Fingerprinting and Identification of Bacteria Present in UASB Granules Used to Treat Winery, Brewery, Distillery or Peach-lye Canning Wastewater

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The effective operation of the anaerobic digestion process in an upflow anaerobic sludge blanket (UASB) bioreactor is dependent on the microbial composition of the UASB granules. The granules contain a consortium of bacteria, with a specific metabolic function for each group, contributing to the overall efficiency and stability of the bioreactor. The aim of this study was to fingerprint and identify the bacteria present in four different types of South African UASB granules that are used to treat winery, brewery, distillery and peach-lye canning wastewaters. This was done by combining conventional microbiological platings with PCR-based denaturing gradient gel electrophoresis (DGGE) and DNA sequence analysis. Each granule type showed distinct PCR-based DGGE fingerprints with unique bands, while other bands were found to be present in all the granules, regardless of the wastewater being treated. Sixty-eight different bacteria (40 pure isolates and 28 clones) were partially sequenced and identified from the winery, brewery, distillery and peach-lye canning granules. Thirty-five percent of the identified bacteria represented the unculturable bacteria and 65% represented the culturable bacteria, which included members of the following genera: *Bacillus*, *Pseudomonas*, *Bacteroides*, *Enterococcus*, *Alcaligenes*, *Clostridium*, *Shewanella*, *Microbacterium*, *Leuconostoc*, *Sulfurospirillum*, *Acidaminococcus*, *Vibrio*, *Aeromonas*, *Nitrospira*, *Synergistes*, *Rhodococcus*, *Rhodocyclus* and *Syntrophobacter*. A DGGE marker was successfully constructed, representing members of the bacterial consortium in UASB granules.

INTRODUCTION

The upflow anaerobic sludge blanket (UASB) process has been widely used for the biological treatment of wastewaters from food and beverage processing (Wolmarans and De Villiers, 2002). These types of wastewaters normally have a high organic content and contain little or no toxic solids, providing the ideal conditions for bioreactor operation (Trnovec and Britz, 1998). The efficiency and stability of UASB bioreactors are dependent on the microbial composition of wastewaters (Roest *et al*., 2005). The microorganisms present in the seeding sludge form dense aggregates or granules through a granulation process characteristic of these bioreactors. The granules that are formed consist of different trophic groups, which are necessary for anaerobic digestion (Sekiguchi *et al*., 1998). These are the acidogenic, acetogenic, homoacetogenic and methanogenic bacteria. These microbial groups are responsible for executing the consecutive stages of the anaerobic digestion process, where the metabolic products of one microbial group are assimilated by the next microbial group (Gerardi, 2003).

Acidogens are responsible for the degradation of organic material to form carbon dioxide, hydrogen, acids and alcohols (Wangnai *et al*., 2004). The acetogenic bacteria convert the fatty acids to acetic acid and hydrogen (Van Andel and Breure, 1984). To prevent hydrogen accumulation, the homoacetogenic bacteria utilise carbon dioxide and hydrogen to form acetate as an end-product. The methanogens convert the acetate and hydrogen to methane and carbon dioxide (Batstone *et al*., 2002). Variations in the composition of one trophic group as a result of changes in substrate composition, reactor temperature, retention time and even pH may influence the entire microbial community structure (Casserly and Erijman, 2003). It is, therefore, important to identify the bacteria present in each granule type so as to better understand and optimise the metabolic activity of the different trophic groups present in the different granules, which will result in the successful operation of a UASB bioreactor.

When treated, different types of wastewaters also have an influence on the microbial consortium present in the granules. Keyser *et al*. (2006) analysed different UASB granules and showed that different methanogenic bacteria were present in the different types. Each type of wastewater favours the growth of specific bacteria that may have an impact on the success of the granulation process and, subsequently, on the successful operation of the

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UASB bioreactor. Each type of bacteria present in the granules performs a specific metabolic role in the granules, and contributes to their performance and ultimately that of the UASB bioreactor. Thus, it is essential to identify the various microorganisms in a bioreactor, since they play a role in its successful operation.

The use of conventional culture-dependent methods to isolate microorganisms from the UASB environment is limited due to the inability of many microorganisms to grow on synthetic media. Over the past few years, culture-independent methods therefore have proven to be of value for the identification of bacteria in complex samples (Roest *et al*., 2005). The aim of this study was firstly to use sequence analysis to identify the mixed microbial community, comprising different culturable and unculturable bacteria, that are present in four different types of UASB granules that had been used to treat either winery, brewery, distillery or peach-lye canning wastewaters. Secondly, PCR-based DGGE was used to obtain the unique fingerprint for each of the granule types in order to construct a DGGE marker representing some of the identified bacteria present in the four different UASB granule types so as to facilitate future rapid identification of UASB microorganisms.

MATERIALS AND METHODS

The PCR-based DGGE analysis used in this study was based on the amplification of a 200 base pair (bp) PCR product using the primers F341 with GC-clamp and R534 (Muyzer *et al*., 1993). DGGE profiles (200 bp) were obtained from the DNA isolated from the four different UASB granules. In addition, 1.5 kilobase (kb) fragments were amplified from the DNA of the different granules using the primers F8 and R1512 (Felske *et al*., 1997), and these were cloned and sequenced to enable identification. In order to confirm that all the microorganisms in the 200 bp DGGE profile had been cloned, sequenced and identified (using the 1.5 kb fragment), a 200 bp PCR fragment was amplified from the 1.5 kb PCR products. The resulting DGGE profile was compared to that of the profile obtained directly from the DNA of the different granules.

Identification of the culturable microorganisms was achieved by obtaining pure isolates from the different UASB granules using microbial platings. DNA was isolated from these pure isolates and a 1.5 kb fragment was amplified and sequenced in order to identify the culturable microorganisms. The DNA from these isolates was also amplified (200 bp), separated using DGGE and compared to the DGGE profiles of the different UASB granules. A DGGE marker was constructed to represent the microorganisms identified from the different UASB granules.

UASB granules

Three of the different types of UASB granules were obtained in 20 kg batches from available and operational industrial-scale UASB bioreactors in South Africa. The granule batches that had been used in the treatment of winery and distillery wastewaters were obtained from Distell, Stellenbosch, Western Cape, RSA. The brewery granules were obtained from the SAB brewery plant in Amanzimtoti, Kwazulu-Natal, RSA. The fourth granule type was originally from the full-scale SAB brewery UASB bioreactor, but had then been used as inoculum for a 60 L laboratory-scale UASB bioreactor treating peach-lye canning wastewater at the Department of Food Science, Stellenbosch University. This 60 L UASB bioreactor was operated for 15 months on the peach-lye canning effluent before granules were taken for analysis.

