FACTORS INFLUENCING THE PREDOMINANCE PATTERNS OF MICROORGANISMS GROWN AS A DEFINED HETEROGENEOUS POPULATION IN CONTINUOUS CULTURE

By

CROSBY WILSON JONES, JR.

Bachelor of Science
Oklahoma State University
Stillwater, Oklahoma
1972

Master of Science
Oklahoma State University
Stillwater, Oklahoma
1974

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of the Oklahoma State University
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Dedicated

to

My Mother and Father

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Thesis Approved:

Elizabeth T. Gaudy
Thesis Adviser

J. C. Lynch

H. L. Barnes

N. P. Maundy

Norman N. Durham
Dean of the Graduate College

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CHAPTER I

LITERATURE REVIEW

The biological treatment of organic wastes is used extensively in the degradation of complex organics to their simpler mineral components. The introduction of these degradation products into a receiving stream results in a minimal effect upon the dissolved oxygen levels. This represents the primary goal in any waste treatment process. The biological method of treatment varies from municipality to municipality and among industries, depending upon such factors as type of waste, volume of waste per day, and economics. Three of the most commonly used methods of treatment are the trickling filter, oxidation pond, and the activated sludge process. Each has its advantages and disadvantages in the treatment of a particular waste. The activated sludge process has attained enormous dimensions since it was developed by Ardern and Lockett (1914). Rapid removal of organics as well as the relatively easy process of separating purified water from the biological floc are two of the most important advantages in using the activated sludge process for biological waste purification.

Activated sludge consists of biological flocs which are the result of growth of bacteria and other microorganisms in the presence of dissolved oxygen. These flocs remove soluble matter as well as some suspended materials. After the removal of the waste has been achieved, the sludge flocs are allowed to settle. This results in the separation

of the microbial mass from the purified waste water. An effluent freed of 90-95 percent of its organic matter is not uncommon in this treatment process. A portion of the settled sludge is recycled to the aerator where purification is an ongoing process. A discussion of the biological concepts in the design and operation of the process has been presented by Gaudy and Gaudy (1971).

An important aspect of any biological waste treatment process is the ecology of the biomass involved. The interactions between the various microbial components is as important as the growth characteristics of the individual species. Growth of the biomass is a result of the utilization of the organic waste for carbon and energy. A typical biomass in a biological treatment process is very heterogeneous. The rate of waste utilization as well as the rate of microbial growth is dependent upon both the growth characteristics of the individual components of the biomass and the design and operation of the treatment plant. Emphasis upon growth characteristics has grown since the classic work with pure cultures by Monod (1942). His review (1949) defined the principles upon which much subsequent work was based. The development of the continuous culture technique in the succeeding years was dependent upon much of the information gleaned from early growth experiments in batch culture. Discussions on the uses and theory of continuous culture have been presented by Monod (1950), Novick and Szilard (1950), Herbert, Ellsworth and Telling (1956), James (1961), and Kubitschek (1970). This technique has proven invaluable in such diverse microbiological research as microbial genetics, physiology, and ecology. The relevance of continuous culture to waste purification is most evident in the activated sludge process which is dependent upon a perpetual

microbial population actively stabilizing incoming organic wastes. Although the activated sludge process has been available for over fifty years, maximum potential is only now being realized as a result of extensive research in defining the engineering and biological parameters of the process.

Specific members of the biological population can achieve importance during waste treatment. Some have a beneficial effect, as exemplified by the excellent floc-forming characteristics of the bacterium Zoogloea ramigera (Heukelekian and Littman, 1939). Conversely, they may be a detriment to the process as are the poor settling characteristics of the bacterium Sphaerotilus natans (Smit, 1934). The heterogeneous nature of the microbial population allows greater flexibility in the variety of organics which are treatable by activated sludge. It also represents, to some extent, a buffer against perturbances of the process commonly known as shock loads. However, because the process is composed not only of beneficial microbes but also those capable of disrupting the process or causing disease, the plant operator prefers that the system be subject to some engineering control so that the effect of the detrimental microorganisms is minimized. Unfortunately, research has stressed the engineering aspects of the process while neglecting the important biological considerations. Many of the problems encountered during the operation of an activated sludge treatment plant may be corrected by an engineering manipulation but an ever-increasing percentage of plant failures can be attributed directly to some biological disorder. These are often difficult to explain, let alone correct by engineering means. The types of microorganisms present, the interrelationships between the various

species, microbial growth kinetics, response to quantitative and qualitative changes of incoming wastes as well as effect of temperature and other physical parameters upon the mixed microbial population are some of the areas awaiting further study. Information obtained from such studies is necessary to improve an already vitally important method of organic waste treatment.

Bacteria are the basic biological units in aerobic waste treatment systems. Most of these are either obligate aerobes or facultative anaerobes. Allen (1944) found that most of the organisms in activated sludge were Gram-negative rods belonging to the genera Pseudomonas, Flavobacterium, and Achromobacter. Bacteria of fecal origin as well as spore-forming species were found only in small numbers. These results agree with those of van Gils (1964) for activated sludge flocs grown on sewages of domestic origin. He found that the majority of the bacteria isolated were from the genus Achromobacter with fewer representatives from the genera Flavobacterium, Pseudomonas, Alcaligenes, and Arthrobacter. The latter genus is the only Gram-positive one reported with any regularity in most activated sludges. Sludges derived from dairy waste waters are often reported to have a substantial number of Gram-positive bacteria. Adamse (1964) reported a succession of predominant genera developing upon a new dairy waste activated sludge as it progressed from its initial to its optimal stage. Among the bacteria he isolated were representatives from the groups Achromobacteraceae, Pseudomonadaceae, and a coryneform group. The pseudomonads which were initially present in large numbers ultimately represented the smallest of the three major groups. About 85 percent of the coryneform bacteria were identified as Arthrobacter-like bacteria.

Another important biological unit in activated sludge is the protozoan group. These organisms play a very important role in the proper functioning of the process. McKinney and Gram (1956) have shown that there is a definite succession of protozoan classes as activated sludge is formed and becomes more efficient. They stress the importance of the ciliates as indicators of the efficiency of the treatment process. These protozoa live upon the large bacterial populations naturally found in activated sludge. An equilibrium develops between the bacteria and the ciliates which results in a clearer effluent, a more stable effluent, a more rapid rate of oxygen uptake, and greater flocculation efficiency. The basis for each of these effects is not completely understood, but research has been stimulated in most of these areas. Much of this research has involved either batch or biological oxygen demand (BOD) studies rather than activated sludge. For example, the increased uptake of oxygen has been studied in BOD bottles by Bhatla and Gaudy (1965). They presented evidence in support of a major oxygen uptake by the protozoa during ingestion and utilization of the bacteria for growth. When there was a significant time difference between the peak in bacterial numbers and the period of rapidly increasing protozoan numbers, a plateau in oxygen uptake was evident. Explanations for the effect upon clarity of the effluent have been presented by Pillai and Subrahmanyan (1944). They showed the protozoa were responsible for reducing the number of free-swimming bacteria thus aiding in producing a clarified effluent. The effects upon effluent stability and flocculation efficiency can also be visualized as being the result of the elimination of free-swimming bacteria. By reducing these bacteria, oxygen-consumers are not released into the receiving stream.

Therefore, this stable effluent prevents exertion of an excessive oxygen demand. Also, with the elimination of free-swimming bacteria, selection for floc-forming bacteria should result in greater flocculation efficiency. The importance of protozoa to the activated sludge process is unquestionable. Further studies on the ecological relationships involved is warranted.

The presence of viruses in activated sludge is important from a human health standpoint although there are undoubtedly significant numbers of bacteriophages present in the system. The effect of the latter upon the efficiency of the activated sludge process has drawn little attention. However, the persistence of pathogenic viruses in activated sludge has been examined by a number of workers. The efficacy of the activated sludge process in removing viruses from sewage under field or operating plant conditions has been studied by Clarke, et al. (1961), and Mack, et al. (1962). The data indicate that the process is quite effective in removing viruses from sewage. Comparisons to trickling filters have shown the superiority of the activated sludge process for removal of viruses (Isherwood, 1965, and Clark and Chang, 1975). The mode of virus removal or inactivation has been examined by several groups. Cliver and Hermann (1972) observed an inactivation of enteroviruses presumed to be partially attributable to destruction of the virus capsid by bacterial proteolytic enzymes. Mose, et al. (1970) observed inactivation of influenza and vaccinia virus by living and dead cultures of the ciliated protozoan Tetrahymena pyriformis. Presumably, this resulted from the release of proteolytic enzymes by both living and dead protozoa. Viruses have been shown to adsorb to the slime in trickling filters, but attempts to disassociate the viruses

from the slime were unsuccessful, indicating either the association was very strong or the viruses were inactivated (Clarke and Chang, 1975). The result in either case is the reduction in effluent virus concentrations.

Fungi also play an important role in the stabilization of organic wastes since, like bacteria, they can metabolize almost every type of organic compound found in influent wastes. They perform a major role in the efficiency of trickling filter processes (Taber, 1976). However, under normal environmental conditions, fungi are of secondary importance in activated sludge (McKinney, 1957, and Hawkes, 1963). The presence of fungi in activated sludge was not described until 1943 by Lackey and Dixon, who frequently observed branching fungi in several activated sludge plants. Among the first fungi isolated from activated sludge was the predacious fungus Zoophagus insidians. Cooke and Ludzack (1958) isolated this organism from an experimental activated sludge unit treating cyanide wastes. Reports of the isolation of other species were soon forthcoming. However, the systematic study by Cooke and Pipes (1970) was the first such study to describe quantitatively the fungal population in activated sludge. They examined 19 different activated sludges for numbers and types of fungi. The fungi most commonly found belonged to the genera Penicillium, Trichosporon, and Geotrichum. A total of 44 species or species groups were described in addition to the three most common members. Of all the fungi isolated, only Geotrichum and Penicillium were isolated from all 19 activated sludge units examined. Most of the fungi isolated were considered to belong to the ecological group of soil fungi known as sugar fungi. This distinction was given to those fungi using simple carbohydrates rather than complex

organic substrates. Since few wastes are predominately simple carbohydrates, the ubiquitous presence of fungi in activated sludge is probably in most cases not a function of the concentration of simple carbohydrates present. Furthermore, bacteria with their greater surface to volume ratio would be expected to compete more favorably than fungi for any available simple carbohydrate. Although Geotrichum and Penicillium are sugar fungi, they have other metabolic characteristics which may explain their ubiquity in activated sludge. Geotrichum candidum is known to have the ability to utilize many complex organic substrates such as motor oil and amyl acetate (Cooke, 1957), organic alcohols and acids, glycerides and fats (Pipes and Jones, 1963), and spent sulfite liquor (Cooke, 1961). The ability to use such a diversity of substrates for growth may explain the persistence of G. candidum in activated sludge. Penicillium may maintain its place in activated sludge by a similar means or possibly by the synthesis of organic acids and antibiotics. Little work on the importance of the latter to the ecology of activated sludge has been attempted.

Other organisms reported to be in activated sludge include algae, rotifers, nematodes, and other higher animals. Algae might be expected to be important only on the sides of aerators or other light-exposed areas. The presence of higher animals has been reported to occur only in highly stabilized systems where an excess of oxygen occurs at all times (McKinney, 1957). Furthermore, rotifers were reported to be useful as indicators of a very high degree of purification, since they appear to predominate after all of the protozoa have died for lack of food. The importance of these organisms in primary purifications is virtually nil.

The activated sludge process with its many advantages for waste stabilization is not problem-free. Obtaining good results from the process requires a sludge with the ability to separate organic matter from the waste in a short time and one which is itself easily and efficiently separable from the clarified waste. Problems may arise when the former requirement is not met. This represents a biological treatability problem which can be prevented at the outset if the composition of the waste is known. Steps may be taken to ensure that all nutrients required for waste assimilation are present. The toxicity and recalcitrance of the waste may also be determined by preliminary studies. With this information, the feasibility of installing an activated sludge plant may be assessed. Even those plants with a sound experimental basis prior to installation are sometimes beset with treatability problems. This invariably occurs in plants treating influent wastes of unpredictable nature. An example of this type of waste is represented by combination municipal-industrial sewage. While day-to-day experience in treating this waste may allow some measure of predictability, this type of waste is inherently more variable due to the diversity of waste sources.

In some cases, microorganisms will grow quite well in the waste thereby satisfying the treatability requirement, but will grow in a manner which affects the separation of the floc from the waste water by sedimentation. As the primary goal of any waste treatment process is to remove substances with the potential of exerting an oxygen demand on the receiving stream, activated sludge in the effluent represents a major malfunction of the process when it occurs. There are many different phenomena which can prevent activated sludge from settling properly

in the clarifier. An all-encompassing term, "bulking," has been used to describe sludges with poor settling characteristics. However, Pipes (1967) has made a number of distinctions between phenomena which can be truly called bulking and those which cannot. He defines bulking sludge as sludge which settles slowly and compacts poorly, leaving a small amount of clear supernatant. This definition distinguishes bulking sludge from other sludges with settling problems such as pin point, billowing, rising, anaerobic, overaerated, and floating sludges. Each of these arises from either floc formation or density problems, whereas bulking sludges arise from floc compaction problems. Bulking sludge appears to arise from excessive filamentous microbial growth. In most cases, bulking sludge appears to be similar to normal sludge except that the filaments extending from the flocs are much more numerous and much longer. The causes of various settling problems have been defined in some instances. However, in these as well as in those which are not so well understood, such as bulking, there are questions as to the number of different mechanisms causing the problem, the identity of the microorganisms involved and the proper methods of controlling the growth of the causative organisms. Some of the parameters reported to have an effect on bulking include organic and inorganic waste composition, toxic compounds, shock loads of various types, pH, temperature, and dissolved oxygen.

A survey of proposed mechanisms leading to bulking conditions has been presented by Pipes (1967). Verification of any of these hypotheses is dependent upon a more extensive knowledge of the ecology of normal activated sludge which is, unfortunately, lacking. Comparisons of normal versus bulking sludges have led to the isolation of a number

of microorganisms believed to be involved in the bulking of sludge. The organism historically described as the cause of bulking is the filamentous bacterium Sphaerotilus natans. Ruchhoft and Watkins (1928) were among the first to isolate and properly identify this organism from a bulking activated sludge. The presence of S. natans in bulking sludge was subsequently confirmed by a number of other workers (Smit, 1934, Lackey and Wattie, 1940, Tkachenko and Droblyanets, 1959). Although there is little doubt that S. natans is responsible for some cases of filamentous bulking, in the vast majority of cases bulking has been attributed to this organism on the basis of microscopic examination alone. Misidentification is not uncommon.

Other bacteria sometimes implicated in the bulking of sludge include members of the genus Bacillus. Pipes (1967) isolated bacteria from three different samples of bulking activated sludge. These isolates initially grew as filaments on agar medium, but subsequently broke into spore-forming single cells characteristic of Bacillus. He also described an earlier study by Shive and Buswell (1928) who reported producing a growth which looked very much like filamentous bulking sludge in a pure culture of Bacillus subtilis. The importance of Bacillus to most reported cases of bulking is probably negligible, since nutrient limitation often causes these organisms to sporulate accompanied by cellular autolysis. The presence of a variety of filamentous bacteria in addition to Sphaerotilus natans and Bacillus has been demonstrated by van Veen (1973). Resemblances of these isolates to members of the genera Microscilla, Flexibacter, and Cyanophyceae were noted. The role of these microorganisms in the bulking of activated sludge was not demonstrated although their presence in the sludge creates the

potential for causing such problems.

Microscopic examination of samples of bulking activated sludge often-times makes it clear that some cases of bulking are caused by fungi and not by filamentous bacteria. With the variety of fungi normally present in activated sludge (Cooke and Pipes, 1970), a major fungal role in bulking might be expected to occur under certain conditions. There have not been many attempts to identify the fungi associated with bulking, but of the available reports it is obvious that a major role is played by the fungus Geotrichum candidum. Smit (1934) was one of the first to identify a bulking fungus as a species of Geotrichum. Activated sludge plants experiencing bulking conditions in Yardley, England, and Providence, Rhode Island, were subsequently shown by Hawkes (1960) and Jones (1964), respectively, to be the result of excessive growth of Geotrichum candidum. This fungus is quite ubiquitous in nature including activated sludge (Cooke and Pipes, 1970). It belongs to the class Fungi Imperfecti because of its apparent lack of a sexual phase of growth. Reproduction occurs asexually by fragmentation of mycelia into arthrospores which may vary in length from 2.7 microns up to 21.6 microns (Jones, 1964). Growth in agitated culture results in spontaneous fragmentation of the hyphae into arthrospores. The degree of sporulation in G. candidum varies markedly with specific growth rate, and has also been shown to be affected by various environmental factors including nutrient concentration (Robinson and Smith, 1976), and oxygen levels (Robinson and Griffith, 1977). Trinci (1972) measured specific growth rates (μ) in a defined medium containing vitamins by both dry weight and turbidity, and reported a value of 0.61 hr^{-1}. This was reported to be the fastest specific growth rate known for any

eukaryotic cell. Another experiment in minimal medium yielded a specific growth rate at 30C of 0.53 hr^{-1} by turbidity, and 0.46 hr^{-1} by dry weight. The wide range of growth rates at which G. candidum is able to grow may confer a selective advantage upon this fungus when competing with other fungi as well as bacteria.

The fragmentary nature of the mycelium is a critical factor in allowing the establishment of steady states in continuous flow studies. Steady states of non-fragmentary fungi in continuous flow are probably only rarely obtained and only when the shearing forces exerted by the impellar cause mycelial fragmentation (Fiddy and Trinci, 1975).

With the importance of G. candidum as a potential plant and human pathogen (Beneke and Rogers, 1970), as well as its role in the bulking of activated sludge, it is a tremendous advantage to be able to grow this organism under steady state conditions in a chemostat. The unique advantages offered by the continuous flow theory may be applied to the study of this fungus (Fiddy and Trinci, 1975, and Robinson and Smith, 1976).

Much information has been accumulated on the nutrition and physiology of G. candidum (Bobrov, 1951, Carmichael, 1954, and Jones, 1964). Nutritionally, a wide variety of complex and simple organic compounds are utilized for carbon and energy. Both inorganic and organic nitrogen compounds will serve as sole nitrogen sources. Growth will occur in media having pH values in the range of 3 to 12. This represents an advantage over most other fungi as well as bacteria. Furthermore, the wide range of temperatures which permits growth (6C to 39C) and the even wider range which permits survival (0C to 60C) may, in part, explain the ubiquity of this organism (Jones, 1964). Its competitiveness

in activated sludge has been shown to be enhanced in the presence of certain metals as well as in the presence of moderately high chloride ion concentrations (Poon and Bhayani, 1971, and Poon and Wang, 1973). With the application of continuous culture to the growth of this fungus, many of the previous studies performed in batch may be confirmed or expanded upon in the future.

It is often the case that the presence of a specific species of microorganism results in the bulking of activated sludge. Therefore, research into the characteristics and physiology of these organisms may find direct application toward solution of the problem. However, as activated sludge is by nature a highly heterogeneous population of microorganisms, the problem becomes a question of species predomination. This necessarily requires studies of a heterogeneous population to establish conditions under which the nuisance organism may be expected to predominate and thereby create the potential for problems. Investigations into the mechanisms of species predomination can ideally be made with mixtures of known species. Such information can then be checked for validity to a natural heterogeneous population such as activated sludge.

The study of defined mixed cultures can provide information concerning many aspects of microbial ecology. The competition among two or more species of microorganisms for a single substrate is a common experimental approach in this type of study. The results can be defined mathematically provided there is no interaction among the organisms (Powell, 1958). Predictions of this sort make it clear that the coexistence of competing species is not possible unless biological constants for the competitors are identical. Experimental verification

of the theoretical description of such studies has been achieved in several laboratories. Oberhofer and Frazier (1973) demonstrated the competitive exclusion principle with a mixed culture of Escherichia coli and Staphylococcus aureus in which E. coli with a greater specific growth rate (μ) successfully eliminated S. aureus. Verstraete, et al. (1975) showed the dominance of an amylolytic Pseudomonas over several other amylolytic bacteria and yeasts when grown as a two-member culture in medium containing soluble starch as the limiting nutrient. The principle of competitive exclusion was used by Jannasch (1967) to enrich for seawater bacteria in continuous culture. He reported that the predominance of one species increased with time; that growth advantages were based upon microbial growth parameters and enrichments could be carried out in the presence of extremely low concentrations of limiting substrate. Meers (1973) showed, using a series of carbon-limited mixed culture experiments, that displacement of one species of microorganism by another occurred with four different species regardless of which two were selected as a mixed culture.

The coexistence of competing species under continuous flow conditions may be attained in various ways. Taylor and Williams (1975) have presented a theoretical discussion on the stability of mixed microbial populations competing for several growth-limiting substrates. They concluded that such populations cannot coexist unless the number of growth-limiting substrates is equal to or greater than the number of species. Maigetter and Pfister (1975) reported the coexistence of two species of bacteria in a chemostat when growth was limited by citrate. This result was related to the niches which the two organisms occupied. One species resided primarily on the culture vessel wall, and the other

grew in liquid suspension. Other interactions allowing coexistence include predation, synergism, and commensalism. Canale (1973) was able to establish coexistence of a protozoan Tetrahymena pyriformis and its prey, Aerobacter aerogenes, together in a chemostat. An oscillatory relationship was established as periods of increasing bacterial numbers were quickly followed by an increase in numbers of the protozoa thereby resulting in reduction of bacterial numbers. This, in turn, resulted in reduced levels of the protozoa and the cycle was repeated. Evidence for true synergism in defined mixed cultures is lacking. However, Pipes (1966) has proposed that the microbial community in activated sludge is in a state of synergism of the whole population. Verification of this theory would appear very difficult if not impossible. There is doubtless a significant amount of commensalism occurring in such a population. Laboratory demonstrations of this type are numerous. Shindala, et al. (1965) have shown such a relationship between Proteus vulgaris and Saccharomyces cerevisiae in continuous culture. An essential niacin-like factor elaborated by the yeast and required by the bacterium caused a dependence of the bacterium on the growth of the yeast. Pickard, et al. (1975) demonstrated the utilization of triaryl phosphates by a mixed bacterial population which was dominated by a bacterium believed to be responsible for much of the initial breakdown. However, attempts to grow this organism as a pure culture in medium using triaryl phosphates as sole carbon source failed. In order for this organism to exert a major role in triaryl degradation, it appears some function provided by the mixed culture was necessary. The basis for this effect was not theorized. Similarly, an experiment by Brunner, et al. (1969) in which E. coli and Serratia marcescens were shown to

exist in common steady state at different growth rates cannot be explained without assuming some undefined interaction was taking place between the two microorganisms (Meers, 1973). One of the few reports describing a defined mixed culture composed of mold and bacterial components was presented by Elliker (1949). An example of commensalism was described between the bacterium Streptococcus lactis and the fungus G. candidum in which the latter was shown to metabolize lactic acid produced by the bacterial population.

Much work remains in the field of microbial ecology to define the relationship between microorganisms and hopefully apply this knowledge to solving practical problems such as the bulking of activated sludge. The present investigation was undertaken to study predominance patterns of the fungus G. candidum when grown in mixed continuous culture with bacteria. The culture has three known bacterial components, a Pseudomonas species, Serratia marcescens, and Escherichia coli. Various physical and nutritional parameters were varied in an attempt to define the conditions under which the mold predominates. Information obtained from this study should prove useful from a practical standpoint in the prevention of bulking during operation of the activated sludge process. Furthermore, the ecological relationships between the bacterial components should provide some insight into the ecology of a somewhat heterogeneous population without the complexity of a population whose components are for the most part unknown.

CHAPTER II

MATERIALS AND METHODS

Organisms

The organisms used in these studies included three bacterial species as well as the fungus Geotrichum candidum Link ex Pers. The latter was isolated on Sabouraud's dextrose agar (Difco) from an activated sludge plant located in Oklahoma City, Oklahoma. Tentative identification was made on the basis of comparisons with published illustrations. Positive identification was made after obtaining a culture of this fungus from the American Type Culture Collection (ATCC #16635) for use in comparative studies. All mixed culture studies included the ATCC culture of the fungus rather than the isolate. Three species of bacteria were isolated from either sewage or a soil suspension. They were identified as Escherichia coli, Serratia marcescens, and Pseudomonas mendocina on the basis of a series of biochemical tests. A species identified as P. aeruginosa was also isolated and used in one of the temperature studies. However, because of its apparent antagonism toward E. coli, it was not used in further experiments.

Identification of the Bacteria

The Eighth Edition of Bergey's Manual (1974) was consulted to select the various differential media used in identifying the organisms.

These included glycerol, glucose, lactose, and sucrose fermentation broths containing bromcresol purple as pH indicator. Other broths used were Koser's citrate, nitrate, tryptone, and methyl red-Voges Proskauer. These were used respectively to test the organisms' ability to use citrate as sole carbon and energy source, for nitrate reduction, indole production, and pattern of fermentation. All incubations were at 30C. Readings were taken at 24 and 48 hours for all fermentation broths. Citrate utilization was checked in 48 hours, indole production in 24 hours, nitrate reduction and the methyl red portion of the MRVP test in five days, and the VP portion of the MRVP test in two days. Gelatin and starch plates were also inoculated and examined for hydrolysis in 48 hours. These tests were supplemented with oxidase tests, Gram stains, wet mounts, and flagella stains.

Media

A minimal salts medium was used throughout these studies in both the continuous flow and the batch systems. Composition of the medium per 900 ml of distilled water was: $(NH_4)_2SO_4$, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.1 g; $FeCl_3 \cdot 6H_2O$, 0.5 mg; $CaCl_2 \cdot 2\ H_2O$, 7.5 mg; KH_2PO_4, 3 g; Na_2HPO_4, 6 g; yeast extract (Difco), 1 mg, and tap water, 100 ml. For the continuous flow studies, the carbon and energy source was used in a limiting concentration of 1000 mg/l in most studies. For the batch studies, the same concentration was used in the determination of the growth constants, μ_m and K_s, in which various lesser concentrations were used. All ingredients of the medium were autoclaved separately, cooled, and mixed aseptically.

For plating purposes, Tryptic Soy Agar (Difco) and Mycosel^{TM} Agar

(BBL) plates were prepared by rehydrating the powdered media with distilled water followed by dissolving and autoclaving at 121C for 15 minutes. The Tryptic Soy Agar (TSA) plates were used for obtaining total counts and the Mycosel plates for obtaining mold counts. The plates contained approximately 30 ml of medium and were dried at 37C before use. The plates were inoculated by the spread plate technique.

Another set of plates was made for one experiment by including 20 g/l of agar (Difco) in minimal medium of the composition described previously. The carbon and energy source was omitted.

Cultivation of the Organisms

Stock cultures of each of the microorganisms used in these studies were transferred to new TSA slants every two months. The inoculated slants were incubated at 30C for 18-24 hours and stored at 4C. For most experiments, cells used as inoculum were grown in minimal medium over night at 30C on a reciprocal shaker.

Determination of Growth Constants

Three biological growth constants were determined for the cultures at various points during these studies. These included maximum specific growth rate (μ_m), saturation constant (K_s), and substrate yield (Y). Each of these constants and their manner of determination have been described previously by Monod (1942) and many others. Substrate yield is defined over any finite period of growth as the dry weight of cells formed per weight of substrate used. The specific growth rate is defined as the rate of concentration increase per unit of organism concentration. In the majority of cases it is proportional to substrate

concentration to a limiting saturation value as described by the so-called Monod equation

$$\mu = \mu_m\left(\frac{S}{K_s + S}\right) \qquad (1)$$

where μ_m is the value at which further increases in initial substrate concentration (S) cause no further increase in μ. K_s is numerically equal to the substrate concentration at which $\mu = \frac{1}{2}\mu_m$.

Yield measurements were obtained by taking samples of approximately 20 ml from either continuous flow or batch systems. The cells were filtered through tared Millipore membrane filters (pore size 0.45 μm) for determination of cell concentration (mg/l dry weight). The cells were washed twice on the filter with distilled water, and the filters were dried to a constant weight in an oven over night at 105C. Dry weight determinations were made on an analytical balance. The filtrate was removed and placed into a prepared chemical oxygen demand (COD) flask and analyzed for total organic matter by oxidation with sulfuric acid-potassium dichromate (COD test, APHA, 1971). Yield was determined by dividing the dry weight of the sample by the difference between the initial feed COD and the sample filtrate COD. A less precise but simpler method of determining total organic matter was used in the bulk of the mixed culture studies (Neish, 1952). This method also involves acid dichromate for chemical oxidation of the organic matter. Two ml of sample was pipetted into an 18 by 250 mm test tube and 5 ml of the sodium dichromate mixture was added. A control was run on distilled water and also on a sodium bisulfite solution. The tubes were heated in a boiling water bath for 20 minutes, cooled to room temperature and

read spectrophotometrically at a wavelength of 650 nm on a Coleman Junior spectrophotometer, Model D. The water control was used to zero the instrument, and the bisulfite control gave the percent transmittance corresponding to complete reduction of the dichromate which was about 0.5 milliequivalents (meq) reduced. A graph of percent transmittance versus meq of dichromate reduced was made on single-phase semi-log paper by joining the two points obtained from the controls. The meq of dichromate reduced by unknown samples was determined by reference to this graph. This value was divided by two since two ml of sample was used. The resulting value was multiplied by 7.26, which is the mg of glucose per meqs of dichromate reduced. From these calculations, organic matter is expressed as mg/ml.

The constants K_s and μ_m were determined from batch growth experiments using the same medium employed in the continuous flow apparatus except the concentration of the limiting nutrient was varied from 100-1000 mg/l. The cells were grown in 250 ml sidearm flasks using 25 ml of medium. These flasks were inoculated with 0.5 ml of a previously grown log phase culture. Incubation was carried out at the appropriate temperature in a Warner-Chilcott shaking water bath at 105 cycles/min with a 1" stroke length. Growth was followed on the Coleman Junior Spectrophotometer at 540 nm by tilting the flasks so that the liquid culture entered the sidearm portion of the flask where absorbance was measured. Specific growth rate was determined by plotting absorbance versus time on semi-logarithmic paper to determine the culture doubling time which was substituted into the equation $\mu = 0.693/t_d$ originally derived by Monod (1942). K_s and μ_m were determined by measuring μ at various initial substrate concentrations. A straight-line form of the

Monod equation, $1/\mu = (1/\mu_m) + K_s/\mu_m S$ (Monod, 1942) was used to determine values for each of these constants.

Sole Carbon and Energy Sources

To select carbon and energy sources which could be used as a sole source by G. candidum, a series of agar plates was prepared. These were prepared after autoclaving by combining all components of the previously described minimal medium except carbon and energy source. The medium was inoculated before pouring by cooling to 45C followed by the addition of the inoculum prepared by washing with distilled water a 24-hour slant of the desired culture. After solidification, a few grains of various organic compounds were placed at different points on the seeded agar to test for utilization as sole carbon and energy sources. A ring of growth around the compound after incubation at 30C for up to five days was indicative of utilization. This method was used for testing cultures of G. candidum and S. marcescens.

Standard Curves

During the course of these experiments a standard curve relating cell numbers to percent transmittance was referred to for purposes of estimating cell numbers so that proper dilutions could be made for each plate count determination. The curve was prepared by growing S. marcescens at 30C in 25 ml of glucose minimal medium on a shaking water bath. Growth was followed spectrophotometrically and at various points during the growth phase, 0.1 ml samples were taken and diluted for plate count determinations.

A curve relating absorbance to dry weight was also prepared using

the same organism. A series of flasks each containing 25 ml of minimal medium was inoculated and placed at 30C on the shaker. Growth was followed spectrophotometrically and at various absorbances the entire contents of a flask was taken for dry weight determination, as described previously.

For the mixed culture continuous flow studies, reference was made to a standard curve for the proper pump setting yielding the desired dilution rate. This curve was prepared by setting up the continuous flow apparatus exactly as used during the actual studies. Distilled water was pumped through the lines to a graduated cylinder located adjacent to the feed inlet of the growth-tube. Measurements of water delivered were converted to ml/hr and further expressed as dilution rate, which is defined as flow rate divided by working volume. Various pump settings were selected and flow rates were noted in each case. A standard curve was derived by plotting ml/hr versus pump setting.

Mixed Batch Studies

As a preliminary study to the mixed culture continuous flow studies, each of the species of microorganisms subsequently utilized in these experiments was tested in batch for its ability to antagonize the growth of a faster-growing species when grown as a mixed culture. Various combinations of microorganisms were used and growth rate measurements were performed spectrophotometrically. The growth medium was glucose minimal medium. Sidearm flasks containing 25 ml of medium were inoculated with 0.1 ml of a log-phase culture of each species to be included in the mixed culture. Incubation was at 30C on a shaking water bath.

Continuous Flow Equipment and Determination

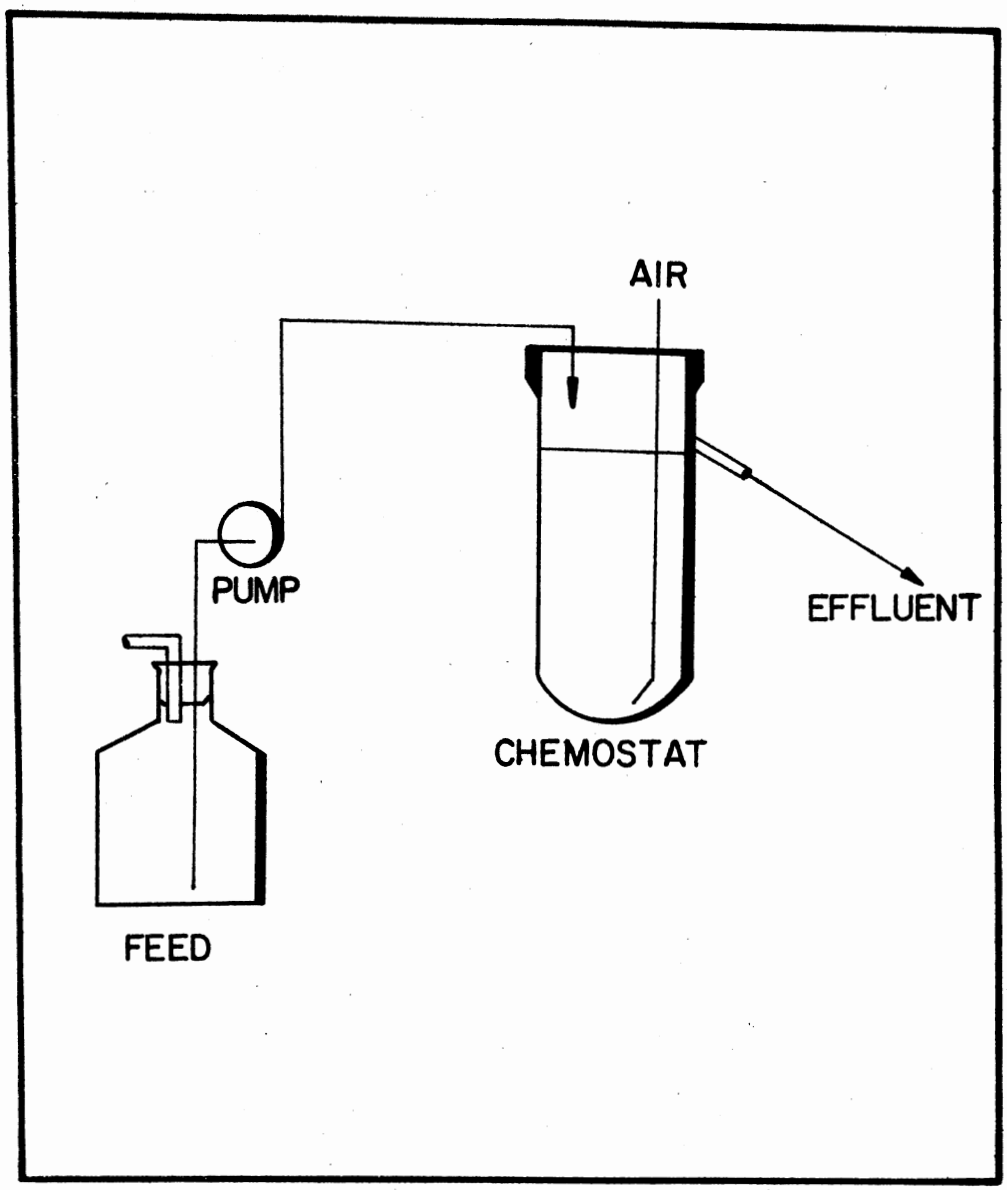
A 2.5-liter chemostat with a working volume of one liter was used throughout the continuous flow studies. It was fitted with a cap possessing several inlets for medium introduction, inoculation, aeration, and air outlets. A carborundum air sparger was fitted to the cap by rubber tubing for aeration. An air jet was used as a source of aeration. Aeration rate was monitored by a Gelman air flow meter. In all studies, the rate of aeration was 1.5-2.0 liters/minute, which was sufficient to provide both aeration and adequate mixing. Contamination of the growth chamber was prevented by passing the air through a previously sterilized cotton-filled 500 ml Erlenmeyer flask. An air filter apparatus containing a membrane filter of 0.45 μm pore size was also used in the air lines.

