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NOT TO BE TAKEN FROM THIS ROOM

GROWTH OF PROTEIN-RICH MICRO-ORGANISMS IN PETROLEUM

by

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INTRODUCTION

As the population in the undernourished countries increases the inadequacy of their diet, and particularly the protein deficiency, is becoming pronounced.

In the year 2000 it is estimated that the world population will be around six and a half billion. To feed this population properly the production of present protein should be at least trepled. If we assume that today we consume 20 million tons of protein per year, in the year 2000 we must have 60 million tons of protein to consume.

A rough estimation shows that intensive cultivation of earth's all arable land will feed 10 billion people inadequately. Plant life utilizes the carbon in CO_2 of the air manufactures organic substances including proteins. Of the twenty odd amino acids required by the body man must get eleven in his food because his body does not synthesize them. The meat of ruminant animals, which with the help of bacteria convert vegetable food into proteins containing a full stock of amino acids, is men's main present means of attaining a balanced diet.

These considerations apply when we contemplate increasing our harvest of protein from the seas. The rich fishing grounds of the cold seas could be exploited more fully than they have been, but the fish life is not unlimited.

In recent years articles like this are seen in every publication pointing out to the serious situation arising.(Ref.4). Reading such articles everywhere and examining the underdeveloped countries, especially, every sound mind sees the necessity of struggling against this emergent problem. Since the present means of improving farming, fishing seem not promising much against the doubling world population in the next 30 years, a new means must be found. This new means must be a quick way of preparing the es-

sentials for our diet, mainly carbohydrates, fats and proteins. This quick way of producing can't be nothing but synthesizing these substances. Among the three protein inadequacy is most important since human body can't synthesize most of them and at the same time can't store them. So there must be a constant supply of protein to the body from outside. Therefore protein synthesis must be considered for the survival of mankind.

It is known that yeast fungi are grown on carbohydrates (most commonly molasses) and they produce vitamins and proteins comparable to those of animals. The microorganisms grow very rapidly doubling their weight every five hours or less which is several thousand times faster than farm animals synthesize protein. However carbohydrates are one of man's diet. So it is not very wise to use one food to produce another food.

Carbohydrates have carbon, hydrogen and oxygen in their structure. Couldn't a substitute be found for the carbohydrates. The first thing that comes to mind is the use of hydrocarbons. Hydrocarbons lack the oxygen, the carbohydrates have, but this may be furnished from the oxygen of the air or some other source easily.

Since petroleum is the most abundant natural source of hydrocarbons the studies may be directed to biosythesize protein by growing protein rich fungi in petroleum. There are various facts, such as the presence of some fungi type microorganisms in the sludge of petroleum storage tanks, that favor above idea.

Infact there are various groups of scientists in the world studying on this subject right now. Research laboratories, located at Marseilles, France belonging to the French BP, have advanced their studies to a great extent and have received some patents as a result of these studies. Some of these studies will be referred later in the thesis.

The purpose of this thesis is to start the biosynthesis of protein from petroleum in Turkey since it is one of the underdeveloped countries that have inadequate nourishment. In Turkey today 14 grams of protein is taken by each person everyday while a normal human being needs 30 grams of protein per day. The studies will be directed to perform the experiments that might have already been done by French or other advance guards with some manipulations to obtain better results in the way to biosynthesize protein. Since not much is known about the studies that are being done all over the world this thesis will have the procedure of a research at the same time.

The study will begin by some fundamentals of fermentation, bacteriology, biochemistry of fungi and proteins and petroleum chemistry. It will proceed by adapting some of the experiments done abroad on the subject to our conditions.

It will come to a conclusion by the results of these experiments.

REVIEW OF LITERATURE

A) Principles of Fermentation[1]

1-General Rules:

Biochemical processes may be defined as those in which enzymes are used to affect chemical reactions. Fermentation processes may be characterised by their employing micro-organisms as the enzyme source. Indeed the pre-eminence of fermentations among the commercial biochemical processes is largely attributable to the ease and rapidity with which some major microorganisms can be grown, and the engineering aspects of fermentation are essentially concerned with the provision of a suitable environment for mass-culturing the organisms.

To understand the chemical engineering problems involved in the process biological properties of the microorganisms must be considered. The most outstanding feature of microorganisms is their small size. Most of the bacteria are of order of one to five microns in length and half to one microns in width. Fungi are larger than bacteria, but they like the bacteria have much larger surface-volume ratios than most plants or animals. The importance of the large surface area is that it facilitates the passage of nutrients and metabolic products between the organism and the culture medium. It has been shown,for instance, that a particular lactose fermenting organism can break down several thousand times its own weight of lactose in an hour. Some bacteria will reproduce and thus double their numbers every 20 minutes under optimal conditions, and although this rate of reproduction is soon limited by the exhaustion of the nutrient medium, the accumulation of toxic end products of metabolism or some such factor, it is possible for one organism to give rise to many millions in a few hours. It is this high surface-volume ratio, intense metabolic activity, and rapid reproduc-

[1]Most of this part is taken from reference: I7

tion which makes industrial fermentation practicable.

In fermentation practice micro organisms are grown and maintained under such conditions that they will effect one or more commerciably desirable chemical transformations. The transformations are effected by enzymes which are organic catalysts elaborated by the living organisms. A single organism has a variety of these enzymes which are easily inactivated by certain physical and chemical agents like heat and for optimum activity they require certain ranges of temperature, pH and substrate concentration. Enzymes may be excreted by the living cell and act extracellularly to bring about changes in the surrounding medium, or they may be intracellular and act only on substances absorbed into the body of the organism. The complex of intracellular enzymes enable the organism to derive energy from its food materials and also to carry out the synthesis of protoplasm and other constituents of its living body. No two organisms have the same enzyme complex.

An organism will carry out its life process as determined by its hereditary constitution and the conditions of its environment. An organism used for commercial purposes will be selected for its ability to carry out a desired transformation and its environment will be controlled to ensure that culture functions efficiently.

There are three primary factors governing the life and growth of micro-organisms; these are their water relationships, their nutrients required and the temperature of the culture. Water is required by micro-organisms for a variety of reasons. In all organisms it is required for certain chemical reactions which take place in the living cell,as a reactant in hydrolysis, etc, and as a reaction medium. Most organisms can grow in fluid media water then functions as a carrier of nutrient media and as a repository for some of the end products. Water should be plentiful and free from toxic or ot-

her undesirable materials. Nutritionally, micro-organisms are classified as autotrophs, which require CO_2 and inorganis sources of N_2, sulfur and other elements on which to live and the heterotrophs, which are unable to synthesize certain substances necessary for their metabolism and require to have them provided in the medium. Between these two extremes there is a spectrum of organisms which are more or less fastidious with regard to their food requirements.

Some organisms obtain their vital energy from light;they are called phototrophic. Industrial fermentation is based on use of chemotrophs which obtain their energy by the oxidation of exogenous materials.

Potassium, phosphorus and calcium may be added to the medium as inorganic compounds. Attention must be paid that they are not toxic to the organism.

pH is important and most micro organisms will grow best between 5.5 and 7.5. It is important too that the pH of the medium should be favorable throughout the fermentation. It is necessary to make the further adjustments from time to time. In some cases optimum pH is not constant throughout the fermentation because two or more stages are involved.

The oxidation-reduction potential is another important factor. Culture media, in general, are reducing but it is sometimes necessary to ensure that oxygen is excluded from the culture when the organism is sensitive to a high oxidation-reduction potential. This problem does not arise in large scale anaerobic fermentations where the physical conditions minimize the oxidation of the medium.

Bacteria are mainly of two types: aerobic which is capable of using oxygen in their metabolic activity and anaerobic which is not capable of using oxygen in their metabolic activity. Some bacteria which are normally aerobic can live anaerobically; they are

called facultative. Some anaerobic bacteria are not sensitive to oxygen and can live anaerobically under aerobic conditions. A few organisms are aerobic but sensitive to too much oxygen, they are called microaerophilic.

All fermentation processes are exothermic and cooling is a problem.

Three basic issues which govern the design and operation of pure culture fermentation units are: 1)Starter cultures must be prepared in the laboratory with sound bacteriological techniques to ensure the complete exclusion of contaminants, 2)The air and media provided for the cultures must be sterile, 3)Plant must be so constructed that all parts of it can be sterilised and so designed that all the necessary operations (inoculation, transfer etc) can be carried out without any risk of introducing contamination from outside.

2-Fermentor Vessels:

a)Mash Attemperation

Fermentations are exothermic and the heat of fermentation may almost equal to the heat of combustion of the carbon source. This has to be cooled. Since fermentation temperatures are usually between 23° and $38^{\circ}C$ tap water can't cool because of the small temperature difference. Therefore it is necessary to use refrigerated water. As will be seen later there was no need for cooling in our case.

b)Hydrogen-Ion Concentration Control

Suitable equipment was found recently. A Beckman no:8990-90 glass electrode and a Beckman no:19700 reference electrode are appropriate. The electrodes can be steam sterilized and the sterility of KCl can be maintained with one percent benzyl alcohol. However

as will be seen later, since these were not available here, pH measurement was made by taking one milliliter samples and measuring the pH by an ordinary pH-meter or paper pH-indicators.

To decrease acidity in most fermentations calcium carbonate is added. Since ammonium compounds serve as the nitrogen sources ammonium hydroxide may also be used to increase the pH. However sterilization of ammonium hydroxide is somewhat difficult.

c)Method of Aeration

Very nearly all fermentations employ the submerged fermentation technique in which sterile nutrient solution is placed in a large, deep vessel and inoculated. This solution is aerated by forcing air into the base of the vessel through some sort of sparger with or without supplementary agitation.

There are various kinds of spargers used to distribute the air evenly in the form of very small bubbles to be well dispersed into the medium. Diaphragm sparger is a variation of sintered glass or porous stone sparger. It may consist of a rubber diaphragm pierced regularly with needle holes held on a backing plate. This has an additional advantage: the mycelial growth tends to block air passages but the elasticity of rubber allows it to stretch and clear itself. Tubes in different shapes with small holes on them may also serve as a sparger.

d)Need For Agitation

Agitation decreases the inter-clump resistances. It disperses air into small bubbles. It also creates currents in the liquid and lengthens the mean bubble bath hence increases the contact time between bubble and liquid. Agitation also facilitates the passage of dissolved nutrients to the cells. It may be in the form of an impeller situated a short distance above the sparger giving radial flow instead of axial flow.

e)Standard Equipment

It is a deep fermentation vessel with an air sparger somewhere near to the bottom and an agitator with an impeller situated a short distance above the sparger. There are outlets and inlets to take out and put in materials without contamination. Temperature and pH measurement are made possible by sparing place for the needed apparatus.

3-Aseptic Techniques:

a)Sterilization of Air

Since oxygen of the air is needed in supplying the oxygen absent in hydrocarbons in great amounts air will be used in fermentation of hydrocarbons. In fact oxygen is essential for the growth of micro-organisms in all aerobic fermentations. Examination of the proper fungi will show later that aerobic conditior is necessary. So air sterilization is necessary in this fermentation. There are various possibilities for air sterilization:
a)Electrostatic precipitation of dust but no available equipment, b)Ultraviolet light is not suitable for rapid sterilization of large volumes of air, c)Wet scrubbing can be effective. The wetting of micro-organisms in the air is the key to the satisfactory performance d)Appropriate heat treatment is the best method. If air is heated to 300^{o}C or over sterilization is virtually instantaneous, e)All large scale air sterilization is based on a filtration process, the filtration media of which includes slag wool and granular carbon and alumina. The efficiency of filters is related to the number of contacts which the air borne particle makes with the filter. It is common practice to sterilize air filters periodically. It only requires a few organisms in several million cubic feet of air to make it unsuitable for fermentation purposes.

A mutant or contaminant will only become an appreciable fraction

of the population if it is capable of growing more rapidly than the organism that is grown on the substrate in use.

b)The Pasteurization and Sterilization of Media

Thermal death time (TDT) of the organisms at each of several sterilization temperatures give a straight line graph on semilog paper where TDT are plotted on a log scale and corresponding temperatures on a linear scale.

Pasteurization is distinquished from heat sterilization since it involves heating a material and holding it at a predetermined temperature for long enough to kill most of the actively growing or vegetative organisms, while sterilization is carried out either at high temperatures or for longer time or both, so that even heat resistant organisms and spores are killed.

At temperatures of 80° to 85°C pasteurization is almost instantaneous. Having foam in pasteurization is dangerous since it may protect some of the micro-organisms. Similarly a stout, may be pasteurized to stop fermentation.

In fermentation both mash and equipment are sterilized by heat and steam is the usual heating agent. The precise sterilization conditions will depend on such factors as the pH, the presence of solid particles and the degree of contamination, but it is generally found that mash which has been held at 120°C for 10 minutes or 115°C for 15 minutes is sterile. If the nutritive value of a mash is not seriously harmed by the process of sterilization itself it is possible to devise a method of cooling which will be sufficiently rapid to prevent the mash from being damaged.

Sterelization by dry heat is mostly confined to laboratory equipment. In this case we heat them at least for an hour at about 160°C. This is because bacterial spores are more resistant to heat if no moisture is present.

c)Aseptic Transfer

Vessels and lines are totally enclosed and can therefore be sterilized by steam under pressure usually at 15 to 20 psia. Using steam raises three issues which call for attention: First the steam must be saturated and free from air. Superheated air imposes dry conditions while air mixed with steam reduces the partial pressure of the steam in the system, and limits the temperature to which the equipment may be heated. It is necessary to flush all air out when the steam is injected. Secondly provision must be made to remove condensate from the system. All the lines and equipment should be designed to drain towards the point from which the condensate should continuosly withdrawn.

d)The Inoculation of Seed Vessel Mash

It is important that the characteristics of the original strain should be maintained in all the sub-cultures and it is therefore usual to store cultures under conditions which reduce the growth of organism, but enable it to remain viable. In this way there is less risk of genetic or other variation taking place in the strain. Among the methods most used are: paraffin oil sealing, freezing, freeze-drying and the preparation of stocks of dry spores.

We must take advantage of that the final inoculum may consist of fresh or recently germinated spores.

In laboratory scale of inoculation a considerable amount of micro-organisms can be prepared in several petri dishes by inoculation from the original living strains which are stored in a test tube kept in the refrigerator. Passages are made monthly to a new test tube that has a fresh nutritive medium. As a precaution to the loss of original characteristics of the strain a freeze-dried portion is always stored. Since growth and genetic variation can't be prevented in a petri dish organisms produced in petri dish should be

used as soon as possible. Generally it is proper to use them 48 hours after the inoculation from the original test tube. Maximum time is one week after which it is not preferable to use the contents of the petri dish. The contents of the petri dish is transferred aseptically to the pre-sterilized laboratory scale fermentor that has the sterile nutritive medium. Fermentation starts as soon as the transfer is completed.

4-Recovery of Fermentation Products:

There are various methods for the recovery depending on the characteristic and future use of the product. Some of these methods are: 1)Clarification and stabilization of the fermented liquid.(beer) 2)Seperation of living cells (yeast), 3)Distillation of volatile products (acetone), 4)Total evaporation of fermented liquid (riboflavin), 5)Precipitation of insoluble complexes (polymyxin), 6)Solvent extraction (penicillin), 7)Adsorption by ion-exchange resins, 8)Concentration and crystallization (citric acid).

B) Bacteriological Laboratory Techniques[1]

Proper bacteriological laboratory techniques are necessary a)for the storage of the living strains of the original micro-organism b)to grow the seed micro-organisms in sufficient amounts for the inoculation of the fermentor, c)for the aseptic transfer of these micro-organisms into the fermentor, d)to work under sterile conditions to keep both the micro-organisms and the handlers from contamination, e)to stop fermentation and examine the products formed and micro-organisms grown.

The bacteriological techniques necessary were studied one by one to fulfill the above requirements:

1-Cleaning and Sterilization of All Apparatus:

Infected apparatus is disinfected and cleaned by the below steps: a.plunge infected apparatus immediately after use into glass jars containing a 2 percent solution of lysol, and allow them to remain therein for some days, b.remove them from the jar and drain, boil in water to which a little soap has been added for thirty minutes, rinse thoroughly in cold water, c.immerse in five percent nitric acid for an hour, d.rinse again in running water to remove all traces of acid, e.boil in a soapy water for twenty minutes, f.rinse with running water first and then with distilled water, g.run acetone and drain as completely as possible, h.dry in an oven at 80°C.

Before sterilization all test tubes and flasks must be carefully plugged with cotton wool: a.for a test tube or a small flask, tear a strip of wool cotton some ten centimeters long by two centimeters wide, b.turn in ends neatly and roll the strip of wool lightly between the thumb and fingers of both hands to form a long cylinder, c.double this at the center and introduce the now rounded end into the

[1]Most of this part is taken from reference: 23

open mouth of the tube or flask, d.now,whilst supporting the wool between the thumb and fingers of the right hand, rotate the test-tube between those of the left, and gradually screw the plug of wool into its mouth for a distance of about 2.5 cm., leaving about the same length of wool projecting.

The plug must be firm and fit the tube or flask fairly tightly, sufficiently tightly in fact to bear the weight of the glass plus the amount of medium the vessel is intended to contain, but not so tightly as to prevent it from being easily removed by a screwing motion when grasped between the fourth, or third and fourth fingers, and the palm of the hand.

For a large flask similar but larger strip of wool must be taken; the method of making and inserting the plug is identical.

After this the apparatus are sterilized in the proper way. Some of this was mentioned in the fermentation part. For the lab apparatus dry heat sterilization is used. The apparatus is kept in a drying oven for 1-2 hours at 160°C. The test-tubes are placed on a rack. The apparatus whose outside walls must be sterilized as well as the inside walls are placed in the oven in containers. Therefore the pippettes are placed in a closed container whose top is closed. Graduated cylinder of two liters whose top was closed by cotton were used for this purpose in our case. If this is not possible the apparatus is made into a package tightly by a proper paper, the proper paper being torn carefully just before using the apparatus.

Apparatus containing the nutritive medium etc. are plugged with cotton and the mouth of the container is wrapped with paper and placed in an autoclave. In the autoclave we have the wet sterilization under pressure. (120°C. for 15 minutes) Pasteurization is possible as described before at lower temperature if the substances may be damaged by the higher temperatures.

2-Method of Cultivation of the Micro-Organism:

a)Tube Cultures

Apart from the freeze-dried stock of the micro-organisms, the living strains are always kept on hand in a test tube which has the nutritive medium. The nutritive mediums are numerous with the numerous micro-organisms. The carbohydrate medium used for the special fungus of the experiment is named "Saboraud". The "Saboraud" medium is prepared as follows: 1)Emulsify peptone in distilled water to make a five percent solution, 2)Weigh out powdered agar and emulsify it in distilled water to make a six percent solution, 3)Mix the two solutions and sterilise at 115°C in the autoclave, 4)Filter from a piece of cotton and sterilise again, 5)Weigh out glucose in an amount to be four percent of the final weight of the solution, dissolve it in distilled water, sterilize and at to the formerly prepared mixture, 6)Bring down the temperature to 35°-40°C and add 0.5 % streptomycin and 1 % penicillin, 7)Tube and sterilize.

The nutrient media in aerobic tube cultures may be either liquid or solid. The solid media explained above are stored in tubes in one of two ways: a-straight tube in which the medium forms a cylindrical mass in the lower portion df the tube and presents an upper surface which is at right angles to the long axis of the tube, b-oblique tube or slanted tube, in which the medium has been allowed to solidify whilst the tube was retained in an inclined position, so forming an extensive surface of medium extending from the bottom of the tube almost to the mouth.

Once a month inoculation to a new tube with fresh nutrients is made to prevent the change of genetic structure of the culture. This transfer is made as follows: 1)Flame the cotton plug of the tube containing the cultivation, 2)Hold the two tubes, side by side, between the left thumb and the first and third finger, seperating the mouths of the tubes by the tip of the second finger, keeping the tubes as

nearly horizontal as possible, 3)Sterilize the platinum loop and allow it to cool, 4)Remove the plugs of both tubes by grasping the plug between the little finger and the palm of the hand, 5)Pass the platinum loop into the tube containing the culture-do not allow the loop to touch the sides of the tube, or the handle to touch the medium-and remove a small portion of growth, withdraw the loop from the tube, keeping the infected side of the loop downward, 6)Pass the loop into the new tube and draw two lines on the medium starting from the bottom of the tube and going upwards, make motions by the loop to spread out the micro-organisms, withdraw the loop, 7)Flame the mouth of both tubes and replug, 8)Sterilize the platinum loop, 9)Label the new tube by the name of the organism and the date of inoculation, 10)Incubate at the proper temperature. This above outlined method is infact the procedure in all tube inoculations.

b)Plate Cultures

The micro-organisms that are to be harvested and transferred to the fermentor in considerable amounts are grown in petri dishes. The petri dish cultures are prepared as explained below,the inoculation being made each time from the original living strains in the tube culture: 1)Liquefy a tube of nutrient agar and pour it into a petri dish and allow to solidify, 2)Invert the dish, raise the bottom half and rest it obliquely on its inverted cover and place in this position into an oven at $60^{o}C$ for 45 minutes to have a firm dry surface, 3)When quite cold raise the bottom of the plate, revert it and deposit a drop of the inoculum (whether a fluid culture or an emulsion from solid culture) upon the surface with a platinum loop, 4)Raise the bottom of the petri dish in the left hand, and holding it vertically, smear the drop of inoculum all over the surface with the short arm of the sterilized spreader by a rototory motion. Replace the dish in its cover, 5)Sterilize the spreader and label and incubate the petri dish.

The plate cultures as mentioned before is used within one week. To harvest the micro-organisms from a petri dish five milliliters of 0.9% NaCl solution is poured on the petri dish, The micro-organisms are made into a suspension by rubbing the surface gently by the sterile end of a small test tube. This suspension is withdrawn by a pipette, put in a test tube and centrifuged. The solution is decanted and the micro-organisms left in the tube is washed with the same amount of NaCl solution. It is centrifuged and washed in the same way three times. After the last washing it is made into a suspension and added to the medium in the fermentor by a pipette.

