Torulopsis petrophilum and Surface Activity

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Torulopsis petrophilum can synthesize either a glycolipid surfactant or a protein emulsifier depending on the substrate used. These compounds were not produced to facilitate the uptake of an insoluble carbon source. The glycolipids produced were identical to the mixture isolated from T. bombicola.

Many bacteria produce extracellular lipids when grown in media containing water-insoluble substrates such as hydrocarbons (1, 4, 5, 8–10, 16; R. Rapp and F. Wagner, Abstr. 5th Int. Ferm. Symp. 1976, 133). There are also examples of polymeric emulsifying agents produced by microorganisms growing at the expense of hydrocarbons (5, 7, 11, 12, 13, 19, 20). This has led to the conclusion that these compounds are produced in response to the insoluble substrate to emulsify or disperse this phase into the water and make it available to the organisms (1, 5, 7–9, 12, 17–20; R. Rapp and F. Wagner, Abstr. 5th Int. Ferm. Symp. 1976, 133).

Emulsification of an insoluble substrate cannot be the only reason for the production of surface-active compounds by microbes because there are some exceptions to the usual results. Bacillus subtilis produced high yields of a very active surfactant when the growth medium contained a carbohydrate as the carbon source (3). Corynebacterium fascians CF15 was able to stabilize emulsions of water and hydrocarbon even if grown on a medium which did not contain hydrocarbon (2).

Few of the data about microbial surface-active agents have been from studies with yeasts. The notable exceptions are the glycolipids produced by Torulopsis species which have recently been shown to be surface active (9, 10, 16). Torulopsis petrophilum was isolated as an organism capable of degrading fractions of crude oil, but there are no reports of its ability to produce surface-active agents (15).

T. petrophilum ATCC 20225 was maintained on nutrient agar at 4°C. The media used all had a basic mineral salts composition of KH_{2}PO_{4} (0.1%), MgSO_{4} (0.02%), CaCl_{2} (0.0002%), FeSO_{4} (0.0001%), and Na_{3} EDTA (0.0002%). The carbon sources used were either 4% glucose or 4% hexadecane. For glycolipid production, the substrate used was 10% glucose plus 9.5% corn oil. Yeast extract was added to most of the media, and the nitrogen source was NH_{4}NO_{3} (0.2%). Biomass was determined as dry weight. Surface tensions were determined with a Fisher Autotensionmat (1, 2).

Emulsification tests were done in one of two ways. A quick test has already been described (1, 2) which used a test tube with water and an oil phase. A more sensitive test was developed to measure mean droplet diameter, which is related to the stability of an emulsion. One hour after being vortexed 1 ml of emulsion was added to 14 ml of 1.5% gelatin, stirred gently, and poured into a 100-mm petri dish to solidify. Microscopic examination gave a size distribution of the drops, and this was used to determine a surface mean diameter.

The glycolipids were collected by washing the spent culture medium twice with equal volumes of ethyl acetate (9, 10, 16). The crude product was washed three times with equal volumes of hexane.

Thin-layer chromatography was done with Fisher Redi-Gel plates. The developing solvent was chloroform-methanol-water (65:15:2). Components were visualized with a solution of α-naphthol (4).

Protein was determined with Coomassie brilliant blue G dye (14). Carbohydrate was determined by the phenol test (6). The standards were bovine serum albumin and glucose. To determine the nature of the emulsifier, we performed a series of emulsion extractions. Biomass was removed by centrifugation at 104 × g for 10 min. The supernatant was vortexed with an equal volume of kerosene. The unemulsified lower aqueous phase was removed, a small sample was removed for analyses, and the remainder was emulsified with fresh kerosene. The process was repeated. Analyses for protein and carbohydrate were performed.

Table 1 contains typical data of studies of T. petrophilum grown with media containing glucose. Small amounts (0.1%) of yeast extract are necessary for the synthesis of appreciable amounts of the emulsifier. A fraction of the
emulsifying activity (ca. 15%) is removed with the biomass, but most of the activity is associated with the supernatant after centrifugation. If the experiment was repeated with hexadecane instead of glucose, there was no growth or emulsification.

The use of a mixed substrate containing 10% glucose, 9.5% sunflower oil, and 0.5% yeast extract resulted in growth of the yeast, but no emulsifier was produced. This fermentation did yield 10 g of glycolipid per liter which had no ability to stabilize emulsions. Thin-layer chromatography identified a mixture of six glycolipids present in the relative amounts (Rf values) 0.62 > 0.52 > 0.60, 0.50, 0.39, 0.19. This enriched medium was the same one used for optimum production of glycolipids by *Torulopsis bombicola* (D. G. Cooper and D. A. Paddock, submitted for publication). The composition of the glycolipids was identical for these two yeasts, but there was a large difference in the maximum yields. *T. bombicola* consistently converted 35% of the substrate to glycolipid, but *T. petrophilum* yielded only 10% product. However, *T. bombicola* did not produce any type of emulsifier with any growth media.

