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FINAL RESEARCH REPORT

(MAY 1975— APRIL 1978)

PRODUCTION OF SINGLE CELL PROTEIN FROM INDUSTRIAL WASTES

Food Technology and Fermentation Division,
Pakistan Council Of Scientific and Industrial
Research Laboratories Lahore-16.

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FINAL RESEARCH REPORT

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Signature of the Principal Investigator.

(Dr. N. Yaqub Chaudry)
Senior Research Officer

Departmental Head
(Dr. F.H. Shah)
Chief Scientific Officer,
Food Technology and Fermentation Division.

Institutional Head
(Dr. M.K. Bhatty)
Director
PCSIR Laboratories,
Ferozepur Road, Lahore-16.

The following scientists actively contributed to the completion of the project:

1) Dr. Muhammad Yaqub Chaudry, Principal Investigator.

2) Dr. F.H. Shah, Co-Principal Investigator.

3) Mr. Muhammad Akram Shah, Research Officer.

4) Miss Azra Baqai, Senior Scientific Assistant.

List of Laboratory and Technical Staff Associated With the Progress of this Project*

		Period of Service
1)	Mr. Safdar Ali, Laboratory Assistant	May, 1975 - April, 1978
2)	Mr. Amjad Iqbal, " "	May, 1975 - Sept., 1975
3)	Mr. Muhammad Khan, " "	Oct., 1975 - Oct., 1976
4)	Mr. Imdad Hussain, " "	Mar., 1977 - Apr., 1977
5)	Mr. Naveed Akram, " "	Apr., 1977 - Sept., 1977
6)	Mr. Rahim Din, Laboratory Attendant	Oct., 1977 - Dec., 1977
7)	Mr. Muhammad Din, " "	Jan., 1978 - Apr., 1978

* Apart from the above mentioned staff of the PSF scheme, the following workers of the PCSIR have been of great help and assistance in carrying out this work. Their services are greatly acknowledged.

1) Mr. Mahboob Ali Qureshi, Junior Technical Officer.
2) Mr. Muhammad Waleed, Senior Technician.
3) Mr. Muhammad Khan, Technician.
4) Mr. Muhammad Yasin, Stenographer.

CONTENTS

TENTATIVE YEAR-WISE PLAN OF WORK

FIRST YEAR

MICROORGANISMS

Screening and selection of microbes possessing higher growth rates and ability to utilize the concerned substrate rapidly.

SUBSTRATES

Industrial wastes such as carbohydrates (molasses), petroleum fractions, sulphite waste liquor, corn steep liquor

SHAKE FLASK EXPERIMENTS

SECOND YEAR

Optimization of culture conditions such as nitrogen and substrate (Quality and quantity) temperature, pH, oxygen supply etc.

SEMI PILOT SCALE EXPERIMENTS

Cultivation of microorganisms batch as well as continuous in 50 to 100 litre fermentors. Quality of the biomass as regards amino acid, vitamin, protein, fat, etc. to be determined.

Evaluation of the Product.

THIRD YEAR

PILOT SCALE STUDIES

Similar type of experiments as above would be carried out in 2000 to 5000 litre fermentors.

SUMMARY

Selection of a suitable microbial culture and choice of substrate and their characterization are the two main pre-requisitions of an economical process.

Yeasts capable of assimilating industrial wastes as the sole source of carbon were isolated from a wide variety of natural sources during investigation on yeast production by enrichment culture techniques and were purified by conventional streak method.

Newly isolated yeast strains along with stock cultures were tested for their affinity towards industrial wastes such as kerosene oil and diesel oil of petroleum fractions, sulfite waste liquors, hydrol or maize 'gur' and molasses as sole source of carbon. During cultivation of yeasts on the above mentioned substrates, growth parameters such as substrate and N_2 concentration both quantitative as well as qualitative, were studied. Their influence on the biomass formationn, % yield and quality of biomass has been reported.

Apart from substrate and N_2 concentration special euphasis has been given to the availability of oxygen and its influence on yield of biomass.

Taxonomical studies revealed that these new sixteen isolates included one strain of Saccharomyces cerevisiae, five

of Pichia membranaefaciens, two of Candida tropicalis, four of C. guilliermondii, two of C. rugosa, one of C. parapsilosis and one of Rhodotorula mucilaginosa.

These industrial wastes were analysed chemically and then used as sole source of carbon.

After the characterization of cultures and substrate come most important step is the fermentation stage in the production of Single Cell Protein (SCP). Growth parameters such as medium composition, carbon and nitrogen concentrations, pH, temperature and supply of oxygen have been studied in shake flask experiments.

The growth of Candida parapsilosis ($H\text{-}D_5$) was much faster than that of Kerosene oil. Maximum cell concentration of 8.1 g/l was observed in case of $(NH_4)_2SO_4$ when compared to 7.2 and 6.4 g/l with NH_4Cl and NH_4NO_3 respectively while studying influence of different nitrogen sources on the growth of Candida lipolytica using diesel oil as the sole source of carbon.

Effect of oxygen availability on the growth of Candida parapsilosis ($H\text{-}D_5$) using diesel oil as the sole source of carbon showed a remarkable increase in biomass synthesis with a corresponding increase in the availability of oxygen to the culture broth and thus resulted in better utilization of the substrate.

The biomass obtained in sulfite waste liquor (SWL) fermentation were 3.4, 2.8 and 2.4 g/l respectively in case of

cultures Candida rugosa (A), C. tropicalis (8_3) and C. parapsilosis ($H\text{-}D_5$) when the inoculum size was 0.8 g/l. The increase in inoculum size upto 6.4 g/l, improved the biomass formation from 3.4 to 16.0 g/l, 2.8 to 14.2 g/l and 2.4 to 11.8 g/l respectively.

Biomass formed ranged between 41.3 to 42.5 % protein and 6.0 to 7.0 % lipid. Maximum biomass formation was observed in Medium II when compared to Medium I while cultivating Candida rugosa (A) in different concentration of SWL.

Effect of different concentrations of NH_4Cl on the growth of C. rugosa (A) using maize 'gur' as the sole source of carbon showed that maximum biomass formation was obtained (12.72 g/l) when nitrogen level was 4 g/l.

Biomass was found to contain 37.5 % and 35 % protein in case of molasses and glucose experiments with Rhodotorula glutinis when the lipids were 22 % and 48.5 % respectively.

Biomass formation was increased from 5.8 to 16.3 g/l after 120 hrs while cultivating C. rugosa with 6 % molasses. The yield of biomass was calculated to be 50 % on the basis of sugar consumed and protein content was 45.4 %.

Regarding scale up studies with laboratory scale fermentors, the following experiments were carried out in 4 l and 60 l working volume fermentors.

Batch cultivation of Candida lipolytica was carried out in 4 l working volume fermentor using diesel oil as carbon source. Biomass increased from 1.14 to 9.85 g/l after 96 hrs.

The biomass formation was observed while cultivating Candida rugosa (A) and Rhodotorula glutinis (R_{44}) with 6 % maize 'gur' in a 3.5 l working volume and found to be 9.5 and 11.5 g/l respectively after 120 hrs. Yield was calculated to be 46 % on the basis of sugar consumed.

Effect of different concentrations of maize 'gur' on the growth of Candida rugosa under constant aeration (1l/l/min) showed that with the increase in sugar concentration in the medium, yield of biomass is decreasing.

Biomass increased from 2.0 to 20.8 g/l after 96 hrs while cultivating C. rugosa using molasses (4 % sugar content) in a 60 l working volume fermentor.

Regarding quality of biomass obtained from hydrocarbon and molasses experiments was analysed for protein, lipid, RNA and some essential amino acids. The amino acid profile of these yeasts protein indicates that it could serve as a good source of food and feed protein.

INTRODUCTION

Food is an important basic need to all people. Approximately 75 grams per person per day requires a supply of 100,000,000 tons per year of protein to satisfy the world's protein needs for food. Additional protein is needed for cattle and poultry feeding. The future food supply picture is uncertain, but shortage of protein already exists. The population of the world is increasing at higher rates (as shown below) than the food production. As a consequence, the difference between protein production and protein required increases.

World Population and Rate of Increase

Years	World population (in billions)	Rate of increase (Million/10 yrs.)
1850	1	-
1925	2	134
1960	3	285
1975	4.5	1000
2000	6.5	1000

In 1955, the Protein Advisory Group was created to help the World Health Organization (WHO) to advise FAO and UNICEF on the safety and suitability for human consumption of new protein foods. The Groups' attention was first directed to the development of protein-rich food mixtures based on dried skim milk, soya flour or locally available sources of vegetable protein. Later, interest developed in the potential of microbially-produced protein as an animal feed

supplement and, possibly, as a human dietary constituent. The global shortage now anticipated is not merely of proteins but rather of food of all kinds.

Many socio-economists have investigated this problem and have proposed many possible solutions. Among those, one which most investigators have agreed upon is the development of completely new sources of high quality protein.

The increasing demand of food products owing to the population growth in developing countries, has motivated scientists and engineers from several countries to state that the industrial production of single cell protein (SCP), might be a practical solution to this problem (Bhattacharjee, 1970; Laine, 1974; Humphrey, 1969). The development of SCP production could be suggested as an additional and/or complementary route to food conversion by domestic animals, but not necessarily as the exclusive way of solving the problem of food demand and consumption. This is in agreement with what has been established by the Protein Advisory Group of the United Nations, that "the protein from microorganisms of the best hope for a new source of major protein, independent from agriculture and climatic conditions". However, the protein derived from microorganisms is not in itself enough to satisfy the total demand; it is only a complement to protein derived from animal and vegetable sources.

Microbial proteins may be expected to play a significant part in efforts to make good the protein component of world food shortages, initially as a cheap and rapidly manufactured protein feed for farm livestock such as cattle and poultry. Certainly, the microbial

conversion of human non-acceptable substrates such as molasses, hydrocarbons etc. for use in food and feeds, is a very efficient and rapid process because of its high metabolic rate as compared to plants and higher organisms.

Single cell microorganisms, yeast in particular, have been known for a long time to be concentrated sources of high quality protein. Recently, SCP produced by yeast and bacteria on various non-edible carbohydrates and hydrocarbons has attracted attention as one means of meeting the demand for new sources of protein. Such proteins obtained by microbiological methods with the aid of living organisms, predetermines the high biological merits of the product and its affinity to animal proteins.

For the production of single cell proteins for feed purposes from industrial wastes and by-products, proper selection of microbial culture is most important. Criteria for selection can be that it should be

a) capable of rapid growth on low cost culture media,

b) yield biomass with a high protein content,

c) produce protein which is palatable and non-toxic.

Yeasts have an advantage over bacteria in the fact that they are easy to harvest by centrifugation or filteration due to larger cell diameter. Yeast, as SCP, is psychologically more palatable for human consumption. Although their useful protein contents of bacteria and yeasts are similar, bacteria have a higher content of nucleic acids which is undesirable to animals in large amounts.

After the selection of a desired culture and substrate for the production of SCP, the most important step is the fermentation stage where the raw materials, sugar or paraffins are consumed by microbes and converted into proteins. Protein production for cattle and direct human consumption involves both biochemical and technological problems. On the biochemical side the substrates, carbohydrates and hydrocarbons, are taken up by the living systems from the medium and catabolic as well as anabolic reactions take place under suitable environmental conditions, the microbes synthesize SCP. In case of carbohydrates, the biochemistry is well established and pathways for their degradation and utilization by cells can be found in text books of biochemistry. Molasses, both cane and beet contain 50% of fermentable sugars which are consumed by the microorganisms as carbon and energy sources. Usual yields of biomass are found to be 50% on the basis of sugar consumed. The composition of such proteins is reported to be:

Proximate Analysis of Food Yeast (Molasses)
(g/100 g dry weight)

Protein	50
Fat	6
Moisture	5
Ash	7
Sodium	0.3

For the production of yeast from carbohydrate substrates, molasses and maize 'gur' serve not only as a source of fermentable sugars but also of minerals like K, Mg, P, Zn, Fe, Cu; vitamins like biotin, pantothenic acid, pyridoxin, thiamin and of amino nitrogen

mainly asparagin, aspertic acid, glutamic acid. Beet molasses is richer in total organic nitrogenous compounds than cane, but half is betaine which is not assimilated by yeast. Cane molasses is substantially richer in Mg^{++}, Ca^{++}, and in vitamins like biotin, pyridoxin, pantothenic acid and thiamin. A detailed composition of beet and cane molasses is given below. The composition and properties of molasses show considerable degree of variations according to geographic region of its origin, processing factory, season and conditions of storage.

Molasses composition

Analysis	Average value	
	% Beet molasses	% Cane molasses
Invert sugar (%)	57	59
Non-fermentable (%)	2.1	3.5
Ash (%)	6.3	5.9
P_2O_5	0.02	0.1
Ca O	0.5	0.8
Mg O	0.1	0.7
K_2O	3.7	2.2
Vitamins (mg/g)		
Biotin	0.08	0.7
Thiamin	0.6	1.0
Pyridoxine	5.5	35
Nitrogen (%)	1.6	0.4
Betaine	0.8	0
Amino nitrogen	0.4	0.15

From: Peppler, H.J. IN: The Yeasts Vol.3, ed. A.H. Rose and J.S. Harrison. Academic Press, p.449 (1970).

From metabolic point of view yeasts can be divided into two groups i.e. fermentative and non-fermentative. Distiller's, brewer's and baker's yeasts belong to the first category, and food and feed yeasts come under the second category. The selection of a yeast strain depends on its physiological competence for the intended application. For example, a distiller's yeast is selected on the basis of its high ethanol tolerance and its maximum conversion efficiency of sugar-carbon to ethanol; baker's yeast is judged by its keeping quality, stability, CO_2 producing characteristics and its osmophilic nature; food and feed yeasts on the other hand should have a stable aerobic metabolism so that maximum amount of sugar-carbon could be converted to biomass and it should have high protein and vitamin contents and high specific growth rate.

The utilization of various hydrocarbons by microorganisms has received a great deal of attention during the last decade. (Miller and Johnson, 1966; Mimura, 1970; Moo-Young, et al., 1971; Einsele,et.a 1975). Paraffinic fractions of petroleum are attacked by microbial cultures especially by yeasts and bacteria. Energy thus released as a result of enzymatic oxidation is stored in the form of chemical energy and used when required by the living system for its normal functions and biosynthetic purposes. The terminal carbon atom of an n-alkane undergoes enzymic oxidation. Fatty acids thus formed by the action of oxygenases and oxygen enter the energy cycle of the living system i.e. TCA cycle after going through β-oxidation and are utilized for biomass synthesis (Leadbetter and Foster, 1959; Peterson, 1967; Davis, 1956). Hydrocarbons are selected as the

substances of primary choice of SCP production because of their availability in large volumes, low price and high cell yields. Crude oil or gas oil fractions with 20-30% of medium chain length n-paraffins when used as substrate yield an additional advantage in that gas oil is upgraded through microbial dewaxing.

