STUDIES ON THE PHYSIOLOGY OF HETEROGENEOUS BACTERIAL POPULATIONS GENERATED IN A CHEMOSTAT

by

GORDON W. MOFFATT

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To My Wife Sherratt,

and

My Mother

In Memory of My Father

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ABSTRACT

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The physiological potential of heterogeneous microbial populations grown in laboratory chemostats was studied with respect to the development of a biodegradation potential screening test. It is envisaged this test would involve challenging resting cell suspensions prepared from the chemostat cultures with test substrates and following their subsequent transformation. Three methods were evaluated as parameters for monitoring the substrate stabilization, namely, following residual substrate in the suspension, measuring the rate of O_2 consumption and measuring the rate of CO_2 production. Three different nutritional formulations, (1) the chemically-undefined commercially available medium, peptonized milk, (2) the chemically-defined medium C of Sundman and Carlberg, and (3) the semi-chemically-defined synthetic sewage medium of James, were studied with respect to their ability to generate in the chemostat mixed microbial populations possessing the broadest physiological spectrum. The substrates utilized in this investigation included: a number of simple organics - acetate, pyruvate and butyrate, a more complex aromatic possessing toxic properties - phenol, and four synthetic organic anionic surfactants - linear alkylbenzene sulfonate, coconut alcohol ethoxylate sulfate, tallow alcohol sulfate and tallow ethoxylate sulfate.

The peptonized milk medium proved to be superior to both medium C and synthetic sewage for generating populations with broad physiological capacities. Peptonized milk-grown cells possessed the ability to degrade all of the above substrates with the exception of tallow

alcohol sulfate and tallow alcohol ethoxylate sulfate. Medium C-grown cells were capable of metabolizing all of the natural organic substrates and linear alkylbenzene sulfonate but lacked the ability to stabilize the latter three surfactants. However, the rates were generally much slower than those obtained in experiments exploiting peptonized milk-grown cells. The populations generated on synthetic sewage degraded only the natural organic substrates.

Enrichment of the medium with the test substrate generally increased the response of the populations when challenged with that substrate. It also resulted in a reduction of the substrate inhibition which occurred in tests utilizing the substrates butyrate and phenol. An interesting secondary observation of the enrichment experiments was a measurable decline in the K_m of the metabolism of most of the substrates. This was attributed to selection in the mixed culture for species which could more efficiently utilize the test substrate thereby possessing a competitive advantage. These results demonstrate that the physiological potential of the mixed culture is definitely affected by prior conditioning.

Direct analysis of residual substrate proved to be the superior method for measuring the stabilization activity of the populations as compared to the indirect methods of measuring O_2 consumption or CO_2 production.

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INTRODUCTION

INTRODUCTION

The increased use of commercially available products which contain recalcitrant synthetic organic compounds has placed a great deal of stress on our environment. The ecological hazard created by these compounds and their residues as a result of their resistance to degradation and resulting persistence in the environment are well documented (2, 47). For this reason it is of the utmost importance to limit the widespread use and distribution of recalcitrant chemicals. However, before such controls can be implemented standard methods must be developed to screen new and existing chemicals for their biodegradation potentials.

In this light numerous studies (1, 8, 28, 34, 53, 54, 55, 58, 83, 89, 92, 95, 98, 104, 105, 106) of the biodegradation potentials of certain recalcitrant molecules have been undertaken. These tests involve field tests or laboratory experiments which utilize naturally occurring microbial populations as the source of active biomass. Two of the principal limitations of the majority of these tests are the absence of a uniform heterogeneous microbial population and the time required to achieve measurable results. As a possible solution to these technical problems it was decided to exploit laboratory-grown heterogeneous bacterial populations generated in a chemostat. Such a procedure would provide large volumes of biomass with high cell density and constant physiological potential.

Rerie (80) simultaneous with this investigation demonstrated it was possible to generate heterogeneous populations in laboratory chemostat cultures, however, a true steady-state population was not

realized. Taylor and leB Williams (96) in a theoretical analysis concluded growth of stable mixed populations in the chemostat is, in theory, possible. Therefore, an extension of Rerie's work is required in order to determine the conditions necessary to promote steady-state growth of heterogeneous populations.

The primary objective of this investigation was to assay the physiological capacities of the heterogeneous populations developed by Rerie (80) in order to ascertain the extent of their physiological potential. In addition the effect of enriching the growth medium with the test substrate was studied to determine if it was possible to select populations which were more readily able to metabolize the enriching substrate.

HISTORICAL

HISTORICAL

With increasing frequency environmental protection agencies are facing pollution problems resulting from the release into the environment of recalcitrant synthetic organic compounds (82). The ecological hazards created by these compounds and their residues are well documented in the literature (2, 36, 47). Their resistance to degradation and their persistence in the environment has also been widely reported (1, 28, 52, 53, 54, 55, 95, 104, 105, 106). Fleming and Maines (28) found an average of 44% residual DDT in soils 8 years after the initial application. Lichtenstein (55) recovered 14.3% of initial DDT concentration in soils after 11 years. More than 50% Dieldrin, Lindane and DDT have been recovered from the soils after 6 years (83). Residual Chlordane and Lindane were recovered from soils 11 years after their application (54). For this reason considerable research over the past two decades has been directed toward the development of new methods to screen chemicals for their recalcitrant properties. Currently, however, no generally accepted universal screening method exists although several methods have been proposed.

Hammerton (34) studying the degradation of selected anionic detergents suggested a river water die-away test in which the detergent, added directly to a river water sample, was incubated in a static position at room temperature. The time required to bring about complete degradation varied with detergent up to approximately 280 days. Swisher (92) using this method compared the

biodegradability potentials of a number of alkylbenzene sulfonate homologs. The maximum rate of biodegradation occurred for straight-chain homologs of 10 to 12 carbon atoms. However, the time required to complete degradation varied with homolog from 10 to 30 days.

Bunch and Chambers (9) in their study of the biodegradation of anionic and nonionic surfactants and various phenols modified the method of Hammerton (34). Rather than using river water as a source of active biomass they employed 10% settled sewage in biochemical oxygen demand (BOD) dilution water containing 50 mg/l yeast extract and 20 mg/l test compound. Flasks were incubated at room temperature in a static position and subcultured weekly to promote adaptation of the sewage microflora to the test compound. The degree of degradation varied with substrate from a minimum of 0% to a maximum of 99.5% removal over the third seven day incubation period. This method also provided substantial information with respect to the time required for the cultures to adapt to the test detergent.

Ludzack *et al.* (58) developed a river water die-away test to monitor the biodegradation of organic cyanides. Since no chemical analysis existed at the time to accurately measure nitrile concentration in water, they used an indirect method involving CO_2 production to follow degradation. Dry, CO_2-free air was fed into a 5-gallon oxidation unit containing river water and the substrate. CO_2 produced by oxidation of the nitriles was flushed from the system by the air-flow and absorbed by a train of $Ba(OH)_2$ scrubbers. These samples were analyzed and the data plotted to represent cumulative CO_2, less control, as % theoretical CO_2 possible after

complete oxidation vs time.

The method of Ludzack *et al*. (58) was modified by Thompson and Duthie (98) who studied the biodegradation of trisodium nitrilotriacetate (NTA). Settled raw sewage diluted in BOD dilution water was used rather than a natural river water sample as a source of active biomass. In addition to monitoring cumulative CO_2 production they also followed NTA disappearance and inorganic N production to confirm degradation. Complete breakdown of the NTA required 25 days under these conditions.

Sturm (89) modified the method of Thompson and Duthie (98) to study the biodegradation of nonionic surfactants. The oxidation unit was scaled down from 20 litres to 6 litres and a 14-day detergent-acclimated sewage-derived culture diluted in BOD dilution water was used as the inoculum. Acclimation was achieved by incubating a 10% settled raw sewage sample in BOD water containing 50 mg/l yeast extract and 20 mg/l test detergent. The cumulative CO_2 produced over a 30-day period showed the degradation varied with detergent from approximately 5% to 100%.

Radiotracer methodology has also been used in biodegradation studies. Beam and Perry (5, 6) studying the biodegradation of cycloparaffinic hydrocarbons showed production of $^{14}CO_2$ from ^{14}C-labelled hydrocarbons by soil and marine mud samples and a number of pure bacterial cultures.

Some researchers have devised scale model or bench top activated sludge plants to study the ability of laboratory-grown activated sludge to break down various complex organic compounds in the hope the data would be used to predict the fate of these compounds in water

treatment processes. Sweeney and Foote (91) used model activated sludge plants fed continuously with domestic sewage spiked with detergents to study surfactant biodegradation. In 150 individual tests on a variety of surfactants they found residual detergent levels varied from 0.2% to 80% of the initial concentration. Swisher *et al.* (93) studying the biodegradation of NTA used both semicontinuous and continuous-flow activated sludge units. They found substantially complete degradation of 20 to 500 mg/l NTA in 24-hour cycle semicontinuous-flow systems and 3- and 6-hour retention time continuous-flow units. Young *et al.* (108) used a semicontinuous-flow system inoculated with settled raw sewage to study the biodegradation of selected aliphatic, aromatic and nitrogenous compounds. Biodegradation was monitored by O_2 consumption, chemical oxygen demand (COD), and total organic carbon (TOC) analysis of the culture effluent.

In order to shorten the time required to appraise biodegradability potentials some researchers have turned to the use of resting cell suspensions as a source of active biomass. These non-proliferating suspensions are capable of much more rapid degradation simply because of the high cell densities employed. Carlson and Polkowski (12) were perhaps the first to use resting cell suspensions in biodegradation experiments. They used suspensions prepared from activated sludge to demonstrate the biodegradation of amino acids. Both O_2 uptake and substrate disappearance were monitored as indices of breakdown. Prakasam and Dondero (78) compared the breakdown of eleven aromatic compounds using resing cell suspensions prepared from activated sludge, laboratory-grown activated sludge and

substrate-acclimated laboratory-grown activated sludge. Ishaque (43) characterizing the physiological capacity of a sewage lagoon showed resting cell suspensions of lagoon bacteria degrade a number of naturally occurring organic compounds including; glucose, acetate, palmitate, amino acids, egg albumin, urea, creatinine and some synthetic organic compounds including a few detergents and some organophosphorus and chlorinated insecticides. Rich (81) and Bashucky (4) employed resting cell suspensions of lagoon bacteria to study the effect of O_2 tension on the rate of stabilization of a number of organic compounds. Lian (51) used resting cell suspensions of lagoon bacteria to illustrate the effect of temperature on the stabilization of a number of anionic surfactants. Kay (48) utilized resting cell suspensions of pure bacterial cultures to study CO_2 production from various simple and complex organics. Abbott and Casida (1) found resting cell suspensions of glucose-grown *Nocardia salmonicolor* PSU-N-18 were able to oxidize hexadecane to a mixture of internal monohexadecenes.

Information gained through the use of the methods hereto described provides valuable insight into the biodegradability potentials of recalcitrant organic compounds. However, all of these methods possess one or both of the following disadvantages; 1) lack of reproducibility resulting from variation in biomass and 2) time required for biodegradation to be completed. Variation in biomass occurring as a result of fluctuations in species composition and concentration in the population, their previous history and acclimation, concentration and nature of organic substrates and growth factors present, O_2 tension and absorptive materials and toxic

impurities adversely affect the reproducibility of these experiments. Only if successive experiments can be reproduced is the information acquired useful. If the results vary dramatically it is impossible to relate the data to natural phenomena. It is also useful in a screening procedure that the time required to demonstrate biodegradation be as short as possible. Further research is therefore needed to develop rapid methods to assess reproducible biodegradation potentials.

In order to solve the first of these problems it was decided to exploit continuous culture as opposed to relying on natural samples or batch culture as a source of active biomass. Use of the chemostat was expected to provide controllable quantities of biomass possessing a constant reproducible physiological capacity.

The methods of continuous culture as first proposed by Monod (66, 67) and Novick and Szilard (69) were reviewed by Herbert *et al.* (39), Brock (8) and more recently by Tempest (97). The method involves the continuous pumping of fresh medium into a completely mixed growth vessel while the effluent containing cells, spent medium and metabolic products is removed at the same rate. As bacteria grow they deplete the nutrients until growth becomes limited by the concentration of a single nutrient in the environment. Monod (66, 67) was the first to show the relationship between specific growth rate and concentration of the growth-limiting substrate. He defined this relationship by the following Michaelis-Menten type equation:

$$\mu = \mu_{max} \left(\frac{s}{K_s + s}\right) \qquad [1]$$

where μ is the specific growth rate of the organism at the growth-limiting substrate concentration s, μ_{max} represents the maximum growth rate possible at saturating levels of growth-limiting substrate, and K_s is the "saturation constant" numerically equal to the growth-limiting substrate concentration at $\frac{1}{2}\mu_{max}$. Thus the specific growth rate is proportional to the growth-limiting substrate concentration since both μ_{max} and s are constants for individual species.

It is essential in continuous culture that one fully understands the relationship between the growth rate of the organisms and the dilution rate. In the culture vessel organisms are growing and are simultaneously being washed out. The change in cell concentration (x) with respect to time (t) is given by:

$$\frac{dx}{dt} = \mu x - Dx \qquad [2]$$
$$= x(\mu - D)$$

where D is the dilution rate. Thus the rate of change in x is equal to the increase in x due to growth minus the removal of organisms from the culture vessel by dilution. If $\mu > D$, $\frac{dx}{dt}$ is positive and the concentration of cells in the culture will increase. If $\mu < D$, $\frac{dx}{dt}$ will be negative and the concentration of organisms will decrease with time until washout. Only when $\mu = D$ will $\frac{dx}{dt} = 0$ and x will remain constant with time, i.e., the culture will be at steady-state and

$$\mu = D = \mu_{max} \left(\frac{s}{K_s + s}\right) \qquad [3]$$

It also follows that s, the growth-limiting substrate concentration, is a function of the dilution rate. If the steady-state value of s is designated $\bar{s}$, rearranging the above equation shows

$$\bar{s} = K_s \left(\frac{D}{\mu_{max} - D}\right) \qquad [4]$$

Since μ_{max} and K_s are constants the principal effect of varying the dilution rate is to change the growth-limiting substrate concentration in the growth vessel, thereby producing a change in the specific growth rate of the organisms.

The theoretical aspects of continuous culture discussed above, were developed to describe pure culture growth only and must be cautiously applied when describing the growth of heterogeneous populations. Growth of mixed cultures is not merely the composite of the growth of the same species in pure culture, but is rather a complex system of interactions between the species present.

Growth of bacteria in mixed continuous culture has been the subject of intense investigation much of which is reviewed by Bungay and Bungay (10), Veldkamp and Jannasch (102), Jannasch and Mateles (46) and Meers (62).

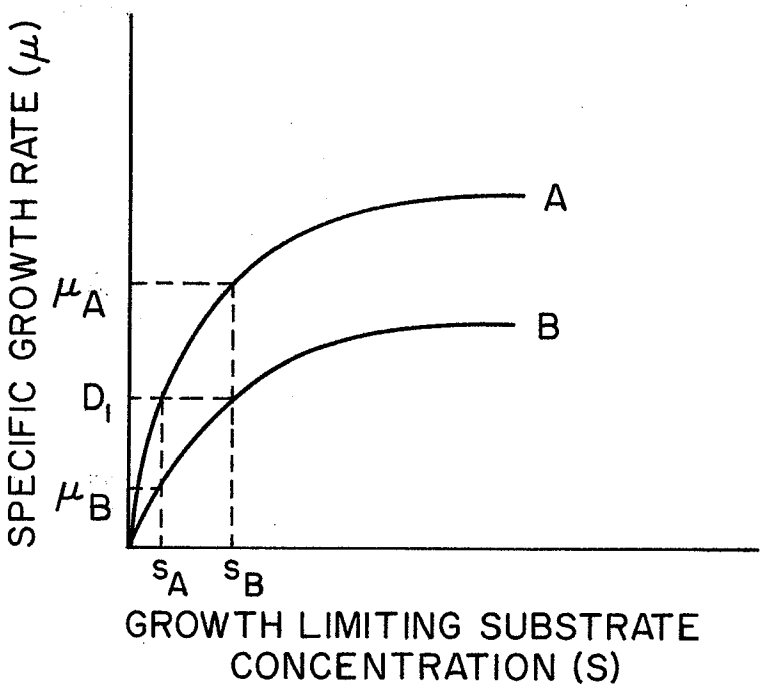
Competition for essential nutrients in mixed culture is perhaps the single most important phenomenon in determining the microbial population which develops in the chemostat. Competition was first studied with respect to the fate of contaminants and mutants in continuous culture. Novick and Szilard (69) demonstrated that when a bacterium *Escherichia coli* B/1, was grown for an extended period in continuous culture a mutant, *E. coli* B/1/f, arose which possessed a more rapid growth rate than the wild type under the

culture conditions and eventually replaced it. Powell (77) in his theoretical analysis of the fate of contaminants and mutants concluded that if the invading organism has a faster specific growth rate than the initial organism under the experimental conditions, the former would replace the latter provided the contaminant or mutant was not washed out before its first few cell divisions. Based on this premise Novick *et al.* (40, 68) have used continuous culture as an effective means of selecting for certain desirable types of mutants.

