

Copyright is owned by the Author of the thesis. Permission is given for a copy to be downloaded by an individual for the purpose of research and private study only. The thesis may not be reproduced elsewhere without the permission of the Author.

The Adhesion Force Study of Dairy Thermophile *Anoxybacillus flavithermus* CM with Atomic Force Microscopy

A thesis presented in partial fulfilment of the requirements for the degree of

Master of Engineering
in
Chemical & Nanotechnology

at Massey University, Manawatu,
New Zealand

Mohd Salihin Mohd Saidi

2014

Abstract

Anoxybacillus flavithermus is a common species of thermophilic bacteria discovered in most milk powder manufacturing plants through out New Zealand. The contamination of it's spores into the finished milk powder is an on-going problem as these spores are able to survive the sterilization process. Cheating death, *A. flavithermus* spores were then believed to attached on the stainless steel surface piping of the production line and germinate into a mature bacteria. A single surviving spore could grow to produce more spores that eventually dislodged from the colony and deposited together with the packaged milk powder. Over the storage time, the contaminated product will gives an off flavor as it deteriorates from bacterial action within.

Currently, the applied cleaning method is by rinsing the target section with 1% sodium hydroxide & acid solutions before being flushed out to remove any microorganisms attached on the interior surfaces. However, it is not very effective in removing spores and there is very little information on the value of the spore's adhesion force on a stainless steel surface. With that in mind, the aim of this study is to determine a proper adhesion force value between a dairy strain spore, *A. flavithermus* CM and stainless steel surface using the Atomic Force Microscopy (AFM) system. Meanwhile, *Geobacillus stearothermophilus* ATCC 2641 which is also a thermophilic organism was used over the study for comparison purpose.

To measure the adhesion force under an Atomic Force Microscopy (AFM), the crude suspension was first purified using two-phase separation method. Polyethylene glycol (PEG) and phosphate buffer were used as the phase separation chemicals while 0.1% polysorbate 20 was added to the freshly purified spores' suspension to aid the imaging sequence under the AFM. All AFM imaging and force measurements were done in air and conducted using the silicon type CSG 11/Au cantilever. The crucial Force-Volume imaging was done on a 32x32 grid scan size (1024 samples) on a scan rate of 0.5 Hz.

It was calculated that a single *A. flavithermus* CM spore has an adhesive force value of 16.8 μN when attached on a stainless steel surface. It has a stronger localize adhesive value of 3.9 nN than a *G. stearothermophilus* ATCC 2641 spore with just 3.6 nN. However, *G. stearothermophilus* ATCC 2641 has a larger adhesive force of 21.1 μN on a stainless steel surface due to it's larger spore size. It was also found that spore's hydrophobicity does not dictates the magnitude of it's adhesion on any surface.

The results from this study have provide the dairy industry an extra sight on the quantitative value of the adhesion force of thermophilic spores, particularly *A. flavithermus* CM. This will help the dairy industry to design strategies in preventing spores from adhering to its production lines.

Contents

| | |
|---|----|
| Table of Figures..... | V |
| 1 Introduction: Literature Review..... | 1 |
| 1.1 Milk powder manufacture | 1 |
| 1.1.1 Milk powder manufacturing steps..... | 1 |
| 1.1.2 Hygiene in manufacturing plant – Clean-In-Place (CIP) | 3 |
| 1.2 Thermophilic contamination of milk powder in manufacturing plant..... | 3 |
| 1.2.1 Source of thermophiles | 3 |
| 1.2.2 Thermophilic spores \ endospores | 3 |
| 1.2.3 Dairy fouling | 5 |
| 1.2.4 Biofilms | 6 |
| 1.2.5 Pasteurization | 6 |
| 1.3 The phylogeny of genus Bacillus | 6 |
| 1.3.1 Anoxybacillus flavithermus..... | 6 |
| 1.3.2 Geobacillus genus: G. stearothermophilus and G. thermoleovorans..... | 7 |
| 1.4 Adhesion forces between bacterial spores & dryer bed | 7 |
| 1.4.1 Hydrophilic and hydrophobic surfaces | 7 |
| 1.4.2 Ionic charges / surface charges | 8 |
| 1.4.3 Cell-surface proteins | 8 |
| 1.4.4 Force on stainless steel..... | 8 |
| 1.4.5 Existing methods on removing or preventing spores in dairy product | 9 |
| 1.5 The Atomic Force Microscopy (AFM)..... | 9 |
| 1.5.1 Background and AFM today | 9 |
| 1.5.2 Biological Application of AFM | 10 |
| 1.5.3 AFM for imaging..... | 11 |
| 1.5.4 Imaging Substrates | 12 |
| 1.5.5 Imaging in air and liquid | 13 |
| 1.6 Adhesion force measurement with AFM..... | 13 |
| 1.6.1 Force spectroscopy | 13 |
| 1.6.2 AFM application in endospore studies | 14 |
| 1.7 Research Objectives: Atomic Force Microscopy on Anoxybacillus flavithermus & Geobacillus stearothermophilus..... | 15 |
| 2 Materials and Methods | 16 |

