STUDIES ON SOPHOROLIPIDS PRODUCTION AND PRETREATMENT OF HIGH FATS AND OILS CONTAINING DAIRY WASTEWATER USING *CANDIDA BOMBICOLA*

A THESIS

submitted by

ACHLESH DAVEREY

for the award of the degree

of

DOCTOR OF PHILOSOPHY

DEPARTMENT OF BIOTECHNOLOGY
INDIAN INSTITUTE OF TECHNOLOGY GUWAHATI

AUGUST 2010

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Dedicated to my Parents

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DEPARTMENT OF BIOTECHNOLOGY

STATEMENT

I do hereby declare that the matter embodied in this thesis is the result of investigations carried out by me in the Department of Biotechnology, Indian Institute of Technology Guwahati, Guwahati, India, under the supervision of Dr. Kannan Pakshirajan.

In keeping with the general practice of reporting scientific observations, due acknowledgements have been made wherever the work described is based on the findings of other investigators.

Date: ………………. **Achlesh Daverey**

INDIAN INSTITUTE OF TECHNOLOGY GUWAHATI

DEPARTMENT OF BIOTECHNOLOGY

CERTIFICATE

It is certified that the work described in this thesis entitled "**Studies on Sophorolipids Production and Pretreatment of High Fats and Oils Containing Dairy Wastewater Using *Candida bombicola***" by Mr. Achlesh Daverey for the award of degree of Doctor of Philosophy is an authentic record of the results obtained from the research work carried out under my supervision in the Department of Biotechnology, Indian Institute of Technology Guwahati, India, and this work has not been submitted elsewhere for a degree.

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Date: ……………… **Achlesh Daverey**

ABSTRACT

Dairy industry is one of the major food industries in most of the countries of the world including India and this industry has grown rapidly due to the high demand of milk and milk products. Dairy industry generate large amount of wastewater which is very rich in biodegradable organics and nutrients. It also contains high level of fats and oils which are not easily biodegradable and often interfere with the normal biological treatment process. Therefore, pretreatment of dairy wastewater is essential to remove these fats and oils from the wastewater before subjecting it to final biological treatment.

In comparison to the existing physical, chemical and biological methods for pretreaing the dairy wastewater biological pretreatment methods using microorganisms and/or their products are found to be the most effective and successful. Biosurfactant facilitated biodegradation of fats and oils, primarily by increasing its solubility has been identified to be a potential method to deal with high fats and oils in wastewaters.

The present work focused on pretreatment of high fats and oils containing dairy wastewater by the sophorolipids (SLs, a glycolipids type of biosurfactant) producing yeast *Candida bombicola*. To reduce the SLs production cost two agro-industrial wastes namely cheese whey and sugarcane molasses were tested as a low cost hydrophilic carbon source in place of costly glucose for its production by the yeast in batch shake flasks as well as in a laboratory scale bioreactor.

Initially, medium based on deproteinized whey was evaluated for SLs production by the yeast. But the yeast produced very low amount of SLs (5.98 g/l). Addition of small amount of glucose to the medium containing deproteinized whey, however, improved the yield to 23.29 g/l in batch shake flasks. The production further increased to 33.32 g/l when the experiment was carried out in a fermentor operated under batch mode with pH control. To further enhance the SLs production, the bioreactor was operated under fed-batch mode using oleic acid as the feeding substrate, and the SLs yield slightly improved from 33 g/l to 40 g/l.

Sugarcane molasses as a cheap raw material was evaluated in the study and was successfully demonstrated as an alternative low cost fermentative substrate for the production of SLs by *C. bombicola* in batch shake flasks as well as in a laboratory scale bioreactor. Optimization of physical (temperature, agitation, inoculum size and age) and chemical variables (sugarcane molasses and soybean oil concentration) influencing the SLs production by the yeast using sugarcane molasses was carried out by employing the statistically valid Taguchi orthogonal array and experimental design technique. At the optimized conditions of the process parameters the yeast produced 60 g/l of SLs in the bioreactor under controlled pH environment which was substantially higher than the production obtained using deproteinized whey. Kinetic parameters estimated by fitting the experimental data to certain logistic models existed in the literature suggested that conventional medium containing glucose can very well be replaced with the low cost fermentative medium based on sugarcane molasses.

The major SL produced using deproteinized whey and oleic acid was purified by silica gel column chromatography and its structural characterization was carried out by FTIR, ^{1}HNMR and MS. The purified SL was identified to be (17-hydroxyoctadecenoic)-1′4″-lactone-6′6″-diacetate SL. The properties of the produced SLs namely critical micelle concentration, minimum surface tension, interfacial tension, emulsification activity, emulsification stability and fats and oils solubilization efficiency were carried out and found to be very good compared to literature reported values. The biosurfactant was highly stable over wide range of temperature, pH and ionic strength. All these properties of the SLs revealed very high potential in environmental applications.

Synthetic dairy wastewater prepared in the laboratory was tested for SLs production by the yeast and its pretreatment. The yeast produced maximum 39 g/l of SLs when synthetic dairy wastewater was supplemented with sugarcane molasses and soybean oil in batch shake flasks. Pretreatment of the synthetic wastewater in a fermentor was carried out under batch, fed-batch and continuous modes. Results showed that the yeast was capable of not only utilizing all the fats and oils present in the wastewater, but also removed more than 95% of COD from the wastewater Finally, wastewater collected from a local dairy was characterized and its pretreatment carried out in a fermentor under different modes of operation after removing the suspended solids. Results from the batch experiments with the real wastewater revealed complete utilization of fats and oils present in the wastewater within 72 h with more than 96% COD removal efficiency. The yeast was, however, able to pretreat the wastewater more quickly and efficiently under fed-batch mode of operation than under batch operated condition in the same fermentor. Continuous experiments with the wastewater for retention time of 40 h in the reactor revealed very good performance of the system in complete utilization of fats and oils with COD removal efficiency of more than 95%. The present study proved excellent potential of the biosurfactant producing yeast in pretreating high fats and oils containing dairy industry wastewater.

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ABBREVIATIONS AND NOTATIONS

Abbreviations

^{1}HNMR	proton nuclear magnetic resonance
^{13}CNMR	carbon nuclear magnetic resonance
2,4,5-T	2,4,5-trichlorophenoxyacetic acid
2,4-D	2,4-dichlorophenoxyacetic acid
ANOVA	analysis of variance
ATP	adenosine triphosphate
BOD	biological oxygen demand
$CDCl_3$	deuterated chloroform
CFC	continuous flow configuration
CMC	critical micelle concentration
CO	carbon monoxide
CO_2	carbon dioxide
CoA	acetyl-coenzyme A
COD	chemical oxygen demand
CPCB	central pollution control board
C_S	any surfactant solution concentration
$C_{S,CMC}$	surfactant solution at its CMC
DAF	dissolved air flotation
db	decibels
DDT	dichlorodiphenyltrichloroethane
DNS	dinitro salicylic acid
ESI	electrospray ionization

Abbreviations

FAO	food and agricultural organization
FTIR	Fourier transform infra red
HCH	hexachlorocyclohexane
HPLC	high-performance liquid chromatography
HRT	hydraulic retention time
IAI	international animal industry
LCFA	long chain fatty acids
LDU	liquid-dosing unit
MEOR	microbial enhanced oil recovery
MS	mass spectroscopy
mST	minimum surface tension
NAPL(s)	non-aqueous phase liquid(s)
NOx	nitrogen oxide
OA	orthogonal array
OD	optical density
PAH(s)	polycyclic aromatic hydrocarbon(s)
PBR-USAB	packed bed reactor - upflow anaerobic sludge blanket reactor
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SL(s)	sophorolipid(s)
TLC	thin layer chromatography
TMS	tetramethyl silane
TPS	tilted plate separators
UDP	uridine diphosphate
USAB	upflow anaerobic sludge blanket reactor

Abbreviations

USDA	United States Department of Agriculture
VFA	volatile fatty acids

Notations

°C	degree centigrade
g	gram
g	gravitational acceleration
h	hour
k_d	decay constan
D	dalton
C_S	Any surfactant solution concentration
$C_{S,CMC}$	surfactant solution at its CMC
g/l	gram per liter
min	minute
ml/min	milliliter per minute
P	SLs concentration (g/l)
P_{max}	maximum concentration of SLs (g/l)
P_r	ratio between the initial volumetric rate of product formation and the initial product concentration P_0 (g/l)
R^2	regression coefficient
rpm	rotational per minute
s	second
S/N	signal-to-noise ratio
S_C	crude oil solubility at any surfactant solution concentration (C_S)
$S_{C,CMC}$	crude oil solubility in surfactant solution at its CMC ($C_{S,CMC}$)
SR	solubilization ratio
w/v	weight/volume
X	yeast biomass concentration (g/l)
X_0	initial biomass concentration (g/l)

Notations

X_{max}	maximum concentration of yeast biomass (g/l)
$Y_{P/M}$	SLs yield due to molasses (g/g),
$Y_{X/M}$	biomass yield due to molasses (g/g)
S_M	molasses concentration (g/l)
S_{M0}	initial molasses concentration (g/l)
$Y_{P/S}$	SLs yield due to soybean oil (g/g)
$Y_{X/S}$	biomass yield due to soybean oil (g/g)
S_S	soybean oil concentration (g/l)
S_{S0}	initial soybean oil concentration (g/l)
μ	specific growth rate (h^{-1})

CHAPTER 1

INTRODUCTION

1.1. Generalities

Dairy industry compared to other industries is fast growing in most countries of the world including developing countries like India due to the steady rise in demand for milk and milk products (Ramasamy et al., 2004; Kushwaha et al., 2010). According to International Animal Industry Expo (IAI Expo) India 2010 report, India is among the world's largest milk producing nation with 104 million tons per annum (in the year 2007-2008), which is about 15 % of the total milk production in the world. The annual growth rate of milk production in India is estimated to be 4%. In India, the market size of milk and milk products in value terms is USD 47.6 billion (INR 2000 billion) and is growing at nearly 7.5% annually (IAI Expo, India, 2010 report). The share of dairy sector in the total agricultural output is nearly 16 %. However, according to World Bank Group Environmental, Health and Safety Guidelines 2007, there are several environmental issues associated with dairy processing facilities including the following:

(a) Wastewater

The dairy industry generates high strength wastewater characterized by high chemical oxygen demand (COD) and biochemical oxygen demand (BOD) (Omil et al., 2003) due to the presence of high concentrations of nutrients and organic contents. The wastewater also contains pathogens and relatively large load of suspended solids (0.4–

2 g/l). In addition, the wastewater varies largely in pH (in the range 4.2 – 9.4) (Kosseva et al., 2003; Kushwaha et al., 2010). It is estimated that about 2% of total milk processed in the industry is wasted in the form of wastewaters (Munavalli and Saler, 2009), which, therefore, predominantly contains milk and milk products (e.g. protein, fat, carbohydrates and lactose) (Omil et al., 2003).

Whey, a by-product of cheese processing industry may also contribute to high organic loads in wastewater. Salting activities during cheese production may result in high salinity levels in the wastewater. In addition, the wastewater may also contain acids, alkali and detergents with a number of active ingredients and disinfectants, including chlorine compounds, hydrogen peroxide and quaternary ammonium compounds. In some cases, dairy industry wastewater have shown microbial load consisting of pathogenic viruses and bacteria (USDA – SCS, 1992). The amount of wastewater generated is estimated to be in the range of 0.2–10 L of effluent (average = 2.5 L) per litre of processed milk (Ramasamy et al., 2004).

(b) Solid waste

Solid wastes from dairy industries are quiet low but require costly handling (Vesilind and Pereira, 1980) and mainly originate from production processes and include nonconforming products and product losses (e.g. milk spillages, liquid whey and buttermilk), grit and filter residues, sludge from centrifugal separators and wastewater treatment. Solid wastes also contain packaging waste (e.g. discarded cuts, spent ripening bags, wax residues from cheese production) arising from incoming raw materials and production line damage (World Bank Group Environmental, Health and Safety Guidelines 2007).

(c) Emissions to air

Exhaust Gases: Exhaust gas emissions (carbon dioxide [CO_2], nitrogen oxides [NOx] and carbon monoxide [CO]) in the dairy processing sector result from the combustion of gas and fuel oil or diesel in turbines, boilers, compressors and other engines for power and heat generation.

Dust: Emissions of dust during dairy processing activities include fine milk powder residues in the exhaust air from spray drying systems and bagging of product.

Odor: The major sources of odor emissions in dairy processing facilities are related to on-site wastewater treatment facilities, in addition to fugitive odor emissions from filling / emptying milk tankers and storage silos.

(d) Energy consumption

Dairy processing facilities consume considerable amounts of energy. Typically, approximately 80% of the energy requirements are for thermal uses to generate hot water and produce steam for process applications (e.g. pasteurization, evaporation, and milk drying) and cleaning purposes. The remaining 20 percent is used as electricity to drive processing machinery, refrigeration, ventilation and lighting.

Of particular importance, among the above concerns, is the wastewater generated by the industry owing to high fats and oils content together with large BOD and COD values and large volume (Mohan et al., 2010; www.fao.org/documents/).

1.2. Sources of Wastewater in Dairy Industry

Based on the Food and Agricultural organization of the United Nations (FAO) corporate document repository report (www.fao.org/documents/), wastewater from dairy industry may originate from the following sources:

***Milk receiving*:** Wastewater results from tank, truck and storage tank washing, pipeline washing and sanitizing. It contains milk solids, detergents, sanitizers and milk wastes.

Whole milk products: Wastewater from this source is mainly produced during cleaning operations. Especially when different types of product are produced in a specific production unit, clean-up operations between product changes are necessary. In developing countries such as India, the main problem is pollution through spoilage of milk (www.fao.org/documents/).

Cheese/Whey/Curd: Waste results mainly from the production of whey, wash water, curd particles etc. Cottage cheese curd for example is more fragile than rennet curd which is used for other types of cheese. Thus the whey and wastewater from cottage cheese may contain appreciably more fine curd particles than that from other varieties of cheese. The amount of fine particles in the wash water increases if mechanical washing processes are employed by the industry.

Other than the above, butter washing in the industry also produces wastewater containing buttermilk.

1.3. Treatment of Dairy Wastewater

Dairy wastewater usually does not contain conventional toxic chemicals like those listed under EPA's Toxic Release Inventory. However, it has high concentration of dissolved organic components like whey proteins, lactose, fat and minerals that leads to high BOD and COD (Mukhopadhyay et al., 2003). Another problem is the volume of wastewater; according to an estimate, about 300 million tons of dairy wastewater is generated annually from dairy industries in India (Mohan et al., 2010).

Table 1.1 presents typical composition of wastewater generated from dairy industry in India (Garg and Garg, 1996; Gavala et al., 1999) and maximum permissible limit of the parameters set by Central Pollution Control Board (CPCB), India, for discharge of such wastewaters into the environment.

Table 1.1: Typical composition of wastewater generated by a dairy industry in India and permissible limit set by CPCB, India (G.S.R. 475(E), EPA, 5th May, 1992 and G.S.R. 801 (E), EPA, 1986, 31st Dec, 1993).

Sl. no.	Contents	Quantity present in wastewater	Permissible limit set as per CPCB, India
1	BOD	800 – 1000 mg/l	30 mg/l
2	COD	3000 – 50000 mg/l	250 mg/l
3	Heavy fat and oil	200 – 300 mg/l	10 mg/l
4	Dissolved solids	1000 mg/l or so	10 mg/l
5	Suspended solids	800 mg/l or so	150 mg/l
6	Nitrogen (mainly proteins)	Very high, though biodegradable	-

It is clear from the above table that in order to meet the wastewater discharge standard set in the country, treatment of the dairy wastewater is necessary, which normally involves several steps as shown in Fig. 1.1. The first step in dairy wastewater treatment is equalization which consists of holding the wastewater for some predetermined time in a continuously mixed basin to produce a uniform composition in the wastewater. After equalization, wastewater is neutralized by adding alkali or acid to the wastewater, which is then followed by physical treatment to separate the fats and oils using gravity oil separators. A large number of pretreatment methods other than gravity oil separators such as tilted plate separators, dissolved air flotation and physical-chemical treatment can also be employed to remove fats and oils from dairy wastewater prior to biological treatment. However, the cost of these methods is high and removal efficiency of fats and oils is low (Tano-Debrah et al., 1999; Demirel et al., 2005; Cammarota et al., 2006; Tchamango et al., 2010).

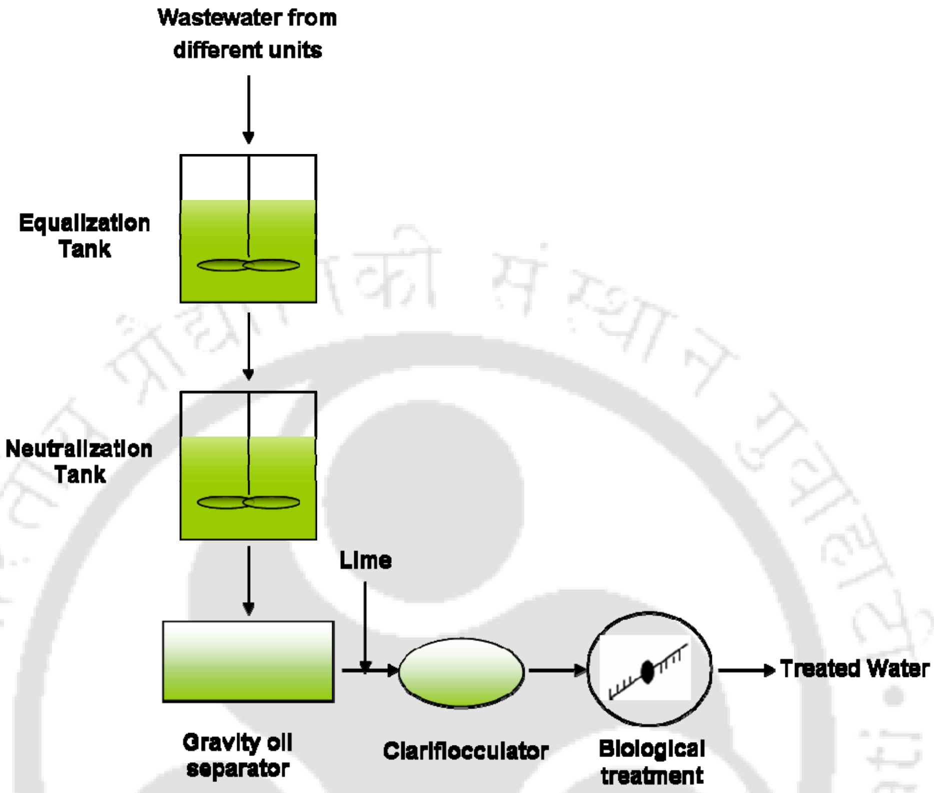


Fig. 1.1: A general wastewater treatment scheme adopted in a dairy industry.

After separating the fats and oils, the wastewater is treated with chemicals such as lime to precipitate the suspended solids which is later subjected to a clariflocculator where the precipitates are allowed to form bigger clumps for easy removal in the unit. Biological treatment by either aerobic or anaerobic microorganisms is finally applied before the wastewater is made suitable for discharging into the environment.

The main problem with the treatment scheme is the inefficiency of the physical-chemical unit operations involved in the removal of high fats and oils present in the wastewater, which often interfere with the biological treatment system.

In order to develop suitable pre-treatment method for removing fats and oils from such wastewaters, there have been few studies on degradation of fats and oils by alkaline/acid/enzymatic hydrolysis (Masse et al., 2001; Sivalingam et al., 2003). However, the main drawbacks of these methods have been either the high cost involved in the process or low removal efficiency of fats and oils. At this juncture, the use of surface active agents – surfactants – has been suggested for removing the fats and oils from wastewater by solubilization of these insolubles (Wipa et al., 1996; Liu et al., 1998; Matsui et al., 2005). Compared to chemical surfactants, biosurfactants can be used to solubilize fats and oils in wastewater due to its several added advantages of being environmentally safe, efficient, non-toxic and biodegradable. However, the utility of biosurfactants or biosurfactants producing organisms has not been fully explored for such wastewater treatment.

Sophorolipids (SLs) is a group of extracellular biosurfactant produced by several non-pathogenic species of yeast *Candida* (Van Bogart et al., 2007). Being a surfactant molecule it can interact with the phase boundary in heterogeneous systems and can solubilize a wide array of lipophilic compounds. However, the production cost of SL is considered to be still very high and can be kept low by utilizing low cost substrates and optimization of physical and chemical variables involved in the process. Further, by *in situ* production of the biosurfactant using suitable organism, pretreatment of dairy wastewater mainly to remove high fats and oils can be achieved.

1.4. Objective and Scope

The aim of the present research work is to develop low cost media for the production of SLs by the yeast *Candida bombicola* and to demonstrate its utility in the pretreatment of high fats and oils containing dairy wastewater.

To achieve the above objective, following investigations were carried out:

- Screening and optimization of media constituents containing low cost raw materials and process parameters for optimal and enhanced production of SLs by the yeast in batch shake flasks and its validation.
- Evaluation of SLs production by the yeast grown on low cost media in a laboratory scale bioreactor.
- Analyses and characterization of the produced SLs.
- Investigations on the properties of SLs like critical micelle concentration (CMC), surface tension, interfacial tension and emulsification activity and stability, solubilization of fats and oils.
- Pretreatment of synthetic wastewater containing high fats and oils using the SLs producing yeast under batch, fed batch and continuous modes of operation in a laboratory scale bioreactor.
- Characterization and pretreatment of real dairy industry wastewater in a bioreactor using the SLs producing yeast.

1.5. Organization of the Thesis

The presentation of the work has been divided into five chapters. The current **Chapter 1** gives a general introduction, objective and scope of the present work. While the literature concerning the present work is presented in **Chapter 2**, details of the materials and methods adopted in the research study are described in **Chapter 3**. **Chapter 3** essentially details the procedures for SLs production from two agro-industrial wastes - deproteinized whey and sugarcane molasses in batch shake flasks and in fermentor, isolation, purification and characterization of SLs produced by the yeast, pretreatment of synthetic as well as real dairy wastewater containing high fats and oil. It also details the analytical methods followed in the above investigations.

Chapter 4 contains the results and discussions, where the results of SLs production by the yeast using deproteinized whey and sugarcane molasses, optimization of process parameters and medium constituents for enhanced production of SLs are presented and thoroughly discussed. This chapter also discusses the results of SLs purification, characterization and properties, such as CMC, surface/interfacial tension reduction, emulsification activity and stability and fats and oils solubization efficiency, of the SLs produced in the study. Finally, the pretreatment results of both synthetic and real dairy industry wastewater using the biosurfactant producing yeast in a fermentor operated under batch, fed-bath and continuous modes are presented and discussed.

Chapter 5 draws summary and appropriate conclusion based on this work. This chapter also provides some useful recommendations for researchers to carry out further work in this field.

CHAPTER 2

LITERATURE REVIEW

2.1. Wastewater

Water that has been used by people and disposed into the environment with altered physical and/or chemical parameters is defined as wastewater. If only the physical parameters of the water were changed, e.g., resulting in an elevated temperature after use as a coolant, treatment before final disposal into a surface water may require only cooling close to its initial temperature. If the water, however, has been contaminated with soluble or insoluble organic or inorganic material, a combination of mechanical, chemical, and/or biological purification procedures may be required to protect the environment from periodic or permanent pollution or damage. For this reason, legislation in industrialized and in many developing countries has reinforced environmental laws that regulate the maximum allowed residual concentrations of carbon, nitrogen, fats and oils, suspended solids, phosphorous and other hazardous compounds in treated wastewater, before it is disposed into a river or into any other receiving water body (Gallert and Winter, 2005). Wastewater originates predominantly from water usage by residences and commercial and industrial establishments, together with groundwater, surface water and storm water. Following section discusses about the wastewater generated from dairy industry, its composition and its pretreatment methods.

2.2. Dairy Wastewater

Wastewater from dairy industry is generated mainly from milk or cheese producing units and contains complex organics, such as lactose, proteins (mainly casein) and lipids (fats and oils) (Gough et al., 2000; Ramasamy and Abbasi, 2000). Usually these wastewaters are associated with high organic load and a high concentration of fermentable substrates with persistently unpleasant odor. The very nature of the different operations involved in dairy, irrespective of the products size, generate wastewater of different magnitude. Table 2.1 presents the composition of wastewater discharged by various units in a dairy plant.

Table 2.1: Composition of wastewater discharged by various units in a dairy plant*.

Constituents	**Cheese manufacturing unit**	**Milk receiving and pasteurization unit**	**Casein preparation unit**	**Butter, butter oil and ghee manufacturing unit**	**Pooled dairy effluent**
Total solids	2250	3620	650	3400	1650
Color	White	White	Clear	Brown	White
Chlorides	100	95	70	100	115
Volatile solids	25	75	55	65	60
Suspended solids	600	1300	100	2200	650
Phosphates	12	10	5	2	10
pH	6.7	8.2	7.7	7.1	6.1
Calcium carbonate	480	500	460	420	530
Absorbed oxygen	480	400	10	85	--
BOD	2150	1620	200	1250	810
COD	3130	2600	370	3200	1340
Fat/oil and grease	520	690	--	1320	290
COD:BOD ratio	1.46	1.43	1.85	2.56	1.65

*Source: www.dairyforall.com

Due to high demand of dairy products, there is a steady rise in the number of dairies and, therefore, large amount of wastewater is generated from these industries. Nearly 2.5 times the volume of the milk processed is generated as wastewater in the form of spent wash or spillage (Mohan et al., 2010). The high concentrations of fats and oils present in dairy wastewater create several operational and biological problems during the biological treatment. Several physical, chemical, phsico-chemical and biological methods are developed to remove these fats and oils from the wastewater before subjecting the wastewater to biological treatment. In this section, the problems associated with fats and oils in wastewater and different pretreatment methods to remove fats and oils are discussed:

2.2.1. Issues with biological treatment of effluents with high fats and oils content

Biological treatment of dairy wastewater can be aerobic or anaerobic, and fats and oils present in wastewater create several operational problems in both types of biological treatment. During aerobic treatment of wastewaters, fats and oils block the oxygen transfer required for the biological degradation. It forms a lipid coat around the microbial cells and reduces the oxygen transfer rates to the biological microbial consortium (Chao and Yang, 1981; Lemmer and Baumann, 1988; Grulois et al., 1993). These fats and oils also cause problems in the pumping and aeration systems thereby reducing the dissolved air concentration which leads to development of filamentous microorganisms such as *Sphaerotilus natans, Thiothrix, Beggiatoa, Nocardia and Microthrix* genuses. These microorganisms are involved in the formation of scum and stable foams on the surface of the aeration tank which ultimately hinder the biomass flocculation and sedimentation, a phenomenon known as bulking (Jenkins et al., 1993). High contents of fats and oils in wastewater also generate agglomerates or pellets inside the secondary sludge flocs, which hinders sedimentation, generating unpleasant odors and reducing the efficiency of the treatment station (Eckenfelder, 2000).

Several aerobic treatments have been used extensively in the dairy industry. These includes activated sludge processes (Shack and Shandhu, 1989; Stephenson, 1989), rotating biological contactors (Radick, 1992), trickling filters (Walsh et al., 1994), membrane sequencing batch reactor (Bae et al., 2003) and aerated lagoons (Carta-Escobar et al., 2005). However, the energy requirements for the aeration in these installations are high and problems such as bulking and excessive biomass growth frequently occur under these conditions (Timmermans et al., 1993).

Carta-Escobar et al. (2005) studied the organic matter removal kinetics of dairy wastewater in two reactor configurations (the first system was a single reactor with a working volume of 80 L and the second one was a three-stage reactor cascade with a total working volume of 30 L) and made general observations concerning the performance of the reactors in series. At the volumetric organic loads studied (0.24–0.70 kg COD/m^3·d), frequent episodes of foaming with sludge floating on the surface of the reactors were observed by the authors. Clogging problems also occurred in the diffusers due to the formation of very viscous films. In addition, the samples exhibited pronounced resistance to filtration, and the filter membranes became fouled by viscous films. These foaming problems and difficulties in obtaining optimal filtration are frequent occurrences in the treatment of dairy wastewaters (Danalewich et al., 1998; Bae et al., 2003).

In anaerobic biological processes, fats and oils may solidify at lower temperatures and thus cause operational damage associated with clogging and unpleasant odors (Grulois et al., 1993; Vidal et al., 2000). Several authors have studied anaerobic treatment of synthetic and real dairy wastewaters using upflow anaerobic sludge blanket (UASB) reactors (Hansen and Hwang, 1990; Rico et al., 1991; Hawkes et al., 1995), hybrid UASB reactors (Ozturk et al., 1993; Jeganathan et al., 2007), expanded granular sludge bed (EGSB) reactors (Petruy and Lettinga, 1997), and reactor based on anaerobic filters (Mendez et al., 1989; Viraraghavan and Kikkeri, 1990; Veiga et al., 1994). These studies have demonstrated that anaerobic treatment can be effectively used in treating dairy effluents, despite the various operational problems described in the literature, such as sludge flotation and toxicity/inhibition of the process. However, in many of these studies only synthetic steady-composition wastes, with dairy waste being simulated by powdered milk, were investigated which contain much less fats and oils than real effluents. Infact, real effluents also contain additives such as disinfectant and cleaning agents that may jeopardize the biological treatment process. In a work by Ramasamy et al. (2004), for example, the authors established the feasibility of upflow anaerobic sludge blanket (UASB) reactors in treating dairy wastewaters. Authors reported COD reduction rates greater than 90% at hydraulic retention times (HRT) of 3 and 12 h and at COD loading rates ranging from 2.4 to 13.5 kg/m^3·d. For these experiments, 1 g of powdered milk per liter of water with a COD of 1440 mg/L was used and the stock solution was diluted several times to obtain effluents of a range of COD concentrations.

Despite the results reported in the literature on biological treatment of dairy wastewater development and flotation of sludges with varying physical characteristics or poor activity is known to cause biomass loss through reactor’s outflow, decreasing its quantity inside the reactor and the treatment efficiency (Rinzema et al., 1993; Perle et al., 1995). The fats and oils adsorbed on the surface of anaerobic sludge may limit the transport of soluble substrates to the biomass and consequently reduce the substrate conversion rate (Rinzema et al., 1994). Petruy and Lettinga (1997) showed that 70% of lipids in wastewater were adsorbed by the granular sludge, within approximately one day, and thereafter, the remaining lipids were slowly converted into methane gas. All these findings have led to the conclusion that to improve wastewater treatment efficiency the levels of fats and oils in the effluent must be reduced to a considerable extent.

2.2.1.1 Effect of long chain fatty acids on biological treatment

Fats and oils are essentially triglycerides consisting of long chain fatty acids (LCFA) attached, as esters, to glycerol. The component fatty acids of edible fats and oils vary considerably, differ in chain length, may be saturated or unsaturated and may contain an odd or even number of carbon atoms (Wakelin and Forster, 1997).Therefore, fats and oils present in wastewater often produces glycerol and LCFA (saturated fatty acids with 12–14 carbon atoms and unsaturated fatty acids with 18 carbon atoms) during the hydrolytic step. Although glycerol is found to be a non-inhibitory compound (Perle et al., 1995), LCFA can exert toxic effects and inhibit anaerobic bacteria (Hanaki et al., 1981; Angelidaki and Ahring, 1992). Also, the biodegradation of lipids is difficult due to their low bioavailability (Petruy and Lettinga, 1997). Several authors have reported that LCFA not only inhibits the activity of various microorganisms (Hanaki et al., 1981; Koster, 1987; Angelidaki and Ahring, 1992; Rinzema et al., 1994; Ching-Shyung et al., 1996) but also decrease the availability of adenosine triphosphate (ATP) (Hanaki et al., 1981; Perle et al., 1995). The inhibitory effect increases with the number of double bonds and cis-isomers that are abundant in natural lipids (Rinzema, 1988). As in the case of volatile fatty acids (VFA), the toxicity of LCFA seems to be related to the unionized form of these acids, namely the long chain free fatty acids.

Studies carried out by Hanaki et al. (1981) showed that LCFA content affects the amount of hydrogen produced by acetogenic bacteria, responsible for the β-oxidation of LCFA. The inhibition of acetogen and acetotrophic methanogens causes a pronounced lag phase in batch experiments. In addition, the inhibition of hydrogenotrophic methanogens causes a decrease on the hydrogen conversion rate (Rinzema et al., 1994).

Vidal et al. (2000) evaluated the influence of absolute and relative concentrations of carbohydrates, fats and proteins on the anaerobic biodegradability of dairy wastewaters. They found that the anaerobic biodegradation rate of wastewaters rich in fat was slower than that of wastewaters with a low fat content due to the reduced rate of the fat hydrolysis step. However, this reduced hydrolysis rate prevented volatile fatty acids (VFAs) accumulation and therefore, the presence of fats in the wastewater prevented the periodic production of high concentrations of VFAs, which may adversely affect the process.

Overall these findings also indicate that to improve wastewater treatment efficiency pretreatment is necessary to reduce the levels of fats and oils in the wastewater. Different pretreatment methods to remove fats and oils from the wastewater along with their merits and demerits are discussed in the following section.

2.2.2. Pretreatment methods to remove fats and oils from dairy wastewater

Pretreatment methods can be divided into physical, chemical, physico-chemical and biological methods.

2.2.2.1. Physical pretreatment

Typically, the first stage of pretreatment is the use of physical processes on individual wastewater streams to recover free oils and fats. Such treatment is therefore applied separately to barometric water, acid water, margarine processing wastewater, and, in certain circumstances, to water in bunded oil tank areas.

The most commonly used physical separation processes for the treatment of separable oils and fats are fat traps, tilted plate separators (TPS) and dissolved air flotation (DAF) units. In addition, centrifuges and electro-flotation systems are occasionally used.

Fat trap method

The main technique for separating fats and oils from wastewater is the fat trap method. Because fats or oils are separated due to their gravity, this technique is also called gravity oil separation technique. Fig 2.1 shows a schematic diagram of fat trap method. The method is carried out in a rectangular or circular vessel or tank through which the wastewater passes under laminar-flow conditions at a rate that allows the fat/oil particles to rise to the surface by the time they approach the outlet end of the trap. As shown in the figure the fats/grease/oils are accumulated at the top and the solid wastes are accumulated at the bottom of the tank. The separation principle is based on Stokes' law relating rising velocity of a particle to its diameter, and in theory separation efficiency is independent of depth:

$$V_c = \frac{g(\rho_s - \rho)d^2}{18\mu} \tag{2.1}$$

where V_c is the terminal velocity of fats or oils, g is the acceleration due to gravity, ρ_s is the density of the particle, ρ is the density of the fluid, d is the diameter of the particle, and μ is the absolute viscosity of the fluid. This method, however, frequently becomes unaesthetic and even causes air pollution in the vicinity of the treatment facility (Willey, 2001).

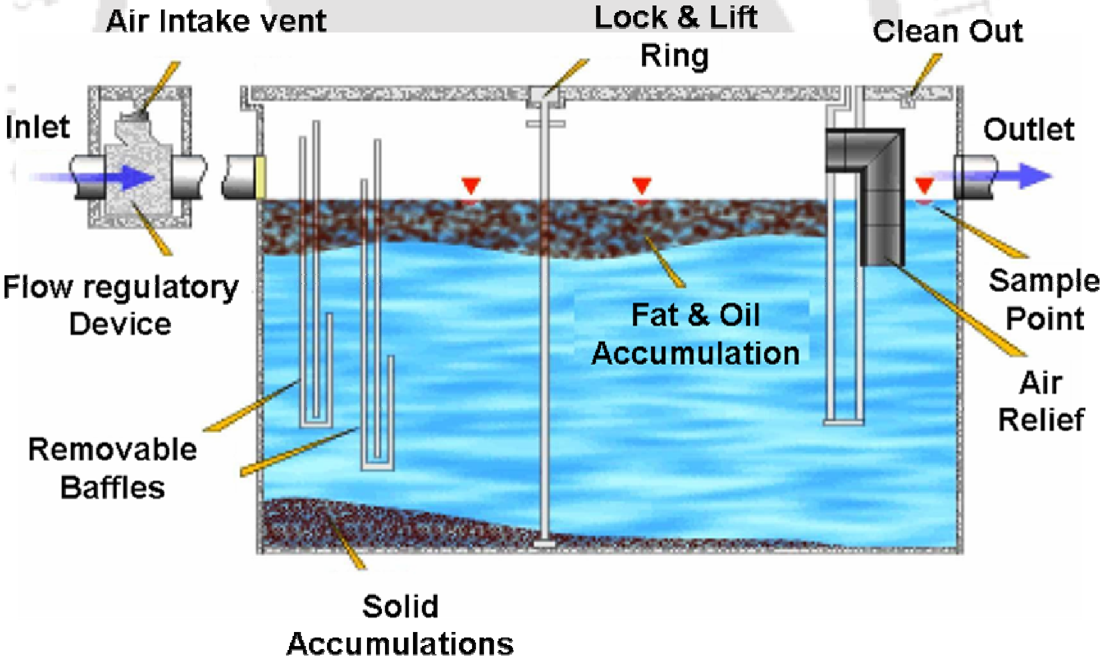


Fig. 2.1: Schematic diagram of fat trap method.

(Source: http://www.co.kent.de.us/Departments/PublicWorks/oil_and_grease_interceptor_and_t.htm).

Tilted plate separators (TPS)

In TPS several inclined plates introduced into a vessel provide many parallel plate gravity separators with low liquid depth, but a high surface area. Typically, a TPS occupies less than 10% of the area of a conventional fat trap. Fig 2.2 shows schematic diagram of TPS. This technique has several advantages over the fat trap method:

1. Low space requirement
2. Very low maintenance cost as there are no moving parts
3. High efficiency and capacity combined with compact volume
4. Low installation cost as units are mostly prefabricated
5. Efficiently handling of shock loads of flow without any effect on effluent quality
6. Continuous operation without major down time
7. Insensitive to weather condition, variation in temperature and composition of incoming liquid

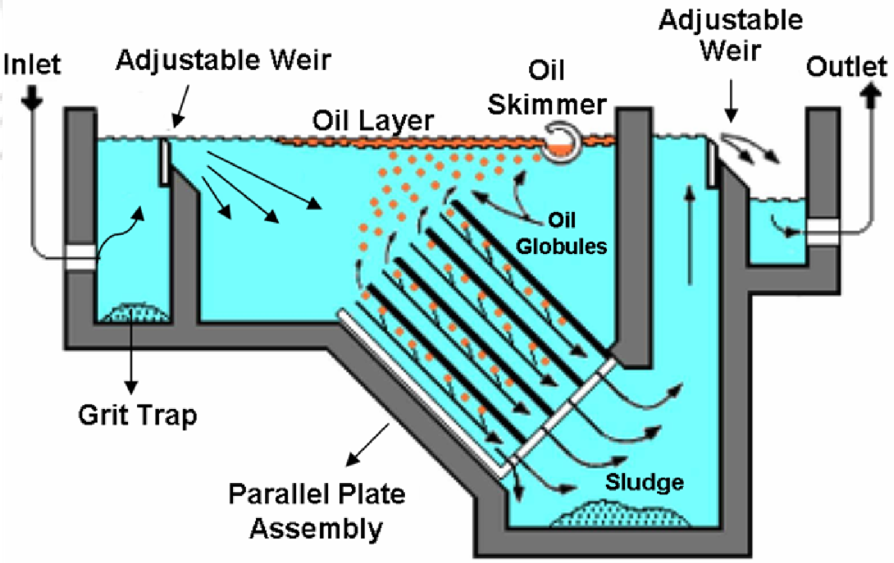


Fig. 2.2: Schematic diagram of tilted plate separator.

(Source: http://en.wikipedia.org/wiki/API_oil-water_separator)

There are, however, certain problems with edible oils and fats that must be considered when using TPS such as (Willey, 2001):

(1) The narrow gaps (20 mm) between the plates may be susceptible to fouling if solid or semisolid fat is present in the effluent.

(2) Removal of the plate pack for cleaning is time consuming and requires the use of a crane.

(3) Careful selection of pumps and flow control are required at the site where the wastewater is pumped into the unit to avoid surging and undesirable fluctuations in liquid depth.

Dissolved air flotation systems (DAF)

DAF utilizes the property of micro-bubble adherence to fats and oils, increasing the tendency of the particles to float. After allowing the fats and oils to float on the surface, mechanical scrapers can be used to skim the solids from the surface into a collecting bin.

Fig 2.3 shows schematic diagram of DAF unit. In the system, air is dissolved into wastewater under pressure (approximately 6 bar). The air-saturated liquor is subsequently passed through nozzles drop and the dissolved air is released from the solution in the form of microbubbles (60–70 μm diameter) clouds. The microbubbles attach to the surface of the fat/oil particles and increase their rise rate. As air solubility is substantially reduced at elevated temperatures (Willey, 2001), wastewater temperature is an important consideration in the design of DAF systems. The major disadvantage of this method is its cost and DAF unit is considered to be very expensive to run due to the compressed air requirement.

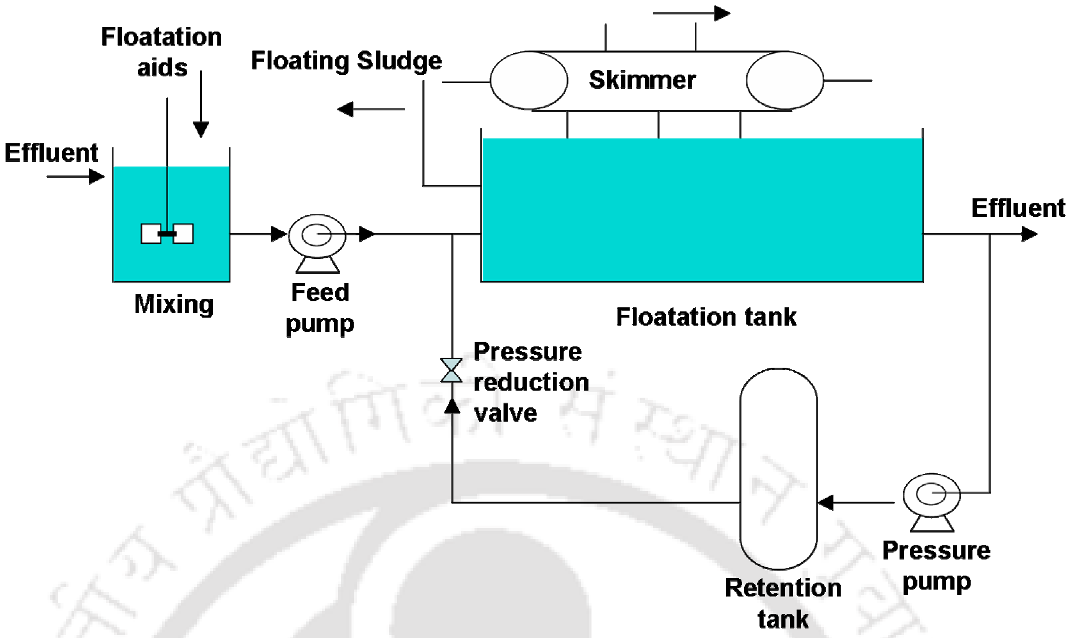


Fig. 2.3: Schematic diagram of DAF. (Source: http://www.fao.org/).

