

Studies on the Growth
and Metabolism of
Eikenella corrodens.

**Studies on the Growth and Metabolism
of *Eikenella corrodens*.**



**A thesis
submitted in fulfilment of the requirements for
the admission to the degree of
Doctor of Philosophy**

by

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Signed Statement

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Summary

Eikenella corrodens, a Gram-negative rod, is a normal inhabitant of the human oral cavity. It is one of the most commonly detected cultivable bacteria from sub- and supra-gingival plaque and is often isolated in elevated proportions from sites exhibiting destructive periodontal disease compared with healthy sites.

Although the contribution of this organism to the disease process remains unclear, existing evidence suggests that *E. corrodens* is a member of the group of microorganisms, often referred to as putative periodontopathogens, which increase in number and proportion at diseased sites. These observations suggest that the putative periodontopathogens are relatively non-competitive with other members of the resident flora in the environmental conditions encountered at healthy sites. It has been proposed that an environmental change, favouring growth of this previously non-competitive group, is required to disturb the healthy steady-state community.

Interest in *Eikenella corrodens* has increased over the past twenty years, primarily due to its potential as an opportunistic human pathogen, but information about its nutritional requirements and growth characteristics is limited and, at times, contradictory. The primary aim of this study was to investigate the metabolism of *E. corrodens*, with particular reference to energy generation, and to determine the effect of physical environmental parameters on its growth.

Following analysis of samples from continuous culture in chemically defined media, and experiments in which individual amino acids were incubated with *E. corrodens* cells in the presence of nitrate, it was established that the organism generates energy primarily through the oxidative deamination of specific amino acids coupled to dissimilatory nitrate reduction to nitrite. The utilisation of glutamate and glutamine, serine and especially proline, either as free amino acids or in peptide form, resulted in high nitrate reduction rates. The importance of proline in ATP generation by the organism is reflected in molar growth yields, which showed that biomass production per mole of this amino acid was significantly higher than that for glutamate and serine. The degradation of amino acids also appears to supply the cell with essential carbon skeletons.

The organism was found to express, constitutively, the enzyme proline iminopeptidase, which releases proline from the N terminus of peptides. The enzyme was partially purified and characterised and found to exist as a 35 kDa monomer in the cytoplasm. Inhibition studies showed that the enzyme, although classified as a serine protease, also appears to require thiol groups for activity, a finding which is consistent with previous reports. The enzyme obeyed Michaelis-Menten kinetics and was found to have a K_m value of 0.223 mM for the substrate proline-*p*-nitroanilide.

The optimal growth conditions for *E. corrodens*, determined in this study, indicate that the organism will produce increased biomass in an environment which is more alkaline, and which provides elevated levels of amino acids, (particularly proline, glutamate, glutamine and serine), than that found in

periodontally healthy sites. These environmental characteristics are consistent with those that develop at periodontally diseased sites and so this study provides an ecological basis for the observed increase in numbers of this organism at these sites.

Contents

Signed Statement	iii
Acknowledgments	iv
Summary	v
1. INTRODUCTION	1
1.1. The Species <i>Eikenella corrodens</i>	2
1.2. Prevalence	3
1.3. Morphology.....	4
1.4. Cell Structure	5
1.4.1. Adherence and Co-aggregation	6
1.5. Species Diversity.....	7
1.6. Extra-oral Infections	9
1.7. Association with Periodontal Diseases	11
1.7.1. Animal Model Studies.....	15
1.8. Ecology of Plaque Bacteria	17
1.8.1. Ecological Plaque Hypothesis	18
1.8.2. Continuous Culture Studies.....	18
1.9. Biochemistry	22
1.10. Summary	26
1.11. Aims.....	28
2. DETERMINATION OF GROWTH PARAMETERS.....	29

2.1. Amino Acid Utilisation Patterns.....	30
2.1.1. <i>Materials and Methods</i>	30
2.1.2. <i>Results</i>	31
2.1.3. <i>Discussion</i>	33
2.2. Strain Selection	34
2.3. Development of a Chemically Defined Medium	34
2.3.1. <i>Materials and Methods</i>	36
2.3.2. <i>Results and Discussion</i>	38
2.4. Optimum Growth Temperature and pH	45
2.4.1. <i>Materials and Methods</i>	46
2.4.2. <i>Results and Discussion</i>	47
2.5. Effect of Atmospheric Conditions on Growth of <i>E. corrodens</i>	60
2.5.1. <i>Materials and Methods</i>	60
2.5.2. <i>Results</i>	61
3. AMINO ACID METABOLISM AND BIOMASS PRODUCTION	62
3.1. Glutamine Metabolism.....	63
3.1.1. <i>Materials and Methods</i>	63
3.1.2. <i>Results and Discussion</i>	63
3.2. Threonine Metabolism	64
3.3. Chemostat Studies in a Minimal Chemically Defined Media.....	65
3.3.1. <i>Materials and Methods</i>	65
3.3.2. <i>Results and Discussion</i>	67
3.4. Amino Acid Metabolism and Nitrate Reduction	73
3.4.1. <i>Materials and Methods</i>	73
3.4.2. <i>Results</i>	74
3.5. Aminopeptidase Activity	78

3.5.1. <i>Material and Methods</i>	78
3.5.2. <i>Results</i>	79
3.6. <i>Utilisation of Peptides and Nitrate Reduction</i>	80
3.6.1. <i>Materials and Methods</i>	80
3.6.2. <i>Results</i>	81
3.7. <i>Proline Iminopeptidase Activity</i>	84
3.7.1. <i>Materials and Methods</i>	85
3.7.2. <i>Results</i>	85
3.7.3. <i>Discussion</i>	86
3.8. <i>The Effect of Growth Medium on Proline Iminopeptidase Activity</i>	86
3.8.1. <i>Materials and Methods</i>	87
3.8.2. <i>Results</i>	88
3.9. <i>Effect of Proline on Growth of E. corrodens 33EK(L)</i>	88
3.9.1. <i>Materials and Methods</i>	89
3.9.2. <i>Results</i>	89
4. PARTIAL PURIFICATION AND CHARACTERISATION OF <i>E.</i>	
<i>CORRODENS</i> PROLINE IMINOPEPTIDASE	92
4.1. <i>Partial Purification</i>	93
4.1.1. <i>Cell Production</i>	93
4.1.2. <i>Determination of PIP Activity</i>	94
4.1.3. <i>Protein Determination</i>	95
4.1.4. <i>Ammonium Sulphate Fractionation</i>	95
4.1.5. <i>Hydrophobic Interaction Chromatography</i>	96
4.1.6. <i>Ion Exchange Chromatography</i>	96
4.1.7. <i>Results</i>	97
4.2. <i>The Effect of Enzyme Inhibitors and Metal Ions on PIP Activity</i>	98
4.2.1. <i>Material and Methods</i>	99

4.2.2. Results	99
4.3. Molecular Weight Estimation	102
4.3.1. Materials and Methods.....	102
4.3.2. Results	103
4.4. Enzyme Location	104
4.4.1. Materials and Methods.....	104
4.4.2. Results	105
4.5. Extracellular Expression of PIP	105
4.5.1. Materials and Methods.....	105
4.5.2. Results	106
4.6. Enzyme Kinetics	106
4.6.1. Materials and Methods.....	106
4.6.2. Results and Discussion.....	107
5. DISCUSSION.....	109
5.1. Physical Growth Parameters	110
5.2. Nutrient Requirements	115
5.3. Energy Generation From Key Amino Acids.....	120
5.3.1. Possible catabolic pathways for glutamate, glutamine and proline	121
5.3.2. The generation of energy from serine degradation	123
5.3.3. Consumption of lysine	124
5.4. Carbon Skeletons from Amino Acid Catabolism	125
5.5. Peptide Utilisation.....	126
5.5.1. <i>E. corrodens</i> proline iminopeptidase (EC 3.4.11.5).....	127
5.6. Oral sources of proline.....	130
5.7. Summary	133
5.8. Future Studies	134

6. APPENDIX 1 - MATERIALS AND METHODS.....	136
6.1. Strains	137
6.2. Composition of YT medium	138
6.3. Determination of Growth Parameters	139
6.3.1. Optical Density.....	139
6.3.2. Dry Weight Estimation	139
6.3.3. Protein Estimation.....	140
6.3.4. Viable Count.....	142
6.4. Amino Acid Analysis.....	143
6.4.1. Detection of Proline	145
6.4.2. Detection of Cysteine	146
6.5. End-product and Glucose Estimation	147
6.6. Composition of EDM-1	148
6.7. Media Filtration	149
6.8. Composition of EMMG1	150
6.9. Nitrite Determination.....	151
6.10. Composition of BM1	153
6.11. Amino Acid Compositions of EMML, EMMM and EMMH.....	154
6.12. Column Chromatography.....	155
6.12.1. Instrumentation	155
6.12.2. Buffers	155
6.12.3. Gradient Conditions.....	156
7. APPENDIX 2 - RESULTS.....	157
7.1. Optical Densities After Batch Growth in YT Medium.....	158
7.2. Amino Acid Utilisation Patterns of <i>E. corrodens</i> Strains Grown in YT Medium Under Batch Conditions	159

7.3. Determination of Maximum Growth Rate of 23834 ^T in EDM-1	160
7.4. Amino Acid Utilisation by 23834 ^T Grown in EDM-1	161
7.5. Maximum Growth Rate of 23834 ^T in Modified EDM-1	162
7.6. Determination of Optimal pH - Growth Parameters	163
7.7. The Effect of Growth pH - Amino Acid Utilisation	164
7.8. Determination of Optimal Growth Temperature - Growth Parameters ..	165
7.9. The Effect of Growth Temperature - Amino Acid Utilisation.....	166
7.10. The Effect of Amino Acid Utilisation on Nitrate Reduction.....	167
7.11. The Aminopeptidase Activity of Resting Cells of <i>E. corrodens</i> 33EK(L)	168
7.12. The Effect of Various Peptides on Nitrate Reduction in Resting Cells of <i>E. corrodens</i> 33EK(L)	169
7.13. Figure 7-1. Standard Curve of OD ₄₁₀ versus ρ-Nitroanilide Concentration	170
7.14. Figure 7-2. The Effect of Growth Medium on PIP Activity	171
7.15. Determination of Enzyme Kinetics.....	172
7.16. Extracellular Expression of PIP Activity	173
7.17. Standard Curve for Molecular Weight Estimation	174
8. BIBLIOGRAPHY	175
9. PUBLICATIONS ARISING FROM THIS STUDY	195

1. Introduction



1.1. The Species *Eikenella corrodens*

The first reported isolation of micro-organisms exhibiting characteristics similar to those of what is now known as *Eikenella corrodens* was by Henriksen [1]. He described the organism as a slow-growing, anaerobic, Gram-negative rod. Shortly after the studies of Holm [2, 3], the organism was called the “corroding bacillus” because of its ability, when grown on solid medium, to pit or corrode the agar surface, producing colonies that grew in depressions.

Eiken was the first to investigate this species in detail when, in 1958, he characterised 21 strains of fastidious Gram-negative anaerobic rods isolated from sputum or pathological specimens from the human oral cavity [4]. The organisms were rather inactive in conventional biochemical tests and produced characteristic colonies which appeared to corrode or pit blood agar surfaces. Under the erroneous assumption that the organisms were obligate anaerobes, Eiken proposed a new species name, *Bacteroides corrodens*, to incorporate these strains.

Henriksen realised, in subsequent studies [5], that the organism described as *Bacteroides corrodens* by Eiken was not an obligate anaerobe but included both facultative and obligate anaerobes.

Subsequently, Jackson and Goodman [6], in a genetic, biochemical and serological study, demonstrated that the name *B. corrodens* had been applied to

two genotypically dissimilar groups of organisms each of which warranted separate taxonomic classification; even though both groups shared the common characteristic of pitting the surface of agar media. They proposed assignment of the facultatively anaerobic Gram-negative rod, previously classified as *B. corrodens*, to a new species, *Eikenella corrodens* and placed it within the family Brucellaceae. The obligate anaerobes retained their assignment to the genus *Bacteroides* and were assigned a new species, *Bacteroides ureolyticus*.

Furthermore, *Eikenella corrodens* also became the accepted designation for the “HB-1” organisms described by King and Tatum [7], because these isolates were shown to be biochemically and serologically identical [8].

1.2. Prevalence

Eikenella corrodens is commonly isolated from the human oral cavity and upper respiratory tract and is probably an indigenous oral organism. In the oral cavity, its predominant ecological niche appears to be sub- and supra-gingival plaque. In numerous studies, *E. corrodens* has been isolated from the oral cavity of healthy subjects as well as from periodontitis patients. A typical example of this finding includes that of Chen *et al*, who reported that the prevalence of *E. corrodens* in the plaque of periodontally healthy patients was 70%, and 100% in periodontitis patients [9].

Although the predominant site of colonisation in the oral cavity appears to be both sub- and supra gingival plaque, in subjects with adult periodontitis, the organism may be found on the surfaces of the tongue, the tonsils, cheek mucosa and in saliva [9, 10].

The presence of *E. corrodens* in the mouth and gingival surfaces appears to be associated with the adherence of the organism to human buccal epithelial cells. Yamazaki *et al.* [11, 12] have shown that the adherence of *E. corrodens* to human buccal epithelial cells may require the interaction of lectin-like proteins on the bacterial cell surface with galactose-like receptors on the surface of epithelial cells.

As various studies have shown that patients with periodontitis have more sites infected with higher levels of *E. corrodens* than healthy subjects, it has been suggested that this organism may be associated with the pathogenesis of periodontitis [13, 14].

1.3. Morphology

E. corrodens typically exhibits corroding colony morphology which is characterised by a circular and irregular margin with a diameter typically $\leq 3\text{mm}$. Such colonies contain a rough, non-refractile, opaque perimeter; a moist umbonate centre; and appear to pit the blood agar surface. Strains with the corroding colony morphology may yield non-corroding colony variants, which are usually smaller,

exhibit a circular margin and a smooth colony surface, and lack the granular perimeter. The typical corroding colony is quite distinct and can easily be distinguished from other species on blood agar. However, *E. corrodens* is not the only bacterial species with corroding colony morphology. Definitive species identification requires additional biochemical tests.

1.4. Cell Structure

E. corrodens possesses a typical Gram-negative cell envelope consisting of an inner membrane, a peptidoglycan layer and an outer membrane which may be covered by an additional polysaccharide slime layer [15, 16]. It is not certain whether this species is fimbriated. In an early report, Henrichsen and Blom [17] reported that *E. corrodens* possessed fimbriae with a diameter of about 5 nm. Although these authors were unable to determine the origin of the fimbriae, they concluded that these processes were most likely polar. In addition, they found that the presence of the “polar” fimbriae correlated with the corroding morphology, as well as the twitching motility of the organism.

In contrast, using antibody to stabilise the surface structures and a staining procedure to reveal exopolysaccharide structures, Progulske and Holt [16] demonstrated that *E. corrodens* was covered by a loosely organised fibrous slime layer. They noted the presence of fibrillar structures on the surface of *E. corrodens* but concluded that the fibrils may have been an artefact. In agreement with this conclusion, the absence of fimbriae was also reported by Ebisu *et al.* [18].

Interestingly, the “polar” pili as shown by Henrichsen and Blom resembled the fibrillar structures noted by Progulske and Holt.

More recently Shiozu [19], in an electron microscopic study of fourteen human oral cavity isolates of *E. corrodens*, reported that although in some strains straight fibrils were observed on the surface of the outer membrane, none of the strains examined possessed a fimbrial structure. It is possible that the “pili” may represent an artefact from the dehydration of slime layers during preparation for electron microscopic examination. However, it is also possible that the pilus expression may vary between *E. corrodens* strains, or may be influenced by growth conditions.

1.4.1. Adherence and Co-aggregation

Specific bacterial adhesins, which bind to host surface receptors (cryptitopes), provide a strong selective advantage for a micro-organism attempting to colonise a host surface. In a study of the adherence of *E. corrodens* to human buccal epithelial cells (BEC) *in vitro*, Yamazaki *et al* [11] found that neuraminidase treatment of the BEC enhanced adherence. It was also shown that the adherence was inhibited by sugars containing D-galactose and N-acetyl-D-galactosamine. The results of this study indicate that the adherence of *E. corrodens* to human BEC requires the interaction of lectin-like proteins on the bacterial surface with galactose-like receptors on the surface of epithelial cells. It is known that epithelial cells and enamel pellicle contain mucins with

oligosaccharide side chains possessing terminal sialic acid. It is likely that oral bacteria, such as *A. naeslundii*, which produce neuraminidase, an enzyme which removes sialic acid and exposes the penultimate galactosyl residue, facilitate the binding of *E. corrodens* to epithelial cells *in vivo*.

Subsequently, Ebisu *et al* [20] investigated the mechanism of aggregation of *E. corrodens* with *E. corrodens* aggregating factor (EcAF), a substance purified from submandibular-sublingual (SM-SL) saliva. They found that aggregation was inhibited by the addition of N-acetyl-D-galactosamine (GalNAc) and saccharides which contain a galactose residue at the non-reducing end. Neuraminidase treatment of EcAF increased its ability to aggregate *E. corrodens*, suggesting a similar binding mechanism as that to BEC. EcAF was also shown to aggregate 16 strains of oral bacteria including periodontopathic bacteria such as *Porphyromonas gingivalis* and *Actinobacillus actinomycetemcomitans*. However, those aggregations were not inhibited by N-acetyl-galactosamine, suggesting that EcAF has more than two types of bacterial binding site which play important roles in accumulation of dental plaque by forming a complex network of plaque bacteria, including periodontopathic strains.

1.5. Species Diversity

Initial findings indicated that genetic divergence is not a marked characteristic of *E. corrodens* strains. When the DNA of *E. corrodens* strains was

analysed their % G+C content was found to vary from 56 to 58%. Some authors have characterised them as a genetically homogeneous group [21, 22].

In one study, DNA fingerprinting of 47 independent isolates *E. corrodens* was performed [14]. Each isolate, from a separate subject in different geographic areas, was from either dental plaque or extra-oral infections. The DNA restriction endonuclease digests from these strains demonstrated extensive genetic heterogeneity, the majority of strains exhibiting unique DNA fingerprints (40 fingerprints from the 47 tested). Multiple strains from four individuals were also analysed.

One periodontitis patient was colonised with six different clonal types of *E. corrodens*. Further, two different clonal types of *E. corrodens* were recovered from a single periodontal pocket in this patient. The three other subjects were also found to harbour *E. corrodens* strains exhibiting more than one DNA fingerprint. These studies suggest that individuals are simultaneously infected with several clones of *E. corrodens*. It is yet to be determined whether some clonal types are more virulent than others, and whether the virulent clones are found in periodontal pockets.

Moreover, phenotypic analysis has revealed an outer membrane and lipopolysaccharide heterogeneity among *E. corrodens* isolates [23-25]. More recently, as molecular methods have become more powerful, studies employing restriction enzyme analysis of genomic DNA have been used to differentiate between *E. corrodens* strains.

In the restriction endonuclease study by Chen *et al* [14], 40 restriction patterns were distinguishable among the 47 isolates. Steffens *et al* [26], in a more recent restriction endonuclease analysis of genomic DNA, employed pulse field electrophoresis in their study. Ten *E. corrodens* strains isolated from oral and extra-oral infection sites in different individuals were analysed. The restriction enzymes BamHI and BglII seemed to be the most suitable for pulsed-field gel electrophoresis analysis. These enzymes cleaved the genomes of all the above strains into 15-20 fragment bands that were clearly separated by pulsed-field gel electrophoresis conditions. Eight individual pulsed-field gel electrophoresis patterns from the 10 strains analysed were revealed. However, only 4 identical outer membrane protein profiles were differentiated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis.

The data obtained in these analyses show clonal divergence among members of the *E. corrodens* species, at the same time displaying the resolving power of modern molecular techniques to reveal differences between isolates once thought to be genetically identical.

1.6. Extra-oral Infections

Although *E. corrodens* has generally been regarded as an organism of low virulence, it has become increasingly recognised as a cause of human infection [27-32]. Human infections usually result from some predisposing factor, such as trauma to a mucous membrane surface, that compromises the normal host defence

mechanisms and allows the organism to gain access to the surrounding tissue.

Once primary infection occurs, hematologic dissemination may establish foci of infection in other parts of the body.

In normal human hosts, *E. corrodens* is usually involved in mixed bacterial infections, often with the viridans group streptococci, and less frequently with various members of the Enterobacteriaceae [33, 27]. Infections frequently involve the head, neck or abdominal area [29, 31, 32].

E. corrodens is also responsible for 7 to 29% of human hand bite-wound infections, [34-39] as well as clenched-fist injuries, which are frequently complicated by bone resorption and osteomyelitis. The organism may also be the sole infecting pathogen and has been reported in cases of endocarditis [40], meningitis [41], subdural empyema [42], septic arthritis [43-47], pneumonia [48] [49-51] post-surgical infections and soft tissue diseases [52-54].

Several reports [30-32] have shown that *E. corrodens* may have an accentuated potential to cause disease in immunocompromised patients, and, as such, may serve as an opportunistic pathogen in this patient population.

In vitro antimicrobial susceptibility studies [55-57] indicate that *E. corrodens* infections can be successfully treated with a wide variety of agents including penicillin, ampicillin, cephalosporins, moxalactam, imipenem and tetracycline. Clindamycin is one of the few drugs to which *Eikenella* isolates are universally resistant.

1.7. Association with Periodontal Diseases

Periodontally diseased sites are characterised by a migration of the junctional epithelium at the gingival crevice towards the root of the tooth to form a periodontal pocket. This may arise from what appears to be one of a number of conditions, often related to clinical descriptors (eg. age group of patient, rate of progress and distribution of lesions), commonly termed “periodontal diseases”. In all forms it appears that the pathology is a combined result of action by plaque bacteria and the damaging side-effects of the inflammatory host immune response to these micro-organisms.

Since *E. corrodens* is predominantly found in supra- and subgingival plaque and its potential pathogenicity is indicated by the fact that it is able to cause serious extra-oral infections, it is considered by many to be a putative periodontal pathogen.

The earliest evidence indicating the involvement of *E. corrodens* with the development of periodontitis appears to derive from clinical studies by Newman and Sims [58] and Tanner *et al* [59]. In these studies, elevated numbers of this organism were found in bacterial samples from patients with periodontitis or periodontal abscesses.

Savitt and Socransky [60] employed selective media to enumerate nine commonly encountered subgingival species in subgingival plaque samples from periodontally healthy, gingivitis, and adult and juvenile periodontitis subjects. Their results indicated that *E. corrodens* and *Fusobacterium nucleatum* were usually elevated in proportions in sites with gingivitis or destructive periodontal disease.

In a study of plaque microflora isolated from sound gingiva, gingivitis and marginal periodontitis, Pfister [61] found that in clinically healthy gingiva, the periodontium is colonised mainly by Gram-positive bacteria. Streptococci represented approximately 80% of micro-organisms present at these sites. The predominant microflora of the sub-gingival plaque in early marginal periodontitis consisted, however, of Gram-negative bacteria such as *Bacteroides*, *Fusobacterium*, *E. corrodens*, *Haemophilus*, *Actinobacillus*, and *Capnocytophaga*. These findings indicate that, in the progression from healthy periodontium to marginal periodontitis, changes in the complex flora take place from predominantly Gram-positive organisms into a characteristic Gram-negative, anaerobic, sub-gingival microflora.

As noted above, Chen and colleagues [9] studied the prevalence and distribution of the putative periodontal pathogen *E. corrodens* in the human oral cavity. A total of 508 oral bacterial samples were taken from ten periodontally healthy adults, 11 adult periodontitis patients, and six localised juvenile periodontitis (LJP) patients. From each subject, samples of supra- and sub-gingival plaque were obtained from six to eight teeth as well as samples from

buccal mucosa, lateral and dorsal surfaces of tongue, tonsil, and saliva. *E.*

corrodens was cultured from 70% of healthy subjects and 100% of periodontitis patients.

In healthy subjects, *E. corrodens* was found in 26% and 31% of supra- and sub-gingival plaque samples, respectively. The organism was rarely found in other sites in these patients; the ecological niche in healthy individuals therefore appears to be dental plaque. Plaque samples from diseased individuals showed an increase in the number of sites infected with *E. corrodens*. It was isolated in 59% of both supra- and sub-gingival plaque samples from adult periodontitis subjects, as well as 48% and 64% of supra- and sub-gingival plaque samples of LJP subjects.

In contrast to healthy subjects, *E. corrodens* was found on the buccal mucosa, tongue, tonsil and in the saliva of patients with periodontitis. The micro-organism was found to constitute, on average, 1 to 2% of the total cultivable bacteria in supra- and sub-gingival plaque samples. Also the prevalence of *E. corrodens* in plaque samples was higher in adult periodontitis and LJP subjects and was significantly different from healthy subjects. Within the adult periodontitis group, the prevalence of *E. corrodens* in sub-gingival plaque was significantly higher from sites with Gingival Index scores greater than or equal to 2. The authors suggested that *E. corrodens* is an indigenous oral micro-organism which may be an opportunistic pathogen associated with gingival inflammation.

In 1990, Choi *et al* [62] noted elevated IgG responses to *E. corrodens* in serum samples from recurrent periodontitis patients compared to those of

periodontally healthy control subjects. Wolff *et al* [63], in a study involving 938 patients with primary gingivitis and early periodontitis, found *E. corrodens* in the sub-gingival plaque of 49% of those sampled.

Lippke and co-workers [64] employed DNA probes to detect the prevalence of *E. corrodens* in sub-gingival plaque samples from adults with untreated periodontitis or gingivitis and in healthy controls. They detected the organism in 62% of the control sites and 81% of the periodontitis sites. Soder *et al* [65], also employing species-specific DNA probes, determined the prevalence of *E. corrodens*, as well as six other putative pathogens in sub-gingival plaque from deep pockets of patients with advanced periodontitis. The subjects were 20 patients with severe adult periodontitis. For each subject, 9-10 sub-gingival sites with the deepest probing depths from each quadrant were sampled by the paper point method; a total of 198 sites. *E. corrodens* was found in all subjects and in 72.9% of the 198 samples. The predominant combination of bacteria consisted of *P. intermedia*, *T. denticola*, *E. corrodens*, *F. nucleatum*, and *W. recta* in 89.5% of the subjects and 46.8% of the sites. This study indicates that severe destructive adult periodontitis is most likely a multi-bacterial infection and that certain combinations of periodontopathogens may be important in the pathogenesis of the disease.

Chan and Chien [66] employed anaerobic culture, direct microscopy, indirect immunofluorescence, and biochemical tests to detect specific micro-organisms in sub-gingival plaque from 336 sites in both healthy individuals and patients with periodontal inflammation. Once again, *E. corrodens*, amongst other

organisms, was detected in a significantly higher proportion in the periodontitis group.

1.7.1. Animal Model Studies

Animal models in which microbiological and immunological aspects of periodontal disease can be studied prospectively have been conducted. The rat bears much resemblance to man with respect to periodontal anatomy, development and composition of dental plaque, histopathology of periodontal lesions, and basic immuno-biology. Furthermore, reproducible methods are available for assessment of periodontal disease in rats, and detectable periodontal destruction can be induced in a few weeks in these animals without traumatising periodontal tissues with ligatures [67].

Heijl *et al* [68], investigated the development of periodontal disease in germfree rats infected with micro-organisms associated with human gingivitis, periodontitis and periodontosis lesions. After weaning the animals, the experimental groups were infected with strains of one of the following organisms; *Actinomyces naeslundii*, *E. corrodens*, *Bacteroides asaccharolyticus* and *Capnocytophaga sputigena*. They found that germfree rats mono-infected with different bacteria associated with human periodontal diseases may suffer severe breakdown of the periodontal tissues. The degree of breakdown tended to increase over time. Periodontal tissue destruction was obviously related to the impaction of

hair and bedding material. Only in areas with severe impaction of foreign material was gross destruction of the supporting apparatus recognised.

Samejima and colleagues [69] investigated the periodontopathic ability of *E. corrodens* on ligature-induced periodontal defects in rats. Alveolar bone resorption of ligated rats was enhanced by the implantation of *E. corrodens*, although the number of total cultivable bacterial cells from ligated sites was not changed by the implantation of the organism. Without ligatures, high inoculum doses of *E. corrodens* did not result in periodontal destruction. These results indicate that it is possible to establish *E. corrodens* in conventional rat flora with a ligature and that the organism causes osteoclastic bone resorption in this model.

In a review of the microbiological and immunological aspects of experimental periodontal disease in rats, Klausen stated [67] that “experimental periodontitis studies in germ-free rats have confirmed the pathogenicity of several suspected periodontal pathogens (*A. actinomycetemcomitans*, *P. gingivalis*, *C. sputigena*, *E. corrodens*, and *F. nucleatum*).” The study also suggested that the number of periodontal pathogens may be higher than generally believed, since species like *Streptococcus sobrinus* and *Actinomyces viscosus* are associated with periodontal bone loss in the rats.

Klausen, in his review [67], also stated that, “Studies in rats with congenital or induced immune defects indicate that generalised or selective immunosuppression at the time of infection with periodontal pathogens may aggravate periodontal disease. Studies in immunised rats indicate that periodontal

disease can be prevented by immunisation against periodontal pathogens. However, it is also possible by immunisation to induce periodontal destruction; ie., the immune system has a destructive potential which should not be overlooked. In the future, the rat model may prove valuable for initial screening of antigen preparations and immunisation regimens in the search for a periodontitis vaccine.”

It is apparent that an explanation for the development of the various manifestations of periodontal disease is complex. A review of the literature shows *E. corrodens* is only one of many species of micro-organisms which may, as a member of various bacterial consortia, be implicated in this process.