Taylor and leB Williams (96) published a theoretical analysis of the growth of mixed microbial populations in the chemostat. They concluded "in theory, stable mixed populations of competing species obeying Monod/Michaelis-Menten growth kinetics (extended to more than one limiting substrate) cannot co-exist unless the number of growth-limiting substrates is equal to or greater than the number of competing species." However, this is an idealistic treatment of the theory and does not incorporate into the model "direct positive or negative interactions between species."

The general rule of one species per independent growth-limiting substrate proposed by Taylor and leB Williams (96) is supported by the work of Meers (60) and Meers and Tempest (63) studying competition between *Aerobacter aerogenes, Pseudomonas fluorescens, Bacillus subtilis, Bacillus megaterium, Staphylococcus epidermidis* and *Torula utilis* in two-membered chemostat cultures limited by magnesium, potassium or carbon. In particular using *B. megaterium* and *T. utilis* in magnesium-limited cultures they demonstrated no combination of species could co-exist in the presence of only one growth-limiting

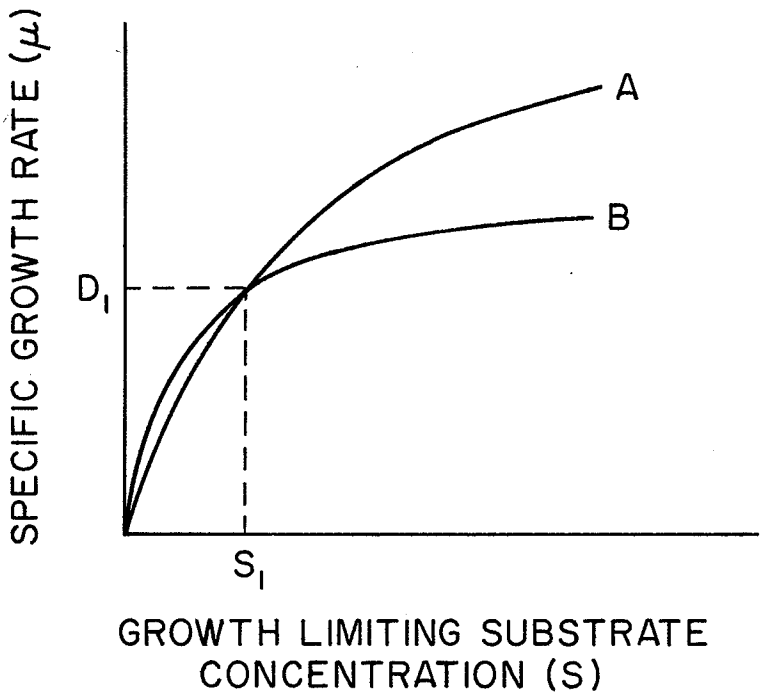
substrate. This result is best explained with the aid of the following illustration which shows the saturation growth curves of two competing species A and B.



If species B was growing at steady-state in a chemostat at a rate D_1 the growth-limiting substrate concentration would be s_B. If a small number of organisms of species A were inoculated into the chemostat they would grow at a rate μ_A governed by the growth-limiting substrate concentration s_B. Since $\mu_A > D$, species A will increase in number and reduce the growth-limiting substrate concentration to s_A where the specific growth rate of organism A equals the dilution rate. At this growth-limiting substrate concentration organism B will grow at a rate μ_B which is $< D_1$ and therefore will be washed out. At all dilution rates the specific growth rate of

species A is greater than that of species B and as such will always be the successful competitor.

It is, however, possible that the saturation growth curves of the two species cross-over as illustrated below.



In this example at dilution rates above D_1 species A possesses the faster growth rate and would therefore be the successful competitor in competition with species B. At dilution rates below D_1 species B possesses the faster growth rate and would be the successful competitor. If the chemostat was operated at a dilution rate equal to D_1 theoretically both species could co-exist if neither had another selective advantage. However, the possibility of this phenomenon

occurring is extremely remote.

The crossing-over of saturation growth curves has in fact been demonstrated. Meers (61) studying the effect of dilution rate on competition between *T. utilis* and *B. subtilis* showed the outcome of competition to be dependent on dilution rate over the range 0.05 hr^{-1} to 0.08 hr^{-1}. At 0.05 hr^{-1} *T. utilis* dominated the population while at 0.08 hr^{-1} *T. utilis* washed out and *B. subtilis* developed into the dominant species. Jannasch (45) showed by varying the dilution rate or growth-limiting substrate concentration in the medium reservoir it was possible to enrich for different species from a heterogeneous inoculum which he explained was due to crossing-over of saturation growth curves.

Competition between species in mixed culture may also be influenced by extracellular products produced by one species which promote a more rapid uptake of the growth-limiting nutrient by that species. Meers and Tempest (63) demonstrated this property in competition experiments between *T. utilis* and *B. megaterium* in magnesium-limited chemostats. However, only at very high cell densities of the bacterium (95% in the initial inoculum) did this product accumulate sufficiently to provide the bacterium with a competitive advantage.

The Monod equation [1] cannot be used to describe growth of an organism if its growth rate can be altered by an extracellular product. For this reason Meers and Tempest (63) proposed the following modification to the Monod equation:

$$\mu = \mu_{max} \left(\frac{1 + \lambda p}{1 + p}\right) \left(\frac{s}{K_s + s}\right) \qquad [5]$$

where p is the concentration of the growth-promoting substance and λ is a constant. In the culture, if p approaches 0 then $(\frac{1 + \lambda p}{1 + p})$ approaches 1 and the formula reverts to the standard Monod equation in which growth is a function of the maximum specific growth rate and the substrate concentration in the reactor.

Competition by mixed bacterial species for two growth-limiting substrates has also been investigated (14). Chemostats fed with mixed glucose-lactate or glucose-butyrate media inoculated with river water and run at various dilution rates developed populations consisting mainly of coliforms and pseudomonads which preferentially used the glucose over the second carbon source. This diauxie effect appeared to be the result of catabolite repression and/or inhibition, as a metabolic product, acetate, began to accumulate in the medium at the same dilution rate at which the utilization of the second carbon source was reduced.

Abiotic factors are important parameters affecting chemostat competition experiments. Temperature was shown by Harder and Veldkamp (35) to influence the outcome of competition between an obligate and a facultative psychrophile. Contois and Yango (21) illustrated the effect of pH on the competition between two strains of *Aerobacter aerogenes* co-existing in a chemostat on only one growth-limiting substrate.

Tolerance to toxic substances present in the environment has been shown to play an important role in competition (101, 102). Sensitivity to sulfide by two purple sulphur bacteria, a *Thiocystis* sp. and a *Chromatium* sp., influenced the outcome of chemostat competition experiments between the two species. Only at very low

sulfide concentrations was the *Chromatium* species the successful competitor.

The ability of bacteria to become attached to surfaces in the chemostat also influences competition. *Sphaerotilus natans* possessed a distinct advantage in the chemostat over other organisms in a heterogeneous inoculum because of its ability to become attached to glass surfaces in the culture vessel (22). Topiwala and Hammer (99) concluded bacteria adhering to surfaces in the chemostat will persist beyond the calculated critical dilution rate (maximum specific growth rate) of the organism.

Other types of interactions between bacterial species are possible when they are grown simultaneously in continuous culture. These include amensalism, commensalism and mutualism.

Amensalism refers to the relationship between species in which the growth of one species inhibits the growth of others. Such a relationship may result from the production of toxic products both organic and inorganic. Acid production has been reported as an amensalistic mechanism which is capable of working either directly as a toxic compound (38) or indirectly by decreasing the pH of the environment (21, 60). Parker and Snyder (72) described an amensalistic relationship between *Streptococcus salivarius* and *Veillonella alcalescens* but did not attempt to resolve the mechanism of interaction.

Commensalism describes the relationship between species in which the growth of one species promotes the growth of a second while the growth of the former is in no way altered by the presence of the latter. This relationship usually involves the production of growth-

promoting substances such as a utilizable carbon source or growth factors. Chao and Reilly (15) described a commensalistic relationship between *Acetobacter suboxydans* and *Saccharomyces carlsbergensis* depending upon the production by the bacterium of a utilizable carbon source for the yeast. Shindala *et al.* (85) demonstrated commensalism between *Saccharomyces cerevisiae* and *Proteus vulgaris* involving the essential growth factor niacin produced by the yeast and required by the bacterium. Tsuchiya *et al.* (29, 64) reported commensalism + competition, pure competition and mutualism + competition relationships between *S. cerevisiae* and *Lactobacillus casei* due to the production of riboflavin by the yeast which is required by the bacterium. Cappenberg (11) simulated in a chemostat a naturally-occurring commensalism + amensalism relationship between *Desulfovibrio desulfuricans* and a *Methanobacterium* sp. Growth of the *Methanobacterium* sp. was dependent upon acetate production, contiguously, from the oxidation of lactate by *D. desulfuricans*. However, the H_2S produced by *D. desulfuricans* inhibited the growth of the *Methanobacterium*. The degree of inhibition increased with increasing lactate concentration in the medium as a result of the more rapid growth of *D. desulfuricans* and corresponding increase in H_2S production.

Mutualism describes the relationship between two species in which both benefit from the presence of the other. These relationships vary from loose co-operation between species involving non-essential growth factors, called protoco-operation, to obligate associations upon which both species depend for co-existance. Yeoh *et al.* (107) described an example of an obligate mutualistic relationship between *P. vulgaris* and *Bacillus polymyxa* which involved two

essential growth factors, niacin and biotin. The mixed culture experiments also showed an unexpected oscillation in total population density which was due to an inhibitor produced by *P. vulgaris*. Iannotti *et al.* (42) reported a mutualistic relationship between two rumen bacteria, *Ruminococcus albus* and *Vibrio succinogenes*. *V. succinogenes* was dependant on the H_2 production by *R. albus* which in turn resulted in changes in the metabolism of the latter.

The preceding discussion of mixed culture interaction demonstrates the dynamics of heterogenous populations and the extent to which the populations can fluctuate under conditions which promote steady-state growth of pure cultures. Therefore, it is essential to determine if steady-state growth of mixed cultures can actually be achieved. Gaudy *et al.* (31, 32) and Chiu *et al.* (19) using continuous flow experiments run for up to 36 days reported fluctuations in biological solids concentration in the chemostat while both effluent COD and substrate concentration remained constant. Chui *et al.* (20) also demonstrated variations in the ATP pool of the reactor which did not parallel fluctuations in biological solids indicating species fluctuations not reflected by suspended solid determinations. Cassell *et al.* (13) reported fluctuations in alcohol-soluble pigments, suspended solids and effluent COD in their heterogeneous continuous-flow system. Variation in pigment type and concentration closely parallelled changes in the predominant species of the culture as determined microscopically. Mateles and Chian (59) observed fluctuations of 10-20% in cell density after 15 retention periods.

Although these groups failed to demonstrate steady-state this

does not mean heterogeneous populations cannot be grown at steady-state. Taylor and leB Williams (96) concluded steady-state growth of heterogeneous populations was theoretically possible, however, only if the number of growth-limiting nutrients in the medium was at least as great as the number of different species and if there was no interaction between species in the population. Therefore, a medium selected for the growth of heterogenous populations should contain a number of potential growth-limiting nutrients to promote the growth of a number of species and a variety of growth factors to minimize interaction between species.

As steady-state growth of the heterogenous populations used in this investigation was essential three media were selected for growth of the populations. Peptonized milk contains a variety of potential growth-limiting nutrients and growth factors and was demonstrated by Larkin (50) to promote growth of a substantially greater number and wider variety of soil bacteria than either soil extract or Trypticase-soy medium. Medium C of Sundman and Carlberg (90) contains five different carbon sources, each a potential growth-limiting nutrient, but lacks growth factors. However, it was shown to grow 68.3% of the bacterial species isolated from soil. The synthetic sewage medium of James (44) contains a variety of growth factors and organic carbon sources and possesses a BOD equivalent to that found in raw sewage.

The ability of these media to support heterogeneous microbial populations in continuous culture was investigated in this laboratory by Rerie (80). He successfully isolated eight and seven different organisms from the peptonized milk culture and synthetic sewage

medium respectively. From the chemically-defined medium C he was able to isolate only three different species. The diversity of these mixed cultures compared favourably with published data on the number of organisms isolated from natural environments. Prakasam (79) isolated six different organisms from activated sludge while Cherry (16) reported an average of eight to ten isolates from natural ponds and streams.

Rerie (80) also studied the steady-state growth of the populations exploited in this investigation by monitoring dry weight, viable cell count and O_2 uptake on casamino acids as his parameters. He discovered all three populations attained steady-state growth with respect to dry weight, however, viable cell count and O_2 uptake experiments revealed fluctuations in the steady-state growth condition not revealed by dry weight analysis. Variations in O_2 consumption of 12% were recorded by populations grown on peptonized milk while even greater instability was found in the medium C (28.8%) and synthetic sewage (17.1%) popultions. Similar discrepancies between methods of determining steady-state have been reported by Blok (7) and Bashucky (4).

Although none of the three media promoted growth of true steady-state heterogenous populations the peptonized milk population approached steady-state much more closely than did the others. This was likely the result of the variety of possible growth-limiting nutrients and growth factors in the medium which would serve to minimize interactions between the species present.

As resting cell suspensions of heterogeneous chemostat populations were employed in this investigation and degradation of

recalcitrant molecules requires lengthy time periods it is essential that one fully appreciates the limitations of these preparations. Rerie (80) has investigated the physical and physiological stability of the mixed populations as resting cell suspensions. After six days of starvation 50% of the peptonized milk-, and medium C-grown cells survived as compared to only 23% of the cells grown on synthetic sewage. The physiological stability of the cells determined by their ability to metabolize casamino acids, agreed remarkably well with the survival of the cells. Peptonized milk-, and medium C-grown cells displayed a linear decline in O_2 uptake activity while they retained 50% and 74% of their initial activity respectively after twelve days. The synthetic sewage-grown cells exhibited a more curvilinear decline in activity retaining only 33% of their initial activity during the same period. The decline in casamino acid-oxidizing ability followed the same trends established by monitoring survival of the cells. However, in each case the O_2 consumption was somewhat higher than was expected when activity was predicted on the basis of cell survival. This result was attributed to the cells' ability to retain catabolic activity after losing their reproductive capacity.

Kay (48) has shown viability and physiological stability of mixed populations in resting cell suspensions could be greatly enchanced by the addition of an adjuvant substrate. In the presence of 0.5 mg/ml acetate or casamino acids more than two thirds of the initial O_2 consumption was retained after 120 hours compared to at best 10% without the adjuvant. Thus viability and catabolic activity of resting cells utilized in the biodegradation experiments

may be even greater than indicated by Rerie.

Rerie (80) also demonstrated the loss of viability and activity is not always linear over the starvation period. Thus no single rate constant can be used to correct for these losses after any given incubation period.

Rerie (80) concluded that of the three media formulations peptonized milk proved superior in promoting growth of heterogeneous populations in the chemostat at or near steady-state and were more stable physically and physiologically in resting cell suspensions.

As a continuum of the investigation by Rerie it was the primary objective of this investigation to study the biodegradation capacity of these populations in order to ascertain their physiological potentials.

Another objective of this study was to employ enrichment culture techniques to select for populations which degrade the test substrates more rapidly. Enrichment culture is a technique used to select from a mixed population those species which exhibit a desired property. Selecting conditions which favor the growth of species exhibiting a desired property results in their predominance in the population. In heterogeneous continuous culture the species comprising the population are those which are the most efficient at substrate uptake at the dilution rate. Therefore, by appropriately designing the nutrient composition of the medium species exhibiting a desired property may be enriched.

Jannasch (45) using continuous culture techniques was able to select a variety of marine species from the same inoculum simply by altering the dilution rate and/or the reservoir substrate

concentration in lactate-limited chemostats. Novick *et al.* (69) employed chemostat enrichment to select lac constitutive mutants in populations of lac inducible cells.