The alkanes are organic compounds of the general formula C_nH_{2n+2}. The alkanes which have been studied more extensively are those between C_{10} and C_{21} which are the less desirable part of petroleum and high quality fuels. They have melting points around room temperature and they are poorly soluble in water and their solubility decreases as their molecular weight increases (Humphrey, 1967).

In petroleum based single cell protein processes, hydrocarbon serves as the primary carbon and energy source for cell growth. It occurs as an insoluble dispersed phase in the aqueous growth medium. Cell growth occurs mainly at the interface between the oil and water phases. Other chemicals necessary for cell growth include nitrogen in the form of cheap NH_4^+ salt, K^+, Mg^{++}, Cl^-, SO_4^{--}, PO_4^{---} and trace minerals. Oxygen must also be supplied. This is accomplished by mechanical means, i.e., aeration and agitation. It is distributed to the cells via contact with all three phases, i.e. aqueous, oil and gas phases.

Amongst the technological problems supply of oxygen and heat removal from the fermentation medium especially under temperate climatic conditions are the two major factors which affect the economy of the product to a remarkable extent (Darlington, 1964;

Guenther, 1965). Solution to these problems have been reported in finding strains which give high yields of biomass (with better quality of protein) and are capable of growing at a higher temperature (Humphrey, 1968).

Both batch and continuous systems of cultivating organisms are being applied in the industry. Continuous system has advantage over batch system because the productivity (g/l/hr.) is increased to about 3 times resulting in the economization of the process. Pakistan produces large amounts of industrial wastes and by-products as given below:

INDUSTRIAL WASTES AVAILABLE IN PAKISTAN

Waste type	Quantity
Molasses	10,00,000 tons/year
Maize 'gur'	40,000 tons/year(approx.)
Sulphite waste liquor) Corn steep liquor)	Substantial amount
Fuel oil	3,50,000 tons/year
Wax	50,000 tons/year
Naptha	80,000 tons/year
Extract (mixture of alphatic and aromatic compounds)	50,000 tons/year
Kerosene	600 barrels/day
Sui gas	20.7 mill.mill.Cft.

These by-products are not finding any valuable use except that they are used as fuel or dumped into the soil, thus causing pollution. With the increase in population and expanding poultry and livestock industry, there is a great demand for proteinaceous substances which at present are being met from expensive fish-meal

and blood meal. Utilization of the above mentioned raw materials for the production of SCP not only provides a check on pollution but also boosting up animal and poultry production in the country.

Keeping in view the demand for proteinaceous substances and the availability of large amounts of raw materials in the country we undertook the studies on the production of SCP. Main attention has been given to the characterization of microbial cultures and substrates, growth parameters, scale up studies and the quality of the biomass.

REVIEW OF LITERATURE

Since mid sixties after the publication of book entitled "World Food Problems" from White House, Washington gave gloomy picture of supply and demand of proteins especially in the developing Countries like India, Pakistan, Brazil etc. Efforts were concentrated on exploring unconventional sources of proteins.

Single-cell protein (SCP, as it is often called) is the protein contained in micro-organisms capable of independent existence as single cells - in particular, yeasts, bacteria, fungi and algae. The cells of micro-organisms contain carbohydrates lipids, mineral and vitamin in addition to proteins. These micro-organisms especially yeasts are capable of utilizing readily available organic materials as their carbon and energy substrate, to covert cheap inorganic nitrogen compounds into nutritionally valuable cellular proteins to replace the more expensive plant and animal proteins used in animal and human diets.

The amount of interest shown in the production of SCP by various Companies, Governments and Organizations of the world can be assessed from the following teble (Moo-Young, 1976).

A Survey of SCP Production Units in World*

Organisms	No. of Plant
Yeast	42
Bacteria and Molds	18

*Ranging from pilot to industrial scale

Substrate	
Hydrocarbon	14
Molasses	9
SWL	7
Methanol	5
Ethanol and Acetic Acid	12
Miscellaneous such as whey, Co_2, plant and Coffee wastes etc.	13

As clear from the table, yeasts are preferred organisms as compared to bacteria and molds. Regarding substrates almost all types of raw materials of carbohydrates and hydrocarbons have been used.

For the production of SCP for food and feed purposes from industrial wastes and by-products, proper selection of microbial culture is the most important step. Yeasts which have simple growth requirements and need only a mixture of inorganic salts and an organic source of carbon (substrate) are selected. It is essential that yeasts should be able to utilize cheap readily available substrates if a process for SCP is to be economical. After the selection of a desired culture and substrate for the production of single cell protein, the most important step is the fermentation stage where the raw materials, sugars or paraffins are consumed by microbes and converted into proteins. During the fermentation stage of the process, optimization of cultural conditions i.e. the physico-chemical parameters such as aeration and agitation, sterilization of air, foam control, pH, temperature etc. are necessary in order to maximize the conversion of substrate into proteins.

In microbial protein production, the desired cell composition is high protein, low carbohydrate, low nucleic acid, low lipid. The product has to compete economically with plant-derived proteins and should therefore contain favourable balances of lysine, methionine and tryptophan, which plant proteins often lack. Before microbial protein product can be used in animal feeds or as a human dietary constituent, comprehensive testing is necessary to ensure that it is devoid of toxicity or other harmful effects.

i) Microbial Cultures for SCP

Many yeasts can utilize cheap, inorganic nitrogen compounds such as ammonium salts for their growth, together with cheap carbon and energy substrates, producing a biomass of useful protein content. White (1954) mentioned that many organisms have been investigated, and among others the following species have been used: Saccharomyces Cerevisiae (many strains), S. lactis, S. logos, Endomyces vernalis, Torulopsis utilis var. major, T. utilis. var. thermophila, T. candida T. lipofera, T. lactosa, Comdida pulcherima, C. arborea, C. tropicalis, Mycotorula lipolytica, Hansenula anomala, H. suaveolens, Oidium lactis, and Rhodotorula gracilis. He also stated that some of the organisms named above are able to utilize a wider variety of carbohydrates than Saccharomyces as carbon sources. Also Peppler (1970) stated that several strains of the following yeast species: S. carlsbergensis, S, fragilis, C. lipolytica, C. tropicalis, and C. utilis. He also mentioned that

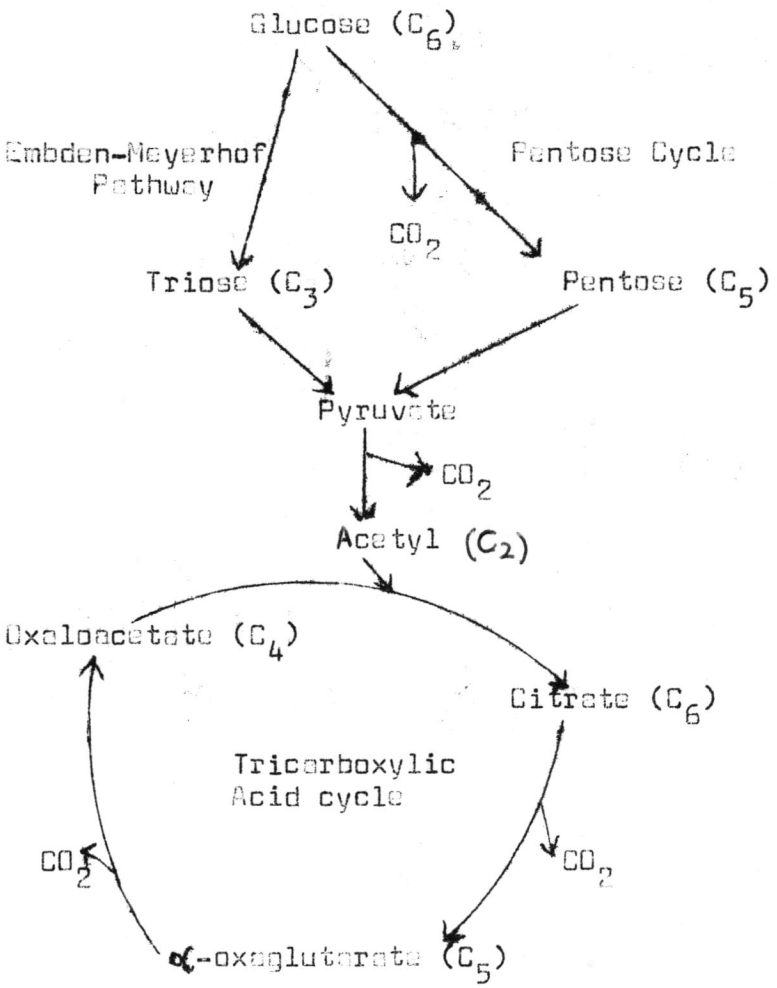
for production of food yeast by ~~primary~~ culture, usually in molasses media, S. cervisiae is preferred.

2) Substrates for SCP

Molasses is a by-product of the cane and beet sugar industry. These carbohydrates such as molasses and maize 'gur' serve not only as a source of fermentable sugars but also of mineral like K, Mg, P, Zn, Fe, Cu; vitamins like biotin, pantothenic acid, pyridoxin, thiamin and of amino nitrogen mainly asparagin, aspartic acid, glutamic acid. Beet molasses is richer in total organic nitrogenous compounds than cane, but half is betaine which is not assimilated by yeast. (Composition of Molasses already given in introduction). Cane molasses is substantially richer in Mg^{++}, Ca^{++}, and in vitamins like biotin, pyridoxine, pantothenic acid and thiamin.

The sulphite waste liquors from the paper pulp industry contain sugars derived from the hydrolysis of hemicelluloses in wood. Their composition varies according to the wood used. Angiosperms give sulphite liquors containing 3% sugars of which some 70% are pentoses (principally xylose), where as gymnospersms produce liquors containing about 2% sugars, some 75% of which are hexoses (principally mannose). Acid hydrolysis of wood cellulose itself may give 65-85% fermentable sugars.

The oxidation of glucose to carbon dioxide and water involves a considerable number of steps. A general scheme for the degradation of glucose is given below.



The history of hydrocarbon microbiology is a little different. It had its begining in 1895 when Myoshi observed that Botrytis cinerea attacked paraffins. But it was in the early 50's that researchers started studying the mechanisms of hydrocarbon attack by microorganisms. Until recently the primary industrial concern of hydrocarbon microbiology with oil prospecting, corrosion problems and the formation of microbial sludges in jet fuel tanks. In 1963, Champagnat started studying the production of edible yeast from oil fractions (Humphrey, 1967).

Gas oil (crudely fractioned [redacted] petroleum oil) contains 10-25% of $C_{10} - C_{21}$ paraffin hydrocarbons which are those most easily usable by microorganisms. Hydrocarbons having the general formula C_nH_{2n+2} may have linear (normal) or branch chain - molecules (iso -): the latter are degraded very slowly, if at all, by microorganisms. In a petroleum fraction such as gas oil there may be other hydrocarbons (cyclic paraffins and aromatic compounds) which are usually less readily metabolized. It is possible to produce microbial biomass by growth on a petroleum fraction and, at the same time, make use of the organisms's substrate specificity. Thus, the yeast Candida lipolytica metabolizes normal paraffins only; after its growth on a petroleum oil fraction, a material enriched in isoparaffins is left. Since the isoparaffins have more desirable properties as fuels, this is a useful contribution

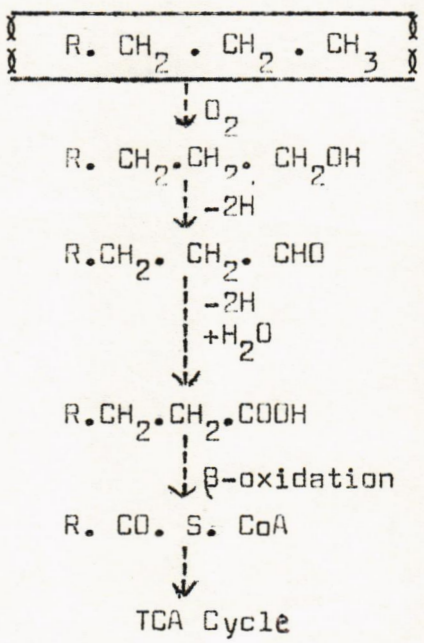
to the refining process. Alternatively it is possible to isolate the normal paraffins of gas oil by various physical methods, such as molecular seiving and use the purified material as substrate for biomass production.

Hydrocarbons have now become important as carbon and energy sources for the growth of microorganisms, mainly in the production of feed protein i.e. SCP. Among the yeasts, cryptococcaceae seem best able to use hydrocarbons; perhaps their cell wall structure is resistant to the solvent properties of hydrocarbons. Species of the genus Candida, such as C. lipolytica, have given the best results from an economic point of view. It can be seen that these yeasts do not assimilate alkanes shorter than C_9 and that the alkanes C_{11} and C_{18} appear to be most widely assimilated. It is possible that alkanes of low molecular weight are toxic, [redacted], being capable of dissolving lipids, they destroy the phospholipid micelles of Cell membrances.

In mixtures of alkanes, those having the shorter chain length are degraded more rapidly. Hydrocarbons have both advantages and disadvantages as growth substrates, when compared with the carbohydrates. They have melting points around room temperature and they are poorly soluble in water and their solubility decreases as their molecular weight increases(Humphrey, 1967). Their low solubility in water makes it possible to recover, by centrifugation, any proportion or fraction which has not been used for microbial growth. On the other hand, this low solubility

causes problems with the transport of substrate from the medium to the cell. It is likely that alkanes enter the cell both as dissolved and dispersed hydrocarbon. Cells of yeasts growing on alkanes have a characteristics morphology: the cytoplasmic membrane grows thicker and shows numerous invaginations. Transport through the cell wall is presumably, passive and only transport across the cytoplasmic membrane is an active process requiring metabolic energy. Because the enzymes involved in the first stages of oxidation are localized on the outside of the cytoplasmic membrane it is believed that active transport is of fatty acids.

The degradation of n-alkane is usually accomplished by oxidation of a terminal methyl group to form the corresponding primary alcohol. This product is then oxidised to a fatty acid which in turn is oxidised by the β-oxidation cycle leading to formation of ATP from oxidation of acetyl-CoA as shown below:



3) Physico-Chemical Environments

In fermentation using hydrocarbon as substrate, microbial oxygen demand is much higher than when carbohydrate is used as reported by many authors (Einsele et. al., 1975; Miller and Johnson, 1966; Mimura, 1970). Sufficient supply of oxygen for microorganisms is a critical problem for design or operation of the fermentor. The culture system, which is composed of aqueous, gaseous and oil phases, and microbial cells, is more complicated than in the case of carbohydrate fermentation, because of an additional immiscible liquid. Growing cells have a strong affinity for oil and aggregate in or on the oil to form flocks composed of oil and cells.

