SELECTIVE EXTRACTION AND PARTIAL PURIFICATION OF BIOMOLECULES USING REVERSE MICELLE BASED SYSTEM

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BY

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CONTENTS

LIST OF TABLES

LIST OF FIGURES

DECLARATION BY THE CANDIDATE

I hereby declare that the thesis entitled, **"Selective extraction and partial purification of biomolecules using reverse micelle system"** submitted by me for the degree of doctor of philosophy is the record of work carried out by me under the guidance of Dr. B. D. Kulkarni and has not formed the basis for the award of any degree, diploma, associateship, fellowship, titles in this or any other university or other institute of higher learning. I further declare that the material obtained from other resources has been duly acknowledged in the thesis.

Mr. Raghavendra P. Gaikaiwari

Research Student

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LIST OF ABBREVIATIONS

AOT	Aerosol OT (bis(2-ethylhexyl) soldium sulfosuccinate)
CTAB	Cetylmethylammonium bromide
g	Gram
HPLC	High performance liquid chromatography
h	Hour (s)
M	Molar
min	Minutes
ml	Mililiter
L	Liter
O/W	Oil in water
pI	Isoelectric pH
SDS	Sodium dodecyl sulphate
TLC	Thin layer chromatography
TS	Total solid (s)
T80	Polysorbate (Tween) 80
µl	Microliter
W/O	Water in oil

ABSTRACT

Several natural products have found applications as neutraceuticals, food supplements, pharmaceutical intermediates and cosmetics. Processes need to be developed and optimized for the extraction of such products from their natural sources. Problems common to many such products include (i) low initial concentrations of the products of interest in a complex matrix, (ii) the presence of several impurities of similar chemical composition in the original source and (iii) low stability even at room temperatures. Several reported liquid-liquid and solid-liquid extraction processes suffer from the disadvantages of low selectivity, multiple steps and long processing times. In this study, we have focused on microemulsion based techniques, an area that has remained largely unexplored for natural product extraction. We have chosen three small molecules, *viz*., solanesol (a trisesquiterpenoid), lycopene (a carotinoid) and 11-α-hydroxy canrenone (a steroid) and two microbial enzymes, *viz*., lipase and tannase. Solanesol and lycopene are sourced from tobacco dust and tomatoes, respectively, while the rest of the products are from fermentation broths.

The parameters optimized in this study include the type of surfactant, concentration of surfactant, relative ratio of the two phases, pH, temperature, time of extraction, back-extraction conditions, etc. We find that the microemulsion based systems, when optimized, show several fold improvement in extraction efficiency and product purity for all the products considered in this study. The process involves only a single step, affords shorter processing times and higher yields. Note that the optimal surfactant type and extraction conditions varied from product to product.

Microemulsion based extraction of solanesol from tobacco dust offered a single step process and a 2.7-fold increase in purity compared to a conventional multi-step extraction process. For lycopene extraction from tomato paste, the extraction yield improved from 160 mg per kilogram of paste for conventional extraction to 440 mg per kg for microemulsion based system. Likewise, extraction of 11 hydroxy canrenone from fermentation broth was developed to obtain optimum yield and purity of 95% and 92%, respectively. To the best of our knowledge, the use of ethyl acetate-aqueous phase-surfactant microemulsion system for extraction of steroids has been reported for the first time and can be extended to the recovery of other steroid bioconversion products form fermentation broths. These three examples demonstrate the applications

of microemulsion based extraction of three small molecule natural products from their natural source and advantages over conventional extraction process.

Reverse micellar extraction process has been developed and optimized for extraction of lipase and tannase from fermentation broths. Lipase was extracted using reverse micellar system of AOT in iso-octane to obtain 15.6-fold purification with 80% recovery. Likewise, tannase was purified using cationic surfactant CTAB to obtain enzyme recovery of 60 % and 20-fold purification. These values compare favorably with the conventional multi-step purification processes. Conventional protein purification is a multi-step, time consuming process, with loss of product at every stage of purification. The surfactant based extraction process enables single step purification as well as concentration of enzymes with high recovery and multi-fold purification when compared to the conventional processes. The lessons learnt regarding the effects of pH, ionic strength and surfactant concentration and extraction time on extraction efficiency would be useful in developing RME based extraction systems for other proteins of commercial interest.

CHAPTER 1

Introduction and Review of Literature

Several natural products have important applications as nutraceuticals, food supplements, pharmaceutical intermediates and cosmetics. The products are typically found in roots, leaves, fruits or trucks of plants. The products are found in low initial concentrations in a complex matrix. Additionally, the products may be labile to high temperatures or harsh extraction conditions. Extraction and purification of such products from their natural sources constitutes a commercial activity that is growing at a rapid pace. Therefore, there is an interest in developing novel and commercially viable processes for the extraction of such natural products.

Literature and patent survey shows over a hundred commercially important natural products. In this study we have focussed on three small molecule and two macromolecule natural products. These are solanesol, lycopene and 11-α-hydroxy canrenone and lipase and tannase, respectively. Lycopene, which has high nutraceutical and cosmetic value, is found in tomatoes, watermelons, carrots and some other varieties of fruits. Solanesol, a 45 – carbon isoprenoid alcohol is found in leaves of various members of solanaceae. Of these, highest content has been detected in tobacco leaves. Solanesol is the starting material many of high value biochemicals such as vitamin K and its analogues and co-enzyme Q10. Another phenomenon of recent past is the growing use of bioconversion processes for producing commercially significant chemicals. In such cases the product of interest may be present in either the cell mass or in the extracellular medium. In the former case, process or operation similar to the ones in the case of lycopene or Solanesol have to be adopted for recovering the chemical products of interest from bio-mass. 11-α--hydroxy canrenone and enzymes such as lipase and tannase are significantly important chemicals under this category.

The most widely used operation for recovery of a product from a solid mass (roots. leaves, biomass) is contacting the solid man with a liquid (water or solvent) or CO_2 at supercritical conditions. This operation of solid liquid contacting is referred to as leaching or extraction. Many investigations have been carried out using extraction aided

by enzymes, microwave etc. However, very little work is reported on use of microemulsions in leaching/extraction operation.

Microemulsions, as discussed in later part, are known to offer many distinct advantages for leaching and extraction. The present investigation is regarding comparative study of use of micelle or reverse micelle based systems and other more conventional methods in recovery of the five products i. Solanesol(from tobacco leaves), Lycopene(from tomato paste),11-hydroxy Canrenone (from fermented broth), bacterial lipase (fermented broth) and fungal tannase (fermented broth).

The present chapter has been divided into two parts. Part A provides a brief introduction to each of the five biomolecules in terms of their characteristics and applications. This part also describes the currently available extraction and purification techniques for these biomolecules. Part B describes microemulsions and their general applications with emphasis on their phase behaviour.

PART A: Review of biomolecules

1.1. Solanesol

Solanesol is a 45-carbon trisesquiterpenoid alcohol predominantly found in tobacco leaves (Fig. 1.1). Solanesol was isolated for the first time from flue-cured tobacco by Rowland *et al* (1956). It is an important precursor of tumorigenic poly nuclear aromatic hydrocarbons (PAHs) of tobacco smoke.

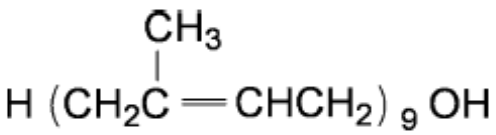

Fig. 1.1: Molecular structure of solanesol

1.1.1. Uses of Solanesol

(i) Solanesol acts as the starting material for many high-value bio-chemicals such as vitamin-K analogues and coenzyme Q10. Coenzyme Q10 is useful in cosmetics and the treatment of heart diseases, cancers and ulcers. Researchers emphasize that introduction of Solanesol radical into the structure of some of the mentioned medicines increases their effect noticeably.

(ii) Solanesol is also used as the main coenzyme against cardiac insufficiency, muscular dystrophy and anaemia.

(iii) Solanesol can be used as lipid antioxidant and in the preparation of cardiac drugs, due to its cardiac stimulant properties. It is also stated that Solanesol possesses antibacterial, anti-inflammation and anti-ulcer properties.

(iv) Solanesol derivatives are found to be novel hybrid compounds which can be used as wound healing agents (Srivastava *et al.*,2009).

1.1.2. Methods for recovery/extraction of solanesol

Significant attention has been directed towards the extraction and purification methodologies to obtain purified solanesol. Although solanesol is mainly primarily found in tobacco leaves, there are reports of Solanesol extraction from other sources such as potato leaves (Asahina *et al*., 1977). Several methods for solanesol extraction have been reported in the literature, which can be divided into two broad categories:

a. Extraction using solvents
b. Extraction using super-critical carbon dioxide

Here we discuss some of the recent innovations in solanesol extraction and their advantages and disadvantages.

Microwave assisted solanesol extraction

Microwave-assisted extraction of biologically active compounds is advantageous over the traditional heat-flux extraction method because of shortened extraction time and lower consumption of solvents (Zhou and Liu, 2006). Microwave energy penetrates the samples and produces a volumetric distribution of heat due to molecular friction resulting from dipolar rotation of polar solvents and from the conductive migration of dissolved ions. The localized temperature and pressure created can cause migration of target compounds from the material to the extraction solvent at a rapid rate.

This invention employed microwave induction to enhance the efficiency of Solanesol extraction from tobacco leaves. The efficiency was improved further by addition of NaOH to the solvent system. For sample preparation, tobacco leaves were dried under uniform temperature and pulverized to powder by a mortar, and then again kept at ambient temperature. The solvent used for extraction was hexane and ethanol (1:3 v/v). Hexane was used as solvent because of high solubility of Solanesol in hexane and ethanol being a good absorber of microwave energy ($\varepsilon = 25.7$). The optimum concentration of NaOH reported was 0.05M. In these experiments the household microwave was modified by adding magnetic stirrer, water condenser, temperature measurement and time control (see Fig. 1.2). The microwave power required for this

sample suspension was 700W. The highest percentage extracted of Solanesol was 0.91% in 40 min.

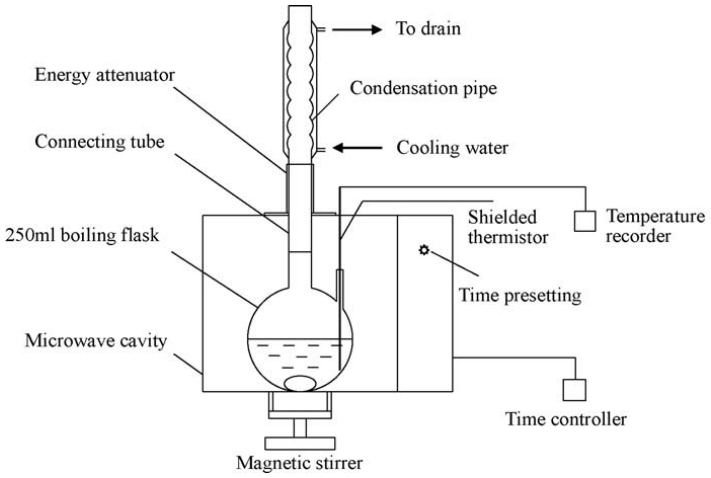

Fig. 1.2: Microwave assisted extraction set up (Source: Zhou and Liu, 2006)

The drawback of this system is that it is an energy intensive process and difficult to scale-up. Microwave systems at commercial scale are very expensive.

Solanesol extraction using bubble column extractor

This method reported by Zhao *et al.* (2009) employed a bubble column device consisting of a glass cylindrical vessel, a sampling port, a flow meter, air inlet, and a pump as shown in Fig. 1.3. The internal diameter of the bubble column was 15 cm, with working volume of 50 L. From the bottom of bubble column gas was sparged in the form of bubbles. This increased the turbulence and facilitated efficient extraction of the target compound. Air flow was controlled by a valve and measured by flow meter. The optimum conditions reported were 80% ethanol used as extraction solvent, particle size less than 350 µm, an extraction time of 54 min, a liquor to material ratio of 13 L/kg and an air flow of 75 L/min. The extraction time required was 54 minutes. However the overall performance of the method depended upon the extraction solvent used, particle size of the sample, extraction time, air flow and liquor to material ratio. The yield of solanesol reported was 4.2 mg/gram of tobacco dust and it was equivalent to percolation and soxhlet extraction methods. The only advantage reported was time required was 54 minutes as against 24 hours for percolation and 6 hours for soxhlet extraction.

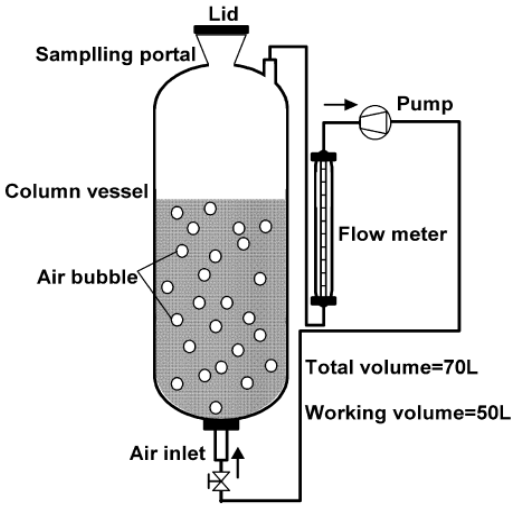

Fig. 1.3: Schematic diagram of the bubble column extractor (Source: Zhao *et al., 2009)*

Supercritical Fluid extraction of solanesol

This method was earlier employed for refining of Solanesol from the crude extract. This method has also been used for extraction of nicotine from tobacco. It has the following features: an extraction cell of 14 cc placed in an oven with temperature controller; at the exit of the system heated back pressure regulator is present which controls the pressure inside the extraction cell; extracts were recovered in a glass cold trap inside an ice bath; the CO_2 pneumatic pump is also present and the CO_2 mass flow rate was measured by two mass-flow meters (see Fig. 1.4).

The sample preparation involved freeze-drying in lyophilizer, followed by final drying at 0°C, milling and sieving. The final powder form of the tobacco leaves is collected. Results revealed that high-pressure CO_2 extraction could be used to obtain a Solanesol even when working at low extraction temperatures. Solanesol/nicotine ratios of 12.20 at 40°C and 15MPa, and 18.91 at 25°C and 8MPa were recorded (Ruiz-Rodriguez *et al*., 2008).

The drawback of the process was that purity of solanesol 20% pure, with contamination of nicotine in the range of 3 to 20%. Further, the process would be very expensive and capital intensive due to use of lyophilization and supercritical extraction steps.

Solid phase extraction of solanesol

This method involved sample preparation in which the flue-cured tobacco leaves were dried, ground and extracted with petroleum ether at 50°C for 2 h and then filtered. This

residue was concentrated in a rotary vacuum evaporator at 40°C to get semi-solid material. Silica gel column, preconditioned by petroleum ether was used for the solid phase extraction (Tang *et al*., 2007). On validation, the mean recovery for Solanesol was found to be 97.5%. Thus it was concluded to be an efficient method for extraction. However nothing was mentioned about the use of this method on industrial scale.

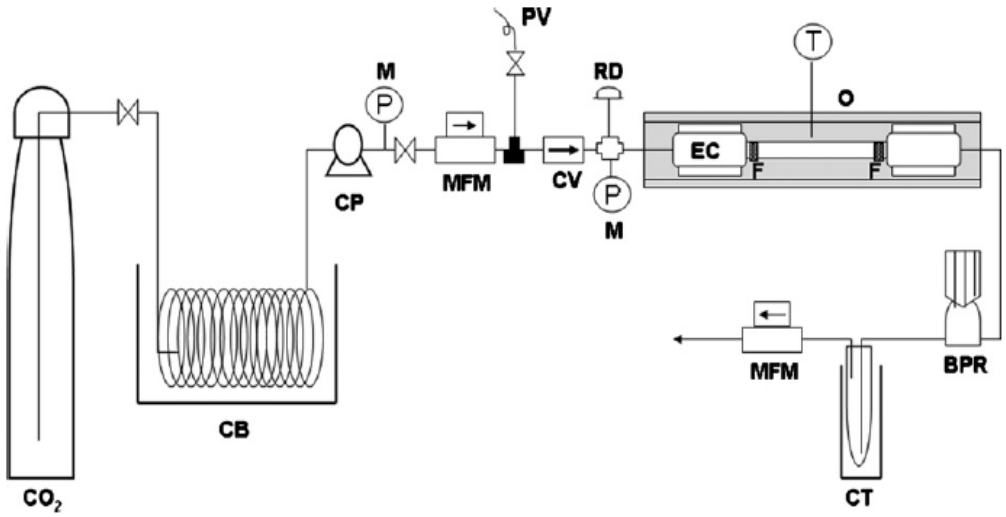

Fig. 1.4: Schematic diagram of the supercritical fluid extraction system (Source: Ruiz-Rodriguez *et al*., 2008)
(CB – cooling bath ; CP – CO2 pump ; M –manometer ; MFM – mass flow meter ; CV- check valve ; PV –purge valve ; RD – rupture safety disk ; EC- extraction cell ;BPR – back pressure regulator; F-filters ; O -oven)

1.2. Lycopene

Lycopene is a naturally occurring bright red carotenoid pigment and it possesses a high nutraceutical value. It is an acyclic isomer of beta-carotene. This highly unsaturated hydrocarbon contains 11 conjugated and 2 unconjugated double bonds, making it longer than any other carotenoid (Fig. 1.5). Lycopene in nature occurs primarily in the trans-isomeric form. However, as a polyene, it undergoes cis-trans isomerisation induced by light, thermal energy, and chemical reactions. Lycopene obtained from plants tends to exist in an all-trans configuration, the most thermodynamically stable form.

The richest source of lycopene is tomato (*Lycopersicon esculentum*). Lycopene is also found in other sources such as watermelon, papayas, carrots grapefruit, and guava (Table 1.1). Lycopene is a carotenoid consumed in greatest amounts in western diets. More than 85% of the lycopene in North American diets comes from tomato products.

More than 20 carotenoid pigments such as α, β, γ-, and ε-carotenes, phytoene, phytofluene, neurosporene, and lutein have been identified and quantified in tomatoes. The lycopene concentration in fresh tomatoes ranges from 20 to 200 ppm with an average of 50 ppm on a fresh weight basis.

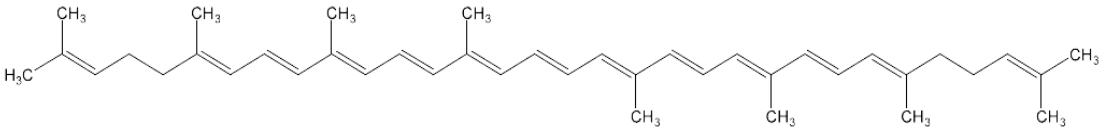

Fig. 1.5: Structure of Lycopene

Table 1.1: Lycopene content of various foods

Source	**Lycopene content (mg/100gm wet basis)**
Tomato fresh	0.72-20
Tomato juice	5.00-11.6
Tomato sauce	6.2
Tomato paste	5.2-150
Tomato soup condensed	7.99
Ketchup	9.9-13.44
Pizza sauce canned	12.76
Spaghetti sauce	9.3-18.2
Barbecue sauce	7.6
Watermelon	2.3-7.2
Pink guava	5.23-5.5
Pink grapefruit	0.35-3.36
Papaya	0.11-5.3
Carrot	0.65-0.78
Pumpkin	0.38-0.46
Sweet potato	0.02-0.11
Apricot	0.01-0.05

Lycopene is synthesized by plants and microorganisms but not by animals. Humans cannot produce lycopene and must ingest fruits, absorb the lycopene, and process it for use in the body. In human plasma, lycopene is present as an isomeric mixture, with 50% as cis isomers (Rao and Agarwal, 1999).

1.2.1 Uses of Lycopene:

a. Lycopene is an important and most powerful biological antioxidant that has been shown to neutralize free radicals; especially those derived from oxygen. Free radicals can cause damage to both the structure and function of cell membranes, DNA, and proteins. This damage has been linked to the onset of many degenerative diseases such as cancer, atherosclerosis, cataracts, associated coronary artery disease and age related macular degeneration, as well as to premature aging. Preliminary research also suggests that lycopene may reduce the risk of macular degenerative disease, serum lipid oxidation, and cancers of the lung, bladder, cervix, and skin. The free radical quenching constant of lycopene was found to be more than twice that of β-carotene (Rao and Agarwal, 1999).
b. Lycopene reduces LDL (low-density lipoprotein) oxidation and helps reduce cholesterol levels in the blood, thereby helping to prevent vascular damage (Omoni and Aluko, 2005).
c. Lycopene in the blood has been shown to be inversely proportional to the incidence of prostate tumours (Giovannucci, 2002).
d. Research also shows that carotenoids may provide protection against damage from ultraviolet radiation from sunlight, the major cause of sunburn, photo damage, and non melanoma skin cancer (Omoni and Aluko, 2005).

1.2.2 Methods for manufacturing/extraction of lycopene

From the extensive literature survey, it has been observed that there are two main approaches for manufacture of lycopene.

i. Manufacture of Lycopene by chemical synthesis method.
ii. Extraction of lycopene from its natural sources.

Chemical synthesis of lycopene

Lycopene has been reported to be synthesized by double Wittig olefination of the corresponding C15-phosfonium salts with the C10-dialdehyde (Fig 1.6) (Ernst, 2002).

The starting material used for industrial lycopene syntheses was (E/Z)-pseudoionone having 13 carbon atoms. Pseudoionone also acted as the precursor in industrial vitamin A processes. From the mixture of (E/Z)-isomers, pure (E)-pseudoionone was isolated by fractional distillation.

The C-15 building block (E)-vinyl Pseudoionol could be obtained by two methods.

i. Ethynylation of compound 11 to compound 13 and its subsequent partial hydrogenation.
ii. Compound E-12 was obtained in one step by the reaction of pseudoionone with a vinyl Grignard reagent (vinyl-MgCl).

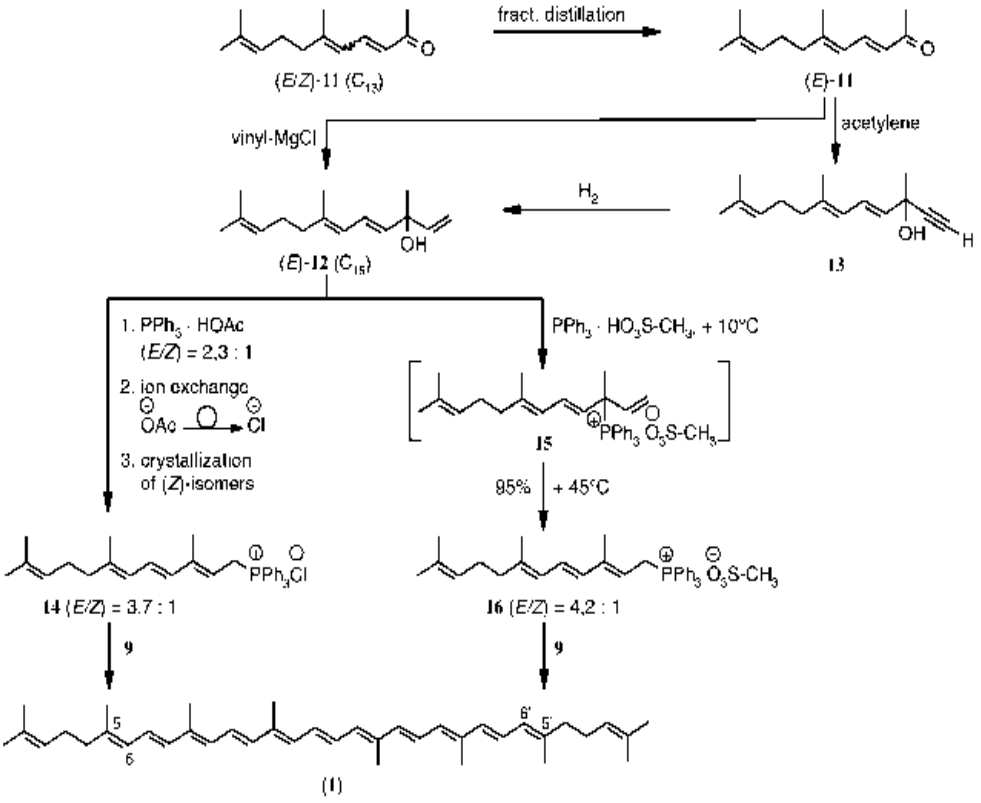

Fig. 1.6: Industrial method for synthesis lycopene (Ernst, 2002)

The most crucial step in the synthesis of lycopene was the rearrangement of (E)-vinyl pseudoionol to form lycopene C15-phosphonium salt. At the same time, as far as possible the tendency of (Z)-isomers to form at the trisubstituted C (5)/C (6) and C(5′)/C(6′) double bonds in the polyene chain must be suppressed. This was achieved by three methods:

i. Triphenyl phosphane and acetic acid- Compound 12 was treated with a mixture of triphenylphosphane and acetic acid (PPh_3.HOAc) to produce the C15-phosphonium acetate in high yield but with an (E/Z)-selectivity of only 2.3:1.
ii. Ion exchange- Enrichment of the (all-E)-isomer by anion exchange of acetate for chloride 14 (ΘOAc $\longrightarrow$ O + ΘCl) was preferred.
iii. Crystallization of (Z) - (Z)-isomers of compound 14 was depleted by crystallization.

If triphenylphosphonium sulfonates (e.g., methanesulfonate) were used the stereoselectivity can be raised to a value of 3.7:1. So, the (E/Z)-isomer ratio in the mother liquor could be raised to a value of 3.7:1 as shown in the figure. NMR analysis indicated that E-(12) passed through an intermediate tertiary phosphonium salt 15. In this second step, compound 15 rearranged at elevated temperature into the desired primary phosphonium salt 16. In this step, isomerisation of the critical double bond could largely be suppressed. By careful controlling of temperature, the (E/Z)-selectivity of the process could be raised to the ratio of 4.2:1. The yield of compound 16 was reported to be 95 %. Compound 16 without further enrichment could react in solution under classic Wittig conditions with C10-dialdehyde to form lycopene.

Drawbacks- The mentioned process involved the utilization of highly toxic chemical solvents, thus being in contrast with the increasing interest in the use of environmental friendly processes for industrial production.

Supercritical Extraction of lycopene

There are reports to show that supercritical CO_2 has been used for the extraction of lycopene from ripe tomatoes (Cadoni *et al*., 2000) and tomato processing waste (Baysal *et al*., 2000; Ollanketo *et al*,., 2001; Rozzi *et al*,., 2002; Sabio Rey, 2009; Topal *et al*., 2006; Vasapollo *et al*., 2004) in recent years. These studies principally focus on optimizing supercritical CO_2 extraction conditions to obtain higher yields of lycopene by altering the temperature, pressure, flow rate, or by adding a modifier or a co solvent.

Baysal *et al* (2000) evaluated the effects of temperature (35, 45, 55, and 65°C), pressure (20, 25, and 30 MPa), addition of ethanol as co solvent (5, 10, and 15%), extraction time (1–3 h), and CO_2 flow rate (2, 4, and 8 kg/h) on the recovery of lycopene by SC-CO_2 fluid extraction. The results showed that the best conditions to obtain a maximum of 54% of lycopene was extraction for 2 h (flow rate of 4 kg/h) at 55°C, and 30 MPa, with the addition of 5% ethanol as co solvent. Ollanketo *et al* (2001) applied various modifiers and different temperatures in SC-CO_2 fluid extraction and achieved 94%

recovery of total lycopene at 110°C and 40 MPa in 15 min. Rozzi *et al* (2002) found that a temperature of 85°C and a pressure of 34.47 MPa at a flow rate of 2.5 mL/min could result in extraction of 61% of the lycopene. Sabio *et al* (2003) studied SC-CO_2 extraction of lycopene and β-carotene from tomato skins and seeds at pressures of 25 and 30 MPa, temperatures of 60 and 80°C, and flow rates of 0.792 and 1.35 kg/h. It was found that the yield of lycopene obtained by supercritical CO_2 extraction at 276 bars increased with temperature in the range of 60-80°C. The phase behaviour has been shown in Fig 1.8. At the lower flow rate studied and extraction conditions of up to 300 bars and 80°C, it was possible to extract 80% of the lycopene and 88% of the β-carotene from the raw tomato waste material (only skins). Vasapollo *et al* (2004) described that the presence of vegetable oil as co solvents improved the yields and contributed to the stability of lycopene. Gómez-Prieto *et al* (2003) studied the optimum conditions for obtaining the most stable isomer (all-trans form) of lycopene. The result showed that most stable isomer exists at 40°C. However, these studies estimated only one factor at a time, and thus, the interactions of other factors were ignored. Fig. 1.7 has illustrated supercritical fluid exraction of lycopene.

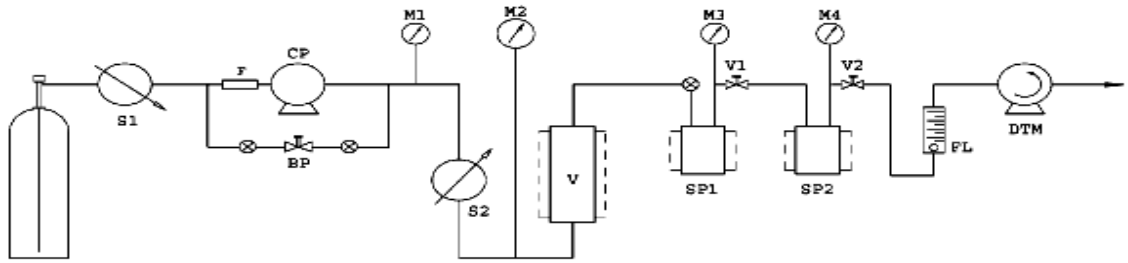

Fig. 1.7: Supercritical fluid extraction apparatus for lycopene (Source: Rozzi *et al*,., 2002) S1, ice cooler; F, filter; CP, circulating pump; BP, back-pressure regulator; M1-M4, manometers; S2, heat exchanger; V, extraction vessel; SP1 and SP2, separators; V1 and V2, valves; FL, rotameter; DTM, dry test meter.

Disadvantages: For financial benefits and to achieve higher yields, industries tried extraction using supercritical CO_2 at higher temperature but due to instability of lycopene at high temperature conditions this could lead to degradation or isomerisation of the lycopene-rich extracts or compounds, and it potentially affects the antioxidant activity of the extract also (Shi and Le Maguer, 2000). Cadoni *et al.* (2000) reported

that data obtained from SC-CO_2 fluid extraction of lycopene from tomatoes were poorly reproducible because of the decomposition and isomerisation of lycopene.

However, so far, few studies have reported on the effects of SC-CO_2 the fluid extraction process on the stability and bioactivity of lycopene-rich extracts. The use of supercritical fluids is quite expensive, making the entire procedure economically unsuitable for a large-scale process.

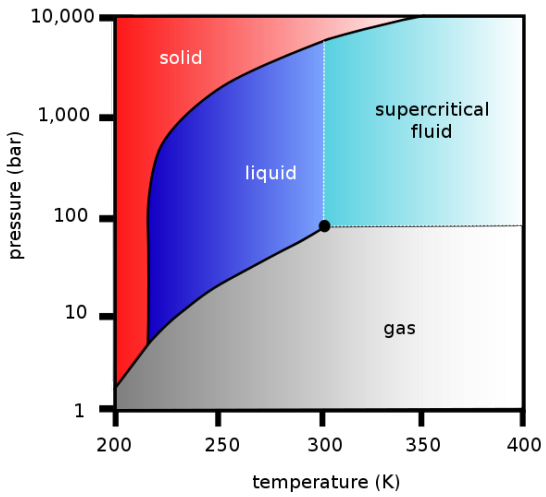

Fig. 1.8 Phase diagram for carbon dioxide (Source: Shi *et al.*, 2009)

Phospholipid aided solvent extraction of lycopene

This particular invention relates with the extraction of desired biomolecule with the help of solvents, such as aliphatic, aromatic and halo-hydrocarbons, with help of co-solvents such as phospholipids.

The tomato fruits were homogenized with a knife mill and resulting slurry was heated to 70-100°C for 15-60 minutes and then cooled to temperature between 50-70°C. These steps were performed under sterile condition. In one representation of this invention, the starting plant material was completely dried and to this dried part another half part by weight of water was added. The added water helped to lyse the cells of tomato, thus facilitating the release of lycopene. The suspension was then centrifuged or filtered to obtain residue containing about 20% of water. The residue was then suspended in sufficient amount of selected solvents, such as n-hexane containing phospholipids (soy lecithin) at varying concentrations (0.01 to 0.100 w/v). In such conditions, solvents extract lycopene in highly selective way, leaving others carotenoids like β-carotenoids

in the biomass. Phospholipids acted as surfactant and stabilizing agent so that the extracts were fractionated to the desired lycopene concentration. The extract was concentrated such that the lycopene content is about 10% by weight in the final formulation.The process was repeated several times to maximize the extraction of lycopene. The extract(s) were concentrated by removing portions of the extraction medium (Bomberdelli and Morazzoni, 1999).

The next step was the purification of the obtained extract, either by chromatographic separations or super critical fluid extraction. Chromatographic separation was performed using acidic alumina column wherein lycopene is eluted with a mixture of solvents such as n-hexane and methylene chloride. In case of supercritical extraction, the purification was carried out using supercritical CO_2 at temperature between 35-60°C and at a pressure between 150-200 bars.

Extraction of lycopene using Naviglio extractor

This was a novel method of lycopene extraction in which the tomato products were subjected to a high pressure process in a Naviglio Extractor (Fig. 1.9).

Fig. 1.9: Naviglio Extractor ® laboratory model (Source: Naviglio, 2003)

The device was a rapid dynamic solid–liquid extractor that worked on the following principle: in a suitable liquid, generating a negative pressure gradient and letting it to go to equilibrium between outside and inside of a solid matrix containing compounds that could be extracted in the solvent, followed by rapid restoration of equilibrium conditions, a forced extraction of compounds not chemically bound contained in the solid matrix was produced. The Naviglio extractor recovered a solid lycopene fraction in a paracrystalline form from tomato waste, using the pressure and depressure effect that operated in the solid–liquid extractor device (Fig. 1.10). The extractor consisted of an extracting chamber equipped with a cylinder and a piston. A porous set at the bottom allowed liquid-phase to pass through and blocked the solid particles.

The solid raw material was placed in the chamber, which was filled with the organic, inorganic or mixture of solvents. The system reached equilibrium (static phase) at a pressure of 8 atmosphere. In dynamic phase the piston moved from its equilibrium position and this step was completed when the piston reaches the lower point of the chamber. The static and dynamic step alternated until the final pressure value was reached.

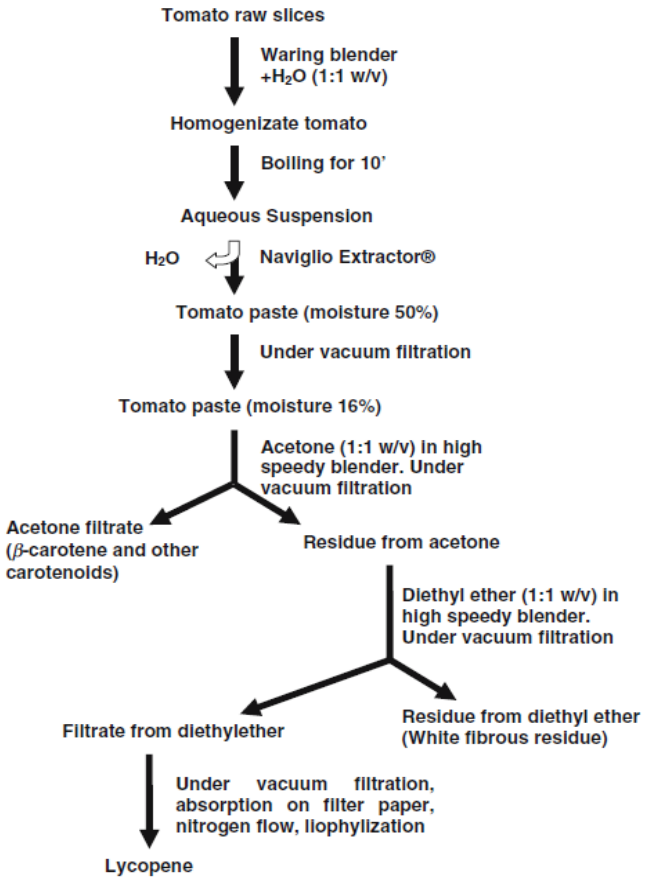

Fig. 1.10: Procedure for extraction of lycopene from raw tomatoes (Source: Naviglio, *et al.*, 2008)

One extraction cycle was formed by one static and one dynamic step. Complete extraction of the solid matrix can be obtained by repeating these operations. When the maximum value set for the pressure was reached, the device stops for few min, to allow the outside and inside part of the solid matrix to equilibrate (static phase) with the solvent pressure. After equilibration, the piston moved and air rapidly left the system, causing lowering of the pressure inside the extraction chamber. This induced the start of the dynamic phase. At the beginning of this step the substances that were soluble in the

solvent and substances not chemically bonded to the matrix were extracted from the solid matrix and transferred to the solvent.

Further purification was performed on column chromatography using SPE-C18 column, where trans-lycopene was separated from other pigments. Lycopene was obtained in the all-trans form with very high purity (approximately 98% (w/w), but average recovery of 14% (w/w) (Naviglio 2003; Naviglio *et al.*, 2008)

Drawbacks- The efficiency of this procedure was lower as compared with other procedures proposed in the literature, because very less quantity of lycopene was present in paracrystalline form in tomato waste. Though the purity obtained was high (>98%) but the overall efficiency was low.

Enzyme aided lycopene extraction

Enzymatic cell wall lysis employing hydrolytic enzymes has been a widely reported method for the extraction of various kinds of biomolecules. For example, enzymes were employed for the extraction of capsaicinoids and carotenoids from chilli (*Capsicum annuum* L.) using ethanol as solvent (Santamaria *et al.*, 2000).

Since tomatoes cell wall is made up of cellulose and pectins, enzymes cellulases and pectinases were used for this purpose. Cellulase acted on cellulose, which is present in the primary wall beneath the first layer of middle lamella of the plant cell wall. Primary wall consists of a rigid skeleton of cellulose embedded in a gel-like matrix composed of pectic compounds, hemi-cellulose and glycoprotein. The Cellulase enzyme catalyzes the breakdown of cellulose into glucose, cellobiose and higher glucose polymers.

Pectinase being pectolytic and hemicellulolytic has the ability to disintegrate pectic compounds and pectin, the latter a polymer of 100-200-galacturonic acids, found in the middle lamella and primary walls.

The method described by Choudhari and Ananthanarayan (2007) used whole tomatoes, tomato peel, laboratory pulper waste and tomato industrial waste as the raw materials. Cellulase at 6% w/w and pectinase at 0.5% w/w proved to be very effective in extracting a high amount of lycopene. Pectinase was shown to be more effective for treating whole tomatoes (224% increase in yield), tomato peel (206% increase in yield). Cellulase was more effective in treating fruit pulper wastes (yield increase of 23%) and treating samples of industrial wastes (yield increase of 61%). Of all the tomato fractions and wastes studied as a substrate of lycopene, tomato peel was found to show the

highest increase in lycopene yield using pectinase enzyme. However this method has not been used on a commercial scale.

Solvent extraction of lycopene

Several methods of extraction of lycopene have been reported in the literature. Zelkha and Mordechai (1997) reported a lycopene extraction process consisting of crushing of tomatoes, heating the crushed tomatoes, separation of the crushed tomatoes into serum and pulp and finally subjecting the pulp to solvent extraction to obtain oleoresin enriched with lycopene.

The commonly used solvent for lycopene extraction is ethyl acetate. This solvent extracts β-carotene and lutein more effectively, when compared to the all-trans isomer of lycopene. However, ethyl acetate is highly inflammable and also considered non-ecofriendly.

Several studies conducted recently have used ethyl lactate as an environmentally benign solvent that can be used for lycopene extraction. Ethyl lactate is produced from fermentation of carbohydrate feed stocks from corn and soybean industries. Also the US FDA has approved its use is food products. Ethyl lactate is completely biodegradable into CO_2 and water along with it being miscible with hydrophilic and hydrophobic compounds. The use of ethyl lactate as an eco-friendly solvent or lycopene extraction has been patent protected (Ishida and Chapman, 2009). The process had all the limitations of solvent extraction and was energy intensive.

1.3. 11-Hydroxy canrenone (11-OHC)

11α-Hydroxy Canrenone is a hydroxyl derivative of canrenone, a steroid molecule, which is the precursor manufacture of Eplerenone (Fig 1.11). Eplerenone is an Active Pharma Ingredient (API) used in formulations for treatment of cardiac related disorders. In many countries, Eplerenone is available under the name “Inspra”.

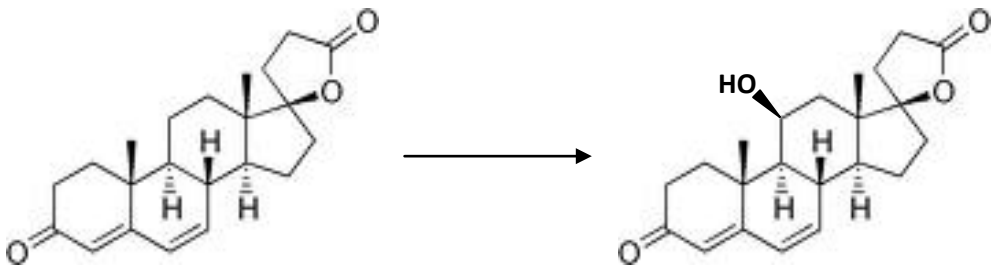

Fig. 1.11: Conversion of canrenone to 11 OHC

To introduce epoxy functional group in any steroid, induction of hydroxyl group is mandatory. This type of transformation is practically impossible by conventional synthetic chemistry. Fungal biotransformations were found to be useful in this conversion and since 1960's steroidal bioconversions have been used for site specific introduction of hydroxyl groups. In the synthesis of Eplerenone, hydroxylation at 11-α position is required because of double bond introduction between Carbons 9 and 11. This is done by making an epoxide between Carbon 9 and 11 from 11-hydroxy derivative of Canrenone.

1.3.1 Characteristics of 11 OHC

Some of the characteristics and / or properties of 11 OHC are as follows:

Chemical name	(11a, 17a) – 11, 17 dihydroxy – 3 – oxo – pregna – 4, 6 – diene – 21 carboxylic acid
Molecular formula	$C_{22}H_{128}O_4$
Molecular mass	356.46 g/mole
Melting point	221 – 225°C
Solubility	Acetone, ahloroform, methanol

1.3.2 Biotransformation

Biotransformation is a process in which a substrate is converted to product by using whole cell mass or enzyme preparation. All these transformations can be carried out by cells at different stages of growth. When the substrate is converted using whole cell mass, it is either added to the growing cell mass or the cell mass is grown along with the substrate. The steroid can be added to spore suspension also (Sebek and Perlman, 2006). The industrially important steroidal bioconversion processes and products have been summarized in Table 1.2.

Steroid biotransformation

Naturally occurring steroids possess remarkable hormonal properties which are of critical importance to human well being. They are all derived from tetracyclic hydrocarbon and differ primarily in a number, type and location of the substituent and in the number and position of the double bonds. Thousands of derivatives of these compounds have been synthesized and tested for their endocrine and other activities (Sebek and Perlman, 2006). Steroidal bioconversion is very important industrial

bioprocess because it is very difficult to convert the steroid chemically. The complex structure of steroid molecule requires complicated multistep schemes for chemical synthesis of steroid compounds. It often involves the preparation of intermediate derivatives with protected groups and their subsequent regeneration, once the intended reaction has occurred, limiting the overall process yield and making it expensive and time consuming.

Table: 1.2 Typical microbial transformations of steroids (Source: Sebek and Perlman, 2006)

Type of reaction	Example	Microorganism involved
Conversion of secondary alcohol to ketone	Cortisol to prednisolone	*Arthrobacter simplex*
Introduction of primary hydroxyl on steroid side chain	Progesterone to 11 α hydroxy progesterone	*Rhizopus nigricans*
Introduction of secondary hydroxyl on steroid nucleus	Progesterone to prednisolone	*Aspergillus sps.*
Introduction of tertiary hydroxyl on steroid nucleus	Stigma sterol to cortisol	*Rhizopus Sps*
Dehydrogenations of ring A of steroid nucleus in positions 1(2) and 4(5)	Cholesterol to Androstedienedione	*Morexella* sp.
Aromatization of ring A of the steroid nucleus	Compesterol to Androstedienedione	*Rhodococcus* sp.
Cleavage of side chain of pregnane at C17 to form ketone	Stigma sterol to progesterone	*Myxobacterium* sp.
Cleavage of side chain of pregnane at C17 and opening of D ring to form testololactone	Cholesterol to testosterone	*Lactobacillus bulgaricus*
Cleavage of side chain of steroid to form carboxyl group	21-Acetoxy-3β-hydroxy-5-pregene-20-one to 21-Hydroxy-7pregene-3,20- dione	*Corynaebacterium mediolanum*
Cleavage of side chain of pregnane steroids at C17 to form secondary alcohol	β-Sitosterol to Androstedienedione	*Mycobacterium* sps

Downstream processing of fermented broth after steroid biotransformation

The addition of steroid for bioconversion is usually at low concentration range of 10-20 g/L. The steroids have very low water solubility and many times water immiscible organic phase is added into the fermentation medium as substrate carrier and also for in

situ product recovery. Downstream processing of the fermentation broth involves number of unit operations. Substrate added into the fermentation medium is suspended in the fermented broth as well as major part is deposited on the cell mass especially in case biotransformation mediated by fungal strains.

The first step in the operation is biomass separation from the fermentation broth. Usually cell free broth as well as separated biomass is extracted with the same solvent separately. The extraction of solid mass with the solvent is a simple solid liquid extraction and the liquid phase after extraction separated either by centrifugation or filtration. Liquid liquid extraction of cell free broth with the organic solvent creates some problem because of natural surfactants present in the fermentation broth. This causes emulsion formation and improper separation of the phases. To overcome this problem liquid liquid extraction is followed by centrifugation in liquid liquid separator at a high 'g' value. Both the organic phases are then mixed and concentrated. If the solubilization of the substrate in the solvent is temperature dependant then concentration, followed by cooling, causes precipitation of substrate of interest. If the substrate is highly soluble in the organic phase then evaporation to dryness and selective leaching out of substrate of interest is the only alternative to purify biotransformed substrate.

In two phase immiscible liquid system, the product will be distributed in a definite ratio between these two phases. The distribution coefficient K is defined as a ratio of concentration in lipophilic phase and concentration in hydrophilic phase (Belter, 2006).

When the product formed is structurally very different from the substrate used, the selectivity of the solvent can be manipulated. But when substrate and product are structurally very similar, with slight difference in functional group, then the organic phase extracts both the substrate as well as the product. Purifying the product from such extracts requires further purification steps such as column purification, adsorption chromatography, preparative HPLC etc.

Structurally close substances with minor difference can be selectively extracted /picked up if surfactants are used in the extracting medium. An exhaustive review of the literature has failed to highlight any reference to the use of surfactant based systems for extraction of steroid biotransformation products.

Biotransformation of canrenone

The route used for production of 11 hydroxy canrenone is bioconversion (fermentation) process using canrenone as starting material. The 11 OHC formed was present in the

biomass. The conventional process for recovery of 11 OHC was extraction of 11 OHC from the biomass using a suitable solvent. 11OHC in the extract was further recovered and purified through solvent recovery followed by crystallization. Given the advantages of the surfactant based system, and the inherent disadvantages associated with conventional solvent extraction, it was decided to investigate the use of surfactant based systems for extraction and purification of a model biotransformation based product from the fermentation broth. The results of these studies are given in the next chapters.

