AN ABSTRACT OF THE THESIS OF

Matthew P Ryder for the degree of Honors Baccalaureate of Science in Bioengineering presented on May 23, 2008. Title: Functionalization of Surfaces with Nisin in a Poly-ethylene Oxide Brush Layer.

Abstract Approved: _____

Joseph McGuire

Infections in hospitals account for over 100,000 deaths per year. These infections occur at the hospital from complications following bacterial adhesion to intravenous catheters, coronary stents and other implanted devices. Another common problem is protein adsorption to the surface of the device and subsequent blood clotting. Methods for combating these issues have been previously studied using antimicrobial coatings consisting of a tri-block polymer with a hydrophobic base and two hydrophilic tails called Pluronic® F108 and an antimicrobial agent, nisin; however studies have not been conducted to determine how long such coatings are effective. Experiments were designed to test the long term efficacy of an F108 coated, nisin loaded surface for killing the Gram positive bacteria, *Pediococcus pentosaceus*. The overall experimentation, consisting of aging nisin-coated microspheres for selected periods of time, followed by assaying bioactivity to determine nisin effectiveness, lasted 28 days, with samples taken from the microsphere suspensions once each week. These samples were plated on MRS agar plates and *P. pentosaceus* was allowed to grow for 48 hours, after which the colony forming units were counted for each sample. The F108-coated, nisin loaded layers showed greater activity retention in comparison to layers prepared with nisin in the absence of F108, but only in the final week of the study.

Key Words: *Pediococcus pentosaceus*, nisin, F108

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Functionalization of Surfaces with Nisin in a Poly-ethylene Oxide Brush Layer

by

Matthew P Ryder

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I understand that my project will become part of the permanent collection of Oregon State University, University Honors College. My signature below authorizes release of my project to any reader upon request.

Matthew P Ryder, Author

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Introduction

Infection in hospitals is the fourth largest killer in the United States today, resulting in over 100,000 deaths each year. These infections are most often contracted while at the hospital, typically from implanted devices such as coronary stents or catheter tubes [1]. There are three main issues associated with implanted devices; clot formation, bacterial adhesion, and cell proliferation. This experiment dealt with only the first two of these problems. Currently, the most prevalent way to counteract these complications is to keep patients loaded with heparin, an anti-clotting drug, and on antibiotics to kill bacteria [2].

These problems are directly related in that they are both initiated by adsorption. In clot formation, blood proteins adsorb to the surface of the device and instigate the clotting cascade. If the clot is dislodged, it can cause many complications, including stroke. In the other issue, bacteria attach, mediat6ed by protein adsorption, and form a film on the surface of the device that can lead to infection. The solution seems to be, then, to remove the opportunity for adsorption as well as kill bacteria on contact. The goal of this research was to explore methods and effectiveness of changing the surface chemistry of material typically found in implantable devices.

Essentially, this involves using a hydrophobic surface, for this research polystyrene was used, and add a hydrophilic, polymer "brush" layer to reduce the incidence of adsorption to the surface, and then to add an agent to kill any bacteria that may approach the surface.

The brush layer is made up of a tri-block co-polymer known as Pluronic® F108. This product consists of a hydrophobic base of poly[propylene oxide] and two longer hydrophilic tails of poly[ethylene oxide]. In solution, the hydrophobic base of the F108 will spontaneously and preferentially locate at the hydrophobic surface of the polystyrene beads used for experimentation (see Figure I1). The hydrophilic tails stick out into solution and repel

approaching proteins or bacteria. After the F108 has preferentially located at the surface of the bead, it is entropically unfavorable for it to desorb, and remains on the surface. Once the F108 has been added to the beads, the lantibiotic nisin is added to the solution in contact with the F108 layer. Nisin is a relatively small protein, made up of 34 amino acids, that acts to kill Gram positive bacteria. Nisin kills by forming open pores in the surface of a bacterium; it takes eight nisin proteins to form a single pore (see Figure I2) [3].

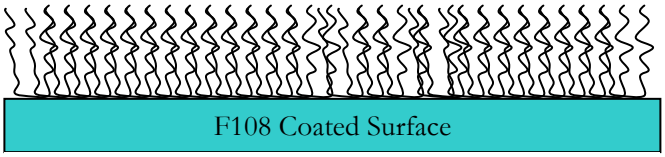

Figure I1 *shows the F108 preferentially located at a hydrophobic surface.*

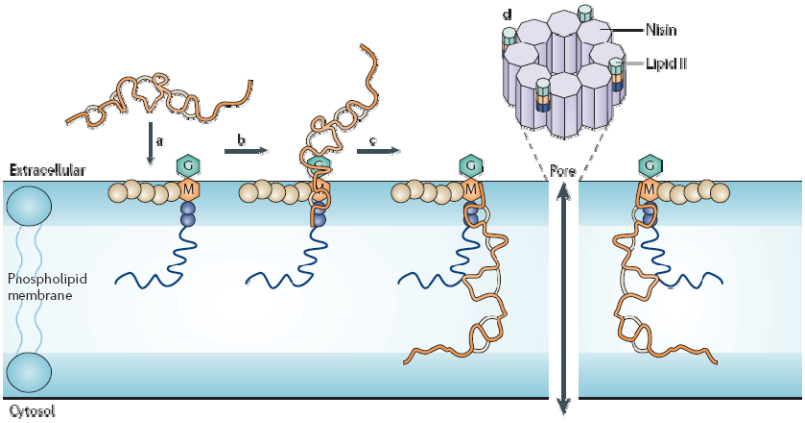

Figure I2 *shows the mechanism by which Nisin forms pores in a target bacterium. (a) nisin approaches cell surface at Lipid II interface. (b) nisin attaches to Lipid II molecule. (c) nisin enters the cell through interaction with Lipid II. (d) 8 nisin molecules aggregate with 4 Lipid II molecules to form a pore in the bacterium surface, killing the cell.*

Contrary to the function of the F108 coated surface, the nisin is able to infiltrate the brush layer and in fact preferentially locates there in aqueous solution (see Figure I3) [4]. This research aimed at dealing with how efficacious this approach is at killing bacteria over time, so the mechanism for why nisin is able to do this was not explored.

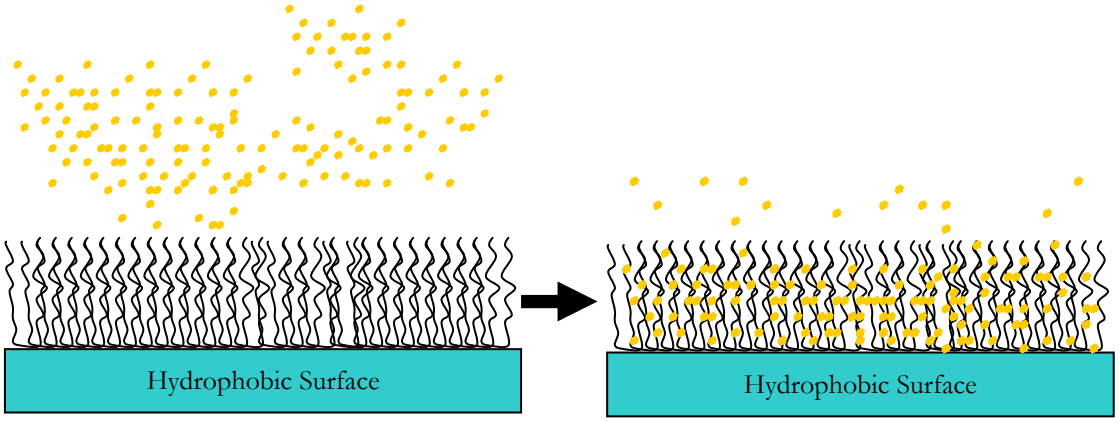

Figure I3 *shows the Nisin (yellow) infiltrating the F108 brush layer. It is important to note this goes against the function of F108.*

Previous work has been done on the potential benefits of this technology on hydrophobic surfaces [3, 5]. In that work, two important aspects were discovered. First, from ellipsometry data it was shown that nisin imbeds into the brush layer by non-specific adsorption as shown in Figure I3. The other important aspect was to show that nisin, in association with the F108 does in fact protect against biofilm formation and protein adsorption to the surface, and is much more effective than nisin coated surfaces alone, especially when challenged with typical blood proteins derived from blood plasma.

Although that study showed the F108 layer is in fact effective, it did not consider the long term effectiveness of such an approach. This research aims to investigate how effective the F108 brush layer is over time, and provide background knowledge on how these systems behave in a solution more similar to physiological conditions. Tai's work investigated the brush layer in a solution of low ionic strength (10 mM sodium phosphate buffer, pH 7). This study is

conducted in solution of intermediate ionic strength (0.01M PBS buffer with 0.15M NaCl) in order to simulate physiologic conditions. Because patients typically have devices implanted for periods longer that 4 days or one week, this study tests the effectiveness of such a system over a 28 day period.

Background

Advances in biomaterials engineering have led to a number of well known and effective technologies in cardiovascular medicine and surgery, orthopedics, ophthalmology and dentistry. While the use of biomaterials has improved the lives of millions of people, there are still problems with blood coagulation, infection, degradations and rejection associated with currently used materials. Controlling biological interactions with biomaterials is the most important aspect of design of temporary and permanent implants, as well as cell and tissue scaffolds and diagnostic probes. The ability to control, in particular, the interaction of materials with bacteria is extremely important. Most materials used in medical devices are susceptible to bacterial adhesion. Once bacteria adhere to the solid surface of an implanted device, they proliferate and imbed themselves within an extracellular matrix, forming a biofilm, which makes them very difficult to combat. Once the bacteria have formed a biofilm, both the host immune system and antimicrobials become much less effective against the bacteria due to difficulty in penetrating the matrix and/or inactivation upon penetration. To reduce morbidity and mortality due to device related infections, new methods are needed to prevent biofilm formation on a multitude of material types and configurations.