| | | |
|-------|---|------------------------------------|
| 2.1 | Source of bacterial isolates | 16 |
| 2.2 | Bacteriological methods | 16 |
| 2.2.1 | Media preparation and storage | 16 |
| 2.2.2 | Spore preparation and crude spore suspension | 16 |
| 2.2.3 | Spore isolation | 16 |
| 2.2.4 | Spore management and storage..... | 17 |
| 2.3 | Light Microscopy | 17 |
| 2.4 | Atomic Force Microscopy | 18 |
| 2.4.1 | Sample fixation..... | 19 |
| 2.4.2 | Height and deflection imaging..... | 19 |
| 2.4.3 | Force-Distance imaging | 20 |
| 2.4.4 | Force-Volume imaging | 21 |
| 2.5 | Data analysis and statistics | 23 |
| 2.5.1 | Measuring the adhesion force..... | 23 |
| 3 | Trials study – Results and discussion..... | 25 |
| 3.1 | AFM study of B.subtilis on stainless steel | 25 |
| 3.1.1 | Preparing monolayer spore lawn on substrate..... | 25 |
| 3.1.2 | Observing the stability of spore lawn under imaging condition | 27 |
| 3.2 | Purification of bacterial spores | 30 |
| 3.2.1 | Initial observation of crude spore suspension | 30 |
| 3.2.2 | Purification: Two-phase separation process | 31 |
| 3.3 | Spore size comparison of different species | 34 |
| 3.4 | Summary | 35 |
| 3.5 | From Force-Curve to Force-Volume imaging | 35 |
| 4 | Force-Volume imaging of A.flavithermus CM & G. stearothermophilus ATCC 2641 – Results and discussion | 40 |
| 4.1 | Force-Volume imaging of Anoxybacillus flavithermus CM with various tips | 40 |
| 4.2 | Imaging & Force-Volume of Anoxybacillus flavithermus CM..... | 44 |
| 4.2.1 | Imaging results..... | 44 |
| 4.2.2 | Statistical analysis..... | 50 |
| 4.3 | Imaging & Force-Volume of Geobacillus | stearothermophilus ATCC 2641 51 |
| 4.3.1 | Imaging results..... | 51 |
| 4.3.2 | Statistical analysis..... | 57 |
| 4.4 | Imaging & Force-Volume of clean glass substrate | 58 |

