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## Optimization of the production of the lantibiotics mutacin 1140 in modified M9 media

Neeti Dahal

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OPTIMIZATION OF THE PRODUCTION OF THE LANTIBIOTICS MUTACIN 1140  
IN MODIFIED M9 MEDIA

By  
Neeti Dahal

A Thesis  
Submitted to the Faculty of  
Mississippi State University  
in Partial Fulfillment of the Requirements  
for the Degree of Master of Science  
in Biological Sciences  
in the Department of Biological Sciences

Mississippi State, Mississippi

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Neeti Dahal

2009

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IN MODIFIED M9 MEDIA

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Mutacin 1140, a class 1 bacteriocin, is produced by *Streptococcus mutans* and belongs to the type A lantibiotic family. Experiments were done to optimize production of mutacin 1140 in minimal media enabling a more cost efficient downstream purification method. The development of a small volume fermentation method enabled a rapid screen of several variables in a standard shaking incubator. This method provided a fast approach for determining components that promote mutacin 1140 production in minimal media broth. Lactose was determined to be the optimal carbon source for mutacin 1140 production. High concentrations of CaCl<sub>2</sub> (0.3% w/v) and MgSO<sub>4</sub> (0.77% w/v) promoted an increase in mutacin 1140 production, while ZnCl<sub>2</sub> and FeCl<sub>3</sub> appeared to impair production. Optimization of mutacin 1140 production in minimal media resulted in more than a 100-fold increase in production compared to the base medium used to begin our optimizations. The yield has been estimated by RP-HPLC to be 10 mg/L.

Key words: Mutacin 1140, Lantibiotic, *Streptococcus mutans*, fermentation, minimal medi

## DEDICATION

I would like to dedicate this research to my parents Nirmal Prasad and Nisha Dahal, my parents-in-law, Hom Nath and Kamala Nepal, my husband, Prakash, and my daughter, Himangi.

## ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to my advisor Dr. James L. Smith, thank you for countless hours of effort and guidance to support me in the completion of this research without which completion of this research would have not materialized. You have always inspired me to work hard. I hope I am able to fulfill your expectations. I would like to thank my committee members Dr. Donna Gordon, Dr. Janet Donaldson and Dr. Todd Pharr for their valuable advice and support without which this research work would have been incomplete.

I would specially like to thank my husband, Prakash for giving me strength, for supporting me in hard times and for his never ending love. I am indebted to my mother-in-law for her inspiration, encouragement and for easing me in my hard times especially during tight schedules of the semesters. Words are not enough to express my thanks to my daughter, Himangi. She brings a smile to my face when I am down. She is meaning to life. My special thanks to my parents for bringing me into this world, my sister, Swati for being such a wonderful sister and friend.

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## CHAPTER I

### INTRODUCTION

Today's world is facing a problem of bacterial antibiotic resistance. To solve this problem it is necessary to find new and better antimicrobial substances. A large body of research and investigation has been going on over the past few decades for new antimicrobials with novel mechanisms of action. Bacteriocins are a group of antibiotics which are proteinaceous in nature [1]. There are different kinds of bacteriocins which have been characterized to date and among them Class I are characterized by being small peptide inhibitors containing modified residues. Class II are characteristically larger, approximately 40 amino acids, and the mechanism of action is generally by disrupting bacterial membranes. These two classes of bacteriocins are most studied due to their abundance and their potential use for industrial applications [2]. Of interest are bacteriocins produced by *Streptococcus mutans* which are known as mutacins [3]; in particular, a class I bacteriocin called mutacin 1140 (Figure 1). Mutacin 1140 belongs to a subgroup of class I bacteriocins called lantibiotics. Lantibiotics are defined as lanthionine-containing antibacterial peptides with dehydrated amino acid residues and thioether bridges resulting from posttranslational modifications [1,4]. The term lantibiotic comes from the presence of unusual amino acids called lanthionine (Lan) and  $\beta$ -ethylanthionine (MeLan) (Figure 2). In addition to Lan and MeLan residues, there are other modified amino acids found in mutacin 1140 which include 2,3-

didehydroalanine (Dha), 2,3 didehydrobutyrine (Dhb), and the unsaturated lanthionine derivatives such as S-amino vinyl-D-cysteine (AviCys). Mutacin 1140 is composed of four lanthionine rings. The C-terminal lanthionine rings C and D overlap, which adds to the complexity of the structure of this antimicrobial peptide.

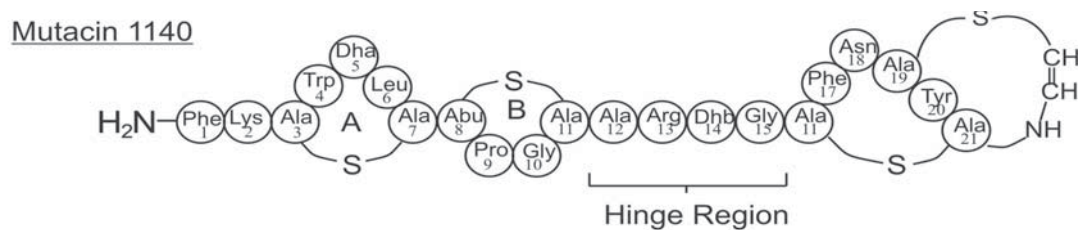


Figure 1. Covalent structure of mutacin 1140 [5].