Microbial isolations

Randomly selected granules from each of the four different types of UASB granules were homogenised with a sterile pestle in 9 mL of sterile saline solution (0.85% (m/v) NaCl) and a dilution series (10^{-1} to 10^{-8}) was prepared. Each dilution was plated on nutrient agar (NA) (Biolab, supplied by Merck, Cape Town, South Africa) and on deMan, Rogosa and Sharpe agar (MRS) (Biolab, supplied by Merck, Cape Town, South Africa). The plates were incubated aerobically and anaerobically at 35°C for two days using the Anaerocult A system (Merck, Cape Town, South Africa). Although MRS is selective for lactic acid bacteria, it facilitates the growth of many bacteria (Van der Merwe and Britz, 1994). All the bacterial colonies obtained were streaked until pure isolates were obtained. The pure isolates were Gram-stained and microscopically examined to confirm isolate purification. These pure isolates were subjected to PCR amplification, purification and sequencing.

Genomic DNA extraction

DNA was extracted from the four different types of UASB bioreactor granules using the method of Van Elsas *et al*. (1997) as modified by Keyser *et al*. (2006). All the DNA extractions were done in duplicate.

PCR-based DGGE analysis

PCR amplifications

PCR reactions were performed using the primers F341 (5' CC TAC GGG AGG CAG CAG 3') with GC-clamp (5' CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G 3') and R534 (5' ATT ACC GCG GCT GCT GG 3'), as described by Muyzer *et al*. (1993). The primers amplify approximately 200 base pairs (bp) of the V3 variable region within the 16S ribosomal RNA (rRNA) gene (corresponding to positions 341 to 534 of the 16S rRNA gene of *Escherichia coli*). PCR reactions and conditions are as described by Keyser *et al*. (2006), using 1 µl 99% (v/v) dimethyl sulphoxide (DMSO) (Merck, Cape Town, South Africa) instead of bovine serum albumin (BSA).

In order to obtain sufficient DNA sequences to identify the bacteria present, a 1.5 kb fragment of the 5' end of the 16S rRNA gene was amplified from the different granule DNA using the primers F8 (5' CAC GGA TCC AGA CTT TGA TYM TGG CTC AG 3') and R1512 (5' GTG AAG CTT ACG GYT AGC TTG TTA CGA CTT 3') (Felske *et al*., 1997). PCR reactions were performed in 50 µl reaction volumes, containing 1 µl *Taq* DNA polymerase (5 U/µl) (Promega), 5 µl 10 x PCR reaction buffer, 2 µl of each of the primers (10 µl), 2 µl dNTPs (10 mM) (Promega), 2 µl 99% (v/v) DMSO (Merck, Cape Town, South Africa) and 2 µl of the extracted DNA (~30 ng.µl^{-1}). The PCR amplification conditions were as follows: initial denaturation was at 92ºC for 3 min; followed by denaturation at 92ºC for 30 sec; primer annealing at 54ºC for 30 sec; and chain elongation at 68ºC for 1 min. These three steps were repeated for 35 cycles. Final chain elongation was performed at 72ºC for 7 min (Felske *et al*., 1997). All the amplified PCR products were separated on 1% (m/v) agarose gels containing ethidium bromide and visualised under UV light (Vilber Lourmat).

A PCR reaction amplifying a 200 bp part of the 5' end of the 16S rRNA gene was performed on the amplified 1.5 kb PCR frag-

ments, using the primers F341 and R534 (Muyzer *et al*., 1993). This was done to confirm that each 200 bp band in the DGGE fingerprints was represented by a 1.5 kb PCR product that was sequenced.

DGGE

The 200 bp PCR fragments were separated using DGGE, performed with the BioRad DCode™ Universal Mutation Detection System (BioRad Laboratories, USA). The PCR products were applied to 8% (m/v) polyacrylamide gels with a gradient of between 45 and 70% (containing 0 to 100% denaturant (7M urea and 40% (v/v) formamide)) as described by Keyser *et al*. (2006).

Cloning

The 1.5 kb PCR fragments obtained from the amplification of the DNA extracted from the different granules were cloned into the pGemT-Easy Vector System II (Promega), since the PCR product contained a mixture of fragments with different DNA sequences. All the transformed cells were screened for the correctly sized insert using the primers T7 and SP6. Amplification reactions were performed in a total reaction volume of 50 µl, containing 1 µl *Taq* DNA polymerase (5 U/µl) (Roche Diagnostics), 5 µl 10 x PCR buffer, 2 µl of each of the primers (10 µM) and 2 µl dNTPs (10 mM) (Promega). The PCR amplification conditions consisted of an initial denaturation at 92ºC for 3 min, followed by 35 cycles of denaturation at 92ºC for 30 sec, annealing at 54ºC for 30 sec, elongation at 68ºC for 1 min, and a final 7 min elongation at 72ºC.

PCR purification

All the amplified 1.5 kb PCR fragments that were sequenced were purified using the High Pure PCR Product Purification Kit (Roche Diagnostics) according to the manufacturer's instructions.

DNA sequencing

All the obtained and purified 1.5 kb PCR fragments were sequenced using the ABI PRISM 377 DNA Sequencer (Perkin-Elmer) at the DNA Sequencing Facility at Stellenbosch University. The sequences obtained were compared to 16S rRNA gene sequences available in the National Centre for Biotechnology Information (NCBI) database using the BLASTn search option (Altschul *et al*., 1990).

Phylogenetic analysis

The 16S rRNA gene sequences were manually aligned by inserting gaps and the phylogenetic analyses were conducted using MEGA 2.1 (Kumar *et al*., 2001). The percentage similarities were calculated to identify the species.

DGGE marker

Eighteen DGGE bands were selected from the profiles obtained for the different granules. These bands represented cloned PCR fragments, as well as isolates from the different granules. The PCR products of the selected DGGE bands were separated using DGGE by loading 2 µl of each reaction mixture in the same well.

RESULTS AND DISCUSSION

PCR-based DGGE fingerprinting of the different UASB granules

The initial 200 bp PCR amplification products that were obtained were successfully separated using DGGE to produce unique fingerprints for each of the granule types evaluated. PCR-based DGGE fingerprints of duplicate DNA extractions of each granule type were found, as shown in Figure 1A and B, to be reproducible. Distinct fingerprints, containing unique bands, were observed for the four different UASB granules.

Definite bands were in the same position on the gel and present in all the granules, suggesting that a section of the microbial composition, under the conditions employed in this study, was identical and constant, irrespective of the wastewater being treated (Figure 1A and B). This suggests that the organisms represented by those bands are constant, irrespective of the wastewater being treated.

Certain bands in the fingerprints from the peach-lye canning granules were similar to bands found in the fingerprints of the brewery granules. Other bands present in the fingerprints of the peach-lye canning granules were not observed in the fingerprints of the brewery granules. These similarities and differences between the peach-lye canning and brewery granules are clear indications of changes in the microbial consortium when granules are subjected to a change in wastewater composition. This confirms that the composition of the wastewater being treated has an impact on the microbial species present in the granules.

