DEGRADATION OF ORTHOCHLOROPHENOL BY AN AQUATIC ACTINOMYCETE

by

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CHAPTER I

INTRODUCTION

The adaptation of growth and the utilization of resistant organic substances, including phenolic compounds, by bacteria and other microorganisms have been reported by a number of investigators (2, 4, 5, 9, 18, 19). However, few investigations have been conducted to evaluate the ability of actinomycetes to degrade organic compounds that are usually regarded as biologically resistant. Aquatic actinomycetes are normal inhabitants of surface waters and are especially numerous in benthic muds associated with these waters. They are commonly mentioned in the literatures in relation to taste and odor problems in potable water supplies. Some of the general characteristics of aquatic actinomycetes, such as--their activeness in decomposition of a wide range of organic materials, their aerobic nature, and their ability to survive under extreme variations in physical environmental conditions --suggest that this group of microorganisms might be used effectively for treating persistent organic wastes.

Chlorinated phenols that appear in lakes and streams are discharged primarily by industries, though runoff from agricultural lands also contributes. These substances may cause taste and odor problems in water supplies, and they may be concentrated by aquatic organisms in passing through the food chain to cause more serious problems.

The objective of this study was to evaluate the extent of o-chlorophenol degradation by an aquatic actinomycete and, if positive results could be demonstrated, to determine which growth stage is the most effective.

CHAPTER II

LITERATURE REVIEW

Phenolic Wastes and Treatment

Phenolic wastes include a broad variety of compounds and materials. Basically, the phenols are compounds that have a hydroxyl group attached directly to an aromatic nucleus, but their reactions differ from those of the alcohols. The basic chemical structure permits formation of a great variety of chemical substitution products and derivatives (20); hence, they are highly reactive compounds.

Some general physical properties of phenolic compounds are: the boiling points increase as the molecular weight increases or as substitution group are added to the phenolic molecule, and the water solubility increases with the addition of hydroxyl groups. The dissociation constant for most phenolic compounds is about 10^{-10}. The latter property is variable because the addition of halogen or nitrogen groups increases the dissociation constant (7).

The phenolic compounds found in surface waters arise mainly from agricultural runoff containing pesticides, herbicides or decaying vegetable matter, from industrial effluents --especially those from coal refining plants, chemical works such as steel mills, petroleum refineries, gas production plants, and tar distilling plants. In addition, phenols are

discharged from industries that synthesize them and also from those that manufacture phenol plastics. The wastewaters from all these industries contain phenols that differ in quantity and type. Even domestic wastes contain a small quantity of phenols. Urine has been reported to contain about 30 milligrams per liter (29).

The toxicity of phenolic wastes to many types of aquatic biota has been established by several investigators (14, 17). Lammering and Burbank (14) reported that the 24- and 48-hour median tolerance limits of bluegill sunfish for o-chlorophenol at 20 degrees Centigrade are 8.2 and 8.1 milligrams per liter, respectively. The permissible concentration for phenols in a water supply is 0.001 milligrams per liter and is based on the threshold concentration for objectionable tastes and odors associated with phenols (21).

The method for the determination of phenolic compounds is detailed in Standard Methods for the Examination of Water and Wastewater (25). The Emerson Reaction, the color reaction of phenols with 4-aminoantipyrine (1-phenyl-2,3-dimethyl-4-amino-5-pyrazolone) and an oxidizing agent, has been considered to be the most suitable method for the quantitation of phenols because the test is highly sensitive.

References in the literature to phenolic waste treatment are numerous and only a selected review is presented here. Chemical treatment of phenolic wastes is common with oxidizing materials--including chlorine, chlorine dioxide,

ozone, and hydrogen peroxide. Activated carbon is used in water treatment plants to remove small amounts of phenols that cause tastes and odors in surface waters. Generally, the capital and operating costs of chemical treatment are higher than those for biological oxidation (3). In many cases involving chemical oxidation, the effluent is toxic to aquatic organisms and is aesthetically objectionable. Recently, it has been reported that the electrolytic decomposition of phenols is an extremely effective treatment technique (13).

Biological treatment of phenolic wastes is usually by the activated sludge or trickling filter process. For a number of years, Phenolic wastes have been treated in dripping towers, oxidation ditches, and aeration tanks with bacteria specially cultivated for the decomposition of phenols. Constant temperature, pH, and supply of nutrients are all necessary to maintain optimum living conditions for these sensitive phyla of microorganisms (29). Temperature has been found to be a major factor in the regulation of the accommodation time of activated sludge to phenol (16). Phenol removal values were increased to 97% with an applied dose of 300 milligrams per liter phenol when the influent feed BOD (biological oxygen demand) to nitrogen ratio was decreased to 20:1 by the addition of diammonium phosphate. In other words, nutrients carbon and nitrogen would affect biological phenol removal.

Laboratory investigations into the ability of various microorganisms to degrade or tolerate phenolic compounds are numerous (1, 2, 4, 9, 18, 19, 22). Organisms studied include Pseudomonas, Vibrio, actinomycetes, yeasts, slime molds, and some anaerobic soil bacteria. The mechanism of biochemical decomposition of phenols has been postulated to be oxidative in nature (4, 24, 27).

The biodegradability of phenolic compounds appears to be a function of the type, number, molecular size, complexity, and position of the substituents groups on the benzene ring. Para-substituted phenols are more bioresistant than either the meta- or ortho-substituted forms. In general, as the molecular weight of the substituent group increases, the biodegradability of the compound is decreased. Dichlorophenols were shown to be more resistant to biological oxidation than monochlorophenols (1).

Actinomycetes

Actinomycetes are filamentous organisms that produce true branches and mold-like spores or conidia (23). These characteristics relate these organisms to the molds, though actinomycetes generally have been regarded as a taxonomically uncertain group. They have been catagorized either as bacteria or as fungi from time-to-time (28). However, the diameter of the actinomycete hyphae varies from 1 to 5 microns and their

length is seldom more than a few millimeters whereas the hyphae of true molds are between 10 and 20 microns in diameter and are often several inches in length.

The cell wall of the actinomycetes is chemically more similar to that of the bacteria than to that of the molds. The cell walls of both bacteria and actinomycetes contain mucopolysaccharide whereas mold cell walls contain chitin or cellulose. In addition, both bacteria and actinomycete cell walls contain muramic and diaminopimelic acids, both of which are absent in molds. The composition of the cell wall is one of the distinguishing features between the mold-like bacteria, the Actinomycetales, and the true molds, the Eumycetes (6, 15).

All actinomycetes are gram positive, most are coenocytic, and only one or two species are motile. For the most part, actinomycetes are harmless saprophytes, widely dispersed in nature. They can be found in soil, in the excreta of animals, and in both marine and fresh waters. They are active in decomposing a wide range of organic materials (6). Both extracellular and endocellular oxidative enzymes have been isolated from actinomycete cultures (10, 11, 24, 27, 28). Phenol oxidase and catalase are produced by several species of Streptomyces (4, 10, 24).

The greatest impetus for studying actinomycetes was given by the discovery that they produce many antibiotics of immense value in human and veterinary medicine, in the control of plant diseases and spoilage by molds and bacteria, and in

scientific research (6). Since that discovery, much research has been conducted into the actinomycetes' mode of growth, their nutritional requirements, and their biochemistry (28). Many forms produce musty-earthy odors during their life cycle and, hence, are known to cause taste and odor problems in water (23). One of the odor-causing compounds--a neutral, volatile oil named geosmin--has been recently isolated and characterized (6). There are two phases of growth in the aquatic actinomycetes life cycle (9, 23). The primary stage of growth is restricted to the subsurface in both solid and liquid media. The dissolved oxygen requirement is minimal during this stage, and, in fact, primary growth is anaerobic or microaerophilic. The secondary phase, which is aerobic, was found physiologically active by Hemphill (9), while Silvey did not agree (23).

CHAPTER III

EXPERIMENTAL METHODS AND MATERIALS

This study was conducted in two phases, the first of which was devoted to the development of suitable culturing conditions for the particular actinomycete used. During this phase of study, detailed observations were recorded regarding growth characteristics. The second phase entailed an evaluation of the ability of the actinomycete to degrade o-chlorophenol. The biomass development at various concentrations also was determined during this phase. All of these studies were conducted with batch cultures of actinomycetes growing in enriched organic culture media to which the o-chlorophenol had been added.

Growth and Development Studies

The aquatic actinomycete strain used for the study was a pure culture of *Streptomyces*, Number 5146, obtained from the Water Laboratory of the Department of Biological Sciences, North Texas State University, Denton, Texas.

Synthetic and enriched organic media, both as broth and as agar, were used for the study of growth development and observation. The composition of the culture media are listed in Appendix A. The media were prepared and sterilized by autoclave at 121 degrees Centigrade, 15 pounds pressure

for 15 minutes. Small samples of organism were transferred by loop from the agar slant onto modified Emerson's agar slants and plates and into synthetic and organic broth media. Cultures were incubated, under static conditions, at room temperature and at 30 degrees Centigrade. During the incubation period, the developing organisms were examined microscopically and macroscopically. The appearance of the developing organisms, their gross cultural characteristics, and the sequence of development were noted. In addition, samples of developing cultures were examined for the following characteristics: gram stain, microscopic and macroscopic morphology, pigment formation, odor, and the optimum pH and temperature for growth.

Seventeen-Day o-chlorophenol Degradation Study (Run 1)

The preliminary study consisted of observations of actinomycete growth as a function of o-chlorophenol concentrations, Residuals of o-chlorophenol were determined at the end of a seventeen-day growth period. Tolerance of the actinomycete to o-chlorophenol was evaluated by culturing the organism in media containing varying concentrations of o-chlorophenol, the pattern of growth and development was noted at each concentration.

The study was initiated by adding o-chlorophenol to modified Emerson's media. Fourteen 250-milliliter Erlenmeyer flasks, each containing 50 milliliters of brown sugar medium

(modified Emerson's medium), were sterilized by autoclave and then inoculated with one milliliter of spore suspension in phosphate buffer, pH 7.2. Sufficient o-chlorophenol stock solution was added to each flask to produce concentrations of 0, 10, 50, 100, 250 and 500 milligrams per liter. The pipets, glassware, and containers for o-chlorophenol stock solution were sterilized by autoclave. The o-chlorophenol (purified grade, Fisher Scientific Company) and the dilution solvent, 75% ethanol, used for making o-chlorophenol stock solution were not sterilized, as it was assumed that there were no viable cells in these materials.

A series of uninoculated controls containing the various o-chlorophenol concentrations was prepared. These controls were analyzed to determine if there was o-chlorophenol reduction during the test interval for reasons other than actinomycete utilization. Four other controls were inoculated with one milliliter of spore suspension but did not contain o-chlorophenol. These served to detect any substances produced by the actinomycete that might give a positive o-chlorophenol test.

All cultures were incubated at 30 degrees Centigrade for seventeen days. During this period, the development of growth was observed daily. On the seventeenth day, all cultures were analyzed for o-chlorophenol by the aminoantipyrine method cited in Standard Methods for the Examination of Water and Wastewater, 13th Edition (25). This procedure involves a

petroleum ether extraction of the phenols from an acidified aqueous sample, an alkaline aqueous extraction of the phenols from the petroleum ether, and subsequent color development with 4-aminoantipyrine and potassium ferricyanide at pH 7.9. A Klett-Summerson photoelectric colorimeter was used to evaluate the color intensity, and sample values were compared against a standard curve.

The aminoantipyrine method is not specific for phenol, and the various ortho-, meta-, and para-substituted isomers can be detected. Standard Methods (25) stipulates 2,4-dichlorophenol as the reference standard, and the test was designed for analysis of water samples. Throughout this investigation, however, samples of media, rather than water, were subjected to the analysis, so appropriate control tests were performed to insure that no interfering substances were present in the medium. Another modification of the standard method was that o-chlorophenol was used as the reference standard.

