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# Sophorose Lipids From Torulopsis Bombicola

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# SOPHOROSE LIPIDS FROM TORULOPSIS BOMBICOLA

by

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Faculty of Engineering Science
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Chemical and Biochemical Engineering

Submitted in partial fulfilment
of the requirements for the degree of
Doctor of Philosophy

Faculty of Graduate Studies
The University of Western Ontario
London, Ontario
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ABSTRACT

Sophorose lipids are a class of biosurfactants which have several industrial applications, such as in lubricants, humectants, personnel care products, soaps and detergents. Environmental considerations are the driving force behind the use of biosurfactants because of their perceived advantages such as lower toxicity, biodegradability and ecological acceptability over chemical surfactants. The key factors that will determine the usage of a given biosurfactant include the development of an economic process for its production, the use of low-cost raw materials and high product yields. The objective of this study was to develop a fermentation process to produce high value biosurfactants from low- or negative cost sugars, fat and oil substrates with the yeast *Torulopsis bombicola*. A wide variety of sugar, nitrogen, fat and oil substrates were examined as potential feedstocks to obtain very effective carbon sources for sophorose lipids production. Experiments were carried out in shake flasks, 1-L Bellco stirred reactors and 20-L fermenter at 450 rpm and 30°C using batch and fed-batch experiments. The results showed that sugar, nitrogen, fat and oil were interactive control parameters which influenced the structure, yield, growth-

phase, and also the metabolic pathways leading to glycolipid production. It was found that the production of sophorose lipids increased with increasing concentrations of both oil and sugar, and was profoundly influenced by the sources and concentration of nitrogen. Corn steep liquor, yeast extract and peptone were shown to be very effective nitrogen sources for sophorose lipids production. It was found that optimal production of sophorose lipids occurred when nitrogen sources became limited. The maximal production of sophorose lipids could reach 150-160 g/L in the optimal medium consisting of 10% glucose, 10.5% canola oil, 0.1% urea, and 0.4% yeast extract in a 1-L Bellco fermenter stirred at 450 rpm, an air flow rate of 2 vvm and 30°C. Similar yields of sophorose lipids were also obtained in a 20-L bioreactor. About 73% of the apolar sophorose lipid, which has been described as 17-L-([2′-O-β-D-glucopyranosyl-β-D-glucopyranosyl]-oxy)-octadecanoic acid 1′-4′′-lactone 6′,6′′-diacetate (SL-1) group, was found in the mixture of sophorose lipids produced under the above conditions. When only lactose was present as the sugar source in the medium, the yeast *Torulopsis bombicola* did not grow. However, in the presence of both lactose and olive oil, intra- and extracellular lipids were synthesized. By fed-batch cultivation. it could be seen that the

maximization of yields, productivity and the minimization of production costs of sophorose lipids were obtained because the production phase was extended and substrates and enzymes were utilized very effectively. Sets of kinetics models for batch and fed-batch fermentation processes for the growth and sophorose lipids by *Torulopsis bombicola* were proposed, which could successfully describe, control and predict the kinetics of cell growth and sophorose lipids production.

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Working with a number of authors over the years has taught me, no thesis can be written without the help and support of a large body of people. This one is no exception. Naturally, it has not been possible to name all of them who directly or indirectly contributed to this thesis, but I am however grateful for their kindness, helps and supports. Without them, this thesis would not exist.

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NOMENCLATURE

ATCC = American Type Culture Collection

B = nongrowth component of specific product appearance rate

BOD = biochemical oxygen demand

CSTR = Continuous Stirred Tank Reactor

CSTBR = Continuous Stirred Tank Biological Reactor

E = volumetric effluent flow rate, volume/time

F = volumetric feed rate, volume/time

$k_d$ = cell death-rate constant

$K_m$ = Monod coefficient corresponding to the component S

m = appreciable maintenance factor

$P_E$ = concentration of product in the effluent

P = concentration of product in the reaction mixture

$P_o$ = concentration of product in the feed (usually zero)

$R_p$ = specific rate of production formation

$\mu$ = specific rate of biomass formation

$\mu_{max}$ = maximum specific growth rate

$R_{vs}$ = specific rate of substrate uptake

$R_{vb}$ = volumetric rate of biomass formation

$R_{vp}$ = volumetric rate of product formation

$R_{vs}$ = volumetric rate of substrate uptake

S = concentration of substrate in the reaction mixture

$S_E$ = concentration of substrate in the effluent

$S_f$ = substrate at the beginning of second batch stage of the repeated fed batch mode

$S_o$ = concentration of substrate in the feed

$S_r$ = concentration of substrate at the beginning of first batch stage

$t_d$ = the time required to double the population of cells

$V_R$ = volume of the reaction mixture

vvm = volume of air per volume of liquid per minute

X = concentration of cells in the reaction mixture

$X_E$ = concentration of cells in the effluent

$X_f$ = the biomass at the beginning of second batch stage of the repeated fed batch mode

$X_r$ = the biomass at the beginning of first batch stage of the repeated fed batch mode

$X_o$ = concentration of cells in the feed, mass per unit volume

$Y_{p/s}$= product formed per unit of substrate consumed (g/g)

$Y_{p/x}$= product formed per unit of biomass formed (g/g)

$Y_{s/x}$= substrate consumed per unit of biomass formed (g/g)

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# 1. INTRODUCTION

The industrial requirement for surfactants is constantly growing. Surfactants possess both hydrophilic and hydrophobic structural moieties, which in turn impart many properties, including an ability to lower the surface tension of water. The surfactant industry has grown about 300% within the U.S. chemical industry during the last decade. The U.S. surfactant industry shipments in 1989 were approximately $3.65 billion, 14% higher than the previous year. United States production was estimated to be 15.5 billion lb. per year (Desai and Desai, 1992).

Soap, one type of surfactant, has been used since the dawn of civilization. Although the oldest synthetic surfactant, sulfonated castor oil or turkey red oil, was produced over a century ago, synthetic surfactants based upon either fats or petroleum have been developed industrially only during the past six decades. During this period the synthetics gradually replaced soap so that at present the latter has practically disappeared from all products used for cleaning, laundry, textile scouring, and so forth. The synthetic surfactants of petrochemical origin gradually attained a dominant position.

Environmental considerations are the driving force behind the use of biosurfactants because many of the areas using conventional surfactants are today dominated by concerns of bio-compatibility, toxicology, and eco-toxicity. The late 80s and early 90s have shown an ever-increasing public awareness and concern over environmental issues. Governments and regulatory agencies all over the world have been sensitized to these issues and are taking legislative steps to ensure human and ecological safety. Biosurfactants have been attracting attention as natural and promising surfactants because they have special advantages over chemical surfactants as they are less toxic, biodegradable and ecologically acceptable (Zajic and Panchal, 1976; Zajic et al., 1977; Finnerty and Singer, 1984; Rosenberg, 1986; Kosaric et al., 1987).

Biosurfactants are produced as metabolic by-products by bacteria, yeasts, and fungi. They are produced extracellularly or as a part of the cell wall. The term "biosurfactants" also helps to distinguish them from natural surfactants like alkylphenol glycols (APGs) or alpha-sulphomethyl esters (ASMEs) and synthetic surfactants such as linear alkylbenzenesulfonates (LAS) or alkylphenol ethoxylates (APE). Mostly they exhibit the typical amphiphilic character of

lipids, and high specificity and are consequently suited to new applications. Basically there are six major classes of biosurfactants: (1) glycolipids, (2) lipopeptides/lipoproteins, (3) phospholipids, (4) neutral lipids, (5) substituted fatty acids, and (6) lipopolysaccharides. Most biosurfactants are either anionic or neutral. Only a few are cationic (those containing amine functions). The hydrophobic part of the molecule is based on long-chain fatty acids, hydroxy fatty acids, or α-alkyl-β-hydroxy fatty acids. The hydrophilic portion can be a carbohydrate, amino acid, cyclic peptide, phosphate, carboxylic acid, or alcohol. A wide variety of microorganisms can produce these compounds as shown in Table 1.1. Most biosurfactants are produced from hydrocarbons. Production is most often growth associated. However, biosurfactants are also produced from carbohydrates. Some biosurfactants can be produced from a variety of simple potentially less expensive substrates *n*-alkanes, carbohydrates, vegetable oil, and wastes (Kosaric et al., 1984; Zhou et al., 1992). For example, rhamnose lipids can be produced by *Pseudomonas aeruginosa* from $C_{11}$ and $C_{12}$ alkanes, succinate, pyruvate, citrate, fructose, glycerol, olive oil, glucose, and mannitol (Desai and Desai, 1992). Biosurfactants are usually secondary metabolites, being

TABLE 1.1 MAJOR TYPES OF BIOSURFACTANTS BY MICROORGANISM

| Biosurfactants | Microorganisms |
|---|---|
| **A. Glycolipids** | |
| Thehalose mycolates | *Rhodococcus erythropolis* |
| | *Arthrobacter paraffineus* |
| | *Mycobacter fortitum* |
| Trehalose esters | *Micromonospora* spp. |
| | *Mycobacter smegmatis* |
| | *Mycobacter paraffinicum* |
| | *R. erythropolis* |
| Mycolates of mono-, di, and trisaccharide | *Mycobacter smegmatis* |
| | *Arthrobacter* spp. |
| Rhamnolipids | *Pseudomonas* spp. |
| Sophorolipids | *Torulopsis bombicola* |
| | *Torulopsis petrophilum* |
| | *Torulopsis apicola* |
| **B. Phospholipids & Fatty acids** | *Candida* spp. |
| Phospholipids and fatty acids | *Corynebacterium* spp. |
| | *Micrococcus* spp. |
| | *Acinetobacter* spp. |
| Phosplipids | *Thiobacillus thiooxidans* |
| | *Aspergillus* spp. |
| **C. Lipopeptides & Lipoproteins** | |
| Gramicidens | *Bacillus brevis* |
| Ornithine-lipid | *Pseudomonas rubescens* |
| | *Thiobacillus thiooxidans* |
| Cerilipin | *Gluconobacter cerinus* |
| Lysin-lipid | *Agro. tumefaciens* |
| | *Str. sioyaensis* |
| Surfactin | *B. subtilis* |
| Peptide-lipid | *B. licheniformis* |
| **D. Polymeric Surfactants** | |
| Lipoheteropolysaccharide | *Arthrobacter calcoaceticus* |
| Heteropolysaccharide | *A. calcoaceticus A2* |
| Polysaccharide-protein | *A. calcoaceticus strains* |
| | *Candida lipolytica* |
| Manno-protein | *S. cerevisiae* |
| Carbohydrate-protein | *Candida petrophilum* |
| | *Endomycopsis lipolytica* |
| Mannan-lipid complex | *Candida tropicalis* |
| Mannose/erythrose-lipid | *Shizonella melanogramma* |
| | *Ustilago maydis* |
| Carbohydrate-protein | *Pseudomonas fluorescens* |
| lipid complex | *Debaryomyces polymorphus* |
| **E. Particulate Biosurfactants** | |
| Membrane vesicles | *Acinetobacter* sp. H01-N |
| Fimbriae | *A. calcoaceticus* |

produced during late logarithmic and stationary growth phases from carbohydrate or hydrocarbon.

In the personal-care sector penetration by biosurfactants is expected to be more rapid. It is already widely predicted that by 2000 all cosmetic products will be "bio-cosmetics", including color bases (eg. bio-lipsticks with fermentation derived "shikonin"). Sophorose-lipids from *Torulopsis bombicola* KSM 35 are already used by Kao Corp in Japan as a high value skin moisturizer (Inoue, 1988).

In industrial processing, the petroleum industry currently offers the largest prospects for biosurfactants utilization. There are several applications (some already commercialized by Petroferm's EMULSAN). The most important application areas for biosurfactants, including those produced in-situ, are in Microbial Enhanced Oil Recovery (MEOR) with some 200 wells in the larger European region and in the cleaning up of near-shore oilspills. The use of biosurfactants for the cleaning of oil tankers was demonstrated as economically rewarding in Kuwaiti field trials (without the biosurfactant-produced by Petrogen's Pet 1006 genetically-engineering micro-organism-having to be separated from the broth). Other commercial

applications include the extraction of bitumen from tar-sands (Canada), the pumping of crudes through use of bio-emulsifiers, and the demulsification of EOR crudes (North America) and viscosity reduction of heavy crudes like the Venezuelan crudes (Hayes et al., 1986; Kosaric, et al., 1987).

The food industry is attractive since lecithin is already the largest used food emulsifier. Sophorose lipids are used as flour additives for quality improvement of bakery goods; rhamnolipids by *Pseudomonas* BOP 100 were used to obtain liposomes (Ohata and Kamata, 1986, Kitahata et al., 1988).

Antibiotic effects of biosurfactants and the inhibitory effect toward the growth of AIDS virus in WBC have been reported recently (Desai and Desai, 1992). The pulmonary surfactant, essential for normal respiration, is a phospholipid protein complex; many premature infants suffer respiration failure because of the deficiency of this surfactant. The human gene for production of the protein molecule of this surfactant has been isolated and cloned in bacteria (While et al., 1985); this has opened up the possibility of larger-scale production of this surfactant for medical application.

Potential applications for biosurfactants will be in pharmacology, biocosmetics, textiles, food, pulp and paper, coal beneficiation, and ore processing (Zajic and Panchal, 1976; Zajic et al., 1977; Finnerty and Singer, 1984; Rosenberg, 1986; Kosaric et al., 1987) ).

The most often isolated and most thoroughly studied biosurfactants are the structurally homogenous glycolipids which are carbohydrates in combination with long-chain aliphatic acids or hydroxy aliphatic acids. Glycosyl diglycerides present in the cell membranes of a wide variety of bacteria are the most common glycolipids. *Torulopsis bombicola* is one of the few yeasts known to produce biosurfactants (sophorolipids). Glycolipids can be classified as (1) trehalose lipids, (2) rhamnolipids, and (3) sophorolipids.

Up to now, microbial surfactants have not been successful in substituting for chemical surfactants because of their high production cost. The key factors governing the success of a biosurfactant will be the development of a cheaper process, the use of low-cost raw materials, a high product yield, and

superactive, highly specific, and selective biosurfactants for specific applications.

## 2. GENERAL AND SPECIFIC OBJECTIVES

The interest in biosurfactants is increasing because they can be produced in a wide variety and with different properties. Their production through microbial synthesis can be simpler and cheaper using cheap substrates. The most important advantage of biosurfactants is that they are biodegradable and less toxic than synthetic surfactants and they can be produced from wastes. The key factors that will determine the usage of a given biosurfactant include the development of an economic process for its production, the use of low-cost raw materials and high product yields. The objective of this work is to develop an economic process to produce high value sophorose lipids from low cost substrates by the yeast *Torulopsis bombicola* as follows:

1) Biosynthesis of sophorose lipids is studied because the knowledge on metabolic pathways involved in biosurfactant production is essential for biosynthesis control.

2) To develop sets of kinetics models for batch and fed-batch fermentation processes for the growth and production of sophorose lipids by *Torulopsis bombicola* in order to describe,

control and predict the kinetics of cell growth and sophorose lipids production.

3) To optimize cultivation conditions for the production of sophorose lipids such as temperature, cultivation pH, sources and concentration of nitrogen, aeration, and agitation. The cultivation conditions must be optimized to achieve optimum production rates and economic yields.

4) A wide variety of sugars, nitrogen sources and vegetable oils are examined as potential feedstocks to obtain very effective carbon sources for sophorose lipids. The choice of inexpensive raw materials including carbon, and nitrogen sources is very important to the reduction of sophorose lipids cost. Sugar, fat and oil, and nitrogen are interactive control parameters which influence the structure, yield, growth-phase, and also the metabolic pathways leading to the biosynthesis of sophorose lipids.

5) To study the cultivation modes including batch and fed-batch for the production of sophorose lipids to obtain the

maximization of yields, productivity and the minimization of sophorose lipids cost.

# 3. LITERATURE SURVEY

## 3.1 Classes of Biosurfactants

Synthetic surfactants are usually classified according to the nature of their polar groups. However, microbial surfactants are commonly differentiated on the basis of their biochemical nature and the microbial species producing them. Table 1.1 shows the various important biosurfactants and the producing microbial species. Biosurfactants can be classified as following:

(1) glycolipids

(2) phospholipids

(3) fatty acids

(4) lipopeptide/lipoproteins

(5) polymeric surfactants, and

(6) particulate surfactants

Most biosurfactants are either anionic or neutral. Only a few are cationic (those containing amine functions). The hydrophobic part of the molecule is based on long-chain fatty acids, hydroxy fatty acids, or α-alkyl-β-hydroxy fatty acids.

The hydrophilic portion can be a carbohydrate, amino acid, cyclic peptide, phosphate, carboxylic acid, alcohol, and so on.

Biosurfactants are synthetized by bacteria, yeasts, and fungi during cultivation on various carbon sources. The largest group of biosurfactants characterized to date are glycolipids. Glycolipid biosurfactants are commonly considered as sugar-containing lipids in which both moieties may be linked either glycosidally (i.e., as an ether or more correctly as a hemiacetal link) as in the sophorose, rhamnose, and cellobiose lipids, or via acylation (i.e., as an ester link) as in the acylpolyols, trehalose lipids, and sugar mycolates. The so-called biosurfactant precursors like fatty acids, fatty alcohols, sugars, glycerides, and phospholipids are not the subject of this section. Amino acid containing lipids with small and great quantities of amino acids, lipopolysaccharides and lipoteichoic acids are also excluded.

Glycolipids with hydrophilic-lipophilic balance (HLB) values near 10, which is calculated on the basis of the relative percentage of hydrophilic to hydrophobic groups in the surfactant molecule, are able to reduce the surface tension

down to <30 mN/m. The interfacial tension between water and *n*-hexadecane was lowered to values <1 mN/m; the critical micelle concentrations (CMC) were in the range of 5 to 200 mg/L. Examples of these excellent properties are some single metabolites after chromatographic purification (rhamnose lipids R1-R4, trehalose-2,2',3,4-tetraester, sucrose-monocorynomycolate) or glycolipid mixtures themselves (cellobiose lipids, rhamnolipids).

In the following section, structures and properties of glycolipids are reviewed. The focus is placed on those of sophorose lipids.

### 3.1.1 Glycolipids

Glycolipids, the most commonly isolated and studied biosurfactants, are carbohydrates in combination with long-chain aliphatic acids or hydroxy aliphatic acids. Glycosyl diglycerides present in the cell membranes of a wide variety of bacteria are the most common glycolipids. The best examples of glycolipids studied from the point of view of surfactant characterization and properties are:

(1) trehalose lipids, (2) rhamnolipids, and (3) sophorose

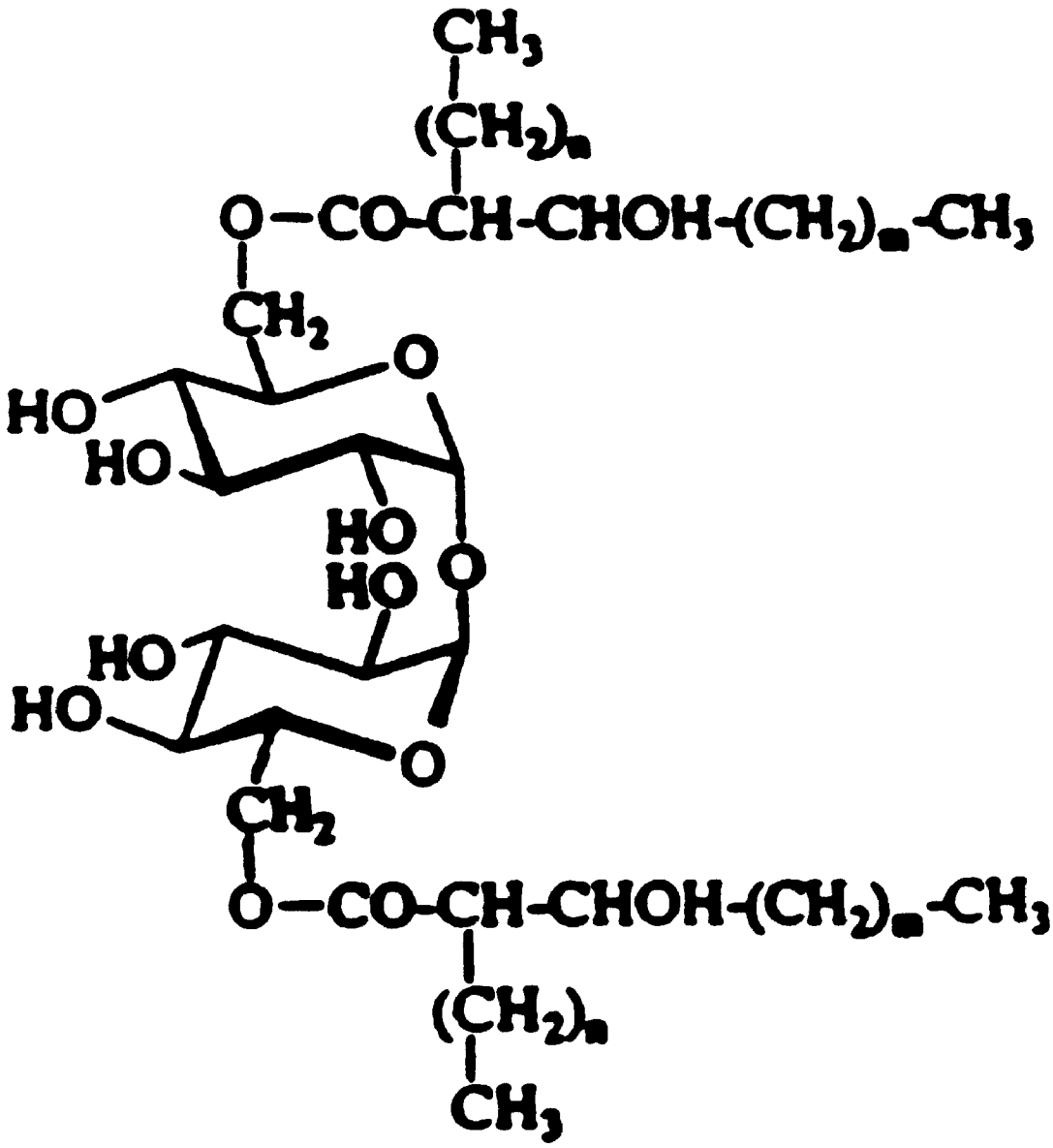
lipids.

### 3.1.1.1 Trehalose Lipids

Several types of trehalose lipids are found to be widely distributed. Figure 3.1 shows the structure of trehalose-6,6'-dicorynomycolate from *Rhodococcus erythropolis* (Rapp et al., 1979).

Mycolic acids with the disaccharide trehalose linked at C6 and C6' are associated with the cell wall structure of most species of the genera *Mycobacterium*, *Nocardia*, and *Corynebacterium*. Mycolic acids are long-chain, α-branched, β-hydroxy fatty acids and the chain length synthesized is a characteristic of producing genera. Trehalose diester produced by *Rhodococcus erythropolis* and *Arthrobacter paraffenium* have been extensively studied by Rapp et al. (1979) and Suzuki et al. (1969). *Mycobacterium phlei* (Prome et al., 1976) produces trehalose 6-O monomycolates, whereas *Mycobacterium fortuitum* and *Micromonospora sp. F3* produce trehalose esters of straight-chain nonhydroxylated C16 and C18 fatty acids (Vilkas et al, 1968). On the other hand, *Mycobacterium smegmatis* produces trehalose esters fully acetylated by polyunsaturated

**Figure 3.1 The Structures of Trehalose-6,6′-Dicorynomycolate from Rhodococcus erythropolis (Rapp et al., 1979)**



acids (Asselineau et al., 1972). Production of a novel nonionic trehaloselipid from *Mycobacterium paraffinicum* (Batrakov et al., 1981) and an anionic trehalose lipid from *R. erythropolis* (Ristau and Wagner, 1983) have been isolated and characterized. Production of mono-, di-, and trisaccharides have been reported using various species of *Corynebacteria, Mycobacteria,* and *Arthrobacter* (Brennan et al., 1970; Suzuki et al., 1974).

In most cases these nonionic trehalose lipids are all wall-associated. They contain a homolog mixture of α-branched-β-hydroxy fatty acids which are esterified with the 6- and 6'-hydroxyl groups of the trehalose unit. The number of carbon atoms varies: Mycolic acids contain 60-90 carbon atoms, nocardomycolic acids have 40-60 carbon atoms, and corynomycolic acids possess 25-40 carbon atoms. The trehalose diesters of *Arthrobacter paraffineus KY* 4303 (Suzuki et al., 1969) and *Rhodococcus erythropolis DSM* 43215 (Rapp et al., 1979) are examples for corynomycolic acid-containing glycolipids.

Wagner and coworkers have extensively studied surface and interfacial activities of trehalose lipids. Trehalose lipids

from *R. erythropolis* have been shown to reduce surface tension to 25-30 mN/m and interfacial tension to 1 mN/m. Corynemycolates of various mono- and disaccharides from *Arthrobacter* spp. have also been reported to lower the surface tension to 33-40 mN/m and interfacial tension to 1-5 mN/m (Lang and Wagner, 1987).

### 3.1.1.2 Rhamnose Lipids

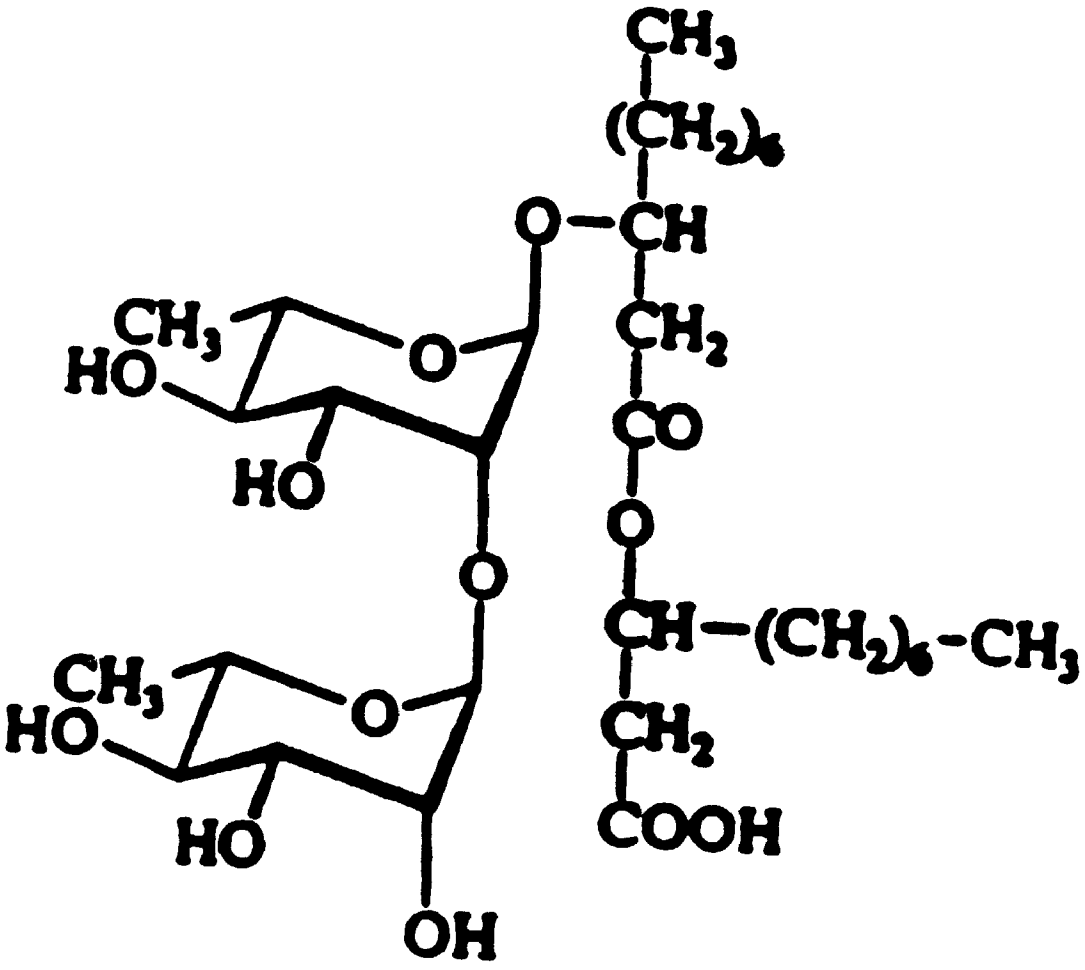
It has been known that glycolipids are produced by certain species of *Pseudomonas*, which contain one or two molecules of rhamnose linked to one or two molecules of β-hydroxydecanoic acid. These are the rhamnolipids R1 and R2, which can be produced from carbohydrates or hydrocarbons. R1 contains two rhamnoses attached to β-hydroxydecanoic acid, whereas R2 consists of one rhamnose connected to the identical hydroxy fatty acid (Jarvis and Johnson, 1949; Edward and Hayashi, 1965; Hisatsuka et al., 1981; Itoh and Suzuki, 1972;). Various carbon sources such as $C_{11}$ and $C_{12}$ alkanes, succinate, pyruvate, citrate, fructose, glycerol, olive oil, glucose, and mannitol could be used for the rhamnolipids production by *P. aeruginosa* (Robert, et al., 1989). Several examples of waste stream utilization have been demonstrated. It was reported

that chicken fat could be used for the production of *P. aeruginosa* SB1 rhamnolipids (Chakrabarty, 1985). Koch et al. (1988) have developed a strain of *P. aeruginosa* to utilize whey for the production of rhamnolipids.

The glycolipid (R1) contains two rhamnose units and two β-hydroxydecanoic acid units. Hisatsuka et al. (1981) found the same 2-O-α-L-rhamnopyranosyl-α-L-rhamnopyranosyl-β-hydroxydecanoyl-β-hydroxydecanoate when they grew *P. aeruginosa* $S_7B_1$ on n-hexadecane, n-paraffin mixtures, or glucose at 30°C for 6 days. A rhamnolipid R2 with two fatty acids and only one sugar named L-α-rhamnopyranosyl-β-hydroxydecanoyl-β-hydroxydecanoate was observed by Itoh et al. (1971) when they cultivated *P. aeruginosa* KY 4025 on 10% n-paraffin at 30°C for 55 hr. Figure 3.2 shows the structure of rhamnose lipids containing one or two rhamnose units, and one or two units of fatty acid (Syldatk et al., 1985).

The rhamnose lipids so far reported possess one or two rhamnose units and in general two β-hydroxydecanoic acid residues. Syldatk et al. (1985) isolated two new rhamnolipids R3 and R4 with only one fatty acid.

Figure 3.2 The Structure of Rhamnose Lipids Containing One or Two Rhamnose Units, and One or Two Units of Fatty Acid from *Pseudomonas aeruginosa (Syldatk et al., 1985)*


CH3
(CH2)6
O
CH
CH2
CO
O
CH-(CH2)6-CH3
CH2
COOH
CH3
HO
O
HO
O
CH3
HO
O
HO
OH

There are several evidences available to show that depending on the pH and salt concentration, pure rhamnolipids from *Pseudomonas* spp. can lower the interfacial tension against n-hexadecane to around 1 mN/m and surface tension to 25-30 mN/m (Itoh and Suzuki 1972; Parra et al., 1989; Lang and Wagner 1987).

### 3.1.1.3 Sophorose Lipids

*Torulopsis* sp. are the examples of the few yeasts known to produce biosurfactants. It was found that biosurfactants heavier than water and consisting of the dimeric carbohydrate sophorose linked to long-chain hydroxycarboxylic acids are produced by using *Torulopsis bombicola* (Gorin et al., 1961; Tulloch et al., 1962; Cooper and Paddock, 1984; Gobbert et al., 1984; Inoue and Itoh, 1982, Zhou et al, 1992), *Torulopsis petrophilum* (Cooper and Paddock, 1983) and *Torulopsis apicola* (Tulloch et al., 1967). These biosurfactants are a mixture of at least six to nine different hydrophobic sophorosides. Recently, Hommel et al.(1987) have investigated the production of a mixture of water-soluble sophorolipids from yeasts. Cutler and Light (1979) showed that *Candida bogoriensis* produces glycolipids in which sophorose is linked to

docosanoic acid diacetate. Sophorose lipids are produced in the late logarithmic phase of growth or stationary phase. A wide range of carbohydtates and hydrocarbons could be used for sophorose lipids production. The optimal production was obtained when both carbohydrate and vegetable oil were used (Inoue and Itoh, 1982; Cooper and Paddock, 1984; Zhou et al., 1992).

## A. Nature of the Microorganism

The first yeast to produce sophorose lipids was isolated in 1954 from nectar and found that it produced moderate yields of an insoluble heavier-than -water liquid. However, the product was not characterized, and the yeast was identified only as a species of *Torulopsis*. A few years later, it was reported that the main components of the product were sophorolipids consisting of 2-O-β-D glucopyranosal-D-glucopyranose units linked β-glycosidically to 17-L-hydroxyoctadecanoic and 17-L-hydroxy-9-octadecenoic acids, the sugar moieties being partly acetylated (Gorin et al., 1961).

Several *Torulopsis* species were isolated from natural habitats including flowers, honeycomb, leaf surface of the shrub, fruit

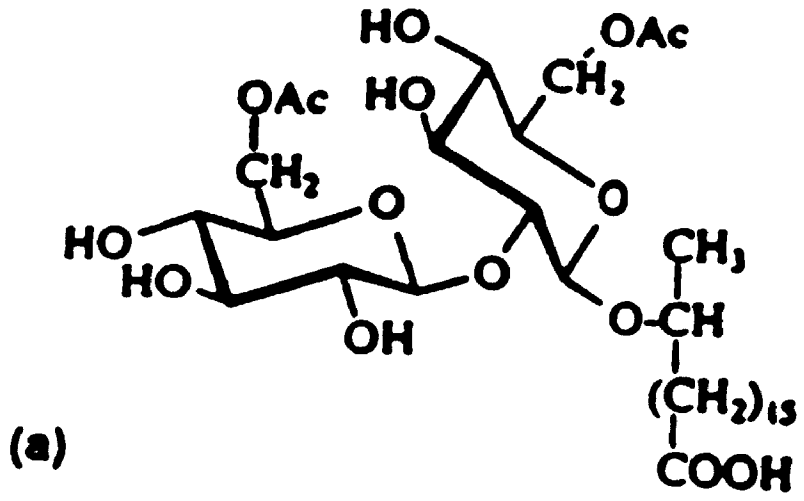
and soils. These yeasts were identified as *Torulopsis apicola, Torulopsis bombicola, Torulopsis apis* and *Torulopsis magnoliae, Candida bombicola, Torulopsis petrophilum* and *Torulopsis gropengiesseri* (Gorin et al., 1961; Tulloch et al., 1968; Jones, 1967; Spencer et al., 1979).

B. Structures of Sophorose Lipids

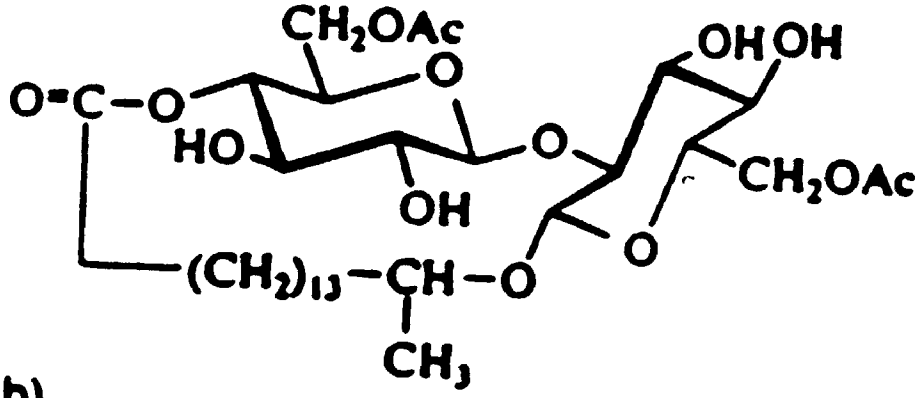
Sophorolipids have two main structures which are the typical amphiphilic structures of surfactants (Gorin et al., 1961, Tulloch et al., 1967). The main components of the sophorose lipids consist of partly acetylated 2′-O-β-D-glucopyranosyl-D-glucopyranose units (sophorose) attached β-glycosidically to 17-L-hydroxy-9-octadecenoic acids. The lipophilic portion of sophorolipids is 17-L-hydroxy-9-octadecanoic acids unit, and hydrophilic portion of sophorolipids is 2′-O-β-D-glucopyranosyl-D-glucopyranose disaccharide unit. The schematic representation of the sophorolipids structures is shown in Figure 3.3 (Gorin et al., 1961) and Figure 3.4 (Tulloch et al., 1967). In Figure 3.3, the sugars of sophorolipids are partly acetylated, but the positions are not determined. As shown in Figure 3.4, sophorolipids have unusual macrocyclic structures in which the carboxyl group is attached

(a) Figure 3.3 The Structure of Acidic Sophorose Lipids from *Torulopsis bombicola (Gorin et al., 1961)*

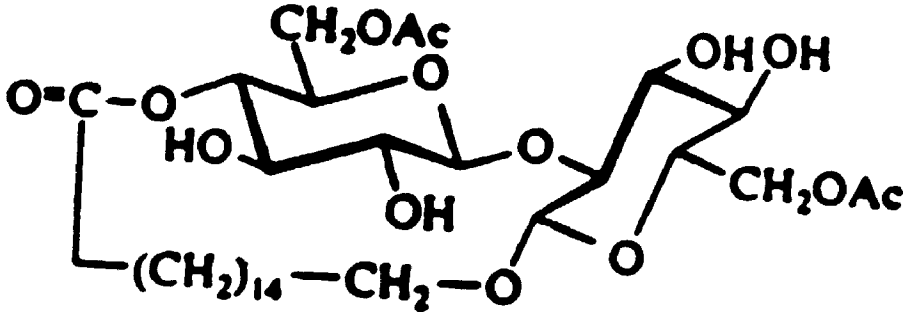
(b) Figure 3.4 The Structure of Lactonic Sophorose Lipids from *Torulopsis apicola* (Tulloch et al., 1967)


HO
OAc
HO
CH2
OAc
CH2
O
O
HO
HO
O
CH3
O-CH
OH
(CH2)15
COOH

(a)


CH2OAc
O
OH OH
O-C-O
HO
O
CH2OAc
OH
O
(CH2)13-CH-O
CH3

(b)


CH2OAc
O
OH OH
O-C-O
HO
O
CH2OAc
OH
O
(CH2)14-CH2-O

to the sugar portion to form a macrocyclic lactone ring and the disaccharide sophorose is an intergral part of the macrocyclic ring. The principal lactonic component has acetate groups at the 6 and 6′ positions and the fatty acid carboxyl group is linked to the 4′ position to form a macrocyclic lactone ring.

In the unusual macrocyclic lactone and the acidic glycoside, the two primary 6-hydroxyl groups are acetylated, and the lactone differs from the acid only in that the 4-hydroxyl group of sophorose ring B has been esterified by the fatty acid carboxyl group. Structure of lactone was determined by a series of oxidation studies and nuclear magnetic-resonance spectroscopy by locating the diol groups in rings A and B. The acidic glycoside, which crystallizes much less readily than the lactone, was isolated by column chromatographic separation of the mother liquors remaining after isolation of lactone. The structure of acid was established by procedures similar to those used for the lactones (Tulloch et al., 1968).

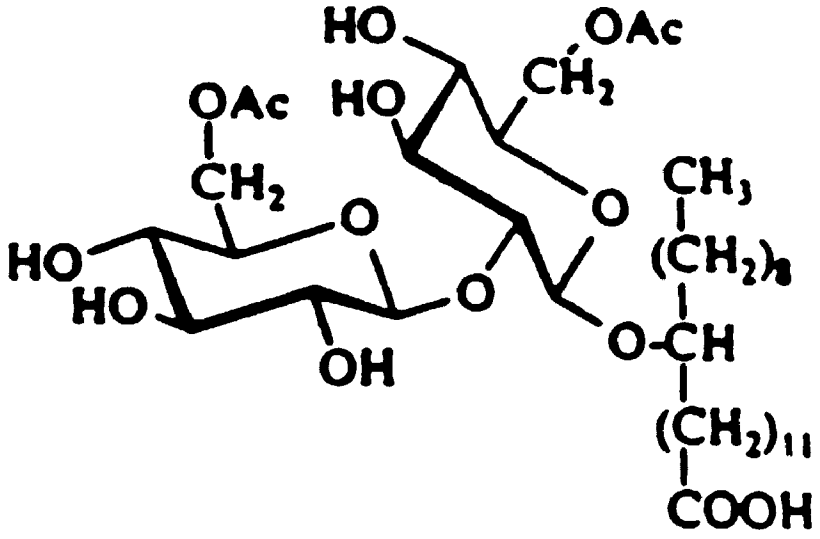
An oleyl alcohol ester of the sophoroside of 17-hydroxyoleic acid was formed by strain 319-67 of *Torulopsis bombicola* on glucose and oleyl alcohol (Tulloch and Spencer, 1972). When

yeast *Torulopsis bombicola* was incubated in the presence of different mono-, di, and trisaccharides or hydrocarbon substrates, a lactonic sophorose lipid containing a mono-unsaturated 17-hydroxyoctadecanoic acid was obtained (Gobbert et al., 1984).

It was reported that *Candida bogoriensis* produced at least three glycolipids identified as the sophoroside 13-(2′-O-B-D-glucopyranosyl-B-D-glucopyranosyloxy)docosanoic acid 6′,6′′-diacetate and the corresponding monoacetylated and unacetylated sophorosides (Tulloch et al., 1968b, Esders and Light, 1972). *Candida bogoriensis*, a basidiomycetous yeast, was isolated from leaves of an Indonesian shrub and shown to be a new species (Deinema, 1961). The structure of this glycolipid is shown in Figure 3.5. As in the structure of acidic glycoside shown in Figure 3.3, both primary sugar hydroxyl groups are acetylated, but the hydroxy fatty-acid portion is 13-hydroxydocosanoic acid, quite different from the fatty acid of the structure of acidic glycoside shown in Figure 3.3.

The structure was again determined by hydrolysis, oxidation, acetobrominolysis and nuclear magnetic resonance spectroscopy

**Figure 3.5 The Structure of Sophorose Lipids from *Candida bogoriensis* (Gorin et al., 1961)**


HO
OAc
HO
CH2
OAc
CH2
O
CH3
(CH2)8
HO
HO
O
O
O-CH
OH
(CH2)11
COOH

(Tulloch et al., 1968). The hydroxy acid of this glycolipid is unusual, since the hydroxyl group occurs near the centre of the carbon chain. Most of these compounds have the hydroxyl group or groups at the end of the chain remote from the carboxyl group, or are 2- or 3-hydroxylated. If increased yields of materials of this nature could be obtained, it would be possible to obtain a new series of unusual hydroxy fatty acids. However, efforts to date to obtain increased yields and incorporation of precursors into the product, as was done with *Torulopsis bombicola*, have been unsuccessful (Spencer et al., 1979).

## C. Properties of Sophorose Lipids

Sophorose lipids are easily hydrolyzed by alkaline or acidic solvents: the ester linkages of sophorose lipids are broken by saponification, the O-glycosidic bonds are split by acidic conditions. Other chemical reactions such as oxidations or esterifications, can easily be carried out (Lang and Wagner, 1987).

### (1) Solubility

In general nonpolar glycolipids like the nonionic trehalose-6,6'-dicorynomycolates are soluble in hydrophobic solvents like chloroform. The more polar glycolipids like anionic trehalose tetraester are soluble in $CHCl_3/CH_3OH$ mixtures. Sophorose lipids were shown to be soluble in ethyl acetate and $CHCl_3/CH_3OH$ mixtures.

(2) Surface and Interfacial Active Properties

Measurements of the surface activity of sophorose lipids were first reported by Inoue and Ito (1982). It was reported that a crude sophorolipid mixture, extracted by ethyl acetate, was able to reduce the surface tension of distilled water to under 35 dynes/cm. At pH 5.6 the critical micelle concentration was 320 mg/L. At pH 4 the critical micelle concentration of the sophorolipids was 82 mg/L, and the minimum surface tension 37 mN/m. The minimum interfacial tensions against hexadecane or any of the substrate vegetable oils were all between 1 and 2 mN/m. The surface tension and interfacial tension were insensitive to concentration of either NaCl or $CaCl_2$ up to 100g/L. The lipid mixture was not able to stabilize emulsions of water and hydrocarbon or water and vegetable oil (Cooper and Paddock, 1983).

One strain, *Torulopsis apicola IMET 43747*, was studied both on n-alkanes and on carbohydrates (Hommel et al., 1987). The product was shown to have remarkable interfacial activities and surface-tension values around 30 mN/m and interfacial tension below 1 mN/m.

It was also shown that sophorose lipids can lower the surface tension to 33 mN/m. Both lactonic and acidic sophorolipids lowered the interfacial tension between *n*-hexadecane and water from 40 to 5 mN/m and show a remarkable stability toward pH and temperature changes (Cooper and Paddock, 1984; Inoue and Itoh, 1982; Zhou et al., 1992).

In the personal care market, biosurfactants are attractive because of their low toxicity, excellent moisturizing properties, and skin compatibility. The addition-polymerized products of 1 mole of sophorolipid and 12 moles of propylene glycol have specific compatibility to the skin and have found commercial utility as skin moisturizers (Inoue, 1988).

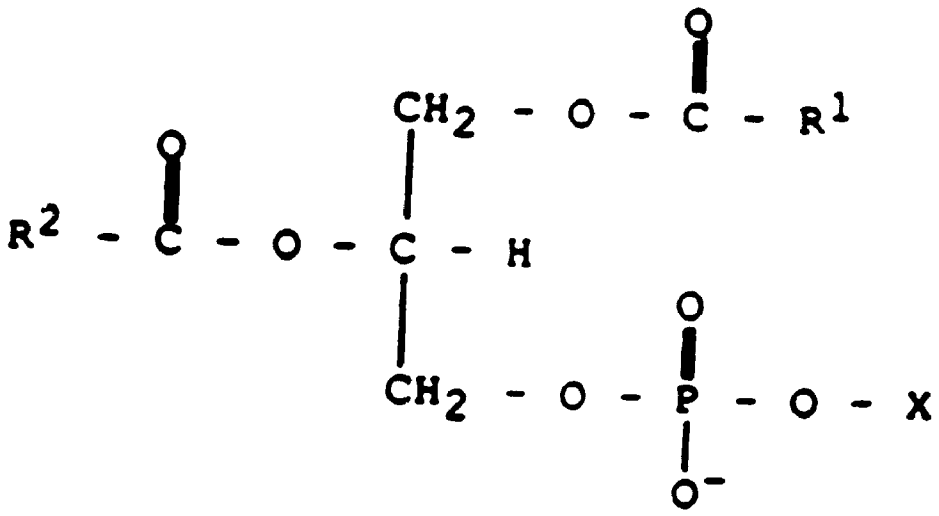
### 3.1.2 Phospholipids and Fatty Acids

Phospholipids are ubiquitous components for all living

microorganisms, as they form the major constitutent of all cell membranes-both of the outer cell envelope and of many intracellular structures, which represent between 2 and 16% of the dry weight of many microorganisms. Phospholipids and fatty acids could be produced from certain hydrocarbon-degrading bacteria and yeasts grown on *n*-alkanes (Asselineau et al., 1972; Cirigliano and Carman, 1985). These surfactants are able to produce optically clear microemulsions of alkanes in water. It was reported that phospholipids by *Thiobacillus thiooxidans* had the ability to wet elemental sulfur (Beeba and Umbreit, 1971). It was reported that phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine, and a cardiolipin-like compound could be synthesized by *Cladosporium resinae* from *n*-alkanes (Siporin and Cooney, 1978). Figure 3.6 shows the generalized phospholipid structures (Ratledge and Wilkinson, 1989; Harwood and Russell, 1984).

