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**DEVELOPMENT OF AN OXALATE BIODEGRADATION PROCESS
USING A ROTATING BIOLOGICAL CONTACTOR**

by

LEE ANDREW BEAUDETTE, B.Sc.

A thesis submitted to
the Faculty of Graduate Studies and Research
in partial fulfilment of
the requirements for the degree of

Master of Science

Department of Biology

Carleton University

Ottawa, Ontario

September 15

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The undersigned recommend to the Faculty of Graduate studies
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USING A ROTATING BIOLOGICAL CONTACTOR"**

submitted by Lee Andrew Beaudette, B.Sc.
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Thesis Supervisor

Chair, Department of Biology

Carleton University

October 12, 1989

ABSTRACT

The development of an oxalate biodegradation process was investigated. Enrichment of bacteria from the rhizosphere of Rheum rhaponticum and Dieffenbachia picta using Oxa-1 medium, resulted in the isolation of an oxalate degrading bacteria, B-1, which degrades 4500 ppm and 16000 ppm oxalate in 18 and 50 hours respectively. B-1 could tolerate up to 8 g/L sodium ion, a pH range between 6 and 9 and temperatures between 5 and 40°C. Data on B-1 suggests that it belongs to the genus Pseudomonas.

Two types of rotating biological contactors (RBC's) were employed using the oxalate degrading bacteria as an inoculum. The first RBC (RBC-1) had a porous plastic support media and was inefficient due to the porosity of the support media. The second RBC (RBC-2) had a solid plastic support media and operated with a residence time of 5 hours degrading 5000 ppm oxalate ion from both synthetic medium and industrial effluent. Biomass on the solid support media was unaffected by temporary exposure to high concentrations of oxalate.

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TABLE OF CONTENTS

	<u>Page</u>
ACCEPTANCE SHEET	ii
ABSTRACT	iii
ACKNOWLEDGEMENTS	iv
TABLE OF CONTENTS	v
LIST OF TABLES	xi
LIST OF FIGURES	xii
LIST OF APPENDICES	xv
LIST OF ABBREVIATIONS	xvi
INTRODUCTION	1
LITERATURE REVIEW	4
I. Toxicity of Oxalate	4
II. Natural Occurance of Oxalate	5
III. Oxalate Metabolism in various Bacteria	6
1. The Oxidation of Oxalate	6
2. Synthesis from Oxalate	10
VI. Energy Production and Growth of <u>Pseudomonas oxalaticus</u> OX1 on Oxalate and Formate	21
1. ATP Required for Cell Material	21
2. Proton Translocation Associated with the Oxidation of Oxalate and Formate	24
V. Evidence for a Membrane-bound Formate dehydrogenase	26
VI. Ruminal Oxalate Degradation by Bacteria	26
VII. Oxalate Degradation by Fungi	29

	<u>Page</u>
VIII. Rotating Biological Contactors (RBC)	30
1. Process Description	30
2. Industrial Waste Treatment	33
MATERIALS AND METHODS	36
1. Materials	36
11. Methods	40
1. Isolation of Oxalate Degrading Microorganisms	40
A. Initial Isolation Procedure	40
B. Subsequent Isolation and Growth Medium using Oxalic Acid	41
C. Subculturing Oxa-1 Grown Organisms on Oxa-1 Plates and Nutrient Agar Plates	41
2. Oxalate Assay (Indole Method)	42
3. Reconstitution of <u>Pseudomonas oxalaticus</u> ATCC 11833	42
4. Kinetics of Oxalate Degradation	43
A. Oxalate Degradation by Six Mixed Cultures	43
B. Oxalate Degradation by Mixed Culture C and F	43
C. Oxalate Degradation by Pure Cultures Obtained from Mixed Cultures C and F	44
5. Determination of the Optimal pH and Yeast Extract Concentration for Mixed Culture F	44
6. Isolation and Identification of Pure Cultures from Mixed Cultures C and F, and the Rotating Biological Contactors	45
A. Isolation	45

	<u>Page</u>
B. Identification	46
7. Isolate B-1 Studies	46
A. Photomicrographs	46
1. Transmission Electron Micrograph (TEM) - Negative Staining	46
2. Transmission Electron Micrograph (TEM) - Thin Sectioning	46
B. Physiological, Biochemical and Toxicity Tests	47
C. Oxalate Degradation by Isolate B-1	48
D. Tolerance of B-1 to High Levels of Oxalate	48
E. Sodium Analysis of Oxalic Acid Standards	48
8. Rotating Biological Contactor (RBC)	49
A. Chemdisk Rotating Biological Contactor (RBC-1)	49
1. Operation of RBC-1	49
2. Bacterial Adherence to Various Materials	49
B. Operation of RBC-2 using Oxalic Acid	50
C. Operation of RBC-2 using Sodium Oxalate	51
1. Analysis of Industrial Sodium Oxalate	51
2. Shake Flask Study using Sodium Oxalate Prior to use in RBC-2	51
3. Operation of RBC-2 using Sodium Oxalate	53

	<u>Page</u>
9. Tolerance of Mixed Culture F from RBC-2 to Chemical and Physical Factors	53
A. Sodium Tolerance	53
B. Effect of Initial pH	53
C. Sodium Oxalate Tolerance	54
D. Temperature	54
10. Shock Loading RBC-2 with Sodium Oxalate	54
11. Analysis of RBC-2 Sloughed Off Biomass	54
12. Synthetic Flocculation of RBC Effluent	55
A. Preparation of the Synthetic Flocculants	55
B. Comparison of Six Flocculants on Settling Rate of RBC-2 Effluent	55
C. Effect of Percol 757 Concentration on Settling Behaviour of Biomass	55
13. Minimization of Sodium Oxalate Medium Constituents	56
RESULTS AND DISCUSSION	57
I. Isolation of Oxalate Degrading Microorganisms	57
1. Initial Isolation Procedure	57
2. Subculturing Oxa-1 Grown Organisms on Oxa-1 Plates and Nutrient Agar Plates	58
II. Oxalate Assay (Indole Method)	59
III. Reconstitution of <u>Pseudomonas oxalaticus</u> ATCC 11833	59
IV. Kinetics of Oxalate Degradation	60
1. Oxalate Degradation by Six Mixed Cultures	60
2. Oxalate Degradation by Mixed Culture C and F	60

	<u>Page</u>
3. Oxalate Degradation by Pure Cultures Obtained from Mixed Cultures C and F	64
V. Determination of the Optimal pH and Yeast Extract Concentration for Mixed Culture F	65
VI. Isolation and Identification of Pure Cultures from Mixed Cultures C and F, and the Rotating Biological Contactors	69
VII. Isolate B-1 Studies	72
1. Photomicrographs	72
A. Transmission Electron Micrograph (TEM) - Negative Staining	72
B. Transmission Electron Micrograph (TEM) - Thin Sectioning	72
2. Physiological, Biochemical and Toxicity Tests	72
3. Oxalate Degradation by Isolate B-1	76
4. Tolerance of B-1 to High Levels of Oxalate and Sodium Analysis of Oxalic Acid Standards	76
VIII. Rotating Biological Contactor (RBC)	79
1. Chemdisk Rotating Biological Contactor (RBC-1)	79
A. Operation of RBC-1	79
B. Bacterial Adherence to Various Materials	82
2. Operation of RBC-2 using Oxalic Acid	82
3. Operation of RBC-2 using Sodium Oxalate	84
A. Analysis of Industrial Sodium Oxalate	84
B. Shake Flask Study using Sodium Oxalate Prior to use in RBC-2	84
C. Operation of RBC-2 using Sodium Oxalate	87

	<u>Page</u>
IX. Tolerance of Mixed Culture F from RBC-2 to Chemical and Physical Factors	89
X. Shock Loading RBC-2 with Sodium Oxalate	91
XI. Analysis of RBC-2 Sloughed Off Biomass	91
XII. Synthetic Flocculation of RBC Effluent	91
1. Comparison of Six Flocculants on Settling Rate of RBC-2 Effluent	94
2. Effect of Percol 757 Concentration on Settling Behaviour of Biomass	94
XIII. Minimization of Sodium Oxalate Medium Constituents	94
CONCLUSIONS	101
BIBLIOGRAPHY	103

LIST OF TABLES

	<u>Page</u>
Table 1. ATP required for the formation of microbial cells from glucose, oxalate and carbon dioxide as the carbon source in mineral salts medium (Dijkhuizen <u>et al.</u> , 1977a).	23
Table 2. Analysis of Industrial sodium oxalate cake.	86
Table 3. Analysis of biomass from the effluent of RBC-2.	93
Table 4. Visual comparison of six synthetic flocculants added to RBC-2 effluent at a concentration of 2.5 mg/L.	95
Table 5. Effect of various concentrations of Percol 757 on the settling behaviour of biomass from the effluent of RBC-2.	96

LIST OF FIGURES

	<u>Page</u>
Figure 1. Pathway derived by Quayle <u>et al.</u> (1961) for the conversion of oxalate into glyoxylate.	15
Figure 2. Conversion of oxalate into hydroxypyruvate (Blackmore and Quayle, 1970).	17
Figure 3. Top view of RBC showing the serpentine movement of medium through the chambers.	32
Figure 4. Rotating Biological Contactor No.1 (RBC-1) purchased from CMS Rotordisk Inc., Mississauga, Ontario.	37
Figure 5. Rotating Biological Contactor No.2 (RBC-2) fabricated at Energy, Mines and Resources, Ottawa, Ontario.	38
Figure 6. Industrial sodium oxalate.	52
Figure 7. Oxalate degradation of six mixed cultures monitored over a 48 hour time period.	61
Figure 8. Oxalate degradation, growth, and pH of mixed culture C monitored over a 47 hour time period.	62
Figure 9. Oxalate degradation, growth, and pH of mixed culture F monitored over a 47 hour time period.	63
Figure 10. Oxalate degradation, growth and pH of mixed culture F monitored over a 27 hour time period. No yeast extract was added to Oxa-1 medium and the initial pH of graph (a) and (b) was 7.0 and 8.5, respectively.	66
Figure 11. Oxalate degradation, growth and pH of mixed culture F monitored over a 27 hour time period. 0.001% yeast extract was used in Oxa-1 medium and the initial pH of graph (a) and (b) was 7.0 and 8.5, respectively.	67

	<u>Page</u>
Figure 12. Oxalate degradation, growth and pH of mixed Culture F monitored over a 27 hour time period. 0.01% yeast extract was used in Oxa-1 medium and the initial pH of graph (a) and (b) was 7.0 and 8.5, respectively.	68
Figure 13. Oxalate degradation and growth of isolate B-1 monitored over a 22 hour time period.	71
Figure 14. Transmission electron micrograph of isolate B-1 grown on oxalic acid medium. Preparation was negatively stained (Magnification = 35,350x)	73
Figure 15. Transmission electron micrograph of isolate B-1 grown on oxalic acid medium. Preparation was thin sectioned (Magnification = 50,000x)	74
Figure 16. Tolerance of isolate B-1 to high levels of oxalate from oxalic acid (Oxa-1) medium.	77
Figure 17. Sodium ion analysis of varying concentrations of oxalic acid medium standards performed on an Atomic Absorption Spectrophotometer	78
Figure 18. RBC-1 disks showing very little growth on the disks and the porosity of the disks.	80
Figure 19. Bar diagram showing the levels of oxalate ion in the influent, RBC chambers and effluent in RBC-1. Oxalic acid medium was run through the RBC-1 with a residence time of 3.5 days and disk rotation speed of 3 rpm.	81
Figure 20. Bar diagram showing the levels of oxalate ion in the influent, RBC chambers and effluent in RBC-2. Oxalic acid medium was run through the RBC-2 with residence times of 28, 14 and 7 hours with a disk rotation speed of 0.6 rpm.	83
Figure 21. Bar diagram showing the levels of oxalate ion in the influent, RBC chambers and effluent in RBC-2. Oxalic acid medium was run through the RBC-2 with a residence time of 5 hours and a disk rotation speed of 0.6 rpm.	85

- Figure 22. Biomass on RBC-2 disks using sodium oxalate. Oxalate concentration was 5000 ppm with a residence time of 5 hours and a disk rotation speed of 3 rpm. 88
- Figure 23. Bar diagram showing the levels of oxalate ion in the influent, RBC chambers and effluent in RBC-2. Sodium oxalate medium was run through the RBC-2 with a residence time of 5 hours and a disk rotation speed of 3 rpm. 90
- Figure 24. Bar diagram showing the levels of oxalate in the influent, RBC chambers and effluent, before, during and after shock loading. Sodium oxalate medium (Na-Ox) was run through the RBC-2 with a residence time of 5 hours and a disk rotation speed of 3 rpm. 92
- Figure 25. Minimization of sodium oxalate medium (Na-Ox) constituents. Each of the following medium constituents were reduced individually from the medium: (a) Ammonium phosphate, (b) Ferrous sulfate, (c) Magnesium sulfate, and (d) Yeast extract. An inoculum was obtained from RBC-2. 98
- Figure 26. Sodium oxalate (Na-Ox) medium constituents were reduced to half the normal concentration. An inoculum was obtained from RBC-2. 99
- Figure 27. Sodium oxalate medium (Na-Ox) constituents were reduced to half the normal concentration and run through the RBC-2 with a residence time of 5 hours and a disk rotation speed of 3 rpm. The bar diagram shows the levels of oxalate in the influent, RBC and effluent. 100

LIST OF APPENDICES

	<u>Page</u>
APPENDIX I	
CHEMICAL REACTION OF INDOLE WITH OXALIC ACID	108
APPENDIX II	
NEGATIVE STAINING OF ISOLATE B-1 FOR TRANSMISSION ELECTRON MICROSCOPY	109
APPENDIX III	
THIN SECTIONING OF ISOLATE B-1 FOR TRANSMISSION ELECTRON MICROSCOPY	110
APPENDIX IV	
METHODOLOGY FOR SODIUM ANALYSIS ON AN ATOMIC ABSORPTION SPECTROPHOTOMETER	111
APPENDIX V	
CALCULATIONS TO OBTAIN 5000 PPM OXALATE FROM SODIUM OXALATE CAKE	112
APPENDIX VI	
PREPARATION OF SAMPLES FOR INDUCTIVELY COUPLED PLASMA SPECTROSCOPY	113
APPENDIX VII	
RESULTS OF BIOCHEMICAL TESTS PERFORMED AT HEALTH AND WELFARE, CANADA, AND THE AMERICAN TYPE CULTURE COLLECTION, MARYLAND, USA	114

