The isolation and characterisation of *Acinetobacter* strains present in the liquid phase of an anaerobic fixed bed digester, while treating a petrochemical effluent

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Abstract

Twenty nine Gram-negative, oxidase-negative, non-sporeforming rods or diplococci, resembling *Acinetobacter*, were isolated from the liquid phase of an anaerobic fixed bed digester, while treating a petrochemical effluent. Six reference strains, twelve *Acinetobacter* strains from clinical specimens, six *Acinetobacter* isolates from an activated sludge process and two strains isolated from river sediment were included in the study. The similarities between the different strains were calculated using Sokal and Michener's similarity coefficient and single linkage clustering techniques. The fifty three strains were recovered in four clusters. The *Acinetobacter* strains in the major cluster consisting of two large subclusters, linked at or above the 93% S-level. *Acinetobacter* strains isolated from the anaerobic digester and from the activated sludge process were included in the same subcluster, and identified as *Acinetobacter calcoaceticus* var. *lwoffi*, while all the strains obtained from clinical specimens were clustered together and were representative of *Acinetobacter calcoaceticus* var. *anitratus*. The presence of *Acinetobacter* strains in the anaerobic digester indicated that these aerobic organisms had a functional role to play in the primary anaerobic digestion reactions.

Introduction

The present understanding of the microbial populations in anaerobic digestion systems is still rather limited and based mainly on bacterial analyses of sewage sludge, animal manure digesters and the rumen. The anaerobic digestion process involves a mixture of interacting microbial species, most of which do not produce methane. Four major microbial groups can be distinguished, of which the acidogens are the largest group, comprising 90% of the population (Zeikus, 1980).

Improvements in the isolation techniques have revealed that most of the acidogenic bacteria present in digesters are strict anaerobes. In spite of these findings, the aerobic and facultative anaerobic bacteria still form a significant and constant portion of the total digester population (Hobson and Shaw, 1974; Iannotti *et al.*, 1982). Their main role is probably the rapid utilisation of oxygen, thereby restoring anaerobic conditions suitable for growth of strict anaerobes (Toerien, 1967), as well as the fermentation of sugars (Hobson and Shaw, 1974). The bacterial population in a digester can usually be divided into functional bacteria, bacteria accidentally introduced, and harmful bacteria (Kistner, 1960). However, the development of typical digester populations is stimulated and dependent upon the specific substrate and carbon source with which it is fed (Zeikus, 1980).

In the literature it has been shown that *Acinetobacter* strains are part of the acidogenic population of polluted waters, sewage sludge, anaerobic digesters, soil, food and infections (Baumann *et al.*, 1968; Rosenthal and Freundlich, 1977; Snodgrass and Koburger, 1967; Warskow and Juni, 1972). De Haast and Britz (1986) also reported the presence of *Acinetobacter* in anaerobic digesters and 2 to 3% of the aerobic and facultative anaerobic bacteria in an anaerobic fixed bed digester, treating a petrochemical effluent were found to resemble *Acinetobacter* (Meyer, 1985).

The genus currently consists of only a single species, *Acinetobacter calcoaceticus* (Juni, 1984; Lessel, 1971), which is divided into two biochemically dissimilar groups (Baumann *et al.*, 1968). However, only two taxa, *Acinetobacter calcoaceticus* and *Acinetobacter lwoffi*, are listed in the Approved List of Bacterial Names (Skerman *et al.*, 1980). More resently Bouvet and Grimont (1986) proposed three new species, *Acinetobacter baumannii*, *A. johnsonii* and *A. junii* and emended the descriptions of *A. calcoaceticus* and *A. lwoffi* into a new combination, *A. haemolyticus*.

Most of the available knowledge on *Acinetobacter* has, however, been obtained from clinical specimens (Hendriksen, 1973) and as a result little is known about *Acinetobacter* strains present in anaerobic systems. In view of this lack of information on *Acinetobacter* and their possible role in anaerobic digestion systems, the main objectives of this study were thus to isolate and characterise *Acinetobacter* strains present in an anaerobic digester, and compare them to strains isolated from an activated sludge plant, clinical specimens and reference strains using standard API systems and numerical methods.

