THESIS

ROBERT COLLEGE GRADUATE SCHOOL

BEBEK, ISTANBUL

PAGE

PROTEIN BY MICRO-ORGANISMS

A Thesis Submitted to the Graduate
Faculty in Partial Fulfillment of the
Requirements for the Degree of
Master of Science
in
Chemical Engineering

by

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JANUARY 15, 1969

ROBERT COLLEGE

BEBEK-İSTANBUL

ACKNOWLEDGEMENTS

I wish to express my thanks to the people that helped me during this work. In particular to:

Dr. Turgut Noyan (Chairmen of the Chemical Engineering Department)
Dr. Güniz Günay (Chemistry Department)
Dr. Mohammed Shana'a (Chemical Engineering Department)
Dr. Arden Slotter (Chemistry Department)
Mr. Ünsel Birecikli
Miss Sevinç Yongaçoğlu
Miss Işın Mutluay
Mr. Ömer Bilgin
Mr. Pınar Kılıç

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I. INTRODUCTION

Proteins, together with carbohydrates and fats, construct, maintain, and operate the body. All three substances undergo chemical changes within the human system and are converted into energy. The human body is built such that it can store carbohydrates and fats but not proteins. Therefore, constant renewal of protein from an outside source is needed.

Protein is required to sustain the living tissues of the body. It is provided in the required form through the digestive processes which break it down to its constituent amino-acids. These are then absorbed by the blood stream and are recombined to make different kinds of proteins required by various types of cells. All proteins are combinations of more than twenty amino-acids. Some of these amino-acids can be synthesized from food chemicals by the human organism. There are eight essential amino-acids which cannot be produced within the body. If these are not provided by food, the protein building mechanism cannot operate satisfactorily.

The problem of inadequate diet is acute today. It is estimated that it will be world-wide in the year 2000. Production of microbial protein seems to be the solution of this problem. Intensive research on this subject is being done in France, Japan, Russia, England, and U.S.A.

The object of this work was to obtain a substantial quantity of yeasts rich in proteins. Microbial protein productions which have been carried out before (1,2,3,4) have been on pure alkanes and gas oil. In this work kerosene was used as the substrate.

The work was carried out at the laboratories of the Chemical Engineering Department of Robert College. It was found that the production of microbial protein by the method used is possible. On the basis of the observations made, throughout the experiments, on the apparatus designed, it has been possible to propose a

convenient apparatus for future work. It is estimated that the proposed apparatus would result in greater growth ratos and, thus, would increase the yield.

II. PROTEIN FROM PETROLEUM

It is clear that the world nutrition problem is essentially a question of proteins. Approximately half of the world's population has a poorly balanced diet which retards normal growth. They live on grains and tubers which may provide enough calories, but lack the amino-acids present in the animal protein.

The situation will be worse in the future. In 1958, the total world production of animal protein was about twenty million tons, of which fourteen million were consumed by less than one billion people of the advanced countries. The remaining six million tons were consumed by the two billion inhabitants of the underdeveloped countries. Champagnat (1) estimated that in order to feed properly the population of about 6.3 billion in the year 2000, the production of high quality protein should be tripled to at least 60 million tons per year.

The primary conventional source of proteins is plants. However, the proteins from most of the common vegetable foods lack some of the essential amino-acids. There are plant products, such as soybeans, that contain well balanced proteins, but these sources are not yet developed into major foods.

Ruminant animals produce protein by converting vegetable food into balanced proteins with the help of intestinal bacteria, but raising animals is a highly expensive method of producing food. Masswise, it is less efficient than raising plant crops. It takes seven calories of plant carbohydrate to produce one calorie of animal protein. Raising animals by feeding them with crops makes the production of animal protein expensive, since the investment required to produce crops is also important.

To increase the protein production by conventional methods requires extensive organization and capital. Therefore a quicker and less expensive method has to be found.

Protein by micro-organisms:

The idea of producing protein by micro-organisms from carbon containing sources is not new. Yeasts are grown on

carbohydrates to produce vitamins and protein. The attractive aspect of the protein production by yeasts is the rapid rate of growth. A common comparison (2) is that a bullock weighing 500 kg synthesizes only 0.5 kg of protein every 24 hours; whereas, in the same time, same weight of yeasts can produce 1250 kg. This rate does not apply to every organism on a suitable substrate, but it gives an idea about the rate of production.

The idea (1,3) was to grow micro-organisms on hydrocarbons instead of carbohydrates since yeasts grow on petroleum. Yeasts are found in the bottom of oil tanks and in oil impregnated soil. A research was made to deparaffinate gas oil using micro-organisms, and it was observed that considerable amounts of protein-rich micro-organisms were produced. Then, the aim of the research changed from deparaffination to production of proteins.

Most of the quantity of food yeast produced is used as animal feed, because it is hard to make people consume microbial food. The acceptability of food by human beings is linked with flavor, color, texture, and habits.

Studies of the economy of protein production from petroleum (2) have shown that the price of microbial protein can compete very easily with that of animal protein. Other advantages of the process can be summarized as:

a) the process does not depend on seasons and climate,
b) it does not depend on irrigation,
c) it does not require great space and labor,
d) it gives constant quality product,
e) the production can be adjusted to the need,
f) raw materials are relatively abundant and evenly distributed,
g) the process can be started anywhere near a refinery in a short time, and
h) the production rate is rapid compared to the traditional ways of production.

III. THE YEASTS

The yeasts are true fungi since they possess no chlorophyll. They represent the group of micro-organisms which lie between the bacteria and higher fungi with respect to the average size of the single cell. Yeasts cannot be characterized as microbes because they always occur as single cells.

Yeasts are widely distributed in nature. Their habitats may include the upper layers of soil and many forms of organic matter of plant origin where carbohydrates are found. Yeasts are carried into the air with dust or by insects and, thus, are widely distributed.

Yeast cells (5) are usually spherical, ovoid or ellipsoid in form. Egg shape is also possible. The shape of the active cell is not an exact means of identifying the species. Variety in form does not mean that the species are contaminated. Yeasts do not posess flagella, so the individual cells are not mobile.

Cell dimensions may vary depending on the species, the nutrition, and the age. Usually, they vary from a little over 1 to 9 or more microns in width and from about 2 to 20 or more microns in length.

The cell wall covers and protects the cytoplasm and the nuclear material. It has selective permeability. The protoplasm is a semifluid mass. Within the protoplasm, there is a nucleus, one or more vacuoles, which are the sites of reserve food, and volutin. The vacuoles are not conspicious in young cells, but appear in mature or old cells.

Cells which have unusual resistance to adverse conditions are called "durable" cells. These cells are thick walled, much enlarged and densely packed with reserve. Stored foods are normal constituents of mature cells, especially of older cells.

Sporulation in yeasts is the basis of reproduction. It has a function in the making of new hybrids. It has also a role in maintaining the viability of the cells during adverse changes of the environment.

Sporulation may be initiated by a deficiency in the food supply and the accumulation of toxic end products. It will not proceed if conditions are not favorable.

The environmental factors affecting the activity of micro-organisms can be grouped as physical and chemical factors. There is an interrelationship between some of the environmental factors. Varying one factor can affect the response of an organism to other factors.

A chemical factor can act as a nutrient to promote some activity of the organism, can be an antimicrobial compound which would hinder the growth of the micro-organism, or it can have no effect whatsoever on the micro-organism. Agar is an example of the last group of compounds. There is no precise classification of nutrients and antimicrobial compounds. A compound that serves as a nutrient to a particular organism can act as an antimicrobial compound to another. A compound can act as a nutrient or as an antimicrobial compound depending on the concentration of the compound in the environment.

Nutrients are the compounds that must be present in the environment for the satisfaction of the requirements of the organism for biosynthetic raw materials and energy.

All chemical reactions taking place in living organisms need an aqueous environment. That is why water constitutes 80 to 90 % of the weight of the micro-organism.

Living organisms require a supply of energy for growth and activity. A minority of the micro-organisms can use solar radiation as an energy source. Others obtain their energy from chemical compounds.

Usually one compound supplies carbon, hydrogen, and oxygen to the micro-organism. Relatively high concentrations of these compounds are required. They account for the bulk of the dry weight of the micro-organism. Common carbon sources are carbon dioxide and carbohydrates, particularly hexoses and amino-acids. Since micro-organisms are often impermeable to organic acids, these compounds cannot be used as carbon sources.

A nitrogen source is needed for the micro-organism to synthesize compounds containing nitrogen. Nitrogen atom is incorporated in the micro-organism in the form of ammonium ion. Therefore, the nitrogen supplied to the micro-organism is preferred to be in the same form.

