Production of Sophorolipids by Candida bombicola

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Abstract

Sophorolipid production was studied using *Candida bombicola* ATCC 22214. Solid and liquid alkanes or fatty acids were used as lipophilic substrates in shake flask experiments. With a few of these substrates, there was a significant amount of direct incorporation into the sophorolipid products. Of all substrates tested, only hexadecane and heptadecane resulted in the exclusive production of crystalline, diacetylated lactone sophorolipids that contained a single type of hydroxy fatty acid. It was determined that the presence of a single type of sophorolipid structure was necessary to obtain crystalline products.

It was also possible to obtain the crystalline product using larger scale experiments with hexadecane. The direct incorporation of hexadecane was shown to result in increased sophorolipid production efficiency with hexadecane being used as the sole source of the hydroxy fatty acids needed for sophorolipid production and the hydrophilic substrate being used as a source of energy. With the other lipophilic substrates, this efficiency was lost because there was appreciable *de novo* synthesis of the fatty acids.
Résumé

La production de sophorolipides par le champignon ascomycète Candida bombicola ATCC 22214 a été étudiée. Des alcanes et des acides gras sous forme liquide et solide ont servi de substrats lipophiles dans des expériences menées avec des flasques agités. De tous les substrats testés, seuls l'hexadecane et l'heptadecane ont incité la production exclusive de sophorolipids diacétylés cristalline lactone contenant une seule sorte d'acide gras hydroxy1. Les expériences démontrée que la formation d'un produit cristalline exigéait la présence de sophorolipids ayant la même structure. Cette structure uniforme se retrouvait quand les sophorolipids contenaient tous la même sorte d'acide gras hydroxy1.

Un produit cristalline a également été produit à grande échelle utilisant l'hexadecane comme substrat. Il a été démontré que l'incorporation directe de l'hexadecane augmentait l'efficacité de la production de sophorolipids lorsque l'hexadecane fournissait d'une source unique d'acides gras hydroxy1 tandis que le substrat hydrophile servait de source d'énergie. Cette efficacité se perduait avec les autres substrats lipophiles, puisque il y avait une partie appréciable de novo des acides gras.
Acknowledgements

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1.0 Introduction

1.1 Metabolic Pathways Involved in Sophorolipid Production

Yeast in the genus Candida (formally Torulopsis) such as C. apicola, C. gropengiesseri, and C. bombicola are known to produce extracellular biosurfactants known as sophorolipids. These compounds have surfactant properties because they possess both hydrophilic and lipophilic moieties. The hydrophilic portion is the sugar sophorose. The lipophilic portion is a hydroxy fatty acid. Sophorolipids are produced as a mixture of acidic and lactonic forms (Figures 1.1 & 1.2). Up to nine different structural classes of sophorolipids have been observed\(^2,10\). Both acidic and lactonic forms can contain differences in the length of the fatty acid component, the presence of unsaturations in the fatty acid chain, and the degree of acetylation in the 6' and 6'' positions.

The initial steps of the enzymatically-catalyzed oxidation of lipophilic substrates by Candida yeasts are shown in Figure 1.3. Lipophilic substrates (alkane, alcohol, or aldehyde) are first oxidized to fatty acids. At this point there are two possible pathways by which hydroxy fatty acids are produced. In the first pathway, the acid is broken down via the β-oxidation oxidation pathway (Figure 1.4). The oxidized substrate is metabolized two carbons at a time, releasing acetyl CoA each time. Acetyl CoA is then involved in a number of functions. It may be used in cellular respiration or it may be used to build larger biomolecules. Of interest here, is the formation of new long chain fatty acids. A comprehensive description of the fatty acid synthesis process can be found
elsewhere\textsuperscript{34}. The synthesized acid may then be hydroxylated to a hydroxy fatty acid. This hydroxy fatty acid can then be incorporated into the sophorolipid product. Hydroxy acids produced in this manner are said to be synthesized \textit{de novo}.

There is an alternative way in which hydroxy fatty acids can be produced. In this case, the oxidized substrate is not broken down but is instead immediately hydroxylated. The resulting hydroxy fatty acid will have the same chain length as that of the substrate and can be incorporated into the sophorolipid. This pathway is referred to as direct incorporation.

There is a great deal of evidence that Candida yeasts generate hydroxy acids by both direct incorporation and \textit{de novo} synthesis\textsuperscript{3,4,36,46,47}. Also, the likelihood of direct incorporation occurring may be increased when an additional substrate such as glucose is provided that can be used to maintain cellular respiration\textsuperscript{6,25,36}.

Unlike hydroxy fatty acids, there is no evidence of the direct incorporation of sugars into the glycolipids produced by \textit{Candida bombicola}. Studies have been performed where sucrose and fructose were provided as hydrophilic substrates\textsuperscript{16,40}. However, regardless of the type of sugar substrate, sophorose was the only sugar observed in the glycolipid product. The sophorose sugars are therefore synthesized \textit{de novo}.

Less is known concerning the final steps of sophorolipid synthesis. However, the scheme shown in Figure 1.5 has been proposed based on structural elucidation studies\textsuperscript{2}. It is believed that the sophorose and hydroxy fatty acid components are synthesized simultaneously and then attached via a hemiacetal bond. The next steps are likely acetylation and lactonization. The exact enzymes involved in these final steps have not
been identified. However, an acetyl-CoA dependent acetyltransferase has been purified from *C. bogoriensis*, and it is likely that similar enzymes are present in other *Candida* yeasts (*C. apicola, C. bombicola*).

The precise reason for sophorolipid production by *Candida* yeasts is unknown. One theory that has been forwarded is that the sophorolipids are produced expressly to aid in the assimilation of alkanes\textsuperscript{30,31}. This theory is based on the observation that the presence of n-alkanes stimulates the growth of *C. bombicola* whereas other lipophilic substrates do not. Other studies have indicated that the enzymes responsible for sophorolipid production are regulated by the sugar substrate provided in the medium\textsuperscript{24}. However, the exact details of this regulation have not been worked out.
Figure 1.1: An acidic sophorolipid produced by *C. bombicola*.

Figure 1.2: A lactonic sophorolipid produced by *C. bombicola*.


**Figure 1.3**: Enzymatically catalyzed steps of alkane oxidation by *Candida bombicola*.
Figure 1.4: Through the β-oxidation pathway fatty acids are metabolized two carbons at a time releasing Acetyl CoA each time.
Sophorose
+ Hydroxy fatty acid

Figure 1.5: Final steps in sophorolipid synthesis as proposed by Asmer et al\textsuperscript{2}. 
1.2  **Relationship Between Medium Composition and Product Quality**

Much of the work that has been done involving sophorolipid production was carried out using a semi-complex media that was originated by Tulluch *et al.*[^45][^46]. This medium was as follows:

**Table 1.1**: The composition of a standard medium used in a number of studies with *Candida bombicola*.

<table>
<thead>
<tr>
<th>Medium Composition</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Yeast Extract</td>
<td>10 g/L</td>
</tr>
<tr>
<td>Glucose</td>
<td>100 g/L</td>
</tr>
<tr>
<td>Urea</td>
<td>1 g/L</td>
</tr>
<tr>
<td>Various lipophilic substrates</td>
<td></td>
</tr>
</tbody>
</table>

This medium has since been used by a large number of researchers and has been shown on numerous occasions to result in the overproduction of sophorolipids by various *Candida* species. Some studies have suggested that it is possible to selectively produce one or more of the sophorolipid structural classes by manipulating the relative concentrations of the medium components. Studies using this same medium showed that the yeast extract concentration had an effect on the degree of lactonization of the product. Specifically, lower yeast extract concentrations favored lactone production[^34][^48]. However, a study by Casas *et al.* produced a contrary result. Their investigation of the effects of culture media on sophorolipid production showed that decreasing the yeast extract in the medium favored the acidic form[^5]. Most of the studies with *Candida bombicola* used various vegetable oils, oleic acid, or other relatively inexpensive materials.
lipophilic substrates\(^5, 7, 8, 10, 12, 13, 38, 39, 46, 48\). Hydrocarbons were used much less frequently. However, an extensive overview of potential lipophilic substrates has been performed by Davila \textit{et al.}\(^11\). The paper stated that, when hexadecane or octadecane were used as substrates, the resulting sophorolipid consisted of a high percentage of lactonic forms (up to 80\%). However, no data was presented.