Materials and methods

Digester and operating conditions

An anaerobic downflow fixed bed digester (Nel *et al.*, 1985) was used to isolate the bacterial strains. The digester was fed with an acetate rich petrochemical effluent, generated during the Fischer-Tropsch Sasol coal-to-oil process. The effluent consisted mainly of monocarboxylic acids (1,0 to 1,3% $m.v^{-1}$), small amounts of emulsified oils, phenols, alcohols and ketones and had a chemical oxygen demand (COD) of 11 660 $mg.\ell^{-1}$ and a volatile fatty acid concentration of 7 600 $mg.\ell^{-1}$. The fatty acids were mainly acetic (67%), propionic (16%), iso-butyric (3%), n-butyric (8%), iso-valeric (2%) and n-valeric acid (4%). Before using the petrochemical effluent, it was filtered, KH_2PO_4 (500 $mg.\ell^{-1}$) and urea (500 $mg.\ell^{-1}$) were added, the pH was adjusted to pH 3,95 with equal amounts of 6N NaOH, 6N KOH and 3N $Ca(OH)_2$ (Nel and Britz, 1986) and boiled. A sterile trace element solution, as recommended by Nel *et al.* (1985) was added. The petrochemical effluent was fed at a loading rate of 8,33 kg COD ($m^3.d^{-1}$) at an hydraulic retention time (HRT) of 1,4 days. The COD and fatty acid removal efficiencies were above 90% and a methane yield of 0,306 m^3 (kg COD removed.d^{-1}) was obtain-

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ed. The digester effluent pH was 6,98 and the digester operated at 37°C.

Isolation of bacteria

Bacterial strains used in the study were obtained by taking samples from the liquid phase, using sterile syringes, from three different levels (2 cm, 20 cm and 50 cm depths). The samples were immediately serially diluted and processed on different media. A modification of the selective medium of Mandel *et al.* (1964) as composed by Holton (1983), as well as the enrichment medium of Baumann (1968) were used. Nutrient agar, to which 100 U.$m\ell^{-1}$ penicillin G was added (Juni, 1984) and MacConkey agar, with the lactose replaced by acetate, were also used as isolation media. Duplicate plates were aerobically incubated at 35°C for 72 hours. All non-pigmented mucoid colonies were purified by repeatedly streaking onto nutrient agar and pure cultures were maintained on agar slants at 4°C.

Morphological and biochemical tests

Cellular morphology was determined by bright field microscopy of the Gram-stained preparations. Motility was determined using the hanging drop method on 18 h cultures and bacterial flagella were observed with bright field optics.

The API 20E, API 20NE and API 50CHE microtube kits were used to determine the biochemical characteristics of the isolates according to instructions of the manufacturer (API System SA, La Balme Les Grottes, 38390 Montalieu Vercieu, France) and their standard abbreviations were used throughout this study. The following additional tests were also performed: oxidase, catalase, endospore formation and growth on MacConkey agar. The utilisation of acetate and propionate (0,05 % m.v^{-1}) in the basal medium of Aragno (1978), was determined gas chromatographically.

Chemical analyses

The utilisation of volatile fatty acids was determined using a Model 5830A Hewlett Packard gas chromatograph, equipped with a flame ionisation detector and a 1,8 m × 1,5 mm ID stainless steel column packed with Porapak Q, 80-100 mesh. The column temperature was 195°C, the detector temperature 250°C and the inlet temperature 180°C. Nitrogen was used as carrier gas at a rate of 60 $m\ell.min^{-1}$. Gas composition determinations were made using a Model 820 Perkin Elmer gas chromatograph equipped with a thermal conductivity detector, and a 4,0 m × 0,3 mm ID stainless steel column packed with Porapak N, 80-100 mesh. The oven temperature was set at 50°C and H_2 used as carrier gas at a flow rate of 30 $m\ell.min^{-1}$.