| | | |
|-------|---|----|
| 4.4.1 | Imaging results..... | 58 |
| 4.4.2 | Statistical analysis..... | 61 |
| 4.5 | Discussion..... | 62 |
| 4.5.1 | Adhesion force between A.flavithermus CM & G.stearothermophilus ATCC 2641 | 62 |
| 4.5.2 | Adhesion force between thermophilic spores & glass substrate..... | 62 |
| 4.5.3 | Adhesion force between clean glass substrate & sample glass substrate..... | 63 |
| 4.5.4 | Determining spore hydrophobicity to capillary effect..... | 64 |
| 4.5.5 | AFM silicon tip relative to stainless steel use in dairy plant..... | 65 |
| 4.6 | Anoxybacillus flavithermus CM : The Findings..... | 66 |
| 4.6.1 | Adhesion force of A.flavithermus CM on stainless steel surface..... | 66 |
| 5 | General Discussion..... | 68 |
| 5.1 | Summary..... | 68 |
| 5.2 | Future Directions..... | 70 |
| 5.2.1 | Improving the methodology of Force-Volume imaging on spores..... | 70 |
| 5.2.2 | Multiple dairy strains study..... | 70 |
| 5.2.3 | The effect of milk processing variables on the spore's adhesion behaviour..... | 70 |
| 5.2.4 | Study of spore-substrate's interaction with different substrates..... | 70 |
| 6 | Bibliography..... | 71 |

Table of Figures

| | |
|--|----|
| Figure 1: Schematic of milk powder manufacturing process (adapted from Pearce, 1996) | 2 |
| Figure 2 : Stages in the development of bacterial spores(de Hoon, Eichenberger, & Vitkup, 2010)..... | 5 |
| Figure 3 : Basic principle of AFM (adapted from Meyer 1992) | 10 |
| Figure 4. Topographical image and Surface map image of similar sample ((Morris, Kirby, & Gunning, 1999)..... | 12 |
| Figure 5 : Example of biological force spectroscopy.Total adhesion image (left) and topography image (right) of a mixed ayer of group A and O cells (Eaton & West, 2010). | 14 |
| Figure 6 : Layer of highly concentrated spores between the PEG and crude suspension phase appears after centrifugation. | 17 |
| Figure 7 : A s | 18 |
| Figure 8 : The anatomy of a silicon cantilever probe. | 18 |
| Figure 9 : Height & Deflection images of <i>B.subtilis</i> spores using a sharp end probe. | 19 |
| Figure 10 : A force-curve plot generated in the Display monitor | 20 |
| Figure 11 : Top image shows the raster-scan of a targeted spore and the bottom image is its Height & FV of 32x32 grids image. | 21 |
| Figure 12 : A model of force-distance curve. Dotted line is recorded value during the approach while the solid line is the retraction data. | 22 |
| Figure 13 : two different sets of force-distance curve from a glass sample.(top) Δd is similar value to Δz . (bottom) Only Δz is salvageable since the values upon retraction is clipped. | 23 |
| Figure 14 : Representation on surface interaction affecting spore lawn's stability. Left image is when most spores adhered fully to the SS substrate while only partially adhered on the substrate on the Right image. | 25 |
| Figure 15 : Height and deflection images of spore lawn on SS substrate. Samples were prepared using spores suspension under different concentration; a (10^8 cfu/ml), b & d (10^4 cfu/ml), c&e 10^2 cfu/ml). Sample a, b & c was immersed for 1 hour while d & e were immersed for 2 hours. Each image varies from $5 \times 5 \mu\text{m}$ to $10 \times 10 \mu\text{m}$ | 26 |
| Figure 16 : Height (left) & Deflection (right) images of clean SS 316 substrate under a liquid environment. Each image is $10 \times 10 \mu\text{m}$ | 27 |
| Figure 17 : Boxplot - Adhesion force of stainless steel substrate (1) and <i>B.subtilis</i> spores (2) | 27 |
| Figure 18 : Boxplot - Adhesion force of fresh (1) and old (2) | 28 |
| Figure 19 : Stained spores under light microscopy. <i>G.stearothermophilus</i> ATCC 2641 b) <i>A.flavithermus</i> CM c) <i>G.stearothermophilus</i> D1 d) <i>B.subtilis</i> e) <i>G.stearothermophilus</i> P3. | 29 |
| Figure 20 : Purified spores from two=phase separation; a) <i>G.stearothermophilus</i> ATCC 2641 b) <i>A.flavithermus</i> CM c) <i>G.stearothermophilus</i> P3 d) <i>B.subtilis</i> | 30 |
| Figure 21 : Light microscopy images of <i>A.flavithermus</i> CM spores from different phases of the two=phase separation method; a) crude suspension-BEFORE b) pure spore layer c) crude suspension-AFTER d) phosphate buffer e) PEG phase | 31 |
| Figure 22 : Boxplot of endospore sizes on various thermophilic species. | 32 |
| Figure 23 : The hit rate for probe to measure spore surface is higher in a smaller scan area (left) compared to a larger scan area (right) | 34 |
| Figure 24 : Surface area measured using a silicon tip (a) and pleateau tip (b). Closely packed purified spores for an easy force measurement (c). | 35 |