There are two pathways for the synthesis of phospholipids depending on whether they are in bacteria or yeasts. In bacteria, primarily phosphatidylserine and phosphatidylethanolamine are synthesized. In yeasts, diacylglycerol is a key intermediate in the synthesis of triacylglycerols or phosphatidylethanolamine. Diacylglycerol

Figure 3.6 The Structures of Generalized Phospholipid $R^1$, $R^2$ alkyl substituents are usually $C_{15}$ or $C_{17}$. X could be H, phosphatidic acid; $-CH_2CH_2NH_2$, phosphatidyl ethanolamine; $-CH_2CH(NH_2)COOH$, phosphatidyl serine; $-CH_2CH_2N(CH_3)_3$, phosphatidyl choline (lecithin); $-CHOHCHOHCH_2OH$, phosphatidyl glycerol; -inositol, phosphatidyl inositol; -phosphatidylglycerol, diphosphatidylcerol (cardiolipin) (Ratledge and Wilkinson, 1988, 1989)



may also be converted directly to phosphatidylcholine when choline is provided exogenously. Lysophospholipids, that is phospholipids without an acyl group at either the 1 or 2-position are much better surface active materials than phospholipids themselves. For example, lysolecithin is used commercially as an emulsifying agent (Hommel and Ratledge, 1992).

Various reports demonstrate the ability of different microorganisms growing on alkanes or other water-insoluble substrates to synthesize large amounts of extracellular free fatty acids. The saturated fatty acids in the range of $C_{12}$ to $C_{14}$ and the complex fatty acids containing hydroxyl groups and alkyl branches are suitable as biosurfactants (Kretschmer et al., 1982; MacDonald et al., 1981).

### 3.1.3 Peptides and Amino Acid Containing Lipids

*Bacillus subtilis* produces surfactin, a cyclic lipopeptide with exceptional surface activity which can lower the surface tension from 72 to 27 mN/m and the interfacial tension against hexadecane to less than 1 mN/m (Arima et al., 1968). It was reported that surfactin inhibited the formation of fibrin

clots and lysed erythrocytes and several bacterial spheroplasts and protoplasts. The lipopeptide contains a carboxylic acid (3-hydroxy-13-methyl tetradecanoic acid) and seven amino acids (Glu-Leu-Val-Asp-Leu-Leu). A yield of surfactin of up to 0.8 g/L was obtained in a batch cultivation by continuously removing the product by foam fractionation. Biosurfactants with similar structural and physicochemical properties to surfactin have been isolated from *B. subtilis* QMB (Berheimer and Avigad, 1970) and *B. licheniformis* JF2 (Javaheri et al., 1985). *B. licheniformis* 86 produced a biosurfactant which lowered surface tension of water to 27 dynes/cm and interfacial tension between water and n-hexadecane to 0.36 dynes/cm (Horowitz et al., 1990). The surfactant was stable at a wide range of pH, temperatures, and NaCl concentrations of solution (Horowitz et al., 1990) and promoted dispersion of colloidal 3-silicon carbide and aluminum nitride slurries (Horowitz and Currie, 1990).

It was reported that decapeptide antibiotics (Gramicidins) produced by *Bacillus brevis* (Marahiel et al., 1979) and lipopetide antibiotics (Polymyxins) by *B. polymyxa* (Suzuki et al., 1965), possess remarkable surface-active properties. Also, some peptide-containing lipids were found to exhibit

biosurfactant activity including ornithine-containing lipids from *P. rubescens* (Wilkinson, 1972) and *T. thiooxydans* (Knoche and Shively, 1972), cerilipin, and ornithine- and taurine-containing lipid from *Gluconobacter cerinus* IFO 3267 (Tahara et al., 1976), and lysine-containing lipids from *Agro. tumefaciens* IFO 3058 (Tahara et al., 1976) and *Str. sioyaensis* (Kawanami et al., 1968).

The spectrum of lipopeptides produced by *Bacillus spp.* is broad, but only a few have been reported in relation to their surface activity. Most of them are known because of their antibiotic properties. The bifunctional properties exhibited by lipopeptides (surface and antibiotic activity) are equally well known for many other biosurfactants, such as rhamnolipids from *Pseudomonas* as well as glycolipids of *Torulopsis apicola*. Within the peptide antibiotic group, the lipopeptides of *B. licheniformis* show analogous structures as representatives of the iturin group (iturin, mycosubtilin, bacillomycin) or of the lactone group (esperin, surfactin, polypeptin). Most of them show similarities in the hydrophilic part, consisting of a seven-amino acid cyclic peptide which is linked by a hydroxy or an ester peptide linkage to the fatty acid part.

### 3.1.4 Polymeric Biosurfactants

Biopolymers with high molecular weight generally exhibit many different properties as compared to low molecular weight biosurfactants such as high viscosity, tensile strength, and resistance to shear, which enables the polymeric biosurfactants to have a variety of industrial uses. Polysaccharide emulsifiers usually do not lower interfacial tension much below 10 dynes/cm. However, their preference for the interface makes them excellent candidates for emulsion stabilizers. The best studied of these biosurfactants are emulsan, liposan, mannoprotein, and other polysaccharide-protein complexes (Panchal et al., 1979; Kosaric et al., 1987).

#### 3.1.4.1 Emulsan

The best studied of these biopolymers is emulsan, the bioemulsifier produced by the oil-degrading bacterium *Acinetobacter colcoaceticus* RAG-1 (Rosenberg et al., 1979). It is an extracellular lipoheteropolysaccharide polyanionic bioemulsifier and is both a cell-associated capsule and a cell-free product. Emulsan with the mean molecular weight of

$9.9 \times 10^5$, has been characterized as a polyanionic amphipathic heteropolysaccharide (Rosenberg et al., 1979). The heteropolysaccharide backbone contains repeating trisaccharide of N-acetyl-D- galactosamine, N-acetylgalactosamine uronic acid, and an unidentified N-acetyl amino sugar (Zukerberg et al., 1979). In addition, fatty acids, which constitute about 10%-15% of the dry weight are shown to be covalently linked to the polysaccharide through O-ester linkages (Zukerberg et al., 1979; Shabtai and Gutnick, 1985). Emulsan does not appreciably reduce the interfacial tension, but it is a very effective emulsifying agent for hydrocarbons in water even at a concentration as low as 0.001%-0.01%. It is one of the most powerful emulsion stabilizers known today and resists inversion even at a water-to-oil ratio of 1:4 (Gutnick and Shabtai, 1987).

Emulsan has been commercialized for enhanced oil recovery and for transportation of crude oil. It is very effective in reducing the interfacial tension between oil and water *in-situ*, reducing the viscosity of the oil by removing water from emulsions prior to processing and releasing bitumen from tar sands. Emulsan which can stabilize emulsion, is separated into two layers when it is centrifuged. Emulsanosol which

represents an upper cream layer, contains approximately 70%-75% oil in the bulk aqueous phase. Emulsanosols can remain stable for months and can withstand enormous shear without any inversion (Zosim et al., 1986). Interestingly, addition of small amounts of alkanols or alcohol derivatives has been shown to alter the hydrocarbon substrate specificity of emulsan (Zosim et al., 1986). The enzyme responsible for the depolymerization of emulsan has been isolated and has been found to act by transelimination (Shoham et al., 1983).

### 3.1.4.2 Biodispersan

Biodispersan is an extracellular, nondialyzable dispersing agent synthesized by *A. calcoaceticus* A2 (Rosenberg et al., 1988). An anionic heteropolysaccharide is the active component of biodispersan, which has an average molecular weight of 51,400 and four reducing sugars, namely, glucosamine, 6-methyl aminohexose, galactosamine uronic acid, and an unidentified amino sugar (Rosenberg et al., 1988).

### 3.1.4.3 Liposan

*C. lipolytica* produces liposan, which is an extracellular water-soluble emulsifier grown only on hydrocarbons (Käppeli and Fiechter, 1977; Cirigliano and Carman, 1984). Recently, the structure of liposan was elucidated (Cirigliano and Carman, 1985). It showed that liposan contains 83% carbohydrate and 17% protein. No fatty acids were detected in liposan. The preparation was capable of emulsifying a wide range of hydrophobic materials including oils, alkanes, and aromatics. The carbohydrate portion is a heteropolysaccharide consisting of glucose, galactose, galactosamine, and galacturonic acid. The partially purified liposan stabilized the emulsion formed from many commercial vegetable oils and water (Cirigliano and Carman, 1985).

### 3.1.5 Other Polysaccharide Protein Complexes

It was found that *A. calcoaceticus* BD. and its mutant minicapsulated derivative *A. calcoaceticus* BD413 (Sar and Rosenberg, 1983) grow on hydrocarbons and produce extracellular emulsifiers. Both strains produced extracellular polysaccharides containing a molar ratio of rhamnose to

glucose of 3:1, which was similar in composition to the cell-bound capsular material. When the capsular polysaccharide of BD4 is released as an extracellular polymer during growth, the product is active as an emulsifier. However, when the capsule is mechanically removed from the cell surface by shearing, the polysaccharide is not active as an emulsifier).

A benthic cyanobacterium *Phormidium* J-1 has been shown to produce an extracellular emulsifier termed Emulcyan. This material accumulated in the medium during the stationary phase of growth. Emulcyan, which appeared to have a moelcular weight greater than 100, 000, was found to bring about the detachment of *Phormidium* from hexadecane droplets or phenyl sepharose beads (Desai and Desai, 1992).

It was reported that high amounts of mannoprotein emulsifier were produced by *S. cerevisiae*. The purified emulsifier contains 44% carbohydrate (mannose) and 17% protein, which emulsifies many oils, alkanes, and organic solvents, and the emulsions are stable at extreme pH, temperatures, and salt concentrations (Cameron et al., 1985).

### 3.1.6 Particulate Biosurfactants

Extracellular emulsifier production is relatively widespread in the genus *Acinetobacter*. Extracellular membrane vesicles have 20-50 nm diameters and a buoyant density of 1.158 g/cm$^3$ by *Acinetobacter* sp. HO1-N cells (Käppeli and Fiechter, 1979). The vesicles partition hydrocarbons in the form of microemulsion and play an important role in the alkane uptake by the cells. The purified vesicles contain a phospholipid at a concentration five times higher and a 360-fold higher polysaccharide content as compared to its concentration in the outer membrane of the same organism.

It was shown that most hydrocarbon degraders such as strains of cyanobacteria, and pathogenic bacteria possess surfactant activity (Rosenberg, 1986). The surface components that contribute to the surfactant activity include M-protein and lipoteichoic acid on *Streptococci* group-A, protein-A of *Staphylococcus aureus*, layer-A of *Aeromonas salmonicids*, prodigiosin of *Sarratia* spp., gramicidins in *B. brevis* spores, and thin fimbriae in *A. calcoaceticus* RAG-1 (Rosenberg, 1986; Zajic and Seffens, 1984).

## 3.2 Biosynthesis of Biosurfactants

### 3.2.1 General Features of Biosynthesis

Biosurfactants reported in literature are nonionic or anionic. Only a few are cationic biosurfactants (those containing amine functions). Biosurfactants can be synthesized according to the following schemes (Syldatk and Wagner, 1987):

1. *De novo* synthesis of hydrophilic and hydrophobic moieties by two independent pathways followed by their linkage to form a complete biosurfactant molecule.

2. *De novo* synthesis of the hydrophilic moiety and the substrate-dependent synthesis of the hydrophobic moiety and its linkage.

3. *De novo* synthesis of the hydrophobic moiety and the substrate-dependent synthesis of the hydrophilic moiety followed by its linkage.

It is also possible that the syntheses of both hydrophobic and hydrophilic moieties depend on the substrates. It was found

that in the case of herbicollin A. both lipid and peptide moieties were synthesized from carbohydrates. Yield and structure of the biosurfactants were affected by addition of amino acids or fatty acids (Greiner and Winkelman, 1991). Hydrophilic moieties of biosurfactants involve a number of biosynthetic pathways and show a greater degree of complexity. The carbon substrate and the cultivation conditions have a great influence on the composition of biosurfactant by *Pseudomonas* spp.. However the hydrocarbon substrate of a different chain length has no effect on the chain length of the fatty acid moiety of glycolipids (Syldatk et al., 1985). A similar result in *P. aeruginosa* has been observed (Edmonds and Coonay, 1969). Sophorose lipids by *T. bombicola* and cellobiose lipid by *U. zeae* are examples of *de novo* biosynthesis. The biosynthesis of trehalose mono- and dicorynomycolates by *R. erythropolis* is *de novo* synthesis of the sugar moiety and the substrate-dependent synthesis of the lipid moiety. Glycolipids by *A. paraffenium* depend on the sugar substrates. When grown on fructose, fructolipids are obtained (Itoh and Suzuki, 1974) and when grown on sucrose and glucose, sucrose lipids are obtained (Suzuki et al., 1974).

### 3.2.2 Regulation by Catabolic Repression

Catabolic repression of biosurfactant synthesis by glucose or primary metabolites is one of the important regulatory mechanisms in microorganisms. The first enzyme in the oxidation of *n*-alkane has been found to be catabolically repressed (Dalhoff and Rehm, 1979). *Actinobacter catcoaceticus* (Neufeld et al., 1983) and *A. paraffenium* (Duvnjak et al., 1982) fail to synthesize surface-active compounds when grown on organic acids and glucose, respectively. It was found that a factor responsible for *n*-alkane oxidation in *P. aeruginosa* was biosynthesized during the growth on hydrocarbons and not on glucose, glycerol, or palmitic acid as the substrates (Hauser and Karnovsky, 1958). Rhamnolipid synthesis decreased drastically with the addition of glucose, acetate, and tricarboxylic acids to the growth medium containing glycerol. A similar observation was noted for liposan synthesis in *C. lipolytica* (Cirigliano and Carman, 1984). However, *B. subtilis* can utilize glucose to produce surfactin which was inhibited by the addition of hydrocarbons in the medium (Cooper et al., 1981). When grown on glucose, *P. aeruginosay* could produce a rhamnolipid. However, *P. aeruginosay* did not grow on *n*-alkanes (Parra et al., 1989). When *B. subtilis* (Suf-1) grew on

glucose, higher production of biosurfactant was obtained (Mulligan et al., 1989).

### 3.2.3 Effects of Substrate Sources

#### 3.2.3.1 Nitrogen Sources

After the carbon sources, the sources of nitrogen supplied to a culture are probably next in importance in determining how the cell regulates its metabolic machinery. The nitrogen requirements for optimal production of biosurfactants are species-dependent. They may be fulfilled by either organic nitrogen sources or inorganic nitrogen sources. Organic nitrogen sources include corn steep liquor, peptones, fish meal, corn germ or gluten meal, urea, yeast, or yeast hydrolysates. Inorganic nitrogen sources include gaseous ammonia, ammonium hydroxide, ammonium sulfate, ammonium nitrate, and so on. Amino acids, purines, pyrmidines, proteins, DNA, and RNA are also sources of nitrogen.

Yeast extract, corn steep liquor, and peptones (from casein or meat) are good sources of free amino acids (lysine, leucine, isoleucine, valine, threonine, and phenylalanine),

nucleotides, and vitamins. Corn steep liquor is the least expensive source, because it is a by-product in the production of starch and sugar syrups from corn.

The ratio of carbon to nitrogen as well as nitrogen source itself are also very important in biosurfactant production during the middle of microbial growth and stationary phases. The highest rhamnose lipid yields by *P. aeruginosa* were obtained when nitrate rather than ammonium was used. The optimal yields of biosurfactant by *Nocardia erythropolis* occurred when 0.02% yeast extract was supplied to the medium. Ustilagic acid production by *Ustilago zeae* was optimized at 0.06% corn steep liquor (Syldalk and Wagner, 1987). Among the inorganic salts, ammonium salts and urea are preferred nitrogen sources for biosurfactant production by *A. paraffenium* (Duvnjuk et al., 1983). It was also found that ammonium nitrate was a better nitrogen source than ammonium chloride or sodium nitrate for surfactin production by *B. subtilis* (de Roubin et al., 1989). Increase in concentration of the ammonium nitrate from 0.4% to 0.8% results in an increase in the surfactin. Yeast extract is a very good nitrogen source for sophorose lipids production by *T. bombicola* (Cooper and Paddock, 1984; Zhou et al., 1992) but

was very poor for *P. aeruginosa* (Guerra-Santos et al., 1984).

The limitation of nitrogen sources results in the overproduction of biosurfactants. The enzyme activities for synthesis of biosurfactants are switched on as nitrogen becomes limiting. Overproduction of rhamnolipid by *P. aeruginosa* occurred under nitrogen limitation (Syldalk and Wagner, 1987). It was found that the production of glycolipid by *Nocardia* SFC-D started when nitrogen was exhaused. Similar observations were obtained from the biosurfactant production by *U. maydis,* and *N. erythropolis* (Brennan et al., 1970). Nitrogen limitation not only resulted in the overproduction of biosurfactant but it also altered the composition of the biosurfactant (Syldatk et al., 1985a). Growth of *N. erythropolis* under normal conditions produces only nonionic trehalose corynomycolates, whereas under nitrogen limitations it produced only anionic trehalose tetraesters.

### 3.2.3.2 Nutrient Sources

Limitation of multivalent cations also results in overproduction of biosurfactant in *Pseudomonas* spp. (Itoh and Suzuki, 1972). It was shown that limitation of magnesium,

calcium, potassium, sodium, and trace elements resulted in overproduction of rhamnolipid by *P. aeruginosa* DSM 2659 (Guerra-Santos et al., 1986). Iron limitation is known to stimulate the biosurfactant production by *P. fluorescens* (Rubinovitz et al., 1982). Conversely, production of surfactin by *B. subtilis* (Cooper et al., 1981) was stimulated by the addition of iron and manganese to the growth medium.

Phosphate limitation is another way to influence the metabolism of *P. aeruginosa* (Mulligan et al., 1989). In an inorganic phosphate-limited medium of proteose peptone/glucose/ammonium salts, biosurfactants production was induced upon phosphate depletion. The change in activity of several intracellular enzymes (alkaline phosphatase, glucose-6-phosphate dehydrogenase, and transhydrogenase) that are also dependent on phosphate levels indicated a shift in metabolism as biosurfactant production occurred. Thus, regulating phosphate levels is a means of enhancing rhamnolipid production.

## 3.3 Production of Biosurfactants by Resting Cells

Glycolipids are usually produced at the end of the logarithmic

phase and during the stationary phase. They are considered as secondary metabolites. The biosurfactants can be produced by resting cells. The cells used are harvested from the surfactant-producing state and maintained in the same state. The resting cells do not multiply but continue to utilize the carbon source for the synthesis of biosurfactants. Examples of production of biosurfactants by resting cells include sophorose lipids by *T. bombicola*, cellobiose lipids by *U. maydis* and rhamnolipid by *Pseudomonas* sp..

### 3.3.1 Sophorose Lipids by Resting *T. bombicola* Cells

It was reported by Gobbert et al. (1984) that resting *T. bombicola* cells could be used for production of sophorose lipids. A further intention was to replace either the sophorose or the fatty acid unit by another carbohydrate or lipophilic moiety. However, the experiments indicated the presence of the same molecular structure which is a sophorose, acetylated in position 6′ and 6′′ and linked to 17-hydroxy-octadecenoic acid.

### 3.3.2 Cellobiose Lipids by Resting *U. maydis* Cells

A modified mineral salts medium without nitrogen source was found to enhance the production of cellobiose lipids by resting cells of *U. maydis* from renewable resources (Frautz et al., 1986). The production of cellobiose lipids depended on various carbon substrates. The highest production of 0.71 g/g cellobiose lipids was obtained from coconut oil at concentration of 2%.

### 3.3.3 Rhamnose Lipids by Resting *Pseudomonas* sp. Cells

It was reported that rhamnolipids could be produced by resting free and immobilized cells of *Pseudomonas* sp. DSM-2874. Immobilizing the resting cells of *Pseudomonas* sp. DSM-2874 in hydrophilic polymer, better stability of the cells has been demonstrated (Syldalk and Wagner, 1987) and production of rhamnolipid has been increased by 5- to 6-fold. The optimal production of rhamnolipids from *n*-alkanes by resting cells was obtained at pH 6.6 and 37°C (Syldatk et al., 1985b). Two more rhamnolipids were synthesized by resting cells. Ramana and Karanth (1989) reported a twofold increase in rhamnolipid production using resting cells of *P. aeruginosa* CFTR-6 in the

medium without phosphate.

## 3.4 Production of Biosurfactants

The largest group of microbial surface active compounds characterized to date are glycolipids. The hydrophilic moieties of these compounds are mono- and disaccharides. Glycolipids can be further subdivided on the basis of the type of the saccharide within their molecules.

### 3.4.1 Trehalose Lipids

*Rhodococcus erythropolis* is known to produce lipid derivatives of trehalose. Depending on the particular strain, the mixture of trehalose mono- and dicorynomycolates (Kretschmer et al., 1982) or the mixture of mono- and disuccinoyl dialkanoyl trehalose is produced (Uchida et al., 1989). The latter seems to possess properties as surfactants (Ishigami et al., 1987). Moreover, a yield of approximately 40 g/L was achieved when *Phodococcus erythropolis* SD-74 was grown on *n*-hexadecane in a high osmotic equivalent culture broth (Uchida et al., 1989).

### 3.4.2 Rhamnose Lipids

Rhamnose lipids were first isolated and reported by Edwards and Hayashi (1965) and subsequently shown to be produced by two species namely *Pseudomonas fluorescens* and *P. aeruginosa* when grown on n-alkanes, glucose, or glycerol (Itoh et al., 1971; Yamaguchi et al., 1976). The lipids are produced in considerable amounts (0.18 g/g n-C-14,15-alkanes) during the stationary phase (Syldatk et al., 1984). Experiments on medium optimization for the growth of *Pseudomonas* sp. indicated that the overproduction of rhamnolipids is caused either by limitation of nitrogen or by multivalent cations. Both the growth and the product kinetics indicated that rhamnose lipids are not formed in substantial amount as long as ammonium ions are presented in the media. In contrast, when ammonia becomes limited, the *n*-alkane consumption and the production of rhamnolipid increases up to 140 h. A further increase in rhamnolipid production was obtained by limitation of Fe, Mg, or Ca cations, which are all essential for growth of *Pseudomonas* sp. (Syldatk et al., 1985a; Guerra-Santos et al., 1984). Reiling's group (1986) developed a continuous process by culturing *P. aeruginosa* DSM 2659 on glucose. The main characteristics of the media were carbon and phosphorus excess

as well as nitrogen and iron limitation. The biosurfactant yield on glucose was 0.077 g/g h, and the productivity was 0.147 g/L h (Guerra-Santos et al., 1984; Reiling et al., 1986).

Although *P. aeruginosa* 44T1 is able to grow and produce rhamnolipids from glycerol, mannitol, and glucose, olive oil supported the highest amount of growth and biosurfactant production (Robert et al., 1989).

### 3.4.3 Sophorose Lipids

Sophorose lipids, produced by yeasts of the genus *Torulopsis*, e.g., *T. magnoliae, T. bombicola* (now belonging to the genus *Candida*) were first reported by Gorin and his collaborators in 1961 (Gorin et al., 1961). The discovery of this compound evoked great interest when its biosurfactant activity was noted. Originally, it was assumed that yeast lipids consisted mainly of triglycerides, phospholipids and sterols. However, the presence of sphingolipids, which in yeasts are derivatives from phytosphingosines and hydrocarbons (Baraud et al., 1967), polyprenols and other intracellular non-glycerides and non-sterols has been detected. Bacterial-type glycolipids

(glycosyl-diglycerides) were also reported (Spencer et al., 1979).

Production of sophorose lipids was first reported using media containing 20% glucose, 1.25% yeast extract and 0.2% of urea agitated at 385 r.p.m. for 8-10 days with an air flow rate of 500 ml/min, and incubated at 30°C in 5-l New Brunswick fermenter (3-l working volume). The maximum yield of the oil was about 5% of the volume of the medium and was recovered by allowing it to stand in a separatory funnel until it all settled out (Gorin et al., 1961).

The yield of the sophorose lipids was increased three- to five-fold by the addition of suitable compounds to the growing culture. The supplement, which can be a long-chain acid, ester, hydrocarbon, or glyceride is hydroxylated and converted to hydroxy fatty acid sophorosides. When fatty acid esters with $C_{16}$ -$C_{19}$ chain length were used, the precursors were incorporated directly into the product without alteration of length or structure of the carbon chain. Also in case of straight-chain alkanes (Tulloch et al., 1962), branched-chain alkanes (Jones, 1968), methyl- or hydroxy-modified, within limits, by the addition of fatty acid esters, hydrocarbons, or

glycerides (Spencer et al., 1962).

*Torulopsis* species are capable of growing and producing sophorolipids on simple mineral media containing glucose as the only carbon source and yeast extract as nitrogen source.

Optimization of the medium formulation has usually been performed by changing nutritional parameters, individually or in combination. The most frequently used carbon source is glucose. Studies in several laboratories proved that glucose, or substrates which produce glucose after hydrolysis, are a satisfactory carbon sources for sophorolipids production from *Torulopsis* yeasts (Gorin et al., 1961; Tulloch et al., 1968; Inoue and Itoh, 1982; Cooper and Paddock, 1984). High concentrations of glucose are required for maximal sophorolipids production (Cooper and Paddock, 1984).

Fatty esters of all chain lengths from $C_{16}$ to $C_{22}$, including several unsaturated esters, and even-numbered hydrocarbons from $C_{16}$ to $C_{24}$ were readily fermented. Shorter-chain compounds were used poorly or not at all. With compounds of 16 to 18 carbon atoms, hydroxylation occurs at the terminal or penultimate carbon atom, depending on the degree of

unsaturation and chain length. Substrates of more than 18 carbon atoms were mainly reduced in chain length by one or more carbon units and hydroxylated, giving rise to $C_{17}$ or $C_{18}$ acids with the hydroxyl group on the penultimate carbon atom.

Esters and hydrocarbons, containing 14 and 15 carbon atoms, are converted to the hydroxy fatty acid portions of glycosides by *Torulopsis apicola* in yields of 10-20%. When C-15 compounds are fermented, almost half of the hydroxy acids which are produced are 16-hydroxy C-17 acids. The carbon chain of the substrate is first lengthened by two carbon atoms and then hydroxylated. Direct hydroxylation also occurs, to a lesser extent, giving both 14-hydroxy-and 15-hydroxypentadecanoic acids. Similar results are obtained when C-14 compounds are used. Lengthening of the chain followed by hydroxylation gives rise to hydroxy C-16 acids and direct hydroxylation produces 13-hydroxy-and 14-hydroxytetradecanoic acids. Primary and secondary C-14 and C-15 alcohols were also isolated from the products of hydrocarbon fermentation (2.5-5% yield). Methyl palmitoleate is converted to hydroxy fatty acids in yields of 40-70%, the major component of which is 16-hydroxy-cis-9-hexadecenoic acid (Tulloch et al., 1968).

The degree of ω-hydroxylation increased as the length of the substrate molecule decreased. ω-Hydroxylation does not occur with stearic acid (25.05 $A^0$), but with the shorter oleic (24.22 $A^0$) and heptadecanoic acids (23.80 $A^0$) it occurs to a small extent (13%). Elaidic acid (24.91 $A^0$), very close to stearic acid in length, is also not terminally hydroxylated. With linoleic (23.31 $A^0$) and palmitic (22.55 $A^0$) acids, which are both shorter , considerable ω-hydroxylation occurs but to a lesser extent with palmitic than with linoleic acid, suggesting that palmitic acid is shorter than the most favorable length for ω-hydroxylation. Pentadecanoic and myristic acids are apparently too short for appreciable hydroxylation of any type to occur. Since linolenic acid (22.51 $A^0$) is about the same length as palmitic acid it is probable that it is not hydroxylated readily because the 15,16-double bond is too close to the site of the reaction. Also, 12-hydroxyoctadecanoic acid probably does not react easily because the hydroxyl group is too near to the reaction site. However, substituents in the 10- and 8-positions appear to be too far away to interfere (Spencer et al., 1979).

## 3.5 Isolation and Purification of Biosurfactants

There are many methods applicable for the isolation of lipids. The method used for the isolation of certain product depends on the nature of this compound (whether it is water-soluble or not, anionic or noniogenic, cell wall-bound or extracellular). Most of the lipids can be isolated by "classic" methods such as extraction, precipitation, or crystallization, after separating the cells by centrifugation. Most biosurfactants are secreted into the medium, and they are isolated from either culture filtrate or supernatant obtained after removal of the cells.

Solvent extraction is the most commonly used technique for the recovery of biosurfactants (Cooper and Zajic, 1980). Solvents used for this purpose include ethyl acetate, ether, pentane, hexane, butanol, chloroform-methanol, or dichloromethane-methanol. After solvent removal by rotary evaporation, the crude product can be purified by column chromatography, preparative thin-layer chromatography, and crystallization.

The examples of biosurfactant recovery by solvent extraction

procedure are the recovery of sophorose lipids from the culture broth (Cooper and Paddock, 1984; Gobbert et al., 1984; Zhou et al., 1992), the recovery of rhamnolipids from the culture broth (Yamaguchi et al., 1976; Hisatsuka et al., 1981; Itoh and Suzuki, 1974), and the recovery of trehalose lipids from the culture broth (Suzuki et al., 1974).

The sophorose lipids can be recovered from the fermentation broth by gravity separation since they are denser than the broth. Contamination by medium components is minimal. Recovery costs are reduced by not separating the individual surfactants. This approach is acceptable since many synthetic surfactants are sold as mixtures. More complete recovery of sophorose lipids can be performed by addition of charcoal to the cell-free supernatant and by mixing. Charcoal is removed by filtration and the lipid is desorbed from the dried charcoal by chilled ethyl acetate. Further purification, if desired, can be achieved by liquid chromatography on silica gel (Stuwer et al., 1987).

Ustilagic acid by *Ustilago zeae,* mannosylerythritol lipids by a *Candida* species and sophorose lipids by *Torulopsis* species can be settled down as heavy oil on centrifugation. The

impurities of these biosurfactants can be removed by addition of ethanol or methanol.

It is possible to recover biosurfactants by precipitation. Some of the classic uses of ammonium sulfate precipitation include the isolation of emulsan (Rosenberg et al., 1979; Shabtai and Gutnick, 1985) and biodispersan (Rosenberg et al., 1988). Other precipitation techniques including acetone and acid precipitation have been used for the isolation of biosurfactants.

"In situ recovery" can be used for a continuous biosurfactant recovery during microbial cultivation. Surfactin by *Bacillus subtilis* (Cooper et al., 1981) and biosurfactants by *Clostridium pasteurianum* (Cooper and Zajic, 1980) can be isolated by flotation and foam fractionation. The foam, which contains the products, is collapsed after flotation by acid precipitation and extracted with different solvents. A lipopeptide surfactant by *Candida petrophilum* (Iguchi et al., 1969) or rhamnolipids by *Pseudomonas* sp. DSM 2874 (Syldatk et al., 1984) can be recovered by "in situ recovery" which is the adsorption of biosurfactants to ion exchange resins or other suitable adsorbents. Another example of "in situ

recovery" is the in-situ extraction of cell wall-bound compounds (Duvnjak and Kosaric, 1981). Biosurfactants can be isolated from the cells in 2-4 hr by addition of dodecene or hexadecene to the culture medium. In the future, "in situ recovery" will probably become the main method for the recovery of biosurfactants because the method have several advantages such as avoiding end-product inhibition, minimizing product degradation, reducing wastewater treatment costs and increasing productivity of biosurfactants.

## 3.6 Application of Biosurfactants

Interest in biosurfactants are increasing for industrial applications because they are biodegradable, often nontoxic, and can be produced by microbial processes, in which simple substrates such as n-alkanes, vegetable oils, and carbohydrates or even industrial waste products can serve as substrates. While many biosurfactants show good physicochemical properties such as low critical micelle concentrations in the range of a few mg/L, reduction of the interfacial tension between water and hydrocarbons to less than 1 mN/m even in high-salinity solutions, and temperature stability in a wide temperature range, their production costs

are often the limiting factor which prevents their commercial use.

Several industrial applications of biosurfactants have been envisioned. Biosurfactants have shown particular promise in the clean-up and dispersion of oil spills. It has been suggested that a biosurfactant enhances the metabolism of the oil by either acting as a growth stimulant, or an emulsifier. As an emulsifying agent, the emulsifier disperses the oil and promotes the accessibility of the oil substrate to the microoganism. Biosurfactants have also shown potential in promoting the release of bitumen from tar sands. Hayes et al. (1986) have demonstrated the emulsification of Boscan, Venezuelan heavy crude oil, using emulsan in which the viscosity of oil is reduced from 2,000,000 to 100 centipcise. Furthermore, they showed that by this treatment it is feasible to pump this oil to a distance comparable to 26,000 miles in a commercial pipeline.

In recent years, the use of biosurfactants in cosmetics became very attractive because of their low level of irritation, antimicrobial activity, and good foaming properties. A large number of new biosurfactants have been commercially applied in

all types of cosmetic products where they could function as emulsifiers, solubilizers, foaming agents, wetting agents, and detergents. The successful application of sophorose lipids and their acylated and alkoxylated derivatives in cosmetics could make them competitive alternatives over synthetic surfactants because of their low irritancy or antiirritating effects and compatibility with skin, both properties demanded by today's market. Kao Co in Japan developed a new cosmetic product of 1 mole of sophorolipids and 12 moles of propylene oxide which were shown to have specific compatibility to the skin and found commercial utility as skin moisturizers (Inoue, 1988). Several applications of esters and hydroxylakyl ethers of sophorose lipids in cosmetics were suggested, such as lip stick makeups (Kono et al., 1980) and especially moisturizers such as octyl, lauryl, and oleyl esters or propolylated derivatives for skin and hair care products (Abe et al., 1980).

Biosurfactants have also been used commercially as an additive to dewater fuel grade peat. The clean-up of a natural site contaminated with petroleum has been accomplished using biosurfactants or biosurfactant-producing microbes. Applications are also being considered in industries

involving pulp and paper, coal, textile, and uranium ore processing and ceramic processing (Kosaric, 1992).

In food industry, biosurfactants are attractive since lecithin is already the largest used food emulsifier. In principle, biosurfactants vary from industrial surfactants only by their origin. The function of biosurfactants is to partition on interfaces of immiscible phases and lower the interfacial tension. The food industry does not yet use biosurfactants as food additives on a large scale as many biosurfactant properties and regulations regarding the approval of new food ingredients have to be solved. Elaborate testing and evaluation of any new food ingredient is required according to the U.S. Food and Drug Administration regulations, and this process can be quite long and expensive. A prerequisite for the use of new substances as additives in food is to make sure that no toxic intermediates or products of their metabolism appear in the organism during degradation or excretion. Sophorose lipids have a great potential for the application in food industry because of their lower toxicity. Similar products produced from sucrose and fatty acids have been known and used for a long time (Ohata and Kamata, 1986). Besides, sophorose [or cyclic (1→2)-β-D-glucan] is already being used

as a sweetening agent (Kitahata et al., 1988). In Japan, sophorose lipids are patented (Ohata and Kamata, 1986) as flour additives for quality improvement and better shelf life of bakery goods (e.g., 0.1 parts of sophorolipids and/or their lactones to 70 parts of flour).

Every time we breathe out, our lungs are prevented from collapsing by a unique lipoprotein mixture-pulmonary surfactant. This lipoprotein is absorbed at the alveolar air/liquid interface where it lowers the surface tension and, therefore, reduces the contractile force at the surface and the work of lung expansion. Collapse is prevented by the formation of a solid film on the alveolar surface during expiration. A dramatic example of its importance is provided by the disease *acute respiratory distress of the newborn*. In this condition, premature infants who have not yet begun to synthesize pulmonary surfactants are unable to expand their lungs properly and suffer from various complications which, even with the best modern treatment, result in death for over 25% of affected individuals (Gurr and Harwood, 1991).

Pulmonary surfactant can be isolated by carefully washing out lungs repeatedly with isotonic saline. Such material only

represents a proportion of the total lung surfactant. Pulmonary surfactants are lipid-rich lipoproteins which are dominated by dipalmitoylphosphatidylcholine. Thus, pulmonary surfactant has a composition particularly suited to the rapid formation of stable lamellar structures. The small amounts of unsaturated phosphatidylcholine believed to help in the rapid spreading of surfactant at body temperatures and phosphatidylglycerol may assist in the morphological dissolution of lamellar bodies in the aqueous sub-phase to provide a constant source for renewal of the surface monolayer. The proteins of surfactant are also thought to be important in its function. Two types are found generally: the so-called surfactant apoprotein which is a glycoprotein present in several species with varying amounts of glycosylation giving molecular weights in the 28-35 kDa range. This protein is thought to be involved in the control of surfactant secretion from its site of synthesis in the alveolar type II cells. In addition, a low molecular weight protein of about 10 kDa is present. Although the function of this protein has not been demonstrated convincingly, it is believed to help in the surface properties of the surfactant. The type II cells which are present at the alveolar surface are the exclusive site of surfactant synthesis. In these cells

the necessary proteins and lipids are made on the endoplasmic reticulum from where they are assembled into lamellar bodies. Surfactant is then released from the latter by exocytosis. The use of bovine-based surfactant replacement therapy using preparations derived from organic solvent extracts of bovine surfactant becomes a very promising treatment for the disease of *acute respiratory distress of the newborn*. Surfactant lipid extracts containing the two hydrophobic peptides as the sole apoproteins have been used for therapy in hyaline membrane disease in newborn babies (Gurr and Hardwood, 1991). The human gene for production of the protein molecule of this surfactant has been isolated and cloned in bacteria, which opened up the possibility of larger-scale production of this surfactant for medical application (While et al., 1985). Antibiotic effects of biosurfactants and the inhibitory effect toward the growth of AIDS virus in WBC have been reported recently (Desai and Desai et al., 1992).

## 3.7 Safety of Biosurfactants

Biosurfactants have a special advantage over chemical surfactants such as biodegradability and low toxicity. Zajic and Gerson (1978) tested crude biosurfactants for toxicity

using *Daphnia magna*. The results showed that biosurfactants were sources of nutrition to the *Daphnia* and the death rate of *Daphnia* with biosurfactants decreased. The brine shrimp *Corophium volutator* was found more sensitive to chemical surfactants. From the bioluminescence inhibition test, it was conluded that biosurfactants were less toxic than the synthetic surfactants (Lang and Wagner, 1992). The results of acute (Ikeda et al., 1986a) and subacute (Ikeda et al., 1986b) toxicity tests for sophorose lipids: oral $LD_{50}$ (rat, mouse) of 10-16 g/kg, no eye irritation (rabbit) or abraded skin (guinea pig) at 50% concentration, no mutagenicity in tests with *S. tyhimurium* TA 98 and TA 100 on rats fed 53 mg/kg $d^{-1}$ (0.06% in their diet). The results of toxicity test for the disodium salt of spiculisporic acid showed that $LD_{50}$ was 2,168 mg/kg for mouse (Ohata and Kamata, 1986).

# 4. BIOSYNTHESIS OF SOPHOROSE LIPIDS

## 4.1 Biosynthesis Pathway for Sophorose Lipids

Organisms need energy, reducing power and precursors produced by catabolism to synthesize the molecular components which they require for growth and reproduction. Microbial metabolism is an intergrated process that requires the coordinated activity of several enzymes. Generally, biosurfactants are the microbial metabolites with a typical amphiphilic structure, where the hydrophobic moiety is a long-chain fatty acid, hydroxy fatty acid, or α-alkyl-β-hydroxy fatty acid and the hydrophilic moiety can be a carbohydrate, amino acid, cyclic peptide, phosphate, carboxylic acid, or alcohol. Metabolic pathways for the synthesis of these two groups of precursors are diverse and utilize a specific set of enzymes. In many cases, the first enzyme is unique to the biosynthetic pathways for the synthesis of these precursors which are regulatory enzymes. There are some common features for biosynthesis of biosurfactants and their regulation. The general schemes of glycolipid synthesis from triglycerides and glucose are outlined in Figure 4.1 and Figure 4.2.

**Figure 4.1 The General Scheme of Glycolipid Synthesis from Triglycerides**

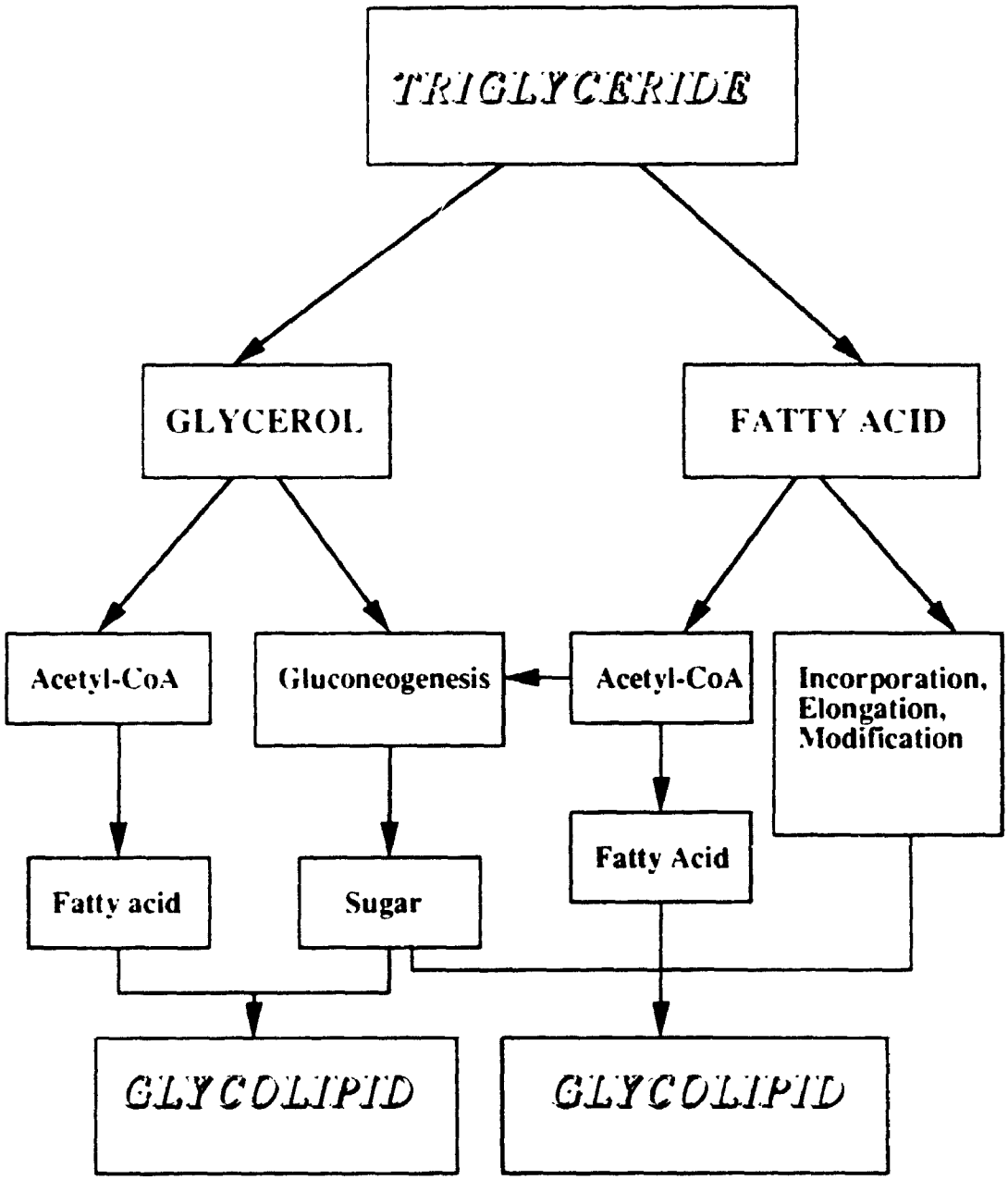
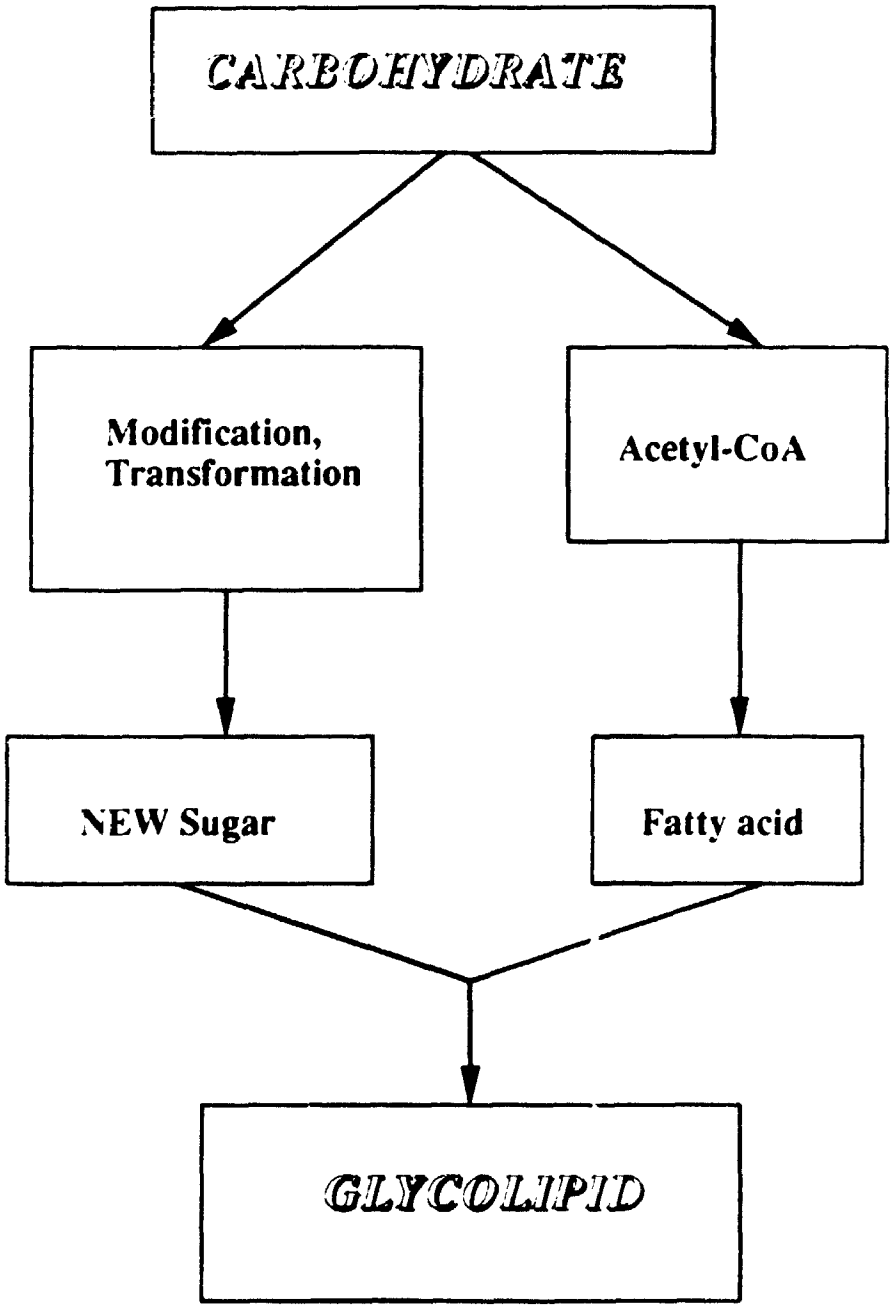

TRIGLYCERIDE
GLYCEROL
FATTY ACID
Acetyl-CoA
Gluconeogenesis
Acetyl-CoA
Incorporation, Elongation, Modification
Fatty acid
Sugar
Fatty Acid
GLYCOLIPID
GLYCOLIPID

Figure 4.2 The General Scheme of Glycolipid Synthesis from Glucose


CARBOHYDRATE
Modification, Transformation
Acetyl-CoA
NEW Sugar
Fatty acid
GLYCOLIPID

In oleaginous microorganisms, lipid synthesis occurs when an essential nutrient is limited and carbons exceed the cell requirements. Although lipids can be synthesized by various carbon sources, the most important carbon sources are water-insoluble substrates such as an alkane or fatty acid (Haferburg et al., 1986). The main factors controlling the amounts of the precursors available for biosurfactant synthesis are those that regulate the levels and activities of the enzymes.

