The addition of a *Pseudomonas* species ensures degradation of butter by a bioaugmentation product
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A commercial bioaugmentation product, comprising strains of the genus *Bacillus*, was evaluated for its ability to degrade fats, oils and greases (FOGs) in the laboratory. Addition of a *Pseudomonas putida* strain to the commercial mixed population was tested for optimization of FOG degradation by the product. Fat degradation was investigated in aerobic batch culture, at 30°C and 150 rpm for 13 days incubation. Two culture media were investigated, a minimal medium and an enriched nutrient medium, supplemented with 1% (w/v) butter. Fat removal was determined gravimetrically and the lipid content was analyzed using thin layer chromatography (TLC) and gas chromatography (GC). The strains were isolated, identified and tested for their lipolytic activity. No degradation of butter by the product was recorded after 13 days of incubation, while up to 97% degradation was observed when the *Pseudomonas putida* was added to the bioaugmentation product. All the *Bacillus* isolates produced lipase but not the *Pseudomonas putida*. TLC and GC results suggested that while the *Bacillus* spp. hydrolyzed the fat to fatty acids and glycerol, complete metabolism of the breakdown products only took place in the presence of the *Pseudomonad* sp.

**Keywords**: *Bacillus*, bioaugmentation, biodegradation, FOG, mixed microbial populations, *Pseudomonas*

**Introduction**

The removal of fats, oils and greases (FOGs) from wastewater is critically important to ensure that wastewater is disposed of efficiently and economically avoiding blockages of sewers and problems in municipal wastewater treatment plants (Chipasa and Medrzycka, 2006). In Ireland, all food producing facilities must comply with the Water Pollution Act 2007 which limits FOG discharge to less than 100mg/L. FOG may be intercepted at source using grease traps. The FOG is then removed and disposed of to a licensed disposal facility or it may be treated biologically *in situ* using bioaugmentation. Bioaugmentation is an environmentally desirable approach and involves the introduction of suitable microorganisms to the grease trap (Rashid and Imanaka, 2008). The degradative capacity of a microbial inoculum is normally greater than that of any single strain within it because of the cooperative activities of the microorganisms (Huban and Plowman, 1997; Loperena *et al.*, 2009) and the several kinds of extracellular enzymes that microbes produce (Rashid and Imanaka, 2008). Lipid hydrolysis is catalyzed by specific enzymes, called lipases. Lipases (triacylglycerol
acylhydrolase, EC 3.1.1.3) catalyze the hydrolysis of triacylglycerols to diacylglycerols, monoacylglycerols, fatty acids and glycerol under suitable conditions (Sharma et al., 2001; Snellman and Colwell, 2004). The genus Bacillus is of considerable importance as it includes important lipase-producing species. Biodegradation of FOGs is difficult due to their low bioavailability and the hydrophobic properties of the fatty acids they contain (Cammarota and Freire, 2006). Therefore, bioaugmentation is a very desirable process that utilises microbial cells, which not only break down the lipids to fatty acids and glycerol by the activity of lipases, but also assimilate the produced fatty acids to carbon dioxide and water through the β-oxidation process. Several fat-degrading microorganisms such as Bacillus spp., Acinetobacter spp., and Pseudomonas spp. have been isolated from a variety of sources and have been studied for their degradation ability on a laboratory scale (Matsumiya et al., 2007; Loperena et al., 2009). Others have studied mixed microbial populations including Tano-Debrah et al., (1999) who designed an inoculum containing a mixed-culture of 15 bacteria capable of the degradation of high levels of fats, oils and proteins. The biodegradation of FOG is a challenge however the biodegradation of hard fats is a particular challenge. In this study, the biodegradation of a hard fat, butter, by a commercial bioaugmentation product is investigated.

Methods

Inocula preparation
The bioaugmentation product used was in powder form and was prepared according to the manufacturer’s instructions. The product (10% w/v) was dissolved in tap water agitated for up to 1h and 1% (w/v) inoculum was used. Pseudomonas putida was grown overnight in nutrient broth and washed in phosphate buffer saline (pH 7). A 1% inoculum OD 1 at 660nm was used.