1.4 Lipase

Lipases are reported to be monomeric proteins having molecular weights in the range of 16,000 to 670, 000 Daltons. They exhibit a tendency to form high molecular weight aggregates. Lipases have found applications in many diverse fields. Lipase used in the present investigation is alkaline lipase having molecular weight of 18 kD.

1.4.1 Applications of lipases

Lipolytic enzymes have attracted enormous attention because of their biotechnological potential and constitute the most important group of biocatalysts (Hasan *et al.,* 2006). Lipases (triacylglycerol acylhydrolase, EC 3.1.1.3) act on carboxylic ester bonds and hydrolyze triglycerides into diglycerides, monoglycerides, fatty acids and glycerol. Lipases also can catalyze esterification, interesterification and transesterification reactions in nonaqueous media. These properties make lipases enzyme of choice for potential applications in the food, detergent, pharmaceutical, cosmetic, textile, paper and leather industries (Houde *et al.*, 2004).

In pharmaceuticals

Chiral intermediates and fine chemicals are in high demand for the preparation of bulk drug substances and agricultural products. Lipases are used to resolve the racemic mixtures and to synthesize the chiral building blocks for pharmaceuticals, agrochemicals and pesticides (Hasan *et al.,* 2006). A lipase from porcine pancreas was reported to resolve (R,S) glycidyl hydrate to glycidol which has been used in the synthesis of drug propanolol (Wu *et al.*, 2003). Patel (2000) reported stereoselective acetylation of racemic 7-[N, N'-bis (benzyloxy-carbonyl)N-Guadinoheptanoly]-ahydroxy glycine to corresponding S-(-) acetate a key intermediate for total chemical synthesis of (-)-15-Deoxyspergualine, an immunosuppressive agent and anti-tumour

antibiotic. *C. antarctica* lipase was documented for resolution of 1-(2- thienyl) alkanols to prepare stereospecific derivatives (Sundby *et al.*, 2001).

In Biodiesel production

The trans-esterification activity of the lipase from P. aeruginosa LX1 indicated its potential biocatalytical application for biodiesel production (Ji *et al.*, 2010). Many types of lipases have been reported for biodiesel production from various sources such as from waste cooking oil (Saifuddin *et al.*, 2009), from side stream refining products (soap stocks, acid oil and deodorized distillates) (Echim *et al.*, 2009). Booth *et al.* (2006) reported triglycerides treatment with lipases to produce biodiesel from Tallow which was termed as Tallodiesel.

In food industry

Most of the commercial lipases produced are utilized for flavour development in dairy products and processing of other foods, such as meat, vegetables, fruit, baked foods, milk product and beer e.g. application of phospholipases in egg yolk treatment for the production of mayonnaise and other emulsifiers, in lecithin modification, and for the oil-degumming step in the refining of vegetable oils. Lipase has also been documented for its application in fermented foods such as Koji and Tempeh where oil from soybean is hydrolyzed by lipase (Aravindam *et al.*, 2007). Lipases have been extensively used in the dairy industry for the hydrolysis of milk fat and to enhance the flavors of various cheeses, acceleration of cheese ripening and the lipolysis of butter, fat and cream (Sharma and Chisti, 2001). A natural antioxidant, oleate of cinnamic acid was reported to be synthesized using Novozyme 435 lipase (Lue *et al.*, 2005). Similarly synthesis of mono and diacylglycerol (MAG, DAG), valuable biosurfactants used in food and pharmaceuticals using *Candida antarctica* lipase was reported by (Adamczak, 2003).

In cosmetic industry

Lipases have been used in cosmetic industry for high quality products e.g. manufacturing of wax esters, preparation of water soluble retinol derivatives by immobilized lipase, also it has been used in hair waving preparation and as a component of topical anti-obesity cream (Hasan *et al.*, 2006; Maugrad *et al.*, 2002). Most of the perfume esters with high purity has been synthesized by using lipases e.g. geranyl butyrate by using *Candida rugosa* lipase (Zorica *et al.*, 2008).

Other applications

C. rugosa lipases have been documented for their applications in the food and flavor industry and production of single cell protein (Benjamin and Pandey, 1998). Application of lipase as lipid biosensor has been reported by where lipase was immobilized onto pH/oxygen electrodes in combination with glucose oxidase to be used in triglycerides and bold cholesterol determinations. Conventionally, in leather industry degreasing of skins and hides involved organic solvents and surfactants while alkaline and acid lipases represent eco-friendly and efficient method to remove fat and grease from skins and hides (Hasan *et al.*, 2006).

1.4.2 Purification of lipases

Purification methods used for most of the enzymes depended on non specific techniques such as precipitation (using salts or water soluble organic solvents), dialysis, and concentration using ultrafiltration. For further purification certain chromatographic techniques are used such as hydrophobic interaction chromatography, gel filtration and ion exchange chromatography (Sharma and Chisti, 2001). *P. aeruginosa* extracellular lipase was purified using ammonium sulphate precipitation and successive chromatographic separations on hydroxyl appetite to achieve 518 fold purification (Sharon *et al.*, 1998). A four step purification of *Bacillus* lipase was reported by Sharma *et al.* (2002) where ultrafiltration was followed by ammonium sulphate precipitation, ion exchange chromatography and gel filtration, where 201.15 fold purification was achieved. An extracellular lipase from *Acetobacter calcoceticus* was purified to homogeneity using hydrophobic interaction fast performance liquid chromatography (FPLC) (Kok *et al.*, 1995). Dandavate *et al* (2009) reported application of gel permeation chromatography to achieve 43 fold purification and acetone precipitated Burkholdia multivorans lipase. Similarly R. oryzae lipase was purified to 1200 fold by using ammonium sulphate precipitation and series of chromatography steps (Hoil *et al.*, 2000).

1.5 Tannase

Tannase or Tannin acyl hydrolase (EC 3.1.1.20), an induced enzyme, catalyses the hydrolysis of the ester bonds present in hydrolysable tannins and gallic acid esters. It also acts on depside linkages (acyl bonds i.e C-O) in gallotannins. Though tannases are reported to be present in plants and animals, they are mainly produced by

microorganisms. Number of bacteria (*Bacillus, Klebsiella, Pseudomonas and Lactobacillus*) and fungi (*Aspergillus, Penicillium* and *Fusarium*) are reported ot produce intra as well as extracellular tannases. Tannases are high molecular weight glycoproteins (59-320 kD) having pI between 3.5 to 8.0 (Belmares *et al.,* 2004; Lekha and Lonsane, 1997; Van de Lagemaat and Pyle, 2005).

Tannase finds its major application in manufacture of instant tea. Use of tannase is found to be cost effective in treatment waste water streams from tanneries. Tannase also finds application in treatment of fruit juices, wine and beer.

1.5.1 Applications of tannase

Tannase plays an important role as biocatalyst in the bioconversion of hydrolysable tannins to simple phenolic acids such as gallic acid, ellagic acid. Tannase has always been an enzyme of industrial importance because of its wide applications in tannery, pharmaecuticals, alcohol and beverage industries (Purohit *et al.,* 2006). Most of the commercial applications of tannase have been in the manufacture of instant tea, acorn liqour and gallic acid production.

Tannase has been widely employed to remove water insoluble precipitates called 'tea cream' during the instant tea manufacturing. Tannase deesterifies tea polyphenols in non converted green tea leaves which enhances the natural levels of gallic acid and epigallochatechin and favors the formation of epitheaflavic acids which are responsible for bright reddish color and very good cold water solubility (Aguilar and Giterrez-Sanchez, 2001; Lekha and Lonsane, 1997). Gallotannins such as Caesalpinia spinosa, Quercus infectoria yield gallic acid on hydrolysis by tannase. The major application of gallic acid has been a precursor for synthesis of broad spectrum antibacterial agent, trimethoprim. It also has been used in preparation of high grade leather tannins, in the manufacture of inks, paints and colour developers and as a photosensitive resin in the semiconductor production (Anderson *et al.,* 1980; Misro *et al.,* 1997; Mukaherjee and Banerjee, 2003; Ow, 2003).

Tannase is used to make acorn wine from Korean acorns (Quercus spp.). It has been used to prevent discoloration and haze development during beer storage and also to treat grape must and juice along with lactase to remove phenolic substances for stabilizing and increasing the quality of wine (Lekha and Lonsane, 1997). Tannase has been reported for its use in enymatic debittering of fruit juices. It reduces the tannin contents responsible for haze, sediment, color, bitterness and astringency of the juice upon

storage. Tannase has been employed as calrifying agent in the preparation of coffee flavoured soft drinks and in the stabilization of malt polyphenols (Aguilar *et al.*, 2007; Das Mohapatra *et al.*, 2006; Rout and Banerjee, 2006; Sabu *et al.*, 2005).

The antinutritional effects of tannin containing food and feed were overcome with pretreatment with tannase thus by improving digestibility and nutrient availability of food and feed (Lekha and Lonsane, 1997). Application of tannase to lentil flours has also been documented for production of bioactive polyphenolic compounds (Duenas *et al.*, 2009). The use of tannase has been an effective and cheap treatment for the removal of high quantities of tannins in tannery effluent which pose serious pollution problems (Aguilar and Guieterrez-Sanchez, 2001; Belmares *et al.*, 2004). Tannases are also applied in preparation of high grade leather tannin (Das Mohapatra *et al.*, 2006).

Tannase used in the present investigation has molecular weight of 96 KDa, monomeric protein having isoelectric pH of 4.5.

1.5.2 Purification of tannase

Cell free broth has been directly used as a source of extracellular tannase. While intracellular enzyme has been prepared by lysis of cells by grinding in sand or crushing of frozen biomass in mortar or homogenizer or congelation – decongelation and extraction of lysate in appropriate buffer (Aguilar *et al.*, 2007; Barthomeuf *et al.*, 1994; Bhardwaj *et al.*, 2003; Sharma *et al.*, 2000). Barthomeuf *et al* (1994) has reported the use of chitinase for the hydrolysis of mycelia to obtain mycelia bound enzyme.

Hamdy (2008) has purified tannase from *F. subglutinans* by 65 % ammonium sulphate precipitation followed by ion exchange chromatography on DEAE cellulose and further fractionation on Sephadex G_{150}. With this method 27 fold purification of the enzyme was achieved. Similarly 19 fold purification was achieved when *Verticillium* tannase was purified by 80 % ammonium sulphate saturation and then by DEAE cellulose column chromatography (Kasieczka-Burnecka *et al.*, 2007). Partial purification of tannase from *A. foetidus* was carried out by aqueous two phase extraction using various molecular weights of PEG (polyethylene glycol) (4000 - 9000) and its concentrations (15 – 30 %). 25 % PEG 6000 was found to show yield of 82 % with 2.7 fold purification of tannase (Naidu *et al.*, 2008). The color impurities contributed by polyphenolic compounds present in *Paecilomyces variotii* tannase preparation was reported to be removed by active charcoal treatment at 4°C without loss in activity before initial purification step. The broth was then purified 30 fold by 70 % ammonium

sulphate precipitation, DEAE cellulose and Sephadex G 200 chromatography (Mahendran *et al.*, 2006).

Acetone was used to precipitate and concentrate the *A.awamori* tannase which was then purified using GF 250 column (Mahapatra *et al.*, 2005). *A.oryzae* tannase was cloned and expressed in *Pichia pastoris*. The recombinant tannase was concentrated by ultrafiltration using 30 kDa cut-off membrane and purified on DEAE sepharose fast flow column with 51 % yield (Zong *et al.*, 2004). Ramirez-Coronel *et al.* (2003) has reported a slightly different protocol for purification of *A. niger* tannase where they used preparative isoelectofocusing as initial purification step for 50 kDa ultrafiltered sample, followed by ion exchange chromatography using Mono Q column and Sephadex G-100 chromatography. The SDS PAGE of purified tannase showed two polypeptides with molecular weights 90 and 180 kDa. Two step pH differential protein precipitation using 0.1 % tannic acid and 0.1 % PEG 6000 was successfully reported for purification of *Aspergillus japonicus* tannase. The first step was at pH 3.0 where undesirable proteins precipitated and in the second step at pH 4.5 tannase precipitated with 7.82 fold purification and 50 % enzyme yield (Gupta, 1997). Barthomeuf *et al.* (1994) has reported the application high pressure size exclusion chromatography followed by ultrafiltration to purify *A. niger* tannase. Application of affinity chromatography on ConA Ultrogel combined with elution with methyl D glucose has been reported for separation of esterase and depsidase activities of tannase by Van De Lagemaat and Pyle (2005). Whereas one step purification of recombinant Lactobacillus plantarum overexpressed in *E. coli* has been documented by using metal chelate affinity chromatography on poorly activated Ni-IDA 6 % agarose gels by Curiel *et al.* (2010). The purification was 15 fold with 95 % yield.

PART B: Microemulsions - structure, transport phenomenon and applications

1.6 Microemulsion – general introduction

This section presents brief overview of the self-organized surfactant systems and discusses the various investigations carried out under micellar / microemulsion conditions. Super molecular interactions in solutions were first introduced by Emil Fisher in 1894. Since then, there has been a growing interest in the research on organized surfactant assemblies. Micelles and microemulsions, vesicles, self-assembled

monolayers, bilayers and supramolecular hosts such as zeolites are the self organized structures observed due to surface active agents in the monophasic, biphasic as well as multi component systems. Among them micelles and microemulsions exhibit a wide unusual properties, such as high interfacial area, low viscosity, high solubilizing capacity etc., which can offer the high potential for numerous applications in industries dealing with catalysts (for manufacture of nano particles), pharmaceuticals (for formulations), biotechnology (protein purification), petrochemicals (for tertiary oil recovery), and many more applications.

1.6.1 Surface active agents (surfactants)

A surfactant molecule has a hydrophilic and hydrophobic region. In the structural representation the hydrophilic head group region of the molecule is generally depicted as a circle and the tail group, the hydrophobic region of the molecule, is depicted as a straight or wavy line. The tail consists of one or more hydrocarbon chains, usually with 6-22 carbon atoms, which may be linear or branched. Fig. 1.12 illustrates the schematic diagram of a typical surface active molecule.

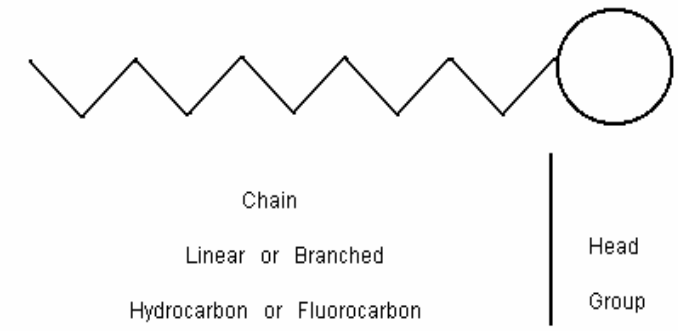

Fig. 1.12: Schematic representation of surface active molecule (Source: Ghosh, 2009)

Surfactant molecules are also called amphiphiles from the greek words "amphi" and "philo" which means both and loving respectively. This dual nature is responsible for the phenomenon of surface activity, and of micellization and solubilization (Nagraj, 1986). They can get adsorbed on to surfaces and interfaces of the dispersed and continuous phase and bring about a change in the interfacial energy (Damodaran, 1990; Ghosh, 2009; McBain and Hutchinson, 1955; Nagrajan and Ruckenstein, 1991). Based on the head group-charge, surfactants are divided into four categories i) cationic, ii) zwitterionic, iii) non-ionic and, iv) anionic (Cosgrove, 2010).

In aqueous solution, the anionic surfactant dissociates giving an anion carrying the amphiphilic properties and an inactive cation (e.g. Na+ or K+). In aqueous solutions, cationic surfactants are ionized in a cation carrying the amphiphic properties, and an inactive anion, such as Cl- or Br-. The cationic group is most often a quarternary ammonium group. Non-ionic surfactants as the name implies do not give ions in solution. The hydrophilic part of their molecule contains polar groups such as ether, alcohol, carbonyl or amino groups. Amphoteric surfactants are ionic surfactants containing positive and negative charges on the same molecule. Table 1.3 lists the representative class of surfactants.

In several important applications, ionic surfactants are used in conjunction with a co surfactant such as a medium chain-length alcohol. The co-surfactant is an uncharged entity and its adsorption is not impeded by the electric field. It therefore provides the additional lowering of interfacial tension necessary for microemulsion formation. Co-surfactants are usually alcohols or amines ranging from C-4 to C-10 and helps in the formation and stabilization of micelles/microemulsions. The lower molecular weight alcohols and amines make the microemulsion flexible and rigidity of the micelle increases with increasing molecular weight of the surfactant (Mathew and Juang, 2007) The co-surfactant provides a "dilution effect" in addition to that of the surfactant and causes a further decrease of the interfacial tension. Salts also have an effect on microemulsions. They tend to partially neutralize the surface potential and decrease coulombic repulsion between adjacent head groups, which allows the formation of larger micelle.

1.6.2 Micellar structure and properties

The characteristic concentration of surface active agents (surfactants) in solution above which the appearance and development of micelles brings about sudden variation in the relation between the concentration and certain physico-chemical properties of the solution is (such as the surface tension) known as critical micellization concentration (CMC) (Cosgrove, 2010).

Above the CMC the concentration of singly dispersed surfactant molecules is visually constant and the surfactant is at essentially its optimum of activity for many applications. CMC values for commonly used surfactants range from about 10^{-4} - 10^{-2} M. The CMC is dependant on a large number of parameters. Usually the more surface active the amphiphilic monomer, the higher is the tendency for micellization and,

hence, the lower the CMC of the micelle produced. Addition of salt to the solution usually decreases the CMC.

Table 1.3: Types of surfactants

Type	Chemical Name	Molecular Formula	Abbreviation(s)
Anionic	Sodium dodecylsulfate	$C_{12}H_{25}OSO_3Na$	SDS
	Sodium p-dodecylbenzene sulfonate	p-$C_{12}H_{25}(C_6H_4)SO_3Na$	SDBS
	Sodium bis(2-ethylhexyl) sulfosuccinate	$C_{20}H_{37}O_7SNa$	AOT
Cationic	Cetyltrimethylammonium bromide	$C_{16}H_{33}N(CH_3)_3Br$	CTAB
	Didodecyldimethylammonium bromide	$(C_{12}H_{25})_2N(CH_3)_2Br$	DDAB
	Dodecytrimethylammonium bromide	$C_{16}H_{33}PyBr$	DTAB
	Cetylpyredenium bromide monohydrate	$C_{21}H_{38}BrN.H_2O$	HDPB
Zwitterio-nic	Tetradecyldimethylamine	$C_{14}H_{29}$-N^+ $(CH_3)_2O^-$	TDMAD
	Hexadecylsulfo betaine	$C_{16}H_{33}$-$N^+Me_2(CH_2)_3SO_3$	SB3-16
	N-Dodecyl-N,N-diemethyl glycine	$CH_3(CH_2)_{11}N^+(CH_3)_2COO^-$	--
Non-ionic	Polyoxyethylene (23)-dodecyl ether	4-(C_9H_{19})-C_6H_4O-(CH_2CH_2O)4-CH_2CH_2OH	Igepal C0-520
	Sorbitan monolaurate	C11H23CO-OCH2CHOH-(C_4H_5O)-$(OH)_2$	Span 20
	Polyoxyethylene (23)-dodecyl ether	$CH_3(CH_2)$11(OCH_2CH_2)n OH N~23	Brij-35
	1-(1,1-Dimethyl-3,3-dimethyl butane) 4-polyoxyethylene (9.5)	$(CH_3)_3CCH_2C(CH_3)_2$ -(C_6H_4)-(C_2H_4O)9.5OH	TritonX-100

A schematic two-dimensional representation with different regions of micelle/ micro-droplet is shown in Fig. 1.13. Electrical charge on the micelle is neutralized by counter-ions in the electrical double layer around it. The first layer immediately adjacent to its surface is called the Stern Layer (Cosgrove, 2010).

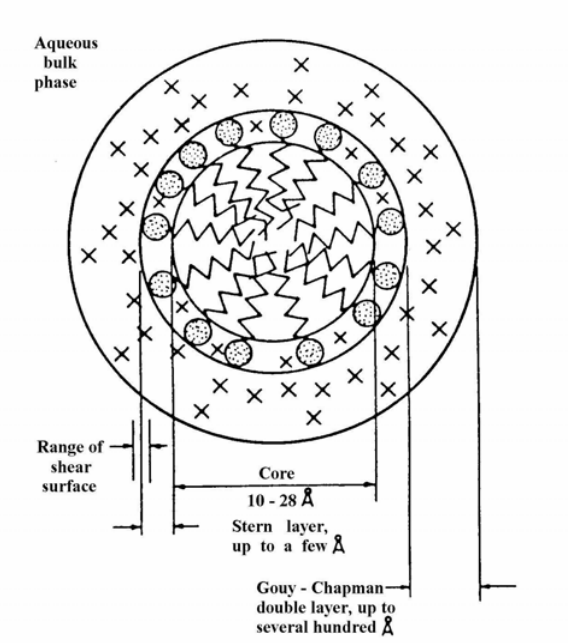

Fig. 1.13: A two dimensional schematic representation of the regions of a spherical micelle. (The head group (O), the hydrocarbon chain (/\/\) and counterions (X) are schematically indicated to denote their relative locations) (Source: Khomane, 2002)

In this layer the counter-ions are adsorbed so strongly that there is no thermal agitation and they migrate together with the colloidal micelle in an electrical field. According to the most widely accepted model, head groups of surfactant molecules also situate in this layer. The remainder of the double layer is called the diffuse (Gouy-Chapman) layer since the ions are diffused into the bulk solution as a consequence of the thermal motion.

The decrease in counter-ion concentration with the distance from the micellar surface has an exponential form (Shinoda and Lindman, 1987). The core radius is about the length of the fully extended alkyl chain of the amphiphile. The core is believed to consist of two regions, namely the inner and outer core.

The outer core contains approximately the first four methylene groups. There is also another defined region within micelles called palisade layer (mantle) which includes the head groups and the first few methylene groups. On the basis of Hartley model, the overall volume of a micelle is approximately twice that of Stern Layer (Bunton *et al.*, 1978; Mahieu *et al.*, 1993; Shelly 1997). Hydrophobic core of the micelles have diameters of about 10-30 A. The charged coat of ionic micelles, called the stern layer is usually 60-90% neutralized by counter-ions in aqueous surfactant solutions without added salt.

1.6.3 Micelles and reverse micelles

It is well known that water and oil do not mix because of high surface tension. However, two immiscible liquids one polar such as water, glycols or diethyl formamide and non-polar hydrocarbon can be converted into an optically transparent emulsion by adding a surfactant. J. H. Schulman coined a term microemulsion for this thermodynamically stable emulsion (Ghosh, 2009; Gradzeilski, 2008; Hoar and Schulman 1943; Schulman *et al.*, 1956). The main difference between micellar solution and microemulsion is their size: micelles are smaller (≤ 10 nm) than microemulsion droplets (≤ 100 nm). Usually for the formation of microemulsion there is a need of a co-surfactant, but in some cases co-surfactants are not required, for example microemulsions are formed in AOT-iso-Octane-Water system without co-surfactant.

The solubilization capacity of microemulsions is generally much higher than that of micellar solutions (Ghosh 2009; Shelly 1997). A microemulsion consists of two phases (i) dispersed phase (the phase broken into fine droplets) and (ii) continuous phase (the liquid surrounding the droplets). Depending on the proportion of components and the hydrophilic-lipophilic balance (HLB) value of the surfactant used, the formation of microdroplets can be in the form of oil swollen micelles dispersed in water as oil-in-water (o/w) microemulsion (Fig. 1.14 b and Fig. 1.15 c) or water swollen micelles dispersed in oil as for water-in-oil (w/o) microemulsion (Fig. 1.14 f and Fig. 1.15 b), also called reverse microemulsion. The w/o and o/w types are inverted form of one type to the other and can be achieved by adding excess of one phase or by changing the emulsifier. Phase behaviour of the microemulsion has been discussed separately. In the intermediate phase region between w/o and o/w microemulsions, there may exist bicontinuous microemulsions whose immiscible phases are interconnected randomly in the form of sponge-like microstructures (Fig. 1.14 c).

Usually bicontinuous phases are viscous-gels (semi-solids). The visco-elastic gel stage (adjacent to the w/o microemulsion) comprises of a hexagonal array of water cylinders and lamellar phase (Fig. 1.14 d, e and Fig. 1.15 d) of swollen bimolecular leaflets (adjacent to the o/w microemulsion). The phase of the gel stage is also called liquid crystalline or myelinic stage (Fig. 1.14 d, e). Bicontinuous microemulsions have both oil and water as continuous phases with surfactant residing at extended oil-water interfaces (Fig. 1.14 j).

In addition to the single-phase microemulsions, multiphase microemulsion containing systems, namely Winsor systems, are also important 27 A Winsor I (W I) system

consists of an o/w microemulsion which is in equilibrium with an excess oil phase, while a Winsor II (W II) system is a w/o microemulsion which is in equilibrium with an excess aqueous phase. The Winsor III (W III) system is a bicontinuous microemulsion, which coexists with oil and water phases containing low concentrations of droplets (w/o and o/w respectively) while a macroscopically single phase microemulsion is denoted as Winsor IV (W IV) system (Fig. 1.14 e, f ,g).

1.6.4 Mass Transport and solubilization phenomena in micelle

One of the most important processes leading to micellar effects on reactions is the solubilization of substrates in to the surfactant micelles. (Shelly, 1997) It is possible to solubilize water insoluble substances or to increase the solubilities of slightly soluble ones in aqueous micellar solutions. They penetrate towards the hydrocarbon-like cores of the micelles. The pathways and the rates of the reactions in micellar systems depend to a great extent on how deep the solubilized species are located within the micelle. The solubilized molecules microemulsion interact with the polar head groups of a micelle and penetrate towards the core. They reside in the inner core, outer core, palisade layer or between the polar head groups. Sometimes micellar effect can also be observed as a result of the solubilization of substrates as counter ions, i.e., without solubilization, with the substrates not hydrophobic enough to be solubilized in the micellar interior (Fletcher, 1996; Minana-Perez *et al.*, 1995).

This depends on different factors affecting the solubilization such as, (i) concentration of the surfactant and co-surfactant; generally above C.M.C., the solubility increases with the surfactant and co-surfactant concentrations, (ii) structure and chain length of the surfactant; if the solubilization occurs in the hydrophobic portion of the surfactant, this will increase with increase in the size of that group, (iii) nature and structure of the solubilizate: molecular size, shape and structure, polarity and polarizability, chain branching, (iv) temperature: in most cases solubilization increases with increase in temperature, (v) nature of the counter ion; in general the solubility increases with increase in size and charge of counter ions, (vi) electrolyte; addition of electrolytes to ionic surfactants usually causes an increase in the C.M.C. and hence an increase in the solubilization capacity. The solubilization capacity of micellar systems, generally follow the order: non-ionics > cationics > anionics for the amphiphiles with same hydrophobic moiety.

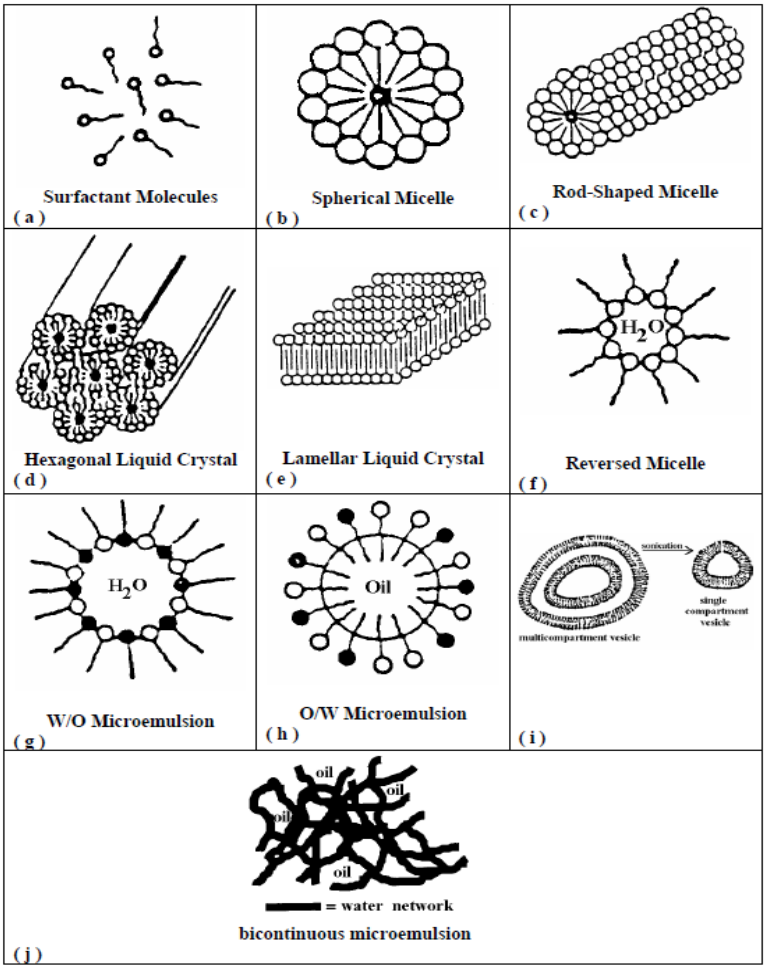

Fig. 1.14: Schematic representation of organized colloidal aggregates (Source: Khomane, 2002)

It is also noted that micelle formation is a dynamic equilibrium process, and hence solubilization process is also called as dynamic solubilization. Schematic view of the four different types of solubilization are shown in Fig. 1.15.

The micellar interaction is not static or rigid and consequently a solubilized substrate is relatively mobile. Few studies also indicate that the solubilization is, on the average, uniformly distributed in the micellar interior (Gradzielski and Hoffman, 1994; Kahlweit *et al.*, 1994; Schomacker and Strey, 1994).

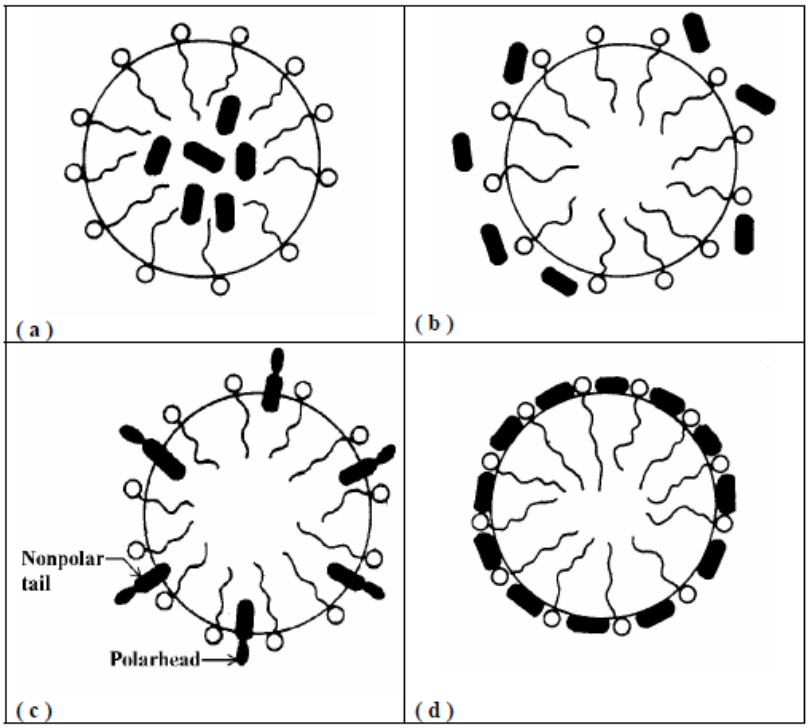

Fig. 1.15: Schematic representation of the four possible solubilization sites in surfactant aggregates organized: (a) micelle interior, (b) outer hydrophilic region, (c) oriented in the micellar surface, (d) adsorbed in the interface. (Source: Khomane, 2002)

1.6.5 Molecular interactions and surfactant adsorption

The interactions between molecules with increasing polarity order are van der Waals forces, hydrogen bonding interaction, and electrostatic forces (Cosgrove, 2010). The van der Waals forces cover a variety of short distance range interactions, whose intensity decreases with the sixth power of the distance between two molecules. They include the London or dispersion forces between non-polar molecules, the Debye forces between the permanent dipole of the molecule and induced dipole of another molecule and the Keesom forces or dipole-dipole interactions between two polar molecules. The order of magnitude of van der Waals forces is 2kJ/mol for polar and moderately polar molecules, and less than 1kj/mol for non-polar molecules. A hydrogen bond is the strong attraction between the hydrogen atom covalently bonded to an electronegative atom, and another electronegative atom. Only N, O and F are sufficiently electronegative to take part in hydrogen bonding in neutral molecules. The strength of the hydrogen bond is about ten-fold higher than any van der Waals forces. It is about 20 kJ/mole for the O-H-O. Ion-ion interactions are long distance range forces, decreasing

with the square of the distance between two ions. The ion-ion interaction strength is tenfold higher than the hydrogen bonding force, in the 200 kJ/mol range. They are very important in the comprehension of the micelle formation of ionic surfactants.

The notion of hydrophobic interaction was well developed by Tanford (1973). When a non-polar solute is dissolved in water, some hydrogen bonds are disrupted. The solute tends to locally distort the water structure and to restrict the motion of the water molecules. Thus, a large entropy increase in the water molecule is associated with the removal of the non-polar solute from aqueous solution. This entropy increase is responsible for the surface activity and micelle formation of surfactant molecules.

Surfactant Adsorption: The amphiphilic character of surfactant molecules explains their trend to adsorb at any interface. Two phases of different polarity are separated by an interface. This polarity difference attracts surfactant molecules because this can minimize the entropy change by putting their polar part in the more polar phase and their non-polar part in less polar phase.

1.6.6 Microemulsion phase behaviour

The main features of microemulsion phase behaviour have already been described by Winsor in the 1950s (Winsor, 1948). Many applications have been reported for single phase (Winsor IV) microemulsions (Paul and Moulik, 2001). However, many investigators have carried out studies on conditions required to produce three phase equilibrium (Winsor III) for a variety of systems. The results of these investigations have led to significant progress in the understanding of phase behaviour of microemulsions. Shinoda has reported regarding effect of temperature on phase behaviour of water-oil system with polyoxyethylene type non ionic surfactant (Shinoda and Lindman, 1987). As reported by Shinoda, at lower temperatures [less than TL] the system existed as O/W emulsion, Winsor I type system. At higher temperatures [more than TH] the system existed as W/O emulsion, Winsor II type system. At intermediate temperatures, [between TL and TH] the system existed as 3 phase system, Winsor III type. Based on these findings, Shinoda introduced the 'Hydrophile-Lipophile-Balance' (HLB) temperature, THLB concept to quantify this behaviour.

Microemulsions show various types of phase behaviour and consistency such as viscous solutions, lamellar liquid crystals, thin and thick gels, single phase, two phase and three phase regions due to effect of temperature, nature of surfactant, co-surfactant, the oil

and their concentrations (Fontell *et al.*, 1983; Koper *et al.*, 1995; Moulik and Paul 1998).

It can be seen that with single chain ionic surfactant systems, microemulsions can be formed by adding a co surfactant; inorganic electrolytes are also added to obtain three-phase equilibria. In these systems the phase sequence Winsor I to Winsor III to Winsor II is obtained by increasing the electrolyte and/or co surfactant concentration. The three phase behaviour (Winsor III) is, in general, temperature-sensitive in single surfactant systems regardless of the type of surfactant: ionic or non-ionic. Studies have been carried out to form temperature-insensitive microemulsions with high solubilization capacity by mixing two surfactants. The phase behaviour in these systems is more complicated because the surfactants partition differently between oil and water due to their different solubilities in both oil and water (Kunieda *et al*., 2000) have shown that the distribution of the surfactants in the different domains of bicontinuous microemulsions can be quantified, even though the particular microstructure of these systems has not been completely elucidated.

The phase behaviour and the conditions needed to produce microemulsions with standard ionic or non-ionic surfactants and aliphatic or aromatic oils have been studied exhaustively by Kunieda *et al.* (2000) and Kahlweit *et al.* (1994).

1.6.7 Applications of microemulsion and micelles

There has been a revolution in the last two decades in the utilization of microemulsion systems in a variety of chemical and industrial processes. A brief account of processes and applications is presented here in order to demonstrate their significance and potential.

Enhanced oil recovery

The most spectacular application of microemulsion is found in the tertiary oil recovery due to low interfacial tension and good wetting properties. Roughly 20 % of the otherwise unrecoverable underground oil can be obtained by the enhanced oil recovery process. The oil remains trapped in the capillary of the rocks of the reservoir because of high interfacial tension (about 20-25 mN/m) between the crude oil and reservoir brine. If the interfacial tension can be reduced to around 10-3 mN/M, a substantial fraction of the residual oil in the porous media in which it is trapped can be mobilized. Low interfacial viscosity of the system is also advantageous. Usually an aqueous or a microemulsion slug of petroleum surfactant is injected in to the reservoir and is

followed by polymer solution (for mobility control) and excess water. The injected slugs are generally miscible with the reservoir oil and produce a microemulsion of a new composition which mobilizes the residual oil (Bansal and Shah, 1977). The Enhanced Oil Recovery (EOR) is becoming an affordable method due to availability of the commonly used surfactants, polymers and alkanols at reasonable price. The advancement in the equipment to monitor the interfacial tension at high temperature and pressure has helped continued refinement of mixed recovery techniques (Paul and Moulik, 2001).

Role of microemulsions in fuels

One of the direct advantages of microemulsion-based fuels is the presence of water in a stable microemulsion and they are successfully used to reduce soot formation. When the water is vaporized during the combustion, this will lower the heat released and the combustion temperature. As a direct consequence, the emission rate of gases like nitrogen oxides (NO_x) and carbon monoxide will decrease. The presence of water is also supposed to cause improved fuel atomization, minimization of particulate emission and sooting, and improved fuel economy in terms of price and miles/volume of the fuel. Another interesting feature of microemulsion-based fuel is their capacity to increase the octane number of diesel oils. Octane number improvers includes formamide, glycols, urea etc. (Paul and Moulik 2001; Schwab *et al.*, 1982)

Pharmaceuticals and cosmetics

Thermodynamic stability, higher degree of homogeneity and solubilization capacity give rise to the various applications of microemulsions (particularly gel or liquid crystalline phases) in pharmaceuticals and cosmetics. The easy formation, remarkable environment independent stability, excellent solubilization capacity, etc. favor microemulsions to be a better proposition over other compartmentalized systems. For example, the solubilization of strongly hydrophobic fluoro-alkanes are used as short-time blood plasma substitutes to maintain a patient's oxygen supply. Ointments and creams also use emulsions and microemulsions to improve healing conditions of wounds, treatment of skin disease as well as to slow down the aging process of the skin. These systems are mostly gel phased (semisolid) emulsions or microemulsions under ambient conditions and are transformed to the liquid state when sheared during the application on the skins. Microemulsions are promising delivery systems to allow sustained or controlled drug release for precutaneous, peroral, topical, transdermal and

parenteral administration (Khomane, 2002; Kumar and Katare, 2005; Mueller *et al.*, 1994).

In case of cosmetic formulations, to avoid fatty component from the formulations, microemulsion based systems were designed and various natural extracts such as Witch Hazel extract were solubilized (Manning, 2007). Microemulsions may have found applications in field of drug delivery systems. For topical drug delivery, lecithin organo-gel system was used as formulation base for formulations containing Ketoprogen, Diclofenac, Ondensetron, Micronized Testesterone and Flurcetine for different applications (Kumar and Katare, 2005). AOT has been reported to increase the intestinal absorption of many drugs and an oral drug delivery system based on AOT microemulsion has been developed (El-Laithy, 2003)

Role of microemulsions in cutting oil and lubricants

Microemulsion or reverse micellar solutions are in use as lubricants, cutting oils and corrosion inhibitors for several decades. These liquids are usually reverse micellar solutions of certain compositions where surfactant shows effects viz. (a) corrosion inhibition by the formation of self-assembled monolayer on the surfaces and (b) the increased water content (compared to pure oils) leads to high heat capacity which helps to cool down the engine. In microemulsions, water with much higher thermal conductivity, imparts higher heat capacity to the system. Such formulation can be used in cutting oil; the oil lubricates the cutting surface, and the water helps to remove the frictional heat generated during the cutting process (Khomane, 2002; Paul and Moulik, 2001).

Washings and separations

Surfactant-based separation techniques have become significant in industrial applications. The removal of contaminants from solid surfaces or tissue capillaries (particularly non-ionic surfactants) is significantly improved by the extremely low interfacial tensions in micro emulsion systems. The advantages of these techniques are requirement of less energy as well as many of the surfactants used are eco-friendly and less toxic. The potential of surfactant based technique for separation has been documented in removal of heavy metals from industrial wastewater streams and removal of organic solutes such as phenol from aqueous solutions (Khomane, 2002; Tung *et al.*, 2002).

Soil remediation via soil washing is also based on a separation technique. In conventional soil washing, organic pollutants are detached from large soil particles by

mechanical energy input which causes increased adsorption of contaminants at the fine-grain fraction (which must then be deposited or burnt). In soil washing with microemulsions, the detached pollutants are solublilized in the oil fraction of the microemulsions as an extractive step in addition to washing.

Micro emulsions have large variety of applications. Few of the applications have been summarized in the **Table 1.4**.

Table 1.4 Applications of microemulsion and micelles

1. Remediation of (pesticide) contaminated soil
2. Agrochemicals
3. Biotechnology – protein extraction from fermentation liquid, enzyme purification, immobilization of protein, biocatalysis for esterification
4. Industrial coating and textile finishing
5. Nano particle synthesis and morphology altering of particles
6. Electrochemical and electro catalytic reactions
7. Analytical applications such as microemulsion electro kinetic chromatography
8. Sensors
9. Magnetic media
10. For solubilisation systems of flavours and fragrances
11. Food and beverages

Source: Chanamai, 2007; Conte, 2005; Ke-Long *et al.*, 2007; Lopez-Quintela *et al.*, 2004; Moulik and Rakshit, 2006; Paul and Maulik, 2001; Pratap and Bhowmick, 2008; Soni and Madamwar, 2001; Vlad *et al*. (2010); Zheng and Wong, 2010

1.6.8 Role of reverse micelle in protein extraction

The application of reverse micelle for solubilisation and purification of proteins was first reported by 1970 (Goto, 2006). This system was developed further by Dekker *et al*. (1986) and Goklen and Hatton (1985 and 1987). Extraction of the protein in the reverse micelle is termed as forward extraction which is a transport of protein from aqueous to organic phase. The forward extraction is influenced by charge on the protein and ionic surfactant. The hydrophilic groups of ionic surfactants become more active at oil water interface and mobilize protein across the interface. On the other hand non ionic surfactants do not carry charged groups and they are considered good for stabilizing proteins in aqueous phase.

Forward extraction depends mainly on electrostatic interaction between surfactant of

micelle and protein. Hydrophobic interaction between tail group of surfactant and hydrophobic domain of protein though less significant, is another factor that influences the forward extraction. Isoelectric pH (pI) of the protein determines its charges in the aqueous phase in which it is solubilised. When the protein solution has pH above pI, the net charge on protein is negative and in that case cationic surfactant such as CTAB, TOMAC, BDBAC are used for solubilising the protein. While anionic surfactant such as AOT, DOLPA are used for solubilising the protein when pH of the protein solution is less than pI of the protein (Lee *et al.*, 2004; Nandini and Rastogi, 2009; Naoe *et al.*, 2007; Pessoa Jr and Vitolo, 1998; Yu *et al.*, 2003).

This is not true when size of reverse micelle is limiting factor for solubilisation. For instance haemoglobin of molecular weight 64.5 kDa is a large molecule having pI of 6.8. Theoretically forward extraction should happen when pH of the medium is below 6.8 in presence AOT reverse micelle system. But due to the smaller size of micelle formed forward extraction does not take place (Goto *et al.*, 1998). Effect of size on extraction has been discussed in detail by Dekker *et al.* (1989). According to the authors if protein size is smaller than water pool inside a reverse micelle, solubilisation occurs as soon as the net protein charge is opposite to that of the reverse micellar interface.

Other factors affecting forward extraction are surfactant concentration, contact time, ionic strength of the protein solution, protein concentration in the aqueous phase and temperature. The effects of the various parameters have been discussed in detail by Tonova and Lazarova (2008). Forward extraction is responsible for mass transfer and selectivity of the protein (Dekker *et al.*, 1991).

The backward extraction is extraction of protein back in an aqueous phase from its solubilised reversed micelle phase and responsible for maximum recovery of protein in the fresh aqueous phase. It is influenced by ionic strength and pH of stripping solution, cosolvent such as alcohols and amines (C4-C10), contact time and temperature (Mathew and Juang, 2007).

A vacancy solution theory has been proposed on partitioning of the protein in reverse micellar system and aqueous phase. This theory has a powerful potential for prediction of protein partitioning in the multicomponent mixtures particularly for solutions containing more than one protein (Haghtalab and Osfouri, 2003).

Need of the present study

The forgoing literature review revealed that though many methodologies have been evolved for extraction and purification of biomolecules, the referred processes do have shortcomings. In case of solanesol, supercritical and solvent extraction produces crude solanesol which is very low in purity and requires subsequent steps such as saponification to produce partially purified product. In case of lycopene the processes such as microwave assisted solvent extraction and supercritical extraction suffers the drawback of scalability and economic feasibility. Conventional solvent extraction has shortcomings such as low yield and environmental hazards.

Similarly extraction of hydroxylated steroids from fermentation broth yields non selective extraction when industrially accepted solvent extraction is implemented in downstream processing. All these molecules are present in low concentration in feed stocks. Being low in concentration as well as commercially significant all these molecules are economically sensitive and of high value. Another class of biomolecules is enzyme which has gained commercial significance in this era of biotechnology. Conventional methodologies of protein extraction and purification, though scalable, are time consuming and yield wise inefficient.

Microemulsions are unique systems which have been extensively studied and explored in various fields in last few decades. These systems are unique due to high surface area and high solubilising properties. Interestingly due to their nano structures they show selectivity towards the molecules. However, surprisingly these systems have not been explored for extraction and purification of biomolecules for unknown reasons.

The aim of the present investigation was to explore this unique system of microemulsion to extract and partially purify the diverse phytomolecules viz. Solanesol (a trisesqui terpenoids), lycopene (a carotenoid) and fermentation product, 11 OHC (a steroid), lipase (a hydrophobic protein) and tannase (a hydrophilic protein).

The objectives of the present investigation were as follows

1. To study and optimize parameters for selective extraction and partial purification of these five biomolecules using microemulsion/reverse micelle based system.
2. To study and optimize parameters for selective extraction and partial purification of these five biomolecules using systems without surfactants/ conventional methods.

3. Compare the performance, in terms of yield and purity of the respective molecules obtained, by the use of the aforementioned two types of systems.
4. Highlight the process economics aspects.