The feed reservoir was either a 10- or 20-liter carboy depending upon the experiment. The medium was pumped from the reservoir and into the chemostat by a Sigmamotor peristaltic pump. The pump was fitted with a vacuum rubber tubing having a 1/4" diameter and a 1/8" bore. A schematic representation of the continuous flow apparatus is shown in Figure 1.

Temperature was held constant during these studies by placing the chemostat in a constant temperature water bath. The temperature used in the majority of the experiments was 30C.

Inoculation of the chemostat was carried out by transferring aseptically five ml from a 24-hour culture of the appropriate organism to the growth chamber. This was done for each species utilized in the experiment. Approximate numbers of cells introduced at each inoculation

Figure 1. Representation of the Continuous Flow Apparatus


AIR
PUMP
EFFLUENT
CHEMOSTAT
FEED

were determined for many of the experiments. Values of 1.5×10^9 cells/ml for S. marcescens and E. coli, 1.7×10^8 cells/ml for Pseudomonas mendocina, and 5×10^6 cells/ml for G. candidum were quite constant for each experiment. The inoculum source was prepared by inoculating 25 ml of glucose minimal medium from a TSA slant of the appropriate organism. Incubation was carried out on a shaking water bath at 30C. One liter of sterile medium was pumped into the inoculated chemostat and aeration was begun. The pump was turned off until, by visual examination, the culture appeared to be growing adequately. The pump was then reactivated and set at the desired pumping rate which yielded a dilution rate of either 0.125 or 0.041 hr^{-1}, depending upon the experiment.

Growth was followed on a daily basis by taking samples from the outflow and measuring absorbance on the spectrophotometer. These samples were also used for plate count determinations. After reference to the standard curve relating absorbance to cell numbers, an appropriate dilution of the sample was made using 0.85 percent saline as diluent. Depending upon the dilution required, 0.05-0.2 ml were plated in triplicate onto TSA and Mycosel Agar plates. The plates were incubated at 30C for 24 hours. Total cell numbers as well as numbers of individual species were recorded. The species utilized in these experiments were easily distinguishable at 24 hours. Graphs relating the log of total, as well as individual species numbers, to days were prepared for each experiment. At the conclusion of each experiment, several samples of approximately 20 ml were taken from the chemostat for both dry weight determination and a Neish analysis for organic matter remaining. The sterile medium was also analyzed by the Neish method to determine the initial substrate concentration.

Mixed Culture Continuous Flow Studies

These studies were performed with a mixed culture of the four organisms described previously. Data were taken over a period of approximately one week at two different dilution rates, 0.125 and 0.041 hr^{-1}. Growth was followed spectrophotometrically and by plate count determinations. With these criteria, an assessment of predominance could be made. Special attention was paid to the presence of G. candidum. In addition to plate counts on Mycosel Agar, growth of the fungus was followed by direct visual observation both by the unaided eye and microscopically. Attempts to quantify the fungus on an indirect basis by subtracting the bacterial dry weight (average bacterial cell weighing 1.1×10^{-12}g, Luria, 1960) from total dry weight proved unreliable under certain conditions. However, when total bacterial counts remained relatively constant and G. candidum expressed a minimum amount of clumping the method was employed for quantification.

Temperature Studies

The temperatures used in these series of experiments were 37C, 30C, and room temperature, which was relatively constant at about 20C. The medium used in these experiments was glucose minimal medium containing 1 gm/l glucose. Batch experiments in glucose minimal medium were performed at room temperature in conjunction with those in continuous flow to obtain the specific growth rate of each of the cultures at this temperature. In addition, specific growth rate for G. candidum at 26C was also determined from a batch study for purposes of comparing it to those reported in the literature. One group of mixed culture

temperature studies involved the use of a pigment-producing Pseudomonas. Another study involved a mixed culture including a non-pigment producing species of Pseudomonas. This organism was used in all subsequent experiments.

Nitrogen Studies

Ammonium sulfate concentration was varied in this series of experiments. Concentrations of 50, 100, 150, and 500 mg/l were utilized as part of the glucose minimal medium containing 1 gm/l glucose. As a corollary to these experiments, K_s determinations for each of the cultures were performed in batch. Concentrations of ammonium sulfate employed in these studies were 25, 50, 100, and 500 mg/l. The temperature used in both the continuous flow and batch studies was 30C.

Carbon and Energy Source Studies

Several carbon and energy sources were used at a concentration of 1000 mg/l to test their effect on the predominance patterns of the mixed culture. These included glucose, fructose, sucrose, lactose, galactose, and acetate. Specific growth rates were determined in batch at 30C for each of the cultures prior to growth in continuous flow. In some cases, one or more of the organisms was unable to utilize the organic compound for growth. However, inoculation of the chemostat was carried out as usual in an attempt to determine if these microorganisms had the ability to persist in the system possibly by metabolizing by-products of the actively growing population.

pH Study

This study was performed at a dilution rate of 0.041 hr^{-1} and a temperature of 30C. Glucose minimal medium was used containing 100 mg/l of Na_2HPO_4 and 50 mg/l of KH_2PO_4. These concentrations were sufficient to keep essential ions such as potassium and phosphorous in excess but were low enough to serve only as a weak buffer. The initial pH of the medium was 6.9. Data were taken as before on a daily basis with the exception that pH was determined at least once and sometimes twice a day. pH determinations were gathered through the use of both pH paper and a Beckman Zeromatic pH meter.

Glucose Concentration Studies

A series of experiments was run in which the concentrations of the glucose in the minimal medium was varied. Concentrations of 100, 250, 500, and 1000 mg/l were used in these experiments. The temperature in these studies was kept at 30C.

Yield Studies With *Citrobacter intermedius* and *E. coli*

The following set of experiments served as a follow-up to an earlier investigation on the relationship between yield and maintenance energy (Jones, 1974). This study showed that substrate-based yields in *E. intermedia* (now known as *Citrobacter intermedius*) varied depending upon the rate at which the cells were grown. It was found that growth of cells in a chemostat at different dilution rates somehow programmed yield characteristics into the cells. Two different dilution rates resulted in cells with two different yields as measured from batch

growth studies in which the specific growth rate was identical for the two cultures. Three theories were presented including a change in the maintenance coefficient, an uncoupling of energy-producing and energy-consuming reactions and selection for a low-yield mutant. The latter two theories were examined to some extent in the present studies. In addition, an identical experiment was performed with E. coli to assess the generality of the observation made with C. intermedius.

Uncoupling Studies with C. intermedius

A flask containing 20 ml of glucose minimal medium was inoculated from a 24-hour slant of C. intermedius and incubated at 30C. Growth was followed spectrophotometrically until mid-log phase was reached at which time several more flasks were inoculated by introducing one ml of the growing cells into 20 ml of fresh medium. All of these flasks were assayed for yield with the exception of one which was to be used as inoculum for another series of flasks. The latter flask was allowed to enter stationary phase where it remained for seven hours as determined by absorbance. At this point, four flasks containing fresh medium were inoculated from the stationary-phase cultures. Near the end of log-phase, the flasks were assayed for yield. A final series of flasks were inoculated from a culture that had been in the decline phase of growth for 10 days (as measured by viability). These flasks were also analyzed for yield after growth neared the end of log phase. All yield determinations were performed as described previously.

Isolation of Yield Mutant

Using E. coli, an attempt was made to isolate a yield mutant. A

culture of E. coli was grown in glucose minimal medium at 30C to an absorbance of 0.28. A 0.1 ml aliquot of this suspension was diluted by 10^{-6} into 0.85 percent saline. The 10^{-2} dilution was kept at room temperature over several days to serve as the source of starving cells. Two plate count determinations were performed daily from this tube to assess viability. After five days of starvation, a final plate count determination was made and five of the smallest colonies developing on the plates were selected as potential mutants. These were labelled ECS I, II, III, IV, and V. A stock culture was made of each on TSA slants. Specific growth rates were determined for each of these cultures and ECS III was selected for yield studies on the basis of its slower growth rate. The stability of the mutational lesion conferring this characteristic on the culture was tested by establishing log growth in batch and using this as inoculum for another flask. This recycling was repeated several times and growth rate was assessed for any tendencies toward returning to a wild type specific growth rate. A series of yield determinations was performed on both the mutant and wild type E. coli to determine what if any differences existed between the two cultures. The procedure used in these determinations was outlined earlier.

Effect of Specific Growth Rate on Yield of E. coli

The experimental protocol for these experiments has been presented in an earlier study with C. intermedius (Jones, 1974). Briefly, E. coli was grown at two different dilution rates in continuous culture. After steady state was obtained, 0.5 ml samples were taken from the

chemostat for inoculation of a series of batch flasks containing the same medium. Determination of the yield constant was performed on 20 ml samples from the chemostat as well as from batch. In this manner, batch growth yields could be evaluated as to whether they represented a function of previous growth rate or alternatively a function of maximum specific growth rate which is approached in batch. Dilution rates of 0.041 and 0.17 hr^{-1} were employed in these studies. A temperature of 30C was used for both batch and continuous flow studies.

CHAPTER III

EXPERIMENTAL RESULTS

Identification of the Organisms

The microorganisms used in these studies included the fungus Geotrichum candidum, which was obtained from the American Type Culture Collection. Also, four species of bacteria were isolated and identified as Serratia marcescens, Escherichia coli, Pseudomonas mendocina, and Pseudomonas aeruginosa. The criteria upon which these identifications were based are shown in Table I. The organisms identified as E. coli, P. mendocina and P. aeruginosa were shown to have identical characteristics to those reported under the same names by Bergey's Manual. P. mendocina grew very weakly on geraniol as sole carbon and energy source. The organism identified as S. marcescens had aberrant methyl red and Voges-Proskauer reactions. Final identification of the latter was based upon pigmentation, citrate utilization, and glycerol and glucose fermentation without the production of gas.

The uncoupling studies reported in the present work were done with an organism identified as Citrobacter intermedius. The identifying tests for this organism were presented in an earlier work in which it was given the name Escherichia intermedia (Jones, 1974). Both names designate the same organism and reflect the changes in nomenclature arising in the more recent Bergey's Eighth Edition.

TABLE I

IDENTIFICATION OF THE BACTERIA

Test	Results			
	S. marcescens	*E. coli*	*Ps. medocina*	*Ps. aeruginosa*
Glucose fermentation	A	AG	-	-
Lactose fermentation	-	AG	-	-
Sucrose fermentation	A	AG*	-	-
Glycerol fermentation	A	A*	-	-
Indole production	-	+	-	-
Citrate utilization	+	-	+	+
Geraniol utilization	nd	nd	+*	nd
Nitrate reduction	-	+(n)	+(d)	+(d)
Methyl Red	+	+	-	-
Voges Proskauer	-	-	-	-
Gelatin hydrolysis	-	-	+	+
Starch hydrolysis	-	-	+	-
Oxidase	-	-	+	+
Pigmentation	Red	None	Yellow	Green
Gram Stain	- rods	- rods	- rods	- rods
Flagella	per	per	pol	pol

Abbreviations: (-), negative, (+), positive; (A), acid; (G), gas; (d), denitrifies; (n), to nitrite; (nd), not determined; (*), small amount; per, peritrichous, and pol, polar.

Geraniol utilization as sole carbon and energy source was checked by streaking the organism on minimal salts agar containing no carbon and energy source. A filter paper soaked with geraniol was placed in the lid and incubated for seven days.

Determination of Growth Constants

Since many of the studies reported herein involved growing the mixed culture in glucose minimal medium at 30C, it was deemed necessary to determine the growth constants of the individual components of the mixed culture under these conditions. Figures 2-6 show the effect of initial glucose concentration on the rate of microbial growth. Specific growth rates were determined at each substrate concentration. These data were used to determine the growth constants, K_s and μ_m, for each of the cultures by plotting specific growth rate versus initial substrate as double reciprocals upon arithmetic graph paper. Figure 7 shows the resulting straight-line approximations for each of the individual experiments. The inverse of the Y-axis intercept value represents the μ_m. K_s is determined from the slope of the line multiplied by μ_m. The values obtained for K_s ranged from a low of 19 mg/l for P. mendocina to a high of 137 mg/l for G. candidum. For μ_m, the low was 0.48 hr^{-1} for P. mendocina, and the high was 0.83 hr^{-1} for E. coli and S. marcescens. A summary of the effect of initial substrate concentration upon specific growth rate as well as a tabulation of individual values for μ_m and K_s are presented in Table II. The values obtained for the growth constants were substituted into the Monod equation to obtain the curves illustrated in Figure 8 for various substrate concentrations. The data points shown were obtained from batch experiments and are presented to verify the predicted approximations shown as curves. The curves obtained for E. coli and S. marcescens are nearly identical although the curve for E. coli is somewhat steeper. G. candidum would be expected to be the least competitive of the organisms

Figure 2. Effect of Initial Glucose Concentration, S_o on the Specific Growth Rate of S. marcescens in Batch at 30C

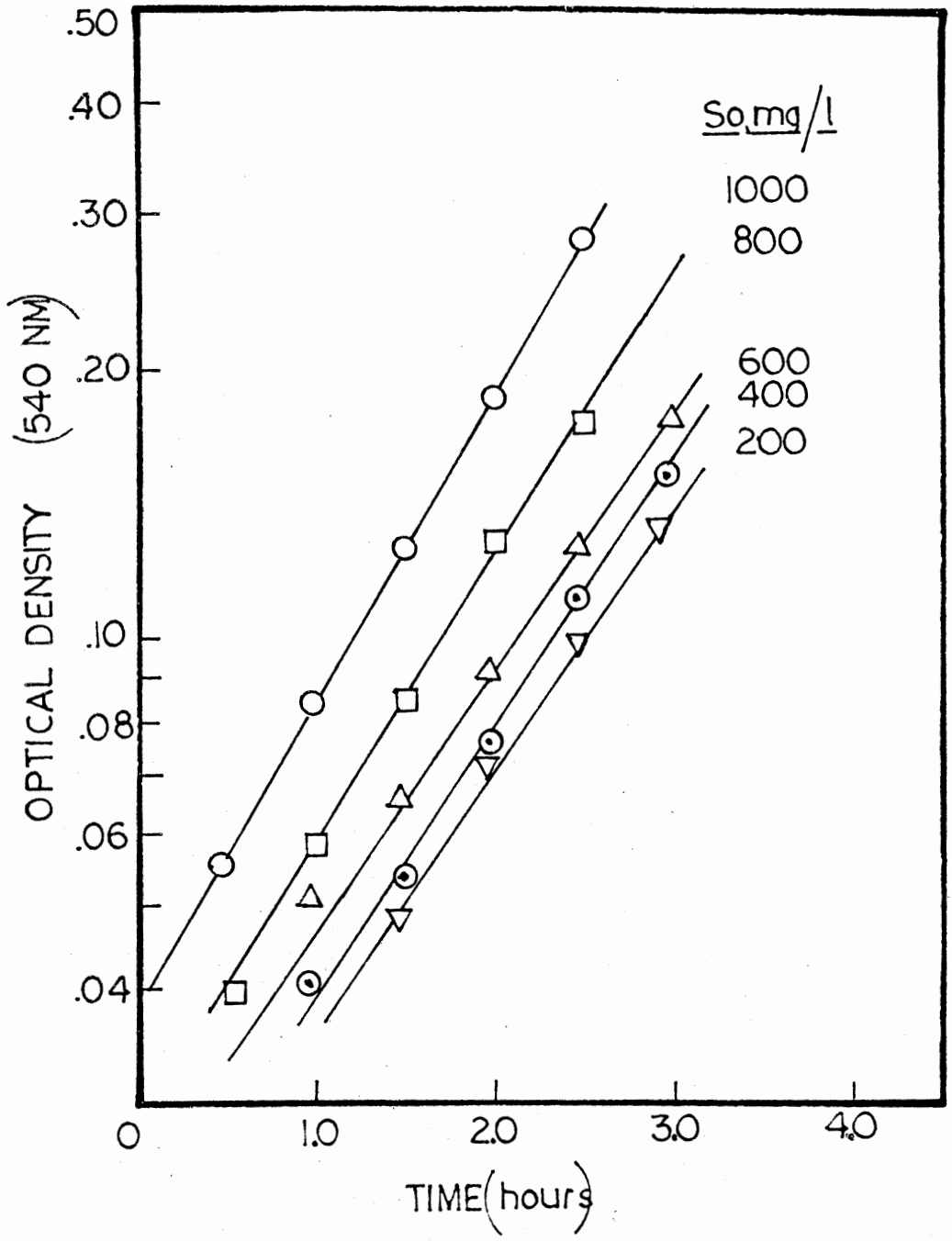

.50
.40
.30
.20
.10
.08
.06
.04
OPTICAL DENSITY (540 NM)
So mg/l
1000
800
600
400
200
0
1.0
2.0
3.0
4.0
TIME(hours)

Figure 3. Effect of Initial Glucose Concentration, S_o, on the Specific Growth Rate of *E. coli* in Batch at 30C

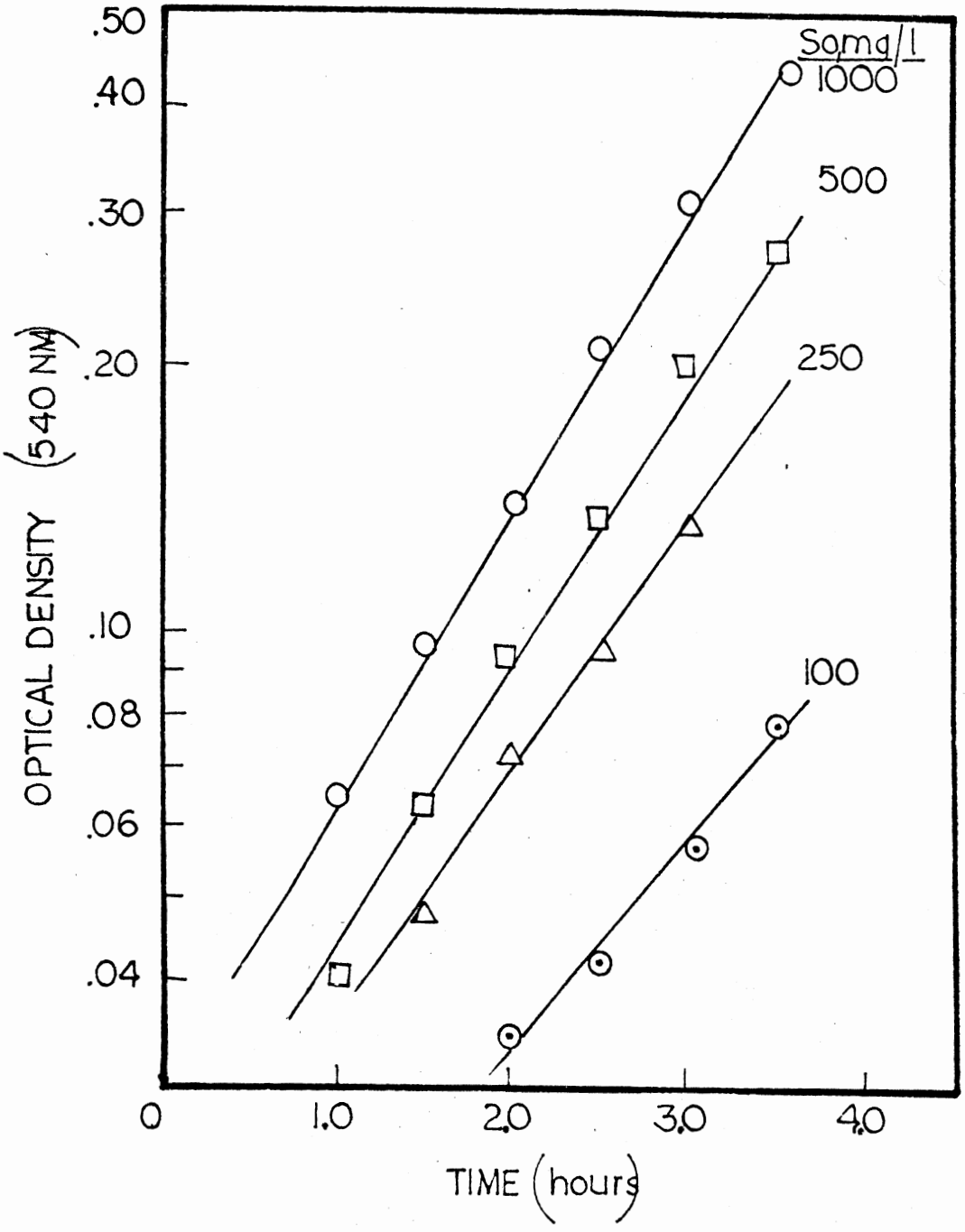

.50
.40
.30
.20
.10
.08
.06
.04
OPTICAL DENSITY (540 NM)
0
1.0
2.0
3.0
4.0
TIME (hours)
50mg/l
1000
500
250
100

Figure 4. Effect of Initial Glucose Concentration, S_o, on the Specific Growth Rate of G. candidum in Batch at 30C

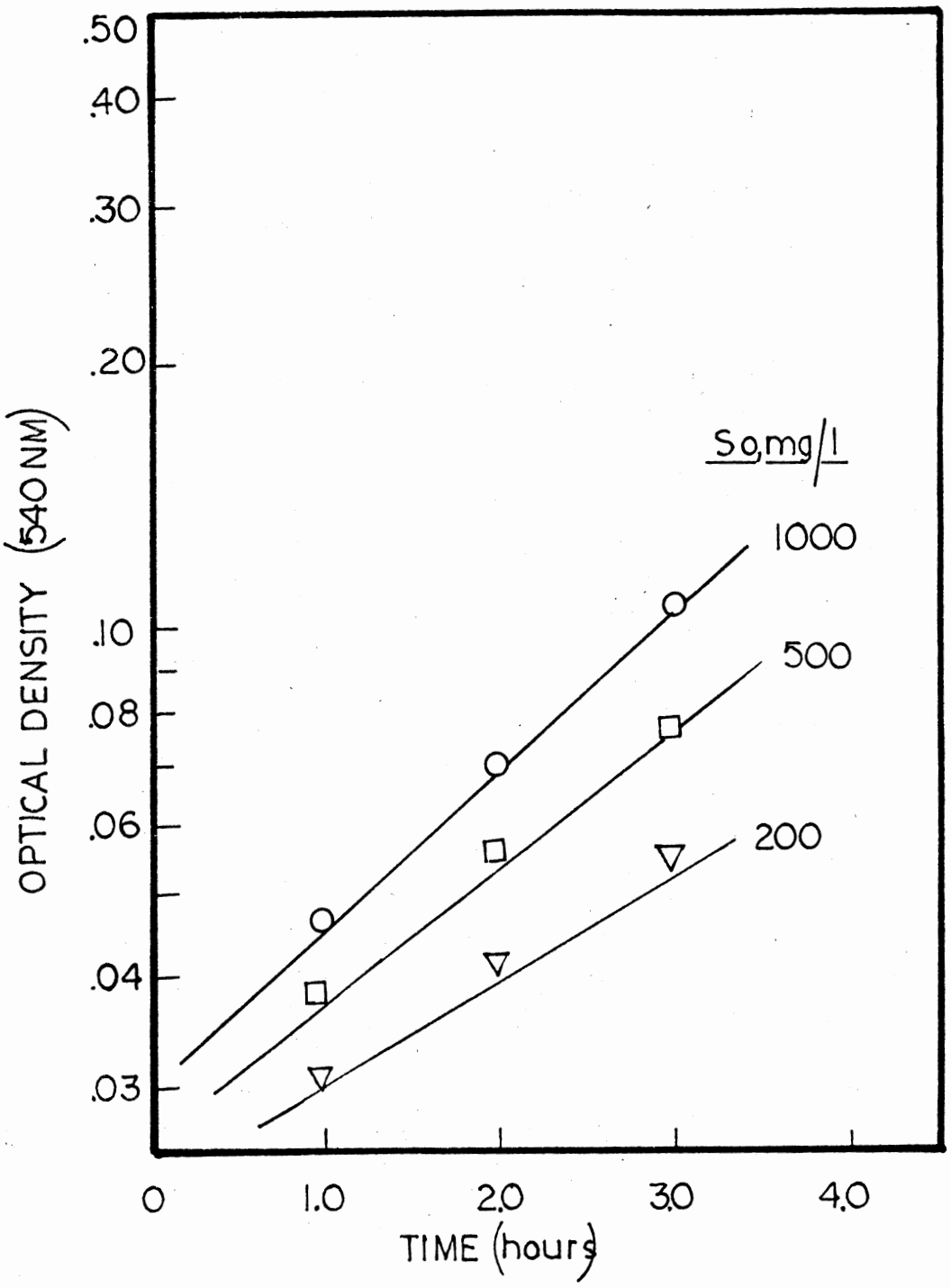

.50
.40
.30
.20
.10
.08
.06
.04
.03
OPTICAL DENSITY (540 NM)
So, mg/l
1000
500
200
0
1.0
2.0
3.0
4.0
TIME (hours)

Figure 5. Effect of Initial Glucose Concentration, S_o, on the Specific Growth Rate of P. aeruginosa in Batch at 30C

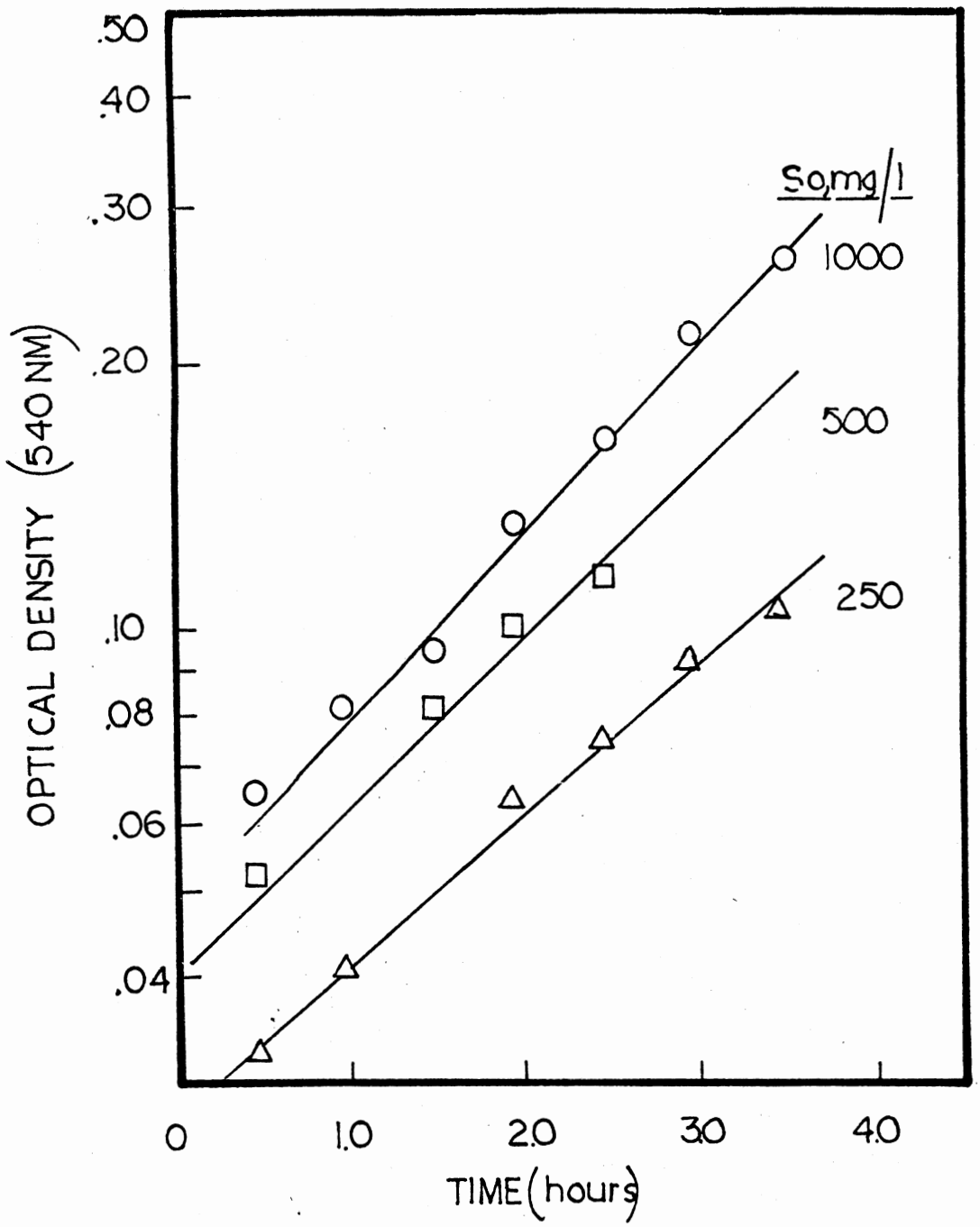

.50
.40
.30
.20
.10
.08
.06
.04
OPTICAL DENSITY (540 NM)
0
1.0
2.0
3.0
4.0
TIME (hours)
S0 mg/l
1000
500
250

Figure 6. Effect of Initial Glucose Concentration, S_o, on the Specific Growth Rate of P. mendocina in Batch at 30C

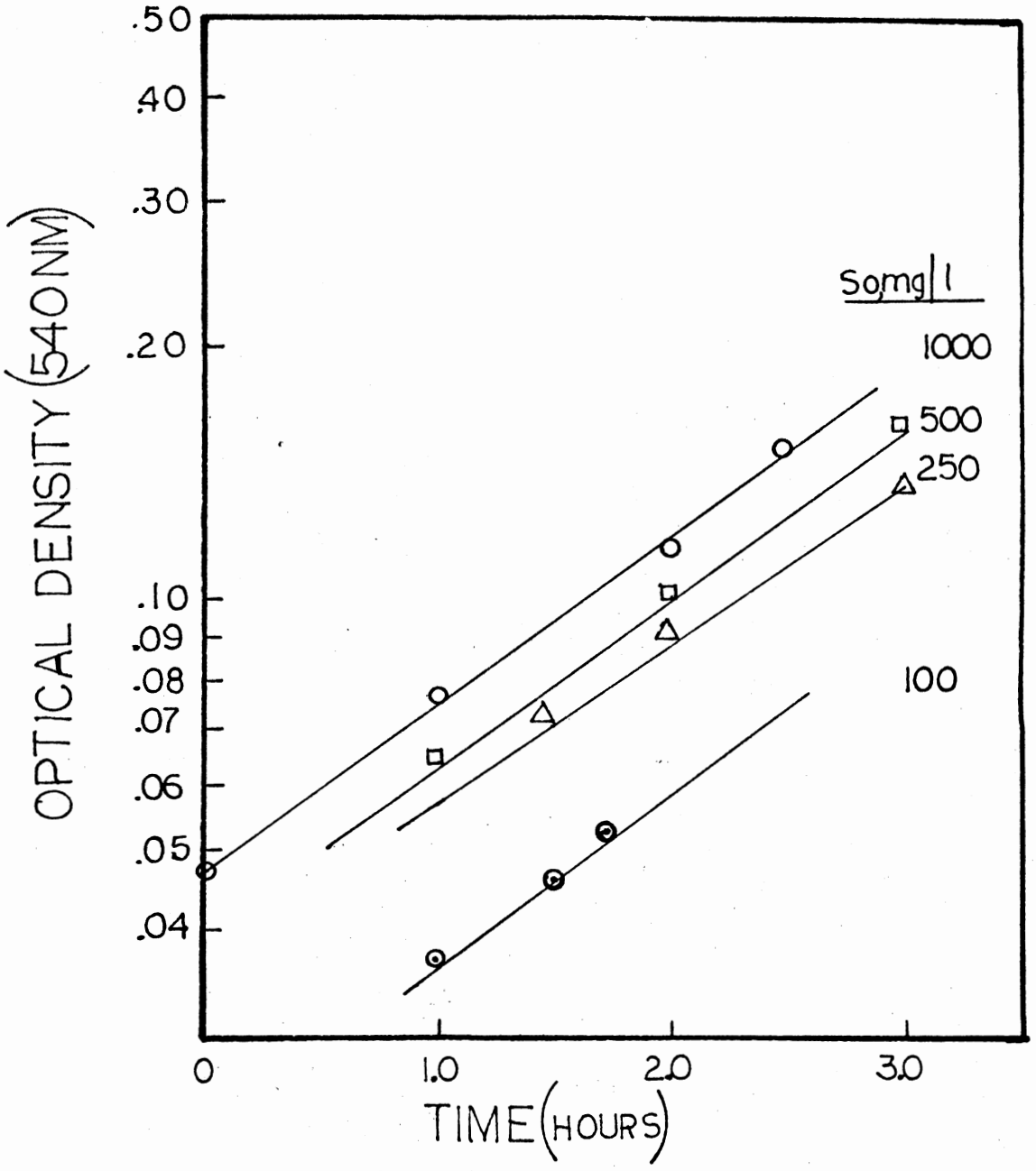

.50
.40
.30
.20
.10
.09
.08
.07
.06
.05
.04
OPTICAL DENSITY (540 NM)
0
1.0
2.0
3.0
TIME (HOURS)
Somg/l
1000
500
250
100

Figure 7. Variation of Specific Growth Rate, μ, With Initial Glucose Concentration, S_o, Plotted as Double Reciprocals to Estimate K_s and μ_m for Individual Cultures in Batch at 30C

