CHAPTER II

LITERATURE SURVEY

2.1. MICROBIAL GROWTH ON HYDROCARBONS :

In the past three decades, considerable research work was done on utilization of paraffinic hydrocarbons as carbon substrates for the growth of different microorganisms which led to the development of processes for the production of single cell proteins reviewed by Wiley,* 1954; Champagnat, 1963; Decerley, 1969; Laine, 1974, 1975; Litchfield, 1979, Solomon, 1986. The concept of growing microorganism in a fermenter vessel on water insoluble hydrocarbons was quite revolutionary in the early 60's since assimilation of paraffinic hydrocarbons present in the middle distillate petroleum fractions (250 - 350^{0}C) by the yeast was found advantageous for the following reasons :

1) Production of high quality proteins (SCP) a suitable supplement for conventional proteins e.g., Soyacake, fishmeal etc in the cattlefeed.
2) Dewaxed oil having better flow properties.

Based on this concepts British Petroleum developed a process (BP) for the production of SCP and bulk operation was carried out at Lavera Refinery, France using gas oil fraction (300-380^{o}c) as feed stock containing 20% paraffin wax (Laine, 1974,1975). Although the process contributed to producing dewaxed gas oil which was reused by the Refinery, and enough attention had been paid towards the acceptability of SCP produced from gas oil and improvement of other process parameters such as yield coefficient of biomass, nutritional requirement and oxygen transfer etc. Very

*P.S. References cited in this chapter are listed in page No.178 onwards.

little work has been reported on the recovery and final treatment of dewaxed oil, its properties and possible end uses.

Another process was developed by BP using pure n-paraffins as feed stock, seperated from gas oil by molecular sieves or Urea adduction process. Large scale production (4000t/y) of SCP was started at Gangemouth, Scotland by BP (Laine, 1974).

The flow diagram of both the SCP production processes as developed by BP are shown in Fig.2.1 and 2.2.

Following the sucess of BP, several other oil companies e.g., ANIC, Sun oil, Gulf oil, Dianippon and Kanegafuchi, Esso-Nestle, etc. also entered into commertial application this technology (Solomon, 1986). However choice of feed stocks had been restricted to either n-paraffins or gas oil and the comparative advantages and disadvantages have been summarised by Litchfied, 1975. It was shown that high productivity and less operating and utility costs are involved in the SCP production process from n-paraffins compared to the gas oil based process which can be operated in non asceptic conditions.

However restricting the scope of SCP process linked with the dewaxing of petroleum fractions, different aspects of microbial growth on hydrocarbon raction have been reviewed in the following sections.

2.1.1 MICROORGANISM :

The ability to degrade and/or utilize hydrocarbon subtrate is exhibited by a wide variety of bacterial and fungal genera. Fuhs, 1961 reviewed the spectrum of microorganisms capable to grow on hydrocarbons. Reviews and Reports on hydrocarbon degradation by bacteria and fungi have appeared several times. (Lodder, 1970; Boss and DE Bruyn, 1973; Shennan and Levi, 1974; Davis, 1975; Bos, 1975; Rehm and Reiff, 1981; Floodgate, 1984). The specific characteristics of any petroleum degrading micro organism postulated by Gutnick and Rosenberg, 1977,are as following :

1. Efficient hydrocarbon uptake system containing receptor sites for binding hydrocarbons and production ofbio-emulsifier that assists in emulsification and transport of hydrocarbon into cell.
2. Induction of the enzyme system e.g., oxygenases to introduce molecular oxygen into the hydrocarbon molecule and subsequently enter common energy yielding catabolic pathways.
3. Positive response of the organism to petroleum and its constituents . inducing the first two systems. Various means by which over 200 diferent species of eukaryotic and prokaryotic hydrocarbon utilising microorganisms to express the above characteristics have been described (Mimura, 1973; Zobell and Prokop, 1966; Walker and Colwell, 1976; Raymond, 1976; Westlake, 1974).

Procaryotic microorganism e.g., bacteria and actinomycetes grow readily on n-alkanes and play a vital role in degradation of naturally occuring and spilled hydrocarbons (Lahey and Colwell, 1990).A list of hydrocarbon utilising bacteria are given in Table-2.1.

The hydrocarbon assimilating bacterial species e.g., *Artho bacter, Brevibacterium, Corynebacterium, Nocardia* have been widely applied for biotransformation of hydrocarbons (Reymond , 1976). Production of glutamate from alkanes by *Corynebacterium* was carried out by Yamada et.al., 1968. Production of biosurfactants and lipids from hydrocarbons have been reported by Kehm and Reiff, 1981; Ratledge, 1968. However, some methane utilising bacteria e.g., Methylomonas methylotrophs have been used for SCP production using methane or methanol as subtrate and ICI developed a large-scale SCP process using methanol as subtrate. The enumeration of hydrocarbon degrading bacteria was developed by Colwell et.al., 1973; Ward and Brock, 1976 and the enrichment culture media was discribed by Horowitz,Rosenberg and Gutnick, 1975.

Eucaryotic microorganisms with the ability to utilise hydrocarbons are to be found mainly among yeasts and molds which are listed in Table-2.1.

Yeasts have been most extensively studied for the development of SCP processes and microbial dewaxing .They belongs to the genera *Cryptococcoudae, Tosulopsis Saccharomycetaelde, Lodderomyces* and *Candida.* The historical background of yeasts accounts for their general acceptability for use as food in baking, brewing and

wine making .Levi,(1974) reported 40 to 50 members of the genus-*Candida* which can assimilate hydrocarbons. However, among most of the species, *Candida lipolytica, Candida tropicalis, Candida glulliermondii, Candida intermedia* are well known (Perlman, 1979).

Bos and de Bruyn, 1973 concluded that *Saccharomyces, Kluyveromyces* and *Hansenula* are completely deviod of ability to assimilate hydrocarbons.

Filamentous fungi contain many species capable of hydrocarbon oxidation (Table 2.1). However, they have seldom been extensively studied in the context of SCP production or dewaxing of petroleum fraction. Generally the growth rate and yield coefficient of fungi is less compared to yeast and bacteria.

Cladosporium resinae, known as Kerosene fungus was identified as contaminant of petroleum products (Devis, 1975). Filamentous fungi have also been used to produce different types of metabolites from alkanes media (Fukui and Tanaka, 1980) e.g., Streptomyces were found to produce different kinds of peptide antibiotics from alkanes.

The following factors are most important for selection of any hydrocarbon degrading microorganism for the dewaxing of petroleum fraction and SCP production.

1. Non toxic to the cattle and human.
2. High growth rate and yield coefficient based on n-paraffin content of the petroleum feed stocks.
3. High optimum growth temperature and wide pH range.
4. Ability to grow in simple media without any growth factor.

5. Easier separation process involved for the separation of the strain from culture broth.

6. Stable under continuous operating conditions.

7. High protein content with balanced aminoacid patteren.

The above factors are favourable for selection of yeast as a potential microorganism to study microbial dewaxing of lube fraction and SCP production.

