Abstract:

It seemed to me that though the unaided eye cannot detect it each species of bacteria might have a distinctive coloring. If so, measuring the colors and creating an accurate catalog of standard measurements for comparison could make the lengthy process of bacteria identification much faster and therefore less expensive. I used a spectral radiometer available to me first at Rampage Systems, Inc. then directly from Photo Research Inc. to measure the "color" of bacteria that I grew at the Lexington High School science laboratory.

I measured the reflectance and transmittance spectra of various species of bacteria. I graphed the spectra using my own computer program and compared the graphs by eye. I have found that there are definite patterns of spectral graphs for each species.

By determining what factors in the bacteria's growth affect their spectra and in what ways, I have developed a consistent method for growing bacteria. A viable, consistent method of bacteria growth is a key element required if spectral analysis is to be practical for bacterial identification.

I have also developed a computer program that uses mathematical algorithms to compare the similarity between a spectral measurement of an unknown species of bacteria and the spectral measurements of various known species. By finding the most similar known curve the program can identify the species of a bacterial culture grown under standard conditions.

Using the techniques described I found that it is possible to identify the species of certain bacterial cultures by spectral analysis. I plan to expand the database of known bacterial spectra.

Purpose:

To speed the identification of an unknown species of bacteria through use of a computerized process.

Hypothesis:

Every species of bacteria has characteristic reflectance and transmittance spectra and it is possible to identify a bacteria's species by recognizing its spectra.

Materials:

laboratory glassware

sterile glass or plastic petri dishes Difco dehydrated nutrient agar

broth media

autoclave

freeze dried bacteria

- Escherichia Coli
- Streptococus Lactis
- Seratia Marcescens WCF
- Seratia Marcescens 933
- Seratia Marcescens D1
- Bacillus Megaterium
- Bacillus Cereus

Bunsen burner

inoculating loop

35° C. incubator

25° C. incubator

Photo Research® spectral radiometer model PR®-703A/PC

SPECTRAVIEW computer program

SPECPLOT computer program

SCALEALL computer program

IDENTIFY computer program

Procedure:

A. Media Preparation

1. Reconstitute agar by heating it in a flask with an appropriate proportion of water until liquid is clear.
2. Autoclave agar in a covered airtight flask.
3. If necessary melt the agar again.
4. Allowing as little air contact as possible, pour liquid agar into petri dishes. Pour three different amounts to provide three different agar thicknesses.
5. Allow agar to cool in covered petri dishes.

B. Inoculation and Incubation

1. Following directions on package of freeze dried bacteria, grow bacteria on broth provided in the package.
2. In a sterile environment heat an inoculating loop in a Bunsen burner flame until the loop is hot enough to glow red.
3. Quickly dip the loop into the broth until there is a bubble of bacteria laden broth in the loop.
4. Quickly spread the bacteria and broth on the agar in the petri dish in a small condensed area.
5. Repeat step one through step four for each species and strain of bacteria.
6. Incubate the petri dishes for two 24 hours and 48 hours at 25° C. and 35°C.

C. Spectral Measurement

1. Measure a standard white sample under the spectral radiometer with the lights shining from above the sample. The white standard sample has a nearly perfect reflectance, and we assume that it has a 100% reflectance at all measured wavelengths.
2. Remove the cover of the petri dish and place it on a black surface under the lens of the spectral radiometer.

3. Measure the colonies of bacteria in the dish with the lights shining from above. Select areas for measurement that show robust growth and that have an appearance that is representative of the colony.
4. Save measurements on computer disk.
5. Carefully record what measurements have been taken.
6. Repeat steps 2 - 5 for each petri dish.
7. Set bacteria on a stand, shining the spectral radiometer's lights through the bacteria, agar, and petri dish.
8. Measure the light transmitted through the bacteria and agar. Measure the light transmitted through only the agar from each dish.
9. Save measurements on computer disk.
10. Carefully record what measurements have been taken.
11. Repeat steps 8 - 10 for each petri dish.
12. Dispose of bacteria appropriately.

