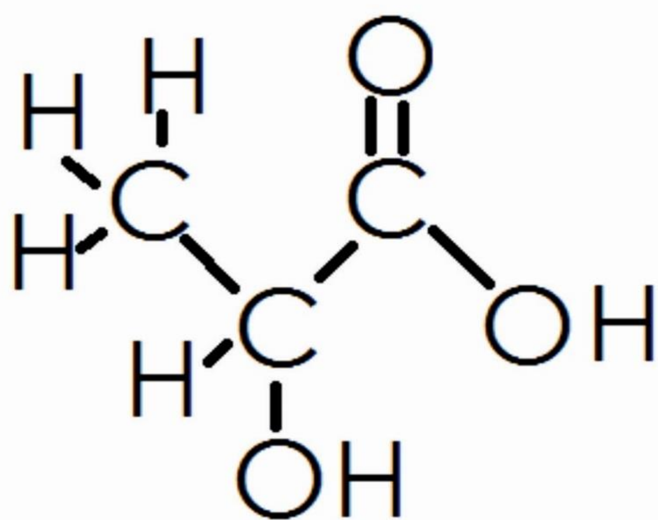
STUDY OF LACTIC ACID FERMENTATION



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Kripa Drishti Publications, Pune.

STUDY OF LACTIC ACID FERMENTATION

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PREFACE

Lactic acid, also known as milk acid, is a chemical compound that plays a role in various biochemical processes and was first isolated in 1780 by the Swedish chemist Carl Wilhelm Scheele. Lactic acid is a carboxylic acid with the chemical formula C3H6O3. It has a hydroxyl group adjacent to the carboxyl group, making it an alpha hydroxy acid. In solution, it can lose a proton from the acidic group, producing the lactate ion (to be specific, an anion due to being negatively charged with an extra electron) CH3CH (OH) COO–.

Compared to acetic acid, its pKa is 1 unit smaller, meaning lactic acid deprotonates ten times as easily as acetic acid does. This higher acidity is the consequence of the intramolecular hydrogen bridge between the a-hydroxyl and the carboxylate group, making the latter less capable of strongly attracting its proton. Lactic acid is miscible with water or ethanol, and is hygroscopic. Lactic acid is chiral and has two optical iso-mers. One is known as L-(+)-lactic acid or (S)-lactic acid and the other, its mirror image, is D-(-)-lactic acid or (R)-lactic acid. In animals, L-lactate is constantly produced from pyruvate via the enzyme lactate dehydrogenase (LDH) in a process of fermentation during normal metabolism and exercise. It does not increase in concentration until the rate of lactate production exceeds the rate of lactate removal, which is governed by a number of factors, including monocarboxylate transporters, concentration and isoform of LDH, and oxidative capacity of tissues. The concentration of blood lactate is usually 1–2 mmol/L at rest, but can rise to over 20 mmol/L during intense exertion. In industry, lactic acid fermentation is performed by lactic acid bacteria. These bacteria can also grow in the

mouth; the acid they produce is responsible for the tooth decay known as caries. In medicine, lactate is one of the main components of lactated Ringer's solution and Hartmann's solution. These intravenous fluids consist of sodium and potassium cations along with lactate and chloride anions in solution with distilled water, generally in concentrations isotonic with human blood. It is most commonly used for fluid resuscitation after blood loss due to trauma, surgery, or burn injury. Lactic acid was discovered in 1780 by Swedish chemist, Carl Wilhelm Scheele, who isolated the lactic acid from sour milk as an impure brown syrup and gave it a name based on its origins: 'Mjölksyra'. The French scientist Frémy produced lactic acid by fermentation, and this gave rise to industrial production in 1881.Lactic acid is produced by the fermentation of sugar and water or by chemical process and is commercially usually sold as a liquid. Pure and anhydrous racemic lactic acid is a white crystalline solid with a low melting point. Lactic acid has two optical forms, L (+) and D (-). L (+)-lactic acid is the biological isomer as it is naturally present in the human body.

Summary

Some Aspects of Search on Lactic Acid Fermentation:

Lactic acid, also known as milk acid, is a chemical compound that plays a role in various biochemical processes and was first isolated in 1780 by the Swedish chemist Carl Wilhelm Scheele. Lactic acid is a carboxylic acid with the chemical formula $C_3H_6O_3$. It has a hydroxyl group adjacent to the carboxyl group, making it an alpha hydroxy acid.

In solution, it can lose a proton from the acidic group, producing the lactate ion (to be specific, an anion due to being negatively charged with an extra electron) CH_3CH (OH) COO–. Compared to acetic acid, its pKa is 1 unit smaller, meaning lactic acid deprotonates ten times as easily as acetic acid does. This higher acidity is the consequence of the intramolecular hydrogen bridge between the a-hydroxyl and the carboxylate group, making the latter less capable of strongly attracting its proton.

Lactic acid is miscible with water or ethanol, and is hygroscopic. Lactic acid is chiral and has two optical isomers. One is known as L-(+)-lactic acid or (S)-lactic acid and the other, its mirror image, is D-(-)-lactic acid or (R)-lactic acid.

