CHAPTER - II

MATERIALS AND METHOD

Curiosity is the wick in the candle of learning.

Reading without reflecting is like eating without digesting.

Activity is the only road to knowledge - Bernard Shaw.

II. MATERIALS AND METHODS

II. 1. Materials

II.1.1. Organisms :

1. The strain belonging to the species " Candida tropicalis " was isolated from the soil collected from one of the tank bottoms of the Gujarat Refinery, using enrichment cultivation method (237). The pure strain which was designated 'IIP-4' through shake flask experiments was adapted to grow on hydrocarbons. This was characterized by the method of Lodder and Kreger - van Rij (238).

2. Another yeast strain supplied by French Institute of Petroleum, France and was designated IFP-29, was characterized by the method of Lodder and Kreger - van Rij (238) and belongs to "Candida lipolytica species".

II.1.1. A. Preservation and Maintanance of Pure Culture :

Stock cultures of these two strains are maintained at 10 ± 2°C on slants of the following medium in

Roux bottles :

Glucose 2% (W/V), yeast extract (Difco) 0.5% (W/V), peptone (Difco) 1% (W/V), Agar 2% (W/V), Sub culturing is done once a month.

Cultures after transfering into fresh slants are incubated at 35 ± 1 °C for 16 to 18 hours.

II.1.2.1. Growth media for shake flask experiments :

For batch cultures the basal medium is prepared from the following solutions of the composition shown in Table (6). (197).

II.1.2.2. Growth media for Bench Scale fermentation studies :

The composition of the medium for continuous bench scale studies is given in Table (7). (197). pH of the medium is adjusted to 4 with IN - H_2SO_4 before it is sterilized at 1.03kgcm^{-2} for 20 minutes.

Table 6. Composition of medium for shake flask experiments.

Name of the constituent		Concentration (g/l)
$KH_2 PO_4$	:	3.4
$Na_2 HPO_4$	:	1.5
$Mg SO_4 7H_2O$	:	0.7
$(NH_4) SO_4$	:	4.0
$Ca Cl_2$	:	0.01
$Fe SO_4 7H_2O$	:	0.001
$Cu SO_4 5H_2O$	:	0.00005
$Mn SO_4$	:	0.001
$H_3 BO_3$	:	0.0001
$2n SO_4$	:	0.0001
$Co (ND_3)_2$	:	0.0001
$(NH_4)_6Mo_7O_{24}4H_2O$	:	0.001
Yeast Extract	:	0.1

The pH of the medium is brought down to 4. It is autoclaved at 1.03 $Kg.cm^{-2}$) for 20 minutes, and cooled.

Table 7. Composition of medium for bench scale fermentation studies

Name of the constituent		Concentration (g/l)
$(NH_4)_2 SO_4$	:	2.00
$KH_2 PO_4$	:	1.26
$Na_2 HPO_4$	:	0.75
$Mg SO_4 7H_2O$	:	0.70
$Ca Cl_2$	:	25.00
$Fe SO_4 7H_2O$	:	16.00
$Zn SO_4$	:	04.40
$Cu SO_4 5H_2O$	:	00.30
$Mn SO_4$	:	00.50
$Co (NO_3)_2$	:	00.50
$(NH_4)_6 Mo_7 O_{24} 4H_2O$	:	00.50
$H_3 BO_3$	:	00.50

II. 1.3. Carbon Substrates :

Petroleum hydrocarbons :

1. Kerosine fraction from Ankleshwar, North Gujarat and Bombay High and Imported Crudes boiling range as determined by ASTM distillation method is given in Table (8).

2. N-heptane from Gujarat Refinery Unit.

3. N-octane.

4. High purity n-hexadecane (C_{16}) from Phillips Petroleum Company Ltd.

5. Kerosine fraction from Ankleshwar crude is subjected to Urea dewaxing experiments (239). The n.paraffinic hydrocarbon fraction is thus collected.

6. Following two cuts of n-paraffinic hydrocarbons produced at Indian Petrochemical Corporation Limited (IPCL) by Molex process (8) from kerosine fraction using molecular sieve 5A as adsorbent.