| | |
|--|----|
| Figure 25 : A spore mounted on a tipless cantilever (left) and setup to measure adhesion force between the surfaces of spores and stainless steel (right)..... | 36 |
| Figure 26 : 32x32 grid map on a 3x3 μm scan area. It should provide an enough scanning area to obtain a complete data from a whole spore..... | 37 |
| Figure 27 : <i>A.flavithermus</i> CM spores imaging using a soft CSG11/Au probe (0.03 N/m). a) Deflection b) Height & elasticity grid c) Adhesion Force's deflection | 40 |
| Figure 28 : <i>A.Flavithermus</i> CM spores imaging using a soft CSG11/Au probe (0.1 N/m). a) Deflection b) Height & elasticity grid c) Adhesion Force's deflection | 41 |
| Figure 29 : <i>A.flavithermus</i> CM, Sample 1. 32x32 grid covering 4 μm length..... | 43 |
| Figure 30 : <i>A.flavithermus</i> CM, Sample 2. 32x32 grid covering 4 μm length..... | 44 |
| Figure 31 : <i>A.flavithermus</i> CM sample 3. 32x32 grid covering 4 μm length..... | 45 |
| Figure 32 : <i>A.flavithermus</i> CM sample 4. 32x32 grid covering 4 μm length. | 46 |
| Figure 33 : <i>A.flavithermus</i> CM sample 5. 32x32 grid covering 5 μm length. | 47 |
| Figure 34 : Boxplot on adhesion forces of AFM tip with <i>A.Flavithermus</i> CM spore surface. . | 48 |
| Figure 35 : <i>G.Stearothermophilus</i> ATCC 2643, Sample 1. 32x32 grid covering 5 μm length. 50 | |
| Figure 36 : <i>G.Stearothermophilus</i> ATCC 2641, Sample 2. 32x32 grid covering 5 μm length. 51 | |
| Figure 37: <i>G.Stearothermophilus</i> ATCC 2641, Sample 3. 32x32 grid covering 5 μm length. 52 | |
| Figure 38 : <i>G.Stearothermophilus</i> ATCC 2641, Sample 4. 32x32 grid covering 4 μm length. 53 | |
| Figure 39 : <i>G.Stearothermophilus</i> ATCC 2641 sample 5. 32x32 grid covering 4 μm length. 54 | |
| Figure 40 : Boxplot on adhesion forces of AFM tip with <i>G.Stearothermophilus</i> ATCC 2641 spore surface..... | 55 |
| Figure 41 : Clean glass sample 1. 32x32 grids covering 5 μm lengths. (Top; Deflection, Bottom; Adhesion force map) | 57 |
| Figure 42 : Clean glass sample 2.32x32 grids covering 5 μm lengths. (Top; Deflection, Bottom; Adhesion force map) | 58 |
| Figure 43 : Boxplot on adhesion forces of AFM tip with clean glass substrate..... | 59 |
| Figure 44: Histogram of adhesion force on <i>A.flavithermus</i> CM spores (left) and <i>G.stearothermophilus</i> ATCC 2641 spores (right)..... | 60 |
| Figure 45 : Histogram of adhesion force on spores (left) and glass substrates (right) | 61 |
| Figure 46 : Histogram of adhesion forces measured on clean glass surface (left) and glass substrate layered with spores (right) | 62 |
| Figure 47 : Interpretation on low capillary force between hydrophilic tip - hydrophilic spores (left) and tip-glass substrate which is both hydrophilic (right)..... | 63 |
| Figure 48 : A representative model in relation to the theoretical scanning area of an AFM probe..... | 65 |