The Comparative Effects of the Homoeopathic Remedy Staphylococcinum, and Penicillin G, on the Growth Parameters and Enzyme Producing Ability of Staphylococcus aureus.

By: Anton Hans de Waard

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Supervisor: Mrs D Macdonald
Qualifications: M.Sc. (U.N.) B.A.Hons. (Unisa)

Signed: [redacted] Date: 29.06.95

I, Anton de Waard, hereby declare that this Dissertation represents my own work.

Sign[redacted] Date: 29/06/95

DEDICATION

I dedicate this work to my parents, Pieter and Shiela de Waard, whose support through my years of study has been more than any person could wish.

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ABSTRACT

The purpose of this study was to investigate the action of the homoeopathic remedy, Staphylococcinum and the antibiotic Penicillin G, on Staphylococcus aureus, with respect to it's growth parameters and enzyme producing ability, in order to determine the effectiveness of homoeopathic remedies on living organisms and to attempt a better understanding of how these remedies work. The hypothesis put forward was that the homoeopathic remedy would have an effect on the growth parameters and enzyme producing ability of the organism, but that the effect would be more subtle than that of Penicillin G.

The method used in this research was the experimental method, and the experiment was repeated a total of three times. The sample used was a non Penicillin-resistant culture of Staphylococcus aureus from the culture banks of the Technikon Natal Microbiology laboratories. A culture of the organism was incubated for 24 hours and then equal amounts transferred into growth media treated with the test substance. Five test cultures were used, a control with sterile water, a Penicillin G treated culture, and three cultures treated with three different potencies of homoeopathic remedy; 5CH, 9CH, and 15CH respectively. These cultures were incubated in a water bath and samples were aseptically removed for the viable cell counts and the enzyme tests. At times 0 hours, 3 hours, 6 hours, 9 hours, 12 hours, 24 hours, and 48 hours, 1 ml of culture was removed from each tube and the procedure of serial-dilution-and-plating was followed to obtain the number of colony forming units per milliliter (cfu/ml). At times 1 hour, 11 hours, 23 hours and 47 hours, samples were taken and tests done for the presence of the enzymes Phosphatase, DNAse, Coagulase and Haemolysin.

From the number of cfu/ml at various time intervals, the growth rates and specific growth rates were calculated for each culture, then the maximum specific growth rates and maximum viable cell counts and percentage differences between the results of the test cultures and the control cultures.

Penicillin G was found to be the most effective at reducing the growth of Staphylococcus aureus, it decreased the maximum specific growth rate by an average of 211.31% when compared to the control, and the maximum viable population by an average of 94.15%. The penicillin treated culture, over the 48 hour period, showed total loss of all the enzymes tested for. The only homoeopathic potency to give a consistent significant effect on the growth of the organism was the 15CH of Staphylococcinum. The 15CH treated culture showed, on average, a 17.69% decrease in maximum specific growth rate when compared to the control, and a 45.63% average reduction in the maximum viable population. None of the potencies of homoeopathic remedy showed any effect on the enzyme production of the organism, the results being the same as the control.

Thus Penicillin G was found to be far more effective at decreasing the growth of Staphylococcus aureus than any of the potencies of Staphylococcinum, even though the 15CH potency did show an effect on the growth of the organism. It can also be concluded that the Homoeopathic remedies effectiveness in treating Staphylococcal infections in vivo is not due to an effect on the enzyme producing ability of the organism, but probably by increasing the generation time of the organism, or most likely though a direct action upon the immune system of the patient. Therefore, Staphylococcinum cannot be recommended as an alternative to Penicillin in decreasing the growth of Staphylococcus aureus in vitro, but recommendations on its further use in vivo can only be made after further tests on patients with Staphylococcal infections.

UITTREKSEL

Die doel van hierdie navorsing was om die aksie van die homoeopatiese medisyne, Staphylococcinum, en die van die antibiotike Penicillin G, op Staphylococcus aureus te ondersoek, met verwysing na sy groei inligting en sy vermoe om ensieme op te lewer, om die indruk van homoeopatiese medisyne op lewende wesens te bepaal en om te probeer om 'n beter begrip te kry van hoe hierdie medisyne eintlik werk. Die hipotese wat voorgesit is, was dat die Staphylococcinum 'n effek op die groei en ensiem oplewerende vermoe van die bakterie sou he, maar dat die effek van die Penicillin G ver grooter sou wees.

Die metode wat in hierdie navorsing gebruik is, was die proefondervindelikke metode, en die navorsing was 'n totaal van drie keer herhaal. Die bakteriese monster wat gebruik is, was 'n nie Penicillin weerstaanbare verbouing van Staphylococcus aureus, wat in die Microbiologiese laboratorium van Technikon Natal gevind was. 'n Verbouing van hiedie bakterie was toegelaat om te groei vir 24 ure en dan was gelyke deele hiervan in elk van die toets bottles gesit, warin daar groeimedia was wat met die medisyne behandel was. In totaal was daar vyf toets bottles, een, 'n beheer bottle, met net water bygevoeg, 'n ander, met Penicillin G behandel, en drie ander met die verskillende sterktes van die homoeopathiese medisyne (5CH, 9CH, en 15CH) behandel. Hierdie verbouings is in n' waterbad gelaat en monsters was op beperkte tydperke gevat om die lewensvatbare sel tellings en ensiem toetse vir elke bottle te doen. Op die volgende tydperke; 0hr, 3hr, 6hr, 9hr, 12hr, 24hr, en 48hr was 1 ml van elke verbouing gevat om opeenvolgende-verdunnings-en-plaating te doen om die Kolonie Formeerende Eenhede per Milliliter (cfu/ml) te vind. Op die volgenge tydperke; 1hr, 11hr, 23hr, en 47hr is monsters gevat om die toetse vir die aanweesigheid van die ensieme, Phosphatase, DNAse, Coagulase, en Haemolysin te doen. Van die nommer cfu/ml was die groei syfer,

die spesifieke groei syfer, die maksimum spesifieke groei syfer, die maksimum lewens vatbare sel telling, en die persentasie van verskil tussen die resultaate van die toets opbouings en die van die beheer opbouing, bereken.

Dit is gevind dat Penicillin G die mees effektief was om die groei van die bakerie te verminder. Dit het die gemiddelde resultate van die maksimum spesifieke groei syfer van die bakterie met 211.31% verminder in vergelyking met die beheer opbouings, en die van die maksimum lewensvatbare sel telling met 94.15% verminder. Die Penicillin behandelde opbouings het oor die 48 uur tydperk die vermoe verloor om al vier ensieme op te lewer. Die enigste sterkte van homoeopatiese medisyne wat n konsekwente, en betekenisvolle effek op die groei van die bakterie getoon het was die 15CH Staphylococcinum. Die 15CH behandelde opbouings het die gemiddelde resultate van die maksimum spesifieke groei syfer van die bakterie met 17.69% verminder in vergelyking met die beheer opbouings, en die van die maksimum lewensvatbare sel telling met 45.63% verminder. Nie een van die sterktes homoeopatiese medisyne het enige effek op die ensiem oplewerende vermoe van die bakterie getoon nie (al die resultate was positief).

Dus, is dit gevind dat Penicillin G baie meer effektief as enige sterkte homoeopatiese medisyne was, om die groei van Staphylococcus aureus te verminder (selfs al het die 15CH sterkte 'n effek op die bakterie gewys). Die volgende gevolgtrekkings kan gemaak word; die homoeopatiese medisyne se doeltreffendheid, in die behandeling van pasiente met Staphylococcal infeksies, is nie 'n resultaat van 'n effek op die ensiem oplewerende vermoe van die bakterie nie, maar waarskynlik omdat dit die generasie

tyd van die bakterie verhoog, of, selfs meer waarskynlik, dat dit 'n direkte effek op die immuunsisteem van die persoon het. Daarom, kan Staphylococcinum nie aanbeveel word as n alternatief vir Penicillin om die groei van Staphylococcus aureus in vitro te verminder nie, maar aanbeveelings oor die verdere gebruik van Staphylococcinum in vivo can net gemaak word na verdere toetse op pasiente met Staphylococcal infeksies.

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INTRODUCTION

Homoeopathy, due to its subjectivity, has long been faced with the need to provide reliable evidence of the impact of remedies on living organisms. The scientific and medical worlds have continually demanded scientific proof that homoeopathy works. Microbiological laboratory experimentation provides a reliable and valid form of research, and thus a result that can stand up to scientific scrutiny.

Many studies have been done both formally and informally (in the form of research, clinical trials and homoeopathic provings) on the effectiveness of homoeopathic remedies on human and animal subjects i.e. in vivo. Very few studies have, however, been done in vitro on bacteria, yeast, plants etc. and none (that could be located) on the effects of Staphylococcinum in particular.

Past research using homoeopathic remedies in an in vitro model on yeast cells and wheat coleoptiles have had conflicting results, some researchers reported strong effects on growth rate (Steffen,1984; Jones and Jenkins,1983) and others very little effect (Baker et al,1985). This research attempts to provide knowledge specifically on the action of Staphylococcinum and generally to supplement existing research involving homoeopathic remedies in vitro.

Information on how homoeopathic remedies act on living organisms is very scarce, and most of the theories available are very subjective. Another of the aims of this research was to provide a little more understanding in this area. Does the remedy work directly on the organism, does it affect it's growth rate, it's growth time or its ability to produce extracellular enzymes?

Staphylococcus aureus is commonly found on the skin and on the mucous membranes of the mouth, nose and gastrointestinal tract of many people (Thomas, 1988). It is the most common cause of pus-producing infections in man (Thomas, 1988). It is an opportunistic pathogen which is capable of causing a number of diseases in man if given the opportunity, e.g. osteomyelitis, pneumonia, pyelonephritis, and 'food poisoning' (Wilson et al, 1979). Staphylococcus aureus also has the ability to produce some extracellular enzymes which increase its virulence as a pathogen, e.g. enzymes to break down blood clots and lyse blood cells(Burrows,1973; Atlas, 1988). It is the major cause of post surgical nosocomial infections, and is becoming increasingly resistant to most antibiotics(Jamison, 1984; Burrows, 1973).

Penicillin, which used to be the mainline treatment for Staphylococcal infections is no longer effective against most pathogenic strains of Staphylococcus and this necessitates the use of other antibiotics (Garrod et al, 1981).An alternative treatment might be shown in the homoeopathic remedy Staphylococcinum. Staphylococcinum is known to be effective in treating patients with Staphylococcal infections (Julian, 1982); the question is whether it will work in vitro or not?

Thus, the purpose of this research was not only to determine the effectiveness of a homoeopathic remedy in vitro but also, by comparing it to Penicillin, attempt to elucidate why and how the remedy acts.

CHAPTER ONE

1.0 THE PROBLEM AND IT'S SETTING

1.1 THE STATEMENT OF THE PROBLEM

The purpose of this study is to investigate the action of the homoeopathic remedy, Staphylococcinum and the antibiotic, Penicillin G on the organism Staphylococcus aureus , with respect to growth parameters and production of some specific enzymes (haemolysin, coagulase, DNAse and phosphatase) of the cultures, with the aim of better understanding the action of the homoeopathic remedy on the organism.

1.2 THE STATEMENT OF THE SUB PROBLEMS

1.2.1 SUB PROBLEM 1

To describe and measure the growth parameters and some specific enzyme production tests of an untreated batch of the microorganism culture, in order to establish a baseline from which to work.

1.2.2 SUB PROBLEM 2

To describe the effects of the antibiotic (Penicillin G) on the organism, with respect to growth parameters and specific enzyme production tests, with the aim of confirming the well documented action of penicillin on the organism (blocks cell wall replication, therefore preventing further division and promoting lysis of the cell)(Davis,1973).

1.2.3 SUB PROBLEM 3

To describe the effects of the homoeopathic remedy Staphylococcinum, in a range of potencies, on the microorganism, with reference to its growth parameters and specific enzyme production tests.

1.2.4 SUB PROBLEM 4

To compare the effect of Staphylococcinum and Penicillin G on the microorganism, with respect to growth parameters and specific enzyme production tests, with the aim of trying to elucidate the action of the remedy on the organism.

1.3 THE HYPOTHESES

1.3.1 HYPOTHESIS 1

The first hypothesis is that Penicillin G will have an effect on the organism with respect to its growth parameters (will interfere with cell replication) and therefore will affect it's extracellular enzyme production.

1.3.2 HYPOTHESIS 2

The second hypothesis is that Staphylococcinum (in 5, 9, and 15CH potencies, chosen as a convenient spread across the potency range) will have an effect on the growth characteristics and enzyme producing ability (i.e. those to be tested) of Staphylococcus aureus.

1.3.3 HYPOTHESIS 3

The third hypothesis is that the homoeopathic remedy will have a more subtle effect on the organism than that of Penicillin G i.e. that it will not dramatically affect the growth characteristics, but rather that it might cause more minor changes (either up or down) in the rate of growth of the organism, the level of growth or the production of enzymes. Penicillin G's effect on the other hand will be more on the growth characteristics of the organism (killing it by preventing cell wall synthesis), but this effect may also impede enzyme production.

1.4 THE DELIMITATIONS

In this study all experiments will be carried out using only one strain of Staphylococcus aureus and using only one batch of antibiotic, and one Staphylococcinum mother tincture.

The study will only examine those aspects of the organism's growth characteristics and the production of specific enzyme as defined in section 1.6 of this proposal.

The study will take place under laboratory conditions and will occur under strictly controlled growth conditions as mentioned in the methodology (Chapter Three).

All potencies of the remedy will be made up by hand and in water by the same qualified person.

1.5 THE ASSUMPTIONS

1.5.1 FIRST ASSUMPTION

It is assumed that all potencies of the remedy have been made up correctly according to Hahnemanian principles and that remedies made in water are as effective as those in alcohol.

1.5.2 SECOND ASSUMPTION

It is assumed that the stock culture of Staphylococcus aureus will remain genetically stable for the duration of the experiments.

1.5.3 THIRD ASSUMPTION

It is assumed that the amount of growth taking place between the time the culture is removed from the water bath, and the time that the measurements on the culture are complete, will be minimal.

1.5.4 FOURTH ASSUMPTION

It is also assumed that all instrumentation will be functioning correctly and that there will be no measurement errors in that regard.

1.5.5 FIFTH ASSUMPTION

It has been shown over the years, by documented and repeated homoeopathic experimentation i.e. provings and clinical trials (Julian, 1982), that the homoeopathic remedy Staphylococcinum has a curative effect on Staphylococcal infections in vivo, thus it is assumed to have an effect on the organism in vitro. Other experiments on

microorganisms using homoeopathic remedies support this hypothesis, and also support the hypothesis that each of the potencies will have a different effect of either increasing or decreasing the growth rate of the organism.

1.6 THE DEFINITIONS

HOMOEOPATHIC REMEDY : A remedy or medicine made according to the principles of homoeopathic dilution and succusion.

POTENCY(IES) : A stage of dilution and succusion according to the method laid down by Samuel Hahnemann.

POTENCY SCALE: A scale or series of homoeopathic dilutions and succusions as laid down by Samuel Hahnemann.

IN VITRO: Within a glass; observable in a test tube; in an artificial environment.

IN VIVO : Within the living body.

CULTURE : A single group or batch of microorganisms all from the same origin and grown in vitro (Dorland, 1977).

OPTIMAL GROWTH CONDITIONS : The conditions under which the organism grows best. For laboratory purposes the best proportions of growth media, incubation times and temperatures have already been established.

BATCH OF REMEDY : One amount of remedy made in the same container at one specific time.

INOCULATION : The process by which smaller quantities (usually in standardized amounts) of substance (e.g. homoeopathic remedy or bacterial culture) are introduced into a larger body of substance (e.g. liquid growth media).

ASEPTIC TECHNIQUE : A specified technique of doing things in a microbiology laboratory (e.g. inoculation of broths or plates)that ensures that the risk of cross infection of the cultures, and the spread of microbes in the laboratory (and consequent risk of infection of staff) is minimalised.

CENTICIMAL HAHNEMANIAN (CH) : A potency scale used by Hahnemann based on a centicimal or 1/100 scale of dilution.

STAPHYLOCOCCINUM : A homoeopathic remedy made from a living, viable culture of Staphylococcus aureus.

STAPHYLOCOCCUS AUREUS : A genus of gram positive bacteria (family Micrococcaceae) often present on the skin and in the upper respiratory tract and the most common cause of suppurative infections. Specifically it is a pigmented, coagulase positive pathogenic form of the Micrococcaceae (Dorland, 1977).

ANTIBIOTIC : A chemical substance produced originally by a microorganism, which has the capacity to inhibit the growth of or kill other microorganisms (Dorland, 1977).

PENICILLIN G : The first antibiotic developed industrially for medical use, effective against gram positive bacteria (except penicillinase resistant Staphylococci), gram negative cocci, Treponema pallidum, Actinomyces israelii, and in treating various other infections. Used mainly in the form of it's sodium, potassium, benzathine, and procaine salts (Dorland, 1977).

GROWTH PARAMETERS : As defined in this study growth parameters refer to the growth rate, specific growth rate, and maximum number of organisms in the culture, as well as the time taken to reach the maximum specific growth rate.

SPECIFIC ENZYME TESTS : For the purposes of this study the enzymes to be tested for will be Coagulase, Phosphatase, DNAse, and Haemolysin.

MICROORGANISM : A microscopic organism e.g. bacteria, viruses, fungi etc. (Dorland, 1977).

EXTRACELLULAR ENZYME : A protein released by the microorganism which is capable of producing some change in a substrate for which it is often specific (Dorland, 1977).

PROVINGS : A method by which a homoeopathic remedy is tested in low potency on a chosen group of people in order to elicit a comprehensive symptom picture for that medicine.

MOTHER TINCTURE : The base substance from which most homoeopathic potencies are made. It is the concentrated and in most cases undiluted essence of the remedy.

BROTHS : Liquid growth media for microorganisms used in microbiology laboratories.

VIABLE: Able to maintain an independent existence, or capable of growth and replication if supplied with the correct conditions (Dorland, 1977).

WATERBATH: A piece of laboratory apparatus containing water which is heated in order to keep something at a constant temperature.

RESISTANT ORGANISMS : Microorganisms which have over time developed a resistance to certain antibiotics to which they are commonly exposed and the antibiotic is no longer as effective on them. This occurs due to many different genetically inherited mechanisms e.g. the formation of an enzyme (penicillinase) by certain strains of Staphylococcus aureus that breaks down many of the penicillins and their relatives.

NOSODE : This is a homoeopathic remedy made from tissues, organs, secretions and excretions of an animal origin (either diseased or healthy).

MUCOUS MEMBRANES : Membranes, linings or mucosa of the body that are kept moist by the secretions of mucus forming cells.

COLONY(IES) : A discrete group of organisms, derived from the replication of a single cell, as in a collection of bacteria on a growth plate.

µm : A metric measurement of distance, meaning one times ten to the negative six.