2.1.2 PETROLEUM FRACTION AS SUBSTRATE FOR BIOMASS PRODUCTION :

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

a) Gaseous hydrocarbons : Utilisation of methane gas as carbon subtrate for biomass production was reported by Hammer, 1967; Annon, 1976. Use of gaseous hydrocarbon as feed stock is advantageous because of adopting easy separation process for the recovery of biomass. However, the productivity of SCP by methane oxidiing microorganism was limited by the rate of oxygen and methane transfer from gaseous phase to microorganism (Hammer and Topiwala, 1975).

Gibson, 1971 demostrated growth of biomass on volatile aromatic hydrocarbons which are toxic in liquid phase. It was observed that presence of hydrocarbon in the enrichment medium frequentlybrings about a selective enrichment in situ for hydrocarbon utilizing microorganism. It was also reported that the supplementation of certain ecosystems, particularly oil polluted marine environment, with nitrogen and phosphorous may increase the relative number of hydrocarbon oxidisers (Atlas and Bartha, 1973;

Gutnick and Rosenberg, 1977; Reisfield, Rosenberg and Gutnick, (1972).

b) Liquid Hydrocarbons : Most of the process development work on production of SCP was conducted on liquid hydrocarbons as feed stocks. The n-paraffins of carbon range (C_{12}-C_{20}) distributed in kerosene (150-250^{o}c) and gas oil (250-350^{o}c) represents potential carbon source for SCP production (Levi, 1979). Consequently, if the n-paraffins present in the oil fraction around 20-40%, which is generally observed in gas oil and lube fraction obtained from different crudes, microbial dewaxing becomes favourable.

The lower homologues of the n-alkanes, which are liquids at room temperature (C_5 to C_9) usualy do not support microbial growth. It was observed that these shorter chain n-alkanes exert inhibitory effect on the growth of the microorganism (Johnson, 1964; Klug and Markovetz, 1971). The lag phase of the yeast, *C. tropicalis* increased exponentially when lower fraction of kerosene obtained from Bombay high crude used as the carbon substrate. The C_7-C_9 alkanes act as delipidizing agent on the yeast cells (Adhikari, 1990).

c) Solid hydrocarbons : Although gas oil (250-380^{o}c) was used as feed stock for SCP production (Laine, 1974) and dewaxing by yeast strains, heavier petroleum fractions i.g; lube fraction as feed stock for the purpose either for SCP production or dewaxing remained unexplored perhaps due to their physical properties such as pour point, density,and viscosity.

Microbial degradation of pure solid hydrocarbons with water solubilities less than 10^{-7}M is not well documented. Shaw et.al., 1970 reported that uptake from the solid phase via

TABLE 2.1 HYDROCARBON ASSIMILATING MICROORGANISMS

Classification	Genus
1. Procaryotes	
Phototrophs	*Rhodospirillum*
Gram negative aerobic	*Alcaligenes*
Rods and Cocci	*Pseudomonas*
Gram negative facultative	*Aeromonas*
Anaerobic rods	*Chromobacterium*
	Klebsiella
Gram negative Cocci	*Acinetobacter*
	Anthrobacter
	Brevibacterium
	Corynebacterium
Actinomycets	*Actinomyces*
	Streptomyces
	Nocardia
2 Eukaryotic	
Ascomycetes (yeast)	*Debaryomyces*
	Endomyces
	Lodderomyces
	Pichia
Basidiomycetes(yeast)	*Leucosporidium*
	Rhodosporidium
Fungi Imperfecti (yeast)	*Candida*
	Rhodotorula
	Torulopsis
	Trichosporon
3.Filamentous fungi	
Mucorales	*Cunninghamella*
	Mucor
Fungi Imperfecti	*Aspergillus*
	Fusarium
	Cladosporium

pseudo-solubilization would be harder to achieve. Dispersion of heavier petroleum fractions in the aqueous medium becomes dificult compared to liquid hydrocarbons there by restricting the availability of surface to volume ratio (Davis and Gibbs, 1975).

n-Alkanes up to 44- carbon atoms were slowly metabolised by soil microorganisms as reported ny Haines ,1974.

Biodegradation of tar balls which is generated by large aggregates of weathered and undegraded spilled oil in sea, remains inaccessible to the microorganism because of their limited surface area (Colwell & Walker, 1978)

2.1.3 MICROBIAL SELECTIVITY FOR HYDROCARBON METABOLISM :

Zobell (1946) formulated four rules for the specificity of utilisation of hydrocarbons by yeast. These rules were reformulated by Shennan and Levi, 1974.

Rule 1. Aliphatic-compounds are more susceptible to microbial assimilation than aromatic compounds. Lodder, 1970 reported that aliphatic hydrocarbons are assimilated by most strains of yeast except *Saccharomyces, Kluyveromyces and Hansunella.*

Rule 2. Long chains are degraded preferentially to short chains. Shennan and Levi, 1974 concluded that subtrate specificity with regard to chain length is probably a function of the species or strain of the organism tested. However, most suitable range of carbon chain of n-alkanes as carbon substrate for the growth of the microorganism was found to be C_{10}-C_{20}. n-Alkanes of chain length shorter than C_9 are not usually assimilated by yeast but

may be oxidized by bacteria and fungi.

Rule 3. Unsaturated compounds are degraded more readily than saturated compounds but the yield is much lower than that obtained with the corresponding saturated n-alkanes.

Rule 4. Branched chain alkanes are not degraded more readily than unbranched molecules. This does not hold true for hydrocarbon assimilating organisms in general, a branched chain alkanes are also attacked by microorganism if there is a straight chain portion of the molecule of length more than C_9 Carbon atoms.

2.1.4 MECHANISM OF HYDROCARBON-UPTAKE :

The assimilation of hydrocarbons by microorganism when used for biomass prouction has been identified as following :

1. Uptake of the exogenous alkanes by the cells and the further transport to the site where the alkanes undergo the initial oxidation.
2. Oxidation of alankes to the corresponding fatty acids.
3. Incorporation of fatty acids to the synthesis of the Tricarboxilic acid (T C A) cycle intermediates via acetyl-CoA or propenyl-CoA (Coenzymes).
4. Synthesis of biomass from the TCA cycle intermediates.

Biological growth process on hydrocarbon involves four phases (i)-gas (air) phase, (ii)solid (cells) phase, (iii) liquid (aqueous medium) phase and (iv) the liquid or solid hydrocarbons.

Transport of alkanes is generally mass transfer controlled phenomenon involving physical interactions among the four

different phases . Three different hypothesis on uptake of liquid hydrocarbons have been advocated by three groups of researchers as follows :

1. The uptake of dissolved hydrocarbon in the aqueous phase is emphasised by Erdstieck and Rietema , 1969; Yoshida , 1971 . They observed the growth of *C. lipolytica* and *C . tropicalis* on C_6 to C_{12} hydrocarbons.
2. Direct contact of the cells with large oil drops at the oil /water interface is emphasised by Erickson, Humphrey and Prokop , 1969; Wang and Ochoa, 1972; Katinger , 1973; Mimura, watanabe and Takeda, 1971; Horowitz, 1975 . They studied the uptake of gas oil fractions by the organism *C.lipilytica* , *C. intermedia* and *C . petrophilum* .
3. Direct contact of the cells with the submicron (20-60μm) oil dropletes generated from large oil drops in presence of an extra cellular emulsifying agent. This phenomenon was emphasised by Aiba et al ,1969; Moo-young, Shimizu and White Worth, 1971; Goma et al, 1973 ; Chakarvarty et. al., 1975 ; Reddy et al., 1983; Einsele et al.,1973; based on the growth of *C.guillermondi, C . tropicalis, C. lipolytica* and *pseudomonas* species on liquid n.paraffin .