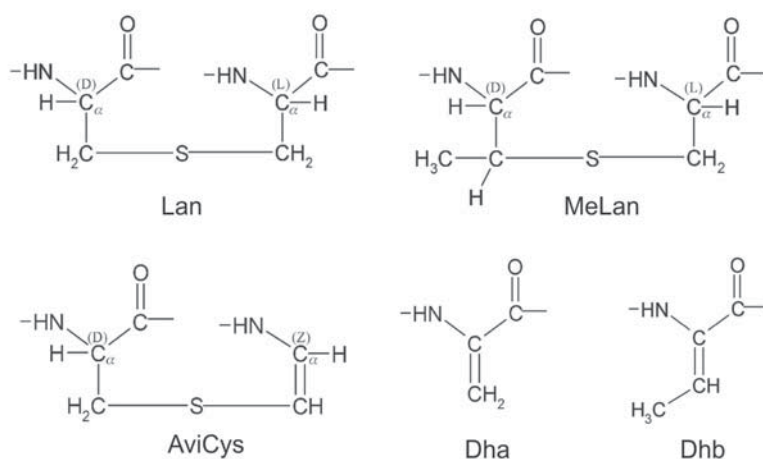


Figure 2. Diagram of the modified amino acid residues present in mutacin 1140[5].

There are two groups of lantibiotics: Type A and Type B, which are grouped based on their structural and functional features. Type A lantibiotics are elongated, cationic peptides up to 34 residues in length. Type A lantibiotics which include nisin, gallidermin, epidermin, and mutacin 1140 act by disrupting the membrane integrity of target organism via a specific interaction to the membrane component lipid II [6-23]. Type B lantibiotics, such as actagardine, are globular peptides containing up to 19

residues in length. They act through disrupting enzyme function e.g., inhibition of cell wall biosynthesis [24,25].

A series of enzymes act on a ribosomally synthesized prepeptide to produce a mature lantibiotic molecule (Figure 3, Step1) [5]. In mutacin 1140, *mutA* gene (LanA gene) encodes a ribosomally synthesized prepeptide containing serine and threonine residues. The *mutB* enzyme (LanB) is responsible for the dehydration of the serine and threonine residues to give Dha and Dhb, respectively. Cysteine residues located upstream of the dehydrated residues form a thioether linkage to these dehydrated amino acids [5]. *MutC* enzyme (LanC) is responsible for the subsequent addition of cysteine sulfhydryl groups to the didehydro amino acids, which results in the thioether ring formations (Figure 3, Step 2). The *mutD* enzyme (LanD) catalyzes the oxidative decarboxylation of the C-terminal cysteine, which gives a C-terminal S-aminovinyl-D-cysteine (AviCys) residue (Figure 3, Step 2). *MutT* (LanT), an ABC transporter, transports the modified peptide outside of the cell where *mutP* (LanP) an extracellular protease cleaves the leader sequence of the modified prepeptide (Figure 3, Step 3) [5].

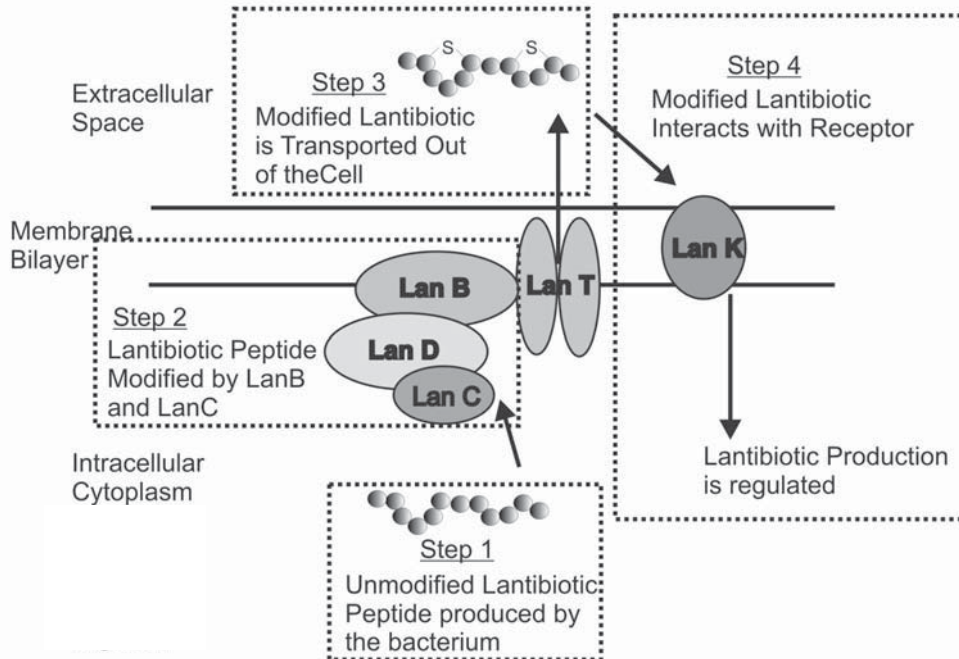


Figure 3. Diagram of the enzymatic modification taking place for mature mutacin 1140 production [5].

Mutacin 1140 has significant commercial value and broad applicability and practical methods for its production would have a significant economic impact. Mutacin 1140 has activity against essentially all tested Gram-positive bacteria and in particular certain medically important Gram-positive bacteria such as *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Enterococcus faecalis* and *Listeria monocytogenes*. Mutacin 1140 has also been shown to have potential oral applications for fighting tooth decay and is a key feature in the development of replacement therapy [26-29]. The producing strain of mutacin 1140, *S. mutans* JH1140, was engineered to make alcohol instead of lactic acid, which prevents the bacterium's ability to erode tooth enamel, thus preventing the formation of cavities. This strain has been shown to displace disease-causing strains of *S.*

*mutans* from the teeth of experimental animals due to its ability to produce mutacin 1140, and thereby indicating possible life-long protection against tooth decay [27,28].

