STABILIZATION OF SELECTED ORGANIC SUBSTRATES BY SEWAGE LAGOON BACTERIA AS A FUNCTION OF DISSOLVED OXYGEN CONCENTRATION

BY

WILLIAM STEVEN BASHUCKY

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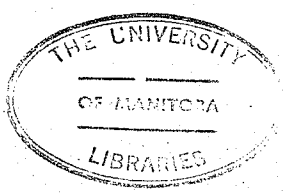

ABSTRACT

The effect of dissolved oxygen (DO) concentration on the stabilization of organic compounds by bacteria indigenous to a mid-continental sewage lagoon was studied. Resting cell suspensions of bacteria recovered from lagoon water for both summer and winter conditions were subjected to automatically controlled dissolved oxygen in the presence of selected substrates. Dissolved oxygen levels that would be non rate-limiting for oxidative breakdown were determined.

For summer samples at 25°C the highest DO concentration required of the substrates tested was $\simeq$ 2 ppm for propionate oxidation. A DO concentration of 0.5 ppm did not limit degradation of butyrate and ethanol. LAS detergent, phenol and acetate utilization was not affected by a DO concentration of 0.24 ppm.

The DO requirement of winter samples at 2°C for propionate was lower, being $> 1.0 \leqslant 1.5$ ppm. A concentration of 0.5 ppm DO did not retard the rate of breakdown of acetate, butyrate, ethanol, phenol and benzoate. LAS was not metabolized by winter bacteria.

When a suspension of psychrotrophic lagoon bacteria was challenged with a mixture of substrates under fully aerobic conditions there was an indication of a diauxic effect.

A manometric procedure to correct for fluctuations in biomass provided a reasonable estimate of bacterial activity. The method was capable of generating an activity constant for the rate of breakdown of test substrates by lagoon bacteria.

The effect of DO concentration upon sewage lagoon management as a function of seasonal change is discussed.

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TO DEBBIE

TABLE OF CONTENTS

Table of Contents Continued

LIST OF TABLES

LIST OF FIGURES

List of Figures Continued

List of Figures Continued

INTRODUCTION

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Strict aerobic microorganisms require certain minimum or "critical" levels of dissolved oxygen (DO) in the suspending medium in order to carry out various oxygen-dependent metabolic functions. The greater proportion of information in this area has been obtained from studies employing pure cultures. In wastewater treatment processes the resident microflora represents a great diversity of physiological and taxonomic types. Each species will demonstrate a particular oxygen requirement for each oxygen-dependent substrate it is able to metabolize. But it is of great practical importance that the overall oxygen demand of the heterogeneous population be met to achieve efficient wastes purification where a wide variety of substrates is available.

The practice of artifically aerating sewage lagoons to augment the supply of oxygen has increased in recent years. The operation and design of such systems has been controlled mainly by engineering technology with little consideration given to the actual biological requirement for dissolved oxygen. The present study was

undertaken to explore the oxygen requirements of mixed cultures of sewage lagoon bacteria. Experiments were carried out to determine the minimum dissolved oxygen levels that would support maximum rates of breakdown of selected substrates by bacteria indigenous to a sewage lagoon.

HISTORICAL

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In biological oxidations molecular oxygen acts as a principal terminal electron acceptor in the energy yielding metabolism of substrates by microorganisms. Since dissolved oxygen (DO) concentration is one of the parameters frequently controlled in industrial operations involving microbial activity such as waste treatment processes it is desirable that the oxygen requirements of the microbial agents be known.

Considerable research effort in both basic and applied fields has been expended in studying the effect of DO concentration on microbial growth and metabolism. Early workers (21, 48, 79) using manometric techniques with resting cell suspensions of bacteria and yeast observed that at oxygen concentrations below a threshold value the rate of oxygen uptake by cells decreased. The point at which respiration rate became limited by oxygen concentration was termed the "critical oxygen tension" by Gerard and Falk (30). Wide variations in critical tensions reported were later partly attributed to measurement of partial pressure of oxygen in the gas phase over the culture rather than dissolved oxygen. It

is now known (38) that in systems of this type, resistance to oxygen diffusion at the gas-liquid interface can result in dissolved oxygen tensions in solution being lower than the partial pressure in the gas phase.

