# Enumeration and isolation of facultative anaerobic bacteria in an upflow anaerobic sludge blanket reactor treating wastewater from a gelatine industry

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**ABSTRACT.** This experiment measured levels of facultative anaerobic bacteria in an Upflow Anaerobic Sludge Blanket (UASB) reactor treating wastewater from a gelatin industry. The microorganism quantification showed similar values in granular sludge bed and fluidized zone. In the settling compartment of reactor, the bacteria were two logs lower. These communities are composed mainly of Gram-negative rods. The most abundant genera isolated were *Acinetobacter, Aeromonas*, *Citrobacter, Escherichia, Klebsiella* and *Stenotrophomonas*. The significance of the bacteria isolated from UASB reactor for public health is unknown. Some species are permanent residents; other are found only in a fraction of the population, and still other species are opportunistic pathogens that cause human infections. In the present study, none of the predominant bacteria belonged to the group of strict pathogens.

**Key words:** UASB-reactor, wastewater, gelatin, facultative anaerobic bacteria, Gram-negative rods.

**RESUMO. Enumeração e isolamento de bactérias anaeróbias facultativas num reator de fluxo ascendente e manta de lodo tratando efluente de uma indústria de gelatina.** Neste trabalho foram determinados os níveis de bactérias anaeróbias facultativas em Reator de Fluxo Ascendente e Manta de Lodo (UASB) utilizado no tratamento de efluente de industria de gelatina. A quantificação dos microrganismos apresentou similar valor na manta de lodo granular e na zona de fluidização. No compartimento de sedimentação do reator as bactérias foram dois logs menores. Estas comunidades são compostas principalmente de bacilos Gram-negativos. Os mais abundantes gêneros foram *Acinetobacter, Aeromonas, Citrobacter, Escherichia, Klebsiella* e *Stenotrophomonas.* O significado para a saúde pública das bactérias isoladas no reator UASB é desconhecido. Algumas espécies são residentes permanentes, outras são encontradas em somente uma parte da população, e ainda outras espécies são patógenos oportunistas que causam infecção humana. No presente estudo, nenhuma das bactérias predominantes pertence ao grupo dos patógenos estritos.

**Palavras-chave:** reator UASB, efluente industria de gelatina, bactérias anaeróbias facultativas, bacilos Gram-negativos.

## Introduction

The upflow anaerobic sludge blanket reactor has become the most popular high-rate reactor for anaerobic biological treatment of industrial and city wastewater. The reactor is typically divided into four compartments: the granular sludge bed, the fluidized zone, the gas-solids separator, and the settling compartment. The granular sludge bed is located in the bottom of the reactor. The wastewater is pumped into the bottom of the reactor and passes upward through the granular sludge bed. Here the organic compounds are biologically degraded and biogas is produced. In the top, just above the granular sludge bed, a fluidized zone is developed, due to biogas production. In this zone, further biological degradation may take place. The biogas is separated from the liquid in the gas-solids separator. Granules with good settling abilities settle back through the fluidized zone to the granular sludge bed. In UASB-type reactors, the biomass retention is promoted by bacterial self-aggregation into dense granules. The aggregation enhances the performance of high-rate anaerobic reactors, since the good settling of granules minimizes the biomass washout and the close cell packing optimizes the interspecies metabolites exchange. (Schmidt and Ahring, 1996).

The end of the treatment sequence involves disposal of the remaining stabilized effluent. Effluent disposal poses a serious, world-wide problem. Because of the increased environmental awareness and the stringent environmental standards governing the disposal set by different environmental protection agencies, the utilization of effluent in agricultural production has been gaining interest and attention in recent years. It offers economic and nutrient recycling advantages over the traditional disposal options, such as incineration for dry sewage and sea disposal (Stone *et al.,* 1998). Nevertheless, potential risks from accumulation of heavy metals and organic compounds, as well as pathogen contamination, must be considered.

The present study aims to (i) determine the predominant facultative bacteria found in the UASB, (ii) to determine the proportion of each predominant type in the total population, and (iii) to determine whether there were potential pathogens among these predominant organisms.

