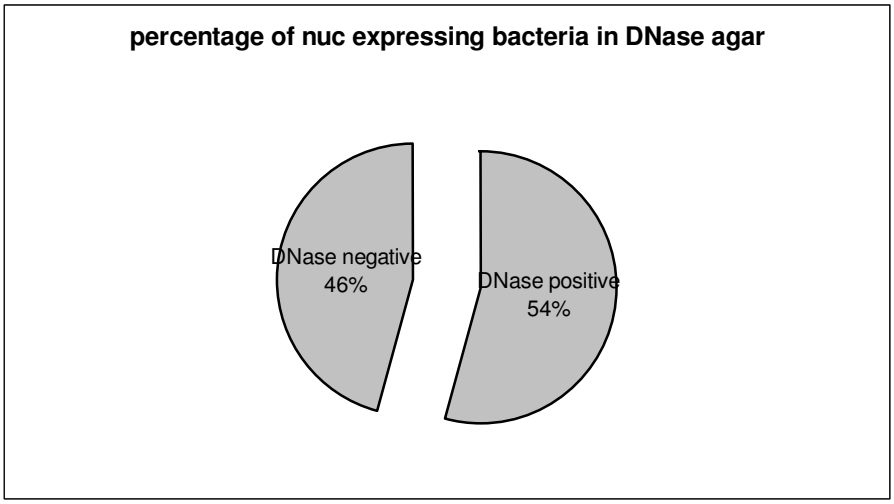

Figure 3.14. The percentage of the thermonuclease (*nuc*) positive bacteria in DNase agar.

The amplifications were visualized with the translimunator and the results are shown on Figure 3. 15, 3. 16 and 3. 17.

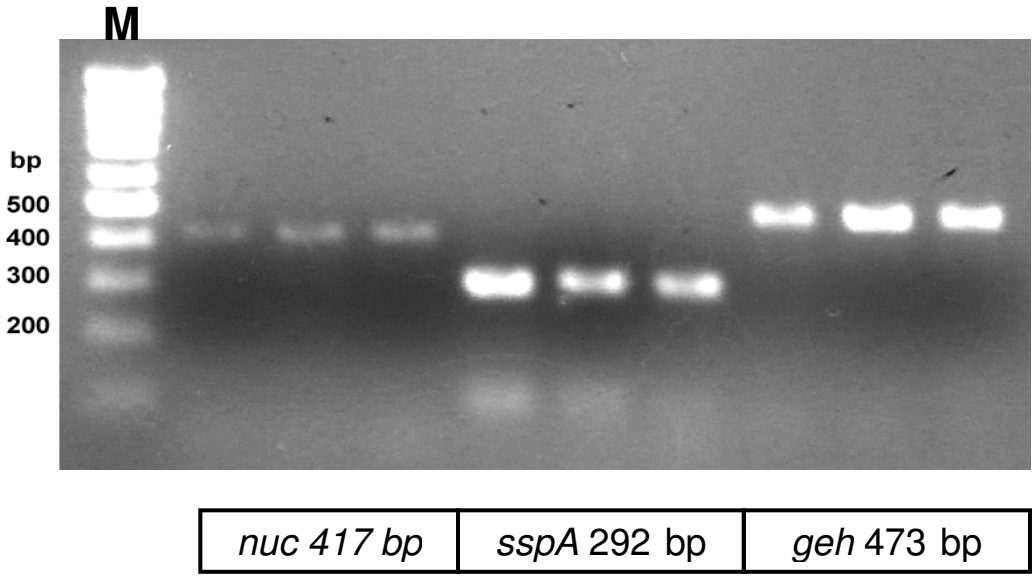

Figure 3. 15. The PCR products of *nuc*, *sspA* and *geh*.

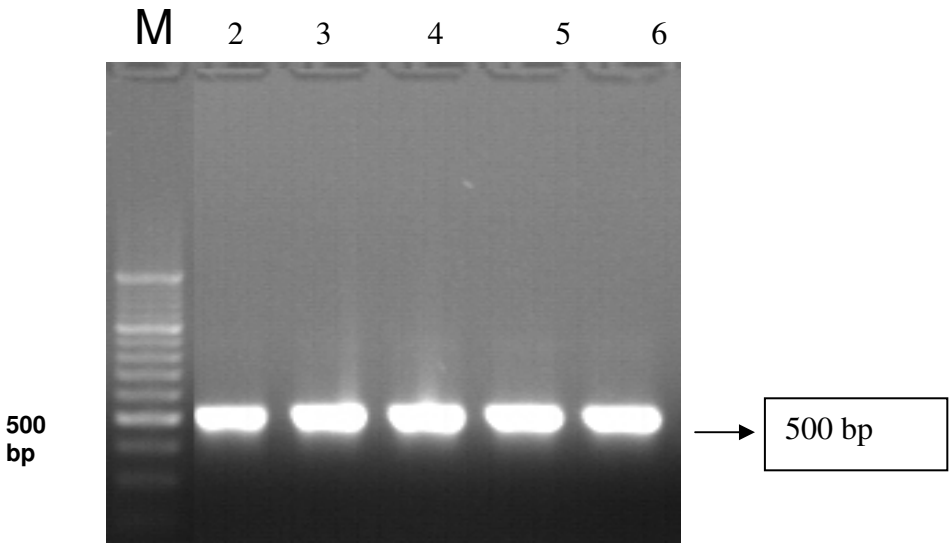

Figure 3.16. PCR products of *sspB*.

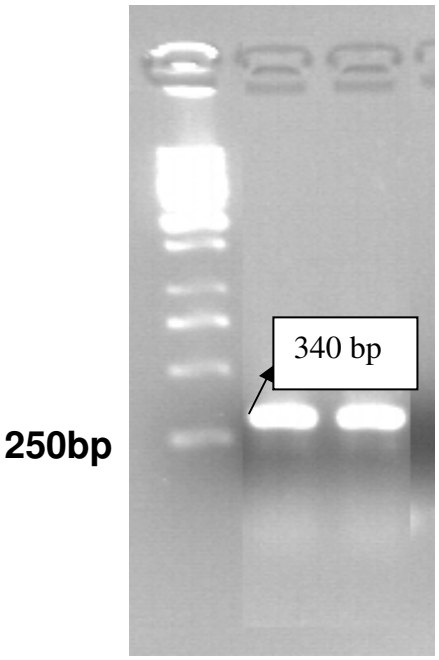

Figure 3.17. PCR products of *aur* .

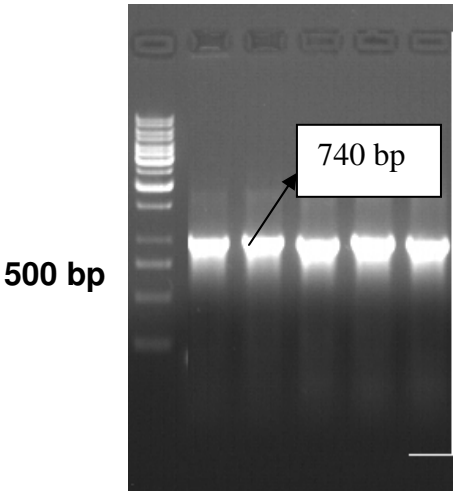

Figure 3.18. PCR products of *prt.*

3.5.2. 16S rRNA- ITS (Internally Transcribed Spacer) RFLP (Restricition Fragment Length Polymorphism) Analysis of the Bacteria

3.5.2.1. Amplification of Bacteria by 16S rRNA– ITS

The 16S rDNA-ITS amplification was done for 70 isolates. The forward primer used was 5'-AGAGTTTGATCCTGGCTCAG-3' and it was complementary to the 5'-end of the 16S rRNA (Mora *et al.*, 1998) and the reverse primer 5'-CAAGGCATCCACCGT -3' was complementary to the downstream sequences of the ITS region (5' end of 23S rRNA) (Jensen *et al.* 1993). The amplification of the isolates with these primers yielded a product of 2000 bp in length (Figure 3.19).