GRAM POSITIVE : Microorganisms which when stained during a routine staining procedure known as Gram's stain, retain the crystal violet and iodine when washed with ethanol. This procedure differentiates microorganisms into two main categories according to the properties of their cell walls.

AGAR: A dried colloidal substance extracted from various species of red algae, it is used as a solidifying agent in solid culture media for bacteria and other microorganisms.

NOSOCOMIAL : A disease pertaining to or originating in a hospital.

CHAPTER TWO

2.0 THE REVIEW OF THE RELATED LITERATURE

2.1 INTRODUCTION

Staphylococcus aureus is a microbe that most of us are in close contact with every day of our lives. It is one of the major pus forming organisms and is responsible for the majority of skin infections and boils, and often causes other more dangerous diseases. It is susceptible to many antibiotics as forms of treatment, one of which is Penicillin G (one of the drugs of choice in treating non-resistant strains of Staphylococcus aureus).

Homoeopathically one of the ways of treating a skin infection or a boil (and the only way of specifically treating a Staphylococcus aureus infection) is with the nosode of the organism, Staphylococcinum.

There are many different methods of counting microorganisms and measuring growth rates, and the most basic methods of direct counting, serial dilution and plating, and spectrophotometry are three of the most established procedures. Qualitative enzyme tests using the effects of the enzymes on specific substrates or media are useful in identifying (medically active) bacteria.

2.2 STAPHYLOCOCCUS AUREUS

Staphylococci were first observed by Van Leeuwenhoek in 1676. Pasteur in 1880 described their presence in pus, but it was not until 1884 that Rosenbach correlated their constant presence to suppurative wounds, abscesses, and

other similar disease processes.(Gerbhardt and Nicholes, 1975).Staphylococcus means clusters of organisms; aureus refers to the slightly golden or yellow colour of the colonies of the organism (Burrows,1973). Staphylococcus aureus is the most pathogenic of the Staphylococci, and it is commonly found on the skin and on the mucous membranes of the mouth, nose, sinuses, respiratory tract, and gastrointestinal tract. It is found on the anterior nasal mucosa of 30-50% of the general population, and at higher rates among hospital staff and patients (Thomas,1988). It is also found in water, soil, milk, and air (Gerbhardt and Nicholes,1975).

2.2.1 MORPHOLOGY.

Staphylococcus aureus is a coccoidal or spherical organism, about 0.8μm in diameter (about one tenth the size of a human red blood cell). The majority of the organisms tend to cluster together and single or double cellular arrangements are the exceptions to the rule. They are strongly gram positive. The colonies when grown on agar plates are opaque, smooth and have a glistening appearance, with a characteristic golden colour (Bergey,1964; Gerbhardt and Nicholes,1975; Burrows,1973). The morphology of a broth culture is turbid, becoming clear, with a yellowish ring around top and sediment at the bottom (Bergey,1964).

2.2.2 PHYSIOLOGY

The Staphylococci are relatively more resistant to heat, and, to a certain extent disinfectants, than the vegetative forms of most bacteria. Temperatures of more than 60 degrees C (e.g. 80C) and times of up to an hour or longer are required to kill Staphylococcus aureus, for most bacteria 30 minutes at 60 degrees is sufficient (Burrows, 1973). They are also resistant to drying, can remain

infectious for extended periods, and are able to grow in relatively high salt (sodium chloride) concentrations (about 10%) (Burrows,1973). They are sensitive to Penicillin and broad spectrum antibiotics such as Tetracyclines, but are insensitive to Streptomycin and other anti Gram negative drugs. Staphylococci seem to be able to develop resistance to antibiotics relatively easily, and this has resulted in many Penicillin, Ampicillin and other antibiotic resistant strains of Staphylococcus aureus being relatively troublesome to the medical profession (Burrows,1973; Smith,1977).

2.2.3 CULTIVATION

Staphylococci are easily cultivated in the laboratory using simple nutrient broth or agar. Blood containing media support a more luxuriant growth, and the red blood cell haemolysin secreted by pathogenic strains of Staphylococcus aureus cause large clear areas around the colonies (Gerbhardt and Nicholes,1975). Better growth is obtained under aerobic or oxygen rich conditions, though some anaerobic strains have been demonstrated (Bergey,1964; Burrows,1973). The organisms grow at a wide temperature range (10 to 40 degrees Celsius) but optimum is 30 to 37 degrees (Bergey,1964).

2.2.4 ENZYMES AND TOXINS

Several toxins are produced by Staphylococcus aureus, most of which play some role in the organism's pathogenic virulence. These exotoxins are haemolysins, coagulase, fibrinolysin, hyaluronidase, leucocidins, necrotizing toxin and enterotoxin (Burrows,1973; Atlas,1988). Also produced are DNAse and Phosphatase (Cowan and Steel,1993).

As far as the haemolysins (Staphylolysins) are concerned, five have been isolated from the cell-free filtrate of cultures (a, b, d, g and j lysins). These toxins do not only enable the organism to lyse red blood cells of various animals (including humans) but are also dermonecrotic, leucocidal and toxic if released into the blood stream. These toxins are what cause the clear area around Staphylococcus aureus cultures on blood agar (Burrows,1973; Atlas,1988).

The toxin, coagulase gives Staphylococcus aureus the ability to clot blood. It activates fibrinogen, causing the blood plasma to clot. This provides a test specific for Staphylococcus aureus (See methodology for details). Coagulase contributes to the virulence of the organism by allowing pieces of fibrin to coat the outside of the organism thus interfering with phagocytosis by macrophages (Gerbhardt and Nicholes,1975; Burrows,1973; Atlas,1988).

The leucocidins (two have been isolated) inhibit phagocytosis, cause aggregation of leukocytes and even kill them by rupturing their cell membranes. This explains Staphylococcus aureus's pyogenic (pus producing) ability. The fibrinolysins allow the organism to break down blood clots and thus to move more freely into the body. The hyaluronidases help in the same manner, but their destructive properties are not limited to fibrin. Hyaluronidase can break down the ground substance of most tissues thus being one of the main agents causing necrosis (Burrows,1973; Atlas,1988).

Two other enzymes produced by many strains of pathogenic Staphylococcus aureus are DNAse and phosphatase (Cowan and Steel,1993). The DNAse enzymes are tested for by the addition of DNA to a medium and inoculating it with the organism(as explained in the methodology). The phosphatase enzyme breaks down free or bound phosphates (Cowan and Steel,1993), as an additional food source, thus increasing the virulence by being able to break down phospholipids.

2.2.5 PATHOGENICITY FOR MAN

Staphylococcus aureus is the most common cause of acute pyogenic (pus producing) infection in man. It has a marked predilection for the skin and surface structures and causes boils, carbuncles, pustules, septic fingers, styes, impetigo, and sticky eyes in babies (Thomas,1988). It also causes breast abscesses and is the commonest cause of secondary infection in wounds and burns (Thomas,1988).

The organism can also cause very serious diseases e.g. acute osteomyelitis (an infection of the marrow and bone substance), Staphylococcal pneumonia (one of the most serious bacterial pneumonia's, because its toxins produce haemorrhagic, narcotizing lesions and lung abscesses), also periosteitis, meningitis, enterocolitis, pyelonephritis, and endocarditis (Wilson et al,1979). The organism may also invade the blood stream causing septicemia (a serious disease characterized by multiplication of bacteria in the bloodstream) which results in a fatality rate of nearly 75% (especially in the immunocompromised)(Burrows,1973; Gerbhardt and Nicholes,1975). Septicemia or blood borne spread from a local focus of infection can cause multiple metastatic abscesses in various organs of the body (Burrows,1973).

It is a frequent cause of sinus infections and is probably partially responsible for the inflammation and mucopus produced in the common cold. It is also one of the causes of infected tonsils, or tonsillitis. It may also cause kidney and bladder infections (Gerbhardt and Nicholes,1975).

Staphylococcus aureus is also one of the major causes of nosocomial infections in a hospital environment. If correct precautions are not taken, then post surgical or wound Staphylococcal infections are a relatively common result. The main problem with this is that most hospital strains of Staphylococcus aureus are resistant to most common antibiotics, they are capable of incorporating pieces of genetic material called plasmids, which may confer new properties to the organism and in this way they become resistant and the patients are usually in a weakened state anyway (Jamison,1984; Burrows,1973).

A fairly common characteristic of this organism is the production of pus, which consists of dead leukocytes, tissue debris, and coagulated plasma. The pus is usually thick and fairly free of blood and can be found in large amounts if the infection is extensive. This is easily seen in the common skin conditions produced e.g. pimples, abscesses, ulcers, acne, and impetigo (Gerbhardt and Nicholes,1975; Wilson et al,1979).

Another of the diseases that Staphylococcus aureus is commonly associated with is the commonly termed "Staphylococcal food poisoning". This is actually an intoxication and not an infection. The food, often meat, is contaminated, usually due to unhygienic processing. The organism multiplies in the food releasing toxin.The toxin is heat stable and not destroyed by boiling, it causes vomiting, and sometimes diarrhoea, 2-8 hours after eating. (Jamison,1984).

2.3 PENICILLIN G

2.3.1 PHYSICAL AND CHEMICAL PROPERTIES

Penicillin can be prepared in quantity only by the original process of cultivating a mold forming it in a suitable liquid medium. In the early stages it was found that four different penicillins were being formed, known as F, G, X and K. Of these G, or benzyl penicillin, had the most desirable properties, and its almost exclusive formation is ensured by adding the appropriate "precursor", phenyl acetic acid, to the medium. As formed penicillin is an unstable acid, in production it is converted to a salt, that of either potassium or sodium, which is more stable. These salts are what is commonly known as 'soluble' or 'crystalline' penicillin (because of their high solubility in water and rapid absorption and excretion), both unsatisfactory terms, because they apply to any penicillin (Garrod et al,1981).

2.3.2 STABILITY

Penicillin is stable when dry, but deteriorates slowly in solution, this process being accelerated by heat. Among many incompatible chemicals the most important is acid, since the action of gastric acid accounts for the loss of most of a dose of penicillin G if it is swallowed. It is also destroyed by penicillinase, an enzyme formed by various bacteria, including some species of Staphylococcus various Bacillus species, some species of Proteus, coliforms, and the tubercle bacillus (Garrod et al,1981).

2.3.3 ANTIBACTERIAL ACTIVITY

Species susceptibility

At one time bacteria were classed simply as sensitive or resistant to penicillin, but they exhibit degrees of sensitivity over a wide range and the fact that large doses can be safely given enables successful treatment of

even some moderately resistant organisms. Basically, the penicillin sensitive organisms include all the gram positive pathogens and some of the gram negative (Garrod et al,1981). There are some exceptions to the rule however.

Abnormal resistance

In some species naturally resistant strains occur, including the following:
Streptococcus viridans
Staphylococcus aureus
Neisseria gonorrhoeae
Streptococcus pneumoniae
(Garrod et al,1981).

Type of antibacterial action

In nutrient medium (i.e. where bacterial growth can occur, but not otherwise) penicillin is bactericidal. About four hours is required to produce high mortality, and this may proceed to extinction, or there may be a few survivors.The effect is best exerted by a concentration 5-10 times greater than the minimum inhibiting growth (minimum inhibitory concentration for Staphylococcus aureus is 0,02-0,05 micrograms per ml (Korzybsk et al,1967)), and no increase above this level will accelerate it. Against many strains of two species, Staphylococcus aureus and Enterococcus faecalis , such an increase actually reduces the death rate, the so-called paradoxical zone phenomenon (Garrod et al,1981).

Penicillin blocks replication by preventing cell wall synthesis ; it blocks the cross linking of the peptidoglycans of the cell wall. Lysis by penicillin does not result simply from explosion of the growing protoplast through an intact peptidoglycan layer; rather it appears to be promoted by the action of autolytic enzymes in the regions of wall growth (Davis,1973).

2.3.4 PHARMACOKINETICS

The salts of benzyl penicillin are freely diffusible, after intramuscular injection absorption occurs within a few minutes to produce a high concentration in the blood. Diffusion takes place into the foetal circulation and into serous cavities. Lower concentrations are found in glandular secretions, and still lower in the cerebro-spinal fluid, but these are raised in meningitis owing both to the presence of penicillin in the exudate, and to it's more ready diffusibility though the walls of dilated capillaries (Garrod et al, 1981).

Concentrations two to five times that of the blood are found in the bile, but excretion is mainly renal accounting for about 60% of the dose, some of which is apparently destroyed in the body. This excretion is mainly tubular and exceedingly rapid, much more so than any other drug with an anti-microbial action (Garrod et al,1981).

The usual way of overcoming this difficulty is simply to give very large doses: the initially very high concentrations produced in the body are not a problem because penicillin is virtually non-toxic. It is important, however to recognize that doubling the dose does not double the duration of the effect (Garrod et al,1981).

It has often been said that blood level is not what matters, but the level in the lesion. Penicillin diffuses into the lesion when the blood level is high and persists there longer. This has been shown to be true of wound exudates, but not true of inflammation in a vascular area without tissue destruction, and it is to this category that acute inflammations belong. One way to prolong the action of each dose is to also administer probenecid, or to administer a long-acting form of benzyl penicillin (e.g.

benethamine penicillin and benzathine penicillin) where a single dose might last for days (Garrod et al,1981).

2.3.5 USE IN TREATING STAPHYLOCOCCAL INFECTIONS

More antibiotics have been produced for the treatment of Staphylococcal infections than for any other bacterial disease, but it is generally agreed that Penicillin G is the most effective Staphylococcal drug provided no penicillinase organisms are active (Kagen,1970; Smith,1977).

Staphylococcal infections by penicillinase producing organisms can obviously not be treated with penicillin. Other agents used are Methacillin, Cloxacillin, Oxacillin, Cephalothin, Erythromycin, Vancomycin and Fucidic acid (for severe infection and should not be used alone but has good penetration into tissues e.g. bone) (Smith,1977).

2.3.6 DRUG LEVELS

Aqueous Penicillin G

ROUTE	Newborn	Infant or small child	Older child	Adult
I.M. orI.V.	25000-50000 units/kg every 12 hr.	50000-500000 units/kg every 4 hr.	300000-3000000 units every 4hr.	20000000-60000000 units per day
Intrathecal Intra-articular	1000 units	1000-4000 units	2000-10000 units	

1mg. Penicillin = 1600 units
(Kagen,1970).

2.4 PRINCIPLES OF HOMOEOPATHY

Before starting to explain the remedy Staphylococcinum, it might be prudent to explain a few of the principles of homoeopathy, to aid in the furtherment of the understanding of the remedy.

The word Homoeopathy is derived from two Greek words :
Homeo meaning "like" and
Pathos meaning "suffering" (Jouanny,1991).

Homoeopathy is a therapeutic method which clinically applies the principles or laws discovered by its founder Dr. Samuel Hahnemann some 200 years ago. (Jouanny,1991).

There are two main laws in homoeopathy :The law of similars and The law of infinitesimal doses.

- The law of similars has been recognized as far back as Hippocrates' time but Hahnemann was the first to incorporate it into a philosophy of medicine.

Basically it states that "The same things which cause a disease, cure it." (Hahnemann,1989). e.g.

White Hellebore, which toxicologically produces a cholera like diarrhoea, is successfully used to treat cholera (Jouanny,1991).
OR
Staphylococcinum, the remedy made from a lysate of Staphylococcus aureus is used to cure Staphylococcal infection and other conditions exhibiting similar symptoms.

- The law of minimum dose was developed due to the toxicity of many of the substances used in homoeopathic medicine. It was very dangerous giving doses of Arsenic to sick people. Thus Hahnemann found that the curative effects were preserved when very weak or infinitesimal doses were used (Jouanny,1991). Thus started the practice of diluting the substance.

In an effort to increase the effectiveness and the duration of action of the medicine Hahnemann began succussing (shaking) his medicines 100 times between each dilution. He believed the therapeutic results were so much greater that Homoeopaths have been succussing their medicines ever since (Vithoulkas,1990).

In homoeopathy it is believed that low potencies (4 or 5CH) act on physical conditions and acute problems, medium potencies (7 or 9CH) act on disorders of function and modalities, and it is believed that high potencies (15 or 30CH) act on a nervous system or behavioral level (Jouanny,1991).

It is also believed that in certain cases low potencies stimulate activity, medium potencies regulate, and high potencies slow down. e.g.

> In the case of treating abscesses with the remedy Hepar Sulph it is seen that the administration of low potencies stimulates suppuration, medium potencies could go either way (regulate the activity according to the bodies needs), and high potencies stop suppuration (Jouanny,1991).

Thus it might be that the effects of the different potencies of the remedy on the organism might be totally different (not enough information on the subject to be able to make a conjecture).

Homoeopathic remedies do not work as other medicines do i.e. measurable blood levels and absorption and excretion rates, but instead they stimulate the patient directly or they "act on the basis of a particular physical state transmitted to the organism electively by the sublingual route." (Jouanny,1991). To put it simply they stimulate the bodies own immune system hopefully causing a cure. Thus the effectiveness of the remedies in in vitro situations is of great interest as it might confirm or detract from the above belief.

Other research into the field of in vitro homoeopathy has had varied success: some studies show marked effects on the growth rates of yeast (Steffen,1984) and wheat coleoptiles (Jones and Jenkins,1983). These studies show markedly different effects of each of the different potencies of the remedy (Pulsatilla) on the growth of the organism, while other studies, based on the above (Baker and Smith,1985), show no major effects of the remedy on the organism. Thus this experiment is necessary to provide further data on the subject.

2.5 STAPHYLOCOCCINUM

2.3.1 DEFINITION

"Staphylococcinum is a biotherapeutic nosode prepared from a lysated suspension of a culture corresponding to 10 milliards in a cm cubed of Pyogenoes aureus Staphylococcus." (Julian,1982).

2.3.2 PREPARATION OF THE LYSATE

The culture medium used is nutrient broth which has been autoclaved to sterilize it. This is then inoculated with two cultures of Staphylococcus aureus and 5 cm cubed of 9% sodium chloride solution to kill off any other organisms. It is then subcultured and incubated for 48

hours. The resulting suspension is adjusted with distilled water so that there are 20 milliards of germs per cm cubed. It is then heated at 60 degrees C for 1 hour and then refrigerated (Julian, 1982).

The killed suspension is then incubated for 5 days to check sterility and make sure that no viable organisms remain; then centrifuged and the floating suspension of toxins is collected, filtered on seitz EKS filters and refridgerated. To this suspension is added equal quantities of sterile water (thus the concentration of is now 10 milliards per cm cubed) and is ready for aseptic distribution in ampoules (Julian,1982).

2.3.3 CLINICAL PATHOGENESIS

(The clinical symptoms that Staphylococcus aureus causes and that the remedy Staphylococcinum will cure)

1) **Generalities :**
Pre diabetic conditions.
Syndrome of acute septicaemia : oscillating fever with great chill, general condition is weakened, splenomegaly.