3-Method of Identification of the Micro-Organisms:

There are a variety of methods to identify a suspected organism. for the identification the micro-organisms are examined a)macroscopically, b)by microscopical methods, c)by chemical methods d)by physical methods, e)by inoculation methods. In fermentation if the growth of micro-organisms in a certain medium is suspected radioactive nutrient salts are put into the medium and the radioactivity of the product cells is detected. If they are radioactive,it is certain that growth has occurred in the media utilizing the radioactive salts.

All of these tests are cumbersome. Besides many of these tests won't be useful in the fermentation case since they will identify the seed micro-organisms, inoculated at the beginning of the fermentation, although they have not grown. So best method is to take the paste of micro-organisms harvested from the medium and to compare them to the original living strains under the microscope. To be sure samples from various portions of the paste may be taken and examined. If the means are available the method of radioactive tracers may be used to detect the growth of micro-organisms in a certain medium.

The microscopical examination of the micro-organisms may be done by stained or unstained micro-organisms. The unstained micro-organisms

are examined as follows: 1)Clean and dry a three by one glass slip. Deposit a drop of distilled water or a drop of one percent solution of KOH on the center of the slip by means of the platinum loop, 2)Open the test tube under sterile conditions as described before and remove a small portion of the growth by touching the loop on the surface of liquid or solid media of growth, withdraw the loop without touching the medium or the glass side of the tube. Replug the mouth of the tube, 3)Mix the contents of the loop thoroughly with the drop of water on the slide, 4)Sterilize the loop, 5)Remove a cover slip that is sterilized in alcohol, burn the excess alcohol and lower this cover slip on to the surface of the drop of fluid gently avoiding the inclusion of air bubbles, 6)Examine microscopically.

During the microscopical examination, stains and other reagents may be run in order to see some aspects better. This is done by placing a drop of the reagent in contact with one edge of the cover-glass and applying the torn edge of a piece of blotting paper to the opposite side. The reagent added may then be observed to flow across the field and come in contact with such of the micro-organisms as lie in its path.

The non-toxic basic dyes most generally employed for the intra-vitam staining of the micro-organisms are: Neutral red, quinoleine blue, methylene green, and vesuvin. The are a great variety of dyes used for the purpose. The dyes are applied in 0.5 percent aqueous solutions. The success of stains used varies from micro-organism to micro-organism.

C) Biochemistry of Fungi

1-Fungal Structure and Organization:

The fungi comprise a large, heterogeneous, and ubiquitous group of heterotrophic organisms living as saprophytes or parasites, or associating with other organisms as symbionts. They are characterized by a distinctive, filamentous, multinucleate vegetative structure known as the mycelium. It consists of a branching system of walled tubes, the hyphae, which contain protoplasm and continually extend by apical growth and lateral branching. Food material is absorbed over the whole surface of the mycelium. After a period of growth reproduction occurs with the formation of spores, uni- or multicellular bodies that become detached from the parent and give rise to new individuals.

Fungi belong to the class of heterotrophs which do not possess chlorophyll and are incapable of photosynthesis. In the course of their metabolism, the heterotrophs, by processes which include both breakdown and synthesis, adapt and modify food materials into molecules suitable for their own cell structure and reproductive activities. These activities require energy, which is derived from oxidative processes, by which a part of the food is converted into carbon dioxide and water, any excess energy being dissipated as heat. The fungi produce in addition to carbon dioxide, water, and all the complex materials required for the cell structure and the vital processes a large number of substances of varying complexity whose function is still obscure. The products of the fungi, each catalyzed by an enzyme, will normally be elaborated within the cell, but, once formed, most of these products when not forming part of the cell substance or otherwise completely utilized, are able to escape from the cell. If they are soluble in water they will be found mainly in the aqueous medium from which they may be obtained by processes of distillation, extraction, chromatography. Amino acids, proteins, nucleic acids and steroles are common in all fungi as in all living matter. The protein in

fungi has superiority over the protein of edible animals, milk and wheat. Table I below shows this superiority.

Table I[1]

	Wheat	Cow Meat	Milk	Candida Yeast Concentrate
% of protein	13.2	59.4	33.1	43.6
Amino acids in 100 grams protein				
Leucine	7.0	8.0	11.0	7.0
Isoleucine	4.2	6.0	7.8	3.05
Valine	4.1	5.5	7.05	8.40
Threonine	2.7	5.0	4.7	9.10
Methionine	1.5	3.2	3.2	1.2
Cystine	1.9	1.2	1.0	0.1
Lysine	1.9	10.0	8.7	11.6
Arginine	4.2	7.7	4.2	8.0
Histidine	2.2	3.3	2.6	8.1
Phenylalanine	5.5	5.0	5.5	7.9
Tryptophane	0.8	1.4	1.5	1.17

The great variety of the other complex substances is seen in the study of the cell wall.

2-Constituents of the Cell Wall:

The gross morphology of fungi is , to a great degree, dependent upon the conformation of their cell walls. The walls of fungi, possessing considerable rigidity, lead to stability of the shapes of vegetative hyphae and various reproductive structures. In the fungi as in green plants, the cell walls are composed of polysaccharides to a great extent. In most fungi the role of cellulose to provide a skeletal framework for the cell wall is assumed by chitin. The reaction of I_2-KI with the cell wall and cytoplasmic constituents has been employed as a technique for the classification of higher fungi. Table II next page shows the cell wall composition for a selected fun

[1]Table I is taken from reference:51, page:4

gi.[1] Values are percentage of dry weight of the walls.

Table II

	Saccharomyces Cerevisiae (Baker's yeast)	Allomyces Macrogynus	Mucor Rouxii
Nitrogen	2.1	5.5	--
Phosphate	0.31	--	23.3
Lipid	8.5	--	7.8
Protein	13.0	10.0	6.3
Chitin	1.0	58.0	9.4
Chitosan	--	--	32.7
Glucan	28.8	16.0	--
Mannan	31.0	--	3.8
Other carbohydrates	--	--	9.5
Ash	--	8.0	2.0

Comparison of table I and table II indicates that most of the fungal protein is in the cytoplasm instead of the cell wall. Table I indicated that candida yeast concentrate contained 43.6 percent protein while Table II indicates that an average yeast cell wall contains about 10 percent protein. This means three quarters of the yeast protein is located in the cytoplasm. The overall analysis of the fungal cell is shown in Table III.

Table III[2]

	Analysis percent by weight
Moisture	7.03
Dry Matter	92.97
Total nitrogen	6.92
Proteins	43.6
Lipids	18.5
Carbohydrates	21.9
Ash	4.43
Calcium	0.211
Phosphorus	1.250
Potassium	0.500
Sodium	0.060

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[1] Table II is taken from reference:2 , page:58, table I

[2] Table III is taken from reference:15 , page:13, table I
This table shows the composition fungi used in BP laboratories in Marseilles in the researches to get protein from petroleum.

Besides these substances indicated in Table III, British Petrol yeast concentrate contains water soluble vitamins. This fact makes this particular fungi more valuable as a food product. Below table IV[1] indicates these vitamins as milligrams per kilogram of yeast.

Table IV

	Thiamine B_1	Riboflavine B_2	Nicotinic acid	Pantothenic acid	Pyridoxine B_6	Cobalamine B_{12}
Daily requirements for adult	2	3	15	3	2	0.01
Beef	1-3	2	40-100	7-21	1-4	--
Milk	0.3-0.7	1-3	1-5	1-4	1-3	--
Cereals	0.5-7	1-1.5	10-30	5-20	3-6	--
Dry Yeast	2-20	30-60	200-500	30-200	40-50	--
BP yeast concentrate	3-16	75	180-200	150-192	23	0.11

All of the information about the constituents of the fungal cell prove that yeasts can compete with most of the foods of our diet. Infact they are superior in some cases. The availability of fungi for this purpose will be shown later as the growth requirements and media of growth is discussed.

The elements in the structure of the fungal cell will be reflected in the study of the chemical environment needed for growth.

3-The Chemical Environment for Fungal Growth:[2]

Fungi are dependent upon the medium, or substrate, for all the elements and compounds they require or utilize except molecular oxygen and, possibly, a little carbon dioxide, which are obtained from

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[1] Table IV is taken from reference:15 , page:14, table: 3.

[2] Most of this part is taken from reference: 2

the atmosphere. From these elements and compounds they synthesize the cellular constituents and obtain the energy necessary for their life processes. Some compounds, such as vitamins and some amino acids, enter the cell without modification. The composition of the fungus cells may be made to vary widely by the substitution of the elements of the same group. Therefore fungus cells don't have an unvarying composition however some elements are needed more than the others. Such elements are named as the essential ones. The minimal composition of a medium or substrate must include all the elements that are essential. In addition, the essential elements must be present in compounds that can be utilized. The concentration of an essential ion required may depend also upon the concentration of other ions in the medium. For example it was found that the amounts of cobalt and nickel accumulated by Candida Utilis was dependent on the magnesium concentration of the medium. If the ratios among the constituents of a medium are such that the constituents are exhausted at the same time, such a medium may be considered to be balanced.

a)Classification of Media

Media may be classified as natural, semisynthetic, or synthetic. In addition, media may be classified as liquid or semisolid since fungi may be grown as a surface film on a static medium or submerged in an agitated medium. Example of liquid media is seen in the microbial oxidation of n-paraffinic hydrocarbons by the fungi Candida Tropicalis and Nocardia which is explained in reference:58. Example of semisolid is seen in reference:74 which explains the microbial degradation of asphalt. For the preparation solid asphalt film in this case asphalt was made into a solution by benzene. Evaporation of benzene later left a thin film of asphalt.

Natural Media: Media of this class are composed of natural products of plant and animal origin. The exact composition of such a media is unknown being very complex. It follows that fungi find at hand many

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metabolites that are suitable for use without modification. Roberts (Reference: 61) in studying amino acid transformation in Candida Utilis found that certain amino acids served as parents of a family of amino acids.

Semisynthetic Media: Media in this category are composed in part of natural products and in part of chemical compounds of known composition. Reference:33 describes a medium that contains 0.5 gr KH_2PO_4 , 0.2 gr $MgSO_4$ $7H_2O$, 0.1 gr NaCl, 2.5 gr $(NH_4)_2SO_4$, traces of iron, 8 gr petroleum fraction boiling between 230° - 290°C and water to make 1000 milliliters. This medium is a good example of semisynthetic medium since it contains salts of known amount and composition and a mixture of petroleum of unknown composition at the same time.

Synthetic Media: The ideal synthetic medium is one in which every constituent and its concentration is known. This ideal situation is never attained in practice because of natural substances, like vitamins required.

b) Aeration

Oxygen, the one essential element not added to media, is obtained from the atmosphere. Since the solubility of oxygen in water is low and the rate of diffusion slow, the area-volume ratio is important in determining rate and amount of growth in still cultures. The deeper the medium, the longer it takes to establish surface growth. Deep media provides long contact time with an air bubble until it reaches the surface. The oxygen requirement for cell growth on any substrate is easily calculated by the formula A / Y - B = C where A is amount of oxygen required for the combustion of one gram of substrate to carbon dioxide and water, Y is the cells produced from one gram of substrate, B is the amount of oxygen required for the combustion of 1 gram of cells to carbon dioxide and water. Thus C is the amount of oxygen used during the production of one gram of cells. (Reference:36 , page: 1535) Thus if the substrate is an alkane, the

formula becomes C (millimoles oxygen per gram cell) = $\frac{107.1}{Y}$ - 41.7 Although such calculations are very useful, they are very seldom taken advantage of. Since the gram of cells produced changes as fermentation proceeds the oxygen requirement is not constant throughout the fermentation. The same reference reports that in an experiment growing Candida species on an alkane substrate 50 milliliters per minute (0.1 volumes oxygen per volume of medium) air was supplied in the first 25 hours, then it was increased to 100 milliliters per minute and after 30.5 hours it was increased to 150 milliliters per minute which was still insufficient after 40 hours.

Another experiment (reference: 33) using petroleum as the hydrocarbon source supplied 400 liters air per hour while a similar experiment in reference:58 used 100-200 milliliters of air per minute.

c)Hydrogen-Ion Concentration

Most fungi grow within the pH range 4-8. Many fungi will grow over a wider range, and a few have been reported to have a narrower range. Reference:7 reports that the particular fungi Candida Lipolytica grows between pH 2.5 and pH 7.6 , it mentions that a good yield was obtained by culturing Candida Lipolytica at pH 5.6-5.8. Reference:36 describes the growth of a Candida intermediate on a petroleum containing medium at pH 5.5. There are other sources about the pH requirements of the special fungus Candida Lipolytica that grows on petroleum fractions. Reference:15 mentions growing Candida Lipolytica on a petroleum containing medium whose pH is four.

The hydrogen ion concentration does not remain constant throughout the fermentation process. The change in pH of the medium results from two types of metabolic activity: (1)the production of organic acids or the release of ammonia, and (2)the unequal utilization on excretion of cations and anions.

Precipitates may form in neutral and alkaline media, especially

when they are autoclaved. The conversion of certain constituents into insoluble compounds alters the composition of the medium. In this case the formation of insoluble zinc and calcium phosphates becomes the greatest problem since phosphorus in the form of phosphate and zinc and calcium are all essential elements. Ammonium tartrate was reported to dissolve calcium and zinc phosphates.

d)Constituents of the Media

The medium contains essential nonmetallic elements, essential metallic elements, microelements and some non-essential metallic or nonmetallic elements.

Essential Nonmetallic Elements:

Hydrogen: On the atom percent basis hydrogen is the most abundant element in the cells of fungi and other organisms. Presumably, an average value of hydrogen for fungus cells would lie between six to eight percent.

Besides that fungus cells are mainly water. Water content of various sporophores ranges up to 73.7 percent. Water exists in fungus cells in two forms. Free water is that removed from cells by drying at room temperature over a desiccant in a vacuum. Bound water is that removed by additional drying at elevated temperatures (80°-110°C). As a basic requirement, the water used should not contain toxic materials Some well and tap waters are suitable for preparing natural media. For exacting work, deionized distilled water, or distilled water from quartz or glass stills may be required.

Reference:6 describes a process for the manufacture of yeasts from hydrocarbons where soft water is used as the water source. Another reference (reference:11) where yeast molds and bacteria are cultivated in suspension or solution form in a feed of C_{10} or higher hydrocarbons uses 200 milliliters of tap water for each liter of the medium, the rest of the medium being distilled water.

Oxygen: An estimate for the oxygen content of the fungus cells, based on carbohydrate, protein and fat content would lie in the range 25 to 35 percent. Most of the organic compounds found in cells contain oxygen The aspects of supplying oxygen to the medium and itsamount was discussed before in the aeration part.

Carbon: This is another abundant element in the fungi. The extreme carbon values for Aspergillus were 56.6 percent and 43.5 percent. Penicillum (Scopulariopsis brevicaulis) contains 62.9 percent carbon. Study of fungal metabolic patterns has shown that most, if not all, species degrade the carbon compound added to the medium and form mycelium from the metabolic products. There appear to be no reports of investigation designed to show that the carbon source added to the medium is directly incorporated into mycelial carbohydrate, lipid or protein.

The formation of parent amino acids by various enzymatic reactions during the active growth and protein biosynthesis may lead to a serious drainage of carbon moieties from the cycle. This would necessiate reactions to provide carbon fragments vital to the continuous function of the cycle. It has long been realized that carbon dioxide is necessary for growth of bacteria, yeasts and filamentous fungi. In many studies fixed carbon dioxide was found in aspartic and glutamic acids.

Various carbon compounds have been used such as carbohydrates, amino acids, organic acids, polycyclic compounds and alkoloids as the carbon source. The characteristic of this research is the choice of hydrocarbons as the carbon source. Petroleum will be the source. The problem in this case is the insolubility of petroleum fractions in aquous media. When fungi are grown in madia containing this insoluble material, the mycelium tends to form balls around the particles, a phenomenon which leads to poor growth. To solve this problem emulsifying agents may be used to make an emulsion of petroleum in water but this time foaming and interference of emulsifying agent in growth becomes the problem. Emulsifying agent forms a coating around the hy-

drocarbon molecule and thus interferes with growth. Solubility measurements of a number of n-alkanes gave the below results:

Table V[1]

Solubility of n-alkanes in water at 25°C

n-alkane	Molar concentration of saturated solution
Hexane	1.1×10^{-4}
Octane	5.8×10^{-6}
Decane	3.1×10^{-7}
Dodecane	1.7×10^{-8}
Tetradecane	9.8×10^{-10}

Fitting this data into a straight line on a semi-log graph we get a general expression for the solubility of n-alkanes in water at 25°C. This equation is: log solubility = 4.526 - 0.588 n where "n" is the number of carbon atoms in the chain.

These show that low molecular weight alkanes are more preferable since they are more soluble. This assumption proves to be wrong when we study the growth rates of micro-organisms in alkanes. Low molecular weight liquid alkanes, like other good lipid solvents, are well known to be toxic to many organism. In a number of laboratories investigating the growth of micro-organisms on low molecular weight hydrocarbons, it has been observed that while relatively few organisms will grow in a medium containing, for example, liquid hexane, many more will grow if the hexane concentration in the medium is kept below the saturation level. Besides this it will be seen that as the length of the substrate carbon chain increases, the generation time decreases. This fact is shown in table: VI next page. The only hydrocarbon with an odd number of carbon atoms tried was heptadecane. It gave a lower cell yield than either hexadecane or octadecane. The low yield was probably not due to incomplete oxidation of the heptadecane since there was no accumulation of ether-extractable acids.

[1] Table V is taken from reference:36 , page:1532, table II.

It will be noted that in every case the carbon recovery was below 100 percent. The nature of the missing carbon atom is not known.

Table VI[1]

Growth of Candida on normal alkanes

Substrate	Generation Time (hr)	Cell yield on substrate %	CO_2 produced per mole of substrate	Carbon recovered as cells and CO_2
$C_{12}H_{26}$	7.0	59.8	4.6 moles	72.7 %
$C_{14}H_{30}$	6.5	83.0	5.2	84.2
$C_{16}H_{34}$	6.5	81.7	7.5	93.8
$C_{17}H_{36}$	5.0	72.9	4.3	67.6
$C_{18}H_{38}$	4.5	82.5	5.4	76.6

The temperature was 30°C, tha pH was controlled at 5.5. The cell concentration at the end of growth period was from 10 to 15 grams per liter.

Table VII gives the data on the composition of the cells produced. It will be noted that with the exception of dodecane and heptadecane, each substrate gave about the same conversion of ammonia nitrogen into cell nitrogen.

Table VII[2]

Composition of cells grown on normal alkanes

Substrate	N added as NH_4OH per 100 grams of cells	Cell N (%)	Crude protein yield on substrate(%)	Cell lipid %
$C_{12}H_{26}$	9.52 gr.	7.10	26.6	5.0
$C_{14}H_{30}$	9.20	7.27	37.7	7.5
$C_{16}H_{34}$	8.60	7.21	36.8	7.2
$C_{17}H_{36}$	8.24	7.26	33.2	10.3
$C_{18}H_{38}$	8.32	6.85	35.5	8.3

[1]Table VI is taken from reference:36 , page: 1534, table:IV

[2]Table VII is taken from reference:36 , page: 1534, table:V

Examination of these tables shows that alkanes with C_{14} and C_{16} carbon atoms are the most suitable fractions to grow protein rich microorganisms. Cell yield and crude protein yield are the best on these alkanes. Experiments made by E. Azoulay and J. Senez, in the year 1964 in Marseilles, by growing Candida Lipolytica on hydrocarbons proves the above statement also. Table VIII, below, which is the result of these experiments, shows this:

Table VIII[1]

Growth of C.Lipolytica on different alkanes

Substrate	Substrate Consumed mg/liter	percent yield %	Molecular yield	time of division
$C_{10}H_{22}$	730	35	50	1 hr 35 min
$C_{12}H_{26}$	766	57	98	1 hr 42 min
$C_{14}H_{30}$	765	68	133	1 hr 40 min
$C_{16}H_{34}$	775	90	205	1 hr 40 min

Molecular yield is given as grams of cells produced per mole of substrate consumed.

Reference:63 , which explains the growth of Candida Lipolytica utilizing several coal derived fractions as major sources of carbon and energy also mentions the success of experiments done with n-hexadecane (C_{16}). There is a list of similar experiments using gas oil (kerosene) fractions for the best yields. Alfred Champagnat uses gas oil in reference:14 . Another one, reference:6 , describes the manufacture of yeasts from hydrocarbons with the simultaneous removal of straight chain paraffins from hydrocarbons and the lowering of pour points in gas oils. Reference:44 explains the utilization of gas oils for the same purpose. Gas oils is the fraction of petroleum that boils between 175°-290°C. It contains the alkanes from C_{10} to C_{16} .

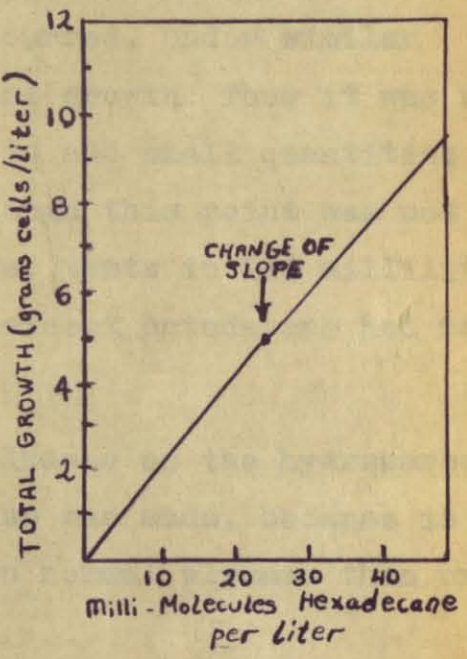
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[1] Table VIII is taken from reference:7 , page:527, Tableau:III

Hoerburger in an industrial laboratory grew Candida Tropicalis in 450 ml mineral salts containing 1.7 percent of a hydrocarbon "cut" (boiling point 230°-290°C). With high aeration (400 liter per hour) he was able to increase the amount of yeast cells from 1 gram to 2.65 grams in 22 hours. Since it is between 230°-290°C his fraction was the higher fractions of kerosene.

