A TECHNIQUE FOR THE MASS CULTURE OF AQUATIC ACTINOMYCETES

APPROVED:

[Signatures]

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A TECHNIQUE FOR THE MASS CULTURE OF AQUATIC ACTINOMYCETES

THESIS

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MASTER OF SCIENCE

By

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CHAPTER I

INTRODUCTION

The problem of tastes and odors in water supplies is almost universal. In areas where natural waters are polluted, actinomycetes may also flourish, resulting in more than one variety of offensive odor producing compounds in the water supply. In the Southern climes one frequently encounters putrefactive tastes and odors, as well as those produced by the decay of underwater vegetation. These tastes and odors should certainly not be confused with those produced by the aquatic actinomycetes (10). Fishy, hay-like, marshy, musty, potato-bin, woody, and earthy odors are produced in sequence and can be detected during the life cycle of the aquatic actinomycetes.

The ability to mass culture aquatic actinomycetes under laboratory conditions will have practical value. Since the actinomycete spores are continuously available in the water, the organisms may be cultured from the water samples, and the taste and odor compounds collected. These compounds can be added to large volumes of water, and complete pilot plant tests can be run to determine the most efficient methods that a regular water plant can employ for reducing or removing the taste and odor compounds from the raw
water in order to furnish a palatable item to the water con-
sumer (14).

History

The investigation of the aquatic actinomycetes in this
country was first considered by McCoy and her students (15)
at the University of Wisconsin with particular reference to
members of the genus Micromonospora. No apparent observa-
tions were made concerning the tastes and odors produced by
the organisms (12). Additional references to these organ-
isms in the literature appear to be sparse (1, 3, 6, 9, 13,
16, 17).

The aquatic actinomycetes were first isolated from
impoundments serving as water supplies in the central area
of the State of Texas. Since those water supplies had
continuous taste and odor problems, and since the plank-
tonic algae were present in maximum concentrations of
140 areal units per milliliter, it appeared that the
source of the tastes and odors must be from organisms other
than algae. In 1948, the first group of these organisms
was isolated from Lake Waco (10).

Silvey, et al. found that in many instances where water
supplies contained taste and odor compounds, the isolated
cultures of actinomycetes, proved to be the causative
agents of tastes and odors. In most instances confirmation
was received indicating that the aquatic actinomycetes
were responsible for producing the woody, earthy, hay-like, marshy, musty, manurial, potato-bin, or bitter tastes and odors in the water supplies (10).

Thorough investigation of the morphology, taxonomy, and ecology of the aquatic actinomycetes was undertaken after the offensive tastes and odors found in many water supplies was attributed to these organisms. Silvey, et al. (11, 14) have completed research on the life history of the aquatic actinomycetes in which observations have been made that indicate an alternation in life history stages. Apparently, the aquatic forms are primarily aquatic because of the requirements of the primary hyphal stages. These actinomycetes, known as members of the *Waksmara*, initiate their aquatic life history from spores that arise either as pyrospores or moniliospores. The spores germinate in an aquatic environment into very small branching filaments about 0.5 μ. The filaments associate themselves with some source of nutrition. This source may be algae, higher plants, or organic matter in the shallow areas of streams, rivers, lakes, and reservoirs. It appears that the hyphae form in a fluid medium at variable depths, depending upon the nutrients, the concentration of oxygen, and the thermal conditions. In an absence of oxygen, the spores will not germinate. Temperatures below 7° C. inhibit development, while the optimum for most species is 25° C. Nitrogen and
potassium appear to be essential for the complete development of the primary stages (11). These primary filaments first produce fairly high concentrations of tastes and odors during their early stages of growth. Usually, during the early stages, fishy odors are produced from two to ten days, depending upon the temperature and the nutrient source. The primary hyphal filaments grow rapidly and simultaneously give rise to a strong grassy taste and odor in the raw water. This type of taste and odor will continue for two to ten days, again depending upon nutritional and thermal conditions. If proper nutrients are available for the actinomycetes, their metabolism alters and the taste and odor produced becomes marshy. This type of taste and odor production exists for approximately the same length of time as the fishy and grassy tastes and odors. If the actinomycetes continue to grow, potato-blin or slightly musty tastes and odors are produced (14). "If the primary stages of the actinomycetes have consumed the available nutrients in the period of time, they will die or give rise to small sex cells that unite to form a zygote or actinomycete 'seed' (14)." At this point the odor may disappear. However, if the source of nutrition is adequate, the primary filaments produce sex cells which develop into large secondary filaments about 0.6 to 1 μin diameter.
If the life cycle is to continue, a nutrient source such as a blue-green algae (*Polycystis*, *Aphanizomenon*, or *Anabaena*) must be available. These blue-greens are high in available nitrogen and also are subject to forming floating mats. As the nutrition of the blue-green algae becomes poor, the secondary hyphal filaments of the aquatic actinomycetes grow abundantly on the algal remains (14). At this time the formation of pyrospores or moniliospores begins on the hyphae of the actinomycetes.

