Generation of Surface-Active Substances from Fungal Systems

A Thesis Submitted

In partial fulfillment for the award for the

Degree of

Masters of Science in Microbiology

Submitted by:
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Frequent repetition of the same types of thoughts creates your beliefs and attitudes. What you belief comes true for you.

– Brahma Kumaris

Candidate's Declaration

I hereby declare that the work which is being presented in the dissertation entitled **" Generation of Surface-Active Substances from Fungal Systems"** in partial fulfillment of the requirements for the award of the degree of Masters in Science in Microbiology, Department of Biotechnology and Environmental Sciences, Thapar University, Patiala is an authentic record to my own work during a period of 6 months from January 2013 to June 2013, under the supervision of Dr.N.Tejo Prakash, Department of Biotechnology and Environmental Sciences, Thapar University, Patiala. I have not submitted the matter embodied in this dissertation for the award of any other degree or diploma.

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Date: 15.07.2013

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This is to certify that the above statement given by the above candidate is correct and true to the best of my knowledge.

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Certificate

This is to certify that the project entitled **" Generation of Surface-Active Substances from Fungal Systems"** being submitted by Ms.Vasudha Hardatt in partial fulfillment of the requirement for the award of degree for the Master of Science in the department of Biotechnology and Environmental Sciences, Thapar University, Patiala is a bonafide work carried out under our guidance and supervision and that no part of this project has been submitted for the award of any other degree.

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Needless to say errors and omissions are solely mine.

VASUDHA HARDATT

Summary

Surface-active substances of biological origin (biosurfactants) have only been described in the past few decades. They have advantages of biodegradability and causes low toxicity to the environment. They have gained importance in research field and their applications are becoming wider. So far, limited reports are there over the generation of surface-active substances from fungal systems. Keeping this in view, we explored two oil tolerant fungal strains belonging to genus *Aspergillus* (RBD01 and RBD02) and determined the emulsification and surface activity of its cell free supernatant. The cell free supernatant of fungal stain RBD02 showed remarkable reduction in the surface tension of water. Crude biosurfactant of this strain was extracted by acetone precipitation method. The crude biosurfactant was further analyzed for lipolytic activity and was found to have ability of breaking down of triglycerides into free fatty acids (FFA). This indicated that extracellular lipase was also generated by the RBD02 with biosurfactant. The fungal stain RBD02 have ability of generating good quality of biosurfactant by utilizing oil as a carbon source and in addition extracellular lipase enzyme was also generated by RBD02 strain which had application in hydrolysis of oil. This observation is being noted first to be reported for any fungal strain, to the best of our knowledge.

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1.0
Introduction

1.0 Introduction

In today's era world population is increasing in a very magnificent rate and results in the building up of more industrial hubs. Due to wide industrial setup across the cities the waste effluent and the various synthetic goods manufactured by industries, the environmental conditions of the surrounding regions are "sailing under false colors" and leads to the overall harm to the whole consortium of that region. Such kind of pollution has created a drastic harm to the human health in different forms. Due to this reason, now-a-days, major emphasis is given towards the use of eco- friendly products which are biodegradable and harmless to human health.

Surfactants constitute an important class of chemical compounds being used by variety of industries. Surfactants may acts as detergents, wetting agents, emulsifier, foaming agents and dispersants. The addition of surfactant directly into the two immiscible substances results in increase of the apparent solubility and desorption rate of the hydrophobic compound to the aqueous phase by reducing the interfacial tension between the aqueous and non-aqueous phases and regulates the emulsion stability of the mixture. They are widely used in various industries like food industries, pharmaceutical industries, cosmetics field, leather and textile industries, etc. Due to the purely chemical nature of surfactants, they accumulates in the environment and cause environmental pollution leading to increase in accumulation of toxic substances in land and water. This is dominantly due to non-biodegradable and recalcitrant nature of the compounds. Anionic surfactants are mainly found in soil as a result of sludge application, waste water irrigation, etc.

To counter the environmental issues concerning chemical surfactants, biologically generated surface active compounds known as biosurfactants have gained considerable interest in recent years. Biosurfactants have various advantages over chemical surfactants as they are highly biodegradable in nature, have lower toxicity, ability of working at extreme conditions such as low pH and variable temperature ranges. They can also be produced by cheap renewable resources or industrial waste products like various used frying oils, soapstock (a gummy, amber colored by-product of oil seed processing), molasses, starch rich wastes, olive oil mill effluent. By this the prospects of the biosurfactants research has increased at a great pace for exploring its wide application in petroleum industry.

Biosurfactants, mainly generated as an activity of the microbial cell surface and are secreted as emulsifiers when grown on water immiscible hydrophobic substrates. This mechanism of production of biosurfactants by selected microbial species is generally carried out for utilizing hydrocarbons as a carbon source have proved a beneficial aspect for treating oil contaminated regions such as fields, industrial waste water containing land, etc. Like surfactants, biosurfactants also have ability of lowering down the surface tension between two immiscible compounds. Due to their remarkable properties, the overall demands for the eco-friendly compounds, has tremendously increased in recent years and is estimated to go higher in the much higher in near future.

The annual global production of surfactants was 13 million metric tons in 2008, and the annual turnover reached US$24.33 billion in 2009, nearly 2% up from the previous year. The market is experiencing growth by 2.8% annually to 2012 and by 3.5-4.0% thereafter ("Market Report: World Surfactant Market" Acmite Market Intelligence). Specialists expect the global surfactant market to make revenues of more than US$41 billion in 2018 – resulting in an average annual growth of 4.5% (Market Study on Surfactants by Ceresana Research, USA). Currently, the biosurfactants that are commercially well known include ZONIX (bio-fungicide made from rhamnolipids), cellobiose lipids, sophorolipids, mannosylerythritol lipids.

Till date, majority of the studies have been focused towards determining the capability of the bacterial systems for the production of biosurfactants. In this context, one of the major hindrances coming in the path for the commercialization of the discovered biosurfactants is the difficulty in the maintenance of efficacy of strains in scale up process and costly downstream processing of the desired product recovery. To combat with such problems recently research has been started by taking up the fungal species and exploring their efficiency for the production of surface active compounds. Fungal systems have ability of overcoming all the hurdles that is shown by the bacterial systems, i.e they are easy to handle, requires less maintenance as compared to the bacterial cultures and mainly downstream processing is much easier as that in the case of bacterial systems. Until now, very few fungal biosurfactants have been reported with many more remaining to be explored.

Keeping this in view the present study has been carried out on two fungal strains RBD01 and RBD02 selected on the bases of their oil tolerant capability and the cell biomass of both the strains showed hydrolysis and transesterification activity. The nature of the surface active

substances produced by these strains extracellularly, in reducing the surface tension and potentially hydrolyzing the oil was examined.

2.0

REVIEW OF LITERATURE

2.0 Review of literature

Increasing public awareness of environmental pollution influences the search and development of technologies that help in clean up of organic and inorganic contaminants such as hydrocarbons and metals. Organic pollutants and metal contaminants in environment persist because of either unavailability to degrading microorganisms or due to low solubility of these pollutants or the degrading microbes are not able to carry out the necessary catabolic reactions. With the advantage of biodegradability, production on renewable resources and functionality under extreme conditions biosurfactants are gaining prominence over chemical surfactants which causes damage to environment. Biosurfactants enhance the accessibility and bioavailability of hydrophobic chemicals by forming stable emulsions and lowering the surface tension. Although the type and amount of the microbial surfactants produced depend primarily on the producer organism, factors like carbon and nitrogen, trace elements, temperature, and aeration also affect their production by the organism.

The biosurfactant accumulates at the interface between two immiscible liquids or between a fluid and a solid. They reduce the surface and interfacial tension between phases and by this the repulsive force gets reduced between two dissimilar phases and the two phases mix and interact with each other more easily. (**Figure 1**)

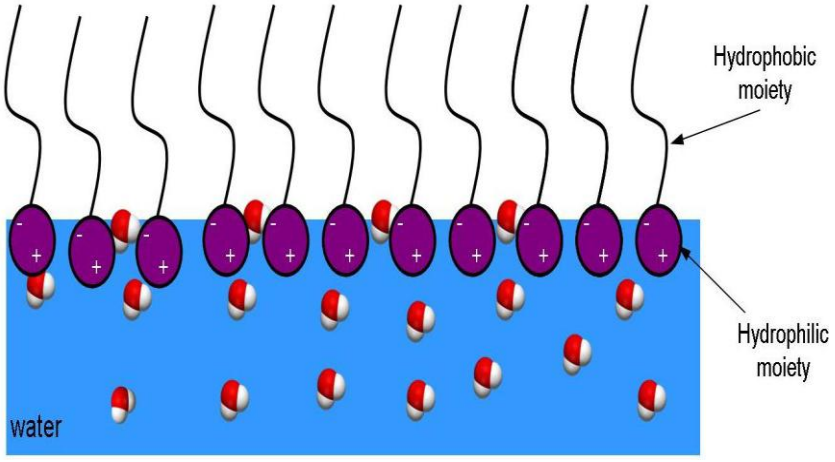


Figure 1. Biosurfactants at the interface between hydrophilic and hydrophobic region
(Pacwa *et al.*, 2011)

2.1. Biosurfactants

The term biosurfactant refers to any surface-active agents produce microorganisms that have ability of lowering down the interfacial tension between the two liquids. Biosurfactants are a heterogeneous group of surface active molecules produced by microorganisms, which either adhere to cell surface or are excreted extracellulary in the growth medium (Fietcher, 1992; Zajic and Stiffens, 1994; Makker and Cameotra, 1998). These molecules reduce surface tension and critical micelle dilution (CMD) in both aqueous solutions and hydrocarbon mixtures. These properties create microemulsion in which micelle formations occur where hydrocarbons can solubilize in water or water in hydrocarbons (Banat, 1995). Since biosurfactants and bioemulsifiers both exhibit emulsification properties, bioemulsifiers are often categorized with biosurfactants, although emulsifiers may not lower surface tension.

The worldwide biosurfactants market was worth USD 1,735.5 million in 2011 and according to the recent market report published by Transparency Market Research it is expected to reach USD 2,210.5 million by the year 2018, corresponding to an average annual growth rate of 3.5% from 2011 to 2018. With the increased awareness among consumers for environmentally friendly compounds, a number of surfactant manufacturers in the market have entered into the biosurfactant industry. Rhamnolipids have recently introduced by *AGAE* technologies Ltd, USA while USA based Jeneil Biosurfactants is already a successful entrepreneur in this field. Other major manufacturers include Fraunhofer IGB (Germany), Cognis (Germany and USA) dealing with production of glycolipid surfactants, cellobiose lipids and mannosylerythritol lipids Saraya (Japan), Ecover Belgium (Belgium), Groupe Soliance (France) and MG Intobio (South Korea) are responsible for manufacturing sophorolipids. Cognis recently made introduction about the production of green surfactant alkyl polyglucoside APG®, which is made from vegetable oil or starch. Commercially available bisurfactants Jeneil biosurfactants, USA are selling are ZONIX, which is a bio-fungicide made from rhamnolipids and RECO, is used in cleaning and recovering oil from the storage tanks (Sekhon *et al*., 2011).

2.1.1. Classification

Surfactants are amphiphilic molecules with both hydrophilic and hydrophobic moieties present within the same molecule. Hydrophilic moiety is composed of amino acids, cyclic peptide, phosphate, carboxylic acid, anions or cations or mono or di or poly-saccharides and a

hydrophobic moiety usually contains long chain saturated, unsaturated and hydroxylated fatty acids and fatty alcohols. A characteristic feature of biosurfactant is a hydrophilic-lipophilic balance which specifies the portion of hydrophilic constituents in surface active substances. Due to amphiphilic structure, biosurfactants increase the surface area of hydrophobic water insoluble substances. Surface activity makes surfactants excellent emulsifier, foaming and dispersing agents. Microbial biosurfactants are generally classified (**Table 1**) on the basis of their biochemical nature. Various types of biosurfactants produced by different types of microorganisms on the bases of their chemical composition are classified as (Gapke *et al.*, 2008).