To summarize fungi, especially Candida species, grows with a good yield on alkanes between C_{10} and C_{16}. The yield is best on C_{16}. If petroleum is to be the hydrocarbon source kerosene is the fraction that contains those alkanes. From the boiling point consideration petroleum fraction boiling between 175°-290°C is containing kerosene. As an approximation petroleum fraction boiling between 200° and 290°C is suitable for Candida growth. The more near is the lower limit to 290°C the more C_{16} is present in the fraction since the boiling point of hexadecane C_{16} is 287.05°C.

The more dilute the medium, the more efficiently the carbon source is converted into mycelium. The economic coefficient (it will be discussed later) usually decreases as the culture ages. In commercial fermentations where the substrate is converted into a product, a high concentration of carbon source is used and the economic coefficient is very low. There are various references stating the percent of hydrocarbon in typical experiments where protein-rich micro-organisms are grown in petroleum. One such reference gives the figure on right where Candida Lipolytica is grown on hexadecane. The curve changes slope at 5.6 grams per liter. This means at 5.6 grams per liter hexadecane concentration gives optimum growth.



[1] Figure:1 is taken from reference: 7 page: 527, figure: 2.

Reference:30 explains the growth of o.200 grams Candida Lipolytica in a 200 milliliter media containing mineral salts and 10 grams of crude oil as the carbon source. Thus crude oil concentration was five percent in the medium.

Reference: 56 explains the giving of Candida Tropicalis the highest cellular mass (9.0 grams/liter/three days) in a series of experiments using 15 percent by volume light oil.

Reference :67 mentions the use of one percent hexadecane in growing a gram-negative coccus on a gyratory shaker at 250 RPM at room temperature.

All of these show that there is no strict amount of hydrocarbon in any substrate, however no success is reported from mediums using over 15 percent hydrocarbon. So we can use up to 15 percent hydrocarbon in the medium if we are successful in agitating it. There is no need to mention that as the hydrocarbon concentration increases aeration and thus agitation becomes a problem more difficult to solve. These suggest that it would be better if the hydrocarbon is added in small portions as it is eaten up by the micro-organisms. This point was made apparent in the microbial oxidation of n-paraffinic hydrocarbons by R.L.Raymond in reference 58 . He says, "If the ratio of n-octadecane to cells was 20:1 or greater no growth occurred. Under similar circumstances a ratio of 7:1 yielded excellent growth. Thus it was necessary when starting with a small inoculum to add small quantities of substrate early in the fermentation. Although this point was not specially tested for the yeasts in fermentors, tests in 250 milliliter indented shake systems indicated that five percent octadecane had no adverse effects."

Up to this point discussion of normal alkanes as the hydrocarbon and petroleum as the source for normal alkanes was made, because in general, micro-organisms grow more readily on normal alkanes than on branched chain alkanes.

Any bituminous material may serve as the hydrocarbon source to the extent of it content of normal alkanes. In that respect the action of micro-organisms on asphalt fractions is mentioned in reference:73 .

Reference:46 indicates that one might hope to produce 10 tons of protein from 2 000 000 cubic feet of natural gas since methane provides all the energy needed for its conversion to protein. High yield of micro-organisms were obtained in simple salt solutions supplied with air and methane.

Pittsburgh Coal Research Center gives the yields for the growth of protein-rich fungus Candida Lipolytica on coal tar fractions. The results are given in the table below:

Table IX [1]

Growth yields of yeasts on pure hydrocarbons and fractions derived from coal and petroleum

Substrate	milligrams C.Lipolytica grown per gram of substrate added
Hexane soluble distillate from low temperature tar	14
Hexane soluble distillate free from phenols	73-83
44% n-paraffin, 56% olefine fraction from coal tar	113
88% n-paraffin, 12% olefine fraction from coal tar	259-272
Petroleum derived fraction consisting C_9 - C_{14}	280-342
1-Octadecene	357
n-Hexadecane	719

This table further proves the fact that the micro-organisms grow best on normal alkanes that has more than ten carbon atoms. The reason of this better yield may be the mechanism of primary attack of

[1] This table is taken from reference: 63 , page:736, table:1.

the micro-organism on the hydrocarbon. There is a great deal of evidence that in almost every case, the alkane is attacked at the terminal methyl group. The methyl group is oxidized to a carboxyl, and further oxidation occurs via the usual β-oxidation reactions. There are apparently two mechanisms for the primary attack on the terminal methyl group. The first of these involves participation of molecular oxygen. An obvious hypothesis is that the primary alcohol is produced from the hydrocarbon by some reaction involving molecular oxygen, part of the alcohol is further oxidized, via the aldehyde, to palmityl CoA, which then reacts with part of the remaining alcohol to yield an ester. The second mechanism does not involve molecular oxygen. This is the anaerobic mechanism.

An attractive hypothesis is that the organism attaches itself to a droplet of alkane, and the long alkane chains become a part of the phospholipid micelle of the cell membrane, and that a lyophobic pathway exists from the outside of the cell membrane to the locus of the enzyme responsible for the primary attack on the hydrocarbon.

There are a number of methods used to determine the efficiency of conversion of carbon sources to mycelium such as (1)total weight of mycelium formed and correlation of this with the amount of carbon source utilized, (2)the amount of carbon in the medium converted to mycelium carbon, (3)the amount of substance directly incorporated into the mycelium.

The first method, often termed the "economic coefficient" is the most widely used. It is defined by the formula

$$\frac{\text{mycelium dry weight (gr)}}{\text{amount of carbon compound consumed (gr)}} \times 100$$

Another formula to measure the efficiency, taken again from reference:51 is:

$$\frac{\text{mycelium dry weight (gr)}}{\text{amount of carbon compound treated (gr)}} \times 100$$

An idea about the economic coefficients of the processes utilizing

hydrocarbons as the carbon source may be gotten by examining some of the experiments in the available references. In such experiments the economic coefficient is very high. Reference:14 mentions that under favorable conditions a kilogram of hydrocarbon will produce a kilogram of yeast. It must be indicated here that the second formula on the last page may give a low efficiency although the first formula gives a high efficiency. Table VI on page 31 gives the cell yield on substrate as 83 % at optimum conditions. Table VIII gives 90 % yield at the optimum conditions. Another reference where growth of Candida Lipolytica in Bernard Laine's experiment is explained (reference: 44) mentions that cultivation of 20 grams of Candida Lipolytica in a 40 liter medium containing 8 liters gas oil (added in portions) resulted in 7.4 kilograms of yeast paste at the end of 15 hours. It must be added at this point that the best yield using carbohydrates as the carbon source is generally below50 percent.

As will be seen later the economic coefficient is affected considerably by the efficiency of aeration (if mixing is not a problem higher economic coefficients are obtained when minimal aeration is used than when high aeration is used), incubation temperature, the presence in the media the required elements and vitamins. Adaptation of culture to substrate is also important. All of these will be discussed later in detail.

Nitrogen: The nitrogen content of fungus cells is variable and depends on species, age and composition of the medium. Rhodotorula gracilis was grown in media containing one and ten grams of asparagine per liter. The nitrogen content of the cells was 1.34 and 9.72 percent in the low and high asparagine media, respectively.

Fungi usually utilize simple inorganic nitrogen compounds. Thus ammonium salts or nitrates are suitable sources of nitrogen for most fungi although with nitrate there may be a lag in growth before the

necessary enzymes concerned in nitrate reduction are produced. Fungi use nitrate in one of two ways: (1)assimilation into cell nitrogen, or (2)dissimilation or nitrate respiration, where nitrate acts as an alternative hydrogen acceptor to oxygen. Fungi oxidize ammonia to nitrate at a slow rate.

Fungi are usually more tolerant of nitrous acid than are bacteria presumably because they are able to grow at a lower pH. Nitrite is used readily by some fungi. Thus S.brevicaulis takes up nitrite at 1.2 milligram nitrogen per gram dry weight per hour, which compares closely with the uptake of nitrate or ammonia under similar conditions

Ammonia is another nitrogen source which enters cells of many organisms by passive diffusion of its undissociated molecule. The entry of ammonia into cells is dependent on the concentration difference of the molecule between the cell sap and the external solution and on the supply of cell constituents that combine with ammonia.

When ammonium nitrate is supplied there is presumptive evidence that ammonia is used first because of a sharp drop in pH during early growth. In some fungi the assimilation of nitrate is somehow blocked by ammonia.

Fungi may be divided into four classes according to their assimilation of different nitrogen compounds.

Table X[1]

Class	N_2	NO_3^-	NH_4^+	Organic
1	yes	yes	yes	yes
2	-	yes	yes	yes
3	-	-	yes	yes
4	-	-	-	yes

[1]This table is taken from reference:2 , page: 474

Potassium, sodium, and calcium nitrates have been used as sources of nitrate N. Sources of ammonium N include ammonium chloride, ammonium sulfate, and ammonium phosphates (diammonium hydrogen phosphate loses ammonia on exposure to air). Of the organic nitrogen sources peptone and casein hydrolyzate are suitable complex sources.

Varying the type and concentration of nitrogen affected both the cell yields and lipid formation. The results using hydrocarbon substrate are similar to those observed for sugars for maximum lipid production by other micro-organisms, i.e, high carbon:nitrogen ratios favor lipid synthesis, and low carbon:nitrogen ratios increase protein synthesis. R.L. Raymond studied the effects on Nocardia cells more thoroughly than the yeast in reference: 58 . Below table:XI shows the results of this study.

Table:XI[1]

Effect of Nitrogen Sources on Nocardial Cell Yield and Lipid Formation
(Substrate 824 mg n-octadecane)

	% N in compound	C_{18} utilized in mg	Dry cell weight mg	Conversion to cells %	Cell Lipid %
$(NH_4)_2SO_4$ (0.1%)	21	606	445	74	72
$(NH_4)_2SO_4$ (0.3%)	21	293	224	83	43
NH_4NO_3 (0.3%)	35	293	207	70	46
KNO_3 (0.1%)	14	394	252	64	70
KNO_3 (0.3 %)	14	795	530	67	65
Urea (0.1%)	47	808	603	75	64
Urea (0.3%)	47	772	522	68	27
Asparagine (0.1%)	21	512	369	72	78
Asparagine (0.3%)	21	791	586	74	69

Initial pH was buffered to neutrality. 144 hours incubation at 30°C in a 100 milliliters medium in 250 ml bottles on gyrotary shaker.

[1]This table is taken from reference:58 , page:29, table:3

Phosphorus: This element is usually the most abundant nonmetallic element found in fungus ash. Analytical results in the literature are often expressed as percentages of phosphorus pentoxide (P_2O_5 contains 43.64 % phosphorus). The salt uptake of Aspergillus niger was studied. The phosphorus content of mycelium grown in medium containing 1/2500 mole KH_2PO_4 per 100 milliliters was 0.014 milimoles/gram; when the phosphate content of the medium was increased tenfold, the mycelium contained 0.095 milimole of phosphorus per gram. Increasing the phosphate content of the medium to still higher levels increased the phosphorus content of the mycelium slightly.

Any soluble nontoxic phosphate may be used as a source of phosphorus. The most commonly used forms are potassium dihydrogen phosphate or dipotassium hydrogen phosphate or both. Dox (1911-1912) found that the phosphorus in sodium phosphite and sodium hypophosphite was not used by Aspergillus niger. The two potassium phosphates also serve as sources of potassium. The necessity of adjusting the pH of media may be avoided by using mixtures of these phosphates.

Sulfur: The sulfur content of fungus cells is variable; it is dependent on the concentration of this element in the medium, or substrate and on other factors. Some investigators found Rhodotorula gracilis (a Candida species) contained 0.21 % sulfur when the medium contained 0.5 gram of sulfur per liter. When the sulfur content of the medium was reduced to 0.0025 gram per liter, the sulfur content of the cells was 0.10 %.

The sulfur containing compounds found in mycelium include enzymes and other proteins, the amino acids cysteine and methionine, the tripeptide glutathionine, and two vitamins, thiamine and biotin. It was found that the nitrogen content of the medium influenced the sulfur content of the cells of Rhodotorula gracilis. Presumably, this was due in part to the increased protein content of the cells since most

proteins contain methionine and cysteine.

Fungi that are able to utilize sulfate sulfur are designated as euthiotrophic, and those that require reduced sulfur as parathiotrophic. Most species utilize sulfate sulfur.

Other Nonmetallic Elements:

Fungi metabolize, or accumulate non-essential elements. Silicon, selenium, arsenic and chlorine are some of them.

Essential Metallic Elements:

Many of the essential metals may be assumed to function as enzyme activators or as constituents of enzymes. The effects of suboptimal concentrations are manifested in various ways: reduced growth, decreased sporulation, increased or decreased concentrations of various enzymes in the cells, and increased or decreased synthesis of various metabolites. Many of the essential elements are toxic in too high a concentration.

Potassium: Spores and mycelium usually contain more potassium than any other metal. Rennerfelt (1934) found spores of Aspergillus niger to contain 4.9 times as much potassium as did mycelium. The highest potassium content of mycelium was found to be 2.95 percent. Nakamigawa and Okuda (1960) found Mucor mandschuricus to accumulate pyruvic acid in a medium lacking thiamine, but containing potassium or rubidium ions. If pantothenic acid and thiamine were added to a medium containing rubidium, pyruvic acid did not accumulate.

This element is frequently supplied as a phosphate. Other sources include potassium chloride, potassium sulfate, and potassium nitrate.

Magnesium: This element appears to be essential for all fungi. Nicholas and Fielding (1951) found the growth of Aspergillus niger to be proportional to the magnesium content of the medium: 20 miligrams per

liter was required for maximum production of mycelium. Vail and Lilly (1961) found Phycomyces blakesleanus to tolerate magnesium concentrations of 820 miligrams per liter without inhibition of growth, although carotene production was depressed at this concentration. Allomyces arbuscula required magnesium for growth; 9 mg/liter sufficed for maximum growth and 200 mg/liter was toxic.

Various soluble nontoxic magnesium salts are used. The most common probably is magnesium sulfate heptahydrate. Magnesium chloride hexahydrate and nitrate hexahydrate are deliquescent.

Microelements:

Iron, zinc manganese, copper and molybdenum are called micro, minor or trace elements because they are required in low concentrations. Calcium and perhaps other elements may be included in this group. Numerous reports state that this or that microelement is not required by a particular fungus. Judgment is required to evaluate negative results.

Iron: This element is essential for all fungi. As little as 0.1 micro gram of iron in 50 milliliters of medium may be detected by Aspergillus niger. The classical method of determining a requirement for iron is supported by the functional approach, since this element is a constituent of various enzymes including the cytochromes, cytochrome oxidase, catalase and others.

Ferrous or ferric ions satisfy the iron requirements of most fungi. Most commonly used iron source is $Fe(NO_3)_3 \cdot 9\ H_2O$.

Zinc: This element, like iron, appears to be essential for all fungi. Zinc is a constituent, or activator, of a number of enzymes. Grimm and Allen (1954) found a zinc concentration of 0.001 ppm to be sufficient for optimal growth of Ustilago sphaerogena. (Reference: 31). The reduced growth and sporulation of fungi cultured in media containing suboptimal concentrations of zinc (and other microelements) is

readily apparent. Most commonly used zinc source is $ZnSO_4.7\ H_2O$.

Manganese: A requirement for manganese is difficult to establish for some fungi using classical methods. Nicholas (1952 Reference:53) found the mycelial weight of Aspergillus niger to be little affected by the omission of manganese from the medium, but very few spores formed in the absence of added manganese. Donald et al. (1952 reference:20) reported a 50 percent reduction in growth of A.Niger when manganese was omitted from media purified by certain techniques.

A common source of manganese is $MnSO_4.H_2O$.

Copper: The essential nature of copper has been demonstrated by classical methods. Steinberg (1950 reference: 65) found that omission of copper from a highly purified medium resulted in a reduction of growth of Sclerotium rolfsii etc. Growth of Aspergillus niger was proportional, within limits, to the copper concentration of the medium. As little as 0.05 microgram per 50 milliliters could be detected. A.niger changes from yellow to brown and then to black as the copper concentration is increased.

A number of enzymes, including tyrosinase and laccase, contain copper. A most suitable copper compound is again a sulfate, $CuSO_4.5H_2O$

Molybdenum: Steinberg,using the classical method, found that Aspergillus niger required more molybdenum when cultured on a medium containing nitrate nitrogen than when ammonium nitrogen was used. Extremely low concentrations are detected. Nicholas and Fielding (1950 reference: 54) found the addition of 0.1 microgram of molybdenum to 50 milliliters of purified medium resulted in an increase of 57 milligrams of mycelium.

Molybdenum is known to be required for the formation and activation of nitrate reductase in fungi and other organisms. Nicholas and Nason (1954 reference: 55) showed that Neurospora crassa nitrate re-

ductase is a flavoprotein containing molybdenum. Molybdenum was not replaced by iron, zinc, manganese, nickel, silver, tungsten, chromium, vanadium, cobalt or boron.

Medina and Nicholas (1957, reference:49) summarized the steps in the reduction of nitrate to ammonia by Neurospora crassa as follows:

Nitrate $\xrightarrow{Mo}$ nitrite $\xrightarrow{Cu,Fe}$ hyponitrite $\xrightarrow{Cu,Fe}$ hydroxylamine $\xrightarrow{Mn}$ ammonia

Commonly used molybdenum compounds are $Na_2MoO_4 . 2H_2O$ and $(NH_4)_6Mo_7O_{24} , 4 H_2O$.

Calcium: The calcium requirements of fungi was investigated and it was found that Aspergillus niger or Fusarium oxysporum did not require it. Calcium doesn't appear to be required for the growth of Chaetomium species, but it frequently increases the formation of perithecia. More investigation will be required to determine the role of calcium in fungi. To be on the safe side traces of calcium may be added to the medium in the form of $CaCl_2.6 H_2O$ usually.

Scandium, Vanadium, and Gallium: Some species of fungi require these elements and show reduced growth in highly purified mediums not containing these elements.

Essential Elements not Required by Fungi:

Boron, fluorine, sodium, chlorine, iodine, sodium, cobalt, and vanadium are essential for various organisms, but none of them appear to be required by fungi.

Nonessential Metals:

Often fungi may absorb non-essential metals if they are present. Presumably, non of these metals are inert, and in sufficient concentration they may be toxic.

Examples of media from different references will be given later.

Vitamins and Other Organic Growth Factors:

The term growth factor, means an organic substance which in minute amounts is necessary or stimulatory for growth and does not serve simply as an energy source. The growth factors are to be considered indispensable for all fungi, just as vitamins are for animals. A fungus can be completely incapable of synthesizing a certain growth factor, or the biosynthesis capacity may be more or less reduced. The capacity to synthesize a certain growth factor may change with time in one strain as a result of changes in the genetic or metabolic mechanisms of the cells. The possibilty of inducing such mutations artificially is studied extensively by the modern branch of biology called biochemical genetics. It should also be mentioned that variations in biosynthetic ability may occur during the life cycle of a fungus. Below table shows four common growth factors.

Table:XII[1]

A Comparison of the Activity of Four Common Growth Factors

Growth factor	Amount of growth factor (µg) necessary for growing 1 mg mycelium	Lowest concentration of growth factor giving maximum growth rate (µg/ml)
Biotin	0.00025	0.0001
Thiamine	0.0004-0.0025	0.001
Pyridoxine	0.0003-0.0007	0.001
Inositol	1.1-1.7	0.5

Other common growth factors are riboflavine, pantothenic acid, p-aminobenzoic acid and the folic acid group, fatty acids, nucleotide constituents and substances active in low concentrations together with some unidentified growth factors. The requirement differs from fungus to fungus. However there are some regularities like being able of the fungi to synthesize the necessary folic acid coenzyme usually and the thiamine requiring of every biotin deficient fungus.

[1]This table is taken from reference: 2 , page:492, tableI

In studying the effects of corn steep liquor and thiamine on glutamic acid fermentation of hydrocarbons (reference: 70) natural nutrient were added to the medium and the results were as follows. A newly isolated strain identified as corynebacterium was used in the experiments.

