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BIOLOGICAL CONTROL OF OLIVE GREEN MOLD IN THE CULTIVATION OF *AGARICUS BISPORUS*

by

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ABSTRACT

The Canadian mushroom industry is experiencing the same problems as are encountered worldwide - attacks of diseases and pests (Ingratta, 1980). Successful methods to control the damaging weed mold - *Chaetomium olivaceum* (Olive green mold) in mushroom beds are presently not known. This thesis investigation attempted to control *C. olivaceum* by biological means. A thermophilic Bacillus sp (resembling *B. coagulans* - resistant to 0.02% sodium azide, acidophilic) which showed dramatic activity against *C. olivaceum* on TSY (Trypticase soy agar + 0.4% Yeast extract) agar plates was isolated from commercial mushroom compost (phase I). When inoculated into conventional and hydroponic mushroom beds, the Bacillus not only provided a significant degree of protection from *C. olivaceum* but also increased yields of *Agaricus bisporus*. This is the first isolation of a microorganism inhibitory to Olive green mold.

The Bacillus was shown to produce an extremely potent and stable antibiotic (named Chaetomacin) effective over a wide range of both pH (2-10) and temperature (-15°C to 150°C). Chaetomacin is soluble in most polar solvents and insoluble in non-polar solvents. This antibiotic produced at mesophilic temperatures is also active against other Bacillus species and various eukaryotes - but demonstrates no activity against Gram negative organisms or Gram positive cocci. Final purification of Chaetomacin was accomplished through thin layer chromatography on

Silica gel analytical plates. Amino acid analysis revealed the antibiotic to be a peptide, acidic in nature. Examination of the literature reveals no other previously isolated antibiotics which are identical to Chaetomacin.

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INTRODUCTION

The cultivation of the common white mushroom, *Agaricus bisporus* Lange (*Agaricus brunnescens*) has expanded tremendously in recent years. The British Columbia Ministry of Agriculture (1981) states that 8820 tons of *A. bisporus* were produced within this province in 1981 (representing approximately a 300 ton increase over 1980). Mushrooms continue to rank second in economic importance as a vegetable crop in B.C. (third overall in Canada). Furthermore, British Columbians lead the continent (and possibly the world) in mushroom consumption per capita, consuming twice that of fellow Canadians and three times as much as U.S. consumers (Anon., 1981; Potter, 1980). It is likely that the mushroom industry will become of greater importance in the production of enzymes, antitumor compounds, and antibiotics, as well as food and feed (Kurtzman, 1979). However, irrespective of public demand and food values, the future prospects for continued expansion and mass production largely concerns the economics of production methods (Hayes & Nair, 1975).

Traditionally accepted as a horticultural crop, mushroom production is in principle a fermentation process (Hayes, 1974; Kurtzman, 1979). Presently mushroom culture represents the only major process in biotechnology which successfully converts cellulosics into useful foods and byproducts. One cycle consists of distinct procedures: namely substrate preparation, inoculation, incubation,

growth, and terminal disinfection; all of which can be identified with standard laboratory methodology in microbiology and in the methods employed in the fermentation industry (Hayes, 1974; Hayes & Wright, 1979). The principles involved in cultivation are common to other industrial processes involving microorganisms, the guarantee of purity of culture which in turn provides for the guarantee of edibility - is the basis on which the industry was founded.

The methods used in this solid-state fermentation have not changed significantly over the years. The main substrate which supports mushroom growth continues to consist of animal manures, plant materials, chemical fertilizers, and other agricultural residues. It must be kept in mind that mushroom compost is a rich nutrient substrate in which there is an association of competitive microorganisms distinguished by differences in physical requirements, products of metabolism, and various nutritional requirements (Lambert, 1938). Moreover, with the high labor input and the pure culture essentiality, the profit margin leaves little to offset crop error and failure due to diseases and pests.

Fungi which grow in competition with the mushroom mycelium are referred to as weed molds and are considered serious disease causing agents (Lambert & Ayers, 1953). One such organism is *Chaetomium olivaceum* more commonly known as Olive green mold. Olive green mold is a weed mold which frequently occurs after pasteurization of the compost and severely inhibits mushroom mycelial development by competing for nutrients (Beach, 1937). Consequently, mushroom

yields may be completely destroyed. One reason for the invasion of *C. olivaceum* into mushroom beds is the presence of free ammonia in the compost after the phase II pasteurization (Rettew, 1948). Insufficient oxygen during phase II leads to anaerobic decomposition of the compost. This produces compounds toxic to mushrooms while encouraging colonization of the compost by Olive green mold (Nair, 1980). As of yet, there are no known methods to successfully control this pest. (Vedder, 1978).

Economic yields require that a grower use the best techniques pertaining to fungal physiology and disease protection. From earlier research (Huhnke, 1970; Nair & Fahy, 1972; Stanek & Rysava-Zatecka, 1970; Townsley, 1974), it was suggested that a certain degree of protection from invasion of the mushroom compost by disease causing organisms may be obtained by prior fermentation with selected microorganisms. Huhnke (1970) states that, by inoculating specific thermophilic microorganisms into sterilized compost the cultures are capable of causing a selective protection of the substrate against diseases and pests. Hence the objectives of this thesis investigation were to select specific thermophilic microorganisms which would not only support the mushroom, but protect it from damage from Olive green mold. Furthermore, it was hoped that this thermophile(s) could be used in the preparation of a hydroponic system utilizing synthetic substrates (thereby eliminating the potential hazards of compost) as a growth medium for *Agaricus bisporus*. If successful, the results of this research should facilitate the emergence of the mushroom industry into a level of technology experienced in modern industrial fermentations.

LITERATURE REVIEW

A. CURRENT METHODS OF MUSHROOM PRODUCTION

1. Phase I

Commercial mushroom production always starts with the production of a suitable mushroom compost (Hatch & Finger, 1979; Kurtzman, 1979; Royse & Schisler, 1980). The substrate materials usually consist of manure, straw, corn cobs, organic nitrogen, phosphates, gypsum and other agricultural residues. A wide range of animal manures are used, including chicken, duck, pig, sheep, goat, yak, buffalo, mule, and elephant (Hayes, 1974). Tree bark and municipal refuse (Block, 1964; Chang, 1980b) can also be utilized to a limited extent as substrate as well. There is no standard pattern in compost formulation - it is based mainly on the availability and price of the raw materials and supplements in the particular growing region (Kinrus, 1974). The raw materials must be subjected to a composting process since no way has yet been found to prepare an uncomposted medium capable of large scale production where fast growing contaminants have access to them (Royse & Schisler, 1980). The nature of the substrate and its preparation, more than all other aspects of growing, dictates the method by which mushrooms are grown. Virtually all of the mushroom compost used in British Columbia is prepared in bulk by the Fraser Valley Mushroom Growers Association (B.C. Ministry of Agric., 1980).

Composting in its broadest defintion may be defined as "incomplete

microbial degradation of organic wastes" (Muller, 1965). Composting is a dynamic bacteriological process in which the organic raw components are progressively broken down and transferred in a series of biological and chemical changes by thermophilic microorganisms to a form which can be utilized readily by the mushroom mycelium (Burden & Peterson, 1972; Huhnke, 1970). The repression and elimination of parasites and pathogenic competitor organisms of the mushroom take place at the same time (Fordyce, 1970). When properly prepared it (compost) supplies all the organic, mineral, and moisture requirements needed for the production of a satisfactory crop. The essential feature of the system is to subject the manure to an aerobic thermophilic fermentation until it is well decomposed. A raw compost will still be an excellent medium for many of the micro-organisms active or present during the phase I and which may be later antagonistic towards the mushroom (Muller, 1965)

Modern techniques of composting are modelled on systems devised by Sinden & Hauser (1953). In this method, there are two main phases: an outdoor composting/fermentation (phase I), followed by an indoor controlled pasteurization (phase II). During the initial stages of composting, mesophilic decomposition is quite rapid, causing temp-eratures to rise to the thermophilic region (70-80°C) (Smith, 1969) and stay there for an extended period (3-4d). Easily transformed materials such as sugars and starch (Hatch & Finger, 1979), hemicelluloses, poly-saccharides, and protein are broken down preferentially (Waksman & Nissen, 1932a,b). As the easily decomposed material is being used, the microorganisms begin to attack the more resistant part of the substrate,

such as cellulose and lignin. The compost piles are turned four times over a period of 8-9d to maintain aerobic conditions within the stack. The duration of time spent on phase I will depend on many local factors - air temperature, type of composting substrates, moisture, and microflora present.

The literature describing the progressive chemical changes accompanying decomposition of organic manures has been extensively reviewed (Burrows, 1951; Gerrits et al, 1965; Gray et al, 1971; Kurtzman, 1979; Maeder, 1960; Mattingly, 1952; Muller, 1965; Schisler, 1980; Schobinger, 1958; Waksman & Cordon, 1939; Waksman et al,1939; Waksman & Iyer, 1932a,b; Waksman & McGrath, 1931; Waksman & Nissen, 1931, 1932). Cellulose, hemicellulose, and solubles all decrease while lignin increases over the course of composting. Since both lignin and insoluble proteins are readily available to mushroom mycelium (Waksman & Nissen, 1932b) and not so readily useable to rival microorganisms, it seems probable that the build up of these products at the expense of the more easily decomposed carbohydrates and soluble proteins is an important component in the complex phenomena responsible for the finished compost attaining a balance advantageous for the mushroom mycelium.

The objectives of the mushroom grower is to regulate chemical and physical conditions during fermentation so that the finished compost will be suitable for mushroom mycelium to predominate over all of the competitive microbial flora present (Fordyce, 1970; Lambert, 1938; Royse & Schisler, 1980; Schisler, 1980). The carbohydrates that are easily broken down must be removed so that other fungi will be less apt

to grow in competition (Vedder, 1978). Hayes (1969) and Fermor et al (1979) state that this can be accomplished by encouraging the growth of large thermophilic microbial populations to utilize the simple carbohydrates which would otherwise be available to be used by undesirable mesophilic fungi. Because of the lower mesophilic numbers, less cellulose and hemicellulose are utilized by them; consequently there is a net conservation of nutrients which support mushroom growth.

The understanding of the components which function in composting led to experiments designed to shift the pattern of the microbiological sequence in the composting fermentation in favor of the organisms which are unable to break down cellulose and hemicellulose. Increased mushroom yields were obtained from several workers (Hayes, 1969; Hayes & Randle, 1969a,b; Laborde et al, 1972; Smith, 1974) by the addition of a carbohydrate supplement at the beginning of phase I. The addition of nutrients at phase I is directed towards the feeding of microbial populations in the compost rather than direct nutrient addition for the mushroom. Sucrose supplementation (Hayes, 1969, 1977) favored high populations of bacteria, at the expense of actinomycetes, with corresponding increases in the levels of lignin, cellulose, and hemicellulose; a conservation of nutrients which was associated with increased yield. Furthermore, supplementation probably caused an increase in bacterial biomass (ie. extracellular polysaccharide capsules) which is a major source of nutrients for *A. bisporus* (Eddy & Jacobs, 1976; Stanek, 1972). San Antonio (1966) concluded that microorganisms may account for erratic results obtained, when different amounts of supplements were added.

Bacteria are probably more important to the mushroom grower than to producers of any other crop (Fletcher, 1979). They play a significant role during the preparation of the compost, initiation of fruitbodies, and may be a major problem because of the diseases they produce. All major groups of microorganisms are active during composting, especially thermophilic bacteria, fungi, and actinomycetes, and ideally each group is dominant at different stages.

Waksman & Cordon (1939) and Waksman et al (1939b,c) were the first to emphasize the importance in mushroom composting of a mixed microflora of thermophilic actinomycetes, bacteria, and fungi. Since then, there have been a great deal of studies concerning the role of microorganisms in the preparation of compost (Hayes, 1969, 1977; Oliver & Guillaumes, 1976; Stanek, 1972) and of the predominant types and succession of each (Chang & Hudson, 1967; Fergus, 1964; Fermor et al, 1979; Fordyce, 1970; Hayes, 1969, 1977; Laborde et al, 1968; Renoux-Blondeau, 1959; Stanek, 1967, 1972; Stanek & Zatecka, 1967).

Hayes (1969) examined the bacterial populations during phase I and recorded the succession and ecological cycle of the major types. At the beginning of composting *Bacillus subtilus* and a Flavobacterium predominated, but after the third day *B. stearothermophilus* and Pseudomonas sp. became the major types. Thermophilic fungi remain active throughout the process and they condition the vegetable matter for later utilization by the mushroom (eg. Humicola, Mucor, Stilbella). He noted that all composts, within narrow limits, are of a given microbiological composition. Oliver & Guillaumes (1976) found that the occurrence of peak

populations of bacteria varied in time with the individual composts examined; reaching a maximum population in the first and/or second week. They reported differences in types of bacteria during composting, but similar total populations were reached in the composts they examined. Stanek (1972) noted a similar succession of bacterial types to those reported by Hayes (1969) and all three groups of workers stressed the importance of Bacillus sp., especially during the first week of composting.

The thermophilic bacteria and actinomycete populations are well known, but the mesophilic and thermophilic activity of the fungi have not been well documented (Eicker, 1980; Fergus, 1978; Kurtzman, 1979). Cailleux (1973) recorded fifteen genera of mesophilic fungi occurring in compost, from the beginning of composting to the start of phase II. The possible role of these fungi in compost and their influence on the mushroom are not yet fully understood. Eicker (1980) reported with regards to mesophilic fungi, a clear successional colonization pattern is evident. She concluded that mesophilic fungi (with high cellulolytic activity) play a more important role in composting and substrate colonization than is generally realized.

Bels-Koning et al (1962) demonstrated that the thermophilic species of Humicola are important in the composting process. The heat resistance of the thermophilic fungi have been studied by Fergus (1971). The competition among thermophilic fungi for the available nutrients determine, to some extent, the type of compost produced. Forms such as Rhizopus, Aspergillus, and Graphia are hazardous (Pope et al, 1962).

Lambert (1941) demonstrated that compost produced under aerobic conditions at temperatures between 50-60°C was the most beneficial for mushroom cultivation - this suggests a direct relationship with the activity of specific groups of microorganisms. Hayes (1969) and Fordyce (1970) have shown that the early increase and late decreases of temperature during phase I are paralleled by similar changes in numbers of microorganisms. In a study by Stanek (1967a), the exchange of microbial groups in the substrate during fermentation was affected not only by changes in the substrate temperature, but also by change in composition of available nutrients containing nitrogen. Approximately 80% of the total nitrogen remaining after composting is fixed in the humus-lignin fractions (ie. lignin-N complex) (Muller, 1965; Schisler, 1980). The remainder is probably fixed in microbial protein. The mushroom mycelium consumes these complexes and the microbial protein. It is apparent that high bacterial activity during the early days of composting releases complex organic compounds which are then available for mushroom nutrition (Fletcher, 1979).

The exact role of bacteria in the nutrition of the crop is not completely clear. In addition to their role in conserving nutrients by reducing fungal competitors, thermophilic bacteria might also serve directly in the nutrition of the mushroom (Fermor et al, 1979). In experiments by Stanek (1968), many thermophilic microorganisms isolated from mushroom compost actively formed vitamins and amino acids. Also it may be the unique nature of the enzymes and proteins which confer the property of heat stability on thermophiles which may be the link

between mushroom growth and a heat producing fermentation. Furthermore, the majority of lipids used by the mushroom from the compost appear to be intracellular lipids of thermophilic microfloras (Schisler, 1980). The fatty acid composition of thermophilic fungi substantiates this. It has been suggested that protein and acetate units are obtained by the mushroom primarily from the biomass of thermophiles built up during the phase I stage (Hayes, 1974).

A number of thermophiles have been added to compost in efforts to increase the speed of phase I or to increase the yield of mushrooms. Pope et al (1962) added thermophilic fungi during the composting process and Renoux-Blondeau (1959) added actinomycetes after phase II - both groups reported increases in yield. Stanek & Rysava-Zatecka (1970) added mixed cultures of thermophilic actinomycetes and bacteria to sterilized compost. They found mushroom cultures were not contaminated by competitive microorganisms and mushroom mycelium grew well in their presence. Furthermore, Huhnke (1970) states that by inoculating specific thermophilic microorganisms into sterilized compost, the cultures are capable of causing (through production of metabolic by-products, enzymes, etc.) a selective protection of the substrate against diseases and pests.

It would appear that there is a great effort to find ways to reduce the amount of time required for composting (Fermor et al, 1979; Kurtzman, 1979). However, most of the rapid composting methods at some time have had difficulties with disease and weed fungi (Chanter & Spencer, 1974). It appears that either they can not reproducibly remove the easily

metabolized nutrients or that there are some naturally occurring antibiotics produced during composting, which are not adequately produced in the rapid methods. Many attempts have been made to accelerate the fermentation by careful preparation of ingredients, mechanical breakdown of vegetable matter (Laborde & Delmas, 1969), and carbohydrate supplements (Hayes, 1969). Recent studies in the United States have shown how mushroom production can be greatly speeded up by inoculating fresh compost with 1% compost which was at the height of its activity.

Till (1962) demonstrated that *A. bisporus* did not require a composted substrate for growth, if the spawn-run in non-composted materials was performed aseptically. *A. bisporus* produced cellulolytic enzymes (Turner, 1977) and should thus be able to break down a suitable substrate unaided by other organisms. However, it is essential for these substrates to be composted before they can be successfully colonized by the mushroom when competing with a natural flora of other microorganisms (Fordyce, 1970; Hayes, 1969). This method has been studied further (Kurtzman, 1979) but presently does not seem to be feasible due to the high costs - particularly for sterilization equipment.

Phase I is complete as soon as the raw materials have become pliable, are capable of holding water, the odor of ammonia is sharp, and the dark brown color indicating carmelization and/or browning reactions have occurred (Schisler, 1980).

2. Phase II

Phase II is also called pasteurization, sweat-out, or peak-heating. It is, in essence, the second solid state fermentation in the production of Agaricus mushrooms (Kurtzman, 1979). Phase II has two main purposes: final conditioning of compost so that it will be mushroom specific (elimination of ammonia and readily available carbohydrates) and pasteurization to eliminate insects, nematodes, competitive fungi and seeds of higher plants (Burden & Peterson, 1972; Schisler, 1980; Smith, 1969). Mushroom production on pasteurized compost is essentially a continuation of the composting process, in which under such parameters that we may predict the cultivated mushroom to be the strongly dominant organism (Muller, 1965). A reduction in mesophilic fungi and bacteria is also achieved, but it is useless to try to eliminate all the mesophiles in the melice of a mushroom facility.

Once the beds or trays are filled, the compost begins to heat through the growth of the microbes and residual nitrogen is ammonified by urea bacteria such as Proteus, Micrococci and Aerobacter (Royse & Schisler, 1980). Steam is initially added to bring the temperature to about 55-60°C. This temperature is held from a few hours up to 2d depending on the practices of the grower (Barret, 1956). Available microbial foods in the compost, at filling (eg. sugars, hemicellulose, starches, lipids), are utilized by the dynamic microbial populations during the phase II. This results in bacterial products at spawning, which are ultimately utilized by the mushroom during spawn-run. The fermentation (phase II) must remove the last of the easily available

nutrients - producing a compost which is a selective medium for the mushroom (Eddy & Jacobs, 1976). Schisler (1980) states that conversion of ammonia products to microbial protein is best accomplished by thermophilic bacteria, actinomycetes (eg. Streptomyces, Thermonopsora) and fungi (Torula, Mucor). By so doing, the ammonia is incorporated into microbial cells and ultimately is available to the mushroom (Sinden & Hauser, 1953). However, the possible production of metabolites or compost degradative products that improve or reduce ultimate mushroom yield is also theoretically possible (Fergus, 1964).

Often the compost becomes whitened by the development of thermophilic actinomycetes ("fire-fang") (Renoux-Blondeau, 1959). However, other workers have reported the presence of molds in the compost (Bels-Koning et al, 1962; Pope et al, 1962). The mold and actinomycete flora of mushroom compost during phase II has been investigated previously (Renoux-Blondeau, 1959; Fergus, 1964; Tendler & Burkholder, 1961). However, much work remains to be done - the identity of the microorganisms still has to be resolved. Hayes (1977) demonstrated that the actinomycetes, *per se*, are of little importance except to indicate that other essential changes have occurred.

Attempts to control and develop a specific microflora (Pope et al, 1962; Renoux-Blondeau, 1959) have so far only been preliminary. Various workers (Hayes, 1970, 1977; Hayes & Randle, 1970) fumigated composts with methyl bromide during phase II. This caused a stimulation in numbers of bacteria and an enhanced effect on productivity. This suggests a strong association between bacterial activity in the

compost and the overall nutrition of the crop. The improved yields were attributed to the destruction of actinomycetes and fungi which utilize valuable cellulosic foods (Hayes, 1969). Further studies on controlling the microflora population in compost will undoubtedly continue.

The major problem in peak-heating is that compost is a relatively good insulator; hence, unless there is a considerable heating from the metabolic activities in the compost, it is nearly impossible to heat the compost to the desired temperature (Kurtzman, 1979). In a study by Ross (1976), at temperatures above 55°C and at 40°C or below, the final composts were not selective and supported the growth of competitive fungi as well as the mushroom. Selective compost were produced over the range of 45-55°C. Recent results by Gerrits (1980) also indicated that the temp for maximum mushroom yield may be below 50°C. Furthermore, in preliminary work by Ross & Harris (1982), it was found that ammonia disappeared rapidly in phase II at much lower temperatures than those conventionally used. Prolonged phase II has been shown to affect adversely the nutrients in the compost, resulting in lower yields (Hatch & Finger, 1979).

Once the smell of ammonia is gone, the compost temperature can be dropped to 25°C for spawning. One of the most urgent needs in mushroom cultivation is a simple quantitative measure of decompostion after phase II to supplement the pH value, and the appearance, smell and feel of the compost now used as indicators of its suitability for mushroom mycelium (Lambert, 1938).

3. Spawning and Spawn-run

Once the phase II composting is complete, the compost bed is ready for inoculation. It is inoculated with a pure culture of mushroom mycelium on cereal grain (spawn), originally obtained from selected growths from multispore germinations (Hatch & Finger, 1979; Hayes, 1974). Spawn is also an excellent medium for a large number of fungus species (Christianson & Kaufman, 1969; Eicker, 1980) and many of the species recorded in the colonized substrate during investigations are known pathogens of grain spawn (Bitner, 1972; Ivanovich-Biserka, 1972). Except under pure culture conditions, mushroom mycelium must always be considered as competing with other microorganisms (Lambert, 1938). It is essential that the mushroom mycelium develop as soon as possible; otherwise competitive molds will begin to grow and to hamper the development of the mushroom mycelium (Vedder, 1978).

Stanek (1972) states that the function of individual kinds and groups of microorganisms, their mutual relations and their influence on the growth of the mushroom mycelia are largely unstudied. Stanek (1974) demonstrated that when mushroom mycelium colonized a compost, the number of bacteria and fungi decreased. At almost the same time as the mesophilic microorganisms begin to assert their influence on the enviro-ment, the thermophilic population declines (Fordyce, 1970) and Agaricus, which is known to produce an antibiotic (Eger, 1962, 1972; Sinden, 1971) becomes dominant. Other mesophilic actinomycete populations are relatively stable. However, Fergus (1971) reports that not all molds are inhibited

by Agaricus, or if so, only in very close proximity to the mycelia. Some fungi, unaffected by the antibiotics, may compete for the available substrate and may even kill the mushroom mycelia with their metabolites (Royse & Schisler, 1980). Furthermore, it was shown (Stanek, 1974) that in the vicinity of mushroom hyphae - Gram negative bacteria predominated (causing stimulative effects), whose properties were different from bacteria occurring in the compost without mushroom mycelia. It is considered probable that the advantageous interaction between mushroom mycelium and hyphosphere microorganisms enables mushroom mycelium to colonize a compost rapidly after spawning.

Examination of the literature reveals that relatively few studies have been conducted to determine if any fungi are growing in the compost with the mushroom mycelia during the spawn-run; and the effects that they may have (eg. toxic products, increased temperature). Also, the effects of these organisms (eg. competition) on the substrate itself and *A. bisporus* are largely unknown (Eicker, 1980). LaTouche (1949) listed some unidentified fungi of varying or doubtful status in compost. In addition Fordyce (1970) reported finding mesophilic species (unidentified) of the genera Aspergillus, Fusarium, Mucor, and Penicillium in the first two weeks following spawning. Recently, Fergus (1978) isolated and identified 50 species of fungi during mushroom spawn-run. The fungi found at the end of spawn-run included many known "weed-molds" capable of generating metabolic temperatures that can not be tolerated by *A. bisporus*. Moreover, the thermophilic molds, actinomycetes, and bacteria causing thermogenesis in phase II can also become active after

the inoculation of spawn, and can raise the temperature high enough to injure or kill the mycelium (Sinden, 1971). In a later study by Fergus (1982), it was demonstrated that due to the poor heat resistance of most fungi - if fungi are to grow in the cooled compost after spawning, they must be introduced into the compost at the time of spawning or thereafter. Trigiano & Fergus (1979) demonstrated that most of the fungi during spawn-run have the capability of utilizing cellulose, starch, and lipids as food sources from the compost (through production of extracellular enzymes). There is evidence that many of these fungi may be capable of degrading lignin. Further studies are needed on the enzymatic abilities of compost fungi so their role in mushroom culture could be more clearly understood.

Extracellular polysaccharides are secreted in the form of slime layers or capsules on the exterior of bacterial and many fungal cells during growth. Eddy (1976) showed that the selectivity of the mushroom compost for mushroom mycelial growth was closely related to the composition of the slime. Eddy & Jacobs (1976) and Stanek (1972) demonstrated that bacterial polysaccharides were 2-9 times better utilized by the mycelium than glucose alone. Chemically, this substance consisted of glucose, fructose, mannose, uronic acid and nitrogen (0.26%) (Hayes, 1977). It was suggested that other components of the bacterial cell may also have important roles to play and it is interesting to note that *A. bisporus* can synthesize the necessary enzymes to degrade fungal and bacterial cell walls (Turner, 1977).

The fermented substrate from a Streptomyces sp. and a Pseudomonas sp.

were used by Stanek (1972) as a medium to cultivate *A. bisporus*. The aerated substrate from the Streptomyces sp. supported the growth of the Agaricus mycelium, but allowed contamination; on the other hand, the substrate from the Pseudomonas supported little growth but did not allow contamination. When the two substrates were mixed, contamination did not occur and increased mycelial growth was evident.

Studies by Stanek & Zatecka (1967) and Treschow (1942), have shown that most strains of thermophilic cellulose decomposing actinomycetes produced pantothenic and nicotinic acids, biotin, thiamin, and vitamin B_6. These substances stimulated the growth of the mushroom mycelia. The B vitamins were also formed by thermotolerant fungi of the genus Humicola. Grabbe (1969) found that humic acids formed by microorganisms in compost were a poor source of nutrients for mushroom mycelium. However, amino acids produced by various thermophilic actinomycetes (eg. *Streptomyces thermovulgaris*) could be utilized by the mushroom as a source of nitrogen (Bohus, 1959; Park, 1971). It is quite evident that the exact role of bacteria in the nutrition of the crop is not completely clear (Fletcher, 1979). Further research is obviously needed to establish the precise contribution of the microbe to the life cycle of the cultivated mushroom.

Ten to fourteen days after spawning, the spawn will have grown fully through the compost and will appear as a greyish white growth over the surface of the compost (Hayes & Wright, 1979). This is the time to lower the temperature and apply the casing soil.