1.8. Ecology of Plaque Bacteria

It is apparent, from numerous publications, that the predominant bacterial consortia isolated from sites characteristic of the various forms of periodontal diseases are markedly different from those prevalent in the healthy crevice. As seen in the previous section, existing evidence suggests that *E. corrodens* is a member of this group of microorganisms, often referred to as putative periodontopathogens; they increase in number and proportion at diseased sites. This leads to the conclusion that, in the environment present at healthy sites, putative periodontopathogens are relatively non-competitive with other members of the resident flora. It would appear that some change in the environment,

favouring growth of this previously non-competitive group, is required to disturb the healthy steady-state community.

1.8.1. Ecological Plaque Hypothesis

Marsh has proposed the “ecological plaque hypothesis” to explain the relationship between the plaque microflora and the host in health and disease [70]. He argued that a breakdown of the healthy homeostatic environment leads to a shift in the balance of the microflora resulting in sites more predisposed to disease. This may result from the accumulation of plaque around a gingival margin, triggering an inflammatory immune response by the host which, in turn, leads to an increased flow of gingival crevicular fluid. This site, now receiving an increased nutrient supply preferred by asaccharolytic, relatively anaerobic, proteolytic species, becomes more favourable for growth of this group of micro-organisms. This results in a shift in predominant species in this micro-environment from Gram-positive to Gram-negative.

1.8.2. Continuous Culture Studies

The coexistence of bacteria in natural environments can often be explained in terms of competition for a growth-limiting substrate(s), and the outcome of such competition depends upon relevant growth parameters such as substrate affinity and yield. For example, dental plaque bacteria are frequently carbon and energy limited and such limitations result in these organisms not dividing for

extended periods, and then at rates much slower than their genetically determined maxima.

It is known that microorganisms can express marked phenotypic differences when cultured at different growth rates and, because of this phenomenon, the use of traditional batch culturing methods to investigate cellular properties may give rise to misleading results. The use of batch culture to investigate cellular metabolism is also less than ideal because of the difficulty in adequately controlling both physical and chemical variables.

Consequently, the chemostat, which allows bacteria to be grown in continuous culture, and by its design conveys stricter control over growth conditions, has been used extensively to study a variety of aspects of microbial growth characteristics and parameters by numerous investigators in the past 50 years.

The chemostat has been frequently employed by researchers studying aspects of the growth of oral microbes in both axenic and mixed culture experiments. Examples of the use of this culturing method in the study of (significant) oral bacteria include the investigation by Hamilton and Ellwood [71] of carbohydrate metabolism by *Actinomyces viscosus* grown under either carbon or nitrogen limitation. Hamilton *et al* also [72] used continuous culture to study the influence of pH and fluoride on the metabolism of an oral strain of *Lactobacillus casei*. Marsh *et al* [73] used cells grown in a chemostat to study carbohydrate metabolism by *Streptococcus sanguis* under glucose or amino acid limitation over

a range of growth rates. Continuous culture has also been used to determine the stability of outer-membrane protein and antigen profiles of a strain of *Bacteroides intermedius* grown at varying pH values and growth rates [74].

As stated previously, continuous culture has also been frequently employed to investigate the effects of various environmental influences on mixed cultures of oral bacteria. In a study of the effects of growth rate and nutrient limitation on the microbial composition and biochemical properties of a complex mixed chemostat culture of oral bacteria originating from a dental plaque sample, Marsh *et al* [75] demonstrated the utility of continuous culture as a tool capable of modelling dynamic population interactions *in vitro*.

Bowden and Hamilton [76], in an investigation of competition between *Streptococcus mutans* and *Lactobacillus casei* in mixed continuous culture, found that environmental pH determined which organism would predominate in the population. In a similar study, on the effects of carbohydrate pulses and environmental pH on a complex, mixed culture comprising nine different oral species, Bradshaw *et al* [77] showed that the pH generated from carbohydrate metabolism was responsible for observed shifts in composition and metabolism of the oral microflora, rather than carbohydrate availability *per se*.

Marsh [78] and Bradshaw *et al* [79] also used a similar mixed culture system to investigate the effects of the antimicrobial agents triclosan and zinc citrate on bacterial population dynamics. The effects of sugar alcohols sorbitol and xylitol were also investigated [80].

In the past, our laboratory has similarly employed continuous culture to investigate the effects of various physical and chemical parameters on the growth and metabolism of a numbers of oral species, both in pure and mixed culture.

In a study of growth characteristics of seven oral *Streptococcus* species and one *Actinomyces viscosus* strain, employing continuous culture conditions, Rogers *et al* [81] showed that arginine was completely consumed by *S. milleri* and *S. sanguis* strains. Further investigation, in this laboratory, of the influence of arginine availability on the coexistence of *S. milleri* and *S. mutans* in glucose-limited mixed continuous culture [82] demonstrated the ability of strains of *S. milleri* to metabolise arginine as an energy source, additional to carbohydrate. This provides an explanation for the observed increase in proportion of such organisms in the plaque of subjects consuming diets almost devoid of fermentable carbohydrate.

This work also showed the utility of the chemostat, when used in conjunction with a simple chemically defined growth medium, to provide valuable nutritional information about micro-organisms and how this information can be employed, *in vitro*, to change the steady-state proportions of coexisting species and to mimic *in vivo* observations. The realisation of the possible use of arginine as an alternative energy source for *S. milleri* and *S. sanguis* led to an investigation of arginine uptake [83], protease activity [84], and utilisation of arginine containing peptides in *S. sanguis* [85].

A similar approach, employing continuous culture in a chemically defined medium, was used in this laboratory in a study on aspects of the growth and metabolism of *F. nucleatum* [86]. The response of the organism to varying pH, nutritional environment and growth rate was studied and the results illustrated that, *inter alia*, *F. nucleatum*, an organism often referred to in published literature as asaccharolytic [87], was indeed capable of generating energy from the metabolism of glucose. It was also shown that the organism obtained energy via the metabolism of specific amino acids. Later, investigation of the peptidase activity of *F. nucleatum* was undertaken [88], and led to the characterisation of an aminopeptidase of possible nutritional importance, given that the survival of this micro-organism in the sub-gingival environment is dependent on obtaining energy via the fermentation of a small number of peptide-derived amino acids.

1.9. Biochemistry

Interest in *E. corrodens* has increased since the late 1970's due to the increasing isolation of this organism from a variety of pathogenic lesions, either from studies demonstrating elevated levels of *E. corrodens* in periodontally diseased sites in humans or from a variety of extra oral infections. However, little is known about the nutritional requirements or growth characteristics of members of this genus.

E. corrodens exhibits very few positive reactions in biochemical tests and this may pose difficulty in distinguishing this species from other phenotypically-similar bacterial species. The organism is unable to liquefy gelatin, hydrolyse starch or casein, produce catalase, hydrogen sulphide, urease or indole. The species is capable of both aerobic and anaerobic growth. It is positive for oxidase and possesses lysine and ornithine decarboxylase activity as well as the ability to reduce nitrate. Little is known about the organism's carbon and energy sources and its preferred atmospheric conditions are not well defined, even though it is routinely cultured in CO₂-enriched environments.

Limited information regarding the growth characteristics of *E. corrodens* can be found in the studies by Hill *et al* [89]. Jackson *et al* [90], noted that the organism grew poorly in liquid media, while James and Robinson [91] observed that the organism's metabolic reactions usually increased the pH of growth medium and suggested that the organism's low metabolic rate limited the value of other biochemical tests. Indeed, these authors expressed "the need for more information on how *E. corrodens* derives its energy to support growth and reproduction before biochemical reactions can be related to a complete metabolic pattern."

Eiken's [4] early study indicated that the organism was asaccharolytic, while the work of Bottone *et al* [92] indicated nitrate was essential for its growth. Deusek and Badger [93] developed a basal defined medium which supported growth of *E. corrodens* and found that fractionated yeast extract gave better growth than standard complex media. The growth-enhancing fraction was found to

be of low molecular weight, dialysable, water soluble and acid labile. The authors suggested that the active factor was a peptide-like compound as the growth-promoting property of this fraction was lost upon treatment with proteolytic enzymes.

In a study on growth characteristics and nutritional requirements of the Type strain of *E. corrodens* ATCC 23834, Progulske and Holt [94] determined that atmospheric CO₂ was not a growth requirement for this strain. They also confirmed that nitrate was essential for growth and that biomass was promoted by the addition of yeast extract. After partial purification of this growth promoting substance from yeast extract, the authors found that it was of low-molecular weight and water soluble. Realising the similarity between their findings and those of Dusek and Bagder, Progulske and Holt felt that their results indicated that *E. corrodens*, an organism apparently unable to metabolise glucose, may have an absolute requirement for one or more peptides as a source of carbon and energy.

Keudell et al [95] investigated the amino acid requirements of a number of strains of *E. corrodens*, including the Type strain ATCC 23834, as well as a number of clinical isolates. Experiments involving single amino acid deletions performed in a chemically defined medium indicated that the organism required arginine, cysteine, histidine, lysine and proline and had a partial requirement for tyrosine. These authors developed a chemically-defined medium containing these six essential amino acids plus aspartic acid, glutamic acid, glycine, inorganic salts and vitamins; it supported growth of *E. corrodens*. The authors noted that their results indicated, in contrast to those of previous workers, that the organism was

not dependent on the presence of peptides and that amino acids were utilised as both carbon and energy sources.

A review of published literature reveals, that, although considerable and growing interest in *E. corrodens* has developed over the past twenty years, little quantitative information is known about its nutritional requirements and growth characteristics. Also, the few studies in this area appear somewhat contradictory with respect to peptide requirement for *E. corrodens* growth.

1.10. Summary

In summary, the published evidence indicates that *E. corrodens* is probably an indigenous member of the oral microflora of humans and is frequently present in the sub- and supra-gingival plaque of individuals with healthy gingiva. It is also an opportunistic pathogen since it is capable, when transferred to a variety of extra-oral sites, of causing disease.

The evidence in favour of a role for the involvement of *E. corrodens* in the aetiology of periodontal diseases, although suggestive, is not conclusive.

Numerous studies have shown that, in disease, the proportion of *Eikenella* in the microbial community at these sites tends to increase. However, the elevated levels of this organism may simply be a reflection of a significant nutritional and/or physical change in the micro-environment which is more favourable for its growth.

As already stated, knowledge concerning the growth requirements and metabolism for *E. corrodens* is relatively limited and, at times, conflicting in nature. Therefore, it is not possible to identify those changes resulting at disease sites which provide a selective advantage to this species.

As Progulske and Holt stated in the introduction to their preliminary growth study of the organism, “as a prerequisite to cell fractionation studies to

ascertain the role(s) of *E. corrodens* and its various cell components (ie., outer membrane, lipopolysaccharide, and exopolysaccharide) in disease quantitative growth studies are essential to an understanding of this organism's growth characteristics" [94].

1.11. Aims

The present study represents an attempt to explain the increase in numbers of *E. corrodens* in diseased sites by investigating its nutritional and other environmental requirements. As stated previously, if its proportions in diseased sites increase, one may postulate that the environment for growth of the organism at these sites becomes more favourable. It is hypothesised that determination of this organism's growth requirements could provide an explanation for its proportional increase at diseased sites and, in turn, provide evidence to confirm the physical and chemical change(s) thought to occur in these environments. This data may well reinforce the "ecological plaque hypothesis" proposed by Marsh.

The first aim of this study was to grow the organism successfully in continuous culture. It was also proposed to develop a relatively simple chemically-defined medium in order to refine and investigate the growth characteristics of *E. corrodens*. If possible, a number of strains, including clinical isolates, would be investigated to determine the degree of variation in the species. The organism's proteolytic activity would also be investigated due to its likely reliance on amino acid metabolism as a supply of energy.

2. Determination of Growth Parameters

2.1. Amino Acid Utilisation Patterns

The purpose of this experiment was to establish the amino acid utilisation patterns, for a number of *E. corrodens* strains, after batch growth in a complex medium. Keudell *et al* [95] showed that the amino acid deletion patterns of six strains of *E. corrodens*, including ATCC 23834, were similar. Nevertheless, it was felt important in the present study to establish any heterogeneity in amino acid utilisation patterns for a number of clinical isolates and to determine whether they differed from that of the Type strain.

2.1.1. Materials and Methods

The medium, designated YT, a complex medium containing yeast extract and tryptone and based on that of Kuedell *et al* [95] was employed for initial batch culture experiments to determine the amino acid utilisation pattern for all strains. (The composition of this medium is shown in Appendix 1, Section 6.2, p. 138). The strains used in this experiment were ATCC 23834, the Type strain, hereafter referred to as 23834^T, and the clinical isolates 33EK(L), 35EK and 39EK, obtained from Dr. R. P. Allaker, London, U.K.

Each strain was grown in YT medium, under batch conditions, at 37°C, in an anaerobic jar containing 5% CO₂ 95% N₂. After 48 hours, growth was determined by measurement of the optical density of the culture at 560 nm. The

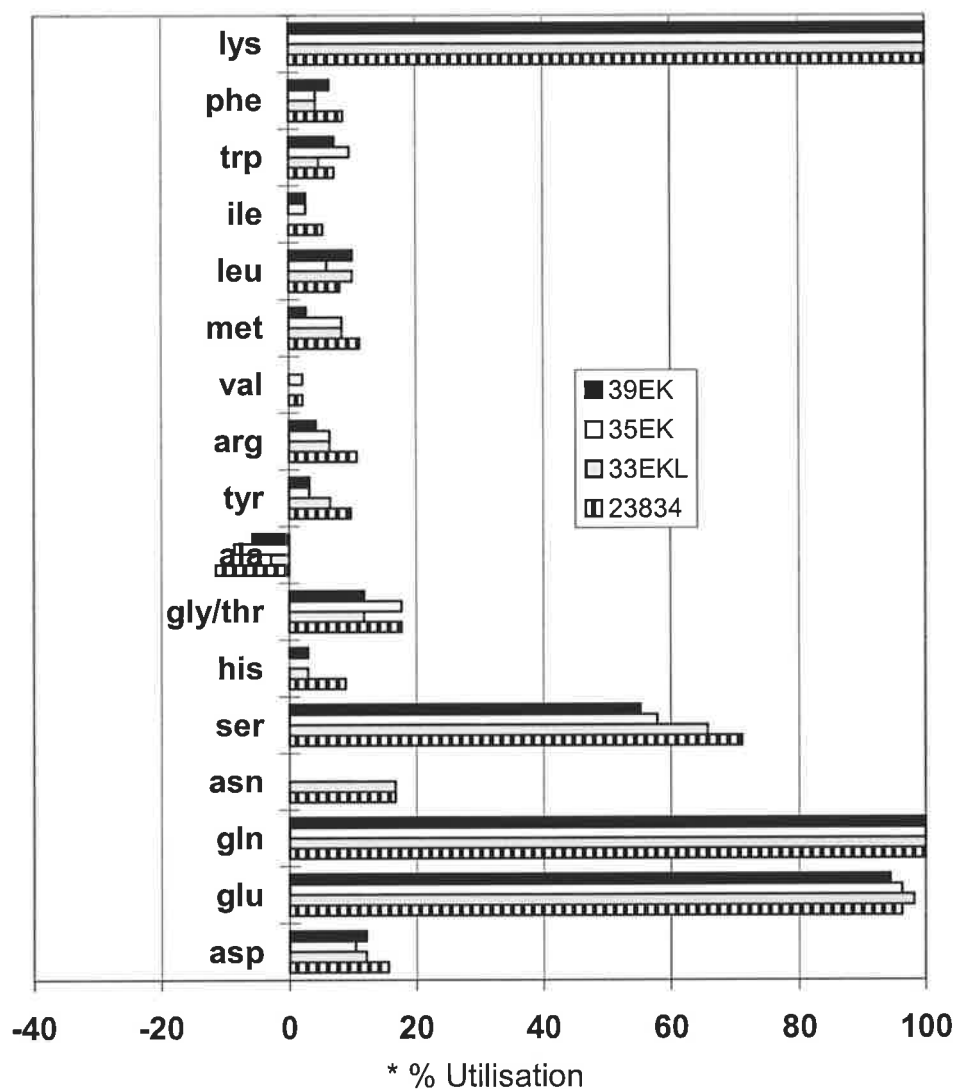
values obtained ranged between 0.69 and 0.78 (see Appendix 2, Section 7.1, p.158). Cultures were smeared, Gram-stained and purity was confirmed by microscopic examination.

Once culture purity was established, the cultures were centrifuged at 3,500g for 30 minutes and 0.5 ml of culture supernatant was removed and passed through a 25 mm diameter, 0.2 µm filter (Minisart, Sartorius AG, Gottingen, Germany) to remove any residual cells. These filtrates were subsequently analysed for amino acid content using reverse phase high performance liquid chromatography (RP-HPLC) of o-phthaldialdehyde (OPA) derivatives based on the method of Hill *et al* [96] (for detailed method see Appendix 1, Section 6.4, p.143). An aliquot of sterile YT medium was similarly analysed to quantify free amino acid levels in the absence of growth.

2.1.2. Results

The results of this experiment are shown in Figure 2-1 (also see Appendix 2, Section 7.2, p.159). It should be noted that this method of amino acid analysis does not allow for detection of secondary amino acids so that proline utilisation could not be established. Quantitative detection of cysteine is not reproducible using this method and so data for this amino acid are not presented. Also, the chromatographic conditions did not allow for complete resolution of the amino acids glycine and threonine; therefore data for these two amino acids are expressed in a combined form.

Figure 2-1. Amino Acid Utilisation Pattern for Strains of *E. corrodens* Grown in YT Medium Under Batch Conditions.



* The ratio free amino acid consumed as compared to that present in uninoculated YT medium, expressed as a percentage. (Actual data shown in Appendix 2, Section 7.2, p.159)

Overall there were only minor variations in the patterns of amino acid utilisation between all of the strains tested. The following amino acids were consumed in significant amounts: lysine, glutamate, glutamine and, to a lesser extent, serine. All other amino acids were utilised in relatively small quantities, with the exception of alanine which, under these conditions, appeared to be produced by all strains.

2.1.3. Discussion

It is important to note that only the utilisation of free amino acids, following growth in a complex medium containing a mixture of amino acids and small peptides, was assayed. This result can be used as a guide in determining which amino acids may be important metabolically but it provides no information as to whether peptides are also capable of providing a source of energy or if key peptides are essential for growth of the organism.

All strains were capable of consuming relatively large amounts of key amino acids during the production of biomass. It is likely that the catabolism of at least some of these would be linked to ATP production in these strains.

It should be noted that the consumption of all available free glutamine may be misleading as the level of this amino acid in YT medium is relatively low when compared to other amino acids utilised at high rates. Also, as this amino acid is not particularly stable in solution, uncatalysed deamination to glutamate during the

culture period may be a contributing factor in its apparent high rate of consumption.

2.2. Strain Selection

The primary aim of the previous experiment was to determine whether the utilisation of amino acids during growth by the Type strain ATCC 24834^T of *E. corrodens*, originally isolated from sputum, was significantly different from that of clinical oral isolates and also the degree of variation between these isolates. It was felt important to establish that these patterns were similar before proceeding with more extensive growth studies using the Type strain. The selection of ATCC 23834^T also allows direct comparison with results of previous metabolic investigations [94, 95].

2.3. Development of a Chemically Defined Medium

The advantage of culturing organisms in a relatively simple chemically defined medium, capable of supporting growth in continuous culture, is apparent from previous studies in this laboratory [81, 82, 86, 97]. The aim of this work was to produce a synthetic medium capable of supporting growth of the organism in continuous culture.

The medium, designated E16TBN, by Keudell *et al* [95] was used as a basis for the development of our medium, EDM-1. Its composition is identical,

with the exception of some variation in amino acid content. The composition of EDM-1 and a comparison with E16TBN is detailed in Appendix 1, Section 6.6, p.148.

The amounts of glutamate and serine present in EDM-1 are significantly higher than in E16TBN, in accordance with the organism's capacity to utilise these amino acids, (see Figure 2-1, p.32). Conversely, the amount of lysine was reduced significantly (from 69 to 10 mM), as, although the organism appeared capable of metabolising this amino acid, the biomass produced in YT medium, which contains 2.1 mM lysine, was comparable to that produced in E16TBN (containing 69 mM). It seems unlikely, therefore, that this extremely high level of lysine is required for biomass production.

The amino acids glutamine and asparagine were not included in EDM-1 in order to simplify amino acid analysis. However, study of the organism's ability to metabolise glutamine was noted for future study. As stated previously, the HPLC method employed for analysis of amino acids was unable to detect proline and so the concentration of this amino acid was retained at the same level, (22 mM), as that of E16TBN.

Hemin was not included in EDM-1 in order to establish whether this compound is required for growth of the organism under these conditions. It is, however, often included in growth media by researchers when culturing *E. corrodens*. For example, Progulske and Holt included hemin in their BA and BY broths, the compositions of which were based on a previous study [93]. On the

other hand, Kuedell *et al* [95] omitted hemin in E16TBN and this medium supported growth of all six strains tested.

2.3.1. Materials and Methods

EDM-1, the composition of which is shown in Appendix 1, Section 6.6, p.148, was prepared by the addition of components to distilled water; the pH was adjusted to 7.2 and the solution filter sterilised (see Appendix 1, Section 6.7, p.149). It should be noted that it was necessary to autoclave the stock thiamine solution prior to its addition, in order for this medium to support growth of the organism. This result confirms a previous study on the thiamine-related requirements of this strain [98].

An inoculum for chemostat culture was prepared by anaerobically culturing 23834^T in 20 ml of YT medium at 37°C for 48 hrs. Once grown, this broth was transferred aseptically to the culture vessel of a model C-30 BioFlo chemostat (New Brunswick Scientific, Edison, NJ) with a working volume of 365 ml. The culture was continuously gassed with a N₂:CO₂ (95:5) mixture and culture pH was maintained at 7.2 by the automatic addition of 2 M KOH. Culture temperature was maintained at 34°C.

Culture samples were removed at intervals, thus enabling the maximum growth rate ($\mu_{\max}$) in EDM-1 to be determined by measurement of the change in OD_{560nm} of the culture during exponential growth. Once $\mu_{\max}$ was calculated the

medium flow was adjusted to give an imposed dilution rate equivalent to $\mu_{\text{rel}} = 0.3$ (i.e. 30% of maximum growth rate). The organism was allowed to grow under continuous culture conditions until steady-state was reached - a period of 10 generations - at which time cell samples were removed for analysis. The culture was allowed to continue under steady-state conditions and samples for analysis were collected daily for a further 48 hours.

Cellular biomass was estimated by determining the dry weight of the culture (see Appendix 1, Section 6.3.2, p.139) and total cellular protein was estimated using the modified Biuret method of Stickland [99] (see Appendix 1, Section 6.3.3, p.140). An aliquot of culture supernatant was filter sterilised for amino acid analysis by RP-HPLC (see Appendix 1, Section 6.4, p.143) to determine the utilisation pattern of 23834^T grown in EDM-1. The utilisation of proline was determined using the modified derivatisation method of Cooper *et al* [100] (Appendix 1, Section 6.4.1, p.145). Cysteine was determined by using a method based on that of Cooper and Turnell [101] (Appendix 1, Section 6.4.2, p.146) and nitrite determination was performed using the method of Canney *et al* [102] (see Appendix 1, Section 6.9, p.151).

To establish whether nitrate was essential for growth, a batch of EDM-1 lacking nitrate was prepared. In a separate experiment to investigate glucose metabolism by *E. corrodens*, another batch of EDM-1, supplemented with 10 mM glucose, was prepared. Detection of glucose and acid end-products in culture filtrates was performed by ion exclusion HPLC (iexHPLC) based on the method of Guerrant *et al* [103] (see Appendix 1, Section 6.5, p.147).

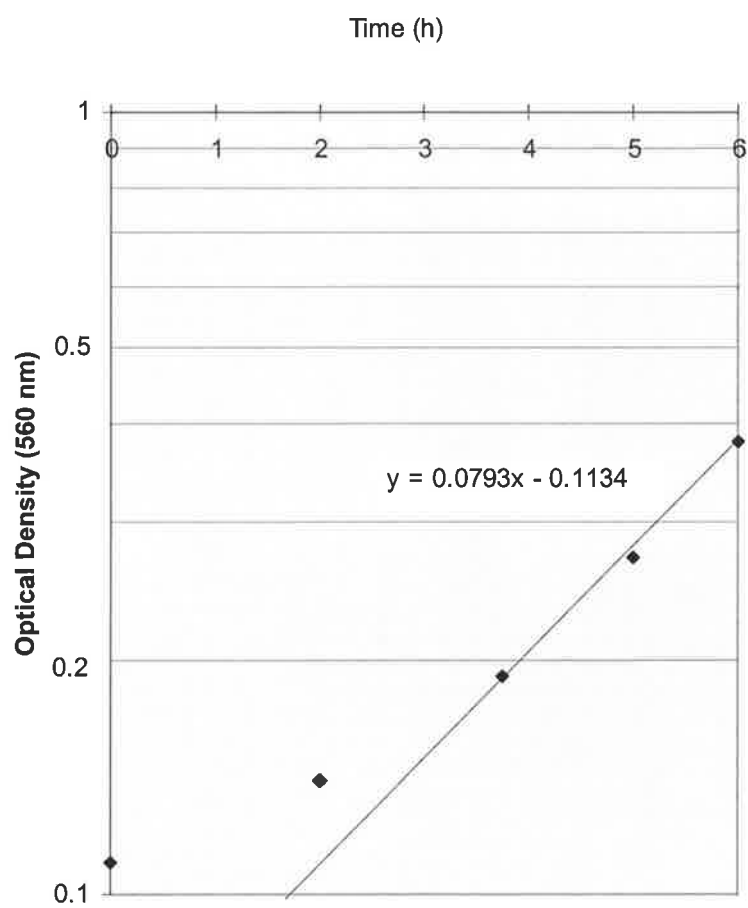
2.3.2. Results and Discussion

The results of measurement of maximum growth rate of the organism in EDM-1 are expressed in Figure 2-2 (see Appendix 2, Section 7.3, p.160, for data). This plot of the log of culture optical density at 560 nm versus time was constructed to determine doubling time. It should be noted that the data points used to determine $\mu_{\max}$ represent those recorded from samples after an apparent lag period, post inoculation, of approximately three hours. The doubling time (t_d) of the organism under these conditions was approximately 2.1 h, reflecting a $\mu_{\max}$ of 0.33 h^{-1} .

Accordingly, the medium flow rate for continuous culture was adjusted to generate a doubling time of approximately 7 h ($D = 0.1 \text{ h}^{-1}$, $\mu_{\text{rel}} = 0.3$).

Figure 2-2. Plot of log OD versus Time for Determination of Maximum

Growth Rate of *E. corrodens* 23834^T in EDM-1



The t_d , when grown in EDM-1 was comparable to that of 2.38 h obtained by Kuedell *et al* [95] when this strain was grown in E16TBN. However, it was significantly shorter than that, published by Progulske and Holt [94], for growth in a complex yeast extract based medium, BY, of 4.2 h.

Estimations of cellular biomass at steady-state were as follows;

$$\text{dry weight} = 0.29 \text{ g L}^{-1}$$

$$\text{cellular protein} = 0.24 \text{ g L}^{-1}$$

$$\text{OD}_{560\text{nm}} = 0.65 \text{ AU}$$

These results represent the average value for each parameter from three separate samples collected at steady-state conditions over a 48 h period.

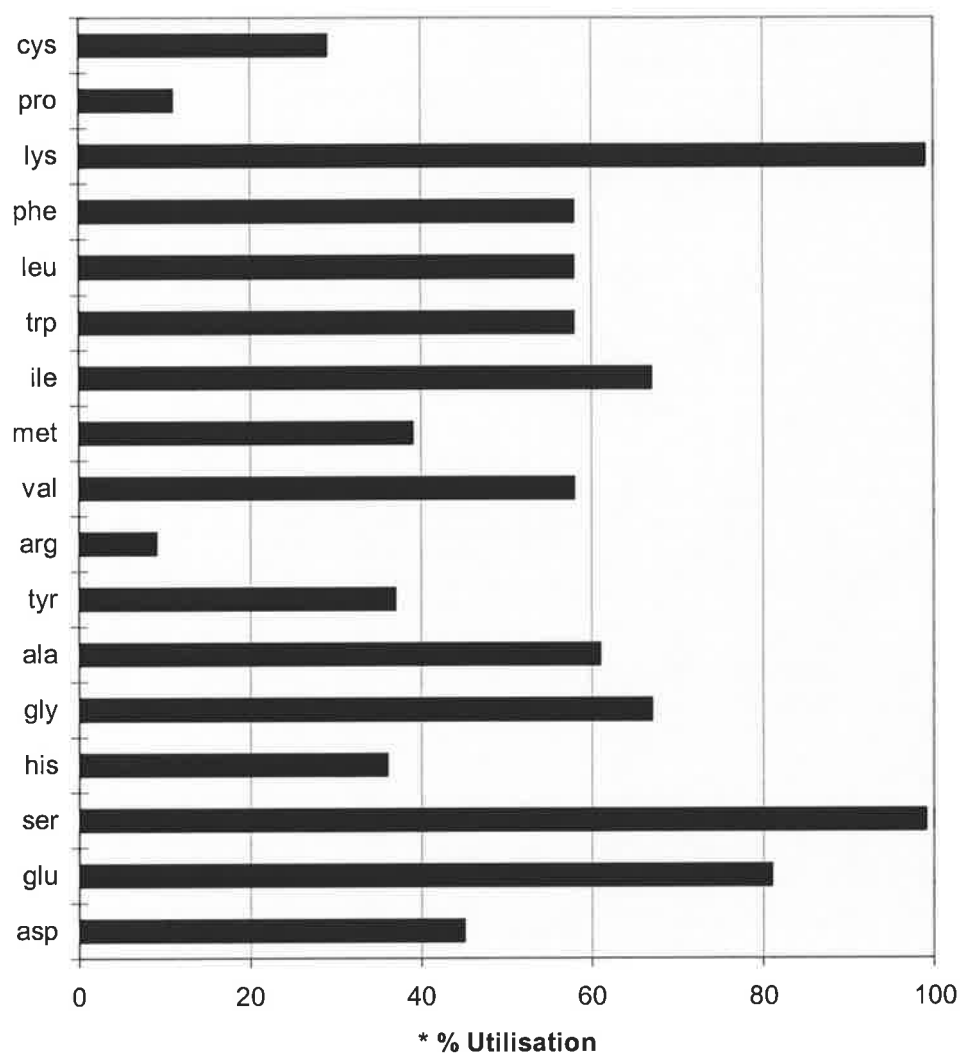
Again, growth in EDM-1 was comparable to that in E16TBN as the published figure for optical density at maximal growth is 0.72, although it should be noted that Keudell *et al* [95] measured optical density at 540 nm. A comparison with BY shows a significant difference in yield, growth in EDM-1 resulted in a dry weight of 0.29 g L^{-1} whereas growth in BY produced only 0.118 g L^{-1} . This indicates that BY is relatively nutritionally poor when compared to either EDM-1 or E16TBN.

