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Selection of microbial consortia for treating metal-working fluids

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The aim of this research was to determine the effectiveness of a strategy for constructing microbial consortia for treating chemically mixed industrial effluent, based on a more thorough understanding of communities within waste metal-working fluids (MWFs). Complementary phenotypic and genotypic methods revealed that the microbial communities in spent MWFs had low diversity and were very similar in species composition in samples originating from different locations and uses. Of 65 bacterial isolates studied, only 9 species were identified using fatty acid methyl ester (FAME) analysis. The results of genotypic analysis by denaturing gradient gel electrophoresis (DGGE) were congruent with observations made using FAME analysis. The metabolic potential of the isolates was assessed in terms of assimilation ability and tolerance of co-contaminants. The three isolates, selected (*Clavibacter michiganensis*, *Methylobacterium mesophilicum*, and *Rhodococcus erythropolis*) to form a consortium, were representative of three of the four most abundant populations and when combined could utilise or tolerate all of the individual MWF components, including the biocide and the recalcitrant compound benzotriazole.
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Introduction

Bioaugmentation with microorganisms with desired catabolic traits can be effective for bioprocessing of pollutants. It has been shown to be effective for enhancing rates of water treatment when indigenous degradative activity is slow or even absent [6,20,21]. In addition to stimulating degradation, bioaugmentation, in some cases, can facilitate the establishment of indigenous populations that also benefit waste treatment [14]. However, despite these successes there are many instances in which bioaugmentation has proven to be ineffective and so its potential remains uncertain [5,4]. One area where there is considerable promise for bioaugmentation is its use in bioreactors for the treatment of chemically mixed industrial wastes. An essential requirement for such systems is that they perform reliably, a feature that is not characteristic of microbial communities that colonise industrial wastes. Studies of the microbiology of fluidised bioreactors suggest that microbial communities in such systems are typically dynamic and unstable [8]. Bioaugmentation with carefully selected consortia may improve the opportunity to recreate more reproducible systems that both enhance degradative ability, but also by pre-emptive colonisation, prevents invasion by opportunistic strains with little degradative ability or are pathogenic.

Successful bioaugmentation for the treatment of chemically mixed wastes requires that suitable strains be introduced. Two features are paramount; the strains (1) are able to degrade the individual chemical components of the waste and (2) they are able to survive and be active in the target waste, particularly when in the presence of toxic co-contaminants. Although strains used for bioaugmentation have typically been selected on the basis of their ability to degrade specific chemical constituents, little consideration is given to their interactions with co-contaminants or their predisposition to survive in the target habitat. In a previous study, we showed that selection of strains, based on an improved understanding of the composition of indigenous microbial communities in the target habitat, was an effective way of selecting for inocula that persisted [10]. The aim of this study was to construct a microbial consortium for treatment of an industrial waste, based on the selection criteria of their degradative ability, tolerance to co-contaminants and their natural abundance in spent and decomposing metal-working fluids (MWFs).

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Materials and methods

The metal-working fluid

The industrial waste selected for study was synthetic MWFs. MWFs were chosen because they are chemically mixed, containing environmentally toxic mixtures of lubricants, antifoaming agents and anticorrosives, and so are representative of many industrial wastes. Disposal of MWF safely and economically is difficult because of the large quantities produced worldwide annually (22×10^9 l year^{-1}) [7]. The synthetic MWF investigated (Castrol, Pangbourne, Berks, UK) is used as a coolant and lubricant in large-scale continuous metal-working processes to machine tungsten carbide and steel. The fluid is comprised of eight main chemical constituents including a formaldehyde-based biocide; benzotriazole (metal passivator); dodecanedioic acid, lauric acid, sebacic

acid and an unspecified amine (corrosion inhibitors); glycerol and propylene glycol (lubrication agents). It is supplied as a concentrate and is typically diluted with water to form, a 6% v/v working fluid, prior to use in machining operations.