Direct measurement of DO was first achieved by Baumberger (2) in 1939. He employed a dropping mercury electrode in studying washed yeast suspensions and concluded that oxygen uptake was independent of oxygen concentration. Winzler (93), using the same technique on yeast cells, found the critical DO concentration in the absence of an energy source to be very low being 0.01 mg/l DO at 30°C (0.2 mmHg)[1], while with substrate present it was ten times higher. He believed the rate-limiting process for respiration at low DO concentrations to be the rate of combination of oxygen with the oxygen transferring enzyme, presumably cytochrome oxidase.

From the determination of apparent K_m (Michaelis constant) values for oxygen in intact cells and cell-free extracts of large bacteria (Bacillus megaterium) and small bacteria (Klebsiella aerogenes), Longmuir (50) concluded that diffusion of oxygen through the cell material to oxidative sites was at least partly rate-

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[1]The concentration of DO may be expressed in mg/l which is ≡ ppm; the corresponding equivalent partial pressure of DO is in mmHg and is referred to as DO tension.

limiting at low oxygen concentrations.

It was later recognized by Johnson (46) that at higher DO levels when uptake is independent of concentration, respiration is controlled by some factor other than cytochrome saturation. This factor, which was termed the "oxygen demand of the cell" is regulated in part by the supply of intermediary metabolites such as adenosine diphosphate (ADP) and reduced nicotinamide adenine dinucleotide ($NADH + H^+$). Johnson also pointed out that the quantity of respiratory enzyme, which is likely to vary with growth conditions, could restrict the ability of the cells to utilize oxygen. He predicted, as had earlier workers (2, 13, 50, 93), that the regulation of metabolism by dissolved oxygen would be in the form of a single enzyme-catalyzed reaction. That is, when the DO falls below the critical level, the lack of saturation of the respiratory enzyme with oxygen results in a lowered reaction velocity so that the rate of reductant supply is not sufficient to meet the oxygen demands of the cell. He maintained that when low levels of oxygen are present, respiration might become limited by the resistance to oxygen diffusion presented by some intracellular barrier. It has been shown (4) that extracellular diffusion of oxygen from the bulk of the

suspending medium to cell surfaces could be regarded as being negligible in most agitated microbial systems.

Recently, Harrison (38) has proposed that data leading to Johnson's hypotheses could equally well be explained by the multi-enzyme model presented by Chance (14) which takes the interactions of the whole respiratory chain into account. In this model, the first component of the chain, the terminal oxidase, starts to become reduced at oxygen concentrations above the critical level and the second and third components also display some reduction. The fourth component, however, remains more or less in a steady state until the critical oxygen level is reached.

Investigation into the adaptive response of growing microorganisms to DO concentration was greatly aided by the application of continuous culture techniques and the development of reliable dissolved oxygen electrodes. This led to work in 1966 by Maclennan and Pirt (53) who outlined a method for automatically controlling the DO concentration in stirred microbial cultures over the range of 0.01 - 1.4 mg/l DO at 30^{o}C (0.26 - 30 mm$Hg0_2$) by changing the partial pressure of oxygen in the gas phase while keeping the total flow constant. It was not until 1964 that the problems of instability and low output currents associated with earlier oxygen

probes (10, 51) were overcome. In that year Mackereth (52) described an improved membrane bound galvanic cell which was selectively permeable to oxygen in solution and which remained stable for long periods of time. An autoclaveable version of the electrode has also been developed (89).