The amino acid utilisation pattern was determined for this medium and is shown in Figure 2-3 (see Appendix 2, Section 7.4, p.161, for raw data). As the component amino acid concentrations are not equivalent in EDM-1, a more meaningful representation of these results is to express the quantity of amino acid

2. Determination of Growth Parameters

consumed (mmoles) per gram dry weight of cells produced. Results expressed in this form are shown in Figure 2-4.

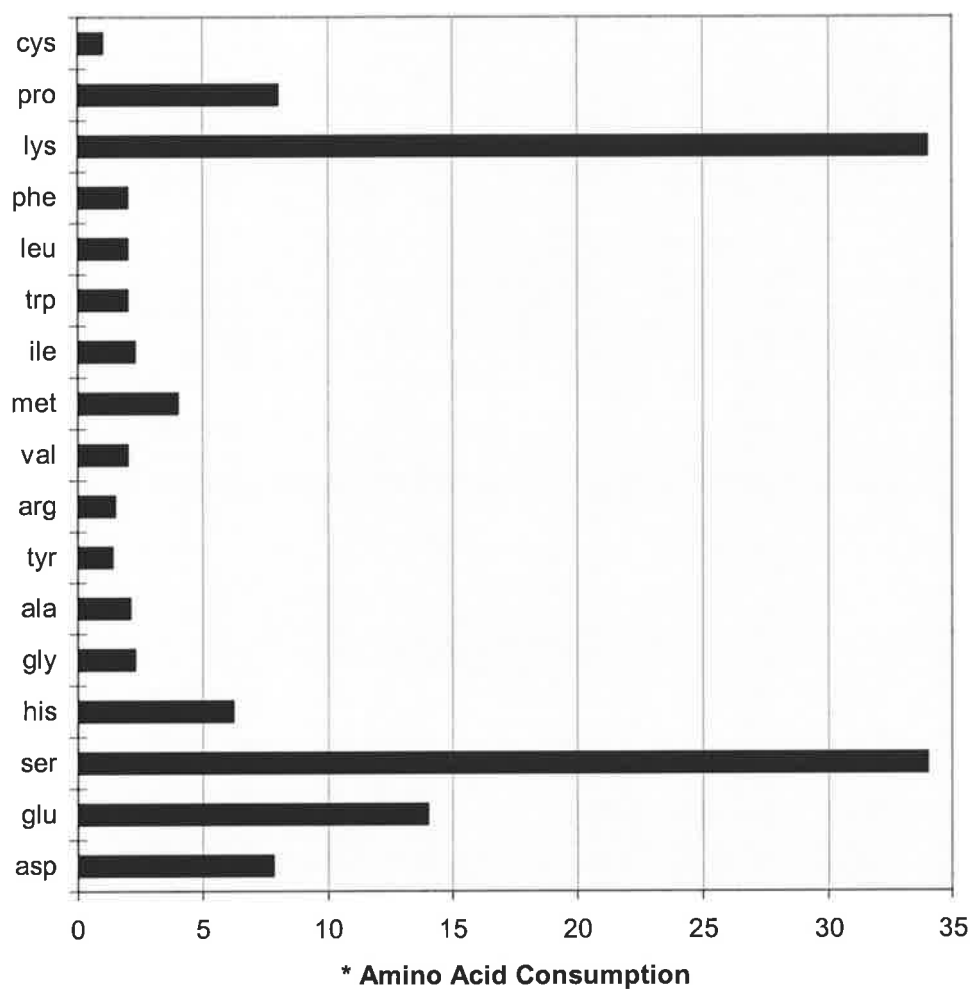
Figure 2-3. Amino Acid utilisation of *E. corrodens* 23834^T Grown in Continuous Culture in EDM-1.



*Expressed as a percentage of the free amino acid consumed as compared to that available in uninoculated YT medium.

(Actual data shown in Appendix 2, Section 7.4, p.161)

Figure 2-4. The Amount of Amino Acid Consumed per Gram Dry Weight of *E. corrodens* 23834^T Grown in Continuous Culture in EDM-1.



Expressed as mmoles of amino acid consumed (g dry weight cells)⁻¹.

(Actual data shown in Appendix 2, Section 7.4, p.161)

The pattern of amino acid utilisation by 23834^T, grown in EDM-1 under continuous culture conditions, was essentially the same as that for free amino acids after batch growth in YT medium.

It is important to note that these results confirm the observations of Keudell *et al* [95] that this organism derives its energy via the metabolism of amino acids and does not appear to require specific peptides, or other factors found in yeast extract, for growth.

The predominant amino acids consumed during growth were glutamate, serine and lysine at levels of 14, 34 and 34 mmoles per gram dry weight, respectively. Other amino acids appeared to be utilised to a greater extent than that when grown in YT. However, quantitative comparisons between these experiments are difficult as there was no analysis of the utilisation of amino acids from peptides during growth in YT medium.

End-product analysis revealed that acetate and formate were the only short-chain fatty acids detectable in the culture filtrate after growth in EDM-1. These metabolic products were present at concentrations of approximately 10 and 6 mM, respectively.

The omission of threonine from this synthetic medium did not appear to have a significant effect on the cell yield, confirming the work of Keudell *et al* [95], who showed that this amino acid was not essential for growth of the

organism and indicating that the catabolism of this amino acid does not appear to contribute significantly to energy production.

The addition of 10 mM glucose to medium EDM-1 failed to elicit an increase in any of the growth parameters measured, again confirming that the organism is unable to derive energy from this carbohydrate. Furthermore, an analysis of cell free culture supernatants by ion exchange HPLC showed no detectable utilisation of glucose from the medium, indicating that the organism may lack a functional transport system for this compound.

An analysis of culture filtrates after growth in EDM-1 revealed a stoichiometric reduction of all available nitrate to nitrite indicating that growth of the organism, under these conditions, may be nitrate limited.

Finally, in an experiment to determine whether nitrate was essential for growth of the organism in this medium, the batch of EDM-1 lacking nitrate was unable to support growth of the organism, either in continuous culture or under batch conditions. This finding confirms that of previous researchers [94], indicating that nitrate is essential for growth of the organism under these conditions.

2.4. Optimum Growth Temperature and pH

An understanding of the organism's ability to grow at ranges of temperature and pH may be important in understanding how change(s) in its physical environment may alter its proportion in a microbial community. A shift to more favourable environmental conditions compared to those of its competitors may lead to a selective advantage and therefore an increase in the numbers of the organism at the site.

2.4.1. Materials and Methods

The strain ATCC 23834^T was again cultured in the chemostat; the culture medium was EDM-1. As the utilisation of proline in EDM-1 was only of the order of 10%, a modification to the medium formula was made, reducing the concentration of this amino acid to 2 mM from 22 mM. A maximum growth rate ($\mu_{\max}$) experiment was again performed, using this modified EDM-1, to establish whether a decrease in proline concentration altered the organism's $\mu_{\max}$.

To determine the optimal growth pH of the organism the culture pH was adjusted to the desired value and maintained at this level by the automatic addition of either 2M KOH or 2M HCl. Samples were removed for growth parameter and HPLC analysis once the culture reached steady-state conditions (10 volume changes), and at 24 and 48 hours subsequently. The results for each pH value, ranging from 6.5 to 9.0, represent the average of the three samples under each condition. Growth temperature for this experiment was set at 34°C.

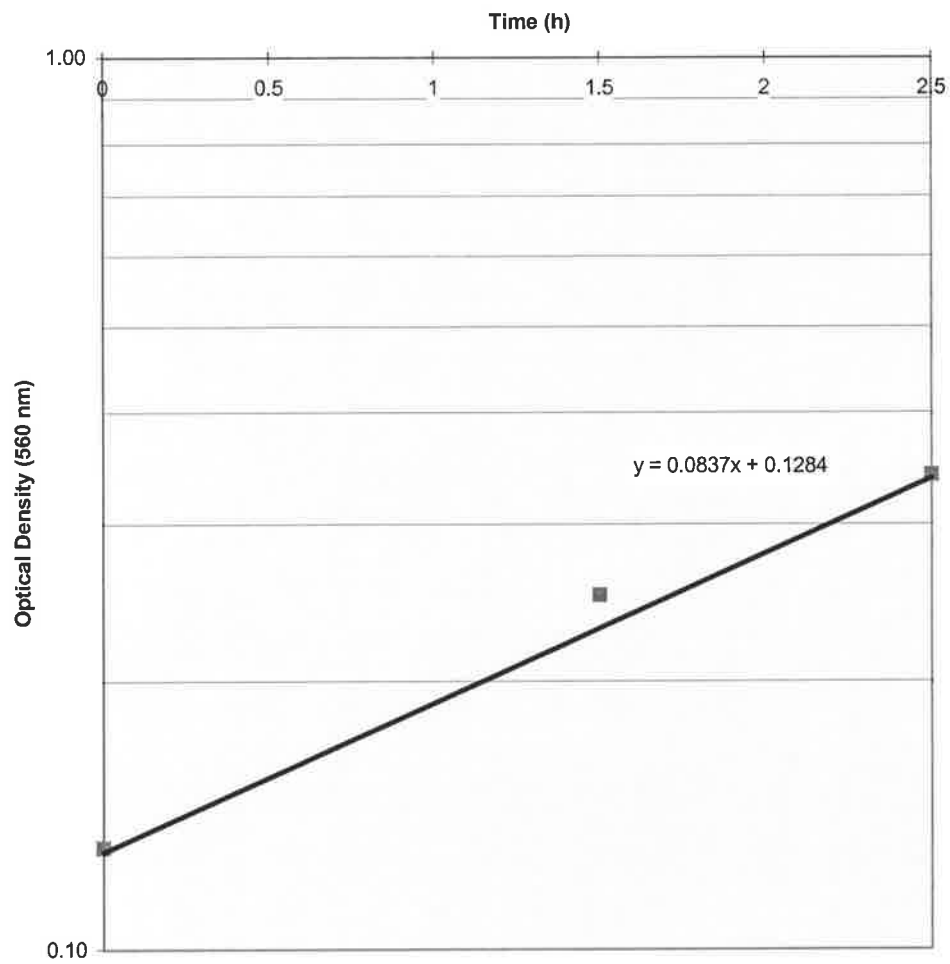
Determination of the optimal growth temperature employed the same methodology as above, except that the culture pH was maintained at 7.2 throughout and culture temperature was controlled thermostatically. Growth at temperatures of 30, 34 and 36°C was assessed in this fashion.

2.4.2. Results and Discussion

The results for determination of maximum growth rate of 23834^T in EDM-1 are shown in Figure 2-5. It should be noted that the slope of the regression line is comparable to that in Figure 2-2 (cf. 0.0837 vs 0.0793). Thus, the doubling time in modified EDM-1 (which contained a reduced proline concentration), although slightly longer, does not appear to be significantly different to that in EDM-1.

It is apparent from Figure 2-5 that, on this occasion there is little or no lag phase compared to that which occurred when medium EDM-1 was used (Figure 2-2). An explanation for this is that on this occasion the organism was inoculated into modified EDM-1 and allowed to grow for 24 hours in the chemostat prior to $\mu_{\max}$ determination. At the commencement of the experiment approximately 90% of the culture volume was removed and replaced with fresh medium. Using this method, the organism did not have to respond to a significant medium change, thus reducing the lag period before growth at the maximum rate occurred. The lag period (Figure 2-2) may have been the result of organisms, cultured in YT medium, responding to a changed nutritional environment.

Figure 2-5. Plot of OD versus Time for Determination of Maximum Growth Rate
of 23834^T in Modified EDM-1



(Actual data shown in Appendix 2, Section 7.5, p.162)

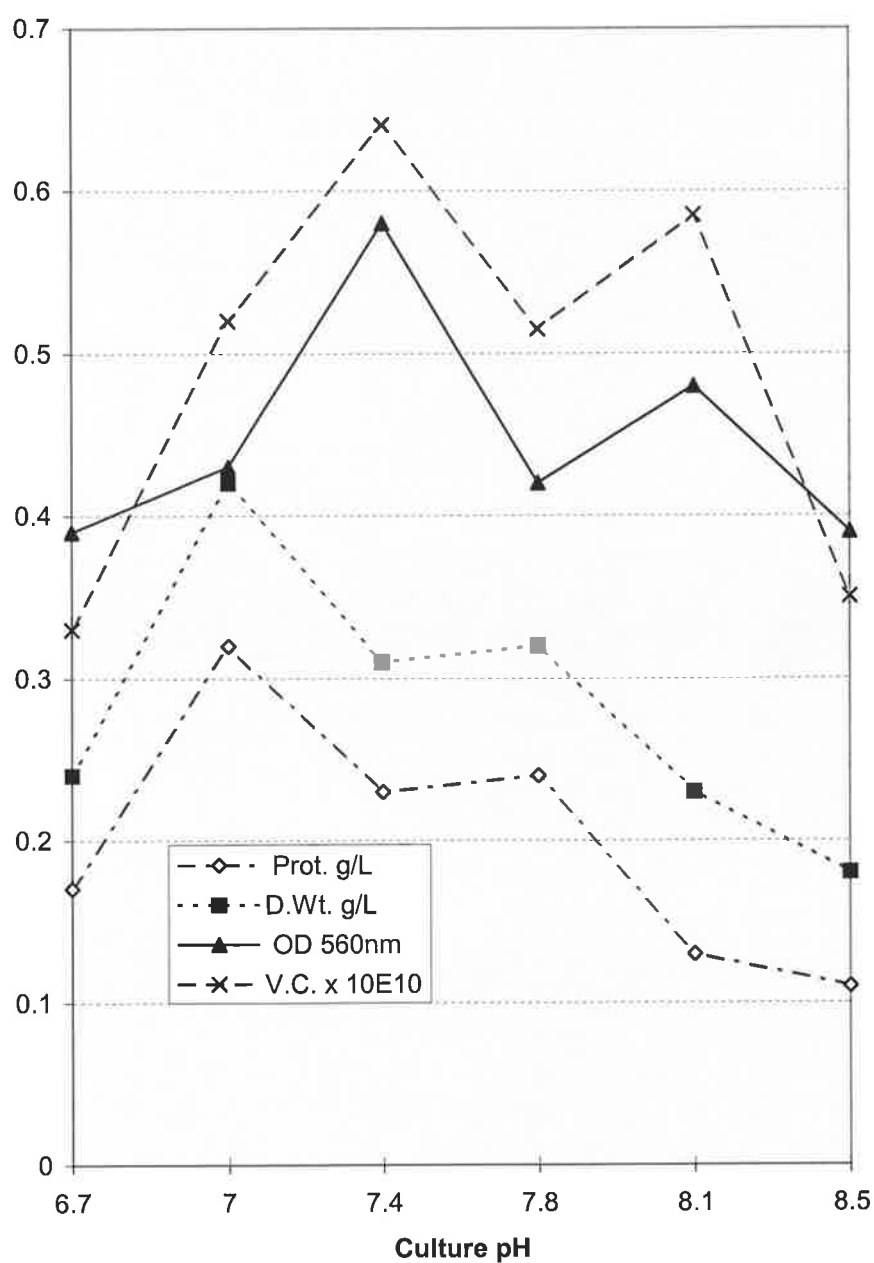
The results of the effect of pH on biomass production, (Figure 2-6), indicate that the optimal environmental pH for this organism lies somewhere between 7 and 7.4. Although measurements of optical density and viable count were maximal at pH 7.4, cell dry weight and protein estimations peaked at 7.0. A possible explanation for this observation is that Gram stained smears of the organism revealed a decreased cell size when culture pH was 7.4 and above. This may have resulted in an increase in optical density and viable count but not in a concomitant increase in biomass. It seems likely that the pH optimum lies between 7.0 and 7.4 and so all future culturing was performed at pH 7.2.

A more significant ecological finding is that the range of pH tolerance for the organism is biased towards alkalinity. An attempt to establish steady-state growth at pH 6.5 resulted in a culture with a flaky macroscopic appearance and growth of the organism was not sustained under the imposed dilution rate of $D = 0.1 \text{ h}^{-1}$. The organism was found, however, to survive at this dilution rate at an environmental pH as high as 8.5 and all growth parameters were greater at pH 7.8 when compared to those at 6.7.

This tolerance of relatively high environmental pH values is probably related to the organism's reliance on the catabolism of amino acids for energy generation as well as a supply of carbon skeletons for anabolic processes. The oxidative deamination of amino acids results in the release of ammonia, the excretion of which produces an elevated pH level in the micro-environment.

Therefore, tolerance to relatively high physiological pH values is a likely essential trait of this organism.

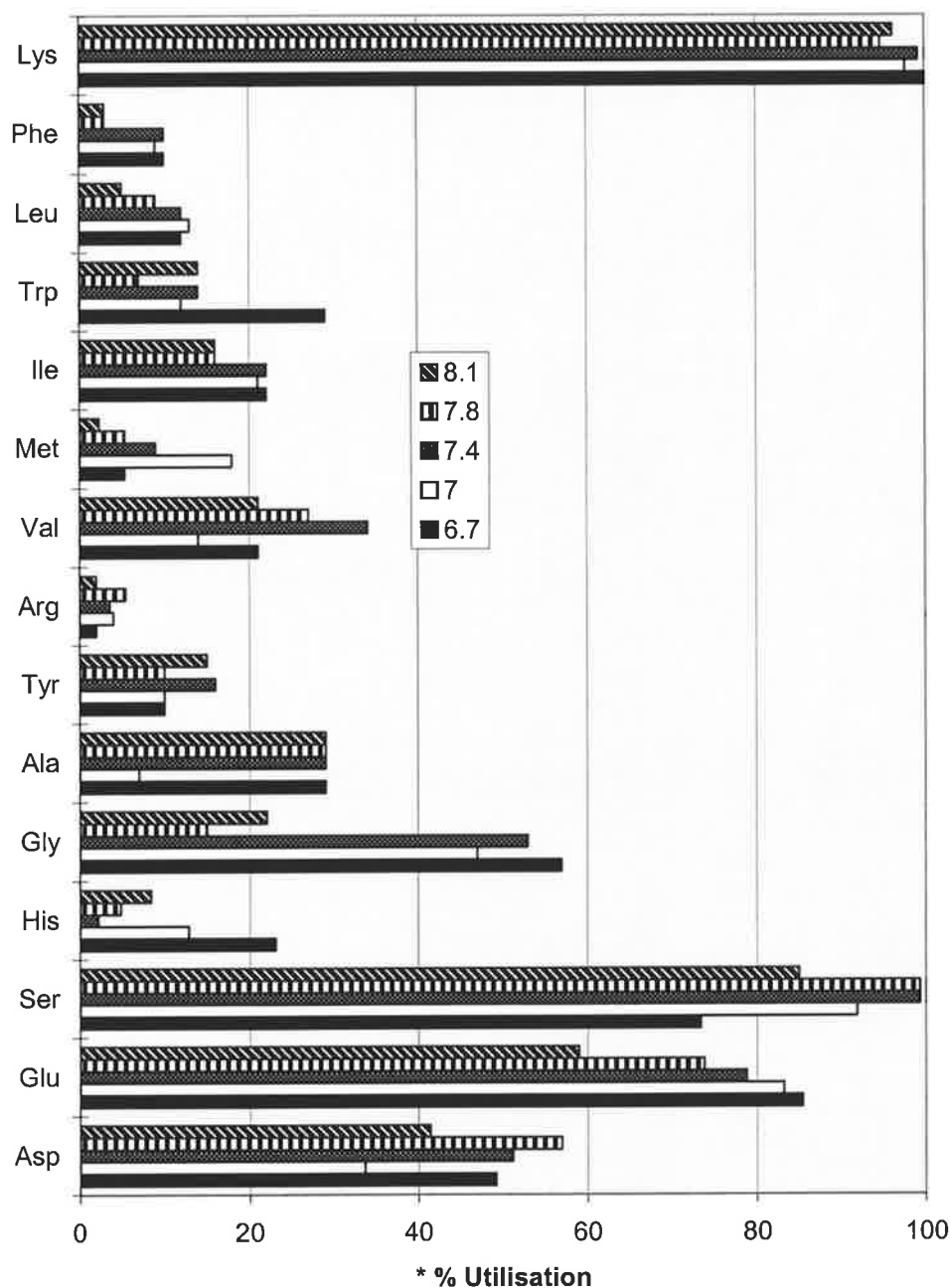
Figure 2-6. The Effect of Culture pH on Growth of *E. corrodens* 23834^T



(Actual data shown in Appendix 2, Section 7.6, p.163)

The utilisation of amino acids of cultures grown at the various pH values appears in Figure 2-7 and the consumption of amino acids over this pH range, expressed per gram dry weight of cells, is shown in Figure 2-8.

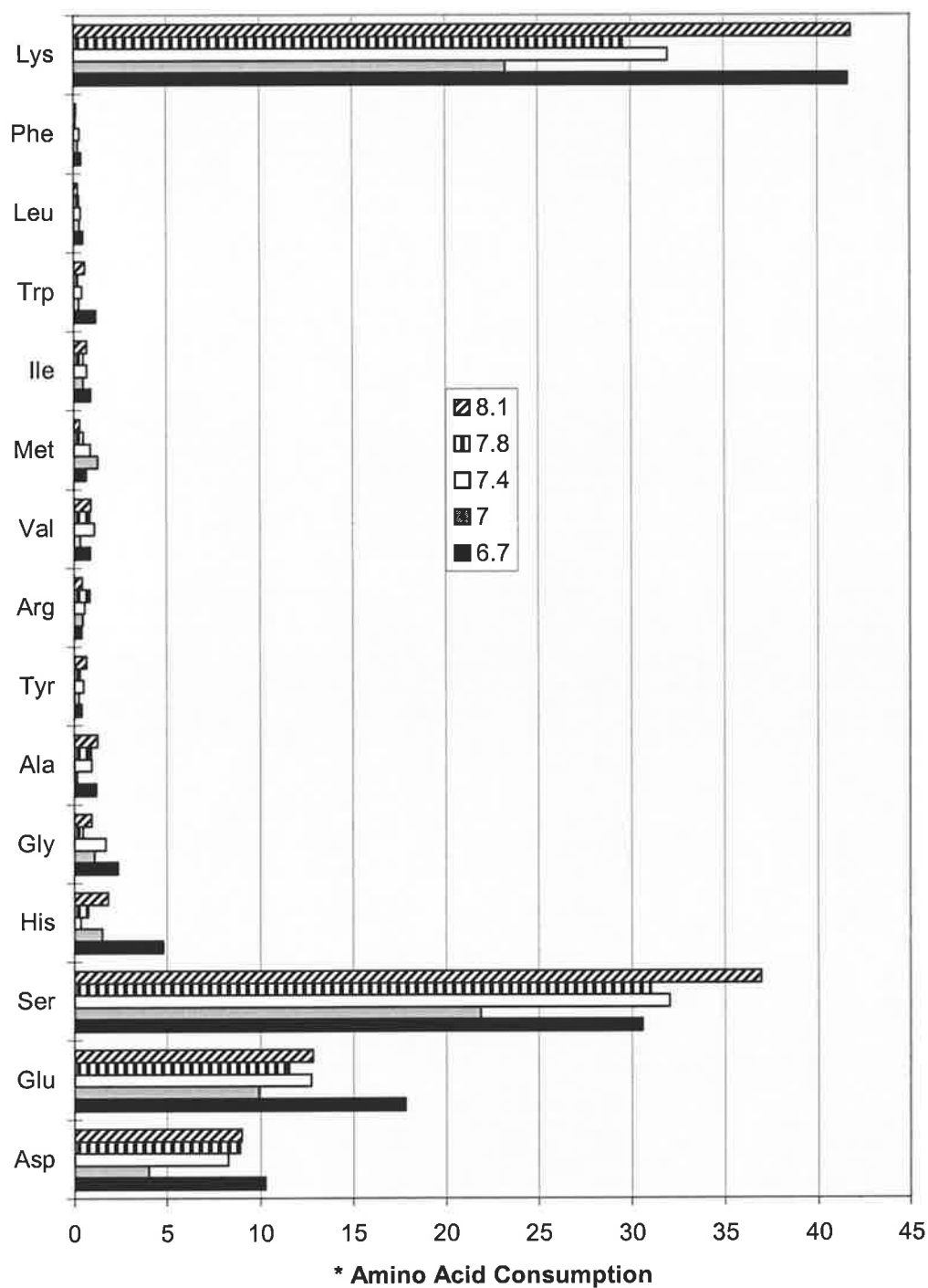
Figure 2-7. The Effect of Culture pH on Amino Acid Utilisation by *E. corrodens* 23834^T



*Expressed as a percentage of the free amino acid consumed as compared to that available in uninoculated EDM-1.

(Actual data shown in Appendix 2, Section 7.7, p.164)

Figure 2-8. The Effect of Culture pH on the Amount of Amino Acid Consumed per Gram Dry Weight of *E. corrodens* 23834^T



* Expressed as mmol of amino acid consumed (g dry weight cells)⁻¹.

(Actual data shown in Appendix 2, Section 7.7, p.164)

As seen previously, of those amino acids analysed in culture filtrates, glutamate, serine and lysine were utilised to the greatest degree. This was true for all growth pH values tested, although some minor, but possibly significant, changes to glutamate consumption over the pH range were observed. The utilisation of this amino acid was maximal at pH 6.7 and declined somewhat as the culture pH was elevated. This shift is not as evident from Figure 2-7, but it is apparent from this data that the amount of glutamate consumed per weight of cells produced appears significantly higher than that for other growth pH values (Figure 2-8). Also, the pattern of utilisation for the amino acids glutamate, serine and glycine following growth at pH 6.7 appears quite different to that for growth at pH 8.1, even though both conditions resulted in similar growth yields; 0.24 and 0.23 g dry weight L⁻¹ respectively. Utilisation of these amino acids was lower at the higher pH value.

The cause of these slight metabolic changes by growth pH is not known but it is noteworthy that the consumption of amino acids, *in toto*, for all pH values tested is comparable. This metabolism results in a quantum of energy which, at optimum pH, is allocated to biomass production. A small shift downward in external pH (to 6.7) results in a reduction in dry weight of almost 50%, indicating that a significant portion of this energy has been diverted from biomass production to cell maintenance. The energy burden at a slightly acidic environmental pH may be the result of the increased energy required for maintenance of a critical intracellular pH. The channelling of available energy towards maintenance of internal pH is also apparent at growth pH values above neutrality, although the

organism is able to withstand a shift towards an alkaline environment to a greater extent.

The effect of growth pH on the formation of acidic end-products is shown in Table 2-1. Once again, the only detectable acid end-products were acetate and formate. The amounts of these, at pH 6.7 and 7.0, were comparable to those found previously at pH 7.2. At higher growth pH values the amounts of these two metabolites decreased. At pH 8.5 the production of acetate and formate was reduced by 45% and 79%, respectively, compared to that at pH 7.0.

