

CHAPTER - III

RESULTS AND DISCUSSION

The Rung of a ladder was never meant to rest upon
but only to hold a man's foot long enough to put
the other some what higher - Thomas Huxley.

III. RESULTS & DISCUSSION

Nutrient Requirement :

Most of the studies on microbial nutrition have primarily been concerned with the identification of growth factors and choosing the optimal medium based on a qualitative approach, that is adding different substrates in more or less arbitrary amounts. The ultimate aim is to achieve optimal growth conditions with defined media.

The nutrients required for growth apart from energy sources may be classified into the following groups.

- (i) sources of the major elements C, H, O and N
- (ii) sources of the minor elements P, K, S, Mg
- (iii) sources of the trace elements
- (iv) vitamins and hormones

Most of the bacterial groups are heterotrophic that is they obtain their cell carbon from organic compounds. Organisms require a source of energy to synthesize cell material for growth and for so-called maintenance functions.

Hydrocarbon as carbon energy source :

A salient feature of hydrocarbon as substrate is their insolubility. The solubility of n-alkanes reaches a peak of about 600 mg/l with chain length from C₂ to C₄ and then decreases with chain length according to the expression.

$$\log H = 4.526 - 0.588n$$

Where H is the solubility in mg/litre and n is the number of carbon atoms in the molecule (90).

Hydrocarbons can be divided into three different kinds of feedstock used for biomass production.

Metabolism of gaseous hydrocarbons which has served advantages in the ultimate stage of separation of non-metabolised substrate from the cells is yet to create an impact in Research and Development programmes. Many researchers have succeeded in the use of methane, ethane and propane and their mixture with a view to utilize ultimately the natural gas as feed stocks. The range C₄ - C₉ does not appear to have proved useful. On the

contrary it disturbs the growth. They proved to be lipid extracting solvents. It is also a costly fraction in view of its use in motor gasoline production.

The most commonly used substrates are gas oil and n-paraffins separated therefrom. Although hydrocarbon fermentation was started and extensively carried out using gas oil, n-paraffinic hydrocarbons separated, from gas oil and further purified to remove aromatic hydrocarbons, are now most commonly used.

Separation of n-paraffins is affected by Urea adduction or adsorption on molecular sieves. Molecular sieves of suitable quality useful for treatment of gasoil have been of recent origin, although they have been available for several years for treatment of kerosine and naphtha fractions. Several commercial processes using 5 A type molecular sieve are available for n-paraffinic hydrocarbon separation. Separation of n-paraffinic hydrocarbons from kerosine and gas oil is not normally required to be done in petroleum refinery producing fuel products, unless it is also connected with the production of petrochemicals

like synthetic detergents. In fact removal of n-paraffins from gas oil within limits helps to reduce pour point and improve pumping characteristics in cold climate.

The characteristics of the kerosine fraction of different crudes in the fermentation work as well as $C_{14} - C_{18}$ cut n-paraffinic produced by molecular sieve adsorption method are given in Table (8).

Other nutrients :

Nitrogen requirements : The nitrogen sources which can be utilized by different organisms include most, if not, all of the inorganic forms of nitrogen. The nitrogen is metabolized to provide mostly protein, nucleic acids and cell wall polymers. The amino acid pool in the cytoplasm accounts for 0.25 to 5% of the dry biomass (244, 245). The cell nitrogen constitutes up to 12% of the dry weight of bacteria.

Nitrogen limited growth : The proteins content of the biomass is lower with nitrogen limited growth than with carbon limited growth, for example in yeast is 30%

proteins with ammonia limitation compared with 50% with glycerol limitation (246). The low protein content with excess carbon sources could reflect to accumulation of energy reserve such as glucogen in the biomass.

Phosphorous requirement : Phosphorous is usually supplied in the forms of inorganic phosphate. The phosphate is mostly incorporated into the nucleic acids, phospholipids and cell wall polymers. Only a small fraction of the total phosphate appears in the form of diffusible organic phosphorous such as ATP.

The phosphorus content of bacterial cell is about 1.5% of the dry biomass, however the content increase with growth rate and varies inversely with temperature. The variation largely reflect the RNA content of the cells (247).

Potassium, Sodium requirements : The Potassium requirements for the growth of microbial biomass corresponds to a growth yield of roughly 60 g dry biomass per gram potassium. Much of the potassium seems to be

bound with the RNA (247) so that the potassium requirements is increased by factors such as growth rate which increase the RNA content of the biomass.

A requirements for sodium ions in microbial growth has rarely been demonstrated. This may be largely because it is difficult to produce media free from sodium ions.

Magnesium requirement :

The growth yield from magnesium varies from about 300 to 900 g dry biomass per gram magnesium and is inversely proportional to the amount of RNA in the biomass. (247).

Sulphur requirements :

The growth yield assimilated sulphur is about 300 g dry biomass per gram sulphur. Sulphur is commonly provided in one of its inorganic forms usually sulphate or in the form of cystine and methionine.

Sulphur sources are assimilated mostly to provide sulphur for the amino acids cysteine and methionine.

Smaller amount of sulphur sources are required to provide sulphur groups in some co-enzymes such as biotin, co-enzyme A, Ferredoxin, Lipolic acid and thiamine. Growth limitations by sulphur may decrease the synthesis of these key sulphur compounds and affect their functions in the cell. An example is sulphate limited growth of yeast which causes loss of one site of oxidative phosphorylations in the respiratory chain, the effect being similar to that of iron limited growth (246).

Trace elements :

It is usually difficult to demonstrate requirements for the trace elements because often they are present in sufficient amount as contaminants in the medium constituents. Rough estimates of the quantitative requirements for some of the more important trace elements are :

(g/100 g)

Ca	:	0.10	Zn	:	0.005
Fe	:	0.015	Cu	:	0.001
Mn	:	0.005	Co	:	0.001

Iron is required for the many cofactors of enzymes with redox function in the cell. The restrictions of supply can disrupt the synthesis of these co-factors and impair their functions in the cell (246). Iron deficiency causes excretion of iron binding compounds by some bacteria & fungi (248). Iron deficiency stimulates riboflavins production by certain yeast. (249).

Cobalt forms a part of the molecule of vitamin B₁₂ or cobalamin which is synthesised by procaryotes.

Copper is present in the terminal oxidase of the respiratory pathway in yeast. A deficiency of copper or iron cause enlargement of mitochondria in yeast (250).

Copper deficiency in a yeast culture selects a mutant which lacks the normal terminal oxidase (251).

Borate was found essential for growth of a *Candida* species in n-paraffin (17) so also Iodide ions are necessary for growth of a *Candida* species on n-paraffins (252).

The composition of the mineral mix depends upon the strain. Typical composition have been mentioned in patents and reviewed by Noyes (253).

The amount and nature of constituents of culture media are determined by the yield of products and the growth rate required. Estimates of the growth yields can be made from the elementary composition of the biomass and the energy source requirements may be estimated from knowledge of the ATP yield.

The main factors influencing the stability of a medium are the nature of the constituents their reaction with each other, temperature, particularly during heat sterilizations ; pH of the mediums, oxygen and the light.

III. 2. Parameters of Growth of Microorganisms

2. 1. Growth Curve :

The requisite conditions for growth of biomass in a culture are :

- (i) viable inoculum
- (ii) energy source
- (iii) nutrients to provide the essential materials from which the biomass is synthesised.
- (iv) absence of inhibitors which prevent growth.
- (v) suitable physicochemical conditions.

When a small quantity of living cells is added to a liquid solution of essential nutrients at a suitable temperature and pH, the cells grow. For unicellular organisms which divide as they grow increase in biomass (mass of living matter) is accompanied by increase in the number of cells present.

Associated with cell growth are two other processes :

Uptake of some material from the cells environments and release of metabolic end products into the surroundings. The rates of these processes vary as growth occurs.

In a typical batch process the number of living cells varies with time. After a lag phase where no significant increase in cell numbers is evident a period of rapid growth ensues during which the cell number increases exponentially with time which is often called logarithmic phase or exponential growth phase which is followed by stationary phase.

Lag phase :

The length of the lag phase observed when fresh medium is inoculated depends on both the changes in nutrients composition if any experienced by the cells and the age and size of the inoculum. In order to minimize the lag phase the following are observed :

- the inoculation culture is as active as possible and the inoculum carried out with cells in the exponential growth phase.
- the culture medium used to grow the inoculum is as close as possible to the final full scale fermentation composition.

Different batch fermentation runs were given with the following which serve as carbon source :

I - Kerosine fractions from four different crudes :

- (i) Ankleshwar
- (ii) North Gujarat
- (iii) Bombay High
- (iv) Imported

The characteristics of these fractions are given in Table (8).

II- Pure n-paraffinic hydrocarbons :

- n.heptane -C₇; n.octane -C₈; n.nonane -C₉;
- n.decane -C₁₀;
- n.paraffinic hydrocarbons - C₁₁ - C₁₃;
- n.paraffinic hydrocarbons - C₁₄ - C₁₈;
- hexadecane - C₁₆ and nonadecane - C₁₉.

From the ASTM distillation data presented in Table (8) one can predict that both North Gujarat and Ankleshwar Kerosine fractions contain C₉ and C₁₀ n.para-ffinic hydrocarbons to the extent of not more than 5%

where as in the case of Bombay High and Imported crude Kerosine fraction these lower paraffinic hydrocarbons could be present to the extent of nearly 35%.

Ankleshwar and North Gujarat Kerosine fractions are likely to contain predominantly C_{14} n.paraffinic hydrocarbons and nearly 20% of C_{15} n.paraffinic hydrocarbons. In the case of Kerosine fraction from Bombay High and Imported crudes 80% distills off below $242^{\circ}C$ and contain mostly n.paraffinic hydrocarbons C_{13} and below.

With batch fermentation runs given with these different kerosine fractions it is observed that the lagphase of the organism IIP-4 remained close to 15 to 17 hours in the case of kerosine fraction from Ankleshwar and North Gujarat crudes. However this lag phase increased to nearly 120 hours when kerosine fraction from imported crude was tried and no growth was observed even after 168 hours when kerosine fraction from Bombay High was tried as carbon substrate.

In the case of pure n.paraffinic hydrocarbons C_{16} as well as $C_{14} - C_{18}$ cut wide range n.paraffinic hydrocarbons the lag phase has been observed to be 2 to 3 hours.

Similar results are obtained with gas oil fraction having initial boiling point above 250°C which are likely to contain C_{14} and above carbon range hydrocarbons. The presence of unassimilable non n.paraffinic hydrocarbons i.e. naphthenic and aromatic hydrocarbons present to varying extent depending upon the type of crude, has not affected the lag phase.

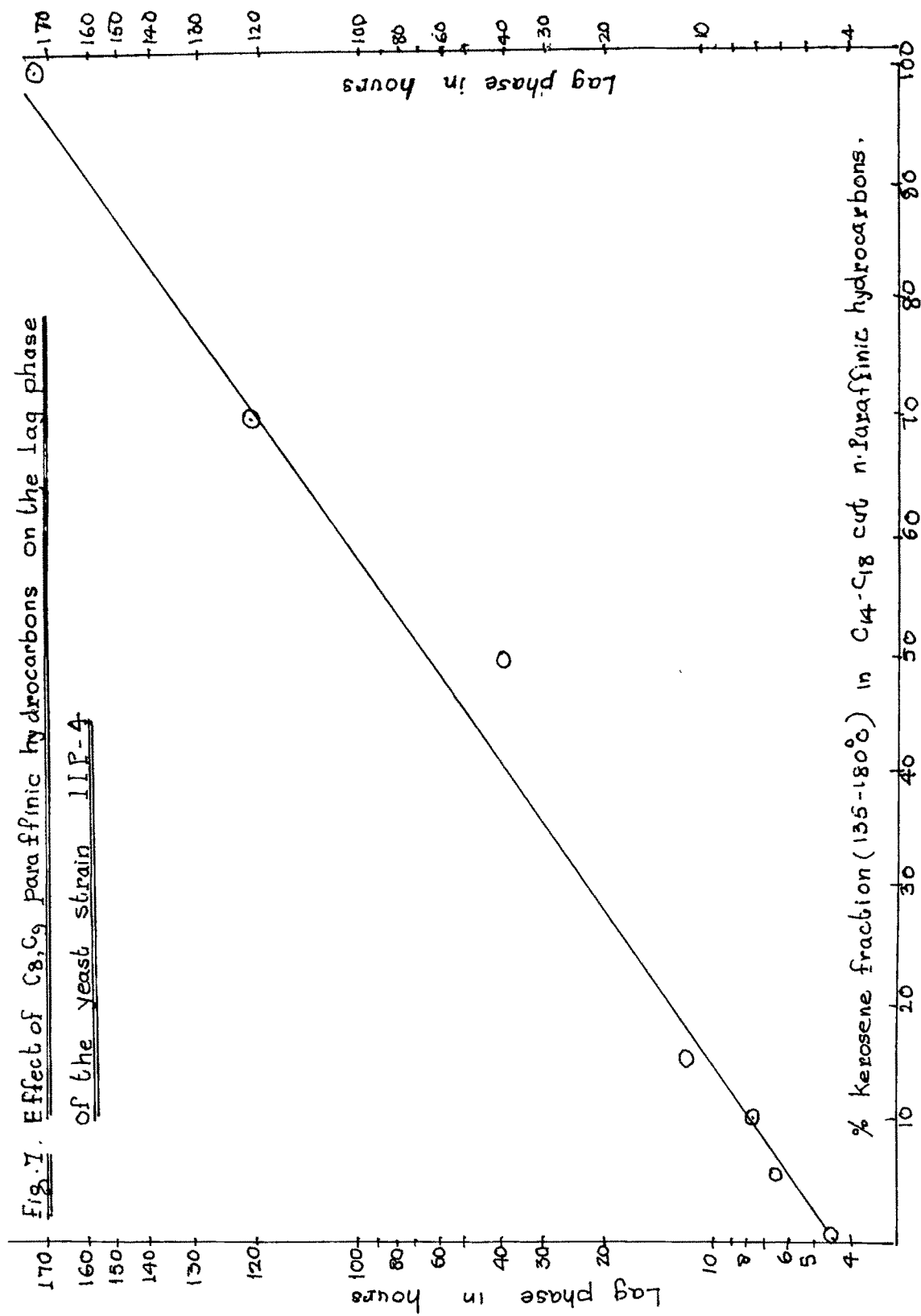
In order to study the effect of C_8 and C_9 n.paraffinic hydrocarbons on the lag phase of the growth curve of the said microorganism IIP-4 different experiments are conducted with $C_{14} - C_{18}$ cut n.paraffinic hydrocarbons mixed with different proportion of the C_8 and C_9 n.paraffinic hydrocarbons. It has been observed that the lag phase increased exponentially with the linear increase of C_8 and C_9 n.paraffinic hydrocarbon in the feed (fig 7).