LIST OF ABBREVIATIONS

AA	Atomic Absorption Spectroscopy
ADP	Adenosine Diphosphate
AMP	Adenosine Monophosphate
ATCC	American Type Culture Collection
ATP	Adenosine Triphosphate
CoA	Coenzyme A
DC	Dieffenbachia soil from Carleton University
DCPIP	2,6 - Dichlorophenol Indophenol
DE	Dieffenbachia soil from EMR
EMR	Energy, Mines and Resources, Canada
FAD	Flavin Adenine Dinucleotide
H ⁺ /O	Protons translocated per mole of organic oxidized
ICP AES	Inductively Coupled Plasma - Atomic Emission Spectroscopy
K _m	Michaelis - Menten Constant
NAD	Nicotinamide Adenine Dinucleotide
NADP	Nicotinamide Adenine Dinucleotide Phosphate
Oxa-1	Oxalic Acid Medium 1
Oxa-2	Oxalic Acid Medium 2
Oxa-3	Oxalic Acid Medium 3
Oxa-4	Oxalic Acid Medium 4
PGA	3-Phosphoglyceric Acid
Pi	Inorganic Phosphate
PMS	Phenazine Methosulphate

P/O	Moles of inorganic phosphate esterfied per g-atom of oxygen taken up
RBC	Rotating Biological Contactor
RBC-1	Rotating Biological Contactor 1
RBC-2	Rotating Biological Contactor 2
rpm	Revolutions per Minute
rRNA	Ribosomal Ribonucleic Acid
RU	Rhubarb soil from Embrun, Ontario
RUBPCase	Ribulose Bisphosphate Carboxylase
TEM	Trasmission Electron Microscope
TPP	Thiamine Pyrophosphate
Y[ATP]	Dry weight of organism produced per g-mole ATP
Y[MAX/ATP]	Maximum dry weight of organism produced per g-mole ATP
$1/Y_{ATP} = m_e u + 1/Y_{MAX/ATP}$ - Modified Pirt Equation, where	
m_e	Maintenance coefficient (g-moles ATP/g dry weight of organism/hr)
u	Specific growth rate (h^{-1})

INTRODUCTION

Oxalate is a very ubiquitous chemical found throughout the animal and plant kingdoms. In humans, oxalic acid and its salts are present in urine, blood and is a major constituent in kidney stones (Jakoby and Bhat, 1958). The actual synthesis of oxalic acid in humans is minimal, therefore it is reasonable to suppose that the bulk of oxalate in animals originates from the oxalate ingested with plant material. It has been found that the oxalate in leaves can reach about 20% of the dry weight of the plants (Jakoby and Bhat, 1958). Several species of fungi, such as Penicillium and Aspergillus, convert sugar into calcium oxalate with 90% yields under optimum conditions (Merck Index, 1983). It is also a product of the metabolism of many molds. Some industries, other than manufactures of oxalic acid, produce oxalate as a by-product in their process.

Animals do not catabolize oxalate very well, and although higher plants and a few mosses metabolize it to some extent, the majority of oxalate degradation is left to the bacteria and fungi. Only a limited number of organisms are known to utilize oxalic acid or oxalate as a sole carbon source. Aerobic oxalate degradation can be carried out by certain bacteria such as Vibrio oxaliticus (Bhat and Barker, 1948), Streptomyces sp. (Khambata and Bhat, 1954), Pseudomonas oxalaticus (Quayle and Keech, 1959a; Blackmore et al., 1968;

Dijkhuizen et al., 1977a), Thiobacillus novellus (Chandra and Shethna, 1977), a Spirillum sp. (Van Der Kooij and Hijnen, 1984), and several Alcaligenes and Pseudomonads species (Khambata and Bhat, 1954; Chandra and Shethna, 1975b; Friedrich et al., 1979), and a number of fungi (Vaisey et al., 1961; Emiliani and Bekes, 1964; Lillehoj and Smith, 1965; McLung, 1954) have been known to degrade oxalate. Anaerobic oxalate degradation has been observed in the rumen (Dawson et al., 1980) and in lake sediments (Smith and Oremland, 1983).

To date, much of the research into the microbial degradation of oxalate has focused on the elucidation of pathways and enzymes involved. Many industries produce large quantities of oxalate as a by-product and thus the use of oxalate degrading organisms in a practical sense would aid these industries in getting rid of the oxalate efficiently and inexpensively. All one has to do is obtain an apparatus, such as a rotating biological contactor (RBC), to house the organisms for a continuous oxalate degrading process. An RBC was first described in 1929, however it was not until 1960 that units became commercially available for municipal waste treatment (Pike et al., 1982). Today, RBC's are being used for municipal waste treatment as well as industrial wastes produced from food processing, leather, pulp and paper, refinery, petrochemical and textile industries (Borghei, 1981; Lee et al., 1975; Mudder and Whitlock, 1984; Anderson and Duarte, 1981).

The objective of the research paper was to isolate oxalate degrading bacteria from the environment, and using these bacteria, develop a biological oxalate degrading process. Specific process requirements must be met in order to make the process attractive to industry. The process must be aerobic, continuous, simple, efficient and have low operating and capital cost.

LITERATURE REVIEW

I. Toxicity of Oxalate

Oxalate poisoning is fundamentally complex and poorly understood. Insoluble oxalates in the diet are not absorbed and are excreted. Soluble oxalates may be absorbed rapidly in nonruminants, however, ruminants appear to be relatively resistant to oxalate poisoning even though most cases of oxalate poisoning occur in sheep and cattle (Kingsbury, 1964). The main reason for the oxalate poisoning is probably due to the time of oxalate consumption and the amount of oxalate consumed.

Oxalic acid (ethanedioic acid) is corrosive to skin and mucous membranes (Kingsbury, 1964). Ingestion of oxalate can cause severe gastroenteritis with vomiting, diarrhea and melena (Merck Index, 1983). Adsorption of soluble oxalates into the bloodstream correlates directly with a drop in the level of ionic calcium in the serum. Removal of calcium ion by precipitation not only produces effects due to calcium loss, but also to imbalances in mono- and divalent cations and perhaps to pH changes. These include nervous symptoms, reduced coagulability of the blood, and acute nephritis (Kingsbury, 1964; Libert and Franceschi, 1987). In severe cases, convulsions, coma and even death can occur as a result of cardiovascular collapse (Merck Index, 1983). One of the main problems observed however, is the kidney's reaction to soluble

oxalates in the circulation. Small amounts are excreted, but large concentrations result in the precipitation of oxalate crystals in the kidney tubules (Kingsbury, 1964; Libert and Franceschi, 1987). The lumen of all the tubules can become plugged and symptoms of renal insufficiency result. The lethal dose of oxalic acid for man varies from 2 to 30g (Libert and Franceschi, 1987).

II. Natural Occurrence of Oxalate

Oxalate is a common constituent of plants. Depending on the species, oxalate accumulates primarily as a soluble oxalate, insoluble calcium oxalate, or a combination of these two forms (Libert and Franceschi, 1964). It has been suggested that the majority of the species of only 11 out of 93 orders of higher plants do not store oxalate (Libert and Franceschi, 1964). This gives one an idea of how wide spread the occurrence of oxalate in plants is in the world. A person can go virtually anywhere and obtain plants that contain oxalate. The following is a short list of plants that contain oxalate in Canada and the United States (Kingsbury, 1964; Stephens, 1980; Libert and Franceschi, 1987):

Plant Species	Common Name
<u>Beta vulgaris</u>	Beet, Mangold
<u>Dieffenbachia picta</u>	Dumbcane
<u>Halogeton glomeratus</u>	Halogeton
<u>Oxalis</u> spp.	Sorrel, Soursob
<u>Portulaca oleracea</u>	purslane
<u>Rheum rhaponticum</u>	Rhubarb
<u>Rumex</u> spp.	Sorrel, Dock
<u>Salsola kali</u>	Russian thistle
<u>Sarcobatus vermiculatis</u>	Greasewood

III. Oxalate Metabolism in Various Bacteria

1. The Oxidation of Oxalate

In the introduction, it was shown that a variety of microorganisms have the ability to degrade oxalic acid, however in 1956, very little was known about the details of oxalic acid metabolism. A simple route for the oxidation of oxalate would be a decarboxylation to formate followed by the oxidation of this compound to water and carbon dioxide (Kornberg and Elsdon, 1961).

Initial research into elucidating the pathway for oxalate degradation was not successful. Bacterial extracts, not supplemented with cofactors, appeared incapable of either

decarboxylating oxalate anaerobically or of oxidizing oxalate in the presence of oxygen or artificial electron acceptors. This was puzzling to researchers because microbial extracts readily catalyzed the oxidation of formate with concomitant reduction of nicotinamide adenine dinucleotide (NAD) (Kornburg and Elsdén, 1961):

In 1956, Jakoby et al. attempted to establish the mechanism of oxalate degradation at the enzymic level using a bacterium, of the genus Bacterium, isolated from the soil. They observed that oxalate was decarboxylated anaerobically by extracts of the bacterium, grown in the presence of ^{14}C -oxalate, if substrate quantities of adenosine triphosphate (ATP) and catalytic amounts of acetate, coenzyme A, Mg^{2+} , and thiamine pyrophosphate (TPP) were added. It was found that acetate and ATP could be replaced by substrate quantities of acetyl-coenzyme A or of acetyl phosphate and coenzyme A, which indicated that the overall reaction was as follows:

Equation 3 was the sum of the equations 4,5 and 6:

In a separate study by Quayle et al. (1961), it was found that crude cell free extracts of oxalate grown Pseudomonas oxalaticus OX1 decarboxylated oxalate with the addition of ATP. It was also observed that the quantities of carbon dioxide and formate formed from oxalate could exceed the amounts of ATP added. When the extracts were purified (Jakoby et al., 1956), the catalytic effect of ATP was lost; the decarboxylation of oxalate required substrate quantities of ATP and catalytic quantities of acetate, coenzyme A, and ThPP. Also, the decarboxylation rates were very low when compared to the crude extract reaction. Obviously something had been lost in the purification. The key element that was lost during purification was succinate. Similar to observations presented above that acetate, ATP and coenzyme A could be replaced by acetyl-coenzyme A, Quayle et al., found that succinyl-coenzyme A could replace succinate, ATP and coenzyme A. Also, the carbon dioxide evolved from oxalate was stoichiometrically related to the amount of acetyl-coenzyme A added in Jakoby's

et al. experiment, whereas in Quayle's et al. experiment, succinyl-coenzyme A promoted a much greater oxalate decarboxylation in catalytic quantities. This suggested that oxalyl-coenzyme A is synthesized by transferase action between succinyl-coenzyme A and oxalate (reaction 8). Oxalyl-coenzyme A is decarboxylated to formyl-coenzyme A, the coenzyme A grouping of which can be transferred back to oxalate either directly, or indirectly via succinate.

The new cycle overcomes problems found in reactions 4, 5 and 6 with the requirement for substrate amounts of ATP and acetyl-coenzyme A. The requirement was due to the inability of acetate to act as the acceptor molecule for coenzyme A from formyl-coenzyme A (Jakoby et al., 1956). The oxidation of formate from oxalate is probably the main source of energy for organisms grown on oxalate, thus requirements for substrate quantities of ATP in formate formation could lead to a loss of a third of the possible energy yield in the complete oxalate oxidation. This loss can be avoided by the

substitution of the transferase reaction (reaction 9) for the hydrolytic reaction (reaction 6) (Kornberg and Elsdén, 1961).

Other findings by Quayle et al. (1961) which supported the above idea were that whole cells of P. oxalaticus oxidized both formate and oxalate, however oxalate oxidation was preceded by a rapid evolution of carbon dioxide suggesting that the oxidation proceeds via formate. Cell free extracts contained high activities of NAD-linked formic dehydrogenase (FAD) but no NAD-linked oxalate dehydrogenase was found. During the decarboxylation of ^{14}C -oxalate, small quantities of both ^{14}C -oxalyl-coenzyme A were detected as their hydroxamic acids.

2. Synthesis from Oxalate

Jakoby and Bhat (1958) suggested that since the major energy yielding reaction is obtained from the oxidation of formate, that formate would also act as the major carbon source. Although this sounds reasonable, there is evidence against the view that growth on oxalate is synonymous with growth on formate.

Quayle and Keech (1959a, 1960) observed that when P. oxalaticus was grown on formate, isotope from labelled formate and carbon dioxide was rapidly incorporated into cell biomass. When the same organism was grown on oxalate as a sole carbon source, negligible quantities of ^{14}C from labelled formate or carbon dioxide were found incorporated. The distribution of

labelled compounds incorporated into the cell differed between ^{14}C -formate grown cells and ^{14}C -oxalate grown cells. Labelled formate grown cells were found to incorporate the label into 3-phosphoglycerate (PGA), whereas the labelled oxalate grown cells were found to incorporate the label into glycine. By using cell free extract of formate grown cells of P. oxalaticus, Quayle and Keech (1959b) found high activities of carboxydismutase, phosphoriboisomerase, and phosphoribulokinase, enzymes characteristic of microorganisms employing autotrophic modes of carbon utilization. Extracts of the organism grown on oxalate were devoid of these enzymes (Quayle and Keech, 1961). Carboxydismutase is a key enzyme in the ribulose diphosphate cycle (Calvin cycle) for carbon dioxide fixation (Zubay, 1983). When cells of P. oxalaticus were grown on oxalate and then transferred to formate, carboxydismutase was found, however when the reverse was done, the enzyme was diluted out (Quayle and Keech, 1960). This showed that oxalate grown cells differ from cells grown on formate or carbon dioxide in the first steps of the reaction. It also showed that other pathways must operate for oxalate grown cells to obtain cellular materials from oxalate.

Quayle and Keech (1960) observed that oxalate grown cells of P. oxalaticus incorporated isotope from oxalate into glycine (65%) and 3-phosphoglyceric acid (25%) after incubation for 3 seconds. After 45 seconds, they observed the isotope in alanine, glutamate, malate and phosphates of

glucose, fructose and sedoheptulose. They proposed the following scheme:

Using cell-free extracts, studies indicated that the transformation of oxalate into PGA occurred by reduction of an activated oxalate to glyoxylate, condensation and decarboxylation to 1 molecule of a C₃ compound which is then reduced and phosphorylated to PGA. Thus glycine results from a side reaction and PGA is the main pathway of cell biosynthesis. An enzyme capable of the formation of glyoxylate from oxalate was purified from oxalate grown cells of P. oxalaticus and Pseudomonas OD1 (Quayle et al. 1961; Kornberg and Elsdon, 1961). The enzyme, glyoxylic dehydrogenase, catalyzed the oxidation of glyoxylate with concomitant and stoichiometric reduction of nicotinamide adenine dinucleotide phosphate (NADP) in the presence of catalytic amounts of coenzyme A (reaction 12):

oxalate and NADPH are the only products of this reaction. The reaction is optimal at pH 8.6 and has a K_m for glyoxylate of $5.7 \times 10^{-4} M$. Research performed by Quayle, Keech and Taylor (1959a, 1959b, 1960 and 1961) resulted in a reaction scheme for the conversion of oxalate into glyoxylate (Figure 1).