METHODS AND MATERIALS

METHODS AND MATERIALS

Continuous Culture Procedure

The heterogeneous bacterial populations used throughout this investigation were generated by exploiting continuous culture techniques. The continuous culture apparatus developed by Kay (48), is illustrated in Fig. 1. The reactor (A) was a modified 1-litre water-jacketed Bellco spinner flask (Bellco Glass Inc., Vineland, N.Y.). The glass dome was replaced by a plexiglass plate with 3 holes. The center hole, connected to a sterile cotton filter, doubled as an air inlet port and as a guide for the magnetic stirrer shaft. The second hole was used as a medium inlet port while the third hole, stoppered with a rubber bung, was reserved for sampling. The fresh medium contained in 20-litre Nalgene carboys (E) (Nalgene Labware Div., Nalge, Rochester, N.Y.) was pumped to the respective reactors by a MHRE 22 DELTA Watson Marlow pump (D) (Fred A. Dungey, Agincourt, Ont.) equipped with a multi-channel head. The dilution rate was arbitrarily set at 0.04 hr^{-1} providing a retention time of 25 hr. A flow meter (H) attached to each feed line provided a method for periodic monitoring and adjustment of the dilution rate.

To prevent grow back in the feed line each line terminated in a stainless-steel tube (200 mm x 2.5 mm I.D.) surrounded by three 10-ohm porcelain-coated resistors (G) joined in series to a step-down transformer and an adjustable rheostat. A rheostat setting between 22 and 26 was sufficient to heat the influent medium to 55° - 60°C.

The culture volume in the reactor was maintained at 1 litre by

Fig. 1 Continuous Culture Apparatus

A. Water-jacketed reactor containing heterogeneous bacterial culture

B. Magnetic stirrer

C. Constant temperature circulating waterbath

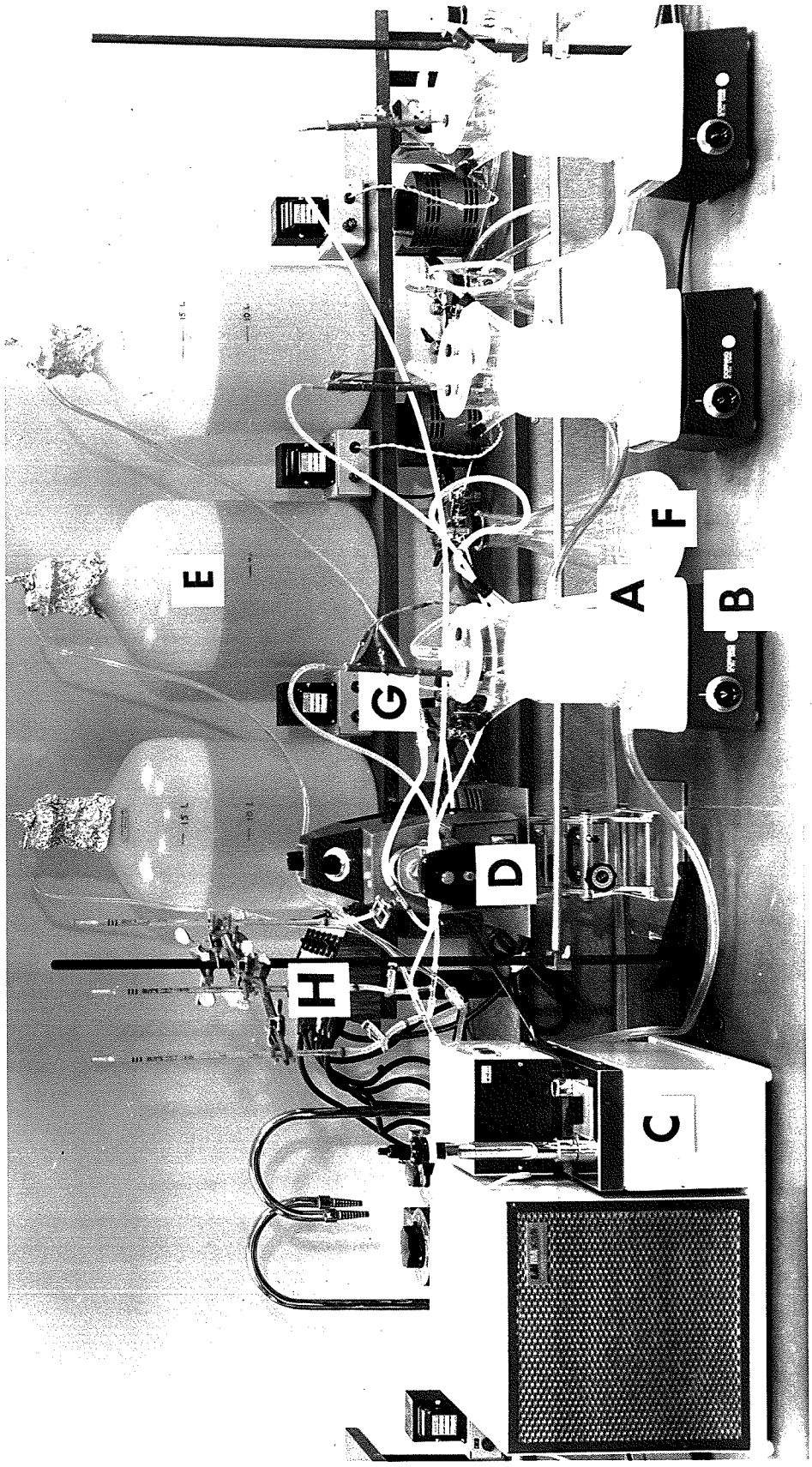
D. Pump with multi-channel head

E. Medium reservoir

F. Effluent collection vessel

G. Porcelain-coated resistors surrounding stainless-steel tube on feed line

H. Flow meter


A
B
C
D
E
F
G
H

drawing off the effluent, through a glass tube extended to the culture surface, into a 2-litre vacuum collection flask (F). The suction also provided a constant air flow to the culture by drawing fresh air through a sterilizing cotton filter attached to the top of each growth vessel.

The chemostat cultures were continually mixed by placing the reactors on Corning PC-353 magnetic stirrers (B) (Corning Labware Products, Corning, N.Y.). This provided a uniform suspension and adequate aeration for growth.

The growth temperature was maintained at 20° ± 0.05° by a Lauda K-2/R circulating waterbath (C) (Lauda Instruments Div., Westbury, N.Y.).

Media

i. Standard Growth Media

Three different nutrient formulations for growth of the heterogeneous microbial populations were used throughout this investigation; i) a chemically-undefined commercially-available medium, Bacto-peptonized milk (Difco Laboratories, Detroit, Mich.), ii) a semi-chemically-defined medium, James' synthetic sewage (44) and iii) a chemically-defined medium, medium C of Sundman and Carlberg (90).

i) Bacto-peptonized milk medium is a proteolytic enzymatic digest of fresh skim milk containing the hydrolytic products of the albumins and globulins of the milk. The medium was used at a concentration of 0.1% w/v in double distilled water as recommended by Larkin (50).

ii) The composition of the synthetic sewage medium was as

follows:

Peptone (Bacto)	1.88 gm
Beef extract	3.75 gm
Dextrose	4.69 gm
$(NH_4)_2SO_4$	0.375 gm
NaCl	0.056 gm
KCl	0.0188 gm
$CaCl_2 \cdot 2H_2O$	0.0413 gm
$MgSO_4 \cdot 7H20$	0.188 gm
$NaH_2PO_4 \cdot H_2O$	19.808 gm
Na_2HPO_4	86.265 gm
Double distilled water	15 litres
pH adjusted to 7.4	

The BOD_5 (20°C) of the medium was found to be 394 mg O_2/l (51). All chemicals were of analytical grade.

iii) The composition of medium C of Sundman and Carlberg was as follows:

Glucose	7.5 gm
Sodium acetate	7.5 gm
Sodium lactate	7.5 gm
Sodium citrate	7.5 gm
Glycerol	7.5 gm
$(NH_4)_2HPO_4$	7.5 gm
K_2HPO_4	6.0 gm
$MgSO_4 \cdot 7H_2O$	0.75 gm
NaCl	1.5 gm
$FeCl_3$	0.15 gm

Double distilled water 15 litres

Final pH adjusted to 6.8

All chemicals were of analytical grade.

All media, prepared in 20-litre carboys, were autoclaved for 80 minutes at 121°C and 15 psi.

ii. Enrichment Media

The standard growth media (above) were modified by the addition of 0.5% w/v of either acetate, pyruvate, butyrate or phenol during the enrichment experiments.

Mixed Culture Inoculum

Garden topsoil served as a source of organisms for all mixed culture experiments. The soil was first passed through a 2 mm mesh sieve to remove stones and debris. Ten ml of a 10^{-4} dilution, prepared from 10 gm sieved soil in 0.1% sterile peptone water, was used to inoculate 1 litre of sterile medium for initial batch culture growth. Actidione (Calbiochem., San Diego, Calif.), a fungal inhibitor, was added to the starter culture at a final concentration of 100 µg/ml to prevent fungal contamination. Two or three days of batch culture growth was normally adequate to establish the bacterial population before dilution was initiated.

Assessment of the Biodegradation Potential of the Resting Cell Suspensions

i. Preparation of the Resting Cell Suspensions

One litre of effluent from each culture vessel was collected over 24 hours in ice-packed receiving flasks (temperature, 4°C). Each suspension was centrifuged at 10,000 rpm (16,300 x g) for 15 minutes

in a Sorvall RC2-B superspeed centrifuge (Ivan Sorvall Inc., Norfolk, Conn.) equipped with a large head. The supernatant liquid was discarded and the pellets resuspended in 100 ml of 50 mM potassium phosphate buffer, pH 7.0. These washed suspensions were centrifuged at 10,000 rpm for 15 minutes and the pellets resuspended in 25 ml of 50 mM phosphate buffer, pooled and broght to a final volume of 20 ml with phosphate buffer. The final suspension was stirred vigorously for 2 hours prior to use.

ii. Substrates

The substrates selected for biodegradation tests were all organic compounds known to require molecular oxygen for microbial breakdown. Acetate and pyruvate were chosen as representatives of simple organic carbon compounds which are important prime intermediates in a number of oxidative pathways. Butyrate was selected as representative of short chain aliphatic hydrocarbons, which in addition is known to possess toxic properties (17, 18, 26, 84). Phenol was chosen as an example of more complex aromatic substrates whose breakdown is mediated by induced enzyme systems (27, 70). Four anionic surfactants; a commercial linear alkylbenzene sulfonate (LAS)[1], tallow alcohol sulfate (PG1)[2], coconut alcohol ethoxylate sulfate (PG2)[3] and tallow alcohol ethoxylate sulfate (PG3)[4] were selected as examples of synthetic organic chemicals known to be biodegradable.

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[1]LAS: average chain length 11.3 carbon atoms

[2]PG1: general structure: $R\text{-}OSO_3Na$ where $R = C_{17}$ (average)
general analysis: 48.23% active, 47.5% water, 1.76% unreacted alcohol, balance $NaSO_4$

PG2: general structure: $R\text{-}O(CH_2CH_2O)_3OSO_3NH_4$ where $R = C_{12}$ (average)
general analysis: 23.58% active, balance water

PG3: general structure: $R\text{-}O(CH_2CH_2O)_3OSO_3Na$ where $R = C_{17}$ (average)
general analysis: 57.22% active, 3.93% unreacted alcohol, 0.42% NaSO , balance water

iii. Standard Experiment Procedure

The biodegradation potentials of the test substrates being metabolized by the resting cell suspensions were assessed by monitoring; i) the rate of breakdown by direct analysis of residual substrate concentration, ii) the initial rate of O_2 uptake and iii) the initial rate of CO_2 production. All experiments conformed to the following protocol.

After initiating dilution the continuous cultures were allowed about seven retention periods to attain steady-state. Following this period effluent was collected and resting cell suspensions were prepared as described above. Cells destined for use in biodegradation experiments involving monitoring residual substrate were harvested during the 8th to 11th retention period (one retention period/substrate tested), those to be used for CO_2 production experiments during the 12th retention period and those involving O_2 uptake during the 13th retention period.

Procedures for the standardization of biomass by dry weight determinations were as follows. Duplicate 5-ml samples of each resting cell suspension were pipetted into preweighed aluminium drying dishes and dried at 105°C for six hours in a drying oven then reweighed on a Mettler H 10T Precision balance (Mettler Instruments, Zurich, Switzerland). The dry weight was calculated as mg dry weight/ml resting cell suspension.

iv. Rate of Substrate Degradation

The actual rate of substrate breakdown was determined by following the disappearance of the substrate at different initial concentrations with time after its addition to the resting cell suspension. Resting

cell suspensions, 25 ml in 125-ml Erlenmeyer flasks, were incubated at 20° ± 0.05°C at 60 oscillations/min on a rotary shaker in a temperature-controlled incubator (Puffer Hubbard Refrigerator Div., Grand Haven, Mich.). After a 30 minute preincubation period to allow temperature, pH and O_2 tension equilibration, 0.5 ml substrate representing 1.0, 5.0, 10.0, 20.0 and 50.0 mM initial concentrations was added to each suspension. At zero time and intervals thereafter 2-ml samples were taken for analysis of residual substrate. In detergent degradation experiments 50 ml of the resting cell suspensions in 125-ml Erlenmeyer flasks were used and 3-ml samples were taken for residual detergent analysis.

Treatment of samples varied with substrate. The 2-ml samples containing pyruvate were added directly to 10 ml cold 10% trichloroacetic acid (TCA) and centrifuged at 10,000 rpm for 10 minutes in a Sorvall Centrifuge. The supernatant was decanted into screw-cap tubes and frozen until analyzed. Samples containing acetate, butyrate and phenol were acidified to pH 1.0 with 6.0 N HCl to stop the reaction, centrifuged at 10,000 rpm for 10 minutes and the supernatant decanted into screw cap tubes and frozen until analyzed. Samples containing detergents followed the same procedures as those containing acetate, butyrate and phenol but with the deletion of the acidification step.

v. Analytical Methods for Measuring Residual Substrates

i) Acetate, ii) Butyrate, iii) Phenol

Quantitative analysis of these substrates was performed by gas-solid chromatography using a Varian Aerograph model 2100 Gas Chromatograph (Varian Aerograph, Walnut Creek, Calif.) equipped with a flame ionization detector. The column, a 1.85 m x 4.0 mm I.D. glass "U", was packed with Chromosorb 101, 80/100 mesh (Johns-Manville Products

Corp., Celite Div., Manville, N.Y.). Nitrogen was chosen as the carrier gas and the flame was supported by a constant flow of hydrogen gas (35 cc/min) and compressed air (350 cc/min). The various operational characteristics used for analysis of each substrate are given in Table 1. The 10-µl syringe was used for on-column injection of 5-µl samples. The resultant peaks were recorded on an Omniscribe recorder (Houston Instruments, Bellaire, Tex.) equipped with an integrator circuit. The amount of residual substrate in each sample was determined by measuring the area under the peak, in integrator units, and relating the value to a standard curve.

Prior to injection the pH of the acidified samples was neutralized (pH 7.0) by 6N NaOH.

iv) Pyruvate

Pyruvate was measured by the method of Friedemann and Haugen (30). This method involves the formation of a 2-4 dinitrophenylhydrazone between the residual pyruvate and 2-4 dinitrophenylhydrazine. The hydrazone was extracted with toluene and again with 10% $NaCO_3$. This solution turns a red-brown color on the addition of 1.5N NaOH. The intensity of the color, measured with a Klett-Summerson photoelectric colorimeter (Klett Manufacturing Co., Inc., New York, N.Y.) using a green filter, was proportional to the pyruvate concentration.

v) Anionic Surfactants (LAS, PG1, PG2 and PG3)

The quantitative analysis of the anionic surfactants was performed using the standard "methylene blue" method (88). The anionic surfactant reacts with methylene blue forming a complex of very low water solubility which is easily extracted with chloroform. The intensity of the color was measured with a Klett-Summerson photoelectric

TABLE 1

Operational characteristics of the Model 2100
Varian Aerograph gas chromatograph

COMPOUND	CARRIER GAS FLOW (cc/min)	TEMPERATURES (°C)			SENSITIVITIES	
		INJECTOR	COLUMN	DETECTOR	ATTENUATION	RANGE (amp/mv)
Acetate	35	250	200	250	10^{-12}	128
Butyrate	115	250	220	250	10^{-11}	64
Phenol	135	250	220	250	10^{-12}	16

colorimeter using a red filter. A colorimetric calibration curve prepared from tetrapropylene alkylbenzene sulfonate (ABS) served as a reference standard. Surfactants measured by this method are commonly designated "methylene blue-active substance" (MBAS).

vi. Initial Rate of O_2 Uptake

The initial rate of O_2 uptake by resting cell suspensions metabolizing the test substrates was assessed by standard manometric procedures (100) using a Bronwill Warburg Respirometer (Bronwill Scientific Inc., Rochester, N.Y.) modified with a copper cooling coil in the waterbath through which cold tap water was circulated. Each Warburg flask contained 2 ml resting cell suspension, 0.5 ml 50 mM phosphate buffer, pH 7.0, a small fluted filter paper with 0.2 ml 20% w/v KOH in the centre well and 0.5 ml substrate, or in the case of the controls 0.5 ml 50 mM phosphate buffer, in one side-arm. The flasks were then attached to their manometers with their stopcocks open to the atmosphere, placed in the thermoregulated waterbath at 20° $\pm$ 0.05°C and shaken at a rate of 60 oscillations/minute. After a 20 minute preincubation period to allow temperature, gas and liquid phase equilibration the stopcocks were closed and the substrate tipped into the cell suspension. Manometer readings were taken at zero time and at 10 minute intervals over a two hour period. The data obtained were used to calculate µl O_2 consumed/min/mg dry weight resting cell suspension.

vii. Initial Rate of CO_2 Production

The initial rate of CO_2 production by the resting cell suspensions metabolizing the substrates was measured by the method of Kay (48)

involving infrared gas analysis. The apparatus is shown in Fig. 2. The reaction vessel (A) was a water-jacketed chamber equipped with a serum-stoppered side-arm and a glass top with inlet and outlet ports. The top and bottom were connected by a ground glass joint sealed with vacuum grease. The resting cell suspension, 2 ml, in the reaction vessel was continually stirred by a magnetic stirrer (B) (Corning Labware Products, Corning N.Y.). The temperature of the reaction was maintained at 20° $\pm$ 0.05°C by a Neslab model RTE - 3 circulating waterbath (C) (Neslab Instruments Inc., Portsmouth, N.H.). The gas phase was circulated through the system by means of interconnecting silicone tubing and employing a Masterflex model 7071 peristaltic pump (D) (Cole-Palmer Instruments Co., Chicago, Ill.). A Beckman model 215B infrared CO_2 analyzer (E) (Beckman Instruments Inc., Fullerton, Calif.) with a range of 0 - 600 PPM was used to measure the amount of CO_2 produced.