2.1.5. BIOEMULSIFIER AS MEDIATOR IN HYDROCARBON UPTAKE :

Investigations by various researchers supported the fact that uptake of liquid hydrocarbons is primarily through Pseudo-solubilization i.e; formation of submicron size(0.1-0.5μm) droplets.This process of pseudosolubilization of hydrocarbon drops

is built up by the spontaneous excretion of emulsifying factor from the organism (Suzuki et.al., 1969; Hisatsuka et.al., 1971; Zajic, 1977; Roy et.al., 1979; Rosenberg, 1979; Kappeli and Fiechter, 1980) It was established that lipopolysaccharides isolated from the walls of hydrocarbon utilizing microorganism play an important role in the process of hydrocarbon uptake. Reddy et.al., 1983 isolated the emulsifying and solubility factors produced by the *Pseudomonas* sp. during growth on pristane(C_{24}).They observed that emulsifying activity increased with the chain length of the alkanes. Very high emulsifying activity was obtained with Pristanedodecyl benzene and xylene.

The surfactants may assist in solubilization of hydrocarbon molecules and aid their passage through the cell membranes to the final site of oxidation. Studies on the ultrastructure of hydrocarbon assimilating yeast cells (*Candida tropicalis*) indicated that thickened cell walls are traversed by channels (Meissel et.al., 1976). These channels may have an altered chemical nature by which it facilitating the transport.

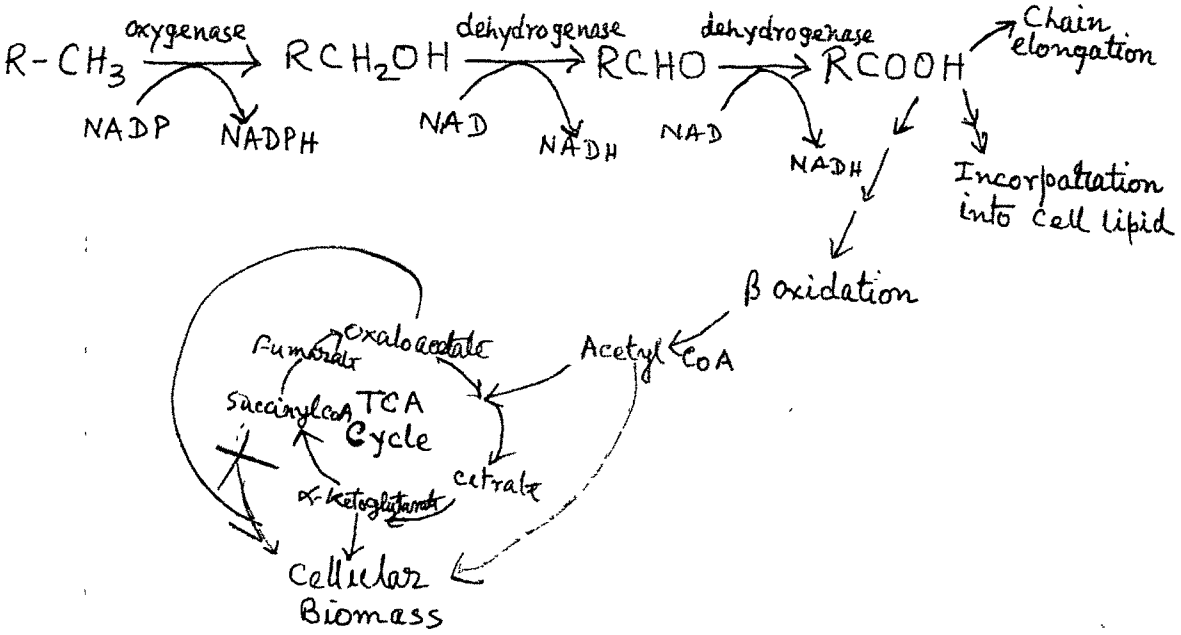
2.1.6. OXIDATION OF ALKANS :

Rehn and Reiff, 1981 presented excellent summary of the oxidation steps of the alkanes,Fukui and Tanaka, 1981 reviewed the diversity in alkane utilization pathway in the yeast. The location of different alkane oxidizing enzymes and their regulation in alkane metabolism in microsomes, mitochondria and peroxisomes were discussed.

The first step of alkane oxidation is catalysed by an oxygenese enzyme either involving cytochrome P-450 or without it. The straight chain alkanes are more readily oxidized than branched ones because only those molecules which can assume a more or less planar conformation have access to the active centre of the alkane oxidising enzymes. Further the oxygenese and hydroxylase enzymes are dependent on NADPH (Nicotonamide Adenine Deoxyribose Phosphate) reduced as observed in *Cadida tropicalis*.

Metabolic Pathway

The overall n-alkane metabolic pathway followed by the yeast to produce biomass may be summarised as following:



There are two major pathways in metabolism of alkanes. One is the terminal oxidation pathway and other is the subterminal pathway. One or both of the terminal methyl groups are oxidized to corresponding fatty acids in the terminal oxidation pathway (Klug and Markovetz, 1971; Einsele and Fiechter, 1971 and Rehm and Reiff, 1981).

In subterminal oxidation, ω-methyl group of the alkane chain oxidizd to corresponding fatty acids and alcohol.

Different pathways followed by the bacteria, yeast and fungi were summerised by Rehm and Reiff, 1981.

i). Bacteria, mainly *Psendomonas* sp., *Acenetobacter* sp., *acromobacter* sp. follow monoterminal pathway where as Bacillus sp. follow subterminal pathway.

ii). Yeast, mainly *Candida* sp. follow predominantly monoterminal and diterminal pathways.

iii) Molds, e.g., *Aspergillus, Penicillum, Fusarrium,* sp. follow monoterminal and subterminal pathways.

Most of the yeasts have the capacity to oxidize alkanes faster than molds because they follow the terminal pathway. The oxidase enzymes, molecular oxygen and electrons supplied by NADPH or NADH via a flavoprotein, known as Cytochrom P-450 reductase results the oxidation of alkanes (Kapeli, 1986) as following:

$$RH + H^+ + NADPH + O_2 \text{---} \rightarrow ROH + NADP^+ + H_2O \quad (1)$$

$$H^+ + NADPH + O_2 \text{ -----} \rightarrow NADP^+ + H_2O_2 \quad (2)$$

$$R_1OOH + ROH \text{ ----------} \rightarrow ROH + R_1OH \quad (3)$$

Most of the hydrocarbon degrading yeast strains, e.g., *Candida tropicalis, Candida lipolytica, Candida Guilliermondii* are capable to induce the monooxygenase enzyme when hydrocarbons are used as sole carbon substrate.