   C_{11} - C_{13} cut lower n-paraffinic hydrocarbons

   C_{14} - C_{18} cut heavier n-paraffinic hydrocarbons.

Table No. 8. Characteristics of Kerosine fractions of different Crude Oil

ASTM distillation data	Ankleshwar °C	North Guj. °C	B'bay High °C	Imported °C
IBP	158 (C_9)	180 (C_{10})	135 (C_9)	140 (C_9)
5%	190 (C_{10})	215 (C_{11})	145 (C_9)	150 (C_9)
10%	208 (C_{11})	234 (C_{12})	158 (C_9)	164 (C_9)
20%	222 (C_{12})	248 (C_{13})	170 (C_9)	178 (C_{10})
30%	238 (C_{13})	254 (C_{14})	178 (C_{10})	191 (C_{10})
40%	245 (C_{13})	258 (C_{14})	190 (C_{10})	200 (C_{11})
50%	253 (C_{14})	263 (C_{14})	208 (C_{11})	209 (C_{11})
60%	260 (C_{14})	267 (C_{14})	215 (C_{11})	220 (C_{12})
70%	267 (C_{14})	271 (C_{14})	228 (C_{12})	231 (C_{12})
80%	272 (C_{15})	276 (C_{15})	242 (C_{13})	242 (C_{13})
90%	286 (C_{16})	285 (C_{15})	255 (C_{14})	255 (C_{14})
FBP	286 (C_{16})	285 (C_{16})	268 (C_{14})	268 (C_{14})
Figures within ( ) indicate predominant carbon number of n-paraffins.				
Density $g.cm^{-3}$	0·8015	0 8291	0 8525	0·8091
Smoke point mm	26	23	21	24
n-Paraffin content %	40	30	25	20
After microbial dewaxing				
Density $g.cm^{-3}$	0·8405	0·8391	No Growth	No Growth
Smoke point mm	17	19	—	—

II. 1.4. Solvents :

1. Acetone - Industrial grade - from National Organic Chemicals Ltd. (NOCIL)

2. Isopropanol- Industrial grade-from National Organic Chemicals Ltd. (NOCIL)

3. Naphtha- Petroleum fraction with the boiling range 60-80°C from Gujarat Refinery

4. Chemicals- Unless otherwise stated all chemicals used were of reagent grade.

II. 2. Experimental set up :

II.2.1.Shake flask experiments :

250 ml Erlenmeyer flasks fitted (fused) with a side tube of uniform diameter are used for shake flask experiments. The side tube can be inserted in a photo electric colorimeter so as to measure the Optical Density (OD) of the medium in the flask. This arrangement helps to conduct growth studies of the required microorganisms under sterile condition. To provide agitation of the system the shake flasks are kept on rotary shaker rotating

at 200 rpm and 2.5 cm radius of its rotation. Oxygen transfer rate (OTR) of the set up is 10 to 12 milli moles of Oxygen/litre/hour (240).

II. 2.2. Bench Scale set up :

Mechanically agitated glass fermentor of total volume 2.5 litres and working volume of 1.5 litre stirred with a turbine type stirrer at 1200 rpm is used for this study. Compressed air filtered through steel filter of mesh size 200 is used to provide aeration. Aeration rate is determined with the help of caliberated rotameter.

Temperature is maintained by circulating water at required temperature from a constant temperature bath through the coil incorporated from the top plate of fermentor and immersed in the broth medium.

To maintain a constant pH, a sterilizable combination glass electrode of 'Ingold' type in combination with Metrohm instrument (Metrohm A G CH - 9100 Herisan) is used. The electrode operates through an electronic pulse controller and in combination with amplifier of the

Metrohm instruments. The controlled flow of 2N ammonia solution, prepared out of commercial grade ammonia gas, through a solenoid valve helps to maintain the desired pH.

For continuous culturing the micro metering pump (Mettering Pump Ltd., London) and precision pump UNITA - 1 (B. Braun Melsunger A.G.W. Germany) are employed to feed medium and the substrate respectively at constant and desired flow rate. Liquid level in the fermentor is maintained through over-flow device connected through a suction pump.