2.2.2.2. Chemical and physical-chemical pretreatment

Chemical treatment reduces the organic load by protein and fat precipitation or flotation with different chemical compounds. However, these chemical methods further require physical methods to remove the floated protein and fats. Generally fats and oils are less soluble under extreme acid and extreme base conditions. Commonly, sulfuric acid is used; however, lime at a pH of 8.4 is also effective. Truly soluble oils are difficult to render insoluble with acids or bases alone, and chemical coagulation at an appropriate pH is usually required. Studies show that alum in the pH range of 8.0 to 9.0 and ferrous sulfate in the pH range of 8.0 to 10.0 are effective in coagulating soluble oil (Liu and Liptak, 1997). Because controlling the pH in this range with lime is more difficult, wastewater treatment facilities generally use soda ash (Na_2CO_3) to raise the pH to the required level. If the facility uses chemical coagulation at an elevated pH, prior treatment with acid to improve separation is not recommended because this treatment requires large amounts of alkali for final pH adjustment. The settlement or flotation rate can frequently be improved by a second-stage flocculation. This involves the addition of low levels of polyelectrolyte (0.5–5.0 mg/L) to the wastewater once coagulation has taken place (Liu and Liptak, 1997; Willey, 2001).

Few studies are reported in literature for the chemical coagulation of suspended and colloidal materials including fats and oils from dairy wastewater (Namasivayam and Ranganathan, 1992**;** Selmer-Olsen et al., 1996; Mukhopadhyay et al., 2003; Hamdani et al., 2004; Sarkar et al., 2006; Kushwaha et al., *in press*).

Sarkar et al. (2006) employed coagulation by chitosan followed by adsorption with powdered activated carbon as pre-treatment steps before treating a dairy wastewater by membrane separation method and found 57% reduction in COD at 10–50 mg/l chitosan dosage. Recently, Kushwaha et al. (*in press*) studied pretreatment of a simulated dairy wastewater by inorganic coagulants such as poly aluminum chloride, ferrous sulphate ($FeSO_4$) and potash alum. At an optimum concentration of poly aluminum chloride, $FeSO_4$ and potash alum, 69.2, 66.5 and 63.8% COD removal efficiencies, respectively, from the wastewater were found in 30 min.

The widespread utility of physical–chemical treatments, however, is questionable because the cost of reagents is high, the removal efficiency of the dissolved fat and oil is low and highly problematic sludge is produced when flocculating agents such as polyelectrolytes and salts are used (Karpati et al., 1995).

2.2.3. Biological pretreatment methods

Biological methods involve the use of microorganisms and/or their products such as enzymes for the pretreatment of fats and oils. There are quit a few reports describing the use of biological methods for the pretreatment of dairy effluents with high fat and oil concentrations (Banu et al., 2008; Loperena et al., 2009).

Banu et al. (2008) studied pretreatment of dairy wastewater by anaerobic hybrid upflow anaerobic sludge blanket reactor followed by final treatment using solar photocatalytic method. The anaerobic pretreatment method was able to remove 84% of COD with the solar photocatalytic treatment removing the residual COD up to 95%.

Loperena et al. (2009) isolated milk fat/protein degrading microorganisms from different locations of a dairy wastewater treatment plant. Among the isolates, eight isolates were identified by 16S rRNA gene sequence analysis as those belonging to the genera *Bacillus*, *Pseudomonas* and *Acinetobacter*. These isolates were tested for their potential in treating synthetic dairy wastewater having an initial COD of 3000 mg/l. The best isolate in the study was able to remove only 75% fats and 57% COD from the synthetic dairy wastewater. A low COD removal indicated that this treatment method further requires secondary treatment before discharging the wastewater into the environment.

Few other authors have also tested biological methods to pretreat wastewater containing high fats and oils, which are discussed further.

2.2.3.1. Use of hydrolytic enzymes

Several reports are available in literature where enzymes (mainly hydrolytic such as lipases) and pure cultures producing hydrolytic enzymes have been used to increase hydrolysis of fats and oils during or prior to biological treatment process (Cail et al., 1986; Aoki and Kawase, 1991; Cammarota et al., 2001; Bhumibhamon et al., 2002; Mongkolthanaruk and Dharmsthiti, 2002; Leal et al., 2002; De Felice et al., 2004; Lanciotti et al., 2005; Jeganathan et al., 2006; Leal et al., 2006; Mendes et al., 2006; Jeganathan et al., 2007; Brozzoli et al., 2009; Gonçalves et al., 2009; Nabarlatz et al., 2010).

Cail et al. (1986) studied the treatment of wool scouring wastewater by *B. subtilis* in an anaerobic digester, operated semi-continuously in order to retain high concentrations of biomass in the digester with or without pretreating with the commercial enzyme Actizyme^{TM}. They found that enzymatic pretreatment of the scouring effluent showed significantly improved treatment rates in terms of removing fats and COD from the wastewater. Addition of Actizyme^{TM} increased the COD removal efficiency from 59% in the control to 78%, increased grease removal efficiency from 47% to over 70% and improved the efficiency of solids reduction from 34% to over 70%.

Cammarota et al. (2001) studied treatment of high fats and oils (868 mg/l) containing dairy wastewaters in an UASB inoculated with sludge collected from an anaerobic reactor from a dairy industry. The results showed only 50% of COD removal but after pretreatment with 0.1% (w/v) of fermented babassu cake containing *Penicillium restrictum* lipases, the COD removal efficiency improved to 90%. Also the concentration of fats and oils in the treated wastewater were reported to be within the permissible limit (50 mg/l).

Leal et al. (2002) studied the hydrolytic enzymes (fungal lipase) as coadjuvants in the anaerobic treatment of synthetic dairy wastewater. They studied the different conditions for fat hydrolysis by this enzymatic extract and reported that hydrolysis should be carried out with 10% v/v enzymatic extract at 35ºC, without agitation, for 12 hours. Both crude and hydrolyzed effluents were then submitted to an anaerobic biological treatment. They observed that efficiency of the anaerobic treatment was significantly improved when the effluent was enzymatically pretreated. For the highest fat content tested by the authors (1,200 mg/l), removal efficiencies of 19 and 80% were attained for crude and hydrolyzed effluents, respectively. In addition, they observed a tenfold increase in the removal rate of COD from the hydrolyzed effluent compared to the crude effluent. Leal et al. (2006) studied the biological treatment of a synthetic dairy wastewater containing high levels of oil and grease (1000 mg/l) using UASB reactors. The UASB was continuously fed with wastewater hydrolyzed with enzymatic extract (fungal lipase) 35ºC for 14 h, which resulted in 90% COD removal efficiency.

Unfortunately, the utilization of commercial enzymes or production of crude enzyme followed by isolation to hydrolyze fats in wastewater being viewed as non-economical because the viability of this technique is strongly dependent on the amount of enzyme necessary for the hydrolysis step and more importantly on the cost of the enzyme preparation (Cammarota et al., 2006). To overcome this problem, Mendes et al. (2006) used a low-cost commercially available lipase preparation from animal source to perform enzymatic hydrolysis pretreatment of lipid-rich wastewater from a dairy industry. For an optimum hydrolysis time of 12 h the pretreatment enabled high-biogas formation along with significant organic matter removal (78.2%). In another study, Jeganathan et al. (2006, 2007) immobilized *C. rugosa* lipase in calcium alginate beads to overcome the cost problem by reusing the immobilized lipase for pretreatment of high fats and oils containing pet food industry wastewater in 1 L flasks and in a hybrid packed bed reactor - upflow anaerobic sludge blanket reactor (PBR-UASB). Results of experiments in flasks showed that approximately 50% of the fats and oils were hydrolyzed due to the enzyme activity. In the hybrid reactor experiment, fats and oils removal efficiencies were slightly improved to 64% with 65% COD removal efficiency.

From the above studies it is apparent that the use of hydrolytic enzymes is most extensively and successfully investigated by several authors. But, the major problem with this method is the high cost of hydrolytic enzymes as the best results were obtained only with pure hydrolytic enzymes. Further, when pure enzymes were replaced with crude enzymes produced using low cost materials or with immobilized enzymes the fat removal efficiency decreased. Moreover, hydrolysis products obtained using such enzymes sometimes inhibited microorganisms used in biological treatment of wastewater (Hanaki et al., 1981; Angelidaki and Ahring, 1992).

2.2.3.2. Use of hydrolytic enzyme producing microorganisms

Use of hydrolytic enzyme producing microorganisms in place of pure enzymes to pretreat high fats and oils containing wastewaters is a better approach and has been tested by several authors. Anon (1994) proposed a method for the treatment of grease-containing restaurant wastewater and other fats and oil containing wastewaters by the direct cultivation of lipophilic yeasts such as *Candida intermedia, C. schatavii, C. visuvanathii, C. fluvatilis, C. pseudolambica* or *C. hellenica* in the wastewater. Use of thermophilic conditions for the biological treatment of fats and oils containing wastewaters can be advantageous because high temperature makes these fats and oils more accessible to microorganisms. Both diffusion coefficients and the solubility of fatty acids in aqueous media increase significantly with rising temperatures, allowing a better mass transfer (Thomas, 1987). However, the major problem with this method is the availability of the thermophilic microorganisms.

Wakelin and Forster (1997) investigated the microbial treatment of waste from fast-food restaurants for the removal of fats, oils and greases by culturing pure and mixed microbial flora. The pure cultures were *Acinetobacter* sp., *Rhodococcus rubra*, *Nocardia amarae* and *Microthrix parvicella*, and the results were compared with those obtained using a mixed culture isolated from a grease trap and from activated sludge. The effectiveness of these cultures was assessed in terms of their grease removal efficiency, biomass production and yield coefficients. The authors reported that the *Acinetobacter* was the most effective among the pure cultures, typically removing 60-65% of the fatty material whose initial concentration was 8 g/l.

Becker et al. (1999) studied biodegradation of olive oil and treatment of a lipid-rich wool scouring wastewater under aerobic thermophilic (65ºC) conditions using a novel isolated strain *B. thermoleovorans* IHI-91. They found that in a continuously operated laboratory-scale stirred-tank reactor olive oil was degraded to more than 90% but severe inhibition in the biomass growth was observed when the feed olive oil concentration was increased to more than 4 g COD/l. Also, lipid removal from the highly loaded wool scouring wastewater (COD 77,000 mg/l) was 20-30% at a residence time of 10-20 h while the COD removal was 15-20%. Compared with data from mesophilic processes the authors found that the lipid degradation rates obtained under thermophilic conditions are extremely high. This may be due to the fact that the biology of thermophilic aerobic systems differs from conventional activated sludge systems in that nitrifying bacteria, floc-forming organisms and higher organisms that aid flocculation are not present, which affects sludge sedimentation (Lapara and Alleman, 1999).

Mongkolthanaruk and Dharmsthiti (2002) evaluated a mixed culture composed of *Pseudomonas aeruginosa* LP602, *A. calcoaceticus* LP009 (both lipase-producing bacteria) and *Bacillus* sp. B304 (an amylase and protease producing bacterium) to remove both lipid content and BOD from a lipid-rich domestic wastewater. Based on the results obtained, the authors suggested that consortium of these three bacterial cultures could be used successfully to treat lipid-rich wastewaters.

Gonçalves et al. (2009) reported biological treatment of olive mill wastewater by non-conventional yeasts such as *C. rugosa*, *C. cylindracea* and *Yarrowia lipolytica* in batch cultures. The authors reported that these yeasts produce lipases and could able to reduce 50 – 60% COD from the olive mill wastewater.

2.2.3.3. Use of biosurfactants

Use of biosurfactants for removing fats and oils in wastewater is an alternative to physical, chemical and other biological pretreatment method. Biosurfactants due to their surfactant properties can increase the solubility of fats and oils in aqueous phase by reducing the surface/interfacial tension between fats/oils and aqueous phase. Once solubilized, these fats and oils can be easily utilized by the degrading microorganisms. There are no reports available in literature where surfactants have been used for pretreating dairy wastewater. However, few reports are available in literature where surfactants have been applied to pretreat industrial effluents containing high fats and oils (Nakhla et al., 2003; Matsui et al., 2005; Jacobucci et al., 2009).

First, Nakhla et al. (2003) successfully demonstrated the effectiveness of a biosurfactant derived from cactus (BOD-Balance^{TM}) in pretreating high strength oil and grease laden wastewater. Matsui et al. (2005) studied the effect of different surfactants including saponin biosurfactant on the continuous treatment of salad oil-containing synthetic wastewater by an activated sludge. Recently, Jacobucci et al. (2009) studied the utilization and COD reduction of oily soap industry effluent by two biosurfactant-producing bacterial strains *Planococcus citreus* and *Pantoea agglomerans* and suggested that *in situ* biosurfactants production by the microorganisms can be a potential approach for pretreating such oily wastewaters.

Combination of the above biological methods was also suggested by Mendes et al. (2005). The authors studied the use of a mixture composed of emulsifiers, microorganisms and enzymes (protease, amylase, lipase, cellulase and pectinase) for lipid removal from wastewaters produced by cleaning of industrial machines. The microorganisms included in the study were *B. subtilis* (a protease and amylase producing bacterium) and *Aspergillus niger* (a lipase, cellulase and pectinase producing fungus). In this mixture, the enzymes were present at concentrations between 1 and 200 mg/l. However, utility of the suggested method for practical reasons is doubted due to the addition of different enzymes, its stability and requirement of different optimum conditions for the enzyme activity.

Overall, it is quite apparent that although use of biosurfactant has been found effective in solubilization of fats and oils for pretreating wastewater, it has not been explored in detail, particularly the use of biosurfactant producing microorganisms. The following section discusses the literature concerning biosurfactant, their properties, production and environmental applications.

2.3. Biosurfactants

Microbial surfactants, commonly known as biosurfactants, are surface active agents produced by a variety of microorganisms when grown on water miscible or oily substrates (Bongolo, 1999; Mukherjee et al., 2006). Similar to chemical surfactants these contain hydrophilic and hydrophobic moieties. Also, they have tendency to reduce surface tension and interfacial tension (Bognolo, 1999).

Biosurfactants of microbial origin have the following properties when compared with chemical surfactants-

a) One or more functional groups and chiral centers
b) High ionic strength tolerance
c) Temperature tolerance
d) Higher biodegradability and lower toxicity
e) Lower critical micelle concentration and higher surface activity
f) Gradual adsorption and enduring activity
g) Superior ability to form molecular assembly and liquid crystal
h) Biological activity

2.3.1. Types of biosurfactants

All biosurfactants are of either nonionic or anionic type. There are no literature reports on cationic biosurfactant; however, presence of nitrogen containing group is known to impart a certain degree of cationic character in the compound. Produced by different types of microorganisms such as bacteria, yeast and fungi, biosurfactants are structurally very diverse and can be classified based on their structure (Bongolo, 1999; Banat et al., 2010). Table 2.2 presents the different types of biosurfactants along with their microbial sources. Biosurfactants may be classified into five groups (Bongolo, 1999) and the chemical structure of important biosurfactant from each class is given in Fig. 2.4.

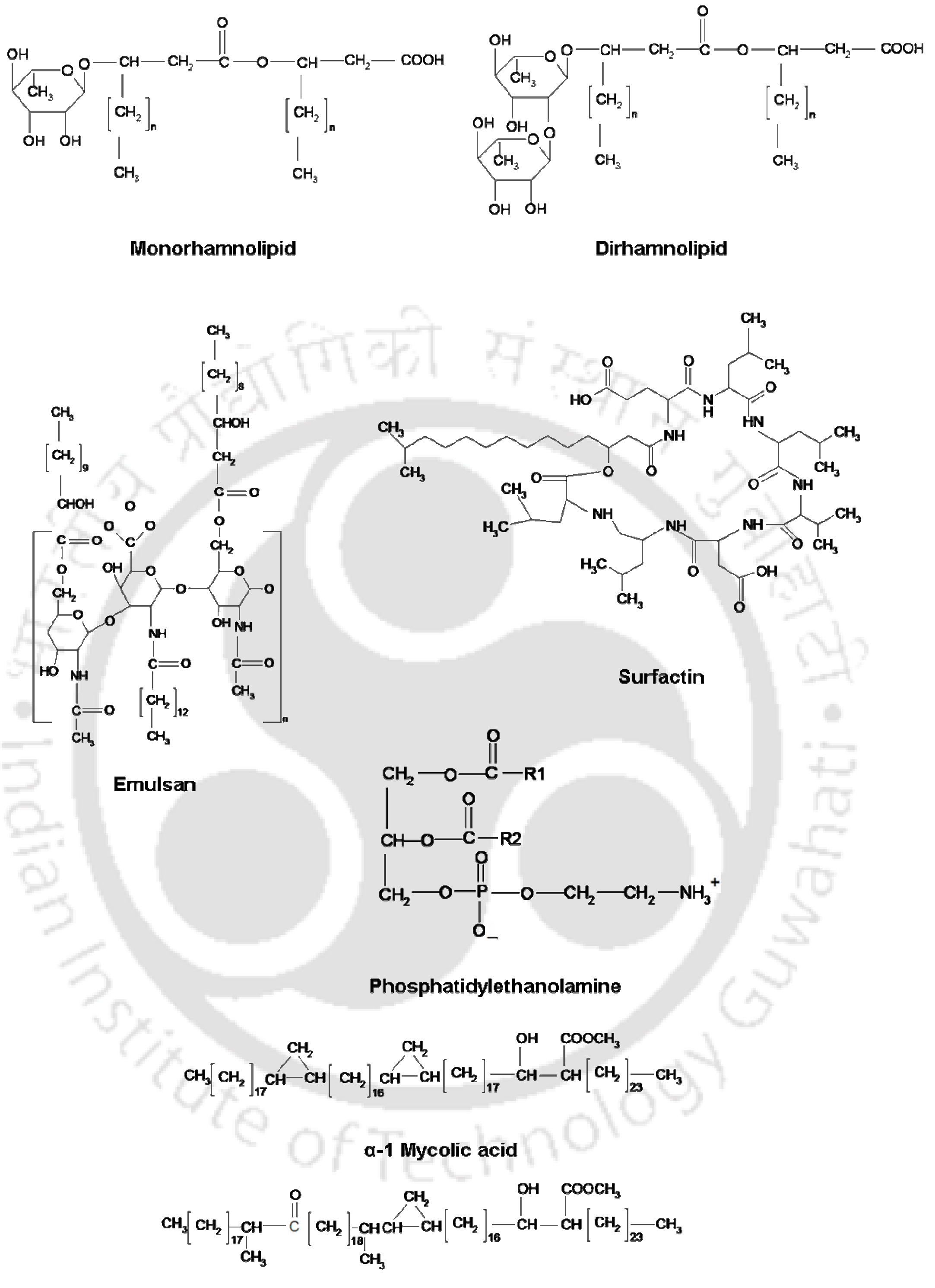
2.3.1.1. Glycolipids

Glycolipids are the most common and most widely studied biosurfactants (Desai and Banat, 1997; Muthusamy et al., 2008; Banat et al., 2010) and it consists of a carbohydrate (mannose, rhamnose or sophorose) and a fatty acid linked by either an ester or an ether group. These are involved in the uptake of low polarity hydrocarbon by microorganisms. Examples of glycolipids include trehalose lipids, sophorose lipids (sophorolipids, SLs), rhamnose lipids (rhamnolipids) and mannosylerythritol lipids.

Structurally, rhamnolipids compose of one or two molecules of rhamnose sugar linked to one or two molecules of β – hydroxydecanoic acid. This group of biosurfactants is produced by few *Pseudomonas* species and was first reported by Jarvis and Johnson (1949). SLs, are mainly produced by few yeasts of *Candida* species and consists of a sophorose linked by a glycosidic bond through a hydroxyl group located at the penultimate position of an 18-carbon fatty acid (Van Bogart et al., 2007). Gorin et al. (1961) were the first to report extracellular sophorolipids using the yeast *Torulopsis magnoliae* (later identified as *C. apicola*).

Table: 2.2: Type and microbial origin of biosurfactants.

Type of surfactant	Microorganism	Reference
Rhamnolipids	*Pseudomonas sp., P. aeruginosa, Serratia rubidea*	Mulligan (2005); Banat et al. (2010)
Sophorose lipids	*Candida apicola, C. bombicola, C. lipolytica, C. bogoriensis, Wickerhamiella domercqiae, C. batistae, Pichia anomala*	Van Bogart et al. (2007); Konishi et al. (2008); Thaniyavaran et al. (2008); Banat et al. (2010)
Trehalose lipids	*Arthrobacter paraffineus, Corynebacterium spp., Mycobacterium spp., Rhodococus erythropolis, Nocardia sp.*	Cooper et al. (1981); Desai and Banat (1997); Mulligan (2005)
Surfactin	*Bacillus subtilis*	Arima et al. (1968)
Mycolic acids	*Mycobacterium, Nocardia, Rhodococcus* and *Corynebacterium* species	Shimakata et al. (1984)
Corynomucolic acid	*Rhodococcus erythropolis*	Kretschner et al. (1982)
Phospholipid	*Acinetobacter* sp., *Thiobacillus thiooxidans, Aspergillus* sp.	Beeba and Umbreit (1971); Kappeli and Finnerty (1979)
Viscosin	*P. fluorescens*	Mulligan (2005)
Surfactin	*B. subtilis*	Arima et al. (1968)
Emulsan	*Acinetobacter calcoaceticus*	Rosenberg et al. (1979)
Liposan	*C. lipolytica*	Kappeli and Fiechter (1977); Cirigliano and Carman (1984)
Alasan	*Acinetobacter radioresistens*	Barkey et al. (1999)
Lichenysin A, Lichenysin B	*Bacillus licheniformis*	Mulligan (2005)
Lipopolysaccharides	*Acinetobacter calcoaceticus (RAG1), Pseudomonas sp.*	Mulligan (2005)


Monorhamnolipid

Dirhamnolipid

Emulsan

Surfactin

Phosphatidylethanolamine

α-1 Mycolic acid

keto-1 Mycolic acid

Fig. 2.4: Chemical structure of most studied biosurfactants from each class: rhamnolipid – a glycolipids; emulsan – a liposaccharides; surfactin – a lipopeptides; mycolic acid – a fatty acid. (Source: Desai and Banat, 1997; Mederos et al., 2007; Banat et al., 2010).

In case of trehalose lipids, disaccharide trehalose sugar is linked to mycolic acid (long chain α-branched-β-hydroxy fatty acid) and these are mainly produced by *Mycobacterium*, *Nocardia*, *Corynebacterium, Rhodococcus* and *Arthrobacter* species (Asselineau and Asselineau, 1978; Kretschmer et al., 1982; Li et al., 1984; Cooper et al., 1981; Desai and Banat, 1997).

2.3.1.2. Liposaccharides/Polymeric biosurfactants

A large number of bacterial species from different genera produce exocellular polymeric surfactants composed of polysaccharides, protein or complex mixture of these biopolymers. The high molecular weight, water-soluble extracellular emulsifiers are produced by hydrocarbon degrading bacteria like *Acinetobacter calcoaceticus* (emulsan). Examples of polymeric biosurfactants are emulsan, liposan, mannoprotien and polysaccarides – protein complexes (Desai and Banat, 1997; Rahman and Gapke, 2008; Satpute et al., 2010; Amoabediny et al., *in press*)

2.3.1.3. Lipopeptides

Lipopeptide belong to a particular antibiotic class of biosurfactant produced by *Bacillus sp.* General structure of lipopeptides consist of peptide cycle of seven (surfactin and iturin) or ten amino acids (fengycin) linked to a fatty acid chain, which vary from C_{13} to C_{16} chain length in surfactin, from C_{14} to C_{17} in iturin and C_{14} to C_{17} in fengycins. Surfactin (cyclic lipopeptide), produced by *B. subtilis*, is the most thoroughly studied biosurfactant in this group (Cooper et al., 1981; Besson and Michel, 1992), and was first reported by Arima et al. (1968). Recently, Kim et al. (2010) reported a novel *Bacillus* strain isolated from soil, *B. subtilis* CM32 capable of producing three types of antifungal lipopeptides namely surfactin A, iturin and fengycin.

2.3.1.4. Phospholipids

Phospholipids are esters formed between alcohol groups on lipids and phosphates present in membrane structure of the microbes. Phospholipids are major component of microbial membrane and the most notable biosurfactants of this class is produced by *Corynebacterium lepus* (Desai and Banat, 1997). *Acinetobacter* sp. strain HO1-N produces phosphatidylethanolamine, a phospholipid surfactant when grown on *n*-alkanes. Besides *Acinetobacter* sp., *Aspergillus* sp. (Kappeli and Finnerty, 1979) and *Thiobacillus thiooxidans* (Beeba and Umbreit, 1971) have also been reported to produce phospholipids in large amounts.

2.3.1.5. Fatty acids and neutral lipids

Fatty acids produced by microorganisms may be simple straight chain fatty acid or may be complex in nature containing OH groups and alkyl branches (Rahman and Gapke, 2008). Mycolic acids are long-chain, β-hydroxy fatty acids substituted at the α-carbon atom with a moderately long aliphatic chain and are mainly produced by *Mycobacterium*, *Nocardia*, *Rhodococcus* and *Corynebacterium* species (Shimakata et al., 1984). Corynomucolic acid is another example of complex fatty acid biosurfactants produced by *Rhodococcus erythropolis* (Kretschner et al., 1982). Triacylglycerols found in all eukaryotic cells (yeasts, molds, plants and animals) and esters produced by *Acinetobatcer* sp. related bacteria such as *Moraxella* and some eukaryotic algae are examples of neutral lipids.

2.3.2. Important properties of biosurfactant

Biosurfactants have several properties similar to chemical surfactants. The most important properties are discussed below:

2.3.2.1. Formation of micelle/ reverse micelle and reduction of surface/ interfacial tension

All surfactants have some common properties such as micelle formation, surface/interfacial tension reduction, critical micelle concentration, emulsification activity etc. And surfactants or biosurfactants are evaluated based on these properties (Parkinson, 1985). When these surfactants are present in a liquid at low concentrations, the surfactant moieties exist separately and are of such a size as to be sub-colloidal. As the concentration of surfactant increases, aggregation occurs over a narrow concentration range. These aggregates, which may contain 50 or more monomers, are called micelles. When present in aqueous phase the non-polar (hydrocarbon chains) tail face inward into the micelle to form their own hydrocarbon environment. On the other hand, when surfactants are present in non-polar liquids, the aggregates formed has different orientation, with the polar head (hydrophilic) facing inwards. This type of surfactant aggregation is called reverse micelle (Martin, 2001). Fig. 2.5 shows the general structure of surfactant and its properties to form micelle and reverse micelle.

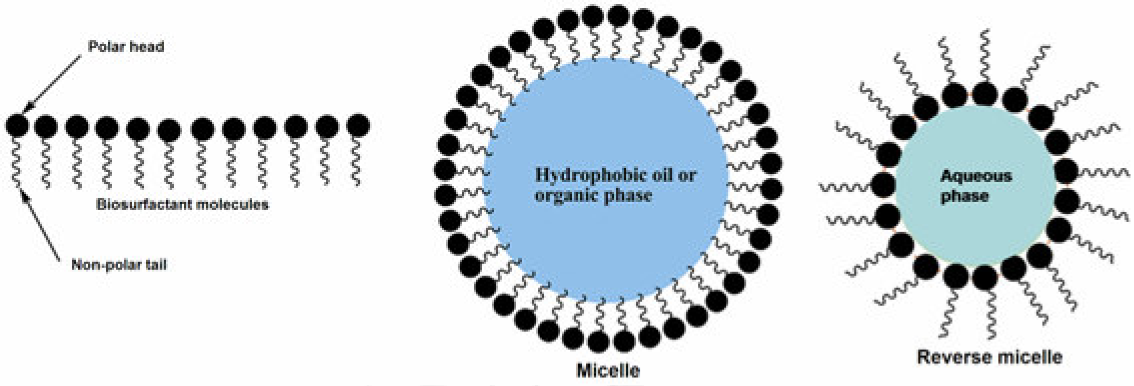


Fig. 2.5: General structures of biosurfactant, micelle and reverse micelle.

The effect of these biosurfactants on different interfaces such as air/water, oil/water or water/solid interface is most readily seen as a reduction in the surface/interfacial tension. The surface tension at air/water and oil/water interfaces (often simply referred to as surface tension and interfacial tension, respectively) can be quantitatively determined by easily available methods. CMC is normally referred to the concentration of surfactant at which micelles begin to form and at this point surface tension of the medium in which the surfactant is dispersed takes up a minimal value (Parkinson, 1985). A lower CMC indicates that less surfactant is needed to saturate interface between either air-liquid or liquid-liquid and form micelles. Thus CMC is the most commonly used measure of surfactant efficiency. At concentrations higher than the CMC, it is supposed that additional surfactant molecules aggregate into micelles in the bulk phase and do not contribute to significant further changes on the interface. Surface and interfacial tensions due to different biosurfactants reported in the literature are presented in Table 2.3. In general, biosurfactants are more effective and their CMC is about 10–40 times lower than that of chemical surfactants, i.e. compared to chemical surfactants quantity of biosurfactants required to reduce surface tension is very less (Desai and Banat, 1997).

Table 2.3: Properties of some important biosurfactants (adopted from Desai and Banat, 1997)

Biosurfactant	Organism	Surface tension (mN/m)	CMC (mg/l)	Interfacial tension (mN/m)
Rhamnolipids	*P. aeruginosa*	29	0.1-10	0.25
Trehalolipids	*R. erythropolis*	32-36	4	14-17
Sophorolipids	*C. bombicola*	33	60	1.8
Surfactin	*B. subtilis*	27-32	23-160	1
Viscosin	*P. flurescens*	26.5	150	

2.3.2.2. Emulsification and de-emulsification activity

Stable emulsions with a lifespan of months and years can be produced using biosurfactants (Velikonja and Kosaric, 1993). Also, biosurfactants can stabilize (emulsifiers) or destabilize (de-emulsifiers) emulsions, and high molecular-mass biosurfactants are in general better emulsifiers than low-molecular-mass biosurfactants (Muthusamy et al., 2008).

2.3.2.3. Biodegradability

One of the major advantages of biosurfactants over synthetic surfactants is their biodegradability and, therefore, they are particularly suitable for bioremediation (Mohan et al., 2006). Zeng et al. (2007) studied the co-degradation of synthetic surfactants (CTAB, Triton X-100 and SDS) and rhamnolipid with glucose by *P. aeruginosa*, *B. subtilis* and compost microorganisms in liquid culture media. In addition to being recalcitrant to degradation by these microorganisms, CTAB inhibited the organisms from utilizing the readily available carbon source glucose from the media. Although the nonionic surfactant Triton X-100 was found to be non-toxic to the microorganisms and hence did not inhibit the growth of the microorganisms, it was recalcitrant to biodegradation. Anionic surfactant SDS also showed no toxicity towards the microorganisms but could be co-degraded as carbon source along with glucose. The biosurfactant rhamnolipid was a distinct type of surfactant that was not only non toxic but also was well degraded by *B. subtilis* and compost microorganisms; however, it could not be utilized by its producer organism *P. aeruginosa*. Biodegradability of SLs produced by *C. bombicola* was also studied by different authors and is reported it to be a readily biodegradable biosurfactant (Hirata et al., 2009; Lo and Ju, 2009).

2.3.2.4. Low toxicity

Microbial surfactants are generally considered as low or non-toxic products and, therefore, more suited for pharmaceutical, cosmetic and food applications (Flasz et al., 1998; Muthusamy et al., 2008). A report suggested that a synthetic anionic surfactant (Corexit) displayed an LC_{50} (concentration lethal to 50% of test species) against *Photobacterium phosphoreum* ten times lower than rhamnolipids, hence demonstrating the higher toxicity of the chemical-derived surfactant (Poremba et al., 1991). Similarly, sophorolipids displayed low cytotoxicity towards human fibroblasts, human keratinocytes cell line HPK II and normal human epidermal keratinocytes (Krivobok et al., 1994; Otto et al., 1999; Hirata et al., 2009).

2.3.3. Environmental applications of biosurfactants

Due to its ability to reduce the surface/interfacial tension between two phases and form micelle, biosurfactants play an important role in the bioremediation of soil and water contaminated with hydrophobic pollutants and also in removal of heavy metals form contaminated soil. The role and mechanism of biosurfactants in bioremediation of sites contaminated with various pollutants are discussed below.

2.3.3.1. Hydrocarbons

Hydrocarbons are organic compounds and as the name suggests it consists of only hydrogen and carbon. They can be classified as saturated hydrocarbons, unsaturated hydrocarbons, cycloalkanes and aromatic hydrocarbons. These are mainly used as a source of fuel/energy and if present in the soil and water they are toxic to the environment and human health due to their mutagenicity, carcinogenicity and tendency to bioaccumulate in the food chain.

The low water solubility of hydrocarbons increases their sorption to surfaces and limits their bio-availability to microorganisms, which is a potential problem for bioremediation sites contaminated with hydrocarbons. Biosurfactants can enhance growth on bound substrates by desorbing them from surfaces or by increasing their apparent water solubility. Several authors have successfully studied the effect of different biosurfactants to increase the biodegradation of hydrocarbons (Schippers et al., 2000; Arun et al., 2008; Gottfried et al., 2010; Sponza and Gok, 2010). Fig. 2.6 shows the mechanism of polycyclic aromatic hydrocarbon (PAH) removal by biosurfactants. The first step is micelle formation, the hydrophobic part of the biosurfactant attaches to the PAH through hydrophobic interactions and make micelle containing the PAH. The micelle easily contacts the cell membrane which is highly hydrophilic in nature and increases the membrane porosity for the PAH to enter into the cell. Once inside the cell, PAH is attacked by the necessary enzymes and therefore gets degraded (Schippers et al., 2000). Arun et al. (2008) isolated the biosurfactant producing *Pseudomonas* strain form oil spilled soil and found that it can degrade 90% of pyrene.

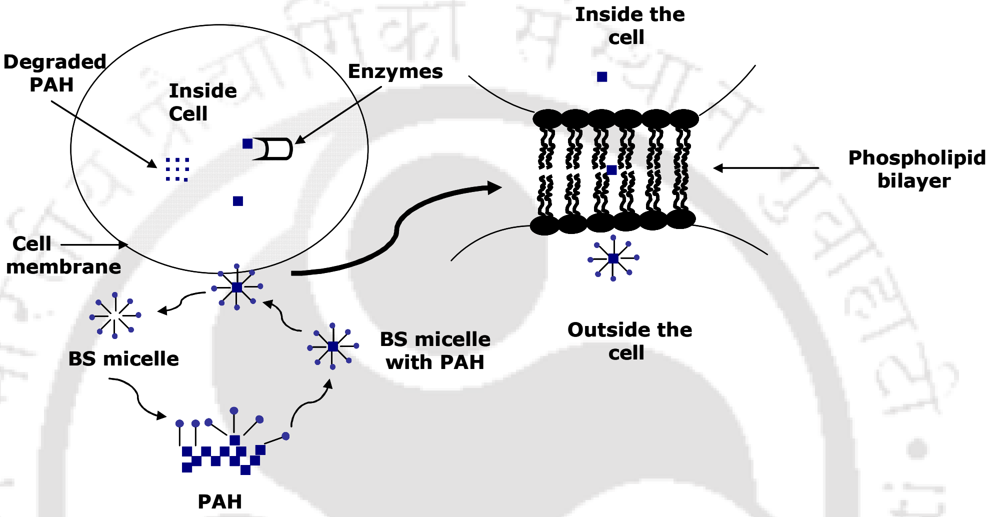


Fig 2.6: Mechanism of PAH degradation by microorganisms in presence of biosurfactant.

Mohanty and Mukherji (2008) studied the biodegradation rate of hydrocarbons by two biosurfactant producing bacterial cultures, *Exiguobacterium aurantiacum* and *Burkholderia cepacia*. Authors reported that these strains were capable of utilizing diesel oil as the sole source of carbon and energy. Also, these cultures showed good degradation characteristics for diesel range n-alkanes (C9–C26) and pristine.

Gottfried et al. (2010) reported that addition of rhamnolipid increases the apparent aqueous solubility of phenanthrene and overall degradation by at least 20% when combined with salicylate or glucose in liquid solution as compared to solutions that contained salicylate or glucose with no biosurfactant.

Sponza and Gok (2010) studied the degradation of PAHs in a petrochemical wastewater in a continuous stirred tank reactor and reported that addition of rhamnolipid increased the removal efficiencies of PAHs from 72 to 80%. The rhamnolipid treatment caused a significant increase in the degradation of five and six ring containing PAHs. Table 2.4 summarizes the various literature reports on biosurfactant aided degradation of different hydrocarbons by microorganisms.

Table 2.4: Hydrocarbons degraded by different microorganisms using biosurfactants.

Hydrocarbon	Microorganism	Biosurfactant	% Biodegradation	Reference
Naphthalene	*Pseudomonas* sp.	Rhamnolipid	100	Vipulanandan and Ren (2000)
Phenanthrene	*P. putida*	Sophorolipid	99.4	Schippers et al. (2000)
Pyrene	*Pseudomonas* sp.	Multi component surfactant consisting of protein and polysaccharides	92.3	Arun et al. (2008)
Acenaphthene			28	
Fluorene			24.4	
Phenanthrene	*P. putida*	Rhamnolipid (0.25g/l)	86	Gottfried et al. (2010)
		Rhamnolipid (1g/l)	91	
		Rhamnolipid (5 g/l)	92	
Mixture of PAH containing petrochemical industrial wastewater	Activated sludge	Rhamnolipid	80	Sponza and Gok (2010)
Phenanthrene	*Pseudomonas aeruginosa* co-culture with *P.* strain R	Rhamnolipid	50	Dean et al. (2001)
Mixture of hydrocarbons	Soil population	Sophorolipid	95	Oberbremer et al. (1990)
Gasoline	Soil sample	Rhamnolipid (0.1%)	67	Rahman et al. (2002)
		Rhamnolipid (1 %)	78	

2.3.3.2. Pesticides and herbicides

Pesticides such as dichlorodiphenyltrichloroethane (DDT), 2,4-dichlorophenoxyacetic acid (2,4-D) and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), plasticizers, pentachlorophenol, polychlorinated biphenyls, among others, are examples of halogenated aromatic compounds. Their stability and toxicity are causes of great concern for the environment and public health. The halogenated aliphatic compounds, position, and number of halogens are important in determining the rate and mechanism of biodegradation (Mulligan, 2005). Similar to PAHs, these pesticides and herbicides also have poor aqueous solubility and biosurfactants are suggested to increase their aqueous solubility, thereby increasing their biodegradation.

Robinson et al. (1996) reported that rhamnolipids at 4 g/l showed capability to increase the mineralization of 4,4′ chlorobiphenyl by an acclimated culture of *Alcaligenes eutrophus* up to 213 times than the control. Mata-Sandoval et al. (2001) studied the rhamnolipids and Triton X-100 aided biodegradation of trifluralin and coumaphos in liquid cultures and soil slurries by microbial consortia isolated from contaminated cattle dip. They found that both the surfactants enhance the biodegradation of trifluralin and coumaphos. A study conducted by Wattanaphon et al. (2009) proved that biosurfactants are more active in solubilizing pesticides compared to synthetic surfactants. Sharma et al. (2009) found that rhamnolipids produced from the isolated strain of *P. aeruginosa* WH-2 improved the aqueous solubility of hexachlorocyclohexane (HCH) isomers, highly toxic, carcinogenic and endocrine disrupters pesticide, suggesting the potential role of biosurfactants in bioremediation of pesticides and herbicides.

2.3.3.3. Heavy metal

Soils contaminated with metals are mainly due to direct contact with industrial discharge, improper disposal of wastes, incidence of spills and failure of land disposal facilities. Metals such as lead, chromium, cadmium, arsenic, copper, and zinc have been detected in most of the sites, with mercury and nickel present in some of the sites (Forstner, 1995; Kim and Vipulanandan, 2006). Metals, unlike many hazardous organic constituents, cannot be degraded or readily detoxified. The existence and fate of the metals in soil is a matter of concern not only because of their potential impact on microbial communities but also because of the potential for groundwater contamination and hence toxicological impact on human health (Kim and Vipulanandan, 2006). Metal contaminants in the environment are usually tightly bound to colloidal particles and organic matter. This represents a major constraint on their removal using currently available *in situ* remediation technologies (Juwaker et al., 2008). Biosurfactants can improve the metal removal and their mechanism is different from solubilization, mobilization, sorption and emulsion formation etc. which have been postulated for hydrocarbon removal. Muligan et al. (1999b) suggested a possible mechanism of biosurfcatnts in metal removal from the soil and is represented in Fig 2.7. Table 2.5 summarizes the various studies that have successfully demonstrated removal of metals from contaminated systems using biosurfactants. Due to the anionic nature of rhamnolipids, it aids in removal of metals from soil and ions such as cadmium, copper, lanthanum, lead and zinc due to their complexation ability (Tan et al., 1994; Herman et al., 1995; Ochoa-Loza, 1998; Mulligan, 2005).

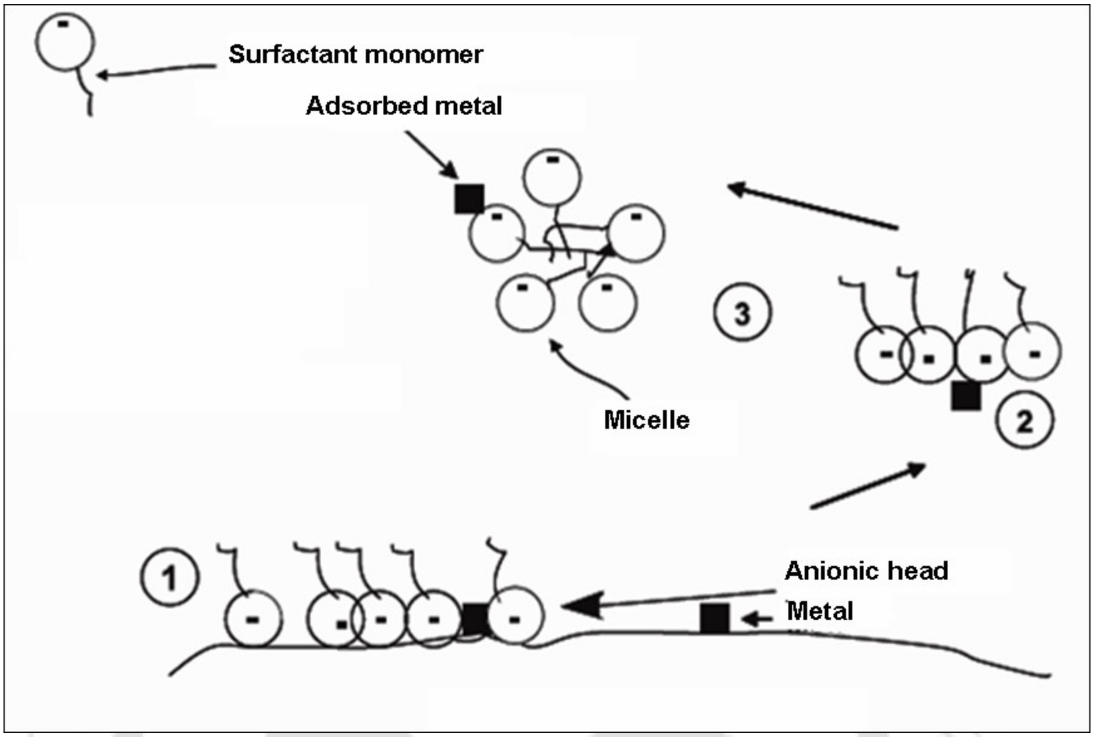


Fig. 2.7: Three step mechanisms for metal removal by biosurfactant (adopted from Mulligan et al., 2005). Step (1) accumulation of surfactant as hemimicelles or admicelles at soil interface, step (2) removal of metal by lowering of interfacial tension and electrostatic attraction, step (3) incorporation of metal into micelle.