Same phenomenon is responsible for increase of lag phase to 120 hours when kerosine fraction from imported crude is used as carbon substrate. Cells grown on kerosine samples from Ankleshwar and North Gujarat have lag phase less than 16 hours due to low concentrations of C_8 and C_9 hydrocarbons.

It is during the lag phase preceding the exponential growth phase that the nodes of control and regulation of enzymes activity that promotes and adaptive characteristic when presented with a new nutrients is achieved by cell production of new enzymes. Thus transfer of a glucose bred culture in its exponential phase to a hydrocarbon medium will necessarily result in a time interval of insignificant cell division rate while the enzyme and co-factors for the hydrocarbon metabolic pathway are synthesised in the cell. Similarly variation in the concentration of nutrients may cause a lag phase.

According to Barnet et al (254) the lag phase is the time required to produce the extra cellular products that would form micelles. Early micells formation

Fig. 7. Effect of C₈, C₉ paraffinic hydrocarbons on the lag phase
of the yeast strain IIP-4



shortens the lag phase. According to them the cells produce liphophillic materials that act upon the hydrocarbon substrate to produce micelles, the large hydrocarbon droplets essentially serving only as hydrocarbon reservoirs.

According to Zagic et al (255, 256) an extra cellular polymer was produced by continuous fermentation in kerosine in a 24 litre reactor and this polymer was composed of lipid proteins and carbohydrates. They have shown Curynebacterium hydrocarboclastus culture isolated in the laboratory to produce extra cellular biopolymer with emulsifying properties on a mixture of linear hydrocarbon.

According to Duvnjak and Koosavie (257) Corynebacterium lepus grown on glucose as its only carbon source synthesised and stored glycolipids intracellularly and if alkanes were added the glycolipid was excreted into the fermentation medium. Yoshida and Co-workers (258) demonstrated actual utilization of alkanes accommodated in the aqueous phase as submicron droplets.

A hydrocarbon solubilizing factor from Pseudomonas aeruginosa was identified as a rhamnolipid by Hisatsuka et al (259).

The same authors isolated protein like activator which has formed in the presence of n.alkanes but not of glucose and this component had emulsifying abilities and stimulated the oxidation of alkanes (260).

According to Ito et al (261) the yeast Torulopsis bombicola produced extracellular sophorolipids. These emulsifiers stimulate the growth on alkanes and turned out to be specific only for this Torulopsis species.

Nakahara et al (262) isolated a mutant capable of excreting a surface active metabolite when growing on n.alkanes from a yeast strain which originally lacked this ability.

Dunlap and Perry (114) proposed that on the basis of the solubility of hydrocarbon the cellular lipids play an important role in hydrocarbon assimilation, intermediates of alkane degradation provided a solvent for the

insoluble alkane. Vestal and Perry (117) suggested that the increased lipid content was necessary for the uptake and the accumulation of lipophilic substrates. When grown on hydrocarbons the yeast Candida tropicalis contained twice as much lipid as when grown on glucose. In the transient culture phase, following a substrate change from glucose to hexadecane, adaptation occurred. The lipid concentration per cell increased during the transient phase. The cause of that adaptation phase was assumed to be due to both induction of the enzyme required for hexadecane oxidation and the necessity of transporting this substrate to the site of enzymatic action.

With the yeast strain IIP-4 belonging to the species "Candida tropicalis" the following findings are made and some of them are in tune with those cited in the literature.

- There is a lag phase when cells grown on glucose are transformed to hydrocarbon medium.
- No growth of this yeast strain on C_8 and C_9 hydrocarbons, The presence of these lower hydrocarbons has increased

the lag phase, depending upon their concentration, when higher hydrocarbon are used as carbon substrate.

- Experiments conducted for extraction of lipids from the yeast cells using C_8 and C_9 n.paraffinic hydrocarbons as solvent indicate delipidisation of yeast cells take place.
- Yeast cells grown on hydrocarbon contain more than 15% lipids and the same yeast strain on glucose was found to contain around 6 to 7%.
- White slimy emulsifying agent could be isolated during hydrocarbon fermentation.
- These emulsifying agents disperses the hydrocarbon as fine droplets which could be seen under microscope and perhaps called micelles and may be acting as hydrocarbon solubilising agents.

III.2. 2. Exponential Growth Phase

Determination of Specific Growth Rate :

The nature of activities of microorganisms is such that the pH of the environment of a metabolising culture will not remain constant for long. The change of pH are associated with the uptake of certain cations and anions. If nitrogen is supplied as an ammonium salts the utilized ammonia leaves in the medium a corresponding amount of free acid. When an organism is grown aerobically on a limiting carbon such as carbohydrate or hydrocarbon then the ammonia uptake is likely to be the major contributor of hydrogen ion to the medium. During growth one can register on a paper the variation of pH if the paper moves with a constant speed. If pH is adjusted with the help of ammonia solution between two choosed values pH_a and pH_b , the speed of acidification of the medium at any particular instant is given by :

$$\frac{pH_a - pH_b}{t_a - t_b} = \frac{\Delta(pH)}{\Delta t}$$

The speed of acidification is proportional to the growth rate of the culture provided the nitrogen contents of the cells remain constant during this period. The consumption of nitrogen of the ammonium salts present in the medium of the culture is proportional to the growth of yeast cells, maintaining the concentration of nitrogen of the cells constant. However with the consumption of ammonium nitrogen for cellular growth, lowering of pH takes place and ammonium hydroxide consumed to maintain constant pH is thus a measure of the growth of the said micro-organism. A plot of logarithm of total alkali added versus time indicate the growth pattern and the exponential growth yields a straight line, the slope of which indicates the specific growth rate. Dry weight of the yeast cells present in the broth drawn every hour from the fermentor when plotted in the logarithmic scale against time of withdrawal yields growth curve. This curve is parallel to the one obtained with logarithmic of total alkali added versus time.

Batch fermentation runs given with the yeast strains

(1) IIP-4 belonging to the species Candida tropicalis

and (2) IFP-29 belonging to Candida lypolytica species, on pure hexadecane (C_{16}) yielded curves which are shown in fig (8).

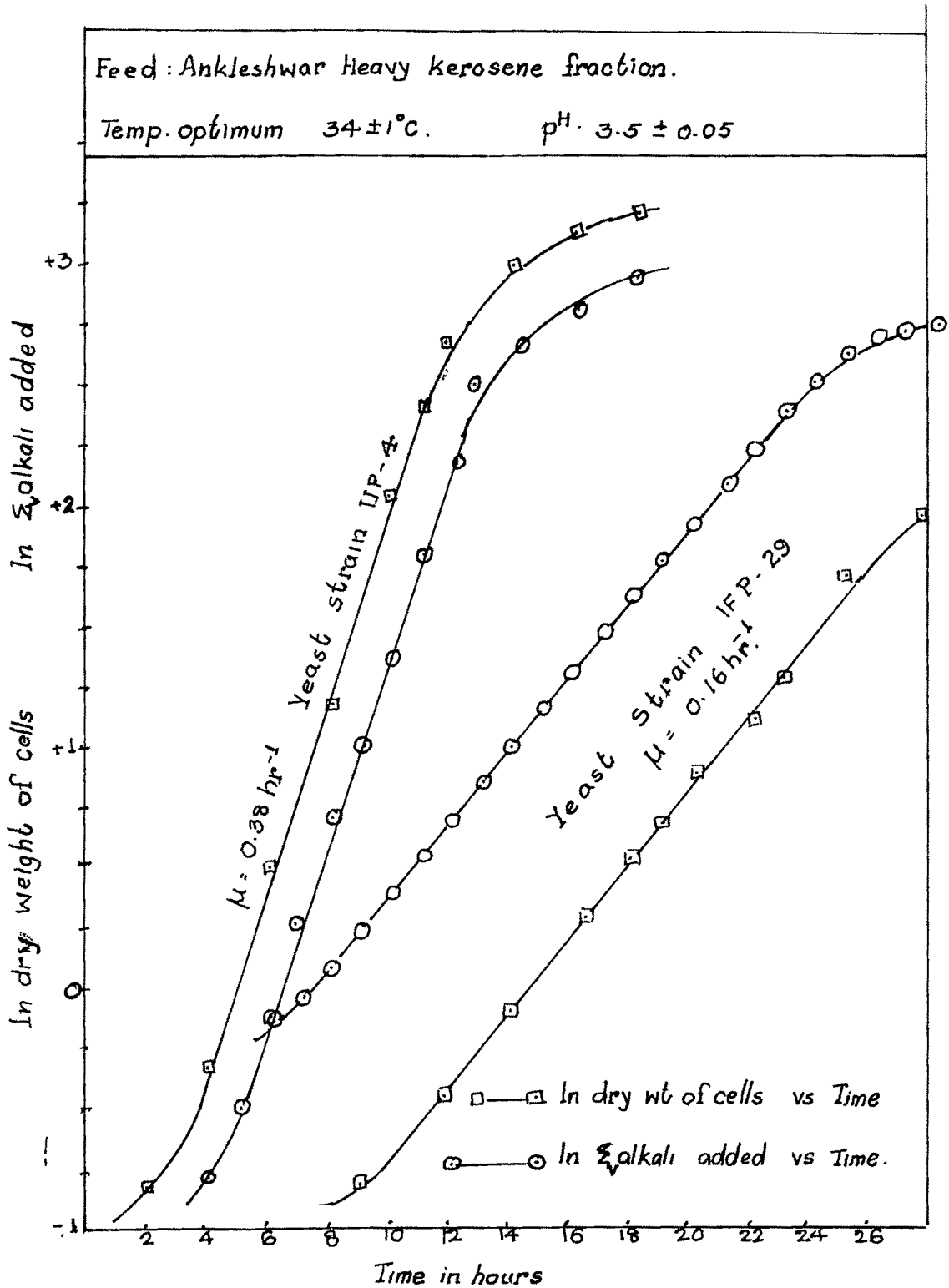
It is clear that logarithms total alkali versus time curve is parallel to the logarithm dry weight versus time curve in both the yeast strains studied. Specific growth rate in the case of IIP-4 as calculated from the slope is 0.38 h^{-1} and that of IFP-29 is 0.16 h^{-1} .

Moo Young et al (96) conducted experiments with an yeast strain Candida lypolytica with n.dodecane as sole carbon source under batch and continuous fermentation conditions and they observed that the specific growth rate during logarithmic growth rate was dependent not only on hydrocarbon concentration but also on the size of the dispersed oil droplets. They also observed that the specific growth rate decreased with increasing droplet diameter.

Blanch and Einsele (76) investigated with pure n.hexadecane as dispersed phase and observed two distinct growth phases the first exponential growth phase which was

Fig. 8

Batch Growth Curve of Two Yeast Strains



independent of stirrer speed; then a linear growth phase. The specific growth rate in the exponential period of batch has been reported to be function of impeller speed. At higher speed the dependence was not marked; μ_{\max} was not affected, only the saturation constant k_s being dependent on rpm of the stirrer.

It has been observed that above 0.01% of hydrocarbon substrate in the nutrients medium the specific growth rate of the yeast strain ' IIP-4 ' in the bench as well as pilot scale fermentor is independent of assimilable hydrocarbon concentration. Due to certain practical problems the specific growth rate could not be measured at low hydrocarbon substrate concentration. The observed growth kinetics appears to depend strongly on the drop size distribution of the dispersed phase. A large number of smaller drops are present as cultivation proceeds presumably due to the production of extracellular substances aiding the emulsification.

III. 2. 3. Maximum Specific Growth Rate : μ_{\max} :

In fermentation kinetics the specific growth rate μ is very important characteristic of microorganism. For higher productivity the organism should have higher specific growth rate μ which is specific for every organism and medium. It is desirable to know the value of maximum specific growth rate of the organism on a particular medium. A fundamental relationship between the specific growth rate μ and substrate was proposed by Monod. Of the same form as the standard rate equation for enzyme catalysed reactions with a single substrate (263) and Michaelis and Mentan (263) the Monod equation states.

$$\mu = \mu_{\max} \frac{S}{K_s + S}$$

Here μ_{\max} is the maximum growth rate achievable when $S \gg K_s$ and the concentration of all other essential nutrients are unchanged.

K_s is that value of the nutrient concentration at which the specific growth rate is half its value; it is

deviation between the lower concentration range where μ is strongly (linearly) dependent on S and higher range where μ becomes independent of S. The K_s value is inversely related to the affinity of the organism for the substrate. Rearranging the equation :

$$\frac{1}{\mu} = \frac{K_s}{S \mu_m} + \frac{1}{\mu_m}$$

A plot of $1/\mu$ against $1/S$ gives a straight line with an intercept on the abscissa at $1/K_s$ and an intercept on the ordinate at $1/\mu_m$. The linearity of the double reciprocal plot forms a convenient test of the validity of the hyperbolic relation. The values of K_s are extremely low often below the sensitivity of chemical assay methods and unless the sampling or assay is instantaneous the substrate level may fall substantially below the sample is assayed.

