# PRODUCTION OF CITRIC ACID FROM TURKISH MOLASSES BY FERMENTATION

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

by
IŞIN MUTLUAY



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ROBERT COLLEGE
BEBEK-İSTANBUL

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# I. INTRODUCTION

Citric acid is one of the widely employed organic acids which is found in various sources in nature, mostly in citrus fruits. It is mainly used in the food industry. Its high solubility, mild sour taste and palatability make it useful as an acidulant in carbonated beverages, jams, jellies and other food products. Another large outlet is in the medicine field where ready assimilability of citric acid makes it useful as a buffer in the manufacture of citrates and effervescent salts. In industry, citric acid is used as a sesquestering agent and as a constituent in plastic industry.

There are two methods of producing citric acid. The first one is the precipitation of citric acid in the form of its calcium salt from juices of citrus fruits. Citric acid obtained by this method is called "natural citric acid". Since growing citrus fruits is an expensive process, citric acid produced by this method is also very expensive. The second method is the fermentation of sucrose containing media with suitable molds. This process yields an inexpensive product which is called "fermentation citric acid".

The consumption of citric acid has been constantly increasing in Turkey because of the increase in the production of soft drinks and other food products. All of citric acid needed in Turkey is imported, and it is estimated that around $ 800,000 per year are spent for this purpose. In contrast to this, there is a large surplus of sucrose and molasses in Turkey. The production cost of sucrose is high in Turkey, so it is not possible to export sucrose since it cannot compete with world prices. Therefore, producing citric acid in Turkey would be a good help to Turkey's balance of payments.

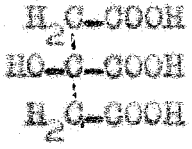
The object of the present work was to find suitable molds which could produce citric acid by fermenting Turkish sucrose and Turkish molasses, and also to determine the most favorable

conditions for this fermentation process. The work was done at the Chemical Engineering laboratories of Robert College. Good yields of citric acid were obtained in the various experiments carried out.

Consumption of sucrose and molasses for production of citric acid will create a new outlet for these substances. The experiments may serve as a basis for further studies on this field in Turkey.

## II. CITRIC ACID AND ITS PROPERTIES

Citric acid (2-hydroxy-1,2,3-propanetricarboxylic acid) is a polyfunctional acid with the structural formula:



It crystallizes from cold aqueous solutions as the monohydrate. The monohydrate crystals, which belong to the orthorhombic system, are colorless and translucent. Citric acid monohydrate is stable in air of normal humidity, but it loses water in dry air or in vacuum over concentrated sulfuric acid. On gentle heating, the monohydrate crystals soften at 70°- 75°C with loss of water and finally melt completely in the range of 135°- 152°C. With rapid heating, crystals melt at 100°C, solidify as they become anhydrous, and melt sharply at 153°C. The specific gravity of citric acid monohydrate is 1.542.

When crystallized from hot, concentrated aqueous solutions, anhydrous citric acid is formed. Mean transition temperature from monohydrate to anhydrous crystals is 36.6 ± 0.15°C. Anhydrous citric acid crystals are also colorless and translucent. These belong to the holohedral class of monoclinic system, melt at 153°C, and their specific gravity is 1.665.

Citric acid is optically inactive and manifests no piezoelectric effect. It is a strong organic acid, as indicated by its dissociation constants. At 18°C, the dissociation constants for the removal of the first, second, and the third acidic hydrogens are $8.2 \times 10^{-4}$, $1.77 \times 10^{-5}$, and $3.9 \times 10^{-6}$, respectively. Citric acid is very soluble in water, as seen from the data in Table 1. It is moderately soluble in alcohol, but is insoluble in ether, chloroform, benzene, carbon disulfide, carbon tetrachloride, and toluene.

Citric acid was first isolated and crystallized from lemon juice by Scheele in 1784(1). The functional groups, that is the

hydroxyl and the carboxyl groups were determined by Liebig in 1838(1). First commercial citric acid production was done in England in 1860, from calcium citrate imported from Italy. Grimoux and Adam (1) obtained the first synthetic citric acid in 1880 starting with symmetrical dichloroacetone obtained from glycerol.

TABLE 1

Solubility of Citric Acid in Water(1)

| Temperature($^{o}$C) | Per cent by weight |
|---|---|
| 10 | 54.0 |
| 20 | 59.2 |
| 30 | 64.3 |
| 40 | 68.6 |
| 50 | 70.9 |
| 60 | 73.5 |
| 70 | 76.2 |
| 80 | 78.8 |
| 90 | 81.4 |
| 100 | 84.0 |

Increasing demand for citric acid has led to research on the means of producing citric acid inexpensively. Manufacture of citric acid by fermentation was first proposed by Currie (2) in 1917 and research on the subject has reached considerable proportions since then.

## III. GENERAL INFORMATION ON MOLDS

The micro-organisms used in the fermentation processes to produce citric acid are molds. The molds are members of the division of the plant kingdom known as Thallophyta. They do not posess chlorophyll. They are widely distributed in nature, especially in the soil.

The mold structure is made up of mycelium and spores. The mycelium is a collection or an aggregate of hyphae, which are thread-like filaments of protoplasm. Hyphae are of two main types: fertile hyphae, which are concerned with the production of reproductive cells, the spores, and vegetative hyphae, the function of which is to secure nutrient substances from the solutions.

Molds grow by the extension of tip cells or by division of the cells in any part of the hypha. The young cells of mold are usually full of dense cytoplasm, but the old cells contain many vacuoles and reserve food materials, such as fat and glycogen. The cell walls of most of the molds contain chitin, a polymer of N-acetylglucosamide.

Molds reproduce through spores. Spores may be asexual or sexual in nature. From the standpoint of industrial microbiology, the term mold is given to aerobic saphrophytes that grow on organic matter or solutions with the formation of large masses of mycelium. These masses may be thin and superficial in character or may occur as felted masses of tough or semi-gelatinous matter. The mycelia can penetrate the substance for some distance, especially when growing on cellular tissues or amorphous masses.

The growth of molds is affected greatly by the environmental factors which may be chemical or physical. A chemical compound can have three effects on the micro-organism. It may act as a nutrient in promoting some activity of the organism, it may hinder growth of the organism, or the organism may be completely insensitive to the presence of the compound. Included

in the third category are substances such as agar, gelatin and silica gel that are used to solidify the media. Nutrients are defined as compounds that are needed by a micro-organism in order to satisfy the requirements of the organism for biosynthetic raw materials and for energy.

Water accounts for approximately 80-90 per cent of the weight of the mold. All chemical reactions taking place in living organisms require an aqueous environment; therefore, water must be available in the environment if the organism is to grow and reproduce.

Carbon, hydrogen, and oxygen are frequently made available to the mold in the form of one compound. They account for the bulk of the dry weight of the molds. If growth is to take place, a utilizable compound containing these elements must be available in the environment in relatively high percentages, usually one to five per cent by weight.

Molds obtain their energy essentially from carbon-containing compounds such as carbohydrates, alcohols, and acids. A large amount of energy is liberated when complete breakdown of a carbohydrate to carbon dioxide and water occurs. This type of energy reaction is the one commonly associated with fermentation.

A source of nitrogen must be present in the environment in order for the mold to survive. The preferred form of the nitrogen atom is the -3 oxidation state which is found in ammonium ion, and the nitrogen atom is incorporated into organic compounds in the form of this ion. In general, ammonium salts, nitrates, proteins, peptones, amino acids, and urea are satisfactory sources of nitrogen.

Traces of mineral elements are required for the growth of all micro-organisms. For molds, iron, zinc, copper, manganese, molybdenum, and gallium are important elements for growth, especially for Aspergillus Niger. Mineral elements function in the micro-organism's microbial metabolism mainly as activators of various enzymes. Magnesium is of more interest, since in addition to activating certain enzymes, it has the important role

of regulating internal functions of a cell.

Molds utilized industrially are aerobic; therefore, they need oxygen to function properly. Small amounts of diseolved oxygen in a certain culture, and atmospheric oxygen assimilated by surface mycelium are sufficient for the growth of molds, because they do not require large amounts of oxygen for this purpose.

All micro-organisms require the presence of hydrogen ions for growth. The optimum concentrations required are usually low. In high concentrations, the ion can have a toxic effect on the micro-organisms. Molds favor acid conditions for favorable growth. Their selectivity of acid concentrations makes it possible to grow molds on non-sterile media without contamination, because the high acid concentration is toxic to other forms of micro-organisms.

The most important physical environmental factor which affects the growth of micro-organisms is heat. A certain amount of thermal energy is required for the activity of all living organisms, and any excess heat has a harmful effect. Micro-organisms are killed above 100°C. Most micro-organisms are seriously affected by heat when temperatures reach even 50°C. This is the basis for thermal sterilization. Growth of a particular organism os favored only within a restricted temperature range from 22° to 30°C, and in this range, there is an optimum temperature.

Taking into consideration all the above mentioned factors, an optimum growth medium for the molds, called the Czapek's Medium, was found (3). There are many minor variations that can be made on this recipe, but the following composition is the best medium with minimum number of constituents:

| | | |
|---|---|---|
| Sucrose..... | 30.0 | gr |
| Sodium Nitrate($NaNO_3$)..... | 2.0 | gr |
| Potassium Acid Phosphate ($K_2HPO_4$)..... | 1.0 | gr |
| Magnesium Sulfate($MgSO_4.7H_2O$) | 0.5 | gr |

| | |
|---|---|
| Potassium Chloride(KCl) .. | 0.5 gr |
| Ferrous Sulfate($FeSO_4 \cdot 7H_2O$) | 0.01 gr or trace |
| Agar ..... | 12-20 gr |
| Water ..... | 1000 ml |

The final reaction of the medium is slightly acidic. Acidity of the medium can be increased by using potassium dihydrogen phosphate ($KH_2PO_4$) in place of the potassium monohydrogen phosphate($K_2HPO_4$).

