Co-aggregation among Rhodococcus and Acinetobacter from the food industry

Shahab Sharifzadeh

Norwegian University of Life Sciences
Department of chemistry, biotechnology and food science
Master Thesis 60 credits 2013

ACKNOWLEDGMENT

The work presented here was submitted to Department of Chemistry, Biotechnology and Food Science, Norwegian University of Life Sciences as a requirement for the M.Sc. degree. This work was done in close collaboration with Nofima, the food research institute, ÅS.

I would like to express my deep gratitude to my supervisor at Nofima, Dr. Trond Møretrø for his patience, motivation, useful comments, remarks and engagement through the steps of this work. His guidance helped me in all steps of this study and also writing the thesis. I am also grateful to my supervisor Dr. Solveig Langsrud for spending time and providing useful suggestions through the work. Furthermore, I would like to thank Professor Helge Holo, my main supervisor at Norwegian University of Life Sciences for his constructive support.

All laboratory works of this thesis was done in Nofima. I would like to thank, Tove Maugesten, Anette Wold Åsli and Merete Rusås Jensen, who shared their experience with me in the process of this work.

Finally I would like to thank my loved ones especially Azadeh Borghan. Without her support, I would not have been able to complete this work. I also thank my family who have always believed in me and have been so supportive.

Ås, august 2013

Shahab Sharifzadeh

ABSTRACT

Microorganisms' attachment to surfaces and consequently biofilm formation creates problems in food production industries. Such problems include contamination of equipment and products. Biofilm development is the result of cell-cell interaction between pairs and different groups of bacteria. Co-aggregation is one of the main cell-cell interactions, which result in bacterial adherence and biofilm development.

This study was conducted to find out about the effects of physiochemical factors on co-aggregation between *Rhodococcus* and *Acinetobacter* from the food industry. In addition, it was intended to characterize the mechanisms that mediate co-aggregate formation between these two strains. Co-aggregation between *Rhodococcus erythropolis* and *Acinetobacter calcoaceticus*, isolated from food production environments, was studied by a visual assay and an optical density assay. Strains were tested under different physicochemical conditions such as various growth temperatures (20^oC, 30^oC), growth mediums (R2A, TSB), and physiological culture status. These tests showed that the highest co-aggregation ability could be reached when the cells were grown in TSB at 30^oC and harvested either in the exponential phase or early stationary phase.

To find out the effect of various washing solutions on co-aggregation ability, the cells that were washed with dH_2O and co-aggregation buffer (three times) were compared. In both cases the cells showed equal co-aggregation ability. To study the effect of different solutions and buffers on co-aggregation, the cells of *Rhodococcus erythropolis* MF3727 and *Acinetobacter calcoaceticus* MF3293 were re-suspended in H_2O, co-aggregation buffer, 0.85% NaCl, TSB, and R2A. Highest co-aggregation ability was registered for those cells which were re-suspended in Co-aggregation buffer and 0.85% NaCl.

In the current study, the co-aggregation possibility between three different strains of *Rhodococcus* and eight strains of *Acinetobacter* was also tested. The achieved results showed that only *Rhodococcus erythropolis* MF3727 + *Acinetobacter calcoaceticus* MF3293, *Rhodococcus erythropolis* MF3727 + *Acinetobacter calcoaceticus* MF3627, *and Rhodococcus erythropolis* MF3727 + *Rhodococcus erythropolis* MF3803 had the ability to form co-aggregates.

Enzymatic treatment (proteinase K) and heat treatment (85^{o}C, 30 min) could inhibit the co-aggregation in all cases where MF3293, MF3627, and MF3803 had been treated. These treatments had no effect on MF3727 co-aggregation ability. These results suggested that MF3293, MF3627, and MF3803 have protein adhesins on their cell surface which mediates their co-aggregation with MF3727. To indicate present sugars in the structure of MF3727 co-aggregation receptors, co-aggregation was tested in the presence of simple sugars such as lactose monohydrate, D (+) galactose, α-L-fucose, N-acetyl-D-galactosamine, D (+) glucose and D-mannose. None of these sugars could completely inhibit co-aggregation between pairs. The strongest inhibitory effect was observed for N-acetyl-D-galactosamine, which managed to inhibit coageration between MF3727 and MF3627 by 46%. These results could suggest the existence of multiple co-aggregation mediating receptors on the cell surface of MF3727.

In this study, the effects of pH on co-aggregation between MF3727 + MF3293, MF3727 + MF3627 and MF3727 + MF3803 in pH range from 1 to 14 were examined. The results showed that all pairs had the ability to form co-aggregation in pH between 3 and 11. According to the results, MF3727 + MF3803 obtained the highest percentage of co-aggregation at pH of 3 and 4. On the other hands, MF3727 + MF3293 and MF3727 + MF3627 showed a wider optimum pH range. To investigate the prevalence of co-aggregation between other food related bacteria, 466 possible combination pairs from a total of 78 strains were tested under standard conditions. No co-aggregation was observed between any of the pairs.

SAMMENDRAG

Festing og biofilmdannelse av mikroorganismer skaper problemer i næringsmiddelindustrien. Forurensning av utstyr og produkter er noen av disse problemene. Biofilm utvikling er resultatet av celle-celle interaksjoner mellom forskjellige bakteriegrupper. Koaggregering er en av de viktigste celle-celle interaksjoner som resulterer i bakteriell festing og biofilm utvikling.

Denne studien ble gjennomført for å undersøke effekten av fysiokjemiske faktorer på koaggregering mellom *Rhodococcus* og *Acinetobacter* relatert til næringsmiddelindustrien. Studiens andre formål var å karakterisere mekanismene bak koaggregering mellom overnevnte stammene.

Koaggregering mellom *Rhodococcus erythropolis* MF3727 og *Acinetobacter calcoaceticus* MF3293, ble studert ved et visuelt assay og en optisk tetthet assay. Stammene ble testet under ulike fysiokjemiske betingelser, som ulike vekst temperaturer (20^o C, 30^o C), vekstmedium (R2A, TSB) og ulik vekstfase. Høyest koaggregerings evne ble observert når stammene var dyrket i TSB ved 30^o C og høstet enten i eksponentiell fase eller i tidlig stasjonær fase. For å undersøke effekten av ulike vaskeløsninger på koaggregering, ble celler av *Rhodococcus erythropolis* MF3727 og *Acinetobacter calcoaceticus* MF3293 vasket tre ganger henholdsvis med dH_2O og koaggregerings buffer. I begge tilfeller viste cellene lik koaggregerings evne. For å studere effekten av ulike løsninger og buffere på koaggregering, ble cellene resuspendert i dH_2O, koaggregerings buffer, 0,85 % NaCl, TSB og R2A. Høyest koaggregerings evne ble observert for de cellene som ble resuspendert i 0,85 % NaCl og koaggregerings buffer.

Koaggregerings mulighetene mellom tre ulike stammer av *Rhodococcus* og åtte ulike stammer av *Acinetobacter* testet. Det ble observert koaggregering for tre par stammer: *Rhodococcus erythropolis* MF3727 + *Acinetobacter calcoaceticus* MF3293, *Rhodococcus erythropolis* MF3727 + *Acinetobacter calcoaceticus* MF3627 og *Rhodococcus erythropolis* MF3727 + *Rhodococcus erythropolis* MF3803. Disse parene ble tatt i bruk i videre undersøkelser i studien.

Enzymatisk behandling (proteinase K) og varmebehandling (85^o C, 30min) hadde ingen effekt på MF3727 koaggregerings evne. Derimot hemmet behandlingen koaggregerings evnen til MF3293, MF3627 og MF3803. Disse resultatene antydet at MF3293, MF3627 og MF3803 har protein adhesiner på deres celleoverflate som er involvert i koaggregering med MF3727.

For å undersøke strukturen av MF3727 koaggregerings reseptor, ble koaggregering testet ved tilstedeværelse av enkle sukkerarter som laktosemonohydrat, D (+) galaktose, α-L-fucose, N-acetyl-D-galaktosamin, D (+) glukose og D-mannose. Ingen av overnevnte sukkerartene kunne hemme koaggregering fullstendig mellom parene. Den sterkeste hemmende effekt ble observert for N-acetyl-D-galaktosamin, som hadde evne til å hemme koaggregering mellom MF3727 og MF3627 opp til 46 %. Disse resultatene kan indikere eksistensen av flere koaggregering reseptorer på celleoverflaten av MF3727.

Effekten av pH på koaggregering mellom parene i pH skala fra 1 til 14 ble undersøkt. Alle parene hadde evnen til å danne koaggregater i pH fra 3 til 11. Ifølge resultatene hadde, MF3727 + MF3803 høyest koaggregerings evne ved pH 3 og 4, mens MF3727 + MF3293 og MF3727 + MF3627 viste et bredere optimalt pH-område. For å undersøke koaggregering mellom andre bakterier fra næringsmiddelindustri, ble 466 mulige kombinasjons par fra 78 stammer testet. Koaggregering ble ikke observert mellom noen av parene.

TABLE OF CONTENTS

1. INTRODUCTION

1.1 Background and aim of the study

Different types of cell-cell interaction between bacteria results in the formation and development of biofilm. Co-aggregation is one of the main cell-cell interactions that bind bacteria together and results in biofilm development. This phenomenon has previously been observed between oral bacteria, lactic acid bacteria, and fresh water bacteria. Co-aggregation in general is described as co-aggregate formation between genetically distinct bacteria. The binding between the bacteria results from binding between ligand and receptor. Various physicochemical factors such as cultivation medium, growth phase, pH, and different buffers may have an influence on co-aggregation.

In a joint study (2011) between Nofima and Alexander Rickard and his group at the University of Michigan, the co-aggregation among *Rhodococcus erythropolis* MF3727 and *Acinetobacter calcoaceticus* MF3293 was observed. The aim of the current work was to study:

- The co-aggregation between *Rhodococcus* and *Acinetobacter* isolated from various food processing environments.
- The effect of environmental conditions on the co-aggregation ability between *Rhodococcus* and *Acinetobacter* from the food industry.
- The mechanisms behind the co-aggregation of *Rhodococcus* and *Acinetobacter* from the food industry.

2. LITERATURE REVIEW

2.1 Food safety

The ever rising population has led to a need for higher volume of food production. As a result, more complicated production procedures are employed and the period of time between production and consumption has been extended. The special focus on production of healthy food has led to great pressure on manufacturers to reduce the use of chemical preservatives (Solveig Langsrud et al. 2003).With this in mind, ensuring safe food with high quality and longer shelf-life requires more precise control of the production process. As a result, a daily regular cleaning and disinfection schedule is considered as necessary in factories in order to achieve the aforementioned objectives (Hood & Zottola 1995).

The main objective of disinfection would be to remove microorganisms from the various surfaces and prevent the contamination of raw materials and product by pathogens and spoilage organisms. Disinfectant concentration, temperature, exposure time and cleaning process are the most important factors which should be considered to achieve the best results to eliminate microorganisms (Langsrud et al. 2003). Despite all of the caution and accuracy employed, there is always the possibility of transferring the remaining microorganisms (on different surfaces of process areas, such as walls, floors and conveyor belts) to products. The microorganisms present in these sites may cross contaminate the products during production and could also re-contaminate the products in storage. By the manufacturers and in legislature, there is always a serious focus on pathogenic bacteria, such as *Escherichia coli*, *Listeria monocytogenes* and *Pseudomonas* spp. (Bagge-Ravn 2003).

However, the food-borne bacteria are not the only harmful organisms in the food production process. Several problems caused by non-pathogenic and food spoilage bacteria have been frequently reported. These bacteria are often referred to as the general microflora or background flora (Langsrud et al. 2012). Although it is possible to detect these bacteria, in general there is little focus on their identity. Therefore there is little information available to producers regarding their growth requirements and the damage they can cause. All of the above mentioned bacteria

can find their way to the production and maintenance environment through a variety of internal and external pathways, such as raw materials, consuming water and labors (Langsrud et al. 2012).

2.2 Bacteria in the food production environment

2.2.1 The bacterial microbiota

Only a minor part of the bacteria found in food processing plants are pathogenic. There is a large variation in microbial background flora and their presence can be variable depending on products and environments in which the processing takes place. Bagge-Ravn (2003) study on different fish industries showed the existence of high amounts of *Pseudomonas* in such process plants. Based on their findings, lactic acid bacteria, *Acinetobacter*, *Neisseriaceae*, *Listeria*, *Enterobacteriaceae* and *Staphylococcus* were also isolated from different environments of the industry. Bore et al. (2005) were able to isolate *Rhodococcus*, *Methylobacterium* and *Sphingomonas* from cheese production environment in their research. Schirmer et al. (2013) studied five Norwegian small scale cheese production sites and succeeded in isolating 1314 bacteria from different environments such as storage surface, drains, doors and floors. These bacteria belonged to 55 different genera.

2.2.2 Pathogenic bacteria

Foodborne pathogens may be present in food processing environments. Hence, one of the main challenges in the food industry is to avoid contamination of raw materials and the products with pathogenic organisms (Langsrud et al. 2006; Bore & Langsrud 2005). *Listeria monocytogenes* is a psychrotrophic bacterium with the ability to grow at refrigeration temperature (> 1^{o}C) and in a wide pH range (pH 4-9). The bacteria find their favorable growing conditions in floors, drains and equipment used in production. Although these areas are routinely cleaned and disinfected, it is sometimes possible to isolate the bacteria from such places (Carpentier & Cerf 2011).

Listeria monocytogenes is the biggest challenge when it comes to ready-to-eat food production, and cross-contamination during production is the main source of the bacteria (Lis & Sharon 1973). Listeriosis is a bacterial infection caused by *Listeria monocytogenes* and primarily causes infections of the central nervous system (meningitis) among immunocompromised people. In light of this, it could be harmful to people who consume immunosuppressive drugs, pregnant

women, children and cancer patients (Swaminathan & Gerner-Smidt 2007). Møretrø et al. (2004) cited 21 studies in their review which showed the presence of *Listeria monocytogenes* in industries such as shrimp production, ice cream production, sauce production and cold smoked salmon. The presence of this bacterium is also reported in raw chicken, meat, vegetables and chees (Granum 2008).

Salmonella sp. are pathogenic bacteria which are transmitted through food. Salmonellosis is a food-borne infection caused by *Salmonella* bacterium. In most cases the people who are infected with *Salmonella* develop diarrhea, fever and abdominal cramps (Granum 2008).The bacteria are able to adhere to the food processing equipment and cause contamination. Several reports have been provided on cross-contamination of food by these bacteria, all of which indicate the presence of different strains of *Salmonella* in a certain production environment (Sinde & Carballo 2000).The presence of *Salmonella* sp. is also a well-known problem in the animal-feed chain. Although heat treatment of animals' feed is one of the main processes for feed safety, some reports still suggest the insufficiency of thermal treatment when it comes to removal of these species (Habimana et al. 2010).

Escherichia coli is another pathogenic bacteria which can cause serious food poisoning in humans. *E.coli* which causes gastroenteritis in humans are classified into six pathogenic groups. Shiga toxin-producing *E.coli* (STEC), a food-borne human pathogen, is responsible for sever gastrointestinal disease (Granum 2008). Several studies have shown that this bacterium has the ability to attach to different surfaces and form biofilm (Rivas et al. 2007; Gilbert et al. 1991). Marouani-Gadri et al. (2009) also showed that residential microflora increased *E.coli* O157:H7 colonization on solid surfaces under static conditions.

2.2.3 Spoilage bacteria

Bacteria from the food production environment can result in sever changes in term of the quality of products if cross contamination take place during production. These changes could be physical or chemical such as changes in color, smell and taste which may result from growth and metabolism of bacteria in the food (Gram et al. 2002). Damage rate caused by spoilage bacteria depends on the type of food, type of process and type of bacteria (Lis & Sharon 1973). *Pseudomonas* spp., *Acinetobacter* spp. and a few gram-negative psychrotrophic organisms can

dominate proteinaceous foods such as fish, milk and meat (Gennari et al. 1992; Bore & Langsrud 2005; Bagge-Ravn 2003).

In products such as meat and fish, the changes in atmosphere by vacuum packing, can prevent the growth of respiratory microorganisms and change microflora to lactic acid bacteria and *Entrobacteriaceae* (Gram et al. 2002). Lactic acid bacteria in general cause spoilage in vacuum packed meat products such as liver sausage, ham and bologna sausage (Samelis et al. 2000). Carbon dioxide packing of fish is also suitable for the growth of CO_2 resistant bacteria such as *Photobacterium phosphoreum* (Gram et al. 2002).

2.2.4 Control of bacteria in the food processing environment

As previously mentioned, cleaning and disinfection are the methods most often used by manufacturers to remove and control these bacteria. However, such a disinfection and removal does not always eradicate all bacteria in the environment, since the cleaning and washing of some equipment or environments is very difficult (Lis & Sharon 1973). In addition, most of the disinfectants used in the food production industry are not able to eliminate bacterial spores (Langsrud et al. 2003). It has been shown that bacteria attached to surfaces are much more resistant to sanitizing compounds than free-living cells. Another group of bacteria which show resistance and are able to survive in the environment are those which colonize surfaces and produce biofilm (Langsrud et al. 2003; Hood & Zottola 1995).

2.2.5 Genus *Acinetobacter*

According to the most recent taxonomic studies, the genus *Acinetobacter* belongs to subclass gamma proteobacteria, family *Moraxellaceae* and comprises a complex and heterogeneous group of bacteria (Visca et al. 2011). Using DNA-DNA hybridization it has been shown that the genus *Acinetobacter* contains at least eighteen genomic species (Claeys et al. 1995). The member of this genus are coccobacilli, Gram-negative, non-motile, aerobic, catalase positive, oxidase negative and is known to have high efficiency to natural transformation (Vaneechoutte et al. 1995; Medigan et al. 2009). Many species of this genus are metabolically varied and can easily grow in a simple microbiological culture media. Some strains of *Acinetobacter* can utilize a range of sugars as carbon source (Medigan et al. 2009).Colonies are usually 2mm in size and are pale yellow or gray. Some strains have force-generating pili, which help them to move a distance of 1-5μm (Visca et al. 2011).

Some of the species, such as *Acinetobacter calcoaceticus*, are capable of twitching motility due to presence of polar fimbriae on their surface (Seruminstitut & Henrichsen 1975).This species can also attach to hydrocarbons or other hydrophobic surfaces due to the fact that it has thin fimbriae (Rosenberg & Bayer 1982). Studies done based on DNA-DNA hybridization showed that *Acinetobacter baumannii*, *Acinetobacter calcoaceticus*, *Acinetobacter pittii* (genomic species 3) *and Acinetobacter nosocomialis* (genomic species 13TU) are closely related. Therefore recognizing them by phenotype or chemotaxonomic criteria would be very difficult (Visca et al. 2011; Martí et al. 2011). For simplicity's sake, many laboratories have called them *A. calcoaceticus – A. bumannii* (ABC) complex.

However, such naming and grouping is not welcome from a clinical point of view, since among them, *A. baumannii* and *A. nosocomialis* (genomic species 13TU) are responsible for many infections in humans. In contrast, *A. pitti* (genomic species 3) is less associated with disease. In addition, *A. calcoaceticus* is an environmental microorganism which can be commonly isolated from the skin of healthy individuals or soil, and has rarely been seen to cause infection in humans (Visca et al. 2011; Martí et al. 2011).

It has been reported that different strains of *Acinetobacter* could be a causing agent of spoilage of meat, sea food and poultry. Some reports also indicated that these bacteria are able to grow at chill temperature, and are therefore able to damage meat, chicken and fish kept in the refrigerator and storages (Barnes & Thornley 1966; Barnes & Impey 1968; Fraser & Sumar 1998). Certain strains, such as *Acinetobacter calcoaceticus*, *Acinetobacter johnsoii* and *Acinetobacter lwoffii* have been isolated from different food processing environments in the dairy, meat and fish industries (Lewis et al. 1989; Habimana et al. 2010). It has been previously reported that strains isolated from the milk transfer pipeline section were able to adhere to stainless steel (Lewis et al. 1989). It was also reported that *Acinetobacter nosocomialis, Acinetobacter pittii, Acinetobacter johnsoii* and *Aacinetobacter lwoffi* were able to create biofilms in air-liquid and solid-liquid interfaces (Martí et al. 2011).

2.2.6 Genus *Rhodococcus*

Over recent years classification of the genus *Rhodococcus* has changed dramatically. However based on the latest reports there are currently 12 established *Rhodococcus* species (Bell et al. 1998). The members of the genus *Rhodococcus* are aerobic, Gram-positive, non-motile, non-sporulating and nocardioform. The term nocardioform refers to mycelial growth with fragment into rod-shaped or coccoid elements (Medigan et al. 2009; Bell et al. 1998).

Rhodococcus species have been isolated from widespread sources such as soil, marine sediments and insects' intestine (Medigan et al. 2009; Bell et al. 1998). There are also reports which have shown that some *Rhodococcus* species have been isolated from some food industries, such as milk and cheese industries (Aaku et al. 2004; Bore & Langsrud 2005). Mobilization of large linear plasmids and the existence of multiple enzymes in catabolic pathways have led to metabolic diversity in this genus (Carvalho & Fonseca 2005). One of the important properties of the *Rhodococcus* species is degradation of hydrocarbons. In addition, the ability of these bacteria to produce certain products, such as surfactants, flocculants, amides and polymers has led to a special view of biotechnology to this genus (Bell et al. 1998; Carvalho & Fonseca 2005).

The presence of enzymes such as alcohol dehydrogenase and hydrolase has been reported in *Rhodococcus erythropolis*. These bacteria are also capable of oxidation of higher liquid alkanes, aromatic compounds and cholesterol (Carvalho & Fonseca 2005). Recent studies have shown that some *Rhodococcus* species have caused infection in humans. There have been many reports indicating infection by *Rhodococcus equi* in patients with impaired immune systems, especially people with HIV(Bell et al. 1998; Doig et al. 1991; Weinstock & Brown 2002).

In their review, Weinstock et al. (2002) stated that this bacterium can be largely isolated from agricultural soils, pigs and horses, and can also be transmitted to humans through breathing, wound or mucous membrane. Some species of *Rhodococcus* isolated from different environments of food processing showed high resistance against disinfectants. For example, *Rhodococcus erythropolis* isolated from the dairy industry showed strong resistance against Oxonia active (peracetic acid) and Titan hypo (hypochlorite).

These bacteria also revealed a tendency to attach to stainless steel (Bore & Langsrud 2005).There are some reports regarding the ability of *Rhodococcus erythropolis* to produce

biofilms on the surface of polystyrene and glass. This bacterium has a polysaccharide with amphiphilic properties on its surface, which facilitates its adherence to the surfaces (Carvalho et al. 2009; Carvalho 2007).

2.3 Bacterial attachment

Bacterial attachment to a solid surface typically either occurs actively or passively. In passive mode, which is typically due to gravity or diffusion, the bacteria are transferred to the surface so that the initial attachment occurs easily (Chmielewski & Frank 2003). Forces involved in this initial attachment include electrostatic forces, hydrophobicity and Van der Waals forces (Palmer et al. 2007). In active mode, however, bacterial cell surface properties such as fimbriae, adhesins, protein structures, surface charge and extracellular polymeric substances (EPS) facilitate the attachment (Chmielewski & Frank 2003).

Due to their high proportion of hydrophobic amino-acid residues, fimbriae play an important role in cell surface hydrophobicity and attachment. The fimbriae can facilitate attachment by overcoming the initial electrostatic repulsion barrier existing between the bacterial cell and substratum (Donlan 2002). Studies have shown that proteolytic enzymes cause the release of attached bacteria (Danielsson et al. 1977). The results of these studies indicate the involvement and role of proteins in the attachment.

EPS production also leads to large changes in bacterial functionality. The EPS-matrix changes the charge on the surfaces, enabling the bacteria to capture nutrients, and potentially facilitating bacterial attachment (Poulsen 1999). It can also cause the formation of micro-colonies, the biofilm structure and increase bacterial resistance to certain environmental stresses such as anti-microbial agents (Poulsen 1999). The main components of EPS consist of polysaccharides, proteins, phospholipids, teichoic acid and nucleic acid (Shi & Zhu 2009; Donlan 2002).

The composition of EPS can vary depending on gram-positive or gram-negative bacteria (Poulsen 1999). For instance, in gram negative bacteria, the polysaccharides are polyanionic due to the presence of uronic acids or ketal-linked pyruvates in their structures. Conversely in gram positive bacteria, the chemical composition of EPS may be primarily cationic (Donlan 2002). Studies of Skillman et al. (1999) showed that EPS mediates bacterial initial adhesion events and also has an important role in dual species biofilm development. Their results showed that the

interactions which result in biofilm formation were more successful when the bacteria were mixed than when they were isolated. They also observed increased biofilm formation due to EPS production and increased resistance against disinfections.

Extracellular DNA (eDNA) is also a major structural component of the EPS (Böckelmann et al. 2006). Earlier, the eDNA was not supposed to be an important component in EPS structure, and it was always assumed that this DNA originated from lysed cells. However, later research on *Pseudomonas aeruginosa* showed that the bacteria were able to produce large amounts of eDNA which were necessary for initial biofilm establishment of the strains (Böckelmann et al. 2006).

It also became clear that eDNA, together with other molecules such as proteins and EPS, form a matrix which helps to hold bacterial biofilm together(Whitchurch et al. 2002). In their investigation, Böckelmann et al. (2006) indicated for the first time that eDNA is a major structural component of the EPS. In addition, in their work, Nishimura et al. (2003) degraded eDNA by DNAse I. Through this they found that the eDNA structure is double stranded but completely different from intracellular DNA.

2.4 Biofilms

Biofilm are defined as the accumulation of microorganisms adhering to the surface (Shi & Zhu 2009). Leeuwenhoek (1684) with his very simple microscope, for the first time managed to observe that microorganisms have the ability to adhere to the tooth surfaces. This observation may be attributed to the first discovery of biofilms (Donlan 2002). Zobell (1943) also reported that bacteria have the ability to adhere to the surfaces. It was observed in those studies that such adhesion is time-dependent, so the number of bacteria attaching to the surface and producing biofilms increased over time (Hood & Zottola 1995). A modern interpretation defines a biofilm formed of surface-associated microbial cells wich are covered with extracellular polymeric substances (EPS).

Biofilms can be composed of a single or multiple species of bacteria that have formed together a single layer or a three-dimensional structure (Poulsen 1999; Chmielewski & Frank 2003).
Biofilms can form on various surfaces such as water transport pipes, different surfaces in the industries or on a living tissue (Donlan 2002).

Many species of microorganisms, including pathogenic or spoilage microorganisms can form biofilms (Poulsen 1999; Shi & Zhu 2009). The attachment of organic molecules such as proteins to the surfaces may play an important role in attachment and biofilm formation by microorganisms. These molecules are able to change some physical-chemical properties of surfaces, such as hydrophobicity or electrostatic charge. In general, the formation of biofilms is a very complex process that starts with a primary attachment.

Microorganisms can either directly attach to the surface, or can gather on the remains of products on equipment surfaces. These cells may be capable of persisting in the environment after cleaning and sanitation, and may begin to grow. In the next phase, they form a thicker layer by producing EPS and contribute to the progress of biofilm formation (Chmielewski & Frank 2003; Shi & Zhu 2009). At this stage, it is possible that microorganisms individually participate in biofilm formation or they may provide the possibility of the entry of other bacterial species into the biofilm tissue by special interaction such as co-aggregation, which results in the creation of a multi-species biofilm (Rendueles & Ghigo 2012; Palmer et al. 2007).

2.5 Multi-species biofilm

Bacterial species are capable of forming multispecies communities in different environments. Interaction between these species sometimes leads to the formation of a heterogeneous structure known, as multispecies biofilm (Rendueles & Ghigo 2012). The interaction between species usually consists of two important parts, including communication and metabolic cooperation. The communication normally occurs through quorum sensing. In metabolic cooperation, one species uses a metabolite produced by a neighboring species (Elias & Banin 2012). These synergistic interactions can improve several beneficial phenotypes which result in the promotion of biofilm formation (Elias & Banin 2012). There are two very important factors when it comes to biofilm formation and development, namely the type of bacterial species which participates, and the interaction between them.

There are species which are incapable of forming biofilm on their own, although they could participate in the creation and development of a multispecies biofilm through an interaction called co-aggregation (Rickard et al. 2003). S*treptococcus gordonii*, a first colonizer on the surfaces of tooth, is able to create conditions which late colonizers such as *Porphyromonas gingivalis* will adhere to. Such a co-colonization is not a passive process and is caused by the

expression of several genes by *Streptococcus gordonii* for the synthesis of extracellular polymers needed in the co-aggregation process (Kuboniwa et al. 2006). In general, the ability of certain species to form co-aggregation will allow them to join other species and lead to the promotion of biofilm (Elias&Banin2012).

2.6 Co-aggregation

The formation of co-aggregates was first reported by Gibbons et al. (1970). They observed that the strains isolated from the oral plaque were capable of adhering together. They tested 23 different strains to investigate the possibility of co-aggregation among them. Only 5 of the 253 possible pairs showed co-aggregation. All these pairs consisted of *Streptococcus* spp. and *Actinomyces* spp. Co-aggregation is defined as an adhesion process between genetically distinct bacteria mediated by their surface molecules. The process of recognition of surface molecules occurs immediately and leads to the formation of a mixed-cell aggregate. It is usually visible by eye and occurs shortly after the cells have been mixed (Kolenbrander 1988).

Research conducted on co-aggregation among microorganisms from dental plaque, indicated the very important role of co-aggregation in creating multi species biofilm communities (Rickard et al. 2003). Generally, biofilm development involving co-aggregation occurs in two forms. In the first form, an initial colonizer adheres to the surface, later another genetically distinct organism attaches to the pre-existing biofilm. This form of co-aggregation which occurs frequently among human oral bacteria is known as co-adhesion (Busscher et al. 1995).

In the second form, initially two individual planktonic organisms are attached together in a suspension and form an aggregate. The formed aggregate would then join a pre-existing biofilm and become a part of the biofilm (Busscher et al. 1995; Rickard et al. 2003). Aggregating bacteria in the form of a mixed biofilm can cause different interactions between existing bacteria. In this way, they can provide conditions to promote the survival of other members as a result of being together.

