

Winning Against Antibiotic Drug Resistance

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Abstract

Effective treatment of communicable diseases, including tuberculosis, malaria, and HIV infection, is often hindered by drug resistance because microbes readily mutate to allow resistant forms to emerge through selection pressure when populations are exposed to antimicrobial drugs. Pharmaceutical companies try to keep a step ahead of resistance by supplying new drugs, but their pipelines deliver a scant flow. This proposal seeks to explore novel sources of drugs. Dr. Thomas Butler discovered that carbohydrate agar, derived from seaweed, when heated to around 150 degrees Celsius, produced an antibacterial substance that was acidic and had a small molecular weight. It needs to be characterized further for its spectrum of antibacterial activity as well as its chemical structure. Research methods of this proposal will be growth of standard strains of bacteria on agar plates and in liquid broth. Antimicrobial substances will be placed into agar, onto nitrocellulose disks, and into broth for observing effects on bacterial growth. Expected outcomes are that antibacterial substances will be found that may have application in treatment of communicable diseases. These carbohydrate derived substances promise to be safe drugs because they come from seaweed that are routinely cooked and ingested by people throughout the world without ill health effects.

Objectives

1. Finding antibacterial substance for future drugs and test it out on different bacteria to discover which bacteria it affects.
2. Characterize the chemical composition of the antimicrobial substance using different chemical tests.
3. Using data from the previous step, we expand on the usage of this antimicrobial substance on other bacteria.

Background

Bacteria have proven extremely adaptive at developing resistance to new agents. The development of resistance may arise from natural selection pressures, chromosomal mutations, whole genome mutations, transposons, etc. These will give novel properties to the bacteria which will enable it to survive the antimicrobial drugs by various techniques such as pumping the drug out of its cell, secreting enzymes that modify or destroy the drug, or by altering the entry at the target site. Needless to say that when a successful antimicrobial drug is found eventually it proves to be of considerable importance. Thomas Butler M.D., Professor of Microbiology in Alfaisal University, College of Medicine, accidentally discovered such an antibacterial substance a few years ago in his laboratory in Texas, USA.

An interview with Dr. Thomas Butler:

*I discovered this by an accident. I had an agar plate and prepared it by pouring heated agar solution into a petri dish and I had it stand at room temperature to cool down. As often happens due to humidity, some water vapor condensed on the surface of the agar gel. I was in a hurry to streak the plates but because of the condensed water on its surface I decided to heat it up to dry. I opened the petri dish and passed it through the flame of a bunsen burner. Some parts of it passed so close to the flame that there developed a roughened corrugated portion on the agar gel. When I took the bacteria (Salmonella *1) and streaked the plate, the area that received the excessive heat did not support the growth of bacteria. Furthermore, to confirm my finding, I gradually heated agar to 150 degrees Celsius and poured it into a petri dish and after cooling it I tried to raise a colony of bacteria on it. I was unsuccessful in doing so, proving that the agar was acting as an antimicrobial agent.*

Agar comes from seaweed or red algae. It is made up polymers of galactose that are found in the cell wall of these aquatic organisms. It is a commonly used medium for the growth of bacteria.

*1 Salmonella is a genus of gram negative bacteria

Methods and Materials

Step 1: Finding zones of inhibition as a primary measure of detection of antimicrobial compound

- > Agar is heated gradually to 150 degrees then cooled
- > Some of this agar is scraped onto a nitrocellulose disk
- > This nitrocellulose disk is then placed on another agar petri dish that already has a healthy colony of Salmonella growing
- > The dish is incubated overnight with the nitrocellulose disk
- > In the morning it should be checked whether there has developed any zones

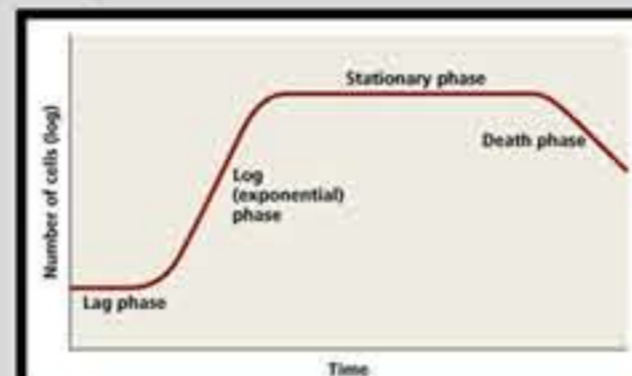
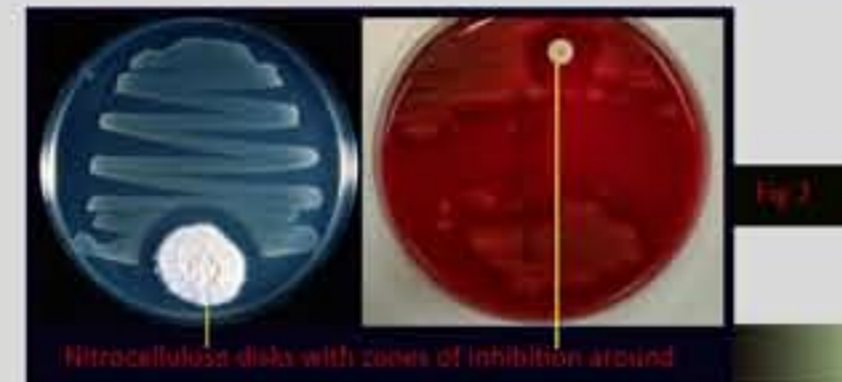