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CHAPTER 2

Extraction of solanesol from tobacco dust using microemulsion/ reverse micelle

2.1 Introduction

Solanesol is found to be present in tobacco dust, the fines obtained during processing of tobacco leaves. The major application of solanesol is in food and pharmaceuticals, which requires solanesol of highest purity considering the quality, safety and efficacy of the finished products. Therefore, it is important to develop processes that can selectively separate and purify solanesol from the crude extracts of tobacco leaves. Solanesol content in tobacco dust ranges from 0.3 % to 3.0 % (w/w) (Rao *et al*., 2007; Rao *et al*., 2008). Several methods have been described in literature for extraction, isolation and purification of solanesol from tobacco (Rao *et al*., 2008). Most of these methods involve multiple step procedures, are non-specific, tedious and time consuming. Generally maceration, percolation, ultra sonication, soxhlet and bubble column have been used for extraction of phytochemicals from the plant materials (Doig *et al* 2005; Rao et al., 2008). The first two techniques are not only time consuming but also give low yields of the desired products. Microwave-assisted extraction (MAE) coupled with saponification was reported to be effective for extraction of solanesol from tobacco leaves (Zhou and Liu, 2006). However, MAE and ultra sonication are useful for high value added products but are very energy intensive for use at commercial scale. Soxhlet has limited industrial applications and not suitable for handling of bulk quantities of tobacco leaves/dust. Tang *et al.* (2007) proposed an extraction procedure with petroleum ether under reflux at 50°C followed by silica gel column chromatography for isolation and purification of solanesol from tobacco leaves. However, the heat-reflux processes involve lengthy operations, require large quantities of solvents and may ultimately lead to thermal decomposition of the target compounds. Recently high-speed countercurrent chromatography (HSCCC) for isolation of solanesol from the crude extracts of tobacco was reported in which the crude extract instead of raw tobacco was used as a feedstock for purification of solanesol (Rao *et al*., 2008). The purity of solanesol thus obtained was less than 95%.

A slow rotary counter current chromatography (SRCCC) involving a non-aqueous two-

phase solvent system of sunflower oil-ethanol was also used to produce food grade solanesol at a commercial scale (Zhao *et al.,* 2007). However, the process is not cost effective and the purity of solanesol was only 26%.

The process of extraction (heat-reflux) of solanesol from tobacco leaves by suitable solvent, such as n-hexane is commercially employed to obtain crude solanesol. The hexane extract is evaporated to dryness to recover solvent and obtain residue, which is referred to as crude solanesol (CSA). The crude solanesol (CSA) so obtained is further purified by process of saponification. The crude solanesol, obtained after saponification and evaporation of the solvent is referred to as saponified Crude solanesol (SCS). The SCS is subjected to extraction by mixed solvent which, through partitioning effect, yields partially purified solanesol (CSB) having purity up to 70 %. The CSB is subjected to further purification, typically by column chromatography to obtain purified solanesol, having purity over 95 %.

One of the objectives of the present research was to optimize parameters for developing a commercially viable process for obtaining SCS. The effect of various parameters on the performance of the two process steps – solvent extraction and saponification was studied during the present investigation. Microemulsion based systems are known to yield substantially improved performance in case of solvent extraction. Second objective of the present investigation was to study use of microemulsion system for extracting solanesol from tobacco dust and compare the performance with the process of extraction followed by saponification.

2.2 Materials and Methods

2.2.1 Materials

All chemicals used for the experimentation were of AR and/or GR grade. The solvents viz. n-hexane, ethylene dichloride, n-propanol, ethyl acetate, methanol, sodium hydroxide and precoated TLC plates of silica gel F_{254} were obtained from Merck India Ltd. Standard of solanesol (90 % purity) and AOT was procured from Sigma Aldrich India. CTAB was purchased from SRL India, SDS from Qualigens and T80 from Hi Media. The water used was deionized using Cascada Water Purification System (Pall Life Sciences). Tobacco dust was a kind gift from M/s Phillip Morris, USA.

2.2.2 Methods

2.2.2.1 Extraction of solanesol

All experiments were carried out using 100 g tobacco dust soaked in 200 ml of water. This was considered as a feed. Effect of feed: solvent ratio using n-hexane in various quantities (100-600 ml) as well as effect of temperature (27-60°C/ reflux conditions) were studied in the reaction assembly as shown in the Fig 2.1 under constant stirring conditions. The mixture was then filtered to separate the spent tobacco dust from aqueous- organic layer (Fig 2.2). The aqueous layer was tested for residual solanesol by TLC. The organic layer (n-hexane extract) was further characterized quantitatively by total solid content and HPLC.

2.2.2.2 Saponification of n-hexane extract

The n- hexane extract was concentrated by using rotary vacuum evaporator at 40°C to obtain a residue of semisolid consistency. The residue was dissolved in ethylene dichloride (EDC) followed by the addition of n-propanol and then subjected to saponification. Saponification was carried out by sodium hydroxide: water (1:1, w/w) at different temperatures (27-80°C, under reflux condition as applicable) for 1.5 h with constant stirring. The mixture was allowed to settle and separate into two phases. Aqueous layer was discarded after confirmation of absence of solanesol by TLC. The organic layer (EDC extract) was washed with hot water (60°C) for 0.5 h under constant stirring conditions (Fig 2.1) and the mixture was then allowed to separate into two layers. Aqueous layer was discarded after confirmation of absence of solanesol by TLC. The organic layer thus obtained was partially purified solanesol extract. The content of solanesol in the partially purified extract was analyzed by HPLC. The schematic diagram of extraction of solanesol and its saponification has been shown in Fig 2.2.

2.2.2.3 Extraction of solanesol by using reverse micelle system

The reverse micelle system based extraction of solanesol was carried out using different surfactants. Tobacco dust (100 g) was first soaked in water (200 ml). Surfactant viz. SDS, T80, CTAB was added in different concentrations (0.001 M - 0.075 M) one at a time. n- hexane then was added to the tobacco-water- surfactant mixture (feed). In case of AOT, it was first dissolved in n-hexane and then the solution was added to tobacco – water mixture. The different ratio of tobacco-water-surfactant and n-hexane was extracted at different temperature (27-70°C, under reflux conditions as applicable) in reaction assembly shown figure 2.2 for varying time period under constant stirring conditions. The mixture was then filtered to separate the spent tobacco dust from

aqueous- organic layer (Fig 2.3). The aqueous layer was analyzed for residual solanesol by TLC. The organic layer (n-hexane extract) was further characterized quantitatively by total solid content and HPLC.

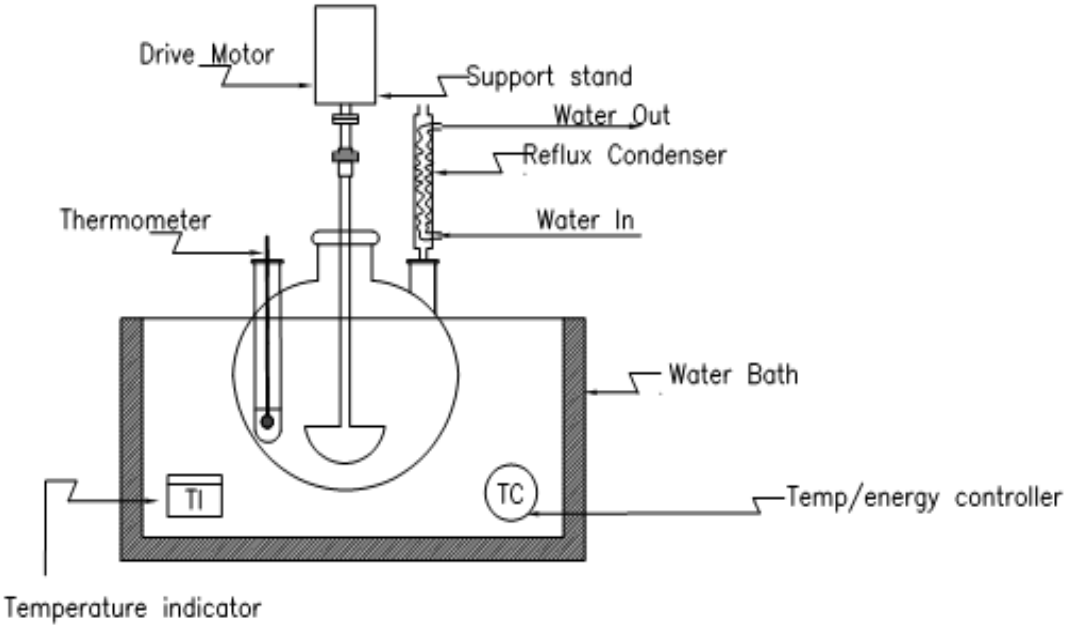


Fig. 2.1: Schematic diagram of the experimental setup for extraction

2.2.2.4 Determination of total solid content

Total solids (TS) content was determined by the method described in AOAC, 1998 with slight modification where n-hexane extract was first evaporated to dryness on water bath at 60°C followed by drying at 60°C in hot air oven till constant weight was achieved. The results were expressed in % w/v or w/w.

2.2.2.5 HPLC analysis of solanesol

The HPLC analysis was carried out using Waters 2487 and BDS Hypersil C18, 250x4.6, 5 µm column. The standard solanesol and samples were injected (50 µl) using isopropanol: methanol (60:40) as mobile phase at 215 nm at 25°C. The results of solanesol content were expressed in % (w/w).

2.2.2.6 TLC analysis of solanesol

TLC was carried out using precoated silica F_{254} plates. Samples were applied on the

plates along with standard solanesol. The plates were developed in ethyl acetate: n-hexane (1:9) and the separated spots were visualized by iodine fume.

2.2.2.7 Presentation of observations

The standard deviation for the results obtained was determined. Only those results for which the standard deviation was within 5 % were accepted as results of the experiment. At least 3 such results of each experiment were selected and arithmetic average (mean) was determined. All such mean values of the results were reported under the table of observations.

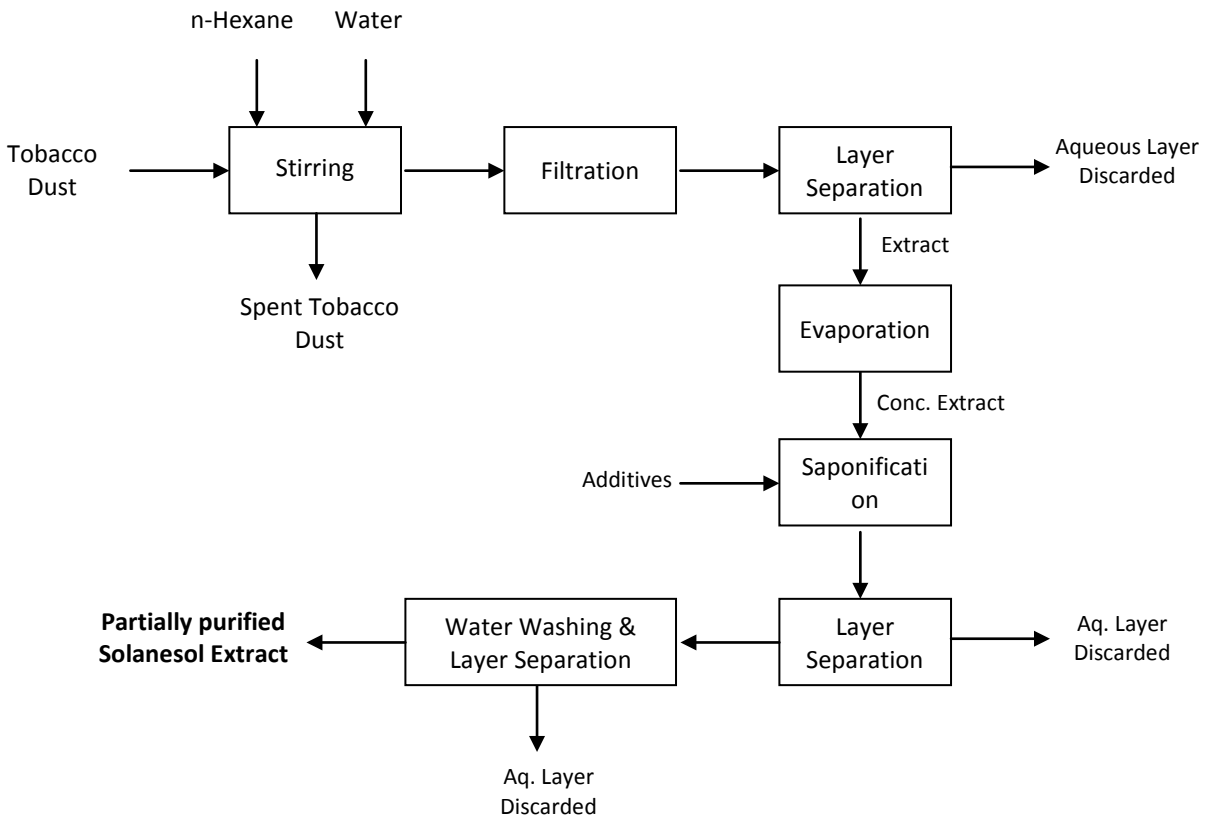
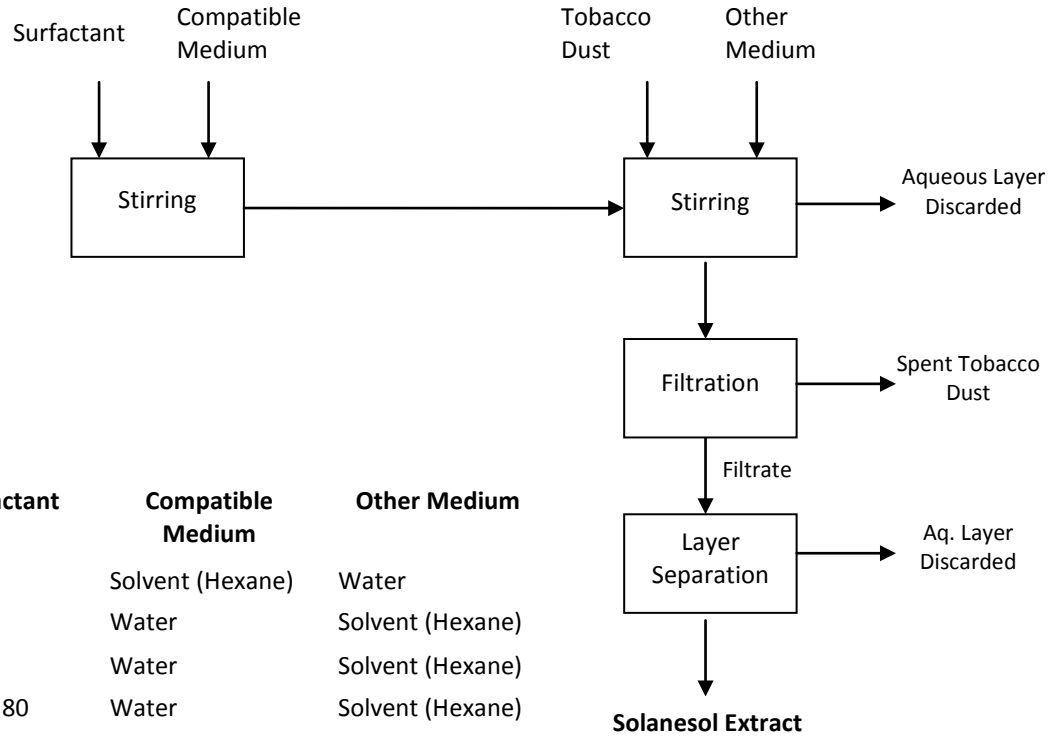


Fig. 2.2: Block Diagram for solanesol extraction and partial purification, system without surfactant



Surfactant	Compatible Medium	Other Medium
AOT	Solvent (Hexane)	Water
CTAB	Water	Solvent (Hexane)
SDS	Water	Solvent (Hexane)
Tween 80	Water	Solvent (Hexane)

Fig. 2.3: Block Diagram for solanesol extraction and partial purification using microemulsion based system

PART A: Extraction of solanesol without surfactant

2.3 Results and discussion

In the present investigation, one of the objectives was to evaluate and compare the performance of the systems without surfactant with the performance of the surfactant based system. Accordingly, in the present investigations preliminary studies were carried out for evaluating the best possible performance of the system without surfactant. As shown in Fig. 2.2, this involves extraction of solanesol from tobacco dust by leaching, concentration of the extract, followed by saponification of the extracted crude solanesol.

2.3.1 Extraction of solanesol by leaching of tobacco dust

The leaching of tobacco dust was carried out in single stage extraction. The extraction of solanesol from tobacco dust, as mentioned earlier, was carried out by first wetting tobacco dust with water. The leaching of solanesol from tobacco dust by n-hexane in presence of water was a rather unique feature of this investigation. The use of water had

the desirable effect of suppressing, extraction into the solvent phase, of the impurities which had solubility in water as well as limited solubility in n-hexane. The effect of quantity of water required for wetting the tobacco dust was investigated. Use of 200 ml water gave optimum results and hence the results with 100 g tobacco dust and 200 ml water are reported here.

During each experiment, samples were drawn at interval of 30 min and analysed to ascertain that the steady state values have been reached. (If the results at 2.0 h and 2.5 h were identical, then 2.0 h was taken as time of reaching steady state condition). For all the experiments, the feed was 100 g tobacco dust in 200 ml water and the solvent used was n-hexane. The time required to reach steady state condition was noted in all experiments. The performance of such leaching and extraction operation, for a given solvent, is governed mainly by two parameters – temperature and solvent to feed (tobacco dust) ratio. The effect of these parameters was evaluated and the results are given in **Table 2.1** and **Table 2.2.**

2.3.2 Effect of temperature

The effect of temperature was studied over a range of 27°C to 60°C, the latter being referred to as the reflux temperature (boiling temperature of solvent at the experimental conditions). The results are given in Table 2.1.

Table 2.1: Effect of temperature on solanesol extraction

Temperature, °C	Total volume, ml	TS, g/l	TS, g	Purity of solanesol, %	Total solanesol, g	Ratio solanesol/ tobacco dust, %
27	562	4.30	2.41	8.7	0.21	0.21
40	560	11.30	6.32	8.7	0.55	0.55
50	561	18.23	10.23	8.8	0.90	0.90
60	560	24.0	13.0	8.7	1.13	1.13

Extraction was carried out using 100 g tobacco dust + 200 ml water + n-hexane as solvent for 2 h. The ratio of solvent to tobacco dust was 6:1 (v/w).

It can be seen that the yield increased steadily as the temperature increased and the highest value of yield (1.13 g) was obtained at the reflux temperature (60°C). The purity was not affected by the increase in temperature. Like all natural systems, this was a multicomponent system. Analysis of number and content of respective component,

other than component of interest (solanesol in this case) was economically unviable proposition and hence not carried out. The behaviour of purity, as observed, could be attributed to nearly similar solubility behaviour of solanesol as well as the impurities and therefore the temperature having no effect on selectivity of the solvent. The behaviour of the yield indicated sharp increase in solubility of solanesol with temperature. For such systems, crystallization by cooling could be feasible method for further purification. However, it was observed during the present investigation that due the solubility behaviour, as discussed earlier, impurities also separated out with solanesol when the extract was cooled. In view of this, the extract was subjected to concentration by evaporation to dryness, prior to its saponification, to optimize the yield.

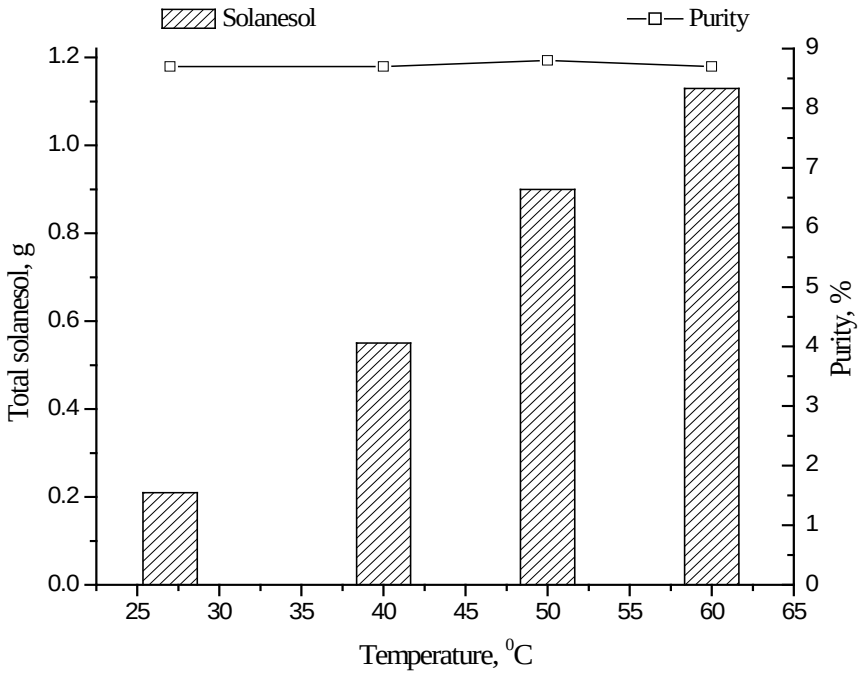


Fig 2.4: Effect of temperature on solanesol extraction

Results related to extraction of solanesol from parts of tobacco plant have been reported earlier. Rowland *et al.* (1956) have reported yield of 0.4 % of the weight of dry leaves (0.4 g per 100 g). They have not reported purity of the solanesol obtained by them or the temperature of extraction. The temperature was apparently ambient, which could be 25°C. Zhou and Liu (2006) have reported results of microwave assisted "heat reflux

extraction", which was carried out at 60°C, with solvents n-hexane, ethanol and mixtures of the two. The highest results of yield reported by them are 0.7 %. (0.7 g solanesol extracted per 100 g of leaves), but the purity of solanesol obtained was not reported. Zhao *et al.* (2009) have reported results for three different methods – percolation, soxhlet and bubble column extractor, using 80 % ethanol as solvent. From the description given by them, it was seen that percolation and bubble column extraction were carried out at ambient temperatures. The soxhlet, as the name suggests, is likely to have been carried out at reflux temperature of the solvent, which has not been mentioned. It can be inferred as about 80°C. The highest yields obtained by all the three methods were reported to be about 4.2 mg/g, which corresponded to 0.42 g per 100 g tobacco leaves. None of these investigators have studied the effect of temperature. During our investigation, the yield obtained at reflux temperature (1.03 %) was significantly higher than the same reported by the other investigators. This may be due to the type and quality of tobacco used for extraction.

2.3.3 Effect of solvent to tobacco dust (feed) ratio

The effect of this ratio was studied over the range 1:1 to 6:1. The results are given in Table 2.2. It is seen from the results that for the increase in ratio up to 4:1, there was a significant increase in yield of solanesol (from 0.36 g to 0.83 g). Further increase in the ratio up to 6:1, increased the yield further (1.13 g).

To check the effect of still higher ratio, an experiment was carried out with 7:1 ratio. However, no further improvement was observed and the purity of solanesol (about 8.7 %) was practically independent of this ratio.

Since the purity was unaffected by the solvent to feed ratio it indicated that the selectivity of the solvent (n-hexane), to solanesol on one hand and the other impurities on the other hand was independent of the ratio. The yield of solanesol was observed to be highest (1.13 g from 100 g tobacco dust) at the feed: solvent ratio of 6:1 and remained unchanged thereafter at higher ratios. This could be attributed to the solubility pattern of solanesol in the given system. The observed behaviour indicated that solanesol was not infinitely soluble in the solvent but had finite solubility. The highest possible percentage extraction was reached at the ratio of 6:1 and thereafter no further improvement was seen when the ratio was increased further. The effect of this ratio on the yield (% extraction) has not been reported by any other investigators. One of the

reasons for the high yield of solanesol obtained (1.13 %) could be optimization of this ratio achieved during the present investigation.

Table 2.2 Effect of solvent to tobacco dust ratio on solanesol extraction

Solvent to tobacco dust ratio, v/w	Total volume, ml	TS, g/l	TS, g	Purity of solanesol, %	Total solanesol, g	Ratio solanesol to tobacco dust, %
1:1	42	98.57	4.0	9.0	0.36	0.36
2:1	115	48.42	5.57	8.8	0.49	0.49
3:1	230	34.68	7.98	8.9	0.71	0.71
4:1	340	27.43	9.32	8.9	0.83	0.83
5:1	450	24.77	11.15	8.7	0.97	0.97
6:1	560	24.0	13.0	8.7	1.13	1.13
7:1	665	19.55	13.0	8.7	1.13	1.13

Extraction was carried out using 100 g tobacco dust + 200 ml water + n-hexane as solvent for 2 h at 60°C (reflux temperature).

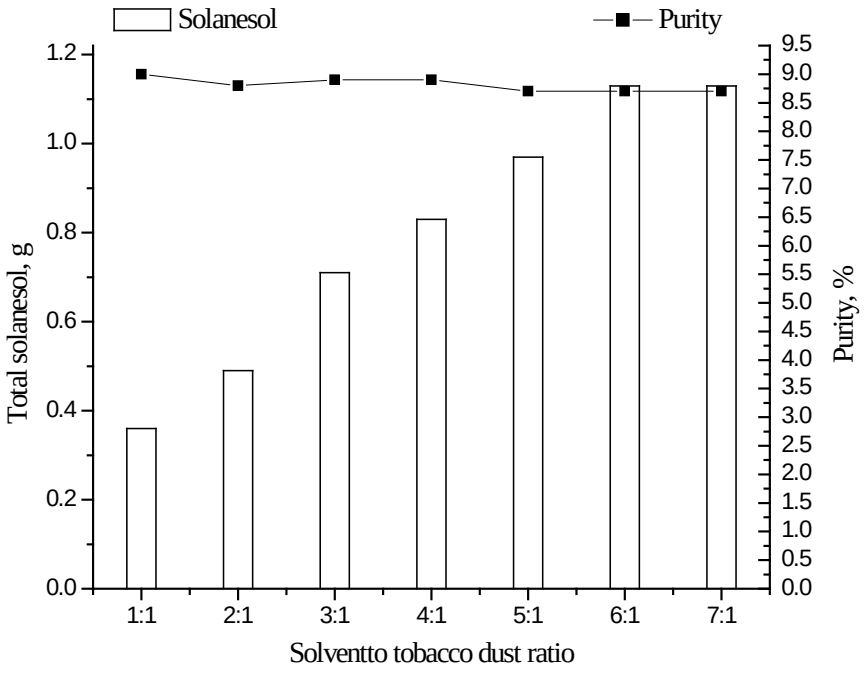


Fig 2.5 Effect of solvent to tobacco dust ratio on solanesol extraction

2.3.4 Saponification of crude solanesol

As discussed earlier, solanesol with purity upwards of 90 % is required for the product to be suitable for commercial application purposes. Multiple stages are required to obtain this level of purity, wherein each stage contributes to some enhancement in purity. Saponification is the stage usually employed to further enhance the purity of the solanesol obtained by extraction (typically 8 % to 9 %). Use of n-hexane as solvent is effective for extraction of solanesol. However, fatty matter present in the tobacco leaves/dust also got leached out due to non-polar nature of n-hexane. Saponification is therefore one of the effective methods of removing the fatty matter from the extract (organic layer) containing solanesol. Saponification of crude solanesol extracted from potato leaves using methanol and NaOH has been reported (Asahina *et al*., 1977). Use of EDC and isopropanol for saponification has been reported for the first time to best of our knowledge. Zhou and Liu (2006) have reported microwave assisted "heat reflux extraction in presence of caustic soda (NaOH). They have also reported increase in yield by presence of NaOH, from about 0.7 % in absence of NaOH to about 0.9 % in presence of NaOH. However reports of both these investigation did not carry any details regarding increase in purity of solanesol as a result of saponification.

In the present investigation, the crude solanesol was saponified as per the protocol discussed under materials and methods section 2.2.2.2.

The performance of the saponification operation was to a major extent governed by temperature of saponification and solvent to crude solanesol ratio. The effect of these two parameters was studied and the results are given in Table 2.3 and Table 2.4.

2.3.3.1 Effect of temperature on saponification

The effect of temperature was studied over the range 27°C to 80°C, the latter being referred to as the reflux temperature. The results have been given in Table 2.3. It could be seen that there was steady but not very substantial increase in purity with increase in temperature. The yield was seen to be nearly independent of the temperature (8.7 g per 100 g crude solanesol, equivalent to about 1.13 g per 100 g tobacco dust).

The results indicated that the best possible performance in terms of saponification is achieved at reflux temperature (80°C). In case of endothermic reactions, the equilibrium conversion increased with increase in temperature. The results obtained, therefore indicated endothermic nature of saponification reactions.

Table 2.3: Effect of temperature on saponification of crude solanesol

(Reflux) Temperature, °C	Total volume EDC, ml	TS, g/l	TS, g	Purity of solanesol, %	Total solanesol, g	TS reduction, %	SEQ*, g
27	590	165.5	97.64	8.90	8.69	2.36	1.12
50	585	156.5	91.58	9.50	8.70	8.42	1.13
60	580	151.5	87.85	9.88	8.68	12.15	1.13
80	570	122.5	69.80	12.45	8.69	30.20	1.13

Saponification of solanesol was carried out using 100 g crude solanesol (purity 8.7 %) + EDC 600 ml + NaOH 25 g + water 25 g keeping n-propanol-crude solanesol ratio 1:1 (v/w) for 1.5 h.
* SEQ is total solanesol in saponified crude solanesol corresponding to 100 g tobacco dust.

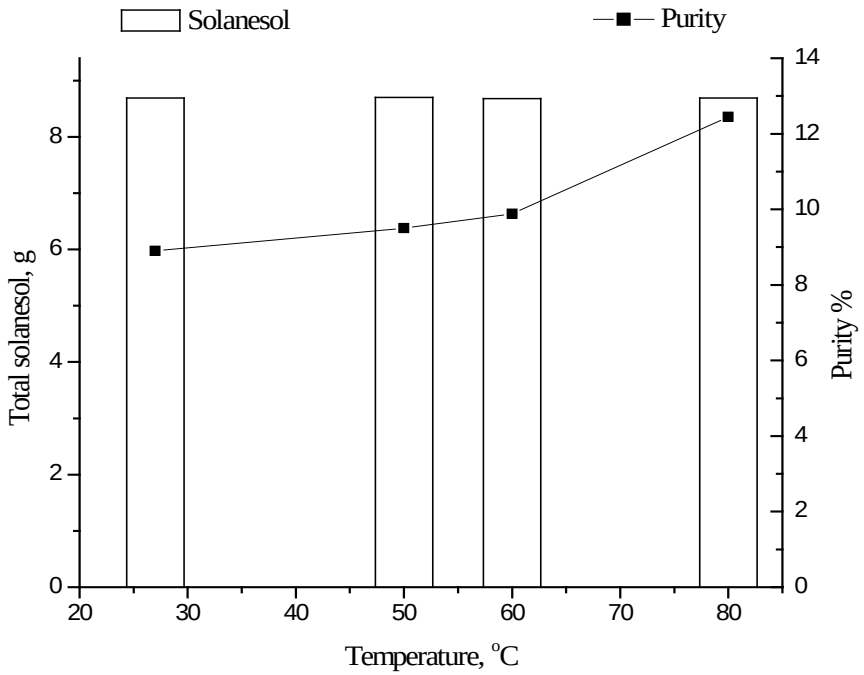


Fig. 2.6 Effect of temperature on saponification of crude solanesol

2.3.3.2 Effect of solvent (n-propanol) to crude solanesol ratio on saponification

The effect of this ratio was studied over the range of 0.75:1 to 3:1. The results have been given in Table 2.4. It was observed that the purity of saponified crude solanesol (SCS) as well as the yield increased with increase in this ratio. Secondly, it was observed that saponification lead to an improvement in purity to approximately 14 % of SCS as against 8.7 % of crude solanesol extracted in n-hexane. This nature of the results indicated a need for optimization of solvent to crude solanesol ratio, since the nature

and composition of fatty impurities was not known. The partitioning of saponified product was favoured at high propanol to crude solanesol ratio.

Table 2.4: Effect of n-propanol to crude solanesol ratio (v/w) on saponification of crude solanesol

n-propanol to crude solanesol ratio, ml/g	Total volume EDC, ml	TS, g/l	TS, g	Purity of solanesol, %	Total solanesol, g	TS reduction, %	SEQ*, g
0.75: 1	575	107.3	78.10	10.14	8.70	24.14	1.12
1:1	570	122.5	69.80	12.45	8.69	30.20	1.13
2:1	565	111.3	62.90	13.80	8.68	37.10	1.13
3:1	570	107.8	61.40	14.10	8.66	38.60	1.13

Saponification of solanesol was carried out using 100 g crude solanesol (purity 8.7 %) + EDC 600 ml + NaOH 25 g + water 25 g at reflux temperature (80°C) for 1.5 h. * SEQ is total solanesol in saponified crude solanesol corresponding to 100 g tobacco dust.

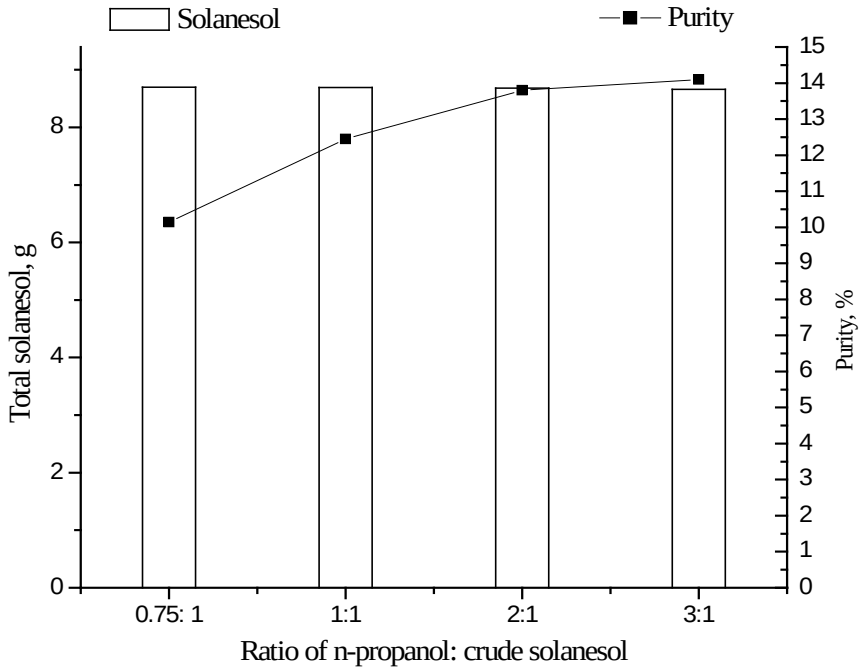


Fig. 2.7: Effect of n-propanol to crude solanesol ratio (v/w) on saponification of crude solanesol

To the best of our knowledge, no published data was available on optimization of the parameters for saponification which was done in the present research. In the present study, saponification of the extract from tobacco dust was been carried out using EDC-

propanol solvent system, since this system was found to be optimum. The process parameters for this system were also optimized. This system was effective in increasing the purity from 8.7 % to 14 %.

Part B: Extraction of solanesol with surfactant

2.4 Results and discussion

In micelle based systems the performance and hence efficacy of extraction process, to a large extent may be governed by the type of surfactant and its concentration. In view of this the effect of type of surfactant and surfactant concentration (0.001 M – 0.05 M) was studied for 3 surfactants viz. AOT (Table 2.5), SDS (Table 2.6) and CTAB (Table 2.7). During these studies other parameters viz. temperature and solvent to tobacco dust ratio were maintained same.

2.4.1 Effect of AOT and its concentration

The results have been given in Table 2.5. The purity of solanesol extracted as well as the yield of extraction increased with increase in AOT concentration up to 0.05 M. Therefore, further investigation was carried out using 0.075M AOT concentration. It was observed (table 2.5) that the increase in AOT concentration (from 0.05 M to 0.075 M) did not show any improvement yield as well as purity.

Table 2.5 Effect of AOT concentration on solanesol extraction

AOT concentration, M	Total volume, ml	Purity of solanesol, %	Total solanesol, g
0.001	540	12.72	0.150
0.0025	542	14.17	0.194
0.0125	539	15.28	0.204
0.025	537	17.51	0.340
0.05	534	20.80	0.340
0.075	534	18.80	0.318

Extraction was carried out using 100 g tobacco dust + 200 ml water + n-hexane as solvent at 60°C (reflux temperature) for 2 h. The ratio of solvent to tobacco dust was 6:1 (v/w).

One disadvantage observed with use of AOT was the lower yield of solanesol, when compared with extraction without surfactant. The highest yield obtained with use of

AOT (0.34 g) was approximately 30 % of the highest yield obtained in system without surfactant (1.13 g).

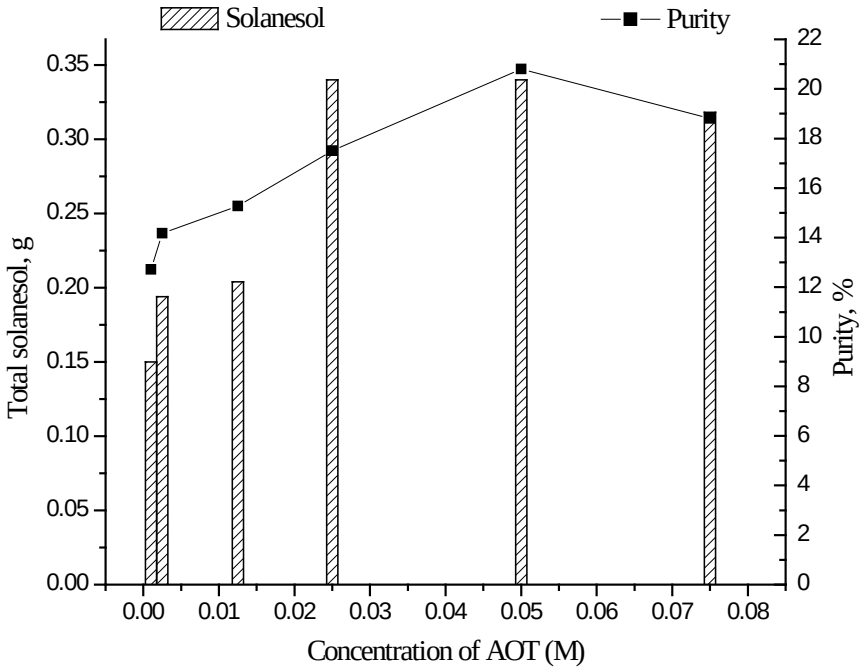


Fig 2.8 Effect of AOT on solanesol extraction

Such adverse effect on the yield indicated adverse effect of surfactant on solubility. To the best of our knowledge, there was no published information available regarding effects of surfactant in case of multicomponent system, such as the one from which solanesol was purified. Another disadvantage in case of AOT was that since AOT was soluble in organic solvent phase (and not in aqueous phase) it would remain in the extract. The process for further purification of solanesol required concentration of the extract by evaporation of the solvent. At this stage if the AOT would have remained in the concentrated extract, one additional operation would be required to remove AOT from the concentrated extract before the extract could be used for obtaining purified solanesol. This consideration restricted the use of AOT for solanesol extraction.

2.4.2 Effect of SDS and its concentration

The results have been given in Table 2.6. The purity of solanesol extracted as well as the yield of extraction was independent of SDS concentration. The results indicated that both selectivity and distribution coefficient of the solvent were not affected by the

surfactant concentration. Secondly the values of purity (about 20 %) and yield (about 0.2 g) were inferior to best results obtained with AOT and CTAB. This may be attributed to the anionic nature of SDS.

Table 2.6 Effect of SDS concentration on solanesol extraction

SDS concentration, M	Total volume, ml	Purity of solanesol, %	Total solanesol, g
0.001	570	18.58	0.213
0.0025	560	18.82	0.192
0.0125	560	19.70	0.272
0.025	562	18.42	0.268
0.05	558	18.18	0.212

Extraction was carried out using 100 g tobacco dust + 200 ml water + n-hexane as solvent at 60°C (reflux temperature) for 2 h. The ratio of solvent to tobacco dust was 6:1 (v/w).

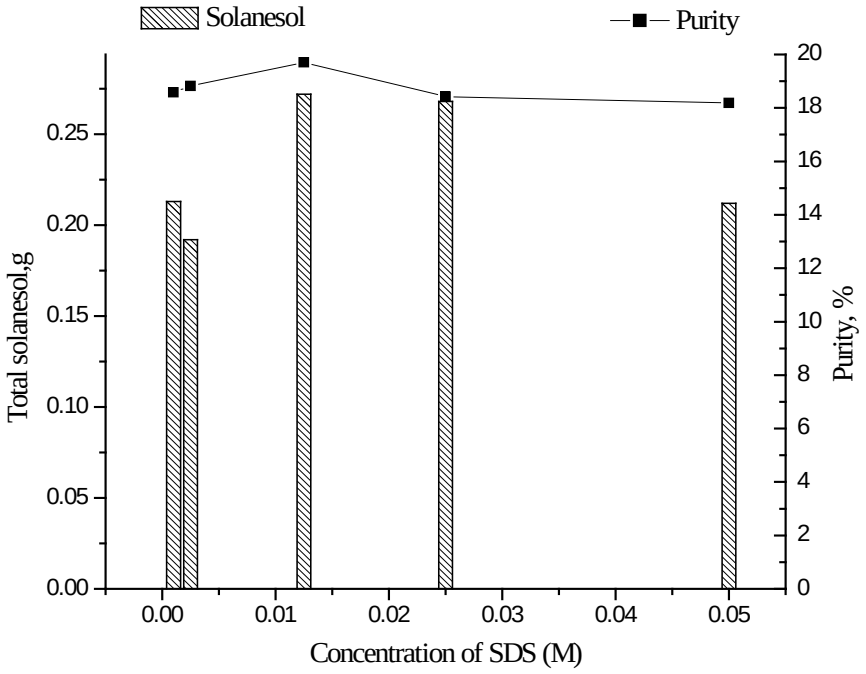


Fig. 2.9 Effect of SDS on solanesol extraction

2.4.3 Effect of CTAB and its concentration

The extraction was carried out at temperature of 60°C. Increase in concentration of CTAB increased purity of solanesol as well as yield. The highest results obtained

(Table 2.7) were 24 % purity and yield of 0.4 g for CTAB concentration of 0.025 M. Further increase in CTAB concentration did not improve the yield as well as purity.

Table 2.7 Effect of CTAB concentration at 60°C on solanesol extraction

CTAB concentration, M	Total volume, ml	Purity of solanesol, %	Total solanesol, g
0.001	568	15.25	0.171
0.0025	540	15.58	0.202
0.0125	542	21.39	0.290
0.025	543	23.98	0.403
0.05	252	13.62	0.308

Extraction was carried out using 100 g tobacco dust + 200 ml water + n-hexane as solvent at 60°C (reflux temperature) for 2 h. The ratio of solvent to tobacco dust was 6:1 (v/w).

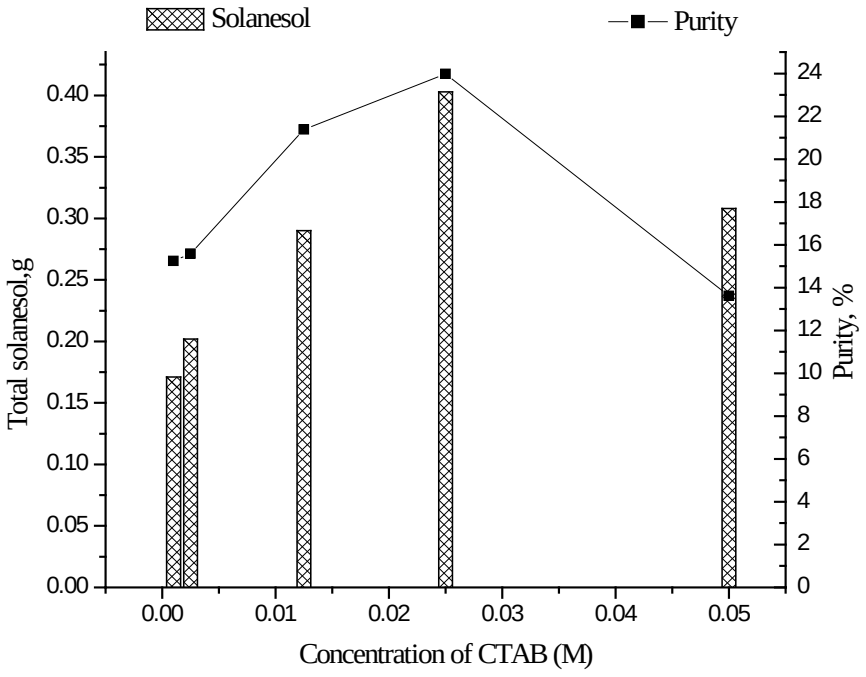


Fig. 2.10: Effect of CTAB on solanesol extraction at 60°C

The highest yield obtained in case of CTAB (0.4 g) was ~ 35 % of the highest yield obtained in system without micelle (1.13 g). However the purity was improved to the level of 24 % (as against the highest value of about 14 % obtained after saponification). The primary reason for this observed behaviour was considered to be the temperature

of extraction, since the performance of the micelle based systems were substantially influenced by the temperature. The effect of temperature at CTAB concentration of 0.025 M was investigated and the results obtained have been given in Table 2.9.

It could be seen that optimum results in terms of comparable yield (1.08 g) with much higher purity (38 %) were obtained at CTAB concentration of 0.025 M and temperature of 40°C. This may be attributed to phase behavior of ionic surfactant systems at different temperatures. The behavior of w/o microemulsion which may co-exist with continuous water phase at low temperature may, at high temperature, reverse to o/w microemulsion which co-exist with continuous oil phase (Solans and Garcia-Celma, 1977). Another contributing factor could be favourable effect of surfactant on leaching of solanesol by the mechanism similar to the one in case of tertiary oil recovery in presence of surfactant (Bansal and Shah,1977; Neuman, 1981).

Considering the improved performance at CTAB concentration of 0.025 M and temperature of 40°C, effect of CTAB concentration was further investigated at the lower temperature of 40°C. The results have been given in Table 2.8. It was observed that best results were obtained at CTAB concentration of 0.025 M.

Table 2.8 Effect of CTAB concentration at 40°C on solanesol extraction

CTAB concentration, M	Total volume, ml	Purity of solanesol, %	Total solanesol, g
0.001	571	19	0.432
0.0025	570	24.2	0.648
0.0125	570	32.1	0.864
0.025	571	38.0	1.08
0.05	569	20.0	0.756

Extraction was carried out using 100 g tobacco dust + 200 ml water + n-hexane as solvent at 40°C for 2 h. The ratio of solvent to tobacco dust was 6:1 (v/w).

Considering the improved results obtained with CTAB at lower temperature, effect of temperature for system with SDS as surfactant was also invetigated. However the results obtained were considerably inferior to those obtained with CTAB and hence are not presented herein.

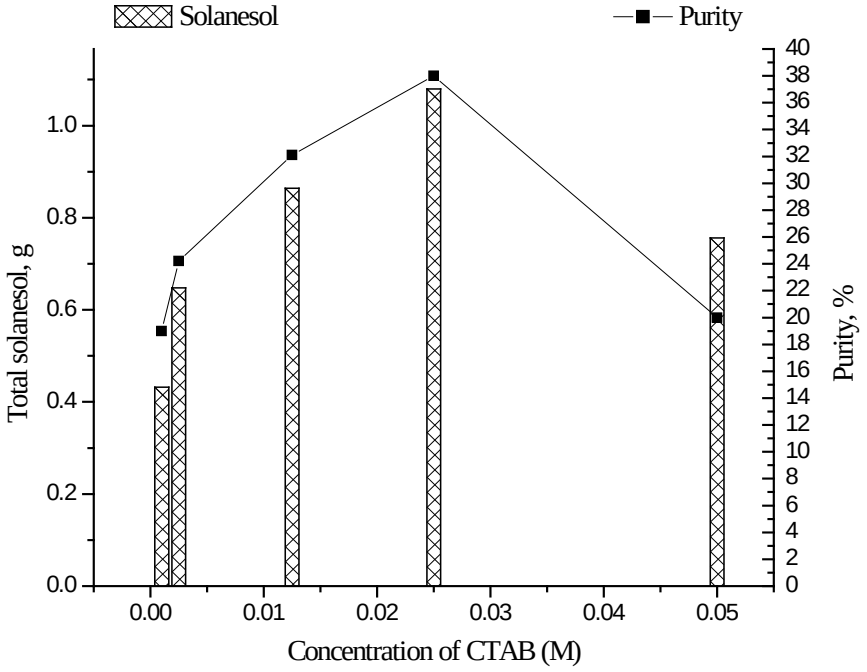


Fig. 2.11: Effect of CTAB on solanesol extraction at 40°C

2.4.4 Selection of Surfactant

As mentioned earlier since AOT required an additional removal step, it was not used in further experimental work, mainly on account of economy related constraint mentioned earlier. SDS was also not used since the results obtained were inferior and only CTAB was used as surfactant in further studies, at the observed optimum concentration of 0.025 M. Another advantage of use CTAB was that CTAB was soluble in water phase and only sparingly soluble in organic phase. Due to this solubility behaviour of CTAB, it was unlikely to affect adversely further purification of solanesol adversely.