The methods used to measure the values of S at different growth rates are as follows :

- (i) instantaneous measurement of the growth limiting

substrate in a chemostat culture at different growth rates, as for oxygen (264).

- (ii) measurement of initial growth rate with different substrate concentration in batch cultures.
- (iii) measurement of the growth rate at end of a batch culture (265, 42).
- (iv) measurement of the critical dilution rates in chemostat cultures with different concentration of the growth limiting substrate in the feed medium (266).

The method - measurement of initial growth rates with different substrates concentrations in batch cultures has been tried. .

Growth Measurement in Shake Flasks :

The chemical method employed to follow the cellular development in suspension is the one based on nephelometry analysis or turbidimetry analysis. A bacterial culture acts as a colloidal suspension blocking and reflecting light that passes through it. Within certain limits the

light absorbed or reflected by a bacterial suspension is directly proportional to the concentration of cell in the culture.

In turbidimetry the light stopping ability of a culture can be expressed as the percentage of light transmitted. Within limits this percentage is directly proportional to the cell concentration. Ordinarily it is more useful to express the turbidity as optical density (OD) which is directly proportional to cell concentration.

In the use of turbidimetry to measure cell growth, the turbidity of bacterial culture is correlated with some other known measure of cell growth. Turbidimeters may be calibrated in terms of the equivalent number of cells of a standard size or the calibration may be referred to the dry weight or nitrogen content of the culture. The turbidity is proportional to total mass has been shown by dry weight determinations.

Many commercial instruments accept only cuvettes of specific size and shape, since these cuvettes are

not only readily adapted to the maintenance of sterility or cultural purity in microbiological work their use requires frequent sampling, usually accompanied by loss of biological material in the sample.

Capped Erlenmeyer flasks of up to 250 ml or even 1 L capacity can be fitted with side arms in such a way that part of the culture may be tipped into the side arm for turbidity reading without loss of material or cultural purity (267, 268). However with hydrocarbon as carbon source due to its immiscibility in nutrient medium turbidity readings obtained are not consistent and it is difficult to get reproducible results. This aspect one could overcome to a very large extent by using mechanical emulsifiers such as ultrazone or cyclomixers.

Using 250 ml side arm Erlenmeyer flasks at very low initial substrate concentration in the medium the specific growth rate has been found to be dependent on the initial substrate concentration. The specific growth rate for the yeast strain IIP-4 at initial concentration of 0.26 g/l was found to be $0.18 \text{ (h}^{-1} \text{)}$. The specific growth

rate increases with the increase of initial substrate concentration in the growth medium and beyond 5 g/l of hydrocarbon concentration in the medium it did not have any affect on the specific growth rate. The results obtained are given in Table (9).

The reciprocal of the specific growth rate $1/\mu$ when plotted against initial substrate concentration ($1/S$) a straight line is obtained and the reciprocal of the value corresponding to the intercept made by the straight line on the Yaxis, according to Line-Weaver. Buck is the maximum specific growth rate and in this case for the yeast strain IIP-4 on pure hexadecane C_{16} has been found to be 0.38 h^{-1} and the K_s value is 0.32 g/l (Fig. 9). For the same strains IIP-4 on different carbon substrate the values of μ_{\max} and K_s obtained are given in Table (10).

Table (9)

Effect of initial hydrocarbon concentration on
specific growth rate.

Strain : IIP-4 Candida tropicalis : C. feed: C₁₄-C₁₈
cut n. paraffins.

S ₀ g/L	μ (h ⁻¹)	1/S ₀	1/μ
4.0	0.34	0.25	2.95
1.33	0.30	0.75	3.33
0.87	0.26	1.15	3.84
0.77	0.26	1.30	3.84
0.67	0.25	1.50	4.00
0.54	0.22	1.85	4.54
0.36	0.20	2.78	5.00
0.26	0.13	3.72	5.56

S₀ : Initial Substrate Concentration

μ : Specific Growth Rate

Fig. 9. Line Weaver Burk Plot for the determination of Saturation constant (K_s) and maximum Specific growth rate (μ_{max})

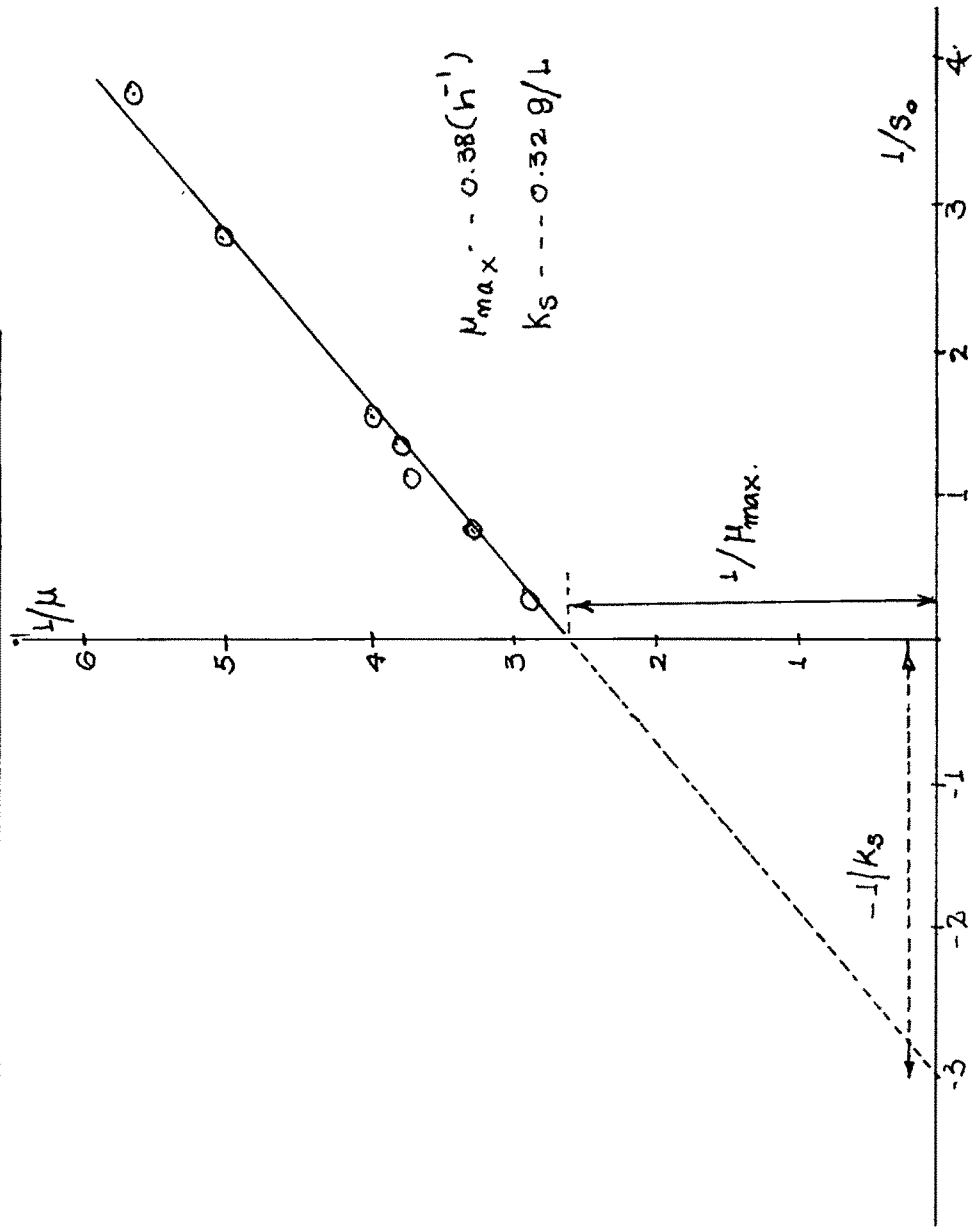


Table 10. K_s and μ_{\max} values of IIP-4 on
various substrates.

<u>C. Substrate</u>	<u>μ_{\max} (h⁻¹)</u>	<u>K_s (g/L)</u>
n. hexadecane	0.38	0.32
C ₁₄ - C ₁₈ n.paraffins	0.38	0.32
glucose	0.94	0.167
sucrose	0.85	0.132
fructose	0.69	0.084
absolute alcohol	0.81	0.042

III. 2.3.1. Growth specificity :

Experimental result of growth rate obtained with the strain IIP-4 on hydrocarbons of varying carbon number and different composition are given in Table (11).

The yeast strain IIP-4 has been isolated from the soil collected from one of the Diesel oil storage tank bottoms and the soil collected is bound to have microbes which can thrive on n.paraffinic hydrocarbons present in diesel fraction. This has a specific growth rate of 0.38 h^{-1} on pure pentadecane hexadecane, $\text{C}_{14} - \text{C}_{18}$ cut n.paraffinic hydrocarbons and on diesel fractions.

The same yeast strain has been found to grow with specific growth rate 0.26 h^{-1} on kerosine fractions from Ankleshwar as well North Gujarat mix. As determined by urea adduction technique (239) the kerosine fraction from Ankleshwar crude was found to contain 40% n.paraffinic hydrocarbons with carbon range $\text{C}_{12} - \text{C}_{17}$ and that of North Gujarat mix around 30% of n.paraffinic hydrocarbons of similar carbon range. Unassimilable non n.paraffinic

Table (11)

Effect of different hydrocarbon feed stock on Specific
Growth rate.

Strain: IIP-4		Temp. °C	36 ± 0.5°C	pH: 4-0 ± 0.05
C.Feed	C-range	Purity	Specific Growth rate μ (h ⁻¹)	Yield $Y = \frac{X}{S} (\frac{g}{g_{sub}})$
Nonadecane	C ₁₉	90%	0.32	--
n.hexadecane	C ₁₆	99%	0.38	0.99
n.Paraffins	C ₁₄ -C ₁₈	99%	0.38	0.85-0.95
n.Paraffins	C ₁₂ -C ₂₅	90%	0.38	0.7
Gas oil fraction	C ₁₂ -C ₂₅	45% n.Paraffins	0.38	0.4
n.Paraffins	C ₁₂ -C ₁₄	90%	0.28	0.5
tridecane	C ₁₃	96%	0.24	0.5
do decane	C ₁₂	98%	0.2	0.4
un decane	C ₁₁	96%	0.12	0.12
decane	C ₁₀	90%	no growth	--
n.Paraffins	C ₈ , C ₉	90%	no growth	--

hydrocarbons i.e. both naphthanic and aromatic hydrocarbons present to the extent of nearly 60% and 70% in the case of Ankleshwar, North Gujarat kerosine fraction respectively has not affected the growth rate of the yeast strain. The lower specific growth rate 0.26 h^{-1} observed is due to lower carbon range n.paraffinic hydrocarbons present in these fractions. Similar observations were made by Ballerini (271) and by Johnson et al (90).

The maximum specific growth rate of the yeast strain IIP-4 on different carbon substrates viz. Molasses, Starch Ethanol are given in Fig (10). (272).

III. 2.3.2. Effect of inoculum size on specific growth rate :

Knowledge of the quantitative effects of inoculum 'age' on the growth lag in cultures is largely based on the study of klebsiella aerogens cultures by Lodge and Hinshel Wood (273). They arbitrarily measured the age of the inoculum from roughly the beginning of the logarithmic phase when the bacterial population was $10^6/\text{ml}$. The age effects depended on whether the medium was minimal