Table XIII[1]

Effect of Natural Nutrients on L-Glutamic Acid Production

	Yeast Extract	Meat Extract	Potato Extract	Peptone	Corn Steep Liquor	No addition
Amount added(mg/l)	10-100	10-100	10-100	10-100	10-100	---
L-Glutamic acid (g/l) 7 days	0.26-0.03	.65-.47	.39-.50	.12-.35	.86-.37	.2-.17
9 days	.51-.03	.6-.97	.35-.72	.19-.57	.81-.76	.24

Since the stimulatory effect of corn steep liquor was apparent effects of biotin and vitamin mixtures were tested and it was found that biotin had very little effect. Then the individual vitamins were tested indicating the high efficiency of Thiamine-HCl. The results are as follows:

Table XIV[2]

Effect of Individual Vitamins on L-Glutamic Acid Production

	p-Aminobenzoic acid	Folic Acid	Inositol	Nicotinic acid	Ca-pantothenate	Pyridoxal HCl	Riboflavine	Thiamine HCl
Amount added µg/liter	20	10	50	10	30	10	50	10
L-Glutamic acid (g/l) 7 days	0.21	0.36	0.30	0.29	0.35	0.28	0.60	2.00

Several references study the same effects on the growth of protein rich fungus Candida Lipolytica in hydrocarbons. Reference: 7 stu-

[1] Table XIII is taken from reference: 70 , page:2, table:1

[2] Table XIV is taken from reference: 70 , page:3, table:3

dies the effect of yeast extract and the effects of the individual vitamins in the yeast extract on the growth of Candida Lipolytica in hexadecane. The result of the studies is tabulated below:

Table XV[1]

Growth of Candida Lipolytica in Hexadecane in the Presence of Various Growth Factors

Growth Factor	Amount added (μg /liter)	% yield	Time of cell Division
None	----	0	---
Yeast Extract	1000	97.7	1 hr 50 min
Biotine	1	84.9	2 " 50 "
Thiamine	100	83.9	2 " 30 "
Riboflavine	10	78.6	3 " 30 "
Ca-Pantothenate	100	84.3	2 " 0 "
p-Aminobenzoic acid	100	69.0	3 " 30 "
Nicotinamide	150	89.3	2 " 0 "
All of the last six factors added together		87.8	1 " 50 "

It is seen that some of the growth factors help the growth more than the others. The important thing to note here is that, most of the growth factors present in the yeast extract don't help the growth as the yeast extract itself even if all of the six factors are present in a medium. Therefore yeast extract is the best growth factor in the growth of Candida Lipolytica in hexadecane. We can generalize this fact by assuming that it applies for the growth of Candida Lipolytica in any other hydrocarbon.

Some Illustrative Examples of Nutritive Media:

Since the chemical constituents of the nutritive media is completed by the vitamins and organic growth factors it is helpful at

[1]This table is taken from reference:7 , page:529, tableau IV

this point to mention some illustrative examples of nutrient media prepared by different references for growing Candida Lipolytica or another Candida species in hydrocarbons.

The nutritive medium in reference: 7 that grows Candida Lipolytica in C_{10} , C_{12} , C_{14} and C_{16} alkanes is composed of the following for one liter of medium: NH_4Cl 2.5 grams; KH_2PO_4 7 grams; Na_2HPO_4 1.2 grams; $MgSO_4.7\ H_2O$ 0.2 grams; yeast extract 1 milligram; alkane 5.6 gr.

The nutritive medium in reference:63 that grows Candida Lipolytica in alkanes derived from petroleum and coal tar is composed of the following per liter of tap water: 2.5 grams K_2HPO_4; 5.0 grams NH_4NO_3; 1.0 gram $MgSO_4.7\ H_2O$; 0.1 gram yeast extract.

The nutritive medium in reference:11 that grows yeasts and bacteria on C_{10} or higher hydrocarbons is composed of the following: 2 grams $(NH_4)_2HPO_4$; 1.15 grams KCl; 0.65 gram $MgSO_4.7\ H_2O$; 0.17 gram $ZnSO_4$; 0.045 gram $MnSO_4.\ H_2O$; 0.068 gr $FeSO_4.\ 7\ H_2O$; 0.025 gram yeast extract; 200 milliliters tap water and rest distilled water to make one liter.Hydrocarbon is added in portions.

The nutritive medium in reference:30 that grows Candida Lipolytica on 10 grams of crude oil per liter is composed of the following besides the crude oil: $(NH_4)_3PO_4$ 0.4 gram ; $(NH_4)_2SO_4$ 0.2 gram; KH_2PO_4 0.2 gram; $MgSO_4$ 0.1 gram in 200 milliliters of water.

4-Physical Environment of the Fungi:

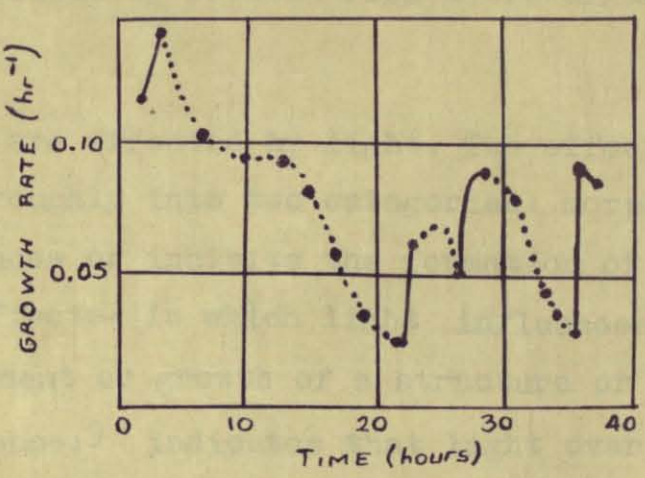
a)Temperature

Temperature has a major effect on all cellular activities. Below 0^oC fungal cells may survive but rarely grow, and above 40^oC most cells stop growing and soon die. Between these temperatures, fungal activities increase and decrease with the degree of temperature because enzymatic reactions, behaving like the ordinary reactions, double or triple their rate with a 10^oC rise in temperature within certain

limits. However, enzymes begin to suffer thermal inactivation at higher temperatures , often as low as 30°C and frequently above 60°C. Under a given set of conditions, an enzymatic reaction achieves a maximum rate at a certain temperature known as the optimum. The incubation temperature for Candida Lipolytica strains is given as 26°C by American Type Culture Collection. At the Hıfzısıhha Institute of Hygiene in Ankara Candida Lipolytica is incubated at the room temperature which varies between 20° and 23°C. The optimum temperatures reported by sources growing Candida species in hydrocarbon containing mediums are generally higher than the room temperature. Reference: 7 gives the optimum temperature to between 32°C and 42°C. Reference: 44 states that the experiments with Candida were done at 30°C ± 1°C. Reference:51 states that 30°C is the optimum temperature for the utilization of hydrocarbons by Candida. The effect of 30°C on growing Candida species in hydrocarbons is best followed from the below graph which shows the growth rate of Candida on octadecane at 30° and 25°C. The temperature was 30°C when the graph is with straight continuous lines and it was 25°C at the regions where the graph is in the form of broken lines. The rise in growth rate is apparent as the temperature is made 30°C. (Taken from reference: 36, page:1535, figure:4)

Figure: 2

Growth Rate of Candida on Octadecane at 30°and 25°C



Low temperatures may be used to preserve viable cultures or spores, but it would be advisable first to make tests with the organism in question. During slow freezing, crystals of ice form around the cells causing dehydration and injury to the cells. Rapid freezing reduce cellular injury.

Most fungal cells are killed by brief exposure to temperatures of 60°C. Thermal death point, which is the lowest temperature required to kill all cells in ten minutes, of six fungi were compared. They ranged from 47°C to 60°C. Moist heat is often more effective in killing cells than dry heat. In moist heat the most resistant fungus was killed in 12 hours at 55°C, but in dry heat this species was not killed by 20 days at 70°C.

b) Hydrostatic Pressure

The parameter of hydrostatic pressure in relation to life processes has generally been neglected. Generally physiological and biochemical studies are made at one atmosphere which is not even mentioned but assumed. However certain studies indicate to the importance of hydrostatic pressure. It was shown that in a certain experiment 4.12 mg ethanol was produced per6ml at one atmosphere. At 600 atmospheres the yield became 0.95 mg/6 ml, whereas it became 0.15 mg/6 ml at 1000 atmospheres. However by the means present effect of pressure on utilization of petroleum by protein-rich micro-organisms won't be tested.

c) Light

Many fungi are effected by light. The effects of light on fungi may be divided roughly into two categories: morphogenetic effects-in which light induces or inhibits the formation of a structure-and non-morphogenetic effects- in which light influences the rate or the direction of movement or growth of a structure or the synthesis of a compound. Reference:9 indicates that light over 200 lux is bad for some fungi.

d)Effects of Radiation

Fungi, like all other organisms, are exposed to the natural radiations of the environment. These radiations may be damaging, beneficial or innocuous depending on the nature of the fungus and the nature of the radiation. Although ionizing radiation kill most of the fungi, there is an extensive body of literature on inducing of mutations with ionizing radiations of various types and energies. These mutations may be important when they change the growth characteristics of the fungus.

It is important to note here the morphological events that occurred in Candida Utilis after ultraviolet irradiation. They observed first a swelling of the vacuole accompanied by a gradual decrease in cell size and suddenly followed by collapse of the cell with apparent precipitation of the cytoplasm. Since sunlight has ultraviolet radiation exposure of Candida strains to sunlight in great quantities may result in the same thing.

5-Kinetics of Fungal Growth:

It is evident that no generalized treatment is possible to the kinetics of fungal growth. Yeasts and presumably other fungi growing in a unicellular manner, can grow exponentially. On the other hand, growth of large fleshy fungi or of fungal pathogens on plant or animal hosts has not been analyzed and is undoubtedly complex. Between these extremes are the situations in artificial culture where fungi grow in essentially undifferentiated form as filamentous organisms. In these cases we can generalize to the extent that growth is apical and that the rate of apical growth, i.e., linear extension, is constant under uniform conditions. The problem arises in attempting to analyze growth in three dimensions. In some fungi, a cubic relation has been shown to obtain. In others, no meaningful, mathematical relation is apparent.

In exponential growth since growth occurs at a constant rate and

since the number of cells present at any time is determined by the number at some preceding time growth can be expressed as $dN/dt = kN$. Integrating and putting in the constant of integration the expression becomes

$$N = ce^{kt}$$

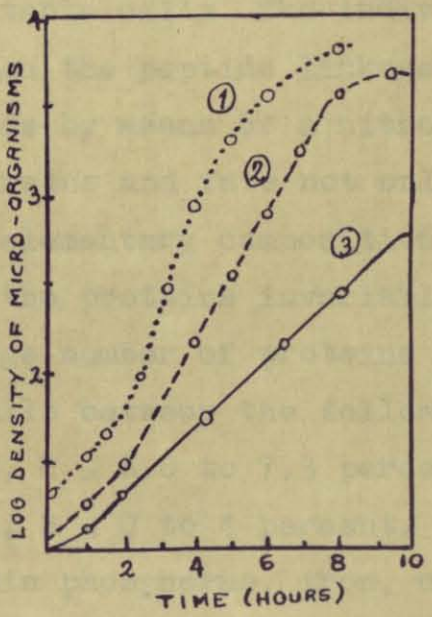
where N is the number of cells at time t, "k" is the proportionality constant and e is the base of natural logarithms. From this it follows that plot of the log of cell number versus time should give a straight line whose slope is "k", is a measure of the growth rate. The constant c represents the intercept on the log N axis and would be the cell number at zero time.

The graph of this relation on arithmetic graph paper shows that first there is a lag phase where no cell division occurs but the cell enlargement and increase in metabolic activity may occur. Then comes the acceleration phase where increase in rate of cell division from zero to some value occurs. After that is the exponential phase where propogation at a uniform rate of cell division occurs. Next phase is characterized by a decreasing rate of cell division and is called the deceleration phase. Next phase is the cessation of cell division called the stationary phase. Here cell division occurs but it is balanced by death of cells. Then there is a phase of decline with a decrease in number of viable cells. A typical relation like this was obtained in the growth of Candida Lipolytica on hexadecane, in reference: 7 . In growing micro-organism on hexadecane Azoulay and Senez used three different variations of Candida Lipolytica as explained in this reference. The first type was a grand colony of the strains while the second was a small colony of the strains and the third was a mixture of the two as identified by the name savage strains (souche sauvage). The results are shown in figure: 3 next page. It is strange to see that the savage strains had a growth rate lower than both of the colonies although it was a mixture of both of these colonies. This result urge isolation of such colonies to get better growth rates in the fu-

ture.

Figure: 3[1]

Growth of Candida Lipolytica on Hexadecane
(Curve 1 grand colony; curve 2 small colony
curve 3 savage strains)



To measure rates of growth, any quantity known to be proportional to growth can be measured. Dry weight measurement is probably the most widely used but it is a destructive test since the organisms are killed during drying. Cell volume determination by using a centrifuge can be convenient in certain specialized cases. Techniques employing changes in number of cells are restricted to those instances where growth occurs as in unicellular organisms. In certain cases growth can be measured indirectly by following the metabolic activity of the organism. Such measerements can include the respiratory activity (CO_2 and O_2 formation), product formation (acid, pigments etc), disappearance of substrate, and enzymatic activity. Cell volume determination and substrate disappearance are very simple methods applicable in any laboratory. Since it is desirable to have populations growing in a constant environment it is necessary to supply fresh medium as the substrate and nutrients disappear in a closed system.

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[1] Figure: 3 is taken from reference:7 , page:528, figure:3.

D) Biochemistry of Proteins

Proteins are defined as nitrogenous, organic substances consisting wholly on in part of alpha amino acids united through their carboxyl and amino groups. They constitute a major part of the living protoplasm in animal and vegetable cells. The individual amino acids are united to each other through the peptide linkage which unites the carbon atoms of the amino acids by means of a nitrogen linkage. The proteins differ from carbohydrates and fats not only in their function in the organism, but also in elementary composition. In addition to carbon, hydrogen and oxygen, the proteins invariably contain nitrogen and generally also sulfur. Large number of proteins from different sources which have been studied falls between the following rather narrow limits: C = 50 to 55 percent, H = 6.0 to 7.3 percent, O = 19 to 24 percent, N = 15 to 19 percent, S = 0 to 4 percent. Proteins have also been described which contain phosphorus, iron, copper, iodine, manganese, zinc and other elements (reference: 32).

Almost all soluble proteins form colloidal solutions of the emulsoid type thus requiring large concentrations of electrolytes for precipitation. Although proteins generally precipitate in an amorphous form from their solution many of them have been isolated in the form of characteristic crystals. The proteins are precipitated from their solutions by salts of heavy metals such as silver nitrate, lead acetate; by acids such as tannic acid and phosphotungstic acid and by many dyes and detergents. These precipitation reactions are probably due, in part at least, to the formation of insoluble compounds of the protein and the precipitating agent and in part to the colloidal character of the protein solutions. To have proteins in colloidal solutions indicates either that the molecules of protein are so large that even when dispersed in molecular solution they have the properties of size and surface which characterize colloidal particles, or else that there is some force connected with protein molecules which

predisposes them to form aggregates of colloidal size. Because of the large size and complexity of the protein molecules their molecular weights cannot be determined with a high degree of accuracy. Their molecular weights are generally between 10 000 and 1 000 000 although as high as 60 000 000 has been reported. Under changing conditions of temperature and concentrations of disperse medium, hydrogen ions, and electrolytes, emulsoids have the property of imbibing large quantities of water and setting into semirigid gels. Substances such as most emulsoids (soaps, proteins etc) which decrease surface tension tend to accumulate at surfaces, while other substances such as sodium chloride which increase surface tension tend to be less concentrated at surfaces.

1-Physical and Chemical Properties of the Proteins:

Pure proteins are generally tasteless, though the predominant taste of protein hydrolysates is bitter. Pure proteins are odorless. When heated they turn brown and char and give off the odor of burning feathers or hair. Besides these major characteristics and the properties mentioned in the introductory part of this section (page:54-55) proteins have other important physical and chemical properties which will give a general idea for their handling, isolation and detection. These properties will be mentioned one by one in this section.

a)Solubility

It is better to discuss the solubility of protein when discussing the individual classes of proteins since the solubility of different classes of properties are different. This will be taken later.

b)Isoelectric pH Values of Proteins

It is believed that proteins behave as molecular solutions of amphoteric electrolytes which exhibit typical colloidal properties because of the large size of the individual molecules. These large molecules contain reactive amino and carboxyl groups which are capable

of entering into true chemical combination with acids and bases. Considered as amphoteric electrolytes, the proteins combine with acids and bases to form salts which ionize into a colloidal protein ion and one or more crystalloidal ions. A protein at its isoelectric point is either entirely without charge-i.e., completely unionized-or else according to the zwitter-ion hypothesis carries equal number of positive and negative charges due to the complete dissociation of equal numbers of acid and base groups in the molecule. In solutions acid to their isoelectric points the proteins exist as positively charged ions, capable of combining with negative ions to form salts, while in solutions alkaline to their isoelectric points proteins exist as negatively charged ions which can combine only with positive ions. The solubility of the proteins, especially those proteins which resemble the suspensoids in their colloidal behavior, is either negligible or at a minimum at the isoelectric points. Other physical properties of the proteins such as viscosity, osmotic pressure, swelling etc are also at a minimum at the isoelectric points. Because of these reasons isoelectric points of proteins are of extreme importance in the protein chemistry. The isoelectric point of common proteins show a great difference varying between pH = 1 and pH = 12 .

c)Crystallization of Proteins

The crystallization of proteins often may be expedited by the addition of a salt such as ammonium sulfate or sodium chloride and adjustment toward the isoelectric pH. The addition of definite amounts of alcohol or acetone is occasionally advantageous. The added substances and adjustment toward isoelectric pH decrease the solubility of the protein. The protein is also least dissociated at the isoelectric pH and may crystallize best in this condition.

d)Hydration of Proteins

While proteins combine with and hold a certain amount of water through the action of poorly defined adsorptive forces of attraction

the peptide chains contain many polar groups ($-NH_2$, -COOH, -OH etc) which tend to combine with water and become hydrated. The swelling of proteins when placed in aquous solutions is definitely associated with hydration of the molecules, though a number of additional processes may be involved.

Despite the fact that the combination of proteins with water represents reversible equilibria, some of the water is held and the removal of the last traces of water from proteins by ordinary drying procedures is very difficult and often imcomplete. When electrolytes, sugars alcohols and other substances which form complexes with water are added to protein solutions, there is competetion for the water, and the degree of hydration of the protein is decreased.

e) Precipitation of Proteins by Salting Out

When increasingly large quantities of very soluble salts such as $(NH_4)_2SO_4$, Na_2SO_4 are added to protein solutions, the solubility of the protein decreases and at some definite salt concentration the protein may be practically completely precipitated from the solution. Below table shows precipitation of serum protein with ammonium sulfate.

Table: ×VI[1]

Fractionation of Serum Protein with $(NH_4)_2SO_4$

Moles of $(NH_4)_2SO_4$ per liter	Type of Protein precipitated	Percent of total protein
1.38	γ-globulins	20
1.64	α-β-γ-globulins	15
2.05	α-β-globulins	14
2.57	crystalline albumin	32
2.80	crys. albumine etc.	14

Although there are exceptions generally the precipitation of protein is most complete at the isoelectric pH. The effect of temperature upon the solubility of proteins in strong salt solutions is highly variable. Horse carboxyhemoglobin is about ten times more soluble at 0° than at

[1] This table is taken from reference: 27, page:19, table:I

f)Precipitation of Proteins with Positive and Negative Ions

The positive ions most commonly used for protein precipitation are ions of the heavy metals such as Zn, Cd, Hg, Fe, Cu, and Pb. These precipitate proteins from solutions alkaline to the protein isoelectric pH.The metal ions may be removed from the metal proteinate by acidification or by precipitation of the metal by hydrogen sulfide or other agent. Metal protein precipitates are dissolved by the addition of strong alkali. The positive ions of organic bases combine with proteins in the same manner and some of these combinations are insoluble.

Negative ions combine with proteins when in the form of protein + to form protein salts some of which are insoluble. Most common ones are tungstic acid, trichloroacetic acid, picric acid and tannic acid.

It has been shown that acid dyes such as naphthylamine brown combine in definite proportions with proteins acid to the isoelectric pH. Similarly basic dyes such as methylene blue combine with proteins on the alkaline side of the isoelectric pH. Since these combinations are often highly insoluble it is possible to determine the amount of dye combined with a given weight of protein and thus plot a titration curve. The combination of proteins with dyes is of much importance in the staining of tissues and micro-organisms for microscopic examination.

g)Common Reactions of Proteins

Since proteins generally contain some free amino groups, they react with nitrous acid to liberate nitrogen, with replacement of amino group by a hydroxyl group.

Acid groups, R-CO- may be introduced into the free hydroxyl and amino groups of proteins by treatment with acid chlorides and anhydrides.

The -S-S- linkages of proteins may be reduced to two -S-H- groups by treatment with reducing agents such as NaCN, cysteine, glutathione.

Some proteins contain free -SH groups which may be readily oxi-

dized by agents such as ferricyanide, porphyrindin, and iodine. They oxidize -SH group to an -S-S- group.

When proteins are treated with alkaline iodine solution (NaOI) iodine is incorporated into the protein molecule. When proteins are treated with alkaline bromine solution the cystine groups are oxidatively destroyed.

Formaldehyde combines with proteins to change their properties markedly. The chemical reactions involved in the action of formaldehyde upon proteins are obscure. Undoubtedly one of the reactions is combination of formaldehyde with various amino groups in a manner similar to its reaction with the amino groups of the amino acids.

h)Color Reactions of Proteins

The Ninhydrin Reaction: To five milliliters of dilute protein solution which must be approximately neutral in reaction, add 0.5 milliliters of a 0.1 percent solution of triketohydrindene hydrate, heat to boiling for one to two minutes, and allow to cool. A blue color devolops if the test is positive. The test gives positive results with proteins, peptones, peptides and amino acids which possess a free carboxyl and α-amino group. In a concentration of one percent the ammonium salts of weak acids react positively, as do the ammonium salts of strong acids in high concentration.

The xanthoproteic reaction: The addition of concentrated nitric acid to protein solution generally causes the formation of a white precipitate which turns yellow upon heating , the color becoming orange when the solution is made alkaline. This reaction is due the nitration of phenyl rings present in tyrosine, phenylalanine and tryptophan.

Millon's test: Millon's reagent is a solution of mercurous and mercuric nitrates containing nitric acid. When added to protein solution a white precipitate is generally first formed which turns red upon heating. Secondary proteoses and peptones give only a red solution. The reaction is not specific for proteins for it is given by phenols in ge

neral. Since tyrosine is the only phenolic amino acid this test proves the presence of tyrosine.

Millon-Nasse reaction: This is an adaptation of the Millon test and can be used in the presence of considerable quantities of inorganic salts especially NaCl. For this test to five milliliters of dilute protein solution add one milliliter of a 15 percent mercuric sulfate solution in 6N sulfuric acid. Place it in boiling water for ten minutes. Cool the contents for five minutes in water add one milliliters of one percent $NaNO_2$. A deep red color indicates tyrosine.

