CHAPTER III

MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 MICROORGANISM :

Yeast strain belonging to the species of *Candida tropicalis* was isolated by the Indian Institute of Petroleum (IIP) from the soil containing residual oily sludge in the Gujarat Refinery at Baroda (India). This strain was screened for the study along with another yeast strain, *Candida lipolytica*, procured by IIP from IFP, France. Both the strains have been studied extensively by IIP to check the stability for continuous culture over 20,000 hrs on n-paraffins (C_{14}-C_{18}). However, comparative adaption capacity of both the strains on heavier hydrocarbon has been checked in shake flask culture.

The strain were preserved at 60^0C in nutrient agar slants of the following composition

Peptone	-	3 g
Yeast Extract	-	3 g
Glucose	-	10 g
Agar (Bacto)	-	25-35 g
Distilled water	-	1000 ml

3.1.2 PETROLEUM FRACTIONS

Two types of vacuum distillates known as lube fraction of different origin of crudes were chosen for the Study

a) Vacuum distillate of the Assam Crude processed in Barauni Refinery, IOC which is being used as the feed stock for the solvent dewaxing unit. However, the dewaxed oil does not meet specification of the lube oil because of the limitation of designed process parameters to handle the

highly paraffinic Assam Crude.

b) Vacuum distillate of the Gulf Crude mix processed in Haldia Refinery, IOC, which produces standard lube oil base stock.

The physical properties of these two feed stocks are given in Table 3.1.2

TABLE : 3.1.2 PHYSICAL PROPERTIES OF THE VACUUM DISTILLATES

Properties		Vacuum distillates	
		Assam Crude(A)	Gulf Crude(B)
1.	Boiling range, ^{o}c	400-490	350-500
2.	Density at 20^{o}c, g/ml	0.9371	0.8806
3.	Pour point, ^{o}c	48	30
4.	K Viscosity Cst, at 100^{o}c	4	10
5.	Colour	dark greenish	dark yellowish
6.	API gravity(60^{o}c)	19.65	18.75
7	Saturates	47	25

3.1.3 MEDIUM

Aqueous solution of inorganic salts of commercial grades, was used to provide nitrogen, phosphorus and other nutrients essential for the growth of the yeast strain. The concentration of the salts maintained is given in Table 3.1.3. The ammonia solution was used as alkali for controlling the pH of the broth during growth which also acted as nitrogen source to the culture during the growth phase. No growth factor e.g., vitamines, amino acids etc. was added in the present study.

TABLE : 313 COMPOSITION OF THE MEDIUM USED FOR CULTIVATION OF YEAST STRAINS FOR MICROBIAL DEWAXING.

	Components	Concentration of salts in culture medium, g/l
i).	Ammonium sulphate	2.00
ii).	Potassium dihydrogen phosphate	1.26
iii).	Disodium hydrogen phosphate. $12H_2O$	0.75
iv).	Magnesium sulphate. $7H_2O$	0.70
v).	Calcium chloride	2.5×10^{-2}
vi).	Ferrous sulphate. $7H_2O$	1.6×10^{-2}
vii).	Zinc sulphate. $7H_2O$	4.4×10^{-3}
viii).	Copper sulphate	3×10^{-4}
ix).	Manganese sulphate	5×10^{-5}
x).	Cobalt nitrate. $6H_2O$	5×10^{-5}
xi).	Boric acid	5×10^{-5}
xii).	Ammonium molybdate. $4H_2O$	5×10^{-5}

3.14 EXPERIMENTAL SET UP

Fermentation experiments were conducted in two stages.

(i) Culture development stage and

(ii) Bio conversion of petroleum fraction under controlled conditions.

i) Culture development in shake flasks :Prior exposure of the yeast strains to lube fraction was conducted by allowing to grow and assimilate hydrocarbons in conical flasks kept on a mechanical shaker. The experiment was designed to screen the potential yeast strain for dewaxing of the lube fraction. Apart from adaptation process in shake flasks, inoculum for larger capacity bioreactor is prepared.

Procedure :

Erlenmeyer flasks of 250 ml capacity containing 50 ml of the medium and petroleum fraction as substrate, 1% (w/v) were sterilized in an autoclave at 1.05 Kg/cm^2 pressure of steam for 15 mins. After cooling, the flasks were inoculated with a loopful of yeast cells taken from agar slants under aseptic conditions. The flasks were incubated at controlled temperature of 35 $\pm$ $1^{o}c$ on a rotary shaker of 400 rpm speed and 2.5 cm eccentric stroke to provide efficient contact between the insoluble substrate medium and cells as well as supply of aerial oxygen . The oxygen transfer rate (OTR) of the set was determined by sulphite oxidation method and was found to be 11.9 m moles of O_2/l.h.