In all of the work mentioned above, sophorolipid morphology has been reported as a denser than water, viscous brown oil. However, there have been some reports of crystalline sophorolipids being produced using the same, or a very similar medium composition.\(^32, 38, 39, 46, 47\). Some ideas have been offered concerning the conditions that bring about sophorolipid crystallization. It has been suggested that pH adjustment and a decreased carbon to nitrogen ratio in the medium have affected product composition and morphology\(^42\). However, an exact relationship between morphology, hydroxy fatty acid content, lactonization and acetylation has not been determined.
1.3 Sophorolipid Applications

Although both acidic and lactonic sophorolipids are usually produced, the various lactonic forms usually represent the largest fraction of the product\(^2,11,47\). These lactones are biodegradable compounds that, because of their bacteriostatic properties, have found application in cosmetics and deodorants. Lactone sophorolipid has also been patented as an anti-dandruff agent\(^37\). They are also sources for compounds that cannot be easily synthesized and sold commercially, such as 15 OH hexadecanoic acid and OH heptadecanoic acids. Lactonic sophorolipids have also been investigated as potential precursors to bio-plastics\(^40\).

Lactone sophorolipids have useful applications and there have been a number of instances where medium composition has seemed to favor their production. Therefore, there has been significant interest in developing processes to produce these compounds in high purity and a number of patents have been taken out to this effect\(^18,19,22,23\). However, a feasible process for the large scale production of high purity lactone has yet to be developed. It is likely that a better of understanding of the relationship between medium composition and product quality will be required for further process development and optimization.
2.0 Objectives

The primary objective of this work was to elucidate the conditions that allow for production of lactone sophorolipids. A secondary objective was to determine how the lipophilic substrate influences the morphology, hydroxy fatty acid content, and structural form of the sophorolipid product.
3.0 Materials and Methods

3.1 Microorganism

3.1.1 Culture Strain

Candida bombicola (formerly Torulopsis bombicola) ATCC 22214 was used in all experimentation.

3.1.2 Culture Maintenance

The culture was maintained at 4 °C on a plate of Difco Agar supplemented with yeast extract. Shake flasks were inoculated by adding cells that were scraped directly from the plate. For inoculation of the sequencing batch reactor (SBR), aqueous cultures were first prepared in shake flasks with a yeast extract medium. These were incubated at 25 °C for 5 days, and then a 5ml sample was added to the SBR. Every 5-6 weeks, the cultures were transferred to fresh agar to maintain viability.
3.2 Medium Preparation

The medium used was a modification of a medium originally used by Tulloch\textsuperscript{46}. The medium composition was as follows:

<table>
<thead>
<tr>
<th>Medium Composition</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>10 g/L</td>
</tr>
<tr>
<td>Yeast Extract</td>
<td>1 g/L</td>
</tr>
<tr>
<td>Urea</td>
<td>0.1 g/L</td>
</tr>
<tr>
<td>Various Lipophilic Substrates</td>
<td>5 g/L</td>
</tr>
</tbody>
</table>

Table 3.1: Medium composition used in shake flask and SBR fermentations.

In both shake flask and SBR experimentation, the yeast extract and urea were sterilized separately from the glucose so as to prevent caramelization of the sugar. Upon cooling, the glucose was aseptically transferred to the other media.

3.3 Fermentation Analysis Methods

3.3.1 Biomass Measurement

The dry weight of the biomass was estimated directly. A 5ml sample of fermentation broth was taken. Remaining lipophilic substrate was removed using pentane. The sophorolipid product was removed with ethyl acetate. The broth was then centrifuged for 5 minutes at 10 000 rpm to obtain a biomass pellet. The supernatent was
then decanted and the pellet was resuspended in distilled water. This water was then added to a pre-weighed aluminum tray and evaporated in a Fisher Model 126G drying oven. When all of the water was evaporated, the tray was weighed again to determine dry weight.

3.3.2 Measurement of Lipophilic Substrates

The concentration of alkane substrates was determined using Gas Chromatography (Hewlett Packard 5890 Series 2). Pentane (2mL), containing 0.01% pentadecane as an internal standard, was used to extract the alkane from a 5ml sample of fermentation broth. The area ratio of the alkane extracted to the internal standard present in the extracting solvent was then determined. A calibration curve was prepared relating area ratio to concentration (Figure 3.1).

For complex lipophilic substrates (kerosene), gas chromatography could not be used to determine concentration. Instead, these substrates were extracted with pentane and added to a pre-weighed aluminum tray. Within five minutes, the pentane evaporated leaving the less volatile, long chain substrate behind. The tray was then weighed again to determine the substrate’s weight. From this value, the substrate concentration in the fermentor could be estimated.
3.3.3 Measurement of Sophorolipid

Sophorolipid concentrations were determined by directly measuring the weight of product in a 5ml sample of broth. The samples were washed twice with pentane to remove residual substrate, and the product was extracted with ethyl acetate. The ethyl acetate was then added to a pre-weighed aluminum tray and allowed to evaporate. Within 20 minutes, ethyl acetate was completely evaporated leaving the sophorolipid product behind. The tray was then weighed to determine the weight of the sophorolipid product. This value was then used to estimate the concentration of sophorolipid in the reactor.

3.3.4 Measurement of Aqueous Phase Carbohydrates

The concentration of carbohydrates in the aqueous phase (glucose + yeast extract) was determined using the anthrone method\textsuperscript{14}. The lipophilic substrate was removed using pentane and the sophorolipid product was extracted using ethyl acetate. The remainder of the sample was centrifuged so that a biomass pellet formed at the bottom. A 0.5 ml sample of the aqueous phase was then removed and diluted 10x with distilled water. A 0.5 ml sample was then removed from the dilute solution. To that sample 0.5ml phenol and 5ml of sulfuric acid was added. The absorbance of this sample was then measured using a Varian UV/Visible spectrophotometer. A calibration curve was then prepared to relate absorbance to glucose concentration (Figure 3.2).
Figure 3.1: Calibration curve for the determination of hexadecane concentration from area ratio.

Figure 3.2: Calibration curve of sophorolipid concentration against absorption at 488nm.
3.4 Analysis of Sophorolipids

3.4.1 Thin Layer Chromatography

The structural classes present in the sophorolipid products were determined using thin layer chromatography. A chloroform/methanol/water (65/15/2) v/v solvent system was used, along with Silica Gel GF (Fisherbrand RediPlate 20 x 20 cm) TLC plates. A compound's position on the TLC plate is characterized by an Rf value. The Rf value is defined as follows:

\[ \text{Rf} = \frac{\text{Distance of the compound from the starting point}}{\text{Distance of the solvent front from the starting point}} \]

A compound will always have the same Rf value in a given solvent system. The various structural forms of sophorolipids had already been elucidated by Asmer et al.\(^2\). The solvent system used in this work was identical to the one used by Asmer et al.\(^2\). Therefore, unknown structures were identified by matching their Rf values with those described by Asmer.

3.4.2 Analysis of Hydroxy Fatty Acids From Sophorolipids

A variation of the methanolysis reaction described by Davila et al.\(^{10}\) was used to isolate the hydroxy fatty acids from the sophorolipid products and to convert them to hydroxy acid methyl esters. The reaction was carried out using 30-50 mg of sophorolipid. The samples were added to 2 ml of 1% H\(_2\)SO\(_4\) and methanol solution along
with 1 ml of toluene containing 2g/L of dodecanoic acid. This mixture was heated for 1 hour at 100 °C. The resulting reaction products were then extracted using cyclohexane. The reaction products were then identified using a gas chromatograph / mass spectrometer (Thermoquest/Finnigan GCQ Plus).
3.5 Sequencing Batch Reactor

3.5.1 Reactor Components

Large scale fermentations were carried out in the sequencing batch reactor (SBR) shown in Figure 3.3. This same system was used in earlier work in which \textit{C. bombicola} was grown on a different medium\textsuperscript{38}. The fermentation broth (1L) was circulated using a March MDX pump. Primary oxygen exchange occurred as the broth trickled down the surface of a glass cyclone reactor. Fresh, water-soluble media was supplied from a 10L polypropylene Nalgene bottle located overhead. Lipophilic substrate was added using a Sage syringe pump (model 335). A harvesting line was located at the lowest point of the reactor on the outlet side of the circulatory pump.

The fermentation temperature was kept constant at 25 °C using a glass, countercurrent shell and tube heat exchanger and a Haake FE2 temperature controller. A condenser was provided at the top of the cyclone to prevent losses due to evaporation. Both the top of the condenser and the air inlet were filtered using a Whatman Vacu-Guard inline air filter.

Dissolved oxygen was measured throughout the fermentations using an Ingold polarographic dissolved oxygen probe (model IL531). The probe signal was amplified by a Cole Parmer 197100 amplifier, digitalized, and sent to an analog strip chart recorder. Probe calibration was carried out by first purging the reactor with nitrogen to determine the 0% saturation point and then by purging the reactor with air to determine the 100% saturation point.
Figure 3.3: Sequencing batch reactor (SBR).
3.5.2 Reactor Sterilization

The reactor system and medium bottle were sterilized separately prior to inoculation. The reactor system was autoclaved at 120 °C for 1 hour. The 10L medium bottle was autoclaved at 120 °C for 3 hours. The medium bottle was allowed to cool for 12 hours. Glucose was then added as described in section 3.2. The line from the medium bottle was then quickly attached to the medium inlet line of the reactor system.

