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*RESEARCH ARTICLE*

# The applicability of the API 20E and API Rapid NFT systems for the identification of bacteria from activated sludge

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**The purpose of this study was to test the applicability of the API 20E and API Rapid NFT systems for the identification of some predominant gram-negative and gram-positive bacteria isolated from lab-scale activated sludge treatment systems. In this study, one lab-scale sequencing batch reactor (SBR) and one lab-scale continuous-flow stirred tank reactor (CFSTR) were setup. After both reactors had reached equilibrium, many pure cultures isolated from the activated sludge in both systems were obtained and many morphological, biochemical, physiological tests were conducted to identify each pure culture. The API 20E system is a standardized, miniaturized version of conventional procedures for rapid identification of *Enterobacteriaceae* and other gram-negative bacteria, and the Rapid NFT kit is used for the identification of the gram-negative, non-fermentative bacteria. Also, a Phillips 300 Transmission Electron Microscope and a Phillips 301 Transmission Electron Microscope were applied to further verify the identification of some genera. According to the results of this study, it has been concluded that some commercial products, such as API 20E system and API Rapid NFT system, can be applied for the identification of microorganisms only at the genus level. Many other additional morphological, biochemical, and physiological tests are always needed to obtain the exact identification of each microorganism at the species level. More advanced technologies such as 16S rRNA may be necessary, however, for a rapid identification of the total bacterial population. In this study, it has also been found that *Brevibacterium acetylicum* and *Pseudomonas vesicularis* are two of the most dominant species in the activated sludge of CFSTR system. Gram-positive bacteria such as members of the genus *Arthrobacter* have shown to be very significant and predominant in the SBR system.**

As a rule, the identification of pure cultures isolated from activated sludge systems can be carried out using Bergey's Manual (Holt, 1986), The Prokaryotes (Starr and Stolp, 1981), and other references (Leifson, 1960; Lighthart and Loew, 1972; Collins and Lyne, 1976; Ward et al. 1986; Atlas et al. 1988; Labeda, 1990). The API 20E (Analytab Products Inc., 1985) and API Rapid NFT systems are two commercial products, made by API Analytab Products, Division of Sherwood Medical, New York, USA. The API 20E system uses 20 miniature reaction compartments (cupules) that produce 23 biochemical reactions, and is a standardized, miniaturized version of conventional procedures for rapid identification of *Enterobacteriaceae* and other gram-negative bacteria. The major advantage of the API 20E system is that it is more convenient and easier to identify gram-negative bacteria than the conventional tests mentioned in the above references. The Rapid NFT kit is used for identification of gram-negative, non-fermentative bacteria. The Rapid NFT system is similar to the 20E system in that a profile number is obtained from standard tests performed on the organisms. Most studies on the population structure of activated sludge are over 30 years old. Several gram-negative bacterial genera, such as *Pseudomonas, Achromobacterium* and *Flavobacterium*, were found to be dominant and responsible for sewage purification by Allen (1943). In 1952, McKinney and Horwood isolated eleven organisms which could produce flocs when aerated in a suitable substrate, and showed that several microorganisms are capable of producing flocs

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similar to activated sludge (McKinney and Horwood, 1952). Anderson and McCoy reported in 1963 that *Pseudomonas* and related genera were predominant in the activated sludge from aeration tanks of municipal waste treatment plants (Anderson and McCoy, 1963), while Dias and Bhat reported in 1964 that *Zoogloea* and *Comamonas* were the predominant bacteria in activated sludge (Dias and Bhat, 1964). These authors also stated that occasionally other genera of bacteria could appear predominant without adversely affecting the system. Several other types of microorganisms were also isolated from the activated sludge. Lighthart and Oglesby (1969) and Lighthart and Loew (1972), presented a large number of microorganisms found to be predominant in activated sludge, and these predominant microorganisms were identified as *Flavobacterium*, *Achromobacter*, and a group classified as *Pseudomanads.* Unz and Dondero (1970) found *Flavobacterium*, *Alcaligenes* and *Pseudomonas* to be the dominant organisms present.