### 4.1.1 Biosynthesis of Disaccharide from Glucose

Glucose is the most universally used carbon source and is often the cheapest source of carbon. Synthesis of the disaccharides such as trehalose, sophorose and cellobiose for the synthesis of many surfactants follows the following pattern of sucrose biosynthesis (Nikaido and Hassid, 1971):

Glucose + UTP → UDP-glucose + $P_i$

UDP-glucose + fructose 6-phosphate → sucrose 6-phosphate + UDP

Sucrose 6-phosphate + $H_2O$ → sucrose + $P_i$

Synthesis of trehalose by *Mycobacterium smegmatis* and *Nocardia* sp. also follows the above pattern. However, in *Streptomyces* spp., GDP-glucose is used as the starting substrate:

GDP-glucose + glucose 6-phosphate → trehalose 6-phosphate + GDP

There exists a specific synthetase in the synthesis of the disaccharides to ensure the correct regiospecific condensation. The phosphorylated disaccharide will then be used as the activated sugar for the formation of the glycolipid surfactant.

Rhamnose is 6-deoxymannose and is synthesized in several bacteria by an NADPH-linked dTDP-D-glucose oxidoreductase for the rhamnolipid surfactants. A multienzyme reaction involves not only the reduction at C6 but also inversion of the configuration at C3, C4 and C5 of glucose (Glazer and Zarkowsky, 1971).

### 4.1.2 Biosynthesis of Disaccharide from Hydrocarbons

In the case of alkanes, fatty acids, or even acetate or ethanol as substrates, the synthesis of sugars is from the pool of intermediates in the central pathways of metabolism. The sugars such as hexoses, pentoses, tetroses, and trioses are all needed either for synthesis of structural entities of the cell or for the biosynthesis of amino acids, proteins, purines, pyrimidines, and nucleic acids. The synthesis of sugars from acetyl-CoA is essentially the reverse of the glycolytic sequence of sugar breakdown and is termed gluconeogenesis. Two new enzymes called phosphoenolypyruvate carboxykinase and fructose biphosphatase which are used for the reaction as that catalyzed by the glycolytic enzymes, pyruvate kinase and phosphofructokinase, are not reversible:

Oxaloacetate + ATP → phosphoenolypyruvate

Fructose 1,6-biphosphate + $H_2O$ → fructose 6-phosphate + $P_i$

The key enzymes for cells to be able to handle acetate, whether used per se as a carbon source, derived from ethanol

by direct oxidation, or derived from degradation of long-chain fatty acids, are isocitrate lyase and malate synthase:

Isocitrate → glyoxylate + succinate

Glyoxylate + acetyl-CoA → malate + CoA

These two enzymes are used to generate a C4 unit (malate) from the C2 acetate and are induced only when the cell is growing on acetate or alkanes.

### 4.1.3 Biosynthesis of Fatty Acids from Glucose

Fatty acids are a class of compounds containing a long hydrocarbon chain and a terminal carboxylate group. Fatty acids have three major physiological roles. First, they are building blocks of phospholipids and glycolipids. These amphipathic molecules are important components of biological membranes. Second, fatty acid derivatives serve as hormones and intracellular messengers. Third, fatty acids are fuel molecules. They are stored as triacylglycerols, which are uncharged esters of glycerol. Triacylglycerols are also called neutral fats or triglycerides.

Fatty acids in biological systems usually contain an even number of carbon atoms, typically between 14 and 24. The 16- and 18-carbon fatty acids are most common with relatively small amounts of shorter ($C_{12}$ and $C_{14}$) and longer ($C_{20}$) acids. Although the biosynthesis of fatty acids is similar in all biological system, there are important differences between some bacterial systems and those of eukaryotic microorganisms (yeasts and molds). Bacteria usually produce 18:1 (*c*11), *cis*-vaccenic acid, whereas yeasts and molds and all other living cells, produce oleic acid, 18:1 (*c*9). These differences occur because of the organization of the enzymes making up the individual fatty acid synthetase complexes. In all cases, however, fatty acid biosynthesis begins with acetyl-coenzyme A (acetyl-CoA) which is formed from pyruvate in mitochondria. As this is the key intermediate for fatty acid biosynthesis and also, via formation of mevalonic acid, for the biosynthesis of all the terpenoid lipids, it is important to consider the metabolic origins of this molecule. This chapter mainly deals with the biosynthesis of sophorose lipids by *Torulopsis bombicola*.

Long-chain acetyl-CoA molecules do not readily traverse the inner mitochondrial membrane, and so a special transport

mechanism is needed. Acetyl-CoA is transferred from the sulfur atom of CoA to the hydroxyl group of carnitine to form acetyl-carnitine which is smaller than acetyl-CoA. This reaction is catalyzed by carnitine acyltransferase I, which is located on the cytosolic face of the inner mitochondrial membrane (Hommel and Ratledge, 1992; Stryer, 1988).

Acetyl-CoA + carnitine → acetyl-carnitine + CoA

Acetyl-carnitine is then shuttled across the inner mitochondrial membrane by a translocase. The acetyl-carnitine is transferred back to regenerate acetyl-CoA.

Another transport mechanism is the citrate translocation and cleavage route. Only a few yeasts and molds required this transport system because it was associated with the phenomenon of oleaginicity whereby lipid contents over 20% of the biomass can be created in yeast and in molds. This system operates in conjunction with the acetyl-carnitine route and not in place of it. Citrate is formed in the mitochondrial matrix by the condensation of acetyl CoA with oxaloacetate. When present at high levels, citrate is transported to the cytosol, where it

is cleaved by citrate lyase:

Citrate + ATP + CoA + $H_2O$ → acetyl-CoA + ADP + $P_i$ + oxaloacetate

Thus, acetyl-CoA and oxaloacetate are transferred from mitochondria to the cytosol at the expense of an ATP.

The formation of malonyl coenzyme A is the committed step in fatty acid synthesis. Fatty acids synthesis starts with the carboxylation of acetyl-CoA to malonyl-CoA. The reaction is irreversible and catalyzed by acetyl-CoA carboxylase, which contains a biotin prosthetic group.

Acetyl-CoA is carboxylated in two stages. First, a carboxybiotin intermediate is formed at the expense of an ATP. The activated $CO_2$ group in this intermediate is then transferred to acetyl-CoA to form malonyl-CoA.

Biotin-enzyme + ATP + $HCO_3^-$ → $CO_2$-biotin-enzyme + ADP + $P_i$

$CO_2$-biotin-enzyme + acetyl-CoA → malonyl-CoA + biotin-enzyme

overall : acetyl-CoA + $HCO_3^-$ + ATP → malonyl-CoA + ADP +$P_i$

The enzyme system that catalyzes the synthesis of saturated long-chain fatty acids from acetyl-CoA, malonyl-CoA, and NADPH is called the fatty acid synthase. The elongation phase of fatty acid synthesis begins with the formation of acetyl-ACP and malonyl-ACP. Acetyl transacylase and malonyl transacylase catalyze these reactions.

Acetyl-CoA + ACP ⇌ acetyl-ACP + CoA

Malonyl-CoA + ACP ⇌ malonyl-ACP + CoA

Malonyl transacylase is highly specific, whereas acetyl transacylase can transfer acyl groups other than the acetyl unit, though at a much slower rate. Fatty acids with an odd number of carbon atoms are synthesized begining with propionyl-ACP, which is formed from propionyl CoA by acetyl transacylase. Acetyl-ACP and malonyl-ACP condense to form acetoacetyl-ACP, a reaction driven by the release of $CO_2$ from

the activated malonyl unit. This condensation reaction is catalyzed by the acyl-malonyl-ACP condensing enzyme.

Acetyl-ACP + malonyl-ACP → acetoacetyl-ACP + ACP + $CO_2$

In the condensation reaction, a four-carbon unit is formed from a two-carbon unit and a three-carbon unit, and $CO_2$ are released.

Acetoacetyl-ACP + NADPH + $H^+$ ⇌ D-3-hydroxybutyryl-ACP + $NADP^+$

D-3-Hydroxybutyryl-ACP ⇌ crotonyl-ACP + $H_2O$

Crotonyl-ACP + NADPH + $H^+$ ⇌ butyryl-ACP + $NADP^+$

The next three steps in fatty acid synthesis reduce the keto group at C-3 to a methylene group. First, acetoacetyl-ACP is reduced to D-3-hydroxybutyryl-ACP. Then D-3-hydroxybutyryl-ACP is dehydrated to form crotonyl-ACP. The final step reduces crotonyl-ACP to butyryl-ACP. NADPH is the reductant in these steps. The butyryl-ACP formed in this way is ready for a

second round of elongation, starting with the addition of a two-carbon unit from malonyl-ACP. Seven rounds of elongation yield palmitoyl-ACP, which is hydrolyzed to palmitate. The synthesis of palmitate requires eight molecules of acetyl-CoA, fourteen NADPH, and seven ATP; seven $HCO_3^-$ play a catalytic role.

Palmitate is the major product of the fatty acid synthase. In eucaryotes, longer fatty acids are formed by elongation reactions catalyzed by enzymes on the cytosolic face of the endoplasmic reticulum membrane. By far the most widespread pathway is by an oxidative mechanism, in which a double bond is introduced directly into the preformed saturated long chain fatty acid with $O_2$ and a reduced compound (such as NADH) as cofactors. This pathway is almost universal and is used by bacteria, yeasts, algae, higher plants, protozoa and animals.

The pathway was first demonstrated in yeast. Cell-free preparations could catalyse the conversion of palmitic into palmitoleic acid (hexadec-9-enoic acid, $9\text{-}C_{16:1}$) only if both a particulate fraction (microsomes) and the supernatant fraction were present. The membrane fraction alone could perform the dehydrogenation provided that the substrate was

the acyl-CoA thiolester. The supernatant contained the acid:CoA ligase to activate the fatty acid. More recently another protein fraction in the soluble cytoplasm has been found to simulate desaturation.

In the conversion of stearoyl CoA into oleoyl CoA, a *cis*-$\Delta^9$ double bond is inserted by an oxidase that employs molecular oxygen and NADH (or NADPH):

$$\text{Stearoyl CoA} + \text{NADH} + H^+ + O_2 \rightarrow \text{oleoyl CoA} + NAD^+ + 2H_2O$$

The reaction is catalyzed by three complex proteins: a flavoprotein, NADH-cytochrome $b_5$ reductase; a haem-containing protein, cytochrome $b_5$ and desaturase itself. The function of the electron transport proteins is to transfer electrons from NADH to the nonhaem iron of the desaturase protein. The reduced iron then binds $O_2$ and the saturated fatty acyl-CoA substrate. A double bond is formed and two molecules of $H_2O$ are released. Two electrons come from NADH and two from the single bond of the fatty acyl substrate.

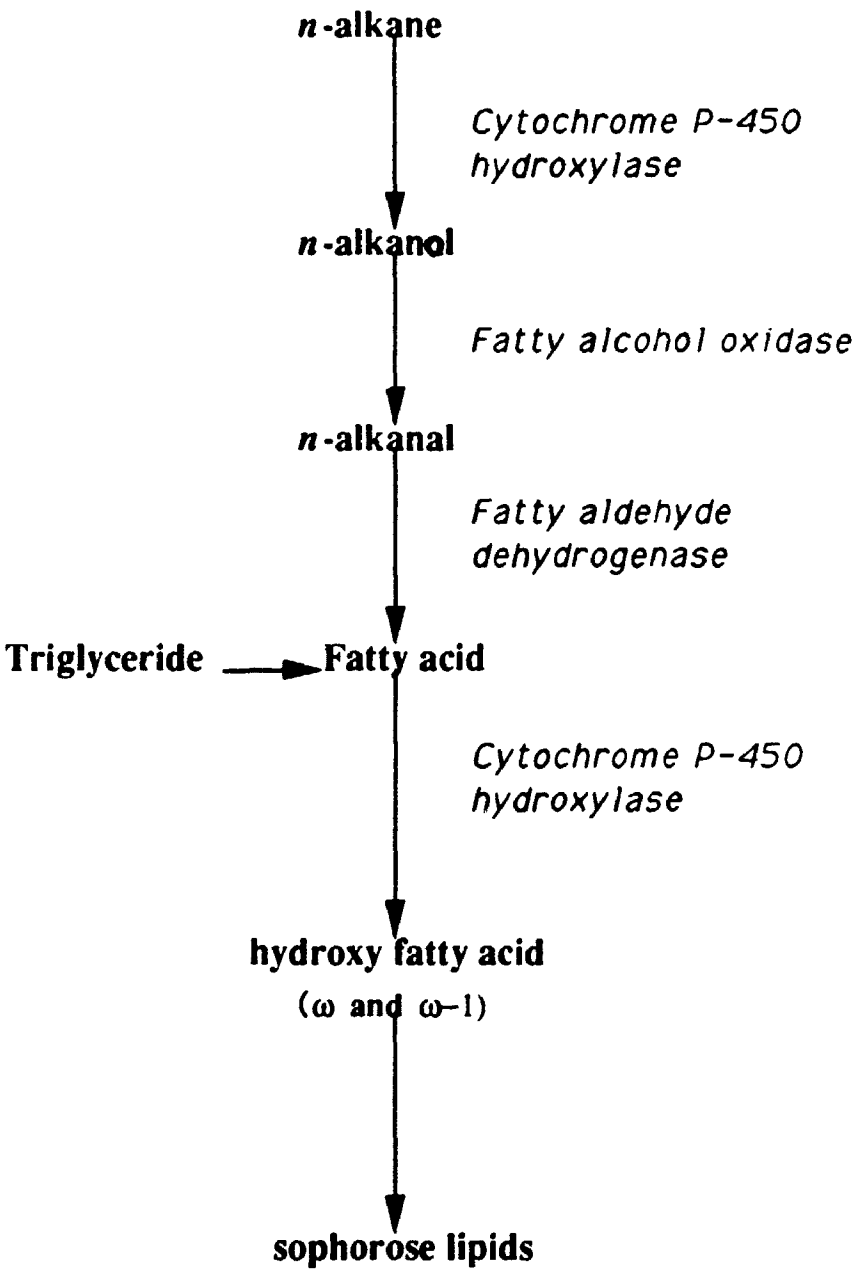
A variety of unsaturated fatty acids can be formed from oleate by a combination of elongation and desaturation reactions. For

example, oleate can be elongated to a 20:1 *cis*-$\Delta^{11}$ fatty acid. Alternatively, a second double bond can be inserted to form an 18:2 *cis*-$\Delta^{6}$, fatty acid. Similarly, palmitate (16:0) can be oxidized to palmitoleate (16:1 *cis*-$\Delta^{9}$), which can then be elongated to *cis*-vaccenate (18:1 *cis*-$\Delta^{11}$).

### 4.1.4 Biosynthesis of Hydroxy Fatty Acids from Alkanes

The scheme of the oxidation of the alkane by *T. apicola* IMET 43747 is proposed as shown in Figure 4.3 (Hommel et al., 1990; Hommel and Ratledge, 1992). Experiments showed that hydroxylation of the fatty acid will be the overall rate-limiting step in *T. apicola*. The yields of glycolipid synthesized under mixed substrate cultivations are determined by the oxidation status of the hydrophobic substrate. It was found that significantly higher yields of sophorose lipids were obtained when triacylglycerols were used as substrate instead of n-alkanes, which indicates that one of the prior reactions is now rate-limiting. Similar kinetics of production of sophorose lipid by *Torulopsis bombicola* as shown in Figure 4.3 suggest comparable pathways and regulation as in *Torulopsis apicola*.

Figure 4.3 The Scheme of the Microsomal Oxidation of n-alkanes to Hydroxy Fatty Acid by *Torulopsis apicola* (Hommel et al., 1990, 1992)

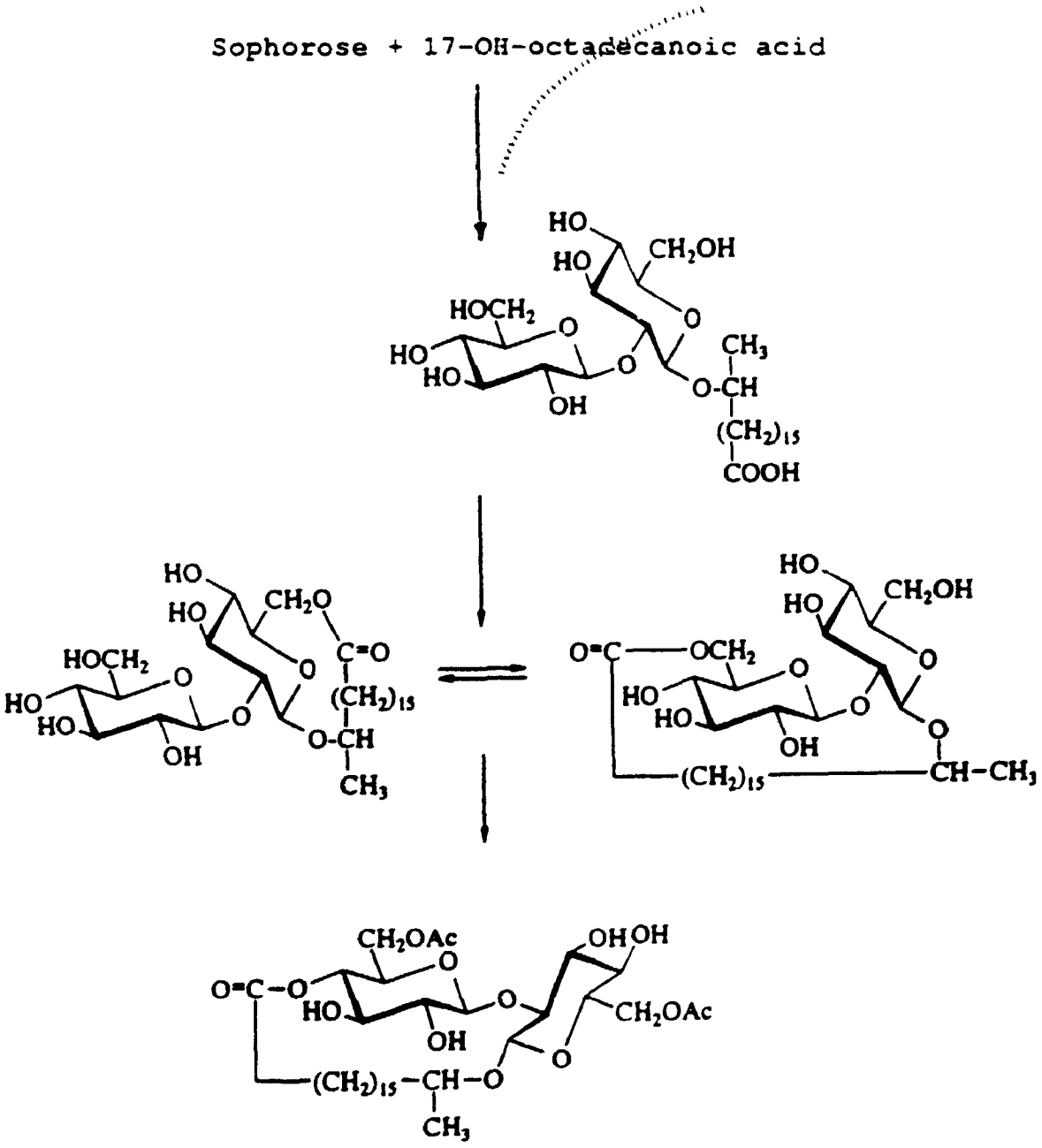

n-alkane
Cytochrome P-450 hydroxylase
n-alkanol
Fatty alcohol oxidase
n-alkanal
Fatty aldehyde dehydrogenase
Triglyceride
Fatty acid
Cytochrome P-450 hydroxylase
hydroxy fatty acid
(ω and ω-1)
sophorose lipids

### 4.1.5 Biosynthesis of Sophorose Lipids

The biosynthesis of sophorose lipids may be as follows: (1) synthesis of the sophorose moiety; (2) synthesis of the fatty acid moiety; (3) linkage of two parts to form complete sophorose lipids. Sophorose lipids are only synthesized by yeasts of the genus *Candida*: *Torulopsis magnoliae, Torulopsis, Torulopsis gropengiesseri, Torulopsis bombicola*. The constitutive carbohydrate backbone of these extracellular glycolipids is sophorose (2′-O-β-D-glucopyranosyl-β-D-glucopyranose), which may be acetylated in position 6 and 6′ and is linked in position 1 with a hydroxy fatty acid. The structure of the carbohydrate moiety in sophorolipids remains the same by changing carbon sources. This suggests that the synthesis of sophorose and its subsequent linkage to the hydroxy fatty acid is the highly specific enzymatic sequences of the *de novo*.

The condensation of the sophorose and 17-OH-octadecanoic acid to sophorose lipids is catalysed by specific synthetases. Possible sequence of last synthesis steps of sophorose lipids by *Torulopsis bombicola* ATCC 22214 was proposed as Figure 4.4 (Asmer et al., 1988). From the Figure, it shows that both the

Figure 4.4 Possible Sequence of Last Synthesis Steps of Sophorose Lipids by *Torulopsis bombicola* (Asmer et al., 1988)


Sophorose + 17-OH-octadecanoic acid
HO
HO
CH2OH
HOCH2
O
HO
HO
OH
O
O
CH3
O-CH
(CH2)15
COOH
HO
HO
CH2O
C=O
HOCH2
O
HO
HO
OH
O
O
(CH2)15
O-CH
CH3
HO
HO
CH2OH
O=C
OCH2
O
HO
HO
OH
O
O
(CH2)15
CH-CH3
CH2OAc
O
OH OH
O=C-O
HO
OH
O
CH2OAc
O
(CH2)15-CH-O
CH3

acetylation and lactonization steps should be involved.

The study of the stereospecific hydroxylation of octadecanoate to 17-L-hydroxyoctadecanoic acid showed that $^{18}O$-labelled molecular $O_2$ was the bridge oxygen atom but not $H_2{}^{18}O$ and $^{18}O$-labelled hydroxy fatty acids (Spencer et al., 1979). There are monooxygenase systems for both the hydroxylation of the alkane and also for the hydroxylation of the opposite terminus in either the ω or ω-1-position. A mixed-function oxidase was found in a cell-free system of *T. bombicola* in the presence of NADPH for the conversion of oleic acid to 17-L-hydroxyoleic acid.

A cytochrome P-450 hydroxylase was found in *T. apicola* IMET 43747 when glucose, or hexadecane or both were used as substrates (Kleber et al., 1989). More recent results with the same strain confirm the lack of a repressible alkane hydroxylase even after alkanes have been totally consumed and glucose becomes the sole remaining carbon source (Hommel et al., 1990). By adding [1-$^{13}C$]palmitic acid to either growing (logarithmic) or glycolipid-producing (stationary) cells of *T. apicola*, it was found by NMR spectroscopy that the recovery of the $^{13}C$ in the sophorolipid was qualitatively the same as that when the compounds were added to stationary phase cells.

However, the stationary phase cells incorporated [1-$^{13}$C]hecadecan-1-ol and [1-$^{13}$C]hexadecanoic acid faster than they oxidized the unlabeled hexadecane as revealed by the enhancements of 10.5 and 13.0 for the alcohol and the acid, respectively. In both experiments the $^{13}$C of the alcohol was found in position 1, thus excluding the diol as a possible intermediate. These results suggest that two different monooxygenase systems are involved in the formation of hydroxyhexadecanoic acids. Furthermore, selective ω-hydroxylation is principally expressed in logarithmic phase cells whereas in stationary phase cells an ω- or[ω-1]-hydroxylating monooxygenase system must be induced. The ratio of ω- to[ω-1]-hydroxylated lactonized products may be shifted from 1:1 to 1:2.3. Two constitutive microsomal fatty alcohol oxidases in *T. bombicola* ATCC 22214, which were able to oxidize long-chain alcohols and diols but not ω-hydroxy fatty acids and not repressed by glucose (Weber et al., 1990).

The overproduction of sophorose lipids occurs when nitrogen is nearly exhaused and carbon is in excess. This suggests that the enzymes are produced during the exponential growth phase and activated during stationary phase for sophorose lipids synthesis.

The biosynthesis pathway for sophorose lipids depends on the carbon source. When only a carbohydrate is used, sophorose lipids synthesis is regulated by both the lipogenic pathway (lipid synthesis) and glycolytic metabolism (the sophorose synthesis) as shown in Figure 4.5 (Ghosh et al., 1985; Hommel and Ratledge, 1992). In this case, sophorose lipids production is limited by the synthesis of the fatty acid moiety. When only a hydrocarbon is used, the pathways are lipolytic and gluconeogenic as shown in Figure 4.6. In this case, fatty acids are readily available from the enzymatic hydrolysis of triglycerides present in the vegetable oil. The limitation to sophorose lipids production results from the synthesis of the sophorose moiety. This can be explained that when both the glucose and vegetable oil were used as carbon sources, the yield of sophorose lipids was increased greatly. In this case, glucose can be enzymatically dimerized to form sophorose, and vegetable oil can be hydrolyzed to supply the fatty acid moiety. By this way, synthesis of sophorose and fatty acid requires considerably less cell energy than synthesis of sophorose and fatty acid from acetate. A further intention was to replace the sophorose unit of lipids by another carbohydrate. However, the precursor experiments always indicated the molecular structure of sophorose in the lipids.

**Figure 4.5 The Pathway of Biosurfactant Precursor Synthesis from Carbonhydrate Substrates**

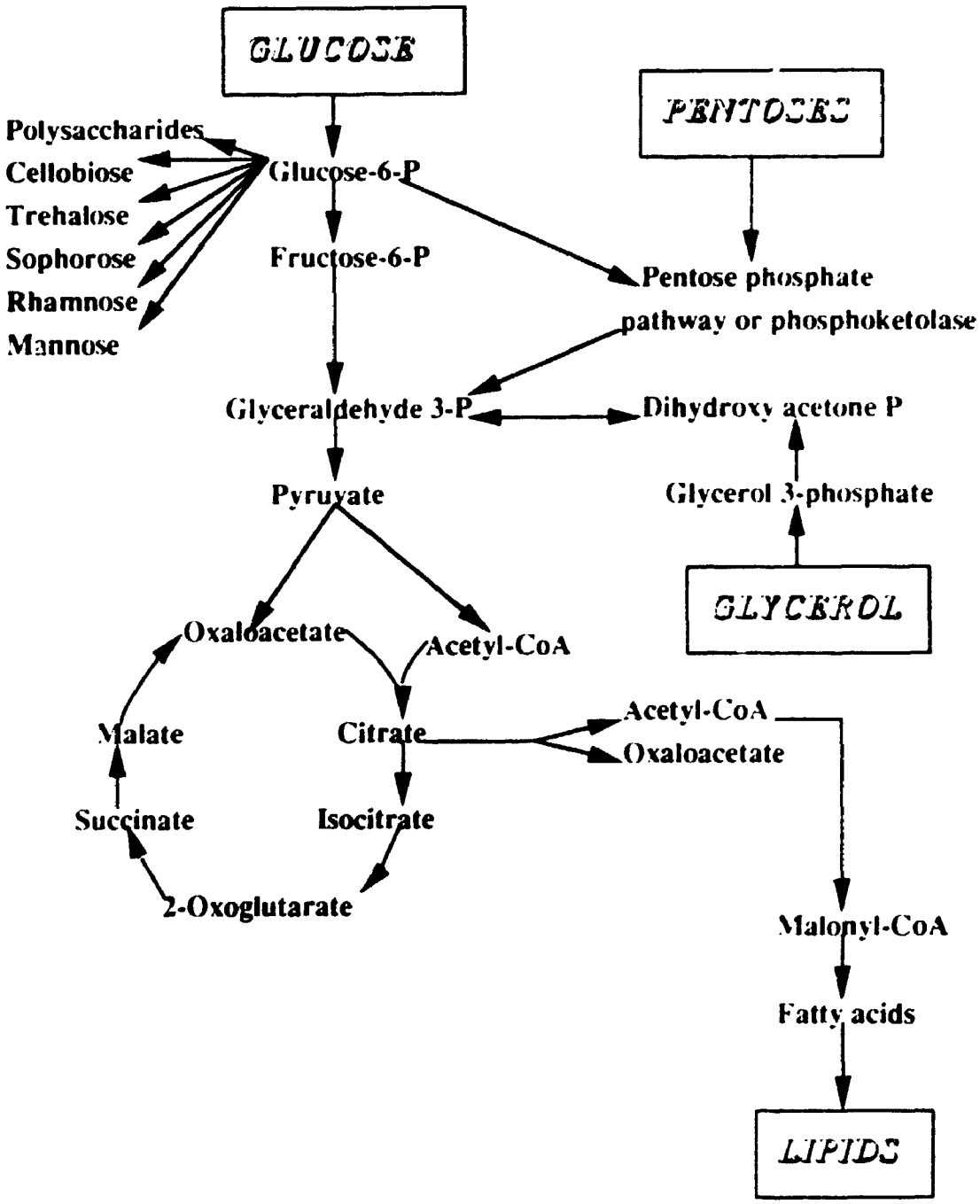
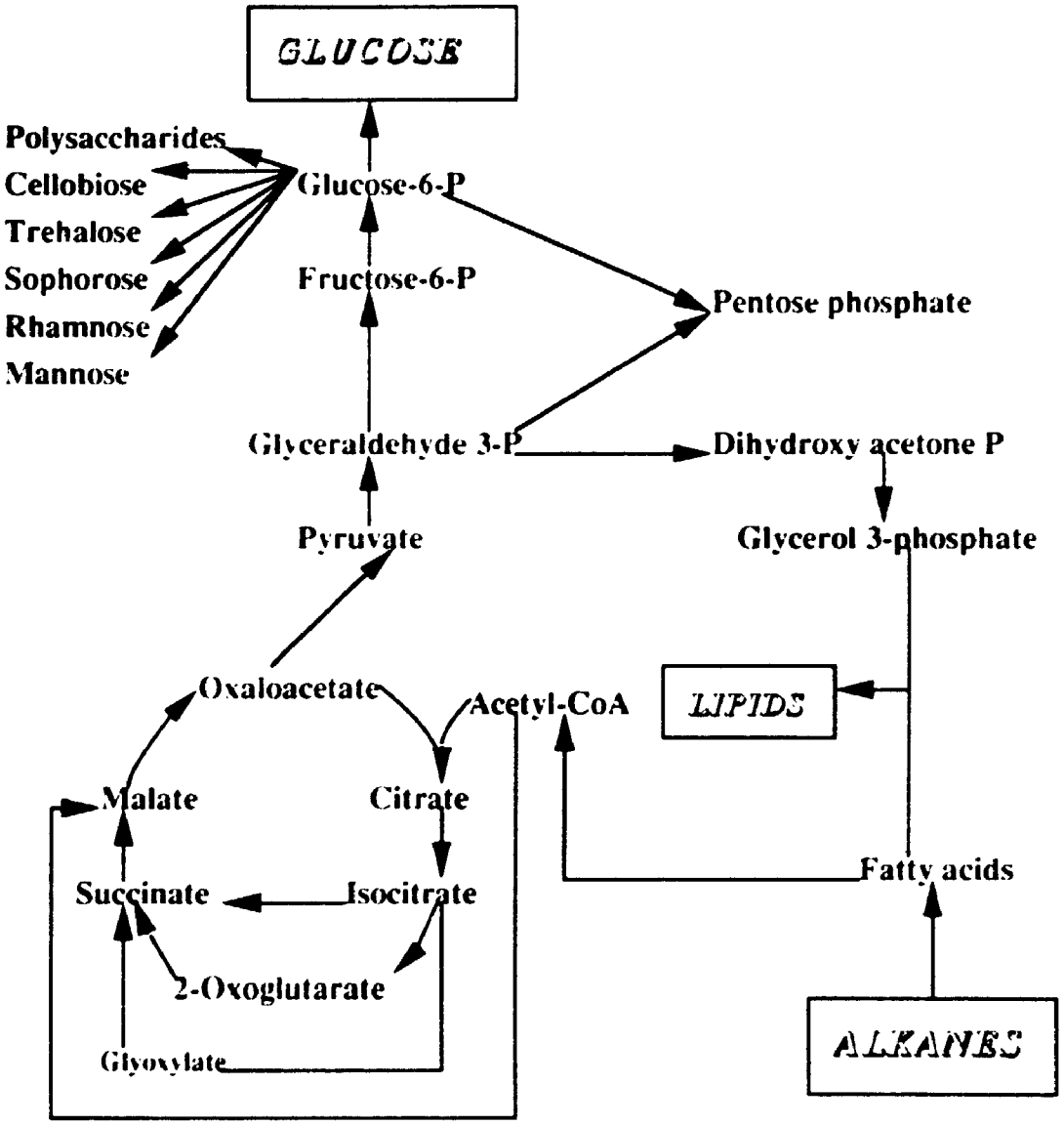

GLUCOSE
PENTOSES
Polysaccharides
Cellobiose
Trehalose
Sophorose
Rhamnose
Mannose
Glucose-6-P
Fructose-6-P
Pentose phosphate
pathway or phosphoketolase
Glyceraldehyde 3-P
Dihydroxy acetone P
Pyruvate
Glycerol 3-phosphate
GLYCEROL
Oxaloacetate
Acetyl-CoA
Acetyl-CoA
Malate
Citrate
Oxaloacetate
Succinate
Isocitrate
2-Oxoglutarate
Malonyl-CoA
Fatty acids
LIPIDS

Figure 4.6 The Pathway of Biosurfactant Precursor Synthesis from Hydrocarbon Substrates


GLUCOSE
Polysaccharides
Cellobiose
Trehalose
Sophorose
Rhamnose
Mannose
Glucose-6-P
Fructose-6-P
Pentose phosphate
Glyceraldehyde 3-P
Dihydroxy acetone P
Pyruvate
Glycerol 3-phosphate
Oxaloacetate
Acetyl-CoA
LIPIDS
Malate
Citrate
Succinate
Isocitrate
2-Oxoglutarate
Glyoxylate
Fatty acids
ALKANES

The precursors are incorporated directly into the product without alteration of the length or structure of the carbon chain in the case of $C_{16}$-$C_{19}$ precursors. Formation of odd-carbon hydroxy acids from odd-carbon substrates showed that the substrate is used directly and not broken down to acetate. Substrates with 16-19 carbons yield hydroxy acids with the same number of carbon atoms but, when substrates with 20 or more carbon atoms are used, the hydroxy acids produced have 17 or 18 carbon atoms. The fatty acid chain is shortened by β-oxidation from the carboxyl end so that erucate (13,14 double bond) and eicos-11-enoate give hydroxyoleic acid with a 9,10 double bond.

Study on protoplasts of *T. apicola* suggests that there are periplasmic vesicles derived from the cytoplasmic membrane which completely separate from the cytoplasmic membrane after protoplasting (Hommel and Ratledge, 1992). These vesicles can be the site of sophorolipid formation and accumulation. The potentially deleterious effect of the sophorolipids within the cell would then be obviated, as such vesicles could secrete the surfactant by a process akin to exocytosis.

When glucose or lactose are substrates along with vegetable

oils. When nitrogen is exhausted, carbon and energy flow are diverted away from protein and nucleic acid synthesis (biomass) and into other pathways including sophorose lipids production. In oleagenous yeast including *Torulopsis bombicola*, citrate accumulates in the mitrochandria upon nitrogen depletion and is transported subsequently to the cytoplasm. The cytoplasmic citrate is cleaved by the enzyme ATP:citrate lyase to oxaloacetate and acetyl-CoA which is the precursor for sophorose lipids synthesis. When sopnorose lipids synthesis is blocked under the conditions, the cells can also accumulate intracellular lipids and have an active ATP:citrate lyase. The long chain acyl CoA intermediates but not acetyl CoA inhibit the ATP:citrate lyase. Whether or not a high enough intracellular concentration of long chain acyl CoA intermediate is obtained to inhibit the citrate cleavage enzyme is dependent upon the relative kinetics 1) for producing such intermediates by mass transfer of extracellular lipids from extracellular to intracellular pools and by *de novo* lipid synthesis resulting from the activity of the ATP:citrate lyase enzyme, and 2) for consuming such intermediates in sophorose lipids production and intracellular triglyceride storage.

When the intracellular concentration of long chain acyl CoA intermediates is low, the ATP:citrate lyase is active. In this case, sophorose lipids are synthesized. The ATP:citrate lyase activity can also be used for the synthesis of oxaloacetate, which is converted to malate. Malate is used for the citrate transport shuttle or is cleaved to pyruvate and $CO_2$ by malic enzyme (malate dehydrogenase). The reaction is accompanied by the reduction of $NADP^+$ to NADPH. The unsaturation of fatty acids is formed by cytosolic desaturase enzymes which use oxygen and NADPH as cofactors. The hydroxyl group at the ω-1 position is likely produced by ω-oxidation which is catalyzed by microsomal enzymes of the oxygenase by the use of NADPH and oxygen.

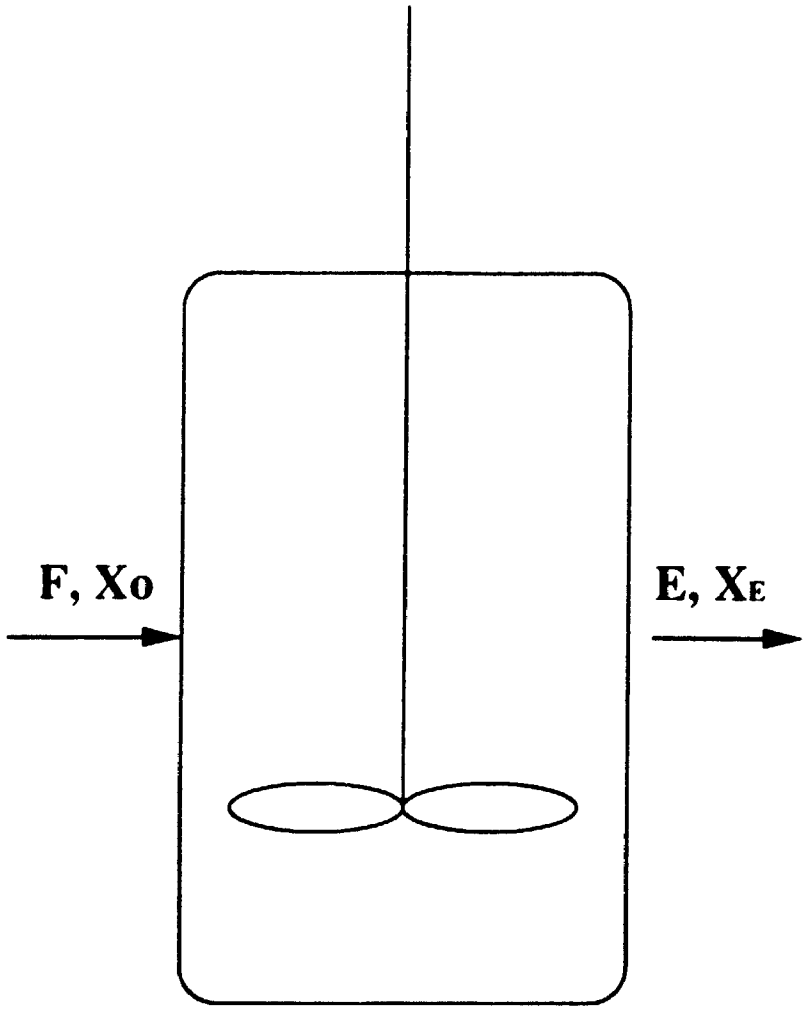
## 4.2 Microbial Kinetics

The CSTR for biological growth systems is the continuous stirred tank biological reactor (CSTBR). Models of the various flows may be developed, starting with that of the basic flow shown in Figure 4.7, the symbols used for this model are:

F = volumetric feed rate, volume/time;

E = volumetric effluent flow rate, volume/time;

Figure 4.7 Single Continuous Stirred Tank Biological Reactor for Biological Growth Systems


F, Xo
E, XE

$X_o$ = concentration of cells in the feed, mass per unit volume;

$X$ = concentration of cells in the reaction mixture;

$X_E$ = concentration of cells in the effluent;

$S_o$ = concentration of substrate in the feed;

$S$ = concentration of substrate in the reaction mixture;

$S_E$ = concentration of substrate in the effluent;

$P_o$ = concentration of product in the feed (usually zero);

$P$ = concentration of product in the reaction mixture;

$P_E$ = concentration of product in the effluent;

$V_R$ = volume of the reaction mixture.

In the general case, both reaction mixture volume ($V_R$) and composition ($X$, $S$, $P$) are variable functions of time. Some simplifying equalities can be stated for the ideal case of a completely mixed reactor by definition:

$$X_E = X,\ S_E = S,\ \text{and}\ P_E = P$$

With the assumption that no reaction mixture specific volume change occurs because of the reaction, a volume balance gives

$$dV_R/dt = F - E \qquad (4.1)$$

A balance on cells is made:

Rate of increase in cell mass = (cell in) - (cells out) + (rate of generation of new cells by multiplication),

(4.2)

or

$$d(V_R X)/dt = FX_o - EX + V_R(dX/dt)_{growth} \quad (4.3)$$

which yields

$$V_R dX/dt + X dV_R/dt = FX_o - EX + V_R(dX/dt)_{growth} \quad (4.4)$$

The rate of change of cell concentration then is

$$dX/dt = F/V_R X_o - E/V_R + (dX/dt)_{growth} - (X/V_R)(dV_R/dt) \quad (4.5)$$

$$(dX/dt)_{growth} = \mu_{max} SX/(K_m + S) = \mu X \quad (4.6)$$

Similar balances can be made on the substrate:

$$dS/dt = F/V_R S_o - E/V_R S + (dS/dt)\text{reaction} - (S/V_R)(dV_R/dt); \quad (4.7)$$

and on the product:

$$dP/dt = F/V_R P_o - E/V_R P + (dP/dt)_{\text{reaction}} - (P/V_R)(dV_R/dt) \quad (4.8)$$

These general equations can now be incorporated into models with specific constraints or enhancements for the various CSTR types.

### 4.2.1 Batch Culture Kinetics

A stirred-tank culture system operated without feed or product stream is an ideal batch reactor. It can be treated as an unsteady-state CSTR with both volumetric feed rate (F) and volumetric effluent flow rate (E) equal to zero. Thus the rate of increase in cell population is solely the result of multiplication (and/or death). Hence the expressions for multiplication limited by growth kinetics, substrate supply,

or mass transport also are the culture rate expressions. Based on the time course of the batch fermentation of *Torulopsis bombicola*, the volumetric rates of substrate uptake $R_{vs}$, the volumetric rate of biomass formation ($R_{vb}$) and the volumetric rate of product formation ($R_{vp}$) can be represented as follows (Aiba et al, 1973: Atkinson and Mavituna, 1991):

$$R_{vs} = -dS/dt \qquad (4.9)$$

$$R_{vb} = dX/dt \qquad (4.10)$$

$$R_{vp} = dP/dt \qquad (4.11)$$

The volumetric rate of change of a component can be obtained from the experimental data by a number of techniques of varying sophistication and accuracy. The dimension of $R_{vI}$ are $M\ L^{-3}T^{-1}$, namely those of the productivity of unit volume of the fermenter.

The specific rate of substrate uptake ($R_s$), the specific rate of biomass formation $\mu$ and specific rate of product formation ($R_p$) can be described as follows:

$$R_s = R_{vs/x} = -(1/X)(dS/dt) \qquad (4.12)$$

$$\mu = R_{vb/x} = (1/X)(dX/dt) \qquad (4.13)$$

$$R_p = R_{vp/x} = (1/X)(dP/dt) \qquad (4.14)$$

The dimensions of $R_s$, $\mu$ and $R_p$ are $M_s M_c^{-1} T^{-1}$, $T^{-1}$ and $M_p M_c T^{-1}$, respectively. The specific rate $\mu$ depends upon the concentration of the components in the medium, for example

$$\mu = f\ (S_c, S_n, S_o, S_p, \ldots) \qquad (4.15)$$

Generally, there exists a single limiting component, which is usually the carbon or nitrogen source or the hydrogen acceptor.

The relationship between the specific growth rate $\mu$ and the limiting component S may be expressed by the Monod equation, that is

$$\mu = \mu_{max} S/(K_s + S) \qquad (4.16)$$

In Equation, $\mu_{max}$ is the maximum specific growth rate and $K_s$ is the Monod coefficient corresponding to the component S. When $S >> K_s$ and the concentrations of all other essential nutrients are unchanged. $K_s$ is that value of the limiting nutrient concentration at which the specific growth rate is half its maximum value; roughly speaking, it is the division between the lower concentration range, where $\mu$ is strongly (linearly) dependent on S, and the higher range, where $\mu$ becomes independent of S. $K_s$ is often rather small and the specific growth rate ($\mu$) is finite. Thus when $S << K_s$, the Monod equation can not be applied because $\mu$ is near zero (Atkinson and Mavituna, 1991).

The Monod equation was originally considered to be purely empirical, but it does have a partially theoretical grounding in enzyme kinetics and carrier-associated transport across cell membranes.

In general, $K_s$ depends on the environmental conditions such as pH, temperature, ionic strength, etc.. For a given set of experimental results, a graph of $\mu$ versus substrate concentration can be plotted to provide a curve such as shown in Appendix 1.

From this graph, approximate values of $\mu_{max}$ and, consequently, $K_s$ may be obtained by visually choosing $\mu_{max}$ and then establishing the value of s that corresponds to $\mu_{max}/2$ (i.e. S′) and then setting $K_s = S'$. The latter relationship follows from the Monod equation, that is

$$\mu_{max}/2 = \mu_{max}\, S'/(K_s + S') \qquad (4.17)$$

However, as may be seen from Appendix 2, it is difficult to establish the exact value of $\mu_{max}$ because the curve is a rectangular hyperbola. A more accurate graphical method for determining $\mu_{max}$ and $K_s$ involves rearranging the Monod equation in the form

$$1/\mu = 1/\mu_{max} + (K_s/\mu_{max})(1/S) \qquad (4.18)$$

and either plotting the data in the form shown in Appendix 2

or by using linear regression. The above equation is used to determine $\mu_{max}$ and $K_s$ by the graphical method for a fermentation process.

Equation 4.17 separates dependent and independent variables but the most accurately known values of $\mu$ are near $\mu_{max}$ and cluster near the origin, i.e. $1/S \rightarrow 0$, and the least accurate will be far from the origin ($1/S \rightarrow$ large). The latter will strongly affect the slope ($K_s/S$). Therefore linear least squares fitting should be avoided.