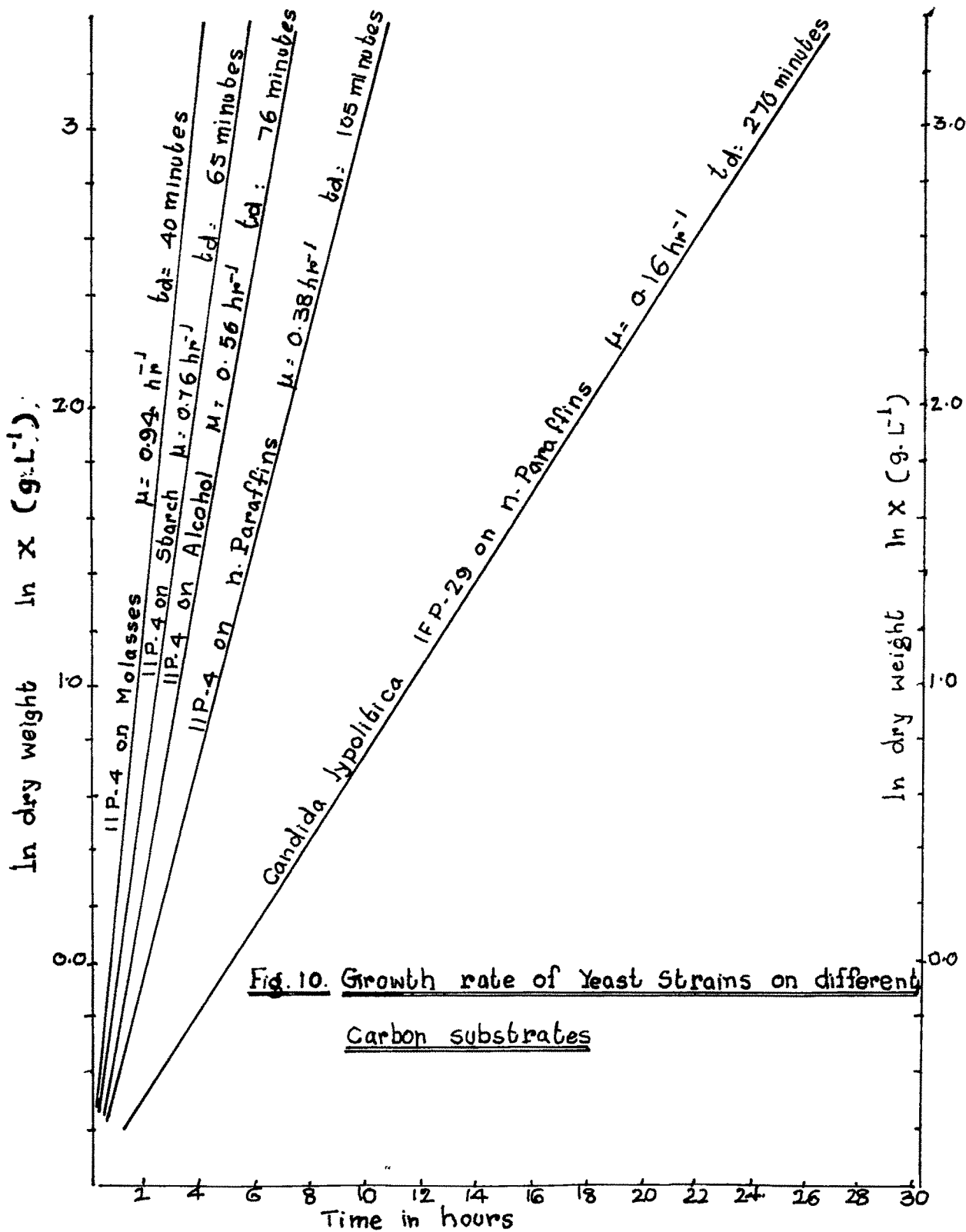


Fig. 10. Growth rate of Yeast Strains on different Carbon substrates

with glucose and ammonia as the sole carbon and nitrogen sources, or whether the ammonia was replaced by asparagine. In the glucose asparagine medium a growth lag began to appear roughly when the inoculum source culture entered the stationary phase and with increasing inoculum age the lag asymptotically approached a maximum. The lag in the glucose ammonia medium was long when the inoculum source was at the beginning of its logarithmic growth phase. The lag reached a minimum when the inoculum source approached the end of the logarithmic phase, then increased with entry of the inoculum source culture into stationary phase. Thus Lodge and Hinshelwood distinguished between the early lag which occurred with inoculum taken from the early logarithmic phase and late lag characteristic of inoculum from a stationary phase culture.

Prokop et al (274) investigated the effect of inoculum size, dispersed phase volume and substrate concentration on the batch growth of *Candida lipolytica* in a model system composed of n-hexadecane dissolved in dewaxed gas oil. Results obtained from experiments with inoculum values varying from 0.45 to 2.75 g cells dry weight/L

indicate the maximum macroscopic specific growth rate decreases from 0.225 h^{-1} to 0.135 h^{-1} as the inoculum size increases from 0.45 to 2.75 g cells dry wt./L.

However the different batch fermentation runs given with the yeast strain ' IIP-4' belongings to the species "Candida tropicalis", varying the inoculum size from 0.1 to 1 gm. dry weight/L did not show any appreciable change in the maximum specific growth rate on n.paraffinic hydrocarbons. This was checked both in 2.5 litre capacity (having liquid volume 1.5 litres) mechanically agitated and aerated ferment or as well as in 1 cubic metre capacity (liquid volume 750 to 800 litres) air lift fermentor. In all these experiments the maximum specific growth rate of the yeast strain IIP-4 on C_{14} to C_{18} cut n.paraffinic hydrocarbons at temperatures 35 ± 1 °C and pH 4 remained at 0.38 h^{-1} .

III. 2.3.3. Effect of temperature on specific growth rate:

Temperature affects the rates of cell reactions, the nature of metabolism, the nutritional requirements and the biomass composition.

Microbial growth and product formation are the result of a complex series of chemical reactions. Like all chemical reactions they are influential by temperature. Growth may be described by

$$\frac{dx}{dt} = \mu x - \alpha X \quad \text{or} \quad \frac{1}{X} \frac{dx}{dt} = \mu - \alpha$$

Where μ is the specific growth rate and α is the specific death rate. Thus the observed specific growth rate $\frac{1}{X} \frac{dx}{dt}$ is a balance of growth and death. Usually microorganisms are grown when $\mu \gg \alpha$ and α can be neglected. However both μ and α are likely to be temperature dependent.

Three typical growth temperature curves are observed. These curves correspond to

(1) Psychophillic (2) Mesophillic and (3) Thermophillic growth. Those with a temperature for maximum growth rate are below 20°C are psychrophillic, those around 30-35 °C are Mesophillic and those above 50°C are thermophillic. As temperature is increased towards the optimal growth temperature, the growth rate doubles over a 10°C range. Above the optimum growth temperature growth

rate declines rapidly with increasing temperature. The declining portion of the curve (temperature above the optimum) is a consequence of an increasing death rate :

Growth may be described by the Arrhenius relationship.

$\mu = Ae^{-E/RT}$ where A is constant dependent on the frequency of formation of activated complex of the reactants and E is a constant known as the activation energy.

The above equation can be written

$$\log \mu = \log A - \frac{E}{2.303} \frac{1}{R.T.}$$

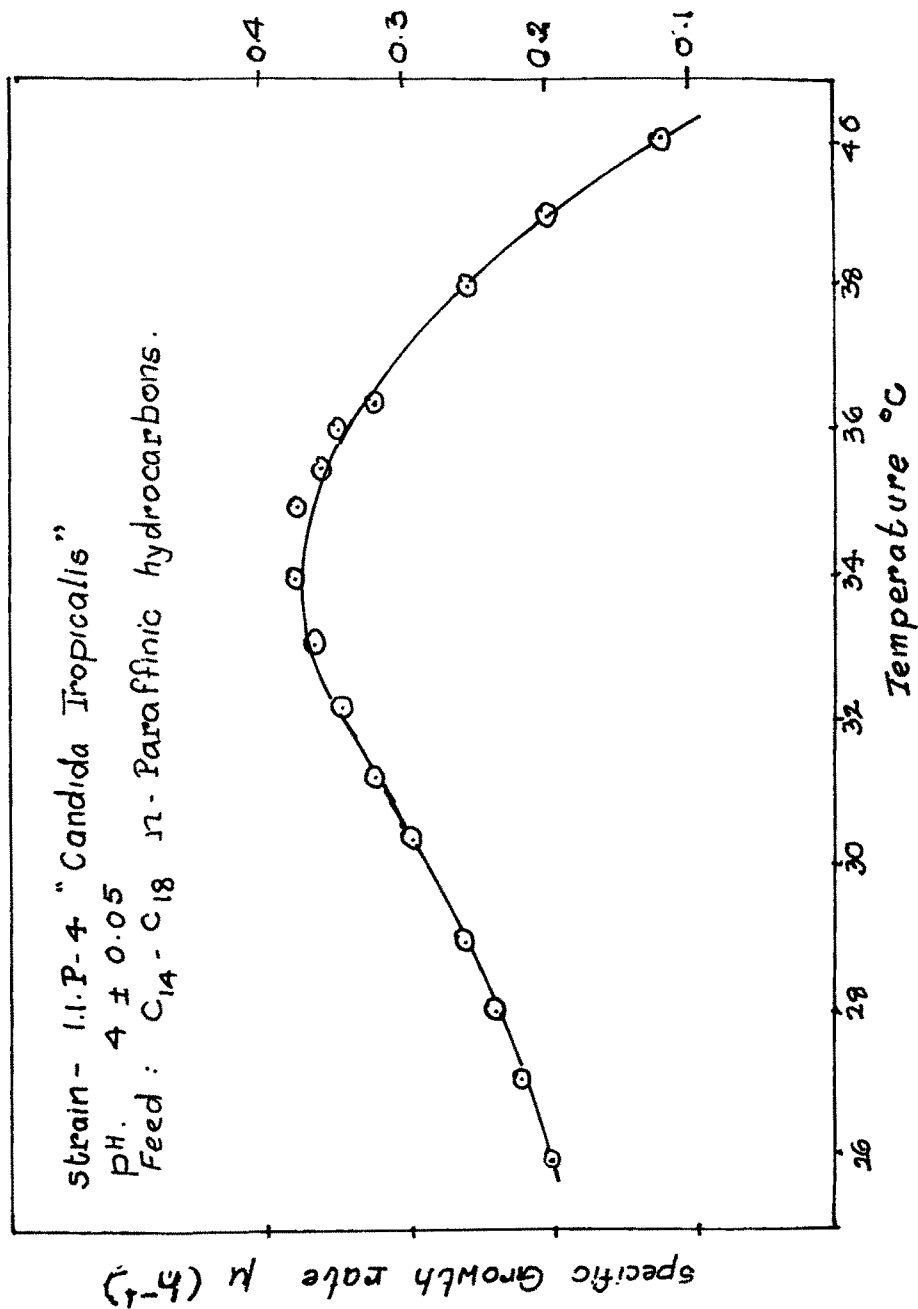
A plot of $\log \mu$ against $\frac{1}{T}$ gives a straight line with slope

$$\frac{E}{2.303} \frac{1}{R}$$

The variation of specific growth rate with temperature in the case of the strain IIP-4 on $C_{14} - C_{18}$ cut n.paraffinic hydrocarbons is shown in fig (11).

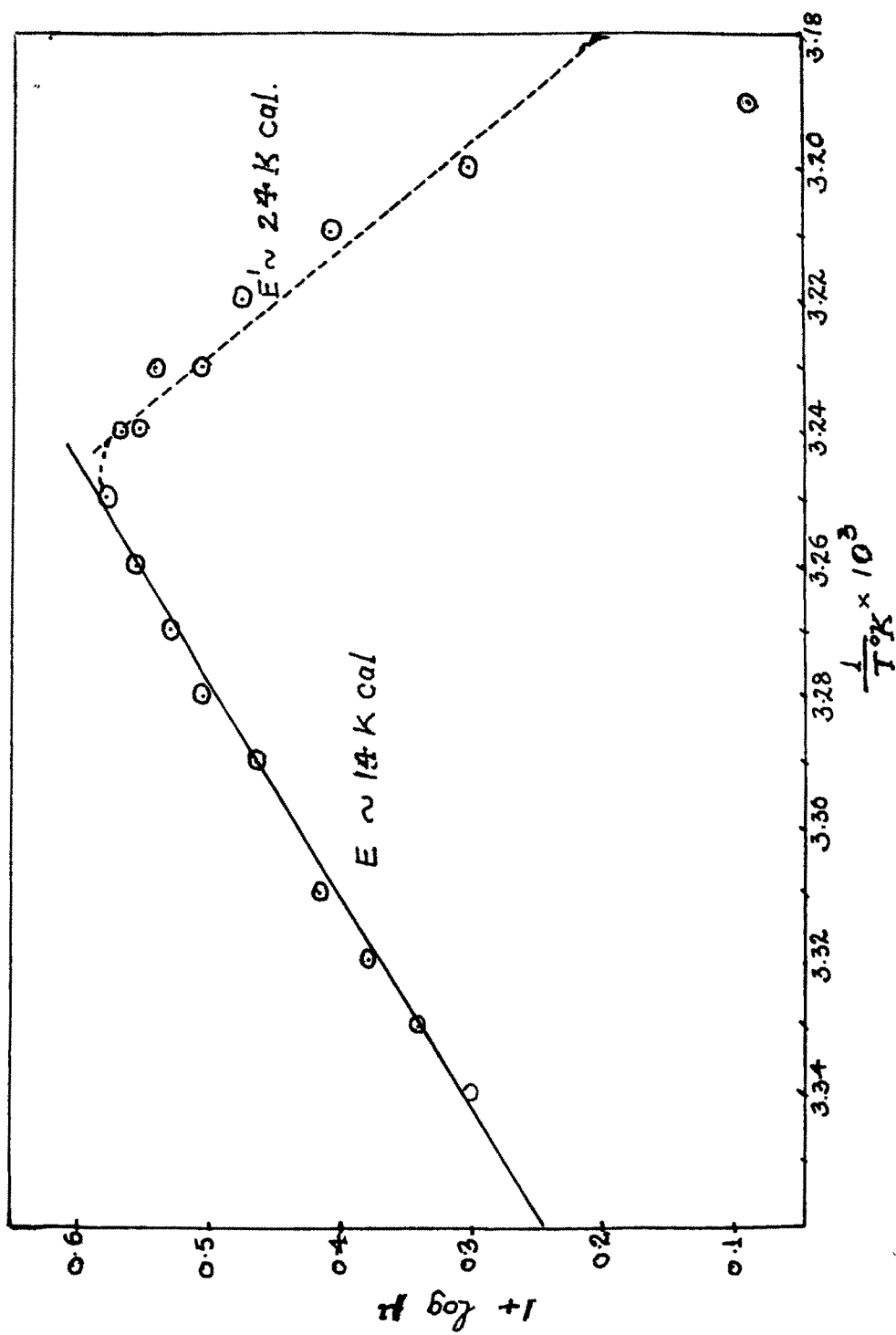
Arrhenius plot and activation energy for growth and death for this microorganism on n.paraffinic hydrocarbons are given in fig (12).

Fig. 11



Effect of Temperature on Specific Growth rate

Fig. 12. Determination of Energy of Activation.



For the yeast strain IFP-29 the maximum specific growth rate of 0.26 was observed at temperature 20°C and the specific growth rate at 34°C was around 0.16 h^{-1} (271). This value coincides with the one obtained on different hydrocarbon at temperature around 34°C.

From the Arrheius plot made the Energy of activation for the strain IIP-4 on n.paraffinic hydrocarbon is 14K Cal/mole and that for the deactivation is 24 K Cal/mole.