In subsequent studies by Quayle (Blackmore and Quayle, 1970), the reduction of oxalate to glyoxylate was shown by the following reactions in P. oxalaticus:

The glyoxylate is converted to glycerate by the glycerate pathway:

The enzyme responsible for the anabolic sequence in reaction 15 is glyoxylate carboligase discovered by Kornberg in 1966. Glyoxylate carboligase in its active state is a tetramer and

flavin adenine dinucleotide (FAD) is required for the formation of the tetramer from inactive dimers (Chung et al., 1971). As opposed to the presence of glyoxylate carboligase in P. oxalaticus, Blackmore and Quayle (1970) showed four oxalate degrading pink-pigmented organisms, Pseudomonas AM1, Pseudomonas AM2, Protminobacter ruber and Pseudomonas extorquens, which have the same enzymes except for glyoxylate carboligase. In contrast, the organisms have an L-serine glyoxylate aminotransferase and an hydroxypyruvate reductase not found in P. oxalaticus. This indicated that the synthesis of C₃ compounds from oxalate by the pigmented organisms occurred by a variation of the serine pathway used by Pseudomonas AM1 during growth of C₁ compounds. An example of a C₃ compound into glycerate is synonymous with the conversion of a C₂ compound by the following:

Figure 1. Pathway derived by Quayle et al. (1961) for the conversion of oxalate into glyoxylate.

Blackmore and Quayle (1970) also showed evidence for this by obtaining a mutant Pseudomonas AM1 which lacked hydroxypyruvate reductase, the key enzyme in reaction 19, which was unable to grow on oxalate. Similar results were obtained by Chandra and Shethna (1975b) with three different organisms. Pseudomonas KOx and Alcaligenes LOx metabolized oxalate by the glycerate pathway involving glyoxylate carboligase and Pseudomonas MOx metabolized oxalate by the serine pathway involving hydroxypyruvate reductase (Figure 2).

Another enzyme present in the cell-free extracts of the pink-pigmented organisms which was not present in P. oxalaticus was serine-glyoxylate aminotransferase (Blackmore and Quayle, 1970).

Blackmore et al. (1968), questioned whether P. oxalaticus was autotrophic or heterotrophic. They examined the utilization of oxalate by formate grown cells after adaptation to growth on oxalate. It has already been shown that formate is the actual source of energy, and if formate is supplied extracellularly the result is autotrophic metabolism. If formate is supplied internally from oxalate, then the result is heterotrophic metabolism. When a formate grown cell is transferred to oxalate medium there is a lag period in which enzymes necessary to decarboxylate oxalate to formate and condense oxalate to phosphoglycerate via glyoxylate are synthesized. During this lag phase the cells still possess the

Figure 2. Conversion of oxalate into hydroxypyruvate (Blackmore and Quayle, 1970).

enzymes for autotrophic growth. The cells have two alternatives: (1) the cell can assimilate carbon from formate autotrophically, or (2) the cell can use the energy of oxidation of the oxalate not only for heterotrophic assimilation of oxalate, but also for autotrophic fixation of carbon dioxide. To answer this question, Blackmore et al. examined the labelling pattern of phosphoglycerate. $^{14}\text{CO}_2$ fixation by the ribulose diphosphate cycle results in labelling of the PGA in the carboxyl group, this pattern would be expected for cells grown autotrophically on ^{14}C -formate or ^{14}C -oxalate. If oxalate was assimilated heterotrophically, the consecutive intermediary metabolites of the biosynthetic pathway (ie. glyoxylate, tartronic acid semialdehyde, glycerate and phosphoglycerate) would be uniformly labelled after a brief incubation on ^{14}C -oxalate.

Their results showed that the phosphoglycerate obtained from cells grown of ^{14}C -formate showed 78% of the radioactivity in the carboxyl group, thus confirming autotrophic assimilation. The labelling pattern of phosphoglycerate obtained from ^{14}C -oxalate grown cells approached uniformity as predicted for heterotrophic assimilation. Phosphoglycerate obtained from cells that had started to grow on ^{14}C -oxalate after adaptation to formate, showed a pattern of uniformity. They also looked at the pattern of $^{14}\text{CO}_2$ incorporation of oxalate grown cells after adaptation to formate and label was

found in the carboxyl group of PGA. They concluded that a mixed heterotrophic-autotrophic metabolism was occurring with the former predominating.

Blackmore et al. (1968) found that the energy requirements for the biosynthesis of one molecule of triose phosphate by the two alternative pathways is very different. Triose phosphate synthesized by the ribulose diphosphate cycle is expressed as:

Triose phosphate synthesis by glycerate pathway of oxalate utilization is expressed as:

The heterotrophic pathway was found to be more energy efficient to the extent of $2\text{NADH} + 5\text{ATP/mol.}$ of triose phosphate formed. Thus, energetically, heterotrophic metabolism would be favoured over autotrophic metabolism.

Chandra and Shethna (1977) and Friedrich et al. (1979) reported that Thiobacillus novellus and Alcaligenes eutrophus, respectively, degraded oxalate heterotrophically. In cell free

extracts of oxalate grown cells there was no evidence of ribulose biphosphate carboxylase (RuBPCase). However the key enzymes of the glycerate pathway, oxalyl-CoA reductase and glyoxylate carboligase, were present. RuBPCase was present in formate, formimide and thiosulfate grown cells indicating autotrophic growth on these substrates.

Dijkhuizen and Harder (1979a) examined autotrophic and heterotrophic growth of *P. oxalaticus* OX1 on oxalate and formate in continuous culture. In a continuous fermenter, the ratio of oxalate to formate could be controlled and the resultant modes of metabolism examined. During growth on mixtures of oxalate and formate, they found the simultaneous utilization of both substrates. Under oxalate limiting conditions, RuBPCase was totally repressed. At fixed oxalate/formate ratios, repression of RuBPCase was greatest at intermediate dilution rates whereas derepression occurred at both low and high dilution rates. Dijkhuizen and Harder suggested that derepression at low dilution rates was attributed to the decreasing concentration of intracellular repressor molecules, parallel to the decreasing concentration of growth limiting substrates in the culture. They also suggested that derepression at higher dilution rates was due to the rate of oxalyl-CoA production from oxalate limited the supply of metabolic intermediates. Additional energy and reducing power generated from formate drains the pools of metabolic intermediates enough to lower the intracellular

concentration of repressors. They concluded that the synthesis of enzymes for autotrophic CO₂ fixation (ie. via Calvin cycle) is regulated by a repression/derepression mechanism.

(IV) Energy Production and Growth of Pseudomonas oxalaticus OX1 on Oxalate and Formate.

In 1977a, Dijkhuizen et al. estimated the efficiency of oxidative phosphorylation in Pseudomonas oxalaticus during growth on oxalate and formate by two methods. In the first method, the amount of ATP required to synthesize cell material of standard composition was calculated during growth of the organism on either of the two substrates. In the second method, the stoichiometries of the respiration-linked proton translocations with oxalate and formate were measured in washed cell suspensions grown on the two substrates.

1. ATP Required for Cell Material

The term [Y Max/ATP] is the dry weight of organisms produced per g-mole ATP after the correction for ATP required for regular maintenance (Stouthamer, 1973). In order to interpret [Y MAX/ATP] values, theoretical [Y MAX/ATP] values were obtained from values calculated for the amount of ATP required for the formation of microbial cells (Table 1)(Dijkhuizen et al., 1977a). The table was calculated from the energy required to synthesis a C₃ compound via the glycerate pathway or via the Calvin cycle followed by the

energy required to synthesis various monomers from 3-phosphoglycerate. In column 1 of Table 1 the energy required for the synthesis of cells from oxalate was calculated on the basis that oxalate is transported across the membrane of *Pseudomonas oxalaticus* by a passive process; column 2 takes into account the energy required to transport oxalate across the membrane (approximately 1 ATP/mole oxalate) (Dijkhuizen et al., 1977a; Dijkhuizen et al., 1977b). The [Y MAX/ATP] theoretical values obtained were 12.5 and 6.5 for oxalate and formate, respectively, and 9.4 if energy is required to transport oxalate across the membrane. Their values indicate that transport of oxalate across the membrane requires about 20% of the total energy. Also, the biosynthesis of cell material from carbon dioxide is far more expensive than from oxalate (Dijkhuizen et al., 1977b). In comparison, glucose grown cells under aerobic conditions have a [Y MAX/ATP] of 28.8 (Stouthamer, 1973). In studies by Stouthamer (1973), it was shown that [Y ATP] values were always less then [Y MAX/ATP] values. To account for this difference they added an uncoupling factor k. As the energy generated from the oxidation of a substrate is not used efficiently for biosynthetic purposes, some of the energy is lost due to uncoupling of energy generation and growth.

Synthesis of	ATP required (mol x 10 ⁻⁴ per g cells)			
	Glucose	Oxalate		Carbon dioxide
		1	2	
Polysaccharides	20.5	92	92	195
Protein	205.0	462	462	907
Lipid	1.5	67	67	172
RNA and DNA	55.1	115	115	198
Turnover m-RNA	14.0	14	14	14
Transport of				
a) carbon source	-	-	261	-
b) ions	52.0	52	52	52
Total	348.0	802	1063	1538
[Y MAX/ATP] Theor.	28.7	12.5	9.4	6.5

Table 1. ATP required for the formation of microbial cells from glucose, oxalate and carbon dioxide as the carbon source in mineral salts medium (Dijkhuizen et al., 1977a).

$$[Y \text{ MAX/ATP}] = k \times [Y \text{ MAX/ATP}]_{\text{thoer.}} \quad (22)$$

The value of k was approximated to be 0.5, therefore the $[Y \text{ MAX/ATP}]$ values were 6.3 and 3.3 for cells grown on oxalate and formate, respectively, and 4.7 for cells grown on oxalate and which required active transport of oxalate (Dijkhuizen et al., 1977b). P/O values, the amount (moles) of inorganic phosphate esterified per g-atom of oxygen taken up, were calculated from the $[Y \text{ MAX/ATP}]$ values using two equations, the modified Pirt equation (23) [where m_o is the maintenance coefficient (g-moles ATP/g dry weight of organisms/hr) and u is the specific growth rate (h^{-1})] and $P/O = Y_o/Y_{\text{ATP}}$ (Stouthamer, 1973; Dijkhuizen et al., 1977b).

$$1/Y_{\text{ATP}} = m_o \cdot u + 1 [Y \text{ MAX/ATP}] \quad (23)$$

The P/O ratios calculated were 1.0, 1.3 and 1.7 and are an indication of the moles of ATP per mole of oxalate, oxalate including active transport and formate oxidized respectively.

2. Proton Translocation Associated with the Oxidation of Oxalate and Formate

Pseudomonas oxalaticus was grown on oxalate and formate, harvested, and then under anaerobic conditions the cells were subjected to a volume of air-saturated with KCl. Immediately, there was a rapid acidification, followed by a

decay back to the original pH with a half time of 60 seconds (Dijkhuizen et al., 1977b). The KCl air also contained potassium thiocyanate to neutralize any electrical charges across the membrane and to maximize the pH difference. With oxalate and formate as electron donors, H^+/O ratios close to 4 were measured which indicated the presence of two energy-conservation sites in the respiratory chain of Pseudomonas oxalaticus during growth on both substrates (Dijkhuizen et al., 1977b). When H^+/O ratios were measured with succinate, ethanol or ascorbate-TMPD as electron donors, values close to 4 and 2 were observed. These results indicates that the phosphorylation sites 2 and 3 in the electron transport chain of Pseudomonas oxalaticus were active (Dijkhuizen et al., 1977b).

The growth yield data gave value of 1 for the number of phosphorylation sites in the case of oxalate whereas the electron translocation data gave a value of 2. Dijkhuizen et al. (1977b) explained the discrepancy by noting the presence of an active transportation site with an energy requirement of 1 mole of ATP per mole of oxalate. They also noted that if 1 mole of ATP per mole of oxalate is required for transport, then the H^+/O ratios with oxalate should be 2 instead of 4, however since the cells were preloaded with oxalate, oxalate transport was not a factor. No active transport system for formate was detected however, the values obtained by both methods were similar.

(V) Evidence for a Membrane-bound Formate Dehydrogenase

Quayle et al (1961) reported that the major energy yielding reaction for P. oxalaticus grown on oxalate and formate was attributed to a soluble NAD-dependent formate dehydrogenase. Dijkhuizen et al. (1979) found evidence that another formate oxidizing enzyme, located in the membrane, was present in P. oxalaticus. The activity was detected spectrophotometrically with artificial electron acceptors phenazine methosulphate (PMS)/2,6-dichlorophenol-indophenol (DCPIP). It was found that the membrane-bound FDH could transfer electrons (via a ubiquinone or cytochrome b) into the respiratory chain in a manner similar to the soluble NAD-FDH.

Friedrich et al. (1979) obtained an organism, Alcaligenes eutrophus strain H16, that grew on formate and oxalate as sole carbon sources. They presented data which indicated that the organism did not assimilate formate directly, but first oxidized it to CO₂ by the action of two formate dehydrogenases. The first is a soluble formate:NAD⁺ oxidoreductase and the second a membrane-bound formate oxidoreductase. Both enzymes were found to be present and are induced in the presence of oxalate and formate.

(VI) Ruminal Oxalate Degradation by Bacteria

Much of the present research into oxalate degradation appears to be associated with ruminal effects. Allison et al.

(1977) fed two sheep and one cow diets of alfalfa hay with increasing concentrations of oxalate from the plant Halogeton glomeratus (which contained 12% w/w oxalic acid). Within 3-4 days the animals adapted to 0.45 moles oxalate/day per animal with no signs of toxicity. Oxalate degradation was negligible in cell-free ruminal fluid from adapted sheep but was associated with fractions that contained bacterial cells. The same results were obtained by Dawson et al. (1980). Enrichment cultures from a medium containing 10% ruminal fluid resulted in the degradation of 45 mM sodium oxalate. One mole of methane was produced per 3.8 moles of oxalate degraded. Molecular hydrogen and formate inhibited oxalate degradation but not methanogenesis. Oxalate degradation by cultures uncoupled from methane production were found to produce 0.93 mol of formate per mol of oxalate degraded. Molecular hydrogen and formate did not inhibit oxalate degradation. Cell yields (g dry weight/mol oxalate degraded) of the primary and uncoupled enrichments were 1.7 and 1.0, respectively. Allison et al. (1977) and Dawson et al. (1980) were unable to isolate oxalate degrading bacteria from the animal's ruminal fluid.