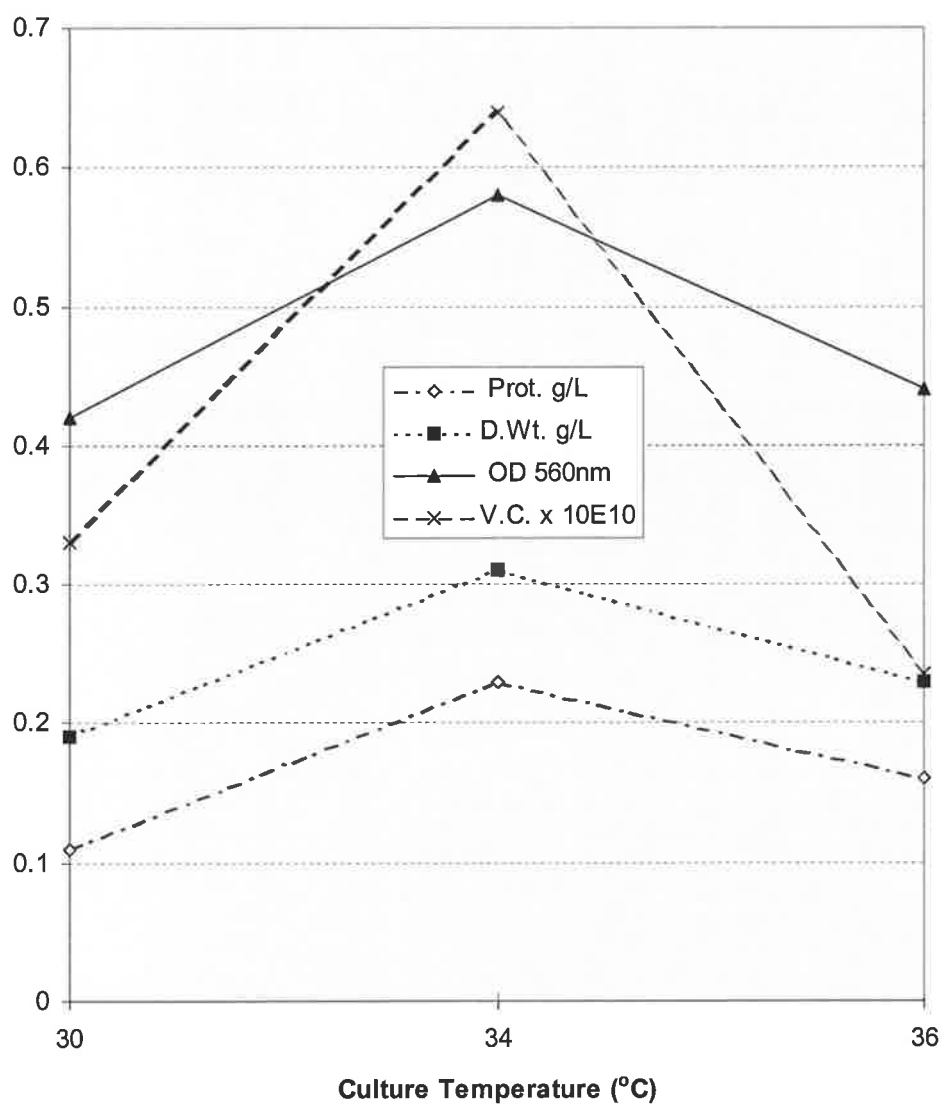
Table 2-1. The Effect of Growth pH on Extracellular End-products in *E. corrodens* 23834^T

Culture pH	[acetate] (mM)	[formate] (mM)
6.7	10*	6.1
7	10	5.7
7.4	9	2.4
7.8	6.2	1.8
8.1	6.8	2.4
8.5	5.5	1.2

* Values represent the average result of triplicate samples, none of which differed by more than 10%.

The effect of temperature on growth of the organism can be seen in Figure 2-9. All growth parameters measured indicated that the optimal growth temperature for the organism is close to 34°C. As a result, all future culturing took place at this temperature.

Figure 2-9. The Effect of Culture Temperature on Growth of *E. corrodens* 23834^T

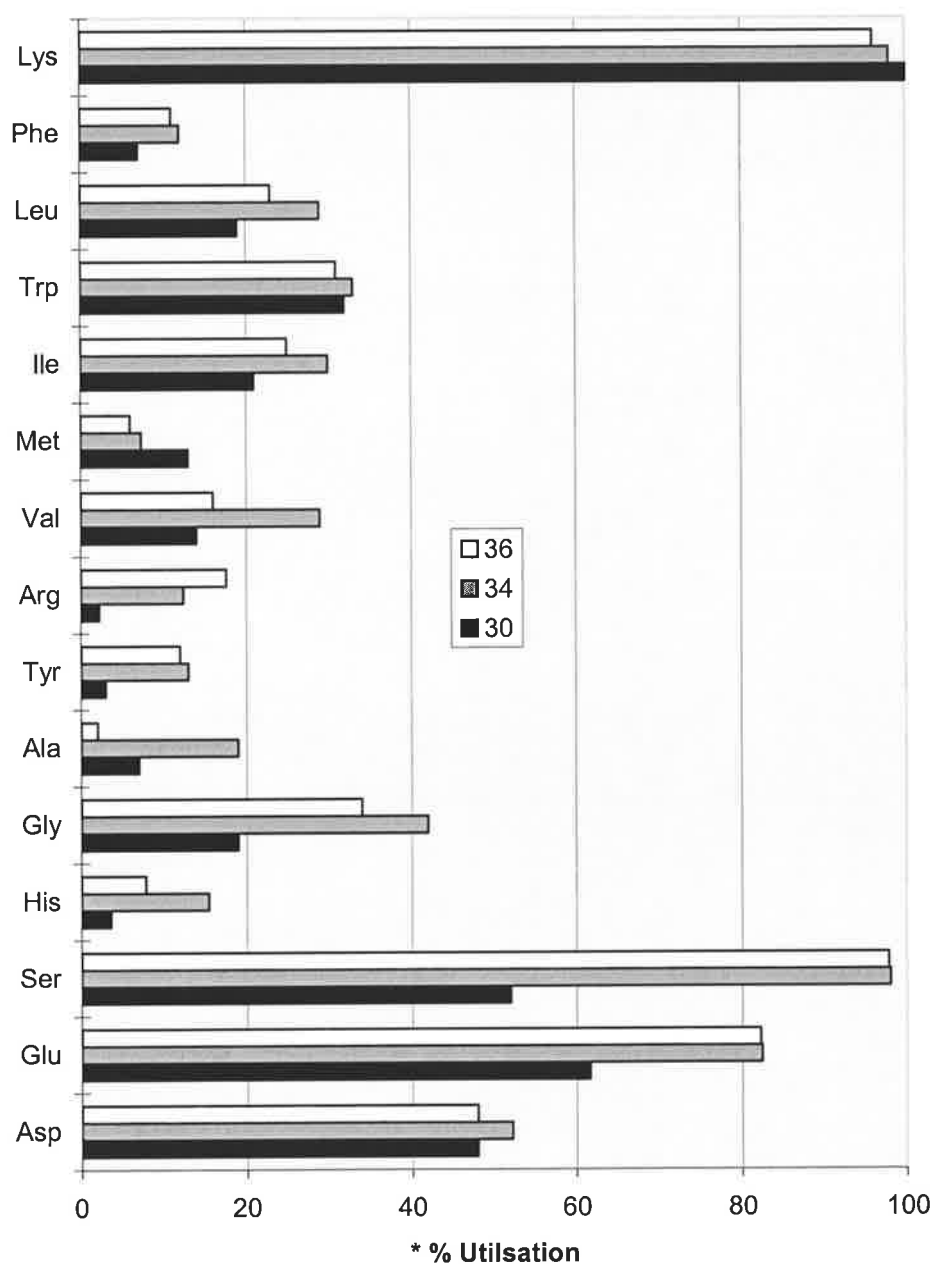


(Actual data shown in Appendix 2, Section 7.8, p.165)

The effect of temperature on amino acid utilisation is depicted in Figure 2-10. These data are also expressed as mmols of amino acid consumed per gram dry weight of cells (Figure 2-11). The pattern of amino acid utilisation for all temperatures was essentially the same as that observed previously for optimal pH determination (Figures 2-7 and 2-8).

Growth at 30°C resulted in the reduced utilisation of all amino acids (except methionine and lysine) compared with growth at higher temperatures. Interestingly, the amounts of glutamate and serine consumed per gram dry weight of cells were significantly reduced at this growth temperature. If the metabolism of these amino acids is involved in energy production, it would appear that the most efficient channelling of this energy into biomass by the organism occurs at a growth temperature of 30°C.

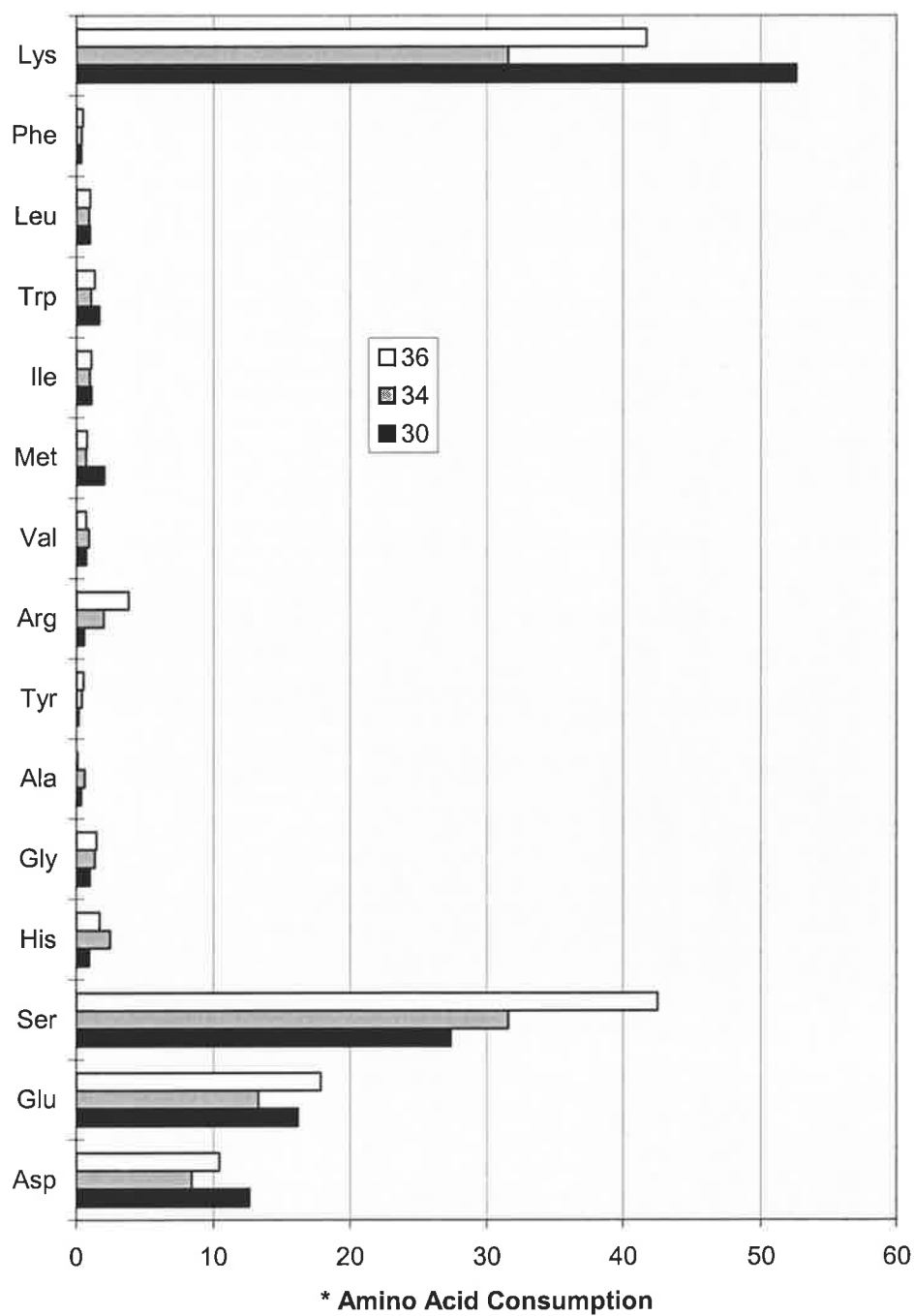
Figure 2-10. The Effect of Culture Temperature on Amino Acid Utilisation by *E. corrodens* 23834^T



*Expressed as a percentage of the free amino acid consumed as compared to that available in uninoculated EDM-1 medium.

(Actual data shown in Appendix 2, Section 7.9, p.166)

Figure 2-11. The Effect of Culture Temperature on the Amount of Amino Acid Consumed per Gram Dry Weight of *E. corrodens* 23834^T



Expressed as mmoles of amino acid consumed (g dry weight cells)⁻¹.

(Actual data shown in Appendix 2, Section 7.9, p.166)

2. Determination of Growth Parameters

To determine the effect of growth temperature on the formation of acidic end-products, culture filtrates from samples grown at the various temperatures were analysed. These results can be seen in Table 2-2.

The amount of acetate produced at 30°C was reduced by almost 50% when compared to growth at higher temperatures and formate was not detected at this temperature. At growth temperatures of 34°C and 36°C acetate concentrations were comparable to those seen previously but formate production was reduced somewhat. The reason for this reduction is not known.

Table 2-2. The Effect of Growth Temperature on Extracellular End-products

in E. corrodens 23834^T

Culture Temp (°C)	[acetate] (mM)	[formate] (mM)
30	4.7*	n.d. ⁺
34	9.0	2.9
36	9.3	1.2

⁺ None Detected

* Values represent the average result of triplicate samples, none of which differed by more than 10%.

2.5. Effect of Atmospheric Conditions on Growth of *E. corrodens*

Although historically this organism has been routinely cultured in an anaerobic environment, often in an atmosphere containing 5% CO₂, little is known about the effect of atmospheric conditions on its growth. To determine the role of the gaseous environment it was decided to grow the organism in continuous culture under different atmospheric conditions.

2.5.1. Materials and Methods

The strain 23834^T was grown in continuous culture, as described previously, in EDM-1 at 34°C, pH 7.2 and $D = 0.1 \text{ h}^{-1}$. It should be noted that the particular atmosphere was introduced into the culture head-space but not actively bubbled through the liquid culture; the exchange of gases therefore took place only at this interface. Initially, the cells were cultured under an imposed atmosphere of 95% N₂ and 5% CO₂. Once steady-state conditions had been reached, a sample of culture was removed; the optical density measured and cell dry weight and protein were estimated using the methods detailed in Appendix 1, Sections 6.3.1, 6.3.2 and 6.3.3 (pp.139 and 140), respectively. Growth was allowed to continue for 48 hours, after initial sampling, with further sampling occurring at 24 hour periods. Cells were also cultured and similarly analysed after growth in the presence of 100% N₂ and in air.

2.5.2. Results

The results for this experiment are shown in Table 2-3.

Table 2-3. The Effect of Atmosphere on Growth of *E. corrodens* 23834^T
in Continuous Culture

Atmospheric Conditions	Protein (g L ⁻¹)	Dry Weight (g L ⁻¹)	Optical Density (560nm)
95% N ₂ + 5% CO ₂	0.22*	0.29	0.56
100% N ₂	0.21	0.27	0.54
Air	0.20	0.28	0.55

* Values represent the average result of triplicate samples, none of which differed by more than 10%.

As seen from the results shown in Table 2-3, growth of the organism was comparable in all atmospheric conditions tested. The presence of O₂ appeared to have no significant effect on cell yields when compared to that obtained when cells were cultured in the presence of either 100% N₂ or 95% N₂ and 5% CO₂. Thus the organism appears tolerant of the higher Eh imposed by the presence of dissolved molecular O₂. Also, it appears that growth is not stimulated by the presence of atmospheric CO₂.

It was concluded from this experiment that future continuous culture could be conducted in a relatively aerobic environment without apparent detriment to cell yield.

3. Amino Acid Metabolism and Biomass Production

3.1. Glutamine Metabolism

As observed in Section 2.1, the organism consumed all available glutamine when grown in YT medium. The following experiment was devised to establish whether the catabolism of this amino acid is linked to the production of energy, in a mechanism similar to that for glutamate.

3.1.1. Materials and Methods

Strain 23834^T was grown in a chemostat at 34°C, pH 7.2 and at a dilution rate of 0.1 h⁻¹ in modified EDM-1 medium that contained 5 mM glutamine instead of 5 mM glutamate. The culture was allowed to reach steady-state (10 generations) and samples were removed at this time and at 24 and 48 hours, subsequently, for measurement of optical density and estimation of cell dry weight and protein content (see Appendix 1, Sections 6.3.1, 6.3.2 and 6.3.3, pp.139 and 140, for detailed methods).

3.1.2. Results and Discussion

The results, presented in Table 3-1, show that the substitution of glutamine for glutamate produced almost identical biomass values. The most likely explanation for these results is that *E. corrodens* is able to deaminate glutamine to

glutamate, which is in turn catabolised, by a process which is directly linked to the generation of cellular energy.

Table 3-1. The Effect of Substitution of Glutamine for Glutamate on the Growth of *E. corrodens* 23834^T

	Protein (g L ⁻¹)	Dry Weight (g L ⁻¹)	Optical Density (560 nm)
EDM-1 (Glutamate)	0.24*	0.29	0.65
EDM-1 (Glutamine)	0.22	0.28	0.59

* Values represent the average result of triplicate samples, none of which differed by more than 10%.

3.2. Threonine Metabolism

During the development of a chemically defined medium it was apparent that the acid end-product propionate was present in culture filtrates when threonine was included in the medium. Unfortunately the OPA RP-HPLC method for analysis of amino acids did not allow resolution of threonine and glycine. As a previous study indicated that glycine was essential for growth of the organism [95], it was decided to exclude threonine from chemically defined media to allow for accurate estimation of glycine utilisation.

It should be noted that small quantities of propionate (2-3 mM) were detected in end-product analysis of culture filtrates taken from growth of the organism in a chemically defined medium containing threonine at a concentration

of 5 mM. Although the metabolism of threonine to propionate is another possible (albeit small) energy source for the cell, no further investigations were undertaken in this area.

3.3. Chemostat Studies in a Minimal Chemically Defined Media

The amino acid utilisation patterns from the experiments involving growth of *E. corrodens*, in relatively simple chemically defined media, under a range of physical conditions, revealed that in all cases glutamate, serine and lysine were consumed in greater amounts than other amino acids. It appeared that the oxidative deamination of these amino acids may supply energy to the organism. Thus, biomass should be able to be modulated by varying the amounts of these key amino acids in the growth medium.

The role of nitrate, possibly acting as the ultimate electron acceptor, was also to be investigated further in this system. To test these hypotheses, the following series of experiments were performed:-

3.3.1. Materials and Methods

A minimal medium, EMMG1 (see Appendix 1, Section 6.8, p.150), capable of supporting growth of 23834^T was developed. This basal medium was designed to allow for the observation and measurement of changes in cellular biomass in response to addition of specific amino acid supplements. Except for the

omission of serine, this medium contained the same range of amino acids, each at a concentration of 1 mM, as did EDM-1. As the focus of this experiment was on the key amino acids glutamate, serine and lysine, threonine was also included, at 1 mM, in the minimal medium formulation.

Cells grown in EDM-1, which contained 20mM nitrate, produced nitrite levels indicating that all available nitrate was reduced to nitrite (see Section 2.3). Accordingly, the concentration of nitrate in this medium was increased to 40mM to decrease the likelihood that this component was limiting for steady-state growth. It should be noted that a previous study on the organism's growth characteristics [94] indicated that media containing up 40 mM nitrate had no apparent adverse effect on growth of the organism.

The organism was cultured in the chemostat, as described previously at pH 7.2, 34°C and $D = 0.1 \text{ h}^{-1}$. Once steady-state conditions were reached, the optical density (560 nm) of the culture was recorded and sufficient culture was removed for dry weight estimation, amino acid and short chain fatty acid analysis, and the production of nitrite. The culture was allowed to grow for a further two days, samples being taken each day.

After final sampling the medium was replaced with EMMG3, a medium identical to EMMG1 except that the glutamate concentration in this medium was increased to 3 mM. The organism was again allowed to reach steady-state conditions and sampled accordingly. This procedure was employed for subsequent medium formulations; EMMG5 (containing 5mM glutamate), EMMG3S

3. Amino Acid Metabolism and Biomass Production

(identical to EMMG3 except for the addition of 10 mM serine) and EMMG3L

(identical to EMMG3 except for the addition of 10 mM lysine).

3.3.2. Results and Discussion

The results for biomass and nitrite production are shown in Table 3-1, and represent the average figure of the three samples for each growth medium.

Table 3-1. Growth parameters for *Eikenella corrodens* 23834^T grown at D=0.1 h⁻¹, pH 7.2, 34°C in chemically defined media.

	Chemically Defined Media ^a				
	EMMG1 ^b	EMMG3 ^b	EMMG5 ^b	EMMG3S ^c	EMMG3L ^d
OD (560 nm)	0.17 ^g	0.32	0.37	0.44	0.32
Dry Weight (g.L⁻¹)	0.035	0.085	0.120	0.185	0.088
Nitrate utilisation (mM)^e	14 (35) ^f	22 (55)	29 (73)	33 (83)	29 (73)

^a All media contained 40 mM nitrate as well as 1 mM each of asp, his, gly, thr, ala, tyr, arg, val, met, ile, trp, leu, phe, lys, pro and cys. The composition of the minimal medium EMMG1 appears in Appendix 1, Section 6.8, p.150.

^b EMMG1, EMMG3 and EMMG5 contained a final concentration of 1, 3 and 5mM glutamate respectively.

^c EMMG3S contained a final concentration of 3 mM glutamate and 10 mM serine.

^d EMMG3L contained a final concentration of 3 mM glutamate and 11 mM lysine.

^e Nitrate utilised was calculated on the basis of residual nitrite detected in culture filtrates.

^f Figures in parentheses represent % utilisation of nitrate.

^g Each figure represents the average of triplicate samples, none of which differed by more than 10%.

It can be seen from the data in Table 3-1 that the additional glutamate in EMMG3 and EMMG5 resulted in significant increases in biomass when growth in these media is compared to that in EMMG1. The additional 2 mM glutamate present in EMMG3 produced optical density and dry weight increases of 88% and 143% respectively, while an additional 4 mM (EMMG5) resulted in optical density and dry weight increases of 118% and 243% of those in growth in EMMG1.

The addition of 10 mM serine also resulted in a biomass increase as the optical density and dry weight values for growth in EMMG3S were 20% and 54% higher than those for growth in EMMG3. In contrast to glutamate and serine, however, the organism was not capable of converting additional lysine into biomass since growth in EMMG3L was not significantly different to that in EMMG3.

The concentrations of residual amino acids in culture filtrates after growth in these media are shown in Table 3-2 from which it can be seen that all available serine and lysine were utilised in media EMMG3S and EMMG3L, respectively.

The organism is capable of metabolising relatively high concentrations of lysine but this process does not appear to be linked to the generation of high energy phosphate currency for the cell. The consumption of all available lysine may be attributable to expression of the enzyme lysine decarboxylase, an enzyme which *E. corrodens* has previously been reported as expressing [104]. Lysine decarboxylase catalyses the conversion of lysine to cadaverine and the expression

of this enzyme may explain the organism's ability to consume relatively large amounts of free lysine in media.

Table 3-2. Concentration of Amino Acids in Culture Filtrates After Growth of *E. corrodens* 23834^T in Various Minimal Media.

Amino Acid	Chemically Defined Media ^a				
	EMMG1 ^b	EMMG3 ^b	EMMG5 ^b	EMMG3S ^c	EMMG3L ^d
asp	0.32 ^e	0.26	0.22	0.25	0.28
glu	0	0	1.1	0	0
ser	*	*	*	0	*
his	0.63	0.55	0.49	0.53	0.57
gly	0.53	0.44	0.42	0.41	0.46
ala	0.39	0.67	0.62	0.59	0.69
tyr	0.63	0.80	0.77	0.74	0.79
arg	0.90	0.88	0.79	0.76	0.74
val	0.42	0.39	0.40	0.38	0.45
met	0.56	0.51	0.50	0.49	0.51
ile	0.66	0.62	0.59	0.55	0.68
trp	0.53	0.51	0.42	0.45	0.55
leu	0.82	0.67	0.59	0.47	0.77
phe	0.82	0.69	0.62	0.64	0.79
lys	0	0	0	0	0

^a All media contained 40 mM nitrate as well as 1 mM each of asp, his, gly, thr, ala, tyr, arg, val, met, ile, trp, leu, phe, lys, pro and cys. The composition of the minimal medium EMMG1 appears in Appendix 1, Section 6.8, p.150.

^b EMMG1, EMMG3 and EMMG5 contained a final concentration of 1, 3 and 5mM glutamate respectively.

^c EMMG3S contained a final concentration of 3 mM glutamate and 10 mM serine.

^d EMMG3L contained a final concentration of 3 mM glutamate and 11 mM lysine.

^e Results represent an average of triplicate samples differing by less than 10% and are expressed as mmol L⁻¹ concentration of residual amino acid.

* These media contained no serine.

While all available glutamate was utilised from EMMG1 and EMMG3, only about 80% of the available glutamate was consumed after growth in EMMG5, indicating that the culture was growth limited by some other factor under these conditions. Molar growth yields calculated from this data for the metabolism of glutamate and serine were 27 and 10 grams dry weight of cells, respectively. Thus, it would appear that the production of energy from the utilisation of glutamate, and subsequent conversion into biomass, is a significantly more efficient process than that for serine.

These results also illustrate the link between nitrate reduction and biomass production, as an increase in nitrite concentration in culture filtrates appears directly related to glutamate utilisation. Additional glutamate supplied in EMMG3 and EMMG5 resulted in an increase in nitrite production of 57% and 107%, respectively, over that produced from EMMG1. The addition of 10 mM serine (EMMG3S) also resulted in increased nitrite (50%) over that produced from the comparable medium lacking this amino acid (EMMG3). However, the quantity of nitrite produced per mmole of amino acid utilised was much lower for serine than that for glutamate.

In contrast, the metabolism of lysine from EMMG3L, although not resulting in any biomass increase, appeared to be linked in some way to nitrate reduction, as growth in this medium produced an increase in nitrite concentration of 33% when compared to that produced in EMMG3. It appears that the

metabolism of lysine, either directly or indirectly, results in nitrate reduction. However, this process provides no net increase in energy available to the cell.

The results of end-product analysis of culture filtrates from growth in each of the minimal media are shown in Table 3-3. The only detectable short-chain fatty acid end-products arising from amino acid metabolism were acetate and formate. The ratio of acetate to formate was generally in the order of 2:1 and the appearance of these two compounds is related to the utilisation of serine. This pattern of end-products is consistent with those found previously in experiments using the medium EDM-1 which contained 10 mM serine.

Table 3-3. End-product Analysis of Culture Filtrates After Growth of *E. corrodens* 23834^T in Various Minimal Media.

End-Product	Chemically Defined Media ^a				
	EMMG1 ^b	EMMG3 ^b	EMMG5 ^b	EMMG3S ^c	EMMG3L ^d
acetate	0 ^e	0	0	9.2	0
formate	0	0	0	3.8	0

^a All media contained 40 mM nitrate as well as 1 mM each of asp, his, gly, thr, ala, tyr, arg, val, met, ile, trp, leu, phe, lys, pro and cys. The composition of the minimal medium EMMG1 appears in Appendix 1, Section 6.8, p.150.

^b EMMG1, EMMG3 and EMMG5 contained a final concentration of 1, 3 and 5mM glutamate respectively.

^c EMMG3S contained a final concentration of 3 mM glutamate and 10 mM serine.

^d EMMG3L contained a final concentration of 3 mM glutamate and 11 mM lysine.

^e Results represent an average of triplicate samples differing by less than 10% and are expressed as mmols L⁻¹ concentration of end-product.

3.4. Amino Acid Metabolism and Nitrate Reduction

Previous experiments indicated that the catabolism of amino acids - primarily glutamate and, to a lesser degree serine - was the source of energy generation in *E. corrodens*. This process also appeared to be linked to the concomitant reduction of nitrate. Therefore, it was decided to further investigate the relationship between amino acid utilisation and nitrate reduction by determining nitrate reduction rates of washed cells incubated with individual amino acids.

3.4.1. Materials and Methods

For future studies it was decided to use the strain 33EK(L), a clinical isolate, obtained by Dr. R. P. Allaker, from a deep (>5 mm) periodontal pocket of a patient presenting for periodontitis treatment.

The growth medium for 33EK(L) was BM1, a complex medium, with a similar composition to that of YT medium, developed by Dr R. P. Allaker [105] (see Appendix 1, Section 6.10, p.153, for its composition). An overnight batch culture of 33EK(L) in BM1 was prepared as an inoculum to initiate chemostat growth. Steady-state conditions, at 35°C, pH 7.2 and a dilution rate of 0.1 h⁻¹ were achieved after 7-10 volume changes. The culture was grown without special atmospheric conditions, since a CO₂-supplemented anaerobic atmosphere was previously found not to be required for growth of this organism (see Section 2.5).

Cell dry weight and total protein were determined as described in Appendix 1, Sections 6.3.2 and 6.3.3, pp.139 and 140. The link between the ability of cells to utilise amino acids and the reduction of nitrate to nitrite was examined as follows: Aliquots of chemostat-grown cells were washed and resuspended in half their original volume of 0.1 M Tris.HCl buffer, pH 7.5, to give a dry weight of approximately 0.4 mg ml^{-1} . To 0.4 ml of this cell suspension was added 2 ml of 10 mM amino acid in 0.1 M Tris buffer, pH 7.5 containing 0.2% (w/v) potassium nitrate. The mixtures were incubated at 37°C and 1 ml samples were removed at 30 and 60 min; each was then immediately filtered to remove bacterial cells.

Following the addition of 1.5 ml of distilled water the samples were assayed for the presence of nitrite by the method described previously (Appendix 1, Section 6.9, p.151).