For the strain IFP-29 on paraffins as reported by Ballerini is 12 K. Cal/mole for activation (271). Values for the activation energy data of Monod for Escherichia coli and of other microorganisms are in Table 12.

Table 12. Values of the activation energy (E)
for microbial growth.

Organism	Temperature Range °C	<u>E</u> K. Cal/mole
Escherichia Coli (265)	23 - 37	13.1
Aspergillus nidulans (275)	20 - 37	14.0
Escherichia Coli (276)	26 - 37	16.2
Klesiella aerogenes (277)	20 - 40	14.2
Candida lipolytica (271)	20 - 29	12.0

The decrease in the growth rate of the upper extreme of the temperature range may reflect either a disruption of metabolic regulation or death of the cells. If death occurs the growth rate of the viable biomass is given by

$$\frac{dx}{dt} = (u - \infty)$$

Specific growth rate and ∞ specific death rate :

The death rate will become predominant at high temperatures if the activation energy for death exceeds that for growth. Topiwala and Sinclair (277) found for Klebsiella aerogenes for which the activation energy for death was 32900 cal/mole compared with a value of 14230 cal/mole for growth. The values as determined for the yeast strain IIP-4 on n.paraffinic for the temperature range are very much in agreement with these values.

III. 2.3.4. Effect of pH on growth rate :

Medium pH is an important parameter affecting growth and product formation. Most organisms function over a pH range of 3 - 4 units. Because pH is so important, it is controlled in most fermentations either by means of a buffer or a pH control systems.

In automatic analysis of the hydrogen ion concentration either the course of the pH changed by cell metabolism is measured and recorded or pH is measured and simultaneously controlled.

In microbial culture pH is generally controlled either by alkali or acid. The control solutions are released from the reservoirs with the aid of solenoid valves. Dosing is gradual and as close as possible to the indication electrode to avoid substantial deviations around the controlled value. The control solutions flow from the reservoirs by their own weight in the case of bench scale unit and with the help of a piston pump in the case of pilot plant.

The pH of the medium can hardly effect the total supply of any of the essential nutrient components, although it may cause wide changes in the availability of some of them. Its principal influence will be exerted upon the cell proteins, and enzyme activities are in fact strongly dependent on it. Gale and Epps (278) have shown that a change in pH during the growth of Escherichia coli causes a change in the enzyme content of the cells.

Controle of microbial growth by pH is a complex process but is presumably bound up with the cells ability to assimilate nutrients and with the activity of autolytic enzymes and of fermentation systems which provide energy not only for growth but for cell maintenance.

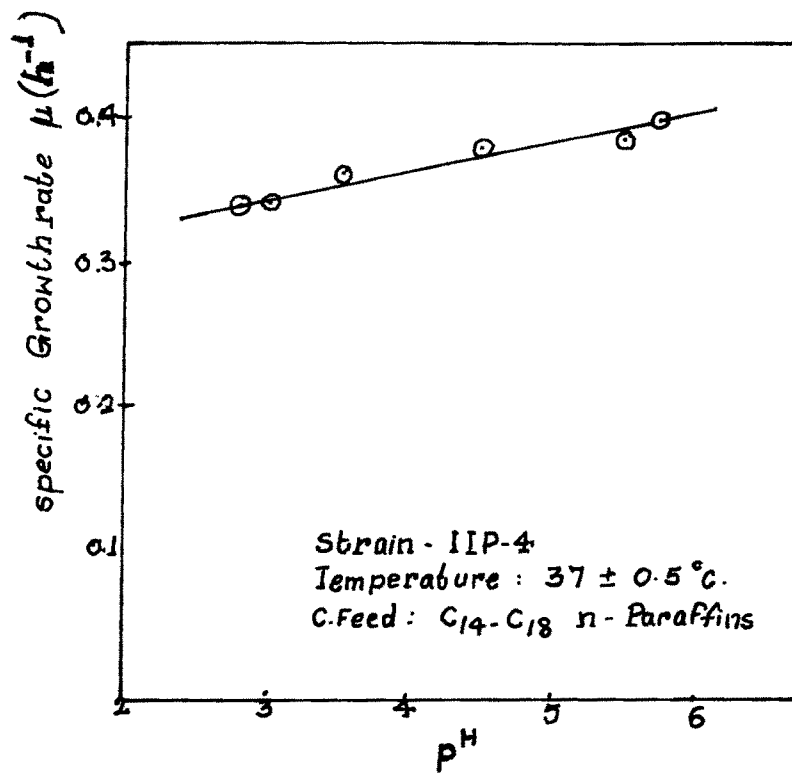
Bacteria usually grow in the range of pH 4 -8. Yeast usually prefers 3 - 6; Molds 3 - 7. As a consequence pH can be used to select preferentially for yeast over bacteria and sometimes to aid in maintaining an environment with minimal susceptibility to contamination. For instance a yeast fermentation at pH between 3 and 4 is unlikely to become contaminated with bacteria. Most

of the experiments are conducted at $\text{pH } 4 \pm 0.05$ thereby avoiding strict sterile conditions.

During a fermentation the pH has a tendency to change for several reasons. When ammonia is nitrogen source pH tends to fall. Ammonia in solution (below pH 9) exists as NH_4^+ ions; the microorganisms incorporate it into the cell as R-NH_3^+ where R is a carbon skeleton. In the process H^+ is left out in the medium. If nitrate is the nitrogen source then hydrogen ions are removed from the medium to reduce the NO_3^- to R-NH_3^- to R-NH_3^+ and the pH tends to rise. If organic amino compounds are used for growth then the pH tends to rise as the compounds are deaminated.

For the yeast strain IIP-4 the variations of specific growth rate with change in pH is given in fig (13).

Fig. 13.



Effect of pH on Specific Growth rate.

III. 2.4. Continuous Culture :

In batch culture the chemical environment is continually changing thereby introducing another varying parameter into what is already a complex situation, it is difficult to vary the growth rate except by altering the carbon and or energy source or by changing the temperature.

Prolonging a culture of microbes by the continuous addition of fresh medium and continuous harvesting of product has been discussed by Ricica (72). Continuous culture is achieved by two main methods.

In the first medium in which all the ingredients are present in excess, is allowed to drip into a vessel from which it is subsequently overflows at such a rate that the cell mass is maintained at the desired level. Under these conditions the growth rate is the optimum for the particular medium and can be only changed by altering the temperature.

In the second method one of the constituents of the medium is kept in such short supply that the growth rate

is a function of the rate at which the medium is added. The apparatus used for the first method is sometimes referred to as a turbidostat (72) and that for the second as a chemostat (72).

A chemostat culture should consists of a perfectly mixed suspension of biomass into which is fed at a constant rate and the culture is harvested at the same rate so that the culture volume remains constant. The fundamental importance of chemostat culture become apparant only after the formation of the basic theory by Monod (279) and Novick and Szilard (280). The theory first indicated that it should be possible to fix the specific growth rate of the biomass at any value from zero to the maximum.

To seek out microorganisms that will perform specific functions, it is necessary to establish a culture environment that will specifically select organisms for the desired function and will not permit or will at least minimize, the growth of other organisms/contaminants.

III. 2.4.1. Contamination :

One of the primary fears associated with continuous culture is the fear of contamination, since it is to maintain sterile conditions for prolonged periods. The need to continually supply sterile fresh air and medium to the culture further increases the probability of contamination. While it is true the probability of contamination increases with time, work done in this area has demonstrated that contaminant free runs of a thousand hours or more are quite feasible and in fact routinely made in many laboratories and industries.

In order to know the problem of contamination in continuous culture it is necessary to understand when and how a contaminant will survive. Let us imagine a carbon limited continuous culture system being operated with a pure culture of organism X. Suppose the culture becomes contaminated by organisms Y, Z or W. The rate of accumulation of these contaminants may be described by the following material balance.

Accumulation of contaminant = Flow of contaminants in - Flow of contaminant out + Contaminant growth

$$\frac{dx^1}{dt} = DX^1_{in} - DX^1_{out} + \mu X^1$$

Where the X^1 refers to the concentration of the contaminants Y, Z or W. The residual concentration of limiting nutrient at dilution rate D is S. The growth rate substrate concentration curves for organisms Y, Z and W are shown compared to the desired organisms X on fig (14). Since the limiting substrate concentration is S, organism Y can only grow at a rate μ_y , which is less than the dilution rate as shown in fig (14 a).

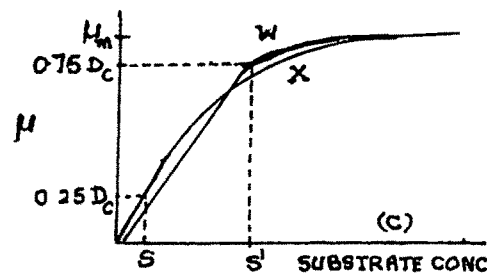
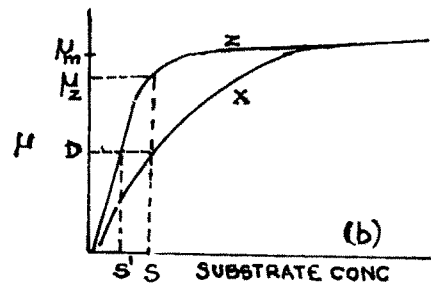
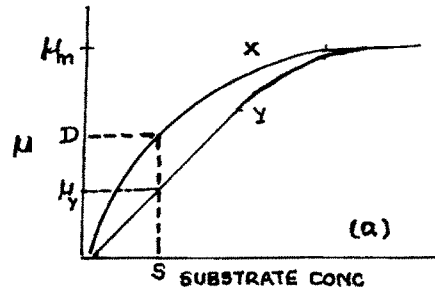
Thus its rate of accumulation given by

$$\frac{dy}{dt} = \mu_y \cdot Y - DY \text{ is negative}$$

As a consequence contaminant Y will not survive in the chemostat.

The relationship of growth rate to substrate concentration for contaminant X however is markedly different

Fig. 14



Growth rate versus substrate curves for a microorganism X
in continuous culture and three types of contaminants
 Y W and Z

than Y. At a substrate concentration of S, organism Z can grow at rate μ_z which is greater than dilution rate. The material balance for organism z become

$$\frac{dz}{dt} = \mu_z Z - DZ$$

with μ_z initially greater than D. Thus $\frac{dz}{dt}$ is positive and organism Z will begin to accumulate. At this point the original organism X will not be able to compete since its growth rate at S^1 is less than the dilution rate.

Organism X will then wash out from the vessel at a rate given by

$$\frac{dx}{dt} = \mu_x X - DX$$

The success or failure of the invasion of the culture by organism W depends on the dilution rate. At a dilution rate $0.25 D_c$ where D_c is the critical dilution rate, organism W cannot compete with X and W will wash out. On the other hand, if the dilution rate is increased to $0.75 D_c$, contaminant W will have a competitive advantage as did Z and will accumulate while the original organism washes out.

In batch culture any contaminant able to grow in the fermentation medium will survive and begin to accumulate. The success or failure of a contaminant in continuous culture depends on its ability to not only to grow but also to compete in the culture environment. For this reason continuous culture offers the advantage of selecting a growth environment, which provides a competitive advantage to the desired organism. Further more it is possible with continuous enrichment culture to select an organism capable of effectively using the limiting nutrients. This selections process should provide the best organisms for the environment chosen, thereby minimising the chance of contamination.

As can be seen from batch curve (fig 8) the strain IIP-4 in on unrestricted medium has a specific growth rate 0.38 h^{-1} at optimum temperature $35 \pm 1 \text{ }^{\circ}\text{C}$ and at $\text{pH } -4 \pm 0.05$. Under the same conditions the strain IFP-29 belonging to Candida lipolytica has a specific growth rate 0.16 h^{-1} . When the strain IFP-29 is subjected to continuous fermentation operation even at a very low dilution

rate within 30 hours of continuous operation uncontrollable foaming was observed which could not be controlled by mechanical defoamer or with antifoaming agents. Usually in continuous fermentation process, any disturbance in the growth of the organism which can happen if the supply of main nutrient fail to reach in time, causes foaming which could be controlled. Uncontrollable foaming observed in the case of the strain IFP-29 during continuous operation has been proved with the help of petridish experiments to be due to the growth of unwanted contaminant. This contaminant was isolated and identified and this belongs to the species "Candida guiermondii". It has been observed that this contaminant grows only in the presence of the yeast strain IFP-29 with a specific growth rate higher than the dilution rate, maintained in the fermentor. Similar study was conducted with the locally isolated yeast strain IIP-4 and no contaminant was observed. Characteristics of contaminants observed during continuous hydrocarbon fermentation is given in Table (13).

Table (13)

Characteristics of contaminants observed during continuous hydrocarbon fermentation.

Microorganism studied	Yeast - <u>Candida tropicalis</u>	Yeast - <u>Candida lipolytica</u>
Carbon feed	C ₁₄ - C ₁₈ cut n.paraffins	C ₁₄ - C ₁₈ cut n.paraffins
Temp. of growth	36 ± 1 °C	32 ± 1 °C
pH	3.5 ± 0.05	3.5 ± 0.05
Problem faced and nature of contaminant	<p>No problem faced</p> <p>Contaminant was observed after 15600 hours of cont. operation. This contaminant belongs to yeast family and remained less than 3% in the fermentor and got washed away. This contaminant yeast strain found to grow on C₁₄-C₁₈ hydrocarbons with specific growth rate 0.24 h⁻¹.</p>	<p>Uncontrollable foaming observed within 50 hours of operation. Petridish experiments indicate the growth of contaminant belonging to Yeast - <u>Candida guilliermondii</u> and this when exceeds 50% creates uncontrollable foaming and further operation.</p>

III. 2.4.2. Continuous fermentation studies-Bench Scale

The stability to grow at different dilutions rates, is checked for nearly 20000 hours in 2.5 litres capacity mechanically agitated aerated Bench Scale fermentor at different dilution rates with the strain IIP-4.

