Isolation, Purification, and Identification of Micro-organisms in Live Miso

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Introduction

Miso is a paste in Japanese cuisine made out of fermented soybeans and is often used as seasoning. Around 98% of miso nowadays is made in factories and depasturized, which kills the micro-organisms in the miso. For the experiment live miso that had been fermented for 3 years was used, which still had micro-organisms from the fermentation.

The purpose of this experiment is to isolate the micro-organisms inside the miso (such as bacteria, yeast, fungi) and identify them by sequencing the 16S rRNA gene, which is present inside almost all bacteria and much more accurate than identification through phenotypes. The DNA of the bacteria will be amplified through PCR (polymerase chain reaction) and gel electrophoresis will be used to observe the state of the DNA.

Isolation & Purification

In order to isolate the organisms inside the miso, three different medias are used as each yeast/fungi/bacteria has different nutritional requirements. The three types of media used are Lactobacillus MRS Agar (MRS), which supports Lactobacillus growth, Glucose Yeast Peptone (GYP), which encourages yeast and fungi growth, and BD Tryptic Soy Agar (TSA), which can support most bacterial growth.

Along with the nutrients, variables like temperature, oxygen levels, and sample size were also regulated. The temperature of the samples could be room temperature, 30°C, and 37°C. The samples could also be grown in an anaerobic environment, though only four samples were due to lack of necessary equipment. In order to prevent dense growth in the petri dish, the amount of the miso that was used for isolation was controlled. To prevent overpopulation, Method I (serial dilutions) and Method II (three-way streaking) were applied.

After the isolation of the micro-organisms was complete, the bacteria/yeast/fungi went through the process of purification, which separated the organisms. Purification was repeated three times to make sure that each petri dish only contained one type of bacteria/yeast/fungi.

A. Isolation
   - I. Method I
     1. Remove small amount of miso with sterile hockey stick (discard and replace hockey stick after each use)
     2. Place the miso in a vial of 1x PBS buffer
     3. Perform a serial dilution on the buffer all the way up to 10^-5
   - II. Method II
     1. Remove small blob of miso with sterile hockey stick (discard and replace hockey stick after each use)
     2. Place the miso on the three prepared medias (TSA, MRS, GYP)
     3. Perform a three-way streak through the miso on the media with sterile sticks (discard and replace sticks after each use)

B. Purification
   - I. Remove desired sample of bacteria/yeast/fungi from petri dish with sterile stick. Choose an isolated colony and do not touch other micro-organisms with the stick
   - II. Place the sample onto a new media that is the same as the one from which the sample was removed
   - III. Perform a four-way streak with sterile sticks (discard and replace sticks after each use)

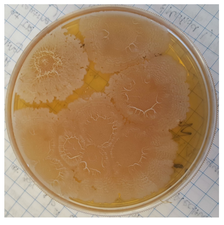
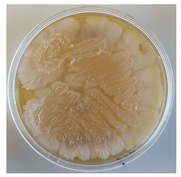


Figure 4. GYP Agar with Method 1 at 37°C and 10^0 dilution

Figure 5. GYP Agar with Method 2 at 37°C



Sample 12 – Bacteria isolated from Figure 4 at different rounds of purification

Figure 6a. Purification #1

Figure 6b. Purification #2

Figure 6c. Purification #3

Identification

1x	8x	Material
5 µL	40 µL	10x buffer
5 µL	40 µL	1% Triton
0.4 µL	3.2 µL	25 mM dNTPs
1 µL	8 µL	F Primer
1 µL	8 µL	R Primer
0.5 µL	4.0 µL	Taq enzyme
37 µL	296 µL	ddH_2O
1	N/A	Small colony

Table 1. Formula for colony PCR with Taq

To sequence the 16S rRNA gene, the DNA of the bacteria/yeast/fungi needs to be amplified through PCR (polymerase chain reaction). To conduct a PCR on a sample, the formula in Table 1 needs to be followed to create a master mix. The primers in the PCR can be either bacterial primers (16S 27F & 16S 1492R) or eukaryotic primers (18S NS1F, 18S NS8R), and both primers were tested on Samples 1-8.

But as seen in Figures 7 & 8, the PCR did not work. The samples were put through gel electrophoresis to see the DNA, but it can be seen that the fluorescent DNA is trapped in the wells and did not move through the gel. The process was repeated with Samples 9-16 with bacterial primers, but similarly the PCR did not work.