Figure 1 is a plot of data collected from a time-dependent growth study of *T. petrophilum* on a medium containing only glucose. The emulsifying ability of samples of the broth increased as the biomass increased. However, the nature of the emulsifier changed dramatically during the fermentation. Initially, the cells stabilized the emulsions. At the end of the exponential-growth phase, a water-soluble emulsifier was present in the supernatant after centrifuging. Forty-eight hours after the beginning of the stationary-growth phase, most of the emulsification ability was due to the soluble emulsifier, not the cells. This product is not necessarily a secondary metabolite. It could be a surface component lost from the older cells.

Attempts to isolate a crude emulsifier were unsuccessful. It could not be precipitated with salt addition, acetone or ethanol additions, or by adjusting the pH of the solution.

An indirect analysis was performed by shaking the supernatant with kerosene to form an emulsion and then analyzing the remaining aqueous layer (Fig. 2). There was no change in carbohydrate concentration, but there was a dramatic decrease in protein concentration as each aliquot of kerosene was shaken with the aqueous phase.

If the emulsion was broken by centrifugation and the aqueous phase was analyzed there was no change in the concentration of protein in the

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**TABLE 1. Growth and properties of *T. petrophilum***

<table>
<thead>
<tr>
<th>Yeast extract added (g liter⁻¹)</th>
<th>Biomass (g liter⁻¹)</th>
<th>Surface tension (mN m⁻¹)</th>
<th>Emulsion after 24 h²</th>
<th>Broth Cells removed</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>2.82</td>
<td>43</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>1.0</td>
<td>5.12</td>
<td>47</td>
<td>70</td>
<td>58</td>
</tr>
<tr>
<td>5.0</td>
<td>9.90</td>
<td>46</td>
<td>65</td>
<td>57</td>
</tr>
</tbody>
</table>

² Percentage of fluid which is an emulsion.

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**FIG. 1.** Growth study of *T. petrophilum*: Symbols: O, biomass; Δ, surface tension; □, percent emulsion after 24 h; ■, percent emulsion of cell-free broth after 24 h.

**FIG. 2.** Analyses of aqueous phase after emulsion extractions; carbohydrates, top of each bar, and proteins, hatched area.
water. Extracting various aqueous samples that exhibited emulsifying properties with ethyl acetate or kerosene did not alter their ability to stabilize emulsions with hydrocarbons.

Figure 3 shows the effect of pH on the surface diameter in the emulsions formed by the cell-free emulsifier solution. The most stable emulsions are those at pH 6. Similar experiments with the addition of salts demonstrated little effect. There were only small increases in surface mean diameter as either NaCl or CaCl₂ were added up to 100 g liter⁻¹.

Although most of the emulsion studies were with kerosene, the emulsifier was also active when pure hydrocarbons or vegetable oil were used. Table 2 contains data for the emulsification of various hydrocarbons with a sample of centrifuged medium. Short-chain alkanes, toluene, cyclohexane, and kerosene were all emulsified much more effectively than the longer-chain alkanes. The water-soluble emulsifier, emulsan (13) from a bacterium, has a marked specificity for the types of hydrocarbons emulsified which is not observed with the T. petrophilum product except that shorter alkanes are more easily emulsified than longer alkanes.

T. petrophilum produced different types of surface-active agents depending on the media used for the growth of this yeast. When the medium contained a water-insoluble vegetable oil, T. petrophilum produced appreciable amounts of glycolipids which were incapable of stabilizing emulsions with either hydrocarbons or vegetable oils. When the medium was a single phase, with no requirement for an emulsifier to make an insoluble substrate more accessible, this yeast produced a potent extracellular protein emulsifier. These results are contrary to conventional arguments that microbial emulsifiers and surfactants are produced to facilitate the uptake of water-insoluble substrates (1, 5, 7-9, 12, 17-20; R. Rapp and F. Wagner, Abstr. 5th Int. Fern. Symp. 1976, 133).

The cost of using hydrocarbons as a growth substrate will prohibit the large-scale use of biosurface-active agents. T. petrophilum can be used to synthesize either a biosurfactant or an emulsifier with renewable substrates. This yeast is as effective as bacteria at generating surface-active agents, but these metabolites are apparently not synthesized to facilitate substrate utilization.

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**LITERATURE CITED**


**TABLE 2. Emulsification of hydrocarbons by cell-free broth of T. petrophilum**

<table>
<thead>
<tr>
<th>Hydrocarbon phase</th>
<th>Emulsion after 24 h¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pentane</td>
<td>70</td>
</tr>
<tr>
<td>Hexane</td>
<td>72</td>
</tr>
<tr>
<td>Dodecane</td>
<td>35</td>
</tr>
<tr>
<td>Hexadecane</td>
<td>24</td>
</tr>
<tr>
<td>Toluene</td>
<td>80</td>
</tr>
<tr>
<td>Cyclohexane</td>
<td>72</td>
</tr>
<tr>
<td>Kerosene</td>
<td>72</td>
</tr>
</tbody>
</table>

¹ Percentage of fluid which is an emulsion.

**FIG. 3. Effect of pH on mean diameter of kerosene droplets in water, stabilized by the soluble emulsifier from T. petrophilum.**