As mentioned above mutacin 1140 has been shown to have desirable characteristics for the treatment of Gram positive infections [28,30]. The principal reason that compounds like mutacin 1140 have not been developed for therapeutic applications is due to the general difficulty of obtaining these molecules in sufficient, cost effective amounts to enable their testing and commercialization. Of the lantibiotics characterized to date [1], only the Type A (I) lantibiotic nisin A, produced by *Lactococcus lactis* has found wide application as a food preservative for the past 50 years. It is important to note that the bacterium *L. lactis* produces nisin A when used as a starter culture and the antibiotic itself has never been purified and directly added as a preservative. Fairly recently, a purification protocol for nisin A has been filed as a US patent application (USPA 20040072333), which utilized a cocktail of expensive proteases followed by column chromatography. There is no published, commercially viable procedure for the purification of nisin A. The problem of finding a commercially viable procedure for purifying nisin A is also true for mutacin 1140, as well as many other lantibiotics.

Fermentations have been optimized for the lantibiotics gallidermin and mutacin NY266 using complex media, such as yeast extract, high (5%) calcium chloride concentrations and using a large inoculum (10% v/v). These authors have reported production levels over 200 mg/L [31-35]. Using similar conditions and media composition about 50 mg/L of mutacin 1140 in a 3L bioreactor controlling temperature, pH, and oxygen is achievable [36]. Extraction of mutacin 1140 from this complex medium by RP-HPLC methods is also achievable, but not a commercially viable

approach because of the high cost for the purification. Synthetic approaches to making lantibiotics is extremely costly involving more than 60 chemical steps [37]. A semi-synthetic approach for producing nisin has been described, in which cell and membrane extracts recovered from the nisin producing organism are mixed with nisin prepeptide made by an *E. coli* expression system [38]. However, the yield of antibiotic produced by this approach is very low. This research will attempt to identify components important for the production of mutacin 1140 in a minimal medium, which will enable future studies aimed at developing a cost effective approach to purifying the mutacin 1140.

## CHAPTER II

### METHODS AND MATERIALS

All media was purchased from Difco Laboratory (Detroit, MI) and chemicals were purchased from Fisher Scientific (Pittsburgh, PA) and were the highest grade, unless otherwise stated.

#### **Bacterial Strains**

Bacterial strains used in this study include: *Streptococcus mutans* JH1140 ATCC 55676 and *Micrococcus luteus* ATCC 272. *S. mutans* was used for the production of mutacin 1140. *M. luteus* was used as an indicator organism for detection of mutacin 1140 production.

*S. mutans* was first screened for colonies that would grow on minimal media and maintain antimicrobial activity. Colonies of *S. mutans* were picked from plates grown on Todd Hewitt-yeast extract (30 g Todd Hewitt broth/L, 3 g yeast extract/L) and plated on modified M9 agar (M9 medium, supplemented with casamino acids (10 g/L), CaCl<sub>2</sub> (5 g/L), glucose (40 g/L), NaHCO<sub>3</sub> (1 g/L), and agar (15 g/L). During our initial screen of medium components, casamino acids were determined to be essential for the antimicrobial activity of *S. mutans*. Individual colonies grown on modified M9 agar were selected and screened for antimicrobial production using a standard deferred antagonism assay as outlined below. Colonies stabbed into a fresh modified M9 agar plate were



overlaid with *M. luteus* indicator strain, while simultaneously plating on a fresh master plate of each colony. Ten colonies producing the largest clearing were analyzed again. The colony that produced the largest zone was selected for further analysis. One possibility, clearing is proportionate to mutacin 1140 production in the minimal media environment, thus this strain was used as the inoculum in the optimization of mutacin 1140 production in minimal media.

### **Rapid procedure for optimizing fermentation conditions**

A stock solution of 100  $\mu\text{L}$  aliquots of purified mutacin 1140 (provided by Oragenics Inc., Alachua FL) in 80% acetonitrile at a concentration of 10 $\mu\text{g}/\text{mL}$  was stored at 4°C and was used to compare mutacin 1140 production over different variables and across different samples. Small volume (20 mL) fermentations using a modified M9 minimal medium (M9 medium, supplemented with casamino acids and a 10% inoculum of *S. mutans* was used as our base medium for optimizing each variable in a shaking incubator at 200 rpm at 37°C for 24 hours. The inoculum was started from a 400  $\mu\text{L}$  glycerol stock (10<sup>9</sup> CFU (colony forming unit)/mL) grown in 40 mL of modified minimal media supplemented with 0.3% yeast extract (to help boost the inoculum growth rate) to an OD<sub>600</sub> of 0.8 at 37°C.

At completion of the fermentation, the samples were centrifuged at 23,000 x g for 20 min and then the supernatants were collected. The collected supernatants were heated for 30 minutes at 65°C to kill any remaining bacteria. The resulting supernatant was assayed for mutacin 1140 production using the modified deferred-antagonism assay described below. Five microliters of the resulting supernatants were stabbed in triplicate

horizontally across a 100 mm Todd Hewitt-Yeast extract plate and the mutacin 1140 stock solution was also stabbed in triplicate across each bioassay plate for comparison. Quantification of mutacin 1140 production was determined by the following formula: Mutacin production = diameter of culture liquor zone (mm)/diameter of mutacin 1140 stock solution zone (mm). The ratio of each zone was averaged and the standard deviation was calculated using the averaged ratios from each fermentation (n = 3). This approach enabled the comparison of each variable tested across numerous plates.