Dahrazma and Mulligan (2007) evaluated the performance of rhamnolipid in a continuous flow configuration (CFC) for removal of heavy metals (copper, zinc, and nickel) from the sediments taken from Lachine Canal, Canada, to simulate a flow through remediation technique. In this configuration, rhamnolipid solution with a constant rate was passed through the sediment sample within a column. The removal of heavy metals from sediments was up to 37% of Cu, 13% of Zn, and 27% of Ni when rhamnolipid without additives was applied. Adding 1% NaOH to 0.5% rhamnolipid improved the removal of copper by up to 4 times compared with 0.5% rhamnolipid alone.

Table 2.5: Removal of different metals by biosurfactants

Metal	**Biosurfactant**	**% Removal**	**Reference**
Cd	Rhamnolipid (0.5%) foam and Rhamnolipid (0.5%) liquid solution	73.2 and 61.7	Mulligan and Wang (2004)
	Rhamnolipid	71.9	Asci et al. (2007)
	Rhamnolipid (0.1%) pH 8	92	Juwarkar et al. (2007)
	Biosurfactant isolated from marine bacterium	97.66	Das et al. (2009)
Ni	Rhamnolipid (0.5%) foam	68.1	Mulligan and Wang (2004)
	Rhamnolipid (0.5%) liquid solution	51	Mulligan and Wang (2004)
Lead	Rhamnolipid (10 mM solution)	15	Neilson et al. (2003)
	Rhamnolipid (0.1%) pH 8	88	Juwarkar et al. (2007)
	Biosurfactant isolated from marine bacterium	100	Das et al. (2009)
Zinc	Rhamnolipid (12%)	19.5	Mulligan et al. (1999a)
	Sophorolipid (4%)	15.8	Mulligan et al. (1999a)
	Sophorolipid (4%) with HCl (0.7%)	100	Mulligan et al. (1999a)
	Surfactin (0.25%) with NaOH (1%)	22	Mulligan et al. (1999b)
Zinc from Na-feldspar	Rhamnolipids (25 mM), pH 6.8	98.83	Asci et al. (2008)
Copper	Rhamnolipid (12%)	25	Mulligan et al. (1999a)
	Surfactin (1%) with NaOH (1%)	70	
	Rhamnolipid (2%)	28	Dahrazma and Mulligan (2007)

Juwarkar et al. (2008) isolated the di-rhamnolipid biosurfactant producing strain *P. aeruginosa* BS2 and further assessed the potential of the biosurfactant as a washing agent for metal removal from multi metal contaminated soil (Cr-940 ppm; Pb-900 ppm; Cd-430 ppm; Ni- 880 ppm; Cu-480 ppm). Between tap water and rhamnolipids, rhamnolipids proved very efficient in mobilization of the metals from contaminated soil; within 36 hours of the leaching study, di-rhamnolipid as compared to tap water facilitated 13 folds higher removal of Cr from the heavy metal spiked soil whereas removal of Pb and Cu were 9-10 and 14 folds higher than with only tap water. On the other hand, leaching of

Cd and Ni from the spiked soil was 25 folds higher using rhamnolipids compared to the use of only tap water. These results showed that leaching behavior of the biosurfactant was different for different metals. The use of wastewater for production of biosurfactant and its efficient use in metal removal have been suggested to make it a strong candidate for bioremediation.

For successful application of biosurfactant, particularly for the environment, it is essential that it is produced by non-pathogenic organism and in large quantities. And compared with other biosurfactants, sophorolipids (SLs) seems to satisfy both these attributes more satisfactorily.

2.4. Sophorolipid

Sophorolipids (SLs) are glycolipid type of biosurfactant produced mainly by the yeast *Candida*. In 1961, Gorin et al. (1961) were the first to describe SLs, an extracellular glycolipid, synthesized by the yeast *Torulopsis apicola*, currently known as *Candida apicola*. In 1968, Tulloch et al. (1968) discovered a new SL from *Candida bogoriensis* (now known as *Rhodotorula bogoriensis*), which differs in hydroxy fatty acid moiety of the SL reported by Gorin et al. (1961). In 1970, Spencer et al. (1970) identified another sophorolipid secreting yeast strain *C. bombicola* (formerly known as *T. bombicola*).

In 1998, Rosa and Lachance (1998) described the novel yeast species *Starmerella bombicola* and introduced it as the teleomorph of *C. bombicola* based on the high 18S rDNA identity between both the strains (more than 98%) and their ability to mate with each other to form ascospores. Chen et al. (2006a) proved SLs synthesis in a new strain of *Wickerhamiella domericqiae*. Although the SLs are produced by different yeasts, the basic structure of the SLs is the same (sophorose sugar linked to a long chain hydroxy fatty acid). Recently, Konishi et al. (2008) reported that *C. batistae* CBS 8550 which is closely related to *S. bombicola* also produces a novel type of SLs. Thaniyavaran et al. (2008) isolated *Pichia anomala* PY1, a thermotolerant strain from fermented food and reported that it produces biosurfactant similar to SLs.

All the SLs producing yeasts are closely related to *C. bombicola* (except *W. domericqiae*) and their mechanisms, characteristics for SLs production, structure and properties can be considered more or less same for all yeast species (Van Bogaert et al., 2007).

2.4.1. SL structure

SLs are amphiphilic molecules; that means they have one hydrophilic head and one hydrophobic tail. Fatty acid chain of 16 or 18 carbon atoms serve as a tail and a sophorose serves as a hydrophilic carbohydrate head in SLs. The most common structure of acidic and lactonic SL is shown in Fig. 2.8. Sophorose is a disaccharide with an unusual β-1,2 bond. The hydroxylated fatty acid is β-glycosidically linked to the sophorose molecule. The carboxylic end of this fatty acid is either free (acidic or open form) or internally esterified at the 4″ or at the 6′- or 6″-position (lactonic form). The hydroxy fatty acid (16 or 18 carbon atoms) can have one ore more unsaturated bonds (Asmer et al., 1988; Davila et al., 1993).

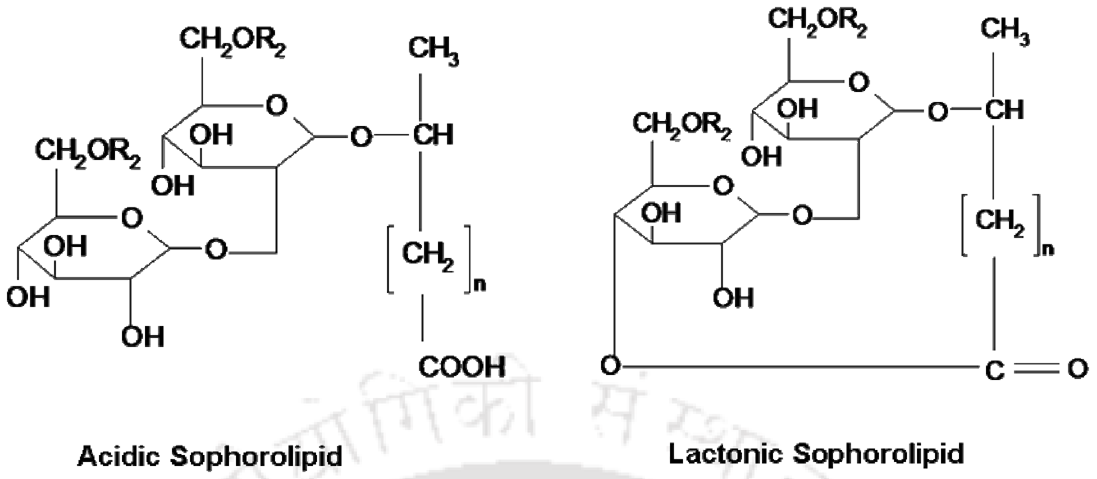


Fig. 2.8: Structure of acidic and lactonic sophorolipids. (Source: Banat et al., 2010)

The SLs synthesized by yeast (*C. bombicola, C. apicola* and *W. domericqiae*) is identified as a mixture of SLs, which differ in fatty acid moiety (chain length, saturation, and position of hydroxylation), lactonization and acetylation patterns (Asmer et al., 1988; Hommel et al., 1994; Chen et al., 2006). Hommel et al. (1994) studied the structure of the crystalline lipid produced by the yeast *C. bombicola* and *C. apicola* by means of NMR spectroscopy. They reported that products of both strains were identical in composition. Asmer et al. (1988) were the first to report the structural variation of SLs produced by *C. bombicola*. They separated the SLs mixture by medium pressure liquid chromatography and thin layer chromatography, and reported 14 different types of SLs. Davila et al. (1993) separated the SLs mixture by a gradient elution high-performance liquid chromatography (HPLC) method and used an evaporative light scattering for the detection of the individual SLs and identified over 20 different types of SLs. Chen et al., (2006) purified the individual SL produced by *W. domericqiae* using HPLC and reported that this yeast produced three different type of SLs.

2.4.2. Biosynthetic pathway of SLs

Fig. 2.9 gives a schematic overview of the biochemical pathways involved in SLs synthesis. The building blocks for conventional SLs synthesis are glucose and a fatty acid. Because SL-producing yeasts strains such as *C. bombicola* and *C. apicola* are capable of growing on alkanes, the organisms possess the enzymes required for terminal oxidation of alkanes, thereby generating fatty acids for further β-oxidation. If hydrophobic substrate is not present in the medium, fatty acids will be formed by de novo starting from acetyl-CoA derived from glycolysis. On the other hand, when concentration of hydrophilic carbon source (especially glucose) is low in the medium, part of the fatty acids will be conducted toward the β-oxidation for cell maintenance instead of SL synthesis (Van Bogaert et al., 2007).

In a first step, the fatty acids are converted to a terminal (ω) or subterminal (ω-1) hydroxy fatty acid through the action of a membrane bound nicotinamide adenine dinucleotide phosphate (reduced form; NADPH) dependent monooxygenase enzyme, cytochrome P450 (Jones, 1968). Lottermoser et al. (1996) identified two cytochrome P450 monooxygenase genes from *C. apicola* (European Molecular Biology Laboratory/GenBank accession numbers X76225 and X87640).

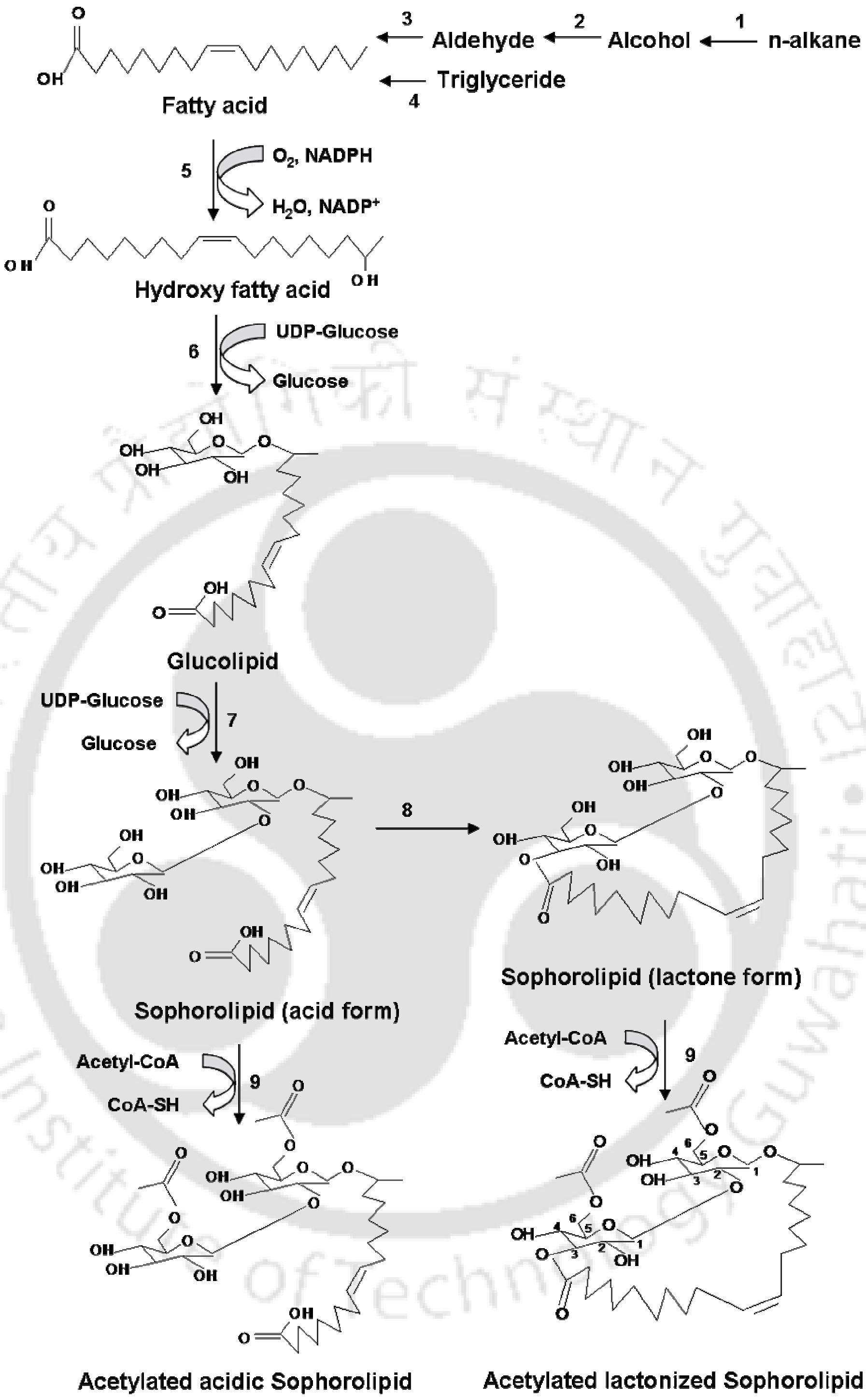


Fig. 2.9: Proposed SL biosynthetic pathway. 1, cytochrome P450 monooxygenase; 2, alcohol-dehydrogenase; 3, aldehyde-dehydrogenase; 4, lipase; 5, cytochrome P450 monooxygenase; 6, glucosyl-transferase I; 7, glucosyl-transferase II; 8, lactonesterase; 9, acetyltransferase (adopted from Van Bogaert et al., 2007).

Based on the amino acid similarity, they were classified into the CYP52 family, which comprises cytochrome P450 enzymes of yeasts capable of hydroxylating alkanes and/or fatty acids (Nelson, 1998). For *C. bombicola*, Van Bogaert et al. (2009) identified three different cytochrome P450 monooxygenase genes belonging to the CYP52 family.

In a second step, glucose is glycosidically coupled (position C1’) to the hydroxyl group of the fatty acid through the action of a specific glycosyltransferase I. Experiments with ^{13}C-labeled glucose pointed out that the bulk of the added glucose first passed through glycolysis, in this way supplementing trioses for the gluconeogenesis of glucose for SLs synthesis (Hommel et al., 1994). The transferase reaction requires nucleotide-activated glucose (uridine diphosphate (UDP)-glucose) as glucosyl donor (Breithaupt and Light, 1982). In a subsequent step, a second glucose is glycosidically coupled to the C2’ position of the first glucose moiety by glycosyltranferase II. Both glycosyltransferases involved in SLs synthesis of *R. bogoriensis* were partially purified but these two enzyme activities could not be separated and highly purified samples exhibit a single major band of 52 kDa on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE; Esders and Light, 1972; Breithaupt and Light, 1982). It is not confirmed yet whether the consecutive glucose transfers are carried out by two different (but co-purified) enzymes or by one and the same (multi)enzyme. It is supposed that SLs synthesis in *C. bombicola* involves analogous enzymes. The SLs obtained after the action of glucosyltransferase II are as such detected in the SLs mixture as the acidic, non-acetylated molecules. The majority of the SLs are however further modified by both internal esterification (lactonization) and by acetylation of the carbohydrate head. Lactonic SLs are formed by an esterification reaction of the carboxyl group of the hydroxy fatty acid with a hydroxyl group of sophorose (Fig. 2.9). The lactones are esterified either at the 4″-position, 6′-position or 6″-position (Asmer et al., 1988) but no esterase has been identified in *C. bombicola* or in other SL producing species. The acetylation at the 6′- and/or 6″-position is carried out by an acetyl-coenzyme A (CoA) dependent acetyl transferase. The transferase from *R. bogoriensis* has been partially purified (Esders and Light 1972; Bucholtz and Light 1976), but the corresponding enzyme has not yet been identified in *C. bombicola*.

2.4.3. Physiological role of SLs

Synthesis of biosurfactants is often associated with the assimilation of hydrophobic substrates (Ito et al., 1980); this theory is however not commonly accepted for SLs formation, especially because the molecules are also formed when no hydrophobic substrate is present and in amounts largely exceeding the concentration required for emulsification. SLs synthesis is associated with nitrogen starvation. It is suggested that formation of glycolipids is some sort of overflow metabolism, by means of extracellular storage material. This hypothesis is supported by the findings of Hommel et al. (1994), regarding the biosynthesis of the sophorose moiety, which resembles the trehalose synthesis of *Saccharomyces cerevisiae* under anaerobic conditions. It was also demonstrated that SLs can be used as sole carbon source (Garcia-Ochoa and Casas, 1997). As *C. bombicola* and *C. apicola* by nature occur in environments with high osmotic strength, SLs production may be a way of dealing with the high sugar concentrations by converting, storing, and making them less available for other organisms. Furthermore, SLs display antimicrobial activity against certain yeasts such as

Candida and *Pichia* species (Ito et al., 1980) and Gram-positive bacteria. The mono- and diacetylated lactones have the strongest inhibitory effect (Lang et al., 1989). Overall, it can be concluded that the physiological role of SLs synthesis is extracellular carbon source storage, combined with dealing with a high-sugar niche and defending it against other competing microorganisms (Van Bogaert et al., 2007).

2.4.4. Fermentative production of SL

Classical commercial fermentation processes for the production of non-growth associated products can be subdivided into three phases (Omstead et al., 1985) and SLs is no exception: (1) the first stage is inoculum development; (2) the second phase is the stage in which SLs are microbiologically synthesized and (3) the third phase is recovery of SLs. However, the SLs yield and its structure is largely depend on the several physical viz. pH, temperature, agitation etc. and chemical viz. carbon source, nitrogen source etc. variables. The following sections will discuss the effect of these variables on SLs production by the yeast:

2.4.4.1. Effect of physical parameters

The physical parameters such as temperature, agitation, aeration etc. greatly influence any type of fermentation including SLs production. Gobbert et al. (1984) found the optimal temperature for SLs production by the *C. bombicola* to be 21°C. Most studies in literature have been performed at 25 or 30°C for the production of SLs by the *Candida* yeasts; as this temperature condition has been found suitable for handling during fermentation e.g., sampling and oil addition.

During the exponential growth phase, pH drops tremendously and must further be maintained at the value of 3.5 by the addition of base (NaOH or KOH) for optimal SLs production (Gobbert et al., 1984). This low pH and the antimicrobial effect of SLs protect the fermentation broth against contamination, even when fed-batch processes of more than 200 h are run.

Oxygen supply by good aeration is also found to be very important throughout the whole fermentation process as the yeast cells are very sensitive to oxygen limitation during their exponential growth. Guilmanov et al. (2002) investigated requirement for aeration in SL production in batch shake flasks and reported that between 50 and 80 $mMO_2/l/h$ gave a high yield of SLs in shake-flasks.

2.4.4.2. Effect of different carbon sources

The production of SLs by the yeasts were found to be very less when the yeast cells are supplied with only one type of carbon source, such as n-alkanes (Jones and Howe, 1968) or glucose (Hommel et al., 1994). However, its production highly increased when the yeasts cells were supplied with two types of carbon sources, hydrophilic (glycidic) and hydrophobic (lipidic). Although, glucose is used as the hydrophilic carbon source, sucrose (Klekner et al., 1991), galactose (Zhou and Kosaric, 1993), lactose (Zhou and Kosaric, 1993; Zhou and Kosaric, 1995) or glycerol (Ashby et al., 2006) can also act as substrates, but the obtained SLs level is lower. To reduce the substrate costs, deproteinized cheese whey (Zhou and Kosaric, 1993; Daniel et al., 1998a; Daniel et al., 1998b; Daniel et al., 1999) and soy molasses (Solaiman et al., 2004; Solaiman et al., 2007) have been proposed as hydrophilic carbon sources. Zhou and Kosaric (1993, 1995) first investigated the production of SLs by the yeast *C. bombicola* with galactose and lactose, the main sugar components of whey. They found that *C. bombicola* was not able to grow when only lactose was present in the medium, but when the medium was supplemented with olive, canola, or safflower oil, growth and SLs formation were observed. They also tested cheese whey and reported that only cheese whey in the medium gives poor growth of the yeast (2.43 g/l of biomass concentration) without any SLs production (Zhou and Kosaric 1993). However, the biomass increased several fold (14 g/l) when cheese whey was supplemented with olive oil. Also, the yeast could able to produce small amount of extracellular SLs (6 g/l). Later, Daniel et al. (1998a) investigated the production of SLs in a medium with deproteinized whey concentrate and rapeseed oil. They obtained high levels of SLs production (280 g/l) under repeated feeding of rapeseed oil over a long period, without consuming lactose. To improve the utilization of lactose present in whey Daniel et al. (1999) first cultivated the oleaginous yeast *Cryptococus curvatus* on whey. The cells, which accumulated a high single cell oil level, were harvested and disrupted and then served as lipidic substrate for the *C. bombicola* cells which produced reduced amount of SLs (12 g/l) in bioreactor under batch mode. However, the SL concentration was improved to 422 g/l when fermentation was carried out for up to 24 d with repeated feeding of rapeseed oil (Daniel et al., 1998b). Soy molasses have also shown to act as glucose substitute, but again, lower yields were observed (Solaiman et al., 2004; Solaiman et al., 2007).

A lot of substrates can act as hydrophobic carbon source such as oils, fatty acids, and their corresponding esters, alkanes, etc. for the production of SLs. The level of SLs formation during fermentations based on alkanes as hydrophobic feedstock largely depends on the chain length of the used substrate. Hexadecane and octadecane seem to yield the best production results as they appear to be directly converted into hydroxy fatty acids and gets incorporated into the SLs molecules (Davila et al., 1994). The yields are higher when compared to the shorter alkanes and even comparable with those obtained using n-C16 and n-C18 (Tulloch et al., 1962; Jones and Howe, 1968).

The above trend in SLs production is observed for fatty acids or fatty acid methyl esters as different carbon source with the best results being obtained for oleic acid (C18:1). It turns out that the fatty acid chain length determines the rate and position of hydroxylation and consequently governs the incorporation into the SL molecule (Van Bogaert et al., 2007).

In addition, oils (especially those of vegetable origin) are widely used as lipidic carbon source. The most common vegetable oils are comprised of saturated or unsaturated fatty acids with chain lengths of 16 or 18 carbon atoms, making them an ideal substrate for direct incorporation and consequent high SLs production and yield. The vegetable oils canola, corn, safflower, sunflower, olive, rapeseed, grapeseed, palm, coconut and soybean oil are reported to be used for SLs production (Cooper and Paddock, 1984; Lee and Kim, 1993; Davila et al., 1994; Zhou and Kosaric, 1995; Rau et al., 1996; Kim et al., 1997; Casas and Garcia-Ochoa, 1999; Cavalero and Cooper, 2003; Pekin et al., 2005). Furthermore, waste streams such as biodiesel by-product streams (Ashby et al., 2005), soybean dark oil (Kim et al., 2005), waste frying oil (Fleurackers, 2006), industrial fatty acid residues (Felse et al., 2007) and restaurant waste oil (Shah et al., 2007) have also been tested for SLs production.

2.4.4.3. Effect of nitrogen sources

Other than carbon source, nitrogen source has also been shown to affect the production of SLs as its synthesis is associated with nitrogen starvation in the medium. Yeast extract is the most preferable nitrogen source for the production of SLs and is essential for both cell growth and SLs formation. Substitution with urea or peptone negatively influenced the biomass and glycolipids yield. Cooper and Paddock (1984) set the optimum concentration of nitrogen source (yeast extract) for SLs production at 5 g/l, whereas Zhou et al. (1992) obtained the highest SLs concentration using only 2 to 3 g/l yeast extract. Casas and Garcia-Ochoa (1999) further decreased the yeast extract content to 1 g/l by pointing out that higher concentration of nitrogen source in the medium favors biomass but decreased the SLs production due to depletion of the carbon sources by cell growth. The partition in structural classes also tends to depend on the level of yeast extract in the medium. A concentration of 1 g/l mainly triggers the lactonic form, whereas 20 g/l lead to the synthesis of acidic SLs (Van Bogaert et al., 2007). The most favorable concentration for SLs production remains unclear.

2.4.4.4. Effect of bioreactor operation mode

Several modes of bioreactor operation are being used in the production of secondary metabolite or non growth associated products such as batch, fed-batch and continuous cultivation. The fed-batch method is more frequently used due to its higher productivity and ease of operation. Fed-batch strategies control the growth rate, prolonging the stationary phase, and overcoming substrate inhibition and metabolite repression. Continuous operation is more efficient than batch or fed-batch operation in terms of processing time, since dead time due to charge and discharge operations, bioreactor preparation and sterilization is avoided. However, productivity is often lowered by genetic variations liable to occur when operation continues over a long time. Also, the probability of contamination is much higher than in batch or fed-batch systems (Saudagar et al., 2008).

Batch and fed-batch modes of bioreactor operation are most commonly studied for the production of SLs by several authors (Klekner et al., 1999; Felse et al., 2007; Konishi et al., 2008; Thaniyavaran et al., 2008; Kim et al., 2009). Other authors have also studied the SLs production by resting-cell method (Gobbert et al., 1984; Casas and Garcia-Ochoa 1999).

When batch culture is used, the yield of SLs depends very much on medium composition and operational conditions (temperature, aeration rate, stirrer speed, etc.) The highest concentration of SLs obtained by this method reported in literature is between 70 and 80 g/l, in 6 d, with a yield of 35% from total carbon sources (Cooper and Paddock, 1984; Lee and Kim, 1993). Asmer et al. (1988) and Davila et al. (1992) used fed-batch mode of operation in a fermentor and reported a SLs concentration of 77 g/l and 190 g/l, respectively. A variation of this method, fed-batch culture with pulse additions, has also been employed by Lee and Kim (1993) obtaining final concentrations of up to 120 g/l. Recently, Kim et al. (2009) developed an easier control method for fed-batch culture of SLs production wherein they reported that the feeding rate of rapeseed oil was dependent on pH, which was calculated by the consumption rate of NaOH and rapeseed oil. The glucose concentration was constantly maintained between 30 and 40 g/l. As a result, they could able to achieve crude SLs up to 365 g/L for 8 days through this feeding-rate-controlled fed-batch process. Self cycling fermentation (SCF) is a semi continuous, computer controlled fermentation operation with the level of dissolved oxygen as the control parameter. This technique was applied by McCafferey and Cooper (1995) to produce SLs from *C. bombicola*.

The resting-cell method consists of growing the yeast to the final exponential or stationary phase, harvesting the cells, washing and inoculation into a fresh production medium. Using this method, Gobbert et al. (1984) obtained a concentration of 16 g/l SLs in 3 d; however, in this study only one carbon source was employed, so the concentration is not comparable with those previously mentioned. Casas and Garcia-Ochoa (1999) studied this method under optimized process conditions and reported over 120 g/l of SLs.

2.4.5. Surfactants properties of SLs

Akin to any biosurfactants, SLs lower the surface tension of water and the value reduced is from 72.8 mN/m to 40 to 30 mN/m. CMC of SLs ranges between 40 to 100 mg/l and the value depends on the substrate used for its production. Shin et al (2010) studied the CMCs of SLs produced from different sources and found that SLs produced using methyl ester erucic acid showed the lowest CMC of 47.5 mg/l and those from methyl ester oleic acid displayed the highest CMC at 70.0 mg/l. The results suggested that the longer hydrophobic chain in SL gives less CMC.

The interfacial tension between water and canola oil due to SLs produced by the *C. bombicola* using glucose and either palmitic acid (SL-p), stearic acid, oleic acid or linoleic acid ranged between 3 and 5 mN/m (Ashby et al., 2008). Although, SLs are able to reduce the surface and interfacial tensions, they were found to be not very good in emulsion formation by Cooper and Paddock (1984). They tested the emulsification activity and stability of the SLs produced by the yeast with hydrocarbons and vegetable oils and reported that the SLs was not able to stabilize emulsions containing water and either the hydrocarbons or the vegetable oils.

2.4.6. Applications of SLs

Due to its surfactants and antibacterial properties, natural mixtures of SLs, pure isolates of individual components and their derivatives have shown several environmental, pharmaceutical and therapeutic applications. Description of SLs applications in different fields are given below:

2.4.6.1. Environmental applications

Oberbremer et al. (1990) added SLs to a 10% soil and 1.35% hydrocarbon mixture and found that 90% of the hydrocarbons were degraded in 79 h compared to 81% in 114 h without the biosurfactant. Mulligan et al. (2001) reported that after single washing of soil by SLs (4%), SLs (4%, 0.7% HCl) and SLs (4%, 1% NaoH) the Zn removal efficiency was shown to be 4%, 16% and 7%, respectively. Schippers et al. (2000) studied the effect of sophorolipids on phenanthrene biodegradation and determined that the concentration of phenanthrene (initial concentration of 80 mg/l) within 36 h decreased to 0.5 mg/l in the presence of 500 mg/l of the biosurfactant compared to 2.3 mg/l without surfactant in a 10% soil suspension. The maximal degradation by *Sphingomonas yanoikuyae* was 1.3 mg/l/h with the SLs instead of 0.8 mg/l/h. The SLs enhanced the phenanthrene concentration in the aqueous medium as shown by fluorescence measurements instead of increasing the biomass concentration. In addition, toxicity of the SLs was low for concentrations up to 1 g/l. Solubilization tests showed that 232 mg of phenanthrene in water and 80.7 mg in soil were solubilized by 1 g of SLs, 10-fold higher than by other surfactants such as sodium dodecyl sulphate (SDS). These experiments have indicated that the SLs enhance biodegradation of the phenanthrene through enhanced solubilization. Recently, Kang et al. (2010) studied the effectiveness of SLs in washing and biodegradation of model hydrocarbons (2-methylnaphthalene, n – hexadecane and pristane) crude oil in soil. Thirty percent of 2-methylnaphthalene was effectively washed and solubilized with 10 g/L of SLs with similar or higher efficiency than that of commercial surfactants. Addition of SLs in soil increased biodegradation of model compounds: 2-methylnaphthalene (95% degradation in 2 days), n – hexadecane (97%, 6 days), and pristane (85%, 6 days). Also, effective biodegradation of crude oil in soil was observed by the addition of SLs, resulting in 80% biodegradation of saturates and 72% aromatics in 8 weeks. These results showed the potentials of SLs as an effective surfactant for soil washing and as an in situ biodegradation enhancer.

However, from literature, it appears that application of SLs in environmental applications has not been explored fully, although its potential is very high. The surfactant properties of SLs can be exploited in the petroleum industry; secondary oil recovery, in removing hydrocarbons from drill material, and in the regeneration of hydrocarbons from dregs and muds (Baviere et al., 1994; Marchal et al., 1999; Pesce, 2002). SLs can also be applied for decontaminating porous media such as soils and groundwater tables polluted by hydrocarbons (Ducreux et al., 1997) and in the removal of heavy metals from soil sediments (Mulligan et al., 2001).

2.4.6.2. Pharmaceutical and medical applications

SLs have been tested for several applications in the field of pharmaceutical and medical due to its anti-cancer activity (Scholz et al., 1998; Chen et al., 2006b; Fu et al., 2008), spermicidal, virucidal and anti-herpes virus properties (Gross and Shah, 2007). It also found application in the treatment of asthma severity (Bluth et al., 2008) and in the treatment of skin diseases (Hillion et al. 1998; Maingault, 1999). Also they were found useful for the synthesis of nanoparticles (Kasture et al., 2007).

2.4.6.3. Other applications

SLs have shown potential for applications in detergent industry (Hall et al., 1996; Futura et al., 2002; Hirata et al., 2009). Hirata et al. (2009) studied the detergency properties of SLs and found that they have low foaming but high detergency activity with low toxicity. SLs have various applications in different cosmetic formulations. The French company Soliance (http://www.groupesoliance.com) produces SL based cosmetics for body and skin. In addition to its role as emulsifier, SLs acts as a bacteriocidal agent in the treatment of acne, dandruff, and body odors (Mager et al., 1987). SLs are a source of difficult to synthesize ω and ω-1 hydroxy fatty acids which have application in the perfume and fragrance industry (Inoue and Miyamoto, 1980). Zerkowski and Solaiman (2006) produced fatty amines starting from SLs derived 17-hydroxy oleic acid and suggested that these fatty amines could be of interest in the preparation of highly functionalized polymers and surfactants.

2.5. Statistical design of experiments

Experiments are always considered a major tool for researchers to practically validate their theoretical hypotheses about scientific knowhow of a process or phenomenon, where the observation is expected to be correlated with some known associated process variable(s). In order to identify important factors and their contribution towards the observed response, simple traditional approach of using “one-variable-at-a-time” requires a large number of experiments to be performed which often fail to explain any significant interaction among the process variables (Ryan et al., 2007).

Statistically designed experiments are a powerful tool for improving the efficiency of experimentation and they allow us to gain knowledge about the system being studied with a minimum number of experiments. Inclusion of replicate test conditions allows the estimation of random, experimental variation. Statistical analysis of data generated from the experiment clearly establishes the relationship between the measured parameter of interest (response) and the process parameters (input factors or factors) being studied. The factors may have individual, simple effects on the response (referred to as main effects) or may have effects that are interdependent (referred to as interaction effects). Since the designed experiments are generated on the basis of statistical theory, confidence in the results obtained and conclusions drawn are clearly defined (Montgomery, 1997; Altekar et al., 2006).

Different types of designs are available; their choice is determined by the objectives of the experiment and the current state of knowledge about the experimental environment. They can be categorized as follows:

- Plackett-Burman

- Taguchi method
- Fractional and full factorial
- Response surface
- Box–Behnken design

Because explaining all the above techniques is beyond the scope of this thesis, a description of Taguchi method employed in the present work is only provided.

Taguchi optimization methodology

This statistical technique was developed by Dr. Genichi Taguchi and it has proved advantageous in both screening and optimizing process conditions. Taguchi method has been used widely in industrial process design, principally in developmental trials. This technique is used to generate enough process information to establish the screening and optimal conditions of parameters to improve process performance using a minimum number of experiments possible (Montgomery, 1997; Taguchi et al., 2000). The basic principle of this method serves as screening filters to examine the effects of many process variables and identify those factors which have major effects on the process using a single trial with a few experiments in which the factors are arranged into an orthogonal array (Dasu et al., 2003). The properties of an orthogonal array are such that between each pair of columns each combination of levels of variables appears an equal number of times. Due to orthogonality of the layout, the effects of the other factors remain balanced and yield a relative value representing the effects of a level compared with the other levels of a given factor. Orthogonal array (OA) experiments minimize the number of test runs while keeping the pair wise balancing property (Byrne and Taguchi, 1987) thus helping in considerable saving of time and cost besides being simple and efficient in approach (Taguchi, 1993; Mohan et al., 2007). The symbolic designation of these arrays indicates the main information on the size of the experimentation, e.g. L18 has 18 trials. The Taguchi methodology has become standard operating procedure in many businesses and it has been successfully applied in the communication and automobile industries (Montgomery, 1997; Taguchi et al., 2000). A few reports are available on the application of Taguchi's method in the field of biotechnology. For instance, Dasu et al. (2003) applied this technique to evaluate the significant parameters for improved production of griseofulvin by *Penicillium griseofulvum* in a batch bioreactor. Chang et al. (2006) optimized media composition for submerged culture of *Ganoderma lucidum* that has been used as a health food and medicine. When the strain was cultivated in the optimal medium under the optimal operating conditions, the mycelium formation was markedly improved from 1.70 to 18.70 g/l. Kammoun et al. (2008) used this technique to optimize nutrient concentration for α-amylase production by *Aspergillus oryzae*. They observed 72.7% improvement in the α-amylase yield by *A. oryzae* under optimized nutrients concentration. Shaligram et al. (2008) optimized the supplement concentrations for production of compactin by *P. brevicompactum* using L25 OA. Lakshmi et al. (2009) studied xylanase production by a newly isolated *Aspergillus terreus* MTCC 8661 using palm fiber in solid state fermentation. Following optimization of the levels of the different parameters, such as incubation temperature, moisture content, medium pH, particle size, incubation time, inoculum size, xylose and sodium nitrate concentrations using the Taguchi methodology, the authors reported 227% improvement in xylanase production. Adnani et al. (2010) optimized lipase-catalyzed synthesis of xylitol ester by Taguchi method and reported 96.10% yield of xylitol ester under optimum conditions.

CHAPTER 3

MATERIALS AND METHODS

3.1. Chemicals and Reagents

Whey protein concentrate was a kind gift from Dynamix Dairy Industries Ltd. India. Sugarcane molasses, milk powder (Amulya, Amul, India), fats (Ghee, Britania, India), soybean oil, sunflower oil, olive oil and kerosene oil, used in the study, were purchased from local market in Guwahati, India. All other chemicals and solvents used in the study were of analytical grade and supplied by either Hi-Media Pvt. Ltd., India or Merck India Ltd.

3.2. Microorganism and its Maintenance

Starmerella bombicola NRRL Y-17069, an equivalent strain of *C. bombicola* ATCC 22214, used in the study was procured from Agricultural Research Service (ARS-Culture collection), United States Department of Agriculture (USDA), Peoria, USA. As per the supplier's instructions, the strain was grown for 48 h at 30°C on agar slants (GYP-agar) containing (g/l): glucose, 10; yeast extract, 3; peptone, 5 and agar, 20. The yeast was enriched in every two weeks and maintained at 4°C in a refrigerator.

3.3. Seed Culture Preparation

The medium used for developing seed culture of the organism contained (g/l): glucose, 100; yeast extract, 10; urea, 1, pH 6.0 (Asmer et al., 1988). 250 ml Erlenmeyer flasks containing 50 ml of seed culture media were autoclaved at 121°C for 20 min and inoculated with a loop full of the microorganism freshly grown on GYP-agar slant. The flasks were then incubated for 48 h at 30°C and 180 rpm in a rotating orbital incubator shaker.

3.4. SLs Production using the Yeast *C. bombicola* NRRL Y – 17069

3.4.1. Production using deproteinized whey

3.4.1.1. Preparation of deproteinized whey

Deproteinization of whey protein concentrate was carried out following the method reported by Roukas and Kotzekidou (1998). In brief, powdered cheese whey was dissolved in distilled water (approx 200 g/l) and heated at 100°C for 20 min. Precipitated proteins were then removed by centrifugation at 4,000 × g for 15 min. The clear supernatant with known lactose concentration was suitably diluted with distilled water and subsequently used in the study with or without adding other medium constituents.

3.4.1.2. Batch shake flasks experiments

Experiments on SLs production by the yeast and its growth using production medium based on deproteinized whey were carried out in 250 ml Erlenmeyer flasks containing 50 ml of the media. The production medium contained (g/l): deproteinized whey, 100; soybean oil, 100; yeast extract, 10; urea, 1; pH 6.0. The flasks were then inoculated with 5 % (v/v) of seed culture mentioned above and incubated at 30°C and 180 rpm in a rotating orbital incubator shaker.

3.4.1.3. Optimization of medium constituents – one variable at a time

For optimum SLs production by *C. bombicola*, the media based on deproteinized whey was optimized as follows. Initial experiments were carried out to study the effect of deproteinized whey on SLs production and yeast growth, in which its concentration was varied from 25 to 125 g/l. The other medium constituents yeast extract, urea and soybean oil were kept constant at 10 g/l, 1 g/l and 100 g/l, respectively. To optimize the total hydrophilic carbon source (glucose and deproteinized whey), while glucose concentration was varied from 10 to 50 g/l, deproteinized whey concentration was varied in the range 50 – 90 g/l so that the total concentration of the hydrophilic carbon source in the medium was maintained at 100 g/l; concentration of the other medium constituents (yeast extract and oleic acid) were kept constant at 10 g/l and 100 g/l, respectively. The optimum yeast extract concentration was investigated by varying its concentration from 2 to 10 g/l by keeping glucose, deproteinized whey and oleic acid at 10 g/l, 90 g/l and 100 g/l, respectively. Similar protocol was followed for oleic acid by varying its concentration from 25 to 200 g/l in the media by keeping the respective concentrations of glucose, deproteinized whey and yeast extract constant at 10 g/l, 90 g/l and 2 g/l.

Finally the effects of vegetable oils (other than the hydrophobic carbon source, soybean oil), namely sunflower and olive oil in place of oleic acid, were studied on SLs production and yeast growth by fixing the medium composition at glucose (10 g/l), deproteinized whey (90 g/l), yeast extract (2 g/l) and vegetable oil (either sunflower or olive oil) (100 g/l). Using an initial pH of 6.0 in all the production media, experiments were carried out in triplicate in 250 ml Erlenmeyer flasks containing 50 ml of the production media. The flasks were incubated at 30°C and 200 rpm in a rotating orbital incubator shaker following inoculation with 5% (v/v) of the seed culture mentioned earlier.

3.4.1.4. Bioreactor experiments

Bioreactor experiments for SLs production was carried out in a 3L fermentor (Biotron, Spectrochem, India) with 1 L working volume under batch and fed-batch modes of operation. The production medium contained (g/l): deproteinized whey, 90; glucose, 10; yeast extract, 2; oleic acid, 100; initial pH 6.0. For operating the fermentor under batch mode, the medium was inoculated with 5% (v/v) seed culture, and the experiment was carried out for eight days at a constant controlled temperature of 30°C; however, the pH was not controlled during the experiment. Agitation and aeration rates were set at 350 rpm and 2 l/min, respectively. Samples were taken periodically for analyzing the concentrations of the yeast biomass and SLs. A second set of batch experiment was carried out using the same fermentor, but under controlled pH condition of 3.5 after allowing an initial drop in the pH from 6.0, as reported by Hu and Ju (2001a).

To study the SLs production under fed-batch mode of operation, the fermentor was initially operated under batch at controlled pH condition with 20 g/l of initial oleic acid concentration in the medium. Oleic acid was then added by means of a peristaltic pump to give a concentration of 40, 40 and 20 g/l in the medium at 36, 96 and 144 h, respectively, in the experiment.

3.4.2. Production using sugarcane molasses

3.4.2.1. Preparation of sugarcane molasses

Sugarcane molasses in the study was obtained as a co-product from a sugar production industry and contained approximately 20% water, 62% sugar, 10% non-sugar and 8% inorganic salts as ash contents (Prescott and Dunn's, 1987). Of the total sugar content, 35% w/v of sugarcane molasses contained sucrose as the major sugar with remaining being composed of glucose and fructose. A blackish homogenous liquid with high viscosity, it also contained mineral and ions such as Mg, Mn, Al, Fe and Zn in variable ratios (Prescott and Dunn's, 1987). For using sugarcane molasses as a hydrophilic carbon source for SLs production in the study it was initially diluted to three times with distilled water to reduce its high viscosity. The resulting mixture was centrifuged at 2000 rpm for 10 min at 25°C to remove any non-volatile suspended solids, and the total carbohydrate concentration in the supernatant was analyzed. Further, the supernatant was suitably diluted with distilled water as required in the experiments.