2.2 SCP PROCESS AND DEWAXING :

During the early 1960`s, British Petroleum Company introduced the concept of using microorganisms,particularly yeasts for production of proteinous biomass known as Single Cell Proteins (SCP) from hydrocarbons (Fig.2.1 & 2.2). Gas oil (250-380^{o}c) and purified n-alkanes (C_{14}-C_{18}) from the gas oil were used as feed stocks for the processes with the objective to utilise n-paraffins for SCP production and dewaxed the petroleum fraction (Laine and duChaffaut, 1975).

The process was scaled upto pilot scale producing 4000 mt/year of SCP in an air-lift fermentor. While the gas oil process was conducted in non sterile condition, aseptic conditions were maintained in n-paraffinic process. The British Petrolium process led to the development of similar yeast based SCP processes utilising hydrocarbon subtrate by Petroleum and Chemical industries throughout the world.

Most of the processes were developed on n-paraffins as feed stocks because the SCP remained free from any carcinogenic chemicals and the easy down stream operation for the recovery of SCP.Einsele and Fiechter, 1971, reported that the n-paraffins containing C_{10} to C_{12} carbon atoms are most suitable carbon source for yeasts. The major production facilities established for the

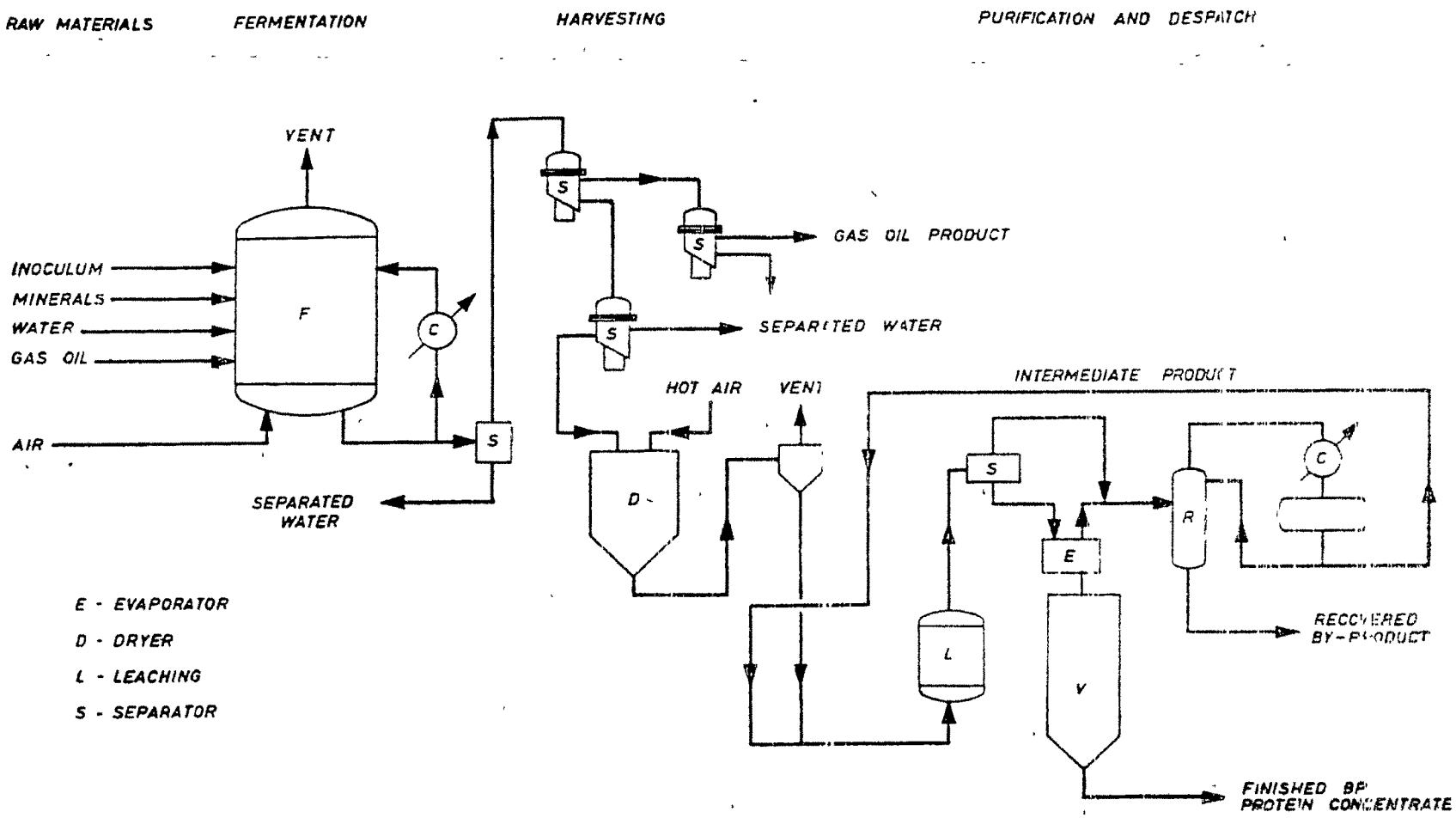


FIG. -2.1 BP SCP PROCESS USING GAS OIL FEEDSTOCK

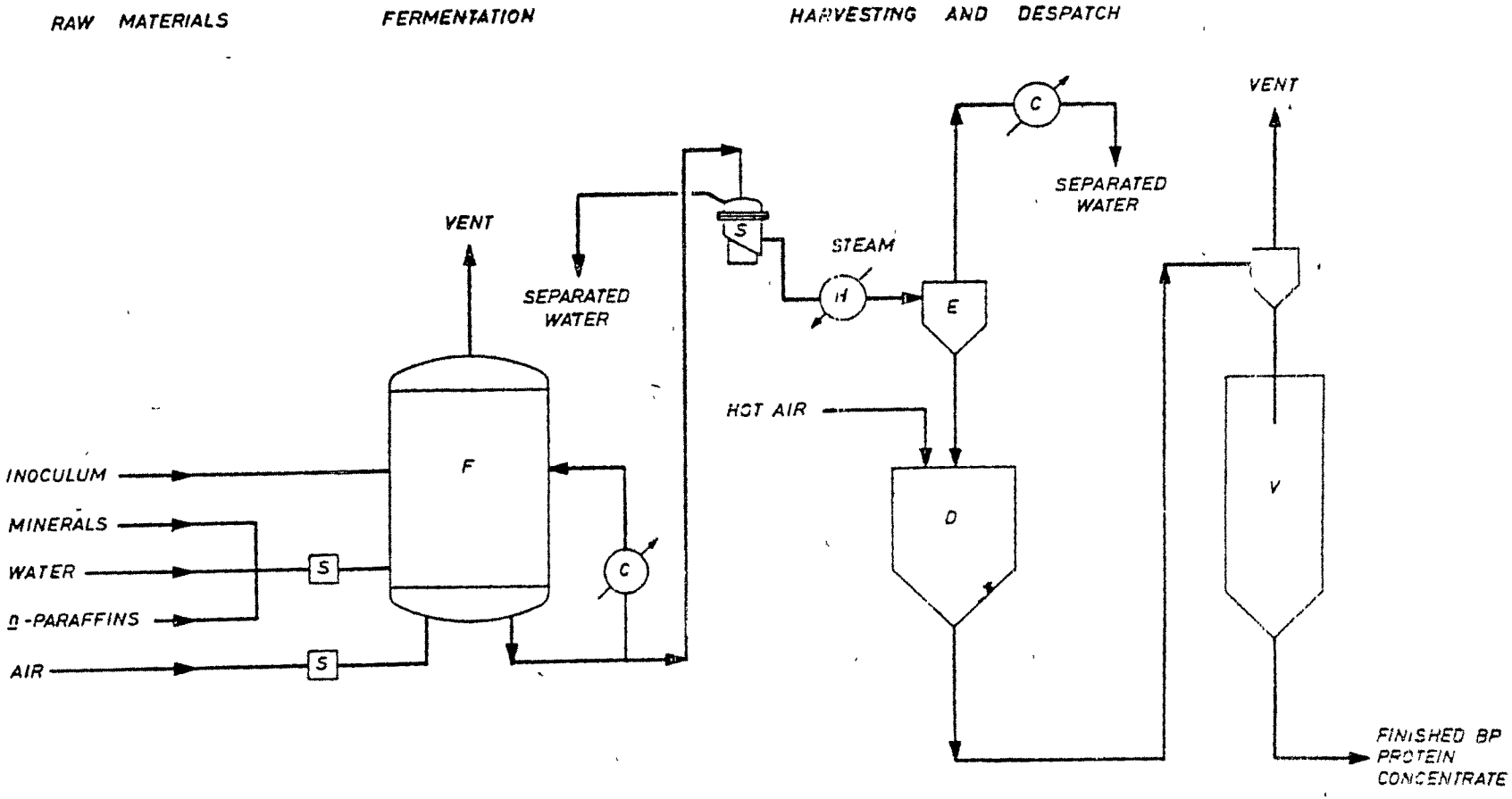


FIG. 2.2 BP SCP PROCESS (n-PARAFFINS AS FEEDSTOCK)

production of SCP from hydrocarbons, summarised by Moogoung, 1976 are given in Table 2.2.

TABLE- 2.2.: SUMMARY OF PRODUCTION CAPACITY OF SCP FROM HYDROCARBONS

Company	Location	Size tonnes/ year	Substrate	Organism
British Petroleum	Gangemouth, U.K.	4000	Paraffin	Yeast
	Lavera, France	1600	Gas oil	Yeast
	Sardinia, Italy (Not operated)	100000	Paraffin	Yeast
LiquiChemica	Reggio Calabria	100000	Paraffin	Yeast
Hoechst/Uhde	Frankfurt, Germany	Pilot scale	Paraffin	Yeast

Apart from these major activities, other processes developed by different organisations in lab. or pilot scale are IFP France, GDR & USSR using gas oil as feed stock and yeast culture for biomass production.

Indian Institute of Petroleum, Dehradun also developed processes for production of SCP using gas oil (250-350^{o}c) and pure n-paraffins (C_{14}-C_{18}) as feed stocks. SCP was produced in pilot scale (40-50 kg/day) in a 1 m^3 airlift fermentor set up at Gujarat Refinery premises, Baroda (Sista, 1989).

The yeast strain *C-tropicalis* was found capable of bringing down the pour point of gas oil fraction (b.p. 250-380)from 15°c to 9°c during continuous Culture Studies (Saini et.al.).The SCP has been accepted as protein substitute (2.5 times better than groundnut cake) in cattle feed.