### **Antimicrobial assay**

A *M. luteus* deferred-antagonism assay is a qualitative assay for bactericidal activity. *M. luteus* is a mutacin sensitive strain with a nanomolar minimum inhibitory concentration (MIC). *M. luteus* was grown in Todd Hewitt-yeast extract to an OD<sub>600</sub> of 0.2. Then, 400 µL of these cells were added to 10 mL of top agar (M9 media, casamino acids 10 g/L, and agar 7.5 g/L). 5 ml of melted top agar containing the standardized suspension were added to each Petri dish containing approximately 20 mL of modified M9 media agar. Before the plates were overlaid with the top agar containing the indicator strain, individual colonies of *S. mutans* were stabbed into the modified M9 medium agar and placed inverted into a candle jar for 48 hours. Following two days of incubation, the stabbed colonies were then overlaid with the top agar containing *M. luteus*. The plates were then allowed to dry before being inverted and placed in a candle jar overnight at 37°C. The following day the plates were checked to determine the relative size of the zone of inhibition created by the stabbed colonies. Zones of inhibitions were measured in units of millimeters. The modified deferred antagonism assay was performed as described

above for the deferred antagonism assay, except that 5 $\mu$ L of the supernatant of the overnight fermentations was stabbed instead of a bacteria colony into Todd Hewitt-yeast extract agar plates and overlaid *M. luteus* in Todd Hewitt-yeast extract top agar (30 g Todd Hewitt Broth/L, 3 g yeast extract/L, and 7.5 g agar/L). Once the top agar on the Petri dish had solidified 5  $\mu$ L of the cell free culture liquor was stabbed in triplicate on the plate. The plates were then allowed to dry before being inverted and placed in a candle jar overnight at 37°C. Zones of inhibitions were measured in units of millimeters. Supernatants from fermentations using our base medium was used as a positive control for mutacin 1140 production and each medium tested (minus the inoculum) was used as a negative control for antimicrobial activity. Colony forming units (CFU) were determined in duplicate by serial dilution and plating method. *S. mutans* grows in long chains, thus the CFU data is slightly variable.

### **Quantification of mutacin 1140**

Following optimization parameters described above, the supernatant of the culture broth was analyzed by RP-HPLC. RP-HPLC was done using a 4.6 x 250 mm C18 column (Grace-Vydac, catalog 201TP54) on a Bio-Rad BioLogic F10 Duo Flow with Quad Tec UV-Vis Detector system. An acetonitrile gradient was established by varying the flow rate of Solvent A (99% acetonitrile-0.1% trifluoroacetic acid (TFA)) relative to Solvent B (Water-0.1% TFA) maintaining a constant flow rate of 1.0 mL/min and monitored at 220 nm. RP-HPLC protocol following a 1 mL injection consisted of a linear gradient (Solvent B, decreased from 95%-30% over a 30 min period), followed by an isocratic flow (Solvent B, 95% over a 5 min period). Mutacin 1140 eluted from the

column at approximately 56% solvent B. Quantification of mutacin 1140 was ascertained from peak volume. MALDI-TOF (ABI 4700 Proteomics Analyzer) was used, along with the *M. luteus* modified deferred-antagonism assay, to verify the HPLC mutacin fraction.  $\alpha$ -cyano-4-hydroxycinnamic acid was used as the matrix for MALDI-TOF.

## CHAPTER III

### RESULTS AND DISCUSSION

#### **Determining optimal concentration of calcium chloride for mutacin 1140 production**

Earlier experiments in the complex medium yeast extract showed that a high concentration of  $\text{CaCl}_2$  (5% w/v) was optimal for mutacin 1140 production [36]. Due to the importance of calcium chloride for mutacin 1140 production in broth,  $\text{CaCl}_2$  was the first component optimized. To determine the optimal concentration for  $\text{CaCl}_2$ , the following percentages (w/v), 0, 0.1, 0.2, 0.3, 0.4, 0.5, 1.0, and 1.5% were first investigated. Figure 4 presents the optimal concentration of  $\text{CaCl}_2$  for mutacin 1140 production. The black columns in the figure correspond to antimicrobial activity, while the grey columns correspond to the CFU values. The optimal concentration for mutacin 1140 production was 0.3%  $\text{CaCl}_2$ . There was approximately a 40% increase in zone diameter as compared to 0.2 and 0.5%  $\text{CaCl}_2$ . Interestingly there appeared to be a narrow window for promoting mutacin 1140 production. No activity was observed at 0.1% and 1%  $\text{CaCl}_2$  concentrations.  $\text{CaCl}_2$  also appeared to be required for cell survival, since there were no CFUs when calcium was inabated from the media. Furthermore, higher concentration of  $\text{CaCl}_2$  appeared to inhibit growth since there was an order of magnitude drop in cell density at 1.0 and 1.5% concentration compared to 0.5%. A 0.3%  $\text{CaCl}_2$  concentration was used as our base medium for the following optimization experiments.