Hopkins-Cole reaction: When a solution of tryptophan or protein is mixed with the reagent (the reagent is prepared by reduction of oxalic acid with sodium amalgam or magnesium powder) and the mixture is layered over concentrated sulfuric acid, a violet ring appears at the interface of the liquids.

Biuret test: If a strongly alkaline solution of biuret is treated with very dilute copper sulfate, a very deep violet color is obtained. This reaction is given by substances containing two $-CONH_2$ groups joined either directly or through a carbon or nitrogen atom. Peptide structures as found in proteins and their derivatives also give the biuret test.

1)Denaturation and Coagulation of Proteins

Coagulation and precipitation of proteins from their solutions by heating is one of the oldest processes of deproteinization of tissue extracts. Most of the proteins are coagulable by heat. Heat coagulation and precipitation occur best near the isoelectric pH of the protein. Heat coagulated protein is generally soluble in acids, alkalies and strong urea and salicylate solutions and reprecipitated as the acid or alkali is neutralized. The presence of water is necessary for heat coagulation. Native protein which has been dried and heated to $100^{\circ}C$ retains its solubility.

Heating the protein causes disorganization of the peptide chains as the result of breaking cross linkages and other actions, with resultant unfolding of the chains and marked changes in properties. This process is called denaturation. When the solution is neutralized, the denatured protein is precipitated at its isoelectric pH which is generally somewhat higher than that of the original protein. Proteins possess more or less characteristic coagulation temperatures which may be altered by changing pH. The coagulation temperature is lowered by the addition of small amounts of acid or alkali, and it can be raised by the addition of much salt such as NaCl.

Protein denaturation involves changes in the molecule which make it insoluble in solvents in which it was originally soluble. Besides heat treatment, treatment with alcohol, acetone and other organic solvent leads to denaturation. Long chain alkyl sulfates (wetting agents) such as sodium dodecyl sulfate are extremely efficient agent for denaturation of proteins, milligram per milliliter solution being sufficient. The initial action of proteolytic enzymes may cause denaturation before any essential peptide hydrolysis occurs. A protein often slowly becomes denatured as a result of standing in solution, even in water, for a protracted period. The denaturation of proteins is caused by various physical agencies besides heat like exposure to x-rays, ultraviolet light and visible light in the presence of a photosensitizer. Violent shaking of a protein solution leads to denaturation as a result of surface action.

Usually denaturation is irreversible since up to this day it has been possible to reverse entirely or partly the denaturation of only a limited number of proteins.

2-Classification of Proteins:

a) Simple Proteins

Simple proteins are defined as those proteins which upon hydroly-

sis yield only amino acids or their derivatives. Simple proteins are further classified according to their solubilities and other characteristics.

Albumins: Soluble in salt-free water and coagulable by heat.

Globulins:Insoluble in salt-free water but soluble in neutral solutions of salts of strong bases with strong acids, such as NaCl; coagulable by heat.

Glutelins: Simple proteins insoluble in all neutral solvents, but readily soluble in very dilute acids and alkalies.

Prolamins: Soluble in 70 to 80 percent alcohol, insoluble in water, absolute alcohol and other neutral solvents.

Albuminoids: All fibrous proteins which have a supporting or protective function in the animal organisms.

Histones: They are soluble in water and insoluble in dilute ammonia, and in the absence of ammonia salts, insoluble even in excess of ammonia. They yield precipitates with solutions of other proteins and a coagulum on heating which is easily soluble in very dilute acids.

Globins: Their solubility characteristics are similar to histones.

Protamins: Simpler polypeptides than the proteins included in the preceding groups. They are soluble in water and uncoagulable by heat. They possess strong basic properties and form stable salts with strong mineral acids.

b)Conjugated Proteins

Conjugated proteins are composed of simple protein combined with some nonprotein substance.

Nucleoproteins: They are composed of simple basic proteins (protamin or histone) in salt combinations with nucleic acids.

Glycoproteins: They are composed of simple proteins combined with some carbohydrate group other than that of a nucleic acid.

Phosphoproteins: They are compounds of protein molecule with phosphoric acid other than a nucleic acid .

Chromoproteins: They are compounds of proteins with a metal containing

proshetic group (proshetic group is the group that contains the nonprotein substance).
Lipoproteins: They are the compounds of protein molecule with lecithin cholesterol, etc.

c)Derived Proteins

This class of proteins includes those substances formed from simple and conjugated proteins. Derived proteins are subdivided into primary derived proteins and secondary derived proteins.

The primary protein derivatives are substances formed from some of the simple and conjugated proteins on denaturation.Their classes are
Proteans: These are insoluble products which apparently result from the incipient action of water, very dilute acid or enzymes. This is probably an early denaturation stage.
Metaproteins: These are products of the action of dilute acids and alkalies whereby the molecule is so far altered as to form products soluble in weak acids and alkalies but insoluble at their isoelectric points.
Coagulated Proteins: They are insoluble products which result when isoelectric solutions of the protein are denatured by heat, alcohol, ultraviolet light, mechanical shaking etc.

The secondary protein derivatives are substances formed during the hydrolysis of the protein molecule. As hydrolysis proceeds the intact molecule is split up into a series of smaller fragments designated by the below groups:
Primary Proteoses: These are soluble in water, noncoagulable by heat, precipitated by concentrated nitric acid, and precipitated by half saturated ammonium sulfate solution.
Secondary Proteoses: These are soluble in water, noncoagulable by heat and precipitated with ammonium sulfate.
Peptones: These are soluble in water, noncoagulable by heat, and not precipitated by saturating their solutions with ammonium sulfate.

peptides: These are characterized as combinations of two or more amino acids, the carboxyl group of one being united with the amino group of the other.

3-Hydrolysis of Proteins:

All proteins yield amino acids when hydrolyzed. Some proteins yield only amino acids when hydrolyzed (simple proteins) while others produce some other type of molecule besides the amino acids. Since proteins are composed of amino acids united with peptide bonds hydrolysis involves the hydrolysis of these peptide linkages. These linkages are resistant to hydrolysis and require prolonged boiling with relatively strong acids or alkalies. The acids most commonly used are hydrochloric 3 to 12 Normal (commonly 6N), sulfuric 4 to 8 N (commonly 8N), and hydroiodic, 57 percent. Among bases 5N sodium hydroxide and 14 percent barium hydroxide are commonly used.

Acid hydrolysis of proteins is generally preferred. Alkaline hydrolysis although efficient in liberating the amino acids, partly converts the pure optically active forms of the proteins into racemic mixtures. Acid hydrolysis doesn't do that. Both acid and alkaline hydrolysis lead to decomposition and loss of some of the more unstable amino acids.

Proteolytic enzymes such as trypsin are frequently used for the hydrolysis of proteins. This process is slow and incomplete but it does not cause racemization or decomposition.

Acid hydrolysis of proteins generally causes the formation of a black or brownish-blach amorphous precipitate called humin or melanin. The production of humin is a complex process as yet incompletely understood. The insoluble humin may be seperated and its nitrogen content may be determined referred as the humin nitrogen.

Hydrolysis of proteins by acids or alkalies splits off more or

less ammonia, the amount depending upon the particular protein concerned. If protein is hydrolyzed with acid the ammonia is retained in solution as the ammonium salt whereas in alkaline hydrolysis it is boiled out of the solution.

4-Classification of Amino Acids:

About two dozen amino acids have been definitely established as occurring in proteins. These amino acids differ in characteristic especially in their solubilities. There are established reactions most of which are color tests to identify the individual amino acid.

a)Aliphatic Amino Acids

Glycine:It is soluble in water and saturated ammonium sulfate, but insoluble in alcohol and ether. It melts with decomposition at 232°C.

Alanine: It is easily soluble in water and quite insoluble in alcohol and acetone. Melts with decomposition at 295°C.

Serine: It crystallizes from water solution as thin irregular plates. It melts at 245°C and is soluble in 23 times its weight of water at room temperature.

Cysteine: It is obtained in the form of its hydrochloride since the free amino acid is readily oxidized in dry state and alkaline or neutral solutions.

Threonine: It can be isolated in pure crystalline form.

Methionine: It is soluble in cold water and alcohol and insoluble in ether.

Valine: It is an aminoisovaleric acid.

Leucine: The pure compound is only slightly soluble in cold water or absolute alcohol but it is quite soluble in hot dilute alcohol.

Isoleucine: It crystallizes like leucine in plates. These melt at 280° and are soluble in 26 parts of water, very soluble in hot glacial acetic acid or hot methyl alcohol.

These are the monoaminocarboxylic acids. The second group of aliphatic amino acids are the monoaminodicarboxylic acids. They are:

Aspartic Acid: It crystallizes in rhombic prisms and has a strong acid taste, but it is rather insoluble in water (1 part in 256 parts water at 10°C and 1 part in 18 parts of water at 100°C).
Glutamic Acid: It crystallizes from water in rhombic tetrahedra which melt at 210°C. The addition of HCl to the acid forms the hydrochloride which is soluble in dilute acid but insoluble in concentrated HCl.

The third group of aliphatic amino acids is the diaminomonocarboxylic acids which are basic in reaction. They are:
Lysine and hydroxylysine.
Arginine: It is easily soluble in water but almost insoluble in alcohol

The fourth group of aliphatic amino acids is the diaminocarboxylic acids which is cystine that crystallizes in characteristic hexagonal plates which are only very slightly soluble in cold water and in alcohol. It dissolves readily in mineral acids or in alkali but it is insoluble in acetic acid.

b)Aromatic Amino Acids

Phenylalanine: It is fairly insoluble in water, crystallizing from warm concentrated solutions in small shiny leaves and as needles from dilute solutions. It is soluble in hot water, but insoluble in cold water as mentioned. It is little soluble in ethyl and methyl alcohols
Tyrosine: Of all the amino acids it is the least soluble in water. It is insoluble in ether, alcohol, acetone and glacial acetic acid, but readily soluble in dilute alkalis and acids.
Diiodotyrosine and thyroxine are the other two members of the class.

c)Heterocyclic Amino Acids

Tryptophan:It is quite insoluble in cold water, in cold and hot absolute alcohol, but it is quite soluble in hot water and pyridine.
Proline: It is easily soluble in alcohol and in cold water.
Hydroxyproline: It is easily soluble in water and difficultly soluble in absolute alcohol.
Histidine: It is slightly soluble in alcohol and crystallizes from a

saturated alcoholic solution in thin platelets.

Although there are other amino acids found in special sources their being a protein constituent has not been proven yet.

5-The Isolation and Purification of Proteins:

The isolation of pure proteins from tissues is often an exceedingly difficult task. Proteins are often denatured and their physical, chemical and biological properties are altered by the chemical agents and physical processes employed.

The first procedure in the isolation of protein from a solid tissue generally is extraction with a suitable solvent such as water, dilute salt, acid and alkali solutions, dilute glycerol, alcohol and acetone. If fresh tissue is quickly frozen denaturation is prevented to a degree in the extraction. The protein may then be precipitated and purified by procedures explained before such as salting-out, adjustment to isoelectric pH, etc. Adsorption of proteins upon such agents as $Al(OH)_3$ or $Ca_3(PO_4)_2$ followed by elution at the proper pH, often is of value in their seperation and purification.

It is often very important to determine when a protein preparation is pure. The purity of protein preparations may be established by physicochemical procedures. The three most important methods of this type are based upon the determination of solubility, of migration rate in the electrophoresis apparatus, and rate of sedimentation in the ultracentrifuge. Solubility of a pure protein is constant and if the molecules are homogeneous they will fall at the same rate during sedimentation.

In reference:44 the extraction of protein, polyprotein and polypeptides from protein-rich micro-organisms is done by autolysis. It is mentioned that the same thing can be done with plasmolysis or hydrolysis. Either of the three operations, performed in a container which has an agitator is sufficient to bring the cellular protein out from

the cells. The autolysis can be done either by the enzymes contained by the cells or by the addition of a salt like sodium chloride which make the cellular protein to come out as a result of the change in the osmotic pressure. The protein diffuses out of the cell because of the difference of osmotic pressure within and outside the cells. The reference explains the autolysis performed at 50°C by the addition of protein rich cells into a sodium chloride solution which contained 13.1 grams of sodium chloride in one liter water. No control of pH was done but the mixture was kept at this temperature for ten hours. If the autolysis is performed by the enzymes control of pH may be necessary depending on the nature of the enzyme that is active. At the end of autolysis the autolyzate contained a solution of proteins, polyproteins, polypeptides etc and cell wall material which was filtered later. The cell wall material was cleaned afterwards by extraction with a hexane-alcohol mixture (80 % hexane + 20 % ethyl alcohol). Since the micro-organisms was grown on hydrocarbons this mixture cleaned the traces of hydrocarbons from the cell wall material. The solution containing the protein may be treated with appropriate reagents to precipitate the protein or it may be hydrolyzed further for the analysis of amino acids.

6-Methods of Seperating Amino Acids:

The seperation and purification of the individual amino acids from complex mixtures such as protein hydrolysates is one of most difficult chemical problems.

A method proposed first by Emil Fischer and developed later consists of three main operations: a)The protein is hydrolyzed with HCl or sulfuric acid and the humin is seperated. If hydrolysis was with sulfuric acid, it is removed by barium precipitation. b)Water is removed from the solution by vacuum distillation followed by repeated evaporation with absolute ethyl alcohol and dry HCl to form the ethyl esters of the amino acids. c)The esters of amino acids are subjected to frac-

tional distillation under vacuum. Each fraction contains esters of several amino acids which are seperated by special procedures.

Another method seperates amino acids by fractionation of copper salts. Copper salts are prepared by heating an aqueous solution of the acids with copper carbonate. The copper salts are dried thoroughly and powdered. By extraction with proper solvents the amino acids can be grouped in three classes each class containing several amino acids. Further seperation involves preparation of various salts of the amino acids and many fractional crystallizations.

Other important methods of amino acid seperation include butyl alcohol extraction, electric transport method, seperation by selective adsorption, and most important of all paper chromatography. Paper chromatography is the easiest one among these seperations.

E) Petroleum Chemistry

Since one of the aims in the area is to utilize petroleum as the hydrocarbon source in growth of protein rich micro-organisms in hydrocarbons a brief introduction to petroleum chemistry showing the rough composition of crude oil and various fractions is necessary.

The major constituent of crude oil are hydrocarbons, oxygen compounds, sulfur compounds, nitrogen compounds and metal salts.

Hydrocarbons: They are the most abundant material in petroleum. However the amount shows great variations from one crude oil to the other. Petroleum like Pennsylvania crude oils contain as high as 97-98 percent hydrocarbons. Heavy California crude on the other hand contains only 50 percent hydrocarbons. Hydrocarbons of petroleum are paraffins, cycloparaffins, and aromatics. Table XVII next page shows the percentages of paraffins, cycloparaffins and aromatics in various crude oils. Olefins and other unsaturated hydrocarbons are not present in petro-

leum, at least in appreciable quantities. Hydrocarbons in high boiling petroleum fraction may have very complex structures, containing in one molecule several aromatic and cycloparaffinic rings, as well as long paraffinic side chains connected with the aromatic and cycloparaffinic rings.

Table: XVII[1]

Distribution of Hydrocarbons in Crude Oil

	Aromatics %	Cycloparaffins %	Paraffins %
Pennsylvania	7	18	75
Mid-Continent	11	31	58
East Texas	9	40	51
California	11	54	35

Oxygen Compounds: Oxygen compounds present in petroleum are of the acidic type. Numerous fatty acids from formic to solid high molecular weight acids have been found in various crude oils in minute amounts. The naphthenic acids occur in some crude oils up to two percent. Phenols and such isomers as cresol and xylene have been detected in minute amounts.

Sulfur Compounds: The sulfur content of crude oil vary in a broad range from 0.1 to 5 percent. Greater part of sulfur is in the form of resinous asphaltic constituents. Sulfur compounds have been identified only in low boiling fractions (gasoline).

Nitrogen compounds: They are present in insignificant quantities usually below 0.3 percent.

In addition crude oils usually contain metal salts, mainly of sodium and calcium dissolved in water emulsified in the crude oil.

Boiling Points of Some Paraffins:

The paraffins boiling between 220°C and 290°C are: Tridecane (C_{13}) 234°C ; tetradecane (C_{14}) 252.5°C ; pentadecane (C_{15}) 270.5°C ; hexadecane (C_{16}) 287°C . Boiling point of commercial kerosene is (175°C - 285°C) .

[1] This table is taken from ref:38 page94

EXPERIMENTS TO GROW PROTEIN RICH MICRO-ORGANISMS IN PETROLEUM

A) Apparatus

1-Apparatus to Prepare Petroleum Fractions:

Petroleum fractions were prepared by simple laboratory distillation. The distillation assembly was glass distillation apparatus consisting of a one liter distilling flask and a liebig condenser. Different condensation methods were used as temperature changed. At very low temperatures at the beginning of distillation light petroleum gases came out which were burned by adapting a bunsen burner to the mouth of the condenser.

2-Fermentor:

a)Aeration Part

To design the air sterilizing system approximate quantity of air needed was calculated. The quantity of air needed would be much more than the theoritical quantity of oxygen required shown by the formula on page:27 because it was decided to agitate the medium by air circulation. Air bubbling from the bottom of the fermentor were found to be the best way of agitation since it was able to mix the medium axially to any desired extent. Amount of air pumped would determine the extent of agitation leaving the experimenter free in the future manipulations. Air agitation caused axial flow which was better than radial flow because of the tube fermentor used (see the part 'Fermentor Design'). This is preferred also because of the coagulation of the micro-organisms in the upper hydrocarbon layer.(See figure:10 on page:86) Axial flow were bringing these micro-organisms down distributing them evenly in the medium. Thus calculations were based on the experimentally found amount of air that was producing the desired agitation. It was found that air flow of four liters per minute (240 liters/hour) would be appropriate most of the time.

The aeration part of the equipment began from the compressor. A compressor equipped with a storage tank able to pump four liters per

hour continuously were available. The maximum capacity of the compressor was 160 psia. The storage tank is an advantage since most of the impurities in air like dust and oil particles remain sticking the walls of the tank.

Air from the compressor were passed through a filter to remove dust and oil particles and other contaminants. Air filter was in the form of a cylinder with a radius of five centimeters and a length of nine centimeters. The filter was filled with sterilized cotton that were sterilized from time to time. From the filter air was carried through 3/8 inches O.D. stainless steel pipe to the heater. Stainless steel were found to be the most suitable metal since it was able to stand oxidation at the high temperatures of sterilization. Other metals, oxidizing easily, would interfere the synthetic medium of fermentation. Infact many of these contaminations would be poisonous.

Besides this all other parts of the apparatus would be of glass or stainless steel since fermentation were to be carried in an acidic medium in the presence of abundant oxygen. Besides micro-organisms constituted another corrosive factor since they were able to utilize many of the metals (see metallic elements page: 41 and microelements page: 42). To keep the medium from such contaminations glass apparatus fitted with stainless steel parts were found to be suitable. Stainless steel pipe is also advantageous by its high heat conductivity. Glass apparatus has the advantage of being transparent. Thus the activities within the fermentor were visible. This was important in checking the efficiency of agitation, disappearance of substrate, rate of growth and control of foaming.

To heat four liters of air per minute to 300^{o}C instantaneously electric power was found to be suitable. To heat the stainless steel pipe from all directions and to minimize the losses to the surroundings, the heater was designed in square prism shape. On four walls of this clay bricks with resistances on them were situated. The stain-

less steel pipe passed from the center. So clay bricks being in between resistances and the walls acted somewhat as insulators. The minimum electric power needed to heat the calculated amount of air from room temperature to 300°C were found from the equation:

$$Q = mC_p (T_2 - T_1)$$

where m is 1.18 grams/liter times 4/60 liters per second, C_p is 7.073 calories per gram mole per degree K, T_2 is 300°C and T_1 is 20°C. To convert grams to gram moles 'm' is divided by 28.9 . The equation gives 5.4 calories per second which is 22.6 watts. This constitutes negligible part of the power needed. The greatest amount of power is needed to maintain the heater at 300°C continuously. This needs much power because of the losses from the heater to the surroundings. Minimum of such a loss is calculated from:

$$\frac{q}{A} = \frac{T_2 - T_1}{\frac{x_1}{k_1} + \frac{x_2}{k_2}}$$

where T_2 is the temperature in the heater (300°C), T_1 is the room temperature (20°C), x_1 is the thickness of brick (1.5 cm), x_2 is the thickness of iron heater wall (0.1 cm), k_1 is the thermal conductivity of brick (2.80×10^{-3} cal-cm / cm^2 sec °K) , k_2 is the thermal conductivity of iron wall (1.242×10^{-1} cal-cm / cm^2 sec °K) and A is the surface area of the heater (1440 cm^2). This gave a 'q' of 745 calorie/second which is equivalent of 3120 watts. So together with the power needed to heat the air 3142.6 watts is needed. As an approximation four resistances, one for each wall, of 800 watts were used. Correction for the losses more than the theoritical amount was not made since there were other factors decreasing the losses that were not considered. One such factor is the thin air layer between the bricks and the iron surface which contributes to the thermal resistance.

As a precaution to stopping of the whole heater by a single open circuit and blowing of fuses in the laboratory power was supplied from three mains. So power from one main was decreased and two mains would be on whenever one was off letting continuous operation of the heater.

Temperature of the air coming out from the heater was measured by taking the wall temperature of the stainless steel pipe by means of a thermocouple. Below figure:4 shows the photograph of the heater. For the complete set up of the heater with its dimensions see fig:7 page:79 .