2.2.1 PROCESS PARAMETERS :

The optimum process parameters observed for the growth of yeast on hydrocarbon have been summarised by Litchfield, 1977; and Einesele and Blanch ,1973 (Table 2.3.).

1) Extent of dewaxing has been reported in terms of pour point of dewaxed oil or the pour point depression ($\Delta t = t^{o}c$ initial - $t^{o}c$ final). In some cases dewaxing has also been quantified in terms of n-paraffin content of the oil (C_o) and dewaxed oil (C_k).Degree of alkane consumption or dewaxing as defined by Katrush,(1981) is following :

$$C_k = \frac{C_o - C_k}{C_o}$$

Though theoretically the microbial dewaxing is growth associated yet the growth curve of the microorganism and the proportional change in pourpoint of the oil has not been reported. Similarly biomass concentration has not been found proportional to the degree of dewaxing during continuous fermentation studies carried out at different dilution rates.(Dostalek and Munk, 1969).

TABLE .2.3. GROWTH PARAMETERS OF YEASTS ON HYDROCARBONS

Organism & Substrate	Mode of operation	Temp. ^{o}c	p^{H}	Sp. growth rate μ^{-1}	Yield g/g	Productivity g/l/h
1. *C-tropicalis* in n-alkanes (C_{14}-C_{18})	Continuous 50,000L 1vvm	30	3.0	0.15-o.24(D)	1.0	1.7-3.0
2. *C-tropicals* (n-hexadecane)	a)Batch 14L 1vvm	30	4.5	0.29	1.0	----
	b)Continuous 14L 1vvm	30	4.5	0.10 (D)	1.0	0.95
3. *C-lpoliplytica* (n-alkanes C_{14}-C_{18})	Batch 14L 1.5vvm ,	25	5.5	0.22	0.90	----
	Continuous 1800L 1.5vvm	32	5.5	0.16 (D)	0.88	3.78
4. *Candida intermedia*	Batch 1L 1525 rpm	25	5.5	0.14-0.23	0.75	----

2) It was observed that phase inversion occured (o/w to w/o) when the oil concentration in the fermentor exceeds 30% (Laine and Chaffant, 1975). The optimum range of gas oil concentration in the fermentor for dewaxing was observed to be 5-10% by Chepigo et.al., 1969. Higher oil concentration limits the oxygen availibility of the microorganism in w/o consequently decreases the specific growth rate of the microorganism. Studies conducted by Dostalek et.al., (1969) on gas oil concentration observed that sp. growth rate of the organism (0.24^{-1}) was unaffected up to15% of the oil concentration in the fermentor beyond which the sp. growth rate of the yeast strain decreaseed to 0.12 h^{-1}.

3) At lower dilution rate dewaxing was observed better where as at dilution nearer to μ_{max} of the yeast strain, *C-lipolytica* poor dewaxing results were observed. Pour point of gas oil decreased from $-12^{o}C$ to $-48^{o}C$ at dilution rate of 0.07 to 0.1 h^{-1} (Chepigo, 1969). However, Dostalek et. al.(1969) reported the dilution rate of 0.11-0.33 h^{-1} for maximum dewaxing of the gas oil using yeast strain *C.lipolytica* having maximum sp. growth rate $0.h^{-1}$.