2.4.5 Effect of different process parameters on solanesol extraction using CTAB

The effect of process parameters viz. contact time, temperature and solvent to tobacco dust ratio in presence of CTAB (0.025 M) was studied to determine conditions for improved performance.

2.4.5.1 Effect of contact time on solanesol extraction

Effect of contact time on recovery of component of interest in reverse micelle systems has been reported by many investigators. The component of interest, in case of most of these reports, was protein or enzyme. Liu *et al.* (2006) have mentioned that contact time

should usually be as short as possible in order to gain maximum activity recovery. They have further mentioned that the phase equilibrium may not be totally achieved. This could be attributed to the effect of surfactant to varying extents on rate of mass transfer for component of interest and for other components present in the feed. This aspect was not investigated during the present study. As mentioned, only those results obtained at the steady state with respect to the component of interest were considered for further analysis and discussion. The equilibrium with reference to solanesol was attained in 2 h.

2.4.5.2 Effect of temperature on solanesol extraction

The effect of temperature (27°C to 60°C) was studied at CTAB concentration of 0.025 M (Table 2.9). The purity and yield of solanesol both showed peak values at temp of 40°C. At temperature of 70°C the emulsion did not separate into 2 phases. When compared with Table 2.1 (system without surfactant) there was a marked improvement in purity of solanesol (increase from approximately 8.7% to 38%) with same yield (about 1.0 g).

Table 2.9: Effect of temperature on solanesol extraction

Temperature, °C	Total volume, ml	Purity of solanesol, %	Total solanesol, g
27	580	33	0.904
40	570	38	1.08
50	560	32.6	0.906
60	540	24	0.904
70	520	20.66	0.87

Extraction was carried out using 100 g tobacco dust + 200 ml water + n-hexane as solvent for 2 h. The ratio of solvent to tobacco dust was 6:1 (v/w). CTAB was used as surfactant at 0.025 M concentration

The yield and purity showed peak values at 40°C that both solubility and selectivity were affected by temperature. As discussed earlier in case of CTAB, lower temperature gave better results in terms of yield and purity. The temperature of 40°C was seen to be optimum for the solanesol system under investigation. At lower temperature, ionic surfactants form w/o microemulsions and at higher temperature, presence of o/w microemulsions are predominant (Maulik and Paul, 1998).

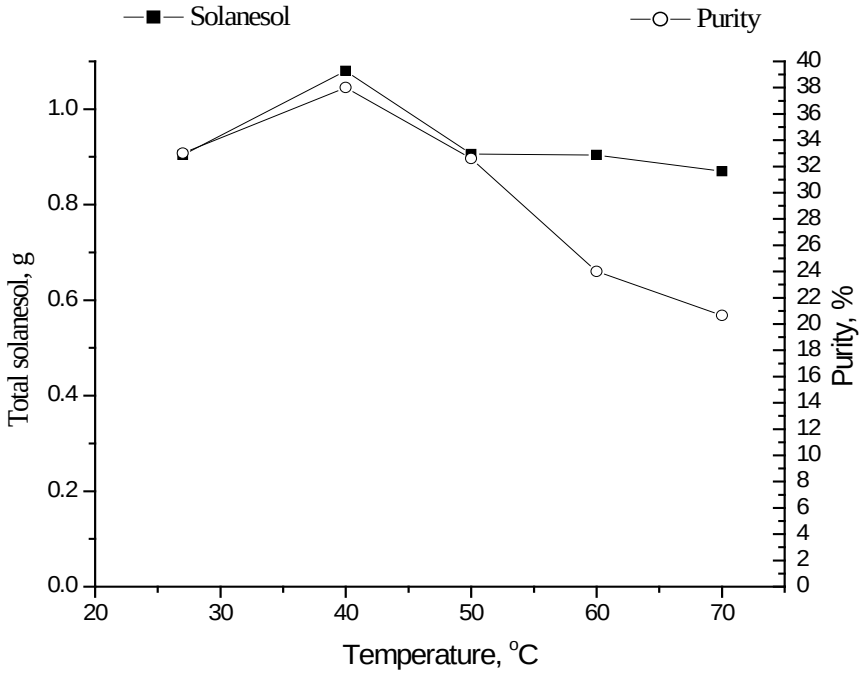


Fig. 2.12: Effect of temperature on solanesol extraction using CTAB

The highest yield at lower temperature (40°C) could therefore be attributed to this phase behaviour characteristics. Secondly marked improvement in purity, in presence of surfactant, may be attributed to favourable effect of surfactant on leaching of solanesol, by mechanism similar to the one in case of tertiary oil recovery in presence of surfactant (Bansal and Shah,1977, Neuman, 1981)

2.4.5.3 Effect of solvent to tobacco dust ratio on solanesol extraction

The effect of solvent to tobacco dust (v/w) was studied for the same CTAB concentration (0.025 M). The results have been given in Table 2.10. The purity of solanesol remained nearly unchanged with increase in the ratio from 1:1 to 6:1 (about 39 %). The yield showed increase from 0.72 g to 0.98 g for increase in the ratio from 1:1 to 3:1 and showed highest value of 1.1 g ratio of 4:1. Further increase in the ratio up to 6:1 did not show any appreciable change. With increasing solvent component, there may have been increase in micellar concentration and it may have contributed to more extraction. Similar observation have been reported in case of protein extractions (Nandini and Rastogi, 2009).

Table 2.10: Effect of solvent to tobacco dust ratio (v/w) on solanesol extraction

Solvent to tobacco dust ratio (v/w)	Total volume, ml	Purity of solanesol, %	Total solanesol, g
1:1	80	39.2	0.72
2:1	170	38.9	0.82
3:1	255	38.0	0.98
4:1	380	38.4	1.10
5:1	475	38.0	1.09
6:1	570	38.0	1.08

Extraction was carried out using 100 g tobacco dust + 200 ml water + n-hexane as solvent for 2h at 40°C. CTAB was used as surfactant at 0.025 M concentration.

When compared with results of Table 2.2, increase in purity of solanesol from 8.7 % to 38 % was observed. The yield of 1.1 g was obtained at solvent to tobacco dust ratio of 4:1 in presence of surfactant whereas this ratio of about 6:1 was required for achieving comparable yield in case of system without micelle.

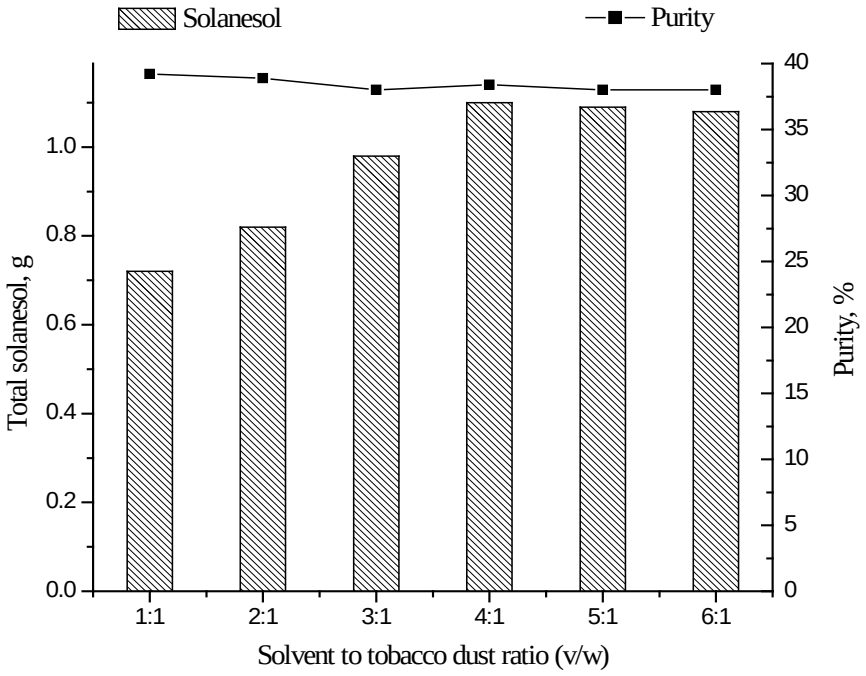


Fig. 2.13: Effect of solvent to tobacco dust ratio on solanesol extraction

As discussed earlier, the marked improvement in purity of solanesol indicated effect of surfactant on the selectivity of the solvent**.** The behaviour of the yield indicated that the distribution coefficient of the solvent for solanesol was influenced by the solvent to

tobacco dust ratio. However, the interesting part of the result was that optimum yield was obtained at lower ratio of 4:1(as against 6:1, as in case of system without surfactant). This would give advantage in terms of lower operating cost in terms of less energy requirements for solvent recovery.

2.5 Conclusion:

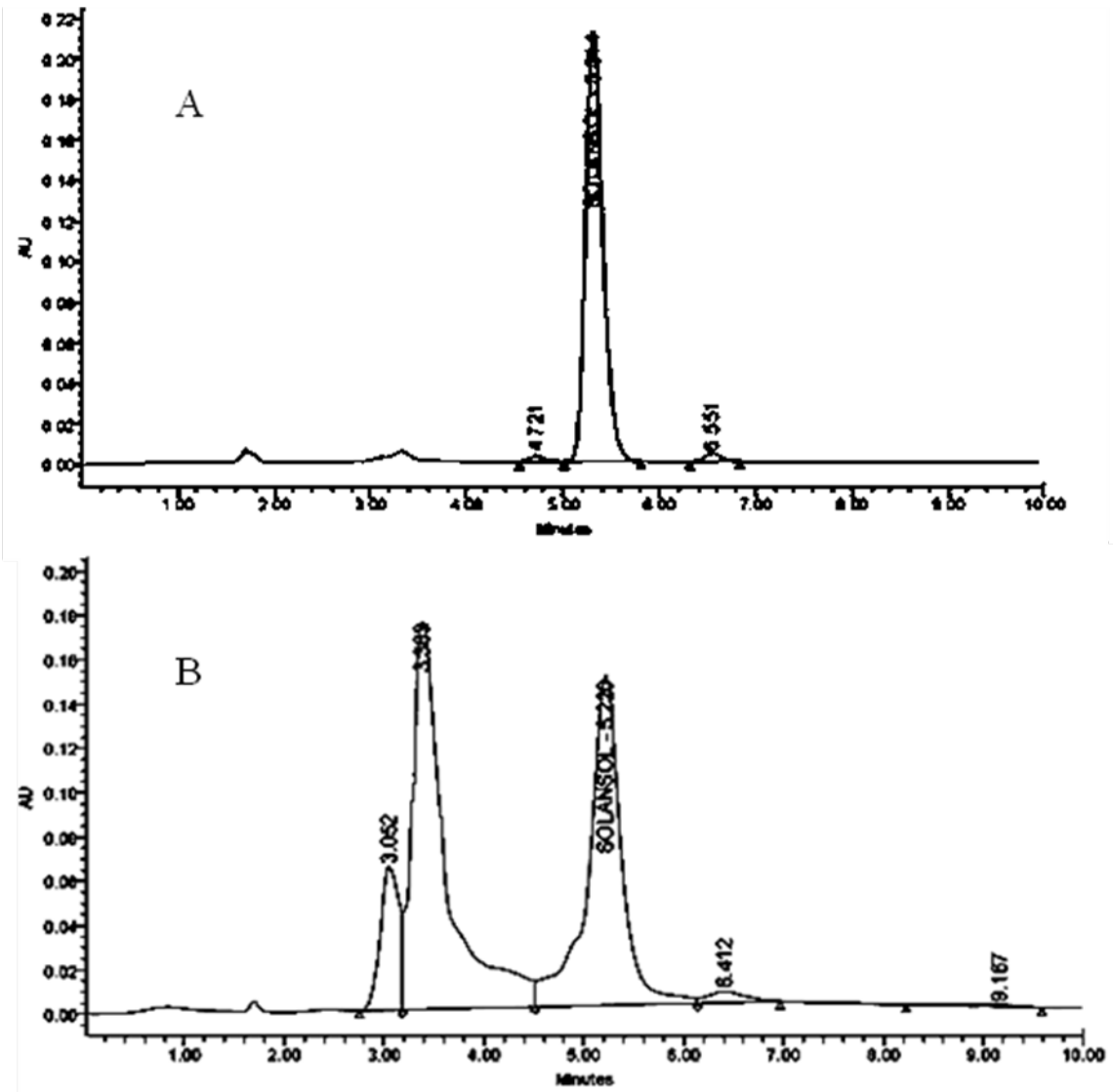


Fig 2.14 A. Standard solanesol **B**. extraction of crude solanesol with surfactant

It was observed from the chromatograph in Fig. 2.14 D that solanesol got enriched after saponification stage, as indicated by the number of impurities (Fig 2.14 C). On the other hand, in surfactant based system there was only one major impurity (at RT 3.36) along with three minor impurities. Therefore it is evident from HPLC analysis of solanesol

(Fig 2.14 B) that system with surfactant was able to extract solanesol selectively. The comparison with the conventional system (extraction and enrichment by saponifacation) showed that the micelle based system in a single stage operation yields significant increase in purity of solanesol (about 38 % as against about 14 %) (Table 2.11). The yield of solanesol however was unchanged at about 1.13 g from 100 g tobacco dust) in both the cases. The highest and comparable yield (about 1.1 g) for micelle based system was obtained at lower solvent to tobacco dust ratio which offers additional advantage in terms of energy saving as discussed earlier.

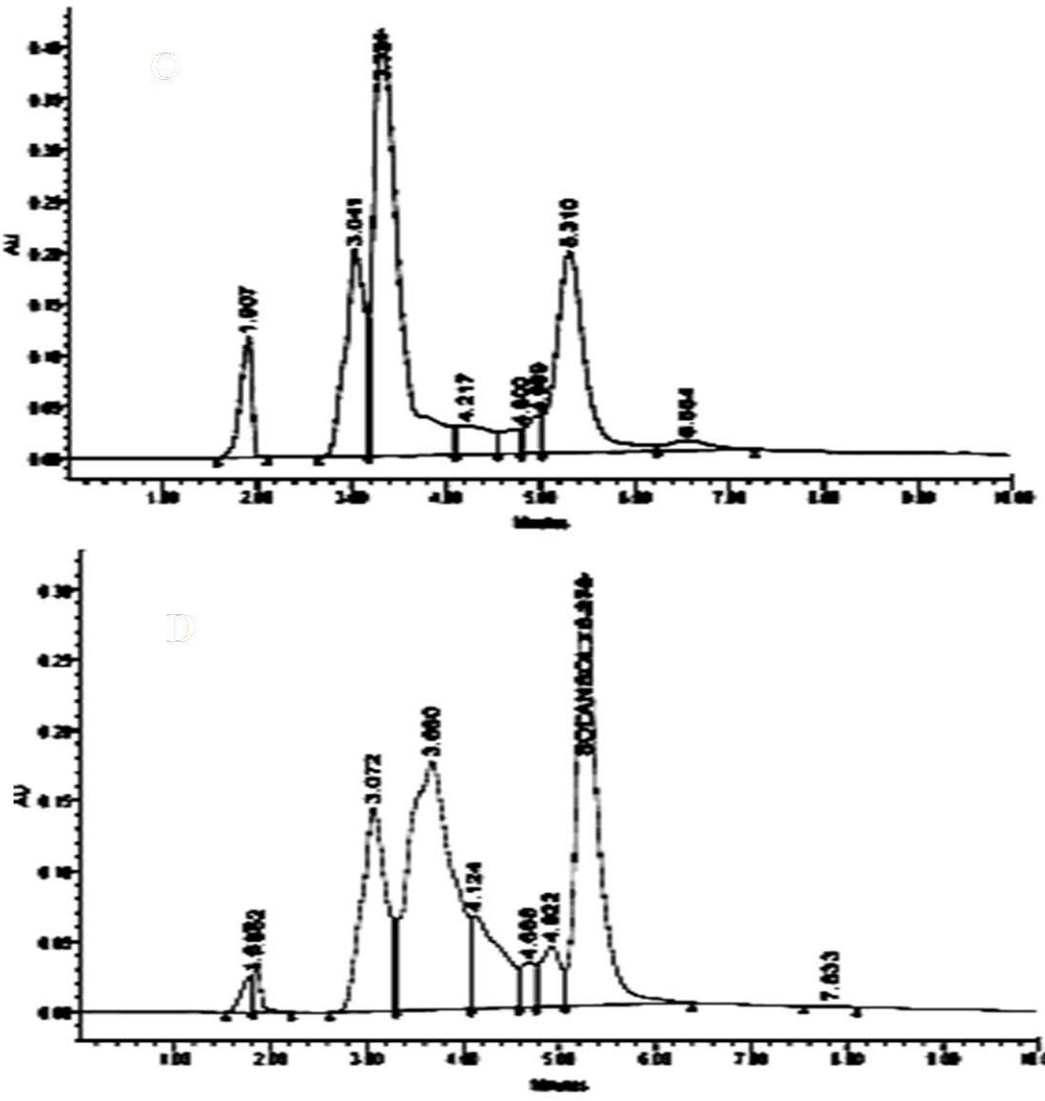


Fig 2.14 C. crude solanesol extract in n-hexane **D.** Saponified solanesol

Table 2.11 Summary of yield and purity of solanesol

	Purity	**Yield/ 100 g Tobacco Dust**
Without surfactant, without saponification	8.7%	1.13 g
Without surfactant and with saponification	14.1%	1.13 g
Surfactant based system	38.4%	1.1g

2.6 Process economics considerations

It can be seen from these results that there was significant increase (from about 14 % to about 38 %) with use of surfactant based system. Moreover this enhanced purity was achieved without the use of saponification step. The saponification step, as can be seen, gave rise to two effluent streams with high COD demand, require high capital and operating cost for effluent treatment. The surfactant (CTAB) based system on the other hand gave rise to only one effluent stream, thereby substantially reducing the operational cost of effluent treatment. Secondly due to high purity of solanesol obtained (by use of surfactant based system) there would be significant reduction in the cost of further purification of solanesol (upto over 90 %) which was essential for the commercial use of this bio-molecule. The use of surfactant based system therefore offered distinct and significant commercial advantage over the conventional system (extraction followed by saponification). All these considerations put together made the surfactant based process more suitable for scale-up to commercial scale operation.

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CHAPTER 3

Extraction of lycopene from tomato paste using microemulsion/reverse micelle

3.1 Introduction

Lycopene is the pigment principally responsible for the characteristic deep red color of ripe tomato fruits and tomato product. It has been found that most of lycopene is found attached to the insoluble fiber portion of tomato (Chaudhary and Ananthanarayan, 2007; Sharma and Le Magure, 1996). Lycopene has attracted considerable attention in nutraceutics as well as cosmetics because of its antioxidant properties. Lycopene exhibits a physical quenching rate constant with singlet oxygen almost twice as high as that of β carotene. Recent study has shown effect of dietary lycopene in reducing the risk of chronic diseases such as prostate and other types of cancer and coronary heart diseases (Rao and Agarwal, 1999). For nutraceutical applications, extract enriched with higher lycopene concentration and pure form of lycopene is a need of the industry.

Lycopene content of tomato has been found to be very low (172 – 200 mg/100 g) (Shi and Maguer, 2000). Along with lycopene tomato contains other carotenoids in the range of 30-34 % which interfere in the extraction of lycopene. The lycopene in tomatoes has been found in all trans form and exposure to high temperature during processing changes all trans form to cis form (Shi and Maguer, 2000). Therefore, it has been a challenge to develop process of extraction which is efficient, selective and non-destructive. Various approaches for extraction have been reported in literature, which can be broadly classified as follows: **a**. Solvent extraction using co-solvents such as phospholipids (Bombardelli *et al*., 1999), **b.** Solvent extraction followed by saponification and subsequent crystallization of lycopene (Ausich and Sanders, 1998), **c**. Extraction using supercritical CO_2, initially to extract crude lycopene followed by selective extraction of lycopene (Schonemann *et al.*, 2010). There are other approaches such as preparation of oleoresin and its application in the formulations as lycopene concentrate (Zelkha *et al.*, 1997). All the processes have been moderately successful. The extractability of lycopene in the solvent has been found to be the limiting factor. Approaches such as enzyme aided extraction have been successful but the use of large

concentration of enzyme in the process has been found to be uneconomical for the final product (Choudhari and Ananthnarayan, 2007). Studies have been conducted in solubilizing lycopene and were found that formulations made using nonionic surfactant (T80) showed higher solubilization of lycopene in organic/oil phase than in the systems without surfactant.

In this study, low temperature extraction of lycopene using cationic, anionic and nonionic surfactants has been conducted. Use of surfactant has been reported for extracting intracellular protein from whole cells (Kilikin *et al*., 2000). This aspect has been considered that surfactant may have more leaching ability to extract lycopene for its attached matrix.

It is seen from review of published literature that no attempt have been made for extracting lycopene using microemulsion based system.

3.2 Materials and Methods

3.2.1 Materials

All chemicals were of AR and/or GR grade. CTAB was purchased from SRL India, SDS from Qualigens, T 80 from Hi Media and AOT and Standard of lycopene (90 % purity) from Sigma Aldrich India. The chemicals/ solvents viz. n-hexane, propylene glycol, methanol, potassium hydroxide and precoated TLC plates of silica gel F_{254} were obtained from Merck India Ltd. The water used was deionized using Cascada water purification system (Pall Life Sciences). Fresh, red tomatoes were procured from local market.

3.2.2 Methods

3.2.2.1 Extraction of lycopene

Fresh, red tomato fruits (3 kg) were first cut into pieces and seeds were separated. The material was homogenized to form paste. The paste was extracted with n- hexane (1:1 w/w) for 2 h with constant stirring at 40-45°C in reaction assembly shown in Fig 2.1. The n-hexane layer was separated by using separating funnel and concentrated under vacuum below 50°C to obtain semisolid oleoresin. The oleoresin was further saponified for lycopene isolation. The block diagram for the procedure followed has been given as Fig 3.1.

3.2.2.2 Saponification of n-hexane extract

The oleoresin was mixed with saponification mixture containing 60 % propylene glycol, 20 % KOH prepared in 45 % methanol and 20 % water and kept at 65°C under gentle stirring for 30 min followed by n-hexane addition. The mixture was washed with warm water to remove saponified matter and excess propylene glycol and KOH. The lycopene crystals formed were filtered through Whatman filter paper no.1 and dried under vacuum. The saponification and washings were carried out in the apparatus **similar to as shown in Fig. 2.1.**

3.2.2.3 Extraction of lycopene by using reverse micelle system

The reverse micelle system based extraction of lycopene was carried out using different surfactants in apparatus as shown in Fig. 2.1.

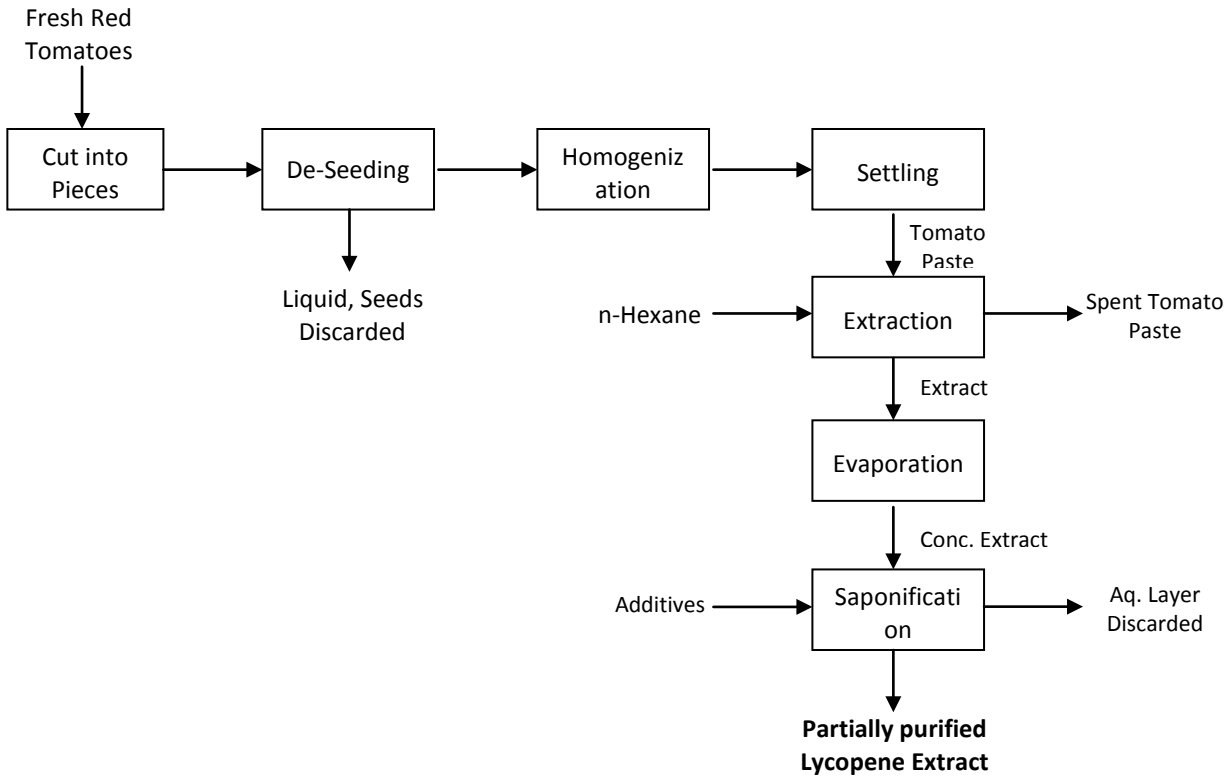
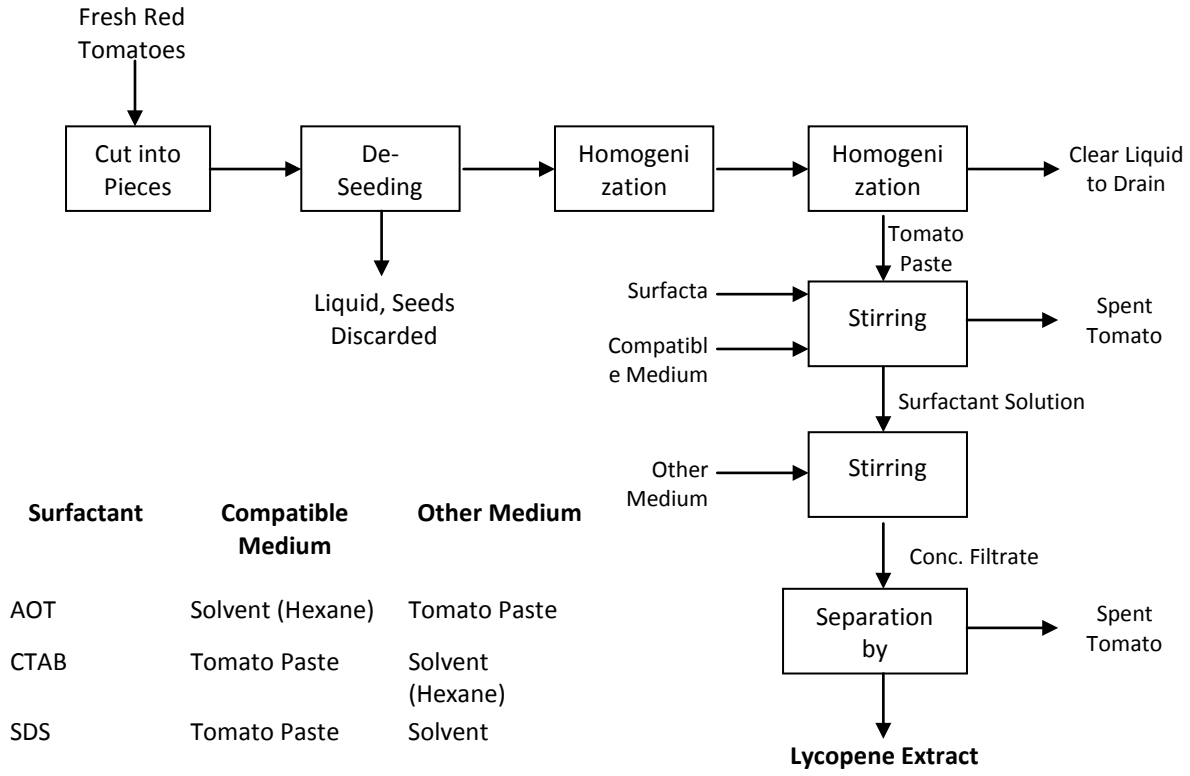


Fig. 3.1: Block diagram for lycopene extraction and partial purification, system without surfactant

Study of effect of various parameters viz. temperature (27-45°C), feed: solvent ratio (1:1-1:3) and concentration of surfactant was carried out one at a time keeping other conditions constant. Typically 100 g tomato paste (feed) was mixed with n-hexane (solvent). Surfactant viz. SDS, CTAB, T 80 and AOT were added in different concentrations (0.001 M – 0.025 M) one at a time keeping feed to solvent ratio

constant. The tomato paste-n hexane-surfactant mixture was kept at stated temperature for stated time period under constant stirring followed by separation of the two layers. The block diagram for the processing scheme has been given in Fig. 3.2. The organic layer (n-hexane extract) was further characterized quantitatively by TS and HPLC.



Surfactant	Compatible Medium	Other Medium
AOT	Solvent (Hexane)	Tomato Paste
CTAB	Tomato Paste	Solvent (Hexane)
SDS	Tomato Paste	Solvent

Fig. 3.2: Block diagram for lycopene extraction using microemulsion based system

3.2.2.4 Determination of total solid content

Total solids (TS) content was determined by the method described in AOAC, 1998 with slight modification where n-hexane extract was first evaporated to dryness on water bath (at 60°C) followed by drying at 60°C in hot air oven till constant weight was achieved. The results were expressed in % w/v or w/w.

3.2.2.5 HPLC analysis of lycopene

The HPLC analysis was carried out using Waters 2487 and BDS Hypersil C18, 250 x 4.6, 5 µm column. The standard of lycopene and samples were injected (50 µl) using isopropanol: methanol (60:40) as mobile phase at 215 nm at 25°C. The results of lycopene content were expressed in % (w/w).

3.2.2.6 TLC analysis of lycopene

TLC was carried out using precoated silica F_{254} plates. Samples were applied on the plates along with standard lycopene. The plates were developed in ethyl acetate: n-hexane (1:9) and the separated spots were observed visually.

3.2.2.7 Presentation of observations

The standard deviation for the results obtained was determined. Only those results for which the standard deviation was within 5 % were accepted as results of the experiment. At least 3 such results of each experiment were selected and arithmetic average (mean) was determined. All such mean values of the results were reported under the table of observations.

PART A – Extraction of lycopene without surfactant

3.3 Results and discussion

In the present investigation, one of the objectives was to evaluate and compare the performance of the systems without surfactant with the performance of the surfactant based system.

Results of investigation on systems without surfactant have been reported by Bortlik (2001) and Zuarro (2008). Bortlik (2001) have reported yield of 74 mg of lycopene for 1 kg of tomato paste. The method employed by them consisted of multistage crossflow type extraction using ethanol of different percentage strengths at each stage. Similarly the details of lycopene extraction process using apolar solvent (n-hexane), protic polar solvent (ethanol) and aprotic polar solvent (acetone) have been reported by Zuarro (2008). The yield of lycopene reported is 70 mg per 1 kg of tomato paste. Preliminary calculation indicated these yields (70-74 mg) were economically very expensive.

In view of the above, in the present investigation preliminary studies were carried out for extraction of lycopene without surfactant. As shown in Fig. 3.1, this involves extraction of lycopene from tomato paste by leaching, concentration of the extract, followed by saponification of the extracted crude lycopene.

3.3.1 Extraction of lycopene by leaching of tomato paste

The leaching of tomato paste was carried out in single stage extraction, using n-hexane. The n-hexane was selected as the solvent since it has low boiling point, which would reduce the thermal degradation of the product and the possibility of achieving lower level of residual solvent in the concentrated extract. During each experiment samples were drawn at interval of 30 min and analyzed to determine if the steady state was

reached. (If the results at 2.0 h and 2.5 h were identical, then 2.0 h was taken as time of reaching steady state condition).

To obtain comparable results for extraction without use of surfactants, as mentioned earlier, preliminary studies were carried out to evaluate effect of temperature and effect of solvent to tomato paste ratio on yield (% extraction) and purity of extracted lycopene. It was observed that both the parameters had negligible effect on the performance of the leaching/extraction operation. We obtained yield of about 160 mg lycopene per kg of tomato paste and purity of about 95 %. The yield and purity were found to be insensitive to temperature and solvent to tomato paste ratio. This could be attributed to inherently very low lycopene content of tomato paste. Although the value of purity (about 95 %) obtained was generally acceptable, the yield (160 mg), as indicated by preliminary calculations, was still economically unviable. Preliminary studies were also carried out for concentration and saponification of the n-hexane extract. There was improvement in purity to the level of about 98%. However, we observed up to 66% loss of lycopene yield during saponification. This could be attributed to degradation of lycopene during the process of saponification. The reduction in yield would further adversely affect the process economics.

Considering the above, detailed studies for system without surfactant, using n-hexane as solvent were not conducted and no detailed results are reported here for such studies, except for the general nature of results, as reported earlier.

PART B – Extraction of lycopene with surfactant

3.4 Results & Discussion

In micelle based systems the performance and hence efficacy of extraction process is, to a large extent, governed by the type of surfactant and its concentration. Hence the effect of type of surfactant and its concentration (0.001 M to 0.05 M) was studied for 3 surfactants viz. AOT (Table 3.1), CTAB (Table 3.2) and SDS (Table 3.3). During these studies other parameters such as temperature and solvent to tomato paste ratio were kept constant.

3.4.1 Effect of AOT and its concentration

The yield of lycopene (mg of lycopene/kg of tomato paste) increased with increase in AOT concentration up to 0.0125 M and then remained nearly unchanged at higher concentrations. The purity of lycopene obtained was nearly the same as that obtained in

system without surfactant. The nature of the results indicated that the selectivity of the solvent for lycopene was not affected by the presence and/or concentration of the surfactant. The results are given in Table 3.1 and Fig 3.3.

Table 3.1: Effect of AOT on lycopene extraction

AOT concentration, M	Total volume, ml	TS, g/l	Purity of lycopene, %	mg of lycopene/kg of tomato paste
0.001	173	0.06	95.23	333.17
0.0025	178	0.14	95.31	392.1
0.005	186	0.32	95.37	387.0
0.0125	189	0.45	95.33	402.2
0.0250	190	0.84	94.48	390.1

Extraction was carried out using 100 g tomato paste + 200 ml n-hexane as solvent at 25°C for 60 min.

The distribution coefficient was however, affected significantly in presence of surfactant and an increase in yield from 160 mg to more than 300 mg was observed.

There was no published information available for effects of surfactant in case of multicomponent system such as the one from which lycopene is obtained. The marked improvement in distribution coefficient in presence of surfactant could be attributed to effect of surfactant on solubility of lycopene and other impurities. Interaction of surfactant molecule with one or more impurities may have lead to formation of new moieties which had lower solubility in solvent as a result of which the selectivity for lycopene was favorably affected.

Another relevant aspect in case of AOT was that AOT being soluble in organic solvent phase (and not in aqueous phase) it would remain in the n-hexane extract. To the best of our knowledge there is no published data or information available on applications of lycopene containing AOT.

However formulations containing AOT with other active pharmaceutical ingredients have been reported for some topical applications (Paul and Maulik, 2001). For applications in cosmetics presence of AOT in lycopene used has been considered to be giving beneficial results. Therefore, even if the enriched lycopene contains traces of AOT, it would be commercially acceptable.

The best results were obtained for 0.0125 M AOT, where total extractability was much higher than the extraction without surfactant, though the % purity was very less.

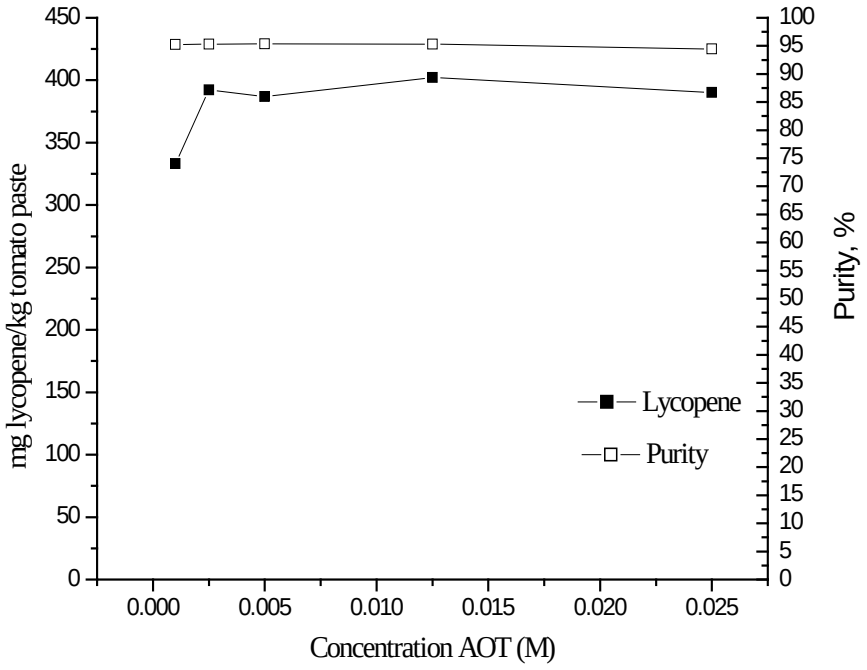


Fig. 3.3 Effect of AOT on lycopene extraction

3.4.2 Effect of CTAB and its concentration

Increase in concentration of CTAB increased the yield of lycopene to about 230 mg at concentration of 0.0025 M. The results are given in Table 3.2. The purity improved marginally further to approximately 98 %. At higher concentration, the mixture of tomato paste and solvent (n-hexane) formed an emulsion which did not separate into two phases, which was necessary for recovery of the lycopene.

This phenomenon could be due to the nature of impurities present in the tomato paste. One of the important aspects to be considered in case of CTAB was that while AOT was soluble in organic solvent phase (and not in aqueous phase), CTAB has been reported to be insoluble in solvent (n-hexane) phase. Therefore, CTAB would remain in the water phase and would not get carried over into the extract and further into lycopene.

For nutraceutical applications lycopene free of surfactant and other impurities is required. Therefore use of CTAB for lycopene extraction would be advantageous for

such applications. Best results were observed at CTAB concentration of 0.0025M. Increase in lycopene recovery with the surfactant concentration upto 0.0025M may be explained by the fact that increase in surfactant concentration increased the surfactant aggregation and reverse micelle formation (Pessoa Jr and Vitolo, 1998), which in turn enhanced extraction. Further increase in the surfactant concentration caused micellar clustering, decreasing the interfacial area available to target molecule, which resulted, in decrease in the extraction capacity of reverse micelle. In addition, the intra-micellar collision may occur because of large population of micelle (Tonova and Lazarova, 2008), resulting in the deassembling/deformation of reverse micelle, leading to decreased extraction (Hebber *et al*., 2007).

Table 3.2: Effect of CTAB on lycopene extraction

Concentration, M	Total volume, ml	TS, g/l	Purity of lycopene, %	mg of lycopene/kg of tomato paste
0.001	197	0.04	96.57	191.73
0.0025	193	0.03	98.08	229.71
0.0125	127	0.03	95.34	41.7

Extraction was carried out using 100 g tomato paste + 200 ml n-hexane as solvent at 25°C for 60 min.

3.4.3 Effect of SDS and its concentration

The purity of lycopene extracted as well as the yield of extraction was nearly independent of SDS concentration.

Table 3.3: Effect of SDS on lycopene extraction

Concentration, M	Total volume, ml	TS, g/l	Purity of lycopene, %	mg of lycopene/kg of tomato paste
0.001	198	0.03	95.56	216.43
0.0025	183	0.03	96.17	195.0
0.0125	32.6	0.1	98.15	31.20

Extraction was carried out using 100 g tomato paste + 200 ml n-hexane as solvent at 25°C for 60 min.

The results in Table 3.3 indicated that both selectivity and distribution coefficient of the solvent were not affected by the surfactant (SDS) concentration. Secondly, both the purity (about 95 %) and yield (about 200 mg) were inferior to best results obtained with

AOT. At SDS concentration higher than 0.0125 M, the mixture of tomato paste and solvent (n-hexane) formed an emulsion which did not separate into two phases, which was necessary for recovery of the lycopene. All these considerations put together did not favour use of SDS as surfactant for the system under investigation.

In view of the above results, further experimental work was carried out using AOT and CTAB as surfactants.

3.4.4 Effect of significant parameters in presence of surfactant (AOT and CTAB)

The effect of other significant process parameters in presence of surfactants viz. CTAB (0.025 M) and AOT (0.0125 M) were studied to determine the conditions for improved performance. The parameters studied were temperature and solvent to paste ratio. The results are given in tables as described in the following part.

3.4.4.1 Effect of contact time for lycopene extraction using CTAB and AOT

Effect of contact time on recovery of component of interest in reverse micelle systems has been reported by many investigators (Liu *et al*., 2006). The component of interest, in case of most of these reports, was a protein or an enzyme. Liu *et al.* (2006) have mentioned that contact time should usually be as short as possible to achieve maximum activity recovery. They have further mentioned to the effect that the phase equilibrium may not be totally achieved. As suggested by Shelly (1996), this could be attributed to the effect of surfactant to varying extents on rate of mass transfer for component of interest and for other components present in the feed. This aspect was not investigated during the present study. As mentioned, only those results obtained at the steady state with respect to the component of interest were considered for further analysis or discussion. For the system under investigation, in presence of surfactants, the steady state conditions were reached after 45 min. Therefore, for all further studies contact time was kept at 45 min.

3.4.4.2 Effect of temperature for lycopene extraction using CTAB

The effect of temperature (25 to 45°C) was studied, at CTAB concentration of 0.025 M and the results have been given in Table 3.4. The purity of lycopene was nearly unaffected for temperature upto 40°C and declined at higher temperature of 45°C. The yield of lycopene showed peak values at temperature of 40°C and declining trend at higher temperatures (Fig 3.4).

Table 3.4: Effect of temperature on lycopene extraction using CTAB

Temperature, °C	Total volume, ml	TS, g/l	Purity of lycopene, %	mg of lycopene/kg of tomato paste
25	193	0.03	98.08	229.71
30	189	0.03	98.68	290.42
35	184	0.031	97.46	220.42
40	178	0.032	96.42	208.43
45	174	0.032	82.11	188.02

Extraction was carried out using 100 g tomato paste + 200 ml n-hexane as solvent and CTAB (0.025 M) at 25°C for 45 min.

The observed behavior of purity indicated that higher temperature had adverse effect on selectivity, which could be attributed to change in solubility behavior of impurities in presence of surfactant. In terms of the yield, the temperature of 30°C was seen to be optimum for the lycopene yield for the system under investigation, which could be due to the degradation of lycopene at higher temperatures. Shi and Maguer (2000) reported loss of lycopene upto about 28 % in n-hexane extract when exposed to temperature of 65°C for 3 h. They have also reported higher extent of degradation at still higher temperatures investigated by them (up to 135°C).

Another aspect may be that w/o microemulsions have been reported to be prevalent at lower temperatures, when ionic surfactants were used (Maulik and Paul, 1998). The distribution coefficient for transport of lycopene from non-organic phase (tomato paste) to organic phase is high when w/o microemulsion is formed. The best results were obtained at 30°C. This could be attributed to predominant presence of w/o reverse micelle.

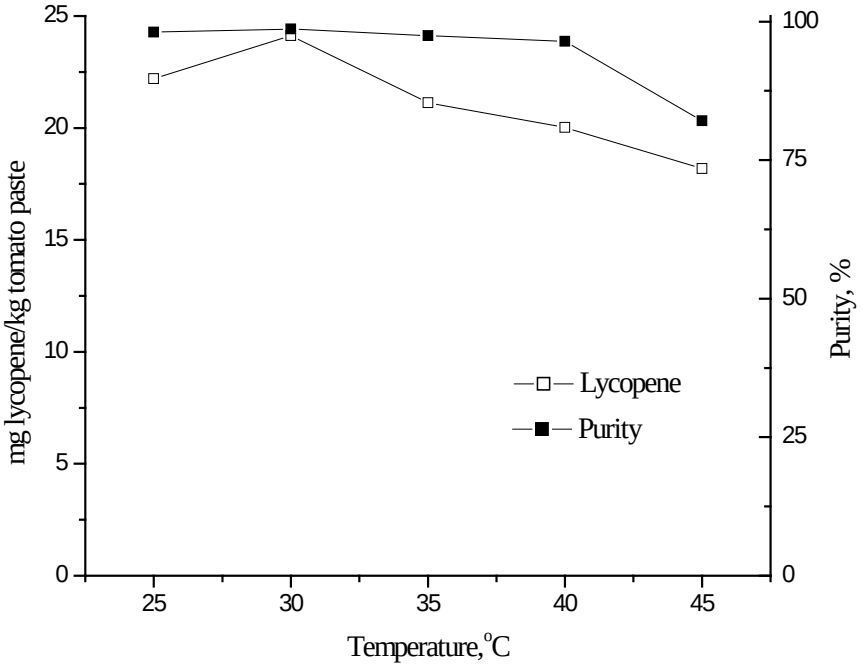


Fig. 3.4: Effect of temperature on lycopene extraction using CTAB

3.4.4.3 Effect of solvent to tomato paste ratio for lycopene extraction using CTAB

The effect of solvent to tomato paste ratio (ml/g) was studied for the same CTAB concentration (0.025 M). The results have been given in Table 3.5. The purity of lycopene remained nearly unchanged with increase in the ratio from 1:1 to 3:1 (about 99 %). The yield showed increase from 190 mg to 290 mg, for increase in the ratio from 1:1 to 2:1. Further increase in the ratio upto 3:1 did not show any appreciable change (Fig 3.5).

Table 3.5: Effect of solvent to tomato paste ratio (v/w) on lycopene extraction using CTAB

solvent: tomato paste	Total volume, ml	TS, g/l	Purity of lycopene, %	mg of lycopene/kg of tomato paste
1:1	74	0.034	98.70	190
2:1	190	0.029	98.68	290
3:1	290	0.021	98.68	278

Extraction was carried out using 100 g tomato paste + 200 ml n-hexane as solvent and CTAB (0.025 M) at 30°C for 45 min.

The behavior of the purity indicated that the ratio did not affect the selectivity of the solvent. The behavior of the yield indicated that the distribution coefficient of the solvent for lycopene was influenced by the solvent to tomato paste ratio, which could be due to the limited solubility of lycopene in the solvent (n-hexane), for the system under investigation. The limiting condition (in terms of solubility) was reached at the ratio of 2:1 and the yield remained unaffected at higher ratios.