In animals, L-lactate is constantly produced from pyruvate via the enzyme lactate dehydrogenase (LDH) in a process of fermentation during normal metabolism and exercise. It does not increase in concentration until the rate of lactate production exceeds the rate of lactate removal, which is governed by a number of factors, including monocarboxylate transporters, concentration and isoform of LDH, and oxidative capacity of tissues. The concentration of blood lactate is usually 1–2 mmol/L at rest, but can rise to over 20 mmol/L during intense exertion. In industry, lactic acid fermentation is performed by lactic acid bacteria. These bacteria can also grow in the mouth; the acid they produce is responsible for the tooth decay known as caries. In medicine, lactate is one of the main components of lactated Ringer's solution and Hartmann's solution.

These intravenous fluids consist of sodium and potassium cations along with lactate and chloride anions in solution with distilled water, generally in concentrations isotonic with human blood. It is most commonly used for fluid resuscitation after blood loss due to trauma, surgery, or burn injury. Lactic acid was discovered in 1780 by Swedish chemist, Carl Wilhelm Scheele, who isolated the lactic acid from sour milk as an impure brown syrup and gave it a name based on its origins: 'Mjölksyra'. The French scientist Frémy produced lactic acid by fermentation and this gave rise to industrial production in 1881.Lactic acid is produced by the fermentation of sugar and water or by chemical process and is commercially usually sold as a liquid.

Pure and anhydrous racemic lactic acid is a white crystalline solid with a low melting point. Lactic acid has two optical forms, L (+) and D (-). L (+)-lactic acid is the biological isomer as it is naturally present in the human body.

Lactic acid is widely used in the food, cosmetic, pharmaceutical, and chemical industries and has received increased attention for use as a monomer for the production of biodegradable poly (lactic acid).

It can be produced by either biotechnological fermentation or chemical synthesis, but the former route has received considerable interest recently, due to environmental concerns and the limited nature of petrochemical feedstocks. There have been various attempts to produce lactic acid efficiently from inexpensive raw materials.

We present a review of lactic acid-producing microorganisms, raw materials for lactic acid production, fermentation approaches for lactic acid production, and various applications of lactic acid, with a particular focus on recent investigations. In addition, the future potentials and economic impacts of lactic acid are discussed.

Keeping in view, the wide range of applications and economic importance of lactic acid and its derivatives in different field of life, the research project has been taken by author to study lactic acid fermentation by Lactobacillus bulgaricus NCIM-2359 exposed to some useful compounds, viz: some active organic molecules and some chemical mutagens.

The Present Thesis has Been Divided in Five Chapters:

Chapter – I: is detailed study of "General fermentations" with special reference to lactic acid fermentation by Lactobacillus bulgaricus NCIM-2359. However, this chapter is a review of previous work done by many workers of this field.

Chapter – II: "General experimental techniques" contains the chemical cleaning and steam sterilization of glasswares and different fermentation medium broth, preparation of culture and production medium and also buffer solution, seeding of culture tubes and inoculation of production medium, incubation of culture tubes and production medium colorimetric determination of lactic cid formed by Lactobacillus bulgaricus NCIM-2359 and molasses sugars left unfermented during the course of present investigation lactic acid fermentation by Lactobacillus bulgaricus NCIM-2359

Chapter – III: "Optimization of parameters" deals with selection of lactic acid producing bacteria, selection of cheapest and easily available economic raw material, optimization of concentration of raw material selected, optimization of temperature, pH and incubation period of lactic acid fermentation process.

In this chapter it has been found that lactic acid fermentation by Lactobacillus bulgaricus NCIM-2359 proceeds best when a molasses solution (raw material) 20% (w/v) is allowed to ferment for 6 days of incubation period at 38°C by maintaining the pH value of the fermentation medium to 6.1 along with other nutritional supplements required by the lactic acid bacteria.

Chapter – IV: "The influence of some AOM (Active Organic Molecule) on lactic acid fermentation by Lactobacillus bulgaricus NCIM-2359 describes effect of 1,3-bis[(2,2-dimethyl-1,3-dioxolan-4-yl) methyl]urea, 5-aminoaorotic acid, mandelic acid, 2-hydroxybutyric acid on lactic acid fermentation by Lactobacillus bulgaricus NCIM-2359.

Study of the influence of 1, 3-bis [(2, 2-dimethyl-1, 3-dioxolan-4-yl) methyl] urea, 5-aminoaorotic acid, mandelic acid 2-Hydroxybutyric acid on lactic acid fermentation by Lactobacillus bulgaricus NCIM-2359 in 6 days of optimum incubation period.