Inorganic phosphates are essential for growth. Phosphorus is present in the micro-organism as phosphate.

Micro-organisms take up sulfur from the environment as sulfate ion. In micro-organisms, the sulfur atom is in an oxidation state of -2 and is found in mercaptan form. (The process of conversion of sulfur from the oxidation state of +6 in the sulfate ion to -2 in mercaptan is known as the "assimilatory sulfate reduction".

Some micro-organisms need a supply of sufficient quantities of organic compounds for the synthesis of new cell material. These compounds are known as "growth factors". The growth factors include vitamins, amino-acids, purines, pyrimidines, and fatty acids.

The function of mineral elements in microbial metabolism is to activate various enzymes (5). These elements are required in trace amounts for growth of all the micro-organisms. They are potassium, magnesium, manganese, iron, zinc, sodium, and chlorine.

Some micro-organisms that resemble higher organisms require molecular oxygen for growth. These are called "aerobic". Others which do not need molecular oxygen are called "unaerobic". Molecular oxygen must be continuously provided for adequate growth of the micro-organism, because it is relatively insoluble in the aqueous medium.

Hydrogen ions are required in the environment for growth. The limits of hydrogen ion concentration for growth of most micro-organisms is about pH 4.0 to 9.0.

The most important physical factor affecting the growth of micro-organisms is heat. Heat is required for the metabolic reactions to proceed at a satisfactory rate. The heat needed is obtained from the environment and from heat generated during metabolism.

Microbial activity can change the chemical composition and the physical state of the environment considerably. Main changes in the chemical composition are due to the accumulation of microbial cell material, removal of the nutrients from the medium, and the excretion of the waste products of the metabolism. The main physical change in the environment is the rise in temperature. It is due to the heat dissipated during metabolic activity.

IV. PREVIOUS WORKS

The work on laboratory scale was mainly concentrated on using pure hydrocarbons as substrates. The work by Azoulay, Cauchoud-Beaumont, and Senez (4) is on the growth of Candida Lipolytica on alkanes. A definite and a complex medium was used. The composition of the complex medium is: 2.5 gr NH_4Cl, 7.0 gr KH_2PO_4, 1.2 gr Na_2HPO_4, 4.0 gr $MgSO_4$, 1 mg of yeast extract (Difco yeast extract), and 1 liter of distilled water. After sterilization, the pH of the medium was between 5.6 and 5.8. The temperature of fermentation was 32°C. Aeration was made by vigorous agitation.

The authors (4) separated two colonies and named them as "grande colonie" and "petite colonie". It was found that the yield increased up to C_{16}. The division time was practically identical from C_{10} to C_{16}. The conclusion was that 1)Candida lipolytica grows aerobically on alkanes with more than ten carbon atoms, 2) the growth rate of "grande colonie" is higher and, 3) growth factors are required.

Klug and Markowetz (6) investigated a number of species of Candida for the ability to use n-alkenes of 9 to 18 carbon atoms. Even numbered n-alkenes of 10 to 18 carbon atoms were also investigated. It was found that a number of species exhibited the ability to assimilate some members of the series employed. Thus the ability to assimilate hydrocarbons is not limited to a few members of the genus Candida. Growth control was made visually. Fermentation time was two weeks. A gyratory shaker turning at 250 rpm was used.

Miller, Lie, and Johnson (7) isolated a micro-organism from soil. This species, called HD-5, was grown on normal alkenes from C_{12} to C_{18}. The growth rate of the organism increased with the length of the alkane chain. The shortest generation time was 4.5 hours with octadecane. Cell yields were high with even numbered alkanes from C_{14} through C_{18}.

The medium of growth (7) per liter contained 5.0 gr KH_2PO_4,

2.5 gr $MgSO_4 \cdot 7H_2O$, 0.5 gr $CaCl_2 \cdot 2H_2O$, 7.0 gr NH_4Cl, 80 μg [illegible], $CuSO_4 \cdot 5H_2O$, 200 μg KI, 400 μg $FeCl_2 \cdot 6H_2O$, 800 μg $MnSO_4 \cdot H_2O$, 400 μg $Na_2MoO_4 \cdot H_2O$, and 800 μg of $ZnSO_4 \cdot 7H_2O$. The fermentor was stainless steel. It was sparged and baffled. An impeller of 1525 rpm was used. The temperature was fixed at 30°C by a water jacket around the fermentor. The pH was controlled automatically within 0.1 pH unit of the set point.

Miller and Johnson (8) investigated the growth of a micro-organism which they called Culture 4 on n-alkanes. The medium used and the fermentor were the same as in the previous studies(7). The growth on solid hydrocarbons were studied. Solid hydrocarbons were solubilized in highly branched hydrocarbons, since highly branched hydrocarbons are not assimilated by the micro-organism.

In the research carried out at Lavéra (1,2,3,9), the fermentor is continuous and of air-lift type. The fermentor has been adapted to the problems of fermentation after extensive studies. It enables extrapolation to greater capacities. Heat of fermentation is dissipated by exterior heat exchangers. The maximum production rate of the pilot fermentor is 500 kg per day of dry product. The concentration of the cells at the outlet changes from 8 to 30 gr per liter according to the conditions. The fermentor is stainless steel. The amount of medium introduced in the fermentor is 40 liters. Composition of the medium is given in Table 1.

Following the medium, 20 liters of inoculum of Candida lipolytica cultured on alkanes of different chain length (C_6 to C_{19}) is introduced, and 15 gr per liter of gas oil is added. This amount is enough to double the cellular density. The cellular density at the beginning is 1 gr of dry material per liter.

The aeration rate is calculated to give 3 millimoles of oxygen per liter of medium per minute. An automatic pH meter keeps the pH at 4.0 by adding 10 N NH_4OH. When 20 ml of base is added, more gas oil is introduced. Addition rate is determined on the basis of the theoretical yield of the culture. A 10 % [illegible] yield is assumed. The yield is calculated as:

$$\text{Yield} = \frac{\text{yeast produced}}{\text{added gas oil}} \times 100$$

A cellular division time of 3 hours is assumed. Addition is continued each hour until the amount of gas oil introduced is 250 gr per liter. In 15 hours, a cellular density of 8 gr per liter is reached.

The effluent of the fermentor is an emulsion of the yeast cells and gas oil in the used medium. Upon centrifuging this emulsion, a cream of yeast with traces of gas oil and an emulsion of gas oil in water are obtained. The yeast cream is diluted with water and recentrifuged. This gives a concentrated cream. The gas oil in the emulsion is recuperated and used as fuel.

Drying of the product is done using drum dryers or atomizers. The temperature is chosen so that it does not change the digestibility and nutrient characteristics of the product.

Using a special procedure, the product is separated from traces of gas oil it contains. The separation is done by solvent extraction. Special care is taken not to change the quality of the product.

Deparaffinated gas oil is returned to the refinery if it is not used as fuel.

TABLE 1

Composition of the Medium (9)

Ammonium Acid Phosphate	2 gr
Potassium Chloride	1.15 gr
Magnesium Sulfate.$7H_2O$	0.65 gr
Zinc Sulfate	0.17 gr
Manganese Sulfate.H_2O	0.045 gr
Ferrous Sulfate.$7H_2O$	0.068 gr
Yeast Extract	0.025 gr
Tab Water	200 gr
Distilled water to take the medium to 1000 ml	

V. THE PROCESS

In the protein production by the micro-organisms, the suitable carbon substrates are(3):

1. Liquid normal alkanes
2. Distillates of paraffin containing media
3. Methane and natural gas

Liquid normal paraffins have been objects of many projects in many countries (4,6,7,8). These substrates have to be of high purity. The method of fermentation of normal paraffins is not very different from that of yeasts on sugar substrates, since a final purification is not necessary. In fact, the carbon substrate is consumed entirely by the micro-organism.

The procedure that uses the distillates of paraffin containing media, preferably gas oil, is used at Lavéra (1,2,9).

Fermentation on methane substrate has not been throughly investigated.

In our experiments, we used kerosene as the substrate. Gas oil would be a better substrate since it has a higher percentage of longer chain normal alkanes. Kerosene was preferred, because it was thought that the separation and the purification of the product would be easier.

The process that is considered is aerobic. The substrate kerosene is insoluble in the aqueous media, therefore the fermentation takes place in a medium composed of two immiscible layers. Aqueous media contains the mineral elements and the growth factors. Kerosene is the carbon source. The yeasts dispersed in this medium have to find what they need for adequate growth, namely mineral elements, growth factors, hydrocarbons, and oxygen. The problem is to mix these two immiscible layers adequately.