Table 1. Major types of biosurfactants produced by microorganisms
(Shekhon *et al.*, 2011)

Biosurfactant type	Microbial species
Glycolipids	
Trehalose mycolates	*Rhodococcus erythropolis*
	Arthrobacter paraffineus
	Mycobacterium phlei
	Nocardia erythropolis
Trehalose esters	*Mycobacterium fortium*
	Micromonospora spp.
	Mycobacterium smegmatis
	Mycobacterium paraffinicum
	Rhodococcus erythropolis
Mycolates of mono, di and tri-saccharide	*Corynobacterium diptheriae*
	Mycobacterium smegmatis
	Arthrobacter spp.
Rhamnolipids	*Pseudomonas spp.*
Sophorolipids	*Torulopsis bombicola/apicola,*
	Candida spp., Torulopsis petrophilum
Rubiwettins	*Serratia rubidaea*
Diglycosyl diglycerides	*Lactobacillus fermenti*
Schizonellins A and B	*Schizonella melanogramma*
Ustilipids	*Ustilago maydis, Geotrichum candidum*
Amino acid lipids	*Bacillus sp.*
Floculosin	*Pseudomonas flocculosa*

Table 1 continued…

Phosphorolipids and fatty acids	
Phosphorolipids and fatty acids	*Candida spp. , corynobacterium spp.* *Micrococcus spp., Acinetobacter spp.* *Thiobacillus thiooxidans* *Aspergillus spp.,* *Pseudomonas sp., Mycococcus sp.,* *Penicillium sp.*
Lipopeptides and Lipoprotiens	
Gramicidins	*Bacillus bravis*
Peptide lipids	*Bacillus licheniformis*
Polymyxin E1	*Bacillus polymyxa*
Ornithin lipid	*Pseudomonas rubescens,* *Thiobacillus thiooxidans*
Viscosin	*Pseudomonas flourescens*
Serrawettin	*Serratia marcescens*
Cerilipin	*Glucunobacter cerius*
Lysine lipid	*Agrobacterium tumefaciens*
Surfactin, subtilysin, subsporin	*Bacillus subtilis*
Lichenysin G	*Bacillus licheniformisIM 1307*
Amphomycin	*Streptomycin canus*
Clamydosin	*Dihetrospora chlamydosporia*
Cyclosporin A	*Tolypocladium inflatum*
Enduracidin A	*Streptomycin fungicidicus*
Globomycin	*Streptomycin globocacience*
Bacillomycin L	*Bacillus subtilis*
Iturin A	*Bacillus subtilis*
Putisolvin I and II	*Pseudomonas putida*
Arthrofactin	*Arthrobacter*
Fengycin	*Bscillus thuringiensis CMB26*
Mycobacillin	*Bacillus subtilis*
Polymeric biosurfactant	
Lipoheteropolysaccharide (Emulsan)	*Acenitobacter Calcoaceticus* RAG-1
Heteropolysacharides (Biodispersan)	*Arethrobacter calcoaceticus*
Polysaccharide protein	*Acenetobacter calcoaceticus* A_2
Manno protein	*Acinetobacter calcoaceticus strains*
Carbohydrate protien	*Saccharomyces cerevisiae*
Mannan lipid complex	*Candida petrophillum,*
Mannose/ Erythrose lipid	*Endomycopsis lipolytica*
Carbohydrate-protein-lipid complex	*Candida tropicalis*
Liposan	*Shizonella melanogramma, Ustilago maydis*
Alasan	*Pseudomonas flourescences,*

Table 1 continued…

Particulate biosurfactants	
Membrane vesicles	*Acenitobacter sp.* H01-N
Fimbriae, whole cell	*Acenitobacter calcoaceticus*

2.1.2 Types of biosurfactants

2.1.2 (a) Glycolipids

Glycolipids consist of mono, di, tri and tetra-saccharides including glucose, mannose, galactose, glucoronic acid, rhamnose and galactose sulphate. Glycolipids are the most common type surfactants. The fatty acid composition of glycolipids is similar to that of phospholipids of microorganisms.

Glycolipids ⟶ Carbohydrates + Long chain fatty aliphatic acids/ Hydroxyaliphatic acids (Desai and Banat, 1997)

Glycolipids are further of various types:

Rhamnolipids - Rhamnolipids constitutes rhamnose and 3-hydroxy fatty acids. It is mainly produced by bacteria of genus *Pseudomonas.* Two molecules of rhamnose sugars and an acetyl group linked with a hydrophobic group forms a disaccharide rhamnolipid. However the lipid part of molecule have ester and carboxyl groups. Rhamnolipids produced by *Pseudomonas aeruginosa* is reported as a mixture of homologous species RL1 ($RhC_{10}C_{10}$), RL2 (RhC_{10}), RL3 ($Rh_3C_{10}C_{10}$) and RL4 ($Rh_2\ C_{10}$) (Syldatk and Wagner, 1987, Lang and Wagner, 1987, Rahman *et al.,* 2002b). Rhamnolipids produced by *Pseudomonas aeruginosa* strains is listed among the most efficient surfactants for the removal of hydrophobic compounds from the contaminated soils (Rahman *et al.,* 2006). Van dyke *et al.,* 1993 reported that they have high affinity for hydrophobic organic molecules and posses low average minimum surface tension of (30-32 mN m^{-1}) ; high average emulsifying activity of (10.4-15.5 U ml^{-1} filtrate) and low critical micelle concentration (CMC) (5-65 mg l^{-1}).

Sophorolipids - This group of glycolipids is produced by *Torulopsis sp.*. Sophorolipids consist of dimeric sugar combined with hydroxyl fatty acids linked by β- glycosidic bond. Sophorolipids are of two types - acidic (non lactonic) sophorolipids and lactonic sophorolipids. In acidic (non lactonic) sophorolipids the hydroxyl fatty acid moieties consist of free carboxylic acid functional group. In lactonic sophorolipids macrocyclic lactone ring is present with 4´´ - Hydroxyl group. Lactonic sophorolipid have been reported of having measurable biocide activity whereas non

lactonic sophorolipid have application in cosmetics as antidandruff, bacteriostatic agents and deodorants (Mager *et al.,* 1987).

Trehalolipids - Due to the presence of trehalose esters on the cell surface serpentine group is seen in many members of genus *Mycobacterium* (Asselineau and Asselineau, 1978). The disaccharide trehalose of C-6 and C-6 to mycolic acid is associated with most species of *Mycobacterium, Nocardia* and *Corynebacterium*. Mycolic acid is made up of long chain α-branched β hydroxyl fatty acids. Trehalose lipids from *Rodococcus erythropolis* and *Arthrobacter sp.* were found to lower the surface tension and interfacial tension from 25-40 and 1-5 mN m^{-1}, respectively (Li *et al.,* 1984). Trehalolipids from various organisms vary in size and structure of mycolic acids, the number of carbon atoms and the degree of unsaturation.

2.1.2 (b) Fatty acids

Fatty acids are produced as a results microbial oxidations from alkanes (Rehn and Reiff, 1981). Microorganisms produce fatty acids in the form of straight- chain, complex fatty acids containing OH groups and alkyl branches. Corynomucolic acid is an example of complex group of fatty acids (Kretschner *et al.,* 1982). The hydrophilic or lipophilic balance balance of fatty acids is dependent on the length of hydrocarbon chain. The most saturated fatty acids for lowering the surface and interfacial tension are in the range of C12-C14 (Rosenberg and Ron, 1999).

2.1.2 (c) Phosphorolipids

Phosphorolipids forms a major component of microbial membarane. By growing certain hydrocarbon degrading bacteria and yeast on an alkane substrate the level of phospholipids increases. Phosphatidylethenolamine produced by *Rodococcus erythropolis* when grown on n-alkane shows the lowering of interfacial tension between water and hexadecane to less than 1mN m^{-1} and CMC of 30 mg l^{-1} (Kretschner *et al.,* 1982). *Thiobacillus thiooxidans* produced phospholipids quantitatively and it is responsible as a wetting elemental sulphur necessary for growth (Beeba and Umbriet, 1971). Phospholipids rich vesicles were produced by *Acenetobacter sp.* HO1-N when hexadecane is used as a growth medium (Kaeppeli and Finnerty, 1979).

2.1.2 (d) Lipopeptides and Lipoproteins

Surfactin, a cyclic lipopeptide are produced by *Bacillus sp.* having seven amino acids linked to carboxyl and hydroxyl groups of a 14-carbon acid. Surfactin have ability to reduce the surface tension from 72-27 mN m^{-1} with concentrations as low as 0.005% providing it abilty of a most powerful biosurfactant (Kakinuma *et al.,* 1969). Huge number of cyclic lipopeptides including decapeptide antibiotics (gramicidin) and lipopeptide antibiotics (polymyxin) is produced by *Bacillus brevis* and *Bacillus polymyxa.* Other examples of being the best studied lipopeptides are orinithine lipids and iturin (Desai and Banat, 1997, Muthusamy *et al.,* 2008).

2.1.2 (e) Polymeric biosurfactants

Polymeric biosurfactants are high molecular weight biopolymers having properties like high viscosity, high tensile strength and resistance to shear. They have ability to alter the reological properties of aqueous solutions at low concentrations. The best studied polymeric biosurfactants are emulsan, liposan, mannoprotein and polysaccharide protein complexes (Desai and Banat, 1997). They are powerful emulsion stabilizers constituting the ability to resist inversion even at a water oil ratio 1:4.

Liposan, an extracellular water soluble emulsifier was synthesised using *Candida lipolytica* which is reported by Ciriglian and Carman (1984). It is composed of 83% carbohydrate and 17% protein, The carbohydrate portion is a heteropolysaccharide consisting of glucose, galactose, galactosamine and galactoronic acid. Emulsan, a potent polyanionic amphipathic heteropolysaccharide bioemulsifier is extracted from *Acinetobacter calcoaceticus* RAG- 1. It is effective against hydrocarbons in water even at concentration as low as 0.001 (Rosenberg *et al*., 1979). A mutant of *Acinetobacter calcoaceticus* BD4 have ability to produce large amount of polysaccharide together with proteins.

Emulsifying protein PA was isolated from *Pseudomonas aeruginosa.* This protein is produced from long chain n-alkanes, 1-hexadecane and acetyl acohol substrate and has MW of 14,000 Da and is threonine and serine rich (Karanth *et al.,* 1999). Surfactants produced by *Pseudomonas* PG-1 are extremely efficient in utilization of wide range of C_xH_y including gaseous volatile and liquid alkanes, alkenes, pristane and alkyl benzenes. Demonstration of production of large amount of mannoproteins by *Saccharomyces cerevisiae* was given by Cameron *et al.,* (1988). The mannoproteins exibit remarkable emulsification activity toward

several oils, alkanes and organic solvents. Some other polymeric biosurfactants such as biodispersan, alasan, food emulsifiers, protein complexes and insecticides emulsifiers have also been reported.

2.1.2 (f) Particulate or whole cell surfactants:

When *Acinetobacter sp.*HO1–N is grown on hexadecane, it accumulates extracellular vesicles of 20 to 50 mm diameter with a buoyant density of 1.158 g/cm^3. These vesicles appear to play a role in the uptake of alkanes by *Acinetobacter sp.*H01-N. Some species of *Corynobacteria* and some pathogens have high affinity for hydrocarbon-water and air-water interfaces. In such cases microbial cell itself is a surfactant (Gobbert *et al., 1984)* (Ito *et al.,* 1982).