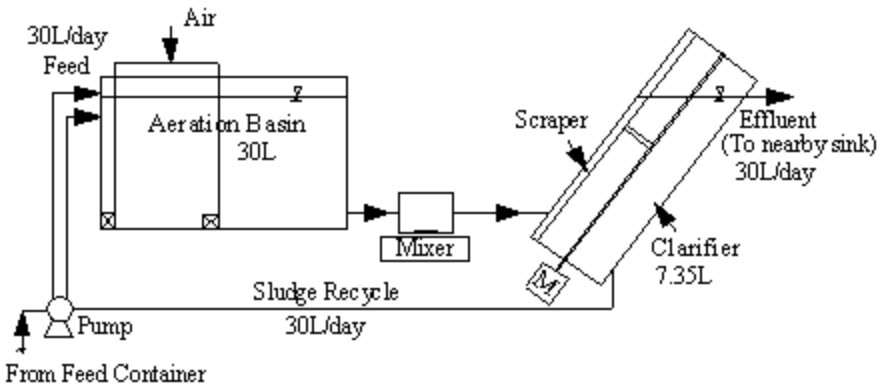


**Figure 1. Bench scale CFSTR system.**

## Materials and Methods

### (a) CFSTR system design

### System design and operating data

The bench scale continuous flow stirred tank reactor is shown in Figure 1, and its operating data shown in Table 1. The feed was pumped to the aeration basin continuously at a rate of 30 liters per day, and the effluent was directly discharged to a nearby sink. The volume of mixed liquor in the aeration basin was maintained at 30 liters. Sludge was recycled continuously through the same pump used for feed and also at a rate of 30 liters per day.

Therefore feed, recycled sludge, and effluent flowed into and out of the reactors, respectively, on a continuous flow basis. By wasting one liter of activated sludge per day, the biological solids retention time was maintained at about 30 days. The cylindrical clarifier was inclined at an angle of 45 degree, so the settled sludge could be easily collected and recycled.

The volume of the clarifier was 7.35 liters, so the hydraulic retention time was about 2.94 hours. A scraper, rotating constantly and slowly at a rate of approximate one revolution per minute, was installed inside the clarifier. The purpose of this scraper was to prevent the sludge from attaching onto the wall.

### Feed composition and seed sludge

The feed composition of the CFSTR is shown in Table 2. The seed sludge of the CFSTR system in this study was originally obtained from one well-operated domestic wastewater treatment plant, called H.C. Morgan Treatment Plant, located at Auburn, Alabama, USA.

**Table 1. CFSTR and SBR operating data.**

| Reactor | CFSTR | SBR |
|---|---|---|
| Volume feed rate (Aeration Basin) | 30.0 (L/day) | 2.5 (L/cycle) |
| Aeration basin volume | 30.0 (L) | 2.94 (L) |
| Clarifier volume | 7.35 (L) | 2.94 (L) |
| Residual basin volume | --- | 0.44 (L) |
| Volume wasted | 1.00 (L/day) | 0.105 (L/cycle) |
| Hydraulic retention time (Aeration Basin) | 1.00 (day) | --- |
| Solids retention time | 30.0 (day) | 28 (day) |
| Sludge recycling rate | 30.0 (L/day) | --- |

### (b) SBR system design

### System design and operating data

The bench scale sequencing batch reactor used in this study is shown in Figure 2, and its operating data is also given in

Table 1. The SBR was operated two cycles per day, and one operating cycle was 12 hours. The mixed liquor was stirred and aerated for a total feed and mix time of about 10.6 hours, and then allowed to settle for 45 minutes. Finally, the sludge remained idle for the remainder of the cycle resulting in a total draw and idle time of 40 minutes.

### Feed composition and seed sludge

The feed composition of the SBR is shown in Table 3. The seed sludge of the SBR system was also originally obtained from H.C. Morgan Treatment Plant.