Species identification

Sixty-eight different bacteria (40 pure cultured isolates and 28 clones) were identified from the winery, brewery, distillery and peach-lye canning granules. Of the 68 bacteria, 24 were representative of uncultured bacteria, constituting 35% of the identified bacteria. The remaining 65% were culturable bacteria. In Table 1, a summary is provided of the number given to each unique DGGE band, whether it was identified from cloned PCR fragments or pure isolates (cultured bacteria), the number of bases of each fragment sequenced and its GenBank accession number, as well as the closest relative and the percentage sequence similarity.

Certain bacteria were specific to a certain granule type, while other bacteria were found to be present in more than one granule. *Clostridium* was only identified in the distillery granules, while *Enterococcus*, *Leuconostoc*, *Aeromonas*, *Vibrio* and species related to *Rhodocyclus*, *Nitrospira*, *Rhodococcus* and *Syntrophobacter* were present only in the brewery granules. *Microbacterium* species were found only in the winery granules. *Sulfurospirillum* and species related to *Acidaminococcus* were found only in the peach-lye canning effluent granules. The most identified species of bacteria present in all four granule types were species of *Bacillus* and *Pseudomonas*. A total of 20 *Bacillus* and 14 *Pseudomonas* were identified in the various granules used in this study.

Bacillus:

Nineteen members of the genus *Bacillus* were found to be present in four different UASB granule types. Seven of these were present in the winery granules, five in the distillery granules, four in the peach-lye canning granules and three in the brewery granules. A comparative analysis was done of the sequences retrieved from GenBank and the percentage similarity can be observed in Table 1. Band W10 showed a 99.2% sequence similarity to an unidentified bacterium (Accession number AY345491) (Table 1) previously isolated from a sediment sample and all the other bands were found to be closely related to *B. cereus*. Band PL4 was

TABLE 1

Identification of bacteria present in different UASB granules.

DGGE band[a] I – Isolate C – Clone	Sequence length (bp)	GenBank accession number (clone/isolate)	% Sequence similarity	Closest relative	GenBank accession number (closest relative)
Bacillus					
W1 – I	510	DQ238239	99.6	*Bacillus pycnus* sp. NRS-1695	AF169535
W2 – I	510	DQ238238	99.8	*Bacillus megaterium* KL-197	AY030338
W3 – I	506	DQ238244	98.2	*Bacillus* sp. TKSP21	AF411341
W6 – I	511	DQ238240	100	*Bacillus cereus* G9667	AY138273
W9 – I	511	DQ238242	100	*Bacillus cereus* J-1	AY305275
W10 – I	510	DQ238243	99.2	Unidentified bacterium clone W4B-B03	AY345491
W20 – I	511	DQ238241	100	*Bacillus subtilis* ATCC21331	AB018487
B6 – I	511	DQ238237	100	*Bacillus cereus* J-1	AY305275
B7 – I	510	DQ239796	100	*Bacillus fusiformis* DSM2898T	AJ310083
B12 – I	510	DQ238236	96.5	*Bacillus sphaericus* PLC-5	AY161044
D1 – I	511	DQ238249	100	*Bacillus cereus* B412	AJ577281
D2 – I	511	DQ238248	100	*Bacillus subtilis* KL-077	AY030331
D3 – I	491	DQ238247	99.8	*Bacillus* sp. 19497	AJ315065
D5 – I	511	DQ238246	100	*Bacillus subtilis* C15	AF274248
D16 – I	511	DQ238251	100	*Bacillus cereus* RIVM BC00068	AJ577283
PL2 – I	511	DQ238255	100	*Bacillus cereus* ATCC535221	AF290551
PL3 – I	511	DQ238254	100	*Bacillus* sp. A24	AF397399
PL4 – I	508	DQ238253	99.6	*Bacillus pumilus*	AF393657
PL6 – I	511	DQ238256	100	*Bacillus* sp. TKSP21	AB017591
Pseudomonas					
B4 – I	682	DQ238235	99.9	*Brevundimonas bullata*	AB023428
D4 – I	739	DQ238233	98.1	Sulphide-oxidizing bacterium N9-1	AF393509
D6 – I	731	DQ238232	97.8	*Pseudomonas* sp. AMSN	AF438148
D10 – C	729	DQ238270	94.7	*Burkholderia pyrrocinia* strain R13058	AJ440714
D13 – C	730	DQ238271	98.5	*Pseudomonas* sp. NZ112	AY014826
PL1 – I	736	DQ238231	99.2	*Pseudomonas fluorescens*	AF094726
Pseudomonas					
PL5 – I	732	DQ238230	99.2	*Pseudomonas fluorescens* bv. C	AF228367
PL8 – I	736	DQ238234	96.0	*Pseudomonas* sp. 7-1	AF521651
PL11 – C	734	DQ238263	99.6	Uncultured Gamma *Proteobacterium*	AB015570
PL14 – C	729	DQ238262	99.6	*Pseudomonas veronii*	AF064460
PL17 – C	733	DQ238260	99.3	*Pseudomonas* sp. NZ024	AY014806
PL19 – C	736	DQ238264	97.8	Uncultured bacterium KM94	AY216460
PL20 – C	734	DQ238259	99.6	*Pseudomonas putida*	D85999
PL22 – C	732	DQ238261	98.4	*Pseudomonas viridiflava*	AF364097
Bacteroides					
W19 – C	886	DQ238265	99.9	Uncultured bacterium clone IIB-29	AJ488088
D9 – C	886	DQ238269	99.3	Uncultured bacterium clone IIIB-28	AJ488099
D15 – C	886	DQ238268	99.9	Uncultured bacterium clone IIB-29	AJ488088
PL16 – C	885	DQ238258	81.9	Uncultured *Bacteroidetes* clone ML635J-40	AF507859
Enterococcus					
B1 – I	738	DQ238227	96.8	Uncultured bacterium clone P-1938-s962-3	AF371532
B11 – I	737	DQ238229	98.1	*Enterococcus* sp. ALE-1	AY017051
B13 – I	740	DQ238228	98.1	*Enterococcus durans*	Y18359
Alcaligenes					
W7 – I	841	DQ238224	99.8	*Achromobacter spanius*	AY170848
B2 – I	855	DQ238226	93.6	Uncultured bacterium clone ZZ14AC10	AY214198
B3 – I	848	DQ238225	99.8	*Alcaligenes faecalis*	AF155147
Clostridium					
D7 – I	894	DQ191233	98.0	*Clostridium butyricum* strain VPI3266	AJ458420
D8 – I	873	DQ191234	97.2	*Clostridium bifermentans*	AF320283
Shewanella					
W8 – I	626	DQ191239	99.5	*Shewanella putrefaciens*	U91551
B8 – I	628	DQ191238	91.2	*Shewanella putrefaciens*	U91553
Microbacterium					
W4 – I	746	DQ191236	99.5	*Microbacterium* sp. PRLIST4	Y15325
W5 – I	742	DQ191237	100	*Microbacterium oxydans*	Y17227
Leuconostoc					
B14 – I	659	DQ191235	85.1	Uncultured *Leuconostoc* sp. clone LabS38	AF335916
Sulfurospirillum					
PL12 – C	577	DQ191240	98.9	*Sulfurospirillum arsenophilum*	U85964
PL13 – C	569	DQ191241	98.1	*Sulfurospirillum halorespirans*	AF218076

TABLE 1 (continued)

Identification of bacteria present in different UASB granules.