3.4.2. Results

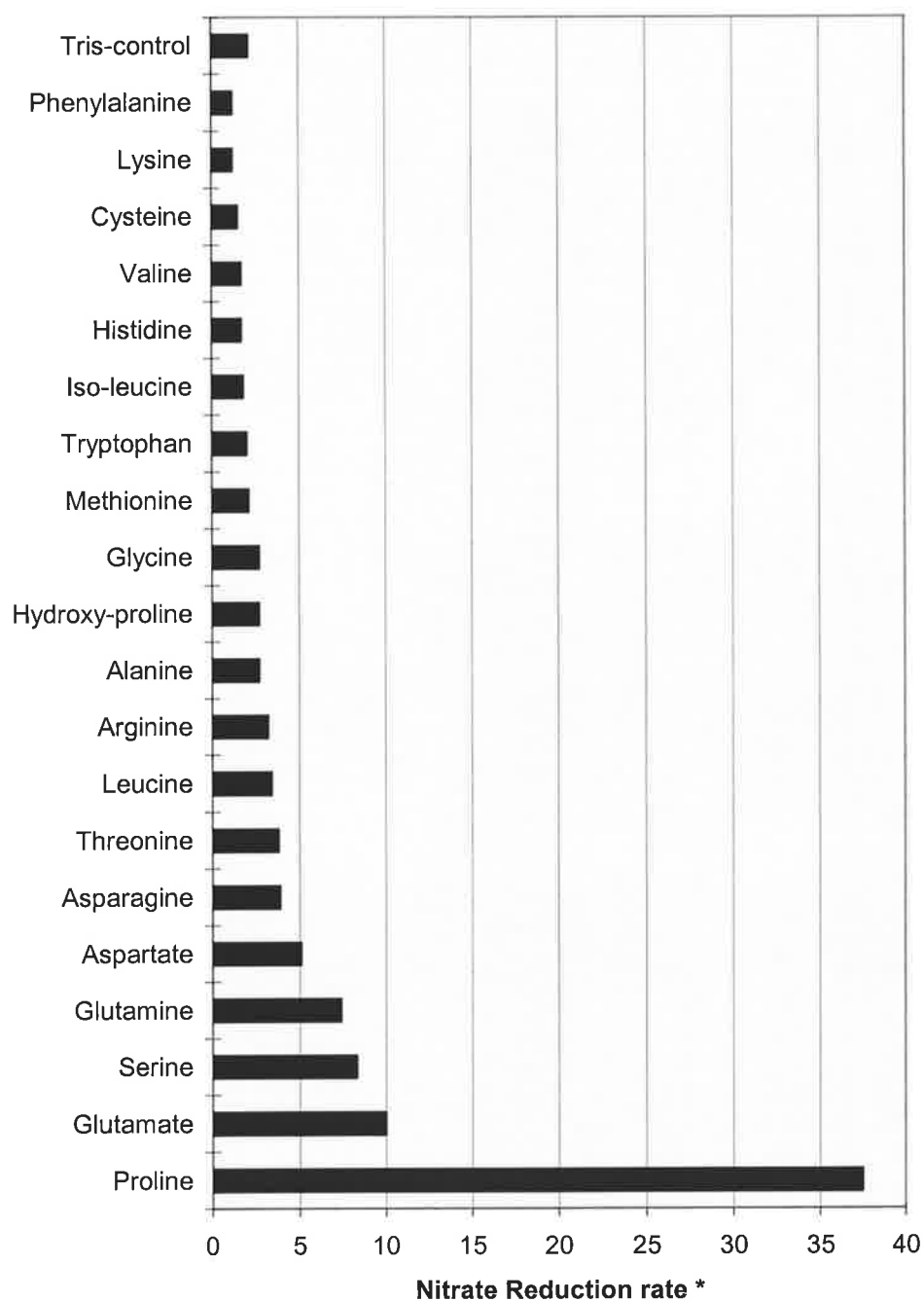
The results of this experiment are expressed in Figure 3-1. It should be noted that the values displayed in this figure represent the means of three separate experiments, reproducible to within 5-10%.

These results again confirmed the link between amino acid catabolism and nitrate reduction in *E. corrodens*. This observation now extends to resting cells of

3. Amino Acid Metabolism and Biomass Production

a clinical isolate. Figure 3-1 shows that the catabolism of proline produced by far the highest rate of nitrate reduction; it was several times higher than that provoked by the addition of glutamate, glutamine, and serine. Most of the other amino acids tested produced rates similar to that of the control.

Figure 3-1. The Effect of Amino Acids on Nitrate Reduction in Resting Cells of *E. corrodens* 33EK(L).



Nitrate reduction rate expressed as $\text{nmol nitrite produced min}^{-1} (\text{mg cell protein})^{-1}$.

Values represent the average result of triplicate samples, none of which differed by more than 10%.

(Actual data shown in Appendix 2, Section 7.10, p.167)

As mentioned previously, derivatisation of proline does not occur under the conditions employed for routine amino acid analysis. Using a modified method for amino acid analysis [100], the utilisation of proline was determined during the development of EDM-1. Reference to Figure 2-3 shows that under these conditions proline was consumed, per gram dry weight of cells, at a relatively high rate compared to that of other amino acids. It should be noted that this medium initially contained 22 mM proline and was modified to 2 mM for studies to determine optimum growth conditions. Because of this, proline, as a possible source of energy for the cell, was not previously recognised.

The finding that glutamine utilisation was associated with nitrate reduction at a significant rate confirms a previous experiment, in which the substitution of glutamine for glutamate in EDM-1 produced no significant difference in biomass (see Section 3.1). This result indicates that the organism is able to produce glutamate via the deamination of glutamine.

As predicted, the addition of serine also resulted in nitrite production at a significant rate. Interestingly, the addition of lysine, under these conditions was not associated with nitrate reduction. Previous data, obtained from the addition of 10 mM lysine to cells in chemostat culture (see Table 3-1), indicated that utilisation of this amino acid may be associated with nitrate reduction.

3.5. Aminopeptidase Activity

Given that *E. corrodens* must derive energy via the catabolism of key amino acids, an understanding of its potential to obtain these amino acids from small peptides, (a biologically relevant energy source), would be valuable. To determine whether the organism possesses aminopeptidase activity, its ability to remove the N-terminal amino acid from a variety of synthetic peptides was assessed.

3.5.1. Material and Methods

Aminopeptidase activity was determined after incubation of cell suspensions with synthetic tri-peptides, with the structure X-gly-gly, as substrates and subsequent measurement of any gly-gly released.

Cell suspensions were prepared as described above (Section 3.4.1) to a dry weight of approximately 0.4 mg ml^{-1} in 0.1 M Tris.HCl buffer pH 7.5. To 0.2 ml of cell suspension, was added 0.5 ml of 7.5 mM peptide and the volume adjusted to 10 ml with Tris.HCl buffer. The mixture was incubated for 1 hour at 37°C.

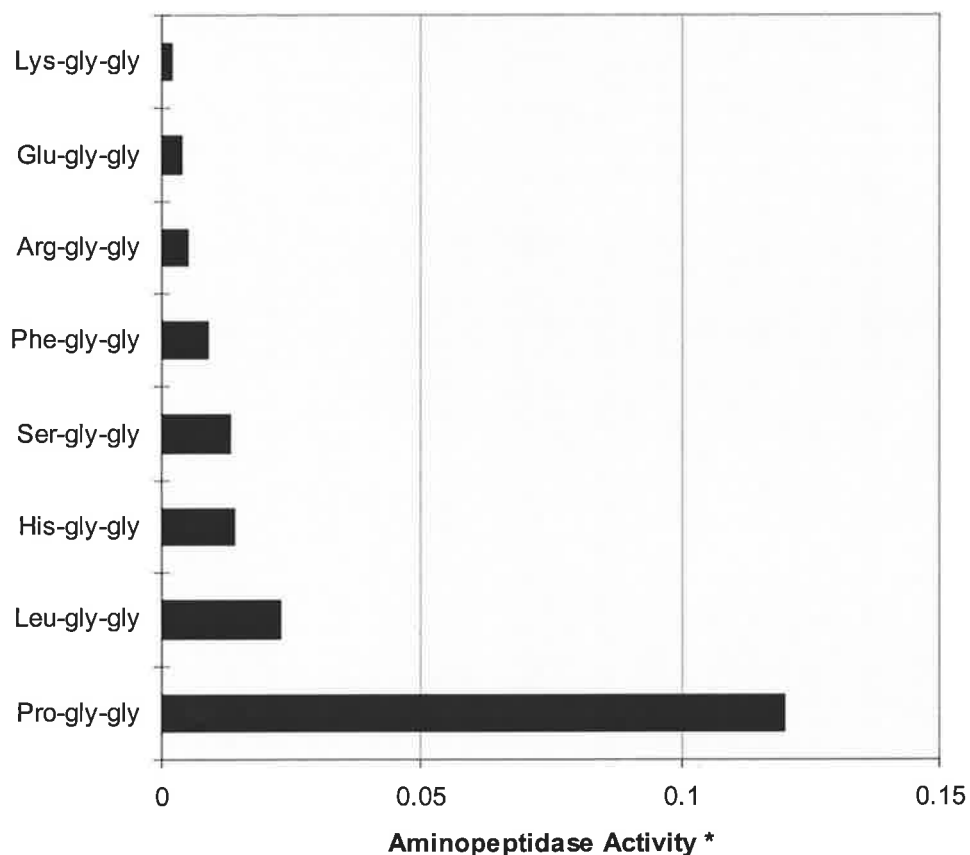
To measure rates of aminopeptidase activity, 1 ml samples were removed at 15 minute intervals and the mixture was immediately filtered to remove cells. A 0.1 ml aliquot of the resultant filtrate was assayed for the presence of gly-gly

employing the OPA RP-HPLC method for amino acid analysis (Appendix 1, Section 6.4, p.143).

3.5.2. Results

The results from this experiment are shown below in Figure 3-2.

Figure 3-2. Aminopeptidase Activity of *E. corrodens* 33EK(L)



* Aminopeptidase activity expressed as $\text{nmol gly-gly released min}^{-1} (\text{mg dry weight cells})^{-1}$.

Values represent the average result of triplicate samples, none of which differed by more than 10%.

(Actual data shown in Appendix 2, Section 7.11, p.168)

It can be seen from Figure 3-2 that the activity against the peptide containing N-terminal proline was, at least, an order of magnitude higher than most of the other peptides. Interestingly, the activity against peptides containing N-terminal glutamate and serine, amino acids whose catabolism result in energy production, was low.

3.6. Utilisation of Peptides and Nitrate Reduction

As the previous experiment demonstrated, the organism has the capacity to release certain amino acid residues from the N-terminus of tri-peptides. The purpose of the following experiment was therefore to determine whether the organism was able to utilise key amino acids, involved in energy production, contained in peptides and, in turn reduce nitrate. It was also designed to establish whether the position in the peptide influenced nitrite production.

3.6.1. Materials and Methods

Peptides were selected for this study on the basis that they contained either proline, glutamate or serine (i.e. amino acids which evoked the highest rates of nitrate reduction; Figure 3-2). Where possible, peptides in which the key amino acid was either N- or C-terminal or an internal residue, were chosen.

Although free histidine did not appear to be involved in nitrate reduction, preliminary experiments, on occasion, produced positive results for this amino

acid. For this reason histidine-containing peptides were also included in the series of peptides tested.

The method employed for this experiment is essentially the same as that used previously for the study on amino acid utilisation and nitrate reduction (Section 3.4.1), except that cells were incubated with a range of peptides at a concentration of 10 mM, instead of free amino acid. The free amino acids proline, glutamate, serine and histidine were tested again.

3.6.2. Results

From the results of this experiment (Figure 3-3) it can be seen that incubation of cells with proline-containing peptides resulted in high rates of nitrate reduction. The cell is able, therefore, to release and catabolise free proline from these peptides. In fact the rate for the dipeptide pro-ala was marginally faster than that for free proline, indicating that, in this case, cleavage of the peptide bond was not the rate-limiting step in the process. In contrast, nitrite production arising from the utilisation of proline in the di-peptide gly-pro occurred at almost twice the rate than that from gly-pro-ala, suggesting that cleavage of the second peptide bond, in this instance, was in fact rate limiting.

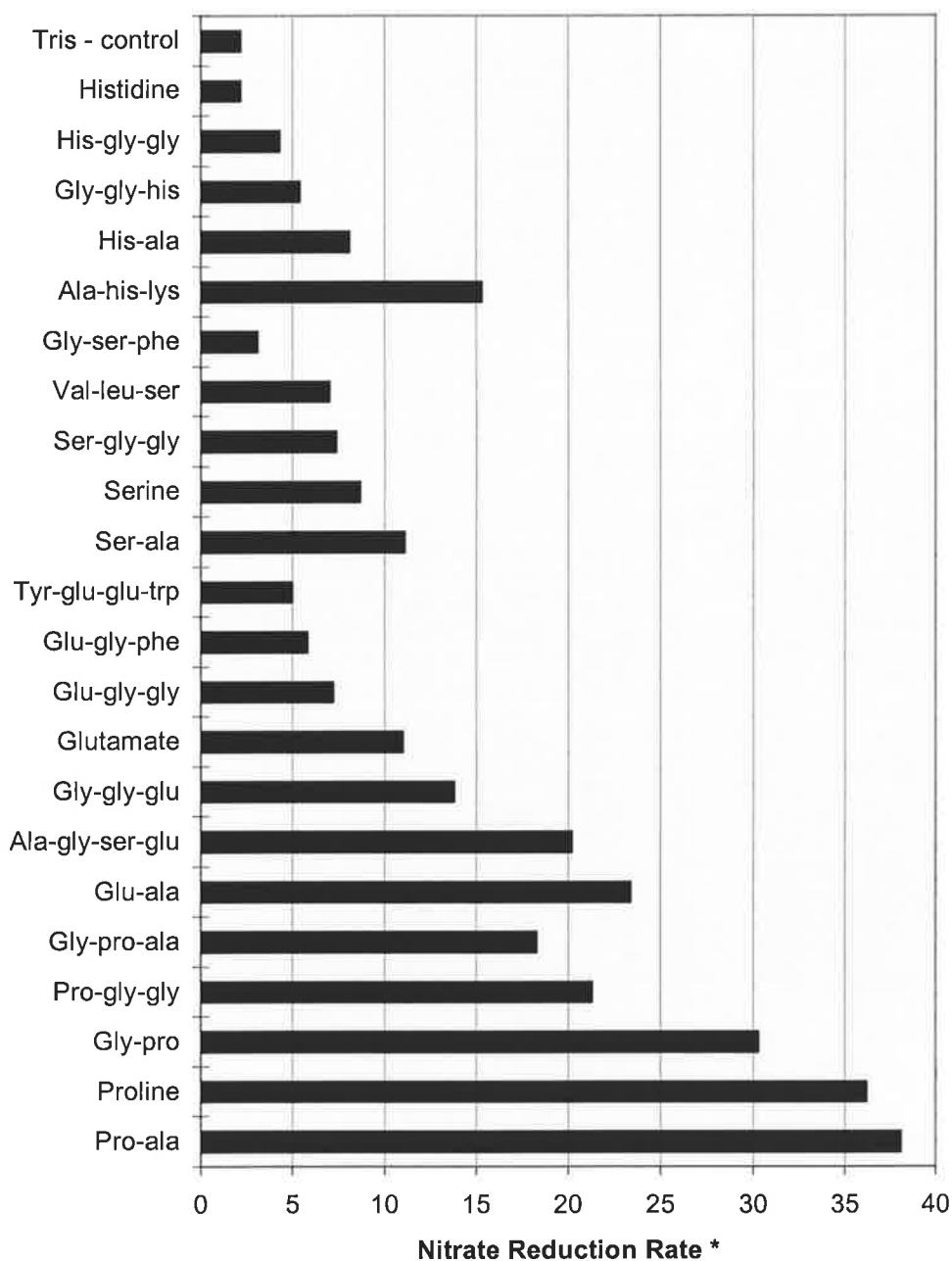
Incubation with both C-terminal glutamate-containing peptides resulted in rates faster than that produced by incubation with free glutamate, whereas N-terminal glutamate-containing peptides produced slower rates than that of free glutamate.

This result for glutamate-containing peptides suggests that the organism may possess carboxypeptidase activity for glutamate and reflects the fact that aminopeptidase activity for residues other than proline is relatively poor (Figure 3-2).

All but one serine-containing peptide produced rates slower than that for free serine. Interestingly the di-peptide ser-ala, in comparison with pro-ala, produced a faster rate than that produced by incubation with the free amino acid, suggesting the organism possesses an efficient uptake system for dipeptides. The rate for the tri-peptide gly-ser-phe, where serine is internal, was slower than those in which this residue was either N- or C- terminal. The relatively slow rates for all serine-containing peptides probably reflects a relatively low removal rate of serine residues from peptides and is most likely accomplished by relatively non-specific peptidases.

Although the addition of free histidine was not linked to nitrite production, the utilisation of histidine-containing peptides resulted in significant rates of nitrate reduction. As with serine, the position of histidine did not seem to influence the rate; in fact the peptide exhibiting the fastest rate was ala-his-lys where histidine was internal.

Figure 3-3. The Effect of Incubation of Peptides with Resting Cells of *E. corrodens* 33EK(L) on Nitrate Reduction



* Rate of nitrate reduction expressed as $\text{nmol nitrite produced min}^{-1} (\text{mg cell protein})^{-1}$.

Values represent the average result of triplicate samples, none of which differed by more than 10%.

(Actual data shown in Appendix 2, Section 7.12, p.169)

Again, this illustrates that peptidase activity is able to liberate, non-specifically, free histidine from peptides and that, once this process occurs, catalysis of the amino acid is involved in nitrate reduction. The reason why incubation with free histidine does not elicit a similar response from the cell is not known but it is possible that the uptake mechanism for histidine in this organism may be deficient. The uptake system for small peptides may allow histidine, in peptide form, to enter the cell and once released catabolism of free histidine occurs.

3.7. Proline Iminopeptidase Activity

Soon after work on the aminopeptidase activity of this organism was commenced, the study by Allaker *et al* [105] on the production of hydrolytic enzymes in *E. corrodens* showed that a number of clinical isolates possessed relatively high levels of proline arylamidase activity. These authors suggested that this activity may reflect the presence of a specific proline iminopeptidase expressed by this organism.

As a previous experiment had shown the presence of aminopeptidase activity relatively specific for proline in this organism, this experiment was designed to confirm the presence of proline arylamidase activity in 33EK(L).

3.7.1. Materials and Methods

Proline arylamidase activity was determined as follows; to 0.2 ml of cell suspension (prepared as described previously; dry weight 0.4 mg ml^{-1}) in 0.1 M Tris.HCl buffer, pH 7.5, was added 0.5 ml of 7.5 mM L-proline- ρ -nitroanilide (pro- ρ -NA) and adjusted to a final volume of 10 ml with Tris.HCl buffer.

The cell suspension was incubated at 37°C and 1 ml samples were removed at 15 minute intervals, over one hour, and filtered to remove cells. The release of free ρ -nitroanilide (ρ -NA) was measured spectrophotometrically at 410 nm. The quantity of free ρ -NA released was calculated by comparing OD_{410} of the sample with values obtained from a standard curve of concentration of ρ -NA versus OD_{410} (see Appendix 2, Figure7-1, p.170).

3.7.2. Results

Proline arylamidase activity, of intact resting cells, as measured by the release of free ρ -NA from pro- ρ -NA, was $16.2 \text{ nmoles } \rho\text{-NA released min}^{-1} (\text{mg dry weight})^{-1}$.

3.7.3. Discussion

It is apparent from previous experiments (3.5 and 3.6) that this strain has the ability to release free proline from peptides where this residue is N-terminal. The activity against the N-terminal proline containing peptide pro-gly-gly was, at least an order of magnitude higher than that of most other substrates. The pattern of these results resembles those published previously [105], where a variety of amino acid ρ -NA substrates were tested against a number of *E. corrodens* isolates. It was found in this study that the level of activity against N-terminal proline was significantly higher ($>2.0 \mu\text{mol}$ substrate hydrolysed $(\text{mg dry weight})^{-1}$) than all other substrates tested ($<0.25 \mu\text{mol}$ substrate hydrolysed $(\text{mg dry weight})^{-1}$).

While the presence of arylamidase activity does not necessarily reflect aminopeptidase activity and *vice versa*, it is apparent that in this case arylamidase activity does, indeed, reflect true aminopeptidase activity when proline is the N-terminal residue in small peptides. Previous experiments in this laboratory with *F. nucleatum* failed to show any detectable activity against a variety of amino acid β -naphthylamide substrates, even though the organism had exhibited aminopeptidase activity against a variety of small synthetic peptides [88].

3.8. The Effect of Growth Medium on Proline Iminopeptidase Activity

To determine whether proline iminopeptidase (PIP) expression was induced by the presence of peptides in the growth medium, enzyme activity was assayed in chemostat-cultured cells from growth in both simple (containing free amino acids only) and complex media containing a mixture of free amino acids and peptides.

3.8.1. Materials and Methods

Strain 33EK(L) was grown in the chemostat at pH 7.2, 34°C and at a dilution rate of 0.1 h⁻¹ in modified EDM-1 containing 2 mM proline. Once the culture had reached steady-state a sample of cells was removed and assayed for PIP activity according to the following procedure; cells were centrifuged at 3,000 g, 4°C for 15 min and the resultant pellet resuspended in sufficient 0.1 M Tris.HCl buffer pH 7.5 to produce a cell suspension with an OD₅₆₀ of 0.3 (dry weight 0.15 mg ml⁻¹). To 3 ml of this cell suspension was added 0.1 ml of a 10 mg ml⁻¹ pro- ρ -NA solution dissolved in Tris.HCl buffer. The rate of ρ -NA production was determined spectrophotometrically by continuous measurement of absorbance at 410 nm for 10 min after the addition of pro- ρ -NA. The change in OD₄₁₀ was linear over this time period and the amount of ρ -NA produced was determined by comparison of OD₄₁₀ to a standard curve for ρ -NA concentration versus OD₄₁₀ (Appendix 2, Figure7-1, p.170).

Cells grown in the chemostat under identical conditions in YT medium were assayed for pro- ρ -NA activity similarly.

3.8.2. Results

The result for cells cultured in a medium containing free amino acids, (EDM-1), - 16.7 nmoles ρ -NA released $\text{min}^{-1} (\text{mg dry weight})^{-1}$ - was not significantly different from that of those grown in the peptide rich conditions of YT, - 15.8 nmoles ρ -NA released $\text{min}^{-1} (\text{mg dry weight})^{-1}$ (see Appendix 2, Figure 7.2, p.171). The figure for cells grown in YT is comparable to that recorded previously for cells grown in BM1 of 16.2 nmoles ρ -NA released $\text{min}^{-1} (\text{mg dry weight})^{-1}$.

As the PIP activity was present, after growth in a peptide-free medium, at a level comparable to that found in cells grown in peptide-containing medium, there is no evidence to indicate that the presence of peptides in growth media results in an induction of PIP activity in cells. It therefore seems that this enzyme is constitutive rather than inducible.

3.9. Effect of Proline on Growth of *E. corrodens* 33EK(L)

As previous experiments indicated that proline catabolism was a potential energy source for the cell, the following experiment was designed to determine whether the biomass of strain 33EK(L) would vary with the amount of this amino acid in a minimal medium; that is, in a pattern similar to that seen previously with 23834^T and glutamate (Section 3.3).

3.9.1. Materials and Methods

Strain 33EK(L) was grown in the chemostat at pH 7.2, 34°C and dilution rate of 0.1 h⁻¹ in each of three minimal media and sampled at steady-state for determination of cellular biomass. The basic medium composition for each was identical except that the amino acid levels in each were different; briefly, EMML contained all amino acids at a concentration of 0.1 mM with the exception of proline, which was 1 mM; EMMM contained all amino acids at a concentration of 1 mM; EMMH contained all amino acids at a concentration of 1 mM with the exception of proline which was at 3 mM. The detailed composition of all media is detailed in Appendix 1, Section 6.11, p.154.

Strains 23834^T and 35EK, a clinical isolate obtained from Dr. R. P. Allaker, were also similarly grown in EMMM to assess the variation in growth yields between strains in this medium. It should be noted that all three strains used in this experiment exhibited a doubling time in EMMM medium comparable to that for strain 23834^T in EDM-1.

3.9.2. Results

The results for biomass production of strain 33EK(L), in each of the three minimal media, are shown in Table 3-4 and a summary of growth of the three strains in EMMM in Table 3-5.

The data in Table 3-4 for strain 33EK(L) shows that steady-state growth of the organism was possible, at a relatively low yield, in medium EMML, which contained proline as the primary energy source. Growth in EMMM, which reflected the effect of a ten-fold increase in all other amino acids to this base medium, resulted in a doubling of biomass. This biomass increase is most likely a result of the catabolism of the additional glutamate and serine present in this medium. It should be noted that growth in this medium also resulted in a proportional increase in nitrite production.

Table 3-4. Growth of *E. corrodens* 33EK(L), in Minimal Media

Medium	Optical Density (560 nm)	Cell Dry Weight. (g L ⁻¹)	Nitrate Utilisation ^d
EMML ^a	0.10*	0.04	7.8
EMMM ^b	0.17	0.08	14.4
EMMH ^c	0.26	0.15	27.7

^a EMML contained all amino acids at a concentration of 0.1 mM, with the exception of proline, which was 1 mM.

^b EMMM contained all amino acids at a concentration of 1 mM;

^c EMMH contained all amino acids at a concentration of 1 mM with the exception of proline which was at 3 mM.

^d Nitrate utilisation expressed as mmol L⁻¹ of nitrite in cell-free filtrate.

* Values represent the average result of triplicate samples, none of which differed by more than 10%.

Table 3-5. Growth of *E. corrodens* strains in EMMM.

Strain	Optical Density (560 nm)	Cell Dry Weight. (g L ⁻¹)	Nitrate Utilisation ^a
33EK(L)	0.17*	0.08	14.4
35EK	0.22	0.10	15.4
23834 ^T	0.17	0.07	13.6

^a Nitrate utilisation expressed as mmols L⁻¹ of nitrite in cell-free filtrate.

* Values represent the average result of triplicate samples, none of which differed by more than 10%.

A three-fold increase in the proline concentration to 3 mM, (in EMMH), resulted in further proportional biomass and nitrite production increases. These results illustrate that, as indicated by previous experiments using stationary cells, proline is a primary energy source for this strain and that the catabolism of this amino acid is associated with the reduction of nitrate.

Steady-state growth of strains 35EK and 23834^T was also sustainable using EMMM as a growth medium and resulted in comparable biomass yields and nitrite production values to those for 33EK(L). These results indicate that the utilisation of proline is likely to be important in energy production in many, if not all, strains of *E. corrodens*.

4. Partial Purification and Characterisation of *E. corrodens* Proline Iminopeptidase

4.1. Partial Purification

E. corrodens has been shown to express a proline iminopeptidase (PIP), an enzyme that cleaves amino-terminal prolyl residues from di- and oligo-peptides. It is also evident that the organism is able to derive energy via the catabolism of proline, a process which results in the reduction of nitrate to nitrite. It is likely, therefore, that PIP expression plays an important nutritional role for this organism *in vivo*.

A method for partial purification was developed to characterise this enzyme and compare its properties to proline iminopeptidases produced by other microorganisms.

4.1.1. Cell Production

E. corrodens strain 33EK(L) was grown in the chemostat at pH 7.2, 34°C and at a dilution rate of 0.1 h⁻¹. The growth medium used was BM1 (see Appendix 1, Section 7.9, p.153) and, after steady-state conditions were reached, cells were collected continuously, on ice, and harvested by centrifugation at 3,000 g for 20 min at 4°C.

The resultant cell pellets were resuspended, in one tenth the original volume, in distilled water, checked for purity microscopically by Gram stain, then

pooled and stored at -20°C. Stored cells after thawing, were disrupted by successive passages through a French pressure cell. The resultant suspension was treated with deoxyribonuclease 1 (from Bovine pancreas) and ribonuclease (from *Aspergillus clavatus*) and stirred for 60 min at 4°C to hydrolyse nucleic acid.

This solution was centrifuged for 45 min (3,000 g, 4°C) to remove remaining intact cells and insoluble debris and a small aliquot of supernatant was assayed for PIP activity. The remainder of the supernatant, designated PPF, was retained for ammonium sulphate fractionation and enzyme localisation studies.

4.1.2. Determination of PIP Activity

The amount of enzyme activity was determined at each stage of purification. As described previously, PIP activity was assayed spectrophotometrically by measuring free ρ -nitroaniline (ρ -NA) released from the synthetic substrate L-proline- ρ -nitroanilide (pro- ρ -NA). 0.1 ml of sample was added to 0.8 ml of Tris buffer (0.1 M, pH 7.5) followed by the addition of 0.1 ml of pro- ρ -NA (10 mg ml⁻¹ in 0.1 M Tris buffer pH 7.5). The change in absorbance at 410 nm of this solution was recorded spectrophotometrically, the rate of change in OD_{410nm} providing a measure of enzyme activity.

4.1.3. Protein Determination

The protein content was determined at each stage of purification by the Pierce BCA method using bovine serum albumin as a standard. (Appendix 1, Section 6.3.3, p.140).

4.1.4. Ammonium Sulphate Fractionation

Thawed supernatant (PPF) (30 ml) was fractionated by ammonium sulphate precipitation from 20 to 60% saturation in three equivalent steps. Initially, ammonium sulphate was added to 20% saturation (0.126 g ml^{-1}) and the solution was stirred for 60 min at 4°C . The solution was then centrifuged at 6,000 g for 30 min and the recovered pellet dissolved in distilled water. Aliquots of both supernatant and dissolved pellet were retained for determination of PIP activity. This procedure was repeated for both 40% and 60% levels of ammonium sulphate saturation.

The dissolved precipitate containing the majority of enzyme activity was desalted against distilled water using a 30 kDa cut-off Centriprep concentrator (Amicon Inc, Beverly, MA, USA), following which an aliquot of this material, (designated PPA), was stored at -60°C for subsequent molecular weight estimation and enzyme kinetic studies. The remainder was then prepared for further purification by hydrophobic interaction chromatography (HIC).