During operation, 2 ml of resting cell suspension was pipetted into the reaction vessel. The system was then flushed with dry CO_2-free air by drawing atmospheric air first through a Drierite column (W.A. Hammond Drierite Co., Xenia, Ohio) and then through an Ascarite column (Arthur H. Thomas Co., Philadelphia, Pa.). After a 20-minute preincubation period which allowed temperature equilibration and the removal of all CO_2 from the system, the endogenous rate of CO_2 production was measured by adjusting the stopcocks to form a closed loop and the rate of CO_2 accumulation was monitored by the infrared analyzer and recorded. The system was then reopened to purge the accumulated CO_2, and the test substrate contained in 50 µl, was injected into the resting cell suspension through the serum-

Fig. 2 Analytical system for measuring CO_2 production by infrared analysis

A. Water-jacketed reaction vessel

B. Magnetic Stirrer

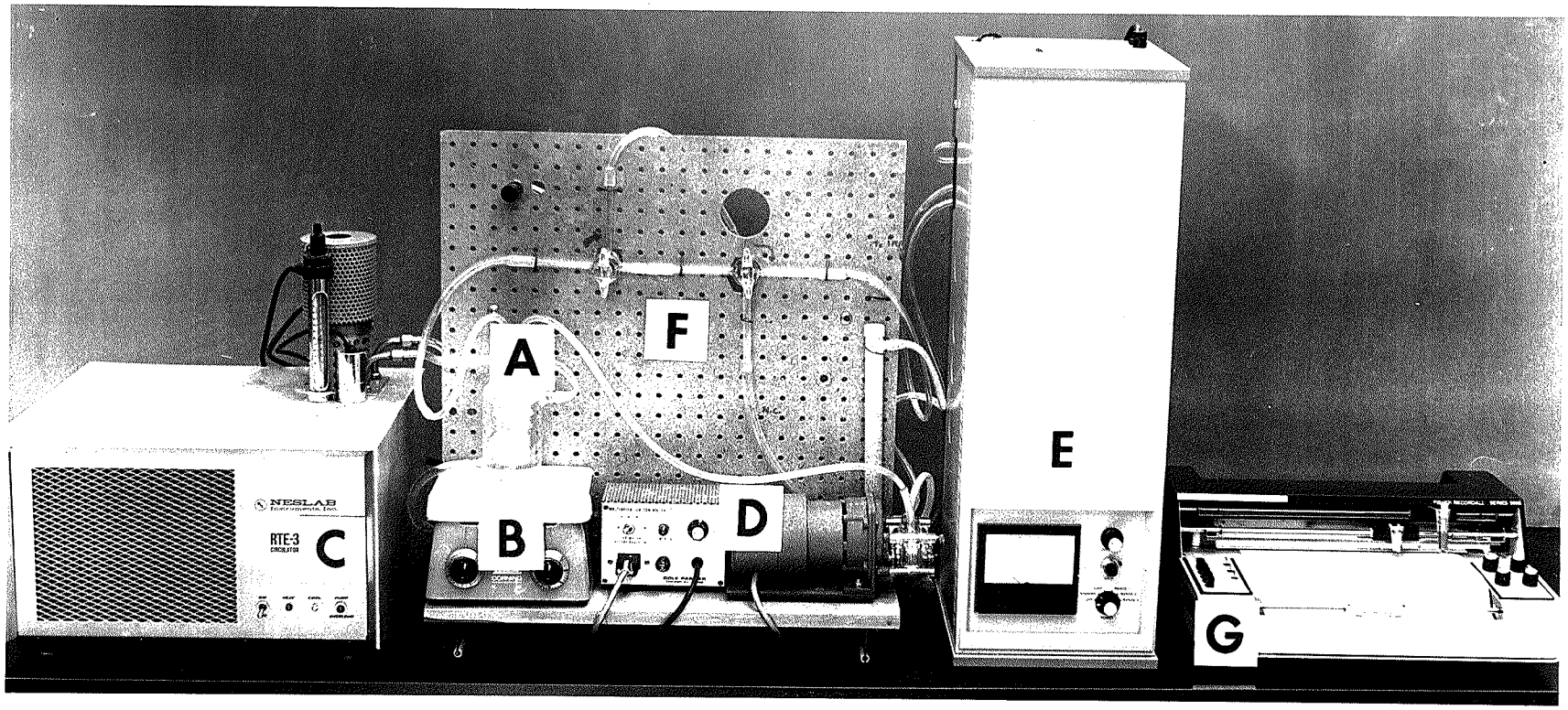
C. Circulating waterbath

D. Masterflex pump with control assembly

E. Infrared analyzer

F. Apparatus support with stopcocks

G. Recorder


A
B
C
D
E
F
G
NESLAB
RTE-3

stoppered side-arm. At intervals thereafter the system was closed to measure the rate of accumulating CO_2 and then reopened to purge the CO_2. The exogenous rate of CO_2 production was corrected for endogenous respiration and calculated as nmoles CO_2 produced/min/mg dry weight resting cell suspension.

RESULTS

RESULTS

Standard Curves for Quantitative Analysis

Standard curves depicting the linear increase in recorder response with increasing amounts of injected materials over the range 0 - 1.0 µg for the determination of acetate, butyrate and phenol by gas-solid chromatography are shown in Fig. 3. The retention time for these compounds under the operational characteristics employed (Table 1) are: acetate, 2 min 45 sec; butyrate, 3 min 10 sec; phenol, 9 min 20 sec.

The standard curve for the quantitative analysis of pyruvate over the range 0 - 100 µg is depicted in Fig. 4.

The standard curve for the colorimetric determination of detergent measured as "methylene blue-active substance" (MBAS) is shown in Fig. 5.

Residual Substrate Concentration as an Indicator of Substrate Transformation

Representative curves of the experiments demonstrating removal of test substrates; acetate, pyruvate, butyrate and phenol, at varying substrate concentrations from a resting cell suspension of peptonized milk-grown cells are provided in Figs. 6 and 7. The rates of transformation determined from these data at 10.0 mM substrate concentration and similar experiments with initial substrate concentrations ranging from 1.0 to 50.0 mM expressed on the basis of cell dry weight, are summarized in Table 2.

Acetate was readily metabolized by the three populations commencing immediately upon its addition to the resting cell suspensions. The most

Fig. 3 Standard curves for the quantitative determination of a) acetate (⊙), b) butyrate (□) and c) phenol (Δ) obtained by gas chromatography analysis.

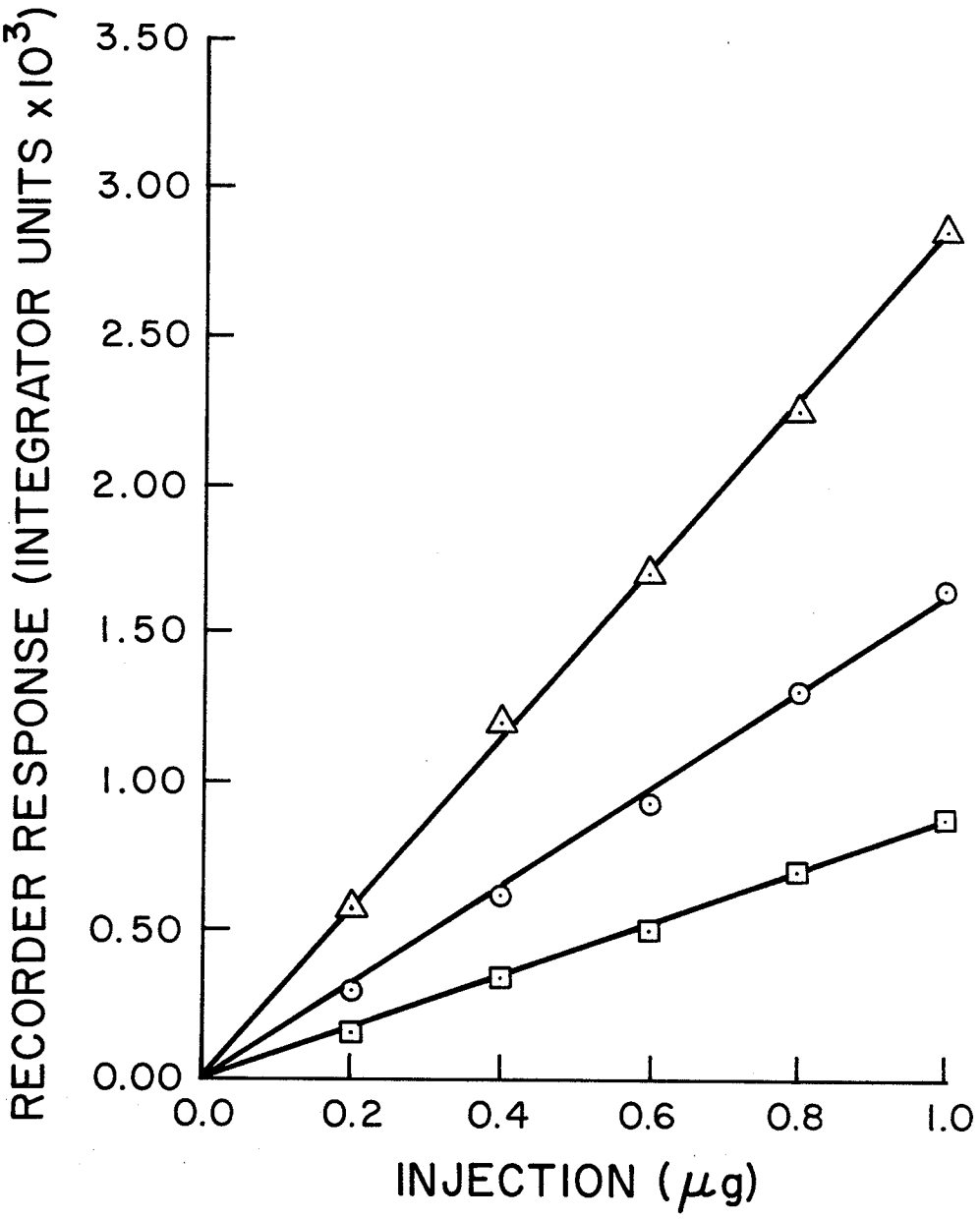
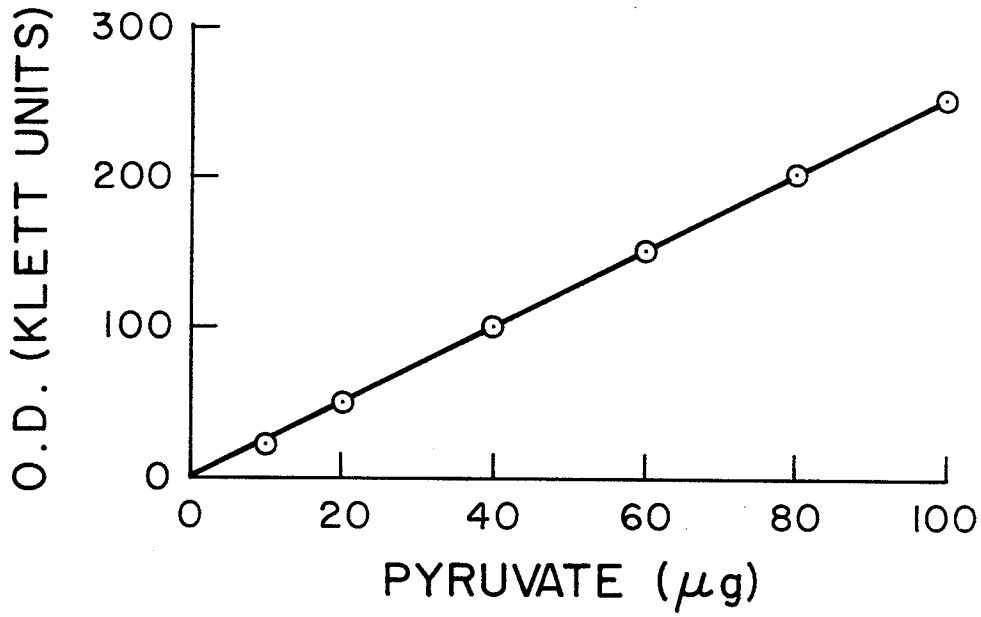

RECORDER RESPONSE (INTEGRATOR UNITS x10³)
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
0.0
0.2
0.4
0.6
0.8
1.0
INJECTION (μg)

Fig. 4 Standard curve for the quantitative determination of pyruvate obtained by colorimetric analysis.

Fig. 5 Standard curve for the quantitative determination of detergents as methylene blue-active substance (MBAS) as determined by colorimetric analysis.


O.D. (KLETT UNITS)
300
200
100
0
0
20
40
60
80
100
PYRUVATE (μg)

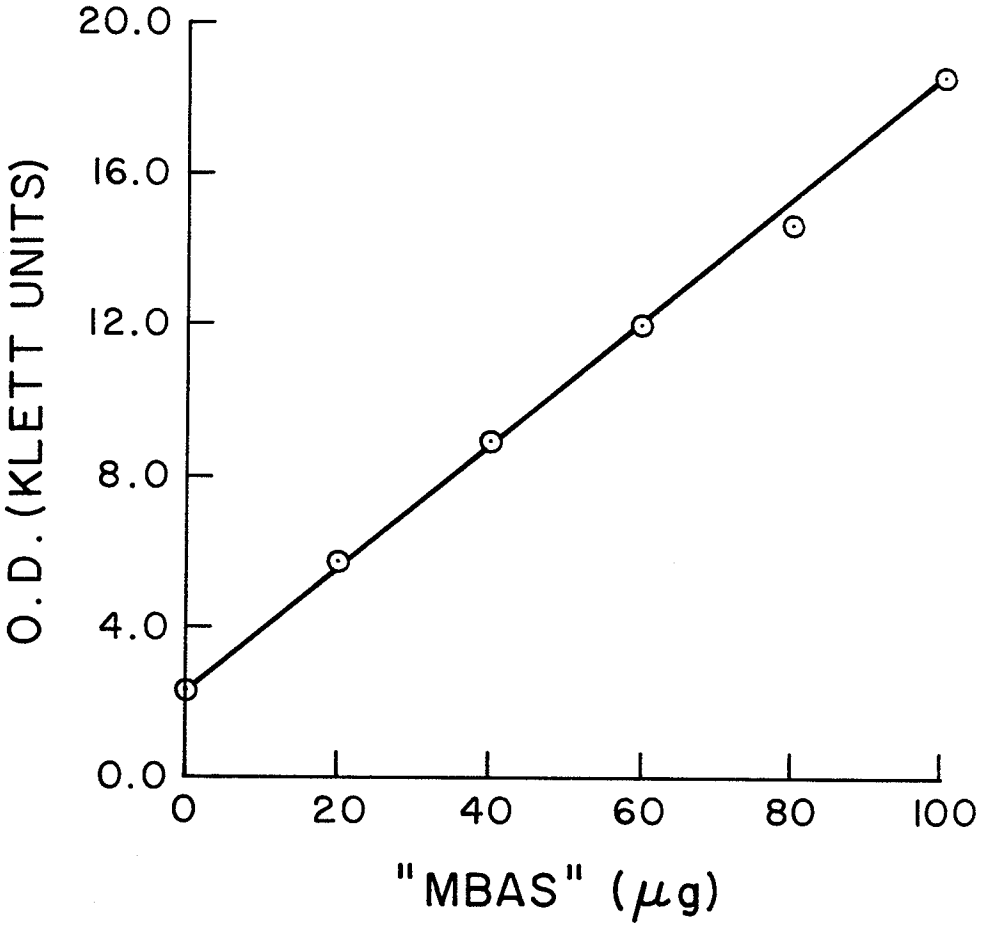

O.D. (KLETT UNITS)
20.0
16.0
12.0
8.0
4.0
0.0
0
20
40
60
80
100
"MBAS" (μg)

Fig. 6 Representative curves illustrating the removal of acetate and pyruvate from a resting cell suspension of peptonized milk-grown cells.