The preliminary o-chlorophenol-utilization study involved the sacrifice of 50-milliliter broth samples after seventeen days of growth. Before the analysis was performed, the volume of each flask was adjusted to 50 milliliters by the addition of phenol-free distilled water, compensating for losses incurred by evaporation. The analitical techniques for determining the concentration of o-chlorophenol, though listed in Standard Methods (25) are briefly summarized in the following statement.

1. Media samples containing o-chlorophenol were diluted with fresh broth, when necessary, to adjust the concentration to 30-70 micrograms per 50 milliliters.
2. A one-milliliter aliquot was diluted with approximately 250 milliliters of phenol-free distilled water in a 500-milliliter separatory funnel. Concentrated hydrochloric acid (5 milliliters) was added, and the sample was extracted three times with 50-milliliter volumes of petroleum ether.
3. The extracts were combined and washed twice with 50-milliliter portions of distilled water. Then, the o-chlorophenol was extracted three times with 0.5 Normal ammonium hydroxide and the extracts combined. These were then mixed with phosphate buffer, pH 6.8. If the final pH was not 7.9 ± 0.1, adjustments were made.
4. The aminoantipyrine and potassium ferricyanide reagents were added, 15 minutes were allowed for color development, and absorbance was measured by use of the colorimeter with a #50 filter. A broth sample containing no phenol was treated in the same manner as the test samples and provided the instrument zero.
5. The sample readings were compared against a standard curve developed from broth media containing various concentrations of a 100 milligrams per liter o-chlorophenol stock solution. The standards were treated in

the same manner as the experimental samples, and the calibration curve was constructed by least-squares regression analysis of the data (Appendix Figure A1). Periodically, the standard curve was checked to insure accuracy.

The analytical procedures just described were the same as were used throughout the entire investigation.

Orthochlorophenol Degradation-Rate Study (Run 2)

Thirty-two 250-milliliter Erlenmeyer flasks, containing 50 milliliters of sterile, modified Emerson's broth were prepared and divided into eight sets of four flasks each. Seven groups were inoculated with o-chlorophenol to produce the following concentrations: 10, 50, 100, 200, 300, 400, and 500 milligrams per liter. No o-chlorophenol was added to the eighth set, and these flasks served as controls. Three of each set of four flasks were inoculated with one milliliter of the actinomycete spore suspension. Therefore, an additional control was provided within each set in order that losses of o-chlorophenol by mechanisms other than biological could be accounted for.

All flasks were incubated at 30 degrees Centigrade. At two-day intervals, several milliliters of media were withdrawn with a sterile hypodermic syringe. These samples were analyzed for o-chlorophenol content by procedures discussed previously.

Volume adjustments to compensate for losses were not possible during this study because the sampling procedures did not permit sacrifice of the entire contents of any given flask.

Orthochlorophenol Degradation-Rate and Biomass Production Study (Run 3)

Sixty-four 99-milliliter milk-dilution bottles, containing 50 milliliters of modified Emerson's broth were sterilized and divided into three groups of twenty each and one group of four. Ten of each group of twenty bottles were inoculated with one milliliter of actinomycete spore suspension. All twenty of each group were inoculated with o-chlorophenol to produce concentrations of 100, 200, and 300 milligrams per liter. The set of four bottles was inoculated with spore suspension only.

At two-day intervals, two bottles from each set containing o-chlorophenol, one inoculated and one uninoculated with actinomycete, were analyzed for o-chlorophenol and biomass concentrations.

The procedure for biomass measurements was made as follows:

1. Twenty milliliters of the sample were withdrawn and filtered through an S & S #589 filter paper (Fisher Scientific Company), 9 centimeter diameter, that had been previously dried at 103 degrees Centigrade

and weighed.

2. The filter paper and hyphae material were washed into a crucible (previously dried at 103 degrees Centigrade and weighed) with 10-milliliter portion of distilled water.
3. The crucible with the filter paper and hyphae material were dried at 103 degrees Centigrade overnight and then cooled in a dessicator for one hour before weighing.
4. Similar procedures were followed using 20 milliliters of the control broth medium.
5. The biomass was determined by subtracting the tare weight of filter paper and crucible from the final weight. The biomass weight was corrected for any contributions from the medium itself.

CHAPTER IV

EXPERIMENTAL RESULTS

The data from the studies of o-chlorophenol degradation were analyzed to determine the toxicity of o-chlorophenol to the aquatic actinomycete, the rate of degradation of the o-chlorophenol, and the relationship between the rate of degradation and biomass production.

General Growth Characteristics

Growth of the actinomycete was rapid on solid media. Twenty-four hours after inoculation, a slimy scum was apparent on the surface of the agar. The width of the primary mycelia ranged from 0.3 to 0.6 microns. At approximately thirty-six hours, the surface growth began to develop into discreet colonies. In the interval between 36- and 72-hours, the ampliation extended, and larger surface features appeared as folds or crests. The width of the secondary mycelia, approximately 1.0 micron, was larger than that of the primary mycelia. Between 36- and 72-hours aerial hyphae developed, and the creamy white colonies spread until the surface of the agar was covered with a mat. The surface appeared to be corrugated.

Other characteristics of this organism were that it is gram-positive, produces musty-earthy odors after approximately three days of development, produces insoluble, creamy-white

pigment, and grows better and more rapidly at 30 to 35 degrees Centigrade than at ambient room temperatures.

The development of this organism in liquid culture media was much slower than on solid media. At 24- to 36-hours in enriched organic media broth, the flocculant, primary mycelia appeared on the bottom of the culture flask. Then the growths became beaded. A sporulating, creamy-white surface growth developed within four or five days after inoculation. It began as a small agglomeration of surface colonies, followed by a membrane-like pellicle that developed and gradually into a thick mat after two weeks incubation. Before the secondary mats were developed completely, a ring of beads often appeared at the periphery of the medium surface. If the culture flask was shaken after the pellicle was formed, the pellicle would sink later, a new pellicle would form on the surface.

The pH of the culture media was an important factor in the development of both primary and secondary growth, as was the incubation temperature. Below pH 6.7, development was retarded and only a small quantity of surface growth appeared. At the optimum pH, which ranged from 7.0 to 7.2, surface growth was extensive.

During the course of study, bacterial contamination of the culture media sometimes caused erratic results. Most of the time, it was a simple matter to distinguish a bacterially contaminated culture from a pure actinomycete culture by the

presence of turbidity in culture media, a change in odor of the broth, or by actually streaking an agar plate and allowing growth to develop so that it can be observed. The pure actinomycete culture was always clear, even with a lot of growth in it.

The Effects of Orthochlorophenol on Growth

Table I summarizes the growth observations made during Run 1. Excellent growth was observed at o-chlorophenol concentrations up to 250 milligrams per liter, but growth was poor at the next highest concentration of 500 milligrams per liter. In Run 2, excellent growth was observed in media containing as much as 400 milligrams per liter, though there appeared to be a reduction in growth rate as the o-chlorophenol concentration increased above 100 milligrams per liter. The reduced rate was especially evident from the length of time required for surface growth to appear in the cultures. There was no visible growth at a concentration of 500 milligrams per liter o-chlorophenol.

Degradation of Orthochlorophenol

The residuals of o-chlorophenol after 17 days in the media comprising Run 1 are shown in Table II. The control (phenol-free) media, both inoculated and uninoculated with

TABLE I

Tolerance of Actinomycete to o-chlorophenol

	Concentration mg/l				
	10	50	100	250	500
Growth	+	+	+	+	0

+ = Normal Growth
0 = No Visible Growth

TABLE II

o-chlorophenol Residuals After 17-Day Incubation and Removal Percentage

0-day Conc. mg/l	17-day Conc. mg/l	Removal Percentage
0	0.00	--
10	0.00	100
50	7.42	86
100	15.12	85
250	87.53	65
500	463.72	8

actinomycete, never gave a positive test with 4-aminoantipyrine. Therefore, no phenolic substances were produced by the organism, at least none that will react with the analytical reagent to produce color.

Figure 1 depicts the average rate of o-chlorophenol degradation during the 17-day incubation period of Run 1 as a function of the initial concentration. The average rate of degradation increased markedly in flasks containing up to 250 milligrams per liter o-chlorophenol, but it decreased markedly in the vessel dosed with 500 milligrams per liter. However, the average degradation rate in the latter culture was higher than that observed in the flask containing only 10 milligrams per liter o-chlorophenol.

Figure 2 presents data for the average degradation rates observed during Run 2 which was conducted for 26 days and which included growth evaluation at o-chlorophenol concentrations of 10, 50, 100, 200, 300, 400, and 500 milligrams per liter. The intermediate dosages between 200 and 500 milligrams per liter permitted a more definitive evaluation of the tolerance of the actinomycete to the o-chlorophenol. The absolute values for the average degradation rates observed for Run 2 were higher than those observed for Run 1, but the incubation period had been extended an additional 9 days for Run 2.

As was true for Run 1, the average utilization rate increased linearly up to concentration of 100 milligrams per

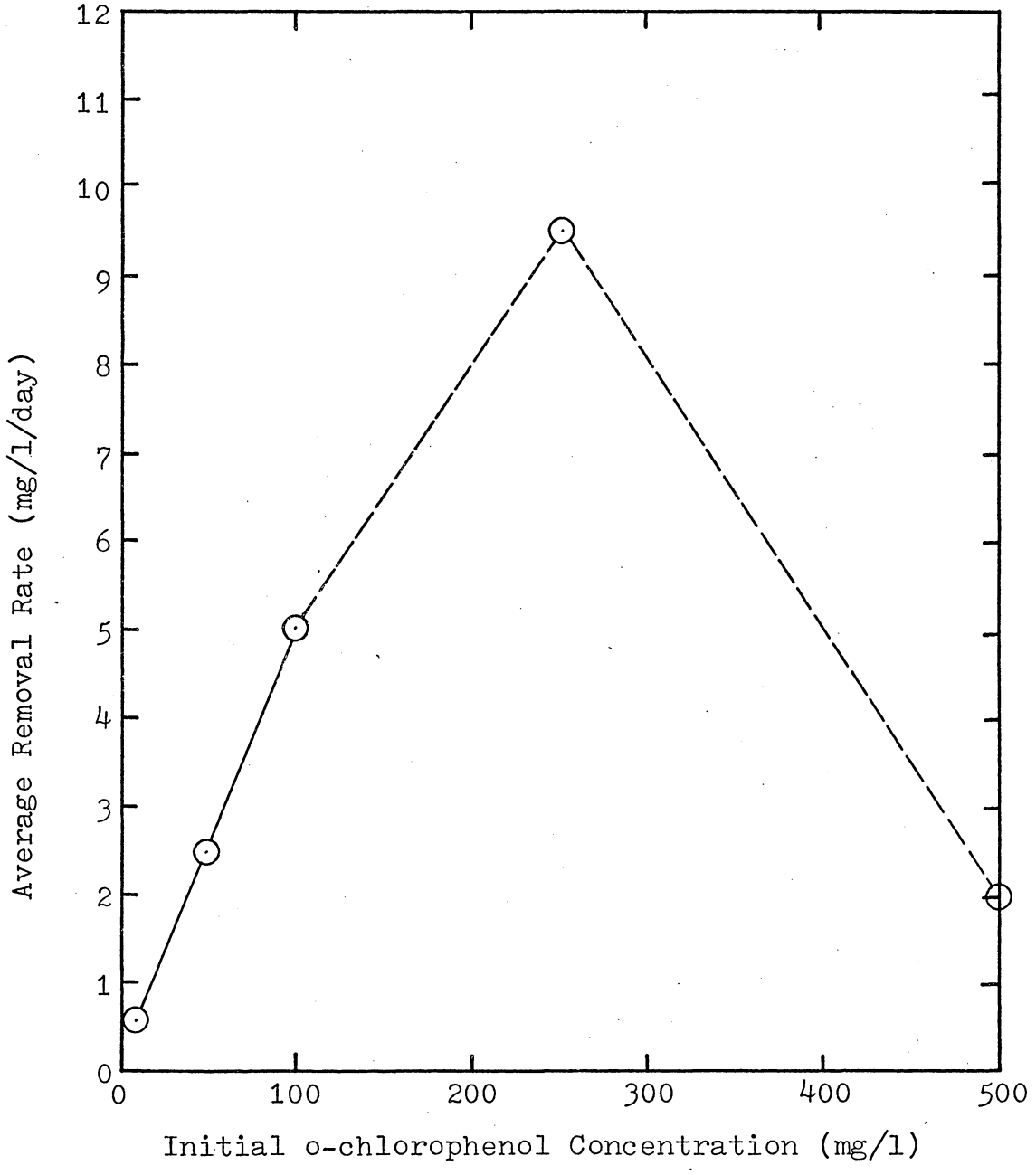

Figure 1 Comparison of o-chlorophenol Removal Rates at Different Initial Concentrations--17-Day Incubation