DGGE band I – Isolate C – Clone	Sequence length (bp)	GenBank accession number (clone/isolate)	% Sequence similarity	Closest relative	GenBank accession number (closest relative)
Acidaminococcus					
PL9 – C	640	DQ191232	91.8	Unidentified eubacterium clone vadinHB04	U81750
PL21 – C	639	DQ191231	94.8	Uncultured bacterium clone ER1_17	AY231317
Vibrio					
B9 – I	852	DQ191248	99.9	*Vibrio parahaemolyticus*	AY245192
Aeromonas					
B10 – I	848	DQ191247	96.2	*Aeromonas salmonicida*	X74681
Syntrophobacter					
B21 – C	794	DQ191246	99.0	Uncultured bacterium clone R1p32	AF482435
Rhodocyclus					
B18 – C	647	DQ191242	95.5	Uncultured bacterium clone HP1B54	AF502232
Rhodococcus					
B16 – C	722	DQ191245	86.1	Uncultured bacterium clone BA149	AF323777
Nitrospira					
B15 – C	661	DQ191243	88.2	Uncultured bacterium DCE29	AF349765
B19 – C	661	DQ191244	99.8	Uncultured bacterium clone SR_FBR_L1	AY340834
Synergistes					
W11 – C	663	DQ238267	98.2	Uncultured bacterium TA19	AF229792
W17 – C	658	DQ238266	85.6	Uncultured bacterium clone SHA-104	AJ306760
D11 – C	675	DQ238272	89.5	Uncultured bacterium clone TTA_B6	AY297966
D12 – C	668	DQ238273	99.7	Uncultured bacterium mle1-42	AF280863
Uncultured					
W18 – C	1069	DQ238245	92.3	Uncultured bacterium clone BSA2B-20	AB175392
B20 – C	972	DQ238252	63.1	Uncultured bacterium clone W31	AY770971
PL15 – C	857	DQ238257	66.8	Uncultured bacterium	AB195900

[a]W = UASB granules used to treat winery wastewater; B = UASB granules used to treat brewery wastewater; D = UASB granules used to treat distillery wastewater; PL = UASB granules used to treat peach-lye canning wastewater.

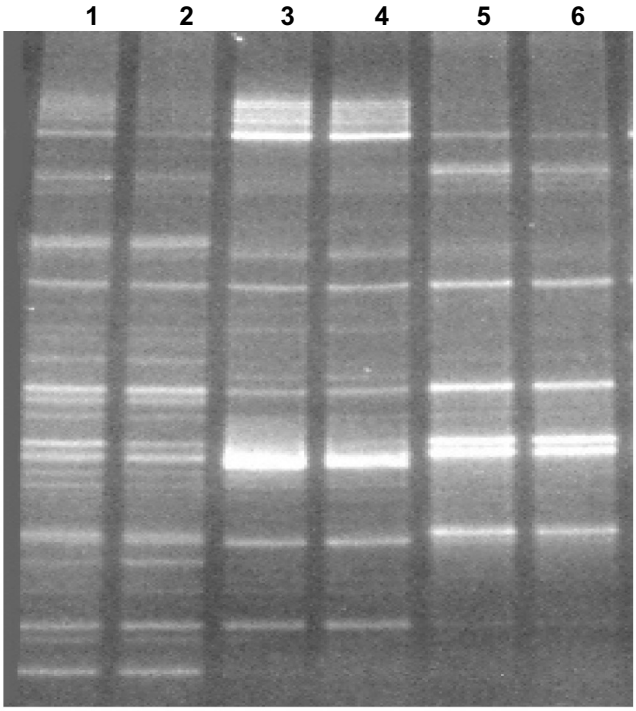

FIGURE 1A

PCR-based DGGE fingerprints of granules from UASB bioreactors used to treat winery wastewater (1 and 2), distillery wastewater (3 and 4) and brewery wastewater (5 and 6). The identical profiles correspond to two independent DNA extractions.

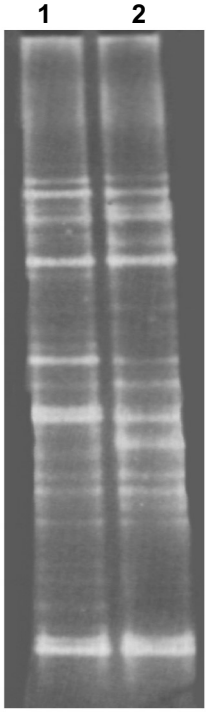

FIGURE 1B

PCR-based DGGE fingerprints of granules from a UASB bioreactor treating peach-lye canning wastewater (1 and 2). The identical profiles correspond to two independent DNA extractions.

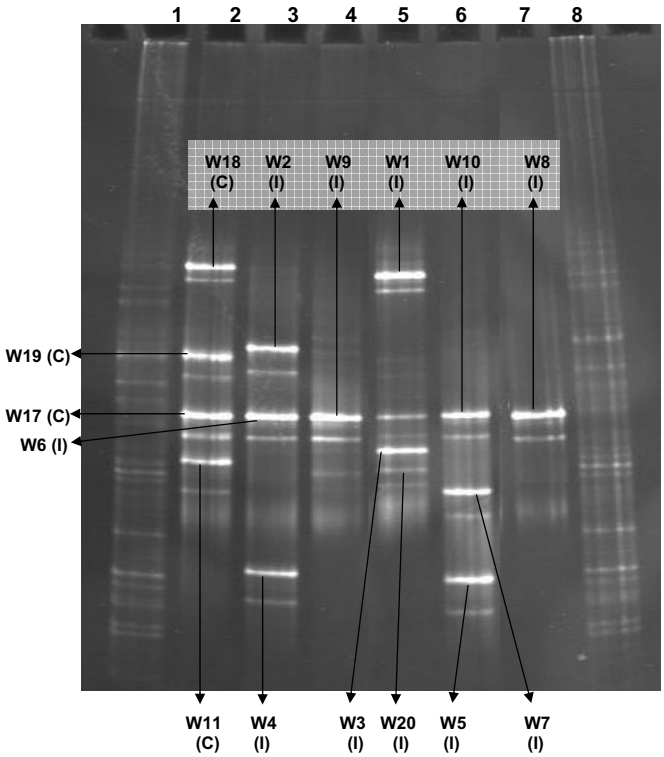

FIGURE 2

Single PCR-based DGGE bands from the cloned inserts (C) and isolates (I) obtained for the winery granules. Lanes 1 & 8: fingerprints of the winery granules that can be used to correlate against the single bands; Lane 2: bands W11, 17, 18 & 19; Lane 3: W2, 4 & 6; Lane 4: W9; Lane 5: W1, 3 & 20; Lane 6: W5, 7 & 10; and Lane 7: W8.