Biosurfactants are also classified according to nature of charge (Gapke *et al.,* 2008). **Anionic surfactants** having negative charge usually due to sulphonate or sulphur group; **Non ionic surfactants** lacking ionic constituents and the majority of all non ionic are polymerization products of 1 2- epoxyethane; **Cationic surfactants** characterized by quarternary ammonium group which is positively charged; and **Amphoteric surfactants** are both positively and negatively charged moieties in same molecule (Ginkel, *1989*).

2.2 Mechanism for hydrocarbon uptake

Hydrocarbons constitutes both aliphatic as well as aromatic compounds and are wide spread xenobiotics to biological systems. Majority of them are biodegradable but few persist in environment for a longer time. Such kind of hydrocarbons comes from crude oil and industrial effluents. The important step required for the hydrocarbon uptake is emulsification of hydrocarbon and water. The emulsified hydrocarbon could be easily absorbed by the cells. The main physiological role of biosurfactant is to allow microorganisms to grow on water immisible substrates by reducing the surface tension at the phase boundary, thus making substrate more readily available for uptake and metabolism, but the molecular mechanism related to substrate uptake is still not fully understood (Desai and Banat, 1997).

There are three pathways proposed by Nakahara *et al.,* (1977) for the uptake of hydrocarbon by microorganisms:

1. Direct contact of the cells with fine droplets

2. Direct contact of the cells with large oil drops.
3. Uptake of dissolved hydrocarbons in the aqueous phase.

2.3. Major applications of biosurfactants

2.3.1 Bioremediation

Use of biosurfactants can be a promising method for bioremediation effectiveness of hydrocarbon contaminated environments. Biosurfactants enhance the bioremediation by two methods. First is by increasing the level of substrate bioavailability for microorganisms and the second is interaction with the cell surfaces which leads to increase in the hydrophobicity of the surface and makes the association of the hydrophobic substrates to the cell surfaces (Mulligan *et al., 2004*). Biosurfactant enhance biodegradation and removal of hydrocarbons by reducing the surface tension and interfacial tension thus, increasing the surface area of insoluble compounds leading to increased mobility and bioavailabilty of hydrocarbons.

The use of sophorose lipids do enhance biodegradation of the hydrocarbons and their chlorinated derivatives in contaminated soil (Kosaric *et al.,* 2001). As an example, the herbicide metholachloro 2-chloro-N-(2-ethyl-6-methylphenyl)-N-(2-methoxy-1-methylethyl) acetamide, was significantly more degraded when sophorose lipids were added to a slurry bioreactor containing soil in suspension. Ermolenko *et al.,* (1997) reported that the strain *Myco-bacterium flavescens* Ex 91 was successfully used for the development of Ekoil, a bacterial preparation for the decontamination of areas polluted with oil. The effect of rhamnolipids biosurfactant on *in situ* biodegradation of hydrocarbons entrapped in porous matrix has been studied and as a result mobilization of hydrocarbons took place at biosurfactant concentration higher than critical micelle concentration (CMC) which has been reported by (Herman *et al.,* 1997).

2.3.1a. Marine contamination

Microbes having ability to degrade hydrocarbon have been isolated from aquatic environments (Brown *et al.,* 1990). *Pseudomonas aeruginosa* SB30 was able to quickly disperse oil into fine droplets, and inferred that it may be useful in removing oil from contaminated sites (Chakarabarty *et al.,* 1985). The environmental technology laboratory University of Alaska Fairbanks, USA reported complete removal of diesel range petroleum hydrocarbons (to the limit of 0.5 mg kg^{-1}) while semi-volatile petroleum hydrocarbons were reduced to the 70% level reaching a removal of 30% (Tumeo *et al.,* 1994). Biosurfactants generated by *Pseudomonas*

aeroginosa has been tested for its ability to remove oil from Alaskan gravel samples under various conditions such as at various concentrations, time of contact, temperature of wash. Increased oil displacement has been reported by (Harvey *et al.*, 1990) as compared to water alone.

2.3.1b. Removal of metal contamination from soils

The addition of biosurfactant may promote desorption of heavy metals from soil in two ways. First by complexation of the metals which are present in the free form in the solution which leads to the decrease in the solution phase activity of the metal and therefore promotes desorption. The second is by reduction in the interfacial tension between the solid-solution interface due to accumulation of the biosurfactant which may allows the direct contact between the biosurfactant and sorbed metal.

Potential of Rhamnolipid biosurfactant produced by *Pseudomonas aeroginosa* ATCC 9027 in the removal of metals from soil contaminated with cadmium was analyzed by (Tan *et al.*, 1994) and reported 92% complexation of Cd^{2+} in a 0.5 mM solution of rhamnolipid (22 µg mg^{-1} rhamnolipid). Similar studies was done by (Herman *et al*., 1995) Rhamnolipids solutions was used at different concentrations 12.5, 25, 50 and 80mM in soil containing sorbed Cd^{2+}, $Pb^{2+}$and a mixture of Pb^{2+} - Cd^{2+} - Zn^{2+}. At 12.5 and 25mM rhamnolipids concentration 78% sorption of to soil and less than 11% soil bound Cd^{2+} and Zn^{2+} was desorbed.

2.3.1c. Removal and degradation of polyaromatic hydrocarbons

The degradation of polyaromatic hydrocarbons (PAHs) with four or more fused rings is limited due to poor availabity of them to microbial systems. This is due to their strong absortive capacity in soil, low hydrophibicity and low aqueous solubility. There are very few microbial species having abilty of degrading the PAHs (polyaromatic hydrocarbons) (Haryama *et al.*, 1997). Increase in the rate of degradation of hexadecane, benzene, toluene , ortho and paracresol and naphthalene in aquous phase bioreactor by the use of rhamnolipids produced by Bacteria has been reported by Churchill *et al.*, (1995). Surfactants degrade PAHs by emulsification or sulobalization to release hydrocarbons and increase the aqueous concentration of hydrophobic compounds resulting in increase in mass transfer rates. The effect of two rhamnolipids biosurfactants was tested on phanathrene bioavailability and increase in both the solubility and degradation rate was reported by (Zhang *et al.*, 1997). Both the surfactants lowers the surface

tension in solution from 72 dynes cm $^{-1}$ to 30 dynes cm^{-1} by using nephthalene and methyl substituted derivatives in crude oil as representative of PAHs content.

2.3.2 Petroleum product and production

2.3.2a. Microbially enhanced oil recovery (MEOR)

Biosurfactants application in microbially enhanced oil recovery is used in recovering out oil remaining in reservoirs after primary and secondary recovery treatment. By using biosurfactants the interfacial tension get reduced at the oil- rock interface and it reduces the capillary forces and prevents oil from moving through oil pores.

Biosurfactants also helps in emulsification of oil and detachment of oil films from rocks. (Banat 1995 a,b). Removal of oil *in situ* is due to multiple effects of the microorganisms having ability to produce biosurfactants on both the environment and oil. These all factors are responsible for reducing the oil viscosity and facilitating oil recovery which includes gas and acid production, reduction in oil viscosity, plugging by biomass accumulation, reduction in interfacial tension and degradation of large organic molecules. Test been conducted by (Behlugil *et al.,*1992) on Turkish heavy oil, by injection of *Clostridium acetobutylicum* into a model reservoir having Turkish heavy oil at 38°C and resulted in overall increase of 12% in microbially enhanced oil recovery effectiveness as compared to control.

2.3.2b. Oil storage tank cleaning

Oil storage tank cleaning is also one of the very useful application of biosurfactants. A gram negative bacteria strain H13A having ability of producing glycolipid surfactant and was reported by (Finnerty and Singer, 1985) to reduce the viscosity of heavy crude oil by 50%. A *Pseudomonas* strain which produce an emulsifying agent and had potential to it emulsify heavy grade oil VI fuel oil was isolated by (Zajic *et al.,* 1974). Banat *et al.,* (1991) investigated the ability of biosurfactants produced by bacterial strain in a pilot field investigation to clean oil tanks for recovering hydrocarbons from the tank. Approximately 91% of 850 m^3 sludge was recovered as resellable crude oil. Such a clean up process is very economical and less hazardous to person involved in these processes maintenance (Lillenberg *et al.,* 1992).

2.3.2c. Therapeutic agents

Biosurfactants have many therapeutic applications. They have properties like antimicrobial properties they can also act as immunoregulators and immunomodulators in

adhesion and desorption and they also play role in signaling. Various biosurfactants produced by various microorganisms have shown antimicrobial activity. Surfactin, a cyclic lipopeptide have various properties such as inhibiting fibrin clot formation and hemolysis (Bernheimer and Avigard, 1970) and formation of ion channels in lipid membranes (Sheppard *et al.,* 1991) and it also have antitumor activity against Ehrlich's ascite carcinoma cells (Kameda *et al.,* 1974), inhibits cyclic adenosine 3′, 5′- monophosphate phosphodiesterase (Hosono and Suzuki, 1983) and also have anti-fungal properties (Vater, 1986b). A probiotic bacteria, *Lactobacillus fermentum* RC-14 and the biosurfactant produced by it have ability to inhibit the implant infection caused by *Staphylococcus aureus* by inhibiting its adherence to surgical implants which is a cause of hospital acquired infections (Gan *et al.,* 2002).

Antimicrobial lipopeptide, isolated from *Bacillus subtilis,* showed a broad spectrum antimicrobial activity against gram anegative bacteria, little activity against gram positive bacteria and also against two test fungi (Bechard *et al.,*1998). A new cyclic depsipeptide, Viscosinamide produced by *Pseudomonas flourescens* DR54 could played a possible role as an antifungal agent (Nielsan *et al.,*1999). A novel series of lipopeptide compounds WF11899s, WF738s, WF14573s, WF16616 and WF22210 was discovered by (Hino *et al.,* 2001) having antifungal properties.

2.3.3 Application in mining process

Biosurfactants can be used in the dispersion of in-organic materials in mining and manufacturing processes. They helps in lowering down the energy required for cleaving the microfractures and also stabilize coal slurries to aid transportation of the coal . Biodispersan had two functions: 1. Enhancing the fracturing of limestone into small particles 2. Acting as dispersant and surfactant. It prevented the flocculation and dispersed 10% limestone in water mixture. Crude biosurfactants derived from *Candida bombicola* were tested for solubulization of coal and partial solubilization of coal took place (Polman *et al.,* 1994).

2.3.4 Agricultural applications

Due to environmental pollution caused by pesticides global concern has been given in finding out the biological substitute for them which would be eco-friendly to the environment

The surface active agents are needed for the hydrophilization of the heavy soils for obtaining good wettability and also for the equal distribution of fertilizers and pesticides in the soils.

A cyclic lipopeptide amphisin produced by *Pseudomonas sp.* DSS73 which was isolated from rhizoplane of sugarbeet seedlings exhibited antagonism towards root pathogenic microfungi *Pythium ultimum* and *Rhizoctonia solani*. A biosurfactant produced by *Pseudomonas aeruginosa* have ability of solubulizing toxic organic chemicals; and increase the solubility and recovery of hexachlorobiphenyl from soil slurries by 31% (Berg et al., 1990). The addition of biosurfactant produced by *Bacillus subtilis* MTCC 2423 enhanced the rate of biodegradation of chlorinated pesticide α and β-endosulfan by 30-40% (Awasthi *et al.,* 1999)

2.3.5 Applications in food industries

In food industries, biosurfactants are used as emulsifiers for the processing of raw materials. Emulsification provides the right consistency and texture as well as phase dispersion. Surface active agents also influence the reological characterisitics of flour and also the emulsification of partially broken fat tissue (Vater *et al* ., 1986a). As reported by Busscher *et al.,* (1996), the biosurfactant produced by thermotolerant *Streptococcus sp.* could be used as foul control of heat exchanger plates in pasteurizers by retarding the colonization of *S. thermophilus* responsible for fouling. The emulsion which remains stable for three months formed by mannoprotein produced by yeast *Kluyveromyces marxianus* on addition in corn oil suggested potential application of biosurfactants as food emulsifier (Lukondeh *et al.,* 2003). The extra cellular carbohydrate rich compound produced by *Candida utilis* is being used as dressings in salads(Shephard *et al.,*1995).