Aeration and agitation mix the flocks with air bubbles producing a peculiar mixture of bubbles, cells, and oil. Under this condition, a smaller amount of hydrocarbon is dispersed in aqueous solution as fine droplets. Much of it spreads between the aqueous and gaseous phases as a thin layer. Einsele et. al. (1975) claimed that microemulsified small particles (0.1 to 1.0 μm) of hydrocarbon are formed by a surface active agent produced by the yeast cell and sometimes cover the whole cell surface.

Under these heterogeneous conditions, oxygen transfer processes are more complicated and cells are presumed to utilize a considerable amount of oxygen from oil which spreads on the surface of air bubbles. Supply of oxygen and heat removal from the fermentation medium especially under temperate climatic

conditions are the two major factors which affect the economy of the product to a remarkable extent (Darlington, 1964, Einsele and Fiechter, 1971; Guenther 1965). Solution to these problems have been reported in finding strains which give high yields of biomass and are capable of growing at a higher temperature which would minimize cooling requirements (Humphrey, 1968).

Air contains microorganisms, mostly bacteria or fungal spores, which may be free or borne on dust particles. In polluted urban areas, where fermentation plant is often situated, it is possible to find an average of 10^3 - 10^4 viable organisms per cubic metre of air.

Both heat treatment and filteration can be used to sterilize air. The two methods can be combined: air is first compressed, the heat produced causing the destruction of at least part of the contaminating flora, and then partly decompressed before being passed through filters.

However it is more usual to employ low-pressure filteration alone. Fibre glass filters, or fibre glass impregnated with resin, are widely used. They function mainly by electrostatic charge effects and are much more effective than cotton filters which were formerly used. Moreover, they can be cleansed and re-used, are less prone to packing effects during use and are resistant to microbial attack.

The intense aeration and agitation in a fermentor may cause abundant foaming of the medium which may overflow through air egress and sampling ports, increasing the possibility of

contamination. Foaming often occurs at a characteristic stage in a fermentation: early foaming is due to medium constituents, later foaming is usually due to microbial products. Antifoam agents such as vegetable or animal oils etc. may be added in response to the rising foam level in the fermentor. An excess of antifoam agent is undesirable, particularly when foam flotation is later to be used for separating cells from the spent medium.

The pH of the medium is generally made acid for yeasts (4.5 - 5.5) and neutral for bacteria (6.0 - 7.5). A low pH in the range of 2.5 to 4.0 minimizes contamination problems in nonsterile system when gas oil is used as the sole source of carbon (Champagnat and Filosa, 1965; Evans, 1968).

The influence of the aqueous medium pH on the yeast growth in media containing liquid hydrocarbons as the main carbon source has received little attention. Miller et. al. (1964) studied the influence of pH on the cultivation of HD-5, a yeast closely resembling Candida intermedia, in media containing pure normal alkanes, but they did not present the growth curve nor the productivity obtained in their experiments. Concone et. al. (1976) studied the influence of the initial pH of the culture medium in shake-flask experiments carried out with Candida guilliermondii Y-8 in media containing Brazilian diesel oil, but the pH was not controlled during cell growth.

The pH values of the culture media were chosen between approximately 3.5 and 5.0, but the effect of pH on the cell growth

was not studied while studying the influence of various experimental conditions on the growth of microorganisms in liquid hydrocarbons (Pilat et. al., 1973; Mimura and Takeda, 1972; Ratledge, 1968). The maximum values of the specific growth rate and productivity were obtained at pH 6.0 and 7.0 respectively by Hiss et. al. (1977) when he studied the influence of the aqueous medium pH on the growth of Candida guilliermondii in media containing diesel oil as the main carbon source.

All microorganisms display an optimum growth temperaure, which is usually close to the maximum allowing growth. The optimum temperature varies according to the strain employed, from 28°C - 40°C. Many strains of yeast used industrially have an optimum temperature of about 30°C. Ueno et. al. (1972) studied that Candida kofuensis MT-Y-8 grew well in a paraffin medium at 37° and pH 3.5 after adaptation treatment and said that it is a very important to select a yeast strain which can grow at high temperature and low pH from the industrial view point of yeast cell production.

However, thermophilic strains, requiring 45 - 65°C, are of considerable interest because such temperatures considerably reduce the risk of contamination.

4) Mode of Cultivation

Industrial fermentations may be carried out usually in two ways:

Batch liquid culture

After a portion of medium has been inoculated with micro-organism in batch culture, a period of time normally elapses before a constant rate of growth is established; this period is called the lag phase. If the microorganisms in the inoculum are already adapted to growing under the conditions obtaining in the fresh medium, then the lag phase may be shortened or even disappear completely. Although there is little or no increase in the number of organisms in the culture during the lag phase, it is nevertheless a period of intense metabolic activity during which the organisms become accustomed to the conditions in the medium in preparation for the period of rapid growth that is to follow. During the lag phase there is a considerable increase in the contents of RNA and total protein in each organism; the DNA content however remains approximately constant. There is also an appreciable increase in the size of many microorganisms during this phase of growth.

When a constant rate of growth has been achieved in batch culture, the microorganisms are said to be in the logarithmic or exponential phase of growth. In this phase, the organisms are growing at the maximum rate possible in the particular medium. As each organisms reaches a certain age, it divides to produce two daughter individuals.

During the exponential phase of growth, which in batch culture of microorganisms in liquid media lasts only a short period of time, the nutrients in the medium become depleted and waste products of metabolism accumulate, so that the medium gradualy becomes less favourable for growth. Ultimately, the culture enters the stationary phase of growth in which the number of organisms in the culture remains constant. This phase can last for a considerable period of time but, sooner or later, it is followed by the death phase in which the number of viable organisms, although not necessarily the total number of organisms, declines.

Continuous Culture

All continuous cultures start their existence as batch cultures, in that the medium in the growth vessel is inoculated with microbes that proceed to grow in batch culture. If, during the exponential phase of growth, fresh medium is added to the culture at a rate sufficient to maintain the culture population density at a fixed value, lower than μ max, then growth should not ultimately cease as it does in a batch culture but continue indefinitely. Obviously, the rate of input of fresh medium would have to increase exponentially, with the increase in biomass, if provision were not made for continuous removal of culture at a rate equal that at which fresh medium was being added.

Continuous system has advantage over batch system because the productivity (g/l/hr) is increased to about 3 times resulting in the economization of the process.

5) Nutrition and Safety

Extensive data is now available on the composition of various SCP products, including proximate analyses and contents of amino acids, nucleic acids, vitamins, and minerals (Waslien, 1975). Also the nutritional value of yeasts grown on hydrocarbons and other substrates have been evaluated in animal species ranging from rodents to domestic livestock including broiler chickens, laying hens, swine, and calves. (Gow et. al., 1975; Litchfield, 1975; Shacklady and Gatumel, 1973).

Yeasts are not rich in sulphur - containing amino acids when compared with bacterial proteins and consequently have a less biological value. Typical Biological Values (BV) for Candida lipolytica strains grown on hydrocarbons are 54 and 61 respectively. After supplementation of the yeast SCP products with 0.3 % DL-methionine, the corresponding Biological Values are 96 and 91, respectively, as compared with 65 and 97, respectively, for soybean protein and dried whole egg supplemented with 0.3% DL - methionine (Shacklady and Gatumel, 1973).

For animal feed applications, SCP products give the best performance in the range 5 to 15% of the ration. At levels above 15% , significant decreases in performance occur in broiler chickens, and this level appears to be the practical limit for use in swine rations (Litchfield, 1975). As a milk replacer for calves, a 7.5% of C. lipolytica grown on either gas oil or purified n-alkanes appears to be the practical limit of use (Shacklady and Gatumel, 1973).

The Protein Advisory Group (PAG) of the United Nations has issued guidlines for SCP products and for evaluation of novel sources of protein including preclinical and human testing(Protein Advisory Group, 1970a, b,c, 1971, 1972, 1974). In addition to these guidlines, regulatory agencies in most countries have requirements for efficacy and safety of food additives that must be met by a SCP product destined for sale in a given country. For example, in the United States the Food and Drug Administration has promulgated regulations for food yeasts and products derived from them (Food and Drug Administration, 1963; Code of Federal Regulations, 1976).

If a SCP product is to be added to food as a major or even supplementary source of protein, then the nucleic acid content of microbial cells which usually contain 6-25 g nucleic acids per 100 g protein, must be reduced to acceptable levels to prevent adverse reactions such as the development of kidney stones and gout in humen consuming these products as a significant portion of the diet. (Scrimshaw, 1975). Processes for reducing the nucleic acid contents of SCP products include precipitation, acid or alkaline hydrolysis, heat shock and incubation for endogenous nuclease action, and use of exogenous nucleases (Litchfield, 1977; Sinskey and Tannenbaum, 1975). The PAG has accepted a 2 gm per day limit of ribonucleic acid (RNA) from an SCP product (Protein Advisory Group, 1972).

Considerable attention has been expressed on the safety of SCP products grown on hydrocarbon substrates (Anonymous, 1973, 1976 a,b).

The major questions relate to the possible presence of traces of polycyclic aromatic compounds or residual n-alkanes in the SCP product. Engel (1973)has conducted extensive studies on rats and mice to determine carcinogenecity, mutagenecity, and teratogenecity of SCP products grown on hydrocarbon substrates, without adverse effects being noted.

The PAG Adhoc Working Group on Single Cell Proteins has reviewed the status of information on n-alkane and aromatic hydrocarbon residues and the presence of odd-carbon fatty acids in SCP products and the safety of hydrocarbon-grown SCP products for use in animal feeding (Protein Advisory Group, 1976 a, b). It was concluded that the low levels of residual n-alkanes present and contents of odd-carbon fatty acids do not present a hazard, and that safe and nutritionally acceptable SCP products for use in animal feeding applications can be produced. Also, conditions used in continuous cultivation of strains of microorganisms used in SCP production would minimize the possibility of mutants arising during growth that might produce toxic substances. Furthermore, incidental contaminants such as arsenic or fluoride may enter the product through mineral nutrients such as phosphates or the water supply, unless raw materials free of these contaminants are used.

Political considerations also enter into safety evaluations. Two large SCP production facilities in Italy are not being operated because of claims of unsafe residues in the product; these claims may be politically motivated (Anonymous, 1976 c).

MATERIALS AND METHODS

1. Substrates

Industrial wastes such as diesel oil and kerosene oil fractions of petroleum, sulphite waste liquor, maize'gur'and molasses were used as the sole source of carbon throughout these studies.

i) Petroleum Fractions

Diesel oil and kerosene oil obtained from National Oil Refinery, Karachi, were analysed for their n-paraffin contents by urea adduct method(Veselov, 1960) as shown in Table I.

ii) Sulphite Waste Liquor (SWL)

SWL was obtained from Packages Limited, Lahore. Its chemical analysis is shown in table II. For clarification, SWL was heated to 100°C and air was passed through it in order to remove the excess of SO_2. After adjusting its pH to 3.8 with conc. H_2SO_4, it was allowed to stand or centrifuged so that the bulk of cationic impurities are removed.

iii) Maize Gur (Hydrol)

Hydrol or "maize gur" a by-product of corn hydrolysing industries such as Glaxo, Rafhan Maize Products was used. Its proximate analysis is given in table III. Maize gur was clarified through double treatment i.e. first with lime to pH 9 and after removing the sediment to pH 5 with concentrated H_2SO_4.

iv) Molasses

Cane and beet molasses were obtained from Crescent Sugar Mills, Faisalabad and Mardan Sugar Mills, Mardan. Their approximate chemical analysis are shown in table IV. Clarification of molasses was done with sulphuric acid treatment and centrifugation.

2. Isolation, Screening and Purification of Yeasts

Yeasts strongly assimilating industrial wastes as the sole source of carbon were isolated from a wide variety of natural sources i.e. oil-soaked soils, waste waters, drain-waters etc. during investigation on yeast production by enrichment culture techniques. Approximately 1 g of solid materials or 2-3 ml liquid materials was added aseptically to 50 ml of screening medium (table V) in 250 ml flask. After incubating on rotary shaker at 30°C for 7 days, the cultures showing good growth were transferred several times to the same medium. Selective isolation was carried out by plating out on a solid medium which was prepared by adding 2 % agar powder to the above medium. The colonies formed within 24 hours at 30°C on the petri-plates, were picked up and then sub-cultured on the slants of the above medium. The growth from the slants was transferred again to petri-plates, by streaking or quantitative dilution method for purification of the cultures. Further purification of single colony can be confirmed by making microscopic slides examinations. All yeast cultures were maintained on MYPG-agar (malt extract 0.3 %, yeast extract 0.3%,

peptone 0.5 %, glucose 1.0 % and agar 2.0 %).

3. Identification of Yeasts

Taxonomical studies of pure cultures obtained by the above procedures were based on the cultural characteristics as well as microscopic examinations. The results obtained were referred to "The Yeast, a Taxonomical Study" (Lodder and Kreger-van Rij, 1952).

Morphological Properties

a) Characteristic of the vegetative reproduction: The cells were cultivated for 3 days at 28°C on MYPG-agar.

b) Shape and size of the cells: The yeast on a young MYPG-agar culture was inoculated into 50 ml MYPG-medium in conical flasks (250 ml). After 3 days incubation at 28°C, microscopic observation was performed by making permanent slides and microphotographs were taken out. Cell size was measured under a microscope using an ocular micrometer as described by Yamagata and Fujita (1971).

c) Ascospore formation: Gorodkowa agar, potato plugs, acetate agar and Anderson-Martin (1975) agar were used as media for sporulation. The cultures were incubated at 28°C and observed after 7, 14 and 28 days by the Schaeffer-Fulton (1933) modification of the Wirtz (1908) method.

d) Pseudomycelium formation: Pseudomycelium formation was observed on Potato-agar medium as described in "The Yeast" (Lodder and Kreger-van Rij, 1952) and microphotographs were taken out after 5 days incubation at 25°C.

e) Ballistospore formation: Ballistospore formation was

examined by the method of Carmo-sousa and Phaff (1962).

f) Macromorphological characteristic of the cultures: The streak culture and the colonies of plate culture on MYPG-agar were observed.