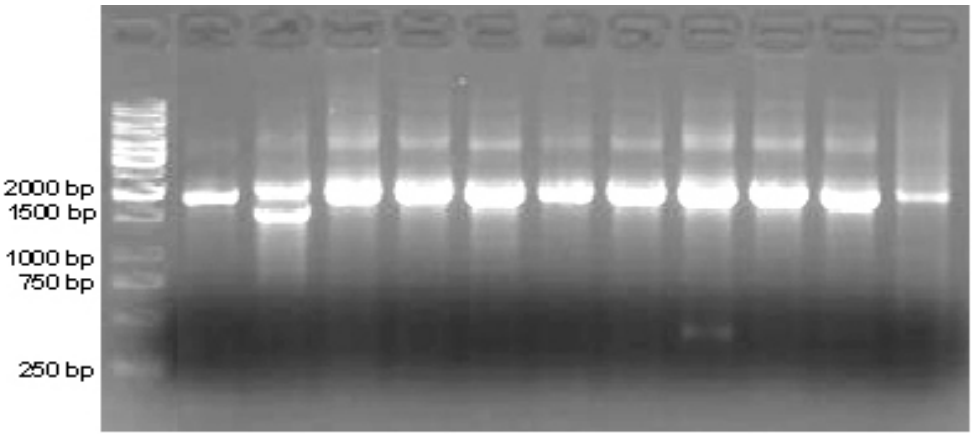

Figure 3.19. 16S- ITS amplification of some isolates .Lane 1 molecular weight marker . 2. Isolate Per1, 3. Isolate Per3, 4. Isolate Per4, 5.Isolate Per5, 6. Isolate Per10A, 7. Isolate Per 19A-1, 8. Isolate GS6, 9. Isolate GS 11, 10. Isolate GS35, Isolate Per 25A.

3.5.2.2. 16S rRNA-RFLP Analysis of Isolates and Reference Strains

The isolates were analyzed by their 16S-ITS rRNA restriction profiles, 54 isolates were digested with the restriction enzyme *Taq1*.

The restriction products were resolved by gel electrophoresis Figure 3.20 .

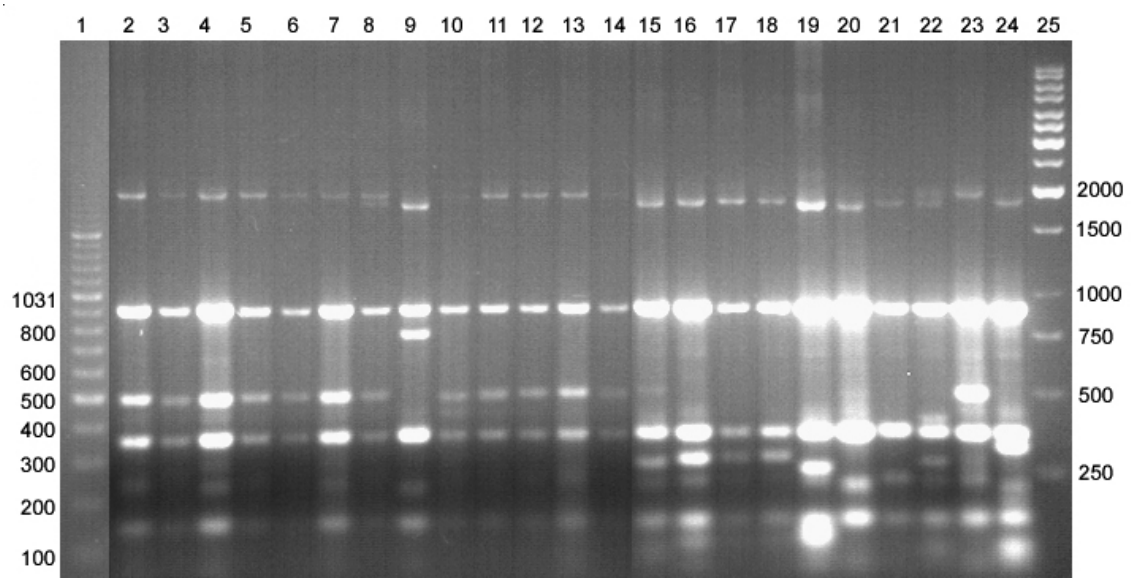

Figure 3.20. *Taq 1* digested 16S-ITS-RFLP profiles of the isolates. Lane 1. DNA ladder, Lane 2. Isolate Per 10B, 3. Isolate Per 19A-1, 4. Isolate Per 25A, 5. Isolate Per1, 6. Isolate Per 3A, 7. Isolate Per 4, 8. Isolate Per 5, 9. Isolate Per 7, 10. Isolate Per 8, 11. Isolate Per 9, 12. Isolate Per 10.A, 12. Isolate Per 13.A, 13. Isolate Per 14, 14. Isolate Per 16.B, 15. Isolate Per17, 16. Isolate Per 20A, 17. Isolate Per 20.B, 18. Isolate Per 21.B, 19. Isolate Per 23, 20. Isolate Per 24, 21. Isolate Per 25B, 22. Isolate Per 26.B, 23. Isolate Per 27, 24. DNA ladder.

The isolates and the references were put into the homology groups according to the *Taq1* digestion by the software. The dendogram was formed on BIO-ID++ Vilber-Lourmat, France.

The dendogram states that there is 90% homology within the two isolates that are Per19 A-1 and Per 10 B, this may be true as they are both DNase positive, *nuc* positive and exept the fact that there is no protein degradation in Per 19 A-1 they share similar properties. Per 19 A- 1 has been detected as *Staphylococcus aureus* by the dry spot test and in this dendogram there is a reference St.02 *Staphylococcus aureus* at the lane 69. In the dendogram this lane is said to hve 93% homology with the lane 68 which is another reference *S. saprophyticus*.

Per 3A, Per 10A, Per13 A, Per 25B, Per26 B, Per 27, GS67, Per38, Per39 a and the reference *S. chromogens* seem to have 85% homology.

Per 17 seemed to have 88% homology with the reference *S. lugdunensis*. But the DNAse test as well as the other biochemical tests do not show any siginificant relationship between these two bacteria.

There are many homologies found within the different reference strains but there is not high homology percentages between the known isolates for example GS 62, Per 19 A-1, GS 59, St. 02, Per 21B are all known to be *Staphylococcus aureus* but showed no homology similarities (Figure 3.21).

There would be some more discriminating tests should be done for example PFGE could beter discriminate these isolates in to subspecies level and besides that could indicate the homologies better than the RFLP .

