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BIOFILM PRODUCTION AMONG PSEUDOMONAS FROM THE FOOD INDUSTRY

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Abbreviations

ATCC	American Type culture Collection
CV	Crystal Violet
CFU	Colony Forming Unit
DNA	Deoxyribonucleic Acid
D/W	Distilled water
EPS	Extracellular Polymeric Substances
MM	Minimal Medium
NaCl	Sodium chloride
OD	Optical Density
PBH	Poly-β-hydroxybutyrate
PNAG	P, N-acetylglucosamine
PPA	Perooxyacetic acid
PPM	Part Per Million
PCR	Polymerase Chain Reaction
QAC	Quaternary Ammonium Compounds
RNA	Ribonucleic acid
RPM	Revolution per minute
TSA	Trypticase Soya Agar
TSB	Trypticase Soya Broth
TSBNG	Trypticase Soya Broth Containing Sodium and Glucose
STDEV	Standard Deviation

Abstract

Pseudomonas are gram-negative bacteria which show great diversity of metabolic activity and are found in a variety of niches. Existences of the *Pseudomonas* spp. in the biofilm forms are posing problems in the food industries due to their potential of contaminating food and the food products. These unique microbial structures are more resistant to physical and chemical stress than the planktonic form of life. Therefore, *Pseudomonas* spp. is of great concern particularly to the food industries.

The study is intended to achieve the greater knowledge on the biofilm formation by *Pseudomonas* spp. and the enzymatic action to prevent it. Twenty- seven different *Pseudomonas* strains from different food processing plants and reference laboratory, were tested. Identification of the strains were performed by API 20NE methodology. These strains were subjected to form biofilm in laboratory conditions. The study focused on capacity of the individual strain to form biofilm and the effectiveness of the disinfectant and enzymes to inhibit the biofilm produced by the strains.

In the study, four *Pseudomonas* strains out of twenty-seven were identified differently from that of 16 s RNA technology. Different *Pseudomonas* species showed different level of biofilm in different cultural conditions but the best biofilm formation have been found in TSBNG medium. *Pseudomonas fluoresecens* (3831) *Pseudomonas putida* (2.12) and Pseudomonas *fragi* (96.6) stood the best among biofilm forming *Pseudomonas* strains.

In addition, Dispersin B, Proteinase K, DNase and Chlorine, were applied to inhibit biofilm produced by the different *Pseudomonas* strains, where activities of these compounds were found to be less effective.

The findings of the present study showed that enzymes are very low effective in inhibition of biofilm produced by *Pseudomonas* strains.

Key words- Biofilm, Enzyme, *Pseudomonas*, Inhibition

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1. Introduction

Communities of microorganisms attached to the surface is defined as biofilm (O'Toole, Kaplan, & Kolter, 2000). Bacteria utilize this phenomenon to survive in different environments. The biofilm formation has complex sequential developmental stages. Biofilm formation initiates with reversible bacterial attachment to the specific surface or stratum. After initial attachment, the bacteria established itself onto the surface and start to form bacterial monolayer, which finally leads to mature biofilm formation (Burmolle et al., 2006). These biofilms are being potential sources of microbial contamination of industrial food products. Biofilm structures are serving as chronic reservoir of microbial contamination in food industries due to their more persistency towards chemical and disinfectants than their planktonic mode of life. So these biofilm might result in reduced quality of food products which may lead to several food borne outbreaks and several illness in human beings and animals.

Several studies have shown that the *Pseudomonas* species have been isolated from surfaces of food industries where they establish themselves in the biofilm form. The reasons for dominance of the species in food industries is still unclear (Sophie & Ford, 2012), however, *Pseudomonas* species are considered as major food spoilage bacteria in food and dairy industries (G. Sundheim, 1998; S. Langsrud, Sundheim, G. and Borgmann-Strahsen, R., 2003). Unique architectural structure of biofilm, diversed metabolic phenomenon, tolerance to several disinfectants and growth in lower temperature may be the important factors for their persistency in food industries. Due to these several reasons, controlling of biofilm has become today's major issue in food industries. So that, understanding of biofilm has become crucial issue in preventing and controlling food related outbreaks and spreading pathogenic bacteria.

The main aim of this study was to examine the biofilms production by different *Pseudomonas* spp. taken from food industries, in different cultural conditions. The 96 well plastic microtiter plates were used for biofilm formation. Initially, good biofilm producing species were screened and the biofilm of the selected strains were exposed to the various concentration of

disinfectants commonly used in food industries. Biofilm production in presence enzymes like dispersin B, proteinase K and DNAse of selected strains were studied to get indirect information about the composition of biofilm matrix. Furthermore, the aim was to investigate the efficiency of certain commercial disinfectants and enzymes in the detachment of biofilm produced on stainless steel coupons. The ultimate goal to achieve greater understanding of bacterial biofilm and how to prevent it.

2 Theory

2.1 Bacteria in food industries

Bacteria can form biofilm on every environment provided with minimum amount of nutrient and moisture. Most of the bacterial species form biofilm under proper environmental condition and persistence of biofilm has been common in food industries. Their existence can be found at all types' surfaces like glass, metal, rubber, plastic and food products (Chmielewski, 2003). Bacterial attachment on surfaces not only pose risk to food spoilage and economic loss but also provide the shelter for food borne pathogen which leads to serious public health problems. In addition, biofilm are responsible for cross contamination and post processing contamination of food products (Hanning I., 2009).

Biofilms have become threat to the industry as well as to the community. Bacteria in the biofilm have been difficult to control and remove. Several evidences have already shown the persistence of the bacteria against several biocides in biofilm mode of life (S. Langsrud, Sundheim, G. and Borgmann-Strahsen, R., 2003). In addition, biofilm provides shelter to unwanted pathogens entering to the system and increases the potential of the pathogen survival which finally leads to further chances of the spreading the pathogen. Such consequences may lead the shutting down of the companies and million lost in revenue (T. E. Cloete, Thantsha, Maluleke, & Kirkpatrick, 2009).

The most common biofilm former bacterial species predominantly present in food industries are *Pseudomonas*, *Listeria, Enterobacter, Flavobacterium, Alkaligens, Staphyllococcus* and *Bacillus* species (Téllez, 2010). *Pseudomonas* spp. especially *Pseudomonas aeruginosa* is an opportunistic human pathogen in immune-compromised individuals and these species are found in food processing environments including floor, drain, fruits, vegetables, meat surfaces, milk and other low acidic food like dairy products. The *Pseudomonas* spp. coexist with other species like *listeria, salmonella* and other pathogens forming multi species biofilm (Golovlev, 2002).

2.2 The role of *Pseudomonas* spp. in spoilage of food in industries

Bacterial spoilage of food products results a huge loss of economy in food industries. Due to large range of growth temperature generally from 2 -35^{o} C, these species can be found in variety of environmental conditions. *Pseudomonas* spp. is predominantly responsible for the spoilage of meat and the dairy products (Walker & Marsh, 2007). Even though these species are destroyed during pasteurization, the food products are spoiled by bacteria which get access after pasteurization process (Rajmohan, Dodd, & Waites, 2002). *Pseudomonas spp*. especially *Pseudomonas fluoresens, Pseudomonas fragi, Pseudomonas lundensis* can grow and form the biofilm in the refrigerated storage (De Jonghe et al., 2011). Many of these species are capable of producing heat stable enzymes like lipases and lecithinases which are responsible for degradation of finished milk products (Marchand, 2012). Thermal stability of these enzymes contributes in the degradation of the finished products . Similarly, *Pseudomonas fragi* is supposed as the principal bacteria for meat spoilage (Labadie1999). In addition, *Pseudomonas* spp predominantly, *Pseudomonas alkaligens, Pseudomonas aeruginosa and Pseudomonas fluorescens* have been found as potential for spoilage of cheese in stored refrigerated condition (S. Arslan, 2011). Even though the predominance of these species are not clearly understood, wide growth temperature range, tolerance to different sanitizing chemicals and the capacity to form biofilm might be important factor for their persistence in food industries.

2.3 History of *Pseudomonas* Species

When Bergey's Manual was published in 1923, for the first time, all the criteria were utilized in an arbitrary way which finally led to mistakes in the classification of bacteria. The limitations are were pointed by other scientists later. In the 1923 edition of Bergey's Manual, a chapter was included for *Pseudomonas* and defined as the gram negative, aerobic, non spore former, motile by means of polar flagella. After the discovery of this genus, it is formed by the large number of species which are increasing with time in large

proportion (Mulet et al., 2008). However, several species initially classified as *Pseudomonas*, have moved to other genera over 50 years during refinement of the criteria to place the bacteria in specified position (Özen AI1, 2012).

After the discovery of the genus *Pseudomonas*, it has undergone several taxonomic modifications. The genus has gone much taxonomic revision over past 100 years from a large and diverse bacterial position to refined specific position (Ussery, 2012). The change of the taxonomic status is not only due to the addition of a variety of species but also due to the criteria applied for definition and delineation. Exhaustive lists of criteria were issued for *Pseudomonas* taxonomy in the current edition of Bergey's manual of systematic bacteriology. Different criteria like cellular morphology, structural composition of cell-wall, pigment types, nutritional and metabolic characteristics, production of antibiotic, and susceptibility to different chemical compounds, pathogenicity, antigenic property, genetic composition and ecological characteristics are utilized to characterize the *Pseudomonas* species. Traditionally, phenotypic characteristics like cell shape, type of flagella, carbon utilization, amino acid utilization, ability to grow in different cultural condition, antibiotic resistivity pattern were used for the identification of bacterial isolates. These criteria are clear, reliable and routinely used in many microbiological laboratories. These criteria are adequate for some strains which are frequently isolated from clinical specimen but it fails sometime to identify environmental species due limited numbers of tests included. However, these criteria are useful for the characterization of the non clinical species not for the identification (Behrendt, Heegaard, & Fornitz, 1999; Peix et al., 2003). The *Pseudomonas* genus recently contains several species assigned to *Pseudomonas* on the registered list of Bacteria. The basis of classification was the 16s RNA, cellular fatty acid and the combination of conventional physiological and the biochemical tests (Ussery, 2012).

2.4 Identification of *Pseudomonas* species by API 20 NE test and 16s RNA technology

API system is a standard system for identification of Gram negative, non fastidious bacteria like *Pseudomonas, Acinetobacter, Flavobacterium, Morexella, Vibrio* etc. The system contains 8 conventional tests and 12 assimilation test. The identification of the organism is done with the help of specific software. In addition to specific 20 tests available in the kit, oxides test should be done separately which is an integral part of the system (Willey, 2008)

The API 20 NE strip consists of 20 micro-tubes which contains dehydrated substrates. The eight conventional tests are inoculated with a saline bacterial suspension which reconstitutes the medium. After incubation, color changes in the medium due to metabolism or by the addition of reagents are noticed. Similarly, assimilation tests done by inoculating the bacteria in minimal medium to see the capability of bacteria to utilize the specific substrate. Some of the tubes are filled with mineral oil to create the anaerobic condition. Finally, the test results are scored according to the reading table and identification is done by the help of software.

It gives the accurate identification based on extensive databases. The system is standard and easy, quick and convenient to use. API test kit is economical and has long shelf life. The software or APIweb^{TM} contains an API database for reliable interpretation of API test result. The reliability of system is very high. The system is limited to specific bacteria like non-fastidious, non-enteric Gram negative rods. It cannot be utilized to identify various other type bacteria. Only, the pure culture of single organisms should be used.

2.5 16 s RNA Technology

There are many molecular techniques available for the evaluating for the phylogenetic relationship. Among them, 16s RNA have proven the best reliable method for the identification of the organism and to establish the relationship among the organisms (Kabiri L, 2013). The 16s RNA genes are present in almost all bacteria. They are very short (1542 bp long) and the function of 16s RNA remained unchanged over time (Abbott, 2007). These

genes can be quickly copied and can be sequenced. The 16s RNA gene share similarities over the terminal regions among different organisms but the sequences are scattered differently in central parts of the gene. The 16s RNA genes are copied from thousand to million times by the PCR techniques. All of this information are stored in the gene libraries and the sequence of the new isolates can be compared with the sequences stored in the libraries. The 16s RNA gene sequences provide the genus identification for isolates that do not fit to any previously accepted biochemical profile. The 16s RNA gene sequencing identifies the strain with high accuracy (> 90%). The technique has also some limitations that it has low resolution power for identification of strains at species level. Additionally, DNA relatedness data are necessary for absolute identification (Mignard, 2006).

2.6 General properties of *Pseudomonas* species

Pseudomonas spp. are straight to curved rods. The cell size measures 0.5 µm -1 µm in diameter by 1.5-5 µm in length. These are Gram -negative and motile by one to several polar flagella. Some of the species are immotile. These are strict aerobes and utilize oxygen as terminal electron acceptor but some of the species used nitrate as alternate electron acceptor which facilitates the species to grow anaerobically. Most of the species shows negative indole and methyl red test while they are oxidase and catalyses positive. The cells are easily lysed by EDTA solution giving the indication of high phosphorous content in the membranes. Most of the *Pseudomonas spp*. produces poly-β-hydroxybutyrate (PBH). Pigment production is another unique property of the *Pseudomonas* spp. Different species produce different types pigment like pyoverdin, pyocyanin, pyomelanin in different environmental conditions.*Pseudomonas* spp. have simple nutritional requirement therefore these species are isolated from different environments. They can be grown in simple laboratory growth medium containing some organic matter at neutral pH Most of the species grow well in the range of 27-30^0 C. *Pseudomonas* species can be grown anaerobically by enriching the medium by the addition of nitrate (NO_3) at 30-40^{0}C. The best growth of *Pseudomonas* spp. is achieved in medium containing organic compounds (0.1-1%w/v) as carbon or energy source

(Delorme et al., 2002). *Pseudomonas* use a wide range of nutritional resources and can even be grown in simple nutritional media without any organic compounds. They stay viable for long times in different habitats and unfavorable conditions. These species can be found in saline water, utensils, pharmaceutical products, disinfectants and natural as well as manufactured food products. Some of the species are psychrotropic. These psychotropic species are primarily responsible for the spoilage of refrigerated meat, fish, shellfish and the dairy products. Several species of the *pseudomonas* have been also isolated from different soil samples (Kwon et al., 2003).

2.7 Bacterial biofilm formation in the food industry

Microorganisms are mainly found in two forms which are planktonic or free floating in water and sessile or attached to a substratum(T. E. Cloete et al., 2009). The attachment to the surface helps to survive the organism in different harsh conditions. The ability of bacteria in attaching to a surface may enhance their persistence during manufacturing and retail and their ability to cause infection. At surface, microorganisms may assemble in large numbers and form a specialized slime layer called biofilm (T. E. Cloete et al., 2009; Kumar & Anand, 1998). This phenomenon of microorganisms seems to be natural while grown in wet environments. Biofilm matrix is composed of extracellular polymeric substances (EPS) that mainly consist of polysaccharides, nucleic acids, lipids and proteins (S. W. Cloete, Misztal, & Olivier, 2009; L. C. Simoes, Simoes, & Vieira, 2010). Additionally, biofilm contains non cellular material like mineral crystals, corrosive particles, clay, silt, and components etc. Biofilm takes place in variety of surfaces therefore the composition of the biofilm also depends upon the nature of the surface environments (Donlan, 2002).