Figure: 4 "Photograph of the Heater"

Since fermentation would take place around 30°C it was necessary to cool the sterilized air. Calculations show that air cools to room temperature after traveling a few centimeters from the heater by diss pating the heat to the room through the steel pipe that has high heat conductivity. From the formula:

$$\frac{q}{L} = \frac{\Delta T_m}{\frac{x_s}{k_s \, 2\pi \, r_m}}$$

where T_m is the mean temperature difference inside and outside the s-

tainless steel pipe, x_s is the wall thickness of the stainless steel pipe (3/64 inches), k_s is the thermal conductivity of the pipe (10 Btu-ft / ft^2 hr ^oF), r_m is the mean radius of the pipe, and L is the length of the pipe. The mean temperature difference was calculated to be 127°C since

$$T_{2m} = \frac{T_2 - T_1}{\ln(T_2/T_1)} = 157^oC$$

In the same way r_m was calculated to be 0.163 inches. Substituting all of these into the formula on page: 75 "q/L" was found to be 50 000 Btu / ft-hr , which is equivalent to 115 calories / cm-sec. As seen on page 74 the heating of air through the same temperature difference needs 5.4 calories per second. Since each centimeter of the tube can dissipate to the room 115 calories through one centimeter of its length each second the cooling of air should be completed within a few millimeters. However some length of the tube becomes hot not because of the heat dissipated by the cooling air but because of the heat carried by conduction through the pipe. Since at least 1.5 to 2 meters of pipe must be extended to the bottom of the fermentor from the heater, part of the tube heated by conduction will also cool and air will reach the fermentor practically at room temperature.

To distrubute the air evenly into the fermentor a vertical tube sparger was fitted to the bottom of the fermentor and sterilized air at room temperature was let through that sparger into the fermentor.

b) Fermentor Vessel

Because of the advantages explained before the fermentor vessel consisted of a pyrex glass tube of 6.9 centimeters outside diameter, 6.5 centimeters internal diameter and 120 centimeters length. Such a glass tube was available and it was made into a fermentor vessel by fitting stainless steel covers with flanges to the bottom and top of the tube. The covers had necessary inlets and outlets.

Top cover has an outlet for the air. A polyethylene tube connected to this outlet leads the exit air into a beaker containing a disinfectant (lysol was used in this case). Thus the micro-organisms escaping by the exit air were captured in the disinfectant. The top cover has an inlet for filling the needed reagents into the vessel. A polyethylene tube, whose end was clipped,was connected to this inlet. Top cover also has a hole having a diameter equal to the diameter of the electrode of a Beckman pH-16 pocket pH meter. This was stoppered by a rubber stopper which was removed when the pH measurement was made (In the first two experiments this pH meter was used. Since it was out of order in the later experiments a paper pH indicator between the range 3.0 to 5.5 was used). The inlet and outlet of a cooler were also situated at the top cover. Since temperature adjustment of the medium was necessary a U-shaped stainless steel cooler (or heater) was extended into the vessel. It would be used as a cooler or heater according to the results of the experiment. Although fermentation was exothermic the medium cooled since time of the reaction was long and heat was lost to the surroundings. This system was used for heating eventually. Hot water at the boiling temperature was taken from the central heating system of the school. The average rate of water through this heater was two liters per minute. Since at most a two liter medium can be used in the fermentor the difference between the entering and exit temperatures of the heating water shows the degrees the medium is heated in one minute. So this system can heat or cool the medium very quickly. However the high temperature of the heating water has the disadvantage of killing the micro-organisms sticking to the walls of the heater. With these the top cover took its final shape (see figure: 5 next page).

The below cover has the outlet for the medium since it is the lowest point of the vessel. The last drops in the vessel drip from this point. To this outlet a polyethylene tube is connected whose end is kept above the liquid level when the fermentor is running.

As mentioned before the air pipe was fitted to the middle of the bottom cover. Bottom cover also has a fitting to insert a thermometer. Thus the final shape of the bottom cover became as seen in figure:6.

Figure:5 "Top Cover"

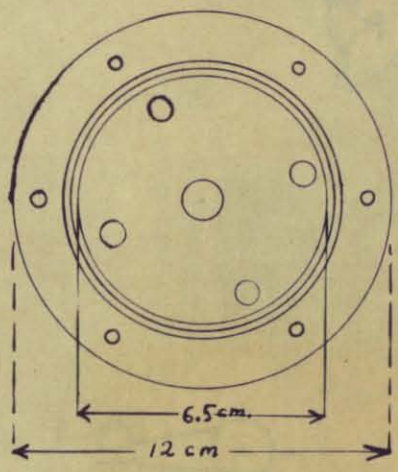
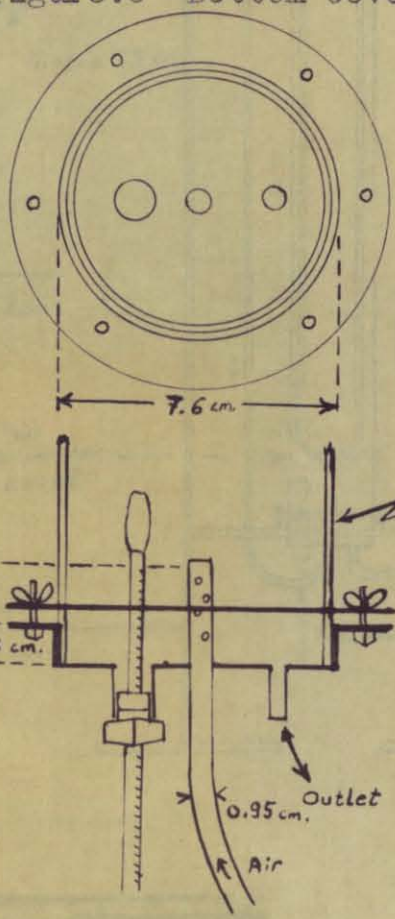
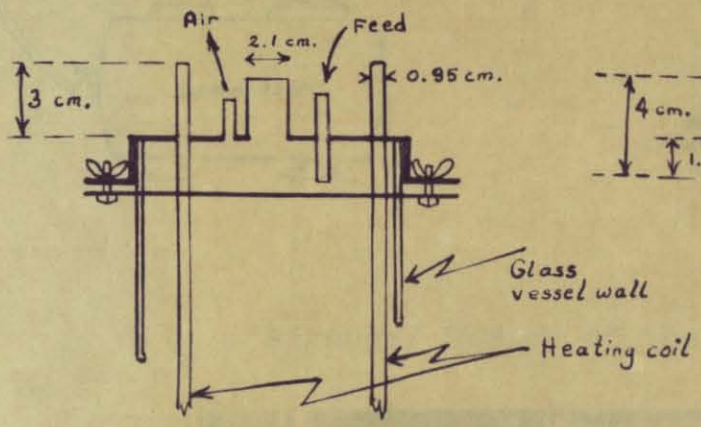


Figure:6 "Bottom Cover"





For the complete fermentor assembly see figure: 7, page: 79.

3-Apparatus for Purification:

During purification many physical, chemical and physicochemical operations were practiced. Most of these were done in simple laboratory apparatus like seperatory funnels, flasks, beakers etc. Washing of cells, hydrolysis and autolysis were tried in the fermentor by air agitation.

Another apparatus used in the purification and identification of the product was a laboratory set up for vacuum distillation. To concentrate the solution containing the micro-organisms, the autolyzate or the hydrolyzate it was necessary to boil the water from the

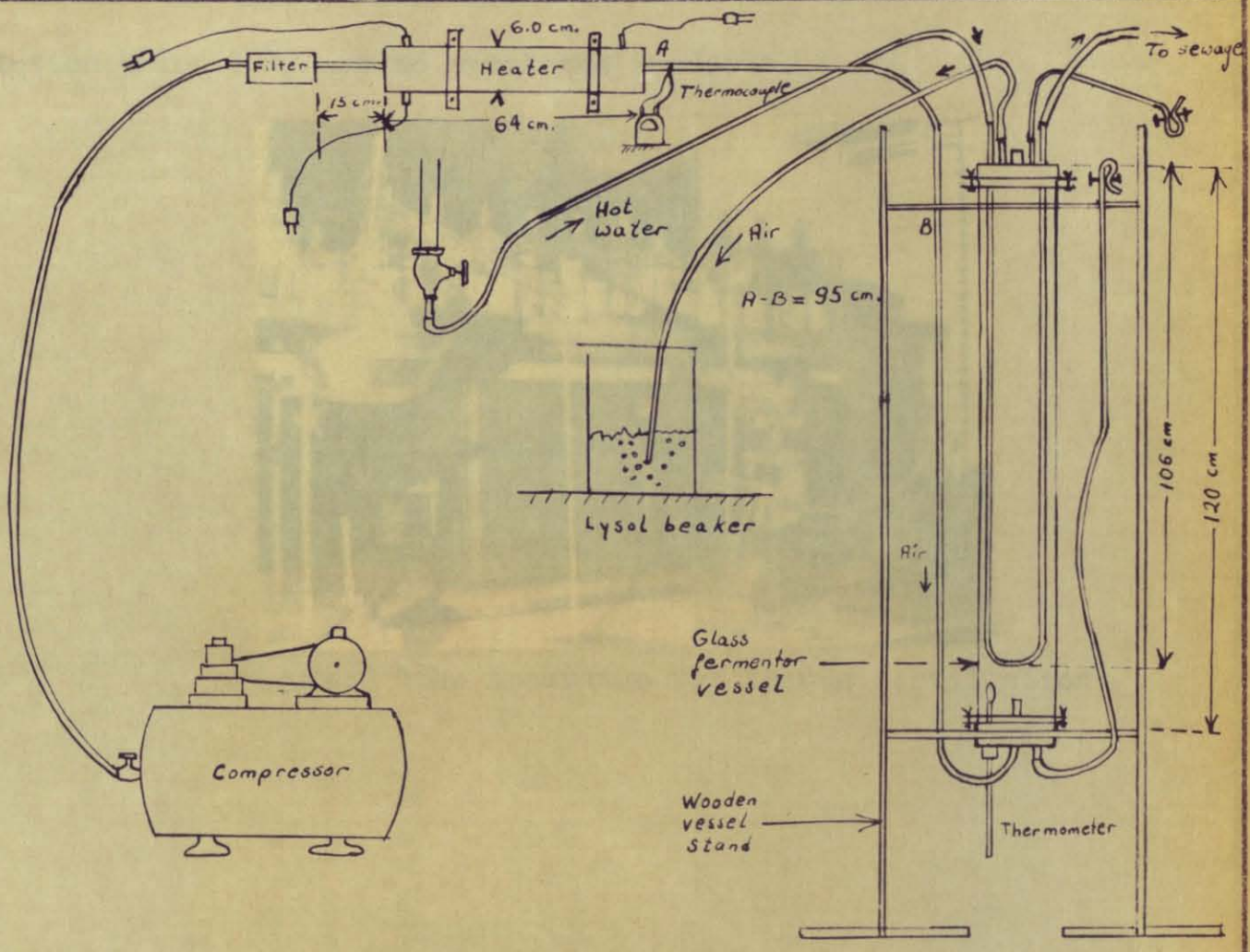


Figure:7 Sketch of the Fermentor

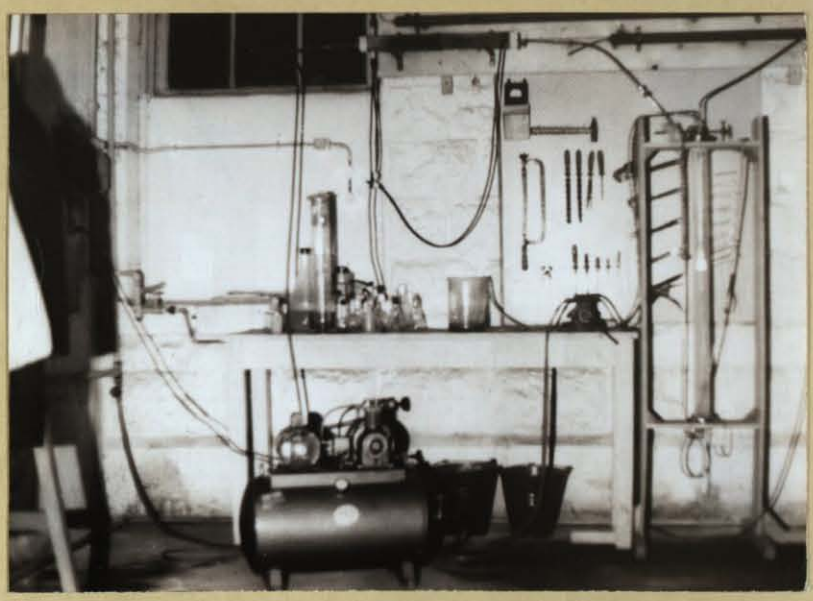


Figure:8 Photograph of the Fermentor

solution below 50°C not to spoil the products.



Figure:9 "The Apparatus for Vacuum Distillation"

B) Experiments

1-Experiment No: I

a) Date of Experiment: February 2, 1967

b) Procedure:

The fermentor was dry heat sterilized at 160°C for two hours at Eczacıbaşı İlaç Fabrikası.

The medium in table XVIII were prepared and sterilized at the same place in an autoclave at 120°C for 15 minutes. (For sterilization of medium see page 16).

Table: XVIII

Medium of Experiment I

$NH_4H_2PO_4$	6.000 gr.
KCl	1.725 gr.
$MgSO_4.7\ H_2O$	0.975 gr.
$ZnSO_4$	0.255 gr.
$MnSO_4.H_2O$	0.030 gr.
$FeSO_4$ dissolved in HCl	0.080 gr.
Yeast Extract[1]	37.5 mg.
Petroleum Fraction (225°C-295°C)[2]	15.0 gr.
Distilled Water to make the solution	1500 ml.

[1] This yeast extract was prepared by weighing 500 milligrams of yeast and making into a solution of 100 milliliters by sterilized distilled water. Keeping this at room temperature for 24 hours causes hydrolysis and the dissolving of water soluble vitamins. 7.5 milliliters of this solution was taken which is the extract of 7.5/100 x 500 =37.5mg of yeast. This yeast extract was not further sterilized not to destroy the vitamins.

[2] In distilling the petroleum clean sterilized apparatus was used and the fraction was not further sterilized since it distilled at elevated temperatures. In fact sterilization by holding at 120°C for long times causes the oxidation of the fraction.

The fraction is distilled from a crude oil taken from İstanbul Petrol Rafinerisi Anonim Şirketi which is identified as a crude from Saudi Arabia (50 %) and Libya (50 %) approximately. The distilled fraction has a specific gravity of 0.840 .

In preparing the medium some of the salts didn't dissolve, but it was sent to sterilization as it was, expecting some of the salts to dissolve in the acidic medium (pH 4) when heated in the autoclave. After the sterilization it was seen that same amount, even more, undissolved salts were present in the medium. It was decided to use the medium for micro-organism growth thinking that micro-organisms would be able to assimilate the element in the form of its salt. The undissolved salts were zinc sulfate, some iron sulfate and an insoluble phosphate formed during heating in the autoclave. The pH was measured and it was found to be four.

Three petri dishes of Candida Lipolytica NRRL-Y 1094 taken from American Type Culture Collection, Maryland-USA were prepared as explained in page 16 and were incubated for 165 hours. These were harvested as explained on page 19 and were taken into test tubes. They were centrifuged at 4830 RPM for 20 minutes. They were washed with five milliliter portions of 0.9 percent NaCl in this way three times. After last centrifuging cell volume was 1.5 milliliters. This was made into a suspension with distilled water.

The medium was transferred into the fermentor under sterile conditions through a sterile funnel (see page 16 for the sterilization of funnel). The suspension of micro-organisms was added to the medium by a pipette.

Aeration began at a rate of 3.5 liters per minute. Air sterilized at 300°C through the heater was used for this purpose. Temperature of the medium was made 30°C.

After four hours of operation under same conditions pH was measured. It was 3.6 . It was made 4.6 by the addition of 1.2 milliliter of concentrated ammonium hydroxide.

After nine hours of operation under same conditions ($T = \pm 3^{\circ}$ 30°C) pH was 4.6 . There was an increase in the amount of foaming at the top of the medium. There was also an increase in the amount of

white greasy micro-organisms sticking to the walls of the fermentor. The turbidity of the medium kept increasing, the medium taking the white color of the micro-organisms.

After eighteen hours of operation turbidity kept increasing. When aeration thus agitation was stopped the colony of micro-organisms sticking to oil droplets were observed floating to the top of the medium.

At the twenty second hour of fermentation the operation was stopped and the medium was transferred to a flask. Lots of micro-organisms stayed in the fermentor sticking to its walls. Some of these were washed down with distilled water, but the washing was not very successful.

The medium was left in this flask for 83 hours. After this time an innoculation was made to a solid Sabouraud medium from this flask. Micro-organisms grew after one day showing that the cells were living in the flask without aeration or agitation.

Then the medium was transferred into an oven and was kept at 65°C for 1.5 hours. After this heat treatment another innoculation to a Sabouraud medium was made. This time no growth occurred proving that these strains are killed by such a heat treatment.

The medium was filtered and was washed with water several times. However, hydrocarbon odor was present after the last washing. After drying these coagulated into lumps of gray color. It is thought that the gray color is due to the presence of carbon particles in the medium as a product of the sterilization of air at elevated temperatures.

A drop of suspension was made with distilled water from these micro-organisms and it was examined through a microscope. Micro-organisms taken from the lump showed the identical shape of Candida species in reference: 23 , page263, figure142d,while the micro-organisms taken from the medium showed the identical shape of Candida Lipolytica cells in reference: 7, page 525.

A general composition test was made to about 100 milligrams of this lump of micro-organisms. The powdered cells were heated in a dry test tube in which was suspended a strip of moistened red litmus paper and across the mouth of which was placed a piece of filter paper moistened with lead acetate solution. As the powder was heated it charred, indicating the presence of carbon; the fumes of ammonia were evolved, turning the red litmus paper blue and indicating the presence of nitrogen and hydrogen; the lead acetate paper was blackened indicating the presence of sulfur, and the deposition of moisture on the side of the tube indicated the presence of hydrogen. At the same time an odor of burning hair was evolved which is characteristic of protein material.

2-Experiment No: II

a)Date of Experiment: March 16, 1967

b)Procedure:

The fermentor was sterilized by passing steam through it for 15 minutes. Steam was supplied by the central heating system of the school. The thermometer of the fermentor showed 100°C during the sterilization.

The salts of the medium in table xix were made into standard solutions containing the appropriate amount of salt per ml of solution such as 10 or 100 milligrams per milliliter. These solutions were sterilised seperately by boiling them for 20 minutes. The medium in table xix was prepared by measuring out the appropriate amounts from these sterilised standard salt solutions. In this way all the salts were dissolved and the formation of precipitates from the ions in the medium during sterilizing were prevented. Needless to say sterilized distilled water was used to make the medium 2000 milliliters. The yeast extract and petroleum fraction were prepared in the same way as explained in footnotes 1 and 2 on page 81. The pH of the medium was brought to 4.5 by the addition of two milliliters concentrated

ammonium hydroxide.

Table: xix

Medium of Experiment II

$NH_4H_2PO_4$	8.000 gr.
KCl	2.300 gr.
$MgSO_4.7\ H_2O$	1.300 gr.
$MnSO_4\ .H_2O$	0.030 gr.
$(NH_4)_6\ Mo_7\ O_{24}$	0.2000gr.
$CaCl_2$	0.040 gr.
Fe (equivalent to 80 mg $FeSO_4$ in HCl)	0.028 gr.
Yeast Extract	0.050 gr.
Petroleum (225°C - 295°C)	20.0 gr.
Distilled Water to make the solution	2000 ml.

Three petri dishes of Candida Lipolytica(CB snr 599 Harrison) taken from the culture collection center in Lausanne were harvested, washed and made into a suspension as explained on page 82 after 48 hours of incubation. Cell volume after the last centrifuging was again 1.5 milliliters.

The medium was transferred into the medium under sterile conditions through a sterile funnel (see page 16 for the sterilization of funnel). The suspension of micro-organisms was added to the medium by a pipette.

Aeration began at a rate of 3.5 liters per minute. Air sterilized at 300°C through the heater was used for this purpose. Temperature of the medium was made 30°C and it was held at this temperature continuously plus or minus 3°C .

(T = 0) Fermentation began in the above explained medium at 30°C, pH 4 , air sterilized at 300°C flowing 3.5 liters per minute.
(T = 3.5 hours) There was little change in the turbidity of the medium, fermentation continued under the same conditions.

(T = 11 hours) Fermentation continued under the same conditions. There was an increase in the amount greasy micro-organisms sticking to the upper parts of the walls of the fermentor. This time the micro-organisms didn't stick to the walls as firmly as in experiment I. They went into the solution with a little amount of liquid poured down the walls of the vessel.

(T = 11.5 hours) Aeration was stopped and the medium was left at room temperature 9 hours. After 9 hours it was started again.

(T = 12 hours, t = 21 hours)[1] While fermentation continued at 30°C, with 3.5 liters per minute air sterilized through the heater, pH measurement was made. pH was made 4.5 by the addition of two milliliters of concentrated NH_4OH. Since there was no visible hydrocarbon layer when the medium was still 8.4 grams of the same petroleum fraction was added.

(T = 19 hours, t = 28 hours) Aeration was stopped and the medium was left at room temperature.

(T = 19 hours, t = 48 hours) The medium was transferred to a flask leaving the top micro-organism plus hydrocarbon layer in the fermentor vessel. (See figure: 10)



Figure: 10
Micro-Organisms Gathering in the Upper hydrocarbon Layer When the Medium is Still

[1] The first T in the parantheses refers to the number of hours fermentation proceeded with aeration, while the second t refers to the time passed since the inoculation of the fermentor vessel which is the sum of times with aeration and non-aeration.