Other bacterial strains

Twelve clinical isolates were obtained from the Department of Medical Microbiology at the University of the Orange Free State. Six *Acinetobacter* strains isolated from an activated sludge plant (Lötter and Murphy, 1985) and two strains isolated from a dam sediment (Du Preez and Lategan, 1977) were also used in this study. The following reference strains obtained from the American Type Culture Collection (ATCC) and the Deutsche Sammlung von Mikroorganismen (DSM) were included: *Acinetobacter calcoaceticus* ATCC 23055; *A. calcoaceticus* ATCC 17925; *A. calcoaceticus* DSM 587; *Klebsiella pneumoniae* ATCC 9997; *Aeromonas hydrophyla* ATCC 9071 and *Achromobacter zerosis* ATCC 14780.

Computer analysis

The 84 unit characteristics used for the numerical study were recorded as either positive, negative or redundant values. Both the Jaccard coefficient (S_J) (Sneath, 1957) and the similarity coefficient of Sokal and Michener (S_{SM}) (Sokal and Michener, 1958) were used to calculate the overall similarity between the different isolates. Clustering was performed, using single linkage clustering techniques (Sneath and Sokal, 1973), on a Sperry Univac 1100 computer. All reference strains were included in the clustering. Six strains, chosen randomly, were examined in duplicate to estimate the average probability of error (P) using the formula of Sneath and Johnson (1972).

Results and discussion

Isolation and enumeration

The average total aerobic counts from the liquid phase of the anaerobic fixed bed digester conducted on nutrient agar, supplemented with 100 U.$m\ell^{-1}$ penicillin G and MacConkey agar, were 2,4 × 10^4 and 1,2 × 10^4 $m\ell^{-1}$ colony forming units respectively. These results are in agreement with counts of aerobic and facultative anaerobic bacteria from fixed bed and conventional digesters (De Haast and Britz, 1986; Hobson and Shaw, 1974; Iannotti *et al.*, 1982).

All non-pigmented, mucoid, smooth colonies (Samuels *et al.*, 1969) obtained on the different media were purified, and 29 Gram-negative, non-motile, oxidase negative, plumb rods or coccobacilli were tentatively identified as members of the genus *Acinetobacter* (Juni, 1978; Warskow and Juni, 1972). Twenty-three of the 29 isolates were identified as *Acinetobacter calcoaceticus* and six as *Klebsiella pneumoniae* by means of the API Index. Seventeen of these *Acinetobacter* strains were obtained from the nutrient agar plates supplemented with penicillin G. Nine of the isolates were recovered from acetate containing MacConkey agar and three isolates were obtained from the enrichment medium by Baumann (1968). The inability to recover large numbers of *Acinetobacter* strains from the selective media by Holten (1983) (O isolates) and Baumann (1968) (3 isolates), was probably due to their low incidence level relative to the other Gram-negative bacteria present in the digester. Another reason might have been that Holton's medium was developed specifically for the isolation of saccharolytically active *Acinetobacter* strains from clinical specimens.

In this study, *Acinetobacter* strains could only be detected in samples from the upper level of the digester. No isolates were obtained from the 20 cm and 50 cm digester levels. This was probably as a result of the low oxygen concentration at the lower digester levels, preventing the growth of the strictly aerobic *Acinetobacter* strains (Juni, 1984). However, one strain was isolated from the digester effluent, suggesting that at shorter hydraulic retention times *Acinetobacter* could be washed out from the digester and thus withstand the anaerobic conditions. The low incidence of *Acinetobacter* in the anaerobic digester is in agreement with results of Seiler (1979) and Baumann (1968) who reported the low occurrence of *Acinetobacter* in water and soil. Similar results from anaerobic fixed bed digesters were found by De Haast and Britz (1986) and Meyer (1985). *Acinetobacter*

strains were also found in low numbers in activated sludge samples (Benedict and Carlson, 1971). In contrast with the low levels of *Acinetobacter* present in anaerobic digesters, *Acinetobacter* was found to be the dominant Gram-negative bacteria in the aerobic basin of the activated sludge process when using the API 20E system to identify viable colonies. High numbers of *Acinetobacter* strains were also isolated from the anaerobic basin of the same process, but these failed to grow anaerobically (Lötter and Murphy, 1985).