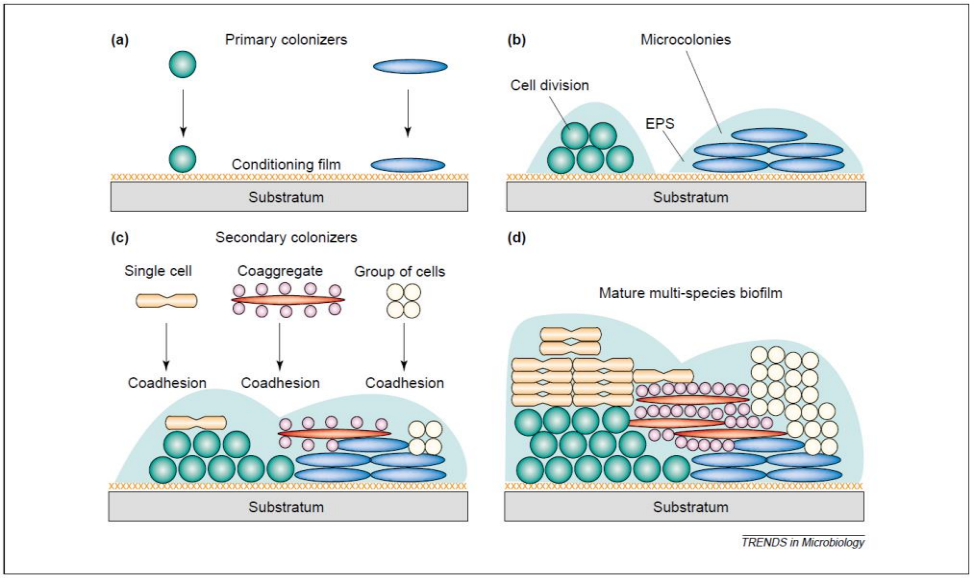


Fig 2.1 Diagram illustrating the possible roles of co-aggregation in the development of multi-species biofilms. . (a) Primary colonization of a substratum covered in a 'conditioning film' composed of polysaccharides and proteins; (b) cell growth, division and production of extracellular polysaccharide (EPS) leading to the development of microcolonies; (c) co-adhesion of single cells, co-aggregated cells and groups of identical cells into the young multi-species biofilm; and (d) maturation and the formation of clonal mosaics within the multi-species biofilm (Source: Rickard et al. 2003).

Jakubovics et al. (2008) previously showed that *Streptococcus gordonii* and *Actinomyces naeslundii* interact metabolically through arginine metabolism. In their investigation, they found that *S. gordonii* is not able to grow in an environment where the concentration of arginine is not sufficient. However, when this strain forms co-aggregation with *A. naeslundii*, it would have the ability to grow. The authors suggest that apparently when these two bacteria co-aggregate, the genes involved in arginine biosynthesis were induced in *S. gordonii* in response to co-aggregation with *A.naeslundii*.

The bacteria can also help each other to increase resistance against antimicrobial agents. In this scenario, one of the strains which participated in co-aggregation forming will induce transient changes in resistance in proximal neighbors. Thereby, the other bacteria can survive under challenging conditions (Kara et al. 2006).

2.7 Co-aggregation among microorganisms in different environments

2.7.1 Co-aggregation between human oral bacteria

Co-aggregation occurs widely among oral bacteria, although only certain types of cells serve as partners (Rickard et al. 2003). Cells incapable of co-aggregation are unable to participate in forming multi-generic aggregates (Kolenbrander 1988). Researchers had been studying dental plaque long before the introduction of the "biofilm term" terminology. For this reason, there is abundance of information available on physiology, ecology and taxonomy of plaque bacteria (Rickard et al. 2003).

Streptococci and gram-positive rods such as *Actinomyces naeslundi* are among the first bacteria that colonize the tooth surface. More than 300 isolates of these genera have been tested in pairwise inter-generic co-aggregation, and more than 90% had the capability to co-aggregate (Kolenbrander et al. 2006). *Streptococcus mitis*, *Streptococcus sangunis* and *Streptococcus oralis* are representatives of the 60-90% of plaque cultivable *Streptococci* during the first 4 hours. Within the subsequent 24 hours, many other bacteria will join them through co-aggregation interaction and form a complex structure such as corn cobs (Kolenbrander et al. 2006).

Partnership between dental plaque bacteria is so specific that the primary colonizers have the ability to create co-aggregation together. However co-aggregation interaction does not normally happen between them and secondary colonizers. This problem is solved by a group of bacteria which is able to create co-aggregation with primary as well as with secondary colonizers. These bacteria are called bridge organisms (Kolenbrander et al. 2006; Rickard et al. 2003).

Some bacterial cells may have several receptors or adhesins on their cell surface. As a result, in a multispecies environment, they can act as a bridge and form a network by forming co-aggregates with multiple different bacteria (Kolenbrander et al. 2006). In their research, Rickard et al. (2002) found that *Streptococcus oralis* plays the role of a co-aggregation bridge among oral bacteria.

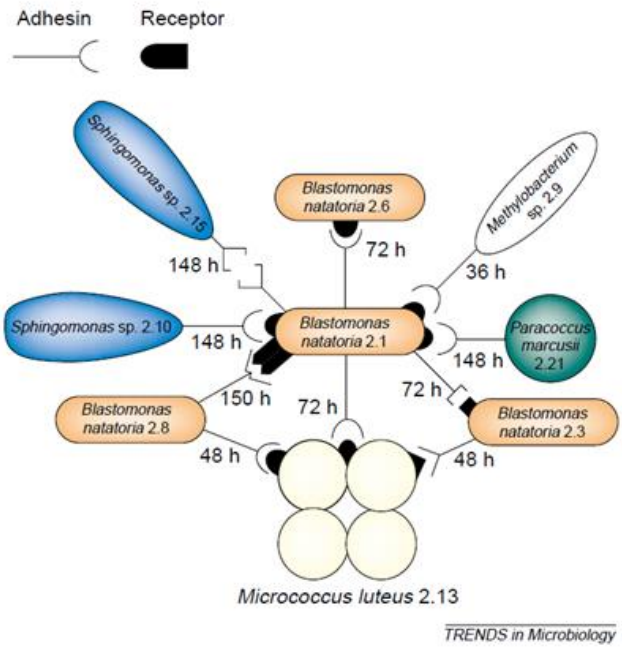


Fig 2.2 Diagrammatic representation of intergeneric and intraspecies co-aggregations between freshwater bacteria. The picture shows the role of *Blastomonas natatoria* as a bridge organism (Source: Rickard et al. 2003).

Studies conducted on co-aggregation between dental plaque bacteria have contributed significantly to understanding the mechanisms used by the bacteria to form biofilms (Gibbons & Nygaard 1970; Kolenbrander & London 1993). These studies also showed that co-aggregation may be created between inter-generic, intra-generic and multi-generic bacteria. The ability of such a wide range of bacteria in co-aggregation forming represents a compilation of different structures of bacteria which contributing to this interaction (Kolenbrander et al. 1990; Kolenbrander 1988).

2.7.2 Co-aggregation among aquatic bacteria

Development of biofilms by co-aggregation is also detected in many aquatic environments. Biofilms may cause corrosion in industrial water pipes as well as drinking water pipes. Rickard et al. (2000) used 16S rRNA gene sequencing for the identification of strains involved in creating biofilms in fresh water. They succeeded to identify six co-aggregation partnerships between five strains of *Blastomonas natatoria* and *Micrococcus luteus*. The authors argue that the mechanisms of co-aggregation between these strains were similar to mechanisms of bacteria which produce

dental plaque, with the exception that co-aggregation interaction among fresh water bacteria was dependent on their growth phase.

Min et al. (2010) showed in their studies that *Sphingomonas natatoria* and *Micrococcus luteus* isolated from a biofilm in fresh water, had the capability to form co-aggregation with each other. In studies on co-aggregation among drinking water bacteria, Simoes et al. (2007) observed that *Acinetobacter calcoaceticus* had the ability to form co-aggregation with five other isolates. They also found that in the absence of *A. calcoaceticus*, no co-aggregation was formed between other strains. They argued that *A. calcoaceticus* played the role of a bridge in the co-aggregation.

Some studies have shown that co-aggregation interaction could increase biofilm development in fast-flowing water system and may also mediate the entry of pathogenic bacteria such as *Campylobacter* into the bacterial community in the biofilm (Rickard et al. 2003; Buswell et al. 1998). Some observations also reported the presence of co-aggregation between certain strains in the activated-sludge. in their research, Malik et al. (2003) observed that *Acinetobacter johnsonii* and *Acinetobacter junii*, two non-flocculating bacteria, had the ability to form co-aggregation with *Oligotropha carboxidovorans*, *Microbacterium esteraromaticum*, and *Xanthomonas* spp. The results of this study suggest the possibility of formation of multigeneric co-aggregates with *Acinetobacter* as bridging organism. The authors argue that co-aggregation interaction is also a mechanism of floc formation in activated sludge.

2.7.3 Co-aggregation among microorganisms in other environments

It should be noted that the ability to form co-aggregates is not only limited to dental biofilm bacteria and aquatic biofilm bacteria. Some recent studies showed that co-aggregation has also been observed between lactobacilli and *E. coli* strains isolated from the human intestinal tract (Drago et al. 1997) and between human vaginal lactobacilli and *E.coli* (Ekmekci et al. 2009).

2.8 Mechanisms involved in co-aggregation

2.8.1 The cell-surface components mediating co-aggregation

Several studies in the field of dental plaque showed that the interaction between adhesins and carbohydrate receptors is responsible for co-aggregation among bacteria. In preliminary research with inactivation of one of the partners by heat and protease treatment, it was found that complementary proteinaceous structures (adhesins) on this partner's surface played an important role in co-aggregation (Kolenbrander 1988). The use of free sugars as inhibitors of co-aggregation also indicated the presence of carbohydrate receptor molecules on the surface of bacteria participating in the formation of the co-aggregates (Kolenbrander 1988).

2.8.2 Co-aggregation adhesins

Many co-aggregation mediating adhesins have been identified on the surface of gram-negative and gram-positive bacteria which are participating in the formation of dental plaque biofilm and aquatic environment biofilm (Rickard et al. 2004; Kolenbrander & Ganeshkumar 1993). The first identified oral adhesin was isolated from *Prevotella loescheii*. This lectin-like protein has a molecular weight of 450,000 and its ability to be involved in co-aggregation was sensitive to β-galactosidase (Kolenbrander & Ganeshkumar 1993).

Most of the co-aggregation adhesins identified later, have been reported for *Streptococcus, Actinomyces* and *Fusobacterium* (Rickard et al. 2003). Studies have shown that *Streptococcus gordonii* carry five different proteins on their surfaces, all of which are involved in co-aggregation interaction (Clemans et al. 1999). One of the most recognized adhesins used by bacteria to form co-aggregation are fimbriae-associated proteins (Kolenbrander & Ganeshkumar 1993). Fimbriae are non-flagellar bacterial appendages other than those clearly involved in the transfer of nucleic acids (Kolenbrander 1988). Adhesins are presented at the tip of these fimbrial structures extending from the cells (Kolenbrander & Ganeshkumar 1993).The distance of these co-aggregation adhesins from the cell surface help the partner organisms to have an active contact together.

Most of the known fimbriae contain a high proportion of hydrophobic amino acids (Kolenbrander 1988). The presence of these amino acids plays an important role in cell hydrophobicity and adherence to other cells. They may facilitate the bacterial adherence by overcoming the initial electrostatic repulsion barrier which exist between cells (Donlan 2002; Kolenbrander 1988). Thus, fimbriae are acting as probes to successfully locate the appropriate receptors on the partners' organism (Rickard et al. 2003).

Takahashi et al. (2002) identified a lectin-like protein with a molecular weight of 203kD associated with fibrillar structures on the surface of *Streptococcus gordonii*. Sandberg et al. (1995) also identified a protein adhesin correlated with fimbriae covering the surface of oral *Actinomyces*. This adhesin mediated the co-aggregation interaction between *Actinomyces* and other bacteria. Adhesins are not only limited to oral bacteria. A co-aggregating protein with a weight of 70kD was isolated from the cell surface of *Blastomonas natatoria*, which is a fresh water biofilm bacterium. This protein structure was very similar to the TonB-dependent receptors structure which facilitates the bacterial adhesion to different surfaces (Rickard & Leach 2002).

2.8.3 Co-aggregation receptors

Unlike adhesins, little knowledge is available about the composition and location of the receptors involved in co-aggregation. All of the research conducted in this regard indicates that diversity among the receptor molecules is limited (Rickard et al. 2003). Through analyses on a large number of co-aggregation mediating receptors, it was found that most of them are polysaccharide and usually composed of structures very similar to each other (Kolenbrander 1988).

In their studies, Abeygunawardana et al. (1991) isolated and characterized the surface carbohydrate receptors that mediated co-aggregation among five *Streptococci*. The results indicated that all of them were linear polysaccharides with N-acetylgalactosamine in their structures, and glycosidic linkage was common to all. The only difference was in the two sugars close to the reducing end that act as adhesin binding sites. Polysaccharide recognition by adhesins leading to co-aggregation formation is called protein-polysaccharide interaction (Kolenbrander et al. 2006; Rickard et al. 2003).

Due to the mentioned differences in the structure of carbohydrates only the complementary adhesins would be able to identify and interact with them (Kolenbrander & Ganeshkumar 1993). As a result, by adding the simple sugars existing in the receptors structure, the adhesins could be blocked and the co-aggregation between partners can be inhibited (Kolenbrander 1988; Kolenbrander & Ganeshkumar 1993).

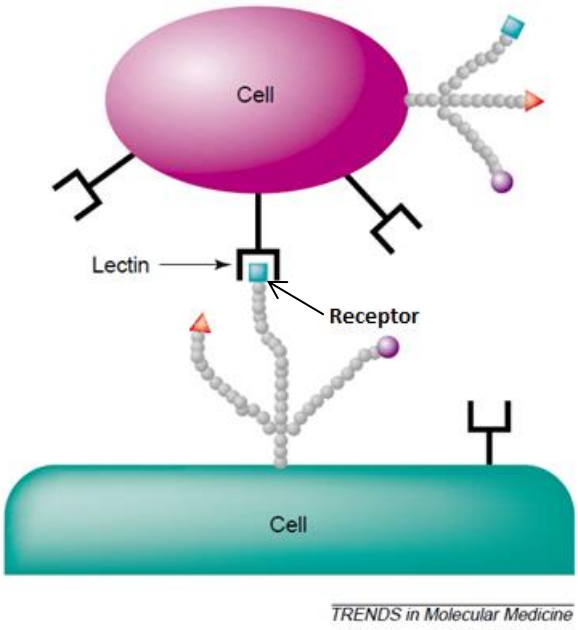


Fig 2.3 Cell–cell interactions mediated by carbohydrate-binding proteins (Source:Nangia-Makker et al. 2002).

Mcintire et al. (1978) previously reported that highly specific co-aggregation between certain *Streptococcus* and *Actinomyces* was inhibited by lactose but not with other sugars, including very similar sugars such a galactose and fructose. In their studies, Grimaudo et al. (1996) used 16 different sugars to investigate the ability of these sugars to inhibit co-aggregation between eight strains of *Actynomyces* and four strains of *Candida albicans*. They found that none of the sugars were able to inhibit the co-aggregation. Malik et al. (2003) argues in this regard that reversibility by simple sugars is not an essential feature of lectin-saccharide interaction.

2.8.4 Surface charge

Bacteria typically have a negative charge on their cell surface at physiological pH values (5-7). The magnitude of this charge varies from species to species, and can sometimes change under the influence of environmental factors such as pH (Poortinga et al. 2002; Palmer et al. 2007). The charge is generated due to the different groups such as carboxyl groups, phosphate and amino groups on the cell wall (Briandet et al. 1999). These charged groups are able to be associated or dissociated in response to environmental changes and also upon approaching to the charged surface of anther bacterium (Poortinga et al. 2002).

Due to the presence of negative charge, the bacteria can experience electric double layer repulsion when they approach other bacteria (Bos et al. 1999; Poortinga et al. 2002). At acidic pH, negative ionized groups can be neutralized by protonation, thus diminishing the strength of repulsive forces between bacteria and leading to increased co-aggregation (Joe et al. 2009; Burdman et al. 1998). Any increase in pH increases the dissociation of acidic groups and conversely increases the negative charge on the bacterial cell surface polymers. With increasing negative charge, the electro repulsion also increases, thus preventing the bacteria from approaching a surface or other bacteria, and results in a decrease in adhesion of bacteria to other bacteria (co-aggregation) or to the surfaces (Lewis et al. 1989). Such an association or dissociation of charged group may sometimes cause changes in the structure of different kinds of surface appendage, such as fimbriae or fibril (Poortinga et al. 2002).

The charge on the bacterial cell surface is determined as its zeta-potential and measured based on the bacterial cell movement in an electric field as a function of pH (Bos et al. 1999; Rickard et al. 2003). The pH value, in which the electrophoretic mobility of cell is equal to zero, is the isoelectric point (pI) of the cell. Using this pI would allow one to determine the molecular composition of cell surface (Poortinga et al. 2002). Rijnaarts et al. (1995) showed that bacteria with a pI lower than 2.8 have anionic polysaccharide on their cell surface. The bacteria with peptidoglycan on their surface have a pI between 3.0 and 4.0, while the bacteria with a pI above 4.0 have proteinaceous appendages on their cell surface.

Previous studies have shown that proteins carry a net positive charge on their surface at pH below their pI (Poortinga et al. 2002).Thereby, a reduction in environmental pH results in increased cell adhesion rate to surfaces with negative charge (Poortinga et al. 2002).

Gilbert et al. (1991) found in their studies that increased negative charge on the surface of the *E.coli* cells led to more resistance in these bacteria against detachment. The results suggest the importance of electrostatic charge in bacterial attachment. Burdman et al. (1998) observed in their studies that pH reduction increased the aggregation ability among various *Azospirillum brasilense* strains.

2.8.5 Hydrophobicity

Studies on bacteria showed that there is a strong relationship between cell surface hydrophobicity and the ability of bacteria cells to adhere to other bacteria (co-aggregation) or other surfaces (Reasoner & Geldreich 1985; Palmer et al. 2007). The hydrophobicity level is different among different bacterial cells, which is due to the properties of the molecules on the bacterial surface, such as proteins and lipids. There is evidence indicating that in many cases the hydrophobicity is related to flagella and fimbriae (Donlan 2002).

The research conducted on fimbriae showed that they contain a high proportion of hydrophobic amino acid residues in their structure. McEldowney et al. (1986) showed that bacteria with a high degree of hydrophobicity contained a higher percentage of nitrogen than carbon. However, bacteria exhibiting a lower hydrophobicity had a higher percentage of oxygen than carbon. These results indicated that the presence of proteinaceous appendages on the cell surface increases the cell surface hydrophobicity.

In his research, Jenkinson (1992) exposed *Streptococcus sanguis* cells to sodium lauroyl sarcosinate in their research, and found that this treatment caused reduced cell hydrophobicity as well as reduced ability of the cells to co-aggregate. The author suggests that a decrease in hydrophobicity resulting in reduced ability to form co-aggregation was due to the loss of polypeptides present on the cell surface (Jenkinson 1992; Jenkinson 1986).

2.9 Effect of environmental conditions on co-aggregation

2.9.1 Ionic strength

Lectin and lectin-like proteins occasionally exhibit activities which are dependent on ions present in the environment. These proteins normally need divalent cations to display a good binding (Dulaney 1978; Lis & Sharon 1973). It was initially thought that the co-aggregation between microorganisms is simply generated by attractive van der Waals forces and acid-base interactions. However, the calcium-mediated co-aggregation between certain bacteria, such as *Streptococci* and *Actinomycetes* strains showed that other factors also can be involved in this interaction (Bos et al. 1999).

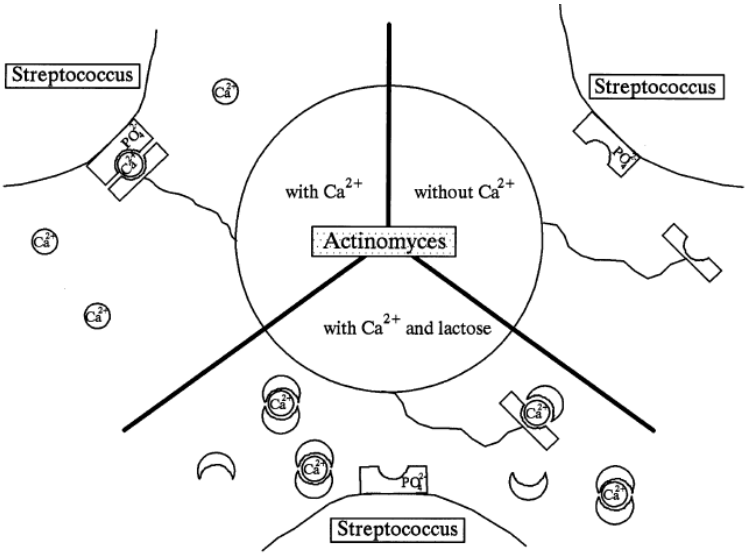


Fig 2.5 Hypothetical model for the calcium-mediated co-aggregation between *Actinomycetes* and *Streptococcus*. Calcium ions are assumed to be adsorbed to the tips of cell surface appendages on the *actinomycetes* to break down the local electrostatic repulsion (Source: Bos et al. 1999).

The existence of cations such as Ca^{2+} in the environment can reduce the electrostatic repulsion between organisms and create a local electrostatic attraction. Bos et al. (1996) found that adding calcium ions to an oral bacteria suspension led to lower negative zeta potential, and thus the bacteria exhibited a better ability to co-aggregate. The authors argued that such a result was due to the reduction of electrostatic repulsive interaction energy by calcium ions.

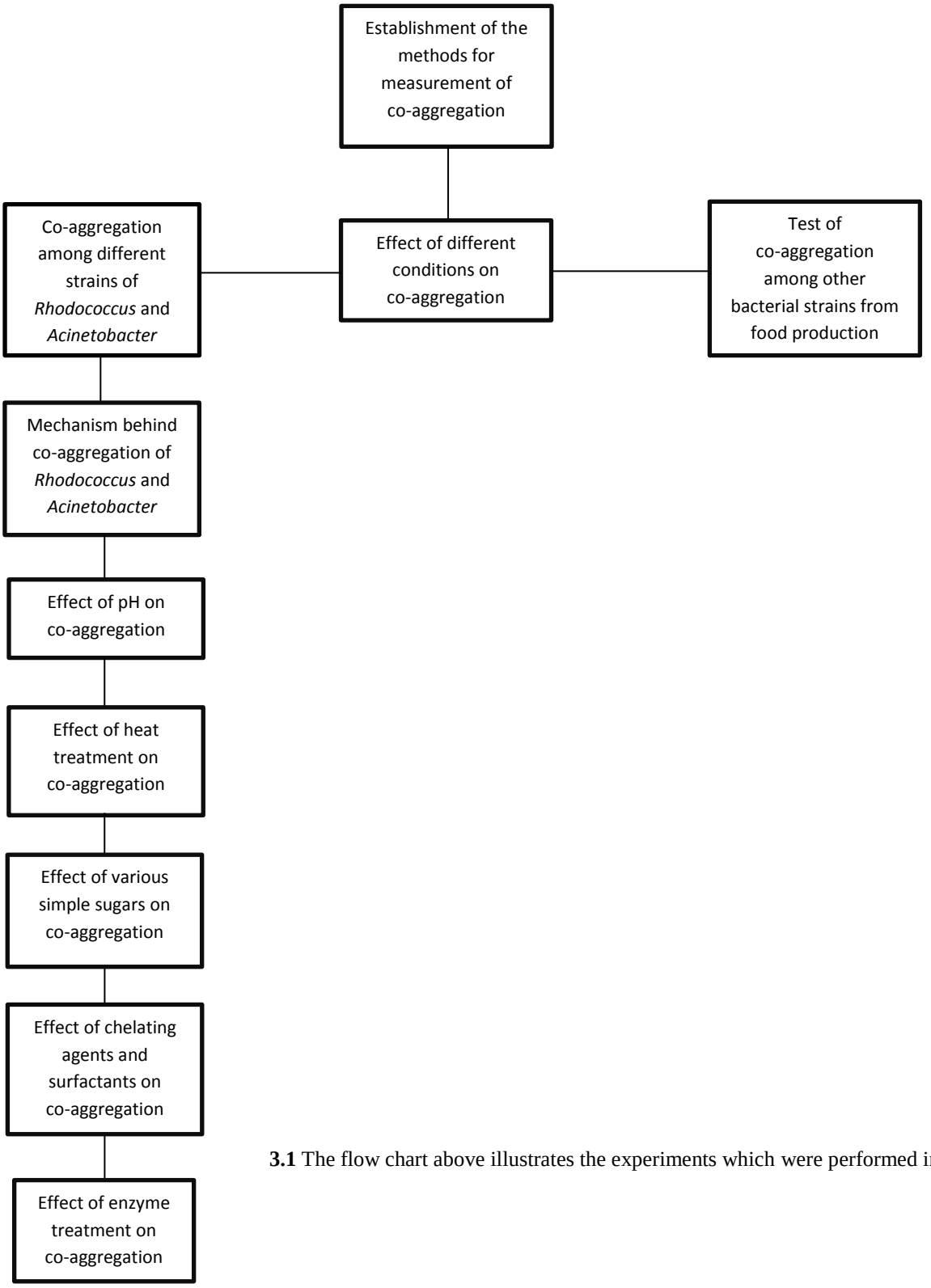
Cisar et al. (1979) also showed that ions play an important role in co-aggregation between oral bacteria. Some previous studies indicated that increased NaCl concentration led to increased re-aggregation ability among bacteria in activated sludge. Moreover, increased co-aggregation ability was seen in the presence of Ca^{2+} (Malik & Kakii 2003). It is important to mention that high environmental ionic strength may result in the association or dissociation of charged groups of the cell-surface polymers. It may also change the structure of polysaccharides, fimbriae and fibrils, which will result in changes in interaction between co-aggregating cells (Min et al. 2010). Cisar et al. (1979) previously reported that an increase in ionic strength could prevent co-aggregation between oral bacteria.

2.9.2 Growth and culture conditions

Adhesion of a bacterial cell to another cell or to a surface can be affected by factors such as growth rate, growth medium and culture conditions (Chmielewski & Frank 2003). All of these factors can affect the cell surface properties and production of extracellular polymeric substances which mediate different interactions (Malik et al. 2004). Some studies showed that the hydrophobicity typically decreases when the growth rate increases (Chmielewski & Frank 2003; Carpentier & Cerf 1993). In some cases, the co-aggregation among bacteria is mediated by specific growth phase dependent interactions. Rickard et al. (2000) found that the lectin-saccharide mediated co-aggregation between *Blastomonas natatoria* and *Micrococcus luteus* completely disappeared in the late stationary phase.

Growth temperature is also a factor which can affect the co-aggregation ability of the bacterial cells. This factor can change cell hydrophobicity and surface electrical properties by affecting the composition of the cell wall or interfering with the electron donor -acceptor systems of the bacterial cells (Chavant et al. 2002). In their studies, Chavant et al. (2002) observed an inverse relationship between *Listeria monocytogenes* cell surface hydrophobicity and incubation temperature. Some studies have also mentioned the growth medium composition as an important factor in co-aggregation formation. Burdman et al. (1998) reported that *Azospirillum brasilense* cells grown in growth medium containing a higher percentage of carbon than nitrogen showed more ability to form re-aggregation compared to cells grown in an environment consisting of less carbon to nitrogen.

3. MATERIALS AND METHODS



3.1 The flow chart above illustrates the experiments which were performed in this study.

3.1 Maintenance and storage of bacteria

The strains that are used in this study had been previously collected from food production environments. These strains after isolation and identification have been stored in the Nofima strain collection at -80^{o}C. One sample from each strain was firstly moved to a petri-dish containing TSA (Tryptone soya agar. Oxoid, Basingstoke, England) and was incubated for 24 $\pm$ 2 hr. at 25 or 30^{o}C (in terms of optimum temperature for each strain).

After the growth of bacterial cells, a single colony was taken from the petri-dish and transferred to a tube containing 5 ml of TSB (Tryptone soya broth. Oxoid). These tubes were kept in a shaker incubator (Innova 4000, England) for one night at the speed of 200 rpm with the temperature of 30^{o}C to obtain the overnight culture. Then, 1 ml of overnight culture with 18 % of glycerol was transferred to a Cryor tube (Nalgene Cryoware Cryogenic Vialas, Thermo Fisher Scientific, USA) and was kept at the temperature of -80^{o}C (Forma ULT Freezer 700s, Thermo scientific ,USA) for this study. The frozen cells (if needed) were cultured on a petri-dish containing TSA, and after the growth of bacteria, plates were stored at 4^{o}C.

3.2 Bacterial strains and batch growth conditions

All of the strains which were used in this study are shown in Table 3.3 and in appendix in tables 9.1 - 9.4. In order to culture bacteria, one single colony of each bacterial strain was first taken from the petri-dish containing TSA and transferred into a tube containing 5 ml of TSB. Then the tubes were incubated in a shaker incubator at the speed of 200 rpm with the temperature of 30^{o}C for one night. 500 µl of the overnight culture was transferred into an Erlenmeyer flask containing 50 ml of TSB which was preheated (at 30^{o}C for 1 hr.) and incubated in a shaker incubator at the speed of 200 rpm with the temperature of 30^{o}C for 18 $\pm$ 2 hr.

3.3 Establishment of methods for measurement of co-aggregation

Two different methods have been employed for the assessment of co-aggregation in this study. First the formation of any co-aggregation was visually observed by using Visual aggregation assay(Cisar & Kolenbrander 1979; Rickard et al. 2004). In addition, the co-aggregation and auto-aggregation percentage was calculated by using Optical density assay(Mcintire et al. 1978).

3.3.1 Preparing the suspensions for co-aggregation assays

45 ml of bacterial suspension grown in the Erlenmeyer flasks containing 50 ml of TSB (see 2.2) was transferred to a centrifuge tube (Centrifuge tubes with screw caps, VWR, LLC, USA). The cells were harvested in the centrifuge (Sorvall, RC5C plus, rotor SH3000, USA) at the speed of 4200 rpm for 20 minutes at 20^{o}C. Then the growth medium was removed and deposited pellet was washed by adding 45 ml of sterile de-ionized water.

Cells were completely re-suspended by the use of a pipette and were centrifuged again at the same condition explained above. The process of washing was repeated three times. Then the cells were re-suspended in 10 ml of co-aggregation buffer which consisted of following: $CaCl_2$ (10^{-4} M), $MgCl_2$ (10^{-4} M) and NaCl (0.15 M) dissolved in 0.001 M Tris (hydroxymethyl) aminomethane, adjusted to pH 8.0 (Kolenbrander & Phucas 1984).This should be done carefully in a way that all cells are totally dissolved in the co-aggregation buffer and do not remain as pellets in the suspension.

The optical density of the suspension was read by a spectrophotometer (Ultrospec 3000, Pharmacia Biotech, England). Using the value obtained, the required volume of cell suspension was calculated to obtain 5ml of cell suspension with an optical density at 650 nm of 1.5 by adding co-aggregation buffer. The obtained suspension has the potential to be used immediately for visual co-aggregation assay and optical density assay, or stored in the refrigerator at 4^{o} C for future works (Cisar & Kolenbrander 1979).