In sugar fermentation, the only amount of oxygen needed is the quantity that corresponds to the energy of combustion in the reactions of the metabolism. There is a need for oxygen in order to form cell material that contains structural oxygen.

Hydrocarbons do not have oxygen in their molecule, so the use of a hydrocarbon substrate makes the required amount of oxygen for adequate growth of the yeasts three times larger than the amount necessary with sugar substrates. However, using hydrocarbons as substrates has an advantage, because the quantity of yeast produced as compared to the consumed substrate is twice the amount obtained when sugar substrates are used.

The fermentation is carried out at constant temperature. In yeast fermentations, the temperature range is between 30°-40°C depending on the species used. In Lavéra (9), using Candida lipolytica as the species, the fermentation temperature was 30°C. Therefore, the fermentation temperatureof the present experiments was chosen as 30°C. It was assumed that the optimum fermentation temperature would be the same, although the substrate of fermentation is different.

The pH of the medium has to be kept constant between 3.5 and 6.5 depending on the species used. In the experiments performed, the pH was taken as 4.0 . This is the same hydrogen ion concentration as that in Lavéra (9).

The medium of the experiments contained water, biosynthetic raw materials, growth factors, mineral elements, oxygen, and hydrogen ions.

Water is the main element of the medium. The reactions of the living organism require an aqueous environment. Therefore, about 95 % of the medium is water. The other required elements, except the substrate, are made available to the micro-organism as solutions in water.

The biosynthetic raw materials in the process considered are: a carbon-hydrogen source, nitrogen, phosphorus, and sulfur.

The carbon-hydrogen source in the medium is the saturated hydrocarbons present in kerosene. The micro-organism grows using the various straight chain hydrocarbons at different rates.

Nitrogen and phosphorus are present in the medium as a water solution of ammonium acid phosphate. Ammonium acid phosphate also acts as a buffer.

Sulfur is present in the medium in the form of the sulfate ion. The sulfate ion concentration is supplied by the sulfates of magnesium, zinc, manganese, and iron.

Most of the mineral elements present in the medium are in the form of their sulfates as listed above. The potassium and the chloride ions are also present, but at higher concentrations than the other mineral elements. Potassium chloride is the source of these ions. Sodium and calcium are present in the tab water used in the medium. When the medium is prepared, the pH is adjusted with hydrochloric acid, which is also another source of the chloride ion.

The growth factors in the medium have a common source which is a solution of yeast extract. This solution was made from "Superlevure", a commercial product. The composition of "Superlevure" is given in Table 2.

TABLE 2

Composition of Superlevure

Factors	Amount in 100 gr
Glucides	32.2 gr
Protides	49.0 gr
Lipids	6.3 gr
Mineral salts	7.0 gr
Vitamin B1	15 mg
Vitamin B2	10 mg
Vitamin B6	3 mg
Panthotenic acid	5 mg
Vitamin PP	50 mg
Folic acid	3 mg

The composition of the aqueous media is the same as in Table 1.

The micro-organism used in the experiments is Candida lipolytica. It is a yeast belonging to the species Cryptococcacaea. Some information about Candida lipolytica is given in Table 3.

TABLE 3

Data Concerning Candida lipolytica (5)

Shape of cells: long oval to elongate, also short oval	
Size of cells (after 3 days on malt extract):	
	(2-4.5) x (4-22) microns
Fermentation on sugars:	-
Assimilation:	
Glucose	+
Other sugars	-
KNO_3 assimilation:	-
Ethanol as carbon source:	no growth or very little growth

VI. THE APPARATUS

A glass fermentor of about 1 liter capacity was available. Since it was planned to work at a higher capacity, the glass fermentor was not used.

It was thought that a polyethylene container could do the job. The polyethylene containers available in the market were of 20 liter capacity.

It was first decided to use a 40 liter container in the present experiments. To have this required capacity, one container was going to be sealed on top of another by epoxy cement. This design would have the desired capacity of 40 liters, and would have a working capacity of about 35 liters.

This type of design had some disadvantages:

1. The height of the liquid medium inside the container was going to be about a meter. This height of solution would result in a high pressure at the bottom of the container. This pressure would have an adverse effect on the air rate. The air rate would be lowered, and since the capacity of the compressor was not high, the air rate could be lowered to an insufficient amount.

2. The temperature of fermentation had to be kept constant, so heaters were required to heat the medium. However, to attain the desired temperature in a small time, a high heating rate would be required. The heating rate is inversely proportional to the volume of the liquid to be heated, so heating difficulties would increase with larger volume. This was a serious problem since the operation was discontinuous.

3. The medium was to be agitated by bubbling air through it. It was thought that because of the pressure at the bottom, the air rate might not be enough for agitation purposes. Therefore mixers had to be used. The mixers available to us had small propellers so that they could not be used efficiently to agitate the volume in consideration.

4. It was difficult to seal the two containers properly.

Polyethylene is not stiff enough to withstand the pressure of the liquid it contains without deformation. Leakages would probably develop as a result of these deformations.

So, before proceeding with such a design, a single container test design was used. Tests on the single container showed that the agitation caused by bubbling air through the medium was not effective enough. Therefore, the idea of two containers was abandoned.

In another test, it was shown that the polyethylene container could stand the pH of the container.

We knew that we could not operate the fermentor continuously. Therefore, when the fermentation was started every morning, we would have to heat the medium from room temperature up to the fermentation temperature. This would require a heating device.

We first decided to heat the incoming air stream by a resistance wound around the metallic pipe through which the air stream would be passing. This would have an advantage in that it would also sterilize the incoming air stream. However, because the heat capacity of air is low, it would have to be heated to relatively high temperatures to obtain the required temperature of the medium. The heat loss of air stream would be considerable if the heating was not done very close to the container. On the basis of the facts mentioned, this design was abandoned.

A different idea was to connect a heating coil of stainless steel to the central heating system. By use of a control device, we could adjust the amount of steam in the coil, and thus control the temperature of the medium. The disadvantage of this was that a precise control of the temperature was not easy, because the control had to be manual. Another disadvantage was that, if a continuous and complete mixing was not maintained, the death of the micro-organisms touching the hot metal surface would lower the yield. We gave up on this design because of the above disadvantages.

A third idea was to place the fermentor in a water bath.

The height of the water in the bath would be a little higher than the height of the medium in the container. The temperature of the medium could be precisely controlled by putting a temperature gradient between the container and the bath. Having a positive or negative gradient, we could heat or cool the medium, and also keep it at a constant temperature. The temperature of the water bath could be increased by placing heating coils in the bath. The design was abandoned on economic basis. The required heaters, and the water bath large enough to contain the fermentor would have a high cost.

Finally, we thought of heating the medium by aquarium heaters. The heaters would be placed below the surface where the organisms are living, thus reduce the chance of death of microorganisms by heat.

To reduce leakage possibilities, it was undesirable to have too many holes in the container, so only one heater was put in. The observed heating rate was slow but appreciable.

Another important problem was to mix the two immiscible layers adequately. The available mixers had very small propellers which were designed for laboratory apparatus of small size. No other mixers being available, we tried to put larger propellers on the same rod to increase the mixing. However, it was observed that these large propellers were too heavy a load on the electric motor, and the continuous mixing had to be avoided because of the high probability of ruining the mixer.

Next, we thought of having an air stream coming in at the maximum possible rate to do the mixing. However, the air rate was limited by the compressor capacity. Continuous operation of the compressor heated the motor excessively. So, on and off control was needed, and this set the limit of the air rate.

Air was blown into the medium from a plastic circular tube, placed at the bottom of the fermentor. The tube was closed at one end and had holes all around to have uniform mixing of the media, so that uniform oxygen supply to all parts of the medium was possible. A closely fitting rubber tubing connected the

plastic tube to the air filter attached to the compressor.

A hole was made in the container and a thermometer was inserted to control the fermentation temperature.

This was the apparatus that we were able to construct. The apparatus that we think would give better results would have a different design, which will be discussed later.