Planktonic -biofilm transition phase is very complex and a highly regulated process (O'Toole et al., 2000). Especially, bacteria are colonized on the surface and covered by extracellular material and the other additional materials trapped within the matrix. Biofilm composed of complex web of different interaction (Burmolle et al., 2006). Water contributes about 80-98%

of biofilm where microorganisms, entrapped organic and inorganic particles contribute the rest percentage.

The biofilm formation process is a complex mechanism in which numerous genetic phenomenon of organisms and physical properties like surface of the substratum are involved. Holes and crevices in the environmental surfaces are the potential residential sites of microorganisms for the initiation of biofilm formation (Xianming Shi, 2009). In addition, plants producing wet products are also potential for the biofilm formation (Xianming Shi, 2009). Different bacteria utilized different mechanisms to initiate attachment to the corresponding surfaces. Most of the bacteria adhere to the surfaces within few hours, however the biofilm formation takes from couple of hours to days. Biofilm formation initiates quickly in unsterilized equipments due to the already existed microorganisms and the bacteria in these biofilms are resistant to the biocidal agents (De Vriendt et al., 2005). Water channels present in the biofilm provides nutrient uptake and waste exchange (K. Sauer, A. K. Camper, G. D. Ehrlich, J. W. Costerton, and D. G. Davies, 2002). The resistance increases with the age of the biofilm also (Carpentier & Cerf, 1993).

2.8 Monospecies and multispecies Biofilm

Biofilms are a closed association of microbial cells with surfaces and are not easily removed by gentle rinsing. Biofilm primarily consist of clay particles, mineral crystals, extracellular components depending on the environment in which the biofilms have been developed. Biofilm formation takes place on variety of surfaces ranging from living tissues to medical devices and industrial piping systems. Biofilm may be monospecies and the multi species. Under natural conditions, multispecies interactions exists and monospecies biofilms are rarely formed (M. Simoes, Pereira, Machado, Simoes, & Vieira, 2006). The formation of multispecies communitiy is a process where surface attachment of one bacterium may have attachment of other bacteria to the same surface or peripheral regions. The heterogeneous, multispecies biofilm communities have a unique complex physiology and metabolism (M. Simoes et al., 2006; M. Simoes, Simoes, Cleto, Pereira, & Vieira, 2008). A single biofilm can

exist of different populations of microorganisms like bacteria, fungi and protozoa. The complexity of biofilm or the microbial life is due to a high degree of interaction among different types of organisms in immobilized form which allows the development of stable mature structure. These consequences lead to synergetic effect within the community. The EPS are main constituent of biofilm and it helps other organism in the attachment or colonization to the biofilm structure (Burmolle et al., 2006). Many studies have proposed different bacterial interactions with in biofilm community. A multispecies biofilm are more stable than the monospecies biofilm community. A range of interactions has been observed among microorganisms within biofilm such as, antagonistic, competitive, mutualistic and commensal. Positive interaction among the organisms may lead to co-aggregation and plasmid conjugation. Every interaction has different impact in biofilm development or maturation. According to the study (M. Simoes et al., 2006) antagonistic association also plays an important role in the maturation and structure of microbial communities.

2.9 Biofilm formation and developmental Stages

Biofilms are aggregate or united solidified structures of microorganisms attached to the surface in the surroundings (Stewart, 2002). The biofilm formation and developments are affected by numerous factors including the specific bacteria strain (Borucki, Peppin, White, Loge, & Call, 2003; Chae & Schraft, 2000) surface properties of substratum and environmental parameters such as pH, nutrient levels and temperature (Donlan, 2002). Biofilm cells are more tolerant to antimicrobial agents than plankotonic life as the biofilm acts a barrier which prevents or lessens the contact with antimicrobial substances (O'Toole et al., 2000).

2.9.1 Initial attachment

Bacteria utilize different mechanisms for the initial attachment to the surface or the substratum. The attachment might be active or passive depending on their motility or the gravitational transportation of their planktonic diffusion (Kumar & Anand, 1998). It is seemed to be a physiochemical process between cell surface and the attachment surface. Van der Walls force , electrostatic interaction and steric forces determine the bacterial adhesion (Michiels, 2010). The attachment process leads to the reversible phase if the attached microorganisms are not yet committed to the differentiation process. A series of developmental as well as morphological changes takes place which leads to biofilm formation. Most of the cells may detach from the surface and return to the planktonic lifestyle (K. Sauer, Camper, Ehrlich, Costerton, & Davies, 2002).

The surface properties of bacteria has significant role in bacterial adhesion. Bacteria may attach to any surfaces like plastic, glass, metal, wood, and food products. Biofilm formation depends mainly upon interaction between the bacterial cells, attachment surfaces and the surrounding environment and nutrient availability (Stoodley, 2002).

Most of the bacteria are motile by the help of peritrichous or polar flagella. The motility is also regarded as the virulence factor for bacterial colonization to the target hosts. Flagella motility plays important role for initial cell to surface contact. In addition, surface appendages such as fimbriae and other physical parameters have been found to be associated with biofilm formation (Ben Abdallah, Chaieb, Zmantar, Kallel, & Bakhrouf, 2009; **Nilsson**, Ross, & Bowman, 2011).

2.9.2 Irreversible attachment or formation of micro colonies

After initial or loose attachment, bacterial cells start to grow by utilizing the nutrient available to the surrounding surface. At the same time, bacteria starts attachment to the surface by the formation of permanent bonding with the help of EPS. Then bacteria start to

multiply and start to intercommunicate with other bacterial cells. This leads to the formation of small bacterial colony (Prakash, Rao, & Parija, 2005). Formation of microcolonies and the subsequent attachment of other bacterial cells to the surfaces stabilize the cells from environmental stress (Chmielewski, 2003).

2.9.3 Biofilm Formation and Maturation

Once the microcolony is established, the bacterial cells start continuous attaching the microcolony and other peripheral structures. Bacterial cells, preformed EPS and environmental debris collectively form the biofilm structure. Complexity and the size of the biofilm depend upon the rate of bacterial replication, generation of extracellular component and inorganic molecules immediate to the biofilm environment. So that the same bacteria might form the different type of biofilm in different environmental conditions. At this stage biofilms are highly resistant to chemicals and disinfectants. Availability of nutrients, removal of waste, internal PH and temperature, flux of the material and surface structure play important role in the maturation of biofilm (Carpentier & Cerf, 1993). In general, it takes eight to ten days to form a mature biofilm (K. Sauer, A. K. Camper, G. D. Ehrlich, J. W. Costerton, and D. G. Davies, 2002; Stoodley, 2002). The architecture of biofilm depends on space and time, due to regular change in external and the internal processes (Donlan, 2002).

2.9.4 Dispersion or Detachment of biofilm

Dispersal of biofilm is the last step in the biofilm and the organisms finally revert into their planktonic life (K. Sauer et al., 2002). Microorganisms from biofilm are dispersed due to several reasons like shearing of biofilm, effect of flow, change of nutrient level or quorum sensing. The true mechanism of cells detachment from actively growing in biofilm is not clearly understood, however, erosion, abrasion and sloughing are actively involved in detachment process. Sloughing is more common than erosion (Donlan, 2001). Treatment

with biocides such as chlorine, surfactants or enzymes also destabilized the biofilm. Detachment of one biofilm helps in the colonization in the new niches (K. Sauer et al., 2002). Surface binding proteins and other endogenous enzymes are also probable reasons of biofilm detachment (Kaplan, Ragunath, Ramasubbu, & Fine, 2003).

2.10 Composition of *Pseudomonas* Biofilm and control strategies

Enzymes are generally used for the degradation of biofilm. Heterogeneity of the biofilm complex may limit the use of one enzyme and a mixture of enzymes are necessary (Mona Augustin, 2004). Many researchers have proposed different models of controlling biofilms. These models are mainly based on the understandings of the development of biofilm structures. Mainly three different strategies are utilized to control the biofilm (Morris, Collyard, & Meyer, 2003), disinfection of biofilm before development (Croes, Stobberingh, Stevens, Knetsch, & Koole, 2011) disinfecting the biofilm by using harsh disinfectants and inhibition of attachment to the surfaces by the modification of the surface structures (Chandra, Zhou, & Ghannoum, 2005). The combination of disinfectants with enzymes greatly enhances the disinfection efficiency than single disinfection only. Several researches have tried to prevent biofilm formation and the development of the broad spectrum molecule (Pan & Ren, 2009). The effective disinfectants towards planktonic microbial cells are less effective against biofilm. The proper understanding of the nature of composition of EPS matrix is crucial for the removal of bioflm (Walker & Marsh, 2007; Xavier Jde, Picioreanu, & van Loosdrecht, 2005). Enzymatic removal of biofilm closely relates to the composition of biofilm composition. Different studies have shown the presence of extracellular DNA, protein, polysaccharides and lipid in the biofilm matrix produced by *Pseudomonas* spp (Brizzolara & Holm, 2006; Hamilton & Dillard, 2006; Larsen, 2002; Steinberger & Holden, 2005; Whitchurch, Tolker-Nielsen, Ragas, & Mattick, 2002). In addition to these major components, other self aggregating components like curli, tafi, fimbriae also contribute in the biofilm formation. Strong association among these components makes the bacteria resistant to harsh environmental stress. Extracellular DNA may be involved in the biofilm formation

and the bacterial colonization (Hamilton & Dillard, 2006). These extracellular DNA are released by living cells and enter the matrix from the lysed cells. Destruction of extracellular DNA of *Pseudomonas aeruginosa* changes the properties of biofilm. Extracellular DNA is a major component in some biofilm of *Pseudomonas aeruginosa* (Ali Mohammed, Nerland, Al-Haroni, & Bakken, 2013). Similarly, P, N-acetylglucosamine (PNAG) acts as polysaccharide adhesion and has the key role in the biofilm formation Psi; Pel and the alginate are the three main component of biofilm formed by *pseudomonas* species. These components have potential role in the cell to cell interaction. Dispersin B isolated from the Actinobacillus actinomycetemcomitans has been found to hydrolyse the PANG (Kaplan et al., 2003). This enzyme rapidly removes the biofilm by endolytically hydrolysing the polyglycosidic linkage of N-acetyl glucosamine (Lequette, Boels, Clarisse, & Faille, 2010). In addition, extracellular proteins are also major components of the biofilm (Ali Mohammed et al., 2013). Similarly, Kumar and his collegues (Kumar Shukla & Rao, 2013) found the potential role of Proteinase K in the enzymatic degradation of protein molecule from the biofilm matrix of gram negative bacteria including *Pseudomonas*.

2.11 Cleaning and Disinfection in the food industry

Cleaning is generally utilized to remove the solid particles in the food industries where as disinfection is utilized to kill the microorganisms. The frequency of cleaning and the disinfection strategy determines the life of biofilm. But environmental surfaces like walls become more prone to biofilm formation due to the untimely cleaning or disinfecting procedure. These surfaces can subsequently contaminate the products contact surfaces through e,g. equipment, water or personnel (G. B. Gibson, Mathias, & Epstein, 1995; L. C. Simoes et al., 2010). The sources of contamination and the recontamination are due to the ineffective or the improper cleaning procedure. As bacteria will be present on most surfaces, the temperature should be kept low as possible and the environment should be dry to avoid unwanted growth of bacteria in food processing plants (Molobela, 2010).

2.11.1 Cleaning

Cleaning is the initial and universal system of sanitation. It improves the quality of sanitation. Microorganisms become more sensitive towards disinfection after they have been detached from the biofilm (Carpentier & Cerf, 1993; L. C. Simoes et al., 2010). Mechanical actions like brushing, high pressure jet are effective in the biofilm removal. Nearly, 90 % of the microorganisms associated with surfaces are removed by the cleaning procedure but the viability of organisms remained uncertain. But the cleaning procedure may relocate the microorganisms to new sites and the bacteria may start replication in that habitat (L. L. Gibson, Rose, & Haas, 1999). Finally, they may form the new biofilm on that particular site depending on time and the nutrient availability. Ineffective cleaning procedures may leave biofilm or food residues on surfaces and do not let the disinfectants penetrate the biofilm resulting in the living cells within the biofilm remain unharmed. The effective cleaning procedures dissolve or disrupt the biofilm matrix so that the disinfectants get access to the live cells (M. Simoes et al., 2006).

Major chemicals used as cleaning agents in food industries are basic alkalis, complex phosphates, surfactants, chelating agents and acid compounds. The combination of the compounds are designed to get complex functions like dispersion, emulsification, penetration, saponifiction, suspension etc (McBain, 2003). Generally, hypochlorite, chlorine dioxide, iodophores, perooxyacetic acid (PPA) and quaternary ammonium compounds (QAC) are used as cleaning chemicals in food industries (Virto, Manas, Alvarez, Condon, & Raso, 2005).

2.11.2 Disinfection

In the disinfection process, antimicrobials are used to kill the microorganisms or to reduce the surface population of the viable cells. The disinfectants should be safe to handle, effective to microorganisms, should not affect the quality of processed food products. Presence of organic substances like carbohydrates, fat and protein in the biofilm, directly affect the effectiveness

of disinfectants. Similarly, other factors like PH, temperature, hardness of water, chemical inhibitors, concentration and the exposure time have direct impact in the potentiality of disinfectants (Bremer, Monk, & Butler, 2002; T. E. Cloete, Jacobs, & Brozel, 1998). There are many types of disinfectants including chlorine, hydrogen peroxide, enzymes, iodine, and ozone, peracetic acid which are utilized in the industrial disinfection procedure (Chmielewski, 2003).

2.12 Stainless steel in the food industries

Stainless steel is a common and routinely used material in food industries. Particularly, 304 and 316 grades are used in industries due to their stability against various chemicals, corrosion and food processing temperature (Wilks, Michels, & Keevil, 2005). Both grades share the same qualities but the grades 316 are more resistant to corrosion due to anticorrosive properties of added molybdenum. Studies done with scanning electron microscopy have proven that many food borne pathogens and spoilage microorganisms gather into biofilms units on stainless steel typically found in food environments. The cracks and crevices and rough appearances are the best suitable environments for the bacteria to survive. Therefore, the equipment should be designed with high hygiene standard which may reduces vulnerable sites like cracks, crevices, corner, gaskets, and joints. Similarly, selection of the disinfectants plays the important role in maintaining the smoothness of the stainless steel.

Hypochlorites are aggressive to stainless steel. The liberation of the free chlorine can cause the pitting which destroys the passive oxide layer and helps in the formation of pits where bacterial attachment takes place. Therefore selection of the chemical disinfectants and the hygienic designing of the equipment plays important role in the biofilm prevention.

3 Material and Methods

Every experiment with microorganisms was performed in a safety bench in the laboratory. Similarly media preparation and other chemical preparations for the experiments were performed in sterile bench**.**

3.1 Media used in the experiment

Compositions of every medium are mentioned in the appendix.