Numerical analysis

The average probability error calculated for six randomly chosen duplicate strains was found to be 0,1% and would not produce serious distortions of the taxonomic structure (Sneath and Johnson, 1972). The similarity levels and clustering sequence obtained when using the Jaccard coefficient (S_J), were in general agreement with Sokal and Michener's coefficient (S_{SM}), but the final linkage was at a lower level. The results of the numerical analysis of the 49 isolates and 6 reference strains are illustrated as a simplified dendrogram (Figure 1) using the S_{SM} similarity coefficient. From the dendrogram it can be seen that the strains could be grouped, at a similarity level (S-level) of 84%, into four major clusters (A, B, C and D). The low final S-level of 66% (Figure 1) was mainly due to the unrelated reference strains (clusters B and D) and the *Klebsiella* strains (cluster C) included in the study.

All the strains in cluster A (75% of the total) were Gram-negative, non-motile plumb rods or coccobacilli usually occurring in pairs or in short chains and becoming coccoid in the latter stages of growth. These strains resisted decolorisation with alcohol during the Gram-staining procedure, formed no endospores, were catalase positive and oxidase negative. All cluster A strains were able to utilise 500 mg.ℓ^{-1} acetate and propionate. Common features for this cluster obtained with the API Systems were: positive for CAP and negative for TRP, ONPG, LDC, IND, H_2S production, TDA and ODC. They were not able to produce acid from glycerol, erytritol, D-arabinose, L-xylose, adonitol, sorbose, rhamnose, dulcitol, mannitol, sorbitol, arbutin, sucrose, trehalose, raffinose, starch, glycogen, xylitol and arabitol.

Cluster B was represented by a single reference strain *Aeromonas hydrophyla*, ATCC 9071. Cluster C contained six isolates in addition to the *Klebsiella pneumoniae* reference strain and was therefore taken as representative of the *Klebsiella pneumoniae* species. Cluster D was represented by a single reference strain, *Achromobacter zerosis* ATCC 14780. The identification of clusters B, C and D were confirmed by comparing the characteristics to the profiles given in the API System. The low similarity of these reference strains to the *Acinetobacter* strains is an indication of the diversity found between these Gram-negative organisms.

Cluster A was representative of the *Acinetobacter calcoaceticus* species and consisted of two major subclusters A1 and A4. The division into two major clusters confirms the results of Henderson (1967), Baumann *et al.* (1968) and Pagel and Seyfried (1976). These workers also divided the genus *Acinetobacter*, on the basis of phenotypic characteristics, into two major groups. Subclusters A1 and A4 were linked at an S-level of 93%, indicating the close relatedness of the two distinct subclusters. The *Acinetobacter* strains isolated from the anaerobic digester and from the activated sludge plant were included in subcluster A1. Subcluster A1 also contained the reference strain *Acinetobacter calcoaceticus* ATCC 17925. The members of this cluster were identified using the Analytical Profile Index as *Acinetobacter calcoaceticus* var. *lwoffi*. This subcluster was therefore taken as representative of *A. calcoaceticus* var. *lwoffi*. All the strains in this subcluster failed to assimilate or produce acids from carbohydrates. This characteristic of the *A. calcoaceticus* var. *lwoffi* species has been the cause of frequent mis-identification. These strains are almost metabolically inert and therefore difficult to place in the genus *Acinetobacter* unless the non-reactivity is relied upon as a differential characteristic.