The first report of anaerobic oxalate degrading bacteria in the cecal contents of rats was reported by Daniel et al. (1987a). The rates of oxalate degradation by the mixed bacterial populations in the cecal contents were found to be between 2.5 to 20.6 $\mu\text{mol/g}$ (dry weight) per hour. The degree of oxalate degradation could be attributed to the source of

the rats. The viable counts of oxalate degrading bacteria in the cecal contents were 10^8 /g (dry weight), which was less than 0.1% of the total population. Nine strains of oxalate degrading bacteria were isolated from wild rats and six isolates (similar to Oxalobacter formigenes) were obtained from one of the laboratory rats.

In a subsequent study by Daniel et al. (1987b) six strains of an anaerobic oxalate degrading bacterium, Oxalobacter formigenes, were examined for their ability to colonize the gastrointestinal tracts of laboratory rat's. Only the strain, OxCR6, that was a natural colonizer of a rats ceca, colonized the ceca and colon of an adult rat fed 4.5% sodium oxalate. Oxalate degradation in the rats colonized by OxCR6 increased 19 to 40 times. Strain OxCR6 would not remained in the cecal contents of rats fed less than 0.3% sodium oxalate.

Oxalate metabolism of Oxalobacter formigenes is similar to P. oxalaticus as both organisms require oxalyl-CoA. O. formigenes differs from P. oxalaticus as formate is the end product of the anaerobic oxalate metabolism. Whereas P. oxalaticus oxidizes formate to CO_2 via a NAD-linked formate dehydrogenase. Baetz and Allison (1989) purified the enzyme oxalyl-CoA decarboxylase from Oxalobacter formigenes. The enzyme is composed of four identical subunits (M_r , 65000) to give the active enzyme (M_r , 260000). The enzyme was found to catalyze the thiamine PPi-dependent decarboxylation of oxalyl-

CoA to formate and carbon dioxide. The maximum specific activity was 13.5 μ M oxalyl-CoA decarboxylated per min per mg of protein and the apparent K_m and V_{max} values were 0.24 mM and 0.25 μ mol/min for oxalyl-CoA and 1.1 pM and 0.14 μ mol/min for thiamine pyrophosphate.

(VII) Oxalate Degradation by Fungi

In 1955, Shimazono discovered white-rot fungi that degraded oxalate. The degradation of oxalic acid under aerobic conditions was due to the presence of oxalate decarboxylase in the mycelium. This was the first evidence of this enzyme in fungi. Emiliani and Pokes (1964) and Lillehoj and Smith (1965) also found oxalate decarboxylase in the fungi Aspergillus niger and Myrothecium verrucaria. The enzyme was found to have a stoichiometry of 1 mole oxalic acid per mole of formic acid and carbon dioxide. Again, the presence of oxygen was mandatory (Lillehoj and Smith, 1965).

In 1961, Vaisey et al. isolated a parasitic fungus, Tilletia contraversa, that oxidized oxalate. Enzyme preparations were shown to degrade oxalate to carbon dioxide and possibly hydrogen peroxide, however no definite conclusions were drawn. The oxidation was maximum at pH 2.6 and was 50% inhibited by fluoride or cyanide. Also, the oxidation was stimulated by riboflavin and flavine mononucleotide, but not by flavine adenine dinucleotide. They postulated the enzyme to be an oxalic acid oxidase. In 1966,

Chiriboga isolated and purified an oxalic acid oxidase from barley seedlings. They found that the system required oxygen and that the K_m was 4.2×10^{-4} M. The system was inhibited by iodoacetate, fluoride and cyanide, however oxidation of oxalate was stimulated by flavins and some chelating agents. The system was also sensitive to changes in ionic strength. The data presented by Chiriboga (1966) suggested that the enzyme was similar to that of the parasitic fungus, Tilletia contraversa.

(VIII) Rotating Biological Contactors (RBC)

1. Process Description

A rotating biological contactor (RBC) consists of a large-diameter plastic support media, which is mounted on a horizontal shaft and placed in a tank. The contactor is slowly rotated while approximately 40% of the surface area is submerged in the wastewater (Figures 3 and 4)(Antonie, 1976). After starting up the RBC, organisms either present in the wastewater or added as an inoculum, adhere to the rotating surfaces and multiply. In 1 to 3 weeks, the entire surface area is covered with a 1- to 4mm thick layer of biomass (Figure 22). The large microbial population achieved in the RBC allows for rapid treatment of wastewater in a short retention time (Antonie, 1976).

While the contactor rotates, a film of wastewater is carried into the air and absorbs oxygen from the air. The

biomass then removes dissolved oxygen and organic materials from this film of wastewater. Further removal of organic materials and consumption of dissolved oxygen occurs as the surfaces continue rotation through the bulk of the wastewater in the tank. Dissolved oxygen in the wastewater film is mixed with the contents of the reactor and maintains a mixed-liquor dissolved-oxygen concentration (Antonie, 1976).

Shear forces exerted on the biomass as it passes through the wastewater cause excess biomass to be stripped from the support medium into the mixed liquor. This process allows for a constant microbial population on the support medium and prevents clogging of the surfaces. The mixing action of the rotating media keeps the stripped solids in suspension until the flow of treated wastewater carries them out of the reactor for separation and disposal. The rotating support medium serves the following functions (Antonie, 1976):

- (1) Provides a surface for the development of a large, fixed biological culture
 - (2) Provides vigorous contact of the biological growth with the wastewater
 - (3) Efficiently aerates the wastewater
 - (4) Provides a positive means of continuously stripping excess biomass
 - (5) Agitates the mixed liquor to keep sloughed solids in suspension and thoroughly mixes each stage of treatment.
- Continuous movement of wastewater through the RBC allows

Figure 3. Top view of RBC showing the serpentine movement of medium through the chambers.

for continual degradation of the wastewater. To prevent short circuiting of the wastewater across the media, the flow of water through the RBC is in a serpentine pattern (Figure 3).

2. Industrial Waste Treatment

Industrial waste must be pretreated prior to treatment in the RBC. In most industrial systems, specific wastes are being disposed of. Therefore, it is important to maintain a constant flow rate, pH, temperature range, organic concentration and nutrient balance. A pretreatment stage allows for: 1) adjustment of the industrial wastewater to meet the requirements of the selected bacteria that degrade the specific waste, 2) if the industrial plant is shut down over a weekend, the pretreated wastewater can be pumped through the RBC to maintain the selected bacterial population and reduce contamination, and 3) if a surge of wastewater is released, the pretreatment stage will inhibit a surge of wastewater to the RBC (Antonie, 1976).

Although there are many examples of industrial wastewater treatment processes using RBC's, a good example is the process developed by Homestake Mining Company, Lead, South Dakota, USA for the treatment of cyanide, thiocyanate, and ammonia (a cyanide degradation by-product) containing wastewater.

The initial stage of development for their process involved gradually acclimatizing a bacterial isolate to higher concentrations of cyanide, cyanide complexes and thiocyanates.

The isolate, Pseudomonas paucimobilis var. mudlock, was obtained from process water at Homestake and was deposited in the American Type Culture Collection (ATCC #39204) (Mudder and Whitlock, 1984). The bacterium oxidizes free and complex cyanides to carbonate and ammonia. Soluble metal ions are adsorbed or are assimilated, metabolized and precipitated.

The Homestake treatment plant design was an aerated, attached growth, biological system using 48 rotating biological contactors followed by clarification and pressure sand filters with dual media beds (Mudder and Whitlock, 1984). The cyanide wastewater is pretreated to maintain constant cyanide concentration, nutrients, pH and temperature. There are five stages of cyanide treatment. In the first two stages removal of the cyanides, thiocyanates and adsorption of the metals is accomplished by P. paucimobilis var. mudlock. The last three stages contain Nitrobacter and Nitrosomonas which convert the ammonia to nitrite and subsequently to nitrate (Mudder and Whitlock, 1984). The RBC effluent is clarified and sand filtered prior to release to a nearby lake.

The Homestake RBC's contain 7.6 m of disks along their longitude axis; each disk is 3.65 m in diameter. The disks are corrugated plastic with surface areas of 9290 m² or 13935 m² depending on the density of the plastic. The disks are 40% submerged and rotate at speeds of 0.5 - 1.5 rpm. To increase aeration, air is sparged into each of the RBC's. The hydraulic loading of the RBC's is 1.5 gpd/ft² which translates to a

retention time of 20 minutes.

The entire process works extremely well in detoxifying the wastewater. Although the system was over designed (5 times too large) and capital costs were very high, operating costs are low and the system is a proven success.

MATERIALS AND METHODS

I. MATERIALS

All of the chemicals used were of reagent grade except for crude sodium oxalate obtained from an industrial source. Soil samples were obtained from three different sources: (1) Rhubarb soil from Embrun, Ontario (Ru); (2) Dieffenbachia soil from Carleton University, Ottawa, Ontario (DC); and (3) Dieffenbachia soil from Energy, Mines and Resources, Canada, Ottawa, Ontario (DE). A culture of Pseudomonas oxalaticus, described by Quayle and Keech (1959), was obtained from Dr. I. Suzuki, University of Manitoba, Canada.

Two Rotating Biological Contactors (RBC) were utilized. The first RBC (RBC-1) was purchased from CMS Rotordisk Inc., Mississauga, Ontario (Figure 4). It is a 30 liter RBC with a disk surface area of 9.3 m^2 . The dimensions are 115 cm x 31.5 cm x 16 cm (L x W x H) with the RBC divided up into three 22 cm chambers and one 41 cm chamber. The second RBC (RBC-2) was fabricated at Energy, Mines and Resources, Ottawa, Ontario (Figure 5). It is a 12 liter RBC with a disk surface area of 3.7 m^2 . The dimensions are 75 cm x 20 cm x 12 cm (L x W x H) with the RBC divided into three 24 cm chambers.

An experiment was performed to look at microbial adherence to different materials (ie. plastic, teflon, rubber, polycarbonate filter cloth, plexiglass, stainless steel wire mesh, nylon cloth and plastic petri dish material), all of

Figure 4. Rotating Biological Contactor No.1 (RBC-1) purchased from CMS Rotordisk Inc., Mississauga, Ontario.

Figure 5. Rotating Biological Contactor No.2 (RBC-2)
fabricated at Energy, Mines and Resources,
Ottawa, Ontario.

these materials were obtained from the laboratory at Energy, Mines and Resources, Canada, Ottawa, Ontario.

Six synthetic flocculants (PMD-30, RMR-30, RMX-30, Nalco 7879, Percol 757 and Percol 788N) and the sodium oxalate cake were obtained from an Industrial source.

Tentative identification of the microorganisms was performed at Energy, Mines and Resources, Canada, Ottawa, Ontario using the Gugol Gram Stain method, Enterotubes and Oxiferm tubes purchased from Hoffman-LaRoche Diagnostics Inc.. Further identification of the microorganisms were carried out by Mr. Charles Martineau, Microbiology Section, Health and Welfare, Canada, Ottawa, Ontario using a Beckman Microcoder IIA System for the identification of Gram negative bacteria and by Judith Frank, Applied Sciences Manager, the American Type Culture Collection (ATCC) Laboratory, Maryland, USA. Electron microscopy, toxicological and biochemical tests were performed by Dr. Wendy Johnson and Pauline Ewan, Laboratory for Disease Control, Health and Welfare Canada; Dr. J.W. Costerton, The University of Calgary; and Judith Frank, Applied Sciences Manager, The American Type Culture Collection (ATCC), Maryland, USA.

II. METHODS

(1) Isolation of Oxalate Degrading Microorganisms

A. Initial Isolation Procedure

Four different media were prepared and dispersed in 100 mL aliquots into 250 mL Erlenmeyer flasks:

i) Medium 1 (Oxa-1) in g/L: $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 0.5; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1; $(\text{NH}_4)_2\text{SO}_4 \cdot 7\text{H}_2\text{O}$, 0.5; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.05; Yeast Extract, 0.1; $(\text{COOH})_2 \cdot 2\text{H}_2\text{O}$, 2.0;

ii) Medium 2 (Oxa-2) in g/L: $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 0.75; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.35; $(\text{NH}_4)_2\text{SO}_4 \cdot 7\text{H}_2\text{O}$, 1.75; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.05; Yeast Extract, 0.1; $(\text{COOH})_2 \cdot 2\text{H}_2\text{O}$, 2.0; KH_2PO_4 , 1.0; NaCl , 0.01;

iii) Medium 3 (Oxa-1 + Trace elements) in g/L: Oxa-1; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 11.0; $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 5.0; $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 0.05; $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$, 2.0; H_3BO_3 , 0.05; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.007;

iv) Medium 4 (Oxa-2 + Trace elements of Medium 3).

Each of the medium constituents, except oxalic acid, were added to a 2000 litre flask and made up to 1 litre with distilled water. The medium was autoclaved, cooled and oxalic acid was added at room temperature. The medium was then adjusted to pH 7.0 with sterile NaOH and divided into 100 ml aliquots in 250 mL flasks. One gram of soil from each of the soil types was used to inoculate each type of medium. A control flask for each medium was prepared resulting in 16 flasks. The flasks were then incubated at 28°C and 150 rpm for

48 hours. After 48 hours, the cultures obtained were subcultured into their respective media using a 5% inoculum. This was performed two more times. Observations were made after the third passage as to whether or not the organisms could grow in the various media.

B. Subsequent Isolation and Growth Medium using Oxalic Acid

Medium Oxa-1 was employed throughout the thesis unless otherwise specified. The oxalate ion concentration was also changed to 37 mM or 3.264 g/L.

C. Subculturing Oxa-1 Grown Organisms on Oxa-1 plates and Nutrient Agar Plates

Bacteria from the Oxa-1 medium grown for 48 hours at 28°C and 150 rpm, were subcultured onto Oxa-1 agar plates using 0.1 ml inoculum from a 10⁵ serial dilution to determine the bacterial colony counts. The plates were incubated at 28°C for 96 hours.

To ensure that the colonies observed on the Oxa-1 plates were single isolates, the isolates were subcultured onto nutrient agar plates and incubated at 28°C for 24 hours. Also to ensure that the isolates were not utilizing trace carbon sources present in the medium, the isolates were subcultured onto Oxa-1 plates without oxalic acid added.