3-Experiment No: III

a)Date of Experiment: March 18, 1967

b)Procedure:

Since the vessel contained the micro-organisms of the second experiment it was not sterilized. The medium presented in table xix, on page 85 was prepared in the same way, the pH was adjusted to 4.5 and this was transferred to the fermentor vessel under sterile conditions. So experiment III may be considered a continuation of experiment II by removing harmful toxins accumulated in the medium excreted by the micro-organisms and adding the exhausted elements. By changing the medium these toxins were removed and the exhausted elements were replaced. Thus the experiment began with a fresh medium containing a great amount of micro-organisms. Since the micro-organisms double their amounts in a definite time having a great initial amount is advantageous.

Temperature of the medium was made 30°C. Aeration began at a rate of 3.5 liters per minute using air sterilized by passing through the filter (no heat treatment).

(T = 4.5 hours) Fermentation continued under same conditions: pH between 4 and 4.5 while temperature was $\pm 3^{\circ}$ of 30°C. At this time aeration was stopped and the medium was left at room temperature for 15 hours.

(T = 4.5 hours, t = 19.5 hours) Since there were no oil droplets visible when the medium was still 16.8 gr. of 225°C - 295°C petroleum fraction was added. pH was made 4.5 by the addition of one milliliter of concentrated ammonium hydroxide. Temperature was adjusted to 30°C and aeration began at the same rate.

(T = 10 hours, t = 25 hours) 0.5 milliliters of Candida Lipolytica (CB snr 599 Harrison) was added to the medium after harvesting from a petri dish incubated for 125.5 hours and washing followed by centrifuging. Temperature was 30°C and pH was 4.5 .

(T = 14.5 hours, t = 29.5 hours) Aeration was stopped and the medium was left at room temperature for 15.5 hours.

(T = 14.5 hours, t = 45 hours) Aeration was started and the temperature was adjusted to 30°C.

(T = 17.5 hours, t = 48 hours) 8.4 grams of the same petroleum fraction was added. pH was measured and was found to be 4.5 .

(T = 22 hours, t = 52.5 hours) 8.4 grams of the same petroleum fraction was added. 70 milliliters of sterilized distilled water was added and pH was measured to be 4.5 .

(T = 23 hours, t = 53.5 hours) Aeration was stopped and the medium was left at room temperature for 41.5 hours.

(T = 23 hours, t = 95 hours) The medium was drained to a flask leaving the micro-organisms within the hydrocarbon layer in the fermentor (see figure:10, page 86).

Table: xx

Reagents Added During Experiment III

Petroleum (225°-295°C)	33.6 gr.
Distilled Water	70 ml.
NH_4OH	1 ml
Candida Lipolytica (CB snr 599) . .	0.5 ml.

4-Experiment No: IV

a)Date of Experiment: March 22, 1967

b)Procedure:

The top hydrocarbon layer containing the micro-organisms was left in the vessel. Rest of the medium was drained (see figure: 10).Thus the drained medium contains only traces of hydrocarbon leaving rest in the fermentor for the next experiment. The new fresh medium was prepared with little modifications on the medium of experiment three.

Table: xxi

Medium of Experiment IV

$NH_4H_2PO_4$	8.200 gr.
KCl	2.300 gr.
$MgSO_4 \cdot 7\ H_2O$	1.300 gr.
$MnSO_4 \cdot H_2O$	0.030 gr.
Fe (equivalent to 80 mg. $FeSO_4$ in HCl)	0.028 gr.
$(NH_4)_6Mo_7O_{24}$	0.050 gr.
$ZnSO_4 \cdot 7\ H_2O$	0.350 gr.
$CaCl_2$	0.040 gr.
Yeast Extract	0.075 gr.
Petroleum (225°C - 295°C)	8.4 gr.
Concentrated NH_4OH	2 ml.

(T = 0) Fermentation started at T = 30°C and pH 4.5 . This time little petroleum was added to the medium since there was a visible petroleum layer from the last experiment in the fermentor.

(T = 1 hour) One petri dish of Candida Lipolytica (CB snr 599) incubated for 193 hours was harvested, washed, centrifuged and transferred to the medium. The pH was adjusted to 4.5 again by the addition of 0.4 milliliters of concentrated NH_4OH .

(T = 6.5 hours) Aeration was stopped and the medium was left at room temperature for 18.5 hours.

(T = 6.5 hours, t = 25 hours) When the medium was still no oil layer was present indicating decreasing of the substrate. Fifty milligrams of yeast extract, 8.4 grams petroleum fraction (225°C - 295°C), 40 milliliters of distilled water and 0.3 milliliters of concentrated NH_4OH were added to the medium. Aeration started at pH 4.5 and T =30°C

(T = 9 hours, t = 27.5 hours) Turbidity increased considerably in the last two hours. 50 milliliters of distilled water and 0.5 milliliters of concentrated ammonium hydroxide were added. pH was 4.5 and

temperature was 32°C.

(T = 11 hours, t = 29.5 hours) Aeration was stopped and the medium was left at room temperature for 18.5 hours.

(T = 11 hours, t = 48 hours) Aeration began at pH 4.5 and T = 30°C.

(T = 12 hours, t = 49 hours)8.4 grams of petroleum (b.p. 225°C to 295°C) were added.

(T = 14 hours, t = 51 hours) 25 milligrams of yeast extract was added. Temperature was kept around 30°C.

(T = 16 hours, t = 53 hours) 8.4 grams of the same petroleum fraction and 50 milliliters of distilled water were added.

(T = 16.5 hours, t = 53.5 hours) Aeration was stopped and the medium was left at room temperature for 19.5 hours.

(T = 16.5 hours, t = 73 hours) Aeration was started and temperature was adjusted to 30°C .

(T = 18.5 hours, t = 75 hours) 8.4 grams petroleum fraction (boiling point 225°C - 295°C), 2 grams of $NH_4H_2PO_4$ were added. pH was measured and was found to be 4.5 .

(T = 21 hours, t = 77.5 hours) Aeration was stopped and the medium was left at room temperature for 14 hours.

(T = 21 hours, t = 91.5 hours) The medium was taken to a flask leaving the top hydrocarbon layer with the micro-organisms in the vessel. The medium was slightly milky which increases in milkiness as it is mixed with the top hydrocarbon layer containing the micro-organisms. This slightly milky solution became slightly yellow on standing at room temperature for two or three days especially if the room was hot. The top hydrocarbon layer with the micro-organisms was drained to a seperate flask. Experiments and methods followed to recover the cells from this hydrocarbon solution in a as clean manner as possible are presented in the following pages.

5-Seperation and Cleaning of Micro-Organisms From Experiments II, III and IV

The hydrocarbon layer containing the micro-organisms consisted of two phases of hydrocarbon and water from the medium. Although most of the cells floated to the upper hydrocarbon layer some cells were present also in the aquous layer from the medium. When a portion of this solution was centrifuged the cells didn't settle to the bottom even at 4830 RPM for one hour. After centrifuging the test tube contained three distinctly seperated layers and a little amount of cells settled to the bottom. The top layer in the test tube consisted of a bulk of cells, the middle layer was the clear hydrocarbon and the lower layer was the aquous solution from the medium of fermentation. Since it was thought that the cells floated because of the hydrocarbon surrounding their walls it was decided to get rid of this hydrocarbon. The methods tried are listed below.

a)One and a half milliliters of an emulsifying agent (Lissapol Nx in 28.5 milliliters of water was added to 10 milliliters of hydrocarbon layer. After shaking a milky solution was obtained which seperated into two layers after one hour. After this seperation the transparent lower layer was centrifuged leaving the milky upper layer in the seperatory funnel. Centrifuging resulted in settling of some micro-organisms, a clear solution and a milky ring adhering to the walls of the test tube at the upper layer. It is thought that this ring is constituted by the emulsifying agent or the by the protein denatured by the emulsifying agent. The settled micro-organisms to the bottom of the test tube had a black line formed by a black precipitate. It is thought that this black precipitate is the humin (see the part 'Biochemistry of proteins').

b)One or two pieces of a detergent was foamed in 100 milliliters of water and 10 milliliters of hydrocarbon layer was mixed with this. After 20 minutes the foams disappeared and the solution was centri-

fuged. The result of centrifuging was the same as in part a, the emulsifying agent case. Only this time a hydrocarbon layer was present above the milky ring.

c)Ten milliliters of the hydrocarbon layer was added to a mixture of 40 milliliters n-hexane and 10 milliliters of isopropyl alcohol. A few minutes after mixing and shaking two clear layers seperated. The yellowish hydrocarbon layer was discarded and the contents of the alcohol layer was centrifuged since most of the cells stayed in the alcohol layer. Centrifuging for 20 minutes gave three layers. The bottom layer was the alcohol and the top layer was the remaining hydrocarbon in hexane. In between the two layers a layer of cells was present. The top layer was discarded easily but the recovery of cells was not possible again.

d)Ten milliliters of n-hexane was added to 10 milliliters of hydrocarbon layer. Two layers were formed: the hydrocarbon dissolved in hexane constituted the top layer with the micro-organisms while the aquous solution from the medium was at the bottom. This bottom layer was discarded since there were not many micro-organisms present in it. Ten milliliters of isopropyl alcohol was added to the top layer. One phase mixture was produced and the micro-organisms sank to the bottom. These were centrifuged and about one milliliter of cells were recovered. Since the hydrocarbon layer drained from the vessel was about 100 milliliters approximately 10 milliliters of cells were present in the fermentor vessel after the fourth experiment.

6-Protein Color Reactions

a)General Composition Test: A few pieces of the recovery of cells as explained in the last paragraph were heated giving off the odor of burning hair. The wet litmus paper at the mouth of the test tube became blue indicating the presence of ammonia. The filter paper wetted with lead acetate charred indicating the presence of carbon, and mois-

ture collected on the test tube's wall indicating the presence of hydrogen.

b)xanthoproteic test: One or two pieces of cells recovered in paragraph d, page 93 was made into a suspension with five drops of water and five drops of concentrated nitric acid was added. The solution became yellow with white precipitates in it. Heating the solution dissolved some of the precipitates. After cooling ammonium hydroxide was added until the solution was alkaline. The color turned deep orange indicating the presence of protein.

c)Millon's reaction: Two pieces of the cells recovered in paragraph d, page 92 was made into suspension with five drops of water and five drops of Millon's reagent was added. Millon's reagent was prepared by digesting one part by weight of mercury with two parts of nitric acid and diluting the resulting solution with two volumes of water. After addition of Millon's reagent the solution was heated and it turned pinkish red indicating the presence of protein. For the theory of these reactions see the part 'Biochemistry of Proteins'.

7-Experiment No: V

a)Date of experiment: March 27, 1967

b)Procedure:

The medium on table XXII, page:95 was prepared in the same manner as before. It was transferred to the fermentor vessel containing some micro-organisms sticking to its walls from the last experiment.

(T = 0) Aeration started at pH 4.5 and T = 30°C.

(T = 4.5 hours) Aeration was stopped and the medium was left still at room temperature for 19 hours.

(T = 4.5 hours, t = 23.5 hours) The medium was observed to be very clear with small amount of cells at the top of the medium. Aeration was started at 20°C.

(T = 6 hours, t = 25 hours) Temperature was made 30°C.

(T = 8.5 hours, t = 27.5 hours) Aeration was stopped and the medium was left at room temperature for 20 hours.

(T = 8.5 hours, t = 47.5 hours) The medium had still low turbidity and small amount of micro-organisms. Aeration was started and temperature was made 31°C.

(T = 14.5 hours, t = 53.5 hours) Turbidity increased considerably in the last three hours. The medium became very milky. When aeration is stopped colonies of cells were seen floating to the top resembling a snowstorm. In the former experiments same thing had happened with the difference that this time the snow particles are small and numerous while in the former experiments they were bigger but smaller in number. Last time they had coagulated around oil droplets, this time the oil droplets were not visible, at least they were smaller in size letting effective aeration and growth. Fifty milliliters of sterilized distilled water was added.

(T = 19 hours, t = 58 hours) Aeration was stopped and the medium was left still at room temperature for 13.5 hours.

(T = 19 hours, t = 71.5 hours) Aeration was started and the temperature was made 30°C.

(T = 21.5 hours, t = 73 hours) 4.2 grams of the petroleum fraction (b.p 225°- 295°C) and 50 milliliters of distilled water were added. pH was made 4.5 by the addition of 1 milliliter of concentrated NH_4OH

(T = 26.5 hours, t = 78 hours) Aeration was stopped and the medium was left at room temperature for 18 hours.

(T = 26.5 hours, t = 96 hours) The medium was drained to a flask leaving the top hydrocarbon layer in the fermentor vessel with the micro organisms. These micro-organisms were to constitute the seed for the next fermentation operation. For the medium used in experiment no: V and the substances added during the fermentation see the table on page: 95

Table xxii

Medium of Experiment V

$NH_4H_2PO_4$	8.000 gr.
KCl	2.300 gr.
$MgSO_4 \cdot 7\ H_2O$	1.300 gr.
$MnSO_4 \cdot H_2O$	0.090 gr.
Fe (equivalent to 80 mg $FeSO_4$)	0.028 gr.
$CaCl_2$	0.040 gr.
$ZnSO_4 \cdot H_2O$	0.350 gr.
Yeast Extract	0.075 gr.
Petroleum Fraction (225°-295°C)	8.4 gr.
Distilled Water to make the solution	2000 ml.
NH_4OH conc. to adjust the pH	2 ml.

Reagents Added During Experiment V

Petroleum Fraction (225°-295°C)	4.2 gr.
NH_4OH conc.	1 ml.
Distilled Water	100 ml.

8-Experiment No: VI

a)Date of Experiment: April 1, 1967

b)Procedure:

Medium shown in table xxiii, page:97 was prepared in the manner described before and was transferred to the fermentor vessel under sterile conditions. The vessel contained the hydrocarbon layer with the micro-organisms from the last experiment.

(T = 0) Aeration was started at pH 4.5 and temperature was made 30°C .

(T = 5.5 hours) Aeration was stopped and the medium was left at room temperature for 40.5 hours.

(T = 5.5 hours, t = 46 hours) Aeration was started and temperature was made 32°C.

(T = 8 hours, t = 48.5 hours) Fifty milliliters of distilled water and 4.2 grams of the same petroleum fraction were added. pH was found to be 3.5 and it was brought to 4.5 by the addition of 1 ml. conc NH_4OH

(T = 13 hours, t = 53.5 hours) Fifty milliliters of distilled water and 4.2 grams of the same petroleum fraction were added. Temperature was $31^{\circ}C$ and pH was 4.2 .

(T = 15 hours, t = 55.5 hours) Aeration was stopped and the medium was left still at room temperature for 17.5 hours.

(T = 15 hours, t = 73 hours) Aeration was started and temperature was made $30^{\circ}C$.

(T = 19 hours, t = 77 hours) Aeration was stopped and the medium was left still at room temperature for 43 hours.

(T = 19 hours, t = 120 hours) Aeration was started and 4.2 grams of the same petroleum fraction and 100 milliliters of distilled water were added. Temperature was made $32^{\circ}C$ and pH was adjusted to 4.5 by the addition of 0.5 milliliter of concentrated NH_4OH.

(T = 26 hours, t = 127 hours) Aeration was stopped and the medium was left still at room temperature for 18 hours.

(T = 26 hours, t = 145 hours) Aeration was started and temperature was adjusted to $31^{\circ}C$. 4.2 grams of the b.p. $225^{\circ}C$ -$295^{\circ}C$ petroleum fraction and 50 milliliters of distilled water were added.

(T = 30 hours, t = 149 hours) Aeration was stopped and the medium was left still at room temperature for 71 hours.

(T = 30 hours, t = 220 hours) All of the medium was transferred to a flask leaving the top layer in the fermentor. This top layer of hydrocarbon and micro-organisms was taken to another flask. The walls of the fermentor was washed with water draining most of the micro-organisms into the flask. Some remained still sticking on the walls. The sticking ones were to constitute the seed for the next fermentation.

Table xxiii

Medium of Experiment VI

$NH_4H_2PO_4$	8.200 gr.
KCl	2.300 gr.
$MgSO_4.7\ H_2O$	1.300 gr.
$MnSO_4.\ H_2O$	0.090 gr.
Fe (equivalent to 95 mg. $FeSO_4$)	0.033 gr.
$ZnSO_4.\ H_2O$	0.350 gr.
$CaCl_2$	0.040 gr.
Yeast Extract	0.075 gr.
Petroleum (b.p 225°-295°C)	4.2 gr.
NH_4OH concentrated	2 ml.

Distilled water to bring the solution to 2000 ml.

Reagents Added During Experiment VI

Petroleum (b.p 225°-295°C)	16.8 gr.
NH_4OH concentrated	1.5 ml.
Distilled Water	250 ml.

9-Seperation of the Product From Experiments V and VI

After experiment VI the top layer was transferred to a seperatory funnel. This layer contained most of the cells and hydrocarbon of the medium plus some aqueous part of the medium. In the seperatory funnel the cells floated to the surface. Although it was not visible some hydrocarbon floated to the surface with the micro-organisms. The lower aqueous layer was drained to a flask. Fifty milliliters of n-hexane and sixty milliliters of isopropyl alcohol were added to the top layer remaining in the seperatory funnel. After shaking a homogeneous yellow solution resulted with the cells in it. This solution was centrifuged which gave three layers in the test tube after centrifuging for 15 minutes. The top layer was a yellow solution (clear), while the bottom was white milky. In between these two layers the micro-organisms coagulated into a thick, dense layer.

The yellow layer was decanted to the flask containing the aqueous layer from the seperatory funnel. The cells were transferred to another test tube making a volume of 8 milliliters. The milky white solution was also transferred to the flask containing the yellow layer and the aqueous solution.

Protein color reactions explained on page:93 were made on the cells recovered in the way explained in the last paragraph. The tests were positive and proved the presence of protein.

The solutions in flask had a volume of 165 milliliters. The contents of the flask were distilled in a 400 ml. distillation flask. The below fractions were obtained:

1) Fraction boiling between 55°C and 79°C.
Amount : 51 milliliters, Odor : The typical hexane odor.

2) Fraction boiling between 79°C and 97°C.
Amount : 50 milliliters, Odor : The typical isopropyl alcohol odor.

3) Fraction boiling between 97°C and 101°C.
Amount : 38 milliliters, Odor : Slightly the isopropyl alcohol odor.

4) Fraction left in the distilling flask which couldn't be distilled because of smoke and bumbing produced after 101°C. It had the odor of the b.p 225°-295°C petroleum fraction. Its amount was 24 milliliters.

The fourth fraction was centrifuged giving two layers- 19 milliliters of petroleum fraction added during the experiment at the top layer and 5 milliliters of a viscous slightly yellow solution, at the bottom. It is worth mentioning here that 40 milliliters of the petroleum fraction was added to the vessel for the experiments V and VI.

Since the five milliliter viscous solution was suspected to be protein that came out from the cells by the action of the alcohol or some other thing protein color tests were made to this solution.

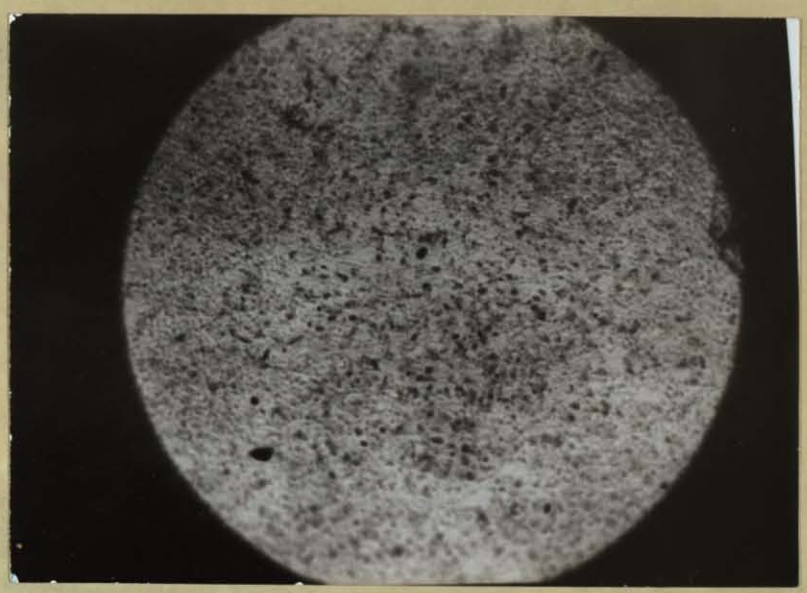
xanthoproteic test: To half milliliter of the solution one milliliter of distilled water was added to have a dilute solution. To this

one milliliter of concentrated nitric acid was added. The precipitate formed immediately dissolved giving a yellow solution. After cooling the solution was made alkaline by the addition of ammonium hydroxide. The solution became orange as it became alkaline. This is a proof of the phenyl group of the protein molecule (see the part 'color reaction of proteins').

Millon's Reaction: Half milliliter of the viscous yellowish solution was diluted with one milliliter of distilled water. To this four drops of Millon's reagent (see page 93, paragraph:c for the preparation of Millon's reagent). A white precipitate formed which turned pinkish red upon heating. This is a proof of the hydroxyphenyl group in the protein molecule.

Biuret Test: To one milliliter of the viscous yellowish solution one milliliter of 10 % sodium hydroxide was added. After mixing 0.5 percent copper sulfate solution was added drop by drop mixing thoroughly between each drop. A pinkish violet color was produced indicating the presence of protein.

10-Microscopic Examination of the Product From Experiments V-VI



Microphotograph was taken by the author.

Figure: 11 Microphotograph of the Product From Experiments V and VI

To examine the product from the experiments V and VI and to take the microphotographs presented on the last page and on this page the cells collected by centrifuging as explained on pages 97 and 98 were made into a suspension and put between a slide and a cover glass as explained on page 20 . The cells were stained by methylene blue as explained on page 20. The cells then were examined microscopically at different magnifications.