With the exception of two strains isolated from river sediment (Du Preez and Lategan, 1977), subcluster A4 consisted only of isolates from clinical specimens, and constituted 26% of the total strains studied. Strains of subcluster A4, with a final S-level of 95%, were positive for ADI, CIT, PAC, negative for VP and were able to produce acids from L-arabinose, ribose, D-xylose, mannose, amygdalin, maltose, melibiose and D-fucose. Strains of this group were identified as *Acinetobacter calcoaceticus* var. *anitratus* by means of the API system. No reference strain was grouped in this cluster and as a result of the API identification these strains were taken as representative of *A. calcoaceticus* var. *anitratus*.

The reference strain *A. calcoaceticus* DSM 587 and type strain *A. calcoaceticus* ATCC 23055 (clusters A2 and A6 respectively), as well as two isolates from an activated sludge plant (clusters A3 and A5), were unclustered. The similarity between clusters A1, A4 and the type strain *A. calcoaceticus* ATCC 23055 (cluster A6) was fairly low with several incontestable differences. These include the ability to reduce nitrate, positive reactions with PNPG, URE, ability to produce acid from inositol and negative reactions with CAP and MLT. Baumann *et al.* (1968) also found the type strain *A. calcoaceticus* ATCC 23055 to be unclustered.

It is known that a number of phenotypic groups exist in the genus *Acinetobacter* (Juni, 1984). Baumann *et al.* (1968) divided *Acinetobacter* into two major groups on the basis of nutritional versatility. Clusters A1 and A4, as found in this study, could easily be separated by means of their ability or inability to produce acid from most carbohydrates. If the separation of Baumann *et al.* (1968) is applied to the results of this study, subcluster A1 corresponds to their subgroup B2, subcluster A2 to their subgroup B4, and subclusters A3 and A4 to their subgroup A1. Subcluster A5 and A6 cannot be assigned to any of the specific groups of Baumann *et al.* (1968).

Assigning *Acinetobacter* strains to taxonomic positions as proposed by Bouvet and Grimont (1986), is more difficult. They described three new species and emended the description of the currently accepted species, using a more stringent method of hybridization and a systematic search for identification characteristics. They also recognized 12 genospecies, two of which had valid names (*A. calcoaceticus* and *A. lwoffi*), and required only emended descriptions. The subcluster A1, as found in this study, corresponded to their genospecies 8, the emended *A. lwoffii* species. Subcluster A2 corresponded to their genospesies 4, a new species *'Acinetobacter haemolyticus'*, on the basis that gelatin is hydrolyzed. Subclusters A3 and A4 corresponded to their genospecies 2, a new species *'Acinetobacter baumannii'*. Subclusters A5 and A6 which include the type strain, could not be assigned to a Bouvet and Grimont (1986) genospecies.

In the numerical taxonomic study of Baumann *et al.* (1968), which was based on nutritional properties, the authors did not identify any characteristics which could be used for species separation, and therefore the work of Bouvet and Grimont (1986) filled a huge gap in the taxonomy of *Acinetobacter*. Their results, however, will only be confirmed in the future when strains, similar to ungrouped strains, are available for study. Although there has been little accordance on the taxonomy of the genus, the current generic description (Juni, 1984) allows unambiguous