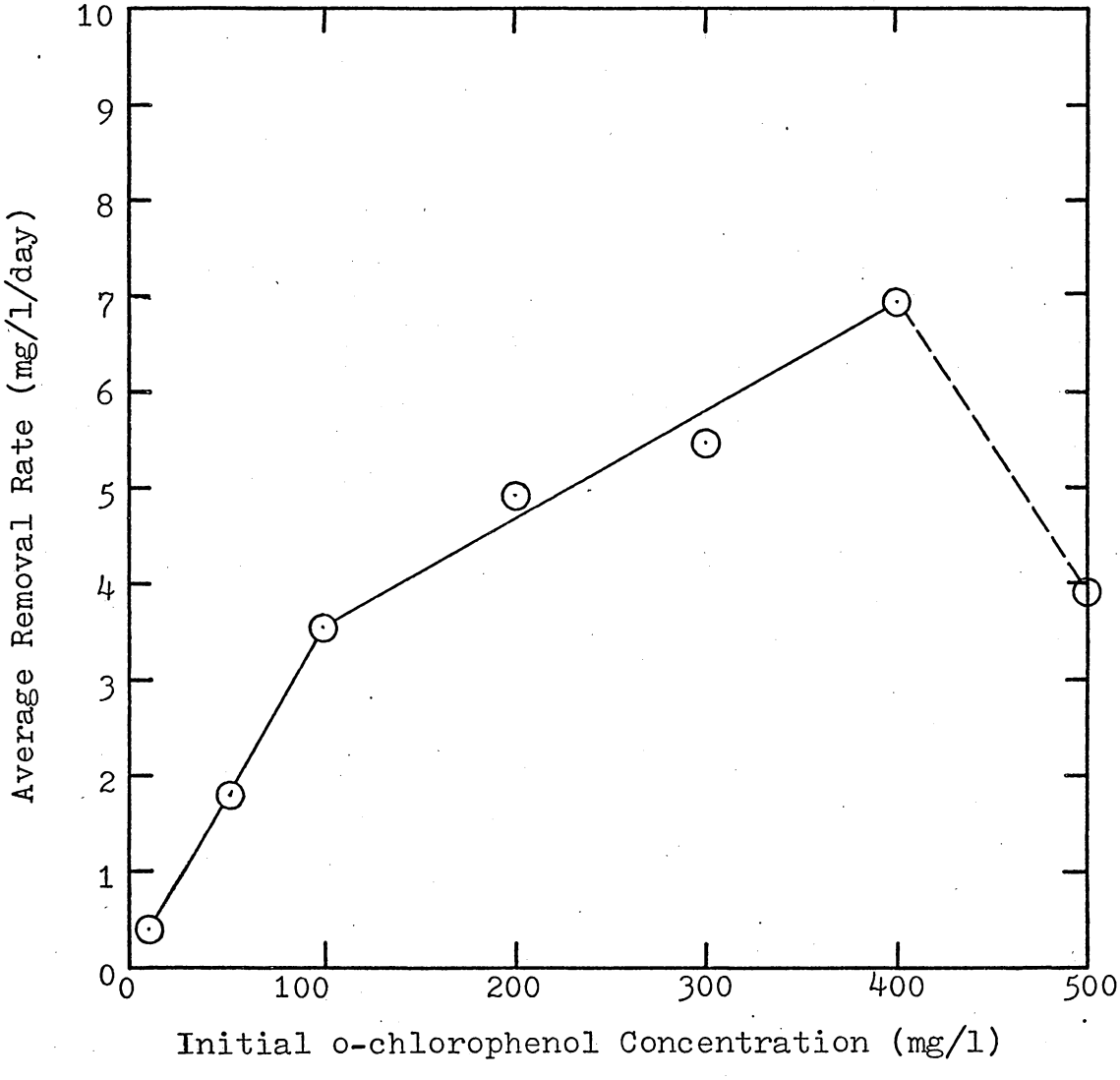

Figure 2 Comparison of o-chlorophenol Removal Rates at Different Initial Concentrations --26-Day Incubation

liter. The average rate appeared to be a linear function of the initial concentration between concentrations of 100 and 400 milligrams per liter, though the incremental change in the rate was less than was observed between 10 and 100 milligrams per liter. Additionally, as in Run 1, the utilization rate of the o-chlorophenol was much lower when the concentration was increased to 500 milligrams per liter, but, again, the rate was not as low as was observed at a concentration of 10 milligrams per liter.

Because the o-chlorophenol concentration in all cultures was determined at frequent intervals during Run 2, it was possible to construct curves relating o-chlorophenol concentration with the time of incubation. Figure 3 is a sample of this type analysis for the culture medium that contained 400 milligrams per liter o-chlorophenol before inoculation with actinomycete. Figures for the other cultures are presented in Appendix Figures A2, A3, and A4. The analytical data used in constructing these figures are tabulated in Appendix B along with the calculated percentages of o-chlorophenol degradation.

During Run 2 it was observed, generally, that the lag growth period was lengthened by increasing the o-chlorophenol concentration. During this phase, the rates of degradation of the o-chlorophenol were low. However, once rapid growth began to occur, the rates of degradation increased markedly. At concentrations of o-chlorophenol above 100 milligrams per

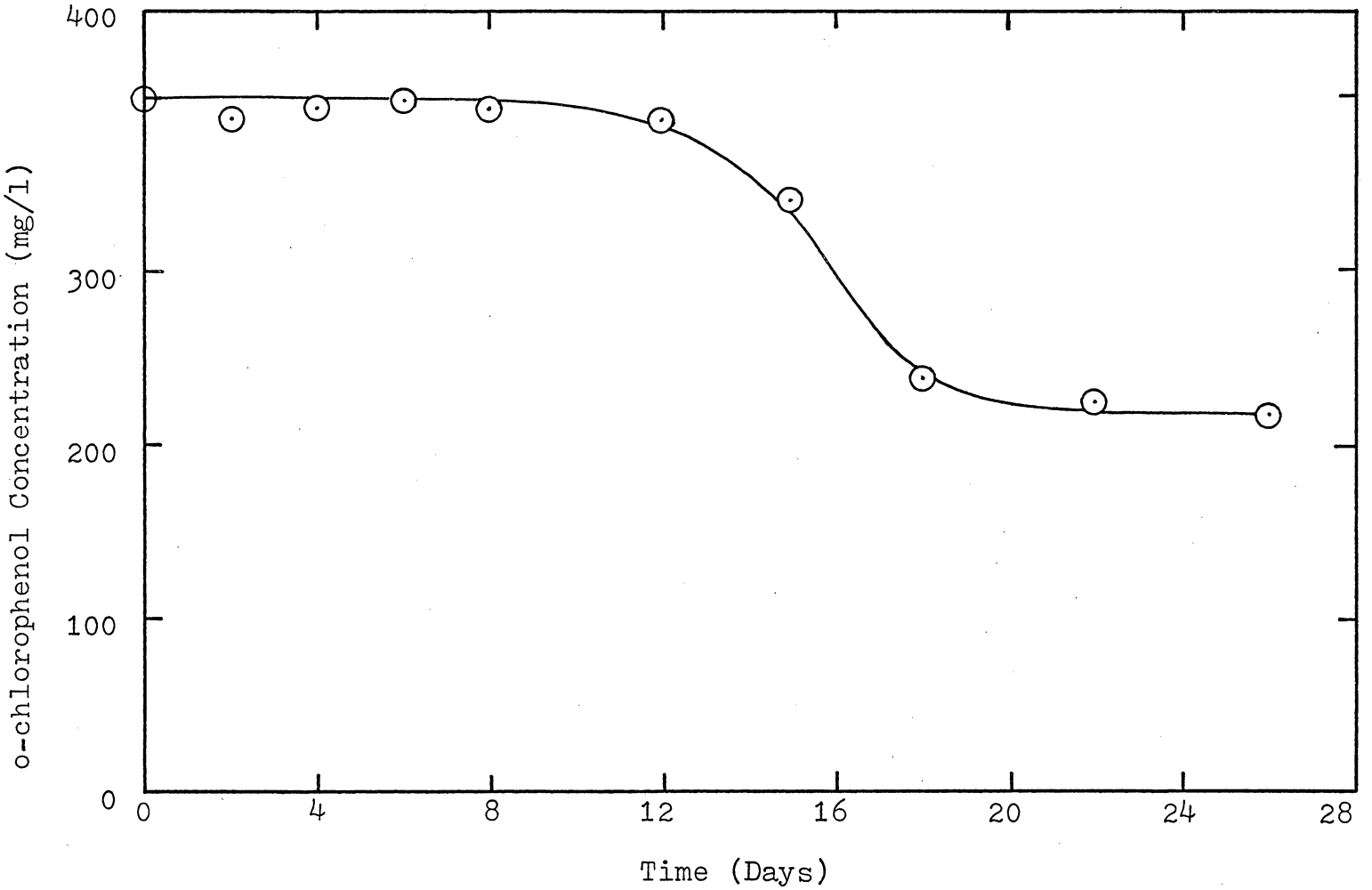

Figure 3 Orthochlorophenol Degradation Rate (C_i=400 mg/l)

liter, the rates of degradation began to slow after approximately 18 days incubation, and this slow rate was manifested throughout the remainder of the test period. Figure 3, previously mentioned, readily illustrates these changes in the rate of o-chlorophenol degradation.

Figure 4 shows the total mass of actinomycete produced after 20 days growth in media containing o-chlorophenol concentrations of 100, 200, and 300 milligrams per liter (Run 3). Figures 5, 6, and 7 were constructed to show the biomass production and the o-chlorophenol degradation as functions of time. The slopes of the curves describe the rates of change of these two parameters, and, generally, the curve that describes the biomass production at a particular o-chlorophenol concentration is a mirror-image of that describing the o-chlorophenol concentration at various times. Figures 5, 6, and 7 show a pattern of o-chlorophenol degradation similar to those observed during Run 2, that is, a slow rate in the early-growth stages, followed by a rapid rate approximately mid-way into the incubation period and a declining rate toward the end.

Figure 8 was constructed to show the relationship between the average rate of degradation during the incubation interval and the initial concentration of o-chlorophenol. There was a direct, linear relationship between the average degradation rate and the initial concentration of the o-chlorophenol.