3.5.3 Dissolved Oxygen as a Control Parameter

Batch runs were carried out until all lipophilic substrate was depleted. At this point, one half of the reactor contents (500ml) were removed and replaced by fresh media from the supply bottle overhead. Removal of the reactor contents was accomplished by measuring the changes in the reactor weight as the contents were removed. The technique was calibrated using broth in the stationary phase to determine the difference in weight that corresponded to the removal of 500ml. Depletion of the lipophilic substrate was indicated by a rapid increase in dissolved oxygen level. The process was automated using an IBM PC interfaced with a data acquisition board. The control algorithm was the same as that described by McCaffrey. Signals from the computer were sent to Skinner V521B1 solenoid valves that regulated both the flow of fresh media and broth removal.
3.6 Experimental Methods

3.6.1 Preliminary Shake Flask Experiments

Preliminary shake flask experiments were carried out whereby the concentrations of the glucose and yeast extract were varied in an effort to determine the best medium composition to use throughout the work. Three medium compositions were examined:

<table>
<thead>
<tr>
<th>Medium Composition</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>10 g/L</td>
<td>50 g/L</td>
<td>10 g/L</td>
</tr>
<tr>
<td>Yeast Extract</td>
<td>1 g/L</td>
<td>1 g/L</td>
<td>10 g/L</td>
</tr>
<tr>
<td>Urea</td>
<td>0.1 g/L</td>
<td>0.1 g/L</td>
<td>0.1 g/L</td>
</tr>
<tr>
<td>Various Lipophilic Substrates</td>
<td>5 g/L</td>
<td>5 g/L</td>
<td>5 g/L</td>
</tr>
</tbody>
</table>

*Table 3.2*: Medium compositions used in preliminary fermentations.
3.6.2 Shake Flask Experimentation

Using Medium #1 from Table 3.1, a number of shake flask experiments were carried out using the following lipophilic substrates.

<table>
<thead>
<tr>
<th>Alkanes</th>
<th>Fatty Acids</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dodecane</td>
<td>tetradecanoic acid</td>
</tr>
<tr>
<td>Tetradecane</td>
<td>hexadecanoic acid</td>
</tr>
<tr>
<td>Pentadecane</td>
<td>octadecanoic acid</td>
</tr>
<tr>
<td>Hexadecane</td>
<td>hexadecenoic acid</td>
</tr>
<tr>
<td>Heptadecane</td>
<td>octadecenoic acid</td>
</tr>
<tr>
<td>Octadecane</td>
<td></td>
</tr>
<tr>
<td>Eicosane</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.3: Lipophilic Substrates Provided in Shake Flask Experiments

The flasks were filled with 100ml of medium and inoculated with *C. bombicola* cells. The experiments lasted 11 days, after which point the contents were removed for analysis. Shake flask experiments were performed in triplicate for each lipophilic substrate used.

3.6.3 Variation of the Volume of Medium in Shake Flasks

Shake flask experiments were also carried out in which the flasks were filled with either 50, 100, or 200ml of medium. Three experiments for each volume were carried out. Hexadecane was provided as a lipophilic substrate in each case.
3.6.4 Sequencing Batch Fermentations

A total of 13 consecutive batch runs were carried out using the sequencing batch reactor described in section 3.6. From one run to the next, the type of lipophilic substrate was changed. Thus, for a fermentation following a substrate transition, there was likely to have been residual substrate from the previous fermentation. However, by the second fermentation following the substrate transition most of the residual substrate was washed out and there was only a single substrate present in the reactor. The following lipophilic substrates were used during experimentation:

<table>
<thead>
<tr>
<th>Alkanes</th>
<th>Complex Substrates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dodecane</td>
<td>Mineral Oil</td>
</tr>
<tr>
<td>Tetradecane</td>
<td>Kerosene</td>
</tr>
<tr>
<td>Hexadecane</td>
<td></td>
</tr>
</tbody>
</table>

*Table 3.4: Lipophilic Substrates Provided in Sequencing Batch Fermentations*
4.0 Results

4.1 Shake Flask Fermentations

4.1.1 Sophorolipid Morphology and Yields

Sophorolipid products obtained from shake flasks had two general morphologies appearing either as brownish colored oil (Figure 4.1) or as white crystals (Figure 4.2). Upon emptying the shake flask contents into a separatory funnel at the end of the fermentation, a significant amount of oily product settled after approximately 20 minutes. This sophorolipid could be recovered by sedimentation. However, attempts to rinse the lipid product with distilled water resulted in its resuspension. Therefore, 15-20 minutes were allowed following each rinsing so that the sophorolipid could settle and the dirty water could be removed. Dealing with the product in this manner proved to be time consuming and resulted in significant losses. Moreover, the oily sophorolipid passed freely through filters and proved difficult to recover in this manner. To avoid significant losses, it was preferable to extract the oily sophorolipid from the fermentation broth using ethyl acetate.

On the other hand, the crystalline sophorolipid was much simpler to work with. Upon emptying the shake flask into a separatory funnel, the sophorolipid settled in its entirety in just under 5 minutes. The crystals also settled rapidly in between rinsings, allowing the product to be cleaned very quickly. The crystals could then be recovered by vacuum filtration without significant losses.
Figure 4.1: Oily sophorolipid morphology.

Figure 4.2: Crystalline sophorolipid morphology.
The variation in sophorolipid morphology with hydrophilic medium composition is presented in Table 4.1. Only the leanest medium resulted in the production of crystalline products.

Sophorolipid morphology varied depending on the hydrocarbon or fatty acid substrate that was used in a given the shake flask fermentation. These results are presented in Table 4.2. The alkane substrates dodecane, tetradecane, and pentadecane all resulted in the production of a brown, oily sophorolipid. When hexadecane or heptadecane were used as substrates, the crystalline form was obtained. However, hexadecene, the only unsaturated aliphatic hydrocarbon used as a substrate, resulted in a brown oil. When octadecane and eicosane were substrates, it became difficult to determine the product morphology due to the presence of significant amounts of undegraded octadecane and eicosane.

In an attempt to clarify the effects of the solid hydrocarbons on sophorolipid morphology, octadecane and eicosane were mixed with hexadecane so as to put them in liquid form. Both the hexadecane / octadecane and hexadecane / eicosane mixtures resulted in the production of oily sophorolipids.

Solid fatty acid fermentations resulted in minimal sophorolipid production and poor degradation of the substrates. In this case it was impossible to liquefy these fatty acids by mixing them with another compound. Determination of morphology was difficult since the product yields were low and recovered products consisted primarily of undegraded substrate. In Table 4.2 these sophorolipids are registered as having an undetermined morphology.
When hexadecenoic acid was used as a substrate, oily sophorolipids were produced. The fermentation of octadecenoic acid, however, gave rise to a crystalline product coexisting with oil. This result was unique in that the products from fermentations of all other substrates resulted in either only oily or only crystalline products.

In general, liquid lipophilic substrates were nearly completely degraded by the yeast while solid lipophilic substrates were degraded to a much lesser extent.

Chromatograms of the products from the fermentations of solid substrates (i.e. octadecane, eicosane, tetradecanoic acid, hexadecanoic acid, octadecanoic acid) revealed that large amounts of undegraded substrate were present. This can be seen in the chromatogram of product from a hexadecanoic acid fermentation (Figure 4.3).

Sophorolipid yields also varied significantly depending on the lipophilic substrate that was used. As can be seen in Figure 4.4, yields increased as alkane chain length increased from C12 up to a maximum at C16. The use of alkane substrates with chain lengths exceeding C16 resulted in significantly reduced yields. Alkanes dodecane to hexadecane, as well as hexadecene, were nearly completely consumed when used as substrates. However, the amount of unconsumed substrate remaining at the end of the fermentation increased dramatically from heptadecane to eicosane. This reduced substrate consumption coincides with the physical differences in the substrates as they go from a wax (heptadecane) to a solid (octadecane).

As shown in Table 4.2, yields of sophorolipid using solid, fatty acid substrates such as tetradecanoic acid, hexadecanoic acid and octadecanoic acid were very low. Denser than water, pasty substances were recovered at the end of fermentations using
fatty acid substrates. However, these pasty substances proved to consist primarily of undegraded substrate and contained very little sophorolipid. Due to the mixing of product and substrate, sophorolipid yields could not be accurately determined.

When liquid fatty acids such as octadecanoic acid and hexadecanoic acid were fermented, they were almost completely degraded. Sophorolipid yields were also much improved, though not as high as the best yields obtained using hydrocarbons (Table 4.2).