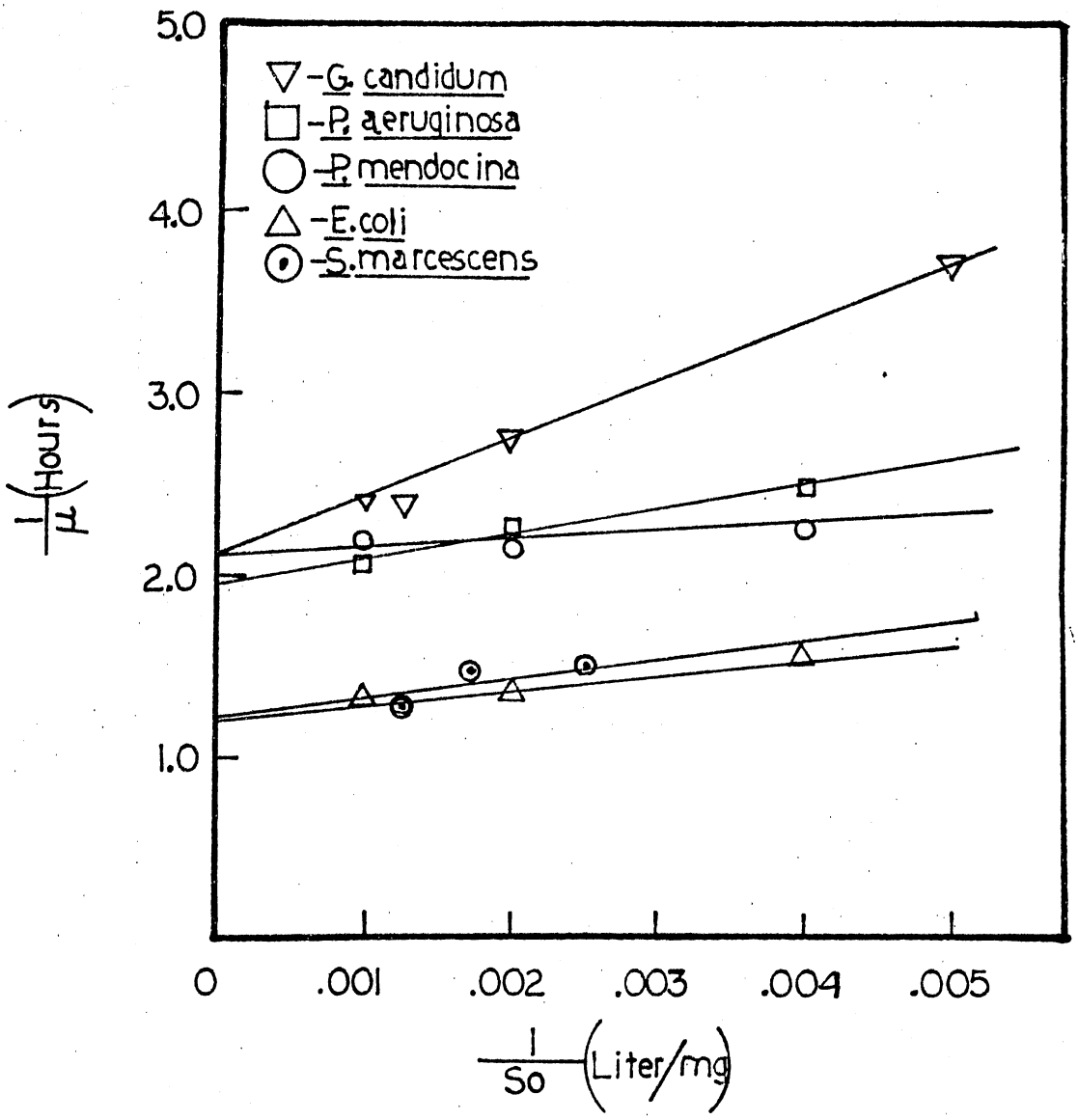

5.0
4.0
3.0
2.0
1.0
▽-G. candidum
□-P. aeruginosa
○-P. mendocina
△-E. coli
⊙-S. marcescens
1/μ (Hours)
0
.001
.002
.003
.004
.005
1/So (Liter/mg)

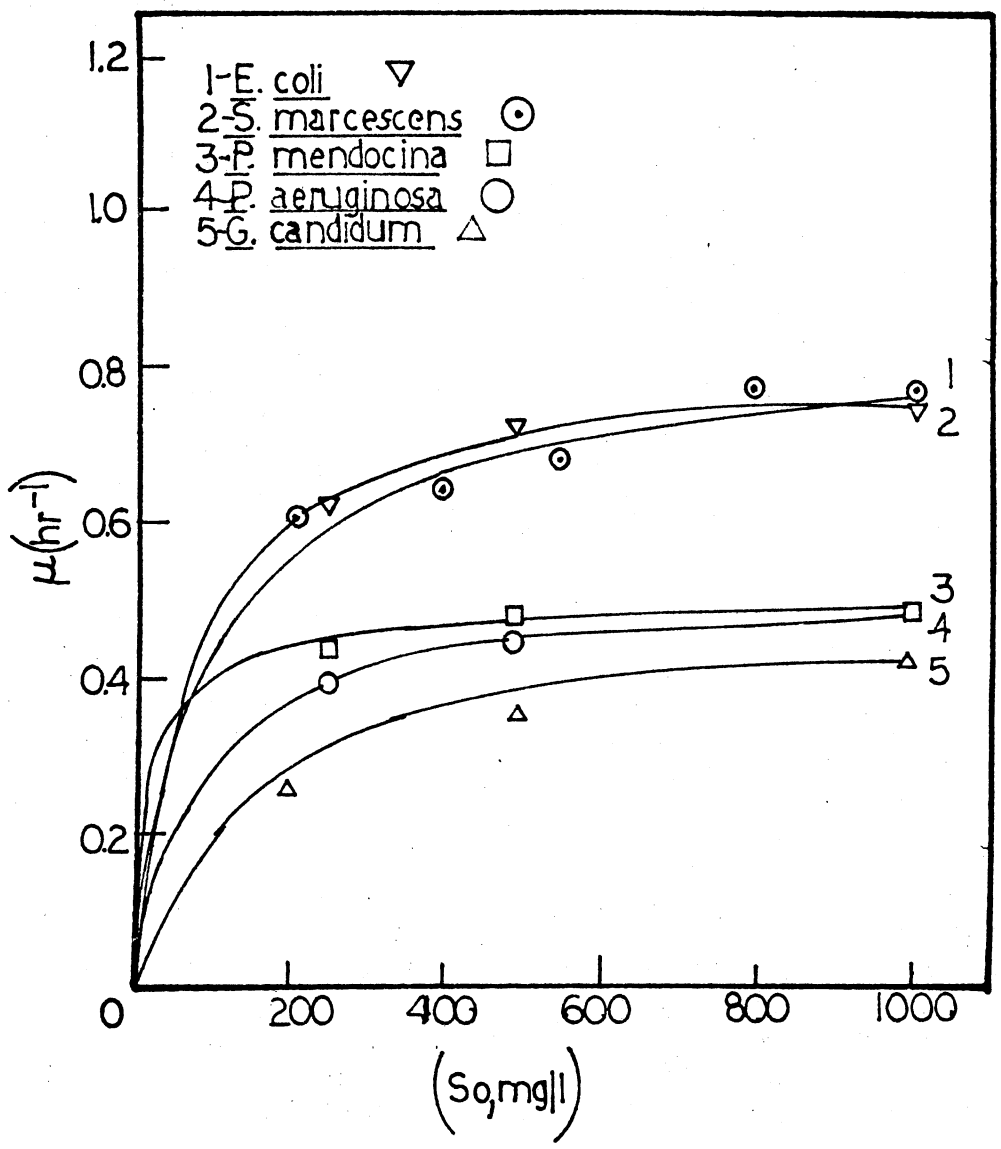
TABLE II

SUMMARY OF GROWTH CHARACTERISTICS IN BATCH AT 30C

Organism	Initial Glucose Concentration (mg/l)	Specific Growth Rate (hr^{-1})	Constants μ_m (hr^{-1})	Constants K_s (mg/l)
P. mendocina	-	-	0.48	19
	250	0.44		
	500	0.46		
	1000	0.46		
P. aeruginosa	-	-	0.52	80
	250	0.40		
	500	0.45		
	1000	0.48		
S. marcescens	-	-	0.83	95
	200	0.62		
	400	0.66		
	600	0.68		
	800	0.77		
	1000	0.77		
E. coli	-	-	0.83	74
	100	0.58		
	250	0.63		
	500	0.73		
	1000	0.76		
G. candidum	-	-	0.49	137
	200	0.27		
	500	0.36		
	1000	0.43		

Figure 8. Variation of Specific Growth Rate, μ, With Initial Glucose Concentration, S_o, for Batch Experiments With Individual Cultures at 30C

The curves shown represent the calculated curves obtained by substituting individual K_s and μ_m values into the Monod equation and solving for μ as a function of various initial glucose concentrations. The points included on the graph are the experimentally derived points.


1-E. coli
2-S. marcescens
3-P. mendocina
4-P. aeruginosa
5-G. candidum
1.2
1.0
0.8
0.6
0.4
0.2
0
μ(hr⁻¹)
200
400
600
800
1000
(So, mg/l)
1
2
3
4
5

since its curve falls somewhat below that of all other species for virtually all initial substrate concentrations. P. aeruginosa and P. mendocina represent an intermediate level. However, P. mendocina, owing to its low K_s value, has a steeper curve at very low substrate concentrations. Its growth rate under these conditions even exceeds that of E. coli and S. marcescens. The value for coexistence of these three species appears to be in the vicinity of 75 mg/l glucose.

Sole Carbon and Energy Sources

Numerous organic compounds were tested for utilization by G. candidum and S. marcescens as sole carbon and energy sources. These two organisms were originally intended as the only members of the mixed cultures. As a consequence, this experiment was performed to select a compound upon which G. candidum would grow and S. marcescens would not. A list of those compounds and the results after 18, 72, and 144 hours are presented in Tables III and IV. A wide variety of organic compounds was utilized by S. marcescens. These included sugars, sugar alcohols, organic acids, and amino acids. Many of these yielded only slight growth as compared to the heavy growth obtained with such compounds as glucose and sucrose. Several of the compounds resulted in growth without the characteristic red pigment being formed. These included glutamic acid, aspartic acid, serine, histidine, glycerophosphate, gluconic acid, ribose, mannitol, and galactose. Moreover, glycerophosphate and galactose appeared to be somewhat inhibitory as growth occurred only as a ring around the site of placement of the few crystals. G. candidum utilized fewer of the compounds than S. marcescens, but growth was more unequivocal than with the bacterium.

TABLE III

SOLE CARBON AND ENERGY SOURCES FOR S. MARCESCENS

	Results					
	Hours				Hours	
Compound	18	72	144	Compound	18	72	144
Urea	-	-	-	Propionate	-	-	-
D-Glucose	+	+	+	Maltose	+	+	+
D-Fructose	+	+	+	Inositol	+	+	+
Dulcitol	-	-	-	D-Gluconic acid	+	+	+
Isoleucine	-	-	-	Raffinose	-	-	-
Glutamic acid	-	-	-	Inulin	-	-	-
Phenylalanine	-	-	-	Aldonitol	+	+	+
Aspartic acid	-	±	±	Melibiose	-	-	-
Serine	-	±	±	D-Mannose	+	+	+
Allantoin	-	-	-	D-Ribose	±	±	±
Threonine	-	-	-	D-Mannitol	±	±	±
Methionine	-	-	-	L-Sorbose	-	-	-
Leucine	-	-	-	D-Trehalose	+	+	+
DL-Malic acid	+	+	+	Sucrose	+	+	+
Histidine	±	±	±	Galactose	±	±	±
Xanthine	-	-	-	D-Sorbitol	+	+	+
Glyceric acid	-	-	-	D-Xylose	-	-	-
Uric acid	-	-	-	L-Rhamnose	-	-	-
DL-Glycerophosphate	±	±	±	Acetate	-	-	±
Lactose	-	-	-	Citrate	+	+	+

Key to symbols: (-), no growth; (+), growth, and (±), slight growth.

TABLE IV

SOLE CARBON AND ENERGY SOURCES FOR G. CANDIDUM

Compound	Results Hours 18	72	144	Compound	Hours 18	72	144
Urea	-	-	-	Propionate	-	+	+
D-Glucose	+	+	+	Maltose	-	±	±
D-Fructose	+	+	+	Inositol	-	-	-
Dulcitol	-	-	-	D-Gluconic acid	-	-	-
Isoleucine	-	±	+	Raffinose	-	-	-
Glutamic acid	+	+	+	Inulin	-	-	-
Phenylalanine	-	-	-	Aldonitol	-	-	-
Aspartic acid	±	+	+	Melibiose	-	-	-
Serine	-	-	-	D-Mannose	+	+	+
Allantoin	-	-	-	D-Ribose	-	-	-
Threonine	-	-	-	D-Mannitol	-	-	+
Methionine	-	-	-	L-Sorbose	-	-	-
Leucine	-	-	-	D-Trehalose	-	-	-
DL-Malic acid	±	+	+	Sucrose	-	-	-
Histidine	-	-	-	Galactose	+	+	+
Xanthine	-	-	-	D-Sorbitol	-	-	-
Glyceric acid	-	-	-	D-Xylose	+	+	+
Uric acid	-	-	-	L-Rhamnose	-	-	-
DL-Glycerophosphate	-	-	-	Acetate	+	+	+
Lactose	-	-	-	Citrate	-	-	-

Key to symbols: (-), no growth; (+), growth, and (±), slight growth.

Standard Curves

Several standard curves were prepared for use at various points in these studies. Figure 9 illustrates the relationship between cell numbers of S. marcescens and percent transmittance at 540 nm. This relationship was linear in the interval from approximately 30 to 70 percent transmittance which allowed one to predict cell numbers accurately. Furthermore, the curve was very useful in estimating total bacterial cell counts in the mixed continuous flow studies as well. Figure 10 shows the linear relationship between optical density and dry weight for S. marcescens. Figure 11 depicts the calibration curve for the pump used in the mixed culture continuous flow studies. The delivery was extremely accurate over the desired range of pumping speeds. To achieve dilution rates of 0.041 hr^{-1} and 0.125 hr^{-1}, the pump was adjusted to 9 percent and 23 percent, respectively. Figure 12 represents the standard curve used to estimate organic matter by the Neish method. The curve gave fairly accurate values for organic matter compared to the more accurate COD test. It was not precise enough for the more exacting yield determinations, but was acceptable for the mixed culture studies as a range indicator for substrate utilization.

Mixed Batch Studies

Comparisons of specific growth rates between pure cultures and two-member mixed cultures were made to evaluate whether any interactions were evident between the various microbial species. Figure 13 represents the logarithmic portion of the growth curves of the various two-member batch cultures. Estimations of specific growth rates were

Figure 9. Standard Curve Relating Cell Numbers of S. marcescens to Percentage Transmission

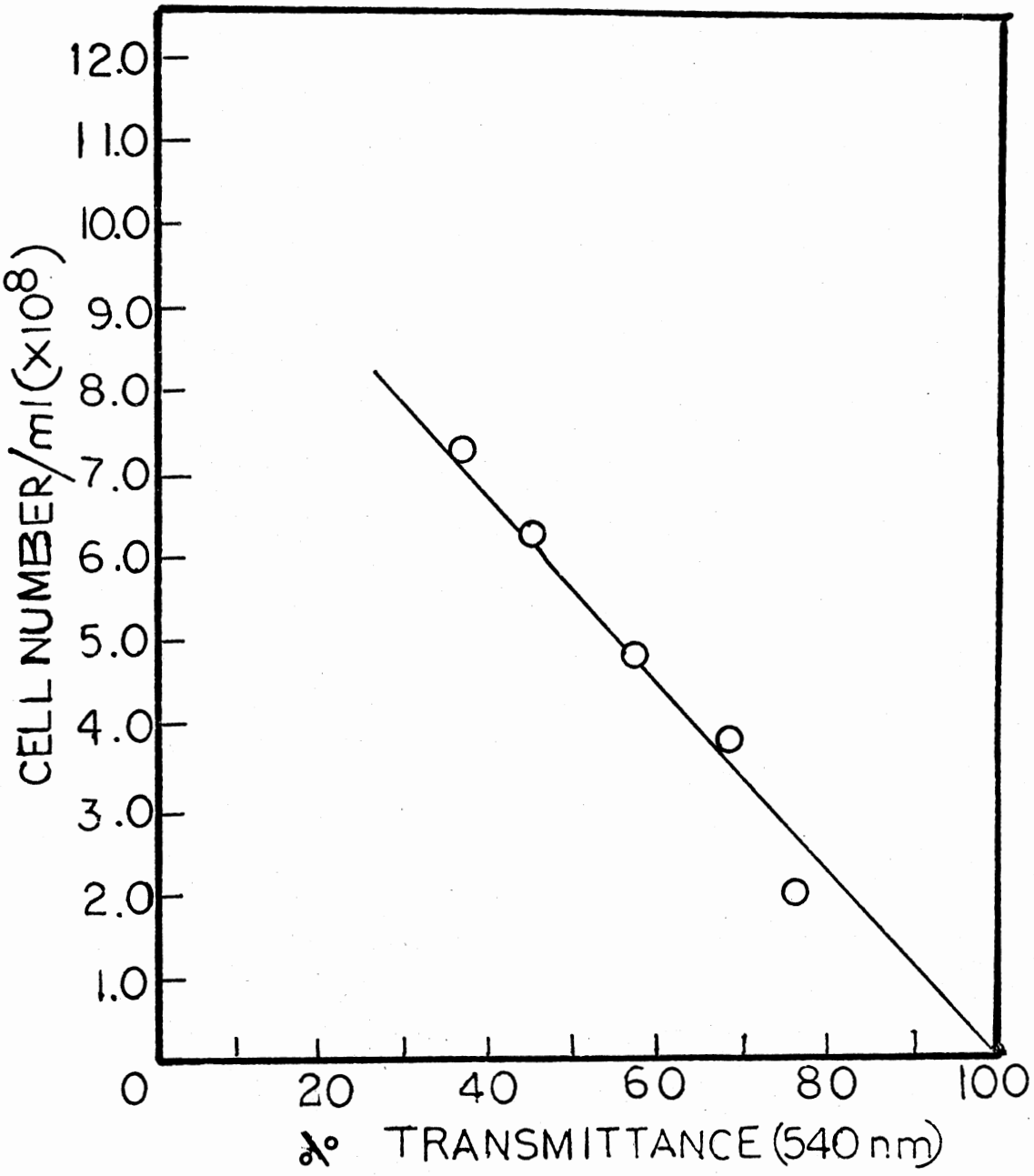

12.0
11.0
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0
CELL NUMBER/ml (X10⁸)
20
40
60
80
100
% TRANSMITTANCE (540 nm)

Figure 10. Standard Curve Relating Optical Density to Dry Weight of S. marcescens

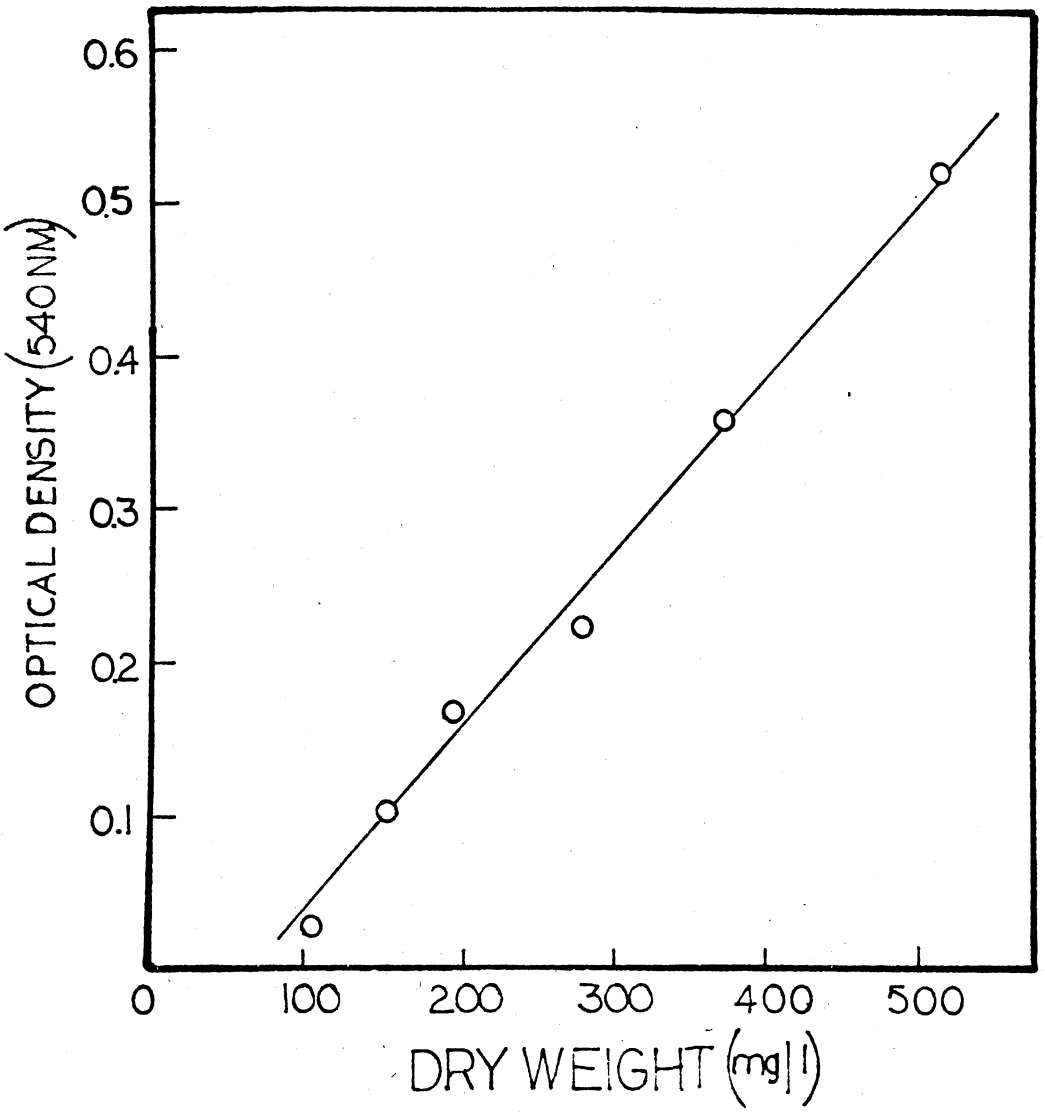

0.6
0.5
0.4
0.3
0.2
0.1
OPTICAL DENSITY (540 NM)
0
100
200
300
400
500
DRY WEIGHT (mg/l)

Figure 11. Calibration Curve for the Sigmamotor Pump

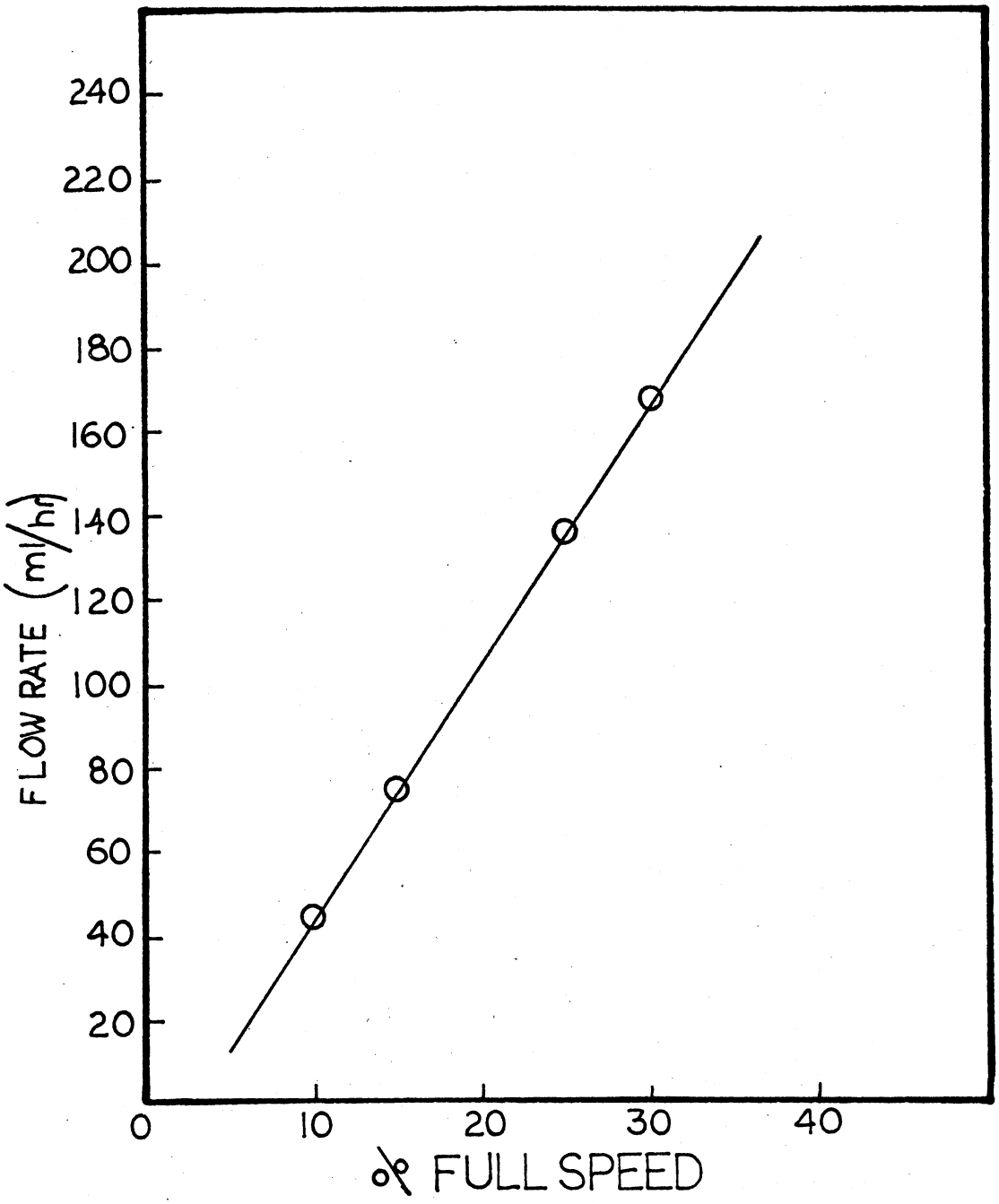

240
220
200
180
160
140
120
100
80
60
40
20
FLOW RATE (ml/hr)
0
10
20
30
40
% FULL SPEED

Figure 12. Standard Curve for the Neish Method of Determination of Organic Matter

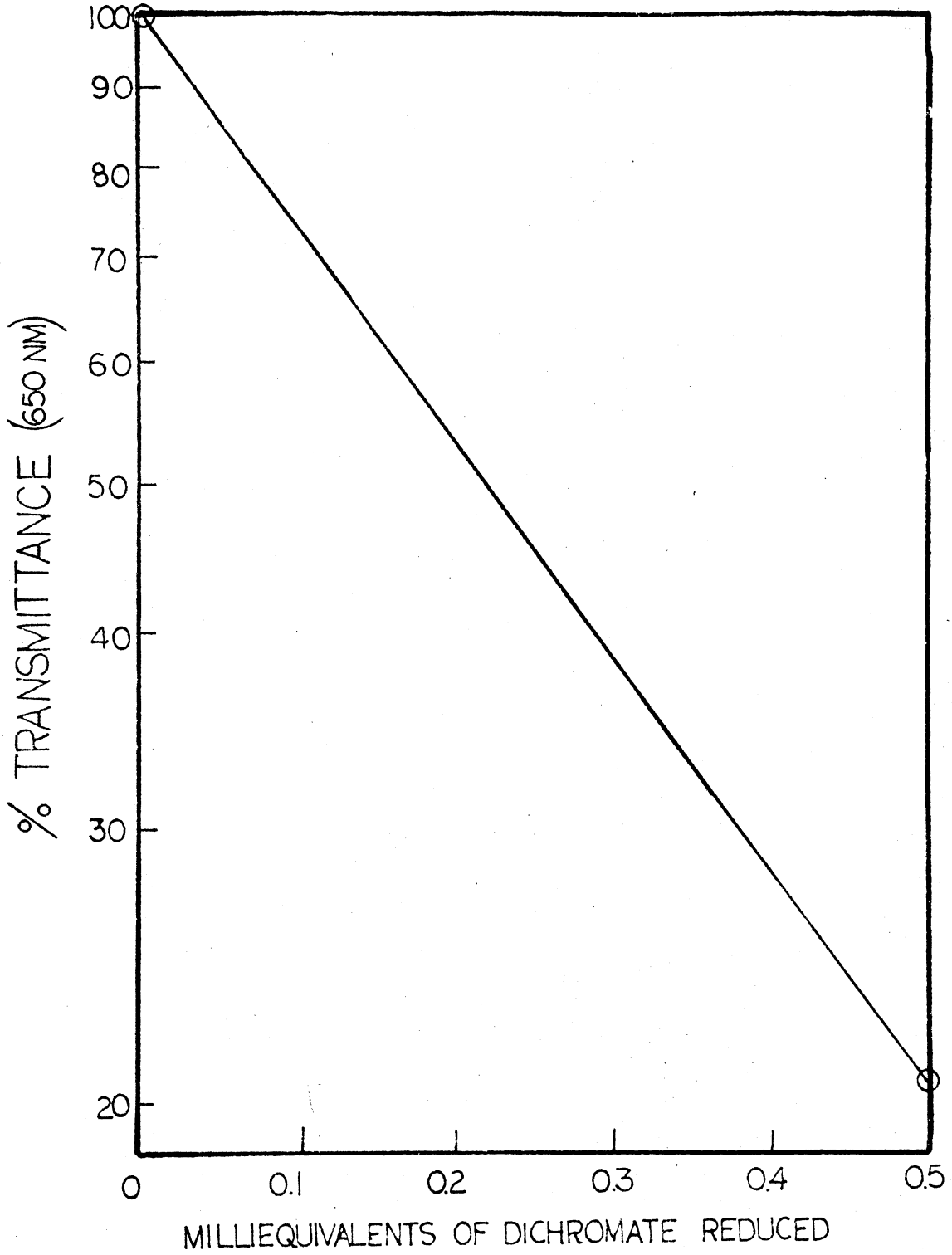
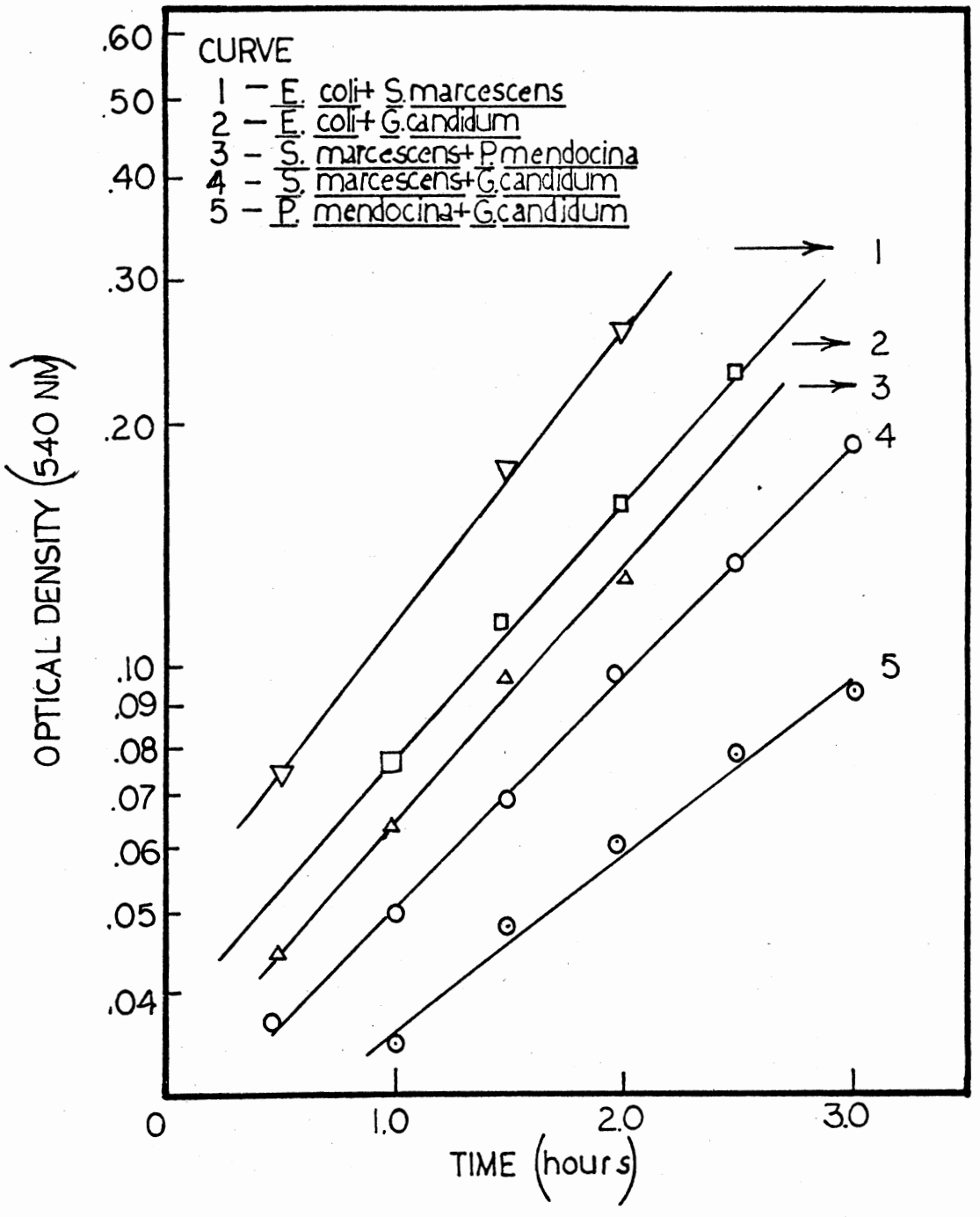

100
90
80
70
60
50
40
30
20
% TRANSMITTANCE (650 NM)
0
0.1
0.2
0.3
0.4
0.5
MILLIEQUIVALENTS OF DICHROMATE REDUCED

Figure 13. Logarithmic Growth of Two-member Mixed Cultures Grown in Glucose-minimal Medium as Batch Cultures at 30C


CURVE
1 – E. coli + S. marcescens
2 – E. coli + G. candidum
3 – S. marcescens + P. mendocina
4 – S. marcescens + G. candidum
5 – P. mendocina + G. candidum
OPTICAL DENSITY (540 NM)
.60
.50
.40
.30
.20
.10
.09
.08
.07
.06
.05
.04
0
1.0
2.0
3.0
TIME (hours)
1
2
3
4
5

determined from these plots and are summarized in Table V. This table also includes the specific growth rates for each species grown as a pure culture in batch. A comparison of these values leads to the conclusion that no substantial interactions, either positive or negative, occur among the four microorganisms. The mixture of E. coli and S. marcescens yielded a somewhat higher growth rate than either one as a pure culture. Furthermore, the same effect is noted in the mixture of P. mendocina and G. candidum. When either of the slower growing species (i.e., G. candidum or P. mendocina) was mixed with E. coli or S. marcescens, the resulting growth rate of the mixed culture was reduced below that of the faster growing species as expected. There was a more pronounced effect on the cultures containing S. marcescens as one of the components than on the cultures containing E. coli. Streak plates were made at the end of growth for each flask. These yielded no unexpected results. Both species were always present albeit at different ratios at the end of the growth phase.