D. Spectral Analysis

1. Use the measurements obtained from the spectral radiometer to calculate the reflectance and transmittance spectra of the bacteria. For a reflectance spectrum compare the reflected light from a bacterial measurement to the white standard measurement of the same day. For a transmittance spectrum compare the transmitted light from a bacterial measurement to the agar measurement from the same dish.
2. Graph the reflectance or transmittance spectrum using SPECPLOT and SCALEALL.

   Note: The spectral radiometer measures the absolute power of the light received by its lens at each of 171 wavelengths. These wavelengths fall within the range of 390 to 730 nm, approximately the range of human vision. It reports this power in units of watts/steradian/meters^2/nanometer. Reflectance is the ratio of the power of light reflected by a surface to that received by the surface. Transmittance is the ratio of the power of light transmitted through a sample to that received by the sample.

3. Make a file named STDLIST.DAT that includes the file name of a measurement of a species known culture for each species that it is possible for the unknown measurements to be. Start the IDENTIFY.EXE program. Enter the name of the unknown file in the appropriate window and begin the identification. By comparing the unknown measurement file to each of the known files, the program will determine the closest matching known species. This process is completed five times using five algorithms.

4. **Algorithm 1:**

   This algorithm calculates the fraction of difference between the greatest and least value of each measurement file. Comparing the standard files' fractions and the unknown file's fractions at each point, the algorithm computes the average difference between the fractions. The algorithm selects the standard file with the least average difference.

   Algorithm 2:

   This algorithm calculates the fraction of difference between the greatest and least value of all of the comparison files. The algorithm compares the standard files' fractions to the unknown file's fractions at each point and computes the average difference between the fractions. The algorithm selects the standard file with the least average difference.

   Algorithm 3:

   This algorithm computes the wavelength of the greatest and least value for all files. The algorithm then calculates the number of nanometers of wavelength between the standard files' maximum and minimum and the unknown file's maximum and minimum. The standard file with the least difference from the unknown file is the one selected by the algorithm.

   Algorithm 4:

   This algorithm computes the concavity of the curve created by each point, the nearest wavelength range end, and the point that number of nanometers of difference on the opposite side of the point in question for the unknown and each of the standard files. The standard file with the most wavelengths having the same concavity as the unknown file is the one selected by the algorithm.

   Algorithm 5:

   This algorithm computes the concavity of the curve created at each point for each range from the nearest wavelength range end, to the point in question, to the point that number of nanometers of difference on the opposite side of the point in question to the point nearest the point in question on each side and the point in question for the unknown and each of the standard files. The standard file with the most wavelength ranges having the same concavity as the unknown file is the one selected by the algorithm.

Results:

Many variables affect a species' spectrum. It is important to understand what effect each variable has and how that variable can be controlled. Some variables that have a significant effect on the culture of bacteria and therefore on spectral measurements are listed below:

1. In reflectance measurements the thickness of the agar is significant. After light passes through the bacteria it reaches the agar. Some light reflects from the surface of the agar, passes through the bacteria, and is measured. Other light enters the agar, is scattered by the agar, and some of the scattered light passes back through the bacteria and is measured. The thickness of the agar changes the amount of scattered light that will become part of the resulting spectrum.
2. In transmittance measurements the thickness of the agar also affects the amount of light that passes through the sample. This should not affect the spectrum very much because the transmittance spectrum is the ratio of light through bacteria and agar versus the light through agar alone. The agar thickness effect of the agar should largely cancel out. It will not cancel entirely however because some light will be reflected off the bacteria, back into the agar where it will be scattered, and some of the scattered light will then go through the bacteria and be measured. Agar thickness variations will change the amount of scattered light.
3. The temperature at which a sample is incubated has an effect on the spectra. If a species of bacteria can't grow at 35° C. then it can't be measured by this method. If it can grow at 25° C. or 35° C. then the temperature will affect the density of colonies. This affects the amount of light that reflects from and passes through the sample.
4. The length of time for which the bacteria is incubated affects the amount of growth that takes place, which in turn affects the density of the bacterial colonies. The density of growth also affects how much light passes through or reflects from the sample.
5. The location within a colony of bacteria from which a measurement is taken has an effect on the spectrum recorded. The percentage of live culture at one location and the thickness of the colony at that location will be different from that of other locations.