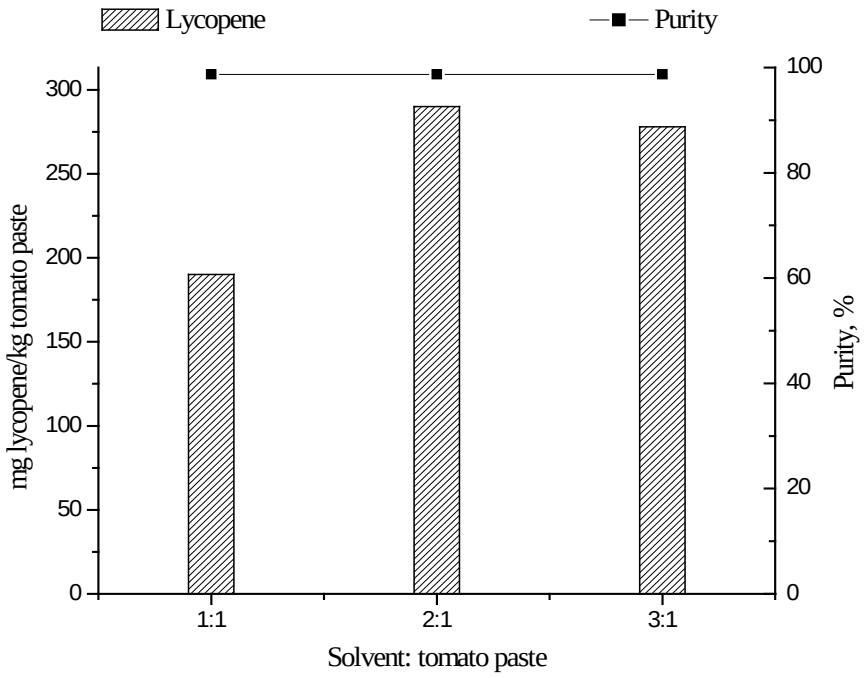


Fig. 3.5: Effect of solvent to tomato paste ratio on lycopene extraction using CTAB

3.4.4.4 Effect of temperature on lycopene extraction using AOT

The effect of temperature (25 to 45°C) was studied, at AOT concentration of 0.0125 M (**Table 3.6**). The purity and yield of lycopene, both showed, moderately declining trend with increase in temperature (Fig 3.6).

The purity and yield, as observed, indicated the adverse effect of higher temperature on selectivity and distribution coefficient. This behavior may be considered to be somewhat similar to the one observed in case of CTAB, as described earlier. AOT being an ionic surfactant, at higher temperatures may form o/w microemulsions, while at lower temperatures the w/o microemulsions would be predominant. That may be a

reason for higher transfer of lycopene to organic phase at room temperature. The effect, as observed in case of AOT, was rather moderate. This could be attributed to change in solubility behavior of impurities in the organic (solvent) phase, the phase in which AOT was soluble.

Table 3.6: Effect of temperature on lycopene extraction using AOT

Temperature, °C	Total volume, ml	TS, g/l	Purity of lycopene, %	mg of lycopene/kg of tomato paste
25	185	4.5	96.33	443
30	184	4.56	94.33	442
35	183	4.6	92.12	420
40	180	4.8	90.10	390
45	177	4.9	84.11	340

Extraction was carried out using 100 g tomato paste + 200 ml n-hexane as solvent and AOT (0.0125 M) for 45 min.

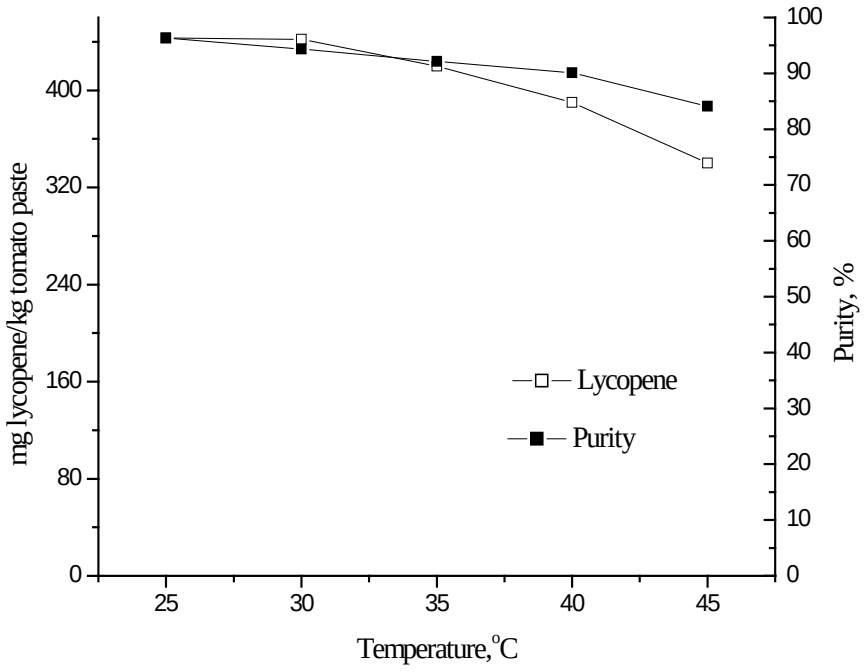


Fig. 3.6: Effect of temperature on lycopene extraction using AOT

3.4.4.5 Effect of solvent to tomato paste ratio for lycopene extraction using AOT

The effect of solvent to tomato paste ratio (ml/g) was studied for the AOT concentration of 0.0125 M (Table 3.7).

Table 3.7: Effect of solvent to tomato paste ratio (v/w) on lycopene extraction using AOT

Solvent to tomato paste ratio, v/w	Total volume, ml	TS, g/l	Purity of lycopene, %	mg of lycopene/kg of tomato paste
1:1	85	8.7	96.4	370
2:1	186	4.5	95.31	443
3:1	289.5	2.0	95.37	442
4:1	390	1.9	94.34	420
5:1	489	1.4	94.30	415

Extraction was carried out using 100 g tomato paste + 200 ml n-hexane as solvent and AOT (0.0125 M) at 25°C for 45 min.

The purity of lycopene remained nearly unchanged (about 95 %) with increase in the ratio from 1:1 to 3:1. The yield showed increase from 370 mg to 440 mg, for increase in the ratio from 1:1 to 3:1. As shown in Fig 3.7, further increase in the ratio up to 3:1 did not show any appreciable change.

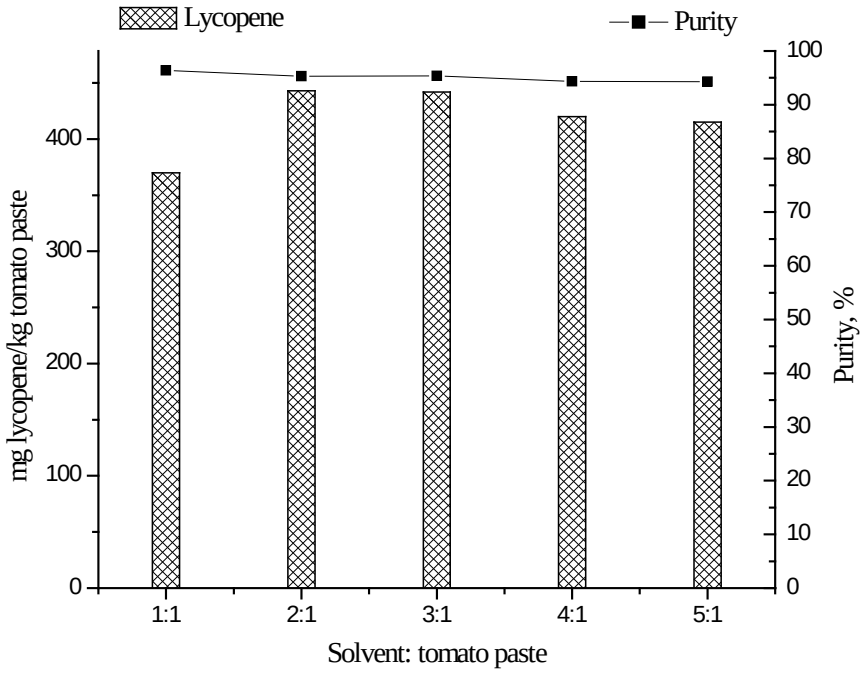


Fig. 3.7: Effect of solvent to tomato paste ratio on lycopene extraction using AOT

The behavior of the purity indicated that the ratio did not affect the selectivity of the solvent. The higher yield indicated that the distribution coefficient of the solvent for lycopene was influenced by the solvent to tomato paste ratio. This could be attributed to lycopene having finite and limited solubility in the solvent (n-hexane), for the system under investigation. The limiting condition (in terms of solubility) was reached at the ratio of 2:1. As such, the yield remained unaffected at higher ratios. The highest yield of lycopene with AOT was 443 mg per kg of tomato paste, with purity of 95.31 % when extraction was carried out at 30°C and at ratio of V_{aq} to V_{RM} was 1:2. The higher quantity of organic phase gave higher concentration of micelle which may be a reason for higher transfer of lycopene with increase in the organic phase.

3.5 Conclusion

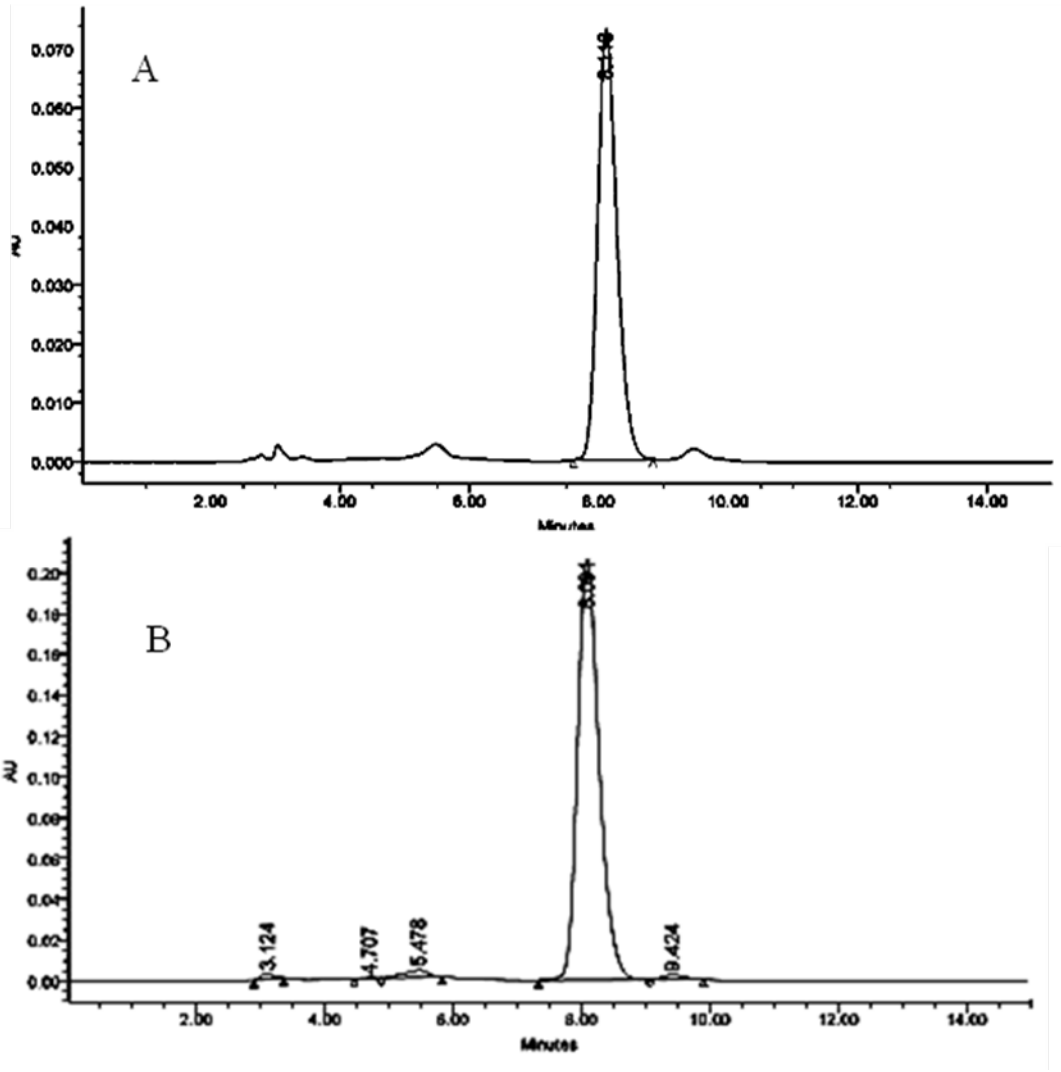


Fig 3.8 A. Standard lycopene **B**. Extraction of lycopene using surfactant

Table 3.8: Summary of purity and yield of lycopene

	Purity	**Yield** mg/kg tomato paste
System without surfactant	95 %	160
System with AOT (0.0125 M)	95 %	440
System with CTAB (0.025 M)	99 %	290

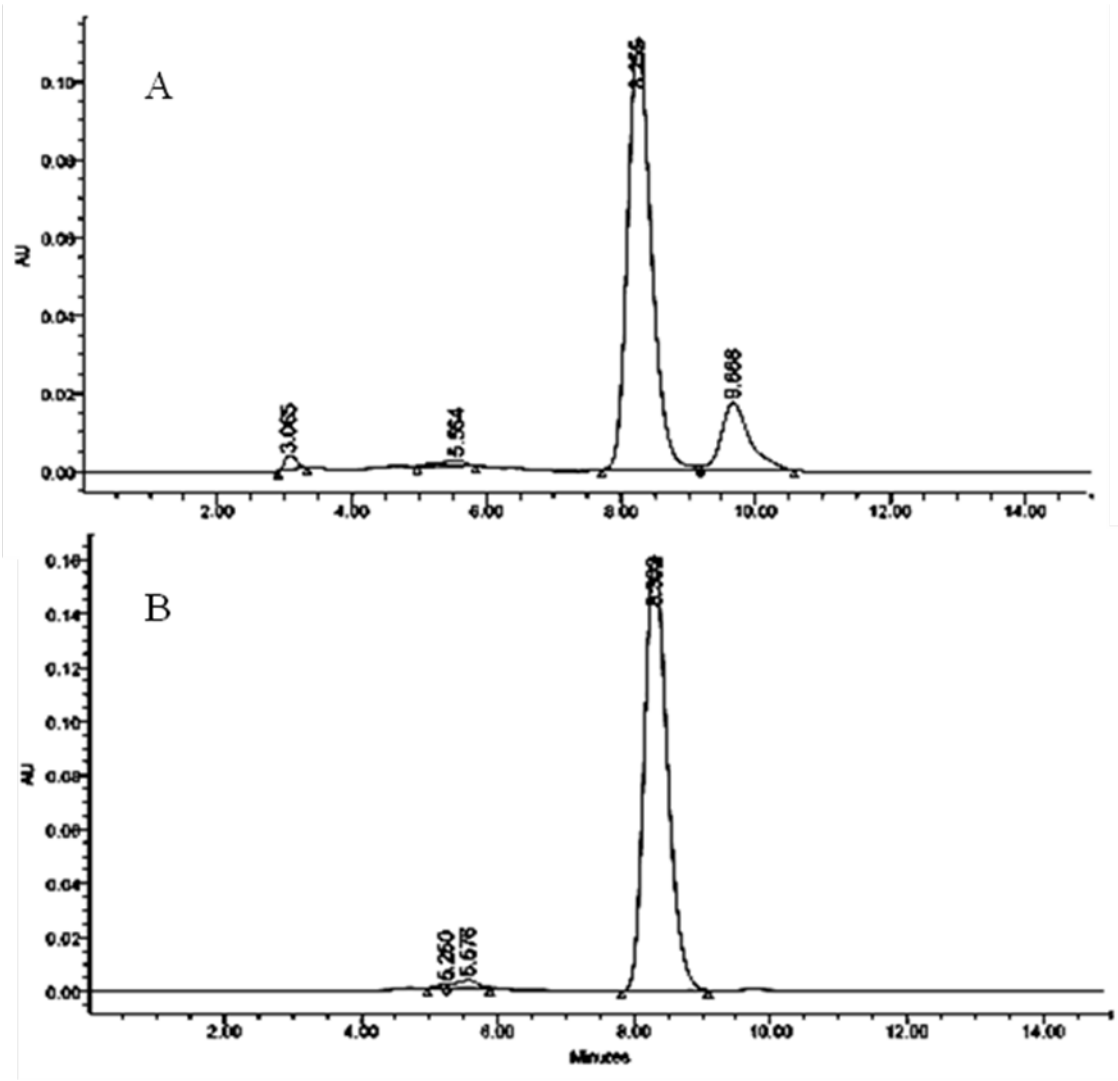


Fig 3.9 A. Crude lycopene extract in n-hexane **B**. Saponified lycopene

Table 3.8 has shown the comparative results of lycopene extraction using system without surfactant and system with surfactant. It is evident from HPLC chromatograms (**Figs 3.8**) that the lycopene yield is much greater in systems with surfactant

than that in systems without surfactant (Fig 3.9). Yield in case of AOT is maximum (440 m/kg of tomato paste) but purity (95 %) is inferior to that with CTAB (purity 99 %). The comparison with the conventional system (extraction and enrichment by saponification) showed that the micelle based system in a single stage operation yields significant increase in yield of lycopene.

3.6 Process economics considerations

The use of n-hexane as solvent has given considerable improvements in terms of yields as well as purity of lycopene. However, the yields obtained without use of surfactants are low and hence the process cost is high. The yields obtained with use of AOT were almost 1.5 times those obtained by use of CTAB, with purity comparable to the conventional process. On the other hand, the purity obtained by using CTAB was superior to that obtained conventional methods. Preliminary calculations indicated that the yields obtained with use of CTAB as well as AOT were economically viable due to higher yields, lesser extraction time, energy efficient process and lower quantities of solvent required (Feed to Solvent Ratio of 1:2 as against 1:6 for conventional process) The product grade obtained by using AOT as surfactant can find use in cosmetics topical applications and the product grade obtained by using CTAB as surfactant can find use in nutraceutical applications.

3.7 References

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CHAPTER 4

Extraction and enrichment of 11α-hydroxy canrenone (11 OHC) from fermentation broth using microemulsion /reverse micelle

4.1 Introduction

Steroid biotransformation essentially involves the conversion of water insoluble substrate to its derivative(s) in presence of microorganism and/or its enzyme(s). The bio transformed intermediates/ products have number of potential applications in the healthcare industry. 11 hydroxy canrenone is one of the major biotransformation intermediates used as a starting molecule for manufacturing of eplerenone – a cardiac drug. Canrenone gets oxidized and converted to its hydroxyl derivative at 11^{th} position as 11 hydroxy canrenone (11-OHC) in presence of microorganisms especially *A. ochraceous*, *Aspergillus niger*, *Rhizopus stolonifer*. The reported range of canrenone concentration in the fermentation was 11 g to 40 g/L while the conversion efficiency was 90-95 % (Wiersma and Meijden, 2000). Steroidal bioconversion processes suffer from some drawbacks such as lengthy time cycles for bio-conversion step (120 to 240 h) and hence more prone to contamination, impact of particle size of substrate on bioconversion, need for precise control of process parameters (for nutrient addition, pH monitoring) and tedious downstream processing. All these tend to make the process economically unviable (Fernandes *et al.*, 2003). In the bioconversion of canrenone though the substrate gets converted to its hydroxyl derivatives, the possibility of selective extraction of the derivative (11-OHC) is very low. This is because of the structural similarity between substrate and its product. In the extraction, because of similar solubility profile in the extracting solvent, both product and substrate get leached out in equal proportion. This requires another unit operation of preparative chromatography which is a tedious process and rather difficult to scale up.

The focus of the present investigation was the separation of structurally similar canrenone and 11 hydroxy canrenone from each other through single step extraction. To best of our knowledge there were no reports on selective extraction of hydroxylated steroids from the fermentation broth. This chapter describes work on the separation of

these two molecules and their yields by conventional method as well as by microemulsion.

4.2 Materials and Methods

4.2.1 Materials

All chemicals viz. dextrose, yeast extract, KH_2PO_4, $MgSO_4$, NH_4NO_3, CTAB, SDS and T80 were of AR/ LR grade and procured from Merck India, SRL and Hi Media. AOT was purchased from Sigma Aldrich India. Canrenone and standard of 11 hydroxy canrenone was a kind gift from Glenmark Pharmaceuticals. The solvents viz. n-hexane, ethyl acetate and precoated TLC plates of silica gel F_{254} were obtained from Merck India Ltd. The water used was deionized using Cascada water purification system (Pall Life Sciences).

4.2.2 Methods

4.2.2.1 Organism

Aspergillus ochraceous NCIM 1140 was used for biotransformation of canrenone to 11 hydroxy canrenone. The organism was maintained on slants of potato dextrose agar at 4°C and routinely subcultured every 30 days.

4.2.2.2 Production of *A.ochraceous* mycelial mass

The spore suspension of *A.ochraceous* was prepared in sterile T80 water, inoculated in potato dextrose broth (potato infusion, 200 g; dextrose, 10 g per L; pH 5.6 – 6.0) and incubated at 28°C and at 180 rpm for 48 h to prepare seed. The seed (12 % (v/v)) was then inoculated in the (300 ml) optimized biotransformation medium (dextrose, 40 g; yeast extract, 15 g; KH_2PO_4, 5 g; $MgSO_4$, 1 g; NH_4NO_3, 5 g per L; pH 5.5) and incubated at 28°C and at 180 rpm for a period of 60 h to achieve 30 % (w/v) packed mycelial volume (PMV).

4.2.2.3 Biotransformation of canrenone

Substrate, canrenone (3 g) was prepared in 10 ml water-ethyl alcohol (7:3) containing 0.1 % T 80 water (w/v). Canrenone was added into the 60 h old fermentation broth as a single dose and incubated further at 28°C at 180 rpm till the desired conversion. Solutions of dextrose (20 %, w/v), yeast extract (10 %, w/v) and KH_2PO_4 (0.05 %) were added periodically every 8 h. The PMV of *A. ochraceous* was maintained at 35 % (w/v)

±3 and pH was maintained between 5.0–6.0 throughout the biotransformation period. Samples were withdrawn every 24 h and checked for cell morphology, pH, PMV, total sugar content, total nitrogen content and conversion (by TLC and HPLC).

4.2.2.4 Downstream procedure to recover 11 OHC

When 70 % to 80 % conversion was observed, the cell mass was separated from fermentation broth by filtration. The separated solids [biomass] and cell free broth were processed as per the scheme shown in Fig. 4.1. The cells and cell free broth were extracted separately in ethyl acetate in 1:3 proportion at room temperature under constant stirring conditions for 2 h using experimental set up shown in Fig 2.1. The organic layer from both the extractions was separated from aqueous layer, pooled and concentrated in rotary vacuum evaporator to obtain powdered mixture of 11-OHC and canrenone. The aqueous layer was checked for presence of 11-OHC and discarded.

4.2.2.5 Extraction of 11 OHC by using microemulsion system

The microemulsion system based extraction of 11-OHC was carried out using different surfactants. The production of mycelial mass of *A. ochraceous* and biotransformation of canrenone was carried as described in section 4.2.2.3. The feed broth was prepared as per procedure shown in Fig. 4.1. Surfactant solution was prepared by adding the surfactant to appropriate phase, as shown in Fig. 4.2. The extraction with surfactant solution (micelle system) was carried out in experimental set-up as shown in Fig. 2.1. Further processing after extraction was done as shown in Fig. 4.2. The organic layer was further characterized quantitatively by TS and HPLC as described in section 4.2.2.6.

4.2.2.6 Analytical procedures

Determination of PMV

A sample containing mycelia were taken (10 ml) in graduated centrifuge tubes. The samples were centrifuged at 3000 g for 30 min. The supernatant and packed mycelia were measured and PMV was calculated as follows:

(Initial volume of sample – Final volume of supernatant) ÷ Initial volume of sample x 100

Determination of total solid content

Total solids (TS) was determined by the method described in AOAC, 1998 with slight modification whereby n-hexane extract was first evaporated to dryness on water bath (at 60°C) followed by drying at 60°C in hot air oven till constant weight was achieved. The results were expressed in % w/v or w/w, as applicable.

HPLC of 11-OHC and residual canrenone

The HPLC analysis was carried out using Waters 2487 and Inertsil ODS 3V C18, 250x4.6, 5 µm column. The standards of 11-OHC and canrenone and samples were injected (50 µl) using acetonitrile: buffer (0.05 % o-phosphoric acid in distilled water) (60:40) as mobile phase, absorption at 283 nm at 25°C. The results of 11 hydroxy content and residual canrenone were expressed in % (w/w).

TLC analysis of 11-OHC and residual canrenone

TLC was carried out using precoated silica F_{254} plates. Samples were applied on the plates along with standards of 11-OHC and canrenone. The plates were developed in ethyl acetate: n-hexane (1:9) and the separated spots were visualized by iodine fume.

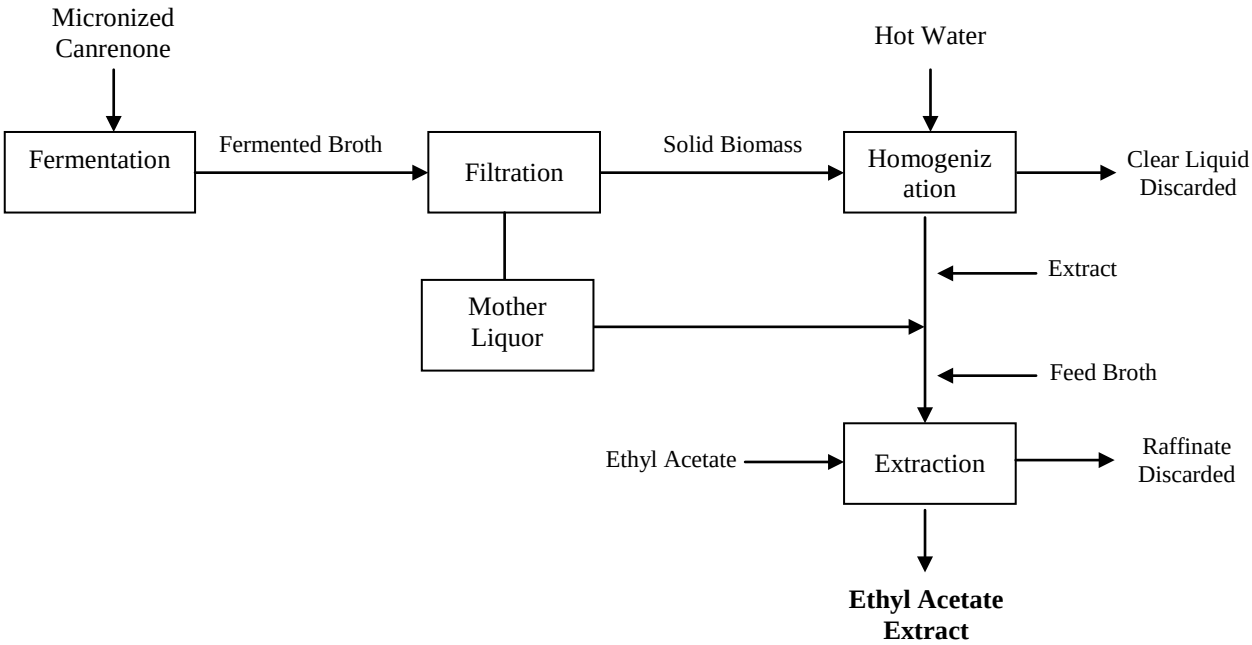
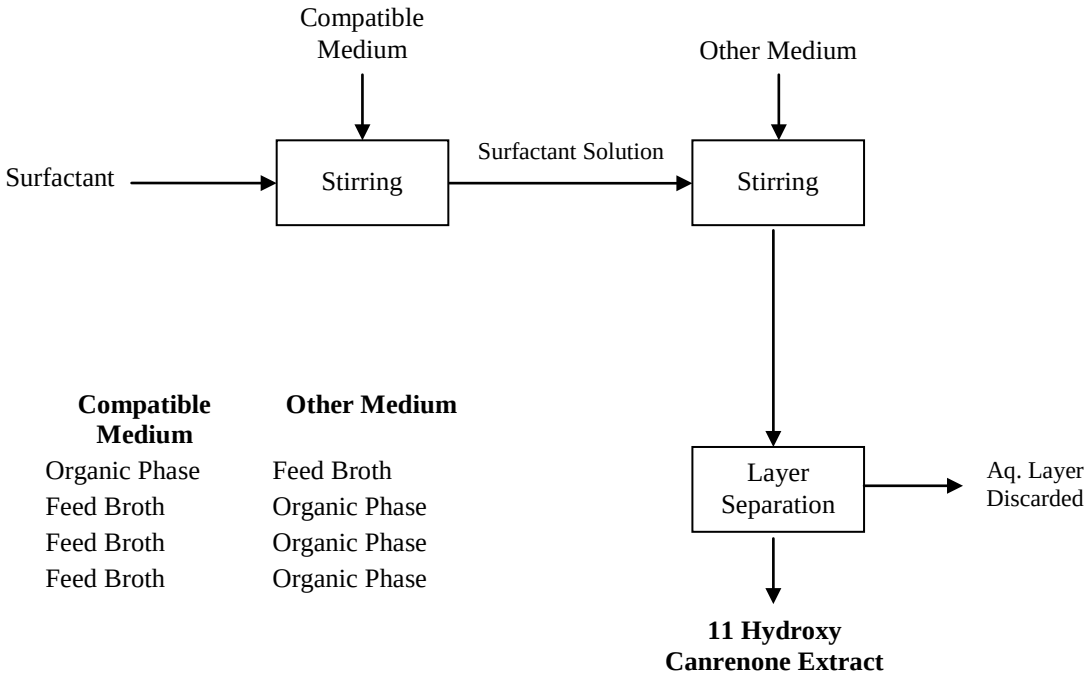


Fig 4.1: Schematic diagram for extraction of 11 OHC, without surfactant



Surfactant	Compatible Medium	Other Medium
AOT	Organic Phase	Feed Broth
CTAB	Feed Broth	Organic Phase
SDS	Feed Broth	Organic Phase
Tween 80	Feed Broth	Organic Phase

Fig. 4.2: Block diagram for 11 OHC extraction using surfactant

PART A: Extraction of 11-OHC without surfactant

4.3 Results & Discussion

In the present investigation, one of the objectives was to evaluate and compare the performance of the systems without micelle with the surfactant based system. Accordingly, studies were carried out to evaluate the best possible performance of the system without micelle. As shown in Fig. 4.1, this involved extraction of 11-OHC from the feed broth by ethyl acetate. In the conventional system understood to be employed on industrial scale operation, the ethyl acetate extract was concentrated and cooled to obtain 11-OHC with purity 90 % and higher by crystallization. The purity of the crystallized 11-OHC was constrained by the purity of 11-OHC in the fermentation broth. Ng *et al.* (2002 and 2005) have reported use of ethyl acetate as solvent to extract various steroids including canrenone from mycelia as well as cell free broth.

4.3.1 Extraction of 11-OHC from the feed broth by ethyl acetate

The extraction from the feed broth was carried out by single stage extraction process. In all the experiments, 100 g feed broth, duly characterized for TS and contents of 11-OHC and canrenone was used as starting material and extracted with ethyl acetate. Samples were drawn at 30 min interval to ascertain the reaching of steady state (if the

results at 1.5 h and 2.0 h were identical, then 1.5 h was taken as time of reaching steady state). The performance of such extraction operation, for a given solvent, is typically governed mainly by two parameters viz. temperature and solvent to feed broth ratio. The effect of these parameters was evaluated and the results are given in Tables 4.1 and Table 4.2.

4.3.2 Effect of temperature

The effect of temperature was studied over a range of 27°C to 75°C, the latter being referred to as the reflux temperature (boiling temperature of solvent at the experimental conditions). The results have been given in Table 4.1. It can be seen that the purity of 11-OHC in extract was nearly independent of the temperature. The % extraction showed significant increase from 11 % to 89 % with increase in temperature from 27°C to reflux temperature (75°C) (Fig 4.3).

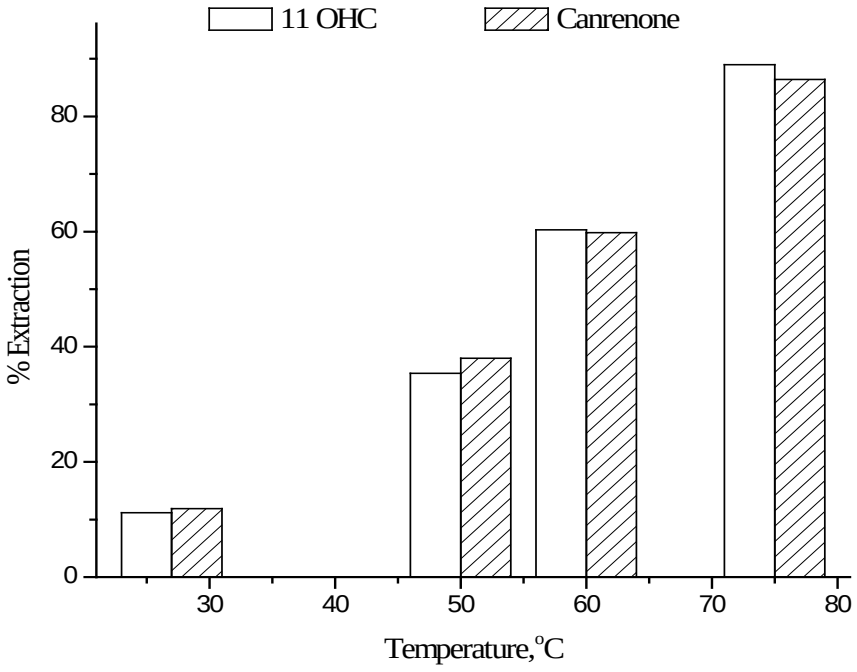


Fig. 4.3: Effect of temperature on extraction of 11 OHC

The observed purity may be due to the selectivity of the solvent, as discussed earlier. The % extraction indicated that the solubilities of 11-OHC as well as canrenone for the given system, increased with temperature. The sharp increase in solubility with

temperature makes 11-OHC amenable to recovery and purification by the route of crystallization by cooling.

4.3.3 Effect of solvent to feed broth ratio (v/v)

The effect of this ratio was studied over the range 1:1 to 6:1. The results have been given in Table 4.2. It can be seen that purity of 11-OHC in extract is nearly independent of the ratio (about 72 %). The % extraction of 11-OHC increased from 62 % to 90 % as the ratio was increased from 1:1 to 4:1. Further increase in the ratio upto 6:1 did not lead to any further improvement (Fig. 4.4).

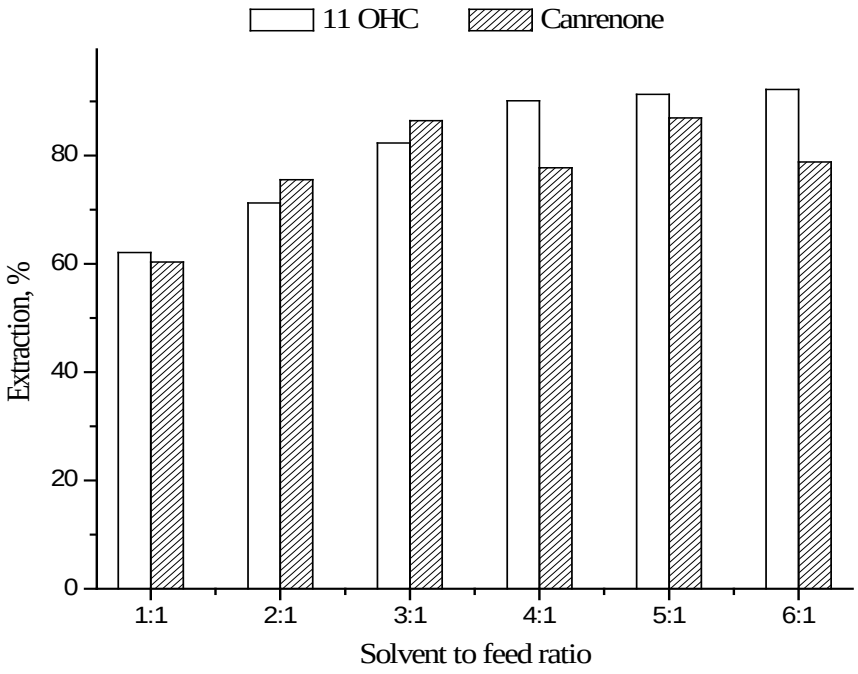


Fig. 4.4: Effect of solvent to feed ratio (v/w) on extraction of 11 OHC

Like many natural systems, the feed broth was a multicomponent system. Analysis of number and content of respective component, other than component of interest (11-OHC and canrenone in this case) was not a feasible proposition. The purity of 11-OHC was unaffected by the solvent to feed ratio indicates that the selectivity of the solvent (ethyl acetate), to 11-OHC on one hand and canrenone and other impurities on the other hand was independent of the concentration. The yield of 11-OHC was observed to be highest at the ratio of 4:1 (extraction 90 %) and unchanged thereafter at higher ratios.

This indicated the independence of distribution coefficient at higher concentration. In case of systems wherein the solvent and feed were immiscible, the % extraction was given by the expression, % extraction = km/(1+km), where m is distribution coefficient and k is ratio of solvent to feed. Yield unchanged at higher ratios is therefore indicated dependence of distribution coefficient (m) on concentrations in extract and raffinate.

Table 4.1: Effect of temperature on 11-OHC extraction

(Reflux) Temperature, °C	Total volume, ml	TS, g/l	TS, g	Purity of 11 –OH canrenone, %	Total 11 – OH canrenone, g	Residual canrenone %	Residual canrenone total g	% extraction	
								11 OH canrenone	canrenone
27	390	0.284	0.111	74.2	0.082	20.1	0.022	11.2	11.9
50	390	0.910	0.355	73.1	0.260	19.6	0.070	35.4	38.0
60	385	1.558	0.600	74.1	0.444	18.4	0.110	60.3	59.8
75	385	2.330	0.897	73.1	0.655	17.7	0.159	89.0	86.4

100 ml broth of 1.90 g TS was used as feed. Feed broth contained 7.36 g/l 11 OH canrenone and 1.84 g/l canrenone. Extraction was carried out using ethyl acetate as a solvent for 3 h. Solvent to feed ratio was 4:1 (v/v).

Table 4.2: Effect of solvent to feed ratio (v/w) on 11 OHC extraction

Solvent to feed ratio, v/v	Total volume, ml	TS, g/L	TS, g	Purity of 11 OHC, %	Total 11 OHC, g	Residual canrenone %	Residual canrenone, g	% extraction	
								11 OHC	canrenone
1:1	96	6.417	0.616	74.1	0.456	18.1	0.111	62.1	60.33
2:1	191	3.644	0.696	75.2	0.522	20.1	0.139	71.2	75.54
3:1	286	2.926	0.837	72.2	0.603	19.1	0.159	82.3	86.41
4:1	385	2.324	0.895	74.1	0.662	16.2	0.143	90.1	77.71
5:1	475	1.985	0.943	71.2	0.670	17.1	0.160	91.3	86.95
6:1	520	1.860	0.967	70.1	0.677	15.2	0.145	92.2	78.80

100 ml broth of 1.90 g TS was used as feed. Feed broth contained 7.36 g/l 11 OH canrenone and 1.84 g/l canrenone. Extraction was carried out using ethyl acetate as a solvent at 75°C (reflux temperature) for 3 h.

Table 4.3: Effect of CTAB on 11 OHC extraction

Concentration, M	Volume, ml	TS g/L	Purity 11 OHC, %	Total 11 OHC, g	Residual Canrenone %	Total g	% extraction	
							11 OHC	Canrenone
0.001	97	1.12	52.12	0.012	0.28	0.0020	16.30%	1.08%
0.005	96	2.16	53.62	0.125	0.89	0.0066	16.98%	3.5%
0.025	96	5.46	52.42	0.218	1.06	0.0079	29.63%	4.2%
0.050	95	6.64	58.29	0.386	1.42	0.0107	50%	5.8%
0.10	* 40	9.42	59.12	0.248	1.62	0.120	33.6%1	65.1%

100 ml broth at 1.90 g % TS was used as feed. Feed broth contained 7.36 g/l 11 OH'C and 1.84 gm/lit Canrenone. Extraction was carried out using ethyl acetate as a solvent for 2 h at 75 ^{0}C (reflux temp). Solvent to feed ratio was 1:1 (v/v). *The phase separation was not very clear and hence the results may be erroneous.

Part B – Extraction of 11 OHC using surfactant

4.4 Results and Discussion

In the surfactant based systems, the performance, and hence efficacy of extraction process was, to a large extent, governed by the type of surfactant and its concentration. In view of this, the effect of surfactant and surfactant concentration (0.001 M – 0.05 M) was studied for 3 surfactants viz. T 80, CTAB (Table 4.3) and SDS (Table 4.4). During these studies all other parameters viz. temperature, contact time and solvent to feed broth ratio were maintained constant.

4.4.1 Effect of T 80 and its concentration

In case of T 80, interface matrix to a considerable extent was formed at a surfactant concentration higher than 0.05 M. At lower concentration, the total solids extracted were very low and 11-OHC and canrenone ratio in extract was nearly same as that in the feed broth. As such, there was no added advantage from the use of this nonionic type of surfactant.

4.4.2 Effect of CTAB and its concentration

It can be seen from Table 4.3 that % extraction of 11-OHC increased with increase in concentration of CTAB, reaching a highest value of 50 % for concentration of 0.05 M. The corresponding % extraction of canrenone also showed a similar trend. As discussed later, the extraction of 50 % was too low, making the operation economically unviable.

4.3 Effect of SDS and its concentration

The purity of extracted 11-OHC increased steadily from 85 % to 92 %, with increase in concentration of SDS from 0.01 M to 0.06 M (Table 4.4). The variation in the % extraction of 11-OHC was observed as follows. It was nearly unchanged (80 %) for SDS concentration up to 0.04 M. Thereafter, it showed a peak value of about 95 % at SDS concentration of 0.05 M and decreased to 92 % for SDS concentration of 0.06 M (Fig. 4.5). The change in SDS concentration also showed effect on extraction of canrenone. The residual canrenone was nearly same (0.96 %) for SDS concentration upto 0.05 M and increased to 2.4 % at SDS concentration of 0.06 M. Similarly, the % extraction of canrenone which was nearly same (4 %) increased to 9.73 % at SDS concentration of 0.06 M.

Table 4.4: Effect of SDS on 11 OHC extraction

Concentration of SDS, M	Total volume, ml	TS, g/l	TS, g	Purity of 11 –OH canrenone, %	Total 11 – OH canrenone, g	Residual canrenone %	Residual canrenone total g	% extraction	
								11 OH canrenone	Canrenone
0.01	96	7.167	0.688	85.45	0.588	0.96	0.0066	79.89	3.59
0.02	94	7.287	0.685	85.48	0.586	0.95	0.0065	79.62	3.59
0.03	95	7.158	0.680	87.10	0.592	0.97	0.0066	80.43	3.59
0.04	95	7.200	0.684	88.20	0.596	0.98	0.0067	80.98	3.60
0.05	96	7.937	0.762	92.12	0.702	0.96	0.0073	95.38	3.96
0.06	94	7.925	0.745	91.45	0.681	2.40	0.0179	92.53	9.73

ND: not detected under given set of conditions. 100 ml broth of 1.90 g TS was used as feed. Feed broth contained 7.36 g/l 11 OH canrenone and 1.84 g/l canrenone. Extraction was carried out using ethyl acetate as a solvent at 75°C (reflux temperature) for 2 h. Solvent to feed ratio was 1:1 (v/v). SDS was used as a surfactant at a concentration of 0.05 M.

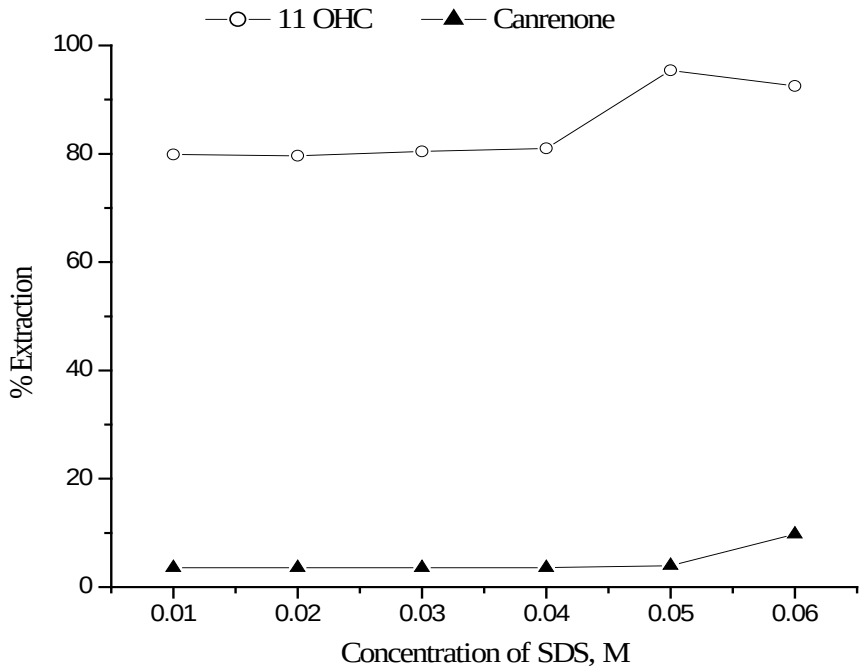


Fig. 4.5 Effect of SDS on extraction of 11 OHC

4.4.4 Selection of surfactant

The comparative performance of the two surfactants has been given in Table 4.5. It can be seen that the highest values of % extraction (95 %) as well as purity (92 %) were obtained with use of SDS. Use of CTAB yielded much inferior values, both for purity as well as % extraction (Fig 4.7). In view of these results, only SDS was used as surfactant in further studies. Moreover, from Table 4.4, optimum concentration of SDS has been seen as 0.05 M. This concentration of SDS (0.05 M) was used in further experimental work.

Table 4.5: Effect of type of surfactant on 11 OHC extraction

Type of surfactant	Total volume ml	TS, g/l	TS, g	Purity of 11 OHC %	Total 11 OHC, g	Residual canrenone %	Residual canrenone total g	% extraction	
								11 OHC	Canrenone
CTAB	95	6.64	0.631	58.29	0.368	ND	ND	50.0	NIL
SDS	96	7.94	0.762	92.12	0.702	0.96	0.0073	95.38	4.13

ND: not detected under given set of conditions. 100 ml broth of 1.90 g TS was used as feed. Feed broth contained 7.36 g/l 11 OH canrenone and 1.84 g/l canrenone. Extraction was carried out using ethyl acetate as a solvent for 2 h at 75°C (reflux temperature). Solvent to feed ratio was 1:1 (v/v). All surfactants were used at concentration of 0.05 M.

4.4.5 Effect of contact time for extraction of 11-OHC

Effect of contact time on recovery of component of interest in reverse micelle systems has been reported by many investigators. The component of interest, in case of most of these reports, was a protein or enzyme. Liu *et al.* (2006) have mentioned that contact time should be usually as short as possible in order to gain maximum activity recovery. They have further mentioned that the phase equilibrium may not be totally achieved. As suggested by some of the researchers, this could be attributed to the effect of surfactant to varying extents on rate of mass transfer for component of interest and for other components present in the feed. This aspect was not investigated during the present study. As mentioned, only those results obtained at the steady state with respect to canrenone and 11-OHC were considered for further analysis or discussion. Some observations during the course of present study suggest that shorter contact time could prove to be more beneficial in case of 11-OHC extraction, as studied in present investigation.