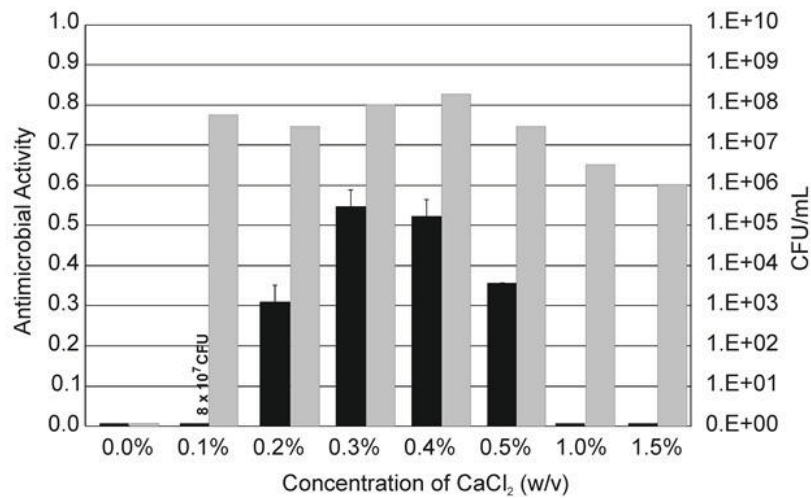


Figure 4. Optimization of mutacin 1140 production by varying CaCl<sub>2</sub> concentrations ranging from 0.0 to 1.5 %.

### Determining optimal concentration of biologically important metals for mutacin

#### 1140 production

Several other inorganic salts can be tested for their effect on mutacin 1140 production, but for the scope of this study the inorganic salts tested were MgSO<sub>4</sub>, ZnCl<sub>2</sub>, and FeCl<sub>3</sub>. Figure 5 presents the optimal concentration of MgSO<sub>4</sub> for mutacin 1140 production. The black columns in the figure correspond to antimicrobial activity, while the grey columns correspond to the CFU values. Magnesium ions are required in a variety of enzymatic reactions, including DNA replication. Mutacin 1140 production was absent when magnesium ions were not added (Figure 5). Interestingly, there was cellular growth without the addition of magnesium. Presumably, the small amount of yeast extract added to the inoculum provided a sufficient source of magnesium ions for growth. There was an interesting decrease in mutacin 1140 production between 60 and 480 µg/mL of magnesium sulfate. These experiments were repeated showing the same

phenomenon. Optimal production of mutacin 1140 occurred at 7,680  $\mu\text{g/mL}$  (0.77% w/v) of  $\text{MgSO}_4$ . However, the benefit of a high concentration of  $\text{MgSO}_4$  has never been mentioned in the production of other lantibiotics. The role of zinc ions had been shown to be important for the production of other lantibiotics [38-40]. Zinc is important for the activity of the post translational modification enzyme responsible for the formation of the lanthionine rings. Interestingly, mutacin 1140 production decreased when  $\text{ZnCl}_2$  was supplemented at concentrations between 20 and 40  $\mu\text{g/mL}$  (Figure 6). The Black columns in the figure correspond to antimicrobial activity, while the grey columns correspond to the CFU values. Given the CFUs at these concentrations, the cells appeared to be healthy since they grew. Also of interest is that mutacin 1140 production was best when no zinc was supplemented in the media. If zinc is a requirement for the formation of the thioether linkages between the cysteine sulfhydryl groups and the didehydro amino acids, then presumably a small amount of zinc may be present in the inoculum which is sufficient for enzymatic activity. Nonetheless, supplementing with additional zinc should be avoided. Iron ions have an important role in the catalytic sites of several bacterial enzymes. Supplementing  $\text{FeCl}_3$  between 0 and 10  $\mu\text{g/mL}$  had no effect on mutacin 1140 production, however at concentrations above 10  $\mu\text{g/mL}$ , a pronounced effect on *S. mutans* viability and mutacin 1140 production was observed (Figure 7). The black columns in the figure correspond to antimicrobial activity, while the grey columns correspond to the CFU values. It is interesting to note that there was a significant amount of mutacin 1140 produced when the media was supplemented with 80  $\mu\text{g/mL}$  given that the cell density was >1000 fold less than the media containing no supplemented iron. Future experiments will explore whether the addition of iron at a later time point in the

fermentation can promote additional mutacin 1140 production. Perhaps bacterial growth is not an important criterion for mutacin 1140 production and that a later time period in which there is a higher cell density may afford more mutacin 1140 production by possibly inactivating other cellular activities that can down regulate mutacin 1140 production.

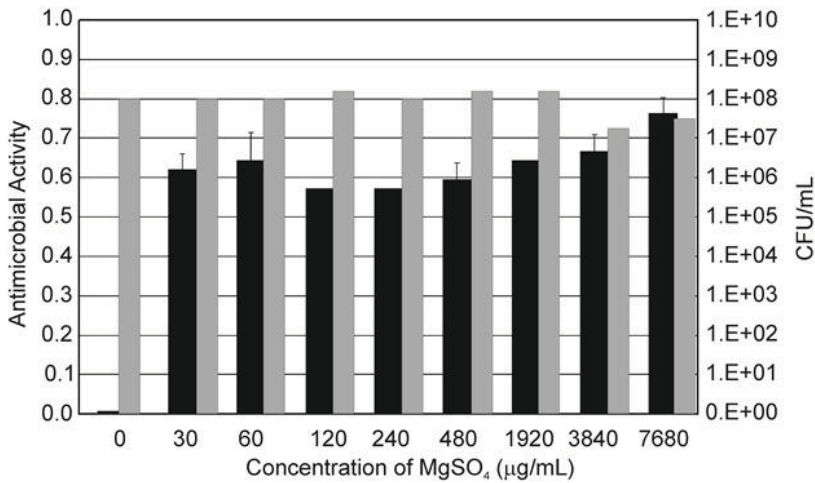


Figure 5. Optimization of mutacin 1140 production by varying MgSO<sub>4</sub> concentrations ranging from 0.0 to 7,680 µg/mL.