4. Casing

The casing layer consists of one inch material with moisture retaining properties which is laid on top of the compost after the spawn has run and is used to help induce fruiting of the mushroom (Dawson, 1977). Neutralized peat is almost exclusively used for casing soil in British Columbia (B.C. Ministry of Agric., 1980). It is now known that the casing layer, although a nutritionally poor substrate, supports a bacterial flora which flourishes under the conditions created by the accumulation of volatile metabolites (Hayes, 1974).

What is the actual mechanism which induces initiation of fruitbody formation? The various factors which affect the development and growth of fruiting bodies are only partially understood. Couvy (1972) used a two medium technique, and suggested that a transition from rich to poor medium is a requirement for primordium formation (ie. casing). The experiments of Eger (1961, 1963), Hayes (1972) and Hayes et al (1969) demonstrated that an unidentified biological factor was in some way involved in sporophore production. Eger (1961) showed that by sterilizing casing soil, fruiting did not occur in sterile conditions although it did very readily if non-sterile casing was used. Hayes & Randle (1969b) postulate that composts without a casing do not allow a buildup of effective populations of stimulatory bacteria. Other workers (Curto & Favelli, 1972; Eger, 1962; Hayes & Nair, 1975; Hume & Hayes, 1972; O'Donoghue, 1965; Park & Agnihotri, 1969; Urayama, 1965) have also examined the role of bacteria in this process. Park & Agnihotri (1969) using sterilized soil showed that a range of soil bacteria (or their

filtrates) would promote fruitbody formation.

Hayes et al (1969) found that certain bacteria of the genus Pseudomonas, in particular, *P. putida* were responsible for the initiation of fruitbodies of *A. bisporus*. Hayes (1974) suggested that *P. putida* strains might act by producing iron chelating compounds (siderochromes), capable of binding iron from the sequestering conditions of the alkaline casing layer. Previously, Hayes (1972) had shown that a substantial increase in the number of pinheads occurred by applying ferrous salts to the casing soil. The extent of the activity of *Pseudomonas putida* has also been shown to be related to mushroom productivity; ie. consistently large numbers of primordia are produced when *P. putida* is grown in association with *A. bisporus* (Hayes & Nair, 1974; Hume & Hayes, 1972). Nair & Hayes (1974) subsequently demonstrated that bicarbonate ions in the casing influenced the populations of Pseudomonads and these, together with a possible role in the release of bound or chelated iron, resulted in sporophore initiation. On the other hand, Wood (1976) found that *Pseudomonas putida*, or suspensions of other bacteria had no effect; neither did iron salts or other iron containing compounds on fruitbody formation.

Experiments by Long & Jacobs (1974) on axenic fruiting of *A. bisporus* showed that any compound involved in fruiting initiation must be of low volatility. Their observations indicated that the role of the casing microflora is more likely to be the removal of one or more self inhibitors of fruiting rather than a positive contribution of fruit-inducing substances.

Eger (1972) states that both the action of CO_2 and volatile organic compounds, and the metabolic activity of bacteria are responsible for fruiting. This was substantiated in the studies by Long & Jacobs (1968) and Nair & Hayes (1974). Bacteria that initiate fruiting are able to utilize volatile metabolites of the fungus as sole carbon sources. Ethyl acetate and acetone may be key metabolites (Hayes et al, 1969) as the soil accumulates these volatiles produced by the mushroom. Other volatiles of interest have been quinones, CO, and ethylene (Kurtzman, 1979). Hayes (1972) found that some hemeproteins and EDTA plus ferrous sulphate stimulated the formation of primordia. Hughes (1963) found that sterols and sterol esters accumulated in developing sporophores. Possibly the bacteria which stimulate sporophore formation supply essential steroidal metabolites. Furthermore, it was shown by Ingratta (1980) that high levels of nematodes at the beginning of the crop tended to result in higher yield. It was postulated that a high population at the beginning of the crop assisted in the distribution of the bacteria thought to be necessary for fruitbody formation.

Hatch & Finger (1979) maintain that fruiting is due to a combination of effects: 1) starvation of mycelium, 2) removal of mycelial metabolites 3) diffusion barrier for mycelial initials, 4) CO_2 gradient, and 5) action of microbial activity. If a specific cause of fruitbody formation can be determined, the need for a casing layer might be eliminated, with obvious advantages to the industry (Ingratta, 1980). Studies on specific stimulators may eventually lead to the development of continous culture systems akin to other industrial processes using microorganisms -

for example, antibiotics and brewing; in which pathogens may be more readily excluded (Hayes, 1974; Hayes & Nair, 1975).

Successful establishment of *A. bisporus* is governed by factors which control the ecology of the two substrates, used in cultivation. Temperature and aeration are the main physical variables. This is ultimately linked to the activity of microorganisms which generally operate by affecting the availability of nutrients (Hayes & Nair, 1975). A degree of chemical control is achieved by providing the correct nutrients in the required proportions to obtain the necessary succession of microorganisms.

The casing layer should therefore be considered as a substrate which not only supports the mushroom but also an associated microflora, which possibly benefits the mushroom and supports its growth through the important transition from mycelial growth to a fruitbody (Hayes & Wright, 1979). Attempts in the future should take into account the possibilites of biological selection of microorganisms essential for sporophore production (Nair et al, 1974).

5. Fruiting

From the developed mycelial aggregates, primordia or pinheads develop, a proportion of which develop further into the characteristic fruits (Chang & Hayes, 1978). The mushrooms appear in "breaks" or flushes and should be ready for picking about 10d after the pinheads first appear (Burden & Peterson, 1972; Toleman, 1979). Mushrooms are then harvested from the beds or trays for a period which ranges from 28-60d depending on the practices of the individual grower (Ingratta, 1980). The total harvest decreases following the third flush (Hayes & Wright, 1979; Vedder, 1978) - relating to the depletion of nutrients and decline of pH (Gerrits, 1965). There is virtually no knowledge of the mechanisms controlling the sequential flushing of mushrooms (Hayes & Nair, 1975).

After approximately the fifth break, mesophilic bacteria and known pathogens of Agaricus are able to colonize the substrate. At this point, the production of mushrooms becomes uneconomical and the spent compost is discarded. On many larger intensive units, the disposal of spent or used compost frequently poses problems. Recent studies have indicated that microorganisms in spent compost have a direct role in the etiology of mushroom worker's lung disease (an extrinsic allergic alveolitis possibly caused by inhalation of spores of thermophilic actinomycetes, mushroom viruses, or chemicals present in compost) (Kleyn & Wetzler, 1981; Kleyn et al, 1981). Therefore increasing attention is being given to systems of recycling/replenishing the nutrients which have been utilized by the mushroom so that they are reused in the

succeeding cycle (eg. hydroponics).

Urayama (1961) demonstrated that by spraying *Bacillus psilocybe*, onto mushroom beds, mycelial density and total production of the fruit-bodies of *A. bisporus* were increased; also initiation of the fruiting was earlier. Curto & Favelli (1972) also found that treatment of *A. bisporus* with certain microorganisms (eg. *Scenedesmus quadricauda*) increased mycelial density, reduced time to first picking, and increased yields. Other workers (Park & Agnihotri, 1969; Park, 1970; Renoux-Blondeaux, 1959) also used selected bacteria to increase yield and decrease time to first picking. Park (1970) tried to stimulate the growth of thermophilic actinomycetes by adding industrial waste to the compost and obtained higher yields of mushrooms in doing so. On the other hand, extracts of Penicillium and Aspergillus species were inhibitory to mushroom yields (Stanek, 1959).

Despite the considerable control that has now been introduced into cultivation of mushrooms, in order to maximize full growth potential of the crop considerable individual skill and judgement are required in management - of maintaining the culture free from harmful competitors, pests and pathogens.

B. WEED MOLDS (*Chaetomium olivaceum*)

Many fungi isolated from mushroom beds act only in the capacity of weeds infesting the compost or casing layer. These organisms are called "weed-molds" and are considered disease-producing agents (Beach, 1937; Lambert & Ayers, 1953). Often, they are known to be serious competitors of *Agaricus bisporus* for various nutrients, or they inhibit normal growth of the spawn by detrimental changes produced in the conditions of the compost. To date, the molecules involved in this inhibitory process in mushroom composts has not been fully characterized (Chang & Hayes, 1978; Hayes & Nair, 1975). There is currently no effective control for weed molds (Nair, 1980). Sinden (1972) predicts that the number of weed molds known to interfere with the growth of the mushroom in some way, will increase in the coming years as the problem receives more attention.

Chaetomium olivaceum, more commonly known as Olive green mold, is one of the most common weed molds occurring in mushroom compost beds (Beach, 1937). Olive green mold is a coprophilous fungus which inhibits the growth of mushroom mycelium by competition for nutrients (Chang & Hayes, 1978; Eddy & Jacobs, 1976), or through some toxic factors. It has been known to reduce yields and often cause complete crop failure (Lambert & Ayers, 1953; Nair, 1980). As of yet there are no known methods to successfully control this pest (Vedder, 1978).

One of the main reasons for the invasion of Chaetomium into mushroom beds is that some ammonia is left in the compost after the phase II cookout (peak-heat) or is formed anew (Bels-Koning et al, 1962; Hayes, 1977; Rettew, 1948; Vedder, 1978). Excessive ammonia in the beds may be due to low amounts of gypsum in the compost during phase I (Gerrits, 1977); peak-heating for too short or too long a time, or at too high temperature (above 55°C) (Kneebone & Merek, 1959; Lambert & Merek, 1959; Lambert, 1953; Sinden, 1955) and humidity; or by too much compaction and moisture (ie. lack of fresh air) (Atkins, 1972; Hayes & Wright, 1979; Schisler, 1980; Vedder, 1978). Furthermore, high concentrations of CO_2 in the compost seems to promote the germination of spores of Olive green mold (Chang & Hayes, 1978). Restriction of the airflow (causing anaerobic decomposition) produces as of yet unknown compounds hazardous to the mushroom but readily accesible to Chaetomium (Schisler, 1980; Sinden, 1971).

Excessive or great variations in temperature (especially during phase II) produces unwanted conversions in the compost (ie. proteins are broken down which can be assimilated by Olive green mold (Lambert, 1953), and it is quite plausible that anhydrous ammonia will be produced from the higher nitrogen compounds already formed (Vedder, 1978). Variations in temperature may occur when the grower attempts to supplement thermogenesis with artificial heat such as live steam. Too moist a mushroom bed will also cause conversions to be pushed in the wrong direction, because sufficient amounts of oxygen are unable to penetrate the compost. In other words, when any of these conditions

(ie. lack of fresh air, high temperatures, etc.) causes the conversion of ammonia into proteinacious compounds to be unsuccessful, the level of *C. olivaceum* will be promoted. Insufficient amounts of available carbohydrates in phase II will lead to incomplete conversion of ammonia and amines and their accumulation in the compost (Chang & Hayes, 1978; Hayes & Wright, 1979). Chaetomium has been shown to tolerate as much as seven times the concentration of ammonia as that tolerated by the mushroom (Vedder, 1978).

Although it does not always predominate, *C. olivaceum* has been isolated from all stages of mushroom cultivation including the spawn-run (Eicker, 1980; Fergus, 1978). Phase I composting does not contribute directly to eradication or exclusion of Olive green mold (Sinden, 1971). There are always cool exteriors on which weed molds can be harbored despite thorough mixing during turning. Furthermore, in a study by Hayes (1977), suppression of actinomycete and fungal activity (eg. excessively wet mixtures) before the near completion of the maturation stage, greatly affected the ability of *A. bisporus* to colonize, favoring the development of Chaetomium species.

The spores of Olive green mold are widely distributed in nature and extremely heat resistant (Beach, 1937; Chang & Hayes, 1978; Eastwood, 1952; Kneebone & Merek, 1959). The spores are very resistant and may survive inadequate pasteurization and soil treatment. Its growth is readily disseminated and unrestricted (Nair, 1980), and has been known to spread into sterilized soil and onto wooden

shelving (Atkins, 1972). It is characterized by spore containing bodies which appear like small, round, olive green to blackish pustules observable on the strands of straw (Figure 1) (Kneebone & Merek, 1959; Rettew, 1948). The mold gives the manure a typical dank or musty odor (Vedder, 1978). As it (*C. olivaceum*) develops more or less densely in the compost, the yield of mushrooms will be influenced to a smaller or greater degree. Consequently if Olive green mold appears in large amounts in a localized area of the compost, no mushrooms will appear at all. Furthermore, spawn growth is retarded or fails altogether (Stanek, 1967). As indicated by Trigiano & Fergus (1979) Chaetomium probably has the ability to produce extracellular enzymes capable of degrading insoluble, organic compounds such as cellulose, starch, lipids, and lignin (factors which aid in its competition). In a study by Chahal et al (1975), Chaetomium species demonstrated the highest carboxymethylcellulase activity and highest degradation of wheat straw when compared to a wide variety of other fungi (all isolated from mushroom compost). Degradation of wheat straw was doubled when it was delignified.

C. olivaceum is capable of growth over a wide variety of pH (Beach, 1937). Its optimum is pH 6.8, but it grows well at pH 8.0, where the growth of spawn is often inhibited. Olive green mold grows most readily on slightly acid materials but it adapts quite readily to very alkaline manure. Beach (1937) has shown that mushroom mycelium was unable to compete with Olive green mold at any point of the pH scale when flasks of manure were inoculated with both fungi.

Figure 1. Extensive contamination of mushroom compost bed by *Chaetomium olivaceum*.

Olive green mold is also found on beds which have been supplemented with nutrients after phase II (Sinden & Schisler, 1962) or during composting (Hayes, 1977; Vedder, 1978). Chaetomium seems to be the limiting factor (in the amount of supplement added) because it competes for the supplement with the mushroom mycelium - with the slow growing mycelium losing out.

Chaetomium is also a serious pathogen of cotton, soybean and sunflower crops (Kanwar et al, 1979; Nik, 1980). It causes biological damage to wool fibers (Sankov et al, 1972), and deteriorates synthetic resins such as urethane rubber (Takeyoshi et al, 1971). Chaetomium is currently known to produce a wide variety of different toxic metabolites (ie. mycotoxins) (Brewer et al, 1970; Brewer & Taylor, 1978; Sekita et al, 1981) such as sterigmatocystin (possible precursor to aflatoxins), oosporein, cochliodinol, chaetomin, and the chaetoglobosins. The latter two products have been the responsible agents in several mycotoxicoses. This organism (Chaetomium) has also been implicated in at least one patient death in the United States (personal communication; Dr. M.G. Rinaldi, Dept. of Microbiology, Montana State University).

C. BIOLOGICAL CONTROL

Garrett (1965) defines biological control as "any conditions under which, or practice whereby, surivival or activity of a pathogen is reduced through the agency of any other living organism (except man himself), with the result that there is a reduction in incidence of the disease caused by the pathogen". In its widest sense, the definition of biological control can be expanded to include integrated control where chemicals and living organisms can be used successfully in conjunction to control pests.(Hudson, 1972).

Microbiological control of plant diseases can be achieved directly through inoculation, or indirectly by changing the conditions prevailing in the plant's environment, and thus the microbiological equilibrium of its ecosystem, or by combination of both systems (Henis & Chet, 1975). Moreover, the inordinately high cost of developing chemicals to control pathogens and the lack of resistance of crop plants to may diseases, has attracted the attention of many scientists and, recently, venture capital companies which see a profitable future (Scroth & Hancock, 1981). A common feature of microbial agents, when compared to chemicals is the widespread resistance they encounter from the receiving biotic environment. Breaking or escaping this resistance is a main condition for the success of biological control (Gindrat, 1979).

Except in a few cases, biological control of plant diseases with antagonistic microorganisms is still restricted to experimental work, despite the large amount of published data and reviews on this subject

(Baker, 1968; Baker & Cook, 1974; Baker & Snyder, 1965; Garrett, 1955, 1965; Hussey, 1969; Henis & Chet, 1975). The extreme difficulty of isolating, correlating, and understanding the many factors that influence microbial activites around root systems and on plant surfaces has impeded the development of biological control practices of commercial benefit (Garrett, 1955; Scroth & Hancock, 1981). Thus, despite the many decades of research, there are only two cases where a biological control agent has been registered for use by a government agency and is commercially used in North America (biological control of insects is excepted).

The probable mechanisms of biological control are that of a living organism acting directly on the pathogen (antagonism) or through the intermediate agency of the host (Baker, 1968). The two main categories of antagonism are antibiosis and competition (Park, 1960). Antibiosis (Scroth & Hancock, 1981) is defined as an interaction between organisms whereby a metabolic agent produced by one organism has a harmful effect on the other. Usually this definition excludes common metabolic products, such as carboxylic acids, ethanol, and CO_2. This topic has also been reviewed rather extensively in the literature (Brian, 1957; Garrett, 1956; Jackson, 1965). Production of antibiotics on growth media is common among soil fungi, actinomycetes and bacilli. However, the function of antibiosis in biological control, their formation and ecological significance in common soil have induced a considerable debate (Baker & Cook, 1974; Jackson, 1965).

Competition refers to the interaction of two organisms striving for the same thing; for example, space, nutrients (Park, 1960; Waksman, 1952) - in other words, active demand in excess of immediate supply of materials or conditions. However, although competition for oxygen and minerals is well known, its role in biological control of plant pathogens is most limited to N_2 (Scroth & Hancock, 1981), which is one of the chief limiting factors in soil.

The biological control of crown gall caused by *Agrobacterium tumefaciens* is the outstanding example of an antagonist that has effectively and economically controlled a major plant disease, has been commercialized and is currently being used in agriculture (Scroth & Hancock, 1981). The application of spores from the fungus *Peniophora gigantea* to control infection by *Fomes annosus* in pine stumps is the other case of a biological control that has been effective, widely tested, and registered for use by the government (Risbeth, 1978).

The commercial production of the mushroom, *Agaricus bisporus* lends itself as an extremely feasible system to apply biological methods of control (Hussey, 1969; Nair & Fahy, 1972; DeTrogoff & Ricard, 1976). Loudon (1850) first suggested that "a toad kept in a mushroom house will eat the vermin, snails, and slugs mentioned, and also worms, and ants and other insects". This interesting statement from an early mushroom grower is an example of the control of pests by biological methods - an approach that is only now, over a century later being considered as potentially valuable in modern cultivation processes (Hayes & Nair, 1975).

The concept of biological control was first utilized in mushroom cultivation for control against insect pests. Hussey (1969, 1972), drew attention to the possibility of using nematodes (*Bradynema* sp and *Tetradonema* sp) for control of sciarids and phorids. In another study by Hudson (1972), it was demonstrated that nematodes (eg. *Tetradonema plicans*) could indeed be potentially effective control agents for the insect pests of mushroom crops. However more work is necessary to exploit this possibility and make it commercially feasible. At present there is not a successful method for mass-production of the nematodes (Richardson, 1981). It would appear that this and similar systems of control could be major methods in controlling the insect pests of mushroom crops in the very near future.

Pseudomonas tolaasii, the cause of brown blotch in mushrooms, is omnipresent in casing soils and is spread in watering. All previous attempts to control brown blotch effectively have failed (Nair, 1974; Nair & Fahy, 1972). Nair & Fahy (1972) have investigated the possibility of biological control of this disease. Their experiments demonstrated that an effective biological control against *P. tolaasii* is to use *P. multivorais*, *P. fluorescens*, or *E. aerogenes* added as a peat culture to the casing soil after it is applied to the compost (mechanism is perhaps by competition for nutrients). Commercial trials with peat inoculants of the bacterial antagonists resulted in successful biological control of the disease, and in some trials increased yields of 8-16% were obtained (Nair & Fahy, 1976).

Verticillium malthousei (causing dry bubble in mushrooms) is probably the most widespread and destructive pathogen of mushrooms. It spreads rapidly through the beds. DeTrogoff & Picard (1976) have shown that spraying Trichoderma propagules on casing soil at the rate of 100×10^6 / l was an effective control of Verticillium.

In a study by Townsley (1974), it was demonstrated that thermophilic fermentation of spawn grain with the pure culture of a thermophile prior to inoculation with a pathogenic Penicillium conferred varying degrees of resistance to the grain against the mold. It was concluded that a degree of protection from invasion of mushroom composts or spawns by disease causing organisms may be obtained by prior fermentation with selected thermophiles.

In a study by Han et al (1974), development of *Mycogne perniciosa* Magn. (wet bubble) was suppressed by unidentified organisms in the casing soil. Infected sporophores occurred when the casing soil was inoculated on the surface with a spore suspension of *M. perniciosa* but not when the inoculum was applied to the surface of the mushroom grain spawn or the middle of the casing soil.

One of the problems of the mushroom industry at the present time is that of toxic chemical treatments and resistance shown by the pest pathogens, so that it may be advantageous to use biological control in conjunction with lower chemical doses in an integrated control programme (Hussey, 1969). There is no doubt that a correctly conducted biological method can achieve more certain and predictable results than that attainable through normal pesticide usage.

METHODS AND MATERIALS

Cultures of *Agaricus bisporus* were received from the Fraser Valley Mushroom Growers Co-op (Langley, B.C.) and maintained on Potato dextrose agar (Difco) at 25°C and 3°C.

Cultures of *Chaetomium olivaceum* were received from Dr. L.C. Schisler, Dept. of Plant Pathology, Pennsylvania State University; *C. olivaceum* was also maintained on Potato dextrose agar at 25°C and 3°C.

I. SELECTION OF THERMOPHILES

Representative (40g) samples of commercial compost which had been passed through the initial standard mushroom composting stage (phase I) prior to delivery to the grower for pasteurization (phase II) were received on a weekly basis from the Fraser Valley Mushroom Growers Compost Division. These samples were immediately incubated upon receipt for two days at 55°C under aerobic conditions and high humidity (Fisher Isotemp incubator). This incubation provided a controlled duplication of the final thermophilic treatment given by the mushroom grower before inoculation with mushroom spawn (Ross & Harris, 1982).

For bacteriological analysis, 10g of the thermophilic compost was mixed with 90ml of sterile distilled water and the sample kneaded thoroughly in order to place sufficient microflora into suspension. Thereafter, tenfold

dilutions using sterile distilled water were prepared to a 10^{-7} dilution. Aliquots of 0.1ml from the 10^{-2} to 10^{-7} dilutions were spread plated in duplicate on TSY agar (Trypticase soy agar (BBL) + 0.4% Yeast extract (Difco)) and incubated at 55°C for 16-20h.

a) Initial screening method for thermophiles antagonistic towards *C. olivaceum*:

Following incubation of plates from above, morphologically different thermophilic colonies were isolated and transferred to new TSY plates for purification. After incubation at 55°C, cultures were transferred to TSY agar slants, incubated until good growth was evident and then refrigerated at 3°C. This procedure was carried out until 45 thermophiles were isolated.

To determine if the isolated thermophiles would inhibit Olive green mold (and support *A. bisporus*) on a synthetic compost medium (see below for components): cultures were first incubated in 25ml of TSY broth for 48h at 55°C in a shaker water bath (Blue M, Illinois). Following incubation, 2.0ml of the culture broth was used to inoculate the synthetic compost media (4 plates/ thermophile). Four control plates containing no thermophile (ie. 2.0ml of TSY broth only) were also prepared. Plates were then incubated at 55°C for 48h under high humidity. After plates had been cooled to room temperature, either a 10mm square agar slab of *C. olivaceum* or *A. bisporus* was then placed on top of each plate and incubated at 25°C. Plates were examined on a daily basis for inhibition of *C. olivaceum* and/or growth of *A. bisporus*.

Because of the poor results associated with this method (see Results & Discussion section) a revised procedure was designed (see (b)).

Synthetic compost medium

The synthetic compost medium was composed of:

$CaCO_3$	1.0g
$(NH_4)_2HPO_4$	0.7g
$MgSO_4$	0.2g
KCl	0.1g
$FeSO_4 \cdot 7H_2O$	10mg
distilled water	1000ml (final pH 7.0)

This nutrients solution was then sterilized using steam for 15min at 15psi. Fifteen ml of the sterile nutrient solution was added to 5.5g of sterile ball-milled wheat straw (in a glass petri dish) and called synthetic compost medium. The nutrient was carefully added at a level just sufficient to evenly moisten the very dry straw.

Lignin analysis of ball-milled wheat straw:

i) Lignin content: the lignin content of the ball-milled wheat straw was determined by the acetyl bromide method of Johnson et al (1961). The lignin content of a standard mushroom compost (previously ball-milled) was also determined for comparison. All experiments were conducted in duplicate.

ii) U.V. spectra: following extraction of the ball-milled wheat straw and compost (50g) with 100% methanol (100ml), samples were evaporated on a Rotavapor R116. The concentrated residues (approx. 10ml) were then scanned through the ultraviolet region on a Canlab Unicam SP. 800B UV Spectrophotometer and spectra recorded.

b) Revised thermophile selection procedure:

Following incubation of the plates from stage I (ie. diluted compost samples) and the appearance of colonies, all TSY plates were sprayed with a spore suspension of *C. olivaceum* (in sterile distilled water) (Figure 2). The treated plates were then incubated at 25°C and examined daily for the presence of zones of fungal inhibition. Thermophilic colonies showing antagonism towards *C. olivaceum* were further purified on TSY agar and later stored on TSY agar slants at 3°C. The 45 previously isolated colonies (from Section a) were also tested in this way.

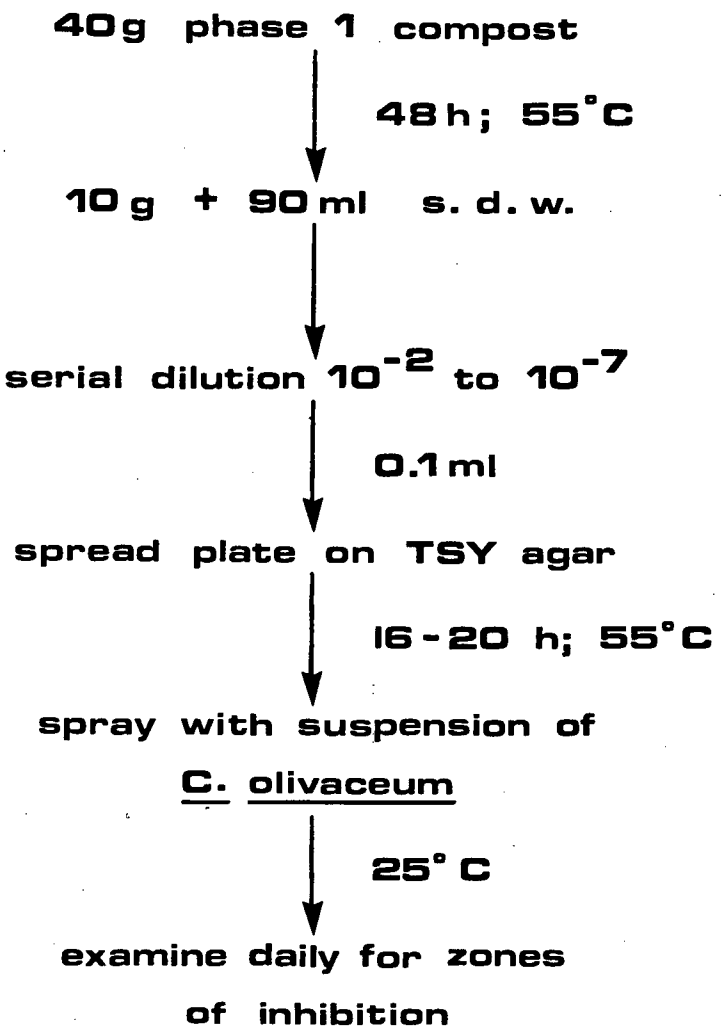


Figure 2. Selection of thermophiles for activity against Chaetomium olivaceum.