Fig. 7 Representative curves illustrating the removal of butyrate and phenol from a resting cell suspension of peptonized milk-grown cells.

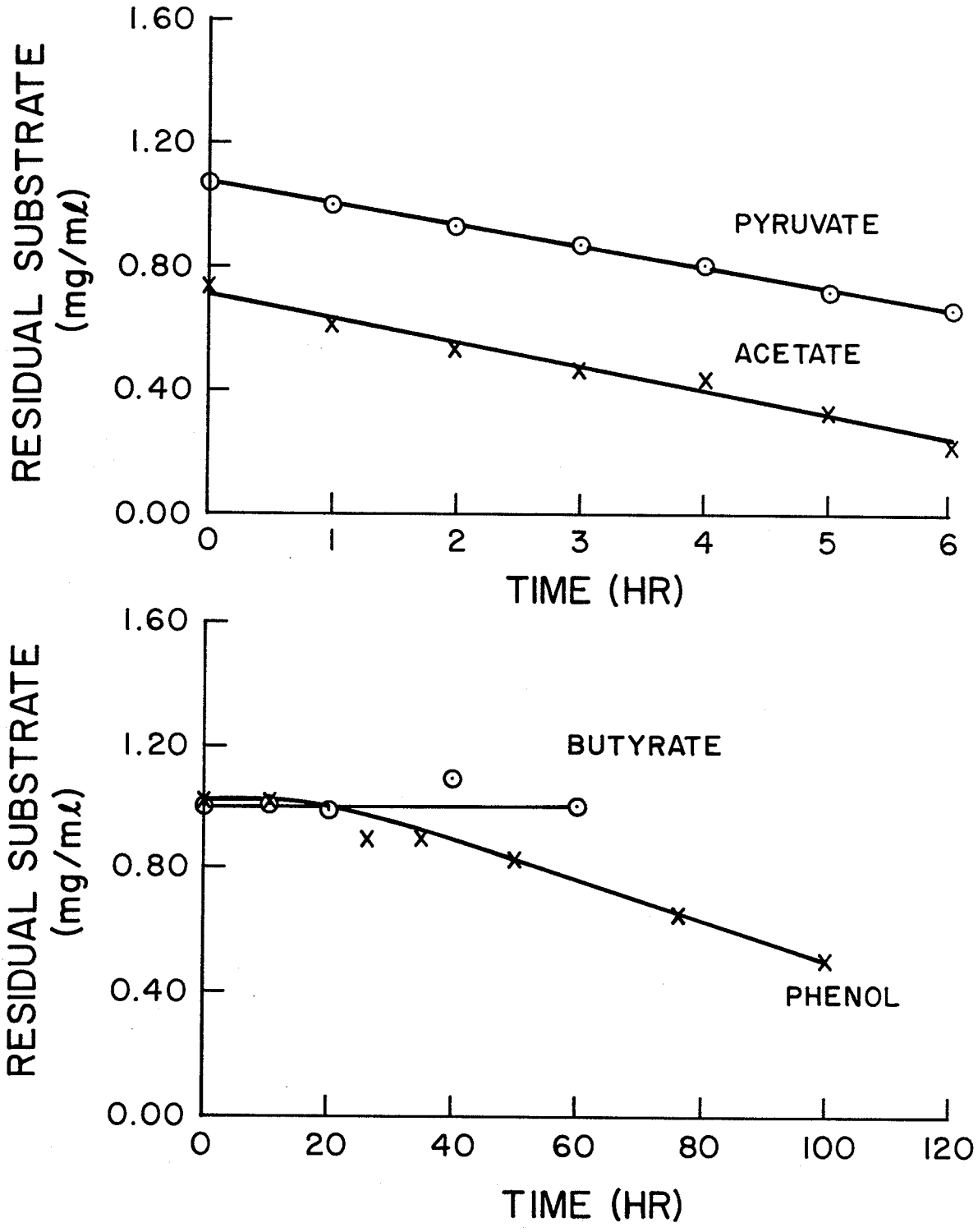

RESIDUAL SUBSTRATE (mg/mℓ)
1.60
1.20
0.80
0.40
0.00
PYRUVATE
ACETATE
0
1
2
3
4
5
6
TIME (HR)
RESIDUAL SUBSTRATE (mg/mℓ)
1.60
1.20
0.80
0.40
0.00
BUTYRATE
PHENOL
0
20
40
60
80
100
120
TIME (HR)

TABLE 2

Rates of substrate removal by resting cell suspensions as an indicator of substrate transformation

SUBSTRATE	MEDIUM*	INITIAL SUBSTRATE CONCENTRATION (mM) 1.0	5.0	10.0	20.0	50.0
Acetate	peptonized milk	-[1]	-	60.0[2]	-	65.9
	medium C	-	-	76.3	-	77.5
	synthetic sewage	-	-	87.2	-	91.3
Pyruvate	peptonized milk	-	-	75.0	-	81.6
	medium C	-	-	26.2	-	30.5
	synthetic sewage	-	-	113.7	-	114.3
Butyrate	peptonized milk	0	0	0	0	0
	medium C	9.16	7.50	0	0	0
	synthetic sewage	7.50	4.00	0	0	0
Phenol	peptonized milk	1.2	3.5	7.8	3.6	4.5
	medium C	7.00	2.40	0	0	0
	synthetic sewage	6.32	8.72	9.49	2.20	0

* - growth medium for the generation of resting cell suspension.

[1] - not determined.

[2] - µgm degraded/hr/mg dry wt resting cell suspension

rapid rates of breakdown determined were 65.9, 77.5 and 91.3 µg/hr/mg dry weight of biomass for peptonized milk-, medium C- and synthetic sewage-grown cells respectively. The rates of acetate degradation increased with increasing concentration and did not achieve their maximum rate over the concentration range of these experiments.

Pyruvate degradation also began immediately after its addition to the suspensions. The fastest rates of transformation recorded were 81.6 and 114.3 µg/hr/mg dry wt of biomass for peptonized milk- and synthetic sewage-grown cells. A much slower rate of removal 30.5 µg/hr/mg dry wt, was found for the medium C-grown cells. The data also indicated the rate of pyruvate breakdown continued to increase with substrate concentration to 50.0 mM in all suspensions regardless of their origin.

Butyrate degradation by the resting cell suspensions was detected only at the lower concentrations and then only after an initial lag period of up to 20 hours. The fastest rate of breakdown was recorded by the medium C-grown cells, 9.16 µg/hr/mg dry wt, while the most rapid rate of removal attained by the synthetic sewage-grown cells was 7.5 µg/hr/mg dry wt. No butyrate degradation was detected for cells grown on peptonized milk even at the lower concentrations during the 60 hour duration of the experiment. The effect of increasing substrate concentration during these experiments was to inhibit totally the breakdown by the medium C-, and synthetic sewage-grown populations at the 10.0 mM and higher concentrations.

Phenol removal from the resting cell suspensions was also preceded by a lag period which increased in duration with increasing substrate concentration from a minimum of 12 hours at 1.0 mM until at 50.0 mM

no breakdown was detected over the 120 hour duration of the experiment. Phenol degradation by resting cell suspensions prepared from peptonized milk-grown cells occurred at all concentrations tested reaching a rate of 7.8 µg/hr/mg dry wt of biomass at the 10.0 mM concentration. Breakdown by medium C-grown cells occurred only at 1.0 and 5.0 mM concentrations with the fastest rate, 7.0 µg/hr/mg dry wt, determined at the 1.0 mM concentration. Synthetic sewage-grown cells degraded phenol at concentrations from 1.0 to 20.0 mM attaining a rate of 9.49 µg/hr/mg dry wt.

Rate of O_2 Uptake as an Indication of Substrate Transformation

The rates of exogenous O_2 uptake by the three resting cell suspensions metabolizing acetate, pyruvate, butyrate and phenol are presented in Table 3.

These experiments indicated acetate was readily metabolized by medium C- and synthetic sewage-grown cells, 27.2 and 27.3 µl O_2 consumed/hr/mg dry wt, respectively while oxidation by peptonized milk-grown cells occurred at a rate approximately 50% slower, 13.1 µl/hr/mg dry wt.

Experiments employing pyruvate as the test substrate showed it was rapidly degraded by synthetic sewage-grown cells, 43.5 µl/hr/mg dry wt of biomass. The rates of degradation recorded by the peptonized milk- and medium C-grown cells were much slower, 24.3 and 13.4 µl O_2/hr/mg dry wt respectively.

O_2 uptake activity indicated butyrate breakdown occurred in resting cell suspensions of peptonized milk- and synthetic sewage-grown cells, however, no exogenous activity was detected in experiments employing

TABLE 3

Rates of O_2 consumption by resting cell suspensions as an indicator of substrate transformation

Substrate	Substrate conc. (mM)	Origin of resting cell suspensions		
		Peptonized milk	Medium C	Synthetic sewage
Acetate	50	13.1[1]	27.2	27.3
Pyruvate	50	24.3	13.4	43.5
Butyrate	20	11.8	0	3.43
Phenol	10	0	0	1.12

[1] µl O_2 consumed/hr/mg dry wt biomass

medium C-grown cells. The rates of O_2 consumption were much slower than those determined in experiments with acetate or pyruvate. Peptonized milk-grown cells demonstrated the greatest respirometric response, 11.8 µl O_2 consumed/hr/mg dry wt of biomass while O_2 uptake by synthetic sewage-grown cells was only 3.43 µl/hr/mg dry wt.

No oxygen uptake activity was detected for either peptonized milk- or medium C-grown cells metabolizing phenol while only negligible exogenous activity, 1.12 µl/hr/mg dry wt, was recorded by the synthetic sewage-grown cells.

Rate of CO_2 Production as an Indicator of Substrate Transformation

Representative Michaelis-Menten (65) and Lineweaver-Burk (56) plots depicting CO_2 production data by freshly prepared resting cell suspensions of peptonized milk origin metabolizing acetate, pyruvate, butyrate and phenol are provided in Figs. 8, 9, 10 and 11. The kinetic data obtained from these and similar experiments with medium C- and synthetic sewage-grown cells are provided in Table 4.

CO_2 production kinetics indicated acetate was degraded by all three cell types. Breakdown by peptonized milk-grown and synthetic sewage-grown cells possessed similar V_{max} 12.7, and 10.9 nmoles CO_2/min/mg dry wt respectively with associated K_m's of 1.67 and 0.78 mM respectively. A much faster V_{max}, 69.0 nmoles CO_2/min/mg dry wt with a K_m of 6.67 mM, was recorded by cells grown on medium C.

Pyruvate also appeared to be readily degraded by all three populations as indicated by CO_2 production data. The V_{max} for CO_2 production by resting suspensions prepared from peptonized milk-grown cells was 14.6 nmoles/min/mg dry wt with a K_m of 1.37 mM. Synthetic sewage-grown

Fig. 8 Representative data of CO_2 production by a resting cell suspension prepared from peptonized milk-grown cells metabolizing acetate.

A: Michaelis-Menten plot.

B: Lineweaver-Burk plot.

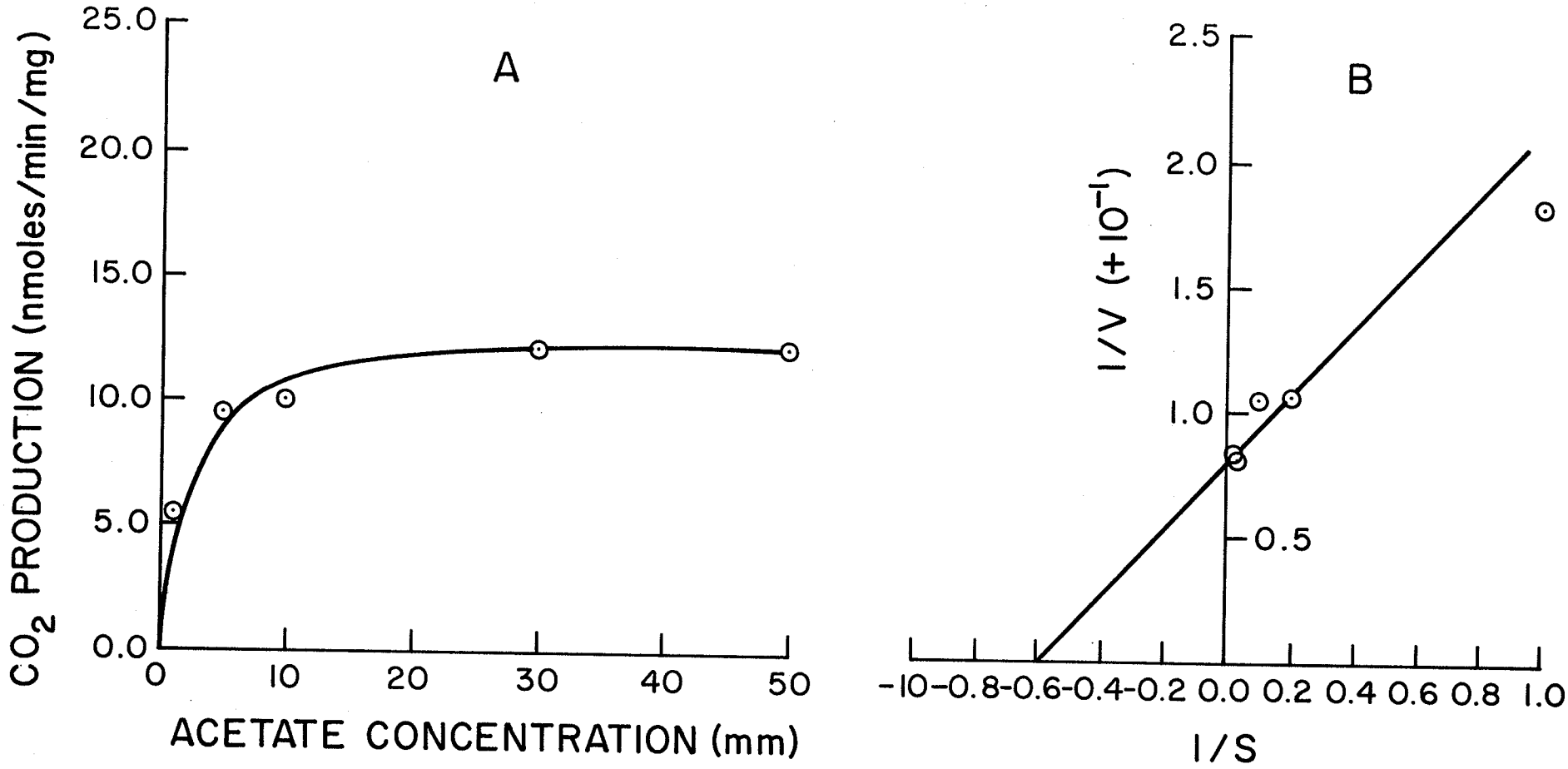
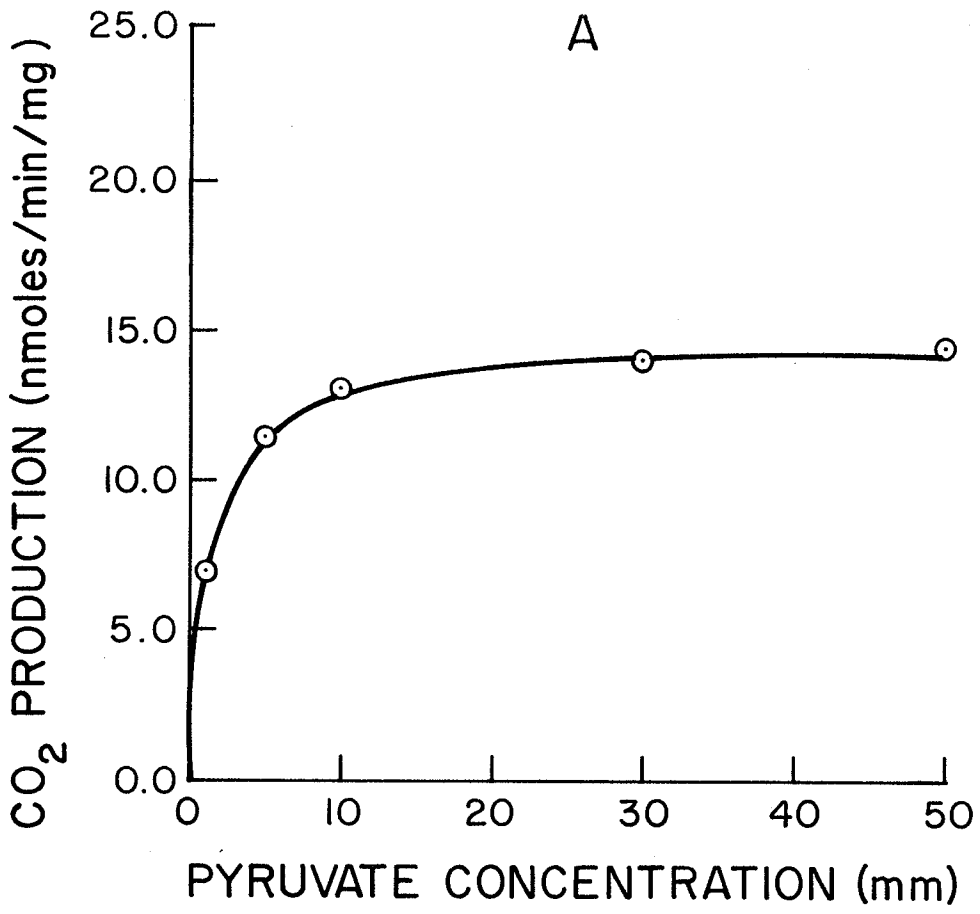

A
B
25.0
20.0
15.0
10.0
5.0
0.0
CO_2 PRODUCTION (nmoles/min/mg)
0
10
20
30
40
50
ACETATE CONCENTRATION (mm)
2.5
2.0
1.5
1.0
0.5
1/V (+ 10^{-1})
-10-0.8-0.6-0.4-0.2 0.0 0.2 0.4 0.6 0.8 1.0
1/S

Fig. 9 Representative data of CO_2 production by a resting cell suspension prepared from peptonized milk-grown cells metabolizing pyruvate.