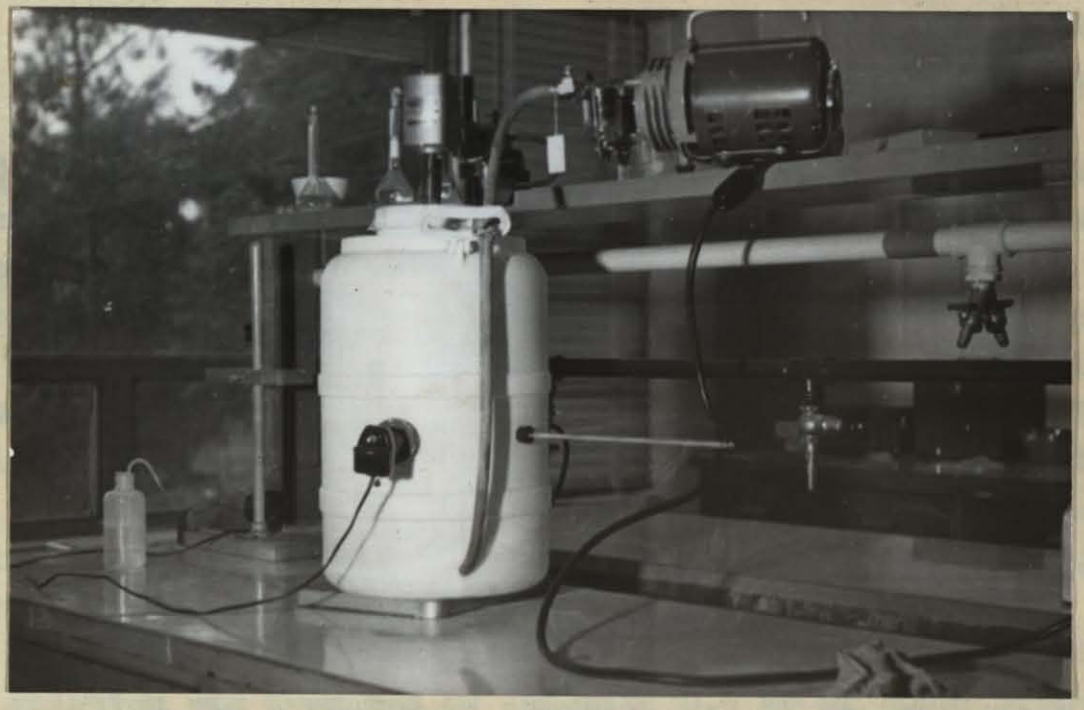

Figure 1
The Apparatus

VII. EXPERIMENTS

The fermentor used was of 20 liters capacity. The working capacity was 15 liters. Empty space was needed in order not to lose some of the medium. A heater of on and off type was used. It took about 4 hours to heat the medium from the room temperature to fermentation temperature. Therefore, the temperature during the experiments was not constant. The temperature versus time graphs of the experiments are given in the Appendix.

The process was discontinuous in all of the experiments. The operation time and the total time data of the experiments are given in the Appendix.

General Procedure: The medium was prepared by weighing the required amounts of salts for a 15 liter medium. These salts were dissolved in 6 liters of sterile distilled water. The distilled water was sterilized by boiling at atmospheric pressure. The concentrated medium was transferred to the fermentor. Hot water was then added to make the medium up to 15 liters. The addition of hot water raised the temperature to 30°C. The heater and the compressor were started at this time. The compressor operated at maximum capacity. The heater was previously adjusted to the fermentation temperature.

The growth factors were added to the medium at this stage. The solution was prepared by weighing 0.5 gr of Superlevure and dissolving this amount in 100 ml of sterile water. The solution was left standing overnight. A 75 ml portion of the solution was added to the medium followed by the addition of the substrate kerosene. The medium was inoculated at this stage.

After 25 hours of operation, the medium was left standing overnight. The upper layer of substrate was separated. The yield was obtained from this layer.

Experiment 1: The micro-organism used was Candida lipolytica NRRL-Y 1094. The inoculum was seeded in petri dishes at the Biochemistry Department of Istanbul University. The cells were harvested by pouring a salt solution into the petri dishes. The

cells were taken into the solution. The solution was centrifuged and the cells were washed several times before being transfered to the fermentor.

At the beginning of the fermentation, 150 ml of kerosene was added to the medium. To compensate the used substrate, and the loss by evaporation, a total of another 150 ml was added in the course of fermentation. To decrease the loss by evaporation, a glass tube was fitted into the container above the level of the medium, and through a rubber tubing, the outlet stream was bubbled in a water bath.

After three hours of fermentation, foaming was observed which continued to increase to the end of fermentation.

At the end of the process, the foamy kerosene layer was separated from the medium. It was placed on filter papers, and as much of the kerosene as possible was removed by pressing the cells on the paper with a spatula. The yield was about 2 gr. An accurate weight was not determined, because the cells were not free of kerosene. Much of the product remained on the filter papers, and therefore, was lost.

Experiment 2: The medium was the same, only no tab water was used.

After the medium of Experiment 1 was removed, the cells that stuck on the walls of the fermentor became the inoculum of Experiment 2. A 100 ml portion of kerosene was added at the beginning of the experiment. Another 250 ml was added during the experiment, of which 100 ml was added before a 20 day step during the experiment. This addition was made to insure that the organism is not left without substrate during this time.

The yeast cells were harvested in the same way. A minimum number of filter papers was used to decrease the loss. A partial removal of kerosene was possible by pressing the cells. The weight of the yield was about 28 gr.

Experiment 3: The inoculum of Experiment 3 was made from cells produced in Experiment 2. The cells were taken from the upper layer of foamy kerosene by a flame sterilized platinium wire and

were transferred to the medium of Experiment 3.

The air providing system of the apparatus was different. Experiments 2 and 3 were carried out at the same time, and the compresser could not provide sufficient amount of air for both experiments. There was no other compressor available; therefore, a vacuum pump was connected to the air outlet of the apparatus. It was thought that this arrangement would work provided that the leaks are minimized. The inlet of air was a rubber tubing. The end of the tubing was kept higher than the liquid level in the fermentor. The result was not bad. Two vacuum pumps were used, in alternation, in order to decrease heating.

The medium was the same as in Experiment 2. The substrate was a mixture of liquid paraffins. At the beginning of the experiment, 50 ml of substrate was added to the medium. No further addition was made, because a visual inspection showed that a sufficient amount of substrate was present. At the fermentation temperature, the loss of substrate paraffin was negligible. The yield after filtration was 2.1 gr.

Experiment 4: The apparatus used for this experiment was the same as the one used in the previous experiments. A different type of Candida lipolytica was used. To remove traces of the previous micro-organism, the fermentor was washed with potassium permanganate.

The first inoculum was made on Czapek's Medium (5). The composition of this medium is given in Table 4.

TABLE 4

Czapek's Medium (5)

Sucrose	30.0	gr
K_2HPO_4	1.0	gr
$NaNO_3$	2.0	gr
$MgSO_4 \cdot 7H_2O$	0.5	gr
KCl	0.5	gr
$FeSO_4 \cdot 7H_2O$	0.01	gr or trace
Agar	15	gr (12-20 gr)
Water	1000	ml

Four drops of growth factors, that is yeast extract, was added to each petri dish to be inoculated.

Czapek's Medium was prepared and boiled for half an hour. It was poured into the petri dishes. Yeast extract was added when the medium was hot. The medium was left to cool until it solidified.

The frozen stock of Candida Lipolytica CB snr 599 Harrison was opened. A platinum wire was flame sterilized. When the wire was cold, a small amount of the stock was spread on the petri dishes, and the dishes were closed. The mouth of the stock was burned and closed. The petri dishes were placed so that the medium was in the upper part. This prevented the forming drops of water to fall on the micro-organism. The dishes were kept at 25°C until the micro-organism developed. A second transfer was made in the same way.

The medium used, and the harvesting done were the same as in Experiment 1. Kerosene of 250 ml volume was added as the inoculate. The yield was about 3 gr, one gram of which was obtained from the filtration of the aqueous layer.

Experiment 5: The micro-organism used was Candida lipolytica CB snr 599 Harrison. The substrate was kerosene. The medium was the same, except the amount of growth factors was doubled.

Changes were made in the apparatus. The compressor used did not have a storage tank, and it could not operate continuously due to excessive heating. Another means of mixing was required during the stops of the compressor. A propeller was used for this purpose. Using the mixer was inconvenient, because the arrangement to decrease the loss of kerosene by evaporation could not work.

To avoid excessive heating of the mixers due to operating at high rpm, two, sometimes three alternating mixers were used.

To heat the medium to fermentation temperature, a quicker method was used. Some of the medium was drained and was heated to about 90°C. This was put back into the fermentor. Kerosene (100 ml) was added during the operation.

The amount of concentrated NH_4OH used to maintain the pH

was plotted against time to see if the resulting figure would be similar to growth rate curves. The result is given in Figure 2.