3.3.2 Visual co-aggregation assay

The assay used in this study is the one presented by Cisar et al. (1979) with slight modifications. To assess the ability of co-aggregation between *Rhodococcus erythropolis* (MF3727) and *Acinetobacter calcoaceticus* (MF3293), a visual co-aggregation test was used. 200 µl of the prepared suspension (3.3.1) from each of the pair of strains to be tested were added into a Silica Durham tube (Borosilicate Glass 12x75ml, Fisher brand, USA).

The tube with the suspension was vortexed, rolled slowly for ten seconds and then left for thirty seconds at room temperature. Then the Durham tubes were investigated visually under magnifying lamp (eaBelysning, Spektra lupelampe 8066D2, Norway) immediately, after 1 and 2hr. If co-aggregation occurs, the bacterial cells stick together and result in a variety of solutions ranging from turbid solution without any sedimentation to a relatively transparent solution with high sedimentation of flocs. To address this variety, the co-aggregation score rating scheme presented by Cisar et al. (1979) was used.

The rating criterion which is presented in this scheme is as follows: Score "0" shows condition in which no observable flocs are formed. Score "1" shows conditions in which very small and concentrated masses are formed. Score "2" shows conditions with observable flocs that are not sedimentary and can form a turbid solution can be seen in the suspension. Score "3" is conditions in which generated flocs are sedimentary but can form a turbid solution. Score "4" is conditions in which generated flocs have large size and have enough sedimentary potential to lead to a solution that is very transparent in its upper parts. This scheme is one of the most prestigious and credible schemes that generally have been used to study the levels and ratings of co-aggregation (Min et al. 2010).

3.3.3 Optical density assay

To calculate the co-aggregation percentage between *Rhodococcus erythropolis* (MF3727) and *Acinetobacter calcoaceticus* (MF3293) an optical density assay was used. After preparing the suspensions (3.3.1), 0.5 ml of each strain to be tested was transferred into a cuvette with total volume of 1 ml and mixed by pipette for ten seconds. Then the absorbance (650nm) was read by spectrophotometer immediately and also after leaving the suspension completely still for 1 and 2hr.

The co-aggregation percentage was calculated according to the equation that is presented as below:

$$\%\ \text{Coaggregation} = 100 \times \frac{(\text{OD}_1 + \text{OD}_2) - 2(\text{OD}_3)}{(\text{OD}_1 + \text{OD}_2)}$$

Where OD_1 is the optical density of strain 1 at 650nm after 2 hr. (for calculation of co-aggregation after 2 hr.), OD_2 is the optical density of strain 2 at 650nm after 2hr and OD_3 is the optical density of strain 1 and strain 2 at 650nm after 2hr (Ekmekci et al. 2009).

3.3.4 Auto-aggregation assay

Each individual strains was tested for the presence of auto-aggregation. After preparing cell suspensions (3.3.1) a mixture containing 200 µl of the bacteria (OD_{650}=1.5) and 200 µl of de-ionized water (according to the visual co-aggregation assay) was prepared for each strain and was put under close observation. The visual rating of auto-aggregation level was carried out on the basis of the co-aggregation rating (3.3.2).

For calculating the percentage of probable auto-aggregation, 1ml of each strain (after preparing cell suspension) was separately transferred into a cuvette. The suspension was mixed by using pipette for ten seconds and then, the absorbance level was read immediately, after 1 and 2hr exactly similar as described for co-aggregation. The auto-aggregation percentage was calculated according to the equation that is presented as below:

$$\%\ \text{Autoaggregation} = 100 \times \frac{\text{OD}_{\text{initial}} - \text{OD}_{\text{final}}}{\text{OD}_{\text{initial}}}$$

Where $OD_{initilal}$ is the optical density at 650nm at initial time (t=0) and OD_{final} is the optical density at 650nm, 2hr after beginning auto-aggregation assay (Tomás et al. 2005). To ensure the results reliability, three separate experiments carried out for each co-aggregating pair.

3.3.5 Scanning electron microscopy (SEM)

Principle

The scanning electron microscope (SEM) benefits much higher resolution than traditional microscopes. The SEM also allows more of a specimen to be in focus at one time and provides detailed surface information. This technique was employed to observe the pattern *Rhodococcus erythropolis* MF3727 and *Acinetobacter calcoaceticus* MF3293 used to form co-aggregates.

Procedure

Because the SEM utilizes vacuum conditions and uses electrons to form an image, special preparations was done to the samples. First a small round cover slip was coated with 1mg/ml poly-l-lysine for each sample. Then the cover slips were placed on a filter paper to air dry. The next step was attaching the bacteria to the cover slips. Right before attaching process, it was required chemical fixation of bacterial cells to preserve and stabilize their structure. Fixation was performed by incubation bacterial cells in a solution of 2.5% glutaraldehyde in PBS buffer (phosphate-buffered saline). To attach the bacteria, the cover slip was leaved in the cell suspension for 30 minutes. Afterwards the cover slip was taken up and putted into a small sample glass containing 1ml sterile de-ionized water for a couple of seconds for rinse off the excess bacteria.

Then the water was removed and 1ml 70% ethanol was quickly added to the sample glass (to avoid air-drying that causes collapse and shrinkage of cells). After 5 minutes the 70% ethanol was removed and 1 ml 90% ethanol was added quickly and left for 5 minutes. Then 90% ethanol was removed and 1ml 96% ethanol was quickly added and left for 5minutes. Afterwards the 96% ethanol was removed and 1 ml 100% ethanol was quickly added and left for 5 minutes.

The last step was repeated for three times. In the next step the ethanol was replaced with liquid carbon dioxide by critical point dryer (BAL-TEC CPD 030, BAL-TEC AG, Blazers, Germany). Then the carbon dioxide was also removed so that no gas-liquid was present longer within the samples during drying. Later the dry samples were sputter-coated with (5-7nm) gold/ palladium (Sputter Coater, Polaron SC 7640, Quorum Technologies Ltd, East Sussex, UK) before

examination in the microscope (Zeiss EVO-50-EP, Carl Zeiss SMT Ltd, Cambridge, UK). The microscopy was performed by Elin Ørmen at microscopy lab, Norwegian University of Life Sciences.

3.4 The effect of growth medium, cultivation time and growth temperature on the co-aggregation capability

Principles

To assess the effects of various physicochemical factors on co-aggregation capability, three factors of cultivation time, growth temperature and nutritional requirements were considered. The main goal of this experiment was to determine the most appropriate growth medium, optimum growth temperature and cultivation time in such a manner that bacterial cells demonstrate their highest capability for co-aggregation. The best result obtained from this experiment was employed as a standard growth condition in all of the further steps of this study.

Experiment design

Rhodococcus erythropolis MF3727 and *Acinetobacter calcoaceticus* MF3293 were cultivated under following conditions (Table 3.1) during this experiment.

Table 3.1 The different cultivation conditions tested for *Rhodococcus erythropolis* (MF3727) and *Acinetobacter calcoaceticus* (MF3293).

Growth medium	Growth temperature	Harvesting time*
TSB	20° C	T1, 18 hr., 42 hr.
TSB	30 ° C	T1, 18 hr., 42 hr.
R2A**	20° C	T1, 18 hr., 42 hr.
R2A	30 ° C	T1, 18 hr., 42 hr.

* Harvesting times were chosen based on different phase of growth curve. T1 varied due to the time each strain required to reach an optical density of 0.5 at 650nm (exponential phase). However in other cases harvesting occurred after 18 and 42 fixed hours (early and mid-stationary phase). ** R2A (Difco, Becton, Sparks, USA)

Procedure

Right before harvesting bacterial cells (cultivated at different conditions), samples were taken from each strain to be used for counting viable bacterial cells in each growth phase. First 0.5 ml of each strain suspension was diluted sequentially for five times (1/10-1/100000). The diluted suspensions were plated by Whitley Automatic Spiral Plater (WASP, Don Whitely Scientific Limited, West Yorkshire, England) and then, the plates were incubated for 24 ± 2 hr. with temperature of 30^{o}C. For counting the grown colonies on the plates Colony counter (ProtoCol2, Colony counting and zone sizing system, Cambridge, UK) was used.

After harvesting and washing the cells, the respective suspensions were prepared (3.3.1). To study co-aggregation ability of the strains, cells which were cultivated under similar conditions, except harvesting time, were cross compounded. The strains were examined with both visual co-aggregation and optical density assay. To ensure the results reliability, all of this experiment was independently repeated three times. The conditions that resulted in maximum co-aggregation were selected for further studies. In the rest of this thesis the term Standard conditions refer to cultivation in TSB at 30^{o} C for 18 hours.

3.5 The effect of different solutions and buffers on co-aggregation

Principles

This experiment aimed to study the co-aggregation capability of *Rhodococcus erythropolis* MF3727 and *Acinetobacter calcoaceticus* MF3293 when the different solution and buffers were used to re-suspend the bacterial cells for preparing suspension (3.3.1). The method that was presented by Min et al. 2010 has been employed with some modifications in this study.

Procedure

After cultivation the bacterial cells at standard conditions (TSB, 30^{o}C, 18hr.), the cells were harvested and washed (3.3.1). To prepare the respective suspension for visual co-aggregation and optical density assay, the cells of each strain were individually re-suspended in different solution and buffers mentioned in table 3.2. To study the effect of each solution and buffer on co-

aggregation, the strains that were re-suspended in common solution or buffer were tested. In addition to testing the effect of the different re-suspension solutions, the effect of the washing solution on co-aggregation was also tested. Two samples of each strain were separately cultivated and then harvested (TSB, 30^{o}C, 18hr.).

The cells were washed with sterile dH_2O and co-aggregation buffer (three times), respectively. Finally both the samples were re-suspended in co-aggregation buffer and the suspensions were prepared for visual co-aggregation and optical density assay (3.3.1). To ensure the results reliability, this experiment was independently repeated three times. In the rest of this thesis, standard condition refers also to three times washing in dH_2O followed by re-suspension in co-aggregation buffer.

Table 3.2 Different solution and buffer used to study the effect of them on co-aggregation ability of *Rhodococcus erythropolis* (MF3727) and *Acinetobacter calcoaceticus* (MF3293).

Solution and buffers
1. Sterile de-ionized water
2. Co-aggregation buffer
3. NaCl 0.85%
4. TSB
5. R2A

3.6 Co-aggregation among different strains of *Rhodococcus* sp. and *Acinetobacter* sp.

Principle

Due to the observation of co-aggregation between *Rhodococcus erythropolis* MF3727 and *Acinetobacter calcoaceticus* MF3293, the possibility of co-aggregation between other strains of *Rhodococcus* spp. and *Acinetobacter* spp. was investigated in this study. The purpose of this experiment was to determine the prevalence and the ability of these strains to co-aggregate.

Procedure

Totally three *Rhodococcus* strains and eight *Acinetobacter* strains were chosen to investigate the co-aggregation ability between them (Table 3.3). First, the cells were cultivated, harvested and the respective suspensions were prepared at standard condition. Then, all 55 possible pairs were tested. The co-aggregation ability of these pairs was tested by both visual co-aggregation and optical density method. To ensure the results reliability, this experiment was repeated three times with different batch culture.

Table 3.3 Strains of *Rhodococcus* sp. and *Acinetobacter* sp. tested for co-aggregation.

Bacteria	Strain number*	Origin	Reference
Rhodococcus erythropolis	3727	Drain, small scale cheese producer A	Schirmer et al 2013
Rhodococcus erythropolis	4633	Slicing machine, meat processing plant C	Schirmer, unpublished
Rhodococcus erythropolis	3803	Floor, small scale cheese producer B	Schirmer et al 2013
Acinetobacter sp.	4642	Conveyor belt, meat processing plant C	Schirmer et al 2013
Acinetobacter sp.	4130	Conveyor belt 1, salmon processing plant D	Heir, unpublished
Acinetobacter sp.	4206	Conveyor belt, salmon slaughterhouse E	Heir, unpublished
Acinetobacter johnsonii	4091	Conveyor belt, salmon processing plant F	Heir, unpublished
Acinetobacter calcoaceticus	3293	Disinfecting footbath with hypochlorite, dairy	Langsrud et al 2006
Acinetobacter calcoaceticus	3627	Platform evisceration, meat slaughterhouse	Møretrø et al 2013
Acinetobacter junii	4112	Filet machine, salmon processing plant D	Heir, unpublished
Acinetobacter sp.	4117	Conveyor belt 2, salmon processing plant D	Heir, unpublished

*refer to MF number in Nofima strain collection.

3.7 Mechanism behind co-aggregation of *Rhodococcus* and *Acinetobacter*

3.7.1 Effect of pH on co-aggregation

Principles

This experiment aimed to study the effect of different pH on co-aggregation ability among *Rhodococcus erythropolis* MF3727 + *Acinetobacter calcoaceticus* MF3293, *Rhodococcus erythropolis* MF3727 + *Acinetobacter calcoaceticus* MF3627 and *Rhodococcus erythropolis* MF3727 + *Rhodococcus erythropolis* MF3803. The method that had been presented by Min et al. (2010) was employed to perform this study.

Procedures

After cultivating the co-aggregating cells (TSB, 30^{o}C, 18hr), the cells were harvested. Then, 14 tubes of cell suspensions were prepared (at standard condition) separately for each of co-aggregating strains. Later the pH of each strain suspensions was regulated from 1 to 14 by using NaOH and NaCl with a pH meter (PHM210, MeterLab, Radiometer Analytical, France). Afterwards the suspensions were incubated for 20 minutes at room temperature. To study the effect of pH, strain with identical pH value were chosen to participate in co-aggregation as pair. To investigate the effect of pH on co-aggregation, the pairs were studied by using visual co-aggregation and optical density assay. To ensure the results reliability, this experiment was independently repeated three times.

3.7.2 Effect of heat treatment on co-aggregation

Principles

In this experiment, it was attempted to inhibit the co-aggregation by applying heat pre-treatment on *Rhodococcus erythropolis* strains (MF3727 and MF3803) and *Acinetobacter calcoaceticus* strains (MF3293 and MF3627). This aimed to determine which one of co-aggregating strains uses protein cell-surface polymers to participate in co-aggregation. The method presented by Rickard et al 2004 has been employed in this study with small modification.

Procedure

First the cells were cultivated, harvested and the respective suspensions were prepared at standard condition. Afterwards, suspension of each strain was individually transferred into an eppendorf tube (Micro-Centrifuge Tubes, 1.5ml, VWR, USA) with a volume of 1.5 ml. Later the eppendorf tubes containing suspension were heated (eppendorf, Thermomixer 5436, Hamburg, Germany) at 85^{o}C for 30 minutes. The suspensions were finally left in water bath at room temperature until cooled off. To investigate the effect of applying heat treatment on co-aggregating ability, each member of co-aggregating pairs including heat treated and untreated ones were studied by using visual co-aggregation and optical density assay. To ensure the results credibility, this experiment was independently repeated three times.

3.7.3 Effect of various simple sugars on co-aggregation

Principles

In this experiment, the different sugars (Table 3.4) capability to reverse or inhibit the co-aggregation among *Rhodococcus erythropolis* MF3727 + *Acinetobacter calcoaceticus* MF3293, *Rhodococcus erythropolis* MF3727 + *Acinetobacter calcoaceticus* MF3627, and *Rhodococcus erythropolis* MF3727 + *Rhodococcus erythropolis* MF3803 was studied. This aimed to investigate the cell-surface polymers which participated and intermediated in co-aggregation. The methods presented by Rickard et al. (2004) and Taweechaisupapong & Doyle (2000) has been employed in this study.

Procedures

The cells were cultivated, harvested and the respective suspensions were prepared at standard condition. To investigate about the inhibition of co-aggregation by visual assay, first two Durham tube containing 400µl of co-aggregating pair (individually for each pair) was prepared for each simple sugar. Then solution of respective sugar with a final concentration of 50mM was added to the Durham tube, and the same volume of sterile de-ionized water was added to another

Durham tube (as control). Finally the tubes were vortexed for 10 seconds and studied based upon the visual co-aggregation assay (3.3.2).

To calculate the inhibition of co-aggregation percentage by using optical co-aggregation assay, two cuvettes containing 1ml of co-aggregating pairs were prepared (as described above). Then the solution of respective sugar with a final concentration of 50mM was added to one cuvette and the same volume of sterile de-ionized water was added to another (as control). Later, the suspensions were mixed by a pipette for 10 seconds and the optical density was read according to assay explained in section 3.3.3. The inhibition percentage was calculated according to the equation that is presented as below:

$$\text{Percent inhibition} = 100 \times \frac{\text{A} - \text{B}}{\text{A}}$$

Where A is %co-aggregation without inhibitor and B is %co-aggregation with inhibitor (Taweechaisupapong & Doyle 2000).To ensure the results reliability, this experiment was independently repeated three times.

Table 3.4 Sugars tested for inhibition or reversion co-aggregation between *R. erythropolis* MF3727 + *A.calcoaceticus* MF3293, *R. erythropolis* MF3727 + *A. calcoaceticus* MF3627 and *R. erythropolis* MF3727 + *R.erythropolis* MF3803.

Sugars*	**Fainal concentration (mM)**
Lactose monohydrate	50
D (+) galactose	50
α-L-fucose	50
N-acetyl-D-galactosamine	50
D (+) glucose	50
D-mannose	50

* Lactose monohydrate (SIGMA), D (+) galactose (SIGMA), α-L-fucose (SIGMA) N-acetyl-D-galactosamine (SIGMA), D (+) glucose (Merck), D-mannose (SIGMA).

3.7.4 Effect of chelating agents and surfactants on co-aggregation

Principles

The capability of different chelating agent and surfactants (Table 3.5) to disperse or inhibit the co-aggregation among *Rhodococcus erythropolis* MF3727 + *Acinetobacter calcoaceticus* MF3293, *Rhodococcus erythropolis* MF3727 + *Acinetobacter calcoaceticus* MF3627 and *Rhodococcus erythropolis* MF3727 + *Rhodococcus erythropolis* MF3803 was studied in this experiment. The methods that had been presented by Malik et al. (2003) and Grimaudo et al. (1996) were employed to perform this study.

Procedures

The method explained in section 3.7.3 was used in this experiment. However different final concentration for chelating agents and surfactants were used (Table 3.5). The inhibition percentage was calculated according to the equation that is presented in section 3.7.3. To ensure the results reliability, this experiment was independently repeated three times.

Table 3.5 Chelating agents and surfactants tested for effect on co-aggregation.

Agents*	Final concentration
EDTA	50mM
EGTA	50mM
Citrate	5.1mM
Tween 80	0.2%
SDS	1%

* EDTA (Merck), EGTA (Merck), Citrate (Merck), Tween 80 (SIGMA), SDS (SIGMA).

3.7.5 The effect of enzymatic treatment of cells on co-aggregation

Principles

The enzymatic treatment method was used in this experiment to determine the sensitivity level of polymers on each pair members which participates in co-aggregation. The method used in this experiment was a modified version presented by Rickard et al. (2004). The enzymes used in this study were entitled in Table 3.6 along with needed buffers for each enzyme.

Procedures

First the cells of *Rhodococcus erythropolis* strains (MF3727 and MF3803) and *Acinetobacter calcoaceticus* strains (MF3293 and MF3627) were cultivated (TSB, 30^{o}C, 18hr). Then the cells were harvested and washed three times with dH_2O. Later, for the purpose of treatment by each enzyme, cells were re-suspended in specific buffer needed for respective enzyme (explained in Table 3.6) and regulated to reach a density of about 1.0×10^{8} cell/ml.

In the next step, enzymes were separately added to suspensions containing co-aggregating strains to be tested. The same volume of sterile de-ionized water was added to control suspensions. Afterwards all suspensions were incubated in a shaker-incubator for 60 minutes at 37^{o}C. The cells were later harvested, and the respective suspension for visual and optical test was prepared at standard condition. To investigate the ability of each bacterial strain to form co-aggregates, the treated and untreated (control) strains were combined in pairs and tested by both visual co-aggregation and optical density assay. To ensure the obtained results reliability, the aforementioned experiment was independently repeated three times.

Table 3.6 Enzymes and buffers used for treating the cells of *Rhodococcus erythropolis* strains (MF3727 and MF3803) and *Acinetobacter calcoaceticus* strains (MF3293 and MF3627) prior to co-aggregation test.

Enzyme*	Concentration	Buffer
Proteinase K	1mg/ml	20mM Tris (pH7.5) + 100mM NaCl
Dispersin B	40μg/ml	PBS
DNAse I	0.1mg/ml	150mM NaCl + 1mM $CaCl_2$

* Proteinase K (SIGMA), Dispersin B (SIGMA), DNAse I (SIGMA).

3.8 Test of co-aggregation among various bacterial strains from food production environment.

Principles

Regarding the fact that all the co-aggregating strains which were initially used in this study were isolated from food production environment, this idea came along whether co-aggregation is a general phenomenon among various bacterial strains from food production environment.

Procedure

To investigate prevalence of co-aggregation between bacteria from the food industry, 78 food related bacterial strains were chosen to be used in this work. These strains categorized in four different groups based on sample origin (Appendix, tables 9.1 – 9.4). Strains in each group were only tested against the strains in the same group. The bacterial cells were cultivated, harvested and the cell suspensions were prepared at standard condition. Later 466 possible pairs were tested by visual co-aggregation and optical density method. To ensure the results reliability, this experiment was independently repeated twice with different batch culture.

3.9 Standard deviation

The standard deviations were calculated by Microsoft Excel, version 2010.

4. RESULTS

4.1 Establishment of methods for measurement of co-aggregation

Initially, methods was tested for evaluating co-aggregation between *Rhodococcus erythropolis* MF3727 and *Acinetobacter calcoaceticus* MF3293, a pair of strains where co-aggregation between them previously had been observed in a screening study between bacteria of different origin (Rickard et. Al., unpublished). The testing was performed on strains cultivated in TSB at 30^{o}C overnight. The cells were harvested, washed and respective suspension for co-aggregation assays were prepared (3.3.1).

4.1.1 Visual co-aggregation assay

The cells of *Rhodococcus erythropolis* MF3727 and *Acinetobacter calcoaceticus* MF3293 attached together and created flocs after 30 seconds (Fig. 4.1B). After 20-30 minutes, large flocks made of co-aggregates were formed as deposits at the bottom of the tube and left a transparent supernatant above (Fig. 4.1 E and F). According to the visual scoring scheme (3.3.2), visual co-aggregation of this pair achieved a score of 4. Each of individual strains was also tested in terms of auto-aggregation with the same assay (3.3.4). No aggregation was observed among the individual strains, thus the score for auto-aggregation was 0.

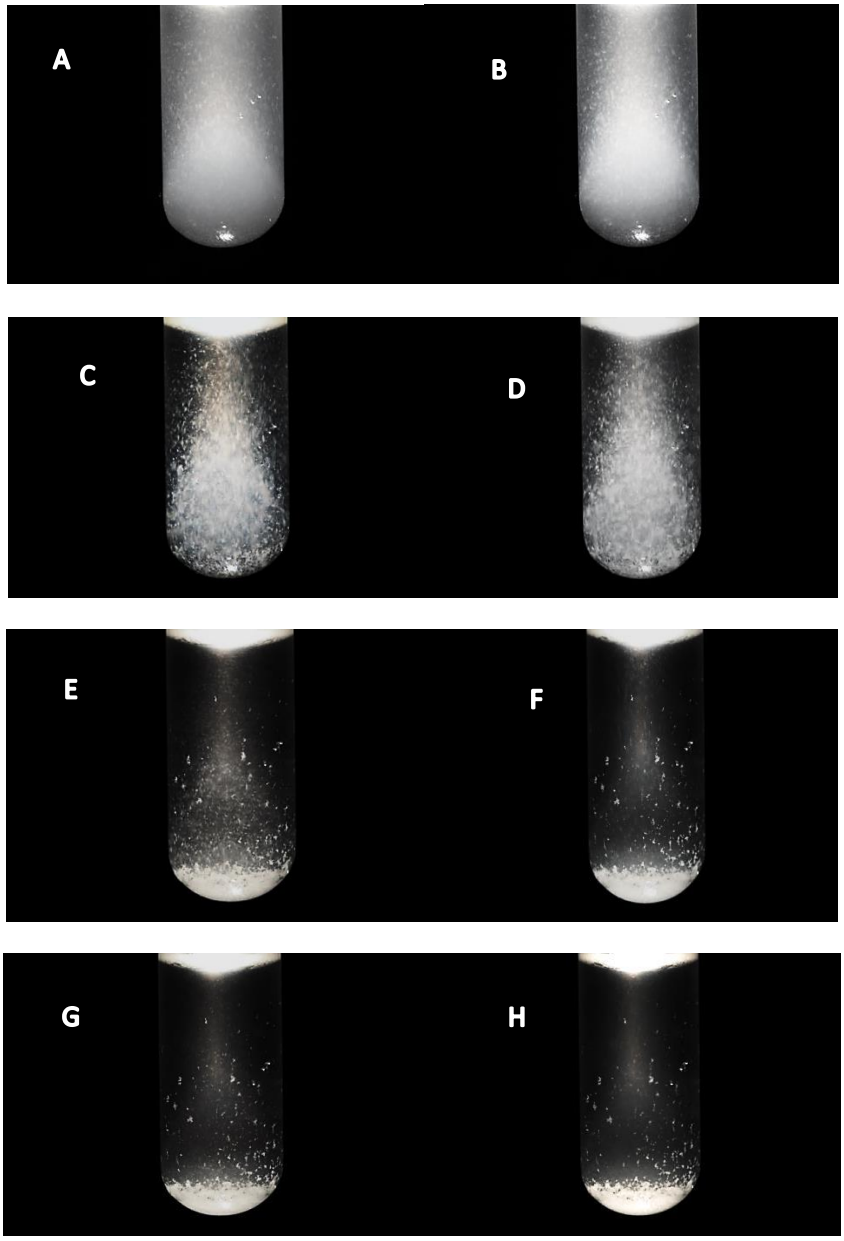


Fig 4.1. Co-aggregation (visual) between *Rhodococcus erythropolis* MF3727and *Acinetobacter calcoaceticus* MF3293. The pictures show co-aggregates formation and sedimentation during 2hr. A: immediately after mixing the strains. B: after 30 sec. C: after 5 min. D: after 10 min. E: after 20 min. F: after 30 min. G: after 1hr. H: after 2hr. The strains were cultivated at (TSB, 30^{o}C, 18hr) and re-suspended in co-aggregation buffer prior to the test. (Photo: Kjell Merok, Nofima).

4.1.2 Optical co-aggregation assay

According to co-aggregation assay, decreased optical density was measured for *Rhodococcus erythropolis* MF3727 and *Acinetobacter calcoaceticus* MF3293 as a co-aggregating pair (3.3.3). The optical density was also measured for each of the strains individually as a measurement of auto-aggregation (3.3.4). As it can be seen in figure 4.2, the optical density decreased over time both for individual and mixed suspension, with stronger decrease in the latter. It was decided to use two hours as standard time when determining co-aggregation.

The visual assay is the most commonly used assay for evaluating co-aggregation. The method is rather subjective and rough as the score is only given as 0, 1, 2, 3 or 4. The optical density method is more objective and more accurately correct for auto-aggregation. It was decided to use both methods in the further investigations of co-aggregation in this study.

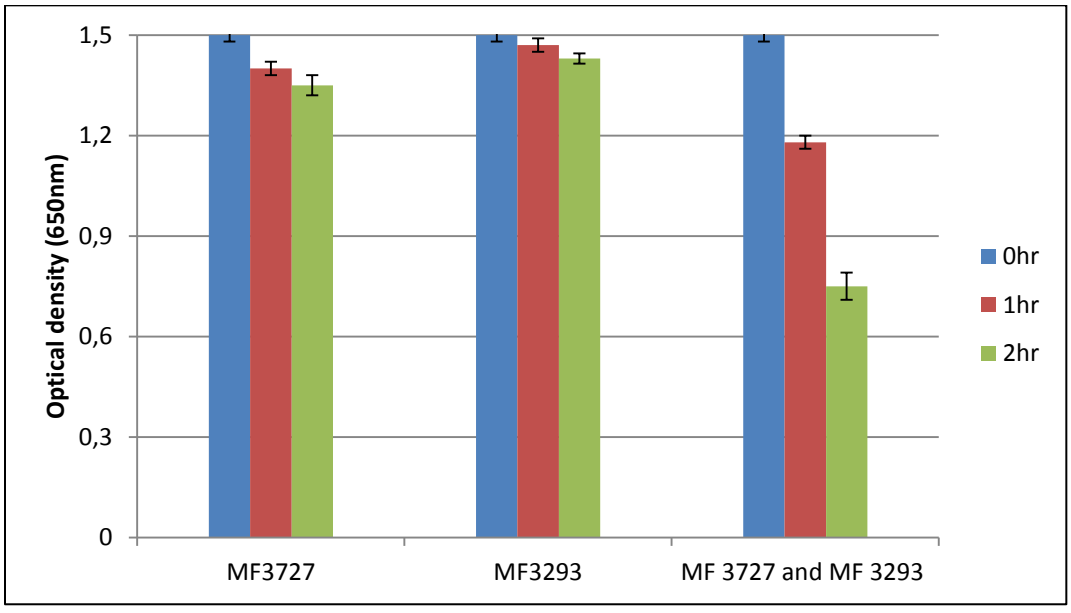


Figure 4.2. Decreased optical density level after incubation. From left, the first and second set of bars show the decrease of optical density after 0, 1 and 2 hours, due to auto-aggregation of *Rhodococcus erythropolis* MF3727and *Acinetobacter calcoaceticus* MF3293, respectively. The last set of bars shows the decrease of optical density due to co-aggregation between these two strains. All strains were cultivated in TSB at 30^{o}C for 18hr. Error bars represent standard deviations from three independent replicates.

4.1.3 Scanning electron microscopy (SEM)

The aim of this experiment was to study the cell surfaces of *Rhodococcus erythropolis* MF3727 and *Acinetobacter calcoaceticus* MF3293 in single culture and during co-aggregation. It was also a purpose to observe structure and size of co-aggregates. The cells appeared as single or pairs of cells in individual suspension (Fig. 4.3), but in the mixed suspension large co-aggregates of cells are seen (Fig. 4.4). *Acinetobacter calcoaceticus* MF3293 possess surface appendages, and those seem to be connecting some of the cells of the co-aggregates to each other (Fig. 4.3B, 4.4B-D).