4.4.6 Effect of other significant parameters in presence of surfactant

The effect of other significant process parameters viz. temperature and solvent to feed broth ratio in presence of surfactant SDS (0.05 M) was studied, with a view to further improve the performance. The results are given in Tables 4.6 and Table 4.7.

4.4.7 Effect of temperature

The effect of temperature was studied over the range of 27°C to 75°C, the latter being referred to as reflux temperature, at SDS concentration of 0.05 M (Table 4.6). The purity of 11-OHC was nearly unaffected (92 %) by the temperature. However, the % extraction increased significantly at the reflux temperature. The % extraction of canrenone also increased significantly at the reflux temperature (Fig 4.6).

In comparison with Table 4.1 (system without surfactant), there was marked improvement in purity of 11-OHC (increase from 74 % to 92 %) with marginal increase in % extraction (increase from 89 % to 95 %) at reflux temperature (75°C).

The effect of temperature on % extraction indicated that in presence of the surfactant, the solubility has not been affected by temperature but the selectivity was improved. The marked improvement in purity of 11-OHC, in presence of surfactant, could be attributed to effect of surfactant on solubility of canrenone and other impurities.

Interaction of surfactant molecule with molecules of one or more impurities could lead to formation of new moieties, which might have higher solubility in water. As a result, the selectivity would be favorably affected.

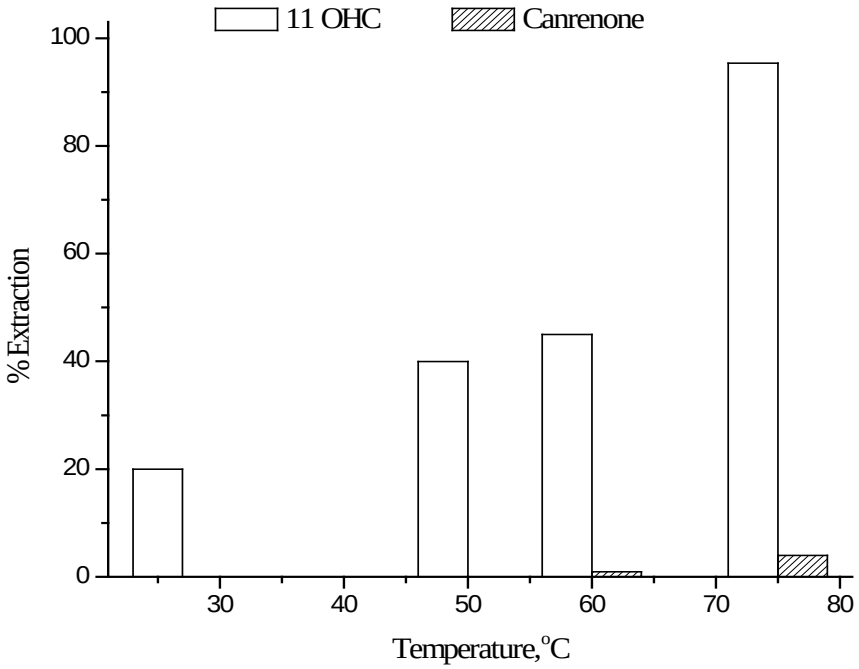


Fig. 4.6 Effect of temperature on extraction of 11 OHC

Table 4.6: Effect of temperature on 11OHC extraction

(Reflux) temperature, ℃	Total volume, ml	TS, g/l	TS, g	Purity of 11 OHC, %	Total 11 – OHC, g	Residual canrenone %	Residual canrenone, g	% extraction	
								11 OH canrenone	Canrenone
27	97	0.165	0.160	91.90	0.147	ND	ND	19.97	NIL
50	97	0.329	0.320	91.78	0.294	ND	ND	39.94	NIL
60	96	0.374	0.359	92.10	0.331	0.45	0.0017	44.97	0.92
75	96	7.937	0.762	92.12	0.702	0.96	0.0073	95.38	3.96

ND: not detected under given set of conditions. 100 ml broth of 1.90 g TS was used as feed. Feed broth contained 7.36 g/l 11 OH canrenone and 1.84 g/l canrenone. Extraction was carried out using ethyl acetate as a solvent for 45 minutes. Solvent to feed ratio was 1:1 (v/v). SDS was used as a surfactant at a concentration of 0.05 M.

4.4.8 Effect of solvent to feed broth ratio

The effect of solvent to feed broth (v/v) was studied for the same SDS concentration (0.05 M). It was evident from Table 4.7 and Fig 4.7 that the purity of 11-OHC showed marginal decrease (from 92 % to 87 %) with increase in the solvent ratio from 1:1 to 2:1. Further increase in the ratio did not affect the purity significantly. The % extraction of 11-OHC however showed a different behavior. The yield of 95 % at ratio of 1:1, dropped to 70 %, with increase in ratio from 1:1 to 2:1 and remained fairly unchanged thereafter. Both residual canrenone as well as canrenone extraction showed similar behavior. When compared with results of Table 4.2, there was increase in purity of 11-OHC from 74 % to 92 %, and comparable yield of 92 % was obtained at lower solvent to feed broth ratio of 1:1.

As discussed earlier, the marked improvement in purity of 11-OHC indicated the effect of surfactant on the selectivity of the solvent. The behavior of the yield indicated that the distribution coefficient was adversely affected by the solvent to feed ratio in presence of surfactant. In view of large number of components of the system it was difficult to correlate this adverse effect only to the molecular structure of 11-OHC. However, interestingly optimum yield was obtained at lower solvent to feed broth ratio of 1:1. This would be advantageous in terms of lower operating cost as there would be less energy requirements for solvent recovery.

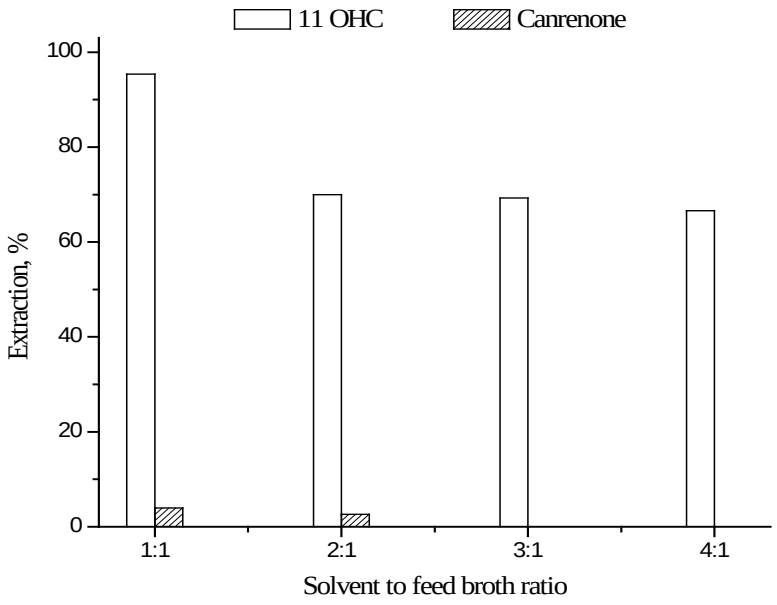


Fig. 4.7 Effect of solvent to feed ratio on extraction of 11OHC

Table 4.7: Effect of solvent to feed ratio on 11 OHC extraction

Solvent to feed ratio, v/v	Total volume, ml	TS, g/l	TS, g	Purity of 11 OHC, %	Total 11 OHC, g	Residual canrenone %	Residual canrenone, g	% extraction	
								11 OHC	Canrenone
1:1	96	7.937	0.762	92.12	0.702	0.96	0.0073	95.38	3.96
2:1	191	3.094	0.591	87.17	0.515	0.82	0.0048	69.97	2.61
3:1	286	2.031	0.581	87.80	0.510	ND	ND	69.29	NIL
4:1	385	1.480	0.570	85.90	0.490	ND	ND	66.57	NIL

ND: not detected under given set of conditions. 100 ml broth of 1.90 g TS was used as feed. Feed broth contained 7.36 g/l 11 OH canrenone and 1.84 g/l canrenone. Extraction was carried out using ethyl acetate as a solvent for 45 minutes. SDS was used as a surfactant at a concentration of 0.05 M.

4.5 Conclusion

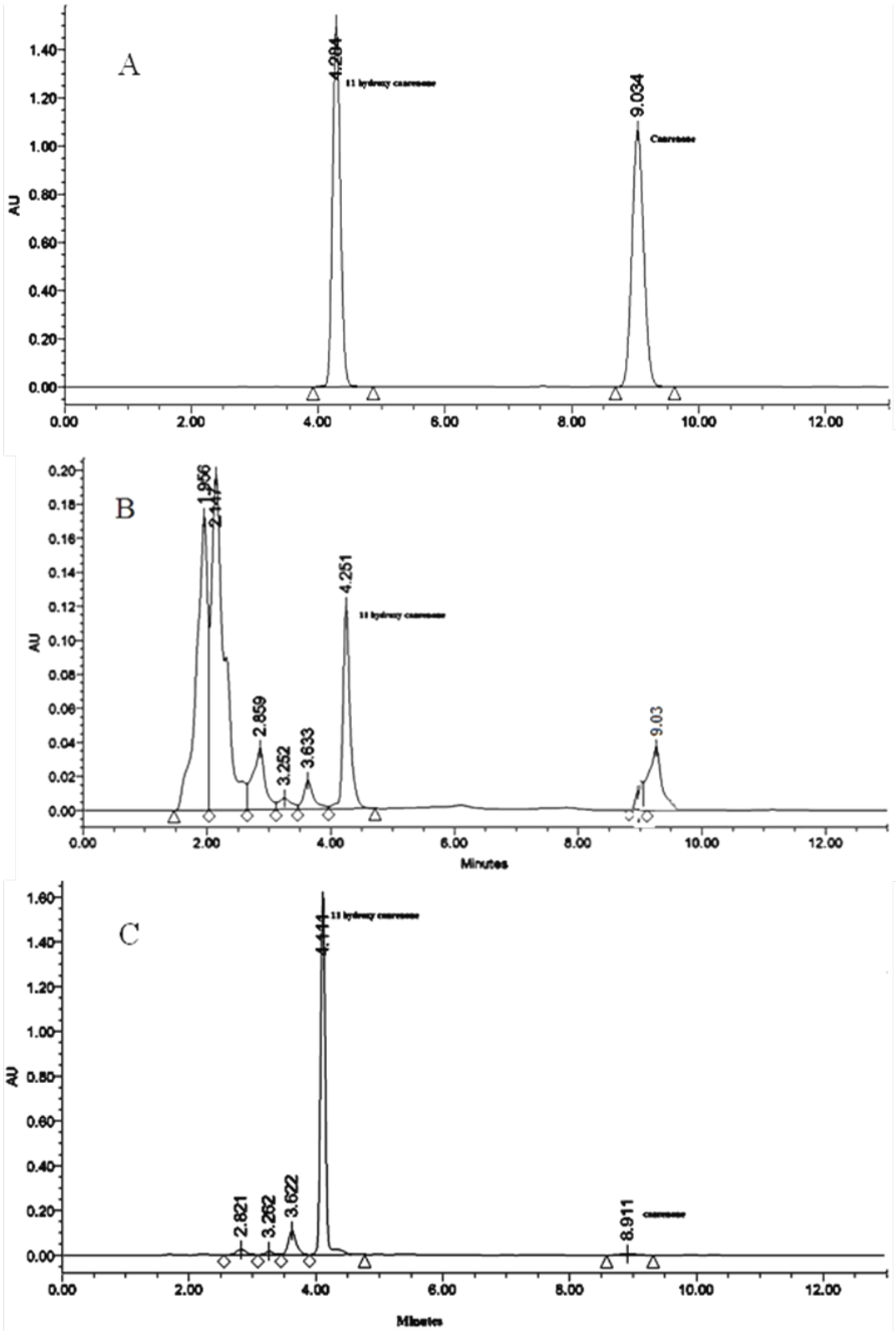


Fig 4.8 A. Standard of canrenone and 11 hydroxy canrenone B. Fermented broth of biotransformation of canrenone C. Extraction of 11 hydroxy canrenone in ethyl acetate

HPLC profile (Fig 4.8) showed that the extract of fermented broth without surfactant has major peak of 11 OHC along with number of impurities and residual canrenone. In Fig 4.8 C, HPLC of extract with surfactant showed major peak of 11 OHC with minor peak of canrenone wheras the other impurities were not observed which clearly reflected selectivity of the surfactant towards 11 OHC.

In comparison, the best results obtained were seen as follows.

	Purity, %	Yield, %
System without surfactant	74%	92%
System with surfactant	92%	95%

A micelle based system comprising ethyl acetate and aqueous phase with anionic surfactant has not been reported to the best of our knowledge. This system was found to be stable at higher temperature and further study will elaborate on mechanism of extraction of water insoluble suspended biomolecules to the organic phase.

4.6 Process economics considerations

It can be seen from these results that there was a significant increase in purity of 11-OHC (74 % to 92 %) by use of micelle based system. On the other hand, the % extraction was not significantly improved by use of micelle based system. However, one distinct advantage offered by the micelle based system was that the improved yield is obtained at much lower solvent to feed broth ratio (1:1 as against 4:1). This would give advantage in terms of lower operating cost in terms of less energy requirements for solvent recovery. The purity of 11-OHC is an important consideration for reason as follows. 11-OHC is precursor for eplerenone. Purity of 11-OHC higher than 95 % is necessary so that it can be used as precursor for eplerenone.

With the micelle based system it is possible to achieve purity of more than 90 %, in the first single stage operation itself. This makes the stage of further purification cost effective. As such, use of micelle based system would lead to significant advantage over the conventional process in terms of energy as well as overall cost savings.

4.7 References:

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CHAPTER 5

Extraction and purification of lipase from fermented broth using reverse micelle

5.1 Introduction

Lipase (Triglycerol hydrolases E.C. 3.1.1.3) catalyzes the hydrolysis of triglycerides at oil-water interface. In recent times, lipase has emerged as an industrially important enzyme. It has found wide applications in the industries as dairy, food and confectionaries and also for manufacturing of biodiesel. The partially purified enzymes have potential applications in the field of biotransformations such as the chiral resolutions and processes viz. esterification, alcoholysis and acidolysis (Echim *et al*., 2009; Joseph *et al*., 2007; Nasratun *et al*., 2009).

Enzyme bio separation, which refers to the recovery and purification of the enzyme from various fermentation feed streams is considered to be one of the most important unit operation in the bioprocess industry. Enzyme purification is classified broadly in three catagories 1. high productivity, low resolution (cell disruption, liquid liquid extraction, ultrafiltration etc) 2. high resolution, low productivity (ultracentrifugation, affinity separation, electrophoresis etc) 3. high resolution, high productivity (fluidized bed chromatography, membrane chromatography etc). (Mathew and Juang, 2007) Reverse micellar extraction (RME) of the protein is an attractive liquid liquid extraction method for the downstream processing for the enzymes. Selective extraction of the target biomolecule from mixture of enzymes/proteins in these reverse micelles can be achieved by varying parameters both in organic phase and in aqueous phase (Chen *et al*., 2006; Dovyap *et al*, 2006; Goto 2006; Hagen *et al*., 2006).

The successful RME includes both forward and backward extraction steps in their optimized conditions. This technique offers many advantages such as higher recovery, enzyme concentration and purification, scalability and continuous operations. RME of lipase has been carried out by many researchers using Aerosol OT (Moniruzzaman *et al*., 2006; Wu *et al*., 2006) or CTAB as surfactants (Nandini and Rastogi, 2009; Shen *et al*., 2005).

The present work was focused on the purification of lipase by conventional approach and reverse micellar based approach. Partial purification of lipase by conventional methods includes multistep operations such as precipitation/concentration, ultrafiltration and (ion exchange, gel filtration, affinity) chromatography whereas RME includes single step extraction using organic solvent and surfactant followed by purification by using suitable stripping agent. The study of effect of various factors (pH, temperature, contact time, solvent: feed ratio) on the forward and backward reactions of RME has also been described in the chapter.

5.2 Materials and Methods

5.2.1 Materials

p- nitrophenyl palmitate (pNPP), AOT, bovine serum albumin and standard of solanesol (90 % purity) were purchased from Sigma Aldrich India. Solvents viz. N-hexane and ethyl acetate and precoated TLC plates of aluminium coated with silica gel F_{254} were obtained from Merck India Ltd. Molecular weight marker kit was from BioRad Laboratories Inc., USA. Coomassie brilliant blue R-250 was purchased from Ubichem Ltd. Sephadex G -100 was from Amersham Pharmacia. All other chemicals viz. CTAB, SDS, T80, Triton X 100, Tris HCl, Acrylamide, N, N- Methylene bis acryamide, Folin Ciaoculteau reagent, Coomassie brilliant blue G250, NaCl, NH_4NO_3, $MgSO_4$, K_2HPO_4, KH_2PO_4 were of AR grade and procured from Merck India, SRL, Qualigens and Hi Media. Soya flour and sesame oil were procured from local market. The water used was deionized using Cascada water purification system (Pall Life Sciences).

5.2.2 Methods

5.2.2.1 Organism

*Pseudomonas sp.*CSD3 isolated in our laboratory was used for production of extracellular lipase. The organism was maintained on slants of nutrient agar at 4°C and routinely subcultured every 30 days.

5.2.2.2 Production of extracellular crude lipase

The culture was grown in the optimized medium (soya flour, 11.12 g; NH_4NO_3, 4.0 g; $MgSO_4$, 1.0 g; K_2HPO_4, 2 g; KH_2PO_4, 5.0 g; sesame oil 5.0 ml per L; pH 6.8 -7.0) at 37°C and 180 rpm for a period of 48 h. At the end of incubation, the bacterial cells in

the fermentation broth were removed by centrifugation at 10000 *g* for 20 min at 4°C. The cell-free supernatant was used as a crude enzyme preparation and purified further as described below.

5.2.2.3 Purification of lipase

Ammonium sulphate precipitation

The extracellular lipase enzyme was concentrated using ammonium sulphate precipitation by 0-30 % fraction. The precipitate was separated by centrifugation (10000 g) at 4°C for 15 min and dissolved in Tris Cl buffer (50 mM), pH 7.0.

Tangential flow filtration (TFF)

The resuspended ammonium sulphate precipitate was diafiltered using TFF unit (Pall Life Sciences, USA) with 10 kDa omega membrane (polyether sulphone) cassette (molecular weight cut off of 10 kD).

Gel filtration chromatography

The TFF enzyme preparation was loaded on a Sephadex G-100 column (2×150 cm) pre-equilibrated with 10 mM sodium phosphate buffer, pH 6.4. The elution was carried out in the same buffer at a flow rate of 0.23 ml/min at room temperature and 4.6 ml fractions were collected. The protein content of fractions was determined by measuring optical density at 280 nm. The protein-containing fractions were assayed for lipase activity.

5.2.2.4 Purification of lipase by reverse micelle system

a. Forward extraction - Preparation of reverse micelle system

Forward extraction of lipase was typically set by mixing crude lipase enzyme in isooctane in presence of surfactant under constant stirring conditions at stated temperature and time. Surfactants viz. AOT, CTAB, Triton X100 and T80 were added at 25 mM concentration. All surfactants except AOT were directly added to crude lipase preparation while AOT was first dissolved in isooctane. Effect of different concentrations of AOT (25-200 mM), pH of fermented broth (5.0-8.5), different contact time (5-40 min) and V_{aq}:V_{RM} (1:1-1:5) was studied. The forward reaction containing microemulsion was incubated at stated temperatures and stated time with constant

stirring. The reaction mixture was then centrifuged at 8000 g for 10 min at 25°C. The two phases (aqueous and organic) were separated and the residual lipase activity in the aqueous phase was checked. Organic phase was subjected further to backward extraction.

b. Backward reaction - Stripping of lipase from reverse micelle system

For the optimization of parameters of forward extraction, the backward extraction was carried out using stripping solution comprising NaCl (0.05 M in 50 mM Tris Cl buffer, pH 7.0) and 15 % isopropanol.

Backward extraction parameters were optimized using stripping solution containing NaCl in different concentration (0.01-1.0 M) and pH (4.0-9.0). The effect of V_{aq}:V_{RM} (1:1-1:8) and different contact time (30-50 min) were also optimized at constant temperature of 25°C. The reaction mixture was centrifuged for 8000 g for 10 min. Lipase activity of stripped aqueous phase was checked. The purification of enzyme was checked by polyacrylamide gel electrophoresis.

5.2.2.5 Enzyme assay

Assay of lipase

Lipase activity was assayed using pNPP substrate as described by Winkler and Stuckman (1979). Suitably diluted enzyme (0.1 ml) was added to freshly prepared substrate emulsion (0.9 ml, 0.468 μM pNPP prepared in 50 mM Tris HCl buffer, pH 7.0, containing 0.44 % Triton X 100 and 0.15 M NaCl) and incubated at 37°C for 30 min. The liberated p-nitrophenol was read immediately at 410 nm. One unit of enzyme activity was defined as 1.0 μmole of p-nitrophenol liberated from p-nitrophnyl palmitate per minute per ml under standard assay conditions.
Unit/ml of enzyme was calculated by the following equation

(A_{410} Test- A_{410} Blank) x 1000 x dilution factor / 9 x 100 x incubation time

Where Blank is enzyme or substrate blank whichever is greater; 9 is milimolar extinction coefficient of p-nitrophenol at 410nm under assay conditions and 100 is volume of enzyme.

5.2.2.6 Estimation of protein

Protein content was estimated according to Lowry *et al*. (1951) using bovine serum albumin (BSA) as a standard.

5.2.2.7 Polyacrylamide gel electrophoresis (PAGE)

Polyacrylamide gel electrophoresis was carried out in 10 % (w/v) polyacrylamide slab gel at pH 8.8. Protein bands were detected using coomassie blue or silver staining method (Laemmli *et al.*, 1970; Sambrook and Russell, 2001).

5.2.2.8 Staining of proteins

Staining by Coomassie blue

The electrophoresed gel was immersed in the staining solution of coomassie brilliant blue R-250 (0.25 % in methanol: acetic acid) and placed on a slowly rocking platform for 3-4 hrs at room temperature. The gel is then removed and destained by soaking the gel into methanol: acetic acid solution on a rocking platform for 3-4 h, changing the destaining solution 3-4 times. After destaining, the gel was stored in deionized water (Sambrook and Russell, 2001).

Staining by silver staining

The electrophoresed gel was incubated in fixing solution (ethanol: acetic acid: water, 30:10:60) overnight at room temperature with gentle shaking to fix the proteins. The fixing solution was discarded and gel was incubated with 30 % ethanol for 30 min at room temperature with gentle shaking. The gel was then removed and incubated deionized water for 10 min with gentle shaking. After the two washes of water, the gel was immersed in freshly prepared silver nitrate solution and incubated for 30 min at room temperature with gentle shaking. The gel was washed with deionized water and incubated in fresh developing solution (aqueous solution of 2.5 % sodium carbonate and 0.02 % formaldehyde) at room temperature with gentle shaking till the stained bands of protein appeared (Sambrook and Russell, 2001).

5.3 Results and Discussion

5.3.1 Purification of lipase by conventional method

Purification of lipase from *Pseudomonas* sp. CSD3 was carried out as described under materials and methods. Initially low titer broth was obtained by using shake flask. The cell free broth was concentrated by fractional ammonium sulphate precipitation. Maximum lipase activity was recovered in 0-30 % fraction of ammonium sulphate. The next step of TFF through 10 kDa cassette membrane achieved desalting of the enzyme as well as removal of low molecular weight impurities. Lipase was purified by 4.3 fold and the yield was 93.20 %. The partially purified protein by TFF was subjected to gel filtration column chromatography as described under materials and methods. Total 50 fractions were collected. The active fractions (no. 8-12) having significant lipase activity was pooled and lyophilized. This step showed 14.98 fold purification (Table 5.1).

Table 5.1: Summary of lipase purification

Step	Total volume, ml	Total protein, mg	Total activity, U	Specific activity, u/mg	Fold purification	Yield, %
Cell free supernatant	100	600	900	1.5	1	100
Ammonium sulphate precipitation and TFF	27	102.91	816.07	7.93	5.29	90.67
Gel filtration	12	21.03	472.53	22.47	14.98	52.50

Similar results have been documented for *P. aeruginosa* extracellular lipase. Primary concentration was done by ammonium sulphate precipitation followed by dialysis and further purified by gel filtration to achieve 12 fold purification with less (16.4 %) yield (Palmeros *et al*., 1994). The multistep purification protocols have been reported in most of the cases of lipase purification where yield is not more than 50 % but fold purification might be more than 100 times. For instance Sharma *et al.* (2002) high fold purification ~201.15 but less yield (19.70 %) for thermophilic *Bacillus* lipase. The partially purified *A. carneus* lipase was reported to have yield of 38.40 % and 24.10 fold purification when subjected to hydrophobic column chromatography (Saxena *et al*., 2003). The multistep purification also was observed to affect the yield significantly from 37 % to 0.96 % though fold purification increased from 13.77 to 43 fold in case of *Bulkholderia multivorans* (Dandavate *et al*., 2009)

5.3.2 Purification of lipase by reverse micellar system

For all experiments, crude lipase preparation (100 ml) of following specifications was used: total activity – 900 U ± 5, total protein 600 mg ± 3 and specific activity – 1.5 ± 0.007.

5.3.2.1 Effect of various parameters on forward extraction

5.3.2.1.1 Effect of surfactant on forward extraction

In the present study, the effect of different surfactants was investigated. A typical forward reaction consisted of crude lipase enzyme, a surfactant and isooctane. Surfactants viz. CTAB, Triton X-100, SDS and T80 were added at concentration of 25 mM in crude lipase enzyme, while AOT (25 mM) was first dissolved in isooctane. SDS was found to denature the crude lipase and hence not used further for forward reaction. The crude lipase enzyme (10 ml) with surfactant was then mixed with 10 ml of isooctane. Out of four different surfactants, anionic surfactant AOT showed maximum recovery of lipase along with 5.61 fold purification at pH 7.0 whereas at pH 4.0 there was significant loss of activity. Lipase activity recovery was 33 % with 4.20 fold purification when the forward reaction was carried out at pH 9.0. With cationic surfactant CTAB and nonionic surfactants viz. Triton X 100 and T80 there was no phase separation observed after completion of forward reaction and hence was not used further. **Table 5.2** shows the effect of AOT in forward extraction.

Table 5.2: Effect of AOT on forward extraction in different pH environment

pH	Total activity, U	Total protein, mg	Specific activity, U/mg	Recovery %		Fold purification
				A	P	
Crude lipase	900	600	1.5	100	100	1
4.0	Nil	40.09	Nil	Nil	-	-
7.0	379.90	45.10	8.42	42.21	7.51	5.61
9.0	300.21	47.58	6.31	33.35	7.93	4.20

A: activity; P: Protein; The reaction mixture (crude lipase, AOT, 25 mM and isooctane) was incubated at 25°C for 30 min under constant stirring conditions. The organic phase was separated by centrifugation at 8000 g for 10 min at 25°C. The lipase recovery was carried out by backward extraction using 0.05 M NaCl in 50 mM Tris Cl buffer, pH 7.0 and 15 % isopropanol (1:1) at 25°C for 30 min.

Similar results have been reported with use of AOT (300 mM) as surfactant for the forward extraction of commercial yeast lipase (pH 4.0) in isooctane where 45 % of

enzyme was recovered (Yu *et al*., 2003). On the contrary, Nandini and Rastogi (2009) have reported 82.72 % enzyme recovery with cationic surfactant CTAB in forward extraction of *A. niger* lipase using isooctane containing n-hexane and n-butanol as cosolvent at pH 7.0. The fold purification was 4.094.

AOT based microemulsion system has been reported to be effective for extracting enzymes. CTAB or any other cationic surfactant often needs cosurfactant which is not a case with anionic AOT. Because of the small alkyl chain in the hydrophobic moiety AOT can extract low molecular weight proteins easily but it has limitations of extracting high molecular weight proteins. This becomes an advantage for the selectivity of the protein (Carvalho and Cabral, 2000).

5.3.2.1.2 Effect of contact time on forward extraction

The effect of contact time on forward extraction of *Pseudomonas* lipase was studied for various time period (5 min to 40 min) in crude lipase (pH 7.0)/AOT (25 mM) /isooctane reverse micelle system.

Table 5.3: Effect of contact time on forward extraction

Contact time (min)	Total activity, U	Total protein, mg	Specific activity, U/mg	Recovery %		Fold purification
				A	P	
5	310.7	18.525	16.77	33.7	3.08	11.18
10	358.0	25.93	13.80	38.91	4.23	9.2
15	382.85	32.11	11.92	41.52	5.35	7.95
20	379.0	37.05	10.24	41.19	6.17	6.83
25	381.68	40.13	9.50	41.48	6.68	6.33
30	380.96	44.46	8.56	41.40	7.41	5.70
35	378.20	44.47	8.50	41.10	7.41	5.65
40	376.34	44.49	8.46	40.89	7.41	5.62

A: activity; P: protein; The reaction mixture (crude lipase, AOT, 25 mM and isooctane) was incubated at 25°C under constant stirring conditions. The organic phase was separated by centrifugation at 8000 g for 10 min at 25°C. The lipase recovery was carried out by backward extraction using 0.05 M NaCl in 50 mM Tris Cl buffer , pH 7.0 and 15 % isopropanol (1:1) at 25°C for 30 min

The ratio of aqueous phase (crude lipase, pH 7.0) (V_{aq}) to organic phase (V_{RM}) was maintained at 1:1 and the extraction was carried out at 200 rpm at 25°C. The mixture was centrifuged at 8000 g for 10 min to reach clear separation of two phases. The increase in time period from 5 to 40 min was found to increase the total protein content and decrease the specific activity gradually (Table 5.3). Fig 5.1 shows the effect of contact time on forward extraction of *Pseudomonas* lipase. The contact time should be long enough to achieve the phase equilibrium. For a real system the contact time usually should be as short as possible to achieve maximum activity recovery where phase equilibrium may not be totally achieved.

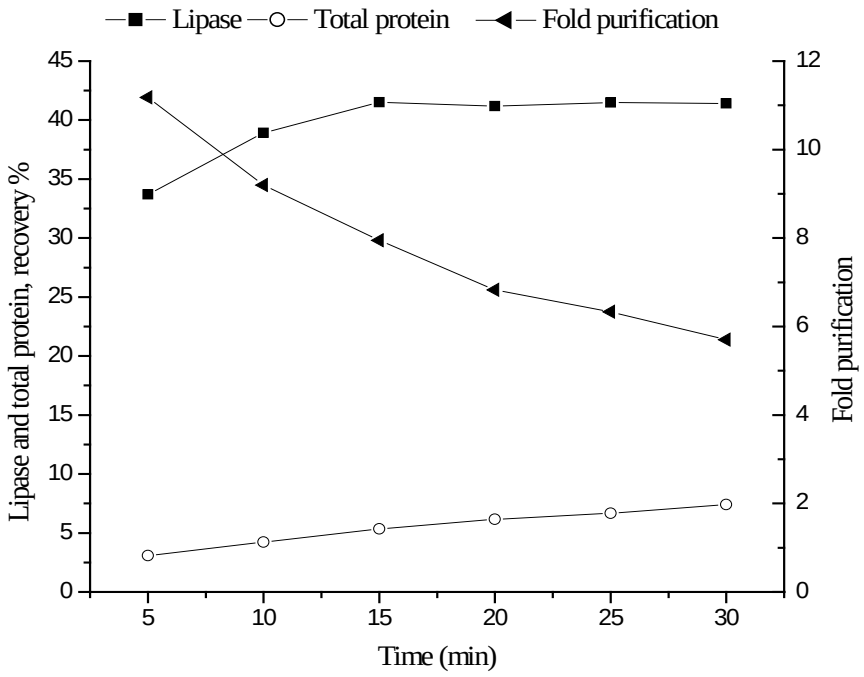

Fig. 5.1: Effect of contact time on forward extraction of lipase

For instance, in case of AOT based reverse micelle extraction of nattokinase from *Bacillus natto*, maximum total protein recovery (39.6 %) was achieved in 12 min whereas maximum enzyme recovery (82 %) was achieved in 8 min before the phase equilibrium reached (Liu *et al*., 2006). In the present studies, extraction of undesirable proteins and consequently decrease in specific activity can be attributed to more contact time. When the extraction was carried out for 15 min the lipase recovery was highest (41.52 %) and remained almost unchanged for further time period. Based on the lipase recovery and specific activity, contact time of 15 min was used for further studies in forward extraction of lipase.

5.3.2.1.3 Effect of AOT concentration on forward extraction

The effect of different concentrations (25 – 200 mM) of AOT was determined for 15 min using a ratio of crude lipase (V_{aq}) (pH 7.0) to reverse micellar phase (V_{RM}) as 1:1. The mixture was centrifuged at 8000 g for 10 min to reach clear separation of two phases. The results are presented in **Table 5.4**. The increase in AOT concentration from that in control (25 mM) to 50 mM resulted into significant increase in the lipase recovery (68.88 %) and specific activity (21.78) than that of control. The fold purification was also increased from 10.60 to 14.52. Further increase in AOT concentration (100 mM and above) did not change the % lipase recovery significantly. Similarly, Yu *et al* (2006) have reported 100 % yeast lipase recovery at 100 mM AOT concentration while with further increase in concentration (up to 300 mM), there was no significant change in lipase recovery. However, in the present investigations there was increase in total protein thereby decreasing the specific activity to 8.63 at 200 mM AOT concentration (Fig 5.2). *A. niger* lipase extraction of 85.49 % at 200 mM CTAB concentration was reported whereas further increase in CTAB concentration to 250 mM, decreased the lipase recovery to 75.69 %. The authors have attributed the decrease in extraction efficiency to micellar clustering which decreased the interfacial area available to the target biomolecule (Nandini and Rastogi, 2009).

Table 5.4 Effect of AOT on forward extraction

AOT concentration, mM	Total activity, U	Total protein, mg	Specific activity, U/mg	Recovery %		Fold purification
				A	P	
25	382	24.0	15.91	42.44	4.0	10.60
50	620	28.4	21.78	68.88	4.74	14.52
100	612	37.93	16.13	68.0	6.32	9.66
150	611	45.53	13.41	67.99	7.58	8.06
200	619	60.43	10.24	68.86	10.07	5.75

A: activity; P: protein; The reaction mixture (crude lipase, AOT and isooctane) was incubated at 25°C for 15 min under constant stirring conditions. The organic phase was separated by centrifugation at 8000 g for 10 min at 25°C. The lipase recovery was carried out by backward extraction using 0.05 M NaCl in 50 mM Tris Cl buffer , pH 7.0 and 15 % isopropanol (1:1) at 25°C for 30 min.

The result of the present study suggested that equilibrium between the organic and the aqueous phases with reference to enzyme recovery was reached at AOT concentration of 50 mM or higher. However, phase equilibrium with reference to protein recovery was not attained even at 200 mM AOT concentration. Presumably the micellar concentration at less than 50 mM might be a limiting factor for enzyme transfer.

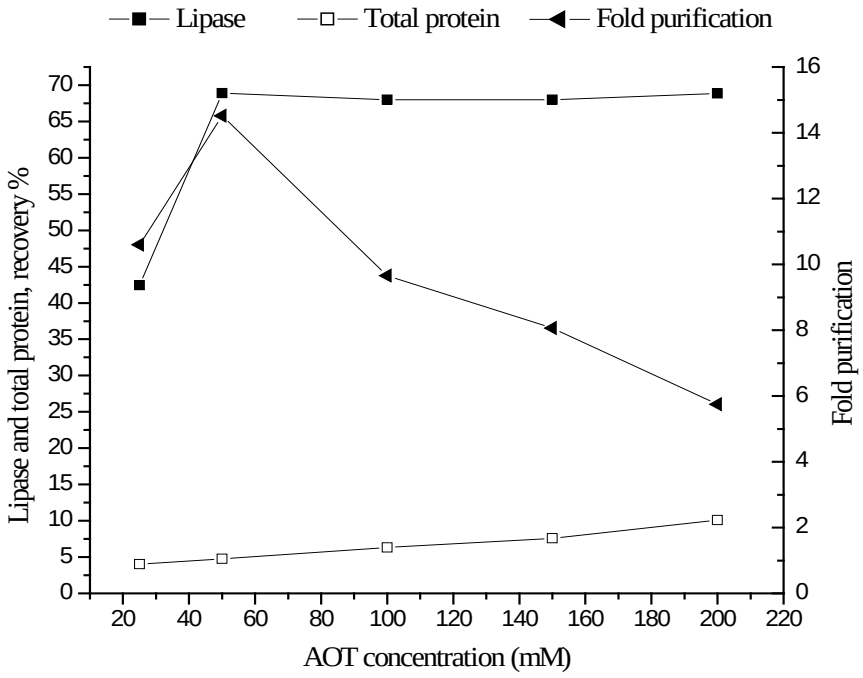

Fig. 5.2 Effect of AOT on forward extraction of lipase

5.3.2.1.4 Effect of pH of fermentation broth

The earlier experiment (5.4.1.1) showed that there was significant lipase activity loss at pH 4.0. The similar trend was observed at pH 5.0 with 90 % loss of enzyme activity. It can be seen from the Table 5.5 and Fig 5.3 that the pH range of 6.0-7.5 was found to be optimal for the maximum recovery of lipase activity i.e. 65 - 70 % of the crude lipase whereas maximum enzyme recovery was found at pH 6.5 with highest fold purification of 14.66.

As the pH increases slightly above the pI of the enzyme, charge on the enzyme becomes negative and below pI it becomes positive (Rawn, 2004). In case of cationic surfactant based reverse micelle system the forward extraction is favoured at pH values greater than pI. This forward extraction is the function of electrostatic interaction between charged protein molecule and charged head groups of reverse micelle (Tonova and

Lazarova, 2008). Nandini and Rastogi (2009) reported *A. niger* lipase recovery using cationic surfactant CTAB. The pI of the lipase was 4.2. At pH 9.0, 84 % recovery of *A. niger* lipase was achieved whereas at pH 7.0 it was only 30 %. The authors attributed the higher % of forward extraction to the increased negative charge on the protein at higher pH. On the other hand, Yu *et al.* (2003) reported 100 % lipase extraction at pH 3.5 which was less than pI of yeast lipase. Under this condition yeast lipase was positively charged and was attracted by anioinic AOT headgroups. In the present investigation, the pI of *Pseudomonas* lipase was 8.2 and the maximum transfer was found at pH 6.5 which was slightly below the pI of the enzyme. This indicated that the electrostatic attraction was playing major role in the extraction. When reverse micelle is formed using ionic surfactant, it is considered that protein solubilization into reverse micelles is governed by the electrokinetics at the phase interface as the ionic interactions are strongest intermolecular forces.

Table 5.5: Effect of pH of fermentation broth on forward extraction

pH	Total activity, U	Total protein, mg	Specific activity, U/mg	Recovery %		Fold purification
				A	P	
5.0	93.75	28.74	3.26	10.41	4.79	2.17
6.0	623.2	28.98	21.50	69.24	4.83	14.33
6.5	625.2	28.40	22.01	69.47	4.73	14.66
7.0	625.2	28.70	21.78	69.47	4.78	14.52
7.5	602.0	30.71	19.60	66.22	5.22	13.06
7.8	550.0	34.11	16.12	61.11	5.68	10.76
8.0	410.2	36.2	11.33	45.12	6.03	7.55
8.5	270.6	37.1	7.29	29.77	6.18	4.86

A: activity; P: protein; The pH of the crude lipase preparation was adjusted to desired pH with 1 M NaOH or HCl. Reverse micellar phase (AOT, 50 mM + isooctane) and aqueous phase were mixed in 1:1 proportion and kept at 200 rpm at 25°C for 15 min. The mixture was centrifuged at 8000 g for 10 min to reach clear separation of two phases. The lipase recovery was carried out by backward extraction using 0.05 M NaCl in 50 mM Tris Cl buffer , pH 7.0 and 15 % isopropanol (1:1) at 25°C for 30 min.

In the present studies at pH 7.5, 7.0 and 6.5, the net positive charge on the protein is higher at pH 6.5. This can be correlated with the results obtained at pH 6.5. For proteins of size smaller than the size of water pool inside a reverse micelle, solubilization occurs

as soon as the net protein charge is opposite to that of the reverse micellar system (Dekker *et al*., 1989). In the present investigation, the maximum transfer of enzyme has been observed in a narrow range of pH i.e. 6.5 – 7.0 which is very close to pI of *Pseudomonas* lipase and this can be attributed to the low molecular weight (18 kDa) of enzyme. Similar behavior has been documented with three low molecular weight proteins (12 – 14 kDa) viz., ribonuclease, cytochrome C and lysozyme (Tonova and Lazarova, 2008).

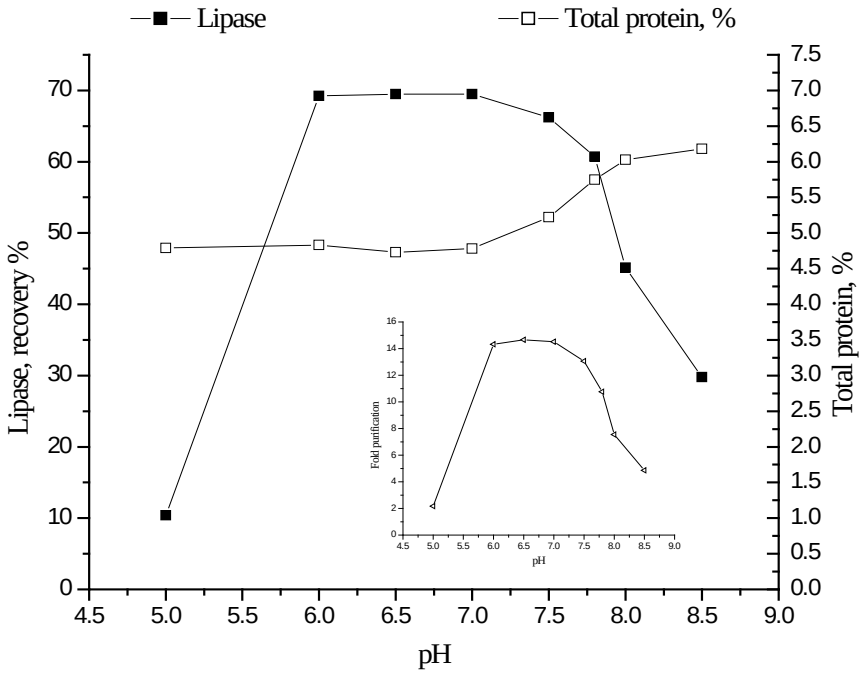

Fig. 5.3: Effect of pH of crude lipase broth on forward extraction (Inset: Effect on fold purification)

The effect of pH in the present study showed enzyme transfer at pH (8.0) near the pI and pH (8.5) slightly above the pI. This behavior might not be explained on the basis of the electrostatic interaction. The possible mechanism could be explained on the basis of the hydrophobic interactions. The solubilization behavior of hydrophobic amino acids and proteins deviated from the model of electrostatic attraction. The hydrophobic interactions between the target protein and hydrophobic tails of the surfactants and/or the solvent play important role in both protein solubilization and recovery. The solubilization of the enzyme in the microemulsion was reported to be mainly governed by nature of the interfacial complex that forms between surfactants and proteins (Aires-Barros and Cabral, 1991; Goto *et al*., 1997; Tonova and Lazarova, 2008).

5.3.2.1.5 Effect of crude broth: organic phase ratio (V_{aq}:V_{RM}) on forward extraction

Liu *et al.* (2006) showed that aqueous to organic phase ratio had significant effect on forward extraction of nattokinase from *Bacillus natto*. In the reverse micellar (AOT + isooctane) extraction, authors obtained maximum nattokinase activity recovery (95 %) and maximum total protein (20 %) when V_{aq}:V_{RM} proportion was 1:3.

Enzyme recovery and total protein was reduced when the proportion was 1:1 where enzyme recovery was 80 % and total protein was 18 % whereas enzyme recovery was only 55 % along with total protein of 14 % when the proportion was 1:0.5.

Similarly in the present studies, the ratio of V_{aq}:V_{RM} (1:1-1:5) showed significant impact on the *Pseudomonas* lipase extraction using AOT surfactant (Table 5.6a). The increase in volume of isooctane resulted in increase in total protein content from 1:1 to 1:5 proportion whereas enzyme recovery was increased only up to proportion of 1:3 (80.67 %) and remained unchanged even after increase in isooctane volume (Fig 5.4). The extraction of undesirable protein with increase in /isooctane led to decrease in specific activity of lipase and in turn fold purification.

Table 5.6 a Effect of ratio of V_{aq}:V_{RM} on forward extraction

V_{aq}:V_{RM}	Total activity, U	Total protein, mg	Specific activity, U/mg	Recovery %		Fold purification
				A	P	
1:1	620.2	28.02	22.12	68.88	4.67	14.74
1:2	689.5	30.28	22.77	76.6	5.04	15.18
1:3	726.10	31.29	23.2	80.67	5.21	15.47
1:4	720.68	34.80	20.95	80.07	5.80	13.97
1:5	716.12	35.2	20.34	79.57	5.86	13.56

A: activity; P: protein. Forward reaction was carried out using crude lipase, pH 6.5, AOT, 50 mM at 25°C for 15 min and The mixture was centrifuged at 8000 g for 10 min to reach clear separation of two phases. The lipase recovery was carried out by backward extraction using 0.05 M NaCl in 50 mM Tris Cl buffer , pH 7.0 and 15 % isopropanol (1:1) at 25°C for 30 min.

The crude broth to organic phase proportion was further optimized using narrow range of 1:2.5, 1:3 and 1:3.5 where maximum enzyme recovery (80.97 %) and maximum fold purification (15.62) was obtained when the proportion was 1:2.5 (**Table 5.6 b**). Based

on these results, the crude broth to isooctane proportion of 1:2.5 was used for further studies.

Increased enzyme extraction with increased isooctane volume was also shown in case of commercial yeast lipase purification using reverse micelle system with AOT as surfactant. The extraction yield was increased with increase in phase volume (isooctane) ratio. Around 80 % of enzyme was recovered when the V_{aq}:V_{RM} was 1:4 and when the proportion was 1:1, the recovery of lipase was reduced to 50 %. The authors have attributed the increase in enzyme recovery along with increase in organic phase to the increase in the number of reverse micelles (Yu *et al*., 2003).

Table 5.6 b: Effect of ratio of V_{aq}:V_{RM} on forward extraction

V_{aq}:V_{RM}	Total activity, U	Total protein, mg	Specific activity, U/mg	Recovery %		Fold purification
				A	P	
1:2.5	728.8	31.10	23.43	80.97	5.18	15.62
1:3	726.10	31.29	23.2	80.67	5.21	15.47
1:3.5	724.30	31.33	23.11	80.47	5.22	15.40

A: activity; P: protein; Forward reaction was carried out using crude lipase, pH 6.5, AOT, 50 mM at 25°C for 15 min and The mixture was centrifuged at 8000 g for 10 min to reach clear separation of two phases. The lipase recovery was carried out by backward extraction using 0.05 M NaCl in 50 mM Tris Cl buffer , pH 7.0 and 15 % isopropanol (1:1) at 25°C for 30 min.