A new group of taste and odor compounds are produced when the secondary filaments begin producing their various types of spores. A musty odor is usually the first odor to be produced, changing after a period of time to a woody or a woody-earthy mixture. This last sequence of tastes and odors is readily detectable, but very difficult to remove from raw water. When the secondary filaments have consumed all of the nutrition sources, the spores are freed into the water, the filaments die, and the odor disappears. If there are available sources of nutrients in the water to support primary hyphae, the pyrospores and/or moniliospores will germinate, and fishy odors will return. This life cycle of events commonly occurs in all surface waters throughout North America (14). The taste and odor sequences described in the life cycle of events may deviate from those mentioned, depending on the aquatic actinomycete species.
A review of available literature revealed some of the attempts that have been made to mass culture other actinomycetes. Howell and Pine (5) grew fifteen strains of terrestrial actinomycetes in 200 milliliter tubes on a synthetic medium containing starch. After inoculating the tubes from stock cultures, the tubes were sealed with rubber stoppers and incubated at 37°C on a rotary shaker set at approximately 228 cycles per minute. Gottlieb and Legator (4) used a similar liquid shake culture to investigate the growth and metabolic behavior of *Streptomyces venezuelae*. As the spores of this organism germinate and give rise to hyphae which first form a loose growth and then a more compact mat, the nitrate ion and carbon source are removed from the medium. Ammonia is produced during the growth stage and the pH of the cultural medium increased. Perlman, et al. (8) successfully grew actinomycetes in cotton plugged 500 milliliter Erlenmeyer flasks containing 100 milliliters of synthetic medium. These flasks were inoculated from stock cultures and placed on a reciprocating shaker at 120 two inch cycles per minute located in a 25°C constant temperature room.

Dill (2) reared batch cultures of *Waksma* sp. on Modified Czapek's Medium. The containers used for growing the organisms were one-gallon duraglass jugs. After these containers were cleaned, they were fitted with cotton
stoppers and sterilized at fifteen pounds steam pressure for a period of thirty minutes. Two liters of modified Czapek's media and an additional 100 milliliters of hot agar solution were added to each of the ten jugs. The pH of the media in each jug was adjusted to seven with 0.01 N sodium hydroxide and the cotton plugs replaced. The jugs containing the media were resterilized at the same pressure for a period of fifteen minutes. The containers with medium were inoculated from pure cultures and placed in an incubation room where the temperature was approximately 25° C, during the entire growth period. After growth became evident, usually in three to four days, the cultures were shaken at forty-eight hour intervals in order to prevent pellicle formation and to agitate the media. The growth period was approximately eighteen days, depending upon the temperature. When the growth of *Wakamara* sp. appeared to have advanced to the maximum by-product formation, the medium containing the organisms and their metabolic products was subjected to distillation in order to recover the chemicals produced during the growth period (2).

McCormick (7) used much the same techniques for mass culturing the aquatic actinomycetes. One-gallon duraglass jugs, containing two liters of modified Czapek's medium, were used to grow large quantities of the actinomycetes.
After inoculating with five cubic centimeters of a broth culture of the Waco strain of actinomycete, the jugs were left at room temperature for thirty to sixty days in order to attain maximum growth. After the incubation period the broth was separated from the mycelium by filtering and the broth containing the actinomycete by-products was then subjected to various chemical tests to ascertain the chemical nature of the taste and odor producing compounds. Some of the chemical tests used were distillation, ether extraction, and carbon adsorption (7).