## Material and methods

The facultative anaerobic population measurements carried out in this study is from an upflow anaerobic sludge blanket reactor, used to treat wastewater from a gelatin industry. The reactor throughput was 46m$^3$ h$^{-1}$ for a retention time of 22.6h. Characterization of influent and effluent were performed according to Standard Methods (Apha, 1995).

The suspended solid or sludge (1mL) from different compartments of the Upflow Anaerobic Sludge Blanket (UASB) was diluted with phosphate-buffered saline to 10ml into 20-mL containing 15 to 20 glass beads of 0.5mm. The serum bottle was placed on a Vortex mixer for 2 min and the bacterial flocs were disrupted. A similar treatment was applied to the fourth successive decimal dilution tube. Samples of 0.1mL of either homogenized or dilutions of these in PBS were spread on Nutrient agar and MacConkey agar plates for colony plate counts, for total culturable and Gram-negative bacteria, respectively. The influent supernatant (IM) clarified by centrifugation was also used in the experiments. Duplicate plates were prepared for each sample. All plates were incubated at 37°C for 24-48h. Following incubation, colony counts were determined, and representative colonies subcultured for identification. Mean bacteria number was calculated as the average of each duplicate set and expressed as colony forming units (CFU) mL$^{-1}$ of sludge. Cellular morphologies were determined by brightfield microscopy of Gram-stained preparations. The microorganisms were identified according to standard methodologies, using biochemical tests (Holt *et al.* 1994) as well as standard techniques (MacFaddin, 1980; Murray *et al.,* 1995) used in our laboratory, and BBL Crystal Identification Systems (BBL Microbiology Systems, Cockeysville, Md).

## Results and discussion

The UASB reactor used to treat the gelatin industry wastewater has a working volume of 46$^3$/h at retention time of 22.6 h, with approximately 290 Nm$^3$/h biogas production, including 22000 ppm of $H_2S$. Chemical oxygen demand (COD) presents an average reduction of 83%, as well as the suspended solids, with reductions of 82% for Volatile Suspended Solids (VSS), 61% for Fixed Suspended Solids (FSS) and 79% for Total Suspended Solids (TSS). The analyses of Total Phosphates, Oils and Greases demonstrate a reduction of 34% and 76% respectively, after the biological treatment. After treatment in the UASB reactor the sulfide concentration increased in 257%, which is a significant increase (Table 1). The average of the reactor fluid pH was 7.8, with a normal range from 7.4 to 8.0.

**Table 1.** Characterization of the influent and effluent of USAB reactor from which granules were obtained for analysis.

| | Concentration* | |
|---|---|---|
| | Influent | Effluent |
| Chemical Oxygen Demand | 6678 | 1133 |
| Volatile Suspended Solids | 1629 | 279 |
| Fixed Suspended Solids | 346 | 134 |
| Total Suspended Solids | 1975 | 412 |
| Total Phosphate | 5.37 | 3.54 |
| Oil and Grease | 1388 | 326 |
| Sulfide | 71 | 253 |

*All values are in mg/l.

Preliminary studies were performed on the sludge samples in order to determine the most suitable medium for counting the bacteria along the suspended solid or sludge. Our attention was focused on enumerating culturable microbial populations, and in all cases the highest numbers were obtained on the nutrient agar (data not shown). Facultative anaerobic bacteria were determined in four separate samples, taken during a 12-month period. The number of culturable cells varied from

$9{,}1\times10^6$ to x $2{,}1\times10^7$ with the mean value of all samples being $1{,}2\times10^7$ viable cells per mL.

The levels of facultative anaerobic bacteria in the different compartments of the Upflow Anaerobic Sludge Blanket (UASB) reactor treating wastewater from a gelatin industry are presented in Figure 1. The quantification of microorganism showed similar values in the granular sludge bed and fluidized zone. Microorganism isolates from UASB samples contained low proportion of Gram-positive, when compared to Gram-negative These communities are composed mainly of Gram-negative rods. The physiological types of bacterial flora in the three compartments and in the effluent varied minimally (data not shown).