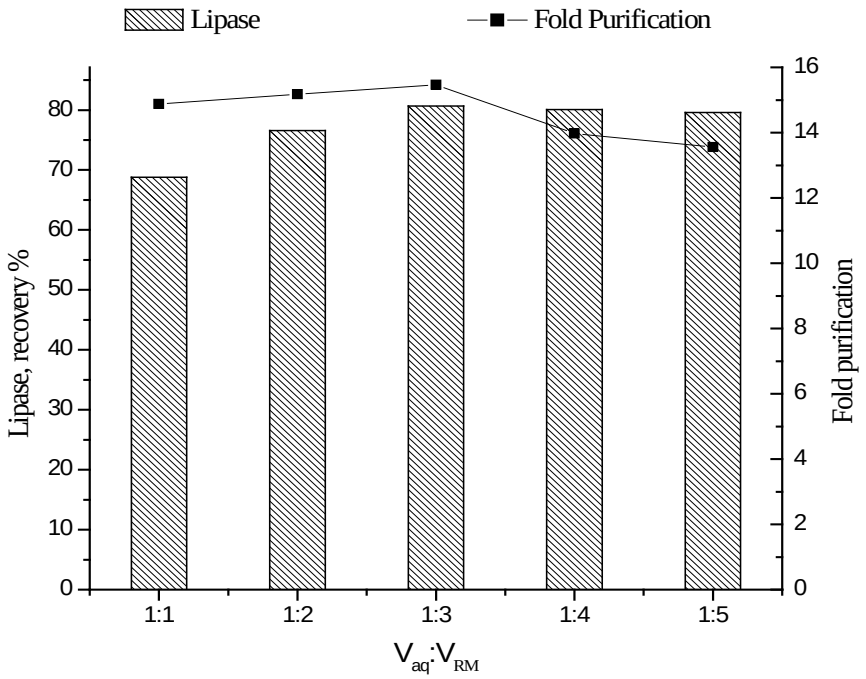

Fig 5.4 Effect of V_{aq}:V_{RM} on forward extraction

5.3.2.2 Effect of various parameters on backward extraction

5.3.2.2.1 Effect of NaCl concentration on backward extraction

Usually the backward extraction is carried out by NaCl as well as KCl. Potassium salts are preferred over sodium salts because K^+ is chaotropes i.e. water structure breaking salt whereas Na^+ is a cosmotropes i.e. water structure making salts (Nandini and Rastogi, 2009)

In the present study, backward extraction was carried out by NaCl as well as KCl at broad concentration range of 0.1 M, 0.5 M and 1.0 M. For all three concentrations used, NaCl showed better enzyme recovery and fold purification than KCl and hence it was used for the further studies.

To extract the protein from reverse micellar system, the stripping solution was prepared containing different NaCl concentration (pH 7.0) of 0.01 M to 1 M. The lipase containing organic phase was added to aqueous stripping solution in the 1:1 proportion and kept at 25°C. The mixture was centrifuged at 8000 g for 10 min to reach a clear separation of two phases.

Backward extraction of protein is extraction of protein from micellar phase to fresh aqueous phase. Use of salts in the extracting aqueous phase is one methodology of stripping protein back from micelle phase. Stripping with high salt concentration i.e. with high ionic strength causes reverse micelle size reduction. This causes expulsion of protein from reverse micelle. This has been observed in the present investigation of *Pseudomonas* lipase. The results are summarized in Table 5.7. The low molarity of NaCl i.e. 0.01 M and 0.05 M was unable to extract the protein from organic phase. It can be seen from the Fig 5.5 that there was a steep increase in the enzyme and total protein recovery as the concentration of NaCl increased from 0.1 M to 0.25 M. At 0.25 M NaCl concentration, the enzyme recovery was highest (80.67 %). Further increase in NaCl concentration to 0.5 M decreased the enzyme recovery significantly though fold purification was changed marginally. At 1.0 M NaCl concentration, 99 % enzyme activity loss was observed. This may be due to conformational change in the protein structure because of squeezing out the enzyme from the reduced size micelle. Deactivation of α amylase and β galactosidase due to this phenomenon during back stripping with higher ionic strength buffer (Lazarova and Tanova, 1999; Shiomori *et al*., 1996).

Pessoa Jr and Vitolo (1998) have reported 0.5 M NaCl as optimum concentration for backward extraction of *Candida kefir* inulinase from BDBAC/isooctane/heanol reverse micelle. Nandini and Rastogi (2009) reported use of KCl in backward extraction. They found 84 % *A. niger* lipase recovery with 1 M KCl stripping solution from reverse micellar phase comprising CTAB, 0.2 M/isooctane/butanol-hexanol. Further increase in molarity of KCl to 1.25 M resulted into less enzyme (40 %) and protein recovery. Authors have attributed less enzyme recovery to the dominance of hydrophobic interactions, aggregation and precipitation of protein during backward extraction.

Table 5.7 Effect of NaCl concentration in stripping solution on backward extraction

NaCl concentration, M	Total activity, U	Total protein, mg	Specific activity, U/mg	Recovery %		Fold purification
				A	P	
0.01	Nil	Nil	Nil	Nil	Nil	Nil
0.05	Nil	Nil	Nil	Nil	Nil	Nil
0.10	174.26	9.01	19.34	19.36	1.50	12.89
0.25	726.10	31.29	23.2	80.67	5.21	15.47
0.50	326.74	17.60	18.56	36.30	2.90	12.37
1.0	7.26	0.84	8.64	0.8	0.14	0.093

A: activity; P: protein; Forward reaction was carried out using crude lipase, pH 6.5, AOT, 50 mM at 25°C for 15 min and The mixture was centrifuged at 8000 g for 10 min to reach clear separation of two phases. The lipase recovery was carried out by backward extraction using NaCl in 50 mM Tris Cl buffer , pH 7.0 (1:1) at 25°C for 30 min.

Need of cosolvent (ethanol) has been reported for the back extraction of yeast lipase from reverse micelle comprising AOT/isooctane-isooctanol. In absence of cosolvent, irrespective of concentration of KCl in stripping solution, lipase was not recovered. Effect of increasing concentration of KCl in the presence of ethanol was prominent, but reasoning for this behavior was not discussed by the authors (Yu *et al*., 2003). Similarly back extraction of *Bacillus* amylase from tomac/isooctane-octanol reverse phase using 0.01 to 1.0 M NaCl (pH 11.30 – 12.50) has been reported. With NaCl concentration higher pH of the stripping solution was required for extraction of amylase. This was attributed to screening effect of NaCl which reduced electrostatic interaction between protein and surfactant headgroup (Brandani *et al*., 1994)

Alcohol used in stripping solution considered to be very effective because of amphiphilic property as a cosurfactant. Mathew and Juang (2005) reported addition of short chain alcohols in the backward extraction of papain from AOT/isooctane/reverse micelle which gave slightly lower back extraction (70 % at 10 % v/v/ alcohol) compared to the branched chain IPA and long chain hexanol (90 % at 10 % v/v alcohol).

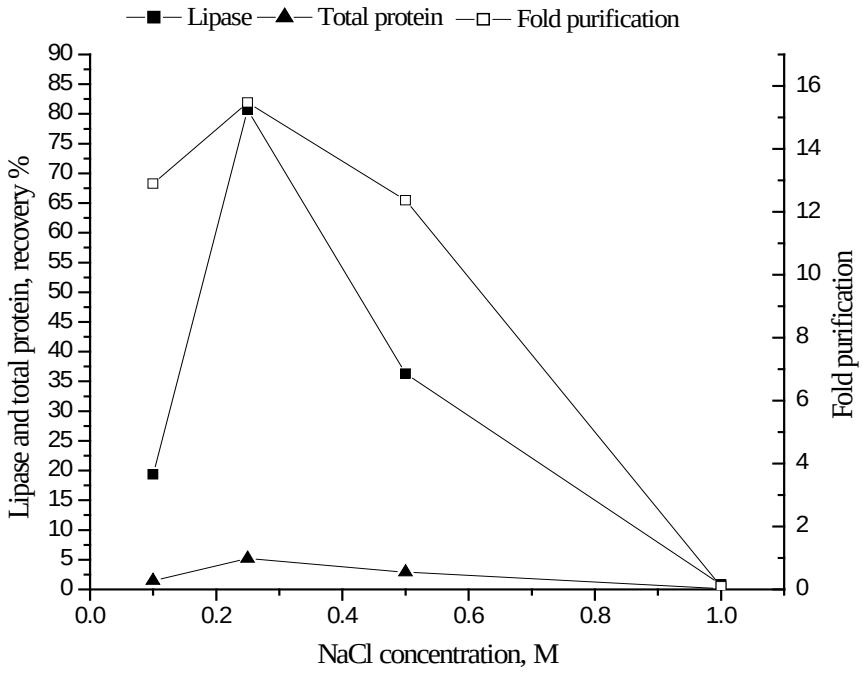

Fig. 5.5 Effect of NaCl concentration (M) on backward extraction of lipase

This back extraction of papain in the presence of alcohol was better as compared to 30 % of back extraction using 0.5 M KCl. This was attributed to interaction between the micelles to form the clusters. The percolation behavior increased in presence of IPA leading to lesser micelle micelle interaction.

In the present investigation, isopropanol (15 %) was used as cosurfactant in the backward extraction of *Pseudomonas* lipase. It has been observed from the Table 5.8 that up to 0.25 M concentration of NaCl, the isopropanol played important role and the extraction was maximum irrespective of NaCl concentration. Later on at the higher salt concentration the protein recovery was comparable but the total activity was less. The reason might be the higher salt concentration causing denaturation of the enzyme.

Table 5.8 Effect of Isopropanol (15 %) in stripping solution on backward extraction

NaCl concentration, M	Total activity, U	Total protein, mg	Specific activity, U/mg	Recovery %		Fold purification
				A	P	
0.10	724.20	31.30	23.13	80.46	5.21	15.46
0.25	727.39	31.02	23.44	80.82	5.17	15.62
0.50	323.0	32.82	9.84	35.88	5.47	6.56
1.0	7.34	32.04	0.22	0.008	5.34	0.14

A: activity; P: protein; Forward reaction was carried out using crude lipase, pH 6.5, AOT, 50 mM at 25°C for 15 min and The mixture was centrifuged at 8000 g for 10 min to reach clear separation of two phases. The lipase recovery was carried out by backward extraction using NaCl in 50 mM Tris Cl buffer , pH 7.0 and 15 % isopropanol (1:1) at 25°C for 30 min.

5.3.2.2.2 Effect of pH on backward extraction

Researchers have studied the effect of pH of stripping solution on the enzyme recovery from reverse micelle systems comprising cationic or anionic surfactants. The pI was found to have significant impact in many of the protein extractions. In the present investigations, the effect of pH on the backward reaction was checked in the pH range 4.0-9.0. In case of anionic surfactants such as AOT, the highest extraction has been reported at pH above pI whereas in presence of cationic surfactants such as CTAB, extraction was found to be highest at pH below pI (Nandini and Rastogi, 2009; Yu *et al*., 2003). Yu *et al.* (2003) also reported maximum extraction of yeast lipase from reverse micelle system comprising AOT at pH 9.0 which was above pI of 8.5. Though the extraction was maximum, pH above pI was documented to be responsible for structural destruction of lipase and hence activity was 15 % while that at pH 8.0 it was 40 %, therefore the authors had used alcohol for bulk enzyme extraction. Similarly in the study of back extraction of papain from reverse micelle containing AOT-isooctane was carried out at different pH values. Activity recovery was 90 %, when pH (around 10.0) of stripping solution (1M KCl) was above pI i.e. 9.6. The 80 % of enzyme activity recovery was observed at pH (around 8.5) less than pI but this behavior was not explained by the authors (Mathew and Juang, 2005).

On the contrary, in the present study, the highest extraction (80.67 %) of *Pseudomonas*

lipase from reverse micelles comprising anionic surfactant (AOT) was obtained at pH 7.0 which was below pI of 8.2 whereas 78.66 % enzyme extraction was obtained at pH 6.0 (**Table 5.9**).

Table 5.9 Effect of pH of stripping solution on backward extraction

pH	Total activity, U	Total protein, mg	Specific activity, U/mg	Recovery %		Fold purification
				A	P	
4.0	Nil	29.02	Nil	-	4.8	-
5.0	104.94	29.04	3.61	11.66	4.84	2.40
6.0	708.10	31.02	22.82	78.66	5.17	15.21
7.0	726.10	31.29	23.2	80.67	5.21	15.47
9.0	65.0	8.12	8.0	7.2	1.3	5.33

A: activity; P: protein; The aqueous phase (crude lipase, pH 6.5) was added to organic phase (isooctane and AOT 50 mM) in 1:2.5 proportion and kept at 25°C on magnetic stirrer for 15 min. The mixture was centrifuged at 8000 g for 10 min to reach clear separation of two phases. The lipase containing reverse micellar solution was added to aqueous stripping solution (containing 0.25 M NaCl in 50 mM Tris buffer) in the 1:1 proportion and kept at 25°C for 30 min.

Enzyme recovery above pI i.e. at pH 9.0 was only 7.2 %. This behavior could not be explained on the basis of pI of the *Pseudomonas* lipase but may be attributed to electrostatic interactions as in case of *A. niger* lipase reported by Nandini and Rastogi (2009). The authors have reported highest activity recovery of lipase (80.55 %) and extraction efficiency of protein (39.29 %) from reverse micelle system (comprising CTAB) at pH 7.0 which was much above the pI (4.2) of *A. niger* lipase. Authors have attributed this to minimum electrostatic interaction at backward extracting aqueous phase.

In another instance, increase in pH showed increase in protein extraction from reverse micelle comprising *Nocardiopsis* protease/AOT-isooctane micellar phase. Stripping solution was buffer of different pH viz. 5.0, 7.0 and 10.0. Enzyme recovery was highest at pH 5.0 (14 %) and reduced to 13 % at pH 10.0 whereas % protein extraction was increased from 45 % at pH 5.0 and 92 % at pH 10.0. Authors have attributed this to protein instability in the organic solvents with high pH but it was not correlated with pI (Monteiro *et al.,* 2005).

5.3.2.2.3 Effect of V_{aq}:V_{RM} on the backward extraction

To attain the concentration of the protein it is important to optimize aqueous to organic volume ratio less than 1 (Aires-Barros and Cabral, 1991). A range of 1:1 through 1:8 was used to study the effect of V_{aq}:V_{RM} on the solubilization of *Pseudomonas* lipase in the present investigation. There was no change in the total recovery of lipase though the volume of reverse micelle phase increased from 1 to 6 times that of the stripping solution (Fig 5.6). The enzyme recovery was 80 % whereas the fold purification was 15 from V_{aq}:V_{RM} of 1:1 to 1:6 (Table 5.10). Though protein recovery was significant (71 %) at V_{aq}:$V_{RM,}$ 1:7, there was no proper phase separation and hence the results can be erroneous. Similarly at V_{aq}:$V_{RM,}$ 1:8, there was no phase separation with less enzyme recovery of 40 % and fold purification of 8. Based on the results, the ratio 1:1 was finalized as V_{aq}:$V_{RM.}$

Table 5.10: Effect of V_{aq}:V_{RM} on backward extraction

V_{aq}:V_{RM}	Total activity, U	Total protein, mg	Specific activity, U/mg	Recovery %		Fold purification
				A	P	
1:1	725.20	31.23	23.22	80.56	5.21	15.40
1:2	726.20	31.24	23.24	80.68	5.21	15.49
1:3	726.14	31.27	23.22	80.67	5.21	15.48
1:4	726.18	31.28	23.21	80.67	5.21	15.47
1:5	726.10	31.28	23.20	80.67	5.21	15.47
1:6	726.18	31.29	23.20	80.68	5.25	15.47
1:7	640.15	28.14	22.74	71.11	4.68	15.16
1:8	360.18	27.16	13.26	40.02	4.52	8.84

A: activity; P: protein; The aqueous phase (crude lipase, pH 6.5) was added to organic phase (isooctane and AOT 50 mM) in 1:2.5 proportion and kept at 25°C on magnetic stirrer for 15 min. The mixture was centrifuged at 8000 g for 10 min to reach clear separation of two phases. The lipase containing reverse micellar solution was added to aqueous stripping solution (containing 0.25 M NaCl in 50 mM Tris Cl, pH 7.0) and kept at 25°C for 30 min.

Pessoa Jr and Vitolo (1998) have reported that with decreasing V_{aq}:V_{RM} ratio in backward extraction of *Candia kefir* inulinase using BDBAC reverse micelle system, the recovery was reduced from 85 % to 80 %. In backward extraction of *A. niger* lipase when V_{aq}:$V_{organic}$ proportion was increased to 1:1.5, recovery was increased to 80.5 %

and further increase in RM phase keeping volume of stripping solution constant the yield was decreased (Nandini and Rastogi, 2009).

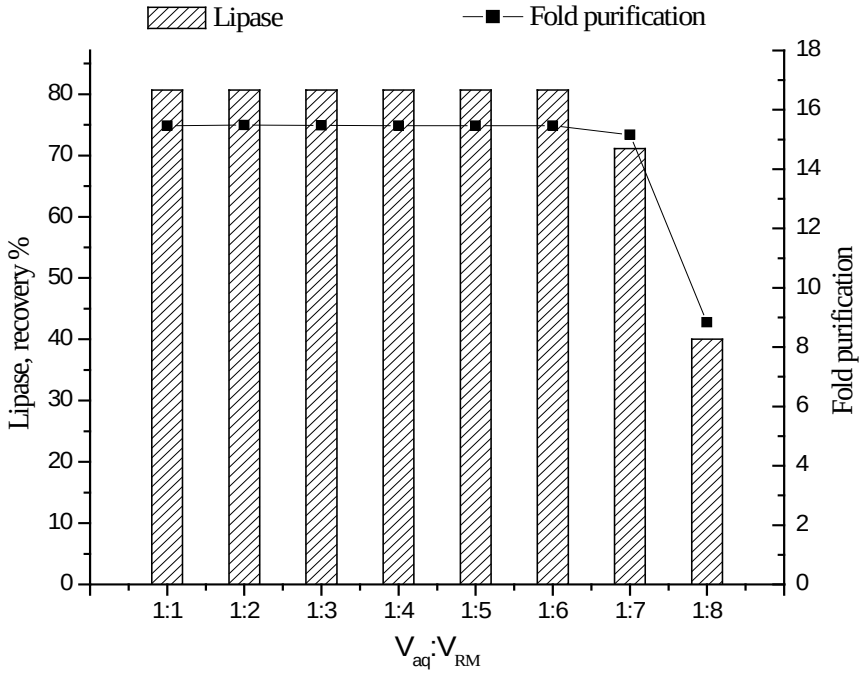

Fig 5.6 Effect of V_{aq}:V_{RM} on backward extraction

5.3.2.2.4 Effect of contact time on backward extraction

The study was carried out to check the effect of contact time on backward extraction lipase when the V_{aq}:V_{RM} was kept at 1:6. The highest lipase recovery was obtained at 30 min contact time with fold purification of 15.47 (Table 5.11). With increasing contact time from 30 to 50 min there was marginal decrease in enzyme recovery.

With increasing contact time, there was marginal loss of the activity which may be attributed to higher contact of the enzyme with organic phase. The extraction of *Pseudomonas* lipase by reverse micelle demonstrated in the present study could be used as an effective enzyme purification tool as it is single step, the time required is less and enzyme recovery is ~80 %. The process is significant especially when the partially purified enzyme is required. On the other hand the conventional purification methodology involved multistep operations and was time consuming. In the present investigations, the conventional purification resulted into 51 % yield with 14.98 fold purification whereas RME system showed 80 % yield with same fold purification.

Table 5.11 Effect of contact time on backward extraction

Time (min)	Total activity, U	Total protein, mg	Specific activity, U/mg	Recovery %		Fold purification
				A	P	
30	726.18	31.27	23.20	80.68	5.21	15.47
40	720.28	31.37	22.96	80.03	5.22	15.30
50	715.36	31.40	22.78	79.48	5.23	15.19

A: activity; P: protein; The aqueous phase (crude lipase, pH 6.5) was added to organic phase (isooctane and AOT 50 mM) in 1:2.5 proportion and kept at 25°C on magnetic stirrer for 15 min. The mixture was centrifuged at 8000 g for 10 min to reach clear separation of two phases. The lipase containing reverse micellar solution was added to aqueous stripping solution (containing 0.25 M NaCl in 50 mM Tris Cl, pH 7.0) in 1:1 ratio and kept at 25°C.

5.4 Conclusion

In the present investigation, the comparative analysis of results of lipase purification by conventional method and by reverse micellar system (AOT-Isooctane) revealed that the reverse micellar system was able to purify lipase more efficiently than the conventional purification protocol. Reverse micellar system gave 15 fold purification, with 80% recovery of *Pseudomonas* lipase, compared to 52% enzyme recovery and 15 fold purification in the conventional system. The analysis by by SDS PAGE confirmed the results. The analysis revealed a major protein band corresponding to ~18 kDa in both, conventionally purified and reverse micellar purified samples (Fig 5.7 c and d).

In reverse micellar extraction the forward extraction was optimized with reference to contact time, AOT concentration, pH of fermentation broth and V_{aq}:V_{RM} and backward extraction was optimized with reference to NaCl concentration in stripping solution, pH of stripping solution, V_{aq}:V_{RM} and contact time.

The overall time required for forward and backward extraction (15 min and 30 min respectively) was much less compared to the conventional method which requires 30 to 40 h.

Thus the reverse micelle based system can be considered favorable for preparation of partial purified lipase in single step and represents a valuable and economical source of lipase purification for application in various fields. The further research work towards

the large scale purification is in progress. A continuous forward backward extractor can be designed and the process can be scaled up at industrial level.

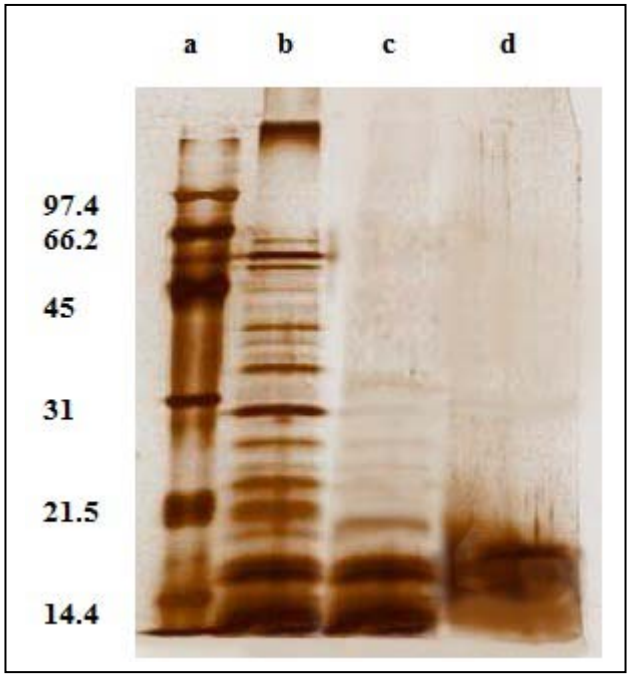

Fig 5.7 SDS PAGE of a. molecular weight marker b. crude lipase broth c. purified lipase by gel filtration d. purified tannase by reverse micelle system

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CHAPTER 6

Extraction and purification of tannase using reverse micelle

6.1 Introduction

Tannin acyl hydrolase (EC 3.1.1.20), commonly known as tannase is widely used for bioconversion of hydrolysable tannins to simple phenolics such as gallic acid and glucose. The tannase from *Aspergillus* has been widely used for production of gallic acid from tannins. Apart from its use in gallic acid production, this enzyme was also found to be industrially important because of its wide applications in tannery, pharmaceuticals, alcohol and beverage industries (Purohit *et al*., 2006). Despite the wide potential and applications, use of tannase at present is limited due to limited large scale studies, inhibitory nature of tannin substrate and in turn high cost (Aguilar *et al*., 2007; Beena *et al*., 2010).

Conventionally tannase has been purified by using multistep approach where precipitation and/or concentration, ultrafitration and chromatographic separation were used for purification. This chapter presents the study of tannase purification from intracellular preparation by conventional methods and by application of reverse micelle system. To the best of our knowledge, there is only one report till date on the reverse micellar extraction of tannase by Barthomeuf *et al.* (1994). Thus there is wide scope for exploration of reverse micellar application for single step purification of tannase and for checking the possibility of scale up at industrial scale.

6.2 Materials and Methods

6.2.1 Materials

Gallic acid, bovine serum albumin, rhodanine, methyl gallate and AOT were procured from Sigma Aldrich India. Molecular weight marker kit was from BioRad Laboratories Inc., USA. Coomassie brilliant blue R-250 was purchased from Ubichem Ltd. DEAE sepharose was from Amersham Pharmacia. Acrylamide, N, N- Methylene bis acryamide, tannic acid, tri sodium citrate, citric acid, sucrose, quinine hydrochloride, Folin Ciaoculteau reagent, CTAB, SDS, n-hexane and ethyl acetate were purchased

from Merck India Ltd. Triton X 100, NaCl, NH_4NO_3, $MgSO_4$, K_2HPO_4, KH_2PO_4, T80, Coomassie brilliant blue G250 were of AR grade and procured from Merck India, SRL and Hi Media.

6.2.2 Methods

6.2.2.1 Organism

Aspergillus allahabadi isolated from pods of *Caesalpinia spinosa* in our laboratory was used for production of intracellular tannase. The organism was maintained on slants of tannic acid glucose agar (tannic acid 10 g, glucose 5 g, K_2HPO_4 0.2, KCl 0.5, agar agar 30, per L) at 4°C and routinely subcultured every 30 days.

6.2.2.2 Production of intracellular crude tannase

The culture was grown in the optimized medium (tannic acid 20 g, $MgSO_4$ 0.5, K_2HPO_4 0.2, KCl 0.5 per L; pH 5.0) at 30°C and 180 rpm for a period of 40 h. At the end of incubation, the fungal mycelia in the fermentation broth were removed by filtration. The mycelia were macerated in liquid nitrogen, resuspended in citrate buffer, 50 mM, pH 5.0 and centrifuged. The supernatant was used as a crude enzyme preparation and purified further as described below.

6.2.2.3 Purification of tannase

Acetone precipitation

Intracellular enzyme was precipitated by 1:3 volumes of chilled acetone (-20°C). The precipitate was kept at 4°C for 3 h for complete precipitation and recovered by centrifugation at 10000 g for 20 min at 4°C. The precipitate was then dried in vacuum desiccator to remove traces of acetone and resuspended in citrate buffer (50 mM, pH 5.0).

Tangential flow filtration (TFF)

Acetone precipitated intracellular enzyme was desalted and concentrated using tangential flow filtration assembly (Pall Life Sciences) with 30 kDa cassette of omega membrane (m-polyethersulphone) (molecular weight cut off 30 kDa) at 4°C.

Ion exchange column chromatography

The concentrated and desalted protein was applied to DEAE sepharose column (1.5 x 27 cm) previously equilibrated with 50 mM citrate buffer, pH 5.0. The elution was carried out with a linear gradient of 0-0.5 M sodium chloride at flow rate 8 ml/h. Fractions (2 ml) were analyzed for protein and tannase activity.

6.2.2.4 Purification of tannase by reverse micelle system

a. Forward extraction (preparation of reverse micelle system)

Forward extraction of tannase was typically carried out by mixing crude tannase enzyme preparation in isooctane in presence of surfactant under constant stirring conditions at stated temperature and time. Surfactants viz. AOT, CTAB, SDS and T80 were added. All surfactants except AOT were directly added to crude tannase preparation while AOT was first dissolved in isooctane. Effect of different surfactants, different concentrations of CTAB (25-200 mM), contact time (5-30 min), pH of fermentation broth (4.5-8.0) and V_{aq}:V_{RM} (1:1-1:4) was studied. The forward extraction containing microemulsion was incubated at stated temperatures and stated time period with constant stirring. The reaction mixture was then centrifuged at 8000 g for 10 min at 25°C. The two phases (aqueous and organic) were separated and residual activity of tannase was checked in aqueous phase. Organic phase was subjected to the backward extraction.

b. Backward reaction (stripping of tannase from reverse micelle system)

For optimization of parameters of forward extraction, the backward extraction was carried out using stripping solution comprising NaCl (0.5 M in 50 mM citrate buffer, pH 5.0) at 30°C. The tannase enzyme was stripped out from organic phase by backward extraction. The parameters for backward extraction were optimized using stripping solution containing NaCl in different concentration (0.25-1.0 M) and pH (3.5-9.0). The effect of V_{aq}:V_{RM} (1:1-1:8) over the time period of 5-30 min was carried out at 30°C. The reaction mixture was centrifuged for 8000 g for 10 min. Tannase activity and total protein of stripped aqueous phase was checked. The purification of enzyme was checked by polyacrylamide gel electrophoresis. The activity of tannase was confirmed by localization in the gel.

6.2.2.5 Enzyme assay

Assay of tannase

Tannase activity was assayed using methyl gallate substrate as described by Sharma *et al.* (2000). Suitably diluted enzyme (0.25 ml) was added to substrate (0.25 ml, 0.01 M methyl gallate in 0.05 M citrate buffer, pH 5.0) and incubated at 30°C for 15 min. The liberated gallic acid was reacted with methanolic rhodanine (0.667 %, 0.3 ml) and further incubated at 30°C for 5 min followed by the addition of potassium hydroxide solution (0.5 M, 0.2 ml) and subsequently distilled water (4.0 ml). Absorbance was recorded at 520 nm. One unit of enzyme activity was defined as the amount of enzyme that liberated one micromole of gallic acid per ml per min under standard assay conditions.

6.2.2.6 Estimation of protein

Protein content was estimated according to Lowry *et al*. (1951) using bovine serum albumin (BSA) as a standard.

6.2.2.7 Polyacrylamide gel electrophoresis (PAGE)

Polyacrylamide gel electrophoresis was carried out in 8 % (w/v) polyacrylamide slab gel at pH 8.8. Protein bands were detected using commassie blue or silver staining method (Laemmli *et al.*, 1970; Sambrook and Russell, 2001).

6.2.2.8 Staining of proteins

Staining by Coomassie blue

The electrophoresed gel was immersed in the staining solution of coomassie brilliant blue R-250 (0.25 % in methanol:acetic acid) and placed on a slowly rocking platform for 3-4 h at room temperature. The gel is then removed and destained by soaking the gel into methanol:acetic acid solution on a rocking platform for 3-4 h, changing the destaining solution 3-4 times. After destaining, the gel was stored in deionized water (Sambrook and Russell, 2001).

Staining by silver staining

The electrophoresed gel was incubated in fixing solution (ethanol: acetic acid:water, 30:10:60) overnight at room temperature with gentle shaking to fix the proteins. The fixing solution was discarded and gel was incubated with 30 % ethanol for 30 min at

room temperature with gentle shaking. The gel was then removed and incubated deionized water for 10 min with gentle shaking. After the two washes of water, the gel was immersed in freshly prepared silver nitrate solution and incubated for 30 min at room temperature with gentle shaking. The gel was washed with deionized water and incubated in fresh developing solution (aqueous solution of 2.5 % sodium carbonate and 0.02 % formaldehyde) at room temperature with gentle shaking till the stained bands of protein appeared (Sambrook and Russell, 2001).

Localization of tannase activity in gel

The electrophoresis was carried out at 50 V for 4 h at 4°C, in Mini Protean II electrophoresis cell (BioRad). The localization of tannase activity in the gel was carried out according to Aoki *et al.* (1979). The gel was washed for 1 h in 100 ml of 2.5 % (v/v) Triton X-100. The gel was then washed with two 45 min washes of 100 ml, 10 mM acetate buffer pH 5.5 with constant shaking. The gel was then incubated in a solution containing 0.5 % (w/v) tannic acid in 0.1 M acetate buffer, pH 5.5 at 30°C with constant shaking. The tannic acid solution was then discarded and replaced with 100 ml of 0.5 % (v/v) quinine hydrochloride in 0.05 M acetate buffer, pH 5.5 at room temperature.

6.3 Results and Discussion

6.3.1 Purification of tannase by conventional method

Purification of tannase from *A.allahabadi* was carried out as described under materials and methods. Initially crude intracellular enzyme was obtained as described under materials and methods section 6.2.2.2.

Table 6.1 Summary of tannase purification

Step	Total volume, ml	Total protein, mg	Total activity, U	Specific activity, u/mg	Fold purification	Yield, %
Crude enzyme preparation	190	93.1	129.2	1.39	1.0	100
acetone precipitation and TFF	15	7.2	67.75	9.40	6.76	52.44
DEAE ion exchange chromatography	10	1.44	41.12	28.55	20.54	31.82

The intracellular enzyme was precipitated by chilled (-20°C) acetone followed concentration by TFF where low molecular weight impurities were also removed. At this step, tannase was purified by 6.76 fold and the yield was 52.44 %. The partially purified protein by TFF was subjected to DEAE ion exchange chromatography. Total 45 fractions were collected. The active fractions (no. 26-29) having significant tannase activity was pooled and lyophilized. This step showed 20.54 fold purification (**Table 6.1**). The purification profile was checked by using native PAGE. Similarly the purification of *P. variotii* tannase, by ammonium sulphate precipitation followed by DEAE cellulose column chromatography showed fold purification of 19.4 but with higher yield of 56 % (Mahendran *et al*., 2006). Zhong *et al.* (2004) reported use of ultrafiltration followed by DEAE sepharose for purification of *A. oryzae* tannase. The purified tannase showed specific activity of 50 IU/mg of the protein. The enzyme was further purified by Sephadex G-200 filtration with 30.5 fold purification and 17% yield.

6.3.2 Purification of tannase by reverse micellar system

There are no reports in published literature seen on reverse micelle application for tannase purification except that of Barthomeuf *et al.* (1994). However there are few reports available on the industrially important hydrophilic glycoproteins of high molecular weight and these reports were discussed in the present studies along with the results of the reverse micelle purification of *A. allahabadi* tannase.

For all experiments, crude tannase preparation (10 ml) of following specifications was used: total activity – 6.8 U ± 0.034, total protein 4.9 mg ± 0.025 and specific activity – 1.39 ± 0.007.

6.3.2.1 Effect various parameters on forward extraction of tannase

6.3.2.1.1 Effect of surfactant

In the present study, the effect of different surfactants was investigated. A typical forward extraction consisted of crude tannase enzyme, a surfactant and isooctane. Surfactants viz. CTAB and Triton X-100 were added at concentration of 25 mM in crude tannase enzyme, while AOT (25 mM) was first dissolved in isooctane. SDS was found to denature the crude tannase and hence not used for forward extraction. The crude tannase enzyme (10 ml) with surfactant was then mixed with 10 ml of isooctane.

The study of effect of surfactant was carried out at three different pH viz. 3.5, 5.0 and 7.0 based on the pI of *A. allahabadi* tannase at 4.5.

Out of four different surfactants, anionic surfactant AOT showed no recovery at different pH (3.5, 5.0, 7.0). There was no transfer of enzyme into the reverse micelle phase while enzyme activity was remained in the aqueous phase with marginal loss and hence AOT was not used for further studies. With nonionic surfactant, Triton X 100 there was little phase separation after completion of forward reaction and more than 80 % enzyme remained in aqueous phase whereas trace enzyme was observed at the interface and hence did not use further. In case of cationic surfactant CTAB, at pH 5.0 and 7.0, the enzyme recovery was around 60 % while at pH 3.5, the recovery was 20 %. Based on the results, CTAB was used for further studies.

6.3.2.1.2 Effect of contact time

In case of reverse micelle system, study of contact time has been considered to be very important because extraction of protein of interest takes place much faster than the time required for phase equilibrium (Dekker *et al*., 1989). The effect of contact time of forward extraction of *A. allahabadi* tannase was carried out for different time period from 5 to 30 min. The results were summarized in Table 6.2.

Table 6.2: Effect of contact time on forward extraction

Contact time (min)	Total activity, U	Total protein, mg	Specific activity, U/mg	Recovery %		Fold purification
				A	P	
5	2.51	0.209	12.09	35.35	4.5	9.15
10	3.66	0.362	10.11	51.55	7.8	8.40
15	3.58	0.397	9.01	50.42	8.6	6.82
20	3.60	0.461	7.80	50.70	10.02	5.90
25	3.62	0.465	7.78	50.98	10.10	5.89
30	3.63	0.471	7.70	51.13	10.23	5.83

A: activity; P: protein; CL: crude lipase. Forward extraction was carried out using crude tannase (pH 5.0) preparation, CTAB (25 mM) and isooctane 1:1 proportion and kept at 30°C. The mixture was centrifuged at 8000 g for 10 min to reach clear separation of two phases. Backward extraction was carried out using 0.5 M NaCl in 50 mM citrate buffer at proportion of 1:1 at 30°C for 30 min.

The reaction time of crude tannase preparation and isooctane containing CTAB was found to be less significant after certain time period. The increase in time, up to 10 min resulted into significant tannase recovery up to 60 %. Further increase in time (15 min and above) resulted into marginal decrease in enzyme extraction. As the reaction time increased from 5 to 30 min, there was increase in total protein content from 4 to 10 % respectively which in turn decreased the specific activity and fold purification (Fig 6.1).

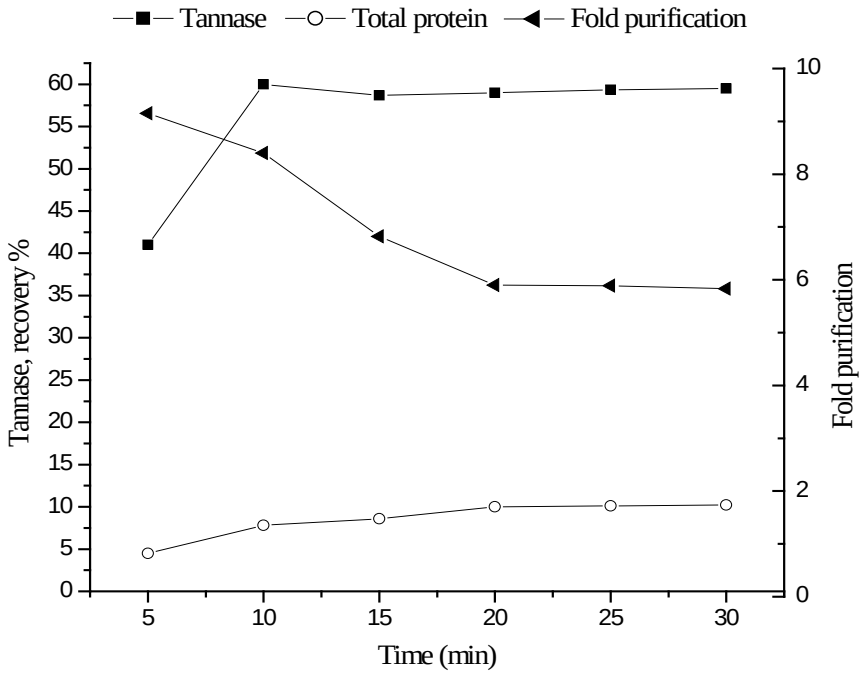

Fig 6.1: Effect of contact time on forward extraction of tannase

Similar findings have been documented in case of fibrinolytic serine protease - nattokinase from *Bacillus Natto*. Nattokinase was extracted using AOT/isooctane reverse micelle system. Enzyme recovery was 82 % within 8 min and then it decreased to 70 % at 12th min. Total protein recovery was increased to 35 % at 8th min and increased further to 40 % in next 4 min (Liu *et al*., 2004). In another instance of chitosanases from *Bacillus cereus* Chen *et al.* (2006) have shown correlation of increase in temperature to decrease in contact time. Authors have reported the highest recovery of chitosanases at 15°C i.e. 80 %, extracted within 80 min and there was reduction in enzyme recovery on further continuation of the extraction. Less activity of enzyme was attributed to the loss of activity due to higher contact of enzyme with organic phase. It

was also shown that on reduction in extraction temperature (10°C) the time required was increased to 120 min for the recovery of 58 % of total activity.

Based on the tannase recovery and fold purification contact time of 10 min was used for further studies.

6.3.2.1.3 Effect of CTAB concentration

Concentration of cationic surfactant CTAB showed significant effect on the forward extraction of tannase (Table 6.3). Barthomeuf *et al.* (1994) reported extraction of tannase of *A. niger* in reverse micelle comprising CTAB and isooctane. Different concentrations of CTAB viz. 50 mM, 80 mM and 100 mM were used. The highest extraction of 84 % of crude tannase was obtained at 80 mM whereas further increase in CTAB concentration resulted into less tannase yield.

Table 6.3: Effect of CTAB on forward extraction

CTAB concentration, mM	Total activity, U	Total protein, mg	Specific activity, U/mg	Recovery %		Fold purification
				A	P	
25	3.64	0.361	10.08	53.52	6.83	7.25
50	4.402	0.371	11.86	62.0	7.0	8.85
75	4.757	0.380	12.50	67.0	7.1	9.33
100	4.68	0.396	11.81	65.9	7.47	8.81
150	4.33	0.380	11.39	60.0	7.17	8.5
200	4.1	0.360	11.38	57.74	6.79	8.49

A: activity; P: protein; Forward extraction was carried out using crude tannase (pH 5.0) preparation, CTAB and isooctane 1:1 proportion and kept at 30°C for 10 min. The mixture was centrifuged at 8000 g for 10 min to reach clear separation of two phases. Backward extraction was carried out using 0.5 M NaCl in 50 mM citrate buffer at 1:1 proportion at 30°C for 30 min.

Similarly in the present investigation, it is evident from Fig 6.2 that tannase recovery was increased from 53 %-67 % as the CTAB concentration increased from 25 mM to 75 mM respectively. This may be attributed to the fact that an increase in surfactant concentration increases surfactant aggregation and reverse micelle (Pessoa Jr and Vitolo, 1998). Further increase in CTAB concentration (100 mM and above) decreased enzyme as well as total protein recovery. This might be correlated with the micellar clustering which decreased the interfacial area available to the target molecule resulting

in decrease in the extraction capacity of reverse micelle. In addition to this, there might have been more intermicellar collision which occurs more frequently because of their large population at a higher surfactant concentration. This results in deassembling/deformation of reverse micelle leading to decreased extraction of enzyme (Pessoa Jr and Vitolo, 1998).

The effect of CTAB concentration on enzyme recovery has been well documented for purification of phenol oxidase (EC 1.14.18.1) from apple. Extraction was carried out in reverse micelle system of a cationic surfactant, CTAB/ isooctane – hexanol. 60 % recovery was obtained at 100 mM concentration while it was 35 % and 40 % at 50 mM and 150 mM respectively. Increased solubilization of enzyme at higher CTAB concentration (100 mM) has been attributed to increased electrostatic interaction. Further increase in CTAB concentration i.e. 150 mM may have caused micellar clustering and thus reduction in interfacial area available to host protein and in turn resulted into less recovery of enzyme (Imm and Kim, 2009).

The fold purification (9.33) of *A. allahabadi* tannase was found to be highest at 75 mM CTAB concentration along with highest tannase recovery (67 %). Based on these results, 75 mM CTAB was used for further experimentation.

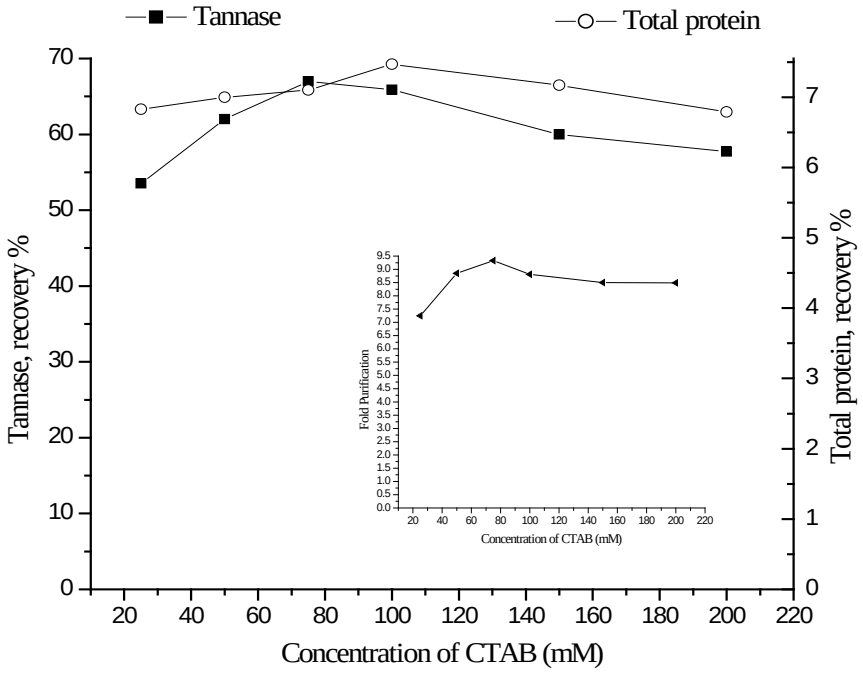

Fig 6.2 Effect of CTAB on forward extraction of tannase

6.3.2.1.4 Effect of pH of fermentation broth

The effect of pH on tannase extraction by reverse micelle system was studied using a pH range of 4.5 – 8.0. The reverse micelle system consisted of cationic surfactant CTAB at 75 mM concentration. The highest enzyme extraction (75 %) was observed at pH 6.0 and 7.0 along with the highest fold purification of 12.0 (Table 6.4 and Fig 6.3).

As the pH increases slightly above the pI of the enzyme, charge on the enzyme becomes negative and below pI it becomes positive (Rawn, 2004). In case of cationic surfactant based reverse micelle system higher pH than pI favours the forward extraction. This forward extraction is the function of electrostatic interaction between charged protein molecule and charged head groups of reverse micelle (Tonova and Lazarova, 2008). In the present study, the effect of pH of crude tannase preparation on tannase recovery could be explained on the basis of model of electrostatic interaction. The pI of *A. allahabadi* tannase was 4.3. Near pI i.e. at pH 4.5, enzyme recovery was 40 % while it was 75 % when the pH was far (6.0 and 7.0) from pI.

Table 6.4 Effect of pH on forward extraction

pH	Total activity, U	Total protein, mg	Specific activity, U/mg	Recovery %		Fold Purification
				A	P	
4.5	2.82	0.295	9.49	40.75	5.62	7.19
5.0	4.750	0.379	12.50	68.74	7.24	9.47
6.0	5.254	0.330	15.92	75.92	6.29	12.06
7.0	5.253	0.331	15.87	75.90	6.31	12.02
8.0	4.321	0.331	13.05	62.43	6.31	9.89

A: activity; P: protein; Forward extraction was carried out using crude tannase preparation, CTAB (75 mM) and isooctane in 1:1 proportion and kept at 30°C for 10 min. The pH of crude tannase preparation was adjusted to the desired pH with 1 M NaOH or HCl. The mixture was centrifuged at 8000 g for 10 min to reach clear separation of two phases. Backward extraction was carried out using 0.5 M NaCl in 50 mM citrate buffer at 1:1 proportion at 30°C for 30 min.

In a lysozyme extraction from freeze dried egg white using reverse micelles formed by the cationic surfactant CDAB (cetyl dimethyl ammonium bromide) Noh and Imm (2005) have found that solubilization of protein molecules was largely governed by aqueous pH of the protein solution. The maximum transfer of lysozyme (65 % of feed lysozyme) was obtained when the pH of aqueous phase was 12.0. When the pH was

maintained between pH 6.0 - 9.0 major egg proteins ovalbumin (pI 4.5) and ovatransferrin (pI 6.0) were transferred to reverse micelle and lysozyme remained in the aqueous phase. At pH 9.0, lysozyme was enriched in aqueous phase by 32.3 fold because all other proteins were transferred to the reverse micelle. This was attributed to the electrostatic interaction between charged protein molecules and polar head of surfactant which drives the protein solubilization.