2) **Neuro-endocrino-psychic System :**
Radiculitis.
Myelitis.
Meningitis.
Abscess of the brain.

3) **Cardio-hemo-vascular System :**
Endocarditis :causes heart murmur of the first or second sound (at the base) later there are organic sounds due to valvular insufficiency.

Pericarditis : Pericardiac rubbing. Myocardia: dull noises. Tension fall (BP) and arrhythmia's.

4) **Respiratory apparatus** :
Disseminated pulmonary condensation, milliary, disseminated or multiple abscesses.
Purulent pleural exudations.

5) **Uro-genital Apparatus** :
Pyuria.
Peri-nephritic Phlegmon.
Prostatitis.
Acute prostatic abscess.

6) **Locomotor System** :
Spondylitis.
Osteitis.
Osteomyelitis.

7) **Skin**:
Follicular pustule.
Abscess of the nasal fossa.
Abscess of the eyelids(styes).
Sycosis of the mustaches.
Folliculitis of the integument.
Hydrosadenitis of the armpits.
Anthrax. Ecthyma.
Panaris. Onyxis. Perionyxis.
Purpura, vesico-purpura.

(Julian,1982).

2.6 MICROBIOLOGICAL METHODS AND TECHNIQUES

2.6.1 THE PREPARATION OF THE MEDIA :

1) Nutrient Broth :

Meat (beef) extract	10g
Peptone	10g
NaCl	5g
Water	1000ml

Dissolve the ingredients by heating in the water. Adjust to pH 8.0-8.4 with 10 M HCl and boil for 10 minutes. Filter while hot through thick filter paper to remove phosphates which are precipitated in alkaline solution at this stage. Adjust to pH 7.2-7.4, and sterilize at 115 degrees C for 20 minutes. Allow to cool before addition of organisms (Cowan and Steel,1993).
Or for premixed broth powder, dissolve 25g/l in water and autoclave for 15 minutes at 121 degrees (off the bottle label (Biolab)).

2) Nutrient Agar :
The same as nutrient broth but 2% agar is added to the ingredients to gel it. Allow to cool to 45-50 degrees C before addition of organisms and/or distribution to tubes or plates (Cowan and Steel,1993).
For premixed agar powder(Plate count agar), mix 23g/l of water, bring to the boil and the autoclave for 15 minutes at 121 degrees C (off label of bottle (Biolab)).

3) Blood Agar :

Defibrinated Blood	50ml
Nutrient agar	950ml

Melt the nutrient agar, cool to 50 degrees C and add the blood aseptically. Mix and distribute to tubes or plates (Cowan and Steel,1993).
Or for premixed agar, mix 40g/l and add 7-10% sterile blood (when base has cooled after autoclaving)(Biolab).

4) DNA Agar :

Dissolve DNA in distilled water; prepare enough to give a final concentration of 2mg/ml in the final medium. Add the DNA solution to nutrient agar base immediately before autoclaving and pour the plates as soon as the medium cools to 50 degrees (Cowan and Steel,1993). For premixed agar, add 39g/l to water and autoclave (Biolab).

5) Phenolphthalein Phosphate Agar :

(Phenolphthalein diphosphate, Na salt 1% aqueous solution. Sterilize by filtration and store at 4 degrees C.)

Phenolphthalein phosphate solution	10ml
Nutrient Agar	1000ml

Melt the nutrient agar and cool to 45-50 degrees C. Add the phenolphthalein phosphate solution aseptically, mix, and distribute into petri dishes (Cowan and Steel,1993).

2.6.2 INOCULATION OF THE MEDIA :

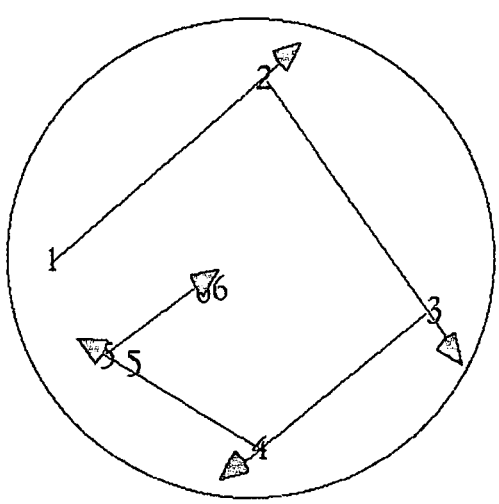
Only the methods of inoculation used in this research are discussed here.

1) Transferring from plate culture to a broth :

An inoculation loop is used for this procedure, the loop must always be sterilized by holding it in the hottest portion of the Bunsen burner flame until red hot. Once sterilized, the loop must never be put down but is held in the hand and allowed to cool for 10 to 20 seconds before use. The required number of loopfuls are transferred in stages, each time the petri dish and the tube or beaker are flamed. The loopful of organisms is shaken slightly to dislodge the organisms, the removed and flamed again before use (Cappuccino and Sherman,1983).

2) Transferal of amounts of broth culture :
In the case of this experiment where 2ml of culture has to be dispensed into each of 5 separate universal bottles, the aseptic technique is different. Presterilised pipettes are used and the transfer takes place in a laminar flow cabinet. The beaker and universal bottles are flamed between uses, and the bottles are flamed before sealing.

3) Streak plate method :
This is a rapid qualitative isolation method. It is essentially a dilution technique that involves spreading a loopful of culture over the surface of an agar plate. Although many methods have been described , the four way or quadrant method is the most commonly used one. It consists of placing a loopful of liquid culture in



area no. 1 on the diagram, the loop is then flamed and cooled. The loop is the streaked from area no. 1 to area no. 2, dragging some liquid with it. Then after reflaming the loop is dragged from 2 to 3 using broader streaks and overlapping area 2. Then after flaming the loop is

dragged from 3 to 4 , overlapping area 3. Then without flaming, the loop is streaked directly from area 4 to 5 in broad streaks. Sometimes the loop is even moved to area 6. Thus the loopful of culture is progressively diluted over the surface of the plate and after incubation hopefully some single colonies will have formed in the last few areas (Cappuccino and Sherman,1983).

2.6.3 SERIAL DILUTION-AGAR PLATING PROCEDURE

This is a method for estimating the number of cells in a bacterial culture sample, and it only counts the live or viable cells in the culture.

Basically the procedure is the following :
The first dilution is made by pipetting 1 ml of the culture into 9 ml of sterile distilled water and mixing to ensure homogenous dispersion occurs. One ml of this 10^{-1} dilution is placed in a test tube containing 9 ml of distilled water to yield a 10^{-2} dilution. This process is continued and the culture is progressively and serially diluted using a decimal scale. After the first dilution the dilutions are done in duplicate.

Thus a bank of up to 10 (or more depending on the estimated growth of culture) serial dilutions is made. Plating can take place from the first to the tenth tube, but if the biomass can be at all estimated, then only the first or last few tubes need be plated. The plating procedure consists of taking 0.1 ml of dilution out of the tube and either putting it into molten agar before the agar sets or aseptically spreading it on the surface of an already set agar plate. This is also done in duplicate for each dilution, thus yielding counts in quadruplicate for each sample.

The plates are then incubated and the plates with accurate, countable growth are chosen for specific counting (between 30 and 300 colonies). Once counted the number of cells is multiplied by the dilution factor to give the number of cells in the original culture, (and by 10 again to get from 0,1ml back to 1ml amounts)(Cappuccino and Sherman,1983).

2.6.4 GROWTH RATE CALCULATIONS :

The growth rate of a culture is simply the rate at which a culture grows, logically it is not the same all the way through the cultures life and most cultures go through a four stage cycle:

- The lag phase is the period taken after inoculation for a culture to get used to it's new environment, there is a slow growth rate.
- The logarithmic phase is the period of time in which cell division is maintained at a constant and maximum rate: it is said to be in balanced growth.
- The stationary phase is when the number of organisms in the culture remains constant. Many cells are dividing but just as many are dying: this is the period of unbalanced growth. The waste products and enzymes produced inhibit growth.
- The death or declining phase is the period when the culture dies. The rates of decline vary from organism to organism (Boyd,1984).

The growth rate can be calculated using the equation :

$$\text{Growth Rate} = \frac{\text{Cell Density 2 - Cell density 1}}{\text{Time 2 - Time 1}} \text{ or } \frac{\text{CD 2 -CD2}}{\text{T 2 - T 1}}$$

The specific growth rate of a culture is calculated in order to eliminate changes in cell numbers between samples. If one sample starts out with a lot of cells and another with just a few then the growth rates of the two samples may appear to be different; for this reason the specific growth rate of the culture is calculated, by using the following formula: (CD = Cell Density)

$$\text{Specific Growth Rate} = \frac{\text{Growth Rate}}{1/2(\text{CD}\,2 + \text{CD}\,1)} \quad \text{or} \quad \frac{\text{Growth Rate}}{\text{Cell Density at time } \frac{T2+T1}{2}}$$

Generation Time: this is calculated in order to see how quickly a culture multiplied. Perhaps there was no difference in biomass between a test culture and the control, yet the test culture could have taken longer to reach that biomass. Therefore the measurement of the time taken between generations of culture is important. Two equations are necessary:

$$G = \frac{t}{n}$$

G= generation time
t = incubation time
n = number of generations

$$b = B \cdot 2^n$$

b = number of cells (time 2)
B = number of cells (time 1)

$$2^n = \frac{b}{B}$$

$$\log 2^n = \log \left(\frac{b}{B}\right)$$

$$\text{thus } n = \frac{\log \left(\frac{b}{B}\right)}{\log 2}$$

(Boyd, 1984).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 THE DATA

The data of this research are of two kinds: primary data and secondary data. The nature of each of these two types of data will be given briefly below.

3.1.1 THE PRIMARY DATA

The primary data used in this study were collected in the experimental manner. Three types of primary data were obtained :

1. The measuring of the normal growth parameters and specific enzyme production tests of the culture before it was treated with anything.

2. The measuring of the growth parameters and specific enzyme production tests of a batch of the culture treated with a dose of Penicillin G.

3. The measuring of the growth parameters and specific enzyme production tests of three equivalent batches of the culture which were treated with Staphylococcinum 5CH, 9CH, and15CH respectively.

3.1.2 THE SECONDARY DATA

The primary data were used as secondary data for subproblem four of the problem statement, where they were compared to fulfill the aim of the study.

3.2 THE SAMPLE

The strain of Staphylococcus aureus used was a non resistant, pathological strain obtained from the Technikon Natal microbiology laboratory (strain serial number American Type Culture Collection 27661.) This strain was known to be Penicillinase negative, Coagulase and DNAse positive, and sensitive to tricyclics.

3.3 THE CRITERIA GOVERNING THE ADMISSIBILITY OF THE DATA

Only data on the growth characteristics and specific enzymes as defined earlier were examined. Only data gathered under the conditions and times stipulated in the method were accepted and used.

Each experiment was repeated three times and the average of the results calculated in order to reduce experimental error.

3.4 ADMINISTRATION

The following steps were taken towards the execution of the study :

Permission to use the Technikon Natal laboratories was granted by the head of the department of Biotechnology.

Meetings were held with the supervisor of this study, Danile Macdonald and with the head of the Biotechnology department, Dr Neethling, to discuss the research.

Orders for all glassware and equipment and supplies used during the study were placed by the researcher through the department of Homoeopathy.

The aid of a Microbiology laboratory technician was enlisted to ensure that procedures and techniques were competently carried out.

3.5 RESEARCH METHODOLOGY

The objective of this study was to determine the effects of Penicillin G and Staphylococcinum on Staphylococcus aureus, in order to contribute towards the understanding of the effect and perhaps the mechanism of action of the remedy on Staphylococcus aureus.

Cell numbers and enzyme activity were monitored over a 48 hour period after the addition of the respective treatments (Penicillin, three potencies of Staphylococcinum and the control (water)). Changes in growth data for each treatment were compared to the data for the control in order to determine whether any of the treatments had a significant effect on Staphylococcus aureus.

3.5.1 PREPARATION OF THE MEDIA

.1)Preparation of the broths:

Nutrient broth was used and was made up according to the package instructions. A few days before each experiment 50ml of broth was placed in a 100ml flask, sealed, and autoclaved. In addition five 30ml universal bottles were each filled with 16ml of nutrient broth, sealed, and autoclaved. All the bottles of broth were stored in the cold room at 0-10 degrees Celsius.

.2)Preparation of the Agar plates:

For the serial-dilution-plate-count procedure Plate count agar was used. This was made up according to the package instructions. Batches of one to three liters were made up at a time, and this was distributed into 500ml bottles, which were then autoclaved. Once sterile the molten agar was stored in an oven at 60 degrees Celsius to keep the bulk of them liquid while the plates were being poured. The plates were poured with the agar at about 50 - 55 degrees Celsius, following strict aseptic technique. The plates, once set, were stored upside down in the cold room till used. About 1200 plates were made for each trial.

Smaller batches of Phenolpthalein Phosphate agar plates, DNAse agar plates, and Blood agar plates were poured from sterile agar made up according to the package instructions and various additives added according to these instructions. Twenty plates of each were used in each trial.

.3)Preparation of the blood plasma:

The day before the trial sterile human blood plasma was diluted 1 in 10 in sterilized saline (1ml plasma to 9ml saline), and this was stored in the fridge.

.4)Preparation of the serial dilution test tubes:

For the serial dilution, glass test tubes with aluminum cap-o-tests were used. Before each experiment about 600 test tubes, numbered for the dilution, were each filled with 9ml of distilled water, autoclaved, and then stored in the cold room for later use.

3.5.2 PREPARATION OF STAPHYLOCOCCINUM

The homoeopathic remedy was made up using Staphylococcinum 4CH as a starting potency. The remedy was obtained from Natura and was from a culture of Staphylococcus aureus. A potency bank of the remedy was made up using 70% alcohol, on the principle of 1 drop in 2 ml 70% = $^{1}/_{100}$ dilution, and the bottles were succussed 100 times in between potencies. Thus one drop of the 4CH was added to two ml of alcohol and succussed 100 times, giving a 5CH. Then one drop of the 5CH was taken and placed in two ml of alcohol, and so on. The bank was only made up to 14CH. From this bank sufficient quantity of remedy in the potencies that were used was made up in distilled, sterile water. e.g. for10ml 9CH, 10 ml water was used and 5 drops of the 8CH (in alcohol) was added and succussed 100 times = 9CH. Ten ml of 5CH, 10ml 9CH and 10ml of 15CH was made. The aqueous potencies were stored in the refrigerator until needed.

3.5.3 PREPARATION OF PENICILLIN G

The Penicillin G used in the experiment was in the powdered form. It was the Sodium Salt of Benzylpenicillin (chemical Formula $C_{16}H_{17}N_3O_4SNa$) and the concentration was 1690 units of Penicillin G base per ml. A final concentration of 50 mg per ml was required in the test culture. Thus a stock solution of 500 mg per ml was made up by adding 0.1 grams of the powder to 20ml of distilled water. 2ml of the stock solution was aseptically injected into the medium before the addition of the organism in order to give a final concentration in each tube of 50 mg per ml.

3.5.4 PREPARATION OF STAPHYLOCOCCUS AUREUS CULTURE

A loopful of Staphylococcus aureus from a slope culture of ATCC 27661 maintained in the Technikon Natal microbiology laboratories was inoculated into 50ml of nutrient broth and incubated at 37 degrees C for 24 hours. The culture bottle was well shaken to ensure uniform distribution of cells before dispensing into universal bottles. From this culture 2ml was pipetted (using separate sterile 5ml pipettes) into each of 5 universal bottles containing 18ml of broth and test substance(16ml broth + 2ml test substance.) The original culture was also kept in the water bath at 37 degrees C and optical density readings were taken at each time period, in case a standard curve of the culture needed to be plotted.

3.5.5 GROWTH EXPERIMENTS

The five, 30ml universal bottles contained the following:

bottle 1	16ml nutrient broth
	2ml Penicillin G solution
	2ml culture
bottle 2	16ml nutrient broth
	2ml sterile distilled water
	2ml culture
bottle 3	16ml nutrient broth
	2ml aqueous 5CH Staphylococcinum
	2ml culture
bottle 4	16ml nutrient broth
	2ml aqueous 9CH Staphylococcinum
	2ml culture
bottle 5	16ml nutrient broth
	2ml aqueous 15CH Staphylococcinum
	2ml culture

All additions of test sustance were done using 0.45 micrometer pore size Millipore filters to ensure sterility. Immediately after inoculation with the organism the lids of the bottles were closed, and the contents mixed with the aid of a vortex stirrer; the tubes were then transferred to a water bath at 37 degrees C. (This was considered to be time zero, T0.)

At times zero hours(T0), three hours(T3), six hours(T6), nine hours(T9), twelve hours(T12), twenty four hours(T24), and finally forty eight hours(T48), 1ml from each of the five bottles was aseptically pipetted respectively (using a 1ml micro pipette with sterile disposable tips) into five test tubes each containing 9ml of sterile distilled water (10^{-1} dilution). Further decimal serial dilution of each sample was performed up to 10^{-6} (for the first few sampling times) and up to 10^{-9} (for later sampling times). After the first dilution(10^{-1}) all dilutions were performed in duplicate.

From each dilution, 0.1ml was pipetted (using an adjustable micro pipette with sterile disposable tips) into each of 2 of the plate count agar plates poured earlier (i.e. done in duplicate).This was then spread over the entire surface of the agar using a sterile glass 'hockey stick' (flamed in between plates). The plates were then incubated at 37 degrees C for 24 hours and the resulting colonies counted (from plates containing between 30 and 300 colonies) to calculate the number of viable bacteria in each of the five bottles at each time of sampling (the average of the four results at each sampling time for each bottle was calculated).

The average results thus obtained were then multiplied by the respective dilution factor and by another 10 (due to only 0.1ml used) to obtain the number of viable organisms in the test culture at that sampling time (in colony forming units per milliliter, cfu/ml) e.g. an average of 62 organisms all counted on the 10^{-6} plate would yield a count of 620000000 cfu/ml in the test culture. These data were then used to plot growth/death curves and to calculate growth rates, specific growth rates, times taken to reach maximum specific growth rates and maximum cell numbers.

At times one hour(T1), eleven hours(T11), twenty three hours(T23), and forty seven hours(T47), loopfuls were aseptically removed from each of the five bottles to do the following enzyme tests (Cowan and Steel,1993):

- Deoxyribonuclease : The already prepared DNA plates were inoculated with the test culture as a streak on the surface of the plate. The plates were then incubated at 37 degrees C for 36 hours. The resulting growth on the plate was stained with a few drops of Toluidine Blue. A positive result was shown by a pink area around the streak of growth, within the blue stained media, when the plate was held up to the light. A negative result was shown by continuous blueness all around the growth.