Oxygen transfer rate under normal condition of agitation and aeration of this set up is 250 m. moles O_2/L.h. schematic diagram of the set up has been given in fig (6A).

II. 2.3. Pilot Fermentor

Pilot plant runs are given in one cubic meter capacity centrally aerated non mechanically agitated air lift type fermentor. In this type of fermentor air is allowed to pass into a sparger which with the help

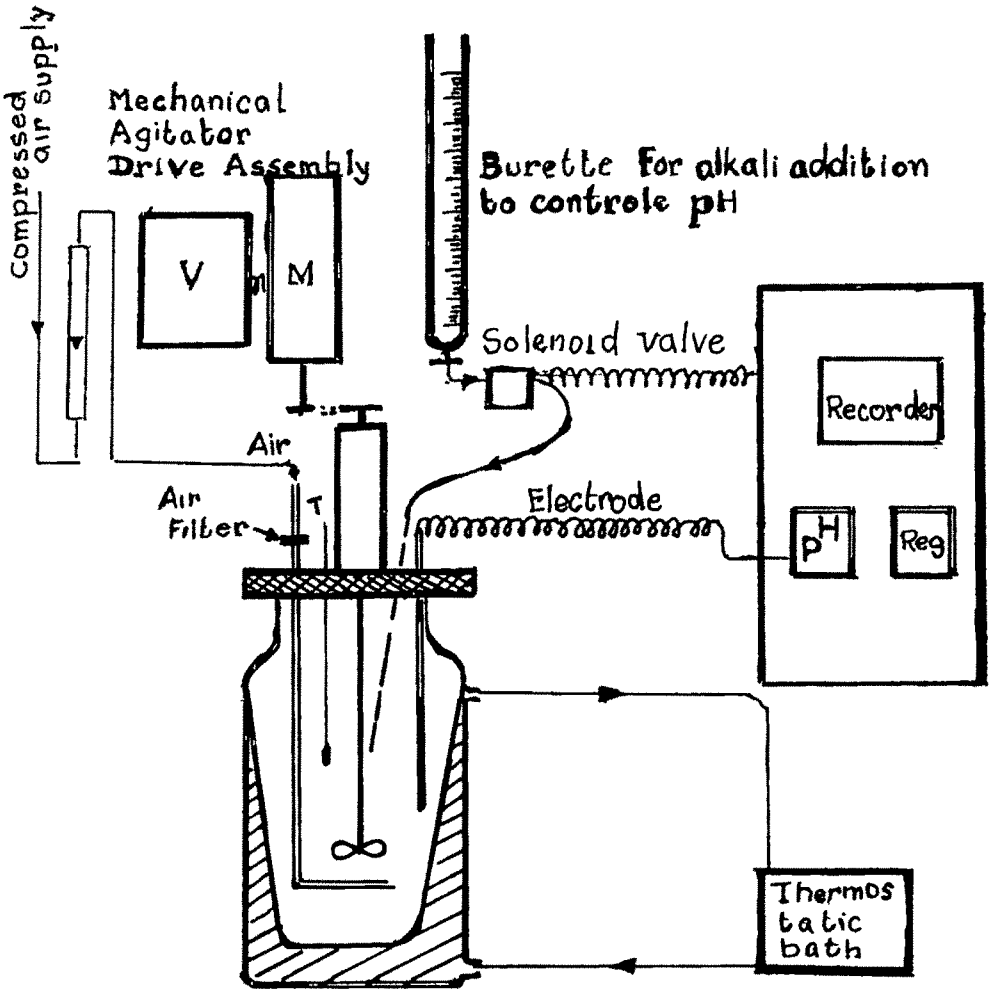
of minute holes allow the air under pressure to escape as thin air bubbles into the liquid medium through the central tube forcing the liquid to rise. This liquid passes through the annular space to the bottom of the fermentor which again is forced by air bubbles to travel through the central tube upwards. This provides both aeration and agitation and does not involve mechanical agitator. The whole fermentor is jacketed to pass water at desired temperature to controle the temperature. There is a provision to insert the ' Ingold' type pH electrode which could be connected to the pH controle system.