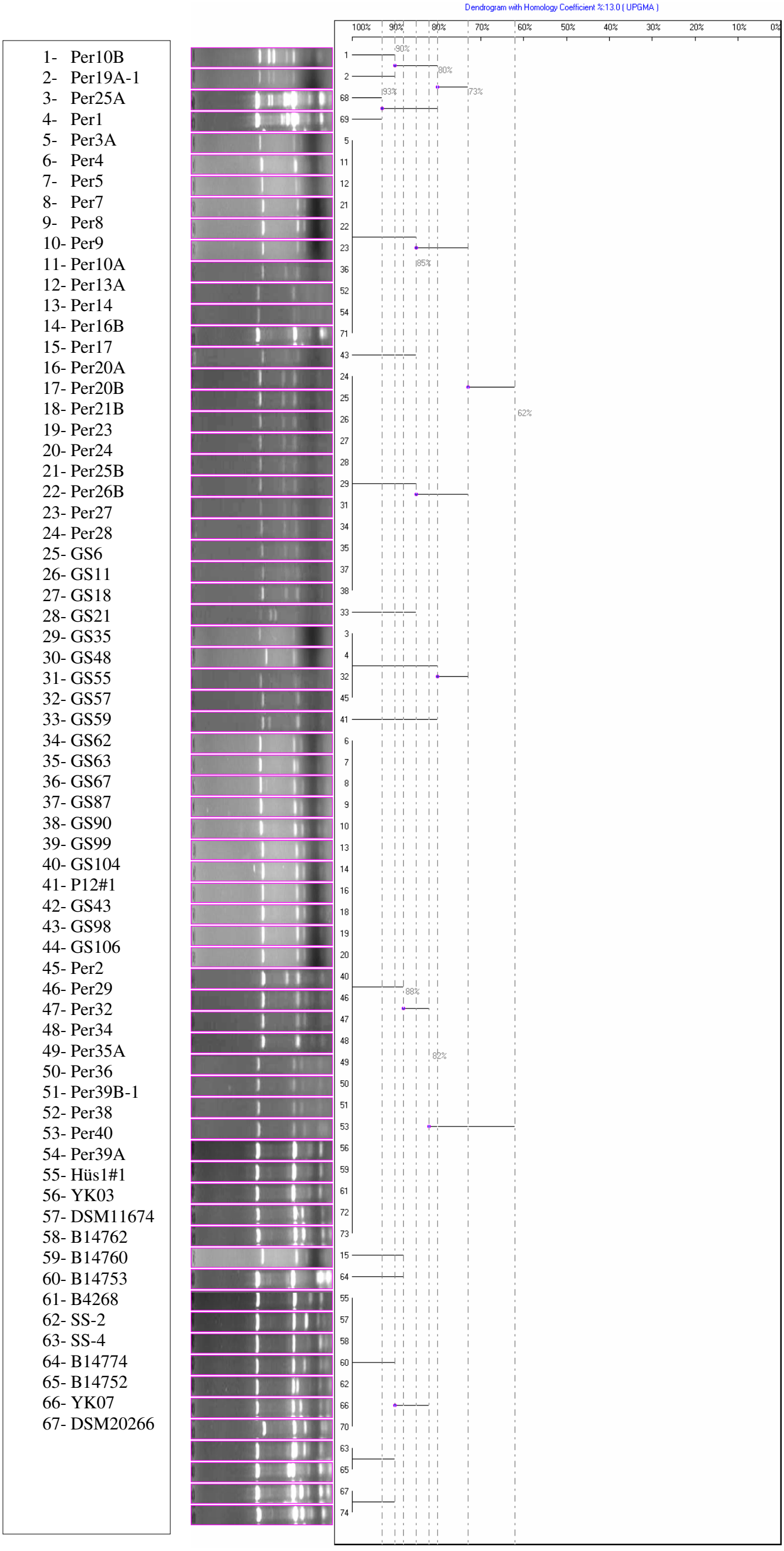

Figure 3.21. Dendogram of isolates and reference strains.

CHAPTER 4

DISCUSSION

Staphylococcus are pathogenic bacteria known to cause diseases among many different organisms. Although not fully described the enzymes proteinase, lipase are studied for their roleplay in the pathogenity and illness process (Arvidson 2000). The lipase and protease coding genes have also taken attention for their roles in the virulence stage so they were thought of being the virulence factors (Carmona et al 1987). So the enzymes and the genes have taken attention for the pathogenity process but neither of the in vitro tests nor the expression of a single gene could explain the mechanism of pathogenesis itself.

There are many extracellular enzymes used for the industrial purposes, it was seen that Staphylococcus produce lipases and proteases more than anyother enzyme group. For the industrial view pathogenic bacteria are not usually used as there would be health risks but if the risks are overcome the enzymes would be purified and used in the industry.

The search of the extracellular enzyme profiles of the different staphylococci would in the future give clues about the site of infection these bacteria have. As an example *S. saprophyticus* is known to have urease activity and in recent years this bacteria is associated with the urinary tract infections of the human.

In this study bacteria were tried to be identified and characterized with the presence of some of the enzyme activities. It was seen that the most abundant enzyme groups are lipase and proteases as stated by many other researches (Arvidson 1973, Ausubel et al., 1994). There were no activities with the enzymes laccases and pectinases and the amylase data was not found to be reliable and these also correlate with the characteristics of this bacteria. Suprisingly there were bacteria having cellulase and xylanase activities which was not stated before.

The enzyme profiles were tried to be correlated together so that a better view about the groups could be get. DNase test is an important tool used to identify coagulase positive staphylococci and mainly *Staphylococcus aureus.* The highest lipase production was found in the tributryin media but the highest lipase production was seen in the Tw20 media within the DNase positive group of the bacteria. The highest

percentage of the proteinase production was seen in the skim milk agar and that was the case with the DNase positive group as well.

The amplifications of the protease and lipase encoding fragments were correlated with the solid media results but there was not a clear relationship found. The expressed form or the enzyme was thought of being the result of some other gene functions.

Staphylococci were tried to be further characterized with the tests, the mannitol phenol red agar is used to identify *Staphylococcus aureus* as the salt tolerence as well as mannitol fermentation were the characteristics of this species. *nuc* amplifications were important signs of the species *Staphylococcus aureus* (Brakstad et al. 1992) and when the reference strains known to be *Staphylococcus aureus* were amplified with *nuc* the results were positive and the DNase test of these bacteria were also positive but some of the *nuc* positive isolates were not able to degrade DNA so the DNase test results were negative.

The RFLP results could not be discriminative for the isolates in this study which was also thought to be the result of someofthe unclear fragments of the RFLP gels. Pulse Field Gel Electrophoresis and DNA sequencing of the suspected fragments would yield better results.

In the future, the role of the extracellular enzymes along with the genes in the pathoghenity process would be identified and this study could serve for a great clue about the relationship between the source of the staphylococci, extracellular enzyme production and some of the PCR amplifications.

REFERENCES

Alexandre, G., Zhulin, I.B. (2000). "Laccases are widespread in bacteria". *Trends Biotechnol.* Vol. 18, p. 41-42.

Alkorta, I., Garbisu, C., Llama, J. M., Serra, J. L. 1998 "Industrial Applications of Pectic Enzymes: A Review, " *Process Biochemistry*. Vol. 1, p. 21-28.

Akbalik, G., Gunes, H., Yavuz, E., Yasa, I., Harsa, S., Elmaci, Z. S and Yenidunya, A. F. 2004. " Identification of extracellular enzyme producing alkalophilic bacilli from Izmir province by 16S-ITS rDNA RFLP", *J Appl Microbiol* Vol. 97, p.766–773

Anvar, A. and Saleemuddin, M. 1998 " Alkaline Proteases: A Review, " *Bioresource technology*. Vol. 64, p. 175-183

Arciola, C. R., Collamati, S., Donati, E. and Montanaro, L. 2001. " A rapid PCR method for the detection of slime-producing strains of *Staphylococcus epidermidis* and *S.aureus* in periprosthesis infections. *Diagn Mol Pathol* Vol. 10, p. 130–137

Arvidson, S. 1973. "Studies on extracellular proteolytic enzymes from *Staphylococcus aureus.* Isolation and characterization of an EDTA-sensitive protease", *Biochim. Biophys. Acta*. Vol. 302, p. 149-157.