Other than all the above mentioned applications of biosurfactants also been used in process such as like in pulp and paper industries (Rosenberg *et al.,* 1989), leather industries, textile industries, ceramic (Horowitz and Currie, 1990) and uranium ore processing (McInerney *et al.,* 1990). A polymeric biosurfactants, Biodispersan produced by *Acinetobacter calcoaceticus* A2, is observed to have potential applications in paint industry. Biosurfactant was also used as dewatering agent in peat pressing (Mulligen *et al.,* 1985).

2.4 Fungi as a source of biosurfactants

For the biodegradation of pollutants present in soil which got added into the environment from petroleum and its by products, some filamentous fungi present in the soil have ability of producing extracellular metabolites or enzymes for utilizing these hydrocarbons as carbon source. These extracellular product helps in the breakdown of hydrocarbons into small particles which further gets utilized by other microflora. The morphology of fungal systems gives them advantage over bacterial systems and make them efficient under adverse conditions such as extreme temperature conditions, pH conditions, limitations of nutrients and low humidity conditions. They are also easy to culture for scale up for solid state fermentation and thus making the large amount of biomass available for production. Till date the production of biosurfactant from the fungal systems was been explored to limited extent. A review of literature also indicated that most of the work has been done on bacteria, yeast and actinomyces. Reports on the fungal biosurfactants are very limited.

The filamentous fungi, *Curvularia lunata IM 2901*, have been able to produce extracellular emulsifying agent on water soluble compounds with a yield of 2.6g/l. The cell free broth have ability of forming emulsion with hydrophobic compounds. The crude biosurfactant was isolated by acetone precipitation The preliminary chemical characterization of the compound showed the bioemulsifier contained 34.0% carbon, 5.7% hydrogen, 1.8% nitrogen, 0.15% sulphur and it was a complex containing 25% proteins and 48% polysaccharide with sugar component being D-glucose (Paraszkiewicz *et al.*, 2001).

A basidiomycete dimorphic fungi, *Ustilago maydis,* known to cause corn smut indicated the possibility of producing metabolites such as laccase and tyrosinase enzymes and glycolipids and indole exopolysacharides which have various applications in food, pharmaceuticals and cosmetics industries. For the production of exopolysacharides, Czapek Dok–sucrose containing high oil oleic sunflower as a sole carbon source was used (Cornejo *et al.*, 2012). Genetic analysis of biosurfactant production was also done. The produced biosurfactant was derivative of two classes of amphipathic glycolipids. First is ustilagic acids which are cellobiose lipids having disaccharide O- glycosidically linked 15,16 dihydroxyhexadecanoic acids. Second is the Ustilipids which are mannosylerythritol lipids derived from acylated ß-D-mannopyranosyl-D-erythritol (Heward *et al.*, 2005).

A fungal strain, *Syncephalastrum racemosum,* also showed the presence of mono and di glycerides in cell free supernatant. The mentioned fungal strain was cultivated on 18% soyabean oil as a sole carbon source in minimal media. The produced biosurfactant product was estimated to play role in treatment of hazardous hydrophobic substances (Bansal *et al.,* 2010).

Based on literature reviewed in the area of biosurfactants , it indicated that very limited reports are there on fungal biosurfactants. Due to the advantages of fungal systems over other biological systems mentioned above there is a required need of exploiting the fungal microflora for making the industrial production and for making the applications of biosurfactants more effective and efficient in varied environmental conditions.

Keeping these lacunae in consideration the following objectives were outlined.

1. Generation and characterization of surface active substances from oil tolerant fungi.
2. To study the properties of biosurfactant generated by the fungus.

3.0

MATERIAL AND METHODS

3.0 Materials and Methods

3.1 Media and reagents

Media

Potato dextrose agar (PDA), potato dextrose broth (PDB), mycological peptone, Bushnell-Haas broth, Muller-Hinton agar (MHA), Muller-Hinton broth (MHB) and Agar powder was procured from Himedia, India. Cotton seed oil was purchased from local market.

Reagents

Acetone was supplied by Loba Chemie, India. Silica gel G, hexane and ethyl acetate extrapure was supplied by SD-Fine Limited, India.

3.2 Cultivation of test cultures

Two fungal strains viz., *Aspergillus sp.* RBD01 and RBD02, previously isolated from biocontaminated clarified butter were considered for the present study due their potential to carry-out transesterification as whole cell biocatalysts (Aulakh et al. 2011). Reculturing of the two strains of were potato dextrose agar (PDA) and conical flasks containing potato dextrose broth (PDB) by inoculating the fungal colonies in respective colonies. Incubation of both plates and conical flasks was carried out for 5-7 days at 28°C for the spore formation and conical flasks were kept on orbital shaker at 120 rpm and 28°C for the growth of fungal biomass.

3.3 Generation of surface – active substances

Biomass of both the strains, RBD01 and RBD02, were inoculated separately in 500ml flask containing 100ml BHB. Media composition in (g/l) of bushnell haas broth includes: magnesium sulphate (0.20); calcium chloride (0.02); monopotassium phosphate (1.00); dipotassium phosphate (1.00); ammonium nitrate (1.00); ferric chloride (0.05); and pH 7.0 ± 0.2. 5% of cotton seed oil was also supplemented in the minimal media before the inoculation of the cultures. After inoculation, both the flasks were incubated on orbital shaker monitored at 120 rpm and temperature regulated at 28°C for 48-72 h.

3.4 Test for emulsification activity

After the completion of incubation time, the respective cell free supernatants (CFS), were recovered and separated out from biomass with the help of coarse filter paper. The recovered CFS was further used for the estimation of emulsification activity by following the method outlined by Bento and Gaylarde (1996). 35 µl of sample (CFS), or 35 µl tris buffer (control) and 50 µl of xylene was added in a glass test tube each having 5ml of 20mM tris buffer (pH 8.0). Vigorous agitation was carried out for 2 min. After vigorous agitation the test samples were then allowed to settle for 20 minutes at room temperature and optical density (O.D) was measured using UV-Vis spectrophotometer (Labindia UV-3200) at 660nm wavelength.

3.5 Measurement of surface tension

20 ml of CFS collected after 48 h and 72 h of growth and were used for the measurement of the surface tension. Surface tension was measured by ring detachment method by using du Noüy ring-type tensiometer with water as standard. The surface tension was measured in dynes/cm. All the surface tension measurements were taken at room temperature. Lesser the reading of the tensiometer more is the efficacy of the test sample.

3.6 Recovery of crude biosurfactant

Based on the efficacy in reducing the surface tension, RBD02 was chosen for further studies. The strain was allowed to grow in triplicates in 500ml flasks containing 200ml BHB with 5% cotton seed oil as a carbon source for 48-72 h at 28°C at 120 rpm shaker. After the completion of the incubation time, the CFS was recovered and crude biosurfactant was derived by acetone precipitation method. In acetone precipitation method, pre-chilled acetone was added into the supernatant in 3:1 ratio and kept at -20°C for overnight. After 12-16 h, the settled precipitates were extracted by centrifugation at 10000 rpm for 15 min at 4°C temperature in refrigerated centrifuge (Hitachi CF15RX II). The supernatant was discarded and the pellet which is a crude biosurfactant was stored in acetone at -20°C temperature.

3.7 CTAB assay for the detection of glycolipids

The method outlined by Siegmund and Wagner (1991) was used for carrying out cetyltri-methylammonium bromide (CTAB) assay. CTAB assay was followed for the detection of glycolipids in the crude biosurfactant. For the CTAB assay, blue agar plates were prepared by adding CTAB (0.2 mg/ml) and methylene blue (5 mg/ml) in agar powder. On setting of agar, wells were drawn by using cork borer of 5mm dia. Three wells were drawn in each plate. In first well, 30 µl of positive control (sodium deodecyl sulphate) was added followed by addition of 30 l of negative control (distilled water) in second well and 30 µl of test sample of crude biosurfactant in third well. Plates were kept undisturbed for 10-12 h at room temperature and zone of clearance were recorded.

3.8 Antimicrobial activity of the crude biosurfactant

Antibacterial activity of the crude biosurfactant was done against test organism *Staphylococcus epidermidis* MTCC 2639. Test organism was reactivated by inoculating loopful of culture in test tubes containing 20ml of Muller-Hinton broth and incubated at 37°C overnight. Muller-Hinton agar plates were prepared and after solidification, four wells were made in each plate with the help of cork borer of 5mm dia. 30 µl of test compounds i.e crude biosurfactant sample, positive control (streptomycin) and negative control (distilled water) were added in respective wells and plates were kept aside for 15 min. for the proper diffusion of the test compounds. After the diffusion, wells were sealed by adding 20 µl molten agar and plates incubated for 10 min. Test organism was adjusted as 0.5 Mcfarland solution and it was spreaded over the whole surface of the above mentioned petri plates with the help of sterilized cotton swabs for determining the efficacy of our test compound. The plates were kept for overnight incubation at 37°C. Zone of inhibition was observed next day.

3.9 Quantification of Free fatty acids (FFA) by hydrolysis of oil on addition of biosurfactant

For the estimation of the lipolytic activity of the crude biosurfactant, 5ml cotton seed oil was taken in round bottom flask and was incubated for 1 h at 35°C. After incubation, 1 g sample of crude precipitate containing biosurfactant was suspended in small amount of sterilized distilled

water and the mixture was kept for stirring on magnetic stirrer (Tarsons Spinot, India). Samples were withdrawn at various time intervals for determining the presence of formation of FFAs and their quantification by thin layer chromatography (TLC) and by nuclear magnetic resonance (^{1}H NMR).

Determination of FFA by thin layer chromatography (TLC)

For examining the formation of FFA, samples were collected at varying intervals. Samples were collected at varying intervals. First sample was withdrawn at 6 h followed by second sample after 17 h and third sample after 22 h. All the samples were suspended in hexane before TLC. Silica gel G was used as a matrix. The solvent system used included hexane and ethyl acetate in 9:1. The spots were visualized by keeping the plate in iodine chamber.

Quantification of FFA by proton nuclear magnetic resonance (^{1}H NMR)

For FFA quantification by proton NMR (400 MHz – JOEL Bruker-Advance II-400) , $CdCl_3$ was used as solvent and chemical shifts were expressed in parts per million (ppm) with trimethylsilane (TMS) as an internal standard. Samples were withdrawn after 6 h after the start of the reaction, Second sample was withdrawn after 17 h and third sample was withdrawn after 22 h. Free fatty acids (FFA) produced by hydrolysis of oil was quantified by using the derivation outlined by Satyarthi et al. (2009). The FFA amount was estimated on the fact that R-CH_2 peaks of fatty acids appear at δ values higher than those of the triglycerides. The difference in chemical shift was due to the higher de-shielding effect of the carboxylic group as compared to that of ester group. The NMR spectrum of the sample containing FFA shows a quartet like spectral pattern in the R-CH_2 region depending on the content of FFA. The area of the unmerged peak of the FFA triplet was calculated by integrating spectral region at 2.37-2.41 ppm. The concentration of the FFA (wt %) was calculated by:

%FFA = [(4 x Area of unmerged peak of αCH_2 of acid) / Total area of acid and ester] x 100

4.0

RESULTS AND DISCUSSION

4.0 Results and discussions

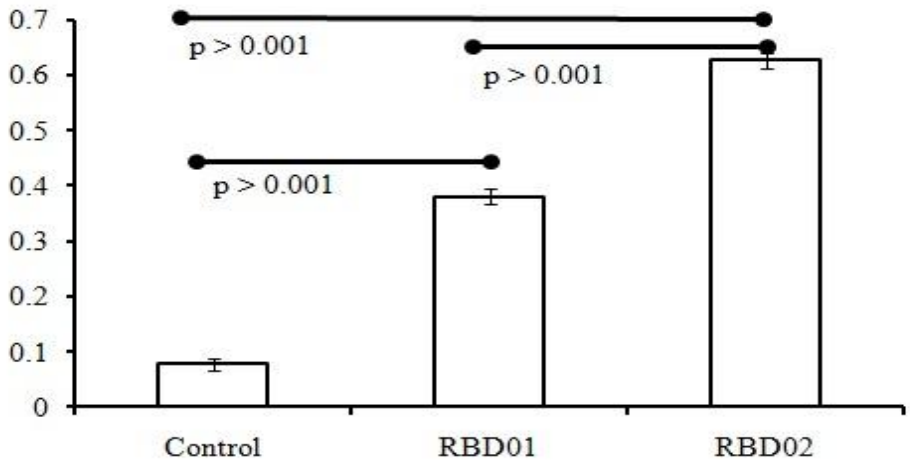
4.1 Emulsification activity of cell free supernatants

Emulsification activity of cell free supernatant which was derived after 72 h of growth of both the fungal species was determined using xylene emulsification method. The emulsification activity depends on the ability of reducing interfacial tension between two immiscible substances. On the addition of xylene in tris-buffer, the presence of biosurfactant results in reduction of interfacial tension of the mixture leading to the formation of stable emulsion between both immiscible substances. The optical density (OD) values for emulsification activity for cell free supernatant of both the fungal strains are outlined in Table 2.