Tests of the IDENTIFY program using the five algorithms incorporated have confirmed that this mehtod can only work effectively when comparing culture measurements that are grown and measured under consistent conditions. Using a mthod that keeps variables as constant as possible and minimizes the effects of variation is essential to the application of this identivication method. I grow bacteria for exactly twenty-four hours on a very thin layer of agar at 27° C.

By following careful procedures it is possible to generat a standard curve for any species of bactiera and then to compare a graph of a sample of an unknown species of bacteria to each of many standard graphs to determine which is the most similar. Through

this process the IDENTIFY comptuer program can identify an unknown culture of bactiera. Spectral Analysis is a viable method for bacteria identification of a limited number of species. When more species have been grown and measured using the standard process this method for identifying bacteria may be effective as a test that could reduce the number of tests currently used as a process of elimination to identify bacteria.

Conclusion:

Different methods of growth affect bacterial health which in turn affects spectra. Some bacteria may not grow at all under conditions in which other bacteria may thrive. Whatever standard conditions of growth are required, it is essential that they be provided with consistency.

When consistent growth and measurement procedures are used, it is possible to identify some species of some bacteria and to distinguish between many others by visual inspection of spectral graphs and by using the IDENTIFY program. When incosistent procedures are used it is not possible to effectively identify species at all.

It is possible to identify some species of some bacteria and to distinguish between many others by visual inspection of spectral graphs. It is not possible to effectively identify species grown under varying conditions using the five algorithms included in the IDENTIFY program.

Different methods of growth affect bacterial health which in turn affects spectra. Some bacteria may not grow at all under conditions in which other bacteria may thrive. An overall identification strategy may have to include several standard conditions of growth.

Whatever standard conditions of growth are required, it is essential that they be provided with consistency.

Discussion:

In reflectance measurements, the light that is received by the spectral radiometer is a combination of light that has bounced directly off bacteria and light that has passed through the bacteria and bounced off the agar layer below. Reflectance of light from agar, as well as the scattering produced by different species of bacteria, affects the spectral measurement. Ideally there would be nothing between the bacteria and a black background. The only way to accomplish that is to remove the bacteria from the agar. That would alter the structure of the bacterial colony and change its spectrum.

In transmittance spectrum measurements, what is computed is the ratio of the power of the light that passes through the agar and the bacteria to the power of the light that passes through the agar alone. In this method, the effect of the agar on the spectrum of the bacteria is largely eliminated, producing a more accurate graph of the bacterial spectrum. A disadvantage of transmittance spectrum measurements is that sometimes the bacteria grows very densely, thereby blocking the passage of much of the light. When the light is made bright enough to be measurable through the bacteria and the agar, it overloads the spectral radiometer when measured through the agar only.

To accurately distinguish between different species of bacteria by their reflectance spectra many variables must be isolated and controlled. Agar must be prepared using the same method for an unknown species of bacteria as is used in obtaining measurements on known bacteria. The bacteria must be prepared and incubated using a consistent method, and the same method must be used when measuring samples with the spectral radiometer. Once variables have been controlled, the resulting measurements should be sufficiently accurate to make bacterial identification by spectral analysis viable.

Using a computer program with mathematical algorithms it is possible to identify bacteria that have been grown under identical conditions. Bacteria grown under varying conditions can not be identified by computer analysis using the current algorithms. I will do work in the future to improve the computer algorithms and to increase the consistency of growth methods. A wider range of species of bacteria will be measured in the future to further define possible uses and possible limitations of this technique.

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Appendix A

Graphs

Appendix B

Measurements List

Appendix C

Spectral Radiometer Certification