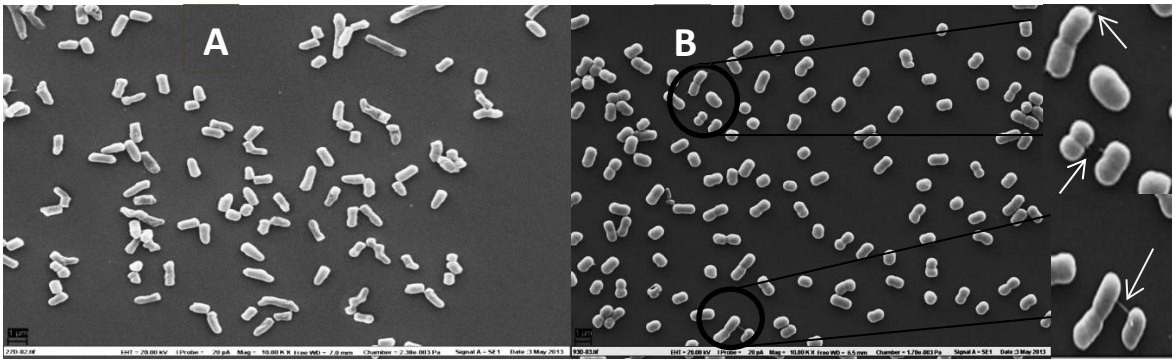


Fig 4.3 Scanning electron microscopy of the cells in single culture. A: *Rhodococcus erythropolis* MF3727 B: *Acinetobacter calcoaceticus* MF3293. The arrows show appendages on the cell surface of *Acinetobacter calcoaceticus* MF3293. The magnifications in both cases are 10.00 KX. (Photo: Elin Ørmen, Norwegian University of Life Sciences)

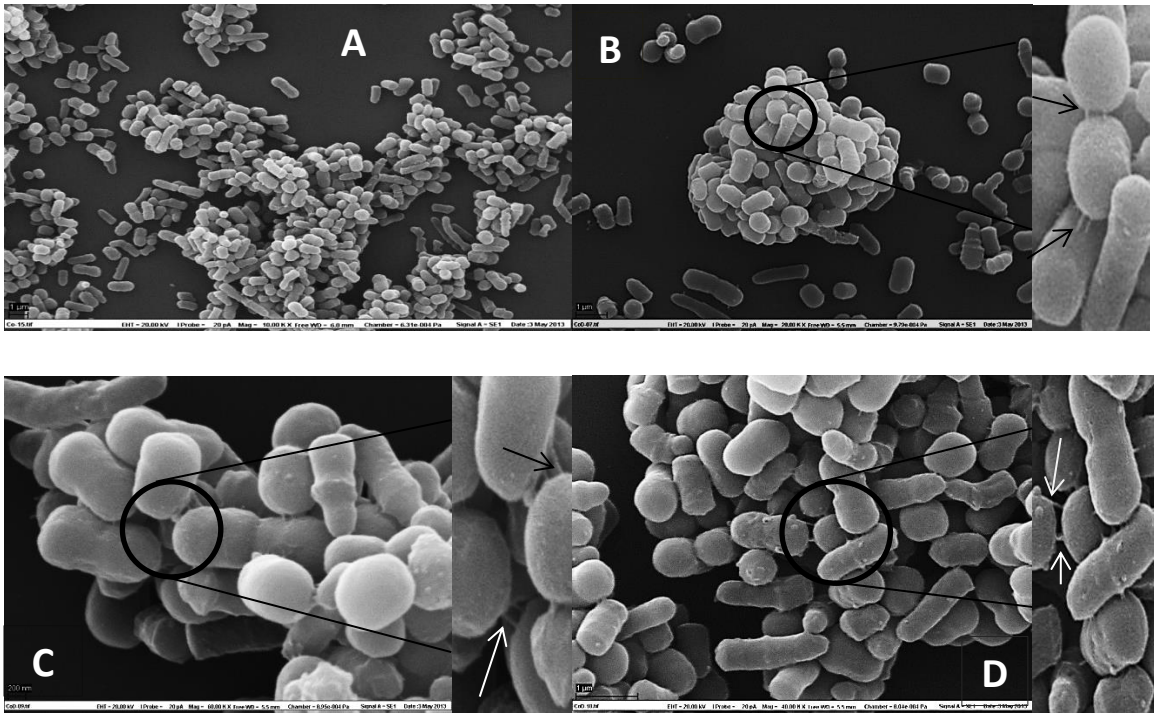


Fig 4.4 Scanning electron microscopy of the co-aggregates belongs to *Rhodococcus erythropolis* MF3727 + *Acinetobacter calcoaceticus* MF3293 with different magnifications (mag). A: 10.00 KX mag. B: 20.00 KX mag. C: 60.00 KX mag. D: 40.00 KX mag. The arrows show appendages on the cell surface of *Acinetobacter calcoaceticus* MF3293. (Photo: Elin Ørmen, Norwegian University of Life Sciences).

4.2 The effect of culturing conditions on the co-aggregation capability of *Rhodococcus erythropolis* MF3727 and *Acinetobacter calcoaceticus* MF3293

The effect of growth medium (R2A and TSB), growth temperature (20^{o} C, 30^{o} C) and growth phase of *Rhodococcus erythropolis* MF3727 and *Acinetobacter calcoaceticus* MF3293 on co-aggregation was tested. After cultivating the strains in different conditions, bacterial cells were harvested after different incubation times.

In order to prepare the suspensions for visual and optical density test, the earlier mentioned preparation method (3.3.1) was used. Afterwards the *Rhodococcus erythropolis* MF3727 and *Acinetobacter calcoaceticus* MF3293 which grew in same common temperature and growth medium but were harvested after different times were tested for co-aggregation in the form of 36

possible pairwise combinations of strains. All of these pairs were tested by using the visual co-aggregation and the optical density assays.

The results of visual co-aggregation method for all possible pairs in this study showed that both MF3727 and MF3293 growing in R2A and TSB demonstrated varying capability for co-aggregation depending on their corresponding temperatures and growth phases. However, the strongest co-aggregation was seen among bacteria grown in TSB. 50% of the tested co-aggregation pairs of TSB-grown cells achieved the maximum visual score of 4, but only 11% of those pairs which grew in R2A reached to score of 4 (Results from TSB is presented in fig 4.5 – 4.10, while results for R2A in Appendix fig 9.1 – 9.6). Higher co-aggregation among TSB grown cells compared to R2A grown cells was confirmed with the optical density assay.

Considering the effect of different growth temperatures on co-aggregation, it was observed that the growth temperature played an important role in co-aggregation ability of MF3727 and MF3293. Pairs grown at 30^{o}C had higher co-aggregation ability in comparison with those grown at 20^{o}C. Based on visual co-aggregation method, 66% of the pairs which grew in TSB at 30^{o}C achieved the score of 4 (Fig. 4.6), but only 33% of those pairs which grew in TSB at 20^{o}C reached to score of 4 (Fig. 4.9). In the case of R2A, only 22% of pairs grew at 30^{o}C reached the score of 4 (Appendix, fig 9.2), While none of pairs grown at 20^{o}C could achieve more than score of 3 (Appendix, fig. 9.5).

The results obtained from optical density method showed that the highest co-aggregation percentage for pairs grown in TSB at 30^{o}C was equal to 50 % (Fig. 4.7). For the pairs which grew in TSB at 20^{o}C, the highest co-aggregation percentage was equal to 46% (Fig. 4.10). Moreover, the results also showed that the highest co-aggregation percentage for pairs grew in R2A at 30^{o}C was equal to 38 % (Appendix, fig. 9.3), and the highest recorded percentage for pairs grew at 20^{o}C was equal to 37% (Appendix, fig. 9.6).

The maximum co-aggregation ability was related to cells that were harvested in exponential growth phase and in the early stationary phase. It is important to mention that the highest level of this effect was among cells that were grown in TSB. By entering mid stationary phase, co-aggregation ability of the cells decreased significantly. The MF3727 cells, which were harvested after 42 hours, had less co-aggregation ability than the MF3293 cells that were harvested at the

same time. Based on the optical density method the maximum recorded co-aggregation percentage was observed for a pair where both MF3727 and MF3293 were grown in TSB at 30^{o}C and harvested in early stationary phase (Fig. 4.7).

After counting the number of viable bacterial cells in different growth phases, it was found that the highest co-aggregation percentage and the highest visual score recorded were at concentration levels of 10^{7}-10^{9} per ml. This number was between 10^{7}-10^{8} per ml for *Rhodococcus erythropolis* MF3727, and 10^{8}-10^{9} per ml for *Acinetobacter calcoaceticus* MF3293. Among all of the results acquired from this experiment, the highest score in visual test and the highest co-aggregation percentage were recorded for a pair in which both of participating strains grew in TSB at 30^{o}C and were harvested after 18hr (Fig 4.6 and 4.7). These conditions were used as the standard growth conditions in the rest of the study.

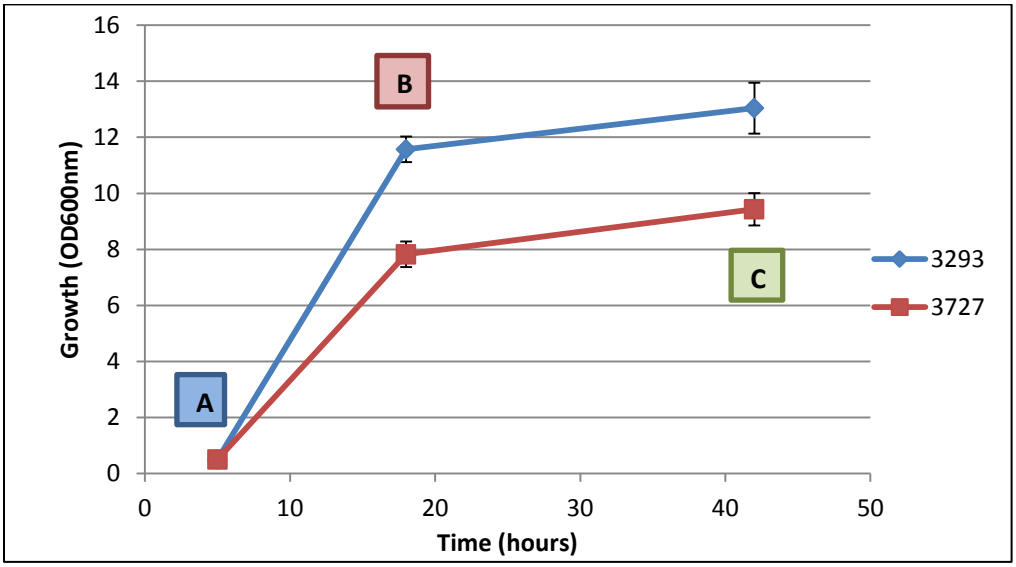


Figure 4.5 Growth curves of *Rhodococcus erythropolis* MF3727 and *Acinetobacter calcoaceticus* MF3293 in TSB medium at 30^{o} C. Letters indicate harvesting of cultures for test of co-aggregation. Error bars represent standard deviations from three independent replicates.

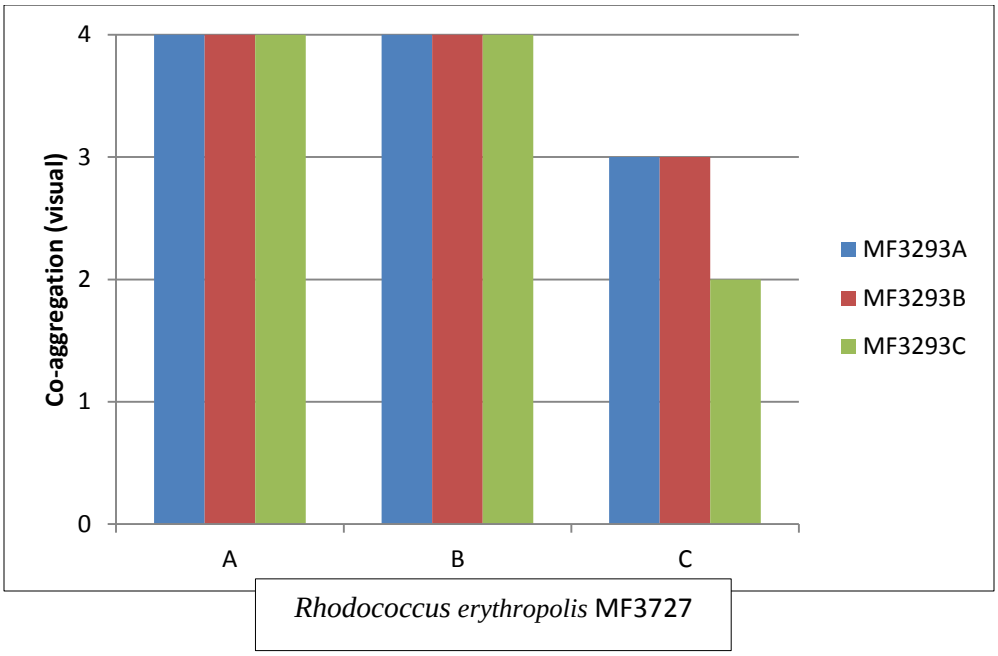


Figure 4.6 Co-aggregation (visual score) between *Rhodococcus erythropolis* MF3727 and *Acinetobacter calcoaceticus* MF3293 sampled from cultures in TSB medium at 30° C in different growth phase. A; OD600nm=0.5, B: 18hr., C: 42 hr. The same results were obtained from three independent replicates.

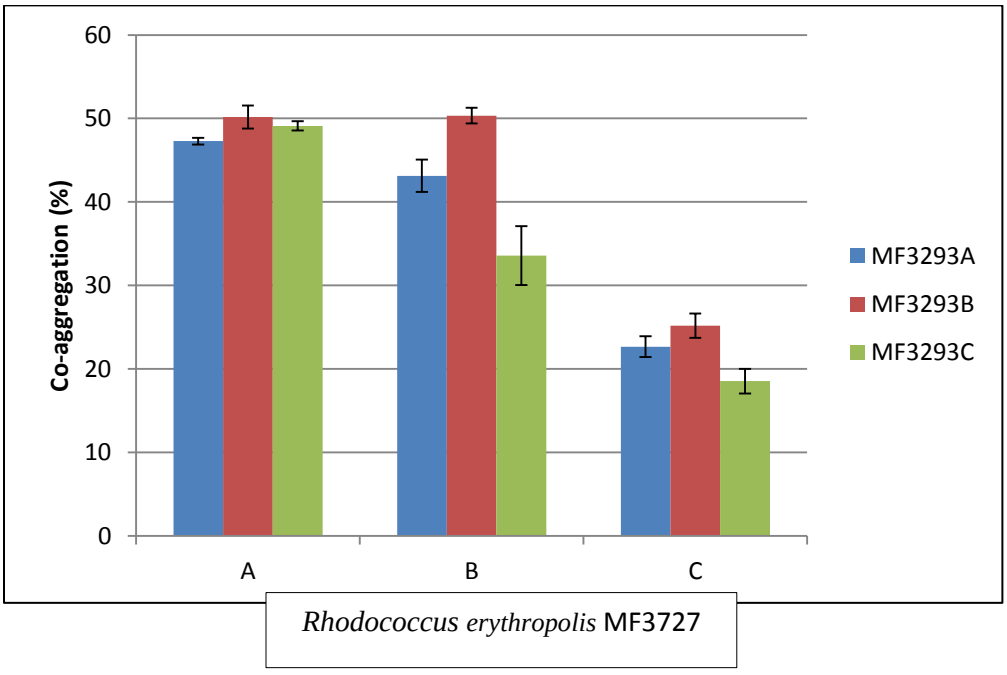


Figure 4.7 Co-aggregation (percentage) between *Rhodococcus erythropolis* MF3727 and *Acinetobacter calcoaceticus* MF3293 sampled from cultures in TSB medium at 30° C in different growth phase. A; OD600nm=0.5, B: 18hr., C: 42 hr. Error bars represent standard deviations from three independent replicates.

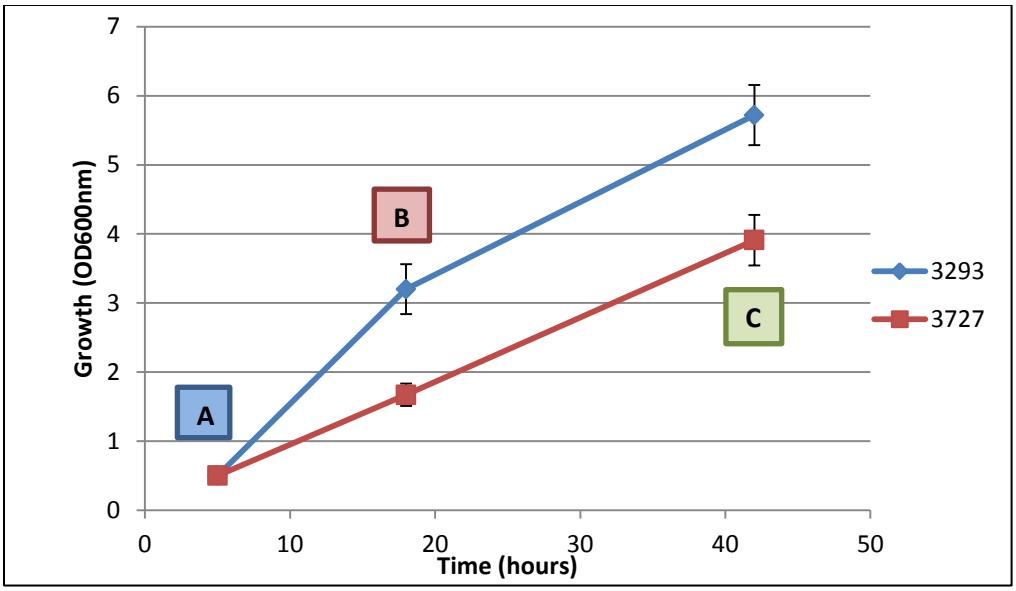


Figure 4.8 Growth curves of *Rhodococcus erythropolis* MF3727 and *Acinetobacter calcoaceticus* MF3293 in TSB medium at 20° C. Letters indicate harvesting of cultures for test of co-aggregation. Error bars represent standard deviations from three independent replicates.

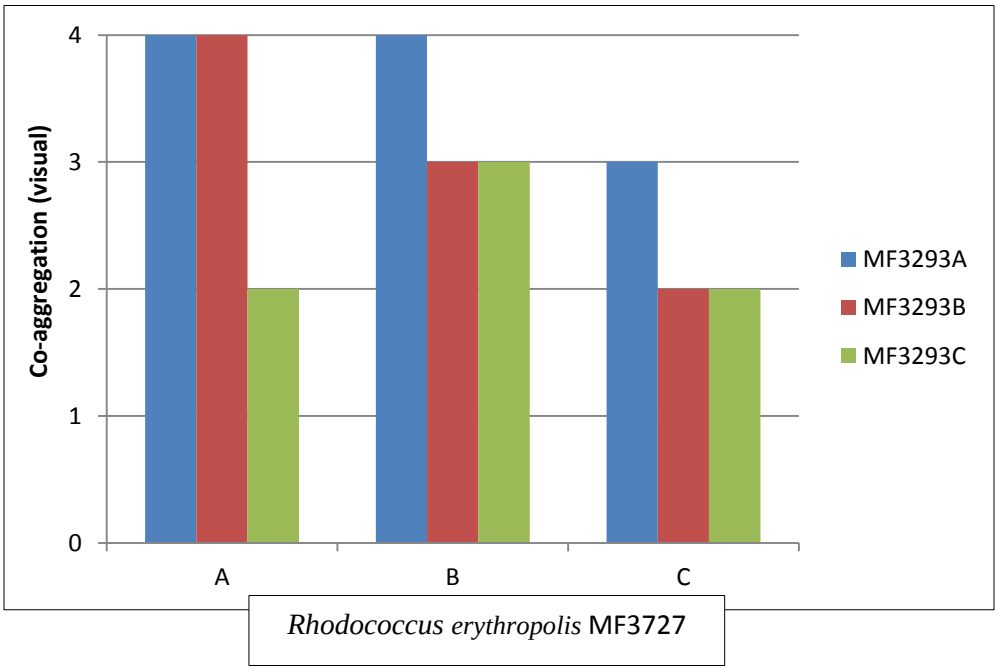


Figure 4.9 Co-aggregation (visual score) between *Rhodococcus erythropolis* MF3727 and *Acinetobacter calcoaceticus* MF3293 sampled from cultures in TSB medium at 20° C in different growth phase. A; OD600nm=0.5, B: 18hr., C: 42 hr. The same results were obtained from three independent replicates.

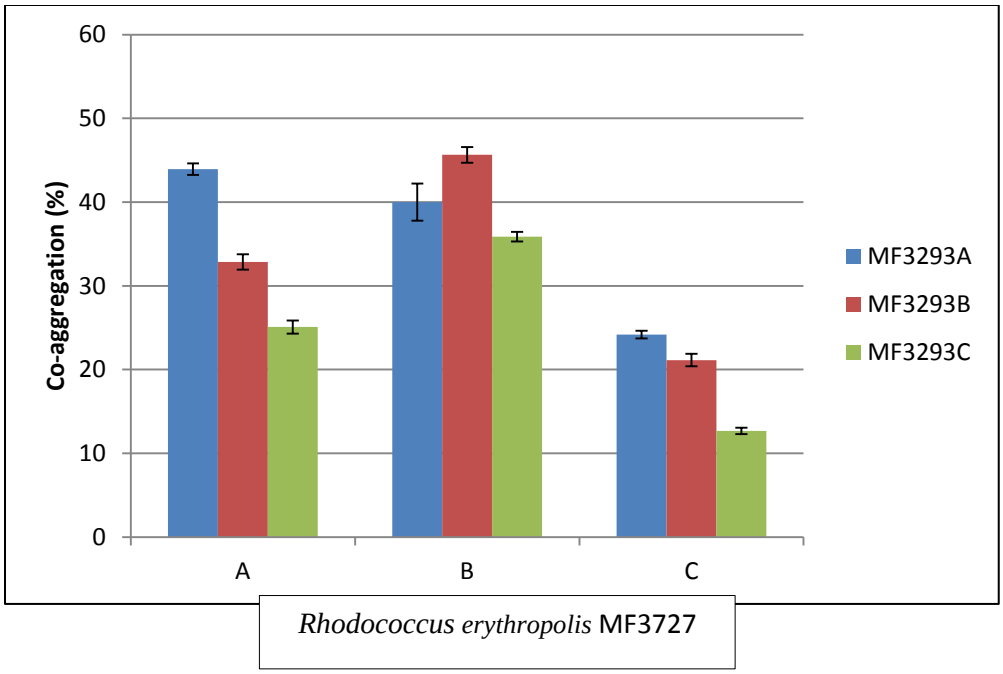


Figure 4.10 Co-aggregation (percentage) between *Rhodococcus erythropolis* MF3727 and *Acinetobacter calcoaceticus* MF3293 sampled from cultures in TSB medium at 20^{o} C in different growth phase. A; OD600nm=0.5, B: 18hr., C: 42 hr. Error bars represent standard deviations from three independent replicates.

4.3 The effect of washing and re-suspension solution on co-aggregation

In order to determine the impact of buffers and solutions on co-aggregation, *Rhodococcus erythropolis* MF3727 and *Acinetobacter calcoaceticus* MF3293 cells were first cultivated at standard condition (TSB,30^{o}C, 18hr). Then the cells were harvested and washed with de-ionized H_2O (dH_2O). Later the cells were re-suspended in different buffers (Table 3.2). Finally, cells which were re-suspended in same common buffers were tested as co-aggregating pairs.

The highest co-aggregation capability was reached when pairs were re-suspended in 0.85% NaCl or co-aggregation buffer, both with a score of 4 according to the visual method. Regarding to the optical method, pairs that were re-suspended in co-aggregation buffer generated slightly higher co-aggregation percentage than pairs re-suspended in 0.85% NaCl (Fig. 4.11).

Less co-aggregation was observed when the cells were re-suspended in dH_2O. When cells were re-suspended in R2A and TSB, the calculation of co-aggregation percentage with the optical density method was not possible, since both supported growth of MF3727 and MF3293, and considerable increase in optical density was observed after 2hr. Hence, the visual assay was the only method that was employed to study co-aggregation of cells re-suspended in TSB and R2A. Pairs which were re-suspended in R2A had a visual score of 3, but those which were re-suspended in TSB demonstrated lower level of capability and had a visual score of 2 (Fig. 4.11).

In addition to testing the effect of the final re-suspension solution on co-aggregation, the effect of the washing solution prior to the last re-suspension were also tested. Two samples of each strain were separately cultivated and then harvested (TSB, 30^o C, 18hr). The cells were washed three times with sterile dH_2O and co-aggregation buffer, respectively. Finally both the samples were re-suspended in co-aggregation buffer and the suspensions were prepared for visual co-aggregation and optical density assay. The results showed no difference between co-aggregation ability of the two different processed samples. Based on the results, further testing of co-aggregation was tested with bacteria washed three times with sterile de-ionized water and re-suspended in co-aggregation buffer (standard conditions).

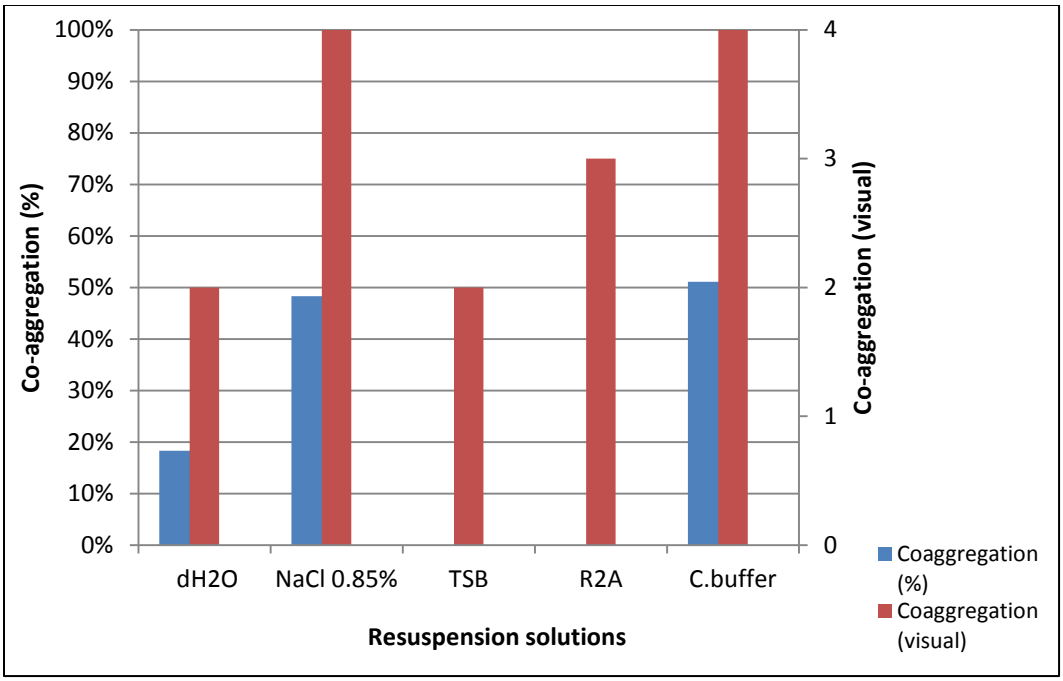


Fig 4.11. Effect of different re-suspension solutions on co-aggregation between *Rhodococcus erythropolis* MF3727 and *Acinetobacter calcoaceticus* MF3293. In the case of cells which were re-suspended in R2A and TSB, the calculation of co-aggregation percentage with the optical density was not possible due to turbidity caused by growth. The standard deviations for co-aggregation percentages are: dH_2O (1.765), 0.85% NaCl (0.425), co-aggregation buffer (0.455). The standard deviations are based on three independent replicates. In the case of visual assay, the same results were obtained from three independent replicates.

4.4 Co-aggregation between different strains of *Rhodococcus* sp. and *Acinetobacter* sp.

After observation of co-aggregation between *Rhodococcus erythropolis* MF3727 and *Acinetobacter calcoaceticus* MF3293, and the establishment of method to study co-aggregation, the possibility of co-aggregation between other strains of *Rhodococcus* sp. and *Acinetobacter* sp. was investigated. Three strains of *Rhodococcus* sp. and eight strains of *Acinetobacter* sp., isolated from various food production environments, were tested (Table 3.3).

The condition used were Standard conditions, which in this thesis refer to cultivation in TSB, 30^{o}C, 18hr, followed by washing of cells in dH_2O and re-suspension in co-aggregation buffer for preparation for visual co-aggregation and optical density test. All possible 55 combinations pairs were tested. In total, co-aggregation (visual score $\geq$ 1) was observed for three different pairs of strains, including the already studied pair of *Rhodococcus erythropolis* MF3727 and

Acinetobacter calcoaceticus MF3293. The results showed that the ability to form co-aggregates was strain-specific as only two strains of *Rhodococcus erythropolis* (MF3727 and MF3803) and two strains of *Acinetobacter calcoaceticus* (MF3293 and MF3627) were involved in formation of co-aggregates.

According to the results showed in table 4.1, *Rhodococcus erythropolis* MF3727 was involved in formation of all three scenarios. This strain had the ability to form co-aggregates with two *Acinetobacter calcoaceticus* (MF3293 and MF3627) as well as another *Rhodococcus erythropolis* (MF3803). All the strains were individually examined with both visual and optical test (more details in appendix, tables 9.5 and 9.6). No auto-aggregation was observed for any of the strains. The mechanisms behind co-aggregation were studied for all the three co-aggregating pairs mentioned in table 4.1.

Table 4.1 Co-aggregation of different strains of *Rhodococcus* sp. and *Acinetobacter* sp.
All strains were cultivated at standard conditions (TSB, 30^{o}C, 18hr) and re-suspended in co-aggregation buffer prior to the test. The standard deviations (in parentheses) are based on three independent replicates. In the case of visual assay, the same results were obtained from three independent replicates.

Pairs*	% Co-aggregation**	Visual score***
R 3727 + A 3293	50.2 (1.57)	4
R 3727 + A 3627	15.2 (1.22)	3
R 3727 + R 3803	9.7 (0.96)	2

*R; *Rhodococcus*, A; *Acinetobacter*. **Co-aggregation percentage based on decrease in $OD650_{nm}$. ***The scores are based on visual co-aggregation score scheme.