A: Michaelis-Menten plot.

B: Lineweaver-Burk plot.


A
25.0
20.0
15.0
10.0
5.0
0.0
0
10
20
30
40
50
CO_2 PRODUCTION (nmoles/min/mg)
PYRUVATE CONCENTRATION (mm)

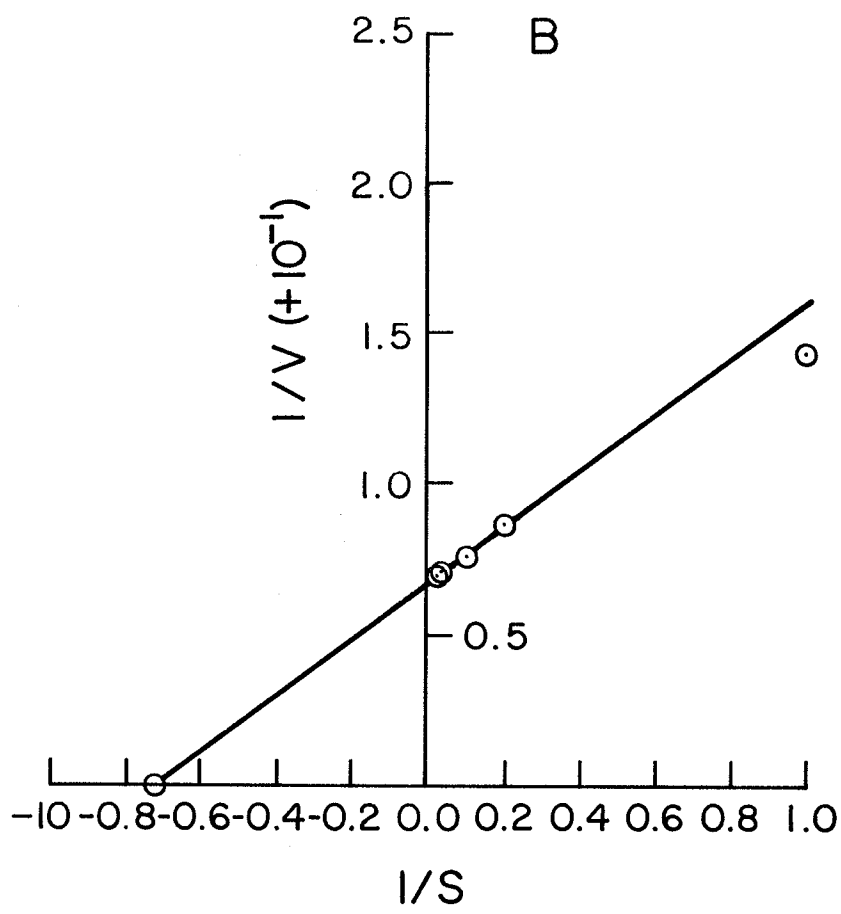
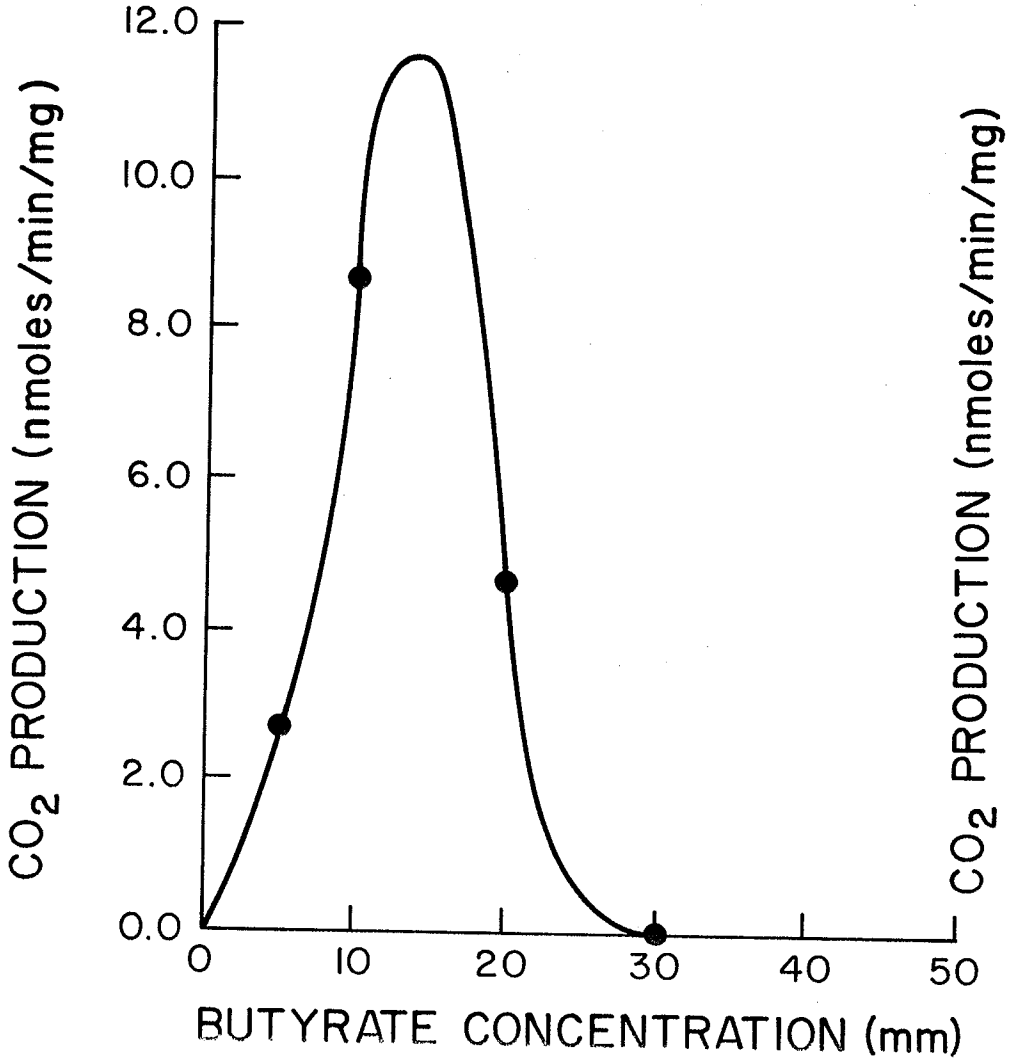

B
2.5
2.0
1.5
1.0
0.5
1/V (+10⁻¹)
-10 -0.8 -0.6 -0.4 -0.2 0.0 0.2 0.4 0.6 0.8 1.0
1/S

Fig. 10 Representative data of CO_2 production by a resting cell suspension prepared from peptonized milk-grown cells metabolizing butyrate. Data are in the form of a Michaelis-Menten plot.

Fig. 11 Representative data of CO_2 production by a resting cell suspension prepared from peptonized milk-grown cells metabolizing phenol. Data in the the form of a Michaelis-Menten plot.


12.0
10.0
8.0
6.0
4.0
2.0
0.0
CO_2 PRODUCTION (nmoles/min/mg)
0
10
20
30
40
50
BUTYRATE CONCENTRATION (mm)

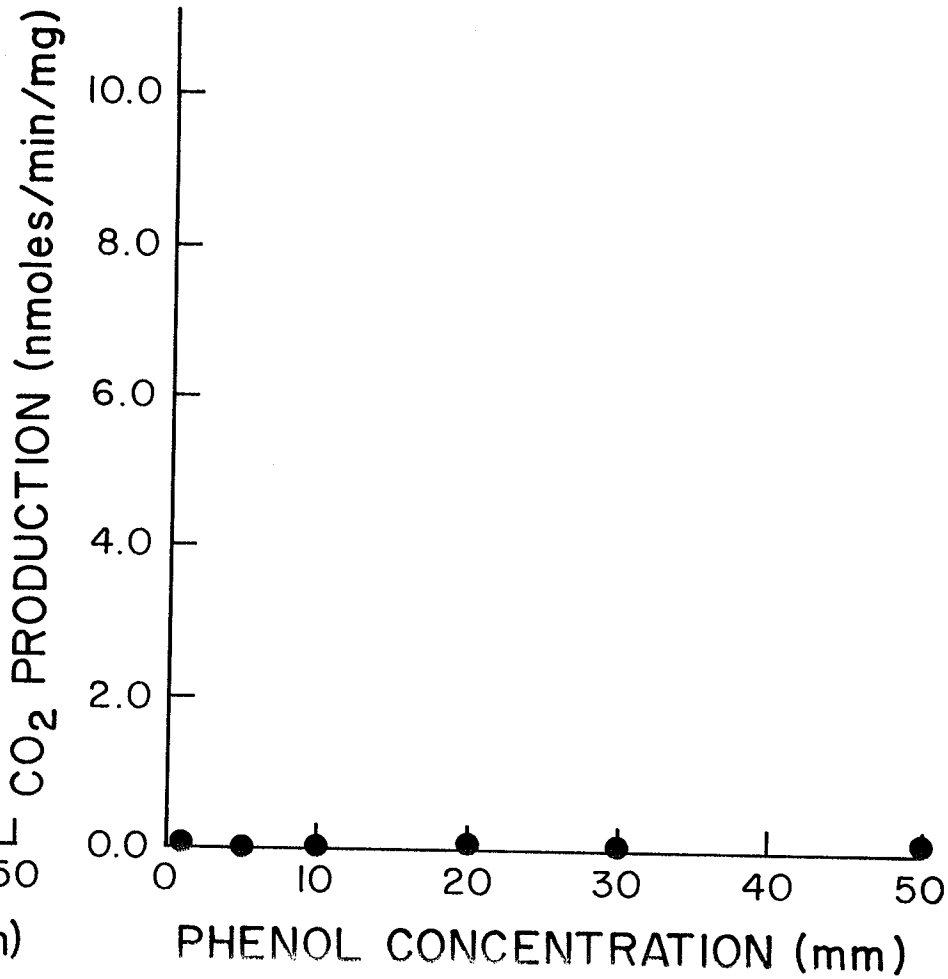

10.0
8.0
6.0
4.0
2.0
0.0
CO_2 PRODUCTION (nmoles/min/mg)
0
10
20
30
40
50
PHENOL CONCENTRATION (mm)

TABLE 4

Rate of CO_2 production by resting cell suspensions as an indicator of substrate transformation

Substrate	Origin of cell suspensions					
	Peptonized milk		Medium C		Synthetic sewage	
	K_m[1]	V_{max}[2]	K_m	V_{max}	K_m	V_{max}
Acetate	1.67	12.7	6.67	69.0	0.78	10.90
Pyruvate	1.37	14.6	8.33	49.3	1.03	34.20
Butyrate	8.0	11.6	0.90	13.0	2.50	12.50
Phenol	-	0	-	0	2.30	2.50

[1] K_m - mM

[2] V_{max} - nmoles CO_2 produced/min/mg dry wt biomass

cells possessed a much more rapid rate of CO_2 production, V_{max} of 34.2 nmoles/min/mg dry wt while the K_m was in the same range, 1.03 mM. Resting suspensions prepared from populations grown on medium C recorded a V_{max} of 49.3 nmoles CO_2/min/mg dry wt with a much higher K_m, 8.33 mM.

CO_2 production rates of the resting cell suspensions metabolizing butyrate were very similar. The V_{max} was 11.6, 13.0 and 12.5 nmoles CO_2/min/mg dry wt respectively for peptonized milk-, medium C- and synthetic sewage-grown cells. The K_m for CO_2 production was high with the peptonized milk-grown cells, 8.0 mM, while the K_m's for the populations grown on medium C and synthetic sewage were much lower being 0.90 and 2.5 mM respectively.

CO_2 production was not detected for peptonized milk- and medium C-grown cells challenged with phenol and only a negligible amount (V_{max} 2.5 nmoles/min/mg dry wt with a K_m of 2.30 mM) was detected during experiments with the synthetic sewage-grown cells.

Effect of Enrichment on the Rate of Substrate Transformation

The effect of enriching the growth media with the test substrate was studied in order to determine if it was possible to select populations in the chemostat that would more rapidly degrade the test substrate when added to the resting cell suspensions prepared from the enriched populations. The data obtained from these experiments are summarized in Tables 5, 6 and 7.

Enrichment with 0.05% sodium acetate (w/v) in the peptonized milk medium resulted in only a 5% increase from 65.9 to 69.2 µg/hr/mg dry wt in the rate of its degradation. O_2 uptake, however, increased

TABLE 5

The effect of enrichment with the test substrate on the rates of substrate removal from resting cell suspensions

Substrate	Medium	Experiment	Initial Substrate Concentration (mM) 1.0	5.0	10.0	20.0	50.0
Acetate	Peptonized milk	Substrate enriched	-	-	68.0[1]	-	69.2
	Peptonized milk	Control	-	-	60.0	-	65.9
	Medium C	Substrate enriched	-	-	45.1	-	42.2
	Medium C	Control	-	-	76.3	-	77.5
	Synthetic sewage	Substrate enriched	-	-	93.2	-	94.4
	Synthetic sewage	Control	-	-	87.2	-	91.3
Pyruvate	Peptonized milk	Substrate enriched	-	-	101.9	-	98.4
	Peptonized milk	Control	-	-	75.0	-	81.6
	Medium C	Substrate enriched	-	-	46.3	-	47.8
	Medium C	Control	-	-	26.2	-	30.5
	Synthetic sewage	Substrate enriched	-	-	72.4	-	78.7
	Synthetic sewage	Control	-	-	113.7	-	114.3
Butyrate	Peptonized milk	Substrate enriched	41.6	44.9	44.1	-	44.9
	Peptonized milk	Control	0	0	0	0	0
	Medium C	Substrate enriched	3.85	8.04	10.5	14.9	0
	Medium C	Control	9.16	7.50	0	0	0
	Synthetic sewage	Substrate enriched	10.9	14.4	10.7	0	0
	Synthetic sewage	Control	7.5	4.0	0	0	0

TABLE 5 (continued)

Substrate	Medium	Experiment	Initial Substrate Concentration (mM) 1.0	5.0	10.0	20.0	50.0
Phenol	Peptonized milk	Substrate enriched	20.5	25.5	20.8	16.6	13.8
	Peptonized milk	Control	1.2	3.5	7.8	3.6	4.5
	Medium C	Substrate enriched	6.4	11.5	8.94	4.81	0
	Medium C	Control	7.0	2.4	0	0	0
	Synthetic sewage	Substrate enriched	10.5	13.5	17.0	11.4	0
	Synthetic sewage	Control	6.32	8.72	9.49	2.22	0

[1] µg/hr/mg dry wt

TABLE 6

The effect of enrichment with the test substrate on the rates of O_2 consumption by resting cell suspensions