Mixed Culture Continuous Flow Studies

Temperature Studies

Two different mixed cultures and their predominance patterns at different temperatures were examined. The cultures differed only in the Pseudomonas species included in the mixed culture. The first group of experiments involved a mixed culture including P. aeruginosa while a subsequent series of studies used P. mendocina. Figure 14 presents bacterial plate count data for the two dilution rates at 30C using the P. aeruginosa mixed culture. At both dilution rates, E. coli was the

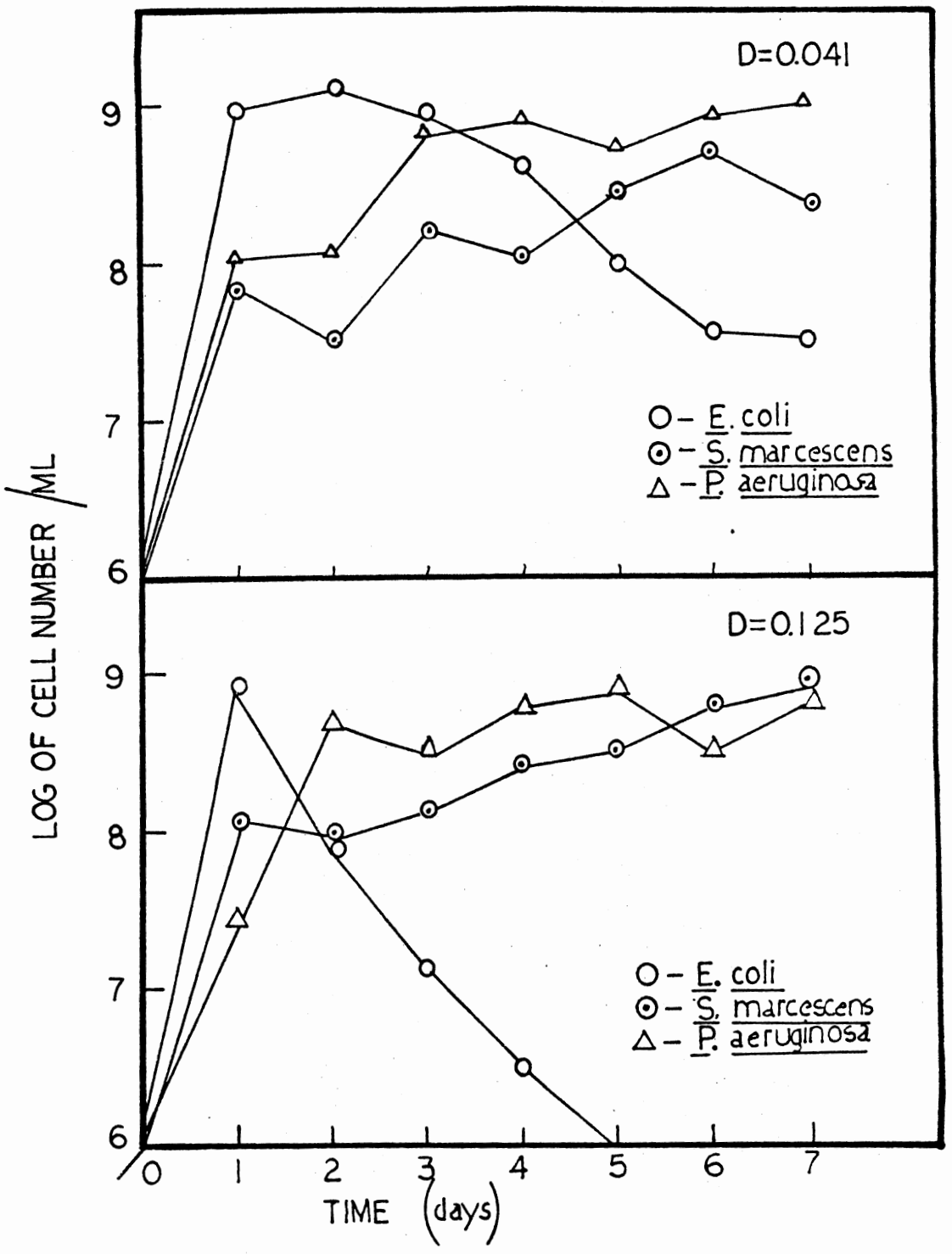
TABLE V

COMPARISON OF GROWTH RATES BETWEEN PURE CULTURES AND TWO-MEMBER MIXED CULTURES

Culture	Specific Growth Rate (hr^{-1})
E. coli (pure culture)	0.76
S. marcescens (pure culture)	0.77
P. mendocina (pure culture)	0.46
G. candidum (pure culture)	0.43
E. coli + *S. marcescens*	0.80
E. coli + *G. candidum*	0.71
E. coli + *P. mendocina*	0.71
S. marcescens + *P. mendocina*	0.69
S. marcescens + *G. candidum*	0.63
P. mendocina + *G. candidum*	0.50

The growth rates were determined using minimal medium containing 1000 mg/l glucose at a growth temperature of 30C

Figure 14. Bacterial Predominance Patterns in a Mixed Culture Continuous Flow Reactor at 30C for Dilution Rates of 0.041 and 0.125 hr^{-1}


D=0.041
D=0.125
O – E. coli
⊙ – S. marcescens
△ – P. aeruginosa
LOG OF CELL NUMBER /ML
TIME (days)
6
7
8
9
0
1
2
3
4
5
6
7

first culture member to reach a maximum number. This occurred by the first day following inoculation. A decline in numbers ensued within two days concomitant with increasing numbers of P. aeruginosa and S. marcescens. The rate of reduction was most rapid at the faster dilution rate. At the close of the experimental run, no singular predominance could be distinguished at either dilution rate as both S. marcescens and P. aeruginosa were still present in quite high numbers. Similar observations were made at 20C and 37C for a dilution rate of 0.125 hr^{-1} as shown in Figure 15. The results nearly parallel those at 30C with E. coli again being eliminated and S. marcescens and P. aeruginosa establishing predominance. However, the latter species seemed to be slightly more competitive at these temperatures, since S. marcescens at six days had yet to equal the level attained by P. aeruginosa. Figure 16 depicts total bacterial and fungal counts for various temperatures and dilution rates. Bacterial counts were highest at 30C for a dilution rate of 0.041 hr^{-1}. The temperature yielding the highest plate counts for G. candidum was 20C at a dilution rate of 0.125 hr^{-1}. Gross visual examination of the culture at all temperatures showed no evidence of excessive filament formation. Likewise, microscopic examination demonstrated an abundance of arthrospores but very few filaments.

Plate counts were performed daily for each experiment. In conjunction with this approach, optical density was also determined on a regular basis. With these data, attainment of cultural steady states could be evaluated. Figure 17 presents "steady state" curves for several temperature and dilution rates. It is evident from these results that temperatures of 20 and 37C resulted in cultures approaching a steady state as measured by this method. However, at 30C there were quite

Figure 15. Bacterial Predominance Patterns in a Mixed Culture Continuous Flow Reactor at 37C and 20C for a Dilution Rate of 0.125 hr^{-1}

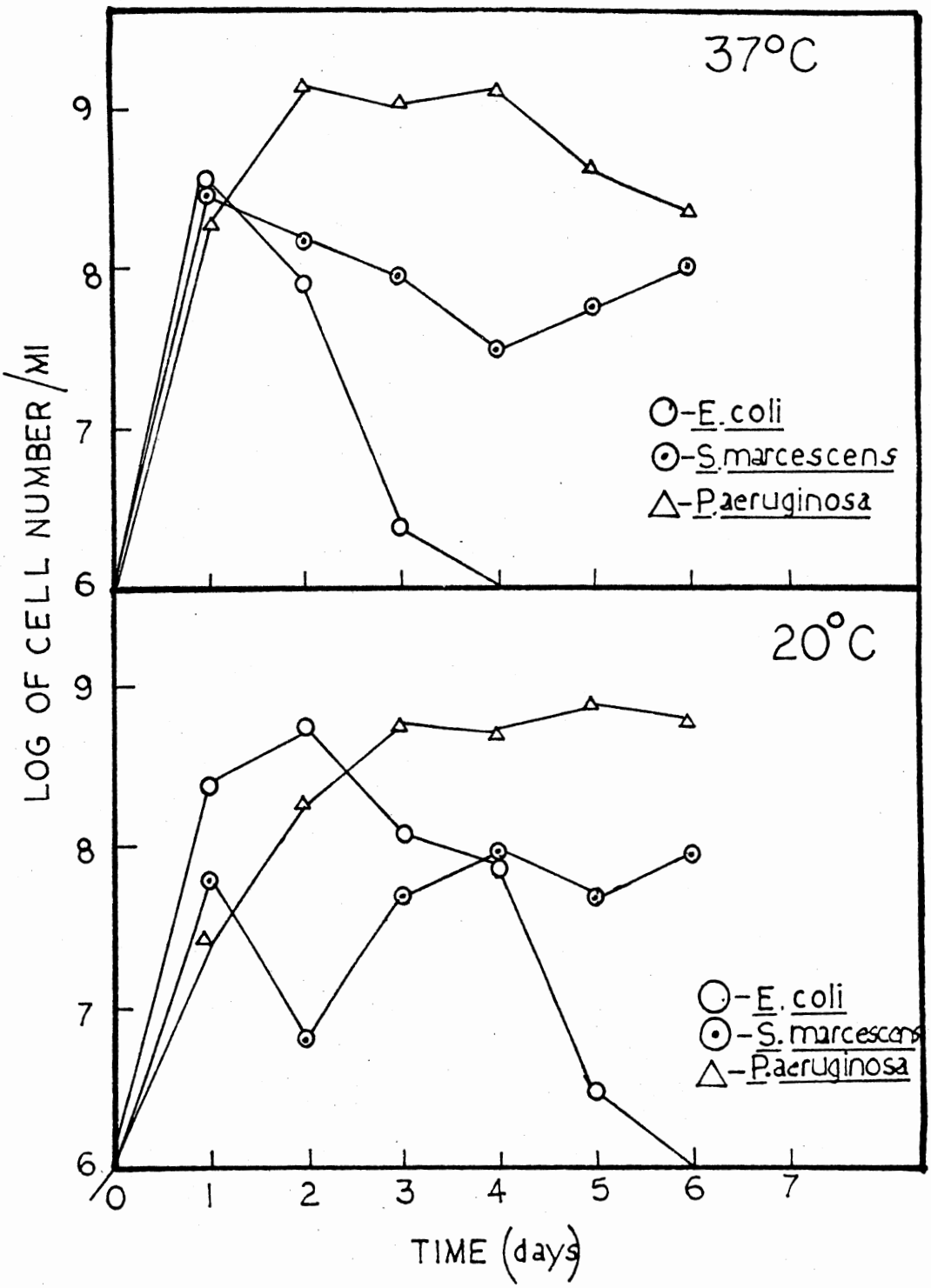

37°C
20°C
LOG OF CELL NUMBER /ML
TIME (days)
O-E. coli
⊙-S. marcescens
△-P. aeruginosa
O-E. coli
⊙-S. marcescens
△-P. aeruginosa
6
7
8
9
0
1
2
3
4
5
6
7

Figure 16. Total Bacterial and Fungal Plate Counts for Mixed Cultures in Continuous Culture at Different Temperatures

The curves shown for total bacterial counts were derived from a summation of the plate counts of S. marcescens, E. coli, and P. aeruginosa on a daily basis. The medium was glucose-minimal medium.

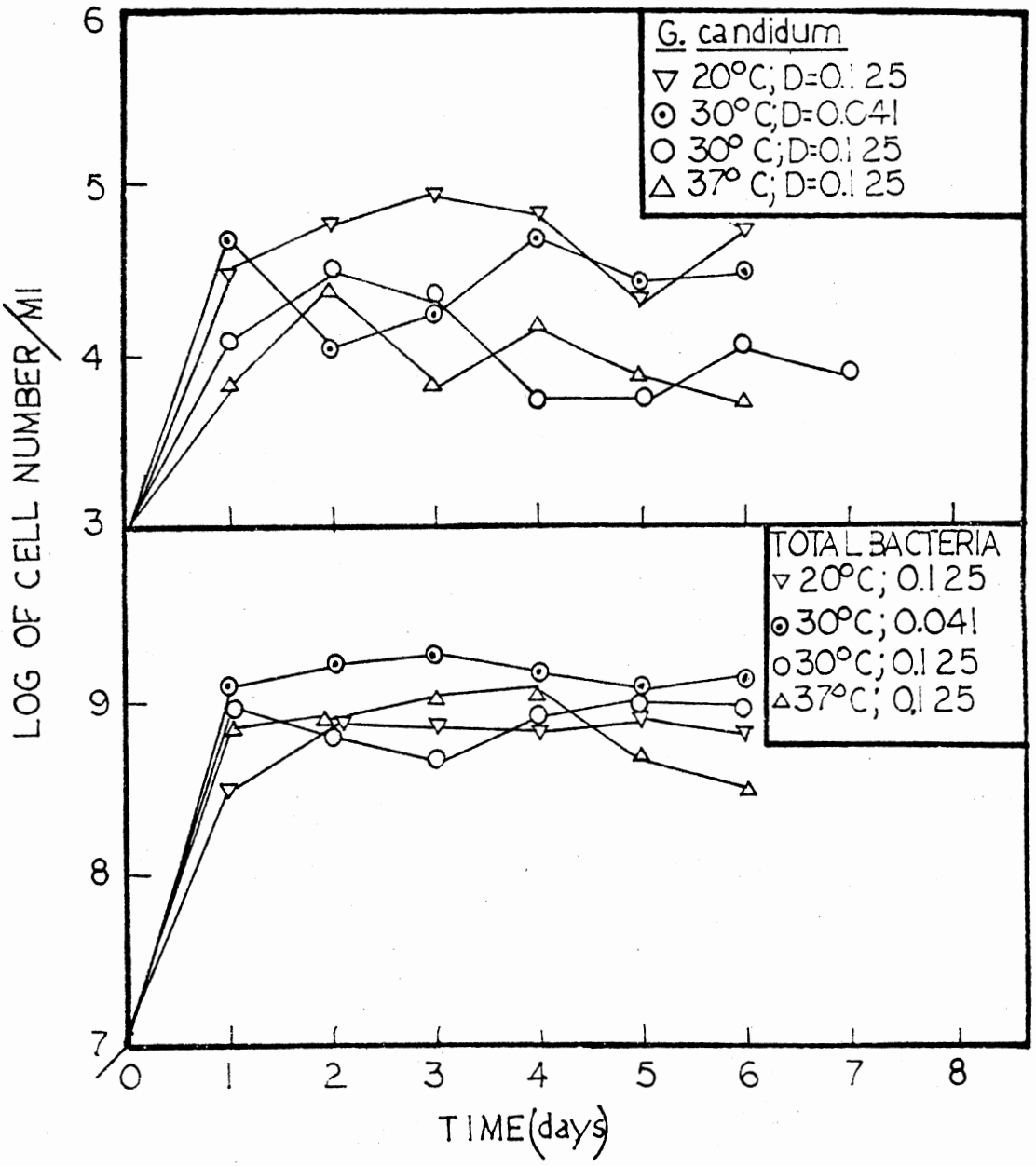
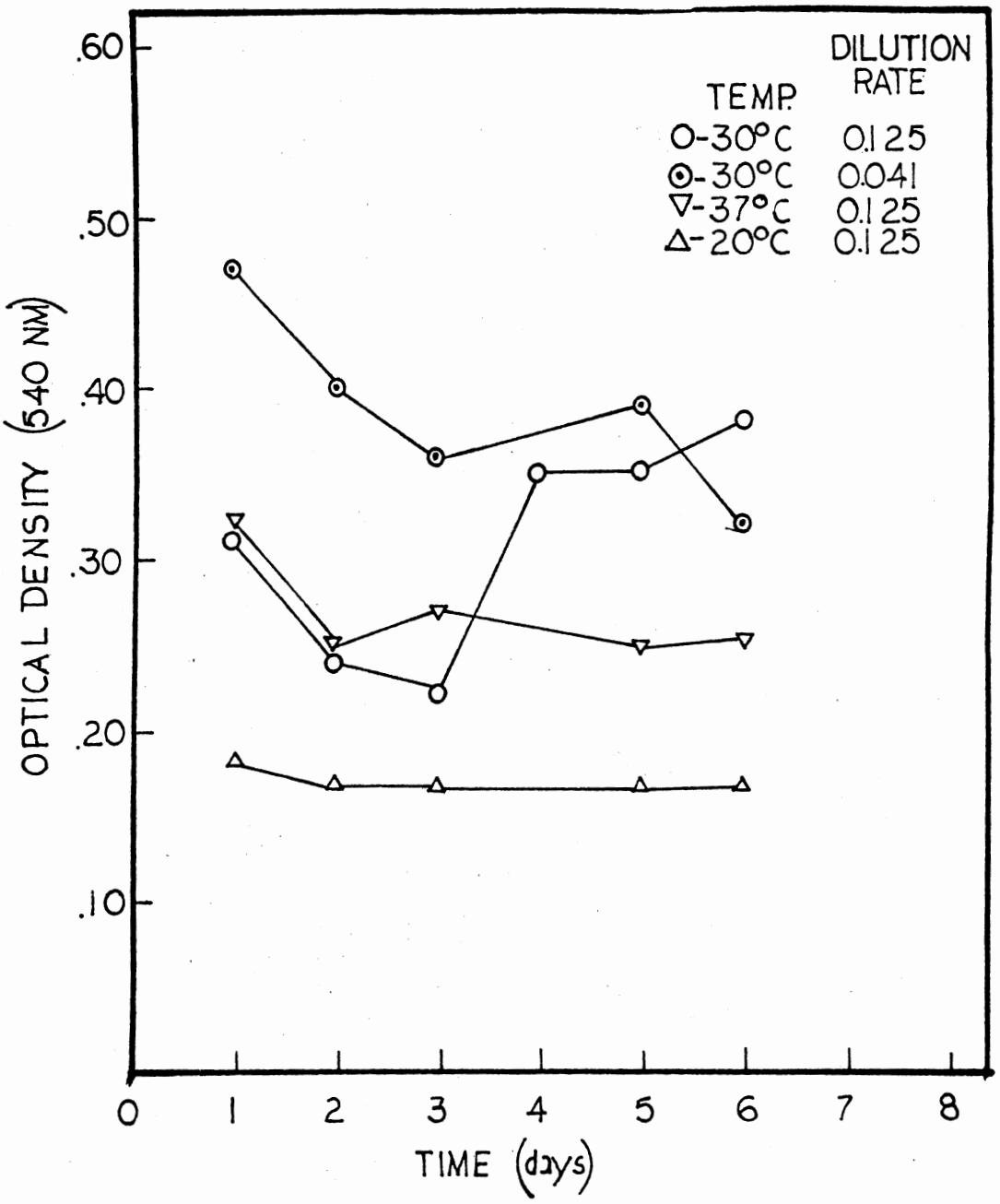

G. candidum
▽ 20°C; D=0.125
⊙ 30°C; D=0.041
○ 30°C; D=0.125
△ 37°C; D=0.125
TOTAL BACTERIA
▽ 20°C; 0.125
⊙ 30°C; 0.041
○ 30°C; 0.125
△ 37°C; 0.125
LOG OF CELL NUMBER/ML
TIME (days)
6
5
4
3
9
8
7
0
1
2
3
4
5
6
7
8

Figure 17. Optical Density of the Mixed Culture at Various Temperatures and Dilution Rates

The mixed culture included G. candidum, S. marcescens, P. aeruginosa, and E. coli. The medium was glucose-minimal medium.


DILUTION RATE
TEMP.
O-30°C 0.125
⊙-30°C 0.041
▽-37°C 0.125
△-20°C 0.125
OPTICAL DENSITY (540 NM)
.60
.50
.40
.30
.20
.10
0
1
2
3
4
5
6
7
8
TIME (days)

significant fluctuations for both dilution rates. Hence, these cultures never attained a level which could be designated a steady state.

Figures 18-20 represent the results of the bacterial plate counts of a mixed culture including P. mendocina at dilution rates of 0.041 and 0.125 hr^{-1}. Immediately apparent is the competitiveness of S. marcescens at all three temperatures. Regardless of the dilution rate, this organism ultimately established predominance in every experiment. The rapidity at which it predominated varied with temperature and dilution rate. At a dilution rate of 0.125, the competition provided by E. coli and P. mendocina was nearly nonexistent as S. marcescens quickly reached levels on the order of 10^9 cells/ml. When a dilution rate of 0.041 hr^{-1} was imposed, the competition between the microorganisms was more obvious. Temperature seemed to play a greater role at this dilution rate. S. marcescens was most competitive at 20C and least competitive at 37C as judged by the number of days elapsed before it established predominance. To reach this point, times of 1, 4, and 8 days were required for 20, 30, and 37C temperatures, respectively. E. coli maintained nearly constant levels at each temperature and was never eliminated from the culture within the time course of the experiments. This is in marked contrast to the effect noted at the faster dilution rate during which E. coli plate counts never reached 10^7/ml. The competitiveness of P. mendocina was the exact reverse of S. marcescens. Figure 21 shows the effect upon total bacterial and G. candidum cell concentrations as a function of temperature at a dilution rate of 0.041 hr^{-1}. Bacterial numbers were little affected by temperature although at 20C a considerably longer period of time was required for counts to reach levels comparable to those at 30 and 37C. The

Figure 18. Bacterial Predominance Patterns in a Mixed Culture Continuous Flow Reactor at 20C for Dilution Rates of 0.041 and 0.125 hr^{-1}

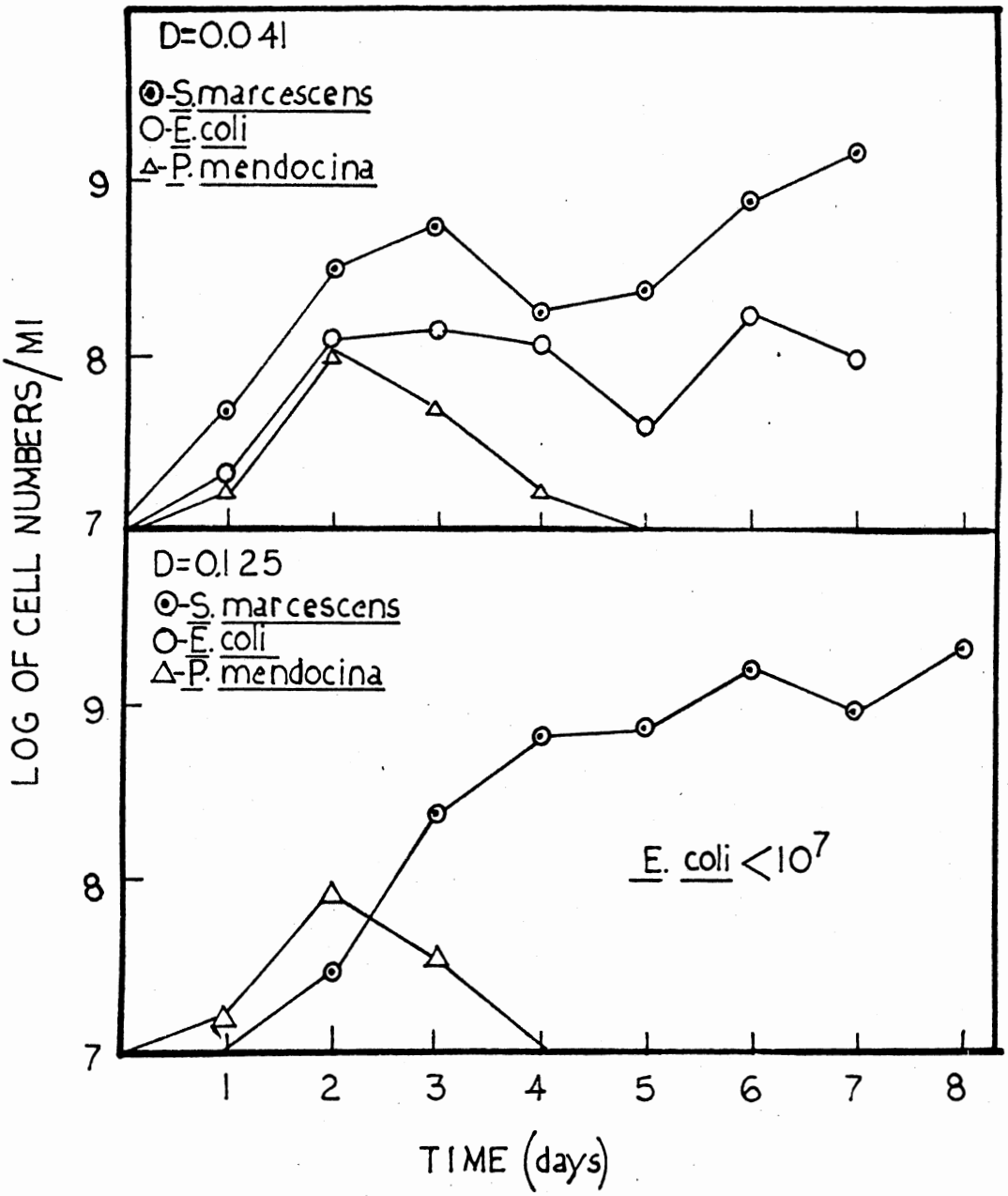

D=0.041
⊙-S. marcescens
O-E. coli
△-P. mendocina
D=0.125
⊙-S. marcescens
O-E. coli
△-P. mendocina
E. coli <10^7
LOG OF CELL NUMBERS/ML
TIME (days)
7
8
9
1
2
3
4
5
6
7
8

Figure 19. Bacterial Predominance Patterns in a Mixed Culture Continuous Flow Reactor at 30C for Dilution Rates of 0.041 and 0.125 hr^{-1}

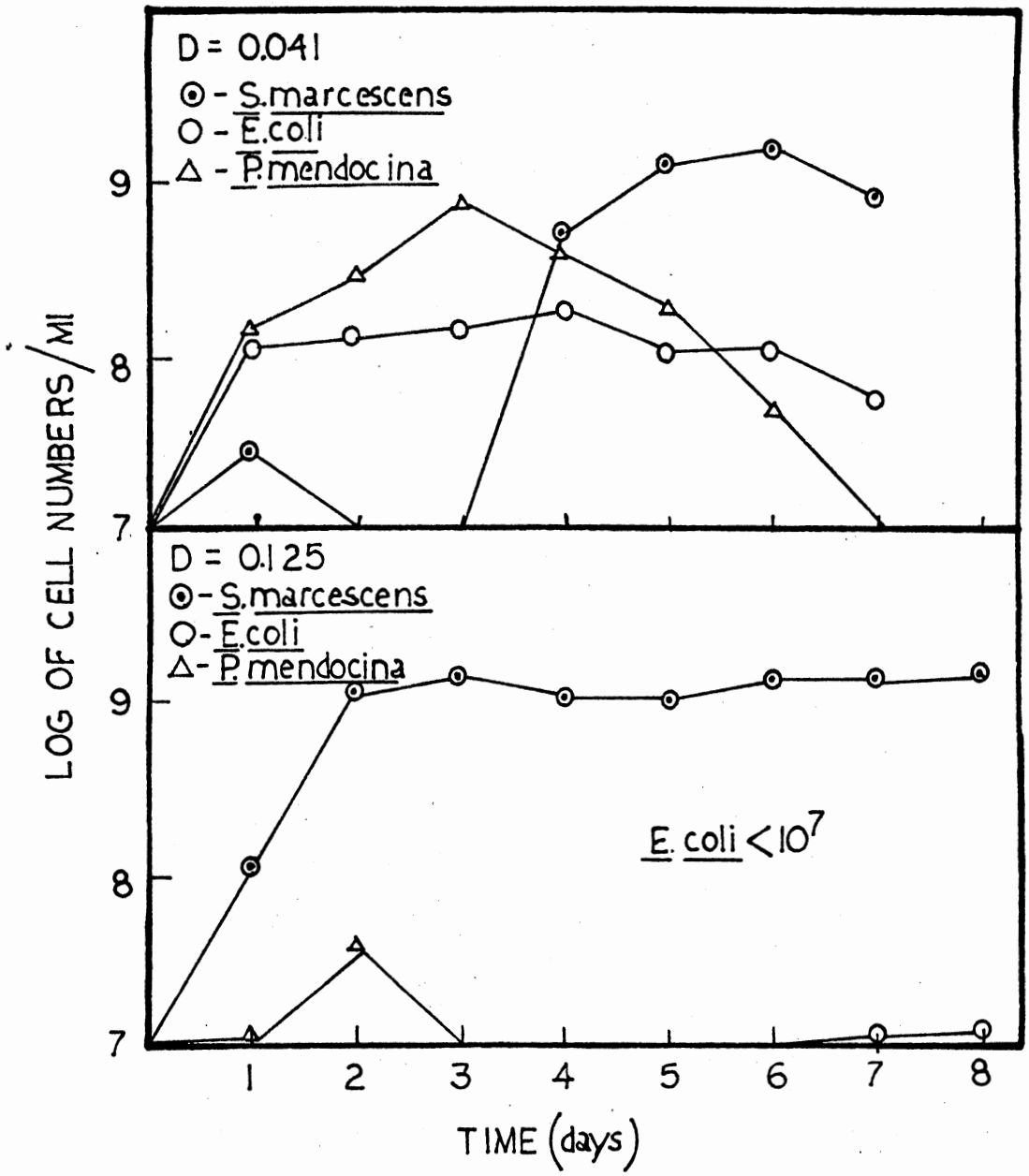

D = 0.041
⊙ - S. marcescens
O - E. coli
△ - P. mendocina
D = 0.125
⊙ - S. marcescens
O - E. coli
△ - P. mendocina
E. coli < 10^7
LOG OF CELL NUMBERS / ML
TIME (days)
7
8
9
1
2
3
4
5
6
7
8

Figure 20. Bacterial Predominance Patterns in a Mixed Culture Continuous Flow Reactor at 37C for Dilution Rates of 0.041 and 0.125 hr^{-1}

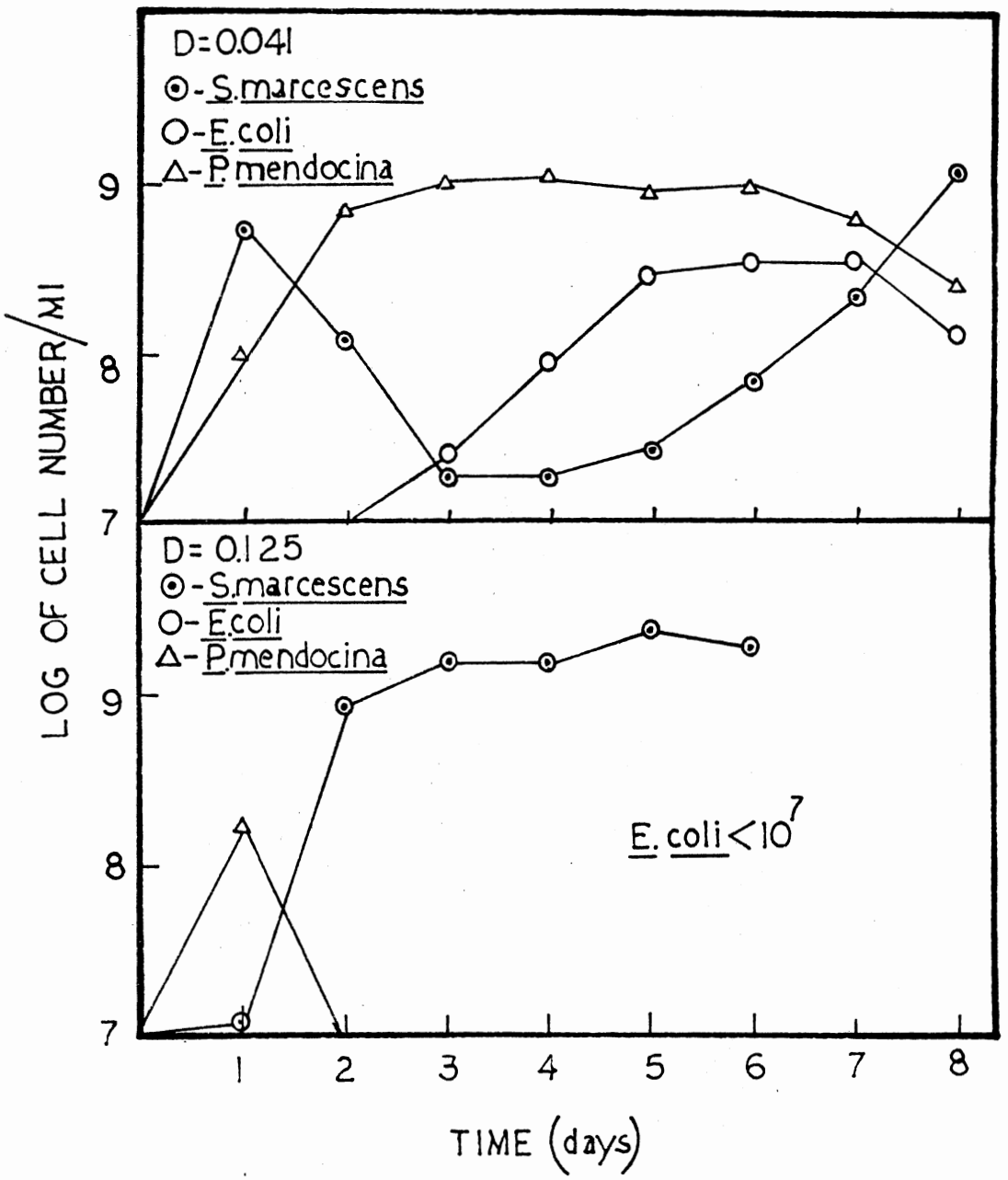
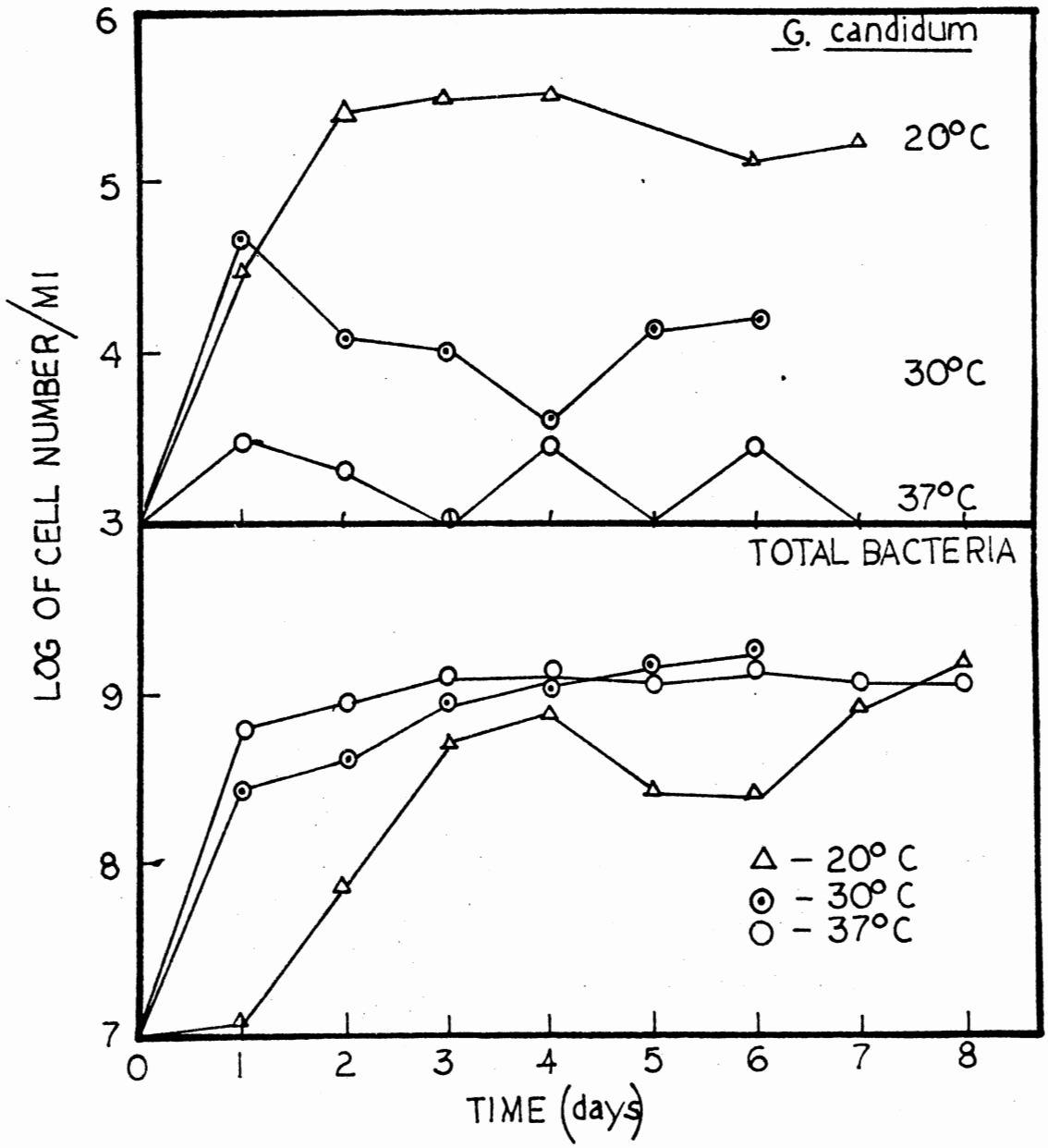

D=0.041
⊙-S.marcescens
O-E.coli
△-P.mendocina
D=0.125
⊙-S.marcescens
O-E.coli
△-P.mendocina
E. coli<10^7
LOG OF CELL NUMBER/ML
TIME (days)
7
8
9
1
2
3
4
5
6
7
8

Figure 21. The Effect of Different Temperatures Upon Total Bacterial and Fungal Plate Counts for a Dilution Rate of 0.041 hr^{-1}

The curves shown for total bacterial counts in this and the following figure were derived from a summation of the plate counts of S. marcescens, E. coli, and P. mendocina on a daily basis. The medium was glucose-minimal medium.