Turbidity developed in the aqueous medium in the flasks was in proportional to the growth of the yeast strains and measured as the optical density of the broth. After 48 hrs, 10 to 15 ml broth was taken for further inoculation to bioreactor.

ii) Hydrocarbon fermentation in bioreactors :

Stirred tank reactors with working capacity of 1.5 litres,and 5 litres attached with automatic control systems to monitor and

control the operating parameters are used in the study.

The photographs of the bioreactors are shown in Fig.3.1.4.

a) The Gallenkamp modular fermentor ,UK (A) comprising of following facilities.

1.5 l capacity glass vessel, dual pH control system by acid and base addition and monitoring by Ingold p^H electrode, temperature control through thermostatic bath (HAAKE) attached with in-situ cooling coil, aeration by external air pump and controlled by rotameter, agitation by variable speed motor attached with the turbine type impeller through agitator shaft.

b) Indigenous glass fermentors (B) of 1.5 L and 5.0 L capacity having similar facilities as available in Gallenkamp fermentor except the dimension of turbine impellers and glass vessels. The power of the agitator motor was higher than that of used in Gallenkamp unit.

c) Microgen II Fermentor, New Brunswick Scientific Co.USA of working volume 5 to 10 l capacity,equipped with GEN II Instrumentation system ,New Brunswick Scientific Co.,USA. The system can control and display pH , temperature, aeration and agitation rate , dissolved oxygen concentration ,internal pressure with the help of ON/ OFF or PID control system.

The Gen II instrument can also be controlled by Televideo Model 910 CRT terminal.

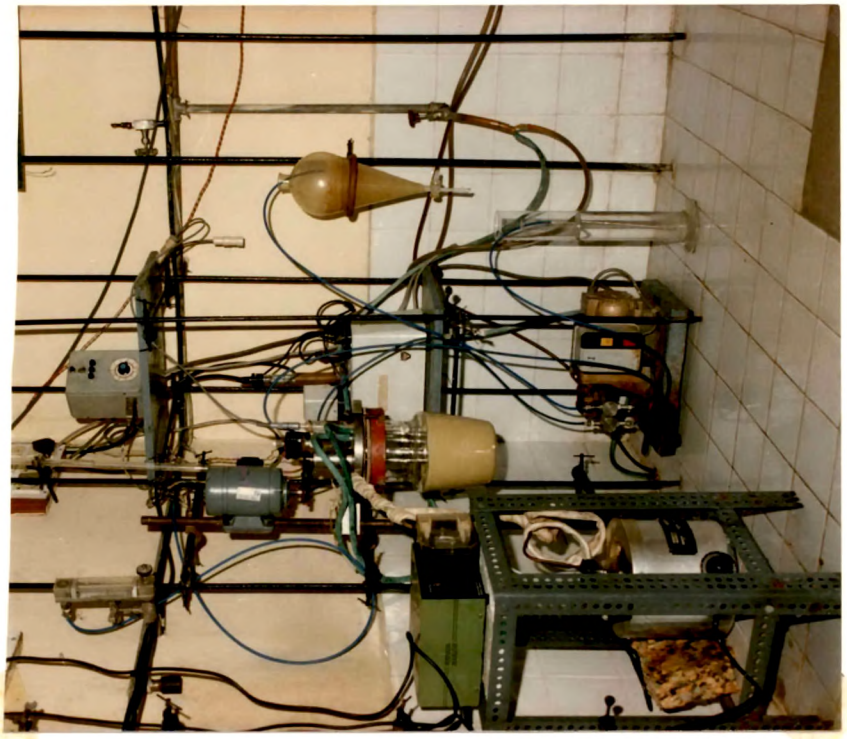

Fig.3.1.4. Bioreactor set up

The relative dimensions of the vessel and the turbine impeller of each bioreactor were measured. The corresponding dimensionless groups were calculated from the data and shown in Table 3.1.4

TABLE : 3.1.4 FERMENTOR DIMENSIONS AND DIMENSIONLESS GROUPS

	Dimension, in cm.	Fermentor		
		A	B	C
1.	Dia of vessel, D	13.0	14.0	17.0
2.	Dia of impeller, dr	6.0	5.5	5.5
3.	Height of blade, hr	1.5	3.5	3.5
4.	Height of liquid, H	11.5	10.0	21.0
5.	Working volume in L	1.5	1.5	5.0
6.	No. of impeller blade, c	4	4	6
7.	$dr^* = dr/D$	0.46	0.39	0.31
8.	$hr^* = hr/d$	0.11	0.25	0.20
9.	$H^* = H/D$	0.88	0.71	1.20

iii) Rotavapour :