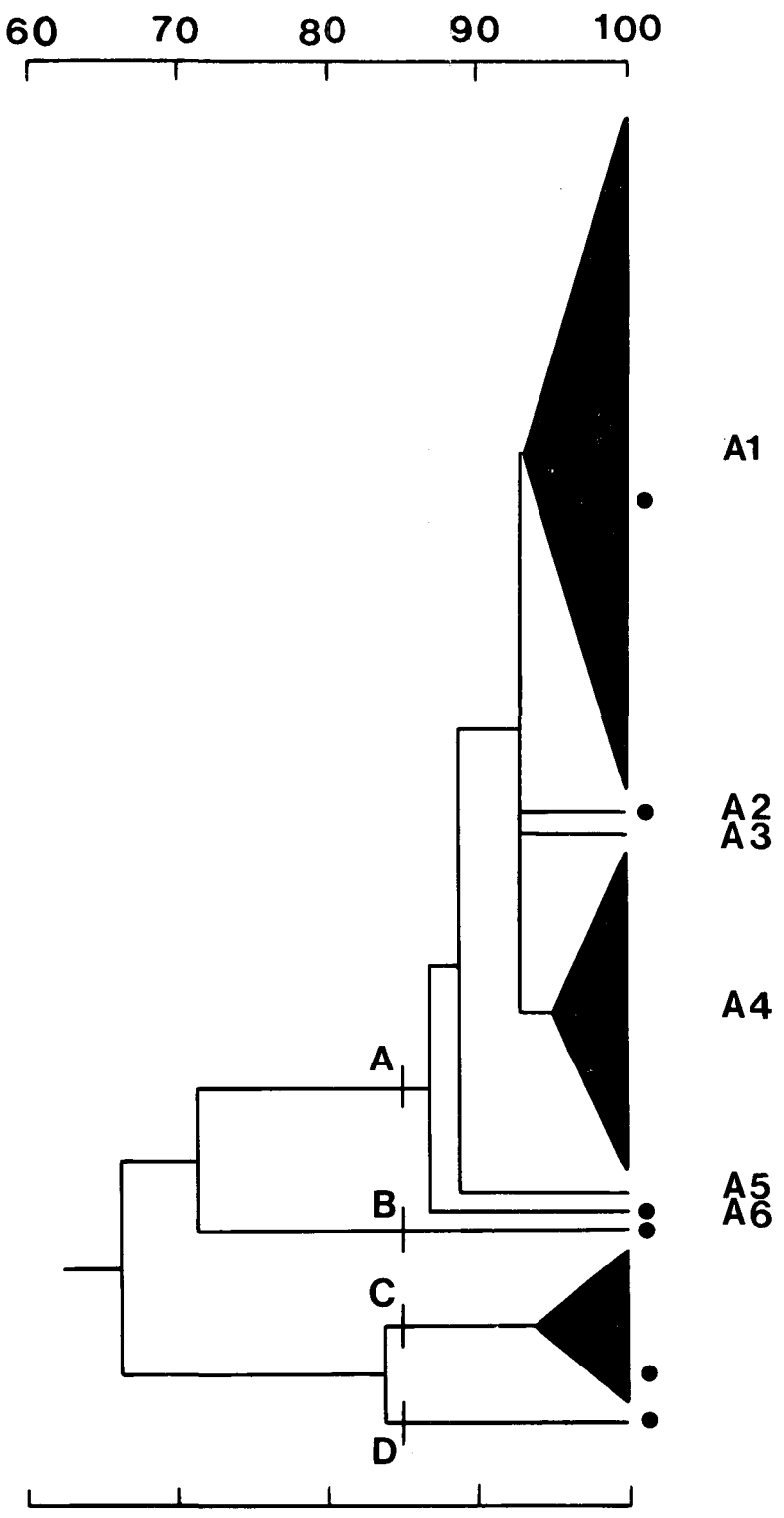

Figure 1
Simplified dendrogram showing the relationship between clusters based on the S_{SM} coefficient and single linkage clustering techniques. (●) = reference strains.

identification to the genus level. Identification to species level is necessary to trace *Acinetobacter* strains in hospitals in view of their role as opportunistic nosocomial pathogens (Rosenthal and Freundlich, 1977) and to differentiate between clinical and environmental strains.