G. candidum
20°C
30°C
37°C
TOTAL BACTERIA
Δ – 20° C
⊙ – 30° C
○ – 37°C
LOG OF CELL NUMBER/ML
TIME (days)
6
5
4
3
9
8
7
0
1
2
3
4
5
6
7
8

effect of temperature upon the numbers of G. candidum was more pronounced. At 20C, counts were greater than 10^5/ml; at 30C they averaged 10^4/ml, and at 37C they were slightly higher than 10^3/ml. The same effect is noted to some extent at a dilution rate of 0.125. These results are shown in Figure 22 as well as the total bacterial counts for this dilution rate. As before, plate counts for the fungus were highest at 20C. However, in contrast to the results at the slower dilution rate, plate counts for 30C and 37C were nearly identical. Furthermore, the fungal plate counts at the 0.125 dilution rate were substantially higher than their counterparts at the 0.041 dilution rate. Total bacterial counts at 0.125 were comparable to those obtained at the slower dilution rate. As before, maximum plate counts were obtained at 20C only after a period of 7-8 days.

Table VI summarizes the effect of temperature and dilution rate upon the conversion of substrate to biomass. Several observations can be made from this information. Mixed culture dry weights were substantially higher at 37 and 30C than at 20C. Substrate-based yields were also higher at these temperatures. Dilution rate apparently influenced yields at 37 and 20C as higher values were obtained for the faster dilution rate compared to the slow.

Table VII compares specific growth rate for each microbial species grown individually in glucose minimal medium at various temperatures. This information was used to explain some of the observations noted in the mixed culture experiments. Of the three temperatures used, 37C was optimal for E. coli and S. marcescens, and 30C was optimal for P. mendocina and G. candidum. However, even at the sub-optimal temperature of 30C, E. coli and S. marcescens both had specific growth rates which

Figure 22. The Effect of Different Temperatures Upon Total Bacterial and Fungal Plate Counts for a Dilution Rate of 0.125 hr^{-1}

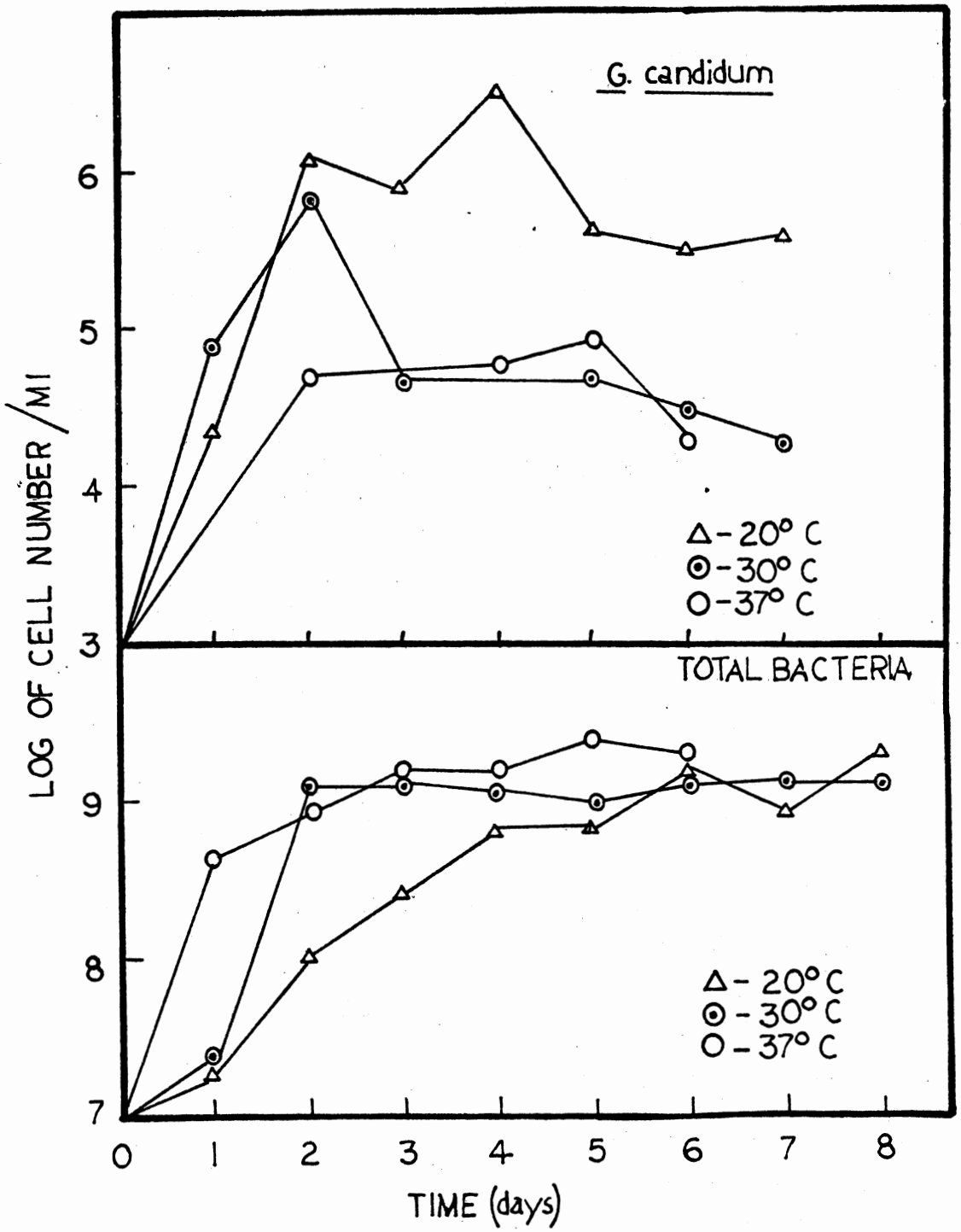

G. candidum
Δ - 20° C
⊙ - 30° C
O - 37° C
TOTAL BACTERIA
Δ - 20° C
⊙ - 30° C
O - 37° C
LOG OF CELL NUMBER /ML
TIME (days)
3
4
5
6
7
8
9
0
1
2
3
4
5
6
7
8

TABLE VI

THE EFFECT OF TEMPERATURE AND DILUTION RATE UPON THE DRY WEIGHT AND SUBSTRATE UTILIZATION OF A MIXED CULTURE

Dilution Rate	Temperature	Dry Weight (mg/l)	Effluent Substrate (mg/l)	Δ Substrate (mg/l)	Yield
0.041	37C	369	91	998	0.37
0.041	30C	425	110	1000	0.48
0.041	20C	221	127	861	0.26
0.125	37C	417	105	875	0.48
0.125	30C	387	100	861	0.45
0.125	20C	279	116	864	0.32

TABLE VII

COMPARISON OF SPECIFIC GROWTH RATES IN BATCH AS A FUNCTION OF TEMPERATURE

Organism	Specific Growth Rates (hr^{-1})			
	20C	26C	30C	37C
E. coli	0.25	-	0.76	0.84
S. marcescens	0.32	-	0.77	0.84
P. mendocina	0.20	-	0.46	0.39
G. candidum	0.24	0.35	0.43	0.25

The medium was glucose-minimal medium containing 1000 mg/l glucose.

exceeded those of P. mendocina and G. candidum. At 20C, all species had reduced growth rates with E. coli being the most affected. Its growth rate was reduced to a value approximating that of G. candidum. S. marcescens maintained a relatively higher growth rate than the other three species at this temperature. A single growth study with G. candidum at 26C yielded a specific growth rate of 0.35 hr^{-1}.

Nitrogen Studies

Figures 23-26 represent the individual bacterial plate count data at both dilution rates for various concentrations of ammonium sulfate at 30C. At 500 mg/l as shown in Figure 23, S. marcescens quickly established predominance at the faster dilution rate. The same experiment performed at the lower dilution rate also resulted in the predominance of S. marcescens although a longer period of time was required for it to do so. Its superiority in numbers seemed to be at the expense of P. mendocina rather than E. coli, since counts of the latter remained relatively constant throughout the experiment while counts of the former fell below a detectible level. Figure 24 represents the plate count data gathered when ammonium sulfate concentration was lowered to 150 mg/ml. At this concentration, S. marcescens no longer predominated at either dilution rate. The behavior of the culture was very similar to that seen at 500 mg/ml although the roles of S. marcescens and E. coli were reversed. E. coli now predominated at both dilution rates and did so very quickly at 0.125 hr^{-1} but less quickly at 0.041 hr^{-1}. Furthermore, at the slow dilution rate, E. coli numbers seemed to be increasing at the expense of P. mendocina as S. marcescens levels remained constant. When the concentration of ammonium sulfate was

Figure 23. Bacterial Predominance Patterns in a Mixed Culture Continuous Flow Reactor Containing 500 mg/l $(NH_4)_2SO_4$ at Dilution rates of 0.041 and 0.125 hr^{-1}

The medium was glucose minimal-medium containing 1000 mg/l glucose. Temperature was 30C.

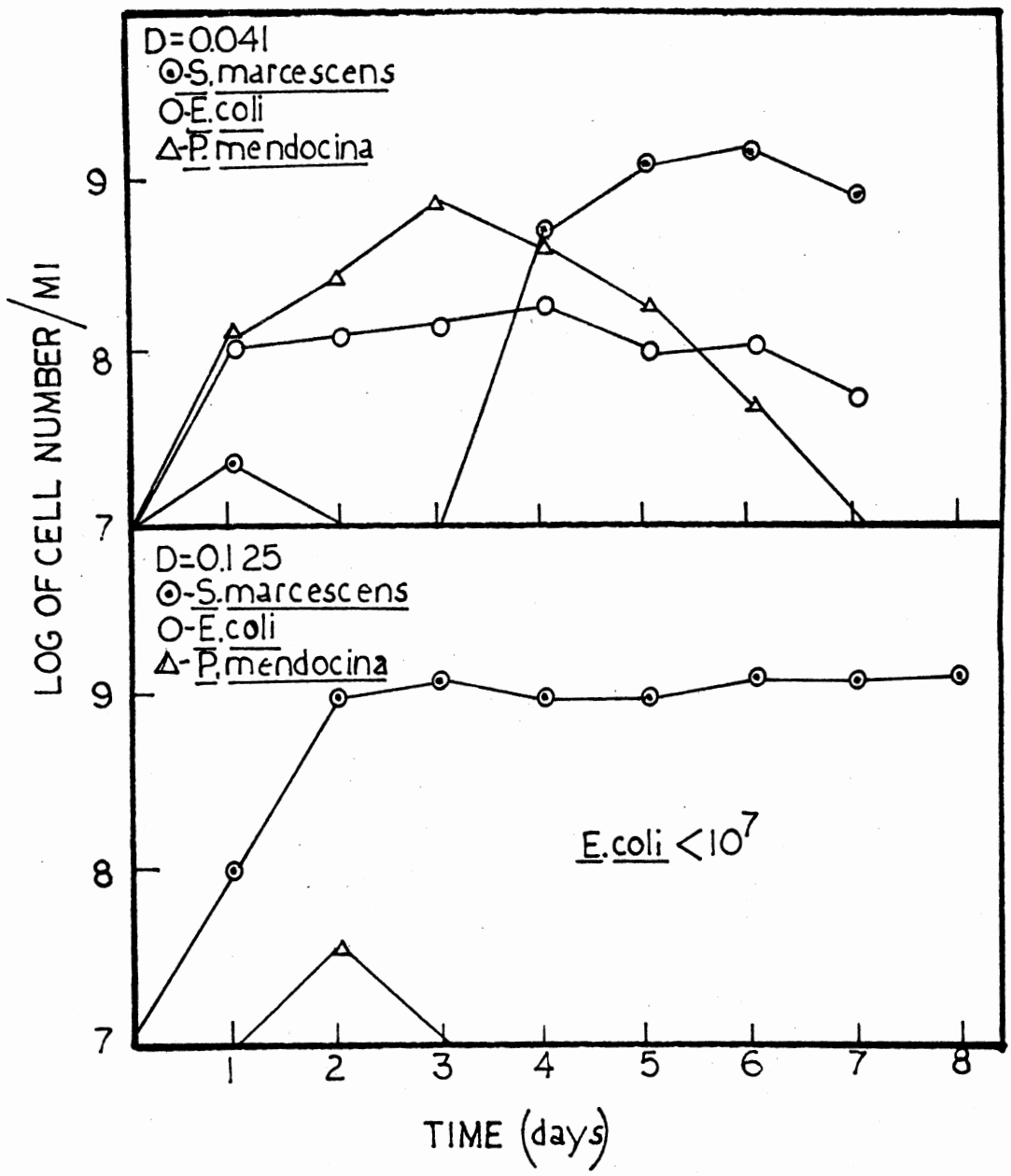
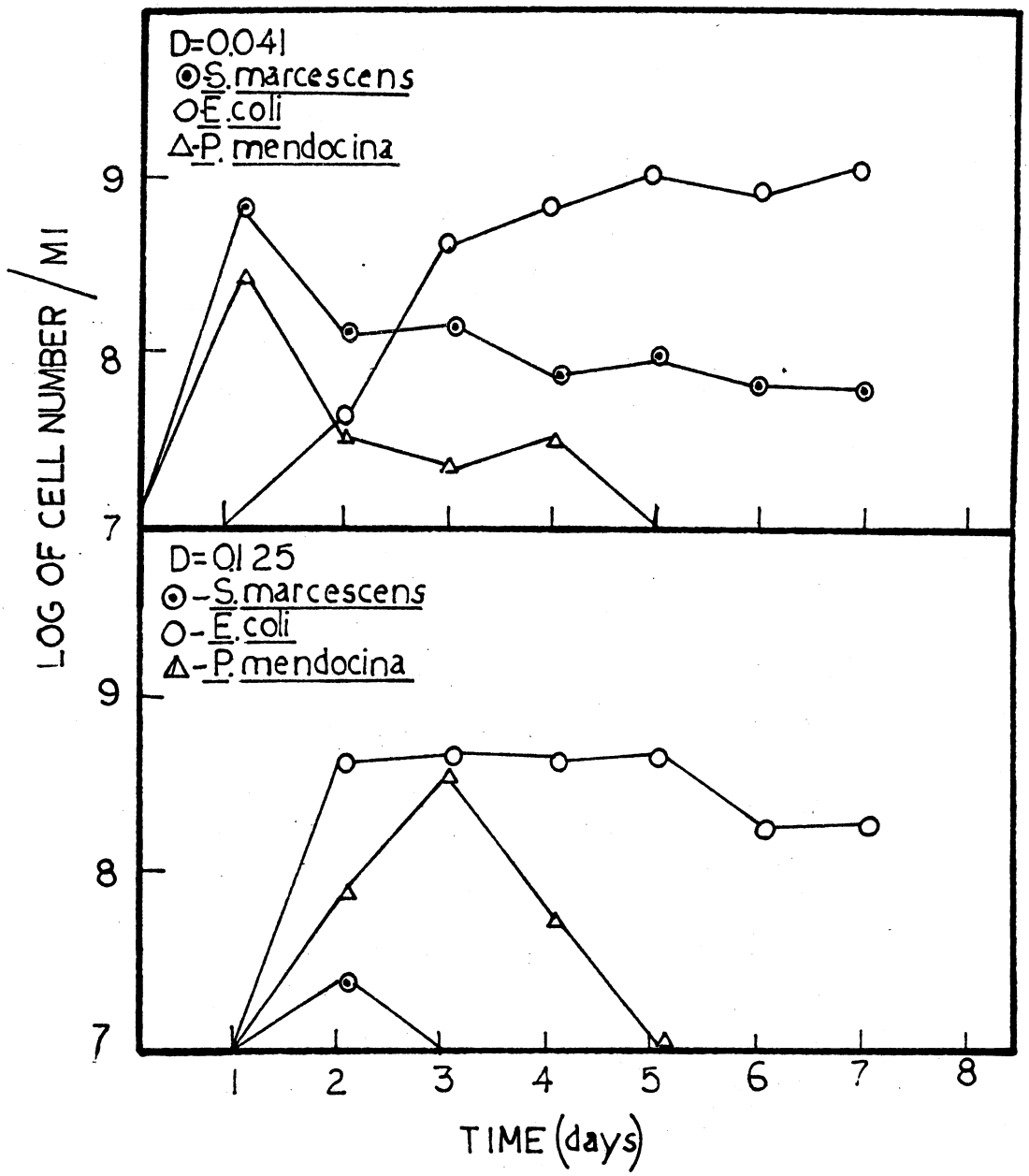

D=0.041
⊙-S. marcescens
O-E. coli
Δ-P. mendocina
D=0.125
⊙-S. marcescens
O-E. coli
Δ-P. mendocina
E. coli <10^7
LOG OF CELL NUMBER/ML
TIME (days)
7
8
9
1
2
3
4
5
6
7
8

Figure 24. Bacterial Predominance Patterns in a Mixed Culture Continuous Flow Reactor Containing 150 mg/l $(NH_4)_2SO_4$ at Dilution Rates of 0.041 and 0.125 hr^{-1}

The medium was glucose-minimal medium containing 1000 mg/l glucose. Temperature was 30C.


D=0.041
⊙S. marcescens
○E. coli
△-P. mendocina
D=0.125
⊙-S. marcescens
○-E. coli
△-P. mendocina
LOG OF CELL NUMBER / Ml
9
8
7
1
2
3
4
5
6
7
8
TIME (days)

dropped to 100 mg/ml, the predominance of E. coli over S. marcescens was much more pronounced as shown in Figure 25. E. coli predominated very quickly at both dilution rates as S. marcescens was diluted below a detectible level. In contrast to the previous experiments, there was little tendency for P. mendocina to be diluted out. It remained at a relatively constant level when the culture was grown at the slow dilution rate. At the faster dilution rate, levels increased over a period of five days then declined somewhat, apparently at the expense of increasing E. coli counts. Figure 26 illustrates further the lack of competitiveness provided by S. marcescens when nitrogen levels were quite low. The concentration of ammonium sulfate used in these experiments was 50 mg/l. A relative increase in competitiveness of P. mendocina was quite evident at the slow dilution rate at which this species exhibited counts surpassing those of the other two bacterial species. Its total count was nearly identical to that obtained at 100 mg/l. However, reducing ammonium sulfate concentration to 50 mg/l resulted in the reduction of E. coli numbers to a level below those of P. mendocina. When the culture was grown at the faster dilution rate, E. coli again established predominance although P. mendocina was not diluted to an undetectible level within the experimental time span.

Figure 27 represents the effect of different ammonium sulfate concentrations upon both total bacterial and G. candidum plate counts at the slow dilution rate. An examination of the total bacterial counts as a function of nitrogen concentration showed an increasing bacterial count with each increase in ammonium sulfate concentration. Cell counts were most drastically affected at 50 mg/l as there was a wide difference between total counts obtained at this concentration and those

Figure 25. Bacterial Predominance Patterns in a Mixed Culture Continuous Flow Reactor Containing 100 mg/l $(NH_4)_2SO_4$ at Dilution Rates of 0.041 and 0.125 hr^{-1}

The medium was glucose-minimal medium containing 1000 mg/l glucose. Temperature was 30C.

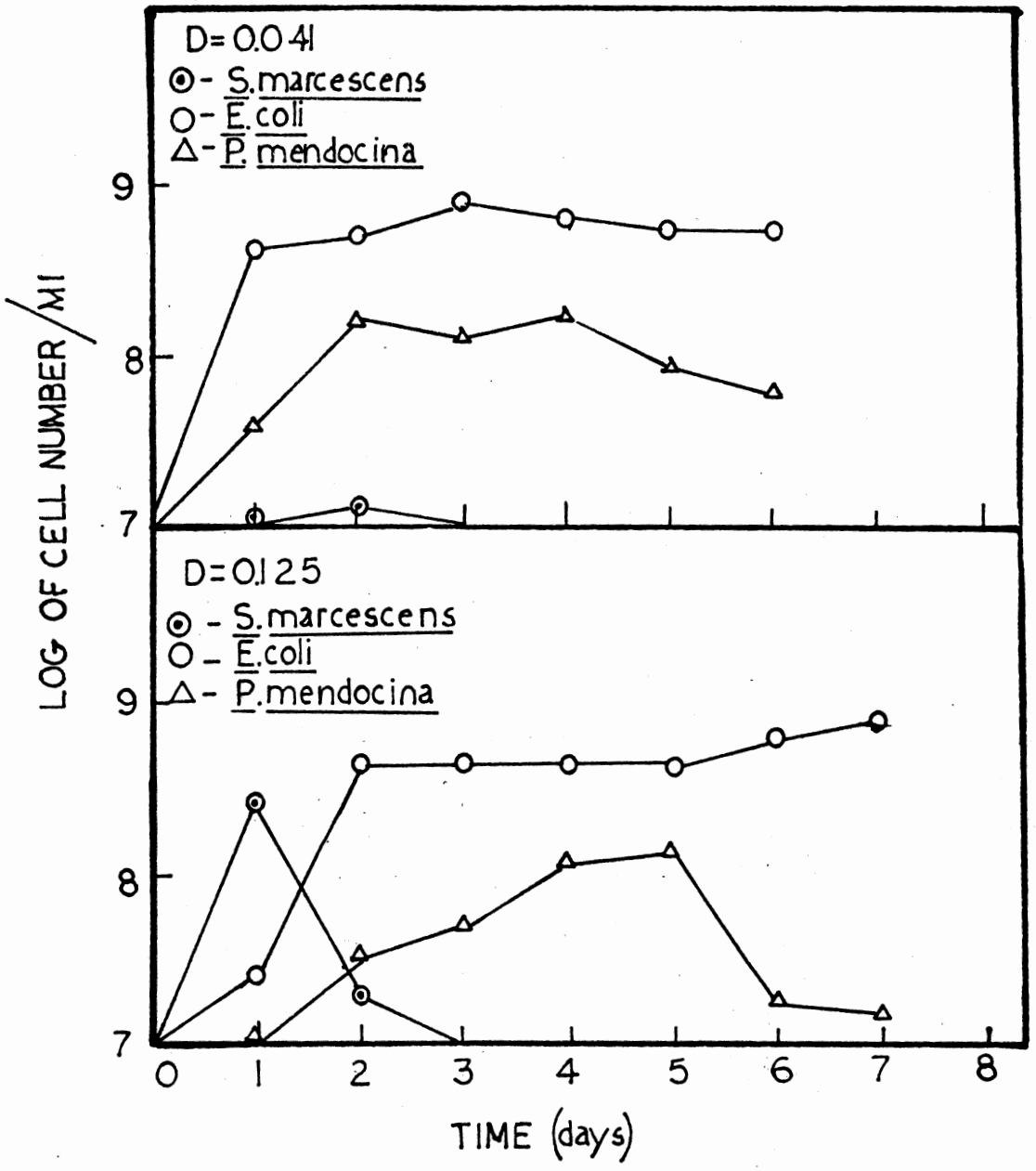

D=0.041
⊙- S.marcescens
O- E.coli
△- P. mendocina
D=0.125
⊙ - S.marcescens
O - E.coli
△ - P.mendocina
LOG OF CELL NUMBER/ML
TIME (days)
7
8
9
0
1
2
3
4
5
6
7
8

Figure 26. Bacterial Predominance Patterns in a Mixed Culture Continuous Flow Reactor Containing 50 mg/l $(NH_4)_2SO_4$ at Dilution Rates of 0.041 and 0.125 hr^{-1}

The medium was glucose-minimal medium containing 1000 mg/l glucose. Temperature was 30C.

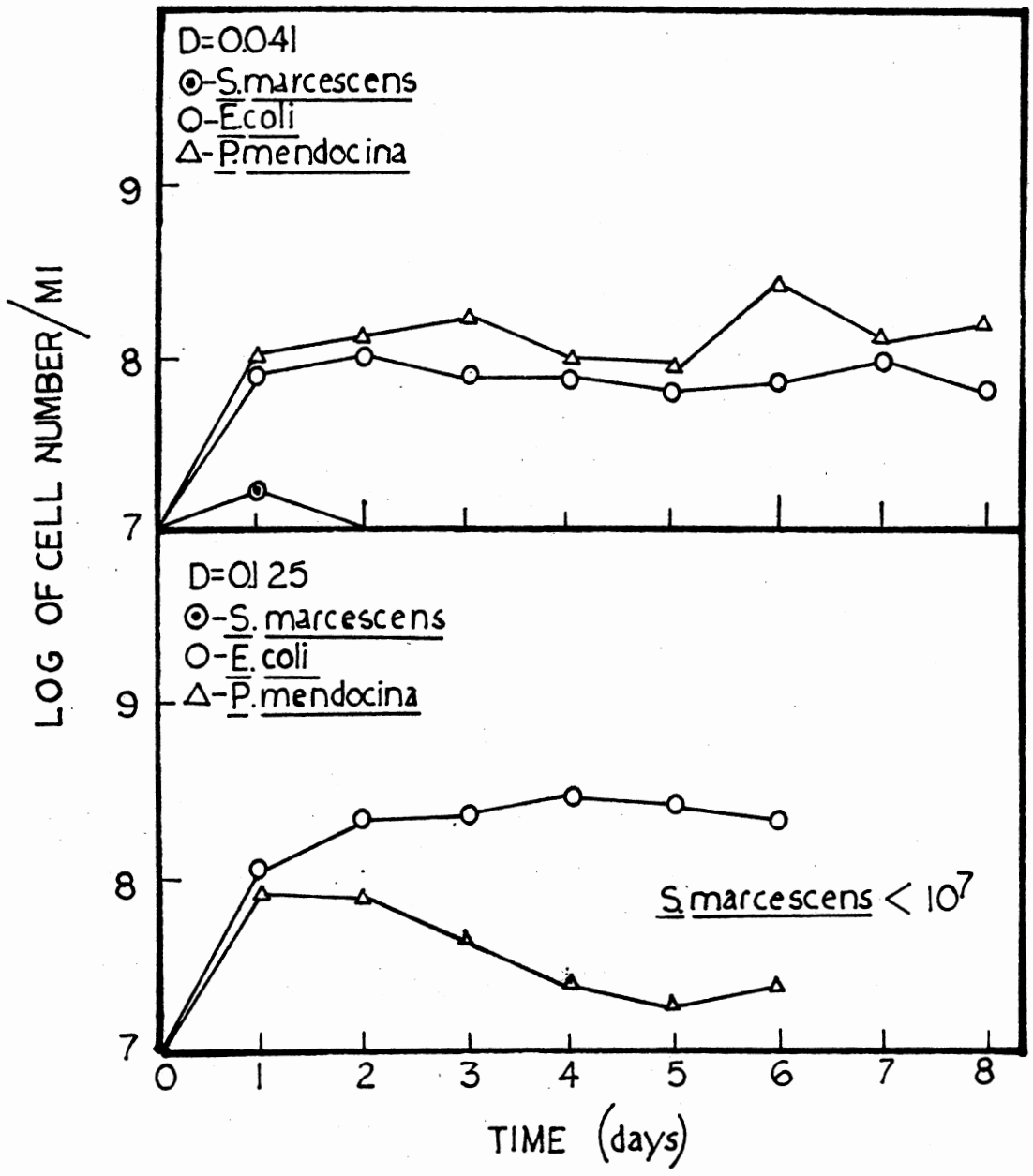
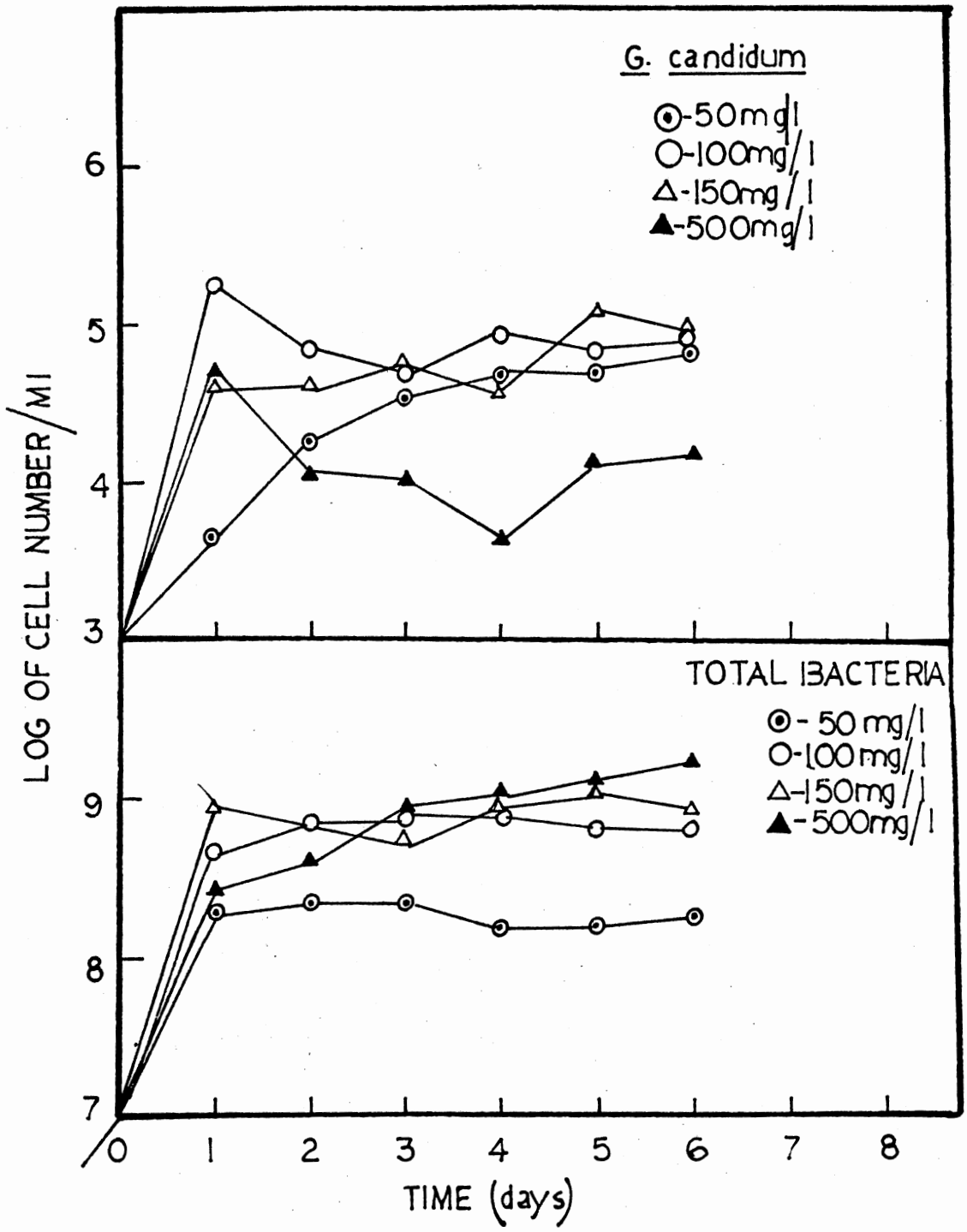

D=0.041
⊙-S.marcescens
O-E.coli
△-P.mendocina
D=0.125
⊙-S. marcescens
O-E. coli
△-P. mendocina
S. marcescens < 10^7
LOG OF CELL NUMBER/ML
TIME (days)
9
8
7
0
1
2
3
4
5
6
7
8

Figure 27. The Effect of Different Concentrations of $(NH_4)_2SO_4$ Upon Total Bacterial and Fungal Plate Counts at a Dilution Rate of 0.041 hr^{-1}

The medium was glucose-minimal medium containing 1000 mg/l glucose. The temperature was 30C.


G. candidum
⊙-50mg/l
O-100mg/l
△-150mg/l
▲-500mg/l
TOTAL BACTERIA
⊙- 50 mg/l
O-100 mg/l
△-150mg/l
▲-500mg/l
LOG OF CELL NUMBER/ML
TIME (days)
3
4
5
6
7
8
9
0
1
2
3
4
5
6
7
8

obtained at 100 mg/l. Total counts of G. candidum at this dilution rate showed that minimal numbers were obtained at 500 mg/l while substantially higher numbers were obtained at lower concentrations of nitrogen. Plate counts ultimately reached a level which was nearly identical for ammonium sulfate concentrations of 50, 100, and 150 mg/l. When the dilution rate was increased to 0.125 hr^{-1}, observations similar to those noted at 0.041 hr^{-1} for total counts were made as shown in Figure 28. Total bacterial counts were again determined by nitrogen concentration. Counts at 50 and 500 mg/l averaged somewhat higher than those at the slow dilution rate. The effect upon G. candidum plate counts was less conclusive than that of comparable studies at the slow dilution rate. Wider fluctuations in plate counts were noted especially at 100 and 150 mg/l ammonium sulfate. Lowest counts were obtained at 500 mg/l, which correlates with a similar observation made at the slow dilution rate.

Upon examination of the culture at the various nitrogen levels, it was evident that little filamentation was being expressed except at 50 mg/l. Visible clumps of growth were seen in the culture at both dilution rates. Microscopic examination of these growths showed them to be composed nearly exclusively of G. candidum in filaments and arthrospores. Because total bacterial plate counts were very constant and G. candidum plate counts were very erratic, quantifying the amount of fungus present was deemed best approached by the indirect method of subtracting estimated bacterial dry weight from total dry weight. Table VIII presents the results of these calculations as a function of dilution rate and ammonium sulfate concentration. The calculated percentage of the culture dry weight represented by G. candidum was at a maximum

Figure 28. The Effect of Different Concentrations of $(NH_4)_2SO_4$ Upon Total Bacterial and Fungal Plate Counts at a Dilution Rate of 0.125 hr^{-1}

The medium was glucose-minimal medium containing 1000 mg/l glucose. The temperature was 30C.