Physiological Properties

a) Pellicle formation: Pellicle formation was observed in MYPG-broth after 3 days at 28°C and finally after one month at room temperature.

b) Sugar fermentation: The fermentation property of yeast was examined using a Durham tube containing 0.45 % yeast extract, 0.75 % peptone and 2 % of the sugar to be tested (Van der Walt, 1971).

c) Sugar assimilation: Sugar assimilation was tested by the auxenographic method of Beijerinck and the results were also compared with liquid culture method as both described in "The Yeast" (Lodder and Kreger-van Rij, 1952)

d) Assimilation of nitrate: The same methods as sugar assimilation test were employed except that liquid medium contained 1 % glucose and 0.078 % KNO_3 or 0.1 % $(NH_4)_2SO_4$ as carbon and nitrogen source instead.

e) Utilization of ethanol as sole source of carbon: Growth in a synthetic medium with 3 % ethanol as sole source of carbon described in monograph (Lodder and Kreger-van Rij, 1952) was examined.

f) Splitting of arbutin: The tests were carried out according to "The Yeast" (Lodder and Kreger-van Rij, 1952).

g) Production of acids: Acid formation was detected by the method of Lodder and Kreger-van Rij (1952) and by clear zone estimation around a growing colony on an agar plate (20 ml) in a petri dish (90 mm), consisting of 5 % glucose, 0.3 % yeast extract, 0.5 % $CaCO_3$ and 2 % agar. The calcium carbonate was added after being sterilized separately.

h) Urease test: Urease activity was tested by employing a solid medium including urea, peptone and phenol red as indicator. The original pH is 6.8 and when urea is split, the colour turns deep red (Christensen, 1946).

i) Production of Starch-like compounds: The test was examined with a medium and a method described in "The Yeast" (Lodder and Kreger-van Rij, 1952).

j) Fat splitting: The tests were carried out according to Eijkman's method described in "The Yeast" (Lodder and Kreger-van Rij, 1952).

4. Estimation of n-alkanes

Urea-adduct method of Veselov (1960) has been used for this purpose. n-alkanes has the tendency to form complex with anhydrous, well ground, urea in the presence of methanol and ether. Difference in weight of urea and complex will give us the amount of alkanes.

5. Estimation of sugars

a) Molasses and Maize 'Gur'

Benedict's solution method has been used for the

estimation of reducing as well total sugars. This is a modification of Fehling solutions and consists of single test solution which does not deteriorate appreciably on standing. In the presence of reducing sugars, the copper of the solution is reduced to cuprous oxide (Benedict, 1911).

For the estimation of total sugars, the sample solution is boiled for ten minutes with 0.5 % conc. hydrochloric acid.

b) Sulphite Waste Liquor

To determine the sugar content in sulphite waste liquor, Fujita and Iwatake (1931) method has been used.

Reagents

A = $K_3Fe(CN)_6$ + K_2HPO_4 + K_3PO_4 + Dist.H_2O

6.6 gm + 70 gm + 21 gm + 1000 ml

B = $ZnSO_4.7H_2O$ + H_2SO_4 + Dist.H_2O

50 gm + 70 ml + 500 ml

C = 15 % KI (w/v) + 0.5 % Na_2CO_3 + Dist.H_2O(250 ml)

D = N/100 N $a_2S_2O_3.5H_2O$

E = Starch solution (Indicator)

Method

Take sample in test tube containing sugar not more than 2.5 mg/ml. Add water to make 5 ml (total volume). Then add 5 ml solution A and heat in boiling H_2O for 15 minutes. Cool under tap water. Add 2 ml solution B + 1 ml solution C. Titrate with solution D till light

yellow colour appears. Add one drop of indicator E and again titrate till colour disappears. Note the volume of $Na_2S_2O_3$ used. From blank, detect volume of $Na_2S_2O_3$ used for the sample.

Reactions

1. $K_3Fe(CN)_6 + KOH \xrightarrow{Glucose} K_4Fe(CN)_6 + 2H_2O + O_2$

2. $KI + H_2SO_4 \longrightarrow K_2SO_4 + 2HI$

3. $2HI \longrightarrow 2H^+ + 2I^-$

4. $I_2 + Starch \longrightarrow Blue$

5. $2Na_2S_2O_3 + I_2 \longrightarrow Na_2S_4O_6 + 2NaI$

   Blue colour disappears.

6. Estimation of Ash Content

Ash content of dried materials was determined by keeping known amount of the sample at 500°C in a muffled furnace for 5-6 hours. (A.O.A.C. 1970).

7. Estimation of Biomass

A gravimetric method has been used. 10 ml of the culture broth is taken in a weighed sintered crucible. Water suction pump is used to remove the water. The cell mass is washed twice with distilled water and kept in the oven at 105°C for overnight. Crucible is then kept in a desiccator, allowed to cool and then weighed. The difference in weight will give us biomass of the sample.

8. Estimation of Nitrogen

The nitrogen present in the sample was estimated by a Micro Kjeldahl Method using SeO_2:$CuSO_4$:K_2SO_4 (0.02:1:9) mixture (A.O.A.C. 1970). A factor of 6.25 was used for conversion of nitrogen into protein content.

9. Estimation of Lipid

For the extraction of fatty acids from cell mass method of Bligh and Dyer (1959) was employed. To a suspension of yeast cream (known amount of cells) in distilled water, concentrated HCl is added so that the solution becomes 1N acidic. This is then refluxed for two hours on low heat using water condenser. When cooled $CHCl_3$ - CH_3OH extraction is used.

Esterified fatty acids form colour complex with hydroxylamine and $FeCl_3$ in an alkaline solution. Colour developed is compared with a standard curve prepared by using triolein in ethanol : ether (3:1).

10. Estimation of oxygen Availability

Oxygen transfer under the experiments was measured by using sulphite oxidation method of Cooper et al (1944).

11. Estimation of Total Amino Acid

i) Preparation of samples

For the estimation of total amino acid content of yeasts except tryptophan, 2.0 gm of dried and defatted samples were separately hydrolysed in 2N HCl by autoclaving

at 115°C for 5 hrs. For tryptophan, the samples were digested with 6 N NaOH instead of HCl. The hydrolysates were then neutralized and diluted to 100 ml with distilled water. Finally 10 ml of this stock solution was further diluted to 100 ml with distilled water.

ii) Microbioassay of Amino Acids

Total amino acids, leucine, isoleucine, valine, methionine, arginine, histidine and tryptophan were determined using the test organism Streptococcus zymogenes NCDO 592 (Ford, 1962). The turbidity was measured using colorimeter Corning-EEL Model 197 Spectra at 580 nm.

12. Estimation of Nucleic Acids

i) Ribose Nucleic Acid

Samples of extract (10-150 µg of RNA) were made up to 1.5 ml with tap water and were mixed with 1.5 ml of orcinol reagent (Schneider, 1957). The tubes were heated for 20 minutes in boiling water bath and cooled, then the absorbancy at 660 nm was related to RNA content by a standard curve prepared with yeast RNA hydrolysed with 1N $HClO_4$.

ii) Deoxyribose Nucleic Acid

One ml of nucleic acid extract was mixed with 2 ml of Diphenylamine reagent (Schneider, 1957) and heated for 10 minutes in boiling water bath and cooled. Then the

intensity of blue colour at 600 nm was related to DNA by a standard curve prepared with yeast DNA hydrolysed with 1N $HClO_4$.

13. Medium and Culture Conditions

The medium used for the cultivation of yeasts has the following compositions for different substrates such as Kerosene oil and diesel oil of petroleum fractions, sulphite waste liquor, maize 'gur' and molasses as shown in table V (a).

Inoculum

Three days old culture of yeast was transferred from the slant to inoculate 50 ml of sterilized cultivation medium in 250 ml Erlenmeyer flask and incubated on rotary shaker 120 rpm at $30^{o}C$ for two days. Two ml of this suspension was used to inoculate 50 ml of fermentation medium.

Propagation

Shake flask cultivation of yeast was carried out in one litre Erlenmeyer flask containing 300 ml cultivation medium and kept on rotary shaker at $30^{o}C$. Samples were taken at an interval of 24 hrs. and analysed for biomass formed, N_2 consumed and change in pH.

For nitrogen source selection, flasks were divided into three sets. In one set $(NH_4)_2SO_4$ (6.93 g/L), in the other NH_4Cl (5.0 g/L) and in the third set NH_4NO_3 (8.40 g/L) was added.

Keeping in view the importance of availability of oxygen in aerobic fermentation, two set of experiments were planned where supply of oxygen varied with the cultivation conditions.

a) Same flasks (capacity 1 l) with varying amount of salt medium i.e., 50, 150, 250 and 350 ml.

b) Same amount of mineral salts i.e 100 ml in flasks of different capacities i.e. 250, 500 and 1000 ml. Batch cultivation of yeast was done in a 4 l and 60 l working volume fermentors. The contents of the fermentor were well agitated by a stirror and an air supply of 1l/l/min.

Table - I

ANALYSIS OF PETROLEUM FRACTIONS USED AS SOURCE OF ENERGY

Name of Products	n-paraffins (%)
Diesel oil	23.30
Kerosene oil	13.46

Table - II

COMPOSITION OF SWL* (Kahi Grass-Saccharum spontaneum) Packages Ltd.

Sugar	3.0 %
Dry wt.	17.0 %
Ash	30.0 %
Nitrogen	0.34 %

* Sulphite Waste Liquor

Table - III

COMPOSITION OF MAIZE "GUR"

Sugar	45.0 %
Moisture	15.38 %
Ash	3.25 %
Nitrogen	0.16 %
Phosphorus	0.25 %

Table - IV

ANALYSIS OF MOLASSES

Type	Total sugar (%)	Reducing sugar (%)	Dry weight (%)	Ash (%)
Cane molasses (Crescent Faisalabad)	58.5	32.4	75.86	11.8
Mixed (Mardan)	55.3	13.9	85.07	15.8
Cane Molasses (Mardan)	52.4	11.1	78.22	10.3
Beet Molasses (Mardan)	61.8	35.4	22.53	10.8

Table - V

COMPOSITION OF ISOLATION MEDIUM

Substrate* (Carbon source)	Variable
KH_2PO_4	0.4 %
K_2HPO_4	0.4 %
$(NH_4)_2SO_4$	0.3 %
$MgSO_4 \cdot 7H_2O$	0.1 %
Tap H_2O	(To make up the volume)
pH	5.0-5.5

* Molasses 5 %
  Diesel oil 10 %
  SWL as such

Table-V(a)

COMPOSITION OF CULTIVATION MEDIA

Component (%)	Kind of substrate				
	Diesel oil@ or kerosene oil (10 %)	S.W.L.* I**	S.W.L.* II***	Maize* 'gur'	Molasses (4%)
K_2HPO_4	0.7	0.4	-	-	-
KH_2PO_4	-	0.4	-	-	-
Na_2HPO_4	0.7	-	-	-	0.6
$(NH_4)_2HPO_4$	-	-	0.3	0.6	-
$(NH_4)_2SO_4$	-	0.3	-	-	0.3
NH_4Cl	0.4	-	-	-	-
$MgSO_4.7H_2O$	0.2	0.1	0.1	0.1	0.1
NaCl	0.1	-	0.01	-	0.05
KCl	-	-	-	0.01	-
Tap H_2O	(To make up the volume)				
pH	(4.5 - 5.5)				
Temperature	(30 ± 2°C)				

* Concentration varied.
** Medium - I
*** Medium - II
@ Hydrocarbon was added after sterilization.

RESULTS AND DISCUSSION

1) Isolation and Screening of Yeasts:-

Yeasts strongly assimilating industrial wastes as the sole source of carbon were isolated from a wide variety of natural sources during investigation on yeast production by conventional streak method.

Newly isolated 16 yeast strains along with 7 strains from culture collections were tested for their affinty towards industrial wastes such as kerosene oil and diesel oil of petroleum fractions and sulfite waste liquor as sole source of carbon. For the qualitative study of growth of yeasts on hydrocarbons, the medium (Table V) was used. These observations have been recorded in Tables VI and VII. It is clear from the Tables that strains B_3, N-y, CBS, H-D_5, D_1, D_2, CLP, D_4 and C have been found to be better utilizers of hydrocarbons.

Yeasts B_3 and CLP were grown on kerosene oil and diesel oil in an inorganic salt medium (Table V). Biomass yields from kerosene oil and diesel oil in shake flask experiments under uncontrolled conditions of temperature and pH were 68% and 77% respectively. The yield may be improved by culturing the yeast strains under control conditions of suitable environments.

Yeasts were also tested for their affinity towards sulfite waste liquor as sole source of carbon using the cultivation medium (V (a)). Yeasts were sub-cultured on slants and incubated at $30^{\circ}C$

Table - VI

AFFINITY OF YEASTS FOR KEROSENE OIL SUBSTRATE

Strain No.	Hours						
	24	48	72	96	120	168	192
S-4	-	-	+	+	+	++	++
S-5	-	-	+	+	+	++	++
S-6	-	-	+	+	++	+++	+++
S-7	-	-	+	+	+	+++	++++
S-9	-	-	+	+	+	+++	++++
S-10	-	-	+	+	++	+++	++++
S-11	-	-	+	+	++	+++	++++
S-21	-	-	+	+	++	++	++
S-31	-	-	+	+	+	++	++
B_3	+	+	+	+	++	+++	++++
N-y	+	+	++	++	+++	+++	++++
$H\text{-}D_5$	+	++	++	+++	+++	++++	++++
CBS (known)	+	+	++	+++	+++	++++	++++
SC "	-	-	+	+	+	+	+
IY "	-	-	+	+	+	+	+
NBC "	-	-	+	+	+	+	+
R_4 "	+	++	++	++	++	+++	++++
CK_4 "	+	+	++	++	++	++	++
D_1	+	++	+++	+++	++++	++++	++++
D_2	+	++	+++	+++	++++	++++	++++
CLP	+	++	+++	+++	++++	++++	++++
D_4	+	++	+++	+++	++++	++++	++++
C	+	++	+++	+++	++++	++++	++++

Table - VII

AFFINITY OF YEASTS FOR DIESEL OIL SUBSTRATE

Strain No.	Hours						
	24	48	72	96	120	168	192
S-4	-	-	+	+	+	++	++
S-5	-	-	+	+	+	++	++
S-6	-	-	+	+	+	++	++
S-7	-	-	+	++	++	+++	++++
S-9	-	-	+	++	++	+++	++++
S-10	-	-	+	++	++	+++	++++
S-11	-	-	+	++	++	+++	++++
S-21	-	-	+	+	+	++	++
S-31	-	-	+	+	+	++	++
B_3	+	+	+++	+++	++++	++++	++++
N-y	+	+	++	++	+++	++++	++++
$H\text{-}D_5$	+	+	++	+++	++++	++++	++++
CBS	+	+	++	++	+++	++++	++++
SC	-	-	+	+	+	+	+
IY	-	-	+	+	+	+	+
NSC	-	-	+	+	+	+	+
R_4	-	+	+	++	++	++++	++++
CK_4	-	-	-	+	+	++	++
D_1	+	+	++	++	+++	++++	++++
D_2	+	+	++	++	+++	++++	++++
CLP	+	++	+++	+++	++++	++++	++++
D_4	+	+	++	++	+++	++++	++++
C	+	+	++	++	+++	++++	++++

for 5 days inorder to see the influence of pre-treatments of SWL on the growth.