Approximately 5 million central venous catheters are used annually in the United States. An estimated 1/3 of catheters fail by infection, and another 1/3 fail due to clot formation creating a blockage for the catheter. For both failure types, the most effective current treatment is replacement of the defective device with another just like it. Medical device-associated infections often result in systemic infections that in the most severe cases lead to multiple organ failure and death, even though the original medical condition was successfully treated. Conservative estimates indicate catheter related blood stream infections (CR-BSI) occur in over 200,000 patients in the US each year. The cost of these infections ranges from $300

million to $2.3 billion. In 2005, CR-BSI resulted in 28,000 deaths [6]. Infection is also a major problem for dialysis patients (>300,000 people in the US) of which 42% rely on AV access grafts. These grafts have an infection rate between 11% to 20% and a mortality rate due to infection of 12-22% [7-9].

CR-BSI infections are becoming increasingly difficult to treat because bacteria such as *Staphylococcus aureus* are becoming increasingly resistant to common antibiotics. In 1974, only 2% of *S. aureous* infections were methicillin-resistant (MRSA). In 2003 the resistance had risen to 57%, and is over 60% today [10].

This work describes new approaches to preventing biofilm formation based on the use of a two part coating that integrates nisin with a copolymer and describes results from experimentation using a *P pentosaceus pentocaceus* as a model bacterial strain. This technology will be useful in the prevention of bacterial adhesion and subsequent proliferation and is expected that such a technology could make a substantial impact on the care of patients needing intravenous catheter placements.

Current approaches to reduce CR-BSI

Several catheter modification approaches have been evaluated for their ability to reduce the incidence of catheter-related blood stream infections (CR-BSI). The approaches can be divided into two categories. In one category, surfaces are modified to prevent bacterial adhesion. Many of these approaches involve minimization of adsorption and adhesion through steric repulsion and/or minimization of interfacial energy. In the case of catheters, adsorption of proteins, particularly fibrinogen, often leads to development of a fibrin sheath or thrombus and eventual occlusion. Several proteins present in blood clotting promote bacteria adhesion and it is well known that there is an association between thrombus formation and CR-BSI [12-14]. On the surfaces of hydrophobic materials, entropically driven hydrophobic interactions dominate

protein adsorption, and many research groups have shown that surfaces grafted with PEO are significantly less prone to protein adsorption and adhesion [15]. Although hydrophilic coatings have been shown to reduce bacterial adhesion, problems with infection still occur.

In the second category, surfaces are modified with agents that actively kill or prevent the growth of bacteria. Two commercially available short term catheters that fit into this category and have been shown to reduce infection rates are chlorhexidine-silver sulfadiazine-impregnated (CSI) and minocyline-rifampin impregnated (MRI) [16, 17]. However these products pose a significant risk for developing drug resistant bacteria and signs of resistance with the use of MRI has been shown *in vitro* and in an animal model [18]. This risk is lower for the antiseptic CSI catheters, but in vitro studies have found that exposure to chlorhexidine can result in increased bacteria resistance to it and other therapeutic antimicrobial agents [19]. Furthermore, CSI is not thought to be effective for longer than 10 days and serious anaphylactoid reactions associated with the use of these catheters have been reported in Japan [20-22]. Although antimicrobial catheters cost more than standard catheters, studies have demonstrated that there is an overall cost benefit of using them in high risk patients due to the high cost of treating CR-BSI. The added cost of antimicrobial catheters is between $25 and $70 per catheter, but the overall cost benefit is about $200 per catheter for CSI [23]. Based on cost benefits and improved patient care, there is a strong motivation to use antimicrobial catheters. However, these pose a very serious risk of furthering the development of resistant bacteria and have been recommended for use only in high risk patients (in the ICU, on total parenteral nutrition, or immunosuppressed) [24]. More recently, they have been recommended for use in patients expected to have a catheter in place for more than 5 days.

The research described in this work show the effectiveness of a material modification that (1) kills bacteria upon contact with the device such that antibacterial agents do not have to

be released into the blood stream or surrounding tissue, (2) is biocompatible and will not adversely affect the patient, (3) can be readily applied to a variety of materials and irregularly shaped objects, (4) prevents blood clots and occlusion, and (5) does not stimulate changes in bacteria that lead to resistance, allowing broad use without compromising the effectiveness of clinical antibiotics.

F108 technology and advantages

Pluronic® surfactants have been successfully used to immobilize bioactive entities at interfaces. This approach utilizes a triblock copolymer (PEO-PPO-PEO) called F108, which self-assembles on hydrophobic materials from aqueous solutions. The hydrophobic PPO center block forms a strong hydrophobic bond with the material while the PEO end blocks remain freely mobile in the fluid phase [25]. Using this approach, a thick PEO brush-like layer is formed at the material surface that serves two important purposes. First, the PEO layer acts as a cushion between the peptide and the substrate preventing peptide denaturation that might otherwise result from surface interactions while retaining peptide mobility [26, 27]. Second, the PEO layer prevents nonspecific adsorption of proteins or cells, which the surface is exposed to during therapeutic use [28].

Previous studies conducted at the University of Utah and *allvivo,* which was AVI's precursor company, demonstrated the advantages of the EGAP technology over other PEO based tethering technologies: it is easily applied to a variety of materials and irregularly shaped objects through dip coating and it provides a mechanism to systematically vary therapeutic biomolecule surface concentration [29, 30]. Medical devices, implants, and tissue engineering scaffolds are prepared from a multitude of materials based on the different mechanical, electrical, and optical properties required by each application. Most materials that display optimal bulk properties for a given application do not have adequate biocompatibility. Direct

chemical modification of materials to improve biocompatibility is complicated and can change bulk material properties. In some cases, direct chemical modification is not feasible based on the material type or the irregular shape of an object. Direct adsorption of biomolecules on such surfaces often leads to denaturation and a change or loss of activity. The EGAP technology provides a simple and versatile solution to these challenges because it can be applied to a number of different materials by dip coating and can be used to immobilize proteins and peptides with retained activity [27, 29, 31, 32].

Lantibiotics and nisin

Nisin has a long history as a potent and safe food preservative. It has been demonstrated that nisin can adsorb to synthetic surfaces, maintain activity, and kill adherent cells *in vitro* [33-37]. While nisin has demonstrated activity against mainly Gram-positive bacteria, it can be an effective inhibitor of certain Gram-negative bacteria when used in combination with other compounds such as chelating agents [38]. *Staphylococcus aureus* and *Staphylococcus epidermidis* are the most frequently encountered biomaterial-associated pathogens, especially for CVC applications, and are both Gram-positive bacteria. Nisin has been shown to prevent microbial adhesion on endotracheal suction catheters *in vitro* (using *Staphylococcus aureus, Staphylococcus epidermidis,* and *Enterococcus faecalis* (*Streptococcus faecalis*)), prompting further *in vivo* studies evaluating nisin-treated intravenous (IV) catheters in sheep and tracheotomy tubes in ponies [39]. Catheters pretreated with nisin for long-term placement (7 days) did not retain antimicrobial activity, while short-term (3-5 h) IV catheters did. The exact duration of nisin activity on IV catheters remains unknown. There were no abnormalities on clinical examination of sheep during the experimental period, and no animal in either group developed catheter-related infection or venous thrombosis. Veins with short-term coated

catheters showed fewer and less severe histologic abnormalities compared with controls, indicating a possible protective effect on vascular endothelium [39].

Lantibiotics are antibiotic compounds that include one or more lanthionine rings. Over 40 lantibiotics are currently known, and more are being discovered each year. The unique physical structure of lantibiotics, (e.g., double bonds, thioether rings, and exotic amino acid residues), makes these antimicrobial peptides highly reactive, and distinguishes their mode-of-action from traditional antibiotics. This suggests that they may remain effective despite the global increase of resistant bacterial strains through antibiotic overuse. Lantibiotics demonstrate a wide variability in their inhibitory spectrums. Some lantibiotics, (e.g., nisin and subtilin) are active against Gram-positive bacteria, while other lantibiotics (e.g., cinnamycin) are active only against Gram-positive rods. Additionally, some lantibiotics have antiviral activity, (e.g., lanthiopeptin) [40], some function as immunosuppressors, (e.g., mersacidin) [41], and some (e.g., duramycin and ancovenin) can inhibit biomedically important enzymes [42].

Many lantibiotic characteristics are valuable for biomedical applications [43]. Unlike typical peptides, lantibiotics contain dehydrated amino acid residues with electrophilic centers that readily react with nucleophilic groups on bacterial DNA and enzymes [44]. The thioether rings found in all lantibiotics increase heat stability, decrease susceptibility to reducing agents, and reduce reactivity toward free radicals as compared to disulfide bonds [45]. Lantibiotics offer a means for limiting the rise of resistant microorganisms, and since their mechanism of action is fundamentally different to that of traditional clinical antibiotics, cross-resistance is highly unlikely. There are reports that repeated exposure to nisin can lead to changes in bacteria that can confer weak resistance to nisin. However, these changes also make the bacteria weak compared to their nonresistant counterparts and more susceptible to antibiotics and the immune response. Nisin has been used broadly and extensively in food products for many years

without problems arising from development of resistance. The use of nisin began around the same time as the use of penicillin, which is not longer effective. The difference is attributed to nisin's multi-tiered mechanism of action as well as concomitant weakening of bacteria with development of nisin resistant traits.