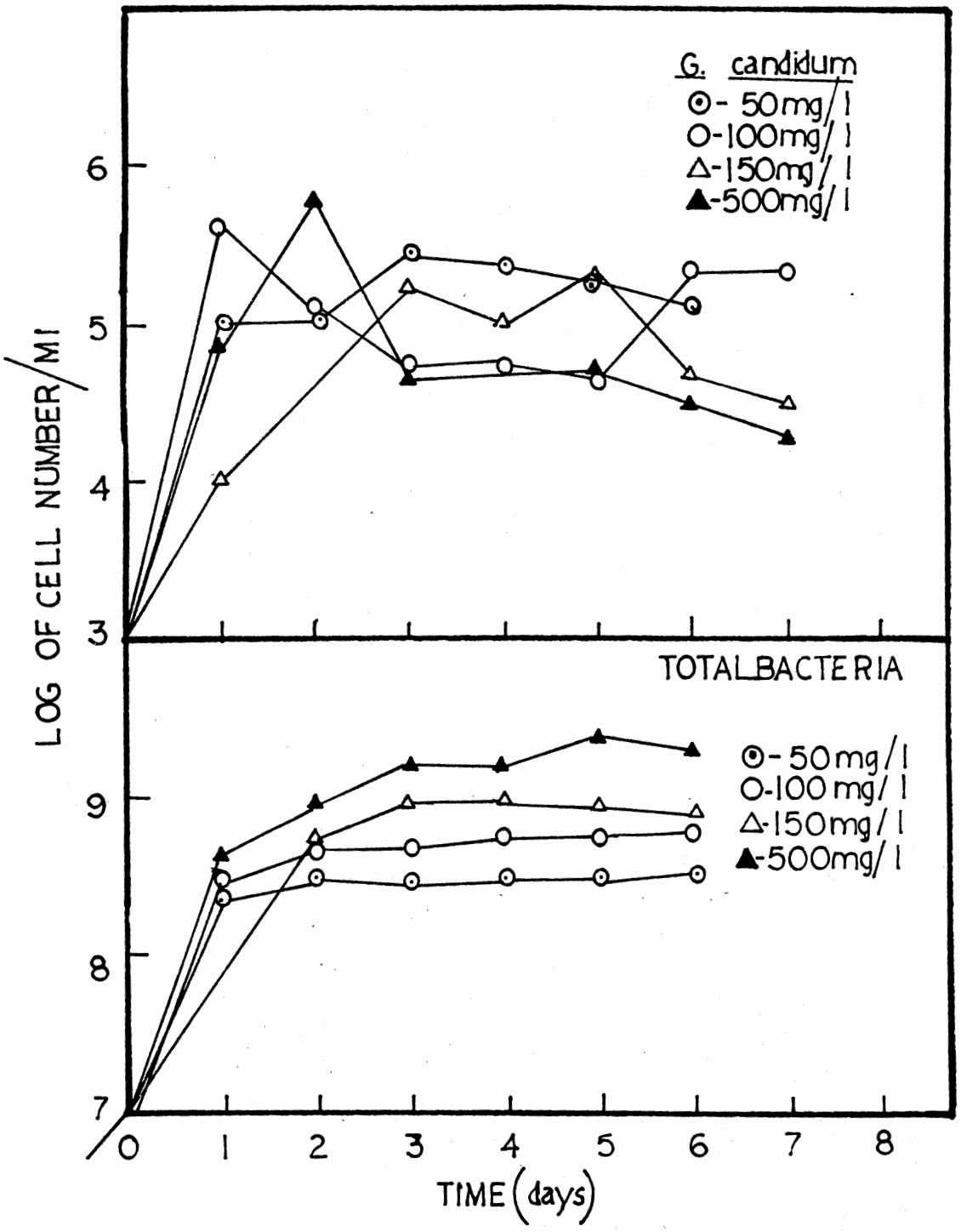

G. candidum
⊙- 50mg/l
O-100mg/l
△-150mg/l
▲-500mg/l
LOG OF CELL NUMBER/ML
TOTAL BACTERIA
⊙- 50mg/l
O-100mg/l
△-150mg/l
▲-500mg/l
TIME (days)

TABLE VIII

PROPORTION OF TOTAL DRY WEIGHT REPRESENTED BY G. CANDIDUM AT VARIOUS NITROGEN CONCENTRATIONS

Dilution Rate (hr^{-1})	$(NH_4)_2SO_4$ Concentration (mg/l)	Total Dry Weight (mg/l)	Bacterial Dry Weight (mg/l)	Fungal Dry Weight (mg/l)	Percent G. Candidum
0.041	50	102	57	45	44
0.041	100	186	124	62	33
0.041	150	266	260	16	6
0.041	500	425	425	-	-
0.125	50	108	66	42	39
0.125	100	204	160	44	22
0.125	150	245	180	65	26
0.125	500	367	292	75	20

Abbreviation: (-), the amount present represented a negligible proportion of total dry weight.

The estimation of percentage G. candidum was obtained from the difference between the total mixed culture dry weight and an estimate of bacterial dry weight. The latter was obtained by multiplying total bacterial plate counts by 2×10^{-13} g which represents the average dry weight of a single bacterial cell.

compared to other nitrogen concentrations. For the slow dilution rate, a percentage of 44 percent G. candidum was obtained while at the faster dilution rate the percentage was 39 percent G. candidum. A difference attributable to dilution rate was noted for concentrations of 100, 150, and 500 mg/l ammonium sulfate. At a dilution rate of 0.041 hr^{-1}, the percentage of the cultural dry weight represented by G. candidum was inversely proportional to the concentration of ammonium sulfate, while at a dilution rate of 0.125 hr^{-1}, the percentage fell from 39 percent at 50 mg/l to a relatively constant level of 20-26 percent for all other concentrations.

Table IX summarizes the efficiency of substrate utilization at various nitrogen concentrations as determined by the Neish method. Efficiency was not significantly different for experiments performed with ammonium sulfate concentrations varying from 100 to 500 mg/l with the possible exception of the experiment performed at a dilution rate of 0.125 hr^{-1} with a concentration of 100 mg/l. This culture utilized 84 percent of the organic matter compared to an average of 89 percent utilization at the other concentrations. When ammonium sulfate concentration was reduced to 50 mg/l, the efficiency of substrate utilization was markedly affected. Only 56 and 44 percent of the organic matter was removed at the slow and fast dilution rates, respectively. Furthermore, the amount of glucose remaining as measured by the glucose oxidase test (Worthington Glucostat) was significantly higher at these concentrations. However, more than 90 percent of the glucose in the medium was utilized even at an ammonium sulfate concentration of 50 mg/l.

Using ammonium sulfate as the growth limiting nutrient, K_s

TABLE IX

SUBSTRATE UTILIZATION BY THE MIXED CULTURE AT VARIOUS NITROGEN CONCENTRATIONS AS MEASURED BY THE NEISH METHOD

Dilution Rate (hr^{-1})	$(NH_4)_2SO_4$ Concentration (mg/l)	Feed Organic Matter (mg/l)	Effluent Organic Matter (mg/l)	Organic Matter Utilized (%)	Glucose Remaining (mg/l)
0.041	50	944	417	56	35
0.041	100	907	91	90	3
0.041	150	951	116	88	-
0.041	500	1000	110	89	-
0.125	50	907	508	44	63
0.125	100	890	145	84	3
0.125	150	907	73	92	0
0.125	500	961	100	90	0

The various estimates of organic matter present were measured by the Neish method except for the glucose determinations, which were determined by the Glucostat method.

determinations for each species were performed in batch to study the relationships between individual specific growth rates and initial concentrations of nitrogen. K_s and μ_m were determined from double reciprocal plots of specific growth rates versus nitrogen concentration, as shown in Figure 29. Exact nitrogen concentrations were calculated from the known amount of ammonium sulfate added in each experiment. Table X summarizes the results of these determinations. The values for K_s ranged from a low of 1 mg/l for G. candidum to a high of 9.5 mg/l for S. marcescens. The estimated μ_m values corresponded quite well with those obtained for carbon limitation although values for P. mendocina and S. marcescens were slightly lower. K_s and μ_m values for nitrogen were substituted into the Monod equation for derivation of the curves shown in Figure 30. The points illustrated on the curves represent actual data points.

Carbon and Energy Source Studies

Five different sugars as well as sodium acetate were tested for their influence upon microbial predominance in continuous culture. As a preliminary study, specific growth rates were determined in batch for each species grown upon each carbon and energy source. The results of these studies are presented in Table XI. Of the compounds tested, glucose yielded the fastest specific growth rates for all species except P. mendocina. This organism grew substantially faster using sodium acetate as sole carbon and energy source. Furthermore, as is characteristic of this species, it was unable to use any of the other sugars as a carbon and energy source. S. marcescens and E. coli used all of the compounds except one, and G. candidum all except two. Galactose seemed

Figure 29. Variation of Specific Growth Rate, μ, With Initial Nitrogen Concentration, S_o, Plotted as Double Reciprocals to Estimate K_s and μ_m for Individual Cultures in Batch

The medium was glucose-minimal medium containing 1000 mg/l glucose. The temperature was 30C.

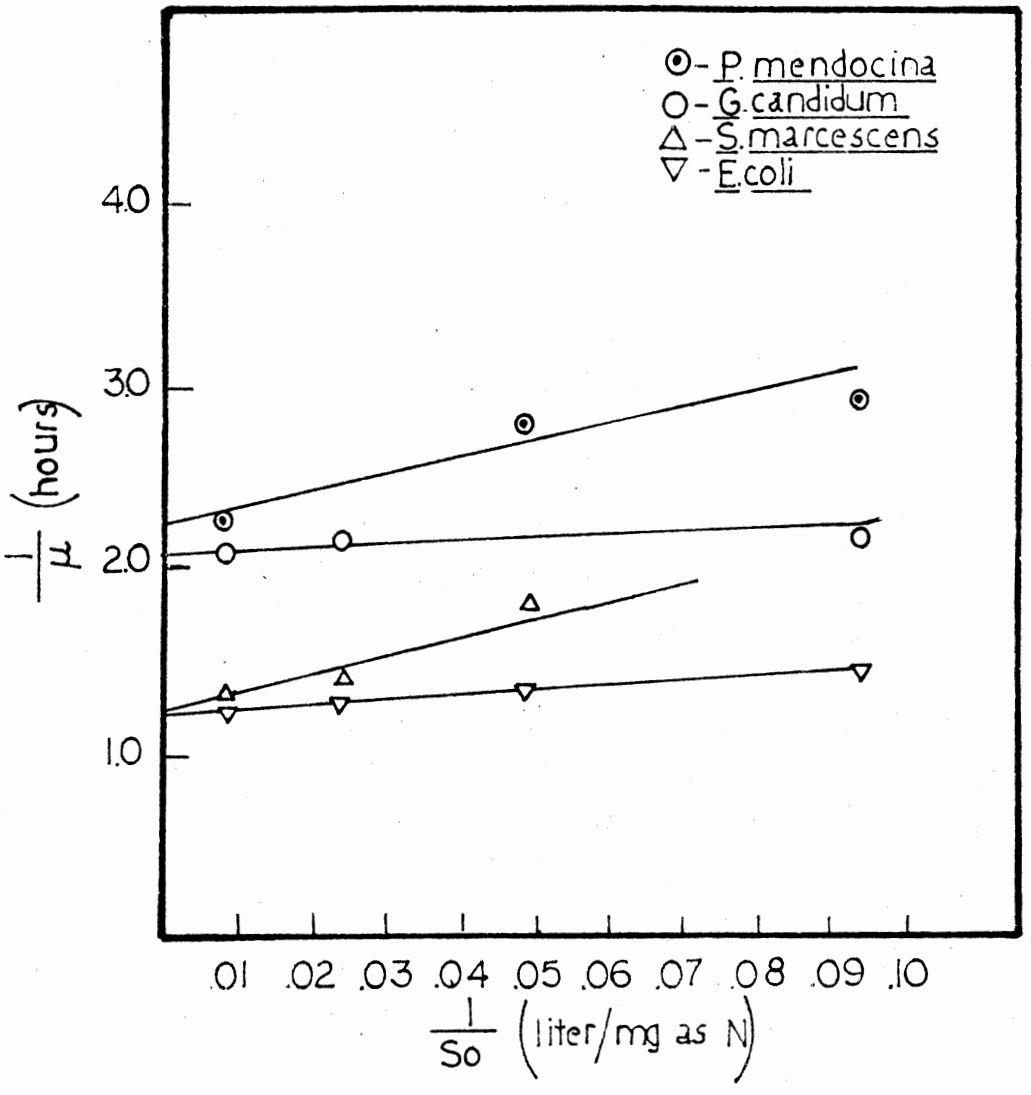

⊙- P. mendocina
○- G. candidum
△- S. marcescens
▽- E. coli
4.0
3.0
2.0
1.0
1/μ (hours)
.01 .02 .03 .04 .05 .06 .07 .08 .09 .10
1/So (liter/mg as N)

TABLE X

SUMMARY OF GROWTH CHARACTERISTICS IN BATCH AS A FUNCTION OF VARYING NITROGEN CONCENTRATIONS

Organism	Ammonium Sulfate Concentration (mg/l)	Nitrogen Concentration (mg/l)	Specific Growth Rate (hr^{-1})	Constants μ_m (hr^{-1})	Constants K_s (hr^{-1})
P. mendocina		-	-	0.44	3.9
	50	10.6	0.34		
	100	21.0	0.36		
	500	106.0	0.44		
S. marcescens		-	-	0.83	9.5
	100	21.0	0.55		
	200	42.4	0.71		
	500	106.0	0.77		
E. coli		-	-	0.83	2.6
	50	10.6	0.69		
	100	21.0	0.75		
	200	42.4	0.77		
	500	106.0	0.80		
G. candidum		-	-	0.49	1.0
	25	5.3	0.41		
	50	10.6	0.46		
	200	42.4	0.46		
	500	106.0	0.49		

Figure 30. Variation of Specific Growth Rate, μ, With Initial Nitrogen Concentration, S_o, for Batch Experiments With Each Culture at 30C

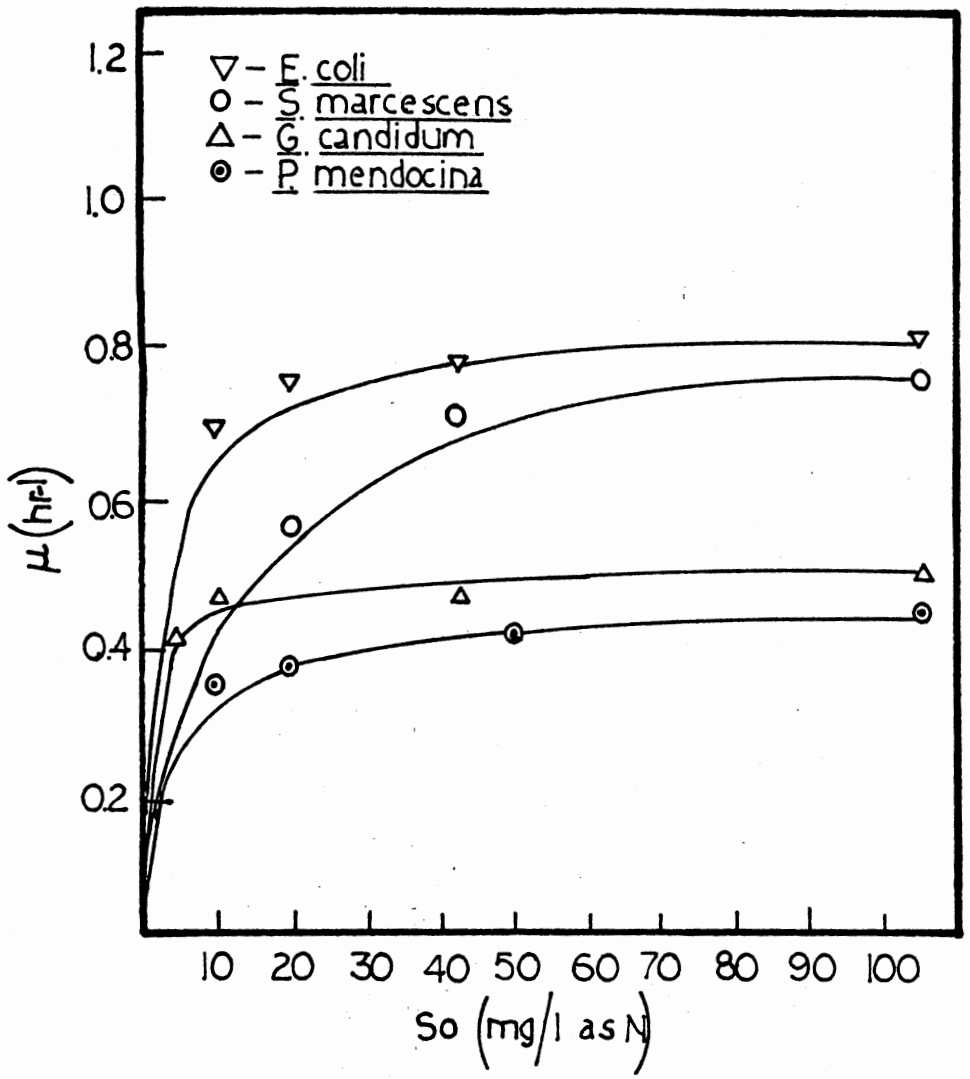

▽ – E. coli
○ – S. marcescens
△ – G. candidum
⊙ – P. mendocina
μ (hr⁻¹)
1.2
1.0
0.8
0.6
0.4
0.2
10 20 30 40 50 60 70 80 90 100
So (mg/l as N)

TABLE XI

SUMMARY OF INDIVIDUAL SPECIFIC GROWTH RATES IN BATCH CONTAINING 1000 mg/l OF VARIOUS CARBON AND ENERGY SOURCES AT 30C

Carbon and Energy Source	Specific Growth Rates			
	G. candidum	S. marcescens	E. coli	Ps. mendocina
Glucose	0.43	0.77	0.76	0.46
Galactose	0.07	0.22	0.60	0.00
Lactose	0.00	0.00	0.73	0.00
Sucrose	0.00	0.64	0.00	0.00
Acetate	0.22	-	0.18	0.55
Fructose	0.39	0.55	0.49	0.00

Abbreviation: (-), a specific growth rate could not be measured although growth occurred.

to be somewhat inhibitory to S. marcescens and G. candidum as very long lag periods followed inoculation of this medium. Four days elapsed before G. candidum began growing and three days were required for S. marcescens. Specific growth rates were markedly lower for this compound than for other compounds supporting growth. Sodium acetate also affected S. marcescens in a manner precluding the measurement of specific growth rate. A three-day lag period occurred after inoculation. Even after growth had commenced, it occurred in a very erratic fashion which was never describable as logarithmic growth. Growth occurred in a discontinuous fashion over 2-3 days. All other species grew over night in acetate medium although G. candidum and E. coli had slow growth rates compared to those obtained in glucose.

The bacterial predominance patterns in a galactose-limited continuous culture are shown in Figure 31 for two dilution rates. As predicted from the batch data, E. coli quickly established predominance with subsequent wash-out of other species. Total bacterial counts were virtually identical at the two dilution rates, as shown in Figure 32. The counts for G. candidum also shown in this figure were quite low for the fast dilution rate and moderately higher for the slow dilution rate. There was no marked tendency toward dilute-out at either growth rate although the specific growth rate of G. candidum in batch was very low. The bacterial predominance patterns noted with galactose as limiting nutrient were very similar to those for lactose-limited continuous cultures as depicted in Figure 33. E. coli again assumed the dominant role as expected, since none of the other species was able to grow in batch as a pure culture in lactose minimal medium. The total bacterial count expressed graphically in Figure 34 was somewhat higher at the slow

Figure 31. Bacterial Predominance Patterns in a Mixed Culture Continuous Flow Reactor Containing 1000 mg/l Galactose in Minimal Salts Medium at Dilution Rates of 0.041 and 0.125 hr^{-1} at 30C

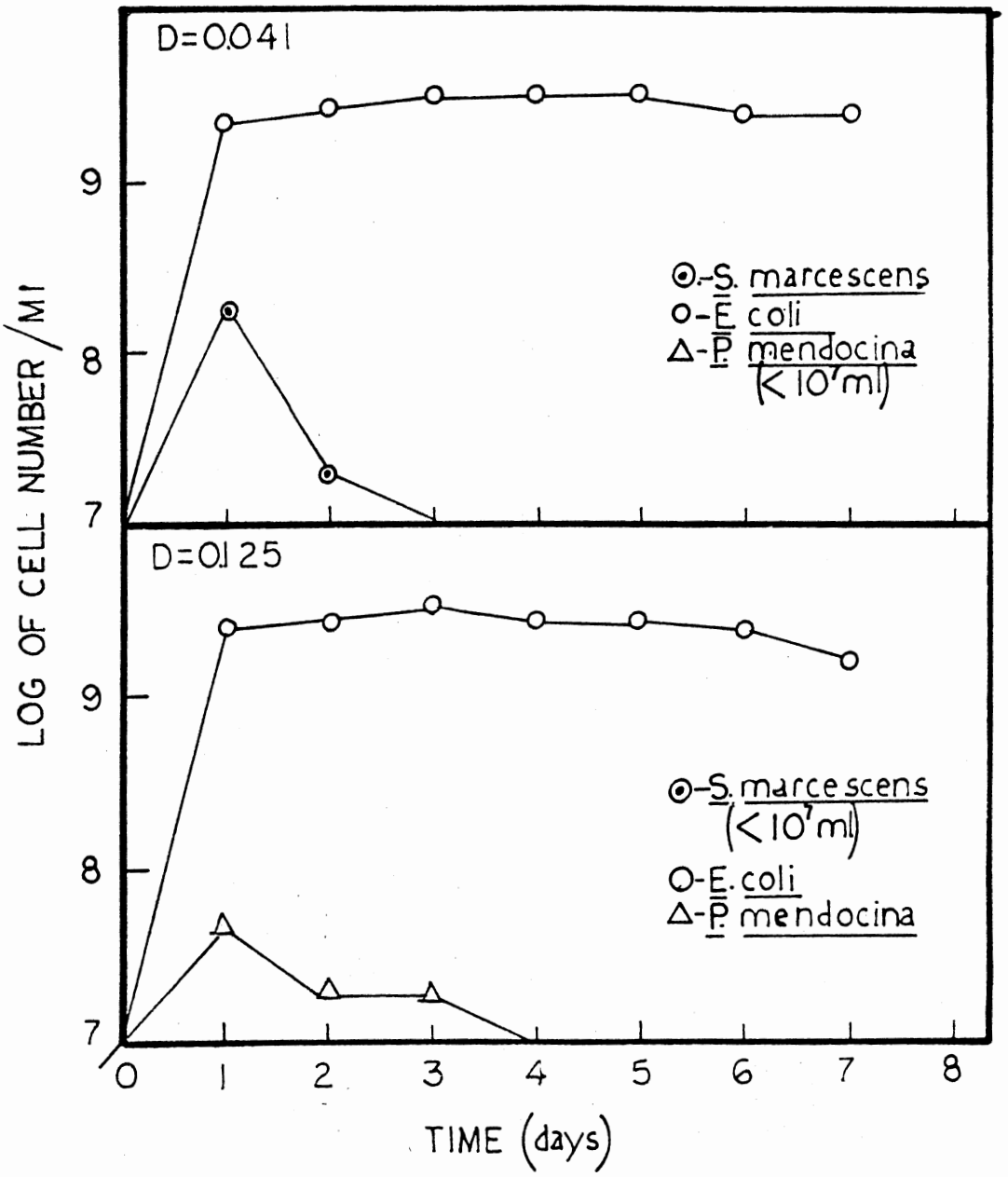

D=0.041
D=0.125
LOG OF CELL NUMBER / ML
TIME (days)
⊙-S. marcescens
O-E. coli
Δ-P. mendocina
(<10⁷ml)
⊙-S. marcescens
(<10⁷ml)
O-E. coli
Δ-P. mendocina
7
8
9
0
1
2
3
4
5
6
7
8

Figure 32. Total Bacterial and Fungal Plate Counts in a Galactose-limited Continuous Flow Reactor at Dilution Rates of 0.041 and 0.125 hr^{-1} at 30C

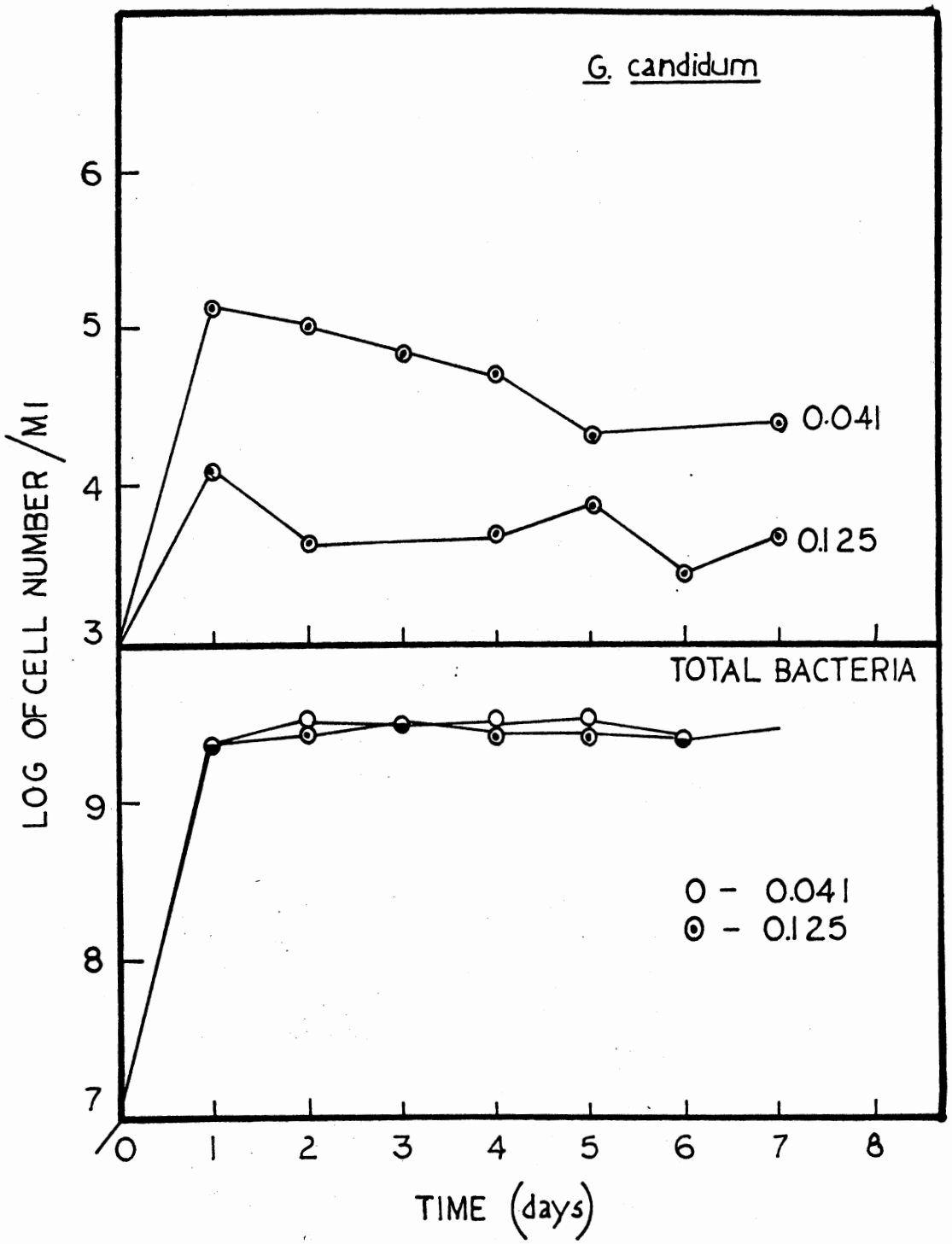

G. candidum
6
5
4
3
0·041
0·125
TOTAL BACTERIA
9
8
7
0 - 0.041
⊙ - 0.125
LOG OF CELL NUMBER /ML
0
1
2
3
4
5
6
7
8
TIME (days)

Figure 33. Bacterial Predominance Patterns in a Mixed Culture Continuous Flow Reactor Containing 1000 mg/l Lactose in Minimal Salts Medium at Dilution Rates of 0.041 and 0.125 hr^{-1} at 30C

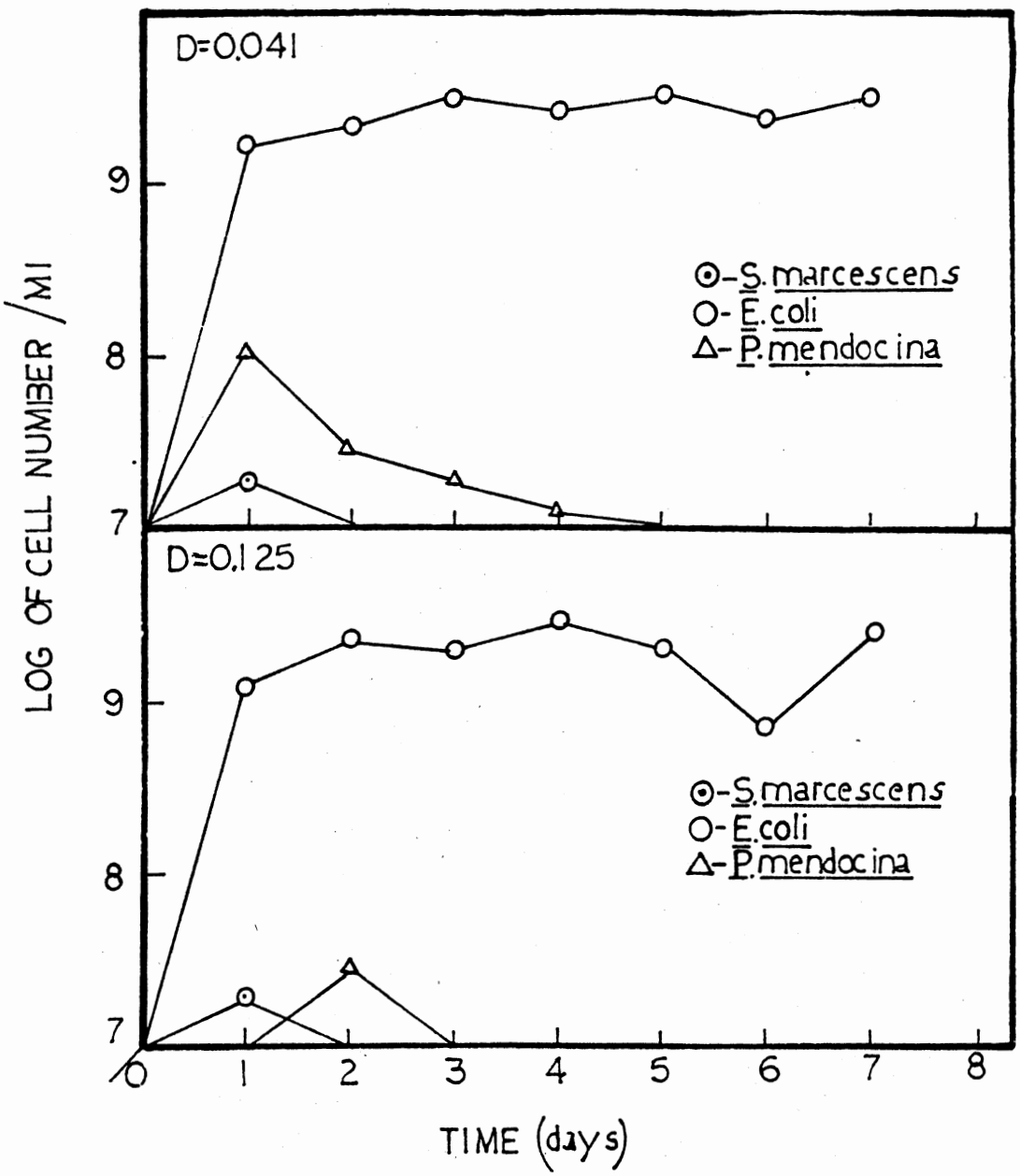
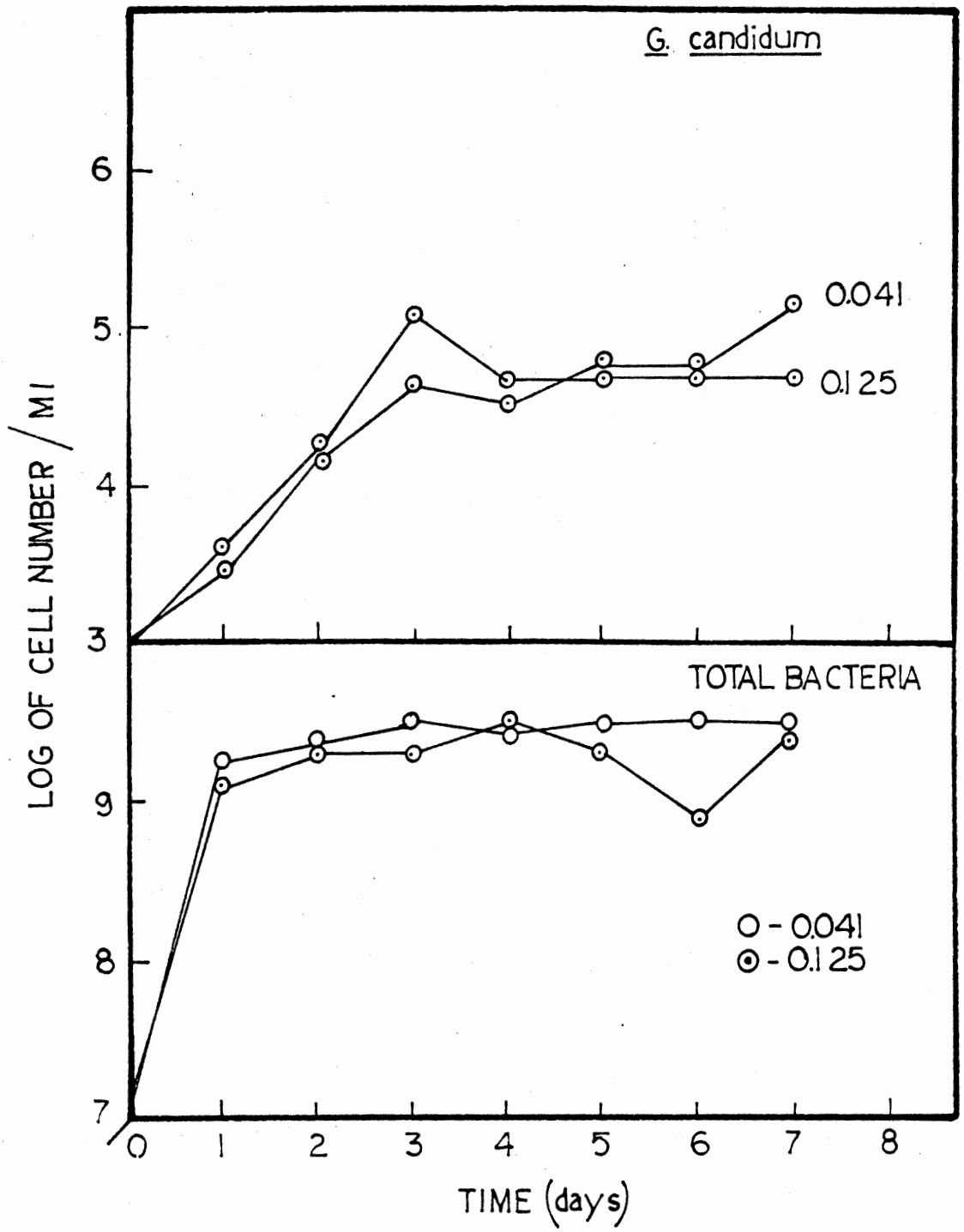

D=0.041
D=0.125
⊙-S. marcescens
○-E. coli
△-P. mendocina
LOG OF CELL NUMBER /ml
TIME (days)
7
8
9
0
1
2
3
4
5
6
7
8

Figure 34. Total Bacterial and Fungal Plate Counts in a Lactose-limited Continuous Flow Reactor at Dilution Rates of 0.041 and 0.125 hr^{-1} at 30C


G. candidum
0.041
0.125
TOTAL BACTERIA
O - 0.041
⊙ - 0.125
LOG OF CELL NUMBER / Ml
TIME (days)
6
5
4
3
9
8
7
0
1
2
3
4
5
6
7
8

dilution rate compared to the fast dilution rate. Surprisingly, counts for G. candidum at both dilution rates were moderately high. Furthermore, there were no indications that dilute-out was occurring during the time course of these experiments. Indeed, counts at 0.041 hr^{-1} were actually increasing at the termination of the experiment.