There is provision to add the concentrated medium and hydrocarbon feed separately with the help of special type of pumps which can operate against pressure of the liquid column in the fermentor and thus to operate continuous fermentation run.

Pilot plant set up include :-

(1) Semi continuous disc type (Westfalia type) centrifugal separator to concentrate the biomass nearly ten times.

Fig. 6.A



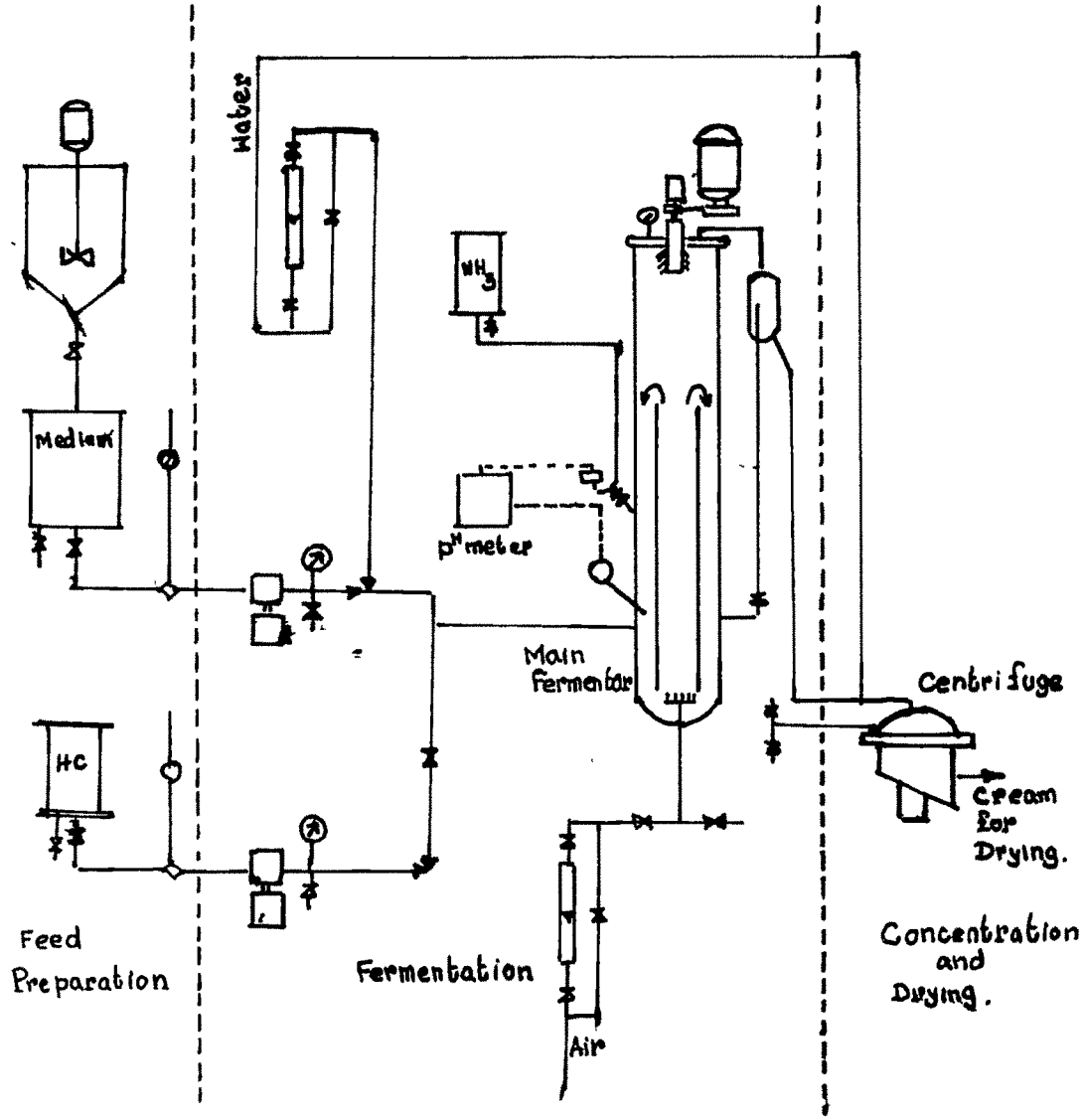
Jacketed Glass Fermentor

SCHEMATIC DIAGRAM OF THE EXPERIMENTAL SET-UP

Frg. 6.B

Flow Diagram of Pilot Fermentation Unit

(Fermentor Capacity : $1m^3$)



(2) Spray Dryer (Anhydro - Make) with water evaporation capacity 25 kg / hour.

Flow diagram of pilot fermentation unit is given in fig (6B).

II. 2.4. Analytical Procedures :

1. Growth monitoring in shake flask experiments :
   In shake flask studies the growth is monitored by measuring the optical density (O.D.) at 610 nm wave length at an interval of 30 minute in a spectrophoto. meter. (Systronics - 105 MK. 1). The natural logarithm of the optical density (OD) is plotted against time. The slope of the exponential phase gives specific growth rate.

2. Oxygen transfer rate (OTR) in the shake flask and in the fermentors is determinal by sulphite oxidation method.

3. Cell concentration in the broth was determined by filtering a known volume of sample through Millipore filteration set up on a pre-weighted 0.45 u membrane

filter paper and subsequently drying the filter cake at 80°C and weighted to a constant weight. Logarithmic cell concentration versus time given growth curve.

Cumulative alkali addition during the fermentation to maintain constant pH of the broth is recorded at half an hour time interval to monitor the growth. Growth curve is obtained by plotting natural logarithm of cumulative alkali addition versus time.

4. Protein content of the dried biomass is determined indirectly by determining its total nitrogen content by micro kjeldhal's method of Ma and Zauzagar (241).

Known quantity of dried yeast cells is digested with concentrated sulphuric acid in presence of catalyst (32 parts of pottasium sulphate, 5 parts of mercuric sulphate and one part of selenium sulphate) on a micro - kjeldahl's digestion unit, till it becomes colourless. The digested sample is diluted with distilled water to a known volume. The ammonia present in an aliquot of diluted smaple was trapped in boric acid solution by steam distillation using sodium hydroxide.