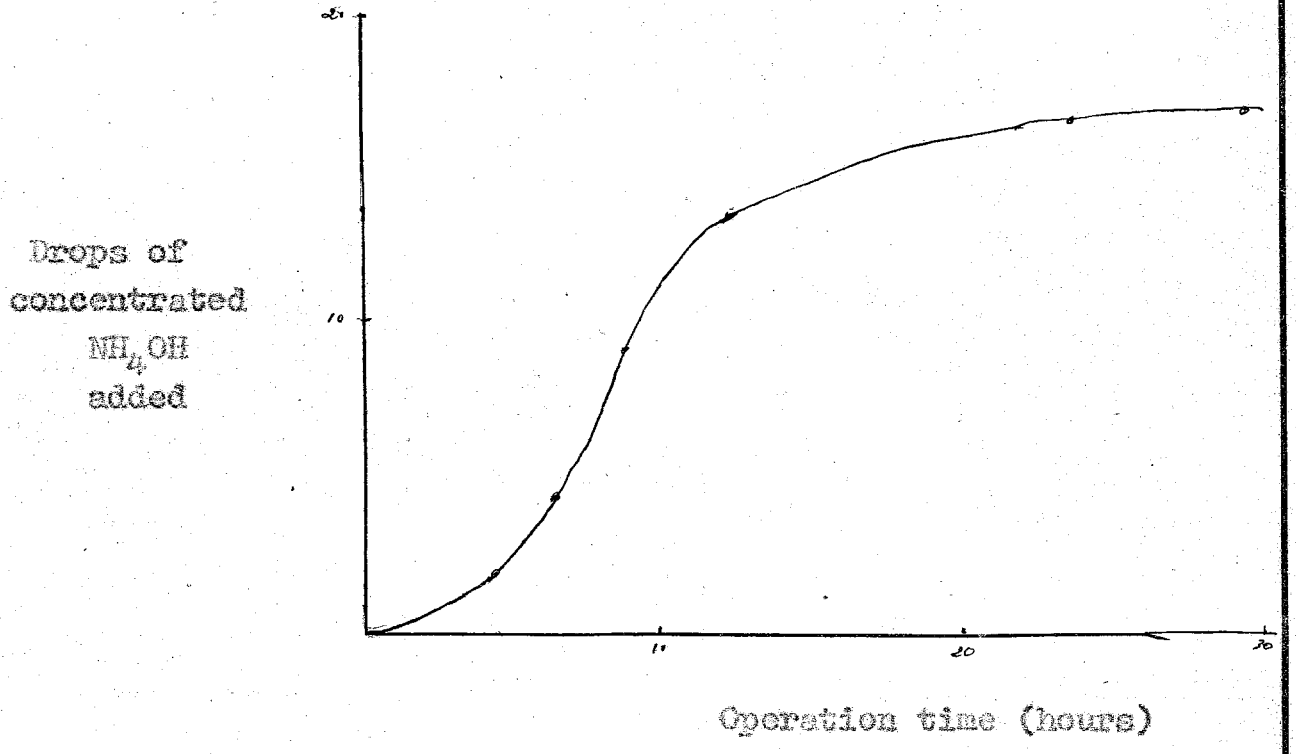

Figure 2
Concentrated NH_4OH added during Fermentation

VIII DISCUSSION

THE APPARATUS

On the basis of the experiments carried out, we can arrive at a conclusion about the performance of the apparatus designed. Although it was the best we could design which served its purpose, it could and should be much more refined for further experiments in the future.

The fallowing changes could be suggested:

a) The fermentor we used was made of polyethylene, a material that could not very well stand the temperatures of sterilization, if sterilization is needed. Another reason for the necessity of changing the material is that when air is bubbled into the vessel, pressure is built inside because of the small outlet for the exit stream. Polyethylene is not very hard, so this pressure inflates the vessel, and makes the hazard of leakage through sealed joints greater. This was the reason why we could not put another heater to the fermentor which would require making another hole of about 5 cm diameter. This would increase the leakage hazard by decreasing the resistance of the vessel to the pressure created by the air stream inside and to the hydrostatic pressure of the liquid medium it contained.

The material to be used in future designs should not have these defects, but any metallic container cannot be used, because the pH of the medium is 4.0. The material to be chosen should stand the acidic conditions without any corrosion. These considerations make stainless steel the best material for the fermentor vessel.

b) As noted before, the micro-organism used in our experiments is aerobic. Therefore, a sufficient amount of oxygen should be continuously provided to the micro-organism, because the oxygen in the liquid medium of fermentation is of a very small amount. Every cell needs substrate, mineral salts, growth factors, and oxygen in solution in the aqueous medium for adequate growth. This requires proper mixing. If this is to be

done by bubbling air through the medium, the amount of air needed is increased.

We did the mixing by bubbling air in, and it was observed that this was not satisfactory. The rate of air has to be greater for better results.

Ideally, we should have an emulsion of the substrate in the aqueous medium, and air bubbles should be as small as possible so that a uniform aeration required for maximum possible growth rate is obtained.

This can be done by passing a large amount of air through a circular steel plate with small holes all over. Another possible design could involve a mixing of the solution so vigorously that the bubbles coming from the bottom of the vessel are broken into smaller ones.

Therefore, the proposed design should be one which would provide a greater amount of air than was available in our experiments. This could be accomplished by using the device with as many small holes as possible which could be placed at the bottom of the tank, and, at the same time, would mix the medium. The bubbling of air would make the mixing more vigorous, and the aeration would be more uniform.

A powerful mixer capable of turning a big propeller at a high rpm would be necessary. A baffled tank would also help uniform mixing. The mixer rod has to be inserted in the vessel in such a way that leakages do not occur. Another design could have the outlet around the mixing rod. Such a device would be needed, because it is desired to pass all of the outlet stream through a measuring device.

c) As it can be seen from the figures showing the relationship of the fermentation temperature to the operation time during the experiments, only a part of the operation is carried out at the optimum temperature for the growth of the microorganism which is $30^{o}C$.

With the heater we had, it was possible to control the temperature at $30^{o}C$ when this temperature was reached. However,

it took 4 - 5 hours to heat the medium from room temperature to the fermentation temperature. Since we could work about 8 hours a day, for more than half of this period, the medium was at a lower temperature than the fermentation temperature. This reduced the amount of the product and the rate of growth considerably.

In favorable conditions for growth, heat is generated by the metabolic reactions of the micro-organism, and this heat has to be removed to keep the temperature constant. This has been pointed out in the literature (9). This was not observed in our experiments. We always had to supply heat to the medium and never removed heat from it. This was perhaps due to the heat loss because of the temperature gradient between the surroundings and the fermentor vessel, or more probably, to the fact that the rate of growth, hence, the rate of heat generation, was far from ideal.

In the future, if other experiments are performed, the heating system should be changed. In view of the fact that we were not able to operate the fermentor continuously, a more powerful heating device is suggested. When the operation is started, the fermentation temperature should be reached as soon as possible. We did this in the last experiment by taking out a portion of the medium, and heating it to higher temperatures and then adding it back to the medium. This seemed to be a quick way of heating the medium, but we do not know if this method has any adverse effects such as the death of some of the cells due to excessive heat during the addition of the hot liquid to the medium.

A water jacket system around the fermentor vessel seems to be the most efficient and quick way of controlling the fermentation temperature. By putting a high temperature gradient, we could heat the medium quickly, and then, by connecting the jacket to a constant temperature water bath, we could control the temperature at this value. Such a system could work for heating, cooling, or keeping the medium at constant temperature.

d) In all five experiments, we could not prevent leakages. This resulted in the loss of substrate, especially when the substrate was kerosene, by evaporation. In the fifth experiment in which a mixing device was used which penetrated into the fermentor vessel through a hole at the top, so that the outlet was open to the atmosphere, the loss of substrate kerosene was very great.

In a more advanced design, all the exit air has to be collected and passed through a device that measures the amount of carbon dioxide in the stream. This is necessary, because the amount of carbon dioxide and its rate of change is a measure of the rate of growth of the micro-organism. A recording type carbon dioxide measuring device could show the growth rate, so that one could spot the latent phase, the exponential phase, and the stationary phase during the experiment. This would allow one to stop the experiment before the stationary phase, or at the beginning of this period. This would increase the average growth rate. Such a device would also be useful in the transfer of cells to another fermentor, because it is advisable to make this transfer at the exponential phase.

e) In our experiments, the pH was controlled at intervals to maintain it at its optimum value which is 4.0. Since control was made at intervals, deviations from this value of hydrogen ion concentration occured during the fermentation.