The volume of broth in the shake flasks also affected sophorolipid yields. As shown in Figure 4.5, shake flasks filled with 200ml of medium (glucose/yeast extract/hexadecane) resulted in higher yields than those filled with 50 or 100ml.
<table>
<thead>
<tr>
<th>Medium Composition</th>
<th>Predominant OH Fatty Acid</th>
<th>Morphology</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose 10g/L, Yeast Extract 1g/L, Urea 0.1 g/L, Hexadecane 4 g/L</td>
<td>hexadecanoic</td>
<td>crystal</td>
</tr>
<tr>
<td>Glucose 10 g/L, Yeast Extract 10 g/L, Urea 0.1 g/L, Hexadecane 4 g/L</td>
<td>octadecanoic</td>
<td>oil</td>
</tr>
<tr>
<td>Glucose 50 g/L, Yeast Extract 1g/L, Urea 0.1 g/L, Hexadecane 4 g/L</td>
<td>hexadecanoic</td>
<td>oil</td>
</tr>
</tbody>
</table>

**Table 4.1:** Results of preliminary shake flask experiments indicate that the nature of the lipophilic medium affects both product morphology and hydroxy fatty acid content.
Figure 4.3: A chromatogram of the products from *C. bombicola* when hexadecanoic acid was used as a substrate. The peak at 14.9 minutes reveals a significant amount of undegraded hexadecanoic acid.
Figure 4.4: Sophorolipid yields (•) from shake flask fermentations with \textit{C. bombicola} in which various alkane substrates were provided.

Figure 4.5: Sophorolipid yields (•) from shake flask fermentations with \textit{C. bombicola}. The volume of medium added to each shake flasks was varied.
Table 4.2: Yields and morphologies of sophorolipids produced when *C. bombicola* was grown on a number of lipophilic substrates. (-) Trace amounts (*) undetermined.
4.1.2 Sophorolipid Composition

4.1.2.1 Characterization of the Fatty Acid Components of Sophorolipid Products

The methanolysis reaction described in Section 3.5.2 was effective with both lactonic and acidic sophorolipids. It was found that silation of the hydroxy methyl esters, as suggested by Davila et al.\textsuperscript{10} was not required since hydroxy acid methyl esters were volatile enough to obtain gas chromatographs.

Identification of the methyl esters of the hydroxy acids, which were obtained by methanolysis of the sophorolipid products, was carried out using a gas chromatograph/mass spectrometer (Thermoquest/Finnigan GCQ Plus). Figures 4.6 to 4.11 relate to esters from the products of fermentations with hexadecane. Only four major compounds were observed (Figure 4.6). The first compound (residence time 18.23) was identified by comparing its mass spectrum (Figure 4.7) with that of a sample of 16-hydroxy hexadecanoic acid that was purchased from Sigma Aldrich (Figure 4.8). An isomer, 15-hydroxy hexadecanoic acid (residence time 17.54), was identified by matching its spectrum (Figure 4.9) with one available on the GC/MS software library. The parent ion of both isomers was indicated by the presence of a strong peak at 287.

The fragmentation patterns of 15 and 16 hydroxy hexadecanoic acid methyl esters were then used in the identification of subterminally and terminally hydroxylated methyl esters of all chain lengths. Dodecanoic, tetradecanoic, pentadecanoic, heptadecanoic, octadecanoic and eicosanic acid methyl esters were identified by molecular weight. Subterminally and terminally hydroxylated isomers were then
identified by their fragmentation patterns which were identical to those of 15-hydroxy and 16-hydroxy hexadecanoic acid methyl ester respectively. However the parent ions were either increased or decreased in size by multiples of 14.

As an example, Figure 4.10 shows the mass spectrum of 14-hydroxy pentadecanoic acid methyl ester. The compound was identified as a hydroxy pentadecanoic acid methyl ester is indicated by the molecular weight of the parent ion (273). Examination of the fragmentation pattern shows the similarity to that of 15 hydroxy hexadecanoic acid. The largest fragment in both cases has a molecular weight that is 18 mass units less than that of the parent ion. The second largest fragment has a molecular weight that is 45 mass units less than that of the parent ion. Thus, it is possible to identify the compound as 14-hydroxy pentadecanoic acid methyl ester.

The other two major compounds in the products from the hexadecane fermentation were identified as hydroxy octadecenoic acid methyl esters by their molecular weights (313). However, their fragmentation patterns were substantially different from those of the saturated compounds (Figure 4.11). Assignment of the identities of these two isomers were made by considering their position on the gas chromatogram. In the case of 15 and 16 hydroxy hexadecanoic acid methyl esters, the subterminally hydroxylated isomer passed though the column faster than the terminally hydroxylated isomer. It was therefore assumed that the unsaturated hydroxy acid methyl esters would behave in the same manner. With this assumption the peaks at 19.0 and 19.2 minutes were characterized as the 17 and 18 hydroxy octadecenoic acid methyl esters respectively.
Methyl esters of other chain lengths were characterized in the same manner. All of the hydroxy fatty acids from the sophorolipid products of various fermentations are presented in Tables 4.3 and 4.4.
Figure 4.6: Chromatogram of sophorolipids produced by *C. bombicola* when grown on hexadecane.

Figure 4.7: Mass spectrum of 16 OH hexadecanoic acid methyl ester isolated from the sophorolipid sample.
Figure 4.8: Mass spectrum of 16 hydroxy hexadecanoic acid methyl ester standard purchased from Sigma Aldrich.

Figure 4.9: Mass spectrum of 15 hydroxy hexadecanoic acid methyl ester isolated from sophorolipid products.
Figure 4.10: Mass spectrum of 14 hydroxy pentadecanoic acid methyl ester isolated from the sophorolipid products produced when pentadecane was supplied as a substrate. The compound was identified based on similarities of its fragmentation pattern with that of 15 hydroxy hexadecanoic acid methyl ester.

Figure 4.11: The mass spectrum of 17 hydroxy octadecenoic acid methyl ester. The fragmentation pattern is distinctly different from those of the saturated compounds.
4.1.2.2 Analysis of Sophorolipid Hydroxy Fatty Acid Content

The hydroxy fatty acid components of the sophorolipid products varied dramatically depending on the lipophilic substrate that was used. As Table 4.3 shows, the sophorolipids from the dodecane fermentation contained hydroxy dodecanoic fatty acids. These hydroxy dodecanoic acids were not present in sophorolipids from any other fermentation. The fermentations of a number of other alkanes gave similar results. Hydroxy tetradecanoic acids were found only in the sophorolipids when tetradecane was fermented. Hydroxy pentadecanoic acids appeared only when pentadecane was fermented. And finally, hydroxy heptadecanoic acids appeared only when heptadecane was fermented. Notably, as the length of the alkane substrate increased from C12 to C17, the percentage of hydroxy acids having the same chain length as the substrate increased (Table 4.3). This phenomenon reached a maximum when heptadecane was provided as a lipophilic substrate.

In each of the alkane fermentations, except for those using heptadecane, the sophorolipid products contained hexadecanoic, octadecanoic, hexadecenoic, and octadecenoic hydroxy acids. The fermentations of heptadecane were unique in that they resulted in the production of sophorolipid containing only odd chain length hydroxy acids. These products contained a high percentage of hydroxy heptadecanoic acids and a small amount of hydroxy pentadecanoic acids. In hexadecane fermentations, the product contained an extremely high percentage of hydroxy hexadecanoic acids. Also, a notably high percentage of hydroxy octadecanoic acids appeared in the sophorolipid from the octadecane fermentation. However, when eicosane was used as a substrate, there was no
trace of hydroxy eicosanic acids in the sophorolipid products. These products consisted of only hexadecanoic, hexadecanoic, and octadecanoic and octadecenoic hydroxy acids.

Although sophorolipid was produced in all of the fermentations of mixed alkane substrates, the only product analyzed for fatty acid content was from the hexadecane / eicosane fermentation. The sophorolipids from this fermentation were shown to contain a significant percentage of hydroxy eicosanic acids (Table 4.4).

Although sophorolipid yields were low in fermentations with solid fatty acids, their hydroxy fatty acid content could still be determined. As shown in Table 4.4, sophorolipids produced in fermentations with tetradecanoic acid, hexadecanoic acid, and octadecanoic acid, contained only hydroxy hexadecanoic and hydroxy octadecanoic acids.

The results of fermentations using liquid fatty acids substrates were markedly different. In both cases the products contained a significant percentage of hydroxy acids with the same chain length as the substrate. As Table 4.4 shows, there was a high percentage of hydroxy hexadecanoic acids in products from the hexadecanoic acid fermentation and a high percentage of hydroxy octadecanoic acids in products from the octadecanoic acid fermentation.

