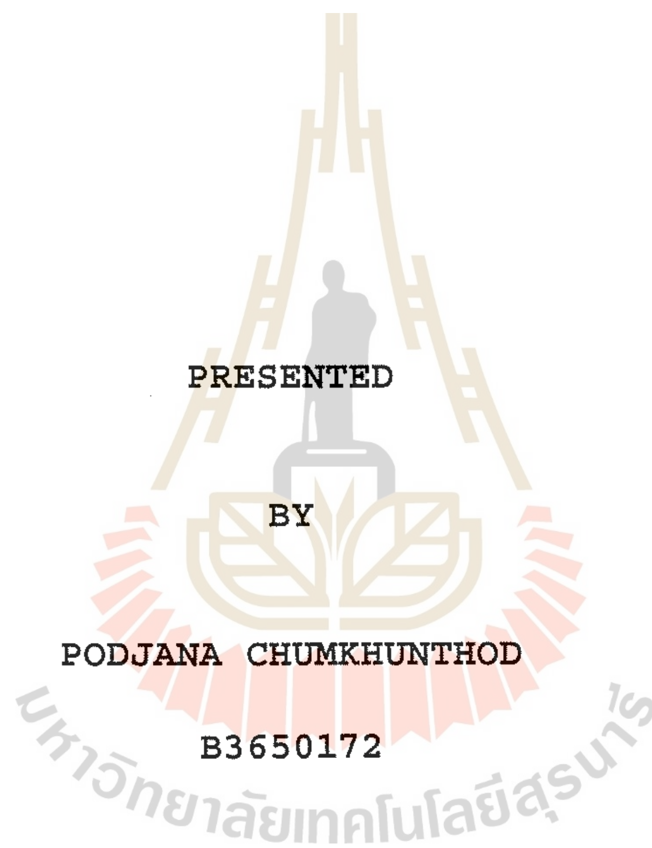
CO-OPERATIVE WORKTERMS REPORT



PRESENTED

BY

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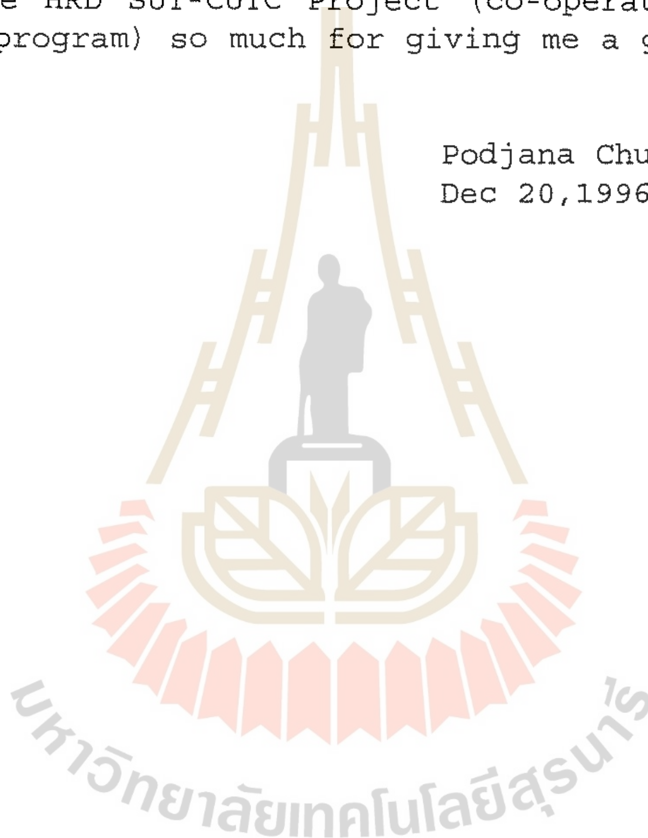
Preface

This report were created during the author was doing training in food science department, University of Guelph, Ontario, Canada in the area of Food Microbiology , Co-operative student program during June 3 to Dec 20, 1996. Most of informations involve about job description, organization of department, the results of work term co-operative, they include experience which the author got during that period. The author think that it will be useful for someone who is interested in Modern technology in Food Microbiology and for the co-operative students of the next generation to gain ideas about the co-operative program.

Thank you The HRD SUT-CUTC Project (co-operative education student exchange program) so much for giving me a good chance to come to Canada.

Podjana Chumkhunthod
Dec 20,1996



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1. The organization of Food Science Deparment University of Guelph, Ontario, Canada.

Chair--Marc LeMaguer
Graduate co-ordinator-Rickey Y.Yada
Graduate secretary--Margaret Walmsley

Graduate Faculty:
Shai Barbut--Associatate Professor
Douglas G. Dalgleish--Professor and Ontario Dairy Council and Industrial Reseach Chair in Dairy Technology
H. Douglas Goff--Associate Professor
Mansel W.Griffiths--Professor and Ontario Milk Marketing Board and Industrial Reseach Chair in Dairy Microbiology
Schraft Heidi--Associate Professor
Linda J,Harris--Associate Professor
Arthur R.Hillnd--Associate Professer
Yukio Kakuda--Associate Professor
Marc LeMaguer--Professor
Robert W.J.Lencki--Assistant Professor
Alejandro G.Marangoni--Assistant Professor
Ronald E.Subden--Professor
Howard J.Swatland--Professor
Marvin A.Tung--Professor and Weston Foundation Food Packaging Technology Chair
Rickey Y.Yada--Professor