For a batch culture follows the Monod rate law with first-order cell death, the cell rate increase is

$$dX/dt = \mu X - k_d X \quad (4.19)$$

Substrate utilization is given by

$$-dS/dt = R_s X \quad (4.20)$$

For a culture with an appreciable maintenance factor, then

$$R_s = Y_{s/x}\mu_{max} S/(K_s + S) + m$$

(4.21)

The product appearance rate then is

$$dP/dt = R_p X, \qquad (4.22)$$

In the case where there is appreciable nongrowth-associated product synthesis,

$$R_p = Y_{p/x}\mu_{max}S/(K_m + S) + B \qquad (4.23)$$

where B = nongrowth component of specific product appearance rate

Numerical integration may be used to solve Equations 4.19-4.23 for S, X, and P as functions of time, t. Simplified cases are solved analytically. For example, the specific multiplication rate, $\mu$, may be essentially constant over a range of substrate and cell concentrations. The values of $Y_{s/x}$ and $Y_{p/s}$ may also be constant. Further, the maintenance factor, m, may be negligible. Taking all these simplifications to be valid yields a case that is easily analyzed:

Initial conditions

$$t = 0;\ X = X_o;\quad S = S_o;\ \text{and } P = 0$$

Reaction rates

$$dX/dt = \mu X \qquad (4.24)$$

$$dS/dt = -Y_{s/x}\, dX/dt \qquad (4.25)$$

$$dP/dt = -Y_{p/s}\, dS/dt \qquad (4.26)$$

Conversion stoichiometry

$$S_o - S = Y_{s/x}(X - X_o) \qquad (4.27)$$

$$P = Y_{p/s}(S_o - S) \qquad (4.28)$$

Integral growth

$$X = X_o e^{\mu t} \qquad (4.29)$$

Combining Equations 4.27 and 4.29 gives

$$S = S_o + Y_{s/x}(X_o - X) = S_o + X_oY_{s/x}(1 - e^{\mu t}) \qquad (4.30)$$

Equation 4.30 may be rearranged to solve for the time required to reach a given substrate concentration:

$$S_o - S = X_oY_{s/x}(e^{\mu t} - 1) \qquad (4.31)$$

$$e^{\mu t} = (S_o - S)/X_oY_{s/x} + 1 \qquad (4.32)$$

$$\mu t = \ln [(S_o - S)/X_oY_{s/x} + 1], \qquad (4.33)$$

and

$$t = 1/\mu \ln[(S_o - S)/X_oY_{s/x} + 1] \qquad (4.34)$$

The time required for a given increase in cell population is derived by integration of Equation 4.24:

$$\ln X/X_o = \mu t \qquad (4.35)$$

or $$t = 1/\mu \ln X/X_o \quad (4.36)$$

$$\mu_{max} = (\ln X_2 - \ln X_1)/(t_2 - t_1) \quad (4.37)$$

$$t_d = \ln 2/\mu \quad (4.38)$$

where $t_d$ is the time required to double the population of cells (doubling time). Note that Equation 4.34 and 4.35 are derived by simple integration and that a limit to X is imposed by substrate supply. If reaching a substrate concentration near zero under the given constraints were possible, Equation 4.27 would impose a limit on X:

$$S_o - 0 = Y_{s/x}(X_{max} - X_o), \quad (4.39)$$

or $$Y_{x/s} = (X_{max} - X_o)/(S_o - 0) \quad (4.40)$$

where $X_{max}$ = maximum value of X under substrate supply limitations.

Then $$X_{max} = S_o/Y_{s/x} + X_o \quad (4.41)$$

$$S_o = Y_{s/x}(X - X_o) \quad (4.42)$$

where $X_o$ is usually equal to zero, $Y_{s/x}$ is constant. Equation 4.42 can be used to determine the minimum value of initial substrate concentration required to produce a given cell population (within limits of validity of initial assumptions) in the batch experiments. For example, in the case of sophorose lipids production, $Y_{s/x}$ is equal to 6 for the optimal sophorose lipids production (Inoue, 1988). For a given cell population of 30 g/L, $S_o = 6 \times (30 - 0) = 180$ g/L. The above batch models also allow us to develop fed-batch models in the following section.

### 4.2.2 Fed-Batch Cultures Kinetics

Unlike the batch and continuous bioreactors, the fed-batch bioreactors may be operated in a variety of ways. The most commonly used fed-batch cultures are constant fed-batch culture, exponentially fed-batch culture, extended culture and repeated fed-batch culture. The constant fed-batch culture, is a batch culture to which the substrate is fed at a constant rate. Exponentially fed-batch culture is a batch culture to which substrate is fed at an exponentially increasing rate. An extended culture is a fed-batch culture to which substrate is fed at a varying rate so as to maintain concentration of the

limiting substrate constant at all times. Fed-batch cultures, where part of the reactor contents are periodically withdrawn are known as repeated fed-batch cultures or cyclic fed-batch cultures.

For the fed-batch system, assume a well mixed bioreactor in which the growth of microorganisms is limited by the concentration of a growth-limiting substrate, where all other nutrients are present in excess. Only the growth limiting substrate or its aqueous solution containing all other necessary nutrients is supplied to the bioreactor at a constant or varied feed rate, and no culture broth is withdrawn from the vessel. With these assumptions, the simplest mathematical model for the fed-batch culture is as follows (Yamane and Shimize, 1984):

$$dV_R/dt = F \qquad (4.43)$$

and Equation 4.4 becomes

$$dX/dt = X_o(F/V_R) + (dX/dt)_{growth} - (X/V_R)(dV_R/dt) \qquad (4.44)$$

Then from Equations 4.43 and 4.44,

$$dX/dt = X_o(F/V_R) + (dX/dt)_{growth} - FX/V_R \quad (4.45)$$

Equation becomes

$$dS/dt = S_oF/V_R + (dS/dt)_{reaction} - (S/V_R)(dV_R/dt) \quad (4.46)$$

$$= S_oF/V_R + (dS/dt)_{reaction} - FS/V_R \quad (4.47)$$

and Equation becomes

$$dP/dt = P_o(F/V_R) + (dP/dt)_{reaction} - FP/V_R \quad (4.48)$$

If the rate-law relationships are known for $(dX/dt)_{growth}$, $(dS/dt)_{growth}$, and $(dP/dt)_{reaction}$, then analytical or numerical integration can be used with these relationships and Equations 4.43, 4.45, 4.46, and 4.48 to solve for X, S, $V_R$, and P as functions of time, depending on the flow rate plan set for feed.

The main objective of using the fed-batch system is to control

substrate concentration. For this control, relationships may be derived from Equation 4.45 as follows:

$$dS/dt = F/V_R\ (S_o - S) + (dS/dt)_{reaction} \quad (4.49)$$

If

$$(dS/dt)_{reaction} = -Y_{s/x}\mu_{max}(SX/(K_m + S) - mX \quad (4.50)$$

Then, setting $(dS/dt) = 0$ for constant S,

$$0 = F/V_R\ (S_o - S) - Y_{s/x}\mu X - mX \quad (4.51)$$

or

$$F = CXV_R, \quad (4.52)$$

where $C = (Y_{s/x}\mu_m S/(K_s+S)+m)(1/(S_o - S)$, or $C = (Y_{s/x}\mu+m)/(S_o-S)$

The required feed rate then is a simple function of reaction mixture volume and cell concentration. The time relationship

for this system is complex, but it can be set up easily for computer control of feed rate without measurement of reaction volume or cell concentration once rate constants have been determined.

In the case of constant X, $X_0 = 0$, m=0

Eauation $dX/dt = F/V_R\ X_o + (dX/dt)_{growth} - FX/V_R$ (4.45)

become

$$0 = 0 + (dX/dt)_{growth} - FX/V_R \quad (4.53)$$

Since $(dX/dt)_{growth} = \mu X$ (4.6)

Then $FX/V_R = \mu X$, (4.54)

$F = \mu V_R$ (4.55)

In a repeated fed-batch cultivation, the cell and substrate concentrations at the beginning of a cycle are equal to that at the end of the previous cycle. Growth was assumed to follow the Monod kinetics.

$$\mu = \mu_{max} S/(K_s + S) \qquad (4.16)$$

$$\ln X/X_o = \mu t \qquad (4.35)$$

or $$t = (1/\mu)(\ln X/X_o) \qquad (4.35)$$

$$\mu_{max} = (\ln X_2 - \ln X_1)/(t_2 - t_1) \qquad (4.37)$$

$$t_d = \ln 2/\mu_{max} \qquad (4.38)$$

$$X_r = X_f \qquad (4.56)$$

and $$S_f = S_r = S_o \qquad (4.57)$$

$$P = Y_{p/s} \times \Delta S'_{total} + \Delta S_{total} \qquad (4.58)$$

$$\Delta S_{total} = N \times (S_r - S'_r) \qquad (4.59)$$

where $S'_r$ is the substrate concentration at the end of each batch and is measured by DNS method. N is the number of repeated batch in a fed-batch mode. $K_s$, $t_d$ and $\mu_{max}$ are constants, $X_r$ is the biomass at the beginning of first batch stage, $X_f$ is the biomass at the beginning of second stage, $S_o$

is initial substrate concentration in the bioreactor, $S_i$ is the substrate concentration at the beginning of second stage, and $S_r$ is the substrate concentration at the beginning of first stage, P is the concentration of sophorose lipids in the reaction mixture. $\Delta S'_{total}$ is the total consumption of substrates at the beginning of stationary phase, which is determined by DNS method (Miller, 1959).

The complex behaviour of cell growth and sophorose lipids production is hardly understood by measurements only. However, the knowledge of mathematical models leads to a better insight into the behaviour of the process. Moreover, an almost precise mathematical model offers the opportunity to research the optimization of the control strategies systematically, without the need of time consuming and expensive experimental studies.

In this work, the objective for the development of the fed-batch models is to design and predict fermentation process for sophorose lipids production. The above fed-batch models allow us to know what the behaviours of the cell growth and sophorose lipids production will be, and how to control substrate concentrations to obtain the maximum of sophorose lipids. Therefore, the above models are very important for the

design of appropriate experiments for sophorose lipids production. Sophorose lipids are secondary metabolites and start to accumulate when the cells reach stationary phase ($X_r = X_t$) under nitrogen limited conditions. For the above fed-batch models, we know that when the substrate concentration is kept constant at stationary phase ($S_o = S_t$), the consumption of substrate is used for sophorose lipids production. In a batch process, the production phase is short, due to the depletion of the carbon-energy source; the subsequent cell autolysis is rapid and severe. From the above fed-batch models, it can be seen that the desired metabolite is still being produced at the end of batch operation. The final production is increased by extending the culture time. Therefore, after transition from growth to synthesizing phase, it is important to maintain a concentration of the carbon-energy source by fed-batch mode where the microorganisms are semi-starved but where enzyme activity for sophorose lipids synthesis is highest.

By now, it should be clear to the reader that why we need to develop the above fed-batch models for fermentation processes. The next question is: how to apply those models to a fed-batch fermentation process or how to solve the above models

equations? To answer those questions we must give an example. The kinetic constants $K_s$, $t_d$, $\mu_{max}$ and $Y_{p/s}$ can be obtained from the experiments or from the literature (Aiba et al., 1973; Atkinson and Mavituna, 1991). Equation 4.18 also allows us to determine $K_s$ and $\mu_{max}$ by measuring $\mu$ and S. $t_d$ can also be determined by equation 4.38. The example for the solution of fed-batch model equations is given as follows:

Solution: (1) Let $K_s$ = 80 g/L, $t_d$ = 1 day. By equation 4.38, we have,

$$\mu_{max} = \ln 2/t_d = 0.693 \text{ day}^{-1}.$$

(2) Let $Y_{s/x}$ = 6, $X_{max}$ = 34 g/L, $X_o$ = 0. By equation 4.42, we have,

$$S_o = 6 \times 34 = 204 \text{ g/L}$$

If both sugar and oil are used as substrates, we have,

$$S_{o\text{-oil}} + S_{o\text{-sugar}} = S_o = 204 \text{ g/L}$$

From the mass balance equation (shown in equation 6.1) and literature (Inoue, 1988; Zhou et al, 1992; 1993), we know that,

$$S_{o\text{-oil}} = 104 \text{ g/L}, \text{ and } S_{o\text{-sugar}} = 100 \text{ g/L}$$

at different t, S can be measured by DNS method (Miller, 1959). Here we assume S on the basis of literature (Asmer et al., 1988; Inoue, 1988; Zhou et

al., 1992; 1993).

(3) By equation 4.16, $\mu$ is determined from S obtained in (2).

(4) By equation 4.35, X can be determined from $\mu$ obtained in (3).

X can also be obtained from literature.

(5) When sophorose lipids synthesis starts at stationary phase,

we have equation 4.56,

$X_r = X_{max} = X_f$.

(6) By the above fed-batch models, it is known that when cell growth stops ( stationary phase, $X_r = X_{max} = X_f$), we start to feed fresh substrates to keep substrate concentration constant, that is,

$S_f = S_r = S_o$

In experiments, because of foaming problem, we keep substrate concentration

$S_r = S_f = 80\% S_o$

(7) The production of sophorose lipids is calculated by equation 4.58. We assume that at the beginning of stationary phase,

concentration of oil = concentration of sugar.

$$P = Y_{p/s} \times \Delta S'_{total} + \Delta S_{total}$$

$$\Delta S_{total} = N \times (S_r - S'_r)$$

An example of the above solution for the fed-batch models is summarized in Table 4.1.

Table 4.1 A summary of The Data obtained from The Solution of Fed-Batch Models

| t (day) | S (g/L, Glucose) | $\mu$ ($day^{-1}$) | X (g/L) | P (g/L) |
|---|---|---|---|---|
| 0 | 100 | - | 0 | 0 |
| 1 | 80 | 0.3465 | 8 | 0 |
| 2 | 60 | 0.297 | 14.5 | 50.4 |
| 3 | 40 | 0.231 | 29 | 100.8 |
| 4 | 80 | - | 34 | 100.8 |
| 5 | 40 | - | 34 | 120.8 |
| 6 | 80 | - | 34 | 140.8 |
| 7 | 40 | - | 34 | 160.8 |
| 8 | 80 | - | 34 | 180.8 |
| 9 | 40 | - | 34 | 200.8 |
| 10 | 80 | - | 34 | 200.8 |

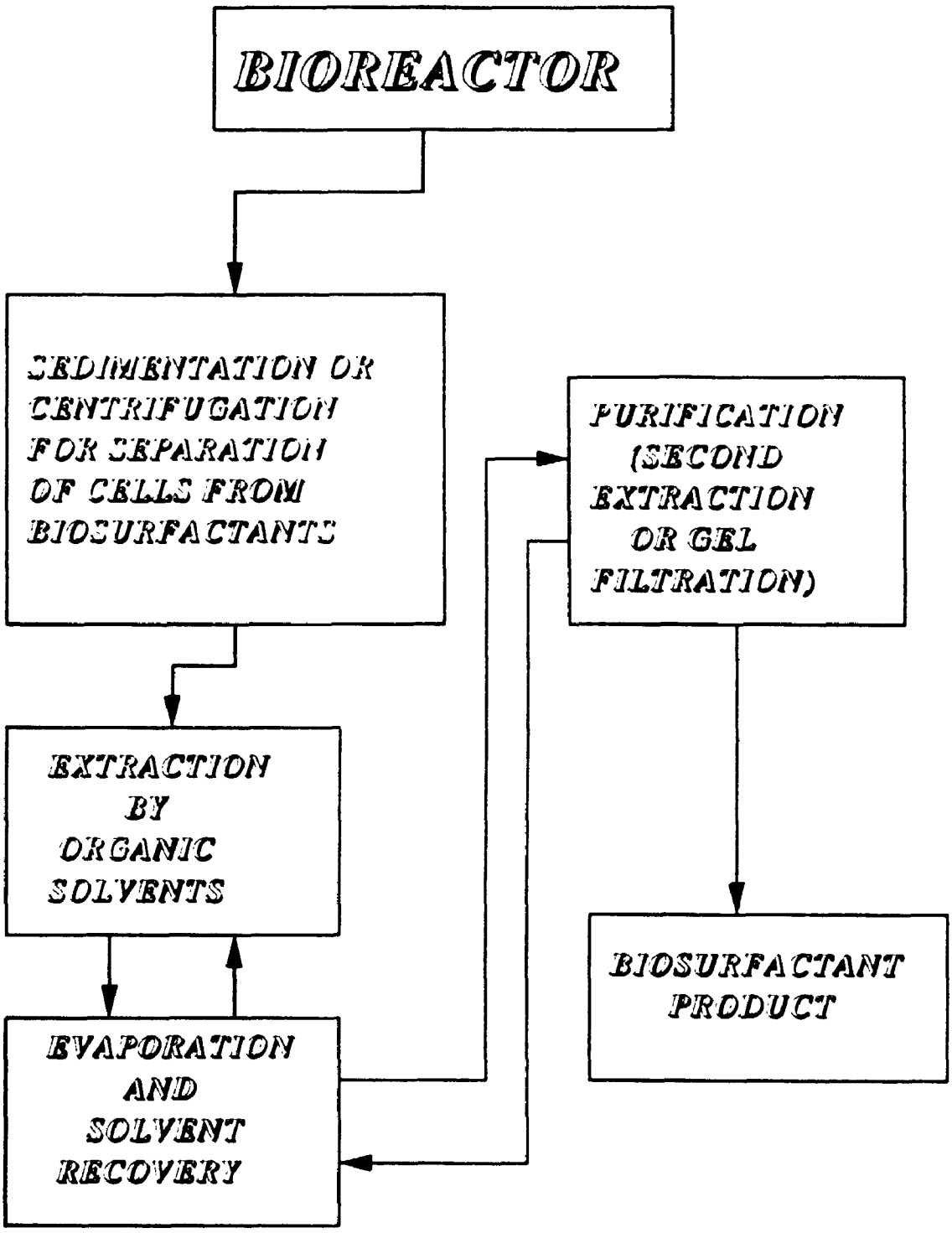
# 5. MATERIALS AND METHODS

A general scheme for the production of sophorose lipids is shown in Figure 5.1. The process involves culture maintenance, fermentation and isolation, which begins with growth on agar slants at 25°C for 2-3 days followed by suspension in sterile distilled water and inoculation into 100 ml of growth medium in 500 ml shake flask culture at 28°C for two days. 1% v/v culture was transferred into 1 L Bellco jar glass fermenters and a 20 L chemap fermenter (Type LF 7/14/20, serial No. NR 365). The analysis methods are given as following:

## 5.1 Culture Maintenance

The yeast *Torulopsis bombicola* ATCC 22214 used to produce sophorose lipids was purchased from the American Type Culture Collection. The *Torulopsis bombicola* ATCC 22214 was maintained on YM agar consisting of 0.3% Bacto-yeast extract, 0.3% Bacto-malt extract, 0.5% Bacto-peptone, 1% Bacto-dextrose, 2% Bacto-agar, made up to one liter with deionized water. The culture was transferred to a fresh agar slant to preserve viability every month at 4°C with caps loosely screwed on.

Figure 5.1 A General Scheme for The Production of Sophorose Lipids


BIOREACTOR
SEDIMENTATION OR CENTRIFUGATION FOR SEPARATION OF CELLS FROM BIOSURFACTANTS
PURIFICATION (SECOND EXTRACTION OR GEL FILTRATION)
EXTRACTION BY ORGANIC SOLVENTS
BIOSURFACTANT PRODUCT
EVAPORATION AND SOLVENT RECOVERY

## 5.2 Optimization of Cultivation Conditions

In this work, the cultivation conditions such as temperature, pH, the sources and concentrations of nitrogen, aeration and agitation, carbon sources, and concentrations of substrates were optimized to achieve maximal production of sophorose lipids. It is assumed that interactions among cultivation conditions can be neglected. It is possible to develop mathematical models to determine the true optimal cultivation conditions for sophorose lipids production. However, the difficulties encountered during the mathematical modelling of this process arise from poorly understood interactions among foaming, color and smell of sophorose lipids, limitation of trace elements, sterilization, yeast stress, microbial physiology, genetics, and so on. Therefore, the optimal conditions obtained with the above assumptions may not be true optimum but they are applicable and acceptable in the process of sophorose lipids production on the empirical basis (Cooper and Paddock, 1983; Yamane and Shimize, 1984).

## 5.3 Composition of Media

The production of sophorose lipids is controlled by medium

composition. It is essential to optimize the growth medium for the maximal production of sophorose lipids. The optimization of medium formulation has been performed by changing nutritional parameters, individually or in combination. Yeast extract and peptone were purchased from Difco Laboratories, Detroit, Michigan. Corn steep liquor was obtained from Casco, London. Glucose, galactose, lactose, olive oil, safflower oil, sunflower oil, cottonseed oil, and soy bean oil were purchased from Sigma Chemical Co., St. Louis, Missouri. Cheese whey and deproteined cheese whey were obtained from Ault Foods, London, Ontario. Crude degummed canola oil was obtained from CanAmera Foods, Russell, Manitoba. The optimized medium for sophorose lipids production is shown in Table 5.1.

TABLE 5.1 The Optimized Medium for Sophorose Lipids

| | |
|---|---|
| $KH_2PO_4$ | 0.1% |
| $MgSO_4.7H_2$ | 0.5% |
| $FeCl_3$ | 0.01% |
| NaCl | 0.01% |
| Yeast extract | 0.4% |
| Urea | 0.1% |
| Canola oil | 10.5% |
| Glucose | 10% |

The pH was adjusted to 4.5 by addition of a 1.5M HCl solution and the medium was always sterilized with all glassware together by autoclaving at 121°C for 20 minutes. In the case of cultivation in the larger fermenter, the time of sterilization was increased to 30 minutes. There was no adjustment of pH during cultivation and pH decreased towards the end of cultivation to between 3.2 and 3.4.

## 5.4 Inoculum Preparation

A loopful of the cream coloured culture from an agar slant was transferred into a 500 ml Erlenmeyer flask containting 100 ml of medium described previously. The culture was incubated aerobically for one day at 30°C on a rotary shaker (250 rpm). 20 ml of culture broth was transferred to a Bellco jar glass fermenter (Bellco Glass Inc.) containing 700 ml medium (1%v/v inoculum). For the inoculation of the 20 L bioreactor (Chemap), 450 ml (1% v/v inoculum) of culture broth in a Bellco jar glass fermenter was used.

## 5.5 Bioreactor Studies

### 5.5.1 Shaker Flasks

Shaker Flask cultures were grown aerobically in 500 ml Erlenmeyer flasks containing 100 ml of medium. The shake flasks were incubated on a New Brunswick Controlled Environment Incubator Shaker with temperature control at 30°C and 250 rpm.

### 5.5.2 Small Scale Fermenters

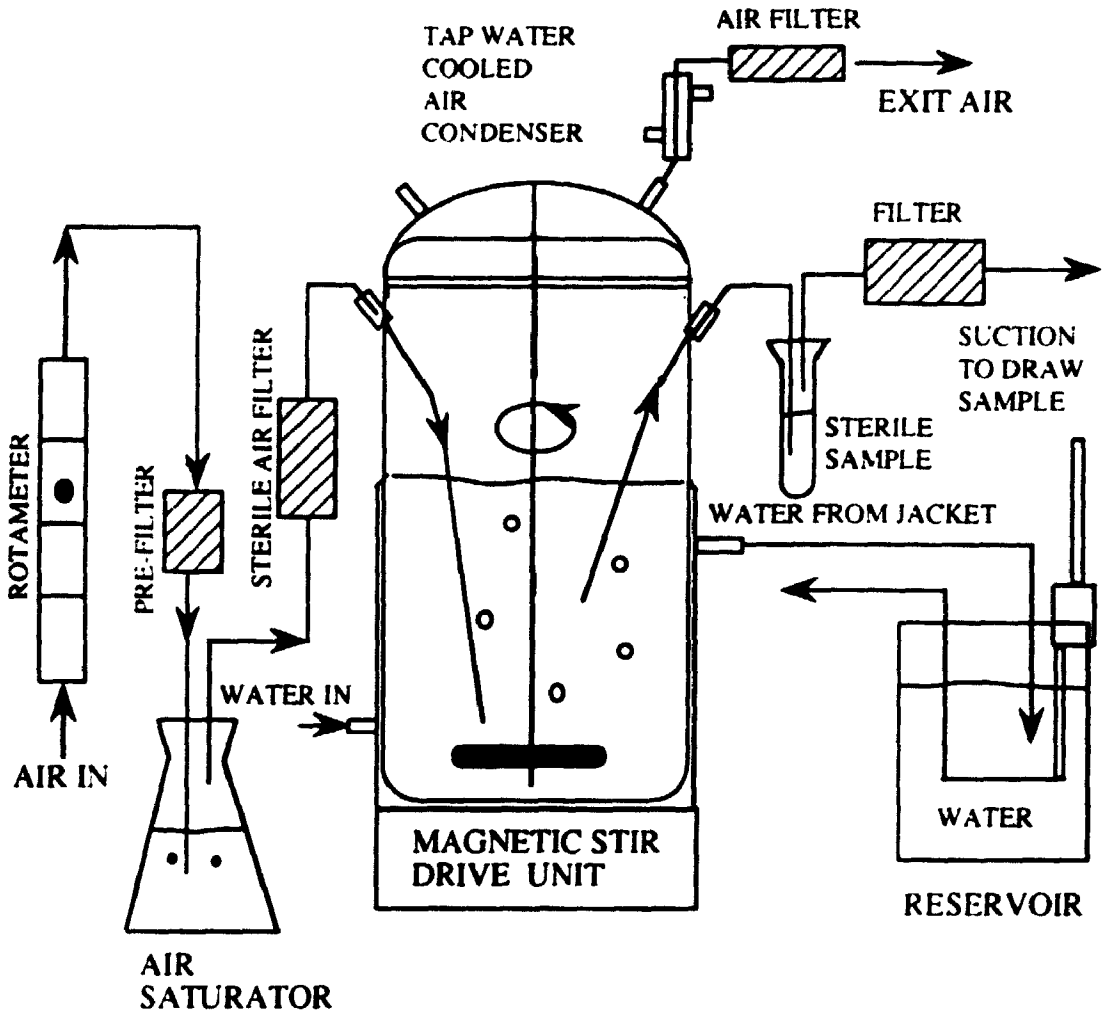
Small scale batch fermentation was performed using Bellco jar glass fermenters containing 700 ml of the medium. The fermenters were magnetically agitated with a Bell-Stir Magnetic stirrer and a magnetic plate at a speed of 450 rpm shown in Figure 5.2. Aeration was set at 2 volume of air per volume of liquid per minute (vvm). The air supplied to fermenters was filtered by a glass column packed with cotton to a depth of 10 cm. The temperature was maintained at 30°C using an external water bath.

### 5.5.3 Large Scale Fermenter

For large scale fermentations, a 20 l Chemap laboratory Pilot Scale fermenter (Type LF 7/14/20, serial No. NR 365) was used with the mechanical antifoam control. The specifications of the fermenter are given in Appendix 3. The fermenter consists of a stainless steel vessel and headplate. Working volume with this fermenter was 15 l of medium. An agitation of 450 rpm was provided by three sets of 4-blade turbine impellers (only two sets of the blades were actually submerged in the culture broth). The aeration was maintained at 0.9 vvm. The

**Figure 5.2 Apparatus for Batch Mode Cultivation by *Torulopsisbombicola* in A 1-L Bellco Jar Glass Fermenter**

# 1 L BELLCO FERMENTOR



temperature was controlled at 30°C and the initial pH was set at 4.5. The fermenter vessel was filled with medium and substrates through a free connection, sterilizing by steam at 121°C for 30 minutes as described in the manual provided by the manufacturer. After sterilization, fermenter was cooled immediately by cooling water to 30°C. Inoculation was carried out with 100 ml of 2-day culture through a steam sterilizeable inoculation port. Samples were taken periodically through a sterilizeable sampling port to monitor biomass, lipid production, substrate, nitrogen, and pH.

## 5.6 Analytical Methods

### 5.6.1 Biomass Determination

At regular time intervals, samples were withdrawn for analyses. The biomass concentration was determined on the basis of its dry cell weight. A 5 ml aliquot of culture broth was mixed with 10 ml of MetOH/$CHCl_3$ (10:1). The mixture was shaken and centrifuged at 2000g for 20 minutes to form a biphasic solution. The upper phase was discarded and the lower phase (solid) was washed with distilled water, placed in an oven at 105°C for 48 h to assure complete drying of the cell,

and then weighed.

### 5.6.2 Lipids Analysis

#### 5.6.2.1 Thin Layer Chromatography/Flame Ionisation Detector (TLC/FID)

The IATROSCAN TH-10 (Iatron Laboratories, Inc., Japan) equipped with an Integrator (Varian Model 4270; S/N 1761) was used for lipids analysis.

The CHROMAROD is a silica rod 0.9 mm diameter and 150 mm long, which has a sintered coating of active adsorbent mixed with glass powder. The coating is uniform throughout the rod's length and has a mean thickness of 75 $\mu$. Thin layer separations can be performed on the CHROMAROD in a manner similar to that used in conventional TLC. Rf values are approximately the same as for plates using the same absorbent.

Quantitative detection of the separated components is obtained by passing the CHROMAROD through a Flame Ionisation Detector (FID). The heat of the hydrogen flame also cleans and re-activates the CHROMAROD.

### 5.6.2.2 Extracellular Lipids Analysis

The sophorose lipids (extracellular lipids) were extracted from 5 ml of culture broth with 10 ml ethyl acetate, thoroughly shaking and centrifuging (2000 g) for 20 minutes. The lipids extract was separated into sophorolipid fractions by thin layer chromatography plates (TLC) on Bakerflex sheets Silica gel IB2-F (J.T. Baker) using chloroform/methanol/water (65:15:2, v/v/v) as the developing solvent. After development, the developed plate was dried and sprayed by α-naphthol to identify the monosophorolipids. The sophorose lipids classes were identified by comparing the spots with the standards (Zhou et al., 1992).

Two methods (A and B) were used for quantitative estimation of sophorose lipids. Method A involves the use of thin layer chromatography/flame ionization detector (TLC/FID). As described above, TLC is performed on a thin rod of refractory and chemically stable material having a bonded, sintered partition medium as an outer coating. 1 μL of sample was applied near to one end of the rod and then developed using the solvent system chloroform/methanol (90:4). The rods were dried to remove the solvent and passed through a FID where the

components separated by the development are detected. The substances on the rod are ionized by passage through a hydrogen flame and the ions produced generate a change in the current between the burner and the collector electrode and then transferred into the recorder. The area under a recorded peak is directly proportional to the concentration of the material component on the rod. Peaks of sophorolipids on TLC/FID were identified by comparison of TLC and TLC/FID of different fractions after partial separation on a SIP-PAK Silica cartridge (Waters). 0.5 ml of sample of isolated SLs in ethyl acetate were applied on SIP-PAK cartridge, washed with hexane and eluted by the TLC solvent mixture. The calibration of FID detector was done by measuring a set of different SLs isolates, correcting for fat content and calculating an average. TLC/FID allows to distinguish between highly hydrophobic diacetyl-SLs (SL-1) and more hydrophilic groups of monoacetyl-SL and/or SL without acetyl, on which basis the two SL groups were quantified.

Method B involves a solvent extraction of sophorolipids with ethyl acetate, separation of lipids and solvent, and drying. A 50 ml sample of fermentation broth was centrifuged at 5000 rpm for 20 minutes. The supernatant was removed and placed

into a 250 ml separatory funnel. 25 ml distilled water and 100 ml ethyl acetate were added to the cell pellet which were resuspended and centrifuged. The supernatant was placed in the same separatory funnel as above. The mixture of the broth and ethyl acetate forms two phases. The upper phase was transferred into a round bottom flask. The lower phase was repeatedly extracted with an equal volume of ethyl acetate three times for complete recovery of sophorose lipids. The round bottom flask was placed on a roto-evaporator (Buchi RotoVapor-R) and the ethyl acetate was evaporated under vacuum at 80$^{0}$C. The residue was washed using hexane three times to remove fatty acids and then dried in the oven until constant weight.

TLC/FID offers an advantage of very small amounts of samples needed for estimation of sophorose lipids and complete separation of sophorose lipids from the oils.

5.6.2.3 Intracellular Lipids Analysis

The intracellular lipids were determined by extraction of biomass with chloroform/methanol (2:1) after extraction of sophorose lipids. The method for quantitative estimation of

intracellular lipids was the same as that for sophorose lipids, but chromatorods were developed in petroleum ether/ether/acetic acid (90:10:1).

### 5.5.3 Sugar Determination

The sugar concentrations were determined by the phenol test method (Dubois et al., 1956). The test was conducted by mixing 1.0 ml fermentation broth with 1.0 ml of 5% (w/v) phenol (Sigma Chemical Co., St. Louis, MO) in distilled water. This mixture was vortexed for 30 seconds followed by rapid addition of 5.0 ml concentrated sulfuric acid. The tubes were vortexed for 30 seconds and incubated at room temperature for 30 minutes. The absorbance was determined at 490 nm with a Varian DMS 90 double beam spectrophotometer.

Another method for the determination of sugars was done in the water phase after extraction of sophorose lipids using the Dinitrosalicylic (DNS) method (Miller, 1959). The composition

of the aqueous reagent for this method is summarized below:

| | |
|---|---|
| Sodium Hydroxide | 10.0 g/L |
| 3,5-dinitrosalicylic acid | 10.0 g/L |
| Phenol | 2.0 g/L |
| Sodium Sulphite | 0.5 g/L |
| Sodium Potassium Tartrate | 200.0 g/L |

The procedure for the DNS determination is as follows:

1. Secure a sample free of biomass
2. Dilute the sample to obtain sugar concentrations in the range 0.0 to 2.0 g/L
3. To a 1 ml sample add 3 ml of the DNS reagent
4. Vortex the solution for ten seconds
5. Place the solution in boiling water for five minutes
6. Cool the solution and add 20 ml of distilled water
7. Vortex the solution for ten seconds
8. Read the absorbance of the solution at 600 nm against the 0 g/L blank
9. Construct a calibration curve using known standards

### 5.6.4 Surface and Interfacial Tension Measurements

The measurements of the surface and interfacial tensions were done with a Fisher Autotensiomat which is a modified duNoüy surface tensiometer equipped with a motorized sample stage and a sensitive strain gauge connected to a platinum ring as shown in Appendix 5. To measure surface and interfacial tensions, the Autotensimat surface tension analyzer employs a silicon strain gauge. This gauge is fixed at one end to the balance beam and the transducer provides a force-summing displacement signal that is proportional to the force or weight measured. There is a simple linear relationship between measured force (F=ma) and surface tension ($\Upsilon = F/2L = ma/2L$). Where m = mass measured in grams, a = acceleration of local gravity usually designated as 980.0 $cm/sec^2$ , and 2L = twice ring circumference in centimeters.

Calibration for readout on either the panel meter or an accessory recorder is easily and quickly accomplished by using the standard platinum-iridium ring and one of the seven analytical balance weights supplied with the instrument.

The sample stage was lowered at 2.0 cm/min for all studies.

Measurements were made at room temperature of 20$^{0}$C. 50 ml of sample was placed in 250 ml glass beaker and the ring was immersed in the aqueous phase. The surface tension was monitored as the ring passed through the liquid-vapour interface.

Interfacial tension measurements are made in a manner similar to surface tension measurements. In fact, the procedure differs only in that the less dense liquid is carefully poured on the surface of the more dense liquid after the ring is submerged in the latter. The results of the surface and interfacial tensions of sophorose lipids obtained from the experiments are shown in Appendix 5.

Each experiment was repeated five times and the results were reported as averages. Statistical evaluation of the data was carried out through analysis of variance using the randomized block design. Comparison of the means was assessed using the Least Significant Difference test (Kreyszig, 1988). The error level is about 1%.

## 5.7 Fed-Batch Mode Studies

Operating a batch reactor involves a large series of preliminary activivities including cleaning, filling with nutrient suspension and, generally, sterilisation of the reactor. After inoculation the bioprocess proceeds through the lag, exponential and stationary phases. The death phase marks the end of the process. The cell mass and /or product are harvested, and the reactor is dismantled. To obtain a second batch of cell and /or product mass requires passing through the entire cycle of events again. Thus, the batch reactor goes through a considerable amount of down-time (time in which the reactor is not operational).

In contrast, the continuous stirred tank reactor (CSTR) does not incorporate the need of restarting a new process after completion of the old one (once started CSTR can operate, theoretically, for indefinite lengths of time and can be run with fairly high biomass concentrations). However, the CSTR operates within a characteristic constant environment. Moreover, the CSTR has the disadvantage of operational complexity (e.g. sterility). The fed-batch process is developed to meet both the requirement of continuous operation

and of a continuously changing environment.

The fed-batch reactor (FBR) is a batch reactor to which, when the nutrients approach depletion, fresh nutrients are added. In other words, the reactor is fed. It is assumed that the concentration of the nutrients added is so high that volume changes are negligible. Periodic addition of fresh culture medium to batch cultures replenishes the nutrients and limits the concentration of waste products.

When a portion of a "fed-batch culture" is withdrawn at intervals and the residual part of the culture is used as an inoculum for the next fed-batch culture, the whole system of operation is called "repeated fed-batch culture". For experiments on repeated fed-batch culture, we used 500 ml shake flasks containing 100 ml of medium containing 10.5% canola oil, 10% glucose and 0.4% yeast extract. After three days growth when the cells had reached stationary phase, 10 ml of fresh medium was added to the culture. Feeding was continued at this rate daily for six more days. A sample was withdrawn at intervals for analysis of cell growth and sophorose lipids production and the results were compared to those found for a 100 ml batch culture which was inoculated at

the same level but not fed. It was shown that the concentration of sophorose lipids in the fed-batch culture was much higher than the level found in the unfed culture. In addition, the production phase of the fed-batch culture was twice as long as that of unfed culture. Experiments were also carried out in the 1-L fermenter. After three days growth when the cells reached stationary phase, 10 ml of fresh medium containing 10.5% canola oil or 10% glucose was added to the culture. Feeding was continued at this rate daily for six days.

## 6. RESULTS AND DISCUSSION

### 6.1 Batch Mode Experiments

In a batch microbial process, all nutrients required during one run of cultivation, except for molecular oxygen are added to the medium before cultivation is started, the final products are only removed at the end of each batch. This section deals wih the possible effects of the cultivation conditions on the cell growth and sophorose lipids production in submerged culture.

The first effect is strongly related to the containment in which growth takes place. Agitation and aeration must meet the requirements for practical performance of the process, e.g., the exclusion of foreign cells (sterility). Temperature and pH play an important role in the cell qrowth and sophorose lipids production. The second effect is medium composition for the cell growth and sophorose lipids production. The availability of neccessary nutrients in media is decisive for the cell. In other words, mass balance and bioreaction kinetics are governed by the chemical nature of the nutrient solution.

Many recipes for media have been developed and described in the literature for yeast cultivation (Suomalainen and Oura, 1971). However there is no medium suitable for all cases. To create a medium for yeast cultivation, one could examine examples in the literature and then add or omit components according to the maximum possible or obtained production rate. The chemical requirements for growth and lipid production of *Torulopsis bombicola* comprise the carbon sources, energy sources, water, nitrogen sources, mineral and vitamins. The search for economically attactive alternative raw materials plays an important part of overall economics because raw materials can account for 40-50 % of the final product cost.

The production of sophorose lipids is controlled by medium composition. Therefore, it is essential to optimize the medium composition for maximal lipids production by using low-cost materials.

The prospects for large scale production of biosurfactants by the yeast *Torulopsis bombicola* largely depend on identifying cheap and abundant feedstocks. The objective in the commercial production of sophorose lipids can be described as "optimum quantity of sophorose lipids at minimum cost". In the bulk product market, production costs are influenced by the price of the feedstocks and other raw materials. The yield of sophorose lipids is also very important for the overall cost, for example, although biosurfactants from *corynebacterium fascians* can be produced on sucrose or hexadecane, yields were over 20 times higher on the latter, offsetting the cost of the hydrocarbon. Various carbon sources can be used for the sophorose lipids production including hydrocarbon, carbohydrate, and vegetable oil sources. It was found that the yeast *T. bombicola* produces a large amount of sophorose lipids from vegetable oils or carbohydrates, and can utilize waste streams such as cheese whey. The optimal yields were obtained when both carbohydrate and vegetable oil were used as

substrates.

Waste streams are the potential source of substrates for sophorose lipids production because they can be obtained either at little or no cost. In addition, waste treatment costs can be offset by the production of valuable products. For example, considerable amounts of whey (4-5% lactose content) are discarded as waste during cheese processing. The biochemical oxygen demand (BOD) value commonly around 60,000-70,000 mg/L results mainly from the lactose present, causing considerable pollution. To utilize the lactose effectively, a chosen organism must be able to consume both the lactose and its breakdown products, glucose and galactose. *T. bombicola* can be such an organism. A wide variety of sugar, nitrogen, fat and oil substrates were examined as potential feedstocks to obtain very effective carbon sources for sophorose lipids production.

The purpose of this study is to establish the culture conditions for optimal production of sophorose lipids by *Torulopsis bombicola*. The results are presented and discussed as follows.

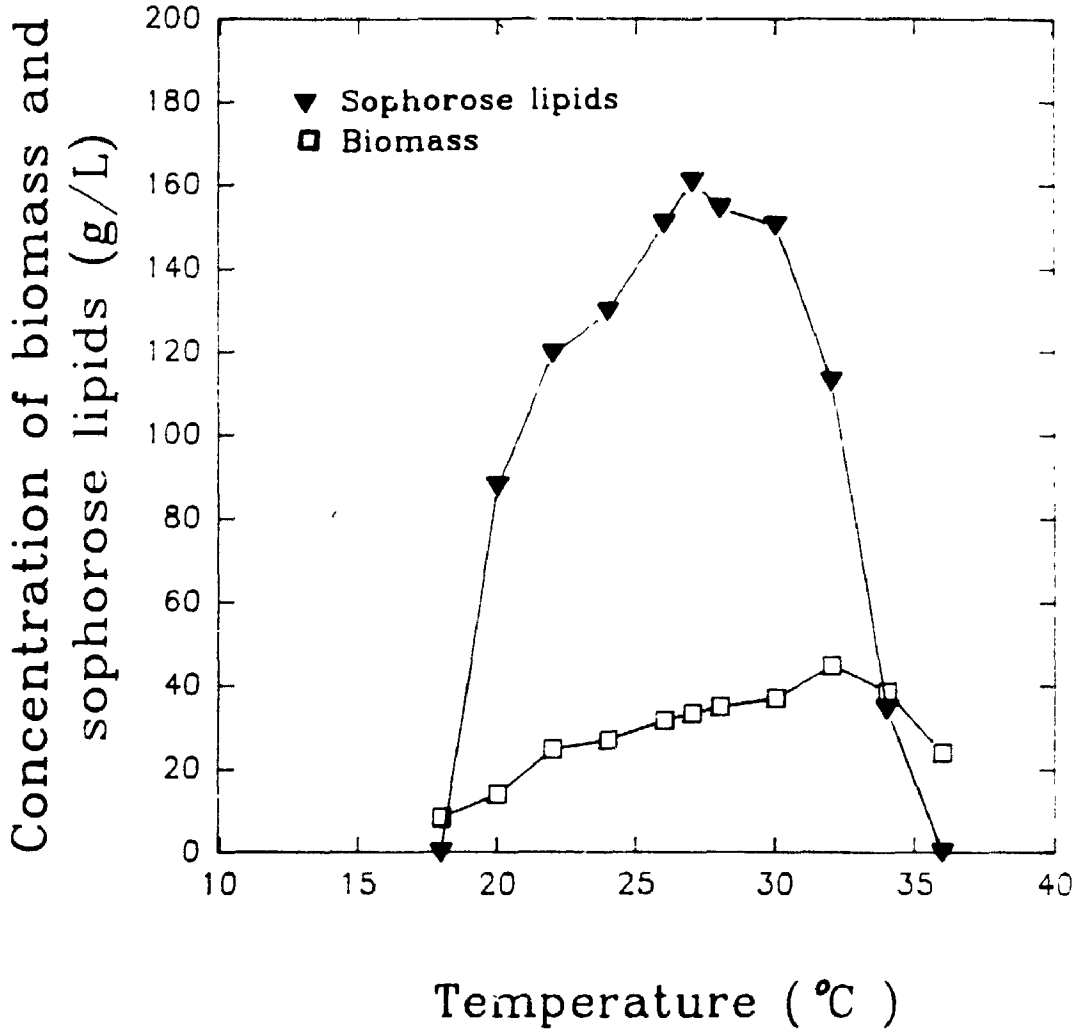
### 6.1.1 Effects of Temperature and pH

Temperature is one of the most important environmental parameters influencing all activities of microorganisms. Yeasts may be classified on the basis of their temperature limits for growth. The terms "psychrophile", "mesophile" and "thermophile", have been used to classify microorganisms into different thermal domains. Obligate psychrophilic yeasts are defined as yeasts with an upper temperature limit for growth at or below 20°C. The term "thermophilic" as applied to microorganisms is usually restricted to those organisms that are capable of growth at temperatures at or above 50°C. It is proposed that all yeasts that are not listed in the above cases be classified as MESOPHILIC. Yeast *Torulopsis bombicola* belongs to this group.

Temperature affects growth rates, enzyme activities and product production of yeasts. In general, growth of yeasts is optimal in the region of 20-30°C for mesophilic and facultative species. Maximum temperatures at which growth occurs for these species are in the range of 30-45°C. Obligate psychrophilic yeasts are unable to grow above a temperature of 20-30°C.

A series of shaking flasks and 1-L fermenters using medium containing 10% glucose and 10.5% canola oil were inoculated with the yeast *Torulopsis bombicola* and cultivated at different temperatures in the range of 20° to 40°C at 450 rpm (250 rpm in a rotary shaker) to study the effect of temperature on the biomass and sophorose lipids production. No significant differences between the two types of bioreactors were noted in sophorose lipids yields. The results obtained from 1-L fermenters are shown in Figure 6.1. It was found that increasing the cultivation temperature from 20 to 40°C significantly affected biomass and sophorose lipids yields. Higher temperatures were beneficial in enhancing biomass from 20 to 32°C. A maximal biomass of 48 g/L was obtained at 32°C. Above 32°C, biomass decreased with increase in temperature. Sophorose lipids production increased with increasing temperature in the range of 20°C to 27°C, then decreased above 27°C. Sophorose lipids production diminished rapidly at temperature above 38°C. The optimal temperature for sophorose lipids production was found to be 27°C, which was different from the optimal temperature for biomass at 32°C. A compromise between the optimum for growth and the optimum for lipids production was 30°C. Increases of the temperature above 38°C ultimately killed yeast cells. It was found that a 95% kill of

Figure 6.1 The Effect of Temperature on The Cell Growth and Sophorose Lipids Production in 1-L Bellco Jar Glass Fermenter at 450 rpm and 2 vvm. Medium contains 10% glucose, 10.5% canola oil and 0.4% yeast extract


Concentration of biomass and sophorose lipids (g/L)
200
180
160
140
120
100
80
60
40
20
0
Sophorose lipids
Biomass
10
15
20
25
30
35
40
Temperature ( ℃ )

cells of yeast *Torulopsis bombicola* occurred in 45 minutes at 42°C, in 18 minutes at 45°C, and in 6 minutes at 52°C.

Enzymes are active only within a narrow range of pH. The optimum, however, is dependent on other factors such as time of reaction, temperature, type of substrates, substrate concentration, chemical properties of the medium in which the reaction is being carried out, ionic strength, type and source of the enzyme. Many microorganisms display an optimum pH for growth at around 7, with the majority favouring the pH range of 5-8. There are differences in pH between different locations covering a pH range of 5.1 to 6.3. Yeasts maintain their internal pH quite well in solutions with pH ranges from 3 to 7. The rate of yeast fermentation between 4 and 6, that is, over a 100-fold range of H ion concentrations, is almost constant. Lower pH levels minimize growth of contaminating bacteria, but enhance adsorption of coloring materials from the sugars.