The ammonia collected in boric acid solution is estimated by titration using hydrochloric acid. From the amount of hydrochloride acid used for titration the total nitrogen is calculated. The nitrogen value thus obtained is multiplied by 6.25 to get the protein content.

5. Determination of crude lipid of the dried yeast cells :

Lipid content of dried yeast cells is determined by solvent extraction using chloroform and methanol as solvent for nearly 12 hours in Soxhlet extraction unit (242).

6. Quantitative - plating method for checking purity of culture :

Quantitative plating method in Petridish is conducted at regular intervals with the sample drawn from the fermentor to determine whether the experimental batch/continuous run is contaminated or not and if it is contaminated to determine its growth rate. To secure a bacterial count the sample is diluted and plated and following incubation the colonies that

develope are counted. The bacterial count of the original sample is then determined by multiplying the number of colonies that develop by the degree of dilution (dilution factor) of the particular plate that is being counted.

7. Properties of petroleum fractions :

ASTM distillation, pour point and density of original petroleum fraction and that after fermentation are determined by the methods described by the Institute of Petroleum, London (243).

II. 3. Methods

II.3.1. Preparation of inoculum :

Inoculum is prepared in shake flask by culturing the pure yeast strain in the medium containing 1% n-paraffinic hydrocarbons at 35± 2°C. The shake flasks are cultured on the shaker to provide agitation for sufficient time for the growth of cells to take place. When the cells are in exponential growth phase, are transfered to another set up of flasks or to the fermentor.

II.3.2. Shake flask studies :

50 ml of growth medium with the desired hydrocarbon is taken in each of 250 ml shake flask provided with side tube of uniform diameter. By maintaining the temperature of the incubation room where the rotary shaker is installed, the temperature of the set up was maintained at desired level ± 2°C.