It is known that the development of typical populations in anaerobic digesters is stimulated and dependent upon the specific substrate and carbon source with which it is fed (Zeikus, 1980). The presence of only one *Acinetobacter* species, *A. calcoaceticus* var. *lwoffi*, in the anaerobic digester shows that the substrate composition influenced the type of bacteria that would survive. Aerobically the *Acinetobacter* strains utilised acetate and propionate, which were the main carbon sources of the petrochemical substrate. The fact that no carbohydrates were present in the sterile fatty acid containing petrochemical substrate used in this study would be advantageous to the development of the acetate utilising *A. calcoaceticus* var. *lwoffi*. The original source of the *Acinetobacter* strains was the sewage which was used as inoculum at the start-up of the digester. Since no further sludge had been added, and a sterile substrate had been used throughout the study, it is difficult to explain their presence as "passengers" alone (Hobson and Shaw, 1974). Furthermore, the bacteria were still present after 4 years of continuous operation, for the last year at an HRT of 1,4 days, indicating that these organisms can survive and grow in the digester environment and might possibly have a functional role in the primary digestion reactions. It also appears as if the *Acinetobacter* strains can survive on the oxygen dissolved in the feed, thereby providing complete anaerobic conditions for the obligate anaerobes to convert the substrate ultimately to methane. It will, however, become necessary in future research to acquire a comprehensive insight into the microbiological and biochemical properties of the acidogenic bacteria governing the anaerobic digestion process. This will enable researchers to increase digester performance by inoculating the digester with preselected, thoroughly identified, organisms.

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References

ARAGNO, M. (1978) Enrichment, isolation and preliminary characterisation of a thermophilic endospore-forming hydrogen bacterium. *FEMS Microbiol. Lett.* **3** 13-15.

BAUMANN, P. (1968) Isolation of *Acinetobacter* from soil and water. *J. Bacteriol.* **96** 39-42.

BAUMANN, P., DOUDOROFF, M. and STAINIER, R.Y. (1968) A study of the *Moraxella* group. II. Oxidative-negative species (genus *Acinetobacter*). *J. Bacteriol.* **95** 1520-1541.

BENEDICT, R.G. and CARLSON, D. (1971) Aerobic heterotrophic bacteria in activated sludge. *Water Res.* **5** 1023-1030.

BOUVET, P.J.M. and GRIMONT, P.A.D. (1986) Taxonomy of the genus *Acinetobacter* with recognition of *Acinetobacter baumannii* sp. nov., *Acinetobacter haemolyticus* sp. nov., *Acinetobacter johnsonii* sp. nov., and *Acinetobacter junii* sp. nov. and emended descriptions of *Acinetobacter calcoaceticus* and *Acinetobacter lwoffii*. *Int. J. Syst. Bacteriol.* **36** 228-240.

DE HAAST, J. and BRITZ, T.J. (1986) Characterization of aerobic and facultative anaerobic bacteria from the liquid phase of an anaerobic fixed-bed digester treating a cheese whey substrate. *Microbial Ecol.* **12** 173-179.

DU PREEZ, J.C. and LATEGAN, P.M. (1977) Microbial treatment of an industrial effluent containing volatile fatty acids. *S. Afr. J. Sci.* **73** 349-351.

HENDERSON, A. (1967) The saccharolytic activity of *Acinetobacter lwoffi* and *A. anitratus*. *J. Gen. Microbiol.* **49** 487-489.

HENDRIKSEN, S.D. (1973) *Moraxella*, *Acinetobacter* and the *Mimae*. *Bacteriol. Rev.* **37** 522-561.

HOBSON, P.N. and SHAW, B.G. (1974) The bacterial population of piggery-waste anaerobic digesters. *Water Res.* **8** 507-516.

HOLTON, J. (1983) A note on the preparation and use of a selective differential medium for the isolation of *Acinetobacter* spp. from clinical sources. *J. Appl. Bacteriol.* **54** 141-142.

IANNOTTI, E.L., FISCHER, J.R. and SIEVERS, D.M. (1982) Characterisation of bacteria from a swine manure digester. *Appl. Environ Microbiol.* **43** 136-143.

JUNI, E. (1978) Genetics and physiology of *Acinetobacter*. *Ann. Rev. Microbiol.* **32** 349-371.

JUNI, E. (1984) Genus III. *Acinetobacter* Brisou and Prévot 1954, p. 303-307. In: Krieg, N.R., and Holt, J.G. (Ed.). *Bergey's Manual of Systematic Bacteriology*, Vol. 1. Williams and Wilkins Co., Baltimore.