4) Pour point depression of $30-50^{o}c$ and n-alkane cosumption of 50-95% was reported when gas oil ($240-360^{o}c$) was used as feed stock (Katrarh,1981). Pour point depression from -3 to $-44^{o}c$ was claimed in a German patent (Klippenstapal et. al., 1978)
A Czech patent (Pilet, 1978) claimed pour point depression from +10 to $-40^{o}c$.

(V) Recovery of dewaxed oil :

Recovery of the dewaxed oil is the important and cost intensive operation in the overall dewaxing process. Usually the fermentation broth after harvesting is allowed to stand and water phase is decanted. The emulsified mass is decanted and subsequently centrifuged to recover oil. British Petroleum obtained a patent on the use of water soluble surfactants for recovery of dewaxed oil and recommended hydrogenation of oil recovered (Laine, 1970). Two extraction stages were recommended in which firstly an azeotrope mixture was formed with alcohol and hydrocarbon and then distillation in the second (Laine, 1971). Use of binary mixture of surface active agents like polyphenyl ethylene, fatty acids C_{12}-C_{18}, hydroxy ethylated sulfated fatty alcohol, alkyl mono sulfonates and amine salts were reported. Kockert et.al.,(1976) recommended the separation of the oil by using a surfactant followed by heating at 350°c for 8 hrs. and subsequent distillation.

Ghosh and Sista, 1968, used a mixture of acetone and petroleun ether (1:3) for extracting dewaxed gas oil from the emulsified fermented broth.The lighter dewaxed fraction so obtained was recomended for hydrofinishing over Co-Mo catalyst. Decrease of surface tension and interfacial tension of water-yeast cells-gas oil system were measured by Srinivasan et.al., 1970.

2.2.2. STATE OF THE ART :

A pilot plant was set up for dewaxing diesel fuel by yeast strain *Loddermyces elenigisporous* in a fermentor having capacity to charge 10 ton of feed (Heinritz et.al., 1985).

Dewaxing of C_{12}-C_{25} range n-paraffins present in light diesel oil using *C-tropicalis* was patented by the Indian Institute of Petroleum (Sista et.al., 1979).

A German group obtained some patents on the dewaxing of distillates producing speciallity products e.g., Transformer Oil (pour point -30°c) by decreasing the n-paraffinic content of the gas oil from 12.5 to 3.5% using C-tropicalis (Gadzhieva, et.al., 1976).

Another patent claimed to produce low pour point transformer oil or electric insulating oil by microbial dewaxing the oil distillate followed by hydrogeneration, sulphuric acid treatment, neutralization and addition of alkylnaphthalene as pour point depressent (Hieke et.al., 1979). Production of high octane Number fuel (82-83) was reported by Hieke et.al., 1979 and Kockret et.al., 1982) by hydrocracking the diesel fuel (234-378°c) which was deparaffinised by yeast strains. Few attempts were, however, made to develope microbial dewaxing technique for lube fraction boiling in the ranges of 350-500°c. Phillips Petroleum obtained a patent on microbial dewaxing of solid hydrocarbon by dissolution in aromatic or cyclic saturated hydrocarbon (Wegner, 1967). However the dewaxed oil produced by any of the above processed did not meet the specifications of lubricating oil. Hence there is ample scope for the development of the microbial dewaxing of lube oil fractions.

2.2.3. AERATION ASPECTS OF HYDROCARBON FERMENTATION :

The growth of microorganisms on alkane is an aerobic process. The oxygen required for the oxidation of alkanes by the microorganism must be provided through air. Oxygen requirement by the cells was observed 2.5 times greater than that required for carbohydrate metabolism. It was observed that the rate of synthesis of cell components was controlled by the overall capacity of the respiratory pathways (Einsele et.al., 1972).

The overall biochemical reaction for the growth of microorganism on both the subtrates can be written by the stoichiometric equations on 1 kg. mole basis as follows :

For hydrocarbons :

$$2n\ CH_2 + 2n\ O_2 + 0.19n\ NH_4^+ \longrightarrow n\{CH_{1.7}\ O_{0.5}\ N_{0.19}\} + 0.8n\ CO_2 + 1.3n\ H_2O + n\ 48000\ KJ \quad --(1)$$

For Glucose :

$$1.8n\ CH_2O + 0.8n\ O_2 + 0.19n\ NH_4^+ \longrightarrow n\{CH_{1.7}\ O_{0.5}\ N_{0.19}\} + 0.8n\ CO_2 + 1.3n\ H_2O + n.19000\ KJ \quad --(2)$$

High oxygen transfer rate in the range of 150 to 400 m.moles $O_2 l^{-1} h^{-1}$ were reported by Einsele et.al., 1972. The critical concentration of dissolved oxygen in the hydrocarbon aqueous system is about 1.0 to 1.2 ppm. Therefore a limitation in aeration or oxygen supply reduces the sp. growth rate and productivity of n-alkane grown microorganism.

There are two possible ways to increase oxygen solubility in the broth.

(a) By increasing its partial pressure through maintaining a positive air pressure inside the bioreactor or by using gas mixture with increased oxygen content.

(b) Through the application of a continuous oil phase system since oxygen is more soluble (65.8 mg/L) in hydrocarbon than water (7.8 mg/L) at 30^{o}c. The interfacial conditions between air and water are disturbed in presence of paraffins by changes in surface tension (Calderbank, 1967).

Addition of paraffin to aqueous sulphite solutions enhances the oxygen transfer rate (Mimura et.al.,1973).

Although oxygen transfer rate determined by sulphite oxidation method does not represent true values in a biological system, it gives close value for a hydrocarbon fermentation system. Einsele et.al., 1981, suggested that any bioreactor which attained sulphite oxidation value upto 1200 m mole O_2/l.h., it could be shown that oxygen is not limiting factor in a hydrocarbon fermentation system. Productivity and optimum dilution rate for continuous culture of yeast on hexadecane is a function of agitation and aeration in the fermentor as observed by Einseley et al ,1973).

The influence of chain length of n-alkane on yield of biomass was observed by Giacobbe, 1973, which indicated that C_{14}-C_{20} chain-length of paraffins provided maximum yield of SCP. It is also evident from the Table2.3. that most of the industrial SCP processes were developed using either n-paraffin of gas oil in the carbon range of C_{14}-C_{20}.

2.2.4. HEAT REMOVAL IN SCP PROCESS :

One of the major problems associated with SCP processes were the removal of large amounts of heat generated during the metabolism of hydrocarbon as shown in following equation. One method estimating quantity of heat evolved during metabolism is based on measuring the heats of combustion of hydrocarbons and of biomass. Assuming hydrocarbons as feed stock and yeast as a product Kanazawa (1975) have established the following correlation by Heat Balance :

$$H = \frac{C_s}{Y_{x/s}} - C_x \qquad \text{---------} \qquad (4)$$

Where, H heat evolved by metabolism (KJ/Kg) of yeast.

C_s heat of combustion of hydrocarbons (KJ/Kg)

C_x heat of combustion of dried yeast (KJ/Kg)

$Y_{x/s}$ dry weight yield of yeast from hydrocarbon (Kg/Kg).