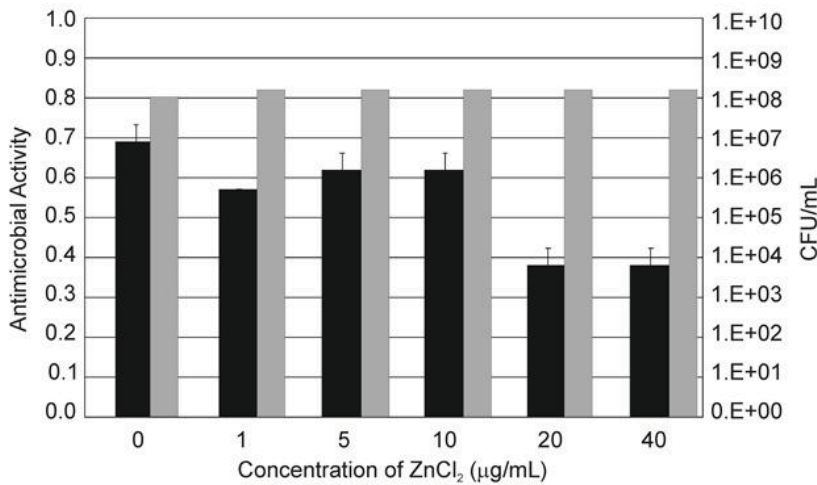


Figure 6. Optimization of mutacin 1140 production by varying ZnCl<sub>2</sub> concentrations ranging from 0.0 to 40 µg/mL.



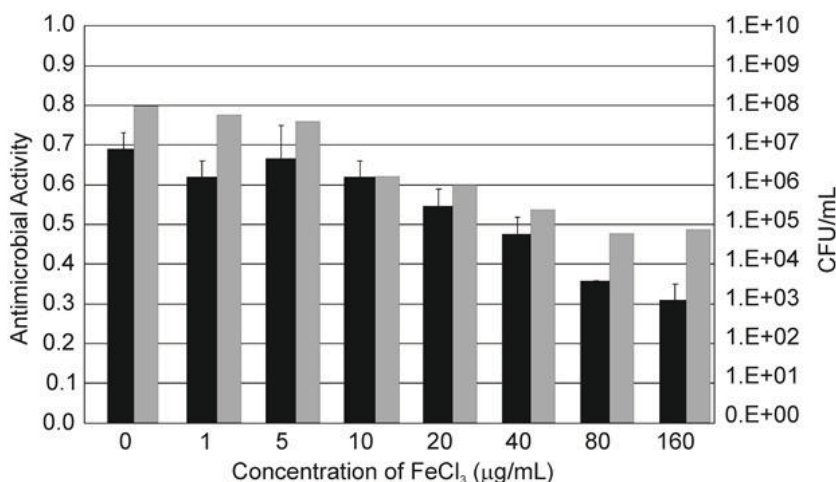


Figure 7. Optimization of mutacin 1140 production by varying FeCl<sub>3</sub> concentrations ranging from 0.0 to 160 µg/mL.

### Screening media supplemented with cystine

Currently, the concentration of casamino acids in the base media is 10 mg/mL. Two amino acids, cysteine and tryptophan, do not survive the hydrolysis procedure of casein that is used for making casamino acids. Given that four of the 22 amino acids found in mutacin 1140 are cysteines, an experiment was designed to determine whether the addition of cystine would have an effect on the production of mutacin 1140. Cysteine was shown to boost the production of the lantibiotic gallidermin when supplemented in the fermentation media [32]. The following concentrations of cystine were tested; 0 µg/mL, 100 µg/mL, 200 µg/mL, 400 µg/mL, and 800 µg/mL. The disulfide link between the two cysteines in cystine is readily reduced by the bacterium to give the corresponding thiol amino acid. The concentration of cystine for Mutacin 1140 production is presented in figure 8. The black columns in the figure correspond to antimicrobial activity, while the grey columns correspond to the CFU values. The addition of cystine to our minimal M9 production medium had no significant effect on the production of mutacin 1140

(Figure 8). Possibly supplementing cystine may be important as the yield of mutacin 1140 is improved and will again be explored in future fermentations.

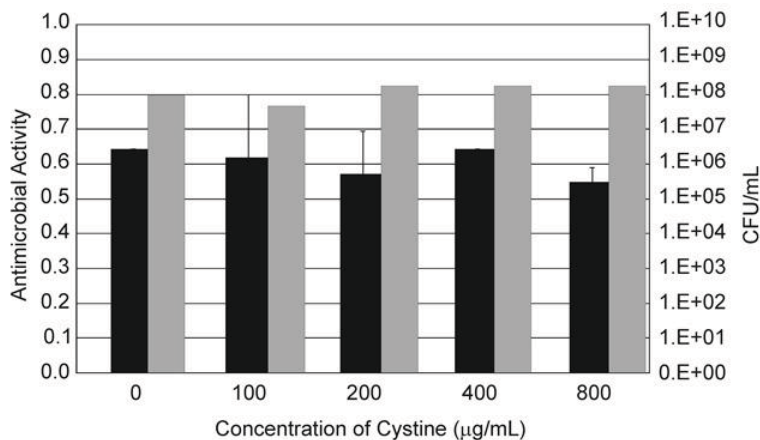


Figure 8. Optimization of mutacin 1140 production by varying cystine concentrations ranging from 0.0 to 800 µg/mL.

