ABSTRACT

Thus far, only monocultures and mixed cultures have been used to generate bioactive compounds. Here, bioactive compound production of a developed *Penicillium* spp.-*bradyrhizobium elkanii* SEMIA 5019 biofilm was compared with monocultures of the two microorganisms. The biofilm was observed to generate a higher number of detectable compounds with relatively high concentrations in comparison to the bacterial and fungal monocultures. This technology can be used to produce different compounds for the discovery of novel compounds that can be used in drug discovery studies, with applications to many fields of biotechnology.

INTRODUCTION

The microbial world has many diverse relationships that play important roles in human lives. Microbially-produced bioactive compounds are attracting increased attention as useful agents for medicine, veterinary medicine, agriculture and as unique biochemical tools, and have been isolated using both mono and mixed cultures of microbes[1-3].

There is a great scope for developing a biofilm technology of producing eco-friendly, beneficial microbial biofilms (EMB) for numerous applications[4], because microbes in their ‘biofilm’ mode have already shown efficient performance in various biotransformation processes[5,6]. Biofilms are multi-cellular consortia of microbial cells (fungal, bacterial, algal and/or other microbial) that closely adhere to each other and are often encased in a self-produced extra-cellular polymeric substance (EPS). Such interactions give the biofilm community meta-
Biofilms and physiological capabilities which are not possible for the individual unattached cells. Although biofilms are generally considered to be problematic, their beneficial aspects have often been overlooked. Previous studies show that organic acids production was increased in biofilms compared to monocultures and non-biofilm forming mixed cultures. Thus, it is apparent that biofilms have a higher potential of producing organic acids, some of which may be bioactive compounds.

There is limited knowledge of interspecies interactions in biofilm communities. Yet, a range of metabolic interactions has been observed among microorganisms in biofilms, including mutualistic and commensal relationships. An understanding of these relationships in biofilms could aid the design of multispecies biofilms for the biosynthesis of specialty chemicals or the biodegradation of xenobiotics that cannot be metabolized efficiently by a single microorganism.

Therefore, in this study we attempt to use the biofilm technology to produce bioactive compounds by using a previously developed fungal-bacterial biofilm. Monosaccharides constitute a major group of bioactive compounds, because they are the starting materials for a number of drugs such as those possessing anti-microbial, anti-tumor, anti-viral, anti-oxidant, free radical scavenging and anti-inflammatory activities. Thus, monosaccharides are used as the test compounds in the present study.

EXPERIMENTAL

Culturing of microorganisms

*Botrytis elkanii* SEMIA 5019 applied as a soybean inoculant was used for this study because microbial metabolism in nature is frequently nitrogen limiting, and this *N₂* fixer has been shown to accumulate atmospheric *N₂* in microbial communities. Cultures were maintained on yeast mannitol broth (YMB) with 0.2 g was hydrolyzed with 0.6 ml of 4M trifluoroacetic acid (TFA) at 105°C for 4 h. It was rested overnight and hydrolysate was filtered through glass fibre filter. It was evaporated to dryness by a stream of *N₂* at 50°C, to remove TFA. One milliliter of methanol was added and evaporated to dryness, and this was repeated. Resulting monosaccharides were reduced to alditols using 1M NaBH₄ in NH₄OH and kept at 25°C for 30 min. To the solution, 0.3 ml acetic acid in methanol was added to decompose excess NaBH₄. The solution was blown to dryness by a flow of *N₂* and above step was repeated. Borohydrate was removed by adding 0.5 ml of methanol and dried under a stream of *N₂*.

Preparation of treatments

One milliliter of a 6-day-old *B. elkanii* SEMIA 5019 culture was inoculated together with 50 μl *Penicillium* spp. spore suspension to 50 ml of concentrated YMB to form a mixed culture. They were separately inoculated to 50 ml of the concentrated YMB to form monocultures. Fifty milliliter of the concentrated YMB without microorganisms was used as the control. Each treatment was replicated four times in 50 ml conical flasks and kept on a rotary shaker at 28°C for 14 days. Biofilms were observed after 7 days of incubation using a phase-contrast microscope with an oil immersion lens and lacto-phenol cotton blue stain. After 14 days of incubation, the cultures were centrifuged at 13,148 x g for 20 min. Supernatants were separated and freeze-dried.