From the industrial standpoint, species of the genera Mucor, Aspergillus, and Pennicillum are the most important molds employed in the fermentation process to obtain citric acid. Although there are references in the literature (4) to the fermentations be Pennicillum and Mucor genera, it has been found through extensive studies (2,5,6,7) that Aspergillus are the most suitable genera for the fermentation of citric acid.

In species of Aspergillus, the mycelium consists of septate branching hyphae, which may be brightly colored or colorless. The mycelium is usually partially submerged in the substrate and partially aerial. The foot cell is a specialised, enlarged, thick walled cell that gives rise to the stalk. The foot cell is usually submerged in the solution. The stalk arises approximately perpendicular to the long axis of the foot cell. Its walls may be smooth, pitted or rough. At the apex, the stalk usually enlarges to form a vesicle. The vesicle which supports the sterigmata is usually a globe. A portion, or all of its face is covered with sterigmata. Sterigmata produce conidia or clusters of other sterigmata. When there are two series of sterigmata, the one adjacent to the vesicle is called the "primary sterigmata".

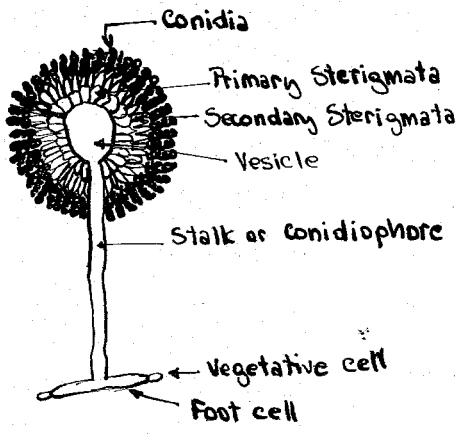



Figure 1
Aspergillus Niger

These give rise to series of "secondary sterigmata". The conidium (the spore) is produced by an elongation and cell division of the sterigma, a crosswall appears and the newly formed cell matures. Spore heads vary with respect to arrangement, color, size, and shape. Heads are black and spherical in Aspergillus Niger, the species employed throughout the present experiments.

Rate of growth of micro-organisms in liquid medium varies with time. A generalized growth curve is shown in Figure 2. After a portion of the medium has been inoculated with a micro-organism, a period of time normally elapses before a constant rate of growth is established. This period is called the lag phase. It is a period of intense metabolic activity during which the organism becomes accustomed to the medium. When a constant rate of growth is achieved in batch culture, the micro-organisms are said to be in the exponential phase of growth. In this phase, organisms grow at the maximum rate possible in that particular medium. During the exponential phase of growth, the nutrients in the medium become depleted and waste products of metabolism accumulate, so that the medium becomes less favorable for growth. Ultimately, the culture enters the stationary phase of growth in which the number of organisms in the culture remain constant. This phase is followed by the death phase.

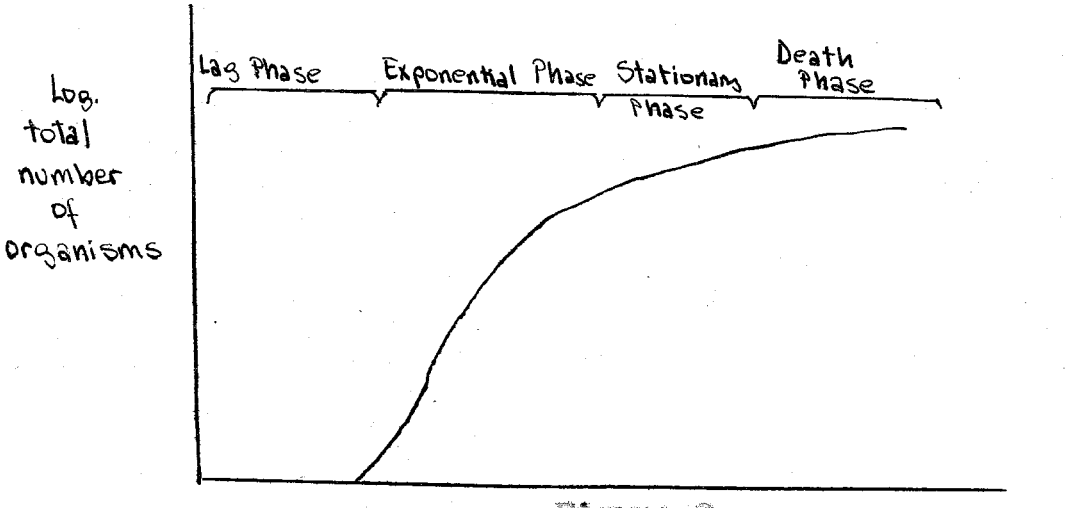



Figure 2
Growth Curve of Micro-organisms(3)

When a hyphe of mold develops in a suitable medium, growth is restricted to the region immediately behind the tip or apex. When molds are grown in static liquid culture, the mycelium forms a mat on the surfece of the medium.

## IV. REVIEW OF THE LITERATURE

Ever since the industrial applications of fermentation products have been increased, interest in all fermentation industries has led to extensive research in the field. The literature is rich in information about citric acid production through fermentation.

Citric acid as a product of mold fermentation was first described by Wehmer (4) in 1893. Wehmer described two molds, Citromyces pffeffarianus and Citromyces glaber in relation to fermentation. Later, he published two sets of results obtained by using Pennicillum luteum and Mucor piriformis.

Currie (2) published his results on production of citric acid in industrial scale in 1917. He fermented sucrose solutions in batch process using Aspergillus Niger. He determined the conditions for favorable fermentations as low nitrogen supply, high concentrations of sucrose, and nitrogen in the form of ammonia salts. The maximum yield of citric acid was usually obtained within 9 to 12 days of incubation.

Citric acid fermentation was next seriously considered by Doelger and Prescott (5) in 1934. They have also used Aspergillus Niger, first transferring the mold on many successive sucrose solutions of varying concentration in order to adapt it to the conditions of fermentation. They used a standard medium of the following composition:

| | |
|---|---|
| Sucrose ..... | 140.00 gr/liter |
| Ammonium Nitrate ..... | 2.23 gr/liter |
| Ammonium Phosphate .... | 1.00 gr/liter |
| Magnesium Sulfate ..... | 0.25 gr/liter |
| pH of the Solution .... | 1.60-2.20 |

The pH of the solution was adjusted by adding 1 N hydrochloric acid.

Doelger and Prescott (5) observed that hydrogen ion concentration was an important factor in fermentation and that hydrochloric acid yielded definitely better results than other

inorganic acids, implying that chloride ion also had an effect. Fermentation was carried out at 24° to 28°C for a period of 20 days. The results revealed that a period of twelve days was sufficient for maximum yields. These results are represented graphically in Figure 3. The experiments were carried out in flasks. Doelger and Prescott (5) reported that temperatures above 30°C retarded the fermentation. Large surface to volume ratio is required for satisfactory fermentations, because enzymic activity can take place only when the mat is in contact with the medium. Shallow aluminum pans were used for fermentation. Aluminum is sufficiently corrosion resistant, and aluminum ions in solution do not affect fermentation.

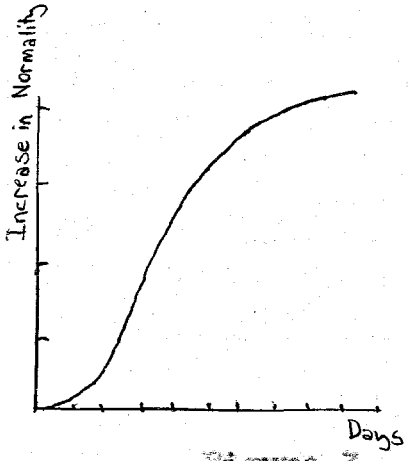



Figure 3
pH Curve for Fermentation of Sucrose Solutions (5)

It was also determined by Doelger and Prescott (5) that the mold must be kept at starving conditions, as far as nutrient minerals and vitamins are concerned, to produce high yields of citric acid.

Cahn (9) in 1935 fermented sucrose solutions impregnated on solid materials, such as bagasse and beet pulp, in a batch culture with Aspergillus Niger. He obtained 50 per cent yield by careful crystallizations. His method was advantageous, because it employed crude sucrose solutions and other sucrose containing media which was not employed previously.

After 1935, molasses was considered as the carbohydrate source for citric acid fermentations. Perlman, Kita and Peterson (6) in 1946 studied citric acid production from cane and beet molasses using Aspergillus Niger. They concluded that both types of molasses were inferior to sucrose in the production of citric acid. Inorganic materials found in molasses inhibited

the formation of citric acid.

In 1949, Oxford (10) discussed the differences observed and the difficulties encountered in the fermentation of cane molasses. He stated that a number of strains of Aspergillus Niger and Aspergillus Wentii were able to ferment cane molasses satisfactorily. The influence of inorganic ions on the fermentation was studied thoroughly. It was determined that relatively high concentrations of the chlorides of magnesium, potassium, and sodium suppressed the formation of oxalic acid to a much greater extent than that of citric acid. The corresponding sulfates in equivalent concentrations had very little effect on oxalic acid formation, while the nitrates had an effect intermediate between that of chlorides and sulfates.

Gerhardt and his associates (7) used two strains of Aspergillus Niger to ferment beet molasses media. They experimented with various concentrations of beet molasses to obtain the optimum conditions, which were as follows:

| | |
|---|---|
| Beet molasses ..... | 340.0 gr |
| Potassium ferrocyanide | 0.6 gr |
| Diatomaceous earth ... | 10.0 gr |
| Water to make up to | 1000 ml |

The final sugar concentration of the medium was about 15 per cent.