Arvidson, S. 2000. "Extracellular enzymes. In: Gram-Positive Pathogens". V.A. Fischetti, R.P. Novick, J.J. Ferretti, D.A. Portnoy, and J.I. Rood. *American Society for Microbiology* Vol.8, p. 379-385.

Arvidson, S.O. 1983. "Extracellular enzymes from *Staphylococcus aureus.* In: Staphhyloccocci and Staphyloccoccal Infections", Vol. 2, p. 745-808.

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., Struhl, K., *Current Protocols In Molecular Biology* Vol.3. (John Wiley and Sons, Inc. 1994).

Beg, Q. K., Kapoor, M., Mahajan, L., Hoondal, G. S. 2001. "Microbial Xylanases and Their Industrial Applications: A Review," *Applied Microbial Biotechnology*. Vol. 56,p. 326-338.

Beguin, P. 1990. "Molecular biology of cellulose degradation", *Annual Review of Microbiology* Vol. 44, p.219-248.

Biely, P. 1985. "Microbial xylanolytic systems", *Trends in Biotechnology* Vol. 3, p. 286-291.

Brakstad, O.G., Aasbakk, K. & Maeland, J.A. 1992. "Detection of *Staphylococcus aureus* by Polymerase Chain Reaction Amplification of the *nuc* Gene", *Journal of Clinical Microbiology*. Vol. 30, No. 7, p. 1654-1660.

Brückner, R., Wagner, E. and Götz, F. 1993. "Characterization of a Sucrase Gene from *Staphylococcus xylosus*", *Journal of Bacteriology*. Vol. 175, No.3, p. 851-857.

Bulut, C., Gunes, H., Okuklu, B., Harsa, S., Kilic, S., Coban, H. S. & Yenidunya, A. F. (2005). Homofermentative lactic acid bacteria of a traditional cheese, Comlek peyniri from Cappadocia region. *J Dairy Res* Vol.72, p.19–24

Carmona, C., and Gray, G.L. 1987. Nucleotide sequence of the serine protease gene of *Staphylococcus aureus*,strain V8. *Nucleic Acids Res*. Vol.15, p. 6757.

Cheung, A.L., Bayer, A.S., Zhang, G.,Gresham, H., Xiong, Y.Q. 2004. "Regulation of viirulence determinants in vitro and in vivo in *Staphylococcus aureus*". *FEMS Immunology and Medical Microbiology*. Vol. 40, p. 1-9.

Claus, H. 2004. "Laccases: structure, reactions, distribution". *Micron*. Vol. 35, p. 93-96.

Costerton, J. W., Lewandowski, Z., Caldwell, D. E., Korber, D. R. and Lappin-Scott, H. M. 1995. "Microbial biofilms", *Annu Rev Microbiol* Vol.49, p. 711–745.

Coughlan, M. P., Hazlewood, G. P. 1993. "β-1,4-D-Xylan-Degrading Enzyme Systems :Biochemistry, Molecular Biology and Applications," *Biotechnology Applied Biochemistry*. Vol. 17, p. 259-289.

Coulter, S.N., Schwan, W.R., Ng, E.Y., Langhorne, M.H., Ritchie H.D., Westbrock-Wadman, S., Hufnagle, W.O., Folger, K.R., Bayer, A.S., and Stover, C.K. 1998. "*Staphylococcus aureus* genetic loci impacting growth and survival in multiple infection environments", *Mol. Microbiol*. Vol.30, p. 393-404.

Cucarella, C., Tormo, M. A., Ubeda, C., Trotonda, M. P., Monzon, M., Peris, C., Amorena, B., Lasa, I. and Penadés, J. R. 2004. "Role of biofilm-associated protein Bap in the pathogenesis of bovine *Staphylococcus aureus*". *Infect Immun* Vol.72, p. 2177–2185.

Drapeau, G.R.1978a. " The primary structure of staphylococcal protease", J. Biochem. Vol. 56, p. 534-544.

Drapeau, G.R., Boily, Y., and Houmard, J. 1972. "Purification and properties of an extracellular protease of *Staphylococcus aureus*", *J. Biol. Chem*. Vol. 247, 6720-6726.

Dubin, G. 2002. "Extracellular Proteases of Staphylococcus spp.", *Biol. Chem*. Vol. 383, p. 1075-1086.

Dubin, G. 2003. “Defense Against Own Arms: Staphylococcal Cysteine Proteases and their Inhibitors”, *Acta Biochimica Polonica*. Vol. 50, No. 3, p. 715-724.

Dufour, P., Jarraud, S., Vandenesch, F., Greenland, T., Novick, R.P., Bes, M., Etienne, J. and Lina, G. 2002. “High Genetic Variability of the agr Locus in *Staphylococcus* Species”, *Journal of Bacteriology*. Vol. 184, No.4, p. 1880-1186.

Eijkman C. 1901 “ Über Enzyme bei Bakterien und Schimmelpilzen ” *Zbl.Bakt. Parasitenk. Infektionskr.*Vol. 29 p. 841–848.

Farrell, A.M., Foster, T.J. and Holland, K.T. 1993. “Molecular analysis and expression of the lipase of *Staphylococcus epidermidis*”. *J Gen. Microbiol.* Vol.139, p.267-277.

Freney, J., Brun, Y., Bes, M., Heugnier, H., Grimont, F., Grimont, P.A.D., Nervie, C. and Fleurette, J.1988. “*Staphylococcus lugdunensis sp*. and *Staphylococcus schleiferi sp.* Two species fom Human Clinical Specimens”. *Int .J. Sup. Bacteriol.* Vol. 38, p. 168-172.

Garcia-Martinez, J., Acinas, S.G., Anton, A.I., Francisco Rodriguez-Valera, F. 1999 “Use of the 16S-23S ribosomal genes spacer region in studies of prokaryotic diversity, ”*Journal of Microbiological Methods.*Vol. 36, p. 55-64.

Gilbert, J. H., Hazlewood, G. P. 1993 “Bacterial Cellulases And Xylanases:Review Article ”*Journal Of General Microbiology*. Vol. 139, p. 187-194.

Gribbon, E. M., Cunliffe, W. J. and Holland, K. T. 1993. “Interaction of *Propionibacterium acnes* with skin lipids *in vitro*”, *J Gen Microbiol* Vol.139, p. 1745-1751.

Godfrey T. and West S, 1996 Industrial Enzymology, Macmillan Press, London p. 3.

Haba, E., Bresco, O., Ferrer, C., Marqués, A., Busquets, M. and Manresa, A. 2000. “Isolation of Lipase-secreting Bacteria by Deploying Used Frying Oil As Selective Substrate”, *Enzyme and Microbial Technology*. Vol. 26, p. 40-44.

Hancock, R.E.W. 2005. “ Mechanisms of Action of Newer Antibiotics for Gram-positive Pathogens”, *Lancet Infection Diseases*.Vol. 5, p. 209-218.

Hedström, S.Å. 1975. “ Lipolytic activity of *Staphylococcus aureus* strains from cases of human chronic osteomyelitis and other infections ”. *Acta Patho. Microbio.* Vol. 83, p. 285-292.

Hedström, S.Å. and Nilsson-Ehle, P. 1983. “Triacylglycerol lipolysis by *Staphylococcus aureus* strains from furunculosis, pyomyosititis, impetigo and osteomyelitis ” *Acta Patho. Microbio.* Vol. 91, p. 169-173.

Heilmann, C., Thumm, G., Chhatwal, G.S., Hartleib, J., Uekötter, A. and Peters, G. 2003. "Identification and Characterization of a Novel Autolysin (Aae) with Adhesive Properties from *Staphylococcus epidermis*", *Microbiology*. Vol. 149, p. 2769-2778.