Table 2. Emulsification activity (O.D) of cell free supernatant of RBD01 and RBD02 after 72 h of growth (n=3)

	Emulsification activity (OD_{600})
Control	0.07 ± 0.011
RBD01	0.38 ± 0.015
RBD02	0.62 ± 0.013

Figure 2. Comparative emulsification activity of RBD01 and RBD02 fungal strains with reference to control



In the present studies, the observed (OD) values show that both the fungal strains (RBD01 and RBD02) exhibit significantly higher ($P<0.0001$) emulsification as compared to control (**Figure 2)**. However, the activity shown by RBD02 was relatively higher and stable as compared to RBD01. The RBD02 strain showed notable and comparable stability of emulsion layer with reference to other reports on bacteria such as *Bacillus stearothermophilus* (Gurjar *et al.*, 1995) and *Bacillus* sp. (Bento and Gaylarde, 1996).

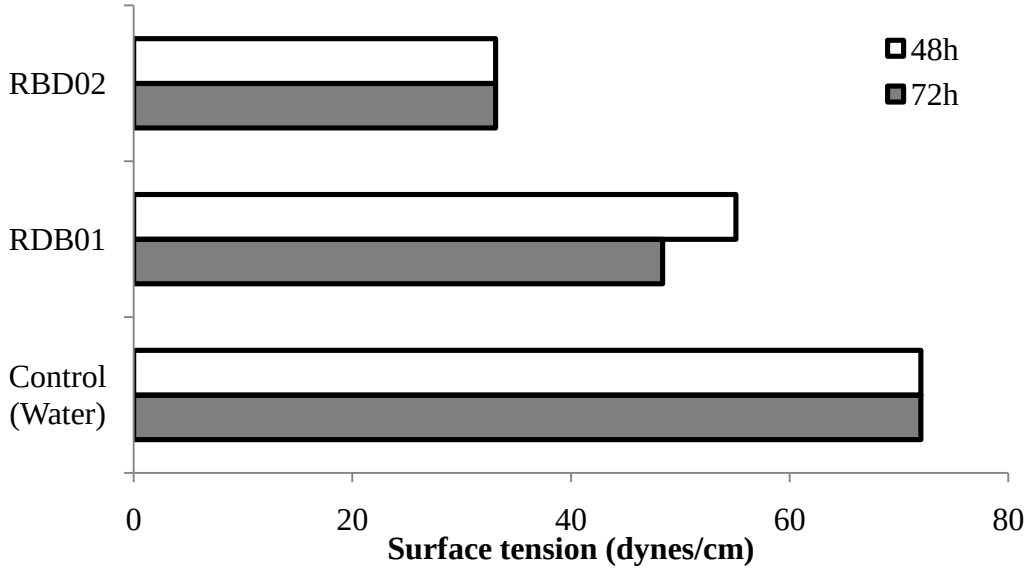
4.2 Surface activity of cell free supernatant

The surface activity of cell free supernatants of both the fungal strains derived at 48 h and 72 h of growth of the fungal biomass were analyzed. Lesser the value of surface tension observed in the tensiometer more was the efficient expected in our test compound. The surface tension is the tendency of molecules in a fluid to be pulled towards the centre of the liquid. Biosurfactants lowers the surface tension of water by interfering with the intermolecular attraction of the water molecule, the surface tension of water gets reduced and results in the mixing up of hydrophobic compounds with water. The surface activity of cell free supernatants of both the fungal strains was measured using De-neuy- Tensiometer by ring detachment method and are outlined in Table 3.

Table 3. Surface tension values of RBD01 and RBD02 strains with 5% cotton seed oil as carbon source

	Time (h)	Surface Tension (dynes cm)
Standard (water)		72.0
RBD01	48 h	48.38
	72 h	55.10
RBD02	48 h	33.12
	72 h	33.12

Figure 3. Profile of surface tension of the CFS at various time intervals of growth with reference to control



The observed values of both the fungal strains indicated that fungal strain RBD02 was efficient in producing surface active substances. It had ability of reducing the surface tension upto 33 dynes/cm which remains constant even when measurement were taken for 72 h CFS derived from RBD02. As reported by Rehman *et al.,* (1996) the air/water surface tension for distilled water at room temperature was 72 dynes/cm and addition of biosurfactant from 5 different bacterial strains to it lowered the interfacial tension to 28 dynes/cm. The surface tension values for fungal strain *Synacephalastrum racemosum* was 42 dynes/cm as reported by Chandni *et al.,* (2007) which were lower as compared to the present study. The surface tension value of 48 h cell free supernatant of RBD01 was 48 dynes/cm which got increased with increase in time and reached at 55 dynes/cm over time. On the basis of above mentioned surface tension results, RBD02 strain was chosen for the further analyses.

4.3 Yield of recovered crude biosurfactant

In the present study by using chilled acetone precipitation method, creamish coloured precipitates were extracted from supernatant of fungal strain RBD02. The yield of crude biosurfactant was noted to be 4.65 g/l approximately. The present reported value was comparably higher than the previously reported biosurfactant fungal strain *Synacephalastrum*

racemosum by our group (Chandi, 2007) which was able to generate approximately 4g/l of crude biosurfactant. The yield of biosurfactant of RBD02 strain increased gradually during exponential phase and started decreasing during the stationary phase similar reports has been given for *Synacephalastrum racemosum* (Chandni *et al.*, 2010) and also for *curvularia lunata* (Paraszkiewicz *et al*., 2002). Smut fungus *Ustilago maydis* DSM 4500 and ATCC 14826, when grown on vegetable oils produced glycolipid biosurfactant at a similar level with *C. lunata* IM 2901 (2 g/l) (Paraszkiewicz *et al.*, 2002).

4.4 CTAB assay for the detection of glycolipids in crude biosurfactant

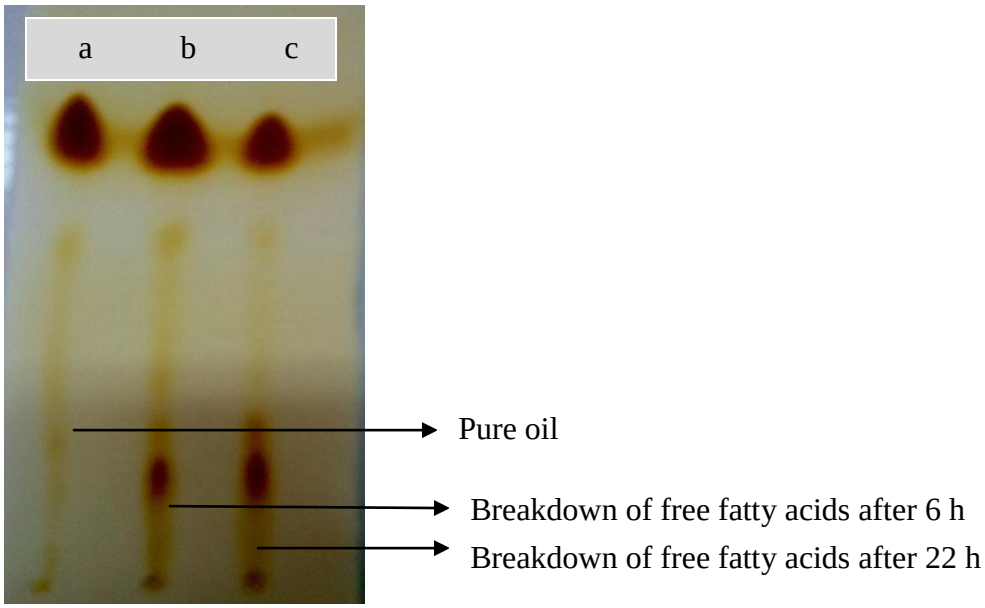
After 12-14 h of incubation of the plates at room temperature, limited zone of clearance was observed around the well containing crude biosurfactant sample whereas, on the other hand blue color zones started appearing after 1 h of incubation around the positive control (SDS). This indicated that there was no clear evidence of generation of extracellular glycolipids or any other anionic surfactant by the test fungi. Extracellular glycolipids production was reported by Nishanthi et al., (2010) where production of extracellular anionic surfactant/glycolipids by bacterial strain lead to blue color zone formation around the bacterial colonies. Till date, there are limited reports on glycolipids/anionic surfactant production by fungal systems. Fungal strain *Ustilago maydis* is reported to produce extracellular glycolipid (ustilipids) (Sekhon *et al.*, 2010). Tomotake et al., (2009) reported that fungal strain *Ustilago scitaminea* NBRC 32730 was able to produce glycolipids in culture broth with sucrose as carbon source. In the present study the crude biosurfactant produced by RBD02 have liquefying property when kept at room temperature. It was hereby, hypothetically inferred that it might be lipoprotein. In addition, RBD02 strain showed very mild activity against test organism as compared to other classes of biosurfactant derived from other bacterial sources.

4.5 Hydrolysis of oil to fatty acids through catalytic activity of crude biosurfactant

The fungal strain RBD02 which was oil tolerant strain isolated our group earlier could grow in oil supplementation to the extent of 70% in the growth medium. Keeping this in view, the crude biosurfactant isolated from this organism was examined for its potential to hydrolyze oil into free fatty acids (FFA). Preliminary screening for lipolytic activity was carried out using

TLC. The generation of free fatty acids produced in the reaction mixture enhanced with increase in the time till 22 h of reaction. Lipase enzyme is necessary for the breakdown of the triglycerides into free fatty acids (FFA). This indicated that RBD02 had ability of producing extracellular lipase enzyme and hence, proved to facilitate hydrolysis of oil to fatty acids. Figure 2 depicts the decrease in the amount of triglycerides in the reaction mixture with increase of time and simultaneous increase in generation of free fatty acids.

Figure 4. Thin layer chromatography showing breakdown of oil (lane a and b) in comparison to pure oil (lane a)



This observation was further confirmed by using ^{1}H NMR technique. The FFA generated during the hydrolytic reaction of CFS was quantified using formula derived by Satyarthi et al. (2009). Due to formation of FFAs, a new triplet of αCH_2 (of FFA) appears adjacent to the triplet of αCH_2 peak of triglycerides. One of the peaks of the triplet of FFA (at 2.38 ppm) remains separate while the other two peaks of the triplet of FFA (at 2.34 and 2.30 ppm) merge with αCH_2 peak of triglycerides at 2.35 ppm and 2.31 ppm. Therefore, a sample containing both triglycerides and FFAs show a quartet-like pattern in αCH_2 region of NMR spectra . Whereas, in case of only tri-glycerides, a clear triplet can be observed at αCH_2 region (**Figure 5**). Appearance of a quartet at 6 h and 22 h, in the present study, clearly indicated the formation of FFAs due to

hydrolysis of oil by the CFS generated by RBD02. The FFA, as quantified using the derivation of Satyarthi et al., (2009), indicated generation to an extent of 5.7% after 6 h of reaction which further increased to 17% in 22 h. In an independent determination, an extended period of incubation (30h) with lesser supplementation of crude biosurfactant (300 mg) resulted in an enhanced lipolytic activity **(Figure 6)** which was indicate of the stability and lipolytic activity of the biosurfactant.