Fig. 3a showing the normal growth curve of bacteria

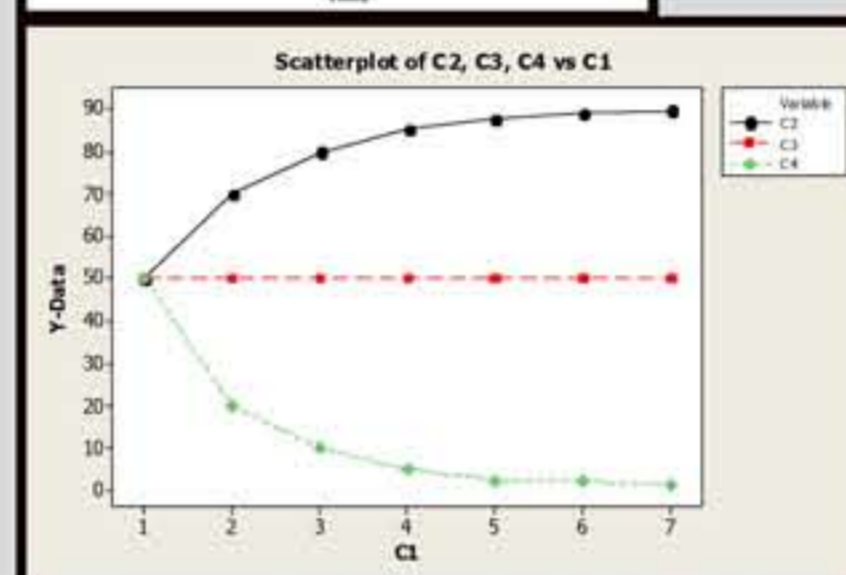


Fig. 3b. Y axis: log of number of bacteria, X axis (C1) = time, C2 = control group, C3 = inhibitory effect, C4 = killing effect

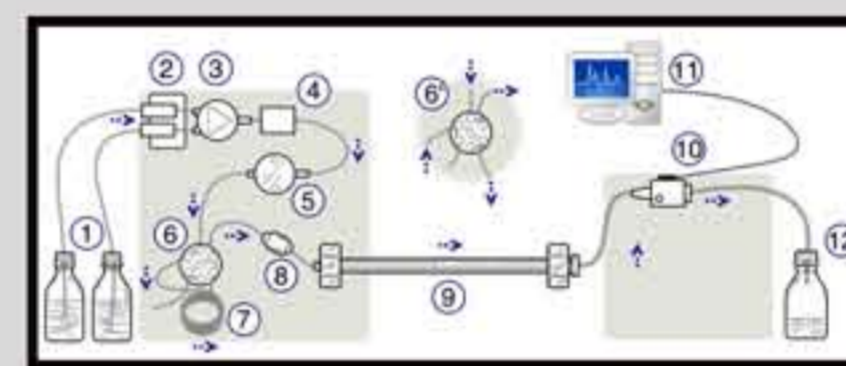


Fig. 4: HPLC
1) Solvent reservoir, 2) Solvent degasser, 3) Gradient valve, 4) Mixing vessel for delivery of mobile phase, 5) High pressure pump, 6) Switching valve (inject position), 7) Switching valve (load position), 8) Sample injection loop, 9) Analytical column, 10) Detector, 11) Data acquisition, 12) Waste/Fraction collector