**Table 2. Feed composition of CFSTR.**

| Material | Conc., mg/l | Material | Conc., mg/l |
|---|---|---|---|
| Nutrient broth | 300.0 | $KNO_3$ | 3.0 |
| $KH_2PO_4$ | 44.0 | NaCl | 100.0 |
| NaOH | 16.7 | $FeCl_3 6H_2O$ | 5.0 |
| $CaCl_2 \cdot 2H_2O$ | 132.4 | $MnSO_4 \cdot H_2O$ | 12.8 |
| $MgSO_4 \cdot 7H_2O$ | 100.0 | $(NH_4)_2\ SO_4$ | 118.4 |
| Glucose | 140.0 | $NaHCO_3$ | 467 |
| Yeast extract | 32.0 | | |

### (c) Confirmation of steady state (equilibrium)

After the system had been operated for more than 2 months, routine effluent samples were taken and filtered through glass fiber filters to check stable operation. Although there was no necessity to filter the accompanying feed samples, both the effluent and feed samples were filtered through 0.45 ìm membrane filters for nitrite and nitrate tests. Many tests were performed following the procedures in Standard Methods (Franson et al. 1985) to ensure that the system had reached equilibrium, such as MLVSS (Mixed-liquor Volatile Suspended Solids), COD, TKN (Total Kjeldahl Nitrogen), $NH_4$-N, $NO_3$-N, $NO_2$-N, pH, and DO. Each test was generally performed for at least four consecutive days and the data were averaged.

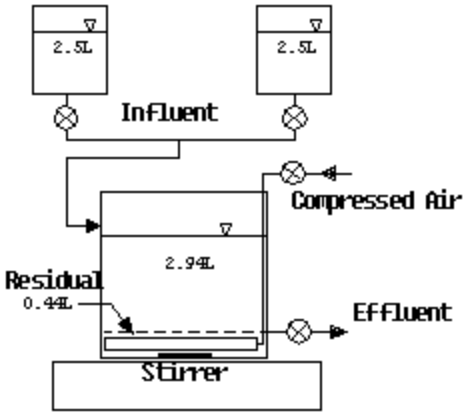


**Figure 2. Bench scale SBR system.**

### (d) Isolation and identification of microorganisms

### Preparation of sludge samples

After the activated sludge system was confirmed to have reached steady state, the sludge was then taken for the identification of predominant microorganisms. For the CFSTR system, samples were always taken directly from the aeration tank. For the SBR system, samples were always taken approximately 2 hours after the start of a new cycle.

**Table 3. Feed composition of SBR.**

| Material | Conc., mg/l | Material | Conc., mg/l |
|---|---|---|---|
| Nutrient broth | 300.0 | $KNO_3$ | 3.0 |
| $KH_2PO_4$ | 44.0 | NaCl | 100.0 |
| NaOH | 20.0 | $FeCl_3 \cdot 6H_2O$ | 5.0 |
| $CaCl_2 \cdot 2H_2O$ | 132.4 | $MnSO_4 \cdot H_2O$ | 12.8 |
| $MgSO_4 \cdot 7H_2O$ | 100.0 | $(NH_4)_2\ SO_4$ | 118.4 |
| Glucose | 140.0 | $NaHCO_3$ | 373.3 |
| Yeast extract | 32.0 | | |

### Preparation of pure culture

It is known that many microorganisms very active in activated sludge are generally slow growers and also mostly do not grow on agar plates. Therefore, the microorganisms isolated from the activated sludge in this study are predominantly the fast growing bacteria. A 10 mL of sample was added to 90 mL of a sodium tripolyphosphate solution [STS (5 mg/L)] and homogenized rapidly in a countertop blender for at least three minutes. Subsequent 1/10 dilutions, which ranged from $10^{-2}$ to $10^{-6}$, were made with the STS and spread on agar plates. For culturing the dominant bacteria, a general-purpose media nutrient agar (Difco) was chosen.

Although probably many strains of organisms present in activated sludge systems could not grow on general nutrient agar media, a special medium, called feed agar, was prepared and used to compare the growing predominant organisms with those growing on nutrient agar. The feed agar had the same relative composition as the feed used for the CFSTR and the SBR systems, but was more concentrated than the feed by a factor of ten.

Two sets of nutrient agar plates and two sets of feed agar plates were spread-plated with each sample dilution and incubated at room temperature (varied from 20ºC to 25ºC) for five to seven days. After incubation, the colonies that appeared visually dissimilar were chosen, counted, and transferred to fresh agar plates. Identification of microorganisms did not commence until it was evident that a pure culture had been obtained. Each pure culture was also transferred to tryptone glucose extract broth or nutrient broth for preservation in case further testing was required.