Effective treatment of beet molasses with potassium ferrocyanide was found to depend on the concentration of ferrocyanide used, the pH of the molasses, and the conditions of sterilization. Relatively small variations from the optimum concentration of ferrocyanide were found to result in reduced yields.

Gerhardt, et.al.(7) employed surface culture methods, fermenting the medium in aluminum pans for a period of ten days. Their yields were 40 to 50 per cent based on the available sugar.

Production of citric acid by surface culture methods has been an industrial procedure in the United States since 1934 (11).

Early efforts to produce citric acid by submerged culture methods invariably resulted in failure. Yields produced by this method were lower than those obtained by surface culture methods.

Occasionally gluconic acid was produced at the expense of citric acid.

The first successful submerged fermentation was carried out by Karow and Waksman (12) in 1947. They concluded that Aspergillus Wentii was the mold most suitable for submerged fermentations. They discovered that manganese favored growth greatly, whereas complex inorganic materials retarded citric acid formation. The concentrations of the nutrient salts must be restricted to a minimum, barely sufficient to allow good mycelial growth, and not so high as to permit maximum growth of the microorganism.

Karow and Waksman (12) fermented sucrose solutions for 10 days. The conversion was about 53 per cent. They also utilized other carbohydrate sources, mainly molasses. They concluded that the organic nitrogen compounds, the inorganic salts, and the gums, whiwh were present in molasses, were the cause of the decrease in citric acid yields.

Szücs (13) employed a strain of Aspergillus Niger in his experiments on submerged fermentation. The mold was first grown in a growth solution and then transferred to the fermentation medium. This medium contained a carbohydrate source and nutrient salts, but no assimilable phosphorus compounds. The presence of small amounts of phosphorus compounds retarded the citric acid formation. He used sucrose solutions as fermenting media. Oxygen was constantly supplied to the medium. The fermentation period was four days and yields were around 70-75 per cent on the basis of sucrose consumed.

Further experiments on production of citric acid by submerged culture methods were carried out by Shu and Johnson (14). They used sucrose as the carbohydrate source and Aspergillus Niger as the fermenting mold. They observed that trace amounts of iron were sufficient for successful fermentations, and that mycelial weight varied directly with iron concentration in the medium. They also showed that the fermentation can be divided into two distinct pheses; an initial growth phase and an acid

producing phase. During the initial growth, sugar was utilized mainly for mycelium production. Throughout the experiments done by Shu and Johnson (14), the average yield of citric acid was about 72 per cent.

Martin and Waters (15) in 1952 worked on the production of citric acid by submerged methods using beet molasses as their carbohydrate source and Aspergillus Niger as the mold. Fermentations were carried out in borosilicate towers flushed with oxygen. Average fermentation period was 30 hours and yields were expressed as 0.93 per cent conversion per hour. Their method produced the best yields of citric acid from sugar beet molasses known so far, but it had the great disadvantage of using up large quantities of oxygen.

A complete survey of literature on fermentation would be very long, because there is extensive research done on the subject all over the world. At this point, one more reference to the work will be given which is concerned with experiments done in Turkey.

The Sugar Technology Research Center in Ankara has been working on citric acid production by fermentation of sucrose solutions since 1957. The molds utilized in the experiments in this work has been obtained from their stocks. They employ batch fermentations of sucrose solutions in aluminum fermenting tanks for a period of 10 to 20 days. Yields up to 80 - 85 per cent on the initial sucrose concentration has been achieved. Their experiments are expected to deal with the fermentation of beet molasses solutions in the near future.

## V. CHOICE OF THE METHOD

Various factors were considered in choosing the experimental method used in the present work. Citric acid is usually produced either by surface culture fermentations or by submerged culture fermentations. The former can serve as a basis for the latter. Once a successful fermentation has been achieved by the first method, it is not difficult to proceed to the second method for further modifications.

Submerged culture fermentation procedure was not considered because of the high cost of equipment and fermentation process. Aeration, especially supplying oxygen, which is an important factor for successful fermentations would have been expensive. In addition, because the solution used in submerged fermentations has a higher pH than that of the surface culture method, sterilization of the medium and the equipment is necessary to prevent contamination of the medium. All submerged culture fermentations on laboratory scale employ borosilicate towers and other auxiliary equipment. Sterilization of the equipment is a costly process.

Some of the significant factors which were considered in selecting the present experimental method are as follows:

Organisms: Since the historic researches of Wehmer (4), it has been shown that a large number of fungi have the ability to produce citric acid. Some of the fungi produce smaller yields, some produce undesirable substances, and some, on account of their unstable cultural characteristics, are not satisfactory for use on a commercial basis. Thus the choice of a strain is a very important factor.

Of all the strains examined by various people (2,4,5,12), Aspergillus Niger has been found to be the most satisfactory for industrial and laboratory applications. Strains of this group of molds produce high yields, posess fairly uniform biochemical characteristics, are easily cultivated, and produce very little undesirable products.

Carbohydrate Source: Many organic substances, principally sugars, may be fermented to produce citric acid. Usually, maximum yields are obtained from sucrose. Molasses can also be used satisfactorily. In general, a high concentration of sucrose is required to produce high yields of citric acid. Solutions with concentrations of 10 to 20 per cent may be used.

Inorganic salts: In addition to carbon, hydrogen, and oxygen supplied by the carbohydrate, nitrogen, potassium, sulfur, phosphorus, and magnesium are indispensable in the fermentation medium. As found by Doelger and Prescott (5), a minimum amount of inorganic salts must be utilized in order to obtain thin mats with slight sporulation which produce high yields of citric acid.

The medium used by Doelger and Prescott (5) was preferred in the present experiments with sucrose. It provided the best medium, with the least number of components, for producing citric acid in an inexpensive way. For the same reason, the medium used by Gerhardt and his associates (7) was used in fermentations with molasses. The determination of the effects of the inorganic salts was very difficult, so the concentrations of these salts have been kept constant throughout the experiments.

pH: The maintenance of a favorable pH is most important for the successful progress and termination of fermentation. The use of a low pH is advantageous in that high yields of citric acid are favored, oxalic acid formation is suppressed, and the danger of contamination is minimized. Sterilization is more readily effected at low pH values. Successful fermentations have been carried out without resorting to heat sterilization of the medium when the initial pH was around 2.00. In general, the best citric acid producing molds are more tolerant to low pH values.

Hydrochloric acid was used to adjust the pH of the medium, because the chloride ion was found to be of distinct value as a constituent of the medium (2,5). The pH range of 1.60 to 2.20 for sucrose solutions, and a pH of 5.5 for molasses solutions were maintained throughout the experiments.

Ratio of Surface Area to Volume: In citric acid fermentation,

the conversion of sucrose to citric acid is brought about by intracellular enzymes and, therefore, takes place within the living cells that make up the mycelial mat. Sugar passes by osmosis into the cells, while the acid diffuses out through the cells. The rate at which the enzymic and diffusion processes proceed will determine the length of the fermentation period. Obviously, in a deep vessel containing a large volume, the progress of acid formation will be relatively small, for the surface area of the mat will be small in comparison with the volume. By using shallow pans, a large surface area of the mycelium is exposed to a relatively shallow layer of medium and conversion of sugar to citric acid proceeds much more rapidly. The surface area to volume ratio which gives the highest yields of citric acid during the shortest fermentation period must be employed. Also a minimum amount of unconverted sugar must remain in the solution.

Oxygen Supply: Although Aspergillus Niger is aerobic, atmospheric oxygen dissolved in the medium, and oxygen supplied by the carbohydrate source were sufficient for satisfactory fermentations.

Temperature: The temperature used depends partly on the organism and partly on the fermentation conditions. Temperatures of $25^{\circ}$ to $30^{\circ}$ are usually employed. Doelger and Prescott (5) suggest $26^{\circ}$ to $28^{\circ}$C as the optimum temperature range. They state that"the amount of citric acid produced will be on a rising scale as the temperatures are increased from $8^{\circ}$ to $28^{\circ}$C. At $30^{\circ}$C. and higher, citric acid production will decrease and a greater proportion of the titrable acidity will be due to the formation of oxalic acid." Throughout the experiments in this work, the temperature was varied between $25^{\circ}$ and $29^{\circ}$C.

Duration of the Fermentation Period: In the production of citric acid by the surface culture methods, the fermentation is usually complete in 7 to 10 days. Fermentations were carried out for 10 to 12 days in the laboratory to ensure maximum conversion of sucrose to citric acid.

## VI. METHODS OF ANALYSIS

A weighed amount of sucrose was dissolved in the fermenting medium in each of the experiments performed, and a polarimetric determination of its concentration was done in each case. The specific rotation, $[\alpha]$, of an optically active substance is, by definition, equal to the observed rotation divided by the weight of substance in grams per milliliter of solution multiplied by the length of the optical path through the solution.

$$[\alpha]_D^t = \frac{\alpha_D^t}{c \times l}$$

In the above equation, $[\alpha]_D^t$ is the specific rotation taken at temperature t using the D line of sodium. $\alpha_D^t$ denotes the observed rotation, c is the concentration expressed in grams per milliliter of solution, and l is the length in decimeters of the tube holding the sample. At the Paris meeting in 1900, the International Comission for Uniform Methods of Sugar Analysis established a standard method for calibrating a polarimeter which involves the measurement of the specific rotation of 26.01 gr of sucrose in a total volume of 100 ml of water determined at 20°C in a 200 mm long tube (16).