Associated Graduate Faculty:
Jose M.Aguilera--Professor, Chemical Engineering, Universidad Catolica de Chile,adjunct Professor
Norman Ball--University of Waterloo
Malcolm C.Bourne--Professor, Food Science and Technology, Cornell University
Carole Buteau
Robert C.Clarke--Health of Animals Laboratory,Agriculture Canada Guelph
Robert H.Coffin--Cavendish farms,Summerside P.E.I.,Adjuct Professor
Valerie J. Davidson--Adjunct professor
Christopher J.Findlay--Compusense Inc., Guelph
Beniot Girard--research Scientist, Agriculture and Agri-Food Canada, Summerland, BC
Elizabelt A.K. Gullett--Adjunct Professor

Michael J.Hincks--ORTECH International, Mississauga
Herbert O.Hultin--University of Massachusetts marine Station,Gloucester
Basil S.Kamel--Vittoria, Ontario
Robin C.Mackellar--Agriculture and Agri food Canada, Ottawa
H. Wayne Modler--Agriculture and Agri Food Canada, Ottawa
Vladimir F.Rasper--Professor Emeritus and Adjunct Professor
David W. Stanley--Adjuct Professor
Samuel Wang--Vineland Research Station Adjunct Professor
O.P. Ward--University of Waterlooo, Adjunct professor

1.1 Organization Chart of Food Science Deparment University of Guelph, Ontario, Canada

Food Science------>

------>**Dairy**: Mansel W. Griffiths
Douglas G. Dalgleish
Arthur R. Hill
H. Douglas Goff

------->**Meat**: Shai Barbut
Howard J. Swatland

------->**Microbiology**: Mansel W. Griffiths
Schraft Heidi
Ronald E. Subden

------->**Chemistry**: Alejandro G. Marangoni
Rickey Y.Yada
Yukio Kakuda

------->**Physic/Material Science**:
DouglasG.Dalgleish
H. Douglas Goff
Marvin A. Tung
David W. Stanley

------->**Engineering**: Marc LeMaguer
Robert W.J. Lencki
Valerie J. Davidson

1.2 Coworkers in Food Microbiology Laboratory

Dr. Mansel Griffiths-Professor
Dr. Heidi Schraft-Assistant Professor
Dr. Jinru Chen-Research Association

Ph.D Students
- Lynn Mcintyre
- Derrick Bautista
- Reem Barakat

M.Sc. Students
- Stacy Favrin
- Andy Buechin
- Stephane Cadieux
- Shirley Lin

Undergraduate Research Student
- Suzanne Smith

Technician
- Yolanda Hirvi
- Ann Toner
- Sukhvinder Kaur

International Co-operative students
- Podjana Chumkhunthod from Thailand
- Hedwig Kasstra from Netherland
- Bianca Brunke from Germany

2. Outline of co-op workterm project

Main Project

"Rapid Monitoring Method to Assess Efficacy of Sanitizers against *Pseudomonas putida* Biofilms"

Part I Biofilm Production.

Part II Evaluation efficiency of 2 biocides to damage biofilm.

Special experiment

"Use of molecular biology to confirm the locations of the lux gene on the bacterial DNA chromosome."

consist of:

- DNA extraction
- Agarose gel electrophoresis
- RNA digestion
- Restriction of DNA
- Southern Blot Membrane
- Southern Hybridization
- Detection of DIG-labeled Nucleic Acids

....

2.1 Biofilm Production

-Test cultures

Five luminescent clone strains of *Pseudomonas putida* were used in this experiment.

objectives:

1. Identify clones with high luminescence and low luminescence.
2. Relate RLU (relative light units) reading to actual cell number by using luminometer and total viable count.
3. Make correlation curve by using Quatto Pro (computer program).

-Biofilm formation

objectives:

1. Try to produce biofilm on rubber gaskets.
2. Test efficiency of 3 different media for biofilm formation.
3. Previous information about clones.

Several methods are used to monitor and check biofilm formation.

- Vortex and ultrasound
- Luminometer
- Epifluorescence microscopy (EFM)
- Scanning electron microscopy (SEM)
- Viable count
- Bactometer
- CCD (charge coupled device)

2.2 Evaluation efficacy of 2 sanitizer to damage biofilm

objectives:

1. Test efficacy of sanitizers commonly used in dairy plant to biofilm of *Pseudomonas putida*.
2. Preform using bioluminescence for rapid microorganism monitoring to access efficacy of sanitizing compare with traditional plate count.

Two types of sanitizer are used for treatment.

- Non-foaming Acidic sanitizer (Divosan Activ)
- Liquid hypochlorite sanitizer (Dibac)

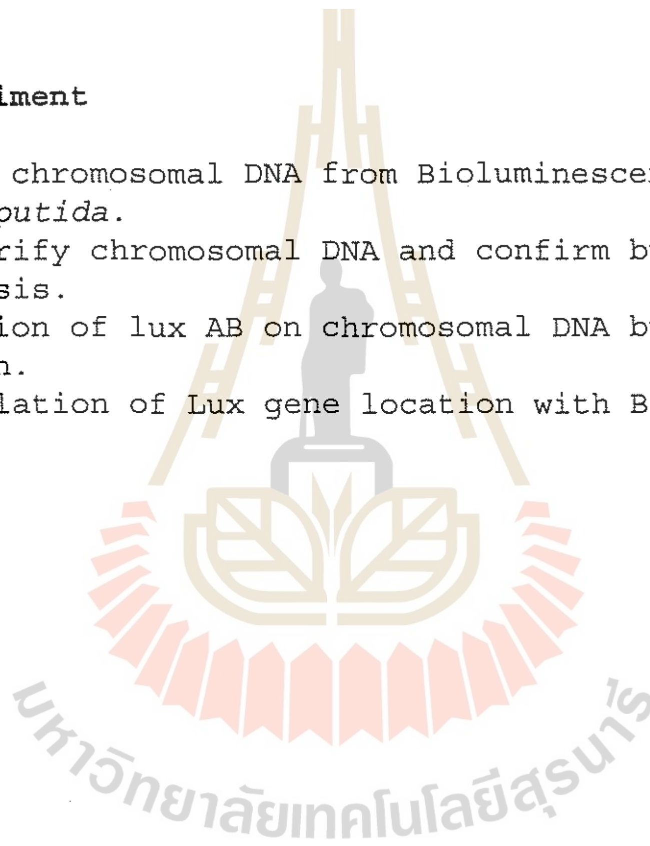
Monitoring methods

1. Bioluminescence
2. Aerobic plate count. In this case, Spiral plating are available.

2.3 Special experiment

Objectives:

1. Try to extract chromosomal DNA from Bioluminescence strains of *Pseudomonas putida*.
2. Isolate and Purify chromosomal DNA and confirm by Agarose Gel Electrophoresis.
3. Identify location of lux AB on chromosomal DNA by Southern Hybridization.
4. Previous Correlation of Lux gene location with Biofilm Production.



3. Rapid Monitoring Method to Assess Efficacy of Sanitizers against *Pseudomonas putida* Biofilms.

3.1 Abstract

Biofilms of luminescent *Pseudomonas putida* were developed on rubber surfaces by incubation in brain heart (BHI) infusion broth. Scanning electron microscopy (SEM) and Epifluorescence microscope (EFM) were used to examine biofilm formation. To test efficacy of two sanitizers commonly employed in dairy plants for CIP (Clean In Place) procedures, bioluminescence and aerobic plating were used to enumerate cell numbers. Immediately after the sanitizer treatments, an apparent 5 log reduction of biofilm associated cells was determined. However, when the samples were resusicitated for 18 h in BHI broth, high numbers of cells were detected which reached levels close to those of non treated controls. Results demonstrated that neither sanitizer could completely eliminate biofilm associated *P. putida*. The novel method, microbial bioluminescence, proved to be the best way for assessing efficiency of sanitizers against microbial biofilms.

Keywords: *Pseudomonas putida*, bioluminescence, biofilm, sanitizers.

3.2 Introduction

Bacterial attachment to solid surfaces and biofilm formation are growing to be a serious concern in the food industry (27). It is recognized that bacterial adhesion can be a potential source for food contamination affecting quality and safety of products (3, 16). Biofilms can be formed on a variety of materials commonly used in food plants. These food contact surfaces include glass, stainless steel, polypropylene, rubber and polytetrafluoroethylene (2, 3, 16, 21, 22, 24, 29). Although these materials appear to be smooth, they may have cervices or small cracks that could harbor bacteria which may shed into the food processing environment (3, 21). It has been documented that biofilm cells have a higher resistance to antimicrobial agents than bacteria in culture suspensions because they are embedded in extracellular substances which protect cells from direct sanitizer exposure. Thus, bacteria may survive on the surface of equipments after cleaning and sanitizing (8, 17, 24, 30).
To assess efficiency of sanitizers against biofilms, bacterial

bioluminescence could be a great choice to monitor survival of microorganisms (28). Bioluminescence is closely related to the basic metabolism of bacterial cells. It involves reducing a flaven mononucleotide which is catalyzed by the enzyme luciferase that is responsible for light emission (4, 10, 20). This reaction needs a long chain fatty acid aldehyde as substrate. Bacteria can be transformed to a luminescent phenotype by using genetic engineering to insert the luciferase gene (*luxAB*). The amount of light emitted by such bacteria is directly proportional to cell numbers and can be rapidly monitored by a luminometer. Many studies have demonstrated that the most dominant genus of the psychrotrophic microflora present in milk processing plants is *Pseudomonas* which is well known as a biofilm producer. In this case study, bioluminescent *P. putida* were used to form biofilms and subsequently treated with sanitizers commonly employed in dairy plants. Efficiency of the sanitizer treatments was evaluated by traditional plate counting and by using the novel method of bioluminescence. The goals of the study were i) evaluation of the efficacy of sanitizers commonly used in dairy plants against microbial biofilms, ii) to examine bacterial attachment of luminescent *P. putida* on Butyl rubber surfaces, iii) to compare advantages and disadvantages of plate counts and bioluminescence as enumeration methods and iv) to examine effect of post treatment resuscitation on the detection of surviving bacteria.

3.3 Materials and Methods

3.3.1 Bacterial cultures

Pseudomonas putida LV 2-4, which had been isolated from biofilm in a milk processing line by Dr. J. Austin, Bureau of Microbial Hazards, Ottawa was used in this study. To obtain a luminescent phenotype, *luxAB* was introduced into the chromosome of *P. putida* LV 2-4 by using the plasmid PT 7-5 according to the procedures described by Chen and Griffiths (5a). One clone, emitting a strong bioluminescent signal while still maintaining its ability to form biofilms was used in this study. Cultures were grown in brain heart infusion (BHI) broth at 30°C overnight.

3.3.2 Biofilm Formation

One mL of an overnight culture of *P. putida* LV 2-4-lux was inoculated into a sterilized flask containing 10 mL of BHI broth

and 3 rubber septa (Butyl rubber/polytetrafluoroethylene, materials commonly used in dairy plants). Inoculated flasks were incubated at 22°C with 70 rpm shaking. Every 12 h the growth medium was replaced with 10 mL of fresh BHI broth (22). After 1, 2, and 3 days, samples were aseptically removed from the flasks and treated with various sanitizers.