- Phosphatase : The already prepared Phenolpthalein Phosphate Agar plates were inoculated to obtain discrete colonies (four way streak), and incubated at 37 degrees C for 18 hours. A few drops of ammonia solution (sp.gr 0.880) were placed in the lid of the petri dish and the plate with growth on it inverted over the lid. Free phenolphthalein liberated by Phosphatase activity during incubation turns bright pink in the presence of the alkaline ammonia, thus indicating a positive result. The Phosphatase negative colonies remained their creamy white colour due to the phenolpthalein remaining complexed with phosphate.

- Haemolysin : A four way streak plate inoculation of the test culture was made onto a Blood Agar plate which was then incubated for 24 hours at 37 degrees C. It was then examined for a clear ring in the blood agar around a single isolated colony when held up to the light (positive test for haemolysins). A negative was shown by no clearing in the blood agar.

Also at the above four time periods one drop of each culture was removed using separate sterile micro pipettes to do the following enzyme test:

- Coagulase : One drop of each test culture was added to nine drops of plasma-saline solution (placed in a sterile test tube with a sterile micro pipette) and incubated at 37 degrees C for 4 hours. The tubes were examined after 1 and 4 hours for coagulation. If the test was negative, the tubes were left at room temperature overnight and re-examined. A positive result was indicated by definite clot formation and a negative by no clot formation.

3.6 THE SPECIFIC TREATMENT OF EACH SUBPROBLEM

3.6.1 SUBPROBLEM 1

The first subproblem was to describe and measure the growth parameters and specific enzyme production tests of an untreated batch of the microorganism culture in order to establish control data to which treatment data could be compared.

NATURE OF THE DATA

The data required were the following:

1) The specific growth rate achieved in normal cultures of Staphylococcus aureus.

2) The maximum cell population achieved in normal cultures of Staphylococcus aureus.

3) The time taken, from inoculation, for the maximum specific growth rate to be achieved in normal cultures of Staphylococcus aureus.

4) The results of the specific enzyme tests for Haemolysins, Coagulase, DNAse, and Phosphatase, in normal cultures of Staphylococcus aureus.

LOCATION OF THE DATA

The data were gathered by laboratory experimentation in the microbiology laboratory at Technikon Natal.

MEANS OF OBTAINING THE DATA

Refer to the detailed method described in Section 3.5.

INTERPRETATION OF THE DATA

The specific growth rate was calculated from the graph of colony forming units against time, as follows: (biomass equals number of cells)

$$\text{growth rate} = \frac{\text{difference in number of cells}}{\text{difference in time}}$$

$$= \frac{CD2-CD1}{T2-T1} \qquad CD = \text{Cell Density}$$

$$\text{Specific growth Rate} = \frac{\text{Growth Rate}}{\text{Biomass at time } \frac{T2+T1}{2}} \quad \textbf{or} \quad \frac{\text{Growth Rate}}{1/2(CD2+CD1)}$$

3.6.2 SUBPROBLEM 2

The second subproblem was to describe the effects of Penicillin G on the organism, with respect to growth parameters and production of specific enzymes with the aim of confirming an established mechanism of action upon the organism.

THE NATURE OF THE DATA

The data required were the following:

1) The specific growth rates achieved in the cultures in the presence and absence of Penicillin G.

2) The maximum cell populations achieved in the culture in the presence and absence of Penicillin G.

3) The time taken for the maximum specific growth rate to be achieved in cultures treated with Penicillin G and in untreated control cultures.

4) The results of the four specific enzyme tests done on the culture in the presence and absence of Penicillin G.

THE LOCATION OF THE DATA

The data was gathered by laboratory experimentation in the microbiology laboratory at Technikon Natal.

MEANS OF OBTAINING THE DATA

Refer to the detailed method described in Section 3.5.

3.6.3 SUBPROBLEM 3

The third subproblem was to describe the effects of the homoeopathic remedy, Staphylococcinum, in a range of potencies, on the microorganism, with reference to it's growth parameters and specific enzyme production tests.

NATURE OF THE DATA

The data needed were the following:

1) The specific growth rates of the cultures both in the presence and absence of different potencies of Staphylococcinum.

2) The maximum cell population of the cultures both in the presence and absence of different potencies of the remedy.

3) The time taken for the maximum specific growth rate of the cultures to be reached, both in the absence of and in the presence of different potencies of the remedy.

4) The results of the four specific enzyme tests done on the samples of cultures, both in the presence and absence of different potencies of Staphylococcinum.

LOCATION OF THE DATA

The data were gathered by laboratory experimentation in the microbiology laboratory at Technikon Natal.

MEANS OF OBTAINING THE DATA

Refer to the detailed method described in Section 3.5.

3.6.4. SUBPROBLEM 4

The fourth subproblem was to compare the effect of Staphylococcinum and Penicillin G on the organism, with respect to growth parameters and specific enzyme production tests with the aim of trying to better understand the action of the remedy on the microorganism.

THE NATURE OF THE DATA

The data required for this subproblem were the data gathered from the above three subproblems. i.e. the specific growth rates, times taken to reach the maximum specific growth rates, maximum viable cell populations, the percentage differences between all the above results and results of the specific enzyme tests on the various treated cultures as described above.

LOCATION OF THE DATA

The location of the data was in the results and comparisons of subproblems 1-3.

MEANS OF OBTAINING THE DATA

The results of the above three subproblems were analyzed.

CHAPTER FOUR

4.0 RESULTS

4.1 SUBPROBLEM ONE

The growth parameters of an untreated or control culture of Staphylococcus aureus.

Table 4.1.1 Mean Viable Cell Count (cfu/ml) Plus Standard Deviations of the Control Cultures of Trials 1 to 3 at Various Times.

TIME (hours)	TRIAL1		TRIAL2		TRIAL3	
	CFU/ML x 10^7	STD. DEV.	CFU/ML x 10^7	STD. DEV.	CFU/ML x 10^7	STD. DEV.
0	3.45	3.70	7.05	7.95	3.98	3.69
3	27.90	4.33	23.93	16.99	12.70	5.24
6	63.75	2.17	59.00	4.58	22.15	7.82
9	14.10	13.92	62.50	4.71	22.48	6.06
12	90.50	10.16	77.75	5.80	32.50	1.11
24	83.50	10.87	83.75	7.22	36.25	1.92
48	195.75	26.01	62.00	6.04	50.75	2.86

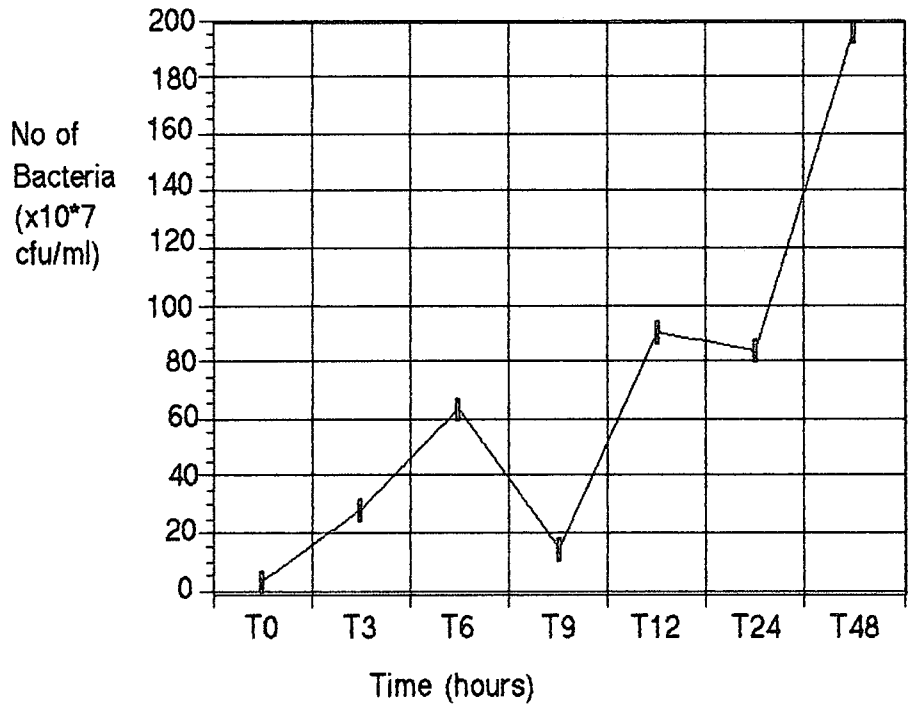


Figure 4.1.1 Colony Forming Units per milliliter against Time forTrial One's Control Culture.

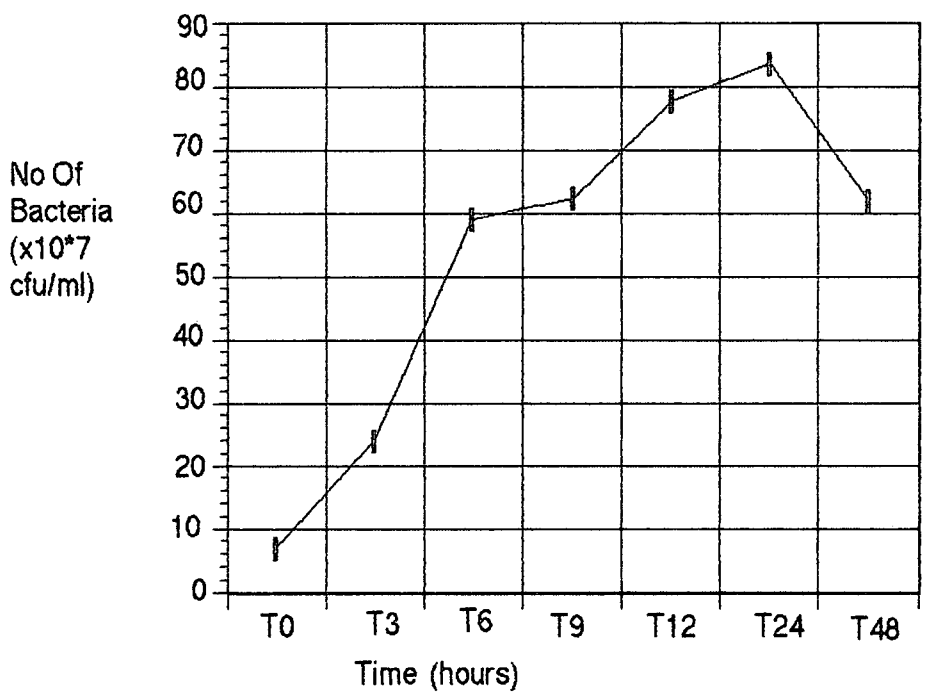


Figure 4.1.2 Colony Forming Units per milliliter against Time for Trial Two's Control Culture.

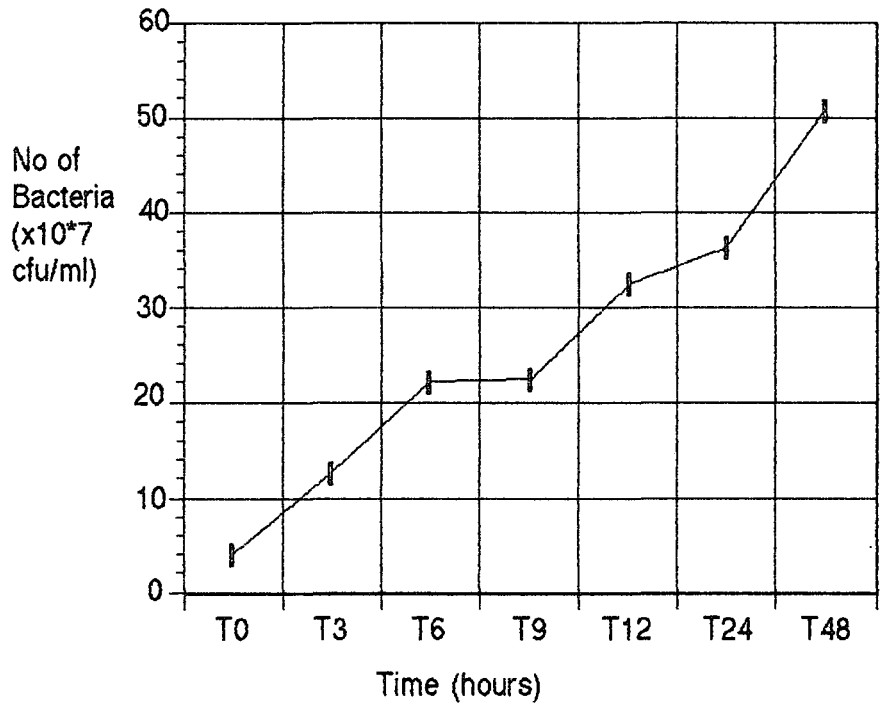


Figure 4.1.3 Colony Forming Units per milliliter against Time for Trial Three's Control Culture.

Table 4.1.2 Specific Growth Rates with Mean and Standard Deviation of the Control Cultures of Trials 1 to 3 at Various Time Periods.

Control	T0-3	T3-6	T6-9	T9-12	T12-24	T24-48
TRIAL1	0.008665	0.004346	-0.007086	0.008115	-0.001117	0.000558
TRIAL2	0.006053	0.004699	0.000320	0.001208	0.000103	0.000207
TRIAL3	0.005813	0.003012	0.000080	0.002026	0.000151	0.000231
MEAN	0.006844	0.004019	-0.002228	0.003783	-0.000287	0.000332
STD. DEV.	0.001291	0.000726	0.003436	0.003081	0.000586	0.000160

Table 4.1.3 The Maximum Viable Population of the Control Cultures of Trials 1 to 3 and the Time Taken to Reach it.

CONTROL	MAX.POPULATION	TIME
TRIAL1	1957500000	48 HOURS
TRIAL2	837500000	24 HOURS
TRIAL3	507500000	48 HOURS

Table 4.1.4 The Maximum Specific Growth Rates with Mean and Standard Deviation of the Control Cultures of Trials 1 to 3, Plus the Time to Reach them.

CONTROL	MAX. SPECIFIC G R	TIME
TRIAL1	0.0086656	3 HOURS
TRIAL2	0.006053	3 HOURS
TRIAL3	0.0058137	3 HOURS
MEAN	0.0068442	3 HOURS
STANDARDDEV.	0.0012915	0.0

Table 4.1.5 Table Showing the Enzyme Test Results for the Control Culture, Trials 1 to 3, in percentages positive.

CONTROL	ENZYME TEST	TIME 1Hour	TIME 11Hours	TIME 23Hours	TIME 47Hours
	HAEMOLYSIN	100%	100%	100%	100%
	PHOSPHATASE	100%	100%	100%	100%
	DNAse	100%	100%	100%	100%
	COAGULASE	100%	100%	100%	100%

4.2 SUBPROBLEM TWO

Table 4.2.1 The Mean Viable Cell Counts Plus Standard Deviations of the Penicillin and Control Cultures of Trial 1.

TIME (hrs)	PENICILLIN		CONTROL	
	CFU/ML x 10^7	STD. DEV.	CFU/ML x 10^7	STD. DEV.
0	5.25	4.61	3.45	3.69
3	2.95	10.21	27.9	4.33
6	0.36	5.95	63.75	2.16
9	0.03075	1.47	14.1	13.02
12	0.06	9.13	90.5	10.16
24	0.009075	17.28	83.5	10.87
48			195.75	26.01

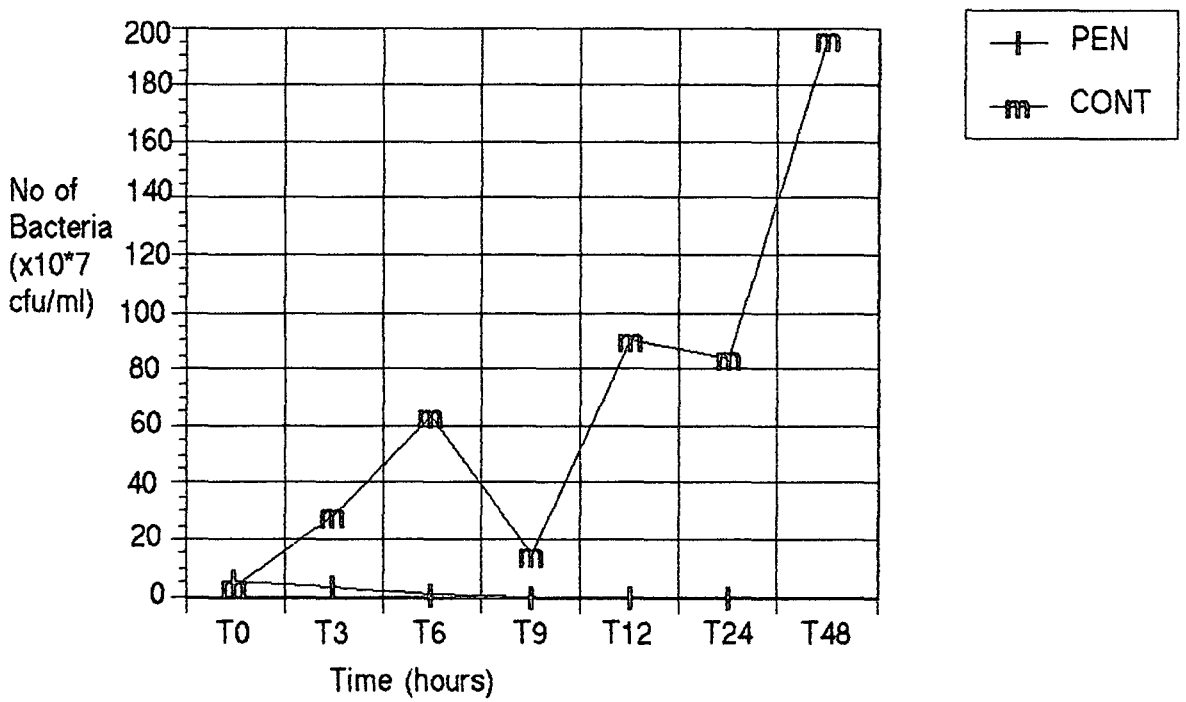


Figure 4.2.1 Colony Forming Units per Milliliter against Time for Trial One's Penicillin and Control Cultures.

Table 4.2.2 The Mean Viable Cell Counts Plus Standard Deviations of the Penicillin and Control Cultures of Trial 2.