(2) Oxalate Assay (Indole Method)

The assay is a modification of that outlined in Snell and Snell (1961). The sample was dissolved in 0.3N H_2SO_4 such that the sample contained 0.2 - 2.0 mg of oxalic acid (2 mL of sample was added to 1 mL of 1N H_2SO_4). 2 mL of 0.1% Indole solution in concentrated sulfuric acid was pipetted down the side of the tube to minimize heat development. After 1 minute, the solution was mixed thoroughly. It was heated at 80 - 90°C for 45 minutes, cooled, and the absorbance was read at 575 nm against a reagent blank. To increase the sensitivity of the assay, readings were taken at 560 nm. (See Appendix I for chemical reaction)

(3) Reconstitution of Pseudomonas oxalaticus ATCC 11833

Pseudomonas oxalaticus, described by Quayle and Keech (1959), was obtained on a nutrient agar slant from Dr. I. Suzuki, The University of Manitoba. The organism was lyophilized in 1959. Pseudomonas oxalaticus was subcultured from a slant into 100 mL of 37 mM oxalate Oxa-1 broth and 100 mL of nutrient broth. The flasks were incubated at 28°C for 72 hours and 150 rpm. After incubation, 0.1 mL of the nutrient broth medium was used to inoculate nutrient agar plates as turbidity of the broth medium was minimal.

In an attempt to revive Pseudomonas oxalaticus, an inoculum from the slant was used to inoculate 100 mL of Pseudomonas broth and agar medium from the DIFCO manual. The

medium composition was as follows (g/L): Bacto peptone, 10; proteose peptone #3, 10; K_2HPO_4 , 1.5; $MgSO_4$, 1.5; agar, 20. The pH is adjusted to 7.0 with NaOH or H_2SO_4 .

(4) Kinetics of Oxalate Degradation

A. Oxalate Degradation by Six Mixed Cultures

Six mixed cultures (A,B,C,D,E,F) were isolated from the following soil samples respectively, DE, DE, DE, DC, RU, RU. 4.2 L of Oxa-1 medium was prepared and divided into 100 ml aliquots in 250 ml flasks. Each of the mixed cultures were inoculated in triplicate into 2 sets of flasks such that duplicate cultures were 12 hours apart. Time 0 was taken as the time of inoculation of the duplicate culture at 12 hours. Immediately after inoculation (time 0), 2 mL samples were taken from each flask to determine the oxalate concentration followed by further sampling at 3, 6, 9, 24, 27, 30 and 48 hours. The flasks were incubated at 28°C and 150 rpm.

B. Oxalate Degradation By Mixed Culture C and F

Two mixed cultures, C and F from the above experiment, resulted in the complete degradation of oxalate. These cultures were studied in detail with respect to growth, changes in pH, and oxalate degradation. Triplicate samples of the mixed cultures of C and F were inoculated into Oxa-1 medium as an inoculum culture. After 72 hours growth a 5% inoculum of the triplicate cultures was subcultured into Oxa-

1 medium in triplicate and 5 ml samples taken at time zero. The pH, absorbance at 540 nm (growth), and oxalate concentration were determined. Further samples were taken at 4, 7, 10, 13, 17, 22, 26, 30 and 47 hours. The flasks were incubated at 28°C and 150 rpm.

C. Oxalate Degradation by Pure Cultures Obtained from Mixed Cultures C and F

An experiment was set up to determine if the individual pure cultures from C and F could degrade oxalate. Oxa-1 broth medium was prepared and inoculated from nutrient agar plates. The organism grew on both nutrient agar and Oxa-1 agar plates. The cultures were monitored for oxalate degradation, growth (O.D. 540 nm) and pH change over a three day period taking samples at 12, 24, 49 and 73 hours. The cultures were incubated at 28°C and 150 rpm. (Note: this experiment was performed four times)

(5) Determination of the Optimal pH and Yeast Extract Concentration for Mixed Culture F

Mixed culture F-1 from the above experiment was used to characterize the culture with respect to growth, oxalate degradation and pH change in Oxa-1 medium at various pH values and with various concentrations of Yeast Extract. The following media were used:

Medium	pH	Yeast Extract (%)
Oxa-1-1	7.0	0
Oxa-1-2	8.5	0
Oxa-1-3	7.0	0.001
Oxa-1-4	8.5	0.001
Oxa-1-5	7.0	0.01
Oxa-1-6	8.5	0.01

Mixed culture F was inoculated into Oxa-1 medium as an inoculum culture and incubated for 72 hours at 28°C and 150 rpm. After 72 hours growth a 5% inoculum of the F culture was subcultured into the above media in triplicate and 5 mL samples of each culture was taken at time zero. The samples were analyzed for pH, absorbance at 540 nm (growth) and oxalate concentration. Further samples were taken at 5, 12, 22, and 26.5 hours. The flasks were incubated at 28°C and 150 rpm.

(6) Isolation and Identification of Pure Cultures from Mixed Cultures C and F, and the Rotating Biological Contactors

A. Isolation

C and F mixed cultures were grown in Oxa-1 broth medium for 72 hours at 28°C and 150 rpm. The above cultures were inoculated onto nutrient agar plates from a dilution series to obtain approximately 100 colonies per plate. The plates

were then incubated at 28°C for 48 hours. After the incubation period, colonies were selected according to their morphology and colour and streaked out on nutrient agar plates and incubated at 28°C for 48 hours to ensure pure cultures.

B. Identification

Each isolate was subjected to the Gram stain and oxidase test. If the organism was Gram negative and oxidase positive the isolate was inoculated into Oxiferm tubes. If the organism was Gram negative and oxidase negative the isolate was inoculated into Enterotubes. Each of the tubes were incubated at 37°C for 48 hours and then identified on the basis of their biochemical reactions.

(7) Isolate B-1 Studies

A. Photomicrographs

A.1 Transmission Electron Micrograph (TEM) - Negative Staining

Isolate B-1 was grown on sodium oxalate and oxalic acid plates for 48 hours at 28°C and sent to Laboratory Centre for Disease Control (LCDC) in Health and Welfare, Canada to obtain transmission electron micrographs of B-1, negatively stained.

(See Appendix II for methodology)

A.2 Transmission Electron Micrographs (TEM) - Thin Sectioning

Isolate B-1 was grown in oxalate medium and nutrient broth at 28°C and 150 rpm until the culture was at mid-

exponential growth phase. At this point the organism was chemically fixed. Two hundred mL of 0.1 M cacodylic acid was adjusted to pH 7.0 with 1N HCl to produce the cacodylate buffer. The solution was divided equally into two flasks and 0.05% ruthenium red was added to one flask.

To make the fixative solutions, 2 mL of 70% glutaraldehyde was added to 26 mL of each of the cacodylate buffer and cacodylate buffer with ruthenium red. Each was stirred until the viscous glutaraldehyde was in solution. Half of each specimen was added to 2-10 mL of each fixative solution and retained fully submerged at room temperature for 2 hours.

After fixation, the fixative solutions were removed by centrifugation and replaced immediately with cacodylate buffer or cacodylate buffer with ruthenium red respectively. The samples were washed twice and allowed to stand for 20 minutes between washes. The fixed specimens were stored at 4°C in the previously described buffers until they could be shipped to Dr. J.W. Costerton, Division of Biological Sciences, University of Calgary, Calgary, Alberta for electron microscopy. (See Appendix III for thin sectioning methodology after fixation)

B. Physiological, Biochemical and Toxicity Tests

Isolate B-1 was grown on sodium oxalate and oxalic acid plates for 48 hours at 28°C and sent to Laboratory Centre for

Disease Control (LCDC) in Health and Welfare, Canada, and to the American Type Culture Collection (ATCC), Maryland, USA.

C. Oxalate Degradation by Isolate B-1

A 5% inoculum of isolate B-1 was added to three, 250 mL flasks containing sodium oxalate medium and incubated at 28°C and 150 rpm. The initial oxalate concentration and turbidity of the sample were recorded at time zero followed by sampling at 10, 13, 16, 19 and 22 hours. The turbidity of the culture was measured spectrophotometrically at 540 nm on a Varian DMS 200 UV-VIS spectrophotometer. The oxalate concentration of the samples was determined by the indole method.

D. Tolerance of B-1 to High Levels of Oxalate

Oxalic acid medium was made up such that duplicate 250 mL flasks contained concentrations of 10000, 12000, 14000, 16000, 18000 and 20000 ppm oxalate ion. Each flask was inoculated with a 5% inoculum from a mid exponential phase culture of B-1. At time zero a sample was taken and an oxalate assay was performed to measure the initial oxalate concentration; samples taken at regular intervals were analyzed for oxalate.

E. Sodium Analysis of Oxalic Acid Standards

Oxalic acid medium standards were prepared such that each volumetric flask contained 10000, 12000, 14000, 16000, 18000

and 20000 ppm oxalate ion. The samples were sent for analysis to the chemistry section at Energy, Mines and Resources, CANMET. Analysis was performed on a Atomic Absorption Spectrophotometer. (See Appendix IV for Methodology)

(8) Rotating Biological Contactors (RBC)

A. Chemdisk Rotating Biological Contactor (RBC-1)

A.1 Operation of RBC-1

RBC-1 was started up in batch mode for 15 days using Oxa-1 medium with an oxalate ion concentration of 3500 ppm and an inoculum from mixed culture F. The disc speed rotation was 3 revolutions per minute (rpm). Half of the oxalic acid medium was removed every second day and fresh medium added to replenish the degraded oxalate. After 15 days the RBC was switched to the continuous mode with a flow rate of 6 mL/minute (residence time of 3.5 days) to increase the biomass on the disks surface. After 12 days, the oxalate concentration was increased to 4900 ppm oxalate ion concentration due to the poor growth on the disks. Eight days after increasing the oxalate ion concentration the RBC was shut down due to insufficient biomass on the disks.

A.2 Bacterial Adherence to Various Materials

During the operation of RBC-1, an experiment was performed to determine if the microorganisms from the RBC would adhere to different types of materials (ie. plastic,

teflon, rubber, polycarbonate filter paper cloth, plexiglass, stainless steel wire mesh, nylon cloth, and petri dish plastic). One litre of Oxa-1 medium was prepared in a Fernbach flasks and inoculated with mixed culture F. After 12 hours growth, the materials were cleaned with 70% alcohol and added to the active stationary culture. Sterile air was sparged through the medium over a six day period. After incubation, the materials were removed, gently rinsed with sterile distilled water and placed individually into separate 250 mL flasks with fresh Oxa-1 medium. These flasks were incubated for 48 hours at 28°C and 150 rpm and observations made.

B. Operation of RBC-2 Using Oxalic Acid

The RBC-2 was initially started up in batch mode for 14 days at room temperature (23°C) containing 12 L of Oxa-1 medium and inoculated with 200 mL of a fresh F mixed culture (1×10^8 cells/mL). Half of the oxalic acid medium was removed every second day and fresh medium added to replenish the degraded oxalate. Fresh oxalate medium was then added to the RBC at a flow rate of 4.0 mL/min. (50 hr residence time) to flush out the spent medium and to prevent evaporative losses in the RBC. The RBC was run for 2-4 weeks in this manner until a film of biomass developed on the discs. Once the biomass had developed, the residence time was slowly reduced to 4-5 hours such that complete degradation of oxalate was observed in the effluent.

C. Operation of RBC-2 Using Sodium Oxalate

C.1 Analysis of Industrial Sodium Oxalate Cake

Analysis of the sodium oxalate cake (Figure 6) was performed by Industry. The content of the cake was analyzed by titrating the acidified solution with 0.1N KMnO_4 . Na, Si, Al, Ca, Fe and V were determined from 0.5 g Cake/100 mL H_2O solution by Inductively Coupled Plasma-Atomic Emission Spectroscopy (ICP AES) technique calibrated with synthetic standard solutions.

C.2 Shake Flask Study using Sodium Oxalate Prior to use in RBC-2

Oxa-1 medium was modified due to the high concentrations of sodium in the sodium oxalate cake. The medium was modified in the following manner (g/L): $\text{NH}_4\text{H}_2\text{PO}_4$, 0.5; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1; Yeast Extract, 0.1; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5; and $(\text{COONa})_2 \cdot ?\text{H}_2\text{O}$, 10-12. The pH was adjusted to 7.0 with NaOH. In some cases the sodium oxalate cake had to be washed prior to use depending on the concentration of NaOH and texture of the cake. The modified medium (Na-Ox) was used in all subsequent studies unless otherwise stated. Na-Ox medium was prepared in 250 mL flasks. Two of the flasks were inoculated with a 5% inoculum from the RBC-2. The flasks were incubated for 24 hours at 28°C and 200 rpm. After 24 hours a second set of flasks were inoculated from the first set of flasks. At the end of each 24 hour incubation period, an oxalate assay was performed to determine if the medium would sustain good growth. (See Appendix V).

Figure 6. Industrial sodium oxalate cake.

C.3 Operation of RBC-2 Using Sodium Oxalate

The RBC was initially started up with oxalic acid medium as mentioned above. Shortly after optimal oxalate degradation was obtained, Na-Ox medium was used as an industrial source of oxalate.

(9) Tolerance of Mixed Culture F from RBC-2 to Chemical and Physical Factors

Experiments were conducted to assess the effects of pH, sodium concentration, oxalate concentration and temperature on oxalate degradation. All the experiments were performed in 250 mL flasks, incubated on a rotary shaker at 150 rpm (and 28°C, if required) and the growth and oxalate degrading ability of the organisms was monitored over a period of time.

A. Sodium Tolerance

In duplicate experiments, total sodium ion levels in oxalic acid medium was set at 0, 4, 8, and 12 g/L using NaHCO_3 as a sodium source. The pH of the medium was initially adjusted to 7.0 using NaOH before addition of the NaHCO_3 and H_2SO_4 after addition of NaHCO_3 .

B. Effect of Initial pH

In duplicate experiments, 10 g/L sodium oxalate cake (approx. 5000 ppm oxalate ion) was used in the modified medium and the pH was adjusted with H_2SO_4 or NaOH over a range of pH from 2 to 12, at 2 pH unit intervals.

C. Sodium Oxalate Cake

Nine flasks were set up with each containing a different concentration of sodium oxalate (ie. 8, 10, 12, ..., 22, 24 g/L).

D. Temperature

5 duplicate sets of flasks were set up with the sodium oxalate medium and inoculated with the mixed culture. The flasks were then incubated in temperatures of 5, 10, 35, 40 and 45°C.

(10) Shock Loading RBC-2 with Sodium Oxalate

When the RBC was running efficiently with 8 g/L sodium oxalate (2500 ppm oxalate ion), a 24 g/L sodium oxalate (13130 ppm) shock loading experiment was performed over a 24 hour period. The residence time of medium running through the RBC was 5.5 hours.