Minimal Medium (MM)

Tryptone soya Broth (TSB)

Tryptone soya Agar (TSA)

Tryptone soya Broth with Glucose and Sodium chloride (TSBNG)

3.2 Chemical and Enzymes used in the experiment

Dispersin B (Kane Biotec Inc. USA)

Proteinase K (Sigma-Aldrich, St. Louis, USA)

DNAse. (Sigma-Aldrich, St. Louis, USA)

Chlorine (Lilleborg,Norway)

Crystal violet (1%)

Acidified ethanol

API Nacl 0.85 % Medium, 5 ml

API suspension medium, 5ml

API 20E reagent Kit

James reagent

VP1+VP2

NIT+VP2,

ZN reagent,

Oxidase reagent

Mineral oil,

Peptone water,

API 20E analytical profile Index or identification software for the identification of tested microbial species.

3.3 Table 1 Bacterial strains used in the experiment and sources of origin

STRAINS PROVIDED	16s RNA IDENTIFICATION	SAMPLE TAKEN FROM
2822	*P. fragi*	Poultry Processing
3603	*P. flourescens.*	Beef slaughter house
2.12	*P sps/P. putida*	Beef slaughter house
96.4	*P fragi*	Salmon Processing
96.6	*P.fluorescens*	Salmon processing
1942	*P.fluorescens*	ATCC 13525
1943	*P fragi*	ATCC 4973
1945	*P. lundensis*	Beef
1946	*P.fluorescens*	Beef
2318	*P tremae*	Poultry Processing
2322	*P.fluorescens*	Poultry Processing
2329	*P.lundensis*	Poultry Processing
2334	*P.fluorescens*	Poultry Processing
2530	*P.aeruginosa*	ATCC 15422
2830	*P.lundensis*	Poultry
2833	*P.fragi*	Polutry
2846	*P.fluorescens*	Poultry
3600	P.fulgida	Beef Slaughter house
3601	*P.aeruginosa*	Beef Slaughter house

3720	*P.putida/flourescens*	Small scale cheese production
3721	*P.putida/fragi*	Small scale cheese production
3796	*P.sps*	Small scale cheese production
3797	*P.aeruginosa*	Small scale cheese production
3831	*P.fluorescence*	ANSES, France
4093	*P.fluorescence*	Salmon Processing
4193	*P.fragi*	Salmon Processing
4757	*P.putida*	Salmon Processing

3.4 Condition for growth of bacterial strains

Provided bacterial strains were stored in glycerol at-80ºC. The strains were plated on TSA (Tryptone soya Agar). The plates were incubated at 30ºC for 24 hours. After 24 hours, the plates were stored in the refrigerator at 4 ºC for frequent reuse proposal**.**

3.5 Formation of biofilm

Bacterial samples were provided in plate from freezer. Plates were incubated plates at 20^0C until growth. Growth of all strains was checked. Colonies were transferred to tubes with 3 ml medium and incubated at 20^oC with agitation of 150 rpm until good growth. Then cultures were freezed down in freezer tubes, at 17-20% glycerol at -80^o C.

Five different Pseudomonas strains were allowed to grow in three different Medias TSB, TSBNG and MM. The selected strains were *P. fluorescence* (3720), *P. aeruginosa* (3601), *P. fragi* (4193), *P.folurescence* (1946) and *P. lundensis* (2329). All five selected strains were allowed to grow for 72 hours and 96 hours at the 12^oC and the 20^oC respectively. Four identical plates were set up for the two corresponding temperatures.ie. 20^oC and 12^oC. Biofilm-forming ability was measured by staining of polystyrene-attached bacteria with crystal violet (CV). 20 µl of bacterial cultures were used as inoculums to obtain

approximately 106 CFU/ml in each well of 96-well, U-bottomed polystyrene plates (BibbySterilin; Bibby Scientific, Staf-fordshire, UK) containing a total 180 µl of medium. Strains were cultured overnight in TSB solution and those overnight cultures were diluted 100 times to obtain the approximately 106 CFU/m as inoculums. Four parallel micro titer plates were used for each strain and cultivation condition. Negative control wells contained 200 µl of TSB/TSBNG/MM only where rest of the tested wells contains 180 µl of medium plus 20 µl of bacterial inoculum. Biofilm formation was tested after incubation at 12/20°C for 3 and 5 days. Total cell mass was measured as absorbance at 600 nm (Titertek Multiskan RC plate reader; Labsystems, Hel-sinki, Finland). Biofilm formation was quantified according to the following procedure. Bacterial suspensions were pipetted off and the remaining biofilm were washed twice with 300 µl distilled water (dH2O), using a semi-automatic microtiter plate washer (Wellwash AC, Thermo Electron Corporation, Waltham, Massachusetts, USA). Surface attached bacteria were dried at 30°C for 15 min and thereafter stained with 200 µl 0.1% CV for 5 - 10 min. After two washes with 300 µl dH2O, surface-bound CV was extracted by addition of 200 µl 33% acetic acid and incubation for 5 min. A volume of 100 µl was transferred to a new microtiter plate and absorbance was measured at 600 nm. Absorbance measurements were subtracted the absorbance values from wells containing TSB/TSBNG/MM only i.e. negative control.

3.6 Effects of enzymes in inhibition of biofilm

For this purpose, the strains *Pseudomonas lundensis* (*2830), Pseudomonas fluorescens* (2846), *Pseudomonas putida* (4757), *Pseudomonas fluorescens* (3831), *Pseudomonas aeruginosa* (2530*), Pseudomonas fluorescens* (96.6), *Pseudomonas putida* (2.12*), Pseudomonas fulgida (*3600), *Pseudomonas fluorescens* (4093*), Pseudomonas lundensis (*2329*), Pseudomonas fluorescens* (2322) were selected.

The selection of the strains were based on good biofilm production capacity, different sources of origin and the species varieties. The main purpose of this experiment was to find out whether the biofilm matrix are composed of polysaccharide, protein and DNA. So that the

enzymes selected were Dispersin B, Proteinase K and DNase. Again the experiment was done in exact manner as in previous biofilm formation but the composition of the medium were changed by application of different concentration of enzymes in the medium which are mentioned as follows.

Control (TSBNG)

TSBNG containing 50 µg/ ml dispersin B

TSBNG containing 100 µg/ml proteinase K

TSBNG containing 100 µg/ml DNase

20 µl of cell suspension of each strain was poured into the well. Plates were incubated at 12^{o}C for 72 hours and the cell concentration and the biofilm were measured at 600_{nm}. *Pseudomonas putida* (2.12), *Pseudomonas fluorescens* (96.6), *Pseudomonas fluorescens* (3831) strains were cultured overnight in TSB at 30^{o}C, were used as inoculum.

3.7 Production of biofilm

The strains were cultivated in TSB at 30°C for 24 hours for the production of biofilm in micro titer plates. Each strain is diluted 100 times in TSBNG. Cell numbers are checked by plating on TSA (two different dilutions). 6 wells plates with stainless steel coupons were added with 5 ml bacterial suspension. (i.e.12 coupons for each strain).

Prior to the experiment, the stainless steel were sterilized. Bacteria are allowed to attach for 3 hours at 12^{o} C. The suspension is then pipetted off and the coupons rinsed gently with distilled water. The water is discarded and the coupons were placed in new wells. Three ml TSBNG is added and the biofilm was grown at 12^{o}C for 96 hours. After 72 hours, two coupons of each strain taken out after 72 hrs for determination of cell numbers in biofilm. Coupons were gently washed with distilled water and they were transferred into the sonication tube containing 6 ml of TSB (sonication and plate spreading on TSA).

3.8 Detachment of the biofilm

The biofilms from previous day was promising (> log 6 cells per plate) so that the detachment experiment was performed further. The suspensions are pipetted off from the experimental plates and the coupons were rinsed gently with distilled water. A volume of 3 ml was added to each well (two wells per treatment, a total of ten coupons per strain)

Control (TSBNG, as before)
TSBNG containing 50 ug/ml Dispersin B
TSBNG containing 100 ug/ml Proteinase K
TSBNG containing 100 ug/ml DNAse
TSBNG containing 0.03% hypochlorite of pH 12 (made by adding NaOH)

3.9 Hypochlorite solution used in the experiment named Klorin, Lilleborg

Hypochlorite used in the experiment was composed of hypochlorite and NaOH, but the sodium hydroxide concentration was too low. Therefore NaOH was used to obtain the PH of 12. Provided concentration of the chlorine was 4.4 percent of PH 12.5. But recommended concentration was 0.03% in the experiment protocol. According to the manufacture Instruction, the PH of the klorin would be low if it was diluted in pure water. Diluting the solution upto the recommended concentration would eventually lowered the concentration. So to avoid the problem, a stock solution of 0.06 percent was prepared in water and adjusted the PH to about 12.1. And water was filled to the stock solution to get the final concentration of 0.03 percent.

To get approximately the same exposure time and to avoid systematic errors (for example that the DNase treatment might be systematically longer than dispersin B), addition of every suspension was done between each five minute interval. (Eg. Control no 1 at 10:00, dispersin no 1 at 10:05 and so forth) and then reverse the order in for the next coupons (hypochlorite no 2 at 10:30, DNase at 10:35.

The biofilms were exposed to the agents for 1 hr at 20^{o}C, rinsed gently and placed in a sonication tube with TSB. The remaining cells were determined by sonication in TSB followed by plate spreading (TSA, 30°C). After sonication for 10 minutes, the solution were serially diluted into 10, 100, 1000 and 10000 folds in peptone water (ie.120 tubes of peptone water for three different strains and five different disinfectants including control). 50 microliter of sample was used in plating. Bacterial Enumeration Whitly Automated Spiral Plater was used for spreading purpose on TSA plates(Don Whitly scientific limited ,14 Otelt Road , Shiply, West Yorkshire, BD 17 7SE, England, WWW.dwscientific.co.uk,). The plates were allowed to dry for some time in the sterile cabin and finally incubated at30°C for 24 hours. After incubation 24 hours, plates were put into the spiral plate automated colony counter for quantification of bacterial number.

3.10 Spiral plate colony counting

It was a standard protocol system provides the automatic counting of the bacteria on these types of plates. The system counts the colony in the different specified segments of the plates. If the total count is less than 20, the colony counter automatically counts the wholly colony in the plate. The colony counter automatically calculates the total no of colonies in a plate. Sometime the colony counter system counts the false colony so manual editing was necessary in these steps.

4 Results

4.1 Identification of the organisms by API test

Four out of the 27 strains were identified as different from that of 16s RNA results (Table no 1 and 2). Total 23 strains were identified as the same in both 16s RNA and the API tests. *Pseudomonas putida* (3720), *Pseudomonas fragi* (3721), *Pseudomonas multicoda* (3796) and *Pseudomonas putida* (4757) were identified as *Burkholderia cepacia*, *Ochrobactrum anthropi*, *Pasteurella multocida* and *Pasteurella multocida.* The four strains were belonging to different species according to two methods (API 20NE and 16 s RNA technology).

Table no 2. API test result of the provided strains

STRAIN	API TEST IDENTIFICATION	16sRNA IDENTIFICATION	% API IDENTITY
2822	*Pseudomonas putida*	*P. fragi*	83.6
3603	*Pseudomonas fluorescens*	*P. flourescens.*	71.0
2.12	*Pseudomonas fluorescens*	*P sps/P. putida*	96.6
96.4	*Pseudomonas putida*	*P fragi*	94.3
96.6	*Pseudomonas fluorescens*	*P.fluorescens*	58.6
1942	*Pseudomonas fluorescens*	*P.fluorescens*	99.8
1943	*Pseudomonas putida*	*P fragi*	99.1
1945	*Pseudomonas alcaligenes*	*P. lundensis*	75.2
1946	*Pseudomonas fluorescens*	*P.fluorescens*	99.3
2318	*Pseudomonas fluorescens*	*P tremae*	99.7
2322	*Pseudomonas fluorescens*	*P.fluorescens*	97.3
2329	*Pseudomonas putida*	*P.lundensis*	97.9
2334	*Pseudomonas fluorescens*	*P.fluorescens*	99.9
2530	*Pseudomonas aeruginosa*	*P.aeruginosa*	55.4
2830	*Pseudomonas fluorescens*	*P.lundensis*	60.0

2833	*Pseudomonas putida*	*P.fragi*	95.2
2846	*Pseudomonas putida*	*P.fluorescens*	61.7
3600	*Pseudomonas fluorescens*	*P.fulgida*	99.1
3601	*Pseudomonas aeruginosa*	*P.aeruginosa*	99.4
3720	*Burkholderia cepacia*	*P.putida/flourescens*	99.0
3721	*Ochrobactrum anthropi*	*P.putida/fragi*	91.9
3796	*Pasteurella multocida*	*P.sps*	95.7
3797	*Pseudomonas aeruginosa*	*P.aeruginosa*	66.7
3831	*Pseudomonas fluorescens*	*P.fluorescence*	69.3
4093	*Pseudomonas fluorescens*	*P.fluorescence*	99.8
4193	*Pseudomonas fragi*	*P.fragi*	90.8
4757	*Pasteurella multocida/ Aeromonas salmonicida masoucida/achromogenes*	*P.putida*	63.7

4.2 Biofilm formation

4.2.1 Effects of nutrient media on biofilm production at 20°C

Biofilm produced by *Pseudomonas* species cultured in three different media at 20°C for 72 hours, was determined. The OD value ranged from 0.9 to 3.0 (Figure 1). All species formed least biofilm while grown in minimal medium and highest biofilm formation in TSB and TSBNG media *Pseudomonas fluorescens* (1946) showed the highest biofilm formation in TSB media.