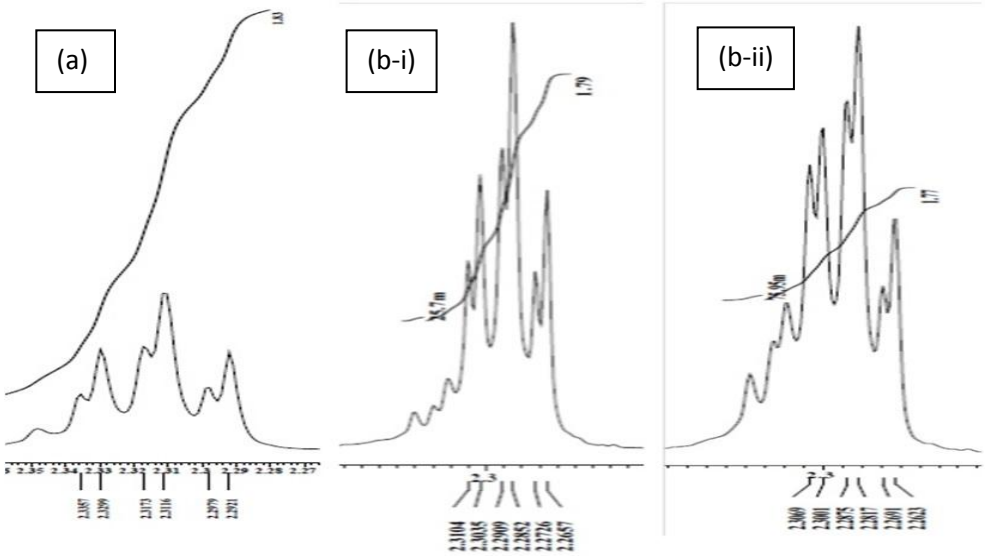


Figure 5. ^{1}H NMR spectra of pure oil (a) and fatty acids generated after 6h (b-i) and 22h (b-ii) incubation

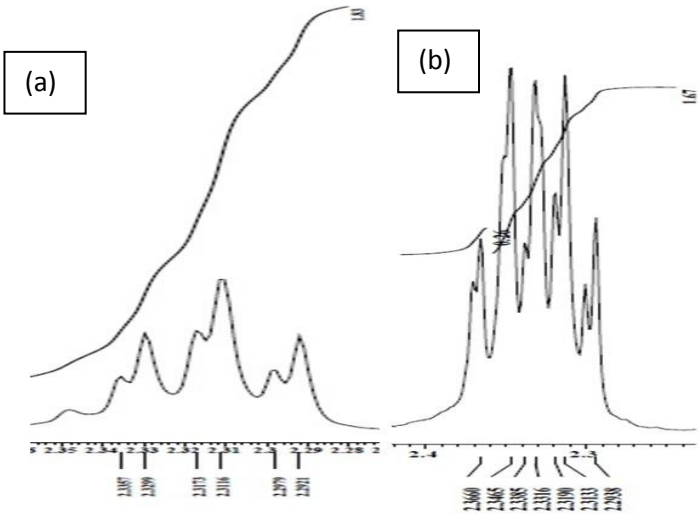


Figure 6. ^{1}H NMR spectra of pure oil (a) and fatty acids generated after 30h (b) incubation

The variations due to enhanced FFA generation at low concentration of biosurfactant during 30h incubation when compared to lower FFA generation at high surfactant concentration is presumed to be due to the presence of optimal concentration of the catalyst and time of incubation when compared to the observation obtained during 22h incubation.

These observations are comparable to studies made by Katsivela et al., (1995) in which the lypolytic activity of crude enzyme of *Ustilago maydis* was tested for the hydrolysis of fatty acids ethyl esters and simple triglycerides and found to 20% against Fatty acid ethyl esters whereas poor against triglycerides as determined by volumetric method. Whereas, in present studies the 17% breakdown of triglycerides was observed in comparison to crude enzyme of *Ustilago maydis* which showed poor hydrolysis of triglycerides. Till date, there are limited reports on the lipolytic activity of biosurfactants. Lipase production in disparate fungi is very dintinct. *Penicillium* and *Rhizopus* strains had capability of producing lipolytic enzyme (Katsivela *et al.,* 1995). Another report on filamentous fungi *Rhizopus microspores* revealed that the fermented solid produced in the solid state cultivation was utilized for the hydrolysis of high fat dairy wastewater and was reported to induce lipolytic activity of around 30 U/gds after 20 h of fermentation. (Alberton *et al.,* 2010).

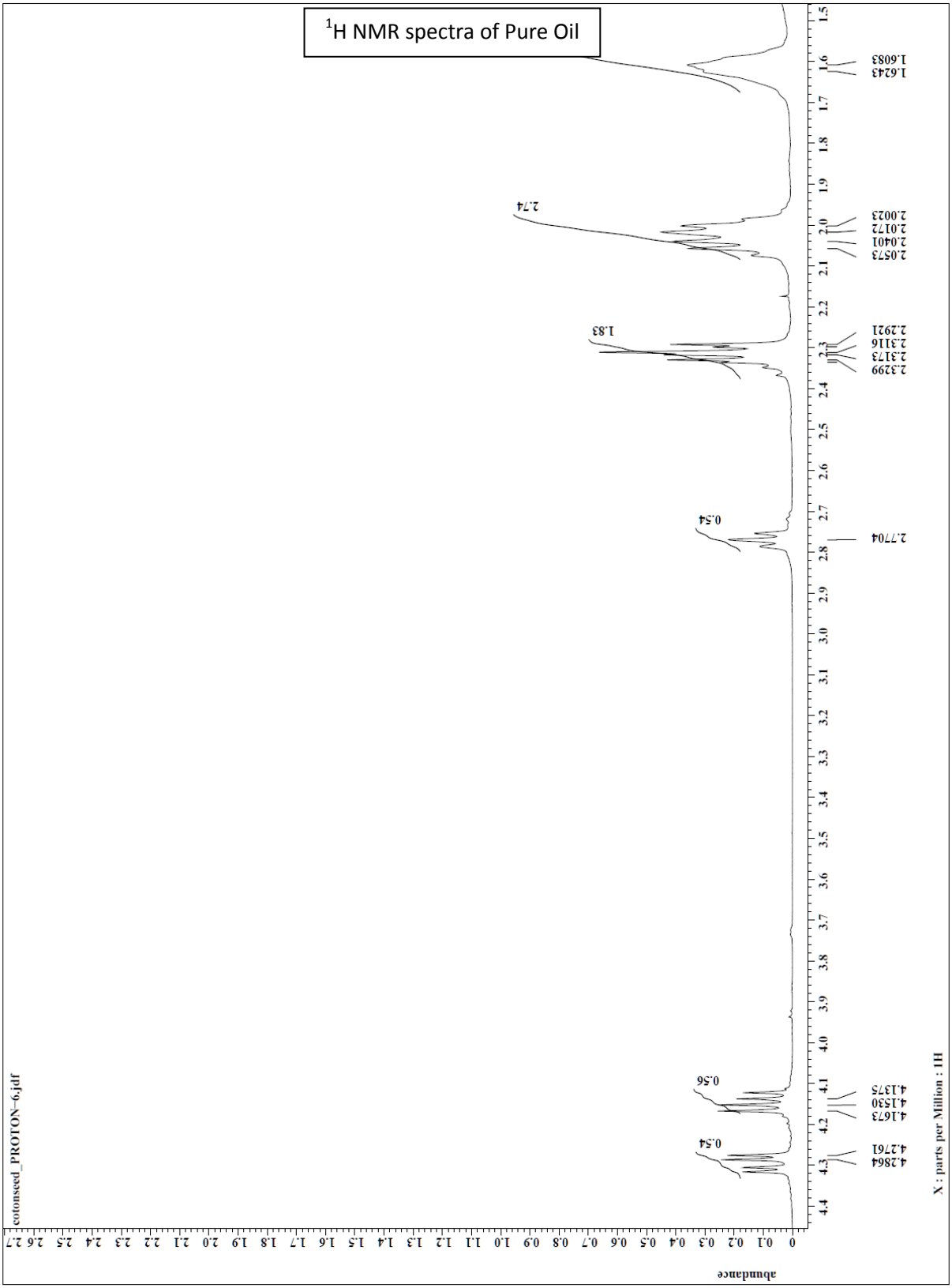
The results obtained in the present study were therefore indicative that along with the production of the surface-active substances, the enzymatic substances were also released which presumably was facilitated through extracellular secreted compounds. The association between the emulsification and lipolysis indicated that the extracted crude surface-active substance could be a lipoprotein.