Holt, J.G. and Krieg, N.R. 1994. "Enrichment and Isolation in Methods for General and Molecular Bacteriology, " *ASM Publications*, pp. 197-200.

Huebner, J. and Goldmann, G.A. 1999. "Coagulase-negative staphyloccocci: role as pathogens. "*Annu Rev Med* Vol. 50, p. 223-236.

Hussain, M., Herrmann, M., Eiff, C., Remington, F.P. and Peters, G. 1997. "A 140-Kilodalton Extracellular Protein is Essential for the Accumulation of *Staphylococcus epidermidis* Strains on Surfaces", *Infection and Immunity*. Vol. 65, No. 2, p. 519-524.

Jaeger, K.E. and Reetz M.T. 1998. " Microbial Lipases from Versatile Tools For Biotecnology", *Tibtech.* Vol. 16, p. 396-403.

Jaeger, K.E., Schneidinger, B., Rosenau F., Werner, M., Lang, D., Dijkstra, B.W., Schimossek, K., Zonta, A. and Reetz M.T. 1997. "Bacterial Lipases for Biotechnological Applications", *Journal of Molecular Catalysis B: Enzymatic.* Vol. 3, p. 3-12.

Jaeger, K., Ransac, S., Dijkstra, B. W., Colson, C., Heuvel, M., Misset, O. 1994 "Bacterial Lipases " *FEMS Microbiology Reviews*. Vol. 15,p. 29-63.

Jarvis, A.W. and Lawrence R.C. 1971. "Production of Extracellular Enzymes and Enterotoxins A, B, and C by *Staphylococcus aureus*", *Infection and Immunity*. Vol. 4, No.2, p. 110-115.

Jean-Pierre, H., Darbas, H., Jean-Roussenq, A. and Boyer, G. 1989. "Pathogenicity in Two Cases of *Staphylococcus schleiferi*, a Recently Described Species". *J.Clin.Microbiol*. Vol.27, p. 2110-2111.

Jensen, M.A., Webster, J.A., Strauss, N.1993."Rapid identification of bacteria on the basis of polymerase chain reaction-amplified ribosomal DNA spacer polymorphisms. " *Appied and Environmental Microbiology*. Vol. 59, p. 943-952.

Joyce, J.G., Abeygunawardana, C., Xu, Q., Cook, J.C., Hepler, R., Przysiecki, C.T., Grimm, K.M., Roper, K., Ip, C.C.Y., Cope, L., Montgomery, D., Chang, M., Campie, S., Brown, M., Mcneely, T.B., Zorman, J., Maria-Litran, T., Pier, G.B., Keller, P.M., Jansen, K.U., Mark III, G.E. 2003. "Isolation, Structural Characterization, and Immunological Evaluation of a High-Molecular-Weigh Exopolysaccharide from *Staphylococcus aureus*", *Carbohydrate Research*. Vol. 338, p. 903-922

Kampen, M.D., Simons, J.-W.F.A., Dekker, N., Egmond, M.R. and Verheij, H.M. 1998. "The Phospholipase Activity of *Staphylococcus hyicus* Lipase Strongly Depends on a Single Ser to Val Mutation", *Chemistry and Physics of Lipids*. Vol. 93, p. 39-45.

Karlsson, A., Saravia-Otten, P., Tegmark, K., Morfeldt, E. and Arvidson, S. 2001. "Decreased Amounts of Cell Wall-Associated Protein A and Fibronectin-Binding Proteins in *Staphylococcus aureus sar*A Mutants due to Up-Regulation of Extracellular Proteases", *Infection and Immunity*. Vol. 69, No.8, p. 4742-4748.

Karlsson, A. and Arvidson S. 2002. "Variation in Extracellular Protease Production among Clinlcal Isolates of *Staphylococcus aureus* Due to Different Levels of Expression of the Protease Repressor *sar*A", *Infection and Immunity.* Vol. 70, No. 8, p. 4239-4246.

Kolganova, T.V., Ermolaev, A.V. and Doyle R.J. 2002. "Effect of Asparaginase and Polyphenol Oxidase on Adhesive Characteristics Of Microorganisms", *Bulletin of Experimental Biology and Medicine*. Vol. 133, No.1, p. 71-74.

Ko, W.H., Wang, I.T. and Ann, P.J. 2005. "A simple Method for Detection of Lipolytic Microorganisms in Soils", *Soil Biology and Biochemistry*. Vol. 37, p. 597-599.

Lem, P., Spiegelman, J., Toye, B. and Ramotar, K. 2001. "Direct Detection of *mec*A, *nuc* and 16S rRNA genes in BacT/Alert Blood Culture bottles", *Diagnostic Microbiology and Infectious Disease.* Vol. 41, p. 165-168.

Longshaw, C.M., Farrell, A.M., Wright, J.D. & Holland, K.T. 2000. "Identification of a second lipase gene, gehD, in *Staphylococcus epidermidis*: comparison of sequence with those of other staphylococcal lipases". *Microbiology*. Vol.146, p. 1419-1427.

Lowe, A.M., Beattie, D.T. & Deresiewicz, R.L. 1998. "Identification of novel *Staphylococcal virulence* genes by in vivo expression technology. " *Mol Microbiol* Vol.27, 967-976.

Mach, P.A. and Lund, M.E. 2000. "Article and Method for Detection of Enterotoxigenic Staphylococci-US Patent 6022682", *Patent Storm*.

Madigan, M.T., Martinco, J.M., Parker, J. "Microbial Growth" *Brock Biology of Microorganisms*, (Prentice-Hall, Inc.,1997), pp 165-168.

Maeda, H., and Yamamoto, T. 1996. " Pathogenic mechanisms induced by microbial proteases in microbial infections " *Bio. Chem.* Vol. 377, p. 217-226.

Maira-Litran, T., Kropec, A., Goldmann, D., Pier, G.B. 2004. "Biologic properties and vaccine potential of the staphylococcal poly-N-acetyl glucosamine surface polysaccharide". *Vaccine*. Vol. 22 p. 872-879.

Marples, R.R., Downing, D.T. & Kligman, A.M. 1971. “Control of free fatty acids in human surface lipids by *Corynebacterium acnes*. ” *J Invest. Dermal* Vol. 56, p.127-131.

Meghji, S., Crean, S.J., Hill, P.A., Sheikh, M., Nair, S.P., Heron, K., Henderson, B., Mawer, E.B. and Harris, M.1998 “Surface-Associated Protein from *Staphylococcus aureus* Stimulates Osteoclastogenesis: Possible Role in *S. aureus*-induced Bone Pathology”, *British Journel of Rheumatology*. Vol. 37, p. 1095-1101.

Menichetti, F. 2005. “Currrent and Emerging Serious Gram-positive Infections”, *Clinical Microbiology and Infection*. Vol. 11, p. 22-28.

Mullarky, I.K., Su, C., Frieze, N., Park, Y.H. and Sordillo, L.M. 2001. “*Staphylococcus aureus agr* Genotypes with Enterotoxin Production Capabilities can Resist Neutrophil Bactericidal Activity”, *Infection and Immunity*. Vol. 69, No.1, p. 45-51.

Naidu, G.S.N., Panda, T. 1998. “Production of pectolytic enzymes- a review”. Bioprocess Engineering. Vol. 19, p. 355-361.

Nicolaides, N. 1974. Skin lipids: their biochemical uniqueness. Science Vol.186, 19-26.

Phillips, W. E. and Kloos, W.E. 1981. “Identification of Coagulase-Positive *Staphylococcus intermedius* and *Staphylococcus hyicus subsp. hyicus* isolates from Veterinary Clinical Specimens”. *J.Clin.Microbiol*. Vol. 14, p. 671-673.