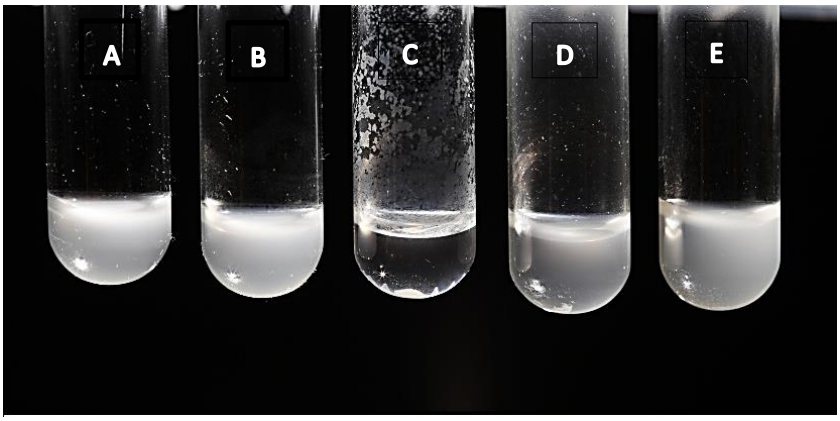


Fig 4.12 Co-aggregation (visual) of different strains of *Rhodococcus* and *Acinetobacter* after 2hr incubation. A: *Rhodococcus erythropolis* MF3727 in single suspension. B: *Acinetobacter calcoaceticus* MF3293 in single suspension. C: *Rhodococcus erythropolis* MF3727 + *Acinetobacter calcoaceticus* MF3293. D: *Rhodococcus erythropolis* MF3727 + *Acinetobacter calcoaceticus* MF3627. E: *Rhodococcus erythropolis* MF3727 + *Rhodococcus erythropolis* MF3803. All strains were cultivated at standard conditions (TSB, 30^{o}C, 18hr) and re-suspended in co-aggregation buffer prior to the test. (Photo: Kjell Merok, Nofima).

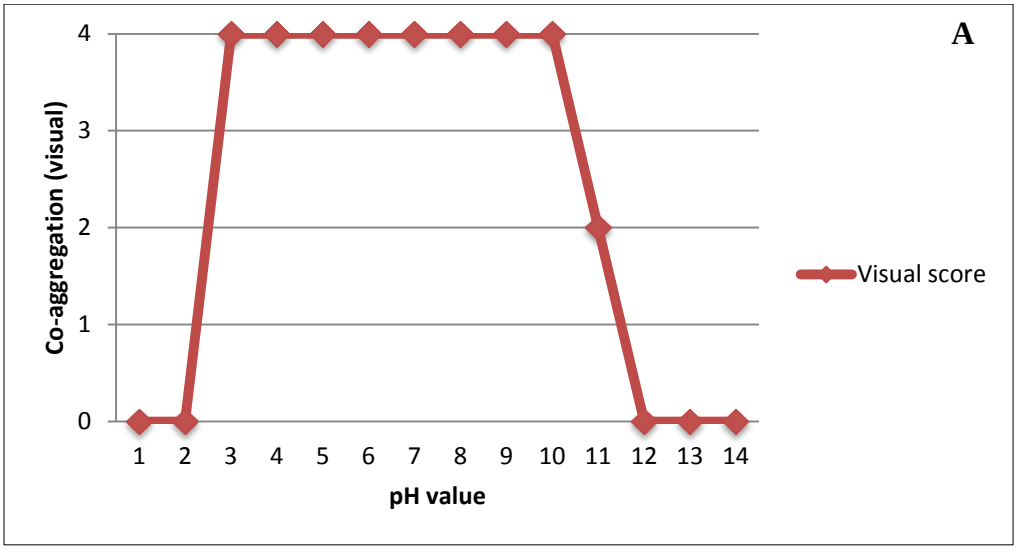
4.5 Mechanism behind co-aggregation of *Rhodococcus* and *Acinetobacter*

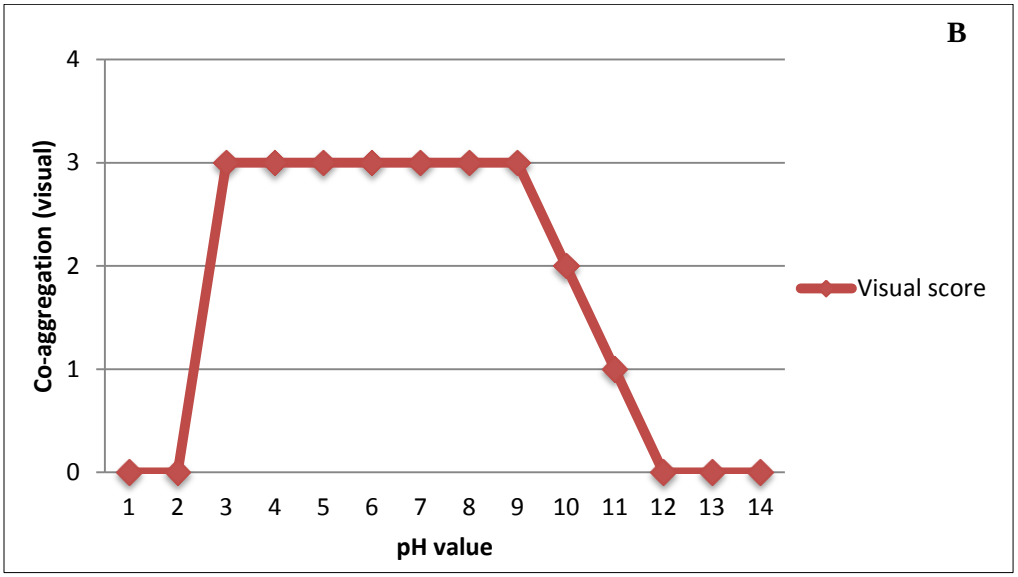
4.5.1 Effect of pH on co-aggregation

To determine the effect of different pH on co-aggregation, cells of *Rhodococcus erythropolis* MF3727, *Rhodococcus erythropolis* MF3803, *Acinetobacter calcoaceticus* MF3293 and *Acinetobacter calcoaceticus* MF3627 was cultivated and harvested (standard conditions). Then 14 cell suspension tubes with OD_{650}=1.5 were prepared for each of co-aggregation strains (standard conditions). Later the pH of suspensions was regulated from 1 to 14. Finally the co-aggregation ability of each pair was evaluated based on visual co-aggregation method. The result showed that all pairs in the pH range of 3 to 11 were able to co-aggregate. On the other hand the cells that were tested out of this range were lysed and no co-aggregation occurred (Fig. 4.13).

Based on the visual assay, it was observed that MF3727 + MF3293 showed highest co-aggregation ability in the range of pH 4 to 10 (Fig. 4.13 A), while for MF3727 + MF3627 the visual score for all pairs in the pH range of 3 to 9 was equal to 3. In the range of 10 to 11 the co-aggregation ability decreased (Fig. 4.13 B). For pair of MF3727 + MF3803 an unexpected increased of co-aggregation ability was observed at the pH of 3 and 4 (Fig. 4.13 C).

The results of optical density method also showed that MF3727 + MF3803 obtained the highest percentage of co-aggregation at pH of 3 and 4. With increase in pH rate, the co-aggregation percentage of the pair gradually decreased. On the other hands, MF3727 + MF3293 and MF3727 + MF3627 showed a wider optimum pH range (Table 4.2).





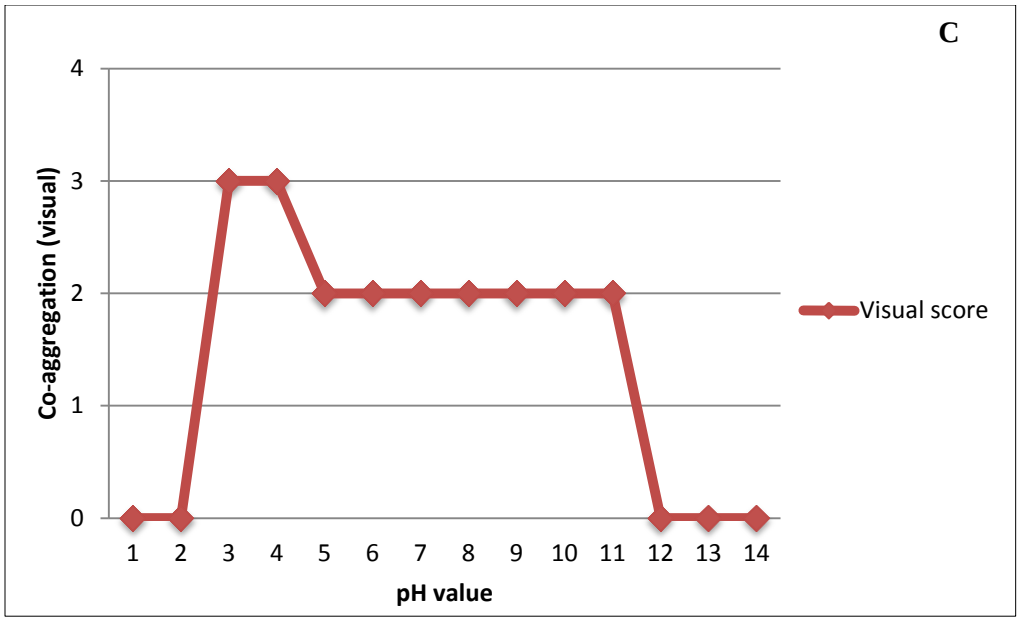


Fig 4.13 Effect of pH on co-aggregation. A: *Rhodococcus erythropolis* MF3727 + *Acinetobacter calcoaceticus* MF3293. B: *Rhodococcus erythropolis* MF3727 + *Acinetobacter calcoaceticus* MF3627. C: *Rhodococcus erythropolis* MF3727 and *Rhodococcus erythropolis* MF3803. All strains were cultivated at standard condition (TSB, 30^{o}C, 18hr). The same results were obtained from three independent replicates.

Table 4.2 The percentage of co-aggregation between *Rhodococcus* and *Acinetobacter* pairs in different pH values. The standard deviations (SD) are based on three independent replicates.

Pairs	pH 3	SD	pH 4	SD	pH 5	SD
MF3727+MF3293	57.3	2.83	55.5	1.60	50.2	2.34
MF3727+MF3627	22.0	2.39	20.4	1.27	14.7	1.40
MF3727+MF3803	21.3	1.95	19.1	1.51	10.8	1.44
Pairs	pH 6	SD	pH 7	SD	pH 8	SD
MF3727+MF3293	49.5	1.40	48.0	1.76	50.2	1.41
MF3727+MF3627	14.2	0.96	14.5	0.70	15.1	0.92
MF3727+MF3803	10.2	1.30	10.4	0.75	9.7	0.70
Pairs	pH 9	SD	pH 10	SD	pH 11	SD
MF3727+MF3293	49.5	1.54	47.1	1.30	21.7	1.32
MF3727+MF3627	13.1	0.72	8.2	0.96	4.6	0.90
MF3727+MF3803	9.7	1.50	8.2	1.11	7.5	0.61

4.5.2 Effect of heat treatment on co-aggregation

To test the effect of heat treatment on the co-aggregation ability of *Rhodococcus erythropolis* (MF3727and MF3803) and *Acinetobacter calcoaceticus* (MF3293and MF3627), cell suspensions of the individual cells were heat treated at 85^{o}C for 30 min. Then they were left at room temperature to cool down.

Later heat treated and untreated bacterial cells were combined in pairs. According to visual co-aggregation results, the heat treatment had no effect on the ability of *Rhodococcus erythropolis* MF3727 to co-aggregate with other member of the pairs. However, heat treatment of the other three strains resulted in complete loss of co-aggregation ability (Table 4.3). The results achieved from the optical density assay are shown in appendix (tables 9.7 – 9.9).

Table 4.3. Effect of heat treatment on co-aggregation. The table shows results for when each partner was pretreated separately with heat (85^{o}C for 30 min). The same results were obtained from three independent replicates.

Co-aggregation score for bacterium with indicated partner type*							
Strains		MF3293		MF3627		MF3803	
		UT	T	UT	T	UT	T
MF 3727	UT	4	0	3	0	2	0
	T	4	0	3	0	2	0

* The scores are based on visual co-aggregation score scheme. T: heat treated; UT: Untreated, no heat treatment.

4.5.3 Effect of sugars on co-aggregation

If a specific sugar is involved in the co-aggregation binding, addition of this sugar to the co-aggregation suspension may lead to a decrease in co-aggregation. The ability of sugars to reverse or inhibit the co-aggregation between *Rhodococcus erythropolis* MF3727 + *Acinetobacter calcoaceticus* MF3293, *Rhodococcus erythropolis* MF3727 + *Acinetobacter calcoaceticus* MF3627,and *Rhodococcus erythropolis* MF3727+ *Rhodococcus erythropolis* MF3803 was determined by adding various simple sugars to the pairs (Table 3.4).

Based on visual co-aggregation method results, only N-acetyl-D-galactosamine was able to decrease co-aggregation ability of MF3727 + MF3627. The achieved score for this pair was 2,

which was one unit less than the score of control pair. On the other hand other sugar variants showed no effect on reversing or inhibiting the co-aggregation between pairs (Table 4.4 – 4.6)

Furthermore, the results from optical density method provided more details. For instance adding N-acetyl-D-galactosamine could decrease the co-aggregation of MF3727 + MF3627 by 46%. Despite the results from other sugars in visual co-aggregation method, the optical density method showed that Lactose monohydrate could inhibit co-aggregation ability for MF3727 + MF3293 by 17% (Table 4.4) and 15% for MF3727 + MF3803 (Table 4.6). In the remaining cases the registered inhibition was less than 10%.

Table 4.4 Inhibition of co-aggregation between *Rhodococcus erythropolis* MF3727 and *Acinetobacter calcoaceticus* MF3293 caused by addition of sugars. Cells were cultivated at standard condition (TSB, 30^{o}C, 18hr). The standard deviations (in parentheses) are based on three independent replicates. In the case of visual assay, the same results were obtained from three independent replicates.

Sugars*	%Inhibition**	Visual score***
Lactose monohydrate	17.0 (1.82)	4
D (+) galactose	1.4 (0.44)	4
α-L-fucose	0.0	4
N-acetyl-D-galactosamine	7.2 (0.56)	4
D (+) glucose	1.4 (0.42)	4
D-mannose	2.8 (0.26)	4
Control		4

*All sugars were tested at a final concentration of 50mM. **Co-aggregation percentage was determined based on decrease in $OD650_{nm}$. ***The scores are based on visual co-aggregation score scheme.

Table 4.5 Inhibition of co-aggregation between *Rhodococcus erythropolis* MF3727 and *Acinetobacter calcoaceticus* MF3627 caused by addition of sugars. Cells were cultivated at standard condition (TSB, 30^{o}C, 18hr). The standard deviations (in parentheses) are based on three independent replicates. In the case of visual assay, the same results were obtained from three independent replicates.

Sugars*	%Inhibition**	Visual score***
Lactose monohydrate	4.8 (0.44)	3
D (+) galactose	9.7 (0.78)	3
α-L-fucose	0.9 (0.20)	3
N-acetyl-D-galactosamine	46.1 (0.92)	2
D (+) glucose	4.5 (0.89)	3
D-mannose	9.0 (0.98)	3
Control		3

*All sugars were tested at a final concentration of 50mM. **Co-aggregation percentage was determined based on decrease in $OD650_{nm}$. ***The scores are based on visual co-aggregation score scheme.

Table 4.6 Inhibition of co-aggregation between *Rhodococcus erythropolis* MF3727 and *Rhodococcus erythropolis* MF3803 caused by addition of sugars. Cells were cultivated at standard condition (TSB, 30°C, 18hr). The standard deviations (in parentheses) are based on three independent replicates. In the case of visual assay, the same results were obtained from three independent replicates.

Sugars*	%Inhibition**	Visual score***
Lactose monohydrate	15.0 (0.70)	2
D (+) galactose	6.8 (0.78)	2
α-L-fucose	0.0	2
N-acetyl-D-galactosamine	7.3 (1.15)	2
D (+) glucose	6.8 (0.56)	2
D-mannose	7.4 (0.92)	2
Control		2

*All sugars were tested at a final concentration of 50mM. **Co-aggregation percentage was determined based on decrease in $OD650_{nm}$. ***The scores are based on visual co-aggregation score scheme.

4.5.4 Effect of chelating agents and surfactants on co-aggregation

To determine reverse and inhibit of co-aggregation ability by chelating agents, cells of *Rhodococcus erythropolis* (MF3727 and MF3803) and *Acinetobacter calcoaceticus* (MF3293 and MF3627) was cultivated and two cell suspensions of each congregating pairs were provided for each chelating agents and surfactants (standard condition).

Chelating agents and surfactants with specified final concentration was added to one of the cell suspensions (Table 3.5), while the other one only contained the same volume of sterile de-ionized water. The co-aggregation ability of the pairs was evaluated by visual co-aggregation and optical density method.

The visual results showed that SDS could completely inhibit co-aggregation between all pairs so that all visual scores were 0. Tween 80 completely inhibited co-aggregation of MF3727 +MF3627 and MF3727 + MF3803 (These pairs reached visual score of 0). Furthermore it was observed that Citrate limited the co-aggregation ability between MF3727+ MF3293 in some extent, but had no effect on co-aggregation between the other pairs. On the other hand EDTA and EGTA did not inhibit co-aggregation between any of the pairs. The optical density method confirmed the results from the visual method (Table 4.7 – 4.9).

Table 4.7 Inhibition of co-aggregation between *Rhodococcus erythropolis* (MF3727) and *Acinetobacter calcoaceticus* (MF3293) by chelating agents and surfactants. Cells were cultivated at standard condition (TSB, 30°C, 18hr). The standard deviations (in parentheses) are based on three independent replicates. In the case of visual assay, the same results were obtained from three independent replicates.

Sugars	Final concentration	%Inhibition *	Visual score**
EDTA	50mM	17.7 (0.56)	4
EGTA	50mM	12.9 (0.79)	4
Citrate	5.1mM	46.8 (1.48)	3
Tween 80	0.2%	25.9 (0.78)	4
SDS	1.0%	96.3 (1.25)	0
Control		0.0	4

*Co-aggregation percentage was determined based on decrease in $OD650_{nm}$. **The scores are based on visual co-aggregation score scheme.

Table 4.8 Inhibition of co-aggregation between *Rhodococcus erythropolis* (MF3727) and *Acinetobacter calcoaceticus* (MF3627) by chelating agents and surfactants. Cells were cultivated at standard condition (TSB, 30°C, 18hr). Cells were cultivated at standard condition (TSB, 30°C, 18hr). The standard deviations (in parentheses) are based on three independent replicates. In the case of visual assay, the same results were obtained from three independent replicates.

Sugars	Final concentration	%Inhibition *	Visual score**
EDTA	50mM	9.7 (0.92)	3
EGTA	50mM	4.5 (0.53)	3
Citrate	5.1mM	14.6 (0.70)	3
Tween 80	0.2%	92.3 (1.21)	0
SDS	1.0%	97.6 (0.90)	0
Control		0.0	3

*Co-aggregation percentage was determined based on decrease in $OD650_{nm}$. **The scores are based on visual co-aggregation score scheme.

Table 4.9 Inhibition of co-aggregation between *Rhodococcus erythropolis* (MF3727) and *Rhodococcus erythropolis* (MF3803) by chelating agents and surfactants. Cells were cultivated at standard condition (TSB, 30°C, 18hr). The standard deviations (in parentheses) are based on three independent replicates. In the case of visual assay, the same results were obtained from three independent replicates.

Sugars	Final concentration	%Inhibition *	Visual score**
EDTA	50mM	8.3 (0.96)	2
EGTA	50mM	9.8 (0.82)	2
Citrate	5.1mM	20.7 (1.08)	2
Tween 80	0.2%	96.4 (0.87)	0
SDS	1.0%	96.5 (0.26)	0
Control		0.0	2

*Co-aggregation percentage was determined based on decrease in $OD650_{nm}$. **The scores are based on visual co-aggregation score scheme.

4.5.5 The effect of enzymatic treatment of cells on co-aggregation

As explained in section 3.7.5, this experiment was designed to investigate the effect of different enzymes on co-aggregation ability of *Rhodococcus erythropolis* (MF3727 and MF3803) and *Acinetobacter calcoaceticus* (MF3293 and MF3627). To control the effect of enzymes, two suspensions of each co-aggregation strains were prepared. Later, enzyme treated and untreated (control) strains were combined in pairs. Finally the cells ability to co-aggregate was assessed by the visual co-aggregation and optical density assay.

The visual results showed that Proteinase K significantly decrease the co-aggregation ability of *Acinetobacter calcoaceticus* (MF3627and MF3293) and *Rhodococcus erythropolis* MF3803. On the other hand this enzyme had no effect on *Rhodococcus erythropolis* MF3727ability to co-aggregate with other strains which participated in co-aggregation. Dispersin B and Dnase I had no effect on co-aggregation of any of the strains (Table 4.10).

The result from the optical density method confirmed the visual results. For instance, co-aggregation percentage of MF3727 + MF3293 dropped significantly, when only MF3293 was proteinase K treated. On the other hand, treating of MF3727 had no effect and the co-aggregation percentage (47%) was similar to the co-aggregation percentage of untreated (control) suspension of the same co-aggregating strains (more details in appendix, tables 9.7 – 9.9).

TABLE 4.10 Effect of Proteinase K on co-aggregation between *Rhodococcus erythropolis* MF3727 and *Acinetobacter calcoaceticus* MF3293, *Acinetobacter calcoaceticus* MF3627 and *Rhodococcus erythropolis* MF3803. All cells were cultivated at standard condition (TSB, 30^{o}C, 18hr). The same results were obtained from three independent replicates.

Visual Co-aggregation score for bacterium with indicated partner type**							
Strains*		3293		3627		3803	
		UT	T	UT	T	UT	T
3727	UT	4	1	3	1	2	0
	T	4	1	3	1	2	0

*The table shows results for when each partner was pretreated separately with Proteinase K. The partners were mixed with either an untreated (UT) or a treated (T) partner. **The scores are based on visual co-aggregation score scheme.

4.6 Test of co-aggregation among other bacterial strains from food production environment

To investigate the prevalence of co-aggregation between other bacteria than *Rhodococcus* and *Acinetobacter* from food production environments, 78 bacterial strains were chosen to be used in this work. These strains could be categorized in four different groups based on origin of the strains (Appendix, tables 9.1 – 9.4). Strains in each category were only tested against the strains in the same category. Bacterial strains were cultivated and the required suspensions for testing co-aggregation were prepared (standard conditions). Later total of 466 combination pairs were tested. These pairs were tested with both visual and optical density assay. However no co-aggregation was observed between any of the pairs. As shown in table 4.11, auto-aggregation was observed for *Variovorax* sp. MF4655, *Micrococcus* sp. MF4649, *Flavobacterium* sp. MF4107 and *Carnobacterium maltaromaticum* MF4109.

Table 4.11 Auto-aggregation among *Variovorax* sp. MF4655, *Micrococcus* sp. MF4649, *Flavobacterium* sp. MF4107 and *Carnobacterium maltaromaticum* MF4109. All strains were cultivated at standard condition (TSB, 30^{o}C, 18hr). The standard deviations (in parentheses) are based on three independent replicates. In the case of visual assay, the same results were obtained from three independent replicates.

Strains	%Auto-aggregation*	Visual score**
MF 4655	62.6 (1.04)	2
MF 4649	60.6 (1.31)	2
MF 4107	86.6 (1.13)	3
MF 4109	80.0 (1.01)	3

*Auto-aggregation percentage based on decrease in $OD650_{nm}$. **The scores are based on visual co-aggregation score scheme.

5. DISCUSSION

In this work, the co-aggregation ability of *Rhodococcus* and *Acinetobacter*, which were isolated from food production environments, were observed *in vitro*. To our knowledge, this is the first time co-aggregation among bacteria from food industry has been reported. Also we are not aware of reports about co-aggregation between *Acinetobacter* sp. and *Rhodococcus* sp., but the ability of *Acinetobacter* to co-aggregate with *Oligotropha carboxidovorans*, *Methylobacterium* sp. and *Staphylococcus* sp. has previously been reported (Malik & Kakii 2003; Simões et al. 2008) .

Co-aggregation occurs between two genetically distinct bacteria and could occur in a variety of ecosystems (Kolenbrander 2000). This mechanism was reported for the first time by Gibbons et al. (1970) between human oral bacteria and was called inter-bacterial aggregation. It was showed that co-aggregation is highly specific and generally occurs between adhesins on one cell type and receptors on the other cell type. Co-aggregation is usually observable by naked eye (Kolenbrander 1988). One could also measure the percentage of co-aggregation occurrence by spectrophotometry (Mcintire et al. 1978).

5.1 Methods to investigate co-aggregation

In this study, two different methods were employed to measure the co-aggregation ability, a semi quantitative visual assay (based on Cisar 1979) and a quantitative optical assay (based on McIntire 1978). As mentioned in the Materials and Methods section, to score visual co-aggregation, the Cisar et al. (1979) scheme was used. As scoring is based on visual observations the result of the visual test would be subjective. One of the critical points in studying co-aggregation was the amount of light and the light angle applied. After trying different lighting possibilities, the best pair observation result was achieved with back angled light. This is another subjective factor in visual tests. Using a magnifying lamp was also very useful to observe co-aggregation pairs.

Co-aggregation was not the only cell to cell interaction that was investigated by visual method in this study. All used strains were also separately tested for their auto-aggregation ability. Opposite of co-aggregation, the auto-aggregation is a cell to cell interaction that occurs between genetically identical strains. Investigating auto-aggregation was useful due to preventing the

false scoring for positive co-aggregation. This was also done for the optical test and was used in the equation to calculate pure co-aggregation percentage (3.3.3). Compared to the visual test, the optical test was much more sensitive in detecting both co aggregation and auto-aggregation. These results agreed with previous works in this area, which approved the sensitivity of the optical density method (Buswell & Herlihy 1997; Shen et al. 2005). As the visual test is fast, simple, and accurate enough for detecting major interactions (compatible with optical density), it was used side by side with the optical density method in this study.

5.2 Effect of environmental conditions on co-aggregation of *Rhodococcus erythropolis* MF3727 and *Acinetobacter calcoaceticus* MF3293

To examine the effect of physical and chemical factors on co-aggregation between *Rhodococcus* and *Acinetobacter*, the effect of growth medium, growth temperature and growth phase was tested. As different experiments were tested in this thesis, reaching a standard condition to use in further experiments was important. This was reached by investigating these three factors. Food production plants, based on their products, could contain blood, meat, and carbohydrates. This would prepare a nutrient rich environment for bacteria.

Cleaning and sanitizing procedures in different times could limit their access to nutrients. To cover these scenarios, TSB (rich growth medium) and R2A (low nutrients medium) was used. The results showed that strains which were grown in TSB and R2A had different co-aggregation ability (dependent on temperature and growth phase). *Rhodococcus erythropolis* MF3727 and *Acinetobacter calcoaceticus* MF3293, which were grown in TSB, showed stronger co-aggregation. This clearly shows that TSB was a better growth medium regarding co-aggregation ability.

The results obtained in this study agreed with the work of Mcintire et al. (1978). They showed that co-aggregation between *Actinomyces viscosus* and *Streptococcus sanguis* occurred when glucose was present in the culture medium. Burdman et al. (1998) also showed that *Azospirillum brasilence* wild type SP7 and mutant type FAJ0204, which were grown in higher C:N medium, had higher co aggregation ability than those which were grown in low C:N medium. However Cisar et al. (1979) reported that the composition of media had no effect on co-aggregation between *Actinomyces naeslundii* and human oral *Streptococci*.

The results also clearly showed that the growth temperature was greatly influencing the formation of co-aggregates between MF3727 and MF3293. Looking at the results, it can be inferred that cells grown at 30°C showed more co-aggregation ability compared to the cells grown at 20°C. As a result, it can be argued that temperature could affect production of temperature-depended macromolecules such as flagella and fimbriae, which sometimes facilitate the bacterial adhesion (Briandet et al. 1999).

Changes in growth temperature may also suppress the expression of structures on cell surface that mediate the forming of co-aggregation (Amano et al. 2001). Another possibility would be that cell surface hydrophobicity would correlate with temperature, which in some condition plays an important role in cell adhesion. These results were in agreement with the studies of Joe et al. (2009) on examining the effect of temperature on co-aggregation between *Azospirillum brasilense* MTCC-125 and *Azotobacter chroococcum* MTCC-446, *Azorhizobiom caulinodans* ORS-571, and *Bacillus megatherium* MTCC-3353. They showed that with increasing temperature from 25°C to 35-40°C, the co-aggregation percentage also increased. In addition, Jenkinson et al. (1990) stated that *Streptococcus sanguis* and *Candida albicans* grown at 37°C showed greater ability to form co-aggregation compared to cells grown at 28°C.

Throughout this study to examine the physiological cell age impact on co-aggregation, the strains of MF3727 and MF3293 were harvested in three different phases, including exponential phase, early stationary phase, and mid stationary phase. Such a strategy led to investigate that whether expression of adhesins and receptors or other cell surface molecules involved in co-aggregation occur simultaneously or not. Also, the expression of these macro molecules can be evaluated over time on the surface of each cell type.

Based on the results, the highest ability to form co-aggregate was related to the cells harvested in the exponential phase and the early stationary phase. By entering the mid stationary phase, the ability of these cells to co-aggregate was reduced. Meanwhile, the harvested MF3727 cells after 42 hours in mid stationary phase, displayed the least co-aggregation ability rate. Based on all the results obtained, it could be argued that the physiological state of the cells plays a very important role in the ability of cells to form co-aggregation. This argument is consistent with the findings of Rickard et al. (2004), demonstrating that the ability of *Blastomonas natatoria* and *Micrococcus luteus* to form co-aggregates was dependent on the growth phase. However, this

effect was irrespective of the cells growth medium in their results. In addition, Burdman et al. (1998) had previously observed that *Azosprillum brasilense* wild type sp7 and the mutant strain FAJ0204, which were at the early to mid-exponential phase of growth, created larger aggregates. Unlike all these arguments, Kolenbrander et al. (1995) believe that none of the factors of physiological aging and calendar aging influence the ability of oral bacteria cells to form co-aggregation.

It could be argued that differences in physiochemical properties of cells grown in different temperature, growth medium and growth phase may affect the synthesis of different molecules and polymers existing on the cell surface and participate in co-aggregation. However, the main aim of the experiment was to specify the conditions in which the cells display their highest capability to form co-aggregates. Based on the results, TSB growth medium, 30°C and harvesting after 18 hours were selected as standard conditions. Considering that all the strains used in this study were isolated from food processing environments, 30°C might not be the appropriate temperature for simulating such conditions. However since the highest co-aggregation capability by MF3727 and MF3293 had been recorded at this temperature, 30°C was used as the standard temperature for further work in this thesis.

To continue the optimization of standard conditions for future studies, the ability to form co-aggregates was examined between MF3727 and MF3293 while re-suspended in different solution and buffers. This experiment provided the opportunity to choose the most suitable buffer for use in standard conditions. Based on the results of this experiment, the studied pair showed different co-aggregation patterns in different environments. However, the highest ability occurred respectively when the cells had been solved in co-aggregation buffer or 0.85% NaCl. Such a high ability to form co-aggregates in the presence of positive ions such as Ca^{+2}, Mg^{2+} and Na^{+} can be explained due to negative charges on the bacterial cell surface. These cations could somehow have reduced the repulsive interaction between the co-aggregating partners (Poortinga et al. 2002).