Biofilm formation was veryfied using scanning electron microscopy (SEM), epifluoresence microscpoy (EFM) and enumeration by plating after removal of bacteria from the surfaces. For SEM, the sample surfaces were prepared by fixing the biofilm cells with standard buffer (0.07 M KH_2PO_4, 0.07 M Na_2HPO_4) containing 2% glutaraldehyde overnight at room temperature. Samples were then rinsed for 10 min. in standard buffer and dehydrated in following an ethanol series. Dehydration was completed by critical control point drying with CO_2. Samples were coated with Au/Pd 30nm with a Hummer VII sputter coater (Anatech JTD Alewandria, VA) and viewed in a scanning electron microscope (Hitachi S-570, Tokyo, Japan) at 50 KV.

For EFM the samples were stained with 1% acridine orange for 1 min. At room temperature and mounted onto a microscope slide. Attached cells were viewed in an epifluorescent microscope with a 100x oil immersion objective.

3.3.3 Enumeration of bacteria

Viable cell counts were determined by surface plating on BHI agar using a spiral plater (Spiral systems). Incubation was at 30°C for 24, 48 and 72 h. Bioluminescence which has a linear correlation to viable cell counts was determined as follows: One mL of the bacterial suspension was thoroughly mixed with with 10 μL of 1% n-decanal and light emission was determined in a luminometer.

3.3.4 Effects of sanitizer treatment on biofilms

Two types of sanitizers obtained from Diversey Inc. (Mississauga, ON) were tested: i) a non foaming acidic sanitizer (Divosan Activ) and a liquid hypochlorite sanitizer (Dibac). Working solutions were prepared according to the directions of the manufacture. Phosphate buffered saline (PBS [0.14M NaCl, 0.003M KCl, 0.01M Na_2HPO_4 and 0.002M KH_2PO_4])+ 2% Tween 80, was used as neutralizer for Divosan Activ. The hypochlorite sanitizer (Dibac) was neutralized with 0.01 M sodium thiosulfate.

At the end of the incubation period, each rubber septum was

aseptically removed from the culture flask and rinsed 10 times in 50 ml of PBS to remove any unattached cells. After rinsing, the rubber septa were cut in 4 sections by a sterile scalpel and each segment was immersed in 10 ml of sanitizer solution for 30 sec at 25°C. Activity of the sanitizer was stopped in neutralizing solution for 5 min. Then the samples were transferred into PBS with 2% Tween 80 solution and biofilm cells were removed from the surface by vortexing for 2 min at maximum speed. The number of viable cells was determined in each sample before and after vortexing by spiral plates and luminometer. To evaluate survival of injured cells, the entire sample after vortexing (rubber septa and PBS+Tween 80 suspension) was transferred into 5 ml of BHI broth and incubated at 30°C. After 2 and 18 h bioluminescence of resuscitated cells was determined. For control samples, sanitizer and neutralizing solutions were replacedwith PBS; otherwise treatments were performed identical to the test samples.

3.4 Results

3.4.1 Biofilm formatiom

Bioluminescent *P. putida* grew well in BHI broth at 30°C and 22°C. Growth rates were identical for the parent and the bioluminescent strain. The cell numbers necessary to detect a positive luminescence signal were at 10^4 CFU. Preliminary experiments using a variety of culture media and conditions had shown that incubation at 22°C with shaking and regularly replacing the culture medium improved biofilm formation (data not shown). Formation of biofilms on the surface of the rubber septa after incubation for 1, 2 and 3 days was visually confirmed by EFM and SEM (Figure 1). Cells present on the rubber septa which had not been removed by rinsing were considered to be adherent. These adherent cells were readily removed by vortexing with PBS+Tween 80 for 2 min. Quantification of attached cells, determined by subtracting the cell numbers before vortexing from the number of cells detected after vortexing, showed the number of biofilm associated bacteria to be between 10^4 and 10^5 CFU/cm². Accordingly, the value of light emission was between 2 and 200 RLU /cm². The number of cells adhering to the surfaces did not significantly change over a 24 to 72 h period. Different ages of biofilms did not result in different numbers of attached cells.

3.4.2 Sanitizer treatment

After biofilm cells were exposed to two types of commercial sanitizers with the maximum concentration recommended by the chemical manufacturer for 30 sec contact time, biofilm associated bacteria were reduced by more than 5 log cycles. No apparent differences in cell reduction were observed for 1-day, 2-day or 3-day old biofilms. There was no visible difference between sanitizer efficiency assessed with CFU or RLU. This would indicate that the sanitizers were effective for eliminating the biofilm associated *P. putida*. However, after the treated biofilm surfaces were resuscitated in fresh media of BHI and incubated at 37°C by shaking for 2 h, luminescence was again detectable. This light emission increased markedly after a prolonged incubation of the samples for 18 h.The RLU values increased to almost as high numbers as the non treated biofilm controls. Different ages of *P. putida* biofilms did not give different results and no marked difference in efficacy was observed between the non-foaming acidic sanitizer and the liquid hypochlorite sanitizer.