Substrate	Substrate Conc. (mM)	Experiment	Origin of cell suspensions: Peptonized milk	Medium C	Synthetic sewage
Acetate	50	Substrate enriched	19.9[1]	10.3	30.8
	50	Control	13.1	27.2	27.3
Pyruvate	50	Substrate enriched	31.6	24.5	53.9
	50	Control	24.3	13.4	43.5
Butyrate	20	Substrate enriched	13.1	2.61	12.3
	20	Control	11.8	0	3.43
Phenol	10	Substrate enriched	9.9	12.0	2.66
	10	Control	0	0	1.12

[1] µl O_2 consumed/hr/mg dry wt biomass

TABLE 7

The effect of enrichment with the test substrate on the rates of CO_2 production by resting cell suspensions

Substrate	Experiment	Origin of cell suspension: Peptonized milk K_m[1]	Peptonized milk V_{max}[2]	Medium C K_m	Medium C V_{max}	Synthetic sewage K_m	Synthetic sewage V_{max}
Acetate	Substrate enriched	0.95	14.6	1.08	46.7	0.13	14.7
	Control	1.67	12.7	6.67	69.0	0.78	10.9
Pyruvate	Substrate enriched	0.84	22.4	1.93	32.7	0.50	24.4
	Control	1.37	14.6	8.33	49.3	1.03	34.2
Butyrate	Substrate enriched	0.39	15.3	2.73	12.4	2.56	10.8
	Control	8.0	11.6	0.90	13.0	12.50	12.5
Phenol	Substrate enriched	0.30	9.0	0.40	3.30	4.30	8.80
	Control	-	0	-	0	2.30	2.50

[1] K_m - mM

[2] V_{max} - nmoles CO_2 produced/min/mg dry wt biomass

45.8% from 13.1 to 19.1 µl/hr/mg dry wt. while CO_2 production increased from 12.7 to 14.6 nmoles/min/mg dry wt or 15.0%. It was also noted that the K_m for CO_2 production was reduced 0.72 mM from 1.67 to 0.95 mM. Similar results were obtained with resting cell suspensions prepared from populations grown on synthetic sewage. The rate of acetate breakdown increased from 91.3 to 94.4 µg/hr/mg dry wt or 3.4% while O_2 consumption increased 12.8% from 27.3 to 30.8 µl/hr/mg dry wt and CO_2 production increased 34.9% from 10.9 to 14.7 nmoles/min/mg dry wt. A substantial reduction in the K_m for the CO_2 production from 0.78 to 0.13 mM in CO_2 was again noted. Enrichment of medium C with acetate resulted in a 41.8% reduction in the rate of acetate breakdown from 77.5 to 45.1 µg/hr/mg dry wt at the same time O_2 consumption was reduced 62.1% from 27.2 to 10.3 µl/hr/mg dry wt and CO_2 production decreased 32.3% from 69.0 to 47.7 nmoles/min/mg dry wt. The K_m for CO_2 production again was dramatically reduced from 6.67 to 1.08 mM.

Addition of the pyruvate to the peptonized milk medium selected a population which degraded pyruvate 24.9% faster than the non-enriched population, an increase in activity from 81.6 to 101.9 µg/hr/mg dry wt. In conjunction, there was a 30.0% increase in the rate of O_2 uptake from 24.3 to 31.6 µl/hr/mg dry wt and a 53.4% increase in CO_2 production from 14.6 to 22.4 nmoles/min/mg dry wt. A decrease of 0.53 mM from 1.37 to 0.84 was noted in the K_m for CO_2 production. Monitoring residual substrate indicated a 76.7% increase from 26.2 to 46.3 µg/hr/mg dry wt in the rate of pyruvate breakdown by the enriched medium C population compared to the control population. There was also a corresponding 82.8% increase in O_2

consumption from 13.4 to 24.5 µl/hr/mg dry wt. CO_2 production, however, decreased 33.7% from 49.3 to 32.7 nmoles/min/mg dry wt. The K_m for CO_2 production decreased from 8.33 to 1.93 mM. Pyruvate enrichment of the synthetic sewage medium resulted in a population which degraded pyruvate more slowly than the control population. The rate of pyruvate breakdown decreased from 114.3 to 78.7 µg/hr/mg dry wt or 31.1%. There was a corresponding 28.7% reduction in CO_2 production from 34.2 to 24.4 nmoles/min/mg dry wt with an associated 0.53 mM reduction in K_m from 1.03 to 0.50 mM. O_2 uptake, however, increased 23.9% from 43.5 to 53.9 µl/hr/mg dry wt.

Enrichment with 0.5% w/v butyrate in the growth medium substantially altered the response of all three populations when challenged with butyrate. The control peptonized milk-grown population lacked the ability to break down butyrate as seen from Table 5, however, the enriched population degraded it at a maximum rate of 44.9 µg/hr/mg dry wt. Although the control population could not degrade butyrate the substrate it appeared to stimulate exogeneous O_2 uptake. Enrichment increased this rate 11.0% from 11.8 to 13.1 µl/hr/mg dry wt. Butyrate also stimulated exogenous CO_2 production by the control population in the absence of breakdown with a maximum rate of 11.6 nmoles/min/mg dry wt with a K_m of 8.0 mM. The V_{max} was increased 31.9% to 15.3 nmoles/min/mg dry wt by enrichment and K_m was reduced from 7.61 mM to 0.39 mM. Butyrate enrichment of the medium C-grown cells increased the maximum rate of its breakdown from 9.16 to 14.9 nmoles/min/µg/hr/mg dry wt or 62.7%. The enriched population was also able to degrade butyrate at higher substrate concentrations as seen from Table 5. O_2 uptake which was not detected in the control population at 20

mM butyrate was observed to occur at a rate of 2.61 µl/hr/mg dry wt in the experiments employing the enriched population. The maximum rate of CO_2 production was only slightly reduced from 13.0 to 12.4 nmoles/min/mg dry wt, or 4.8% by enrichment. The K_m for CO_2 production increased in this experiment which agrees with the breakdown data recorded by monitoring residual substrate. Butyrate enrichment of the synthetic sewage-grown cells resulted in an increase from 7.5 to 14.7 µg/hr/mg dry wt or 96.0% in the maxiumum rate of butyrate degradation. Enrichment also promoted substrate breakdown at higher concentrations than the control (Table 5). O_2 uptake was increased 3.57 times to 12.3 µl/hr/mg dry wt from 3.43 µl/hr/mg dry wt. The effect on CO_2 production by butyrate enrichment was a reduction of 13.6% from 12.5 to 10.8 nmoles/min/mg dry wt with a corresponding reduction in K_m of 9.94 mM to 2.56 mM from 12.5 mM butyrate.

Enrichment of the peptonized milk medium with phenol selected a population that degraded the substrate 3.27 times faster than the control population. This represented an increase in the maximum rate of breakdown from 7.8 µg/hr/mg dry by the control population to 25.5 µg/hr/mg dry wt by the enriched population. The K_m for breakdown was also substantially reduced as seen from Table 7. A lag period prior to breakdown, which was evident at all concentrations in the control experiment, was not detected at concentrations of 1.0, 5.0 and 10.0 mM and the duration of the lag was dramatically reduced at concentrations of 20.0 and 50.0 mM phenol. The rate of O_2 uptake increased from nil by the control population to 9.9 µl/hr/mg dry wt with enrichment. CO_2 production which was not detected during the control experiments increased to 9.0 nmoles/min/mg dry wt with a K_m of 0.30.

Phenol-enriched medium C-grown cells showed a response similar to that of peptonized milk when compared to the control population. There was a 64.3% increase in the rate of phenol degradation from 7.0 to 11.5 µg/hr/mg dry wt. The degree of phenol inhibition of breakdown was again dramatically reduced. No lag phase was evident at 1.0 or 5.0 mM concentrations. Partial inhibition did, however, occur at concentrations of 1.0 and 20.0 mM while total inhibition of breakdown was recorded at 50.0 mM phenol. O_2 uptake increased from nil to 2.61 µl/hr/mg dry wt with enrichment while CO_2 production increased from nil to 3.30 nmoles/min/mg dry wt with a K_m of 0.40 mM phenol.

Synthetic sewage-grown cells showed a 79.1% increase in the rate of phenol degradation when enriched with phenol as compared to the non-enriched population. Total inhibition of degradation at the 50.0 mM concentration was not relieved, however, the lag period prior to breakdown was absent at concentrations from 1.0 to 10.0 mM and was markedly reduced at 20.0 mM. A very low exogenous rate of O_2 uptake, 2.66 µl/hr/mg dry wt, was detected which represented an increase of 2.38 times over the control rate of 1.02 µl/hr/mg dry wt. The V_{max} for CO_2 production increased 3.52 times from 2.50 to 8.80 nmoles/min/mg dry wt with enrichment. The K_m also increased from 2.30 to 4.30 mM.

Transformation of Selected Detergents by Resting Cell Suspensions

One of the long term objectives of this project is to develop a screening test for determining biodegradation potentials of complex organic compounds. To this end four synthetic organic surfactants previously shown to be biodegradable (51) were assayed by the methods outlined in this dissertation. The detergents used in this study were; linear alkylbenzene sulfonate (LAS), tallow alcohol sulfate (PG1), coconut alcohol ethoxylate sulfate (PG2) and tallow alcohol ethoxylate sulfate (PG3).

Fig. 12. indicates only peptonized milk-grown cells possessed the ability to degrade LAS which was 73.1% complete in 100 hours. There was no reduction in LAS concentration by either medium C- or synthetic sewage grown cells. Both medium C- and peptonized milk-grown populations showed the capacity to metabolize coconut alcohol ethoxylate as seen in Fig. 13. In experiments exploiting peptonized milk-grown cells stabilization proceeded without a lag and was 90% complete in 24 hours while in experiments employing medium C-grown cells degradation followed a short lag period and was 90% complete in 85 hours. Experiments utilizing tallow alcohol sulfate and tallow alcohol ethoxylate sulfate proved disappointing as recovery of the detergents from the cell suspensions presented technical difficulties (Tables 8 and 9). Recovery exceeded 50% in only one instance. Acidification of the samples prior to centrifugation did not release the surfactant from the cells.

Fig. 12 Removal of linear alkylbenzene sulfonate from resting cell suspensions of; peptonized milk (●), medium C (⊙) and synthetic sewage (x)-grown cells.

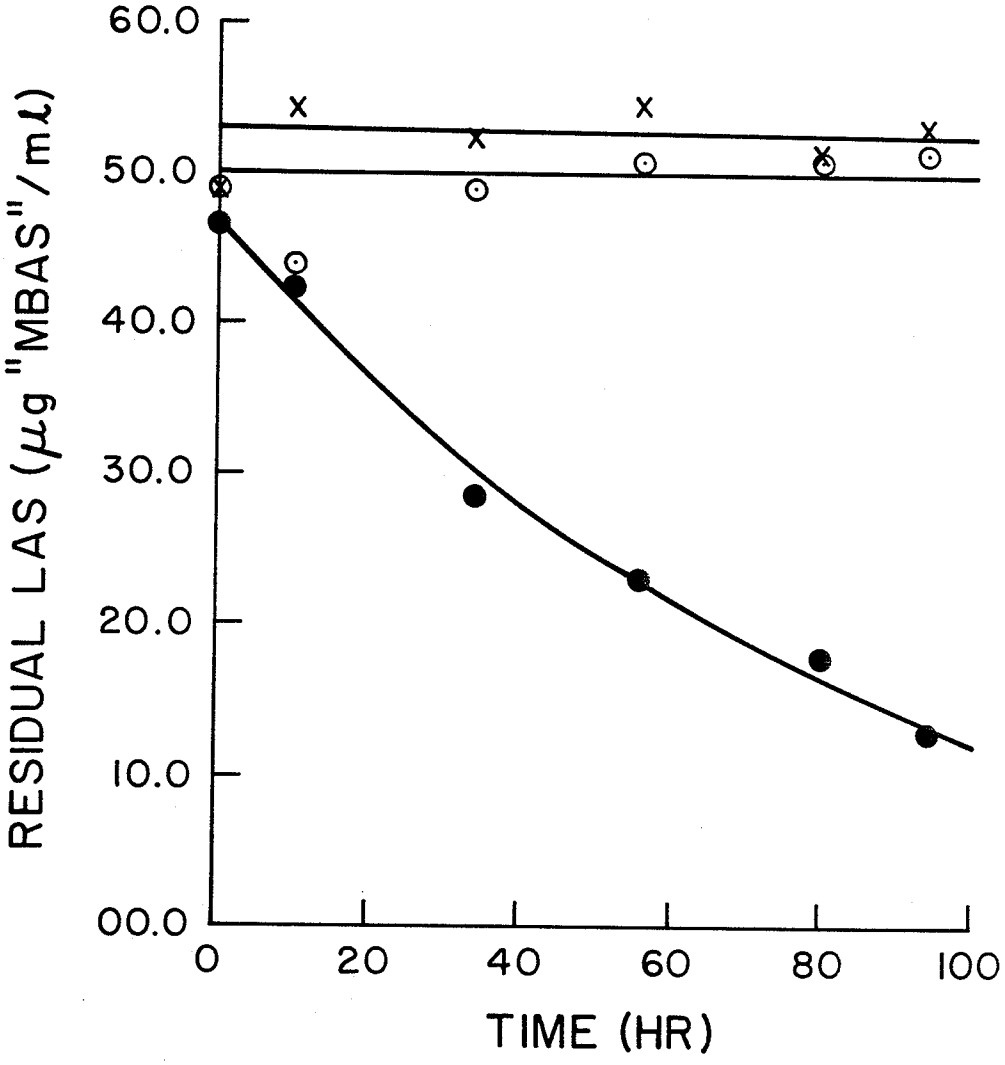

60.0
50.0
40.0
30.0
20.0
10.0
00.0
RESIDUAL LAS (μg "MBAS"/mℓ)
0
20
40
60
80
100
TIME (HR)

Fig. 13 Removal of coconut alcohol ethoxylate sulfate from resting cell suspensions of; peptonized milk (●), medium C (⊖) and synthetic sewage (x)-grown cells.

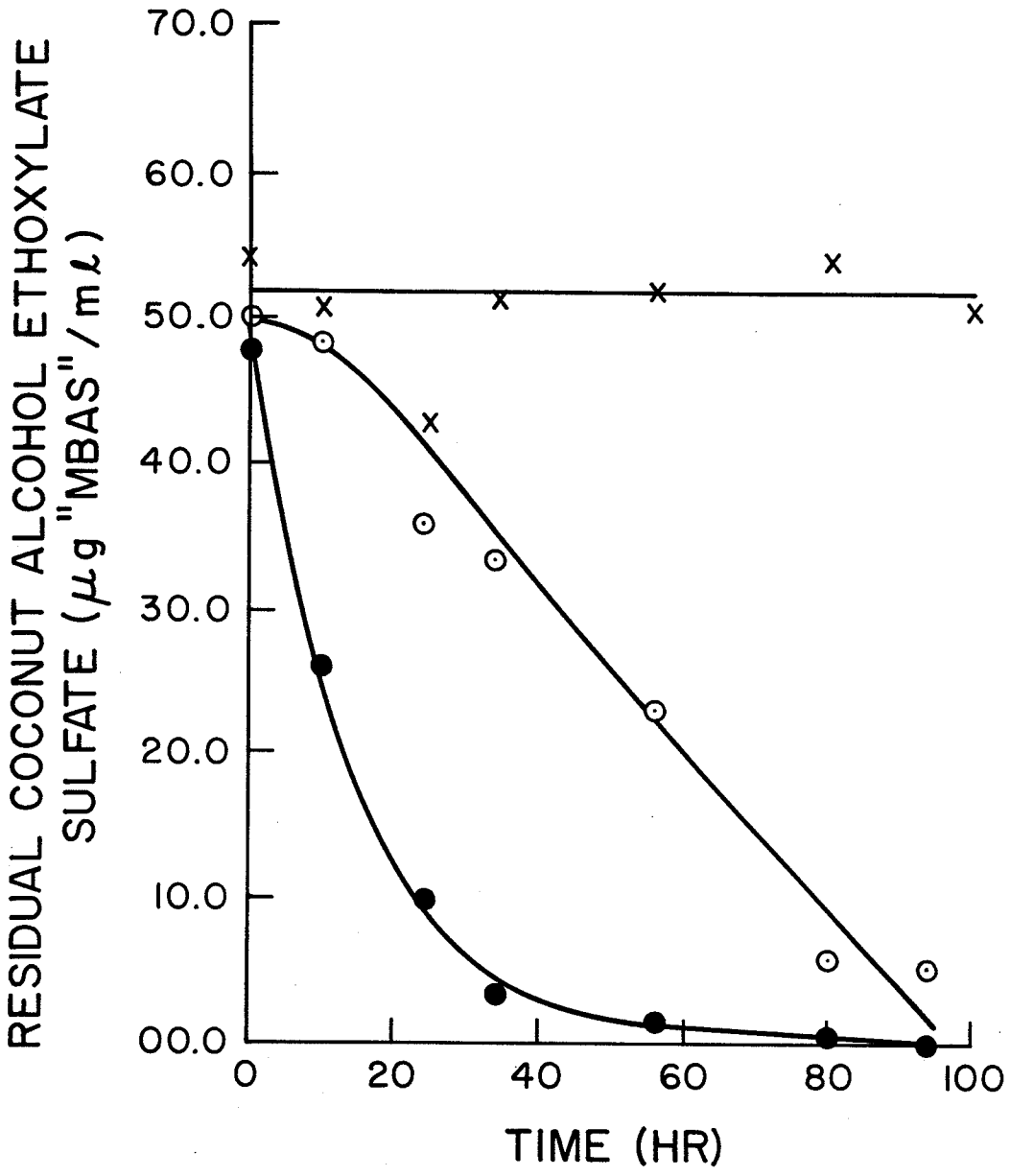

RESIDUAL COCONUT ALCOHOL ETHOXYLATE SULFATE (μg "MBAS"/mℓ)
70.0
60.0
50.0
40.0
30.0
20.0
10.0
00.0
0
20
40
60
80
100
TIME (HR)

TABLE 8

Recovery of tallow alcohol sulfate from resting cell suspensions prepared from peptonized milk-, medium C- and synthetic sewage-grown cells.