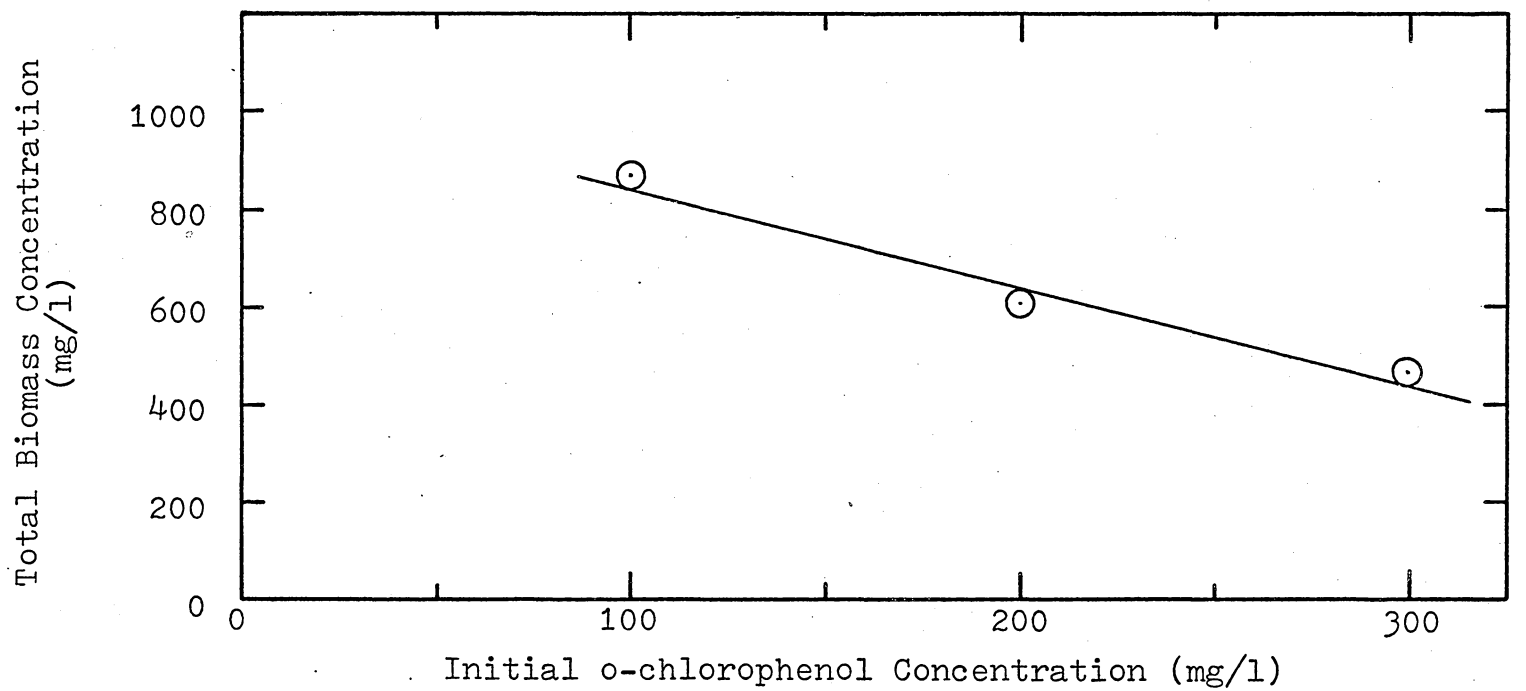

Figure 4 Biomass Production in 20 Days at Different Initial o-chlorophenol Concentrations

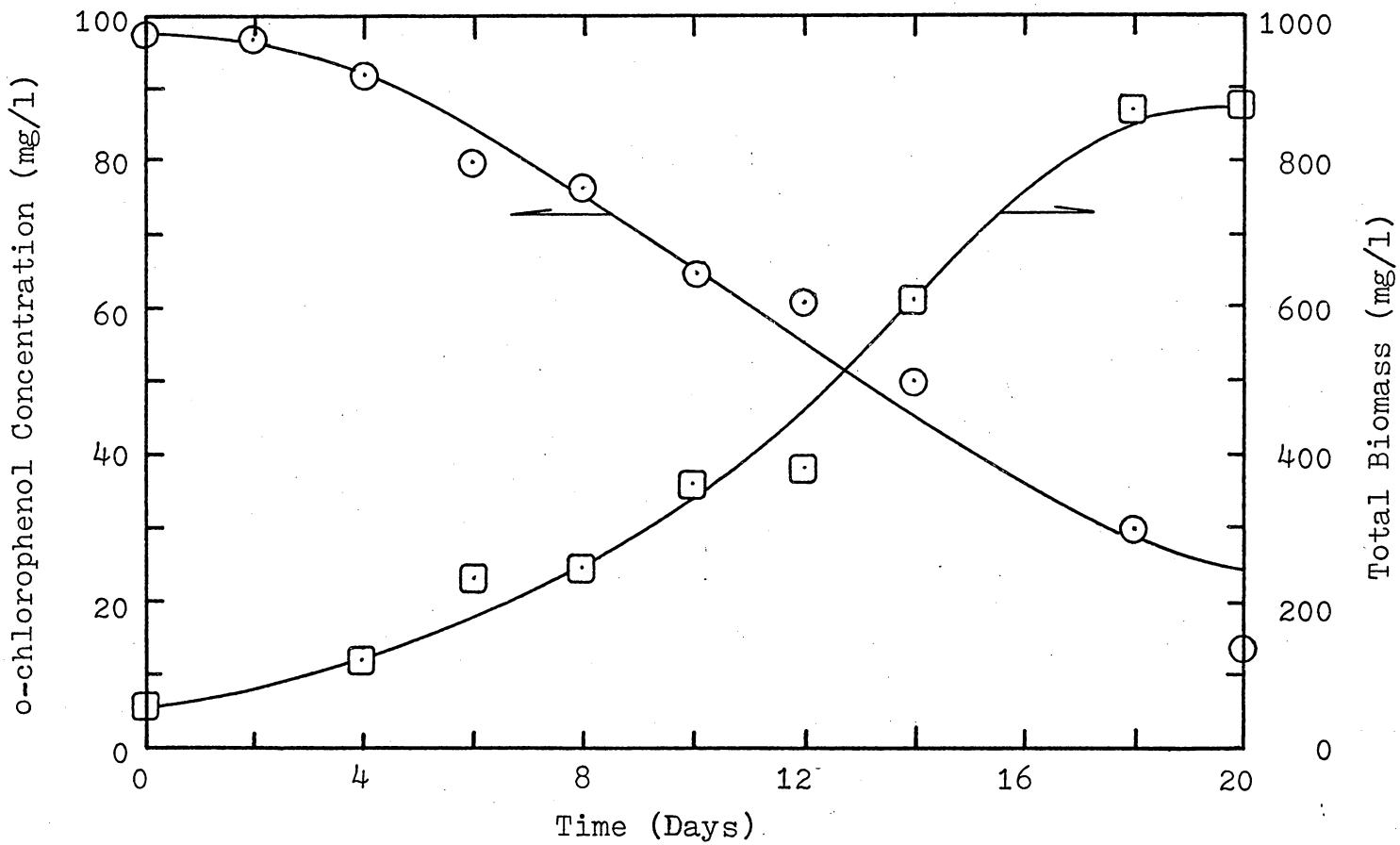

Figure 5 Biomass Production and o-chlorophenol Degradation During 20-Day Period (C_i=100 mg/l)

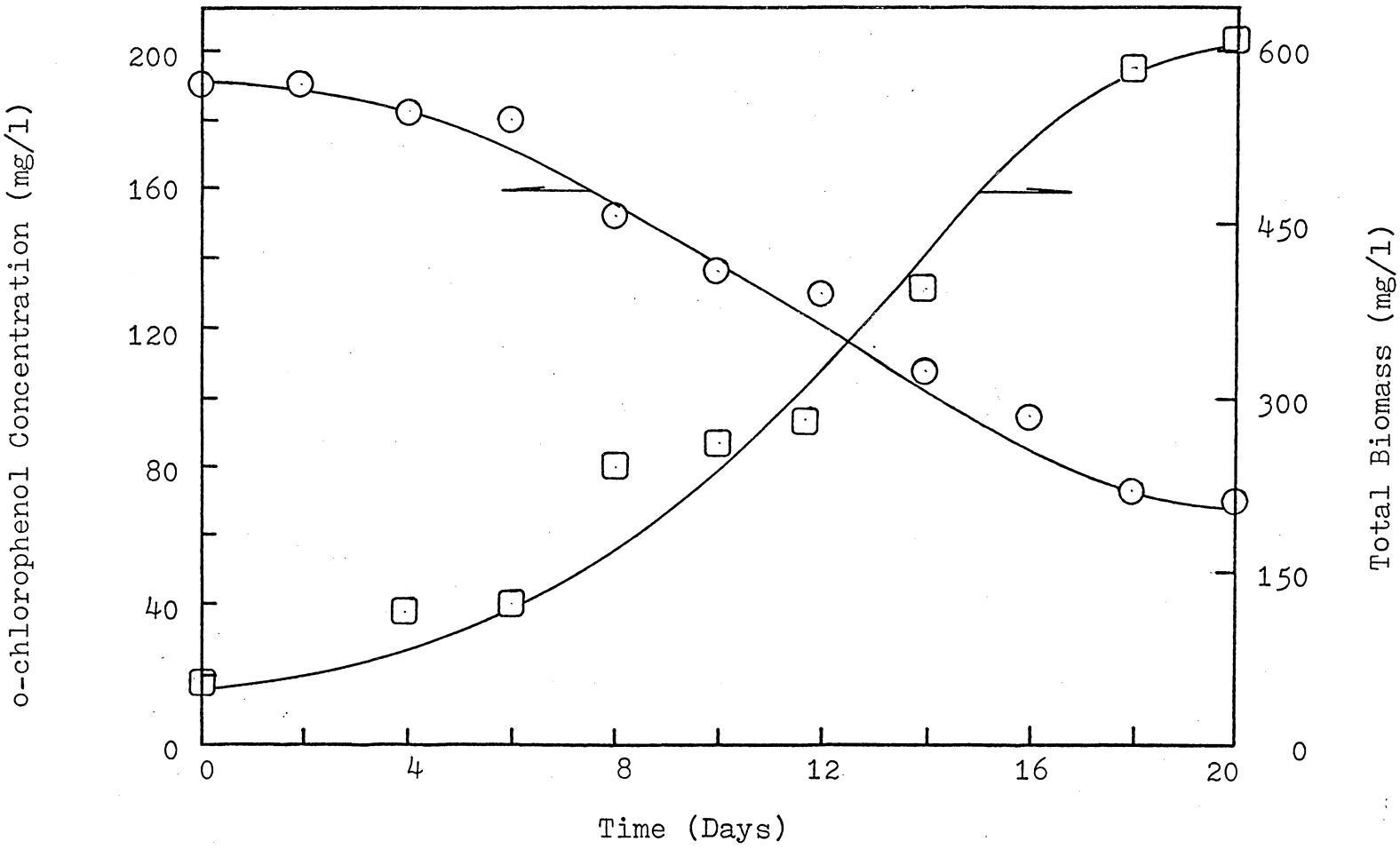

Figure 6 Biomass Production and o-chlorophenol Degradation During 20-Day Period (C_i=200 mg/l)

