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NISIN RESISTANCE OF *BACILLUS CERUS*: PREPARATION OF NISIN

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Abstract

Nisin is a peptide that is made by the bacterium *Lactococcus lactis*. Nisin is a small molecule that kills gram positive bacteria by binding to their membrane and by disrupting the proton motive force. When food is processed it is heated to kill bacteria, but some bacteria still survive. Adding nisin to the food provides a second barrier for the growth of the bacteria. Purified nisin has become quite expensive in the current marketplace. Therefore this study is directed at producing nisin and purifying it. *L. lactis* was grown in five different media (BHI, BHI + 1% glucose, BHI + 1% sucrose, BHI + 3% yeast extract, and skim milk medium). Samples of nisin from these media were filter-sterilized and tested for their ability to inhibit the growth of *Lactobacillus viridescens*, which is susceptible to nisin. It was found that nisin from the media BHI + 3% yeast extract after 8 hours of growth has a similar concentration as the standard, which was 1000 µg/ml. The nisin was then purified. An extraction process using ammonium sulfate was used to precipitate nisin out of the media. It was found that a 50% ammonium sulfate concentration precipitated out all of the nisin. The nisin was dialyzed against distilled water to remove the salts. The Minimal Inhibitory Concentration (MIC) of the extracted nisin, using *Bacillus cereus* and endospores was equivalent to a commercial nisin preparation at 1000µg/ml.

Introduction

Nisin is a peptide that is made by the bacterium *Lactococcus lactis*. It is a small molecule that kills gram positive bacteria by binding to their membranes and by disrupting the proton motive force (1). It appears to be active against the endospores of the food-borne, pathogenic gram positive bacteria, *Clostridium botulinum* and *Bacillus cereus*, and inhibits their outgrowth. In recent years, nisin obtained a GRAS (generally recognized as safe) status for use as a biopreservative in the food industry.

When food is processed it normally goes through a heat treatment. This kills bacteria that are found in food. While vegetative cells of *B. cereus* are killed by heat, the endospores are more resistant. The endospores that survive turn into vegetative cells and start producing toxins. These toxins cause food poisoning. Adding nisin to the food provides a second barrier for the growth of the bacteria.

However, there is concern that bacteria will develop resistance to nisin, just as they did to antibiotics like penicillin. *Listeria monocytogenes*, another gram positive food borne pathogen, develops a resistance to nisin (2). *L. monocytogenes* does not form endospores. Whether endospores can become resistant is not known. However, *C. botulinum* can become nisin resistant and their spore outgrowth inhibited (3).

In order to produce endospores of *B. cereus* it is helpful to grow them on solid agar cultures. Addition of nisin to the culture medium requires large concentrations of nisin. Purified nisin has become quite expensive in the current marketplace. Therefore this study is directed at producing nisin and purifying it. Then it can be used with *B. cereus* cultures for the development of resistance in spores and vegetative cells.

Methods

Medium and stage of growth:

L. lactis was grown in BHI (brain heart infusion) overnight at 30 C. Then a 0.5 ml inoculum was inoculated into 50 ml of five different media (BHI, BHI + 1% glucose, BHI + 1% sucrose, BHI + 3% yeast extract, and Skim Milk medium). The cultures were incubated in a shaker water bath set at 30 C. A 5 ml sample was taken at 4, 6, 8, and 10 hours. The last 5 ml sample was taken at 24 hrs. The samples were centrifuged and filter-sterilized and were tested for their ability to inhibit the growth of *Lactobacillus viridescens*, a very sensitive gram positive bacterium.

Measurement of nisin activity

The test organism, *L. viridescens*, was prepared from an overnight culture and 0.1 ml of the culture was added to 5 ml of Nutrient Agar (NA) and then poured over a layer of 15 ml of NA in a Petri dish. A 5 µl sample of nisin from the five cultures and a sample of a known nisin concentration were dotted onto the top agar. The plates were incubated for 24 hours at 37 C and examined for zones of inhibition. The size of the zone is proportional to the amount of nisin in it. This dot method allowed me to compare all 5 cultures rapidly and has been used by others (4). The time of maximum production and the medium were used to produce more nisin and then purify it.

Minimum inhibitory concentrations (MIC) were also determined by a dilution assay. The nisin was serially diluted in Tryptic Soy Broth (TSB) for the test organism (*Bacillus cereus* and *L. viridescens*) and then the test organism is added to all the

dilutions. The lowest concentration that does not show growth of the test organism is the MIC (5)

Ammonium sulfate was added to the culture supernatant to yield 40, 50, 05 60% saturation and mixed for 24 hours at 15 C. The precipitated material was separated from the supernatant following centrifugation at 10,000 g for 20 minutes. The partially purified nisin was then fed into an ultrafiltration unit (Amicron) with a 10,000 Dalton molecular weight cutoff. The retentate and the filtrate were tested for nisin.

Results

Lactococcus lactis was grown in five different media (BHI, BHI + 1% glucose, BHI + 1% sucrose, BHI + 3% yeast extract, and skim milk medium) to determine the media that would best produce nisin the fastest and at a concentration similar to that of a standard concentration (1000 µg/ml). It was found that nisin from the media BHI + 3% yeast extract after 8 hours of growth has a similar concentration as the standard, which was 1000 µg/ml. Nisin production from the media BHI + sucrose was determined to be just below that of nisin from the media BHI + yeast extract. Nisin from BHI and BHI + glucose were similar to each but was lower than the standard concentration. The production of nisin from the media Skim Milk started out very slowly and then increased quickly. It was still increasing at the termination of the experiment. (Figure 1.)