### Screening sugars for determining optimal carbon source

The base medium for the production of mutacin 1140 contains 4% glucose, which may not be the optimal carbon source for mutacin 1140 production. Several monosaccharide and disaccharide sugars were tested to determine the optimal carbon source. The monosaccharides glucose, galactose, fructose, xylose and ribose, as well as the disaccharides sucrose, maltose and lactose were tested at a 4% concentration (w/v). Production of mutacin 1140 was enhanced with the addition of lactose and galactose. Figure 9 presents the optimal concentration of monosaccharide and disaccharide sugars at 4% (w/v) concentrations. The black columns in the figure correspond to antimicrobial activity, while the grey columns correspond to the CFU values. Carbon sources fructose, xylose, and ribose resulted in no or low mutacin 1140 production, while the CFUs suggested that these carbon sources did support growth. Optimal concentration of lactose and galactose

in the fermentation media was not possible to determine because of the present scope of the research.

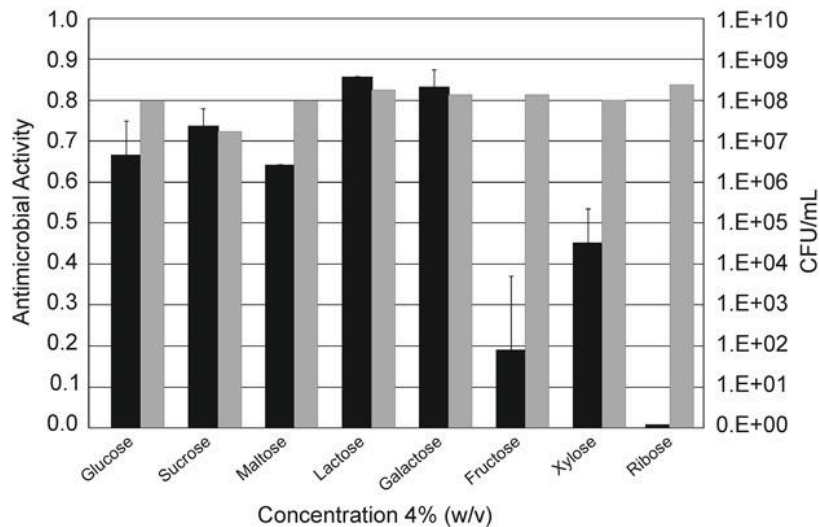


Figure 9. Optimization of mutacin 1140 production by varying monosaccharide and disaccharide sugars at 4% (w/v) concentrations.

### Determining optimal temperature and time for mutacin 1140 production

Mutacin 1140 production was tested at 37, 32, and 27°C. There was minimal production of mutacin 1140 at 32 and 27°C over a 24 hour time period (data not shown). Therefore, fermentations at 37°C using the modified M9 minimal media appeared to be the optimal temperature for determining the effects that the other variables had on mutacin 1140 production. Temperatures between 37 and 32°C as well as higher temperatures were considered for future experiments. There was no difference in mutacin 1140 production between the 24 hour and 48 hour samples at 37°C, which suggests that mutacin 1140 was produced during the 24 hour time period after inoculation. Samples were drawn hourly over a 24 hour time period to determine mutacin 1140 production (Figure 10). pH values of the fermentation broth listed in the figure was for each 2 hour

interval. Interestingly, there was no production until the ninth hour and production stopped at the twelfth hour. The pH over this time period ranged from 5.3 to 4.7. It is interesting to speculate that a lower pH is required for mutacin 1140 production. A 24 hour time period appears to be sufficient for optimizing mutacin 1140 production, since other variables may promote a longer timeframe in which mutacin 1140 is produced and since the compound is stable in the supernatant over 24 hours. Once mutacin 1140 production is completely optimized, scaling the time frame to peak mutacin 1140 production will be reinvestigated.

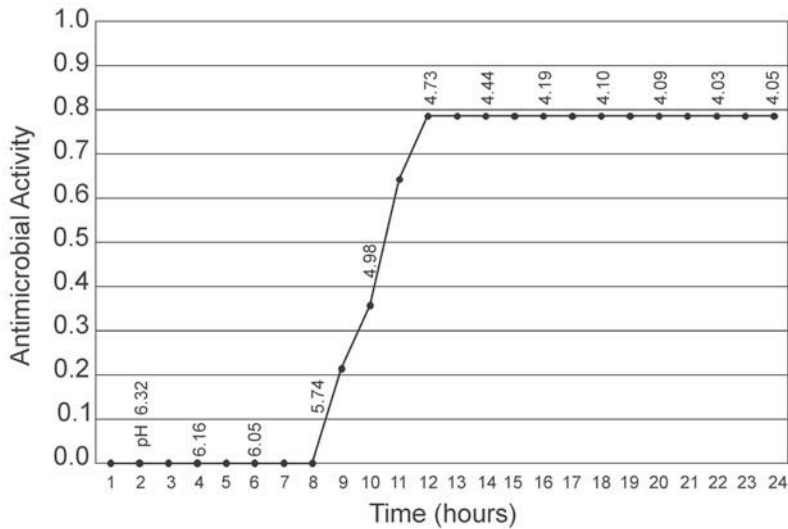


Figure 10. Production of mutacin 1140 over a 24 hour time period.