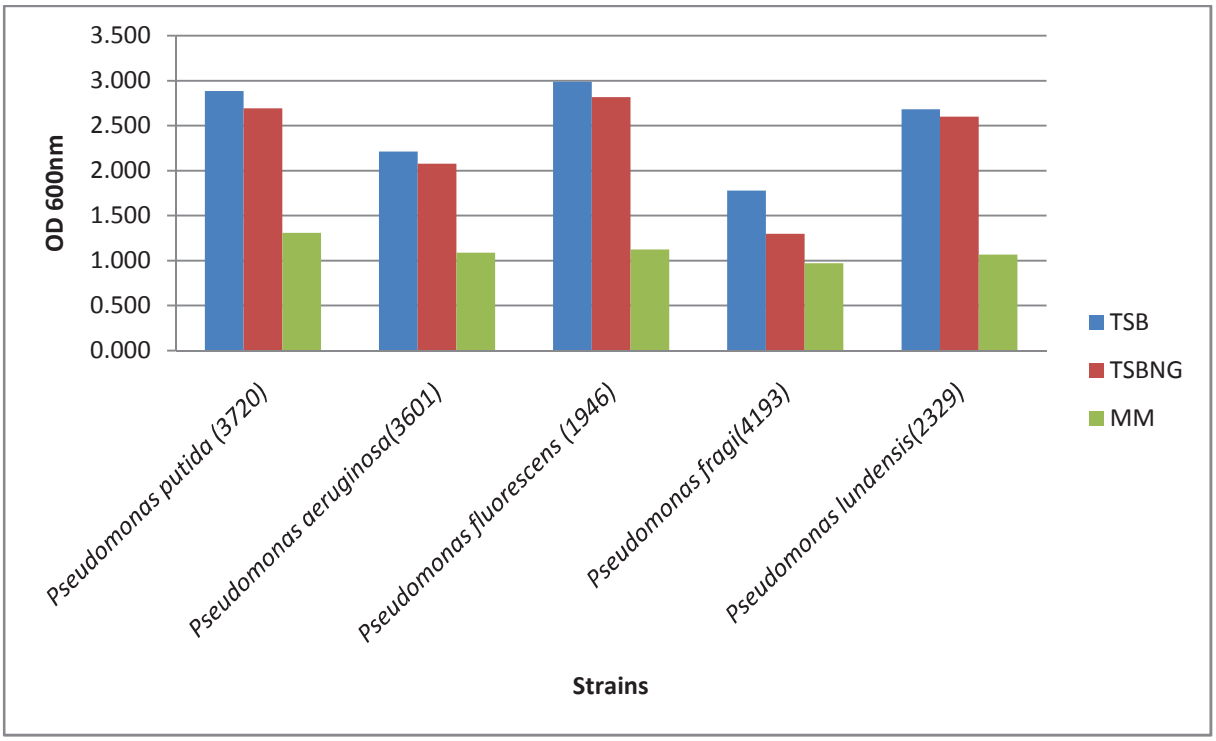

Figure 1 Biofilm formation by five different strains in three different culture media. The *Pseudomonas* strains were cultured at 20°C for 72 hours.

4.2.2 Effect of nutrient media on biofilm production at 20°C

Biofilm produced by *Pseudomonas* species cultured in three different media at 20°C for 5 days, was determined. The OD value ranged from 0.8 to 2.8 (Figure 2). All species formed least biofilm while grown in minimal medium and more biofilm in TSBNG media. *Pseudomonas fluorescens* (1946) showed the highest biofilm formation with OD value 2.8 consequently followed by *Pseudomonas lundensis* (2329) and *Pseudomonas putida* (3720).

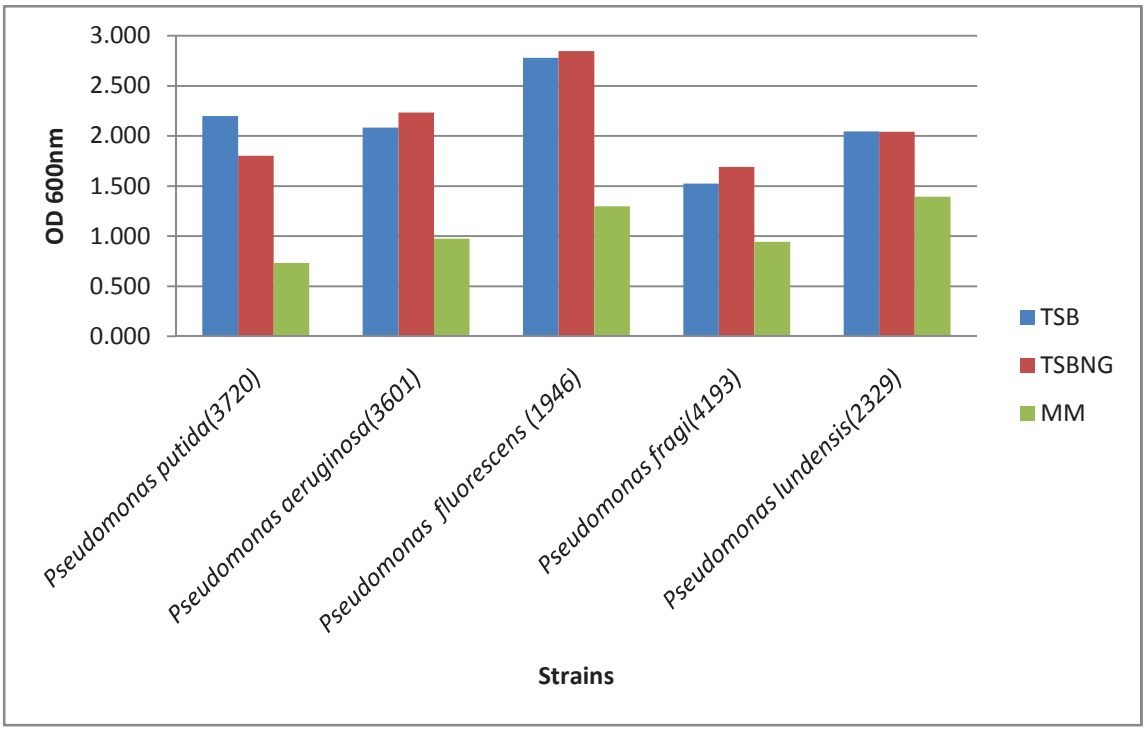

Figure 2 Biofilm formation by five *Pseudomonas* strains in three different culture media. The *Pseudomonas* strains were cultured at 20°C for 5 days.

4.2.3 Effect of nutrient on biofilm production 12^0C

Biofilm produced by *Pseudomonas* species cultured in three different media at 12 ^oC for 72 hours, was determined. The OD value ranged from 0.9 to 3.2 (Figure 3). All species formed least biofilm while grown in minimal medium and higher biofilm in TSBNG media. *Pseudomonas fluorescens* (1946) showed the highest biofilm formation with OD value 3.2.

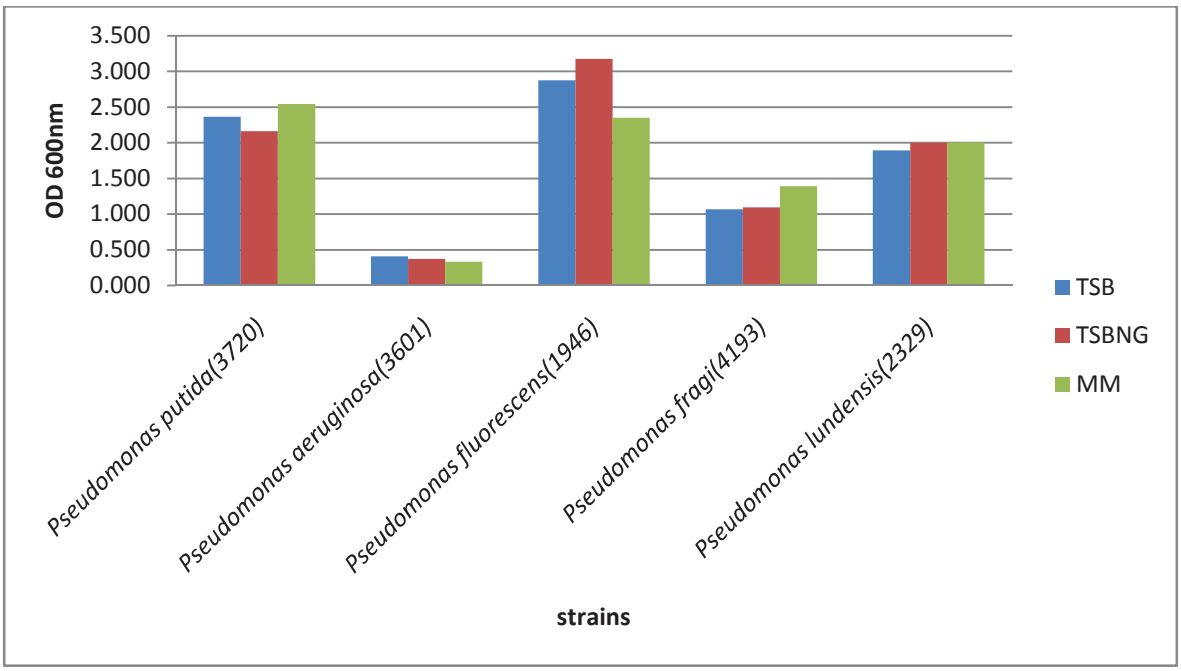

Figure 3 Biofilm formation by five *Pseudomonas* strains in three different culture media. The *Pseudomonas* strains were cultured at 12^oC for 72 hours.

4.2.4 Effect of nutrient media on biofilm formation at 12 °C

Biofilm produced by *Pseudomonas* species cultured in three different media at 12°C for 5 days, was determined. The OD value ranged from 0.3 to 3.0 (Figure 4). All species formed least biofilm while grown in minimal medium and higher biofilm formation in TSBNG media. *Pseudomonas fluorescens* (1946) showed the highest biofilm formation with OD value 3.0, which were followed by *Pseudomonas lundensis* (2329) and *Pseudomonas putida* (3720). *Pseudomonas fragi* (4193) and *Pseudomonas aeruginosa* (3601) formed comparatively very low biofilm in all three media.

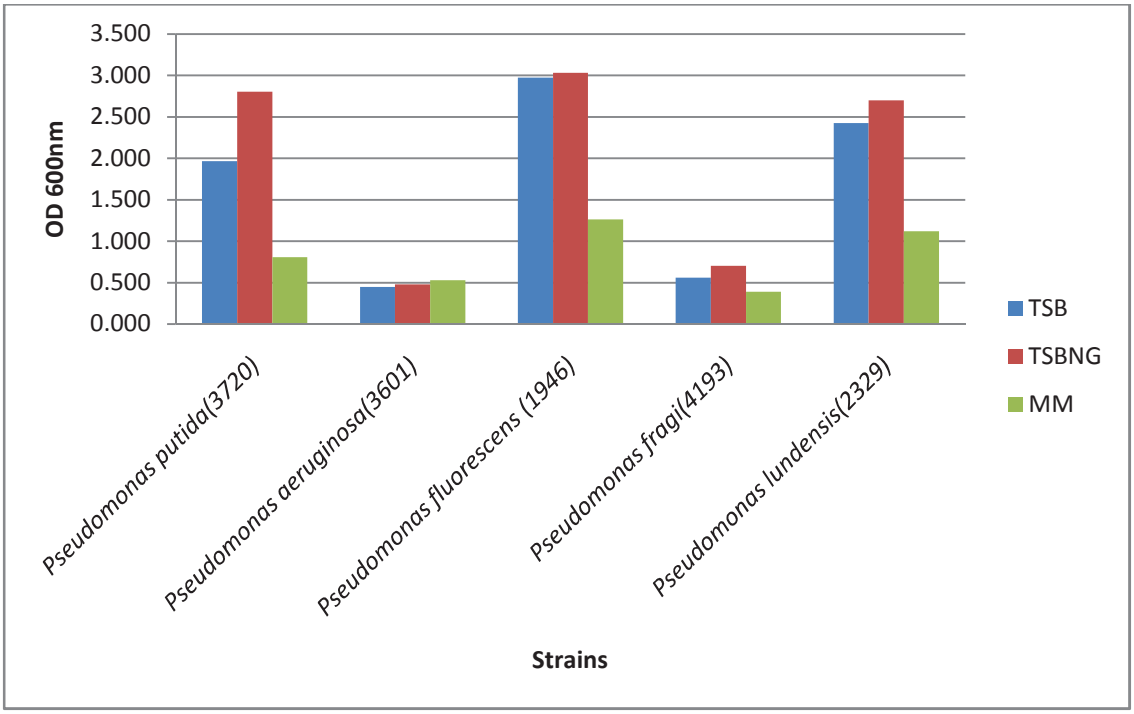

Figure 4 Biofilm formation by five different *Pseudomonas* strains in three different culture media. The *Pseudomonas* strains were cultured at 12^0C for 5 days.

4.3 Biofilm formation by 27 *Pseudomonas* strains cultured in TSBNG media at 12^0C for 72 hours

OD_{600nm} measurement of biofilm produced by *Pseudomonas* species cultured in TSBNG media at 12°C for 72 hours. The value ranged from 0.1 to 3.4. *Pseudomonas fluorescens* (2846) formed thicker biofilm with OD value of 3.4 which were followed by *Pseudomonas fluorescens* (3831) and *Pseudomonas fluorescens* (2334). *Pseudomonas fluorescens* (1942). *Pseudomonas fragi* (1943) and *Pseudomonas fluorescens* (3796) formed very low biofilm in comparison to other strains.

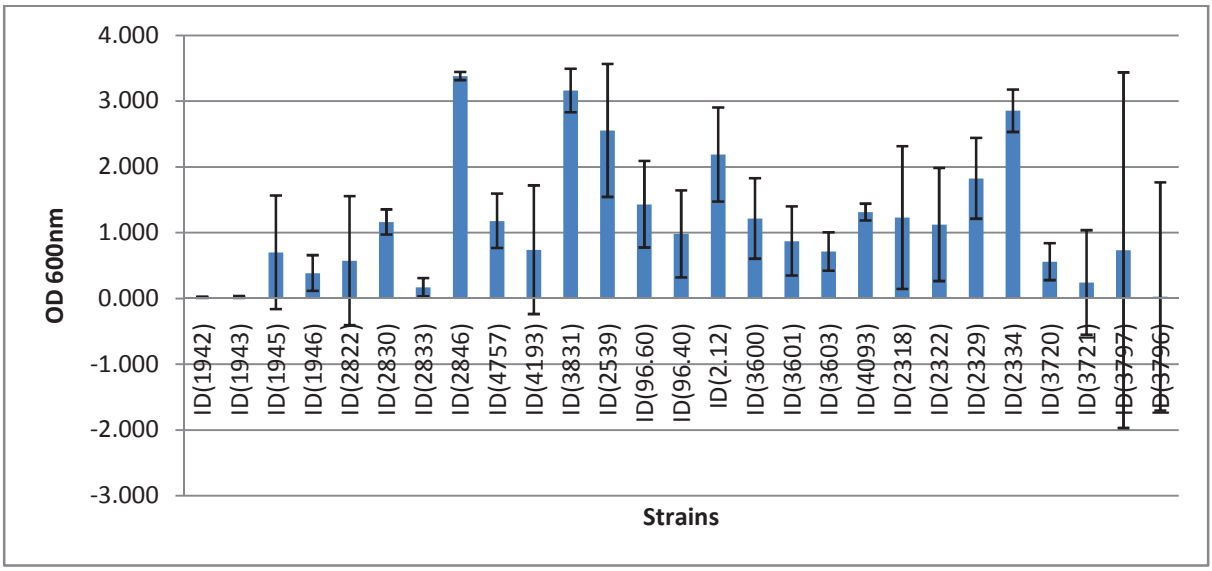

Figure 5 Biofilm formation by different strains in TSBNG media. The mean values and the standard deviation are shown. The strains were cultured at 12^0C for 72 hours.

4.4 Biofilm formation by 27 *Pseudomonas* species cultured in TSBNG media at 20° C for 72 hours

OD_{600nm} measurement of biofilm produced by *Pseudomonas* species cultured in TSBNG media at 20°C for 72 hours. The OD value ranged from 0.2 to 3.4. *Pseudomonas lundensis* (2830) formed thick biofilm with OD value of 3.4 which were followed by *Pseudomonas fluorescens* (3831) and *Pseudomonas fluorescens* (2334). *Pseudomonas fluorescens* (1942) showed very low biofilm in comparison to other strains. All most all species showed competitive biofilm where the value ranged from 1.5 to 3.4.