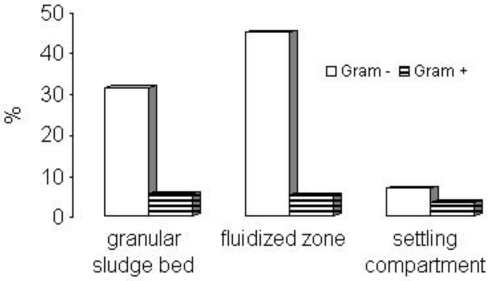

**Figure 1.** Distribution of facultative anaerobic bacteria in the different compartment of the UASB reactor

Figure 1. Angélica M.S. Silva et al. 2002. Facultative Anaerobic Bacteria in a UASB Reactor)

Five Gram-negative genera were isolated and identified in this study, including *Acinetobacter, Escherichia, Klebsiella, Citrobacter, Aeromonas, and Stenotrophomonas* (Table 2). The genera *Acinetobacter* and *Stenotrophomonas* are grouped as aerobic/microaerophilic rods. *Acinetobacter iwoffi* comprised 33% of the total number of isolates. It was suggested that the growth stimulation of *Acinetobacter* by low carbon concentrations reflects the ability of this organisms to use a variety of carbon sources and may in part explain its predominance in wastewater. *Stenotrophomonas maltophila* has been isolated from a number of water sources including rivers, wells, a hypereutrophic lake, bottled water and sewage (Denton and Kerr, 1998).

Members of the genera *Escherichia, Klebsiella, Citrobacter* and *Aeromonas* are grouped as facultatively anaerobic Gram-negative rods, and include a group of bacteria that inhabit human and other animals intestines. Some species are permanent residents, others are found only in a fraction of the population, and still others are present only as agents of disease conditions. *K. oxytoca, K.pneumoniae,* and occasionally other species are opportunistic pathogens which cause human infections. The genus *Aeromonas* has long been known to cause different infections in animals such as fish, reptiles, and amphibians (Hird *et al.,* 1983; Pathak *et al.,* 1988). These pathogens have also been associated with several human infections, such gastroenteritis, peritonitis, endocarditis, urinary tract and wound infection (Krovacek *et al.,* 1994).

Anaerobic digestion is a factor of particular importance for the effluent treatment system because of its potential in waste stabilization. To ensure the degradation of various contaminants present in wastewater, the presence of diversified groups of bacteria in the granules is very important. It is possible to develop granules on a wide variety of wastewater, but there have been several reports on lack of granulation on specific wastewater. Moreover, some researchers have reported sudden disintegration of granules without any obvious reason (Schmidt and Ahring, 1996). The end of the treatment sequence involves the disposal of the remaining stabilized effluent. Effluent disposal poses a serious, world-wide problem. Potential risks from accumulation of heavy metals and organic compounds, as well as pathogen contamination, must be considered.

The significance of the bacteria isolated from UASB reactor for public health is unknown. Some species are permanent residents, others are found only in a fraction of the population, and still others species are opportunistic pathogens that cause human infections. In the present study, none of the predominant bacteria belonged to groups of strict pathogens.

**Table 2.** Gram-negative isolates in sludge from UASB reactor

| Group | Identification | No. of isolates from: | | |
|---|---|---|---|---|
| | | Granular sludge bed | Fluidized zone | Settling compartment |
| Facultatively anaerobic *Gram-negative rods* | *Klebsiella oxytoca* | 23 | 32 | 6 |
| | *Citrobacter freundii* | 6 | 8 | 1 |
| | *Aeromonas veronii* | 6 | 8 | 1 |
| | *Aeromonas hydrophila* | 9 | 13 | 2 |
| | *Escherichia fergusonii* | 1 | 1 | 0 |
| | *Escherichia coli* | 3 | 4 | 0 |
| Gram-negative aerobic/microaerophilic rods | Acinetobacter iwoffi | 23 | 35 | 6 |
| | *Stenotrophomonas maltophila* | 3 | 4 | 0 |

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