	ORIGIN OF CELL SUSPENSIONS					
	Peptonized Milk		Medium C		Synthetic Sewage	
Time (Hrs)	Amount Recovered[1]	% Recovered[2]	Amount Recovered	% Recovered	Amount Recovered	% Recovered
0	7.0	14.0	7.5	15.0	8.5	17.0
10	3.5	7.0	27.0	54.0	14.5	29.0
24	0	0	27.0	54.0	8.5	17.0
34	0	0	8.5	17.0	8.5	17.0
55	0	0	2.5	5.0	2.5	5.0
80	0	0	2.0	4.0	0	0
94	0	0	0	0	0	0

[1]µg MBAS/ml

[2]Initial amount of substrate added 50.0 µg MBAS/ml

TABLE 9

Recovery of tallow alcohol ethoxylate sulfate from resting cell suspensions of; peptonized milk -, medium C- and synthetic sewage-grown cells

	ORIGIN OF CELL SUSPENSIONS					
	Peptonized Milk		Medium C		Synthetic Sewage	
Time (Hrs)	Amount Recovered[1]	% Recovered[2]	Amount Recovered	% Recovered	Amount Recovered	% Recovered
0	9.0	18.0	14.5	29.0	15.5	31.0
10	9.0	18.0	17.5	35.0	22.0	44.0
24	2.5	5.0	11.5	23.0	18.0	36.0
34	1.5	3.0	5.5	11.0	14.5	29.0
55	1.5	3.0	4.0	8.0	12.0	24.0
90	1.0	2.0	3.0	6.0	11.0	22.0
94	1.0	2.0	3.0	6.0	10.0	20.0

[1]µg MBAS/ml

[2]Initial amount of substrate added 50.0 µg MBAS/ml

DISCUSSION

DISCUSSION

The primary objective of this project was to provide some preliminary data on the physiological potential of heterogeneous bacterial populations grown in the chemostat under steady-state conditions in order to assess the possibility of exploiting these populations as the source of active biomass for biodegradation screening tests. Fundamental to a screening test is a continuous source of readily available biomass which possesses the same physiological capacity. In the past, researchers have been forced to use various natural samples as their active biomass. However, these populations varied dramatically with respect to their physiological potential (4, 48) as changes in the growth environment led to variation in species composition and concentration in the samples. These fluctuations invariably resulted in changes in the physiological capacity of cells from samples with the result of providing data which were difficult to correlate. For this reason continuous culture techniques were exploited in this project in an attempt to provide a reproducible source of active biomass. Three media formulations; peptonized milk, Sundman and Carlberg's medium C and James' synthetic sewage, all previously shown to support growth of a wide variety of bacterial species in pure culture (44, 50, 90) and mixed continuous culture (48, 80), were used to generate the mixed populations. Although these media were able to support growth of heterogeneous bacterial populations Rerie (80) contiguous with this investigation demonstrated that the populations did not achieve true steady-state growth in the chemostat. Therefore, as true steady-state growth is fundamental to the screening test

proposed in this manuscript, further research is required to develop a medium which is capable of promoting steady-state growth.

As the main objective of this project was to investigate physiological potentials of the various populations true steady-state was not essential. However, as steady-state was not obtained one must interpret the data on a qualitative basis observing trends rather than on a purely quantitative basis.

The dilution rate, 0.04 hr^{-1}, used throughout this investigation was selected arbitrarily, simply to provide sufficient biomass for use in the biodegradation experiments yet slow enough to provide slower growing cells a chance to develop in the culture. Altering the dilution rate, as illustrated by Meers (61), would have a dramatic affect on the chemostat population. A slower rate would likely moderate any fluctuations appearing in the population and provide a more heterogeneous population as slower growth would provide a competitive advantage to those organisms which were efficient at substrate uptake. In contrast, a faster dilution rate would wash out the slower growing species and would likely result in a less heterogeneous population. A slower dilution rate would also lead to more efficient utilization of the media resulting in a more dense population, whereas, a faster rate would result in a thinner population with a higher concentration of substrate in the effluent. These factors were considered with respect to obtaining sufficient biomass to perform the biodegradation tests.

Resting cell suspensions prepared from the heterogeneous microbial populations described above were utilized as the active biomass in degradation studies rather than actively growing cells. The rationale behind this procedure was resting cell suspensions can be prepared to a

very high cell density thereby reducing the duration of the biodegradation tests. The stability of the cells in resting suspensions was discussed in detail by Rerie (80) who indicated the populations remained suitably stable for up to seven days, retaining approximately 50% of their initial physiological activity at 20°C. However, as the loss of activity was neither linear nor curvilinear the activity rates could not be mathematically corrected for loss of activity with time.

It might be useful at this time to point out the populations utilized in this investigation were standardized on a dry weight basis. Although this is not the most precise method of standardizing active biomass it was selected because of expedience and the lack of a suitable alternative. The use of the Coulter counter or standard plating methods was rejected due to some clumping of cells in the resting cell suspensions. Measurement of cellular components which has been used to assess active biomass (94, 103) was eliminated because these components vary with growth conditions. A number of reports have indicated cellular enzymatic content bears a linear relationship to active biomass (33, 74, 75, 76, 86, 87). This method was rejected because the selected enzyme or enzyme system may not be "universal" to all of the cells comprising the mixed populations. Endogenous respiration has also been used to measure active biomass (24) but use of this technique appears to have a questionable basis since a reduction in respiration results from depletion of energy reserves as well as loss of cell viability. Bashucky (4) used O_2 uptake on casamino acids, a broad spectrum substrate, to standardize mixed microbial populations. However, use of this method was rejected as O_2 consumption per unit biomass on casamino acids varied with the growth conditions used in the investigation.

It has formerly been proposed (3) that cellular ATP is the best method of evaluating active biomass. This is supported, with respect to resting cell suspensions, by the work of Rerie (80) who concluded measurement of ATP was superior to standard plating methods for measuring viable biomass.

Three methods for evaluating substrate breakdown; (1) monitoring residual substrate, (2) measuring O_2 uptake and (3) measuring CO_2 production were compared in this investigation. O_2 uptake experiments were performed at concentrations which would provide maximum activity as indicated by the CO_2 production data in order that situations involving substrate inhibition were not encountered. It was concluded that direct analysis of residual substrate is a better parameter for measuring catabolism than monitoring either O_2 uptake or CO_2 production. The latter two methods did provide accurate information with regard to the readily metabolizable substrates, acetate and pyruvate. However, since these experiments were conducted over the first 2 hours after addition of the substrate to the biomass they did not and will not provide much useful information when lag periods are encountered or when the rate of breakdown is so slow that the exogenous activity is too low to detect. If O_2 uptake or CO_2 production are to be used as a parameter for measuring substrate stabilization then perhaps monitoring total O_2 uptake or CO_2 production over the duration of the experiment should be considered as opposed to monitoring activity during the initial period after addition of the test substrate to the resting cell suspensions.

Monitoring actual residual substrate as the index of decomposition though superior to using indirect methods has two major disadvantages.

The first is that analysis of samples is often very tedious and time consuming. The second and most important disadvantage is that methods for measuring chemicals both natural organic and synthetic organic, often require sophisticated methods and/or expensive equipment which are not always available.

Although the rate of CO_2 production is commonly used in physiological studies little information is available which specifically relates the velocity of CO_2 production to substrate concentration. Kay (48), in the development of the infrared CO_2 analysis technique employed in this investigation, discovered that the relationship between the evolution of CO_2 by resting bacterial suspensions and substrate concentration could be described by Michaelis-Menten enzyme kinetics (65). Application of Michaelis-Menten kinetics to whole cell systems has also been used by Monod (66, 67) to describe bacterial growth, by Parsons and Strickland (73) to measure uptake of organic material by heterotrophs and by Longmuir (57) to study the rate of bacterial respiration as a function of O_2 concentration. The premise behind exploiting Michaelis-Menten kinetics in whole cell studies is that at steady-state in a particular reaction pathway the concentrations of the intermediates are small and remain constant to correspond to the total flow of metabolites, therefore, all reactions must proceed at the same rate. Dixon and Webb (23) and Hearon (37) concluded the velocity of such a metabolic line is determined by the rate of reaction of the slowest step in the sequence. In a series of enzymatic reactions:

$$A \xleftrightarrow{E_1} B \xleftrightarrow{E_2} C \xleftrightarrow{E_3} D \xleftrightarrow{E_4} \ldots$$

if E_3 is slower than E_1, E_2 and E_4 then intermediate C would accumulate.

Accumulation and increase in concentration of C would tend to accelerate conversion of C to D according to the Michaelis-Menten equation

$$V = \frac{V_{max}\ S}{K_m + S}$$

and decelerate E_2 by increasing the rate of feedback inhibition. This leads to a process of equilization of velocities, a conclusion which is compatible with cellular regulatory mechanisms. Thus the rate of the above reaction is controlled by the rate of the key enzyme E_3 while the other reactions merely keep up with it. This is the pacemaker concept of Krebs and Kornberg (49). Each enzyme in the series will have its own characteristic V_{max} and K_m while the amount present in the cell itself will depend upon the environmental conditions. Under any particular environment the enzyme with the lowest V_{max} will become the limiting or pacemaking enzyme in the system and its maximum velocity will become the maximum velocity of the reaction under substrate saturating conditions and the substrate concentration of each preceding or succeeding enzyme will adjust itself to that point on the Michaelis-Menten curve which gives that rate. Pardee (71) reviewing enzyme mechanisms and activity concluded that, in view of the cell's ability to regulate the synthesis and activities of all enzymes, adjustment of enzyme reactions to keep pace with the slowest reaction is conceivable.

Experimental data indicated that of the three populations tested the peptonized milk-grown population possessed the broadest physiological spectrum. These cells were capable of oxidizing all three simple organics - acetate, pyruvate and butyrate, the aromatic, phenol and two of the four surfactants tested. In addition the rates of activity

achieved by the peptonized milk-grown cells were generally greater than the others. The medium C-grown cells not only commonly possessed slower rates of activity but also degraded only one of the four surfactants. The synthetic sewage-grown cells were able to oxidize only the natural organic substrates. As the peptonized milk-grown population was able to degrade, at measurable rates, representatives of simple and complex organic and synthetic organic substrates it was concluded that some laboratory-grown heterogeneous microbial populations possess sufficiently diverse physiologies to be effectively utilized as the source of active biomass in biodegradation screening tests. However, in order for a population to be effectively utilized in the proposed screening test further research is required to develop a medium which is able to promote steady-state growth in the chemostat in addition to generating a population possessing a broad physiological capacity.

Oxidation of acetate, pyruvate and butyrate involve constitutive enzyme systems. However, degradation of phenol and other more complex aromatic substrates requires induced enzyme systems whose induction requires prior exposure of the cells to that substrate or some other related compound (25, 27, 41, 70). As such transformation of these chemicals requires longer periods of time. This extended time frame introduces a technical problem into the proposed screening test, that being loss of catabolic activity of the resting cell suspensions during the period of enzyme induction. In order to solve this problem the effect of enrichment with the test substrate in the growth medium was examined. It was hoped enrichment would both select a population with a greater proportion of species capable of oxidizing the test substrate and promote enzyme induction during the growth phase rather than having to wait until the

cells were exposed to the substrate in the resting suspensions. As steady-state growth in the chemostat was not attained and the physiological capacity of the populations varied (80) absolute quantitative comparisons between the control and enriched populations was not possible.

The effect of enrichment by the simple organics acetate and pyruvate varied. Acetate enrichment of peptonized milk and synthetic sewage increased the rate of its degradation while in experiments with medium C-grown populations enrichment decreased the rate of degradation. However, these changes were not sufficiently great to be attributed to enrichment but rather to variations between the control and enriched populations resulting from the absence of steady-state growth. Pyruvate enrichment of peptonized milk-grown and medium C-grown populations increased their ability to degrade the test substrate and sufficiently great enough to be attributed to enrichment. Enrichment of the synthetic sewage medium resulted in reducing the rate of pyruvate breakdown, however, this change was not attributed to enrichment. As these substrates are chemically very simple, found in many natural environments and are natural metabolic intermediates enrichment was not expected to significantly alter the physiology of the populations with respect to their maximum rates of stabilization.

Butyrate enrichment increased dramatically the ability of all three populations to degrade the substrate. The change was far too great to be attriubted to variations in the steady-state populations. Butyrate enrichment also considerably reduced the inhibitory action observed in experiments with the control population.

Phenol enrichment also dramatically altered the response of the three populations. The maximum rate of breakdown was increased substantially which was supported by O_2 uptake and CO_2 production data.

A second very interesting observation became evident during

enrichment studies. With the exception of the medium C-grown population metabolizing butyrate and the synthetic sewage-grown population degrading phenol, both CO_2 production and where applicable, residual substrate studies indicated substantial reductions in the K_m of substrate being evaluated. That is, the enriched populations were capable of degrading the substrates at lower concentrations and much more rapidly than the controls, thus indicating a selection of species in the culture capable of more effective utilization of the substrate.

It is, therefore, concluded enrichment with the test substrates substantially alters the chemostat population. Although the maximum rate of reaction may not be significantly increased the population becomes much more efficient at utilizing the enriching substrate since the K_m values for the metabolism of the enriching substrate is significantly reduced. Enrichment also served to reduce substrate inhibition and stimulated enzyme induction which is essential if resting cell suspensions are to be used in the proposed screening test as the active biomass.

As an indication of the ability of the laboratory grown populations to degrade the more complex chemicals which are creating the environmental problems of today four representative anionic surfactants; linear alkylbenzene sulfonate, coconut alcohol ethoxylate sulfate, tallow alcohol sufate and tallow alcohol ethoxylate sulfate were assayed for biodegradation by the methods described herein.

Only the peptonized milk-grown population possessed the ability to degrade LAS, the disappearance of which followed the same pattern as that indicated by Ishaque (43) who used a similar experimental procedure but employed resting cell suspensions of lagoon water samples. Both peptonized milk- and medium C-grown cells showed the capacity to degrade coconut alcohol ethoxylate sulfate. Stabilization by peptonized

milk-grown populations occurred with no lag period while a slight lag was observed in the experiments with medium C-grown cells. Lian (51) studying the stabilization of anionic surfactants under psychrophilic conditions demonstrated breakdown of this substrate with a lagoon population, however, only after a lag period of 30 hours. Ishaque (43) had indicated the lag period prior to detergent stabilization could be substantially reduced with increased temperature. We, therefore, conclude stabilization of coconut alcohol ethoxylate sulfate by the laboratory populations would be fairly representative of that by a lagoon population at the same temperature.

Results in experiments utilizing tallow alcohol sulfate and tallow alcohol ethoxylate sulfate indicated the surfactant was binding to the cells in the reaction vessels as less than 50% of the surfactant added to the cell suspension was recoverable at time zero. Reduction in substrate concentration could not be attributed to breakdown rather simply to the detergent binding to the cells and subsequently removed by centrifugation. Attempts to release the substrate from the cells by acidification prior to centrifugation proved unsuccessful.

This study has dealt with only a limited number of test substrates, however, the results indicate that the overall concept and procedure has potential as a biodegradation screening test. The resting cell suspensions possessed the capacity to oxidize some simple and more complex natural organic and some synthetic organic substrates. Experimental data indicate enrichment with the test substrate in the growth medium may increase the rate of stabilization and reduce the duration of lag periods by inducing enzyme systems required to degrade the substrates. Further work is necessary in developing a growth medium that will

promote steady-state growth of heterogeneous populations, in testing a much greater variety of substrates especially complex synthetic organics and in comparing the laboratory data to similar data obtained from natural populations.

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