Fermentation study
The inocula were added to 250 ml Erlenmeyer flasks containing 100 ml of sterile culture medium to which the butter was added (10 g/L equal to 7.5 g/L total fat). Two culture media were investigated, a minimal medium (MM) containing (g/L): NH₄Cl 0.57, KH₂PO₄ 0.43, K₂HPO₄ 1.09, Na₂HPO₄ 1.33, MgSO₄ x 7H₂O 0.023, CaCl₂ 0.028, and FeCl₃ x 6H₂O 0.025 (Loperena et al., 2006) and an enriched nutrient medium (ENM) containing (g/L): yeast extract 0.2, glucose 0.1, KNO₂ 1, MgSO₄ x 7H₂O 0.2, NaH₂PO₄ 0.1, CaCl₂ x 2H₂O 0.001, MnSO₄ x H₂O 0.01, ferric ammonium citrate 0.005 (Brooksbank et al. 2007). The flasks were incubated aerobically at 30°C, 150 rpm for 7 to 13 days. Bacterial growth was monitored using the pour plate technique.

Fat Determination
Remaining fat was extracted three times with hexane and once with chloroform and determined gravimetrically as described by Brooksbank et al. (2007). Fat degradation was studied using thin layer chromatography (TLC) technique on Silica Gel TLC plates with a mobile phase of hexane-diethyl ether-acetic acid (70:28:2, v/v/v) as described by (Cipinyte et al., 2009). Fatty acid identification and quantification was carried out as described by Brooksbank et al (2007). Methyl esters of the fatty acids were analysed by gas chromatography.

Bacterial Identification and characterisation
Bacterial identification was conducted by Gram-staining, spore-formation, cell
morphology, catalase and oxidase production tests (Harley, 1990) followed by biochemical analysis using the API system (Biomerieux). API 20NE was used for the Gram negative bacterium and API 50CHB combined with API 20E for the Gram positive isolates as indicated by Logan and Berkeley (1984). All methods and reagent preparations were carried out according to the recommendations of the manufacturer. Lipase activity of each strain was detected using tributyrin agar plates and clear zones around the colonies were observed as described by Rashid and Imanaka (2008).

Data analysis
All analyses were performed in duplicate. Data were plotted using Microsoft Office Excel Version 2007 showing the mean values with the standard error mean.

Results
There was no degradation of butter when the bioaugmentation product (1x10^6 cfu/ml) was incubated for up to 13 days (Fig. 1) and no bacterial growth was detected. When the bacterial composition of the product was investigated, eight strains were isolated. All the bacteria were aerobic, Gram positive, rod-shaped, spore-forming organisms and were oxidase and catalase positive indicating membership of the genus Bacillus. All the isolates showed 98-99% homology with the respective genus using the API identification system (Table 1). While each bacterial isolate displayed lipase activity, none of the isolates was capable of degrading butter when grown in pure culture. When Pseudomonas putida was added to the bioaugmentation product, 97% butter degradation in minimal medium and 74% degradation in enriched nutrient medium were observed after 7 days incubation (Fig. 2). The Pseudomonas species did not produce lipase.

Figure 1. Butter removal during incubation of the bioaugmentation product in two media.

<table>
<thead>
<tr>
<th>Bacterial Identification</th>
<th>N° of strains</th>
<th>Lipase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacillus circulans</td>
<td>1</td>
<td>+++</td>
</tr>
<tr>
<td>Bacillus megaterium</td>
<td>1</td>
<td>+++</td>
</tr>
<tr>
<td>Bacillus subtilis</td>
<td>3</td>
<td>+++</td>
</tr>
<tr>
<td>Bacillus licheniformis</td>
<td>2</td>
<td>+</td>
</tr>
<tr>
<td>Brevibacillus laterosporus</td>
<td>1</td>
<td>+</td>
</tr>
</tbody>
</table>

Table 1. Identification of the bacteria present in the product and indication of lipolytic activity.