TIME (hrs)	PENICILLIN		CONTROL	
	CFU/ML x 10^7	STD. DEV.	CFU/ML x 10^7	STD. DEV.
0	6.65	4.5	7.05	7.95
3	0.9525	5.71	23.925	16.99
6	0.094	6.20	59.00	4.58
9	0.03875	5.06	62.50	4.71
12	0.0042	10.00	77.75	5.80
24	0.000001	0.0	83.75	7.22
48	0.00	0.0	62.00	6.04

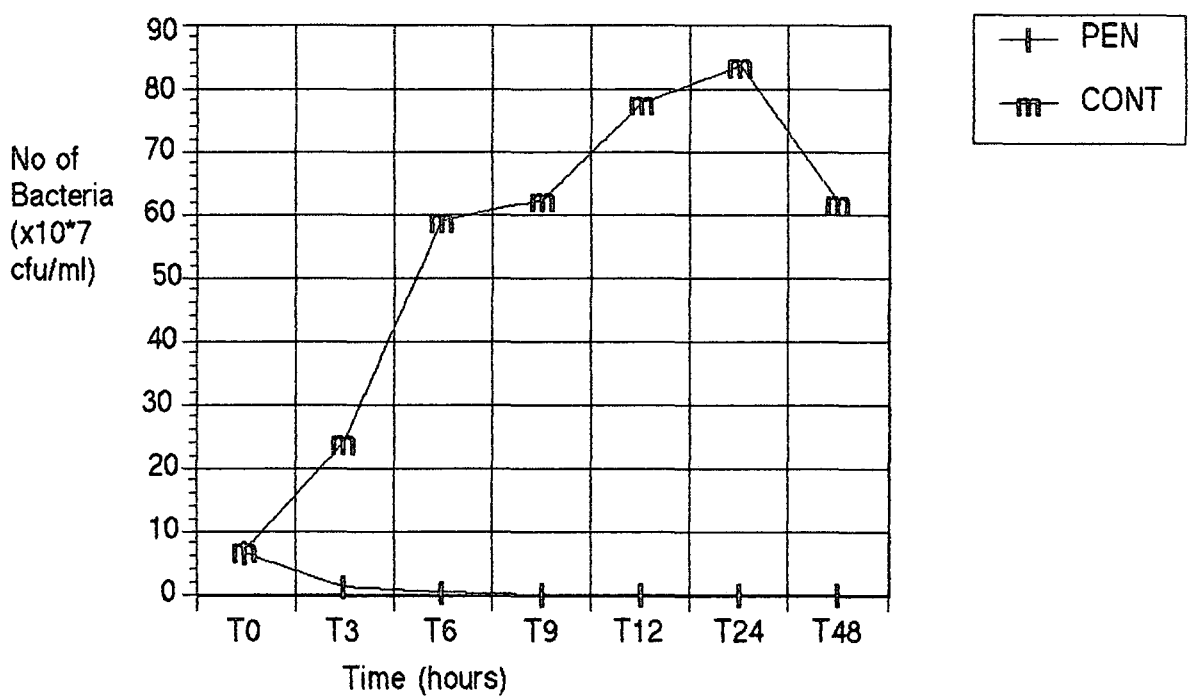


Figure 4.2.2 Colony Forming Units per Milliliter against Time for Trial Two's Penicillin and Control Cultures.

Table 4.2.3 The Mean Viable Cell Counts Plus Standard Deviations of the Penicillin and Control Cultures of Trial 3.

TIME (hrs)	PENICILLIN		CONTROL	
	CFU/ML x 10^7	STD. DEV.	CFU/ML x 10^7	STD. DEV.
0	3.5	5.40	3.975	3.69
3	0.335	2.29	12.7	5.24
6	0.12575	4.66	22.15	7.82
9	0.0163	7.24	22.475	6.06
12	0.0086	3.39	32.50	1.11
24	0.000155	1.5	36.25	1.92
48	0.00001	0.0	50.75	2.86

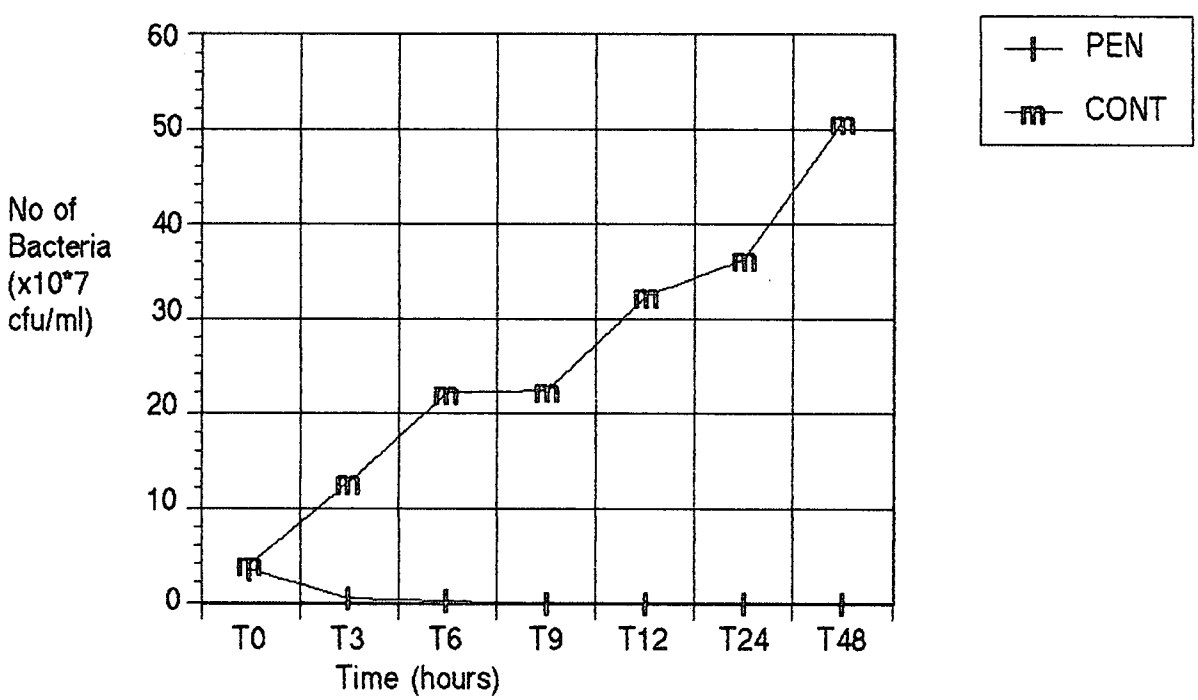


Figure 4.2.3 Colony Forming Units per Milliliter against Time for Trial Three's Penicillin and Control Cultures.

Table 4.2.4 The Specific Growth Rates, Mean Specific Growth Rates and Standard Deviations of the Penicillin and Control Cultures of Trials 1 to 3 for Various Time Periods.

	T0-3	T3-6	T6-9	T9-12	T12-24	T24-48
TRIAL1						
PENICIL	-0.003116	-0.008694	-0.009362	0.0035812	-0.002047	
CONT	0.0086656	0.0043462	-0.007086	0.0081156	-0.001117	0.000558
TRIAL2						
PENICIL	-0.008326	-0.009115	-0.004624	-0.010872	-0.002764	-0.001388
CONT	0.0060533	0.0046996	0.0003200	0.0012081	0.0001031	0.0002072
TRIAL3						
PENICIL	-0.009169	-0.005046	-0.008561	-0.003435	-0.00405	-0.001220
CONT	0.0058137	0.0030129	0.0000809	0.0020261	0.0001515	0.0002314
MEAN						
PENICIL	-0.006871	-0.007618	-0.007515	-0.005963	-0.002954	-0.001304
CONT	0.0068442	0.0040195	-0.002228	0.0037832	-0.000287	0.0003323
STD. DEV						
PENICIL	0.0021950	0.0018276	0.002070	0.0034379	0.0008315	0.000080
CONT	0.0012906	0.0007264	0.004287	0.0030636	0.0005834	0.0001563

Table 4.2.5 The Maximum Number of Viable Cells of the Penicillin and Control Cultures of Trials 1 to 3, with the Percentage Difference Between them and the Time Taken to Reach it.

TRIAL	MAX. NUMBER	TIME (hours)	% DIFFERENCE
TRIAL1			
PENICILLIN	52500000	0	97.31%
CONTROL	1957500000	48	
TRIAL2			
PENICILLIN	66500000	0	92.05%
CONTROL	837500000	24	
TRIAL3			
PENICILLIN	35000000	0	93.10%
CONTROL	507500000	48	

Table 4.2.6 The Maximum Specific Growth Rates of the Penicillin and Control Cultures of Trials1 to 3, with the Mean and Standard Deviation, as well as the Percentage Difference Between Them and the Time Taken to Reach it.

	MAXIMUM SPECIFIC G.R.	TIME (hours)	% DIFFERENCE
TRIAL1			
PENICILLIN	-0.00936	9	208.08%
CONTROL	0.00866	3	
TRIAL2			
PENICILLIN	-0.010872	12	279.61%
CONTROL	0.006053	3	
TRIAL3			
PENICILLIN	-0.009169	3	257.73%
CONTROL	0.005813	3	
MEAN			
PENICILLIN	-0.007618	6	211.31%
CONTROL	0.006844	3	
STANDARD DEVIATION			
PENICILLIN	0.0007608	3.74	29.911%
CONTROL	0.0012898	0.0	

Table 4.2.7 Summary Table of the Mean Results of the Enzyme Tests in Percentages Positive Over the Three Trials for the Penicillin and Control Cultures.

TEST CULTURE	ENZYME TEST	TIME 1Hours	TIME 11Hours	TIME 23Hours	TIME 47Hours
PENICILLIN	HAEMOLYSIN	100%	33.3%	0%	0%
	PHOSPHATASE	100%	50%	33.3%	0%
	DNAse	100%	50%	33.3%	0%
	COAGULASE	0%	0%	0%	0%
CONTROL	HAEMOLYSIN	100%	100%	100%	100%
	PHOSPHATASE	100%	100%	100%	100%
	DNAse	100%	100%	100%	100%
	COAGULASE	100%	100%	100%	100%

4.3 SUBPROBLEM THREE

Table 4.3.1 Table Showing the Mean Viable Cell Counts and Standard Deviations of the 5CH, 9CH and15CH Homoeopathic Remedy Cultures, as well as those of the Control Culture, of Trial 1 at Various Times.

	MEAN CELL COUNT x10^7 cfu/ml				STANDARD DEVIATION			
TIME	5CH	9CH	15CH	CONT	5CH	9CH	15CH	CONT
0 Hours	4.05	6.20	4.64	3.45	7.56	4.95	6.57	3.69
3 Hours	11.15	17.35	18.40	27.90	9.91	3.76	11.40	4.33
6 Hours	31.25	48.50	14.10	63.75	6.05	13.00	1.22	2.16
9 Hours	28.08	20.73	9.98	14.10	25.21	7.22	15.53	13.02
12 Hours	100.50	44.00	9.60	90.50	12.75	10.77	19.55	10.16
24 Hours	71.50	67.50	3.55	83.50	8.04	8.91	4.15	10.87
48 Hours	89.50	112.75	5.33	195.75	18.68	4.82	5.11	26.01

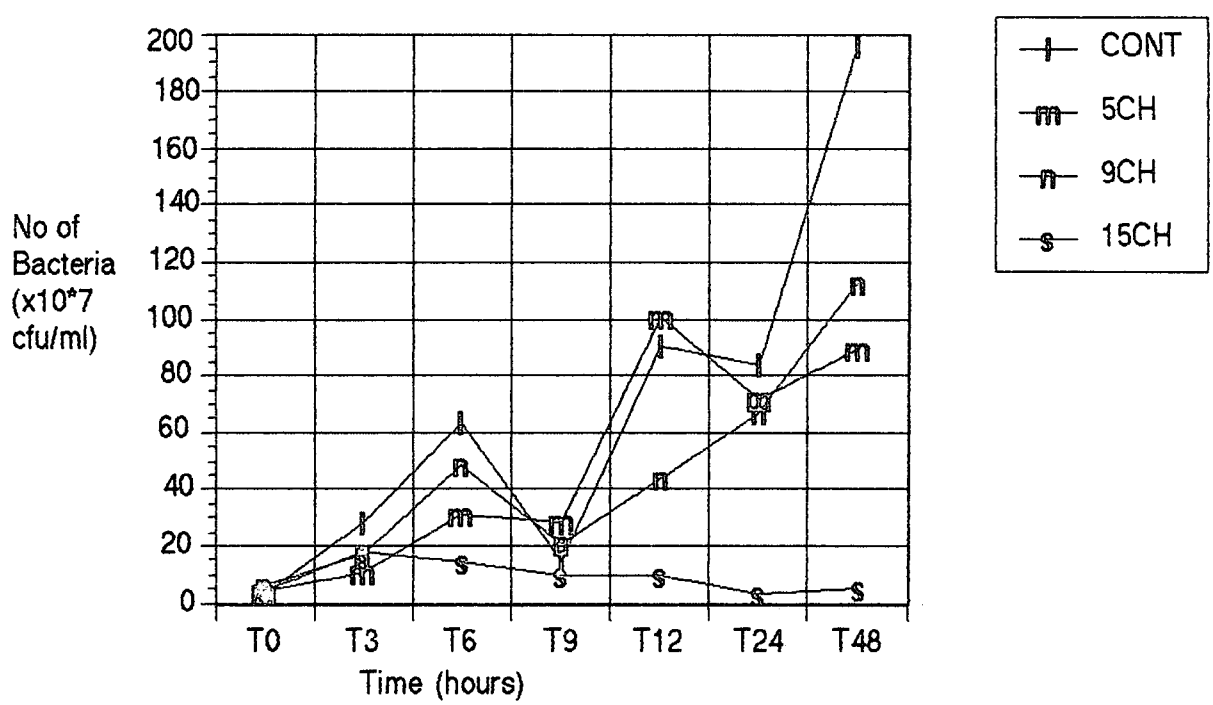


Figure 4.3.1 Colony Forming Units per Milliliter against Time for Trial One's Control, 5CH. 9CH and 15CH Cultures.

Table 4.3.2 Table Showing the Mean Viable Cell Counts and Standard Deviations of the 5CH, 9CH and 15CH Homoeopathic Remedy Cultures, as well as that of the Control Culture, of Trial 2 at Various Times.

	MEAN CELL COUNT x10^7 cfu/ml				STANDARD DEVIATION			
TIME	5CH	9CH	15CH	CONT	5CH	9CH	15CH	CONT
0 Hours	6.95	7.63	6.53	7.05	6.81	12.96	4.38	7.95
3 Hours	21.78	18.40	17.10	23.93	8.31	12.09	8.06	16.99
6 Hours	102.00	64.50	43.50	59.00	4.38	8.61	6.94	4.56
9 Hours	89.00	90.50	42.25	62.50	8.44	7.39	7.73	4.71
12 Hours	80.00	81.00	56.50	77.75	3.74	4.84	6.53	5.80
24 Hours	94.25	98.50	29.88	83.75	5.93	8.38	9.10	7.22
48 Hours	85.25	75.00	32.25	62.00	7.04	3.94	4.76	6.04

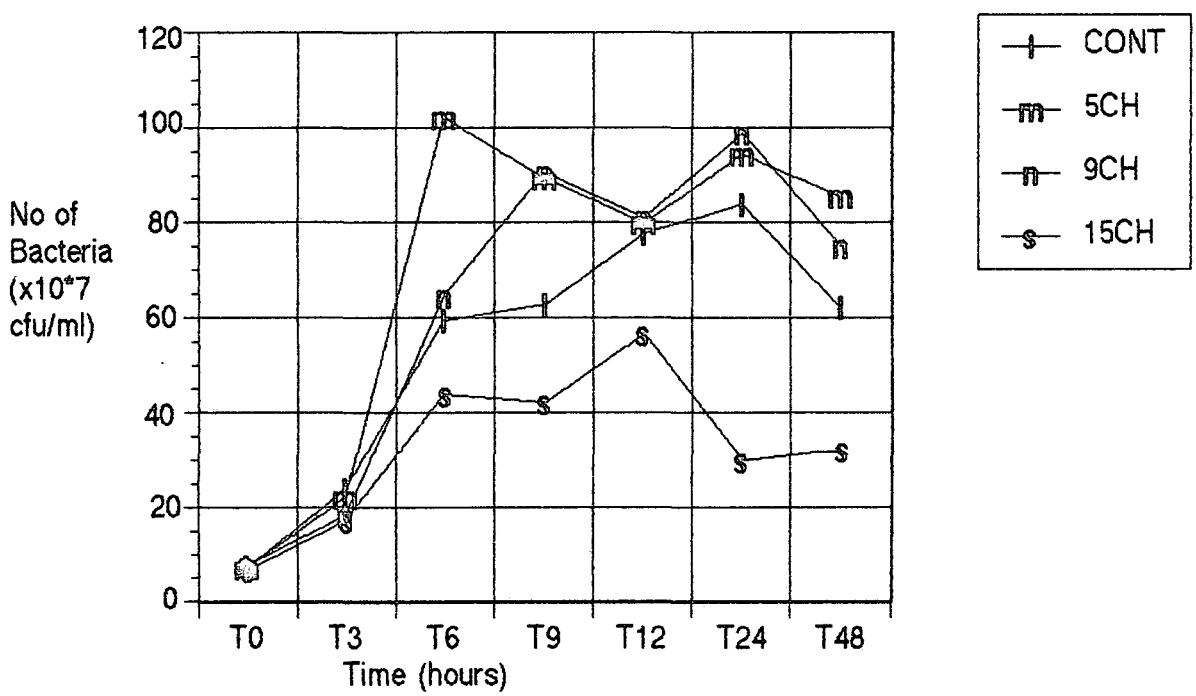


Figure 4.3.2 Colony Forming Units per Milliliter for Trial Two's Control, 5CH. 9CH, and 15CH Cultures.

Table 4.3.3 Table Showing the Mean Viable Cell Counts and Standard Deviations for the 5CH, 9CH and 15CH Homoeopathic Remedy Cultures, as well as those of the Control Culture, for Trial 3 at Various Times.