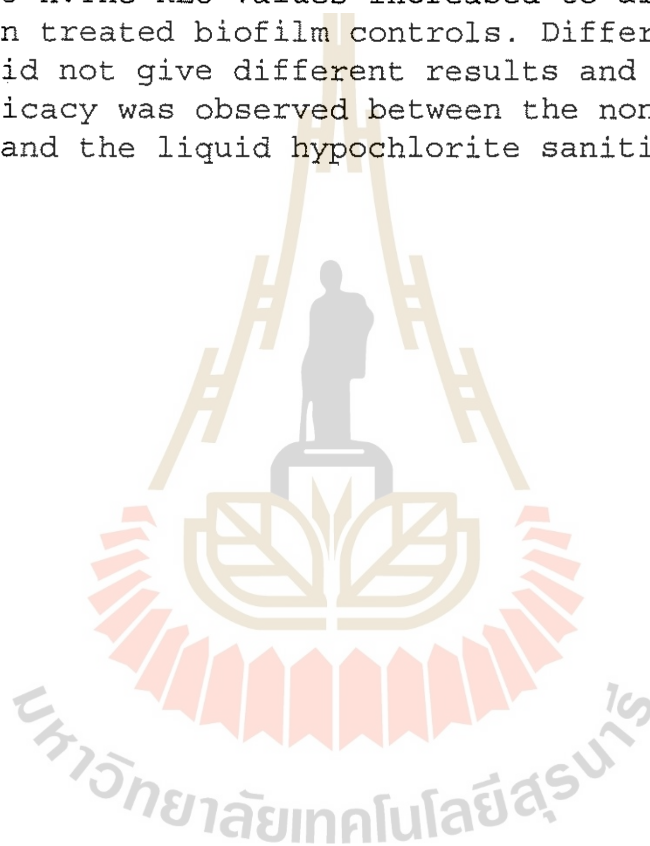


Figure 1: Scanning Electron Microphotograph of *P. putida* biofilm after 1 day incubation

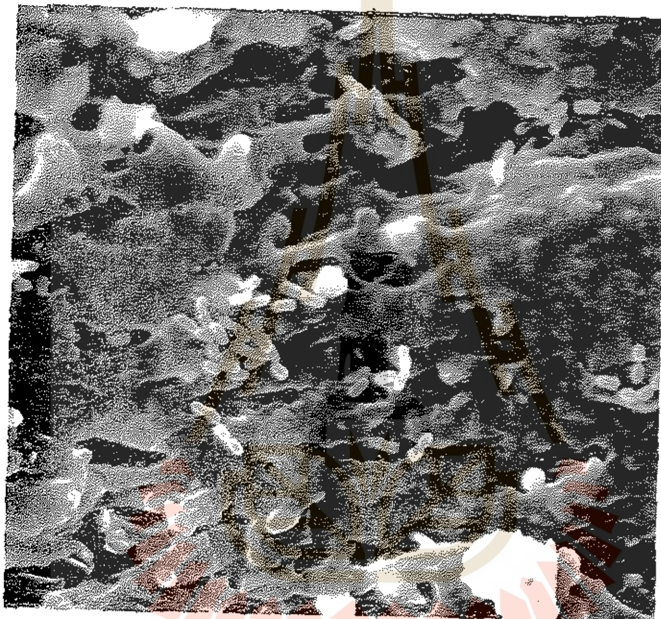


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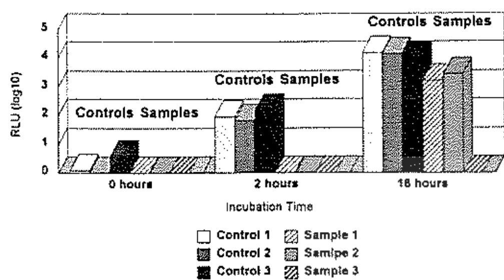
Figure 2: Effect of two commercial sanitizers against biofilm associated *Pseudomonas putida*

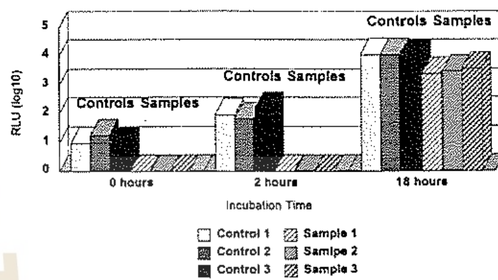
Non-Foaming Acidic Sanitizer

Liquid Hypochlorite Sanitizer

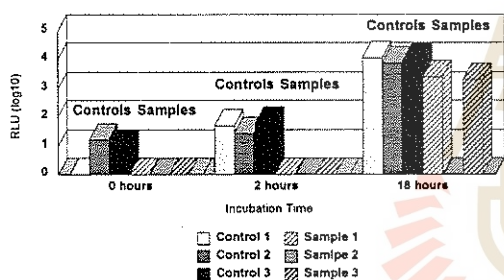
Biofilm 1 day old



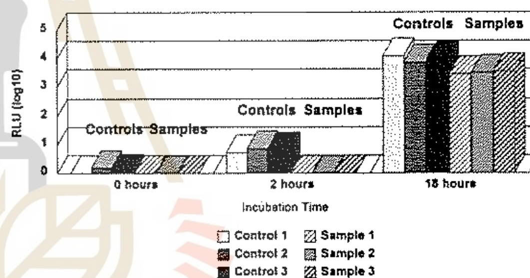
Biofilm 1 day old



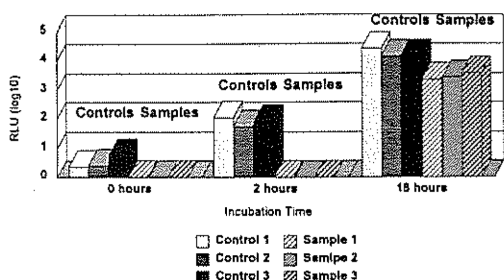
Biofilm 2 days old



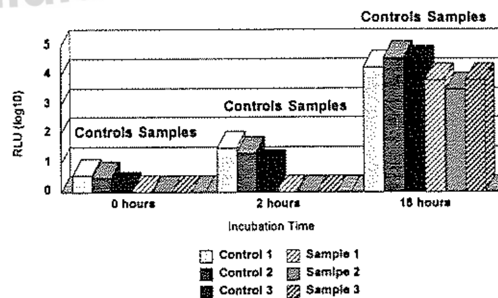
Biofilm 2 days old



Biofilm 3 days old



Biofilm 3 days old



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3.5 Discussion.