Also presented in Table 4.4 is the fatty acid composition of the products obtained when hexadecene was used as a substrate.
## Table 4.3

<table>
<thead>
<tr>
<th>hydroxy fatty acid constituent</th>
<th>Dodecane</th>
<th>Tetradecane</th>
<th>Pentadecane</th>
<th>Hexadecane</th>
<th>Heptadecane</th>
<th>Octadecane</th>
<th>Eicosane</th>
</tr>
</thead>
<tbody>
<tr>
<td>11 hydroxy dodecanoic</td>
<td>6</td>
<td>-</td>
<td>-</td>
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<td>-</td>
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<td>-</td>
<td>23</td>
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</tr>
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<td>-</td>
<td>19</td>
<td>-</td>
<td>8</td>
<td>-</td>
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<td>9</td>
<td>-</td>
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<td>5</td>
<td>4</td>
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</tr>
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</tr>
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<td>-</td>
<td>-</td>
<td>-</td>
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</tr>
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<td>-</td>
<td>-</td>
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<td>-</td>
</tr>
</tbody>
</table>

Table 4.3: Hydroxy acid constituents of sophorolipid products from various alkane fermentations.
### Table 4.4: Hydroxy acid constituents of sophorolipid products from various fatty acid fermentations.

<table>
<thead>
<tr>
<th>Hydroxy fatty acid constituent</th>
<th>Tetradecanoic acid</th>
<th>Hexadecanoic acid</th>
<th>Hexadecenoic acid</th>
<th>Octadecenoic acid</th>
<th>Hexadecene</th>
<th>Eicosane/Hexadecane</th>
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<td>11 hydroxy dodecanoic</td>
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</table>
4.1.3.3 Determination of Sophorolipid Structural Classes

Using thin layer chromatography (TLC), the various structural classes of sophorolipid were identified. Analysis of the products from the preliminary shake flask experiments revealed that the leanest medium resulted in the production of high purity diacetylated lactone (Figure 4.12).

TLC of sophorolipids that were produced using various lipophilic substrates revealed that a high purity, diacetylated lactone was obtained only when hexadecane or heptadecane were used as substrates (Figure 4.13). The fermentations of all other alkanes or fatty acids yielded mixtures of lactonic (diacetylated, monoacetylated, non-acetylated) and acidic sophorolipid forms.

As mentioned in section 4.2.1, sophorolipids from the fermentations of solid fatty acids were produced in low yields and were intermixed with a large amount of undegraded substrate. Consequently, when thin layer chromatography was performed on the samples, there was poor resolution of the various lipids. However, as can be seen in Figure 4.14, the TLC results did indicate the presence of at least two different structural classes.

The more readily degraded, liquid fatty acids resulted in higher product yields with little contamination by residual substrate. TLC spots of the various sophorolipid products from these fermentations could be seen clearly. As Figure 4.15 shows, both the octadecenoic acid and hexadecenoic acid fermentations resulted in sophorolipids consisting of a mixture of structural forms. The products obtained when hexadecene was
provided as a substrate were shown to contain the most diverse mixture of structural classes (Figure 4.16).
Figure 4.12: a) Thin layer chromatography of products from *C. bombicola* grown with a medium containing a) 1 g/L yeast extract, 10 g/L glucose, and 0.1 g/L urea. b) 1 g/L yeast extract, 50 g/L glucose, and 0.1 g/L urea. c) 10 g/L yeast extract, 10 g/L glucose, and 0.1 g/L urea.
Figure 4.13: Thin layer chromatography of sophorolipids produced by C. bombicola when various alkanes were used as substrates. Product purity increases sharply when hexadecane and heptadecane are used as substrates.
Figure 4.14: Thin layer chromatography of the sophorolipid products obtained when *C. bombicola* was grown on a) tetradecanoic acid  b) hexadecanoic acid c) octadecanoic acid. Significant amounts of undegraded substrate obscured the TLC spots of various sophorolipids.
Figure 4.15: Thin layer chromatography products produced by *C. bombicola* when growing on a) octadecenoic acid and b) hexadecenoic acid.
Figure 4.16: Thin layer chromatography of products produced by *C. bombicola* when growing on hexadecene. Hexadecene resulted in the most extensive mixture of structural classes, as indicated by the numerous spots on the TLC plate.
4.2 Sequencing Batch Reactor Fermentations

4.2.1 Fermentations of Single Lipophilic Substrates

A number of fermentations were carried out using pure dodecane, tetradecane and hexadecane. Figures 4.17 through 4.19 show the degradation of glucose and the lipophilic substrate in each of these fermentations. Sophorolipid production is shown in Figures 4.20 through 4.22. Biomass production is shown in Figures 4.23 and 4.25. Sophorolipid yields and morphologies are presented in Table 4.5.
Figure 4.17: Degradation of dodecane (♦) and aqueous phase carbohydrates (■) by Candida bombicola.

Figure 4.18: Degradation of tetradecane (♦) and aqueous phase carbohydrates by Candida bombicola.
Figure 4.19: Degradation of hexadecane (♦) and aqueous phase carbohydrates (■) by Candida bombicola.
Figure 4.20 : Production of sophorolipid (♦) by *C. bombicola* when dodecane is provided as a lipophilic substrate.

Figure 4.21 : Production of sophorolipid (♦) by *C. bombicola* when tetradecane is provided as a lipophilic substrate.
Figure 4.22: Production of sophorolipid (♦) by *C. bombicola* when hexadecane is provided as a lipophilic substrate.
Figure 4.23: *C. bombicola* biomass (●) production using dodecane as a lipophilic substrate.

Figure 4.24: *C. bombicola* biomass (●) production using tetradecane as a lipophilic substrate.
Figure 4.25: \textit{C. bombicola} biomass (\textbullet) production using hexadecane as a lipophilic substrate.
Table 4.5: Sophorolipid yields and morphologies from fermentations in which a single alkane substrate was provided.

<table>
<thead>
<tr>
<th>Lipophilic Substrate</th>
<th>Average Yield (g/L)</th>
<th>Morphology</th>
</tr>
</thead>
<tbody>
<tr>
<td>dodecane</td>
<td>0.41</td>
<td>oil</td>
</tr>
<tr>
<td>tetradecane</td>
<td>0.73</td>
<td>oil</td>
</tr>
<tr>
<td>hexadecane</td>
<td>1.55</td>
<td>crystal</td>
</tr>
</tbody>
</table>
4.2.2 Substrate Transition Fermentations

Fresh medium was supplied to the reactor when chromatographic analysis indicated that the lipophilic substrate was depleted. However, in fermentations where a new type of lipophilic substrate was added, an increase in concentration of the previous substrate was observed. This phenomenon was observed in transitions from hexadecane to dodecane, dodecane to tetradecane, and tetradecane to hexadecane. These results are shown in Figures 4.26 through 4.28. Sophorolipid production and glucose degradation are also shown in Figure 4.29 through 4.31 and 4.32 through 4.34 respectively. Biomass production in these fermentations was no different than in those with single substrates. Sophorolipid yields and morphologies are presented in Table 4.6.
Figure 4.26: The concentration of hexadecane (■) increased upon addition of dodecane (★).

Figure 4.27: The concentration of dodecane (■) increased upon addition of tetradecane (★).
Figure 4.28: The concentration of tetradecane (■) increased upon addition of hexadecane (★).
Figure 4.29: Sophorolipid (●) production by *C. bombicola* when dodecane was used as a lipophilic substrate. There was also some residual hexadecane present.

Figure 4.30: Sophorolipid (●) production by *C. bombicola* when tetradecane was used as a lipophilic substrate. There was also some residual dodecane present.
Figure 4.31: Sophorolipid (♦) production by *C. bombicola* when hexadecane was used as a lipophilic substrate. There was also some residual tetradecane present.
Figure 4.32: Degradation of dodecane (♦) and aqueous phase carbohydrates (■) by Candida bombicola. A small amount of residual hexadecane was also present.

Figure 4.33: Degradation of tetradecane (♦) and aqueous phase carbohydrates (■) by Candida bombicola. A small amount of residual dodecane was also present.
Figure 4.34: Degradation of hexadecane (♦) and aqueous phase carbohydrates (■) by *Candida bombicola*. A small amount of residual tetradecane was also present.
Table 4.6: Sophorolipid yields and morphologies from fermentations in which two alkane substrates were present.

<table>
<thead>
<tr>
<th>Primary Lipophilic Substrate</th>
<th>Residual Lipophilic Substrate</th>
<th>Average Yield (g/L)</th>
<th>Morphology</th>
</tr>
</thead>
<tbody>
<tr>
<td>dodecane</td>
<td>hexadecane</td>
<td>1.2</td>
<td>oil</td>
</tr>
<tr>
<td>tetradecane</td>
<td>dodecane</td>
<td>0.43</td>
<td>oil</td>
</tr>
<tr>
<td>hexadecane</td>
<td>tetradecane</td>
<td>1.3</td>
<td>crystal</td>
</tr>
</tbody>
</table>
4.2.3 Mixed Substrate Runs

Three fermentations were carried out using kerosene and mineral oil. Figures 4.34 and 4.35 show the degradation of glucose and the lipophilic substrate in each fermentation. Sophorolipid production is shown in Figures 4.37 and 4.38. Biomass production is shown in Figures 4.39 and 4.40. Yields and morphologies of sophorolipid products are shown in Table 4.7.
Figure 4.35: Degradation of kerosene (●) and aqueous phase carbohydrates (■) by Candida bombicola.