Cultivation and isolation conditions

Operationally exhausted synthetic MWF samples containing indigenous microbial communities were used as a source of strains from which to develop inocula. These originated from British Steel (Corby, England); Short Brothers (Belfast, Northern Ireland), Jones and Shipman (Swindon, England, two samples) all of which had different application histories. Samples from different locations were selected in order to construct a consortium that would have wide-ranging applications (e.g., varying waste MWF concentration and application). Strains capable of utilising fresh synthetic MWF as a nutrient source were enriched from four operationally exhausted MWF samples, by adding the fluids to 250-ml conical flasks containing 97 ml M9 minimal medium [16], at pH 7.5 with 3 ml fresh MWF concentrate. Samples (2% v/v) of operationally exhausted MWF were added to individual flasks and incubated at 28°C on an orbital incubator shaker at 170 rpm. After 7 days, 2% v/v samples of suspension from each flask were subcultured into fresh media (as described above). After a further 7 days, 2% v/v subsamples from each flask were inoculated into flasks containing M9 minimal medium and individual MWF components at a concentration of 5 mM. The flasks were incubated as described above, and 100-μl aliquots of the medium were serially diluted and 50 μl aliquots plated onto M9 agar (Agar No. 3, Oxoid, Basingstoke, UK) containing individual components at 5 mM concentration. Plates were incubated at 28°C for 3 days. Following the approach of Baecker *et al* [2], the highest serial dilution of samples that produced growth on plates were considered to have consisted of the most common microorganisms in the original samples. It was from these plates that all colonies were taken and repeatedly streaked to obtain pure cultures [2]. The pure bacterial cultures were subsequently transferred onto 10% v/v tryptic soy broth plus agar (TSBA) plates (Oxoid).

Microbial identification and phenotypic assessment

The phenotypic diversity and identification of individual strains isolated from MWF was determined by fatty acid methyl ester (FAME) analysis, essentially as described by Thompson *et al* [24] and van der Gast *et al* [25]. The samples were injected into a Hewlett-Packard model 5890 series II gas chromatograph and fatty acid peaks were named by the Microbial Identification System (MIS) software (Microbial ID, Newark, DE) and isolates identified using the MIS "Aerobe Library." Similarities between isolates were calculated using a coefficient based on the Euclidean distance between pairs of isolates. Cluster analysis was performed with the MIS "dendrogram program" using unweighted pair group method with arithmetic averages (UPGMA) [18,24].

Genotypic analysis of MWF samples by denaturing gradient gel electrophoresis

The genetic diversity of the whole bacterial community contained in operationally exhausted MWF samples and MWF-enriched mixed bacterial populations were determined. Total DNA samples were extracted, PCR amplified and the fragments analysed by denaturing gradient gel electrophoresis (DGGE), as described by van der Gast *et al* [25].

Rapid metal working fluid component utilisation screening

The ability of bacteria isolated from MWF to assimilate components of the fluid as sole carbon sources was assessed by inoculating each isolate into microtitre plate wells containing 100 μl M9 minimal medium and 3% v/v MWF concentrate as sole sources of carbon. The master microtitre plate (containing all 65 bacterial isolates) was incubated at 28°C overnight or until the broth became turbid. Bacteria were transferred from the master microtitre plate to flat-bottom 96-well microtitre plates containing M9 minimal medium, with individual synthetic MWF components (formaldehyde-based biocide, benzotriazole, dodecanedioic acid, lauric acid, sebacic acid, an amine, glycerol and propylene glycol — Section 2.2) added at 5 mM concentration, using 200-μl tips arranged in the same pattern as the wells in the microtitre plates. The plates were covered with Seal-plate film (Sigma, Poole, UK) to prevent cross contamination or evaporation and incubated for 7 days at 28°C. Optical density was measured every 24 h at 620 nm using a LUCY 1 microplate luminometer (Rosys Anthos, Switzerland). This method allowed a rapid screen of all 65 bacterial isolates for tolerance and ability to assimilate individual MWF components as the carbon source.

Results

Origins of the MWF isolates

The final enrichment broths were serially diluted onto corresponding solid media and incubated at 28°C for 3 days. Plates were then examined and colonies counted and determined to be in the range of 10^6 CFU ml^{-1}. All colonies growing on plates of 10^6 dilution were subsequently isolated and identified. Sixty-five bacterial and 17 fungal strains were isolated from four operationally exhausted synthetic MWF samples taken from four different locations in the UK. From initial identification by microscopic examination, all fungal isolates were determined to be the same strain. The fungi were tentatively identified, using identification keys [9], as belonging to the *Acremonium* genus. With the exception of propylene glycol, the fungal isolates were capable of utilising all the individual components of the MWF as a single source of

Table 1 Number and origins of strains isolated on components of the MWF when present as a sole carbon source. Also presented below is isolation frequency for the 65 bacterial isolates. Identifications were based on FAME analysis

Component	Isolates from				All isolates
	Belfast	Corby	Swindon 1	Swindon 2	
Formaldehyde-based biocide	0	0	0	0	0
Benzotriazole	2	1	0	2	5
Dodecanedioic acid	0	3	2	3	8
Lauric acid	7	2	2	2	13
Amine	0	1	4	3	8
Propylene glycol	10	1	1	4	16
Glycerol	2	0	1	2	5
Sebacic acid	1	2	4	3	10
Total no. of isolates	22	10	14	19	65

C. michiganensis (29%), *R. pickettii* (29%), *M. mesophilicum* (15%), *R. erythropolis* (12%), *M. radiotolerans* (6%), *E. faecium* (3%), *A. xylosoxydans* (2%), *C. flaccumfaciens* (2%) and *P. saccharophilia* (2%).