It is evident that majority of the yeast strains studied gave better growth when the concentration of SWL in the medium was 30% (Table VIII). Only Candida rugosa (A) showed better growth on 50 to 70% concentration of SWL. These studies revealed that strains A, B_3, $H\text{-}D_5$ and CBS had greater affinty for SWL and thus were selected for further studies. The poor growth of almost all the strains in higher concentrations (70 to 100%) of SWL seems to be due to toxic effect of SO_2 and other compounds such as lignosulphates in the SWL. This fact is further supported by the observations that heating of SWL for 60 to 90 minutes showed a positive effect on the growth of yeasts (Table IX). All the strains except I-Y, gave better growth when the medium was supplemented with SWL after heating for 60 to 90 minutes and after passing dry air. SWL used as such (no treatment) did not support growth. These observations are in accordance with those reported by Simard and Cameron (1974) who held that decrease in SO_2 contents of medium by dilution (1 : 2) had a positive effect on yeast growth.

Growth of yeasts were also tested at various temperatures with a temperature gradient incubator. It was found that the yeast grew over the range of 28 to 45^{o}C as shown in Table X. The optimum temperature range for majority of strains was 28 to 37^{o}C but only few cultures R_4, SC, I-Y and NSC were unable to grow at 37^{o}C. Some of the yeast strains i.e. S-5, S-6, S-9, S-11, S-31,

Table - VIII

EFFECT OF DIFFERENT CONCENTRATIONS OF S.W.L. ON GROWTH OF YEASTS

Strain No.	Percentage of S.W.L. in medium				
	20 %	30 %	50 %	70 %	100 %
CBS	++	+++	++	+	+
HD_5	++	+++	++	+	+
IV	-	-	-	-	-
Ch_4	+	+	+	+	+
B_3	++	+++	+	+	+
R_4	+	++	+	-	+
A	+	++	+++	+++	++
SC	-	-	-	-	-
S-5	++	+++	++	+	+
S-10	+	++	+	+	+

Table - IX

EFFECT OF DIFFERENT HEATING TIMES OF S.W.L. ON GROWTH OF YEASTS*

Strain No.	Untreated	Minutes				
		15	30	45	60	90
CBS	-	+	++	++	+++	+++
H-D_5	++	++	++	+++	+++	+++
B_3	+	++	++	++	+++	+++
NSC	-	-	+	+	+	+
IY	-	-	-	-	-	-
CK_4	+	+	+	+	+	+
A_1	+	++	++	+++	+++	+++
S-4	-	-	+	+	+	+
R_4	-	-	+	+	++	+
S-5	+	+	++	++	+	+
A	+	++	+++	+++	++++	++

* The medium was boiled for different time intervals by adding conc. H_2SO_4 (0.5 %).

S-21	+	+	-
S-31	+	+	+
N-y	+	+	-
$H\text{-}D_5$	+	+	-
C	+	+	+
B_3	+	+	+
A	+	+	-
B	+	+	-
CBS(Known)	+	+	+
CLP "	+	+	+
R_4 "	+	-	-
CK_4 "	+	+	-
SC "	+	-	-
IY "	+	-	-
NSC "	+	-	-

C, B_3, CBS and CLP were found to be able to grow at 45°C also.

2) Identification of yeasts

Taxinomical studies were based on morphological and physiological characteristics (Tables XI, XII and XIII). These studies showed that six strains were ascospore former and belonged to the family Endomycetaceae. These included one strain of Saccharomyces cerevisiae (Fig. I). and five of Pichia membranefaciens (Figs. IIA and IIB).

The remaining ten strains did not form ascospores, ballistospores or arthrospores. These are the characteristics of asporogenous yeast i.e., family cryptococcaceae. Nine strains had no ogive shaped and no arthrospores but produced well-developed true and pseudomycelia, hence closely resembled with the genus Candida. These were identified as two strains of Candida tropicalis (Figs. III A and III B), four of C. guilliermondii (Figs. IV A and IV B), two of C. rugosa (Figs. V A and V B) and one of C.parapsilosis (Figs. VI A and VI B).

The presence of carotenoid pigments and negative fermentation in the last strain S-4 showed that it belonged to sub-family Rhodotoruloideae and identified as Rhodotorula mucilaginosa (Fig. VII).

3) Hydrocarbon Fermentation:-

Cultivation of Candida parapsilosis ($H\text{-}D_5$) was carried out in shake flasks (1l) containing 300 ml of cultivation medium (V a) containing kerosene oil and diesel oil as the sole source of carbon.

Photomicrographs of cells and pseudomycelia of different strains of yeast

Fig. I Microphotograph of strain S-1

Cells in MYPG after 3 days at 25°C.

Fig.II Microphotograph of strain S-31

A. Cells in MYPG after 3 days at 25°C

B. Pseudomycelia in a slide culture on potato-agar medium at 25°C.

Fig.III Microphotograph of strain C

A Cells in MYPG after 3 days at 25°C

B Pseudomycelia in a slide culture on potato-agar medium at 25°C.

Fig.IV Microphotograph of strain N-Y

A Cells in MYPG after 3 days at 25°C.

B Pseudomycelia in a slide culture on potato-agar medium at 25°C.

Fig.V Microphotograph of strain A

A Cells in MYPG after 3 days at 25°C.

B Pseudomycelia in a slide culture on potato-agar medium at 25°C.

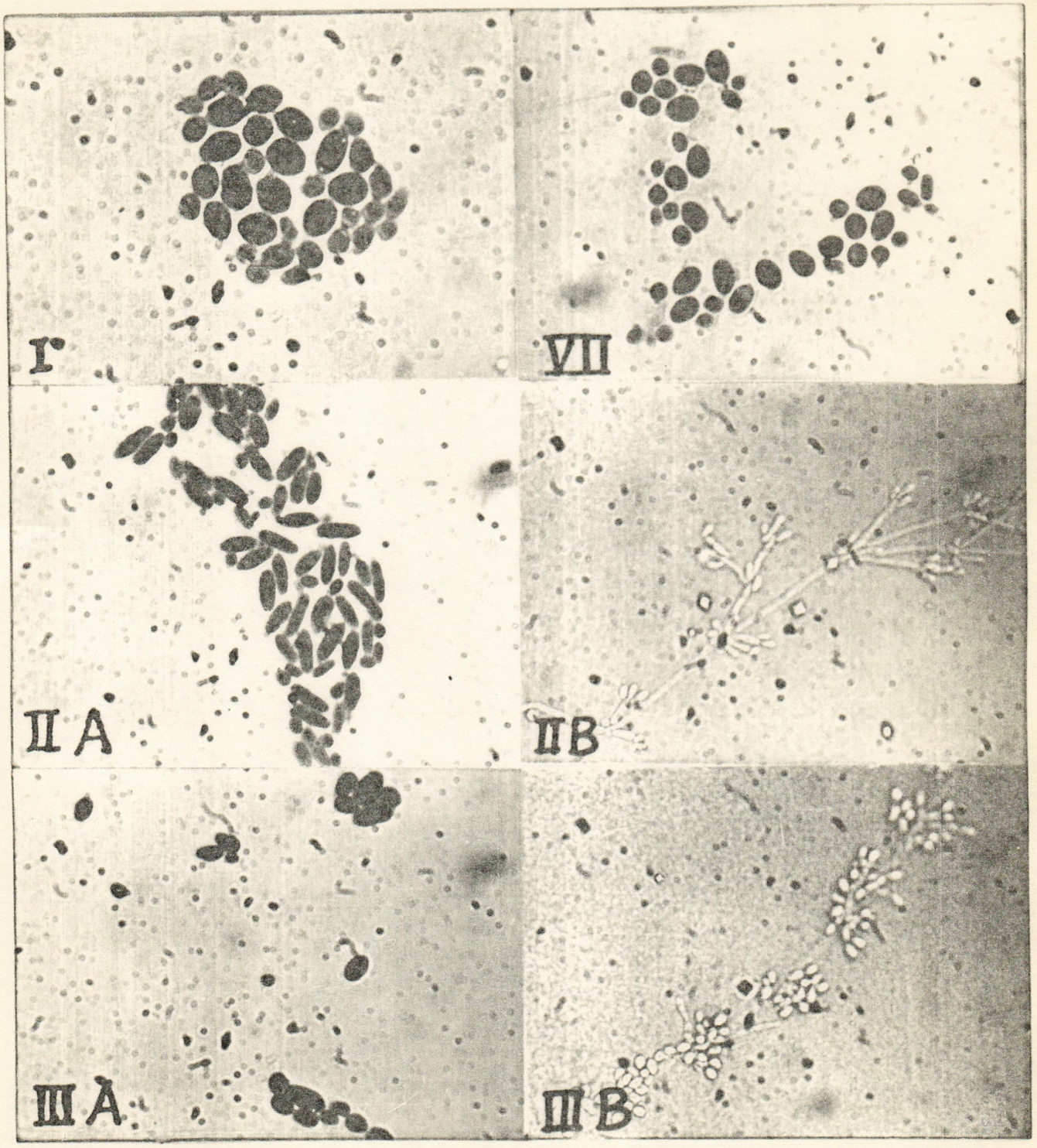
Fig.VI Microphotograph of strain $H\text{-}D_5$

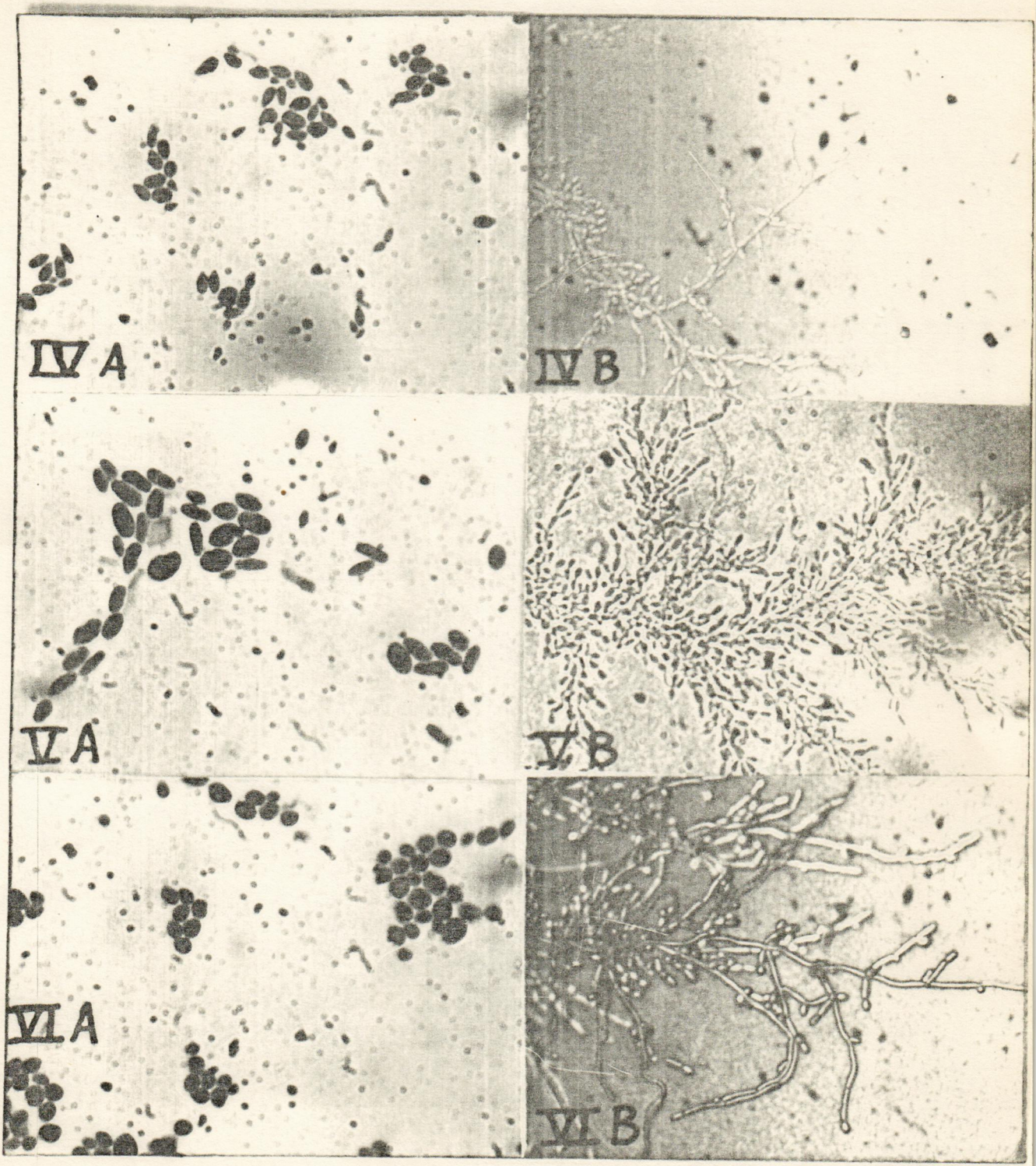
A Cells in MYPG after 3 days at 25°C.

B Pseudomycelia in a slid culture on potato-agar medium at 25°C.

Fig.VII Microphotograph of strain S-4

Cells in MYPG after 3 days at 25°C.


I
VII
II A
II B
III A
III B


IV A
IV B
V A
V B
VI A
VI B

		short oval	
S-5,S-6, S-9,S-11, S-31	Dull white	Short oval to elongate	(0.9-2.7)x(2.7-7
C,B_3	Dull white	Round to short oval to elongate	(1.8-4.5)x(2.7-8.
N-Y,S-7, S-10,S-21	Dull White	Round to short oval	(0.67-2.7)x(0.9-5
A,B	Cream	Short oval to elongate	(1.8-4.5)x(3.6-8.
H-D_5	Dull off white	Round to short oval	(1.35-2.7)x(1.8-4
S-4	Red	Round to short oval	(0.9-2.7)x(1.8-4.