II. IDENTIFICATION OF THERMOPHILES

1. Microscopic appearance

a) Gram stain:

Young cultures of the thermophiles previously grown on TSY agar (12-20h) were Gram stained and observed for size (micrometer) and shape of cells, morphology and particular groupings, and presence/location of spores (using oil immersion microscopy).

b) Spore stain:

Cultures were stained according to the method of Dorner (Doetsch, 1981) and observed for the location and nature of sporebodies (any swelling of the sporangia was also noted). Spores were observed to stain red against a colorless bacterial cell.

2. Macroscopic appearance

Thermophiles were observed for colony morphology such as size, shape, margin, elevation, pigmentation, etc., on TSY and PDA agars.

3. Motility

The motility of young cultures of each thermophile was determined by:

a) direct microscopic observation from wet mounts of the organisms (Smibert & Krieg, 1981).

b) use of semisolid media (0.7% agar + TSY broth); cultures were stabbed to one half the depth of the tube and incubated at 55°C. Motility was indicated by migration of cells through the surrounding medium (Krieg & Gerhardt, 1981).

4. Anaerobic growth

To determine if cultures could grow under anaerobic (obligate or facultative) conditions:

a) thermophiles were stabbed to the bottom of a sloppy agar medium (0.7% agar + TSY broth) in duplicate and incubated at 55°C for 5d. Growth on the surface of the agar (aerobic) and along the length of the stab (anaerobic) was recorded by visual observation.

b) to confirm a), cultures were streaked onto TSY agar plates in duplicate and incubated in anaerobic jars at 55°C. Anaerobic jars were used with a Gaspak (BBL Microbiology Systems) and catalyst.

5. Maximum and minimum temperatures of growth

Cultures were inoculated into TSY broth and incubated in duplicate at:

a) for maximum temperature determination - 55, 60, 65, 70, and 75°C in a waterbath (Blue M, Illinois). Growth of the cultures were determined after 3d (Gordon et al, 1973) by the presence or absence of turbidity. During incubation, the water level of the bath was carefully maintained.

b) for minimum temperature determination - 15, 25, 35, 45, and 55°C. Fifteen centigrade was the lowest temperature that could be maintained accurately. Growth was recorded after 5d at temperatures between 35-55°C and after 21d at 15°C (Gordon et al, 1973).

6. Biochemical reactions

The following biochemical tests were performed on the thermophiles:

a) Catalase test: (Smibert & Krieg, 1981).

b) Methyl red test: (Smibert & Krieg, 1981).

c) Hydrolysis of urea: (Smibert & Krieg, 1981).

d) Hydrolysis of starch: (Gordon et al, 1973).

e) Production of indole: (Smibert & Krieg, 1981).

f) Voges-Proskauer: As the formation of acetylmethylcarbinol (acetoin) is one of the most reliable and useful characters in separating Bacillus species (Gordon et al, 1973), two methods for its detection were used:

i) method of Smibert & Krieg (1981), utilizing a standard MR-VP broth (BBL).

ii) method of Gordon et al (1973), utilizing a revised VP medium.

g) Growth in NaCl: Tubes of nutrient broth containing 0, 5, 7, and 10% ($^{w}/v$) sodium chloride were inoculated with cultures previously grown in nutrient broth. Tubes were incubated at 55°C and growth in the various concentrations of NaCl were recorded at 7 and 14d (Gordon et al, 1973).

h) Growth on Mannitol salt agar: (Smibert & Krieg, 1981).

i) Resistance to lysozyme: (Gordon et al, 1973).

j) Utilization of sodium citrate: (Gordon et al, 1973).

k) Utilization of sodium propionate: (Gordon et al, 1973).

l) Reduction of nitrate to nitrite: (Gordon et al, 1973).

m) Deamination of phenylalanine: (Gordon et al, 1973).

n) Decomposition of tyrosine: (Gordon et al, 1973).

o) Growth at pH 5.7: TSY broth was prepared according to the manufacturer and the pH adjusted to 5.7 with IN HCl. Media was filter sterilized (0.45 um millipore filter) and aseptically distributed into sterile test tubes. Cultures were inoculated in duplicate and incubated at 55°C. Growth was recorded at 7 and 14d of incubation.

p) Growth in sodium azide: Tubes of azide dextrose broth were prepared by two methods;

i) according to the directions of Gordon et al (1973).

ii) azide dextrose broth (Fisher Gram-Pac, Pittsburgh), prepared according to manufacturer's directions.

Both methods result in a final concentration of 0.02% azide in each tube. Cultures were inoculated in duplicate into both types of medium and incubated at 55°C. Growth was observed after 7 and 14d of incubation.

q) Acid from carbohydrates: 10% aqueous solutions of D (+)- glucose (Amachem), L (+)- arabinose, D (+)- xylose, and D(-)- mannitol were filter sterilized (0.45um millipore) and aseptically added to phenol red broth base (Difco) to yield a final concentration of 1% sugar in each tube.

Cultures were inoculated into sugars in duplicate and incubated at 55°C. Acid production was recorded at 2, 7, and 14d.

r) Production of dihydroxyacetone: (Gordon et al, 1973).

s) Milk reactions: i) Litmus milk;

Tubes of litmus milk (Difco) were prepared according to manufacturer's directions and 10ml aliquots were autoclaved for 15min at 15psi.

Cultures were inoculated in triplicate and incubated for 7 and 14d at 55°C (Gordon et al, 1973). Tubes were observed for color (acid/alkaline), reduction of indicator (milk appears white due to discoloration of litmus), formation and type of curd (hard or soft), gas and digestion of casein (proteolysis - milk becomes translucent due to hydrolysis of casein).

ii) Decomposition of casein;

Bacto-Skim milk powder (10g) in 100ml of distilled water, and 2g of agar in 100ml of distilled water were autoclaved (separately) for 15min at 15psi. After cooling to approximatley 45°C, they were mixed together and poured into sterile plastic petri dishes. After allowing plates to dry for 3d, cultures were streaked once across a plate (in duplicate) and incubated at 55°C for 7 and 14d.

After the incubation periods, clearing around and underneath the growth indicated casein decomposition (Gordon et al, 1973).

III. USE OF BACILLUS AOG IN MUSHROOM CULTIVATION

A. Conventional Production of *Agaricus bisporus*

500g of standard phase II mushroom compost was spawned with 9.0g of *Agaricus bisporus* spawn grains (obtained from Fraser Valley Mushroom Growers Association) and placed in plastic growing trays (8 x 15 x 23cm). Elemental analysis of the compost used (Canadian Microanalytical Laboratories, Vancouver) demonstrated it to contain 46.7% Carbon and 2.14% Nitrogen (avg. of 3-50g samples). This spawned compost was then subjected to various treatments:

a) control - no further addition to the compost bed;

b) 100ml of a 10^5/ml thermophilic Bacillus AOG culture grown in TSY broth for 96h at 55°C (New Brunswick Psychrotherm shaker) was added;

c) 100ml of a 10^5/ml thermophilic Bacillus AOG culture grown in TSY broth for 96h at 55°C was added; followed by spraying 2.0ml of a *Chaetomium olivaceum* spore suspension on top of the bed;

or, d) 2.0ml of a *C. olivaceum* spore suspension sprayed on top of the bed only.

All experiments were conducted in triplicate (except d, which was done in duplicate).

The growing trays were then incubated at 25°C for 12d to promote spawn growth. During this incubation, the mycelial diameters of the spawn in each tray were measured on a daily basis (for 7 days) as an indication of the mycelial developments under the various treatments (Brancato & Golding, 1953; Fermor, 1982). After approximatley I week, the spawn spread throughout the bed in most trays and accurate measurements could no longer be recorded. After 12 days, each of the various trays was cased with 2.5cm of a sterile peat/sand/ground limestone (Dolomite) mixture in a ratio of 5:5:1 respectively and placed in a 16°C incubator to promote pinhead formation. Air was pumped in at 550 ml/min to keep CO_2 levels at a minimum (ie. CO_2 is inhibitory to fruitbody development at levels above 0.5%; Long & Jacobs, 1968). Relative humidity was maintained at 80%. As the mushroom fruitbodies appeared (harvested just prior to opening of gills) yields (expressed in grams freshweight) and date of picking were determined over a I month period.

B. Hydroponic Production of *Agaricus bisporus*

2% (^{w}/v) Malt extract (Difco), containing 2% $CaCO_3$ and 0.4% Yeast extract (pH 7.0) or a liquid compost solution (see below) were chosen as the liquid nutrients for mushroom hydroponic culture.

Liquid compost:

Liquid compost was prepared by adding 5L of distilled water to 1kg wet weight of mushroom compost. Three 100g samples of the compost were oven dried at 80°C for 48h to determine total solids present. The mixture was agitated vigorously (584rpm) in a thermophilic waste fermenter (Figure 3) with air being pumped in at 4800 ml/min to maintain aerobic conditions. The compost extract was then collected by filtration through cheesecloth and sterilized using steam at 15psi for 15min. The resulting substrate was called liquid compost. Final pH of this medium was 7.2.

To this liquid compost and to the 2% Malt extract was inoculated the Bacillus AOG culture. These liquid substrates were then incubated at 55°C for 96h to yield a final concentration of 10^4 cells/ml (determined by plate count on TSY agar). During the above 96h incubation, 2ml samples were aseptically removed on a daily basis from the 2% Malt extract medium for total carbohydrate analysis (see below).

Figure 3. Aerobic waste fermenter used for preparation of liquid compost.

The experimental treatments were similar to the solid compost plan previously described (see Section A). Either 500ml of a 10^4/ml Bacillus AOG culture in liquid compost or in 2% Malt extract was added to 180g of sterile vermiculite (inert carrier material no less than 2.0mm in size) within a plastic tray. To the bottom (centre) of each tray was placed a sterile glass wool plug approximately 20cm long to help ensure aerobic conditions within the bed. Following spawning (9.0g of spawn grains/tray), representative trays with or without the Bacillus were sprayed with 2.0ml of a *C. olivaceum* spore suspension and incubated at 25°C for 12d. The rate of mycelial development was determined on a daily basis for each tray during this incubation (for 7d). All trays were then cased with 2.5cm of a sterile peat/sand/ground limestone mixture (5:5:1 respectively) and maintained at 16°C for fruitbody formation. Yields of mushrooms and day of picking were recorded during a 30d cropping period. All hydroponic experiments were conducted in triplicate.

Carbohydrate analysis:

Total carbohydrate analysis of the 2% Malt extract medium was determined by the phenol-sulphuric acid method of Dubois et al (1956). Tubes were read at 490*um* on a Beckman Model DB Spectrophotometer. All tests were conducted in triplicate.

Statistical analysis:

The curves obtained from mycelial diameter data were analyzed for significant differences in slope and level.

Analysis of variance and Student Newman-Keul's multiple range test were performed on data for mushroom yields.

IV. ANALYSIS OF INHIBITOR PRODUCED BY BACILLUS AOG

A. Determination of a pH Change

a) Bacillus AOG was streaked once across 6 TSY agar plates and incubated at 55°C for 16-20h. Following incubation, plates were sprayed with a spore suspension of *C. olivaceum* and incubated for 4d at 25°C (ie. until the zone of inhibition was fully developed on each plate). The pH of each plate was determined throughout the medium with a Fisher Combination Flat-Surface-Polymer Body Electrode. The pH of 4 un-inoculated (control) TSY plates was also measured.

b) 10ml of TSY broth were placed in 18 x 150mm test tubes and sterilzed for 15min at 15psi. The pH of each tube was aseptically adjusted between 2-10 with either 1N HCl or 3N NaOH. *C. olivaceum* was inoculated into all tubes (conducted in duplicate) and incubated at 25°C for 7d. Evidence of growth (of *C. olivaceum*) was indicated by lack or presence of turbidity after 7d.

B. Methods to Extract Inhibitor from Cell-free Extracts of Bacillus AOG

i) Thermophilic conditions:

a) TSY broth: Bacillus AOG was inoculated into 100ml of TSY broth (in duplicate) and incubated for 2-5d at 55°C in a shaker waterbath (Blue M, Illinois). Following incubation, cultures were centrifuged in a SS-34 rotor for 25min at 15,000rpm (27,000 x G), filtered through a 0.22um millipore, and the filtrate then freeze-dried for concentration. The resulting compound was reconstituted in either 1ml of 50% ethanol or 1ml of cold sterile distilled water. Samples (0.1ml) were then soaked onto filter paper discs and placed onto TSY agar plates. Plates were sprayed with a *C. olivaceum* spore suspension and incubated at 25°C for 5d (examined daily for the presence of zones of inhibition).

b) Soybean meal: Bacillus AOG was also grown in 100ml of soybean meal (soybean meal, 4g; $CaCO_3$, 0.5g; starch, 0.5g; distilled water, 1000ml) for 3, 4, 5, 7, and 8d periods at 55°C (in duplicate) and harvested as above. Prior to harvesting, cultures were Gram stained and examined for spore formation. After freeze-drying, cultures were reconstituted in cold sterile distilled water and extracted with n-butanol. The n-butanol extract was then soaked (0.1ml) onto filter paper discs and placed onto TSY agar plates. Plates were sprayed with a spore suspension of *C. olivaceum*, incubated for 5d at 25°C and examined daily for zones of inhibition.

c) TSY agar plates: Experiments were conducted to determine if the antibiotic compound could be extracted/ leached out of TSY agar.

Bacillus AOG was streaked once across a TSY agar plate and incubated at 55°C for 20h. Following incubation, plates were sprayed with *C. olivaceum* and incubated for 4-5d at 25°C (ie. until a good zone of inhibition was present). The zones of inhibition (approx. 1.5cm squares) were then aseptically removed from the agar plates and one of the following experiments were conducted in duplicate (controls of un-inoculated TSY agar was also done):

1) An agar disc was placed in a sterile tube containing a sterile filter paper disc (so as to saturate the disc) and contents allowed to stand for 24h at room temperature.

2) Four agar discs were placed in a sterile tube containing 5ml of sterile distilled water. Tubes were then allowed to stand for 24h at room temperature, +/- vortexed for 1min, and contents then soaked onto a sterile filter paper disc.

3) An agar disc was placed into a glass petri dish containing a filter paper soaked with sterile distilled water on the bottom. A sterile filter paper disc was then placed on top of the agar disc and sterile air was carefully blown over the petri dish for better saturation of the filter disc.

All filter discs were then placed onto TSY agar plates, sprayed with a spore suspension of Olive green mold, and incubated for 4-5d at 25°C (examined daily for zones of inhibition).

ii) Mesophilic conditions:

Since the TSY agar plates showing inhibition against Olive green mold (by Bacillus AOG) had been incubated at mesophilic temperatures (ie. 25°C), the inhibitor may only be produced by Bacillus AOG at this lower temperature range.

Hence, Bacillus AOG was inoculated into 100ml of TSY broth and incubated for 2, 4, 6, and 8d at 25, 37, and 55°C in a shaker waterbath. Cultures were then centrifuged at 8000rpm (10,400 x G; GSA rotor) in a Sorvall RC2-B (0°C) for 35min and filtered through a 0.22*um* millipore. The filtrate was then freeze-dried and reconstituted in either 1ml of cold sterile distilled water or 1ml of n-butanol. Samples (0.1ml) were soaked onto filter paper discs, placed onto TSY agar plates and sprayed with *C. olivaceum*. Plates were examined daily for zones of inhibition after 4d incubation at 25°C.

iii) Evaporation method:

Cultures of Bacillus AOG were incubated in TSY broth for 7d at 25°C, 150rpm (New Brunswick Psychrotherm shaker). Following incubation, cultures were centrifuged at 8000rpm (10,400 x G) for 30min (GSA rotor) and then mixed 1:1 with n-butanol. Extraction (2 times) was carried out at room temperature by agititation in a large separatory funnel. The butanol layer was then removed and concentrated (Brinkman Rotavapor R116) to approximately 10ml. This concentrate was centrifuged at 15,000rpm in a SS-34 rotor (27,000 x G) for 20min and stored at 4°C for further analysis.

C. Temperature Stability

0.1ml of the n-butanol extracted antibiotic was placed into a series of 100 microlitre pipettes and given one of the following treatments in duplicate:

1) heated (dry) at 25, 50, 75, 100, 125, and 150°C for 1, 5, 10 and 15min (Despatch oven).

or, 2) frozen at -15°C for 48h, and 3 months (Viking freezer).

Following treatments, the pipette contents were placed onto filter paper discs on TSY agar and challenged for activity against *C. olivaceum* (25°C). Heat treatments could not be tested at temperatures greater than 150°C (5min) because of evaporation/drying of the pipette contents at the higher temperatures.

D. pH Stability

The extracted inhibitor (in water) was adjusted to various pH levels (2-10) with either 1N HCl or 1N NaOH and tested for activity against *C. olivaceum*, using filter paper discs on TSY agar (25°C).

E. Solvent Solubility

Freeze-dried cultures of Bacillus AOG were reconstituted in cold sterile distilled water as outlined previously. One ml of a particular solvent (water, n-butanol, n-propanol, isopropanol, ethanol, methanol, acetone, pyridine, dioxane, ethyl acetate, chloroform, toluene, hexane, benzene, petroleum ether, carbon tetrachloride, or cyclohexanol) was then added to the 1ml of Bacillus AOG extract. The solvents were all of reagent, A.C.S. or U.S.P. grade.

Samples were then vortexed vigorously for 1min and then centrifuged at 3000rpm in a SS-34 rotor (1085 x G; 0°C) for 15min. Following centrifugation, the pH was determined (Fisher pH paper) of the solvent extracted layer and 0.1ml (of solvent extracted layer) was placed onto a sterile filter paper disc. These discs were let stand in a sterile petri dish for 12h at room temperature in order to evaporate excess solvent. 0.1ml of the solvent only was also added to a control disc. All experiments were conducted in duplicate.

Discs were then placed on a TSY agar plate, challenged with Olive green mold and incubated at 25°C for 4d. Following incubation, zones of inhibition were measured and recorded.

F. Spectrum of Activity of Antibiotic

Bacillus AOG was streaked once across a TSY agar plate and incubated for 20h at 55°C. Following incubation, various test organisms (Table 1) were streaked on the same TSY plate at right angles to the streak of Bacillus AOG. All test organisms had previously been grown on TSY agar for a minimum of 48h.

Plates were incubated in duplicate at both 37 and 25°C for 4d. The antibiotic was concluded to be effective against those microorganisms which demonstrated a lack of growth in the vicinity of the Bacillus AOG streak after incubation.

G. Thin Layer Chromatography

Purification of the antibiotic was initially conducted through thin layer chromatography. The solvents employed were all of reagent, A.C.S, or U.S.P. grade. Various solvent systems were investigated to determine the optimum separation/purification of the crude antibiotic mixture (Table 7).

After extraction of freeze-dried extracts of Bacillus AOG with n-butanol as previously described, 3.0ml of extract was streaked across the entire origin of a 20 x 20cm Silica gel 60 preparative plate (2.0mm thickness) with no indicator (EM Reagents). These plates had been previously activated by heating for 48h at 100°C (Blue M, Illinois). The chromatography

Table 1. Microorganisms tested for susceptibility to antibiotic.

Organism	ATCC
Gram negative	
Serratia marcescens	8100
Proteus vulgaris	13315
Escherischia coli	25922
Klebsiella pneumoniae	13883
Enterobacter cloacae	23355
Gram positive	
Staphylococcus aureus	25923
Streptococcus pyogenes	19615
Streptococcus lactis	19435
Streptococcus cremoris	19257
Bacillus subtilis	28281
Bacillus megaterium	25848
Yeasts	
Candida lipolytica	8862
Candida utilus	9256
Saccharomyces cerevisiae	7753
Saccharomyces cerevisiae	7754
Saccharomyces pastorianus	2339

tanks were allowed to equilibrate with the developing solvent before each run. Development took place at room temperature. The preparative chromatograms were developed with n-butanol - acetic acid (glacial) - water (60:20:20 V/V/V) for a distance of 15cm (approx. 12h). The plates were removed from the tanks, air dried, and biologically active bands (see below) extracted three times with n-butanol.

0.5ml of the biologically active bands were streaked across the origin of separate 20 x 20cm plastic Silica gel analytical plates (0.2mm thickness) with no indicator (Merck Kieselgel 60). The chromatograms were then run in a methanol - chloroform - 17% ammonium hydroxide solvent system (40:40:20 V/V/V) for a distance of 15cm (approx. 90 min) at room temperature.

R_f values of appropriate bands were calculated and recorded.

Location and characterization of TLC bands:

i) air dried chromatograms were examined under both short and long wave ultraviolet light (ChromatoVue; Ultraviolet Products Inc., San Gabriel, Calif.).

ii) biologically active bands - to locate the biologically active bands, 2.5cm of a TLC plate was layered with a thin section (approx. 0.3cm) of TSY agar, sprayed with *C. olivaceum* and incubated at 25°C for 4d. Chromatogram sections were observed daily for the presence of zones of inhibition.

iii) to check ii) - biologically active bands were also extracted from the chromatograms with n-butanol and 0.1ml was saturated onto filter paper discs (in duplicate). Discs were placed onto TSY agar, sprayed with Olive green mold, and incubated for 4d at 25°C. Plates were examined daily for the presence of zones of inhibition.

iv) spray reagents - a) Ninhydrin: 0.15g of 1, 2, 3, indantrione monohydrate (Ninhydrin) (MCB Chemicals) was mixed with 50ml of n-butanol, followed by the addition of 1.5ml of glacial acetic acid (according to the method of Stahl, 1969). This reagent was sprayed across the TLC plate and then the plates were heated at 110°C until optimum color development occurred (approx. 1min). A yellow color indicated the possible presence of proline and hydroxyproline; whereas violet was representative of all other alpha amino acids.

b) Rhodamine 6G: TLC plates were sprayed with a Rhodamine 6G (1mg Rhodamine 6G dissolved in 100ml acetone) solution and observed under longwave ultraviolet light. This spray reagent indicates the possible presence of lipids (Stahl, 1969).

c) Phenol-sulphuric acid: Chromatograms were sprayed with phenol-sulphuric acid reagent to detect the presence of sugars. This reagent was prepared by adding 3g phenol and 5ml concentrated H_2SO_4 in 95ml ethanol. Chromatograms were then sprayed, and heated for 15min at 100°C (Stahl, 1969).

d) *Alpha*-cyclodextrin: Chromatograms were sprayed with *alpha*-cyclodextrin (30% ethanolic solution of *alpha*-cyclodextrin), air-dried, and placed in a closed tank containing iodine vapor. This spray reagent indicates the presence of straight chain lipids (Stahl, 1969).

H. Ultraviolet Spectrum

Purified samples of Bands I (3.1mg) and II (5.0mg) (TLC) in n-butanol were scanned under ultraviolet light (Varian Cary 210 Spectrophotometer) and data was recorded. The absorptivity of the samples were also calculated, using the equation:

$$A = a \cdot b \cdot c$$

where A = absorbance

a = absorptivity

b = light path (cm)

c = concentration (mg/ml)

I. Column Chromatography

i) Ion-exchange chromatography:

a) column preparation: A cation exchange resin - AG 50W X-8 with 100-200 mesh size (Biorad) was hydrated with distilled deionized water and poured into a 2.0 x 25cm glass column.

This resin was converted to the H^+ form by washing with 150ml of 1N HCl and testing for low pH (0.5). The column was then rinsed with 200ml of deionized water for a final pH of 6.5.

b) sample elution: 10ml of the sample (Bacillus antibiotic) in n-butanol was carefully layered onto the column. Elution was performed with a discontinous gradient of 100ml of 0.01N, 0.02N, 0.05N, 0.08N, and 0.1N NH_4OH. A column flow rate of 1ml/min was maintained at room temperature. Samples were collected on an Isco Fraction Collector.

c) detection of sample: The O.D. of all tubes were read at U.V. 275um and 270um and data recorded (Varian Cary 210 Spectrophotometer).

ii) Sephadex LH 20:

a) column preparation: Sephadex LH 20 (Pharmacia Fine Chemicals), with a particle size of 25-100u was swollen overnight in boiled distilled water. Excess water was removed until a thick slurry resulted and this was then deaerated under vacuum. Chromatography was performed in a 2.5 x 30cm glass column.

b) sample elution: 5ml of sample (Bacillus antibiotic) in distilled water was carefully layered onto the column. The sample was eluted with 600ml of distilled water using a flow rate of 1ml/min. at room temperature.

c) sample detection: Each tube was scanned under U.V. light (Varian Cary 210 Spectrophotometer) and its spectrum recorded. Fractions demonstrating a U.V. peak (and similar U.V. scan) were pooled and freeze-dried.

These extracts were then reconstituted with 1ml of n-butanol, saturated onto filter paper discs (0.1ml) and placed onto TSY agar plates. These plates were sprayed with *C. olivaceum*, incubated at 25°C and examined daily for the presence of zones of inhibition.

0.3ml of reconstituted sample was also streaked across the entire origin of a 7 x 20cm plastic Silica Gel 60 plate (0.2mm, no indicator). Chromatograms were then run in a butanol - acetic acid - water solvent system (60:20:20). Location of biologically active bands was performed as previously mentioned (Section G of Methods & Materials).

J. Fluorescent Spectrum

Biologically active bands (from TLC analysis) were scanned on an Aminco-Bowman Spectrophotofluorometer and spectra recorded.

K. Amino Acid Analysis

Amino acid analysis was performed on purified samples of the antibiotic. Following extraction from TLC plates as previously described, appropriate bands (ie. biologically active) were dried under a nitrogen flush. These crystals (lower TLC band, 2.1mg; upper TLC band, 1.3mg) were then hydrolyzed with p-toluenesulfonic acid in the presence of 3-(-2 amino-ethyl indole) for 24h at 110°C according to the method of Liu & Chang (1971). Because of very low sample amounts no SH blocking of cysteine was conducted, and only 1ml of 1N NaOH was added to samples after hydrolysis.

The digested samples were then filtered through an ultrafine sintered glass filter. The filter had previously been reversed flushed with 1N NaOH, distilled water, and neutralized with 1N HCl; a final rinse was conducted with distilled water and then air dried using acetone. 0.5ml of the filtered samples were analyzed on a Phoenix Model 6880 (Phoenix Instruments, Phil.) amino acid analyzer utilizing a single column elution system (Durram Chemical Corp., Palo Alto, Calif.).

RESULTS AND DISCUSSION

I. Isolation of Thermophiles Antagonistic Towards *Chaetomium olivaceum*

Initially, it was decided to isolate thermophilic microorganisms which would both inhibit *C. olivaceum* and support *Agaricus bisporus* using a medium which resembles actual compost substrates (ie. "synthetic compost medium"). The lignin content of the synthetic compost medium (ie. ball-milled wheat straw) was shown to possess 22.2% lignin. The control compost sample (ie. fermented) on the other hand, demonstrated a 30.1% lignin content. These data seemed to support the fact that mushroom composting results in an increase in the amount of lignin (Waksman & Nissen, 1931). The U.V. spectra of methanol extracted lignin from wheat straw and compost are shown in Figure 4. The peak in the U.V. region 275um corresponds to the phenolic content of the lignin (Aulin-Erdtman, 1949; Brauns, 1952).