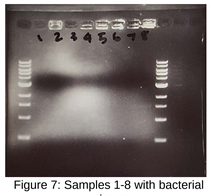


Figure 7: Samples 1-8 with bacterial primers

PCR	Sample
1	#2
2	#5
3	#6
4	#7
5	#8
6	#10
7	#11
8	#13

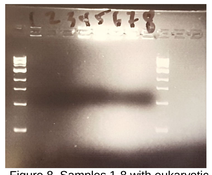


Figure 8. Samples 1-8 with eukaryotic primers

PCR	Sample
1	#13
2	#11
3	#10
4	#8
5	#7
6	#6
7	#5
8	#2

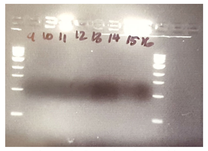


Figure 9. Samples 9-16 with bacterial primers

PCR	Sample
9	M1, T30, 10^0
10	#16
11	#21
12	#19
13	#15
14	#3
15	#9
16	#12

In order to further break down the membrane, PCR samples 9-16 were made into cultures and frozen. A different master mix formula was used for the PCR samples made with cultures, as shown in Table 2. The cultures were frozen and thawed twice so the cell membrane would be weakened by liquid expansion during freezing. In Figure 11, it is seen that some DNA is visible in Sample 16, but freezing the cultures failed to affect the rest of the samples. As the PCR did not succeed, the samples cannot be identified through 16S rRNA gene sequencing, but pictures of the bacteria were taken using a dilution of the cultures.

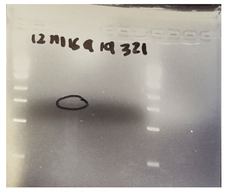


Figure 10. Gel Electrophoresis with cultures (labels correspond to sample #)

1x	8x	Material
5 µL	40 µL	10x buffer
5 µL	40 µL	1% Triton
0.4 µL	3.2 µL	25 mM dNTPs
1 µL	8 µL	F Primer
1 µL	8 µL	R Primer
0.5 µL	4.0 µL	Taq enzyme
36 µL	296 µL	ddH_2O
1 µL	N/A	Culture

Table 2. Formula for culture PCR

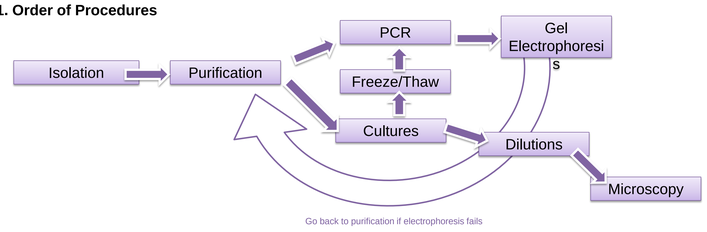
Data

Table 3. Isolation & Purification Samples

Sample #	Media	Temp (°C)	Method	Dilution	Appearance
Sample #1	GYP	30	1	10^-1	Small, round, white, shiny dot
Sample #2	GYP	Room temp	1	10^0	Small plateau-like yeast/fungi
Sample #3	TSA	Room temp	1	10^-1	Gel-like reflective round dot
Sample #4	TSA	Room temp	1	10^0	Yeast/fungi with white coating
Sample #5	GYP	30	1	10^-2	Small, white, round dot
Sample #6	TSA	30	1	10^0	White yeast/fungi w/ white growth in center
Sample #7	TSA	37	1	10^0	White yeast/fungi w/ white circle
Sample #8	MRS	37	1	10^0	Volcano-shaped yeast/fungus & sticky
Sample #9	GYP	Room temp	1	10^0	Dull round substance w/o peaks
Sample #10	GYP	Room temp	1	10^-1	Gel-secreting mound w/ tough membrane
Sample #11	GYP	30	1	10^0	Gel-like reflective substance
Sample #12	GYP	37	1	10^0	Yeast/fungi w/ smooth center & branches on borders
Sample #13	TSA	30	1	10^-2	Yellow-colored gel-like round blob
Sample #14	MRS	30	1	10^-2	Yeast/fungi grown to top of petri dish
Sample #15	MRS	30	1	10^-1	Small white round dot
Sample #16	MRS	37	2	N/A	Yeast/fungi with peaks
Sample #17	MRS	30	2	N/A	Yeast/fungi w/ creases
Sample #18	MRS	Room temp	1	10^-1	Small white round bacteria
Sample #19	GYP	30 (anaerobic)	2	N/A	Very small & unidentifiable colonies of bacteria/yeast/fungi
Sample #20	TSA	30 (anaerobic)	2	N/A	Gel or liquid-like growth of bacteria/yeast/fungi
Sample #21	MRS	30 (anaerobic)	2	N/A	Very small colonies of unidentifiable micro-organisms