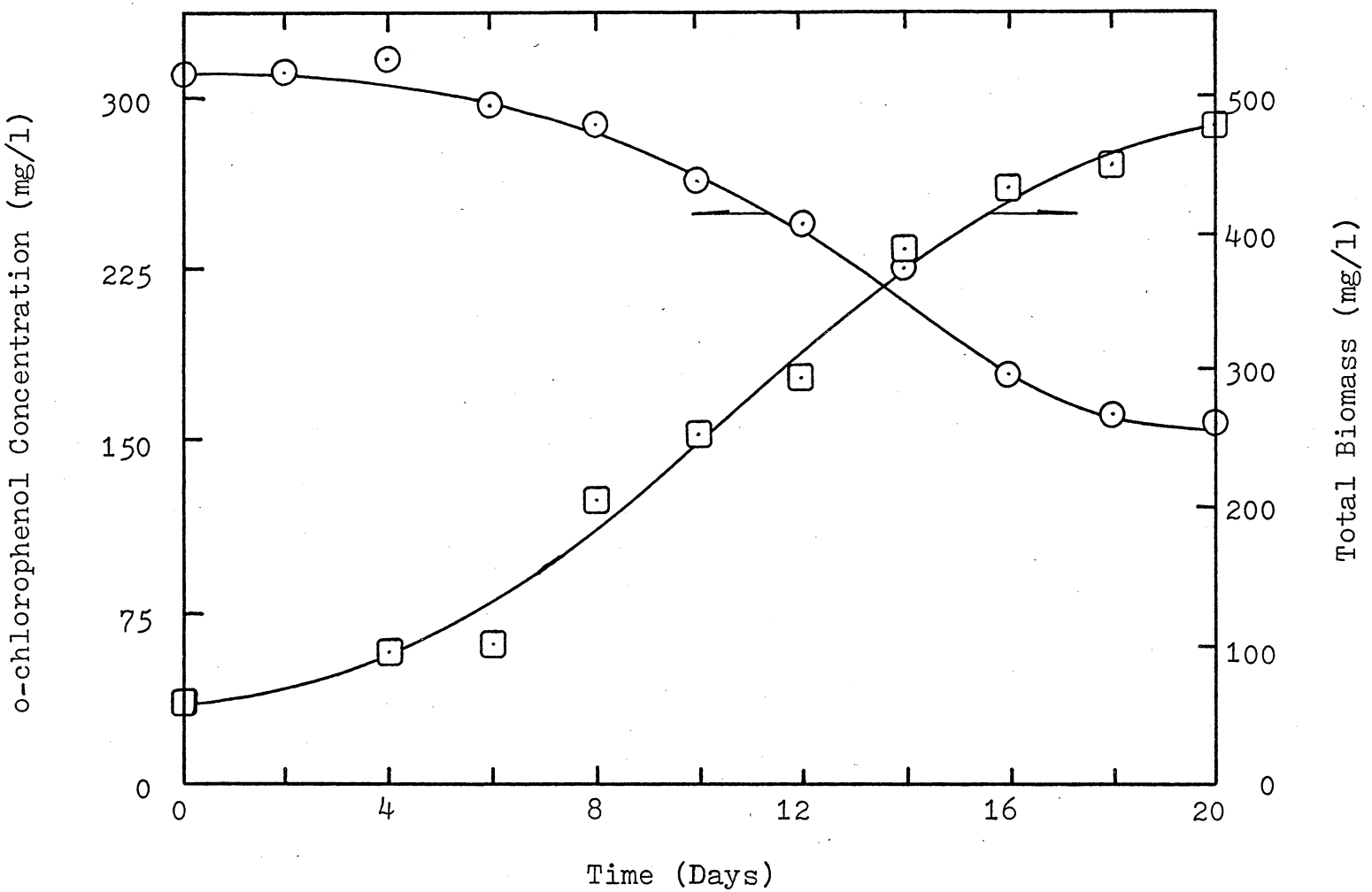

Figure 7 Biomass Production and o-chlorophenol Degradation During 20-Day Period (C_i=300 mg/l)

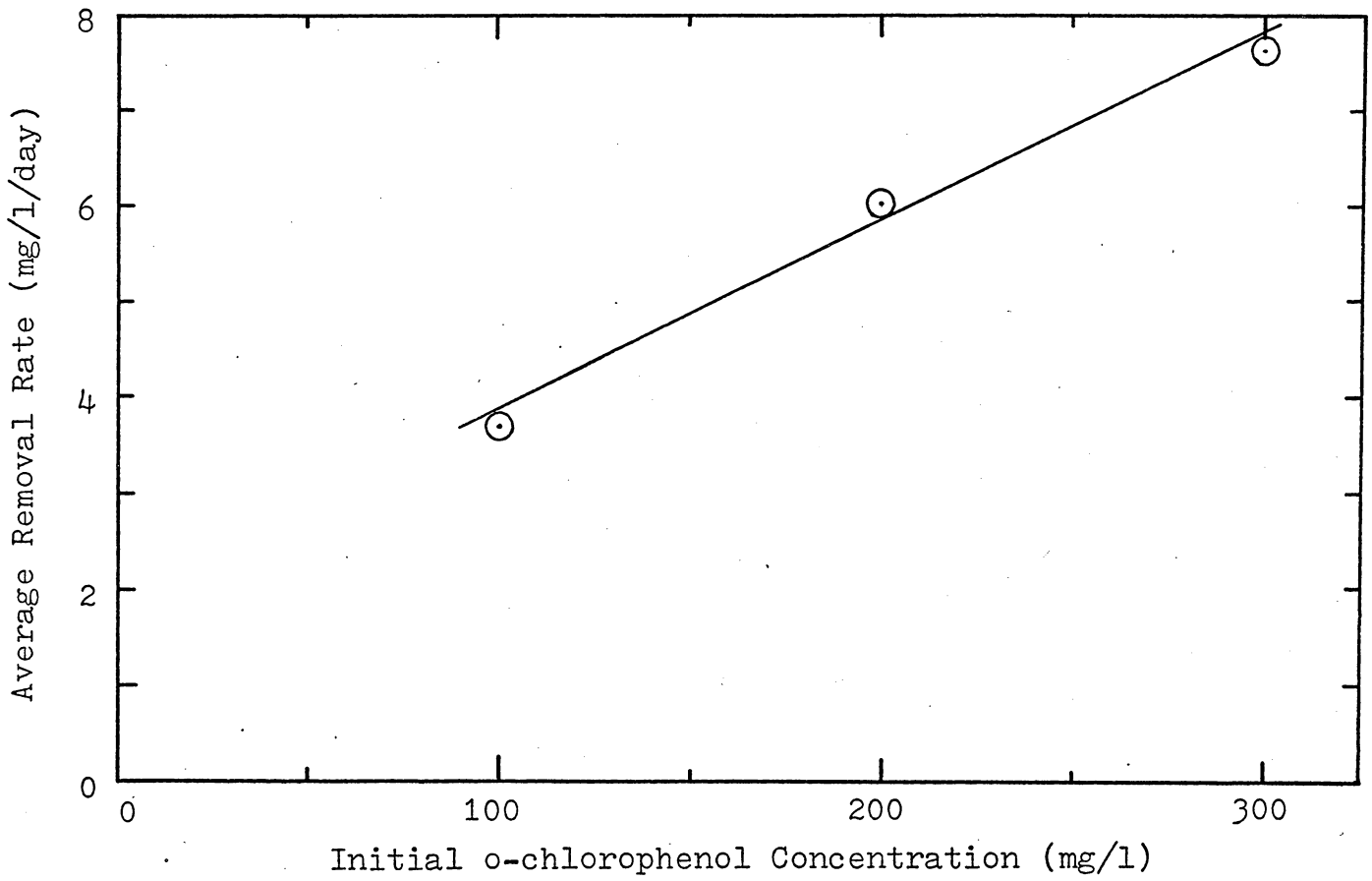

Figure 8 Comparison of o-chlorophenol Removal Rates at Different Initial Concentrations--20-Day Incubation

Figure 9 shows the average degradation rate per average actinomycete biomass concentration as a function of the final o-chlorophenol concentration. This analysis is a modification of that used when an assumption of first-order substrate-utilization kinetics are assumed for a system. The modification was that the average biomass concentration (total mass produced divided by total incubation time) was used in the analysis instead of the total biomass present at the termination of the run. Appendix Figures A5, A6, and A7 show the relationships between o-chlorophenol degradation and biomass production for each experimental culture in Run 3. The slopes of these curves are the rates of o-chlorophenol degradation per unit total biomass produced during the incubation period. The data used in constructing these figures is tabulated in Appendix C.

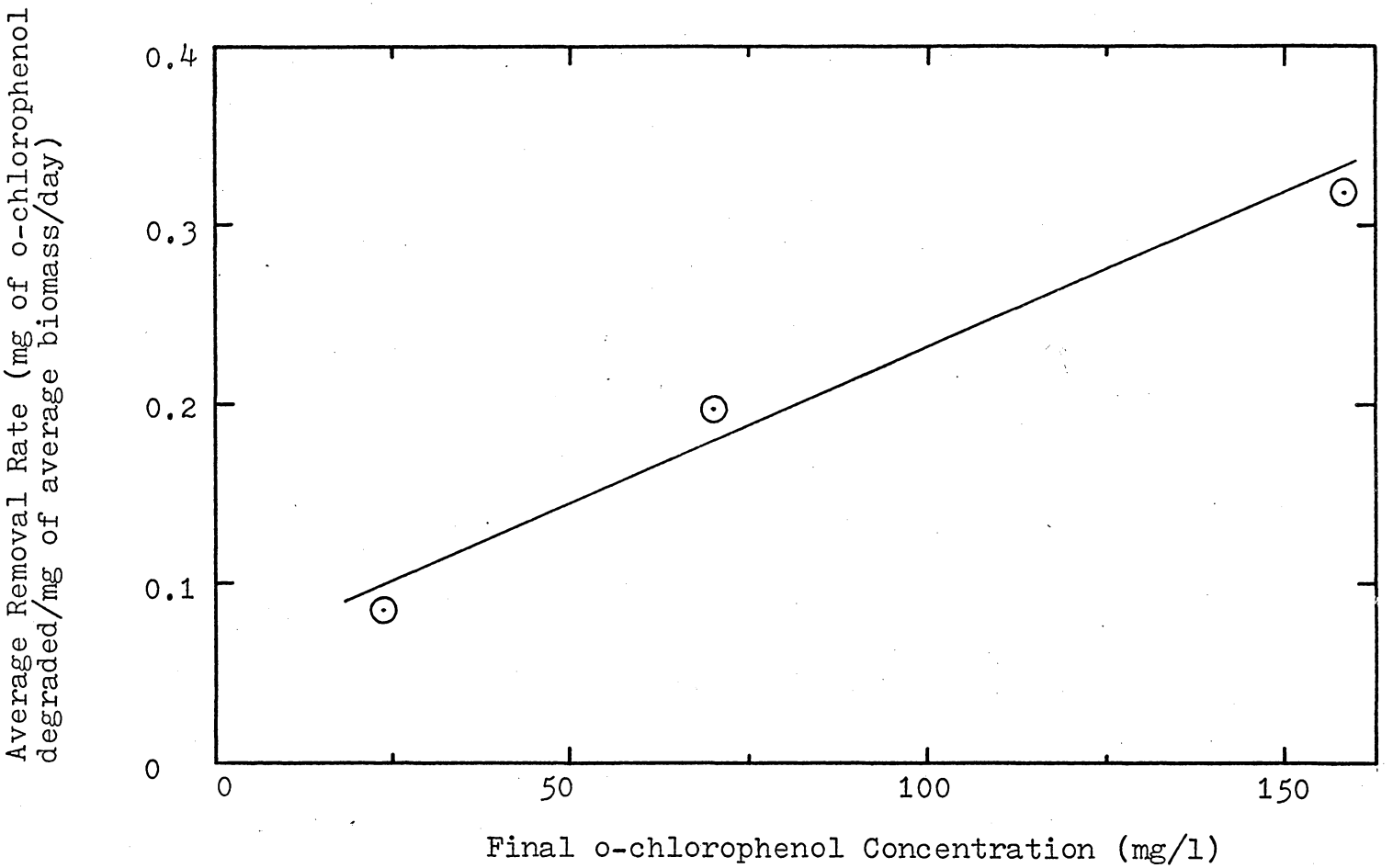

Figure 9 Average Removal Rate of o-chlorophenol Per Average Unit Biomass Produced

CHAPTER V

DISCUSSION OF RESULTS

The general developmental characteristics of the actinomycete used in this investigation were similar to those reported in past studies (9, 15, 23, 28). Both primary and secondary growth stages were observed, and the medium pH of 7.0-7.2 was optimum for growth. However, the optimum temperature for development was observed to be 30-35 degrees Centigrade instead of room temperature as most of the other investigators have mentioned.