Statement of Problem

The purpose of the problem was to develop a laboratory technique for mass culturing of the aquatic actinomycetes. In order to solve the problem, it was necessary to devise a suitable culture chamber that would nurture the various species in both primary and secondary stages. It was also important to provide an adequate medium for growth of these organisms. Finally, the construction of the culture chambers must provide for a continuous exhaust of the taste and odor compounds so that the production of these components could be correlated with growth stages.
CHAPTER I BIBLIOGRAPHY


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CHAPTER II
METHODS

The early methods for the mass culturing of the aquatic actinomycetes, as described in Chapter I, were not entirely successful for the production of the evasive taste and odor by-products. An evaluation of the various early methods indicated that a successful technique for the mass culturing of the aquatic actinomycetes should include these essentials:

1. Precautions must be taken to prevent bacterial contamination. A technique must be devised for the sterilization of the culture chamber as well as its contents. Provisions must be made for the maintenance of sterile conditions within the culture chamber at all times.

2. An air source is necessary for the growth of the aquatic actinomycetes. The air must be sterilized in order to prevent bacterial contamination in the mass culture chamber.

3. Constant temperature conditions should be maintained within the mass culture chambers. The optimum aquatic actinomycete growth is attained in the temperature range from 25°C to 27°C; therefore, some means must be provided for maintaining the desired temperature within the culture chambers.
4. Nutrients must be available for the growth of the aquatic actinomycetes. A culture medium must be formulated which is adequate for the growth of the aquatic actinomycetes, in both the primary and secondary stages. Provisions must be made for sterilizing and introducing the culture medium into the mass culture chambers.

5. Substrates must be used to provide a surface area for the aquatic actinomycete growth. Substrates are not needed during the primary stages of aquatic actinomycete growth; instead, the primary hyphae can grow in a fluid medium. In order for the aquatic actinomycete to complete its life cycle some material must serve as a substrate for the secondary hyphal growth.

6. A method should be developed for spore suspension preparation from laboratory stock cultures of various strains of the aquatic actinomycetes. A technique must be evolved for the inoculation of the mass culture medium with the aquatic actinomycete spore suspensions.

7. Arrangements must be made for taste and odor detection and collection from the mass culture chambers. The tastes and odors produced by the aquatic actinomycetes must be collected for later biochemical studies.

8. Copious taste and odor compounds must be produced by the aquatic actinomycete mass culturing technique. Adequate taste and odor compounds must be available if
successful biochemical investigation is to be completed on the organisms.

A glass column was constructed to meet these requirements for a mass culture chamber.

Hollow Core Vertical Column

**Column Construction**

Seven columns of the type shown in Figure 1 were constructed. These columns were of Pyrex glass tubing, 122 centimeters in length with a diameter of thirty millimeters. A hollow center core of Pyrex glass tubing was used in the column. The hollow core was 135 centimeters in length with a diameter of fifteen millimeters. The columns were fitted with rubber stoppers and assembled on plexi-frame units in a vertical position. Pyrex glass tubing, six millimeters in diameter, served as inlets and outlets to the vertical column. The total assembly is illustrated in Figure 1. At the lower end of the vertical column, Item A served as a sterile air inlet and Item C as a medium inlet and outlet. At the upper end of the vertical, Item F, a rubber-capped tube was used as the injection point for aquatic actinomycete spore suspensions. Item G served as the air outlet. The hollow core (See Figure 1, Item E) was arranged so that water could be circulated through it, resulting in constant thermal conditions of 25° C. to 27° C.
FIGURE 1 - HOLLOW CORE VERTICAL COLUMN
In order to sterilize the column, steam was passed through the center core for one hour each day for three days.

In order to provide substrates for the actinomycetes growth, various materials were placed in the columns. Three of the columns contained various sizes of Beryl ceramic saddles as a substrate for the growth of the colonies of the aquatic actinomycetes. Two columns contained glass helicelles, while two others contained glass wool. Different strains of actinomycetes require different types of substrate on which to grow. All of these materials were sterilized in a hot air oven for forty-eight hours at a temperature of 160°C, prior to being placed within the columns.

Sterile air for the columns was obtained from a ceramic heating chamber (See Figure 2). The sterile air was continuously pumped through copper tubing into the columns to provide an oxygen source. The sterile air from each column was exhausted through a small Pyrex glass tube into an individual scrub bottle. The scrub bottles were filled with water or wet granular activated carbon to obtain the tastes and odors resulting from the aquatic actinomycete metabolic by-products.