After the specific rotation has been determined, the sucrose concentration in commercial sugar is obtained as follows:

$$\text{Constant} = \frac{\alpha_{D_1}^t}{26.01} = \frac{\alpha_{D_2}^t}{c}$$

where c is the grams of sugar dissolved in 100 ml of water.

Before the sucrose content of a molasses solution can be determined, the solution is first treated with potassium ferrocyanide to precipitate the excess salts, followed by reaction with basic lead acetate solution to precipitate matter which interferes with polarimetric determinations. After the solution is filtered, it is placed in the polarimeter, and the final concentration of sucrose is determined by the formula above.

Since citric acid and oxalic acid are optically inactive,

they do not effect the polarimetric determinations. Therefore, the final sucrose concentration in the solution after fermentation can also be determined by polarimetric methods.

In order to determine the acidity of the fermenting solutions, samples are titrated with 1.032 N sodium hydroxide solution at the beginning and at the end of each fermentation, using phenolphtalein as the indicator. The range of phenolphtalein covers all three acid constants of citric acid.

Paper chromotography is a very simple and very effective method for determining the presence of organic acids in the medium. Its important advantage, apart from its simplicity, is that it is extremely delicate, so that only a few micrograms of a substance can be easily detected under suitable conditions.

In one dimensional paper chromotography, a drop of the solution to be analyzed is placed near one end of a sheet of paper, and this end is then dipped into an appropriate solvent. Capillary action draws the solvent through the paper and the substances present in the mixture advance at varying rates behind the liquid front. The rate of movement of a substance depends on a number of factors, such as the nature of the substance, the nature of the solvent, and the temperature. The $R_F$ value of a substance is a measure of the relative rates of movement of solute and solvent.

$$R_F = \frac{\text{distance traveled by the substance}}{\text{distance traveled by the solvent}}$$

The separation of non-volatile, water soluble aliphatic acids is usually accomplished on Whatman No.1 filter paper. The solvent systems most frequently employed are aqueous solutions which contain a "swamp" solution. The "swamp" solution does not affect the general movement of the acids, but does give better resolution, minimizes tailing of the spots, and eliminates "ghost" spots at the starting line. The sensitivity of the method for the location of the acid spots is also improved, because the paper has been freed of soluble acids and reducing impurities. The "swamp" in the solvent system used in the experiments was

ammonia.

Addition of water to the solvent system is necessary to produce useful separations.

Movement in the alkaline solvent is controlled by the number of carboxylic groups present in the solute molecule. Thus, the monocarboxylic acids move much more rapidly than the dicarboxylic acids, and they, in turn, travel further than the tricarboxylic acids.

It has been verified through extensive studies (2,5,7,9,14) that only citric acid and oxalic acid are present in the fermented medium under the conditions of fermentation. The $R_F$ values of citric acid and oxalic acid are given in Table 2 for various solvent systems. It can be seen from this table that the separation of citric and oxalic acids can best be accomplished in solvent systems containing 1-Butanol.

TABLE 2

$R_F$ Values of Citric and Oxalic Acids (17)

| Solvent System | $R_F$ x 100 Citric acid | Oxalic acid |
|---|---|---|
| Phenol:Water:Formic acid (75:25:1) | 26 | 18 |
| 1-Butanol:Formic acid:Water(10:2:15) | 37 | 05 |
| Ether:Acetic acid:Water (13:3:1) | 15 | 12 |
| Ethyl Cellosolve:Ammonia:Water(80:5:15) | 23 | 02 |
| Ethanol: Ammonia (80:20) | 02 | 02 |
| Carbon Tetrachloride:Acetic acid(5:5) | 06 | 05 |

Considering the data in Table 2, the following solvent system was chosen which was effectively used:

| Solvent | Parts by Volume |
|---|---|
| 1-Butanol | 4 |
| Ethyl Alcohol | 4 |
| Ammonia | 1 |
| Water | 3 |

The chromotograms were sprayed with bromothymol blue in

ethyl alcohol. This solution was prepared by dissolving 60 mg of bromothymol blue in 100 ml of ethanol and adjusting the color to blue by adding 1.032 N sodium hydroxide. Organic acids develop yellow spots on a blue background when they react with this reagent.

Qualitative determinations on the chromotograms were made by a comparison of the positions of the test spots with those of standard spots. Standard solutions of citric acid and oxalic acid for chromotograms were prepared by dissolving ten grams of pure citric and oxalic acid in 1000 ml of distilled water. Quantitative determinations were based on the relationship that the area of a spot is proportional to the logarithm of concentration (18). Areas of the standard spots and the test spots were calculated and the concentration of citric acid in test spots was then determined by the following ratio:

$$\frac{\text{Area of Standard Spot}}{\text{Area of Test Spot}} = \frac{\text{Log. Concentration of Standard Spot}}{\text{Log. } x}$$

where x is the concentration of the test spot.

The sucrose consumed during fermentation is converted to citric acid in a 1:2 molar ratio. The concentration of citric acid obtained from paper chromotograms divided by the theoretical amount of citric acid, calculated as above, yielded the per cent of conversion of sucrose to citric acid.

## VII. PRELIMINARY EXPERIMENTS

The greatest difficulty encountered during the preliminary experiments was to obtain the strains of Aspergillus Niger most suitable for fermentation. Since there is no institute in Turkey where isolation and identification of molds is carried out, finding the suitable mold for any fermentation process depends on pure luck. Importing molds from other countries is a costly and time consuming procedure.

Preliminary experiments were carried out with the Aspergillus Niger strains supplied by the Istanbul University. These strains failed to produce any citric or oxalic acid when grown on sucrose medium. Later it was discovered that this sample of the mold contained several strains, none of which being suitable for fermentation. Finally, the strains supplied by the Sugar Technology Research Center in Ankara made the experiments possible. In the experiments on which the results are based, six different strains of Aspergillus Niger have been employed. The nomenclature of these strains is invented by the Sugar Technology Research Center. The strains employed are:

Aspergillus Niger S-3 : Isolated in Turkey from bread and sucrose
Aspergillus Niger I-1: Isolated in Turkey from sucrose crystals
Aspergillus Niger I-2: Isolated in Turkey from sucrose crystals
Aspergillus Niger I-3: Isolated in Turkey from bread
Aspergillus Niger St-Volt: Brought from Germany in December, 1967
Aspergillus Niger CBS-2: Brought from Germany in March, 1968.

A number of fermentation processes carried out in aluminum pans are reported in the literature (5,7). The first experiments to produce citric acid by surface culture methods in the laboratory were carried out in wooden boxes of 30x20 cm covered with aluminum foil to prevent leakage. Large surface to volume ratio was obtained in these boxes. The corrosive nature of the acidic medium caused the aluminum to dissolve in the medium,

however, aluminum does not affect the fermentation. This procedure was abandoned because of the leakages in the pans. Further experiments were carried out in glass flasks or circular cups, and the volumes of the media were chosen to provide the largest possible ratio of surface area to volume. A procedure was then devised for further experiments to obtain the highest yields of citric acid with the available conditions.

## VIII. PROCEDURE

The first step in the procedure was to revive the mold spores and to adapt the mold to the fermentation conditions. Spores from the six strains to be employed in the experiments were seeded on sterile petri dishes containing Czapek's Medium(3). These petri dishes were kept in an incubator at 28°C for 10 days. Fresh spores appeared after the fifth day. The spores were then transferred on a new medium containing one gram per liter of citric acid in addition to the other ingredients of Czapek's Medium (3). The transfer of spores was done by picking mature spores by a flame sterilized platinum wire and placing them on the surface of the new medium. Five such transfers were made. The citric acid content of the medium was increased from one gram per liter to ten grams per liter gradually with each new medium. The aim in this procedure was to adapt the mold to a medium containing citric acid, thereby reducing the lag phase in the fermentation.

The sucrose containing media was prepared by dissolving around 140 gr of commercial sucrose together with 2.23 gr of ammonium nitrate, 1.00 gr of ammonium hydrogen phosphate and 0.23 gr of magnesium sulfate in one liter of distilled water. The final medium was adjusted to pH 2.00 with 1 N hydrochloric acid. The sucrose concentration has been varied between 140 and 150 gr per liter, and the pH has been selected at a value between 1.60 and 2.20 during the course of the experiments. The sucrose concentration before fermentation was determined polarimetrically in each experiment. The initial acidity was determined by titrating a 5 ml sample with 1.032 N sodium hydroxide.

In the first experiments, the medium was placed in 50 ml and 100 ml portions into 500 ml Erlenmayer flasks covered with cotton plugs to prevent contamination. The solution and the flasks were sterilized for half an hour at a saturated steam pressure of 8.5 psi gage in a steel sterilization tank. After sterilization, the surface of the solutions was seeded with the spores grown

as described above. The seeded samples were placed in the incubator at 26° to 28°C and were kept there for ten days.

Mat formation started after the second day of incubation, but spores did not appear until the ninth day. The start of the sporulation was an indication that fermentation was complete. After the tenth day, the solutions were taken out of the incubator and were filtered to get rid of the mycelium. The mycelium was squeezed to recover the medium soaked up in it. Portions of 5 ml from each sample were titrated with 1.032 N sodium hydroxide solution to determine the increase in acidity. The final sucrose concentration was determined polarimetrically.

Samples of 2,5 and 10 micrograms from the fermented solutions were placed on a Whatman No.1 filter paper along with 5 micrograms of standard citric acid and oxalic acid solutions. After the spots were dry, the paper was placed in an ascending chromotography cabinet in such a way that the edge nearest the spots was resting in the solvent. The paper was kept in the cabinet for 12 hours, and then it was dried for 30 minutes at 110°C. The color reagent was then sprayed on the paper uniformly. The ascending type of chromotography was preferred because it produces the least tailing of the oxalic acid spots. After the paper was dry, the position of the spots relative to standard spots, and the average diameter of the spots were determined.