The data from bench experiments have been used for the prediction of conditions of the yeast population in continuous culture. Continuous supply of fresh medium is started when an appropriate biomass concentration is achieved during batch growth. The biomass concentration falls for the first few hours and then becomes stable. Steady state values of the population density (X) and the productivity ($D.X.$) of the yeast strain 'IIP-4' in a single stage continuous system are plotted against dilution rate fig (15). The productivity of cell mass reaches to its maximum values of $2.25 \text{ (g/L h}^{-1}\text{)}$ at dilution rate 0.25 h^{-1} . When the dilution rate is increased beyond this value the productivity falls sharply and has similar pattern to that of the steady state biomass concentration. In these experiments two values of dilution rates are noted, the

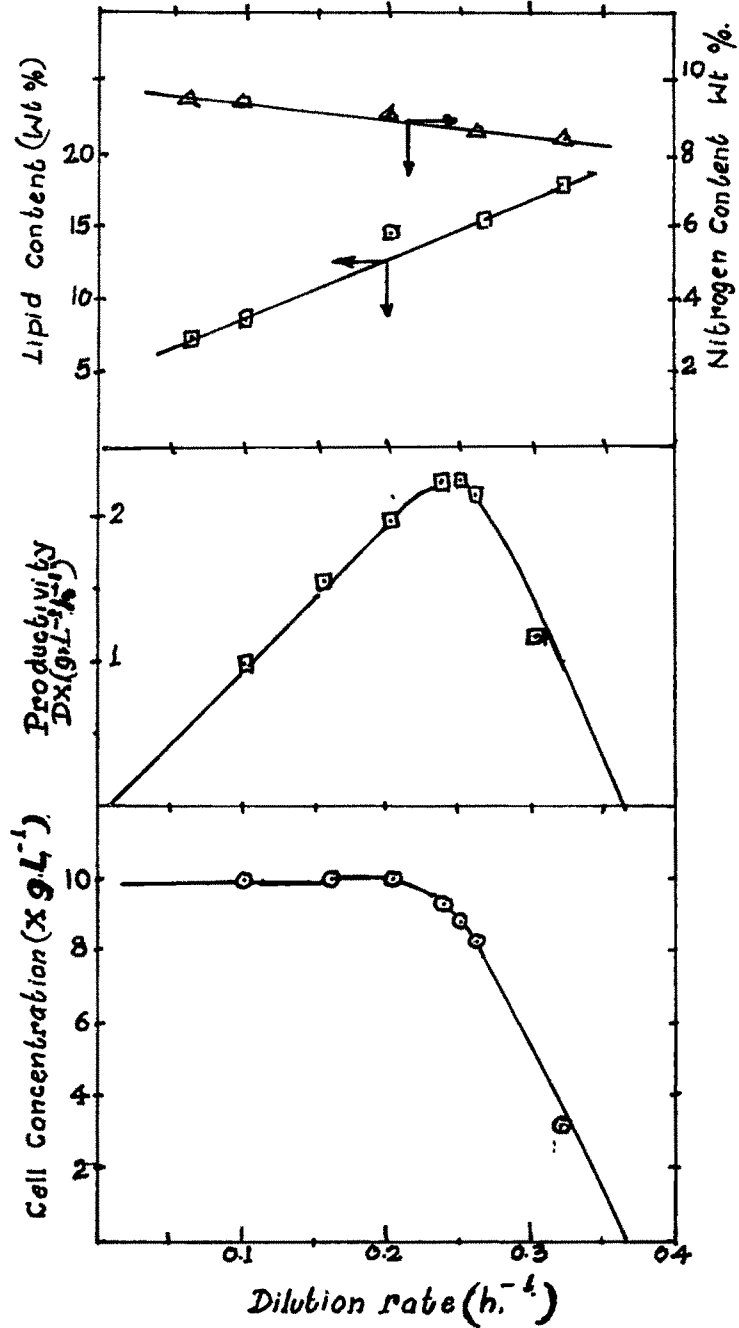
Fig. 15

Continuous Fermentation Studies

Capacity of the fermentor : 2.5 Lts.

Strain - J.I.P-4 p^H 3.5 Temp: $37 \pm 0.5^\circ$

C.feed: $(C_{14}-C_{18})$ n-Paraffins. S_0 : 1% Wt.



dilution rate 0.25 h^{-1} at which the maximum productivity of biomass occurs and the critical dilution rate 0.38 h^{-1} at which the complete wash out of the yeast strain 'IIP-4' takes place.

To study whether the maximum physiological activity of the culture also occurs at the dilution rate at which the maximum biomass productivity is achieved protein and lipid synthetic rates were studied. The results of both nitrogen content and lipid content determined of the biomass collected at different dilution rate are given against the dilution rate. Lipid content increased from nearly 5 wt % to 17% as the dilution rate increased where as the variation in nitrogen content is not that predominant and it decreased from 10% to 8%.

III. 2.4.3. Continuous fermentation studies in Pilot scale:

The results of a typical continuous run given in 1 m^3 capacity mechanically agitated and aerated Pilot fermentor are summarized in Table(14). For the first 326 hours continuous fermentation run was given at a dilution rate

0.17 h⁻¹ maintaining hydrocarbon substrate concentration at 9.5 g/L and the cell concentration obtained is 8 g/L which accounts to a growth yield of 0.84 (gms of cells/ gm of hydrocarbon). From 326 hours to 542 hours at the dilution rate of 0.18 h⁻¹ and hydrocarbon substrate concentration at 10.5 g/L the cell concentration in the fermentor increased to 9 g/L, the growth yield continued to be at 0.84 to 0.85 (gms of cells/ gm of hydrocarbon). The next 200 hours i.e. up ^{to} 758 hours the dilution rate increased to 0.19 h⁻¹ and at hydrocarbon concentration 10.5 g/L the cell concentration in the fermentor continued to be 9 g/L. For the next 170 hours the dilution rate was maintained at 0.2 h⁻¹ and for a hydrocarbon concentration of 11.5 g/L the cell concentration remained constant at 10 g/L giving a growth yield of 0.86. For another 96 hours the run is continued increasing the aeration rate to 140 m³/h⁻¹ to provide a V.V.M. (Volume of air per Volume of liquid per hour) of 2.7 and with a hydrocarbon substrate concentration 12.5 gms/L a cell concentration of 10.5 g/L obtained and the production figure touched 51 kg/day.

In a closed system of batch technique the cells grow in a limited amount of medium as the cells multiply, population changes gradually occur in the biological and chemical properties of cells and in the medium composition as well.

The specific growth rate in batch process increases initially as far as growth limiting nutrient is sufficient enough to support the growth of microorganisms. After attaining a maximum value the exponential growth phase continuous and it starts declining partially due to the exhaustion of the nutrients and partially due to the accumulation of some autotoxic metabolites.

It is obvious that all the features of a batch culture are a function of time and the culture as a whole is in a continuous state of transition. On the other hand in an open system of continuous technique the microorganisms grow in steady condition of growth which are independent of time. The maximum attainable specific growth rate in a single stage, chemostat is hindered by wash out of cells as the value of dilution rate is increased beyond a critical value.

Table (14)

Results of Pilot Plant Continuous Fermentation Studies

Capacity of the fermentor	: 1m ³ Type : Air lift					
Liquid volume of the fermentor	: 850 litres					
Yeast strain	: IIP-4 of 2006 hrs from Bench Scale fermentor					
C.Substrates	: 99% purity n.paraffins (C ₁₄ -C ₁₈) range					
Temperature	: 37 ± 1 °C pH : 3.5 ± 0.2					

Time in hours	-326	-542	-590	-758	-926	-1022
Aeration rate m ³ /hr	110	110	110	130	130	140
V.V.M. (Vol. of air/ Vol. of liquid/minute	2.2	2.2	2.2	2.6	2.6	2.7
Hydrocarbon substrate So g/L	9.5	10.5	10.5	10.5	11.5	12.5
Flow rate of substrate F ₁ L/h	150	160	170	170	175	180
Dilution rate D = F/V = μ . h ⁻¹	0.17	0.18	0.19	0.19	0.2	0.2
Cell concentration in the fermentor X.g/L	8	9	9	9	10	10.5
Productivity DX g.L ⁻¹ . h ⁻¹	1.36	1.62	1.7	1.7	2.0	2.2
Production/day kgs/day	29	35	40	40	46	51

In this case the maximum value of specific growth rate achieved is less than the batch value.

Single stage continuous culture has distinct economic advantages over batch culture.

- Process productivity is unquestionably higher in continuous culture. In case a cubic meter capacity pilot fermentor a productivity of $2.5 \text{ kg/m}^3 \text{ h}^{-1}$ (around 50 kg of single cell proteins/day) could be obtained when operated on a continuous process whereas on batch process even though the productivity is $2.5 \text{ kg/m}^3 \text{ h}^{-1}$ production per day works out nearly 15 kg/hr. In between two batch process minimum 6 hour is lost towards cleaning sterilizing and cooling and another 2 to 3 hour towards lag phase of fresh run.
- Continuous fermentation process can be operated without maintaining strict sterile conditions for many thousands of hours.
- The carbon and energy source can be controlled for complete utilization in continuous culture, thus there

is very little wastages of the substrate in spent medium after cell recovery.

- Product specification from continuous culture is more uniform than that obtained from batch culture.
- Adjustment of medium composition and the concentration of the growth limiting nutrient are easily accommodated.
- Continuous fermentation can be described as a stable and self regulating process.

III. 2.5. Microbial dewaxing :

The yeast strain IIP-4 grows on straight chain paraffinic hydrocarbon and the presence of non-normal paraffinic hydrocarbons i.e. naphthanic and aromatic hydrocarbons do not interfere on its growth. Based on this principle microbial dewaxing technique was applied on kerosene fractions from Ankleshwar and North Gujarat crudes. Properties of the dewaxed oil along with that of original fractions are given in Table (15).

Similar studies are conducted on gas oil fractions having boiling range 220-390°C from Ankleshwar crude. From the boiling point data the approximate carbon pattern of the n.paraffinic hydrocarbons present in the gas oil fraction was determined from " Selected Values of Physical and Thermodynamic properties of hydrocarbons and related compounds " - American Petroleum Institute Research Project -44 and its carbon number ranges between 12 to 24, growth rate of the yeast strain has been found to be 0.38 h^{-1} on this fraction.

Table (15)

Characteristics of Gas Oil fraction before and
after microbial dewaxing.

ASTM distillation data :

IBP	221 °C	60%	335 °C
10%	260	70%	345 °C
20%	295	80%	360 °C
30%	305	90%	371 °C
40%	316	95%	383 °C
50%	323	FBP	388 °C

Approximate carbon range of n.paraffins : $C_{13} - C_{23}$

Properties before and after Microbial dewaxing

	<u>before</u>	<u>after removal of n.paraffins</u>	
		27% removal	37% removal
Density ³⁰ ₄	0.832	0.851	0.861
Pour Point °C	24	15	9
Aniline Point °C	92	86	80
% n.paraffins	44%	17%	4%

Variation of cell concentration in the fermentor with the increase of dilution rate for the same concentration of oil is shown in fig (16). Cell concentration in the fermentor at dilution rates beyond 0.18 h^{-1} starts decreasing and washing out from the fermentor takes at dilution rate corresponding to 0.38 h^{-1} . Removal of n.paraffinic hydrocarbons from the oil fraction is maximum upto the dilution rate 0.18 h^{-1} . The productivity is maximum at dilution rate 0.25 h^{-1} . Variation of cell concentration with the increases of oil concentration in the feed during continuous fermentation for the same dilution rate of 0.18 h^{-1} and at constant temperature $35 \pm 2^\circ\text{C}$ and at pH 4 ± 0.1 is shown in fig (17).

At very low concentration of oil in the feed the cell concentration is not proportional to the n.paraffinic hydrocarbon availability and results obtained are lower. A certain minimum concentration is necessary for the maintenance of the culture and thus the availability of n.paraffin for cellular growth is less than that actually fed, which thereby lowers the cell concentration. From

Fig. 16. Continuous fermentation of Gas oil fraction at different dilution rates.

Strain 11P-4 $p^H. 4 \pm 0.05$ Temp. $^{\circ}C. 35 \pm 0.5$.