Since the highest percentage of co-aggregation was recorded for cells re-suspended in co-aggregation buffer and 0.85% NaCl solution, it would be possible that co-aggregation is cations dependent. In cases that the absence of external cations failed to make much impact on co-

aggregation, it could also be argued that the cations, such as Ca^{2+}, leaked from the cell walls of the co-aggregation partners have helped to maintain the co-aggregation (Malik et al. 2003).

Kakii et al. (1990) previously reported that *Kluyvera cryocrescen*, a floc-forming bacteria from sewage activated sludge, showed a good flocculation ability in the presence of Ca^{2+}. They also showed that re-aggregation of these strains increased with increasing NaCl concentration in the presence of Ca^{2+}. On the other hand, the studies of Malik et al. (2003) showed that the absence of Ca^{2+} or lowering of NaCl concentration did not affect the co-aggregation between *Oligotropha carboxidororans* and *Acinetobacter junii.*

In their studies, Min et al. (2010) showed that co-aggregation between *Sphingomonas natatoria* and *Micrococcus luteus* were completely inhibited when the cells of these partners were re-suspended in co-aggregation buffer. In contrast, the cells re-suspended in KCl buffer showed the highest ability. The authors suggest that co-aggregation occurs optimally in buffers that closely resemble the environmental conditions from which the bacteria were isolated.

5.3 Co-aggregation among food related *Rhodococcus* and *Acinetobacter*

As mentioned earlier, the probability of co-aggregation among other strains of *Acinetobacter* and *Rhodococcus* were also examined (Table 3.3). Among 55 possible combination pairs from a total of three *Rhodococcus* strains and eight *Acinetobacter* strains, only *Rhodococcus erythropolis* MF3727 + *Acinetobacter calcoaceticus* MF3293, *Rhodococcus erythropolis* MF3727 + *Acinetobacter calcoaceticus* MF3627, and *Rhodococcus erythropolis* MF3727 + *Rhodococcus erythropolis* MF3803 pairs were able to form co-aggregates. These results suggest that the ability to form co-aggregates was strain-specific.

If different type of surface polymers could mediate the co-aggregation interaction between each pair, it is possible that MF3727 can play the role of a bridge in an environment where all these strains exist together. In case of shared adhesions or receptors, it is possible that each of the partner strains competes with each other for co-aggregation with MF3727 (Kolenbrander et al. 2006).

It can be seen in the results that the co-aggregation percentage and visual score rate of the three mentioned pairs are different from each other. For instance, MF3727 + MF3293 obtained a score equal to 4 while this score for MF3727 + MF3803 was equal to 2. The provided score cannot

necessarily be a measure of the relative strength of interaction between individual ligand on different cells, because the measured extent of co-aggregation by both assays is effectively a measure of the size and density of formed aggregates. On one hand, the density and size of the co-aggregates depend on the density and size of strains participated in co-aggregates formation. Another crucial point is the formation of co-aggregation between MF3727 and MF3803, given that both are *Rhodococcus erythropolis* but are genetically two different strains. This kind of co-aggregation is called intra-generic co-aggregation (Kolenbrander et al. 1995). Thus, the rule that co-aggregation is the interaction between a pair of genetically distinct partners (Kolenbrander 1988; Min et al. 2010) is still applied in this case.

5.4 Mechanisms behind co-aggregation of *Rhodococcus* and *Acinetobacter*

In recent years, research has been done on factors causing co-aggregation between various bacterial strains. However, factors such as fimbriae, flagella, cell surface hydrophobicity, EPS production, and presence of adhesions and receptors on bacterial cell surface, could play an important role in the formation of co-aggregation (Donlan 2002).

Among all the factors mentioned, the formation of co-aggregates mediated by adhesin (lectin-form) and receptors (polysaccharides) between human oral bacteria and drinking water bacteria has been frequently reported (Kolenbrander 1988; Simões et al. 2008). Most research has been conducted to identify co-aggregating adhesins among dental plaque bacteria and many of them have been identified. Earlier research on *Streptococcus gordonii,* a dental plaque primary colonizer, provided that the bacterium will express five distinct proteins all involved in co-aggregation interaction (Rickard et al. 2003). Also, among the fresh water bacteria, the co-aggregation between 15 different bacterial genera is mediated by adhesin-receptor interaction (Rickard et al. 2004). All these reports indicate that the lectin-saccharide mediated co-aggregation is very common in both oral and aquatic biofilm communities. Therefore, identifying and investigating the factors mediating co-aggregation was very important in this study.

All co-aggregating pars, including MF3727 + MF3293, MF3727 + MF3627, and MF3727 + MF3803 were included in all subsequent works related to characterization of co-aggregation . To investigate whether co-aggregation between which one of the pairs was mediated by adhesions or receptors, heat treatment (85^{o}C, 30min) and proteinase treatment (proteinase K) were used in

this study. Following that all cells were treated with heat, the results showed that co-aggregation was completely prevented in all cases where MF3293, MF3627 and MF3803had been heat-treated. Heat treatment of MF3727 did not affect co-aggregation between any of these pairs. The obtained results clearly indicate that MF3293, MF3627, and MF3803, all have proteinaceous adhesins on their cell surface, which mediate the co-aggregation interaction with MF3727. The protein structure on MF3293 might be the same appendage or fimbriae observed by scanning electron microscopy (SEM).

The results achieved from proteinase K pretreatment of partners also showed that the mentioned enzyme had no impact on MF3727 in formation of co-aggregation, while other strains lost a major part of their ability to form co-aggregation. These results confirm the heat treatment results and show that MF3293, MF3627, and MF3803 have proteinase and heat sensitive protein structures which are involved in co-aggregation. However, the MF3727 likely has a non-proteinaceous receptor that is heat and proteinase stable and mediates the co-aggregation interaction between MF3727 and other strains.

Perhaps, the failure to completely prevent co-aggregation between MF3727 + MF3293 and MF3727 + MF3627 by proteinase K was due to insufficient time for this enzyme to fully digest the proteins (adhesins) present on the surface of MF3293 and MF3627 or insufficient concentration used in the test. Another possibility could be the involvement of binding molecules other than the protein structure.

This finding was in agreement with the results reported by Simoes et al. (2007)They observed that heat treatment (80^{o}C, 30min) and protease treatment could completely prevent the co-aggregation between *Acinetobacter calcoaceticus* and *Burkholderia cepacia* as well as *Methylobacterium* sp. and *Staphylococcus* sp. This only occurred when *Acinetobacter calcoaceticus* cells had been treated and treated or untreated cells of other strains had no effect on co-aggregation. The authors argued that the interaction between the co-aggregation pairs was apparently mediated by heat and protease sensitive adhesins of *Acinetobacter calcoaceticus* and heat and protease stable receptors on the surface of the other bacterium. It is also mentioned in the report that neither heat nor proteinase treatment were able to prevent co-aggregation between *Acinetobacter calcoaceticus* and *Sphingomonas capsulata* and *Mycobacterium mucogenicum*. The authors believed that other types of interactions mediate the formation of co-aggregates

between these partners. Contrary to the results obtained in this study, Malik et al. (2003) research on co-aggregation between *Acinetobacter junii* and *Oligotropha carboxidovorans* showed that proteinase treatment led to inactivation of both bacteria so that none of the treated strains was able to form co-aggregation with their partners. The author suggests that both strains carry protein structure, which mediates the co-aggregation interaction between these two strains.

One of the polymers expressed by bacteria is EPS (extracellular polymeric substances), which acts as an intercellular cement during biofilm formation. This polymer can also act as a receptor in the formation of co-aggregates (Rickard et al. 2003). Dispersin B is a glycoside hydrolase enzyme capable of degrading an N-acetylglucosamine containing EPS (Kolenbrander et al. 2006; Rhoads et al. 2008; Ramasubbu et al. 2005).This enzyme is normally used for facilitation of detachment and dispersion of cells in biofilms (Ramasubbu et al. 2005).

To our knowledge, there is no report suggesting the use of this enzyme to prevent the co-aggregation formation. However, it was used in this study to investigate the possible role of EPS in formation of co-aggregation between the three aforementioned pairs. According to the results, this enzyme does not have the ability to prevent the process, and in all cases, treated and untreated cells had the ability to form co-aggregation with their partners. The results of these experiments can be translated as such that EPS has been generated by none of the strains in any scenarios, or if present, it has not played the receptor role in co-aggregation.

Some previous studies on biofilm formation of bacteria have shown that extracellular DNA (eDNA) plays an important role in the initial establishment of biofilms in bacteria that release DNA (Moscoso et al. 2006). This DNA, associates with other macromolecules such as proteins and exopolysaccharide, which is a complex mixture and holds bacterial biofilms together (Whitchurch et al. 2002). Several studies have shown that eDNA, as a major structural component of EPS, is able to participate in co-aggregation formation (Nishimura et al. 2003; Böckelmann et al. 2006) .

In this study, the possibility for expression of eDNA and its role in co-aggregation between MF3727 + MF3293, MF3727 + MF3627, and MF3727 + MF3803 were examined. For this purpose, all strains were pre-treated with DNAse I, and then, the treated and untreated partners

were combined in reciprocal pairs. The results showed no effect on co-aggregation between any of the pairs. However, Palmen et al. (1995) found that *Acinetobacter calcoaceticus* grown at 30°C has the ability to produce large amounts of eDNA at early and mid-exponential phases. Steinberger et al. (2005) also found that *Rhodococcus erythropolis* was able to produce low amount of eDNA in single species biofilm as well as in dual-species biofilm associated with *Pseudomonas aeruginosa*. Earlier literature showed that eDNA increased the aggregation percentage of *Escherichia coli* dependent on eDNA concentration and length (Liu et al. 2008).

According to the results obtained in this study, it might be argued that eDNA had no role in co-aggregation or its specific complementary receptors were not available on the partner surface. It is also possible that the generated eDNA by these strains has been lost due to harvesting or preparation of suspensions. This argument is based on that in all previous reports, the DNA amount was measured in batch culture (Palmen & Hellingwerf 1995) and biofilm (Steinberger & Holden 2005).

Results from heat and enzyme treatment indicated that co-aggregation between MF3727 and MF3293, MF3627 and MF3803 has been created by an adhesin-receptor interaction. These results also indicated the presence of proteinaceous adhesin on the cell surface of MF3293, MF3627 and MF3803. It was therefore necessary to confirm that MF3727 has a polysaccharide receptor on its cell surface, which is an adhesin binding site.

It is known that simple sugars, by attachment to the adhesins, are able to inhibit adhesin-receptor interaction (Kolenbrander 1998; Kolenbrander & Ganeshkumar 1993). In this study, simple sugars (Table 3.4) with a final concentration of 50mM were tested for possible inhibition or reversal of co-aggregation between these pairs. As can be seen from the visual results, no complete inhibition was obtained by any of the sugars. However, results showed that N-acetyl-D-galactosamine was able to decrease co-aggregation ability of MF3727 + MF3627. The achieved score for this pair in presence of N-acetyl-D-galactosamine was 2, which was one unit less than the score of control pair. The results obtained from optical density also showed that that N-acetyl-D-galactosamine could inhibit the co-aggregation between MF3727 and MF3627 up to 46%. On the other hands, lactose monohydrate had some inhibitory effect on co-aggregation between MF3727 + MF3293 and MF3727 + MF3803. Regarding the obtained results, several possibilities could be considered. The relative inhibition caused by N-acetyl-D-galactosamine

between MF3727 and MF3627, could be explained by the structure of the MF3727 receptor that mediates the co-aggregation between MF3727 and MF3627 are different than the structure of receptors that mediate the co-aggregation between MF3727 + MF3293 and MF3727 + MF3803. This idea could suggest the existence of multiple receptors on MF3727 cell surface and that each of them has different sugars on its structure.

One could also argument that only a limited number of sugars were used in this study. The failure in complete inhibition could indicate that the complementary receptors on MF3727 cell surface lack all of used sugars in their structure. Another possibility to explain the failure of all sugars used to fully prevent the co-aggregation could be due to insufficient final concentrations of the sugars to initiate a complete inhibition. For instance, N-acetyl-D-galactosamine could prevent 46% of forming the co-aggregation between MF3727 and MF3627. Using more final concentration of the sugar could have provided the complete inhibition.

This conclusion is consistent with Malik et al. (2003) studies. They used three different sugars, including glucose, galactose, and mannose to prevent the co-aggregation between *Oligotropha carboxidovorans* and *Acinetobacter junii*. Using final sugar concentration of 50mM, they could somewhat inhibit the co-aggregation between the pair. Moreover in the case of final concentrations being equal to 300mM, the sugars were able to completely inhibit the co-aggregation between these pairs. Rickard et al. (2004) used sugars such as galactose, N-acetyl-D-galactosamine, lactose and methyl-ά-D-galactopyranoside to inhibit the co-aggregation between *Blastomonas natatoria* and *Micrococcus luteus*. However, only galactose could totally inhibit the co-aggregation among all tested sugars.

In addition, Simões et al. (2008) had some research to prevent the formation of co-aggregation between *Acinetobacter calcoaceticus*, *Sphingomonas capsulata* and *Methylobacteruim* sp. They found among D (+)-galactose, D (+)-fucose, D (+)-lactose and N-acetyl-D-galactosamine, only the last listed sugar could not prevent the formation of co-aggregation.

Earlier research showed that *Rhodococcus erythropolis* carries polysaccharides on its cell surface (Rijnaarts et al. 1993; Neu & Poralla 1988) . In addition, Rijnaarts et al. (1995) using the measurement of isoelecteric point of bacterial cell surface in their studies showed that *R. erythropolis* contains amphiphilic polymers on its surface. Studies on electrophoretic mobility

and surface charge of bacterial cells clearly demonstrated that the mentioned polymer is anionic polysaccharides (Poortinga et al. 2002).

Formation of co-aggregation between two co-aggregating partners, when mediated by polysaccharide receptors and proteinaceous adhesins, can be affected by environmental changes such as pH and ionic strength. Since the bacterial cell surface charge strongly changes in response to environmental factors, such changes can lead to more attraction or repulsion (Palmer et al. 2007).

In this study, the effects of pH on co-aggregation between MF3727 and MF3293, MF3627, and MF3803 in pH range from 1 to 14 were examined. The results from visual method showed that all pairs had the ability to form co-aggregation in pH between 3 and 11. According to the optical density results, MF3727 + MF3803 obtained the highest percentage of co-aggregation at pH of 3 and 4. On the other hands, MF3727 + MF3293 and MF3727 + MF3627 showed a wider optimum pH range (Table 4.2).

Different ability of pairs to form co-aggregation indicates that the cell surface charges play an important role in bacterial co-aggregation forming. Since MF3727 + MF3803 showed the highest co-aggregation ability at pH 3 and pH 4, it could be possible that the protein structures of MF3803 that participate in co-aggregation would have an isoelectric point (pI) more than pH 4 (Poortinga et al. 2002; Rijnaarts & Norde 1995). It is known that proteins at pH lower than their pI carry a net of positive charge (Poortinga et al. 2002). Thus, it can be argued that below pH 5, a strong interaction occurs between positively charged proteins on MF3803 and the anionic polysaccharides on MF3727, which leads to enhancement co-aggregation between these pair.

The obtained results showed that by increasing pH values, the co-aggregation percentage between the pairs was gradually reduced. The result can be explained in that due to the gradually increased negative charged of proteins and the anionic polysaccharide (due to pH increase), the protein-polysaccharide interactions changed from attraction to repulsion. Such argument is consistent with Lewis et al. (1989) explanations that any increase in pH results in an increase in dissociation of acidic group and thus an increase in anionic charge on the extracellular polymer molecules.

However, these results were consistent with previous experiments which showed that the co-aggregation percentage of *Azosprillum brasilense* wild type and a mutant strains FAJ0204 increased with pH reduction (Burdman et al. 1998). They found that at pH between 3 and 9, the highest recorded ability to form co-aggregation between these two strains occurred at pH 3. In addition, studies on co-aggregation between *Sphingomonas natatoria* and *Micrococcus luteus* showed that the co-aggregation between these two occurred in the pH range of 3 to 10. Outside this range, no co-aggregation happened and the cells were susceptible to lysis (Min et al. 2010). It has been shown that increased pH resulted in increased numbers of *Acinetobacter* sp. cells detached from stainless steel (Lewis et al. 1989) . On the other hand, a previous study showed that the rate and the degree of co-aggregation between *Actinomyces viscosus* and *Streptococcus sanguis* gradually increased between pH 6 and 8 (Mcintire et al. 1978).

To find out whether molecular interactions other than adhesin-polysaccharide may be involved in the formation of the co-aggregation and influence it, chelating agents and surfactants were also used in this study. The results from chelating agents treatment showed that addition of EDTA, EGTA and citrate to co-aggregating pairs re-suspended in co-aggregation buffer was able to partially prevent the formation of co-aggregates between the pairs. Meanwhile, the highest recorded impact was of citrate. Finding that a metal binding agent such as citrate has the ability to disrupt the formation of co-aggregation between all the pairs suggested the existence of a common mechanism.

Given that earlier mentioned agents had the ability to block divalent cations such as Ca^{2+} and Mg^{2+} (Mcintire et al. 1978; Burdman et al. 1998), the results can be explained that the presence of these two metal ions in the co-aggregation buffer could have facilitated the co-aggregation forming between the pairs. This arguments support previous results of this study, suggesting that the pairs showed their highest co-aggregation ability in co-aggregation buffer and 0.85% NaCl compared to the pairs dissolved in dH_2O.

It is well known that citrate is a much milder chelating agent than EDTA and EGTA (Taweechaisupapong & Doyle 2000). However, it is unclear why it showed a greater ability to inhibit the co-aggregation between the pairs in this study. The results were in agreement with the Taweechaisupapong et al. (2000) observations, in which both EDTA and citrate were able to

prevent the formation of co-aggregation between *Actinomyces naeslundi*, *Streptococcus sanguis*, *Fusobacterium nucleatum* and *Prevotella intermeidia*.

Lewis et al. (1989) found that EDTA present in treatment buffer caused the excessive number of *Acinetobacter* sp. detached from stainless cell surface. This indicates that calcium and magnesium ions were involved in the attachment of these cells. A previous study has shown that the addition of 1mM of EDTA and 1mM EGTA into the co-aggregation buffer, could decrease the co-aggregation between *Azosprillum brasilensense*, *Azorhizobium caulinodans*, and *Baciluus megatherium* as much as 20-40% and 30-35%, respectively (Joe et al. 2009).On the other hands, Burdman et al. (1998) showed that neither EDTA nor EGTA were able to prevent re-aggregation of *Azospirillum brasilense* after disruption by mechanical homogenization.

Compared with the chelating agents, surfactants used in this study more effectively inhibited the co-aggregation between the pairs. For instance, SDS was able to reduce the rate of co-aggregation between all three pairs over 90%.The involvement of proteinaceous structure in the co-aggregation between all these pairs is known from previous results. It can therefore be argued that SDS, which is a protein denaturant (Grimaudo et al. 1996), could have temporary denatured proteinaceous adhesins involved in co-aggregation. Another possibility to be considered is that perhaps this anionic detergent has caused the complete lysis of the bacterial cells, which would result in losing the ability to create co-aggregation.

However, the results of this study were in agreement with Grimaudo et al. (1996) previous observations. They found that SDS was able to completely prevent the formation of co-aggregation between *Candida albicans* and eight different strains of oral *Actinomyces*. In addition, Malik et al. (2005) also observed that SDS was able to completely prevent the formation of co-aggregation between *Acinetobacter johnsonii* and *Oligotropha carboxidovorans.*

The results recorded for Tween 80 were a little different. This surfactant could prevent over 90% of co-aggregation formation between MF3727 + MF3627 and MF3727 + MF3803. In contrast, it showed much less capability against MF3727 + MF3293, and only could inhibit 25% of co-aggregation between this pair. Considering that this nonionic surfactant is an emulsifier (Nitschke & Costa 2007), probably due to increasing the suspension viscosity, it could have prevented the contact of partners, and as a result, it has caused a reduction in the co-aggregation.

The reason for its failure to show high inhibition of co-aggregation against MF3727 and MF3293 is not clear.

Min et al. (2010) previously found an inverse relationship between viscosity and degree of co-aggregation between *Sphingomonas natatoria* and *Micrococcus luteus*. The authors argue that the solutions with high viscosity either prevent or quietly slow down the rate at which the aggregates are formed. McEldowney et al. (1986) found that Tween 80 enhanced the detachment of *Chromobacterium* sp. from polystyrene substrate, but had no effect on co-aggregation between *Candida albicans* and *Actinomyces* sp.

5.5 Co-aggregation among other bacterial strains from food production environments

Based on the fact that all of the *Rhodococcus* and *Acinetobacter* strains able to form co-aggregates were isolated from food processing environments, it came to mind to investigate the possibility of co-aggregation among other bacteria isolated from food plants. Of the 466 possible combination pairs from 78 different strains, no co-aggregation was observed and recorded. Only in four cases, auto-aggregation was observed (Table 3.2). The obtained results can be explained by several hypotheses. The first possibility could be that co-aggregation is not common among food processing bacteria. As another possibility, perhaps the tested standard conditions might not have been appropriate conditions for different bacteria to show good ability to form co-aggregation.

5.6 Implications of co-aggregation in the food industry

The result of the current study indicated that the co-aggregation mechanism between *Rhodococcus* and *Acinetobacter*, isolated from food processing environments, are similar to oral plaque bacteria and fresh water bacteria, which are all mediated by adhesin-receptor interaction. Although *Rhodococcus* and *Acinetobacter*, used in this study, were isolated from different environments, could still co-aggregate to each other.

Although 30^{o}C, as standard temperature in this work (best result), is probably not the realistic temperature in food production environments, it is very important to mention that the co-aggregation among strains was also observed at 20^{o}C, a temperature more realistic in food production environments. TSB medium, as a rich growth environment, could to great extent

simulate the food production environments and showed that the high amount of nutrition would have positive influence on co-aggregation ability among *Rhodococcus erythropolis* and *Acinetobacter calcoaceticus*.

The study of effect of physiological age on co-aggregation would suggest that remaining bacteria, on equipment and surfaces, would have the possibility to form co-aggregation between the regular process of cleaning and disinfection. It is difficult to state how common co-aggregation is among bacteria from the food industry. Although co-aggregation involving other types of bacteria than *Rhodococcus* and *Acinetobacter* was not observed under the single set of conditions tested in this study, it cannot be excluded that they may co-aggregate under other conditions.

Co-aggregation may be beneficial, but due to lack of time in this work, it was not tested whether co-aggregation may lead to higher resistance of bacteria against disinfectants nor whether co-aggregation may improve biofilm formation.

6. CONCLUSION

Evaluation of co-aggregation ability was done in two different methods through this work, Visual assay and optical density assay. The result showed that the visual assay was less sensitive than the optical density assay. In addition the visual assay is subjective and is not free from human error. However this method is simple, fast, and accurate enough for studying co-aggregation. These characteristics make this method a good candidate in addition to optical assay.

To find the effects of physicochemical factors on co-aggregation between *Rhodococcus erythropolis* and *Acinetobacter calcoaceticus*, growth temperature (20^oC, 30^oC) growth medium (R2A, TSB) and physiological culture status was studied. The results showed that the cells of *Rhodococcus erythropolis* and *Acinetobacter calcoaceticus* that grew in TSB at 30^oC and harvested in the exponential phase early and stationary phase had the highest co-aggregation ability. These results clearly indicate that earlier mentioned factors have the ability to affect the co-aggregation ability of these strains. In addition it was found that cations such as Ca^{2+}, Mg^{2+}, Na^{+} and low pH levels have a positive influence on the co-aggregation ability of *Rhodococcus erythropolis* and *Acinetobacter calcoaceticus*.

Furthermore, it was decided to study the ability of co-aggregation between different strains of *Rhodococcus* and *Acinetobacter*. From three Rhodococcus strains and eight Acinetobacter strains, 55 possible combination pairs were tested and only 3 of the pairs (MF3727 +MF3293, MF3727 + MF3627 and MF3727 + MF3803) had the ability to form co-aggregates. All co-aggregating pairs had *Rhodococcus erythropolis* MF3727 strain in common.

Focusing on the reason of adherence between strains, the results of heat treatment (85^oC, 30min) and enzyme treatment (proteinase K) showed that the co-aggregation between pairs was mediated by adhesin- saccharide (receptor) interaction. It was also found that Peroteinaceous adhesins exist on the cell surface of *Acinetobacter calcoaceticus* (MF3293 and MF3627), while receptors would be on the cell surface of *Rhodococcus erythropolis* MF3727. However when it was applied to co-aggregation between *Rhodococcus erythropolis* MF3727 + *Rhodococcus erythropolis* MF3803, the adhesin was on the cell surface of MF3803.

The study of using sugars to inhibit co-aggregation showed that none of the used sugars could completely prevent the co-aggregation. N-acetyl-D-galactosamine could prevent the co-aggregation between MF3727 and MF3627 by 46%, but did not inhibit co-aggregation of the two other tested pairs of bacteria. These results could suggest the existence of multiple receptors on the cell surface of MF3727.

Later during this work, the possibility of co-aggregation between 78 different bacterial strains was tested. However, the results showed that none of the 466 tested pairs showed any ability to form co-aggregation. These results could suggest that the bacterial types from food production environments do not have the ability to co-aggregate, or the lab conditions provided were not adequate for the bacteria to form co-aggregation.

7. FUTURE WORK

Suggestion for further work:

- Study the co-aggregation between different bacteria which are isolated from various food processing environments under different environmental conditions than tested in this study.
- Examine the role of *Rhodococcus erythropolis* MF3727 as a bridge to form mixed species biofilm with other strains.
- Further characterization of adhesins and receptors of *Rhodococcus* and *Acinetobacter*.
- Examining the effect of co-aggregation on increased resistance against different stress factors such as disinfectants.
- Comparing the biofilm formation ability between co-aggregating strains and non-coaggregating mutants.

8. REFERENCES

Aaku, E.N. et al., 2004. Microbiological quality of milk from two processing plants in Gaborone Botswana. *Food Control*, 15(3), pp.181–186.

Abeygunawardana, C., Bush, C. a & Cisar, J O, 1991. Complete structure of the cell surface polysaccharide of Streptococcus oralis C104: a 600-MHz NMR study. *Biochemistry*, 30(35), pp.8568–77.

Amano, a et al., 2001. Altered antigenicity in periodontitis patients and decreased adhesion of Porphyromonas gingivalis by environmental temperature stress. *Oral microbiology and immunology*, 16(2), pp.124–8.

Bagge-Ravn, D., 2003. The microbial ecology of processing equipment in different fish industries—analysis of the microflora during processing and following cleaning and disinfection. *International Journal of Food Microbiology*, 87(3), pp.239–250.

Barnes, E.M. & Thorkely, M., 1966. The spoilage flora of eviscerated chickens stored at different temperatures. *International Journal of Food technology*, pp.113–119.

Barnes, E.M. & Impey, C.S., 1968. Psychrophilic spoilage bacteria of poultry. *The Journal of applied bacteriology*, 31(1), pp.97–107.

Bell, K.S., Philp, J.C., Aw, D.W.J., Christofi, N, 1998. The genus Rhodococcus. *Journal of Applied Microbiology*, 85(2), pp.195–210.

Bore, E. & Langsrud, S, 2005. Characterization of micro-organisms isolated from dairy industry after cleaning and fogging disinfection with alkyl amine and peracetic acid. *Journal of applied microbiology*, 98(1), pp.96–105.

Bos, R & Mei, H. van der, 1996. The role of physicochemical and structural surface properties in co-adhesion of microbial pairs in a parallel-plate flow chamber. *Colloids and Surfaces B: Biointerface*, 7(1996) pp.101-112.

Bos, R, Van der Mei, H.C. & Busscher, H J, 1999. Physico-chemistry of initial microbial adhesive interactions--its mechanisms and methods for study. *FEMS microbiology reviews*, 23(2), pp.179–230.

Briandet, R., Meylheuc, T., Maher, C., Bellon-Fontaine, M.N., 1999. Listeria monocytogenes Scott A : Cell Surface Charge , Hydrophobicity , and Electron Donor and Acceptor Characteristics under Different Environmental Growth Conditions. *Appl. Environ. Microbiol,* 65(12):5328-5333

Burdman, S., Jurkevitch, E., Schwatsburd, B., Hampel, M., Okon, Y., 1998. Aggregation in Azospirillum brasilense: effects of chemical and physical factors and involvement of extracellular components. *Microbiology*,144 (1 998), pp.1989–99.

Busscher, H J, Bos, R & Van der Mei, H.C., 1995. Initial microbial adhesion is a determinant for the strength of biofilm adhesion. *FEMS microbiology letters*, 128(3), pp.229–34.

Buswell, C. & Herlihy, Y., 1997. Coaggregation amongst aquatic biofilm bacteria. *Journal of applied Microbiology*, 83(1996), pp.477–484.

Buswell, C.M., Herlihy, Y.M., Lawrence, L.M., McGuiggan, J.T.M., Marsh, P.D., Keevil, C.W., Leach, S.A., 1998. Extended Survival and Persistence of Campylobacterspp . in Water and Aquatic Biofilms and Their Detection by Staining Extended Survival and Persistence of Campylobacter spp . in Water and Aquatic Biofilms and Their Detection by Immunofluorescent-Antibody. *Appl. Environ. Microbiol,* 64(2):733-741.

Böckelmann, U., Herlihy, Y.M., Lawrence, L.M., McGuiggan, T.M., Marsh, P.D., Keevil, C.W., Leach, S.A., 2006. Bacterial extracellular DNA forming a defined network-like structure. *FEMS microbiology letters*, 262(1), pp.31–8.

Carpentier, B & Cerf, O, 1993. Biofilms and their consequences, with particular reference to hygiene in the food industry. *The Journal of applied bacteriology*, 75(6), pp.499–511.

Carpentier, Brigitte & Cerf, Olivier, 2011. Review--Persistence of Listeria monocytogenes in food industry equipment and premises. *International journal of food microbiology*, 145(1), pp.1–8.

Carvalho, C.C.C.R., 2007. Biofilms: recent developments on an old battle. *Recent patents on biotechnology*, 1(1), pp.49–57.

Carvalho, C.C.C.R. & Da Fonseca, M.M.R., 2005. The remarkable Rhodococcus erythropolis. *Applied microbiology and biotechnology*, 67(6), pp.715–26.

Carvalho, C.C.C.R., Wick, L.Y. & Heipieper, H.J., 2009. Cell wall adaptations of planktonic and biofilm Rhodococcus erythropolis cells to growth on C5 to C16 n-alkane hydrocarbons. *Applied microbiology and biotechnology*, 82(2), pp.311–20.

Chavant, P., Martinie, B., Meylheuc, T., Bellon-Fontaine, M.N., Hebraud, M., 2002. Listeria monocytogenes LO28 : Surface Physicochemical Properties and Ability To Form Biofilms at Different Temperatures and Growth Phases. *Appl. Environ. Microbiol,* 68(2):728-737.