Although these data looked promising for further lignin research, as a selection protocol it (synthetic compost) was found to be an extremely lengthy and involved procedure. The greatest time employed was found to be in preparing the media - for example, ball-milling the wheat straw, having to add the nutrients extremely carefully for even moisture distribution, and lignin analysis; also waiting for the *A. bisporus* mycelium to develop (2-3 weeks) on this medium (possibly due to lower amounts of

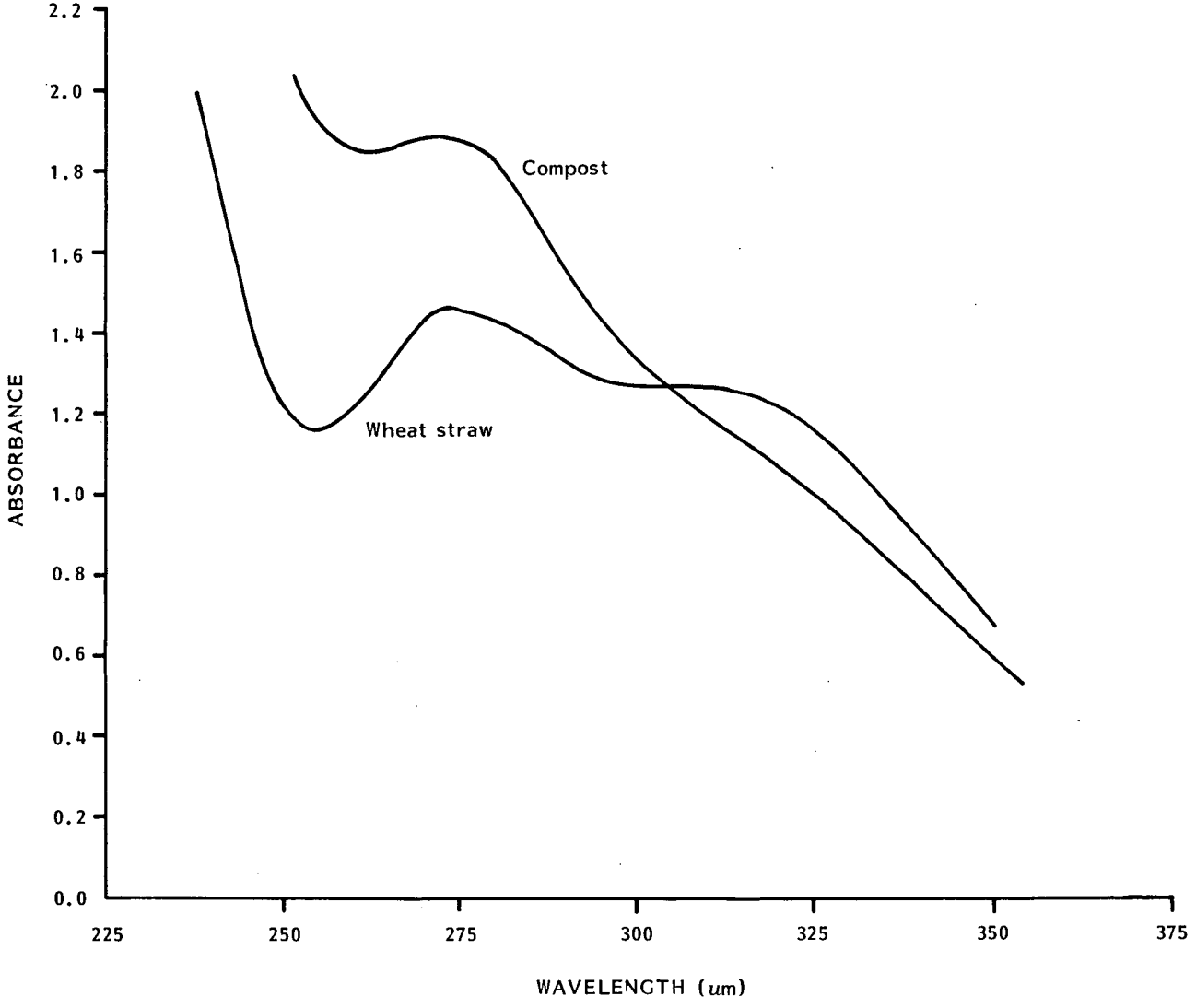
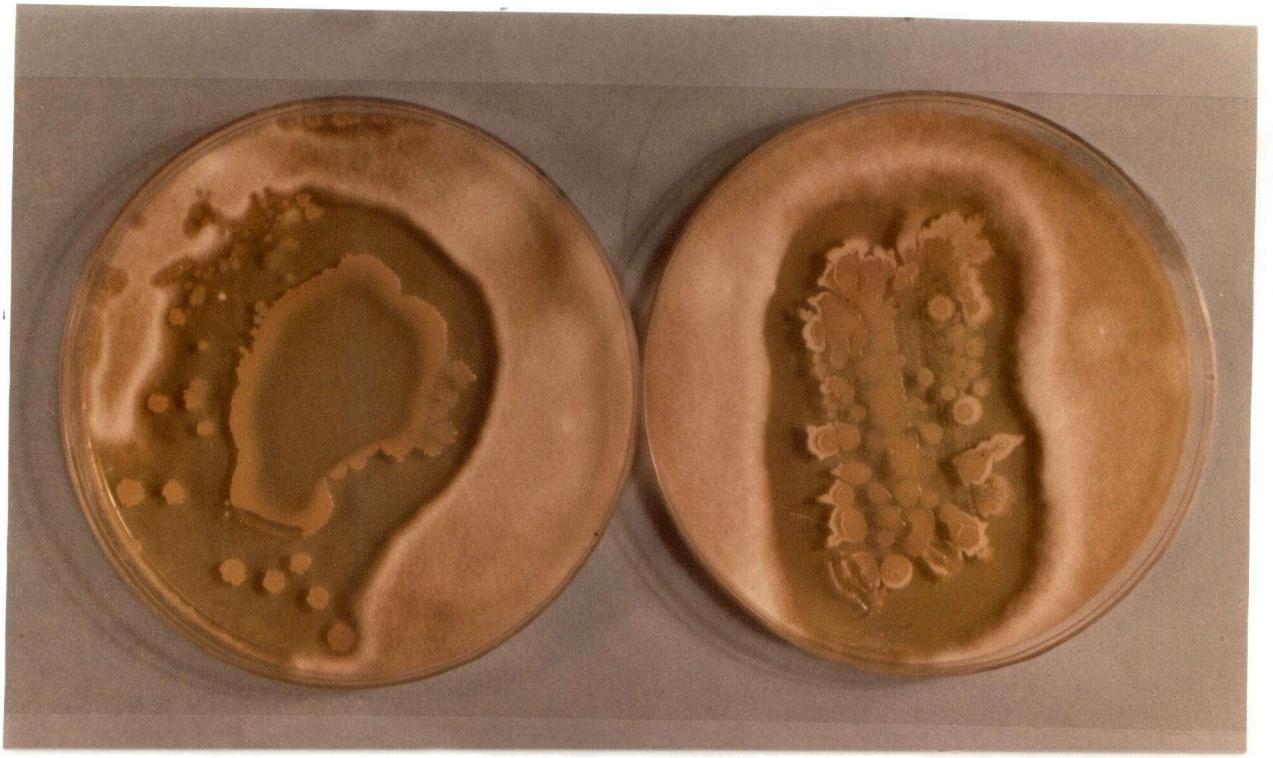


Figure 4. Ultraviolet spectra of lignin extracted from wheat straw and compost.

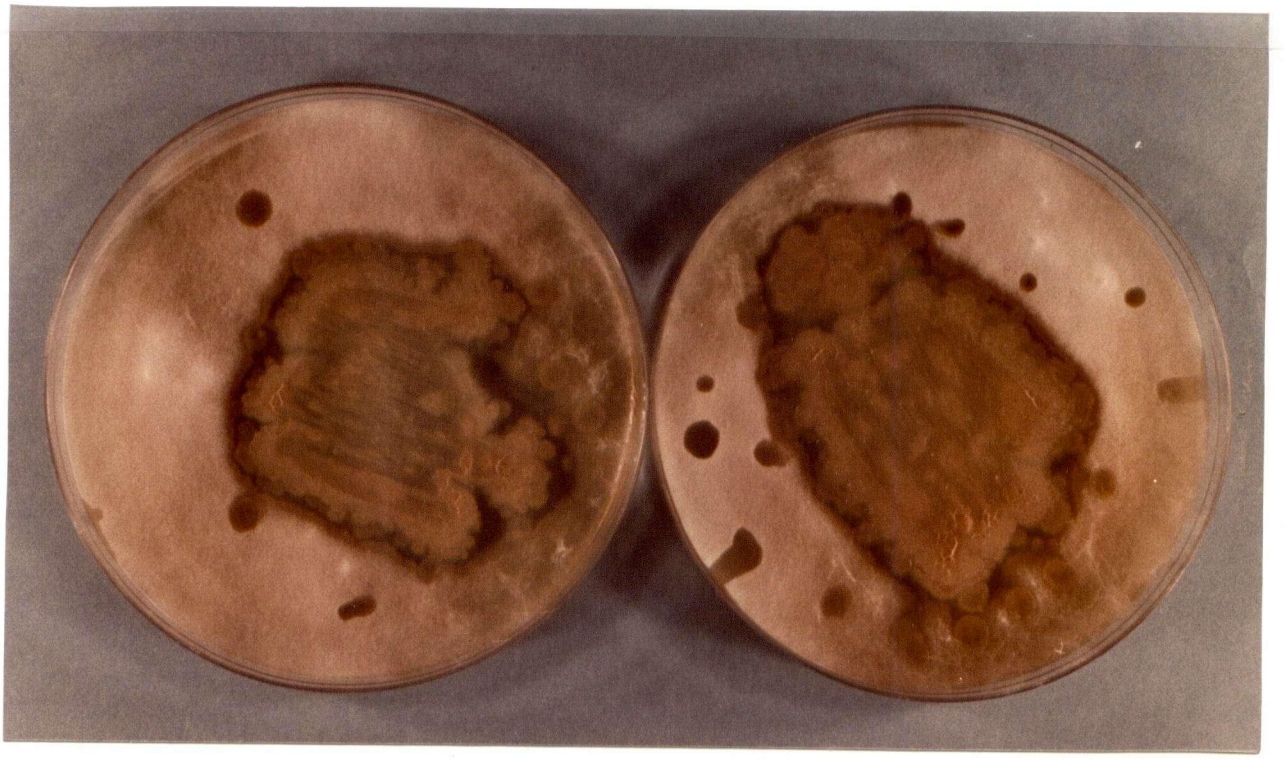
lignin). Although this method was repeated twice, no *C. olivaceum* inhibition resulted with over forty thermophiles tested in this way. These factors forced a reconsideration of the medium and method to a revised selection protocol which resulted in a much more efficient approach to this problem.

The second method involved spraying a spore suspension of Olive green mold directly onto all TSY plates following the initial dilution and incubation of compost samples (see Methods and Materials). This demonstrated an immediate reaction (ie. approx. 3d) between the *C. olivaceum* and any of the thermophiles on the TSY plates. The organisms capability to support *Agaricus bisporus* could be determined at a later time.

After approximately four months of compost examinations using the revised selection procedure, ten thermophiles were isolated which showed varying degrees of antagonism towards Olive green mold on TSY agar plates (Figure 5). Three of the ten thermophiles (referred to as #1-3) were initially isolated during the use of method 1.



a. b.



c. d.

Figure 5. Thermophiles (a - #9; b - #10; c - #4; d - #6) showing varying degrees of antagonism towards *C. olivaceum* on TSY agar.

II. Identification of Isolated Thermophiles

The next stage in this investigation was to determine the identity of the organisms which were causing inhibition of *Chaetomium olivaceum* (as shown on TSY agar). Pure cultures of the thermophiles were first subjected to classification according to their Gram reaction and cellular morphology. Microscopic observations demonstrated the organisms to range from Gram positive to Gram variable (Table 2), rod-shaped (approx. 1 x 4) (Figure 6), occurring generally in singles, pairs, and short chains. Endospore formation was also evident (subterminal to terminal), although the sporangia were not appreciably swollen by the spores in any of the cultures. Macroscopic observations showed the absence of mycelium on any of the solid media tested. These observations implied that the ten isolated microorganisms belonged to the family *Bacillaceae* (Buchanan & Gibbons, 1975).

Furthermore, since the thermophiles were shown to be facultative anaerobes (growth in sloppy agar along top and entire stab; growth in anaerobic jars) as well as being catalase positive (Table 3), it was concluded that they belong to the genus *Bacillus*. Members of the genus Bacillus can be defined (Wolf & Barker, 1968) as "rod-shaped organisms which are spore-bearing, usually Gram positive, catalase producing and capable of sporulating aerobically" (distinguishes Bacillus from some aero-tolerant Clostridia).

The maximum growth temperature of these organisms was observed to

Table 2. Microscopic observations and colony morphology of isolated thermophiles.

	Thermophiles #1-9	Thermophile #10
Gram stain	+,v[1] rods (singles,pairs short chains)	+ rods (singles, pairs short chains)
Size - length (um)	2.0 - 4.8	1.7 - 5.31
width (um)	.78 - 1.0	.78 - .94
spore arrangement	subterminal to terminal	subterminal to terminal
Appearance of colonies		
TSY agar		
size	large (3-10mm)	large (2-12mm)
shape	generally round	round to irregular
margin	irregular	smooth to irregular (undulate)
pigmentation	green/brown	tan to cream (slight green)
colony under reflected light	dull	shiny
transmitted light	translucent	opaque
elevation	relatively flat	umbonate/hilly
other		extremely mucoid; some volcano-like structures
PDA agar		
pigmentation	none	none
colony under reflected light	dull	relatively dull
transmitted light	translucent	opaque
elevation	flat	umbonate
other	no mycelium	no mycelium
Agar slant growth	effuse	effuse

[1]thermophiles #7, 9 were observed to be Gram negative.

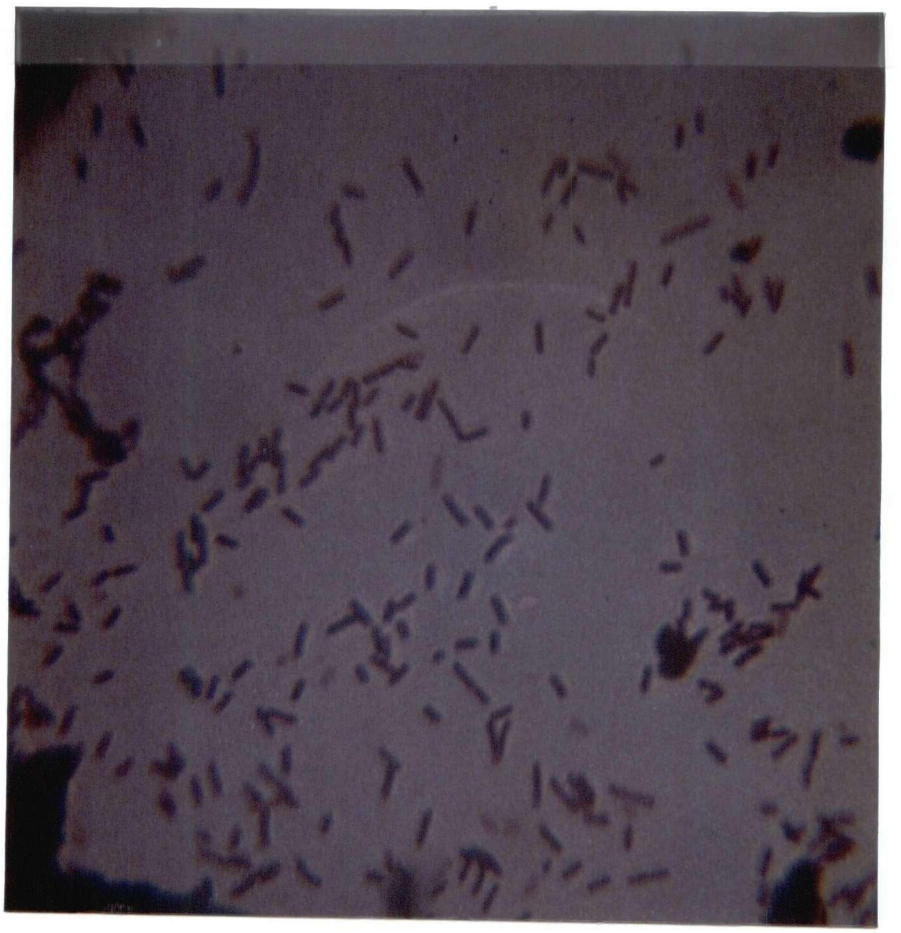


Figure 6. Gram reaction of Thermophile # 10.

Table 3. Properties of isolated thermophiles.

Property	Thermophiles #1-9	Thermophile #10
Motility	+	+
Anaerobic growth	+	+
Temperature Maximum	60°C	65°C
Minimum	15°C	15°C
Catalase	+	+
Voges-Proskauer	+	+
Methyl-red	+	+
Resistance to lysozyme	+	+
Growth in 10% NaCl	+	+
Growth at pH 5.7	+	+
Growth in 0.02% azide	+	+
Acid from glucose	+	+
arabinose	+	-
xylose	+	-
mannose	+	-
Hydrolysis of starch	+	+
Utilization of urea	-	-
citrate	+	-
propionate	-	-
NO_3^- to NO_2^-	+	-
Production of dihydroxyacetone	-	-
indole	-	-
Deamination of phenylalanine	-	-
Decomposition of casein	+	+
tyrosine	-	-
Litmus milk	alkaline clot reduction	alkaline clot reduction
Mannitol salt	+	+

be 60 to 65°C. According to Buchanan & Gibbons (1975) and Gordon et al (1973), there are only five species of the Bacillus genus which are known to be capable of growth over 50°C (Table 4). *B. stearothermophilus* has a maximum growth range of 65 - 75°C; *B. coagulans*, 55 - 60 (65) °C; *B. brevis*, 40 - 60°C; *B. licheniformis*, 50 - 55°C; and *B. subtilus* 45 - 55°C.

Thermophiles #1 through #9 were observed to be the same (or extremely closely related) species of organism - as shown by biochemical and cellular characteristics (Table 2, 3). There was only a difference in Gram reaction with cultures #7 and #9 as compared to the others in this series; this discrepancy may be due to differences in age and/or staining technique. Moreover, thermophiles #1 through #9 all differed from #10 in such properties as maximum temperature of growth, sugar fermentation pattern, utilization of citrate, nitrate reduction, colony morphology and were thus considered distinct species.

It was concluded from the above facts (ie. cellular and biochemical properties) that all of the isolated thermophiles belonged to the species *Bacillus coagulans* (or a closely related variant of *B. coagulans*). This decision is based on the following:

1. *B. coagulans* is the only known thermophile which is capable of growth in 0.02% sodium azide.

2. Ability of isolated thermophiles to grow in acid media.

Table 4. Summary of properties of known thermophilic Bacillus species.

Property	Thermophile				
	B. licheniformis	B. coagulans	B. brevis	B. stearothermophilus	B. subtilus
size - width (u)	0.6 to 0.8	0.6 to 1.0	0.6 to 0.9	0.6 to 1.0	0.7 to 0.8
- length (u)	1.5 to 3.0	2.5 to 5.0	1.5 to 4.0	2.0 to 3.5	2.0 to 3.0
Gram stain	Gram +	Gram +/v	Gram +/v	Gram +/v/-	Gram +
spore formation	central/ paracentral; slight swelling of sporangia	subterminal or terminal; slight to no swelling of sporangia	central / subterminal / terminal; swelling of sporangia	subterminal/ terminal; definite swelling of sporangia	central/ paracentral; slight to no swelling of sporangia
motility	+	+	+	+	+
catalase	+	+	+	v	+
anaerobic	+	+	-	-	-
temperature maximum °C) - minimum	50-55 15	55-60(65) 15-25	40-60 10-35	65-75 30-45	45-55 5-20
V.P.	+	+	-	-	+
resistance to lysozyme	-	-	v	-	v
NaCl 10%	+	-	-	-	+
pH 5.7	+	+	v	-	+
0.02% azide	-	+	-	-	-

Table 4. - Continued

Property	Thermophile				
	B. lichenformis	B. coagulans	B. brevis	B. stearothermophilus	B. subtilus
acid from - glucose	+	+	+	+	+
arabinose	+	v	-	v	+
xylose	+	v	-	v	+
mannose	+	v	v	v	+
starch hydrolysis	+	+	-	+	+
utilization of citrate	+	v	v	-	+
utilization of propionate	+	-	ND	ND	-
NO_3 - NO_2	+	v	v	v	+
dihydroxyacetone	ND	v	-	-	ND
indole	ND	-	-	-	ND
phenylalanine deamination	ND	-	-	-	ND
decomposition of - tyrosine	-	-	+	-	-
casein	+	v	+	v	+

ND - not done
\+ - positive for 90-100% of strains
\- - negative for 90-100% of strains
v - character inconstant

3. *B. licheniformis* and *B. coagulans* are the only thermophiles which are capable of growth under anaerobic conditions.

4. Microscopic and biochemical properties of the isolated thermophiles closely resembles those of *B. coagulans*; exceptions to this are the isolates' ability to grow in high salt concentrations (10% NaCl) and their resistance to lysozyme. These dissimilarities may be due to biochemical variations within the species which are known to occur (Campbell & Sniff, 1959; Humphreys & Costilow, 1957; Marshall & Beers, 1967), variation due to different basal media used during testing (Gordon et al, 1973), plus the observations of Allen (1953) who noted that the characteristics of the thermophilic Bacillus species tended to change on continued culture in the laboratory. *B. coagulans* has been reported to exist in a variety of morphological types (Wolf & Barker, 1968).

Bacillus coagulans was first described in the literature by Hammer in 1915, and has since been classified into two distinct morphological types. Smith et al (1952) defined group I as sporangia not appreciably swollen by oval spores. Hence, the organisms of this study were classified as belonging to group I.

The most fundamental characteristics of *B. coagulans* are its acidophilic and thermophilic properties (Wolf & Barker, 1968). Virtually all types grow at 60°C and are capable of initiating growth at pH 5.3. Growth at

low temperatures is affected both by the medium and nature of the inoculum, spores proving more responsive to lower temperature than vegetative cells. Hence it is the maximum temperature which may be of greatest significance in classification of Bacillus thermophiles (Wolf & Sharp, 1981). Essentially, *B. coagulans* is a facultative thermophile, growing well at 45 - 55°C.

During the period of identification, it was found that thermophilic Bacillus species #1 through #9 demonstrated a much lower antagonism towards *C. olivaceum* on TSY agar. This varied from total lack of inhibition of Olive green mold to extremely minimal antibiotic capability. It seemed that these cultures had or were undergoing a possible mutation process during the cultivation on laboratory medium. Fresh isolates of thermophiles #1 through #9 which had been stored at 3°C on TSY agar slants were regrown at 55°C on fresh medium. These cultures were also soon shown to have lost their antagonism against Olive green mold (usually after a few transfers on TSY plates).

However, thermophilic Bacillus #10 maintained an excellent inhibitory effect against *C. olivaceum* (Figure 7) since its initial isolation and also during the identification protocol. Thus, only *Bacillus coagulans* #10 was used for any further experimentation (hereinafter referred to as Bacillus AOG - "Anti-Olive green").

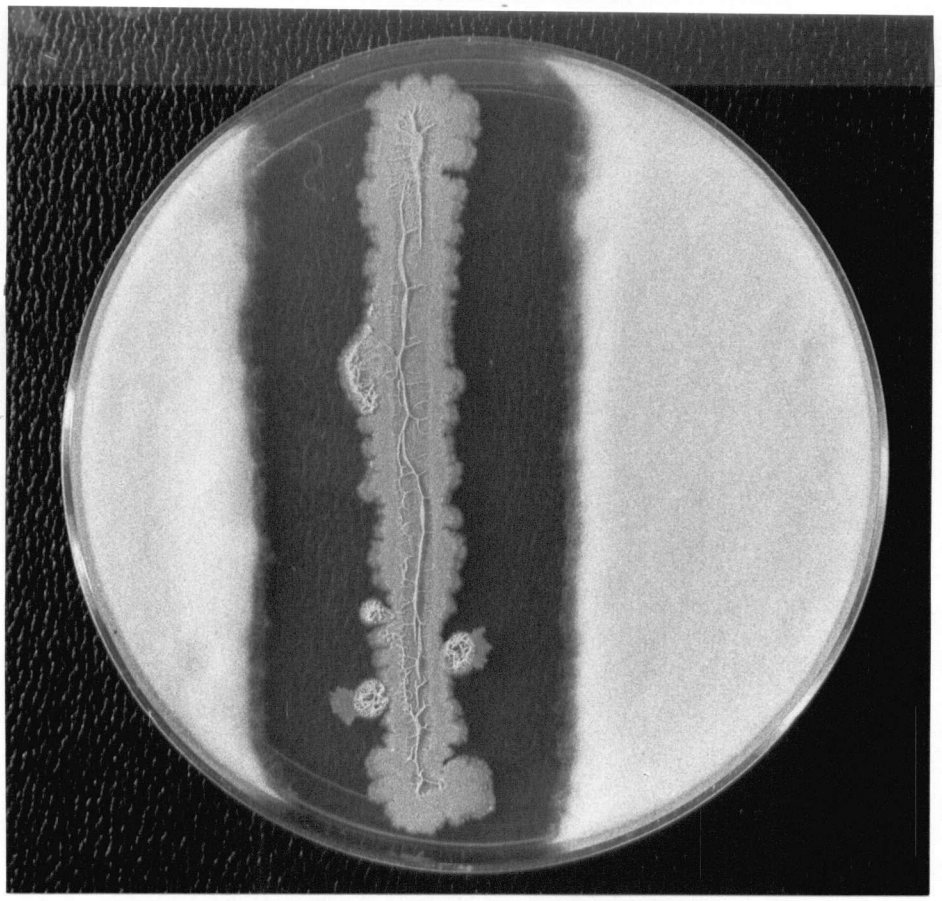


Figure 7. Inhibition of Olive green mold by Bacillus AOG.

III. Cultivation of *Agaricus bisporus* with the Thermophile-Bacillus AOG

To determine if Bacillus AOG would support the mushroom, *Agaricus bisporus*, as well as protect it from damage by Olive green mold, this thermophile was inoculated into two kinds of culture media. One medium was conventional - consisting of standard phase II mushroom compost; and the other was hydroponic - consisting of liquid substrates absorbed onto an inert physical support, vermiculite.