Podbielski, A. and Kreikemeyer, B. 2004. “Cell Density-dependent Regulation: Basic Principles and Effects on the Virulence of Gram-positive Cocci”, *International Journal of Infectious Disease.* Vol. 8, p. 81-95.

Potempa, J., Porwit-Bobr, Z., and Travis, J. 1989. “Stabilization vs. Degradation of *Staphylococcus aureus* metalloproteinase” *Biochim. Biophys. Acta* Vol. 993, p. 301-304.

Potempa, J., Watorek, W., and Travis, J. 1986. “The inactivation of human plasma α1-proteinase inhibitor by proteinases from *Staphylococcus aureus* ” *J. Biol. Chem.* Vol. 261, p. 14330-14334.

Prėvost, G., Rifai, S., Chaix, M.L., and Piėmont, Y. 1991. “ Functional evidence that the Ser-195 residue of staphylococcal exfoliative toxin A is essential for biological activitity. ” *Infect. Immun*. Vol. 59, p. 3337-3339.

Prokešová, L., Porwit-Bóbr, Z., Baran, K., Potempa, J., and John, C. 1988. “Effect of serine proteinase from Staphylococcus aureus on in vitro stimulation of human lymphocytes”. *Immun. Lett*. Vol. 19, p.127-132.

Rao, M.B., Tanksale, M.A., Ghatge, S.M., Deshpande, V.1998. “Molecular and Biotechnological Aspects of Microbial Proteases, ” *Microbiology and Molecular Biology Reviews* Vol. 62, p.597-635.

Reed, S.B., Wesson, C.A., Liou, L.E., Trumble, W.R., Schlievert, P.M., Bohach, G.A., and Bayles, K.W. 2001. "Molecular characterization of a novel *Staphylococcus aureus* serine protease operon." *Infect. Immun*. Vol.69, p.1521-1527.

Rice K, Peralta R, Bast D, Azavedo J, McGavin MJ. 2001 " Description of staphylococcus serine protease (*ssp*) operon in Staphylococcus aureus and nonpolar inactivation of sspA-encoded serine protease. " *Infect Immun*. Vol. 69: 159-69.

Rollof J, Hedström SA, Nilsson-Ehle P.1987 "Lipolytic activity of Staphylococcus aureus strains from disseminated and localized infections". *Acta Pathol Microbiol Immunol Scand [B]*. Vol. 95 No.2 p.109–113.

Ryding U., Renneberg J., Rollof J., Christensson B.1992 " Antibody response to *Staphylococcus aureus* whole cell, lipase and staphylolysin in patients with *S. aureus* infections". FEMS Microbiol. Immunol. Vol.4 p.105.

Saggers, B.A. and Stewart, G.T. 1968. "Lipolytic Esterases in Staphylococci", *Journal of Bacteriology*. Vol. 96, No. 4, p. 1006 -1010.

Said-Salim, B., Dunman, P.M., McAleese, F.M., Macapagal, D., Murphy, E., McNamara, P.J., Arvidson, S., Foster, T.J., Projan, S.J. and Kreiswirth, B.N. 2003. "Global Regulation of *Staphylococcus aureus* Genes by Rot", *Journal of Bacteriology*. Vol. 185, No. 2, p. 610-619.

Sanford, B.A. and Ramsay, M.A. 1986. "Detection of Staphylococcal Membrane Receptors on Virus-Infected Cells by Direct Adhesin Overlay", *Infection and Immunity*. Vol. 133, No.1, p. 71 -74.

Sayari, A., Agrebi, N., Jaoua, S. and Gargouri Y. 2001. "Biochemical and Molecular Characterization of *Staphylococcus Simulans* Lipase", *Biochimie.* Vol. 83, p. 863-871.

Sharma,R. ,Chisti, Y., Banerjee, U.C. 2001 "Production, Purification, Characterization and Application of Lipases, "*Biotechnology Advances* Vol. 19, p. 627-662.

Shaw, L., Golonka, E., Potempa, J. and Foster, S.J. 2004. "The Role and Regulation of The Extracellular Proteases of *Staphylococcus aureus*", *Microbiology*. Vol. 150, p. 217-228.

Smeltzer, M., Hart, M.E. and Iandolo, J.J. 1992. "Quantitative Spectrophotometric Assay for Staphylacoccal Lipase", *Applied and Environmental Microbiology*. Vol. 70, No. 8, p. 4239-4246.

Smibert, R.M. and Krieg, N.R. "Phenotypic Characterization, " in Methods for General and Molecular Bacteriology edited by P. Gerhardt (ASM Publications, 1994) p. 607.

Spare, M.K., Tebbs, S.E., Lang, S., Lambert, P.A., Worthington, T., Lipkin, G.W. and Elliott T.S.J. 2003. "Genotypic and Phenotypic Properties of Coagulase-Negative Staphylococci Causing Dialysis Catheter-Related Sepsis", *Journel of Hospital Infection*. Vol. 54, p. 272-278.

Stehr, F., Kretschmar, M., Kröger, C., Hube, B. and Schäfer, W. 2003. "Microbial Lipases as Virulence Factors", *Journal of Molecular Catalysis B: Enzymatic.* Vol. 22, p. 347-355.

Svendsen, A.2000 "Review-Lipase Protein Engineering, "*Biochimicq et Biophysica Acta* Vol. 1543, p. 223-238.

Travis, J., Potempa, J., and Maeda, H. 1995. "Are bacterial proteinases pathogenic factors? " *Trends in Microbiol*. Vol. 3, p. 405-407.

Umeda, A., Ueki, Y. and Amako K. 1987. "Structure of the *Staphylococcus aureus* Cell Wall Determined by the Freeze-Substitution Method", *Journal of Bacteriology*. Vol. 169, No. 6, p. 2482-2487.

Vadehra, D. V. 1974. "Staphylococcal lipases". Lipids. Vol.9, p. 158-165.

Yavuz, E., Gunes, H., Bulut, C., Harsa, S. & Yenidunya, A. F. 2004a. " RFLP of 16S rDNA-ITS region to differentiate lactobacilli at species level. " *World J Microbiol Biotechnol* Vol. 20, p.535–537

Wittenberger, C.L., Beaman A.J. and Lee, L.N. 1978. "Tween 80 Effect on Glucosyltransferase Synthesis by *Staphylococcus salivarius*", *Journal of Bacteriology*. Vol. 133, No.1, p. 231-239.