Chmielewski, R. a. N. & Frank, J.F., 2003. Biofilm Formation and Control in Food Processing Facilities. *Comprehensive Reviews in Food Science and Food Safety*, 2(1), pp.22–32.

Cisar, J O & Kolenbrander, P E, 1979. Specificity of coaggregation reactions between human oral streptococci and strains of Actinomyces viscosus or Actinomyces naeslundii. *Infect. Immun,*24(3):742-752.

Clemans, D.L., Kolenbrander, P.E., Debabov, D.V., Zhang, Q., Lunsford, R.D., Sakone, H., Whittaker, C.J., Heaton, M.P., 1999. Insertional inactivation of genes responsible for the D-alanylation of lipoteichoic acid in Streptococcus gordonii DL1 (Challis) affects intrageneric coaggregations. *Infection and immunity*, 67(5), pp.2464–74.

Danielsson, A., Norkrans, B. & Björnsson, A., 1977. On Bacterial Adhesion – The Effect of Certain Enzymes on Adhered Cells of a Marine Pseudomonas sp. *Botanica Marina*, 20(1), pp.13–17.

Doig, C., Gill, M.J. & Chqrch, D.L., 1991. Rhodococcus equi-an Easily Missed Opportunistic pathogen. *Scand. J. Infect. Dis,* 23(1991):1-6.

Donlan, R.M., 2002. Biofilms: microbial life on surfaces. *Emerging infectious diseases*, 8(9), pp.881–90.

Drago L, Gismondo MR, Lombardi A, Haen C, G.L., 1997. Inhibition of in vitro growth of enteropathogens by new Lactobacillus isolates of human intestinal origin. *FEMS microbiology letters 153 (1997)*, pp.455–463.

Dulaney, J.T., 1978. Binding interactions of glycoproteins with lectins. *Molecular and cellular biochemistry*, 21(1), pp.43–63.

Ekmekci, H., Aslim, B. & Ozturk, S., 2009. Characterization of vaginal lactobacilli coaggregation ability with Escherichia coli. *Microbiology and immunology*, 53(2), pp.59–65.

Elias, S. & Banin, E., 2012. Multi-species biofilms: living with friendly neighbors. *FEMS. Microbiol.Rev, 36(2012):990-1004.*

Fraser, O.P. & Sumar, S., 1998. Compositional changes and spoilage in fish (part II) - microbiological induced deterioration. *Nutrition & Food Science*, 98(6), pp.325–329.

Gennari, M., Parini, M., Volpon, D., Serio, M., 1992. Isolation and characterization by conventional methods and genetic transformation of Psychrobacter and Acinetobacter from fresh and spoiled meat, milk and cheese. *International journal of food microbiology*, 15(1-2), pp.61–75.

Gibbons, R.J. & Nygaard, M., 1970. Interbacterial aggregation of plaque bacteria. *Archives of oral biology*, 15(12), pp.1397–400.

Gilbert, P., Evans, D.J., Duguid, I.G., Brown, M.R.W.., 1991. Surface characteristics and adhesion of Escherichia coli and Staphylococcus epidermidis. *The Journal of applied bacteriology*, 71(1):72–77.

Gram, L., Ravn, L., Rasch, M., Bruhn, J.B., Christensen, A.B., Givskov, M., 2002. Food spoilage-interactions between food spoilage bacteria. *International journal of food microbiology*, 78(2002):79–97.

Granum, PE. 2008. Matforgifting, Næringsmiddelbårne infeksjoner og intoksikasjoner. Høyskoleforlaget AS-Norwegian Academic Press. 406. ISBN 978-82-7634-625-1.

Grimaudo, N.J., Nesbitt, W.E. & Clark, W.B., 1996. Coaggregation of Candida albicans with oral Actinomyces species. *Oral microbiology and immunology*, 11(1), pp.59–61.

Habimana, O., Heir, E., Langsrud, S., Wold Åsli, A., Møretrø, T., 2010. Enhanced surface colonization by Escherichia coli O157:H7 in biofilms formed by an Acinetobacter calcoaceticus isolate from meat-processing environments. *Applied and environmental microbiology*, 76(13), pp.4557–9.

Habimana, O., Møretrø, T., Langsrud, S., Vestby, L.K., Nesse, L.L., Heir, E ., 2010. Micro ecosystems from feed industry surfaces: a survival and biofilm study of Salmonella versus host resident flora strains. *BMC veterinary research*, 6(1), p.48.

Henrichsen, J., Blom, J., 1975. Correlation between twitching motility and possession of polar fimbriae in Acinetobacter calcoaceticus. *Acta Path. microbiol. Scand. Sec. B*, 83: 103-115.

Hood, S.K. & Zottola, E. a., 1995. Biofilms in food processing. *Food Control*, 6(1), pp.9–18.

Jakubovics, N.S, Gill, S.R., Lobst, S.E., Vickerman, M.M., Kolenbrander, P.E.., 2008. Regulation of gene expression in a mixed-genus community: stabilized arginine biosynthesis in Streptococcus gordonii by coaggregation with Actinomyces naeslundii. *Journal of bacteriology*, 190(10), pp.3646–57.

Jenkinson, H.F., 1992. Adherence, coaggregation, and hydrophobicity of Streptococcus gordonii associated with expression of cell surface lipoproteins. *Infection and immunity*, 60(3):1225-1228.

Jenkinson, H.F., 1986. Cell-surface proteins of Streptococcus sanguis associated with cell hydrophobicity and coaggregation properties. *Journal of general microbiology*, 132(6), pp.1575–89.

Jenkinson, H.F., Lala, H.C. & Shepherd, M.G., 1990. Coaggregation of Streptococcus sanguis and other streptococci with Candida albicans. *Infection and immunity*, 58(5), pp.1429–36.

Joe, M.M., Jaleel, C.A., Sivakumar, P.K., Zhao, C.X., Karthikeyan, B., 2009. Co-aggregation in Azospirillum brasilensense MTCC-125 with other PGPR strains: Effect of physical and chemical factors and stress endurance ability. *Journal of the Taiwan Institute of Chemical Engineers*, 40(5), pp.491–499.

Juárez Tomás, M.S., Wiese, B. & Nader-Macías, M.E., 2005. Effects of culture conditions on the growth and auto-aggregation ability of vaginal Lactobacillus johnsonii CRL 1294. *Journal of applied microbiology*, 99(6), pp.1383–91.

Kakii, K., Hasumi, M., Shirakasi, T., kuriyama, M., 1990. Involvement o f Ca 2 + in the Flocculation of Kluyvera. *Journal of Fermentation and Bioenginering*, 69(4), pp.224–227.

Kara, D., Luppens, S.B.I. & Cate, J.M., 2006. Differences between single- and dual-species biofilms of Streptococcus mutans and Veillonella parvula in growth, acidogenicity and susceptibility to chlorhexidine. *European journal of oral sciences*, 114(1), pp.58–63.

Kolenbrander, P. E., Parrish, K., Andersen, R.N., Greenberg, E.P., 1995. Intergeneric coaggregation of oral Treponema spp. with Fusobacterium spp. and intrageneric coaggregation among Fusobacterium spp. *Infection and immunity*, 63(12), pp.4584–8.

Kolenbrander, P E, 2000. Oral microbial communities: biofilms, interactions, and genetic systems. *Annual review of microbiology*, 54, pp.413–37.

Kolenbrander, P E, Andersen, R.N. & Moore, L. V, 1990. Intrageneric coaggregation among strains of human oral bacteria: potential role in primary colonization of the tooth surface. *Applied and environmental microbiology*, 56(12), pp.3890–4.

Kolenbrander, P E & Phucas, C.S., 1984. Effect of saliva on coaggregation of oral Actinomyces and Streptococcus species. *Infection and immunity*, 44(2), pp.228–33.

Kolenbrander, P., 1988. Intergeneric coaggregation among human oral bacteria and ecology of dental plaque. *Ann. Rev. Microbiol,* 42(1998):627-656.

Kolenbrander, P., 1989. Surface recognition among oral bacteria: multigeneric coaggregations and their mediators. *Critical reviews in microbiology*, 17(2):137-159.

Kolenbrander, P. & Ganeshkumar, N., 1993. Coaggregation: specific adherence among human oral plaque bacteria. *The FASEB journal*. 7(1993):406-413.

Kolenbrander, P. & London, J., 1993. Adhere today, here tomorrow: oral bacterial adherence. *Journal of Bacteriology*, 175(11), pp.3247–3252.

Kolenbrander, P.E., Palmer, R.J., Rickard, A.H., Jakubovics, N.S., Chalmers, N.I., Diaz, P.I., 2006. Bacterial interactions and successions during plaque development. *Periodontology 2000*, 42(5), pp.47–79.

Kuboniwa, M., Tribble, G.D., James, C.E., Kilic, A.O., Tao, L., Herzberg, M.C., Shizukushi, S., Lamont, R.J., 2006. Streptococcus gordonii utilizes several distinct gene functions to recruit Porphyromonas gingivalis into a mixed community. *Molecular microbiology*, 60(1), pp.121–39.

Langsrud, S., Sidhu, M.S., Heir, E., Holck, A.L., 2003. Bacterial disinfectant resistance—a challenge for the food industry. *International Biodeterioration & Biodegradation*, 51(4), pp.283–290.

Langsrud, S., Seifert, L. and Møretrø, T., 2006. Characterization of the microbial flora in disinfecting footbaths with hypochlorite. *Journal of Food Protection*, 69:2193-2198.

Langsrud, S., Møretrø, T., Heir, E., 2012. Bacteria in the food production environment. *Food Science & Technology* 26 (3): 26-30.

Lewis, S.J., Gilmour, A. & Johnston, D.E., 1989. Factors influencing the detachment of a polymer-associated Acinetobacter sp. from stainless steel. *International journal of food microbiology*, 8(2), pp.155–64.

Lis, H. & Sharon, N., 1973. The biochemistry of plant lectins (phytohemagglutinins). *Annual review of biochemistry*, 42, pp.541–74.

Liu, H.-H., Yang, Y.-Z., Shen, X.-C., Zhang, Z.-L., Shen, P., Xie, Z.-X., 2008. Role of DNA in bacterial aggregation. *Current microbiology*, 57(2), pp.139–44.

Madigan, MT., Martinko, JM., Dunlap, PV., ClarkDP. 2009. Brock Biology of Microorganisms. Benjamin Cummings. 1061. ISBN 0-321-53615-0.

Malik, A., Sakamoto, M., Ono,, T., Kakii, K., 2003. Coaggregation between Acinetobacter johnsonii S35 and Microbacterium esteraromaticum strains isolated from sewage activated sludge. *Journal of bioscience and bioengineering*, 96(1), pp.10–5.

Malik, A. & Kakii, K., 2003. Intergeneric coaggregations among Oligotropha carboxidovorans and Acinetobacter species present in activated sludge. *FEMS Microbiology Letters*, 224(1), pp.23–28.

Malik, A., Kimchhayarasy, P. & Kakii, K., 2005. Effect of surfactants on stability of Acinetobacter johnsonii S35 and Oligotropha carboxidovorans S23 coaggregates. *FEMS microbiology ecology*, 51(3), pp.313–21.

Malik, A., Phuong, K. & Suzuki, T., 2004. Effect of cultivation time and growth medium on the coaggregation capability of Acinetobacter johnsonii S35. *World Journal of Microbiology*,20: pp.781–786.

Malik, A., Sakamoto, M., Hanazaki, S., Osawa, M., Suzuki, T., Tochigi, M., Kakii, K., 2003. Coaggregation among nonflocculating bacteria isolated from activated sludge. *Appl.Environ. Microbiol,* 69(10):6056-6063.

Marouani-Gadri, N., Augier, G. & Carpentier, Brigitte, 2009. Characterization of bacterial strains isolated from a beef-processing plant following cleaning and disinfection - Influence of isolated strains on biofilm formation by Sakaï and EDL 933 E. coli O157:H7. *International journal of food microbiology*, 133(1-2), pp.62–7.

Martí, S, Rodriguez-Bano, J., Catel-Ferreira, M., Jouenne, T., Vila, J., Seifert, H., De, E., 2011. Biofilm formation at the solid-liquid and air-liquid interfaces by Acinetobacter species. *BMC research notes*, 4(1), p.1-5.

McEldowney, S. & Fletcher, M., 1986. Variability of the influence of physicochemical factors affecting bacterial adhesion to polystyrene substrata. *Appl. Environ. Microbiol.* 52(3):460-465.

Mcintire, F.C., Vatter, A.V., Baros, J., Arnold, J., 1978. Mechanism of coaggregation between Actinomyces viscosus Mechanism of Coaggregation Between Actinomyces viscosus T14V and Streptococcus sanguis 34. *Infect. Immun. 21(3):978-988.*

Min, K.R., Zimmer, M.N. & Rickard, a H, 2010. Physicochemical parameters influencing coaggregation between the freshwater bacteria Sphingomonas natatoria 2.1 and Micrococcus luteus 2.13. *Biofouling*, 26(8), pp.931–40.

Moscoso, M., García, E. & López, R., 2006. Biofilm formation by Streptococcus pneumoniae: role of choline, extracellular DNA, and capsular polysaccharide in microbial accretion. *Journal of bacteriology*, 188(22), pp.7785–95.

Møretrø, T. Langsrud, S., 2004. Listeria monocytogenes: biofilm formation and persistence in food-processing environments. *Biofilm* (2004) 1, 107-121.

Nangia-Makker, P., Conklin, J., Hogan, V., Raz, A., 2002. Carbohydrate-binding proteins in cancer, and their ligands as therapeutic agents. *Trends in molecular medicine*, 8(4), pp.187–92.

Nesse, L. L., Nordby, K., Heir, E., Bergsjoe, B., Vardund, T., Nygaard, H., & Holstad, G. (2003). Molecular Analyses of Salmonella enterica Isolates from Fish Feed Factories and Fish Feed Ingredients. *Appl.Environ. Microbiol 69*(2), 1075–1081.

Neu, T. & Poralla, K., 1988. An amphiphilic polysaccharide from an adhesive Rhodococcus strain. *FEMS microbiology letters*, 49(1988) :389–392.

Nishimura, S., Tanaka, T. & Fujita, K., 2003. Extracellular DNA and RNA produced by a marine photosynthetic bacterium Rhodovulum sulfidophilum. *Nucleic Acids Research Supplement*, 3(2003), pp.279–280.

Nitschke, M. & Costa, S.G.V. a. O., 2007. Biosurfactants in food industry. *Trends in Food Science & Technology*, 18(5), pp.252–259.

Palmen, R. & Hellingwerf, K.J., 1995. Acinetobacter calcoaceticus liberates chromosomal DNA during induction of competence by cell lysis. *Current Microbiology*, 30(1), pp.7–10.

Palmer, J., Flint, S. & Brooks, J., 2007. Bacterial cell attachment, the beginning of a biofilm. *Journal of industrial microbiology & biotechnology*, 34(9), pp.577–88.

Poortinga, A.T., Bos, R., Norde, W., Busscher, H.J., 2002. Electric double layer interactions in bacterial adhesion to surfaces. *Surface Science Reports,* 47 (2002), 1-32.

Poulsen, L.V., 1999. Microbial biofilm in food processing. *Lebensm.-Wiss. u.-Technol, 3*2(1999), pp.321–326.

Ramasubbu, N., Thomas, L.M., Rangunath, C., Kaplan, J.B.., 2005. Structural analysis of dispersin B, a biofilm-releasing glycoside hydrolase from the periodontopathogen Actinobacillus actinomycetemcomitans. *Journal of molecular biology*, 349(3), pp.475–86.

Reasoner, D.J. & Geldreich, E.E., 1985. A new medium for the enumeration and subculture of bacteria from potable water. *Applied and environmental microbiology*, 49(1), pp.1–7.

Rendueles, O. & Ghigo, J.-M., 2012. Multi-species biofilms: how to avoid unfriendly neighbors. *FEMS microbiology reviews*, 36, pp.972–989.

Rhoads, D.D., Wolcott, R.D. & Percival, S.L., 2008. Biofilms in wounds: management strategies. *Journal of wound care*, 17(11), pp.502–8.

Rickard, A.H., Leach, S.A., Buswell, C.M., Hich, N.J., Handley, P.S., 2000. Coaggregation between aquatic bacteria is mediated by specific-growth-phase-dependent lectin-saccharide interactions. *Applied and environmental microbiology*, 66(1), pp.431–4.

Rickard, a H, Gilbert, P & Handley, P S, 2004. Influence of growth environment on coaggregation between freshwater biofilm bacteria. *Journal of applied microbiology*, 96(6), pp.1367–73.

Rickard, A. & Leach, S.A., Hall, L.S., Buswell, C.M., High, N.J., Handley, P.S., 2002. Phylogenetic relationships and coaggregation ability of freshwater biofilm bacteria. *Appl. Environ. Microbiol,* 68(7):3644-3650.

Rickard, A.H., Gilbert., High, N.J., Kolenbrander, P.E., Handley, P.S., 2003. Bacterial coaggregation: an integral process in the development of multi-species biofilms. *Trends in Microbiology*, 11(2), pp.94–100.

Rickard, A.H., McBain, A.J., Ledder, R.G., Handley, P.S., Gilbert, P., 2003. Coaggregation between freshwater bacteria within biofilm and planktonic communities. *FEMS Microbiology Letters*, 220(1), pp.133–140.

Rijnaarts, H. & Norde, W, 1995. The isoelectric point of bacteria as an indicator for the presence of cell surface polymers that inhibit adhesion. *Colloids and Surfaces B: Biointerface*, 4(1995), pp.191-197.

Rijnaarts, H.H., Norde, W., Bouwer, E.J., Lyklema, J., Zehnder, A.J.B., 1993. Bacterial Adhesion under Static and Dynamic Conditions. *Applied and environmental microbiology*, 59(10), pp.3255–65.

Rijnaarts, H.H.M. 1994. Interaction between Bacteria and Solid Surfaces in Relation to Bacterial Transport in Porous Media.1-166. ISBN 90-5485-252-6.

Rivas, L., Fegan, N. & Dykes, G. a, 2007. Attachment of Shiga toxigenic Escherichia coli to stainless steel. *International journal of food microbiology*, 115(1), pp.89–94.

Rosenberg, M., Bayer, E.A., Delarea, J., Rosenberg, E., 1982. Role of thin fimbriae in adherence and growth of Acinetobacter calcoaceticus RAG-1 on hexadecane. *Appl. Environ. Microbiol,* 44(4):929-937.

Samelis, J., Kakouri, A. & Rementzis, J., 2000. Selective effect of the product type and the packaging conditions on the species of lactic acid bacteria dominating the spoilage microbial association of cooked meats at 4°C. *Food Microbiology*, 17(3), pp.329–340.

Sandberg, A.L. , Ruhl, S., Joralmon, R.A., Brennan, M.J., Sutphin, M.J., Cisar, J.O., 1995. Putative glycoprotein and glycolipid polymorphonuclear leukocyte receptors for the Actinomyces naeslundii WVU45 fimbrial lectin. *Infect. Immun,*63(7):2625-2631.

Schirmer, B.C.T., Heir, E., Møretrø, T., Skaar, I., and Langsrud, S., 2013. Microbial background flora in small scale cheese production facilities does not inhibit growth and surface attachment of *Listeria monocytogenes. Journal of Dairy Science,* accepted.

Shen, S., Samaranayake, L.P. & Yip, H.-K., 2005. Coaggregation profiles of the microflora from root surface caries lesions. *Archives of oral biology*, 50(1), pp.23–32.

Shi, X. & Zhu, X., 2009. Biofilm formation and food safety in food industries. *Trends in Food Science & Technology*, 20(9), pp.407–413.

Simoes, L.C., Simoes, M. & Vieira, M. J., 2007. Intergeneric Coaggregation among Drinking Water Bacteria: Evidence of a Role for Acinetobacter calcoaceticus as a Bridging Bacterium. *Applied and Environmental Microbiology*, 74(4), pp.1259–1263.

Simões, L.C., Simões, M. & Vieira, Maria João, 2008. Acinetobacter calcoaceticus plays a bridging function in drinking water biofilms. *IBB-Institute for Biotechnology and Bioengineering, Center of Biological Engineering, Universidade do Minho, Portugal*, ENGIUM.1-13.

Sinde, E. & Carballo, J., 2000. Attachment of Salmonella spp. and Listeria monocytogenes to stainless steel, rubber and polytetrafluorethylene: the influence of free energy and the effect of commercial sanitizers. *Food Microbiology*, 17(4), pp.439–447.

Skillman, L.C. & Sutherland, I.W., 1999. The role of exopolysaccharides in dual species biofilm development. *Jornal of Aoolied Microbiology Symposium Supplement* , 85, pp.13–18.

Steinberger, R.E. & Holden, P.A., 2005. Extracellular DNA in Single- and Multiple-Species Unsaturated Biofilms. *Appl. Environ. Microbiol* , 71(9):5404-5410.

Swaminathan, B. & Gerner-Smidt, P., 2007. The epidemiology of human listeriosis. *Microbes and infection / Institut Pasteur*, 9(10), pp.1236–43.

Takahashi, Y., Konishi, K., cisar, J.O., Yoshikawa, M., 2002. Identification and Characterization of hsa , the Gene Encoding the Sialic Acid-Binding Adhesin of Streptococcus gordonii DL1. *Infect. Immun,*70(3):1209-1218.

Taweechaisupapong, S. & Doyle, R.J., 2000. Sensitivity of bacterial coaggregation to chelating agents. *FEMS immunology and medical microbiology*, 28(4), pp.343–6.

Vaneechoutte, M., Dijkshoorn, L., Tjenberg, I., Elaichouni, A., Vos, P.D., Clayes, G., Verschraegen, G., 1995. Identification of Acinetobacter genomic species by amplified ribosomal DNA restriction analysis. *J. Clin. Microbiol*, 33(1):11-15.

Visca, P., Seifert, H. & Towner, K.J., 2011. Acinetobacter infection--an emerging threat to human health. *IUBMB life*, 63(12), pp.1048–54.

Weinstock, D.M. & Brown, A.E., 2002. Rhodococcus equi: an emerging pathogen. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America*, 34(10), pp.1379–85.

Whitchurch, C.B., Tolker-Nilsen, T., Rages, P.C., Mattick, J.S., 2002. Extracellular DNA required for bacterial biofilm formation. *Science*, 295, p.1487.

9 APPENDIX

9.1 Mediums and solutions

TSB (Tryptone soya broth)

30 g TSB (Oxoid LTD., Basingstoke, England) re-suspended in 1l dH_2O and sterilized by autoclaving at 121^{o} C in 15 minutes.

TSA (Tryptone soya agar)

40 g TSA (Oxoid) re-suspended in 1l dH_2O and sterilized by autoclaving at 121^{o} C in 15 minutes.

R2A

18.1 g R2A (Difco) re-suspended in 1l dH_2O and sterilized by autoclaving at 121^{o} C in 15 minutes.

Sterile de-ionized water

Water was distilled, deionized and filtered (MiliQ-water, Millipore AB, Oslo, Norway; dH_2O) before being autoclaved at 121^{o} C for 15-20 minutes.

Peptone water

4 g bacteriological peptone (Oxoid) and 340 g NaCl (Sodium chloride, Merck, Darmstadt, Germany) were dissolved in 4l dH_2O. pH was adjusted to 7.2 before sterilization at 121^{o} C in 15 minutes.

PBS (phosphate-bufferd saline)

Dissolve the following in 800ml distilled H_2O.

8g of NaCl, 0.2g of KCl, 1.44g of Na_2HPO_4, 0.24g of KH_2PO_4 .

Adjust volume to 1L with additional distilled H_2O.

Adjust pH to 7.4 with HCl. Sterilize by autoclaving.

Co-aggregation buffer

Dissolved 0.121 g Tris, 0.011g $CaCl_2$, 0.02g $MgCl_2$, 8.77g NaCl in 900 ml dH2O.

Adjusted volume to 1000ml. adjusted the pH to 8.0 with NaOH.

Sterilized by autoclaving.

9.2 Chemicals

SDS 1.0%

Dissolved 1g SDS in 90 ml dH_2O.

Heated to 68°C to assist dissolution.

Adjusted the pH to 7.2 with HCl. Adjusted volume to 100 ml

Sterilized by sterile filter.

0.5M EDTA (pH 8.0)

Added 16,81 g disodium ethylenediamine tetraacetate (EDTA) to 80ml dH_2O.

Added NaOH slowly with stirring until the EDTA dissolved. adjust volume to 100ml.

Adjusted the pH to 8.0 with NaOH. Sterilized by sterile filter.

0.5M EGTA (pH 8.0)

Added 19g Ethylene glycol tetraacetic acid (EGTA) to 80ml dH_2O.

Adjust volume to 100ml. adjusted pH 8.0 with NaOH. Sterilized by sterile filter.

0.85% NaCl

Added 1.7 g NaCl (Merck) to 150ml dH_2O. Adjust volume to 200ml.

Sterilized by sterile filter.

9.3 Bacterial strains tested for co-aggregation

Table 9.1 Bacterial strains from different food production environment (group 1) excluding *Rhodococcus erythropolis* MF3727 and *Acinetobacter calcoaceticus* MF3293.

Bacteria	Strain number*	Origin	Reference
Listerias sp.	4057	Conveyor belt 1, Salmon processing, plant D	Heir, unpublished
Listeria monocytogenes	4602	Conveyor belt 1, Salmon processing, plant D	Heir, unpublished
Flavobacterium sp.	4107	Conveyor belt 1, Salmon processing, plant D	Heir, unpublished
Carnobacterium sp.	4109	Conveyor belt 1, Salmon processing, plant D	Heir, unpublished
Variovorax sp.	4655	Meat processing plant	Schirmer, unpublished
Micrococcus sp.	4649	Meat processing plant	Schirmer, unpublished
Sphingomonas sp.	4670	Meat processing plant	Schirmer, unpublished
Sphingomonas sp.	4632	Meat processing plant	Schirmer, unpublished
Microbacterium	4654	Meat processing plant	Schirmer, unpublished
Rhodococcus erythropolis	3727	Drain, small scale cheese producer A	Schirmer et al 2013
Acinetobacter calcoaceticus	3293	Disinfecting footbath with hypochlorite, dairy	Langsrud et al 2006

*refer to MF number in Nofima strain collection

Table 9.2 Bacterial strains from fish industry (group 2) excluding *Rhodococcus erythropolis* MF3727 and *Acinetobacter calcoaceticus* MF3293.

Bacteria	Strain number*	Origin	Reference
Listeria monocytogenes	3900	Conveyor belt 1, Salmon processing, plant D	Heir, unpublished
Listeria monocytogenes	3930	Conveyor belt 2, Salmon processing, plant D	Heir, unpublished
Listeria monocytogenes	3939	Conveyor belt 3, Salmon processing, plant D	Heir, unpublished
Listeria monocytogenes	3940	Conveyor belt 1, Salmon processing, plant E	Heir, unpublished
Listeria monocytogenes	4237	Drain, Salmon slaughter house plant F	Heir, unpublished
Listeria monocytogenes	4238	Drain, Salmon slaughter house plant F	Heir, unpublished
Psychrobacter sp.	4104	Conveyor belt 1, Salmon processing, plant D	Heir, unpublished
Pseudomonas sp.	4106	Conveyor belt 1, Salmon processing, plant D	Heir, unpublished
Flavobacterium sp.	4107	Conveyor belt 1, Salmon processing, plant D	Heir, unpublished
Acinetobacter junii	4112	Filet machine, salmon processing plant D	Heir, unpublished
Aeromonas sp.	4108	Conveyor belt 1, Salmon processing, plant D	Heir, unpublished
Acenitobacter sp.	4117	Conveyor belt 1, salmon processing plant D	Heir, unpublished
Pseudomonas sp.	4118	Conveyor belt 1, Salmon processing, plant D	Heir, unpublished
Chryseobacterium sp.	4123	Conveyor belt 1, Salmon processing, plant D	Heir, unpublished
Pseudomonas fragi	96.4	Conveyor belt 3, Salmon processing, plant D	Heir, unpublished
Serratia liquefaciens	96.5	Conveyor belt 3, Salmon processing, plant D	Heir, unpublished
Pseudomonas fluorescens	96.6	Conveyor belt 3, Salmon processing, plant D	Heir, unpublished
Stenotrophomonas maltphilia	96.7	Conveyor belt 3, Salmon processing, plant D	Heir, unpublished
Brochotrix thermospacta	96.9	Conveyor belt 3, Salmon processing, plant D	Heir, unpublished
Acinetobacter johnsonii	4091	Conveyor belt, salmon processing plant F	Heir, unpublished
Serratia liquefaciens	3971	Conveyor belt 3, Salmon processing, plant D	Heir, unpublished

Rhodococcus erythropolis	3727	Drain, small scale cheese producer A	Schirmer et al 2013
Acinetobacter calcoaceticus	3293	Disinfecting footbath with hypochlorite, dairy	Langsrud et al 2006

*refer to MF number in Nofima strain collection

Table 9.3 Bacterial strains from meat industry (group 3) excluding *Rhodococcus erythropolis* MF3727 and *Acinetobacter calcoaceticus* MF3293.

Bacteria	Strain number*	Origin	Reference
Listeria monocytogenes	LB1	Shoe washing machine, before producing, plant X	Schirmer, unpublished
Listeria monocytogenes	LB45	Drain, meat processing, plant X	Schirmer, unpublished
Listeria monocytogenes	LB51	Shoe washing machine, during production,plant X	Schirmer, unpublished
Listeria monocytogenes	LB83	Conveyor belt 1, meat processing, plant X	Schirmer, unpublished
Listeria monocytogenes	LB84	Conveyor belt 2, meat processing, plant X	Schirmer, unpublished
Pseudomonas fragi	L1-2	Shoe washing machine, before producing, plant X	Schirmer, unpublished
Aerococcus sp.	L1-3	Shoe washing machine, before producing, plant X	Schirmer, unpublished
Brochotrix thermosphacta	L1-7	Shoe washing machine, before producing, plant X	Schirmer, unpublished
Microbacterium sp.	L1-8	Shoe washing machine, before producing, plant X	Schirmer, unpublished
Pseudomonas fluorescens	L1-10	Shoe washing machine, before producing, plant X	Schirmer, unpublished
Staphylococcus sp.	L83-2	Conveyor belt 1, meat processing, plant X	Schirmer, unpublished
Staphylococcus sp.	L83-7	Conveyor belt 1, meat processing, plant X	Schirmer, unpublished
Staphylococcus sp.	L83-13	Conveyor belt 1, meat processing, plant X	Schirmer, unpublished
Staphylococcus sp.	L83-17	Conveyor belt 1, meat processing, plant X	Schirmer, unpublished
Enterococcus devriesei	L45-7	Drain, meat processing, plant X	Schirmer, unpublished
Brucella melitensis	L45-9	Drain, meat processing, plant X	Schirmer, unpublished
Leuconostoc mesenteroides	L45-17	Drain, meat processing, plant X	Schirmer, unpublished
Bacillus sp.	L45-19	Drain, meat processing,	Schirmer,

		plant X	unpublished
Corynebacterium testudinoris	L45-23	Drain, meat processing, plant X	Schirmer, unpublished
Citrobacter sp.	L84-2	Conveyor belt 2, meat processing, plant X	Schirmer, unpublished
Proteus sp.	L84-5	Conveyor belt 2, meat processing, plant X	Schirmer, unpublished
Kocuria sp.	L51-1	Shoe washing machine, during production,plant X	Schirmer, unpublished
Microbacterium sp.	L51-9	Shoe washing machine, during production,plant X	Schirmer, unpublished
Chryseobacterium sp.	L51-10	Shoe washing machine, during production,plant X	Schirmer, unpublished
Exiguobacterium sp.	L51-13	Shoe washing machine, during production,plant X	Schirmer, unpublished
Epilithonimonas sp.	L51-21	Shoe washing machine, during production,plant X	Schirmer, unpublished
Acinetobacter sp.	L51-22	Shoe washing machine, during production,plant X	Schirmer, unpublished
Unknown**	L51-25	Shoe washing machine, during production,plant X	Schirmer, unpublished
Pseudomonas fragi	L51-26	Shoe washing machine, during production,plant X	Schirmer, unpublished
Rhodococcus erythropolis	3727	Drain, small scale cheese producer A	Schirmer et al 2013
Acinetobacter calcoaceticus	3293	Disinfecting footbath with hypochlorite, dairy	Langsrud et al 2006

*refer to MF number in Nofima strain collection. ** No hits in 16S sequencing.