4.1.5. Hydrophobic Interaction Chromatography

An Econo-Pac Methyl HIC cartridge, bed volume 5 ml (Bio-Rad, Bio-Rad Laboratories, CA, USA) was equilibrated with 1.5 M ammonium sulphate in 0.1 M sodium phosphate buffer, pH 6.8 (Buffer A). The material obtained following ammonium sulphate precipitation (PPA) was equilibrated with Buffer A using a Centriprep concentrator and 1 ml aliquots of this sample were subsequently applied to the column. After sample loading, the column was eluted with a linear gradient of decreasing ammonium sulphate, from 1.5 M to 0 M in 0.1 M sodium phosphate buffer, pH 6.8 over 60 min at a flow rate of 1 ml min⁻¹.

Fractions (1 ml) were collected, commencing at the time of sample loading, and 50 µl aliquots were removed from each for detection of PIP activity. Identification of active fractions was achieved by incubation of 50 µl fraction aliquots with 0.1ml of pro-p-NA (10mg ml⁻¹ in 0.1 M Tris buffer pH 7.5) in a micro-titre tray well. The fractions exhibiting the strongest colour change after incubation at 37°C for 15 minutes were pooled, then desalted and concentrated in a Centriprep concentrator. The resultant material from this purification stage was designated PPH.

4.1.6. Ion Exchange Chromatography

An Econo-Pac Q ion exchange cartridge (Bio-Rad, Bio-Rad Laboratories, CA, USA), bed volume 5 ml, was equilibrated with 25 mM Tris, pH 8 (Buffer A).

The active fraction following HIC, (PPH), was also equilibrated against Buffer A before application of 1 ml amounts to the column. Elution of protein from the column, which commenced after the passage of 10 ml of Buffer A through the column at a flow rate of 1 ml min⁻¹, was achieved by the application of a linear gradient to a final concentration 1 M NaCl (in 25 mM Tris, pH 8) at a constant flow rate of 1 ml min⁻¹.

Fractions (1 ml) were collected and tested for activity against pro- ρ -NA using the same method as that employed above for hydrophobic interaction chromatography. Those fractions exhibiting the highest activity were pooled, desalted and concentrated, and stored at -60°C. This active fraction was designated PPI and was used for experiments on the effect of protease inhibitors and metal ions on its activity.

4.1.7. Results

PIP activity was found in both 40% and 60% pellets, with the majority of the activity (56%) residing in the latter fraction. The 40% pellet (which contained 44% of the total PIP activity) was not used further in this study because, upon addition of distilled water, much of the material remained insoluble, in contrast to the 60% pellet which dissolved readily in distilled water.

Fractions 38 - 46 contained enzyme activity following HIC. Only those fractions containing the highest activity (40 - 44) were retained for further

4. Partial Purification and Characterisation of *E. corrodens* PIP

purification. Following IEC, fractions 27 - 33 contained enzyme activity. Only those fractions containing the highest activity (29 - 31) were pooled, desalted and concentrated to a final volume of 1 ml. This solution was designated PPI and retained for further enzyme characterisation studies.

A summary of the results of the purification method are shown in Table 4-1.

Table 4-1. Summary of the purification of *E. corrodens* PIP.

Purification Stage	Volume (ml)	Protein (mg)	Total activity*	Specific Activity ⁺	Purification Factor (fold)
None	30	72	1,078	14.97	1
(NH ₄) ₂ SO ₄	5	16	561	35	2.34
HIC	2	3.7	341	92	6.14
IEC	1	0.8	218	273	18.24

* Activity expressed as nmoles p-NA released min⁻¹

⁺ Specific activity expressed as nmoles p-NA released min⁻¹ (mg protein)⁻¹

4.2. The Effect of Enzyme Inhibitors and Metal Ions on PIP Activity

In an attempt to characterise the enzyme, the effect of various enzyme inhibitors and metal ions on PIP activity was determined.

4.2.1. Material and Methods

Reaction mixtures containing 25 μ l of partially purified enzyme (PPI) were pre-incubated at 37°C with 25 μ l of inhibitor or metal ion solution and 0.85 ml Tris.HCl buffer (0.1 M, pH 7.5) for 10 min prior to the addition of 0.1ml of pro- ρ -NA (10mg ml⁻¹ in 0.1 M Tris buffer pH 7.5). The reaction was allowed to proceed for 30 minutes, at which time the sample was transferred to a 1 cm quartz cuvette and the OD₄₁₀ recorded spectrophotometrically.

4.2.2. Results

The rate of reaction was determined by measuring the Δ OD₄₁₀ for each reaction mixture and the effect on enzyme activity was expressed as a percentage of the control mixture which contained no inhibitors or metal ions. This data is presented in Table 4-2.

4. Partial Purification and Characterisation of *E. corrodens* PIP

Table 4-2. The Effect of Various Inhibitors and Metal Ions
on *E. corrodens* PIP Activity.

Inhibitor	Concentration (mM)	Relative Activity (%) ^a
Control	-	100*
Ethylene diamine tetraacetate	5	99
Dithiothreitol	5	93
Cysteine	5	95
N-Ethylmaleimide	5	71
Benzamidine	5	99
Leupeptin	1	98
Phenylmethylsulphonyl fluoride	1	68
p-Chloromercuribenzoate	10	16
Bestatin	1	89
Metal ion	Concentration (mM)	Relative Activity (%)
Ca ²⁺	5	111
Co ²⁺	5	39
Co ²⁺	1	77
Co ²⁺	0.5	86
Mg ²⁺	5	88
Mn ²⁺	5	96
Zn ²⁺	5	0
Zn ²⁺	1	6
Zn ²⁺	0.5	20
Cu ²⁺	5	19
Cu ²⁺	1	101
Cu ²⁺	0.5	94
Fe ²⁺	5	77
Fe ²⁺	1	112
Fe ²⁺	0.5	99
Hg ²⁺	1	10

^a Expressed as a percentage of the ΔOD_{410} of the control mixture which contained no inhibitors or metal ions.

* Values represent the average result of triplicate samples, none of which differed by more than 10%.



Enzyme activity was unaffected by the reducing agents cysteine and dithiothreitol, the metallo-aminopeptidase inhibitor ethylene diamine tetraacetate and the specific amino-peptidase inhibitors bestatin and leupeptin. Moderate inhibition was observed with the serine protease inhibitor phenylmethylsulphonyl fluoride (PMSF) and thiol protease inhibitors N-ethyl maleimide (NEM) and significant inhibition with the thiol protease inhibitor ρ -chloromercuribenzoate (PCMB). Of the metal ions tested only Zn^{2+} and Hg^{2+} were found to strongly inhibit enzyme activity.

Members of the proline iminopeptidase family, are classified as serine proteases [106], i.e. possessing a functional serine residue at the catalytic site. However, enzyme activity was only moderately inhibited by the presence of the serine protease inhibitor PMSF. It appears that the enzyme requires active thiol groups for activity as observed by the moderate effect of NEM and the strong inhibition in the presence of the thiol inhibitor PCMB.

These results concur with those of Allaker *et al* [107], who tested the PIP activity of whole cell suspensions of *E. corrodens* against a similar range of inhibitors and found that the enzyme was relatively insensitive to PMSF but inhibited by PCMB. The finding that activity is dependent on active thiol groups has also been reported for other bacterial enzymes in this family [108, 109].

It appears that, while the *E. corrodens* PIP may be a serine protease, it is relatively unaffected by the presence of the serine protease inhibitor PMSF thus

suggesting structural blocking of the inactivation of the active serine residue by this compound.

4.3. Molecular Weight Estimation

In order to obtain an estimate of the molecular weight of the native enzyme, a quantity of partially purified enzyme was applied to a size exclusion chromatography (SEC) column under non-denaturing conditions.

4.3.1. Materials and Methods

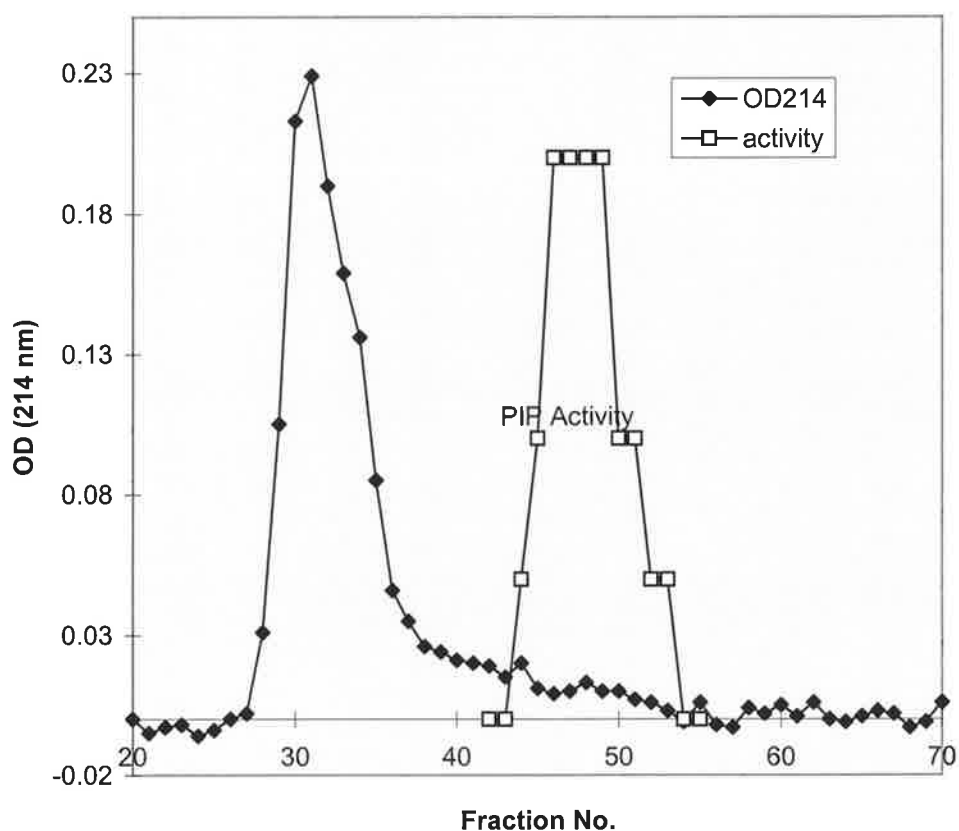
A sample containing 2 mg protein of partially purified enzyme (PPA) was equilibrated with 0.1 M sodium phosphate buffer, pH 7.2 and the volume adjusted to 1 ml. This solution was loaded onto a glass chromatography column packed with Sephadex G-150 (Pharmacia Fine Chemicals AB, Uppsala, Sweden) and eluted with sodium phosphate buffer (0.1M pH 7.2) at 4°C.

Eluted fractions (1 ml) were collected and the OD₂₁₄ recorded for each as well as assaying for PIP activity using the microtitre tray method described in Section 4.1.5.

4.3.2. Results

A plot of the results of SEC is shown in Figure 4-1.

Figure 4-1. Molecular Weight Estimation of Native *E. corrodens* PIP using Size Exclusion Chromatography



Following elution, fractions 44-53 demonstrated activity against pro- ρ -NA, with fractions 46-49 exhibiting the strongest activity. Molecular weight of the native enzyme was estimated to be approximately 35 kDa after reference to a plot

of the molecular mass of protein standards against elution volume (see Appendix2, Section1.17, p.175).

4.4. Enzyme Location

To establish whether PIP is either a membrane associated or cytoplasmic enzyme the following experiment, in which disrupted cells were centrifugally fractionated to separate cytoplasmic contents from cell membrane fragments, was performed.

4.4.1. Materials and Methods

A 20 ml aliquot of PPF, which represented disrupted cell contents (see Section 4.1.1) was centrifuged at 100,000 g for 60 min at 4°C (Model L8-80 Beckman ultracentrifuge, Beckman Instruments, CA, USA). Following centrifugation the supernatant was retained and the pellet was resuspended in its original volume of Tris.HCl buffer (0.1 M, pH 7.5). Both supernatant and pellet fractions were subsequently assayed for PIP activity (see Section 4.1.2 for method).

4.4.2. Results

The supernatant fraction, which reflects cytoplasmic contents, retained 98.6% of the original sample activity ($\Delta OD_{410} = 0.048 \text{ AU min}^{-1}$) in contrast to the pellet fraction which contained no detectable PIP activity. Thus, it appears that the enzyme is not membrane associated and is expressed cytoplasmically.

4.5. Extracellular Expression of PIP

To determine whether significant amounts of the enzyme are expressed extracellularly it was decided to assay both chemostat grown cells and culture supernatant for PIP activity.

4.5.1. Materials and Methods

Samples from chemostat cells grown to steady-state in YT medium (34°C, pH 7.2, $D = 0.1 \text{ h}^{-1}$) were centrifuged (3,000 g, 30 min, 4°C) and the culture supernatant retained for analysis. The resultant cell pellet was washed in distilled water and resuspended in the original volume of Tris.HCl buffer (0.1 M, pH 7.5).

Levels of PIP activity in both the cell suspension and culture supernatant were calculated by the addition of 0.2 ml of sample to 0.5 ml of pro- ρ -NA (0.25% in 0.1 M Tris.HCl buffer, pH 7.5) and the final volume adjusted to 3 ml with the

addition of Tris buffer (0.1 M, pH 7.5). The OD₄₁₀ of each was recorded prior to, and immediately following, incubation for 60 min at 37°C. As a negative control, 0.2 ml of distilled water was used instead of either cells or supernatant sample.

4.5.2. Results

The majority of PIP activity, in cultures grown under these conditions, is cell-associated. After 60 minutes incubation with the synthetic substrate pro- ρ -NA, the supernatant sample generated Δ OD₄₁₀ of 0.073 AU compared with a Δ OD₄₁₀ of 0.835 AU for an equivalent amount of cells (for data, see Appendix 2, Section 7.16, p.173). Under these growth conditions, *E. corrodens* does not secrete significant amounts of PIP into the extracellular environment. It is likely that the organism does not actively export the enzyme at all and that levels of extracellular enzyme activity found in this study may be attributable to cell lysis.

4.6. Enzyme Kinetics

4.6.1. Materials and Methods

The Michaelis-Menten kinetic constants K_m and V_{max} for the enzyme were determined using the following method. The reaction mixture (1 ml) consisted of a fixed amount of enzyme (0.1 ml of PPA, protein concentration 3 mg ml⁻¹) and varying amounts of pro- ρ -NA (in 0.1 M Tris pH 7.5) to produce final substrate

concentrations between 0.07 and 5 mM. The rate of reaction (ΔOD_{410}) was measured over a 30 second period following the addition of pro- ρ -NA.

The reaction temperature was 25°C and absorbance (410 nm) of the reaction mixture was recorded continuously immediately following addition of pro- ρ -NA. The initial velocities (average results of triplicate experiments) from nine substrate concentrations were obtained to determine K_m and V_{max} . The quantity of ρ -NA produced during the reaction was determined using the standard curve of OD_{410} versus known concentrations of ρ -NA (Appendix 2, Section 7.13, p.170).

4.6.2. Results and Discussion

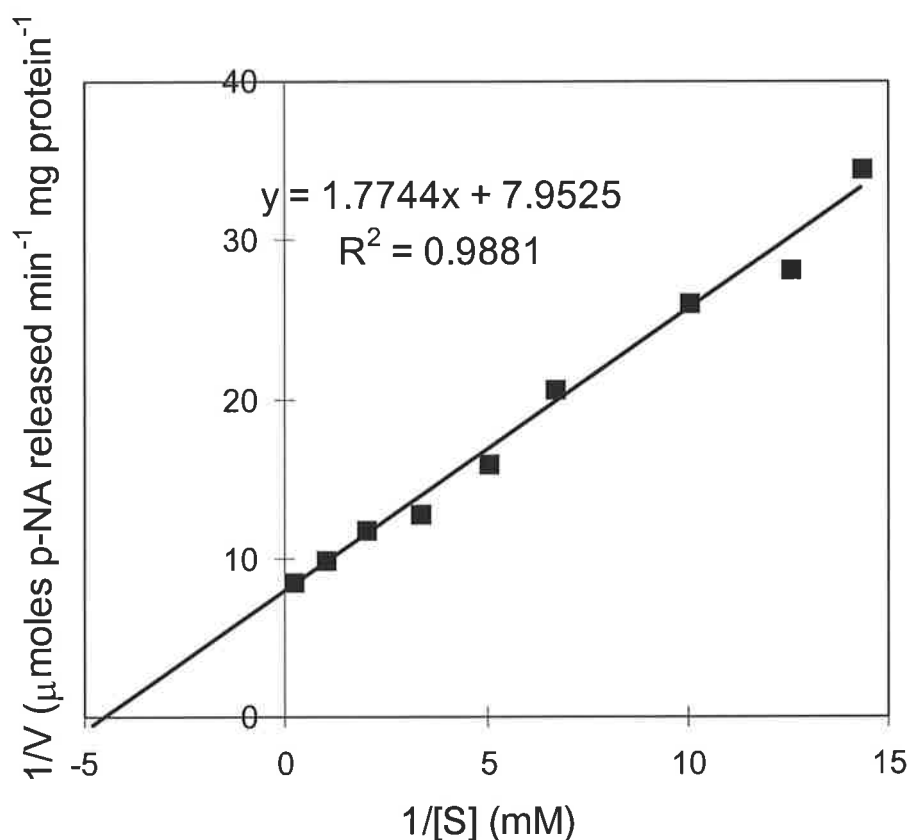
The data obtained from this experiment (see Appendix 2, Section 7.15, p.172) enabled construction of a Lineweaver-Burke plot, Figure 4-2, from which the values of 0.223 mM and $0.126 \mu\text{moles min}^{-1} (\text{mg protein})^{-1}$, for K_m and V_{max} , respectively, were derived. It should be noted that for all concentrations of pro- ρ -NA, the substrate concentration change during the period of initial velocity calculation was less than 10%.

The K_m value calculated for *E. corrodens* PIP of 0.223 mM is comparable with that for PIPs isolated from *L. helveticus* SBT 2171 (0.6 mM) [110] and *L. bulgaricus* CNRZ 397 (0.93 mM) [111]. The V_{max} calculated in this study is an underestimate as the enzyme preparation used for this experiment was only

4. Partial Purification and Characterisation of *E. corrodens* PIP

partially purified. However, it should be noted that the maximum velocity for the reaction was not reached at substrate concentrations as high as 5 mM. This illustrates that the enzyme, while not possessing a particularly high affinity for small peptides containing N-terminal proline, is capable of rapidly releasing free proline from these peptides at relatively high physiological concentrations.

Figure 4-2. Lineweaver-Burke Plot for *E. corrodens* PIP Activity Against the Substrate Proline- ρ -Nitroanilide.



(see Appendix 2, Section 7.15, p.172 for raw data)

5. Discussion

A simple chemically defined medium (EDM-1), capable of supporting growth of the organism in continuous culture, was developed. This medium was suitable for studying of the physical growth parameters of *E. corrodens* and, with modification, was employed to reveal more about the role of the metabolism of key amino acids in energy production.

Batch growth of the ATCC Type strain 23834 in EDM-1 medium resulted in doubling times and biomass values comparable to those obtained by Kuedell *et al* [95] for this strain, following growth in a synthetic medium of similar composition. In contrast, growth in this simple, chemically defined medium resulted in a significantly shorter generation time and a biomass of more than double that for growth in a more complex, yeast extract-based medium [94]. These observations indicate that the yeast extract-based medium is not as energetically rich, as evidenced by the lower biomass yield and that *E. corrodens* is able to derive energy at a faster rate from EDM-1, as cells were able to divide at a faster rate.

As EDM-1 supported continuous culture of *E. corrodens* it was used to investigate the effect of the physical environment and specifically modified to study the organism's physiology.

5.1. Physical Growth Parameters

Microorganisms cannot generally tolerate extreme pH values, since under highly alkaline or acidic conditions some microbial cell components may be hydrolysed or enzymes may be denatured. Even exposure to less extreme conditions can effect cell growth because pH affects the dissociation of protein functional groups; in order to catalyse reactions, enzymes must be in a particular state of dissociation. Therefore, certain pH values will be optimal for activities of specific enzymes.

Thus, the pH of an environment affects microorganisms and microbial enzymes directly and may also influence the dissociation and availability of required compounds (eg. ammonium and phosphate), nutrients that influence the growth rates of microorganisms in ecosystems.

Studies on the effect of environmental pH on biomass showed that the optimum for *E. corrodens* was about pH 7.2 and that chemostat culture of the organism resulted in observable, steady-state growth between the pH values 6.7 and 8.5. This finding of a bias in tolerance towards the alkaline region of the pH spectrum is likely related to the organism's reliance on consumption of amino acids and the excretion of resultant ammonium ions into the extracellular environment. As well as providing an energy source, a large percentage of the acidic end-products of amino acid catabolism is probably intracellularly recycled, providing carbon skeletons for anabolic pathways; a view supported by the

observation of minimal amounts of acidic end-products appearing in culture filtrates.

It is interesting to note that tolerance to relatively high physiological pH values, pH optima above neutrality and sensitivity to environmental pH below 7.0 are physiological traits also shared by both *F. nucleatum* [86, 112] and *P. gingivalis* [113, 112], organisms frequently implicated in the aetiology of periodontal diseases and which also appear to derive energy via the degradation of amino acids.

The relatively sharp drop in growth yields under conditions below pH 7.0 indicate the organism is not well suited to growth in a microbial community actively producing relatively large amounts of acidic end-products from carbohydrate. In contrast to amino acid metabolism, there is no concomitant production of basic compounds to prevent pH fall during carbohydrate metabolism.

As discussed previously, an apparent shift in the microbial composition of dental plaque occurs in the transition from periodontal health to disease. The predominantly Gram positive plaque, comprised of species capable of utilising carbohydrate and in turn producing acidic end-products, at “healthy” sites is transformed, at diseased sites, into a Gram negative-dominated community, consisting of a high proportion of asaccharolytic species relying on the metabolism of amino acids for survival.

It would appear, therefore, that growth of *E. corrodens* would be favoured in a microbial community consisting of organisms metabolising amino acids (peptide derived) as their primary energy source rather than one which predominantly utilises carbohydrate.

Growth pH, although affecting some quantitative differences, did not significantly change the amino acid utilisation pattern of the organism; and at all growth pH values tested, of the amino acids analysed, glutamate, serine and lysine were those consumed in greatest amounts. It should be noted that consumption of the key energy yielding amino acids, glutamate and serine, was most efficient at pH 7.0 and a shift in pH away from this figure resulted in a reduction in biomass which represented that portion of energy diverted into cell maintenance. An increased amount of available energy may be required for maintenance of intracellular pH at non-optimal growth pH and provides an explanation for the observed lower biomass at these pH values. How the cell manages to cope with the chemiosmotically unfavourable ΔH^+ balance at alkaline pH is not known. However, many alkaliphilic bacteria use Na^+/H^+ antiporters in pH regulation and are able to maintain a cytoplasmic pH two or more units below that of their environment by using an electrochemical Na^+ gradient rather than protons to energise solute uptake. Interestingly, ATP production may occur via proton-coupled oxidative phosphorylation in an alkaline environment in the presence of an adverse pH gradient. [114].

A study of the effect of growth pH on the formation of acidic end-products showed that, at all values, only acetate and formate were detected. It appears that the production of these acids is the result of serine catabolism and at elevated growth pH values the amounts of these two metabolites appearing in culture filtrates decreased.

An investigation into the effect of culture temperature on growth of *E. corrodens* showed that, of the three temperatures tested, all biomass parameters were maximal at 34°C. An increase of 2°C in culture temperature from this value resulted in a 25% decrease in dry weight and a similar decline in cell protein levels, indicating that the optimal growth temperature for the organism is closer to 34°C than 36°C.

This finding is interesting as other studies have shown that sub-gingival temperatures, at healthy sites are often approximately 2°C lower than sub-lingual temperatures [115, 116]. Haffajee *et al*, in a study involving 44 individuals, found that the mean of each patient's whole mouth sub-gingival temperature was 1.9°C lower ($34.8 \pm 0.6^\circ\text{C}$) than the mean of sub-lingual temperatures ($36.6 \pm 0.4^\circ\text{C}$) [117] and that sub-gingival temperatures increased only slightly in areas exhibiting inflammation. Perdok *et al* [118] found that the mean maxillary and mandibular sub-gingival temperatures were 34.1 ± 0.6 and $34.7 \pm 0.4^\circ\text{C}$, respectively, and increased by less than 1°C after 14 days in the absence of oral hygiene. Thus, both healthy and diseased sub-gingival environments exhibit temperatures at which growth of the organism is near maximal.

The effect of temperature on growth of microorganisms, although long-established as important, is often overlooked in physiological studies involving pathogens, as cultures are routinely grown at 37°C. In this case it would appear that growth of *E. corrodens*, at this temperature is not only sub-optimal but, more importantly, does not accurately reflect the *in vivo* environment.

Following growth under a variety of atmospheric conditions it is apparent that the organism does not have a requirement for atmospheric CO₂. These results agree with those of Progulske and Holt [94] who found that the organism did not have a requirement for atmospheric CO₂ or incorporate CO₂ into cellular material. Their data indicate that the yield for static growth in air (dry weight = 0.196 g L⁻¹) was only of the order of 10% lower than that for 10% CO₂-enriched air (dry weight = 0.224 g L⁻¹). Quantitative differences between our study and that of Progulske and Holt may be due to differences in both culture method and medium composition.

A previous study reported a CO₂ requirement for initial isolation of *E. corrodens* strains [92]. The higher cell yields observed may have been the result of increased buffering capacity of these cultures due to formation of carbonic acid from dissolved CO₂. The dissociation of carbonic acid to bicarbonate would help balance the pH increase resulting from amino acid catabolism during growth of the organism, thus helping to maintain the culture pH at a value closer to the optimum. Alternatively, it is possible that upon initial isolation, the organism does, in fact, require CO₂ for growth but this requirement is lost following

repeated sub-culturing as observed by Socransky (personal communication to Progulske and Holt; quoted in [94]). This phenomenon, of a diminishing requirement for CO₂ upon sub-culture, has been reported to occur in *Haemophilus* [119] and also in some species of *Neisseria* and *Brucella* [120].

Growth of the organism did not appear to be adversely affected by the presence of oxygen, as cell yields from cultures grown in a relatively aerobic environment were not significantly different from those grown anaerobically in either the presence or absence of CO₂. Recent studies in this laboratory [121] investigating oxygen metabolism by *F. nucleatum*, suggest that the Eh of sterile medium, in an aerobic atmosphere, is approximately +60 mV. This value is similar to those obtained for healthy gingival sulcus by Leke *et al* [122] and Kenny and Ash [123]. *E. corrodens*, although historically referred to as an anaerobe, is tolerant of a much wider range in environmental Eh than either *F. nucleatum* or *P. gingivalis* [112, 122].

5.2. Nutrient Requirements

Attempts to grow the organism in a chemically defined medium lacking nitrate were unsuccessful thus confirming the findings of Progulske and Holt [94] that nitrate is essential for growth of *E. corrodens*. In a study of 595 strains identified as *E. corrodens* all but two shared this property, indicating that nitrate reduction is a trait strongly conserved in this organism and that the two exceptions were misclassified isolates. Also confirmed was the organism's ability to reduce

nitrate to nitrite [6], a metabolite detected in culture filtrates at concentrations which increased proportionally with cell yield.

It appears that cell growth occurs via a metabolism involving dissimilatory nitrate reduction, with nitrate serving as the terminal electron acceptor and the resultant nitrite ions transported into the extracellular environment. This study has shown that these electrons originate from key amino acids, the oxidation of which supplies the cell with a source of both energy and carbon. The finding that cellular biomass from cultures grown in the presence of air were comparable to those grown anaerobically indicate that this form of “anaerobic respiration” does not appear to be significantly affected by the presence of oxygen.

Dissimilatory nitrate reduction involves membrane bound nitrate reductases, multi-subunit enzymes which have been purified from a number of nitrate-respiring bacteria [124]. The nitrate reductase complex uses quinones (redox carriers for electron transport with nitrate as the acceptor), as the physiological electron donor and generate a proton motive force by a redox loop mechanism [125, 126]. This mechanism has been demonstrated for both the formate-nitrate reductase complex of *E. coli* [127] and the NADH-nitrate reductase complex of *K. aerogenes* [128]. A membrane bound subunit oxidises quinones at the cytoplasmic side of the membrane, releasing two protons into the periplasm. Electrons are passed to another cytoplasmic subunit to reduce nitrate with the consumption of two cytoplasmic protons and the resultant nitrite is transported into the periplasm.

The availability of nitrate in the human oral cavity varies greatly [129] and much is converted to nitrite by oral microorganisms before ingestion. The diet supplies the majority of nitrate (approximately 100 mg/day to 1000 mg/day). Leafy green vegetables typically contain high nitrate levels [130] while it appears that a smaller portion is supplied endogenously (approximately 50 mg/day). Although circulating plasma levels of nitrate are of the order of 0.04 mM, it is concentrated in the salivary glands, resulting in salivary concentrations an order of magnitude higher than this [131]. Significant proportional increases in plasma and salivary levels have been observed following the ingestion of nitrate [131] and levels in saliva have been reported to have increased by as much as 60 fold following the consumption of vegetable-rich meals [130]. It has also been demonstrated that salivary nitrate levels may also be influenced by factors other than diet; such as salivary flow rate [132].

It appears, therefore, that the concentration of nitrate in saliva available to oral microbes is variable and may range between approximately 0.5mM and 30 mM. As this nutrient was found essential for growth of the organism, it is likely that while *E. corrodens* may, at times, be nitrate limited *in vivo*, the amount of nitrate found in the oral environment is comparable to that used in this study.