1. Conventional methods:

Figure 8 represents the rate of mushroom mycelial development in standard compost over a seven day period. As can be seen, when the Bacillus was added to the compost, the rate of mycelial development in the trays was enhanced. In addition, the Bacillus AOG exhibited a definite inhibitory effect on the development of Olive green mold (F test on slopes $p < .01$). This biological protection was further indicated by a significant 86.5% increase in mushroom yield of the trays containing Bacillus AOG and Olive green mold over that of the trays containing Olive green mold only (Figure 9) (analysis of variance $p < .05$). Futhermore, trays with only *C. olivaceum* produced fruitbodies a full week later than those with Bacillus AOG and *C. olivaceum*

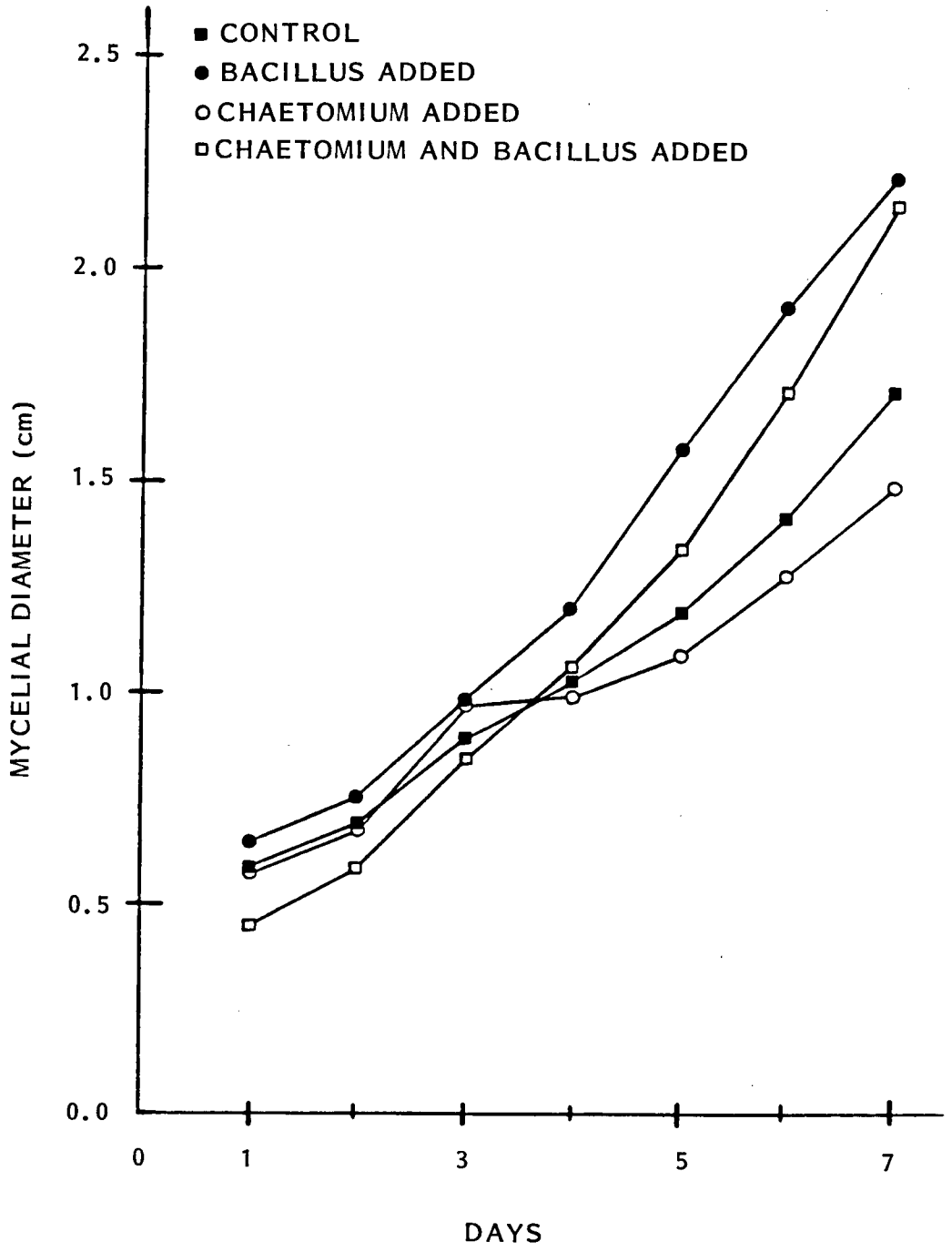


Figure 8. Mycelial development in standard mushroom compost.

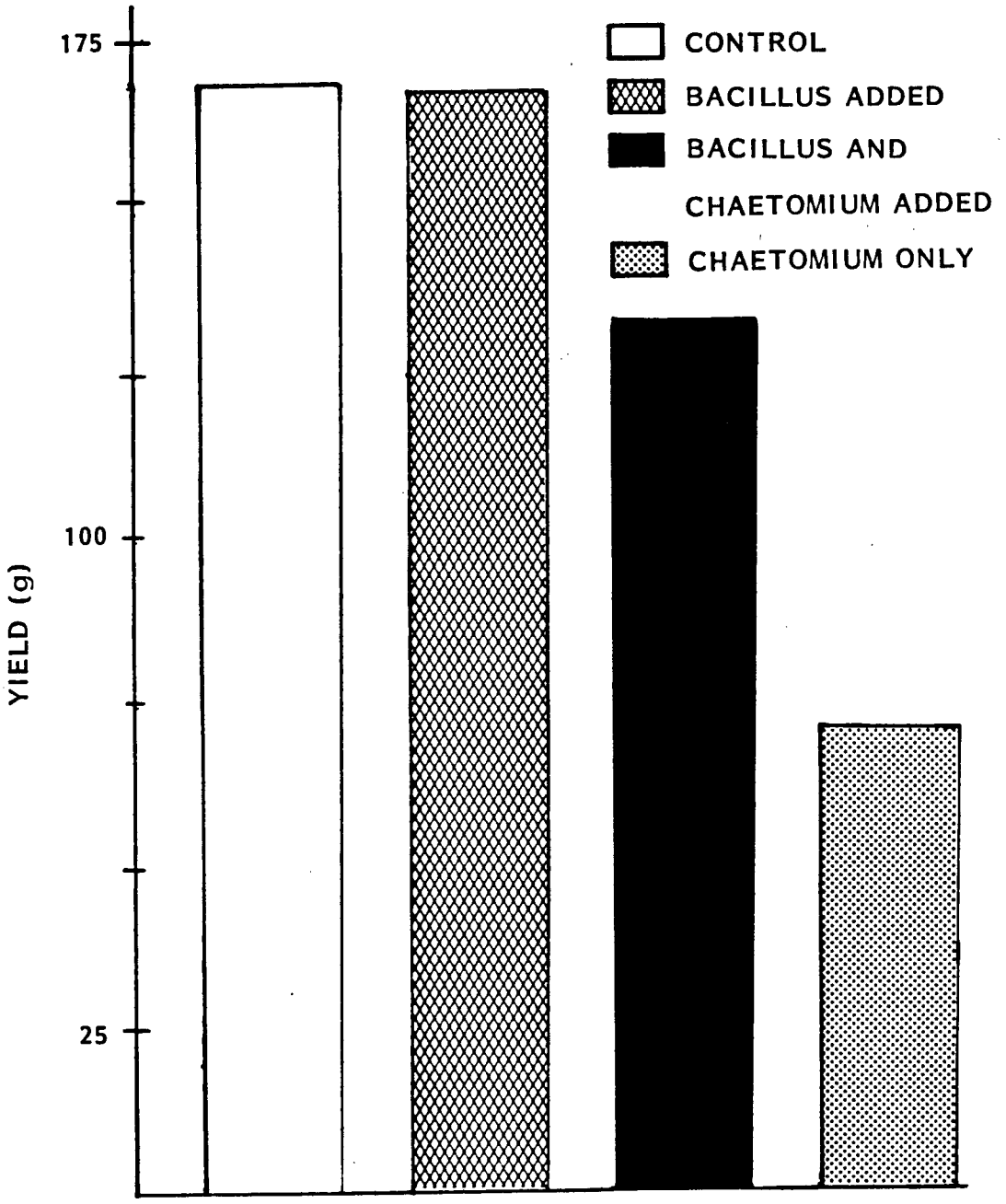


Figure 9. Yield of mushrooms in compost.

together. The observation that initation and development of fruitbodies were not retarded shows that Bacillus AOG had no apparent inhibitory effect on organisms such as *P. putida*, known to stimulate the formation of sporophores (Nair & Fahy, 1972). As demonstrated in these experiments, the application of Bacillus AOG may eventually form an effective biological control method (against Olive green mold) in the commercial production of the mushroom.

2. Hydroponic methods:

The hydroponic series of experiments utilized either 2% (^{w}/v) Malt extract or a liquid compost solution (containing 47% total solids) absorbed onto a carrier material of sterile vermiculite. Vermiculite (hydrated magnesium aluminum-iron silicate) is a common sorbent used in many hydroponic systems (Douglas, 1976; Resh, 1978) because of its excellent qualities. It is lightweight, neutral in reaction with good buffering properties, and is capable of absorbing large quantities of water or nutrients. Also, it has a relatively high cation exchange capacity and thus can hold nutrients in reserve and later release them. Vermiculite contains some magnesium and potassium (essential nutrients for mycelium production and sporophore formation) which are available to mushrooms. These factors seem to make vermiculite

a good choice for hydroponic cultivation of *A. bisporus*.

As can be seen from Figure 10, when the Bacillus was added to hydroponic trays in 2% Malt extract, a remarkable improvement in the rate of mycelial development occurred ($p < .01$). The improved rate of mycelial growth is shown more vividly in the respective photographs of the hydroponic trays. Figure 11 represents a tray with only *Chaetomium olivaceum* added - as can be seen, relatively poor spawn growth is evident; Figure 12 represents the effect of the Bacillus AOG and Olive green mold present at the same time. As these results show, the mushroom mycelia flourished in the presence of the Bacillus.

Mushroom yields from the 2% Malt extract experiments (Figure 13) containing Olive green mold only, showed complete failure of any *A. bisporus* fruitbody formation. Furthermore, all trays without Bacillus AOG (ie. unfermented 2% Malt extract) showed visible signs of contamination by an unidentified blue mold. However, the crop yields from trays containing the Bacillus AOG were maximum - even in the presence of the Chaetomium mold.

The results for hydroponic culture with liquid compost demonstrated significant biological control during both the mycelial growth phase ($p < .01$) (Figure 14) and during fruiting of the mushroom as the yields demonstrate (Figure 15) (analysis of variance $p < .05$). Trays with only the competitor *C. olivaceum* added did not produce mushrooms. However, similar to the 2% Malt experiments, the maximum yield occurred for trays containing the Bacillus AOG and *C. olivaceum* together. Furthermore,

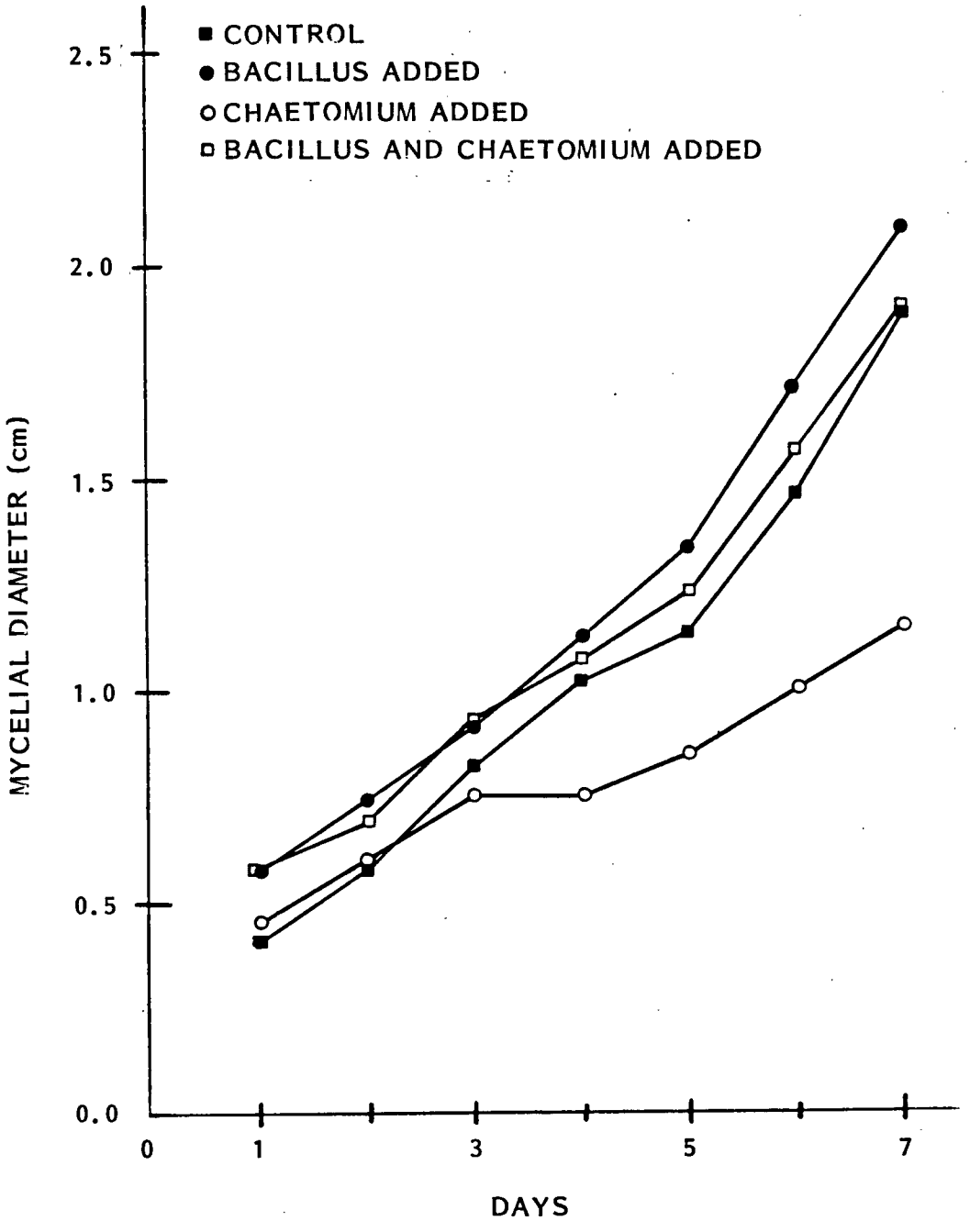


Figure 10. Mycelial development in 2% malt extract.

Figure 11. Poor spawn growth in hydroponic tray with only Olive green mold present.

Figure 12. Improved mycelial development due to the biological protection of Bacillus AOG against *C. olivaceum*.

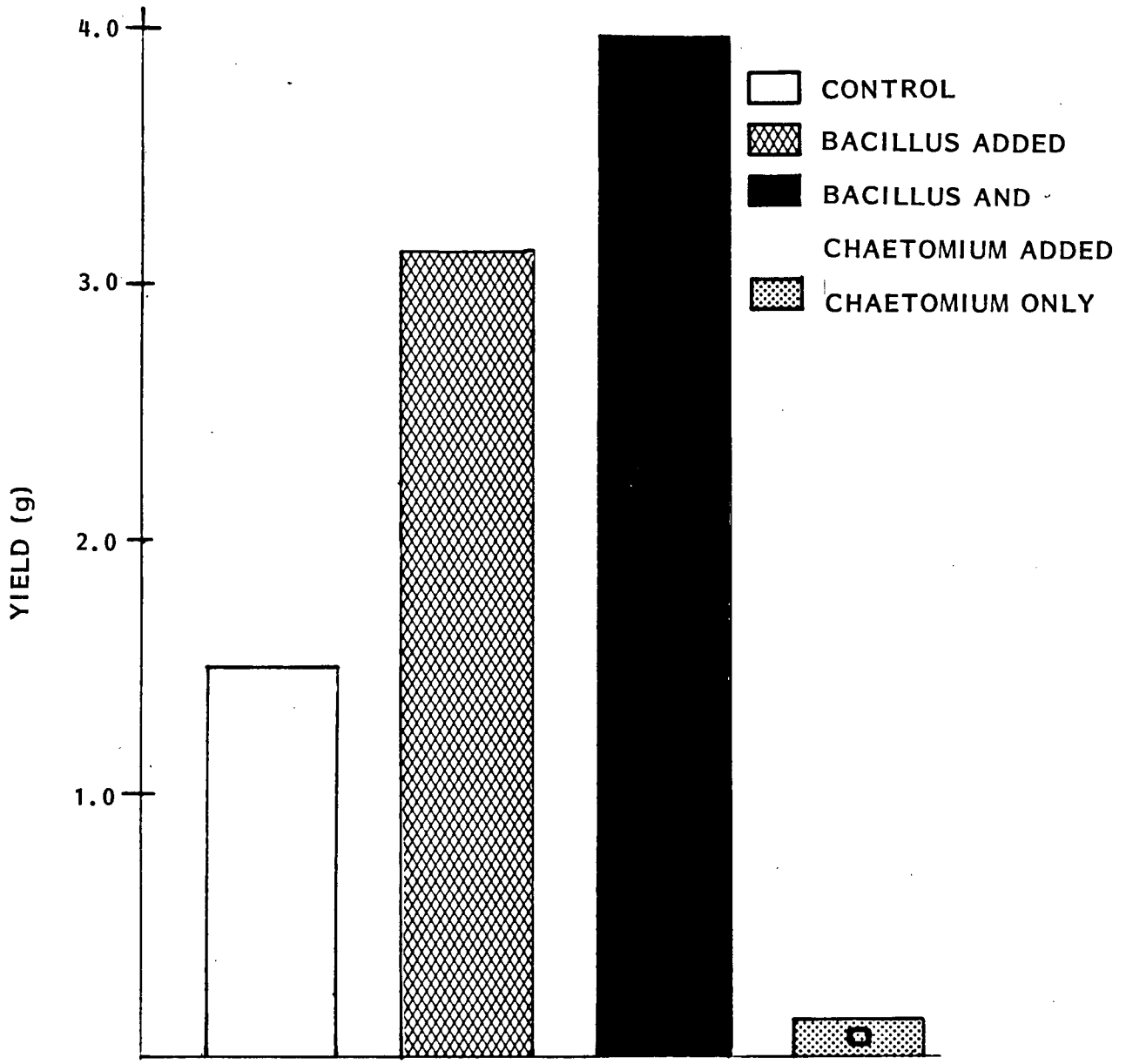


Figure 13. Yield of mushrooms in 2% malt.

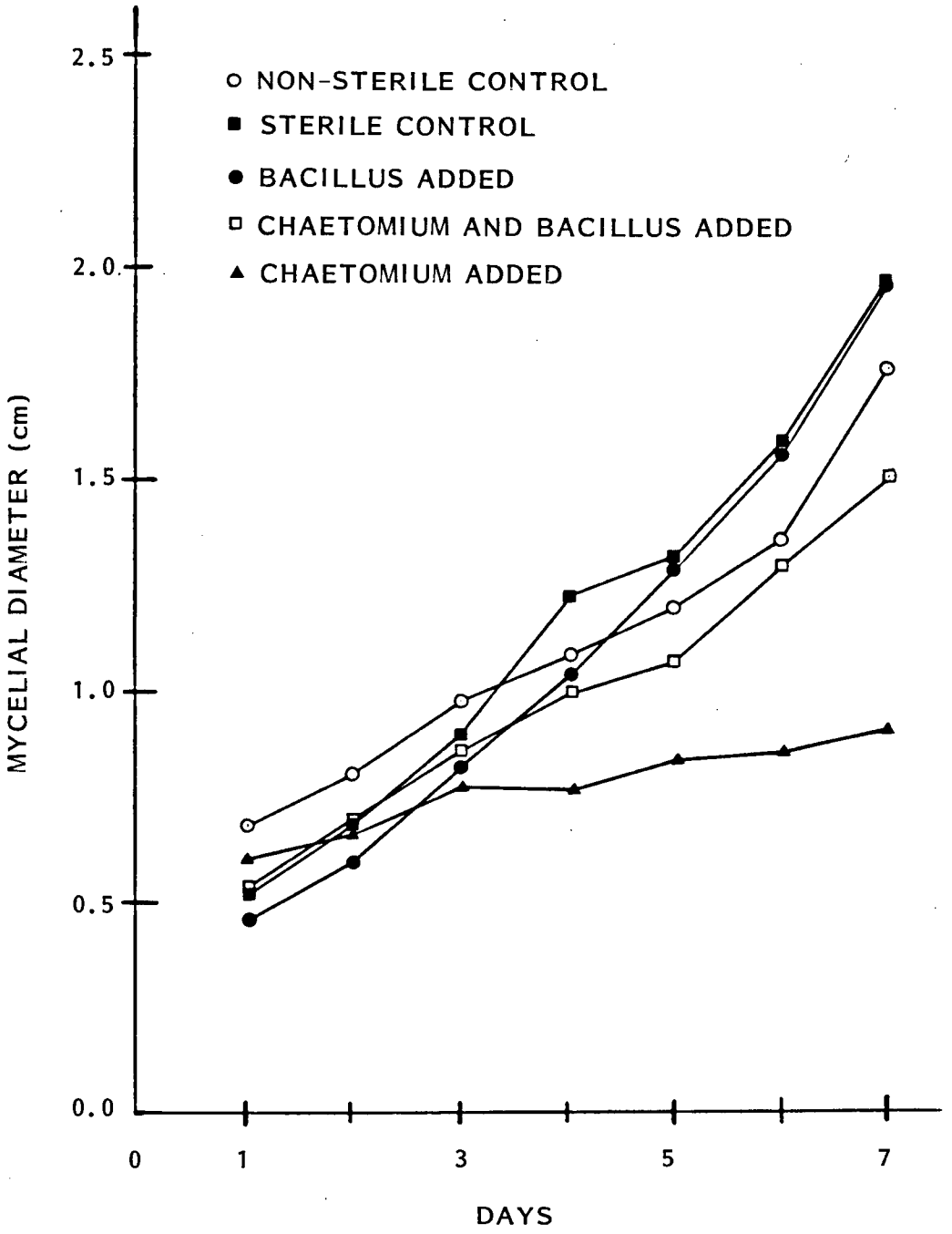


Figure 14. Mycelial development in liquid compost.

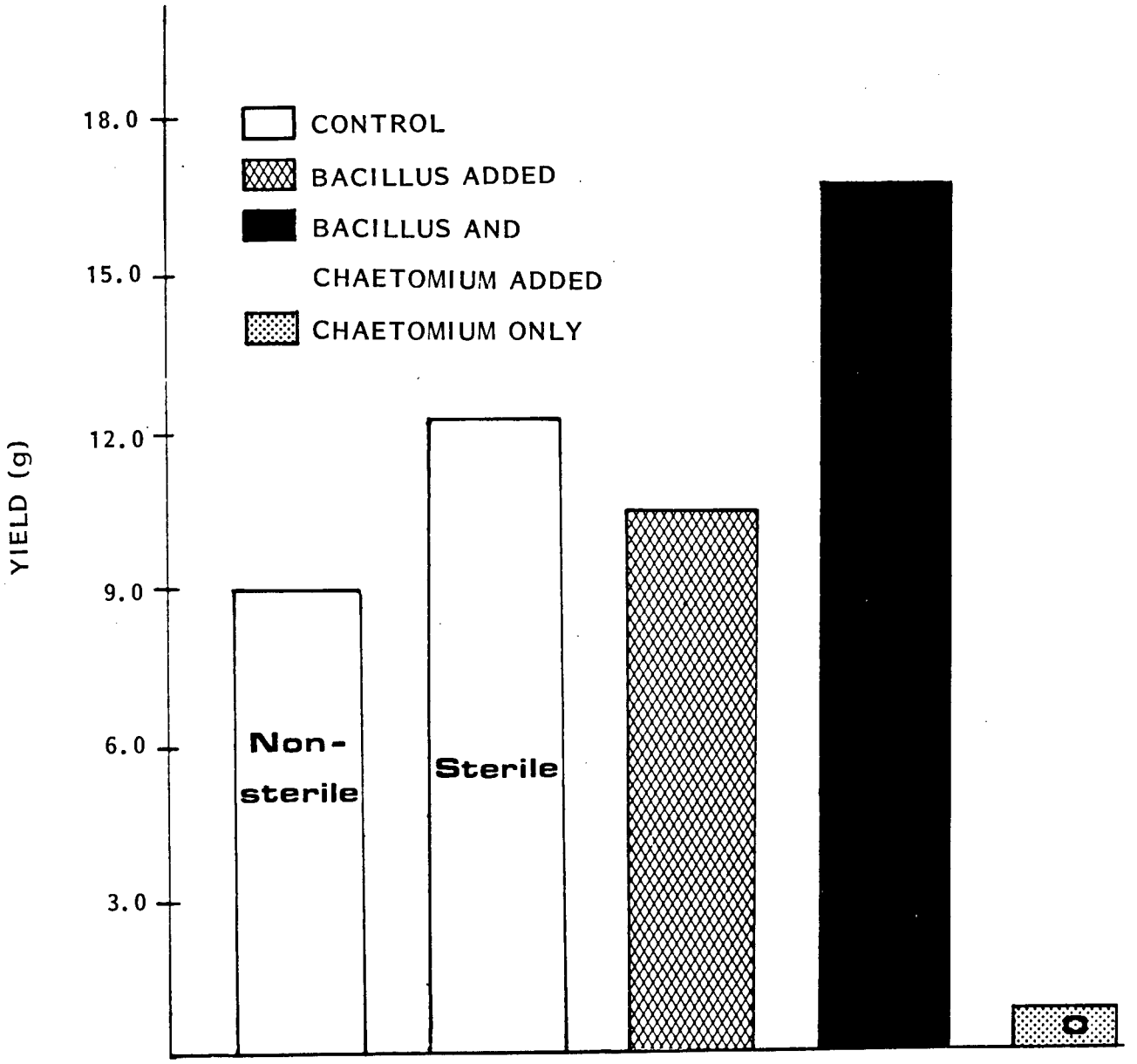


Figure 15. Yield of mushrooms in liquid compost.

trays with Bacillus AOG in liquid compost were shown to produce the earliest occurring flushes as compared to any other medium.

Overall yields in liquid compost were substantially larger than vermiculite trays containing 2% Malt extract. This is probably due to a much greater amount of essential nutrients (in the required form) initially present in the compost - as compared to those in 2% Malt extract (Fermor, 1982). This is substantiated by the carbohydrate analysis of 2% Malt extract during fermentation by Bacillus AOG. As shown in Figure 16, after the fourth day of incubation there is less than 0.05% of available carbohydrate left. Hence, one might conclude that *A. bisporus* obtained its carbon nutrition through utilization of the thermophile Bacillus AOG (or its products). It has been previously proven (Turner, 1977) that Agaricus can synthesize all the needed enzymes for use of microorganisms as a food source. These results clearly show that Bacillus AOG readily supports both mycelial growth and fruiting of the mushroom.

Although liquid compost was the most successful hydroponic medium employed - the yields were still much lower than the conventional experiments. This could be explained by a lack of nutrients during the pinhead formation and/or subsequent fruitbody production stages in the hydroponic medium. In other words, mycelial development was shown to be as good in liquid compost as in the conventional trays (Figures 8 & 14) during the spawn run. Therefore, it seems the initial 500ml of liquid compost/tray (ie. water soluble nutrients of

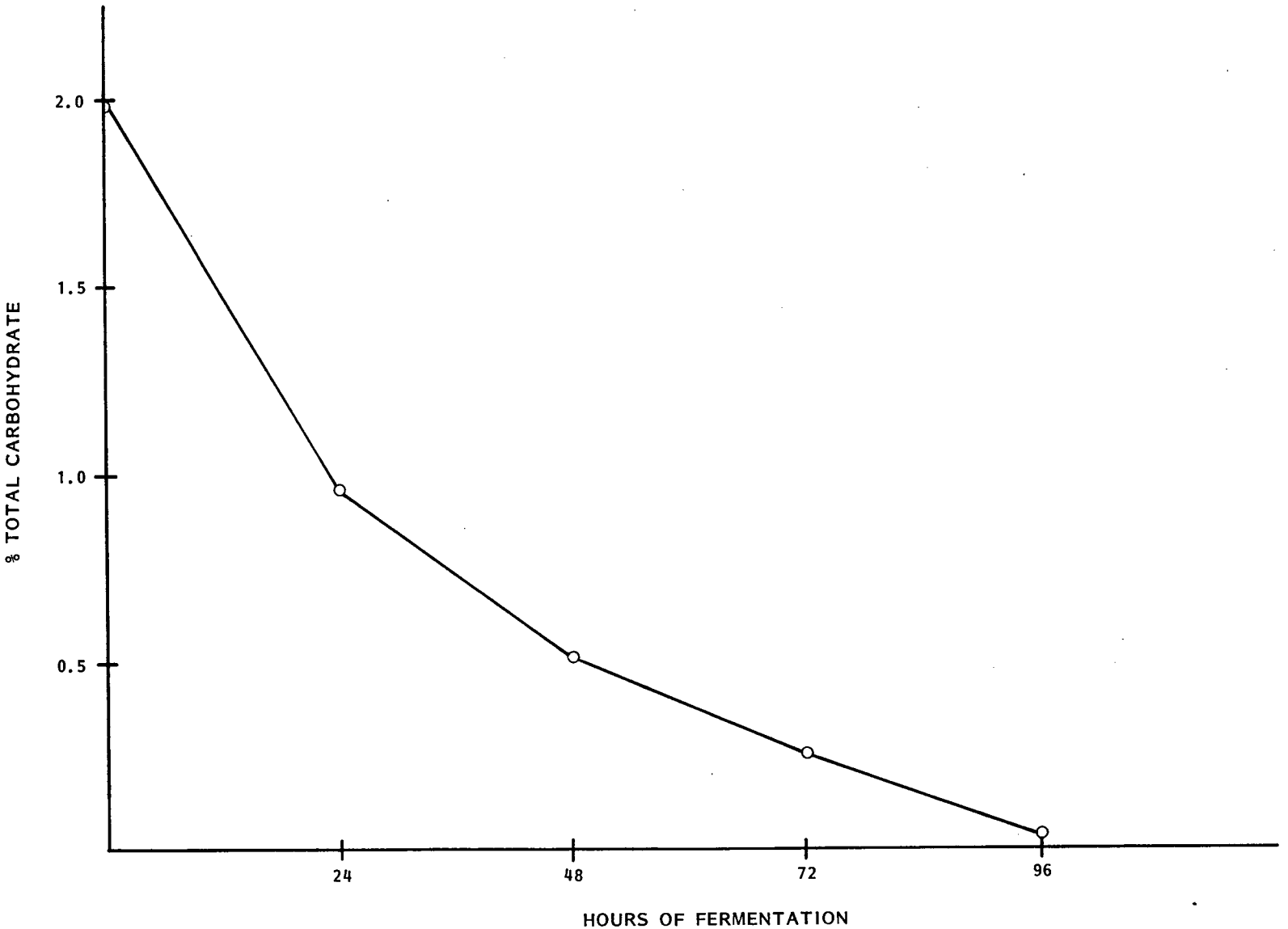


Figure 16. Fermentation of 2% malt extract by Bacillus AOG.

compost) was largely depleted for mycelial development and thus the residual nutrients were insufficient for larger mushroom yields. However, this could easily be corrected in future endeavours by replenishing the vermiculite beds prior to casing with fresh nutrients (or by improving the extraction protocol of the compost).