There are several different mechanisms through which lantibiotics exert their antimicrobial effect [47]. Type A lantibiotics (such as nisin) are strongly cationic, linear molecules. They are highly surface active and kill susceptible bacteria through a multistep process that destabilizes the phospholipid bilayer of the cell and creates transient pores. Targeted bacterium are rapidly killed by efflux of ions and cytoplasmic solutes such as amino acids and nucleotides, and subsequent dissipation of the membrane potential [48]. Depolarization of the cytoplasmic membrane results in instant termination of all biosynthetic processes [49]. Structural analyses have indicated that the hydrophilic groups of nisin interact with the phospholipid headgroups and the hydrophobic side chains are immersed in the hydrophobic core of the membrane [50]. Two models of nisin antimicrobial action have been proposed. The "wedge" model of pore formation [51] proposes that the peptides insert into the membrane without losing contact with the membrane surface, resulting in the formation of a short-lived (milliseconds to seconds) pore. In the wedge model, pore formation is caused by local perturbations of the lipid bilayer, whereby the hydrophobic residues of the peptide are shallowly inserted into the outer leaflet of the lipid bilayer [52]. The "barrel-stave" model [53, 54] proposes that nisin binds as a monomer and inserts into the lipid bilayer. The inserted monomers then aggregate laterally to form pores. Each of these models was proposed when questions remained concerning the involvement of cell surface factors, lifetime of the pore, and the number of molecules required for pore formation [46, 55-57].

Procedure

In this experiment, we started by coating microspheres with F-108 and letting them incubate overnight. Nisin was then loaded on to the microspheres and incubated for another hour. We had five different samples, they were as follows:

- Microspheres, F-108 and Nisin (MFN)
- Microspheres and Nisin (MN)
- Microspheres and F-108 (MF)
- F-108 and Nisin (FN)
- Nisin (N)

2 mL of each sample was taken and added to 8 mL of diluted *P pentocaceus*, the dilution was based on observed absorbance units, diluted to a value 0.65, and then diluted 100x. We then prepared three of each sample and incubated for another 4 hours. Each sample was plated (100 µL) on an agar gel plate and incubated for two days. After two days, we counted the number of colonies that formed on the plate. The experimentation consisted of reproducible 28 day studies, so this sampling method was conducted a total of four times per study, or once per week. Each of the 5 sample types were plated using standard laboratory methods on MRS agar plates in replicates of 3. For each sample period of the study, 20 plates were kept, 3 each of the samples, 3 with only *P pentocaceus*, and 2 agar only samples. Results are shown below. An SOP is available in the Appendix.

Results and Discussion

As stated previously, the goal of this study was to test the effectiveness of F108 adsorbed to a polystyrene coated microsphere on the retention of nisin over time. Samples were taken once a week for four weeks, providing four data sets. We measured this effectiveness by incubating each solution with *P pentosaceus*, diluting this solution and plating on MRS agar plates. After two days we counted the number of colonies on the plates. We determined the Colony Forming Units (CFUs) originally present in the incubation, which are equal to:

$$CFU = Total\ Colonies\ Counted\ \times dilution\ of\ \mathrm{P.pentosaceus}\ after\ addition\ of\ sample.$$

The data for the control tests is shown in Figure D1, sample data is shown in Figure D2. The raw data is shown in Appendix C.

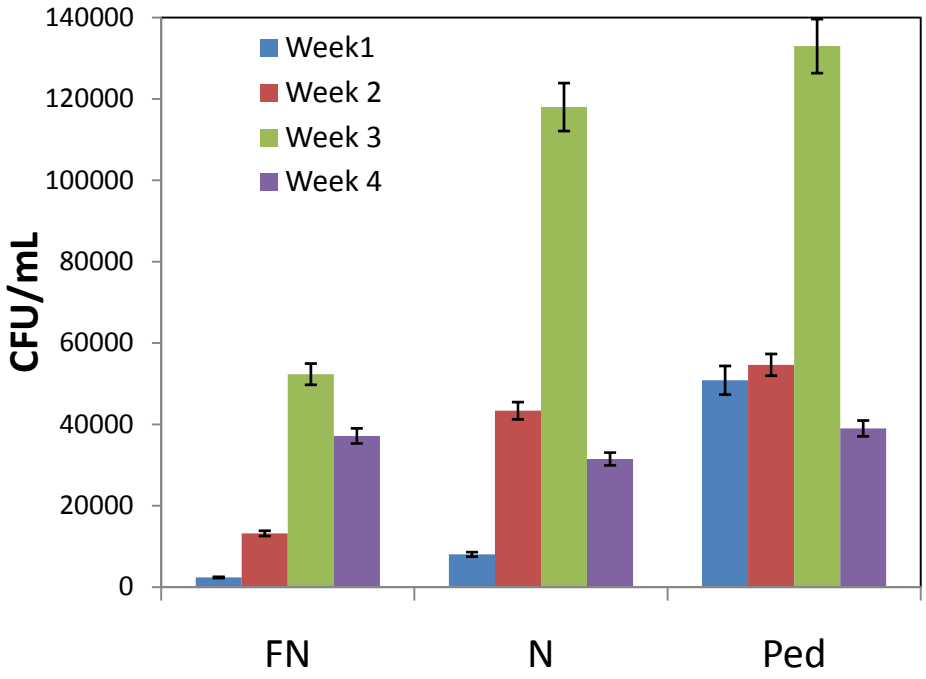

Figure D1: Total growth of *P pentosaceus* on MRS agar plates. Samples were taken once per week. Sample free agar plates were also examined, and exhibited no growth in all cases. Solutions are as follows: FN – F1-8 and nisin; N – nisin; Ped – *P pentosaceus* only.

The data shown in Figure D1 is for the microsphere free controls only. When analyzing the week-four data, the agar of the Nisin-only samples was extremely dry and had split down the middle, this seemed to indicate more harsh conditions for these samples than any of the others, for this reason, the following analysis reflects only the first 3 weeks of data. Notice the amount of colonies formed is less in the FN sample than in the N sample on all test days. This clearly indicates that samples including F108 protected the nisin's activity from being degraded over time. There are a few possibilities as to why this may be. First, nisin experiences its highest potential activity at a pH of around 4; higher pH helps to hydrolyze the nisin and degrade it more quickly. The temperature of 37 °C likely contributes to this degradation. In solution with F108, however, the nisin aggregates with the F108 and other nisin, and is protected from the free protons and hydroxyls in solution. This likely protects the nisin over time and aids the retention of activity. The second possibility is that in the vessel, nisin alone may create a poly-layer on the surface over time, thus reducing the amount of nisin in solution. Again the addition of F108 keeps the nisin from adsorbing in a polylayer to the surface of the vessel and can keep nisin in solution. In either case, this seems to prove the effectiveness of F108 on the protection of nisin over time, and this may likely remain true with the addition of microspheres. The results of that aspect of the experimentation are shown below, in Figure D2

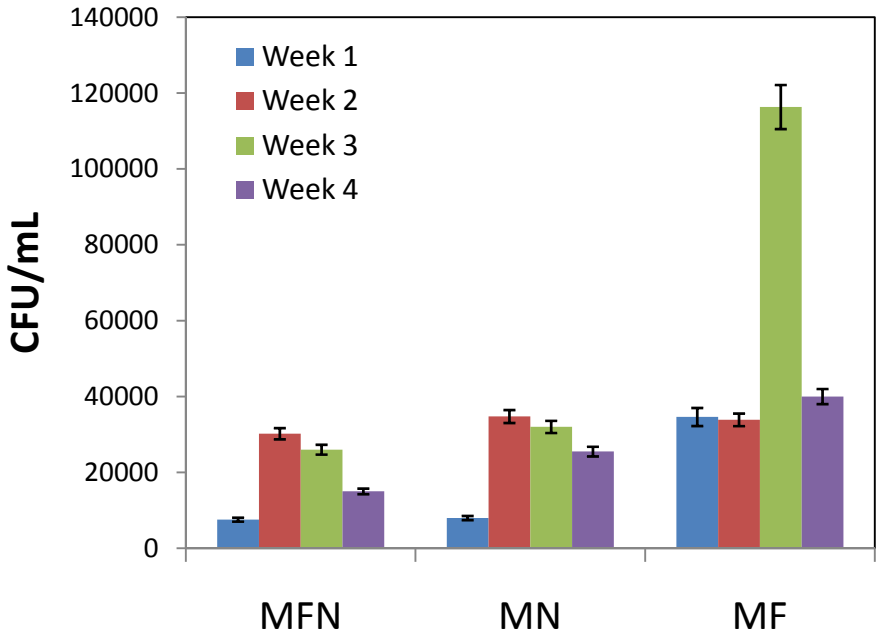

Figure D2: Solutions are as follows: MFN – microspheres, F108, and nisin; MN – microspheres and nisin; MF – microspheres and F108; FN – F1-8 and nisin

As shown in figure D2, there seems to be no apparent difference between the MFN and MN samples. During all test days, the MFN and MN samples seemed reasonably similar and this figure does not explicitly indicated any differences between the antimicrobial activity of these two samples. The sample without nisin would be expected to remain constant over time, given there are no aspects in the sample to kill bacteria. As shown, this is not the case. This is likely related to the colony density on each plate (see "*P pentosaceus* Growth" below). This may mean that a difference between MFN and MN exists, but cannot be seen as the data is graphed. The only data that may be indicative of a growth difference exists in week four. It may be that without blood proteins present in solution, it takes an extended period of time to see a difference between MFN and MN, given there are no protein challenges to encourage the nisin to leave the microsphere in the MN samples. The data was plotted against a theoretical maximum *P pentosaceus* growth, taken to be whatever the MF growth was on that particular test day. This data is represented in Figure D3.