Recovery of dewaxed oil from the oil-solvent mixture obtained as overflow stream from the leaching operation was conducted in Rotavapour unit, Buchi 411 Switzerland attached with thermostatic bath E 465. The unit was operated under vacuum of 150 mm Hg with simultaneous purging of N_2 gas to remove the traces of solvent present in the dewaxed oil.

iv) D.O.Probe :

BIOCHEM DIGITAL OXYGEN ANALYSER M 80 1704, Universal biochem Madurai,was used to determine the dissolved oxygen concentration of the culture broth. The probe was operated using Polythene membrane suplied by the company . The response time of the probe in presence of hydrocarbon was high (2 min) as compared to aqueous phase (8-10 sec).

v) Spectrophotometer

The Systronics spectrophotometer, was used to determine the turbidity of the culture growing in shake flasks. The test tubes supplied by the company to measure the optical density of any sample were fused with the flasks as side arm. The optical density (O.D) of the culture was measured directly by inserting the side arm filled with culture liquid in the instrument. The O.D. values and the percentage transmision were noted from the dial of the instrument. The increase of O.D. values of the growing yeast strains were compared against blank flask containing all the ingradients except the culture. The O.D values were measured at 620 μm at which maximum absorption of incident light was observed.

The increase of O.D. values have indicated the relative increase of growing yeast culture in the shake flask. Actual growth curve of the cells on hydrocarbon in fermentor was determind by alkali consumption rate.

vi) Viscometers :

Two different types of viscometers were used in the study.

a) Suspended level, 3 limbed viscometer made of borosilicate glass marked with SLSC, type-3 No. 1628/03 manufactured by Precision Scientific Works Ltd., U.K. was used for the determination of Kinematic Viscosity of the oil samples.

The constant of the viscometer (No. 6) was determined (0.31) by calibrating with standard calibrating liquid (S-20).

Similarly capillary viscometer (two limbed) marked SKB 1:64, No. - 4 manufactured by IIP, Dehradun was also used.These viscometers are used for the determination of Kinematic Viscosity (ν) of the vacuum distillates and the dewaxed oil samples as prescribed in standard ASTM procedure.

b) The Dynamic Viscosity (η) of the culture liquid in the fermentor was determined by Brookfield SYMCRO-LECTRIC viscometer with UL adapter, model No. - 73261. The rotating spindle No. LVF 1 was found suitable for the determination of viscosity of the culture fluid. The metallic spindle (cylinder type) which can rotate freely inside the metallic adapter, was clamped with the viscometer .The adapter and the spindle was immersed in water bath 7-37^{o}c. Anglo-Swiss digital watch wasused for the measurement of efflux time..

vii) Mass spectorometer: KRATOS MS-50 was used for the analysis of the saturate and aromatic components of the feed and dewaxed oil separately. The result of the analysis indicated the consumption pattern of the two components present in the oil samples

viii) Chemicals used in different experiments are listed below.

a) Acetone (commercial grade) and petroleum ether b.p. 80-100^{o}c, Ranbaxy Lab. were used for the treatment of emulsion (o/w) to seperate the dewaxed oil and biomass.

b) All the inorganic salts used for the preparation of growth medium as mentioned in 8.1.3 were LR grade, manufactured by BDH, Bombay.

Sodium Sulphite, Potassium dichromate, Sodium thiosulphate, Copper sulphate, Hydrochloric acid, all of LR grade and manufactured by BDH were used for the determination of oxygen transfer rate .

c) Chloroform and methanol mixture (1:3)(BDH, LR GRADE) was used for lipid extraction from biomass in the Soxhlet apparatus.

d) n-pentane, ethyl acetate, chloroform, and absolute alcohol were used as eluents for the seperation of saturate and aromatic hydrocarbon present in the vacuum distillate before and after dewaxing.

Bauxite and silica gel (120-200 mesh) were used as adsorbents of the chromatographic coloum.

3.2. METHODS :

3.2.1. Determination of density :

Standard method available for determination of density of semi-solid and solid petroleum products was followed.(IS 1448/P:32). Density of the culture broth, vacuum distillated and the dewaxed oil was determining using pyknometers of 10 and 15 ml capacity selected as per standards.