(11) Analyses of RBC Sloughed Off Biomass

30 g dry weight biomass obtained from 180 L medium was digested using perchloric acid and hydrofluoric acid and chemical analyses were performed using ICP, at Energy, Mines and Resources, Canada, Ottawa, Ontario. (See Appendix VI for preparation of samples for ICP)

(12) Synthetic Flocculation of RBC effluent

A. Preparation of the Synthetic Flocculants

All of the flocculants (RMR-30, RMX-30, RMD-30, Nalco 7879, Percol 757 and Percol 788N) were prepared the same way. 0.118 g of a flocculant was weighed out in a 250 mL flask and 5% ethyl alcohol in distilled water was added to make up 50 mL flocculant solution.

B. Comparison of Six flocculants on Settling Rate of RBC-2 Effluent

Seven, 500 mL graduated cylinders containing 500 mL of RBC-2 effluent were set up for each flocculant and a control. One mL from each flocculant stock was added to the appropriate graduated cylinder, to give 2.5 mg flocculant/ liter of effluent, and the flocculant was dispersed throughout the solution by mixing with a plastic screen. Observations were made at various time intervals.

C. Effect of Percol 757 Concentrations on Settling Behaviour of Biomass

Eight, 500 mL graduated cylinders containing 500 mL of RBC-2 effluent were set up for each Percol 757 concentration and a control. From the stock flocculant solution 1, 0.5, 0.2, 0.1, 0.05, 0.02 and 0.01 mL was added to the graduated cylinders giving flocculant concentrations, in mg/L per effluent, of 2.5, 1.25, 0.5, 0.25, 0.125, 0.05 and .025. Observations were made at various time intervals. After

flocculation was complete, the liquor was tested for its clarity by reading the absorbance at 540 nm on a Bausch and Lomb Model 700 Spectrophotometer.

(13) Minimization of Sodium Oxalate Medium Constituents

Sodium oxalate medium was prepared in which the four different individual medium constituents were varied separately (for example, sodium oxalate medium was prepared with three different concentrations of ferrous sulfate 0.05, 0.025 and 0.0 g/L). The flasks were inoculated with a 5% inoculum of the mixed culture in the RBC and the flasks were incubated at 28°C and 150 rpm. Oxalate concentrations were determined at various time intervals.

Once the optimal degradation rates verses lowest constituent concentration were obtained from above, all of the medium constituents were reduced in one experiment and degradation parameters were compared to those observed with the regular sodium oxalate medium. Again 250 mL flasks were inoculated with a 5% inoculum from the RBC and the flasks incubated at 28°C and 150 rpm.

The final experiment involved running the revised medium through the RBC at a residence time of 5 hours and comparing the degradation parameters to those obtained with the regular medium.

RESULTS AND DISCUSSION

(I) Isolation of Oxalate Degrading Microorganisms

1. Initial Isolation Procedure

Soil samples were obtained from the rhizosphere of Rhubarb and Dieffenbachia, which are plants known to contain oxalate (Kingsbury, 1964; Stephens, 1980; Libert and Franceschi, 1987). Since the plants contain oxalate, one would have to assume that microorganisms are present either on the plant or in the soil that are capable of degrading oxalate. Enrichment culture techniques have been previously used to isolate oxalate degrading bacteria from soil (Bhat and Barker, 1948; Jayasuriya, 1955; Chandra and Shethna, 1975a). In order to enrich for oxalate degrading microorganisms, a simple medium had to be developed to sustain growth for a minimum of three passages using oxalate as a sole carbon source. Jayasuriya (1955) and Chandra and Shethna (1975a) used a defined medium in their isolation procedure however their medium was too complex to be utilized in a cost efficient process. The four media that were developed differed with respect to macronutrient concentration and/or addition of micronutrients. Oxalic acid has a melting point of 101-102°C therefore it was added after autoclaving (Merck Index, 1983). It was also observed that filter sterilization of oxalic acid was not necessary as no contamination was observed in the medium when it was left at room temperature for several weeks.

In fact, if contamination was observed it would have been of interest to isolate the contaminant.

Observations of each of the soil types in the four media revealed that after three passages, each medium could sustain growth of the soil bacteria. Because a simple medium was sought, Oxa-1 medium was used for subsequent experiments and the oxalate concentration was increased to 37 mM.

2. Subculturing Oxa-1 Grown Organisms on Oxa-1 plates and Nutrient Agar Plates

The organisms isolated on the Oxa-1 plates were very small (1-2 mM in diameter) cream coloured colonies from each of the soil types. The limited growth of the colonies are consistent with the observations of Bhat and Barker (1948), Jayasuriya (1955) and Chandra and Shethna (1975a). Individual colonies with different morphologies were subcultured onto Oxa-1 plates and nutrient agar plates. Each of the colonies selected grew well on nutrient agar. Subculturing of the isolates on Oxa-1 medium without oxalate, revealed that the organisms were scavenging on either yeast extract or impurities in the agar such as other carbohydrates. Because growth was observed in the liquid medium, organisms must be present that utilize oxalate as a carbon source. The non-oxalate degrading organisms isolated from the Oxa-1 liquid medium must have utilized waste products or dead biomass from the oxalate degraders. This is common and was observed by Bhat

and Barker (1948).

(II) Oxalate Assay (Indole Method)

The indole method of oxalate determination is limited to a range of approximately 100 to 5000 ppm oxalate. The assay was characterized by a change in colour from yellow to red in the presence of oxalate. A wavelength of 560 nm allowed for an increase in sensitivity at the lower oxalate concentrations on the DMS 200 UV-Vis spectrophotometer. Sampling the RBC's resulted in a standard deviation of less than 100 ppm, whereas in some shake flask studies, standard deviations up to 1000 ppm were observed in exponential phase cultures.

(III) Reconstitution of Pseudomonas oxalaticus ATCC 11833

Growth of Pseudomonas oxalaticus was observed in the nutrient broth as well as in liquid Pseudomonas medium. From each medium, the organism was subcultured on its respective medium and again growth was observed indicating that the organism was still viable. Subculturing the organism from both medium types to Oxa-1 agar plates was ineffective. The organism would not grow on or in oxalate medium. Pseudomonas oxalaticus was one of the main organisms used to elucidate the pathway for oxalate degradation therefore one would expect that it would not have any problems growing in oxalate medium. A possible reason for its inability to degrade oxalate may be the lyophilized storage for more than twenty five years.

(IV) Kinetics of Oxalate Degradation

1. Oxalate Degradation by Six Mixed Cultures

After following the same procedure in 1A for the isolation of oxalate degraders, six mixed cultures were obtained from the various soil types after three passages. Up until now, the presence of oxalate degraders was determined by the observation of growth in oxalate medium. In this experiment, oxalate degradation by the six mixed cultures was followed over a 48 hours period (Figure 7). It was observed that only mixed culture C from Dieffenbachia soil and mixed culture F from Rhubarb soil could degrade oxalate. Although growth was observed in each of the inoculum cultures, the oxalate degraders must have utilized the oxalate which would then allow transient organisms to utilize the dead biomass and waste products of the oxalate degraders. Bhat and Barker (1948) observed the same phenomena where only two of eight mixed cultures degraded oxalate.

2. Oxalate Degradation By Mixed Cultures C and F

Since C and F mixed cultures were the only cultures observed to degrade oxalate, it was of interest to follow the kinetics of oxalate degradation with respect to growth of the culture, pH and oxalate degradation. Figures 8 and 9 show that as growth of the cultures increased, there was a change in pH from 7.0 to 8.25. This was a common phenomenon observed by other researchers dealing with oxalate degrading bacteria

Figure 7. Oxalate degradation of six mixed cultures monitored over a 48 hour time period. A triplicate set of 250 mL flasks containing oxalic acid medium (Oxa-1) were incubated at 28°C and 150 rpm.

Figure 3. Oxalate degradation, growth, and pH of mixed culture C monitored over a 47 hour time period. A triplicate set of 250 mL flasks containing oxalic acid medium (Oxa-1) were incubated at 28°C and 150 rpm.

Figure 9. Oxalate degradation, growth, and pH of mixed culture F monitored over a 47 hour time period. A triplicate set of 250 mL flasks containing oxalic acid medium (Oxa-1) were incubated at 28°C and 150 rpm

(Bhat and Barker, 1948; Jayasuriya, 1955; Chandra and Shethna, 1975a). Mixed culture C degraded 82% of the oxalate whereas mixed culture F degraded 95% of the oxalate. The main problem with these cultures was the extended lag phase. It appeared that non-reproducible lag phases are common and may be dependent on the concentration of yeast extract. Yeast extract has been shown to be essential for oxalate degradation (Bhat and Barker, 1948; Jayasuriya, 1955; Chandra and Shethna, 1975a). These two cultures were used in subsequent experiments in order to isolate the individual bacteria responsible for oxalate degradation and to test their ability to degrade oxalate.

3. Oxalate Degradation by Pure Cultures Obtained From Mixed Cultures C and F

Procedure 1C was used to obtain pure cultures from mixed cultures C and F. Three pure isolates were obtained from C and five pure isolates were obtained from F. Each pure isolate was inoculated into Oxa-1 liquid medium to assess growth, changes in pH and an oxalate degradation curve. After four unsuccessful attempts to get the isolates to grow in Oxa-1 medium, it was concluded that the oxalate degrading organisms were lost during the isolation procedure or that there may be a synergistic effect between two or more of the organisms. The latter possibility is unlikely as no other author has found a synergism between oxalate degrading organisms (Bhat and

Barker, 1948; Jayasuriya, 1955; Chandra and Shethna, 1975a). Oxalate degrading bacteria appear as pin point colonies on oxalate plates (Bhat and Barker, 1948; Jayasuriya, 1955; Chandra and Shethna, 1975a). This is due to the limited energy the organisms obtain from utilizing oxalate as a sole carbon source. As mentioned in the literature review, P. oxalaticus obtains approximately 1.0 mole of ATP per mole of oxalate (Dijkhuizen et al., 1977b). Non-oxalate degrading bacteria metabolizing impurities in the agar would appear as pin point colonies as well. Thus differentiation of the colonies according to size would be very difficult.

(V) Determination of the Optimal pH and Yeast Extract Concentration for Mixed Culture F

As mentioned previously, the lag phase of the cultures was not reproducible. The variance in the lag phases may be dependent on the yeast extract concentration. Yeast extract contains B vitamins, nitrogen and carbon compounds which aid in the initial growth period of bacterial cultures (Pelzar and Chan, 1981). Since the cultures were observed to increase the pH from 7.0 to 8.5, it was of interest to observe the growth rate at an initial pH of 8.5. Figures 10, 11 and 12 show that the concentration of yeast extract does have an effect on the growth rate. Oxalate degradation was observed over a 27 hour period with yeast extract concentrations of 0.01 and 0.001% and a control at two pH values. The culture containing 0.001%

Figure 10.

Oxalate degradation, growth and pH of mixed culture F were monitored over a 27 hour time period. No yeast extract was added to Oxa-1 medium and the initial pH of graph (a) and (b) was 7.0 and 8.5, respectively. A triplicate set of 250 mL flasks were incubated at 28°C and 150 rpm.

Figure 11. Oxalate degradation, growth and pH of mixed culture F were monitored over a 27 hour time period. 0.001% yeast extract was used in Oxa-1 medium and the initial pH of graph (a) and (b) was 7.0 and 8.5, respectively. A triplicate set of 250 mL flasks were incubated at 28°C and 150 rpm.

Yeast Extract = 0.01

— Growth-7.0 — pH-7.0 — Growth-8.5 — pH-8.5

Yeast Extract = 0.01

— Growth-7.0 — Oxalate-7.0 — Growth-8.5 — Oxalate-8.5

Figure 12.

Oxalate degradation, growth and pH of mixed culture F were monitored over a 27 hour time period. 0.01% yeast extract was used in Oxa-1 medium and the initial pH of graph (a) and (b) was 7.0 and 8.5, respectively. A triplicate set of 250 mL flasks were incubated at 28°C and 150 rpm.

yeast extract had a greater lag phase than the culture with 0.01% yeast extract; no growth was observed in the control. These results indicate that the present yeast extract concentration that is used in Oxa-1, is sufficient to allow for optimal growth of mixed culture F. Various yeast extract concentrations have been cited in the literature from 10 to 0.05%. The most common concentration was 0.1% (Bhat and Barker, 1948; Jayasuriya, 1955; Chandra and Shethna, 1975a). Bhat and Barker (1948) also looked at the effect of various concentrations of yeast extract on the growth of Vibrio oxaliticus with different formate concentrations. Their results indicate the optimal concentration of yeast extract was between 0.2 and 0.4%. Although the concentration of yeast extract in Oxa-1 medium is lower than the cited concentration, excellent degradation was observed. One also must keep in mind that the costs must be kept low and that once the RBC is running continuously, there will be no lag period as the system will be in steady state. The initial pH of the medium did not affect oxalate degradation.

(VI) Isolation and Identification of Pure Cultures from Mixed Cultures C and F, and the Rotating Biological Contactors

A number of pure isolate have been obtained from the mixed cultures C and F and from the two RBC's. In the first isolation of C and F, four pure cultures were identified as Pseudomonas stutzeri, Moraxella sp., Citrobacter freundii,

and Pseudomonas cepacia using the Oxiferm and Enterotube method of bacterial identification at Energy, Mines and Resources, Canada. Although eight isolates were mentioned earlier in 4C, some of the isolates were the same. Once the RBC was inoculated with F mixed culture and running with sodium oxalate, a second isolation was performed which resulted in the identification of the following cultures: Acinetobacter lwoffii, Pseudomonas cepacia, Micrococcus sp. and Flavobacterium odoratum. These and subsequent cultures were identified at Health and Welfare Canada. To determine if the culture in the RBC changed over time, a second isolation was performed five months later and the following isolates were obtained: Citrobacter amalonaticus, Aeromonas hydrophila, an unidentified organism (B-1) and Flavobacterium odoratum. There was a continuous change in the microflora within the RBC over a period of time. Again, five months later, organisms were isolated from the RBC and in addition to the above organisms, two other organisms were identified, Enterobacter agglomerans and Pseudomonas fluorescens. All of the isolates were tested for their ability to degrade oxalate. The only organism observed to degrade oxalate however, was the unidentified B-1 (Figure 13). The other organisms must be transient contaminants surviving on the dead biomass of B-1. B-1 has been difficult to identify thus a separate section in the thesis is devoted to B-1.

Figure 13.

Oxalate degradation and growth of isolate B-1 monitored over a 22 hour time period. A triplicate set of 250 mL flasks containing oxalic acid medium (Oxa-1) were incubated at 28°C and 150 rpm.