Influence of pH on the cells growth and sophorose lipids production was investigated using a batch mode of cell cultivation in the culture medium containing 10% glucose and 10.5% canola oil at 30°C and 450 rpm under pH control using 1-

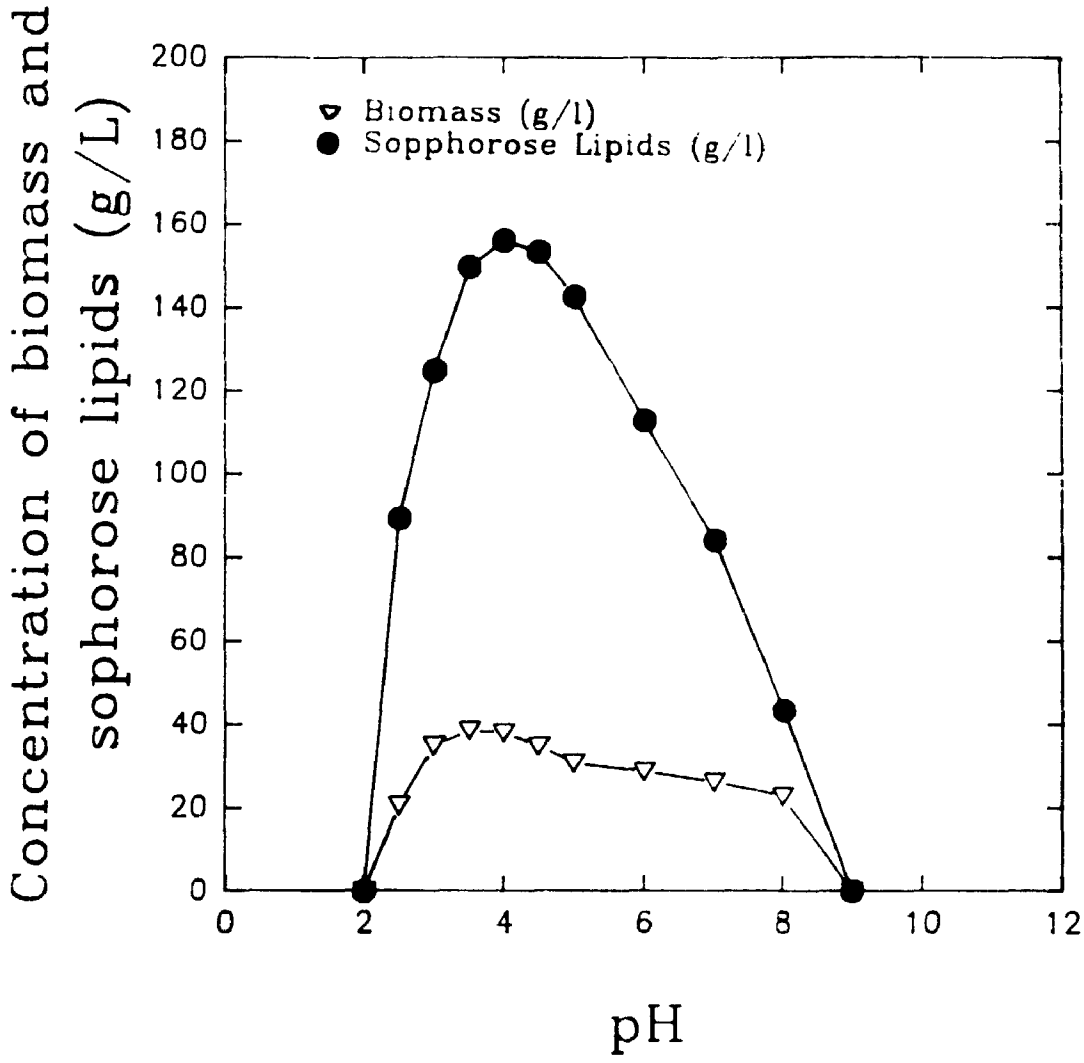
L fermenters. As shown in Figure 6.2, pH affected the cells growth and production of sophorose lipids by *Torulopsis bombicola*. When the pH was over 7 and below 2.5 in the cultivation medium, there was a distinct decrease in sophorose lipids production. Above pH 7.0, deprotonation of the carboxyl and amino groups occurred. The optimal pH for sophorose lipids production was about 4. A pH of 3-5.5 appears to be satisfactory.

With no pH control, it was observed that an intial pH of 6.0 was suitable for sophorose lipids production. pH decreased towards the end of cultivation to between 3.2 and 3.4 because of a high concentration of sophorose lipids produced in the medium. This observation agreed with a pH optimum of 4 for sophorose lipids production.

### 6.1.2 Effects of Sources and Concentration of Nitrogen

0After carbon source and aeration, the source of nitrogen supplied to a culture is probably next in importance in determining how the cell regulates its metabolic machinery. The nitrogen requirements for optimal production of sophorose lipids are species-dependent. They may be fulfilled by organic

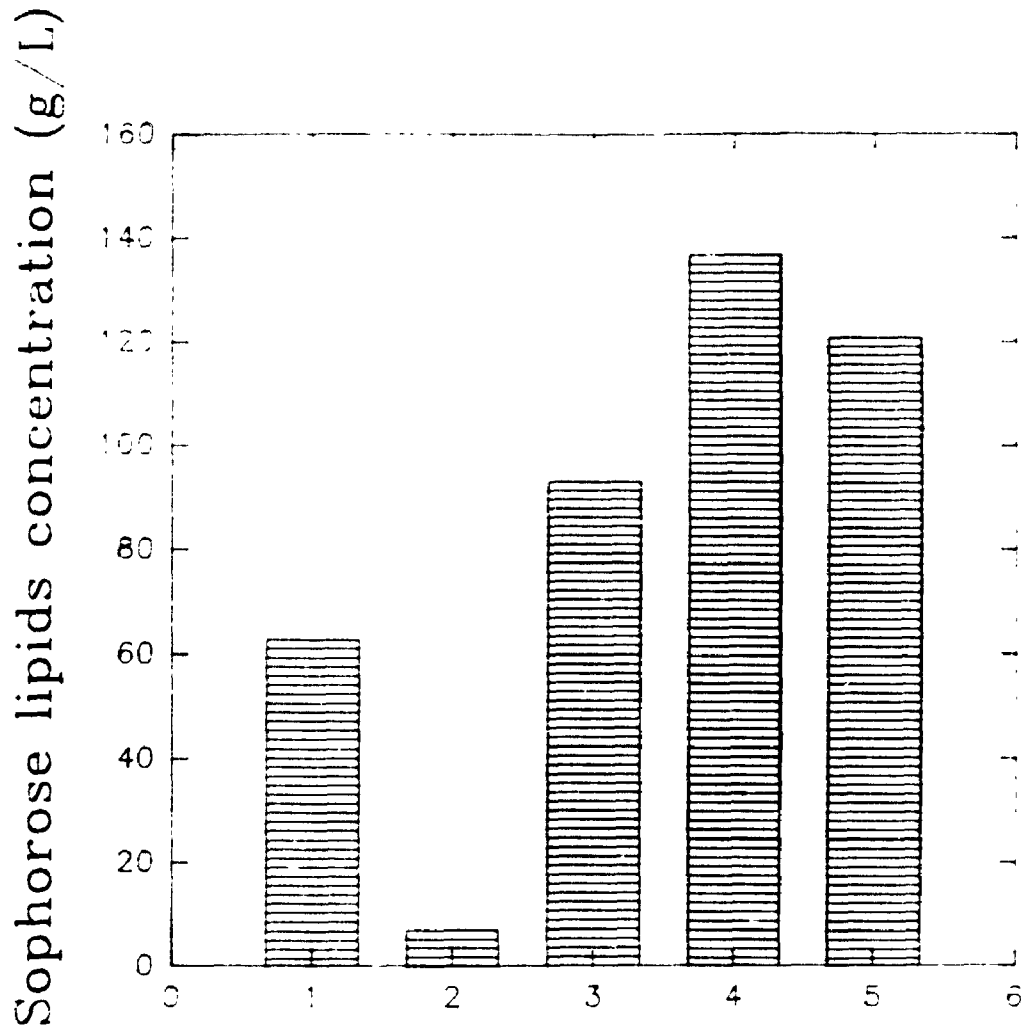
Figure 6.2 The Effect of pH on The Cell Growth and Sophorose Lipids Production in 1-L Bellco Jar Glass Fermenter at 450 rpm and 2 vvm. Medium contains 10% glucose, 10.5% canola oil and 0.4% yeast extract


Concentration of biomass and sophorose lipids (g/L)
Biomass (g/l)
Sopphorose Lipids (g/l)
200
180
160
140
120
100
80
60
40
20
0
0
2
4
6
8
10
12
pH

nitrogen sources, inorganic nitrogen sources and all forms of nitrogen. Organic nitrogen sources include corn steep liquor, peptone, fish meal, corn germ or gluten meal, urea, yeast, or yeast hydrolysates. Inorganic nitrogen sources include gaseous ammonia, ammonium hydroxide, ammonium sulfate, ammonium nitrate, and so on. Amino acids, purines, pyrmidines, proteins, DNA, and RNA are all forms of nitrogen.

Experiments with shaking flasks and 1-L fermenters were performed with several different nitrogen sources using the medium containing 10.5% safflower oil and 10% glucose at 30°C. An effort was made to find the proper nitrogen sources for sophorose lipids production at low cost. Evaluation of a number of nitrogen sources led to a determination that the yeast extract supported the highest sophorose lipids production as shown in Figure 6.3. The reason is that yeast extract is the water-soluble extract of autolyzed yeast and can provide all necessary yeast growth factors: amino acids, purines, pyrimidines and vitamins as well as minerals. Furthermore, yeast extract contains biotin which enhances sophorose lipids production. Biotin takes part in all major biochemical reactions that involve protein synthesis, nucleic acid synthesis, carbohydrate metabolism, the synthesis of

Figure 6.3 The Effect of Nitrogen Sources on Sophorose Lipids Production in 1-L Bellco Jar Glass Fermenter at 450 rpm, 2 vvm and 30°C. Medium contains 10% glucose, 10.5% safflower oil

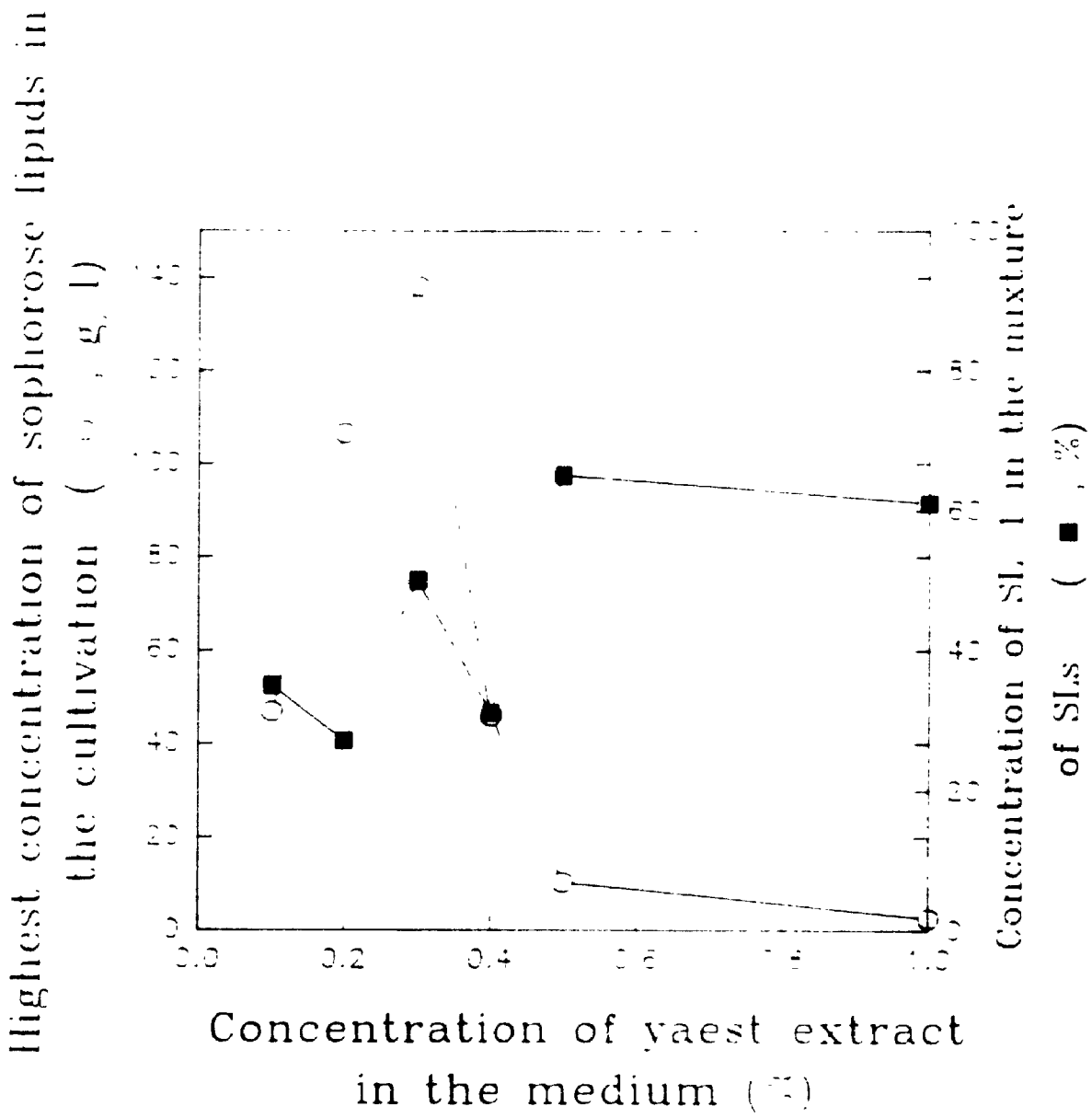



1. Urea 2. Ammonium Nitrate 3. Peptone
4. Yeast Extract 5. Corn Steep Liquor

fatty acids, and enzyme synthesis. Its deficiency is shown by poor growth and damaged plasma membranes. Yeast extract is shown to be a very good nitrogen source for sophorose lipids production. However the cost of it is too high. Nitrate was not as good a nitrogen source for biomass and sophorose lipids production as are the reduced nitrogen species. Peptone could be substituted for yeast extract, but gave lower biomass and sophorose lipids than the yeast extract. If urea was used instead of the yeast extract, growth was poor and very little sophorose lipids were produced. Yeast extract, corn steep liquor and peptone are good sources of free amino acids (lysine, leucine, isoleucine, valine, threonine, and phenylalanine), nucleotides, and vitamins. Attempts to replace yeast extract in the sophorose lipids production media were aimed at finding inexpensive nitrogen sources by using corn steep liquor because it is a by-product of the production of starch from corn which contains a variety of soluble peptide oligomers, inorganic salts, lactic acid and other low molecular weight products. The results showed that corn steep liquor gave a very good yield which was next to yeast extract. Corn steep liquor is a satisfactory nitrogen sources as a substitute for yeast extract from an economic viewpoint.

Experiments with 1-L fermenters containing 10% glucose and 10.5% safflower oil were carried out at 30°C and 450 rpm and different concentrations of yeast extract to determine the effect of nitrogen on the production of sophorose lipids. As seen in Figure 6.4, increasing yeast extract concentration from 0.1 % to 0.25 % led to an increase in the yield of sophorose lipids. The maximum yield of sophorose lipids was obtained at 0.25% of yeast extract. Above 0.25 % of yeast extract, the yields of sophorose lipids decreased with an increase in the concentration of yeast extract. Sophorose lipids are secondary metabolizes and start to accumulate when cells reach stationary phase under nitrogen limitation. Excess-nitrogen containing media presumably suppress sophorose lipids production. In this case, yeast consumes more substrates to produce biomass. However too low nitrogen concentration did not support sophorose lipids production because the growth medium lacked all necessary yeast growth factors: amino acids, purines, pyrimidines and vitamins as well as minerals which was provided by yeast extract. Studies carried out in shaken flasks with yeast extract as a sole source of nitrogen have shown that 0.5 % yeast extract stimulated sophorose lipids production compared with a medium with 0.1 % yeast extract (Cooper and Paddock, 1984). In our

Figure 6.4 The Effect of Concentration of Yeast Extract on Sophorose Lipids Production Using 10.5% Safflower Oil and 10% Glucose in 1-L Bellco Jar Glass Fermenter at 450 rpm, 2 vvm and 30°C.


Highest concentration of sophorose lipids in the cultivation ( , g/l)
Concentration of SL 1 in the mixture of SLs ( , %)
Concentration of yaest extract in the medium ( )
0.0
0.2
0.4

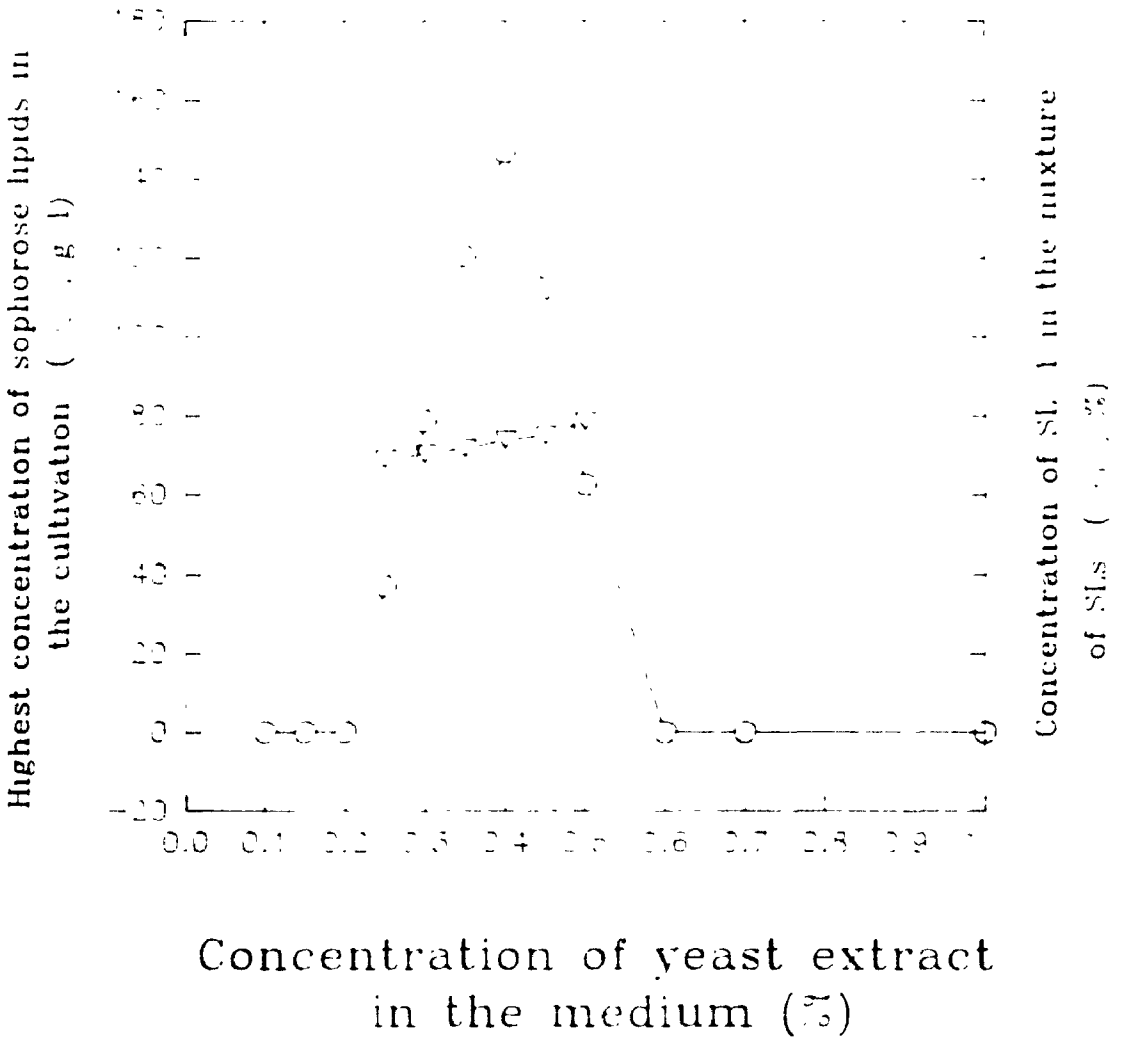
case when urea was used besides yeast extract as a nitrogen source, lower yeast extract concentration was more favourable suggesting that overall concentration of nitrogen in the medium is of importance. However, when content of nitrogen in urea (47 %) and in yeast extract (about 10 %) are compared, it becomes evident that 0.1 % of urea actually substitutes approximately 0.47 % of yeast extract and counts for about 2/3 of total nitrogen in medium. Additional increase in urea concentration is not likely desirable as the production of SLs and growth of *T. bombicola* on sole urea have been found poor. Moreover, deficiency in vitamins like pantothenic acid, thiamin, and pyridoxin that are supplied by yeast extract has been reported to decrease the amount of lipids synthetized by yeast (Zhou et al., 1992).

The composition of SLs mixture as characterized here by the content of SL-1 was constant during a batch cultivation. Nevertheless, yeast extract concentration influenced not only yield but also sophorose lipids composition. As shown in Figure 6.4, it seems that higher yeast extract concentration preferred production of SL-1.

It was confirmed that there was a profound effect of yeast

extract concentration on sophorose lipids production when the yeast *Torulopsis bombicola* was cultivated in the medium containing 10% glucose and 10.5% canola oil. Since canola oil is much cheaper than safflower oil, we use canola oil to replace safflower oil in the carbon mixture. Experiments with 1-L fermenters containing 10% glucose and 10.5% canola oil were carried out at 30°C and 450 rpm and different concentrations of yeast extract to determine the effect of nitrogen on the production of sophorose lipids. It was found that the concentration of nitrogen greatly influenced the yield of sophorose lipids. As shown in Figure 6.5, increase in concentration of yeast extract from 0.1% to 0.4% led to an increase in the yields of sophorose lipids. However, above 0.4% yeast extract, sophorose lipid biosynthesis diminished drastically because excess yeast extract inhibited sophorolipid production. Sophorose lipids are a secondary metabolite and start to accumulate under nitrogen limitation. Upon nitrogen depletion, carbon flow is diverted away from protein and nucleic acid synthesis and into sophorose lipids production. When nitrogen is nearly exhausted, the enzyme activities for sophorose lipids synthesis are switched on. However, it was found that high concentration of yeast extract had a beneficial effect on biomass production. This

Figure 6.5 The Effect of Concentration of Yeast Extract on Sophorose Lipids Production Using 10.5% Canola Oil and 10% Glucose in 1-L Bellco Jar Glass Fermenter at 450 rpm, 2 vvm and 30°C.


Highest concentration of sophorose lipids in the cultivation
Concentration of SL 1 in the mixture of SLs
0.0
0.1
0.2
0.3
0.4
0.6
0.7
0.8
Concentration of yeast extract in the medium (%)

observation is in good agreement with previously published reports (Zhou et al., 1992), confirming the lipids accumulation under nitrogen-limited condition.

### 6.1.3 Effects of Aeration and Agitat' n

Providing an adequate supply of oxygen is essential for the cell growth in culture. Oxygen is not very soluble in the culture media. Consequently, the demand for oxygen in dense cultures must be provided by a constant transfer of oxygen acros? the gas-liquid interface. In small scale cultures, the surface liquid can often provide adequate gas exchange. With increasing scales and in very dense cultures, however, the gas exchange accross the liquid surface becomes insufficient. Oxygen is required for growth of *T. bombicola* on carbon sources and this has to be filtered to free it from dust, bacteria and yeasts. Fibrous filters or membrane filters of controlled pore size are available which perform this function very efficiently. A set of 1-L fermenters experiments using medium containing 10% glucose and 10.5% safflower oil were performed at different aeration and agitation to study the effects of aeration and agitation on sophorose lipids production. The results are shown in Figure 6.6 and 6.7. From

Figure 6.6 The Effect of Aeration on Sophorose Lipids Production Using 10.5% Safflower Oil and 10% Glucose in 1-L Bellco Jar Glass Fermenter at 450 rpm and 30°C.

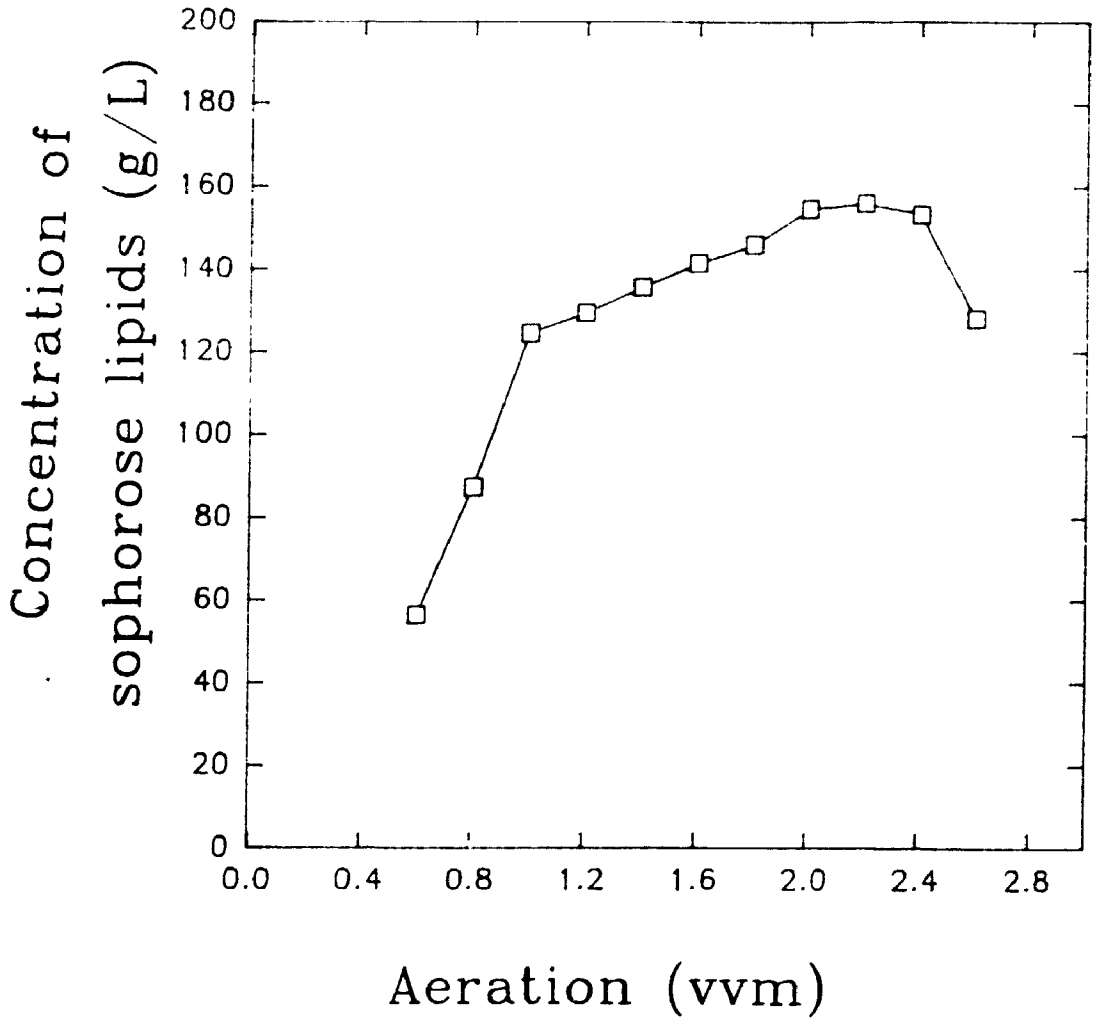

Concentration of sophorose lipids (g/L)
200
180
160
140
120
100
80
60
40
20
0
0.0
0.4
0.8
1.2
1.6
2.0
2.4
2.8
Aeration (vvm)

Figure 6.6. it could be seen that sophorose lipids production increased with increase in aeration and remained approximately the same above 2 volume of air per volume of liquid per minute (vvm). A high oxygen transfer rate was necessary for maximum yield of sophorose lipids. However, when too high agitation is employed, the yield decreases as a result of the loss of sophorose lipids. Another problem resulting from the high aeration rates employed is foaming.

The purpose of agitation is as follows:

- to suspend the cells uniformly throughout the working volume in order to prevent sedimentation or flotation
- to minimize temperature and concentration gradients in the bulk of liquid
- to maintain high mass transfer rates by the uniform distribution of all reactants

The mass transfer capacity of an agitation system is usually evaluated using the $k_La$ values for oxygen. As oxygen is of poor solubility in aqueous solutions (<10ppm), its supply to the cell can become critical in a rather short time (30 s). The influence of agitation on sophorose lipids production is

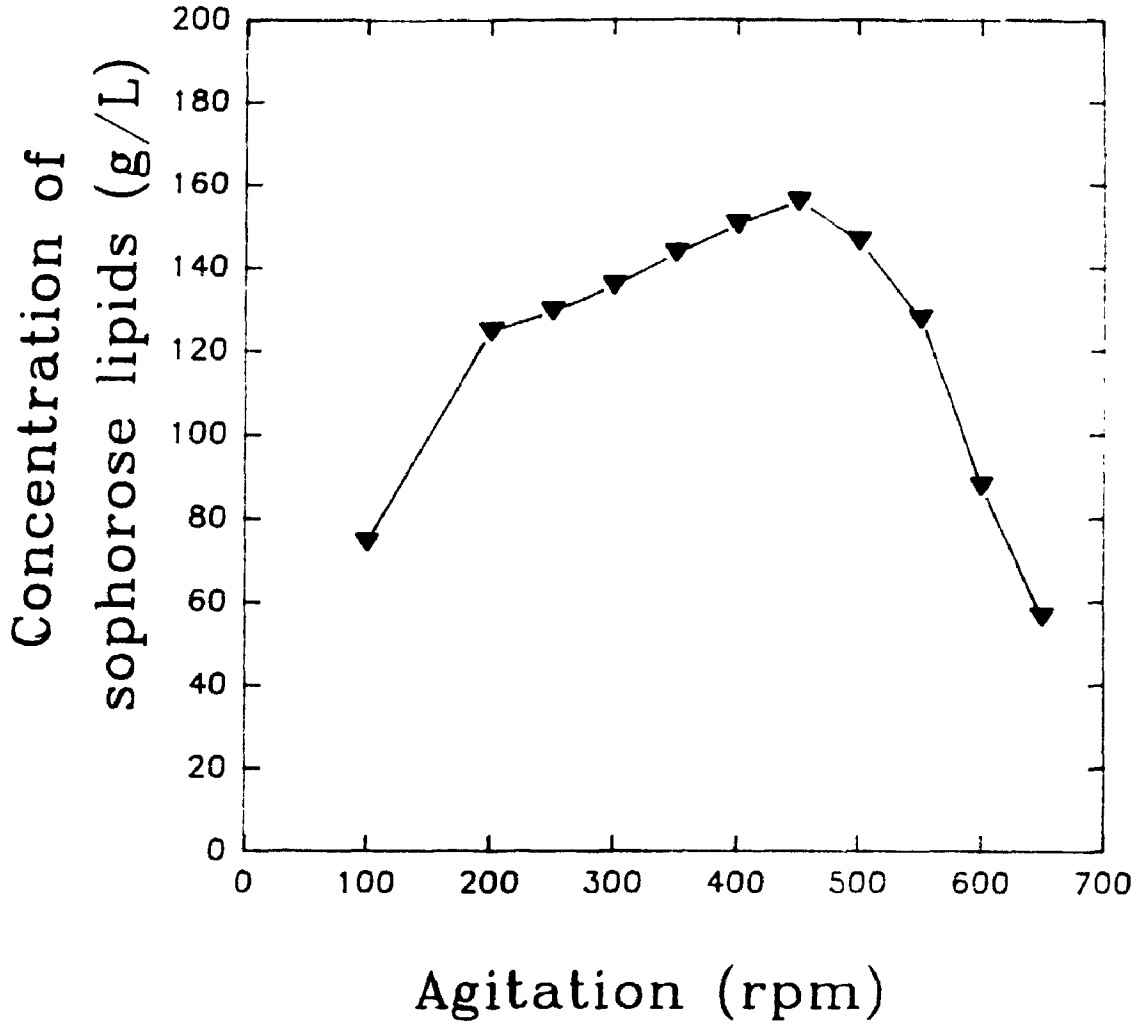
shown in Figure 6.7. It was observed that as the rate of agitation increased from 100 rpm to 450 rpm, sophorose lipids production increased. However, above 450 rpm, sophorose lipids production decreased with increase in the rate of agitation. Agitation had a major influence on the dispersion of the insoluble vegetable oils which could provide the cells with access to substrates. However, too high agitation resulted in cells lysis by shear induced and removal of microbes from the oil droplet surface, shearing of the hydrophobic substrate, surfactants and/or enzymes from the cell surface, and loss of sophorose lipids. The optimal rate for sophorose lipids production was obtained at 450 rpm.

### 6.1.4 Effects of Carbon Substrates

#### 6.1.4.1 Effects of Sugars

Carbon sources have a dual nutritional role: as a source of carbon and as a source of energy. In combination primarily with hydrogen, oxygen and nitrogen, they make up the major structural units of yeast; approximately 50% of yeast (dry weight) is carbon. The most widely utilized carbon sources are the sugars, though the particular sugars which may be

Figure 6.7 The Effect of Agitation on Sophorose Lipids Production Using 10.5% Safflower Oil and 10% Glucose in 1-L Bellco Jar Glass Fermenter at 2vvm and 30°C.


Concentration of sophorose lipids (g/L)
200
180
160
140
120
100
80
60
40
20
0
0
100
200
300
400
500
600
700
Agitation (rpm)

assimilated are variable within species and strain. Several different sugars including glucose, galactose, fructose, lactose, mannose and sucrose are used for the production of sophorose lipids by the yeast *Torulopsis bombicola* because these substrates can be obtained from renewable resources such as agricultural and forestry products at low cost. Since the yeast *Torulopsis bombicola* was isolated from bumblebee honey (Spencer et al., 1979), it is concluded that this yeast favors honey which contains about 50% glucose, about 50% fructose and a small amount of trace elements such as mineral matters. It is believed that those trace elements play a very important role in sophorose lipids production. Therefore honey is also used as a substrate for the production of sophorose lipids. The cell growth and production of sophorose lipids from fructose, glucose, galactose, honey, sucrose, maltose are shown in Figure 6.8 to 6.13. Figure 6.8 shows the time course of cultivation of *Torulopsis bombicola* from honey (12% in the medium) in the 1 L fermenter under optimum cultivation conditions. The concentration of biomass increased with time up to 3 days, then remained constant and decreased slightly after 7 days. Honey was almost exhausted after 7 days. SLs accumulated during both growth and stationary phase of the cultivation up to 7 days. A maximal biomass of 18.45 g/L and

a maximal sophorose lipids of 84.43 g/L were obtained. The accumulation of sophorose lipids is triggered by exhausion of nitrogen rather than the sugar. As shown in 6.8 to 6.13, the patterns of the cell growth and the production of sophorose lipids from honey, galactose, glucose, fructose, sucrose, maltose are quite similar. The comparison of the production of sophorose lipids from various sugars is shown in Figure 6.14. The highest concentration of sophorose lipids was obtained from honey. A slightly lower production of sophorose lipids was obtained when glucose was used as the sole carbon source. It should be noted that no growth was evident when lactose was used as the carbon source. The sugars produce sophorose lipids in order:

honey > glucose > maltose > sucrose > fructose > galactose

The sugar sources appears to be important for the production of sophorose lipids. The results show that honey is the most effective source of sugar for the production of sophorose lipids, followed by glucose. Honey contains a small amount of trace elements such as mineral matters which enhance lipids production. Sucrose and maltose give slightly lower production of sophorose lipids than glucose. However, honey is too

Figure 6.8 The Time Course of Cultivation of *Torulopsis bombicola* from Honey in 1-L Bellco Jar Glass Fermenter at 450 rpm, 2vvm and 30°C.
Medium Contains 12% Honey and 0.15% Yeast Extract

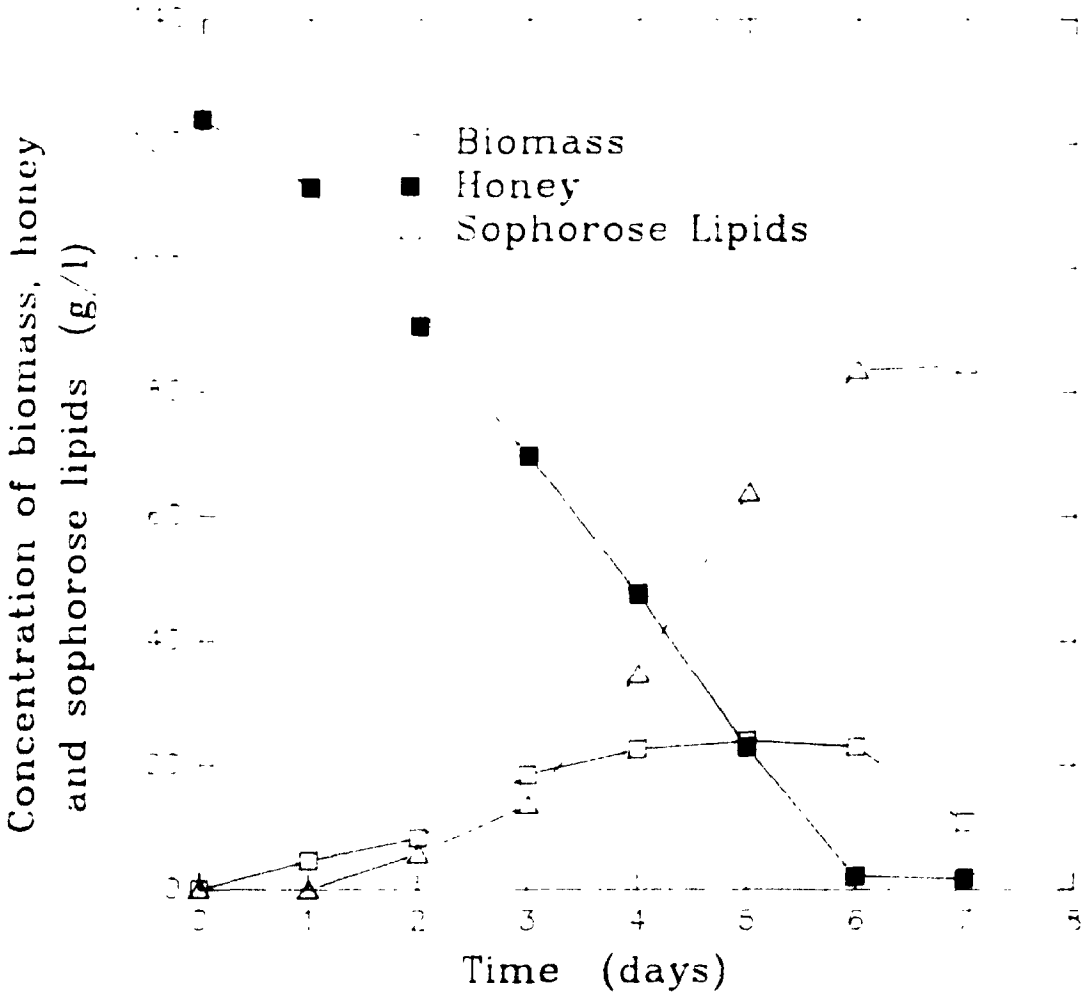

Biomass
Honey
Sophorose Lipids
Concentration of biomass, honey and sophorose lipids (g/l)
Time (days)
0
1
2
3
4
5
6

Figure 6.9 The Time Course of Cultivation of *Torulopsis bombicola* from Glucose in 1-L Bellco Jar Glass Fermenter at 450 rpm, 2vvm and 30°C.
Medium Contains 12% Glucose and 0.15% Yeast Extract

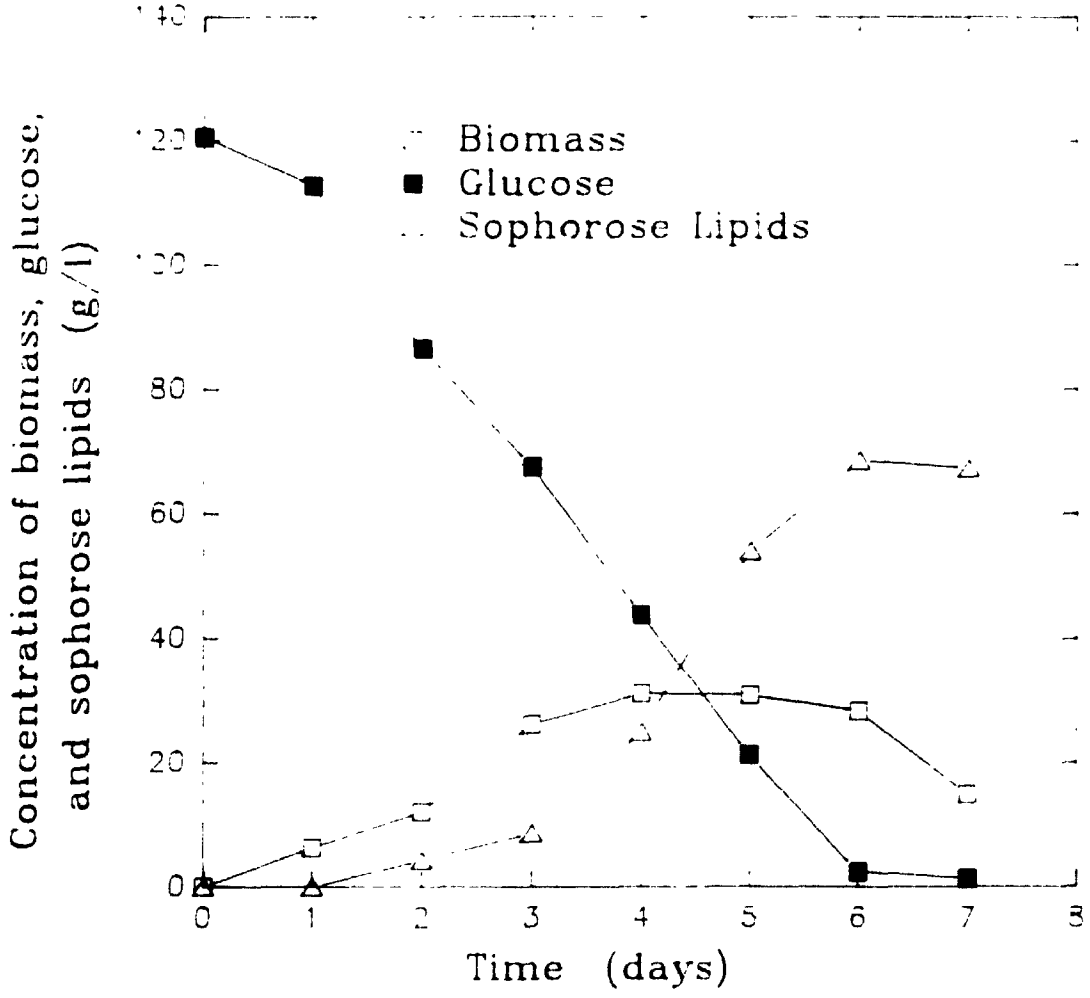

Biomass
Glucose
Sophorose Lipids
Concentration of biomass, glucose, and sophorose lipids (g/l)
Time (days)
0
20
40
60
80
0
1
2
3
4
5
6
7
8

Figure 6.10 The Time Course of Cultivation of *Torulopsis bombicola* from Fructose in 1-L Bellco Jar Glass Fermenter at 450 rpm, 2vvm and 30°C.
Medium Contains 12% Fructose and 0.15% Yeast Extract

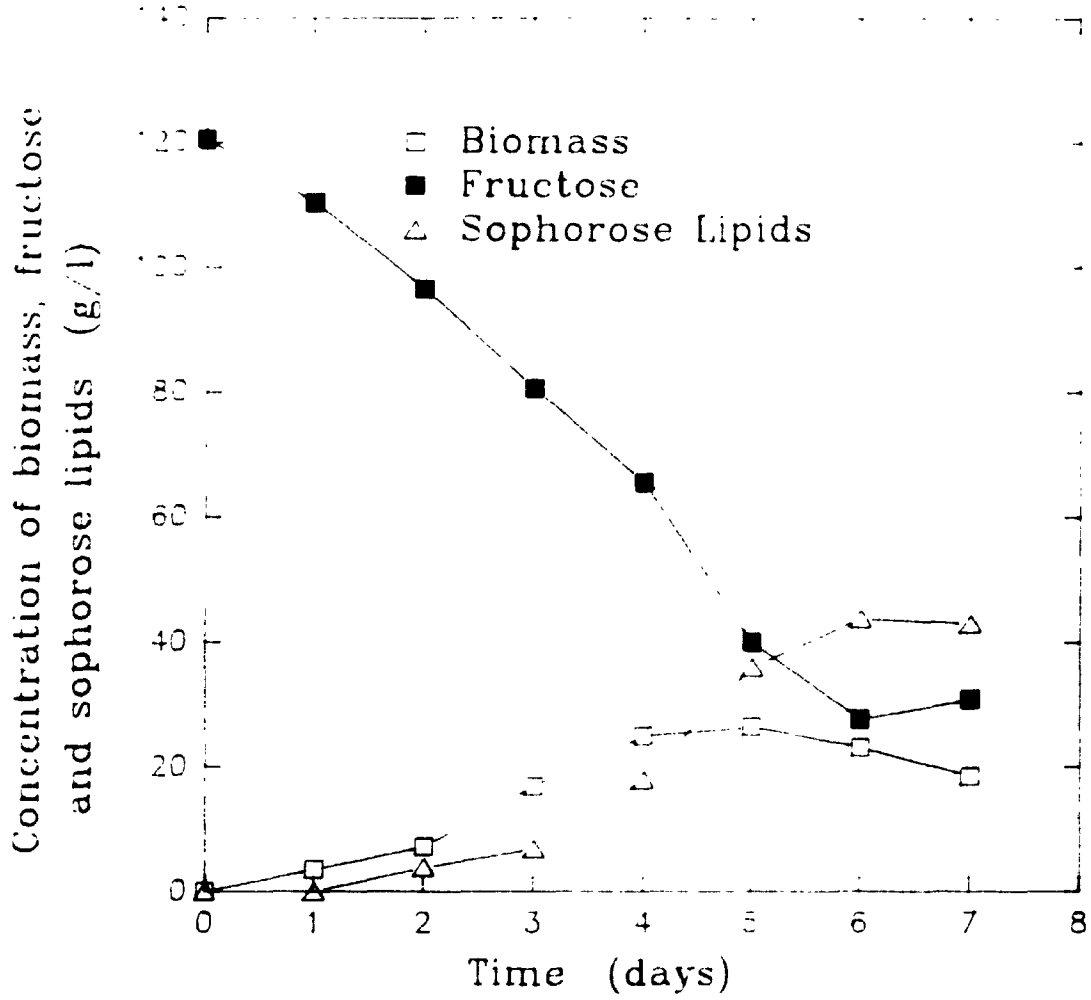

Biomass
Fructose
Sophorose Lipids
Concentration of biomass, fructose and sophorose lipids (g/l)
Time (days)
0
20
40
60
80
0
1
2
3
4
5
6
7
8

Figure 6.11 The Time Course of Cultivation of *Torulopsis bombicola* from Galactose in 1-L Bellco Jar Glass Fermenter at 450 rpm, 2vvm and 30°C.
Medium Contains 12% Galactose and 0.15% Yeast Extract