3.2.2. Determination of pour point :

Pour point in ^{o}C of the vacuum distillates and dewaxed oil was determined in the apparatus obtained as per standard (IS: 1448/IP:10).

3.2.3. Determination of viscosity

a) Kinematic viscosity of oil sample was determined following the standared IS:1448/IP:25 method using either suspended level, 3 limbed viscometer or glass capillary type viscometer as per requirement.

b)Determination of dynamic viscosity :

Rheological properties of the culture fluid was determined by measuring the apparent viscosity (μ_a), known as dynamic viscosity. Since most of the culture fluid showed non-Newtonian behavior, the apparant viscosity of culture fluid can not be determined in glass capillary viscometer because cells and emulsion could not flow homogeneously through the glass capillary.

The rheological parametrs of power law non - Newtonians fluids can be described by the following correlation :

$$Y = K (Y)^{n}$$

The coefficients of the equation was evaluated on variable-shear-rate viscometer e.g., Brookfield viscometer.

Pocedure :

Samples of culture broth was drawn at different intervals and 16 ml of the sample was filled in the adapter of the brookfield viscometer which was used in the study.

The rotating spindle No. LVF 1 was attached with the viscometer and inserted slowly into the adapter. Care was taken to dip the entire spindle with the test liquid. The dial reading of viscometer was noted after 5 minutes of the constant reading of the instrument for each sample. The spindle and the adapter were cleaned before filling up with fresh sample.

Calculation and reporting :

The dial reading was calibrated as shear force (dyne cm) for each rpm of rotating spindle and was multiplied by the corresponding factor obtained from the nomograph supplied by the company. The multiple was reported as dynamic viscosity of the sample in centipoise .

3.2.4. Determination of viscosity index :

Viscosity index of the dewaxed oil sample was calculated following the standard method (ASTM D 2270-79/IP-226/78).
A bath of 50 cm dia fitted with a coil containing ethylene glycol as circulating liquid was used as thermostatic bath for determination of viscosity at desired temperature. The temperature of the bath was controlled by thermostat, VEB MK4LW, PRUFGERATE-WERK, GDR which can be adjusted at any temperature within the range of 10-100^{o}c with 0.2^{o}c precision. The temperature of the bath was monitored with the help of digital thermometer. A diffused light source was provided behind the glass jar to notice the level of test sample in the viscometer.

3.2.5. Power measurement :

The net power transfer by the motor to the agitator of the fermentor was determined with the help of watt meter connected to the agitator shaft through belt and pulley as described in Fig.3.2.

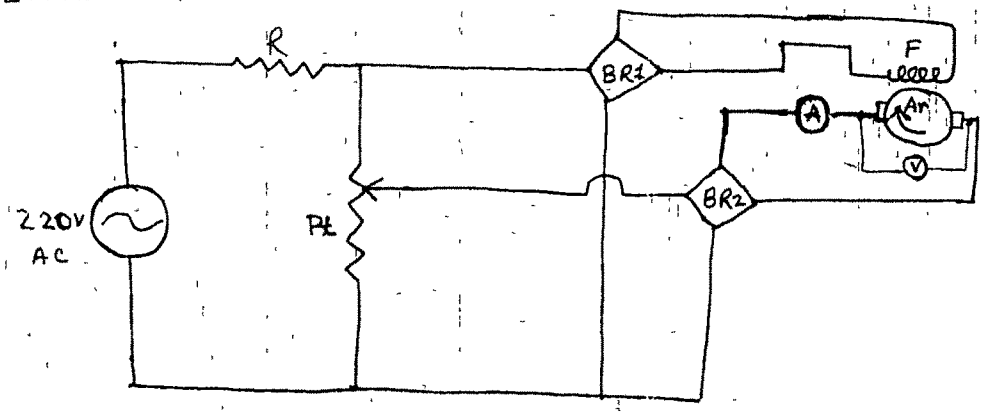

Fig. 3.2. Circuit diagram for the measurement of power drawn by the agitator motor

a) Description of Motor :

D.C.,Shunt, separately excited motor was used for agitation. The power supply (220 V AC) from mains was devided into two parts,one which passed through rectifier (BR 1) for the supply of constant 180 V DC to the field (F) whereas the other part passed through the potentiometer (PT) and rectifier (BR 2) to armature (Ar) coils. The rotational speed of the armature was controlled by varying voltage through potentiometer. A voltmeter (V) was connected parrallel to the armature and the miliammeter (A) was

conected in series to the armature to measure the voltage and current drawn by the agitator during experiment. Power supply was determined as per standard correlations which are described later.