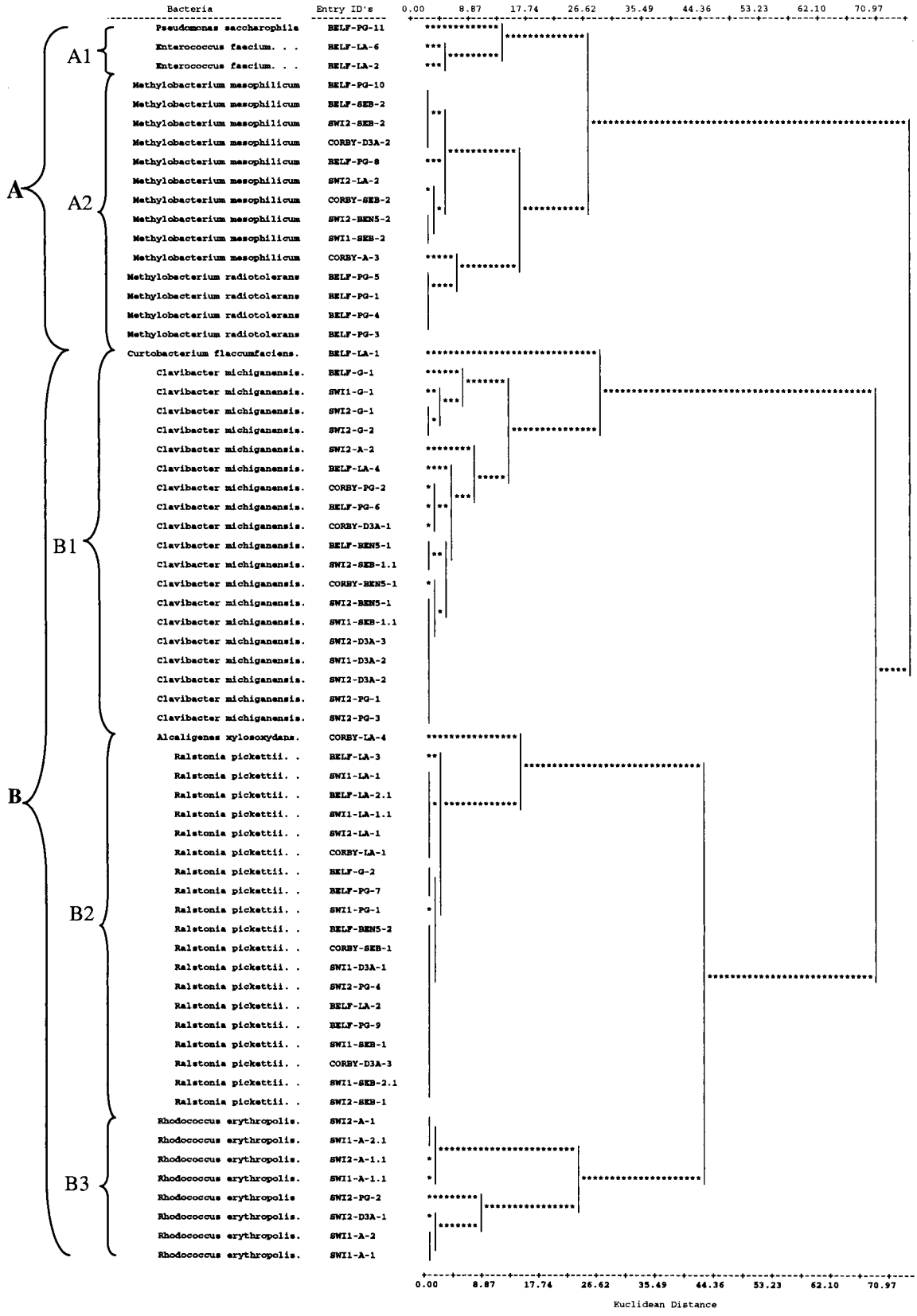

Figure 1 Dendrogram of bacterial strains isolated from MWF components and generated by cluster analysis of FAME profiles, based on Euclidean distance. Each strain was designated with the following codes: SWI1 and SWI2 (Swindon samples), BELF (Belfast) and Corby indicating location. LA, lauric acid; SA, sebacic acid; D3A, dodecanedioic acid; A, amine; PG, propylene glycol; G, glycerol, BEN5, benzotriazole, indicating which substrate strains were isolated.

carbon, at a concentration of 5 mM (including the biocide). However, the fungal strains were isolated only from the used MWF samples taken from Belfast and Corby.