No attempts were made during this study to differentiate between primary and secondary mycelia in broth media on the basis of hyphae diameter. However, others (9, 23, 28) have reported that surface colonies are comprised of secondary mycelium. Using this growth characteristic as an approximate indication of the time of secondary development, it was observed that an increase in the concentration of o-chlorophenol lengthened the time required for the formation of secondary mycelia. However, the media containing the most surface growth were not always those that exhibited the greatest uptake of o-chlorophenol. Therefore, the stage of growth that was the most active in the degradation of the o-chlorophenol could not be determined with certainty.

The results of the rate-degradation studies (Run 2 and 3) demonstrated that the particular species of actinomycete

used in this study was effective in degrading o-chlorophenol at concentrations up to 400 milligrams per liter. At 500 milligrams per liter, utilization did occur, but the rate was much slower than at 400 milligrams per liter, indicating that a lethal concentration was being approached. It is possible that a longer acclimation period might be required at higher concentrations to overcome the inhibitory effects of the phenolic compound. Oxygen-uptake studies would be useful in establishing whether o-chlorophenol was toxic or merely inhibitory at any given concentration.

The inhibitory effects of the o-chlorophenol at various concentrations are indicated indirectly by the fact that the incremental rate of utilization was much less between concentrations of 100 and 400 milligrams per liter than it was between 10 and 100 milligrams per liter. It should be emphasized that these differences may have been merely a reflection of the longer duration of the lag period of growth in samples containing the higher concentrations of o-chlorophenol. It is possible that the utilization rates should be measured only during the log growth phase for comparative studies, but, of course, knowledge of the extended lag phase is important if the actinomycete is ever to be used in field applications of chlorophenol waste treatment.

During Run 3, it was observed that the o-chlorophenol uptake rate per unit of actinomycete biomass increased with increasing o-chlorophenol concentration, even though there

was an inverse relation between the total biomass produced and the concentration of the o-chlorophenol. Though it is not possible to make an analogy between nutrient degradation in static and in continuous-flow studies (biostats or chemostats), some principles involved in the latter may be useful in explaining the results observed in this investigation.

At all times, the available food-to-microorganism ratio was highest in the culture containing the most o-chlorophenol and lowest in the one containing the least. If o-chlorophenol degradation is a first-order reaction, as was assumed by the analysis shown in Figure 9, then one could expect that the rate of removal per unit biomass would increase as the food-to-microorganism ratio increased. This increase was observed for the three concentrations of o-chlorophenol that were studied. The relationship most likely would not have held if first-order kinetics were not operative nor if the o-chlorophenol were toxic at those concentrations.

Other possibilities that should be considered as explanations for the results shown in Figure 9 are:

1. After the initial lag growth period, the organism might be better adapted to the o-chlorophenol and, thus, would be able to utilize it as a primary carbon source.
2. The production of oxidative enzymes, active against o-chlorophenol, might be stimulated by increasing the o-chlorophenol concentration.

CHAPTER VI

SUMMARY AND CONCLUSION

Based on the analytical data derived from this investigation the following conclusions were reached:

1. The pH and temperature for optimum development of the actinomycete used in this study were, 7.0-7.2 and 30-35 degrees Centigrade, respectively.
2. The actinomycete was shown to effectively degrade o-chlorophenol in concentrations up to 400 milligrams per liter in enriched, organic media. A concentration of 500 milligrams per liter was inhibitory but did not prevent degradation by this organism.
3. Increasing the concentration of o-chlorophenol beyond 100 milligrams per liter had the effect of increasing the duration of the lag growth period for the actinomycete. However, the rates of degradation increased as the initial o-chlorophenol concentration increased up to 400 milligrams per liter, indicating that first-order kinetics were operative.
4. It appeared, based on limited evidence, that significant o-chlorophenol degradation did not begin until the actinomycete developed secondary growth. However, no measurements of hyphae were made to substantiate that secondary mycelium had developed. Therefore, which stage in the actinomycete life

cycle that is the most active in degrading the o-chlorophenol cannot be stated with certainty.

5. Though it was established that the actinomycete can effectively degrade a chlorinated phenol, further study needs to be done to determine which growth stage (primary or secondary) is the most active. Too, similar investigations with other chloro-isomers of phenol should be considered.

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APPENDIX

APPENDIX A

Media Composition

Synthetic M_1B_2 Media

Compound	Quantity, gms
Sodium Citrate	10.00
Dextrose	10.00
Calcium Chloride	0.10
Magnesium Sulfate	0.05
Ferrous Sulfate	0.01
Ammonium Nitrate	6.00
Disodium Phosphate	2.00
Dipotassium Phosphate	2.00
Distilled Water	1000 ml

Modified Emerson's Media

Compound	Quantity, gms
Dark Brown Sugar	20
Peptone	8
Beef Extract	4
Yeast Extract	2
NaCl	2
Distilled Water	1000 ml

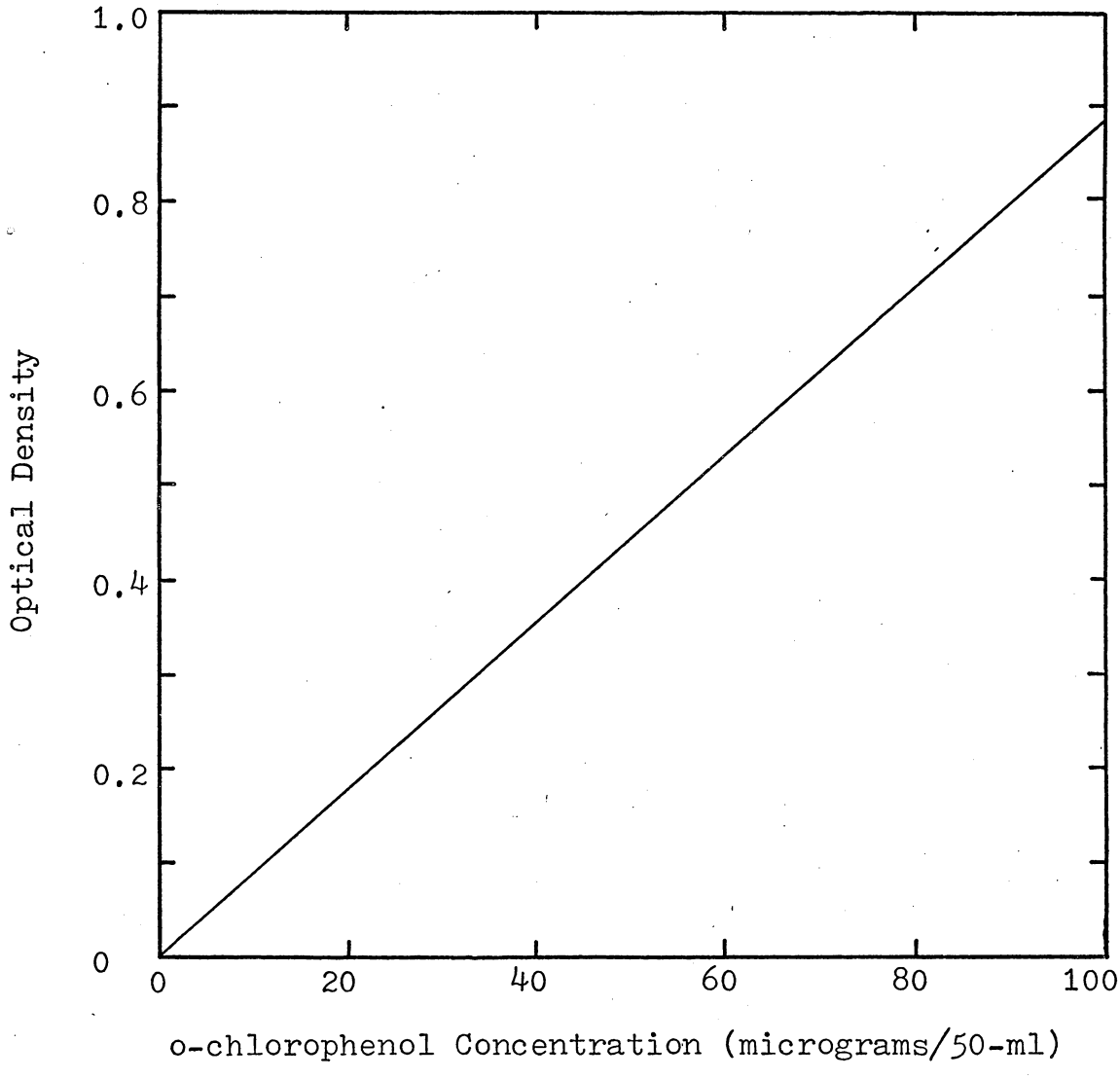

Figure A1 Calibration Curve for Colorimetric o-chlorophenol Determination by 4-aminoantipyrine Method

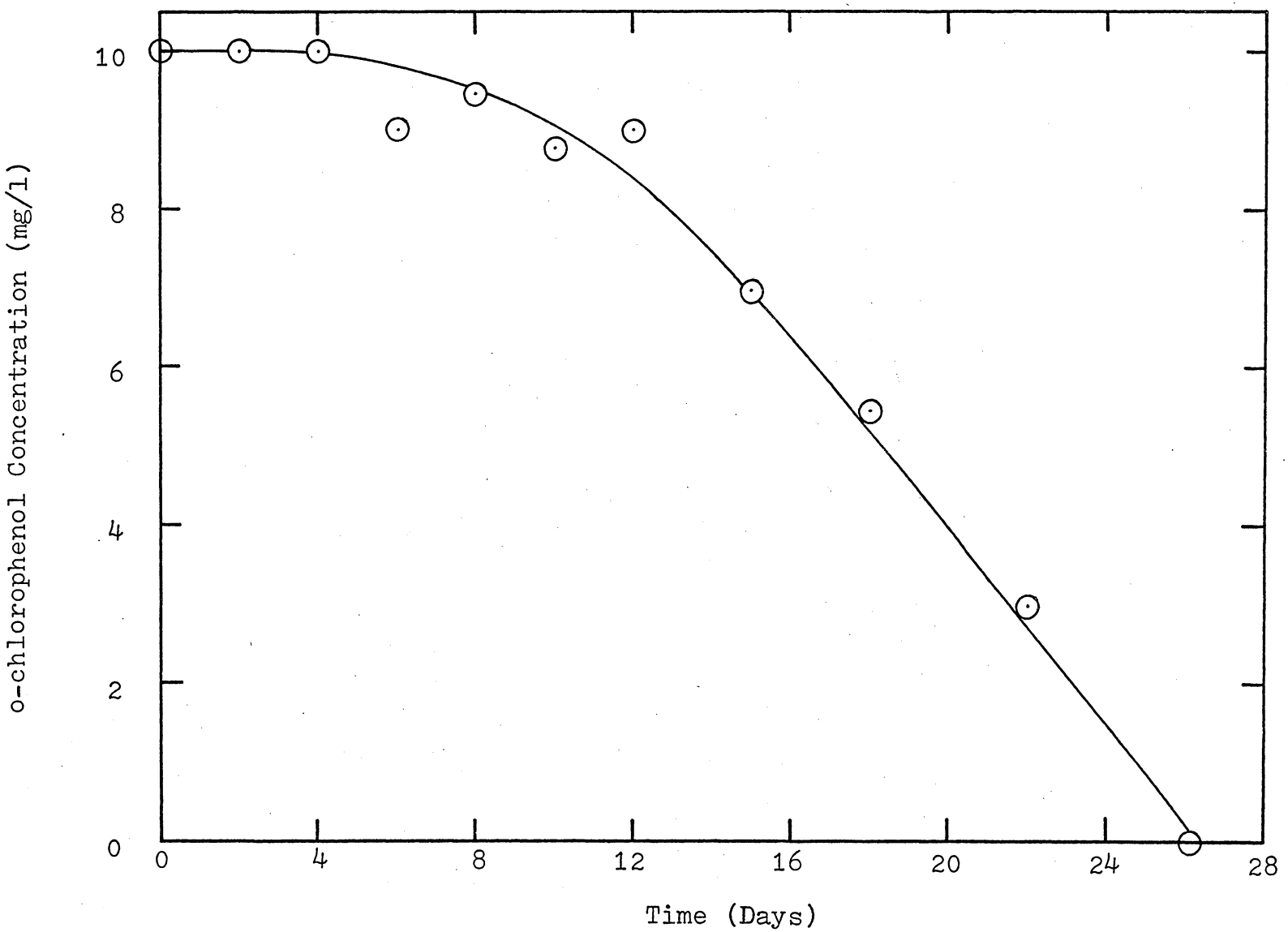

Figure A2 o-chlorophenol Degradation Rate (C_i=10 mg/l)