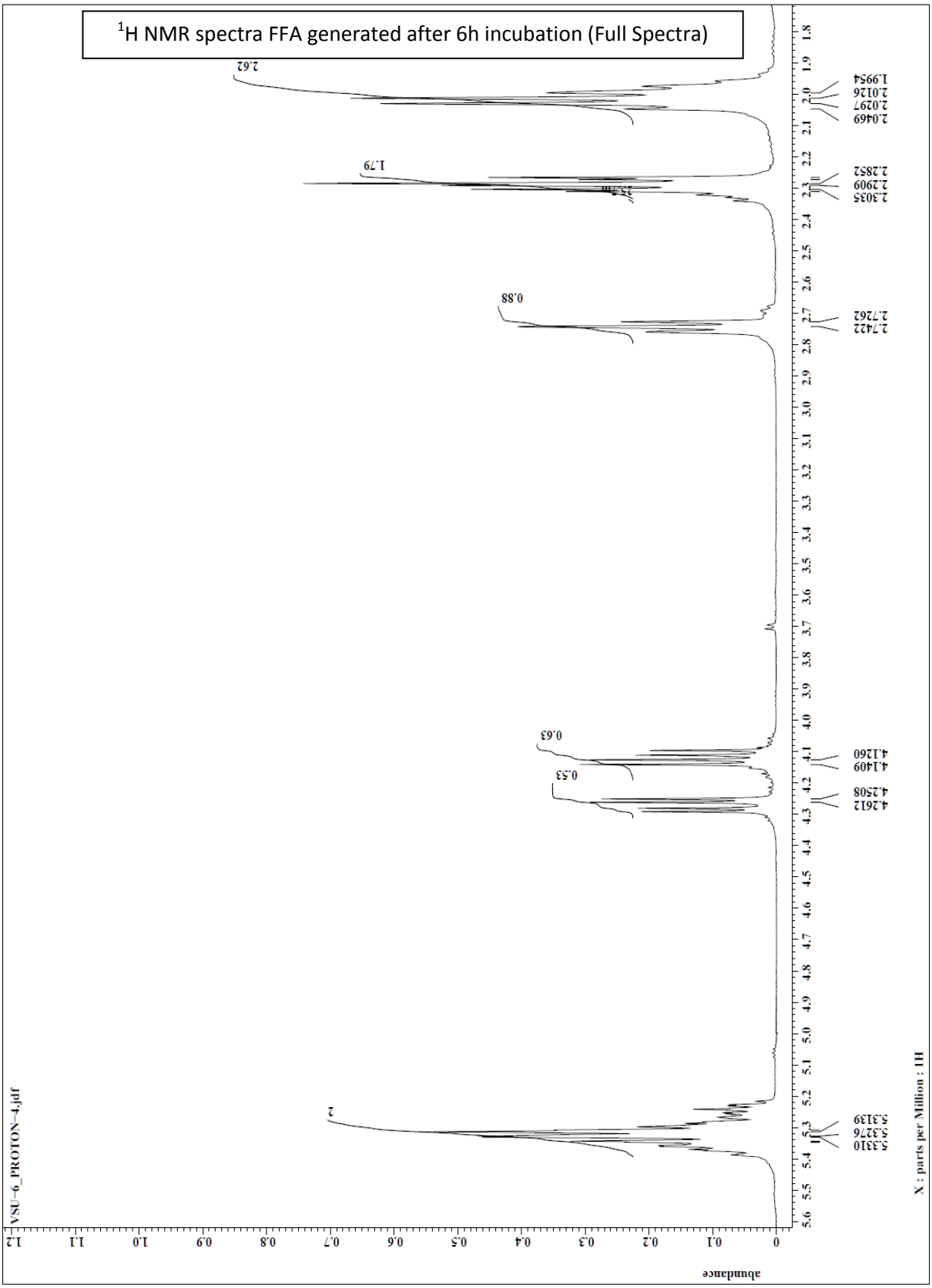
Conclusion

- The present study demonstrates that the in both the fungal strains RBD01 and RBD02, the fungal strain RBD02 is able to produce surface-active substances by utilizing 5% cotton seed oil present in the medium as a carbon source.
- The emulsion formed with from cell free supernatant of RBD02 with xylene was stable for more time as compared to RBD01 strain. This indicates that RBD02 have good emulsification activity.
- The RBD02 showed high ability of lowering down the surface tension upto 33.12 dynes/cm with 5% oil which is quite remarkable and proved the crude biosurfactant as a good quality biosurfactant.
- The yield of the crude biosurfactant derived by acetone precipitation method is very satisfactory as compared to the other fungal biosurfactants yield.
- In addition of ability of lowering down the surface tension the crude biosurfactant, the crude surfactant also showed significant lipolytic activity as demonstrated by the amount of free fatty acid (FFA) generated and examined by thin layer chromatography. The observations clearly indicated the increase in the amount of free fatty acid (FFA) in the reaction mixture and decrease in the amount of oil with increase in time which becomes constant after 28-30 h.
- The quantitative estimation of free fatty acid (FFA) was done by ^{1}H NMR and was observed to be 6% FFA after 6 h and 17% FFA after 22 h.
- An independent observation indicated higher FFA generation at (44%) at relatively lower concentration of biosurfactant (300mg) which showed optimal potential of crude biosurfactant to hydrolyze oils to fatty acids even at lower supplementation.
- This study concludes that the fungal strain RBD02 have noticeable ability of producing surface-active substances and in addition also have ability of producing extracellular lipase enzyme which can hydrolyze oil into free fatty acids .

5.0

^{1}H NMR Spectra


^{1}H NMR spectra of Pure Oil
cotonseed_PROTON-6.jdf
X : parts per Million : 1H
abundance
0.54
0.56
1.83
2.74
4.2864
4.2761
4.1673
4.1530
4.1375
2.7704
2.3299
2.3173
2.3116
2.2921
2.0573
2.0401
2.0172
2.0023
1.6243
1.6083


^{1}H NMR spectra FFA generated after 6h incubation (Full Spectra)
VSU-6_PROTON-4.jdf
X : parts per Million : 1H
abundance
2
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0.63
0.88
1.79
2.62
5.3310
5.3276
5.3139
4.2612
4.2508
4.1409
4.1260
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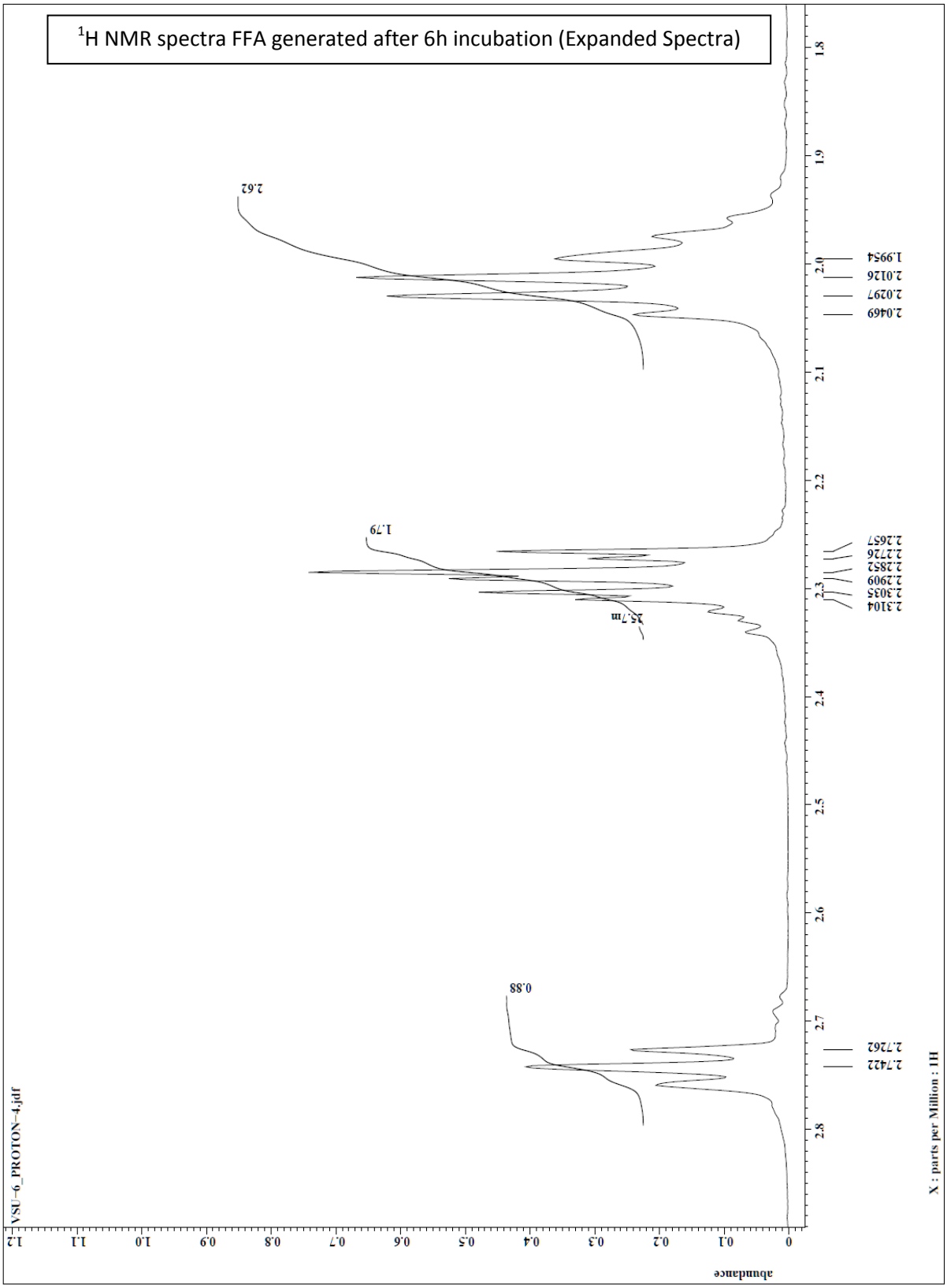
2.2852
2.0469
2.0297
2.0126
1.9954


^{1}H NMR spectra FFA generated after 6h incubation (Expanded Spectra)
VSU-6_PROTON-4.jdf
abundance
X : parts per Million : 1H
0.88
1.79
2.62
2.7422
2.7662
2.3104
2.3035
2.2909
2.2852
2.2726
2.2657
2.0469
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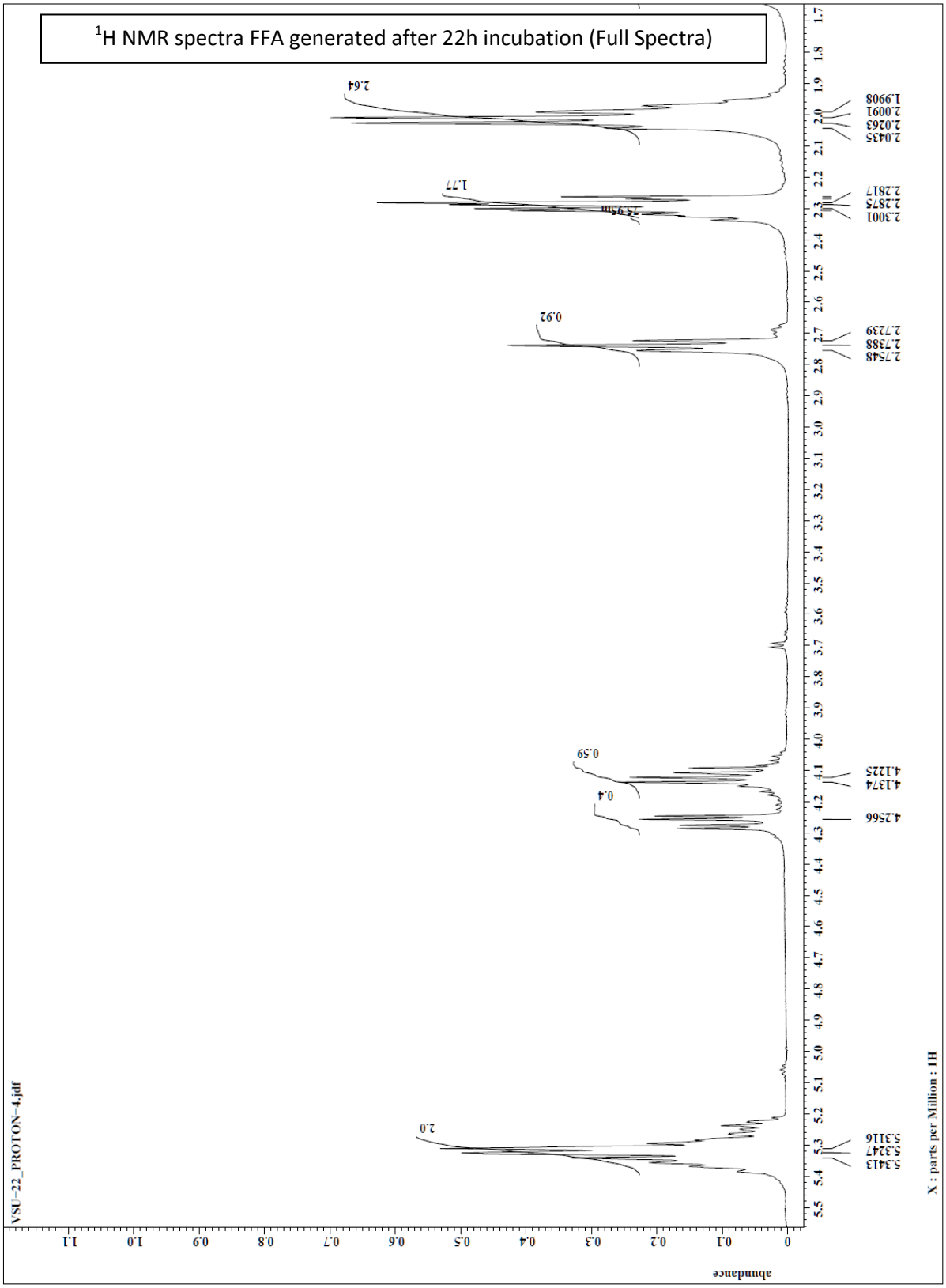
2.0126
1.9954