Bacterial strains were isolated from all components of the MWF, when used as the sole source of carbon, with the exception of the formaldehyde-based biocide (Table 1). Almost half of the bacteria were isolated on minimal media containing either lauric acid (20%) or propylene glycol (25%), as the sole source of carbon. The majority of the bacteria from the Belfast sample were isolated on media containing lauric acid (32%) and propylene glycol (45%) as the sole sources of carbon. Seventy percent of the bacteria from the Corby sample were isolated from the three corrosion-inhibitor organic acids. Nearly 60% of all bacteria from the first Swindon sample were isolated on an amine and sebacic acid. With the second Swindon sample, bacteria were isolated on all components, with the exception of the biocide.

Phenotypic characterisation and identification of bacterial isolates

FAME analysis revealed that the 65 bacterial isolates (Table 1) comprised of nine species. The most abundant isolates were the Gram-negative *Ralstonia pickettii* and the Gram-positive *Clavibacter michiganensis* (Table 1). The FAME data for each species was assessed by cluster analysis (Figure 1) to determine the degree of phenotypic diversity within each population.

Cluster analysis of all 65 bacterial isolates revealed two main clusters (A and B) at Euclidean distance (ED) of above 70.97. Cluster A comprised two subclusters (A1 and A2); the largest subcluster (A2) comprised *Methylobacterium mesophilicum* and *Methylobacterium radiotolerans*. Subcluster A1 comprised two: *Enterococcus faecium* and a *Pseudomomas saccharophila* strains. Cluster B consisted of three subclusters (B1, B2 and B3). Subcluster B1 was comprised solely of Gram-positive bacteria, *C. michiganensis* and one *Curtobacterium flaccumfaciens* that clustered at a Euclidean Distance (ED) of 26.62, whereas the Gram-negative subcluster B2 was comprised of one *Alcaligenes xylosoxydans* and at an ED above 2.5, all 19 *R. pickettii* isolates. The Gram-positive subcluster B3, was comprised of *Rhodococcus erythropolis* isolates that clustered at a 26.6 ED. The cluster analysis revealed a low degree of phenotypic diversity within the isolates, identified by FAME to be the same species, but isolated from different samples. For example, all isolates in the *R. pickettii* subcluster B2 (Figure 1) clustered at 2 or less Euclids even though they originated from different samples and locations. Clustering of two isolates at 2 ED and below is taken to indicate that they are the same genotype [24].

Genotypic analysis of used MWF samples by DGGE

To overcome concerns relating to diversity assessments based on plating methods and to account for the unculturable component, the total community composition of bacteria colonising used and fresh MWF was analysed by DGGE (Figure 2). This confirmed findings obtained by plating with FAME analysis that the bacterial community colonising used MWF was low. DGGE analysis of the amplified 16S rDNA from the four used-fluid samples revealed eight or less bands per sample. Furthermore, as well as low diversity, the composition of the colonising bacterial community after enrichment was highly conserved, as revealed by similar DGGE banding patterns. This community convergence suggests that the MWF exerted a selective pressure that favoured just a few distinct bacterial populations regardless of sample origin.

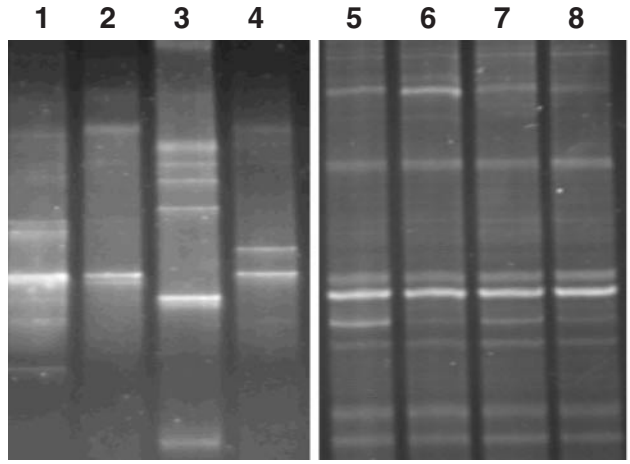

Figure 2 Genotypic diversity (DGGE) of used MWF samples before and after enrichment. Lanes 1 to 4, used MWF samples before enrichment; lanes 5 to 8, after two enrichment steps (sample order 1 to 4 and 5 to 8: Swindon 1, Belfast, Swindon 2, and Corby samples, respectively).