### Identification of pure culture

The major identification tools used in this study were the API 20E system and the API Rapid NFT system. When the gram-negative pure culture was obtained, it was first tested by both API systems incubating at the same temperature it was isolated. However, many other additional tests are always required for further identification tests of the microorganisms.

Through the additional use of Bergey's Manual (Holt, 1986), The Prokaryotes (Starr and Stolp, 1981), and other references (Leifson, 1960; Lighthart and Loew, 1972; Collins and Lyne, 1976; Ward et al. 1986; Atlas et al. 1988; Labeda, 1990), many traditional morphological, biochemical, and physiological tests were selected for this study. The API biochemical test results were then used with the additional traditional tests to collect necessary data needed for an exact identification. Some of these methods used for the identification of microorganisms are listed in Table 4.

Since the API 20E system does not test very well for gram-positive organisms, many conventional tests described in Bergey's Manual have been used for the identification. A Phillips 300 Transmission Electron Microscope and a Phillips 301 Transmission Electron Microscope were further applied to verify the identification of many predominant genera.

Many of the gram-negative and gram-positive isolates were also sent directly to apiLAB, a division of Analytab Products, Inc., for verifying their identities with the results obtained in our laboratory.

**Table 4. Methods used for the identification of microorganisms.**

| | |
|---|---|
| 1. API-20E | 14. Phenol red broth (glucose, lactose, and sucrose) |
| 2. API Rapid NFT | 15. Starch agar |
| 3. Gram stain | 16. Thioglycollate broth |
| 4. Morphology | 17. Urease test |
| 5. Flagella test | 18. Nitrate, nitrite tests |
| 6. Motility test | 19. Oxidase test |
| 7. Heat spore test | 20. Simmon's citrate test |
| 8. Temperature tolerance test | 21. O/F tests |
| 9. Salt tolerance test | 22. O/129 test |
| 10. Catalase test | 23. Gelatin test |
| 11. Carbohydrate tests (glucose, lactose, sucrose, and fructose) | 24. Tryptone glucose extract agar (TGE) |
| 12. MacConkey agar | 25. Tryptone glucose extract agar (TGE) /crystal violet |
| 13. Blood agar | 26. Electron microscope |

## Results and Discussion

### (a) Stability tests

### CFSTR system

After an approximately two-month adaptation period for the CFSTR system, stability tests were performed for five consecutive days to confirm that a stable operation (steady state) had been reached.

Table 5 shows the average characteristics of the feed and the effluent of CFSTR system. From the data shown in Table 5, it is evident that this system operated normally with respect to substrate removal and nitrification. The pH value in the effluent was above 6.0, and the dissolved oxygen in the aeration basin was between 5.6 mg/L and 6.4 mg/L. Therefore, the system was determined to be stable.

**Table 5. CFSTR stability tests.**

| Characteristics | Feed (mg/L) | Effluent (mg/L) |
|---|---|---|
| COD | 389.7 | 33.1 |
| TKN-N | 45.2 | 0.26 |
| $NH_3$-N | 28.4 | 0.17 |
| $NO_2$-N | 0.01 | 0.64 |
| $NO_3$-N | 0.71 | 57.8 |
| pH | 7.8 | 6.41 |
| MLVSS | 2035 | --- |

### SBR system

At the time of these investigations, the SBR system had been operating for more than 6 months in the laboratory. Steady-state tests were performed for 4 consecutive days to confirm that a steady state operation of the SBR system had been reached. Table 6 shows the average values obtained from the steady state tests.

From the data shown in Table 6, it is evident that the SBR system also operated normally with respect to substrate removal and nitrification. The pH value in the effluent was above 7.0, and the dissolved oxygen in the aeration basin was about 6.5 mg/L. Therefore, the system was determined to be stable.

**Table 6. SBR stability tests.**

| Characteristics | Feed (mg/L) | Effluent (mg/L) |
|---|---|---|
| COD | 212.7 | 29.3 |
| TKN-N | 58.7 | 0.7 |
| $NH_3$-N | 24.7 | 0.03 |
| $NO_2$-N | 1.5 | 0.05 |
| $NO_3$-N | 6.2 | 54.1 |
| pH | 7.4 | 7.15 |
| MLVSS | 1560 | --- |

### (b) Bacteriology of CFSTR

The predominant microorganisms isolated from the CFSTR system are presented in Table 7 and Table 8. *Brevibacterium acetylicum* and *Pseudomonas vesicularis* were two of the most dominant species isolated on both nutrient agar and feed agar.