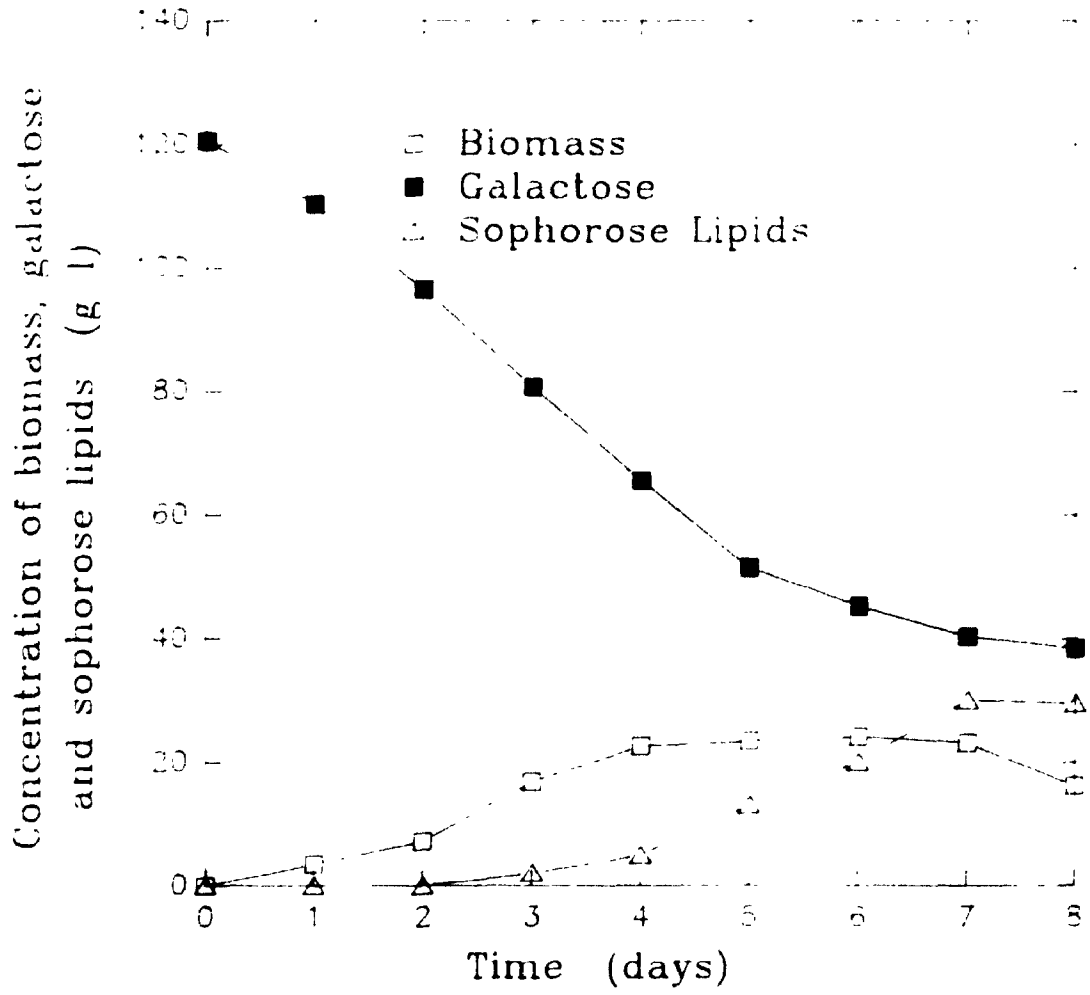

Biomass
Galactose
Sophorose Lipids
Concentration of biomass, galactose and sophorose lipids (g l)
Time (days)
0
1
2
3
4
5
6
7
8

Figure 6.12 The Time Course of Cultivation of *Torulopsis bombicola* from Sucrose in 1-L Bellco Jar Glass Fermenter at 450 rpm, 2vvm and 30°C.
Medium Contains 12% Sucrose and 0.15% Yeast Extract

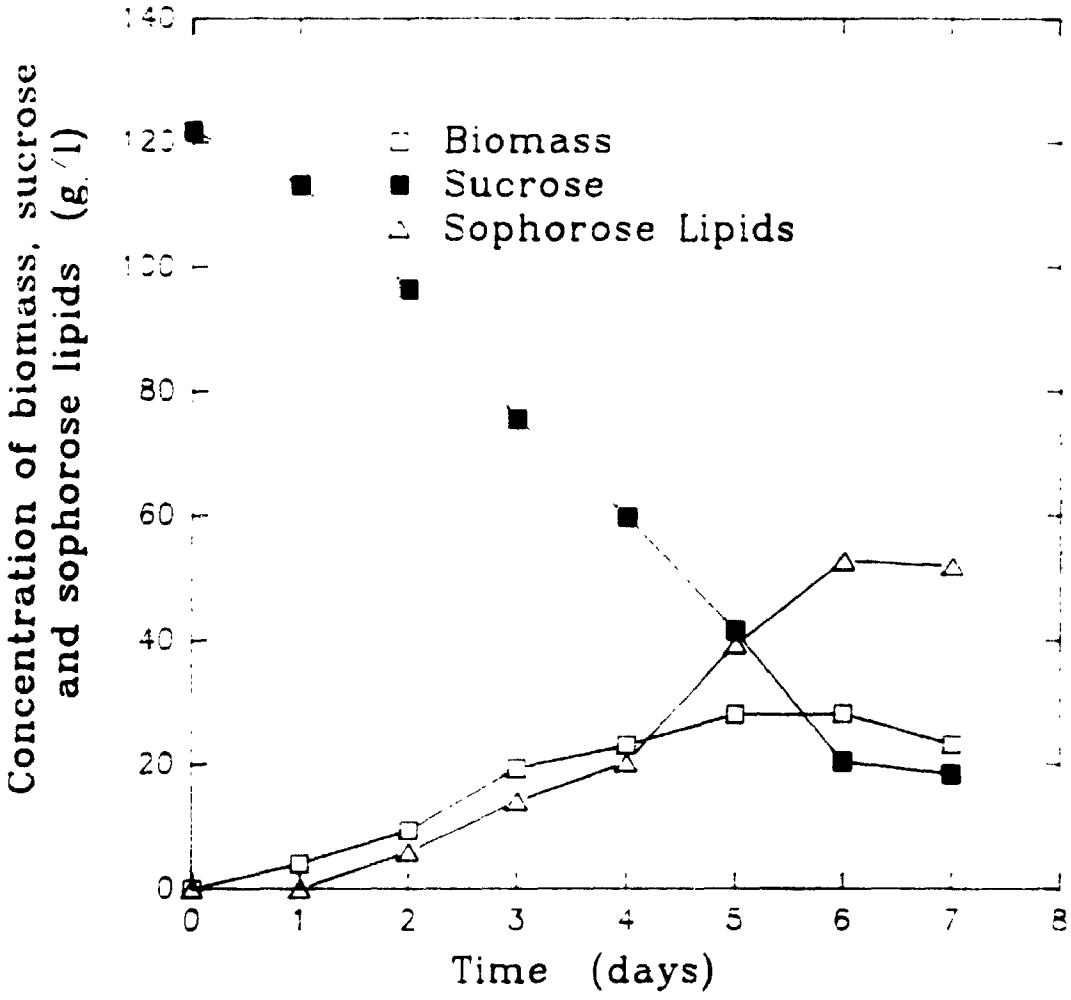

Concentration of biomass, sucrose and sophorose lipids (g/l)
140
120
100
80
60
40
20
0
0
1
2
3
4
5
6
7
8
Time (days)
Biomass
Sucrose
Sophorose Lipids

Figure 6.13 The Time Course of Cultivation of *Torulopsis bombicola* from Maltose in 1-L Bellco Jar Glass Fermenter at 450 rpm, 2vvm and 30°C.
Medium Contains 12% Maltose and 0.15% Yeast Extract

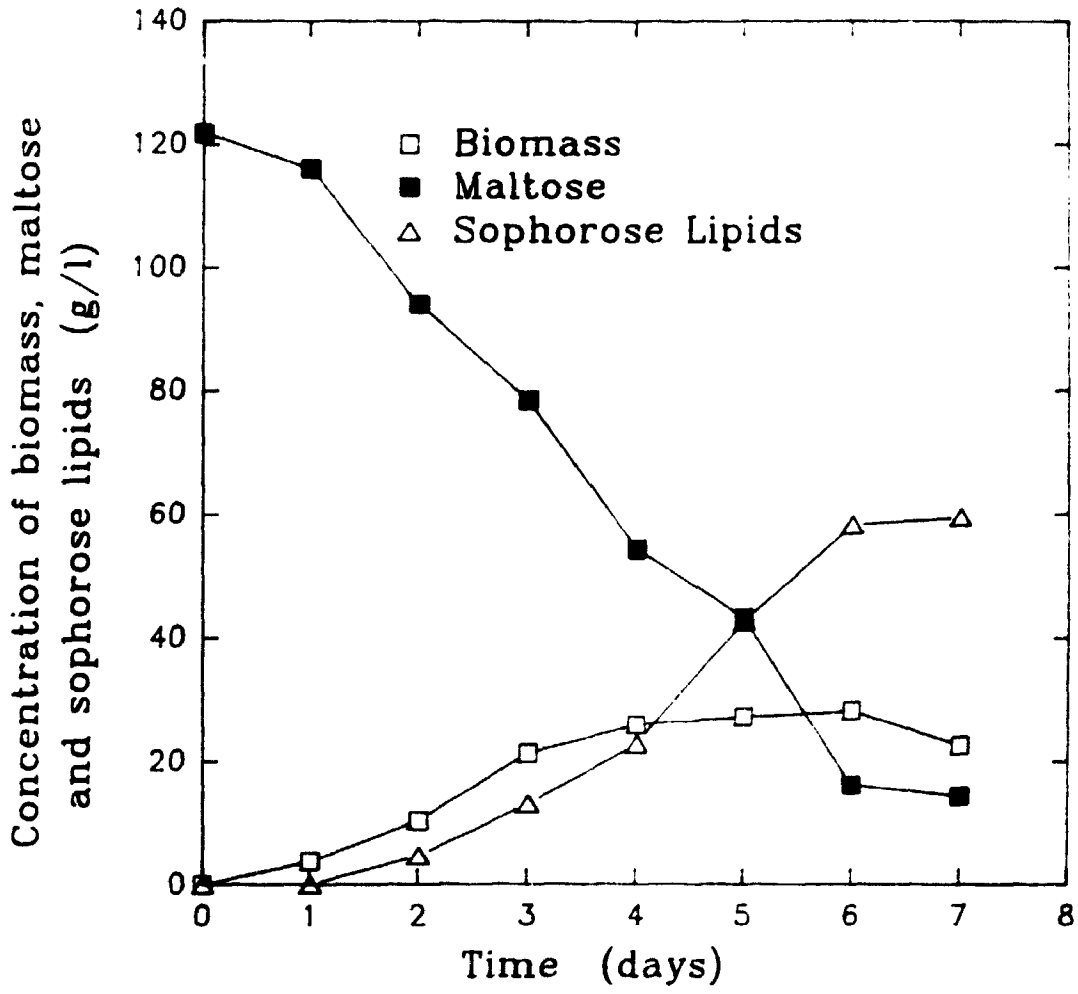
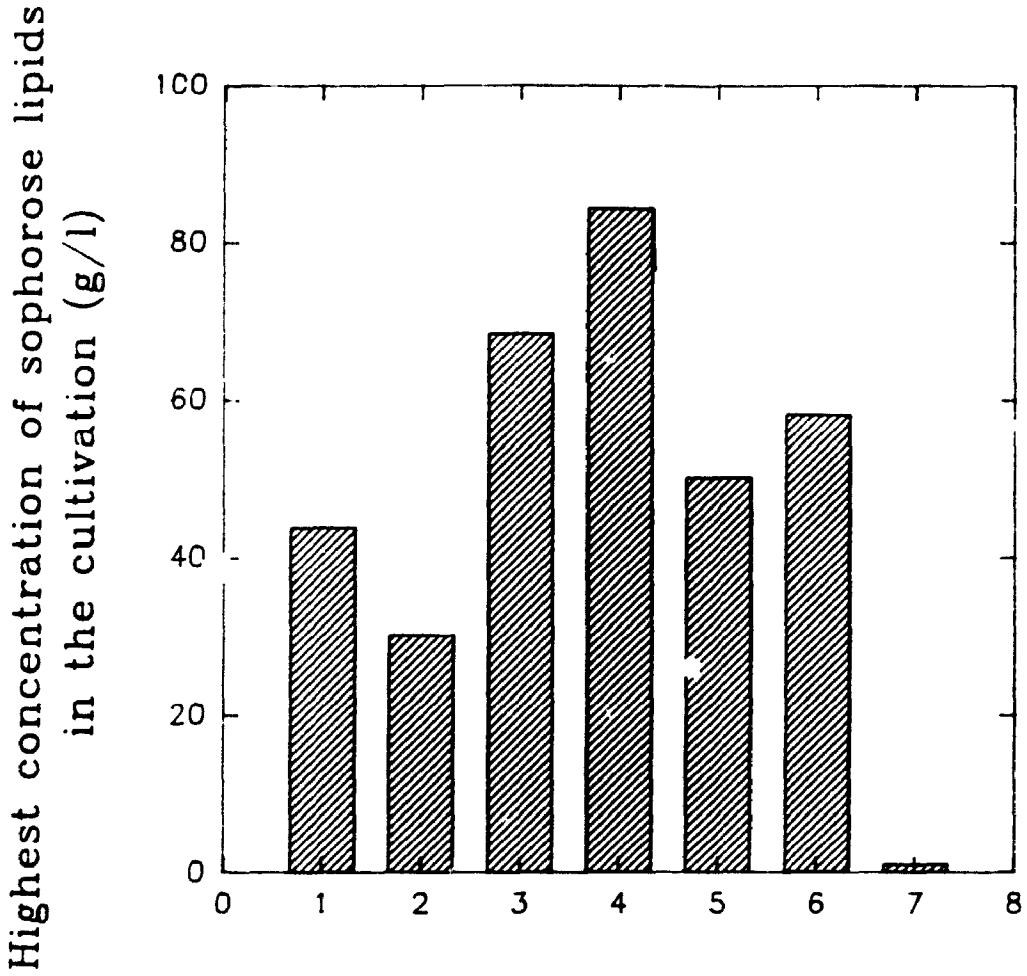

Concentration of biomass, maltose and sophorose lipids (g/l)
140
120
100
80
60
40
20
0
0
1
2
3
4
5
6
7
8
Time (days)
Biomass
Maltose
Sophorose Lipids

**Figure 6.14 The Comparison of The Production of Sophorose Lipids from Various Sugars**




1. Fructose
2. Galactose
3. Glucose
4. Honey
5. Sucrose
6. Maltose
7. Lactose

expensive for the production of sophorose lipids. Sucrose and maltose are very promising substrates for the production of sophorose lipids because disaccharides can be found in larger quantities in wastes at very low cost.

### 6.1.4.2 Effects of Vegetable oils

A set of shake flask experiments and 1-L fermenter experiments were performed with different vegetable oils to investigate the effects of vegetable oils on the production of sophorose lipids. The yeast *Torulopsis bombicola* was cultivated in the medium containing 12% of one of the following vegetable oils as the sole carbon source: canola oil, safflower oil, sunflower oil, olive oil, soy bean oil and cottonseed oil. These vegetable oils contain various amounts of saturated and unsaturated fatty acids shown in Appendix 4 (Rossell and Pritchard, 1991). There were significant differences in sophorose lipids production from different vegetable oils. The effect of various vegetable oils on the production of sophorose lipids is summarized in Figure 6.15. As seen in the Figure, the highest sophorose lipids production of 70 g/L was obtained when canola oil was used as the sole carbon source. A slightly lower production of sophorose lipids was obtained

when safflower oil was used as the sole carbon source. The lowest production of sophorose lipids was obtained with olive oil. The vegetable oils produce sophorose lipids in order:

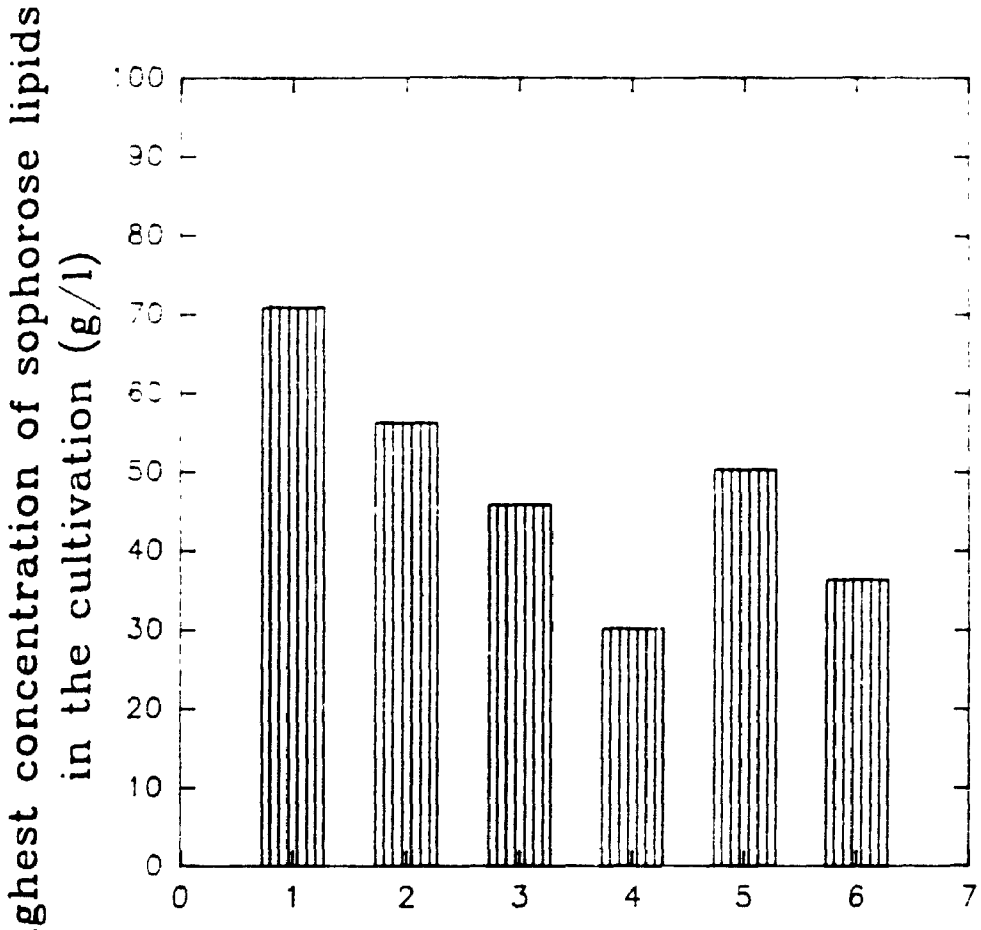
canola oil > safflower oil > sunflower oil > cotton seed oil > soy bean oil > olive oil

Comparing all vegetable oils studied, it can be said that the yeast *Torulopsis bombicola* prefers canola oil for the production of sophorose lipids. The reason is that canola oil contains 25% C18:3, which are easier to synthesize sophorose lipids. Safflower oil, sunflower oil and cotton seed oil gave good production of sophorose lipids.

### 6.1.4.3 Effects of the Mixtures of Vegetable oils and Sugars

During the preliminary screening of sugars and vegetable oils for the cultivation of the yeast *Torulopsis bombicola*, it was observed that canola oil, safflower oil, glucose, and honey were suitable as carbon sources for the production of sophorose lipids. Disaccharides could be considered as very promising carbon sources since they could be readily available at low cost. However, the conversion of substrates to

Figure 6.15 The Comparison of The Production of Sophorose Lipids from Various Vegetable Oils

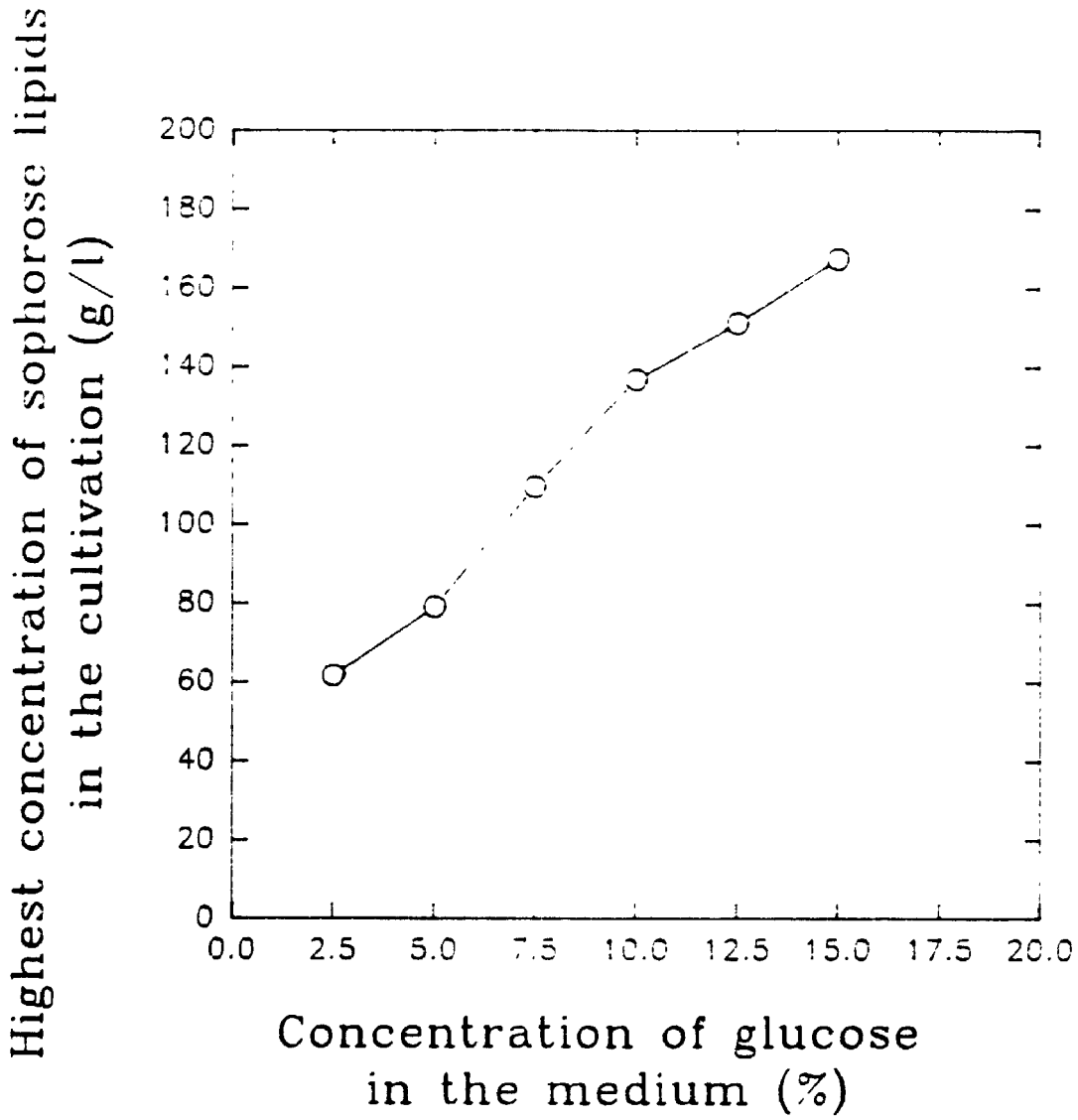



1. Canola Oil
2. Safflower Oil
3. Sunflower Oil
4. Olive Oil
5. Cotton Seed Oil
6. Soy Bean Oil

sophorose lipids is not high when only a single substrate is used. A possible explanation is that when a glucose is used as the sole carbon source, sophorose lipids production is limited by the synthesis of the fatty acid moiety. When a vegetable oil is used as the sole carbon source, the limitation to sophorose lipids production results from the synthesis of the sophorose moiety. The optimal production of sophorose lipids can be obtained when both sugar and vegetable oil are used as the carbon sources. In this case, much less cell energy is required to synthesize sophorose lipids. In an attempt to obtain the optimal production of sophorose lipids, several different mixtures of vegetable oil and sugar were studied for the production of sophorose lipids. The effects of substrate concentration were also studied to find the optimal concentrations of substrates for the production of sophorose lipids.

The optimization of substrate concentrations in the growth medium were performed by changing concentration of sugars and vegetable oils in the growth medium containing the mixed carbon sources. Figure 6.16 shows the effect of glucose concentration on the production of sophorose lipids in the medium containing 10.5% safflower oil, 0.25% yeast extract.

Figure 6.16 The Effect of Glucose Concentration on The Production of Sophorose Lipids in The Medium Containing 10.5% Safflower Oil and 0.25% Yeast Extract in 1-L Bellco Jar Glass Fermenter at 450 rpm, 2vvm and 30°C.


Highest concentration of sophorose lipids in the cultivation (g/l)
200
180
160
140
120
100
80
60
40
20
0
0.0
2.5
5.0
7.5
10.0
12.5
15.0
17.5
20.0
Concentration of glucose in the medium (%)

From the results shown in Figure 6.16, it can be seen that when glucose in different concentrations was added to the medium containing 10.5% safflower oil, the production of sophorose lipids was much higher in comparison to that obtained in previous experiment without glucose.

To determine the effect of safflower oil on the production of sophorose lipids, the experiments were conducted by the addition of safflower oil in concentrations of 1 to 15% to the medium containing 10% glucose and 0.25% yeast extract. As shown in Figure 6.17, the addition of safflower oil tc the growth medium resulted in a substantial increase in the production of sophorose lipids. From Figure 6.16 and 6.17, it is clear that both carbon sources were needed in high concentration in order to obtain a high production of sophorose lipids. However, too high concentration of sophorose lipids caused foaming. Optimum concentrations of substrates for the production of sophorose lipids are 10% glucose and 10.5% safflower oil.

Figure 6.18 shows the time course of cultivation of *Torulopsis bombicola* in 1 L Bellco jar glass fermenter in the medium containing 10% glucose, 10.5% safflower oil and 0.25% yeast

**Figure 6.17 The Effect of The Concentration of Safflower Oil on The Production of Sophorose Lipids in The Medium Containing 10.5% Safflower Oil and 0.25% Yeast Extract in 1-L Bellco Jar Glass Fermenter at 450 rpm, 2vvm and 30°C.**

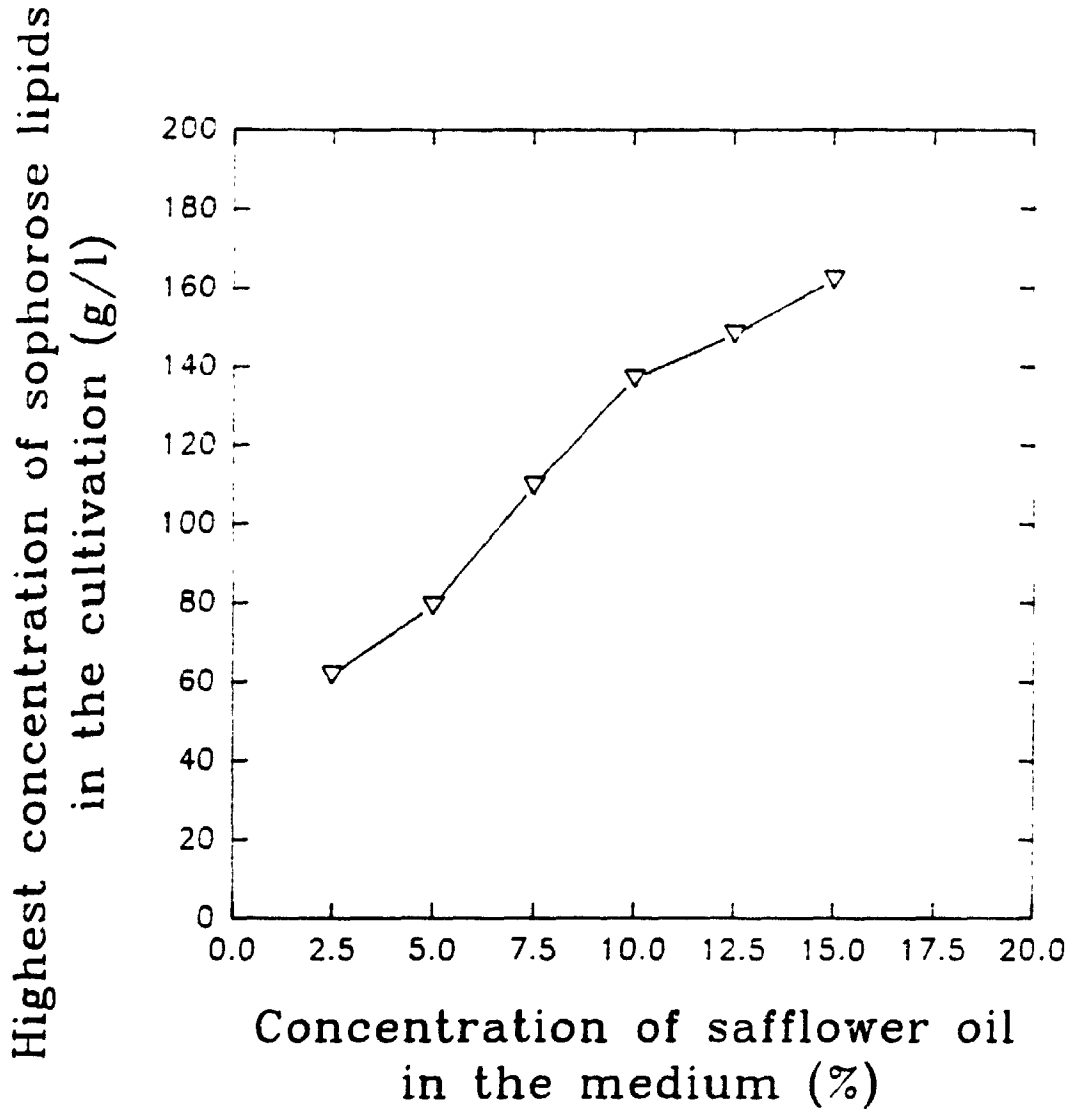
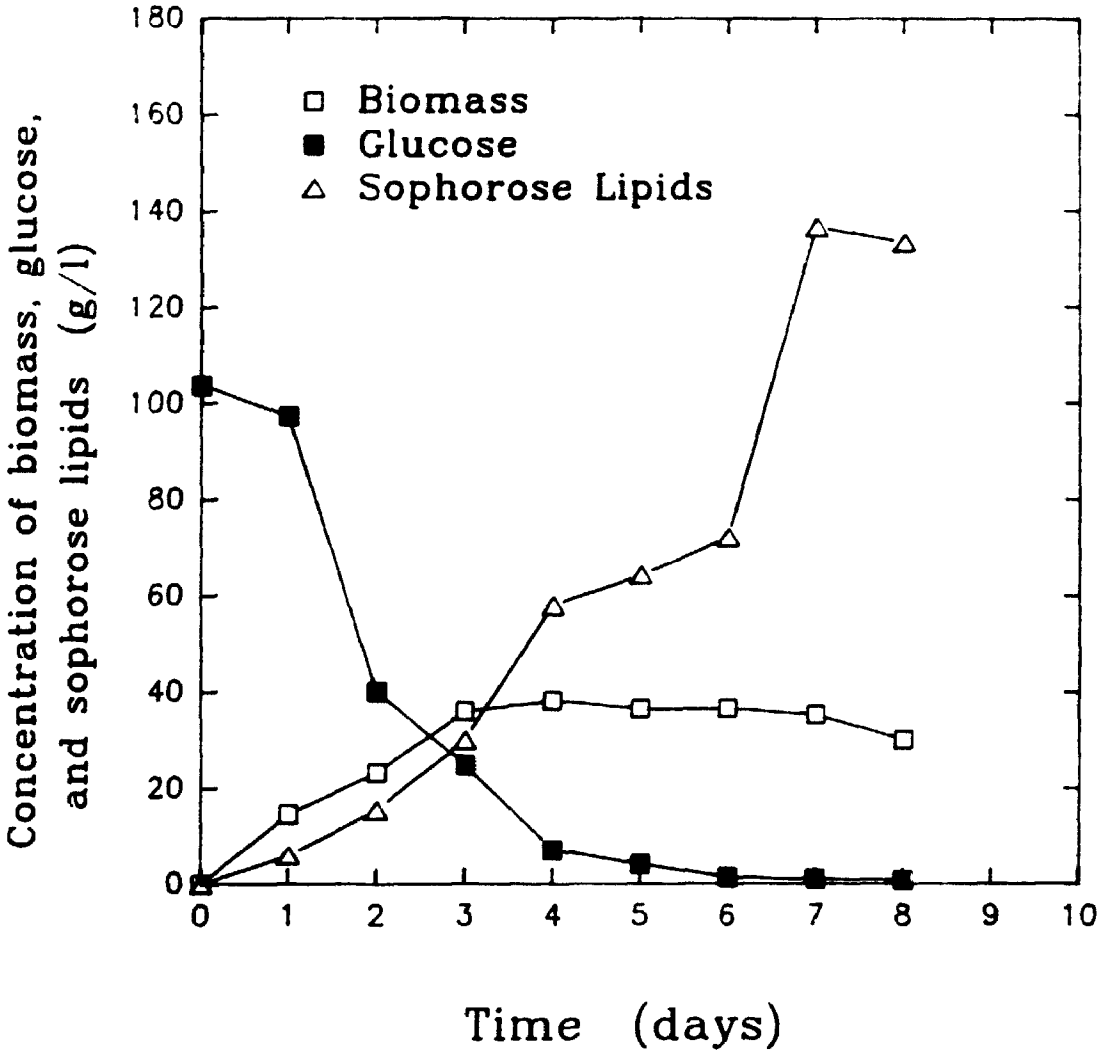

Highest concentration of sophorose lipids in the cultivation (g/l)
200
180
160
140
120
100
80
60
40
20
0
0.0
2.5
5.0
7.5
10.0
12.5
15.0
17.5
20.0
Concentration of safflower oil in the medium (%)

**Figure 6.18 The Time Course of Cultivation of *Torulopsis bombicola* in 1-L Bellco Jar Glass Fermenters from 10% Glucose, 10.5% Safflower Oil, and 0.25% Yeast Extract at 450 rpm, 2vvm and 30°C.**

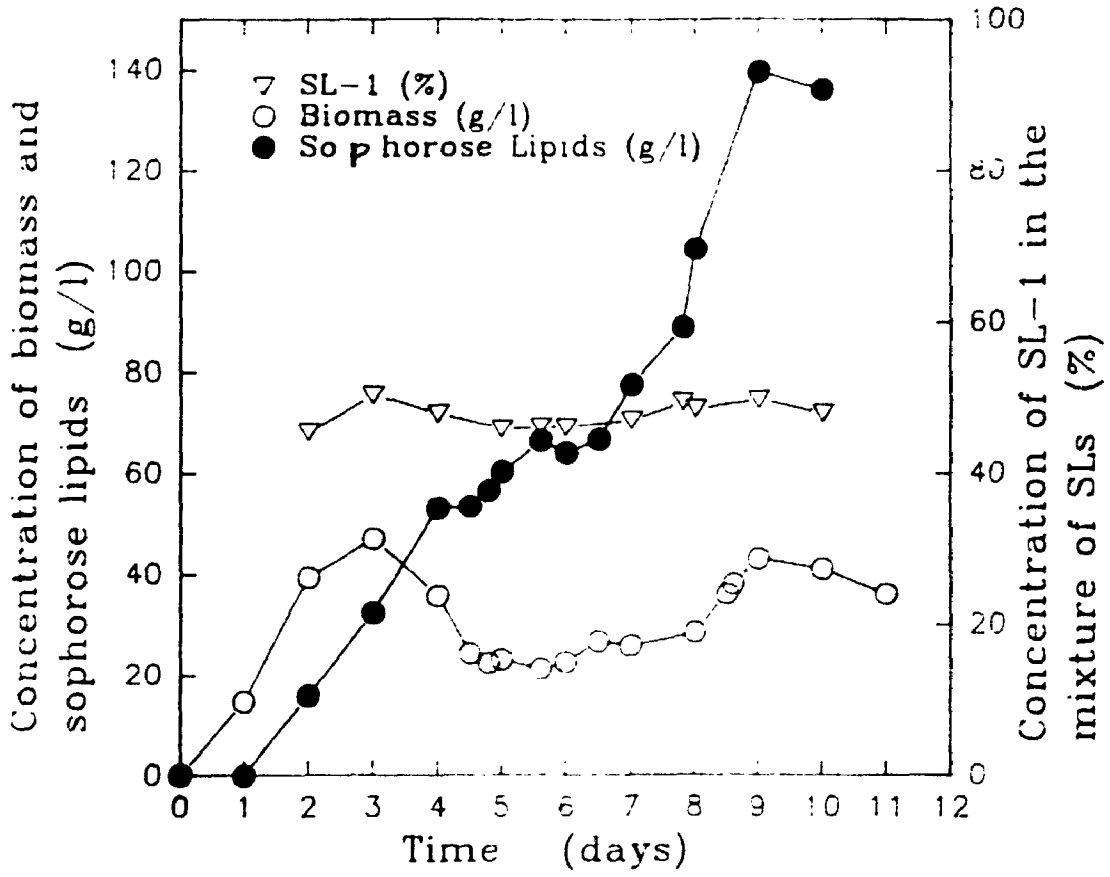

Biomass
Glucose
Sophorose Lipids
Concentration of biomass, glucose, and sophorose lipids (g/l)
Time (days)
0
20
40
60
80
100
120
140
160
180
0
1
2
3
4
5
6
7
8
9
10

extract. The concentration of biomass increased up to 3 days, then remained constant and decreased slightly after 6 days. Glucose was almost exhausted after 4 days. Sophorose lipids accumulated during both growth and stationary phase of the cultivation up to 7 days and reached a maximum of 135 g/l.

To verify the high yields of sophorose lipids on a large scale the cultivation was carried out in a 20 L bioreactor shown in Figure 6.19. The time course of the cultivation was similar to the cultivation in the 1 L Bellco jar glass fermenter although the maximum of 136.6 g/L sophorose lipids was reached after longer time. About 50 % of the apolar sophorose lipid 1′,4′′-lactone 6′,6′′-diacetate (SL-1) was found in the mixture of sophorose lipids produced under these conditions. There is a clear possibility to influence the composition of sophorose lipids by medium composition but this is to be connected to changes in the sophorose lipids yield (Zhou et al., 1992).

From the above experiments, it can be seen that honey is a very good substrate for the production of sophorose lipids. Honey contains a small amount of mineral elements such as phosphorus, magnesium, potassium, sodium, manganese, and sulfur which become integral parts of the protein as

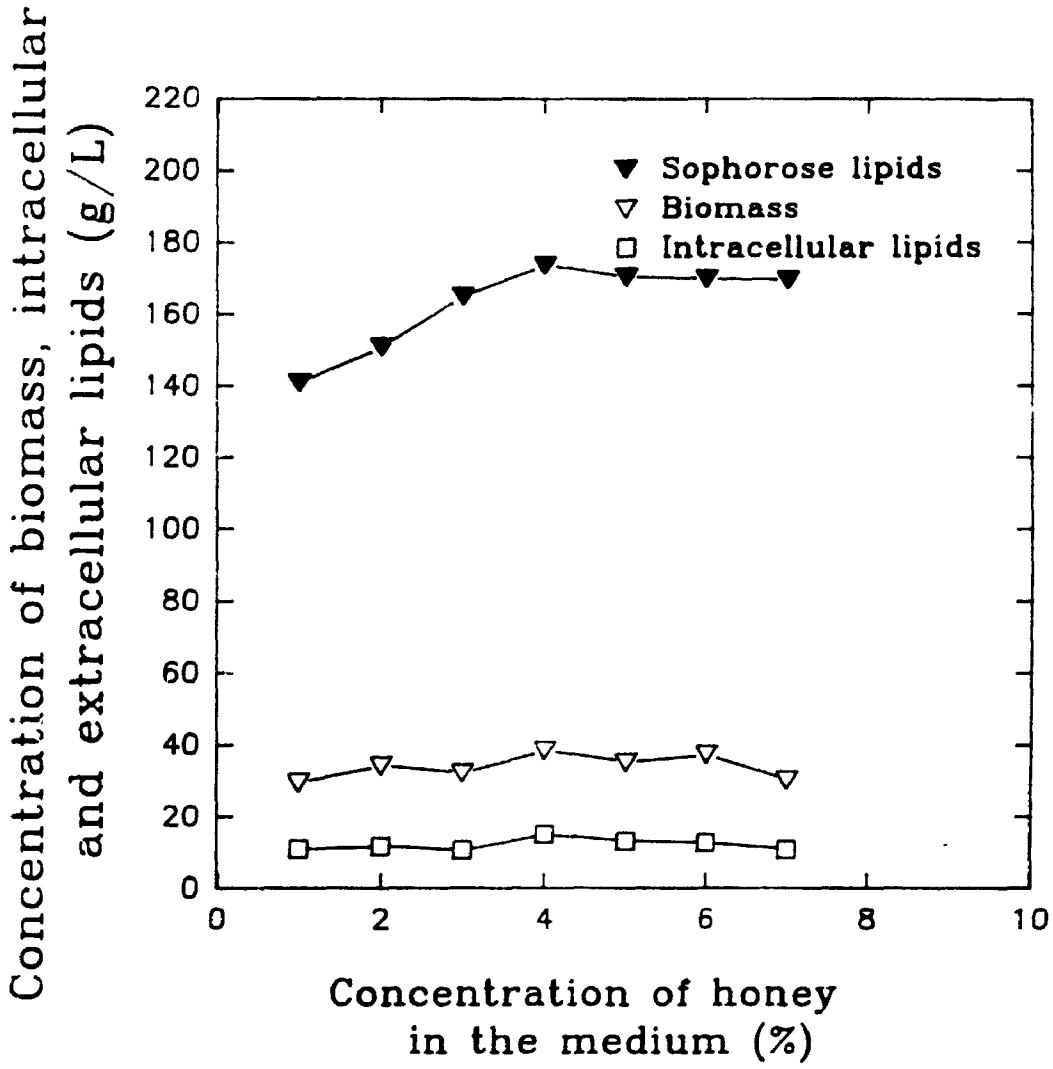
**Figure 6.19 The Time Course of Cultivation of *Torulopsis bombicola* in A 20 L Bioreactor from 10% Glucose, 10.5% Safflower Oil, and 0.25% Yeast Extract at 450 rpm, 0.9 vvm and 30°C.**


SL-1 (%)
Biomass (g/l)
Sophorose Lipids (g/l)
Concentration of biomass and sophorose lipids (g/l)
Concentration of SL-1 in the mixture of SLs (%)
Time (days)
0
20
40
60
80
100
120
140
0
20
40
60
80
100
0 1 2 3 4 5 6 7 8 9 10 11 12

activators of enzymes and function as cofactors in biochemical reactions within the cell. They also function as stabilizers for proteins. However, it was too expensive. Therefore, a small amount of honey was added to the cultivation medium containing mainly glucose and safflower oil. In the experiments, different concentrations of honey were added to the medium of 10% glucose, 10.5% safflower oil, and 0.25% yeast extract. The results are shown in Figure 6.20. It was found that the addition of small amount of honey (4%) to the medium contained 10.5% safflower oil, 10% glucose and 0.25% yeast extract significantly enhanced synthesis of sophorose lipids. The small amount honey can provide enough amount of trace elements which are very important for the cell growth and lipids production.

The effects of lactose and galactose on the production of sophorose lipids are shown in Figure 6.21 and 6.22. From the Figures, it can be seen that the production of sophorose lipids is significantly enhanced by the presence of lactose or galactose in the medium containing 10% glucose and 10.5% safflower oil. Figure 6.21 shows the effect of lactose on the production of sophorose lipids. The different concentrations of lactose were added to the optimum medium of 10% glucose,

Figure 6.20 The Effect of Honey on Sophorose Lipids Production in The Mixed Carbon Sources Containing 10.5% Safflower Oil and 10% Glucose and 0.25% Yeast Extract at 450 rpm, 2vvm and 30°C.


Concentration of biomass, intracellular and extracellular lipids (g/L)
220
200
180
160
140
120
100
80
60
40
20
0
0
2
4
6
8
10
Sophorose lipids
Biomass
Intracellular lipids
Concentration of honey in the medium (%)

10.5% safflower oil, and 0.25% yeast extract for the lipid production. Lactose was shown to promote the biosynthesis of extracellular sophorose lipids. For a lactose concentration of less than 4%, the production of sophorose lipids increased with increasing lactose concentration, with a maximum production of about 117 g/L in shaking flasks experiments (Zhou and Kosaric, 1993) and 170 g/ L in 1-L fermenters shown in Figure 6.21. The effect of galactose on the production of sophorose lipids is shown in Figure 6.22. The results were very similar to those obtained in Figure 6.21. From the above observation, it can be concluded that the addition of lactose or galactose or honey to the medium containing 10% glucose, 10.5% safflower oil and 0.25% yeast extract stimulated the production of sophorose lipids. It can be seen that galactose or lactose in the mixture of glucose and vegetable oil can replace honey in the mixture of glucose and vegetable oil for the optimal production of sophorose lipids. In those cases, the yeast can easily use vegetable oil and glucose to produce sophorose lipids which act as biosurfactants to enhance the galactose and lactose uptake. However, lactose or galactose as the sole carbon source can not replace honey because they do not have good transport systems and do not have enough trace elements.