For a maximum growth rate, an automatic pH control device connected to an ammonium hydroxide source is necessary. Such a device would be more helpful if a recording type instrument that recorded the amount of ammonium hydroxide used versus time was also available. The supplied ammonium hydroxide accounts for the nitrogen used by the micro-organism for growth, and also it neutralizes the acidic compounds produced by the cells during growth. If the amount of base used versus time is plotted, this plot would give the rate of growth of the micro-organism. The amount of ammonium hydroxide used is proportional to the rate of growth.

Such a figure was obtained in Experiment 5, and it is observed that the figure giving the total amount of ammonium hydroxide added to medium versus operation time shows a similar pattern to growth rate curves. It has three separate phases, corresponding to the latent, exponential,and stationary phases of growth.

If such a recording pH meter was available, much more accurate curves would be obtained, and the average rate of growth would be increased.

In using micro-organisms to solve the problem of protein shortage in the world, one faces the problem of process and equipment design. This problem arises because every investment has to give a profit, and, if possible, the maximum profit.

Manufacturing costs are determined by fixed capital investment and operating costs. We can divide these factors into their elements.

Plant investment:

1. Land, buildings, site preparation, etc.
2. Process equipment, piping, etc.
3. Instrumentations and controls
4. Steam, electrical supply and power facilities
5. Water supply and waste disposal facilities

Operating costs:

1. Raw materials
2. Labor and supervision
3. Utilities
4. Plant overhead

In our case of producing protein, some important factors would be:

Materials: In almost all of the fermentation processes on industrial scale, stainless steel is the material used. This is an expensive material and should be avoided if possible to reduce the plant cost. It is known (10) that wooden vessels have been used for antibiotic production with success. It is also noted that specially finished concrete is a very attractive

material. Therefore, these materials should be tried before using stainless steel. It is also possible to use vessels made of synthetic polymers. Our experiments showed that polyethylene vessels could be used on laboratory scale. The possibility of using the same kind of material on a larger scale offers some problems, mainly that of heat transfer. Nevertheless, the material needs to be considered. Apparatus made of glass can be used with success in the laboratory but not on larger scales.

Reactor design: Reactor in a microbial process is the fermentor vessel, and the first step in fermentor design is to establish the basic type and configuration. Microbiological processes usually use the standard cylindrical stirred tank with a height to diameter ratio between 2/1 and 4/1. Since design has to minimize capital investment, other configurations have to be also considered.

It is reported (10) that some recent yeast plants have been designed which have a series of cubical concrete cells. Four of such cells make a single reactor. The material flows from one chamber to the next and each successive cell is lower than the operating level of the preceeding cell.

In this design, the single high power agitator is replaced with four low power agitators.

This design is also favorable if any gradient in the process conditions, such as pH or temperature, is desired.

In our case, since no such gradient was needed, the advantage of cubic cells in series is not so obvious. Investigations of the mixing facilities of one configuration as compared to another have to be made before deciding on the type of fermentor to be used in the process.

Most of the manufacturing cost of a fermentor in a microbiological process is the cost of aeration and the agitator power. Protein production from hydrocarbons present in petroleum requires oxygen, so aeration of the fermentor vessel in the most efficient and economical way is an important factor. In this process, mixing of the two immiscible layers (petroleum fraction-

water) is also important. It would reduce the cost to combine the two operations into a single one. This could be done by mixing the medium by aeration. It has been reported that at Lavéra (9) such a design is used.

Heat transfer problem is another factor that affects the design of the fermentor vessel. The reaction in the fermentor is usually an exothermic reaction. Most of the processes in microbiological industries have to be carried out at a constant temperature. This offers heat removal problems. The fermentation temperature is usually close to room temperature, so the temperature gradient between the medium to be cooled and the outside is small. Efficient cooling by water circulation would require large volumes of water which means high pumping costs.

The problem of heat transfer in our process was much the same. There was an advantage,in that the process needed aeration. If a design could be made that would make it possible to cool the medium by aeration, the operation cost due to power consumed would be much lowered.

Utilities: Like most other plants involving aerobic processes, steam, electricity, and air are required for a microbiological process plant.

Sterilization is also usually required in microbiological process plants. Steam sterilization is the most common form of sterilization. Chemical sterilization is also possible. Sterilization by steam results in high operating costs. If possible, sterilization should be avoided. One must investigate the loss in yield due to working without sterilization, and compare it to the cost of sterilization. This comparison could be the basis of a decision to use or not to use sterile process. Sterilization in a continuous large scale operation is very hard. In the process that we consider, sterile air, sterile water, sterile petroleum fraction, and also sterilization of the reactor are required. A factor favoring non-sterile working conditions is that the medium is specifically prepared for one special micro-organism. So, one expects that contamination, if any, will

not be great. In the process considered, it is possible to use non-sterile process.

Raw materials: A singularity of a microbiological process is that one of the raw materials is a living organism. One must consider the way to gather the micro-organism in amounts and regularity required for an industrial scale process.

Other factors: Storage of the product is another factor to be considered. Since we produce food for living beings, the product stored must not lose its nutritive properties.

The process in consideration requires a complete purification of the product which means that any trace of the medium in which the organism grows must be removed. Since, in our case, there is petroleum fraction in the medium, this purification is important.

Considering the fact that the most important raw material in the process is gas oil, the choice of the plant site should consider easy accessibility to this raw material. The plant should be near a refinery. This is also favorable, because the production of protein also produces deparaffinated gas oil. This by-product could be returned to the refinery. The pumping costs of the raw material to the plant, and of the by-product back to the refinery would be lowered in this way.

THE METHOD

It can be seen from the data given for the composition of the medium in different experiments that the composition used was about the same for all experiments. The only changes were in the concentration of the yeast extract, and in some experiments tab water was used. The composition used was taken from the patent given to BP (9).

No investigation was made on the influence of any metallic salts or the growth factors on the growth of the micro-organism. We know that without the growth factors present in the medium in the form of yeast extract, the growth does not occur. The optimum concentration required was not investigated since we had no means of measuring the growth rate accurately. Another reason for not investigating the influence of any other factor present in the medium is the fact that such an investigation would require a comparison of the rates of the two fermentations. In order to do this, we had to keep every factor, except one, constant and vary the one to see its influence on the growth rate and on the total growth. This was not possible because we could not keep the necessary factors constant. For example, we could not keep the pH and the temperature constant, or, we could not control these factors accurately enough for a sound comparison of the growth rates.

Usually, in industry and research, either gas oil or paraffins are used as substrates. We used liquid paraffin mixture in only one experiment. We thought that the growth rate would increase in this experiment, but this was not so. The reason for this will be discussed later.

In four of the experiments, we used kerosene as the substrate. In an industrial scale research, gas oil would be used, because the product would be less expensive and the product gas oil would have better quality than the raw material gas oil, since it will have been deparaffinated. In our case, the economics of the process was not considered, and our sole aim was to produce

protein rich micro-organisms, so kerosene was preferred to gas oil. The reason for the preference of kerosene was that the separation by filtration and the purification of the product would be much easier.

Due to reasons discussed before, complete sterilization of the fermentor was not made. The air stream, when compressor with storage tank was used, was passed through a filter to remove dust particles. However, because the same operation is carried on industrial scale where complete sterilization is not easy, and on the basis of our observations of favorable results, we can conclude that sterilization is not very important. The cells sticking to the walls of the fermentor were still alive and they constituted the inoculum of the next fermentation, even though the fermentor vessel was left empty and open for about four hours. Since the medium is specially prepared for the growth of the yeast we used, contamination is not probable.

As discussed before, the growth rate of the cells was also limited by the insufficient and non-uniform mixing in our case.

The amount of cells produced also depends on the amount of cells present at the beginning of the operation. In order to have higher yields, we should have greater mass per liter of medium of cells initially present as inoculum. Since the amount of cells double in about two three hours, beginning with a multiple amount of cells as inoculum would increase the final amount considerably. In the experiments we carried out, the amount of inoculum was very small which limited the amount of product obtained. We thought at the beginning that this difficulty could be overcome by making the operation time longer, but this was not so, as indicated by the fact that the stationary phase was reached in about 20 hours of operation. Continuation of the operation did not increase the amount of product considerably. This was due to the poisoning of the medium by the compound produced by the cells during growth.

If we could harvest the living cells from the fermentation vessel and use this amount as inoculum for another fermentation,

the yield would be very much increased. There are two reasons for this:

1) the yield would increase since the amount of inoculum is greater,
2) the yield would increase because the inoculum is grown on the same medium that the fermentation is made, so the cells would be adapted to the medium.

In three of the experiments, the inoculum was made by cells grown on solid medium, and it was observed that this had an adverse effect on the final yield.