The bacterial strain used in this study was obtained directly from a milk processing plant. Thus, the population of *P. putida* used could be considered a significant source of milk contamination. This research demonstrated that under circumstances close to those in food processing plants, *P. putida* can grow and form biofilms on rubber surfaces within 24 h.

Surface irregularities such as roughness, crevices and pits have been shown to increase bacterial adherence by increasing cell attachment and reducing the abilityto remove cells (1). In our study, we chose butyl rubber as contact surface. Visualisation of biofilm by SEM illustrated theoriginal rubber surface displayed a lot of crevices and small holes. In these regions most attached cells were found. It was wondering that very little of extracellular matrix (ECM) which believed to be polysaccharide in nature that may protect cells from antibacterial agents appeared.(24) It was possible that BHI broth are not rich media. Cell were starved and decreased polysaccharide production or that there were many porous on rubber surface which maked it easy for cells attached so it was not necessory to produce much ECM aid for adhesion. The other way was used to viewed biofilm formation is epifluorescence microscope.

Although Scanning electron microscope and epifluorescence were able use for confirm biofilm formation, visual biofilm structure or the presence of ECM. But they did not give quantitive results. So Spiral plating system was investigated for enumeration in this case study. However, viable plate count takes a long time and needs labour. This work we tried to use bioluminescence for asssessing efficacy of sanitizer treatment too. Luminometer was used compare with spiral plate count. Results indicated that bioluminescence had more efficiency than traditional plating because it needed only short time for whole procedure, did not need much labour and could used to measure while there were only small amount of sample left. Beside that, we found that there were some common problem from traditional plating. One was arised when cell were removed from surface is that large clumps. It might be not dispered. This leaded to be a source of error because cells cound not form colony on the agar surface. It was difficult to count accurately cells and the total number of cells might be unestimated.(13) The other point, Our study were unable to get accurratly results from enumeration by spiral plating in rescusitation step more than bioluminescence

method because there were injuried cells unable to grow on BHI agar but still alived.

Results of works conducted by many researchers suggested that the resistance of microorganisms were affected by the ages of biofilm (3, 31). This study 1-day, 2-day and 3-day old biofilms were tested for sanitizer treatment with two commercial sanitizer. After 30 sec exposure time, the results of measuring showed that no different marks of efficiency between non forming acidic and liquid hypochlorite sanitizer on various ages of biofilm, probably the attachment and biofilm development were different in processes and culture.

In conclusion, This work has shown that (a) the behavior of biofilm cells in extracellular production was affected by the type of surface and nutrition media. (b) This research supposed with the earlier reports that the sanitizer efficacy was low in porous surfaces such as rubbers. (c) Engineering a lux gene construction into food isolated of Pseudomonas putida provided rapid monitoring method for assessing efficiency of sanitizer treatment. (d) Both sanitizers seemed to be effective to kill bacteria adherence after treatment but after injuried were enriched with fresh media, they can survived and reproduced themself quickly. So we concluded that non forming acidic and liquid hypochlorite sanitizer could not eliminate the biofilm associated Pseudomonas putida cells.

It's difficult to remove the biofilms completely from the surface and regular cleaning and sanitizing still be the best way to prevent biofilm. So the frequency and adequacy times of sanitizing should be important feasible point for controlling microbial attach in food processing plant.

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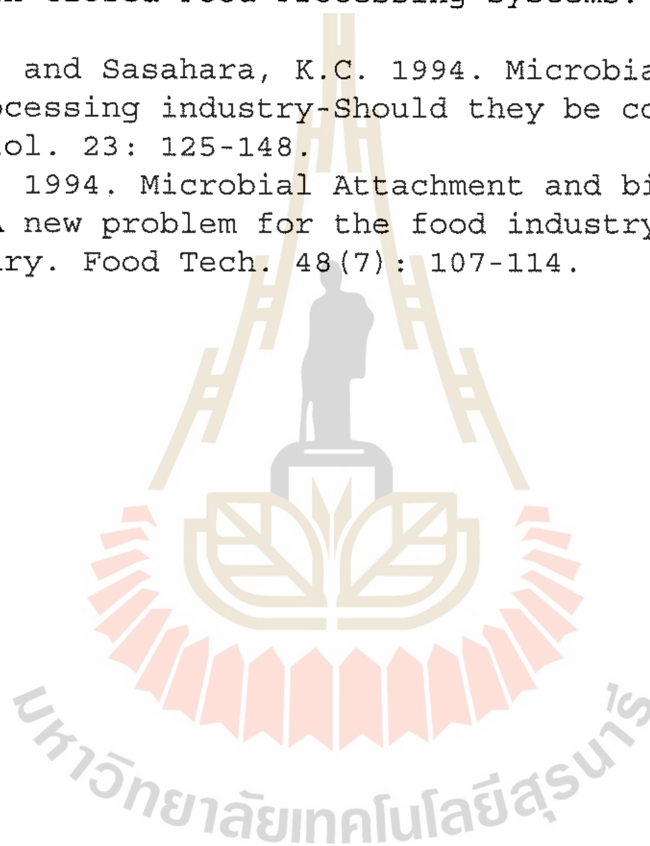

4.Special Experiment

"Use of molecular biology to confirm the location of the lux AB on the bacterial chromosome"

4.1 Objectives

1. Learn about molecular biology
2. Perform several steps to identify the location of lux gene of Pseudomonas putida in clone 4-8.
   2.1 DNA extraction
   2.2 DNA detection by Gel electrophoresis
   2.3 RNA digestion
   2.4 Restriction of DNA
   2.5 Southern transfer
   2.6 Southern hybridization
   2.7 Detection of DIG-labeled nucleic acids
3. Previous information of each clone.
4. Conclusion correlation of location of lux gene with biofilm production.