3.4.2.2. Batch shake flasks and bioreactor experiments

Preliminary investigations on SLs production by *C. bombicola* using sugarcane molasses were carried out in 250 ml Erlenmeyer flasks containing 50 ml of medium containing (g/l): sugarcane molasses, 100; soybean oil, 100; yeast extract, 10; urea, 1; pH 6.0. The medium was inoculated with 5 % (v/v) of seed culture and incubated at 30°C and 180 rpm in a rotating orbital incubator shaker. Medium containing only sugarcane molasses and any of the hydrophobic carbon sources, such as soybean oil, sunflower oil and olive oil was also investigated for SLs production. For comparison, experiments on SLs production by the yeast using glucose as the carbon source was also carried out in batch shake flasks. The composition of the glucose containing medium in these experiments was (g/l): glucose, 100; soybean oil, 100; yeast extract, 10; urea, 1; pH 6.0.

To study the potential of sugarcane molasses as a low cost substrate for SLs production, further experiments were carried out in a 5L fermentor (Biostat B, Sartorius, Germany) with 2L working volume. The medium was inoculated with 5 % (v/v) of seed culture, and the fermentation carried out for 5 days under batch operated condition at a constant controlled temperature of 30°C; however, the pH was not controlled during the fermentation. Agitation and aeration were set at 700 rpm and 3 L min^{-1}, respectively. Samples were taken periodically for analyzing the concentrations of biomass, residual sugarcane molasses, oil and SLs.

3.4.2.3. Screening and optimization of process parameters and medium constituents

A well known experimental design technique, namely L18 orthogonal array (OA) design (Saudagar and Singhal, 2007; Hao et al., 2009) was employed to study the effect of six parameters, viz inoculum size, sugarcane molasses concentration, soybean oil concentration, temperature, inoculum age and agitation on SLs production using the medium based on sugarcane molasses. Essentially, the design consisted of a total of 18 experiments with the six parameters - one parameter at two levels and the other parameters at three levels, i.e. L18 ($2^1 \times 3^5$). Table 3.1 presents the process parameters and their levels assigned in L18 ($2^1 \times 3^5$) OA and Table 3.2 presents the experimental combinations adopted as per the design.

Table 3.1: Process parameters and their levels assigned in L18 ($2^1 \times 3^5$) OA.

Factor		**Levels**		
		1	**2**	**3**
A	Inoculum size (%)	5.0	10.0	-
B	Sugarcane molasses (g/l)	50	100	150
C	Soybean oil (g/l)	50	100	150
D	Temperature (ºC)	20	25	30
E	Inoculum age (d)	2	3	4
F	Agitation (rpm)	150	200	250

Table 3.2: L18 (2^1 x 3^5) OA experimental design matrix showing parameters and their levels used in the screening and optimization study on SLs production using media based on sugarcane molasses.

Experimental run no.	**Parameters**					
	Inoculum size (%)	**Sugarcane molasses (g/l)**	**Soybean oil (g/l)**	**Temperature (ºC)**	**Inoculum age (d)**	**Agitation (rpm)**
1	5 (1)	50 (1)	50 (1)	20 (1)	2 (1)	150 (1)
2	5 (1)	50 (1)	100 (2)	25 (2)	3 (2)	200 (2)
3	5 (1)	50 (1)	150 (3)	30 (3)	4 (3)	250 (3)
4	5 (1)	100 (2)	50 (1)	20 (1)	3 (2)	200 (2)
5	5 (1)	100 (2)	100 (2)	25 (2)	4 (3)	250 (3)
6	5 (1)	100 (2)	150 (3)	30 (3)	2 (1)	150 (1)
7	5 (1)	150 (3)	50 (1)	25 (2)	2 (1)	250 (3)
8	5 (1)	150 (3)	100 (2)	30 (3)	3 (2)	150 (1)
9	5 (1)	150 (3)	150 (3)	20 (1)	4 (3)	200 (2)
10	10 (2)	50 (1)	50 (1)	30 (3)	4 (3)	200 (2)
11	10 (2)	50 (1)	100 (2)	20 (1)	2 (1)	250 (3)
12	10 (2)	50 (1)	150 (3)	25 (2)	3 (2)	150 (1)
13	10 (2)	100 (2)	50 (1)	25 (2)	4 (3)	150 (1)
14	10 (2)	100 (2)	100 (2)	30 (3)	2 (1)	200 (2)
15	10 (2)	100 (2)	150 (3)	20 (1)	3 (2)	250 (3)
16	10 (2)	150 (3)	50 (1)	30 (3)	3 (2)	250 (3)
17	10 (2)	150 (3)	100 (2)	20 (1)	4 (3)	150 (1)
18	10 (2)	150 (3)	150 (3)	30 (3)	2 (1)	200 (2)

All experiments in this study were performed in 250 ml Erlenmeyer flasks by varying the medium composition and culture conditions according to the experimental plan (Table 3.2). The flasks were agitated in an incubator shaker for 8 d. For varying the age of seed culture/inoculum in the experiments, yeast biomass taken in three flasks each containing 50 ml of the seed culture medium was cultured at 30ºC and 180 rpm in a rotating orbital incubator shaker for different times as per the experimental design in Table 3.2. Samples were taken at regular time intervals during the experiments for analyzing the concentrations of biomass, residual sugarcane molasses, oil and SLs in the media. From each experimental run, response was recorded as SLs production, and corresponding signal-to-noise (S/N) ratio was calculated using Equation (3.1) with an overall objective of estimating the effects of various parameters on SLs production, where a large S/N ratio was preferred.

$$\frac{S}{N} = -10 \times \log\left(\frac{1/Y^2}{n}\right) \tag{3.1}$$

where Y is the response and n is the number of experimental runs.

The results obtained from the Taguchi design of experiments were fitted into a first order polynomial of type shown in Equation (3.2):

$$Y = b_0 + b_1 A + b_2 B + b_3 C + b_4 D + b_5 E + b_6 F + (\varepsilon) \tag{3.2}$$

where b_1, b_2, b_3, b_4, and b_5 are estimates of the process parameters, b_0 is constant term, ε is error, A, B, C, D, E, F represents the six parameters and Y is the response (SLs production).

Statistical analysis of the results in the form of analysis of variance (ANOVA) was performed using the statistical software package MINITAB® Release 15.1, PA, USA.

At the optimum values of the parameters obtained from the above study, SLs production was investigated in a 250 ml Erlenmeyer flask with 50 ml working volume and later verified in a batch operated 3 L fermentor (with or without pH control) (Applikon, Holland) of 1 L working volume. Fermentor experiments were carried out as mentioned earlier for SLs production using deproteinized whey.

3.4.2.4. Estimation of kinetic parameters involved in SLs production

Experimental data obtained from the batch shake flasks and batch fermentor (with and without pH) carried out under optimized medium based on sugarcane molasses was used for estimating the kinetic parameters involved in SLs production. Table 3.3 presents the kinetic models applied in this study along with the estimable kinetic parameters from these models. These models are essentially unstructured logistic models originally proposed by Mercier et al. (1992) for describing the kinetics of biomass growth, substrate consumption and product accumulation. For fitting the models to the experimental data, nonlinear regression using the least-squares method was used employing Matlab® 7 and Microsoft Excel Solver 2003. The method followed for the estimation of kinetic parameters from the equations mentioned in Table 3.3 is described in Appendix A1.

Table 3.3: Models applied for the estimation of biokinetic constants involved in the SLs production, yeast biomass growth and substrate utilization by the yeast.

	Kinetic model equation[*]		**Estimable kinetic parameters**[*]
SLs production	$P = \dfrac{P_0 P_{max} e^{P_r t}}{P_{max} - P_0 + P_0 e^{P_r t}}$	(3.3)	P_r, P_0, P_{max}
Yeast biomass growth	$X = \dfrac{X_0 X_{max} e^{\mu t}}{X_{max} - X_0 + X_0 e^{\mu t}}$	(3.4)	μ, X_0, X_{max}
Sugarcane molasses utilization	$(S_{M0} - S_M) = \dfrac{1}{Y_{P/M}}(P - P_0) + \dfrac{1}{Y_{X/M}}(X - X_0)$	(3.5)	S_{M0}, $Y_{P/M}$, $Y_{X/M}$,
Soybean oil utilization	$(S_{S0} - S_S) = \dfrac{1}{Y_{P/S}}(P - P_0) + \dfrac{1}{Y_{X/S}}(X - X_0)$	(3.6)	S_{S0}, $Y_{P/S}$, $Y_{X/S}$

[*] t = time (h), P = SLs concentration (g/l), P_{max} = maximum concentration of SLs (g/l), P_r = ratio between the initial volumetric rate of product formation and the initial product concentration P_0 (g/l), X = yeast biomass concentration (g/l), X_{max} = maximum concentration of yeast biomass (g/l), μ (h^{-1}) = ratio between the initial volumetric rate of biomass formation and the initial biomass concentration X_0 (g/l), $Y_{P/M}$ = SLs yield due to molasses (g/g), $Y_{X/M}$ = biomass yield due to molasses (g/g), S_M = molasses concentration (g/l), S_{M0} = initial molasses concentration (g/l), $Y_{P/S}$ = SLs yield due to soybean oil (g/g), $Y_{X/S}$ = biomass yield due to soybean oil (g/g), S_S = soybean oil concentration (g/l) and S_{S0} = initial soybean oil concentration (g/l).

3.5. Properties of SLs Produced by the Yeast *C. bombicola*

The produced SLs in the study were characterized in terms of its surface/interfacial tension reduction, CMC, emulsification activity and stability. All the experiments for characterizing SLs were carried out in triplicate and results reported are average with standard deviation. Experiments were also carried out to investigate fats and oils solubilization by SLs.

3.5.1. Minimum surface tension, CMC and interfacial tension determination

Minimum surface tension, critical micelle concentration (CMC) and interfacial tension of SLs mixture produced in the study were estimated using a surface tensiometer (DCAT 11, Dataphysics Instruments, Germany) equipped with a liquid-dosing unit (LDU, Dataphysics Instruments, Germany) by either Wilhelmy plate method or Du Nuoy ring method. Wilhelmy plate used was of length 10 mm, width 19.9 mm and thickness 0.2 mm. A platinum ring of diameter 18.7 mm was used for the Du Nuoy ring method. A stock solution (200 mg/l) containing partially purified SLs was prepared using MilliQ water and its surface tension measured at 25°C. CMC value and minimum surface tension were calculated from the relationship between SLs concentration and corresponding surface tension. Interfacial tension measurements were carried out against n-hexane, sunflower oil and olive oil. All measurements of surface/interfacial tensions were performed in triplicate and results reported are the average of triplicate analysis.

3.5.2. Effect of environmental parameters on surface tension reduction

The effect of environmental parameters such as salt concentration, pH and heat on surface activity of the SLs produced by *C. bombicola* was determined by varying the levels of the individual parameters one-at-a-time by keeping the other parameters at a fixed level. To study the effect of salt concentration, NaCl at different concentrations in the range 0 - 20% (w/v) were mixed with aqueous solutions of SLs (200 mg/l of SLs in

MilliQ water) and surface tension measured. Similarly, to study the effect of pH, aqueous solution containing the SLs was adjusted to desired values of pH using either 0.1 N NaOH or 0.1 N HCl and surface tension measured. For observing the heat stability of the biosurfactant, aqueous solutions of the SLs (200 mg/l of SLs in MilliQ water) were heated to 100ºC in boiling water bath for different time intervals ranging from 0 to 120 min and then allowed to cool to room temperature before measurement of surface tension.

3.5.3. Solubilization of fat and oil

Solubilization of fat and oil using the produced SLs were carried out by the method previously reported by Urum and Pekdemir (2004). Briefly, saturated fat and oil solutions were prepared by mixing 20 ml of fat (ghee) and oil (soybean oil) in 100 ml of biosurfactant solutions (27 to 100 mg/l) taken in 250 ml Erlenmeyer flasks. The contents in the flasks were then shaken gently (to avoid formation of emulsions) at 80 rpm in an orbital incubator shaker set at room temperature 25ºC. The contents of the flasks were then taken in a 250 ml separating funnel and allowed to settle for 48 h, which resulted in two distinct phases: upper oil or fat rich phase and lower biosurfactant rich phase. 40 ml of the surfactant rich phase was collected and its oil/fat extracted using n-hexane. The concentration of fat/oil following the hexane extraction was obtained gravimetrically and its solubilization ratio (SR) determined using the following relationship:

$$\mathrm{SR} = \frac{S_C - S_{C,\,CMC}}{C_S - C_{S,\,CMC}} \tag{3.7}$$

where S_C is the crude oil solubility at any surfactant solution concentration (C_S) and $S_{C,CMC}$ is the crude oil solubility in surfactant solution at its CMC ($C_{S,CMC}$).

3.5.4. Emulsification activity and stability

Emulsification activities and stabilities due to the produced SLs towards different non-aqueous phase liquids were measured using the method reported by Cirigliano and Carman (1984). According to this method 1 ml sample containing the SL at 0.5 mg/ml was mixed with 1 ml of non-aqueous phase liquid (NAPL), which was either an oil (kerosene or soybean oil) or an organic solvent like benzene or 1-hexadecene. Thereafter, the mixture was shaken vigorously in a vortex mixer for 2 min and was allowed to sit for 10 min before measuring its absorbance at 600 nm by photo-emission diode spectrometer (SPEKOL 1200, Analytikjena®). Emulsification activity was therefore expressed as the absorbance of the mixture at 600 nm (A_{600}), and one unit of emulsification activity of the biosurfactant was defined as the amount that resulted in an emulsion with OD_{600} of 1.0. Stability of the emulsions formed was also analyzed by allowing the emulsions to stand for about 30 min at room temperature and measuring its absorbance at 600 nm every 5 or 10 min for about 1 h. Stability of the emulsions was thus expressed as the decay constant (k_d) obtained using the following equation (Kim et al., 2000):

$$\log A_{600} = -k_d \times t \tag{3.8}$$

where, ‘A’ is absorbance (OD_{600}) of the emulsion at time ‘t’.

3.5.5. Effect of pH and temperature on emulsification activity and stability by the SLs

For testing the influence of pH on emulsification activity and stability due to the SLs produced, 1 ml SLs solutions (0.5 mg/ml) were prepared in buffer solutions of desired pH values in the range of 4.0 – 8.0 and incubated for 1 h at 30 °C. Soybean oil (0.5 mL) was added into each of these surfactant solutions and vortexed for 2 min; the resulting mixtures were then tested for emulsification activity and stability as described previously. Similarly, for observing the effect of temperature, 1 ml SLs solutions (0.5 mg/ml) were incubated for 30 min at various temperatures in the range 20 - 100 ºC and emulsification activity and stability was than investigated using soybean oil as the substrate.

3.6. Purification and Structural Characterization of SLs Produced by *C. bombicola*

3.6.1. Purification of SLs produced by the yeast using medium based on deproteinized whey by silica gel column chromatography

SLs from the yeast fermented broth were first extracted with ethyl acetate and isopropanol (8: 2) and solvent layer containing the SLs was then separated from the broth. Following evaporation of the solvent, SLs residue was washed with hexane to obtain partially purified SLs. Further purification was carried out by silica gel column chromatography using a glass column packed with slurry containing silica gel (200 – 300 mesh size, Merck India Ltd.) in chloroform (HPLC grade, Merck). For, chromatography the column was loaded with the partially purified SLs dissolved in ethyl acetate (1-2 ml), and its elution was carried out with $CHCl_3$: methanol using gradient system (0 to 20% methanol). Fractions obtained from the column were analyzed by thin layer chromatography (TLC) (silica gel 60 F254, Merck Co., 20×20 cm^2 aluminum sheets) with $CHCl_3$: methanol (9 : 1) as the mobile solvent. Fractions containing identical SL were pooled and the solvent removed by evaporation for further structural characterization.

3.6.2. Structural characterization

The purified SL product using silica gel column chromatography was initially identified and characterized by Fourier transform infra red (FTIR) spectrophotometer (Perkin-Elmer Spectrum-One spectrophotometer, USA) using the KBr pellet method (Silverstaein and Webster, 1998). This method involves forming a small disc of KBr with SL sample incorporated into the disc which was then placed in a spectrophotometer. Further structural characterization was carried out by proton nuclear magnetic resonance (^{1}HNMR) spectroscopy using Mercury Plus 400 NMR Spectrometer, Varian, USA and mass spectroscopy (MS) (Q-TOF Premier^{TM}, Waters, USA). For ^{1}HNMR sample was dissolved in $CDCl_3$ (Deuterated chloroform, spectroscopic grade, Sigma chemicals, India) and an NMR spectrum was recorded. Tetramethyl silane (TMS) was used as an internal standard. For MS analysis, electrospray ionization ESI probe connected to a Micromass ZMD served as the negative ion mode. Similarly, characterization of partially purified SLs from the yeast grown on sugarcane molasses and soybean oil was carried out using FTIR, ^{1}HNMR, ^{13}CNMR and MS. The FTIR and ^{1}HNMR analysis were carried out as mentioned above. However, for MS of the partially purified sample, ESI probe connected to a Micromass ZMD served as the negative ion mode. For ^{13}CNMR, sample was dissolved in $CDCl_3$ and an NMR spectrum was recorded using TMS as an internal standard.

3.7. Feasibility Study Using Synthetic Dairy Wastewater for SLs Production and Pretreatment by the Yeast *C. bombicola*

3.7.1. Preparation of synthetic dairy wastewater

Synthetic dairy wastewater, based on dairy wastewater composition, was prepared in the laboratory as reported by Leal et al (2006) and contained dried milk powder (2 g/l) and fat (0.2 g/l). The major constituents of the commercially available milk powder (Amulya^{TM}, Amul, India) and fats (Ghee, Britania, India) used to prepare the synthetic dairy wastewater are mentioned in Tables 3.4 and 3.5, respectively. The synthetic wastewater had an initial pH 6.0 and COD content of 2800 mg/l.

Table 3.4: Composition of the commercially available milk powder* used in the preparation of synthetic dairy wastewater in the study.

Constituents	Amount per 100 gm
Total fat	20 g
Saturated fat	12.4 g
Cholesterol	52 mg
Sodium	88 mg
Total carbohydrates	50 g
Sucrose	18 g
Protein	20 g
Calcium	1 g

* Brand name: Amulya^{TM}; manufacturer: Amul, India

Table 3.5: Composition of the commercially available fat[*] used in the preparation of synthetic dairy wastewater in the study.

Constituents	**Amount per 100 gm**
Milk fat	99.7 g
Saturated fatty acids	63.0 g
Poly unsaturated fatty acids	1.7 g
Mono unsaturated fatty acids	24.5 g
Trans fatty acids	3.0 g
Cholesterol	0.4 g
Vitamin A	700 µg

[*] Manufacturer: Britania, India; local name: ghee

3.7.2. SLs production using synthetic dairy wastewater in batch shake flasks

Initially the synthetic dairy wastewater was tested for SLs production in batch shake flasks each containing 50 ml of synthetic dairy wastewater with or without other added nutrients. The flasks were inoculated with 5% of seed culture (prepared as mentioned in section 3.3) and incubated for 8 days in a rotating orbital incubator shaker at 30 ºC and 200 rpm. Table 3.6 shows the compositions of the different media based on synthetic dairy wastewater that were tested for SLs production in this study. Samples were collected at different time intervals and analyzed for SLs production, yeast biomass growth and substrate utilization.

Table 3.6: Composition of the various media based on synthetic dairy wastewater with or without any added nutrients.

Medium	Composition (g/l)						
	Dried milk powder	Fat	Sugarcane molasses	Soybean oil	Glucose	Yeast extract	Urea
A	2	0.2	-	-	-	-	-
B	2	0.2	50	-	-	-	-
C	2	0.2	50	50	-	-	-
D	2	0.2	-	100	100	10	1
E	5	1	-	-	-	-	-

3.7.3. Pretreatment of synthetic dairy wastewater in a laboratory scale bioreactor under batch, fed-batch and continuous modes of operation

All experiments to investigate the efficiency of the yeast in pretreating the synthetic dairy wastewater to remove fats were carried out in a 3 L fermentor (Applikon, Holland) with 1 L working volume. For inoculation 5% of the yeast seed culture was used. Based on the prior experiments, a small amount of sugarcane molasses (1%) and yeast extract (0.1%) was added to the synthetic wastewater to support the yeast growth. The agitation and aeration in the bioreactor were set at 300 rpm and 1.5 L/m, respectively. The initial pH of the synthetic wastewater was kept at 6.0 and was not controlled during the experiments. Initial experiment using the fermentor was carried out in batch mode and later on under fed-batch and continuous modes to compare the pretreatment efficiencies in the study. Pretreatment under batch mode was carried out for 5 days. Based on the kinetic parameters (specific growth rate and yeast biomass yield) obtained from the batch experiment, feed rate of the wastewater required for operating the reactor under fed-batch mode was calculated. In fed-batch experiments, the fermentor was run under batch mode with 1 L of wastewater for the first 48 h and later feeding of wastewater was started. After the final volume of the reactor reached 2 L, the reactor was run under batch mode for 62 h. For continuous experiments, the fermentor was initially run under batch mode up to 72 h and then shifted to continuous mode with a wastewater retention time (HRT) of 28.5 h which corresponded to a dilution rate of 0.035 h^{-1}. Samples taken during the experiments were analyzed for fats, yeast biomass, sophorolipids, total carbohydrate concentration and COD.

3.8. Characterization and Pretreatment of Real Dairy Wastewater by the Yeast *C. bombicola* in a Laboratory Scale Bioreactor under Batch, Fed-batch and Continuous Modes of Operation

3.8.1. Wastewater characterization

Dairy wastewater used in the study was collected from a local dairy in Guwahati, India, and was characterized in terms of its pH, total fat content, total solids, total suspended solids, total dissolved solids, total carbohydrate content, total BOD and total COD as per the standard methods for water and wastewater treatment (APHA, 1995).

3.8.2. Pretreatment of dairy wastewater

The suspended solids present in the wastewater were removed by centrifugation (1000 × g, for 10 min) and pH of the wastewater was adjusted before the experiments. Based on the results obtained from the previous experiments, real dairy industry wastewater was supplemented with sugarcane molasses (1%) and yeast extract (0.1%) to support the yeast growth. During the experiments pH of the wastewater was not controlled. The wastewater was pretreated mainly for the removal of fats and oils by the yeast. Experiments were carried out as before for synthetic wastewater using the fermentor operated under batch, fed-batch and continuous modes. The process conditions for batch and fed-batch modes were the same as previously mentioned in section 3.7.3 for pretreating synthetic dairy wastewater. However, for continuous experiment the wastewater retention time (HRT) was 40 h which corresponded to a dilution rate of 0.025 h^{-1}, and the reactor was run up to 9 days under continuous mode. Samples taken during the experiments were analyzed for fats and oils, yeast biomass, SLs, total carbohydrate concentration and COD.

3.9. Analytical Methods

3.9.1. Estimation of yeast biomass concentration and carbohydrate content

For yeast biomass measurement and estimation of sugars, samples were extracted twice with equal volume of ethyl acetate to remove any unutilized oil and SLs in the fermentation broth. Following separation of the two layers, the aqueous layer was centrifuged at 12,000 × *g* for 15 min at 25°C and the cell pellets washed twice with distilled water and dried to constant weight at 80°C for determining the yeast biomass concentration. The resulting supernatant was utilized for analyzing residual sugar concentration in the sample. Sugarcane molasses concentration or total carbohydrate concentration was analyzed by anthrone method (Scott and Melvin, 1953) using glucose as the standard. Glucose or lactose concentration in the sample, if any, was analyzed by

DNS (dinitro salicylic acid) method (Miller, 1959) using the respective as the standard, respectively.

For Anthrone method, 2.5 ml of anthrone reagent was mixed with 0.5 ml of a suitably diluted sample in test tubes and heated in a boiling water bath for 15 min. A suitable blank prepared by mixing 2.5 ml of Anthrone reagent with 0.5 ml of Milli Q water and heating in a boiling water bath for 15 min, was also used for the analysis. The intensity of the colour developed was measured at 620 nm by photo-emission diode spectrometer (SPEKOL 1200, Analytikjena®). A calibration curve prepared using known concentrations of glucose (0.01 to 0.2 g/l), as shown in Fig 3.1 was used for total carbohydrate concentration in the sample.

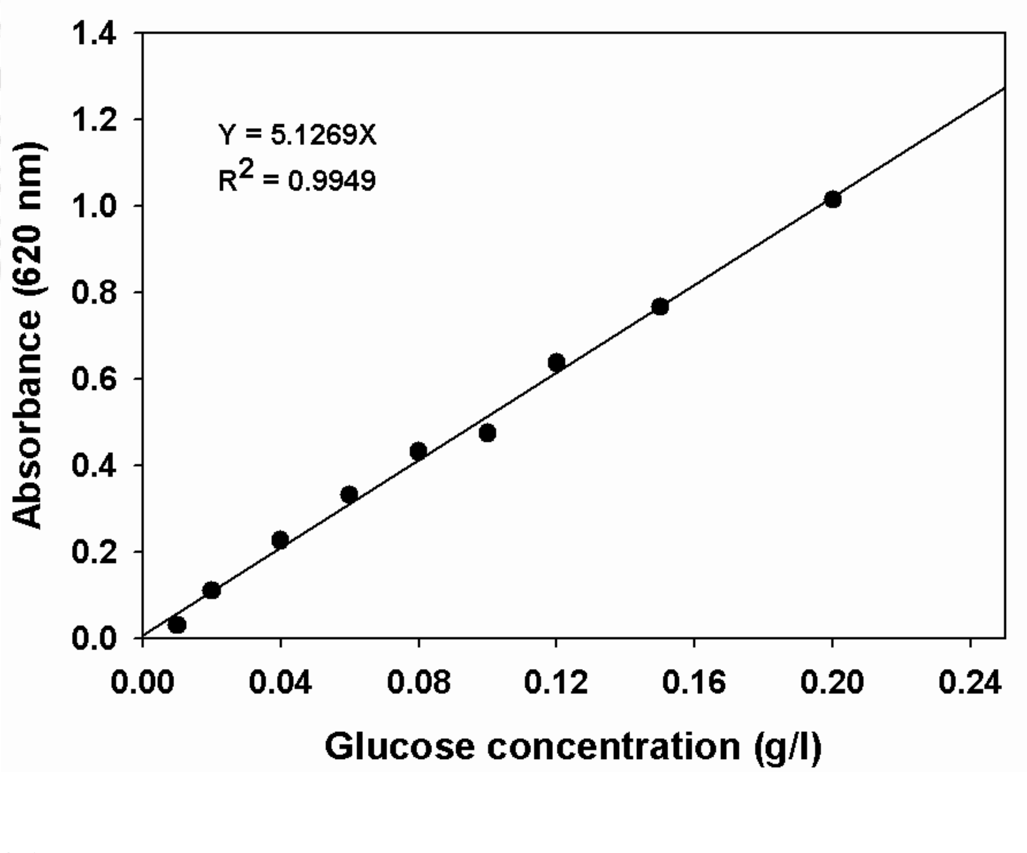


Fig. 3.1: Standard curve of glucose concentration vs. absorbance at 620 nm used in the estimation of total carbohydrate content by Anthrone method.

For DNS method, 1 ml of sample (suitably diluted) was mixed with 1 ml of DNS reagent and heated in a boiling water bath for 15 min. For blank preparation, 1 ml Milli Q water was taken in place of sample. The intensity of the colour developed was measured at 540 nm by photo-emission diode spectrometer. The calibration curves shown in Fig. 3.2 and Fig. 3.3 were used for estimating glucose or lactose concentrations, respectively, in the sample.

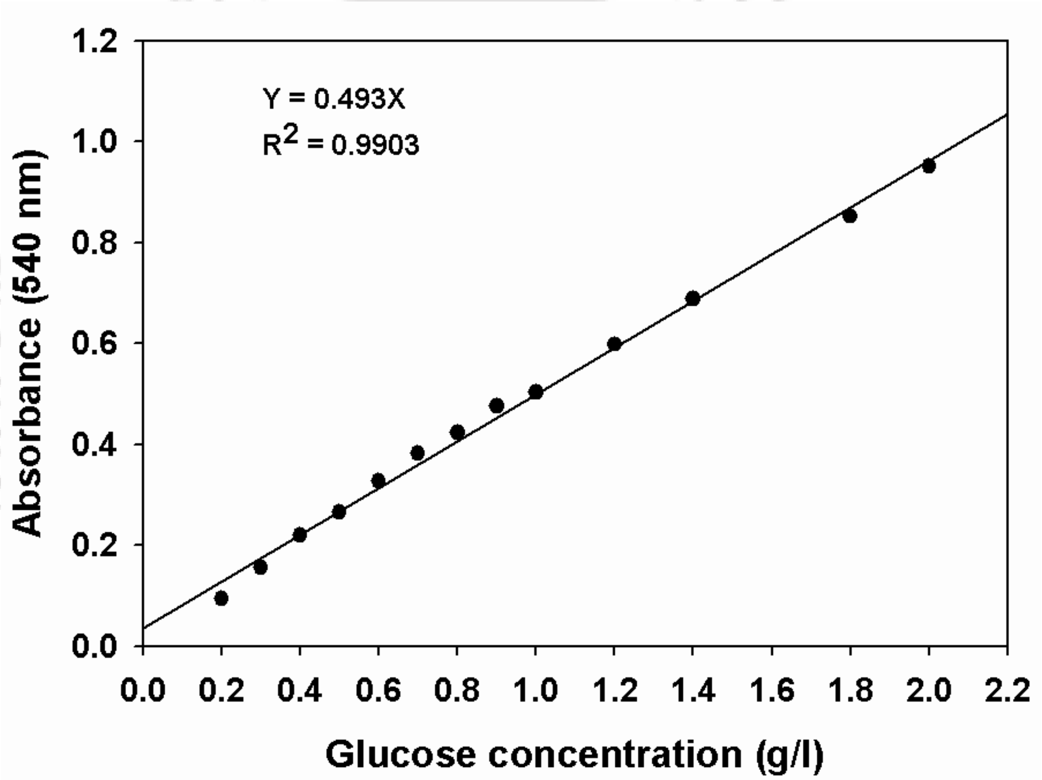


Fig. 3.2: Standard curve of glucose concentration vs. absorbance at 540 nm used in the estimation of glucose content by DNS method.

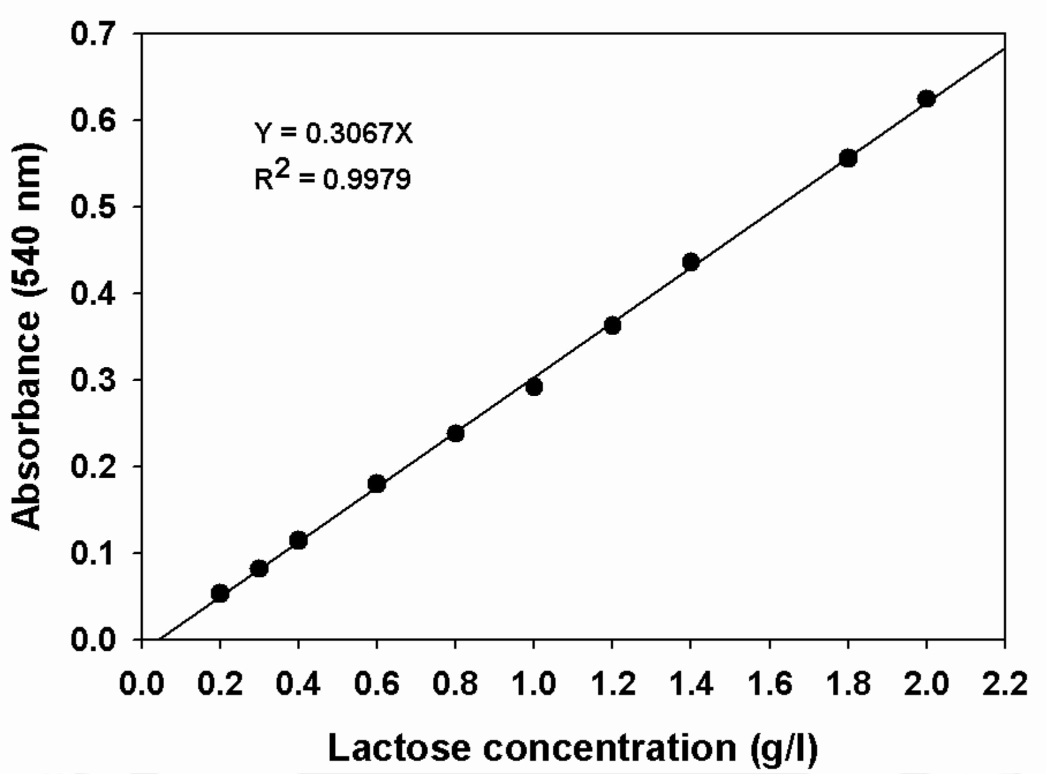


Fig. 3.3: Standard curve of lactose concentration vs. absorbance at 540 nm used in the estimation of lactose content by DNS.

3.9.2. Estimation of SLs, oleic acid and vegetable oils

For SL analysis, the previously obtained ethyl acetate extract (from section 3.9.1.) was vacuum-dried at 40°C to remove the solvent. The residue was twice washed with hexane to remove any remaining hydrophobic substances (Hu and Ju, 2001a). Partially purified SL were thus obtained after vaporizing the residual hexane at 40°C under vacuum and its yield calculated from gravimetric analysis of the compound. Fig. 3.4 is a flow chart showing steps followed in isolating the product from the yeast culture broth. Gravimetric analysis of the n-hexane extract obtained from the washing step in Fig. 3.4 was used in the evaluation of unutilized oleic acid or vegetable oil (soybean oil, sunflower oil and olive oil) in the samples (Hu and Ju, 2001a).

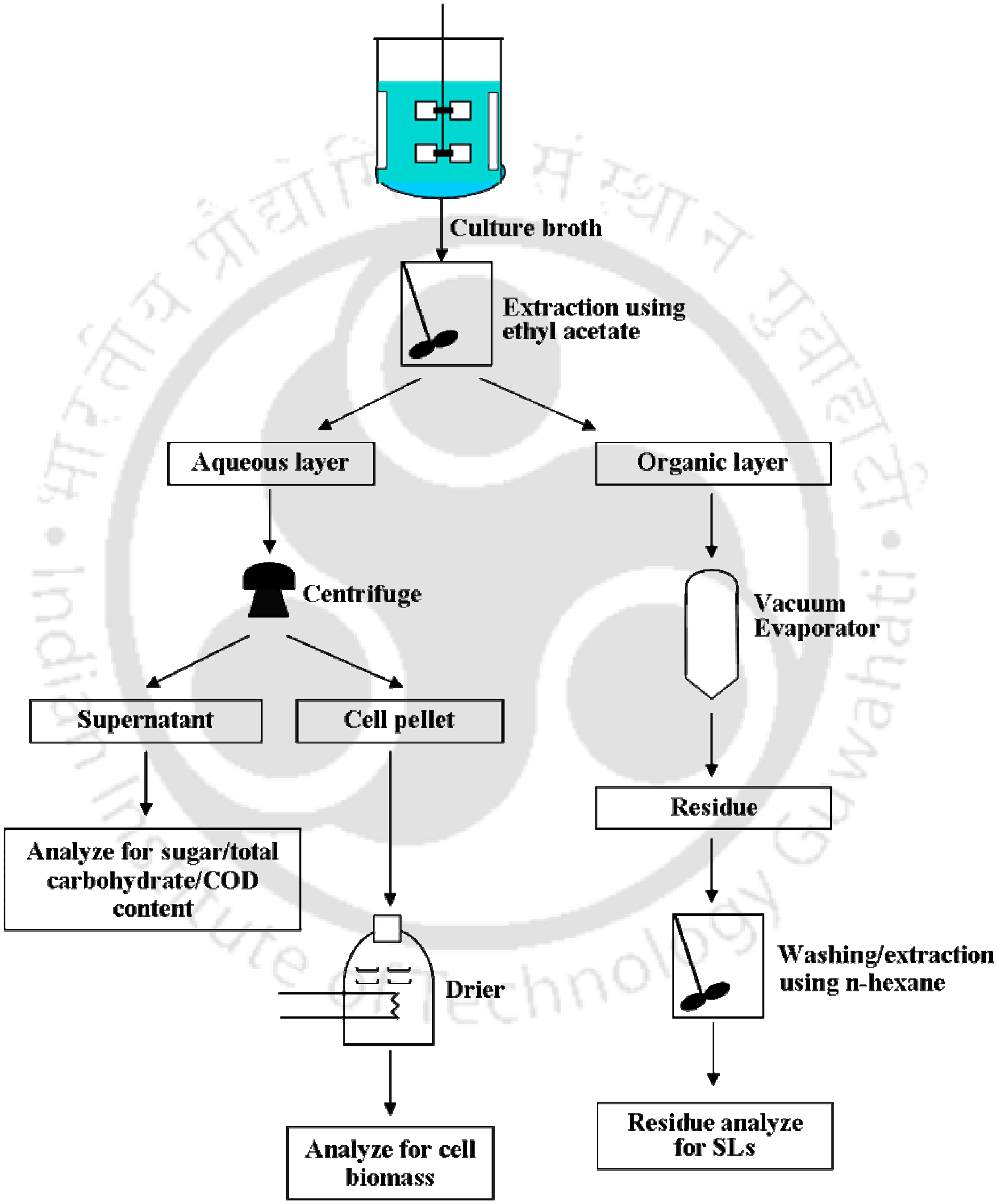


Fig. 3.4: Steps followed in isolation of SLs from the yeast culture broth.

3.9.3. Estimation of fats and oils

Fats and oils present in the wastewater were analyzed as per the standard methods for water and wastewater treatment (APHA, 1995). Briefly, samples collected at different time intervals during the experiments were extracted twice with n-hexane. The n-hexane layer containing fats and oils were separated and vacuum-dried at 40°C to remove the organic solvent. Remaining residue was analyzed for total fats by gravimetric method.

3.9.4. Estimation of suspended solids, dissolved solids and total solids

For suspended solids and dissolved solids analysis in the real dairy wastewater, a well mixed sample was taken and filtered through a preweighed filter paper into a preweighed petri plate. The filter paper was dried to a constant weight in an oven at 105^{o}C. The increase in the weight over that of the initial weight of filter paper represented the total suspended solids. The filtrate obtained was evaporated to a constant weight in an oven at 105^{o}C. The increase in weight over that of the initial weight gave the total dissolved solids content in the wastewater sample.

For total solids estimation, a well mixed sample was evaporated in a preweighed dish and dried to a constant weight in an oven at 105^{o}C. The increase in weight over that of the empty petri-plate represented the total solids content (APHA, 1995).

3.9.5. Estimation of COD

Samples obtained from the experiments were centrifuged at 8,000 rpm for 15 min to separate the yeast biomass and SLs. The supernatants were suitably diluted and COD analyses were carried out by closed reflux, titrimetric method as per the standard methods for water and wastewater treatment (APHA, 1995). In this method, 2.5 ml of a suitably diluted sample taken in COD vials was added with 1.5 ml of standard potassium dichromate digestion solution and 3.5 ml of sulphuric acid reagent, properly mixed and kept in a COD digester (Hach, USA) at 150°C for 2 hours. Later the solution mixture was cooled to room temperature and titrated with ferrous ammonium sulphate (FAS) solution using ferroin as an indicator. A sharp colour change from blue green to reddish brown was considered as the end point. MilliQ water without sample was taken as the blank in the COD analysis. COD in the sample was thus calculated using the following equation and was expressed as mg O_2/l.

$$COD = \frac{(A-B)\times M\times 8000}{ml\ sample} \tag{3.9}$$

where; A = ml of FAS consumed by blank, B = ml of FAS consumed by sample, M = molarity of FAS used, 8000 = milliequivalent weight of oxygen × 1000 ml/l.

3.9.6. Estimation of BOD

A standard BOD_5 method was used to analyze the BOD of dairy industry wastewater (APHA, 1995). According to this method, samples were suitably diluted with dilution water and filled in 300 ml BOD bottle. The BOD bottle was capped properly after measuring its dissolved oxygen (DO) using a LBOD meter (HQ40d meter with IntelliCAL LBOD 101 probe, Hach, USA) and incubated in a BOD incubator for five days at 20°C. After five days, DO was measured, and the 5 day BOD of the wastewater was calculated using the following equation:

$$BOD_5\,(mg/L) = \frac{(D_1 - D_2)}{P}\times df \tag{3.11}$$

where; D_1 = initial DO before incubation, D_2 = final DO after 5 day incubation, P = volume of sample and df = dilution factor.

All analysis of the samples was carried out in triplicate and results reported are average. Average of the above analytical methods was found to be ±3% standard deviation.

CHAPTER 4

RESULTS AND DISCUSSION

Dairy is one of the major contributors among the various food industries both in terms of value and effluent. The relatively high concentration of organic matter in wastewater from dairy industry deserves special mention and contributes to high BOD and COD in the wastewater. Effluent from dairy industry is not allowed to mix up with the municipal waste as it adds up the wastewater load and, therefore, the effluent is best treated prior to its discharge from the industry. Dairy wastewater treatment involves two major steps: pretreatment followed by secondary biological treatment which may be aerobic or anaerobic. Pretreatment of dairy wastewater, in general, includes equalization, neutralization and physical or chemical or physicochemical separation of fats and oils. But separation of fats and oils by these conventional pretreatment methods are costly and found to be less effective compared to biological pretreatment using microorganisms and/or their products such as hydrolytic enzymes (Masse et al., 2001; De Felice et al., 2004; Jeganathan et al., 2006). A novel biological pretreatment method involving *in situ* production of biosurfactant by the yeast *C. bombicola* mainly for the utilization of fats and oils in dairy wastewater by increasing its aqueous solubility is evaluated in this study.

This chapter discusses the results of SLs production by the yeast using deproteinized whey and sugarcane molasses as cheap raw materials. The chapter also discusses the purification, structural characterization and properties of SLs produced in the study. Based on these results, pretreatment of synthetic wastewater in a laboratory scale fermentor was investigated and reported. Finally, efficiency of the biosurfactant producing yeast in treating a real dairy wastewater is reported in this chapter.

4.1. SLs Production by the Yeast *C. bombicola* NRRL Y-17039

Production economy is the major bottleneck with most biotechnological processes including biosurfactant production for environmental applications. Often, the amount and type of a raw material can contribute considerably to the production cost. It is well known that the cost of raw materials contributes up to 75% of the selling price of bioproducts (Lynd et al., 1999). Thus, in the present study, to lower the process cost involved in the cultivating yeast and SLs production for removing high fats and oils from dairy wastewater it was decided to use low-cost fermentative raw materials. One possibility that was explored extensively was the use of low cost and agro-based raw materials as substrates. Therefore, to reduce the cost of SLs production by the yeast *C. bombicola* for its application in dairy wastewater treatment, two agro-industrial wastes namely whey and sugarcane molasses were tested and evaluated as low cost fermentative substrates.