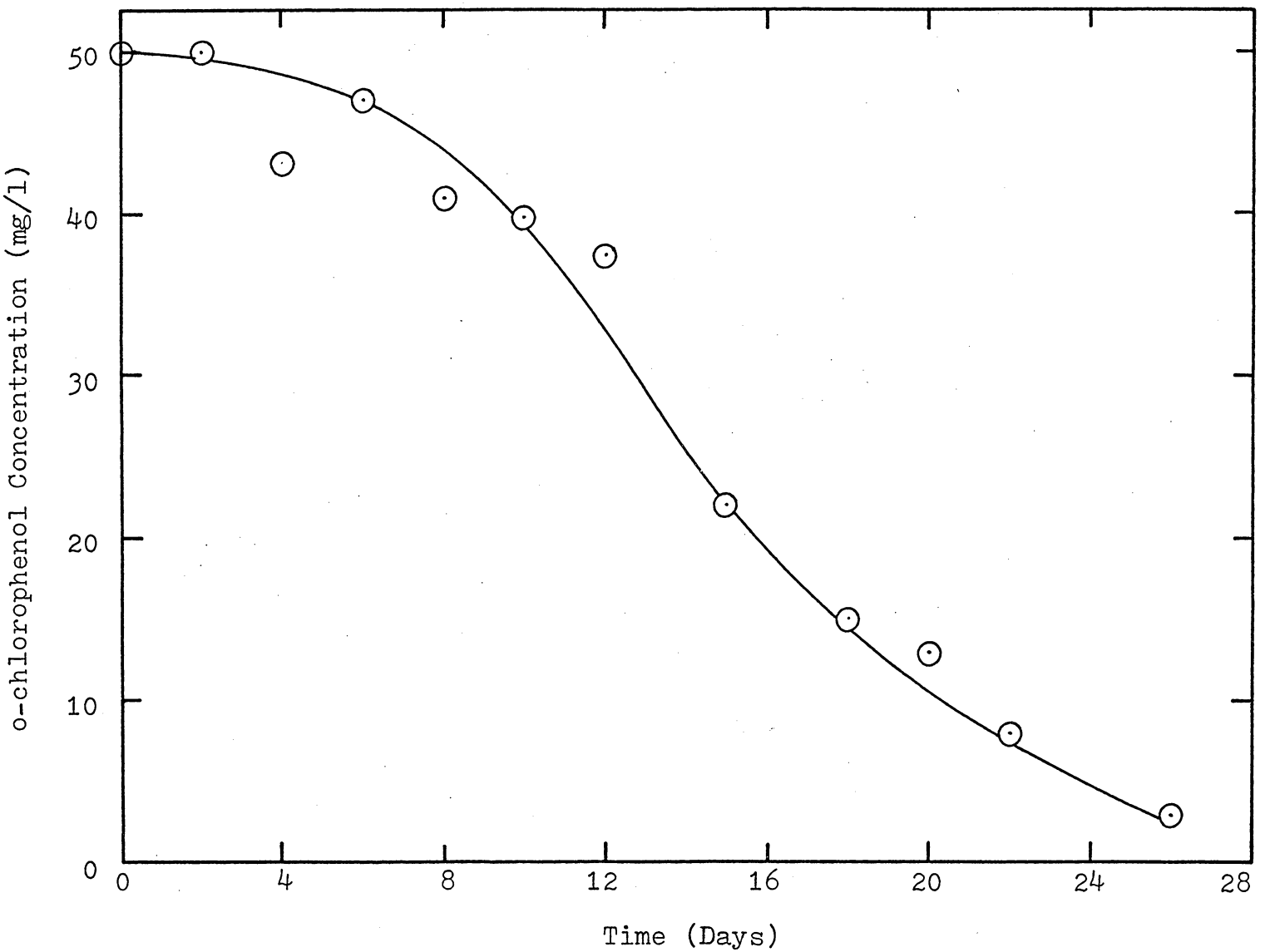

Figure A3 o-chlorophenol Degradation Rate (C_i=50 mg/l)

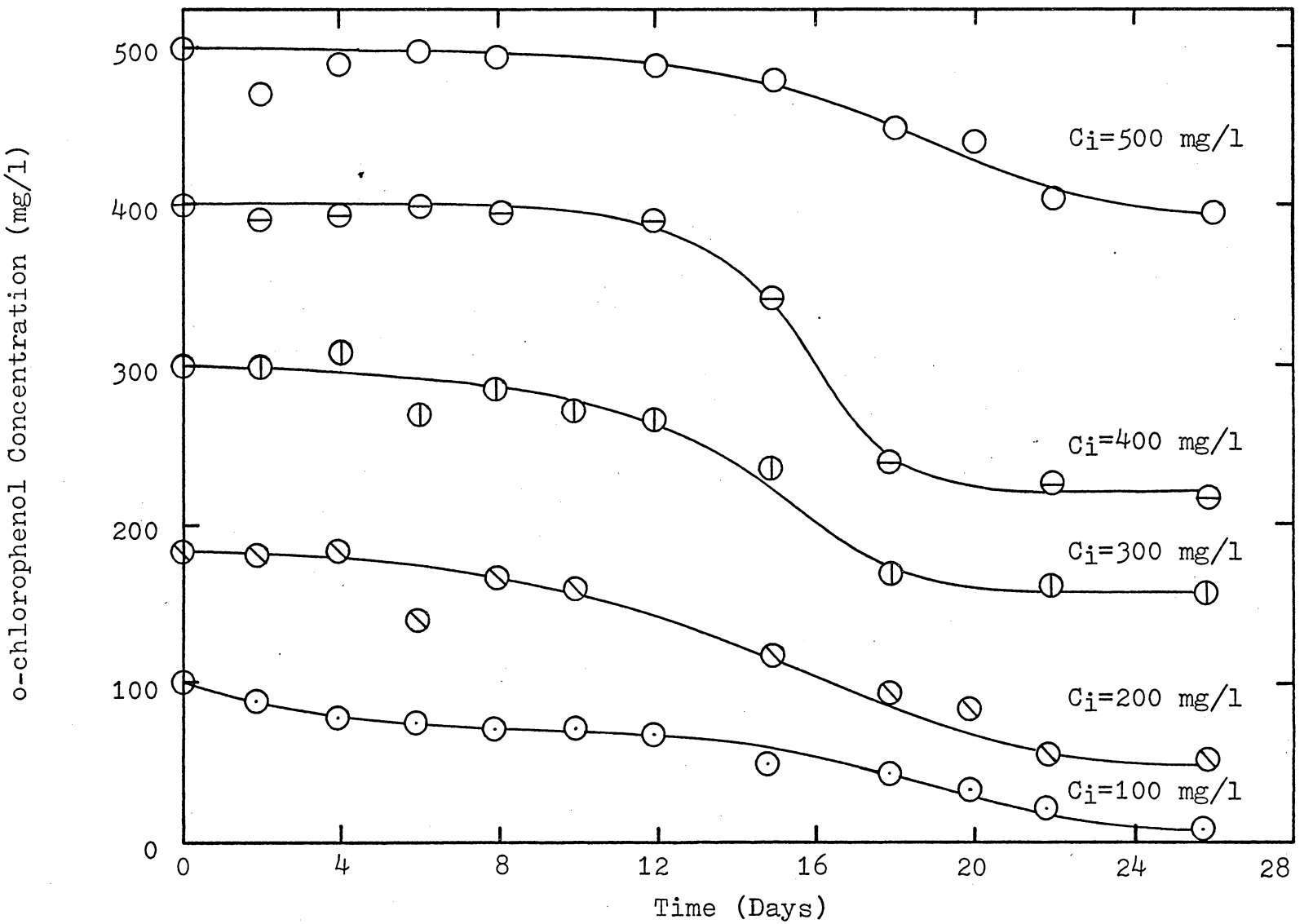

Figure A4 o-chlorophenol Degradation Rate for Various Initial Concentrations

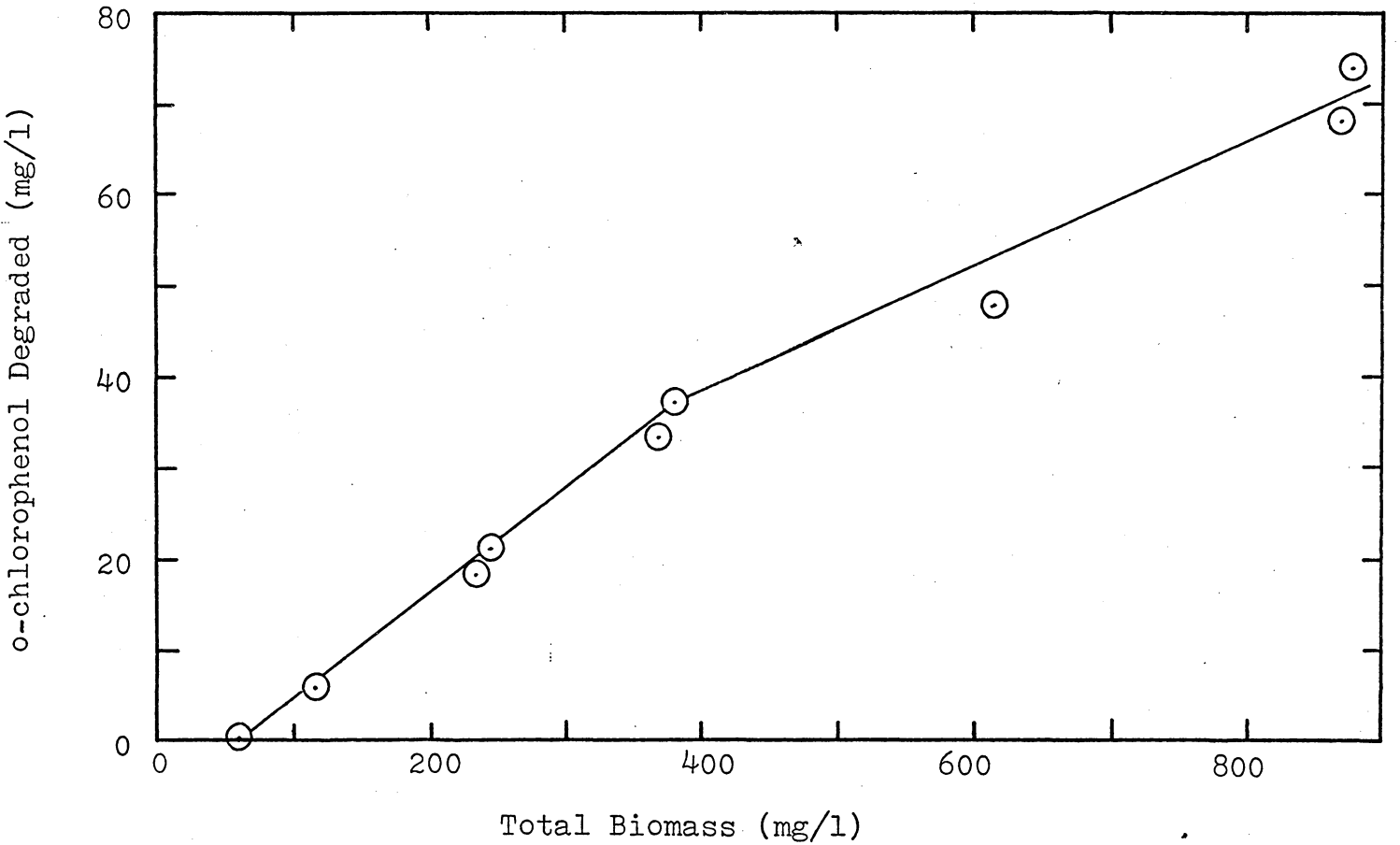

Figure A5 o-chlorophenol Degradation and Biomass Production (C_i=100 mg/l)