The batch data led to predictions that S. marcescens should predominate in a sucrose-limited continuous culture. Figure 35 showing such studies verifies these predictions as S. marcescens is shown to grow to the exclusion of the other two bacterial species. Total numbers of S. marcescens fell below those attained by E. coli in lactose and galactose-limited continuous cultures. Figure 36 shows that the total bacterial counts also fell below the levels obtained in comparable studies with lactose and galactose. Similar to the lactose studies, total bacterial counts were higher at a dilution rate of 0.041 than at 0.125 hr^{-1}. Examination of G. candidum counts also shows a similarity to those obtained in lactose. There was no tendency toward dilute-out at either dilution rate. Counts were moderately high for a dilution rate of 0.125 and somewhat less at 0.041. There also appeared to be oscillations in the daily plate counts particularly at the dilution rate of 0.125 hr^{-1}. This effect was not evident with other growth-limiting substrates.

Figures 37 and 38 present the bacterial predominance patterns, G. candidum plate counts and total bacterial counts for a sodium acetate-limited continuous culture grown at two different dilution rates. The initial high counts of S. marcescens and E. coli represented normal inoculum levels and appeared higher than previous experiments because the acetate experiments resulted in an overall decrease in total

Figure 35. Bacterial Predominance Patterns in a Mixed Culture Continuous Flow Reactor Containing 1000 mg/l Sucrose in Minimal Salts Medium at Dilution Rates of 0.041 and 0.125 hr^{-1} at 30C

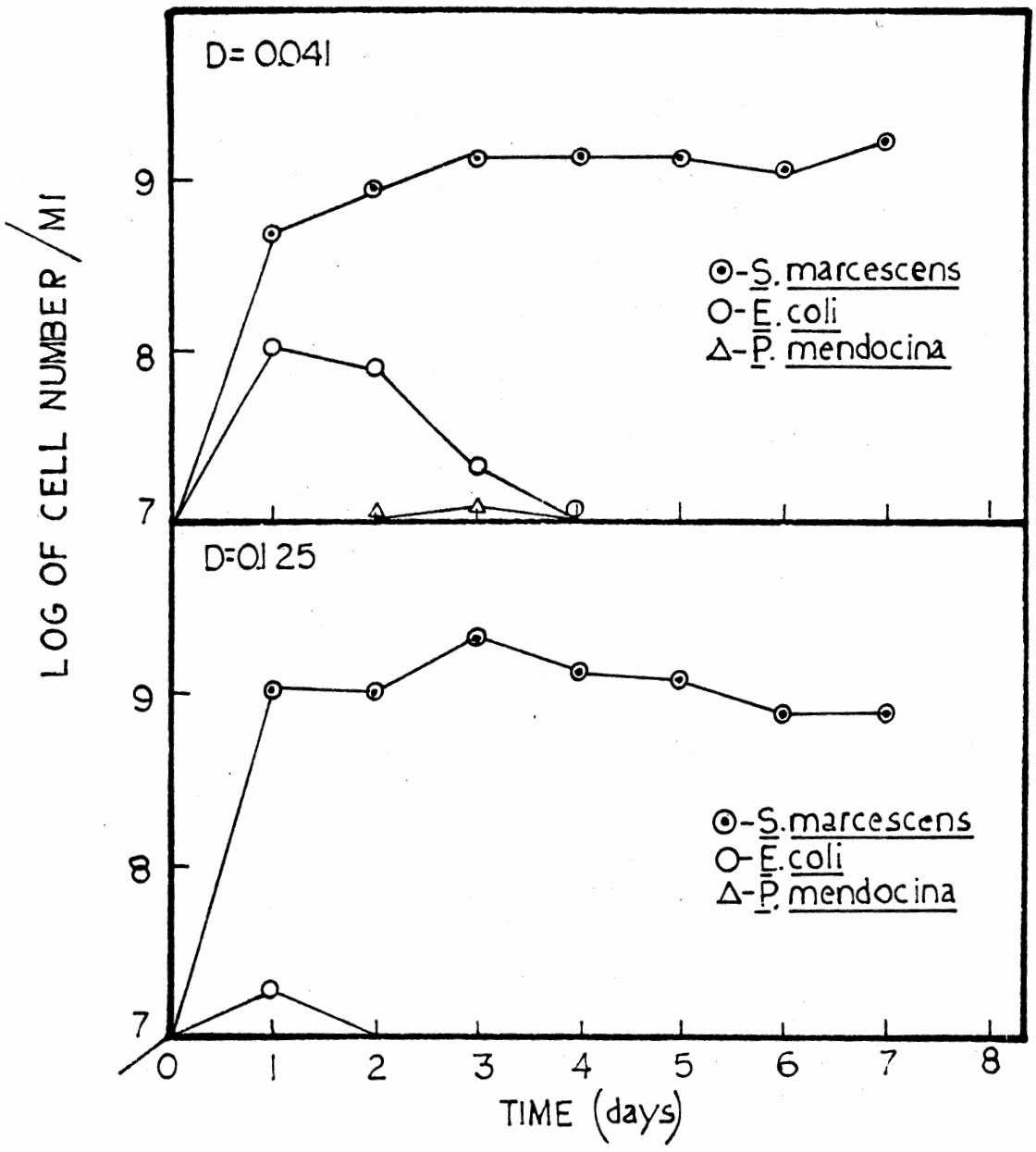

D= 0.041
D=0.125
⊙-S. marcescens
O-E. coli
Δ-P. mendocina
LOG OF CELL NUMBER / ML
TIME (days)
7
8
9
0
1
2
3
4
5
6
7
8

Figure 36. Total Bacterial and Fungal Plate Counts in a Sucrose-limited Continuous Flow Reactor at Dilution Rates of 0.041 and 0.125 hr^{-1} at 30C

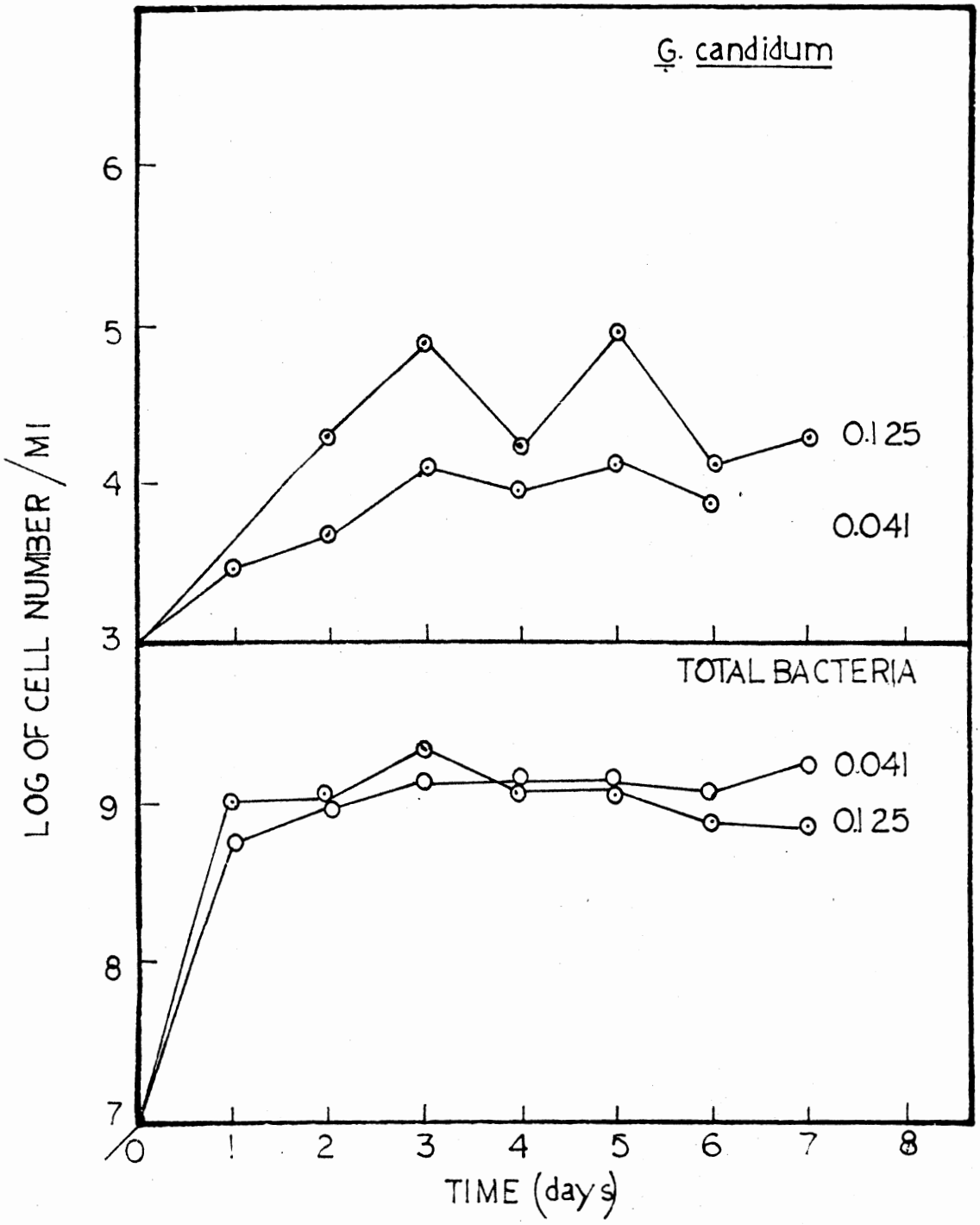

G. candidum
0.125
0.041
TOTAL BACTERIA
0.041
0.125
LOG OF CELL NUMBER / ML
3
4
5
6
7
8
9
0
1
2
3
4
5
6
7
8
TIME (days)

Figure 37. Bacterial Predominance Patterns in a Mixed Culture Continuous Flow Reactor Containing 1000 mg/l Sodium Acetate in Minimal Salts Medium at Dilution Rates of 0.041 and 0.125 hr^{-1} at 30C

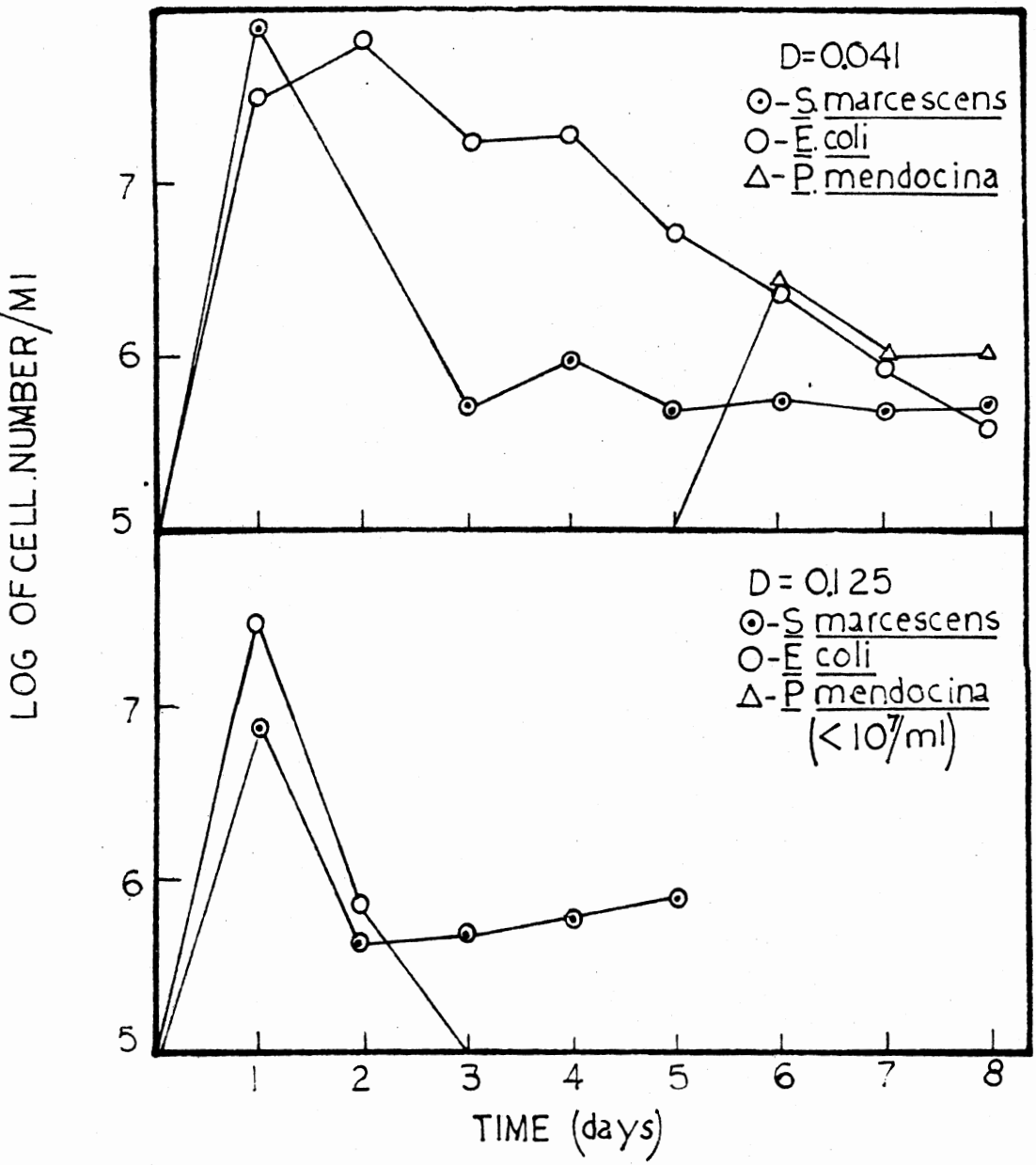
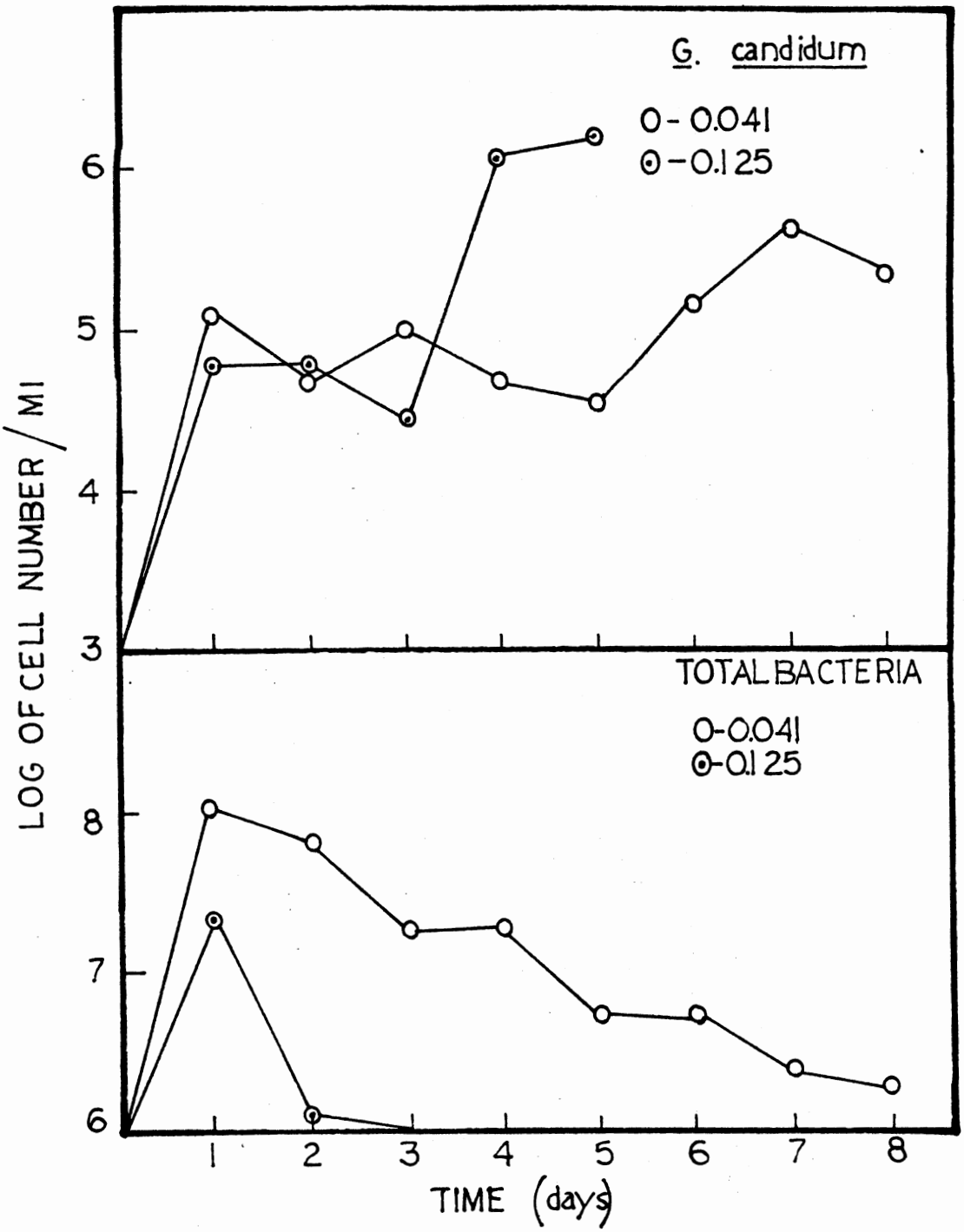

D=0.041
⊙-S. marcescens
O-E. coli
Δ-P. mendocina
D = 0.125
⊙-S marcescens
O-E coli
Δ-P mendocina
(<10⁷/ml)
LOG OF CELL NUMBER/ML
TIME (days)
5
6
7
1
2
3
4
5
6
7
8

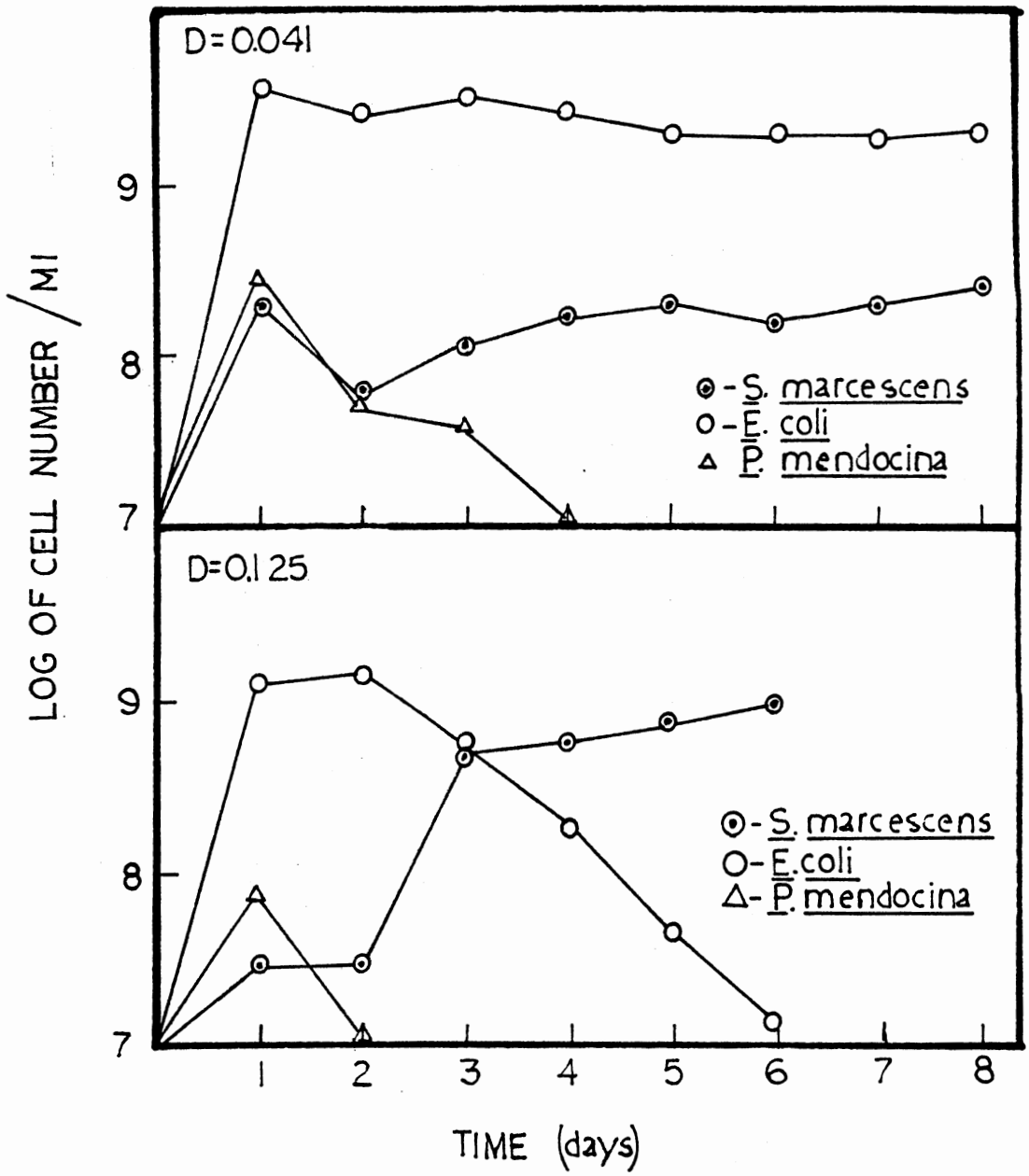
Figure 38. Total Bacterial and Fungal Plate Counts in a Sodium Acetate-limited Continuous Flow Reactor at Dilution Rates of 0.041 and 0.125 hr^{-1} at 30C


G. candidum
0 - 0.041
⊙ - 0.125
TOTAL BACTERIA
0-0.041
⊙-0.125
LOG OF CELL NUMBER / MI
TIME (days)
3
4
5
6
6
7
8
1
2
3
4
5
6
7
8

bacterial levels and subsequently graphic representation was on a level falling below that of the inoculum in contrast to previous experiments. Total bacterial counts never exceeded the inoculum counts. The individual bacterial counts were somewhat ambiguous in light of the data obtained in batch. These data showed that P. mendocina had a rapid growth rate and therefore might be expected to predominate easily in mixed culture. At a dilution rate of 0.041 hr^{-1} it ultimately predominated but only after a lag of five days. E. coli was steadily washed out of the culture and displayed no tendency to grow. S. marcescens was also washed out for two days but reached a lower level at which counts were remarkably constant for five days. When a dilution rate of 0.125 hr^{-1} was imposed, E. coli was quickly washed out of the culture and S. marcescens was again washed out for a short period before establishing a level of counts approximating that at 0.041 hr^{-1}. However, P. mendocina did not appear in detectible numbers at the faster dilution rate although its presence in the culture was demonstrated on a streak plate of the undiluted mixture. A comparison of total bacterial counts with G. candidum counts showed a pronounced tendency toward complete predominance by the fungus. Counts were quite high at both dilution rates and were highest at the point of minimum bacterial numbers. Rounded clumps of growth were much in evidence at both dilution rates throughout both experiments.

Figure 39 represents the bacterial predominance patterns for the fructose studies. A notable difference was evident for the cultures grown at two different dilution rates. At both dilution rates, P. mendocina was washed out of the system as expected, since it was unable to grow in batch fructose medium. S. marcescens, which possessed the

Figure 39. Bacterial Predominance Patterns in a Mixed Culture Continuous Flow Reactor Containing 1000 mg/l Fructose in Minimal Salts Medium at Dilution Rates of 0.041 and 0.125 hr^{-1} at 30C


D=0.041
D=0.125
LOG OF CELL NUMBER / Ml
TIME (days)
⊙-S. marcescens
O-E. coli
△ P. mendocina
⊙-S. marcescens
O-E.coli
△-P. mendocina
7
8
9
1
2
3
4
5
6
7
8

highest specific growth rate in batch predominated at the fast dilution rate. E. coli was initially present in high numbers but was systematically eliminated from the culture concomitant with increases in numbers of S. marcescens. At a dilution rate of 0.041 hr^{-1}, E. coli established itself as predominant member of the culture for the entire time course of the experiment spanning eight days. Counts remained quite constant for the length of the experiment. S. marcescens attained a level somewhat below that of E. coli, and slowly appeared to be increasing in numbers although E. coli remained constant for the latter four days of the experiment. Total bacterial counts, shown in Figure 40, were similar to those noted with lactose and sucrose where a dilution rate of 0.125 hr^{-1} yielded less cell numbers than a dilution rate of 0.041 hr^{-1}. G. candidum plate counts demonstrated a moderately high number at 0.125 hr^{-1} and a somewhat lower count at 0.041 hr^{-1}.

Table XII presents a summary of dry weights and percentage substrate utilization as a function of dilution rate. Immediately evident was the lower dry weights obtained without exception for each sugar at a dilution rate of 0.125 hr^{-1}. The difference is most striking when sucrose and fructose were used as limiting carbon and energy sources. The data for the acetate-limited culture show higher dry weights at 0.125 than 0.041 hr^{-1} in contrast to the sugar data. Substrate utilization was highest at the faster dilution rate for glucose, galactose, and lactose, and less for sucrose and likely fructose. The determination for fructose at 0.041 hr^{-1} was discarded by accident before recording the data but from visual examination alone it represented much more efficient utilization than at 0.125 hr^{-1}. The Neish method could

Figure 40. Total Bacterial and Fungal Plate Counts in a Fructose-limited Continuous Flow Reactor at Dilution Rates of 0.041 and 0.125 hr^{-1} at 30C

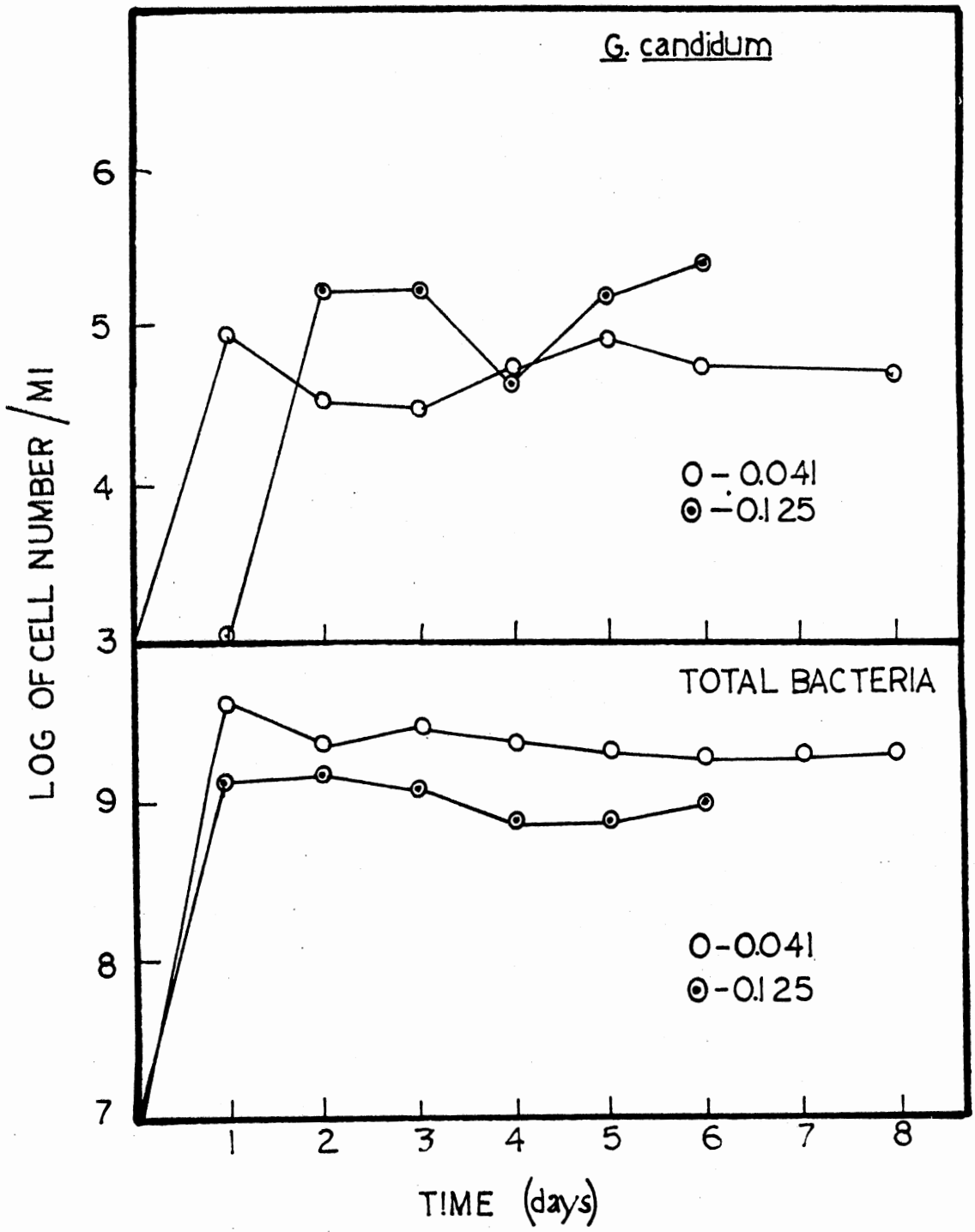

G. candidum
0 – 0.041
⊙ – 0.125
TOTAL BACTERIA
0-0.041
⊙-0.125
LOG OF CELL NUMBER /MI
TIME (days)
3
4
5
6
7
8
9
1
2
3
4
5
6
7
8

TABLE XII

COMPARISON OF MIXED CULTURE DRY WEIGHTS AND SUBSTRATE UTILIZATION AT TWO DILUTION RATES FOR VARIOUS CARBON AND ENERGY SOURCES

Carbon and Energy Source	Dilution Rate (hr^{-1})	Effluent Substrate (mg/l)	Dry Weight (mg/l)	Substrate Utilization (%)
Glucose	0.041	110	425	89
	0.125	100	367	90
Galactose	0.041	127	472	87
	0.125	54	435	95
Lactose	0.041	163	461	83
	0.125	73	420	92
Sucrose	0.041	109	333	89
	0.125	273	156	71
Fructose	0.041	-	420	-
	0.125	363	160	60
Acetate	0.041	-	58	-
	0.125	-	112	-

Substrate utilization was determined by the Neish method. This method could not be used for the determination of acetate.

not be used for acetate determination, resulting in no data for substrate utilization.

Table XIII emphasizes the tendencies of G. candidum to predominate as a function of carbon and energy source as well as dilution rate. Several indices of predomination were selected to provide more complete information as to the nature of predomination. Results were deemed positive if by visual observation clumps or flocs of growth were evident in the culture at any time. Plate counts were positive if they were greater than 10^6/ml. Counts on the order of 10^5/ml were defined as a tendency toward predomination and designated in the table as $\pm$. A fungal dry weight of greater than 50 percent of the culture weight was defined as predominance. A dry weight between 33-50 percent represented a tendency toward predomination. From the table it is apparent that only acetate and fructose-limited cultures allowed enough growth of G. candidum to satisfy any of the criteria for predomination. Although the presence of G. candidum could be demonstrated by plate counts in cultures limited by each of the other sugars, its numbers were less than 10^5/ml and represented a negligible fraction of the total dry weight. Acetate-limited cultures were clearly dominated by fungus. Visible, rounded clumps of growth were apparent after 24 hours of growth and persisted throughout the experiments. Plate count determinations yielded greater than 10^6/ml at 0.125 hr^{-1} and greater than 10^5/ml at 0.041 hr^{-1}. Both are undoubtedly underestimates, as many filaments and clumps were present at the time of plating. Estimates of dry weight yielded values of greater than 99 percent of total dry weight represented by G. candidum at both dilution rates. When the culture was limited by fructose, G. candidum tended to grow in small rounded clumps which

TABLE XIII

THE EVALUATION OF VARIOUS CARBON AND ENERGY SOURCES AS TO THEIR EFFECT UPON PREDOMINATION TENDENCIES OF G. CANDIDUM at 30C

Carbon and Energy Source	Dilution Rate (hr^{-1})	Index of Predomination		
		Visual Observation	Plate Count	Dry Weight
Glucose	0.041	-	-	-
	0.125	-	-	-
Galactose	0.041	-	-	-
	0.125	-	-	-
Lactose	0.041	-	-	-
	0.125	-	-	-
Sucrose	0.041	-	-	-
	0.125	-	-	-
Acetate	0.041	+	±	+
	0.125	+	+	+
Fructose	0.041	+	-	-
	0.125	+	±	-

Abbreviations: (+), positive for predomination; (±). tendency for predomination; (-), negative for predomination.

were visible after approximately two days of incubation. Concentration of these clumps never reached the high levels apparent in acetate-limited cultures. They were most obvious at a dilution rate of 0.125 hr^{-1} but occurred at both dilution rates. Neither plate counts nor dry weight estimations showed any tendency of G. candidum to predominate although plate counts at 0.125 hr^{-1} were slightly higher than 10^5/ml. This apparent anomaly is best explained by considering the manner of fungal growth in fructose-limited cultures. Since growth occurred primarily as small clumps in low density, both plate count determinations and dry weight estimations would tend to be underestimates if the sample taken for analysis did not include a representative amount of these growths.

pH Study

Figure 41 presents the bacterial and fungal predominance patterns for a mixed culture grown in lightly buffered glucose minimal medium at a dilution rate of 0.041 hr^{-1}. There is a continuous rise in G. candidum plate counts in contrast to a continuous wash-out of the bacteria. All indices of G. candidum predominance were met in this experiment. Heavy growth of the mold was clearly visible throughout the culture as well as on the air sparger. Plate counts reached levels greater than 10^6/ml and dry weight of the fungus represented greater than 99 percent of the entire culture. E. coli and P. mendocina were quickly eliminated from the culture, but S. marcescens required seven days for its reduction in numbers to an undetectible level. Figure 42 shows the pH changes occurring during the experiment. From an initial pH of 6.95, the pH quickly fell to 3.9 in 24 hours and slowly leveled off at 3.1

Figure 41. G. candidum Plate Counts and Bacterial Predominance Patterns in Lightly Buffered Glucose Minimal Medium at a Dilution Rate of 0.041 hr^{-1}

The medium contained 1000 mg/l glucose, 100 mg/l Na_2HPO_4 and 50 mg/l KH_2PO_4. The growth temperature was 30C.