These experiments clearly show the benefits resulting from selective protection (biological control) through controlled fermentation of the nutrient substrate. The successful use of hydroponics coupled with pure cultures of microorganisms are important factors in the microbiological development of this fermentation process. Compost manufacture and materials are evaluated to cost 20 - 25% of the total mushroom production expenditures (Royse & Schisler, 1980). The annual consumption of horse manure in the U.S.A. now exceeds 350,000 tons and is quickly approaching the supply limit. This is reflected in a doubling of the horse manure price over the past two years (Hatch & Finger, 1979). These costs imply that the mushroom industry will require continued improvements in culture systems to stay competitive with other agricultural crops. However, the problem worsens by the ever changing composition of the manure (ie. manure from different stables) and seasonal variations in temperature and other environmental changes (Huhnke, 1970). These situations cause control of the already complicated composting process to be extremely difficult; and this leads to the production of varying grades of composts which create considerable fluctuations in the mushroom yield and often to total crop

failure. Furthermore, on many larger intensive units, the disposal of used compost frequently poses serious problems (eg. mushroom worker's lung disease; Kleyn et al, 1981). Out of necessity, less horse manure will be utilized in the future years - new forms of raw materials will have to be substituted. Therefore increasing attention is being given to systems of recycling/replenishing (eg. hydroponics) the nutrients which have been utilized by the mushroom so that they can be reused in the succeeding cycle.

Dramatic improvements in composting will be imperative to contribute not only to standardization and control, but also to the economics of culture. With the use of hydroponic methods, the substrate no longer depends on the seasonal weather, and complete regulation of the substrate composition and its reproducible quality are virtually guaranteed. Cultures are healthier due to total elimination of all diseases and pests originally present in the substrate; moreover there have been several reports in North America of botulism poisoning due to consumption of canned mushrooms (Clostridium spores are world-wide in distribution - in soil and compost). The continous delivery of horse manure is no longer necessary and the grower is independent of its production. The bad smell and fumes of ammonia produced from the compost stacks are eradicated, thus facilitating the employment of workers. As neighbors are no longer disturbed by smell, mushroom enterprises can now be created near residential areas. In view of these advantages, it seems well worthwhile to adop the new procedure (thermophilic bacterial liquid feeding) as an economic approach to the fermentation of *Agaricus bisporus*.

IV. Analysis of Inhibitor Produced by Bacillus AOG

The next stage in this thesis investigation was to determine the nature of the inhibitor produced by Bacillus AOG against *Chaetomium olivaceum* (as seen on TSY agar plates).

A. Effect of pH:

The first step in analysis of the Bacillus AOG inhibitor was to determine if a significant change in pH was involved on TSY media (ie. since *B. coagulans* is a known acidophile). This was done by measuring the pH of TSY agar plates at various locations after growth of Bacillus AOG (and challenge with *C. olivaceum*); and by a separate pH growth study of Olive green mold. The pH of the TSY agar plates was shown to be relatively constant throughout the entire agar media (pH 8.8). Furthermore, *C. olivaceum* was observed to be capable of growth throughout an extensive pH range (4-10) and thus would not be inhibited at pH 8.8. Good growth at both high and low pH levels by Olive green mold has also been reported by Beach (1937). These data seem to clearly conclude that a change in pH was not the responsible agent for inhibition of Chaetomium.

B. Extraction of Antibiotic:

Experiments were now designed to determine if the inhibitor/antibiotic[1] (produced by Bacillus AOG) could be extracted from cell-free extracts of fermentation medium.

i) Thermophilic extraction:

Bacillus AOG was first grown at thermophilic temperatures (55°C) in TSY broth for periods of 2-5d. Following filter sterilization and concentration of the samples, attempts were made to extract the antibiotic compound using distilled water or 50% ethanol. However, when these extracts (on filter paper discs) were challenged for activity against *C. olivaceum*, no inhibition of Olive green mold occurred.

Many Bacillus spp. are known to produce large quantities of antibiotic compounds at the sporulation stage of growth (Sadoff, 1972). Hence, a soybean meal fermentation media was utilized to attempt to stimulate extensive spore formation in Bacillus AOG. After 6-8d incubation at 55°C in soybean meal, Gram staining revealed the presence of large ovoid spores in Bacillus AOG. Extraction of the sterile culture filtrates was carried out with n-butanol. This method of extraction (ie. n-butanol) closely resembles

[1]According to Wakman's definition (Stahl, 1969), antibiotics are substances which are formed by microorganisms and which kill off other microorganisms or inhibit their growth. This definition can thus be applied to the product produced by Bacillus AOG (against *C. olivaceum*).

the procedures commonly employed for isolation of many Bacillus sp. producing antibiotics. However, similar to the above extraction these cell-free extracts also showed no activity against Olive green mold when tested on TSY agar.

From the poor results with broth media, it was decided to try to extract the inhibitor directly from the TSY agar plates. This was done by removing aseptically the inhibition zone produced by Bacillus AOG against *C. olivaceum* (on TSY agar) and subsequently attempting to leach the antibiotic out of the agar onto filter paper discs.

Only the experimental trials using sterile air to concentrate the extract onto the sterile filter discs was reasonably successful (Table 5). Other experiments were found to be either contaminated (ie. due to Bacillus AOG spores present), or demonstrated no antagonism towards *C. olivaceum* (probably due to low amounts of compound absorbed onto discs). These methods were repeated twice with no better yielding results.

ii) Mesophilic extraction:

It was suggested that since plates showing inhibition of Olive green mold by Bacillus AOG had been incubated at 25°C, the production of this antibiotic compound may only be created during mesophilic growth temperatures. Hence, Bacillus AOG was grown in TSY broth at 25 and 37°C for periods ranging from 2-8d. Following

Table 5. Extraction of inhibitor from TSY agar discs.

Experiment	Zone of inhibition [1] (mm)
1) control - uninoculated TSY agar	0
2) agar disc + filter paper only	0
3) agar disc with 5 ml distilled water (+/- vortex)	0
4) saturation of disc utilizing sterile air	22

[1] ave. of 2 plates

harvesting, cell-free concentrates were extracted with n-butanol and saturated into filter paper discs. As shown in Figure 17, the production of the antibiotic was indeed only produced during the lower growth temperatures by Bacillus AOG.

It might be postulated that the organism Bacillus AOG only synthesizes this antibiotic at a mesophilic range of temperature because of certain thermophilic requirements at higher temperatures. In other words, the inhibitor may be a needed component of the cell wall under the thermophilic requirements (55°C) but is not required at mesophilic temperatures (25, 37°C) and thus becomes expelled from the cell.

All further extractions of the antibiotic were conducted with cultures at 25°C in TSY broth for 7d incubation. Extraction and concentration of the antibiotic with n-butanol can also be done directly (ie. without freeze-drying) by evaporation/concentration of the fermentation broth on a Rotavapor apparatus (see Methods & Materials). Successful extraction by evaporation is especially suited for possible industrial applications (ie. excessive costs are employed for freeze-drying).

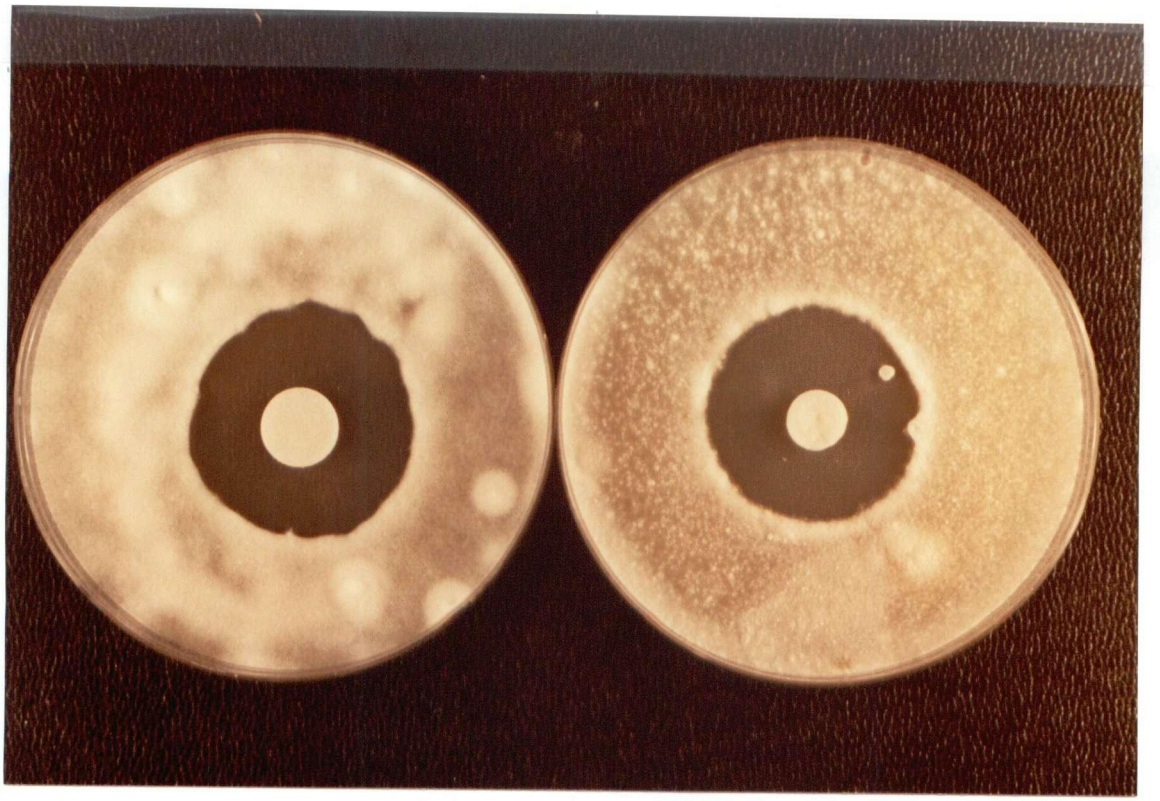


Figure 17. Inhibition of *C. olivaceum* from cell-free extracts of Bacillus AOG.

C. Temperature and pH Stability of Antibiotic:

The extracted antibiotic was shown to be stable over a wide range of both pH and temperature. It was found to be active (towards *C. olivaceum*) over the entire range of pH (2-10) and temperature (-15°C to 150°C) tested. Even after storage for 4 months at -15°C no loss in activity was observed. Hence, the antibiotic appears to be capable of growth over an extensive spectrum of physical conditions.

D. Solvent Solubility:

Studies on the solvent solubility of the antibiotic demonstrated it to be soluble in polar solvents (Table 6); water alone, n-butanol, n-propanol, isopropanol, ethanol, methanol, dioxane, pyridine, and acetone; whereas it is insoluble in chloroform, ethyl acetate, toluene, hexane, benzene, petroleum ether, and carbon tetrachloride. This antibiotic thus seems to be hydrophilic in nature.

Table 6. Solvent solubility of antibiotic.

Solvent	pH	Zone of Inhibition (Avg of duplicate plates) (cm)
water	7.0	3.3
acetone	7.5	3.4
n-propanol	7.5	3.3
pyridine	7.6	3.0
n-butanol	7.0	3.0
isopropanol	7.5	3.0
ethanol (100%)	7.5	3.0
methanol	8.0	2.9
dioxane	7.5	2.9
ethyl acetate	6.5	0.0
chloroform	6.5	0.0
toluene	6.5	0.0
hexane	6.5	0.0
benzene	7.0	0.0
petroleum ether	6.5	0.0
carbon tetrachloride	6.5	0.0
cyclohexanol	6.5	4.0[1]

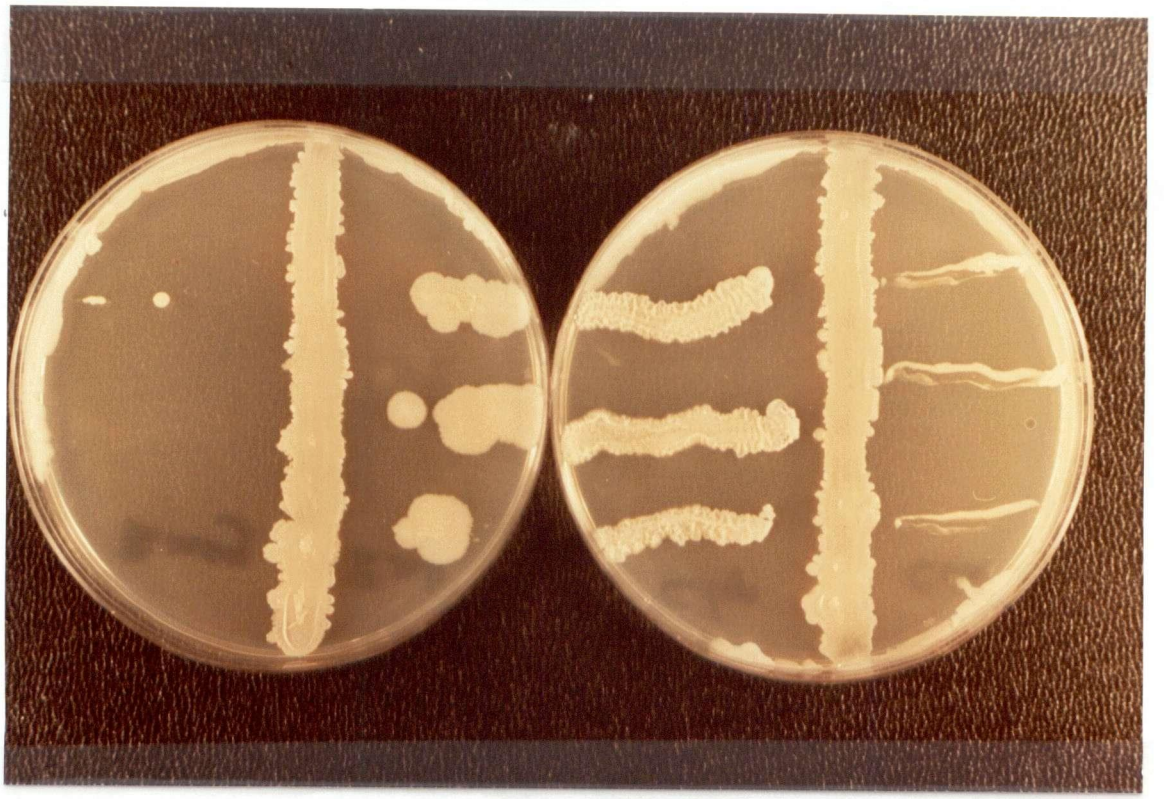
[1]control plates (ie. cyclohexanol only) also inhibited C. olivaceum to the same extent.

E. Spectrum of Activity:

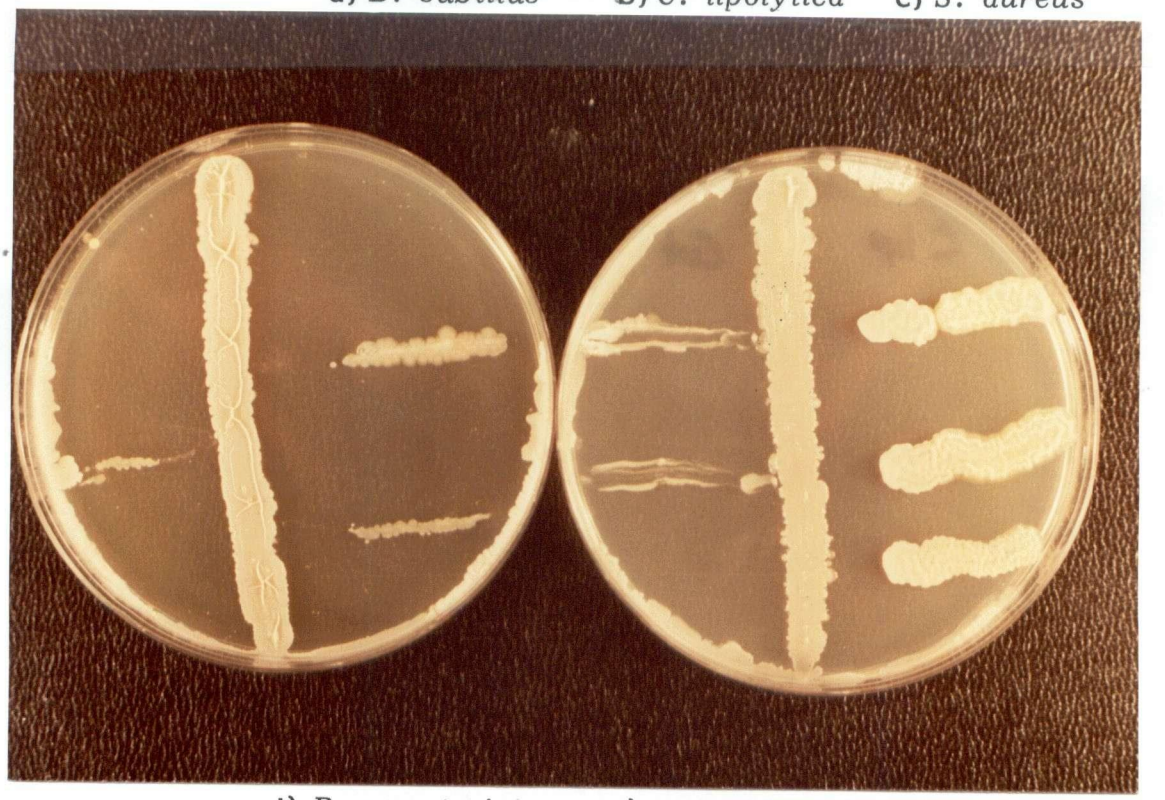
It was found that the antibiotic demonstrated no suppression of growth of any Gram negative organisms or of the Gram positive cocci tested. However, when tested against *Bacillus subtilus* and *B. megaterium* it was found to be quite potent (Figure 18). Furthermore, the growth of the fungus *Candida lipolytica* was also inhibited. Therefore this antibiotic can be used effectively not only against *Chaetomium olivaceum* but other microorganisms as well.

F. Thin Layer Chromatography:

Because of its high resolving power and speed, thin layer chromatography lends itself well to the separation and identification of antibiotics (Stahl, 1969). Therefore, purification of the antibiotic produced by Bacillus AOG was initially carried out by TLC on Silica Gel plates. Table 7 shows the results of various solvent systems used to determine the optimum separation of the crude antibiotic extract. The presence of pyridine in any solvent system was shown to destroy the biological activity of the antibiotic and hence not further used. It was found that the best results occurred with an n-butanol - acetic acid (glacial) - water (60:20:20) solvent system followed by purification in a methanol - chloroform - 17% ammonium hydroxide (40:40:20) TLC system. These solvent mixtures are two of the most widely utilized systems in separation of antibiotics (Stahl, 1969).



a) *B. subtilus* b) *C. lipolytica* c) *S. aureus*



d) *B. megaterium* e) *S. lactis* f) *C. lipolytica*

Figure 18. Spectrum of activity of Bacillus AOG against various microorganisms.

Table 7. Solvent systems investigated to determine optimum TLC separation of crude antibiotic.

Solvent System	Observations
n-butanol:glacial acetic acid:water (60:20:20)	optimum separation
methanol:chloroform:17% ammonium hydroxide (40:20:20)	"
n-butanol:n-propanol:water (60:20:20)	fair to good separation
n-butanol:n-propanol:water:glacial acetic acid (60:20:10:5)	"
n-propanol:water (70:30)	"
methyl ethyl ketone:pyridine:water: glacial acetic acid (70:15:15:2)	pyridine found to cause loss in biological activity
n-butanol:pyridine:glacial acetic acid:water (30:20:6:24)	"
n-propanol:water:pyridine (70:15:15)	"
ethyl acetate:pyridine:glacial acetic acid:water (50:10:10:10)	"
chloroform:methanol (50:50)	no movement of sample from origin
methanol:chloroform (3:97)	"
ethyl acetate:methanol (87:13)	"

As previously mentioned (Methods & Materials), freeze-dried extracts of Bacillus AOG (in n-butanol) were streaked across a Silica Gel 60 chromatogram and run in a butanol - acetic acid - water TLC system. To locate the biologically active bands, a portion of the air dried plate was layered with TSY agar, sprayed with Olive green mold and incubated at 25°C. Two Ultraviolet light positive (visible under both short and long wave - however, brighter under short) bands with R_f values of .46 and .52 (analytical 0.2mm plate) resulted in inhibition of *C. olivaceum*. This inhibition was shown to be extremely stable on the TLC plates. The lower band (Band I) appeared as a broad yellow band under short wave U.V. light; whereas the upper band (Band II) appeared as a narrow light blue band. Furthermore, Band I was observed to produce bubbles when it was layered with TSY agar - this may possibly indicate gas production due to the possible presence of perhaps a carboxyl group or CO_2 group in the lower TLC band.

Bands I and II when sprayed with ninhydrin reagent produced a violet color reaction indicating the possible presence of amino acids (Stahl, 1969). Presence of lipids was not observed in either band when TLC chromatograms were sprayed with either Rhodamine 6G or *alpha*-cyclodextrin. A phenol-sulphuric acid spray resulted in Band I appearing light brown and Band II a medium brown color after heating plates for 15min at 100°C. These color reactions may denote the presence of sugars in the two biologically active bands (Stahl, 1969).

When preparative plates (2.0mm thickness) were used for purification

in butanol - acetic acid - water, chromatograms were air dried after the first run, and developed a second time in this same solvent (in same direction) which resulted in better separation of the bands. R_f values of .66 for Band I and .73 for Band II resulted.

The two biologically active bands (from preparative plates) were then scraped off the chromatogram, extracted in n-butanol and layered across separate analytical Silica Gel plates (0.2mm thickness) for further purification in methanol - chloroform - 17% ammonium hydroxide. Earlier observations showed poor separation had occurred in the methanol - chloroform - 17% ammonium hydroxide system when using a preparative TLC plate.

R_f values in methanol - chloroform - 17% ammonium hydroxide were: for Band I, .27; and for Band II, .32. Bands on these plates were also shown to be biologically active and ninhydrin positive when layered with TSY agar and challenged with Olive green mold; or sprayed with ninhydrin reagent respectively.

Purification of the Bacillus AOG antibiotic by thin layer chromatography was shown to be an extremely long and tedious process (and expensive). This is especially true when analytical TLC plates of only 0.2mm were used for the final purification stage. This resulted in extremely low amounts of pure inhibitor being isolated. For example, approximately 20 analytical Silica plates would result in only perhaps a yield of 1-2mg of antibiotic crystals. Hence, it was decided to utilize column chromatography techniques in an attempt to isolate larger quantities of the antibiotic.

G. Column Chromatography:

i) Ion-exchange:

Because of the possible presence of amino acids in the antibiotic (as indicated by ninhydrin reaction on TLC plates) ion-exchange chromatography was employed as the first column method of separation.

The antibiotic extract (in n-butanol) was carefully layered onto a cation exchange resin (H^+ form) with a high cross linking (X-8) capacity. This high cross linking made it especially suited for separation of amino acid and small peptide compounds (according to manufacturer's suggestion). However, as shown by the elution profile (Figure 19) of the antibiotic extract, the sample did not bind to the column and was washed out during the first elution with distilled water. Non-binding may have been due to the sample having no ionic charge, or possessing the same charge (-) as the functional group of the resin (ie. SO_3^-). In other words, during ion-exchange chromatography, neutral molecules and those having the same charge as the functional group (ie. of the column) flow through the column and are separated from any sorbed ions (ie. those with a + charge in this case). Or, perhaps non-binding may have been due to the sample being a much larger compound than initially believed.

ii) Sephadex LH 20:

Further column chromatography of the antibiotic was conducted with a Sephadex LH 20 type

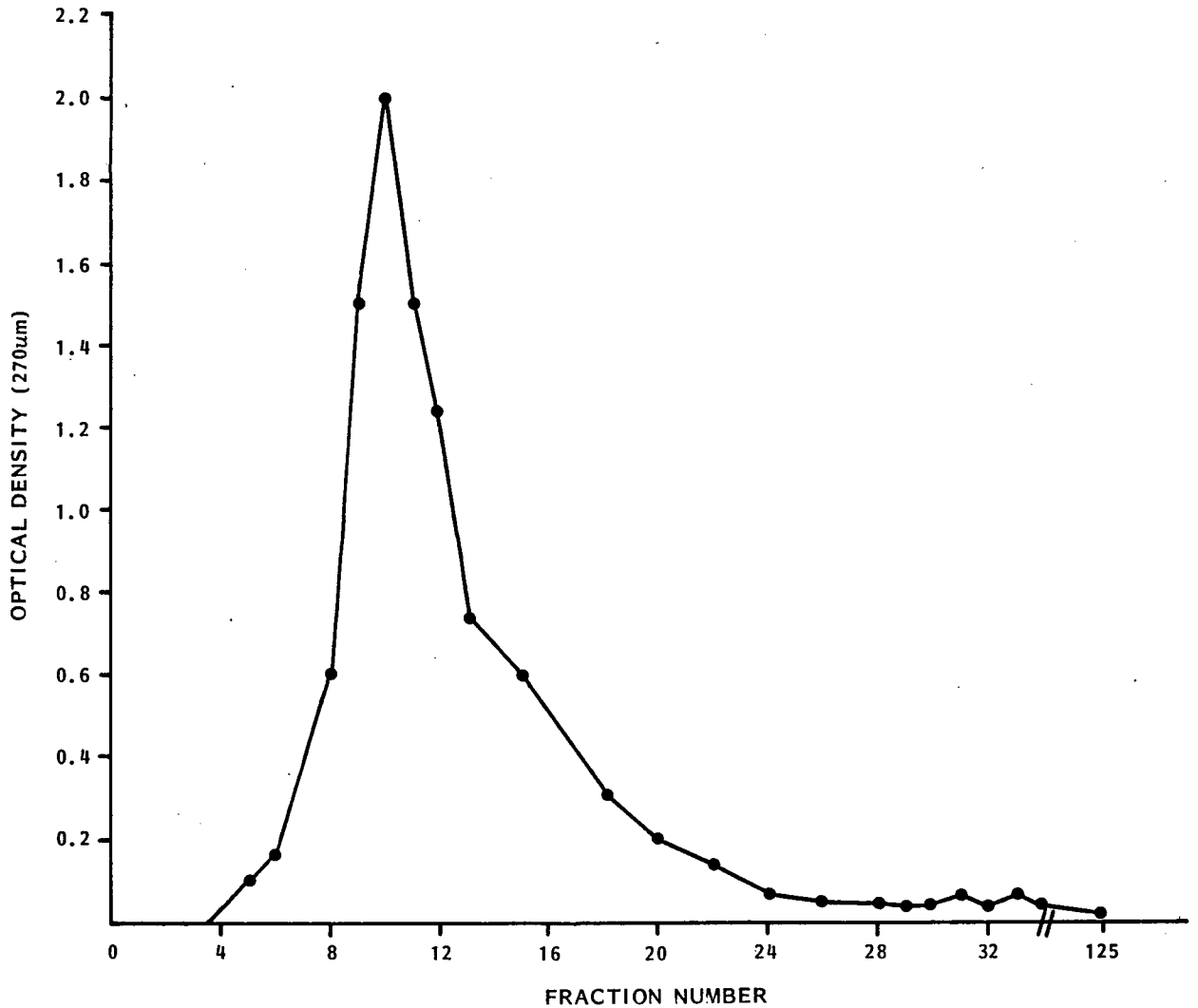


Figure 19. Antibiotic elution profile in cation exchange resin.

resin. Sephadex LH 20 can be used in polar solvents, and is commonly employed for separation of biologically active substances, natural and synthetic polymers, and low molecular weight solutes (Joustra et al, 1967). As well as separating compounds according to size LH 20 possesses both hydrophilic and lipophilic properties, and can be used for partition chromatography.