The cooling requirement in the fermentor can be calculated based on the above equation. In order to achieve constant temperature in the bioreactor, heat from the following sources has to be transferred :

i) heat produced by the exergonic biochemical process.

ii) heat produced by the agitator for mixing and aeration.

Generally yeast based commercial SCP process is carried out in the range of 26-34^{o}c. An increase of process temperature has several advantages such as :

- decrease cost for cooling equipment.
- reduced energy cost for cooling.
- easier handling in regions of warmer climate.
- fewer problems with yeast contaminants.

Estimated heat evolution for the BP Pocess on n-alkane or gas oil is approx. 4.6×10^8 kJ/hr. for a 10^6 t /Year plant (Litchfield, 1977). Therefore, use of cooling water at 29 to30 ^{0}C is not feasible for complete heat removal from the fermentor operating at 30-32^{o}c. Additional refrigeration is required in SCP process.

Two types of heat exchangers are used in SCP production.

a) External circulation of broth in a high capacity heat exchanger against cooling water,

b) Intercooling by circulating water through cooling pipes inside the fermentor.

Fussman,(1973) calculated refrigeration horsepower requirement for plants operating at 34^{o}c. In India, it is about 17240 hp compared to 9100hp in Finland based on the assumption that cooling water temperature available at 35^{o}c and 14^{o}c respectively. However, average cooling water temperature in India varies from 20-30^{o}c in different geographic regions. Considerable economy in steam and cooling water requirements would become possible if the organism grows at higher temperature e.g., 36-40^{o}c. The yeast strain C.tropicalis developed by Indian Institute of Petroleum showed higher growth temperature (36^{o}c) on n-parafins (C_{14}-C_{16}) (Sista, 1989).

2.2.5. NUTRITIONAL VALUE OF SCP :

Yeast cells, like other microorganisms are composed of proteins, lipids, carbohydrates vitamins . The proximate alysis of selected microorganism of interset in SCP production are summerised in Table 2.4. Protein and lipid content of the microorganism, reflect the composition of the medium and growth condition. Yeast, mold and higher fungi have higher cellular lipid contents and lower nitrogen and protein content than bacteria.

However, the lipid content of the cells increased when the cells were grown in nitrogen deficient medium.

TABLE 2.4. PROXIMATE COMPOSITION OF BACTERA AND YEAST USED FOR SCP PRODUCTION [a]

Composition	Bacteria (Acenetobacter Cerifiens)	Yeast (Candida tipolytica)
Carbon substrate	n-Hexadecane	Gas oil
Nitrogen, g/100 g dry cell	11	9.6
Protein, g/100 g dry cell	70	60
Lipid, g/100 g dry cell101		8
Carbohydrate, g/100 g dry cell	24	22
Ashg/100 g dry cell	5	8

a : Shacklady, 1973

Hence the nitrogen content (C/N) of media should always remain at a critical level to achieve a maximum yield of as well as higher protein composition of the cells.

Bacteria accumulates higher concentration of nutritionally undesirable nuclie acid, (20%) compared to yeast (8-12%) due to higher growth rate of bacteria. While these nucleic acids are digested by animals, foods containing much in excess of 1% cannot be safely digested by humans.Hence, their removal is mandatory for human consumption. Protein Advisory Group (PAG) has fixed the guideline for safe limit i.e., 2 mg of nucleic acid per day per person. Cell wall of yeast is also not digestable by human.

The amino acid composition of the yeast protein together with the FAO standard protein for human consumption is presented in Table.2.5.

The amino acid pattern in the SCP from hydrocarbon indicates high lysine content (7.4) which is comparatively low in cereals consumed in bulk by human and animals. But the sulfur containing amino acids are low in yeast protein as compared to cereals supplementation of SCP in appropriate proportion can constitute a balanced protein diet.

TABLE 25 AMINO ACID PROFILE OF YEAST AND OTHER SELECTED FOODSTUFFS [S]

UNIT: GM AMINO ACID/ G NITROGEN

Amino Acid	Feed yeast Toprina (BP Process)	Fish meal	Extracted Soyabean	Egg Protein	FAO Standard
Leucine	7.4	7.3	7.7	8.9	4.8
Isoleuine	5.1	4.6	5.4	6.7	4.2
Valine	5.9	5.2	5.0	7.3	4.2
Threonine	4.9	4.2	4.0	5.1	2.8
Methionine	1.8	2.6	1.4	5.1	2.8
Cystien	1.1	1.0	1.4	2.4	2.0
Lysine	7.4	7.0	6.5	6.5	4.2
Arginine	5.1	5.0	7.7	-	-
Phenylalanine	4.3	4.0	5.1	5.8	2.8
Trypotophan	1.4	1.2	1.5	1.6	1.4
Histidine	2.1	2.3	2.4	-	-
Tyrosine	3.6	2.9	2.7	4.2	2.8
Total Sulfur Containing Acids	2.9	3.6	2.8	7.5	4.8

s : Levi et.al ; 1979.

The direct use of SCP for human food canonly occur in the long term for the reasons that its safety aspects have to be conclusively demonstrated and methods have to be evolved for the effective and economic isolation and conversion of SCP to forms readily acceptable to human .

The nutritional value of SCP produced on hydrocarbons was evaluated in animal species ranging from rodents to domestic livestock, including broiler chickens, laying hens, swines and calves. Typical biological values (BV) for *Candida lipolytica* strain grown on gas oil or n-alkanes are 54 and 61 respectively. These values may be compared with 42 and 90 respectively, for Soyabean Protein and Dried Whole egg. Supplementation of 0.3% DL methionine with the SCP raises the biological value equivalent to soyabean and whole dried egg (Levi, 1979).

Performance of SCP products in cattle feed are evaluated by their nutritional values e.g., metabolizable energy, protein digestibility and feed conversion ratio are usual parameters and these were tested on broiler chickens, swines and calves.

Significant difference between experimental and control diets at the 10% level of SCP in diet of 1 broiler chicken was not served (Gow et.al., 1975). When yeast SCP grown on hydrocarbons was used as a milk replacer for calves and steer at the approximate level of 7.5% in the ration it maintained the calves nourishment (Shacklady, 1974). In the United States, the food and drug administration regulation allows the use of the dried yeasts in foods provided that the folic acid content of the yeast does not exceed 0,04 mg/gm. Young and Scrimshaw, 1975 pointed out that yeasts and algal have low protein digestibility value that can be improved considerably if these SCP products are processed suitably. However nucleic acid content is a limiting factor in the extent to which SCP products can be used in human foods.

2.2.6. TOXICOLOGICAL AND SAFETY ASPECTS OF SCP :

Hydrocarbon based SCP was introduced with the intention of using it as part of animal feed rations. Because of its new and unusual starting material, n-alkanes or gas oil of petroleum origine, its manufacturers realised that they would have to undertake extensive studies on animals to prove the nutritional value of the product and the absence of toxicological symptoms and the safety of manufacture.

ICI reported to have used approxiately 500 tons of SCP in feeding 20,000 animals of different species at a cost of approximate $ 6 million (Anon, 1980). Similar programs have been carried out by BP, Kanegafuchi and Dainippon of their products. IIP also crried out nutritional and toxicological tests in different laboratories and agricultural universities (Nath et.al, 1979; Vijjan et.al., 1978).