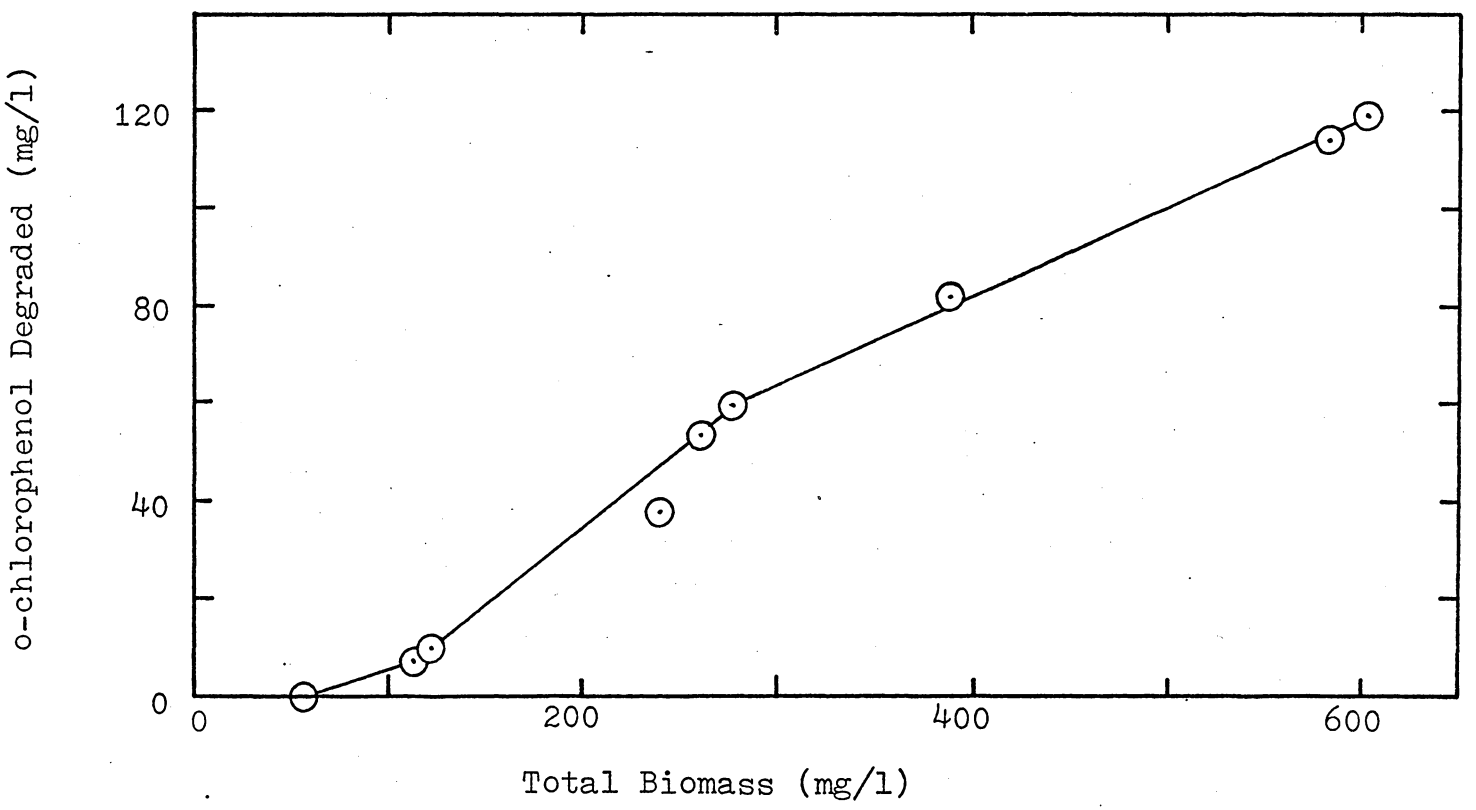

Figure A6 o-chlorophenol Degradation and Biomass Production (C_i=200 mg/l)

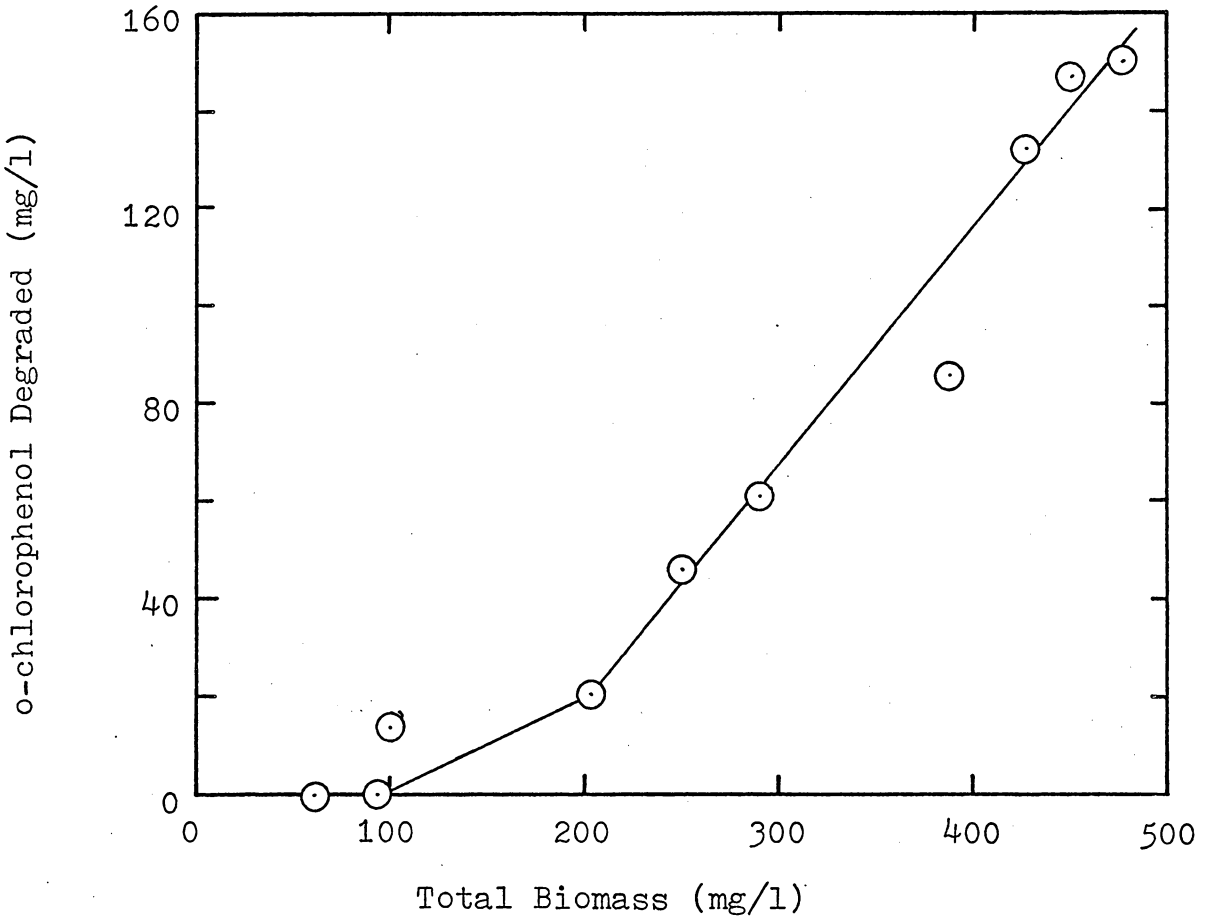

Figure A7 o-chlorophenol Degradation and Biomass Production (C_i=300 mg/l)

APPENDIX B

ORTHOCHLOROPHENOL DEGRADATION (RUN 2)

Time Days	Concentration, mg/l						
	10	50	100	200	300	400	500
2	10	50	90	178	300	390	470
4	10	43	79	180	309	396	490
6	9	47	76	142	271	400	500
8	9	41	74	168	285	395	405
10	9	40	70	160	270	--	--
12	9	38	68	--	268	390	490
15	7	22	49	116	234	340	482
18	5	15	44	96	170	241	448
20	-	13	36	84	--	--	443
22	3	8	24	56	164	227	402
24	-	-	--	--	--	--	--
26	0	3	8	52	157	219	398
Total Removal Percentage	100	94	92	71	68	44	20

APPENDIX C

ORTHOCHLOROPHENOL DEGRADATION
(RUN 3)

	(C_i=100 mg/l)		(C_i=200 mg/l)		(C_i=300 mg/l)	
Time Days	Residuals mg/l	Biomass mg/l	Residuals mg/l	Biomass mg/l	Residuals mg/l	Biomass mg/l
0	98	60	190	53	310	64
2	--	--	192	--	310	--
4	92	117	182	114	318	94
6	80	235	180	120	295	103
8	77	247	152	241	189	305
10	65	368	136	263	263	252
12	61	381	130	280	248	294
14	50	614	108	394	224	390
16	--	--	94	--	177	428
18	30	870	73	586	161	450
20	24	874	70	607	158	478

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DEGRADATION OF ORTHOCHLOROPHENOL BY AN AQUATIC ACTINOMYCETE

by

Yi-ning King Chen

(ABSTRACT)

Many industrial operations produce phenolic wastes which are objectionable to receiving waters by conventional biological treatment. It has been established that some actinomycetes, especially Streptomyces species, are capable of decomposing a wide variety of aromatic compounds, including phenols. The purpose of this investigation was to determine the growth characteristics of an aquatic actinomycete and to evaluate its potential for degrading o-chlorophenol.

Results of the investigation showed that this Streptomyces species has two growth stages, primary and secondary. In the primary stage, growth was restricted to the bottom or subsurface of both liquid and solid media. Secondary growth occurred extensively on the medium surface. Orthochlorophenol degradation was evaluated by adding it in different concentrations to the developing cultures of the organism. The rate of degradation was increased by increasing the dose of o-chlorophenol up to a concentration of 400 milligrams per liter. Growth inhibition was clearly evident at a concentration of 500 milligrams per liter.