The rating of the motor was following :

	Armature		Field		Max. rpm
Max. power supply	Voltage	Current	Voltage	Current	(no load)
	V	A	V	A	
110 W	180	0.85	180	0.08	3000
Cold winding resistance (R_{ac}), ohms	40		2000		

b) Measurement of agitator power requirement

Power consuption of large scale fermentors is measured by Wattmeter. However, strain gauge dynamometer attached with the agitator shaft of test fermentors was found more suitable for precise value of the net power consuption (Aiba et.al., 1969).

In the absence of power of strain gauge dynamometer with the fermentors available for the present study, power requirement was measured with the help of wattmeter in the present Study as described in previous section (3.1.4).

c) Test Procedure :

Swinburn Tests (Cotton, 1961) were carried out to determine the losses of power input in load and no load conditions. Power transmission arrangement to the agitator of the fermentor motor is connected to the agitator shaft of the fermetor through the belt.

The armature current is measured with the help of a DC milliammeter connected in series with the armature circuit. Similarly voltage to armature was also measured by voltmeter connected in parallel to the armature circuit. The rpm of the agitator shaft was measured with the help of a tachometer.

Armature Resistance:

The motor was operated on no load condition and the armature current (I_o) was measured. Then the cold resistance of the armature (R_{oc}) and the field circuit (R_{fc}) were measured by the following equation :

$$\alpha_1 = \alpha_o / (1 - \alpha_o T_o) \qquad (3.1)$$

$$R_t = R_{ac} (1 + \alpha_1 t) \qquad (3.2)$$

where

α_o = Coefficient of expansion, for Cooper = 0.00427.

α_1 = Coefficient of Cooper at experimental temperature.

R_c = Cold resistance.

R_t = Hot resistance (excited condition).

t = Temperature rise, ^oc.

Now

$$\alpha = \frac{\alpha_o}{(1 + \alpha_o T_o)} \qquad (3.3)$$

$$R_t = R_{ac}(1 + \alpha_1 t) \quad (3.4)$$

$$R_f = R_{fc}(1 + \alpha_1 t) \quad (3.5)$$

d) Calculation of power losses

Any motor running in no load condition incurs some losses e.g., frictional losses and iron losses which are known as STRAY LOSSES. The power losses can be calculated as following :

i) Stray losses = Frictional losses + Iron losses

$$= Va\ Io - Io^2 R_{ac} - \frac{V_{fv}}{R_{fc}}$$

ii) Constant losses (Pc) = back emf + stray losses

$$Pc = \frac{V^2}{R_f} + Va\ Io - Io^2 R_{ac} - \frac{Vb^2}{R_{fc}}$$

iii) Total losses (Pt) = Heat losses + Constant losses

$$= Pc + I_a^2 Ra$$

e) Determination of power input to agitator

i) Power input to motor (P_{IN}) = $Va\ Ia + V_b I_b$

ii) Power output of the motor (P_{OUT}) = $P_{IN} - P_t$

iii) Power input to the agitator = P out of the motor assuming negligible frictional loses due to belt pulley arrangement.

Where,

V_a = Armature voltage

I_o = Current drawn by armature in no load conditions

V_f = Field voltage

I_a = Armature current in load conditions

I_f = Field current

The net power consuption by the agitator during fermentation was determined by subtracting the power output value by the motor with load i.e., without the culture broth. Hence the frictional losses occured due to rotation of the agitator was nullified and the actual power drawn by the culture fluid was determined.

3.2.6. Determination of lipid :

The lipid percentage of the biomass was determined by Soxhlet apparatus (AOAC, 14.028 ,1979).

3.2.7. Determination of nitrogen in biomass :

Nitrogen content of the biomass produced by the dewaxing process was determined by the micro-kjeldhal method as described in AOAC ,7.033,1979. The protein content of the biomass samples were determined by multiplying the nitrogen percentage of the samples by 6.25.