II.3.3. Effect of different sub-optimal levels of C. Substrate on the specific growth of IIP 4 yeast strain:

Growth studies are conducted with the growth medium containing different levels of carbon substrate ranging between 0.25 to 4 g/l of hydrocarbon, levels of substrate. After autoclaving the flasks containing the nutrient medium, the hydrocarbons substrate in required quantity is added maintaining the sterile conditions, care has been taken to add the same quantity of inoculum in each flask. Optical density of the medium at regular intervals is measured in each case so as to plot the growth curve.

II.3.4. Bench scale fermentation studies;

II.3.4.1. Batch fermentation : 1.5 l. medium containing

the substrate taken in the 2.5 l. capacity fermentor is autoclaved. After the medium attained the desired temperature, inoculum prepared in shake flasks is added so as to provide around 0.1 g/l of cells growing in exponential phase. During the growth the pH falls and with the help of automatic pH controle system which monitors the 2 N ammonia alkali addition constant pH is maintained at desired value.

Growth studies with the yeast strains : Candida tropicalis (IIP-4) and Candida lipolytica on n-paraffinic hydrocarbons :

Keeping pH at 4 ± 0.2, temperature 34 ± 1 °C, aeration rate 0.6 to 0.8 VVM stirrer speed at 1500 rpm to provide agitation and hydrocarbon substrate level in the fermentor at 1 % by volume, different batch run were given with the two dirferent strains; Candida tropicalis IIP-4 and Candida lipolytica IrP-29. Specific growth rate (μ) of both the strains were calculated by plotting the natural logarithm of dry weight (In X) and/or cumulative alkali addition against time. The slope of the curve during exponential phase is taken as specific growth rate.

II.3.4.2. Growth studies of the strain IIP-4 on different carbon substrates :

Growth studies are conducted in Bench scale fermentor with the yeast strain Candida tropicalis (IIP-4), keeping pH at 4 ± 0.2 temperature 34 ± 1 °C aeration rate 0.6 to 0.8 VVM. Stirrer speed 1500 rpm with different carbon substrate. Carbon substrates studied include, glucose, ethanol molasses and n-paraffins. In each case the substrate concentration in the fermentor is maintained so as to get a cell coneentration of around 10 to 12 gms per litre.

The natural logarithm of dry weight against time as well as cumulative alkali addition against time are plotted to calculate the specific growth rate.

II.3.4.3. Effect of Temperature on the growth rate :

Keeping pH at 4 ± 0.2 aeration rate at 0.6 VVM stirrer speed 1500 rpm n-paraffinic hydrocarbons substrate level in the fermentor at 1% by volume, different batch runs are given with the yeast strain IIP-4 at different

temperature to study the effect of temperature on the growth rate. In each case the specific growth rate is calculated.

II.3.4.4. Effect of pH on the growth rate :

Keeping temperature at 34 ± 0.5 °C, aeration rate 0.6 VVM stirrer speed 1500 rpm, hydrocarbon substrate level in the fermentor at 1% by volume, different batch runs are given with the yeast strain IIP-4 at different pH to study how the pH of the medium in the fermentor affects the growth rate of the microorganism.

II.3.4.5. Effect of hydrocarbon feed on the growth rate :

Microbial dewaxing of kerosine fractions :

Separate batch experiments are conducted keeping temperature at 34 ± 0.5 °C pH at 4, aeration rate 0.6 VVM stirrer speed 1500 rpm with kerisine fractions collected from four different crudes i.e. Ankleshwar, North Gujarat, Bombay High and Imported, maintaining around 0.75 to 1% of n-paraffinic content in the medium.

Separate batch experiments are conducted with C_{11} - C_{13} cut n-paraffinic hydrocarbons, C_{14} - C_{18} cut n-paraffinic hydrocarbons and pure hexadecane. Yield coefficient ($Y = \frac{X}{S}$) is determined from the cell concentration at the completion of the batch experiments in each case.