The Bacillus AOG extract (in distilled water) was layered onto the Sephadex LH 20 column and eluted with distilled water. The use of distilled water as an eluant facilitated the fractions capability of later being freeze-dried for concentration. All tubes were scanned throughout the U.V. spectrum and the fractions from 5 peaks (demonstrating similar U.V. spectra) were pooled and freeze-dried.

Thin layer chromatography of the reconstituted fractions revealed biological activity (against *C. olivaceum*) from only one set of pooled fractions (tubes 16-20). These two biologically active bands were visible under U.V. light and were also ninhydrin positive (ie. similar to previous TLC results). However, it seems Sephadex LH 20 did not separate the two bands (Band I and II) to any greater extent than utilizing a single purification stage with TLC in butanol - acetic acid-water (ie. similar R_f values). Since optimum purification was necessary for further analysis of the antibiotic, it was therefore decided to continue with thin layer chromatography as the method of purification.

One should keep in mind however, that Sephadex LH 20 by no means was proven ineffective as a chromatography technique for the Bacillus AOG antibiotic. This resin could potentially be used in future endeavours

at large scale purification of the antibiotic with perhaps certain modifications. For example, experimental trials with variations in sample size, column size, and type of eluant used might result in better separation of the bands as compared to that witnessed in this initial study.

H. Ultraviolet Spectrum Analysis:

Figure 20 shows the ultraviolet spectra of purified Bands I and II. Band I demonstrated a fairly broad peak at 275mu and a smaller peak at approximately 288mu. Band II was observed to consist of a single peak at 270mu.

The absorptivity coefficients were .835 and .401 $mgml cm^{-1}$ for Bands I and II respectively.

I. Fluorescent Spectrum Analysis:

Figure 21 represents the fluorescent spectrum of TLC Band I. As can be seen, a single peak occurred at 360um - with a relative intensity of 42.

There was no fluorescent spectrum detectable for Band II. This is in agreement with the results of amino acid analysis (Section J) in which tryptophan was not found in Band II (ie. tryptophan is the major amino acid responsible for fluorescence).

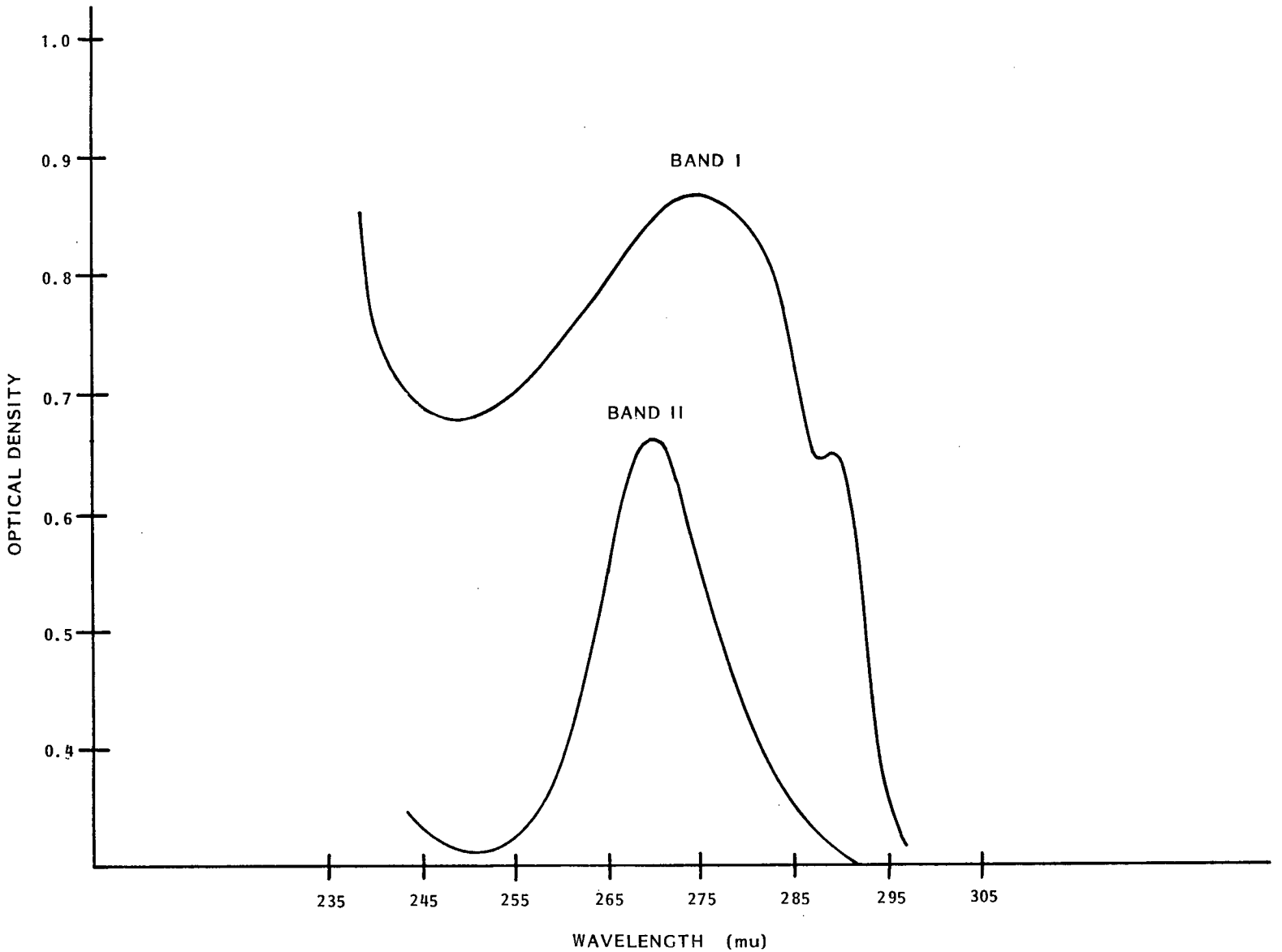


Figure 20. UV absorption spectra of lower (Band I) and upper (Band II) TLC bands.

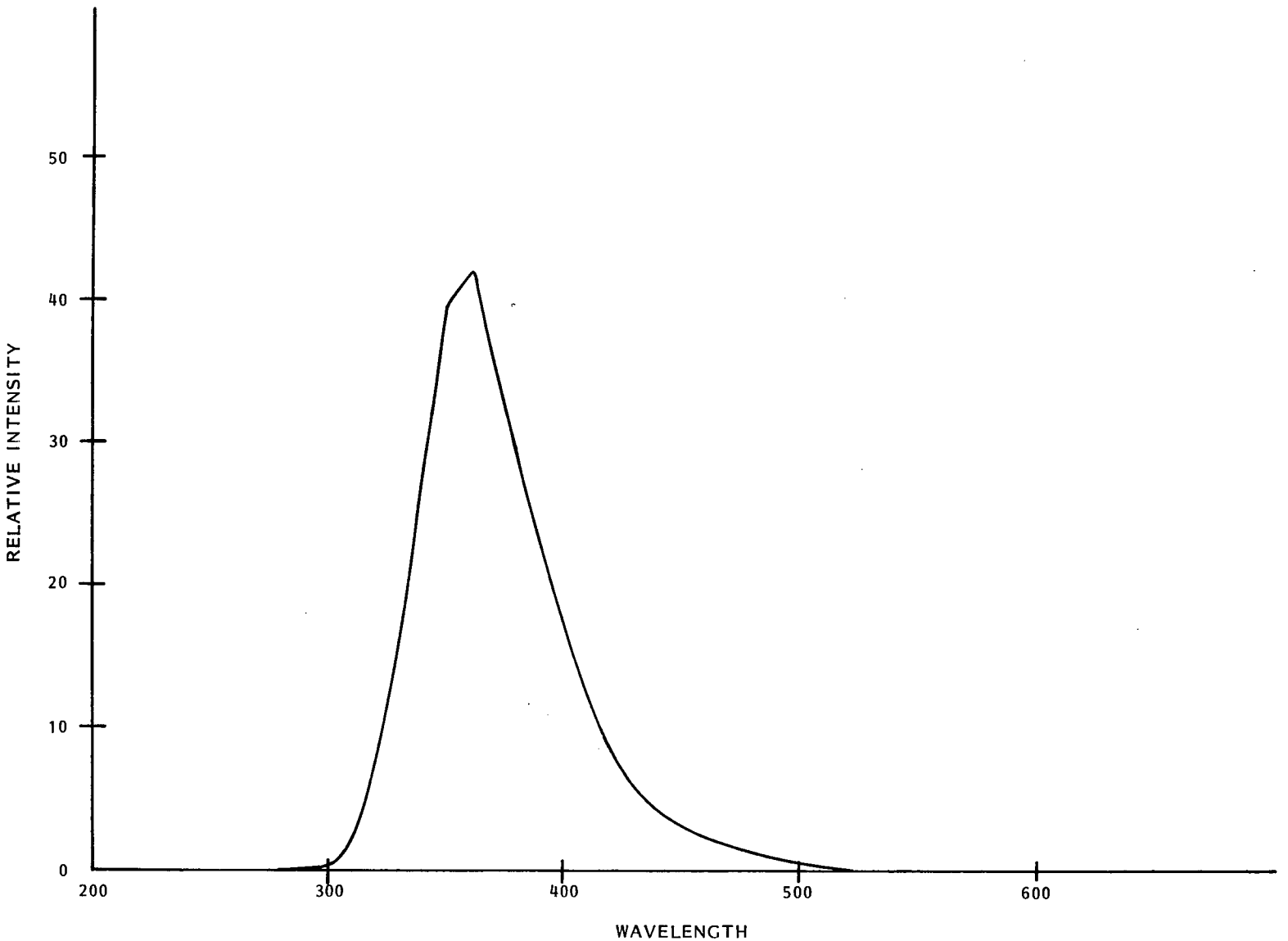


Figure 21. Fluorescent spectrum of TLC band I.

J. Amino Acid Analysis:

Amino acid analysis of Bands I and II from TLC chromatography revealed the inhibitor produced by Bacillus AOG to be a peptide antibiotic (Table 8). Both bands were relatively similar in amino acid composition. They both contained high amounts of tyrosine, serine, aspartic and glutamic acids. Band I however, contained approximately three times the amount of phenylalanine as compared to Band II. Furthermore, Band I contained small amounts of lysine, histidine, tryptophan and proline - these amino acids were not detected in Band II.

Although cysteine was not SH blocked prior to hydrolysis (due to small amounts of compound) not even a small peak was apparent for the presence of this amino acid. Furthermore, the breakdown products of cysteine (cysteic acid) from hydrolysis with p-toluenesulfonic acid were shown not to be present. Unusual amino acids or peaks which could not be accounted for were also not found.

This amino acid profile also indicates the antibiotic to be relatively acidic in nature. This feature might be responsible for the earlier observations (Section G) associated with ion-exchange chromatography (ie. non-binding to resin).

These results pose many interesting questions regarding the peptide antibiotics produced by Bacillus AOG. For example, why does the organism produce two distinct antibiotic structures, why are they synthesized (especially at only mesophilic temperatures), and are these

Table 8. Amino acid analysis of Bands I and II.

	Band I	Band II
	(g/100g amino acid)	(g/100g amino acid)
Aspartic acid	19.90	24.40
Phenylalanine	18.90	5.66
Tyrosine	14.51	17.63
Serine	14.19	17.11
Glutamic acid	12.34	17.06
Threonine	5.72	8.47
Leucine	2.80	1.72
Tryptophane	2.80	0.00
Proline	1.52	0.00
Isoleucine	0.60	0.98
Valine	0.54	0.66
Alanine	0.41	0.33
Glycine	0.40	0.98
Lysine	0.22	0.00
Histidine	0.12	0.00
Total	94.97	95.00

antibiotic compounds unique (ie. have they been isolated before)?

During the past thirty years of antibiotic screening, members of the genus Bacillus have proven to be the most successful of all in the order of Eubacteriales in the exploration for new antibiotics (Meyers et al, 1973). Furthermore, it is generally recognized , these antibiotics are mainly peptide in structure. Shoji (1978) reports that the number of known antibiotics from Bacillus is approximately 117, of which 80 members are peptides. Most of the peptide antibiotics formed by these organisms are composed entirely of amino acids (eg. tyrocidines, gramidicin S), where as others may contain amino acids plus other constituents (eg. ring structures, amino sugars, fatty acids)(Katz & Demain, 1977).

Why would Bacillus AOG produce two antibiotic substances which are relatively similar in amino acid composition? Examination of the literature reveals that in the case of bacilli, this is the rule rather than the exception. Katz & Demain (1977) state that generally a family of closely related peptides rather than a single substance is produced by a Bacillus organism. The members may differ from each other by one, or at most, a few amino acids (Perlman & Bodanszky, 1971). For example, *Bacillus subtilus* produces at least fourteen distinct (but related) antibiotics; *B. brevis* also produces a multiplicity of peptide antibiotics (23) (eg. edeine, gramicidin, and tyrocidine) (Sādoff, 1972). The antibiotics of the Bacillaceae are similar in the following general properties: 1) they are peptides comprised of 6-15 amino acids; 2) they are produced by cells after exponential growth during the course of

sporulation; 3) their direct synthesis does not involve mRNA or ribosomes as in the case of normal protein synthesis, and 4) peptide antibiotics specifically inhibit important cellular processes (eg. DNA synthesis).

Little progress has been made in elucidating the function(s) of antibiotics in the producing organism (Bu'Lock, 1961). Generally members of the Bacillaceae are characterized by their ability to produce resistant endospores and in this course, to elaborate peptide antibiotics (Hodgson, 1970). It thus seems appropriate to assume that the sporulation antibiotics also exert some controlling outcome in the cells from which they have been excreted. As Bu'Lock (1961) reports, antibiotic synthesis is a means of keeping the cellular machinery in working order during the time when cell growth is not possible due to unfavorable conditions.

Hodgson (1970) believes that peptide antibiotics might be used in several ways by an organism such as modifiers of the cell membrane; for example, ion carriers, modifying permeability characteristics. Furthermore, Sadoff (1972) states that the peptide antibiotics produced by the bacilli exhibit effects upon membrane synthesis and function, cell wall synthesis, and nucleic acid synthesis. It was contended that the antibiotic when produced by an organism, may act as selective modifiers of cell function - ie. repressing or inhibiting vegetative cell macromolecular synthesis. The quantities required in sporulation might amount to relatively few molecules per cell. Thus antibiotic production by Bacillus AOG probably represents an amplification of a normal event which occurs to a lesser degree in all

aerobic sporulating bacilli.

Although the mechanism of antibiotic synthesis varies from protein synthesis, there appears to be a competition between these two processes for the amino acids available in the cell. For example, during active cellular growth of *B. licheniformis* with high protein synthesis, practically no bacitracin was produced (Studer, 1967). In contrast, bacitracin synthesis was high when the requirement for protein synthesis was inhibited. It thus seems plausible that at thermophilic temperatures Bacillus AOG may require added protein synthesis for thermophilic requirements (eg. to maintain the integrity of the cell components) and thus excess amino acids are not available for production of peptide antibiotics. At lower temperatures (25°C) Bacillus AOG is no longer in a "stressed-state", less cell wall material is needed, and thus there might be a larger number of available amino acids. An abundance of amino acids at mesophilic temperatures might normally result in cell death due to unbalanced growth (Weinberg, 1971). Bacillus AOG may therefore have a system of detoxifying itself by incorporating the metabolites (ie. amino acids) to antibiotics which are then released from the cell. Hence, the production of antibiotics might be more likely to occur at lower mesophilic temperatures.

Matteo et al (1976) reports that it is quite possible that peptide antibiotic formation is controlled by carbon and nitrogen catabolite repression or is under growth rate control; manipulations which affect those controls (eg. possibly large fluctuations in temperature) would be expected

to modify the temporal relationship between antibiotic synthesis and growth.

Still under current consideration, is the possibility that antibiotics function to kill or inhibit the growth of other organisms in nature, thereby providing a competitive advantage to the producing organism (Gottlieb, 1976). One might speculate that Bacillus AOG has evolved to produce antibiotics at only mesophilic temperatures because there is a much larger number of mesophilic competitive organisms as compared to those at thermophilic temperatures - especially in mushroom compost from where Bacillus AOG was initially isolated.

The three main classes of antifungal peptides (possessing only slight antibacterial activity) produced by Bacillus sp. are the bacillomycins, mycobacillins, and fungistatin antibiotics (Studer, 1967). Similar to the Bacillus AOG inhibitor, they have proven to be extracellular, acidic (fungistatin - basic), contain no sulphur amino acids, and are heat-stable peptides. They are also soluble in polar, and insoluble in non-polar organic solvents (Sharon et al, 1954).. Most of these antibiotics, although isolated many years ago are known only in their qualitative composition. Except for fungistatin (which contains unidentified amino acids), both the bacillomycin and mycobacillin family contain only known amino acids with no other components.

However, closer examination reveals that severe disparities occur between these Bacillus antibiotic classes and the Bacillus AOG compound - and thus can not be grouped into these families. For example, both

mycobacillin and bacillomycin are ninhydrin negative (Sharon et al, 1954), and significant differences in content of specific amino acids (eg. bacillomycin contains only 5 amino acids), U.V. adsorption, and solvent solubility occurs.

A further extensive examination of the literature concerning fungal peptide antibiotics reveals as of yet, no known antibiotics with identical properties (eg. amino acids, U.V. spectra, solvent solubility etc.) as that observed in this thesis investigation of the Bacillus AOG antibiotic. One may reasonably conclude therefore, that the inhibitor produced by Bacillus AOG is a unique antibiotic compound and thus has been given the name Chaetomacin.

CONCLUSIONS

Mushroom cultivation is now one of the most intensive and most technically demanding of all vegetable cultivations practised throughout the world (Smith, 1969). The fact that basidiomycetes convert waste materials into a highly flavored proteinaceous food is clearly relevant to the requirements of both the emerging and technologically advanced countries. The future role of mushrooms will be governed by the economics of production methods and costs relative to other animal and vegetable foods (Hayes & Nair, 1975). However, escalating costs for maintenance and operating continue to plague growers with the high cost of labor being the most significant (greater than 50% of the total production costs).

The commercial mushroom industry has suffered serious crop losses for years by the uncontrollable damage in compost beds caused by the weed mold *Chaetomium olivaceum*. There is currently no known means to successfully control this pathogen. This thesis investigation resulted in the isolation of a thermophilic Bacillus sp (resembling *B. coagulans* - resistant to 0.02% sodium azide, acidophilic) which showed dramatic activity against *C. olivaceum* on TSY agar plates. Studies involving both conventional and hydroponic mushroom cultivation methods demonstrated Bacillus AOG to significantly protect the mushroom from Olive green mold damage as well as to increase yields of *Agaricus bisporus*.

The observation that initiation and development of fruitbodies were not retarded shows that Bacillus AOG had no apparent inhibitory effect on organisms such as *P. putida*, known to stimulate the formation of sporophores. These experiments clearly show the benefits resulting from selective protection through controlled fermentation of the nutrient substrate.

The isolation of a thermophilic microorganism antagonistic towards Olive green mold is an extremely unique and significant finding when considering microbial development of the commercial mushroom industry. The reserve of chemical controls for mushroom pathogens continues to shrink as various materials become ineffective or are removed from usage because of residues or suspected residues of toxic compounds in the marketed product (Ingratta, 1980). This novel finding (Bacillus AOG) could potentially be used as an effective application of biological control in this solid state fermentation process. Mushroom producers now have the capability of protecting their crops from Chaetomium damage for the first time in the history of this industry.

Substrates assume the greatest share of production costs (Hayes & Wright, 1979). The main advantages of hydroponics over composting are: much less labor intensive, more efficient regulation of nutrient composition (ie. completely controlled fermentation with known thermophiles present), efficient use of water (important in arid countries), protection from pathogens, and permanence of medium (compost must be discarded regularly - whereas vermiculite may last for several years

and is very light to facilitate transportation) - possibly leading to eventual continuous culture techniques.

Bacillus AOG was shown to produce a potent antibiotic (named Chaetomacin) at mesophilic temperatures. Extraction of this compound with n-butanol revealed it to be a stable substance, effective against other fungi and Bacillus species. Purification through thin layer chromatography revealed two compounds with close R_f values. Amino acid analysis showed these two bands to be similar in structural compostion. Examination of the literature reveals no other previously isolated antibiotics which are identical to the inhibitors found in this thesis study. Chaetomacin could potentially be extended into the preparation of differential media and in the protection of foods and plants from fungal invasion.

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APPENDICES

APPENDIX A

Statistical analysis of mycelial diameters.

CONVENTIONAL

LINE 1 : NOTH DAY LINE 2 : BAC DAY

	INDIVIDUAL SIMREG LINES		STANDARD ERROR OF ESTIMATE	CORRELATION COEFFICIENT	DEGREES OF FREEDOM
1	A= 0.34857E+00	B= 0.18393E+00	SEY=0.6594E-01	R= 0.9887	DF= 6.
2	A= 0.25714E+00	B= 0.27143E+00	SEY=0.1038E+00	R= 0.9872	DF= 6.
	SIMREG FOR POOLED DATA				
3	A= 0.30286E+00	B= 0.22768E+00	SEY=0.1864E+00	R= 0.9351	DF= 13.

COVARIANCE ANALYSIS

TEST FOR HOMOGENEITY OF RESIDUAL VARIANCES

F, 5./ 5.= 0.403E+00

TEST FOR SLOPES

F, 1/ 10.= 0.142E+02

TEST FOR LEVELS

F, 1/ 11.= 0.141E+02

OVERALL TEST

F, 2./ 10.= 0.226E+02

CONVENTIONAL

LINE 1 : NOTH DAY LINE 2 : B+C DAY

	INDIVIDUAL SIMREG LINES		STANDARD ERROR OF ESTIMATE	CORRELATION COEFFICIENT	DEGREES OF FREEDOM
1	A= 0.34857E+00	B= 0.18393E+00	SEY=0.6594E-01	R= 0.9887	DF= 6.
2	A= 0.60000E-01	B= 0.28071E+00	SEY=0.1104E+00	R= 0.9865	DF= 6.
	SIMREG FOR POOLED DATA				
3	A= 0.20429E+00	B= 0.23232E+00	SEY=0.1437E+00	R= 0.9614	DF= 13.

COVARIANCE ANALYSIS

TEST FOR HOMOGENEITY OF RESIDUAL VARIANCES

F, 5./ 5.= 0.357E+00

TEST FOR SLOPES

F, 1/ 10.= 0.159E+02

TEST FOR LEVELS

F, 1/ 11.= 0.175E+01

OVERALL TEST

F, 2./ 10.= 0.999E+01

CONVENTIONAL

LINE 1 : NOTH LINE 2 : CHAE
DAY DAY

	INDIVIDUAL SIMREG LINES		STANDARD ERROR OF ESTIMATE	CORRELATION COEFFICIENT	DEGREES OF FREEDOM
1	A= 0.34857E+00	B= 0.18393E+00	SEY=0.6594E-01	R= 0.9887	DF= 6.
2	A= 0.45714E+00	B= 0.14286E+00	SEY=0.6761E-01	R= 0.9806	DF= 6.
	SIMREG FOR POOLED DATA				
3	A= 0.40286E+00	B= 0.16339E+00	SEY=0.8118E-01	R= 0.9746	DF= 13.

COVARIANCE ANALYSIS

TEST FOR HOMOGENEITY OF RESIDUAL VARIANCES

F, 5./ 5.= 0.951E+00

TEST FOR SLOPES

F, 1/ 10.= 0.530E+01

TEST FOR LEVELS

F, 1/ 11.= 0.175E+01

OVERALL TEST

F, 2./ 10.= 0.387E+01

CONVENTIONAL

LINE 1 : BAC DAY LINE 2 : B+C DAY

	INDIVIDUAL SIMREG LINES		STANDARD ERROR OF ESTIMATE	CORRELATION COEFFICIENT	DEGREES OF FREEDOM
1	A= 0.25714E+00	B= 0.27143E+00	SEY=0.1038E+00	R= 0.9872	DF= 6.
2	A= 0.60000E-01	B= 0.28071E+00	SEY=0.1104E+00	R= 0.9865	DF= 6.
	SIMREG FOR POOLED DATA				
3	A= 0.15857E+00	B= 0.27607E+00	SEY=0.1309E+00	R= 0.9767	DF= 13.

COVARIANCE ANALYSIS

TEST FOR HOMOGENEITY OF RESIDUAL VARIANCES

F, 5./ 5.= 0.884E+00

TEST FOR SLOPES

F, 1/ 10.= 0.105E+00

TEST FOR LEVELS

F, 1/ 11.= 0.849E+01

OVERALL TEST

F, 2./ 10.= 0.395E+01

CONVENTIONAL

LINE 1 : BAC LINE 2 : CHAE
DAY DAY

	INDIVIDUAL SIMREG LINES		STANDARD ERROR OF ESTIMATE	CORRELATION COEFFICIENT	DEGREES OF FREEDOM
1	A= 0.25714E+00	B= 0.27143E+00	SEY=0.1038E+00	R= 0.9872	DF= 6.
2	A= 0.45714E+00	B= 0.14286E+00	SEY=0.6761E-01	R= 0.9806	DF= 6.
	SIMREG FOR POOLED DATA				
3	A= 0.35714E+00	B= 0.20714E+00	SEY=0.2334E+00	R= 0.8866	DF= 13.

COVARIANCE ANALYSIS

TEST FOR HOMOGENEITY OF RESIDUAL VARIANCES

F, 5./ 5.= 0.236E+01

TEST FOR SLOPES

F, 1/ 10.= 0.302E+02

TEST FOR LEVELS

F, 1/ 11.= 0.123E+02

OVERALL TEST

F, 2./ 10.= 0.376E+02

CONVENTIONAL

LINE 1 : B+C DAY LINE 2 : CHAE DAY

	INDIVIDUAL SIMREG LINES		STANDARD ERROR OF ESTIMATE	CORRELATION COEFFICIENT	DEGREES OF FREEDOM
1	A= 0.60000E-01	B= 0.28071E+00	SEY=0.1104E+00	R= 0.9865	DF= 6.
2	A= 0.45714E+00	B= 0.14286E+00	SEY=0.6761E-01	R= 0.9806	DF= 6.
	SIMREG FOR POOLED DATA				
3	A= 0.25857E+00	B= 0.21179E+00	SEY=0.1900E+00	R= 0.9235	DF= 13.