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4.1.1 DNA Extraction

objective : Extract chromosomal DNA from Microorganism.

Materials & methods

- Bacteria cones 4-8 of *P.putida* are cultured in BHI broth to be sampled
- Take 6-10 ml of bacteria culture in centrifuge tubes and centrifuge at 5 degree c for 15 min.
- Suspend pellet in 800 ml of 6.7% sucrose by vortexing.
- Add 50 microlites of lysozyme (10 mg/ml and shake at 37 degree c for 30 min).
- Add 10 microlites of proteinase K (20 mg/ml and shake at 37 degree c for 30 min).
- Add 1 ml of Phenol/Chloroform/Isoamul alcohol (ratio, 25:24:1) underfumehood, shake quickly and then spin at full speed for 15 min by centrifuge at 5 degree c. After the first spin, the supernatant solution is collected.
- Add 1ml of Phenol/CH3Cl/Isoamyl (mix together).
- Spin full speed at 4 degree c for 15 min.
- 500 microlites are collected and tranferred to lock safe eppendorf vials.
- Add 50 microlites of Na-Acetate, pH 5.2 (0.1*volumn of last volume).
- Add chilled 100% ethanol (2 times of sample).
- Store overnight at -20 degree c
- Check for precipitate, spin down at maximum speed for 15 min at

4 degree c.
- Discard the supernatant
- Wash with 70% ethanol and spin again for 15 min, allow the pillet to dry. Then the pellet is resuspended in TE buffer, pH 7.6.
- Put it in the oven to dissolve for 1-2 hours at 42 degree c.
- Keep it in the fridge.

4.1.2 Agarose gel electrophoresis

Objective: Verify presence of chromosomal DNA.

Materials & Methods
- Agarose gel preparation (0.8% agarose in 1*TAE buffer)
- Add Ethidium bromide (10 mg/ml) and boil until it becomes clear.
- Cool down and pour in the gel chamber (make sure to set the comb ready).
- Immerse the gel in the chamber and cover with 1*Tris-Acetate-EDTA buffer (TAE buffer).
- Drop DNA sample (mixing solution of 2 micorlites of loading buffer, 2 microlites of sample and 5 microlites of distilled water).
- Run the gel at 50-100 volts for 45 min.
- Keep the remaining sample in the fridge for RNA digestion.

4.1.3 RNA digestion

Objective: Digest RNA

Materials & Methods
- Add 0.8 microlites of RNase in thawed sample and spin a little bit.
- Incubate at 37 degree for 2-3 hours.
- Keep at room temperature.
- Run gel electrophoresis for to verify complete digestion of RNA.

4.1.4 DNA restriction

Objective: Cut DNA chromosome into fragments

Materials & Methods
- Add 2 microlites of restriction enzyme (EcoRI), 1.5 microlites of buffer solution and 1.5 microlites of distilled water into tube containing 10 microlites of chromosomal DNA.
- Incubate at 37 degree c overnight
- Prepare agarose gel of appropriate composition (0.8%) a high purity nucleic acid grade agarose and a Tris-Acetate-EDTA

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(TAE) buffer.
- Run the gel. If desired, the gel may be stained with Ethidium bromide to visualize the DNA fragments and confirm subsequent Southern transfer to membrane.

4.1.5 Southern transfer

Objective: Transfer chromosomal DNA fracments from the gel to membrane.

Materials & Methods

- Depurinate for 5 min by submerging the agarose gel in 250 mM HCL while shaking at room temperature.
- Rinse the gel with distilled water.
- Submerge the agarose gel in denaturation solution for 45 min at room temperature. Shake gently. This incubation denatures the DNA target prior to transfer.
- Rinse the gel with distilled water.
- Submerge the gel in neutralizaton solution twice for 15 min at room temperature to neutralize the gel.
- Blot the DNA from the gel by capillary transfer to the membrane (Nylon membrane), using 10*SSC buffer.
- Change tissue after first 1-2 hours before leaving it overnight at room temperature.
- Rinse membrane briefly with distilled water.

DNA fixation

Objective: To fix the DNA on the membrane.

Materials & Methods

- DNA is efficiently bound to the membrane by UV-crosslinking for 5 min.
- The membrane can be used immediately for hybridization. In this case, it was stored dry at room temperature for future use.

4.1.6 Souther Hybridization

Objective: Hybridize DIG-labeled probe with immobilized target nucleic acid.

Materials & Methods

Prehybridization

- Place the blot in roller tubes containing 20 ml standard prehybridization solution and incubate in hybridization oven at 60 degree c for 1-2 hours.
- Heat probe in boiling water for 10 min in order to denature the DNA and chill immediately on ice.
- Discard the prehybridization solution out.

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Hybridization

- Add the hybridization solution containing the DIG-labeled probe and allow the probe to hybridize overnight at 60 degree c.
- At the end of Hybridization, pour the hybridization soluiton from the roller tube into a capped tube. Store the tube at -20 degree c. Hybridization solution can be reused several times.
- Wash the membrane twice, 5 min per wash, in 2x wash solution at room temperature to remove unbound probe.
- Wash the membrane twice, 15 min per wash, in 0.1x wash solution at hybridization temperature (long prove, >100 bp should be washed at 68 degree c).