Harrison and Pirt (39) were the first to use such a membrane electrode in studies into the effect of oxygen on the metabolism of microorganisms in continuous culture. They found that for the facultative anaerobe Klebsiella aerogenes NCTB 8017, there was a critical DO between 0.46 - 0.7 mg/l DO at 30°C (10 - 15 mmHg) above which respiration rate was constant. Below this level the organism demonstrated a stimulation of oxygen uptake and the products of glucose metabolism started to change to those typical of anaerobic metabolism. Increased rates of respiration under conditions of low oxygen concentration have been further reported for Klebsiella aerogenes (41, 42, 43), and also for Escherichia coli (41, 42, 61), Pseudomonas AM1 (55) and Hemophilus parainfluenzae (92). The same phenomenon has also been observed under certain conditions in the fungus Aspergillus nidulans (12) and in the yeast Candida utilis (62).

Prompted by their own observations (39) and those of others, Harrison and Pirt modified the original (30) definition of critical DO tension to become: "the oxygen tension above which the respiration rate of an organism is independent of changes in DO; below this tension the oxygen uptake rate of the organism may increase or decrease in response to a decrease in oxygen tension, according to cultural conditions".

The increase in oxygen uptake at low DO has been ascribed to various causes by different authors, with the recognition that the overall mechanism is one of controlling cellular energy balances. Harrison and Pirt (39) theorized that this stimulation might be caused by a decrease in ATP production resulting from either an alternate electron transport pathway becoming operational or a loss of coupling at a site of oxidative phosphorylation. Harrison and Maitra (43) expanded on this and suggested that energy production via oxidative phosphorylation becomes less efficient at low DO concentrations and that an increased rate of respiration is required to maintain a tight control over the ATP/ADP ratio. Any change in ATP level is then corrected for by a feedback type of response which alters the ratio of substrate oxidized to substrate used for anabolism.

Apparently a high level of ATP favors anabolism and a low level favors catabolism.

Responses of this type under reduced DO concentration, however, are not the usual case reported for the wide majority of studies on oxygen-linked metabolism by various microorganisms. The most predominant observation is that respiration and metabolism proceed independent of DO until very low levels are reached. Decreases in oxygen concentration below this critical level then result in a slowing of metabolic reactions.

Button and Garver (8) found the affinity constant (K_s) for oxygen of a chemostat culture of Torulopsis utilis growing on glycerol to be 0.45 ppm 0_2 (1.4×10^{-5}M). Later work by Johnson (46) on Candida utilis grown on acetate indicated a K_m for oxygen of 0.04 ppm (1.3×10^{-6}M). The results of other workers (6) are in good agreement with the findings of a low oxygen requirement in this yeast. Saccharomyces cerevisiae has been shown to carry out oxidative metabolism at levels as low as 0.06 - 0.12 ppm 0_2 (5, 73).

Carter and Bull (11, 12) used an automatic DO control system (53) in continuous cultures of Aspergillus nidulans growing on glucose and found that the critical level for this fungus was 0.08 mg/l DO at 30°C (1.75 mmHg).

Below this level nitrite accumulated in the medium and changes in morphology were noted.

Harrison, Maclennan and Pirt (40) studying continuous cultures of Pseudomonas DX2, and Maclennan and Pirt (54) using the same organism under automatically controlled DO, determined the growth limiting or "critical" DO concentration was 0.04 - 0.3 mg/l DO at 30°C (1.0 - 7.0 mmHg) for decane as substrate and < 0.04 mg/l with glucose as the carbon source. Their interpretation of the data suggested that for growth of this organism on glucose, two oxygen uptake enzyme systems or two states of the same system were involved. The reaction dominating at low DO below 0.17 mg/l DO (4.2 mmHg) would have an increased affinity for oxygen with an apparent Km of only 0.01 mg/l DO (0.2 mmHg). For growth on decane they found that below the critical level respiration rate decreased with an increase in decane utilization occurring around 0.12 mg/l DO (3 mm Hg). An increase in the formation of extracellular products from the incomplete oxidation of decane was detected at this point. The authors deduced that at low DO there was either an increase in the amount of oxygenase in the cell or that there was some release of inhibition on the enzyme.

Maclennan et al (55) employed essentially the same methods as previous workers (54) on continuous cultures of the strict aerobe Pseudomonas AM1. The critical DO for growth on methanol was 0.28 mg/l DO at 30°C (7 mmHg). Below this value the culture became oxygen-limited and residual quantities of methanol increased.