Utilisation and tolerance of synthetic MWF components

All bacterial isolates collected from MWFs were screened for their ability to grow on and hence assimilate the nine individual components of the synthetic MWFs as their sole source of carbon.

An isolate was assumed to have utilised an MWF component as a sole source of carbon if there was an exponential increase in optical density (OD) over a 7-day incubation period; have tolerated a component if there was steady increase (yet not a logarithmic increase) in OD; and have been suppressed if there was no detectable increase in OD. To condense the large quantity of data produced, component utilisation, tolerance and suppression were represented by, +, 0, or −, respectively (Table 2).

Only one isolate, *R. erythropolis* (Isolate SWI1-A-1.1), tolerated the formaldehyde-based biocide, while all other isolates were suppressed (Table 2). Eighteen percent of isolates including seven *C. michiganensis* isolates, from each of the MWF samples analysed, and single isolates of *M. radiotolerans* and *R. erythropolis*, originating from the Belfast and Swindon-1 samples, respectively, tolerated the metal passivator, benzotriazole. All other MWF components were more widely utilised by the isolated populations. The corrosion inhibitor, an unspecified amine, had no detectable suppressive effect on any of the bacterial isolates.

Selection of bacterial isolates for use in bioreactors

The screening procedure revealed that the combined metabolic ability of three isolates together could either tolerate or assimilate all of the MWF components as nutrients or co-metabolites (isolates highlighted in Table 2). The three isolates were subjected to further screening to determine their ability to tolerate or utilise MWF components, when present at a higher concentration (50 mM), reflecting more the concentrations of working fluids. The results are shown in Figure 3, where growth is presented as the mean percentage increase in optical density over a 7-day incubation period.