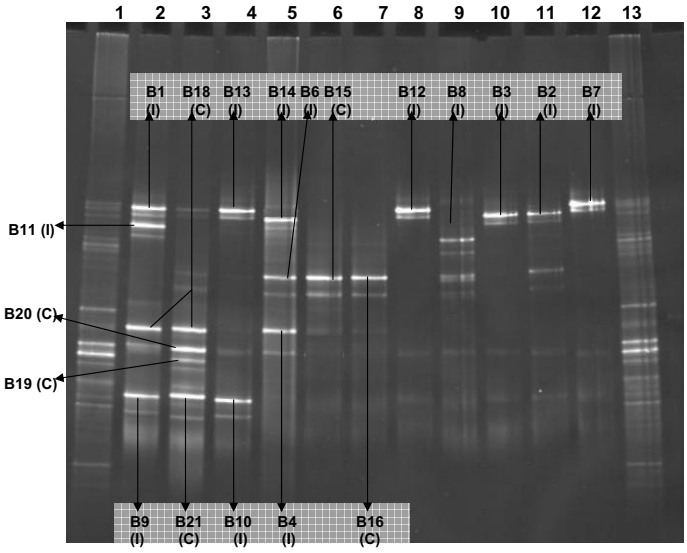

FIGURE 3

Single PCR-based DGGE bands from the cloned inserts (C) and isolates (I) obtained for the brewery granules. Lanes 1 & 13: fingerprints of the brewery granules that can be used to correlate against the single bands; Lane 2: bands B1, 5, 9 & 11; Lane 3: B18, 19, 20 & 21; Lane 4: B10 & 13; Lane 5: B4, 6 & 14; Lane 6: B15; Lane 7: B16; Lane 8: B12; Lane 9: B8; Lane 10: B3; Lane 11: B2; and Lane 12: B7.

found to be closely related to *B. pumilus*, supported by a 99.6% sequence similarity. Bands D3 and W2 showed a 99.8% sequence similarity to a *Bacillus* sp. (Accession number AJ315065) and *B. megaterium,* respectively. Band W1 was found to be closely related to *B. pycnus* and band B7 was identified as *B. fusiformis*, supported by a 100% sequence similarity, while B12 was found to be closely related to *B. sphaericus*. The bands W20, D2, D5 and PL6 were all closely related to *B. subtilis*.

It is generally accepted that most of the bacteria that can survive and be metabolically active in bioreactors are anaerobes, but the aerobic and facultative anaerobic bacteria, such as *Bacillus* species, still form a significant and constant part of the total bioreactor population. Species of *Bacillus* are metabolically active during the anaerobic digestion process and can degrade different types of organic compounds, such as proteins, cellulose, starch or lipids (Gerardi, 2003). The presence of *Bacillus* spp. in a bioreactor may also play a role in the formation of immobilised microbial populations or facilitate their clumping because of their adhesion ability (Petruccioli *et al*., 2000; Gerardi, 2003). Noeth *et al*. (1988) isolated *B. pumilus*, *B. subtilis*, *B. megaterium* and *B. sphaericus* from an anaerobic, fixed-bed bioreactor. They ascribed the growth of the bacilli to sufficient oxygen in the bioreactor that was probably introduced by the substrate. *Bacillus coagulans* and *B. sphaericus* were also identified from a laboratory-scale UASB bioreactor (Thierry *et al*., 2004).

Pseudomonas:

Fourteen members of the genus *Pseudomonas* were found as part of the population of the four different UASB granules (Table 1). Nine *Pseudomonas* species were present in the peach-lye canning granules, four in the distillery granules and one was isolated from the brewery granules. Band B4, from the brewery granules, showed a 99.9% sequence similarity to *Brevundimonas bullata*, while band PL11 was found to be closely related to an uncultured gamma *Proteobacterium* (Li *et al*., 1999). Band PL19 was found to be closely related to an uncultured bacterium previously isolated from wetland sediments (Accession number AY216460). Band PL5 was closely related to *P. fluorescens*, a potential phenol-degrading bacterium (Heinaru *et al*., 2000), and band PL17 showed a sequence similarity of 99.3% to a *Pseudomonas* sp. (Accession number AY014806). PL14 was closely related to *P. veronii*, PL22 to *P. viridiflava* and band PL20 was closely related to *P. putida*. The distillery band D13 was closely related to a *Pseudomonas* sp. (Accession number AY014826), while D4 was found to be closely related to a sulphide-oxidising bacterium, previously isolated from an environmental sample (Accession number AF393509). Bands D6 and PL8 were found to be closely related to these *Pseudomonas* spp. (Accession numbers AF438148 and AF521651, respectively), and PL1 was closely related to *P. fluorescens*. Band D10 was found to be closely related to *Burkholderia pyrrocina*.

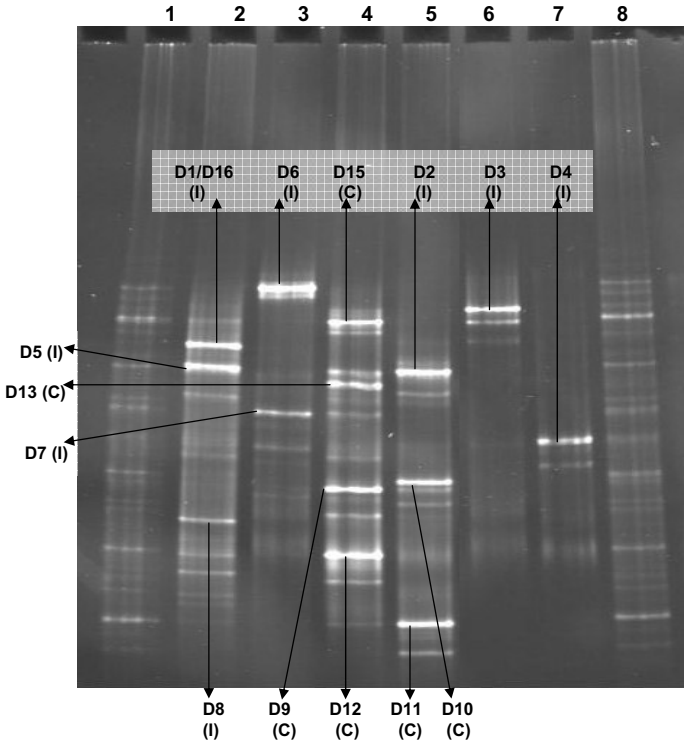

FIGURE 4

Single PCR-based DGGE bands from the cloned inserts (C) and isolates (I) obtained for the distillery granules. Lanes 1 & 8: fingerprints of the distillery granules that can be used to correlate against the single bands; Lane 2: bands D1, 5, 8, 16; Lane 3: D6 & 7; Lane 4: D9, 12, 13 & 15; Lane 5: D2, 10 & 11; Lane 6: D3; and Lane 7: D4.