4.1.1. Production using deproteinized whey

Disposal of cheese whey, a byproduct of cheese processing industry is a continuing and growing problem in the dairy industry, but can be deproteinized to obtain pharmaceutically important proteins such as lactalbumin and lactoferrin. Lactose can also be isolated by crystallization, which represents 70% of the whey solids (Daniel et al., 1999). Because approximately 50 g/l lactose remains in the already deproteinized and unconcentrated waste, the same was evaluated for the production of SLs by the yeast *C. bombicola*. The composition of whey protein concentrate, a kind gift from Dynamix Dairy, is mentioned in Table 4.1.

Table 4.1: Composition and characteristics of whey protein concentrate used for SLs production by *C. bombicola*.

Sl no.	**Testing parameter**	**Test Results***
A	**Organoleptic**	
A.1	Taste and flavour	Pleasant and clean
A.2	Colour	Creamy white
B	**Chemical**	
B.1	Moisture (%)	4.48
B.2	Fat (%)	3.37
B.3	Protein (%)	35.31
B.4	Ash (%)	8.11
B.5	Lactose (%)	48.73
B.6	pH (10 % solution)	6.81
B.7	Scorched particles per 25g	Disc A (7.5 mg)
B.8	Insolubility index (ml)	0.20
C	**Microbiological**	
C.1	Standard plate count per g	1000
C.2	Coliform per g	Absent
C.3	*E. coli* per g	Absent
C.4	Yeast and Molds per g	Absent
C.5	*Staphylococcus aureus* per g	Absent
C.6	*Salmonella* per g	Absent

* Data supplied by Dynamix Dairy Limited, India.

4.1.1.1 SLs production and yeast growth in batch shake flasks

Preliminary experiment was carried out in batch shake flasks to study the SLs production and growth of *C. bombicola* using deproteinized whey (100 g/l) as a hydrophilic carbon source and soybean oil (100 g/l) as a lipophilic carbon source. The profiles of SLs production and yeast biomass growth are shown in Fig. 4.1. The yeast growth started without lag phase and it reached stationary phase after 72 h and the maximum 20.81±1.04 g/l of yeast biomass was found at the end of batch culture.

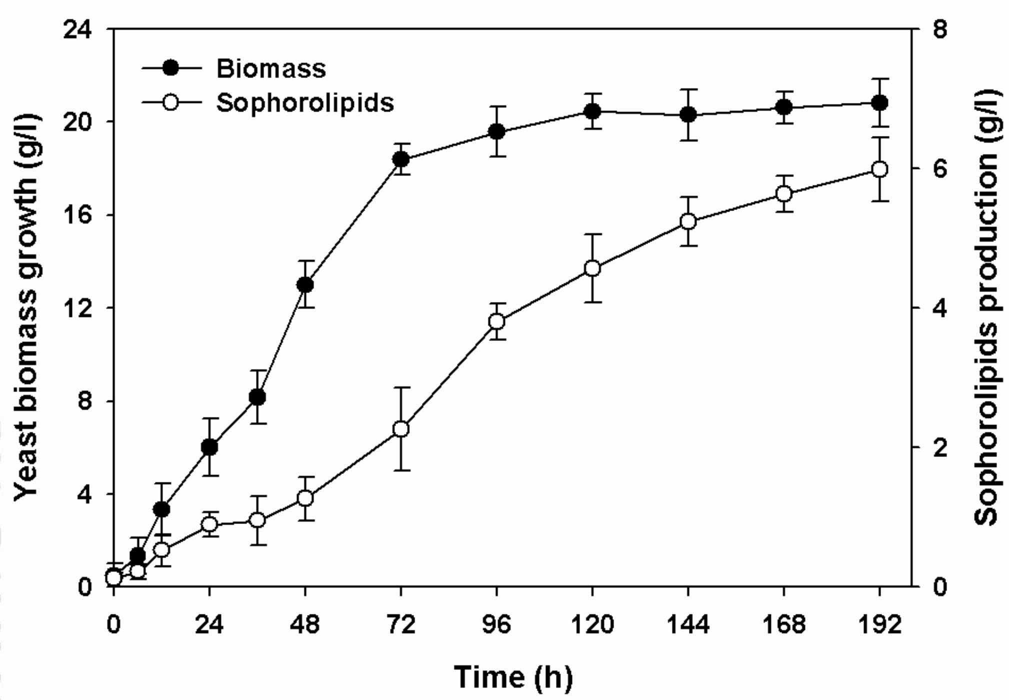


Fig. 4.1: Profiles of yeast biomass growth and SLs production using the medium based on deproteinized whey and soybean oil.

During the exponential phase the SLs production was found to be very less and its production switched on when the yeast reached late exponential phase (Fig. 4.1). The observation that the SLs production occurred during both the exponential and stationary growth phases of the yeast confirm that the biosurfactant production followed a mixed growth and non growth associated phenomenon. It was clear from Fig. 4.1 that at the end of the batch fermentation the yeast could able to produce only 5.98±0.45 g/l of SLs which is very less compared to the literature reported yield of SLs using animal fat (120 g/L), glucose (80 g/L) and soy molasses (53 g/L)

(Deshpande and Daniel, 1995; Kim et al., 1997; Solaiman et al., 2007). Inability of the yeast to utilize lactose present in the whey may be noted as a probable reason for the reduced SLs production in the present study (Daniel et al., 1998a). To enhance the SLs production by the yeast optimization of the medium constituents was carried out.

4.1.1.2. Optimization of medium constituents – one variable at a time

Medium components are known to play a vital role in the production of SLs by the yeast *C. bombicola*, and both hydrophilic and hydrophobic carbon sources are preferred for high yield of the product (Van Bogaert et al., 2007). Compared to hydrophilic carbon source, hydrophobic carbon source in the medium highly influences SLs production by the yeast (Casas and Garcia-Ochoa, 1999; Van Bogaert et al., 2007). Nitrogen source in medium also plays an important role in SLs production as it is well known that the yeast produces biosurfactant under nitrogen limiting conditions. Therefore, the levels of these medium constituents were optimized to enhance the production of SLs in the study.

For optimization, the most simple and conventional method - one variable at a time was adopted. It involves varying one factor levels while maintaining the other factor at an unspecified constant level (Haider and Pakshirajan, 2007). To determine the optimum concentration of deproteinized whey in the study, its concentration was varied from 25 to 125 g/l by keeping the concentrations of yeast extract , urea and soybean oil constant at 10, 1 and 100 g/l, respectively. Fig. 4.2 shows the effect of deproteinized whey concentration on SLs production which reveals that maximum SLs yield of 5.62 g/l was obtained at 100 g/l of whey at the end of 192 h of fermentation. Above 100 g/l of deproteinized whey concentration the SLs production declined probably due to substrate inhibition by whey. Casas and Garcia-Ochoa

(1999) studied the effect of glucose concentration on SLs production and they also found that glucose concentration above 100 g/l in the medium decreased the production level. Zhou and Kosaric (1993) obtained 6.23 g/l of SLs by *C. bombicola* using cheese whey and olive oil as carbon source by carrying out experiments in a 1 L fermentor. Compared to this literature report, SLs production in the present study is slightly less may be because of the hydrophobic carbon source soybean oil used in place of olive oil. The production level is also very less compared to those obtained with glucose as a substrate (Cooper and Paddock 1984; Zhou et al., 1992; Casas and Garcia-Ochoa 1999).

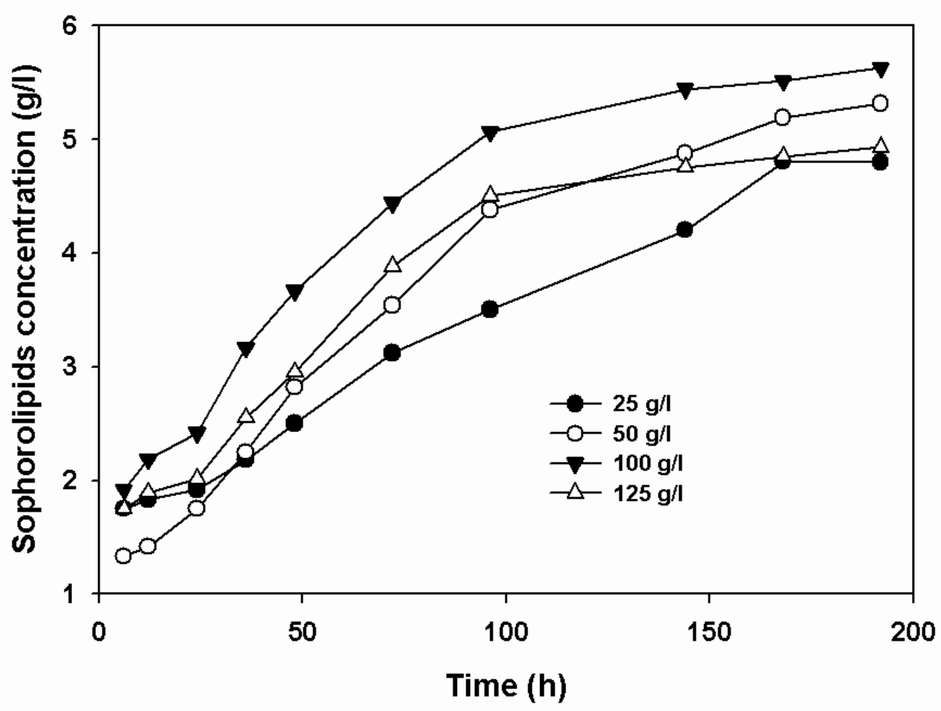


Fig. 4.2: Effect of deproteinized whey concentration on SLs production.

In order to enhance the SLs production by the yeast, glucose was therefore, added to the medium containing deproteinized whey in such way that the final carbohydrate concentration (hydrophilic carbon source) was maintained at 100 g/l. Also, urea was omitted from the medium and in place of soybean oil, oleic acid was used as it was reported to give better yield of the product with glucose by Van Bogaert et al. (2007). Based on this modification in the production medium, results obtained showed that addition of only 10 g/l of glucose in the medium almost tripled the SLs production (14.88 g/l) with 14.75 g/l of yeast biomass, which is clearly seen from Fig. 4.3. The figure also reveals that while the yeast biomass was positively affected, the SLs yield decreased due to raise in glucose concentration in the medium (Fig. 4.3).

The effect of yeast extract as a source of nitrogen on both yeast cell growth and SLs production was next investigated. The concentration of other medium constituents, deproteinized whey, glucose and oleic acid were kept constant at 90, 10 and 100 g/l, respectively. Fig. 4.4 shows that an increase in the concentration of yeast extract in the medium above 2 g/l had a negative effect on the SLs production, and hence, 2 g/l was found to be the optimum concentration of yeast extract in the study.

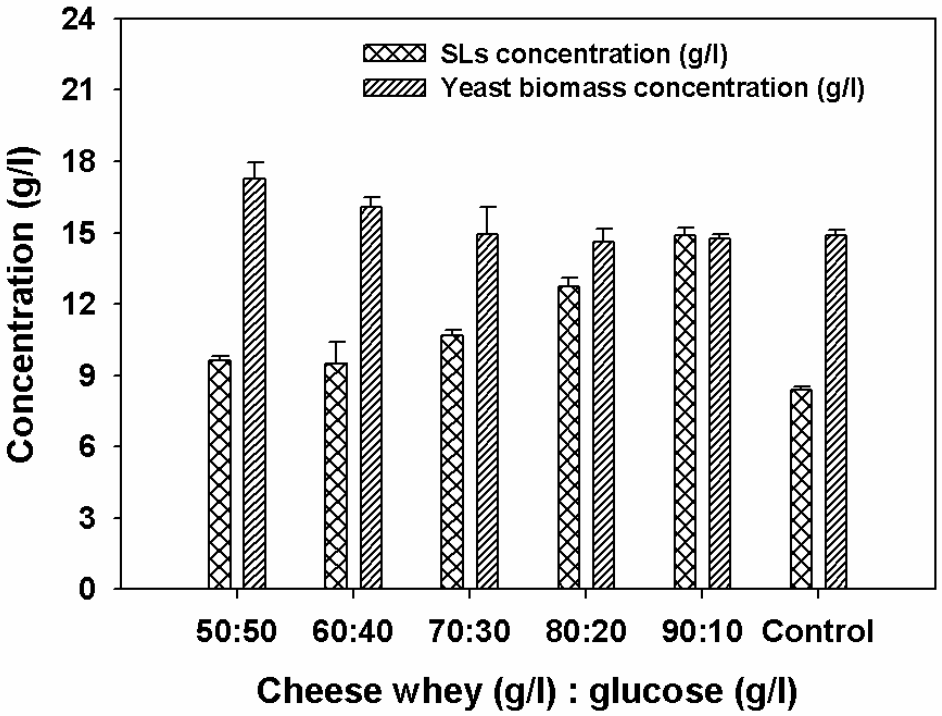


Fig. 4.3: Effect of combined deproteinized whey and glucose concentration on SLs production and yeast biomass growth.

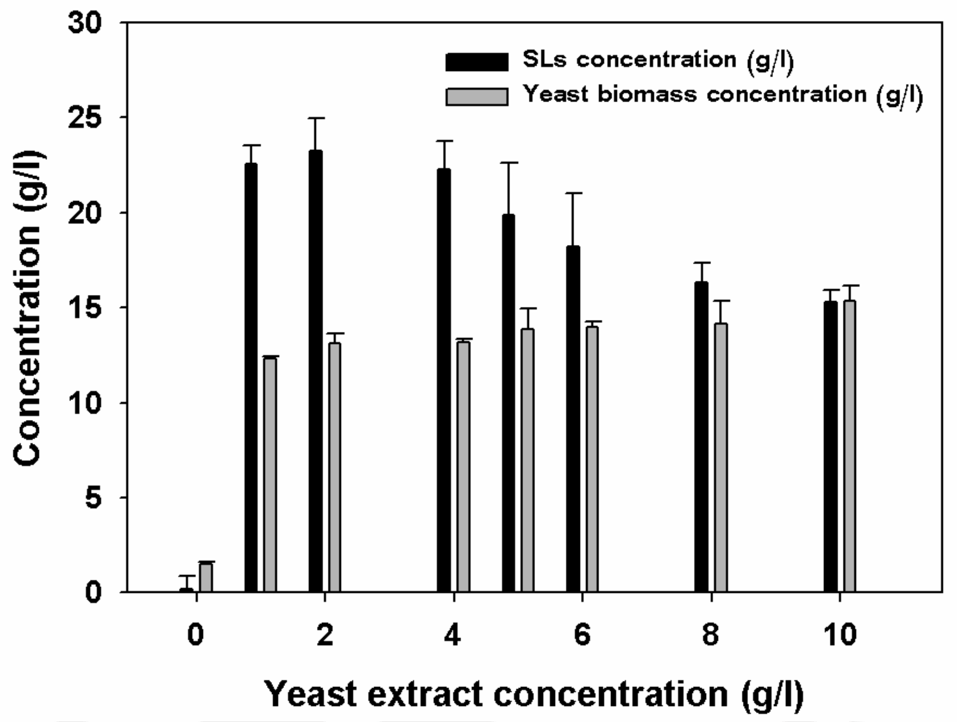


Fig. 4.4: Effect of yeast extract concentration on SLs production and yeast biomass growth.

The result of optimum yeast extract concentration in the study is found to be consistent with those of Casas and Garcia-Ochoa, (1999) and Van Bogaert et al., (2007). The effect of yeast concentration may be easily understood because nitrogen limitation mainly cause a decline in the specific activities of NAD^+ - and $NADP^+$ - dependent isocitrate dehydrogenase, which leads to accumulation of isocitrate and subsequently citrate in the cells. These citrates are cleaved by ATP dependant citrate synthase to produce fatty acid precursor acetyl-CoA but these enzymes are not fully active until the yeast reaches its stationary growth phase. This is supported by the observations that although the yeast biomass concentration was not much affected due to increase in yeast extract concentration, SLs production was enhanced sharply (Fig. 4.4).

Zhou and Kosaric (1995) studied the effect of yeast extract on the production of SLs during cultivation of *C. bombicola* on glucose (100 g/l) and canola oil (105 g/l) and found optimum yeast extract concentration to be 4 g/l. Different hydrophobic carbon source than that used in the present study may be the reason for a slightly different optimum concentration of yeast extract observed by Zhou and Kosaric (1995).

As the SLs production is highly influenced by the hydrophobic carbon source in the medium (Casas and Garcia-Ochoa, 1999 and Van Bogaert et al., 2007), the effect of various concentrations of oleic acid and other vegetable oils, viz. sunflower oil and olive oil, was tested in the study. Fig. 4.5 illustrates the influence of oleic acid on SLs production and yeast growth, which shows that oleic acid at 100 g/l is optimum for both yeast biomass and SLs production. At the optimum concentration of oleic acid, 23.29 g/l of SLs and 13.38 g/l of yeast biomass were produced; concentrations above the optimum level did not improve the SLs production by the yeast.

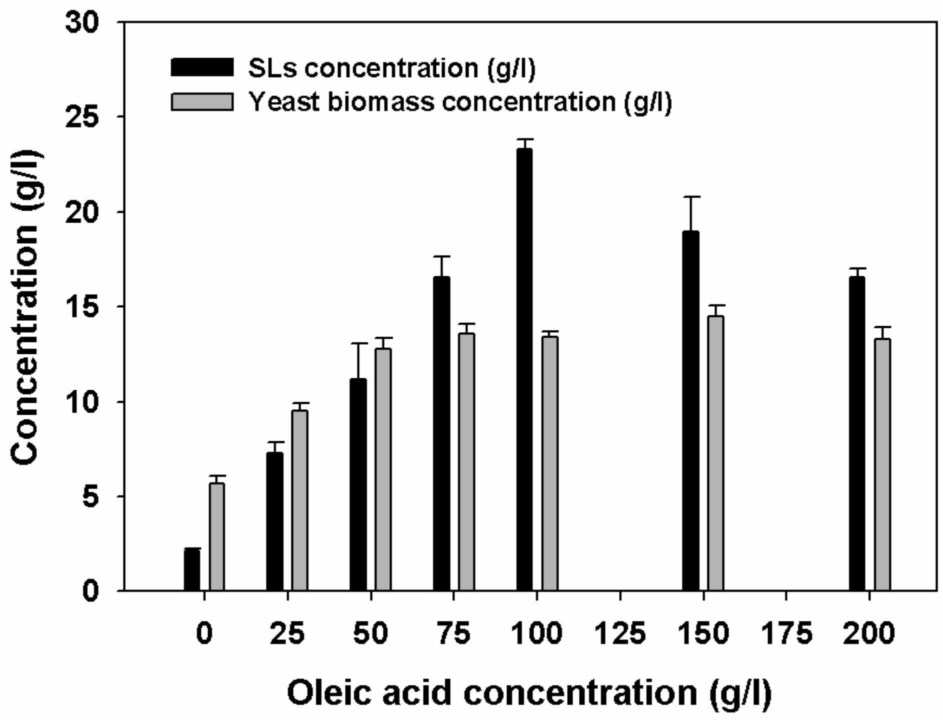


Fig. 4.5: Effect of oleic acid concentration on SLs production and yeast biomass growth.

However, because it is quite understood that oleic acid is a costly lipophilic substrate, attempts were made to replace oleic acid with vegetable oils such as sunflower oil and olive oil but not soybean oil which was already tested. When oleic acid was replaced with either sunflower oil or olive oil, SLs production considerably reduced to 2.6 and 6.2 g/l, respectively (Fig 4.6). The SLs production values were similar to those obtained with soybean oil. As reported in literature, this study further proved that oleic acid (a pure source of C18:1 fatty acid compared to other tested vegetable oils) is the best lipophilic carbon source for SLs production (Van Bogaert et al., 2007).

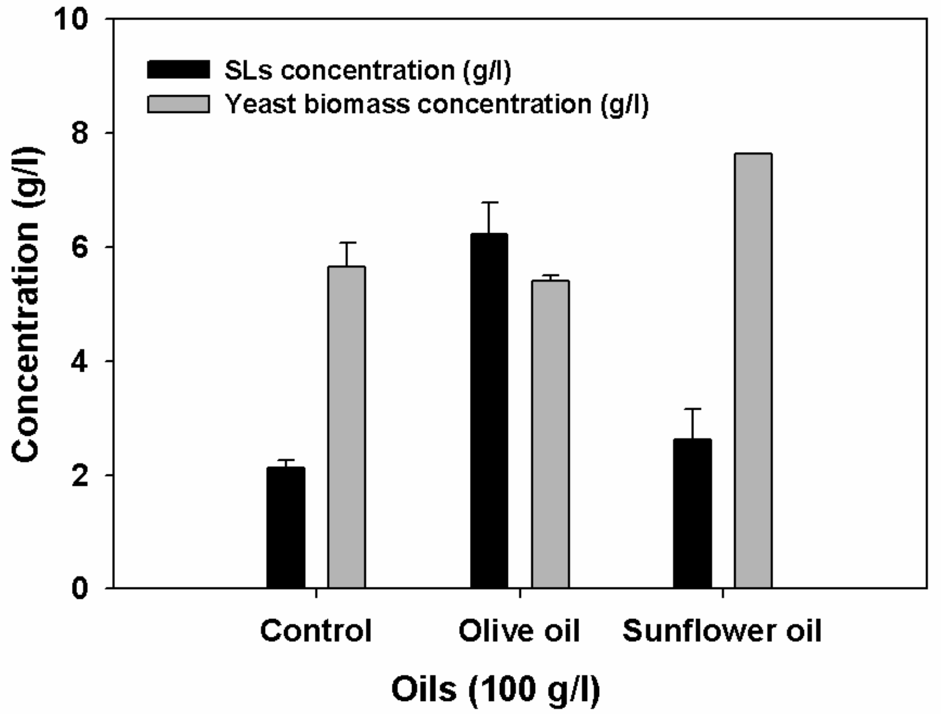


Fig. 4.6: Effect of olive oil and sunflower oil concentration on SLs production and yeast biomass growth

Thus, based on the results obtained, the medium constituents and their optimum levels for maximum SLs production were found to be deproteinized whey (90 g/l), glucose (10 g/l), yeast extract (2 g/l) and oleic acid (100 g/l). These optimum levels were further evaluated by carrying out experiments using a fermentor.

4.1.1.3. SLs production in fermentor

At the previously found optimum concentrations of the medium components based on deproteinized whey and glucose, SLs production was investigated in a batch operated 3 l fermentor (with or without pH control). Fig 4.7 and 4.8 show the profiles of SLs production, yeast biomass growth and oleic acid utilization by the yeast in the fermentor operated without and with pH control, respectively.

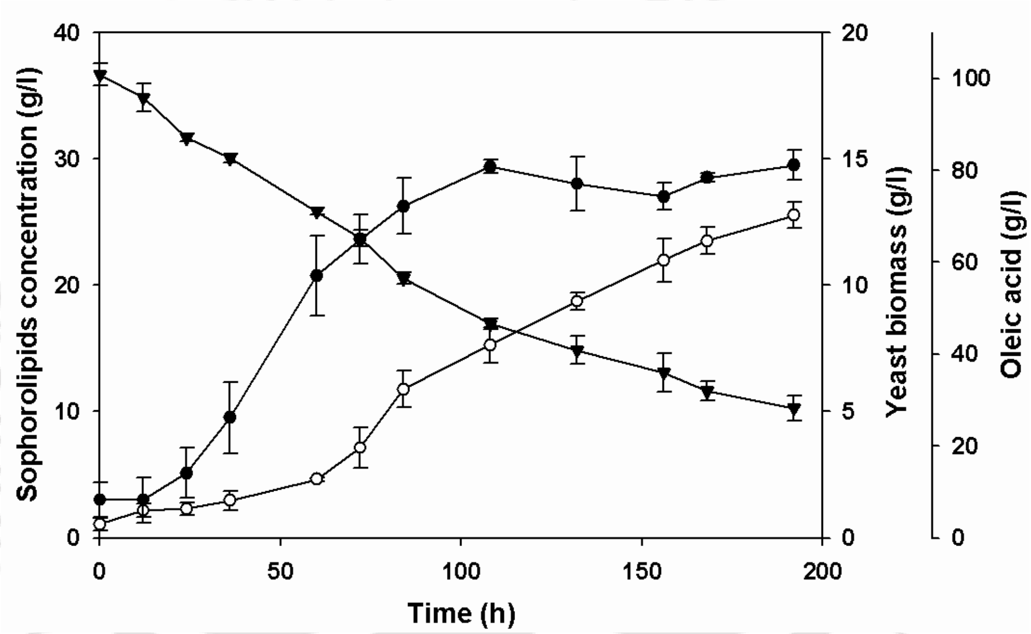


Fig. 4.7: Profiles of SLs production (▬○▬), yeast biomass growth (▬●▬) and utilization of oleic acid (▬▼▬) in the batch operated fermentor without pH control.

It could be seen that in the fermentor operated without pH control, the yeast was able to produce 26 g/l of SLs, which was slightly higher than the value observed from the shake flask experiment, mainly due to better control of operating conditions such as aeration, temperature and agitation in the fermentor. Further, batch fermentation was carried out using the same fermentor, but under controlled pH condition, as reported in the previous chapter (3.4.1.4).

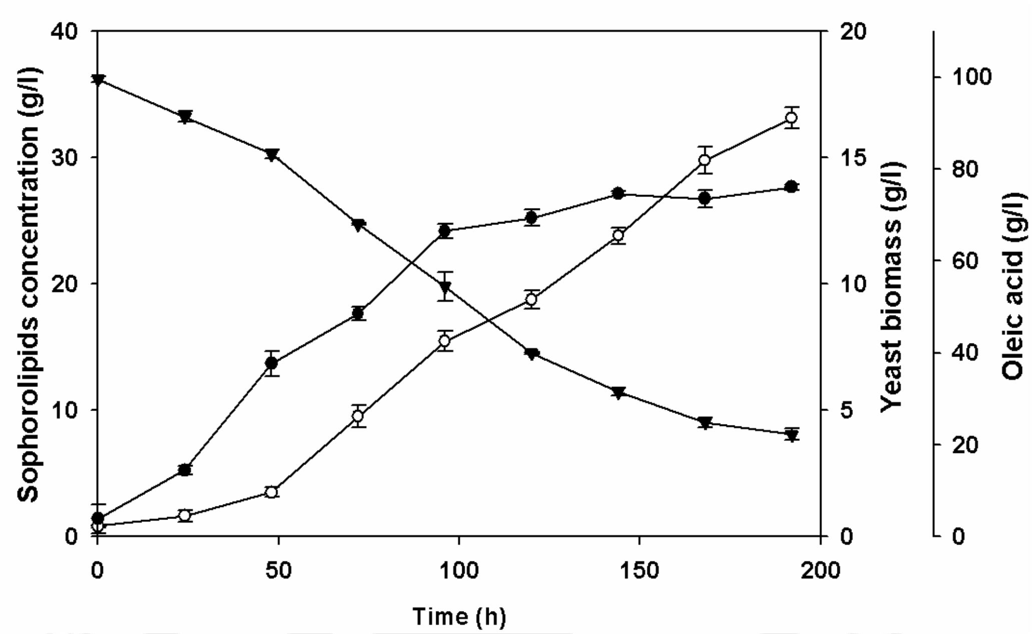


Fig. 4.8: Profiles of SLs production (—○—), yeast biomass growth (—●—) and utilization of oleic acid (—▼—) in the batch operated fermentor with pH control.

The SLs production level under pH controlled condition was found to be significantly improved at 33 g/l however, there was no effect on the yeast biomass. This may be due to the fact that the enzymes involved in the biosynthesis of SLs are most active at this particular pH. Other authors also found that controlled pH conditions improve SLs production by *C. bombicola* (Hu and Ju, 2001a; Solaiman et al., 2004; Solaiman et al., 2007). Daniel et al, (1999) developed a two-step batch cultivation process for SLs production using deproteinized whey in a 100 L fermentor, in which the first step consisted of growing the yeast *Cryptococcus curvatus* ATCC 20509 on deproteinized whey concentrates; in the second step the cultivation broth, disrupted in a glass bead mill, served as medium for growth and SLs production by the yeast *C. bombicola*. The authors reported only 12 g/l of SLs by this method which is almost three times less compared to that obtained in the present study.

Table 4.2 summarizes the literature reported values of SLs production yield in batch bioreactor by *C. bombicola* using whey as the hydrophilic carbon source. It is clear from the table that the SLs yield obtained in the present study is superior compared to those found in the literature, which could be attributed to the fact that the medium constituents used in the batch bioreactor experiment were all present at their optimum levels.

Table 4.2: Comparison of SLs production yield using deproteinized whey in batch bioreactor obtained in the present study with those found in the literature.

Sl. no.	Hydrophilic carbon	Hydrophobic carbon	SLs yield (g/l)	Reference
1.	Deproteinized whey	Soybean oil	5.62	Present study
2.	Deproteinized whey and glucose	Oleic acid	33	Present study
3.	Deproteinized whey	Olive oil	6.23	Zhou and Kosaric, 1993
4.	Deproteinized whey	--	12	Daniel et al., 1999

To further enhance the SLs production the bioreactor was operated under fed-batch mode using oleic acid as the feeding substrate as the other substrate lactose present in the medium was remained largely unutilized. Results of fed-batch experiments revealed that even after 192 h of fermentation the yeast could able to produce only 40 g/l of SLs and that too with the expense of slightly high concentration (140 g/l) of the feeding substrate oleic acid (Fig. 4.9).

Compared to SLs production observed by other authors, the value obtained in the present study is found to be low (Deshpande and Daniel, 1995; Kim et al., 1997; Daniel et al., 1998a; Solaiman et al., 2007). Considering the fact that the optimized medium deproteinized whey, glucose and costly oleic acid did not significantly improve the SLs production by the yeast, even under fed-batch mode of operation, another industrial waste, sugarcane molasses, was tested in place of deproteinized whey as a cheap substrate.

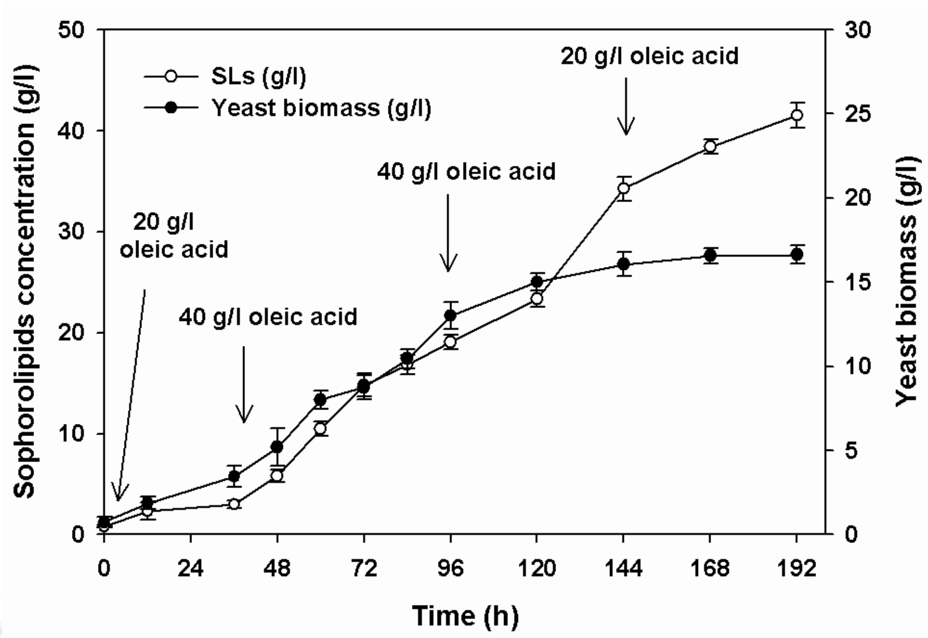


Fig. 4.9: Profiles of SLs production (—○—) and yeast biomass growth (—●—) under fed-batch mode of operation in the fermentor with pH control. Feeding substrate used was oleic acid.

4.1.2. Production using sugarcane molasses

Sugarcane molasses, a by product of sugar industry, is one of the most abundant waste material available in India. It is mainly fermented in distilleries to produce alcohol commercially. It also used to produce several other chemicals such as acetaldehyde, acetic acid, poly vinyl chloride and mono ethylene glycol. However, till date there is no study with sugarcane molasses for the production of SLs.

4.1.2.1. SLs production in batch shake flasks and in fermentor

Initial experiments were carried out in batch shake flasks to compare the SLs production and yeast growth using sugarcane molasses with that produced using glucose. Results from these experiments revealed that the yeast produced more biomass when grown on medium containing sugarcane molasses, soybean oil, yeast extract and urea (17.95 g/l) than when grown on medium containing glucose, soybean oil, yeast extract and urea (14.46 g/l), but the SLs produced using sugarcane molasses was found to be only less than 50% of that produced using glucose (12.67 g/l vs. 29.37 g/l). However, the SLs produced was much higher compared to that obtained using deproteinized whey as the sole hydrophilic carbon source.

Thus, SLs production using sugarcane molasses was further investigated in fermentor under necessary continuous monitoring of pH, dissolved oxygen (DO) and temperature. The profiles of SLs production, total carbohydrate, biomass during the experiment were also monitored over time. The results obtained are presented in Fig. 4.10, which shows that a maximum SLs concentration of 63.7 g/l at 60 h of batch fermentation was produced. After this time period SLs concentration in the medium declined quite gradually up to 34.8 g/l at the end of five days. The decrease in SLs concentration at the end of 60 h could be attributed to the potential inhibition on the cell growth. In a study by Deshpande and Daniel (1995) on SLs production by the yeast in a 10 L fermentor using animal fat in the medium, maximum SLs concentration obtained at 72 h was found to decrease later. The authors suggested the decrease in the product yield due to substrate limitation in the fermentation medium and recommended for harvesting the product before 72 h time period. Kim et al. (1997) reported a maximum SLs production of 80 g/l when the yeast was grown on glucose-soybean oil containing medium in a 5 L fermentor. On the other hand, Solaiman et al. (2004) observed that the yeast produced only 21 g/l of SLs when soy molasses and oleic acid were used in addition to yeast extract and urea in the fermentation medium. The SLs production, however, improved to 53 g/l when the yeast was grown on medium containing only soy molasses and oleic acid (Solaiman et al., 2007). Compared to these yield values of SLs found in the literature, the value obtained using sugarcane molasses in the present study seems more feasible and more attractive for wastewater application as well. Also, the SLs production was significantly high compared to that obtained with deproteinized whey and oleic acid.

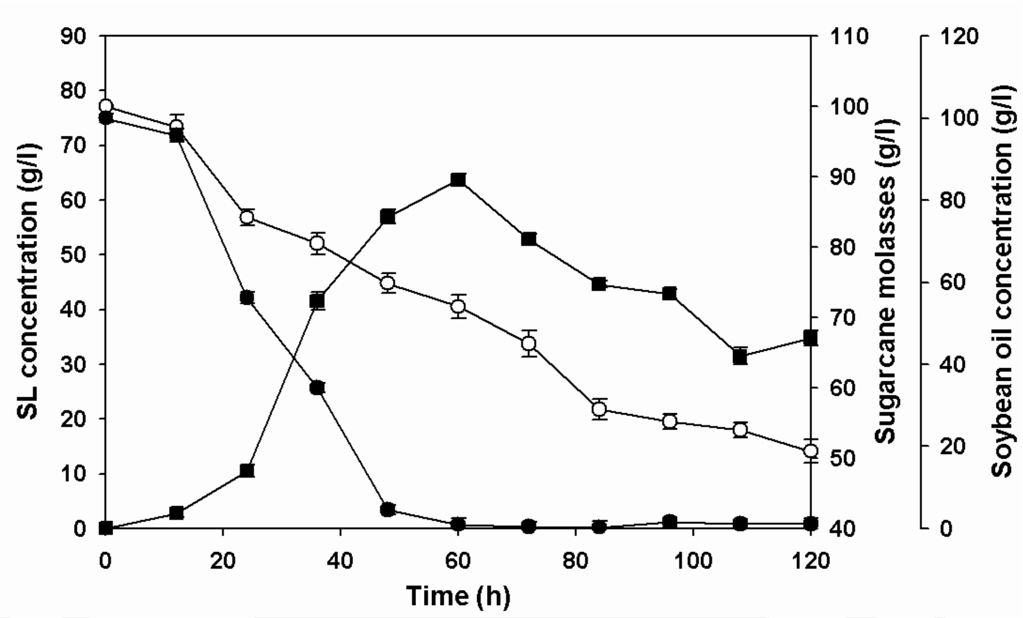


Fig. 4.10: Time course of SLs production (—■—) and substrate utilization (sugarcane molasses, —○—; soybean oil, —●—) by *C. bombicola* in the batch operated fermentor using sugarcane molasses based medium.

Fig. 4.11 shows the relationship between pH, DO and biomass during SLs production by *C. bombicola* in the fermentor operated under batch mode. It could be seen that during the exponential growth phase of the yeast, pH dropped drastically from 6.0 to 3.6, probably due to generation of fatty acids; however, during the later phase of fermentation, the pH was stable after reaching a value of 4.0.

With respect to the yeast biomass, the amount increased very rapidly within the first 36 h and then decreased slightly till it assumed a constant value. These changes in the biomass profile corresponded well with the medium DO, but negatively. The observations on these parameters were also found to be true by Deshpande and Daniel (1995) on SLs production by *C. bombicola* in their study.

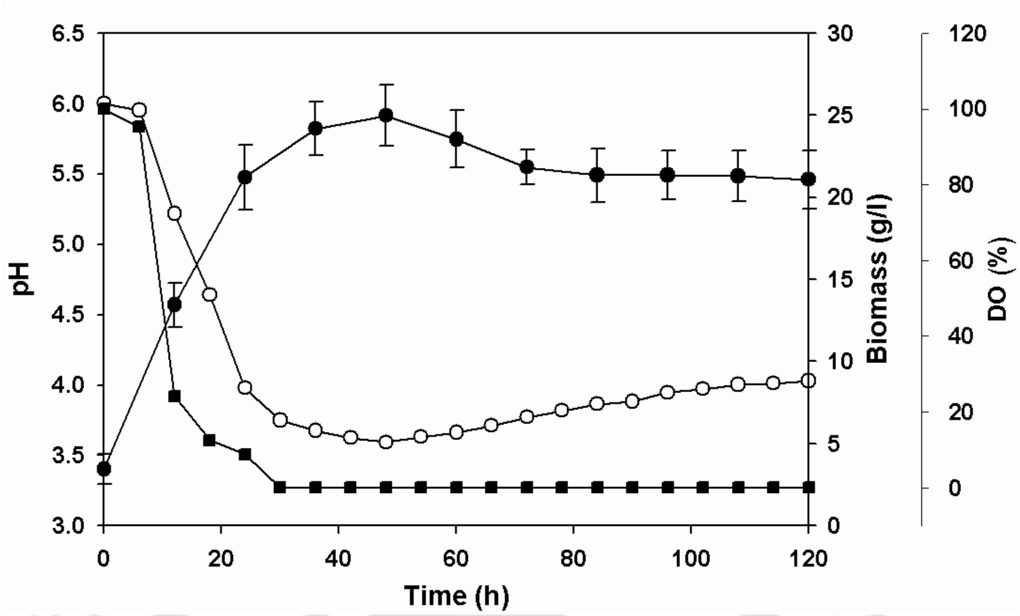


Fig. 4.11: pH (▬○▬), biomass (▬●▬) and DO (▬■▬) profiles during SLs production by *C. bombicola* in the fermentor using sugarcane molasses based medium.

Further, because sugarcane molasses itself contains 1.5 – 2.5 g/l of nitrogen recognized for better production of SLs by the yeast, extra addition of the nitrogen sources yeast extract and urea was omitted from the medium and SLs production by the yeast was evaluated using only sugarcane molasses and a hydrophobic carbon source.

4.1.2.2. SLs production using only sugarcane molasses and a hydrophobic carbon source

In this study, sugarcane molasses as the sole source of carbon and nitrogen source for the production of SLs by the yeast was evaluated along with a lipophilic carbon source. From the previous results of SLs production using deproteinized whey based medium, which showed that lipophilic carbon source played a significant role, different oils (sunflower and olive oil) other than soybean oil were tested in the media containing only sugarcane molasses. At the end of five days of fermentation period, the yeast produced 23.25 ± 1.07 g/l of SLs when grown on medium containing only sugarcane molasses and soybean oil. But when soybean oil was replaced either with sunflower oil or olive oil, the SLs production by the yeast was low at 17.46±1.56 g/l and 18.96±0.44 g/l, respectively. With respect to the yeast biomass growth, the values were found to be very less using only sugarcane molasses and oil. The amount of biomass was found to be 3.67±0.38, 4.25±0.5 and 4.46±0.63 using soybean oil, sunflower oil and olive oil, respectively as the hydrophobic carbon source in the medium. The reduced amount of biomass using only sugarcane molasses and oil in the media could be due to inability of the yeast biomass to utilize the nitrogen source in molasses for its growth. However, this aspect needs to be verified further.

Nevertheless, since the values of SLs production were higher than the SLs production obtained in batch shake flasks using medium that contained yeast extract and urea in addition to sugarcane molasses and soybean oil, media containing only sugarcane molasses and soybean oil can be said to replace both the costly conventional synthetic medium containing glucose and the earlier tested medium based on deproteinized whey for the production of SLs by *C. bombicola*. To further enhance the SLs production using the medium containing sugarcane molasses and soybean oil, process parameters were optimized and the results are discussed in the following section.

4.1.2.3. Screening and optimization of process parameters and medium constituents

Any biochemical process is significantly influenced by physical (temperature, agitation, aeration etc.) and chemical (medium constituents) parameters, and for best results it is necessary to optimize these process parameters. Optimization of process parameters can be done by traditional - one variable at time method or statistical based methods such as response surface methodology or Taguchi method. Compared to traditional one variable at a time optimization method, statistical design methods such as Taguchi method possess the unique advantage that many factors can be examined simultaneously and much quantitative information can be extracted by performing only a few experimental trials.

To improve the SLs yield by the yeast effect of various physical (temperature, agitation, inoculum size and age) and chemical variables (sugarcane molasses and soybean oil concentration) was studied and optimized by employing the statistically valid Taguchi orthogonal array (OA) and experimental design technique.

Influence of individual parameters on the SLs production:

In these experiments, performed as per the OA design presented in the previous chapter (section 3.4.2.3, Table 3.2), SLs production was taken as the response. Table 4.3 presents the SLs production obtained in each experimental run along with the levels of the different parameters, which also depicts the S/N ratio obtained in the experiments.

It is clear from the table that depending upon the combinations of the process variables and their levels, SLs production in each run varied largely thus indicating strong influence of the variables and their levels on the response. Further, to understand which of these variables affected SLs production in a significant manner, the variables were ranked based on the calculated delta S/N ratio. In general, delta value for a factor, calculated by measuring the difference between the highest and lowest characteristic average S/N ratio of the factor, indicates its relative significance over others on a given response: higher value of delta for a factor denoting a larger significant effect than others.

Table 4.3: Experimental design matrix showing parameters and their levels along with the observed response in the media optimization chart.