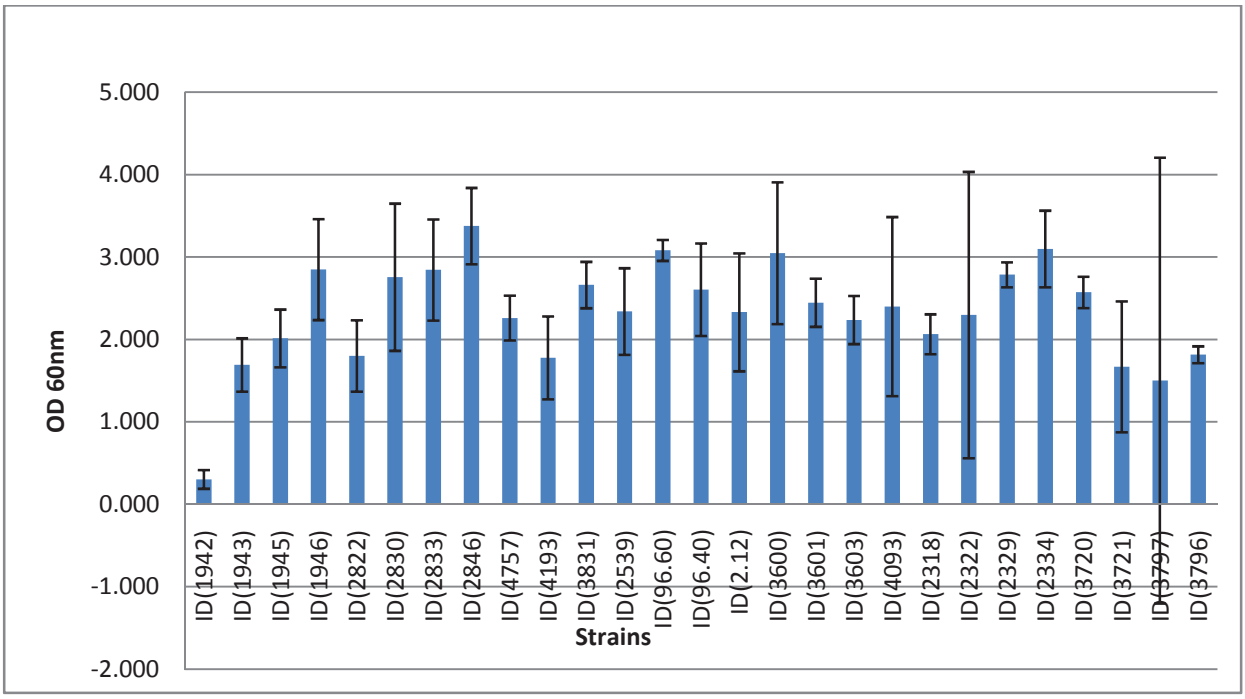

Figure 6 Biofilm formation by *Pseudomonas* strains in TSBNG media. The mean values and the standard deviation are shown. The strains were cultured at 20°C for 72 hours.

4.5 Effects of enzyme on biofilm formation.

4.5.1 Biofilm formation by *Pseudomonas* strains cultured in TSBNG medium containing 50 ug /ml Dispersin B

OD_{600nm} measurement of biofilm produced by *Pseudomonas* species cultured in TSBNG medium containing 50 ug /ml Dispersin B, at 12°C for 72 hours. The OD value ranged from 0.4 to 1.7. *Pseudomonas fluorescens* (2830), *Pseudomonas fluorescens* (96.6) and *Pseudomonas fluorescens* (4093) showed in reduction biofilm formation in the medium containing enzyme than that of control medium.

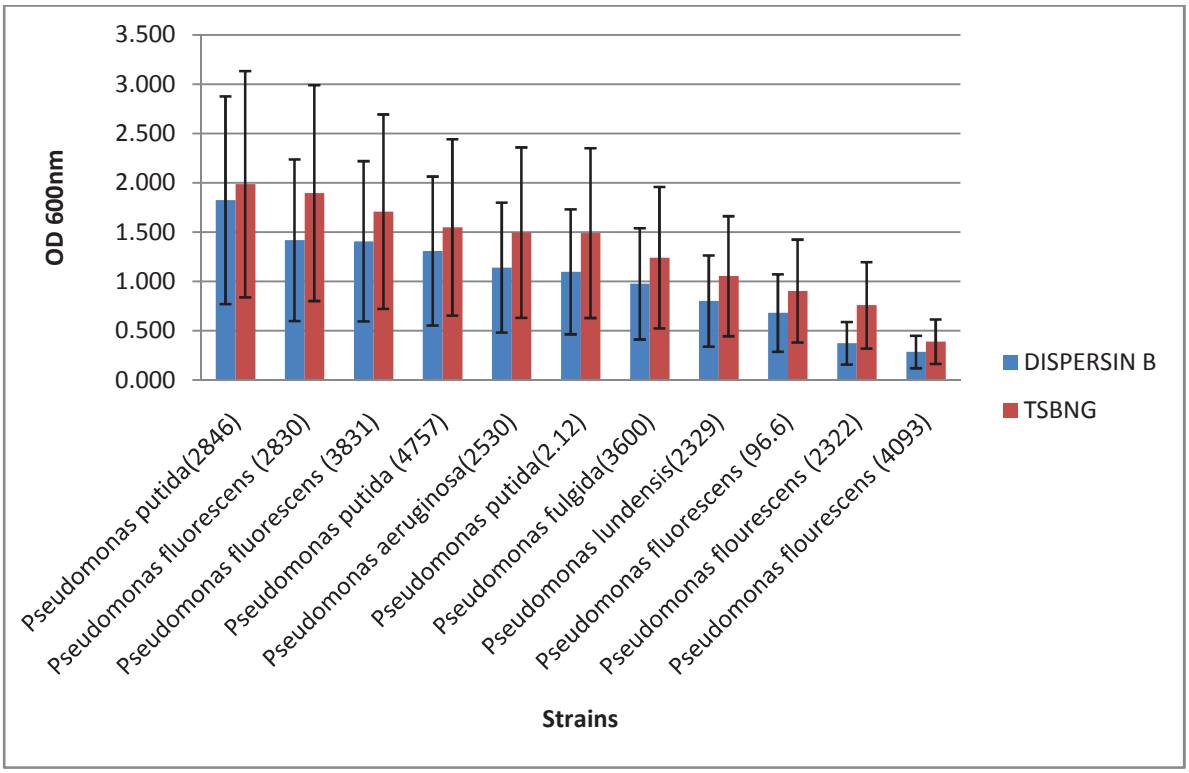

Figure 7 Biofilm formation by *Pseudomonas* strains in presence of TSBNG medium containing 50 ug /ml Dispersin B. The mean values and the standard deviation of the test and the control (TSBNG) are shown. The strains were cultured at 12° for 72 hours.

4.5.2 Biofilm formation by *Pseudomonas* strains cultured in TSBNG medium containing 100 ug /ml Proteinase K

OD_{600nm} measurement of biofilm produced by 11 different *Pseudomonas* species cultured in TSBNG medium containing 50 ug /ml Proteinase K at 12°C for 72 hours. The value ranged from 0.4 to 1.7. *Pseudomonas putida* (2.12), *Pseudomonas fulgida* (2830) and *Pseudomonas fluorescens (*2322) formed less biofilm in medium containing enzyme than that of control.

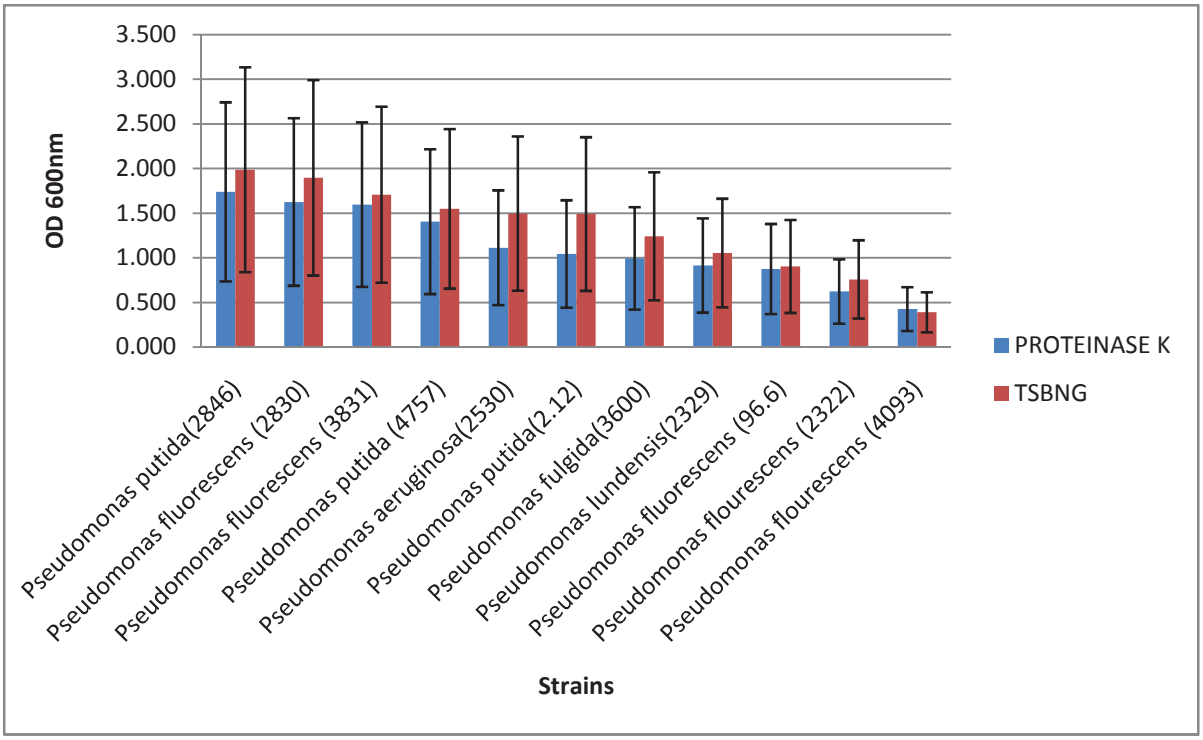

Figure 8 Biofilm formation by *Pseudomonas* strains in TSBNG medium containing 100 ug /ml Proteinase K. The mean values and the standard deviation of the test and the control (TSBNG) are shown. The strains were cultured at 12° for 72 hours.

4.5.3 Biofilm formation by different 11 *Pseudomonas* strains cultured in TSBNG medium containing 100 ug /ml DNase

OD_{600nm} measurement of biofilm produced by *Pseudomonas* species cultured in TSBNG medium containing 50 ug /ml DNase enzyme at 12°C for 72 hours. The value ranged from 0.3 to 1.6. *Pseudomonas putida* (2.12) showed the reduction in biofilm formation which were followed by *Pseudomonas fluorescens (*3831) and *Pseudomonas aeruginosa* (2530).

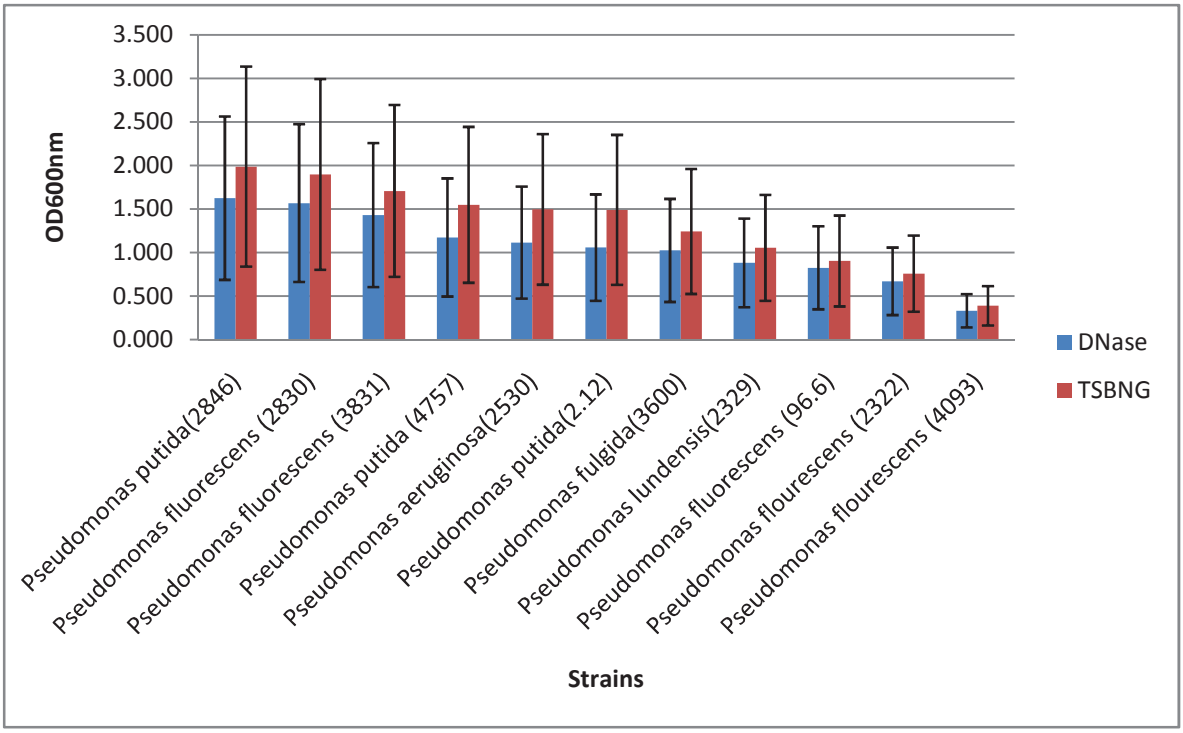

Figure 9 Biofilm formation by 11 different strains in TSBNG medium containing 100 ug /ml DNase. The mean values and the standard deviation of the test and the control (TSBNG) are shown. The strains were cultured at 12° for 72 hours.

4.5.4 Biofilm formation by different *Pseudomonas* strains cultured in TSBNG medium containing mixture of enzymes (Dispersin B, Proteinase K and DNase). The strains were cultured at 12^0C

OD_{600nm} measurement of biofilm produced by *Pseudomonas* species cultured in TSBNG medium containing all three enzymes mixture, at 12°C for 72 hours. The value ranged from 0.3 to 2.1. *Pseudomonas fluorescens* (3831) showed the reduction in biofilm formation which were followed by *Pseudomonas fluorescens* (2830), *Pseudomonas fulgida (*3600) and *Pseudomonas fluorescens* (4757).