In one experiment, the substrate was liquid paraffin. It was thought that the yield would be higher in this case. However, a better yield was not obtained probably because the inoculum was made by using cells grown on kerosene substrate, so they were not adapted to grow on a different substrate.

Investigations of the influence of growing the inoculum for fermentation on a similar medium to that of the fermentation should be made, because this is expected to increase the yields considerably.

Another factor which reduced the yields in our experiments was that our operation was discontinuous. When such an operation is stopped, the growth is diminished or even stopped, and when the operation starts again, there is a time lag until the cells begin to continue their normal growth. That is, every time the operation is started, the cells are in a latent phase which decreases the growth rate.

In the first three experiments, only the upper substrate layer was filtered to obtain the product. Then it was decided to filter the aqueous layer as well, because it was observed that the color of the aqueous layer in the end was different from the color at the beginning of the operation. Filtration yielded a considerable amount of product compared to the other substrate layer.

We think that while the living cells stay in the upper, substrate layer, the dead cells are in the aqueous phase. If

this is true, a design for a continuous operation is possible. In such a design, there would be two containers connected to the fermentor vessel. After 10 hours of fermentation, fresh medium would be added to the fermentor from one of the containers, and at the same time, the used medium would be transferred to the other container at an experimentally determined rate. The living cells in the substrate would stay in the fermentor. The product would be obtained by the filtration of the drained medium continuously.

Such a design would give a considerably higher growth rate and a larger total amount of product because:

a) the operation being continuous, the latent phase would only occur at the beginning of the operation,

b) the poisoning of the medium due to high concentrations of compounds formed during the metabolism could be avoided so that the operation would not reach a stationary phase,

c) only the dead cells would be removed and the living cells would stay in the medium. Thus, the rate of growth would increase with time until reaching a constant value. The rate cannot increase continuously, but the final amount of living cells would be considerably greater than in a discontinuous case such as ours.

In the experiments we have performed, the separation of the product from the medium was made by filtration using a vacuum pump. The filtration rate was slow, since the product formed a cake and stuck on the filter paper.

This rate could be incresed when filter papers were changed, and the filtration rate was slowed down, as in the third experiment, where only one filter paper was used. However, this caused a loss of product, because some of it could not be separated from the filter paper, and, with increasing number of filter papers, the loss increased, too. To decrease this loss, we used only one filter paper and worked with slow filtration rates.

Separation by the use of a centrifuge is also possible, but

this was not done since it was not practical to centrifuge more than 15 liters of medium in test tubes.

We preferred the use of kerosene to liquid paraffin as the substrate, because of the high viscosity of the paraffin. High viscosity resulted in a less uniform mixing and aeration, and in a slower rate of filtration of the product.

Purification of the product was not attempted, but purification is possible by washing or by solvent extraction, fallowed by filtration or centrifugation.

THE PRODUCT

When we compare the amounts of products we obtained from the five experiments, we see that with the exception of Experiment 2, the yields are about 2-3 gr. In Experiment 2, the yield was 28 gr. This seemed peculiar at first consideration, but it can clearly be explained.

In the first experiment, the inoculum was made on a solid medium, and the substrate of the experiment was kerosene. When the experiment was finished, the cells sticking to the walls constituted the inoculum of the second experiment. With the same substrate and in about the same operation time, 15 times more product was obtained in the second experiment. When inoculation was made on Experiment 3, with the cells from Experiment 2, using liquid paraffin as substrate, the amount of product was only about 2 gr.

The reason for the high yield in Experiment 2 is that the cells that were the inoculum in Experiment 2 were grown in Experiment 1, so the micro-organism was adapted to the kerosene substrate. This resulted in an increased growth rate, a smaller latent phase, and a much higher yield.

In the third experiment, the yield was low, because the substrate used was not kerosene, and the micro-organism was not adapted.

In the fourth and fifth experiments, another strain of Candida lipolytica was used, and the yields were about the same as in Experiments 1 and 3; that is, they were low.

In Experiments 4 and 5, the inoculation was made using cells grown on Czapek's Medium (5), so that the cells were not adapted to growth on the kerosene substrate. We can safely assume that, if an inoculation was made from cells grown in Experiment 4 to another fermentation, the yield would have been much greater.

Some of the product from Experiment 2, and all of the product from Experiment 5 were used in analyses for amino-acids. Experiment 5 was done to analyze the fresh product.

The products from all of the experiments were tested for protein by burning and dying with a protein dye; both tests were positive.

There was a slight difference in color between the products obtained from different experiments. There was even a difference between products obtained with the same strain of Candida lipolytica. We assume that this is due to non-identical operation conditions.

After filtration, a slight odor of kerosene remained in the product. The odor disappeared with time due to evaporation of kerosene which left the typical odor of the product behind. The product was preserved in the refrigerator for about 6 months without any appreciable change. It can be better preserved if frozen, as was observed later.

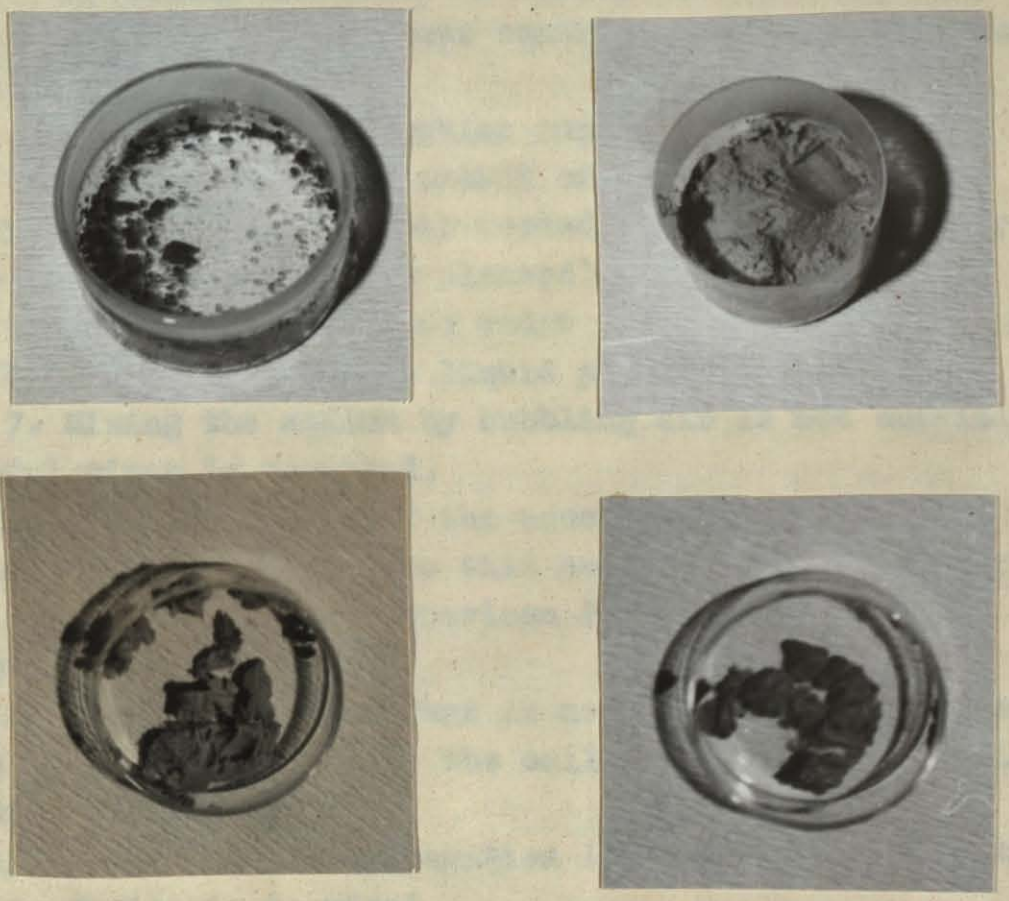

Figure 3
The Products

IX. CONCLUSION

On the basis of the experiments performed, we can conclude that:

1. Although the apparatus used was much defective as compared to the ones previously used for this purpose, the yield was good with respect to the operational conditions.

2. The yield could have been improved by carrying out several transfers of inoculum from one fermentation to another using the same substrate.

3. Even under the conditions of our experiments, the rate of production of protein by the micro-organism was higher than in other means employed.

4. The growth rate could be increased by:
   a) making transfers of inoculum,
   b) having an accurate control of the fermentation conditions,
   c) making the operation continuous, and
   d) increasing the amount of inoculum.