Table 2 Component utilisation profiles for individual bacterial isolates

No.	Isolate name	Isolate code	Formaldehyde based biocide	Benzotriazole	Dodecanedioic Acid	Lauric acid	Amine (unspecified)	Propylene glycol	Glycerol	Sebacic acid
1	*P. saccharophilia*	BELF-PG-11	–	–	–	0	0	0	0	–
2	*E. faecium*	BELF-LA-2	–	–	–	–	0	+	0	–
3	*E. faecium*	BELF-LA-6	–	–	–	–	0	0	0	–
4	*M. mesophilicum*	CORBY-D3A-2	–	–	–	0	+	+	0	–
5	*M. mesophilicum*	SW12-SEB-2	–	–	–	+	0	+	+	–
6	*M. mesophilicum*	BELF-SEB-2	–	–	–	0	0	0	0	–
7	*M. mesophilicum*	BELF-PG-10	–	–	–	–	0	0	+	–
8	*M. radiotolerans*	BELF-PG-1	–	–	–	+	0	0	+	–
9	*M. radiotolerans*	BELF-PG-5	–	–	–	0	+	+	+	0
10	*M. mesophilicum*	CORBY-A-3	–	–	–	–	+	0	0	–
11	*M. mesophilicum*	SW11-SEB-2	–	–	+	–	+	+	0	–
12	*M. mesophilicum*	SW12-BEN5-2	–	–	–	0	0	+	0	–
13	*M. mesophilicum*	CORBY-SEB-2	–	–	–	0	0	0	0	–
14*	***M. mesophilicum***	**SW12-LA-2**	**–**	**–**	**+**	**–**	**+**	**0**	**0**	**+**
15	*M. mesophilicum*	BELF-PG-8	–	–	–	+	0	0	–	–
16	*M. radiotolerans*	BELF-PG-3	–	0	–	0	0	–	–	–
17	*M. radiotolerans*	BELF-PG-4	–	–	–	0	+	–	0	0
18	*C. flaccumfaciens*	BELF-LA-1	–	–	–	0	+	+	+	–
19	*C. michiganensis*	SW12-PG-3	–	–	–	0	+	0	–	–
20	*C. michiganensis*	SW12-PG-1	–	–	–	+	+	+	+	–
21	*C. michiganensis*	CORBY-D3A-	–	–	–	+	+	0	–	–
22	*C. michiganensis*	SW12-A-2	–	–	–	0	0	0	0	–
23	*C. michiganensis*	SW12-G-2	–	–	–	0	0	0	+	–
24	*C. michiganensis*	SW12-G-1	–	–	–	0	+	0	+	–
25*	***C. michiganensis***	**SW11-G-1**	**–**	**0**	**–**	**0**	**+**	**0**	**0**	**+**
26	*C. michiganensis*	BELF-G-1	–	0	–	0	0	–	0	–
27	*C. michiganensis*	SW11-SEB-1.1	–	0	–	0	0	0	–	–
28	*C. michiganensis*	SW12-BEN5-1	–	0	–	–	+	–	–	–
29	*C. michiganensis*	CORBY-BEN5-1	–	0	–	–	+	–	0	–
30	*C. michiganensis*	SW12-SEB-1.1	–	0	+	–	0	–	–	+
31	*C. michiganensis*	BELF-BEN5-1	–	0	0	–	0	0	0	+
32	*C. michiganensis*	BELF-PG-6	–	–	–	+	+	+	+	–
33	*C. michiganensis*	CORBY-PG-2	–	–	–	0	+	+	+	–
34	*C. michiganensis*	BELF-LA-4	–	–	–	–	+	–	0	–
35	*C. michiganensis*	SW12-D3A-2	–	–	–	0	0	–	0	–
36	*C. michiganensis*	SW11-D3A-2	–	–	–	–	0	0	0	–
37	*C. michiganensis*	SW12-D3A-3	–	–	–	–	0	–	0	–
38	*A. xylosoxydans*	CORBY-LA-4	–	–	–	–	0	–	–	–
39	*R. pickettii*	SW11-LA-1.1	–	–	–	0	0	+	+	–
40	*R. pickettii*	BELF-LA-2.1	–	–	–	–	0	+	+	–
41	*R. pickettii*	SW11-LA-1	–	–	–	–	0	+	+	–
42	*R. pickettii*	BELF-LA-3	–	–	–	–	0	+	+	–
43	*R. pickettii*	BELF-BEN5-2	–	–	–	–	0	0	+	–
44	*R. pickettii*	SW11-PG-1	–	–	–	–	+	+	0	–
45	*R. pickettii*	BELF-PG-7	–	–	–	–	+	+	0	–
46	*R. pickettii*	BELF-P-2	–	–	–	–	+	+	0	+
47	*R. pickettii*	CORBY-LA-1	–	–	–	–	0	+	+	–
48	*R. pickettii*	SW12-LA-1	–	–	–	–	0	–	–	+
49	*R. pickettii*	BELF-LA-2	–	–	–	–	0	0	0	–
50	*R. pickettii*	SW12-PG-4	–	–	–	–	+	+	+	–
51	*R. pickettii*	SW11-D3A-1	–	–	–	–	0	0	+	–
52	*R. pickettii*	CORBY-SEB-1	–	–	–	0	0	0	0	–
53	*R. pickettii*	SW11-SEB-2.1	–	–	–	0	0	0	0	–
54	*R. pickettii*	CORBY-D3A-3	–	–	–	–	0	0	0	–
55	*R. pickettii*	SW11-SEB-1	–	–	–	–	0	–	0	–
56	*R. pickettii*	BELF-PG-9	–	–	–	–	0	0	0	–
57	*R. pickettii*	SW12-SEB-1	–	–	–	–	0	–	0	–
58	*R. erythropolis*	SW12-PG-2	–	–	0	0	+	+	+	–
59*	***R. erythropolis***	**SW11-A-1.1**	**0**	**0**	**0**	**0**	**+**	**+**	**+**	**+**
60	*R. erythropolis*	SW12-A-1.1	–	–	–	–	0	+	0	–
61	*R. erythropolis*	SW11-A-2.1	–	0	0	–	+	+	0	+
62	*R. erythropolis*	SW12-A-1	–	–	–	–	0	+	0	–
63	*R. erythropolis*	SW11-A-1	–	0	0	+	+	+	0	0
64	*R. erythropolis*	SW11-A-2	–	0	0	–	0	+	0	0
65	*R. erythropolis*	SW12-D3A-1	–	–	0	0	+	+	0	–

(+) denotes utilisation, (0) tolerance and (−) suppression.
*Isolates selected for further analysis are highlighted in bold. Isolate codes are explained in Figure 2.