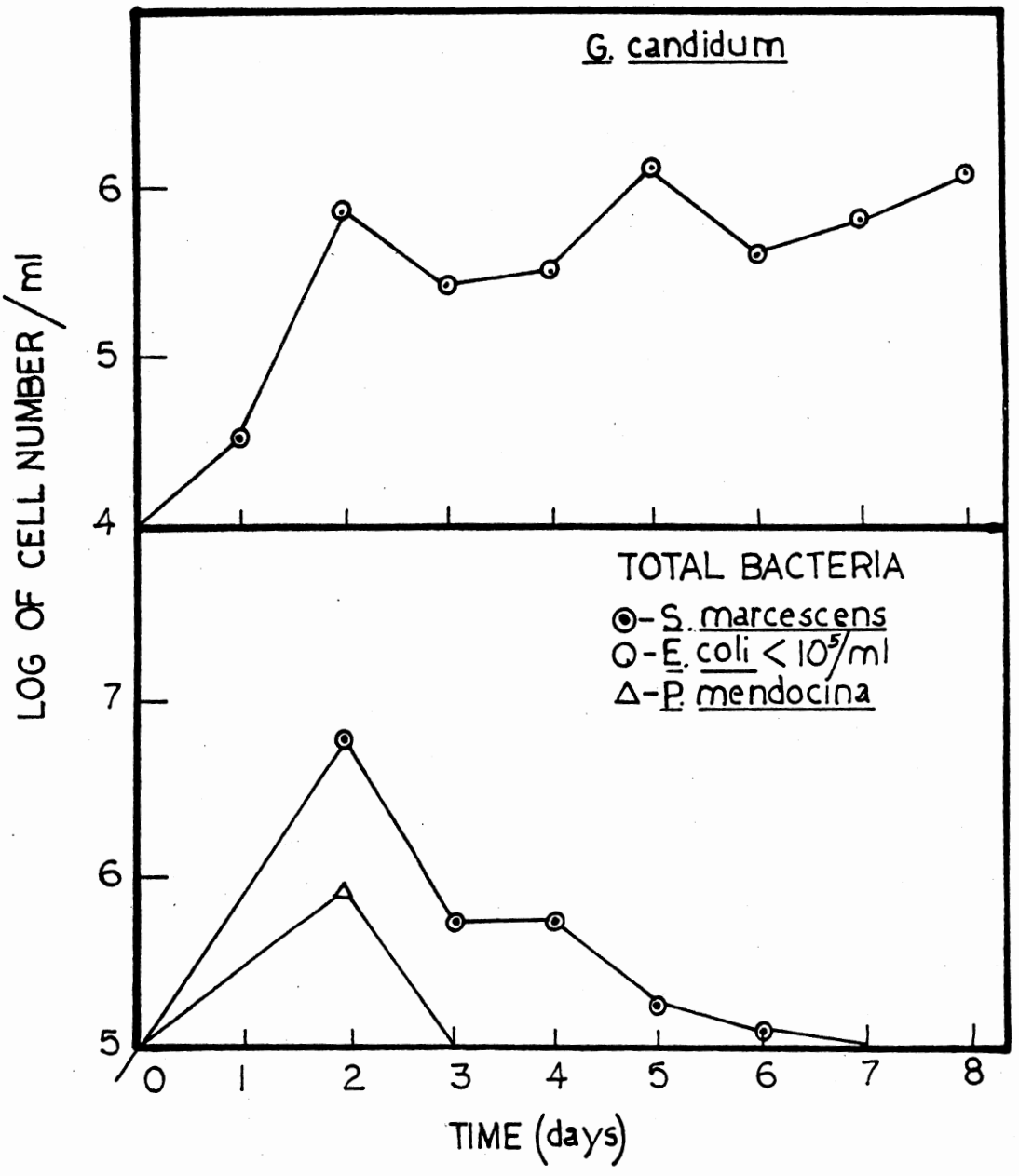
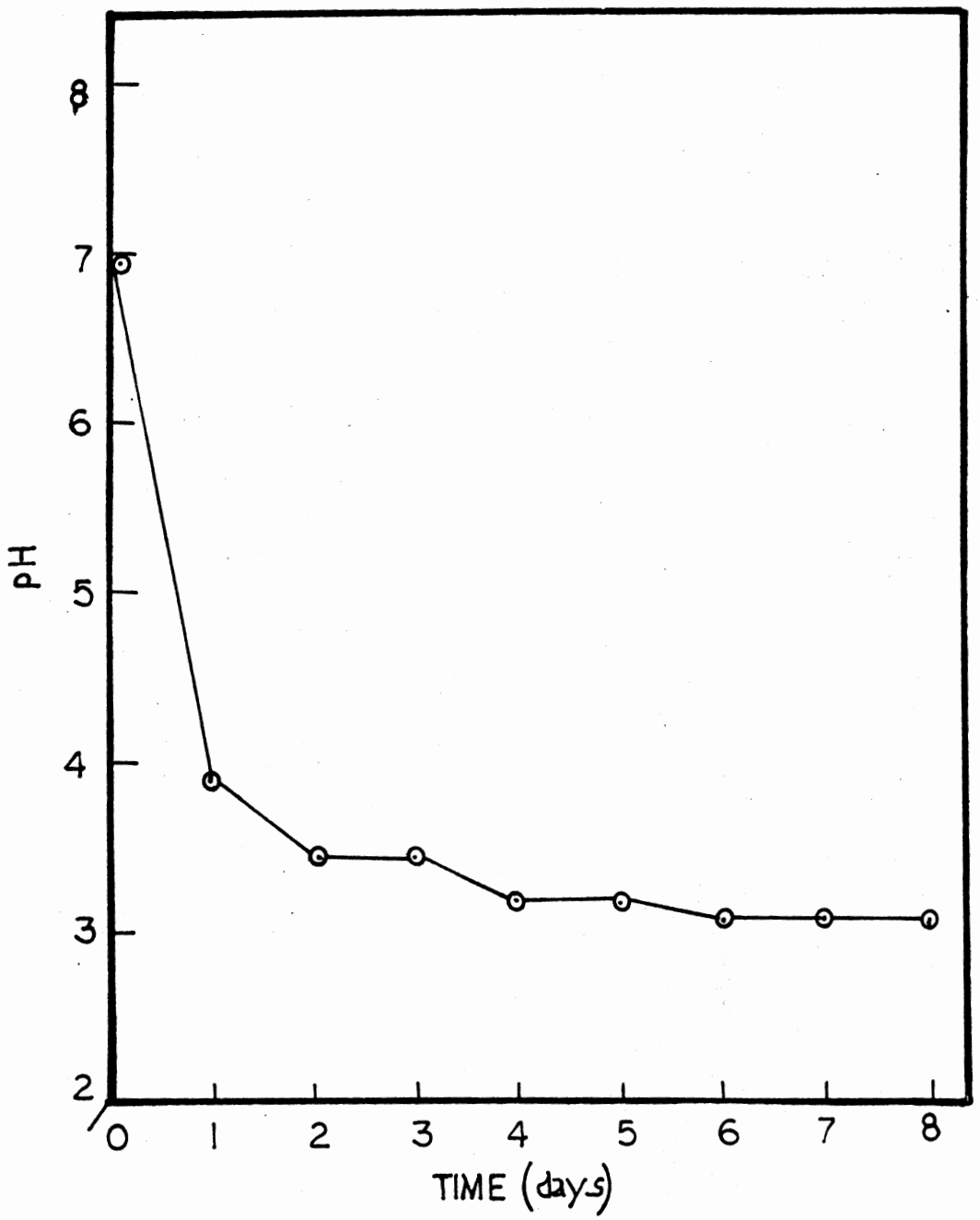

G. candidum
6
5
4
LOG OF CELL NUMBER / ml
TOTAL BACTERIA
⊙-S. marcescens
O-E. coli < 10⁵/ml
Δ-P. mendocina
7
6
5
0
1
2
3
4
5
6
7
8
TIME (days)

Figure 42. pH Changes in a Mixed Culture Grown in Lightly Buffered Glucose Minimal Medium at a Dilution Rate of 0.041 hr^{-1}

The medium contained 1000 mg/l of Glucose, 100 mg/l Na_2HPO_4 and 50 mg/l KH_2PO_4. The growth temperature was 30C.


8
7
6
5
4
3
2
pH
0
1
2
3
4
5
6
7
8
TIME (days)

for several days prior to the termination of the experiment. Substrate removal at this pH was 74 percent of the initial organic matter. Of the remaining organic matter, virtually none was glucose as determined by the Glucostat method. Dry weight represented primarily by G. candidum was 153 mg/l.

Glucose Concentration Studies

A series of glucose concentrations were tested for their effect upon the predominance patterns of the mixed culture when grown at dilution rates of 0.041 hr^{-1} and 0.125 hr^{-1}. As demonstrated earlier in the temperature and nitrogen studies, S. marcescens quickly predominated when glucose was the limiting nutrient at a concentration of 1000 mg/l. When glucose concentration was decreased to 500 mg/l, the results at a dilution rate of 0.125 hr^{-1} were similar to those at 1000 mg/l as shown in Figure 43. S. marcescens was quickly the predominating species. However, as further illustrated by the figure, at a dilution rate of 0.041, E. coli became the predominant organism. S. marcescens was never completely eliminated from the culture, but was substantially reduced in numbers compared to E. coli. P. mendocina at both dilution rates was quickly eliminated from the mixed culture. Figure 44 shows that both total bacterial and G. candidum counts were higher at the faster dilution rate. Total counts of G. candidum were moderately higher for both dilution rates than in corresponding studies at a concentration of 1000 mg/l.

Figure 45 shows the effect of decreasing the glucose concentration to 250 mg/l. In contrast to the results obtained at 500 mg/l, E. coli predominated at both dilution rates. However, S. marcescens was

Figure 43. Bacterial Predominance Patterns in a Glucose-limited Continuous Culture Containing 500 mg/l Glucose in a Minimal Salts Medium for Dilution Rates of 0.041 and 0.125 hr^{-1} at 30C

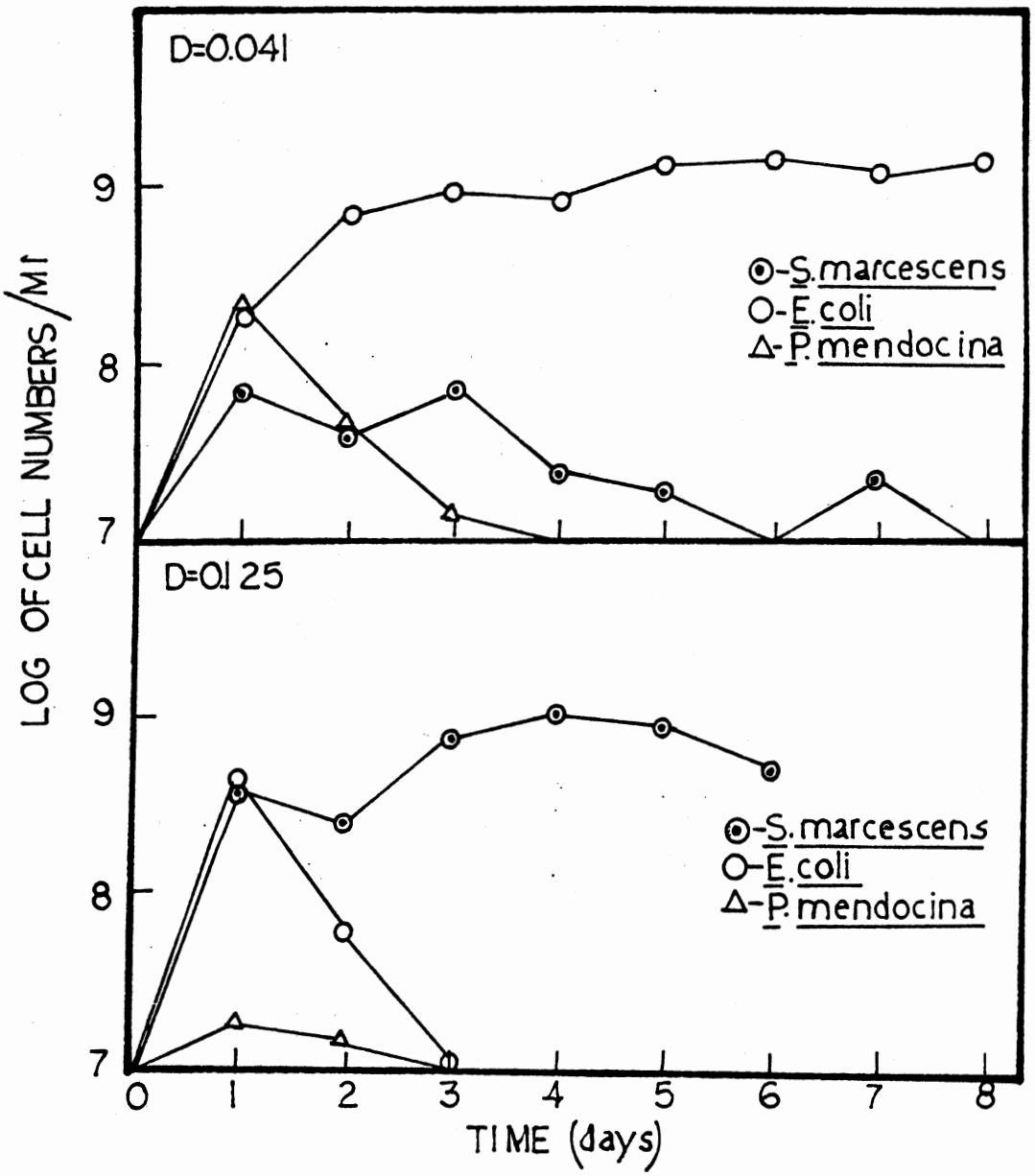

D=0.041
D=0.125
⊙-S. marcescens
O-E. coli
Δ-P. mendocina
LOG OF CELL NUMBERS/ML
TIME (days)
7
8
9
0
1
2
3
4
5
6
7
8

Figure 44. Total Bacterial and Fungal Counts in a Glucose-limited Continuous Culture Containing 500 mg/l Glucose in a Minimal Salts Medium for Dilution Rates of 0.041 and 0.125 hr^{-1} at 30C

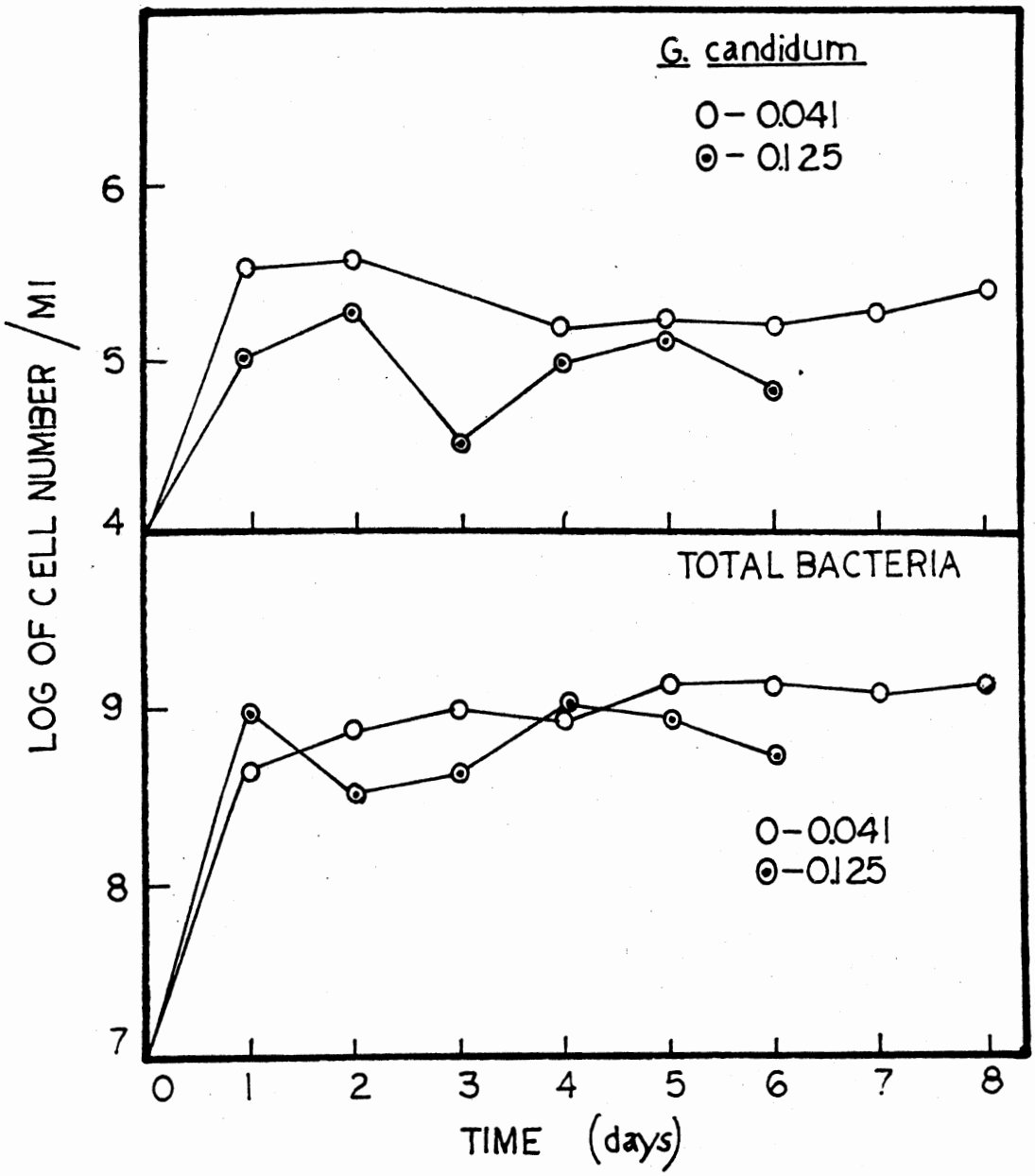
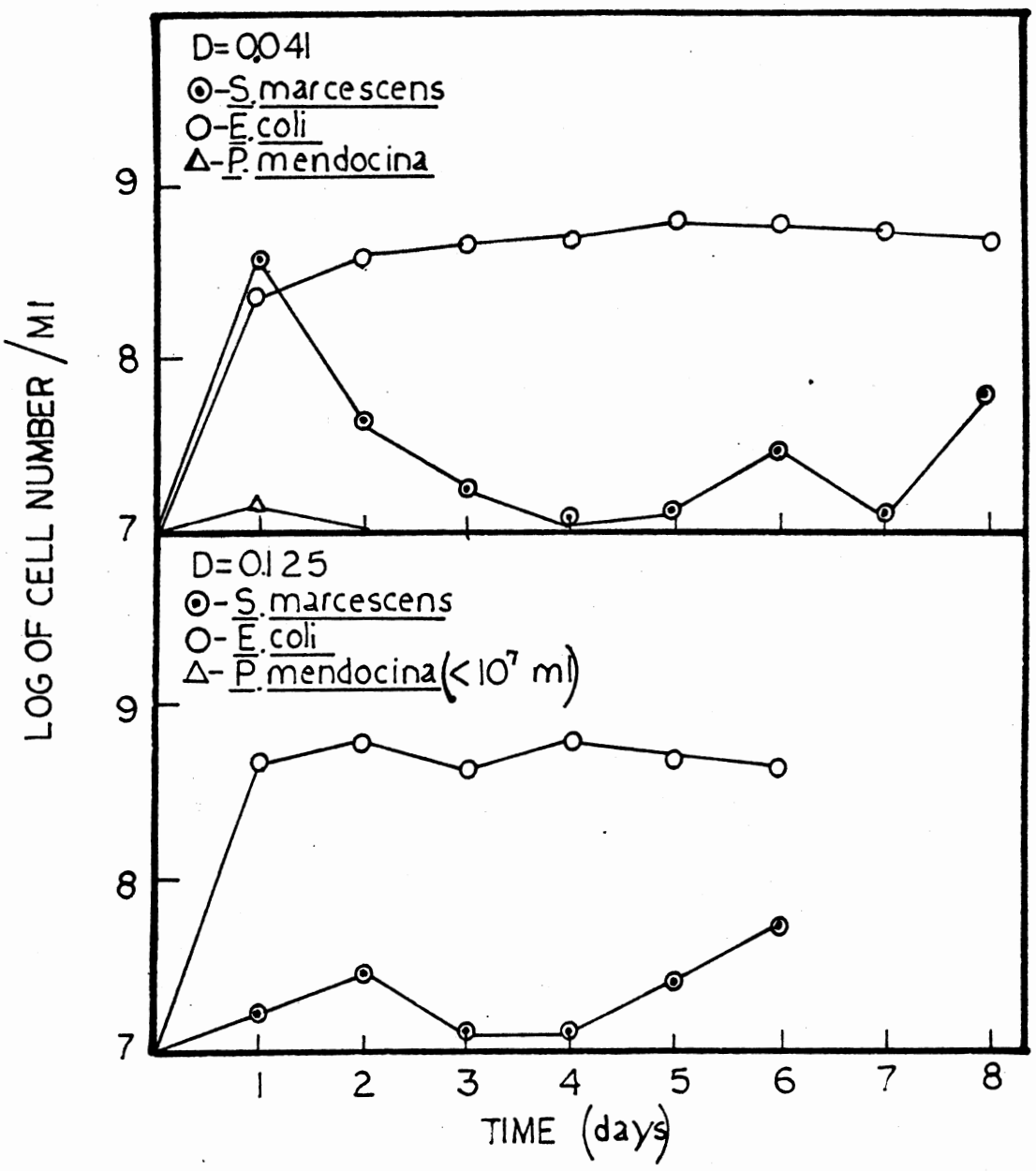

G. candidum
O - 0.041
⊙ - 0.125
TOTAL BACTERIA
O - 0.041
⊙ - 0.125
LOG OF CELL NUMBER / ML
4
5
6
7
8
9
0
1
2
3
4
5
6
7
8
TIME (days)

Figure 45. Bacterial Predominance Patterns in a Glucose-limited Continuous Culture Containing 250 mg/l Glucose in a Minimal Salts Medium for Dilution Rates of 0.041 and 0.125 hr^{-1} at 30C


D=0.041
⊙-S. marcescens
O-E. coli
Δ-P. mendocina
D=0.125
⊙-S. marcescens
O-E. coli
Δ-P. mendocina (< 10⁷ ml)
LOG OF CELL NUMBER / ML
9
8
7
1
2
3
4
5
6
7
8
TIME (days)

present continuously in both cultures throughout the experiments. At both dilution rates, most notably at 0.125 hr^{-1}, it expressed some tendency to increase in numbers at the expense of E. coli although within the experimental time course, E. coli continued to predominate. P. mendocina provided no competition at either dilution rate. Figure 46 shows that the bacterial counts were approximately equal for the two dilution rates but fungal counts were higher at the faster dilution rate at comparable times near the end of the experiments.

When glucose concentration was reduced to 100 mg/l, E. coli was again the predominant species at both dilution rates, as shown in Figure 47. S. marcescens was much less competitive at the slow dilution rate compared to its competitiveness at 0.125 hr^{-1}. It showed no tendency to affect E. coli levels as was noted in previous studies at higher glucose concentrations. However, when the experiment was run at the fast dilution rate, S. marcescens definitely expressed strong tendencies to displace E. coli. Indeed, based upon the plate count data presented in Figure 47, had the experiment been carried another day further, S. marcescens would likely have predominated. As in previous experiments, P. mendocina was of no consequence from a competition standpoint. Figure 48 shows total bacterial counts were higher at the fast dilution rate, and fungal counts were rather variable at both rates.

Table XIV summarizes data from the preceding experiments concerning substrate utilization, dry weights and proportion of dry weight represented by G. candidum. Dry weights were higher at slow dilution rates for both 500 and 1000 mg/l glucose. At glucose concentrations of 250 and 100 mg/l, the dry weights for the two dilution rates were

Figure 46. Total Bacterial and Fungal Counts in a Glucose-limited Continuous Culture Containing 250 mg/l Glucose in a Minimal Salts Medium for Dilution Rates of 0.041 and 0.125 hr^{-1} at 30C

Figure 46. Total Bacterial and Fungal Counts in a Glucose-limited Continuous Culture Containing 250 mg/l Glucose in a Minimal Salts Medium for Dilution Rates of 0.041 and 0.125 hr^{-1} at 30C

Figure 47. Bacterial Predominance Patterns in a Glucose-limited Continuous Culture Containing 100 mg/l Glucose in a Minimal Salts Medium for Dilution Rates of 0.041 and 0.125 hr^{-1} at 30C

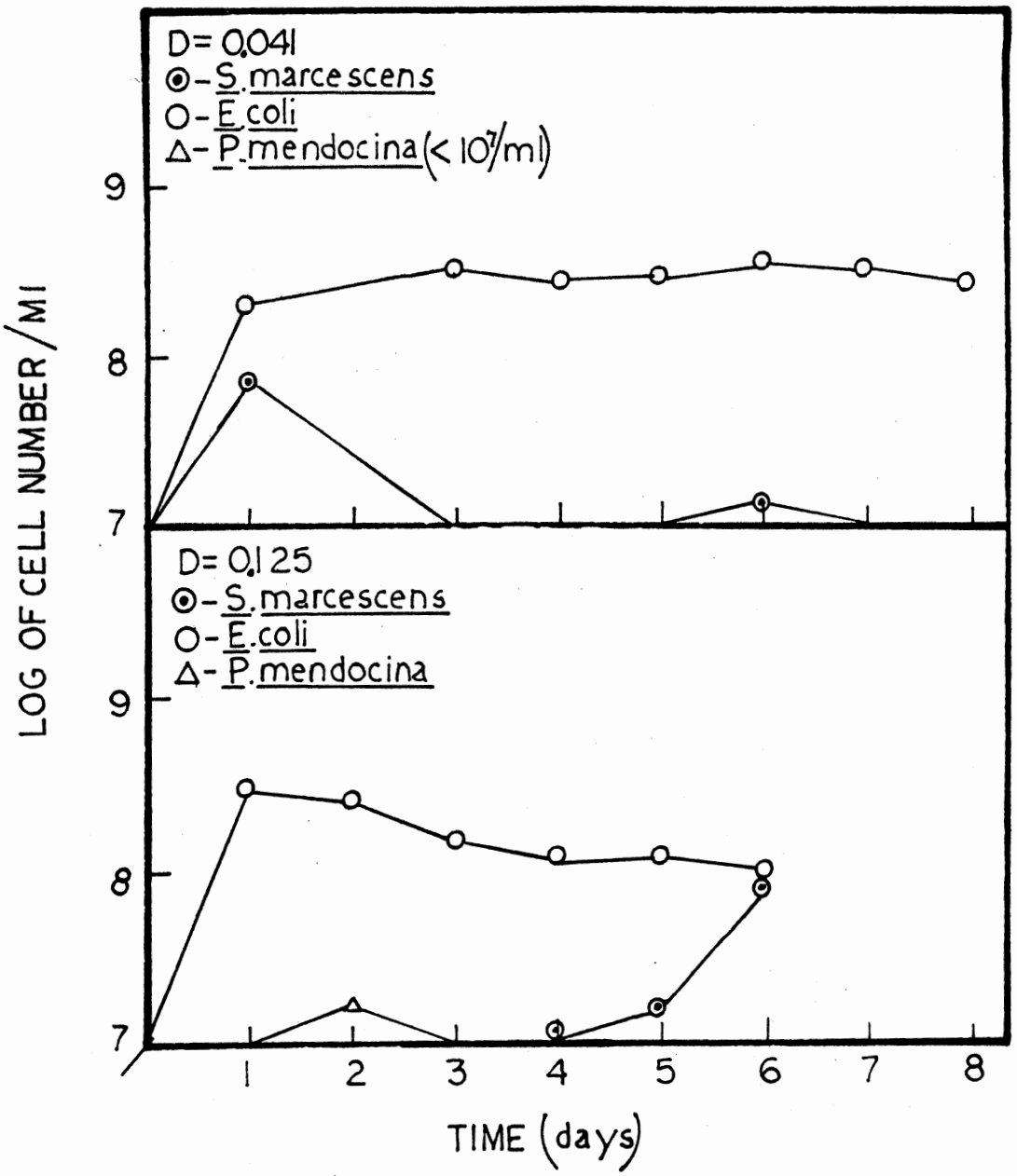

D= 0.041
⊙- S. marcescens
O- E. coli
Δ- P. mendocina (< 10⁷/ml)
D= 0.125
⊙- S. marcescens
O- E. coli
Δ- P. mendocina
LOG OF CELL NUMBER / ML
9
8
7
1
2
3
4
5
6
7
8
TIME (days)

Figure 48. Total Bacterial and Fungal Counts in a Glucose-limited Continuous Culture Containing 100 mg/l Glucose in a Minimal Salts Medium for Dilution Rates of 0.041 and 0.125 hr^{-1} at 30C

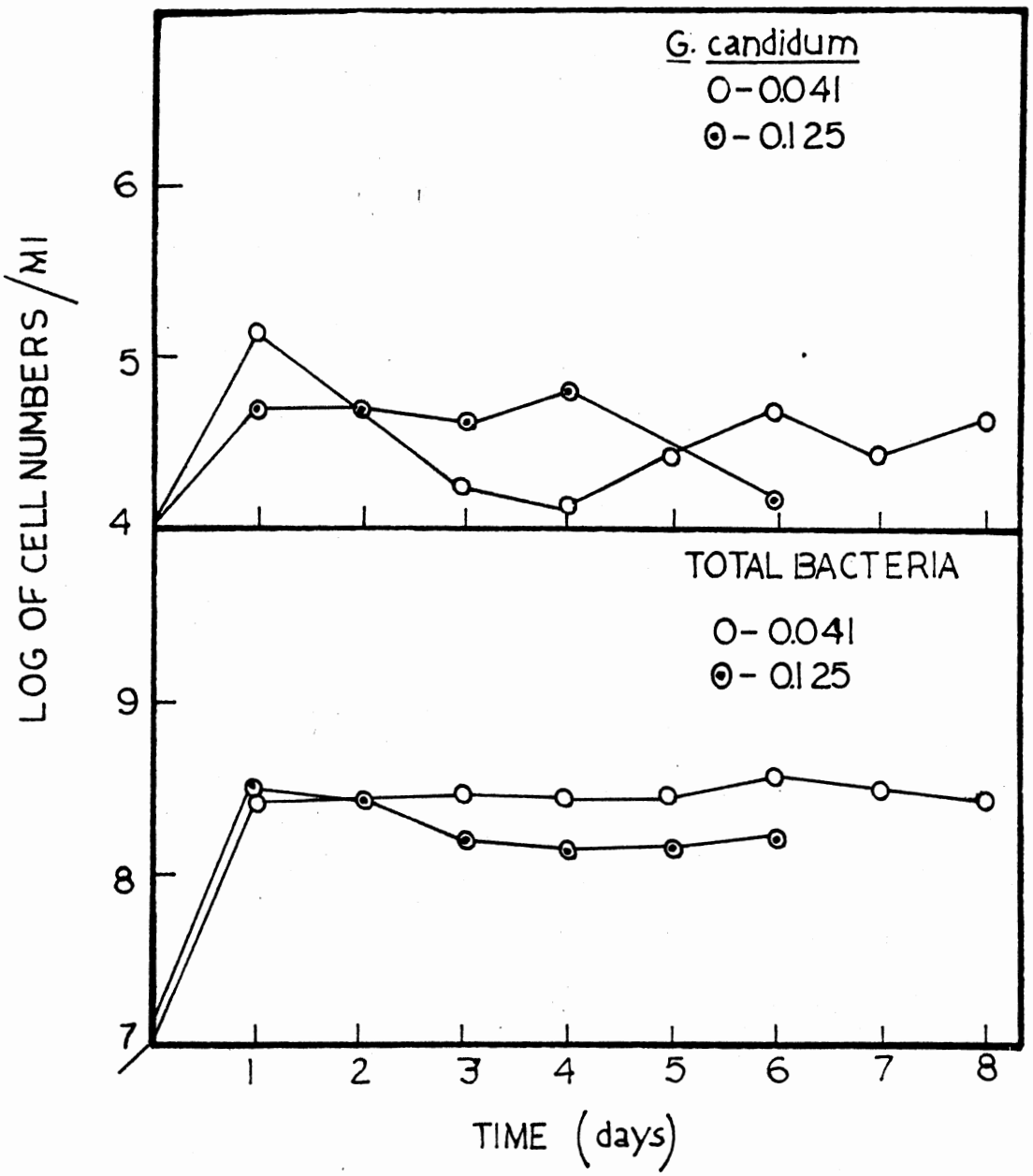

G. candidum
O-0.041
⊙-0.125
TOTAL BACTERIA
O-0.041
⊙-0.125
LOG OF CELL NUMBERS /ML
6
5
4
9
8
7
1
2
3
4
5
6
7
8
TIME (days)

TABLE XIV

SUMMARY OF DATA COLLECTED AT VARIOUS INITIAL GLUCOSE CONCENTRATIONS DURING CONTINUOUS FLOW EXPERIMENTS AT TWO DIFFERENT DILUTION RATES

Glucose Concentration (mg/l)	Dilution Rate (hr^{-1})	Total Dry Weight (mg/l)	Effluent Substrate (mg/l)	Substrate Utilization (%)	Fungus (%)
1000	0.041	425	110	89	-
	0.125	367	100	90	20
500	0.041	295	32	93	-
	0.125	169	45	90	35
250	0.041	116	36	85	5
	0.125	123	37	83	15
100	0.041	68	27	74	18
	0.125	67	27	73	49

Abbreviation: (-), the amount present represented a negligible proportion of total dry weight.

The percentage fungus present was calculated from the difference between the total mixed culture dry weight and an estimate for bacterial dry weight. The percentage substrate utilization was determined by the Neish method.

very nearly the same. Substrate utilization appeared to be most efficient at the higher glucose concentrations. When 100 mg/l of glucose was used, utilization was only 73-74 percent. Glucose utilization was virtually complete in all experiments as demonstrated with the Glucostat method of glucose determination.

Macroscopically visible growth of G. candidum never occurred in any of the preceding experiments. However, presence of the fungus could be demonstrated in every experiment by plate count determinations. Levels never reached the predominance index of 10^6/ml, but the 10^5/ml level was reached when glucose concentration was 500 mg/l. An interesting and fairly consistent result was obtained upon calculating percentage of total dry weight represented by G. candidum. This fungus seemed to compete most favorably on a dry weight basis at the higher dilution rate. Except for the somewhat low value obtained with 250 mg/l glucose, the data also yielded an inverse relationship between glucose concentration and percentage of the total dry weight represented by G. candidum. For a dilution rate of 0.125 hr^{-1}, the percentage of G. candidum rose from 20 percent at 1000 mg/l to 49 percent at 100 mg/l. The effect was much less obvious at a dilution rate of 0.041 hr^{-1} where competition by the fungus was very low. A negligible dry weight was obtained at glucose concentrations of 1000 and 500 mg/l. A small increase to 5 percent occurred at 250 mg/l glucose, and a further increase to 18 percent was noted at 100 mg/l.

A final experiment involving glucose concentration was begun with 50 mg/l. Plate counts were taken over three days to establish bacterial predominance patterns at a dilution rate of 0.041 hr^{-1}. On the third day, a sterile glucose solution was added to the feed reservoir

VITA

Crosby Wilson Jones, Jr.

Candidate for the Degree of

Doctor of Philosophy

Thesis: FACTORS INFLUENCING THE PREDOMINANCE PATTERNS OF MICROORGANISMS GROWN AS A DEFINED HETEROGENEOUS POPULATION IN CONTINUOUS CULTURE

Major Field: Microbiology

Biographical:

Personal Data: Born at Claremore, Oklahoma, on August 1, 1950, the son of Crosby Wilson Jones, Sr. and Mary Jane Jones.

Education: Graduated from Claremore High School, Claremore, Oklahoma, in 1968; received the Bachelor of Science degree in Microbiology from Oklahoma State University, Stillwater, Oklahoma, in May, 1972; received the Master of Science degree from Oklahoma State University in December, 1974; completed requirements for the Doctor of Philosophy degree at Oklahoma State University in December, 1977.

Professional Experience: Graduate Teaching Assistant, Department of Microbiology and School of Biological Sciences, Oklahoma State University, September, 1972, to present; Environmental Protection Agency Bioengineer Trainee, Department of Civil Engineering, Oklahoma State University, June, 1973, to present.