	MEAN CELL COUNT x10^7 cfu/ml				STANDARD DEVIATION			
TIME	5CH	9CH	15CH	CONT	5CH	9CH	15CH	CONT
0 Hours	3.55	3.48	3.60	3.98	3.20	3.03	3.81	3.69
3 Hours	13.53	12.70	10.18	12.70	6.94	7.18	8.84	5.24
6 Hours	23.23	21.40	20.10	22.15	7.08	6.52	6.75	7.82
9 Hours	21.70	21.18	17.13	22.48	9.89	8.55	6.10	6.06
12 Hours	24.20	21.83	19.75	32.50	5.61	8.98	5.36	1.11
24 Hours	33.00	32.25	28.50	36.25	1.58	1.09	5.09	1.92
48 Hours	56.25	48.00	43.75	50.75	4.76	5.00	2.17	2.86

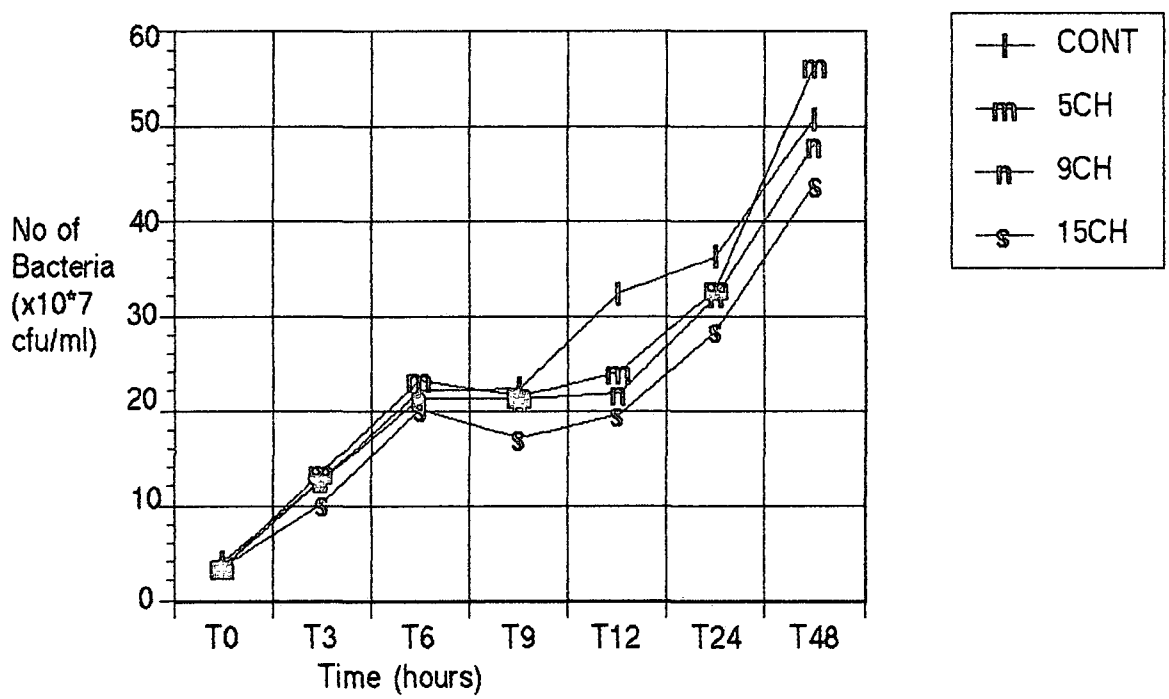


Figure 4.3.3 Colony Forming Units per Milliliter for Trial Three's Control, 5CH, 9CH, and 15CH Cultures.

Table 4.3.4 Table Showing the Specific Growth Rates Plus Means and Standard Deviations of the 5CH, 9CH and 15CH Homoeopathic Remedy Cultures, as well as those of the Control Cultures, of Trials 1 to 3 at Various Times.

	T0-3	T3-6	T6-9	T9-12	T12-24	T24-48
TRIAL1						
5CH	0.005190	0.005267	-0.00059	0.006258	-0.00046	0.000155
9CH	0.005260	0.005256	-0.00445	0.003995	0.000585	0.000348
15CH	0.006628	-0.00147	-0.00190	-0.00021	-0.00127	0.000277
CONT.	0.008665	0.004346	-0.00708	0.008115	-0.00111	0.000558
TRIAL2						
5CH	0.005734	0.007201	-0.00075	-0.00059	0.000227	-0.00018
9CH	0.004600	0.006178	0.001863	-0.00061	0.000270	-0.00018
15CH	0.004973	0.004840	-0.00016	0.001603	-0.00085	0.000053
CONT.	0.006053	0.004699	0.000320	0.001208	0.000103	0.000207
TRIAL3						
5CH	0.006336	0.002932	-0.00037	0.000605	0.000427	0.000361
9CH	0.006336	0.002834	-0.00005	0.000167	0.000535	0.000272
15CH	0.005303	0.003642	-0.00088	0.000790	0.000503	0.000293
CONT.	0.005813	0.003012	0.000080	0.002026	0.000151	0.000231
MEAN						
5CH	0.005753	0.005133	-0.00057	0.002091	0.000062	0.000149
9CH	0.005399	0.004756	-0.00088	0.001182	0.000463	0.000144
15CH	0.005635	0.002337	-0.00098	0.000727	-0.00054	0.000207
CONT.	0.006844	0.004019	-0.00222	0.003783	-0.00028	0.000332
STD. DEV.						
5CH	0.000465	0.001745	-0.00015	0.00298	0.000377	0.000222
9CH	0.000703	0.00137	0.00276	0.00196	0.000135	0.000219
15CH	0.000725	0.00268	0.000713	0.00098	0.000755	0.000108
CONT.	0.001297	0.000726	0.00428	0.00306	0.000583	0.000156

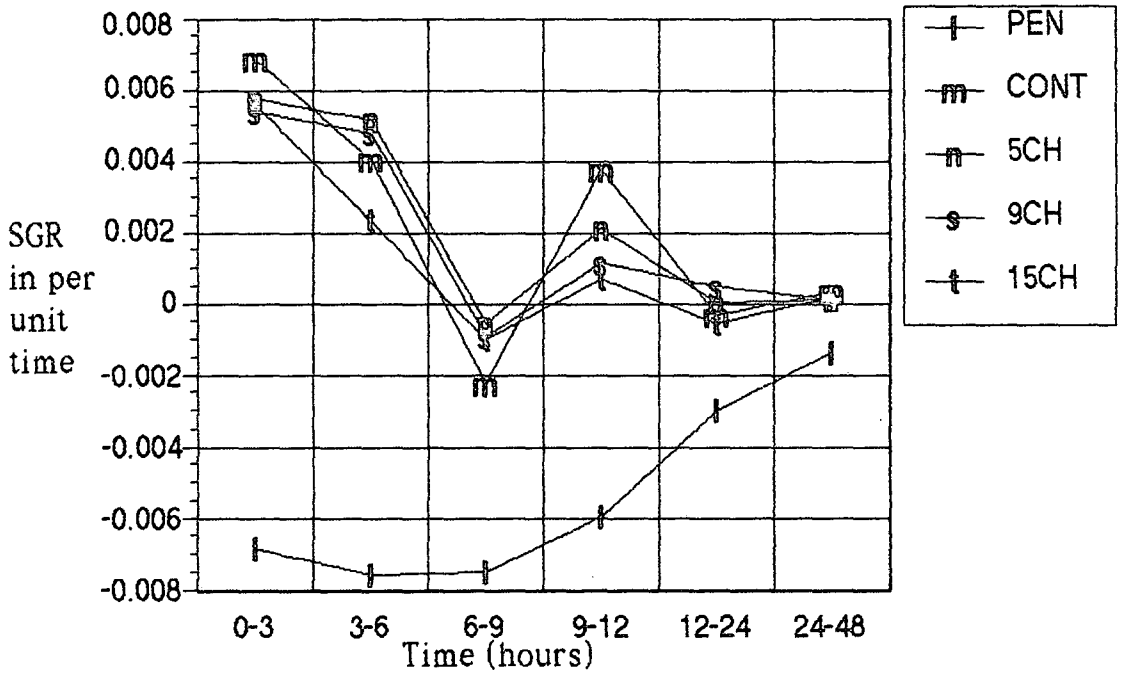


Figure 4.3.4 Graph Plotting the Mean Specific Growth Rates of all Five Cultures in Per Unit Time, against Time.

Table 4.3.5 Table Showing the Maximum Specific Growth Rates of the 5CH, 9CH and 15CH Homoeopathic Cultures, as well as those of the Control Cultures, for Trials 1 to 3, with Mean and Standard Deviation, as well as Percentage Difference Between them and Time Taken to Reach it.

	MAX. SPECIFIC GROWTH RATE	TIME (hours)	% DIFFERENCE
TRIAL1			
5CH	0.006258	12	- 27.77%
9CH	0.005260	3	- 39.29%
15CH	0.006628	3	- 23.05%
CONTROL	0.008665	3	
TRIAL2			
5CH	0.007201	3	+18.96%
9CH	0.006178	6	+ 2.06%
15CH	0.004973	3	- 17.84%
CONTROL	0.006053	3	
TRIAL3			
5CH	0.006336	3	- 8.99%
9CH	0.006336	3	- 8.99%
15CH	0.005303	3	- 8.77%
CONTROL	0.005813	3	
MEAN			
5CH	0.006598	3	- 3.59%
9CH	0.005925	3	- 13.43%
15CH	0.005635	3	- 17.67%
CONTROL	0.006844	3	
STD. DEV.			
5CH	0.000449	4.242	7.68
9CH	0.000478	1.5	16.15
15CH	0.000725	0	5.94
CONTROL	0.001275	0	

Table 4.3.6 Table Showing the Maximum Number of Viable Cells, the Time Taken to Reach this and the Percentage Difference Between the Test Culture and the Control, for the 5CH, 9CH and 15CH Homoeopathic Culture, as well as the Control Cultures of Trials 1 to 3.

	MAX. NUMBER	TIME (hours)	% DIFFERENCE
TRIAL1			
5CH	1005000000	12	- 48.65%
9CH	1127500000	48	- 42.40%
15CH	184000000	3	- 90.60%
CONTROL	1957500000	48	
TRIAL2			
5CH	1020000000	6	+21.79%
9CH	905000000	9	+ 8.05%
15CH	565000000	12	- 32.53%
CONTROL	837500000	24	
TRIAL3			
5CH	562500000	48	+10.83%
9CH	480000000	48	- 5.41%
15CH	437500000	48	- 13.79%
CONTROL	507500000	48	

Table 4.3.7 Summary Table of the Comparative Effects of the 5CH, 9CH and 15CH Homoeopathic Potencies, as well as the Control Cultures on Enzyme Production in Percentages.

	5CH REMEDY	9CH REMEDY	15CH REMEDY	CONTROL
HAEMOLYSIN	100%	100%	100%	100%
PHOSPHATASE	100%	100%	100%	100%
COAGULASE	100%	100%	100%	100%
DNAse	100%	100%	100%	100%

4.4 SUBPROBLEM FOUR

Comparison of the data collected in subproblems 1 - 3.

Table 4.4.1 Table Comparing the Mean Percentage Differences Between the Various Test Cultures and the Control Cultures of Trials 1 to 3, as far as the Maximum Population and Specific Growth Rate, the Time Taken to Reach the Maximum Specific Growth Rate and the Differences in Enzyme Tests Observed.

TEST SUBSTANCE ADDED	PERCENTAGE DIFFERENCE BETWEEN THE TEST AND CONTROL CULTURES		TIME TAKENTO REACH THE MAX. SPECIFIC GROWTH RATE	DIFFERENCE IN ENZYME TESTS OBSERVED (+ OR -)
	MAX. SPEC. GROWTH RATE	MAX. POPULATION (BIOMASS)		
PENICILLIN	-211.31%	-94.15%	N/A	loss
5CH REMEDY	- 3.59%	- 5.34%	3	none
9CH REMEDY	- 13.43%	- 13.25%	3	none
15CH REMEDY	- 17.67%	- 45.63%	3	none
CONTROL	N/A	N/A	3	none

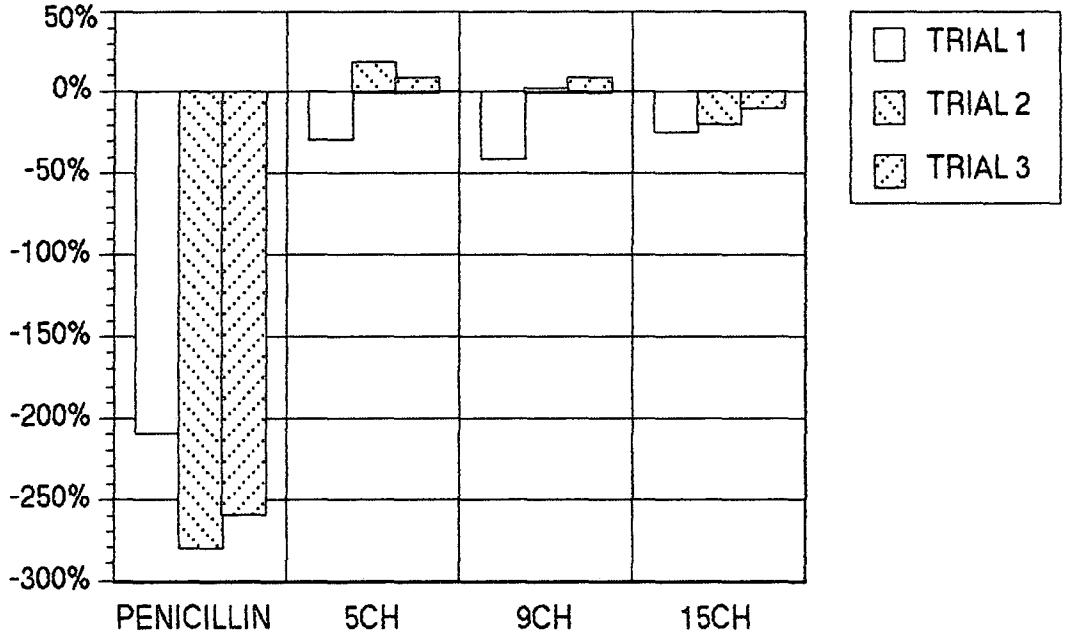


Figure 4.4.1 The Percentage Difference Between the Test and Control in the Maximum Specific Growth Rate.

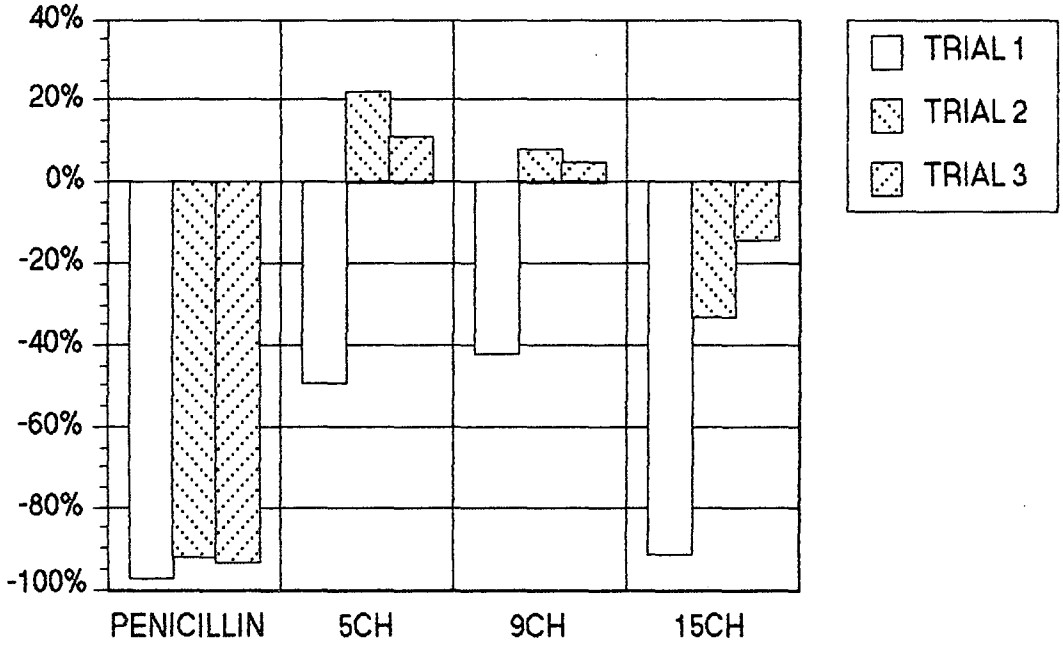


Figure 4.4.2 The Percentage Difference between the Test and Control in the Maximum Viable Cell Population.

4.5 ADDITIONAL RESULTS

Table 4.5.1 Table Showing the Generation Time and Mean Generation Time in Minutes for the 5CH, 9CH and 15CH Homoeopathic Potencies, as well as for the Control Cultures of Trials 1 to 3, at Various Times.

	TIME PERIOD (hours)					
CULTURE	3hours	6hours	9hours	12hours	24hours	48hours
CONTROL						
Trial 1	59	85	265	153	312	492
Trial 2	102	117	171	207	401	914
Trial 3	107	145	215	237	450	781
Mean	89	117	217	199	388	729
5CH REMEDY						
Trial 1	123	122	193	155	346	643
Trial 2	109	93	146	204	382	794
Trial 3	93	132	206	259	446	720
Mean	108	116	182	206	391	719
9CH REMEDY						
Trial 1	121	121	309	254	417	686
Trial 2	141	116	151	210	389	870
Trial 3	96	137	206	271	446	758
Mean	119	125	222	245	417	771
15CH REMEDY						
Trial 1	90	224	487	684	-	14448
Trial 2	129	131	200	230	654	1245
Trial 3	120	145	239	292	481	797
Mean	113	167	309	402	568 *	5497

* Denotes a Statistically significant Result.

Table 4.5.2. Table Showing the Results of the One Way Analysis of Variance of the Average Specific Growth Rates and Biomasses of the Various Cultures.

CONTRAST	SPECIFIC GROWTH RATE			BIOMASS		
	TRIAL 1	TRIAL 2	TRIAL 3	TRIAL 1	TRIAL 2	TRIAL 3
CONTROL+PENICILLIN	****	****	****	****	****	****
CONTROL + 5CH						
CONTROL + 9CH						
CONTROL + 15CH				****		
PENICILLIN + 5CH	****	****	****	****	****	****
PENICILLIN + 9CH	****	****	****	****	****	****
PENICILLIN + 15CH	****	****	****		****	****
5CH + 9CH						
5CH + 15CH					****	
9CH + 15CH						

**** Denotes a Statistically Significant Result

CHAPTER FIVE

5.0 DISCUSSION

5.1 SUBPROBLEM ONE

Control Culture.

From Table 4.1.1 and Figures 4.1.1, 4.1.2, and 4.1.3 it can be seen that the growth of the control cultures of Staphylococcus aureus was not in the shape of a normal logarithmic curve. In fact it seems to be more linear for the first six hours, than logarithmic. The possible reason for this is that, even under optimal conditions, Staphylococcus aureus tends to clump (Bergey, 1964). Thus, despite the mixing each time a sample was removed for dilution and counting, it is possible that the cells on the inside of the cluster did not get the same nutrients as those on the outside, therefore keeping the growth of the culture linear rather than logarithmic.

Another interesting feature noticed when comparing the three graphs, is that there seems to be a tendency for growth to slow down after the 12 hour mark. When looking at the graphs it is important to notice that the scale on the x-axis is not uniform, and that the last two time periods have large times between them (12 hours and 24 hours respectively) and this makes the relative increase in biomass for those time periods even less. It would seem that here the culture has reached its stationary phase (or even its declining phase in Figure 4.1.2). A possible explanation for this, is the fact that the culture was already 24 hours old when the test bottles were inoculated. Thus there were already a large

number of cells in the culture, add to this the large amount of culture used to inoculate the broth (2ml to only 18ml of broth) and it can be understood why it reached its stationary phase after 12 hours. The distinct lack of a lag phase can be explained in the same way, and because the sample of culture was removed from a 24 hour old culture and placed into the same growth medium, there would be no need for the organisms to adapt, and therefore no lag phase.