The vertical columns, containing the various substrates, were sterilized prior to the addition of the medium. Sterilization methods, such as filling the columns
FIGURE 2 - HEATING UNIT FOR AIR STERILIZATION
with alcohol solutions and passing steam through the center core, were used for periods of one hour each day for three days to insure sterile conditions.

**Culture Medium**

The columns were filled with Czapek's modified broth (Appendix) to provide sufficient nutrients for mass culturing of the aquatic actinomycetes. Culture medium was prepared for introduction into the vertical column by autoclaving several two liter containers of the medium for thirty minutes at 121° F., fifteen to seventeen pounds pressure. Prior to autoclaving, the two liter containers of medium were fitted with rubber stoppers containing two Pyrex glass tubes. One of the glass tubes reached to the bottom of the two-liter container to serve as a flow tube when connected by Tygon tubing to the vertical column; the second glass tube served as an air inlet. The Tygon tubing connected to the flow tube of the two-liter container was sealed during the autoclaving period. The air inlet tube in the two-liter container was plugged with cotton saturated with alcohol. At the completion of the autoclaving period the medium containers were placed on a plexiframe stand above the top level of the columns. The Tygon tubing, attached to the flow tube in the two-liter container, was connected to the medium inlet at the bottom of the vertical column. The cotton plug was removed from the air inlet
tube in the two-liter container and a sterile air source was connected at this point. The sterile air entering the two liter container through the air inlet tube forced the culture medium through the flow tube into the vertical column. A method for replacing medium was needed because of evaporation and exhaustion of nutrients; therefore, by leaving the connections attached to the sterile container of medium, a quantity of medium could be added, other than the initial 1500 milliliters used to fill the column. After filling the columns with medium, steam was passed through the center core for one hour each day for three days. This series of heating periods was employed to further insure sterile conditions.

**Spore Preparation and Inoculation**

Inoculum cultures were prepared from North Texas State College stock cultures by introducing twenty cubic centimeters of Emerson's modified broth (Appendix) into Emerson's modified agar (Appendix) slants covered with actinomycete growth. The test tube, containing the broth and actinomycete growth, was agitated until the spores were freed into the broth. A sterile twenty-cubic centimeter syringe and long needle were used to remove the broth spore suspension from the agar tube. The same needle and syringe were employed to inject the spore suspension into the columns through a rubber-capped injection tube (See Figure 1).
Solid Core Vertical Column

Evaluation of the Hollow Core Vertical Columns revealed a lack of sterilization in the culture chambers and failure to maintain sterile conditions during the introduction of medium into the columns; these discrepancies led to the design of a Solid Core Vertical Column.

**Column Construction**

Seven columns of the type illustrated in Figure 3 were constructed. These columns were Pyrex glass tubing, 122 centimeters in length with a diameter of sixty millimeters. A solid glass center core of Pyrex was used in the columns. The solid core was 135 centimeters in length with a diameter of ten millimeters. The rubber stoppers on each end of the columns were fitted with metal clamps which were tightened to insure complete sealing of the chambers.

The columns were mounted on plexiframe units in a vertical position. The completed assembly including air inlets and outlets, medium inlets and outlets, and the injection tube can be seen in Figure 3. The purposes of the inlets and outlets in this type of column were the same as those explained in the Hollow Core Vertical Column.

The solid center core (See Figure 3) was wrapped with asbestos cord. Coils of chromel-resistance wire were wound around the asbestos-wrapped center core. The turns of wire were spaced about one inch apart. The wire was connected
FIGURE 3 - SOLID CORE VERTICAL COLUMN
at each end of the core to fourteen gauge copper wire. Using the copper wire as electrodes, current was passed through the chromel-resistance wire to create heat for column sterilization.

In order to provide substrates for actinomycete growth, two of the columns were filled with various sizes of Beryl ceramic saddles and two columns with glass helicals. In three columns the asbestos core was utilized as the substrate. The various substrates were used to provide the required surface area for the secondary hyphal growth of the aquatic actinomycetes. The substrates were sterilized in the same manner as described in the Hollow Core Vertical Column method.

The sterile air for these columns was obtained from a ceramic heating chamber (See Figure 2) and connected to the columns in the same manner as described in the Hollow Core Vertical Column method. Constant temperature for the cultures were maintained in the laboratory by room air conditioning, rather than attempting to maintain a certain temperature within each column, as described in the Hollow Core Vertical Column method.