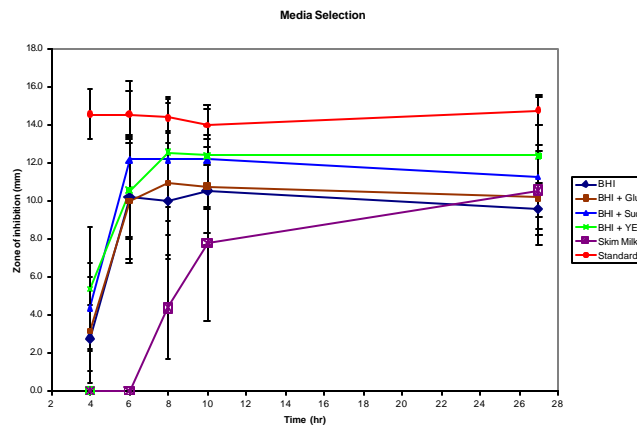


Figure 1. Production of nisin for 27 hours in five media.

Minimum inhibitory concentrations (MIC) were also determined by a dilution assay. The nisin was serially diluted in Tryptic Soy Broth (TSB) and then the test organisms (*Bacillus cereus* and *L. viridescens*) were added to all the dilutions. It was determined that the MIC of the nisin from BHI + yeast extract was consistent between the two test organisms. The MIC for BHI + sucrose was varied between the two test organisms. The Standard nisin also had variable MIC's between the two test organisms. (Figure 2.)

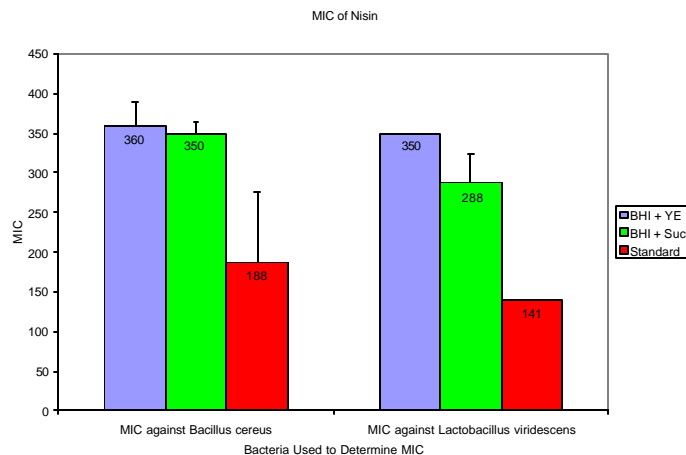


Figure 2. MIC of nisin from BHI + YE and BHI + Sucrose using *B. cereus* and *L. viridescens*.

Ammonium Sulfate Precipitation

An extraction process using ammonium sulfate was used to concentrate nisin from the media. The 40%, 50% and 60% ammonium sulfate concentrations were tested. It was found that a minimum of 50% concentration of ammonium sulfate precipitation was enough to precipitate salts out of the solution (which inhibits the activity of nisin) so the activity was at its highest against *B. cereus*. The Minimal Inhibitory Concentration (MIC) of the extracted nisin, using *Bacillus cereus* was equivalent to a commercial nisin preparation at 1000 μ g/ml. (Table 1.)

Nisin from Extraction using Ammonium Sulfate	
	Activity relative to Standard
Original Culture	0.4
40% Extraction	0.8
50% Extraction	1.3
60% Extraction	1.3

Table 1. Concentrated nisin by ammonium sulfate precipitation.

Filtration

Another purification process that was used was an Ultra Filtration Cell with a membrane that has a molecular cutoff weight of 10,000. The supernatant from the 60% ammonium sulfate extraction was used. The nisin was filtrated through the cell and the MIC's of both the filtrate and the retentate was determined. Also the concentrated nisin (retentate) was diluted to 50% with PBS to simulate the original concentration before the nisin was put through the Filtration Cell. It was determined that the retentate was at a concentration of twice that of the standard, which was expected. The concentration of the filtrate was greater than a thousand, which was also expected. The concentration of the nisin that was diluted to 50% was very similar to that of the standard. (Table 2.)

Nisin From Filtration Cell	
	Activity relative to Standard
Original Culture	1
Retentate	2.5
50% Diluted Retentate	1
Filtrate	< 0.5

Table 2. Filter precipitation of nisin.

Conclusion

BHI + 3% Yeast Extract supported the best production of nisin. The media type did not affect the activity of nisin against the test organisms. Ammonium sulfate extraction increased activity of nisin. The nisin was precipitated in 50% ammonium sulfate and was concentrated three-fold. The retentate from the ultrafiltration retained the nisin. Therefore, as more nisin is produced in the laboratory the filtration appears to be the best for collecting partially purified nisin. Further testing is necessary to determine the purity of the nisin.

Acknowledgements

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Author Biography:

Peter Weber grew up in LeCenter, MN and graduated from LeCenter Highschool in May of 1999. He started attending Minnesota State University, Mankato in the fall of 1999. He is currently majoring in Microbiology with a minor in Chemistry.

Faculty Mentor Biography:

Dorothy Wrigley, a professor in the Biology department has been at MSU for 20 years. Her interests are in food microbiology and the interactions between microbes and their eukaryotic hosts. She also directs the Food Science Technology program.