Sample preparation

Analysis of monosaccharides was done by gas-liquid chromatography of alditol acetates based on the method of Amelung et al. Here, carbohydrates with acid constituents are hydrolysed to constituent monosaccharides, which are subjected to reduction followed by acetylation to yield alditol acetates. Of the freeze-dried crude material, 0.2 g was hydrolyzed with 0.6 ml of 4M trifluoroacetic acid (TFA) at 105°C for 4 h. It was rested overnight and hydrolysate was filtered through glass fibre filter. It was evaporated to dryness by a stream of *N₂* at 50°C, to remove TFA. One milliliter of methanol was added and evaporated to dryness, and this was repeated. Resulting monosaccharides were reduced to alditols using 1M NaBH₄ in NH₄OH and kept at 25°C for 30 min. To the solution, 0.3 ml acetic acid in methanol was added to decompose excess NaBH₄. The solution was blown to dryness by a flow of *N₂* and above step was repeated. Borohydrate was removed by adding 0.5 ml of methanol and dried under a stream of *N₂*.

The upper layer was removed using a Pasteur pipette and filtered through a silica
gel filter. This was re-extracted with 0.5 ml EtOAc and concentrated in a cryo tube.

**Gas-liquid chromatographic analysis**

Gas-liquid chromatography of the monosaccharides was performed with a Shimadzu GC-9 AM gas chromatograph (GC) with a 30 m × 0.25 mm i.d × 0.25 μm film thickness SPB™-1701 fused silica capillary column. Analytical conditions were an initial temperature of 180°C, injector at 220°C and detector at 260°C. Residue obtained from the sample preparation was dissolved in 30 μl EtOAc of which 20 μl was injected into the GC by the split technique. Standards of monosaccharides were also analyzed with similar preparation steps but without TFA hydrolysis.

**RESULTS AND DISCUSSION**

On observing upon incubation, the *Penicillium* spp. mycelium was heavily colonized by *B. elkanii* SEMIA 5019, forming the *Penicillium* spp.-*B. elkanii* SEMIA 5019 biofilm (Figure 1). Detectable quantities of fucose, ribose, arabinose and xylose were produced only by the biofilm. They were not detected in the *B. elkanii* SEMIA 5019 alone or *Penicillium* spp. alone treatment, possibly due to their production below the detection limits. The monocultures produced up to 10 detectable monosaccharides, whereas the biofilm produced 123 of them, though most of them are yet to be identified (TABLE 1).

This clearly shows the very high potential of the biofilm in generating bioactive compounds in higher numbers compared to its constituent monocultures.

**TABLE 1: Concentrations of monosaccharides detected of four replicates of the microbial treatments**

<table>
<thead>
<tr>
<th>Microbial treatment</th>
<th>Fucose</th>
<th>Ribose</th>
<th>Arabinose</th>
<th>Xylose</th>
<th>Number of unidentified compounds</th>
<th>Total number of compounds</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bradyrhizobium elkanii SEMIA 5019</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>0 - 4</td>
<td>0 - 4</td>
</tr>
<tr>
<td><em>Penicillium</em> spp.</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>5-10</td>
<td>5-10</td>
</tr>
<tr>
<td><em>Penicillium</em> spp. - <em>B. elkanii</em> SEMIA 5019 biofilm</td>
<td>38 ± 13</td>
<td>629 ± 112</td>
<td>25 ± 12</td>
<td>34 ± 1</td>
<td>36-123</td>
<td>38-123</td>
</tr>
<tr>
<td>Control (no microbes)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>9</td>
<td>9</td>
</tr>
</tbody>
</table>

Mean ± standard error. ND - not detected. Ranges are the numbers of the four replicates.
SHORT COMMUNICATION

and cell-to-cell communication to achieve optimal conditions. Thus, substrates are more optimally utilized in the biofilm formation, resulting in faster degradation of the primary nutrients. This is further enhanced by the critical cell density dependent quorum sensing which leads to biofilm formation. In previous studies, single species biofilms have been used to produce industrially important chemicals. However, the use of dual-species biofilms have higher quorum sensing and community level gene expression that lead to higher levels of substrate utilization and maximization of products, as observed in the present study.

Conventional drug discovery studies use natural or genetically modified monocultures of microorganisms. But in the biofilm technology interactions of the same microorganisms with different microbes can yield a multitude of results and an endless number of novel compounds. This technology can be used to produce different compounds by the combination of a variety of microbes, by changing the carbon source or altering the culture conditions. Individual compounds with bioactivity can also be optimized by studying the concerned interactions and their culture conditions. Its manipulative nature therefore can be made use to open a myriad of avenues for the discovery of novel, easily produced compounds that can be used in drug discovery studies thus finding solutions to many hurdles in the treatment of diseases. Advantage of this also lies in the reduced time frame compared to isolation of compounds from plants, and conventional methods used. Further studies to optimize this biotechnology and also to produce and detect other compounds of bioactive significance are in progress.

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REFERENCES