Experimental run no.	Parameters						SLs$^{\$}$ Production (g/l)	S/N ratio (dB)*
	Inoculums size (%)	Sugarcane molasses (g/l)	Soybean oil (g/l)	Temperature (ºC)	Inoculums age (days)	Agitation (rpm)		
1	5 (1)	50 (1)	50 (1)	20 (1)	2 (1)	150 (1)	28.62 ± 1.17	29.14
2	5 (1)	50 (1)	100 (2)	25 (2)	3 (2)	200 (2)	32.4 ± 1.98	30.21
3	5 (1)	50 (1)	150 (3)	30 (3)	4 (3)	250 (3)	15.8 ± 1.84	23.97
4	5 (1)	100 (2)	50 (1)	20 (1)	3 (2)	200 (2)	31.2 ± 1.13	29.88
5	5 (1)	100 (2)	100 (2)	25 (2)	4 (3)	250 (3)	14.2 ± 1.27	23.05
6	5 (1)	100 (2)	150 (3)	30 (3)	2 (1)	150 (1)	30.5 ± 0.71	29.69
7	5 (1)	150 (3)	50 (1)	25 (2)	2 (1)	250 (3)	17.78 ± 1.10	25.00
8	5 (1)	150 (3)	100 (2)	30 (3)	3 (2)	150 (1)	16.5 ± 0.85	24.35
9	5 (1)	150 (3)	150 (3)	20 (1)	4 (3)	200 (2)	14.25 ± 1.34	23.08
10	10 (2)	50 (1)	50 (1)	30 (3)	4 (3)	200 (2)	38.67 ± 2.36	31.75
11	10 (2)	50 (1)	100 (2)	20 (1)	2 (1)	250 (3)	12.9 ± 1.13	22.21
12	10 (2)	50 (1)	150 (3)	25 (2)	3 (2)	150 (1)	26.1 ± 0.99	28.33
13	10 (2)	100 (2)	50 (1)	25 (2)	4 (3)	150 (1)	24.3 ± 2.26	27.71
14	10 (2)	100 (2)	100 (2)	30 (3)	2 (1)	200 (2)	36.8 ± 1.70	31.32
15	10 (2)	100 (2)	150 (3)	20 (1)	3 (2)	250 (3)	10.5 ± 2.12	20.42
16	10 (2)	150 (3)	50 (1)	30 (3)	3 (2)	250 (3)	16.3 ± 1.41	24.24
17	10 (2)	150 (3)	100 (2)	20 (1)	4 (3)	150 (1)	13.25 ± 1.06	22.44
18	10 (2)	150 (3)	150 (3)	30 (3)	2 (1)	200 (2)	15.65 ± 0.78	23.89

* Decibels; $^{\$}$ Results are the average of three experiments

While S/N ratio indicates effect of factors on a response, delta S/N ratio can be used as a criterion for ranking factors of their effects on the response (Saudagar and Singhal, 2007).

In this study, based on the delta S/N ratio obtained for each factor, the six parameters were ranked accordingly, and the results were presented in Table 4.4. This ranking of the variables suggests that agitation had the maximum effect while inoculum age had the least effect on SLs production by *C. bombicola*. Because agitation compared to other variables is known to affect necessary transfer of oxygen to the cells for effective growth of the yeast and SLs production, its significance over others is more likely preceding.

Table 4.4: Values of average S/N ratio of the process variables at various levels and their ranking based on delta S/N ratio in the optimization study.

Level	**Inoculum size**	**Sugarcane molasses**	**Soybean oil**	**Temperature**	**Inoculum age**	**Agitation**
1	26.48	27.60	27.95	24.53	26.87	26.94
2	25.81	27.01	25.60	26.37	23.24	28.35
3		23.83	24.90	27.55	25.33	23.15
Delta*	0.67	3.77	3.06	3.02	1.54	5.20
Rank	**6**	**2**	**3**	**4**	**5**	**1**

***** Difference between maximum and minimum S/N ratio values

To validate these findings on the significance of the individual parameters and their contribution on SLs production, analysis of variance (ANOVA) of the results was employed. ANOVA is a well known statistical technique that subdivides the total variation in a set of data into component parts associated with specific sources of variation for the purpose of testing hypotheses on the parameters in a given model (Montgomery, 1997). Table 4.5 presents the ANOVA of SLs production obtained in the study. The mean sum of squares (MS) of a model term is calculated by dividing the sum of squares (SS) to degrees of freedom (df) of the model term, and the Fisher's F value is calculated by dividing the MS owing to the model by the MS owing to error. Table 4.5 shows a term for error, the MS value, which indicates that the amount of variation in the response data that is left unexplained by the model is low.

Table 4.5: Analysis of variance of SLs production in the optimization study.

Source	**DF**[a]	**SS**[b]	**Adj SS**	**MS**[c]	**F ratio**	**P value**	**Confidence level (%)**	**Percent contribution**
Inoculums size	1	2.56	2.56	2.56	0.17	0.695	30.5	0.196
Sugarcane molasses	2	368.47	368.47	184.24	12.18	0.008	99.2	28.148
Soybean oil	2	170.46	170.46	85.23	5.63	0.042	95.8	13.021
Temperature	2	160.75	160.75	80.37	5.31	0.047	95.3	12.28
Inoculums age	2	39.85	39.85	19.92	1.32	0.336	66.4	3.044
Agitation	2	566.95	566.95	283.48	18.74	0.003	99.7	43.310
Error	6	90.76	91.76	15.13				
Total	17	1399.80						100

[a] Degree of freedom, [b] Sum of squares, [c] Mean sum of squares

Table 4.5 also shows the main effects of the factors on the SLs production. In general, low P value of a term in ANOVA indicates high significance of the term; hence, in this study, agitation was found to have a maximum significant effect on SLs production, which was followed by sugarcane molasses concentration. Guilmanov et al. (2002) also reported that agitation largely impacts SLs production compared to any other parameters. Soybean oil concentration and temperature were next found to have a significant effect on the SLs production with P values 0.042 and 0.047, respectively. The other two variables, i.e. inoculum age and size, did not, however, show any significant effect on the SLs production. These findings of the effects of parameters on SLs production by ANOVA are in good agreement with those observed earlier from the factors ranking based on their delta S/N ratio. The determination coefficient R^2 of the model from ANOVA was found to be 0.935, which implied that the model could well explain 93.52% variation in the response. The percent contribution of variance was also calculated by dividing the sum of squared deviations (SS) by the total sum of squared deviations (Oktem et al., 2007) and these are presented for each model term in the ANOVA table (Table 4.5), which shows that agitation and concentration of sugarcane molasses contributed more than 70% of the total SLs production. Hence, based on the ANOVA results, the four variables concentrations of sugarcane molasses and soybean oil, temperature, and agitation speed were further chosen for predicting the maximum SLs production under optimized conditions.

Selection of optimum levels of parameters for enhancing the SLs production

Fig. 4.12 illustrates the effects of the six process variables investigated in the study on SLs production.

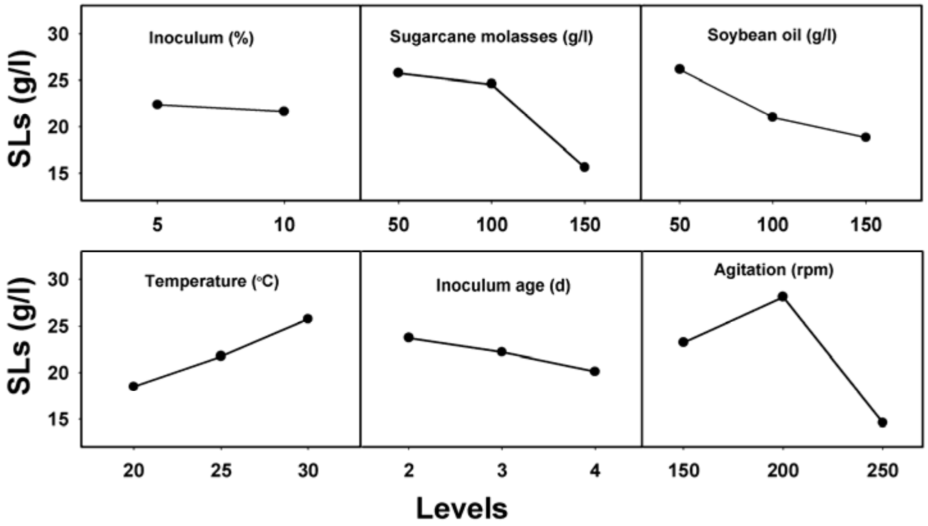


Fig. 4.12: Main effects plot of the process variables in the optimization study.

It could be seen from this figure that for each of the six variables tested at different levels, particular levels of the variables caused significant increase in the mean response compared to other levels of the variables. For e.g., level 1 of each of the variables on inoculum size and age, concentrations of sugarcane molasses and soybean oil, level 3 of temperature and level 2 of agitation were found to exhibit a significant positive effect on the response. These levels of the variables were therefore found to be optimum for maximum production of SLs in the medium (Saudagar and Singhal, 2007). Based on this observation, optimum levels of the process parameters were chosen for enhancing the SLs production, and are presented in Table 4.6.

Table 4.6: Optimum levels of the process parameters chosen for enhancing the SLs production.

Parameter	**Optimum level**
Inoculum size	5%
Molasses concentration	50 g/l
Soybean oil concentration	50 g/l
Temperature	30º C
Inoculum age	2 days
Agitation	200 rpm

The maximum S/N ratio for maximum SLs production was predicted using the optimum levels of process parameters using Equation (4.1):

$$\text{Predicted S/N} = \text{S/Nm} + \{(\text{S/N}_{\text{Molasses}} - \text{S/Nm})\} + \{(\text{S/N}_{\text{Soybean}} - \text{S/Nm})\} + \{(\text{S/N}_{\text{Temperature}} - \text{S/Nm})\} + \{(\text{S/N}_{\text{Agitation}} - \text{S/Nm}) \qquad (4.1)$$

where S/N_m is the total mean S/N ratio and $S/N_{Molasses}$, $S/N_{Soybean}$, $S/N_{Temperature}$, $S/N_{Agitation}$ are the mean S/N ratio at the optimal levels of the variables (Kim et al., 2007). As the variables inoculum age and size did not significantly affect SLs production, they were excluded from the above model equation. The value of S/N_m

calculated from Table 4.3 was 26.15 dB (decibels, and the values of $S/N_{Molasses}$, $S/N_{Soybean}$, $S/N_{Temperature}$, and $S/N_{Agitation}$ were obtained from Table 4.4. Thus, using Equation (4.1), the predicted S/N ratio for SLs production was found to be 33.00 dB and the corresponding SLs production, calculated using Equation (3.2) mentioned in the previous chapter was found to be 44.67 g/l, which is higher than the SLs production values obtained at the un-optimized settings of the parameters (Table 4.3).

To validate the model predicted SLs production value, experiments were carried out both in a 250 ml Erlenmeyer flask with 50 ml working volume and in a batch operated 3 L fermentor (Applikon, Holland) with 1 L working volume (without and with pH control) using the optimized levels of the process variables (Table 4.6).

The profiles of SLs production, yeast biomass growth, utilization of sugarcane molasses and soybean oil in the above three different systems (batch shake flasks, bioreactor operated without pH control and bioreactor operated under pH control) at the optimized process conditions are illustrated in Fig 4.13. It can be seen from Fig. 4.13 (b) and (c) that there was no lag in the yeast growth with an exponential phase of up to 48 h fermentation time, and during this period the amount of SLs produced was less. This observation is also found consistent with our earlier results on the yeast biomass growth and SLs production with deproteinized whey. Later when the yeast entered its stationary growth phase, the SLs yield increased with time (Fig. 4.13). These results are in good agreement with the literature reported results and our earlier results with deproteinized whey that the SLs production by the yeast is a non-growth associate phenomenon (Hu and Ju, 2001a; Guilmanov et al., 2002). On the other hand, compared to SLs production in batch shake flask, where it remained constant after 144 h of fermentation, the results from the fermentor showed that the biosurfactant production could be maintained high even after 192 h (Fig. 4.13).

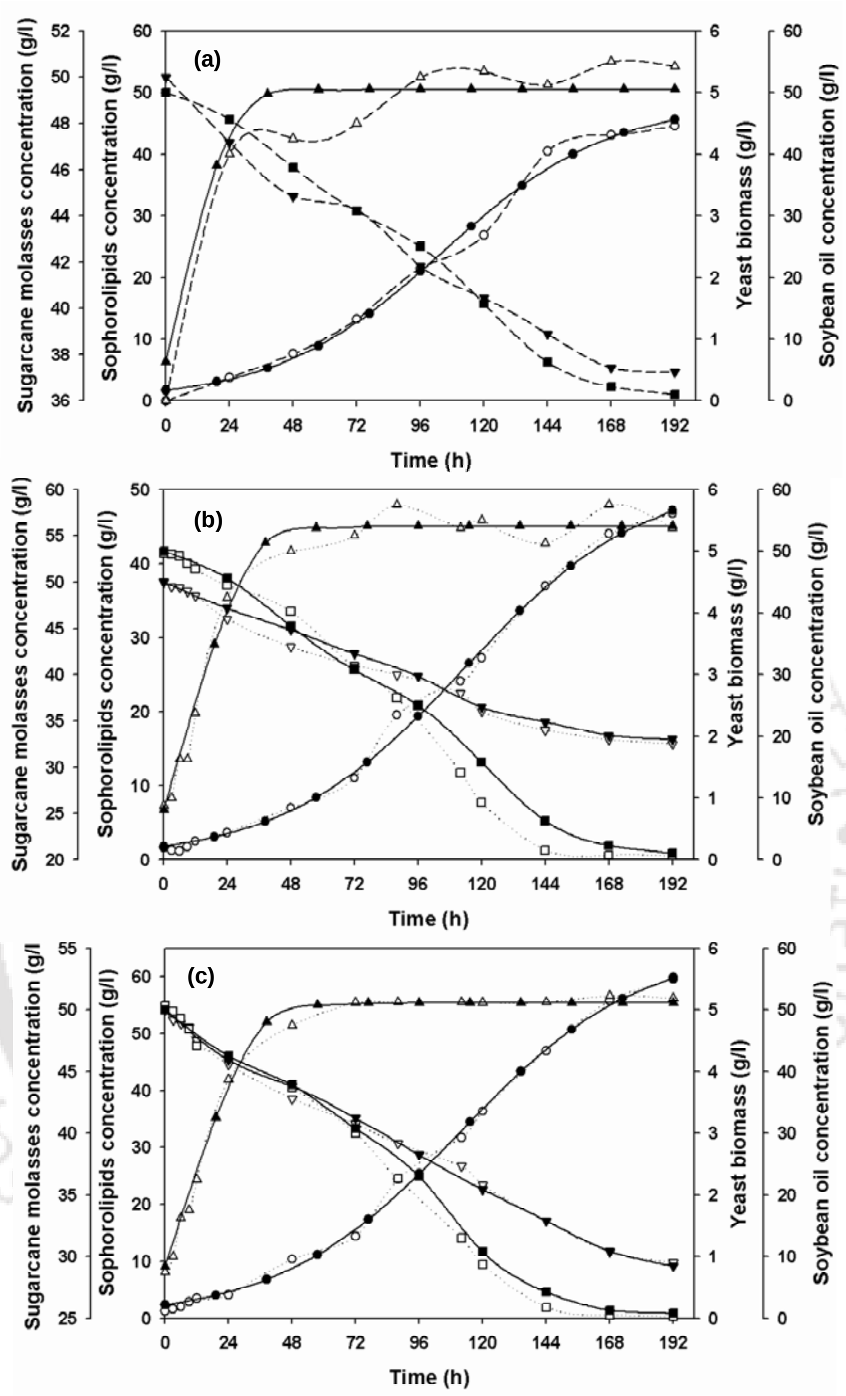


Fig. 4.13: Experimental (dotted line) and model predicted (continuous line) profiles of SLs production (circles), yeast biomass growth (upward triangles), utilization of sugarcane molasses (downside triangles) and soybean oil (squares) in (a) batch shake flask, (b) fermentor operated without pH control and (c) fermentor operated with pH control.

From the utilization profiles of the carbon sources, it was clear that soybean oil compared to sugarcane molasses in all the three systems was completely utilized by the yeast during the experiment.

Fig 4.13 (a) showed that in the simple batch shake flasks, the obtained SLs production value of 45 g/l, which matched accurately well with the predicted value. In the fermentor experiments operated without pH control, the yeast was able to produce 47 g/l of SLs (Fig 4.13b**)**, which was even slightly higher than the value observed from the shake flask experiment, mainly due to better control of aeration, temperature and agitation conditions in the bioreactor. Thus, screening and optimization of process parameters on SLs production by the yeast using media containing only sugarcane molasses and soybean oil resulted in 90.02% enhancement (1.92 fold) in SLs concentration compared to the value obtained at un-optimized settings of process conditions. Further, batch experiment with the same fermentor but under controlled pH condition, as detailed earlier was carried out. Briefly, the initial pH of the production medium was set to 6.0 before autoclaving, and during the fermentation when the pH dropped to 3.5, it was controlled at this value afterwards. Results showed even higher SLs production of 60 g/l, which is 27.6% higher than the value obtained in fermentor operated without control of pH (Fig 4.13c**)**.

Table 4.7 compares the SLs production by the yeast using different media investigated in the study. From the preceding table it is very clear that medium containing only sugarcane molasses and soybean oil can very well be used in place of conventional glucose containing media for the production of the SLs by the yeast. Further, biokinetic constants involved in SLs production using the above mentioned media were determined and its results are discussed in the next section.

Table 4.7: Comparison of the SLs production by the yeast using different media investigated in the study.

Media composition	**Shake flasks/ fermentor**	**SLs production (g/l)**
Deproteinized whey + soybean oil + yeast extract	Shake flasks	5.98
Deproteinized whey + glucose + oleic acid + yeast extract*	Shake flasks	23.29
Deproteinized whey + glucose + oleic acid + yeast extract*	Fermentor	26
Deproteinized whey + glucose + oleic acid + yeast extract*	Fermentor (pH controlled)	33
Deproteinized whey + glucose + oleic acid + yeast extract*	Fermentor (fed batch, controlled pH)	40
Glucose + soybean oil + yeast extract + urea	Shake flasks	29.73
Sugarcane molasses + soybean oil + yeast extract + urea	Shake flasks	12.67
Sugarcane molasses + soybean oil	Shake flasks	23.25
Sugarcane molasses + soybean oil#	Shake flasks	45
Sugarcane molasses + soybean oil#	Fermentor	47
Sugarcane molasses + soybean oil#	Fermentor (pH controlled)	60

* = optimized medium using one variable at a time

\# = optimized medium using Taguchi method

4.1.2.4. Kinetics of SLs production, yeast biomass growth and utilization of carbon source and estimation of kinetics parameters

The objective of this study was to investigate the kinetics parameters such as specific growth rate, substrate consumption rate, SLs production rate and related yields involved in the SLs production and yeast cell growth under optimum process conditions. The information obtained from this kinetic study may be useful to obtain better understanding of the process for large scale application.

To estimate the biokinetic constants involved in the process, models previously mentioned in Table 3.3 (Chapter 3) were fitted to the experimental data on SLs production, yeast biomass growth and utilization of sugarcane molasses and soybean oil. These mathematical models were originally proposed by Mercier et al. (1992) and were chosen as it fairly describes biomass growth, substrate consumption and product accumulation pattern in any process. Also, these models were recently applied by Rodrigues et al. (2006) for explaining the biosurfactant production kinetics in their study. For fitting the models to the experimental data in the present study, nonlinear regression using the least-squares method was used employing Matlab® 7 and Microsoft Excel Solver 2003.

The estimated kinetic parameters values obtained from these models are mentioned in Table 4.8, which also shows that the determination coefficient (R^2) values obtained by fitting the various models to the experimental data were found to be very high (≥ 0.95) thus revealing good accuracy of these models in explaining the experimental data. From the kinetic parameter values presented in the table, it could be seen that the values of P_r and μ were found to be higher in the batch operated fermentor compared to the values obtained in batch shake flasks, which is attributable to better control of physical parameters, mainly temperature, agitation and aeration, in the fermentor than in the simple shake flasks. Also, the values of product yields ($Y_{P/M}$ and $Y_{P/S}$) were found to be higher than those reported by Garcia-Ochoa and Casas (1999) suggesting that the low cost fermentative medium can replace the costly conventional SLs production medium with a better yield of the biosurfactant.

Table 4.8: Kinetic parameters estimated by fitting the various models to experimental data obtained for different systems in the study.

Batch shake flask/ fermentor	**Sophorolipids production**[*]				**Yeast biomass production**[*]				**Sugarcane molasses utilization**[*]				**Soybean oil utilization**[*]			
	P_0 **(g/l)**	P_{max} **(g/l)**	P_r **(1/h)**	R^2	X_0 **(g/l)**	X_{max} **(g/l)**	μ **(1/h)**	R^2	S_{M0} **(g/l)**	$Y_{P/M}$ **(g/g)**	$Y_{X/M}$ **(g/g)**	R^2	S_{S0} **(g/l)**	$Y_{P/S}$ **(g/g)**	$Y_{X/S}$ **(g/g)**	R^2
Batch shake flasks	1.692	48.48	0.028	0.990	0.638	5.054	0.109	0.937	49.99	0.719	2.438	0.999	49.99	0.783	0.431	0.999
Fermentor (no pH control)	1.784	51.88	0.029	0.996	0.804	5.410	0.122	0.988	49.99	0.721	2.625	0.963	49.62	0.785	0.417	0.963
Fermentor (under pH control)	2.373	65.42	0.030	0.997	0.840	5.117	0.114	0.996	49.99	0.730	2.425	0.977	50.60	1.025	0.470	0.977

[*] P_{max} = maximum concentration of SLs (g/l), P_r = ratio between the initial volumetric rate of product formation and the initial product concentration P_0 (g/l), X_{max} = maximum concentration of yeast biomass (g/l), μ (1/h) = ratio between the initial volumetric rate of biomass formation and the initial biomass concentration X_0 (g/l), $Y_{P/M}$ (g/g) = SLs yield due to molasses, $Y_{X/M}$ (g/g) = biomass yield due to molasses, S_{M0} = initial molasses concentration (g/l), $Y_{P/S}$ (g/g) = SLs yield due to soybean oil, $Y_{X/S}$ (g/g) = biomass yield due to soybean oil and S_{S0} = initial soybean oil concentration (g/l)

However, these authors found high values of growth kinetic parameter in their study (μ = 0.14 h^{-1}, X_{max} = 5.98 g/l), which is mainly due to the fact that these authors added extra nitrogen source, i.e. yeast extract, for fermenting the yeast.

It is reported that while hydrophilic carbon source is utilized by the yeast both for its growth and SLs production, lipophilic carbon source (oil) is spent mainly for product formation and for maintenance of the biomass (Garcia-Ochoa and Casas, 1999). This aspect was found to be true in the present study also as it was observed that (a) the biomass yield due to sugarcane molasses ($Y_{X/M}$) is higher than that due to soybean oil ($Y_{X/S}$) and (b) the SLs yield due to sugarcane molasses ($Y_{P/M}$) is less compared to the yield from soybean oil ($Y_{P/S}$) (Table 4.8). These findings on the kinetics parameters from the different models were also in agreement with those of Rodrigues et al. (2006). The kinetic results further suggest the low cost medium composed of only sugarcane molasses and soybean oil can be a substitute for conventional glucose containing medium for production of the biosurfactant.

For any bioproduct, it is necessary to study its properties in order to prove its applicability for a given objective. Hence properties of the produced SLs were evaluated with an aim to establish its potential in solubilizing high fats and oils present in dairy wastewater. The following section discusses the properties of the biosurfactant produced using cheap raw materials in the study.

4.2. Properties of Produced SLs

Biosurfactants are evaluated based on properties such as surface/interfacial tension reduction, critical micelle concentration (CMC), fats and oils solubilization and emulsification activity including its stability over wide range environmental factors such as pH and temperature. Therefore, these properties of SLs produced by the yeast were studied in detail.

4.2.1. Minimum surface tension, CMC and interfacial tension of the produced SLs

Critical to any surfactant are its properties such as CMC, surface tension reduction and interfacial tension reduction. CMC is normally referred to the concentration of surfactant at which micelles begins to form and at this point, surface tension of the medium in which the surfactant is dispersed takes up a minimal value. A lower CMC indicates that less surfactant is needed to saturate interface between either air-liquid or liquid-liquid and form micelles. Figs. 4.14 and 4.15 depict the results of determination of CMC and minimum surface tension (mST) in water due to the SLs produced by the yeast using deproteinized whey and sugarcane molasses as hydrophilic substrates, respectively.

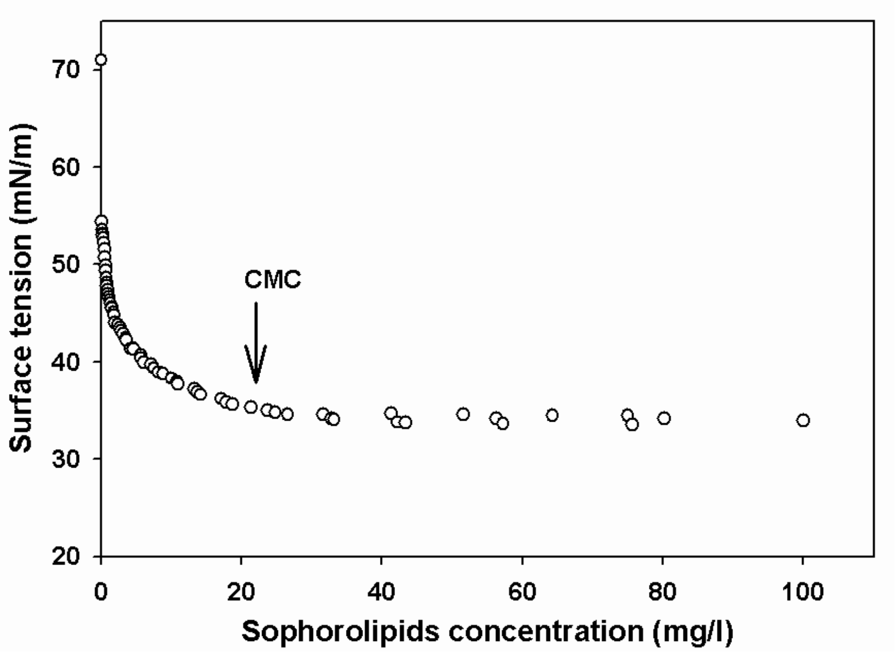


Fig. 4.14: CMC and mST reduction by the SLs produced using deproteinized whey and oleic acid.

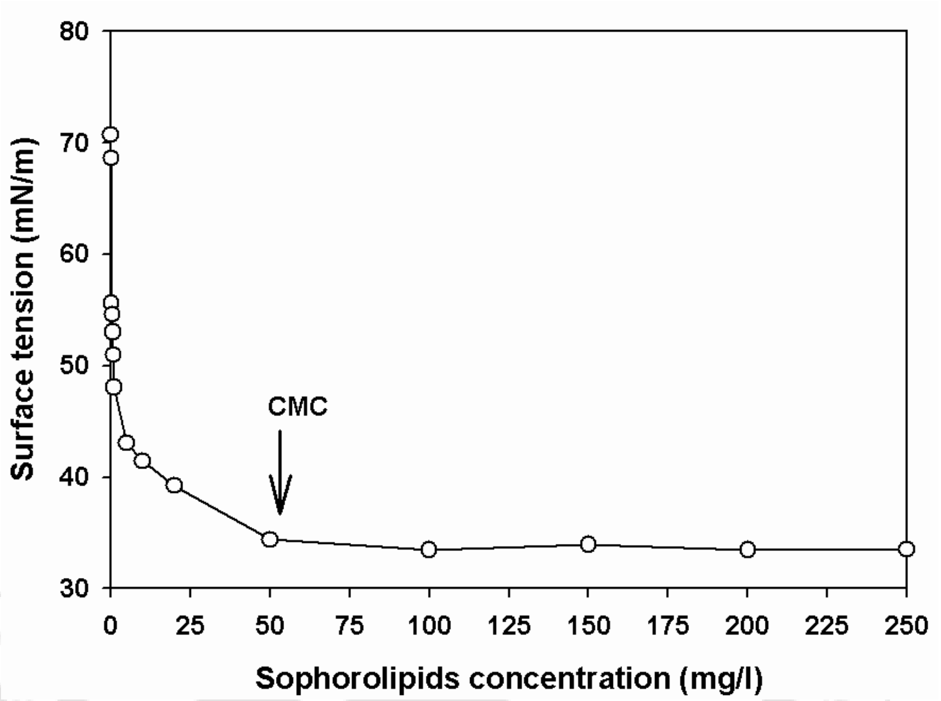


Fig. 4.15: CMC and mST reduction by the SLs produced using sugarcane molasses and soybean oil.

CMC and mST of SLs produced by the yeast using medium based on deproteinized whey and oleic acid were found to be 27.17 mg/l and 34.18 mN/m, respectively. On the other hand, these values obtained using medium based on sugarcane molasses and soybean oil were found to be 59.43 mg/l and 34.15 mN/m respectively. The difference in the CMC values is mainly due to the fact that in the previous case oleic acid was used as a lipophilic substrate which is a pure source of C18:1 fatty acid and reported to give better results compared to vegetable oils (Van Bogaert et al., 2007). Table 4.9 compare the values of CMC and mST of SLs reported in literature by other authors with those obtained in the present study. It is clear from the table that the results obtained in the present study were superior to those reported in the literature. The difference in all these values is mainly because the medium compositions in all the cases are different.

Also the properties of the biosurfactant to reduce the interfacial tension between water and a non-aqueous phase, viz. n-hexane, soybean oil, olive oil, was also tested and found to be 0.99, 3.44 and 4.46 mN/m.

Table 4.9: CMC and mST of SLs produced by *C. bombicola* grown on different substrates.

Sl. no.	Hydrophilic carbon	Hydrophobic carbon	CMC (mg/l)	mST (mN/m)	Reference
1	Deproteinized whey and glucose	Oleic acid	27.17	34.18	Present study
2	Sugarcane molasses	Soybean oil	59.43	34.15	Present study
3	Glucose	Soybean dark oil	150	48	Kim et al (2005)
4	Glucose	Corn oil	82	41	do
5	Glucose	Soybean oil	88	40.5	do
6	Deproteinized whey	Rapeseed oil	130	39	Otto et al (1999)
7	Glucose	Methyl ester of erucic acid	47.5	42	Shin et al. (2010)

Thus, the efficiency of the biosurfactant in reducing the interfacial tension between water and various hydrophobic substances suggests suitability of the product for environmental application such as solubilizing fats and oils present in dairy wastewater, microbial enhanced oil recovery (MEOR) and enhanced oil recovery. However, for environmental applications the produced biosurfactant should remain active at high temperature, ionic strength and wide range of pH. Therefore, the stability of the produced SLs was tested with respect to theses factors and results are presented in the following section.

4.2.2. Effect of environmental parameters on surface tension reduction

For successful industrial applications of any biosurfactant, it is important that it remains stable at extremes of temperature, pH and ionic strength. Hence, the effect of NaCl concentration, heating time and pH on surface tension reduction by the SLs produced in the study was investigated.

Fig. 4.16 shows the effect of these factors on surface tension reduction by the SLs produced using deproteinized whey and oleic acid, from which it is clear that the biosurfactant is highly active at all salt concentrations tested.

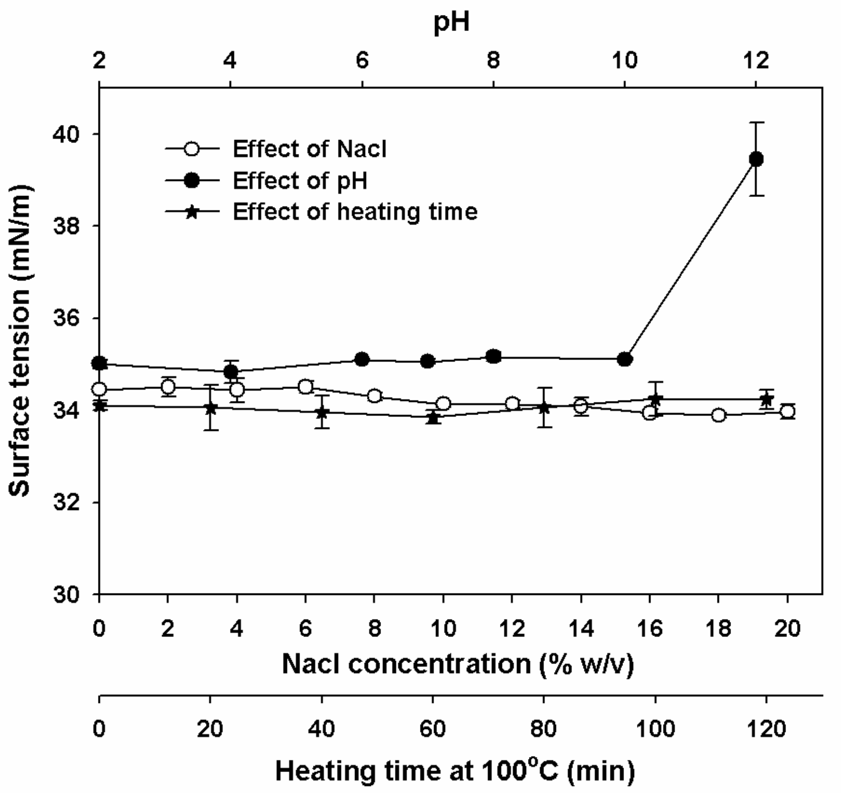


Fig. 4.16: Effect of salt concentration, pH and heating time on surface tension reduction by the SLs produced in the study using deproteinized whey and oleic acid.

In literature, Ghojavand et al. (2008) reported that biosurfactant produced by a member of the *B. subtilis* group (PTCC 1696) showed a maximum surface activity at salt concentrations in the range of only 4–8% (w/v). The difference in the activity of the SLs with respect to the ionic strength compared to that of the biosurfactant produced by *B. subtilis* could be due to the difference in the producer organisms itself and also due to the difference in the composition of the biosurfactant molecule. Further, unhindered surface activity of the SLs in the present study up to 2 h of incubation in boiled water revealed very good thermal stability of the product (Fig. 4.16). The figure also shows that the SLs was highly active over a broad pH range of 2-10; however, above pH 10 its activity decreased sharply and the surface tension increased up to 39.45±0.79 mN/m. This is mainly due to the hydrolysis of ester bond in the SLs at higher pH, particularly above pH 10 (Inoue et al., 1980; Hu and Ju 2001b). Similar profiles were obtained on the effect of these environmental parameters on surface tension reduction by SLs produced using sugarcane molasses and soybean oil.

4.2.3. Solubilization of fat and oil

As SLs produced from deproteinized whey showed similar or slightly better characteristics (minimum surface tension reduction and effect of environmental parameters), than that produced from sugarcane molasses, fat and oil solubilization study was carried out only with SLs produced using deproteinized whey using soybean oil and fat (ghee).

Solubilization ratio, as noted before was determined from slope of a plot between SLs concentrations and oil/fat solubilization beyond its CMC. Fig. 4.17 shows the profiles of solubilization of oil (soybean) and fat (ghee) in the study. From the figure, solubilization ratios were found to be 26.0×10^{-2} and 83.33×10^{-2} for oil and fat, respectively, which implied that 26.0×10^{-2} mg/l of soybean oil and 83.33×10^{-2} mg/l of fats can be solubilized using only 1 mg/l of SLs solution. Because a typical dairy industry wastewater in India may contains 200 – 300 mg/l of fats (Garg and Garg, 1996), approximately only 340 mg/l of SLs is required for solubilizing a maximum amount of 300 mg/l fats in the wastewater. Thus, the results of solubilization ratio obtained in the present study clearly demonstrated a very good potential of the SLs for solubilizing high fats and oils that are common in dairy industry wastewater.

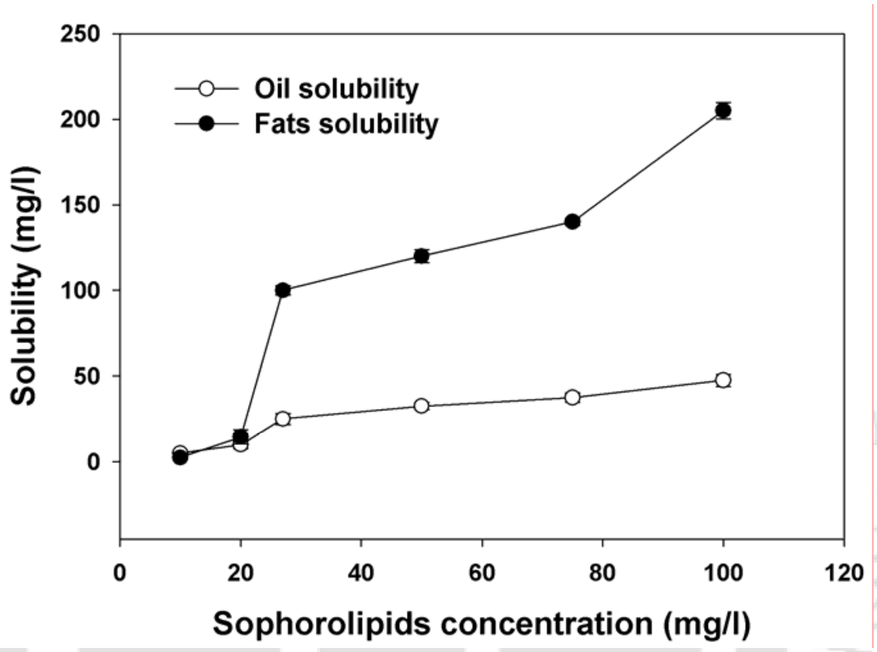


Fig 4.17: Solubilization of fat and oil in aqueous phase using various concentrations of the SLs produced using deproteinized whey and oleic acid.

4.2.4. Emulsification activity and stability

Emulsification activity and its stability with any biosurfactant truly indicate its potential for practical applications. Fig. 4.18 shows the linear form of the emulsion stability profiles towards different oils tested in this study, which were used to calculate the decay constant (k_d) values, as per the relationship in Equation (3.6). Table 4.10 presents the results of emulsification activity and its stability with the produced SLs using various non aqueous phase liquids (NAPLs) as the substrate.

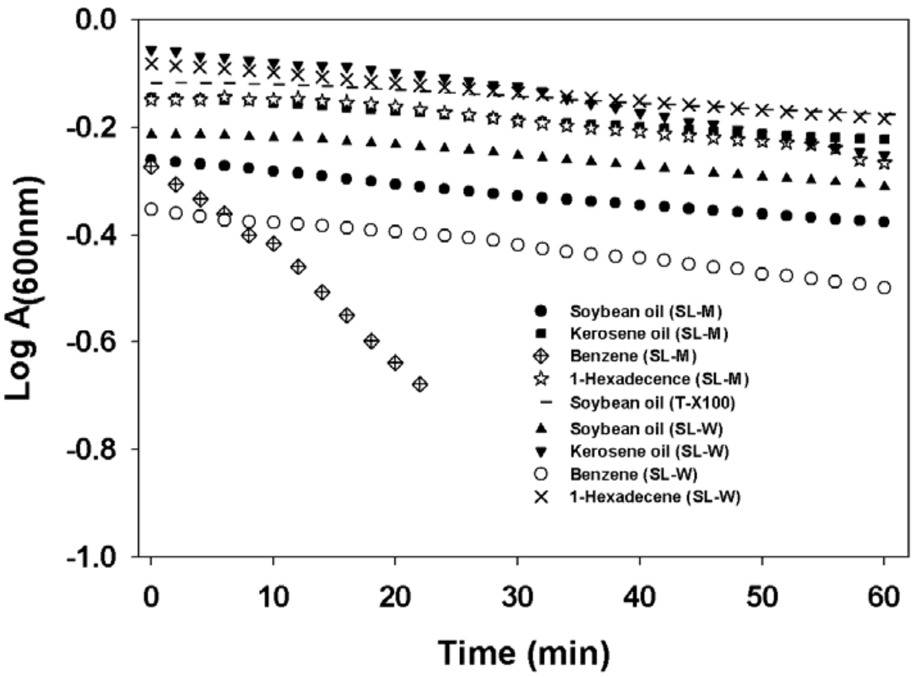


Fig. 4.18: Emulsion stability profile towards different organic solvents due to the produced SLs (SL-M, SLs from molasses; SL-W, SLs from whey and T-X100, Triton X-100).

Table 4.10: Emulsification activity and its stability due to the produced SLs using different non aqueous phase liquids.

Non aqueous phase liquid	**SLs from molasses**		**SLs from whey**	
	Emulsification activity ($A_{600\ nm}$)	**k_d (d^{-1})**	**Emulsification activity ($A_{600\ nm}$)**	**k_d (d^{-1})**
Soybean oil	0.548	-2.88	0.632	-2.592
Kerosene oil	0.716	-1.584	0.880	-4.752
Benzene	0.533	-26.784	0.443	-3.456
1-Hexadecene	0.709	-2.88	0.828	-2.448

All these results showed that the emulsification activities and stabilities of the SLs produced by the yeast using deproteinized whey and oleic acid were found to be slightly better than the values obtained with the SLs produced by the same yeast using sugarcane molasses and soybean oil (Table 4.10). It is understood that using oleic acid as the lipophilic carbon source in the medium results in mainly lactonic type of SLs; on the other hand when soybean oil is used as the lipohilic carbon source, SLs produced are mainly the acidic type. And, because lactonic SLs show better surface tension lowering activity compared to acidic SLs (Van Bogaert et al., 2007). SLs produced using deproteinized whey and oleic acid yielded better emulsification activity compared to those obtained using sugarcane molasses and soybean oil. Among the different NAPLs tested, all the substrates showed comparable emulsification activity and stability, except benzene. And the best results were obtained with kerosene oil.

From Table 4.10, it was concluded that emulsification activity by the SLs from sugarcane molasses followed the order: kerosene oil > 1-Hexadecene > soybean oil > benzene. The stability of emulsion formed using the different NAPLs, also followed the same order. In literature, Mahanty et al., (2006) also reported a similar result of higher emulsification activity towards kerosene oil using an uncharacterized biosurfactant produced by an isolated strain from petroleum contaminated soil.

On the other hand, in case of SLs from deproteinized whey, emulsification activity followed the same order as that due to SLs from sugarcane molasses but the emulsification stability followed the order: 1-Hexadecene > soybean oil > benzene > kerosene oil. Difference in the emulsification activity and stability of the SLs produced using two different carbon sources could be the reason as the composition of SLs changes with different lipophilic carbon source.

The emulsification activity and its stability due to SLs produced using low cost substrates (sugarcane molasses and deproteinized whey) towards soybean oil, was also compared to that obtained using a well known chemical surfactant - Triton X – 100. And the values were found to be 0.762 and -1.584 d^{-1} for emulsification activity and stability, respectively which were close to those obtained using the SLs produced in this work.

SLs produced by the yeast *C. bombicola* were reported by other authors to be a poor emulsifying agent (Cooper and Paddock, 1984; Muthuswami et al., 2008). Cooper and Paddock, (1984) tested the emulsification activity and stability of the SLs produced by the yeast with hydrocarbons and vegetable oils and reported that the SLs was not able to stabilize emulsions containing water and either of the two non-aqueous phase. However, the present study shows that the SLs produced by the yeast when grown on either deproteinized whey or sugarcane molasses as an alternative hydrophilic carbon source to costly glucose had better emulsification activity and stability as compared to those reported by others in the literature (Cooper and Paddock, 1984; Muthuswami et al., 2008). These values were also found to better than the values obtained using different biosurfactants reported in the literature (Mahanty et al., 2006; Lee at al., 2008).