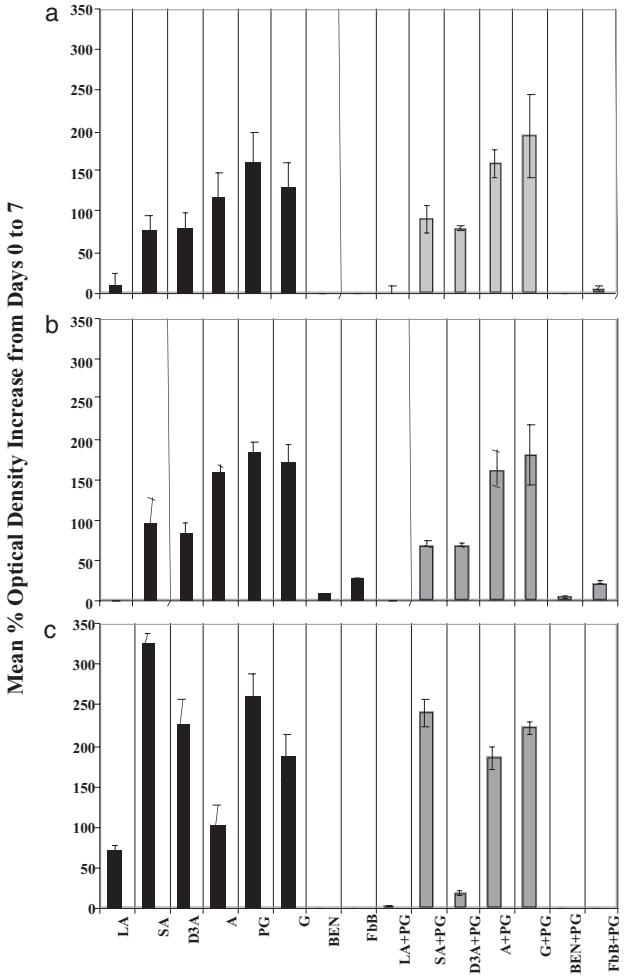

Figure 3 Growth curves of the three members of the selected consortium (based on optical density). (a) *C. michiganensis*, (b) *M. mesophilicum*, (c) *R. erythropolis* in components of the MWF, present as sole carbon sources. Abbreviations: LA, lauric acid; SA, sebacic acid; D3A, dodecanedioic acid; A, an unspecified amine; PG, propylene glycol; G, glycerol; BEN, benzotriazole; FbB, formaldehyde-based biocide. Error bars indicate standard deviation (±) and are the mean of triplicate samples.

Propylene glycol was combined with other MWF components as it was shown to be a readily degradable MWF component.

With the exception of lauric acid, both solely and in the presence of propylene glycol, the *M. mesophilicum* isolate utilised or tolerated all the components of the MWF sources of carbon. It also tolerated the formaldehyde-based biocide, benzotriazole, both solely or in the presence of propylene glycol (Figure 3b). Combinations of the formaldehyde-based biocide, benzotriazole, benzotriazole with propylene glycol, lauric acid with propylene glycol, all suppressed growth of the *C. michiganensis* isolate. However, it tolerated lauric acid and the formaldehyde-based biocide with propylene glycol, and utilised all other test substrates as sole sources of carbon (Figure 3a).

The *R. erythropolis* isolate utilised all substrates tested as sole sources of carbon except: lauric acid with propylene glycol and dodecanedioic acid with propylene glycol, which it tolerated; and the formaldehyde-based biocide, benzotriazole, solely and with propylene glycol, which suppressed growth (Figure 3c). The combined metabolic capabilities of all three isolates were synergistic and greater than the individual isolates. The three isolates together where able to utilise all components of the MWF.

Discussion

Although bioaugmentation, including those for processing chemically mixed wastes, has had some success, the approach is still an unreliable technology and because of this is not widely accepted as an effective approach for treating industrial wastes. The reasons for the failure of bioaugmentation are numerous and complex, but the initial selection of strains is undoubtedly an important factor. In most previous studies, strains have been selected very much on the basis of specific traits, such as the ability to catabolise targeted substrates, with little consideration of their natural abundance within problem substrates or likely survival in the target habitat. In a previous study we demonstrated that selection of strains, based on a thorough knowledge of their diversity and spatial and temporal distribution in the site, was a very effective way of selecting for persistent strains when reinoculated into the site [10]. The aim of this study was to test the effectiveness of this community diversity approach, when combined with knowledge of the assimilation characteristics of isolates, for developing a consortium that was capable of treating operationally exhausted MWF.