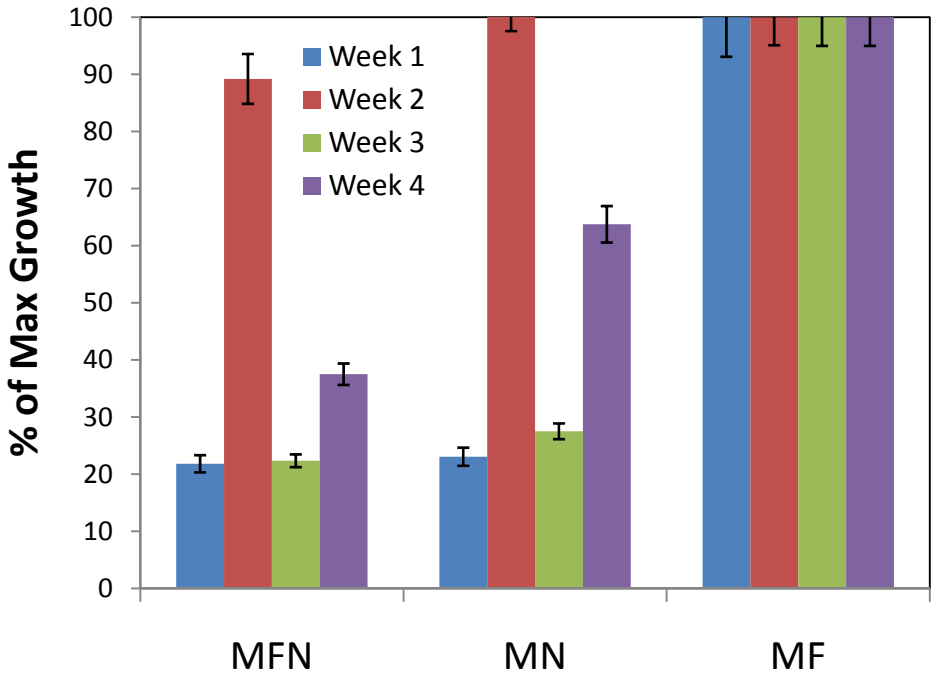

Figure D3: % of assumed maximum *P pentosaceus* growth, taken to be whatever MF exhibited.

This data indicates two things. First, there seems to be some issue with the second week of data. During the experimentation, we incubated all samples with 100x diluted *P pentosaceus*, except in the case of the second week, where we incubated with 400x diluted *P pentosaceus*. This was an oversight during the experimentation that may provide reasoning for the seeming discrepancy. The data also seems to indicate the MFN allows for less growth in terms of percentage over time than the MN sample. Whatever protection may be occurring seems slight, however, and may be the result of experimental error. The results would only be worrisome if the MFN sample exhibited more growth than MN over time. Because the samples were kept in PBS buffer for the duration of the experiment, we would not expect a lot of difference between the samples over time. This is due to the lack of alternative proteins in solution to challenge the nisin for adsorption on the surface of the microspheres. Without that challenge, the nisin would not likely elute from the surface of the microsphere, and the overall activity may not change that dramatically. Over a very extended period of time, however, the

MFN should prove more effective at protecting the activity of the nisin when compared to the F108 free sample, and in fact this difference seems to be evident in the fourth week of data, where we can see a significant difference between the MFN and MN samples. This data seems to indicate the MFN is already proving more effective, so these results are extremely promising for future analysis.

Alternative Analyses

Throughout our research, we have found when working with live cultures, it is extremely important to take extra precaution to insure the system is treated the exact same every time. For this reason, every week one researcher plated the same samples as in the previous week, and so on throughout the duration of the test period. Even so, there are several points throughout the experiment that may have contributed to the deviations seen in our data.

MRS Broth

There are two aspects of the MRS broth that are potentially problematic. First, the expiration date of the broth has passed. Given that this is a dry, granulated product, stored in a dry container, and that the broth need only sustain the bacteria for a maximum of 70 hrs, it is unlikely this is a significant issue in our experimentation. This potential issue is further mitigated since all of the samples are treated with the same broth and all plates are made from the same stock. The other issue concerns how long the broth that was made was used. Typically, a stock solution of broth should not be used for more than two weeks consecutive use. To save time and energy, we used the broth for a total of three weeks. Again, because all samples were treated the same, during each week, this unlikely accounts for any differences between test types on a given week; over time however, this may have contributed to lower cell growth of the *P pentosaceus*. The other concern with this is the potential for contamination. The bottle was stored at 4 °C and was autoclaved at 121 °C before initial use. After that time the bottle

was opened only 4 times, which reduces the potential for contamination, and the majority of the broth removed was again autoclaved before being poured into agar plates.

PBS Buffer

The only potential issue that may have arisen occurred in the middle of the experiment, when more buffer had to be filtered and the pH needed to be adjusted. Because this same procedure was performed every time new buffer was needed, it is unlikely this caused any issues in the samples, however it does represent a point in the experiment where previous samples may have experienced a different environment than later ones.

***P pentosaceus* Growth**

When working with any bacterial strain, the amount of variability from sample to sample can be enormous. For this reason, we plated all solutions in 3 replicates. Even so, Figure D1 shows drastic variability in the viability of the *P pentosaceus* on different dates. One particular issue that may have arisen is the amount of colonies that grew on each plate every week. For the first two samples, the solutions were not diluted at all before being plated, resulting in high cell density, whereas the third sample experienced a 100x dilution before being plated. This high cell density can lead to one of two things. First, it is possible that each colony is representative of more than one CFU, and in fact can be anywhere from 1 to likely 10 CFUs, with no way to tell the difference. The second possibility is that at high cell density, competition for limiting resources kills all but the strongest cells on the plate. Both possibilities result in a lower than expected CFU count, and may account for the discrepancies shown in the data. For best results, a plate should only support between 30 and 300 colonies.

Bead Loss

Because each sample and each vial goes through several washes (each plated sample experiences a total of 6 washes), incremental and nearly immeasurable bead loss will reduce the

surface area available for F108 and nisin adsorption and is variable for each sample type. If the MFN sample had much less surface area than MN, we would expect to see results as shown, and may explain why these samples are so similar in killing power. This bead loss can be due in part to an inability for the microspheres to create a solid pellet during centrifugation or magnetic pelleting.

Beads

The beads used for this experimentation were super para-magnetic iron core microsphers with a polystyrene coating. These were chosen so we could separate the beads from solution using rare earth magnets rather than centrifugation. The beads were specified to be between 1 and 2 μm in diameter, with a specific gravity of about 2. Due to discoloration in the supernatant upon initial bead loading, we suspected that either the beads were leaching iron, or the bead size varied enough so that extremely small beads would not precipitate in the magnetic field, given the time provided. We kept the waste supernatant in all cases, and left over-night with a magnet at the base to see if the fluid contained iron ions or very small beads. Overnight the solution cleared completely, prompting us to look at beads using an SEM. Under SEM we were able to see the actual sizes of the beads, shown in Figure D4.

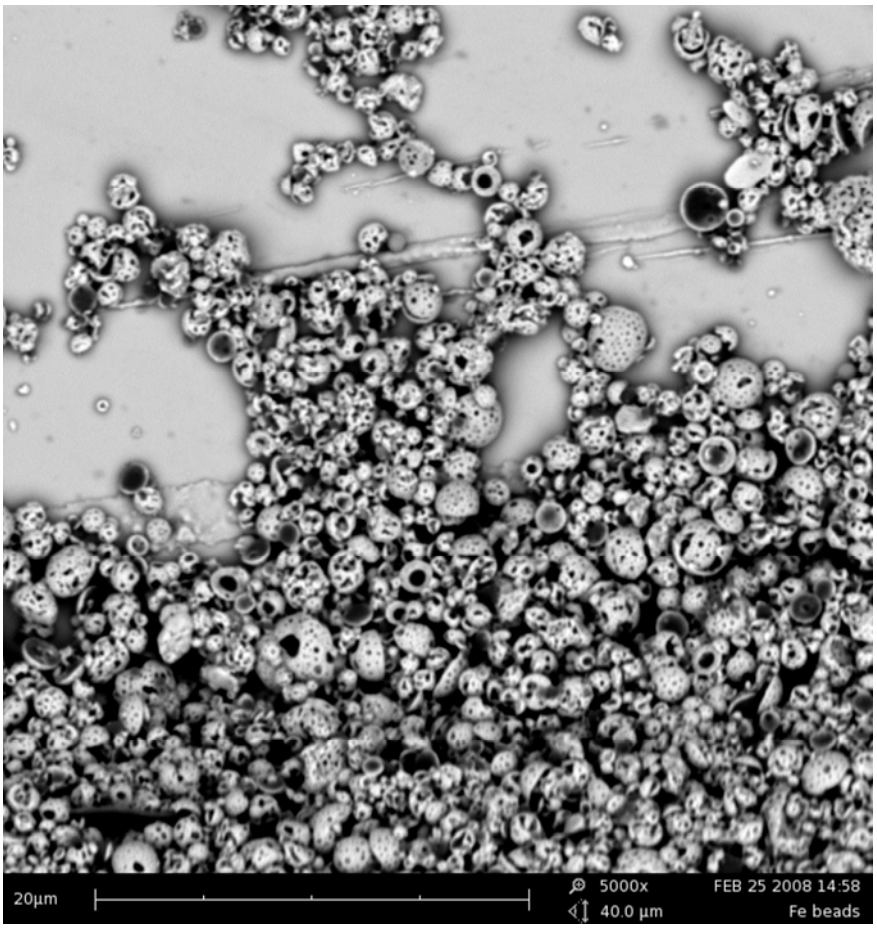

Figure D4: This image shows the varying size and shape of the beads used for experimentation.