### Optimized media

A fermentation at 37°C for 24 hrs using all the optimized variables in the modified M9 media, supplemented with 1% casamino acids, 0.1% NaCOH<sub>3</sub>, 0.3% CaCl<sub>2</sub>, 0.77% MgSO<sub>4</sub>, and 4% lactose resulted in the supernatants having an antimicrobial activity greater than 1.0, demonstrating that production in a shaking incubator exceeded the concentration of the mutacin 1140 standard (10 mg/L) (data not shown). The

procedure was scaled from a 20 mL fermentation volume to a 500 mL fermentation volume in a 1 L bottle, which resulted in the same level of production (Figure 11). Translation of the procedure to 500 mL is important for future optimization studies in a controlled bioreactor. A serial 5 fold dilution assay of the 500 mL fermentation media also resulted in the same level of activity as was observed in the mutacin 1140 standard. The antimicrobial activity units for each five fold dilution of the mutacin 1140 standard and for the 500 mL fermentation are shown in Figure 11. The antimicrobial activity units were calculated by taking the ratio of the diameter of each dilution to diameter of the first zone of the mutacin 1140 standard. This shows that a 125 fold dilution resulted in an antimicrobial activity of 0.36, which was comparable to the activity seen in base modified M9 medium, containing 0.5% CaCl<sub>2</sub> and 4% glucose. Therefore, the small volume fermentations in a shaking incubator method for optimizing mutacin 1140 provided a rapid means of increasing the production by more than 100 fold. Confirmation that the antimicrobial activity observed in the fermentation came from RP-HPLC followed by MALDI mass determination. In Figure 12, the bioactive peak corresponds to the elution profile of mutacin 1140 eluting at 56% Water:Acetonitrile and the mass of the purified compound corresponds to the mass of mutacin 1140 which is 2266 Da.



Figure 11. Modified deferred-antagonism assay of the mutacin standard and the 500 mL fermentation.

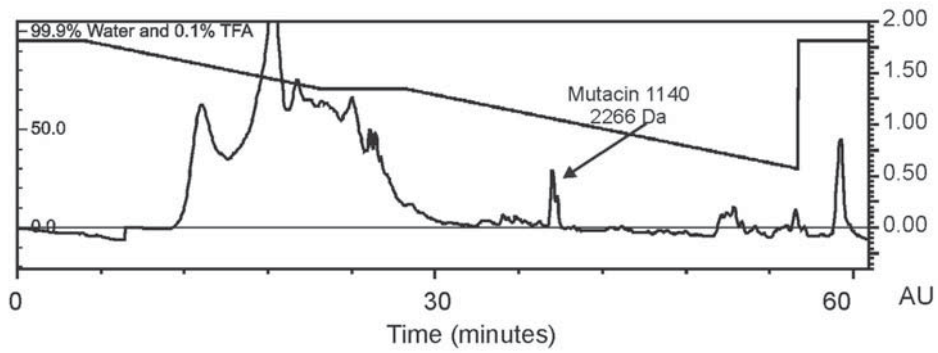


Figure 12. HPLC Chromatogram of purified mutacin 1140.

## CHAPTER IV

### CONCLUSIONS AND FUTURE DIRECTIONS

Several antibiotics have been known for decades but they have not been extensively tested for their potential usefulness for treating infections. The principal reason for this is due to the difficulty of obtaining these molecules in cost effective amounts and purity that would enable their testing for the treatment of infections. One major drawback is the use of complex medium generally used for their production, which obstructs the development of an effective approach to extract and purify them from the culture liquor.

Supplementations of  $MgSO_4$ ,  $CaCl_2$ , and lactose were shown to promote mutacin 1140 production, while  $ZnCl_2$  and  $FeCl_3$  appeared to impair production. The development of a small volume fermentation method enabled a rapid screen of several variables in a standard shaking incubator. Future experiments are planned using a 3L bioreactor. This will enable us to control for oxygen and pH, which should boost our current production [31-34]. Depending on the outcome of these experiments, the minimal medium approach may offer a significant advantage over production in complex medium yeast extract in terms of cost, as well as facilitating a more cost effective downstream purification method. Nevertheless, the small volume fermentation method described in this paper provides a useful method for optimizing the production of mutacin 1140 and may also be useful in the optimization of other antimicrobial substances. Furthermore, the current

yields of mutacin 1140 in minimal media will enable the production of isotopically labeled mutacin 1140 for nuclear magnetic resonance studies aimed at characterizing mutacin 1140's structure and interactions within bacterial mimetic membranes. Also, production in minimal media will enable the use of an IPTG inducible *S. mutans* plasmid for the production of other peptides in the mutacin 1140 producing strain *S. mutans* JH1140 while taking advantage of the lantibiotic transporter and extracellular protease. Solution and solid phase peptide synthesis is the current method of choice for producing peptides. However, the procedure requires expensive automated equipment and reagents. Minimal media environment for the expression of peptides in *S. mutans* would facilitate isolation and purification of recombinant peptides, while taking advantage of the bacterial system to produce the peptides of choice. Small protein/peptides, under 5000 Da, are generally degraded in *E. coli* expression systems, thus, future work will be aimed at developing the lantibiotic producing strain *S. mutans* JH1140 to effectively produce non-native peptides.



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