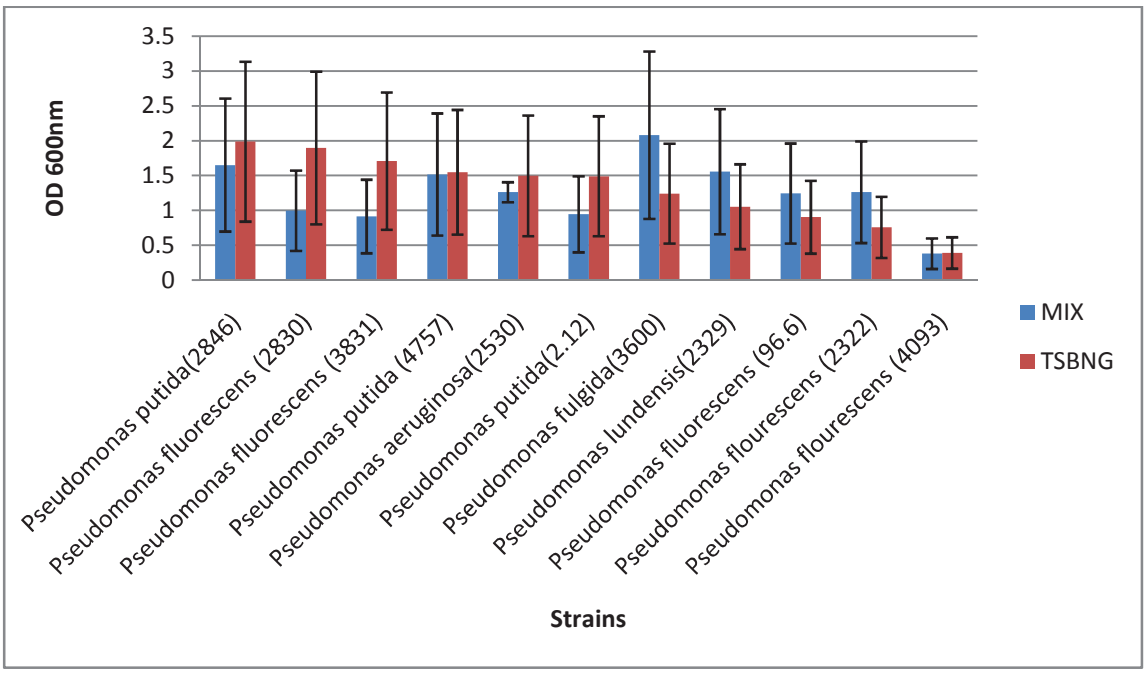

Figure 10 Biofilm formation by *Pseudomonas* strains in TSBNG medium containing mixure of enzymes. The mean values and the standard deviation of the test and the control (TSBNG) are shown. The strains were cultured at 12° for 72 hours.

4.6 Detachment of biofilm

4.6.1 Bacterial count after exposing the biofilm to the enzymes for 1 hour without agitation.

Cell numbers of *Pseudomonas putida*, (3831) *Pseudomonas fluorescens* (2.12) and *Pseudomonas fluorescens* (96.6) were determined after the biofilm had been exposed to the enzymes for 1 hour without agitation. The numbers were also calculated from the different dilution of 10^{-3} and 10^{-4}. The bacterial numbers ranged from $\log_{10}$ 6.2 to 10.0.

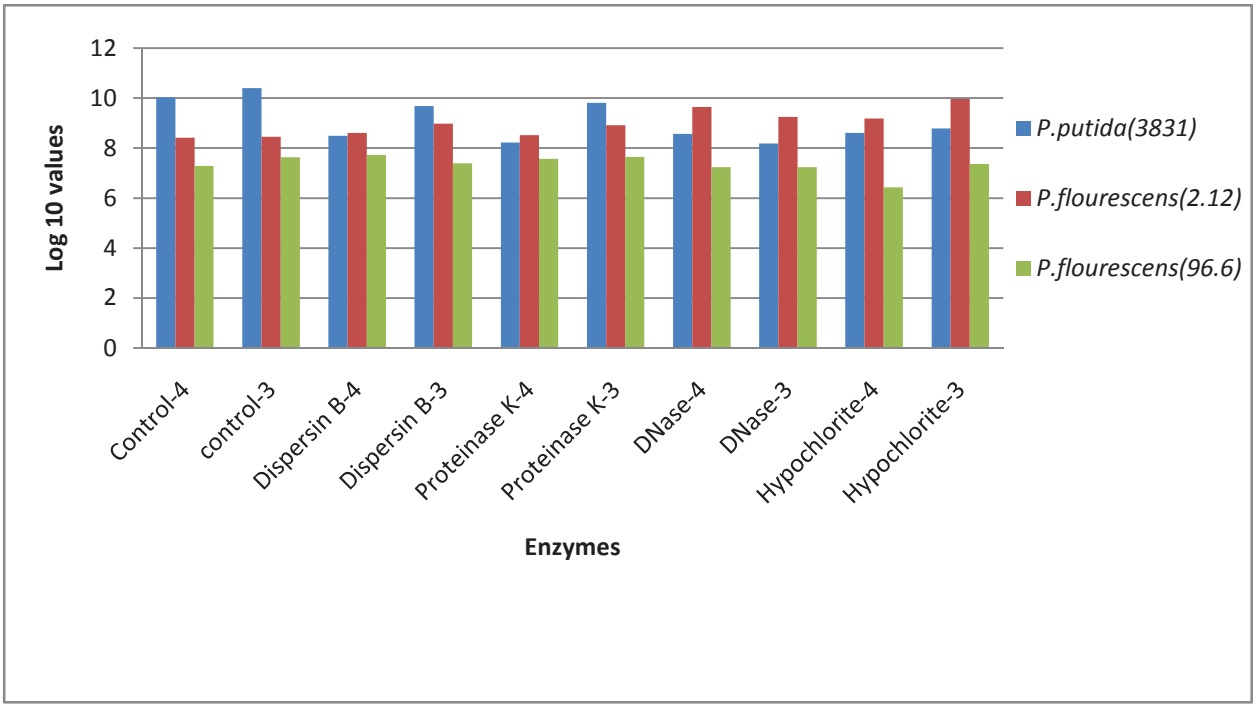

Figure 11 Logarithmic bacterial count of two replicates after exposing the biofilm to the enzymes for one hour without agitation. The first six bars ie.control-3 and control-4 represents the bacteria bacterial count in control(TSBNG) medium.

4.6.2 Bacterial count after exposing the biofilm to the enzymes for one and half hour with gentle agitation

Cell numbers of *Pseudomonas putida* (3831) *Pseudomonas fluorescens* (2.12) and *Pseudomonas fluorescens* (96.6) were determined after the biofilm had been exposed to the enzymes for one and half hour with gentle agitation. The numbers were also calculated from the different dilution of 10^{-3} and 10^{-4}. The bacterial number ranged from $\log_{10}$ 6.2 to 8.9.

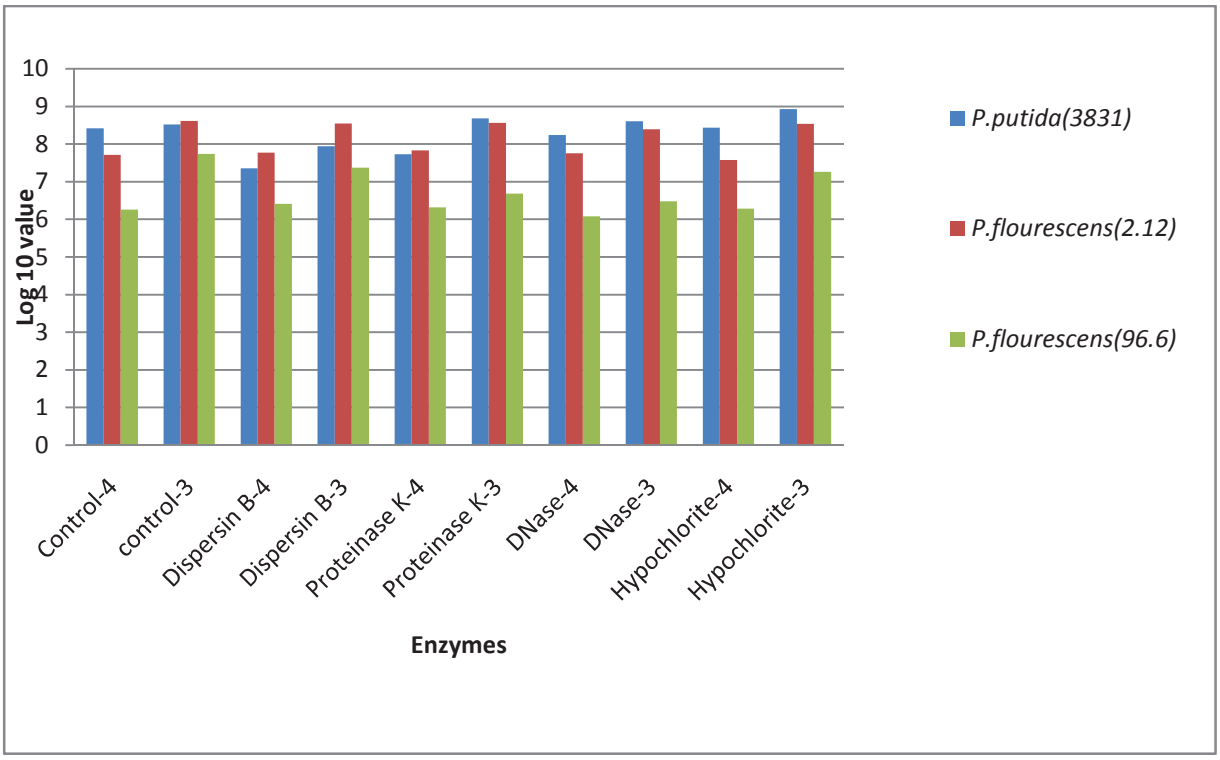

Figure 12 Logarithmic bacterial count of two replicate after exposing the biofilm to the enzymes for one and half hour with gentle agitation. The first six bars ie.control-3 and control-4 represents the bacteria bacterial count in control (TSBNG) medium.

5 Discussion

Microbial colonization or biofilm have been associated with many out breaks and food borne diseases, which have been a threat to human. Organisms in biofilm forms are more resistant to commercial disinfectants and other physical stresses. These properties of the bacteria have become the major issues in the food industries (Sokunrotanak Srey, 2013). Similarly, the predominance presences of *Pseudomonas* biofilm and their inherent resistant to the biocides have become the root of persistent and chronic bacterial infection (Steenackers et al., 2008). Understanding the underlying mechanism of biofilm resistance towards different chemicals is of great importance in the development of new control strategy or treatment. The main purpose of this study was to screen the *Pseudomonas* strains having good biofilm production capacity and utilization of the enzymes and the chemical disinfectants for inhibition of the biofilm.

5.1 Identification of *Pseudomonas* strains by API test

The isolates were identified by API20 NE test and the results were compared to identification based on 16s rRNA technology. 23 out of 27 strains were identified as *Pseudomonas* spp in both 16s RNA and the API test results. Four of stains identified as *Pseudomonas* spp using rDNA were identified as respectively *Burkholderiaeurella cepacia*(99.9%)*, Ochrobactrum anthropi*(91.9%)*, Pasteurella multocida*(63.7%) and *Pasteurella multocida* (95.7%) and Though, the species are identified different from pseudomonas, all the species relates to industrial food spoilage.

Both identification methods API and 16 s RNA, have their own advantages and limitations. A long investigation of 6 years from 1997 to 2003, (Van Houdt, Aertsen, Jansen, Quintana, & Michiels, 2004) concluded the 16 RNA methodology was the most valuable tool in the identification of bacteria. Similarly, another study (Kemp et al., 2013) suggested that isolates should be subjected to sequence analysis by 16s RNA if the accurate identification is concerned. In another experiment (Bosshard et al., 2006), 16 S RNA gene sequencing was

found to be more accurate for the identification of gram negative non-fermentative than API 20 NE. 92 % of the isolates were identified at the species level where 53 % by API test. They concluded the experiments that majority of the strains could not be identified by phonotypic profiling as well as the 16 s RNA gene sequencing as the best alternative for the species identification.. But in another experiment by (Awong-Taylor, Craven, Griffiths, Bass, & Muscarella, 2008) 16s RNA techniques identified only 66 % of bacterial isolates in comparison to 74% by API. The technique has also some limitations that it has low resolution power for identification of strains at species level. Additionally, DNA relatedness data are necessary for absolute identification.

5.2 Biofilm formation in three different media

Different media are used in the literature for culturing Pseudomonas in biofilm and intial experiments were done to compare biofilm formation in various nutrient media. Five different pseudomonas strains *Pseudomonas putida* (3720), *Pseudomonas aeruginosa* (3601), *Pseudomonas fluorescence*(1946), *Pseudomonas fragi* (4193) and *Pseudomonas lundensis* (2329) were cultured in three different media TSB, TSBNG (TSB containing 0.33 % glucose and 0.25% sodium chloride) and the minimal media (MM) at different temperatures(12^0 C and 20^0 C). The biofilm formation was measured after 72 hours and 5 days incubation. In comparison to the TSBNG and TSB, bacteria did not form effective biofilm in minimal medium. However, strain *Pseudomonas lundensis* (2329), *Pseudomonas putida* (3720) and *Pseudomonas fluorescence* (1946) formed the same level of biofilm in minimal media while cultured in12^0 C for 72 hours. *Pseudomonas .aeruginosa* (3601) did not formed significant biofilm while cultured in 12^0 c but the strain formed comparable biofilm as other strains at 20^0 c. In most of the cases, the strains formed the most biofilm while grown in TSBNG medium and more biofilm in 72 hours than 5 days. The exhaustion of the nutrient content might be the reason for less biofilm formation. However, biofilm formation trend was not uniform in all cases but in general the strains formed the best biofilm in TSBNG cultured for 72 hours. Our result is consistent with a recent study (Tekade et al., 2013). They found the some

clinical isolates of staphylococci formed significantly more biofilm in presence of glucose and saline. In addition, they found effective biofilm formation by *Pseudomonas areuginosa* after addition of 0.25% glucose in the culture medium. Similarly they explained the environmental factors like addition of glucose and sodium chloride helped in biofilm formation other than the temperature. They found the addition of sodium nitroprusside enhanced the biofilm formation by *Pseudomonas aeruginosa* and *Burkholderia cenocepacia* which is consistent with our result.