This figure reveals a few important facets of these beads. First, they are very obviously much more variant than 1-2 µm in diameter. These seem to range from 0.5 – 5 µm according to this image. Next, these beads seem to be porous, as indicated by the evenly spaced dark spots shown on the beads. This would indicate that although it may not have occurred, iron leaching is a plausible possibility. Finally, many of these beads seem to be misshapen or broken, this may have occurred during vortexing and sonicating of the samples. In any event, because each of the samples were treated with the same beads and were treated identically in terms of mixing and plating, it seems unlikely these particular issues would have played any role in the growth patterns and CFUs shown in the previous analysis.

Conclusion

The purpose of this experiment was to test the long term effectiveness of nisin retention on F-108 coated microspheres. We ran the experiment for four weeks and took samples each week. The result we were most interested in was the difference between the MFN and MN samples. Over an extended period of time we in fact saw that the MFN sample was more effective at preserving nisin activity than was the MN sample, however, the differences were not significant enough to warrant this matter closed completely. Two issues come to mind. First, it may be that the MN spheres would not tend to show a drastic decrease in nisin activity because there was nothing in solution to challenge their position on the micrsopheres. Second, there may have been complications with our experimental procedures or materials that compromised our experiment beyond analysis.

Our first recommendation would be to run this experiment again with more uniform microspheres, along with more sample dates throughout the 28 day period. The issue with increasing the number of samples is the time constraint. Each sample takes 7 hours, so it is difficult to do more than one sample per week. In order to do this we would need more people working on this research project.

For future research we also recommend introducing blood proteins to the system. Blood serum studies will test the longevity of nisin in near physiological conditions. This test would hopefully show that the F108 brush layer is in fact extremely effective at protecting the activity of the nisin. Many different types of tests can be done to measure this effectiveness, including tests to show the zeta potential of the surface, ellipsometry testing, or maybe simply SEM imaging.

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APPENDICES

Appendix A - Raw Data

week	sample	wedge	counted	+/-	total	+/-	Average	error
1	mfn1	0.5	499	20	998	40	755.3	40.0
	mfn2	0.25	162	10	648	40		
	mfn3	0.25	155	10	620	40		
	mn1	0.25	95	10	380	40	798.0	60.0
	mn2	0.25	189.5	15	758	60		
	mn3	0.25	314	20	1256	80		
	mf1	0.0625	141	10	2256	160	3461.3	266.7
	mf2	0.0625	276	20	4416	320		
	mf3	0.0625	232	20	3712	320		
	fn1	0.5	130	10	260	20	238.0	20.0
	fn2	0.5	99	10	198	20		
	fn3	0.5	128	10	256	20		
	n1	0.5	310	10	620	20	804.7	33.3
	n2	0.25	330	15	1320	60		
	n3	0.5	237	10	474	20		
	ped1	0.0625	380	50	6080	800	5085.3	426.7
	ped2	0.125	421	20	3368	160		
	ped3	0.0625	363	20	5808	320		
2	mfn1	0.25	1031	50	4124	200	3021.3	186.7
	mfn2	0.25	537	30	2148	120		
	mfn3	0.125	349	30	2792	240		
	mn1	0.125	366	20	2928	160	3474.7	186.7
	mn2	0.125	265	20	2120	160		
	mn3	0.0625	336	15	5376	240		
	mf1	0.125	277	30	2216	240	3386.7	186.7
	mf2	0.125	519	20	4152	160		
	mf3	0.125	474	20	3792	160		
	fn1	0.125	167	10	1336	80	1320.0	53.3
	fn2	0.125	171	5	1368	40		
	fn3	0.125	157	5	1256	40		
	n1	0.125	594	40	4752	320	4336.0	240.0
	n2	0.125	582	30	4656	240		
	n3	0.125	450	20	3600	160		
	ped1	0.125	725	15	5800	120	5464.0	146.7
	ped2	0.125	828	20	6624	160		
	ped3	0.125	496	20	3968	160		

3	mfn1	1	14	0.7	14	0.7	26.0	1.3
	mfn2	1	26	1.3	26	1.3		
	mfn3	1	38	1.9	38	1.9		
	mn1	1	3	0.15	3	0.15	32.0	1.6
	mn2	1	55	2.75	55	2.75		
	mn3	1	38	1.9	38	1.9		
	mf1	1	63	3.15	63	3.15	116.3	5.8
	mf2	1	129	6.45	129	6.45		
	mf3	1	157	7.85	157	7.85		
	fn1	1	46	2.3	46	2.3	52.3	2.6
	fn2	1	43	2.15	43	2.15		
	fn3	1	68	3.4	68	3.4		
	n1	1	105	5.25	105	5.25	118.0	5.9
	n2	1	132	6.6	132	6.6		
	n3	1	117	5.85	117	5.85		
	ped1	1	109	5.45	109	5.45	133.0	6.7
	ped2	1	154	7.7	154	7.7		
	ped3	1	136	6.8	136	6.8		
4	mfn1	1	42	2.1	42	2.1	30.0	1.5
	mfn2	1	30	1.5	30	1.5		
	mfn3	1	18	0.9	18	0.9		
	mn1	1	49	2.45	49	2.45	51.0	2.6
	mn2	1	50	2.5	50	2.5		
	mn3	1	54	2.7	54	2.7		
	mf1	1	93	4.65	93	4.65	80.0	4.0
	mf2	1	87	4.35	87	4.35		
	mf3	1	60	3	60	3		
	fn1	1	53	2.65	53	2.65	74.3	3.7
	fn2	1	93	4.65	93	4.65		
	fn3	1	77	3.85	77	3.85		
	n1	1	53	2.65	53	2.65	63.0	3.2
	n2	1	77	3.85	77	3.85		
	n3	1	59	2.95	59	2.95		
	ped1	1	88	4.4	88	4.4	78.0	3.9
	ped2	1	79	3.95	79	3.95		
	ped3	1	67	3.35	67	3.35		

Averages

Week	Sample	Ave/Plate	error	% error	ave %	CFU/mL	Log(CFU)/mL
1	MFN	755.3	40.0	5.30	6.91	7553.33	3.88
	MN	798.0	60.0	7.52		7980.00	3.90
	MF	3461.3	266.7	7.70		34613.33	4.54
	FN	238.0	20.0	8.40		2380.00	3.38
	N	804.7	33.3	4.14		8046.67	3.91
	Ped	5085.3	426.7	8.39		50853.33	4.71
2	MFN	3021.3	186.7	6.18	4.89	30213.33	4.48
	MN	3474.7	186.7	5.37		34746.67	4.54
	MF	3386.7	186.7	5.51		33866.67	4.53
	FN	1320.0	53.3	4.04		13200.00	4.12
	N	4336.0	240.0	5.54		43360.00	4.64
	Ped	5464.0	146.7	2.68		54640.00	4.74
3	MFN	26.0	1.3	0.05	5.00	26000.00	4.41
	MN	32.0	1.6	0.05		32000.00	4.51
	MF	116.3	5.8	0.05		116333.33	5.07
	FN	52.3	2.6	0.05		52333.33	4.72
	N	118.0	5.9	0.05		118000.00	5.07
	Ped	133.0	6.7	0.05		133000.00	5.12
4	MFN	30	1.5	0.05	5.00	15000.00	4.18
	MN	51	2.55	0.05		25500.00	4.41
	MF	80	4	0.05		40000.00	4.60
	FN	74.33	3.72	0.05		37166.67	4.57
	N	63	3.15	0.05		31500.00	4.50
	Ped	78	3.9	0.05		39000.00	4.59

Appendix B – MSDS

Material Safety Data Sheet

Issue Date: 5/7/2007

Section 1: Chemical Product and Company Identification

Cat#: 18190

Part Name: POLYSTYRENE PARA-MAGNETIC MICROPARTICLES 1-2 MICRON

Supplier: Polysciences, Inc.
400 Valley Road
Warrington, PA 18976
Telephone #215-343-6484

Section 2: Composition/ Information on Ingredients

Item#	Name	CAS#	% in product
1	Benzene, ethenyl-, homopolymer	009003536	2.5
2	Iron oxide (Fe3O4)	001317619	0.1
3	Water	007732185	97.5

OSHA (ACGIH) Exposure Limits

		TWA		STEL		CEILING	
		ppm	mg/	ppm	mg/m3	ppm	mg/m3
CAS#: 001317619	IDLH: NE						
OSHA		NE	NE	NE	NE	NE	NE
ACGIH		NE	NE	NE	NE	NE	NE
CAS#: 007732185	IDLH: NE						
OSHA		NE	NE	NE	NE	NE	NE
ACGIH		NE	NE	NE	NE	NE	NE
CAS#: 009003536	IDLH: NE						
OSHA		NE	NE	NE	NE	NE	NE
ACGIH		NE	NE	NE	NE	NE	NE

Section 3: Hazards Identification

Low hazard for usual industrial or commercial handling.