**Figure 6.21 The Effect of Lactose on Sophorose Lipids Production in The Mixed Carbon Sources Containing 10.5% Safflower Oil and 10% Glucose and 0.25% Yeast Extract at 450 rpm, 2vvm and 30°C.**

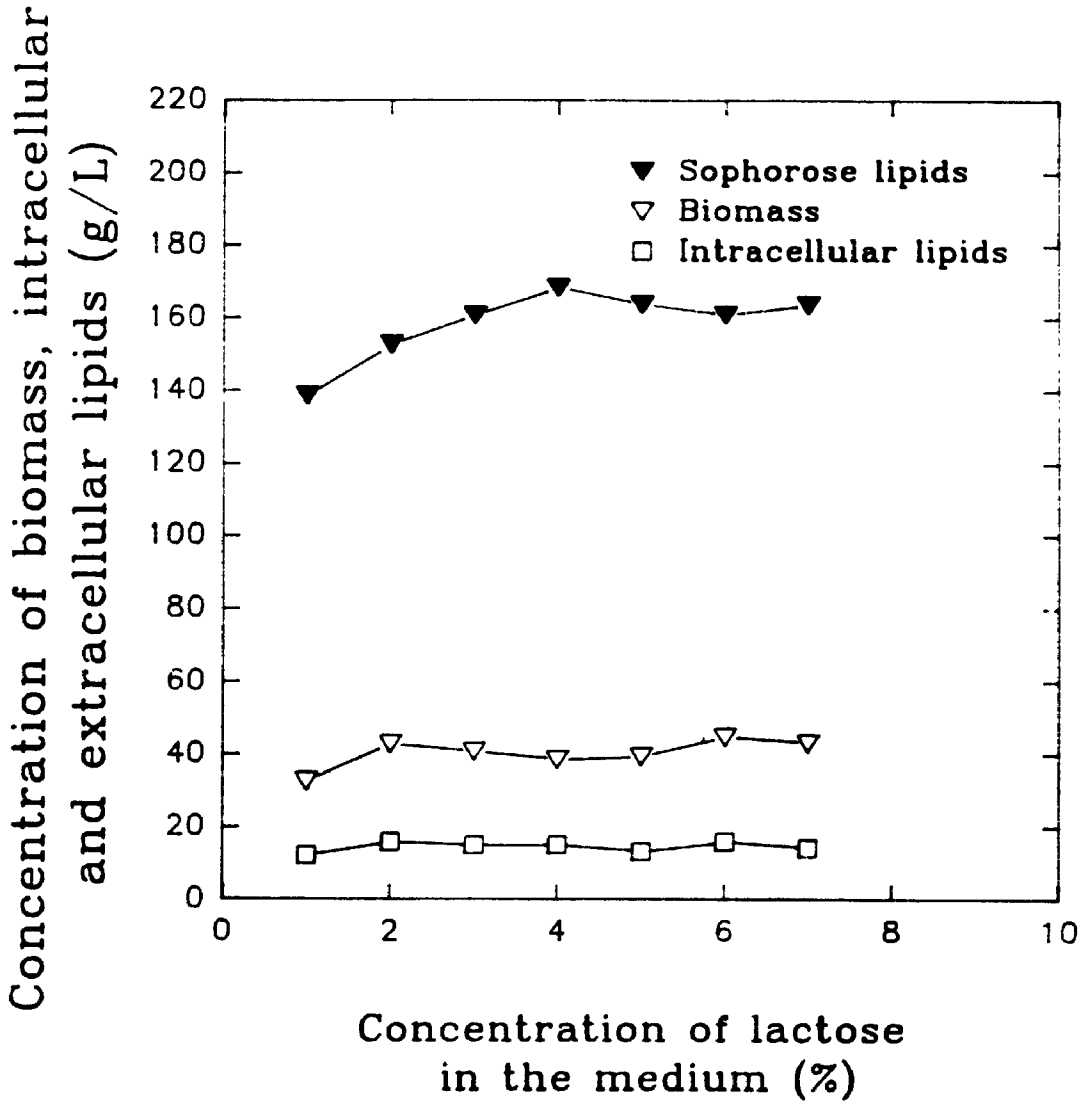
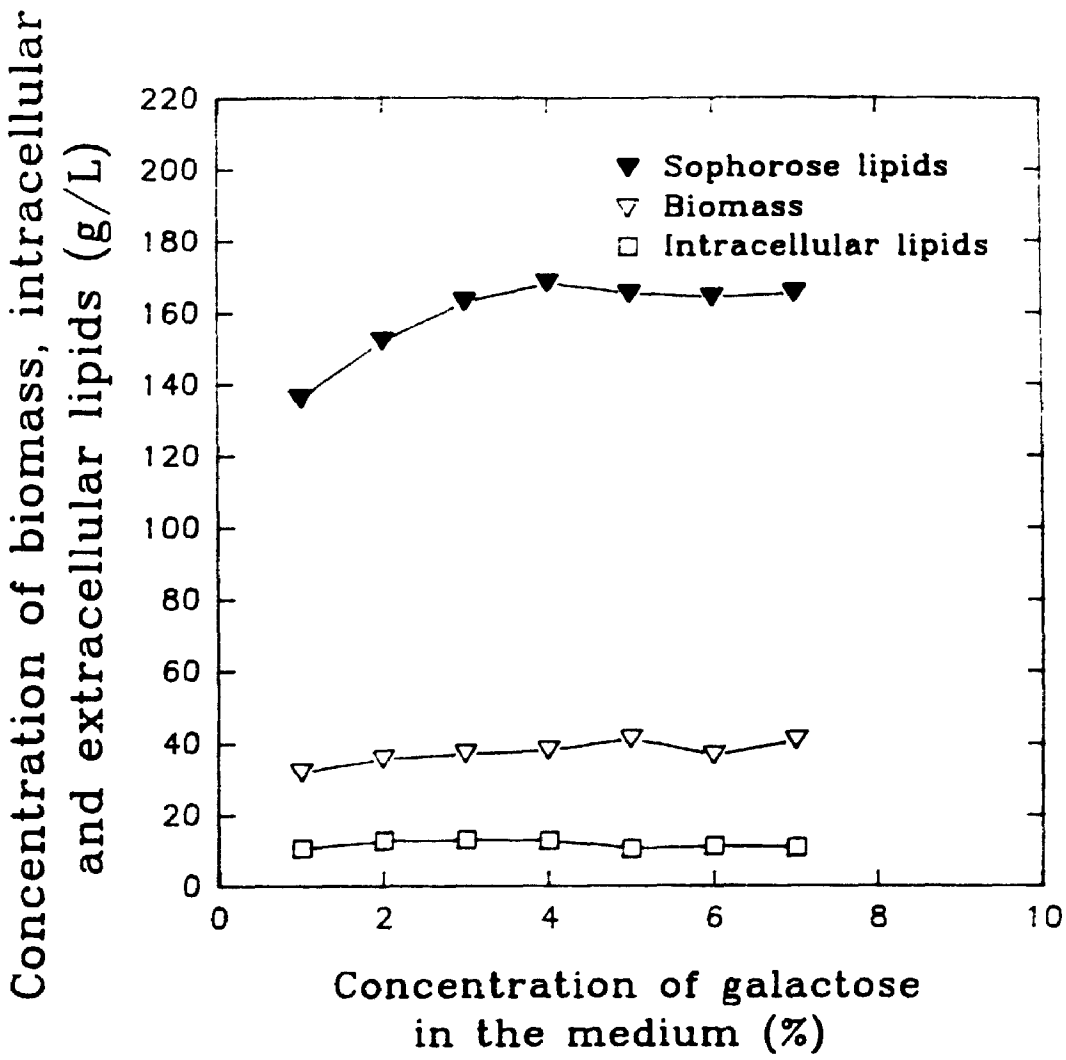

Concentration of biomass, intracellular and extracellular lipids (g/L)
220
200
180
160
140
120
100
80
60
40
20
0
0
2
4
6
8
10
Sophorose lipids
Biomass
Intracellular lipids
Concentration of lactose in the medium (%)

Figure 6.22 The Effect of Galactose on Sophorose Lipids Production in The Mixed Carbon Sources Containing 10.5% Safflower Oil and 10% Glucose and 0.25% Yeast Extract at 450 rpm, 2vvm and 30°C.

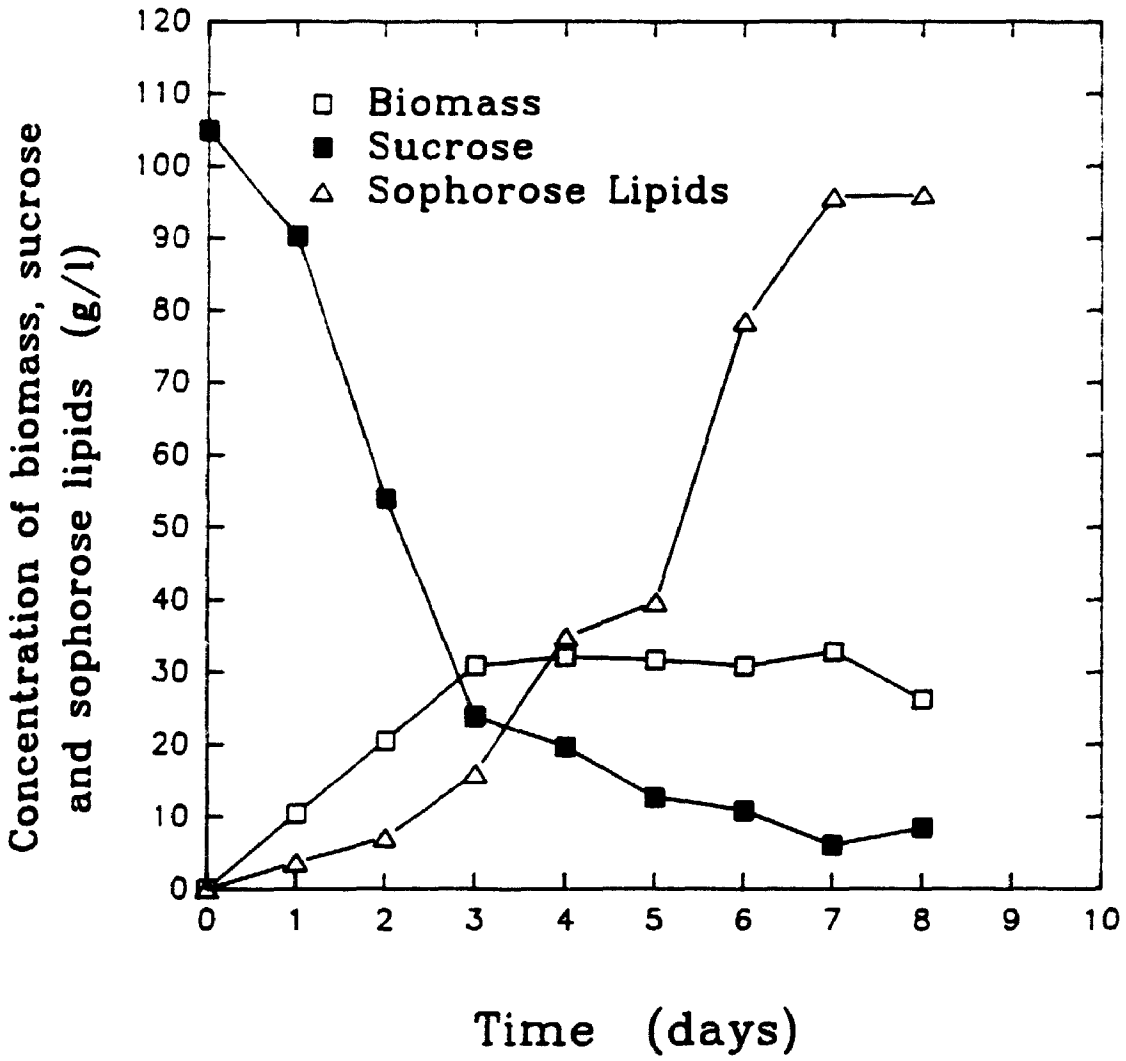

Concentration of biomass, intracellular and extracellular lipids (g/L)
220
200
180
160
140
120
100
80
60
40
20
0
0
2
4
6
8
10
Sophorose lipids
Biomass
Intracellular lipids
Concentration of galactose in the medium (%)

From Figure 6.12, it can be seen that *Torulopsis bombicola* produced sophorose lipids when growing on sucrose as the sole substrate in similar yields as if growing on glucose as the sole substrate. As glucose is rarely found in larger quantities in wastes we investigated the substitution of glucose by sucrose in the mixed medium containing 10.5% safflower oil and 0.25% yeast extract.

To verify the possibility of replacing glucose with sucrose in the mixed medium for the production of sophorose lipids, the yeast *Torulopsis bombicola* was cultivated in 1-L Bellco jar glass ferments for 6-8 days. As shown in Figure 6.23, the highest concentration of 100-110 g/L sophorose lipids was obtained at 7-day cultivation. The biomass was 30 g/L. Comparing to the cultivation on glucose and safflower oil under the same conditions, the production of sophorose lipids from sucrose and safflower oil was lower. On the basis of TLC analysis it can be said that the composition of mixture was not changing during the course of cultivation.

Batch fermentations of *Torulopsis bombicola* with lactose, galactose, olive oil and cheese whey as the carbon sources were studied to develop cost-effective substrates for maximum

**Figure 6.23 The Time Course of Cultivation of *Torulopsis bombicola* in 1-L Bellco Jar Glass Fermenters from 10% Sucrose, 10.5% Safflower Oil, and 0.25% Yeast Extract at 450 rpm, 2vvm and 30°C.**


Concentration of biomass, sucrose and sophorose lipids (g/l)
120
110
100
90
80
70
60
50
40
30
20
10
0
Biomass
Sucrose
Sophorose Lipids
0
1
2
3
4
5
6
7
8
9
10
Time (days)

lipids production and the simultaneous production of intra- and extracellular lipids in a single fermentation step.

The yeast *Torulopsis bombicola* was shown to produce sophorose lipids abundantly under appropriate culture conditions. In the previous experiments, it was shown that optimal productivity of 136.6 g/L sophorose lipids occurs when both 10% glucose and 10.5% safflower oil were simultaneously present as the growth substrates in a 20 l bioreactor (Zhou et al., 1992). Considerable cost reduction might be possible by the use of other cheap or waste materials as substrates (Klekner et al., 1991). Different studies have been carried out to improve the production of extracellular lipids by *Torulopsis bombicola*, however, no literature information is available on the intracellular lipids of this yeast and this is also no report is on using lactose or cheese whey as cheap substrates.

Lactose, 4-o-β-d-galactopyranosyl-D-glucose, a dissacharide composed of galactose and glucose, is the sugar present in milk and is obtained from whey as a by-product of the cheese industry at concentrations between 4.5 and 5% representing approximately 70 % of the whey solids. The disposal of cheese whey is a continuing and growing problem in the dairy

industry. Over 44% of the whey produced in North America is not utilized for human or animal food, but is discarded as waste. The $BOD_5$ value commonly around 60,000-70,000 mg/L, results mainly from the lactose present. About 115 million tons of liquid whey containing 6 million tons of lactose are produced yearly worldwide The rising cost of the disposal of lactose, the need to protect the environment, and cost reduction of lipids production have encouraged studies for lactose utilization as a more efficient and cost-effective substrate for lipids production (Ratledge and Tan, 1990).

It this study, we examined the effects of lactose, galactose, olive oil, safflower oil, and cheese whey on the production of intra- and extracellular lipids by *Torulopsis bombicola*. The results are summarised in Table 6.1. As shown, lactose and olive oil have a great influence on the biosynthesis of intra- and extracellular lipids. When only lactose is present, *Torulopsis bombicola* did not grow. However, in the presence of both lactose and olive oil, intra- and extracellular lipids were synthesized. By using optimum culture conditions (Zhou el al.,1992), the maximum production of 46.46 g/L extracellular lipids, 18.32 g/L intracellular lipids, and 38.42 g/L biomass was obtained with shaking flasks experiments. Galactose and

olive oil have also shown to be good substrates for intra- and extracellular lipid biosynthesis with 12.57 g/L intracellular lipids, 24.41 g/L extracellular lipids and 28.56 g/L biomass. Cheese whey as the sole source of carbon did not support growth, however, addition of olive oil to cheese whey as the carbon sources resulted in the production of small amounts of sophorose lipids and biomass. As shown in Table 6.1, lactose and olive oil were the most effective carbon sources for the simultaneous production of intra- and extracellular lipids in a single fermentation step. Glucose and safflower oil were especially good for sophorose lipids production (extracellular lipids) (Zhou and Kosaric, 1993).

In yeasts, solute transport can be by free diffusion and facilitated diffusion, but active transport is the means by which the vast majority of solutes are transported across yeast plasma membranes. The proteins that are responsible for facilitated diffusion and active transport are referred as "transport systems" or "permease" (Rose and Harrison, 1989). From the above experiments, it was known that the yeast *Torulopsis bombicola* could easily uptake monosaccharides such as glucose, fructose, and galactose, hydrocarbons and vegetable oils. This yeast possesses at least two

TABLE 6.1 Lipid Production by *T. bombicola* Grown on Various Carbon Sources in shaking flasks for 6-8 days

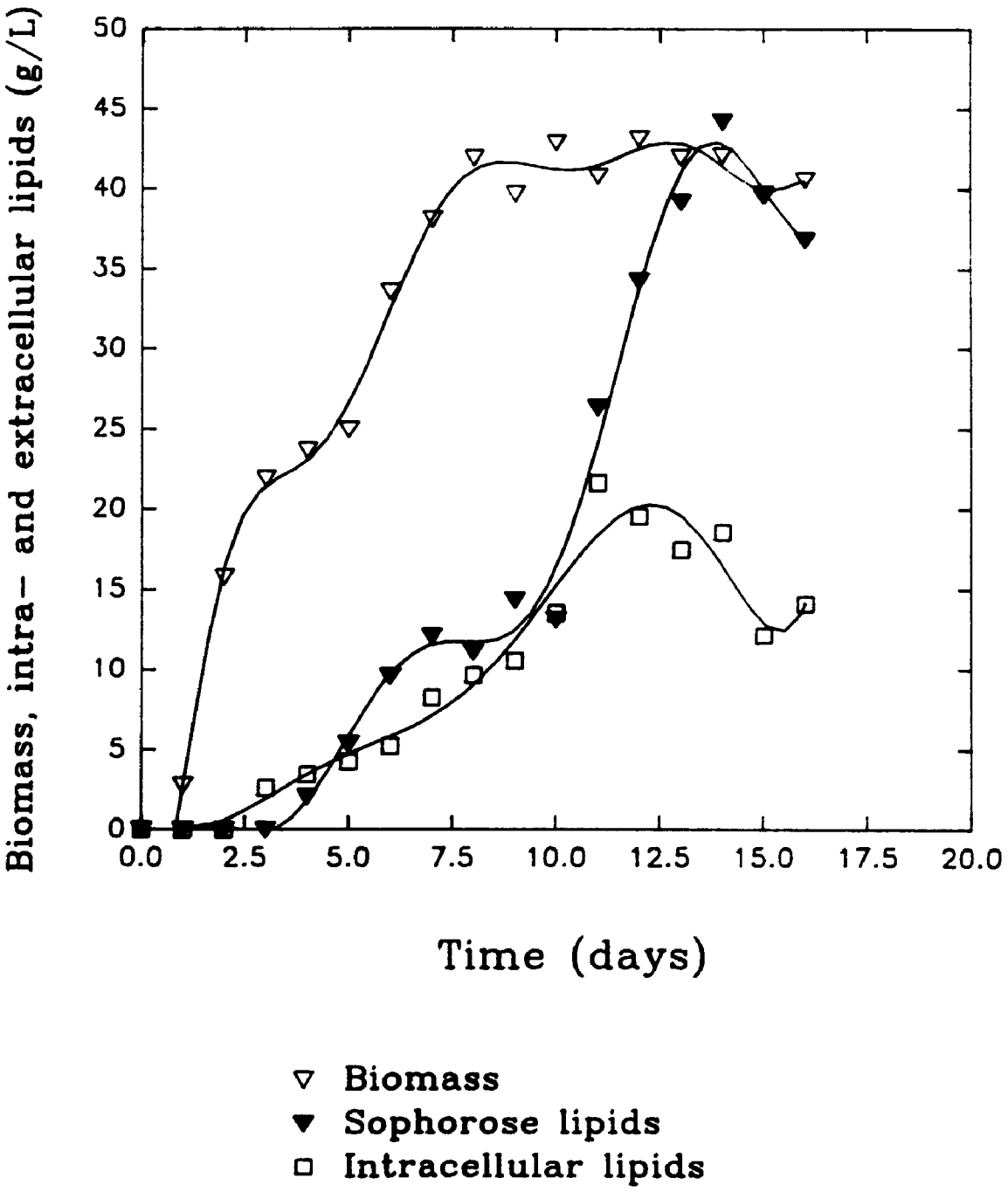
| Carbon source sugar(10%) oil(10.5%) | Biomass (g/L) | Intra-lipids (g/L) | Extra-lipids (g/L) | Sugar utilized (g/L) |
|---|---|---|---|---|
| Glucose | 25.22 | | 30.76 | 60.14 |
| Glu.+Sal. | 34.38 | 10.45 | 110.7 | 98.31 |
| Galactose | 22.59 | 0 | 9.38 | 35.72 |
| Ga.+OL. | 28.56 | 12.57 | 24.41 | 42.05 |
| Lactose | 1.87 | 0 | 0 | 3.78 |
| Lac+OL. | 38.42 | 18.32 | 46.46 | 56.3 |
| Sucrose | 19.45 | 0 | 18.74 | 43.56 |
| Suc.+Sal. | 22.65 | 0 | 58.32 | 66.42 |
| Cheese whey | 2.43 | 0 | 0 | - |
| CW.+OL. | 14.37 | - | 6.23 | - |

Glu. = glucose; Ga.=galactose ; Suc. = sucrose; Lac. = lactose ; CW. = cheese whey; Sal. = safflower oil; OL.=olive oil. Culture conditions as described in the text.

monosaccharide-transport system, a constitutive one for glucose and an inducible galactose carrier. The yeast did not uptake lactose for sophorose lipids production. Lactose is disaccharide and it is very difficult to transport across the plasma membrane. Utilization of lactose by the yeast is believed to require extracellular hydrolysis, or an active lactose transport system, or the enzyme β-D-galactosidase (lactase) (Ratledge and Tan, 1990). However, when using lactose and oil, intra- and extracellular lipids are synthesized. This may indicate that the oil has an effect in enhancing either the lactose transport systems or inducing lactase, or both. *Torulopsis bombicola* uses vegetable oils to produce sophorose lipids which acted as surfactants to facilitate lactose transport across yeast plasma membranes.

The concentrations of lactose and olive oil have a great influence on lipid production which was similar to our previous study (data not shown). The optimum medium composition was 10% lactose, 10.5% olive oil and 0.25-0.3% yeast extract. Figure 6.24 shows the time course of cultivation in 1-L fermenters by using the optimum medium composition.

**Figure 6.24 The Time Course of Cultivation of *Torulopsis bombicola* in 1-L Bellco Jar Glass Fermenters from 10% Lactose, 10.5% olive Oil, and 0.25% Yeast Extract at 450 rpm, 2vvm and 30°C.**

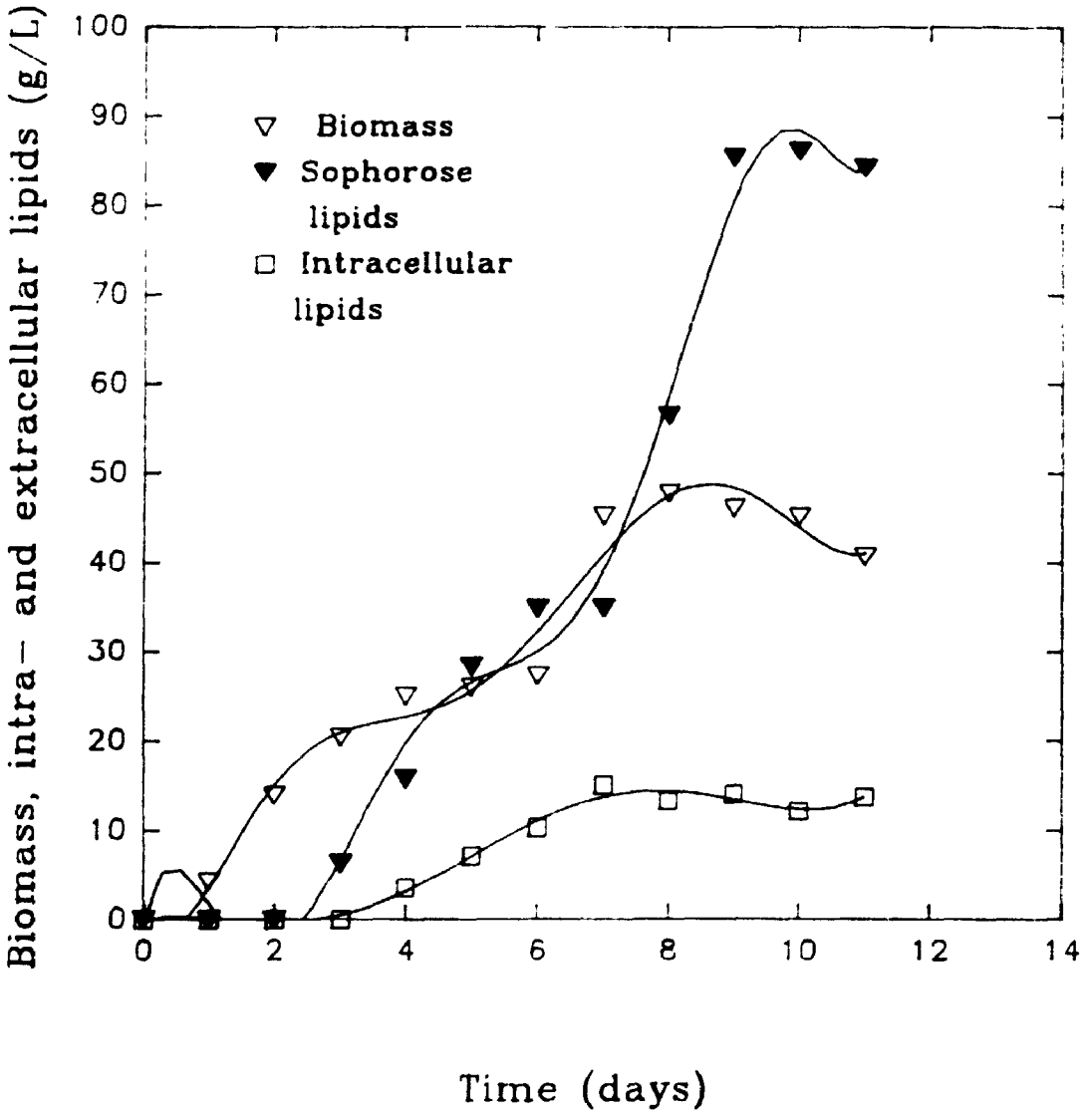

Biomass, intra- and extracellular lipids (g/L)
50
45
40
35
30
25
20
15
10
5
0
0.0
2.5
5.0
7.5
10.0
12.5
15.0
17.5
20.0
Time (days)
Biomass
Sophorose lipids
Intracellular lipids

In Figure 6.24, the concentration of biomass increased for about 6 days and reached about 40 g/L after 6 days of cultivation. Extracellular lipids production started in the middle of the exponential growth phase and rapidly increased in the late exponential growth phase. At this time nitrogen is exhausted (data not shown). In the stationary growth phase the residual carbon source was obviously used for continued extracellular lipids production. The maximum production of 46 g/L extracellular lipids was obtained. The concentration of intracellular lipids increased with time, reached a maximum of 18 g/L and then decreased. When 10.5 % safflower oil was used to replace olive oil in the mixed substrates, higher production of 90 g/ L sophorose lipids was obtained as shown in Figure 6.25.

The reported studies demonstrated that lactose plus olive oil can be used by *Torulopsis bombicola* for simultaneous production of intra- and extracellular lipids in a single fermentation step. The studies also showed the possibility of producing high value product lipid by this yeast from cheese whey.

Under competitive market conditions, the price of canola oil

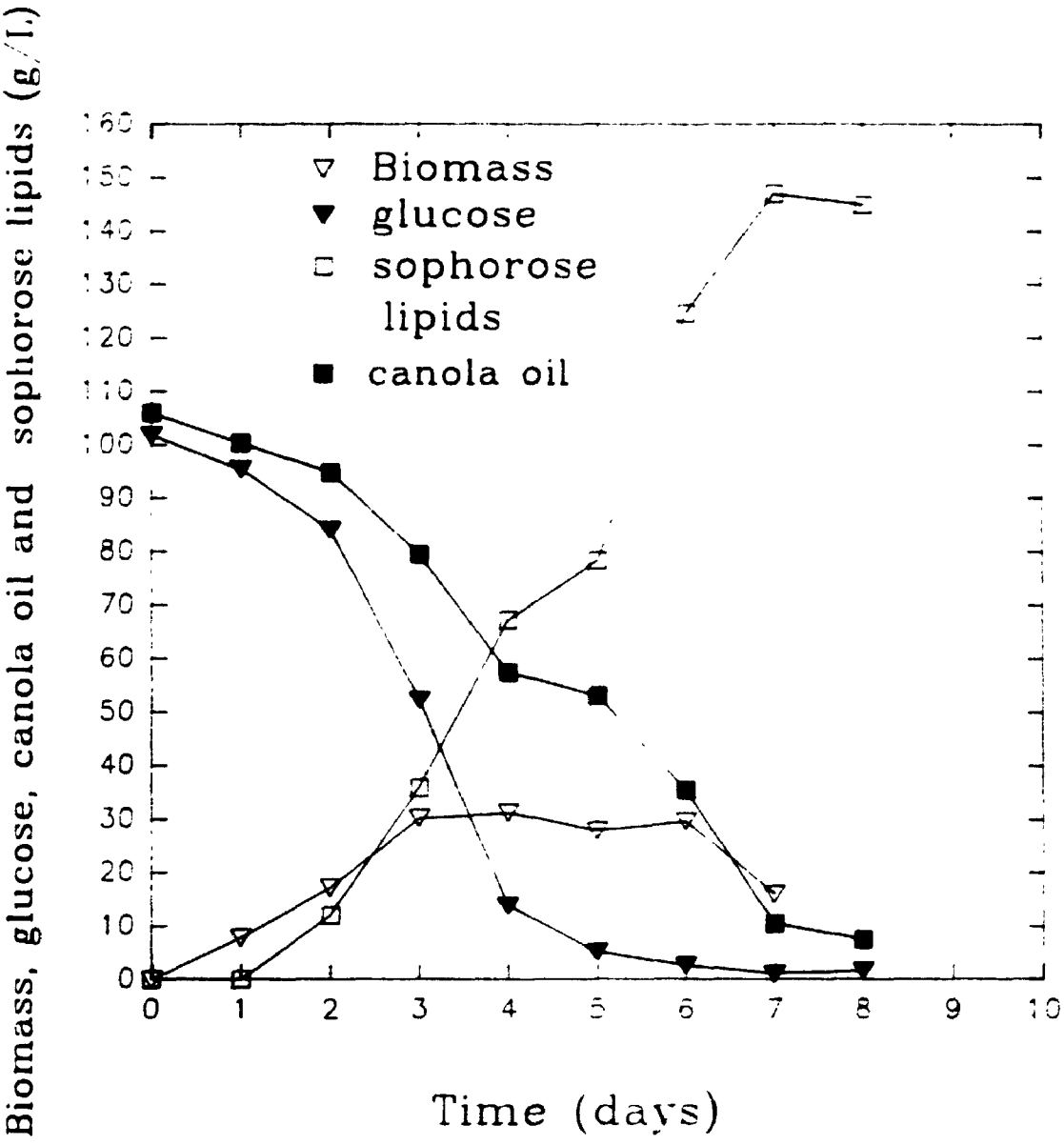
Figure 6.25 The Time Course of Cultivation of *Torulopsis bombicola* in 1-L Bellco Jar Glass Fermenters from 10% Lactose, 10.5% Safflower Oil, and 0.25% Yeast Extract at 450 rpm, 2vvm and 30°C.


Biomass, intra- and extracellular lipids (g/L)
100
90
80
70
60
50
40
30
20
10
0
0
2
4
6
8
10
12
14
Biomass
Sophorose lipids
Intracellular lipids
Time (days)

is expected to decrease in response to the larger supply. In the following experiments, canola oil with glucose, or lactose as carbon sources as cost-effective substrates is utilized to produce sophorose lipids (SLs) by *Torulopsis bombicola*.

Figure 6.26 shows the time course of the cultivation of *Torulopsis bombicola* in medium containing 10% glucose and 10.5% canola oil in the shaking flasks. From the Figure, it can be seen that the lipids accumulation started in the middle of the exponential phase and increased rapidly when growth ceased. At this time nitrogen was nearly exhausted. Glucose was more quickly converted to biomass and sophorose lipids than canola oil. Glucose was nearly completely consumed after 4 days and canola oil was nearly consumed after 7 days. At the beginning of the fermentation, mainly glucose with a small amount of canola oil was utilized for biomass, then was converted to sophorose lipids. The highest yield of about 150 g/L sophorose lipids occurred at 7 days of fermentation. The results suggested that enzymes for biosynthesis of sophorose lipids were produced during the exponential growth phase. When nitrogen was nearly exhaused, the enzyme activities for biosurfactant synthesis were switched on.

**Figure 6.26 The Time Course of Cultivation of *Torulopsis bombicola* in shaking flasks from 10% Glucose, 10.5% Canola Oil, and 0.4% Yeast Extract at 250 rpm, 2vvm and 30°C.**


Biomass, glucose, canola oil and sophorose lipids (g/L)
Biomass
glucose
sophorose lipids
canola oil
Time (days)
0
10
20
30
40
50
60
70
80
90
100
110
120
130
140
150
160
0
1
2
3
4
5
6
7
8
9
10

The above experiments were repeated in the 1-L Bellco jar glass fermenters and the results are shown in Figure 6.27. It can be seen that the biomass production, sophorose lipids production, canola oil and glucose utilization from the growth medium containing 10% glucose and 10.5% canola oil in 1-L fermenter were very similar to those observed in the shake flask cultures. The highest yield of about 160 g/L sophorose lipids was obtained.

Since lactose can be easily obtained from cheese whey, lactose was used to replace glucose in repeating the above experiments in both shake flasks and 1-L fermenters. The results obtained in both shake flasks and 1-L fermenter were very similar. Figure 6.28 shows that the time course of cultivation of *Torulopsis bombicola* in 1-L fermenter in medium containing 10% lactose, 10.5% canola oil and 0.4% yeast extract. Conversion of canola oil and lactose of approximately 55% to sophorose lipids was achieved in 1-L fermenter compared to that of 80% from glucose and canola oil. Sophorose lipids were detected after 3 days of fermentation when near complete depletion of nitrogen source in the growth medium had occurred. The highest concentration of 110-120 g/L sophorose lipids was obtained when the culture was grown in 1-L fermenter after 6 days. The

**Figure 6.27 The Time Course of Cultivation of *Torulopsis bombicola* in 1-L Bellco Jar Glass Fermenters from 10% Glucose, 10.5% Canola Oil, and 0.4% Yeast Extract at 450 rpm, 2vvm and 30°C.**

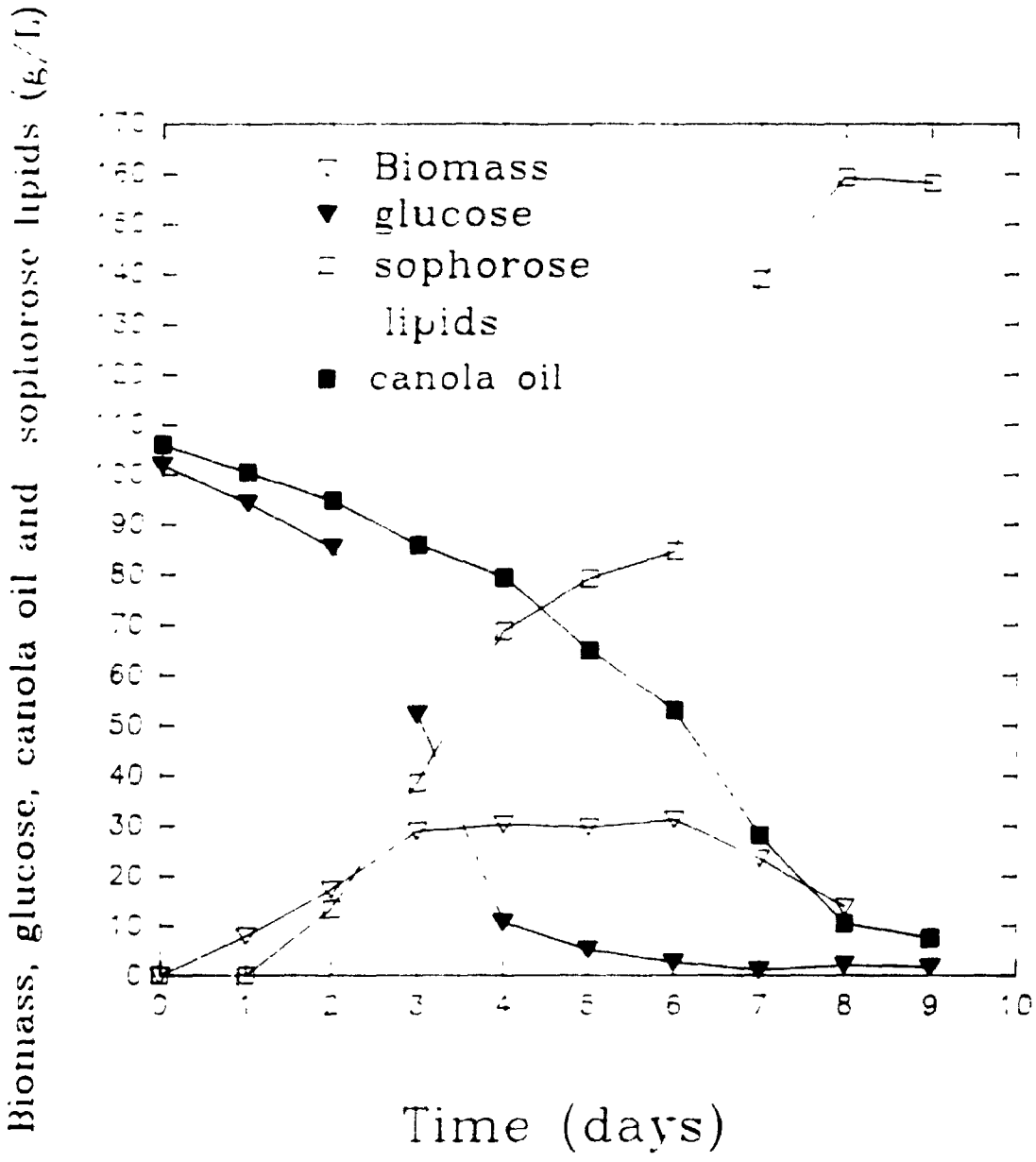
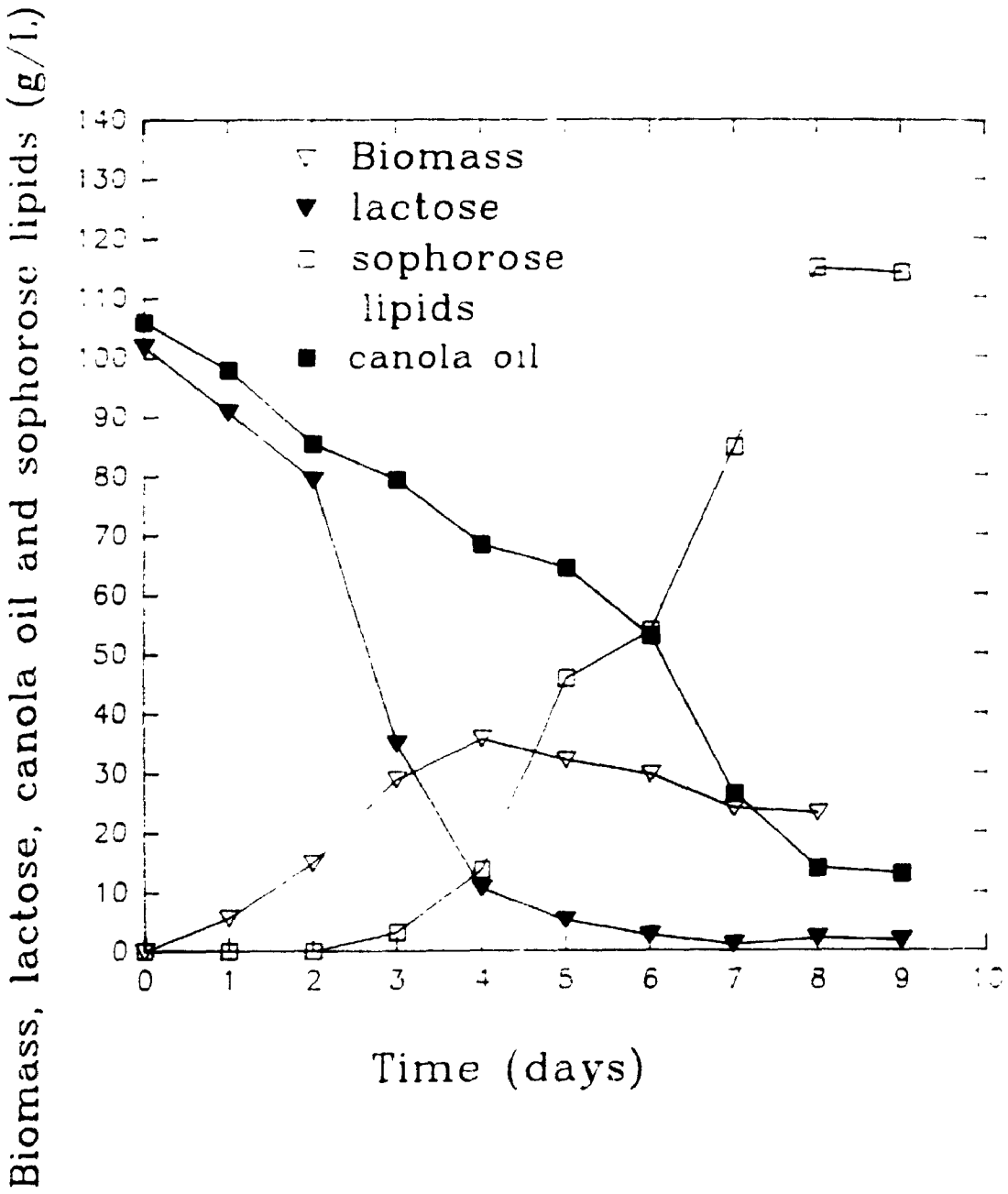

Biomass, glucose, canola oil and sophorose lipids (g/L)
Biomass
glucose
sophorose
lipids
canola oil
0
10
20
30
40
50
60
70
80
90
100
0
1
2
3
4
5
6
7
8
9
10
Time (days)

Figure 6.28 The Time Course of Cultivation of *Torulopsis bombicola* in 1-L Bellco Jar Glass Fermenters from 10% Lactose, 10.5% Canola Oil, and 0.4% Yeast Extract at 450 rpm, 2vvm and 30°C.


Biomass, lactose, canola oil and sophorose lipids (g/L)
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
0
1
2
3
4
5
6
7
8
9
10
Biomass
lactose
sophorose lipids
canola oil
Time (days)

maximum production of sophorose lipids from lactose and canola oil decreased compared to that from glucose and canola oil. However, lactose can be obtained either at little or no cost because considerable amounts of whey are obtained during cheese processing, causing considerable pollution (4-5% lactose content). When using 20% of solid cheese whey (70% lactose) to replace lactose in the above medium, about 12 g/L sophorose lipids were obtained. The production of sophorose lipids could be improved greatly when cheese whey from which 80% of the protein was removed by heat precipitation and ultrafiltration is used together with canola oil. The results are very similar to those obtained when actose and canola oil were used as substrates for the production of sophorose lipids. The reason is that sophorose lipids are secondary metabolizes produced under nitrogen limitation. Protein is a nitrogen sources. When cheese whey is used for sophorose lipids production, production is very low because it contains high concentration of protein which inhibits sophorose lipids production. Therefore deproteinated whey plus canola oil gave a very good lipids production. From the above experiments, the potential for using canola oil, lactose and cheese whey for the commercial fermentation process of sophorose lipids production has been demonstrated.

The most apolar sophorose lipid with highly hydrophobic group that has been described as 17-L-([2′-O-β-D-glucopyranosyl-β-D-glucopyranosyl]-oxy)-octadecanoic acid 1′-4′′-lactone 6′,6′′-diacetate (SL-1) group was increased to 73% from 50% by using canola oil instead of safflower oil in the mixture of sophorose lipids. Use of canola oil has resulted in increased yield of sophorose lipids comparable to the yield obtained in our laboratory when safflower oil was used in the medium (Zhou et al., 1992). Other data from literature yielded 70 g/L sophorose lipids (Cooper and Paddock, 1984), and 120 g/L (Inoue and Itoh, 1982) as shown in Table 6.2.

From the above experimental data, it appears that all mixed carbon sources tested increase the production of sophorose lipids compared to the sole carbon source. It is very important to use both vegetable oil and glucose to obtain a high concentration of sophorose lipids. In this case, sugar is used to synthesize sophorose and vegetable oil is modified to fatty acid. By this way, the cell energy required for the synthesis of sophorose lipids reduces. The mixture of canola oil and glucose was the best substrate for the production of sophorose lipids because canola oil contains 25% C18:3 which is more active to form sophorose lipids.

TABLE 6.2 COMPARISON OF PRODUCTION OF SOPHOROSE LIPIDS BY *TORULOPSIS BOMBICOLA*

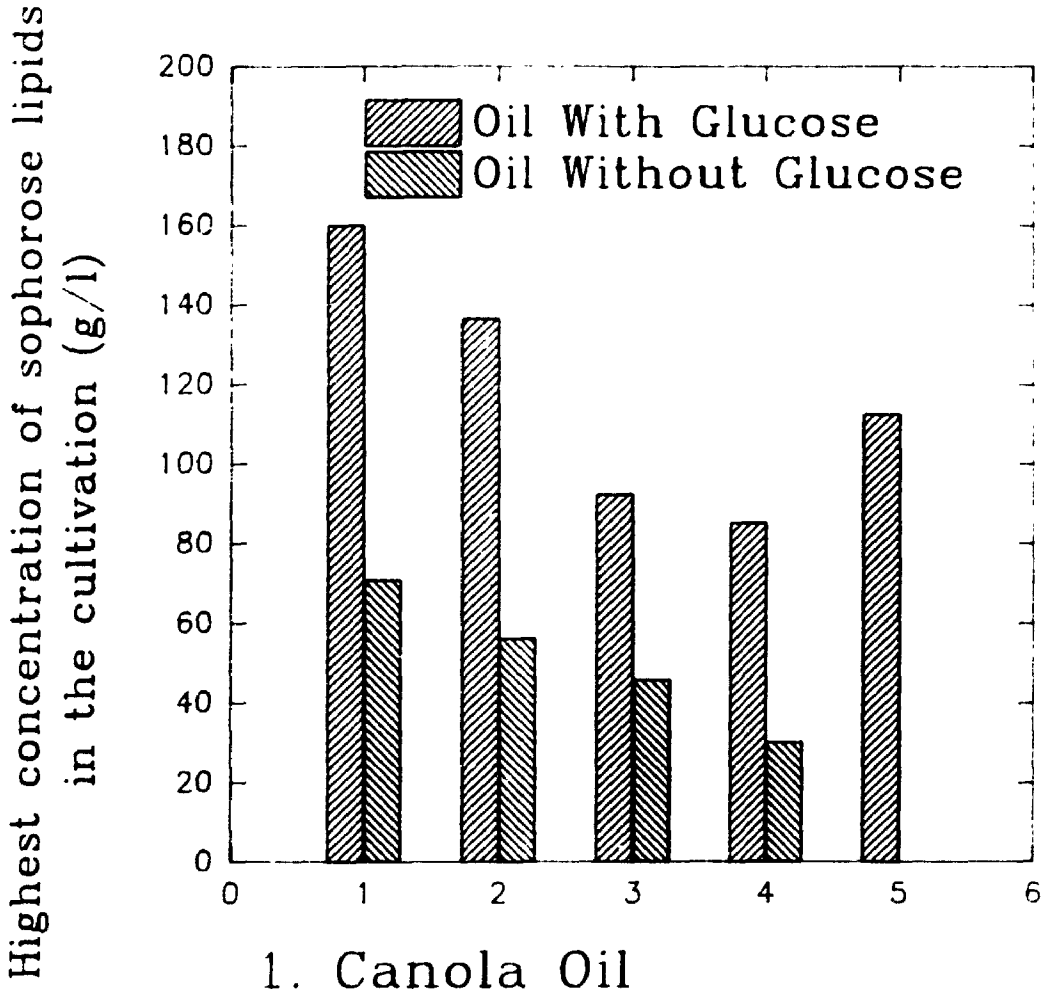
| MEDIUM COMPOSITION | BIOREACTOR VOLUME | CULTIVATION MODE | MAXIMUM YIELD, g/L | REFERENCE |
|---|---|---|---|---|
| 10% glucose<br>14% palm oil<br>0.75% yeast nitrogen base<br>0.2% yeast extract | 500 mL | Batch | 120 | INOUE & ITOH 1982 |
| 10% glucose<br>9.5% sunflower oil<br>0.5% yeast extract | 7 L | Batch | 70 | COOPER & PADDOCK 1984 |
| 13.6% oleic acid<br>1% yeast extract<br>0.1% urea | 30 L | Batch | 74 | ASMER et al. 1988 |
| 10% glucose<br>10.5% safflower oil<br>0.25% yeast extract<br>0.1% urea | 20 L | Batch | 137 | ZHOU et al. 1992 |
| 10% glucose or (10% lactose)<br>10% canola oil<br>0.4% yeast extract<br>0.1% urea | 1 L | Batch | 150-160 (glucose)<br>110-120 (lactose) | ZHOU & KOSARIC 1993 |

Comparison of production of sophorose lipids from various substrates was shown in Figure 6.29. As can be seen from the Figure, the highest production of sophorose lipids appears to be that from canola oil and glucose. The next was from safflower oil and glucose. It also showed that the highest production of sophorose lipids was obtained when both vegetable oil and sugar were used as substrates. Canola oil contains 25% of C18:3 which make it easier to metabolize and synthesize sophorose lipids than other vegetable oils which contain no C18:3.