N: Wrinkled S; Smooth

Table XII

PHYSIOLOGICAL CHARACTERISTICS OF ISOLATED YEASTS

Isolates	Fermentation of Sugars						Assimilation of sugars					Assimilation of Nitrate	Assimilation of Ethanol	Splitting of Arbutin	Acid formation	Urease test	Production of starch like compounds	Production of carotenoid pigments	Fat Splitting	Identified as
	G	Ga	S	M	L	R	G	Ga	S	M	L									
S-1	+	+	+	+	-	+W	+	+	+	+	-	-	+VW	-	-	-	-	-	-	S.cerevisae
S-5,S-6, S-9,S-11 S-31	+W	-	-	-	-	-	+	-	-	-	-	-	+	-	-	-	-	-	-	F.membranaefaciens.
C,B_3	+	+	+	+	-	-	+	+	+	+	-	-	+W	+W	+W	-	-	-	-	C.tropicalis
N-Y,S-7, S-10, S-21	+VW	-	+VW	-	-	+VW	+	+	+	+	-	-	+W	+	+	-	-	-	-	C.guilliermondii
A,B	-	-	-	-	-	-	+	+	-	-	-	-	+W	-	-	-	-	-	-	C.rugosa
H-D_5	+	+W	+VW	-	-	-	+	+	+	+	-	-	+W	+W	+	-	-	-	-	C.parapsilosis
S-4	-	-	-	-	-	-	+	+	+	+	-	-	+W	+	-	+	-	+	-	R.mucilaginosa

G: Glucose Ga: Galactose S: Sucrose M: Maltose L: Lactose R: Raffinose W: Weak VW: Very Weak

Table XIII

CARBOHYDRATES AND CARBOHYDRATE DERIVATIVES ASSIMILATION SPECTRA OF ISOLATED YEASTS

Isolates	Arabinose	Callobiose	Citric Acid	Dextrin	Dulcitol	Fructose	Glycerol	Inulin	Inositol	Lactic Acid	Mannitol	α-Naphthol	β-Naphthol	Oxalic Acid	Ribose	Raffinose	Rhamnose	Sorbose	Sorbitol	Succinic Acid	Xylose
S-1	-	-	-	-	-	+	+VW	-	-	-	-	+	+	+L	-	-	-	-	-	-	-
S-5,S-6, S-9,S-11, S-31	-	-	+	-	-	+	+	-	-	+	-	+	+	+L	-	-	-	-	-	+	-
C,B_3	-	-	+	-	-	-	+	-	-	-	+	+	+	+L	-	-	-	+	+	+	+
N-Y, S-7, S-10,S-21	-	+	+	-	-	+	+	-	-	-	+	+	+	+L	-	+	-	-	+	+	+
A,B	-	-	-	-	-	+	+W	-	-	-	+	+	+	+L	-	-	-	-	+	+	-
$H-D_5$	-	-	+	-	-	-	+	-	-	-	+	+	+	+L	-	-	-	+	+	+	+
S-4	-	-	+	-	-	+	+	-	-	-	+	+	+	+L	-	-	-	-	+	+	+

W: Weak VW: Very Weak L: Latent

Results are given in Tables XIV and XV with kerosene oil and diesel oil respectively. As is obvious from the Tables, the growth on diesel oil is much faster than that of kerosene oil. Poor growth on kerosene oil may be due to the presence of toxic materials, branched - chain and aromatic hydrocarbons in the commercially available kerosene oil. These observations are in accordance with the findings of Shoda and Bas (1966). Tanabe et. al. (1966) also observed the utilization of kerosene oil by yeast but with poor cell mass formation.

The influence of different nitrogen sources such as $(NH_4)_2SO_4$, NH_4Cl and NH_4NO_3 on the growth of Candida lipolytica using diesel oil as the sole source of carbon as in the previous experiment during batch cultivation was shown in Table XVI. Maximum cell concentration of 8.10 g/l was observed in case of $(NH_4)_2SO_4$ when compared to 7.2 and 6.4 g/l with NH_4Cl and NH_4NO_3 respectively.

Results of the effect of oxygen availability on the growth of Candida parapsilosis ($H\text{-}D_5$) using diesel oil as the sole source of carbon were depicted in Figs. VIII, IX and X. Oxygen availability in the range of 3 to 25 m mol O_2 per l/min was obtained by changing the volume of the medium constant or by changing the volume of the medium using the same flasks. These results show a remarkable increase in the biomass synthesis with a corresponding increase in the availability of oxygen to the culture broth and thus resulted in better utilization of the substrate.

0	0.02
24	0.32
48	0.53
72	1.26
96	2.12
120	2.88
144	3.40
168	3.70
192	3.70

* Shake flask

0	0.02
24	0.35
48	0.70
72	1.65
96	3.50
120	5.70
144	6.70
168	7.28
192	7.30

* Shake flask

Table XVI

EFFECT OF DIFFERENT NITROGEN SOURCES ON THE GROWTH OF CANDIDA LIPOLYTICA (SUBSTRATE = DIESEL OIL).

Hours of cultivation	$(NH_4)_2SO_4$			NH_4Cl			NH_4NO_3		
	Biomass g/l	N_2 consumed g/l	pH	Biomass g/l	N_2 consumed g/l	pH	Biomass g/l	N_2 consumed g/l	pH
0	0.02	-	4.5	0.02	-	4.5	0.02	-	4.5
24	0.40	0.02	4.2	0.30	0.04	4.3	0.40	0.04	4.2
48	1.10	0.11	3.8	0.90	0.12	4.0	0.70	0.09	4.0
72	2.70	0.24	3.4	2.45	0.21	3.5	2.05	0.2[illegible]	3.4
96	4.90	0.35	3.0	3.80	0.28	3.[illegible]	3.36	0.29	3.2
120	6.75	0.[illegible]0	2.8	5.70	0.39	2.9	4.60	0.37	3.0
144	7.70	0.60	2.6	6.75	0.52	2.8	5.80	0.45	2.9
168	8.08	0.66	2.5	7.08	0.58	2.7	6.37	0.50	2.8
192	8.10	0.66	2.5	7.20	0.58	2.7	6.40	0.57	2.8

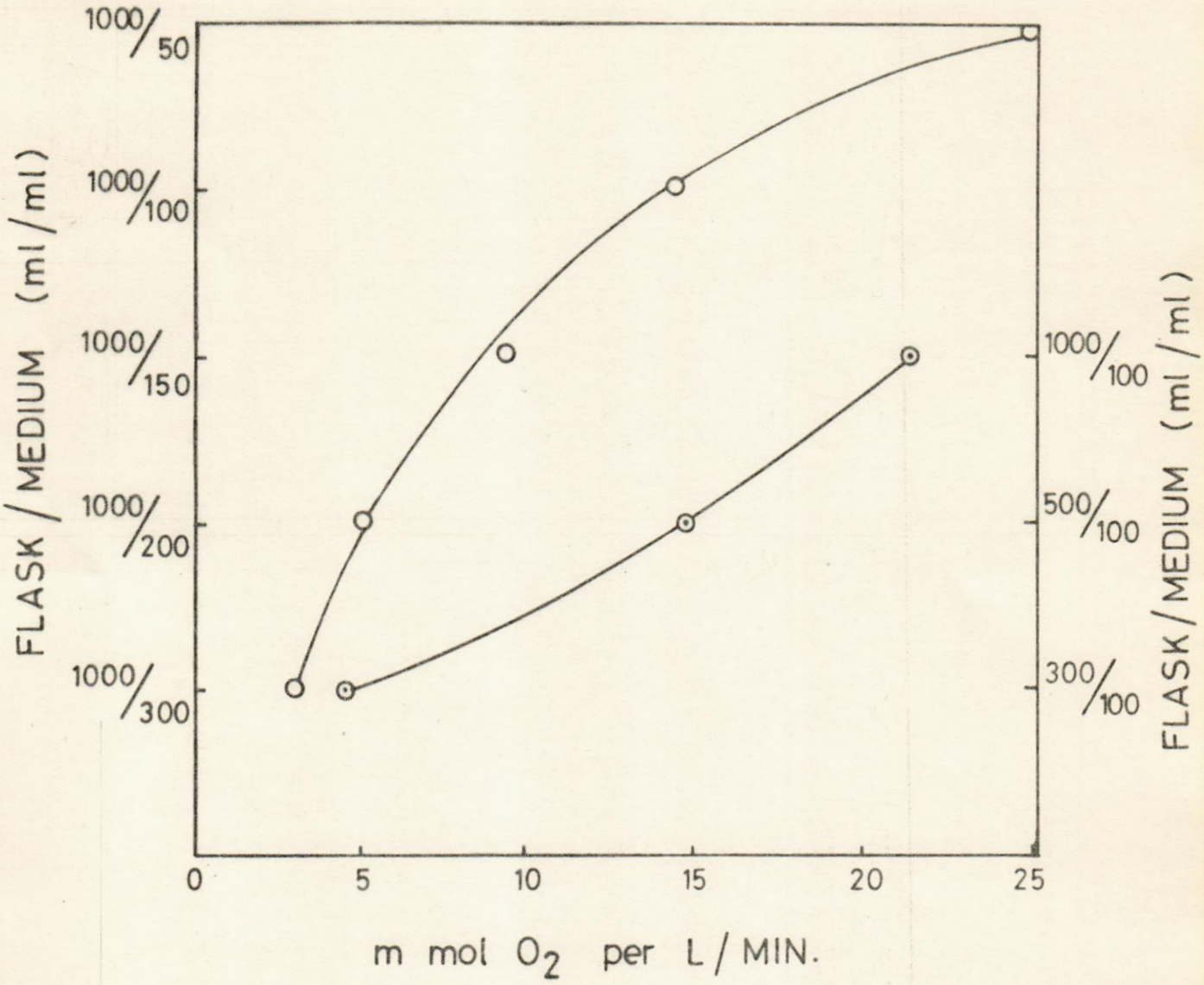


FIG. VIII RATE OF DISSOLVED OXYGEN IN THE CULTIVATION SYSTEM.

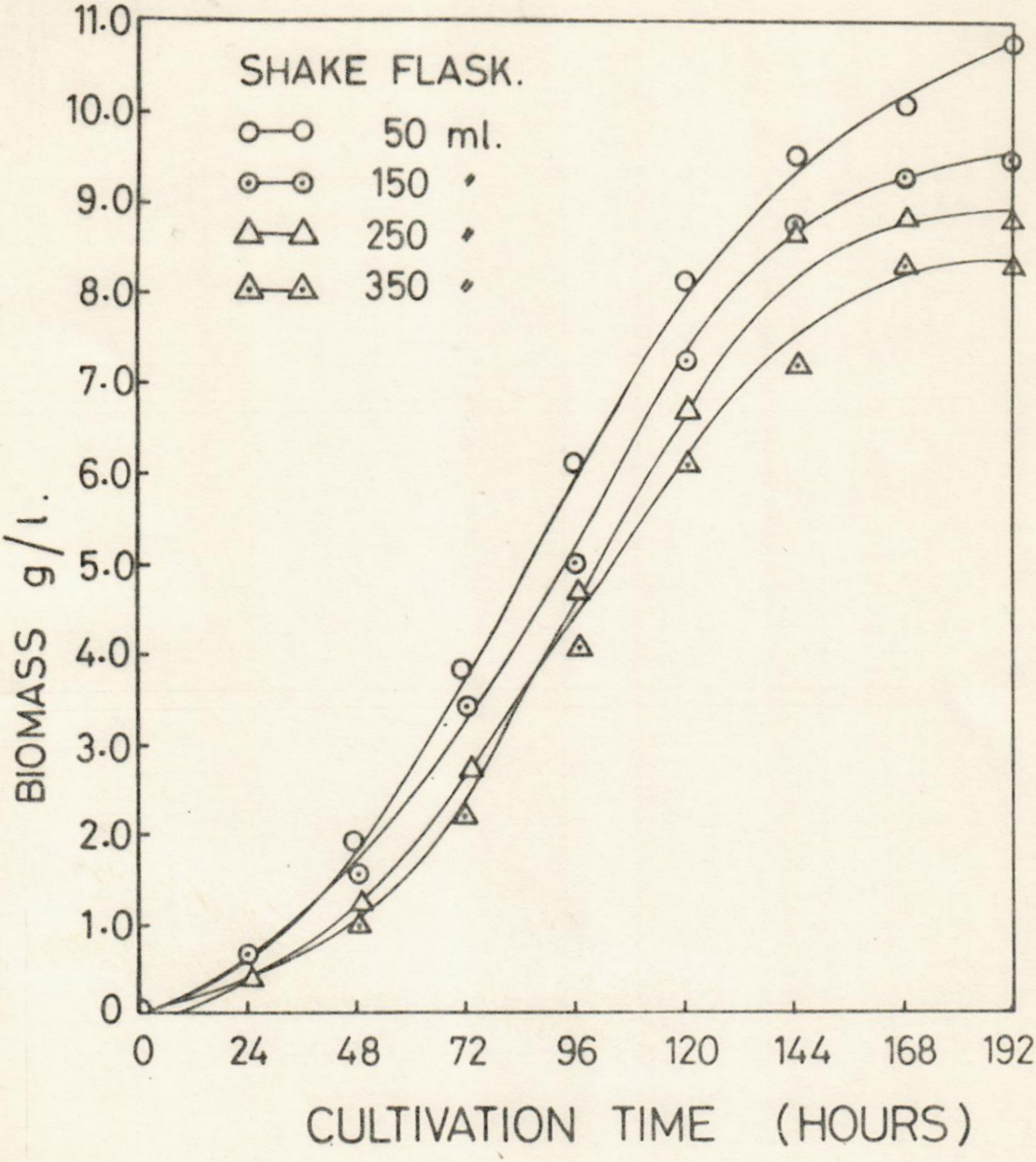


FIG. IX EFFECT OF OXYGEN AVAILABILITY ON THE GROWTH OF CANDIDA parapsilosis ON DIESEL OIL.

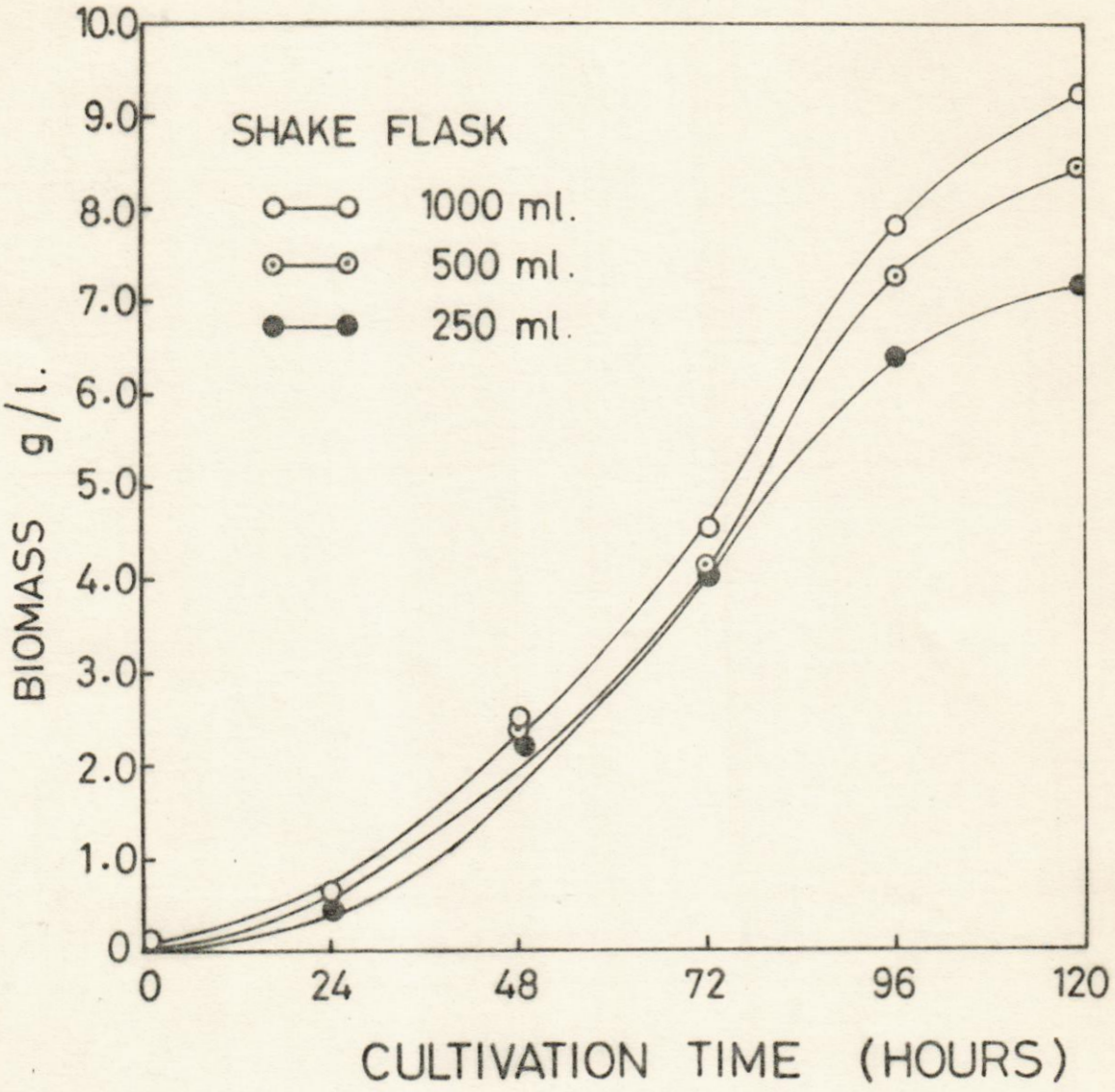


FIG. X. EFFECT OF OXYGEN AVAILABILITY ON THE GROWTH OF CANDIDA parapsilosis ON DIESEL OIL.