Hazard Ratings:
These ratings are Polysciences' Inc. own assesments of the properties of the material using the ANSI/NFPA 704 Standard.
Additional information can be found by consulting in the NFPA published ratings lists (List 325 and List 49).

If no data is listed the information is not available.

Health	Flammability	Reactivity
0	1	0

Section 4: First Aid Measures

Contact medical personnel.

Flush eyes with flowing water for at least 15 minutes.

If swallowed, wash out mouth with water if person is conscious.

Separate eyelids with finger tips.

Wash skin with deluge of water for at least 15 minutes.

Section 5: Fire Fighting Measures

Flash point, deg F.: >200 Method:

UEL: no data LEL: no data Autoignition temperature, deg. F.: no data

Flammability Classification: no data Flame Propagation Rate: no data

Hazardous Combustion Products: no data

Section 6: Accidental Release Measures

Any information listed below is to be considered in addition to internal guidelines for isolation of spill, containment of spill, removal of ignition sources from immediate area, and collection for disposal of spill by trained, properly protected clean up personnel.

No special measures are indicated.

Section 7: Handling and Storage

Keep from freezing.

Section 8: Exposure Controls/ Personal Protection

The use of eye protection in the form of safety glasses with side shields and the use of skin protection for hands in the form of gloves are considered minimum and non-discretionary in work places and laboratories. Any recommended personal protection equipment or environmental equipment is to be considered as additional to safety glasses and gloves.

No special protection is indicated.

Section 9: Physical and Chemical Properties

Formula:	mixture	vapor pressure:	no data
Formula Weight:	nap	vapor density:	no data
boiling point:	212	Specific gravity:	1.0
melting point:	32	ph:	7
solubility:	dispersible	appearance:	opaque brown mobile liquid

Section 10: Stability and Reactivity

Chemical Stabilit stable

Conditions to Avoid: none

Incompatibility with other materials: none

Hazardous Decomposition Products: none

Hazardous Polymerization: will not occur

Section 11:Toxicological Information

Acute Data: no data

Subchronic data: no data

Section 12: Ecological Information

no data

Section 13: Disposal Considerations

The following chart lists the status of the chemical and its components in reference to 40 CFR Part 261.33. If the product is listed by code number the substance may be subject to special federal and state disposal regulations. If no codes are listed the material must be disposed in compliance with all Federal, State and Local Regulations.

CAS#	Waste Code	Regulated Name
001317619	not listed	not listed
007732185	not listed	not listed
009003536	not listed	not listed

Section 14: Transportation Data

Refer to bill of lading or container label for DOT or other transportation hazard classification , if any.

Section 15: Regulatory Information

All components of this product are on the TSCA public inventory.

Prop 65 - Column A identifies those items which are known to the State of California to cause cancer. Column B identified items which are known to the State of California to cause reproductive toxicity.

CAS#	Column A	Column B
001317619	no	no
007732185	no	no
009003536	no	no

State Regulatory Information :If a CAS# is listed below this material is subject to the listed state right-to-know requirements.

CAS#	
001317619	not listed
007732185	not listed
009003536	not listed

SARA Toxic Release Chemicals(as defined in Section 313 of SARA Title III)
This list identifies the toxic chemicals, including their de minimis concentrations for which reporting is required under Section 313 of the Emergency Planning and Community Right-to-Know Act (EPCRA). The list is also referred to as the Toxics Release Inventory (TRI) List.

CAS#	Regulated name	de minimis conc. %	Rep. Thres.
001317619	not listed	not listed	not listed
007732185	not listed	not listed	not listed
009003536	not listed	not listed	not listed

SARA Extremely Hazardous Substances and TPQs
This list includes hazardous chemicals as defined in 29 CFR 1910.1200(c); and extremely hazardous substances regulated under Section 302 of SARA Title III with their TPQs (in pounds), as listed in 40 CFR 355, Appendices A and B.

CAS#	Regulated name	TPQ (pounds)	EHS-RQ(pounds)
001317619	not listed	not listed	not listed
007732185	not listed	not listed	not listed
009003536	not listed	not listed	not listed

CERCLA
The hazardous substances, and their reportable quantities (RQs) are listed in the federal regulations at 40 CFR Part 302, Table 302.4. Release of a CERCLA hazardous substance in an amount equal to or greater than its RQ, in any 24-hour period, must be reported to the National Response Center at (800) 424-8802.

CAS#	Regulated name	RQ (pounds)
001317619	Not listed	Not listed
007732185	Not listed	Not listed
009003536	Not listed	Not listed

Section 16: Other Information

POLYSCIENCES, INC. provides the information contained herein in good faith but makes no representation as to its comprehensiveness or accuracy.
Individuals receiving this information must exercise their independent judgment in determining its appropriateness for a particular purpose. POLYSCIENCES, INC. makes no representations or warranties, either expressed or implied of merchantability, fitness for particular purposes with respect to the information set forth herein or to which the information refers. Accordingly, POLYSCIENCES, INC. will not be responsible for damages resulting from the use of or reliance upon this information.

END OF MSDS

Appendix C - SOP

Nisin Activity on PS Microspheres
Matt Ryder
Revision 4

General Schedule

Day Before: Date_____

- Make MRS Broth (change bi-weekly): Vol _____ Date _____
- Make overnight P pentosaceus culture (16 hr incubation) Time/Date Start_____
  Finish_____; Vol_____; OD_{600}_____; rpm _____
- Incubate microspheres with F108 (16 hr min) Start_____ Finish_____

Day of test: Date_____

- Dilute 100x the overnight P pentosaceus culture: OD_{600}_____
- Make MRS agar. Total # samples: _____; MRS Gel Vol: _____ ml
- Make MRS agar tubes. Total made _____
- Incubate microspheres with nisin (1hr). Date _____

Perform colony count assay. Performed (# and date)

Day 7: _____

Day 14: _____

Day 21: _____

Day 28: _____

Inventory Check

- ☐ PS microspheres, (Iron Core, 1 – 2 μm, 2.5% solid. In refrigerator.)
- ☐ Pluronic F-108 Solution (5%, in refrigerator Lab 300.) if diluted, note here:

  _____
- ☐ South Africa nisin Solution (-80 C freezer)
- ☐ 0.01 M phosphate buffer, 0.15 NaCl. 0.02 μm filtered
- ☐ Screw-top test tubes
- ☐ P pentosaceus Stock Solution (FBB 612, In -80 C freezer)
- ☐ Lactobacilli MRS Broth, (EMD, Product No: 1.10661.0500)
- ☐ Agar, Granulated (Difco, Product No: 214530)
- ☐ Petri Dishes, 100 mm in diameter.

Preparation

- ☐ Make sure incubator is on 37 ºC.
- ☐ Make sure steam valve for autoclave is open 1 hr before use.
- ☐ Wash hands carefully with soap and 70% alcohol. Put on gloves.
- ☐ **MRS Broth:** Add 52.2g of MRS to every liter of RO Water. Heat to dissolve MRS powder, then take to autoclave at 121 ºC for 15 min and 15 min drying.
  Vol made: _____ L
- ☐ **Overnight P pentosaceus Culture:** Take P pentosaceus glycerol stock from -80 ºC freezer. Dip a loop of P pentosaceus in a 50 ml Falcon test tube containing 10 ml of MRS broth. Incubate at 37 ºC for 16 hrs. Store at 4 ºC immediately after use.
  Date/Time Start: _____ Finish _____ OD_{600}_____
- ☐ **100x dilution of P pentosaceus:** Dilute P pentosaceus for necessary total volume.
  Vol made _____ Date _____
- ☐ **MRS Agar Sol'n:** Add 15g agar to every liter of MRS broth. Heat to dissolve powder, then take to autoclave at 121 ºC for 15 min and 15 min drying.
- ☐ **Glassware:** Autoclave necessary # of Screw-top glass test tubes and flasks with agar sol'n.
- ☐ **MRS Agar tubes**: Pipette 12ml of MRS agar sol'n into each autoclaved screw-top tube. Tubes may be stored at 4 ºC for 2 weeks. Date/Time made _____

- ☐ **Seeding P pentosaceus:** Wait until agar sol'n does not burn hand. Add 1 ml of overnight P pentosaceus dilution for every liter of MRS agar solution. Proceed ADT dish steps immediately.

Preparation of Pluronic F108 Coated PS Microspheres:

1. Mark 40 ml screw-top Oakridge tubes to organize for later testing, according to formulation. Add F108 to beads and incubate overnight. Date/Time _____
2. After incubation, vortex the tubes for 30 seconds to well-mix the microsphere solution
3. Spin down microspheres at 3 krpm for 7 min in swing arm centrifuge, Kelly Laboratory.
4. Remove supernatant and fill tubes with 0.01M PBS, 0.15 NaCl up to 25 ml.
5. Vortex tubes to disperse microspheres again.
6. Repeat steps 3-5 two more times.

Preparation of Nisin loaded Microspheres

1. Add Nisin and other excepients according to experiment, except serum. excepients added _____
2. Mount samples on rotator. Incubate for 1 hr at room temperature.

1st Washing

3. Spin down microspheres at 3 krpm for 7 min.
4. Remove supernatant
5. Fill tubes to 25 ml with 0.01 M PBS.
6. Repeat steps 3-5 two more times.
7. Add PBS up to 25 ml to samples. Let nisin coated microspheres stand for 1 hr.
8. Clean incubator with 70% alcohol before incubation.