APPENDIX A

CHEMICALS USED

Table A1 Chemicals Used in Experiments

NO	CHEMICAL	CODE
1	Agar- Agar	Merck 1.01613
2	Bacteriological pepton	Oxoid LP037
3	Yeast Extract	Merck 1.03753
4	Skimmed milk	LabM MC27
5	Glycerol	AppliChem A2926
6	NaCl	AppliChem A2942
7	K_2HPO_4	AppliChem A2945
8	$MgSO_4.7H_2O$	Merck 1.05886
9	Xylan from birchwood	Sigma X-0502
10	Pectin from citrus peel	Fluka 76280
11	Polygalacturonic acid	Fluka 81325
12	Carboxymethylcellulose sodium salt	Fluka biochemika 21902
13	Soluble Starch	Merck 1.01252.0250
14	Disodium hydrogen phosphate	Applichem A2943
15	Ammonium sulfate	Applichem A3485
16	KH_2PO_4	Merck 1.04871
17	Immersion oil	Applichem A0699
18	Cetyl trimethylammonium bromide	Applichem A0805
19	Calcium chloride	Applichem A3652
20	Crystal violet	Sigma C3886
21	Safranin O	Merck 1.15948
22	Nutrient broth	Merck 1.05443
23	Congo Red	Sigma C6767

Table A.1 Chemicals Used in Experiments (Continued)

24	Potassium Iodide	Sigma P8256
25	Urea Base	AppliChem A0124
26	Tween80	AppliChem A1390
27	Tween 20	AppliChem A1389
28	Sodium carbonate	Merck 1.06392
29	Tris Base	Sigma T6066
30	EDTA	AppliChem A2937
31	Isopropanol	AppliChem A3928
32	Proteinase K	AppHChem A3830
33	Ethidium bromide	AppliChem A1151
34	Ethanol	AppliChem A3678
35	Taq DNA polymerase	MBI, Fermentas EP0401
36	dNTP set	MBI,Fermentas,R0181
37	*Taq* I	Fermentas, ER0671
38	Lysozyme	AppliChemA3711
39	Sodium lauryl sulfate	AppliChemA1163
41	Sodium deoxycholate	AppliChemA1531
42	Proteinase K	AppliChemA3830
43	Chloroform	AppliChemA3633
44	Isoamyl alcohol	AppiChemA2610
45	Bromophenol blue	Merck 1.08122
46	Potato Dextrose Agar	AppliChem
47	Blood Agar	Oxoid
48	Mannitol Phenol Red Agar	Merck 014526

APPENDIX B

MEDIA

B.1 MEDIA USED FOR ISOLATION

B.1.1 Solid Media For Dilution Plate Technique

	g/1
Yeast extract	1
Glucose	1
Agar agar	15

All ingredients were dissolved in sterilised deionized water. Medium was sterilised by autoclaving at 121 °C for 15 minutes.

B.1.2 Broth Media Used For Enrichment Technique

	g/1
Yeast extract	1
Glucose	1

All ingredients were dissolved in sterilised deionized water. Medium was sterilised by autoclaving at 121 °C for 15 minutes.

B.2 MEDIA FOR EXTRACELLULAR ENZYME SCREENING

B.2.1 Media Used For Protease Screening

Media 1

	g/l
Nutrient broth	8
Skim milk	10
Agar agar	15

Ingredients except skim milk were dissolved in deionized water. Medium was sterilised by autoclaving at 121 °C for 15 minutes. Skim-milk is autoclaved separately at 110 °C for 5 minutes (two times) and added to the medium.

Media 2

	g/l
Nutrient Broth	8
Casein	10
Agaragar	15

Ingredients except casein were dissolved in deionized water. Medium was sterilised by autoclaving at 121 °C for 15 minutes. Casein is autoclaved separately at 110 °C for 5 minutes (two times) and added to the medium.

B.2.2 Media Used For Amylase Screening

Media 1

	g/1
Yeast extract	1
$MgSO_4.7H_2O$	0.1
K_2HPO_4	7
KH_2PO_4	2
$(NH_4)_2SO_4$	1
NaCl	5
Starch	5
Agar agar	15

All ingredients were dissolved in deionized water. Medium was sterilised by autoclaving at 121 °C for 15 minutes.

Media 2

	g/1
Yeast extract	1
Soluble starch	5
Agar agar	15

Ingredients were dissolved in sterilised deionized water. Medium was sterilised by autoclaving at 121 °C for 15 minutes.

B.2.3 Media Used For Lipase Screening

Media 1

	g/1
Nutrient broth	8
$CaCl_2 2H_20$	0.5
Tween 80	10ml
Agar agar	15

Ingredients except Tween 80 were dissolved in deionized water. Medium was sterilised by autoclaving at 121 °C for 15 minutes. Tween 80 was autoclaved separately and added to the medium.

Media 2

	g/1
Nutrient broth	8
$CaCl_2 2H_20$	0.5
Tween 20	10ml
Agar agar	15

Ingredients except Tween 20 were dissolved in deionized water. Medium was sterilised by autoclaving at 121 °C for 15 minutes. Tween 20 was autoclaved separately and added to the medium.

Media 3

	g/l
Nutrient Broth	8
$CaCl_2.2H_2O$	0,1
AgarAgar	15
Lipid Emultion	10 ml

Ingredients except the lipid emultion were autoclaved at 121 °C for 15 minutes then sterile 10 ml of 10% lipidemultion was added, mixed well.

Media 4

	g/l
Nutrient Broth	8
Yeast Extract	3
AgarAgar	12
Tributryin	10 ml

Ingredients were dissolved in sterilised deionized water. Medium was sterilised by autoclaving at 121 °C for 15 minutes.

B.2.4 Media Used For Xylanase Screening

	g/l
Yeast extract	1
Birchwood xylan	5
Agar agar	15

Ingredients were dissolved in sterilised deionized water. Medium was sterilised by autoclaving at 121 °C for 15 minutes.

B.2.5 Media Used for Cellulase Screening

	g/l
Yeast extract	1
Carboxymethylcellulose sodium salt	5
Agar agar	15

Ingredients were dissolved in deionized water. Medium was sterilised by autoclaving at 121°C for 15 minutes.

B.2.6 Media Used For Pectinase Screening

Media 1

	g/l
Yeast extract	1
Ammonium sulfate	2
Na_2HPO_4	6
KH_2PO_4	3
Polygalacturonic acid	5
Agar agar	15

All ingredients were dissolved in deionized water. Medium was sterilised by autoclaving at 121 °C for 15 minutes

Media 2

	g/l
Yeast extract	1
Ammonium sulfate	2
Na_2HPO_4	6
KH2PO4	3
Pectin from citrus peel	5
Agar agar	15

All ingredients were dissolved in deionized water. Medium was sterilised by autoclaving at 121°C for 15 minutes.

B. 3 Media Used for Staphylococcus Characterization

B. 3. 1 Mannitol Salt Phenol Red Agar

	g/l
Peptones	10
Meat Extract	1
Sodium Chloride	75
D(-) Mannitol	10
Phenol Red	0.025
Agar Agar	15

All ingredients were dissolved in deionized water. Medium was sterilised by autoclaving at 121°C for 15 minutes.

B. 3. 2 Blood Agar

	g/l
Lab Lemco Powder	10.0
Peptone neutralised	10.0
SodiumChloride	5.0
Blood	200 ml
Agar agar	15.0

All ingredients except the blood were dissolved in deionized water. Medium was sterilised by autoclaving at 121°C for 15 minutes. The base was cooled to 50°C and 5% blood was added.

APPENDIX C

PCR-RFLP RECIPIES

C.1 PCR Mixture

Mg free Taq DNA polymerase buffer	5 µl
$MgCl_2$ (25 mM)	3 µl
Sterile deionized water	35µl
Oligo forward 10 picomole/ µl	1 µl
Oligo reverse 10 picomole/ µl	1 µl
dNTP (2mMeach) 10X	5 µl
Taq DNA polymerase	0.25 µl (1.25 U)

C.2 6X Gel Loading Buffer (20 ML)

10X TBE	2 ml
Glycerol	6 ml
Deionized water	12 ml

Bromophenol blue was added with toothpick until obtaining sufficient color of the solution.

C.3 dNTP(10X)

10 µl of each 100mM dATP, dCTP, dGTP and dTTP in separate vials were taken. They were mixed in 0.2 ml PCR tubes and 460 µl sterile deionized water was added. They were mixed gently and 2mM concentration of each was obtained and stored at -20 °C.