Odor detection and collection were accomplished in the same manner described in the Hollow Core Vertical Column method. Wet granular activated carbon, wet powdered carbon,
and water in which the pH was varied from 3.5 to 10.5, were used as odor collection agents in the scrub bottles.

In order to sterilize the columns, 1500 milliliters of Denton tap water were boiled in the columns for thirty minutes each day for three days. At the termination of the sterilization periods, approximately 1000 milliliters of sterile water remained in the columns.

**Culture Medium**

In these Solid Core Vertical Columns various media were tested in an effort to provide sufficient nutrients for the aquatic actinomycetes. Media (Appendix) used in this method were nutrient broth, Emerson's modified broth, Czapek's modified broth, Emerson's-Czapek's modified broth, and a peptone-protease broth. The ingredients of these media were dissolved in 500 milliliters of Denton tap water. In order to sterilize the medium concentrate, it was autoclaved for fifteen minutes at 121°F, fifteen to seventeen pounds of pressure.

As previously mentioned, at the termination of the third sterilization period approximately 1000 milliliters of sterile water remained in the columns. As the columns cooled, the 500 milliliters of sterile medium concentrate were connected, by means of glass tubing, to the medium inlet on the column. The medium concentrate was drawn inside the columns due to a difference in pressure. The mixing
of medium concentrate with the sterile water in the columns resulted in the proper concentration of required nutrients. The 1500 milliliters of medium were sterilized in the columns by boiling for fifteen minutes each day for three days. Unlike the Hollow Core Vertical Column method, no arrangement was made for the addition of medium after the initial filling of the columns.

**Spore Preparation and Inoculation**

The same technique of spore suspension preparation used in the Hollow Core Vertical Columns was employed in the Solid Core Vertical Columns. An additional technique, consisting of the injection of spore suspensions from a twelve inch test tube containing aquatic actinomycetes growing in Emerson's modified broth (Appendix), was also tested. The twelve-inch test tubes (See Figure 4) were half filled with sterile Beryl ceramic saddles, which served as substrates, and 150 milliliters of Emerson's modified broth (Appendix). The isolation tubes (See Figure 4) were fitted with rubber stoppers through which three Pyrex glass tubes were inserted. Two glass tubes, Items A and C, served as the air inlet and outlet, respectively; the third glass tube, Item B, was rubber-capped and used as an injection point for the aquatic actinomycete spores. The air inlet and outlet tubes were plugged with cotton saturated in alcohol; then, the assembled isolation
FIGURE 4 - ISOLATION TUBE
tubes were autoclaved for thirty minutes at 121° F., fifteen to seventeen pounds pressure. Upon completion of the autoclaving period the isolation tubes were mounted in a vertical position on a plexiframe unit. The air inlet was connected to a sterile air source and the air outlet to a small scrub bottle. Ten cubic centimeters of an aquatic actinomycete spore suspension were injected at the rubber-capped injection point into the isolation tube. The actinomycete growth became evident in three to four days and was allowed to continue for approximately eighteen days, or until heavy pellicles were formed on the surface of the substrates. At the end of this period the tube was disconnected from the sterile air source and shaken to free spores into the medium. A twenty cubic centimeter sterile syringe and long needle were used to withdraw the spore suspensions from the isolation tubes and to inject the spore suspensions into the Solid Core Vertical Column.

**Solid Core Horizontal Column**

It was noted that exposure of aquatic actinomycete mats above the surface of the medium, in the Solid Core Vertical Columns, resulted in a greater concentration of by-product odors; a new type of column was constructed to take advantage of this observation. Other changes were incorporated to overcome the excessive media odors and contamination encountered in the Solid Core Vertical Columns.
Column Construction

In the Solid Core Horizontal Column method, seven columns were constructed of the same dimensions and the same materials as the Solid Core Vertical Column method. A difference in these columns, illustrated in Figure 5, was in the positioning of the inlets, outlets, injection points, and asbestos core. These new columns were mounted horizontally on plexiframe units, rather than vertically as in the two previous types of columns. The sterile air source (See Figure 2), the technique of taste and odor collection and detection, and the substrates were identical to those used in the Solid Core Vertical Column method.

Prior to mounting the horizontal columns, an electric heating tape was wound around the outside of the columns. A temperature of 160° C. was maintained inside the columns for a minimum of eighteen hours. The dry sterilization period was followed by wet sterilization periods as described in the Solid Core Vertical Column method. Eight hundred milliliters of Denton tap water were boiled during the wet sterilization periods. At the termination of the third boiling period, 400 milliliters of sterile water remained.