The approach used in this study was based on an improved knowledge of the composition of microbial communities that naturally invade MWF. The results of complementary phenotypic and genotypic analysis confirmed the observation made in previous studies that the microbial diversity within MWF is comparatively very low [2,3,15,25]. For instance, in one study 75 of the isolates from MWF were determined, by phenotypic analysis, to be composed of six genera [2]. In this study, we detected only nine taxonomic groups (species) (Table 1) from the 65 isolates studied. In comparison, FAME analysis of 556 isolates taken from field-grown sugar beet revealed 102 species of bacteria [13]. In the past, comprehensive assessments of the microbial communities has been restricted by the limitations of the traditional culture approach, which typically only enables between 1% and 10% [1] of the total community present to be analysed. The advantage we had over previous studies of MWF was the application of total community DNA fingerprinting (DGGE), which also accounted for that part of the community that cannot be isolated on culture media. The results of DGGE analysis confirm observations obtained using culture approaches, that microbial diversity within the MWF was very low. For instance, DGGE analysis of many individual MWF samples revealed less than 5 bands (Figure 3), in comparison, for soil there are typically over 20 bands [11]. Although we are not yet able to equate the presence of specific bands to species or genera, the low number of DGGE bands detected in spent MWF confirms the low diversity within the substrate.

In addition to the very low diversity of the microbial community, the analyses also revealed that although the individual spent MWFs collected were spatially and temporally separated, the species composition and community structure was very conserved. For instance, 10 of the isolates identified by FAME analysis to be *R. pickettii* clustered at or below 2 Euclids (Figure 2). Isolates that cluster at this level are typically taken to be the same genotype [18,24]. There are other instances and species (e.g., *C. michiganensis* [see Figure 2]) where the same strain occurred in at least

two spatially and temporally separated samples. These results suggest that MWFs are highly selective and encourage the growth of a narrow range of species and even genotypes that can survive the toxic nature of the substrate. Furthermore, evidence for the very selective nature of MWF comes from the DGGE analysis (Figure 3). The DGGE band pattern (band positions) of four MWF samples, prior to enrichment, were distinctive suggesting different species composition, but similar community structure (number of species/bands). After two enrichment steps in minimal broth with MWF as the sole nutrient source, the DGGE banding pattern became converged and almost identical, suggesting the enrichment process was highly selective. The highly selective nature of the MWF is assumed to be due to the very toxic nature of the substrate and is reflected in the fungal community that consisted of just one genus, *Acremonium*.

The results of this study are the first step in a process in which the overall aim is to construct a microbial consortium to process operationally exhausted MWFs. The results suggest that the consortium will be dominated by, if not entirely composed of, selected bacteria. Although fungi were isolated, they were detected in only two of four MWF samples. This confirms the reports of previous workers who suggest that fungi are secondary colonisers of MWFs [17]. Previous reports suggest that bacteria are the primary colonisers of MWFs and it is their activities that create the conditions, such as lowering the pH by the degradation process, that then favour fungal invasion [19,26]. It was the low occurrence of fungi in our samples that determined that the consortium we wished to assemble should consist entirely of bacteria.

The low diversity and highly selective nature of the MWF is a highly encouraging result, in terms of constructing a consortium that will be effective at treating one MWF formulation in bioreactors. We are more likely to be able to assemble an effective consortium if the diversity of the community from which to select strains is very low. The convergent nature of the communities, presumably as a result of the selective nature of the MWF, suggests that it should be possible to construct a consortium that can persist in a broad range of MWF conditions of variable use history and chemistry. Our initial results in test-tube-based analysis of the three-strain consortium are very encouraging. The consortium strains (*C. michiganensis*, *R erythropolis* and *M. mesophilicum*) were selected on the basis of their spatial and temporal distribution, which presumably reflected the tolerance to the MWF chemistry and their ability to assimilate components of the MWF as a nutrient source. The combined use of community composition and assimilation data proved to be a very effective way of selecting bacteria that were likely to survive in MWF, since that was where they were originally isolated, and that they could assimilate it as a sole carbon source. We have found that the combined metabolic ability of the three individual strains, when grown together is greater than the sum of the individual strains when grown separately in spent MWF (data not shown). We also found that *R. pickettii* contributed little, in terms of metabolic ability, and so was excluded from the consortium.

Despite their toxicity, including the presence of biocidal agents, MWFs are degraded by microorganisms, and this can be exploited in bioreactor-based systems [12,22,23]. Indeed, bioreactors established for disposing of MWFs are a prime target for bioaugmentation approaches, since they are currently commonly inoculated with microbial communities from sewage, which is heterogeneous in composition and likely to harbour potential human pathogens. The next challenge in this study is to determine how well the consortium survives in bioreactors for treating spent MWF and its effectiveness in enhancing the degradative ability of the indigenous microbial community.

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