The bacterial count of *B. acetylicum* on nutrient agar (34 x $10^6$ cfu/mL) was about the same as that on feed agar (36 x $10^6$ cfu/mL). However, the count of *P. vesicularis* on nutrient agar was much higher than that on feed agar. Similar results were observed for other less dominant bacteria. For example, *Aeromonas hydrophila*, *Acinetobacter calcoaceticus*, *Pasteurella aerogenes*, *Achromobacter group V D*, and one *Arthrobacter* sp. were only found in nutrient agar, while *P. maltiphila*, *Comamonas testosteroni*, and *Acaligenes faecalis* were only isolated from feed agar.

**Table 7. Predominant bacteria isolated from CFSTR (Nutrient Agar).**

| Nutrient Agar Medium | Counting (cfu/mL) |
|---|---|
| *Brevibacterium acetylicum* | 34 x $10^6$ |
| *Arthrobacter* sp. I [a] | 8 x $10^6$ |
| *Arthrobacter* sp. II [a] | 3 x $10^6$ |
| *Arthrobacter* sp. III [a] | 2 x $10^6$ |
| *Corynebacterium* sp. I [a] | 1 x $10^6$ |
| *Pseudomonas vesicularis* | 35 x $10^6$ |
| *Pseudomonas putrefaciens* | 1 x $10^6$ |
| *Pseudomonas pickettii biovar 2* | 3 x $10^6$ |
| *Aeromonas caviae* [b] | 2 x $10^6$ |
| *Achromobater Group V D* [c] | 6 x $10^6$ |
| *Aeromonas hydrophila* | 2 x $10^6$ |
| *Vibrio fluvialis* | 4 x $10^6$ |
| *Flavobacterium indolotheticum* | 7 x $10^6$ |
| *Acinetobacter calcoaceticus var lwoffi* | 5 x $10^6$ |
| *Pasteurella aerogenes* | 4 x $10^6$ |

[a] "I", "II", and "III" were labeled to represent different species.

[b] Most closely resembling this species.

[c] New name: *Ochrobactrum anthropi.*

Some other bacteria such as *Arthrobacter* spp. II and III, *Corynebacterium* sp. I, *Ps. putrefaciens*, *Ps. pickettii*, *A. caviae*, *V. fluvialis*, and *F. indolotheticum* were all isolated from both agars, although they were not very dominant in the CFSTR system. Therefore, considering the whole population structure of the activated sludge in CFSTR, there was no apparent or significant difference between the results obtained from nutrient agar and those from feed agar

### (c) Bacteriology of SBR

The predominant microorganisms isolated from the SBR system are listed in Table 9, which indicates that gram-positive organisms such as members of the genus *Arthrobacter* appeared to be very significant and predominant in the SBR system. It is obvious that most gram-positive strains were isolated from both nutrient and feed agars, but none of the same gram-negative bacteria were isolated from both agars. *Aeromonas caviae*, *Comamonas testosteroni*, *Pseudomonas maltiphila*, and *Flavobacterium indolotheticum* were only isolated from nutrient agar, and *Achromobacter group V D*, *Pseudomonas diminuta*, and *Aeromonas hydrophila* were only found in feed agar. Therefore, except for the gram-positive genera, there was a significant difference between the results obtained from nutrient agar and feed agar in the SBR system. It may be explained that some gram-negative microorganisms active in the activated sludge of SBR system can be obtained depending on the type of agar plates. However, these bacteria may not be very significant and predominant in the SBR system.