After removing the aqueous medium the concentrated biomass is treated with ten times its volume a solvent mixture containing acetone and n-heptane blended in the ratio 3 : 1.

To replace acetone with 2 - propanol experiments are conducted to plot ternary phase diagram for the system 2-propanol gas oil fraction and water containing nutrient medium.

Yeast cells gets settled after solvent treatment and these settled cells are separated from the solvent mixture by filteration. The unassimilated hydrocarbons which goes along with the solvent mixture is recovered by simple distillation.

N-paraffinic hydrocarbons present in the kerosine sample and in the raffinate are estimated by Urea adduction technique.

II.3.4.6. Continuous fermentation studies :

1. Bench scale fermentation :

The fermentor used for continuous fermentation is the same as in batch fermentations and hence the set up for monitoring the parameters of growth are same as described in batch fermentation studies.

Growth conditions established through batch fermentations for the yeast strain IIP-4 vis : temperature 34 ± 1 °C, pH 4 aeration 0.25 VVM agitation 1500 rpm are maintained to check the stability of both the yeast strains viz IIP-29 belonging to Candida lipolytica species and IIP-4 belonging to Candida tropicalis, for long duration continuous operation.

Maintanence of dilution rate :

Initially batch fermentation run is given and when the cells are in exponential growth phase after it attains a cell concentration of over 5 to 6 g/l in the fermentor, the batch run is converted to continuous run maintaining a very low dilution rate around 0.1 h^{-1}. Continuous withdrawal of the biomass from the fermentor and

simultaneous addition of fresh medium (maintained at the same pH as that in the fermentor, viz at pH : 4) and hydrocarbon feed at desired level with the help of separate pumps are made, maintaining a constant level in the fermentor. Care is taken to change the dilution rate gradually so as to avoid giving shock to the strain and at each dilution rate sufficient time is allowed after it reaches steady state conditions before the cell concentration is determined. Besides determining the residual hydrocarbon content in the broth, protein content and lipid content of the final powder of the biomass collected at each dilution rate are determined.

II.3.4.6. 2. Pilot plant operation :

Nearly 850 litres of medium containing commercial grade salts of composition given in Table (7) and as hydrocarbon C_{14} - C_{18} cut n-paraffinic hydrocarbon so as to provide 8 to 10 gms/litre of initial substrate concentration are maintained at a temperature 36 to 37 °C and pH of the medium at 3.5 $\pm$ 0.05.

This is inoculated with sufficiently large quantity of biomass produced in 2.5 lts. cap. bench fermentor and preserved at 8 to 10 °C after removing the water medium. The quantity of inoculum added is such that it provides an initial cell concentration of 0.075 to 0.1 gm/lt of dry cells. After about 15 hours of operation when the cell concentration in the fermentor reaches around 8 to 10 g/l the batch operation is gradually changed to continuous one. First hydrocarbon pump gets into operation adding hydrocarbon at the rate of 1.8 litre/hour. Concentrated medium (8M of composition as given in Table 7) is added at the rate of 17 to 18 L/h and water at the rate of 125 to 130 L/h. During the operation pH is kept constant at 3.5 with the help of pH controle system. The total addition is maintained so as to provide a dilution rate of 0.16 - 0.17 h^{-1} and the biomass withdrawn through constant level control system is collected in a tank where water medium is decanted.

The concentrated cream along with fresh water enters into the centrifuge and the water washed concentrated

cream containing 100 to 150 g/l cells is passed into the spray dryer with the help of special pump at such a rate so as to maintain the spray dryer bottom temperature between 90 - 95 °C. Hot air comes into contact with the cream which is sprayed through a disc rotating at a speed of 10000 rpm.

Spray dried sample thus collected is subjected to

(i) Nutritional studies at C.F.T.R.I. Mysore

(ii) Toxicological studies at I.T.R.C. Lucknow

(iii) Field Trial Experiments conducted in farm animals at

(1) I.V.R.I. - Izatnagar

(2) Gujarat Agricultural University - Anand

(3) National Dairy Research Institute-Karnal.