2nd Washing

9. Spin down microspheres at 3 krpm for 7 min.
10. Remove supernatant
11. Fill tubes to 25 ml with 0.01 M PBS.
12. Repeat steps 9-11 two more times.
13. Remove supernatant from the tubes of the previous step. Add buffer and/or serum according to formulation.
14. Vortex (1 min) and sonicate (5 min) samples to disperse beads. Put samples on rotator and incubate at 37 ºC until designated test day. Date/Time _____

Assay of Microphere Activity:

Plate Count Method

1. Spin down or magnetically pellet incubated beads and remove supernatants. Wash, as in previous procedure, microsphere samples three times.
2. Add 100x dilute P pentosaceus culture up to 1.5 ml and disperse the 1 ml bead sample by vortexing.
3. Add another 1.5 ml 100x dilute P pentosaceus culture and sonicate for 3 min.
4. Pipette sol'n into 15 ml Falcon plastic tubes.
5. Add 7 ml of 100x dilute P pentosaceus culture to reach a 10 ml volume.
6. Rotate tubes at 37 ºC for 4 hours.
7. Start heating water to melt agar gels, heat to 40 ºC.
8. Take out 4-hr incubated tubes; disperse contents by vortexing.
9. Add 100 µl of the 4-hr culture to 9.9 ml of MRS broth to achieve 1000x dilution.
10. Add 0.5 ml of well-mixed 4 hr, 100x diluted samples at the center of Petri dish.
11. Obtain a MRS agar tube from water bath and pour on top of the microsphere containing sol'n; rotate the Petri dish in either direction to evenly distribute MRS agar on plate.
12. Incubate plates at 37 ºC for 48 hrs. Date/Time _____
13. Count the colonies on the plates. Record data in excel spreadsheet.

Appendix D – Ingredient List

Total sample size	1	ml	1000	
Total volume of Seradyn stock withdrawed	2.50E-08	m^3	2.50E-05	liter
Diameter of microspheres	1.50E-06	m	1.5	um
Radius of microspheres	7.50E-07	m		
Volume of one microsphere	1.77E-18	m^3		
% solid in Seradyn PS microspheres solution	2.5%			
Total volumn of solid in solution	6.25E-10	m^3		
Total number of microspheres	3.54E+08			
Surface of one microsphere	7.07E-12	m^2		
Total surface area of all microsphere	2.50E-03	m^2	25.00	cm^2
EGAP coating density (assume ~ F108)	3.28E+00	mg/m^2		
EGAP MW	1.48E+04	g/mol		
F108 MW	1.46E+04	g/mol		
Calculated coating density of F108	3.24E+00	mg/m^2		
Total amount of F108 needed for coating	8.09E-03	mg	8.09E-06	g
5% F108 in NaPi	0.05	g/ml	50	mg/ml
Final concentration of F108 need in samples	0.005			
Volume of 0.5% F108 needed to coat PS surface	1.62E-04	ml	**0.16**	ul
Total mole of F108 added	5.54E-10	mol		
% of serum protein needed	50%			
volume of serum needed	0.5	ml	500.00	ul
Surface Concentration of Nisin (I hr adsorption)	1.50E-01	ug/cm^2	1.50E-04	mg/cm^2
Total nisin needed	3.75E-03	mg	3.75E-06	g
MW of nisin	3.51E+03	g/mol		
Nisin Conc (mg/ml)	3.64E+00	mg/ml	**Note: Here use 16 ul of 0.5 mg/ml SA nisin**	
Volume need	1.03E-03	ml	**1.03**	ul

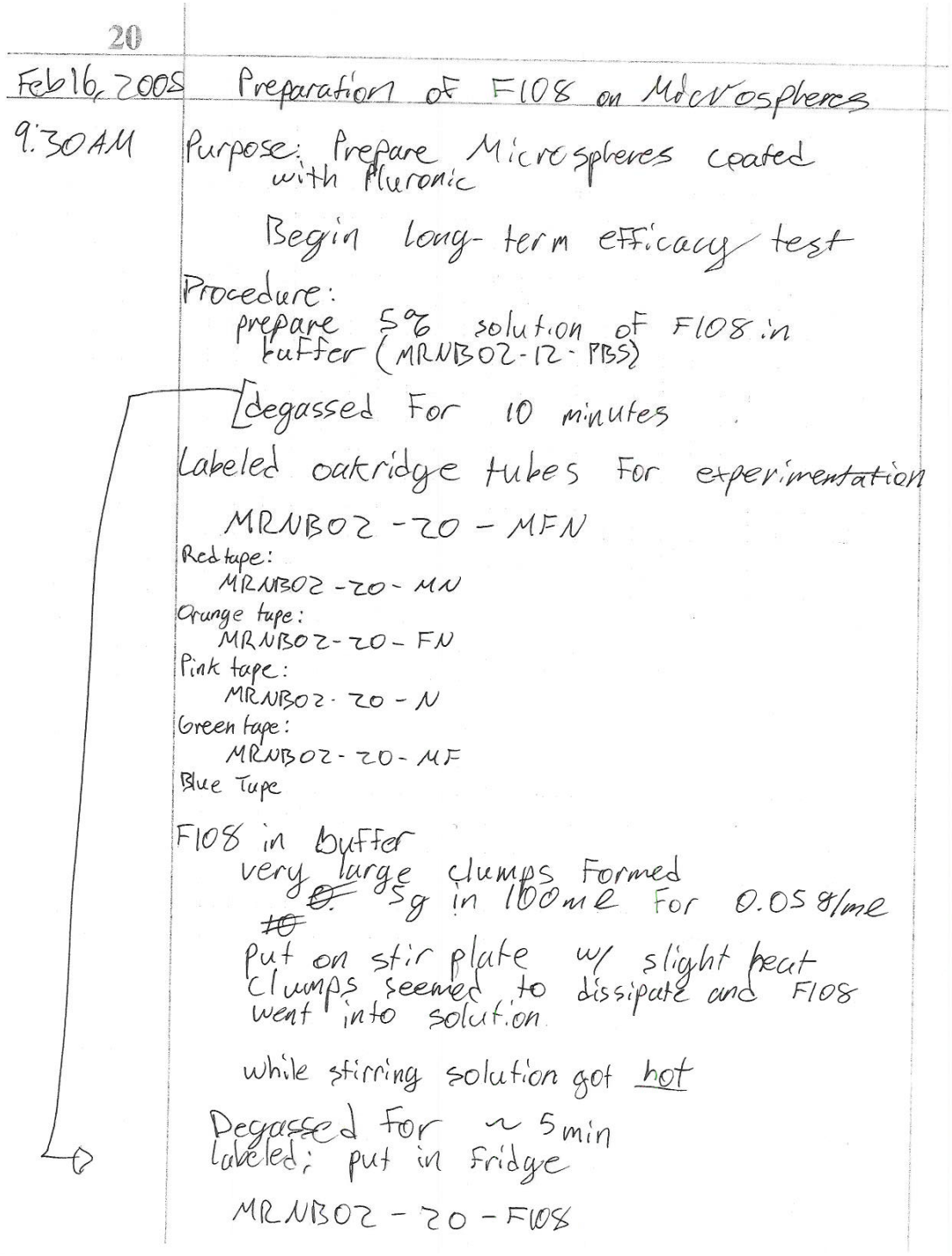
Formulations

	NaPi buffer (ml)	microspheres (ml)	5% F108 (ml)	nisin (0.5 mg/ml) (ml)
microsphere+F108+nisin	25.469	1.631	2.500	0.400
microsphere+nisin	27.969	1.631		0.400
F108+nisin	27.100		2.500	0.400
nisin only	29.600			0.400
microsphere+F108	25.869	1.631	2.500	

	# of sample	Code	Color	Total (ml)
microsphere+F108+nisin	1	MFN	**Red**	30.00
microsphere+nisin	1	MN	**Orange**	30.00
F108+nisin	1	FN	**Purple**	30.00
nisin only	1	N	**Green**	30.00
microsphere+F108	1	MF	**Blue**	30.00

Estimated Excepient Usage		
Microspheres	4.8935	ml
0.5% F108	7.5	ml
0.5 mg/ml nisin	1.6	ml
ms + F108	136.0065	ml

Appendix E – Laboratory Notes



20

Feb 16, 2005 Preparation of F108 on Microspheres

9:30 AM Purpose: Prepare Microspheres coated with Pluronic

Begin long-term efficacy test

Procedure:
prepare 5% solution of F108 in buffer (MRNB02-12-PBS)

degassed For 10 minutes

Labeled oakridge tubes For experimentation

MRNB02-20-MFN

Red tape:
MRNB02-20-MN

Orange tape:
MRNB02-20-FN

Pink tape:
MRNB02-20-N

Green tape:
MRNB02-20-MF

Blue Tape

F108 in buffer
very large clumps Formed
~~0.~~ 5g in 100ml For 0.05 g/ml
~~10~~
put on stir plate w/ slight heat
clumps seemed to dissipate and F108 went into solution

while stirring solution got hot

Degassed For ~ 5 min
labeled; put in Fridge

MRNB02-20-F108

21

Feb 16

Microspheres:
brown in color
2.57% soln
need 2.61 x previous volumes

1.63 ml per tube (Oakridge - autoclaved)

bottled opened on 2-16-08 @ 10:30 AM

had to open 2nd bottle
not all ms were in soln ... should have vortexed it

added FIOS to wrong tube; switch labels on MN & MF. No harm no foul.

Prepared 2 L mrs media

Autoclaved
media
DI W
test tubes for agar

20 min 10 min

Magnet did not work well
centrifuged in Glsn 202 swing arm
5 min 3000 rpm

Magnet ... to pull well ...