Batch cultivation of Candida lipolytica was carried out in a 4l working volume fermentor on a mineral salts medium containing diesel oil as carbon source as shown in Table XVII. Biomass increased from 1.14 to 9.85 g/l after 96 hrs. of cultivation. Growth retarded with the depletion of nitrogen from the medium and unfavourable pH of the medium.

Analysis of the biomass obtained from two strains grown in hydrocarbon medium and their biomass was analysed for estimating the protein, lipid and RNA (Table XVII (a)). It was observed that C. lipolytica resulted in better % age of protein and lipid as compared to C. rugosa RNA was higher in case of C. rugosa when compared to C. lipolytica.

Analysis of the biomass was also carried out to estimate some essential amino acids (Table (XVII (b)). It is clear that biomass of C. rugosa contained high amount of all the essential amino acids as compared to C. lipolytica. While comparing the amino acid content of the biomass of these two cultures with FAO level, it was found that only isoleucine and valine of C. rugosa are compabble to some extent with FAO level.

Good quality protein can be obtained from paraffins contained in the waxy fractions of petroleum such as waxy lubricating oils. Up-grading of the gas oil as a result of microbiological dewaxing will give us a very useful by-product to be used in cattle and poultry feed.

Table XVII

GROWTH OF CANDIDA LIPOLYTICA ON DIESEL OIL
9 L FERMENTOR

Hours of cultivation	Biomass g/l	N_2 consumed g/l	pH
0	1.14	-	5.0
24	2.83	0.11	4.3
48	8.07	0.78	3.5
72	9.40	0.93	3.0
96	9.85	0.98	2.6

Table - XVII (a)

ANALYSIS OF BIOMASS FROM HYDROCARBON UTILIZING YEASTS

Yeast strain	Protein (%)	Lipid (%)	RNA (%)
A*	48.3	12.3	11.3
CLP**	53.1	14.5	9.8

* C. rugosa
** C. lipolytica

Table - XVII(b)

MICROBIOASSAY OF SOME ESSENTIAL AMINO ACIDS CONTENT OF HYDROCARBON UTILIZING YEASTS

Yeast strain	Argi-nine	Histi-dine	Isoleu-cine	Leu-cine	Methio-nine	Trypto-phan	Va-line
A*	3.64	1.52	3.59	5.31	1.43	0.19	4.53
CLP**	1.52	0.38	1.19	1.08	0.34	0.32	2.60

* C. rugosa
** C. lipolytica

4) Sulfite Waste Liquor Fermentation:-

The growth of Candida rugosa (A) on SWL is shown in Table XVIII. In this experiment higher amount of inoculum was used in order to overcome the inhibitory effects of lignin and SO_2 present in the SWL. When lower inoculum (1.5 g/l) was used under similar conditions, an increase in biomass of 5 g/l was observed at 164 hrs as compared to 14.2 g/l in case of higher inoculum.

The growth of different strains of yeast i.e. Candida rugosa (A), C. tropicalis (B_3) and C. parapsilosis ($H\text{-}D_5$) on SWL is shown in Table XIX. After 144 hrs. fermentation in shake flasks, cell concentration was observed as 3.4, 2.8 and 2.4 g/l respectively when the inoculum size was 0.8 g/l.

Results showing the effect of different media on biomass production by Candida rugosa (A) and C. tropicalis (B_3) in different concentration of SWL are presented in Tables XX and XXI. Maximum biomass formation in both the cases has been observed at 100% SWL after 144 hrs. in Medium II as compared to Medium I.

The effect of inoculum size on the growth of different strains of yeast i.e. Candida rugosa (A), C. tropicalis (B_3) and C. parapsilosis $H\text{-}D_5$ was studied and is reported in Table XXII. With increase in inoculum size, a corresponding increase in the accumulation of cell mass has been observed - maximum (16.0 g/l) being with Candida rugosa (A). This seems to be the result of greater number of live cells due to increase in inoculum size which resulted in the production of increased biomass.

Table - XVIII

GROWTH OF Candida rugosa on S. W. L.*

Hours of cultivation	Sugar consumed g/l	N_2 consumed g/l	Biomass g/l
0	-	-	6.0
20	6	0.10	9.0
44	12	0.15	12.0
140	14	0.18	13.3
164	16	0.21	14.2

* Boiled SWL (1½ hr) ; sugar conc. = 3 %

	v/v %	sumed g/l	g/l	v/v %	
48	1.30	5.20	0.12		1.10
96	2.50	13.30	0.24		1.80
144	3.40	18.10	0.35		2.80

* Concentration of SUL = as such and inoculum

Table - XX

EFFECT OF DIFFERENT CONCENTRATION OF SULPHITE WASTE LIQUOR ON BIOMASS PRODUCTION BY Candida rugosa (A).

SWL %	Sugar %	48 h Biomass g/l		96 h Biomass g/l		144 h Biomass g/l	
		MI*	MII*	MI	MII	MI	MII
20	0.6	1.00	1.00	1.20	1.20	1.30	1.32
30	0.9	1.15	1.18	1.45	1.50	1.60	1.65
40	1.2	1.10	1.25	1.60	1.68	1.82	1.88
50	1.5	1.15	1.29	1.69	1.76	1.96	2.00
60	1.8	1.15	1.30	1.80	1.90	2.12	2.25
70	2.1	1.20	1.40	2.06	2.18	2.30	2.42
80	2.4	1.26	1.42	2.22	2.30	2.68	2.74
90	2.7	1.29	1.48	2.40	2.45	3.00	3.10
100	3.0	1.32	1.56	2.60	2.68	3.30	3.45

MI* = Medium I ; MII** = Medium II ; Inoculum = 0.8 g/l

Table - XXI

EFFECT OF DIFFERENT CONCENTRATION OF SWL ON BIOMASS PRODUCTION BY Candida tropicalis (B_3)

SWL %	48 h Biomass g/l		96 h Biomass g/l		144 h Biomass g/l	
	MI*	MII**	MI	MII	MI	MII
20	0.90	0.90	1.00	1.05	1.20	1.30
30	1.00	1.15	1.20	1.30	1.30	1.46
40	1.15	1.30	1.30	1.50	1.45	1.60
50	1.10	1.30	1.43	1.60	1.70	1.80
60	1.10	1.30	1.50	1.65	1.80	2.00
70	1.20	1.50	1.60	1.75	2.00	2.20
80	1.30	1.60	1.65	1.90	2.20	2.30
90	1.34	1.69	1.75	2.10	2.40	2.50
100	1.40	1.80	1.80	2.30	2.60	2.80

* Medium I ; ** Medium II

Table XXII

EFFECT OF INOCULUM SIZE ON BIOMASS PRODUCTION OF DIFFERENT STRAINS OF YEAST DURING CULTIVATION ON SNL.

Yeast strain	C. rugosa (A)			C. tropicalis (B_3)			C. parapsilosis (HD_5)		
Hours of cultivation / Inoculum size g/l	48	96	144	48	96	144	48	96	144
0.8	1.30	2.50	3.40	1.10	1.90	2.80	0.95	1.60	2.40
1.6	2.30	4.00	5.20	2.00	3.70	4.20	1.90	2.40	2.80
3.2	5.80	8.80	10.50	4.50	7.00	9.20	3.60	5.80	6.50
6.4	12.00	14.00	16.00	10.10	12.05	14.20	8.50	9.80	11.80

Biomass obtained was found to range between 41.3 to 42.5 % and 6.0 to 7.0 lipids. The protein contents of biomass were comparatively lower than those reported by Pochland et. al. (1968).

Thus 70-75% of sugar contents of SWL have been successfully utilized and converted to biomass which can be used for feed purposes. Although higher concentrations of sugar utilization have been reported (Ivanyukovich et. al., 1968), however, it may be due to the difference in chemical composition of SWL. The process can be made economical further by supplementation with molasses or maize 'gur'.

5) Maize 'Gur' (Hydrol) Fermentation:-

The growth of Candida rugosa (A) and Rhodotorula glutinis (R_{44}) in stirred, aerated fermentor is shown in Table XXIII. The concentration of maize gur was 6% and the working volume was 3.5 l. From the amount of biomass formed in both the cases i.e. 9.5 and 11.5 g/l after 120 hrs., it is concluded that hydrol could be good substrate for yeast growth. In both the cases the yield is about 46 % on the basis of sugar consumed.

Effect of different concentration of maize gur in the medium is shown in Fig. XI while growing C. rugosa. With the increase in the sugar concentration in the medium, yield of biomass is decreasing. This can be due to oxygen limitation as in all the cases rate of aeration was kept constant i.e. 1l/l/min or due to some inhibitory ions from maize 'gur'whose concentration also increase in sugar content.

Table XXIII

EFFECT OF DIFFERENT STRAINS OF YEAST ON MAIZE GUR

9 L FERMENTOR

Yeast Strain	Candida rugosa (A)					Rhodotorula glutinis (R44)				
Hours of cultivation	Biomass g/l	Sugar consumed g/l	N_2 consumed g/l	pH	Yield %	Biomass g/l	sugar consumed g/l	N_2 consumed g/l	pH	Yield %
0	1.2	-	-	5.0	-	1.2	-	-	5.0	-
20	3.0	6.0	0.12	4.4	30	3.0	7.0	0.09	4.4	43
40	4.5	9.0	0.15	4.0	37	4.4	10.0	0.17	3.9	44
60	6.0	14.0	0.19	3.6	39	6.9	15.0	0.21	3.5	46
80	8.0	16.0	0.25	3.2	40	9.0	20.0	0.29	3.1	45
100	9.0	20.0	0.32	2.8	39	11.0	23.0	0.34	2.6	49
120	9.5	22.0	0.34	2.6	37	11.5	25.0	0.35	2.4	46

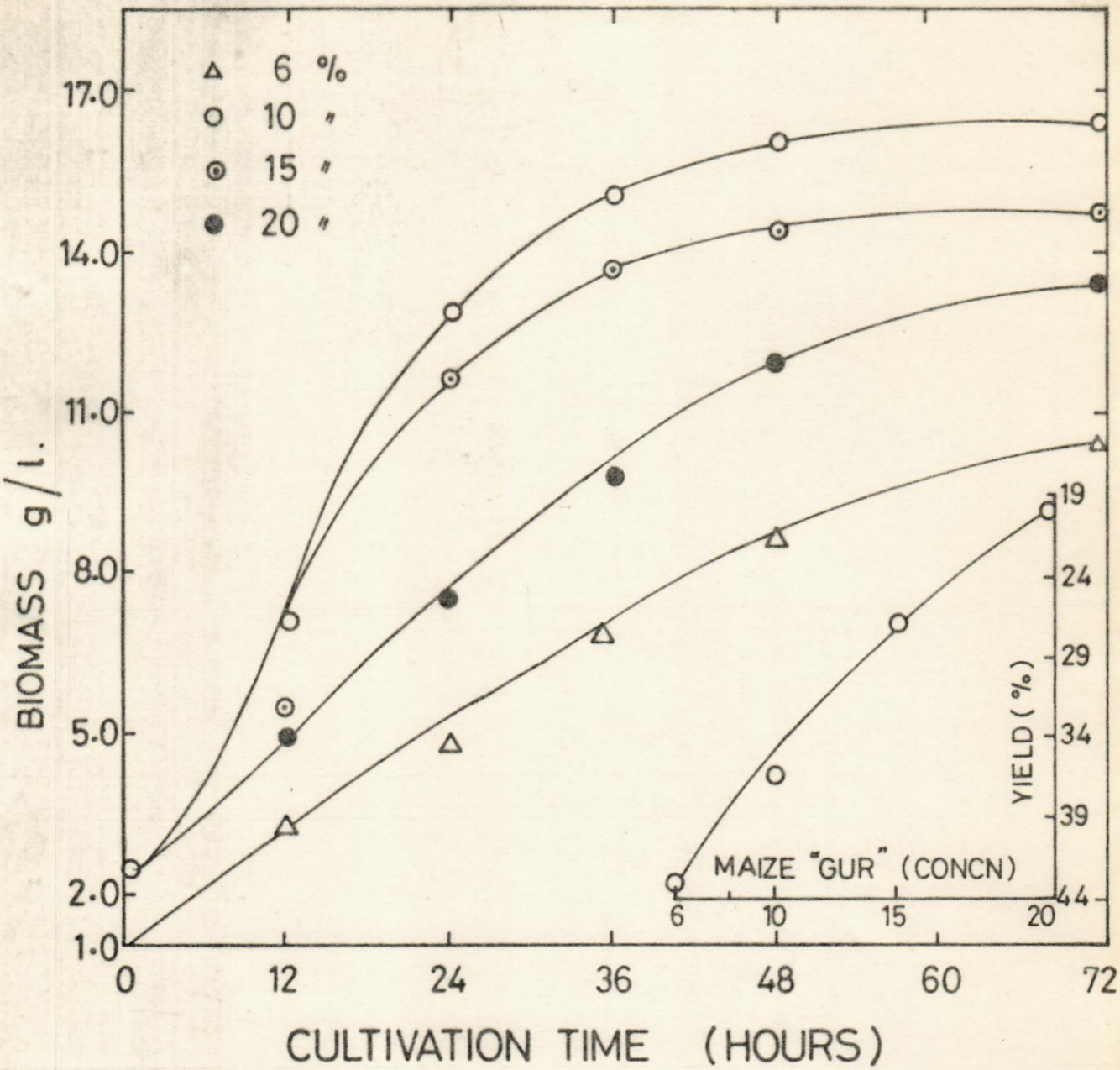


FIG. XI. EFFECT OF DIFFERENT CONCENTRATION OF MAIZE "GUR" ON THE GROWTH OF CANDIDA rugosa.