3.2.8. Determination of Oxygen transfer rate (OTR) in the fermentors

The oxygen transfer rates (OTR) in the fermentors (1.5 L Gallenkamp, 1.5 L glass and 5 L glass fermentors and Microgen ii fermentor were determined by the sulphite oxidation method as described by Linek et al , 1973. The method is based on the fact that sodium sulphite is almost instantaneously oxidised by dissolved oxygen in presence Cu^{++} or Co^{++} as catalyst as per following equations :

$$2\ Na_2\ SO_3 + O_2 \xrightarrow{\text{-----}} 2\ Na_2\ SO_4 \quad \text{-----(i)}$$

$$Na_2\ SO_3 + I_2 + H_2\ O \xrightarrow{\text{-----}} Na_2\ SO_4 + 2\ H\ I \quad \text{---- (ii)}$$

$$2\ Na_2S_2\ O_3 + I_2 \xrightarrow{\text{-----}} Na_2\ S_4\ O_6 + 2\ Na\ I \quad \text{- (iii)}$$

The rate of sulphite oxidation in aerated and agitated fermentors was determined by quantitative estimation of amount of sodium sulphite oxidised at different time intervals, The slope of the straight line obtained by plotting the sulphite concentration Vs time gave the sulphite oxydation rate, which was used to determine OTR value in terms m mole of O_2 consumed per litre per hour using stoichiometry of reaction (i).

3.2.9. Separation of components of lubricating oil fraction :

Separation and determination of saturate and aromatic components present in the vacuum distillate and dewaxed oil was done by Elution Column Chromatograph as described in ASTM method (D 2549-81). 10 gm of oil sample was charged in a column packed with Bauxite on the top and silica gel (Grade 12, 923) of 100-200 mesh at the bottom. The saturates and aromatic hydrocarbons were eluted consequently by adding the elements e.g., n-pentane, diethyl ether, chloroform and ethyl alcohol successively.

3.2.10. Determination of Specific. growth rate ($\mu\ h^{-1}$) :

Determination of cellular dry wt. in hydrocarbon fermentation poses problem of geting representative samples from the fermentor. The cells get attached to the heterogeonus petroleum fraction and uniform cell concentration of the entire culture fluid was not

available. The problem was observed more significantly during the growth study on lube fraction.

An alternative method was followed (Prokop, 1979, Hirth, 1990) to get the accurate growth curve of the culture. Microbial growth in hydrocarbons is associated with the increase of H^+ concentration if ammonium salt is used as nitrogen source. As NH_3 is consumed by the organism H^+ accumulates in the fermentor as follows :

$$NH_4OH \quad <======> \quad NH_3 + 2\,H^+$$

Alkali (NH_4OH) was added to the fermentor to keep the pH of the culture fluid constant. As a result the rate of alkali addition was found propotional to the rate of growth of the biomass which was calculated 6.25 ml of (IN) NH_4OH / increase gm of biomass. The logaritham of cumulative volume of alkali added in every hour was plotted against time and the growth curve of the biomass on lube fraction was obtained.

The slope of the linear portion of the curve was taken as the maximum sp. growth rate ($\mu\ h^{-1}$) of strain.

3.2.11. Determination of yield of biomass :

Biomass yield on the lube fraction was determined by measuring the total wt. of dry biomass obtained from the measured quantity of lube fraction taken in the fermentor. The yield of biomass was expressed as follows :

$$Y = \frac{\text{wt. of dry biomass}}{\text{Total wt. of oil added as substrate}}$$

3.2.12. Determination of yield of dewaxed oil

The yield of dewaxed oil was determined on the basis of lube fraction fed as feed stock in the fermentor.

$$Y = \frac{\text{wt. of dewaxed oil}}{\text{wt. of oil added as substrate}}$$

3.2.13. Recovery of dewaxed oil :

The fermented broth obtained at the end of batch or semi continuous operation was further treated with a solvent for the recovery of dewaxed oi and biomass.

The aqueous phase of the fermented broth was centrifuged at 6000 rpm and the traces of the oily emulsion was recovered from the aquous phase by separating funnel . The aquous phase contained negligible amount of emulsified o/w and cells.

The oily phase was treated with solvent mixture of acetone and petroleum ether (b.p. 80-100^{o}c), mixed in 1:3 ratio for leaching out the emulsified oil in the solvent phase (overflow). The extraction carried out at room temperature 30 ^{o}c in a separating funnel. The volume of solvent added was twice the volume of oily emulsion and was shaken vigorously for 5 minutes with intermittent depressurising the funnel by opening the stop cock. After shaking operation the separating funnels are allowed to stand for 15 minutes to get the clear oil/solvent phase at the top and solvent/water with cells at the bottom. The upper layer was collected and stored for distillation for the recovery of dewaxed

oil.

The bottom phase was further treated 4 times in similar way to get the 95% recovery of the dewaxed oil. Finally the settled biomass in aqueous/acetone phase was filtered and dried to get the protein rich SCP.

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