4.2.5. Effect of pH and temperature on emulsification activity and stability by the SLs produced using sugarcane molasses and soybean oil

pH and temperature are known to be the two most important environmental factors influencing the performance of any biosurfactant or bioemulsifier. Therefore, using soybean oil as the substrate, the effects of these two factors were investigated on emulsification activity and its stability due to the produced SLs. The test was performed only on SLs produced using sugarcane molasses and soybean oil. It showed comparable emulsification activity and stability towards different solvent with those produced using deproteinized whey and oleic acid. While Fig. 4.19 (a) and (b) illustrate the emulsification activity of the produced SLs towards soybean oil at different pHs and temperatures, respectively, the effect of these two parameters on emulsion stability is shown in Fig 4.20 (a) and (b).

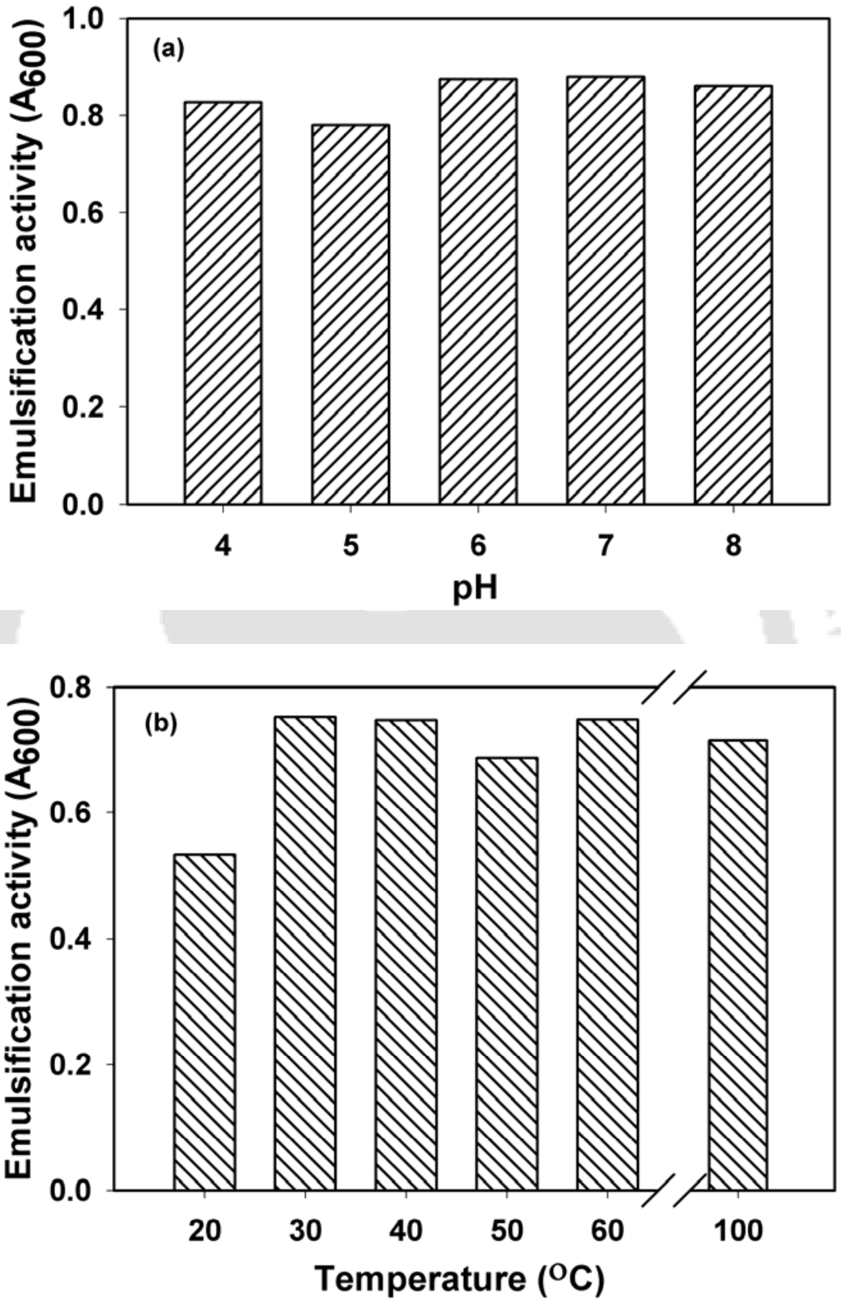


Fig 4.19: Influence of (a) pH and (b) temperature on emulsifying activity of the produced SLs using sugarcane molasses and soybean oil towards soybean oil.

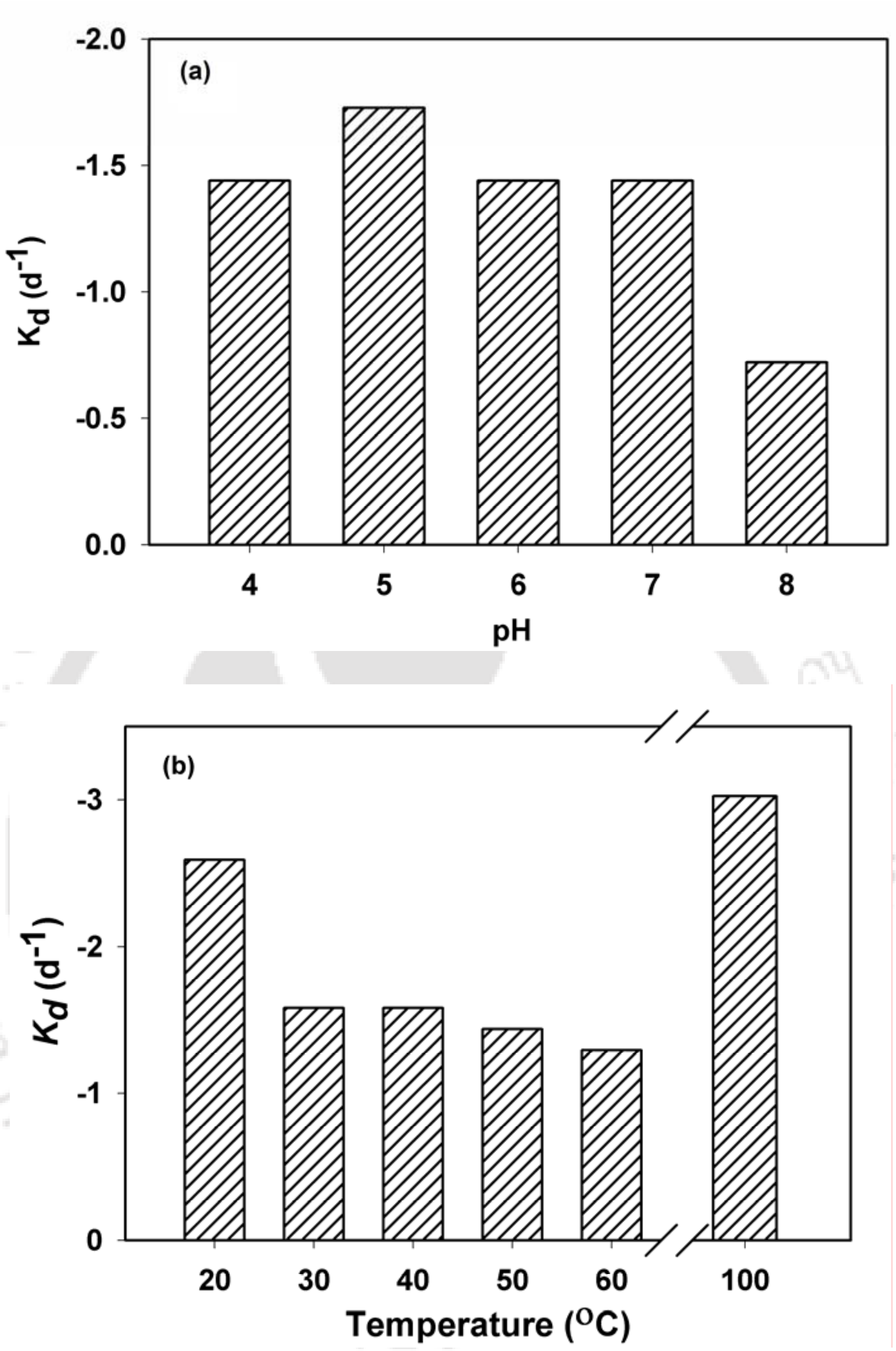


Fig. 4.20: Influence of (a) pH and (b) temperature on emulsion stability with the produced SLs using sugarcane molasses and soybean oil.

It can be seen that the activity of the SLs towards soybean oil was found to be nearly same at the entire pH range (pH 4 – 8) tested with the highest activity shown at pH 7.0 (Fig 4.19a); however, maximum stability of the emulsions formed was found to be at pH 8.0 (Fig. 4.20a). In comparison to liposan, another biosurfactant reported in the literature, these values of emulsification activity and stability at different pH conditions were better.

In case of liposan the emulsification activity was found to be only in a narrow pH range of 2 to 5 (Cirigliano and Carman, 1984). By contrast, alasan, a bioemulsifier from *Acinetobacter radioresistens* reported in the literature, showed maximum activity in a broad pH range of 3.3–9.2 and maximum activity at pH 5.0 (Navon-Venezia et al., 1995). Different nature and sources of these biosurfactants can be cited as the reason for the different optimum pH values for their emulsification activity and stability.

The influence of temperature on emulsifying activity of the produced SLs, depicted in Fig. 4.19 (b), revealed that the activity was low at 20°C than at higher temperatures (30 - 100°C). Similar trend i.e. higher activity of biosurfactants at higher temperatures has also been reported in the literature (Navon-Venezia et al., 1995; Mahanty et al., 2006). On the other hand, Fig 4.20 (b) revealed that the stability of the SLs was found to increase with temperature up to 60°C. This is easily understood since an increase in temperature raise the solubility of SLs in the aqueous phase; but at 100°C the stability of the emulsion decreased probably due to degradation of SLs at this high temperature. This aspect of temperature effect, however, needs further investigations to confirm.

Compared to the literature reports, the present study showed high emulsification activity and stability by the SLs over a wide range of pH and temperature. And, based on the properties of the produced SLs by *C. bombicola* using low cost substrate, it could be well said that the biosurfactant possesses good potential for application in solubilizing high fats and oils present in dairy and such food industry wastewaters, which can be further easily handled by any biological treatment system. Structural characteristics of the biosurfactant were investigated by carrying out its purification and further analysis. The results of structural characterization of the SLs produced using two different cheaply available raw materials are discussed below.

4.3. Purification and Structural Characterization of SLs

Typical structure of SLs consists of a sophorose (dimeric glucose) linked by a glycosidic bond through a hydroxyl group located at the penultimate position of an 18-carbon fatty acid (Gorin et al., 1961; Asmer et al., 1988). Because the biosurfactant occurs as a mixture of compounds containing macrolactone and open-chain or free acid forms, purification of the major SL followed by its structural characterization was carried out in the present study.

4.3.1. Purification by silica gel column chromatography

SLs produced using deproteinized whey and oleic acid showed very good properties, particularly low CMC, compared to that produced using sugarcane molasses and soybean oil. Therefore, SLs obtained from the former was purified using silica gel column chromatography for detailed structural analysis. Silica gel chromatography is a type of adsorption chromatography which is based upon the principle that certain solid materials (collectively known as adsorbents) have the ability to hold molecules at their surface. This adsorption process, which involves weak, non-ionic attractive forces of the van der Waals and hydrogen bonding type, occur at specific adsorption sites. In sililca gel column chromatography, silica is the adsorbent, which has silanol (Si-OH) groups on its surface that are slightly acidic and can interact with polar functional groups of the analyte or eluent. As eluent is constantly passed down the column, differences in these binding strengths eventually leads to the separation of the analyte. The analyte can then be easily detected by thin layer chromatography (TLC) plates (Wilson and Walker, 2010).

For purification of the SLs in the present work, silica column was loaded with SLs mixture and individual SLs were eluted with $CHCl_3$: methanol using gradient system (0 to 20% methanol). Samples were colleted at different time intervals and were spotted to on TLC plates to check for the individual SLs._Fig. 4.21 shows TLC plate of the different fractions collected during the column chromatography. Four different types of SLs were purified and the major SL (denoted as SL -1) was found to be white crystalline in nature, which was further characterized by FTIR, ^{1}H NMR and MS.

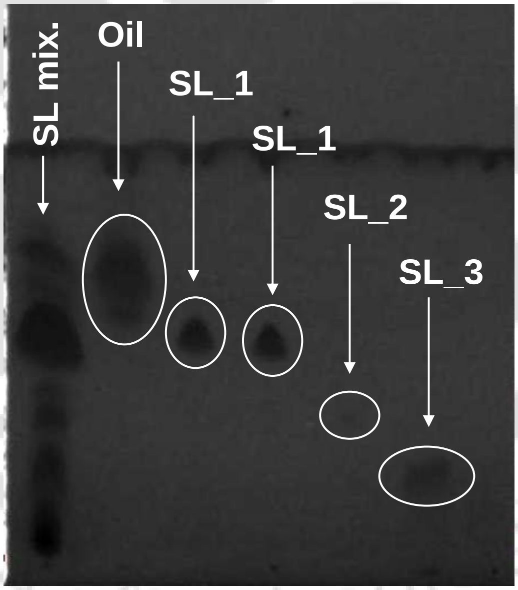


Fig 4.21: TLC plate showing the individual SLs obtained after purification with column chromatography.

4.3.2. Structural characterization of purified SL-1

In general, structural characterization of the organic or inorganic compounds can be done by using Fourier transform infra red (FTIR), nuclear magnetic resonance (NMR) and mass spectroscopy (MS). FTIR relies on the fact that most molecules in a given sample absorb light in the infra-red region (0.8 – 25μm) of the electromagnetic spectrum; this absorption corresponds specifically to the bonds present in the molecule. The sample is irradiated by a broad spectrum of infra-red light and the level of absorbance at a particular frequency is plotted after fourier transforming the data. The resulting spectrum is characteristic of the functional groups of organic molecules present in the sample and is absolutely specific. NMR spectroscopy is another form of adsorption spectroscopy wherein, under appropriate conditions in a magnetic field, a sample can adsorb electromagnetic radiation in the radio frequency region at frequencies governed by the characteristics of the sample. Absorption is a function of certain nuclei (^{1}H, ^{13}C, ^{15}N, ^{19}F, ^{31}P) in the molecule. A plot of the frequencies of the adsorption peaks versus peak intensities constitutes an NMR spectrum. ^{1}H and ^{13}C NMR spectroscopy are the most widely used in biological field to study molecular structure, conformational changes and certain types of kinetic investigation. In MS a mass spectrometer bombard molecules in the vapor phase with a high energy electron beam and records the results of electron impact as a spectrum of positive ions separated on the basis of mass/charge (m/z). This technique is widely used for determination of molecular weight of a given sample. (Silverstaein and Webster, 1998; Wilson and Walker, 2010)

In the present study, SL-1 obtained by column chromatography of the SLs mixture was initially identified and characterized by FTIR. Fig. 4.22 shows the FTIR spectra of the SL-1 which reveals a broad band at 3403 cm^{-1} corresponding to the O-H stretch in its structure. The spectra also reveals that asymmetrical stretching (ν_{as} CH$_2$) and symmetrical stretching (ν_{s} CH$_2$) of methylene groups occurred at 2926 and 2854 cm^{-1}, respectively; further band at 1624 cm^{-1} was observed due to stretching of the unsaturated C=C bonds in the SL-1 molecule (Hu and Ju, 2001b).

While the absorption band at 1747 cm^{-1} indicated the presence of lactone (C=O) group in SL-1, a missing band at 1447 cm^{-1}corresponding to C-O-H in-plane bending of carboxylic acid (–COOH) confirmed the product to be a lactonic type of SL. Further, the stretch of C-O band of C(=O)-O-C in lactones was represented by a band at 1157 cm^{-1}. Moreover, sugar C-O stretch of C-O-H groups was found to be at 1035 cm^{-1} (Hu and Ju, 2001b). All these structural details confirmed the product SL-1 to be lactonic SL.

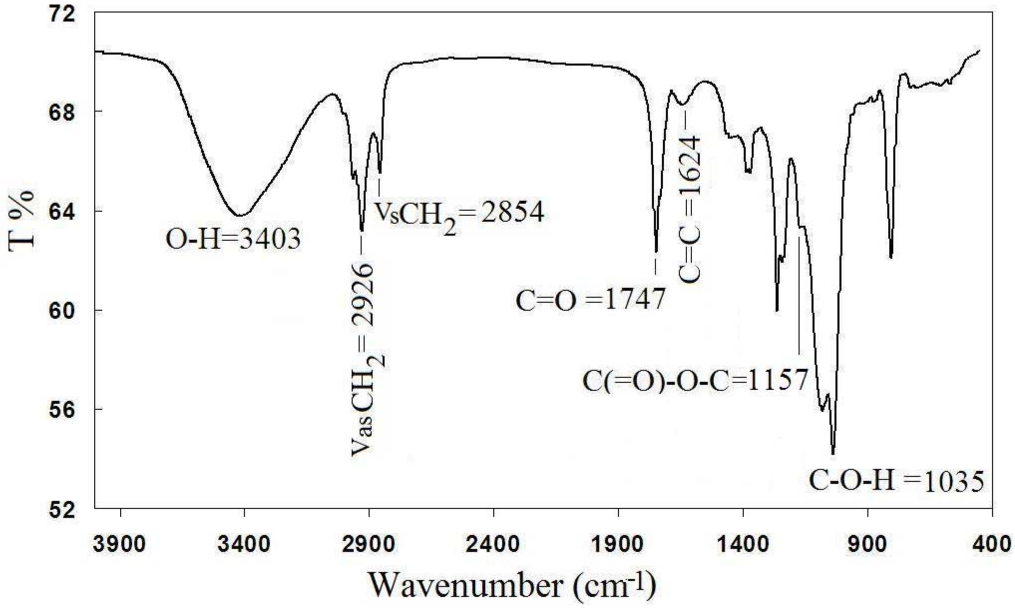
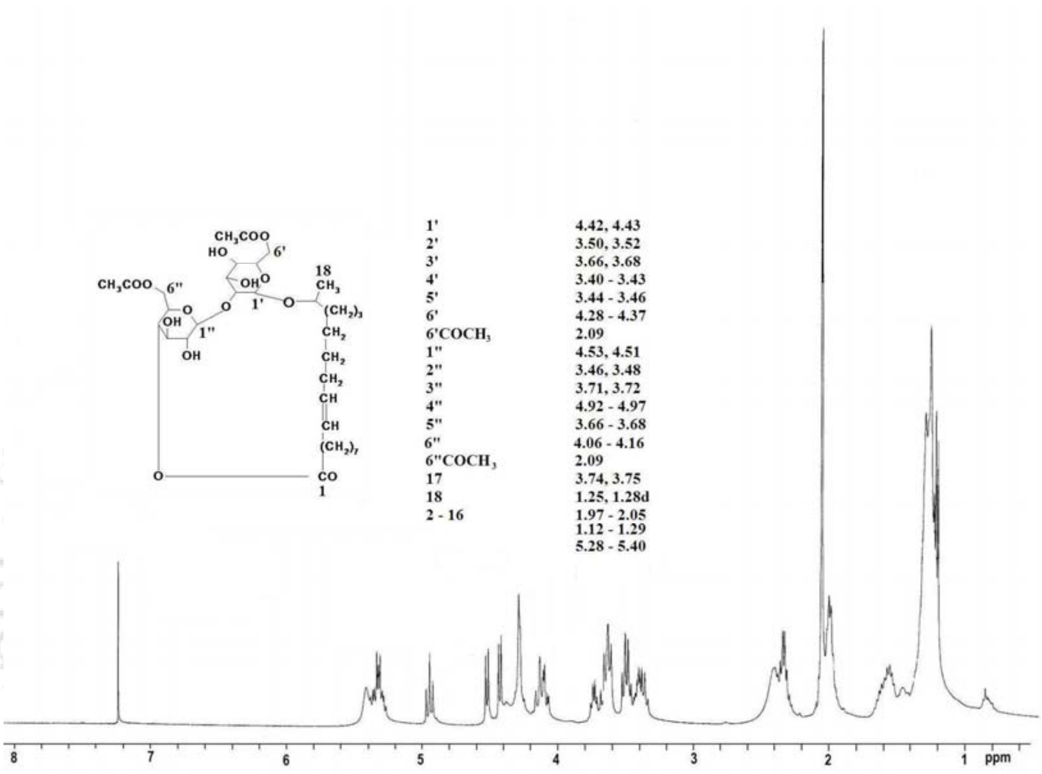


Fig 4. 22: FTIR spectra of the purified SL-1.

An ^{1}H NMR spectrum of the purified SL-1 was taken in CDCl$_3$ and it was assigned to a typical glycolipid-type structure as shown in Fig. 4. 23. The protons of glucose-H-1' and glucose-H-1" (Fig. 4.23) were resonated at 4.42 and 4.43, and at 4.51 and 4.53 ppm, respectively. The other protons of two glucoses were resonated at 3.40–4.50

ppm. Multiple signals of protons at 1.12–1.29 ppm revealed the existence of a fatty acid chain moiety, and signals at 5.28–5.40 ppm revealed –CH=CH– group in the fatty acid chain of SL-1. Also, a signal at 2.09 ppm revealed the presence of (–$COCH_3$) group in SL-1 (Chen et al., 2006).



1'	4.42, 4.43
2'	3.50, 3.52
3'	3.66, 3.68
4'	3.40 - 3.43
5'	3.44 - 3.46
6'	4.28 - 4.37
6'COCH₃	2.09
1"	4.53, 4.51
2"	3.46, 3.48
3"	3.71, 3.72
4"	4.92 - 4.97
5"	3.66 - 3.68
6"	4.06 - 4.16
6"COCH₃	2.09
17	3.74, 3.75
18	1.25, 1.28d
2 - 16	1.97 - 2.05 1.12 - 1.29 5.28 - 5.40

Fig. 4.23: ^{1}HNMR spectra of the purified SL-1.

To elucidate the molecular weight of the purified SL-1, MS analysis was performed. The mass spectrum of the compound is presented in Fig. 4.24. The mass to charge ratio (m/z) at 687.08 (M-1) in the spectra revealed that the molecular weight of SL-1 is 688 D, which is the molecular weight of (17-hydroxyoctadecenoic)-1'4"-lactone-6'6"-diacetate SL (Otto et al., 1999).

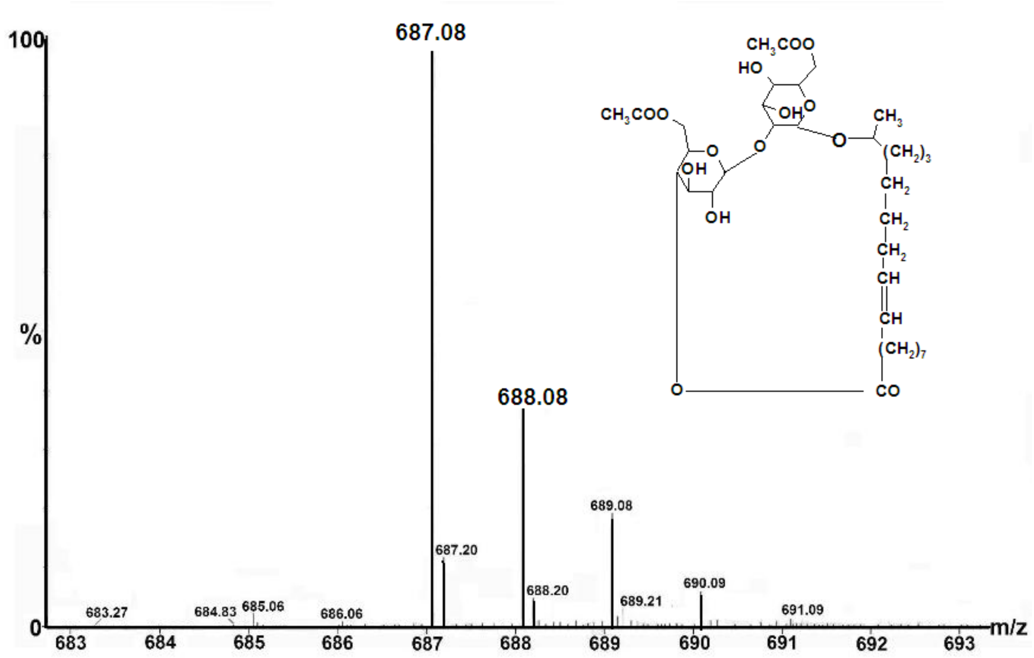


Fig. 4.24: MS spectra of the purified SL-1.

4.3.3. Characterization of SLs produced by the yeast using sugarcane molasses and soybean oil

To investigate whether the characteristics of the SLs produced using two different carbon source (deproteinized whey/oleic acid and sugarcane molasses/soybean oil) were identical or not, partially purified SLs produced using sugarcane molasses and soybean oil were similarly characterized by FTIR, ^{1}HNMR and MS as before, and by ^{13}CNMR. The SLs produced using sugarcane molasses/ soybean oil were not purified by column chromatography because the properties of SLs were inferior to those obtained using deproteinized whey/oleic acid. Fig 4.25 shows the FTIR of the SLs mixture produced by the yeast using sugarcane molasses and soybean oil as substrates which reveals that the spectra is almost identical to that of the purified SL-1 produced using deproteinized whey. However, the sharp peak at 1447 cm^{-1} (which was missing in the FTIR of purified SL-1) corresponding to C-O-H in-plane bending of carboxylic acid (–COOH), indicated that the product contained acidic types of SLs besides the major lactonic type. Thus it was observed that the yeast produced a mixture of acidic and lactonic SLs.

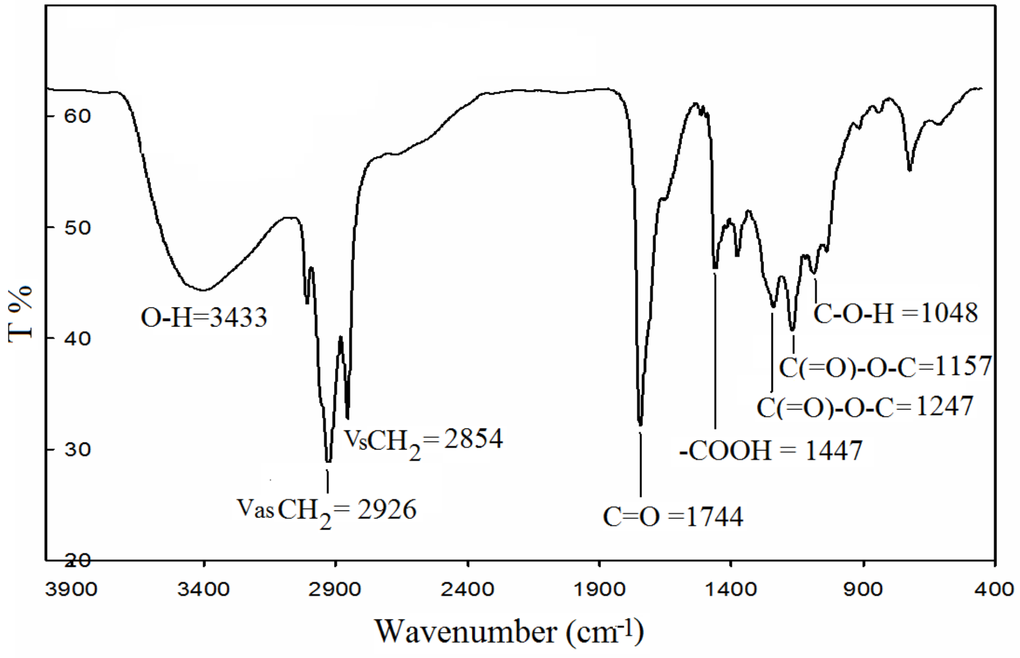


Fig. 4.25: FTIR spectra of the partially purified SLs from the yeast grown on sugarcane molasses and soybean oil.

The ^{1}HNMR of partially purified SLs, was also similar to that of SL-1, also confirmed the presence of SLs in the fermented product (Fig 4.26). Further, ^{13}C NMR spectrum of the SLs (Fig. 4.27) showed the presence of several =CH- groups in the fatty chain moiety resonated between 128 and 130 ppm, which may be probably due to contribution from different SLs in the sample. In addition, several $–CH_2-$ groups in the fatty chain moiety were also resonated at 21.02 - 37.68 ppm, confirming the presence of fatty acid moieties.

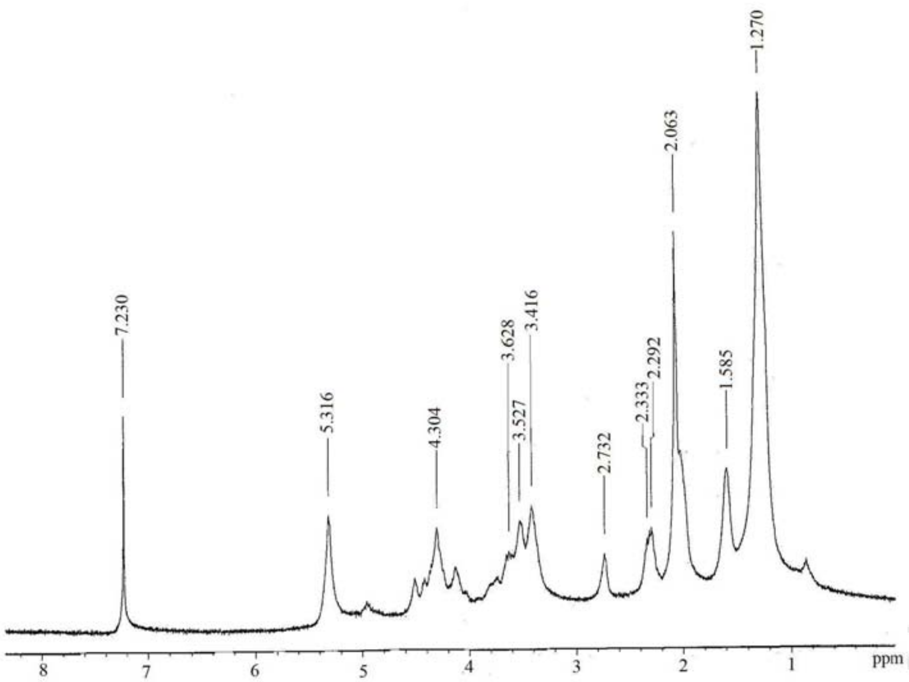


Fig. 4.26: ^{1}H NMR spectra of the partially purified SLs from the yeast grown on sugarcane molasses and soybean oil.

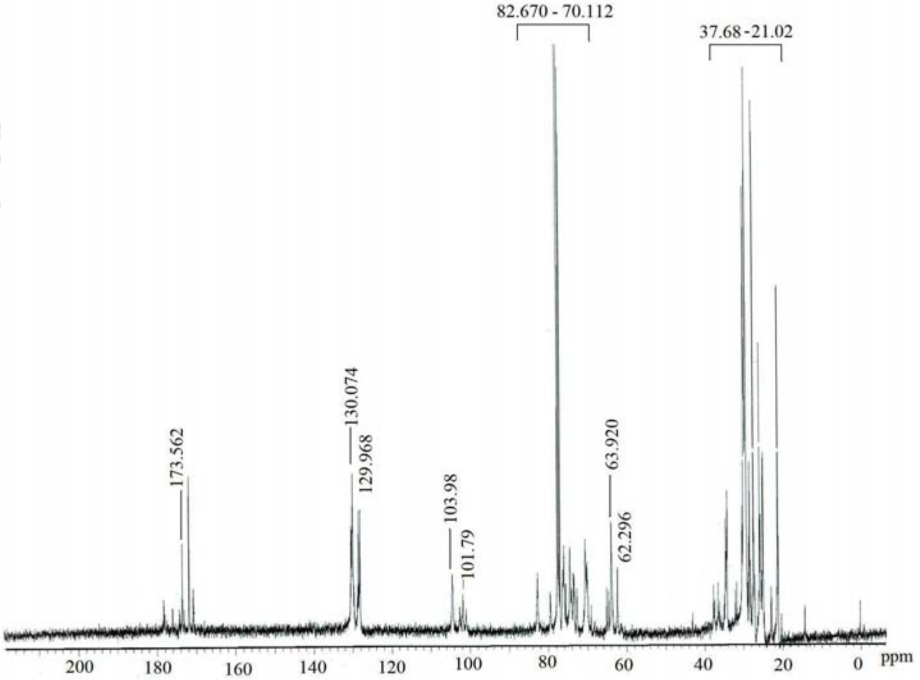


Fig. 4.27: ^{13}C NMR spectra of the partially purified SLs from the yeast grown on sugarcane molasses and soybean oil.

The spectrum also revealed signals of glucose-C-1′ at 103.98 ppm, glucose-C-1″ at 101.79 ppm, glucose-C-6′ at 63.92 ppm and glucose-C-6″ at 62.29 ppm; the other carbon atoms of glucose were resonated between 70.11 and 82.67 ppm (Chen et al., 2006). All these resilts confirmed that the yeast produces multiple SLs (acidic as well as lactonic SLs) during the fermentation.

The mass spectrum of the compound is presented in Fig. 4.28, which also indicates the presence of a mixture of SLs with varying molecular weights. However, the m/z at 717.36 (M+H) in the spectra revealed that the mixture predominantly contains a SL molecule with 716 D molecular weight which is different from the SL-1 produced by the yeast using deproteinized whey/oleic acid as substrates.

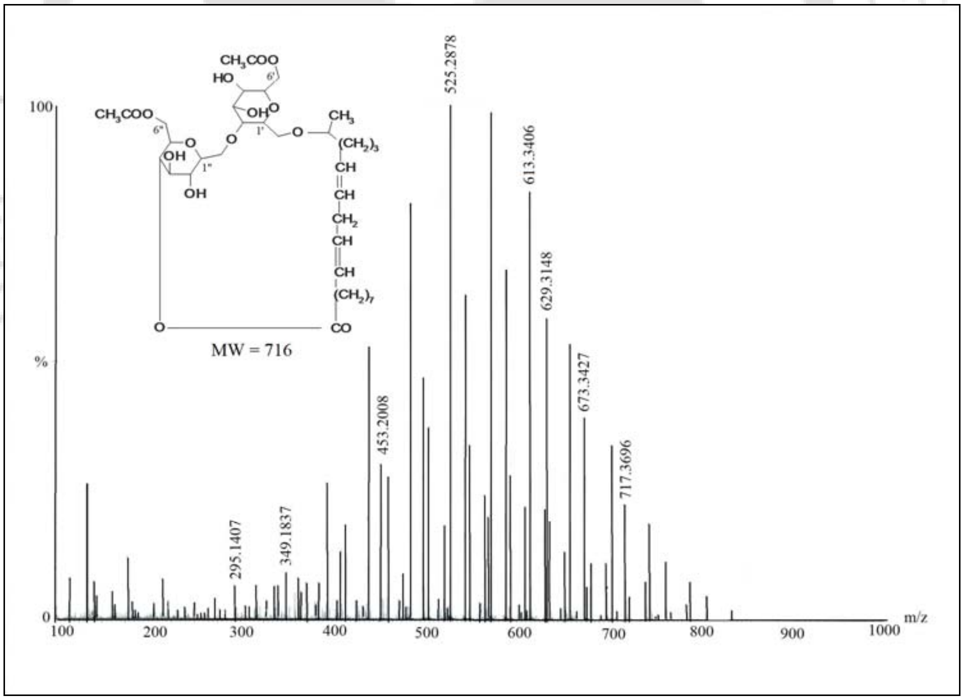


Fig. 4.28: Mass spectra of the partially purified SLs from the yeast grown on sugarcane molasses and soybean oil.

Further, a decrease of m/z from 717.36 to 673.34 and then to 629.31 clearly indicated the fact that the SL molecule was diacetylated (Fig. 4.28). In addition, presence of two sugar moieties in the SL molecule was ascertained by a respective decrease in the m/z at 453 and at 295 of the spectrum (Fig. 4.28). Moreover, peak at 295 m/z in the spectrum revealed a C18 : 2 lipidic moiety in the SL molecule. Similarly, in the literature, Hu and Ju (2001a) reported, from the results of LC-MS analysis of SLs produced by *C. bombicola* using glucose and soybean oil as the carbon sources, that the compound was a mixture of both lactonic (C18:0, C18:1, C18:2 and C16:0) and acidic SLs (C18:0, C18:1, C18:2 and C16:0).

These differences in the structural composition of the SLs produced from two different hydrophilic as well as lipophilic carbon sources could be attribute to the slight difference in the properties of the biosurfactant viz. CMC, emulsification activity and stability towards different NAPLs as noted earlier. Overall, it could be said that the SLs produced by the yeast using different carbon source were quite similar both in terms of the properties and characterization, ideally suited for solubilizing high fats and oils in wastewater. In order to validate this aspect, further investigations were carried out in this direction. The following section discusses the results of SLs production using synthetic dairy wastewater and its pretreatment.

4.4. Feasibility Study on Synthetic Dairy Wastewater for SLs Production and Pretreatment by the Yeast *C. bombicola*

Initial experiments in this feasibility study were carried out by preparing synthetic dairy wastewater for SLs production by the yeast.

4.4.1. SLs production in batch shake flasks

Different compositions of synthetic dairy wastewater with or without extra carbon and nitrogen sources were tested for SLs production and yeast growth in batch shake flasks. For extra carbon and nitrogen source in the wastewater, either sugarcane molasses or sugarcane molasses-soybean oil or glucose-soybean oil-yeast extract-urea was used. Because the yeast could not utilize lactose present in deproteinized whey which would otherwise lead to high COD in the wastewater, the same was not tested in the study. Time course of SLs production during the yeast fermentation using different media based on synthetic dairy wastewater investigated in this study is presented in Fig. 4.29; it can be observed that a maximum SLs production of 38.76±1.3 g/l was obtained using the wastewater supplemented with sugarcane molasses and soybean oil (medium type C), which was however, found to be slightly less compared to maximum 45 g/l of SLs produced by the yeast using only sugarcane molasses and soybean oil in batch shake flasks. This less biomass growth using medium type C could be the possible reason for this.

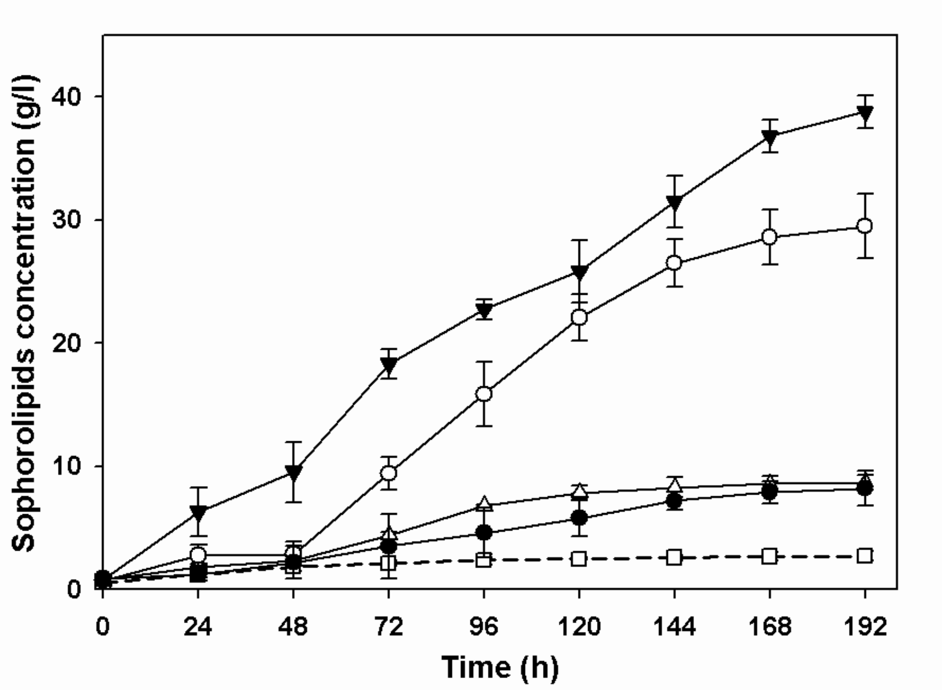


Fig 4.29: Time course of SLs production by the yeast employing the different media types based on synthetic dairy wastewater with and without any added nutrients (--□-- Medium type A; ▬Δ▬ Medium type B; ▬▼▬ Medium type C; ▬○▬ Medium type D; ▬●▬Medium type E).

On the other hand when synthetic wastewater was supplemented with glucose, yeast extract, urea and soybean oil (medium type D) 29.49±2.9 g/l of SLs produced by the yeast was similar to that obtained without synthetic dairy wastewater. The production, however, decreased to 8.71±0.6 g/l when the synthetic wastewater was supplemented with only sugarcane molasses. Further, the SLs production was found to be very less when synthetic wastewater (medium type A) was not supplemented with any external carbon or nitrogen source (2.78±1.29 g/l of SLs). These results confirmed that for optimal SLs production both hydrophilic and lipophilic carbon source is necessary.

With respect to the yeast biomass concentration obtained using synthetic wastewater supplemented with glucose, yeast extract, urea and soybean oil the value was higher compared to other compositions of the synthetic wastewater (Fig. 4.30).

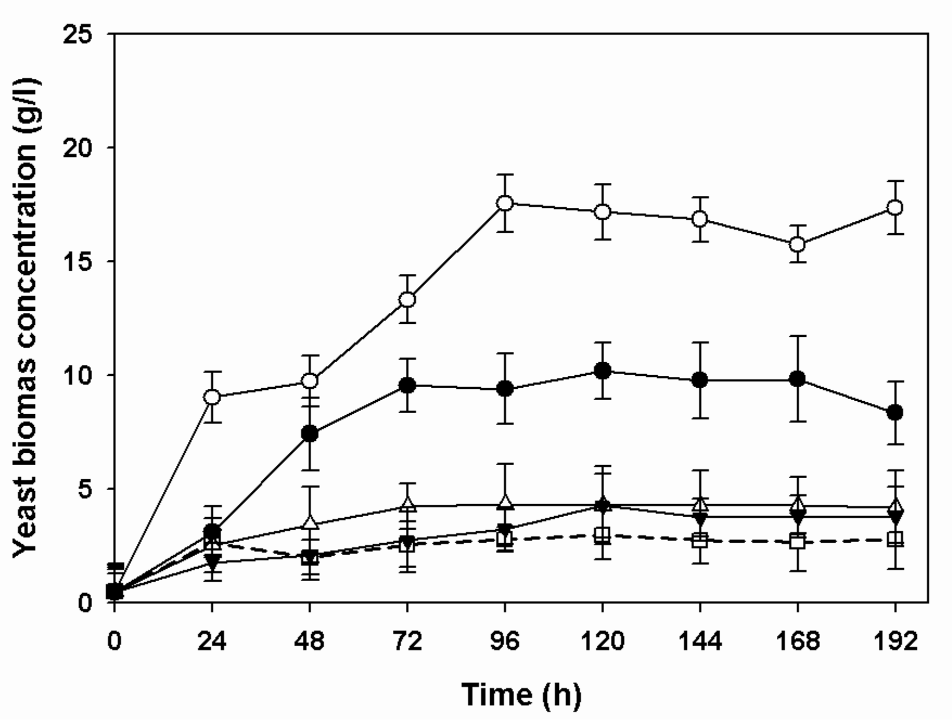


Fig 4.30: Time course of yeast biomass growth obtained using the different media types based on synthetic dairy wastewater with and without any added nutrients (--□-- Medium type A; —Δ— Medium type B; —▼—Medium type C; —○— Medium type D; —●—Medium type E).