5. Sterilization is not needed (an important factor if large scale operations are planned).

6. For experiments made under the same conditions, kerosene is a better substrate than liquid paraffin or gas oil.

7. Mixing the medium by bubbling air is not sufficient, a powerful mixer is required.

8. Change of color of the aqueous medium is due to the presence of cells in it, so this medium has to be filtered.

9. If growth rate comparison is to be made, better instrumentation is required.

10. A powerful centrifuge is needed for the hydrolysis of the cells in order to separate the cell walls and the protein it contains.

11. If a continuous operation is not possible, a better heating device is required.

12. Further work on the same research should concentrate on the purification and the analysis of the product.

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APPENDIX

I. Experiment 1

TABLE 5

Operation Time versus Total Time

t= working time

T= total time

t=0	T=0
t=3	T=3
t=3	T=19
t=7	T=23
t=7	T=24
t=10	T=27
t=10	T=43
t=12	T=45
t=12	T=46
t=15	T=49
t=15	T=67
t=21	T=73
t=21	T=91
t=26	T=96

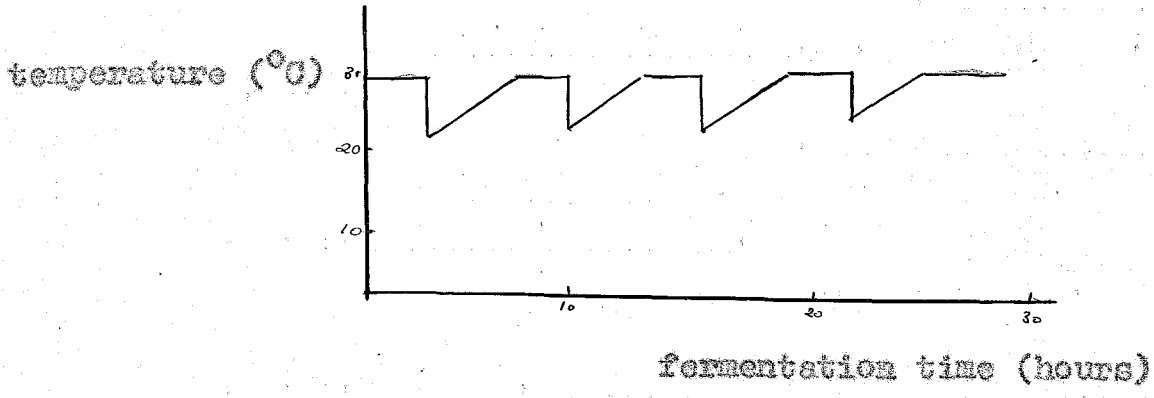

Figure 4

Temperature during Fermentation

II. Experiment 2

TABLE 6

Operation Time versus Total Time

t= working time

T= total time

t=0	T=0
t=2	T=2
t=2	T=5
t=4	T=7
t=4	T=20
t=10	T=26
t=10	T=280
t=16	T=286
t=16	T=302
t=25	T=311

Temperature during fermentation figure is not given since there was a 20 day stop during the experiment.

III. Experiment 3

TABLE 7

Operation Time versus Total Time

t= working time

T= total time

t=0	T=0
t=8	T=8
t=8	T=24
t=16	T=32
t=16	T=48
t=20	T=52
t=20	T=72
t=25	T=77

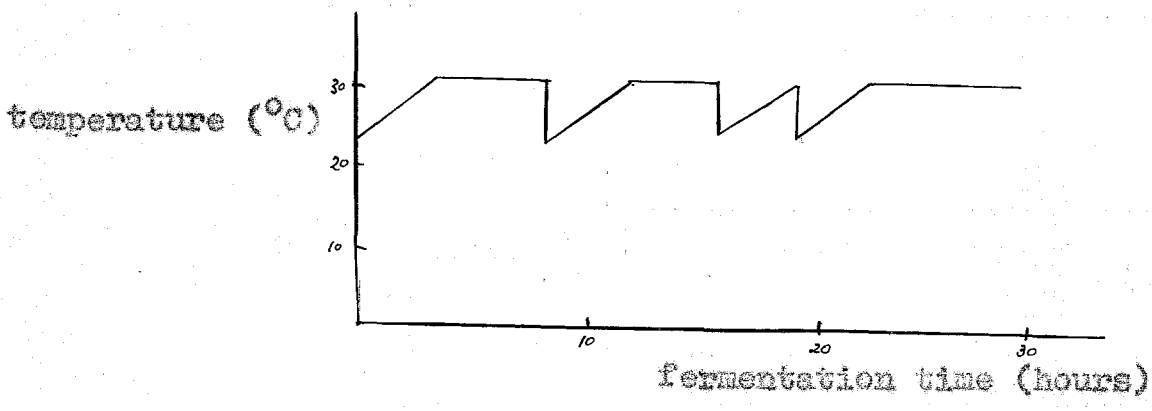

Figure 5
Temperature during Fermentation

IV. Experiment 4

TABLE 8

Operation Time versus Total Time

t= working time

T= total time

t=0	T=0
t=7	T=7
t=7	T=23
t=12	T=28
t=12	T=43
t=18	T=51
t=18	T=119
t=23	T=124
t=23	T=140
t=28	T=145

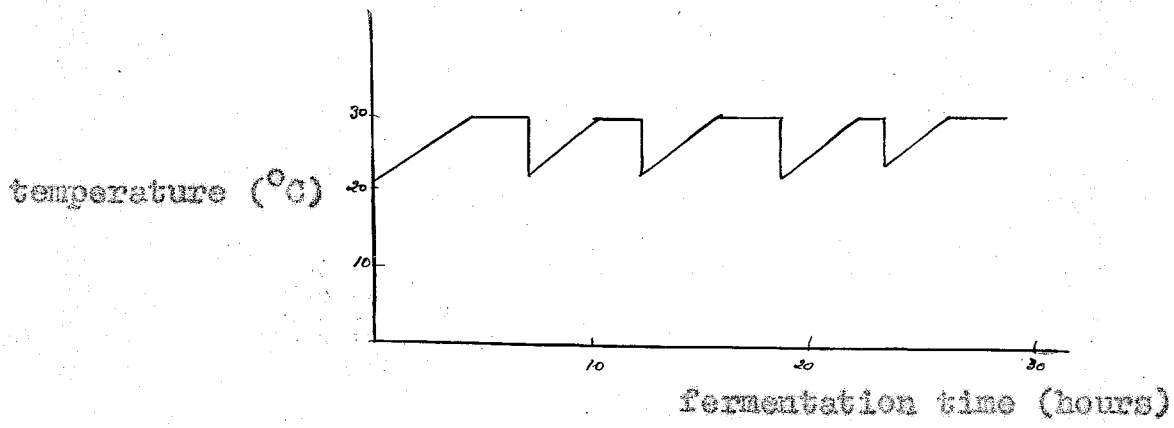

Figure 6
Temperature during Fermentation

V. Experiment 5

TABLE 9
Operation Time versus Total Time

t= working time
T= total time

t=0	T=0
t=5	T=5
t=5	T=21
t=13	T=29
t=13	T=45
t=21	T=53
t=21	T=70
t= 28	T=77
t=28	T=93
t=32	T=101

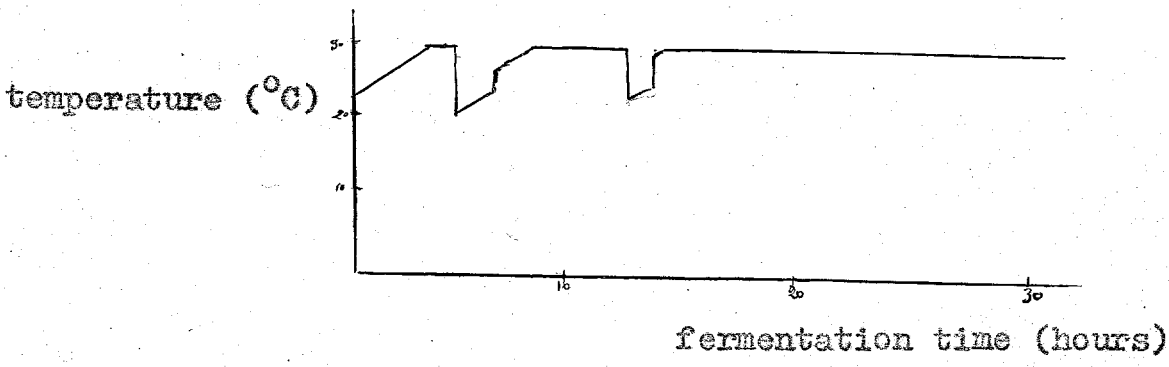

Figure 7
Temperature during Fermentation