Figure 4.36: Degradation of mineral oil (●) and aqueous phase carbohydrates (■) by Candida bombicola.
**Figure 4.37**: Production of sophorolipids (♦) using kerosene as a lipophilic substrate.

**Figure 4.38**: Production of sophorolipids (♦) using mineral oil as a lipophilic substrate.
Figure 4.39: *C. bombicola* biomass (♦) production using kerosene as a lipophilic substrate.

Figure 4.40: *C. bombicola* biomass (♦) production using mineral oil as a lipophilic substrate.
Table 4.7: Sophorolipid yields and morphologies from fermentations in which kerosene and mineral oil were provided as substrates.
5.0 Discussion

5.1 Experimental Error in Shake Flask and Sequencing Batch Experimentation

Standard deviations of sophorolipid yields were calculated only in experiments in which there were not significant amounts of unused substrate remaining at the end of the experiment. These deviations from the average were therefore only presented in figures 4.4 and 4.5 which present the results of experimentation with alkane substrates. All acid substrates remained in significant concentrations at the end of the experiments. This, combined with low sophorolipid production in these cases, did not allow for the calculation of accurate yields. Standard deviations were therefore not calculated in these cases.

In sequencing batch experiments, samples were taken from a system consisting of an aqueous phase, an oil phase (substrate), and a solid phase (sophorolipid). The complexity of this system contributed to significant error in sampling. Sampling errors were especially prevalent at the beginning of a sequence when there was less biomass present to act as an emulsifier. In many cases (Figures 4.20 to 4.31), both the lipophilic and hydrophilic substrates appeared to increase in the first 10 hours, and there were variations in substrate and product as high as 6g/L throughout many of the sequences. However, by conducting a number of sequences with a given substrate, trends in substrate consumption and sophorolipid production become evident. Furthermore, while exact concentrations could not be determined at each sampling instance, the changes in substrate and sophorolipid concentrations from the beginning to the end of the sequences far exceeded the error in sampling.
5.2 Shake Flask Fermentations

5.2.1 Preliminary Shake Flask Fermentations and Medium Selection

It was desirable to select a medium that would promote the production of lactonic sophorolipids. However, there were some inconsistencies in the literature concerning how the medium affected the nature of the sophorolipid product. There have been reports that increasing the yeast extract concentration resulted in an increase in lactonic sophorolipids\textsuperscript{34, 48}. However, Casas \textit{et al.} reported that decreasing the yeast extract concentration favored lactone production\textsuperscript{5}. It was therefore necessary to carry out the preliminary shake flask fermentations described in Section 3.7.1 to facilitate medium selection. Thin layer chromatography was performed on the products of each fermentation and these results are shown in Figure 4.12. These TLC plates indicated that all media resulted in significantly high levels of lactonization. However, the medium with low glucose (10 g/L) and yeast extract (1 g/L) concentrations resulted in the production of a high percentage of diacetylated lactone (Figure 4.1.a). Increasing the glucose concentration resulted in the production of a mixture of sophorolipid forms that can be seen in Figure 4.12.c. This result is consistent with reports by Hommel \textit{et al.} that decreasing the carbon to nitrogen ratio resulted in the production of a high percentage of diacetylated lactone\textsuperscript{27}. Throughout the remainder of this work, the medium containing 1 g/L yeast extract, 10 g/L glucose, and 0.1 g/L urea was used. Various lipophilic substrates were provided.
5.2.2 Direct Incorporation of Lipophilic Substrates

Tracer studies using $^{13}$C have confirmed that Candida yeasts are capable of directly incorporating lipophilic substrates as the lipidic portion of their sophorolipid products. In the 1960's Spencer and Tulloch speculated on the direct incorporation of various long chain methyl esters, fatty acids, and n-alkanes. $C. bombicola$ has also been shown to directly incorporate n-alkanols resulting in the production of novel sophorolipids.

Perhaps the strongest evidence of direct incorporation in the work presented here was the production of sophorolipids containing hydroxy heptadecanoic or hydroxy pentadecanoic moieties when heptadecane or pentadecane were provided as substrates (Table 4.3). Since the yeast does not normally synthesize fatty acids with odd chain lengths, the only explanation for their presence is that heptadecane and pentadecane were directly incorporated. There was also a small amount of hydroxy heptadecanoic acid when $C. bombicola$ was grown on heptadecane. Since this yeast is known to have desaturases, this can still be attributed to direct incorporation.

The amount of direct incorporation of even chain length substrates was estimated by comparison with typical de novo hydroxy acid compositions. To do this, several factors need to be considered. It has been established that $Candida bombicola$ is capable of producing hydroxy hexadecanoic, hydroxy hexadecenoic, hydroxy octadecenoic and hydroxy octadecanoic acids by de novo synthesis. Of this mixture, hydroxy octadecenoic acids are typically present in the highest percentage (40-50%).
In this study, it was assumed that any hydroxy dodecanoic or hydroxy
tetradecanoic acids present in the sophorolipids must have resulted from direct
incorporation because they were only observed when either dodecane or tetradecane were
in the medium. As well, neither of these compounds have been reported to be produced
*de novo* in any of the literature using lipophilic substrates other than these two
hydrocarbons\(^2,5,6,7,8,11,34,35\).

The extent of the direct incorporation of hexadecanoic and octadecanoic acid can
be determined by comparing the percentages of these compounds in sophorolipids to the
percentages that would be expected, if they were synthesized *de novo*. In this work the
products of the eicosane fermentation showed no indication of direct incorporation and
were therefore taken to represent typical *de novo* products. The extent of direct
incorporation of other substrates was then estimated by comparison to the eicosane
products. This method was confirmed by considering other aspects of individual
products. For example, the sophorolipid produced using hexadecane contains an
extremely high percentage of hydroxy hexadecanoic acid relative to the *de novo* product.
This is consistent with the pattern of direct incorporation.

The estimated amount of direct incorporation of hydrocarbon substrates is
presented in Figure 5.1 as a function of chain length of the substrates. There is a
noticeable maximum in the amount of direct incorporation at sixteen and seventeen
carbon alkane substrates. These coincide with the maximum sophorolipid yields (Figure
4.11).
Figure 5.1: Percent direct incorporation of alkanes varies with alkane chain length.
The same analysis was carried out for sophorolipids produced using solid and liquid fatty acid substrates. The solid acid fermentations all resulted in sophorolipid products with hydroxy fatty acid compositions consistent with \textit{de novo} synthesis. However, the liquid fatty acids showed evidence of direct incorporation. The fermentation of hexadecenoic acid resulted in the production of sophorolipids containing a high percentage (65\%) of hydroxy hexadecenoic acid. The fact that hydroxy hexadecenoic acid is produced only sparingly through \textit{de novo} synthesis implies that it must have been directly incorporated in this case to be present in such a high percentage. Similarly, the fermentation of octadecenoic acid results in sophorolipids that contained 95\% hydroxy octadecenoic acid. This percentage is much higher than the 40-50\% that would be expected from \textit{de novo} production (and it can be inferred that at least 35\% of the hydroxy octadecenoic acid must have been directly incorporated). Figure 5.2 indicates that, as with alkanes, fatty acids with 16 carbons are directly incorporated to the greatest extent.

The sophorolipid products from fermentations with hexadecene showed no indication of direct incorporation. Instead of containing a high percentage of hydroxy hexadecenoic acid, these products contained a mixture of hydroxy hexadecanoic, octadecenoic and octadecenoic acids. This result was likely caused by the unsaturation at the terminal end of hexadecene, which precluded either subterminal or terminal hydroxylation.

The narrow range of substrate chain lengths in which direct incorporation occurs suggests the possible involvement of enzyme specificity. \textit{Candida} yeasts contain multiple enzyme systems for each specific function. Multiple fatty alcohol oxidases\textsuperscript{21} and multiple
monoxygenase systems have been detected in *Candida bombicola*. The analysis of all sophorolipid products from all fermentations suggest that the enzymes responsible for both $\omega$ and $\omega-1$ hydroxylation have a stronger specificity towards hexadecanoic and heptadecanoic acids than towards acids of other chain lengths.