In order to provide information and suggest positive guidelines for test procedures, the Protein Advisory Group of United Nations has issued over the course of time, a series of statements and guidelines (PAG Guidelines, 1974). In addition, the International Union of Pure and Applied Chemists (IUPAC) proposed guidelines for testing SCP for animal feed, which were later amplified (IUPAC Guidelines, 1978).

The safety of SCP has to be basically demonstrated on the following aspects :

- It is not carcinogenic
- It does not affect reproduction rate

- The absence of teratogenic effect or deformation producing effect on off-spring
- The absence of mutagenic effect, i.e., the fertility is not impaired

Nutritional and toxicological testing involves short-term studies for accute toxicity leading to long term life-span and multigeneration studies, teratologenic and mutagenic studies, and sensitization and immunoresponse studies. Some of the aromatic hydrocarbons present in petroleum fractions are known for their carcinogenicity and any food material prepared out of petroleum fractions may be connected with cancer in some way or other.

Hence large scale field trial experiments were conducted on SCP. 1,500 tons per annum of Toprina were tested by BP for 7 years after it was observed extensive range of toxicological and nutritional tests that SCP from hydrocarbon is not harmful to animals. Similar extensive testing has been carried out on the Pruteen product of ICI (Waterworth, 1981).

Yeasts grown on mineral oil and pure n-alkanes have been found to contain ample amount of polycyclic aromatic compounds. Compared to baker's yeast these contain 10 fold more of these compounds. Careful control of the feed stock purity or the post fermentation solvent extraction procedure especially in the case of gas oil or heavier petroleum fractions is essential to ensure absence of polycyclic aromatic compounds in the single cell biomass. IUPAC has published recommendation for the polycyclic aromatic hydrocarbon standards of SCP (IUPAC Guidelines, 1978) as shown in Table.2.6.

TABLE 2.6. RECOMMENDED STANDARDS FOR SCP FROM HYDROCARBONS

Benzopyrene	< 5 ppb
Acetyl acetone reagent	< 20 ppm
Total hydrocarbons	< 0.5 %
Total aromatic hydrocarbons	< 0.5 %

2.2.7. SCP AS ANIMAL FEED :

The majority of animal feed consists of cereals , protein ingradients , minerals and vitamines which are available as agriculture byproducts . The protein component of animal feeds are being supplied as solvent extracted soya bean meals or extracted groundnut cake in the country . These agriculture based vegitable proteins are dependent on climatic conditions as well as availability of other input e.g; land , water , fertiliser, etc .

Alternatively SCP containing higher quantity of proteins along with other components e.g; carbohydrates , fats, vitamines, and minerals would be good substitute of those vegitable proteins. With the increase in population of cattles, the production of cattlefeed has gone up from 150 mt/y to 1400 mt/y in the last two decades (Fig. 2.2.3). Hence there is a potential of supply of SCP as protein ingradient in the cattlefeed industry because of its higher productivy and the production process is independent of any climatic conditions as compared to vegitable proteins .

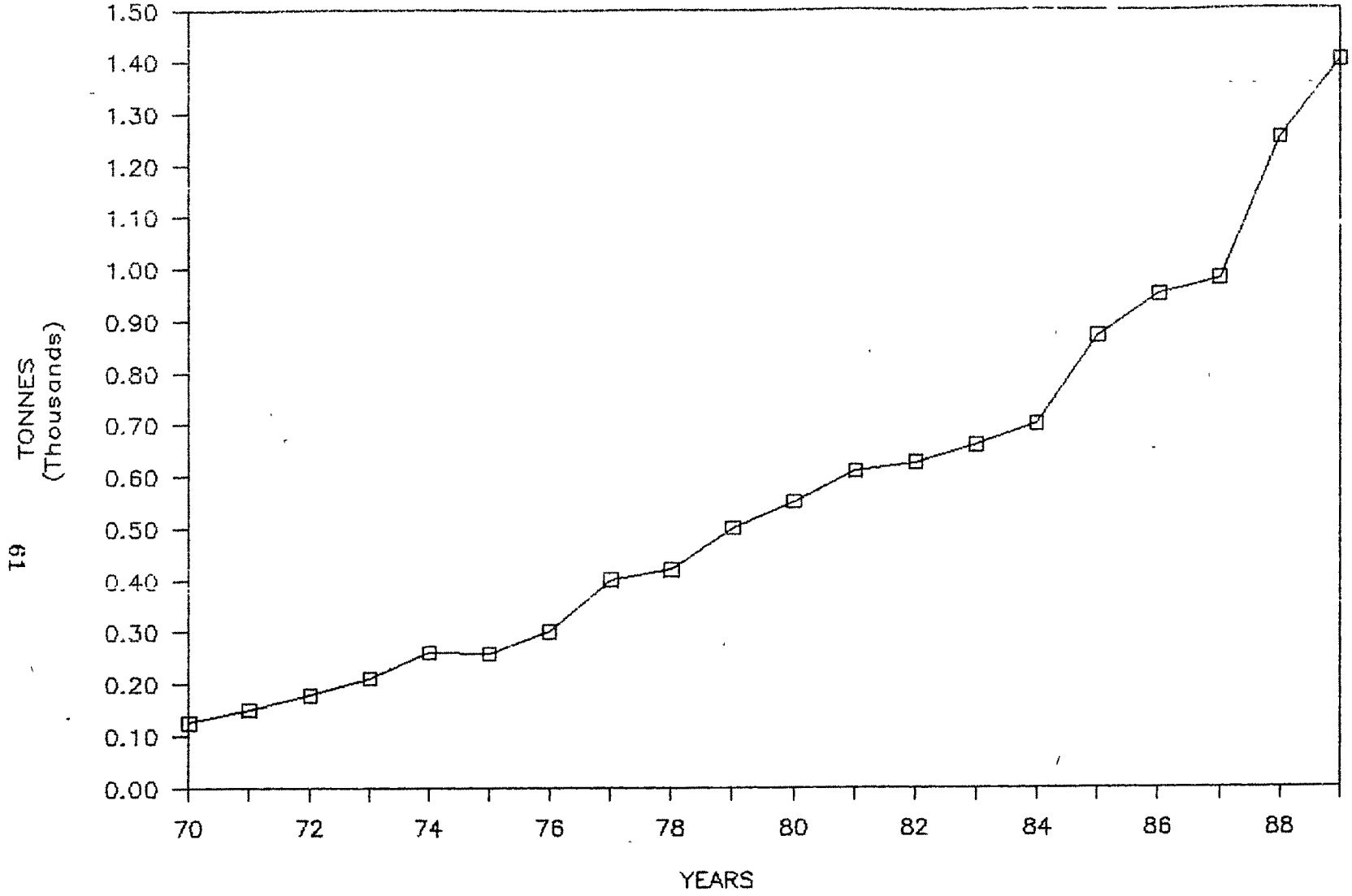


Fig 2.2.3. Cattle feed production in India.