**Table 8. Predominant bacteria isolated from CFSTR (Feed Agar).**

| Feed Agar Medium | Counting (cfu/mL) |
|---|---|
| *Brevibacterium acetylicum* | 36 x $10^6$ |
| *Arthrobacter* sp. II [a] | 1 x $10^6$ |
| *Arthrobacter* sp. III [a] | 5 x $10^6$ |
| *Corynebacterium* sp. I [a] | 5 x $10^6$ |
| *Pseudomonas vesicularis* | 12 x $10^6$ |
| *Pseudomonas putrefaciens* | 2 x $10^6$ |
| *Pseudomonas maltiphila* | 4 x $10^6$ |
| *Pseudomonas pickettii biovar 2* | 3 x $10^6$ |
| *Aeromonas caviae* [b] | 1 x $10^6$ |
| *Comamonas testosteroni* | 2 x $10^6$ |
| *Alcaligenes faecalis* | 1 x $10^6$ |
| *Vibrio fluvialis* | 1 x $10^6$ |
| *Flavobacterium indolotheticum* | 4 x $10^6$ |

[a] "I", "II", and "III" were labeled to represent different species.

[b] Most closely resembling this species.

### Concluding Remarks

API 20E system and API Rapid NFT system can be applied for the identification of the dominating microorganisms, especially gram-negative ones, in the activated sludge wastewater treatment system. Since both API identification systems mostly can identify the gram-negative microorganisms in activated sludge only at the genus level, many additional morphological, biochemical, and physiological tests are always required to further identify them to the species level. In order to obtain a comprehensive view of the total microflora, it would be advisable to use molecular methods, such as 16S rRNA determinations.

The food:microorganism (F:M) ratio and dissolved oxygen concentration is always assumed to be relatively constant in a CFSTR, therefore the dominating microbial population in the system should remain constant. This can be seen from the results of this study. The predominant microbes isolated from the CFSTR have shown more diversities than from the

**Table 9. Microorganisms isolated from SBR.**

| Nutrient Agar | | Feed Agar | |
|---|---|---|---|
| *Arthrobacter* sp. I [a] | 80 x $10^6$ | *Brevibacterium acetylicum* | 3 x $10^6$ |
| *Arthrobacter* sp. II [a] | 130 x $10^6$ | *Arthrobacter* sp. II [a] | 6 x $10^6$ |
| *Arthrobacter* sp. III [a] | 20 x $10^6$ | *Arthrobacter* sp. III [a] | 106 x $10^6$ |
| *Aeromonas caviae* [c] | 50 x $10^6$ | *Achromobacter group V D* [b] | 15 x $10^6$ |
| *Comamonas testosteroni* | 80 x $10^6$ | *Pseudomonas diminuta* | 5 x $10^6$ |
| *Pseudomonas maltiphila* | 10 x $10^6$ | *Aeromonas hydrophila* | 6 x $10^6$ |
| *Flavobacterium indolotheticum* | 10 x $10^6$ | | |

[a] "I", "II", and "III" are labeled to express different species.

[b] New name: *Ochrobactrum anthropi*.

[c] Most likely resembling this species.

SBR (see Table 6, Table 7, and Table 8). For CFSTR system, there was also no apparent or significant difference in the microbial population grown on either nutrient or feed agar. For instance, *Brevibacterium acetylicum*, *Arthrobacter* spp. II and III, *Corynebacterium* sp. I, *Pseudomonas vesicularis*, *Ps. putrefaciens*, *Ps. pickettii*, *A. caviae*, *V. fluvialis*, and *F. indolotheticum* were isolated from both nutrient agar and feed agar, and it could be concluded that these microorganisms must be active and significant in the lab-scale CFSTR system. Especially, *Brevibacterium acetylicum* and *Pseudomonas vesicularis* have been known to be two of the most dominant species isolated on both nutrient agar and feed agar. This also means that both species could be playing very important roles on the substrate removal in the continuous-flow-type of wastewater treatment system, which has a high substrate removal rate and complete nitrification. In SBR, the microorganisms grow under a high F:M ratio condition when the feed enters the reactor and the aeration starts. As time passes, the F:M ratio decreases, therefore the system is never really at equilibrium. Competition for food becomes more intense as the F:M ratio decreases. However, the substrate removal rate was still very high and nitrification was completed in this system. According to the results of this study, there were fewer species of microorganisms isolated from the SBR system than from the CFSTR system. Except for the gram-positive genera, there was a significant difference between the predominant microorganisms obtained from nutrient agar and feed agar in the SBR system.

Therefore it can be concluded that some gram-positive strains, such as *Arthrobacter*, appear to be very significant and predominant in the lab-scale SBR system. The gram-positive genera could be playing very important roles on the substrate removal in the batch-type of wastewater treatment system.

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