Evidence of enzyme specificity can also be seen in this work by examining the products of the hexadecane and heptadecane fermentations (Table 4.3). The hydroxy acids that were incorporated during the hexadecane fermentation were $\omega$ and $\omega-1$ hydroxylated in equal proportions. However, the heptadecanoic acids that were incorporated during the heptadecane fermentation were almost entirely $\omega$-hydroxylated. These observations, which are consistent with findings by Jones, suggest that the enzyme(s) responsible for $\omega$ hydroxylation have a stronger specificity towards heptadecanoic acid.
Figure 5.2: Percent direct incorporation of unsaturated liquid fatty acid substrates.
5.2.3 Degradation of Solid and Liquid Substrates

In general, solid substrates were degraded to a much lesser than liquid ones. As the alkane substrates became increasingly more solid in nature, both sophorolipid yield and substrate degradation decreased. In general, product yields increased and decreased with the degree of direct incorporation. However, the sophorolipid yields from alkane fermentations reach a maximum at hexadecane while the degree of direct incorporation reaches a maximum at heptadecane. This discrepancy was attributed to the waxy morphology of heptadecane which inhibited degradation of the compound. Solid substrate morphologies likely reduced the degree of direct incorporation simply by making the substrate less bioavailable. This is best supported by the fact that eicosane was only directly incorporated after it was liquefied by mixing it with hexadecane.

Tetradecanoic, hexadecanoic, and octadecenoic acids all had relatively high melting points. When used as substrates, they were all poorly degraded and the sophorolipid products showed no sign of direct incorporation. Given the limited degradation of these substrates, it is most likely that the sophorolipids produced during these fermentations contained hydroxy acids that were produced de novo, with direct incorporation being limited by the bioavailability of the solid substrate. The liquid acids, hexadecenoic and octadecenoic, were degraded almost to completion by the yeast and, in fermentations with both of these substrates, significant direct incorporation occurred.

In summary, solid substrates are difficult for the yeast to degrade and the hydroxy acid portions of the sophorolipids must be synthesized de novo. This ultimately limits their utility as lipophilic substrates. For liquid substrates, which are more readily
degraded by the organism, sophorolipid yield is proportional to the extent of direct incorporation.

### 5.2.4 Production of Crystalline Sophorolipids

There are a number of *Candida* yeasts that are recognized as producing sophorolipids. These include *C. bombicola*, *C. apicola* and *C. gropengiesseri*. These yeasts have very similar metabolisms, and many authors tend to use their names synonymously.

Stüwer *et al* were the first to report crystalline sophorolipids by *C. apicola*\(^4^2\). They found that, that by lowering the pH of the fermentation broth by adding sodium citrate, crude crystalline sophorolipids containing 80% of a single structural form were produced by *C. apicola*. It was later found that the major component of this crystalline product was a diacetylated lactone with a lipidic component of either 15 or 16 hydroxy hexadecanoic acid\(^4^3\). It was also reported that pentadecane, heptadecane, octadecane, and various vegetable oils were examined as potential substrates. No products were obtained using pentadecane, crystalline products were obtained using hexadecane, heptadecane and octadecane, and "amorphous" products were obtained from vegetable oils.

The results mentioned above are not entirely consistent with those reported here. In this work, pentadecane resulted in a significant yield of sophorolipids having an, "amorphous" or oily morphology. When octadecane was fermented, enough undegraded solid substrate remained to prevent an accurate determination of product morphology.
However, in fermentations involving the hexadecane/octadecane mixture, the substrates were nearly completely degraded and high yields of an "amorphous" product were obtained. Fermentation of an eicosane/hexadecane mixture also resulted in an "amorphous" product. Thus, in the current work, crystalline sophorolipids were only obtained when hexadecane or heptadecane were used as the sole, lipophilic substrates. The production of crystalline sophorolipids was accomplished without pH adjustment.

Hommel et al., again using a citrate enriched medium, also suggested that decreasing the carbon to nitrogen ratio induced crystallization in C. apicola and C. bombicola. However, the results reported here suggest that it is the type of lipophilic substrate used that ultimately allows for the production of crystalline compounds. Manipulations of pH or the carbon to nitrogen ratio will only bring about crystallization, if an appropriate lipophilic substrate is used.

The differences between the results reported here and those obtained in the studies mentioned above may be, at least in part, due to the differences between C. bombicola and C. apicola. For instance, Stüwer et al. (1987) reported that while pH adjustment profoundly affected the nature of the sophorolipid produced by C. apicola, it had no affect whatsoever on the sophorolipids produced by C. bombicola. Also, Hommel et al. concluded that urea was not effective as a nitrogen source for C. apicola when, for many years, urea had been frequently used in work with C. bombicola. Also, while Cooper and Paddock reported that the nature of the nitrogen source affected C. bombicola biomass formation, Hommel et al. found no such affect in their work with C. apicola.

It is also noteworthy that C. bombicola was first isolated based on differences in carbon assimilation patterns and polysaccharide content from other sophorolipid
producing yeasts of the *Candida* genus\(^4\). Thus, the names of the various *Candida* yeasts should not be used interchangeably, and speculation on results concerning *C. apicola* should not be extrapolated to *C. bombicola*.

Of all the substrates that were provided, only three resulted in sophorolipid products that contained more than 85% of single type of hydroxy fatty acid. These substrates were hexadecane, heptadecane, and oleic acid. Both hexadecane and heptadecane resulted in the production of sophorolipids containing a single type of hydroxy acid and consisting of a single structural form (diacetylated lactone). However, oleic acid resulted in the production of sophorolipids containing a single type of hydroxy acid and consisting of a mixture of sophorolipid forms. These results are important because they help clarify the relationship between hydroxy fatty acid content and overall sophorolipid structure. *Candida bombicola* has been shown to produce crystalline sophorolipids in studies conducted by Rau et al\(^39\). In that work, the lipidic moieties of the sophorolipids were shown to contain 76.7% hydroxy octadecenoic acid and the resulting crystals were shown to contain only 70% lactonic sophorolipid. The results reported here are consistent with those obtained by Rau *et al.* in that the sophorolipid from the oleic acid fermentation was shown to contain 95% hydroxy octadecenoic acid and yet consisted of a mixture of structural forms. Furthermore, the results of the preliminary shake flask fermentations showed that, despite containing a predominance of hydroxy hexadecanoic acids, the product that was obtained from the glucose enriched medium consisted of a mixture of sophorolipid forms. These results ultimately show that having a high content of a single hydroxy fatty acid does not guarantee that the sophorolipid product will consist of a single structural form.
5.3 Larger Scale Sophorolipid Production

Earlier fermentations with *C. bombicola* \(^{46,47,32,33}\), using a medium consisting of hexadecane, glucose, yeast extract, and urea, resulted in direct incorporation, although not to the extent observed here. However, the resultant sophorolipid product was described as a "brown paste" and not crystals. Davila *et al.* also examined hexadecane, among other substrates, for its potential in commercial applications\(^{11}\). In this work a shift to the lactone form of the sophorolipid was observed, but again the product was not crystalline. These two studies reported very different patterns of sophorolipid production using essentially the same medium. The main difference between these two studies and the present work were the fermentation conditions. Furthermore, Klekner *et al.* reported different patterns of sophorolipid production between fermentations carried out in shake flasks and those in large-scale reactors\(^{34}\). All of this information led to questions concerning the scalability of the process. Therefore, a number of experiments were carried out in a sequencing batch reactor (SBR) in order to determine whether sophorolipid crystallization would still occur in larger scale fermentations. The type lipophilic substrate was changed throughout a series of 14 sequences. Two sequences were carried out with each type of lipophilic substrate.
5.3.1 Substrate Transition Fermentations

The fermentations in which a new lipophilic substrate was provided (Figures 4.26-4.28) gave some insight into the nature of the crystallization phenomenon. When the substrate was changed from hexadecane to dodecane, the morphology of the sophorolipid product did not immediately shift from oil to crystals. Instead, the shift occurred approximately 20 to 30 hours into the sequence and coincided with the beginning of sophorolipid production. Similarly, when the substrate was changed from tetradecane to hexadecane, the shift from oil to crystals did not occur until significant sophorolipid production began. The change in morphology only occurred when new product was introduced to the system. Furthermore, crystalline products appeared in fermentations with hexadecane despite the presence of oily products from previous fermentations with other lipophilic substrates. This demonstrated that the change in product morphology was not caused by physical interactions with the product, the new substrate, and the remainder of the media. Oil and crystalline morphologies were, instead, dependant only on the nature of the products themselves.

The SBR work suggests that the biomass may have retained some of the lipophilic substrate without utilizing it (Figures 4.19-4.21). However, it is also possible that the lipophilic substrate was retained in the reactor tubing or by residual sophorolipid in the reactor. When the lipophilic substrate was switched from hexadecane to dodecane, the concentration of dodecane increased from near-zero levels to 0.3 g/L. The same
observation was made in fermentations where the lipophilic substrate was changed from dodecane to tetradecane and tetradecane to hexadecane.