While it is possible that other compounds may serve as terminal electron acceptors *in vivo*, the standard free energy (ΔG°) for the reduction of nitrate to nitrite is -161.2 kJ/2e⁻, a parameter directly proportional to the amount of ATP generated per pair of electrons donated. Thus, the reduction of nitrate with almost

all electron donors is sufficiently exergonic to allow for the formation of 2 ATP per mol nitrate [133]. Fumarate may be used by a variety of organisms as a terminal electron acceptor during anaerobic respiration [134, 135], but, the ΔG° for the reduction of fumarate to succinate by fumarate reductase is -86.2 kJ/2e^- , approximately one half that for nitrate reduction. *E. corrodens* failed to grow following substitution of nitrate with fumarate in the chemically defined medium (unpublished data).

In order to survive, *E. corrodens* must successfully compete for nitrate with other nitrate reductase-positive microbes that colonise the oral cavity. As discussed previously, the supply of nitrate to oral organisms may fluctuate dramatically depending on levels of dietary intake which, in turn, influence both plasma and salivary concentrations. For these reasons it is likely that growth of the organism, in an oral environment containing an adequate supply of amino acids, may be nitrate limited.

The inhibitory effect of nitrite on bacterial cells is usually associated with its conversion to nitric oxide (NO); either by macrophages or in the acidic environment of the stomach. NO and solutions of acidified nitrite, mimicking gastric conditions, have been shown to have antimicrobial activity against a wide range of organisms [136]. It has also been shown that nitrite inhibits active transport and oxidative phosphorylation in aerobic bacteria but does not affect the phosphoenolpyruvate-phosphotransferase system [137]. This work also indicated that nitrite may be inhibitory to a wide range of physiological types of bacteria. In addition, nitrite appears to inhibit respiratory energy coupling in certain bacterial

species [138]. While not all bacterial species appear to be affected by nitrite, another report has suggested that growth inhibition seems to depend primarily on the extent to which the organism derives its energy from electron-transport-mediated processes [139].

These reports may explain why only about 80% of the available glutamate was consumed after growth in medium EMMG5 (Section 3.3.2). Growth in this medium resulted in an extracellular nitrite concentration of approximately 30 mM. It is possible that at this level nitrite inhibits the active transport of glutamate and/or the electron transport system, thus limiting growth of the organism.

Growth of *E. corrodens* in EDM-1, which lacked hemin, confirmed a previous finding [95] that inclusion of hemin in chemically defined media was not an essential nutrient for the organism. This indicates that *E. corrodens* was able to acquire adequate amounts of essential iron for growth directly from the medium. Previous researchers had suggested that the organism may have a hemin requirement [93] and it is often included in culture media [94]. It is possible that upon isolation strains may possess a hemin requirement but this trait is lost upon continued laboratory sub-culturing.

Also confirmed during this study was the observation of Robertson and Keudell that *E. corrodens* is unable to take up exogenous thiamine but is able to overcome this deficiency by utilising the thiamine degradation products 2-methyl-4-amino-5-hydroxymethylpyrimidine and 4-methyl-5-(β -hydroxyethyl) thiazole [98]. The organism may lack transport mechanisms for the uptake of thiamine or

its phosphorylated esters, whereas the uptake and assembly of the component moieties proceeds as in other bacteria. [140].

Thiamine, biotin, nicotinic acid and all of the other B-group vitamins are known to be present in saliva [141]. Robertson and Keudell postulated that thiamine moieties may also be present in saliva since a number of bacterial species are known to produce thiaminase, an enzyme which cleaves thiamine to its component moieties. It is possible that the observed requirement of *E. corrodens* for these compounds, rather than intact thiamine, is advantageous in that its uptake system is devoted to scavenging these moieties rather than competing with other organisms for thiamine, which may be in greater demand.

5.3. Energy Generation From Key Amino Acids

E. corrodens is unable to utilise glucose and analysis of culture filtrates following growth of the organism in glucose-containing medium indicated that the organism does not possess a functional glucose transport system. Cell growth and metabolism is reliant on the catabolism of amino acids as a source of energy and anabolic carbon skeletons.

Studies with a number of strains of *E. corrodens* examined demonstrated utilisation of free amino acids after batch growth in a complex medium containing a mixture of amino acids and small peptides. All strains exhibited similar

utilisation patterns and were found to consume relatively large amounts of key amino acids during growth.

Analysis of samples from experiments of chemostat cultures of *E. corrodens* isolates grown in simple chemically defined media confirmed the amino acid utilisation pattern and provided a link between the utilisation of specific amino acids, biomass production and nitrate reduction. The relationship between the utilisation of individual amino acids and nitrate reduction was further investigated and clearly established that the catabolism of glutamate, glutamine, proline and serine is the primary energy source for the cell.

Numerous studies have shown that proline can provide both energy and carbon to a variety of microorganisms [142-144]. Proline uptake and metabolism has been most extensively studied in *E. coli* [145], which is capable not only of degrading proline to glutamate but also of accumulating the amino acid which serves as an osmoprotectant during growth in media of high osmolarity [146]. Proline uptake is subject to both catabolite repression and specific induction by proline and uptake appears to occur via a single porter encoded by the *putP* gene [147].

5.3.1. Possible catabolic pathways for glutamate, glutamine and proline

It is possible that the catabolism of amino acids glutamate, glutamine and proline results in the production of the Krebs cycle intermediate α -ketoglutarate. The likely pathways involve the preliminary conversion of both proline and glutamine to glutamate which subsequently yields α -ketoglutarate, NH_4^+ and a reduced electron carrier in a reaction catalysed by glutamate dehydrogenase. It appears that NADH is the likely electron carrier as it has this role in most microbes undergoing anaerobic respiration involving nitrate as the terminal electron acceptor [148]. The reduced electron carrier in turn is oxidised, passing electrons to the respiratory chain, ultimately involving the reduction of nitrate and the generation of proton motive force across the cytoplasmic membrane which drives ATP production.

The organism may produce glutaminase, an enzyme which deaminates glutamine and results in its conversion to glutamate. The catabolism of proline is more complex and involves two oxidation steps. Initially proline is oxidised by proline oxidase to the Schiff base Δ^1 -pyrroline-5-carboxylate which undergoes hydrolysis to glutamate semialdehyde. This undergoes further oxidation via glutamate semialdehyde dehydrogenase, producing glutamate and NADH [149].

The conversion of proline to α -ketoglutarate, therefore, involves three oxidation steps, at least two of which result in the donation of electrons to NAD which can be used to generate ATP. Further conversion of α -ketoglutarate to succinate is unlikely as this reaction is not usually carried out by anaerobes [148]. As the respiratory chain involving the conversion of nitrate to nitrite has the

capacity to generate 2 ATP per pair of electrons, degradation of proline has the potential to yield at least 4 ATP, compared to 2 ATP per molecule of either glutamate or glutamine. The net yield of ATP to the cell also depends on the energy expended in uptake of these amino acids. It is not known whether this process requires hydrolysis of ATP although it is known that uptake of acidic amino acids can be driven by the ΔpH component of proton motive force whereas neutral amino acids may be driven by both ΔpH and $\Delta\psi$ in anaerobes [148]. The utilisation by *E. corrodens* of glutamate and glutamine may result in the net production of at least one ATP from each and at least 3 ATP for proline.

Evidence supporting this view is shown in Figure 3-1 (p. 76), which shows the rate of nitrate reduction by *E. corrodens* cells exposed to various free amino acids. It can be seen that incubation of cells in the presence of proline resulted in a nitrate reduction rate between three and four times that of the rate in the presence of glutamate. The rate of nitrate reduction in the presence of glutamine was slower than the rate for glutamate, reflecting the extra step catalysed by glutaminase. It should be noted that this interpretation of the data assumes comparable amino acid uptake rates. Analysis of the data obtained from growth of the organism in chemically defined media with increasing levels of either glutamate (Section 3.3) or proline (Section 3.8) reveals that the growth yield from proline catabolism is close to twice that for glutamate.

5.3.2. The generation of energy from serine degradation

The organism also appears to derive energy from the catabolism of available serine. The pathway for this process has not been investigated in this study but utilisation results in equimolar amounts of acetate in culture filtrates. Serine thus appears to be catabolised to acetate, possibly via pyruvate, with loss of a carbon as CO₂. The generation of ATP from serine degradation appears significantly lower than for glutamate or proline as evidenced by data showing that utilisation of serine resulted in a molar growth yield less than half that for glutamate (section 3.3).

Incubation of *E. corrodens* cells with serine resulted in nitrite production rates comparable to those of glutamate and glutamine. However, an analysis of chemostat culture filtrates following growth in a chemically defined medium containing serine showed that nitrite levels, under these conditions, increased only marginally. An explanation for these observations may be that during the catabolism of serine, electrons are donated to a carrier, (other than NAD), that transfers electrons to a different component of the respiratory chain; one which only supplies 1 ATP per pair of electrons.

5.3.3. Consumption of lysine

The organism demonstrated significant capacity to degrade the amino acid lysine. Although unrelated to energy production, this ability is due to the action of the enzyme lysine decarboxylase, which produces the highly basic polyamine cadaverine. Other studies have reported homology between the lysine

decarboxylase of *E. corrodens* and those of enteric bacteria and that the activity of this enzyme in plaque may slow the turnover of junctional epithelium because of lysine depletion [150].

In *E. coli*, the biodegradative lysine decarboxylases, which synthesise polyamines, are strongly induced at acidic pH [151] and the cadaverine generated from decarboxylation of lysine is not further metabolised for energy but is excreted into the environment [152]. Hence, it has been suggested that this process has a possible role in pH homeostasis. The effect of pH on the growth of *E. corrodens* in this study shows that a drop in environmental pH to 6.5 had a dramatic effect on growth yields, and so the biological function of lysine decarboxylase in this organism may be the same as that for the degradative lysine decarboxylases of enteric bacteria, namely, maintenance of external pH.

5.4. Carbon Skeletons from Amino Acid Catabolism

It is evident from the present study that key amino acids provide *E. corrodens* not only with a source of energy but also of carbon. It appears that the oxidative degradation of proline, glutamine and glutamate to α -ketoglutarate is sufficient to supply the cell with a significant amount of carbon for anabolic processes. Analysis of culture filtrates revealed that the only detectable short-chain fatty acids present in the culture filtrate after growth in defined medium were acetate and formate. The organism has previously been reported to excrete acetate

[104], the production of which, during this investigation, was only detected following growth in media containing significant quantities of serine.

It has recently been demonstrated that the organism also possesses aspartase (Dr. R. Allaker, personal communication), an enzyme which catalyses the conversion of aspartate to fumarate. It is known that many anaerobes, which lack components of the Krebs cycle, utilise aspartate via fumarate, succinate and ultimately succinyl-CoA in the synthesis of essential porphyrins. It is interesting to note that Keudell *et al* [95] were unable to obtain growth of the organism in synthetic minimal media containing only those amino acids found to be essential, unless supplemented with aspartate, glycine and glutamate at concentrations of 500 mg L⁻¹. In the present study, amino acid utilisation patterns for *E. corrodens* indicate that significant quantities of aspartate are consumed during growth of the organism. It is possible, therefore, that *E. corrodens* may also obtain a portion of its carbon requirement from the degradation of aspartate as well as from that of glutamate. It is also worth noting that in both this study and in that of Keudell *et al* [95], growth yields of *E. corrodens* strains were lower in chemically defined media in which “non-essential” amino acids were either absent or in low quantities.

5.5. Peptide Utilisation

It has been shown that the utilisation, by microorganisms, of amino acids derived from peptides may be more efficient than utilisation of the corresponding

free amino acids. [153, 154]. The present study has shown that *E. corrodens* possesses aminopeptidase activity, with particular activity specific to small peptides containing N-terminal proline. Experiments investigating the link between utilisation of small peptides and nitrate reduction in resting cells of *E. corrodens* illustrate the critical biological role that exopeptidase activities play in the organism's metabolism. All tested peptides, containing key energy supplying amino acids, were metabolised at rates comparable to those of the corresponding free amino acid. Such results indicate the possible presence of a glutamate-specific carboxypeptidase and also that the organism, while it may lack a functional uptake system for histidine, is able to metabolise histidine-containing peptides. It is likely that the organism is able to derive energy from histidine following its release from histidine- containing peptides. As histidine is a member of the glutamate family, energy derived from this amino acid probably follows its enzymic conversion to glutamate.

5.5.1. *E. corrodens* proline iminopeptidase (EC 3.4.11.5)

The finding that metabolism of the di-peptide pro-ala resulted in a nitrate reduction rate slightly faster than that for free proline led to further investigation of the proline iminopeptidase (PIP) in *E. corrodens*. This enzyme was initially reported by Allaker *et al* [105] in a study of the hydrolytic enzymes of oral isolates *E. corrodens*. The fact that all twenty isolates, from both periodontally healthy and adult periodontitis patients, expressed PIP activity indicates that the expression of this enzyme may play a crucial role in growth of the organism. In contrast,

expression of PIP in *N. gonorrhoeae* has been reported to be non-essential for growth of this organism *in vitro* [155].

Proline iminopeptidase is an aminopeptidase which can release N-terminal proline residues from peptides and, although first described and purified from *E. coli*, appears to be expressed in a large number of microorganisms. The enzyme is also found in mammalian tissues, the highest levels occurring in the kidney and liver [156]. The subunit structure of the functional enzyme appears to be variable between bacterial species. For example, following an initial estimate of approximately 300 kDa [157], subsequent cloning studies revealed a subunit molecular mass of between 32 and 35 kDa [155, 158, 159]. It has also been reported that native molecular masses vary between 40 and 120 kDa, suggesting that the enzyme may be monomeric or exist as a di-, tri- or tetramer [160]. An estimation of the molecular mass of native *E. corrodens* PIP in the present study indicates that the enzyme exists in the monomeric form and is approximately 35 kDa. The estimation of molecular weight has subsequently been confirmed by others who have cloned and sequenced the PIP gene from *E. corrodens* (Dr. D. Dymock, personal communication).

Although reports of the pH optimum for the enzyme vary between 6.5 and 9.0 [160, 161, 111, 109], studies on *E. corrodens* PIP by Allaker *et al* [107] demonstrated that the optimal pH for the enzyme in this organism was between 7.5 - 8.0. Optimum temperature for activity has been reported to be between 40 - 50 °C, a value comparable to that obtained for this organism [107].

The activity of PIP in *E. corrodens* strain 33EK(L), grown in continuous culture, was determined and found to be comparable to that previously published for this strain grown under batch-culture conditions [105]. Growth of the organism in peptide-free and peptide-containing media resulted in cells expressing similar PIP activities, indicating that the enzyme is expressed constitutively. There does not appear to be significant extracellular expression of the enzyme, as the present study showed that more than 90% of the total activity in chemostat culture was cell-associated. The finding that the enzyme is cytoplasmic is in accord with studies of the enzyme in other bacteria [106] and indicates that the release of proline from peptides is dependent on peptide uptake.

Proline iminopeptidase, often reported as a thiol enzyme due to its inhibition by thiol protease inhibitors such as PCMB and iodoacetamide, is now considered to be a member of the serine protease family [106]. This is due to a number of reports questioning its thiol protease classification [157, 161, 111] and that an active site serine residue, essential for catalytic activity, has been identified as the consensus sequence for serine proteases [158, 159].

Following partial purification characterisation of PIP with various protease inhibitors and metal ions, inhibition of enzyme activity was obtained with the serine protease inhibitor, phenylmethylsulphonyl fluoride (PMSF), and thiol protease inhibitors N-ethyl maleimide (NEM) and p-chloromercuribenzoate (PCMB). As found previously by others [107] these results indicate some thiol involvement in enzyme activity. Also confirmed was the fact that zinc and

mercuric ions also strongly inhibit enzyme activity. *E. corrodens* PIP displayed Michaelian saturation kinetics and the K_m for the synthetic substrate proline- ρ -nitroanilide was determined and found to be comparable to those for the majority of aminopeptidases [106] and similar to that reported for the PIP of *Lactobacillus delbrueckii* [111]. A recent study involving the cloning and sequencing of *E. corrodens* PIP indicates that the enzyme shares much homology with the PIP of *Neisseria gonorrhoeae* (Dr. D. Dymock, personal communication).

5.6. Oral sources of proline

Growth of *E. corrodens* is dependent on a supply of amino acids from its environment as the organism has been demonstrated to have both an auxotrophic requirement for a number of amino acids (including proline) [95] and has been shown, in the present study, to obtain energy and carbon via the degradation of proline, glutamate, glutamine and serine. Providing that the organism has an adequate supply of nitrate, the factors influencing its numbers in a microbial community will essentially be determined by the concentrations of these key amino acids, particularly proline, in its immediate proximity and its ability to compete for them with neighbouring bacterial species.

Researchers have previously commented on the unusually high concentration of bound and free proline in the oral environment [162] and it is known that proline-rich proteins form the largest group of proteins within human parotid saliva [163]. Proline-containing peptides may also be liberated during

breakdown of proline-rich collagen fibres. The increased activity of collagenases in diseased sites thus provides an increased source of energy for *E. corrodens* and a possible explanation for the increase in the numbers of the organism at these sites.

The supply of amino acids to organisms inhabiting the healthy gingival crevice originates primarily from either saliva or gingival crevicular fluid (GCF). Progression to a diseased state involves a deepening of the crevice and development of a periodontal pocket, an associated increased flow of GCF and a significant change to a predominantly asaccharolytic microflora. An increase in numbers of the proteolytic organisms *P. gingivalis* and *B. forsythus*, as well as other amino acid degrading organisms with demonstrable aminopeptidase activities, such as *F. nucleatum* and *E. corrodens*, has been observed [164, 165], [166]. The elevated proteolytic capacity of this community would lead to an increase in amino acids and peptides available to its members.

The ability of organisms, such as *F. nucleatum* and *E. corrodens*, which require amino acids but have poor proteolytic capacity themselves, to survive in this ecosystem, depends on their effectiveness in interspecies competition for essential nutrients and also that requirements of species complement each other to some degree. Studies in this laboratory have shown that *P. gingivalis*, grown in continuous culture in a medium containing protein as the prime source of amino acids, does not utilise proline during growth (Rogers *et al*, unpublished data).

Another possible source of energy for *E. corrodens* may be provided via the metabolism of arginine by *Treponema denticola*, an anaerobe frequently present in the human oral cavity. Cultures of *T. denticola* metabolise this amino acid to proline, via ornithine, the proline being excreted from cells growing in a complex arginine-containing medium [167].

Previous studies in this laboratory [86, 88] have demonstrated that *F. nucleatum* utilises both glutamate and serine for energy production and, therefore, would be in competition for these amino acids with *E. corrodens*. It would seem that organisms such as *E. corrodens* and *F. nucleatum* benefit from the presence of more proteolytic species in the microbial community but only if they are able to compete successfully with other organisms for an adequate nutrient supply. Although competition for common nutrients occurs, such as that between *E. corrodens* and *F. nucleatum* for glutamate, complementation between species — such as *E. corrodens* utilising proline provided by the proteolytic enzymes of *P. gingivalis* or the metabolism of oxygen by *F. nucleatum* which assists the survival of the more oxygen-sensitive *P. gingivalis* (Diaz *et al*, unpublished data) — must be a contributing factor in providing a balance in the relative stability of the complex microbial community.

5.7. Summary

It was originally hypothesised that an investigation of the physical and nutritional factors affecting growth of *E. corrodens* would provide an explanation for its isolation in increased proportions from plaque at periodontally diseased sites.

The optimal growth conditions determined from these experiments indicated that the organism is favoured in a slightly alkaline environment containing a supply of nitrate and a supply of amino acids (particularly proline and glutamate). During the progression of a site from periodontal health to disease, an increase in pH is observed as is the concentration of amino acids and peptides resulting from, the combined effects of, increased gingival crevicular fluid flow and action of proteolytic enzymes. It appears that the oral cavity is a relatively proline-rich environment and the increased activity of proteases on salivary proteins and collagen fibres at diseased sites would thus provide a ready source of carbon and energy for *E. corrodens*.

As a result of the inflammatory process temperatures at diseased sites may be elevated by as much as 2-3°C. It was found that the optimal growth temperature of the organism was below 36°C, a figure comparable to that of subgingival temperatures at healthy sites. The organism does not require carbohydrate for growth and, of the group of organisms commonly referred to as oral anaerobes, is relatively insensitive to the presence of oxygen.

The growth characteristics of *E. corrodens* determined in this study provide an ecological explanation for the presence of this organism in the normal oral flora as well as its increase in numbers at sites of periodontal disease.

5.8. Future Studies

The finding that *E. corrodens* PIP is an intracellular enzyme and that the rate of metabolism of proline from peptides is comparable to that of the free amino acid indicate that the peptide uptake systems play an important role in energy generation in the organism. It is thought that the periodontal environment, in which it is found, is supplied with an increased supply of small peptides generated by the more proteolytic members of the microbial community, which themselves are also reliant on the degradation of amino acids for survival and growth. Information about the mechanism of peptide uptake and affinity for peptides by *E. corrodens* may be valuable in understanding how the organism is able to maintain its numbers in this competitive environment.

A number of possibilities for mixed continuous culture studies arise from the findings of this investigation. For example, knowledge of the organism's growth requirements and those of *F. nucleatum* from previous work and the ability to culture both organisms individually in simple chemically defined media provides a basis for the development of a defined medium allowing stable co-culture of the two. The finding that *P. gingivalis* does not utilise significant

quantities of proline during growth should allow development of a nitrate-containing, protein-based medium capable of supporting stable continuous co-culture of *P. gingivalis* and *E. corrodens*. Recently in this laboratory a stable co-culture of *P. gingivalis* and *F. nucleatum* has been established (Diaz *et al*, unpublished data) and the introduction of *E. corrodens* into this system may also be possible.

Studies of this type would be invaluable in obtaining a more complete understanding of the complex relationships between organisms existing in both periodontally healthy and diseased sites in terms of providing information regarding the nutritional requirements and the effect of physical parameters on bacterial consortia inhabiting these sites. This information is largely missing, or incomplete, for many of the species seen to increase in proportion during the progression to the diseased state. Little is known about the amino acid requirements of many of the oral anaerobes. Although much work has been done investigating the proteolytic activities of *P. gingivalis*, an organism which is also thought to derive energy and carbon from the degradation of amino acids, knowledge of the organism's peptide uptake systems, intracellular peptidases and mechanisms of generating energy is lacking. Studies similar to that undertaken for *E. corrodens* in this investigation, and those previously undertaken in this laboratory with *F. nucleatum*, would be valuable in further understanding the role of *P. gingivalis* and other oral anaerobes in the progression to the diseased state.

6. Appendix 1 - Materials and Methods

6.1. Strains

A number of strains of *E. corrodens* were used in this study. The type strain ATCC 23834^T and was provided by Dr. C. K. Chen, Department of Periodontology, University of Southern California, Los Angeles, California, USA. The clinical isolates 33EKL, 35EK and 39EK were obtained from Dr. R. P. Allaker, Department of Oral Microbiology, The London Hospital Medical College, London, UK.

All strains were maintained by weekly subculture on anaerobic blood agar (Oxoid) and stored long term in 40% glycerol broth at -80°C.

6.2. Composition of YT medium

The components of YT medium per litre were:

yeast extract	20g
tryptone	10g
KNO ₃	2g
KH ₂ PO ₄	0.2g
hemin	5mg

The medium was adjusted to a final pH of 7.3 by the addition of KOH and sterilised by autoclaving.

6.3. Determination of Growth Parameters

6.3.1. Optical Density

Estimation of optical densities of cultures and cell suspensions was accomplished by the measurement of absorbance of the sample in a Spectronic 20 spectrometer (Bausch and Lomb, USA) at a wavelength of 560 nm.

6.3.2. Dry Weight Estimation

Culture dry weight estimation was performed by centrifugation of duplicate 20 ml culture samples at 3,500 g for 30 min at 4°C in a refrigerated benchtop centrifuge (Centra MP4R, International Equipment Company, USA). Resultant pellets were washed twice in distilled water and resuspended in 1 ml distilled water. Cell suspensions were then placed in pre-weighed planchettes, dried to constant weight at 105°C and reweighed using an analytical balance (Model H54AR, Mettler, USA).

6.3.3. Protein Estimation

Cellular Protein

Determination of cellular protein concentration was performed using a modification of the Biuret reaction [99]. In this procedure the bacterial cells are dissolved in 1.0 N NaOH and then CuSO_4 without tartrate is added. The insoluble cellular material and insoluble $\text{Cu}(\text{OH})_2$ are removed by centrifugation (3,000 g, 15 min).

The reagent used for this procedure is prepared by the addition of 1.5 g of cupric sulphate. $5\text{H}_2\text{O}$ to 500 ml of distilled water. To this solution is added, with stirring, 300 ml of a 10% (w/v) solution of NaOH in water. The volume of this solution is then adjusted to 1 litre with distilled water.

The procedure for protein content determination involves the addition of 2.0 ml of alkaline cupric sulphate reagent to 0.5 ml of sample. Reactions are performed at room temperature for 30 min at which time the samples are centrifuged and the optical density at 500nm of the resultant supernatants recorded spectrophotometrically. The OD_{500} of samples were compared to values obtained from a standard curve of protein standards containing bovine serum albumin in the range 0.5 - 5 mg.

Determination of Protein During Enzyme Purification

Protein content at stages of enzyme purification was determined using the Pierce Bicinchoninic Acid (BCA) Protein Assay (Pierce Chemical Co., Rockford, IL, USA). For all protein determinations, known concentrations of bovine serum albumin (BSA) were used to generate standard curves.

Following incubation of samples with BCA reagents, the absorbance was measured spectrophotometrically at 562 nm (Lambda 5 UV/Vis Spectrophotometer, Bodenseewerk Perkin Elmer & Co, Germany).

The protein content at initial stages of enzyme purification was determined using the standard protocol (37°C, 30 min), whereas the enhanced protocol (60°C, 60 min), was used for samples after HIC and IEC fractionation as these samples contained a much lower protein concentration.

6.3.4. Viable Count

Viable counts of chemostat cultures were performed by preparing serial 10-fold dilutions of 0.3 ml of culture in sterile saline. Aliquots (0.1 ml) of 10^{-5} and 10^{-6} dilutions were spread onto duplicate anaerobic blood agar plates (Medvet Science, Adelaide, Australia) and the plates were incubated aerobically at 37°C for 48 hours prior to counting.

6.4. Amino Acid Analysis

Instrumentation:

Analysis of amino acids was performed using the following equipment:

Waters U6K injector,

Two Waters Model 510 pumps,

Waters automated gradient controller, Model 680,

Waters Novapak C-18 reverse phase column (150 mm x 4.6 mm),

Gilson Spectra/glo filter fluorimeter.

Method Overview:

Qualitative and quantitative analysis of amino acids was achieved employing a modified method based on that of Hill *et al* [96], involving pre-column derivatisation with o-phthaldialdehyde, separation using reverse phase chromatography and detection of fluorescent derivatives.

Mobile Phase and Gradient Conditions:

Solution A - 25 mM sodium phosphate buffer pH 7.4

Solution B - 55 % acetonitrile 45 % sodium phosphate buffer pH 7.4 (v/v)

Both solutions were degassed and filter sterilised by vacuum filtration through 0.45 µm filters (type HVLP, Millipore Corp.)

A linear gradient, starting at 80 % solution A and finishing at 10% Solution A, was applied over 30 min at a flow rate of 1 ml min⁻¹. Once gradient conditions were completed the column was allowed to re-equilibrate for 7 min prior to application of the next sample.

Derivatisation Procedure

The derivatising solution (OPA reagent) was prepared by the addition of 20 µl of ethanethiol and 0.5 ml borate buffer (0.5 M, pH 10.5) to a 10 mg ml⁻¹ solution of o-phthaldialdehyde dissolved in methanol. The solution was prepared fresh daily and protected from light.

Samples were derivatised by the addition of 25 µl of derivatising solution to 0.1 ml of sample and the mixture was allowed to react at room temperature for 30 sec prior to injection of 10 µl.

An amino acid standard solution, containing amino acids at a concentration of 0.1 mM, was prepared. It was derivatised and analysed prior to the analysis of unknown samples to determine retention time and relative fluorescence of individual amino acids. Chromatograms were processed by PC based Delta Chromatography Data Systems Software.

6.4.1. Detection of Proline

Detection of proline was performed using the modified derivatisation method of Cooper *et al* [100]. For this procedure the following reagents were required:

Borohydride solution:

250 mM sodium borohydride in 600 mM lithium hydroxide

Chloramine T solution:

12.5 mM chloramine T in 2 ml dimethyl sulphoxide and 8 ml of 200 mM sodium borate buffer pH 10

OPA Reagent:

Prepared as described previously

Derivatisation Procedure

To a sample volume of 50 µl was added 0.2 ml of chloramine T solution pre-heated to 60°C in a water bath. Following incubation for 1 min, 0.2 ml of sodium borohydride solution was added and the mixture incubated for a further 10 min at 60°C. After incubation 50 µl of mixture was removed and added to 0.1 ml of OPA reagent, 10 µl of which was injected for RP-HPLC analysis as described above.

6.4.2. Detection of Cysteine

Detection of cysteine was performed using the modified derivatisation method of Cooper and Turnell [101]. For this procedure the following reagents were required:

PCA-MCE reagent consisting of 70 mM perchloric acid in 25 mM mercaptoethanol

100 mM iodoacetic acid

3 M NaOH

OPA reagent (as described previously)

Derivatisation Method:

To 0.1 ml of sample was added 0.1 ml glutamic acid (0.1 mM) internal standard, 0.5 ml PCA-MCE, 0.2 ml NaOH and 0.2 ml iodoacetate. After mixing 0.2 ml was removed and added to 50 µl of OPA reagent. 10 µl of this mixture was injected for RP-HPLC analysis as described above.