In order to precipitate citric acid and oxalic acid from the fermenting medium, calcium hydroxide was added until the solution was nearly neutral, that is until the pH was 6.8. The solution was allowed to stay for a few hours at room temperature during which the calcium oxalate precipitated. After calcium oxalate was filtered, the decantate was placed on a water bath at 60° to 65°C and was allowed to stand until calcium citrate precipitated completely. Calcium citrate was then filtered, dried, and weighed. Precipitation was carried out with solutions which contained appreciable amounts of citric acid.

The calcium citrate obtained was mixed with a small amount of distilled water to form a sludge. To this mixture, 0.1 N

sulfuric acid was added slowly until the precipitation of calcium sulfate was complete. This procedure was carried out at 70°C. The calcium sulfate was filtered, and the clear decantate was evaporated in a vacuum dessicator. If the decantate was yellow in color, it was filtered once more with active carbon before it was placed in the vacuum dessicator. Citric acid monohydrate crysrallized under these conditions. The product was checked with melting point determinations.

Modifications on this procedure were made when several samples fermented in non-sterile conditions indicated that sterilization was not necessary. The fermentations were then carried out in circular cups of one liter capacity. Samples were placed in these cups in 300 ml portions, and they were covered with aluminum foil. In this way, it was possible to ferment larger quantities of solutions with a good surface to volume ratio. Sterilization was abandoned, and the new solutions were seeded with the spores obtained from the mats of the previous fermentations. Two strains were chosen which were superior in citric acid formation, and gave negligible amounts of oxalic acid. Further fermentations were carried out with only these two strains.

The molasses containing medium was prepared by dissolving 340 gr of molasses together with 0.6 gr of potassium ferrocyanide in one liter of distilled water. Ten grams of diatomaceous earth was mixed with this solution, and the mixture was permitted to stand overnight at about 6°C. The treated medium was decanted. A 50 ml semple was mixed with an equal volume of basic lead acetate solution. The final mixture was filtered to get rid of the precipitated impurities. Initial sucrose concentration of this clear solution was determined by polarimetry. A 5 ml portion of the initial molasses solution was titrated with 1.032 N sodium hydroxide to determine the initial acidity.

Basic lead acetate is the clarifying agent most extensively used. It is formed by the chemical combination of lead acetate, $Pb(C_2H_3O_2)_2$, with litharge, PbO. 430 gr of neutral lead acetate, 130 gr of litharge and one liter of water are boiled for 30 min.

The mixture is allowed to cool and settle, and the supernatant liquid is diluted to one liter with recently boiled distilled water.

Samples of molasses solutions (300 ml) were placed in circular cups of one liter capacity covered with aluminum foil. The samples were seeded with spores of the selected two strains. The spores were obtained from mats formed on sucrose solutions.

The procedure of fermentation of molasses media was identical with that of sucrose media with one exception. After the fermentation was complete, 50 ml sample of the final solution was filtered and treated with an equal volume of basic lead acetate solution. The clear decantate was used for polarimetric determination of the final sucrose concentration. Citric acid was recovered from the remaining solution.

## IX. EXPERIMENTAL RESULTS

The first experiments were carried out with sucrose solutions for the purpose of determining the strains that would yield maximum amount of citric acid with minimum amount of oxalic acid. The increase in acidity was taken as the indication for the formation of citric acid, and the yield of citric acid was calculated on the basis of the results of paper chromotography.

TABLE 3

Change in Acidity due to Fermentation

| Strain | Volume | Change in Acidity | Oxalic Acid |
|---|---|---|---|
| From İstanbul | | | |
| university | 100 ml | -0.003 N | - |
| " | 100 ml | -0.001 N | - |
| " | 100 ml | +0.001 N | - |
| 1/I | 50 ml | +0.035 N | - |
| 1/I | 50 ml | +0.037 N | - |
| 1/I | 100 ml | +0.464 N | - |
| 1/I | 100 ml | +0.477 N | - |
| 1/I | 50 ml | +0.652 N | - |
| 1/I | 50 ml | +0.658 N | - |
| 2/I | 100 ml | -0.006 N | - |
| 2/I | 100 ml | -0.007 N | - |
| 2/I | 50 ml | -0.025 N | - |
| 2/I | 50 ml | -0.017 N | - |
| 3/I | 100 ml | +0.267 N | + |
| 3/I | 100 ml | +0.271 N | + |
| 3/I | 50 ml | +0.252 N | + |
| 3/I | 50 ml | +0.254 N | + |
| S-3 | 100 ml | +0.025 N | - |
| S-3 | 100 ml | +0.029 N | - |
| S-3 | 50 ml | +0.018 N | - |
| S-3 | 50 ml | +0.022 N | - |
| CBS-2 | 100 ml | +0.232N | - |
| CBS-2 | 100 ml | +0.254 N | - |

| Strain | Volume | Additional Acidity | Oxalic acid |
|---|---|---|---|
| CBS-2 | 50 ml | +0.282 N | - |
| CBS-2 | 50 ml | +0.272 N | - |
| St-Volt | 100 ml | -0.022 N | - |
| St-Volt | 100 ml | -0.010 N | - |
| St-Volt | 50 ml | -0.030 N | - |
| St-Volt | 50 ml | -0.028 N | - |

TABLE 4

Yields of Citric and Oxalic Acids

| Strain | Volume | Citric Acid(gr/l) | % Conversion |
|---|---|---|---|
| From İstanbul University | 100 ml | - | - |
| 1/I | 50 ml | 1.36 | 1.44 |
| 1/I | 100 ml | 25.7 | 25.4 |
| 1/I | 50 ml | 31.6 | 30.4 |
| 2/I | 100 ml | - | - |
| 3/I | 100 ml | 27.6 | 36.3 |
| 3/I | 50 ml | 15.9 | 22.1 |
| S-3 | 100 ml | 1.38 | 2.8 |
| CBS-2 | 100 ml | 16.2 | 17.2 |
| CBS-2 | 50 ml | 22.9 | 23.1 |
| St-Volt | 100 ml | - | - |

(The error in these results may be as high as ± 10 % (18))

As seen from the tables above, only the strains 1/I and CBS-2 were successful in producing citric acid in substantial amounts. Oxalic acid was not produced at all in both cases. The following table also gives an indication of yields of citric acid.

TABLE 5

Calcium Citrate Precipitated

| Strain | Volume | Calcium Citrate | Sucrose Consumed |
|---|---|---|---|
| 1/I | 100 ml | 8.1 gr | 9.0 gr |
| 1/I | 100 ml | 9.0 gr | 9.2 gr |
| 1/I | 50 ml | 5.8 gr | 4.5 gr |

| Strain | Volume | Calcium Citrate | Sucrose Consumed |
|---|---|---|---|
| 1/I | 50 ml | 5.9 gr | 4.6 gr |
| CBS-2 | 100 ml | 4.3 gr | 8.4 gr |
| CBS-2 | 100 ml | 4.5 gr | 8.3 gr |
| CBS-2 | 50 ml | 5.3 gr | 4.5 gr |
| CBS-2 | 50 ml | 5.1 gr | 4.4 gr |

In the fermented molasses solutions, the presence of oxalic acid was not detected. The yields of citric acid as increase in normality are given below.

TABLE 6

Increase in Acidity of Molasses Media

| Strain | Volume | Additional Acidity |
|---|---|---|
| 1/I | 360 ml | +0.105 N |
| 1/I | 360 ml | +0.109 N |
| 1/I | 360 ml | +0.128 N |
| 1/I | 360 ml | +0.133 N |
| CBS-2 | 385 ml | +0.099 N |
| CBS-2 | 385 ml | +0.086 N |
| CBS-2 | 380 ml | +0.115 N |
| CBS-2 | 380 ml | +0.121 N |
| CBS-2 | 100 ml | +0.178 N |

Yields can also be expressed as percentage conversion to citric acid based on the amount of sucrose consumed. The results are tabulated below:

TABLE 7

Yields of Citric Acid from Molasses Media

| Strain | Volume | Citric Acid(gr/l) | % Conversion |
|---|---|---|---|
| 1/I | 360 ml | 4.96 | 55.8 |
| 1/I | 360 ml | 1.68 | 18.4 |
| CBS-2 | 385 ml | 3.90 | 43.0 |
| CBS-2 | 380 ml | 5.20 | 58.3 |

(These results may be subject to ± 10 % error (18).)

Citric acid as calcium citrate was only precipitated from

the 580 ml samples fermented by CBS-2.

TABLE 8

Yields as Calcium Citrate

| Calcium Citrate | Sucrose Consumed |
|---|---|
| 7.4 gr | 4.2 gr |
| 7.8 gr | 4.2 gr |

A 100 ml sample of molasses medium was fermented for a period of 12 days to see the increase in acidity with time. The results are plotted in Figure 4 below.