*All TSA media switched to GYP media as purification proceeds

Figure 11. Order of Procedures



Results

Figure 11. Sample #3

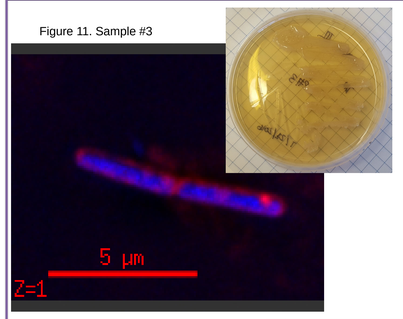


Figure 12. Sample #16

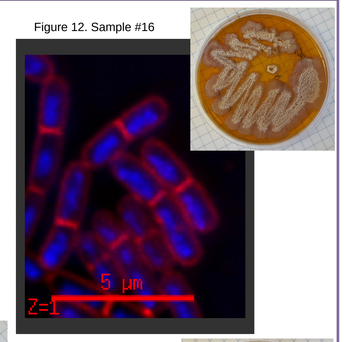


Figure 13. Sample #21

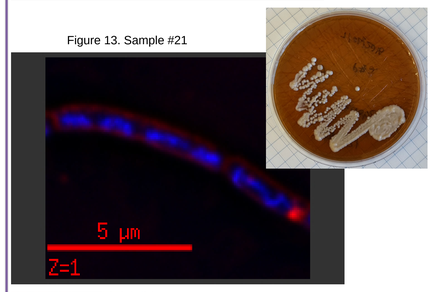


Figure 14. Sample #12

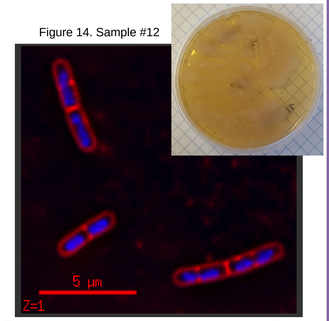
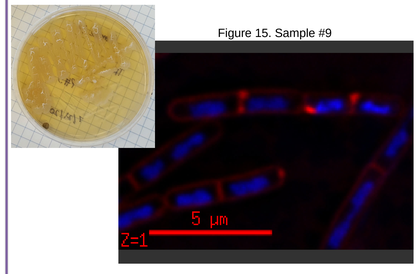


Figure 15. Sample #9



Even though the bacteria were not identified by sequencing the 16S rRNA gene, pictures of the bacteria were able to be taken with an inverted light microscope and fluorescent dyes. The red colored dye is known as FM4-64 and binds with proteins in the cell membrane, while the blue dye is known as DAPI and displays the DNA inside the bacteria. Sample 3, 9, 12, 16, and 21's cultures were diluted with 5mL LB to 100µL 10^0 culture and made into slides. All of the samples are shown to be rod-shaped bacteria, and spores were seen in Sample 9.

Discussion and Conclusions

The main problems during the experiment that prevented the identification of the bacteria was the inability to break open the cell membrane through PCR (DNA amplification) or freezing (liquid expansion). As all the samples viewed under the microscope appeared to be bacteria, so one possible reason for the difficulty with breaking open the cells could be because the samples were gram positive bacteria. Gram positive bacteria have cell walls along with cell membranes, which would be much harder to break than just cell membranes. Reasons that support the presence of gram-positive bacteria are that a common bacteria in fermented foods is *Lactobacillus*, a gram-positive bacteria, and that Sample #9 was seen going through sporulation, which is another trait of gram-positive bacteria.

References

1. Janda, J. Michael, and Sharon L. Abbott. "16S RRNA Gene Sequencing for Bacterial Identification in the Diagnostic Laboratory: Pluses, Perils, and Pitfalls." *Journal of Clincial Microbiology* 45.9 (2007): 2761-764. Print.
2. Onda, T., F. Yanagida, M. Tsuji, T. Shinohara, and K. Yokotsuka. "Time Series Analysis of Aerobic Bacterial Flora during Miso Fermentation." *Letters in Applied Microbiology* 37.2 (2003): 162-68. Print.
3. Onda, Takumi, Fujitoshi Yanagida, Tai Uchimura, Masao Tsuji, Satoshi Ogino, Takashi Shinohara, and Koki Yokotsuka. "Analysis of Lactic Acid Bacterial Flora during Miso Fermentation." *Food Science and Technology Research* 9.1 (2003): 17-24. Print.

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