Constant temperature conditions for the cultures were maintained in the laboratory by room air conditioning, as described in the Solid Core Vertical Column method.
FIGURE 5 - SOLID CORE HORIZONTAL COLUMN

COPPER CONNECTORS MEDIUM LEVEL

SOLID GLASS ROD COVERED WITH ASBESTOS
AND CHROMEL WIRE HEATING ELEMENT.

DEPRESSION POINT DRAIN WITH SCREW CLAMP.

WATER BOTTLE

AIR INLET

INJECTION POINT

PYREX GLASS CULTURE CHAMBER

INJECTION POINT

UPPER MEDIUM OUTLET OR MEDIUM INLET

AIR INLET

FLOW GAGE
additional medium into the columns at the infection points.

sterile and twenty-four hour needled were used to inject the

sterile medium was added when required by the experiment.

day for three days to insure sterility. Add another

hult tillled the columns, were posted for fourteen minutes each

into the columns. The 600 miliometers of medium, which

column method were used to introduce the medium concentrate.

The same technique described in the Solid Core Vertical

the columns at the termination of the third planting period,

were added to the 400 miliometers of sterile water remain in

The 500 miliometers of sterile medium concentration were

preparad as described in the Solid Core Vertical Columns.

five hundred miliometers of medium concentrate were

modified agar (Appendix) was also tested.

Column method were tested. A solid medium, mediumless,

were used in the Solid Core Vertical

of medium, the same types used in the Solid Core Vertical

of an ordinary nitrogen source. In addition to the new type

was created. The basis for this medium was the elimination

Solid Core Vertical Columns, a new type of medium (Appendix)

a mixture of mesquite and actinomyces product dyes in the

In order to overcome the nitrogen media dyes that caused

Culture Medium
better coverage of the medium surface area.

Two injection ports in the portonatal column inserted. The use of rubber-capped injection ports (see figure 1) allowed the spore suspensions to be injected into the column at the two move the broth spore suspensions from the aer tube. The twenty centimeters above and below needed to re-attached until spores were freed into the broth. A sterile tube containing the broth and the accepted growth was placed covered with autoclave cover. The test specimen is inoculated into the appropriate medium as described earlier. The inoculation cultures were prepared by introducing twenty

Spore Preparation and inoculation
were produced they were swept out by air currents and
were produced by the actinomycetes. As these by-products
influenced small amounts of the odours were detected that
and detected with spore suspensions, in three of these
beings spouted were drained, sterilised, filled with medium,
formed the perforated columns. In each case the columns
were made to produce odors in the seven
of the columns were found.

From the column were extracted an actinomycete and bacteria
preparative odor was produced. The laboratory samples drained
were extracted by those and a transparent-like or 
within days after inoculation the fluid in the columns be-
formed the perforated columns. Microscopic examination of fluid drained
the substrates. Microscopic examination of fluid drained
weened, was visible on the sides of the columns and on

The submerged growth, which was composed of primary
after infection evidence of actinomycete growth appeared.
seven hollow core perforated columns, three to five days
apparatus and the spore suspensions of actinomycetes, actinomycetes

Hollow Core Perforated Column

RESULTS

CHAPTER III
At 48-hour exposure above the medium, a greater concentration of
exposed above the surface of the medium. As the mats
attained maturity after 72 hours, these mats were partially
permeated by the mats to maturity. The mats, it was observed,
and finally spores, the liquid in the columns was lowered to
as the organisms began to produce their mature hyphae

Once more, more spores were mixed or malted with mature oocytes.

In most instances the spores were detected at this time. In most instances, these
spores and mature oocytes were spores that had been on the spore
of the medium and around the position of the mature oocytes,
which formed by the mature hyphae were observed on the surface,
strates and mature spores. Spots to 20 cm after inoculation,
were composed of mature mycelium, were visible on the sub-
spore suspension of mature mycelium, which
in 3 to 5 days after inoculation, evidence of
were injected into the columns

Wheat, wheat, and barley, both of which
solid, positive vertetal column

three columns became bacterially contaminated.
verted carbon. Six to eight days after inoculation these
collected in screw bottles filled with wet granular agar

31
Solid Core Horizontal Column

Forty mass culture attempts were conducted in the Solid Core Horizontal Columns. In every instance substantial growth was achieved.