(VII) Isolate B-1 Studies

1. Photomicrographs

1.A Transmission Electron Micrograph (TEM) - Negative Staining

The TEM shows B-1, a rod shaped bacteria with a single polar flagella characteristic of the Pseudomonads (Bergey's Manual of Systematic Bacteriology, 1989)(Figure 14).

1.B Transmission Electron Micrograph (TEM) - Thin Sectioning

The TEM shows B-1, a rod shaped bacteria with multiple membrane layers characteristic of Gram negative bacteria and because of the multiple membranes, characteristic of the Pseudomonads (Costerton et al., 1974; Bergey's Manual of Systematic Bacteriology, 1988)(Figure 15).

2. Physiological, Biochemical and Toxicity Tests

The results obtained from similar tests performed at ATCC and Health and Welfare, Canada are identical. Both institutes had difficulty identifying B-1 on the basis of conventional biochemical and nutritional tests. The biochemical tests at Health and Welfare, Canada showed the organism to resemble a Pseudomonad. Oxalate degrading organisms similar to B-1 have been isolated before, Jayasuriya (1955) and Chandra and Shethna (1975a) isolated Pseudomonas OD1 and Pseudomonas YOx respectively, which were rods (0.5 x 1.5 um) with a single polar flagellum which produced a yellow insoluble pigment.

Figure 14. Transmission electron micrograph of isolate B-1 grown on oxalic acid medium. Preparation was negatively stained. (Magnification = 35,350x)

Figure 15. Transmission electron micrograph of isolate B-1 grown on oxalic acid medium. Preparation was thin sectioned. (Magnification = 50,000x)

ATCC's biochemical and physiological tests showed that B-1 resembled a Xylophilus ampelina, however the growth factors and nutritional spectra are quite different. ATCC stated that X. ampelina forms a subbranch of Pseudomonas acidovorans based on rRNA (ribosomal ribonucleic acid). However, the nomenclature of strains in this rRNA complex is presently being challenged. The fatty acid analysis performed by ATCC was also equivocal because of the slow growth of the strain (See Appendix VII for tests performed by ATCC).

Toxicity tests were performed at Health and Welfare, Canada. The tests were performed using 24 and 48 hour filtrates of cultures from both nutrient broth and oxalate medium. The tissue culture system included Vero (monkey kidney cells), HeLa (human cervical carcinoma cells), and CHO (chinese hamster ovary cells). These cell lines respond to a variety of microbial enterotoxins and cytotoxins. Suckling mouse tests were performed using 3 day old CF1 mice. Weak, non-specific cytotoxicity was observed in all cell lines with the 48 hour oxalate filtrate, however these effects were not observed in the nutrient broth filtrates. Intestinal weight/remaining body weight ratios (IB/BW) in the suckling mice after 4 hours were considered negative for microbial enterotoxins reactive in this bioassay.

3. Oxalate Degradation by Isolate B-1

Complete degradation of oxalate was observed within 18 hours after inoculation with isolate B-1 (Figure 13). This curve is comparable to the kinetics study performed with mixed culture F suggesting that B-1 is the organism responsible for oxalate degradation in the mixed culture.

4. Tolerance of B-1 to High Levels of Oxalate and Sodium Analysis of Oxalic Acid Standards

Isolate B-1 was observed to completely degrade up to 16000 ppm oxalate ion in 50 hours. The highest oxalate ion concentration reported to have been degraded in the literature was 9500 ppm by a Pseudomonas KOx (Chandra and Shethna, 1975a). Isolate B-1 did not degrade 18000 and 20000 ppm oxalate ion to completion, since the remaining oxalate ion concentrations were 2000 and 4000 ppm respectively (Figure 16). The organism could partially degrade the higher levels of oxalate therefore something other than oxalate must have been preventing the organism from completely degrading the the oxalate. It was determined that on adding 16000 ppm oxalate, the sodium levels exceed 8 g/L as a result of the sodium hydroxide required to neutralize the medium (Figure 17). It was observed previously that B-1 could only tolerate up to 8 g/L sodium ion (Section IX).

Figure 16. Tolerance of isolate B-1 to high levels of oxalate from oxalic acid (Oxa-1) medium. Oxalate was monitored over a 145 hour period. A duplicate set of 250 mL flasks were incubated at 28°C and 150 rpm.

Figure 17. Sodium ion analysis of varying concentrations of oxalic acid medium standards performed on an Atomic Absorption Spectrophotometer.

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MICROCOPY RESOLUTION TEST CHART
NATIONAL BUREAU OF STANDARDS
STANDARD REFERENCE MATERIAL 1010a
(ANSI and ISO TEST CHART No. 2)

(VIII) Rotating Biological Contactor (RBC)

1. Chemdisk Rotating Biological Contactor (RBC-1)

1.A Operation of RBC-1

The RBC-1 was started up in batch mode to allow for growth and attachment of the organisms to the disk surface. Half of the medium was changed every second day to replenish the oxalate. After 15 days, growth on the disks was very poor, therefore the RBC was switched to continuous mode with a long residence time of 3.5 days to minimize washout of the bacteria. The poor bacterial growth could have been due to insufficient oxalate supply to the RBC. Once in continuous mode, the oxalate concentration was increased to 7000 ppm oxalate ion to further replenish the oxalate supply. After 35 days operation, the RBC-1 showed little growth, even on the disk closest to the influent port (Figure 18). Oxalate assays of medium from the chambers indicated poor degradation (Figure 19). Poor bacterial growth may be due to: 1) the porosity and composition of the disks did not allow for bacterial adherence and 2) oxalate does not provide sufficient energy or cellular carbon to allow extracellular polysaccharide synthesis which would aid in bacterial adherence to solid surfaces.

Figure 18. RBC-1 disks showing very little growth on the disks and the porosity of the disks.

Figure 19. Bar diagram showing the levels of oxalate ion in the influent, RBC chambers and effluent in RBC-1. Oxalic acid medium was run through the RBC-1 with a residence time of 3.5 days and disk rotation speed of 3 rpm.

1.B Bacterial Adherence to Various Materials

An experiment was performed to rule out the possibility that the oxalate degrading organisms could not adhere to the plastic disks in RBC-1. Eight different types of materials were submerged in a stationary culture of oxalate degrading bacteria, taken out and washed and then individually put back into fresh oxalate medium. Each of the flasks containing the washed materials showed growth after 48 hours incubation. This indicated that oxalate degrading bacteria must have adhered to the materials in order to go through the washing stage and act as an inoculum in fresh oxalate medium. These results suggest that the bacteria in RBC-1 did have the ability to adhere to the disk material.

2. Operation of RBC-2 Using Oxalic Acid

RBC-2 was started in the same manner as RBC-1. When the RBC was switched to the continuous mode there was not much growth on the disks. However as the flow rate was increased (decrease residence time), growth increased starting from the disks closest to the influent port. A gradation in color from dark orange to light orange between the influent and effluent ports developed (Figure 5). The orange colour was due to iron precipitation in the biomass. Samples from each of the chambers revealed that at low flow rates, most of the oxalate degradation was taking place in the first chamber, however as the flow rate was increased, bacterial growth on the disks was

Figure 20.

Bar diagram showing the levels of oxalate ion in the influent, RBC chambers and effluent in RBC-2. Oxalic acid medium was run through the RBC-2 with residence times of 28, 14 and 7 hours with a disk rotation speed of 0.6 rpm.

evenly distributed throughout the RBC and thus oxalate degradation was occurring in all of the chambers (Figure 20). The flow rate was increased to 40 mL/min. (5 hour residence time) resulting in >95% reduction in the oxalate concentration (Figure 21). This RBC had very good biomass development on the disks thus proving that oxalate degrading bacteria could obtain energy and cellular carbon from oxalate to produce extracellular polysaccharides. The results of section B.2 and this section indicate that the problem with RBC-1 was the porosity of the disks.

3. Operation of RBC-2 Using Sodium Oxalate

3.A Analysis of Industrial Sodium Oxalate Cake

The analysis of the sodium oxalate cake (Table 2) shows that the wet sample contains >50% sodium oxalate with lesser amounts of water and sodium (probably in the form of NaOH). The other elements are negligible and should have no effect on the oxalate degrading bacteria.

3.B Shake Flask Study Using Sodium Oxalate Prior to use in RBC-2

Sodium oxalate cake contained 21% sodium and thus substitution of sodium oxalate for oxalic acid in the Oxa-1 medium would increase the sodium ion concentration. To minimize the sodium ion concentration, sodium phosphate and ammonium sulphate were replaced with ammonium phosphate in a

Figure 21. Bar diagram showing the levels of oxalate ion in the influent, RBC chambers and effluent in RBC-2. Oxalic acid medium was run through the RBC-2 with a residence time of 5 hours and a disk rotation speed of 0.6 rpm.

	<u>%</u>
(COONa) ₂	52.00
Na	21.00
H ₂ O	32.00
Si	00.05
Ca	00.13
Fe	00.05
V	00.01

Table 2. Analysis of Industrial sodium oxalate cake. The sodium oxalate cake was analyzed by titrating the acidified solution with 0.1N KMnO₄. The elements were determined from 0.5 g cake/100 mL of H₂O solution by ICP AES technique.

new medium thereafter referred to as sodium oxalate medium (Na-Ox medium). It was also calculated that to maintain the same concentration of oxalate ion as in Oxa-1 medium, approximately 7.15 g/L sodium oxalate had to be used (see appendix 2). The composition of the sodium oxalate cake was inconsistent, therefore the oxalate concentration varied from experiment to experiment due to this industrial substrate.

A shake flask study was set up to test the Na-Ox medium on bacteria from RBC-2. In the first set of flasks, 94% of the oxalate was degraded in 24 hours. On subculturing to a second set of flasks, 98% of the oxalate was degraded in 24 hours. This data indicates that the new medium could be used in the RBC-2 without detrimental effects.

3.C Operation of RBC-2 Using Sodium Oxalate Medium

Sodium oxalate was continuously fed into the RBC with a residence time of 5 hours shortly after optimal degradation was achieved using Oxa-1 medium. The addition of industrial sodium oxalate resulted in a heavier film of biomass on the disks (Figure 22). Better growth was observed in the RBC with Na-Ox medium than with the Oxa-1 medium. The reason for the better growth may be the presence of humates in the sodium oxalate cake.

Figure 22. Biomass development on RBC-2 using sodium oxalate medium. The oxalate concentration was 5000 ppm with a residence time of 5 hours and a disk rotation speed of 3 rpm.

This medium also allowed a unique matrix of silicates, polysaccharides, iron and biomass to form on the disks. From Figure 23, complete oxalate degradation occurs within a 5 hour residence time. Growth was so great with the sodium oxalate medium that biomass sloughed off and clogged the bottom of the trough. This was rectified by increasing the disk rotation speed from 0.6 rpm to 3 rpm which produced more turbulence, allowing the wash out of suspended biomass.

(IX) Tolerance of Mixed Culture F from RBC-2 to Chemical and Physical Factors

In a number of shake flask studies, the mixed culture in the RBC-2 running with Na-Ox medium was tested for its tolerance to sodium ion, pH, sodium oxalate and temperature. The results indicated that in separate studies, the culture could tolerate up to 8 g/L sodium ion, a pH range between 6 and 9, 16 g/L (~5000 ppm) sodium oxalate and temperatures between 5 and 40°C. The pH and temperature range are comparable to oxalate degrading bacteria studied by Bhat and Barker (1948), Jayasuriya (1955) and Chandra and Shethna (1975a). Also the sodium tolerance is normal for a non-halophile (Peczar and Chan, 1981). It is important in the operation of the RBC to maintain optimum conditions that achieve optimum degradation rates. These results indicate the limits to which parameters can be altered, however the closer to the boundary one gets, the lower the degradation rate.

Figure 23. Bar diagram showing the levels of oxalate ion in the influent, RBC chambers and effluent in RBC-2. Sodium oxalate medium was run through the RBC-2 with residence times of 5 hours and a disk rotation speed of 3 rpm.

(X) Shock Loading RBC-2 with Sodium Oxalate

The shock loading experiment in RBC-2 revealed that the mixed culture could not only tolerate high levels of sodium oxalate (13500 ppm oxalate ion), but also degrade approximately 66% of the oxalate in the process (Figure 24). After shock loading, regular sodium oxalate medium was pumped through the RBC with almost complete degradation of the oxalate. No harmful effects were observed on the biomass.

(XI) Analyses of RBC Sloughed Off Biomass

Chemical analyses of the sloughed off biomass from RBC-2 shows very low levels of toxic metals within the biomass (Table 3). Using the biomass as a soil amendment is a possibility, however without the proper bioassays, this is not recommended. Most of the elements identified are required for bacterial growth.

(XII) Synthetic Flocculation of RBC Effluent

The biodegradation of oxalate is of industrial importance and therefore it is important to look at the removal of biomass from the effluent. Flocculation studies were thus carried out on the RBC-2 effluent.

Figure 24.

Bar diagram showing the levels of oxalate in the influent, RBC chambers and effluent, before, during and after shock loading. Sodium oxalate medium (Na-Ox) was run through the RBC-2 with a residence time of 5 hours and a disk rotation speed of 3 rpm.

Element	mg/g Biomass	Element	mg/g Biomass
B	0.6	V	0.14
Ca	17.0	As	0.11
Fe	43.0	Sr	0.12
Mg	12.0	Zn	0.20
P	58.0	Pb	0.22
Si	5.6		

Elements with less than 0.10 mg/g Biomass
Be, Mn, Ti, Ag, Co, Cu, Ga, Ni, Pd, Se, Y, Au, Ba, Pt

Table 3. Analysis of biomass from the effluent of RBC-2. 30 g dry weight biomass/180 L medium was digested using perchloric acid and hydrofluoric acid and chemical analyses performed on ICP.

1. Comparison of Six Flocculants on Settling Rate of RBC-2 Effluent

Six cationic synthetic flocculants were chosen for industrial biomass flocculation. The same flocculant concentrations were added to the RBC-2 effluent and by visual comparison, Percol 757 achieved the fastest settling rate and the clearest liquor (Table 4). The other flocculants and the control achieved similar settling rates.

2. Effect of Percol 757 Concentration on Settling Behaviour of Biomass

The data from Table 5 clearly indicates that the addition of Percol 757 at 2.5 mg/L effluent gave the most desirable results. Large flocs, a faster settling rate and better clarity of the liquor. However, the biomass in the effluent also settles on its own at a comparable rate without the addition of a synthetic flocculant.

The ultimate decision on whether to use the synthetic flocculant would be based on the economics of letting the biomass settle out by itself in a settling tank or adding a synthetic flocculant to improve the settling behaviour.