4.1.7 Detection of DIG-labeled nucleic acids

Objective: To detect DIG-labeled Nucleic acids.

Materials & Methods

- After hybridization and Post hybridization wash, equilibrate the membrane in buffer 1 for 1 min.
- Block the membrane by gently agitating in buffer 2 for 30-60 min.
- Incubate for 30 min in Antibody solution (containing 10 ml of 1% Blocking and 2 microlites antibody fragments)
- Discard the antibody solution twice for 15 min in buffer 1 (buffer 1 plus 0.3% Tween 20).
- Pour buffer 1 off and equilibrate the membrane in buffer 3 for 5 min
- Add lumigen PPD 5'(dilute 1:100 in buffer 3), wet membrane throughly for 5 min.
- Incubate in plastic seal at 37 degree c for 10 min.
- For detection, the membrane is measured by charge coupled device(CCD) camera.

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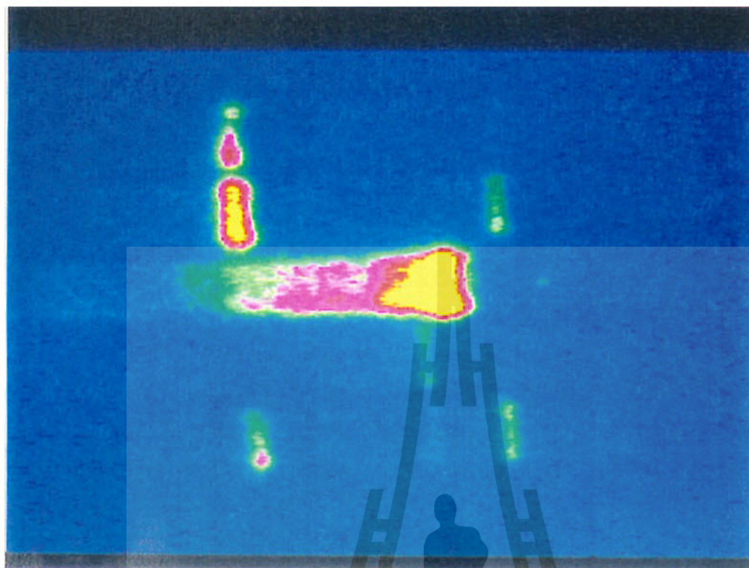
4.2 Results and Discussion

After membrane was visualized by charge coupled device (CCD), the location of lux AB were performed. For each clone the location of lux gene are different spot. They were shown in picture 1.



clone 4
clone 5
clone 6
clone 7
clone 8

Almost every clones were able identify the location of lux AB in chromosomal DNA. Except clone 7, the location was not good performed. It is possible that supernatant collection was not done completely in DNA extraction step so there wer some chromosomal DNA got lost. There are small amount left. When it was detected with DIG-labeled nucleic acids. Low amount of light emission appeared by CCD camera.

4.3 Conclusion

- The location of the lux AB on chromosomal DNA were able to express.
- Each clone of *P. putida* (clone 4,5,6,7,8) have different location of lux AB.

4.4 Reference

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5. Summery all activities during work term.

5.1 Working in Laboratory for main project and special project every day.
5.2 Participate in Laboratory Meeting every two weeks and in the lab party each times.
5.3 Presentation of project in Laboratory Meeting.
5.4 Volunteer in Food Science Conference, Guelph, Ontario, Canada.
5.5 Traveling to Montreal, Quebec and Boston with co-workers from the Labolatory as a vacation.
5.6 Traveling around Ontario during Holidays.

Adventages

- Learning more culture and traditional of other country especially Canada.
- Improving adjustment for new surrounding
- Learning more about laboratory technique.
- Solving various problems with myself.
- Developing idea and knowledge in various areas.
- Improving English language.
- Learning more about using libraly such as using libraly database computer search the specific information.

6. Recommendation for co-op program.

International trainee student should have international student identification card to identify themself.

ACKNOWLEDGEMENT

Thanks you Dr.Mansel Griffith so much for accepting me to be trainee student in his laboratory and support everthing for all project.

Thanks Dr. Heidi Schraft for everthing her teaching and training all the time I was in Canada.

Thank you very much

The author

Appendix

Solutions required for Southern Blotting are listed below. Refer to Appendix C for details on preparing the additionally required solutions.

Solutions	Description
HCl	250 mM HCl.
H_2O	Sterile, distilled water.
Denaturation solution 1	0.5 N NaOH, 1.5 M NaCl.
Neutralization solution 1	0.5 M Tris-HCl, pH 7.5; 3 M NaCl.
20 x SSC buffer	3 M NaCl, 300 mM sodium citrate; pH 7.0.
5 x SSC buffer	750 mM NaCl, 75 mM sodium citrate, pH 7.0.
Standard prehybridization buffer	5 x SSC, 1.0% (w/v) Blocking Reagent for nucleic acid hybridization, 0.1% N-lauroylsarcosine, 0.02% sodium dodecyl sulfate (SDS). When using RNA probes, add formamide to 50% and increase Blocking Reagent to 2 (w/v)*.
Standard hybridization buffer	DIG-labeled probe diluted in standard prehybridization buffer.
2 x wash solution	2 x SSC containing 0.1% SDS.
0.1 x wash solution	0.1 x SSC containing 0.1% SDS.

* Added from the Blocking Reagent stock solution (100 mM maleic acid, 150 mM NaCl, pH 7.5, containing 10% (w/v) Blocking Reagent for nucleic acid and hybridization). See "Preparation of Additionally Required Solutions and Buffers" in Appendix C.

(Boehringer Mannheim Biochemica, 1993)

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