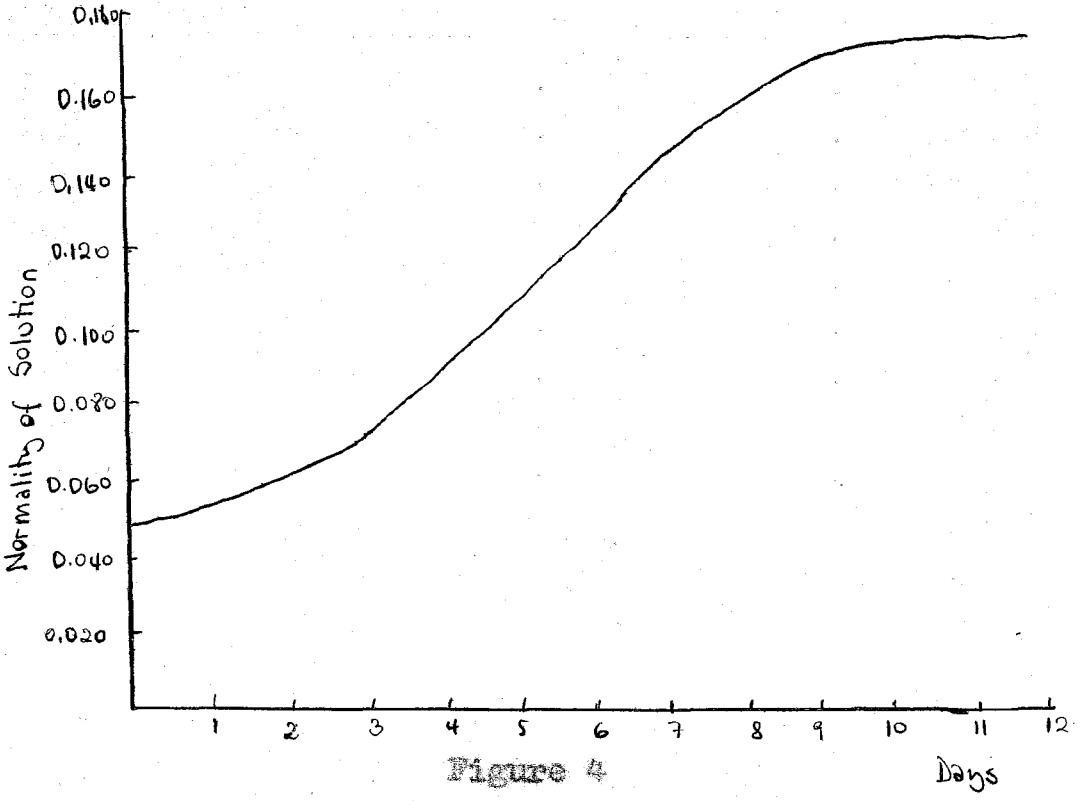



Figure 4

Increase in Acidity During Fermentation

## X. DISCUSSION OF THE RESULTS

Fermentation processes are very difficult to control effectively because of the large number of factors involved. Dealing with micro-organisms means accepting wide variations in results obtained through the same type of experiments. Therefore, results can only be evaluated on an average basis. Duplicity of the results, even under appearently identical conditions, is impossible, as seen from the results of the experiments. Although the medium, and the incubation temperature were identical in the case of two samples of equal volume fermented by the same strain, there was a variation of about 2 per cent in the additional acidity in each case.

Experiments done using sucrose solutions as fermenting media were done with the purpose of finding a suitable strain of the mold for fermentation. Of the six strains of Aspergillus Niger tested, only two, strains 1/I and CBS-2, were found to be effective. These strains produced citric acid from sucrose with negligible amounts of oxalic acid. Two other strains, 2/I and St-Volt, failed to produce any citric or oxalic acid. They consumed large amounts of sucrose (45 gr/l forstrain 2/I and 35 gr/l for strain St-Volt) for growth, and at the end of the fermentation period, formed a thick mat with extensive sporulation. There was a slight decrease in acidity in the media on which these strains grew. This decrease was more pronounced in the 50 ml samples then in the 100 ml samples. This amount of acidity was assumed to be utilized by the mold in the process of growth. Strain 3/I produced appreciable amounts of oxalic acid along with citric acid. Therefore, it was discarded because of the difficulties in separating the two acids. Even though strain S-3 produced pure citric acid, at the end of fermentation, the yields indicated by the increase in acidity were very low, even on pure sucrose solutions. About 50 gr/l of sucrose was consumed and only an increase in acidity of 0.029 N was observed. Therefore it was not economical to use this strain.

Figure 5
Aspergillus Niger on Czapek's Medium

Figure 6
Aspergillus Niger in Liquid Medium

It was interesting to note the properties of the mats formed, and the extent of sporulation since these gave an indication of the fermenting ability of the mold. Strain 1/I formed very thin mats on pure sucrose and molasses media. Sporulation was very scarce and spores appeared in the last three days of fermentation. The mat was white and the fermented solution remained colorless. Strain 2/I formed a thin mat with extensive sporulation. Spores appeared even on the third day of fermentation. The solution remained colorless and the mat was white. Strain 3/I formed thick white mats, but sporulation was scarce. Strain S-3 formed thin yellow mats and sporulation was extensive. The medium fermented also turned yellow after the fourth day of fermentation. Strain CBS-2 formed very thick and rubber-like white mats on sucrose solutions and thin mats on molasses. In the last two days of fermentation, a yellow color appeared in the solution, but this did not affect the purity of the product. Sporulation was very scarce in both cases and spores appeared only in the last two days of fermentation. Strain St-Volt formed thin, yellow mats covered with spores. The medium was also colored yellow.

It is appearent from the above discussion that the coloring of the mats is specific for different strains. Therefore it is possible to separate strains by growing them on solutions and noting the color of the mats formed. The sample of Aspergillus Niger supplied by the Istanbul University did not have a uniformly colored mat. Some parts of the mat were yellow and some parts were white. Therefore, it was concluded that the sample contained several strains, none of which were suitable for fermentation.

The mold should be under starving conditions for successful fermentations. Mats should be thin and sporulation should be scarce. Strains 1/I and CBS-2 were the only two strains that showed these characteristics on sucrose and molasses media. These strains produced the highest conversions to citric acid. The formation of yellow color in media fermented by CBS-2 could not be explained but this had no effect on the citric acid produced.

Figure 7
Mat formed by the Strain CBS-2 on Sucrose Solution

Figure 8
Mat formed by the Strain CBS-2 on Molasses Medium

Sucrose is converted to citric acid through the enzymic activity of the micro-organism. The conversion occurs only on the surface between the mat and the medium. Therefore surface to volume ratio plays a very important role in successful fermentations. As seen from the results, greater surface to volume ratio yielded higher percentage of conversion. Although the initial media were identical, 50 ml samples resulted in higher additional acidity than 100 ml samples when both were fermented in 500 ml Erlenmeyer flasks.

In all the experiments, the mats wrinkled. A larger surface of contact formed between the medium and the mat through wrinkling. The wrinkling was observed especially in molasses media where the sucrose concentration was much lower.

The transfer of spores also played an important part in successful fermentations. The first fermentation carried out with strain 1/I yielded very low conversion, only an increase of 0.035 N in total acidity. The second fermentation, where spores from the previous experiment were employed, yielded an increase of 0.477 N. In the third fermentation, the increase in acidity was 0.658 N. Thus it was observed that the strain adapted itself to the acidic medium and functioned more effectively with each transfer. Along with surface to volume ratio, this factor was an important reason in the increase of the yield of citric acid. But after three to four transfers, during which the strain became wholly adapted to the system, further transfers did not increase the yield. The transfers were useful to keep the spores fresh. Dormant spores, even though adapted to the conditions of fermentation, were less efficient than fresh ones for fermentation.

The fermentations carried out in molasses media were successful. The initial sucrose concentration of molasses medium was low, 17.0 - 18.0 gr/liter and impurities were high. The ratio of calcium citrate precipitated to sucrose consumed in fermentation was higher for molasses fermentations than for sucrose fermentations. Therefore it may be concluded that more sucrose was converted to citric acid in molasses media.

Figure 9
Citric Acid Crystals obtained from Sucrose Media

Figure 10
Citric Acid Crystals obtained from Molasses Media

Ferrocyanide content played an important role in the success of fermentations. Increase in ferrocyanide content resulted in a decrease in the yields. The most favorable ferrocyanide content was 0.55 - 0.60 gr/liter. Less than this amount failed to precipitate the unfavorable impurities in the medium, and more than this amount had a toxic effect on the strain used. Increase from 0.6 gr/liter to 0.8 gr/liter of ferrocyanide in media fermented with strain 1/I resulted in a decrease from 0.120 N to 0.108 N in additional acidity. Decrease from 0.9 gr/liter to 0.55 gr/liter in media fermented with CBS-2 resulted in an increase in additional acidity from 0.093 N to 0.118 N.

No difference between the two strains were observed as far as fermenting ability was concerned.

Comparisons of initially identical samples fermented at different times indicated that molasses fermentations show greater variety than sucrose fermentations. This is probably due to the fact that molasses is not uniform in chemical composition. Since molasses is a very dense fluid, it is very difficult to accomplish uniform mixing. The non-uniform concentrations in different samples may be the reason for varying results.

It was difficult to completely purify the calcium citrate precipitated from the molasses media. Obtaining pure citric acid required recrystallization several times. The losses due to recrystallization and purification were considerable.