6.5. End-product and Glucose Estimation

Determination of acidic end-products and glucose was performed using ion-exclusion chromatography based on the method of Guerrant *et al* [103].

Instrumentation:

Rheodyne 7125 injector

Organic Acid Analysis HPLC Column (Aminex HPX-87H, Bio-Rad Laboratories)

Waters Model 501 HPLC Pump

Waters Model R401 Refractive Index Detector

Waters Model 730 Data Module

Waters Model 2259 Temperature Control Module

Mobile Phase:

This was an isocratic separation using 3.5 mM H₂SO₄ at a flow rate of 1 ml min⁻¹ for elution.

Cell-free culture filtrates were prepared and 20 µl of these were injected for analysis. A standard solution containing the following compounds at a concentration of 10 mM, formate, acetate, lactate, glucose, propionate, succinate, butyrate and iso-butyrate were used to determine peak retention times and area for quantitation.

6.6. Composition of EDM-1

Amino acid	Conc ⁿ . (mM)	Salt Solution	Conc ⁿ . (mg L ⁻¹)
Aspartate	5 *(3.3)	KNO ₃	2,020
Glutamate	5 (0.3)	KH ₂ PO ₄	1,361
Serine	10 (0)	MgSO ₄ .7H ₂ O	50
Histidine	5 (5.4)	NaCl	20
Glycine	1 (6.7)	CaCl ₂ 2H ₂ O	12
Alanine	1 (0)	FeSO ₄ .7H ₂ O	5
Tyrosine	1 (0.6)	MnSO ₄ .H ₂ O	4
Arginine	5 (1.4)		
Valine	1 (4.3)	Vitamins	Concⁿ. (mg L⁻¹)
Methionine	3 (3.4)	Biotin	0.01
Isoleucine	1 (3.8)	Nicotinic acid	1
Leucine	1 (3.8)	Thiamine - HCl*	2
Tryptophan	1 (2.5)		
Phenylalanine	1 (0.6)		
Lysine	10 (69)		
Proline ⁺	22 (22)		
Cysteine	1 (2.9)		

All components were completely dissolved in distilled water prior to pH adjustment.

The pH of medium was adjusted to 7.2 with KOH.

Figures in parentheses indicate the amino acid concentration in E16TBN.

*A stock solution of thiamine was used which had been autoclaved prior to addition to EDM-1.

⁺ Note that modified EDM-1 contained 2 mM proline.

6.7. Media Filtration

Media filtration was performed using a 142 mm diameter pressure filtration system (Millipore Corp., Bedford, Mass, USA).

6.8. Composition of EMMG1

Amino acid	Conc ⁿ . (mM)	Salt Solution	Conc ⁿ . (mg L ⁻¹)
Aspartate	1	KNO ₃	4,040
Glutamate	1	KH ₂ PO ₄	1,361
Serine	0	MgSO ₄ .7H ₂ O	50
Histidine	1	NaCl	20
Threonine	1	CaCl ₂ .2H ₂ O	12
Glycine	1	FeSO ₄ .7H ₂ O	5
Alanine	1	MnSO ₄ .H ₂ O	4
Tyrosine	1		
Arginine	1	Vitamins	Concⁿ. (mg L⁻¹)
Valine	1	Biotin	0.01
Methionine	1	Nicotinic acid	1
Isoleucine	1	Thiamine - HCl*	2
Leucine	1		
Tryptophan	1		
Phenylalanine	1		
Lysine	1		
Proline	1		
Cysteine	1		

All components were completely dissolved in distilled water prior to pH adjustment.

The pH of medium was adjusted to 7.2 with KOH.

* A stock solution of thiamine was used which had been autoclaved prior to addition to EDM-1.

6.9. Nitrite Determination

The presence of nitrite was assayed using the method of Canney *et al* [102].

Reagents

Solution A - 0.2 g N-(1-Naphthyl)ethylenediamine dihydrochloride in 100 ml 1.5 N HCl

Solution B - 1 g Sulphanillic acid in 100 ml 1.5 N HCl

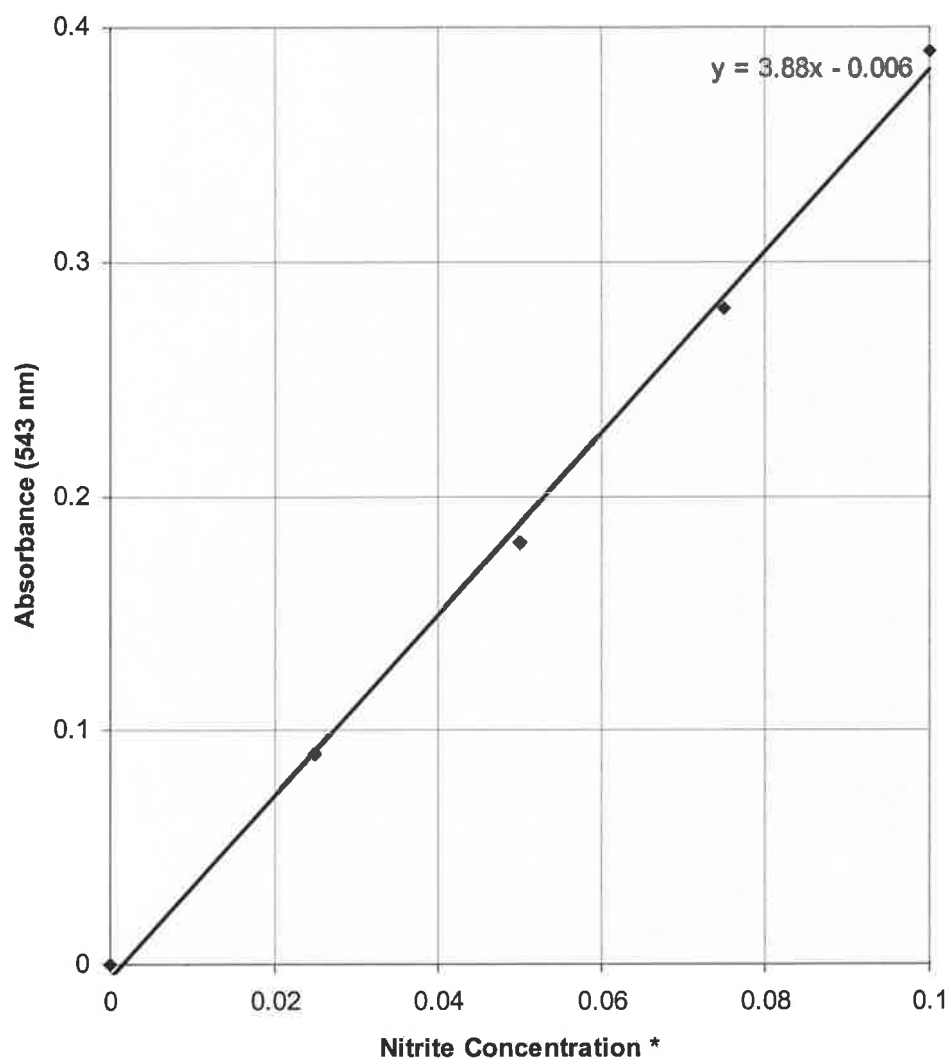
Test Reagent - Equal volumes of solutions A and B were mixed.

Standard NO_2^- solutions were prepared by dissolving 0.13 g of NaNO_2 in 500 ml of distilled water. This solution was used a stock solution and diluted appropriately with distilled water to produce standards of known nitrite concentration for construction of the standard curve.

Procedure

To 0.1 ml of reagent was added to 5 ml of sample and allowed to react for 10 min at room temperature. At this time the absorbance was measured at 543 nm. Using this method the plot of OD_{543} versus concentration of nitrite is linear over the range 0-180 $\mu\text{g ml}^{-1}$ NO_2^- .

Figure 6.1. Standard Curve For Determination of Nitrite



* Nitrite concentration expressed as $\mu\text{g ml}^{-1}$

6.10. Composition of BM1

The components of BM1 medium per litre were;

yeast extract	5g
proteose peptone	10g
KNO ₃	2g
cysteine HCl	0.5g
hemin	10mg
menadione	50µg

The medium was adjusted to pH 7.2 and sterilised by autoclaving.

6.11. Amino Acid Compositions of EMML, EMMM and EMMH

Amino acid	Amino Acid Concentration (mM)		
	EMML	EMMM	EMMH
Aspartate	0.1	1	1
Glutamate	0.1	1	1
Serine	0.1	1	1
Histidine	0.1	1	1
Threonine	0.1	1	1
Glycine	0.1	1	1
Alanine	0.1	1	1
Tyrosine	0.1	1	1
Arginine	0.1	1	1
Valine	0.1	1	1
Methionine	0.1	1	1
Isoleucine	0.1	1	1
Leucine	0.1	1	1
Tryptophan	0.1	1	1
Phenylalanine	0.1	1	1
Lysine	0.1	1	1
Proline	1	1	3
Cysteine	0.1	1	1

6.12. Column Chromatography

6.12.1. Instrumentation

Hydrophobic interaction (HIC) and ion exchange chromatography (IEC) were performed by using a gradient programmer (Isco model 2360, Isco Inc, Nebraska, USA) in conjunction with a peristaltic pump (Ismatec Ms Reglo, Glattbrugg, Zurich, Switzerland) and a fraction collector (Isco model 1200, Isco Inc, Nebraska, USA).

6.12.2. Buffers

The following buffers were employed for HIC:

Buffer A: 1.5 M $(\text{NH}_4)_2\text{SO}_4$ in 0.1 M NaHPO_4 , pH 6.8

Buffer B: 0.1 M NaHPO_4 , pH 6.8

The following buffers were employed for IEC:

Buffer A: 0.25 mM Tris.HCl , pH 8.

Buffer B: 1 M NaCl in 0.25 mM Tris.HCl , pH 8.

6.12.3.Gradient Conditions

HIC: Following sample loading, linear gradient from 100% Buffer A to 100% Buffer B over 60 min, at a flow rate of 1 ml min⁻¹.

IEC: Following sample loading, 100% Buffer A for 10 min at which time a linear gradient to 100% B over 60 min was commenced. The flow rate was 1 ml min⁻¹.

7. Appendix 2 - Results

7.1. Optical Densities After Batch Growth in YT Medium

Strain	OD _{560nm}
23834 ^T	0.73
33EKL	0.78
35EK	0.74
39EK	0.69

7.2. Amino Acid Utilisation Patterns of *E. corrodens* Strains Grown in YT Medium Under Batch Conditions.

Amino Acid	YT Medium	Strain			
		23834 [†]	33EKL	35EK	39EK
asp	5.8*	4.9	5.1	5.2	5.1
glu	5.3	0.2	0.1	0.2	0.3
gln	0.4	0	0	0	0
asn	0.6	0.5	0.5	0.6	0.6
ser	3.8	1.1	1.3	1.6	1.7
his	3.4	3.1	3.3	3.4	3.3
gly/thr	1.7	1.4	1.5	1.4	1.5
ala	3.5	3.9	3.6	3.8	3.7
tyr	3.1	2.8	2.9	3.0	3.0
arg	4.7	4.2	4.4	4.4	4.5
val	4.6	4.5	4.6	4.5	4.6
met	3.6	3.2	3.3	3.3	3.5
leu	5.0	4.6	4.5	4.7	4.5
ile	3.7	3.5	3.7	3.6	3.6
trp	4.2	3.9	4.0	3.8	3.9
phe	4.7	4.3	4.5	4.5	4.4
lys	2.1	0	0	0	0

* Data represented as concentration of amino acid mmols L⁻¹

7.3. Determination of Maximum Growth Rate of 23834^T in EDM-1

Time (h)	OD_{560nm}
0	0.11
2	0.14
3.75	0.19
5	0.27
6	0.38

7.4. Amino Acid Utilisation by 23834^T Grown in EDM-1

Amino Acid	Medium (mM)	Residual (mM) *	Utilisation	
asp	5	2.75	45 ^a	7.8 ^b
glu	5	0.95	81	14
ser	10	0.1	99	34
his	5	3.2	36	6.2
gly	1	0.33	67	2.3
ala	1	0.39	61	2.1
tyr	1	0.63	37	1.4
arg	5	4.55	9	1.5
val	1	0.42	58	2
met	3	1.83	39	4
ile	1	0.33	67	2.3
trp	1	0.42	58	2
leu	1	0.42	58	2
phe	1	0.42	58	2
lys	10	0.1	99	34
pro	22	19.58	11	8
cys	1	0.71	29	1

* Values represent the average result of triplicate samples, none of which differed by more than 10%.

^a Data represents mmoles of amino consumed per litre of culture

^b Data represents mmoles of amino consumed per gram dry weight of cells

7.5. Maximum Growth Rate of 23834^T in Modified EDM-1

Time (hr)	OD(560 nm)
0	0.13
1.5	0.25
2.5	0.34
4.25	0.45

7.6. Determination of Optimal pH - Growth Parameters

Culture pH	Protein Content (g L ⁻¹)	Dry Weight (g L ⁻¹)	Optical Density (560 nm)	Viable Count. cfu x 10 ⁻¹⁰
6.7	0.17	0.24	0.39	0.33
7.0	0.32	0.42	0.43	0.52
7.4	0.23	0.31	0.58	0.64
7.8	0.24	0.32	0.42	0.52
8.1	0.13	0.23	0.48	0.59
8.5	0.11	0.18	0.39	0.35

7.7. The Effect of Growth pH - Amino Acid Utilisation

	EDM-1	Growth pH				
		6.7	7	7.4	7.8	8.1
Asp	5	2.54*	3.32	2.44	2.15	2.93
Glu	5	0.73	0.84	1.06	1.31	2.05
Ser	10	2.66	0.82	0.07	0.07	1.50
His	5	3.85	4.36	4.89	4.76	4.58
Gly	1	0.43	0.53	0.47	0.85	0.78
Ala	1	0.71	0.93	0.71	0.71	0.71
Tyr	1	0.90	0.90	0.84	0.90	0.85
Arg	5	4.90	4.80	4.82	4.73	4.90
Val	1	0.79	0.86	0.66	0.73	0.79
Met	3	2.84	2.46	2.73	2.84	2.93
Ile	1	0.78	0.79	0.78	0.84	0.84
Trp	1	0.71	0.88	0.86	0.93	0.86
Leu	1	0.88	0.87	0.88	0.91	0.95
Phe	1	0.90	0.91	0.90	0.97	0.97
Lys	10	0.00	0.23	0.08	0.53	0.38

* Data represents concentration (mM) of amino acid in culture filtrate

7.8. Determination of Optimal Growth Temperature - Growth

Parameters

Culture Temperature (deg. C)	Protein Content (g L ⁻¹)	Dry Weight. (g L ⁻¹)	Optical Density (560 nm)	Viable Count. (cfu x 10 ⁻¹⁰)
30	0.11	0.19	0.42	0.33
34	0.23	0.31	0.58	0.64
36	0.16	0.23	0.44	0.24

7.9. The Effect of Growth Temperature - Amino Acid Utilisation

		Growth Temperature (°C)		
	EDM-1	30	34	36
Asp	5	2.60*	2.39	2.60
Glu	5	1.92	0.88	0.89
Ser	10	4.80	0.2	0.22
His	5	4.82	4.23	4.61
Gly	1	0.81	0.58	0.66
Ala	1	0.93	0.81	0.98
Tyr	1	0.97	0.87	0.88
Arg	5	4.89	4.38	4.12
Val	1	0.86	0.71	0.84
Met	3	2.61	2.78	2.82
Ile	1	0.79	0.7	0.75
Trp	1	0.68	0.67	0.69
Leu	1	0.81	0.71	0.77
Phe	1	0.93	0.88	0.89
Lys	10	0.00	0.2	0.40

* Data represents concentration (mM) of amino acid in culture filtrate

7.10. The Effect of Amino Acid Utilisation on Nitrate Reduction

Amino Acid	Rate of Nitrate Reduction ^a
Proline	37.5
Glutamate	10.0
Serine	8.3
Glutamine	7.4
Aspartate	5.1
Asparagine	3.9
Threonine	3.8
Leucine	3.4
Arginine	3.2
Alanine	2.7
Hydroxy-proline	2.7
Glycine	2.7
Methionine	2.1
Tryptophan	2.0
Iso-leucine	1.8
Histidine	1.7
Valine	1.7
Cysteine	1.5
Lysine	1.2
Phenylalanine	1.2
Tris-control	2.1

^a Rate of nitrate reduction expressed as nmoles of nitrite produced min⁻¹ (mg cell protein)

7.11. The Aminopeptidase Activity of Resting Cells of *E. corrodens*

33EK(L)

Peptide	Aminopeptidase activity ^a
Pro-gly-gly	0.120
Leu-gly-gly	0.023
His-gly-gly	0.014
Ser-gly-gly	0.013
Phe-gly-gly	0.009
Arg-gly-gly	0.005
Glu-gly-gly	0.004
Lys-gly-gly	0.002

^a Aminopeptidase activity expressed as nmol gly-gly released min⁻¹ (mg dry weight cells)⁻¹.

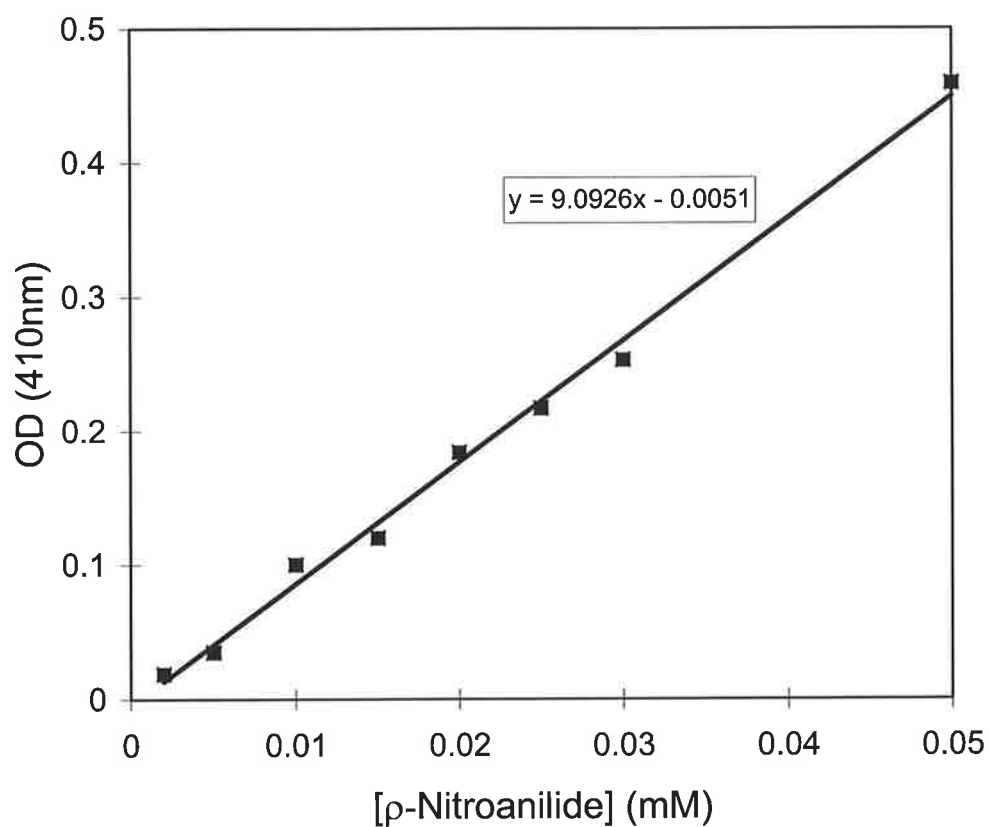
7.12. The Effect of Various Peptides on Nitrate Reduction in Resting Cells of *E. corrodens* 33EK(L)

Amino Acid or Peptide	Rate of Nitrate Reduction ^a
Pro-ala	38.1
Proline	36.2
Gly-pro	30.3
Pro-gly-gly	21.3
Gly-pro-ala	18.3
Glu-ala	23.4
Ala-gly-ser-glu	20.2
Gly-gly-gly	13.8
Glutamate	11.0
Glu-gly-gly	7.2
Glu-gly-phe	5.8
Tyr-glu-glu-trp	5.0
Ser-ala	11.1
Serine	8.7
Ser-gly-gly	7.4
Val-leu-ser	7.0
Gly-ser-phe	3.1
Ala-his-lys	15.3
His-ala	8.1
Gly-gly-his	5.4
His-gly-gly	4.3
Histidine	2.2
Tris - control	2.2

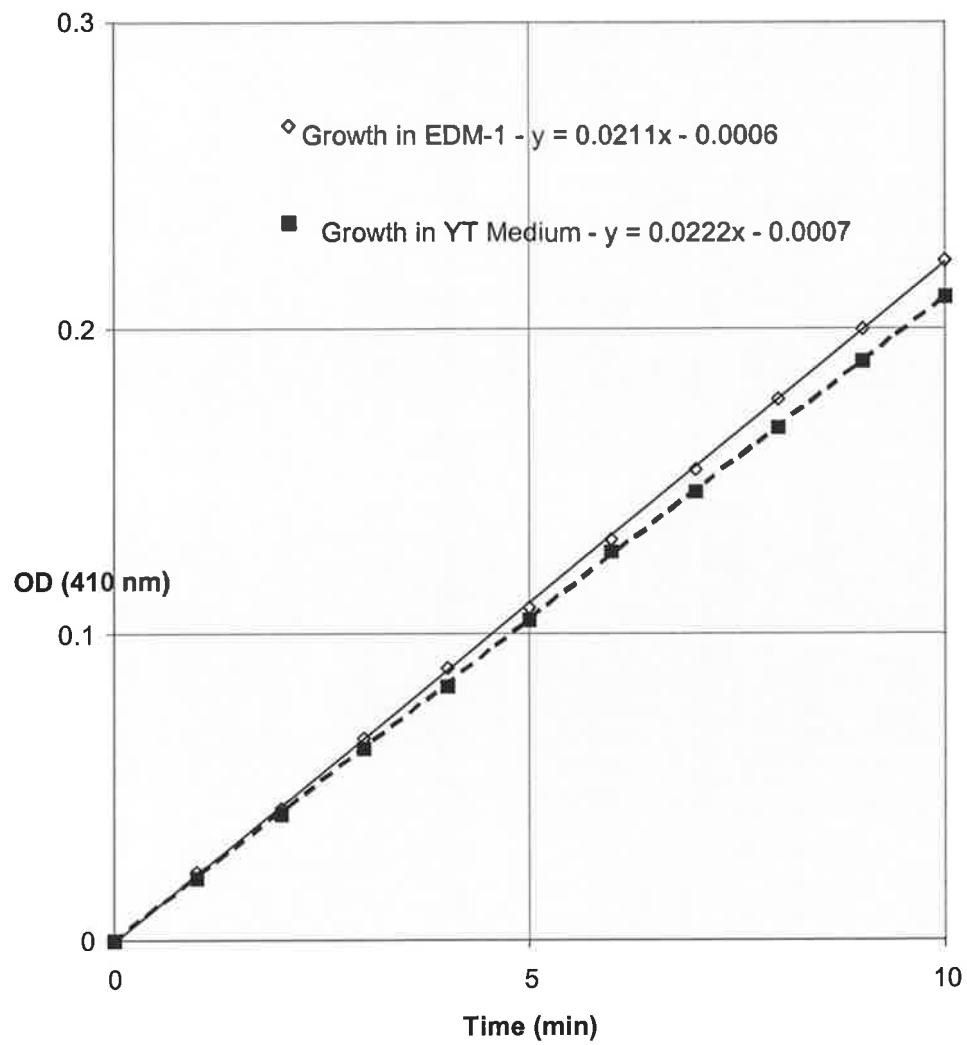
^a Rate of nitrate reduction expressed as nmol nitrite produced min⁻¹ (mg cell protein)⁻¹.

7.13. Figure 7-1. Standard Curve of OD₄₁₀ versus ρ -Nitroanilide

Concentration



7.14. Figure 7-2. The Effect of Growth Medium on PIP Activity



7.15. Determination of Enzyme Kinetics

	$\Delta OD_{410} \text{ sec}^{-1}$			
[pro- ρ -NA] (mM)	Expt. 1	Expt. 2	Expt 3	Average
0.07	0.00109	0.00115	0.00124	0.00116
0.08	0.00142	0.00152	0.00144	0.00146
0.1	0.00159	0.0017	0.00149	0.001595
0.15	0.00187	0.00239	0.00194	0.002067
0.2	0.0025	0.00272	0.00293	0.002717
0.3	0.0036	0.00321	0.00341	0.003407
0.5	0.0038	0.00321	0.00413	0.003713
1	0.00492	0.00413	0.00431	0.004453
5	0.00548	0.00508	0.00509	0.005217

[pro- ρ -NA] (mM)	$\Delta OD_{410} \text{ min}^{-1}$	umoles ρ -NA min^{-1}	umoles ρ -NA min^{-1} mg protein^{-1}
0.07	0.0696	0.008663193	0.02887731
0.08	0.0876	0.010605129	0.03535043
0.1	0.0957	0.011479	0.03826333
0.15	0.124	0.014532155	0.04844052
0.2	0.163	0.018739683	0.06246561
0.3	0.2044	0.023206137	0.07735379
0.5	0.2228	0.025191227	0.08397076
1	0.2672	0.029981336	0.09993779
5	0.313	0.034922484	0.11640828

1/S (mM^{-1})	1/Rate ($1/\text{umoles } \rho\text{-NA min}^{-1} \text{ mg protein}^{-1}$)
14.29	34.63
12.5	28.29
10	26.13
6.67	20.64
5	16.00
3.33	12.93
2	11.91
1	10.01
0.2	8.59

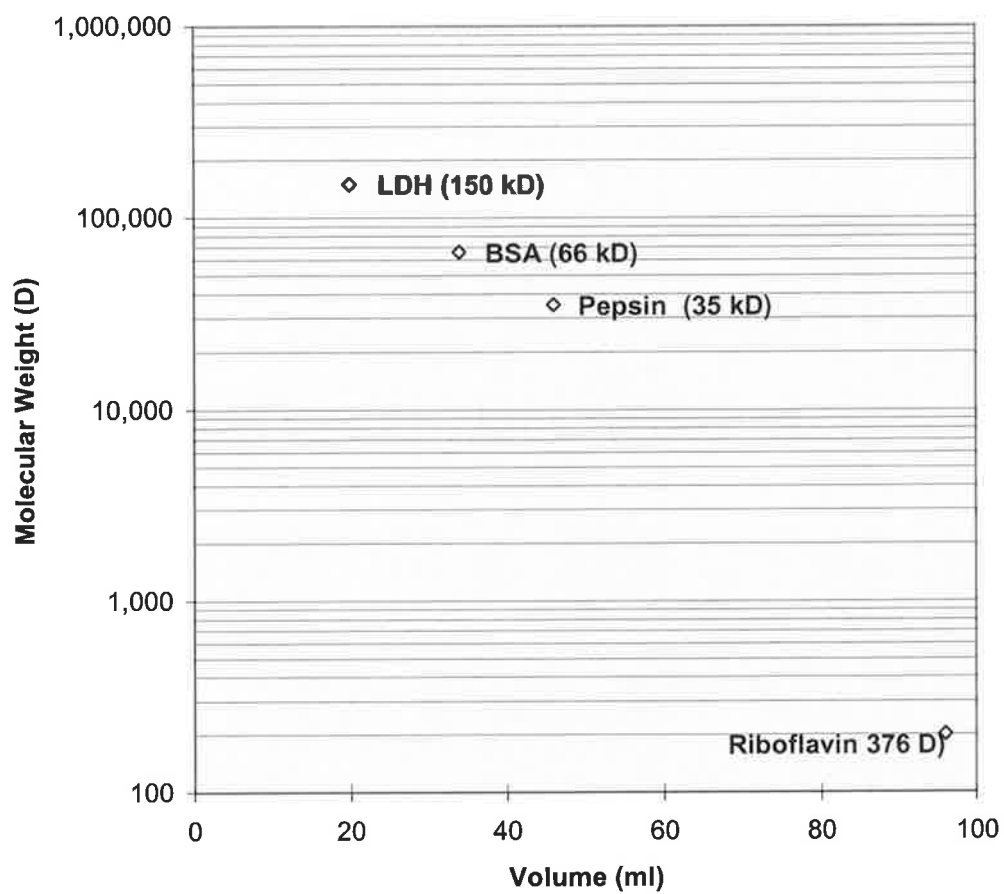
7.16. Extracellular Expression of PIP Activity

Sample	OD _{410nm}	
	0 min	60 min
Control	0.036	0.039
Supernatant	0.075	0.152
Cells	0.143	0.981

Levels of PIP activity in cell suspensions and culture supernatants were calculated by the addition of pro- ρ -NA. The OD₄₁₀ of each was recorded prior to, and immediately following, incubation for 60 min at 37°C. As a negative control distilled water was used instead of either cells or supernatant sample.

Each figure represents the average of triplicate samples, none of which differed by more than 10%.

7.17. Standard Curve for Molecular Weight Estimation



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9. Publications Arising from this Study

N. J. Gully and A. H. Rogers (1995) Some observations on the nutritional requirements of *Eikenella corrodens* ATCC 23834T grown in continuous culture. *Oral Microbiology and Immunology*, v. 10 (2), pp. 115–118, April 1995

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N. J. Gully and A. H. Rogers (1996) Energy production and peptidase activity in *Eikenella corrodens*.

FEMS Microbiology Letters, v. 13 (2/3), pp. 209–213, June 1996

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