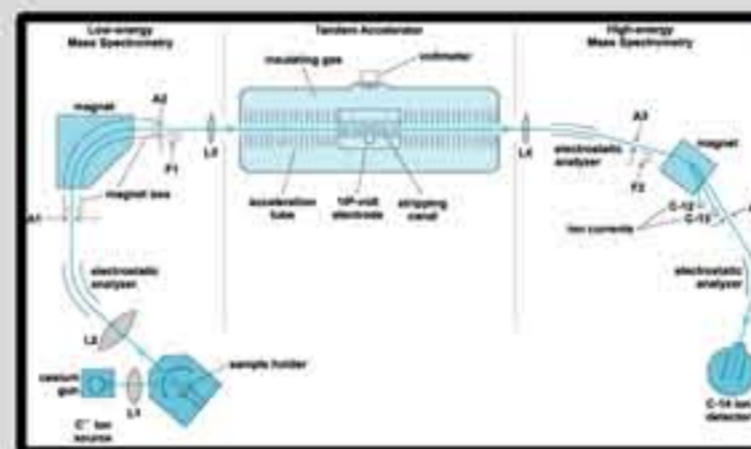


Fig. 5: Mass Spectrometry



Dr. Thomas C. Butler



Fig. 7: Liquid agar

Conclusion

If antimicrobial drug is discovered then mass spectrometry can be used to find its chemical formula by finding the exact ratio of each of the elements in the drug. Also, it will be determined whether it has any chemical properties like that of an organic compound, like aldehyde, alcohol or carboxylic acid etc.

-> of inhibition around the nitrocellulose disk (Fig 2) (*1). It is expected this test will be positive in which case we move to step 2

Step 2: Determining the exact effect of the antimicrobial substance on the growth of bacteria

- > Two identical liquid broths (*2) are prepared containing the bacteria Salmonella, one containing the proposed antimicrobial agar gel and the other with none, acting as the control
- > At equal intervals of time samples from both the broth are measured to count the number of bacteria in each sample, from which the total number of bacteria in the entire broth can be calculated,
- > A growth curve (Fig 3b) is drawn to represent the growth of bacteria, in both the antimicrobial substance broth and the control broth.

Step 3: High Performance Liquid Chromatography (HPLC) for purification of the final product

-> HPLC (*3) (Fig 4) Contains a column that holds a chromatographic packing (stationary phase), a pump that pushes through the column, and a detector that reveals the retention times of the molecules as they are eluted (washed out with a solvent) from the column. Solvents will be varied as mixtures of water and organic liquids in order to get good separation of the elutes. Reverse phase HPLC using a non-polar stationary phase and an aqueous, moderately polar mobile phase will be employed according to the properties of our extracts.

-> The HPLC separates chemicals based on their idiosyncratic polarities (*4) and interactions with the column's stationary phase. The retention time of each chemical compound varies depending on the strength of its interactions with the stationary phase, the ratio/composition of solvent(s) used, and the flow rate of the mobile phase.

Step 4: Mass Spectrometry (MS) for chemical characterisation of the purified product

-> MS (*4) (Fig 5) uses the principle of ionizing compounds to generate charged molecules or fragments and measuring the mass-to-charge ratio (Fig 6). The sample is loaded onto the instrument and vaporized. Then the components are ionized with an electron beam to form positive ions, which are accelerated in a magnetic field. The composition of mass-to-charge ratio of the particles is based on motion of ions as they transit through the electromagnetic fields to reach a detector that quantifies a compound.

-> Determinations can be achieved by MS are molecular mass, molecular structure and sample purity. Each of these questions will require a different experimental procedure. The first step for identification of an unknown compound is to compare its mass spectrum with a library of mass spectra. Software is available for interpretation of fragments of molecules with known decomposition reactions. A computer algorithm called a formula generator calculates all molecular formulas that theoretically fit a given mass with specified tolerance.

*1 Nitrocellulose disk - a disk onto which a solution of experimental compound can be placed. When the disk is placed on an agar gel the experimental compound is introduced to it.

*2 Liquid broth - a liquid growth medium for bacteria which consists of the required nutrient, suitable pH and temperature for the growth of bacteria.

*3 HPLC - A process frequently used to separate, identify and quantify chemical compounds

*4 Mass Spectrometry - A commonly used analytical technique for determining the elemental composition of a sample or molecule, peptides, or proteins.

Theory

It is hypothesized that upon being heated, the chemical bonds between the galactose polymers of agar break down, re-forms with rearrangement of the chemical constituents, giving rise to potentially new substances with antimicrobial properties. The antimicrobial characteristics can manifest in many forms such as:

- > Disrupting the DNA of the bacteria
- > Destroying the cell wall of the bacteria, making it vulnerable to lysis
- > Altering protein synthesis of the bacteria
- > Enzymatic inhibition of the metabolic pathways of the bacteria
- > These effects could either kill the bacteria or simply inhibit the growth.

Results

It is expected that heated agar will show antibacterial properties. If it kills the bacteria it will have the curve of C4 as show in Fig 3b or it will take the form of C3 if it is just inhibiting the growth

Using statistical tests such as Chi squared test and student's T test it will be determined if the results obtained were due to chance, or because of the heated agar, and if so, are the results significant to prove the hypothesized theory.

Future Plan

- = Testing the antibacterial drug on animals to search for toxicity or adverse side effects, keeping in mind animal safety ofcourse
- = Finding out its plasma life, half life, oral and parental dosing forms, interference with other drugs, tissue distribution, effect on the normal flora of the body
- = Develop clinical trials and improve for further specificity and effectiveness for a more extensive species of bacteria.