In columns containing medium composed of peptone, beef extract, or other types of organic nitrogen compounds, odor production was limited. The mixing or masking of actinomycete odors by the odorous materials in these media, prevented the identification of the taste and odor compounds. A new medium, containing varying concentrations of dark brown sugar, potassium chloride, ammonium nitrate, and Denton tap water, was used to achieve successful odor production in fourteen tests. The new medium was formulated after it was observed that primary mycelium would grow in a medium containing inorganic constituents only, while the secondary mycelium would not. Investigation revealed that an organic nitrogen source, such as Emerson's modified agar (Appendix), eliminated the need for using a medium which contained the ingredient in the culture column.

The mass culturing of four strains of aquatic actinomycetes was achieved in the Solid Core Horizontal Columns. The primary and secondary stages of their growth and the sequence of their by-product odor production were observed.
A few hours after inoculation of *Waksamara concurrens*,
the primary mycelia began their growth as small white masses
in the lower fluid layer. Incidental with the beginning
growth, slight fishy odors were detected in the exhausted
air. Apparently, this was the only odor produced during
the primary stage of this species' growth. Secondary growth
appeared in the form of pasty colonies on the asbestos
covered core and a faint marshy odor was observed. This
odor continued for approximately twenty-four hours; at
the termination of that period a musty odor appeared and
masked whatever marshy odor was present. Approximately
ninety-six hours after inoculation the secondary actinomycete
growth became somewhat powdery and woody-earthy
odors appeared. These odors predominated throughout the
growth of the secondary mycelia. It was observed that the
duration of the life-span of the secondary stage depended,
as in many microorganisms, on volume, space, the amount of
nutrient, temperature, and general physical and chemical
conditions. Good concentrations of by-product odors were
obtained for a period of three months. Additional nutrients
added to the columns from time to time expedited the output
of odors for two to four weeks. At the termination of three
months, regardless of added nutrients, the mats had so com-
pletely covered the core of the columns that subsequent
growth did not appear.
The primary stages of *Waksamara glaucopprimenda* were observed a few hours after injection of the horizontal columns. Slight fishy odors were detected at this stage of growth, changing to a hay-like odor in a few hours. Marshy odors were detected during the development of the secondary mycelial stage. It is possible that a marshy odor was not the terminal type of by-product; occasionally, there were indications of woody odors. Earthy odors did not appear at any time. The life-span and production of by-product odors is similar to those observed in *Waksamara concurrens*.

After spore injection of *Waksamara brevipyrosporulata*, primary mycelia developed in small cottony masses and produced hay-like and potato-bin odors for twenty-four to thirty-six hours. In the beginning of secondary mycelia development, woody odors were produced. When heavy sporulation occurred, the woody odor became less evident and an earthy odor predominated. The growth period was approximately four months. It appeared that maximum odor production occurred for approximately two months.

*Waksamara pyrospora* was the fourth species of aquatic actinomycete investigated. The primary mycelia formed rapidly and were easily observed because of their yellowish coloration. They produced strong hay-like odor that persisted for twenty-four to thirty-six hours. Colonies of secondary mycelia appeared on the asbestos core;
eventually, they formed a complete cover on the emergent side of the asbestos core. In the early stages of secondary mycelia growth, the odor was marshy; ultimately, a musty odor with a slight earthy taint was produced. Woody odors were not observed.
CHAPTER IV

DISCUSSION

Eight essentials of a successful mass culturing technique for aquatic actinomycetes were outlined in the early stages of this study. In order to evolve a technique encompassing these essentials, three methods were tested.

The mass culturing of the aquatic actinomycetes by the Hollow Core Vertical Column method was unsuccessful because of the high percentage of contamination. The cause of contamination could not be determined; however, the lack of heating around the top stoppers of the columns and the mode of siphoning medium into the columns were the probable causative factors. A Solid Core Vertical Column was devised in an attempt to overcome the defects of the Hollow Core Vertical Column.

In the Solid Core Vertical Columns contamination was reduced; however, an undesirable degree of contamination was still encountered. Apparently, the area at the upper end of the Solid Core Vertical Columns did not receive enough heat to insure sterilization. Contamination during the introduction of medium into the columns, as experienced in the Hollow Core Vertical Column method, was eliminated
by the technique used in the Solid Core Vertical Column method. Apparently, the aquatic actinomycetes by-product odors were being produced in the Solid Core Vertical Column, but these tastes and odors were mixed or masked with high media odors to such a degree that they could not be detected. The mixing or masking of the by-product tastes and odors was attributed to the types of medium being used and the small amount of exposed medium surface area.