KISTNER, A. (1960) An improved method for viable counts of bacteria of the ovine rumen which ferment carbohydrates. *J. Gen. Microbiol.* **23** 565-576.

LESSEL, E.F. (1971) International committee on nomenclature of bacteria. Subcommittee on the taxonomy of *Moraxella* and allied bacteria. *Int. J. Syst. Bacteriol.* **21** 213-214.

LÖTTER, L.H. and MURPHY, M. (1985) The identification of heterotrophic bacteria in an activated sludge plant with particular reference to polyphosphate accumulation. *Water SA* **11**(4) 179-184.

MANDEL, A.D., WRIGHT, K. and McKINNON, J.M. (1964) Selective medium for the isolation of *Mima* and *Herellea* organisms. *J. Bacteriol.* **88** 1524-1515.

MEYER, L.C. (1985) Anaërobe vertering van 'n petrochemiese fabrieksuitvloeisel met behulp van vastebedkolomreaktors. M.Sc. thesis. University of the Orange Free State. Bloemfontein.

NEL, L.H., BRITZ, T.J. and LATEGAN, P.M. (1985) The effect of trace elements on the performance efficiency of an anaerobic fixed-film digester treating a petrochemical effluent. *Water SA* **11**(3) 107-110.

NEL, L.H. and BRITZ, T.J. (1986) The influence of different substrate pH values on the performance of a downflow anaerobic fixed bed reactor treating a petrochemical effluent. *Biotechnol. Lett.* **8** 293-298.

PAGEL, J.E. and SEYFRIED, P.L. (1976) Numerical taxonomy of aquatic *Acinetobacter* isolates. *J. Gen. Microbiol.* **95** 220-232.

ROSENTHAL, S.L. and FREUNDLICH, L.F. (1977) The clinical significance of *Acinetobacter* species. *Health Laboratory Science* **14** 194-198.

SAMUELS, S.B., PITTMAN, B. and CHERRY, W.B. (1969) Practical physiological scheme for the identification of *Herellea vaginicola* and its differentiation from similar organisms. *Appl. Microbiol.* **18** 1015-1024.

SEILER, VON H. (1979) Taxonomische Untersuchungen über *Acinetobacter* aus Oberflächenwasser. *Arch. Hydrobiol.* **85** 57-71.

SKERMAN, V.B.D., McGOWAN, V. and SNEATH, P.H.A. (1980) Approved lists of bacterial names. *Int. J. Syst. Bacteriol.* **30** 225-420.

SNEATH, P.H.A. (1957) The application of computers to taxonomy. *J. Gen. Microbiol.* **17** 201-226.

SNEATH, P.H.A. and JOHNSON, R. (1972) The influence on numerical taxonomic similarities of errors in microbiological tests. *J. Gen. Microbiol.* **72** 377-392.

SNEATH, P.H.A. and SOKAL. R.R. (1973) *Numerical Taxonomy: the principles and practice of numerical classification.* W.H. Freeman, San Francisco.

SNODGRASS, C.J. and KOBURGER, J.A. (1967) The isolation of the tribe *Mimeae* in foodstuffs. *J. Food Sci.* **32** 589-591.

SOKAL, R.R. and MICHENER, C.D. (1958) A statistical method for evaluating systematic relationships. *Kansas Univ. Sci. Bull.* **38** 1409-1438.

TOERIEN, D.F. (1967) Enrichment culture studies on aerobic and facultative anaerobic bacteria found in anaerobic digesters. *Water Res.* **1** 147-155.

WARSKOW, A.L. and JUNI, E. (1972) Nutritional requirements of *Acinetobacter* strains isolated from soil, water and sewage. *J. Bacteriol.* **112** 1014-1016.

ZEIKUS, J.G. (1980) Microbial populations in digesters. In: Stafford, D.A., Wheatley, B.I., and Hughes D.E. (Eds.). *Anaerobic Digestion.* Applied Science Publishers, London. pp. 61-89.