Similarly Brandini and Giacomo (1993) reported extraction of commercial *Bacillus* α amylase using reverse micellar system consisting of isooctane, octanol (1 %, v/v) and cationic surfactant TOMAC (tri octyl methyl ammonium chloride). It was observed that good extraction yield was obtained at pH values between 9.3 and 10.5. Authors attributed this to the sufficient negative charge on the protein molecule to interact with positive surfactant head groups. At pH higher than 10.5, the enzyme α amylase was denatured, so it was not considered for the study. Partition coefficient K, ratio of protein in reverse micelle phase to fermentation broth was 20 at pH 10.1 when 0.04 M TOMAC was used.

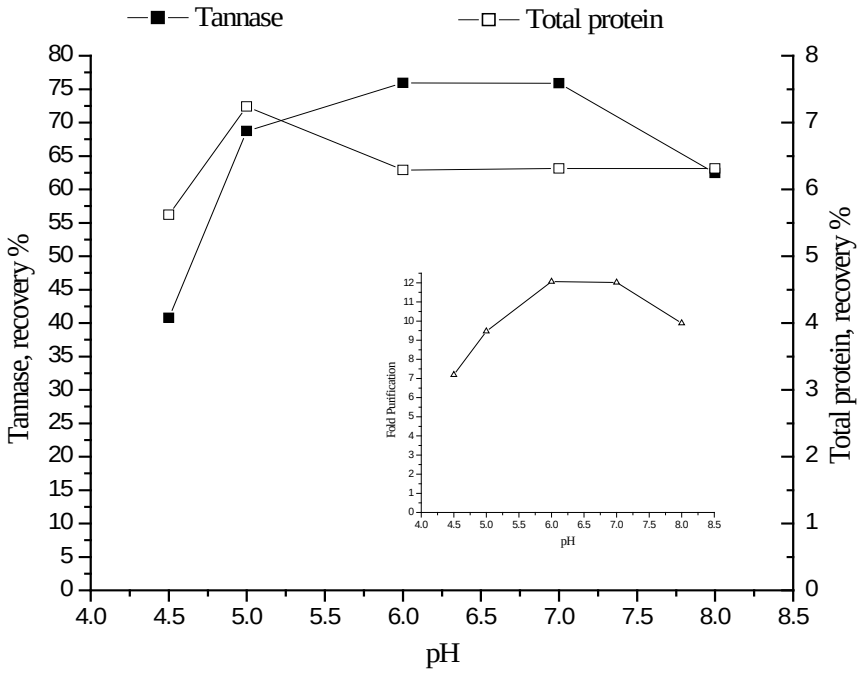

Fig. 6.3: Effect of pH on forward extraction of tannase

6.3.2.1.5 Effect of crude enzyme: organic phase (V_{aq}:V_{RM}) ratio

To study the extent to which enzyme can be enriched in the reverse micelle system, it is important to study the proportion of V_{aq}: V_{RM} higher than 1:1. The inulinase solubilization in BDBAC/isooctane-hexanol reverse micelle system was found to be decreased as the organic phase volume increased and this might be due to the less number of micelles to enfold all the inulinase present in the broth (Pessoa Jr and Vitolo, 1998).

The results of the present study regarding the effect of ratio of V_{aq}: V_{RM} was different than that obtained in case of inulinase (Pessoa and Vitolo, 1998). In the present study, the highest enzyme recovery was obtained at 1:2 proportion and remained unchanged in spite of further change in the ratio of crude enzyme: isooctane (Fig. 6.4).

Table 6.5: Effect of ratio of V_{aq}:V_{RM} on forward extraction

V_{aq}: V_{RM}, v/v	Total activity, U	Total protein, mg	Specific activity, U/mg	Recovery %		Fold purification
				A	P	
1:1	5.251	0.330	15.92	76.71	6.37	12.06
1:2	5.538	0.336	16.48	80.85	6.48	12.48
1:3	5.538	0.338	16.38	80.85	6.62	12.41
1:4	5.538	0.341	16.24	80.85	6.68	12.30

A: activity; P: protein; Forward extraction was carried out using crude tannase preparation (pH 6.0), CTAB (75 mM) and isooctane and kept at 30°C for 10 min. The mixture was centrifuged at 8000 g for 10 min to reach clear separation of two phases. Backward extraction was carried out using 0.5 M NaCl in 50 mM citrate buffer at 1:1 proportion at 30°C for 30 min.

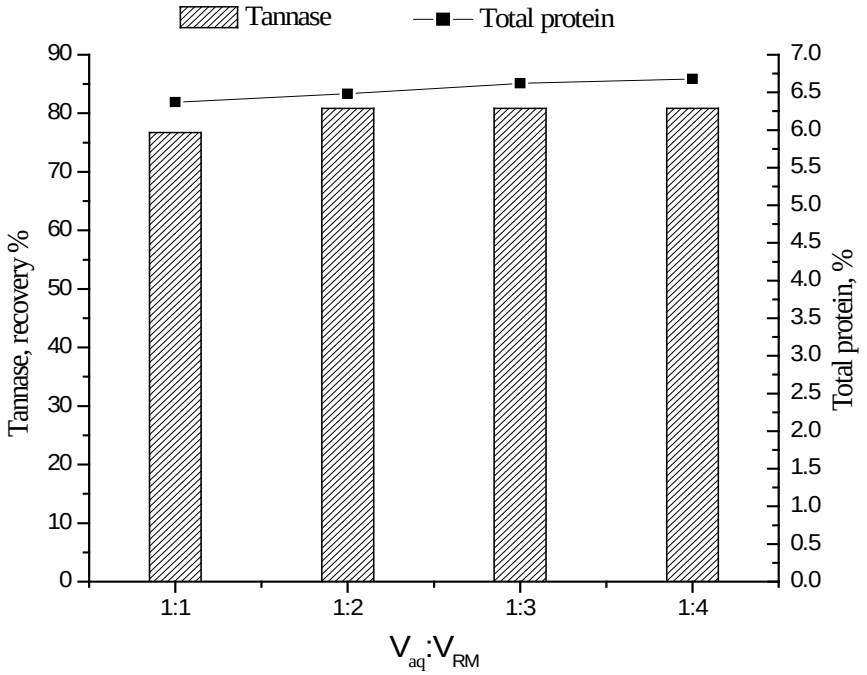

Fig. 6.4: Effect of V_{aq}:V_{RM} ratio on forward extraction of tannase

There was marginal increase in total protein content with increase in isooctane volume. The increase in total protein can be attributed to the undesirable protein extraction by reverse micelle phase. The fold purification was also found to be changed marginally with the increase in isooctane volume (Table 6.5).

6.3.2.2 Effect of various parameters on backward extraction of tannase

6.3.2.2.1 Effect of pH of stripping solution

In case of backward extraction of enzyme from reverse micellar phase pH plays an important role. Though it has not been documented emphatically, in case of most of the hydrophilic enzymes, back extraction is favoured below pI of the enzyme. If pH at which back extraction is done denatures the enzyme then different strategy needs to be adapted (Tonova and Lazarova, 2008). This hypothesis has been supported by the results of back extraction of *B. licheniformis* α amylase from CTAB/isooctane RM phase at pH lower than pI (6.5) (Tonova and Lazarova, 2005).

Table 6.6: Effect of pH of stripping solution on backward extraction

pH	Total activity, U	Total protein, mg	Specific activity, U/mg	Recovery %		Fold Purification
				A	P	
3.5	5.538	0.336	16.48	80.02	6.4	12.48
5.0	2.128	0.246	8.65	30.75	4.68	6.55
7.0	-	0.292	-	-	5.56	-
9.0	-	0.192	-	-	3.6	-

A: activity; P: protein; Forward extraction was carried out using crude tannase preparation, CTAB (75 mM) and isooctane in 1:2 proportion and kept at 30°C for 10 min. The mixture was centrifuged at 8000 g for 10 min to reach clear separation of two phases. Backward extraction was carried out using 0.5 M NaCl in 50 mM citrate buffer at 1:1 proportion at 30°C for 30 min.

We tested back extraction of tannase from *A. allahabadi* from CTAB/ isooctane reverse micelle phase in pH range of 3.5 – 9.0. As expected there was no enzyme recovery in the alkaline pH range (i.e. 7.0 and 9.0) whereas total protein was obtained in the range of 3 – 5 %. The highest tannase recovery (80.02 %) was observed at pH 3.5 with 12.48 fold purification followed by 31 % enzyme recovery at pH 5.0 with 6.55 fold purification (Table 6.6). Our findings were in agreement with the findings of Tonova and Lazarova (2005). The enzyme was stable at pH 3.5 and so use of stripping solution of pH 3.5 did not denature the enzyme. 30 % tannase was recovered at pH (5.0) slightly above pI (4.5) and that may be because of interaction of salt with the RM system. Similarly Barthomeuf *et al.* (1994) reported back extraction of *A. niger* tannase from CTAB (80 mM)/isooctane reverse micelle phase at pH 4.0, much lesser than pI of tannase reported in the literature. But the authors have not mentioned the pI of *A. niger* tannase in the study.

6.3.2.2.2 Effect of NaCl concentration

KCl/KBr, NaCl and $CaCl_2$ are among the most used salts in Winsor II systems (reverse micelle) of ionic surfactants for both protein solubilization and stripping. Usually at the extraction step, more hydrated ion pairs are involved e.g. NaCl, $CaCl_2$ while at the recovery stage, less hydrated ion pairs are used e.g. KCl and KBr (Tonova and Lazarova, 2008).

Alves *et al.* (2003) studied backward extraction of commercial penicillin acylase with 1 M KCl and the organic phase was characterized with low water content (W_0 – moles of water/moles of surfactant). W_0 value of 3.9 after backward extraction was W_0 value of

fresh reverse micelle made for forward extraction. It was an indication of stripping of all protein in fresh aqueous phase. The difference in the osmotic pressures between the reverse micellar core and bulk stripping phase has been considered as driving force of the water transfer by permeation through the surfactant shell. This movement has been two way and led to destabilization of RMS where protein has been back extracted. When low ionic strength has been used, the reverse micelle swelling happened and the protein was released.

To extract the *A. allahabadi* tannase from reverse micellar system, in the present studies, the stripping solution was prepared containing different NaCl concentration (pH 3.5). The aqueous stripping solution was added to the tannase containing reverse micellar solution in the 1:1 proportion and kept for 30 min at 30°C. The mixture was centrifuged at 8000 g for 10 min to reach a clear separation of two phases. It can be seen from Table 6.7 that 0.5 M NaCl resulted into highest tannase extraction (81.40 %) and fold purification (12.88). The tannase recovery observed to be the same with further increase in NaCl concentration of 1.0 M though fold purification was marginally lower (12.84). There was no significant change in total protein extraction over the range of NaCl concentration of 0.25 M – 1.0 M. In the present investigation, for forward extraction no salt was used to transfer the enzyme in RMS. This might be reason that even at lower concentration of NaCl the enzyme was back extracted efficiently.

In a similar study, commercial glucose oxidase, a hydrophilic enzyme was back extracted effectively with 1.0 M NaCl. In the forward extraction of the enzyme, only CTAB/isooctane was used as RM phase and no salt was used (Ferreira *et al*., 2005).

Interesting observation in case of forward and backward extraction of *Candida kefir* inulinase using RM comprising BDBAC in isooctane/hexanol has been reported by Pessoa Jr and Vitolo (1998). For all forward extraction studies, the stripping solution (0.1 M acetate buffer, 0.5 M NaCl, pH 4.5) was kept constant. In forward extraction, when ionic strength was kept very low i.e. no salt was used, 48 % of enzyme recovery was observed whereas at 0.25 M salt concentration, no enzyme activity was recovered. These findings established a correlation that if no salt was used in the forward extraction, even NaCl at lower concentration could be used in the stripping solution. This increased the osmotic pressure and helped to release the protein at interface and then ultimately to stripping solution.

Table 6.7: Effect of NaCl concentration in stripping solution on backward extraction

NaCl concentration, M	Total activity, U	Total protein, mg	Specific activity, U/mg	Recovery %		Fold purification
				A	P	
0.25	5.54	0.336	16.49	78.0	6.33	12.48
0.50	5.780	0.340	17.00	81.40	6.41	12.88
1.0	5.780	0.341	16.95	81.40	6.43	12.84

A: activity; P: protein; Forward extraction was carried out using crude tannase enzyme (pH 6.0), CTAB (75 mM) and isooctane in 1:2 proportion and kept at 30°C for 10 min. The mixture was centrifuged at 8000 g for 10 min to reach clear separation of two phases. Backward extraction was carried out using NaCl in 50 mM citrate buffer, pH 3.5 at 1:1 proportion at 30°C for 30 min.

There are many examples of use of isopropanol in backward extraction of enzymes such as chymotrypsin from AOT/DOPA/isooctane reverse micelle, porcine protease from AOT isooctane reverse micelle, the bovine chymosin from AOT isooctane reverse micelle and hemoglobin from DOPA/isooctane reverse micelle (Mathew and Juang, 2007). In the present study, when different concentration of isopropanol was used in the stripping solution at 0.5 M NaCl, there was no improvement in the extraction of the *A. allahabadi* tannase whereas in absence of isopropanol, same results were observed. Therefore, it was clear that the electrostatic interaction was significant between the salt and headgroup of surfactant of reverse micelle in the backward extraction of protein.

6.3.2.2.3 Effect of Vaq:V_{RM} on backward extraction of tannase and its correlation with extraction contact time

A. Effect of on Vaq:V_{RM} on backward extraction of tannase

Volume ratio of aqueous to RM phase (V_{aq}:V_{RM}) is a critical parameter in extraction and concentration of enzymes. In the forward extraction, the volume of RM phase is equal to or more than the crude intracellular preparation whereas in the backward extraction, there is a possibility of reduction in volume by extracting enzyme in smaller volume from enzyme enriched RM phase.

Table 6.8 a: Effect of V_{aq}:V_{RM} on backward extraction of tannase for 30 min

V_{aq}:$V_{RM,}$ v/v	Total activity, U	Total protein, mg	Specific activity, U/mg	Recovery %		Fold purification
				A	P	
1:1	5.53	0.32	17.28	81.40	6.41	12.86
1:2	5.54	0.32	17.31	81.42	6.41	12.80
1:3	5.53	0.33	16.76	81.38	6.41	12.77
1:4	5.55	0.32	17.76	81.40	6.41	12.78
1:5	5.53	0.331	17.37	81.43	6.39	12.81
1:6	5.54	0.319	17.37	81.40	6.39	12.81
1:7	5.49	0.312	17.60	80.84	6.30	12.78
1:8	5.41	0.32	16.91	79.01	6.24	12.73

A: activity; P: protein; Forward extraction was carried out using crude tannase preparation (pH 6.0), CTAB (75 mM) and isooctane in 1:2 proportion and kept at 30°C. The mixture was centrifuged at 8000 g for 10 min to reach clear separation of two phases. Backward extraction was carried out using 0.5 M NaCl in 50 mM citrate buffer, pH 3.5 at 30°C for 30 min.

Liu *et al.* (2006) studied effect of V_{aq}:V_{RM} in backward extraction of nattokinase in AOT/isooctane reverse micelle system and its correlation with contact time. Authors have shown that decrease in volume of stripping solution (Vaq) increased the contact time. 80 % nattokinase recovery in 18 min was obtained when the proportion of stripping solution (V_{aq}) to RM phase (V_{RM}) (composed of 15 % isopropanol, 0.2 M KCl, pH 8.0, 20 mM phosphate buffer) was 1:1 and when the proportion was 1:6, the time required for the same recovery was 25 min. Further change in proportion to 1:8/1:10 resulted into less recovery (70 % and 60 % respectively) of crude enzyme. Similarly the effect of V_{aq}/V_{RM} in backward reaction was studied in the present investigation. The aqueous stripping solution (containing 0.5 M NaCl, pH 3.5) was added to the tannase containing reverse micellar solution in the 1:1 to 1:8 proportion and kept at 25°C for 30 min under constant stirring conditions. The mixture was centrifuged at 8000 rpm for 10 min to reach a clear separation of two phases. The highest enzyme recovery was obtained when V_{aq}:V_{RM} was 1:5 and 1:6 (Table 6.8a). Because of the advantage of lower stripping volume, V_{aq}:V_{RM} 1:6 was considered for further studies.

Table 6.8 b Effect of V_{aq}:V_{RM} (1:1) on backward extraction of tannase for different time period

Time (min)	Total activity, U	Total protein, mg	Specific activity, U/mg	Recovery %		Fold Purification
				A	P	
5	3.38	0.188	17.98	48.84	3.58	13.62
10	5.548	0.304	18.25	80.16	5.79	13.82
15	5.54	0.314	17.64	80.05	5.98	13.36
20	5.538	0.320	17.31	80.02	6.10	13.11
25	5.54	0.326	16.99	80.05	6.21	12.87
30	5.54	0.337	16.44	80.05	6.42	12.45

A: activity; P: protein; Forward extraction was carried out using crude tannase preparation, CTAB (75 mM) and isooctane in 1:2 proportion and kept at 30°C. The mixture was centrifuged at 8000 g for 10 min to reach clear separation of two phases. Backward extraction was carried out using 0.5 M NaCl in 50 mM citrate buffer, pH 3.5 at 30°C.

Study of correlation of contact time with ratio of V_{aq}: V_{RM} was carried out using 1:1, 1:3 and 1:6 over a time period of 30 min with time interval of 5 min. The results have been shown in Tables 6.8 (b, c and d) and Figures 6.5 and 6.6. Study of contact time is very important because it gives the minimum time required for the equilibrium between two phases. With decreasing V_{aq}:V_{RM} ratio, the time required for the back extraction was increased.

Table 6.8 c Effect of V_{aq}:V_{RM} (1:3) on backward extraction of tannase for different time period

Time (min)	Total activity, U	Total protein, mg	Specific activity, U/mg	Recovery %		Fold Purification
				A	P	
5	1.662	0.075	22.16	24.01	1.43	16.79
10	3.324	0.170	19.55	48.03	3.24	14.81
15	4.54	0.246	18.45	65.60	4.69	13.98
20	5.54	0.316	17.53	80.05	6.02	13.28
25	5.536	0.320	17.3	79.99	6.09	13.10
30	5.540	0.324	17.09	80.0	6.17	12.95

A: activity; P: protein; Forward extraction was carried out using crude tannase preparation, CTAB (75 mM) and isooctane in 1:2 proportion and kept at 30°C. The mixture was centrifuged at 8000 g for 10 min to reach clear separation of two phases. Backward extraction was carried out using 0.5 M NaCl in 50 mM citrate buffer, pH 3.5 at 30°C

For 1:1 proportion, time required for highest tannase recovery was 10 min after which total protein content was found to be increased which in turn decreased the specific activity. The time required for 1:3 and 1:6 proportion was 20 min and 30 min. The exact time may be less than time recorded but for practical reason the reading were taken for time interval of 5 min.

These results are similar to results shown by Liu *et al* (2006) which are discussed earlier. The time required for forward extraction was similar to the time required for backward extraction when the proportion was 1:1.

Table 6.8 d Effect of V_{aq}:V_{RM} (1:6) on backward extraction of tannase for different time period

Time (min)	Total activity, U	Total protein, mg	Specific activity, U/mg	Recovery %		Fold Purification
				A	P	
5	1.55	0.070	22.14	22.18	1.33	16.77
10	2.32	0.113	20.53	33.20	2.15	15.55
15	3.22	0.174	18.50	46.08	3.31	14.01
20	3.98	0.221	18.0	56.95	4.21	13.63
25	4.70	0.268	17.53	67.26	5.11	13.28
30	5.538	0.335	16.53	79.29	6.38	12.52

A: activity; P: protein; Forward extraction was carried out using crude tannase preparation, CTAB (75 mM) and isooctane in 1:2 proportion and kept at 30°C. The mixture was centrifuged at 8000 g for 10 min to reach clear separation of two phases. Backward extraction was carried out using 0.5 M NaCl in 50 mM citrate buffer, pH 3.5 at 30°C

As V_{aq}:V_{RM} proportion decreased, the time required for backward extraction increased. Though the time required for backward extraction was more at lower proportion of 1:6, the enzyme was concentrated 3 times. In the forward extraction, best results were obtained with 1:2 proportion of protein aqueous phase:RM phase. The enzyme was diluted by 2 fold in the forward extraction. When 1:1 proportion was used in backward extraction, this dilution factor remained same. When the proportion of V_{aq}:V_{RM} in backward extraction was reduced to 1:6, the protein was concentrated by 6/2 times i.e. approximately 3 times. When the protein is to be extracted from fermentation broth, the reduction in volume of the broth is a need of downstream processing. This study is useful while working out downstream strategy for enzyme extraction, concentration and

purification. Therefore the best results for backward extraction were V_{aq}:V_{RM} 1:6 for 30 min at pH 3.5.

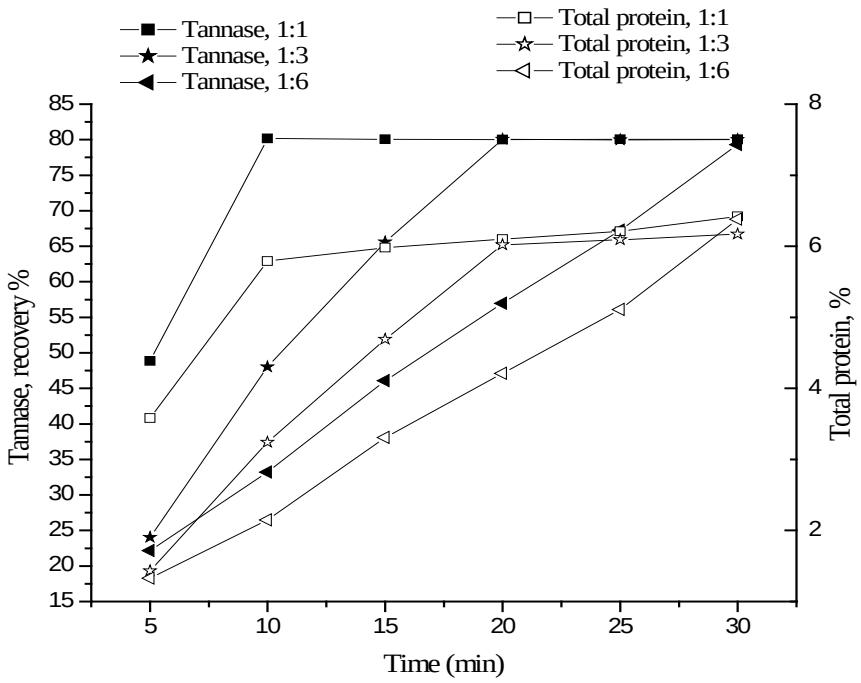

Fig 6.5 Effect of V_{aq}:V_{RM} on backward extraction of tannase and total protein

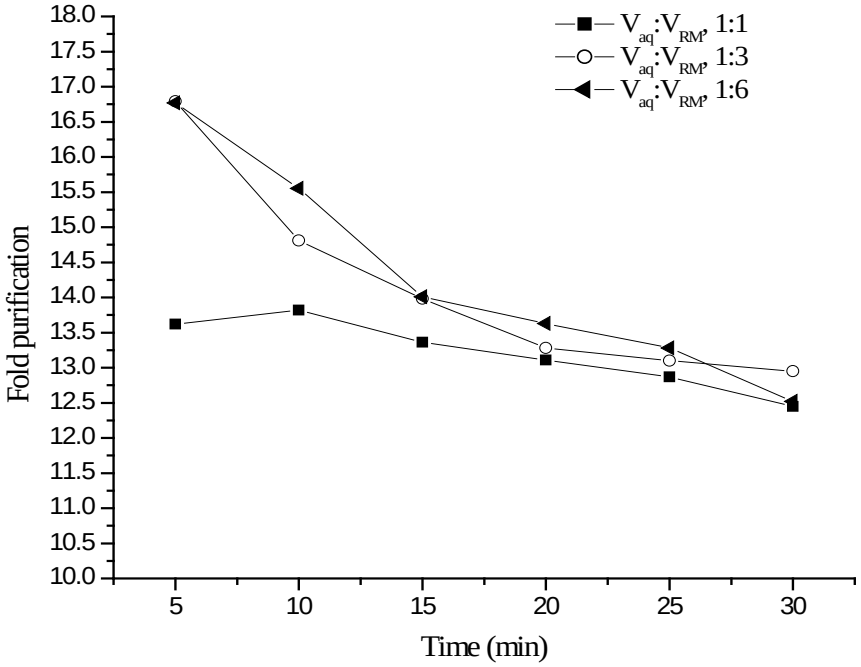

Fig 6.6 Effect of V_{aq}:V_{RM} on backward extraction and fold purification of tannase

B. Effect of extended contact time on backward extraction of tannase

The contact time of 30 min was found to extract highest tannase when V_{aq}:V_{RM} ratio was 1:6. Further the effect of extended contact time up to 60 min on the backward extraction of tannase was studied to check the tannase recovery. The results are shown in Table 6.9.

Table 6.9 Effect of extended contact time on backward extraction

Time, min	Total activity, U	Total protein, mg	Specific activity, U/mg	Recovery %		Fold purification
				A	P	
30	5.780	0.339	17.05	81.17	6.42	12.72
45	5.774	0.375	15.40	81.09	7.10	11.49
60	5.782	0.381	15.17	81.20	7.21	11.32

A: activity; P: protein; Forward extraction was carried out using crude tannase preparation (pH 6.0), CTAB (75 mM) and isooctane in 1:2 proportion and kept at 30°C. The mixture was centrifuged at 8000 g for 10 min to reach clear separation of two phases. Backward extraction was carried out using 0.5 M NaCl in 50 mM citrate buffer, pH 3.5 at 1:6 proportion at 30°C.

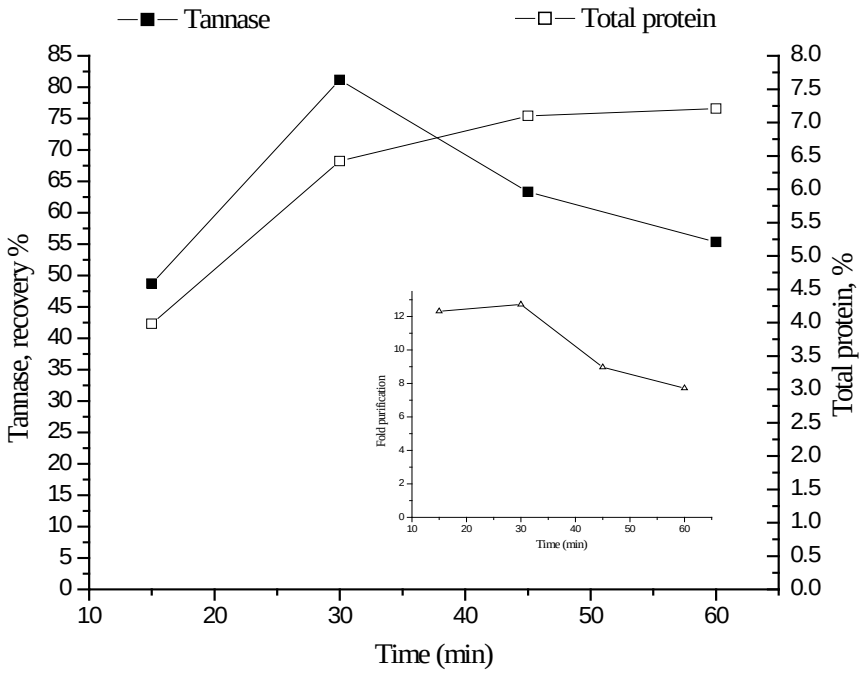

Fig. 6.7: Effect of contact time on backward extraction of tannase

Increase in time to 45 and 60 min did not show significant change in enzyme recovery whereas total protein was observed to increase gradually over the time period (Fig. 6.7). This in turn resulted into decrease in fold purification over a period of 60 min. Based on the highest tannase extraction (81.17 %) and fold purification (12.72), 30 min contact time was found to be optimum for backward extraction of tannase.

6.4 Conclusion

The aim of the present study was to evaluate the reverse micelle based system for tannase purification and comparative analysis of purification using conventional tannase purification methodology. It was evident that reverse micelle based system (CTAB-Isooctane) was efficient than conventional method for tannase purification with 80 % of tannase recovery, compared to only 31 % by the conventional method. However the fold purification of tannase was less (12.74 fold) in case of reverse micelle based system than that of conventional purification system (20.54). The purity was checked by SDS-PAGE and the results were shown in Fig 6.8. The enzyme was localized in the gel and identified as transparent band of tannase against the opaque background (Fig **6.9**). The reverse micellar system is a single step process which requires much less time compared to the conventional multi step process. Various forward extraction (contact time, pH of fermentation broth, CTAB concentration, V_{aq}:V_{RM} ratio) and backward extraction (NaCl concentration, pH of stripping solution) parameters were optimized to achieve the recovery of tannase from 50 % to 81 %. It was also found that with decrease in V_{aq}:V_{RM}, the time required for backward extraction increased (V_{aq}:V_{RM}, 1:6/ 30 min).

In the light of these findings, our study has allowed the better understanding of reverse micelle based system for tannase purification. Although further studies are required to increase the fold purification of tannase, reverse micelle extraction can be considered as a promising tool for partial purification of tannase enzyme with significant recovery.

Fig 6.8 SDS PAGE of a. crude tannase preparation b. tannase purified by reverse micelle system c. tannase purified by DEAE ion exchange chromatography

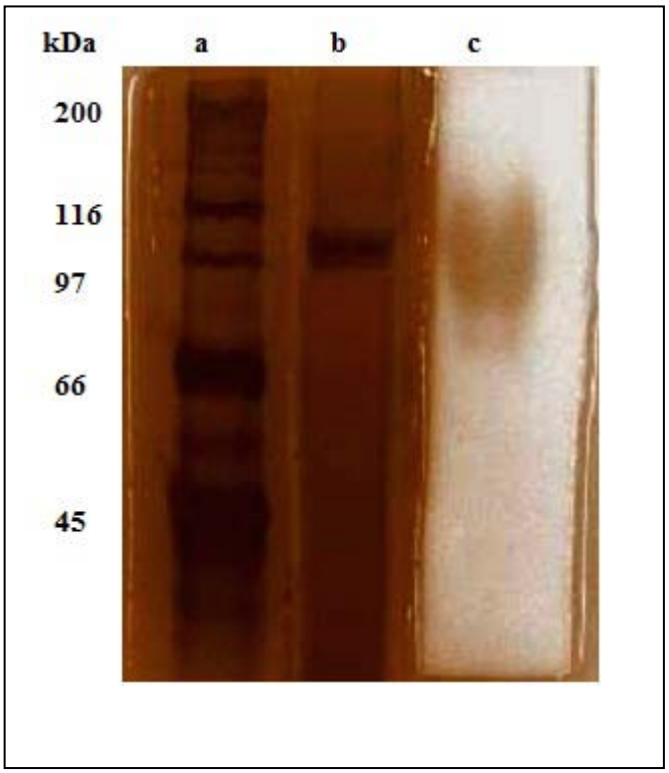

Fig 6.9 SDS PAGE of a. molecular weight marker b. purified tannase by reverse micelle system and c. activity staining of tannase

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CHAPTER 7

Summary and Conclusion

The objectives of the present investigation were to study and evaluate systems for selective extraction and partial purification of bio-molecules of interest, *viz*., solanesol, lycopene, 11 hydroxy canrenone, lipase and tannase. Review of literature indicated that use of reverse micelle based systems (RME) have been investigated for extraction of proteins and enzymes, the class of molecules to which lipase and tannase belong and that encouraging results were obtained by use of such systems. However, very little published information could be found on use of surfactant based systems for extraction or leaching of small molecules from their natural sources. Further, micelle or reverse micelle based systems have not been optimized for extraction and purification of lipase and tannase enzymes. The main objective of this study therefore was to explore and optimize surfactant based microemulsions for extraction and purification of select natural products. Extraction or leaching by using system without surfactant have also been carried out and comparative performance of the two types of systems has been evaluated.

Extraction of solanesol from tobacco dust using microemulsion/reverse micelle

In case of solanesol, leaching of tobacco dust by the solvent n-hexane has been studied with and without surfactant. Significantly, we carried out leaching in presence of water. This suppressed the leaching of water soluble and undesired impurities into n-hexane extract. To the best of our knowledge, this is the first report of extraction and partial purification of solanesol with water-n-hexane system, with or without the use of surfactant. Without surfactant, optimized process showed solanesol purity and yield of ca. 8.7% and 1 g solanesol / 100 g tobacco dust, respectively. The n-hexane extract was further concentrated and subjected to saponification. Another significant feature of the present investigation is use of solvents Ethylene Di Chloride (EDC) and n-propanol for the saponification step. Under optimized conditions, purity of solanesol increased to about 14% with concomitant reduction of fatty material during saponification. The increase in purity obtained in our work is much greater than that reported with methanol as solvent for saponification (Asahina *et al.*, 1977**)**.

For extraction and purification with water-n-hexanol and a surfactant, the investigation was restricted to the first process step of leaching only. We investigated three different surfactants, *viz*., AOT, CTAB and SDS. Among these, best results were obtained with CTAB. Under optimum conditions, the system with CTAB showed solanesol purity and yield of *ca*. 38 % and 1.1 g/100 g tobacco dust, respectively. On the other hand, the system with AOT showed solanesol purity and yield of *ca*. 21 % and 0.34 g/100 g tobacco dust, respectively. The use of AOT had an additional disadvantage that it remained in the solvent phase with solanesol necessitating one extra operation for its removal. CTAB, on the other hand, stayed in the aqueous phase during leaching. The use of SDS showed inferior results with purity and yield of *ca*. 20 % and 0.27 g, respectively. Interestingly, with CTAB, solanesol purity showed maxima at a temperature of 40°C. This could be attributed to the effect of CTAB and temperature on solubility behavior of solanesol as well as impurities. The study of this aspect provides scope for further work in application of surfactant for leaching of solanesol from tobacco dust and other sources.

Extraction of lycopene from tomato paste using microemulsion/reverse micelle

Leaching of lycopene from tomato paste using solvent n-hexane has been studied in the present investigation. Very limited published information is available on the use of solvent n-hexane for this leaching. For system without surfactant, lycopene purity of about 95 % and yield of about 160 mg were obtained. These results were by and large found to be independent of process parameters such as temperature and solvent to tomato paste ratio. Preliminary studies were carried out regarding concentration of the extract and followed by saponification of the concentrated extract. There was improvement in purity to the level of about 98 %. However, there was significant loss of yield due to degradation of lycopene during the saponification process. For extraction with surfactant, we investigated three different surfactants, *viz*., AOT, CTAB and SDS. The investigation was restricted to the first process step of leaching only. With AOT under optimum conditions, the purity of lycopene obtained was about 95 % and yield of about 400 mg/1 kg of tomato paste.

The best results obtained with CTAB were purity of about 98 % and yield of about 230 mg. Use of SDS gave results of purity of about 96 % and yield of 200 mg. Some noteworthy features of the results obtained are as follows. AOT being solvent soluble, it would get carried with lycopene. However, in case of lycopene, presence of AOT is not

very objectionable. Lycopene as a product with some content of AOT may find applications in cosmetic formulation. As such, use of AOT merits further investigation for optimization since AOT gave the highest yield. With use of CTAB, yield was relatively lower but purity obtained (98 %) was very encouraging. Use of SDS was not studied further in view of lower yield as well as lower purity. In view of the results obtained, process conditions for use of both AOT as well CTAB were studied with a view to further optimize the results. The optimum results obtained were as follows. With AOT, yield about 440 mg, purity about 95 %. With CTAB, yield about 290 mg, purity about 98 %. The significant aspects of the results obtained are as follows. With surfactants, there is considerable increase in yield of lycopene. Secondly, the yield and purity of lycopene show declining trend with increase in temperature, indicating limited thermal stability of lycopene. The considerable increase in yield in presence of surfactant could be attributable to penetration of the solvent, facilitated by surfactant into solid matrix holding lycopene inside tomato paste. The phenomenon could be comparable to "enhanced oil recovery" with surfactants. The elucidation of the phenomenon leading to this enhanced recovery of lycopene could constitute scope for further work in lycopene recovery by using system with surfactant.

Extraction and enrichment of 11 α-Hydroxy canrenone (11 OHC) from fermentation broth using miroemulsion/reverse micelle

Being a molecule which has come into prominence only in recent past, very little published information of scientific nature is available in literature. It is understood that for commercial scale operations, ethyl acetate is used as solvent for extraction from the feed broth. The same solvent (ethyl acetate) was used in the present studies in system with surfactant as well as without surfactant. Under optimal conditions, the system without surfactant showed purity and yield of *ca*. 74 % and 92%, respectively. The yield of 11 OHC was found to increase with temperature. The purity was by and large unaffected by the temperature, indicating temperature having no effect on selectivity of the solvent.

For the system with surfactant, we investigated three different surfactants, *viz*., CTAB, SDS and T 80. The investigation was restricted to the first process step of leaching only. The results obtained can be summarized as follows. With CTAB, purity was of about 58 %, yield of about 50 %. With SDS, purity was of about 92 %, yield of about 95 %. With T 80, the results obtained were very poor. In view of the results obtained, only SDS was

used in further optimization studies. The yield increased with increase in SDS concentration and the optimum yield of about 95 % was obtained at SDS concentration of 0.05 M. Further increase in SDS concentration increased extraction of canrenone (an impurity from the broth) and hence decreased the purity of the desired product. Another significant feature of the results is that the yield increased with temperature with the optimum yield being obtained at reflux temperature. The results indicated temperature stability of the surfactant based system. There are many components present in the system other than canrenone and 11 OHC. Their nature and possibility the same leading to temperature stability of the system could constitute scope for further work. Another aspect which could merit a specific mention is as follows. Presence of surfactant is reported to be leading to additional resistance to mass transfer across the O/W interface. As a result it has been reported that extent of mass transfer of different components in given time could be different and this could lead to varying values of purity for different times of contact. This has not been studied in the present investigated, mainly because preliminary results did not show trends of any significance. However, further investigation into this aspect for one or more of the bio-molecules could constitute scope for further work.

Extraction and purification of lipase and tannase using reverse micelle

Reverse Micelle Extraction (RME) has been documented for the purification of number of enzymes such as α amylase, lipase, ribonulease etc. Although proof of concept has been demonstrated in several reports, development of a commercially viable process is a challenge. In the present studies, the application of RME system has been demonstrated as a commercially viable model for purification of two industrially important enzymes viz. lipase and tannase.

Purification of *Pseudomonas* lipase and *A. allahabadi* tannase was attempted via conventional as well as RME system. By a conventional protein purification method, the crude lipase preparation was concentrated by fractional ammonium sulphate precipitation followed by tangential flow filtration (TFF) using 10 kDa membrane cassette and finally purified by gel filtration chromatography. The lipase was purified by 14.98 fold with 52 % enzyme yield. Similarly tannase was purified to 20.54 fold with 31 % yield when it was precipitated by chilled acetone followed by TFF and then purified by DEAE ion exchange chromatography. The lipase enzyme was efficiently purified by forward extraction using RME system containing AOT (50 mM) and

isooctane and then by backward extraction using stripping solution of 0.5 M NaCl while tannase was purified by using CTAB (75 mM)/isooctane RME system and 0.5 M NaCl as stripping solution. We studied the effects of surfactant concentration and other environmental conditions such as pH, contact time, NaCl concentration and V_{aq}: V_{RM} for forward and backward extraction of lipase and tannase.

In presence of cationic surfactant, CTAB, there was no phase separation between the protein rich aqueous phase and isooctane (oil) phase. The interface was stable and it was not separated even after centrifugation and keeping overnight. Anionic surfactant AOT showed maximum recovery of *Pseudomonas* lipase. In case of *A. allahabadi* tannase, anionic surfactant AOT showed no recovery as enzyme activity remained in the aqueous phase with marginal loss but with cationic surfactant CTAB, at pH 5.0 and 7.0, the enzyme recovery was around 60 %. There was little or no phase separation when nonionic surfactants viz. Triton X 100 and T80 were used in case of both the enzymes.

Lipase recovery improved when AOT concentration was increased from 25 mM to 50 mM but did not improve further after increasing the AOT concentration beyond 50 mM and above. This suggested that equilibrium between the organic and the aqueous phases with reference to enzyme recovery was reached at AOT concentration of 50 mM or higher. This could be attributed to increased micellar concentration (Dekkar, *et al.*, 1986). Phase equilibrium with reference to protein recovery was not attained even at 200 mM AOT concentration. Similarly tannase recovery was found to increase from 25 mM to 75 mM (51-67 %) but the enzyme recovery and total protein were decreased on further increase in CTAB concentration from 100 mM and above suggesting decreased interfacial area due to micellar clustering resulting into reduced extraction capacity (Nandini and Rastogi, 2009).

The pH was observed to affect the action of the surfactant in the forward extraction. There was significant loss of lipase activity at pH 4.0 and as well as from pH 7.8 – 9.0. A pH range of 6.0-7.5 was found to be optimal for the maximum recovery of lipase activity i.e. 65 - 70 % of the crude lipase. In case of tannase, 75 % of activity was recovered at pH 6.0 and 7.0. The pI of the lipase (8.2) and tannase (4.5) were thought to play a major role in the extraction of the enzymes. The maximum transfer of lipase was found at pH 6.5 which was slightly below the pI whereas that of tannase was found at pH 6.0 which was above pI.

To achieve maximum activity recovery the contact time at V_{aq}:V_{RM} is usually as short as possible where phase equilibrium may not be totally achieved. The extraction of undesirable proteins was found to increase with increase in time period from 5 to 40 min in RME of *Pseudomonas* lipase whereas the lipase recovery was highest (41.52 %) at 15 min and remained almost unchanged for further time period. In case of tannase the increase in time up to 10 min resulted into significant enzyme recovery up to 60 %. Further increase in time (15 min and above) resulted into marginal decrease in enzyme extraction. The total protein increase was observed as the reaction time increased from 5 to 30 min.

The extent of enzyme enrichment in RME was estimated by studying the ratio of V_{aq}:V_{RM}. Maximum enzyme recovery (80.97 %) and fold purification (15.62) of *Pseudomonas* lipase was obtained at V_{aq}:V_{RM} of 1:2.5 while at V_{aq}:V_{RM} of 1:2, highest tannase (80.85 %) was recovered with 12.48 fold purification. Inspite of further increase in the V_{aq}:V_{RM} ratio up to 1:4, there was no change in the tannase recovery.

Both lipase and tannase were attempted to back extract by NaCl and KCl as stripping agent wherein NaCl showed higher enzyme recovery and fold purification in case of both the enzymes. NaCl concentration 0.1 – 0.25 M resulted into steep increase in lipase extraction and then decreased significantly with increase in NaCl concentration from 0.5 M and above. Highest lipase recovery (80.67 %) was gained at 0.25 M NaCl concentration. The use of isopropanol (15 %) as cosurfactant in the backward extraction resulted into 80 % lipase recovery up to 0.25 M NaCl concentration in turn making NaCl concentration insignificant. On the other hand there was no significant change in tannase enzyme recovery and total protein extraction over the range of NaCl concentration of 0.25 M – 1.0 M. Highest tannase recovery (81.40 %) was obtained at 0.5 M NaCl concentration. In case of tannase recovery, use of isopropanol as co solvent was found to be irrelevant.

The pI mediated effect of pH of stripping solution in the back extraction was studied. The highest lipase extraction was obtained at pH 7.0, below pI of 8.2 contrary to the hypothesis that in presence of anionic surfactant such as AOT maximum recovery takes place at pH above pI. In case of tannase, back extraction with RME comprising CTAB/isooctane resulted into highest recovery at pH 3.5 which was below pI (4.5). Thus supported the hypothesis that back extraction is favored below pI in most of the hydrophilic enzymes.

To attain the objective of concentration of protein in backward extraction the ratio of V_{aq}:V_{RM} was optimized. The maximum i.e. 80 % lipase was extracted when the V_{aq}:V_{RM} was kept at 1:6 for 30 min. In case of tannase a correlation between stripping volume (V_{aq}) and contact time was established. With decreasing V_{aq}:V_{RM} ratio, the back extraction time was increased for example at V_{aq}:V_{RM} of 1:1, time was 10 min whereas at 1:3 and 1:6, it was 20 and 30 min respectively. But at V_{aq}:V_{RM} of 1:6 the protein got concentrated by ~ 3 times which might be a useful to work out the downstream strategy of protein purification and therefore the best protein concentration was achieved at V_{aq}:V_{RM} of 1:6 for 30 min.

In conclusion, tannase was purified to 20 fold by conventional method and 12.72 fold by RME system but there was significant difference in the total recovery of enzyme i.e. 31 % and 81 % respectively. On the other hand, the fold purification of lipase enzyme in both the cases i.e. conventional and RME system was 15 whereas partially purified lipase recovery was greater (80 %) by RME system than that by conventional multistep purification methodology (51.92 %). This shows the feasibility of RME for the large scale recovery and purification of these enzymes.

The salient findings of the thesis can be summarized as follows:

1. The selective extraction by reverse micelle based system was employed to extract and purify the biomolecules of various categories viz. natural products such as solanesol from tobacco leaves and lycopene from tomato, insoluble steroid 11 hydroxy canrenone from fermentation broth and microbial enzymes (lipase and tannase).

2. The comparison of total yield and purity by conventional methods of extraction/purification of biomolecules was carried out with that by the reverse micelle based extraction (RME).

3. This study, for the first time, demonstrated that RME system could be applied as an efficient and time saving methodology for selective extraction of natural products viz. solanesol and lycopene and insoluble steroid, 11 hydroxy canrenone from fermentation broth. Accordingly three patents have been filed.

4. The successful demonstration of RME system for selective extraction of product (11 hydroxy canrenone) directly from fermentation broth suggested that the

process can be potentially useful for recovery of many pharmaceutically important biotransformation products such as 7-α and 9-α hydroxy steroids and could save the capital cost and time involved in the recovery of such product (s).

5. The comparative study in the present investigation showed that the RME system could be as effective for enzyme recovery and fold purification as the conventional protein purification process using two industrially important enzymes viz. *Pseudomonas* lipase and *A. allahabadi* tannase. Moreover RME is single step and less time consuming.

6. Interestingly in case of the *Pseudomonas* lipase, pI was not significant in backward extraction. The effect of cosolvent, isopropanol was more significant in the backward extraction. Electrostatic interaction due to salt was insignificant in comparison with the percolation and undulation effect of cosolvent.

7. The successful RME application for tannase recovery paved the way for further research and exploration of large scale application of RME for this enzyme as there is only one report has been documented on tannase extraction by RME system. The manuscript is under preparation.

Scope of Future Work

The scope of future work, which has been discussed in various parts of the chapters, is summarized below:

1. Determination of microemulsion structure in case of multi component, multi phasic systems, such as CTAB (surfactant)-Water-n-hexane (liquid phase)-tobacco dust (Solid Phase). Similar studies can be carried out in case of AOT-n-hexane-Tomato Paste.
2. Study of micellar system comprising aqueous phase, ethyl acetate (organic phase) and surfactant (such as SDS).
3. Development of a suitable reactor model for simultaneous forward and backward extraction for the proteins and enzymes.
4. Localization study of low and high molecular weight biomolecules in micellar system.

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List of publications/ patents/ presentations/ technology transfer

Publications

Gaikaiwari R.P and Kulkarni B.D. 'Extraction and purification of tannase using reverse micelle', communicated to Bioresource Technology.

Gaikaiwari R.P and Kulkarni B.D. 'Extraction and partial purification of Pseudomonas lipase using reverse micellar system' communicated to Separation and Purification Technology.

Patent

Gaikaiwari R.P. and Kulkarni B.D. Process for Extraction of Solanesol (643/MUM/2011)

Gaikaiwari R.P. and Kulkarni B.D. Process for Extraction of Lycopene (644/MUM/2011)

Gaikaiwari R.P. and Kulkarni B.D. Process for Extraction of 11 Hydroxy Canrenone (884/MUM/2011)