5.3 Effects of enzymes in biofilm inhibition

In this experiment , the selected strains of *Pseudomonas* were allowed to form the biofilm in the TSBNG medium containing 50 ug/ml of Dispersin B, 100 ug/ml of Proteinase K, 100ug/ml of DNase and the mix (containing the equal proportion of the all three enzyme of the respective concentration). The strains were selected on the basis of good biofilm forming capacity that belong to different species and were of different origin. All of these strains formed the good biofilm. All of the biofilm production by these strains range from 2 to 3 OD value where, biofilm produced by *Pseudomonas fluorescens* (2846) showed the best value of 3.2 OD with minimum standard of deviation which are followed by *Pseudomonas fluorescens* (96.6) and *Pseudomonas fulgida* (3600). Compairing the results with the control (TSBNG,), we found the slight decrement in the biofilm production by the species. But the strains *Pseudomonas fluorescens* (3831), *Pseudomonas aeruginosa* (2530), *Pseudomonas putida* (2.12), *Pseudomonas lundensis* (2830) showed reduced biofilm production when cultured in mixed enzymes medium. None of the species showed remarkable reduction in biofilm formation. Our result is consistent with the findings by (Nicholson, Shore, Smith, & Frana, 2013) where Dispersin B had no effect against MRSA when added at the starting of the biofilm growth and at the mature biofilm state. But strains used were not consistent with our study. In spite of that, the Proteinase K and the DNAse were effective to inhibit the biofilm, in the same study. Similarly, (Kumar Shukla & Rao, 2013) reported ineffectiveness of proteinase K to inhibit the biofilm by *Pseudomonas aeruginosa* alone but it seemed to be

effective in combination with other disinfectants. The report is somewhat similar with our results. In another experiment (Ali Mohammed et al., 2013), DNase I and proteinase k had little effect on the biofilm matrix formed by *Pseudomonas* spp. The findings are similar with our result however the condition used for the growth of *Pseudomonas* spp. was anaerobic in their experiment. Similarly Dispersin B have been found to inhibit the biofilm formation by gram-negative bacteria including *Pseudomonas* spp. but the condition of the growth does not relates to our study (K. Sauer et al., 2002).

5.4 Detachment of the biofilm

Pseudomonas putida (2.12), *Pseudomonas fluoresces*(3831) and *Psedomonas fluorescens* (96.6) were selected for investigating detachment of biofilm. The selections of the organisms were based on the good biofilm forming capacity of different sources of origin and strains of different species. The strains were originally taken from beef slaughtering house (2.12), ANSES, France (3831) and salmon processing plant (96.6). The organisms were allowed to attach on stainless steel coupons 3 hours at 12^0 c. and these coupons were exposed to the enzymes (Dispersin B, proteinase K, DNase and hypochlotite) for specified time duration. The bacteria were quantified with the help of spiral plate automated colony counter. The number of bacterial count was changed into log 10 values. In the experiment, enzymes were found to be effective against biofilm produced by the specified strains. However, the strain *Pseudomonas fluoresces* (3831) showed the decreased bacterial count than the control (TSBNG) while exposing the biofilm with enzymes for one hour. But the strain did not show the same trend while exposing the biofilm with enzymes for one and half hours with simple agitation. Taking into the consideration that agitation might contribute to expose the biofilm homogenously with enzymes as well as it helps in the detachment of biofilm by additional mechanical force. However, speed of agitation might contribute differently in detachment of biofilm. In most of the cases, bacterial count after enzymatic treatment seemed to be very lower than that of the control, which signifies the very low effectiveness of the enzymes against biofilm. However, the significant of the effectiveness was uncertain to predict. Only,

two replicates of the same biofilm detachment experiments were performed. More repetitions or the more experimental trials would be necessary to determine the effectiveness of the enzymes. Beside these parameters, the enzymes used in the experiment did not showed the practical inhibitory effects towards the biofilm. The concentration of the enzymes used in the experiment might be insufficient to penetrate the biofilm. Similarly, the exposure time of the enzymes to towards the biofilm might be insufficient even though the concentration was quite effective for inhibition. Poor penetration power of enzymes, nutrient limitation and the adaptive stress response are responsible for biofilm resistance (Stewart, 2002). Exposing the sanitizer to biofilm helps to remove the top layer which exposes the subsequent layer to nutrients that helps further growth and the biofilm formation.(Kumar & Anand, 1998). In the study, (Florjanic & Kristl, 2011) biofilm removal is achieved through the combination of mechanical and the enzymetic methods. They have focused on the formulation and the concentration of sanitizing agent, exposure time, temperature, and the mechanical action. Biofilm itself provides the effective permeability barrier to various chemicals and compounds. Potentiality of disinfectants against bacterial biofilm depends not only the bactericidal activity but also on the bacterial number and architecture of biofilm (Carpentier & Cerf, 2011). Similarly the surface of steel coupons used on the experiment was not very smooth. Washing procedure was supposed to remove the microorganisms but these unnoticeable structures harbors the microorganisms. Bacteria might protect themselves by protecting on the small holes and the crevices. Similarly, the attached bacterial cells are highly resistant to bactericidal agent and these bacteria persists in the surfaces regarded as cleaned (Schirmer, Heir, Moretro, Skaar, & Langsrud, 2013). *Listeria monocytogens* and the commensal bacterial flora such as the pseudomonas as a dominating strain in the abatoir after cleaning and disinfection. Some bacteria survive and develop resistance after frequent exposure to same disinfectants (S. Langsrud, Sundheim, & Holck, 2004).

In this study, selections of the enzymes tested were based on the composition of the biofilm matrix of *Pseudomonas* spp. Several studies have shown that the biofilm produced by *Pseudomonas* spp. mainly consist of polysaccharides, protein and DNA (Borlee et al., 2010; Wang et al., 2006). Some other structures like fimbriae, tafi and curli also contribute in the adhesion of the microbial cells to the environmental surfaces (Latasa et al., 2005;

Ledeboer,Frye, McClelland, & Jones, 2006; Wang et al., 2006). Microorganisms acquire high degree of resistance by mutation and the genetic exchange so the single process of biofilm control strategy may not efficient to control the biofilm alone (L. C. Simoes et al., 2010). Structural composition of the EPS differs even among the bacteria of the same species which may lead to ineffectiveness of the assigned disinfectants with specific formulation (Sokunrotanak Srey, 2013). The capacity of *Pseudomonas* species to alter the EPS composition may be another reason for its intrinsic resistance towards the different antimicrobials (Dynes et al., 2009). In another study (Molobela, 2010), the enzymatic treatment of biofilm by *Pseudomonas fluorescens* remained uneffective. But our findings are contrary with the findings by (Kadouri & Tran, 2013). They found the proteinase K and DNase caused the rapid detachment and inhibition of biofilm. Similarly, in another study (Zuroff, Gu, Fore, Leschine, & Curtis, 2014), found reduction of mature biofilm from 30- 60 % by DNase and proteinse K. This findings have huge contradiction with our result.

In one experiment, chlorine concentration of 1000 ppm were found to be effective against multispecies biofilm by *Lysteria monocytogens* and *Pseudomonas putida* while the concentration of 10 ppm was effective against planktonic cells (Norwood & Gilmour, 2000). The reason of reduced effect on biofilm might be due to the neutralization of the chlorine compound with organic compounds attached in the biofilm surfaces. The active chlorine compound reacts with surface material than it can enter into the biofilm (Xu, Stewart, & Chen, 1996). Altered microenvironment of the biofilm may limit the efficacy of the disinfectants (Augustin, Ali-Vehmas, & Atroshi, 2004).

The heterogeneity of the biofilm may limit the capacity of the single chemical to remove the biofilm but the composition two or more than two enzymes might be necessary for the effective removal. Formulation and the effective concentration of the enzymes in combination of temperature and time of exposure, play important role in biofilm removal. Pseudomonas species repeatedly exposed to antibiotics results in increased synthesis of EPS which results in more proliferous biofilm matrix (Lewis, 2007). In this study, bacterial count from the enzyme treated stainless steel and the control were almost similar, however, some of bacterial counts of enzyme treated coupons are fewer than the control which suggest the

enzymes might be able to degrade the biofilm matrix. But the effectiveness of enzymes in biofilm removal could not be predicted precisely from these observations.

6 Further work

More experiments should be repeated to be confirmed with the results found in this thesis. Similarly, a large no of repetition needed to due to variation of these experiments. Experiments with slight modification like by increasing the concentration of the enzymes in addition with other mechanical application would be better.

It would be better to calculate the result with statistics by repetition of the experiment with sufficient sample size.

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7 Conclusion

In this experiment, the biofilm formation capacity differed among the species in different time duration and temperature range. Some strains formed thick biofilm The result showed that the TSBNG medium found to be the best medium for the biofilm formation than TSB and the minimal medium. Most of *Pseudomonas* strained formed very low biofilm while grown in minimal medium. Biofilm formation trend were different in different experimental condition. Some *Pseudomonas* strains formed very thick biofilm even at lower temperature.

In the experiment, *Pseudomonas* strains cultured in TSBNG media with different concentration of the enzymes did not showed remarkable inhibition in the biofilm formation. However, *Pseudomonas aeruginosa* (2530) and *Pseudomonas fluorescens*(3831)showed reduction in biofilm formation while cultured with mixture of enzymes. However, enzymes, which are commonly used in food industries were not found to be effective in the detachment of the preformed biofilm. The cell numbers calculated after detachment experiment is almost same as that of control (TSBNG) which concludes the ineffectiveness of the enzymes in biofilm degradation.

8 References

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9 Appendix

Composition of the media used in the experiments.

Tryptic soya agar (TSA)

Ingredients Gram/Liter

Casein peptone, pancreatic- 15.0

Soya peptone 5.0

Sodium chloride 5.0

Agar 5.0

Final PH 7.3+/-0.2 at 25°C

Tryptic soya broth (TSB)

Tryptone (Pancreatic Digest of Casein) 17.0 g

Soytone (Peptic Digest of casein) 3.0g

Glucose (Dextrose) 2.5g

Sodium Chloride 5.0g

Dipotassium Hydrogen Phosphate 2.5g

PH7.33± 0.2

Minimal medium (MM):

Stockbuffer: 49 ml 0.2M Na_2HPO_4 + 51 ml 0.2 M KH_2PO_4 (400ml of each solution)

Final medium: 66 ml stockbufferadded d H_2O to tol 400 ml. Autoclave.

Add (these solutions were made individually):

10% $MgSO_4$ 2 ml

10% NH4Cl	4 ml
10% Fe-ammonium citrate	200 ul
10% CaCl2	20 ul
Glycerol	0.4 ml.

API 20 NE test ingredients and enzymes.

TEST	ACTIVE INGREDIENTS	REACTION /ENZYMES
ONPG	2-nitrophenyl-beta-D-galactopyranoside	Beta-galactosidase
ADH	L-arginin	Arginine dyhydrolase
LCD	L-Lysine	Lysine decarboxylase
ODC	L-ornithine	Omithine decarboxylase
H2S	Sodium thiosulphate	Hydrogen disulphide production
URE	Urea	urease
CIT	Sodium citrate	Citrate utility Formentation/oxidation zation
TDA	L-tryptophane	Tryptophane deaminase
IND	L-tryptophane	Indole production
VP	Sodium pyruvate	Acetoin production
GEL	gelatin	Gelatinase
GLU	D-glucose	Formentation/oxidation
MAN	D-mannitol	Formentation/oxidation
INO	inositol	Formentation/oxidation
SOR	D-sorbitol	Formentation/oxidation
RHA	L-ramnose	Formentation/oxidation
SAC	D-sucrose	Formentation/oxidation
MEL	D-melibiose	Formentation/oxidation
AMY	amygdalin	Formentation/oxidation
ARA	L-arabinose	Formentation/oxidation

Table 1 Colony count of the stock culture solution for the biofilm detachment experiment

Strain	Dilution	Total count	Log 10 Value
3831	10^{-7}	67200000.00	7.83
3831	10^{-6}	263070000.00	8.42
2.12	10^{-7}	50670000.00	7.70
2.12	10^{-6}	3868600000.00	9.59
96.6	10^{-7}	39600000.00	7.60
96.6	10^{-6}	139770000.00	8.15

Figure 1 Graph below representing mean total growth (with their standard deviation) of 27 *Pseudomonas* spp. cultured in TSBNG media at 20^0C for 72 hours

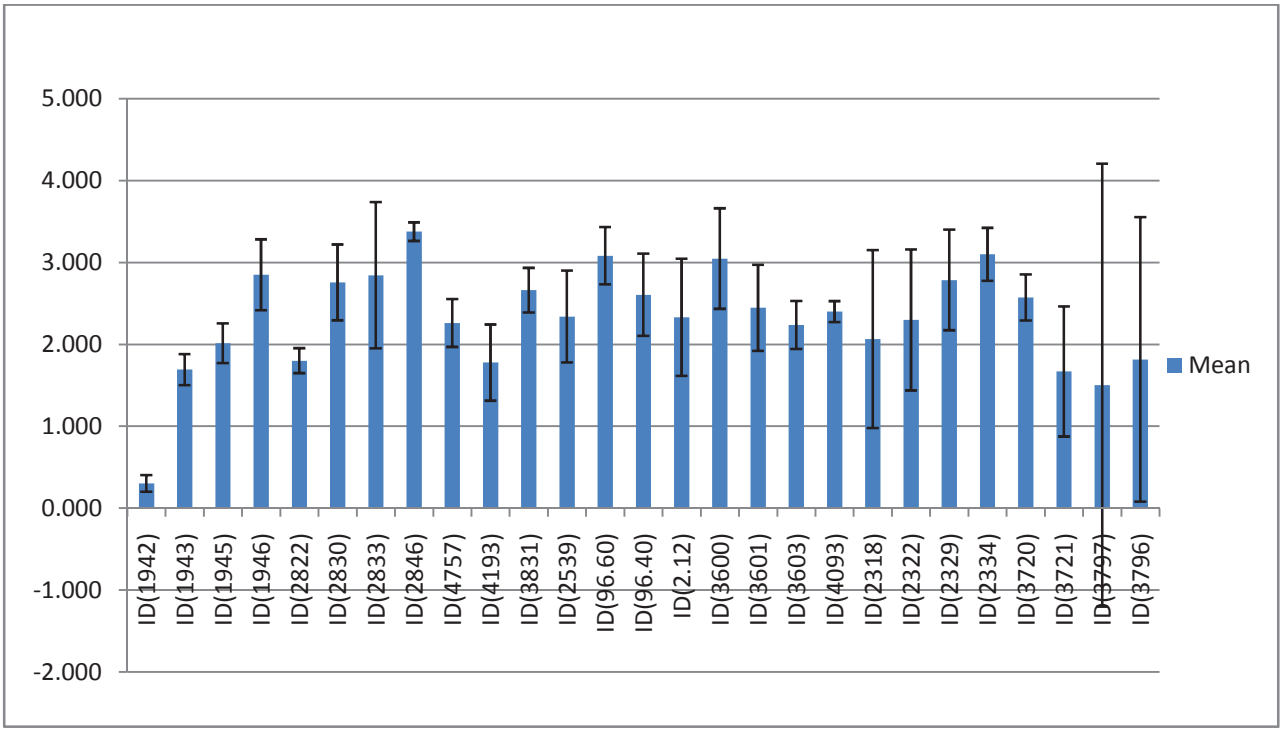

Figure 2 Graph below representing mean total growth (with their standard deviation) of 27 *Pseudomonas* spp. cultured in TSBNG media at 12^0C for 72 hours.