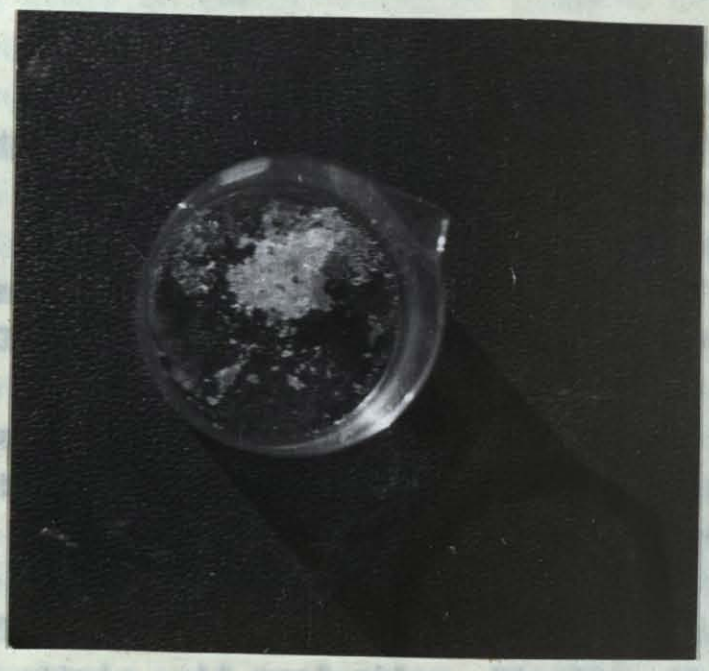


**Figure 11**
**Citric Acid Crystals from Molasses**

## XI. CONCLUSION

It has been shown in the present experiments that it is possible to produce citric acid from Turkish molasses and sucrose by fermentation processes. However, the fermentation of molasses has several disadvantages. First, the low sucrose concentration of molasses makes it imperative to process large quantities of this substance to obtain appreciable amounts of citric acid. Although molasses is very abundant and therefore relatively inexpensive in Turkey, this method would require big fermentation units and larger processing equipment. In addition, larger quantities of heat and energy would be spent to heat this large mass. Secondly, ferrocyanide compounds are required in appreciable amounts when large quantities of molasses are being fermented. These compounds are expensive, so they would raise the cost of citric acid production. Finally, it is difficult to purify citric acid obtained from molasses. Citric acid is mainly used in food industry, therefore, a very pure product is required. Extensive purification would require costly procedures.

It is reported in the literature (6) that ion-exchangers can be used to improve the production of citric acid from molasses. The effect of ion-exchangers is to remove the inhibitory ions from the solution, and thus increase the conversion of sucrose present to citric acid. However, in the samples fermented in this work, a good conversion of sucrose to citric acid was obtained, as indicated by the fact that a very small amount of sucrose remained, and a minimum amount of it was consumed by the mold. Therefore, the use of ion-exchangers for removing inhibitory ions to improve the yields would not be necessary. However, passing the molasses media through ion-exchangers would probably aid the crystallization of calcium citrate from the medium. This may be a further point of study in this fermentation method. It should be kept in mind that ion-exchangers are also rare and expensive in our country.

At the present time, the least expensive process to obtain citric acid would be fermentation of sucrose solutions. Commercial sucrose is produced excessively in Turkey, so the price of sucrose is quite low. Fermentation with a strain such as Aspergillus Niger 1/I would give a conversion as high as 85 per cent without appreciable formation of oxalic acid. The inorganic salts added to the medium are comparatively less expensive than the ferrocyanide salts and are required in smaller amounts. The precipitation of calcium citrate and the recovery of citric acid are easy and quantitative. Sterilization is not necessary because the danger of contamination is very little. When batch process is employed, the equipment necessary is simple and inexpensive. The process would also provide a good outlet to the excess sucrose available in Turkey. Variations in the results would be unimportant when the process is carried out in a large scale.

Further research in the field of fermentation of sucrose to citric acid in Turkey should concentrate on the discovery of new strains of Aspergillus Niger or other molds which would improve the yields even more. Also emphasis should be given on experiments based on submerged fermentation. At present, no research is being done on this labor saving process. However, results from this kind of research may even make surface culture fermentations obsolete.

The results obtained throughout the experiments performed were very hopeful. However, before any attempts are made towards industrial production, an institute for classification and searching of molds should be formed. Once the fermenting micro-organisms can be obtained easily, citric acid production and other fermentation processes can flourish in Turkey.

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## APPENDIX

I. Experiments carried out using Aspergillus Niger supplied by the Istanbul University:

Number of samples: 3
Initial volume: 100 ml
Initial sucrose concentration: 140 gr/l
Initial acidity: 0.112 N
Duration: 10 days
Samples were sterilized.

| | A | B | C |
|---|---|---|---|
| Final volume: | 92 ml | 88 ml | 93 ml |
| Final sucrose conc.: | 80 gr/l | 73 gr/l | 81 gr/l |
| Final acidity: | 0.109 N | 0.111 N | 0.113 N |

II. Experiments carried out using the strains obtained from Research Center in Ankara:

Experiment I

Mold used: 1/I
Number of samples: 2
Initial volume: 50 ml
Initial sucrose conc.: 140 gr/l
Initial acidity: 0.109 N
Samples were sterilized,

| | A | B |
|---|---|---|
| Final volume: | 43 ml | 42 ml |
| Final sucrose conc.: | 56 gr/l | 54 gr/l |
| Final acidity: | 0,144 N | 0.146 N |
| Additional acidity: | 0.035 N | 0.037 N |
| Sucrose consumed: | 84 gr/l | 86 gr/l |

Paper chromotography revealed only citric acid.

Diameter of 5 micron of standard spot: 1.1 cm
" " 5 micron " test spot: 0.4 cm

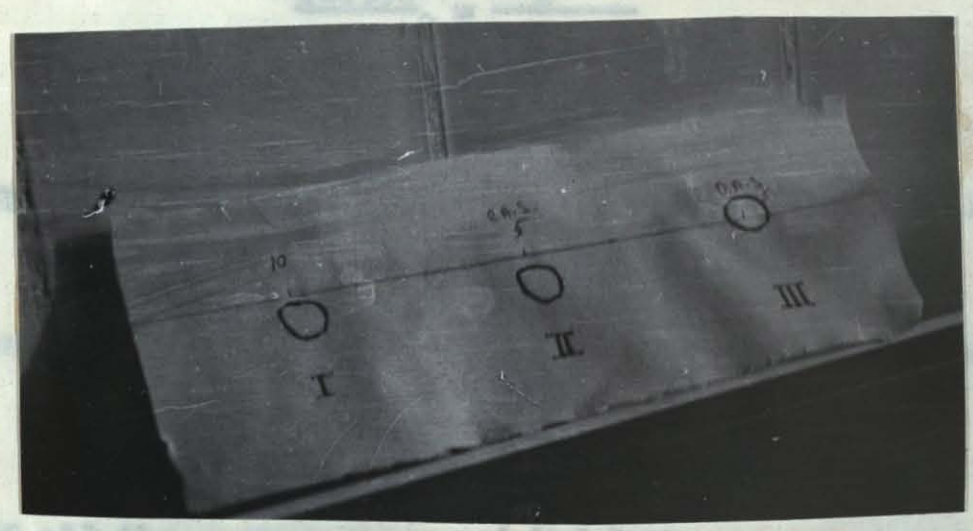


Figure 12
Paper Chromotogram of a Solution
containing only Citric Acid

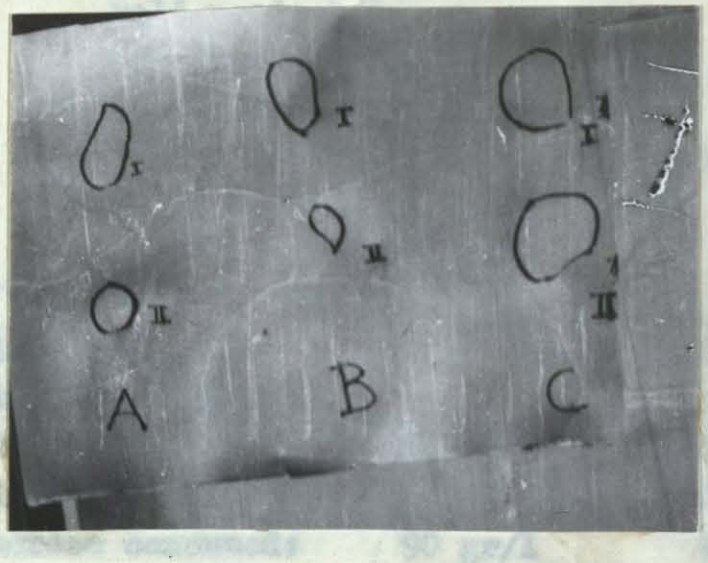


Figure 13
Paper Chromotogram of a Solution
containing both Citric and Oxalic Acids
A and B are two samples being tested and C is the standard,
I shows oxalic acid and II shows citric acid positions.

Sample Calculation:

$$\frac{(0.55)^2}{(0.20)^2} = \frac{1}{\log x}$$

$$\log x = 0.134$$

$$x = 1.36 \text{ gr/l}$$

The concentration of citric acid in the medium is 1.36 gr/l.

Molecular weight of sucrose: 342.30

Molecular weight of Citric acid: 192.12

Assuming 1 mole of sucrose yields 2 moles of citric acid:

$$\frac{84 \text{ gr/l}}{342.30} = \frac{x}{192.12(2)}$$

$$x = 94.4 \text{ gr/l citric acid}$$

If all the sucrose consumed had been converted to citric acid, 94.4 gr/l of citric acid would have been formed. Therefore percentage conversion is:

$$\frac{1.36}{94.4} \times 100 = 1.5 \%$$

Experiment II

Mold used: 1/I

Number of samples: 2

Initial volume: 100 ml

Initial sucrose conc.: 143 gr/l

Initial acidity: 0.134 N

Samples were sterilized.

| | A | B |
|---|---|---|
| Final volume: | 81.5 ml | 83 ml |
| Final sucrose conc.: | 53 gr/l | 51 gr/l |
| Final acidity: | 0.598 N | 0.611 N |
| Additional acidity: | 0.464 N | 0.477 N |
| Sucrose consumed: | 90 gr/l | 92 gr/l |

Paper chromotography revealed only citric acid.