## 6.2 Fed Batch Experiments

Based on the fed-batch models, we know how to design the fed-batch mode experiments. The model allow us to predict the behaviors of cell growth, and lipids production. Figure 6.30 shows the time course for fed-batch mode cultivation of *Torulopsis bombicola* with 10.5% canola oil, 10% glucose, and 0.4% yeast extract. After three days growth when the cells had reached stationary phase, 10 ml of fresh medium containing 10% glucose was added daily to the culture. Feeding was continued at this rate daily for six more day. Production of sophorose lipids increased greatly but biomass was kept constant. It

Figure 6.29 Comparison of Production of Sophorose Lipids by Various Substrates in 1-L Bellco Jar Glass Fermenters Under The Optimal Cultivation Conditions




1. Canola Oil
2. Safflower Oil
3. Sunflower Oil
4. Olive Oil
5. Canola Oil + Lactose

could be seen that excess of carbon was converted to sophorose lipids during the stationary phase. By comparing the data in Table 4.1 and the data in Figure 6.30, it can be seen that both show similar patterns of cell growth, sophorose lipids production and sugar utilization. The models fit the experiment patterns with reasonable success. For example, both show that at the stationary phase, by keeping sugar concentration constant, lipids production phase are extended and productivity are increased. It is important to mention that the data in Table 4.1 can not used for the analysis of experiment data because the kinetic constants are different. However, kinetic modelling for the fed-batch mode results in relatively simple equations which can be used to describe, design and predict experiments. When 10.5% canola oil and 10% glucose were used as fresh medium to repeat the above experiments, the results shown in Figure 6.31 were very similar to the above.

Sophorose lipids are produced during starting early stationary phase. Therefore they are considered as secondary metabolites. The yeast *Torulopsis bombicola* intially rapidly utilizes carbon-energy sources for cell growth and then synthesizes sophorose lipids in the stationary phase. In a batch culture

**Figure 6.30 The Time Course of Cultivation of *Torulopsis bombicola* in 1-L Bellco Jar Glass Fermenters from Glucose and Canola oil with The Addition of Glucose to The Mixed Carbon Sources in The Repeated Fed Batch Mode at 450 rpm, 2 vvm and 30°C.**

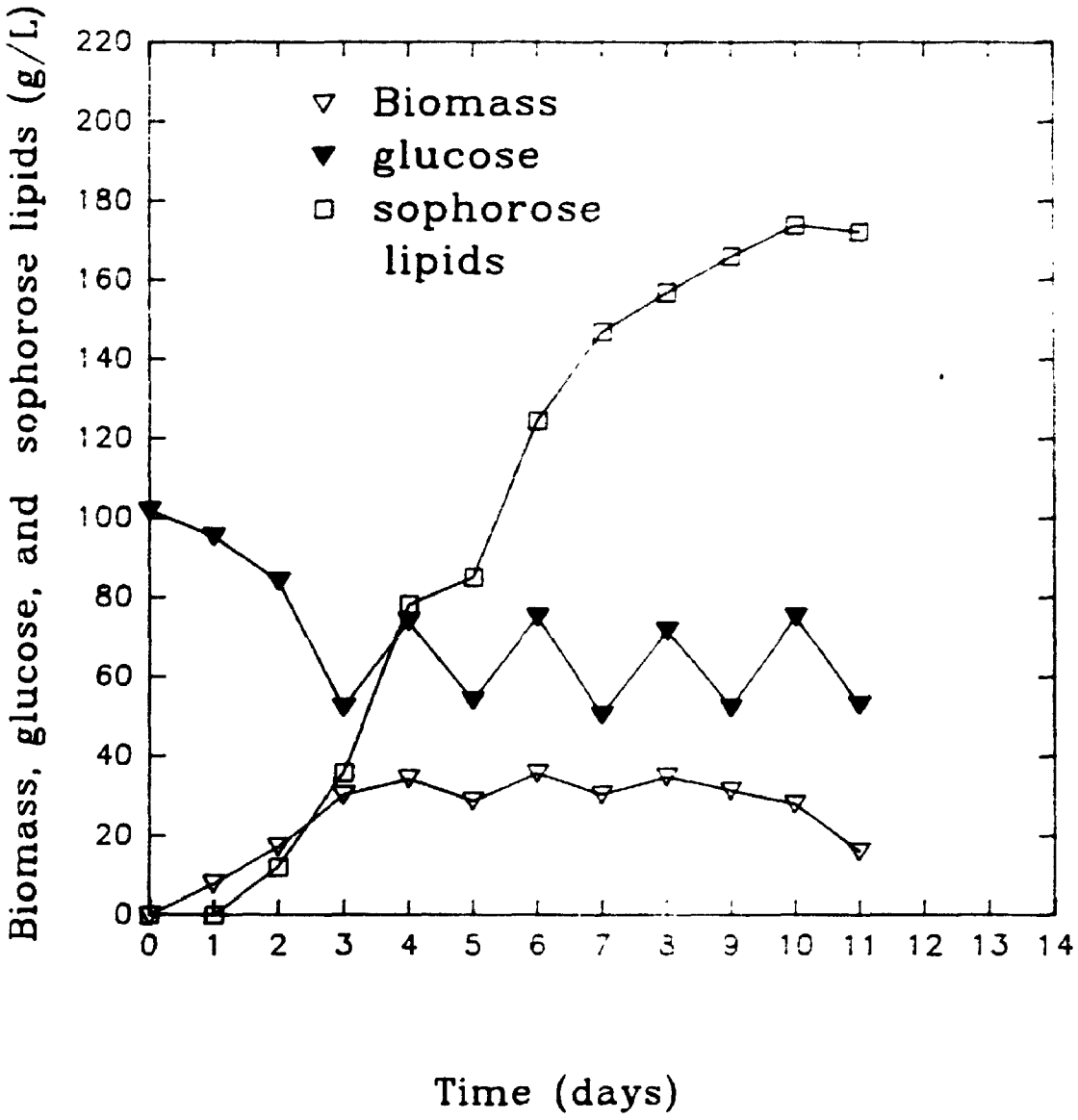
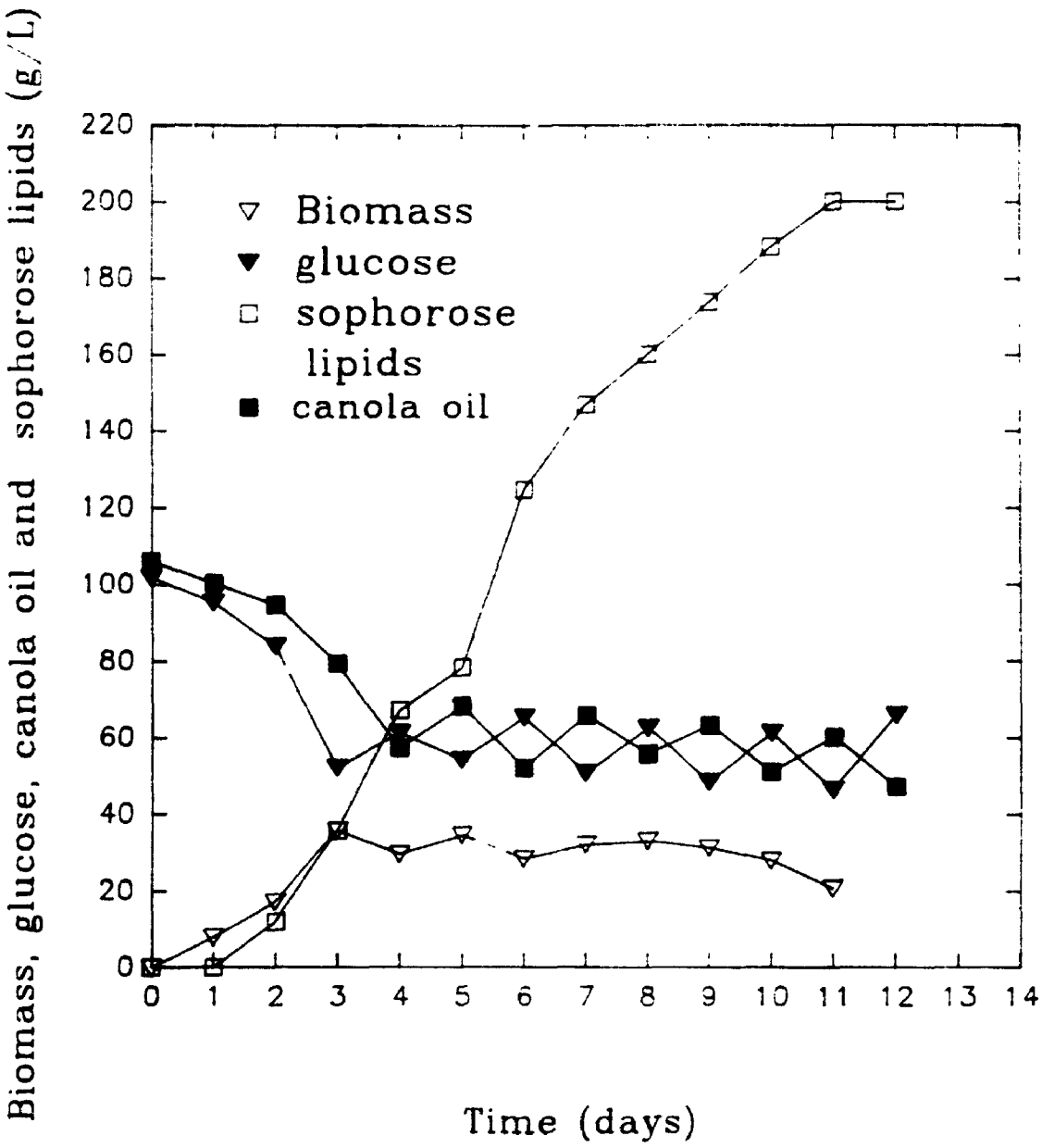

Biomass
glucose
sophorose lipids
Biomass, glucose, and sophorose lipids (g/L)
Time (days)
0
20
40
60
80
100
120
140
160
180
200
220
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14

**Figure 6.31 The Time Course of Cultivation of *Torulopsis bombicola* in 1-L Bellco Jar Glass Fermenters from Glucose and Canola Oil with The Addition of Canola oil and Glucose to The Mixed Carbon Sources in The Repeated Fed Batch Mode at 450 rpm, 2vvm and 30°C.**


Biomass, glucose, canola oil and sophorose lipids (g/L)
220
200
180
160
140
120
100
80
60
40
20
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
Time (days)
Biomass
glucose
sophorose lipids
canola oil

mode, the production phase was short, due to the depletion of the carbon-energy sources. Therefore, after transition from the growth to the synthesizing phase, it is important to maintain a concentration of the carbon-energy source by fed-batch cultivation. Fed-batch cultivation also has the advantage that inocula need not be prepared for the second and subsequent cycles. By fed-batch cultivation, it could be seen that the maximization of yields, productivity and the minimization of production costs of sophorose lipids were obtained because the production phase was extended and substrates and enzymes were utilized very effectively.

## 6.3 Costs Estimates

### 6.3.1 Mass Balance

Stoichimetric equations describing the consumption of substrate and the formation of biomass can be used in various ways.

(1) To obtain relationships between different yield coeffients based on elemental and energy balances describing microbial activity.

(2) To obtain the theoretical limits of the maximum yields of biomass and products formed from a given substrate.

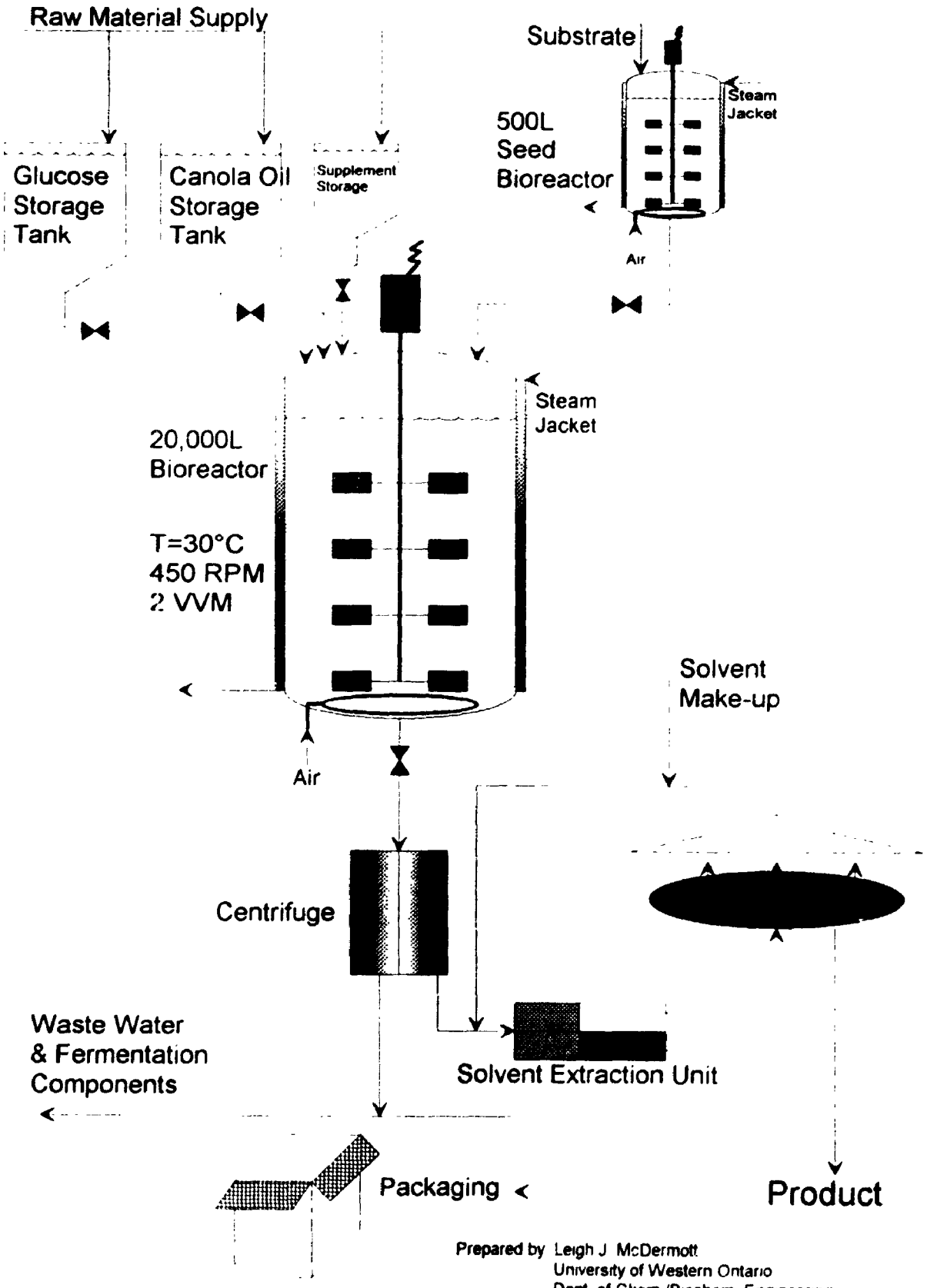
(3) To estimate yield coefficients from experimentally determined quantities such as substrate consumption, biomass production, and nitrogen consumption

(4) To explore reasons for discrepancies between measured yield values and those predicted from the stoichiometry for a given system due to systematic errors in measurement and an incorrect system description, such as the presence of an unknown product or substrate.

(5) To construct figures that allow quick estimates of yield coefficients as functions of experimentally measurable variables.

Figure 6.32 shows sophorose lipids production process. Mass balances for production of sophorose lipids from sugars and vegetable oils can be made by the assumption that the only products of metabolism are cells, sophorose lipids, carbon dioxide and water.

Figure 6.32 Sophorose Lipids Production Process

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Dept of Chem /Biochem Engineering

For aerobic growth of the yeast *Torulopsis bombicola* on the mixture of glucose and safflower oil the following equation can be applied.

$$2.5C_6H_{12}O_6 + 1.6\ C_{17}H_{33}COOH + 7\ O_2 + 1.62\ NH_3 \rightarrow$$
$$1.89C_{4.4}H_{7.3}N_{0.86}O_{1.2} + 1.89CO_2 + 8.7H_2O$$
$$+\ C_{34}H_{58}O_{18} \qquad (6.1)$$

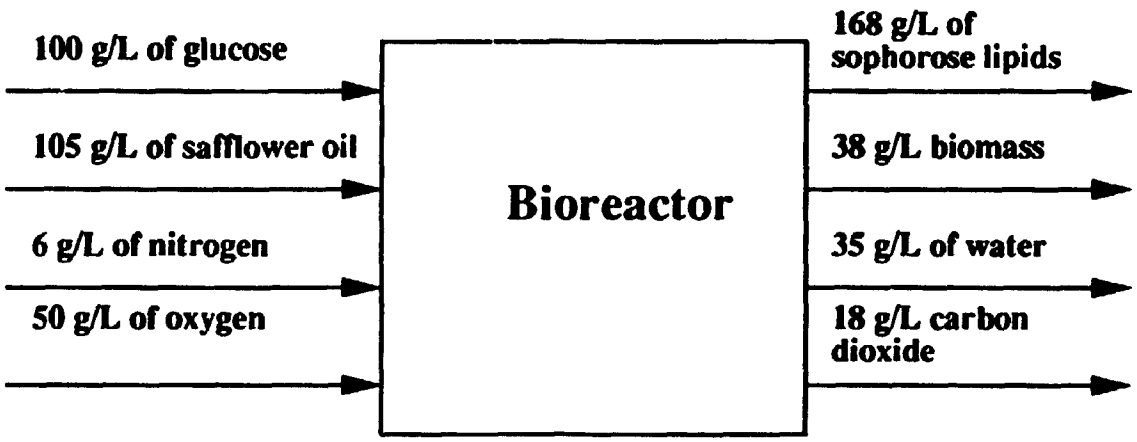
The above stoichiometric equations can be used to estimate the theoretically possible maximum yields on the utilized substrates. According to this equation on a weight basis, 84% of glucose and safflower oil are converted to sophorose lipids and 16% to carbon dioxide, water and biomass. Thus the yield may be expressed as follows:

$$YsL/s = 84\%$$

Based on the consumed substrates of 100 g/L glucose and 105 g/L safflower oil (or canola oil), 50 g/L oxygen and 5 g/L nitrogen, mass balance flow-sheet for Sophorose Lipids Production is made as shown in Figure 6.33. From the mass balance flow-sheet, it can be seen that 38 g/L biomass, 18.29 g/L $CO_2$, 35 g/L water and 168. 71 g/L sophorose lipids can be

Figure 6.33 Mass Balance Flow-Sheet for Sophorose Lipids Production

Mass balance flow-sheet




Basis: 100 g/L glucose

according to the mass balance equation for sophorose lipids production, we have

(a) substrates consumed

safflower oil = 282 x (100/180) (1.6/2.5) = 101 g/L
oxygen = 32 x (100/180)(7/2.5) = 50 g/L
nitrogen = 17 x (100/180)(1.62/2.5) = 6 g/L

(b) products formed

sophorose lipids = 754 x (100/180) = 168 g/L
water = 18 x (100/180)(8.7/2.5) = 35 g/L
biomass = 91.2x (100/180)(1.89/2.5) = 38 g/L
carbon dioxide = 44 x (100/180)( 1.89/2.5) = 18 g/L

Overall mass balance: substrates consumed = product formed + losses

substrates consumed = 100 + 105 + 50 + 6 = 261 g/L
product formed = 168 + 35 + 38 + 18 = 259 g/L

Therefore , substrates consumed nearly equal to products formed
( there is a small amount of losses)

obtained.

These values are the maximum theoretical yields attainable. In practice, yields of only 80-90% of the maximum theoretical value were achieved due to losses in the formation of byproducts.

### 6.3.2 Cost of Sophorose Lipids

The key factor governing a sucessful fermentation process for sophorose lipids by Torulopsis bombicola largely depends on identifying a cheap and aboundant feedstock. A wide variety of carbon sources including sugars and vegatable oils were examined for the sophorose lipids production. The results had been discussed in the above text. It was found that the yeast *T. bombicola* produced a large amount of sophorose lipids in vegetable oils, sugars or mixture of sugar and oil. This yeast could also utilize waste stream such as cheease whey. The optimal yields are obtained when both carbohydrate and vegetable oil were used as substrates.

The main limiting factor for the commercialization of fermentation compounds is the economics of large-scale

production. This is also the case for biosurfactants. Economics is a major determinant in bringing a product to market since a monetary return has to be realized. Biosurfactants must be produced less expensively. Inexpensive substrates could be very important in determining the economic feasibility of producing biosurfactants commercially. The choice of raw material can significantly influence the final product cost. In attempting an analysis of the costs of producing sophorose lipids from various substrates one is faced with a variety of problems. The shortage of relevant data is the main hindrance, as is always the case for systems with which there is only a limited amount of practical experience. Moreover many of the detailed economic appraisals that have been performed for specific projects are not publicly available. For the information that is available, a number of additional problems emerge. Firstly, cost data are rarely presented in a common format. The accounting systems used differ, as do the taxation levels, the depreciation allowances, and the financing methods. This makes it difficult to do a valid comparison between the results from different studies. In view of these problems in analyzing the cost of biosurfactants production, the following simple approach has been taken. This is a simplified method of costing which

allows easy and direct comparison of production costs to be made. In this method, the costs for sophorose lipids production by various substrates may be roughly calculated based on the estimate that carbon sources make up about 50% of the price of biosurfactants. The calculated costs equation is given by

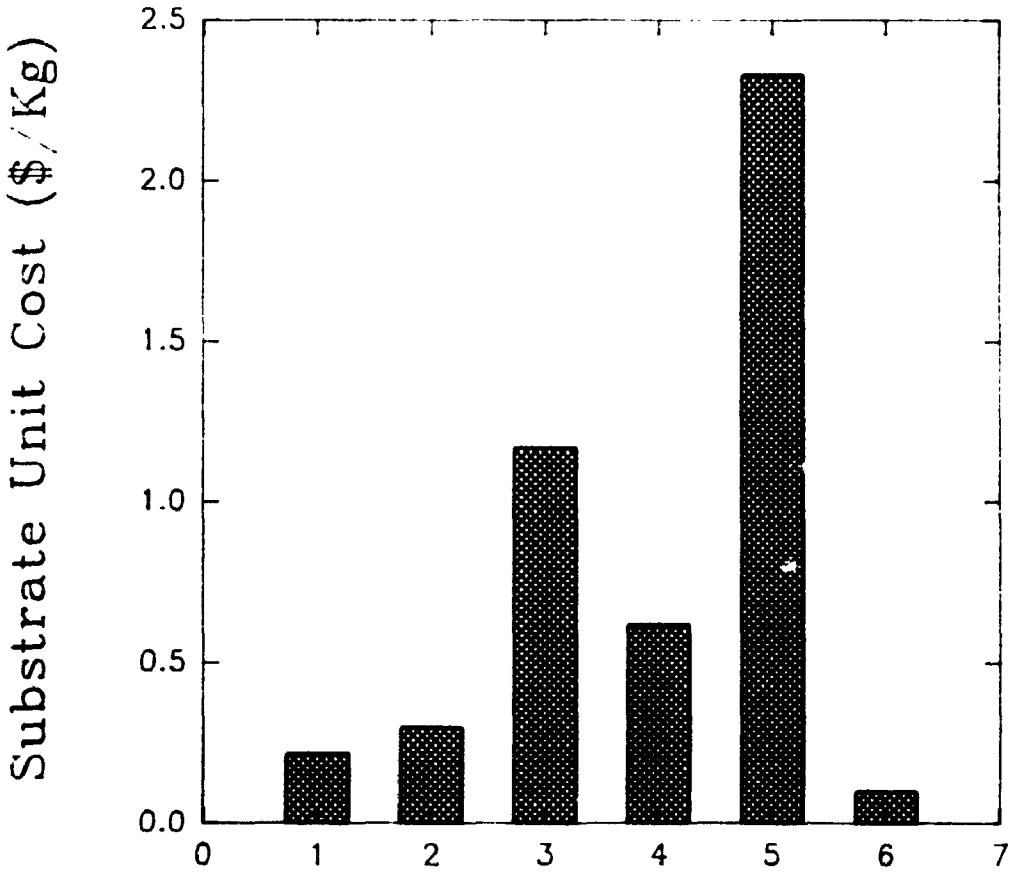
$$T_{cost} = S_{cost}/0.5P = S_{cost}/(0.5X_{cell}Y_{p/x})$$

where $T_{cost}$ is product cost, $S_{cost}$ is substrates cost, $X_{cell}$ is biomass, P is product yield and $Y_{p/x}$ is the yield coefficient. For example, canola oil costs \$0.32/kg, lactose from cheese whey \$0.07/kg, thus, the total cost of substrates being \$0.39, P = 0.9 kg/2kg substrates. The total cost for sophorose lipids is

$$T_{cost} = 0.39/0.5x0.9 = \$0.867/kg$$

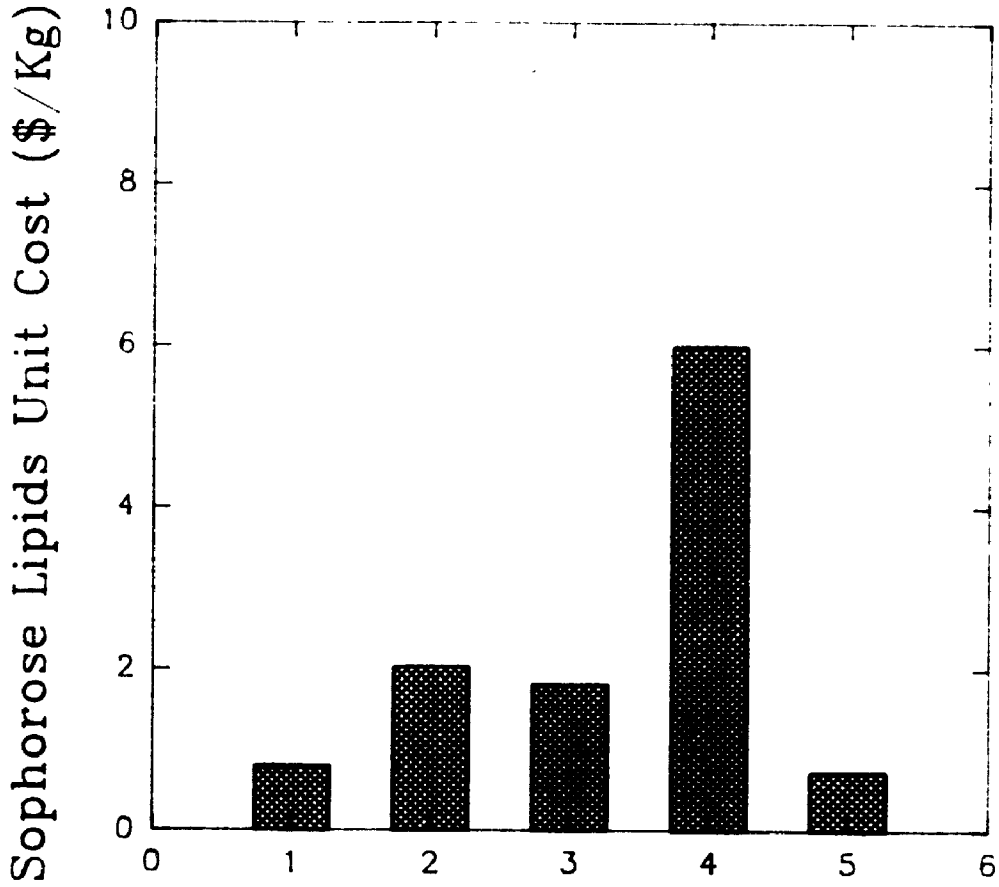
Figure 6.34 shows the costs of various carbon sources. Comparing the cost of various substrates, canola oil and lactose appear to be the least expensive. Glucose is the next least expensive alternative, with olive oil coming last. Based on Figure 6.34 and the above equation, it was possible to culculate the cost of sophorose lipids from various

Figure 6.34 Comparison of Substrate Unit Cost for Sophorose Lipids Production




1. Glucose
2. Canola Oil
3. Safflower Oil
4. Sunflower Oil
5. Olive Oil
6. Lactose from Cheese Whey

**Figure 6.35 Comparison of Sophorose Lipids Unit Cost from Various Substrates**




Sophorose Lipids from

1. Canoia Oil + Glucose
2. Safflower Oil + Glucose
3. Sunflower Oil + Glucose
4. Olive Oil + Glucose
5. Canola Oil + Lactose

substrates. A summary cost comparison of the final biosurfactants by various raw materials is shown in Figure 6.35. These estimates are obviuosly crude but suggest that the sophorose lipids would be competitive with synthetic surfactants in many industries, under present prices, provided they are used as biosurfactants. It is shown that the cheapest cost of sophorose lipids is from canola oil and lactose.

As mentioned, there are a variety of problems in comparing cost estimates for sophorose lipids production. The simple method described above allows direct comparisons to be made without worrying about distortions arising from the use of different interest rates, depreciation allowances, tax rates or other factors. The major disadvantage of this kind of approach is that simplification can lead to errors unless it is done carefully. A much more detailed method in production cost estimates is to use " costing categories". In this method, the costs of biosurfactant production have been broken down into two basis categories. These categories and the types of costs they include are listed as follows:

1. Capital cost

- Plant construction
- Cultivation equipment
- Harvesting equipment
- Miscellaneous

2. Operating cost

- Raw materials
- Labor
- Harvesting
- Utilities

The costs of carbon sources, nitrogen sources, and other components for biosurfactant production are shown in Table 6.3-6.7 (Mulligan and Gibbs, 1992). To do the cost estimate for sophorose lipids, the following assumptions have been made :

1. The lifetime of sophorose lipids plant is 30 years.
2. The volume of batch fermenter is 200, 000 litre.
3. Each batch fermentation needs 6 days.
4. Conversion plant operates 300 day per year = 50 batch

fermentation per year.

5. Utilities including power consumption, steam consumption and air consumption are $80 per day.

Using these assumptions, Table 6.3-6.7 and Figure 6.32, the cost estimate is made as follows:

Capital cost:

| | |
|---|---|
| Plant construction | 100, 000/year |
| 200, 000 liter fermenter | 150, 000/year |
| Harvesting equipment | 30, 000/year |
| Miscellaneous | 100, 000/year |
| the subtotal of capital cost | 380, 000/year |

Operating cost:

Based on Table 6.3-6.7, raw materials cost for a 200,000 liter fermenter can be made :

| Medium composition | | Weight (kg) | Cost (US$) |
|---|---|---|---|
| $KH_2PO_4$ | 0.1% | 200 | 434 |
| $MgSO_4.7H_2$ | 0.5% | 100 | 350 |

TABLE 6.3 The Cost of Various Carbon Sources

| Carbon Sources | Unit Cost($/kg) |
|---|---|
| **Carbohydrates** | |
| Corn syrup (95% glucose) | 0.25 |
| Glucose derived from corn | 0.22 |
| Fructose derived from corn | 0.48 |
| Corn starch | 0.20 |
| Black strap molasses | 0.08 |
| Ethanol | 0.52 |
| Methanol | 0.12 |
| Citric acid | 1.64 |
| Lactic acid (88% | 2.27 |
| Succinic acid | 9.57 |
| Mannitol | 7.31 |
| Glycerol | 1.61 |
| Dextrose | 0.52 |
| Fructose | 0.86 |
| Sucrose | 0.79 |
| Lactose | 0.48 |
| **Hydrocarbons** | |
| Kerosene | 0.49 |
| n-Paraffins | 0.51 |
| Hexane | 0.30 |
| Xylene | 0.38 |
| Hexadecane (87%) | 4.50 |
| **Vegetable oils** | |
| Soybean oil | 0.52 |
| Corn oil | 0.62 |
| Safflower oil | 1.17 |
| Sunflower oil | 0.62 |
| Olive oil | 2.33 |
| Canola oil | 0.35 |
| Cottonseed oil | 0.50 |

TABLE 6.4 The Cost of Nitrogen Sources

| Nitrogen Source | %N | Unit Cost($/kg) | $/kgN |
|---|---|---|---|
| Ammonia | 82.3 | 0.12 | 0.15 |
| Ammonium hydroxide | 24.2 | 0.26 | 1.08 |
| Ammonium nitrate | 33.5 | 0.14 | 0.42 |
| Ammonium sulphate | 14.2 | 0.14 | 0.99 |
| Soybean meal | 8.0 | 0.18 | 2.25 |
| Cottonseed meal | 5.2 | 0.18 | 3.46 |
| Urea | 47.7 | 0.15 | 0.31 |
| Yeast extract | 8.0 | 3.30 | 41.25 |
| Hy-Soy | 9.5 | 10.89 | 114.63 |
| N-Z-Case | 13.1 | 10.89 | 83.13 |
| Corn steep water | 6.7 | 0.14 | 2.09 |
| Sodium glutamate | 9.5 | 1.67 | 17.58 |
| Brewer's yeast | 8.0 | 2.40 | 30.00 |
| Whey | 1.5 | 0.60 | 40.00 |
| Black Strap molass | 1.5 | 0.08 | 5.32 |

TABLE 6.5 The Cost of Phosphates

| Phosphate Source | Unit Cost($/kg) |
|---|---|
| Ammonium phosphate | |
| Monobasic | 0.17 |
| Dibasic | 0.18 |
| Sodium phosphate | |
| Monobasic | 1.35 |
| Dibasic | 1.32 |
| Potassium phosphate | |
| Monobasic | 2.17 |
| Phosphoric acid | |
| 52-54% | 0.29 |
| 70% | 0.34 |

TABLE 6.6 The Cost of Other Component

| Solvent | Unit Cost($/kg) |
|---|---|
| Magnesium sulfate | 0.35 |
| Manganese sulfate | 0.48 |
| Sodium chloride | 0.06 |
| Ferrous chloride | 0.01 |
| Acids | |
| Hydrochloric | 0.055 |
| Sulfuric | 0.075 |
| Phosphoric | 0.27 (54%) |
| | 0.35 (75%) |
| Bases | |
| Potassium hydroxide | 0.29 |
| Ammonium hydroxide | 0.26 |
| Caustic soda | 0.29 |
| Potassium bicarbonate | 0.63 |
| Sodium bicarbonate | 0.38 |

TABLE 6.7 The Cost of Various Solvents Used for Biosurfactant Recovery

| Solvent | Unit Cost($/kg) |
|---|---|
| Butanol | 0.84 |
| Ethyl acetate | 0.90 |
| Hexane | 0.30 |
| Chloroform | 0.79 |
| Methanol | 0.12 |
| Dichloromethane | 0.64 |
| Ethyl ether | 1.13 |

| | | | |
|---|---|---|---|
| $FeCl_3$ | 0.01% | 20 | 0.2 |
| NaCl | 0.01% | 20 | 1.2 |
| Yeast extract (corn steep liquor) | 0.4% | 800 | 2, 640 |
| Urea | 0.1% | 200 | 30 |
| Canola oil | 10.5% | 21, 000 | 6, 720 |
| Glucose | 10% | 20, 000 | 4, 400 |

Each batch fermentation $14, 575.4

50 batchs per year $728, 770/year

Harvesting

| | | | |
|---|---|---|---|
| Ethyl acetate | 50% | 100, 000 | 90, 000 |
| Methanol | 50% | 100, 000 | 12, 000 |
| Chloroform | 5% | 10, 000 | 7, 900 |

subtotal of harvesting $109, 900

Labor 5 x $30, 000 = $150, 000/year

Utilities 300 x $80 = $24, 000/year

the subtotal of operating cost $1, 012,670/year

total cost for sophorose lipids production

= the subtotal of capital cost + the subtotal of operating cost

= 380, 000/year + $1, 012, 670/year

= $1, 392, 670/year

The conversion of carbon sources to sophorose lipids is about 80%,

the yield sophorose lipids =(20, 000 + 21, 000) x 80% x 50

= 1, 640, 000 kg/year

The cost of sophorose lipids = $1, 392, 670/1, 640, 000 = $0.85/kg

The estimated cost for sophorose lipids using the above two approaches are about the same. Obviously, the accuracy of these estimates is not very great since the major assumptions have been made in the analyses. Within its terms of reference, however, this study has attempted to go some way in answering the basic questions" are sophorose lipids competive with synthetic surfactants?". It is hoped that the simplification that have been made in the cost estimates can provide very useful information in choosing very effective substrates for the production of biosurfactants.

# 7. CONCLUSIONS

The commercial interest in sophorose lipids production has triggered our research effort towards the synthesis of this high value biosurfactant. The key to the successful commercial production of sophorose lipids lies in using cheap and plentiful substrates since substrates make up 50% the final cost of product. Various low or negative cost substrates were used to produce sophorose lipids to reduce the final product costs. It showed that a wide range of carbon sources including agricultural renewable resources like sugars and oils are suitable carbon sources to produce sophorose lipids with good surfactant properties. The cost for production of sophorose lipids can be very low if appropriate substrates are used. Probably the most exciting aspect of this study is how to obtain high production of sophorose lipids by low and negative cost substrates. The results obtained thus far reveal many possibilities for the economic production of biosurfactants. The most attractive route would be to use plentiful and low or negative cost substrates for the production of biosurfactants. Moreover utilization of waste materials in producing sophorose lipids also helps to prevent overwhelming pollution problems. For example, Cheese whey is a source of cheap raw materals and

potentially has important features from environmental viewpoints. In the near future, sophorose lipids will partially replace the synthetic surfactants.

*Torulopsis bombicola* is one of the few yeasts known to produce biosurfactants. Sophorose lipids can lower the surface tension to 33 mN/m which are potentially useful in various industries including food, cosmetic, soap, detergent, bioremediation of contaminated land sites. It is expected that biosurfactants will substantially contribute to the cosmetic industry because they have the advantages of extremely low irritancy or antiirritating effects and compatibility with the skin, both properties demanded by today's market. Moreover, they are produced from cheap or waste materials. Probably, the most important advantage of biosurfactants over chemical surfactants is their ecological acceptability. Many chemically synthesized surfactants cause ecological problems owing to their resistance to biodegradation, toxicity, and accumulation in natural ecosystems. On the other hand, biosurfactants are biodegradable. From the experimental results, it can be concluded that:

1. The carbon sources and concentrations have a profound

effect on sophorose lipids production. High carbon concentrations enhance sophorose lipids production. The optimal production of sophorose lipids was obtained when both sugars and vegetable oils were used as the mixed carbon sources.

2. The nitrogen sources and concentration have a profound effect on sophorose lipids production. Excess of nitrogen repressed sophorose lipids production. The highest concentration of 150-160 g/L sophorose lipids in the batch mode culture was achieved under the nitrogen limitation using 0.4% yeast extract, 10.5 % canola oil and 10% glucose in the growth medium.

3. The addition of small amounts of honey, lactose and galactose to the medium containing 10% glucose, and 10.5% safflower oil increased greatly the production of sophorose lipids. Honey is not essential to the high production of sophorose lipids in the mixed medium and it can be replaced by lactose and galactose in the mixed carbon sources for the high production of sophorose lipids.

4. It was shown that deproteinated cheese whey was a very

effective substrate for sophorose lipids production.

5. The productivity of sophorose lipids could be improved by the use of fed-batch cultivation.

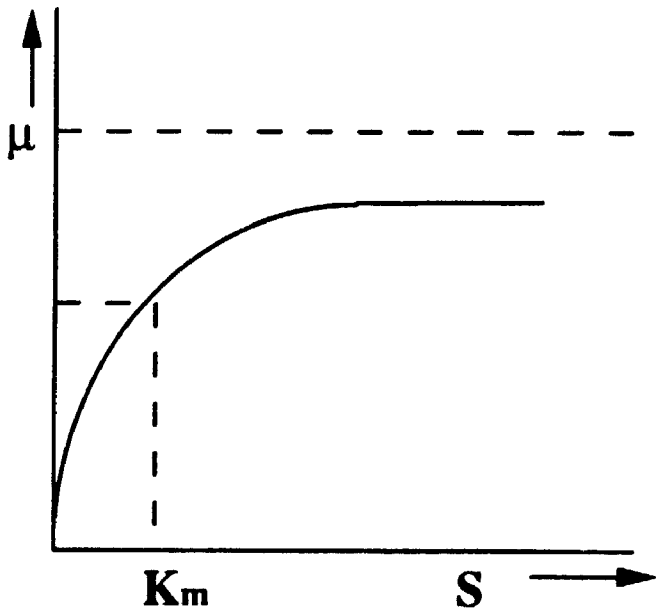
# 8. RECOMMENDATIONS

This study has demonstrated an economic process by using low cost and waste materials to make biosurfactants economically competitive with synthetic surfactants. There still is much work to be done in this area. There are several areas which clearly require further research to improve the prospects for commercialization of sophorose lipids. It is recommended to use bulk, readily available, and waste materials to produce biosurfactants. Waste streams should be investigated more thoroughly as no-cost substrates as a way to reduce treatment costs. Further improvements in reactor design, such as the two-stage continuous system can greatly enhance the productivity of sophorose lipids and it should be the main concern of research efforts. It may be possible to use ultrafiltrati membrane systems for the recovery of sophorose lipids to reduce the cost of product recovery. Large-scale production of sophorose lipids should be carried out for the commercial application. Application of sophorose lipids in medicine can be studied to exploit their potential application of sophorose lipids. In addition, a better understanding of the relationship between cellular organization and secondary

metabolism is needed to more clearly define the requirements for the design of bioreactors.

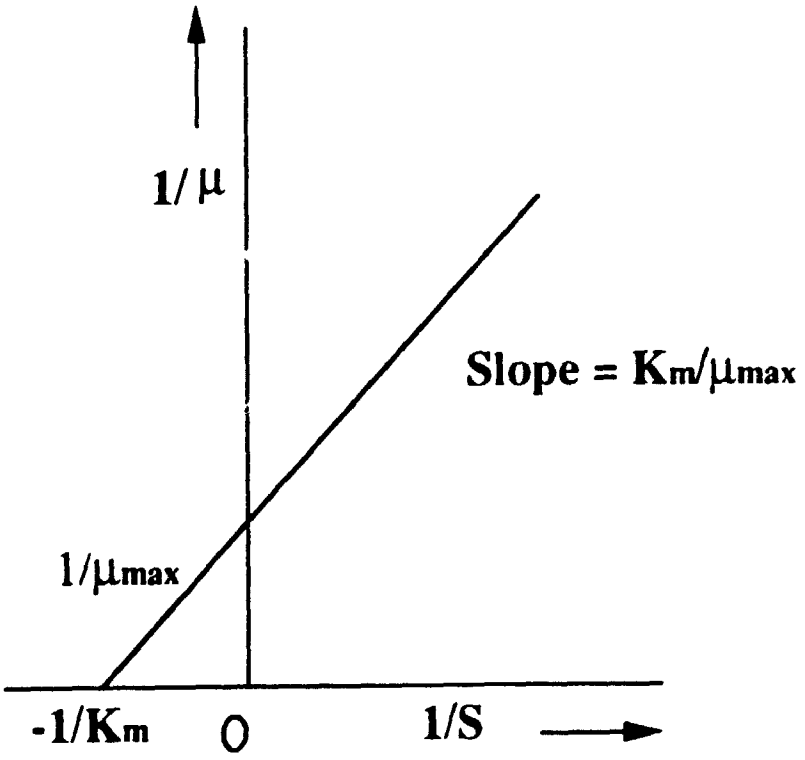
# APPENDIX 1

## SPECIFIC GROWTH RATE VERSUS SUBSTRATE CONCENTRATION



# APPENDIX 2

## LINWEAVER-BURK PLOT



# APPENDIX 3

## THE SPECIFICATIONS OF 20 L FERMENTER

### 20 L Chemap Laboratory Pilot Fermenter

| | |
|---|---|
| Model | LF .7/14/20 |
| Serial No. | NR 365 |
| Inside diameter (Dt), cm | 25 |
| Total height, cm | 40.5 |
| Capacity, liters | 20 |
| Impeller type | Flat blade turbine |
| No. impeller, blades/set | 4 |
| No. impeller sets | 3 |
| Impeller outside diameter, cm | 8 |
| Blade width, cm | 1.5 |
| Baffle assembly: | |
| No. baffles | 4 |
| width of baffles, | 3 |
| Sparger | Single orifice |
| Antifoam control | Mechanical |

# APPENDIX 4

## FATTY ACID COMPOSITIONS OF SOME VEGETABLE OILS (% m/m)

| Fatty acid | Canola | Cotton-seed | Olive | Soyabean | Sunflower seed | Safflower seed |
|---|---|---|---|---|---|---|
| C14:0 | - | 0.7-1.0 | - | - | - | - |
| C16:0 | 3.4-6.0 | 21.4-26.4 | 12 | 9.9 | 5.6-7.4 | 5.3-8.0 |
| C16:1 | 0.2-0.6 | 0.3-1.1 | 0.5 | - | - | - |
| C18:0 | 1.1-2.5 | 2.3-3.2 | 2.5 | 3.6-5.4 | 3.0-6.3 | 12.1-2.9 |
| C18:1 | 52-66 | 14.7-21.2 | 75.5 | 17.7-25.5 | 14.0-34.0 | 8.4-21.3 |
| C18:2 | 17-25 | 46.7-57.7 | 8.5 | 50.5-56.8 | 55.5-73.9 | 67.7-83.2 |
| C18:3 | 6.5-15 | - | - | 5.5-9.5 | - | - |
| C20:0 | - | - | - | - | - | - |
| C20:1 | 1.2-3.4 | - | - | - | - | - |
| C22:0 | - | - | - | - | - | - |

# APPENDIX 5

## SURFACE AND INTERFACIAL TENSION MEASUREMENTS

One of the most important characteristics of the sophorose lipids is its ability to reduce the surface and interfacial tensions of oil/water interfaces. The surface and interfacial tensions were evaluated with a Fisher Autotensiomat.

At room temperature, the surface tension of water is approximately 72 mN/m; this is the highest surface tension of any biologically relevant liquid, and it results primarily from the effects of hydrogen bonding. Many surfactants can reduce surface tension to approximately 25-35 mN/m depending on concentration and the surfactant type. In the case of sophorose lipids, increasing the concentration of sophorose lipids reduced the surface tension of water. Sophorose lipids which produced glucose and safflower oil lowered the surface tension of water from 72 mN/m to 33 mN/m. The critical micelle concentration of sophorose lipids was 82 mg/L. It was also found that sophorose lipids obtained from glucose or lactose plus canola oil could lower the surface tension of water (air-water interface) from 72 mN/m to 33 mN/m and the interfacial tension (oil-water interface) for water against n-hexadecane from 40 mN/m to 1 mN/m. Sophorose lipids are good biosurfactants because they have hydrophilic and hydrophobic moieties which act as a bridge between the two materials meeting at the interface to lower surface and interface tension. The hydrophilic portion of sophorose lipids is imbedded in the aqueous phase, whereas the hydrophobic portion faces the air.

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