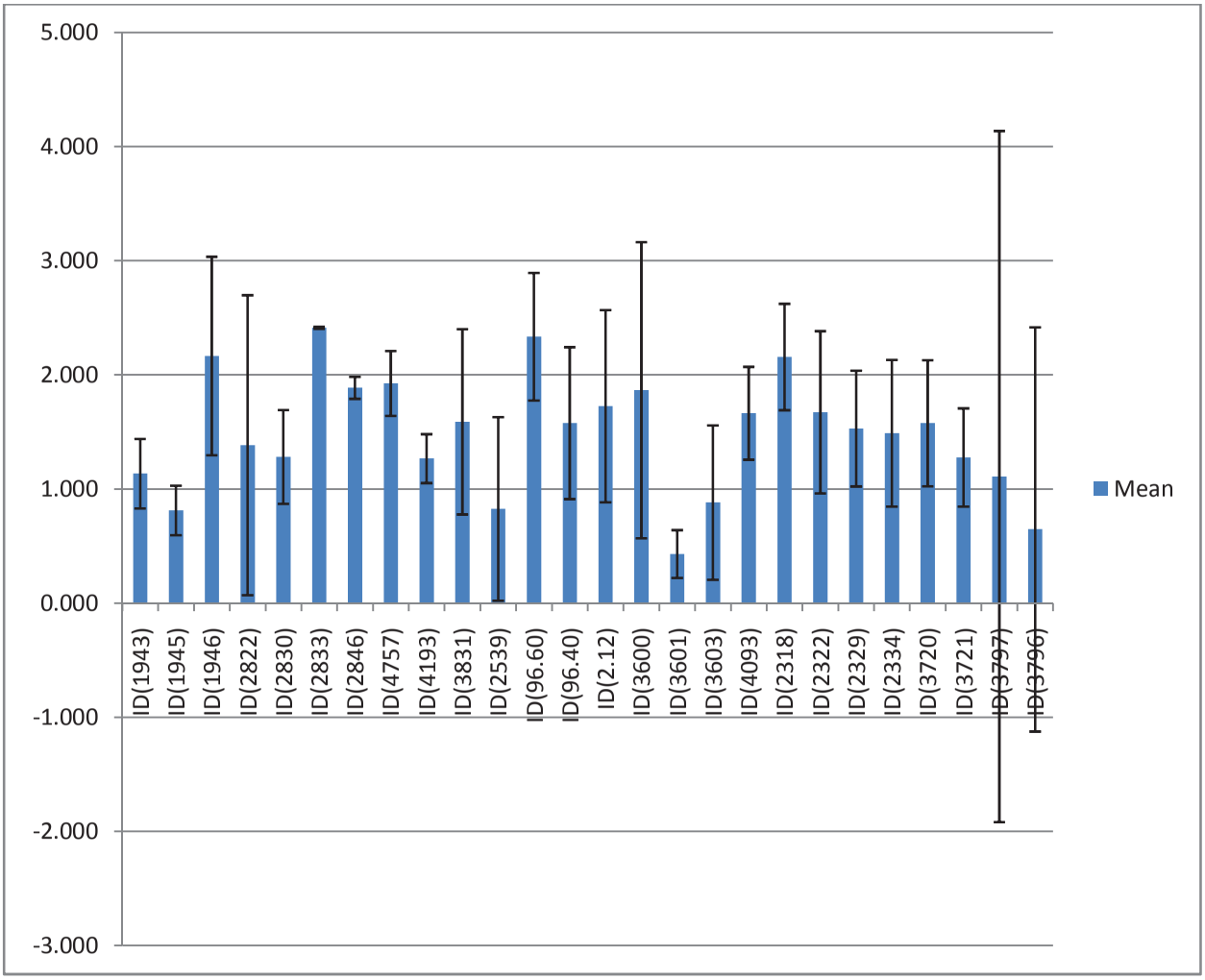

Figure 3 Total growth of the selected *Pseudomonas* strains cultured in TSBNG as well as the TSBNG containing different concentration of enzymes.

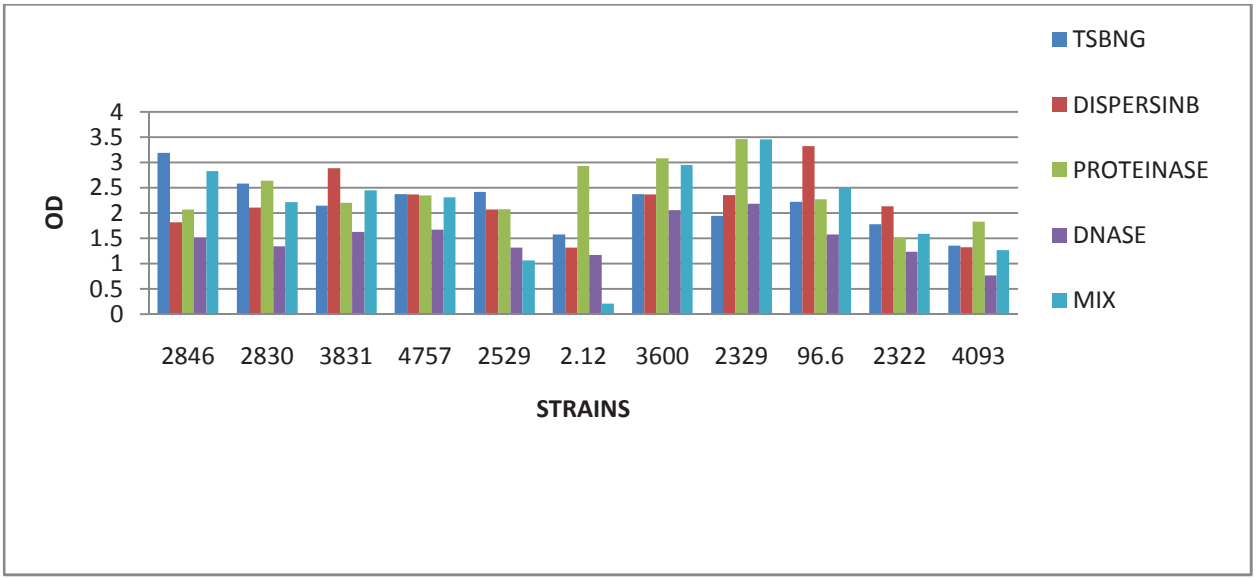

Figure 4 Total growth of the selected *Pseudomonas* strains cultured in TSBNG as well as the TSBNG containing different concentration of enzymes.

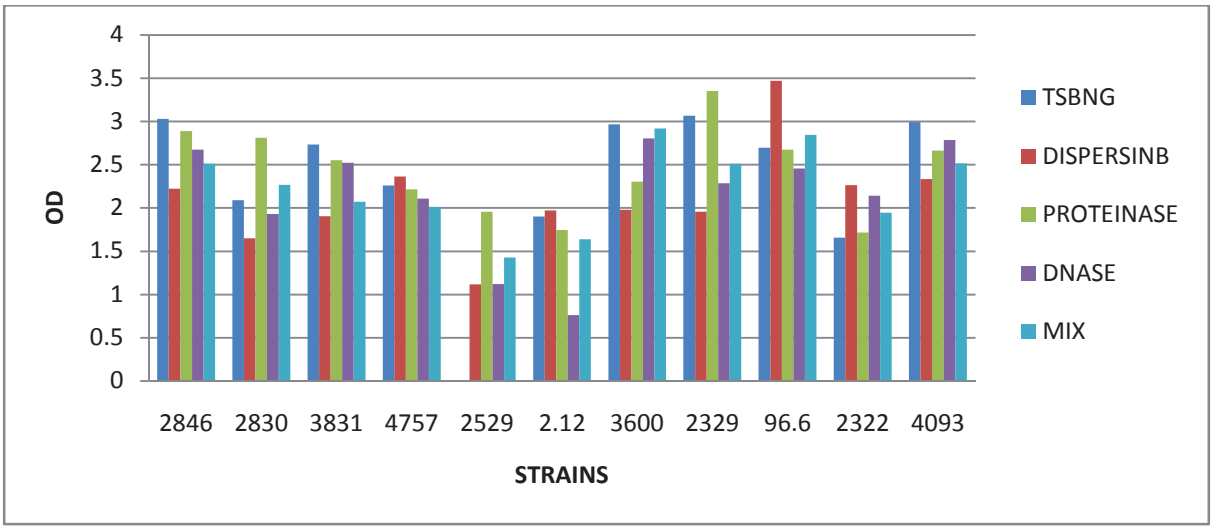

Figure 5 Total growth of the selected *Pseudomonas* strains cultured in TSBNG as well as the TSBNG containing different concentration of enzymes.

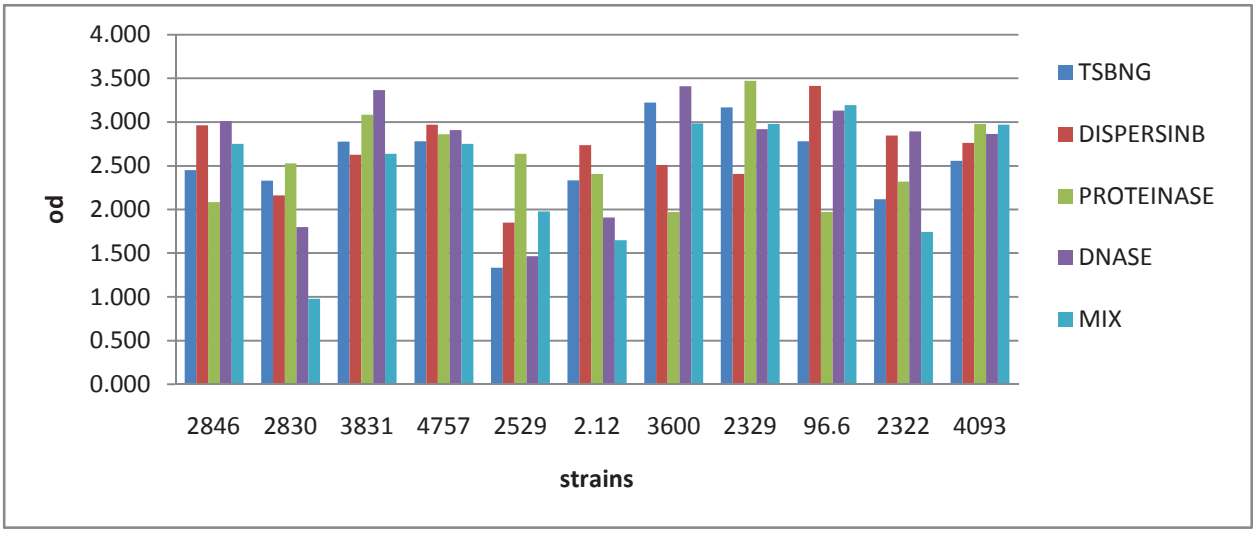

Figure 6 Total growth of the selected *Pseudomonas* strains cultured in TSBNG as well as the TSBNG containing different concentration of enzymes.

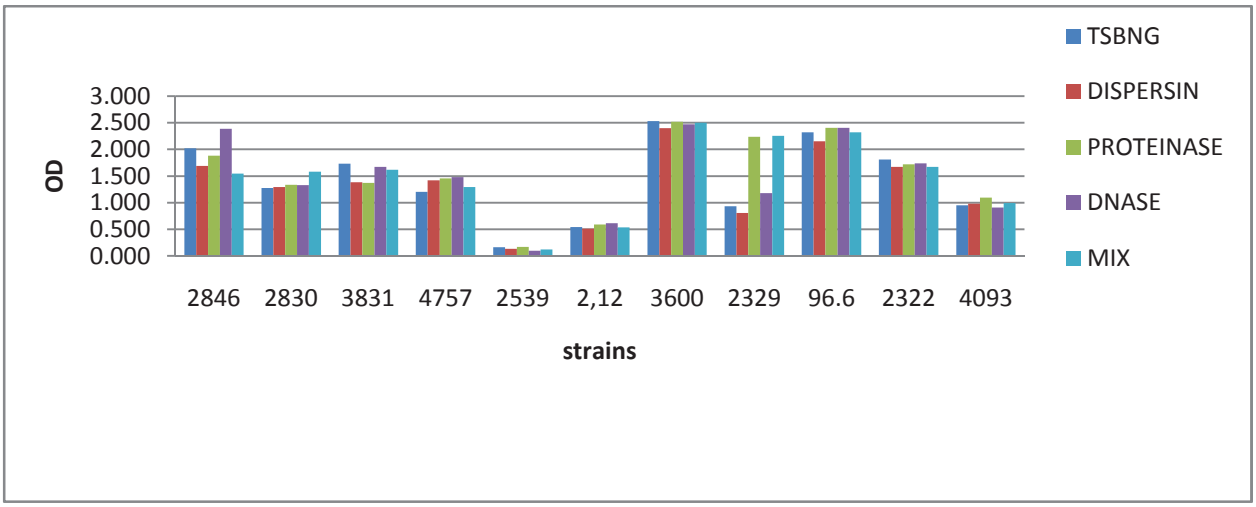

Table no 2

The table showing the bacterial number after exposing the biofilm to the enzymes for 1 hour.

Enzyme	Dilution	Strain 3831(Mean values)	Log 10 values	Strain 2.12(Mean values)	Log 10 values	Strain 96.6(Mean values)	Log 10 values
TSBNG (control)	10^{-4}	10802970000	10.034	256233000	8.409	19440000	7.289
TSBNG (control)	10^{-3}	25202160000	10.401	280290000	8.448	42402000	7.627
Dispersin B	10^{-4}	311190000	8.493	399990000	8.602	52116600	7.717
Dispersin B	10^{-3}	4819920000	9.683	950250000	8.978	24525600	7.390
Proteinase K	10^{-4}	166200000	8.221	327390000	8.515	37226400	7.571
Proteinase K	10^{-3}	6520950000	9.814	820200000	9.914	44233800	7.646
DNAse	10^{-4}	370110000	8.568	4460520000	9.649	17080200	7.232
DNAse	10^{-3}	153300000	8.186	1767600000	9.247	17080200	7.232
Hypochlorite	10^{-4}	39840000	8.600	1526700000	9.184	2715000	6.434
Hypochlorte	10^{-3}	614640000	8.789	9103890000	9.959	20682000	7.316

Table no 3.

The table showing the bacterial number after exposing the biofilm to the enzymes for one and half hour with gentle agitation .

Enzyme	Dilution	Strain 3831(Mean values)	Log 10 values	Strain 2.12(Mean values)	Log 10 values	Strain 96.6(Mean values)	Log 10 values
TSBNG (control)	10^{-4}	262200000	8.419	51651000	7.713	1860000	6.270
TSBNG (control)	10^{-3}	332364000	8.522	413868000	8.617	5433000	7.735
Dispersin B	10^{-4}	22680000	7.356	59304000	7.773	2580000	6.412
Dispersin B	10^{-3}	86745000	7.938	3482174000	8.542	23760000	7.376
Proteinase K	10^{-4}	53547000	7.729	68301000	7.834	2100000	6.322
Proteinase K	10^{-3}	476844000	8.678	363504000	8.561	4800000	6.681
DNAse	10^{-4}	172335000	8.236	56604000	7.753	1206000	6.081
DNAse	10^{-3}	402918000	8.605	249636000	8.397	3030000	6.481
Hypochlorite	10^{-4}	272991000	8.436	3789000	7.579	192000	6.283
Hypochlorte	10^{-3}	844446000	8.927	34160400	8.534	18180000	7.260