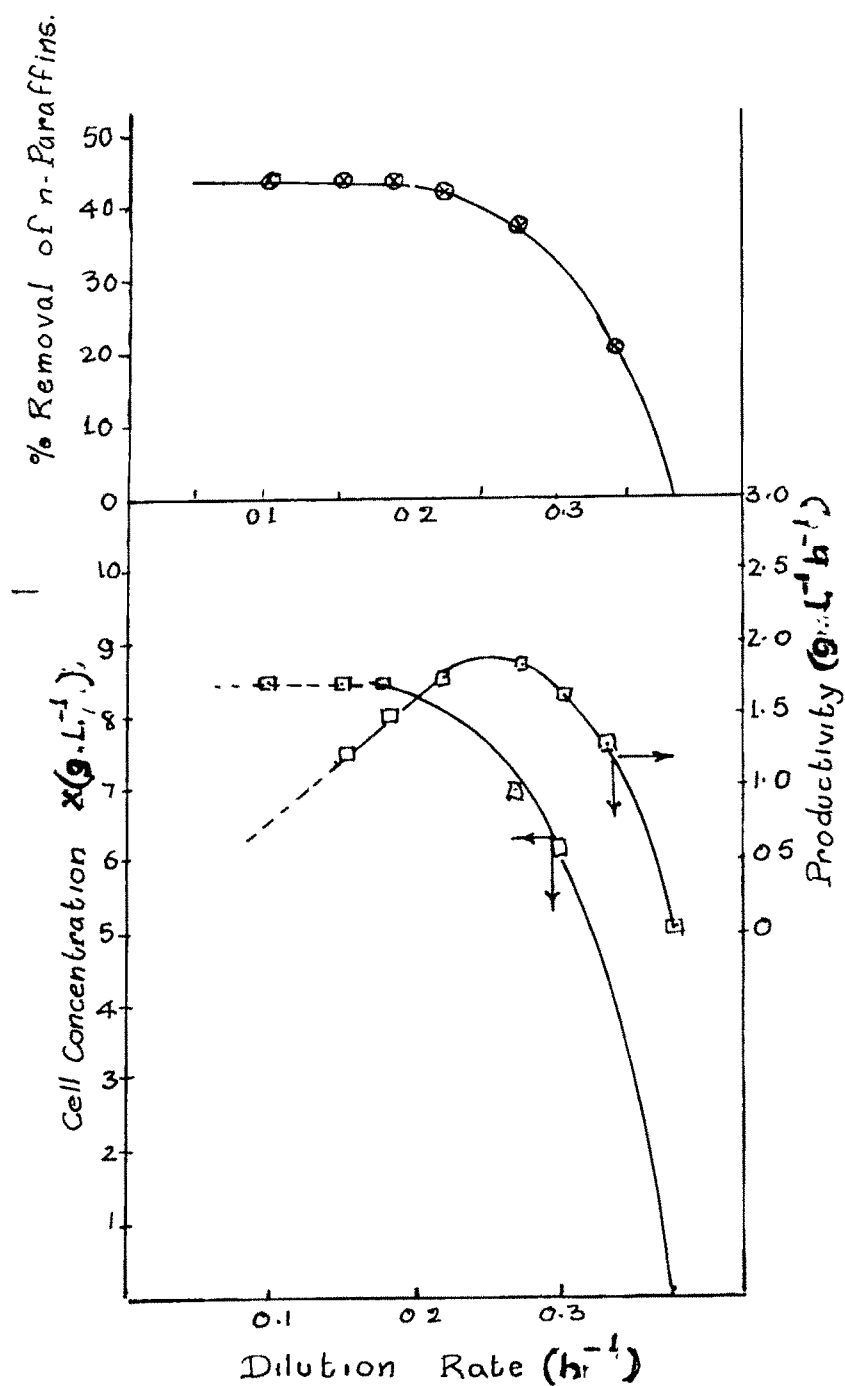
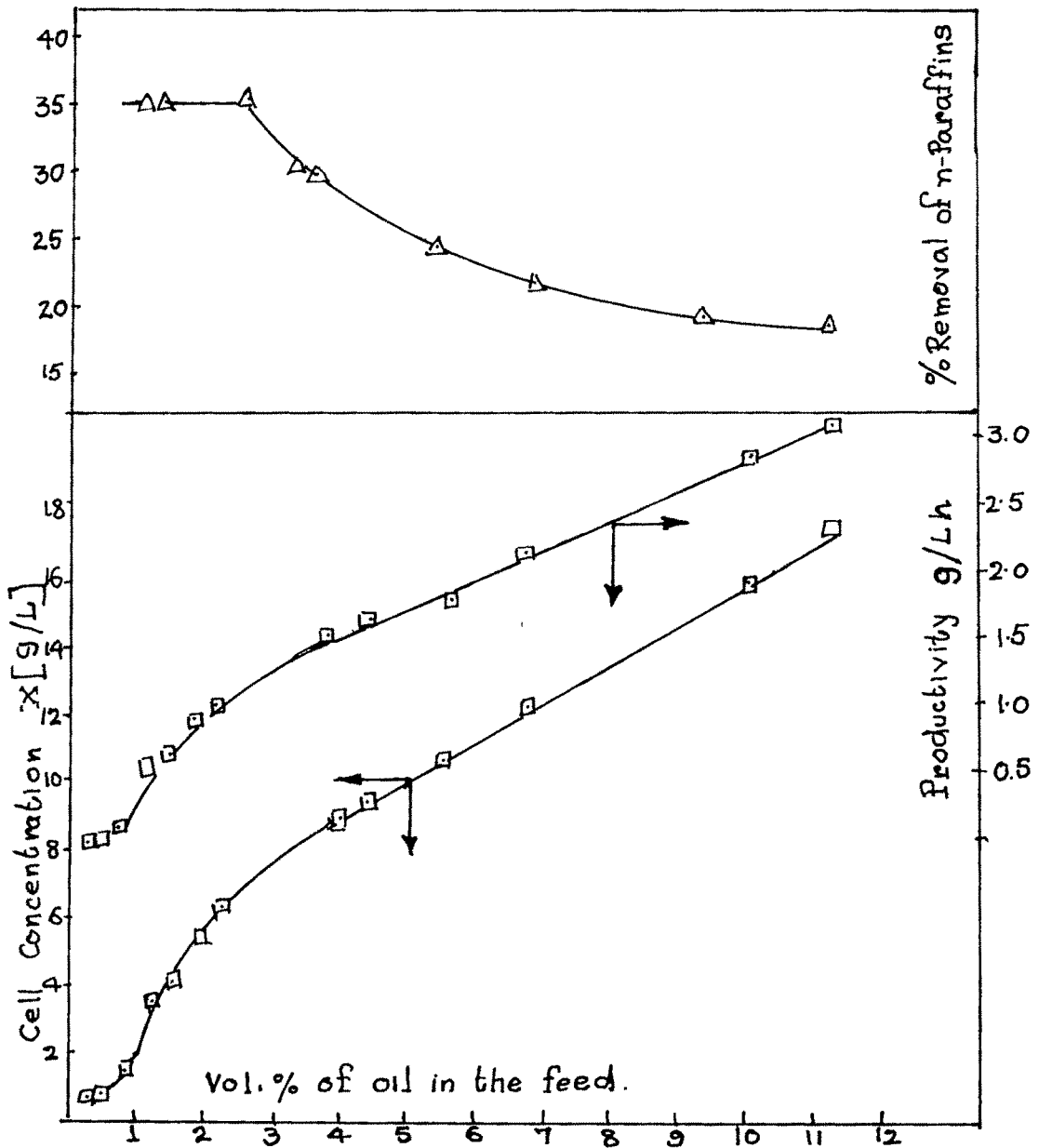


Fig.17. Effect of oil concentration in the feed on
n-paraffin removal from Gas oil fraction



Strain - IIP-4. Dilution rate, 0.18 h^{-1} .

pH: 4 ± 0.1

Temp $^{\circ}\text{C}$: $35 \pm 0.5^{\circ}\text{C}$.

Vvm: 3. (aeration)

nearly 1 upto 2.7 Vol. percent of oil in the feed, maximum removal of n.paraffinic hydrocarbons from the gas oil fraction amounting to nearly 35% taken place. At oil concentration above 2.7 vol. percent oil in the feed cell growth is no longer proportional to the n.paraffin availability. Beyond 7 to 8 g/L cell concentration in the fermentor perhaps due to oxygen limitation, paraffinic feed that was available was not fully utilized for cellular growth.

Properties of oil collected after microbial dewaxing.

The acidity of the oil recovered from the fermentor has been found to be more and is mostly due to organic acids. Unless alkali wash is given to neutralize these acids, it cannot meet the specification. Density , pour point, aniline point are some of the properties determined on the dewaxed oil and the variation of these properties with increase of oil concentration in the feed is shown in fig (18).

Thus Microbial dewaxing technique developed can be applied to conduct dewaxing of kerosine and Diesel oil

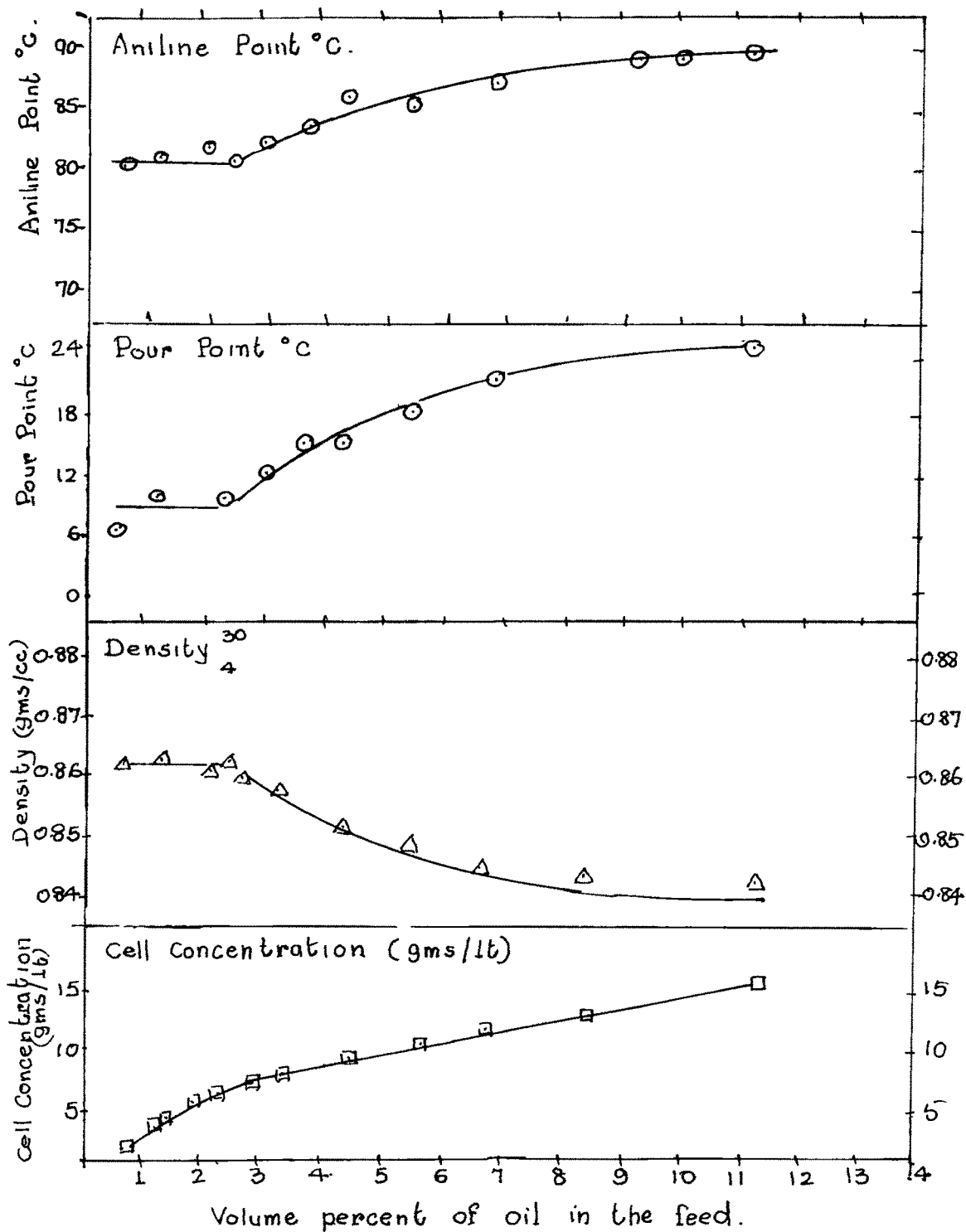


Fig. 18: Effect of oil concentration in the feed on the properties of dewaxed oil

fractions on batch as well as continuous basis to produce deparaffinized oil having better flow properties and instead of waxy components almost equivalent quantity of Single Cell Proteins.

III. 3.1. Treatment of Biomass.

Yeast cells range in size from 5 to 8 μm and have a density of $1.04 - 1.09 \text{ gm/cm}^3$. They can be recovered readily from the growth medium by continuous centrifugation. Usually, the cells are centrifuged in a first stage to initially dewater, yielding a yeast cream. This is followed by the subsequent washing and centrifugations. The final washed yeast cream usually contains 15 - 20% solids. This procedure is typical of that used in the recovery of *C. utilis* after growth on sulfite waste liquor (188, 189). In the case of *Candida* sp. grown on n.alkanes a wash with a surfactant may be required to remove traces of hydrocarbon following initial centrifugation (168, 171).

With gas oil, a much more complex separation and clean up procedure is used to remove hydrocarbon. Decantation, phase separation with solvents, working with surfactants and solvents extraction have been used alone or in combination.

Relatively cheap solvents such as food grade hexane, acetone or isopropyl alcohol can be employed to extract

almost all the unassimilated hydrocarbons present in the biomass obtained from the fermentation unit. Solvent residues should be below the level of detection by taste or smell. Substances capable of producing taste or smell as a result of storage should be absent.