Figure 2. Bacterial growth and butter degradation by the bioaugmentation product combined with Pseudomonas putida in two media.
Analysis of the extracted fat by TLC showed the release of free fatty acids by the activity of lipase. The hydrolysed fat in the two treatments (with and without the *Pseudomonas* sp.) is illustrated in Figure 3 (b and a, respectively), using representative samples. Figure 3(a) shows analysis of samples at days 3, 5, 9, 11, 13 and figure 3(b) shows samples at days 1, 3, 5, 9.

TLC analysis showed that the fat was hydrolyzed after 24 hours incubation and the hydrolysis products were rapidly consumed in the presence of *Pseudomonas putida* but not in its absence. Quantified analysis of the fatty acids by GC showed that the main fatty acids present in the butter were lauric acid, myristic acid, palmitic acid, stearic acid and oleic acid. While assimilation of these fatty acids did not occur in the presence of the product, all five fatty acids with the exception of stearic acid, were readily assimilated in the presence of the *Pseudomonas* sp. (Figure 4, a and b.)
Discussion

The optimum microbiological composition of a bioaugmentation product is critical for effective performance. In the case of FOG, effective biodegradation of hard fats is not widely reported. Brooksbank et al. (2007) reported that 40% degradation of lard (10ml/L) was obtained by a commercial bioaugmentation product after 21 days compared with the degradation of up to 97% butter in 7 days in this study. Saravia et al. (2004) studied the degradation of butter by a bioaugmentation product and reported 82-95% degradation of butter after 20-40 hours incubation, however they used lower concentrations of fat (0.2-1 g/L) and the butter was homogenized prior to biodegradation. In the current study, TLC analysis showed that it took 11 days of incubation for the fat to be hydrolysed when the product alone was used. However, following the addition of Pseudomonas putida to the product good hydrolysis of the fat was observed within 1 day of incubation and total hydrolysis was observed within 3 days of incubation. Cipinyte et al. (2009), made a similar observation. They studied the degradation of lipids by a mixed culture of Gram negative and Gram positive strains (Enterobacter aerogenes and Arthrobacter sp.). Very good degradation of the fat was noted after 3 days incubation. However, they used a softer fat, sunflower oil, and at a lower concentration (0.5%) to that used in this study. Analysis using gas chromatography confirmed the complete metabolism of the fatty acids in the presence of the Pseudomonas species. There was a preferential use of oleic acid over stearic acid. Metabolism of the oleic acid gave rise to a temporary rise in the levels of stearic acid on day 7. By day 11 all fatty acids were metabolised. This has been also reported by Brooksbank et al. (2007) and Sun and Wakeham (1994) demonstrating the preferential degradation of unsaturated fatty acids over saturated fatty acids of the same carbon chain length. The increase in the levels of stearic acid suggests production of stearic acid following the degradation of oleic acid a results also observed by Chipasa et al. (2008) and Pereira et al. (1998). This suggests a cooperative activity between the Bacillus spp. and the Pseudomonas putida. The Bacillus spp. could hydrolase the fat, through the activity of the lipase, but not assimilate the breakdown products while the Pseudomonas sp., incapable of degrading the fat could assimilate the breakdown products generated by the Bacillus spp.

Conclusions

No degradation of butter was observed by the bioaugmentation product containing only Bacillus spp, while up to 97% butter degradation was achieved after the addition of the Pseudomonas putida in the bioaugmentation product.

Degradation products were glycerides fractions and free fatty acids which were only consumed after the addition of the Pseudomonas sp. in the bioaugmentation product.

The TLC and GC results suggests cooperative activity between the Bacillus spp. and the Pseudomonas putida.

The findings in this study show that an effective bioaugmentation product can be designed by using the correct combination of bacterial species.
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**Disclosures**
The authors have nothing to disclose.