For the facultative anaerobe Beneckea natriegens respiration and glucose oxidation were recently (49) found to be independent of DO down to 0.08 mg/l DO at 30°C (2 mmHg). Only when the oxygen supply rate fell behind the oxygen demand rate was there a switch to fermentative metabolism.

Studies on the oxygen requirements of species of aerobic psychrotolerant Pseudomonas and Achromobacter have shown that the oxygen concentration of the suspending medium could be lowered to 0.17 mg/l DO at 22°C (2% saturation) without noticeably inhibiting growth rate (18).

It is of interest to note that in many instances (12, 18, 92) when microorganisms are grown under low oxygen concentrations an adaptive response in the form of a decreased requirement for oxygen (lowered critical DO) becomes apparent. The synthesis of cytochromes by both obligate aerobes and facultative organisms is

strongly influenced by the environment, and in particular, by the degree of aeration. Many reports of maximum respiratory enzyme content under low DO concentration have appeared in the literature (20, 40, 61, 62, 63, 75, 77, 80). Meyer and Jones (59, 60) estimated the efficiency of energy production via oxidative phosphorylation in several species of bacteria selected on the basis of their cytochrome oxidase composition. In summarizing their results they concluded that, at low levels of DO, oxidases associated with respiratory chains of low energy conservation efficiency were synthesized in greater amounts; that oxidases with increased affinities for oxygen became operative so that energy production could proceed, although inefficiently, at very low oxygen concentrations was yet another possibility.

Information on the response of mixed cultures to DO concentration is of practical importance in the field of water pollution control. The three basic methods presently employed in waste treatment are the trickling filter (7, 58), the activated sludge process (15, 82), and the oxidation or stabilization pond (68). One of the more recent innovations is the aerated lagoon (57, 76), a system initially developed to supplement the supply of oxygen during the period of spring break-up, and to alleviate nuisance odours by artificially aerating stabilization ponds. Although specialized engineering structures and

mechanical equipment for the variations in these methods vary widely, the fundamental process of aerobic biological decomposition of organic wastes remains basically unchanged from system to system.

A sewage lagoon might best be described as a continuous-flow enrichment culture of microorganisms. A wide variety of microbial types are present in mixed culture with the predominating species being determined by the characteristics of the waste input and environmental influences such as climate and operational procedures. It is known that bacterial numbers, activities and biotic types vary in response to seasonal changes (36) and other parameters (85). In a lagoon system several factors mediate the amounts and types of substrates present but the overall homogeneity of the organic loading in lagoons serving domestic communities results in a fairly constant substrate supply. The pH in this instance has been shown to fluctuate only moderately (36).

Two of the most important physical factors affecting the efficiency of aerobic wastes stabilization would appear to be temperature and oxygen supply. The efficiency of a waste treatment process is usually reported as the percent removal of biochemical oxygen demand over a five-day test period at 20°C (BOD_5). Temperature

has been shown to effect BOD_5 reduction in varying degrees for activated sludge units, stabilization ponds and aerated lagoons (19, 22, 34, 70). Vennes and Olson (90) found no difference in BOD_5 reduction between 0^oC to 20^oC in an aerated lagoon operating with a detention time of 40 days under much the same conditions as the lagoon described in the present study. They concluded that the limiting parameter in such a system would be delivered or biologically utilizable oxygen.

While it is generally assumed that the concentration of dissolved oxygen plays a vital role in the purification of wastes, relatively little information is available on the actual biological requirement for oxygen in the breakdown of organics. Much of the applied work done to date has been directed towards the activated sludge process where the microorganisms responsible for stabilization grow in flocculated masses. In this system the amount of oxidation is dependent on the diffusion of oxygen into the microbial floc.

Mueller et al (65) have reported that for Zoogloea ramigera floc particles, the DO concentration that would become limiting for respiration was in the range 0.6 - 2.5 ppm and was dependent on the size and activity of the floc. They also found that the critical oxygen concentration for dispersed cells of the same organism was below 0.1 ppm DO. The authors suggested that for most