The decrease in growth between 6 and 9 hours so apparent in Figure 4.1.1 is difficult to explain. It is possible that it was due to poor technique, and an error in measurement on behalf of the researcher, this is supported by a high standard deviation at this point. It is possible that after nine hours of growth the clumping of Staphylococcus aureus makes it very difficult to withdraw a representative aliquot for testing since a homogenous mixture is difficult to achieve even if vortexing is used to mix the tube prior to sampling. Other possible explanations are that perhaps the equipment had been tampered with, or that there was a short power failure during this time period.

It can also be seen in all the graphs (figures 4.1.1, 4.1.2, and 4.1.3) that the slopes of the graphs become more even and smooth from trial 1 to 3. This is put down to the researcher becoming more familiar with the procedures and becoming more adept at the microbiological techniques. Thus it is recommended, for any further research in this particular field, that the trials be run more than three times, between five and eight times would provide more reliable results. Another recommendation for future researchers is that any further trials be carried out as blind or double blind studies to prevent any prejudice or expectation on behalf on the experimenter. Finally it is recommended that the culture is monitored more frequently than every three hours, this would give more information as to what is happening in the culture from moment to moment.

When studying Table 4.1.2, it can be seen that there is consistency between the trials. The specific growth rates of all three trials start out high, then slowly tail down to a low point at the period of between 6 to 9 hours,(thus perhaps there was some sort of temporary lack of nutrients or build up of toxins at this time) and then all pick up again between 9 and 12 hours. Then in all three cases the specific growth rates decrease to almost zero between 12 and 48 hours. This supports the theory that the cultures reached a stationary phase after 12 hours. Again the most likely explanation for the 6 to 9 hour dip is experimental error, the pipette not being 100% accurate and the clumping of the bacteria provide some variation in the number of organisms that was in the final 0.1 ml that was placed on the plate. The fact that it occurred in all three trials may indicate that some other factor was involved. Perhaps the decrease in specific growth rate was due to cells dying (perhaps weaker or more susceptible to toxins), and after they have died other stronger cells can divide more rapidly and fill the gap, causing the increase between 9 and 12 hours.

From Table 4.1.3 it can be seen that the maximum biomass was attained in the final stages of the culture. In trials 1 and 3 the cultures were still building biomass until the last reading, and in trial 2 maximum biomass was attained after 24 hours, perhaps indicating some evidence of a declining phase here.

Table 4.1.4 shows that the highest specific growth rate in the case of all three trials occurred between zero and three hours, when the organisms were freshly introduced to the test broth and thus still had plentiful nutrients and minimum toxic waste product build up.

Table 4.1.5 shows that all the enzyme tests on the control cultures are positive, and thus indicates that the organism, even after repeated culturing in the microbiology laboratories did not loose the ability to produce any of these enzymes.

5.2 SUBPROBLEM TWO

The Effect of Penicillin G.

From Tables 4.2.1, 4.2.2, and 4.2.3. and from Figures 4.2.1, 4.2.2, and 4.2.3. it can easily be seen how the Penicillin G had a constant and significant effect on the test cultures over the three trials. In all three cases the number of viable cells in the cultures decreased dramatically, and steadily, to almost zero by the end of each experiment. This shows that the Penicillin G was having its desired effect and that the concentration of Penicillin G in the cultures was strong enough. The missing observation at the 48 hours time period of trial one was due to contamination by what was later found to be a Gram Negative bacillus, but the observations present showed enough of a strong and definite trend towards the death of the culture that the final observation was not crucial.

Table 4.2.4 shows that in all three trials the Penicillin G caused not just a decrease in the specific growth rate, but an almost constantly negative specific growth rate. This indicates that there was a steady decrease in the biomass of the culture, or that the culture was dying. In fact in trial 2 the culture was totally dead after 48 hours. The effect of Penicillin G on the specific growth rate can be seen in figure 4.3.4, a comparison of the average specific growth rates; the Penicillin treated cultures are constantly negative, while the others are mostly always positive.

Table 4.2.5 shows that the maximum number of viable cells, in all the cultures exposed to penicillin G occurred at zero hours (i.e. the starting number.) It also shows the percentage difference between the Penicillin-treated cultures' and the control cultures' Maximum Biomass, all of the results being above 90% (average

of 94.15%); thus the difference here is great. This is obviously because the control cultures reached their maximum biomass after between 24 and 48 hours of growth, while the Penicillin G cultures were almost dead by then.

In Table 4.2.6 it can be seen that the maximum (negative) specific growth rate for the Penicillin G cultures occurred between three hours and twelve hours, thus the different cultures decreased at their greatest rate at these times. The percentage differences between these readings and those of the control culture are great (all readings above 200% difference with an average of 211.31% difference). Thus the Penicillin G had a very marked effect on the maximum specific growth rate of the culture.

Table 4.2.7 shows that the Penicillin G had an effect on the extracellular enzymes of Staphylococcus aureus. The Coagulase enzyme was the first to be affected, and this one was no longer present after just one hour's exposure to the Penicillin G. The next enzyme to disappear was Haemolysin, the enzyme reaction was weakened after 11 hours, and was totally lost in all cases after 23 hours. In most cases the Phosphatase and DNAse enzyme reactions were weakened after 11 hours and were in all cases absent after 47 hours. Thus it can be assumed that the Coagulase enzyme system was the most sensitive and perhaps the Penicillin's effect on the cell wall of the organism prevented or inhibited its release. The other enzyme systems only started being affected later on, and the reason for this might be that Penicillin G only kills dividing cells, thus the cells that survived for a time (i.e. did not divide) were still able to produce enzymes, also the clumping effect of Staphylococcus aureus may have protected the inner cells while dividing, thus allowing them to go on producing enzymes for a while. Thus Penicillin did not seem to have such a strong effect on the enzyme producing ability itself for these enzyme systems.

One way analysis of variance was undertaken using the average specific growth rate for each culture in each trial (Table 4.5.2). From this it was found that according to the average specific growth rates the only cultures to show statistically significant difference from the control, were the Penicillin G cultures in each of the three trials.

5.3 SUBPROBLEM THREE

The Effect of Staphylococcinum.

From Table 4.3.1 and Figure 4.3.1 it can be seen that the 5CH, 9CH and the control grew along a relatively linear pattern. The Dip in the growth of all three between six and nine hours could be due to experimental error as explained in section 5.1. The culture exposed to 15CH Staphylococcinum shows much less growth than the others. This trend is followed throughout all three trials, the 5CH, 9CH and control cultures being similar, whereas the 15CH culture exhibits growth retardation, albeit only very slightly in trial three, as see in Tables 4.3.2 and 4.3.3, as well as Figures 4.3.2 and 4.3.3.

A similar trend is seen in Table 4.3.4, whose mean results are graphically represented in Figure 4.3.4. It can be seen that the specific growth rates of the Staphylococcinum treated cultures and the control culture all start out high, then drop steadily to their lowest point at time 6 to 9 hours, rise again between 9 and 12 hours and then drop again. The result of the 15CH treated culture is mostly slightly lower than the rest.

Table 4.3.5 shows the maximum specific growth rates of the three trials. In trial one it can be seen that the percentage difference between the maximum specific growth rates of the 5CH, the 9CH and the 15CH homoeopathic potency cultures and the control are not that great. All the results are less than the control, being between 23% and 39% less (the lowest being the 15CH.) In trial two the results are

slightly different, the maximum specific growth rates of the 5CH, and the 9CH culture are higher than that of the control (+19% and +2% respectively), while the 15CH's result is 18% lower than the control. Trial three is similar to trial two; 5CH and 9CH more than the control (both +9%), and the 15CH less (-9%). On average the results are all lower than the control, 15CH showing the greatest difference at -18% and 5CH the least at -4%. Thus it seems that the culture exposed to the15CH Staphylococcinum showed a specific growth rate which was consistently less than that of the control. The 5CH and 9CH's results were not consistent enough to reliably indicate a trend.

In Table 4.3.6 the maximum number of viable cells can be seen. In trial one the results for 5CH, 9CH and 15CH are all less than the control, 5CH and 9CH similar at 49% and 42% respectively, while the 15CH cultures result is quite significant at 91% less than that of the control. For trial two the results are different, the 5CH and 9CH results are greater than the control at (+22% and +8% respectively) and the 15CH result is 33% less than the control. For trial three the 5CH result is greater than the control by +11%, and the 9CH and 15CH results are less than the control at 5% and 14% differences respectively. Thus here too a trend is evident, the 15CH 's maximum biomass seem to be consistently and in some cases significantly lower than those of the control. The results for the 5CH and 9CH were not consistent enough to reliably indicate a trend.

From Table 4.3.7 it can be seen that the results of the enzyme tests for all the cultures treated with the homoeopathic potencies are the same as those for the control cultures. Thus it can be said, from the results of the qualitative enzyme tests used, that the homoeopathic remedy Staphylococcinum did not have any effect on the enzyme producing ability of Staphylococcus aureus.

5.4 SUBPROBLEM FOUR

Comparison of Data from Previous Subproblems.

Table 4.4.1 is a comparison of the percentage differences calculated from the results of the above three subproblems, it was created by calculating the means of the three trials results (see Apendix D for each trials results separately). From this table it can be seen that Penicillin G has a dramatic effect on the maximum specific growth rate and on the maximum biomass of Staphylococcus aureus achieved. The next greatest effect in both cases is shown by the 15CH Staphylococcum cultures, then the 9CH and finally the 5CH. It might be said that the term maximum specific growth rate does not apply to the Penicillin treated culture, as the culture did not grow at all, and the -211.31% difference in maximum specific growth rate compared to that of the control refers to death and not growth of the culture.

When one way analysis of variance was undertaken for the average biomass of each culture in a trial (Table 4.5.2.), it was found that the Penicillin treated cultures of all three trials consistently showed statistically significant differences from the control cultures. Other findings of interest were that for the average biomasses of trial one, the 15CH treated culture showed statistically significant difference from the control culture, and a lack of difference to the Penicillin treated culture. It was also found, for the average biomasses of trial two, that there was a statistically significant difference between the 5CH and 15CH treated cultures.

The data in Table 4.5.1 further indicate that the 15CH Staphylococcum did have a significant effect on the Staphylococcus aureus culture. It can be seen from this table that the generation time for the test cultures and the control, not only increases over time, but seems to be higher in the 15CH cultures. One way analysis of variance of the results of Table 4.5.1 shows

that the only statistically significantly different group of results are the results for the 15CH cultures, at the 24 hour time period. The results for the 15CH cultures at 48 hours would also probably have been statistically significant were it not for the high variance within the group. Thus there was a significant increase in the time taken for the culture exposed to 15CH Staphylococcinum to reproduce, but the increase in generation time only became significant after 24 hours.

From the Figures 4.4.1. and 4.4.2. drawn from the previous summaries, it can be seen that as far as maximum specific growth rate and maximum biomass are concerned the only two cultures that showed consistent results were those treated with the Penicillin G and 15CH Staphylococcinum. The other culture's results were neither consistent, nor significant.

Thus it can be concluded that Penicillin G is very effective at killing Staphylococcus aureus, as expected. The only potency of homoeopathic remedy that seemed to have a reliable effect on the growth parameters of the organism was the 15CH potency of Staphylococcinum. The probable reason for this is that the higher potencies 15 or 30CH are known to inhibit or slow down certain physiological processes (Jouanny, 1991), so perhaps the physiological processes of the organism were slowed down (thus the increase in the generation time in the cultures exposed to Staphylococcinum), causing the decrease in the growth parameters.

Table 4.5.2. shows the one way analysis of variance for the average specific growth rates and average biomasses of the various cultures. When the average specific growth rates are taken for the whole trial, then no other results other than those of the Penicillin G are statistically significantly different from the control. When the average biomass is considered over the

duration of each trial, significant differences were shown between Penicillin and every other culture (with one exception). Apart from the differences with the Penicillin culture there were also differences noted within trials one and two.

One of the differences noticed in trial one was between the control culture and that of the 15CH culture, this is explained because the biomass of the 15CH in trial one remained constantly lower than the control. The other significant result from trial one was a lack of significant difference between the Penicillin culture and the 15CH culture, i.e. that the two cultures could be considered similar on average biomass, this was so because of the constantly low readings from the 15CH culture in this trial. The significant results from trial two were, that the 5CH and the 15CH results showed difference, this was because in trial two the 5CH showed very high growth (higher than the control) and the 15CH was consistently low.

Thus this research conforms with the hypothesies that were proposed; the Penicillin G was effective in killing the organism, the homoeopathic remedy did show an effect on the growth parameters of the organism (if only in the 15CH potency), and the effect of the remedy on the organism was more subtle than that of the antibiotic. The results of this study also seem to provide some evidence as to how homoeopathic remedies work (or do not work), it has been shown that the remedy had no effect on the enzyme producing ability of the organism, therefore it's effectiveness in treatment of Staphylococcal infections is not likely to be due to an effect on this aspect of the organism. It also seems that the remedy increases the generation time of the organism, therefore slowing down it's growth. Thus this research does provide scientific evidence of some effect of homoeopathic remedies on living organisms.

Unlike the results of Baker et al (1985), this study has shown that at least one potency of the homoeopathic remedy did have a constant effect on the growth parameters of a micro-organism. But the results of this study still do not match the consistent high levels of significance claimed by Jones and Jenkins (1983). As far as the further use of Staphylococcinum in vitro goes, it cannot be recommended as an anti Staphylococcal agent as Penicillin was far more effective in killing the organism.

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APPENDIX A

Table's A.1-4 The Enzyme Test Results of the Test and Control Cultures of Trial One at Various Times.

Enzyme Experiment 1 , Time 1 hour.

	PEN	CONT.	5CH	9CH	15CH
Blood	+	+	+	+	+
Phosphate	+	+	+	+	+
DNA	+	+	+	+	+
Coagulation	-	+	+	+	+

Enzyme Experiment 2 , Time 11 hours.

	PEN	CONT.	5CH	9CH	15CH
Blood	+	+	+	+	+
Phosphate	+	+	+	+	+
DNA	+	+	+	+	+
Coagulation	_	+	+	+	+

Enzyme Experiment 3 , Time 23 hours.

	PEN	CONT.	5CH	9CH	15CH
Blood	_	+	+	+	+
Phosphate	+	+	+	+	+
DNA	+	+	+	+	+
Coagulation	_	+	+	+	+

Enzyme Experiment 4 , Time 47 hours.

	PEN	CONT.	5CH	9CH	15CH
Blood	_	+	+	+	+
Phosphate	_	+	+	+	+
DNA	_	+	+	+	+
Coagulation	_	+	+	+	+

Table's A.5-8 The Enzyme Test Results of the Test and Control Cultures of Trial Two at Various Times.

Enzyme Experiment 1 Time 1 hour.

	PEN	CONT.	5CH	9CH	15CH
Bld	+	++	++	++	++
PPA	+	+	+	+	+
DNA	+	+	+	+	+
Coag	-	+	+	+	+

Enzyme Experiment 2 , Time 11 hours.

	PEN	CONT.	5CH	9CH	15CH
Bld	-	+	+	+	+
PPA	0.25	+	+	+	+
DNA	0.5	+	+	+	+
Coag	-	+	+	+	+

Enzyme experiment 3, Time 23 hours.

	PEN	CONT.	5CH	9CH	15CH
Bld	-	+	+	+	+
PPA	-	+	+	+	+
DNA	-	+	+	+	+
Coag	-	+	+	+	+

Enzyme Experiment 4 , Time 47 hours.

	PEN	CONT.	5CH	9CH	15CH
Bld	-	+	+	+	+
PPA	-	+	+	+	+
DNA	-	+	+	+	+
Coag	-	+	+	+	+

Table's A.9-12 The Enzyme Test Results of the Test and Control Cultures of Trial Three at Various Times.

Enzyme Experiment 1 , Time 1 hour.

	PEN	CONT.	5CH	9CH	15CH
Bld	+	+	+	+	+
Phos	+	+	+	+	+
DNA	+	+	+	+	+
Coag	-	+	+	+	+

Enzyme Experiment 2 , Time 11 hours.

	PEN	CONT.	5CH	9CH	15CH
Bld	-	+	+	+	+
Phos	-	+	+	+	+
DNA	-	+	+	+	+
Coag	-	+	+	+	+

Enzyme Experiment 3 , Time 23 hours.

	PEN	CONT.	5CH	9CH	15CH
Bld	-	+	+	+	+
Phos	-	+	+	+	+
DNA	-	+	+	+	+
Coag	-	+	+	+	+

Enzyme Experiment 4 , Time 47 hours.

	PEN	CONT.	5CH	9CH	15CH
Bld	-	+	+	+	+
Phos	-	+	+	+	+
DNA	-	+	+	+	+
Coag	-	+	+	+	+

APPENDIX B

Table's B.1-7 The Growth Experiment Results of the Test and Control Cultures of Trial One at Various Times.

Growth experiment 1, Time 0 hours.

	PEN	CONT	5CH	9CH	15CH
- 2	tntc	tntc	tntc	tntc	tntc
- 3	tntc	tntc	tntc	tntc	tntc
- 4	tntc	375	tntc	tntc	tntc
		382			
- 5	47	30	32	55	39
	58	39	47	60	52
	56	32	49	68	54
	49	37	34	65	41
Average	**52.5**	**34.5**	**40.5**	**62**	**46.5**

Growth Experiment 2, Time 3 hours.

	PEN	CONT	5CH	9CH	15CH
- 3	tntc	tntc	tntc	tntc	tntc
- 4	281	tntc	tntc	tntc	tntc
	291				
	298				
	309				
Average	**295**				
- 5	31	283	125	171	168
	28	277	110	173	182
	29	271	117	181	200
	27	275	98	176	186
Average		**279**	**111.5**	**173.5**	**184**
- 6	2	26	14	18	18
	1	27	9	15	16
	2	33	10	15	15
	3	29	11	13	21

Growth Experiment 3, Time 6 hours.

	PEN	CONT	5CH	9CH	15CH
- 3	tntc				
- 4	46		tntc	tntc	tntc
	35				
	32				
	31				
Average	**36**				
- 5	3	tntc	245	234	124
	5		326	260	162
	4		267	259	141
	3		292	233	137
Average					**141**
- 6	0	61	30	29	12
	0	63	23	46	11
	0	67	40	47	11
	2	64	32	72	14
Average		**63.75**	**31.25**	**48.5**	

Growth Experiment 4, Time 9 hours.

	PEN.	CONT	5CH	9CH	15CH
- 2	tntc	tntc	tntc	tntc	tntc
- 3	31				
	30				
	29				
	33				
Average	**30.75**				
- 4	12	tntc	tntc	tntc	tntc
	2				
	3				
	4				
- 5	0	127	272	213	82
	1	160	246	204	93
	0	136	290	197	118
	0	141	315	215	106
Average		**141**	**280.75**	**207.25**	**99.75**
- 6	0	9	35	10	15
	0	3	40	40	0
	0	4	28	8	0
	0	7	36	54	9

Growth Experiment 5, Time 12 hours.