C.4 Restriction Enzyme Mixture

Restriction enzyme buffer	5 µl
Sterile deionized water	35
DNA	10 µl
Restriction Enzyme	0.5 µl (5U)

C.5 Oligonucleotide Primers

L1: 5'- CAAGGCATCCACCGT -3'

350 µg primer L1 was dissolved in 175 µl of sterile deionized water to obtain 2 µl / µg stock solutions. Four microliter of stock solution were then taken and mixed with 96 µl sterile deionized water. Therefore 100 µl, 10 picomole / µl working solution was obtained. Stock and working solutions were stored at -20 °C.

EGE 1 5'- AGAGTTTGATCCTGGCTCAG -3'

590 ug primer EGE 1 was dissolved in 295 ul of sterile deionized water to obtain 2 jag / ul stock solutions. Five microliter of stock solution were then taken and mixed with 95 ul sterile deionized water. Therefore 100 ul, 10 picomole / ul working solution was obtained. Stock and working solutions were stored at -20 °C.

The same procedure was followed with *sspA*, *sspB*, *geh*, *nuc*, *aur* and *prt* primers.

APPENDIX D

STAINS AND INDICATORS

D.1 Solutions for Gram Staining

D.1.1. Crystal Violet Staining Reagent

Solution A

Crystal violet	2g
Ethanol (95%)	20 ml

Solution B

Ammonium oxalate	0.8g
Distilled water	80ml

Solution A and B were mixed to obtain crystal violet staining reagent.

D.1.2. Iodine Solution

Iodine	1g
Potassium iodide	2g
Distilled water	3000ml

Iodine and potassium iodide were grinded. Water was then added slowly and the solution was stirred until the iodine was dissolved.

D.1.3 Safranin Solution

Safranin (2,5% in 95% alcohol)	10ml
Distilled water	100ml

APPENDIX E

Buffers and Stock Solutions

E. 1. 50XTAE

242 g Tris base was dissolved in deionized water, 57.1 ml glacial acetic acid and 100 ml 0.5 M EDTA (pH 8.0) were added. Volume was adjusted to 1000 ml with deionized water.

E. 2. 1XTAE

20 ml of 50X TAE buffer was taken and the volume was adjusted to 1 liter with deionized water to obtain 1 liter IX TAE buffer.

E. 3. 1M Tris-HCl pH 7.2

121.1 g Tris base was dissolved in 800 ml of deionized water. pH was adjusted to 7.2 with concentrated HC1. Volume is brought to 11 with deionized water.

E. 4. 1M Tris-HCl pH 8

121.1 g Tris base was dissolved in 800 ml of deionized water. pH was adjusted to 8 with concentrated HC1. Volume is brought to 11 with deionized water.

E. 5. M EDTA pH 8.0

186.12 g EDTA was dissolved in 800 ml of deionized water and pH was adjusted to 8.0 with 10 N NaOH. Volume was brought to 1000 ml with deionized water.

E. 6. IX TE

10 mM Tris, pH 8, lmM EDTA

E. 7. Sodium Acetate (3M, pH 5.2)

408.1 g sodium acetate (3 H_2O) was dissolved in 800 ml deionized water and pH was adjusted to 5.2 by glacial acetic acid. Volume was brought to 1000ml.

E. 8. Ethidium Bromide Stock Solution (10 mg/ml)

0.5 g ethidium bromide was dissolved in 50 ml of deionized water.

E. 9. Chloroform-Isoamyl Alcohol Solution

48 ml of chloroform was mixed with 2 ml of isoamyl alcohol.

E. 10. Phenol

Liquified phenol firstly, was removed from the freezer. After allowing to warm at room temperature, it was melted at 68 °C. Equal volume of buffer (usually 0.5 M Tris.Cl pH 8 at room temperature) was added to the melted phenol. The mixture was stirred for 15 minutes. When the two phases have separated, the aqueous (upper) phase was removed using a separation funnel. Equal volume of 0:1 M Tris.Cl pH 8 was then added to the phenol. The mixture was again stirred for 15 minutes. The aqueous phase was removed as described before. The extractions were repeated until the pH of the phenolic phase was > 7.8. The pH was measured by using pH paper. After the phenol was equilibrated, the mixture was divided into aliquots and they were stored at -20 °C. Before use, the phenol was melted at room temperature. Hydroxyquinoline and p-mercaptoethanol were added to a final concentration of 0.1% and 0.2 %, respectively. The phenol solution can be stored in this form at 4 °C.

E. 11. Phenol: Chloroform: Isoamyl Alcohol (25:24:1)

Equal volume of phenol and chloroform isoamyl alcohol (24:1) solution were mixed.

E. 12. CTAB/NaCl Solution

4.1 g NaCl was dissolved in 80 ml water. lOg CTAB was added slowly while heating and stirring. If necessary the solution was heated to 65 °C to dissolve. The final volume was then adjusted to 100 ml.

APPENDIX F

PCR Results of the Isolates

Isolates	sspA	prt	aur	sspB	geh	nuc
Per1	+	-	-	-	-	-
Per2	-	-	+	-	-	-
Per3A	+	-	+	-	+	+
Per4	+	-	-	-	+	+
Per5	-	-	-	-	-	-
Per7	-	-	-	-	-	-
Per8	+	-	-	-	-	+
Per9	+	-	-	-	+	+
Per10A	+	-	-	-	-	-
Per10B	-	-	-	+	-	+
Per13A	-	-	-	-	-	-
Per14	+	-	-	-	+	+
Per16B	-	-	-	-	-	-
Per17	+	-	-	-	-	-
Per19A	-	-	+	+	-	+
Per20A	+	-	-	-	-	+
Per20B	+	-	-	-	+	+
Per21B	+	-	-	-	-	-
Per23	-	-	-	-	-	-
Per24	-	-	+	-	-	+
Per25A	-	-	-	-	-	+
Per25B	-	-	-	-	-	+
Per26B	-	-	-	-	-	-
Per27	+	-	-	-	-	+
Per28	-	-	-	-	-	-
Per29	-	-	-	-	-	-

Per30	-	-	-	-	-	-
Per32	-	-	-	-	-	-
Per33	-	-	-	-	-	-
Per34	+	-	+	-	+	-
Per35A	-	-	-	-	-	-
Per36	-	-	-	-	-	-
Per37A	-	-	-	-	-	-
Per38	-	-	-	-	+	-
Per39A	+	+	+	-	-	+
Per39B	-	-	+	-	-	-
Per40	-	+	-	-	-	-
Hüs1.2	-	-	-	-	-	-
P12.1	-	+	+	-	-	-
GS6	-	+	-	-	-	-
GS11	-	+	-	-	-	+
GS16	-	-	-	-	-	-
GS18	-	+	-	-	-	+
GS21	+	+	-	-	-	+
GS27	-	-	-	-	-	-
GS35	-	+	-	-	-	-
GS43	-	-	-	-	-	+
GS48	-	+	-	-	-	-
GS55	-	+	-	-	-	-
GS57	-	+	-	-	-	-
GS59	-	-	-	-	-	+
GS62	-	-	-	-	-	-
GS63	-	-	+	-	-	+
GS67	-	-	-	+	-	-
GS87	-	+	-	+	-	-
GS90	-	+	-	-	-	-
GS98	-	-	-	-	-	-
GS99	-	+	-	-	+	-
GS104	-	-	-	-	-	-

GS106	+	-	-	-	-	-
P8.1	-	-	-	-	-	-
P10.4	-	-	-	-	-	-
P20.1	-	+	-	-	-	-
BÇ-1	-	-	-	-	-	-
P10.2	-	-	-	-	+	-
Bid 1.1	-	-	-	-	-	-
Hüs1.1	+	-	+	-	+	-
EY1	-	+	-	-	-	-
EY2	-	+	-	-	-	-
H3.2	-	-	-	-	-	-