Diameter of standard spot: 1.1 cm

Diameter of test spot: 1.7 cm

Experiment III

Mold used: 1/1
Number of samples: 2
Initial volume: 50 ml
Initial sucrose conc.: 145 gr/l
Initial acidity: 0.134 N
Samples were not sterilized.

| | A | B |
|---|---|---|
| Final volume: | 38.3 ml | 37.8 ml |
| Final sucrose conc.: | 56 gr/l | 58 gr/l |
| Final acidity: | 0.786 N | 0.792 N |
| Additional acidity: | 0.652 N | 0.658 N |
| Sucrose consumed: | 89 gr/l | 93 gr/l |

Paper chromotography revealed only citric acid.
Diameter of standard spot: 0.9 cm
Diameter of test spot: 1.1 cm

Experiment IV

Mold used: 2/1
Number of samples: 2
Initial volume: 100 ml
Initial sucrose conc.: 140 gr/l
Initial acidity: 0,119 N
Samples were sterilized.

| | A | B |
|---|---|---|
| Final volume: | 92 ml | 94 ml |
| Final sucrose conc.: | 95 gr/l | 97 gr/l |
| Final acidity: | 0.113 N | 0.112 N |

Paper chromotography revealed no citric or oxalic acid.

Experiment V

Mold used: 2/1
Number of samples: 2
Initial volume: 50 ml
Initial sucrose conc.: 152 gr/l
Initial acidity: 0.141 N
Samples were not sterilized.

| | A | B |
|---|---|---|
| Final volume: | 38 ml | 40 ml |
| Final sucrose conc.: | 100 gr/l | 98 gr/l |
| Final acidity: | 0.116 N | 0.124 N |

Experiment VI

Mold used: 3/I
Number of samples: 2
Initial volume: 100 ml
Initial sucrose conc.: 140 gr/l
Initial acidity: 0.124 N
Samples were sterilized.

| | A | B |
|---|---|---|
| Final volume: | 88 ml | 89 ml |
| Final sucrose conc.: | 72 gr/l | 74 gr/l |
| Final acidity: | 0.391 N | 0.395 N |
| Additional acidity: | 0.267 N | 0.271 N |
| Sucrose consumed: | 68 gr/l | 66 gr/l |

Paper chromotography revealed both citric and oxalic acids.
Diameter of standard citric acid spot: 1.0 cm
Diameter of standard oxalic acid spot: 1.1 cm
Diameter of test citric acid spot: 1.2 cm
Diameter of test oxalic acid spot: 0.6 cm

Experiment VII

Mold used: 3/I
Number of samples: 2
Initial volume: 50 ml
Initial sucrose conc.: 138 gr/l
Initial acidity: 0.139 N
Samples were not sterilized.

| | A | B |
|---|---|---|
| Final volume: | 39.5 ml | 41 ml |
| Final sucrose conc.: | 71 gr/l | 75 gr/l |
| Final acidity: | 0.391 N | 0.393 N |
| Additional acidity: | 0.252 N | 0.254 N |

Experiment VIII

Mold used: S-3
Number of samples: 2
Initial volume: 100 ml
Initial sucrose conc.: 140 gr/l
Initial acidity: 0.124 N
Samples were sterilized.

| | A | B |
|---|---|---|
| Final volume: | 90.5 ml | 91 ml |
| Final sucrose conc.: | 94 gr/l | 93 gr/l |
| Final acidity: | 0.150 N | 0.153 N |
| Additional acidity: | 0.026 N | 0.029 N |
| Sucrose consumed: | 46 gr/l | 47 gr/l |

Paper chromotography revealed only citric acid.

Experiment IX

Mold used: S-3
Initial volume: 50 ml
Initial sucrose conc.: 143 gr/l
Initial acidity: 0.126 N
Samples were not sterilized.

| | A | B |
|---|---|---|
| Final volume: | 42 ml | 40 ml |
| Final sucrose conc.: | 91 gr/l | 88 gr/l |
| Final acidity: | 0.144 N | 0.148 N |
| Additional acidity: | 0.018 N | 0.022 N |
| Sucrose consumed: | 52 gr/l | 55 gr/l |

Experiment X

Mold used: CBS-2
Number of samples: 2
Initial volume: 100 ml
Initial sucrose conc.: 140 gr/l
Initial acid conc.: 0.136 N
Samples were sterilized.

| | A | B |
|---|---|---|
| Final volume: | 79 ml | 82 ml |
| Final sucrose conc.: | 55.6 gr/l | 57 gr/l |
| Final acidity: | 0.368 N | 0.381 N |
| Additional acidity: | 0.232 N | 0.245 N |
| Sucrose consumed: | 85 gr/l | 83 gr/l |

Paper chromotography revealed only citric acid.

Experiment XI

Mold used: CBS-2
Number of samples: 2
Initial volume: 50 ml
Initial sucrose conc.: 145 gr/l
Initial acidity: 0.130 N
Samples were not sterilized.

| | A | B |
|---|---|---|
| Final volume: | 41 ml | 42 ml |
| Final sucrose conc.: | 55 gr/l | 56 gr/l |
| Final acidity: | 0.412 N | 0.402 N |
| Additional acidity: | 0.282 N | 0.272 N |
| Sucrose consumed: | 90 gr/l | 89 gr/l |

Paper chromotography revealed only citric acid.

Experiment XII

Samples: 2
Mold used: St-Volt
Initial volume: 100 ml
Initial sucrose conc.: 140 gr/l
Initial acidity: 0.119 N
Samples were sterilized.

| | A | B |
|---|---|---|
| Final volume: | 89 ml | 91 ml |
| Final sucrose conc.: | 101 gr/l | 106 gr/l |
| Final acidity: | 0.097 N | 0.099 N |
| Sucrose consumed: | 39 gr/l | 34 gr/l |

Paper chromotography revealed no citric or oxalic acid.

Experiment XIII

Mold used: St-Volt
Number of samples: 2
Initial volume: 50 ml
Initial sucrose conc.: 150 gr/l
Initial acidity: 0.160 N
Samples were not sterilized.

| | A | B |
|---|---|---|
| Final volume: | 34 ml | 35 ml |
| Final sucrose conc.: | 103 gr/l | 100 gr/l |
| Final acidity: | 0.130 N | 0.132 N |
| Sucrose consumed: | 47 gr/l | 50 gr/l |

Paper chromotography revealed no citric or oxalic acid.

III. Experiments carried out with molasses

Experiment XIV

Mold used: 1/I
Number of samples: 2
Initial volume: 360 ml
Initial sucrose conc.: 17 gr/l
Initial acidity: 0.010 N
Ferrocyanide content: 0.6 gr/l
Samples were not sterilized.

| | A | B |
|---|---|---|
| Final volume: | 328 ml | 335 ml |
| Final sucrose conc.: | 1 gr/l | 1 gr/l |
| Final acidity: | 0.133 N | 0.128 N |
| Additional acidity: | 0.123 N | 0.118 N |
| Sucrose consumed: | 16 gr/l | 16 gr/l |

Paper chromotography revealed the presence of only citric acid.

Experiment XV

Mold used: 1/I
Number of samples: 2
Initial volume: 385 ml
Initial sucrose conc.: 18 gr/l

Initial acidity: 0.020 N
Ferrocyanide content: 0.8 gr/l
Samples were not sterilized.

| | A | B |
|---|---|---|
| Final volume: | 307 ml | 313 ml |
| Final sucrose conc.: | 2 gr/l | 2 gr/l |
| Final acidity: | 0.125 N | 0.129 N |
| Additional acidity: | 0.105 N | 0.109 N |
| Sucrose consumed: | 16 gr/l | 16 gr/l |

Chromotography revealed only citric acid.

Experiment XVI

Mold used: CBS-2
Number of samples: 2
Initial volume: 385 ml
Initial sucrose conc.: 18 gr/l
Initial acidity: 0.023 N
Ferrocyanide content: 0.90 gr/l
Samples were not sterilized.

| | A | B |
|---|---|---|
| Final volume: | 316 ml | 321 ml |
| Final sucrose conc.: | 2 gr/l | 2 gr/l |
| Final acidity: | 0.112 N | 0.109 N |
| Additional acidity: | 0.099 N | 0.086 N |
| Sucrose consumed: | 16 gr/l | 16 gr/l |

Paper chromotography revealed only citric acid.

Experiment XVII

Mold used: CBS-2
Number of samples: 2
Initial volume: 380 ml
Initial sucrose conc.: 18 gr/l
Initial acidity: 0.053 N
Ferrocyanide content: 0.55 gr/l
Samples were not sterilized.

| | A | B |
|---|---|---|
| Final volume: | 342 ml | 338 ml |
| Final sucrose conc.: | 2 gr/l | 2 gr/l |
| Final acidity: | 0.168 N | 0.174 N |
| Additional acidity: | 0.115 N | 0.121 N |
| Sucrose consumed: | 16 gr/l | 16 gr/l |

Paper chromotography revealed the presence of citric acid.

Experiment XVIII

Mold used: CBS-2
Number of samples: 1
Initial volume: 100 ml
Initial sucrose conc.: 18 gr/l
Initial acidity: 0.053 N
Ferrocyanide conc.: 0.58 gr/l
Sample was not sterilized.

| Days | Acid concentration |
|---|---|
| 1 | 0.053 N |
| 2 | 0.054 N |
| 3 | 0 .071 N |
| 4 | 0.105 N |
| 5 | 0.118 N |
| 6 | 0.129 N |
| 7 | 0.142 N |
| 8 | 0.157 N |
| 9 | 0.168 N |
| 10 | 0.175 N |
| 11 | 0.176 N |
| 12 | 0.178 N |

Final acidity: 0.178 N
Additional acidity: 0.127 N
Final sucrose conc.: 2 gr/l
Sucrose consumed: 16 gr/l

Additional acidity is only due to citric acid.