22

Feb 16, 2008

MN - difficult to resuspend

MFN
MF } easy to resuspend

MN - vortex vigorously ~2min
sonicated 2min

vortexed vigorously ~1min
sonicated 2min

Microspheres are in sol'n :)!!

centrifuged

3000 rpm
6 min

MN easier to get back in sol'n but not fantastic.

I suspect some bead loss as there is iron in the supernatants.

supernatants are ORANGE :(

getting more clear, but not water-like; still orangy

centrifuged

3000 rpm 6 min

MN has ms on surface

23

2-16-
Feb 16,

MRS Broth
made 2L (104.4 g mrs solids)

MRNB 02-23- MRS

autoclaved DI water
DIW 2-16-08

Today's Observations

work time 9am - 1pm

supernatant of solutions was orange in color
possible Iron leaching
non-pelleted microspheres
- some packing sol'n

possible effects
iron coats rather than FIOS or NiSm
may affect pediococcus

Magnet only worked once pellet was formed... may need stronger magnet
Allison Fry suggested a cow bell

Centrifuge
Christine's centrifuge (swing arm) worked well @ 3000 rpm for 6 min

MS seemed to coat surface of cartridge tube, may represent microsphere loss.

Total washes: 3

24

Feb 17, 2005 Loading Nisin on spheres

Prepared 0.5 mg/mL of Nisin using DI water (sterile) from yesterday

↳ made ~~with~~ by diluting from 10 mg/mL in 10mL to 0.5 mg/mL in 10mL

---

00 MN from yesterday has coated surface of tube, supernatant is clear; this is problematic

Nisin dilution

weighed 100 mg (0.1g) on scale in glass 300.

mass Nisin 0.1057 in 10mL sDI

need 20x dilution for 0.5 mg/mL

so 0.5 mL 10 mg/mL solution + 9.5 mL DIW = 10mL of 0.5 mg/mL soln

---

added nisin to reeessary tubes.

sonicated MN for 2 min to get beads off sides

rotate @ rt 1 hr

started @ 12:20 PM

25

Feb 1

centrifuged

MN & MFN for 6 min @ 3000 rpm
in 6ISN 202

since the addition of Nisin & ~2 hr rotation

MN seems to have spun down much better than microspheres alone.

Also, supernatant is much clearer today

Vortexed 1 min

Sonicated 1 min

centrifuged 6 min 3000 rpm

lost lots of microspheres this wash & centrifuge longer, pull out sooner

vortexed/sonicated
centrifuged 6 min 3000 rpm

centrifuged again right after
7 min 3500 rpm

Vortexed/sonicated: into 37 °C incubator

prepped 3 vials 10 mL pediococcus
in mrs media
in 50 mL falcon tubes

26

Th Feb 18, 2008 Day 1 of testing

~~Fem~~

Purpose: Plate samples on agar plates

T of Pediococcus incubator ~38°C

A of Ped culture

1) 1.257 @ 600nm
2) 1.234
3) 1.216

dilute all to 0.7 absorbance as base solution

created 700 mL 100x dilute pediococcus

Final absorbance 0.635

centrifuged samples on table top cent for 3 min @ 13.2 kppm

~~susped~~ Suspected bead loss too great 1 wash only

incubate 4 hrs started @ 1:30 PM ;)

27

Feb 18

Preparation of agar

added 15 g/L to 400 mL media

15 + 0.4 = 6g

autoclaved 20-10 on liquid setting.

Poured 20 plates in UV Flow hood in blsn 204

Observations

MN samples very hard to pellet

plating

Katie

MN - tip broke in MN1, sat for a bit,
MF = tip broke, tore gel left dent
FN =

Mat

MFN -
N -
Ped - } all went Fine, One hockey stick broke in frame

plated 100 mL of each sample + 100+ Pediococcus
Left two empty (just agargel)

Counting Colonies

Count colonies on plates to compare with previous and subsequent Data

Sample	Wedge Fraction	#counted	plate total
MFN 1	1/2	499 ± 20	998 ± 40
MFN 2	1/4	162 ± 10	648 ± 40
MFN 3	1/4	155 ± 10	620 ± 40
MF 1	1/16	141 ± 10	2256 ± 160
MF 2	1/16	276 ± 20	4416 ± 320
MF 3	1/16	232 ± 20	3712 ± 320
MN 1	1/4	95 ± 10	380 ± 40
MN 2	1/4	91 – 288 ± 15	364 – 1152
MN 3	1/4	314 ± 20	1256 ± 80
FN 1	1/2	130 ± 10	260 ± 20
FN 2	1/2	99 ± 10	198 ± 20
FN 3	1/2	128 ± 10	256 ± 20
N 1	1/2	310 ± 10	620 ± 20
N 2	1/4	330 ± 15	1320 ± 60
N 3	1/2	237 ± 10	474 ± 20
Ped 1	1/16	380 ± 50	6080 ± 800
Ped 2	1/8	421 ± 20	3368 ± 160
Ped 3	1/16	363 ± 20	5808 ± 320

29

Feb 20,

Observations:

"Blanks" had zero colonies, as expected

Ped 1 - so many colonies, very small
consider values as estimations only

Ped 2 - some streaking; looks like possible contamination of gel (~~has~~ colony formation in gel)

Ped 3 - slight tear in gel - not where counted

larger counts have larger variability
due to smaller sizes and more
convergence of colonies

Procedural changes for next test

increase dilutions for next sampling

$d_0 = 100x \Rightarrow \boxed{d_1 = 400x}$

Pediococcus
→ shoot for 0.635
grow 3 vials on Saturday
dilute each separately

Matt
MFN
N
Ped

Katie
MF
MN
FN

30

2/23/2008 Pediococcus culture

Purpose: start growing Pediococcus culture for sampling on 2-24-2008

Also MV vortexed & centrifuged

2/24/2008 2nd test day

Purpose: Plate test samples for data collection on Feb 26

Katie: Preparation of Agar and dilute Pediococcus

Matt: wash ~~##~~ test samples

Pediococcus: Temp was 36 °C

Filtered more buffer; adjusted pH to ~ 7.4

buffer seems to hold stable bubbles, which makes no sense
- protein contamination?
- surfactant on 0.2 um filter?

MRNB02-30-PBS

While washing

Magnets work!

however bead loss may still be prevalent, so only two washes were conducted

31

Plate Counts / Data Collection — Feb 26,

Purpose: collect data by counting colonies on plates.

Sample	Wedge Frac	# counted	plate total
MFN1	1/4	1034 ± 50	4124 ± 200
MFN2	1/4	537 ± 30	2148 ± 120
MFN3	1/8	348 ± 30	2792 ± 240
N1	1/8	594 ± 40	4752 ± 320
N2	1/8	582 ± 30	4656 ± 240
N3	1/8	450 ± 20	3600 ± 160
Ped1	1/8	725 ± 15	5800 ± 120
Ped2	1/8	828 ± 20	6624 ± 160
Ped3	1/8	446 ± 20	3968 ± 160
MF1	1/16 1/8	277 ± 30	2216 ± 240
MF2	1/8	519 ± 20	4152 ± 160
MF3	1/8	474 ± 20	3792 ± 160
MN1	1/8	366 ± 20	2928 ± 160
MN2	1/8	265 ± 20	2120 ± 160
MN3	1/16	336 ± 15	5376 ± 240
FN1	1/8	167 ± 10	1336 ± 80
FN2	1/8	171 ± 5	1368 ± 40
FN3	1/8	157 ± 5	1256 ± 40

"Agar-only" has no growth!

very patchy growth throughout.

32

1, 2005 Culturing Pediococcus

Incubation started @ 8:29 PM

7, 2005 Taking Samples, week 3

Purpose: take samples and plate them for counting on Mar 4

took 3ml from each sample, prepared 3 replicates of 1ml samples of each vial

Red caps - MFN
Blue caps - MF
Orange caps - MN
Green caps - N
purple caps - FN

First vortexed and sonicated MN

Created 200 mL of 100x pediococcus

$A_{600} = 0.617$

completed 3 washes

MN seems to hold to the microfuge tubes much more than any other sample
MFN does slightly, but MN shows strong interaction.

33

Plate Counts ~~&~~ / Data collection — Mar 4,

Purpose: collect data by counting colonies on plates

Sample	Wedge Frac	# counted	plate total
MFN1	1	14	14
MFN2	1	26	26
MFN3	1	38	38
N1	1	105	105
N2	1	132	132
N3	1	117	117
Ped1	1	109 ± 5	109 ± 5
Ped2	1	154 ± 5	154 ± 5
Ped3	1	136 ± 5	136 ± 5
MF1	1	63	63
MF2	1	129 ±5	129
MF3	1	157	157
MN1	1	3	3
MN2	1	55	55
MN3	1	38	38
FN1	1	46	46
FN2	1	43	43
FN3	1	68	68

Agar-only has no growth

35

Plate Counts / Data Collection

Purpose: collect data by counting colonies on plates

Sample	wedge	# counted	plate total
MFN1	1	42	42
MFN2	1	30	30
MFN3	1	18	18
N1	1	53	53
N2	1	77	77
N3	1	59	59
Ped1	1	86	86
Ped2	1	74	74
Ped3	1	67	67
MF1	1	93	93
MF2	1	87	87
MF3	1	60	60
MN1	1	44	44
MN2	1	50	50
MN3	1	54	54
FN1	1	53	53
FN2	1	93	93
FN3	1	77	77

Agar - only has no growth!