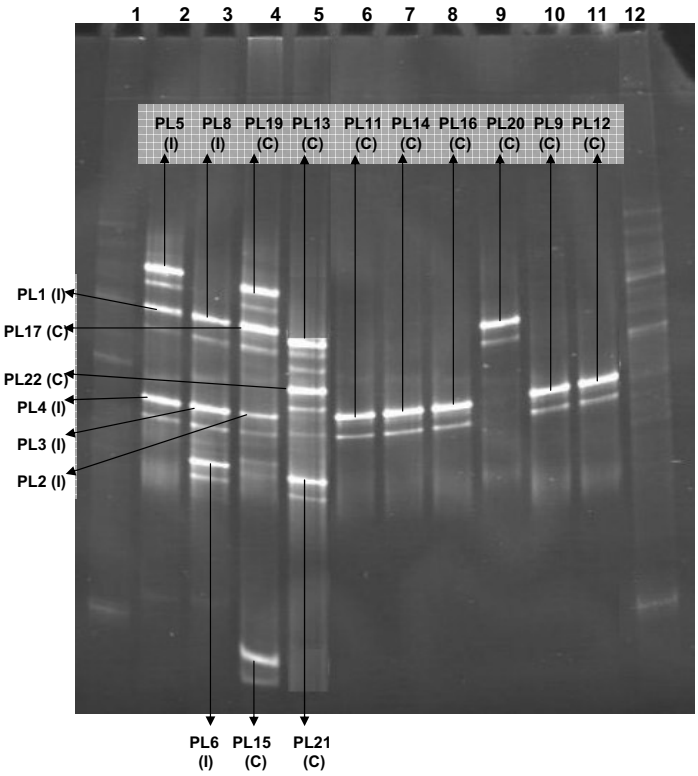

FIGURE 5

Single PCR-based DGGE bands from the cloned inserts (C) and isolates (I) obtained for the peach-lye canning granules that can be used to correlate against the single bands. Lanes 1 & 12: fingerprints of the peach-lye canning granules; Lane 2: bands PL1, 4 & 5; Lane 3: PL3, 6 & 8; Lane 4: PL2, 15, 17 & 19; Lane 5: PL13, 21 & 22; Lane 6: PL11; Lane 7: PL14; Lane 8: PL16; Lane 9: PL20; Lane 10: PL9; and Lane 11: PL12

Several members of the *Pseudomonas* are known for their ability to degrade aromatic compounds and to produce exo-polysaccharides. This ability might explain why *Pseudomonas* may play an important role in granulation (Petruccioli *et al*., 2000). *Pseudomonas stutzeri*, *P. aeruginosa* and *P. putida* have been isolated from UASB bioreactors in the past (De Haast and Britz, 1986; Muthumbi *et al*., 2001; Pereira *et al*., 2002; Thierry *et al*., 2004). *Pseudomonas* was identified from the brewery, distillery and peach-lye canning granules, but since most of these bacteria were identified from the peach-lye canning granules, it is apparent that the *Pseudomonas* found the environmental conditions in these granules favourable for growth. A possible explanation for their presence might be that these identified *Pseudomonas* are halotolerant (Mioni *et al*., 2003; Lo Nostro *et al*., 2005) and also could withstand the alkaline environment (pH 8.5) caused by the lye in the wastewater (Sigge *et al*., 2001). Although *Pseudomonas* is not classified as alkalophiles, it seems that these identified *Pseudomonas* was able to metabolise and grow in more alkaline environments.

Bacteroides:

Four *Bacteroides* species were identified from the UASB granules that were analysed (Table 1) and two were present in the distillery granules, one in the winery granules and one in the peach-lye canning granules. Comparative analysis of the sequences retrieved from GenBank show that band D9 showed the highest sequence similarity (99.8%) to an uncultured bacterium (Accession number AJ488099). The DGGE band PL16 was shown to be closely related to an uncultured *Bacteroidetes* bacterium (Accession number AF507859), while the bands W19 and D15 were found to be closely related to an uncultured bacterium previously isolated from environmental samples (Accession number AJ488088).

Gram-negative *Bacteroides* spp. are acidogenic, anaerobic bacteria (Krieg and Holt, 1984). They can metabolise carbohydrates and peptone to form acetate, lactate, formate or propionate. Members of this genus have been isolated from sewage (Krieg and Holt, 1984), anaerobic bioreactors (Joubert and Britz, 1987; McHugh and O'Flaherty, 2004) and activated sludge (Liu *et al*., 2005), and include the species *B. fragilis*, *B. distasonis*, *B. uniformis*, *B. splanchnicus* and *B. forsythus*.

Enterococcus:

Three *Enterococcus* species were isolated from the brewery granules (see Table 1). Band B1 showed a 96.8% sequence similarity to an uncultured bacterium closely related to the genus *Enterococcus* (Leser *et al*., 2002), while bands B11 and B13 represent species that are closely related to *Enterococcus* sp. (98.1% sequence similarity) (Chee-Sanford *et al*., 2001) and *E. durans* (98.1% sequence similarity) (Collins *et al*., 1984) respectively.

Enterococcus is Gram-positive, facultatively anaerobic and ferments carbohydrates to mainly form lactic acid. *Enterococcus*

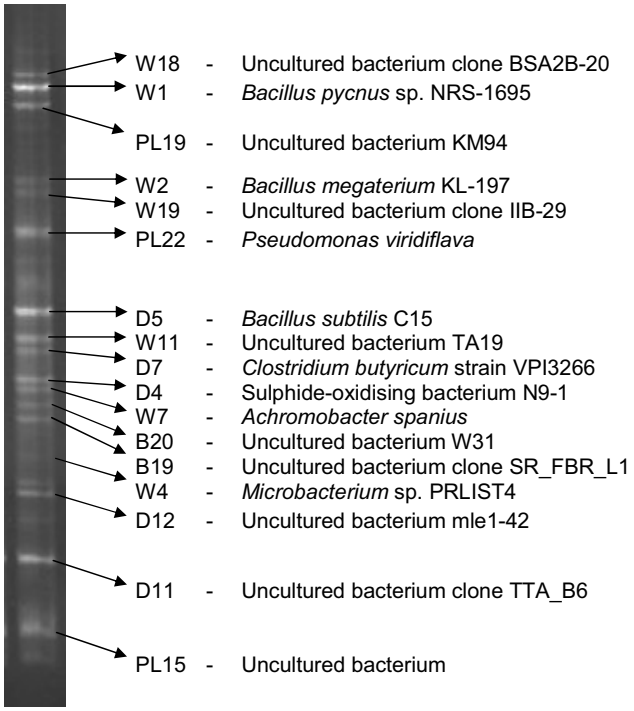

FIGURE 6

DGGE marker constructed from the different bacteria present in the UASB granules that were used to treat winery, brewery, distillery and peach-lye canning wastewaters.

durans, *E. avium* and *E. faecium* were isolated from raw baker's yeast effluent (Van der Merwe and Britz, 1994). Chan *et al.* (2001) studied the microbial community of granular sludge for treating brewery wastewater, but did not identify any *Enterococcus* species.