In each of these cases, the displaced lipophilic substrate was degraded by the end of the fermentation and did not reappear in subsequent runs. It can therefore be assumed that it had been completely metabolized or rinsed from the reactor by that point. It was observed that dodecane displaced hexadecane, tetradecane displaced dodecane, and hexadecane displaced tetradecane. These results offer no pattern of preferential alkane absorption by *C. bombicola*. In each case, the lipophilic substrate present in highest concentration displaced the one present in lower concentration.

5.3.2 Product Quality

5.3.2.1 Effect of Oxygen Limitation on Sophorolipid Yields

In all of the SBR fermentations sophorolipid yields (g SL / biomass, g SL / L, g SL / (Oil +Glucose) ) were significantly lower than those from the shake flask fermentations (Tables 4.6,4.7,4.8). The major difference between the SBR and shake flasks is that the SBR provides superior mixing and oxygen exchange. The experiment described in Section 3.7.4 was used to determine the effects of changing the dissolved oxygen concentration.

The results of this experiment are shown in Figure 4.5. Significantly higher yields were obtained from the shake flasks containing 200ml of medium. The yields were presumably increased when the organism was subjected to oxygen limiting
conditions. The plausibility of oxygen limitation affecting the yields in this manner is supported by a number of facts concerning the metabolism of *Candida* yeasts.

Cytochrome P-450 dehydrogenase is thought to be responsible for the initial hydroxylation of alkanes and the terminal or subterminal hydroxylation of fatty acids. Reports indicate that the P-450 enzyme is induced in a number of *Candida* species under oxygen limiting conditions. Also, Hommel *et al.* reported that P-450 levels in *C. apicola* drop off significantly when the organism enters the stationary growth phase. Therefore, it is plausible that by providing less oxygen to *C. bombicola* during the stationary phase, P-450 may be induced sufficiently so that fatty acids are immediately hydroxylated and incorporated into the sophorolipid product. The resultant increase in production efficiency could account for improved yields. It is also possible that oxygen limitation promotes sophorolipid production by directly inhibiting the β-oxidation pathway. With no way to break down fatty acids, the yeast may have no option but to hydroxylate them and incorporate them into sophorolipid.

The apparent affect of oxygen limitation observed here warrants further inquiry. The simple experiment that was performed was flawed in that the dissolved oxygen concentration was indirectly varied. In further experiments it would be ideal to keep the degree of physical mixing constant and to control dissolved oxygen directly.

### 5.3.2.2 Sophorolipid Production Efficiency

In the SBR fermentations, substrate degradation patterns varied significantly depending on the substrate used (Figures 4.11-4.12 and 4.25-4.27). In all fermentations
with hexadecane and aqueous phase carbohydrates, both of these substrates were
degraded simultaneously. This degradation pattern is significantly different than those in
fermentations with dodecane, tetradecane and kerosene where aqueous phase
carbohydrates degradation essentially ceased when the degradation of the lipophilic
substrate began. Furthermore, the period of simultaneous aqueous phase carbohydrate
and hexadecane degradation coincided with the rapid production of crystalline
sophorolipid. Hexadecane resulted the higher yields than the other substrates tested in
the larger reactor. Maximum sophorolipid levels when hexadecane was used were in the
vicinity of 6-8 g/L compared to 4-6 g/L in all other fermentations.

A theory concerning sophorolipid production efficiency has been proposed by
Linton\textsuperscript{35}. When the yeast is provided with only a lipophilic substrate or only aqueous
phase substrates there is a high ATP demand in sophorolipid synthesis. This was
attributed to the need for the organism to produce either a carbohydrate moiety from a
lipophilic substrate or a fatty acids from a carbohydrate substrate. The ATP demand may
be significantly reduced when both lipophilic and hydrophilic substrates are provided if,
as proposed by Hommel \textit{et al.}, aqueous phase carbohydrates are used primarily in cellular
respiration and in sophorose synthesis while the lipophilic substrate is used exclusively in
the production of the lipidic portion of the sophorolipid. The net result is increased
production efficiency when both lipophilic and hydrophilic substrates are provided. The
results of the SBR fermentations are consistent with this theory. Higher production in
hexadecane fermentations can be attributed to increased efficiency. The hexadecane can
be directly converted to the hydroxy fatty acid at the same time as the aqueous phase
carbohydrates are being used. In fermentations with dodecane, tetradecane and kerosene
decreased production would have occurred since the substrates were essentially used sequentially.

The results discussed in Section 5.2.2 concerning direct incorporation are directly related to the results of the SBR fermentations. In theory, an increase in production efficiency can be achieved by providing both hydrophilic and lipophilic substrates so long as the lipophilic substrate is reserved exclusively for sophorolipid production. For this to be successful, it then becomes necessary that the lipophilic substrate be directly incorporated instead of being broken down and used as a source of energy. The shake flask fermentations discussed in Section 5.2 have shown that direct incorporation occurs to a much greater extent with hexadecane than with alkane substrates with shorter chain lengths. When hexadecane is directly incorporated, it is precluded from being used in other metabolic pathways and the yeast is forced to use glucose as an energy source. The other alkanes substrates did not exhibit significant amounts of direct incorporation and therefore stand a greater chance of being used as both an energy source and as a lipidic constituent of the sophorolipid. Thus, sophorolipid production efficiency is ultimately reduced.

The results of the mineral oil fermentation clearly show the detrimental impact on production efficiency that occurs when only a single carbon source is provided. As Figure 4.29 shows, the yeast did not degrade mineral oil. In the absence of a usable lipophilic substrate, the yeast responded by degrading aqueous phase carbohydrates more rapidly than in any other fermentation. Although aqueous phase carbohydrate degradation was extensive, there was no observable increase in sophorolipid concentration during the
fermentation. This demonstrates the difficulty of producing the lipophilic components from a carbohydrate precursor\textsuperscript{6,36}.

Regardless of the lipophilic substrate, the biomass concentration leveled off at approximately 2g/L in all of the fermentations. This result is not surprising considering the degradation patterns that were observed. The patterns fell into two general categories. In the first case, the aqueous phase carbohydrate substrates and lipophilic substrate were degraded sequentially so that only a single carbon source could be used for biomass production. In the second case, both aqueous phase carbohydrates and the lipophilic substrate were degraded simultaneously, but the additional (lipophilic) carbon source was used in sophorolipid production, leaving aqueous phase carbohydrates to be used for biomass production. Therefore, biomass concentrations always reached the same level regardless of the degradation pattern of the two substrates.
6.0 Conclusions

High yields of crystalline sophorolipids were obtained in shake flasks only when hexadecane or heptadecane were used as lipophilic substrates. The appearance of crystals can be related to the degree of variation in the products. The crystalline sophorolipids were shown to be exclusively diacetylated lactones containing a high percentage of hydroxy hexadecanoic acids or hydroxy heptadecanoic acids, respectively. In other experiments oily products were obtained when using oleic acid, or elevated glucose concentrations, and these were shown to be mixtures of structural types of sophorolipids even though these were shown to contain a single type of hydroxy fatty acid. Thus, a single structural class appears to be essential for the formation of crystals.

The lactone crystals were much simpler to collect and wash than oily mixtures. Having a high yield of lactone sophorolipid was also desirable because the compound has a number of useful industrial applications.

Solid substrates were poorly degraded by *C. bombicola*. When these solid substrates were provided, the hydroxy acids observed in sophorolipid products must have been synthesized *de novo*, resulting in poor yields. Moreover, the sophorolipids produced were oily, consisted of a mixture of structural forms, and were difficult to recover. Solid alkanes and fatty acids can therefore be considered inappropriate as substrates for *C. bombicola*.

In comparison to shake flask fermentations, significantly lower yields of sophorolipid were achieved using the sequencing batch reactor. In this respect, the scale-up was not entirely successful, but these yield data did help to elucidate the system.
Hexadecane still resulted in higher yields in the SBR than the other lipophilic substrates and it was shown that only hexadecane was degraded simultaneously with glucose. This could be attributed to enzyme specificity of the yeast's monoxygenase systems towards hexadecanoic acid, which, in turn, resulted in the direct incorporation of this compound. Since hexadecane was being directly incorporated into the sophorolipid, *C. bombicola* must have been using glucose as an energy source and, consequently, both substrates were degraded simultaneously. This increased efficiency in substrate utilization ultimately resulted in improved sophorolipid yields relative to those with all of the other hydrocarbons used in the large-scale growth studies.

There was some indication that the higher concentration of dissolved oxygen in the SBR played a role in reducing the yields relative to those from the shake flasks. However, further experimentation is required to either confirm or reject this hypothesis.

A very narrow range of substrates can be used to produce high purity, crystalline, diacetylated lactone sophorolipids. This precludes the use of many, less expensive substrates that contain mixtures of alkanes or fatty acids. However, given the numerous applications for this lactone, it remains worthwhile to pursue further process development to increase yields during scale up while maintaining the desirable aspects of the shake flask experiments.
7.0 References