	PEN	CONT.	5CH	9CH	15CH
- 3	50	tntc	tntc	tntc	tntc
	67				
	52				
	71				
Average	60				
- 4	8	tntc	tntc	tntc	tntc
	3				
	6				
	5				
- 5	0	tntc	tntc	tntc	83
	0				74
	1				125
	0				102
Average					96
- 6	0	106	91	34	12
	0	93	85	38	11
	0	80	115	62	7
	0	83	111	42	4
Average		90.5	100.5	44	
- 7	0	10	4	3	0
	0	6	11	4	1
	0	9	7	4	1
	0	10	7	7	0

Growth Experiment 6, Time 24 hours.

	PEN	CONT.	5CH	9CH	15CH
- 1	tntc				
- 2	70				
	87				
	88				
	118				
Average	**90.75**				
- 3	8				tntc
	9				
	10				
	6				
- 4	1				317
	1				346
	0				297
	0				350
- 5	1	tntc	tntc	tntc	35
	0				40
	0				29
	0				38
Average					**35.5**
- 6	0	76	75	53	3
	0	71	83	60	1
	0	88	63	72	3
	0	99	65	75	2
Average		**83.5**	**71.5**	**67.5**	
- 7	0	6	6	2	1
	0	6	7	6	0
	0	9	10	5	0
	0	7	9	4	0
- 8	0	0	0	1	0
	0	0	0	1	0
	0	1	0	0	0
	0	2	1	0	1

Growth Experiment 7, Time 48 hours.

	PEN	CONT	5CH	9CH	15CH
- 4					tntc
- 5		tntc	tntc	tntc	61
					51
					47
					54
Average					**53.25**
- 6		219	83	190	6
		157	72	108	6
		187	82	183	4
		220	121	177	5
		195.75	**89.5**	**112.75**	
- 7		18	12	9	1
		10	7	16	2
		12	3	15	0
		17	14	12	0
- 8		1	2	1	0
		0	2	2	0
		0	0	0	0
		0	1	1	0

Table's B.8-14 The Growth Experiment Results of the Test and Control Cultures of Trial Two at Various Times.

Growth Experiment 1, Time 0 hours.

	PEN	CONT	5CH	9CH	15CH
- 4	tntc	tntc	tntc	tntc	tntc
- 5	68	83	78	98	64
	71	61	65	65	71
	68	68	61	59	67
	59	70	74	83	59
Average	**66.5**	**70.5**	**69.5**	**76.25**	**65.25**

Growth Experiment 2 , Time 3 hours.

	PEN	CONT	5CH	9CH	15CH
- 3	tntc	tntc	tntc	tntc	tntc
- 4	98	tntc	tntc	tntc	tntc
	92				
	88				
	103				
Average	**95.25**				
- 5	7	254	229	181	184
	8	258	232	172	168
	10	226	219	205	170
	10	219	191	178	162
Average		**239.25**	**217.75**	**184**	**171**

Growth Experiment 3 , Time 6 hours.

	PEN	CONT	5CH	9CH	15CH
- 2	tntc	tntc	tntc	tntc	tntc
- 3	92				
	93				
	104				
	87				
Average	**94**				
- 4	5				
	6				
	4				
	8				
- 5	0	tntc	tntc	tntc	tntc
	0				
	0				
	0				
- 6	0	56	103	77	32
	0	66	92	62	45
	0	60	102	66	45
	0	54	111	53	51
Average		**59**	**102**	**64.5**	**43.5**

Growth Experiment 4 , Time 9 hours.

	PEN	CONT	5CH	9CH	15CH
- 2	tntc				
- 3	32				
	42				
	36				
	45				
Average	**38.75**				
- 4	3				
	2				
	4				
	5				
- 5	0	tntc	tntc	tntc	tntc
	0				
	0				
	0				
- 6	0	70	80	102	53
	0	62	77	92	32
	0	57	91	87	39
	0	61	108	81	45
Average		**62.5**	**89**	**90.5**	**42.25**
- 7	0	8	6	6	7
	0	5	11	8	6
	0	6	7	5	7
	0	7	9	8	5

Growth experiment 5 , Time 12 hours.

	PEN	CONT	5CH	9CH	15CH
- 1	32				
	52				
Average	**42**				
- 2	14				
	11				
	5				
	7				
- 3	1				
	0				
	0				
	0				
- 4	0				
	0				
	0				
	0				
- 5	0	tntc	tntc	tntc	tntc
	0				
	0				
	0				
- 6	0	71	86	89	59
	0	76	78	76	55
	0	77	80	80	47
	0	87	76	79	65
Average		**77.75**	**80**	**81**	**56.5**
- 7	0	6	9	4	3
	0	5	5	4	5
	0	3	6	7	7
	0	3	4	3	7

Growth Experiment 6 , Time 24 hours.

	PEN	CONT	5CH	9CH	15CH
- 1	1				
	1				
Average	1				
- 2	0				
	0				
	0				
	1				
//////	//////	//////	//////	//////	//////
- 5	0	tntc	tntc	tntc	283
	0				297
	0				284
	0				331
Average					298.75
- 6	0	84	102	88	22
	0	88	88	93	28
	0	72	98	104	20
	0	91	89	109	41
Average		83.75	94.25	98.5	
- 7	0	9	7	8	7
	0	6	5	11	5
	0	5	11	12	6
	0	8	11	9	4
- 8	0	1	2	2	2
	0	0	1	3	4
	0	2	0	0	1
	0	1	2	2	1

Growth Experiment 7 , Time 48 hours.

	PEN	CONT	5CH	9CH	15CH
- 1	0				
	0				
- 2	0				
	0				
	0				
	0				
//////	//////	//////	//////	//////	//////
- 5	0	tntc	tntc	tntc	tntc
	0				
	0				
	0				
- 6	0	52	87	81	27
	0	65	78	76	31
	0	63	96	71	31
	0	68	80	72	40
Average		**62**	**85.25**	**75**	**32.25**
- 7	0	6	11	6	3
	0	5	10	8	2
	0	7	9	8	3
	0	3	8	6	1
- 8	0	1	2	2	1
	0	2	1	1	0
	0	0	1	0	0
	0	0	3	0	1

Table B.15-21 The Growth Experiment Results of the Test and Control Cultures of Trial Three at Various Times.

Growth Experiment 1, Time 0 hours.

	PEN	CONT	5CH	9CH	15CH
- 4	tntc	tntc	tntc	tntc	tntc
- 5	43	38	32	33	34
	50	35	40	36	31
	38	45	33	31	41
	36	41	37	39	38
Average	**35**	**39.75**	**35.5**	**34.75**	**36**
- 6	2	2	3	3	5
	1	1	3	4	2
	3	1	5	4	3
	3	3	4	4	3

Growth Experiment 2 , Time 3 hours.

	PEN	CONT	5CH	9CH	15CH
- 3	TNTC				
- 4	32	tntc	tntc	tntc	tntc
	31				
	34				
	37				
Average	**33.5**				
- 5	3	130	132	129	109
	2	133	147	136	94
	4	119	133	116	92
	2	126	129	127	112
Average		**127**	**135.25**	**127**	**101.75**
- 6	0	13	10	12	8
	0	10	10	11	9
	0	11	11	11	9
	0	15	12	10	11

Growth Experiment 3 , Time 6 hours.

	PEN	CONT	5CH	9CH	15CH
- 2	tntc				
- 3	132				
	125				
	119				
	127				
Average	**125.75**				
- 4	11	tntc	tntc	tntc	tntc
	9				
	12				
	12				
- 5	0	218	221	205	207
	0	211	240	211	206
	0	232	232	218	190
	0	225	236	222	201
Average		**221.5**	**232.25**	**214**	**201**
- 6	0	18	21	20	19
	0	22	20	22	19
	0	19	18	17	16
	0	20	18	18	17

Growth Experiment 4 , Time 9 hours.

	PEN	CONT	5CH	9CH	15CH
-1	tntc				
- 2	161				
	169				
	170				
	152				
Average	**163**				
- 3	16				
	17				
	12				
	11				
- 4	0	tntc	tntc	tntc	tntc
	0				
	1				
	0				
- 5	0	234	223	198	179
	0	217	201	212	173
	0	223	217	221	162
	0	225	227	216	171
Average		**224.75**	**217**	**211.75**	**171.25**
- 6	0	19	17	19	15
	0	22	19	19	18
	0	21	15	21	14
	0	16	20	21	17
- 7	0	0	1	0	0
	0	1	0	2	1
	0	0	2	0	1
	0	0	0	2	1

Growth Experiment 5 , Time 12 hours.

	PEN	CONT	5CH	9CH	15CH
- 1	tntc				
- 2	82				
	91				
	84				
	87				
Average	**86**				
- 3	9				
	10				
	8				
	8				
- 4	1	tntc	tntc	tntc	tntc
	0				
	0				
	0				
- 5	0	343	236	233	189
	0	tntc	242	217	197
	0	tntc	239	214	203
	0	tntc	251	209	201
Average			**242**	**218.25**	**197.5**
- 6	0	34	23	20	18
	0	32	24	21	19
	0	31	23	23	20
	0	33	25	21	20
Average		**32.5**			
- 7	0	3	2	1	2
	0	2	2	1	2
	0	2	1	1	3
	0	1	1	2	2

Growth Experiment 6 , Time 24 hours.

	PEN	CONT	5CH	9CH	15CH
- 1	17				
	14				
Average	**15.5**				
- 2	1				
	1				
	1				
	1				
- 3	0				
	0				
	0				
	0				
- 4	0				tntc
	0				
	0				
	0				
- 5	0	tntc	tntc	tntc	289
	0				291
	0				281
	0				279
Average					**285**
- 6	0	35	35	31	27
	0	39	34	34	25
	0	34	31	32	29
	0	37	32	32	28
Average		**36.25**	**33**	**32.25**	
- 7	0	3	2	2	1
	0	1	2	2	1
	0	2	1	3	2
	0	2	2	3	1

Growth Experiment 7 , Time 48 hours.

	PEN	CONT	5CH	9CH	15CH
- 1	1				
	1				
- 2	0				
	0				
	0				
	0				
- 3	0				
	0				
	0				
	0				
- 4	0				
	0				
	0				
	0				
- 5	0	tntc	tntc	tntc	tntc
	0				
	0				
	0				
- 6	0	50	61	49	44
	0	55	60	41	43
	0	47	55	55	47
	0	51	49	47	41
Average		**50.75**	**56.25**	**48**	**43.75**
- 7	0	5	5	5	4
	0	5	6	4	4
	0	4	5	3	3
	0	5	6	4	2
- 8	0	2	2	1	1
	0	1	2	0	1
	0	0	0	0	0
	0	0	1	0	0

APPENDIX C

Table C.1 The Cell Numbers, Times, Growth Rates, and Specific Growth Rates of the Test and Control Cultures for Trial One.

TRIAL 1	CFU/ML	TIME (MIN)	GROWTH RATE	SPECIFIC G R
PENICILLIN	52500000	0	-127777.78	-0.0031165
	29500000	180	-143888.89	-0.0086942
	3600000	360	-18291.667	-0.0093623
	307500	540	1625	0.00358127
	600000	720	-707.29167	-0.0020479
	90750	1440		
CONTROL	34500000	0	1358333.33	0.0086656
	279000000	180	1991666.67	0.00434624
	637500000	360	-2758333.3	-0.0070863
	141000000	540	4244444.44	0.00811557
	905000000	720	-97222.222	-0.0001117
	835000000	1440	779513.889	0.00055829
	1957500000	2880		
5CH	40500000	0	394444.444	0.00519006
	111500000	180	1116666.67	0.0052673
	312500000	360	-176388.89	-0.0005947
	280750000	540	4023611.11	0.00625878
	1005000000	720	-402777.78	-0.0004683
	715000000	1440	125000	0.00015528
	895000000	2880		
9CH	62000000	0	619444.444	0.00526067
	173500000	180	1730555.56	0.00525605
	485000000	360	-1543055.6	-0.0044581
	207250000	540	1293055.56	0.00399554
	440000000	720	326388.889	0.00058545
	675000000	1440	314236.111	0.00034867
	1127500000	2880		
15CH	46500000	0	763888.889	0.0066281
	184000000	180	-238888.89	-0.0014701
	141000000	360	-229166.67	-0.0019038
	99750000	540	-20833.333	-0.0002129
	96000000	720	-84027.778	-0.001278
	35500000	1440	12326.3889	0.00027778
	53250000	2880		

Table C.2 The Cell Numbers, Time, Growth Rates, and Specific Growth Rates of the Test and Control Cultures of Trial Two at Various Times.

TRIAL 2	CFU/ML	TIME (MIN.)	GROWTH RATE	SPECIFIC G R
PENICILLIN	66500000	0	-316527.78	-0.0083269
	9525000	180	-47694.444	-0.009115
	940000	360	-3069.4444	-0.0046244
	387500	540	-2129.4444	-0.0108728
	4200	720	-5.8194444	-0.0027646
	10	1440	-0.0069444	-0.0013889
	0	2880		
CONTROL	70500000	0	937500	0.00605327
	239250000	180	1948611.11	0.0046997
	590000000	360	194444.444	0.00032007
	625000000	540	847222.222	0.00120816
	777500000	720	83333.3333	0.0001032
	837500000	1440	-151041.67	-0.0002073
	620000000	2880		
5CH	69500000	0	823611.111	0.00573446
	217750000	180	4456944.44	0.00720169
	1020000000	360	-722222.22	-0.0007563
	890000000	540	-500000	-0.0005917
	800000000	720	197916.667	0.00022716
	942500000	1440	-62500	-0.0000696
	852500000	2880		
9CH	76250000	0	598611.111	0.00460028
	184000000	180	2561111.11	0.0061788
	645000000	360	1444444.44	0.0018638
	905000000	540	-527777.78	-0.0006155
	810000000	720	243055.556	0.00027081
	985000000	1440	-163194.44	-0.0001881
	750000000	2880		
15CH	65250000	0	587500	0.00497354
	171000000	180	1466666.67	0.00484048
	435000000	360	-69444.444	-0.000162
	422500000	540	791666.667	0.00160338
	565000000	720	-369791.67	-0.0008562
	298750000	1440	16493.0556	0.00005309
	322500000	2880		

Table C.3 The Cell Numbers, Time, Growth Rates, and Specific Growth Rates of the Test and Control Cultures of Trial Three at Various Times.

TRIAL 3	CFU/ML	TIME (MIN.)	GROWTH RATE	SPECIFIC G R
PENICILLIN	35000000	0	-175833.33	-0.0091699
	3350000	180	-11625	-0.0050461
	1257500	360	-6080.5556	-0.0085611
	163000	540	-427.77778	-0.003436
	86000	720	-117.29167	-0.0026794
	1550	1440	-1.0069444	-0.0012205
	100	2880		
CONTROL	39750000	0	484722.222	0.00581376
	127000000	180	525000	0.00301291
	221500000	360	18055.5556	8.0921E-05
	224750000	540	556944.444	0.00202617
	325000000	720	52083.3333	0.00015152
	362500000	1440	100694.444	0.00023148
	507500000	2880		
5CH	35500000	0	554166.667	0.00649097
	135250000	180	538888.889	0.00293273
	232250000	360	-84722.222	-0.0003772
	217000000	540	138888.889	0.00060518
	242000000	720	122222.222	0.00042735
	330000000	1440	161458.333	0.00036181
	562500000	2880		
9CH	34750000	0	512500	0.00633694
	127000000	180	483333.333	0.0028348
	214000000	360	-12500	-0.0000587
	211750000	540	36111.1111	0.00016796
	218250000	720	144791.667	0.00053552
	322500000	1440	109375	0.00027259
	480000000	2880		
15CH	36000000	0	365277.778	0.00530349
	101750000	180	551388.889	0.00364254
	201000000	360	-165277.78	-0.000888
	171250000	540	145833.333	0.00079096
	197500000	720	121527.778	0.00050374
	285000000	1440	105902.778	0.00029316
	437500000	2880		

APPENDIX D

Table D.1 Table Comparing the Percentage Differences Between the Various Test Cultures and the Control Culture of Trial 1, as far as the Maximum Population and Specific Growth Rate, the Time Taken to Reach the Maximum Specific Growth Rate and the Differences in Enzyme Tests Observed.

TEST SUBSTANCE ADDED	PERCENTAGE DIFFERENCE BETWEEN THE TEST AND THE CONTROL		TIME TAKEN TO REACH THE MAX. SPECIFIC GROWTH RATE (hrs)	DIFFERENCE IN ENZYME TESTS OBSERVED (+ OR -)
	MAX. SPEC. GROWTH RATE	MAX. POPULATION		
PENICILLIN	-208.08%	- 97.31%	N/A	loss
5CH REMEDY	- 27.77%	- 48.65%	12	none
9CH REMEDY	- 39.29%	- 42.40%	3	none
15CH REMEDY	- 23.05%	- 90.60%	3	none
CONTROL	N/A	N/A	3	none

Table D.2 Table Comparing the Percentage Differences Between the Various Test Cultures and the Control Culture of Trial 2, as far as the Maximum Population and Specific Growth Rate, the Time Taken to Reach the Maximum Specific Growth Rate and the Differences in Enzyme Tests Observed.

TEST SUBSTANCE ADDED	PERCENTAGE DIFFERENCE BETWEEN TEST AND AND THE CONTROL		TIME TAKEN TO REACH THE MAX. SPECIFIC GROWTH RATE	DIFFERENCE IN ENZYME TESTS OBSERVED (+ OR -)
	MAX. SPEC. GROWTH RATE	MAX. POPULATION		
PENICILLIN	- 279.61%	- 92.05%	N/A	loss
5CH REMEDY	+ 18.96%	+ 21.79%	3	none
9CH REMEDY	+ 2.06%	+ 8.05%	6	none
15CH REMEDY	- 17.84%	- 32.53%	3	none
CONTROL	N/A	N/A	3	none

Table D.3 Table Comparing the Percentage Differences Between the Various Test Cultures and the Control Culture of Trial 3, as far as the Maximum Population and Specific Growth Rate, the Time Taken to Reach the Maximum Specific Growth Rate and the Differences in Enzyme Tests Observed.

TEST SUBSTANCE ADDED	PERCENTAGE DIFFERENCE BETWEEN TEST AND AND THE CONTROL		TIME TAKEN TO REACH THE MAX. SPECIFIC GROWTH RATE	DIFFERENCE IN ENZYME TESTS OBSERVED (+ OR -)
	MAX. SPEC. GROWTH RATE	MAX. POPULATION		
PENICILLIN	-257.73%	- 93.10%	N/A	loss
5CH REMEDY	+ 8.99%	+ 10.83%	3	none
9CH REMEDY	+ 8.99%	- 5.41%	3	none
15CH REMEDY	- 8.99%	- 13.79%	3	none
CONTROL	N/A	N/A	3	none