Alcaligenes:

Two species of this genus were identified from the brewery granules and one from the winery granules (Table 1). Band B2 showed a 93.6% sequence similarity to an uncultured bacterium (Accession number AY214198) related to the genus *Alcaligenes*. Band B3 showed a 99.8% sequence similarity to *A. faecalis*. *Alcaligenes* spp. can utilise a variety of organic acids and amino acids as carbon sources and certain strains are also capable of anaerobic respiration in the presence of nitrate or nitrite, although *A. faecalis* can only reduce nitrite (Krieg and Holt, 1984). *Alcaligenes faecalis* has also previously been isolated from bioreactors (De Haast and Britz, 1986) and the presence of this bacterium was ascribed to the sewage sludge used as inoculum at the start-up of the bioreactor.

Winery band W7 was found to be closely related to *Achromobacter spanius*, supported by a 99.8% sequence similarity. Coenye *et al.* (2003) found that *Achromobacter spanius* resembled *Alcaligenes faecalis* phenotypically, but protein and fatty acid analyses showed it to be distinct to *Achromobacter*. *Achromobacter* spp. can reduce nitrate and metabolise gluconate, acetate, propionate, butyrate, iso-butyrate and succinate during acetogenesis (Coenye *et al.*, 2003). It may be that the winery granules in this study favoured the growth of *Achromobacter*, and that the brewery granules favoured the growth of *Alcaligenes*.

Clostridium:

Two *Clostridium* species were isolated, only from the distillery granules (Table 1). Band D7 was found to be closely related to *C. butyricum* and band D8 to *C. bifermentans*. Clostridia produce organic acids and alcohols from carbohydrates or peptones (Sneath *et al.*, 1986) and are regarded as acidogenic or homoacetogenic bacteria in anaerobic bioreactors. *Clostridium bifermentans* was previously isolated from oil mill wastewater (Chamkha *et al.*, 2001) and *C. butyricum* was a prevalent hydrolytic bacterium in an anaerobic bioreactor treating cheese whey (Chartrain and Zeikus, 1986). Distillery granules favoured the growth of species belonging to the genus *Clostridium*. This can possibly be ascribed to a favourable redox potential for the development and growth of Clostridia.

Shewanella:

In this study, band W8 was isolated from the winery granules and band B8 from the brewery granules (Table 1), and they were found to be part of the genus *Shewanella*. Band W8 showed a 99.5% and band B8 a 91.2% sequence similarity to *Shewanella putrefaciens*. All *Shewanella* species are Gram-negative and can reduce nitrate to nitrite. *Shewanella putrefaciens* can reduce trimethylamine N-oxide and sulphur and can produce hydrogen sulphate from thiosulphate (Venkateswaran *et al.*, 1999).

Microbacterium:

Two *Microbacterium* species were isolated, only from the winery granules (Table 1). Band W4 was found to be closely related to a *Microbacterium* sp. (Accession number Y15325), supported by a 99.5% sequence similarity, and band W5 was identified as *M. oxydans* (100% sequence similarity). *Microbacterium oxydans* can produce acid from glucose, fructose, galactose, mannose, sucrose, maltose, mannitol, glycerol, salicin and dextrin (Schumann *et al.*, 1999). Therefore, the bands W4 and W5 formed part of the acidogenic population in the winery granules.

Leuconostoc:

One *Leuconostoc* sp. was isolated from the brewery granules (Table 1). Band B14 was found to be related to an uncultured *Leuconostoc* sp. (Accession number AF335916), supported by a sequence similarity of 85.1%. This *Leuconostoc* sp. showed a 99.8% sequence similarity to *Leuc. mesenteroides* and it may be possible that B14 is related to *Leuc. mesenteroides*.

Leuconostoc species are Gram-positive, facultative anaerobes and growth is dependent on the presence of amino acids and fermentable carbohydrates, such as glucose (Sneath *et al.*, 1986). Chartrain and Zeikus (1986) found that *Leuc. mesenteroides* formed part of the hydrolytic bacteria in an anaerobic bioreactor, making band B14 a member of the acidogenic bacteria in the brewery granules.

Sulfurospirillum:

Two *Sulfurospirillum* species were cloned, only from the peach-lye canning granules (Table 1). Band PL12 showed a 98.9% sequence similarity to *S. arsenophilum* (Stolz *et al.*, 1999) and band PL13 showed a 98.1% sequence similarity to *S. halorespirans*. Lactate, pyruvate and fumarate can be used by *S. arsenophilum* and *S. halorespirans* as electron donors, but when acetate is used as the carbon source, hydrogen and formate serve as the electron donors (Luijten *et al.*, 2003).

Acidaminococcus:

Two uncultured species, related to the genus *Acidaminococcus*, were identified from the peach-lye canning granules (Table 1). Band PL9 showed a 91.8% sequence similarity to an unidentified eubacterium (Accession number U81750), which was found to be related to *Acidaminococcus fermentans*, previously found in anaerobic bioreactors (Godon *et al*., 1997) and paper mill wastewater (Roest *et al*., 2005). Band PL21 showed a 94.8% sequence similarity to an uncultured bacterium (Accession number AY231317) previously detected in an anaerobic bioreactor, which showed a 92% sequence similarity to *A. fermentans*.

Vibrio:

Band B9 showed a 99.9% sequence similarity to *Vibrio parahaemolyticus* (Table 1). *Vibrio parahaemolyticus* has a fermentative metabolism (Krieg and Holt, 1984), thus it may be possible that band B9 was part of the acidogenic bacteria in the brewery granules.

Aeromonas:

Band B10 showed a 96.2% sequence similarity to *Aeromonas salmonicida* (Table 1). *Aeromonas* has been isolated from winery wastewater (Petruccioli *et al*., 2000) and raw baker's yeast wastewater (Van der Merwe and Britz, 1994). Species of *Aeromonas* were found to be partly responsible for phosphorus uptake and release in bioreactors (You *et al*., 2002; Li *et al*., 2003).

Syntrophobacter, Rhodocyclus, Rhodococcus, Nitrospira:

Brewery bands B21, B18, B16, B15 and B19 were all identified as uncultured bacteria. Brewery band B21 showed a 99% sequence similarity to an uncultured bacterium (Accession number AF482435) (Table 1), previously found in granular sludge, and a sequence similarity of 89.8% to an uncultured bacterium SJA-172 (Accession number AJ009502). Roest *et al*. (2005) also identified uncultured bacterium SJA-172 in a UASB bioreactor and suggested that this bacterium might be involved in the oxidation of propionate. Propionate oxidation is an energetically unfavourable reaction and microorganisms involved in the degradation of propionate play a crucial role in the anaerobic degradation process in methanogenic bioreactors. Uncultured bacterium SJA-172 was found to be closely related to *Syntrophobacter wolinii*. It is, therefore, possible that band B21 and the uncultured bacterium R1p32 may be related to the genus *Syntrophobacter*.