The appropriate magnification to take the photographs was found to be 43x5 . The photograph on figure: 11 was taken it that way. The photograph shows individual Candida Lipolytica cells and colonies of the cells. At higher magnifications the spores were also apparent. The photograph last page shows the colonies and the individual cells. The photograph has a network that also absorbed the stain. This network is typical of protein solutions. This shows that there was dissolved protein together with the cells. As proved by the distillation operation and protein color reactions on pages 97-99 this protein is dissolved from the harvested cells during the seperation and cleaning operations. This dissolving is mainly due to the action of alcohol on the micro-organisms. The stained protein network becomes more apparent as it is enlarged a little bit (see figure: 12).



Microphotograph was taken by the author.

Figure:12 Microphotograph of C.Lipolytica Cells and Protein Network

11-Experiment No: VII

a) Date of Experiment: April, 10, 1967

b) Procedure:

The medium shown in table: xiv was transferred under sterile conditions into the fermentor vessel containing some micro-organisms left from the last experiment sticking to the walls of the fermentor vessel.

Table: xiv

Medium of Experiment VII

$NH_4H_2PO_4$	8.200 gr.
KCl	2.300 gr.
$MgSO_4 \cdot 7\ H_2O$	1.300 gr.
$MnSO_4 \cdot H_2O$	0.090 gr.
Fe (equivalent to 80 mg $FeSO_4$ in HCl)	0.028 gr.
$ZnSO_4 \cdot H_2O$	0.350 gr.
$CaCl_2$	0.040 gr.
$Co(NO_3)_2 \cdot 6\ H_2O$	20 µg.
Yeast Extract	0.075 gr.
Kerosene (as sold in the market)	5 ml.
Concentrated NH_4OH	1.2 ml.
Distilled Water to make the solution 2000 ml.	

Reagents Added During Experiment VII

Kerosene	24 ml.
NH_4OH concentrated	1.1 ml.
Distilled Water	420 ml.

(T = 0) Aeration at the rate 4 liters per minute started while the pH was 4.5 and the temperature was 25°C.

(T = 5.5 hours) Aeration was stopped and the medium was left at room temperature for 15.5 hours.

(T = 5.5 hours, t = 21 hours) Aeration started and the temperature

was made 25°C.

(T = 10 hours, t = 25.5 hours) Six milliliters of kerosene and 60 milliliters of distilled water were added. pH was measured and was found to be 3.5 . To increase the pH 0.6 milliliters of concentrated NH_4OH was added.

(T = 13.5 hours, t = 29 hours) Aeration was stopped and the medium was left at room temperature for 40 hours.

(T = 13.5 hours, t = 69 hours) Aeration was started and the temperature was made 25°C.

(T = 19 hours, t = 74.5 hours) Nine milliliters of kerosene and 100 milliliters of distilled water were added.

(T = 21.5 hours, t = 77 hours) Aeration was stopped and the medium was left at room temperature for 65.5 hours.

(T = 21.5 hours, t = 142.5 hours) Aeration was started and the temperature was made 25°C.

(T = 23.5 hours, t = 144.5 hours) Aeration was stopped and the medium was left at room temperature for 25 hours.

(T = 23.5 hours, t = 169.5 hours) Aeration was started and the temperature was made 25°C. Nine milliliters of kerosene and 160 milliliters distilled water were added. pH was measured and was found to be 3.5. It was made 4.5 by the addition of 0.5 milliliter of NH_4OH.

(T = 31 hours, t = 177 hours) Aeration was stopped and the medium was left at room temperature for 38.5 hours.

(T = 31 hours, t = 215.5 hours) Aeration was started and the temperature was made 25°C. Hundred milliliters of distilled water was added.

(T = 37 hours, t = 221.5 hours) Aeration was stopped and the medium was left at room temperature for 120 hours.

(T = 37 hours, t = 341.5 hours) Aeration was started and the temperature was made 25°C.

(T = 41 hours, t = 345.5 hours) Aeration was stopped and the medium was left at room temperature for 88 hours.

(T = 41 hours, t = 433.5 hours) 1820 milliliters of the medium was drained into a flask. The rest of the medium contained the top hydrocarbon layer together with the micro-organisms. The rest of the medium was drained to a seperatory funnel from where it was led to the funnel of a suction flask, the funnel having a filter paper. Thus the cells were seperated from the medium by filtration. The cells were washed with n-hexane and hexane was evaporated by leaving at room temperature for some time. Then the cells were washed with water. After the last washing they were free of the kerosene odor. They were dried afterwards and weighed. The dry weight of the micro-organisms harvested from the experiment was 3.359 grams.

12-Analysis of the Medium of Experiment VII

After the seperation of the micro-organisms the filtrate was added to the 1820 milliliters medium making a solution of 2105 milliliters (it is greater than 2000 milliliters because of the washings from the purification of cells). The solution had a milky yellowish color. It was evaporated in a distilling flask. Eighty four percent of the medium evaporated at 100°C. Since the concentration of salts increased in the medium as the solution evaporated boiling point was raised and 12 percent of the medium evaporated up to 130°C. Thus 4 percent of the medium was left as a dirty brownish solution. Upon centrifuging this gave a precipitate of cell wall residue leaving a clear yellow solution with a small hydrocarbon layer on top of it. The hydrocarbon layers were taken to a test tube by means of a pipette. The non-utilized kerosene amounted to 3 milliliters.

To the clear yellow solutions concentrated NH_4OH was added gi-

ving a white precipitate. This precipitate was probably the non-utilized salts in the medium. After centrifuging out this precipitate the solution was still slightly yellow. The solution part was 30 milliliters. This was further evaporated at 100°C. When the boiling temperature became 120°C five milliliters of the solution was left in the distilling flask. This final solution had a dirty yellow color with lots of carbon black in it. Carbon is probably from the decomposition of some organic matter. The organic matter probably was something other than protein since amino acids don't decompose at such low temperatures. Protein might be present in the five milliliters solution but this wasn't tested since the solution was full of salts unsuitable for protein color reactions.

13-Nitrogen Determination of the Cells From Experiment VII

A dried sample of cells weighing 0.829 grams from experiment VII was digested in 25 milliliters of concentrated sulfuric acid, 15 grams of K_2SO_4 and a small crystal of copper sulfate as catalyst. After two hours of digestion the solution became clear, light yellow solution indicating the end of digestion.

After digestion 250 milliliters of distilled water was added to the cool digested solution. The solution was made alkaline by the addition of 45 grams NaOH in 75 milliliters water. By the addition of a few pieces of granulated zinc the Kjehdahl-Gunning apparatus was set and distillation into 0.218 N HCl began. After half of the solution was evaporated distillation was stopped and the HCl solution was titrated with standard NaOH to calculate the milliequivalents of HCl neutralized by the ammonia evolved from the solution .

The calculations showed that the original cell sample contained 39.8 milligrams of nitrogen which was 4.8 % of the weight of the cells. This nitrogen amount is equivalent to 39.8 x 6.25 = 249 milligrams of protein which is 30.0 % of the weight of the cells.

RESULTS AND DISCUSSION

A) Results

Since this study was a research almost every step of the study proved an important fact. The facts proven varied in their importance in relation to the central object of growing protein-rich micro-organisms in petroleum. The protein content of the micro-organisms was very important in relation to the object of the study while the evaporation of water from the medium during aeration was a result of minor importance.

The results may be best followed by examining the procedure of the experiments and manipulations done in the procedures as the experiments proceeded. These results have the form to guide the experimentors in a similar study in the future. The most important results are listed below in the order of their importance.

---Two types of the fungus Candida Lipolytica (CB snr 599 Harrison and NRRL-Y 1094) utilized petroleum fractions (kerosene and the fraction b.p. 225^{o}-295^{o}C) as the carbon source in mediums containing appropriate amounts of nitrogen, phosphorus, potassium, magnesium salts, iron, calcium, cobalt, zinc, manganese, molybdenum trace elements and yeast extract as an organic growth factor. The nitrogen, phosphorus, potassium salts and organic growth factors were present in all experiments while some of the trace elements were neglected from time to time. The apparent results of neglecting some trace elements were as follows:

There was no stimulating effect of molybdenum in growth. In fact 100 milligrams $(NH_4)_6Mo_7O_{24}$ per liter inhibited the growth somewhat (see experiment II).

Presence of zinc considerably increased rate and yield of growth. This is apparent by comparing the growth rates in experiments III and IV.

Addition of cobalt to the medium increased rate of growth (see experiment VII).

---The optimum concentration of nitrogen, phosphorus, potassium salts

trace elements and organic growth factors were as presented in table xiv, page 101 of experiment VII..

---The optimum hydrocarbon concentration was about 5 grams per liter. If the concentration was less than that there was no visible adverse effects (it was less than 5 grams per liter at the beginning of experiment VII). On the other hand hydrocarbon concentration more than 5 grams per liter reduced the growth rate.

---Kerosene was as successful as the petroleum fraction (b.p 225°C-295°C) in being a carbon source for the fungus Candida Lipolytica. In fact the result of experiments VI and VII favors the use of kerosene. The amount of hydrocarbon utilized was 47.5 percent of the charged petroleum fraction in experiments V and VI while it was 90 percent of the kerosene charged in experiment VII.

---The pH of 4.5 and temperature of 30°C were appropriate for growth. Growth rate was reduced slightly at 25°C.

---The hydrogen ion concentration changed during fermentation. The pH decreased as the fermentation proceeded. Adjusting the pH by NH_4OH addition was appropriate.

---Aeration of 4 liters air per minute and the agitation produced by the flow of this air through the tube fermentor of 7 centimeters diameter were sufficient for growth.

---Water evaporated from the medium considerably by the action of the bubbling air. Therefore water was added as it decreased not to change the concentration of the medium.

---The turbidity of the medium increased during the fermentation becoming more milky as the cells increased in number. The growth was most rapid between the eighth and thirteenth hours of each experiment. When aeration was stopped the cells gathered in the upper hydrocarbon layer (see figure 10, page 86), leaving the medium as a clear solution. After the tenth hour of each experiment the still medium attain-

ed a slightly milky condition, too. After harvesting of cells this slightly milky medium became yellowish upon standing at room temperature for 2-3 days. The parts of the harvested cells in contact with air became reddish upon standing at room temperature for 2-3 days after harvesting.

---Since the medium was a very special one, containing a carbon source non-utilizable by most of the micro-organisms aseptic techniques not to contaminate the medium during the experiment proved to be of less importance. Air sterilization by passing air through sterilized cotton filter and sterilization of medium and fermentor by pasteurization was effective and sufficient not to introduce mutants and contaminants interfering the growth of Candida Lipolytica on petroleum.

---The Candida Lipolytica cells stayed alive in a still medium at room temperature with no aeration and agitation for 3-4 days.

---The Candida Lipolytica cells were killed by a heat treatment at 65°C for one and a half hour.

---The cells were seperated from the medium by filtration through a filter paper. Washing with hexane, evaporating it and then washing with water was an effective way of purifying the cells.

---Extraction with isopropyl alcohol caused the rupturing of cells and dissolving of cellular protein in alcohol. The presence of protein outside the cells was detected by the protein color reactions made on the alcoholic extract from the harvested cells (see the seperation experiment on pages 97-99). The presence of protein outside the cells was also proven by the microscopic examination of cells purified by using alcohol (see microphotograph on figure:12).

---The presence of protein in harvested cells seperated both by filtration and alcoholic extraction was proven by general composition test and protein color reactions.

---Kjehdahl nitrogen determination showed that the protein content of

the cells was 30.0 percent. In experiment VII 24.5 grams of kerosene was charged, 2.5 grams of kerosene was non-utilized. From the utilized kerosene 3.359 grams of cells were produced which contained 30.0 percent protein. So 22.0 grams of kerosene gave 1.0 gram of protein.

---The apparatus designed fitted very well the laboratory scale experiments. Only stainless steel heating tube in the fermentor vessel lost its shiny surface as the number of experiments performed increased. The corrosion was most apparent at the upper parts of the medium where the micro-organisms were present in concentrated amounts. The vicinity of corrosion is indicated below by arrows on the picture of the fermentor vessel.

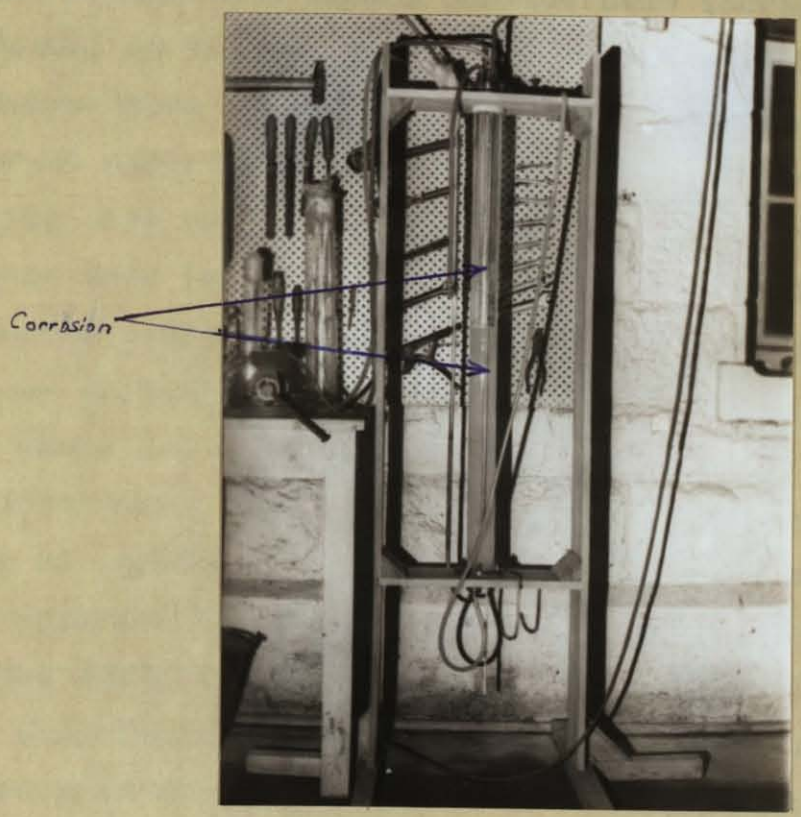


Figure: 13
Photograph of the Fermentor Vessel with the Arrows Indicating the Place of Corrosion

B)Discussion of Results and Conclusion

The central object of the thesis was accomplished by growing protein rich micro-organisms in petroleum. However, as described in the introduction part the ultimate aim is feeding the increasing world po-

pulation from such a synthetic protein source. There are two questions Is the protein produced in the experiments pure and metabolizable enough to be utilized by the human beings? Can it be produced industrially in amounts sufficient and prices low enough for such an aim? The results of the experiments don't give positive answers to these questions yet. The study was a promising start only.

What are the next steps in reaching the ultimate aim? How far did the study approach to these steps? Why it was not possible to proceed further? First of all to approach such steps is beyond the limits of the time given by a master's thesis. To reach the ultimate aim the yield and growth rate need a considerable increase. Growth rate and yield depend on the substances in the medium, their concentration, pH and temperature of the medium, aeration and agitation of the medium. Different experiments must be done keeping everything constant and changing only one of the numerous factors above. Thus the effect of changing this factor is measured. Thus the optimum pH, temperature, substances needed and their optimum concentration, appropriate aeration and agitation and most suitable micro-organism may be determined. Since there are about ten possible substances plus the micro-organism, pH, temperature, aeration and agitation making a total of 15 factors trying at least five different cases of each variable needs at least 75 experiments to be run. Since an experiment at least, takes one week with the evaluation of results a minimum of 75 weeks is needed for these experiments. Besides the time factor inadequacy of some materials and apparatus needed to run the experiments made the study difficult. There was no appropriate device for heating the medium and holding it at that temperature during the experiment. The heating was done by using steam from the central heating system of the school which heated the walls of the heating tube in the fermentor to at least 80°C. Thus there was the possibility of killing the micro-organisms near to the heating tube, interfering with growth and reducing

growth rate. Besides that since the heating tube became so hot that it was difficult to adjust the medium to the temperature desired. A difference of $\pm$ 4°C was obligatory by such a system. In the future a thermostat equipped with an electric heater must be used to control the temperature.

The pH measurement was made with pH paper after the second experiment. Since the paper was in the range pH 3.0 to 5.5 it gave approximate results. For exact control of pH a continuous pH measuring device that can be sterilized should be employed. In the recent years such pH meters are used in some fermentation industries (see reference: 3).

There was no good way to measure growth rate. Since bacteriological count per milliliter is beyond the experience of a chemical engineer the easiest way to measure growth is the turbidity measurement. A turbidity measurement device must be used to measure the growth rates and direct the experiments accordingly in the future.

An efficient way of seperation of cells and dissolved protein from the medium is centrifuging in the ultracentrifuge. Since ultracentrifuge was not available especially seperation of protein was not possible.

Leaving aside the discussion of what were not possible to be done the results obtained deserve some discussion, too. Beginning from the fermentor it must be said that the fermentor operated continuously as good as it was desired. It fulfilled all of the functions needed only the rate of evaporation from the medium was great. The rate of evaporation of water is seen from the continuous addition of water in every experiment. The evaporation kerosene from the medium was made apparent by the yield results on page 109. In experiment VII 22 grams of kerosene disappeared from the medium giving a product of 3.359 grams of cells. Maybe some kerosene had gone in forming enzymes and organic toxins excreted by the cells but this can't be more than the weight

of the cells produced. These assumptions show that about 60 % of the kerosene charged had disappeared. Probably most of this was due to its evaporation from the medium. This fact must be considered in future fermentor designs. Another thing that needs to be changed is the stainless steel heating tube. It corroded at the parts where the cells gathered in concentrated amounts. Since stainless steel contains most of the trace elements utilized by the fungi corrosion is possible but this fact needs further testing. If this is verified the only change needed is making the heating coil of glass which is the safest material. The top and bottom stainless steel covers were not affected since the micro-organisms were not concentrated at those regions. Another fact to remember in the design of the future fermentors is to make an air filter sterilization system instead of the air heating sterilization system because the chance of mutants and contaminants is low as explained in the results.

The micro-organisms selected utilized petroleum fractions. The possibility of finding another micro-organism utilizing the petroleum fractions more effectively exists but since much work has been done in this area by the experienced bacteriologists and Candida Lipolytica was found to be the most appropriate one, testing for different micro-organisms is the last thing to be done in this research especially if the research is carried by a chemical engineer.

On the other hand there is much work to be done in selecting the appropriate elements and their concentrations in the medium. The few results in this area have been reported among the results part. Kerosene seemed more efficient than the petroleum fraction b.p 225^{o}-295^{o}C. A comparison of experiment VII with the other experiments shows that 47.5 % of the charged petroleum fraction disappeared while 90 % of the charged kerosene disappeared from the medium. However the kerosene might have disappeared more quickly due to its higher rate of evaporation from the medium. It must be added here that the observed rate of

growth by the experimentor was greater in the experiment utilizing kerosene. The reason for this is probably the smaller cyclic hydrocarbon content of the kerosene. The hydrocarbon concentration effects the utilization. The inhibitory effect of more than five percent hydrocarbon was apparent in experiments IV and V. Experiment IV and the experiments following it proved the need for zinc in the medium. Experiment VII showed the stimulatory effect of cobalt in the medium. However nothing sure about these can be said since cobalt or zinc addition were not the only variables in these experiments. Experiment VII differed from VI by cobalt addition, substitution of kerosene for the petroleum fraction and the difference of fermentation temperature. So we can't be sure which one of the changes caused the stimulatory effect. At least it was apparent that cobalt had no adverse effects on growth at that concentration. On the other hand molybdenum had inhibiting effect if it was present as 100 milligrams $(NH_4)_6Mo_7O_{24}$ per liter. In like manner the effect of the inclusion of Ca in the medium in experiment II was not verified due to other changes in the medium made. Besides that the inclusion of some elements in the medium and their concentration has effects on the composition, amino acid and vitamin content of the cells. As mentioned in the biochemistry of fungi the carbon-nitrogen ratio in the medium determines the protein and lipid synthesis (see page 39). The amino acid determination was not made due to the lack of apparatus. However one thing is apparent from all of these studies: The best growth rate and yield was obtained from the medium of experiment VII as presented on table xiv, page 101. So the beginning step for the future experiments must be this medium.

The effect of pH was not measured and observed. Since fungi lives from pH 2 to 7 the advisable pH of 4.5 for Candida Lipolytica, which is in between the above range, was practiced. As mentioned before to measure the effects of pH a continuous pH measurement device, a rate of growth measuring technique must be employed. Since these were not available the effects of pH was not measured. Only the decrease of pH

after good growth hours was apparent and the adjustment of pH by ammonium hydroxide addition was found to be appropriate.

In like manner effect of temperature was not measured due to the lack of apparatus and time. However the decrease of growth at 25°C instead of 30°C was observed. Was this again due to changes in the medium etc ? Nothing can be said.

Agitation and aeration were related, their rate depending greatly on the capacity and heating up of the compressor. Aeration greater than 4 liters per minute produced undesirable effects like foaming and shaking of the fermentor vessel and wasnot tried therefore.

Seperation proceses described in part 5 on pages 91 to 93 seem to be inadequate due to the action of alcohol on cells and not settling of the cells in the centrifuge. The best method was the method applied after experiment VII, namely filtering and washing. However washing techniques need lots of improvement to make the cells free from hydrocarbons and other impurities. Several washing techniques must be employed in the future.

Protein color reactions proved the presence of protein and Kjehdahl nitrogen determination method gave the approximate amount of protein in the cells from experiment VII. The protein content being 30.0 percent is much lower than the protein content of Candida yeast concentrate which is given as 43.6 percent in table I, page 22. This somewhat low protein content is due to the incomplete drying of the cells before the Kjehdahl determination. The cells still were oily after the drying making stains on a filter paper. This makes one to overestimate the weight of the cells resulting in a low protein percent.

Examining the results and discussion of the results it is seen that the study was a good start in the field. The results make it a promising start but still there is a long way to go.

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