(XIII) Minimization of Sodium Oxalate Medium Constituents

To minimize operating costs, it was important to reduce the medium constituents to a minimal concentration without affecting the degradation rate. Four graphs were generated by

Synthetic Floc.	Complete Settling Time (min.)	Liquor
RMR-30	12	cloudy
RMD-30	12	cloudy
RMX-30	15	cloudy
Nalco 7879	15	cloudy
Percol 788N	15	cloudy
Percol 757	5	less cloudy
Control	15	cloudy

Table 4. Visual comparison of six synthetic flocculants added to RBC-2 effluent at a concentration of 2.5 mg/L.

mg Percol 757 per Liter Effluent	Complete Settling Time (min.)	Absorbance (540 nm)
2.5	5 - 7	0.40
1.25	7 - 10	0.50
0.50	10 - 12	0.55
0.25	10 - 12	0.57
0.125	10 - 12	0.58
0.05	10 - 12	0.59
0.025	10 - 12	0.59
Control	10 - 12	0.77

Table 5. Effect of various concentrations of Percol 757 on the settling behaviour of biomass from the effluent of RBC-2. Absorbance was measured on a Bausch and Lomb 700 spectrophotometer.

reducing the individual constituents in the sodium oxalate medium (Figure 25).

Each graph showed that at half the constituent concentration, the rate of oxalate degradation was comparable to that at the regular concentration. At less than half the constituent concentration, the rate of oxalate degradation decreased substantially. The results indicated that all of the medium constituents could be decreased by half. Figure 26 shows that half medium constituents medium followed the same rate of oxalate degradation as the regular sodium oxalate medium after inoculation with the mixed culture from the RBC. Complete degradation of oxalate was observed in the RBC when the revised medium was tested (Figure 27).

a) Ammonium Phosphate conc. (g/L)

b) Ferrous Sulfate Conc. (g/L)

c) Magnesium Sulfate conc. (g/L)

d) Yeast Extract (g/L)

Figures 25.

Minimization of sodium oxalate medium (Na-Ox) constituents. Each of the following medium constituents were reduced individually from the medium: (a) Ammonium phosphate, (b) Ferrous sulfate, (c) Magnesium sulfate, and (d) Yeast extract. An inoculum was obtained from RBC-2 and the duplicate set of 250 mL flasks were incubated at 28°C and 150 rpm. Oxalate degradation was monitored over time.

Figure 26.

Sodium oxalate (Na-Ox) medium constituents were reduced to half the normal concentration. An inoculum was obtained from RBC-2 and a duplicate set of 250 mL flasks were incubated at 29°C and 150 rpm. Oxalate degradation was monitored over 40 hours.

Figure 27.

Sodium oxalate medium (Na-Ox) constituents were reduced to half the normal concentration and run through the RBC-2 with a residence time of 5 hours and a disk rotation speed of 3 rpm. The bar diagram shows the levels of oxalate in the influent, RBC and effluent.

CONCLUSION

An industrial process was developed using oxalate degrading bacteria and a rotating biological contactor (RBC). Microorganisms that degrade oxalate are ubiquitous in the environment as there are many natural vegetative sources of oxalate. Enrichment of the organisms proved to be time consuming because both non-oxalate degrading bacteria, metabolizing impurities in the medium, and oxalate degrading bacteria, appear as pin point colonies on the solid oxalate medium. Thus differentiation was difficult. The oxalate degrading bacterium isolated, B-1, was found to degrade the highest oxalate concentration to date, 16000 ppm. The highest previously reported concentration tolerated by a bacterium was 9500 ppm. Identification of B-1 has been difficult because it is a new organism and it is fastidious. The data obtained to date on B-1 suggests that it belongs to the genus Pseudomonas.

Two rotating biological contactors were employed as continuous aerobic reactors because of their simple design, easy maintenance, low capital cost and low operating costs. RBC-1 was found unsuitable for oxalate degradation due to the porosity of the disks (0.6 cm opening), however RBC-2 worked very well resulting in the degradation of 5000 ppm oxalate with a 5 hour retention time. For such a system to work efficiently, the residence time should be low with a relatively high organic concentration in the influent to the

RBC. The oxalate degradation process met these requirements with oxalic acid, sodium oxalate and the revised sodium oxalate medium. In fact, the biomass on the RBC disks could tolerate very high transient concentrations (shock loading) of oxalate and still maintain efficient oxalate degradation after shock loading.

Industrial interests are currently determining the feasibility of scaling up the process to a commercial scale.

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APPENDIX I

CHEMICAL REACTION OF INDOLE WITH OXALIC ACID

The chemical reaction of indole with oxalic acid results in a colour change from yellow to red.

APPENDIX II

NEGATIVE STAINING OF ISOLATE B-1 FOR TRANSMISSION ELECTRON MICROSCOPY

A drop, from an oxalate grown liquid culture of B-1, was added to a drop of distilled water in a parafilm sheet using a standard bore pasteur pipet. A carbon-stabilized, formvar-coated 400 mesh copper grid was then floated on the drop, film side down, for 60 seconds. The grid was then removed from the drop, excess fluid was removed by touching the edge of the grid with filter paper and the grid was allowed to dry. A drop of potassium phosphotungstate, 0.25 %, aqueous, pH 6.8 was then added as a negative stain. After a constant time of approximately 1-2 seconds, excess stain was removed by touching the edge of the grid with a filter paper and the preparation was allowed to air dry.

The grid was examined in a Zeiss EM 10c transmission electron microscope and representative cells were recorded photographically.

APPENDIX III

THIN SECTIONING OF ISOLATE B-1 FOR TRANSMISSION ELECTRON MICROSCOPY

Fixation of isolate B-1 was performed by the author and is therefore found in the methods section. After fixation, the specimen was dehydrated in a series of acetone-distilled water solutions (30%, 50%, 70%, 90%, 100%, 100%) followed by two washes in propylene oxide, and then infiltrated with a 3:1 mixture of propylene oxide and low viscosity resin (24) and left for 24 hrs. The samples were transferred to fresh resin and left an additional 24 hrs. The specimens were embedded in fresh resin in polyethylene capsules and polymerized for 16 hrs at 60°C. Thin sections were cut on an LKB ultratome III and stained with uranyl acetate and lead nitrate. The copper 400 mesh grids were carbon backed using a Balzers 360 freeze etching apparatus. The samples were viewed with an Hitachi H-600 transmission electron microscope.

APPENDIX IV

METHODOLOGY FOR SODIUM ANALYSIS ON AN ATOMIC ABSORPTION SPECTROPHOTOMETER

Sodium, in the oxalic acid medium, was determined by Atomic Absorption Spectroscopy in an oxidizing air/acetylene flame. The wavelength and spectral band width were 589.6 and 0.2 nm, respectively. The calibration range was 0.5 to 2.0 ug/mL. Potassium chloride was added to the calibration standards and samples to give a potassium concentration of 2000 ug/mL.

APPENDIX V

CALCULATIONS TO OBTAIN 5000 PPM OXALATE FROM SODIUM OXALATE CAKE

The oxalate in 5000 ppm oxalic acid is 3491 ppm, therefore the concentration of sodium oxalate required was calculated out to be 5134 ppm dry weight.

M.W. oxalate	88.0199 g/L	=	x g/L
M.W. oxalic acid (hydrated)	126.066 g/L		5.000 g/L

x = 3.491 g/L or 3491 ppm oxalate in 5000 ppm oxalic acid

M.W. oxalate	88.0199 g/L	=	3.491 g/L
M.W. Na-oxalate	133.98 g/L		x g/L

x = 5.314 g/L or 5314 ppm dry weight Na-oxalate to achieve 3491 ppm oxalate

Because 32 % of the sample is water and 2.6 % is aluminum, one must add on the additional weight of 1.835 g/L. The theoretical weight to get 3491 ppm oxalate is therefore 7.15 g/L or 7150 ppm sodium oxalate cake.

APPENDIX VI

PREPARATION OF SAMPLES FOR INDUCTIVELY COUPLED PLASMA SPECTROSCOPY

0.5g of dried biomass was weighed out and moistened with distilled water. 15 mL of HCl was added and the sample boiled for 10 minutes. While hot, 10 mL of HNO₃ was added and the solution boiled for an additional 20 minutes. 10 mL of HClO₄ (perchloric acid) was then added and heated to fumes followed by addition of 5 mL HF (hydrofluoric acid). Once the sample approached dryness, it was removed from the heat, cooled, and 10 mL HNO₃ added. The sample was heated to boiling followed by addition of 100 mL of distilled water. Again the sample was boiled until all of the salts had dissolved. The sample was then transferred to a volumetric flask and brought up to 200 mL with distilled water to give a 5% HNO₃ solution. The solution was then sent for ICP analysis.

APPENDIX VII

RESULTS OF BIOCHEMICAL TESTS PERFORMED AT HEALTH AND WELFARE,
CANADA, OTTAWA, AND THE AMERICAN TYPE CULTURE COLLECTION,
MARYLAND, USA

1. COMPARISON OF XYLOPHILUS AMPELINA TO ISOLATE B-1

Tests	<u>X.ampelina</u>	Isolate B-1
Flagella monotrichous	+	+
Growth at 4oC, 37oC	-	-
Yellow pigment	+	+
Very slow sparse growth	+	+
Gelatin	-	-
Nitrate reduction	-	-
Phenylalanine deamination	N.G.	N.G.
Catalase	+	+
Oxidase	-	slow & weak
Arginine, ornithine, lysine decarboxylation	-	-
Acid from:		
O-F D-xylose	-	-
D-ribose	-	-
L-rhamnose	-	-
D-glucose	-	-
D-mannose	-	-
D-fructose	-	-
sucrose	-	-
trehalose	-	-
cellobiose	-	-
lactose	-	-
maltose	-	-
dulcitol	-	-
D-mannitol	-	-
D-sorbitol	-	-
Utilization of Sole Carbon Source:		
benzoate	-	-
gluconate	-	-
propionate	-	-
malonate	-	-
DL-malate	+	+
succinate	+	+

N.G. = No growth

2. SIGNIFICANT DIFFERENCES BETWEEN X.AMPELINA AND ISOLATE B-1

Tests	<u>X.ampelina</u>	Isolate B-1
Utilization of oxalate as sole carbon source	-	+
Acid from O-F arabinose	+	-
Urease	+	-
Growth factor requirements	+	-

3. COMPARISON OF ISOLATE B-1 TO PSEUDOMONAS

Tests	<u>Pseudomonas</u>	Isolate B-1
Gram negative	+	+
Aerobic metabolism	+	+
Polar flagella	+	+
Motility	+	+
Oxidase	+ or -	weak & slow
Catalase	+	+

4. PHYSIOLOGY AND BIOCHEMISTRY OF B-1

Tests	+/-	Tests	+/-
Gram positive	-	Gelatinase	-
Gram negative	+	Tween 20 hydrolysis	+
Gram variable	-	Tween 80 hydrolysis	-
Motile at RT	+	Indole	-
Flagella peritrichous	-	Simmons citrate growth	W
Flagella lophotrichous	-	Urease	-
Flagella monotrichous	+	Nitrate to nitrite	-
Flagella lateral	-	Nitrite reduction	-
4oC growth	-	Nitrite to nitrogen gas	-
25oC growth	+	Hydrogen sulfide (TSI)	-
30oC growth	+	Lead acetate strip	+
37oC growth	-	Lysine decarboxylase	-
41oC growth	-	Arginine (Mollers)	-
Fluorescien produced	-	Ornithine decarboxylase	-
Pyocyanine produced	-	Phenylalanine deamination	NG
Diffusible orange	-	Phosphate	-
Diffusible yellow	-	Catalase	+
Diffusible purple	-	Oxidase slow and weak	+
Non-diffusible green	-	Gluconate oxidation	-
Other non-diff. pigment	Y	Growth on malonate as SCS	-
Melanin pigment prod.	-	Tyrosine degradation	-
pH 6.0 growth	+	dl-hydroxybutyrate growth	-
3% NaCl growth	+	Growth on 0.05% cetrimide	-
6.5% NaCl growth	-	Growth on acetate as SCS	-
MacConkey agar growth	+	Testosterone degradation	-
Skim milk agar growth	W	Mucoid growth on Glucose	-
Aesculin hydrolysis	-	0.1% TTC growth	-
Casein hydrolysis	-	0.02% TTC growth	+
starch hydrolysis	-	Litmus milk acid	-
		Litmus milk peptonized	-

W = weakly positive

Y = yellow

TTC = Triphenyl Tetrazolium chloride

5. REACTIONS OBSERVED WHEN ISOLATE B-1 IS GROWN IN HUGH AND LIEFSON O-F MEDIUM

Tests	Reaction
Acid from:	
L-arabinose	K
cellobiose	K
ethanol	K
D-fructose	K
D-glucose A02	K
D-glucose A102	-
Alkaline pH in D-glucose	+
Acid from:	
glycerol	K
inositol	K
lactose	K
maltose	K
D-mannitol	K
D-mannose	K
L-rhamnose	K
D-ribose	-
sucrose	K
trehalose	K
D-xylose	K
adonitol	K
dulcitol	K
D-galactose	K
inulin	K
salicin	K
D-sorbitol	K
Control	K

K = alkaline
 - = no change
 + = acid

6. GROWTH OF ISOLATE B-1 ON SOLE CARBON SOURCES IN STANIER'S MINERAL BASE (Growth factors not required)

Sole Carbon Source	+/-	Sole Carbon Sources	+/-
L-arabinose	-	2-ketogluconate	+
cellobiose	-	DL-lactate	+
D-fructose	+	L-malate	+
D-glucose	-	pelargonate	-
lactose	-	propionate	-
maltose	-	quinat	-
D-mannitol	-	succinate	+
L-rhamnose	-	L-+-tartrate	-
D-ribose	+	valerate	-
D-sorbitol	-	B-alanine	-
sucrose	-	D-A-alanine	+
trehalose	-	betaine	-
D-xylose	-	glycine	-
adonitol	-	L-histidine	-
erythritol	-	DL-norleucine	-
glycerol	-	L-proline	-
ethanol	-	D-tryptophan	-
geranitol	-	L-valine	-
inositol	-	DL-arginine	-
sebacic acid	-	benzylamine	-
acetamide	-	butylamine	-
adipate	-	putrescine	-
benzoate	-	mesaconate	-
butyrate	-	DL-glycerate	+
citraconate	-	L-tryptophane	-
D-gluconate	-	Methanol	-
M-hydroxybenzoate	-	Oxalate	+

W = weakly positive

* = became positive after 4 weeks

END

1|2|0|4|9|1

FIN