COVARIANCE ANALYSIS

TEST FOR HOMOGENEITY OF RESIDUAL VARIANCES

F, 5./ 5.= 0.267E+01

TEST FOR SLOPES

F, 1/ 10.= 0.318E+02

TEST FOR LEVELS

F, 1/ 11.= 0.262E+01

OVERALL TEST

F, 2./ 10.= 0.208E+02

THERE ARE 5 VARIABLES AND 6 PAIRS OF LINES

(F3.0,4F6.2)

CONVENTIONAL

LINE 1 : NOTH DAY LINE 2 : BAC DAY

	INDIVIDUAL SIMREG LINES		STANDARD ERROR OF ESTIMATE	CORRELATION COEFFICIENT	DEGREES OF FREEDOM
1	A= 0.34857E+00	B= 0.18393E+00	SEY=0.6594E-01	R= 0.9887	DF= 6.
2	A= 0.25714E+00	B= 0.27143E+00	SEY=0.1038E+00	R= 0.9872	DF= 6.
	SIMREG FOR POOLED DATA				
3	A= 0.30286E+00	B= 0.22768E+00	SEY=0.1864E+00	R= 0.9351	DF= 13.

COVARIANCE ANALYSIS

TEST FOR HOMOGENEITY OF RESIDUAL VARIANCES

F, 5./ 5.= 0.403E+00

TEST FOR SLOPES

F, 1/ 10.= 0.142E+02

TEST FOR LEVELS

F, 1/ 11.= 0.141E+02

OVERALL TEST

F, 2./ 10.= 0.226E+02

CONVENTIONAL

LINE 1 : NOTH DAY LINE 2 : B+C DAY

	INDIVIDUAL SIMREG LINES		STANDARD ERROR OF ESTIMATE	CORRELATION COEFFICIENT	DEGREES OF FREEDOM
1	A= 0.34857E+00	B= 0.18393E+00	SEY=0.6594E-01	R= 0.9887	DF= 6.
2	A= 0.60000E-01	B= 0.28071E+00	SEY=0.1104E+00	R= 0.9865	DF= 6.
	SIMREG FOR POOLED DATA				
3	A= 0.20429E+00	B= 0.23232E+00	SEY=0.1437E+00	R= 0.9614	DF= 13.

COVARIANCE ANALYSIS

TEST FOR HOMOGENEITY OF RESIDUAL VARIANCES

F, 5./ 5.= 0.357E+00

TEST FOR SLOPES

F, 1/ 10.= 0.159E+02

TEST FOR LEVELS

F, 1/ 11.= 0.175E+01

OVERALL TEST

F, 2./ 10.= 0.999E+01

CONVENTIONAL

LINE 1 : NOTH DAY LINE 2 : CHAE DAY

	INDIVIDUAL SIMREG LINES		STANDARD ERROR OF ESTIMATE	CORRELATION COEFFICIENT	DEGREES OF FREEDOM
1	A= 0.34857E+00	B= 0.18393E+00	SEY=0.6594E-01	R= 0.9887	DF= 6.
2	A= 0.45714E+00	B= 0.14286E+00	SEY=0.6761E-01	R= 0.9806	DF= 6.
	SIMREG FOR POOLED DATA				
3	A= 0.40286E+00	B= 0.16339E+00	SEY=0.8118E-01	R= 0.9746	DF= 13.

COVARIANCE ANALYSIS

TEST FOR HOMOGENEITY OF RESIDUAL VARIANCES

F, 5./ 5.= 0.951E+00

TEST FOR SLOPES

F, 1/ 10.= 0.530E+01

TEST FOR LEVELS

F, 1/ 11.= 0.175E+01

OVERALL TEST

F, 2./ 10.= 0.387E+01

CONVENTIONAL

LINE 1 : BAC DAY LINE 2 : B+C DAY

	INDIVIDUAL SIMREG LINES		STANDARD ERROR OF ESTIMATE	CORRELATION COEFFICIENT	DEGREES OF FREEDOM
1	A= 0.25714E+00	B= 0.27143E+00	SEY=0.1038E+00	R= 0.9872	DF= 6.
2	A= 0.60000E-01	B= 0.28071E+00	SEY=0.1104E+00	R= 0.9865	DF= 6.
	SIMREG FOR POOLED DATA				
3	A= 0.15857E+00	B= 0.27607E+00	SEY=0.1309E+00	R= 0.9767	DF= 13.

COVARIANCE ANALYSIS

TEST FOR HOMOGENEITY OF RESIDUAL VARIANCES

F, 5./ 5.= 0.884E+00

TEST FOR SLOPES

F, 1/ 10.= 0.105E+00

TEST FOR LEVELS

F, 1/ 11.= 0.849E+01

OVERALL TEST

F, 2./ 10.= 0.395E+01

CONVENTIONAL

LINE 1 : BAC DAY LINE 2 : CHAE DAY

	INDIVIDUAL SIMREG LINES		STANDARD ERROR OF ESTIMATE	CORRELATION COEFFICIENT	DEGREES OF FREEDOM
1	A= 0.25714E+00	B= 0.27143E+00	SEY=0.1038E+00	R= 0.9872	DF= 6.
2	A= 0.45714E+00	B= 0.14286E+00	SEY=0.6761E-01	R= 0.9806	DF= 6.
	SIMREG FOR POOLED DATA				
3	A= 0.35714E+00	B= 0.20714E+00	SEY=0.2334E+00	R= 0.8866	DF= 13.

COVARIANCE ANALYSIS

TEST FOR HOMOGENEITY OF RESIDUAL VARIANCES

F, 5./ 5.= 0.236E+01

TEST FOR SLOPES

F, 1/ 10.= 0.302E+02

TEST FOR LEVELS

F, 1/ 11.= 0.123E+02

OVERALL TEST

F, 2./ 10.= 0.376E+02

CONVENTIONAL

LINE 1 : B+C DAY LINE 2 : CHAE DAY

	INDIVIDUAL SIMREG LINES		STANDARD ERROR OF ESTIMATE	CORRELATION COEFFICIENT	DEGREES OF FREEDOM
1	A= 0.60000E-01	B= 0.28071E+00	SEY=0.1104E+00	R= 0.9865	DF= 6.
2	A= 0.45714E+00	B= 0.14286E+00	SEY=0.6761E-01	R= 0.9806	DF= 6.
	SIMREG FOR POOLED DATA				
3	A= 0.25857E+00	B= 0.21179E+00	SEY=0.1900E+00	R= 0.9235	DF= 13.

COVARIANCE ANALYSIS

TEST FOR HOMOGENEITY OF RESIDUAL VARIANCES

F, 5./ 5.= 0.267E+01

TEST FOR SLOPES

F, 1/ 10.= 0.318E+02

TEST FOR LEVELS

F, 1/ 11.= 0.262E+01

OVERALL TEST

F, 2./ 10.= 0.208E+02

TWO PERCENT MALT

LINE 1 : NOTH LINE 2 : BAC
DAY DAY

	INDIVIDUAL SIMREG LINES		STANDARD ERROR OF ESTIMATE	CORRELATION COEFFICIENT	DEGREES OF FREEDOM
1	A= 0.12143E+00	B= 0.23250E+00	SEY=0.1052E+00	R= 0.9822	DF= 6.
2	A= 0.22286E+00	B= 0.24536E+00	SEY=0.1008E+00	R= 0.9853	DF= 6.
	SIMREG FOR POOLED DATA				
3	A= 0.17214E+00	B= 0.23893E+00	SEY=0.1259E+00	R= 0.9715	DF= 13.

COVARIANCE ANALYSIS

TEST FOR HOMOGENEITY OF RESIDUAL VARIANCES

F, 5./ 5.= 0.109E+01

TEST FOR SLOPES

F, 1/ 10.= 0.218E+00

TEST FOR LEVELS

F, 1/ 11.= 0.830E+01

OVERALL TEST

F, 2./ 10.= 0.396E+01

TWO PERCENT MALT

LINE 1 : NOTH DAY LINE 2 : B+C DAY

	INDIVIDUAL SIMREG LINES		STANDARD ERROR OF ESTIMATE	CORRELATION COEFFICIENT	DEGREES OF FREEDOM
1	A= 0.12143E+00	B= 0.23250E+00	SEY=0.1052E+00	R= 0.9822	DF= 6.
2	A= 0.26857E+00	B= 0.21643E+00	SEY=0.8924E-01	R= 0.9852	DF= 6.
	SIMREG FOR POOLED DATA				
3	A= 0.19500E+00	B= 0.22446E+00	SEY=0.1011E+00	R= 0.9789	DF= 13.

COVARIANCE ANALYSIS

TEST FOR HOMOGENEITY OF RESIDUAL VARIANCES

F, 5./ 5.= 0.139E+01

TEST FOR SLOPES

F, 1/ 10.= 0.380E+00

TEST FOR LEVELS

F, 1/ 11.= 0.268E+01

OVERALL TEST

F, 2./ 10.= 0.145E+01

TWO PERCENT MALT

LINE 1 : NOTH DAY LINE 2 : CHAE DAY

	INDIVIDUAL SIMREG LINES		STANDARD ERROR OF ESTIMATE	CORRELATION COEFFICIENT	DEGREES OF FREEDOM
1	A= 0.12143E+00	B= 0.23250E+00	SEY=0.1052E+00	R= 0.9822	DF= 6.
2	A= 0.36429E+00	B= 0.10714E+00	SEY=0.4629E-01	R= 0.9837	DF= 6.
	SIMREG FOR POOLED DATA				
3	A= 0.24286E+00	B= 0.16982E+00	SEY=0.2082E+00	R= 0.8697	DF= 13.

COVARIANCE ANALYSIS

TEST FOR HOMOGENEITY OF RESIDUAL VARIANCES

F, 5./ 5.= 0.516E+01

TEST FOR SLOPES

F, 1/ 10.= 0.333E+02

TEST FOR LEVELS

F, 1/ 11.= 0.900E+01

OVERALL TEST

F, 2./ 10.= 0.344E+02

TWO PERCENT MALT

LINE 1 : BAC LINE 2 : B+C
DAY DAY

	INDIVIDUAL SIMREG LINES		STANDARD ERROR OF ESTIMATE	CORRELATION COEFFICIENT	DEGREES OF FREEDOM
1	A= 0.22286E+00	B= 0.24536E+00	SEY=0.1008E+00	R= 0.9853	DF= 6.
2	A= 0.26857E+00	B= 0.21643E+00	SEY=0.8924E-01	R= 0.9852	DF= 6.
	SIMREG FOR POOLED DATA				
3	A= 0.24571E+00	B= 0.23089E+00	SEY=0.9977E-01	R= 0.9806	DF= 13.

COVARIANCE ANALYSIS

TEST FOR HOMOGENEITY OF RESIDUAL VARIANCES

F, 5./ 5.= 0.128E+01

TEST FOR SLOPES

F, 1/ 10.= 0.129E+01

TEST FOR LEVELS

F, 1/ 11.= 0.184E+01

OVERALL TEST

F, 2./ 10.= 0.159E+01

TWO PERCENT MALT

LINE 1 : BAC DAY LINE 2 : CHAE DAY

	INDIVIDUAL SIMREG LINES		STANDARD ERROR OF ESTIMATE	CORRELATION COEFFICIENT	DEGREES OF FREEDOM
1	A= 0.22286E+00	B= 0.24536E+00	SEY=0.1008E+00	R= 0.9853	DF= 6.
2	A= 0.36429E+00	B= 0.10714E+00	SEY=0.4629E-01	R= 0.9837	DF= 6.
	SIMREG FOR POOLED DATA				
3	A= 0.29357E+00	B= 0.17625E+00	SEY=0.2771E+00	R= 0.8085	DF= 13.

COVARIANCE ANALYSIS

TEST FOR HOMOGENEITY OF RESIDUAL VARIANCES

F, 5./ 5.= 0.474E+01

TEST FOR SLOPES

F, 1/ 10.= 0.435E+02

TEST FOR LEVELS

F, 1/ 11.= 0.198E+02

OVERALL TEST

F, 2./ 10.= 0.699E+02

TWO PERCENT MALT

LINE 1 : B+C DAY LINE 2 : CHAE DAY

	INDIVIDUAL SIMREG LINES		STANDARD ERROR OF ESTIMATE	CORRELATION COEFFICIENT	DEGREES OF FREEDOM
1	A= 0.26857E+00	B= 0.21643E+00	SEY=0.8924E-01	R= 0.9852	DF= 6.
2	A= 0.36429E+00	B= 0.10714E+00	SEY=0.4629E-01	R= 0.9837	DF= 6.
	SIMREG FOR POOLED DATA				
3	A= 0.31643E+00	B= 0.16179E+00	SEY=0.2284E+00	R= 0.8371	DF= 13.

COVARIANCE ANALYSIS

TEST FOR HOMOGENEITY OF RESIDUAL VARIANCES

F, 5./ 5.= 0.372E+01

TEST FOR SLOPES

F, 1/ 10.= 0.331E+02

TEST FOR LEVELS

F, 1/ 11.= 0.206E+02

OVERALL TEST

F, 2./ 10.= 0.569E+02

LIQUID COMPOST

LINE 1 : NOTH LINE 2 : BAC
DAY DAY

	INDIVIDUAL SIMREG LINES		STANDARD ERROR OF ESTIMATE	CORRELATION COEFFICIENT	DEGREES OF FREEDOM
1	A= 0.45429E+00	B= 0.16679E+00	SEY=0.9208E-01	R= 0.9738	DF= 6.
2	A= 0.23143E+00	B= 0.23607E+00	SEY=0.7203E-01	R= 0.9918	DF= 6.
	SIMREG FOR POOLED DATA				
3	A= 0.34286E+00	B= 0.20143E+00	SEY=0.1102E+00	R= 0.9694	DF= 13.

COVARIANCE ANALYSIS

TEST FOR HOMOGENEITY OF RESIDUAL VARIANCES

F, 5./ 5.= 0.163E+01

TEST FOR SLOPES

F, 1/ 10.= 0.983E+01

TEST FOR LEVELS

F, 1/ 11.= 0.837E+00

OVERALL TEST

F, 2./ 10.= 0.567E+01

LIQUID COMPOST

LINE 1 : NOTH DAY LINE 2 : B+F DAY

	INDIVIDUAL SIMREG LINES		STANDARD ERROR OF ESTIMATE	CORRELATION COEFFICIENT	DEGREES OF FREEDOM
1	A= 0.45429E+00	B= 0.16679E+00	SEY=0.9208E-01	R= 0.9738	DF= 6.
2	A= 0.12286E+00	B= 0.24679E+00	SEY=0.8545E-01	R= 0.9895	DF= 6.
	SIMREG FOR POOLED DATA				
3	A= 0.28857E+00	B= 0.20679E+00	SEY=0.1187E+00	R= 0.9665	DF= 13.

COVARIANCE ANALYSIS

TEST FOR HOMOGENEITY OF RESIDUAL VARIANCES

F, 5./ 5.= 0.116E+01

TEST FOR SLOPES

F, 1/ 10.= 0.114E+02

TEST FOR LEVELS

F, 1/ 11.= 0.299E-01

OVERALL TEST

F, 2./ 10.= 0.571E+01

LIQUID COMPOST

LINE 1 : NOTH DAY LINE 2 : B+C DAY

	INDIVIDUAL SIMREG LINES		STANDARD ERROR OF ESTIMATE	CORRELATION COEFFICIENT	DEGREES OF FREEDOM
1	A= 0.45429E+00	B= 0.16679E+00	SEY=0.9208E-01	R= 0.9738	DF= 6.
2	A= 0.35857E+00	B= 0.15929E+00	SEY=0.4890E-01	R= 0.9917	DF= 6.
	SIMREG FOR POOLED DATA				
3	A= 0.40643E+00	B= 0.16304E+00	SEY=0.9594E-01	R= 0.9648	DF= 13.

COVARIANCE ANALYSIS

TEST FOR HOMOGENEITY OF RESIDUAL VARIANCES

F, 5./ 5.= 0.355E+01

TEST FOR SLOPES

F, 1/ 10.= 0.145E+00

TEST FOR LEVELS

F, 1/ 11.= 0.110E+02

OVERALL TEST

F, 2./ 10.= 0.516E+01

LIQUID COMPOST

LINE 1 : NOTH LINE 2 : CHAE
DAY DAY

	INDIVIDUAL SIMREG LINES		STANDARD ERROR OF ESTIMATE	CORRELATION COEFFICIENT	DEGREES OF FREEDOM
1	A= 0.45429E+00	B= 0.16679E+00	SEY=0.9208E-01	R= 0.9738	DF= 6.
2	A= 0.57286E+00	B= 0.48214E-01	SEY=0.2401E-01	R= 0.9786	DF= 6.
	SIMREG FOR POOLED DATA				
3	A= 0.51357E+00	B= 0.10750E+00	SEY=0.2389E+00	R= 0.6970	DF= 13.

COVARIANCE ANALYSIS

TEST FOR HOMOGENEITY OF RESIDUAL VARIANCES

F, 5./ 5.= 0.147E+02

TEST FOR SLOPES

F, 1/ 10.= 0.435E+02

TEST FOR LEVELS

F, 1/ 11.= 0.201E+02

OVERALL TEST

F, 2./ 10.= 0.706E+02

LIQUID COMPOST

LINE 1 : BAC LINE 2 : B+F
DAY DAY

	INDIVIDUAL SIMREG LINES		STANDARD ERROR OF ESTIMATE	CORRELATION COEFFICIENT	DEGREES OF FREEDOM
1	A= 0.23143E+00	B= 0.23607E+00	SEY=0.7203E-01	R= 0.9918	DF= 6.
2	A= 0.12286E+00	B= 0.24679E+00	SEY=0.8545E-01	R= 0.9895	DF= 6.
	SIMREG FOR POOLED DATA				
3	A= 0.17714E+00	B= 0.24143E+00	SEY=0.8123E-01	R= 0.9881	DF= 13.

COVARIANCE ANALYSIS

TEST FOR HOMOGENEITY OF RESIDUAL VARIANCES

F, 5./ 5.= 0.710E+00

TEST FOR SLOPES

F, 1/ 10.= 0.257E+00

TEST FOR LEVELS

F, 1/ 11.= 0.260E+01

OVERALL TEST

F, 2./ 10.= 0.134E+01

LIQUID COMPOST

LINE 1 : BAC DAY LINE 2 : B+C DAY

	INDIVIDUAL SIMREG LINES		STANDARD ERROR OF ESTIMATE	CORRELATION COEFFICIENT	DEGREES OF FREEDOM
1	A= 0.23143E+00	B= 0.23607E+00	SEY=0.7203E-01	R= 0.9918	DF= 6.
2	A= 0.35857E+00	B= 0.15929E+00	SEY=0.4890E-01	R= 0.9917	DF= 6.
	SIMREG FOR POOLED DATA				
3	A= 0.29500E+00	B= 0.19768E+00	SEY=0.1396E+00	R= 0.9505	DF= 13.

COVARIANCE ANALYSIS

TEST FOR HOMOGENEITY OF RESIDUAL VARIANCES

F, 5./ 5.= 0.217E+01

TEST FOR SLOPES

F, 1/ 10.= 0.218E+02

TEST FOR LEVELS

F, 1/ 11.= 0.104E+02

OVERALL TEST

F, 2./ 10.= 0.259E+02

LIQUID COMPOST

LINE 1 : BAC DAY LINE 2 : CHAE DAY

	INDIVIDUAL SIMREG LINES		STANDARD ERROR OF ESTIMATE	CORRELATION COEFFICIENT	DEGREES OF FREEDOM
1	A= 0.23143E+00	B= 0.23607E+00	SEY=0.7203E-01	R= 0.9918	DF= 6.
2	A= 0.57286E+00	B= 0.48214E-01	SEY=0.2401E-01	R= 0.9786	DF= 6.
	SIMREG FOR POOLED DATA				
3	A= 0.40214E+00	B= 0.14214E+00	SEY=0.3043E+00	R= 0.7103	DF= 13.

COVARIANCE ANALYSIS

TEST FOR HOMOGENEITY OF RESIDUAL VARIANCES

F, 5./ 5.= 0.900E+01

TEST FOR SLOPES

F, 1/ 10.= 0.171E+03

TEST FOR LEVELS

F, 1/ 11.= 0.124E+02

OVERALL TEST

F, 2./ 10.= 0.188E+03

LIQUID COMPOST

LINE 1 : B+F DAY LINE 2 : B+C DAY

	INDIVIDUAL SIMREG LINES		STANDARD ERROR OF ESTIMATE	CORRELATION COEFFICIENT	DEGREES OF FREEDOM
1	A= 0.12286E+00	B= 0.24679E+00	SEY=0.8545E-01	R= 0.9895	DF= 6.
2	A= 0.35857E+00	B= 0.15929E+00	SEY=0.4890E-01	R= 0.9917	DF= 6.
	SIMREG FOR POOLED DATA				
3	A= 0.24071E+00	B= 0.20304E+00	SEY=0.1295E+00	R= 0.9590	DF= 13.

COVARIANCE ANALYSIS

TEST FOR HOMOGENEITY OF RESIDUAL VARIANCES

F, 5./ 5.= 0.305E+01

TEST FOR SLOPES

F, 1/ 10.= 0.221E+02

TEST FOR LEVELS

F, 1/ 11.= 0.323E+01

OVERALL TEST

F, 2./ 10.= 0.158E+02

LIQUID COMPOST

LINE 1 : B+F DAY LINE 2 : CHAE DAY

	INDIVIDUAL SIMREG LINES		STANDARD ERROR OF ESTIMATE	CORRELATION COEFFICIENT	DEGREES OF FREEDOM
1	A= 0.12286E+00	B= 0.24679E+00	SEY=0.8545E-01	R= 0.9895	DF= 6.
2	A= 0.57286E+00	B= 0.48214E-01	SEY=0.2401E-01	R= 0.9786	DF= 6.
	SIMREG FOR POOLED DATA				
3	A= 0.34786E+00	B= 0.14750E+00	SEY=0.2896E+00	R= 0.7400	DF= 13.

COVARIANCE ANALYSIS

TEST FOR HOMOGENEITY OF RESIDUAL VARIANCES

F, 5./ 5.= 0.127E+02

TEST FOR SLOPES

F, 1/ 10.= 0.140E+03

TEST FOR LEVELS

F, 1/ 11.= 0.772E+01

OVERALL TEST

F, 2./ 10.= 0.123E+03

LIQUID COMPOST

LINE 1 : B+C LINE 2 : CHAE
DAY DAY

	INDIVIDUAL SIMREG LINES		STANDARD ERROR OF ESTIMATE	CORRELATION COEFFICIENT	DEGREES OF FREEDOM
1	A= 0.35857E+00	B= 0.15929E+00	SEY=0.4890E-01	R= 0.9917	DF= 6.
2	A= 0.57286E+00	B= 0.48214E-01	SEY=0.2401E-01	R= 0.9786	DF= 6.
	SIMREG FOR POOLED DATA				
3	A= 0.46571E+00	B= 0.10375E+00	SEY=0.1762E+00	R= 0.7861	DF= 13.

COVARIANCE ANALYSIS

TEST FOR HOMOGENEITY OF RESIDUAL VARIANCES

F, 5./ 5.= 0.415E+01

TEST FOR SLOPES

F, 1/ 10.= 0.116E+03

TEST FOR LEVELS

F, 1/ 11.= 0.109E+02

OVERALL TEST

F, 2./ 10.= 0.121E+03

APPENDIX B

Statistical analysis of mushroom yields.

VARIABLE NAMES - YIELD

DATA FORMAT (I2,4X,F6.1)

YIELD - CONVENTIONAL

ANALYSIS OF VARIANCE - YIELD

SOURCE	DF	SUM SQ	MEAN SQ	ERROR	F-VALUE	PROB
TREAT	3	14500.	4833.4		12.244	0.35742E-02
ERROR	7	2763.4	394.77			
TOTAL	10	17264.				

GRAND MEAN 144.60

STANDARD DEVIATION OF VARIABLE 1 IS 41.550

FREQUENCIES, MEANS, STANDARD DEVIATIONS

****

TREAT	1	2	3	4
MN YIELD	172.2	170.8	138.0	73.80

STUDENTIZED RANGES FOR NEWMAN-KEUL'S TEST, ALPHA=0.05
3.344 4.165 4.681

THERE ARE 2 HOMOGENEOUS SUBSETS (SUBSETS OF ELEMENTS, NO PAIR OF WHICH DIFFER BY MORE THAN THE SHORTEST SIGNIFICANT RANGE FOR A SUBSET OF THAT SIZE) WHICH ARE LISTED AS FOLLOWS
(4)
(3, 2, 1)

TIME FOR MULTIPLE RANGE TESTS IS 0.1180E-01 SECONDS.

ANALYSIS COMPLETE.

VARIABLE NAMES - YIELD

DATA FORMAT (I2,4X,F6.1)

YIELD - TWO PERCENT MALT

ANALYSIS OF VARIANCE - YIELD

SOURCE	DF	SUM SQ	MEAN SQ	ERROR	F-VALUE	PROB
TREAT	3	27.189	9.0631		4.5619	0.38232E-01
ERROR	8	15.893	1.9867			
TOTAL	11	43.082				

GRAND MEAN 2.1250

STANDARD DEVIATION OF VARIABLE 1 IS 1.9790

FREQUENCIES, MEANS, STANDARD DEVIATIONS

****

TREAT	1	2	3	4
MN YIELD	1.500	3.067	3.933	0.0

STUDENTIZED RANGES FOR NEWMAN-KEUL'S TEST, ALPHA=0.05
3.261 4.041 4.529

THERE ARE 2 HOMOGENEOUS SUBSETS (SUBSETS OF ELEMENTS, NO PAIR OF WHICH DIFFER BY MORE THAN THE SHORTEST SIGNIFICANT RANGE FOR A SUBSET OF THAT SIZE) WHICH ARE LISTED AS FOLLOWS
(4, 1, 2)
(1, 2, 3)

TIME FOR MULTIPLE RANGE TESTS IS 0.8255E-02 SECONDS.

ANALYSIS COMPLETE.

VARIABLE NAMES - YIELD

DATA FORMAT (I2,4X,F6.1)

YIELD - LIQUID COMPOST

ANALYSIS OF VARIANCE - YIELD

SOURCE	DF	SUM SQ	MEAN SQ	ERROR	F-VALUE	PROB
TREAT	4	444.98	111.25		23.377	0.46429E-04
ERROR	10	47.587	4.7587			
TOTAL	14	492.57				

GRAND MEAN 9.6733

STANDARD DEVIATION OF VARIABLE 1 IS 5.9316

FREQUENCIES, MEANS, STANDARD DEVIATIONS

****

TREAT	1	2	3	4	5
MN YIELD	9.000	12.30	10.57	16.50	0.0

STUDENTIZED RANGES FOR NEWMAN-KEUL'S TEST, ALPHA=0.05

3.151 3.877 4.327 4.654

THERE ARE 3 HOMOGENEOUS SUBSETS (SUBSETS OF ELEMENTS, NO PAIR OF WHICH DIFFER BY MORE THAN THE SHORTEST SIGNIFICANT RANGE FOR A SUBSET OF THAT SIZE) WHICH ARE LISTED AS FOLLOWS

(5)
(1, 3, 2)
(4)

TIME FOR MULTIPLE RANGE TESTS IS 0.8568E-02 SECONDS.

ANALYSIS COMPLETE.