Effect of different concentration of NH_4Cl on the growth of Candida rugosa using maize 'gur' as sole source of carbon during shake flask experiment was shown in Table (XXIV). Different concentrations of NH_4Cl were tried and their effect on the culture growth was studied. Maximum biomass formation of Candida rugosa (A) was obtained (12.72 g/l) when nitrogen level was 4 g/l. There was a distinct relationship between concentration of NH_4Cl and biomass formation. Growth of yeast culture went on increasing with increase of nitrogen concentration. The possible reason of resulting quite satisfactory biomass at 4 g/l was the ionization and easy availability of nitrogen to the culture from NH_4Cl. It can be assumed that if concentration of NH_4Cl exceeds 4 g/l, the biomass is greatly affected because of the culture does not have the capability of utilizing the excess nitrogen present in the medium.

6) Molasses and Glucose Fermentation:-

Rhodotorula glutinis was cultivated in shake flasks using variables of commercial glucose and molasses as sole carbon source. Different / $(NH_4)_2SO_4$, commercial glucose and molasses were used. Results are given in Tables XXV and XXVI. Biomass was found to contain 37.5% and 35% protein in case of molasses and glucose experiments when the lipids were 22 % and 48.5% respectively.

The growth of Candida rugosa (A) was carried out in shake flask experiment with 6% molasses as sole source of carbon. Biomass increased from 5.8 to 16.3 g/l after 120 hrs. cultivation. The

Table - XXIV

EFFECT OF DIFFERENT CONCENTRATION OF NITROGEN SOURCE ON THE GROWTH OF Candida rugosa USING MAIZE 'GUR'*

Hours of Cultivation	Concentration of NH_4Cl											
	4 g/l			3 g/l			2 g/l			1g/l		
	Biomass g/l	N_2 consumed g/l	Sugar consumed g/l	Biomass g/l	N_2 consumed g/l	Sugar consumed g/l	Biomass g/l	N_2 consumed g/l	Sugar consumed g/l	Biomass (g/l	N_2 consumed g/l	Sugar con g/l
0	3.16	-	-	3.16	-	-	3.16	-	-	3.16	-	-
12	5.60	0.19	12	5.85	0.14	12	5.50	0.25	14	5.32	0.07	12
24	6.90	0.33	19	6.70	0.24	19	6.92	0.42	15	5.90	0.11	17
36	7.80	0.41	20	7.02	0.28	21	8.10	0.42	20	6.48	0.18	22
48	11.10	0.65	28	9.12	0.49	24	9.22	0.56	38	6.80	0.18	26
60	12.72	0.77	39	10.47	0.64	35	9.50	0.61	42	8.00	0.00	28

* Concn. of Maize Gur 5 %
(Shake flask)

Table - XXV

PERCENTAGE OF LIPIDS PRODUCED BY RHODOTORULA GLUTINIS UNDER CHANGING AMOUNTS OF GLUCOSE AND $(NH_4)_2SO_4$.

Glucose (5%) $(NH_4)_2SO_4$ (0.3%)	Glucose (10%) $(NH_4)_2SO_4$ (0.3%)	Glucose (15%) $(NH_4)_2SO_4$ (0.3%)	Glucose (15%) $(NH_4)_2SO_4$ (0.1%)
16.0	17.0	20.0	48.5

Table - XXVI

PERCENTAGE OF LIPIDS STORED BY RHODOTORULA GLUTINIS IN A MEDIUM CONTAINING MOLASSES AND $(NH_4)_2SO_4$.

Molasses (6 %) $(NH_4)_2SO_4$ (0.1%)	Molasses (12 %) $(NH_4)_2SO_4$ (0.1 %)	Molasses (12 %) $(NH_4)_2SO_4$ (0.3 %)
13.3	18.0	22.0

Table - XXVII

GROWTH OF CANDIDA RUGOSA ON MOLASSES*

Hours of cultivation	sugar consumed g/l	Nitrogen consumed g/l	Biomass g/l	Yield %
0	-	-	5.8	-
20	8	0.03	8.6	35
44	12	0.14	9.2	28
68	16	0.16	10.5	29
72	18	0.22	12.3	36
120	21	0.33	16.3	50

* 6 % molasses + mineral salts.

yield of biomass was calculated to be 50% on the basis of sugar consumed. The protein content was 45.4%. These results are comparable with those generally found in literature.

Batch cultivation of Candida rugosa on molasses (4% sugar content) was done in a 60l working volume fermentor. The contents of the fermentor are well agitated (500 r.p.m.) and an air supply of 0.8 l/l/min. Results are shown in Table XXVIII. Biomass increased from 2.0 to 20.8 g/l after 96 hrs. of cultivation. Biomass obtained after centrifugation is washed with tap water and analysed for protein, lipid, RNA and essential amino acids.

Two strains of yeast such as Candida rugosa (A) and Saccharomyces cerevisiae (SC) were grown in molasses medium and their biomass was analysed for estimating the protein, lipid and RNA (Table XXIX). It was observed that C. rugosa resulted in better % age of protein and lipid as compared to S. cerevisiae. but RNA was higher in case of C. rugosa as compared to S. cerevisiae. C. rugosa responded in the medium satisfactory due to essential amino acids and other accessory factors such as vitamins present in the molasses. However, S. cerevisiae showed poor growth as culture was sensitive towards some amino acids and vitamins which were absent in the medium.

Analysis of the biomass obtained from two different strains grown in molasses medium was also analysed for some essential amino acids. Results are reported in Table XXX. It is clear

Table - XXVIII

GROWTH OF CANDIDA RUGOSA ON MOLASSES

100L Fermentor

Hours of cultivation	Biomass g/l	Sugar consumed g/l
0	2.0	-
4	2.8	3.8
8	4.6	6.5
12	7.8	13.0
16	10.9	20.9
20	15.2	28.1
24	20.5	38.5
28	20.8	40.0

Table - XXIX

ANALYSIS OF BIOMASS FROM MOLASSES UTILIZING YEASTS

Yeast strain	Protein (%)	Lipid (%)	RNA (%)
A*	51.2	7.85	11.32
Sc**	48.0	5.72	10.40

* C. rugosa

** S. cerevisiae

Table - XXX

MICROBIOLOGY OF SOME ESSENTIAL AMINO ACIDS CONTENT OF MOLASSES UTILIZING YEASTS (g/100 g dry yeast)

Yeast strains	Argi-nine	Histi-dine	Isoleu-cine	Leucine	Methio-nine	Trypto-phan	Valine
A*	3.72	1.42	3.64	4.92	1.23	0.21	4.64
Sc**	5.34	2.84	4.26	3.45	2.45	0.72	2.64

* C. rugosa

** S. cerevisiae

that biomass of S. cerevisice contained high amount of arginine, histidine, isoleucine, methionine and tryptophan as compared to C. rugosa where as valine and leucine were present in higher concentration in the biomass of C. rugosa as compared to S. cerevisae. While comparing the amino acid contents in the biomass of these two cultures with FAO level, it was found that isoleucine present in both biomass of the culture was quite near to the values of FAO level, where as others such as leucine, methionine and valine were not upto the FAO level.

CONCLUSIONS

Results obtained so far regarding growth characteristics of yeasts, nutrient requirements, yield and quality of biomass indicate that Single Cell Protein (SCP) will be available at a competitive price when compared with other protein supplements available in the feed industry market. SCP production from industrial wastes will result in an additional minimization in pollution.

Carbohydrates such as molasses and maize 'gur' are the good substrates for SCP because they contain 50-55 % fermentable sugar and these can be easily recovered in the form of useful product i.e. protein for feed industry.

Experiments with molasses under nitrogen limitation will form a base for an ambitious hypothesis i.e. simultaneous production of protein and microbial fat. By manipulating the addition of nitrogen or carbon during fermentation, we can shift the priority to lipids or protein production.

Pretreatment of sulfite waste liquor (SWL) is necessary in order to remove inhibitory effects of SO_2 before utilization. Thus 70-75 % of the sugar contents of SWL have been successfully utilized and converted to biomass which can be used for feed purposes. To make the process economical supplementation of SWL with molasses or maize 'gur' is quite necessary as this will bring the level of fermentable sugar to a strength where maximum cell

mass production capacity of the plant can be exploited.

Good quality protein can also be obtained from paraffins contained in the waxy fractions of petroleum such as waxy lubricating oils. Up-grading of the gas oil as a result of microbiological dewaxing will give us a very useful by-product to be used in cattle and poultry feed.

SUGGESTIONS AND RECOMMENDATIONS FOR FURTHER WORK

1) Scale up studies on a pilot plant (1000 gallon working capacity) are to be carried out for bulk production of biomass for a period of six months whereas the cell mass thus formed can be used in poultry feed as a protein supplement.
2) Experiments regarding lipid formation along with protein by-product could be taken up on a larger scale and economical feasibility report be prepared as we are importing oils at the expense of huge amounts of foreign exchange.
3) Presently we are passing through a phase of energy crises. It is highly desirable for our scientists and technologists to develop indigenous resources make alternate arrangements depending upon local raw material. One of these can be the use of alcohol as a source of energy. This can only compete in the market if it is produced at a competitive price. Cellulosic raw materials along with molasses will be the raw

materials. Semi-anaerobic conditions will not only give us alcohol but also a useful by-product in the form of protein.

4) Continuous studies for new cultures and their development will be carried out so that cultures with better and good yields are added to our existing stocks. Mutants of yeasts with better amino acid profiles and low nucleic acid contents will be most welcome.

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APPENDIX

Construction and Fabrication

Some of the major equipment and items added under the PSF Project to the already existing facilities in the Fermentation Pilot Plant are:-

1) Purchased a fermentation unit for pilot plant consisting of 4 fermentors (9 l capacity) with controls from Antibiotics' (Private) Ltd. Iskandarabad (Daudkhel) and installed.

2) Construction of a room/shed (32' x 24') in the pilot plant building.

3) Heavy duty compressor OP Osaka, Japan.

4) Designing and fabrication of a 500 gallons working capacity fermentor especially suited for heterogenous system.

5) Local fabrication of stainless steel filter press for pilot plant (order placed with M/s. Steel Valve Engineering Ltd. Lahore).

6) Mixer grinder.

7) Medium tanks, empty drums and other containers for the storage of medium and substrate.

FIG. XII. PILOT PLANT PRODUCTION OF SINGLE CELL PROTEINS

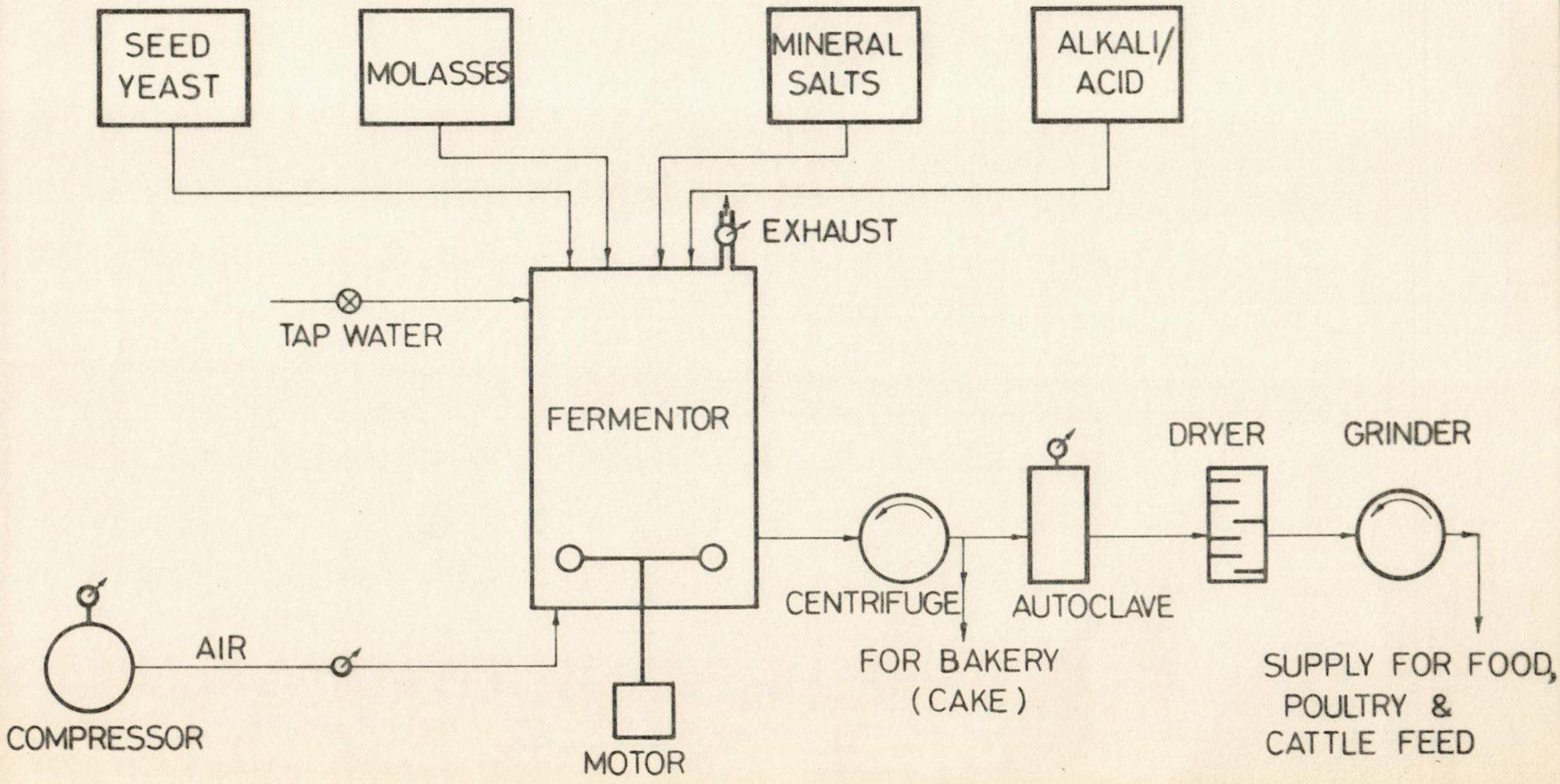

FIG. XIII. 2 Cu. METER FERMENTOR.