Band B18 showed a 95.5% sequence similarity to an uncultured bacterium (Accession number AF502232) previously present in activated sludge with high phosphorus content. This uncultured bacterium was found to be closely related to a species belonging to the genus *Rhodocyclus,* which was associated with phosphorus removal in sludges (McMahon *et al*., 2002). This supports the finding that band B18 may be related to the genus *Rhodocyclus*. The growth rate of *Rhodocyclus* species can be increased in the presence of complex organic nutrients or even yeast extract (Staley *et al*., 1989). Since band B18 was found in UASB granules used to treat brewery wastewater, it is possible that these bacteria could have used the yeast cells for their metabolism.

Band B16 showed an 86.1% sequence similarity to uncultured bacterium clone BA149 previously found in environmental samples (Accession number AF323777). Bacterium clone BA149 was found to show a sequence similarity of 74.1% to *Rhodococcus rhodochrous*, which can produce acid from dextrine, ethanol, fructose, glucose and sucrose (Sneath *et al*., 1986) and may have played an important role during acidogenesis in the brewery granules. Hawari *et al*. (2000) found that *Rhodococcus* produced formaldehyde or methanol as end-products during the anaerobic biodegradation of anaerobic sludge.

Band B15 showed an 88.2% sequence similarity to uncultured bacterium DCE29 (Gu *et al*., 2004) and B19 showed a 99.8% sequence similarity to uncultured bacterium SR_FBR_L1 (Kakosen *et al*., 2004) (Table 1). Gu *et al*. (2004) reported that the uncultured bacterium DCE29 used hydrogen as an electron donor and could be affiliated with the genus *Nitrospira*. Uncultured bacterium SR_FBR_L1 was identified as part of a lactate-utilising sulphate-reducing fluidised-bed bioreactor bacterial community (Kakosen *et al*., 2004).

Synergistes:

Four uncultured bacteria were identified from the winery and distillery granules and found to be related to the genus *Synergistes*. These are acidogenic bacteria associated with anaerobic bioreactors and soil (Godon *et al*., 2005) (Table 1). Band W11 showed a 98.2% sequence similarity to the uncultured bacterium TA19, previously identified in a UASB bioreactor (Wu *et al*., 2001) and found to be closely related to *Synergistes jonesii*. Band D12 showed a 99.7% sequence similarity to the uncultured bacterium mle1-42, which was found to be present in bioreactors (Lapara *et al*., 2000; Pereira *et al*., 2002). Uncultured bacterium mle1-42 formed part of a cluster containing *S. jonesii* (Lapara *et al*., 2000). Band W17 showed a high sequence similarity of 94.4% to DGGE band D11, which also showed an 89.5% sequence similarity to the uncultured bacterium clone TTA_B6. This bacterium was found to be the second "most dominant" bacteria in an anaerobic reactor (Chen *et al*., 2004). This organism was closely related to an environmental clone MUG10 (Sekiguchi *et al*., 1998).

Other uncultured bacteria:

Band W18 showed a 92.3% sequence similarity to the uncultured bacterium clone BSA2B-20 (Accession number AB175392). Bands B20 and PL15 showed a sequence similarity of 63.1% and 66.8%, respectively to an uncultured bacterium clone W31 (Accession number AY770971) and an uncultured bacterium (Accession number AB195900). The sequence similarities of 63.1% and 66.8% are low and the identification of these two bacteria is uncertain.

A 200 bp PCR fragment was amplified from each 1.5 kb PCR fragment from the pure isolates and the cloned inserts, using the primers F341 and R534 (Muyzer *et al*., 1993). This was done to confirm that each band in the DGGE fingerprints was sequenced and identified. It was observed that many of the DGGE single bands were accompanied at close distance by another band. Such shadow bands also occurred with the cloned sequences. However, similar observations of artifactual 'double bands' have been reported by other researchers (Janse *et al*., 2004). These shadow bands could be formed during PCR cycling, when secondary products are formed due to prematurely halted elongation. Although Janse *et al*. (2004) decreased the intensity of the artifactual bands by extending the final elongation step, it did not

have the same effect in this study. In Figures 2 to 5, the single bands obtained for the winery, brewery, distillery and peach-lye canning granules can be observed. In Figure 2, more than one PCR product was loaded in lanes 1, 2, 5 and 6 specifically. The separate PCR products (2 µl of each product) were loaded in one well in order to accommodate all the bands of the winery granules in one gel. The same was done for the brewery, distillery and peach-lye canning granules. The identified microorganisms where then visually correlated to the corresponding DGGE band in the profiles to enable the construction of a DGGE marker.

DGGE marker

The developed DGGE marker was constructed using selected DGGE bands from the profiles obtained for the winery, brewery, distillery and peach-lye canning effluent granules (Figure 6). This marker represents the bacteria that were most present in the four different granules used in this study, and can be of great value for the possible identification or indication of members of the microbial consortium in UASB bioreactors.

Since it is an extremely time-consuming process to identify microorganisms, especially those that are not readily cultured, the use of the DGGE marker can be of great assistance to provide a quick method to verify the presence of these microorganisms where each bacterium has a specific role to play during anaerobic digestion. Knowledge of the composition of the microbial consortium can be of great value during the start-up of a new bioreactor, and the marker can be used as a reference to monitor the various microorganisms during their adaptation period in a new bioreactor or a bioreactor treating a new type of wastewater.

CONCLUSIONS

During the past decade, advances in molecular biology have provided better insight into the structure of complex microbial communities. In this study, PCR-based DGGE proved useful to fingerprint the various UASB granules. It is evident from the results obtained that the use of culture-independent molecular techniques is essential, since a total of 35% of the identified bacteria were unculturable bacteria. This study therefore clearly shows the value of integrated culture-dependent and culture-independent research.

The different bacteria that were isolated and identified from the different granules emphasise the fact that the composition of each type of wastewater has a major impact on the microbial species present in the granules. Fingerprinting and identification of the complex microbial bacterial community in UASB granules may lead to a better understanding of the influence that the treatment of various wastewaters may have on the structure of the different populations present in the UASB granules. A better understanding of the diversity of bacteria in different UASB granules can improve the stability of the anaerobic process and the performance of the bioreactor. The metabolic activity of the different groups of bacteria plays a major role during anaerobic digestion and, if the bacteria are identified, it is possible that tailor-made granules could be used to enhance bioreactor process stability. Tailor-made granules may also be used to reduce the start-up period. The survival of these incorporated microorganisms used to enhance bioreactor efficiency may be monitored by using the DGGE marker that was constructed in this study.

A major advantage of this DGGE marker is that it could be complemented by additional DGGE bands found in UASB bioreactors. The DGGE marker can also be used to assist in the monitoring of selected species during bio-augmentation or enrichment of granules for the treatment of specific wastewaters. The DGGE marker has to be used in combination with sequence analysis when analysing new granule batches. It is possible that some overlapping of the microorganisms can take place in the DGGE profiles under certain conditions.

The data obtained in this study should be of value in the future identification of microbial communities present in anaerobic digestion studies, as well as for the process optimisation of UASB bioreactors.

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