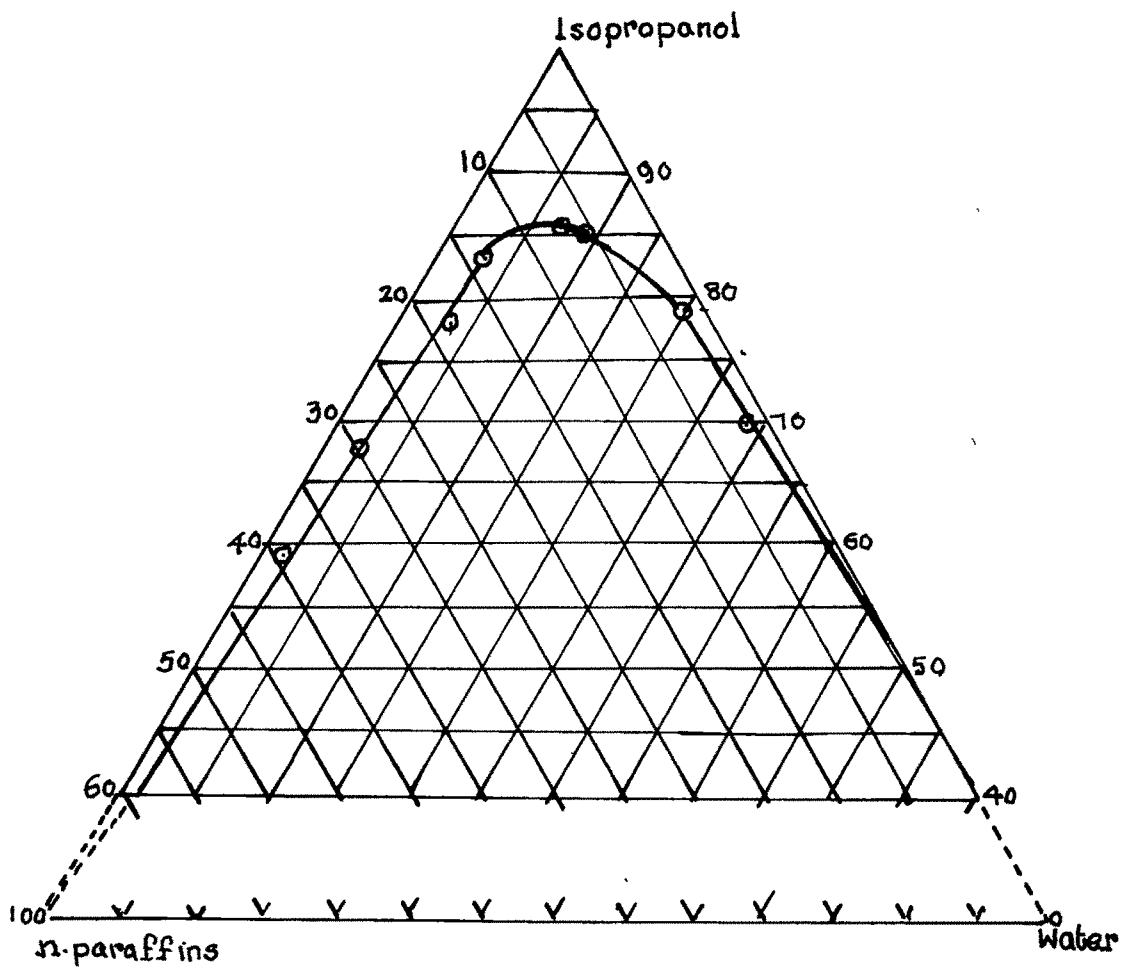
Earlier work concentrated on the solvent acetone (199). In the production of fish protein concentrate there have been several commercial proposals to use acetone to extract both water and fat and the economics of the process including recovery are attractive (282). It has been reported that acetone invariably leaves, the final product contaminated with traces of strongly flavoured condensation components which cannot be removed below the level of taste detection even by subsequent extraction with other solvents, or by any normal desolventizing procedure. Isopropanol has been tried successfully in place of acetone to treat the biomass.

Ternary phase equilibrium was studied using normal paraffinic hydrocarbons as one component and water and 2 propanol as the other two components and the results are

shown in fig (19). The ternary phase diagram was drawn using the data obtained by titration method (281). The quantity of biomass containing yeast cells, unassimilated hydrocarbons and water with the residual salts, for further treatment with solvent depends on the residual hydrocarbons content in the fermentor. It will be uneconomical to run the fermentor, by keeping more than 0.05% of the residual hydrocarbons on the fermentor. When pure n.paraffinic hydrocarbons are used, as carbon source, if the conversion is good then in general the residual hydrocarbons content is less than 0.05% in the fermentor and the cell volume in the biomass reduces and settles to about 1/10 of the original biomass volume and the water containing less than 1% of the cells could be removed, by decantation.

In the case of microbial dewaxing of kerosine or Diesel fraction or any petroleum fraction after fermentation the biomass is likely to contain more than 2 to 3 % of unassimilable non n.paraffinic hydrocarbons as most of the petroleum fractions of different crude oils contain not more than 25% of assimilable n.paraffinic hydrocarbons.

Fig . 19.



Phase equilibrium diagram for n-paraffins ($C_{14}-C_{18}$)-Isopropanol-
-water system

In that case one can calculate from the ternary phase diagram the quantity of solvent required for removal of the residual hydrocarbons from the biomass.

Thus the ternary phase diagram could be used to calculate the exact quantity of solvent required for any system containing different quantities of hydrocarbons and water.

Using the solvent 2 propanol, the biomass obtained from fermentation could be treated to get yeast cells free from residual hydrocarbons. The separated cells can either be drum or spray dried. Drum drying if not carefully controlled, may result in darkening of the product. According to Labuza the colour and finished characteristics of the final yeast product produced by spray drying are superior to those obtained after drum drying (171). The yeast cells are subjected to spray drying to get cream coloured odourless, tasteless Single Cell Protein in fine powder form. Using the solvent dimethyl sulphoxide which has been proved good to extract aromatic hydrocarbons

present in traces and the techniques spectrophotometric and gaschromatography, developed a method to test the final product for residual hydrocarbons (283).

III.3.2. Solvent Recovery :

By simple fractional distillation one cannot completely remove water from Isoproponol water mixture as water forms minimum boiling homogeneous mixture with 2 proponol containing 13% by weight of water in the mixture (284) (Table 17).

Azetropic distillation using Naphtha as an entrainer :

After standardizing the length of laboratory distillation column experiments have been carried out to determine the quantity of naphthe required in order to use as an entrainer for the complete removal of water by azetropic distillation and the results are given in Table (18).

Advantageous of using isopropanol as solvent :

Isopropanol is most effective against unwanted

Table (17)

Binary and Ternary systems which exhibit constant boiling points (Azeotropes)

Components	Azeotrope					
	B.P.°C	B.P.°C	Azeo- trope	Composition (Int %) in	Relative Volume of Layers	Specific gravity
Compounds				Upper Layer	Lower Layer	
2 Propanol	82.5	80.4	87.5	---	---	---
Water	100	--	12.2	---	---	---
Benzene	80.1	65.7	72.0	77.5	0.5	U 93.6 0.855
2 Propanol	82.3	--	19.8	20.2	14.4	L 6.4 0.966
Water	100.0	--	8.2	2.3	83.1	---
Naphtha	80-120	74.0	81.1	91.6	3.0	U 88 0.739
2 Propanol	82.5	--	10.4	5.8	43.6	L 12 0.883
Water	100	--	8.5	2.6	53.4	---

microorganisms such as staphylococcus aureus E-Coli,
Bacterium Anthrax and subtilis (285). Thus it acts
as germicidal and antiseptic agent. It has further
advantage as it does not have any residual taste and
unpleasant odour as observed with acetone. Further it
has been noted that when cells are treated with the solvent
isopropanol, it reduces the lipid content of the cells,
thus increasing the proteins content. While ingestion of
2 propanol has an inaction on central nervous system
similar to that of alcohols causing drowsiness head ache
of varying intensity narcosis, repeated administration has
apparently no injurious action on liver as in the case of
ethyl alcohol and no untoward effect on vision as in the
case of methyl alcohol (286).

III. 4. Nutritional Quality and the safety of Single
Cell Protein.

It is recommended that a number of procedures suggested by the protein advisory group of FAO/WHO/UNICEF and given in guideline No.6 entitled " Guidelines for preclinical testing of Novel sources of Protein " are to be used for evaluation of Single Cell Proteins from n.paraffins for animal feeding ingredient.

Investigations according to the above mentioned guideline include :

Microbiological examination : Viable and non viable contaminants - Pathogenic organisms of toxin producers.

Chemical analysis : Protein - Amino acid composition - nucleic acids - lipid content - hydrocarbon residues - heavy metals.

Protein Quality Studies : Digestibility, Protein Efficiency Ratio, Net Protein Utilization.

Animal safety tests : Acute, sub-acute toxicity studies - Chronic Toxicity - Carcinogenicity - Reproductions and

lactation Multigeneration - Teratogenicity -
Mutagenicity.

Additional feeding tests using farm animals such as chickens, pigs.

III.4.1. Nutritional studies :

Nutritional studies carried out at Central Food Technological Research Institute (CFTRI), Mysore on Single Cell Proteins (SCP) produced in IIP Proteins Projects pilot plants compares well with high-quality protein sources such as egg, milk, meat and fish in terms of the amount of " crude " protein that the product can supply as well as in the overall pattern of nutrients. This is evident from the figures shown in the Table (16).

Amino acid analysis of food stuff :

The pattern of amino acids in SCP is reasonably good when compared to high quality proteins such as those of egg and milk. Yeast proteins is a good source of lysine and has sufficient quantities of other essential amino acids such as tryptophane and threonine, the concentrate

Table 16. Proximate composition of food stuff and SCP.

<u>Material</u>	<u>Protein</u>	<u>Fat</u>	<u>Carbo Hydrate</u>	<u>Ash</u>	<u>Water</u>
Egg	48.8	44.5	2.8	4.2	74.0
Milk	26.9	30.0	37.7	5.4	87.0
Meat	57.1	37.1	2.0	3.1	65.0
Fish	55.4	37.9	--	8.2	67.2
Corn	10.2	3.9	82.5	1.3	12.0
Wheat	13.6	1.5	84.1	1.4	12.0
<u>Soy flour</u>	46.7	7.1	40.9	5.3	9.0
SCP from IIP	59.9	7.8	24.4	7.9	8.0

of which is low in cereal proteins. Yeast proteins is however deficient in sulphur containing amino acids. The E/T (Essential Total) ratio varies between 2.94 and 3.26 which places yeast protein among the better quality proteins (287).

III.4.1.1. Proteins Efficiency Ratio :

At 10% level of proteins PER experiments on rats are carried out. Unsupplemented SCP gave a PER value 1.8 which when supplemented with 0.3% methionine the PER value increased to 3, slightly better than that could be obtained with casein protein 2.8 (287). Supplementary value of yeast grown on Petroleum hydrocarbons to poor diets based on Rice and Ragi (Eleusine Coracana) has been studied (288).

4.1.2. Studies on Broilers and layers : (287)

The SCP has been tested on broilers and layers chickens of a particular strain to ascertain its effects at different levels as the dietary source of protein in comparison with conventional diet fish, meal and groundnut cakes.

Studies on 300 Broilers have shown that SCP incorporated in diets at 5% and 10% levels resulted in better growth rate, feed conversion and carcass yield than controls containing fish, meal and groundnut cake.

Feeding of Single Cell Proteins at 10% and 20% levels in feed to 300 layer birds showed slightly higher egg production than the control diet. In corporation of SCP at 10% level gave better egg production than the 20% level.

III.4.2. Toxicity Studies :

Industrial Toxicology Research Centre (ITRC), Lucknow conducted toxicity studies on Single Cell Proteins samples supplied by IIP-Proteins Project (289, 290). Single Cell Protein was fed to rats at the dietary protein level of 20% for 24 months. (Two control diets were used one 20% casein diet and other Hindustan Lever Standard Pellet, rat diets). In the 2 years study no signs of clinical toxicity were observed. Growth rate was comparable to casein diet. Food intake was comparable to

controle diets. During the 2 year study period no adverse effects were demonstrated on growth mortality and hematology.

Blood glucose, serum urea, uric acid allantoin and cholesterol were within normal limits and were comparable to casein diet group of animals. There was slight rise in liver nucleic acid contents at the terminal end of experimental yeast, like any rapidly growing cell has a relatively higher concentrations of ribonucleic acid (RNA), which in human is excreted as uric acid. Excessive level of later in body tissue may increase the risk of gout and kidney stones in susceptible individuals. The nucleic acid content of SCP food can be reduced by simple processing procedures. Allantoin and uric acid in blood were not high in the present experiment indicating non toxic effects even after 24 months of SCP feeding. There was no change in urine composition and did not reveal presence of abnormal constituents serum alkaline phosphatase, GOT and GPT activities were comparable to control group of animals.

None of the experimental animals showed pathological changes in any of the vital organs that could be attributable to the feeding of SCP and cytogenetic assay of bone marrow and spermatogonial cells did not reveal any kind of chromosomal aberrations. A 2 year feeding study of Single Cell Protein to albino rats of both sexes did not reveal any kind of deleterious effects.

III.4.3. Field Trial Experiments on Farm Animals with IIP - produced SINGLE CELL PROTEIN.

Field trial experiments were carried out on Lamb and Sheep at Indian Veterinary Research Institute (IVRI), Izatnagar, Goat heifers at National Dairy Research Institute (NDRI), Karnal and Kankrej bullocks at Gujarat Agricultural University (GAU), Anand.

Experiments conducted at IVRI, Izatnagar on Lamb and Sheeps have indicated the possibility of incorporating Single Cell Protein (SCP) in place of conventional groundnut cake in the concentrate mixture without any adverse effects on health or milk production (291), (292). Protein of SCP has high digestibility of 87.6%.

Parmerkar Subhash and Mudgal (293) of National Dairy Research Institute, Karnal investigated to evaluate the relative feeding value of Single Cell Protein and groundnut cake as an ingredient in the concentrate mixture of cross-bred goats and no significant difference in balance of these nutrients was found.

An attempt to compare the efficiency of milk and milk substitute containing SCP as a major protein supplement for growth and nutrient utilization in cross bred kids during their early growth period was made and Parmerkar and Mudgal (294) concluded that goat kids could maintain almost as good growth on milk substitute (SCP as major protein source) as that for those on goat milk itself.

Chemical composition of Single Cell Protein and its feeding effect on nutrients utilization and rumen fermentation in goats were studied by Singhal and Mudgal (295).

G.A.U., Anand based on their work on Kankrej bullocks concluded that the crude protein digestibility varied from 90 - 99% indicating that SCP is highly digestible for ruminants (296).