That increase in yeast biomass is mainly due to high nitrogen concentration and easy utilization of glucose than sugarcane molasses or the synthetic wastewater by the yeast. The yeast biomass produced was very less (2.78±1.29 g/l) when synthetic dairy wastewater was used as a medium without any extra carbon and nitrogen source (medium type A).

The time course of carbohydrate utilization by the yeast is presented in Fig. 4.31, which shows that the yeast was able to consume all the carbohydrate in medium type D, which contained glucose as extra carbon source in the wastewater. It is also clear from Fig. 4.31 that the yeast was able to utilize the carbohydrates present in the synthetic dairy wastewater (media type A and E). On the other hand approximately 60% of the carbohydrate remained unutilized when the synthetic wastewater was supplemented with sugarcane molasses.

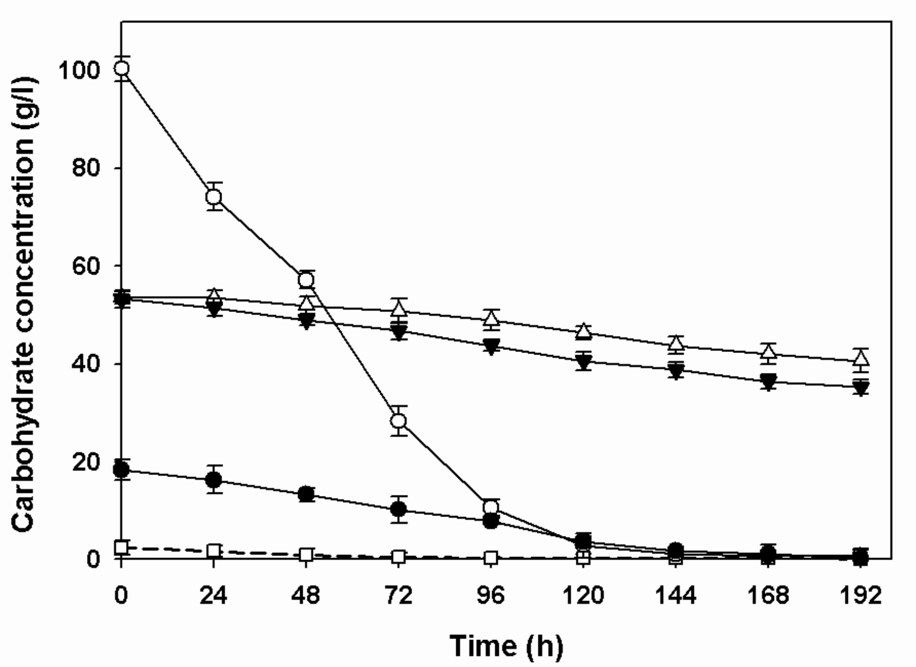


Fig 4.31: Time course of carbohydrate utilization by the yeast employing the different media types based on synthetic dairy wastewater with and without any added nutrients (--□-- Medium type A; ▬Δ▬ Medium type B; ▬▼▬Medium type C; ▬○▬ Medium type D; ▬●▬Medium type E).

Overall, from Figs.4.29 – 4.31 it was concluded that although the yeast is able to grow, produce SLs and utilize carbohydrate in the wastewater; however, when the wastewater is supplemented with additional carbon and nitrogen sources, the yeast could produce more biomass and SLs than without supplementation.

4.4.2. Pretreatment of synthetic dairy wastewater

For pretreatment under batch mode, initial experiments were carried out with 1 L of synthetic wastewater prepared in the laboratory in a 3 L fermentor under non-sterile condition. The results of initial batch fermentor experiment carried out using the synthetic dairy wastewater showed that the yeast could able to remove only a maximum of 50% of initial COD even after 120 h with very less utilization of the fats present in the wastewater (Fig. 4.32).

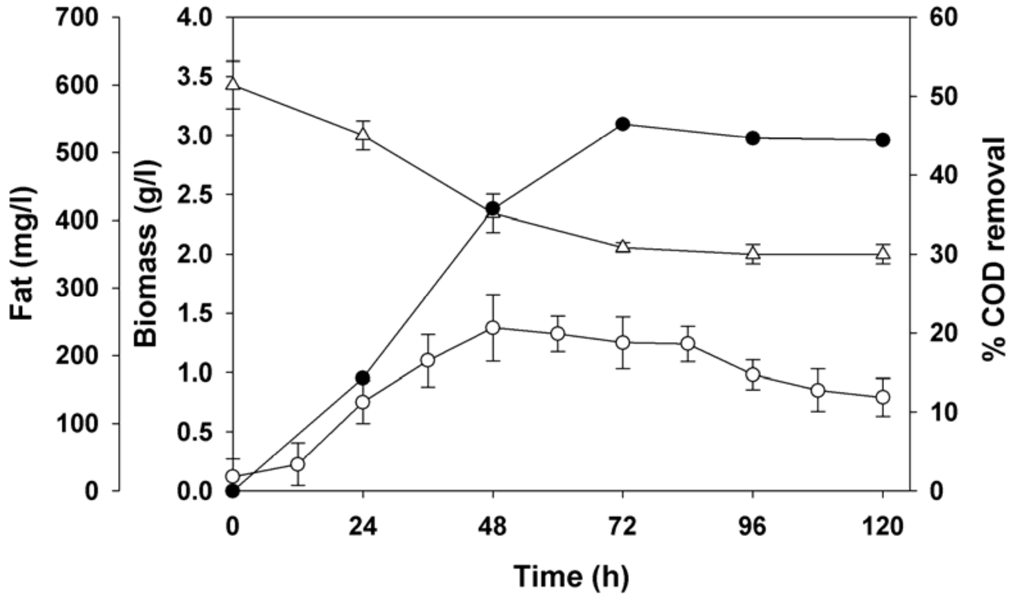


Fig. 4.32: COD reduction, fat utilization and yeast biomass growth by *C. bombicola* using the synthetic dairy wastewater in the fermentor operated under batch mode (▬○▬ Yeast biomass, ▬Δ▬ Fat, ▬●▬ % COD removal).

The produced SLs was also found to be below detectable limit most probably due to poor growth of the yeast. Thus, to investigate pretreatment of the wastewater using the yeast, sugarcane molasses and yeast extract were added in small amounts of 1% (w/v) and 0.1% (w/v), respectively, to support the yeast growth and SLs production.

All experiments to pretreat synthetic dairy wastewater supplemented with sugarcane molasses and yeast extract by the yeast was carried out using a fermentor operated under different modes of operation viz. batch, fed-batch and continuous. Because each mode of operation has its own advantages and disadvantages, all three were tested in the study.

4.4.2.1. Pretreatment under batch mode of operation

Batch mode is the simplest mode of operation and is characterized by growth of microorganisms without supply of additional substrate (wastewater) after starting the culture. In other words, it is a closed system, where all of the wastewater required for pre/treatment by the microorganism is contained within the vessel at the start of the process (Krahe 2007; Macauley-Patrick and Finn 2008).

From the previous results, it was established that for better handling the wastewater, supplementation with minimum amount of hydrophilic carbon and nitrogen source is necessary. Hence wastewater pretreatment was carried out by adding 1% (w/v) of sugarcane molasses and 0.1% (w/v) of yeast extract to the wastewater. Moreover, because it was shown that lactose present in deproteinized whey could not be well-utilized by the yeast, even though SLs produced using deproteinized whey exhibit slightly better properties than shown by those SLs produced by using sugarcane molasses, deproteinized whey was not tested for pretreating the wastewater.

Fig. 4.33 shows the results of pretreating the synthetic wastewater supplemented with sugarcane molasses (1%) and yeast extract (0.1%) in the batch operated fermentor which reveals complete utilization of fats in the wastewater within 96 h; also, the total carbohydrate remaining to be utilized was negligible with a maximum biomass concentration obtained at 72 h (4.53 g/l). Further, the yeast showed very high COD removal efficiency of more than 93% in the system (Fig. 4.33). The amount of SLs produced by the yeast was, however, very less (1 – 2 g/l) owing to the low amount of the hydrophobic carbon source (fats and oils) present in the wastewater. Nevertheless, the amount was sufficient enough to completely solubilize the fats present in the wastewater.

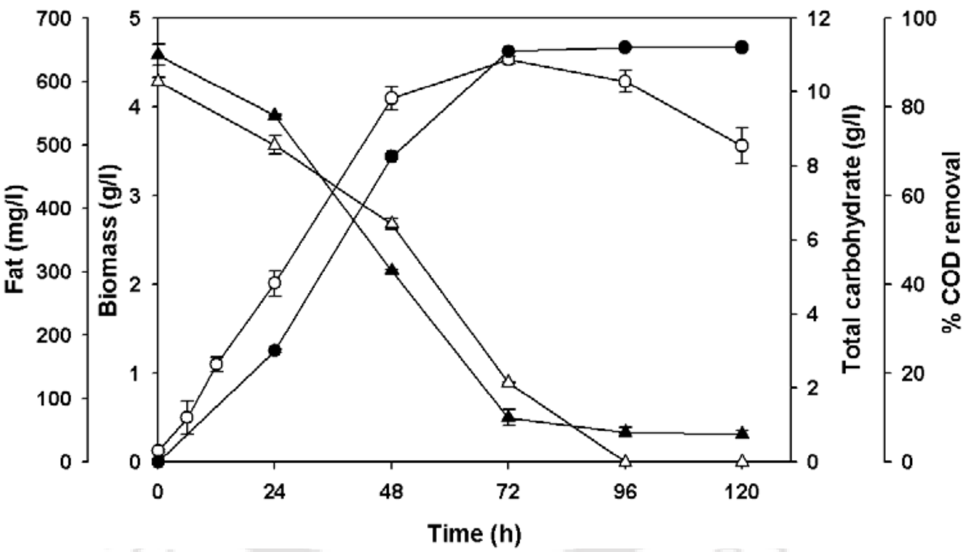


Fig 4.33: COD reduction, fat solubilization, carbohydrate utilization and yeast biomass growth by *C. bombicola* using synthetic dairy wastewater in the fermentor operated under batch mode (—○— Yeast biomass, —▲— Total carbohydrate, —Δ— Fat, —●— % COD removal). (The SDWW was supplemented with sugarcane molasses and yeast extract).

Following batch experiments in the fermentor, fed-batch and continuous modes of operation were further evaluated to investigate the efficiency of the biosurfactant producing yeast in pretreating the synthetic dairy wastewater.

4.4.2.2. Pretreatment under fed-batch mode of operation

Fed-batch operation of an aeration tank is a rather new concept in wastewater treatment technology and only few reports are available in wastewater treatment (Kargi and Dincer, 1996; Bali and Şengul, 2002). Fed-batch operation involves slow addition of highly concentrated wastewater (or nutrient media) into an aeration tank (or reactor) with no effluent removal until the tank is full. An aeration tank contains a large volume of highly active and dense organisms at the beginning of the operation. With slow feeding, concentrated or toxic wastewater gets diluted inside the reactor, thereby resulting in less inhibition and higher COD/BOD removal rates.

Therefore, in this study to increase the efficiency of the system in pretreating synthetic dairy wastewater fed-batch experiment was carried out in a 3 L fermentor with 1 L initial working volume. For operating the fermentor under fed-batch mode, feed-rate of the wastewater was calculated as per the following equation (Yamane and Shimizu, 1984):

$$F = \frac{\mu X_0 V_0 e^{\mu t}}{Y_{X/s} S_0} \tag{4.2}$$

where, F is the feed rate of the wastewater (1/h); X_0 is the yeast biomass concentration at the end of batch operation (g/l); V_0 is the volume of wastewater in the reactor at the end of batch; S_0 is total carbohydrate concentration (g/l) taken as input to the wastewater.

The biokinetic constants specific growth rate, μ (1/h), and yield of yeast biomass, $Y_{X/S}$, were estimated from the batch data using the following equations:

$$\mu = \frac{1}{X}\frac{dx}{dt} \tag{4.3}$$

$$Y_{X/S} = \frac{X_m - X_0}{S_0 - S_m} \tag{4.4}$$

In Equation (4.4) X_m represents maximum cell concentration (g/l) at time (t), X_0 is the initial cell concentration (g/l) at initial time (t = 0), S_m is the total substrate concentration (g/l) at time (t) and S_0 the total substrate concentration (g/l) at initial time (t = 0). For calculating μ, linearized form of Equation (4.3) was used. The method followed for calculating μ is described in Appendix A2.

Based on the values of the biokinetic constants obtained (μ, 0.072 h^{-1} and $Y_{X/S}$, 0.675) feeding was started at the end of 48 h of batch and later switched over to batch. Fig. 4.34 shows the results of COD reduction, fats utilization, yeast biomass and carbohydrate utilization in the synthetic wastewater in the fermentor operated under fed-batch mode.

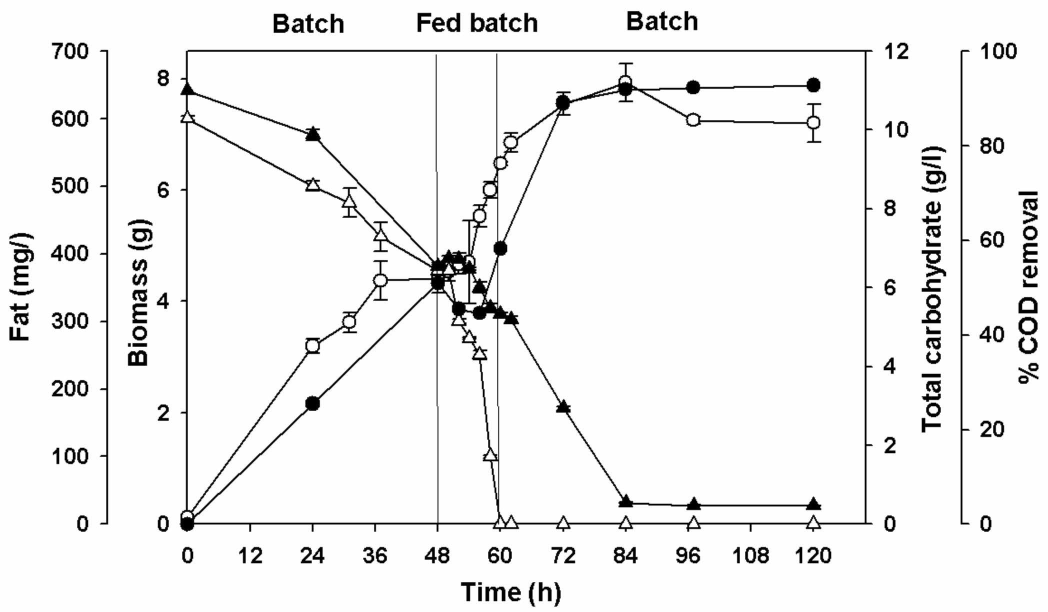


Fig. 4.34: COD reduction, fat solubilization, carbohydrate utilization and yeast biomass growth by *C. bombicola* using synthetic dairy wastewater in a fermentor operated under fed-batch mode of operation (—○— Yeast biomass, —▲— Total carbohydrate, —△— Fat, —●— % COD removal). (The SDWW was supplemented with sugarcane molasses and yeast extract).

All these profiles indicate complete utilization of fats in the synthetic wastewater within 10 h of fed-batch operation with 93% COD removal efficiency at the end of 84 h. Similar to batch experiments approximately 2 g/l of SLs obtained in fed-batch experiments. Further, in these experiments the amount of wastewater treated was twice compared to that treated in the batch experiments. These results clearly indicate that fed-batch mode of operation is more advantageous than batch. The yeast could able to pretreat the synthetic dairy wastewater more quickly and efficiently than under batch operated condition. However, because continuous mode of treatment is more preferred due to its high productivity compared with batch and fed-batch treatment system, the same was evaluated further in the study.

4.4.2.3. Pretreatment under continuous mode of operation

For performing continuous experiments using the fermentor, the reactor was initially run under batch mode up to 72 h and then shifted to continuous mode with a synthetic wastewater retention time (HRT) of 28.5 h which corresponded to a dilution rate of 0.035 h^{-1} in the reactor. Fig. 4.35 depicts the results obtained from the continuous experiments. It could be seen from the figure that throughout the continuous operation period of 72 h no trace of fats/oils was found in the synthetic wastewater and the yeast biomass concentration also remained constant. Similar to the previous batch and fed-batch experiments, the yeast showed 93% COD removal from the synthetic wastewater in this continuous reactor study. Also, the yeast produce small amount of SLs (2-3 g/l) after 72 h of operation and its level was remain constant during the continuous operation period.

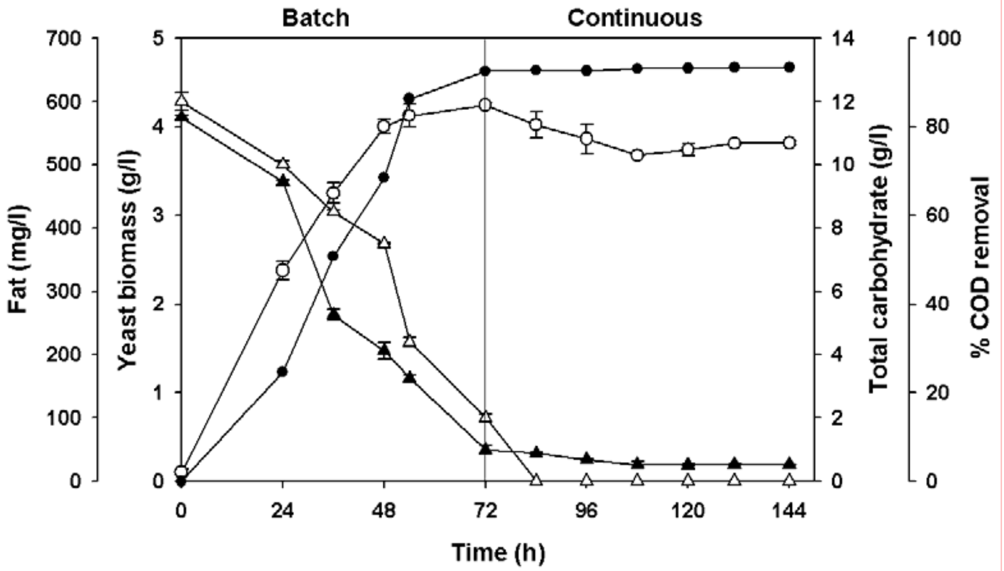


Fig. 4.35: COD reduction, fat solubilization, carbohydrate utilization and yeast biomass growth by *C. bombicola* using synthetic dairy wastewater in a fermentor operated under continuous mode of operation (▬○▬ Yeast biomass, ▬▲▬ Total carbohydrate, ▬Δ▬ Fat, ▬●▬ % COD removal). (The SDWW was supplemented with sugarcane molasses and yeast extract).

Overall the SLs producing yeast could not only completely utilize the fats present in the synthetic wastewater, but also showed very high COD removal efficiency, both for a very low HRT of little longer than 1 d. These aspects clearly demonstrate very good potential of the biosurfactant producing yeast in removing fats and oils and biological load from real dairy wastewater. To validate this aspect experiments were carried out with a real dairy industry wastewater.

4.5. Pretreatment of Real Dairy Wastewater by the Yeast *C. bombicola*

Besides the milk contents (lactose, fat and oils) real dairy wastewater may contain detergents used for floor washing and variety of sterilizing agents (Bakka, 1992). Also, it may contain grease removed from instruments and machines during the washing and cleaning steps. This make a real dairy wastewater more complex than synthetic wastewater and such real wastewater need to be tested for establishing the potential of the whole work. Real dairy wastewater collected from a local dairy in Guwahati was initially characterized and later tested for pretreatment by the SLs producing yeast.

4.5.1. Composition of dairy wastewater

Table 4.11 presents composition of the dairy wastewater which reveals that the wastewater contained high BOD and COD. The high organic load is mainly owing to the dissolved milk protein, sugars and fats and oils present in the wastewater. Fig 4.36 is a photograph showing general appearance (colour) of the dairy wastewater used in the study.

Table 4.11: Composition of the raw dairy wastewater.

Parameter	**Value**
pH	5.94
Total solids content	2970 mg/l
Total suspended solids	350 mg/ l
Total dissolved solids	645 mg/l
Total fat content	407 mg/l
Total carbohydrates	5.6 g/l
BOD	740 mg/l
COD	2136 mg/l
Colour	Dark grey
Smell	Pungent

Fig. 4.36: Photograph showing wastewater sample collected from the dairy industry.

As reported in literature by other authors (Gavala et al., 1999; Rajeshkumar and Jayachandran, 2004; Sarkar et al., 2006) the pH of the dairy wastewater was slightly acidic, i.e. near 6.0 which is, however, best suited for the yeast *C. bombicola*. Sarkar et al (2006) studied the characteristics of diary wastewater collected from an industry in Hyderabad, India, and found similar composition of the industrial effluent.

According to the Central Pollution Control Board (CPCB), India, the maximum permissible limit of BOD, COD and fats and oils in wastewater for discharge into natural stream are 30 mg/l, 250 mg/l and 10 mg/l, respectively. It is clear from the wastewater composition in Table 4.11 that it is essential to treat such wastewater before discharging it into the environment. Further, the high amount of fats and oils (407 mg/l) present in wastewater may interfere in the biological treatment which require that pretreatment of dairy wastewater is necessary.

4.5.2. Pretreatment of dairy wastewater in fermentor

Prior to experiments with the dairy wastewater using a fermentor, suspended solids in the wastewater were removed by centrifugation (1000 × g, for 10 min). The pH of the wastewater was not adjusted before and during the experiments. Also, experiments were carried out under non-sterile conditions in the fermentor with the yeast culture.

According to literature reports, estimation of BOD is not ideally suited for studies on process design, treatability, control of treatment plants, setting of standards for treated effluents and assessment of the effect of polluting discharges on the oxygen resources of receiving waters and rather removal of COD to access efficiency of a treatment system is suggested (Montgomery, 1967; Rajeshkumar and Jayachandran, 2004). Hence, in the present study, COD instead of BOD was analyzed to determine the efficiency of the SLs producing yeast in pretreating the wastewater. Similar to previous experiments carried out using synthetic wastewater, real dairy wastewater was supplemented with sugarcane molasses (1%) and yeast extract (0.1%) to support the yeast growth. Also, experiments were performed using 1 L of wastewater in a 3 L fermentor under different modes of operation. During the experiments fats and oils, COD, SLs concentration, carbohydrate concentration and yeast biomass were monitored.

4.5.2.1. Pretreatment under batch mode of operation

Fig 4.37 shows the results of batch experiments using real industry wastewater supplemented with sugarcane molasses (1%) and yeast extract (0.1%) which reveals that the yeast was able to produce maximum biomass (3.5 g/l) at 66 h, able to utilize all the fats and oils present in the wastewater after 72 h and reduce 96% of COD. Also, during the experiment the yeast produced approximately 2-3 g/l of SLs which was more than sufficient to solubilize and remove the high fats and oils present in the wastewater. These results were found to be similar or even better compared with those obtained with synthetic wastewater.

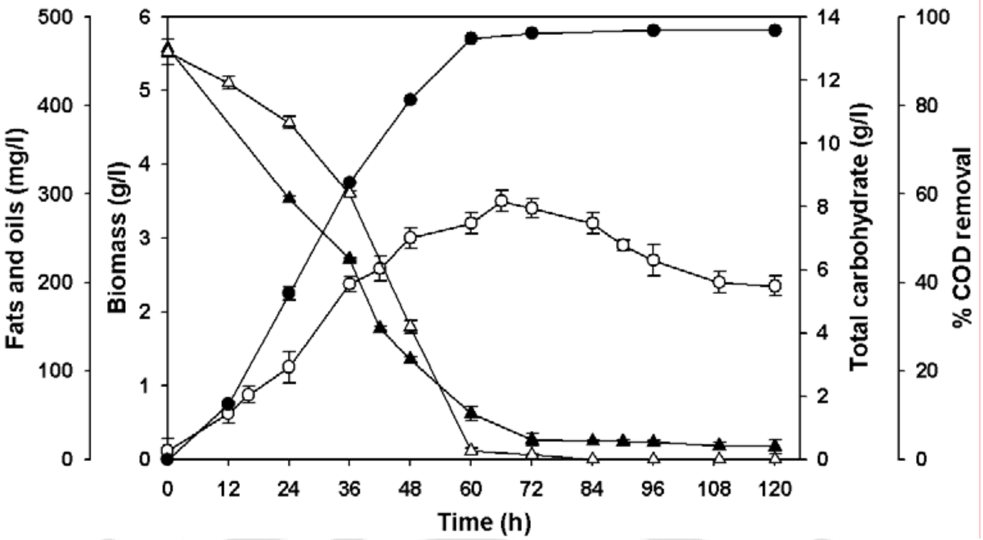


Fig. 4.37: COD reduction, fats and oils utilization, carbohydrate utilization and yeast biomass growth by *C. bombicola* using the dairy wastewater supplemented with sugarcane molasses (1%) and yeast extract (0.1%) in the fermentor operated under batch mode (▬○▬ Yeast biomass, ▬▲▬ Total carbohydrate, ▬Δ▬ Fat, ▬●▬ % COD removal).

In literature, Loperena et al. (2009) reported that some milk fat/protein degrading microorganisms isolated from a dairy wastewater treatment plant was able to remove only 75% of fats and 57% of COD from the synthetic dairy wastewater having an initial COD of 3000 mg/l. In another study, the potential of two biosurfactant producing strains *Planococcus citreus* and *Pantoea agglomerans* in reducing COD of oily soap industry effluent was reported by Jacobucci et al. (2009). These strains were shown to remove 76% and 70% COD of the oily soap industry effluent, in batch experiments. Compared to these reports, the present study showed excellent results in terms of COD removal and fats utilization under batch mode of operation by the yeast *C. bombicola*. As fed-batch mode of operating the fermentor previously showed good efficiency to pretreat synthetic dairy wastewater, the same was tested for pretreating the real dairy wastewater also.

4.5.2.2. Pretreatment under fed-batch mode of operation

Similar to the experiments on pretreatment of synthetic wastewater under fed-batch mode, feeding rate of the real wastewater was calculated from the Equation (4.2). Initially, fermentor was run under batch mode, and after 48 h, feeding of wastewater was started. The kinetic parameters μ and $Y_{X/S}$ were calculated to be 0.055 h^{-1} and 0.39, respectively, from the batch experiment using Equations (4.3) and (4.4).

Fig. 4.38 shows the results of COD reduction, fats and oils utilization, yeast biomass, and carbohydrate utilization in the wastewater in the fermentor operated under fed-batch mode which shows complete utilization of fats and oils within 72 h and 97% COD removal efficiency at the end of 96 h. These results indicated slightly better efficiency of the fed-batch operated system over the batch system.

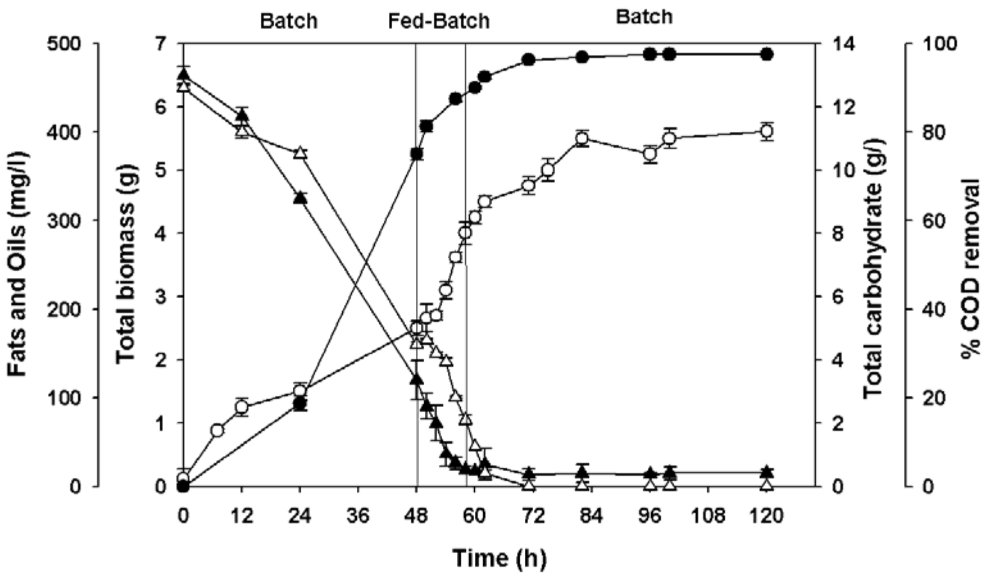


Fig. 4.38: COD reduction, fats and oils utilization, carbohydrate utilization and yeast biomass growth by *C. bombicola* using the dairy wastewater supplemented with sugarcane molasses (1%) and yeast extract (0.1%) in the fermentor operated under fed-batch mode (▬○▬ Yeast biomass, ▬▲▬ Total carbohydrate, ▬Δ▬ Fat, ▬●▬ % COD removal).

Also, similar to results obtained with synthetic wastewater the amount of wastewater treated in the fed-batch experiment was twice that treated in the batch experiment. During the feeding stage, although biomass increases exponentially and total biomass was 5.16 g in the reactor, SLs production did not improve mush and remained within 2-3 g/l. This value of yeast biomass was slightly less compared with values obtained in synthetic wastewater pretreatment, probably due to the fact that real dairy wastewater also contain variety of sterilizing agents and various acid and alkaline detergents which may affect the growth of the yeast (Bakka, 1992).

4.5.2.3. Pretreatment under continuous mode of operation

For improved productivity of the treatment systems continuous operations are always preferred. In this study, for operating the fermentor under continuous mode, the reactor was initially run under batch mode up to 72 h and then shifted to continuous mode with an HRT of 40 h which corresponded to dilution rate of 0.025 h^{-1} in the reactor. Fig 4.39 shows the results of pretreating the dairy industry wastewater under continuous mode of operation. The pretreatment under continuous mode was tested for 9 days. It could be seen from the figure that yeast was successful in pretreating wastewater during the continuous mode of operation. However, the SLs production remained constant (2-3 g/l) throughout the experiment due to insufficient hydrophobic carbon source (fats and oils) in the wastewater. Fig. 4.40 is a photograph of the experimental setup showing dairy wastewater pretreatment by the yeast in the fermentor under continuous mode. These results confirmed the potential of the biosurfactant producing yeast in pretreating real dairy industry wastewater.

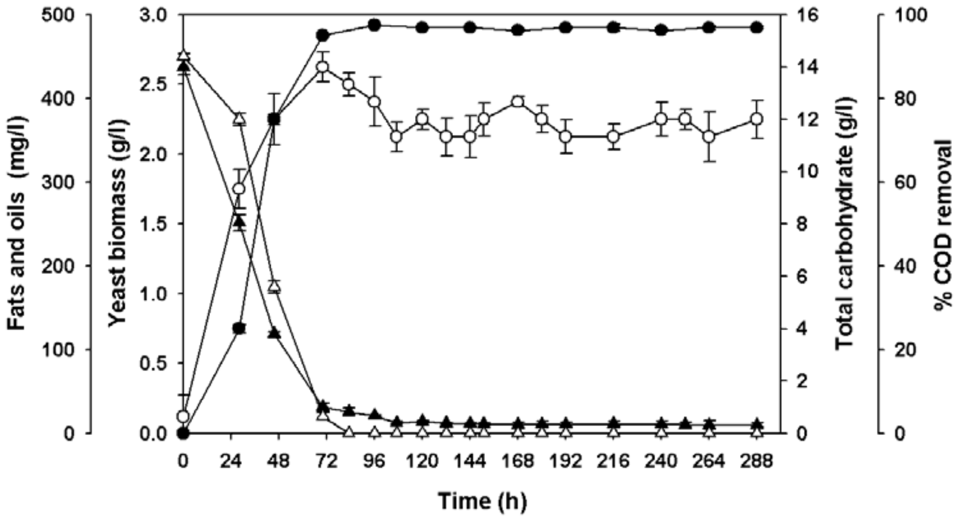


Fig. 4.39: COD reduction, fats and oils utilization, carbohydrate utilization and yeast biomass growth by *C. bombicola* using the dairy wastewater supplemented with sugarcane molasses (1%) and yeast extract (0.1%) in the fermentor operated under continuous mode (—○— Yeast biomass, —▲— Total carbohydrate, —Δ— Fat, —●— % COD removal).

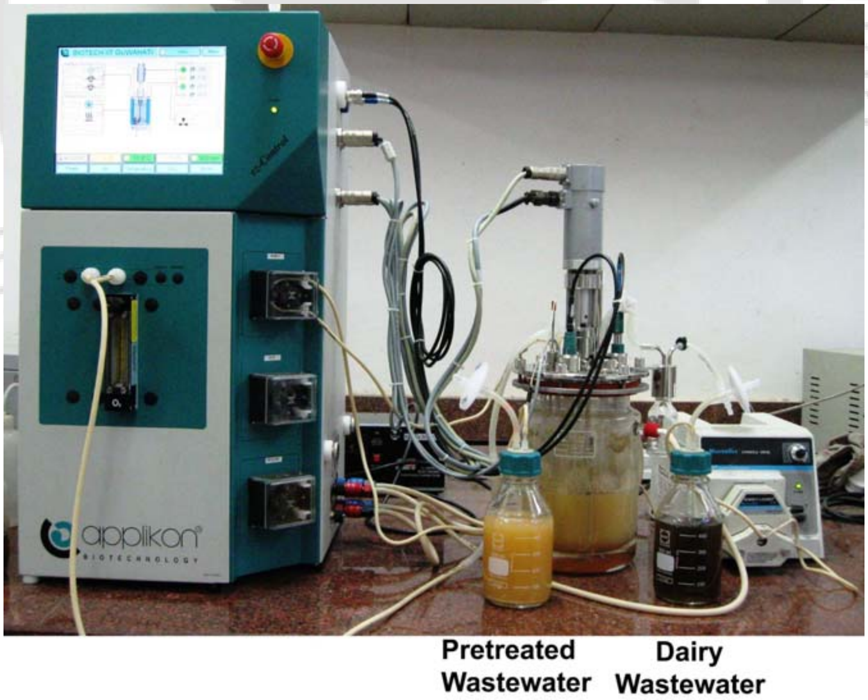


Fig. 4.40: Photograph of the experimental setup showing pretreatment of dairy wastewater in the fermentor operated under continuous mode.

Nakhla et al. (2003) studied the effect of commercial biosurfactant (BOD-Balance^{TM}) on the treatment of pet food wastewater by anaerobic digestion system and found that the biosurfactant at doses in the rage 130–200 mg/l decreased oil and grease concentrations from 66,300 to 10,200 mg/l, besides reducing the initial COD to 40.86% for an operation period of over two months. The effect of different surfactants including saponin biosurfactant on the continuous treatment of salad oil-containing synthetic wastewater by an activated sludge was studied by Matsui et al. (2005). They found that the outlet oil concentration in the wastewater remained quite low at 30 mg/l in presence of surfactants compared to more than 100 mg/l without any added surfactant. Recently, Banu et al. (2008) studied pretreatment of dairy wastewater by anaerobic hybrid upflow anaerobic sludge blanket reactor followed by final treatment using solar photocatalytic method. The anaerobic pretreatment method was able to remove 84% of COD with the secondary solar photocatalytic treatment removing the COD up to 95%. However, all these methods were reported to suffer from drawbacks such as requirement of *ex situ* addition of biosurfactant/coagulant/adsorbent which enhanced the process cost, low COD removal efficiency and longer pretreatment time. Compared to these reports, the present study shows complete utilization of fats and oils with high COD removal efficiency by the SLs producing yeast *C. bombicola*.

CHAPTER 5

SUMMARY AND CONCLUSION

The present research work successfully investigated pretreatment of dairy wastewater containing high fats and oils and production of SLs using two agro-industrial wastes (deproteinized whey and sugarcane molasses) by the yeast *C. bombicola.*

Compared to deproteinized whey, sugarcane molasses was found more suitable as hydrophilic carbon source for SLs production by the yeast, which followed a mixed growth and non-growth associated phenomenon. The low production level with deproteinized whey was observed mainly due to the inability of the yeast to utilize lactose present in the whey. To enhance the SLs production by the yeast using deproteinized whey-oleic acid small amount of glucose as a supplement was added into the medium which improved the SLs production both in batch shake flaks and in batch fermentor experiments (without pH control). Slight improvement in the SLs production were however, achieved under pH controlled conditions using the reactor operated under batch and fed-batch modes.

Compared to these results, when sugarcane molasses along with soybean oil, yeast extract and urea were used in the medium for SLs production in batch shake flasks a maximum SLs concentration of 12.67 g/l was obtained. On the other hand, the production level was significantly improved when yeast extract and urea were omitted from the medium. However, oil other than soybean oil reduced the yield of the product. To further improve the SLs produced by the yeast using only sugarcane molasses and soybean oil, various physical (temperature, agitation, inoculum size and age) and chemical variables (sugarcane molasses and soybean oil concentration) were optimized by employing the statistically valid Taguchi OA and experimental design technique. Under the optimum levels of the process variables the yeast produced 45 g/l and 47 g/l of SLs in batch shake flasks and batch fermentor without pH controlled condition, respectively, which were ~91% higher than the value obtained under un-optimized process conditions. Using batch fermentor operated under controlled pH condition, even higher SLs production of 60 g/l was achieved. Biokinetic parameters in the study were also estimated by fitting the experimental data on SLs production, yeast biomass growth and utilization of sugarcane molasses and soybean oil to suitable models found in the literature. The values of SLs yields ($Y_{P/M}$ and $Y_{P/S}$) were found to be higher than those reported in literature using costly glucose based medium suggesting that the low cost fermentative medium containing only sugarcane molasses and soybean oil can replace the costly conventional SLs production medium with a better yield of the biosurfactant.

The major SL produced using medium based on deproteinized whey-oleic acid was purified by silica gel column chromatography and its structural characterization was carried out by FTIR, ^{1}HNMR and MS. The purified SL was identified to be (17-hydroxyoctadecenoic)-1′4 ″-lactone-6′6″-diacetate SL with a molecular weight of 687 D. In case of SLs produced using sugarcane molasses-soybean oil, the molecular weight of the major SL was also nearly the same. The structural details of the biosurfactant further revealed that *C. bombicola* produces a mixture of acidic and lactonic SLs.

Properties of the biosurfactant produced using different carbon sources viz. deproteinized whey-oleic acid and sugarcane molasses-soybean oil, revealed that although the mST values were the same, the CMC values were different probably due to the two different hydrophobic carbon source used in the medium. However, the biosurfactant produced from both the carbon sources was found to be stable over wide range of pHs, temperature and salt concentrations. Also, the produced SLs were found to be highly active in solublizing fats and oils in aqueous phase

Further, the results of emulsifying activity and stability towards different NAPLs tested showed comparable emulsification activity and stability and the best results were obtained with kerosene oil. Emulsification activity and stability by the SLs from sugarcane molasses-soybean oil followed the order: kerosene oil > 1-Hexadecene > soybean oil > benzene. In case of SLs from deproteinized whey-oleic acid, emulsification activity followed the same order as that due to SLs from sugarcane molasses but the emulsification stability followed the order: 1-Hexadecene > soybean oil > benzene > kerosene oil. Environmental effects of pH and temperature on emulsification activity and stability due to the SLs produced using sugarcane molasses-soybean oil towards soybean oil was also investigated. While the highest activity was shown at pH 7.0 and temperature 60°C, the emulsion stability was more at pH 8.0 and temperature 60°C than other values of the environmental factors. Overall, the surfactant properties of the SLs produced using both the carbon sources (sugarcane molasses-soybean oil and deproteinized whey-oleic acid) namely CMC, mST, interfacial tension, emulsification activity, emulsification stability, oil solubilization activity and its stability over wide range of temperature, pH and ionic strength revealed very high potential of the biosurfactant in environmental applications.

The biosurfactant producing yeast *C. bombicola* was highly successful in pretreating synthetic as well as real dairy wastewaters containing high fats and oils in a laboratory scale fermentor operated under batch, fed-batch and continuous modes. However, the wastewater was supplemented with minimum amount of sugarcane molasses and yeast extract for the yeast biomass to initially produce the biosurfactant for pretreating the wastewater. Although the SLs production was found be very low due to insufficient hydrophobic carbon source (fats and oils), the system was not only highly efficient in complete removal of the fats and oils present in the wastewater but also in removing the wastewater COD with an efficiency of >95% in all the experiments. Thus, the study proved very good potential of the SLs producing yeast in removing fats and oils and to significant extent COD from contaminated wastewater.

Scope for Future Work

The present research work focused on SLs production the yeast *C. bombicola* using deproteinized whey-oleic acid and sugarcane molasses-soybean oil as cheap raw materials for pretreating dairy wastewater in a laboratory scale fermentor. The following are suggested as future work to continue in this area of research:

1) Pretreatment of dairy wastewater by the SLs producing yeast under different operating conditions such as wastewater hydraulic retention time, shock loading conditions etc. Other wastewaters such as wastewaters from slaughter house, restaurants and oil processing industries can also be tested.
2) Performance evaluation of the yeast system using bioreactors other than the fermentor in pretreating dairy wastewater.

3) Evaluation of other low cost industrial wastes such as wastes from potato processing industry and cassava wastewater for the production of SLs and its properties.
4) Enhancing the SLs production by the yeast using dairy or other wastewaters and recovery of the product.
5) Metabolic engineering studies for improved production of SLs by the yeast using cheaply available raw materials.

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APPENDIX

A.1. Determination of biokinetic parameters involved in the SLs production

Kinetic parameters (P_r, P_0 and P_{max}), involved in SLs production were determined from the series of experimental data on SLs concentration vs. time using the models mentioned in Equation (3.3) by regression analysis method using the Gauss-Newtonian method run in MATLAB. The initial values of the model parameters were guessed on the basis of experimental results. Similarly, kinetic parameters (μ, X_0, X_{max}) involved in the yeast biomass growth were calculated from the series of experimental data on yeast biomass concentration vs. time using the model Equation (3.4) by regression analysis method using the Gauss-Newtonian method run in MATLAB.

On the other hand, the kinetic model parameters S_{M0}, $Y_{P/M}$, and $Y_{X/M}$, involved in sugarcane molasses utilization and parameters S_{S0}, $Y_{P/S}$, and $Y_{X/S}$ involved in soybean oil utilization were determined from the series of experimental data on sugarcane molasses vs. time and on soybean oil vs. time using the models mentioned Equations (3.5) and (3.6), respectively by regression analysis method using the Gauss-Newtonian method run in MS Excel program.

A.2. Evaluation of specific growth rate

The growth of *C. bombicola* with both the low cost substrates (sugarcane molasses and deproteinized whey) followed a typical microbial growth pattern with lag, exponential and stationary phase, however, the lag phase was very short. To calculate the specific growth rate (μ) of the yeast grown on synthetic and real dairy wastewater under batch mode was identified. This was done by using MS Excel program, which calculates (1/X)(dX/dt) at discrete time interval for the experimental data. The value of μ was obtained from the maximum value of (1/X)(dX/dt).

LIST OF PUBLICATIONS

Published in Referred International Journals

1. **A. Daverey** and K. Pakshirajan (2010). Sophorolipids from *Candida bombicola* using mixed hydrophilic substrates: production, purification and characterization. *Colloids and Surfaces. B, Biointerfaces*. 79, 246-253.
2. **A. Daverey** and K. Pakshirajan (2010). Kinetics of growth and enhanced sophorolipids production by *Candida bombicola* using a low cost fermentative medium. *Applied Biochemistry and Biotechnology*, 160 (7), 2090 – 2101.
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