^{1}H NMR spectra FFA generated after 22h incubation (Full Spectra)
VSU-22_PROTON-4.jdf
X : parts per Million : 1H
abundance
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5.3247
5.3116
4.2566
4.1374
4.1225
2.7548
2.7388
2.7239
2.3001
2.2875
2.2817
2.0435
2.0263
2.0091
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1.77
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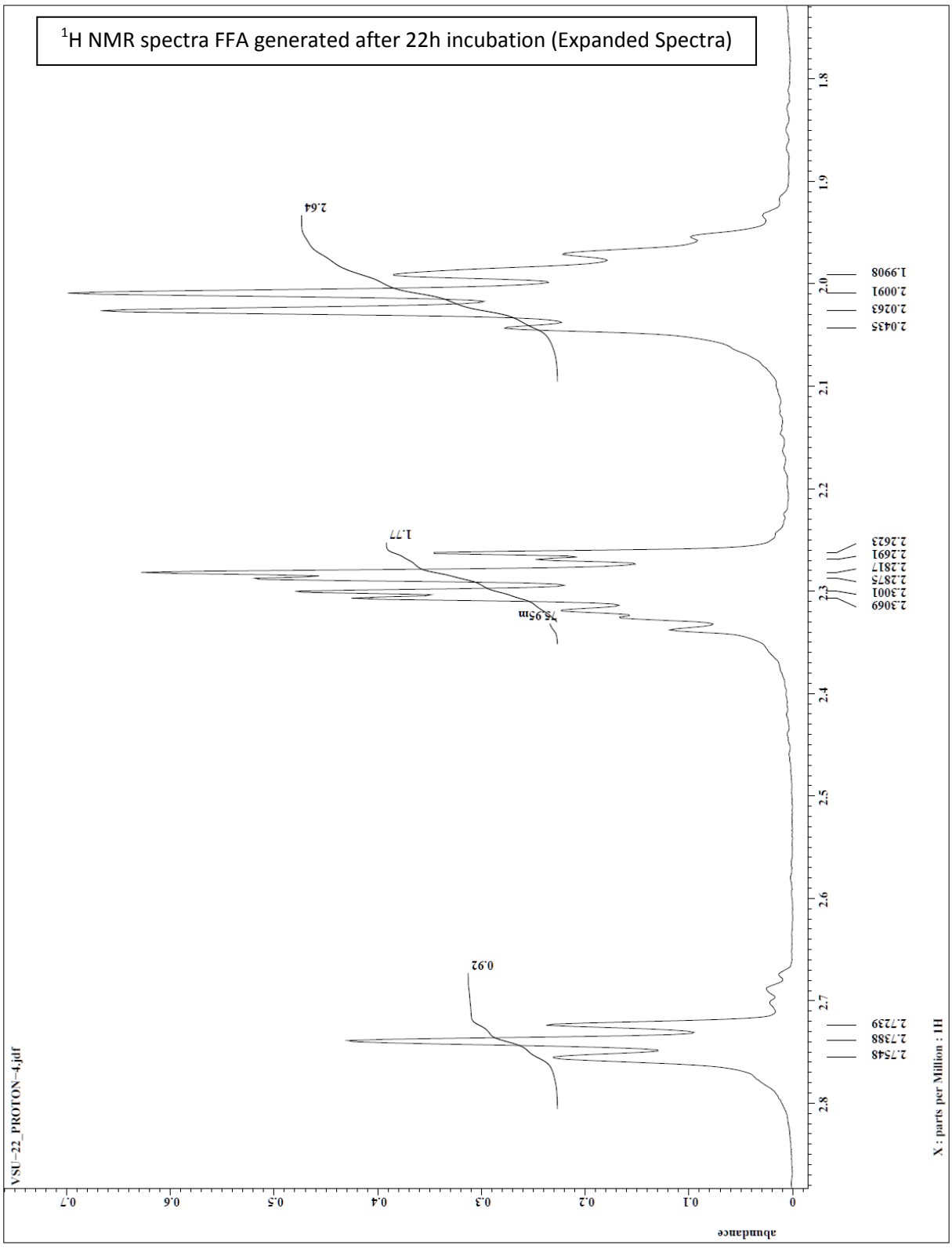
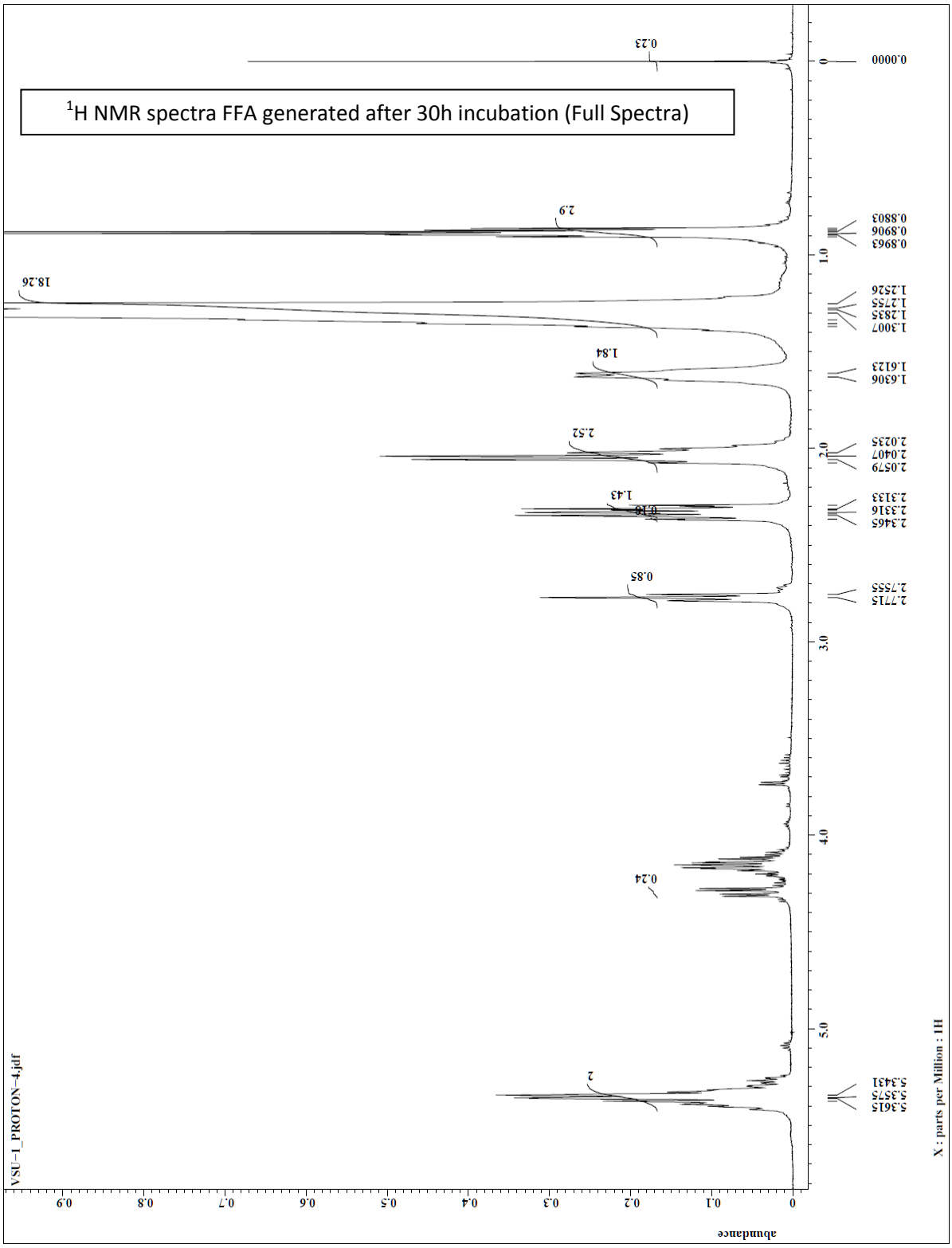
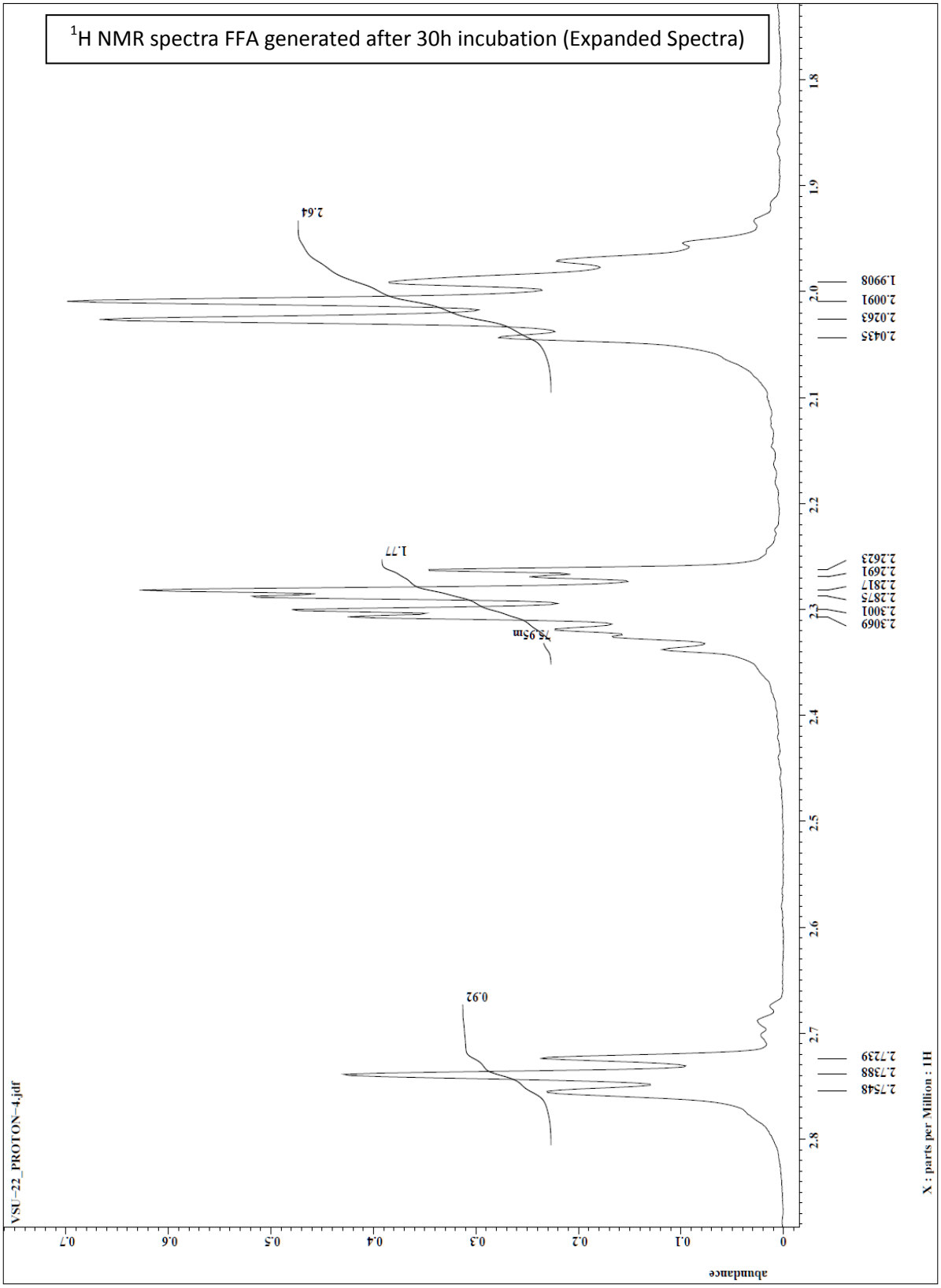
0.59
0.4
2.0

^{1}H NMR spectra FFA generated after 22h incubation (Expanded Spectra)




^{1}H NMR spectra FFA generated after 30h incubation (Full Spectra)
VSU-1_PROTON-4.jdf
X : parts per Million : 1H
abundance

^{1}H NMR spectra FFA generated after 30h incubation (Expanded Spectra)



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