The Solid Core Vertical Columns were useful in the investigation of the primary stages of the aquatic actinomycete growth; however, investigation of the secondary stages was hindered. The secondary mats did not develop completely when they were submerged as they require either greater quantities of oxygen or lesser quantities of water than were provided in a vertically mounted column. In fresh water environments the aquatic actinomycetes are observed to attach themselves to floating mats of algae or aquatic vegetation that is partly emergent; since this condition could not be obtained in the vertical columns, taste and odor production by the aquatic actinomycetes was limited.

The Solid Core Horizontal Column met the requirements proposed for a mass culturing technique for the aquatic actinomycete. In these columns contamination was eliminated
by the combination of a dry sterilization period and wet sterilization periods. Successful taste and odor production was achieved in the Solid Core Horizontal Column as a result of the larger medium surface area offered for secondary mycelial growth. Apparently, the partial submergence of the secondary mycelia in the vertical solid core and hollow core columns was a major growth and odor inhibiting factor. The aquatic actinomycete mats attained maturity earlier when partially exposed above the surface of the medium; therefore, the horizontal mounting of the culture chamber proved vastly superior to vertical mounting.

Summary and Conclusions

A laboratory technique for the mass culturing of the aquatic actinomycetes was developed. A glass, horizontally mounted culture chamber was used successfully to nurture four strains of aquatic actinomycetes, Waksmara concurrens, Waksmara brevipyrosporulata, Waksmara glaucopprimenda, and Waksmara pyrospora. The primary and secondary stages of their development and the sequence of their growth and by-product odor production were observed.

Temperature, nutrition, and many other factors are important environmental conditions which contribute to the growth of the aquatic actinomycetes. These factors may be controlled in the laboratory by using a Solid Core Horizontal Column as a culture chamber. This chamber will
probably prove to have practical value since it is possible to get samples from a surface water supply at a time when no tastes and odors are present. The actinomycete spores are continuously available in the water; therefore, the organisms may be cultured from the water samples, grown in the columns, and the taste and odor compounds collected. These compounds can be added to large volumes of water and complete pilot plant tests can be used to determine the most efficient methods for removing the taste and odor compounds from the raw water in order to furnish a palatable item to the water consumer.
## APPENDIX

### Czapek's Modified Broth
- Dark brown sugar... . .90 gms.
- Sodium nitrate... . .6 gms.
- Potassium phosphate... . 3 gms.
- Sodium chloride... . .3 gms.
- Peptone... . . . . . 3 gms.
- Tyrosine... . . . . .0.15 gms.
- Microelements solution... .3 gms.
- (Contains zinc, iron, and copper. Total concentration of 10-15 milligrams per liter.)
- Denton tap water... . 3000 gms.

### Emerson's Modified Broth
- Dark brown sugar... .80 gms.
- Peptone... . .8 gms.
- Beef extract... . .4 gms.
- Yeast extract... . .2 gms.
- Sodium chloride... . .3 gms.
- Denton tap water...1000 gms.

By adding 20 grams of agar-agar to the ingredients listed above, Emerson's Modified Agar can be prepared.

### Emerson-Czapek Modified Broth
- Dark brown sugar... .90 gms.
- Sodium nitrate... . .6 gms.
- Potassium phosphate... . 3 gms.
- Sodium chloride... . .3 gms.
- Peptone... . . . . . 3 gms.
- Tyrosine... . . . . .0.15 gms.
- Beef extract... . .12 gms.
- Yeast extract... . .3 gms.
- Microelements solution... .3 mls.
- Denton tap water... .3000 mls.

### Peptone-Protease Broth
- Dark brown sugar... .10 gms.
- Peptone-protease... .10 gms.
- Sodium chloride... . .3 gms.
- Denton tap water...1000 mls.

### New Nutrient Medium
- Dark brown sugar... .80 gms.
- Ammonium nitrate... . .4 gms.
- Potassium chloride... . .2 gms.
- Denton tap water... .1000 mls.

### Nutrient Broth
- Peptone... . . . . .3 gms.
- Beef extract... . .5 gms.
- Denton tap water...1000 mls.
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