Table 9.4 Bacterial strains from fish feed producing plants (group 4) excluding *Rhodococcus erythropolis* MF3727 and *Acinetobacter calcoaceticus* MF3293.

Bacteria	Strain number*	Origin	Reference
Salmonella kentucky	4728	Fish feed production, plant D	Nesse et al 2003
Salmonella senftenberg	4736	Fish feed production, plant D	Nesse et al 2003
Staphylococcus piscifermentans	4713	Cooler sampling 1 plant Y	Habimana et al 2010
Staphylococcus piscifermentans	4714	Coater sieve sampling 1 plant Y	Habimana et al 2010
Staphylococcus saprophyticus	4698	Coater sieve sampling 2 plant Y	Habimana et al 2010
Staphylococcus saprophyticus	4699	Cooler sampling 1 plant Y	Habimana et al 2010
Pantoea conspicua	4700	Mixer sampling 2 plant Y	Habimana et al 2010
Pantoea agglomerans	4715	Mixer sampling 1 plant Y	Habimana et al 2010
Corynebacterium ammoniagenes	4716	Coater sieve sampling 1 plant Y	Habimana et al 2010
Pseudomonas fluorescens	4702	Extruder plant Y	Habimana et al 2010
Paenibacillus glucanolyticus	4718	Coater sieve sampling 1 plant Y	Habimana et al 2010
Bacillus silvestris	4704	Cooler sampling 1 plant Y	Habimana et al 2010
Bacillus subtilis	4705	Coater sieve sampling 2 plant Y	Habimana et al 2010
Pediococcus pentosaceus	4706	Cooler sampling 1 plant Y	Habimana et al 2010
Psychrobacter faecalis	4707	Extruder plant Y	Habimana et al 2010
Psychrobacter pulmonis	4708	Mixer sampling 2 plant Y	Habimana et al 2010
Acinetobacter baumannii	4719	Extruder plant Y	Habimana et al 2010
Rhodococcus erythropolis	3727	Drain, small scale cheese producer A	Schirmer et al 2013
Acinetobacter calcoaceticus	3293	Disinfecting footbath with hypochlorite, dairy	Langsrud et al 2006

*refer to MF number in Nofima strain collection

9.4 Tables of pairs of different *Rhodococcus* and *Acinetobacter* strains tested for co-aggregation

Table 9.5 Visual co-aggregation score. The same results were obtained from three independent replicates.

Strain number*	3727	4633	3803	4642	4130	4206	4091	3293	3627	4112	4117
3727											
4633	0										
3803	2	0									
4642	0	0	0								
4130	0	0	0	0							
4206	0	0	0	0	0						
4091	0	0	0	0	0	0					
3293	4	0	0	0	0	0	0				
3627	3	0	0	0	0	0	0	0			
4112	0	0	0	0	0	0	0	0	0		
4117	0	0	0	0	0	0	0	0	0	0	

*refer to MF number in Nofima strain collection.

Table 9.6 Co-aggregation percentage with optical density assay.

Strain number*	3727	4633	3803	4642	4130	4206	4091	3293	3627	4112	4117
3727											
4633	0.4										
3803	9.7	0									
4642	1.0	0	0								
4130	0.0	0.7	0	0.3							
4206	0.0	0.3	0.3	0	0.3						
4091	0.7	0	0	0.7	0.3	0.7					
3293	50.2	1.4	0.7	0	0.7	0.3	0.3				
3627	15.2	0	0	0	0	0.3	0.3	0			
4112	0.0	1.1	0	0	0.4	1.4	0.7	1.8	0.3		
4117	0.7	1.0	0	0	0.3	0.6	0.7	0	0	0	

*refer to MF number in Nofima strain collection.

9.5 Effect of enzymatic treatment of cells on co-aggregation

Table 9.7 Effect of different enzyme on co-aggregation between *Rhodococcus erythropolis* (MF3727) and *Acinetobacter calcoaceticus* (MF3293). All cells were cultivated at standard condition (TSB, 30oC, 18hr). The standard deviations (in parentheses) are based on three independent replicates.

	% Co-aggregation*			
Enzymes / Pairs	3727UT+3293UT	3727UT+3293T	3727T+3293UT	3727T+3293T
Proteinase K	48.0 (1.42)	8.9 (1.22)	47.3 (1.42)	5.3 (0.70)
Dispersin	49.5 (1.02)	46.6 (0.98)	48.7 (1.14)	47.3 (0.62)
DNAse	48.7 (0.72)	49.5 (0.62)	49.5 (1.14)	48.0 (1.49)

*Co-aggregation percentage was determined based on decrease in $OD650_{nm}$. T: heat treated; UT: Untreated, no enzyme treatment.

Table 9.8 Effect of different enzyme on co-aggregation between *Rhodococcus erythropolis* (MF3727) and *Acinetobacter calcoaceticus* (MF3627). All cells were cultivated at standard condition (TSB, 30^{o}C, 18hr). The standard deviations (in parentheses) are based on three independent replicates.

	% co-aggregation*			
Enzymes / Pairs	3727UT+3293UT	3727UT+3293T	3727T+3293UT	3727T+3293T
Proteinase K	14.5 (0.87)	2.5 (0.26)	15.2 (0.60)	2.0 (0.43)
Dispersin	15.9 (0.62)	15.9 (0.61)	15.2 (0.62)	15.9 (0.26)
DNAse	14.5 (0.46)	14.5 (0.78)	13.8 (0.70)	14.5 (0.40)

*Co-aggregation percentage was determined based on decrease in $OD650_{nm}$. T: heat treated; UT: Untreated, no enzyme treatment.

Table 9.9 Effect of different enzyme on co-aggregation between *Rhodococcus erythropolis* (MF3727) and *Rhodococcus erythropolis* (MF3803). All cells were cultivated at standard condition (TSB, 30^{o}C, 18hr). The standard deviations (in parentheses) are based on three independent replicates.

	% co-aggregation*			
Enzymes / Pairs	3727UT+3293UT	3727UT+3293T	3727T+3293UT	3727T+3293T
Proteinase K	9.7 (0.60)	0.7 (0.26)	8.9 (0.65)	0.4 (0.10)
Dispersin	10.4 (0.70)	9.7 (0.50)	9.7 (0.46)	9.0 (0.89)
DNAse	10.4 (0.53)	9.7 (0.62)	9.7 (0.61)	9.0 (1.13)

*Co-aggregation percentage was determined based on decrease in $OD650_{nm}$. T: heat treated; UT: Untreated, no enzyme treatment.

9.6 The effect of culturing conditions on the co-aggregation capability of *Rhodococcus erythropolis* MF3727 and *Acinetobacter calcoaceticus* MF3293

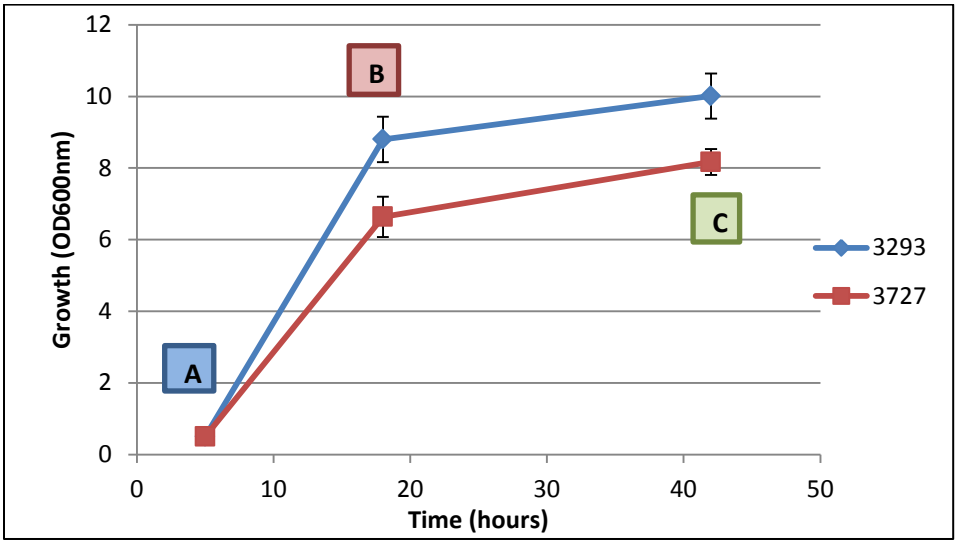


Figure 9.1 Growth curves of *Rhodococcus erythropolis* MF3727 and *Acinetobacter calcoaceticus* MF3293 in R2A medium at 30° C. Letters indicate harvesting of cultures for test of co-aggregation. Error bars represent standard deviations from three independent replicates.

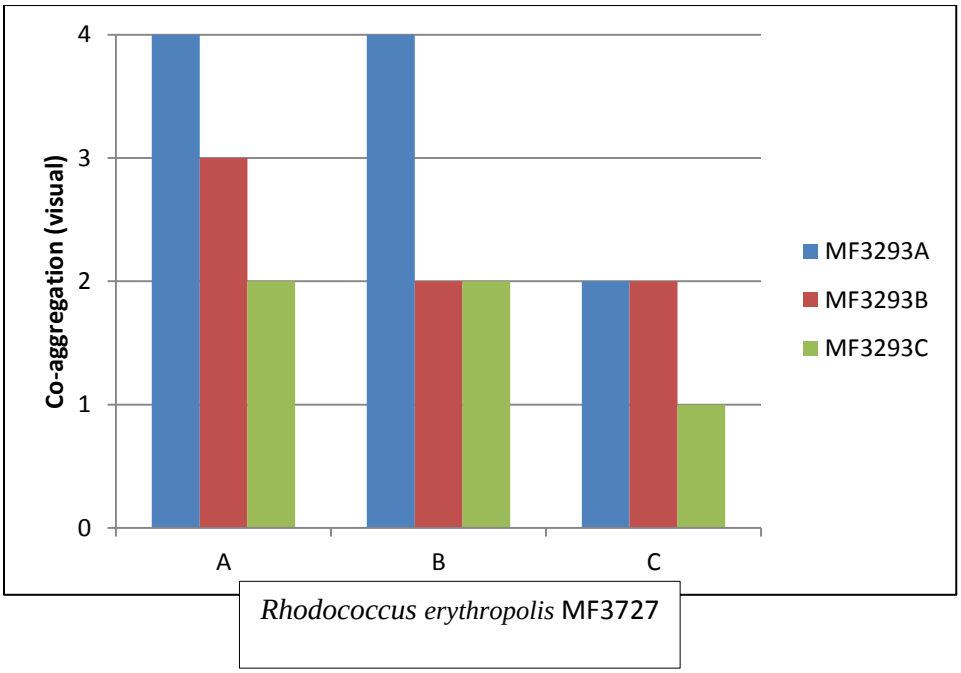


Figure 9.2 Co-aggregation (visual score) between *Rhodococcus erythropolis* MF3727 and *Acinetobacter calcoaceticus* MF3293 sampled from cultures in R2A medium at 30° C in different growth phase. A; OD600nm=0.5, B: 18hr., C: 42 hr. The same results were obtained from three independent replicates.

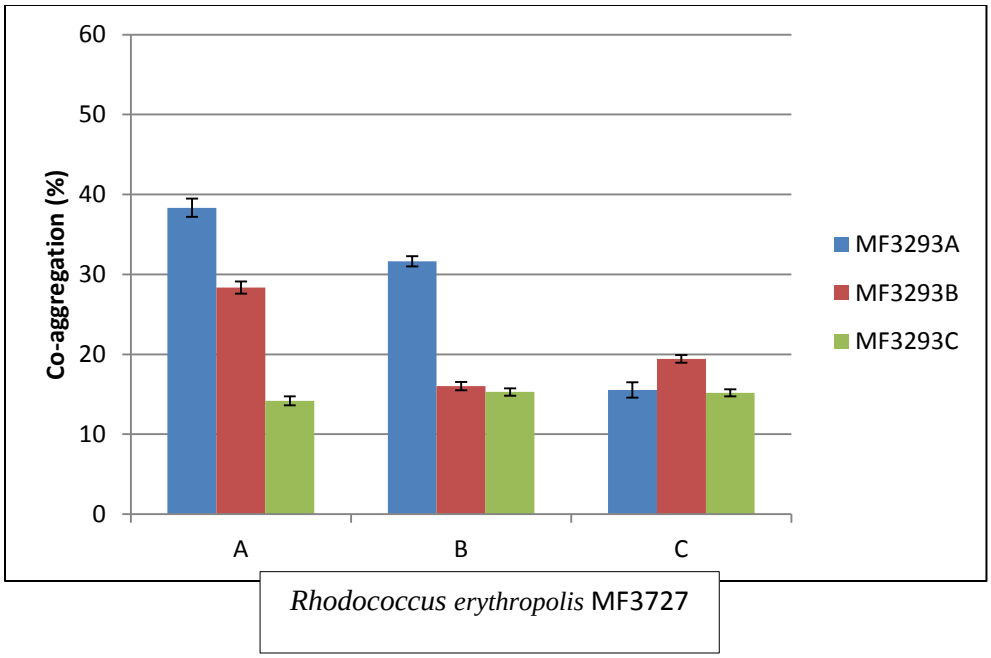


Figure 9.3 Co-aggregation (percentage) between *Rhodococcus erythropolis* MF3727 and *Acinetobacter calcoaceticus* MF3293 sampled from cultures in R2A medium at 30° C in different growth phase. A; OD600nm=0.5, B: 18hr., C: 42 hr. Error bars represent standard deviations from three independent replicates.

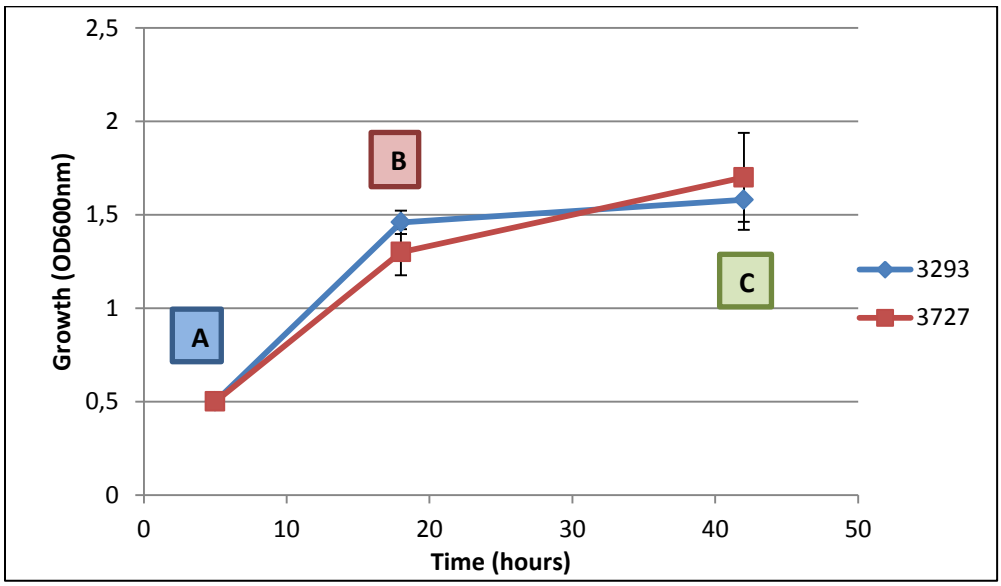


Figure 9.4 Growth curves of *Rhodococcus erythropolis* MF3727 and *Acinetobacter calcoaceticus* MF3293 in R2A medium at 20° C. Letters indicate harvesting of cultures for test of co-aggregation. Error bars represent standard deviations from three independent replicates.

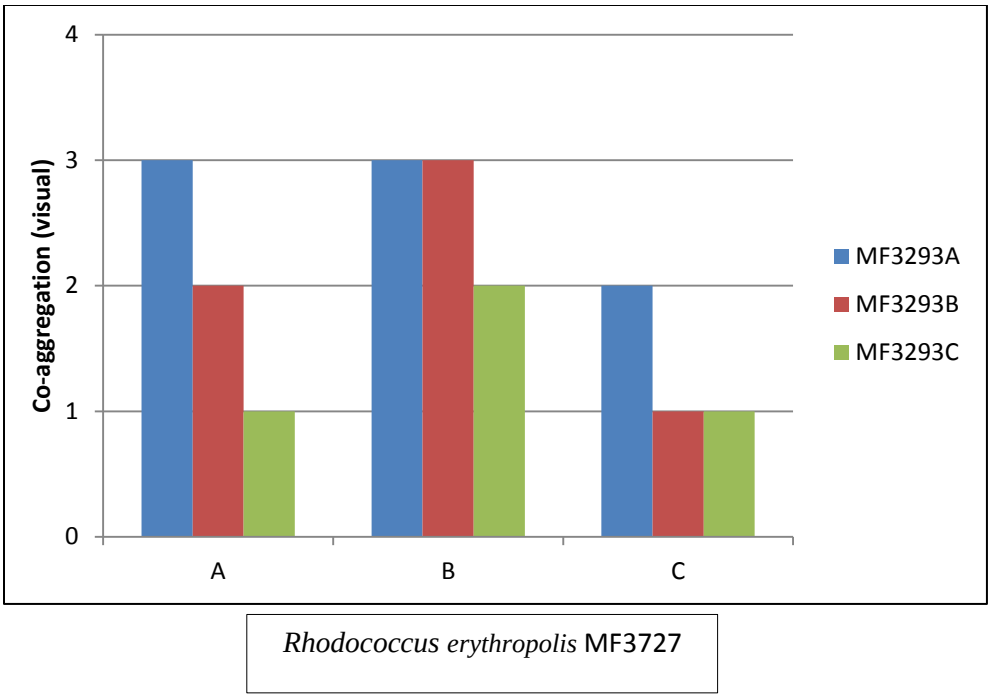


Figure 9.5 Co-aggregation (visual score) between *Rhodococcus erythropolis* MF3727 and *Acinetobacter calcoaceticus* MF3293 sampled from cultures in R2A medium at 20^{o} C in different growth phase.
A; OD600nm=0.5, B: 18hr., C: 42 hr. The same results were obtained from three independent replicates.

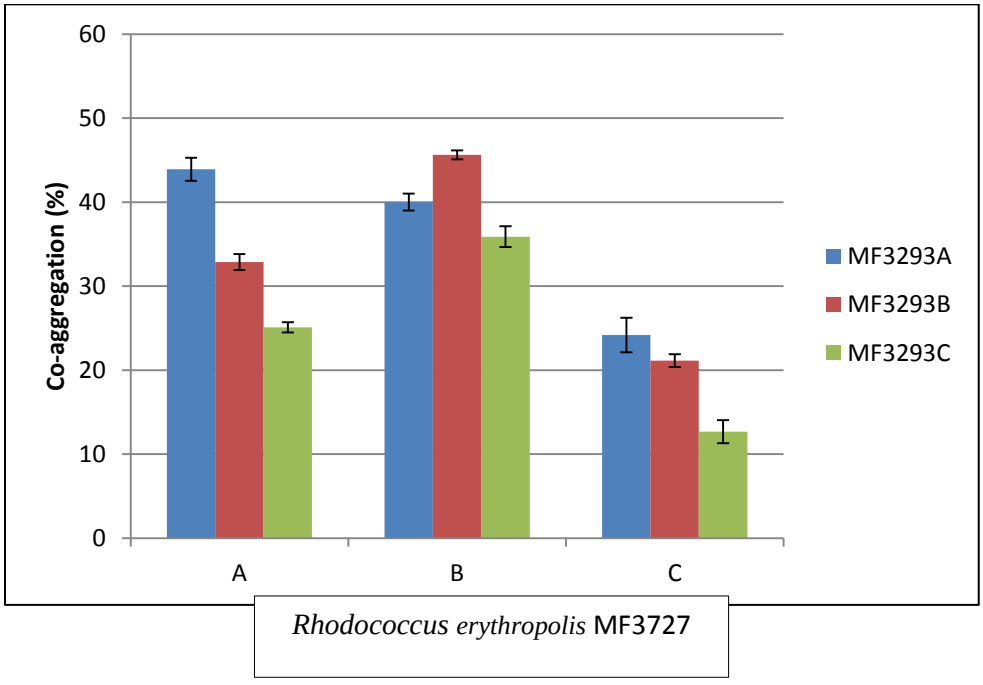


Figure 9.6 Co-aggregation (percentage) between *Rhodococcus erythropolis* MF3727 and *Acinetobacter calcoaceticus* MF3293 sampled from cultures in R2A medium at 20^{o} C in different growth phase. A; OD600nm=0.5, B: 18hr., C: 42 hr. Error bars represent standard deviations from three independent replicates.

9.7 Effect of heat treatment on co-aggregation

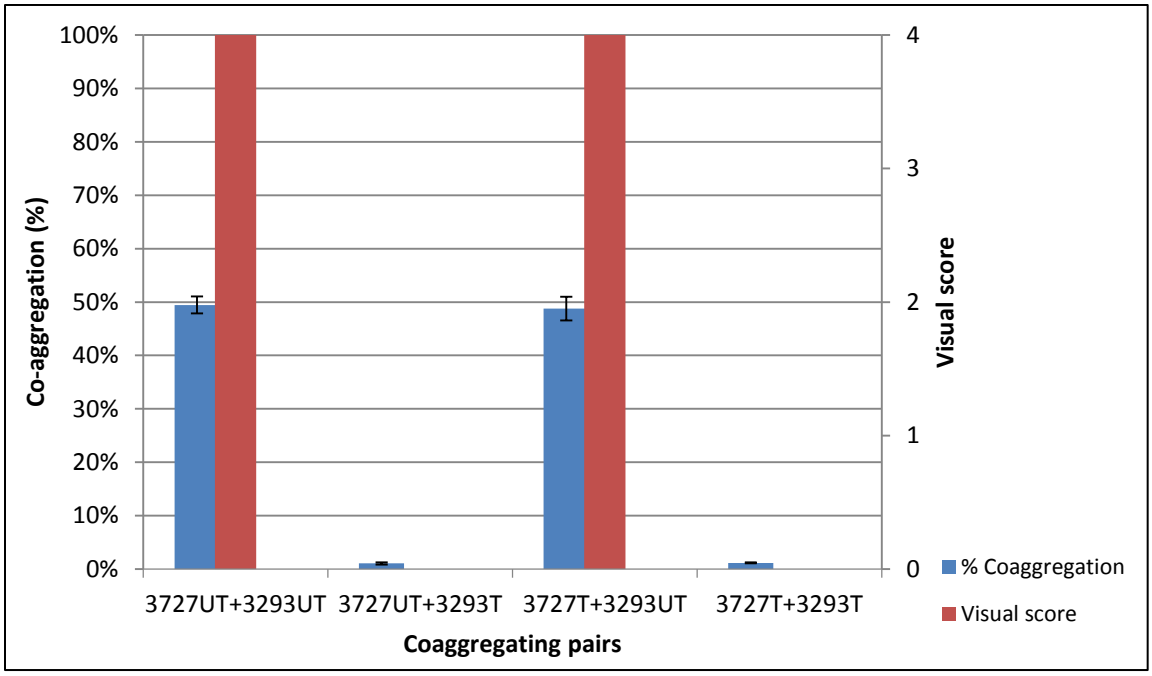


Figure 9.7 Effect of heat treatment on co-aggregation between *Rhodococcus erythropolis* MF3727and *Acinetobacter calcoaceticus* MF3293. All cells were cultivated at standard condition (TSB, 30^{o}C, 18hr) and re-suspended in co-aggregation buffer prior to the test. T: heat treated; UT: Untreated, no heat treatment. Error bars represent standard deviations from three independent replicates. In the case of visual assay, the same results were obtained from three independent replicates.

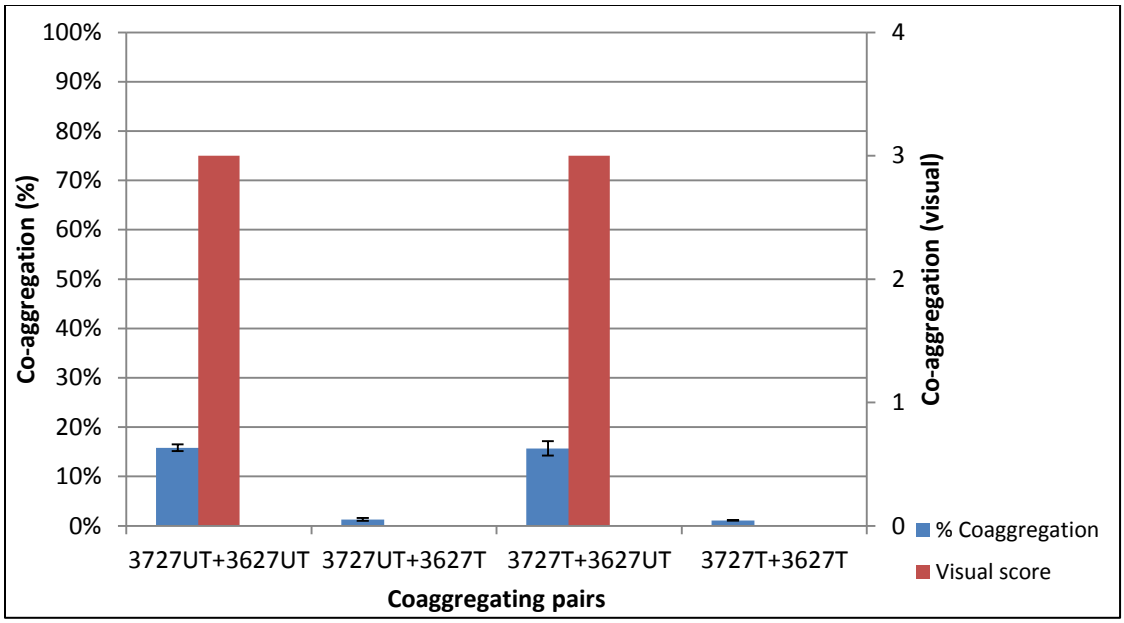


Figure 9.8 Effect of heat treatment on co-aggregation between *Rhodococcus erythropolis* MF3727and *Acinetobacter calcoaceticus* MF3627. All cells were cultivated at standard condition (TSB, 30°C, 18hr) and re-suspended in co-aggregation buffer prior to the test. T: heat treated; UT: Untreated, no heat treatment. Error bars represent standard deviations from three independent replicates. In the case of visual assay, the same results were obtained from three independent replicates.

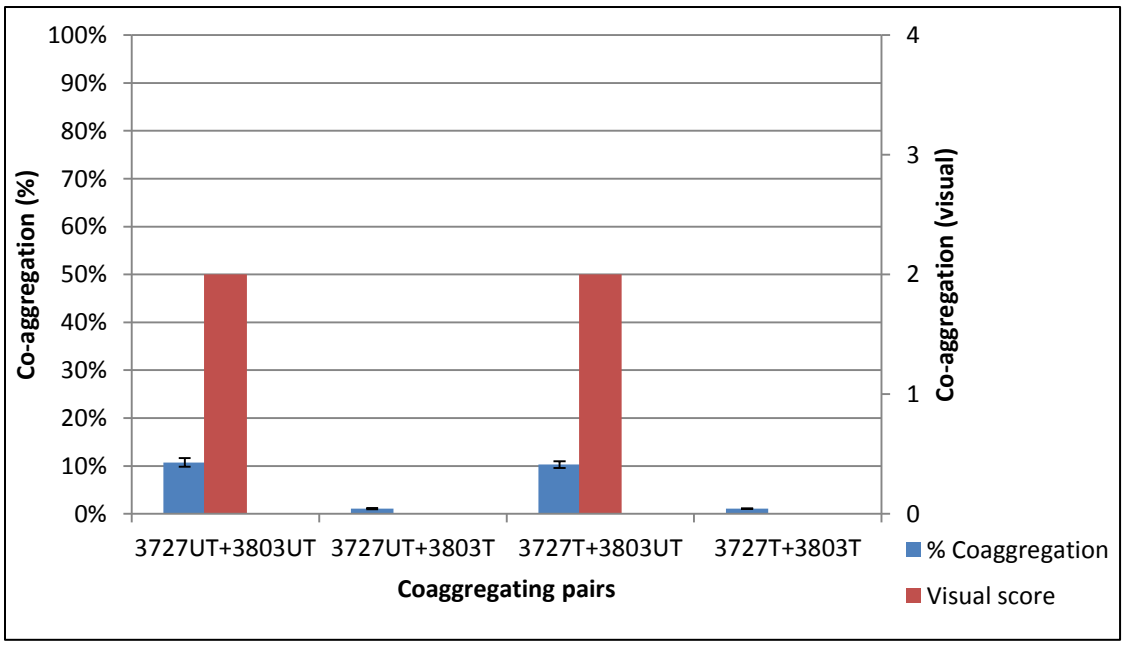


Figure 9.9 Effect of heat treatment on co-aggregation between *Rhodococcus erythropolis* MF3727and *Rhodococcus erythropolis* MF3803. All cells were cultivated at standard condition (TSB, 30°C, 18hr) and re-suspended in co-aggregation buffer prior to the test. T: heat treated; UT: Untreated, no heat treatment. Error bars represent standard deviations from three independent replicates. In the case of visual assay, the same results were obtained from three independent replicates.