AOM Used	Optimum Concentration of the AOM Used.	Max. Yield of Lactic Acid* In Control Flasks In g/100ml	Max. Yield of Lactic Acid* in The Presence of AOM in g/100ml	% of Lactic Acid Increase or Decrease in 6 Days of Incubation Pd.
1	6.0×10^{-5}M	7.9115967	8.8380566	(+) 11.7101507
2	5.0×10^{-5}M	7.8926956	8.6267162	(+) 9.2999988
3	4.0×10^{-5}M	7.9347015	8.1497429	(+) 2.7101384
4	4.0×10^{-5}M	7.8869820	8.0062844	(+) 1.5126495

* Each value represents mean of three observations

(+) Values indicates % increase in the yield of lactic acid.

Experimental deviation (+) 2.5 to 3.5%.

a. 1, 3-bis [(2, 2-dimethyl-1, 3-dioxolan-4-yl) methyl] urea
b. 5-aminoaorotic acid
c. Mandelic acid
d. 2-Hydroxybutyric acid

It may be summarized that 1,3-bis[(2,2-dimethyl-1,3-dioxolan-4-yl) methyl] urea and 5-aminoaorotic acid enhances the lactic acid fermentation by Lactobacillus bulgaricus NCIM-2359 at all concentrations used; while mandelic acid and 2-hydroxybutyric acid antagonizes the course of lactic acid fermentation by Lactobacillus bulgaricus NCIM-2359 at their higher concentrations used, i.e., 4.0×10^{-5}M and 4.0×10^{-5} respectively retarding thereby the yield of lactic acid.

However, it was interesting to point out that1,3-bis [(2, 2-dimethyl-1, 3-dioxolan-4-yl) methyl] urea was very effective amongst the active organic molecule used which could increase significantly the yield of lactic acid to an extent of 11.7101507% in comparison to control fermentor flasks, on the other hand 5-aminoaorotic acid was also found effective for the lactic acid fermentation by Lactobacillus bulgaricus NCIM-2359 but it could enhance the yield of lactic acid only to an extent of 9.2999988% in the same experimental conditions.

Chapter – V: deals lactic acid fermentation by Lactobacillus bulgaricus NCIM-2359 exposed to some chemical mutagens, viz: benzidine, 3, 3’-dichlorobenzidine, 1-methyl-3-nitro-1-nitrosoguanidine, N-methyl-N-nitrosoethyl carbamate.

Study of the influence of benzidine; 3, 3’-dichlorobenzidine; 1-methyl-3-nitro-1-nitrosoguanidine and N-methyl-N-nitrosoethyl carbamate on lactic acid fermentation by Lactobacillus bulgaricus NCIM-2359 in 6 days of optimum incubation period.

Chemical Mutagens Used	Optimum Concentration of the Mutagens Used.	Max. Yield of Lactic Acid* in Control Flasks in g/100ml	Max. Yield of Lactic Acid* in the Presence of Mutagens	% of Lactic Acid Increase in 6 Days of Incubation Pd.
1	6.0×10^{-5} M	8.1579630	8.9259145	(+) 9.4134957
2	4.0×10^{-5} M	8.2346156	8.9685928	(+) 8.9133146
3	7.0×10^{-5} M	8.3540970	9.4245315	(+)12.813283
4	4.0×10^{-5} M	8.1273413	8.4657369	(+) 4.1636691

+ve values indicate % increase in the yield of lactic acid

a. Benzidine
b. 3, 3'-dichlorobenzidine
c. 1-methyl-3-nitro-1-nitrosoguanidine
d. N-methyl-N-nitrosoethyl carbamate

It may be summarized that benzidine; 3, 3'-dichlorobenzidine and 1-methyl-3-nitro-1-nitrosoguanidine stimulates and enhances the lactic acid fermentation by Lactobacillus bulgaricus NCIM-2359.

It has been observed that 1-methyl-3-nitro-1-nitrosoguanidine has influenced the production of lactic acid significantly to a great extent while benzidine and 3,3'-dichlorobenzidine were approximately equally effective for lactic acid fermentation by Lactobacillus bulgaricus NCIM-2359 and could increase the lactic acid production nearly in the range of 9.4143957 and 8.9133146 in comparison to control.

However, the N-methyl-N-nitrosoethyl carbamate was found to be inhibitory at higher concentration which deactivates lactic acid fermentation by Lactobacillus bulgaricus NCIM-2359.

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Chapter 1

General Introduction, Scope and Importance of Lactic Acid Fermentation

1.1 Introduction:

Lactic acid, also known as milk acid, is a chemical compound that plays a role in various biochemical processes and was first isolated in 1780 by the Swedish chemist Carl Wilhelm Scheele. Lactic acid is a carboxylic acid with the chemical formula $C_3H_6O_3$. It has a hydroxyl group adjacent to the carboxyl group, making it an alpha hydroxy acid (AHA).

In solution, it can lose a proton from the acidic group, producing the lactate ion (to be specific, an anion due to being negatively charged with an extra electron) $CH_3CH(OH)COO^-$. Compared to acetic acid, its pKa is 1 unit smaller, meaning lactic acid deprotonates ten times as easily as acetic acid does. This higher acidity is the consequence of the intramolecular hydrogen bridge between the α -hydroxyl and the carboxylate group, making the latter less capable of strongly attracting its proton. Lactic acid is miscible with water or ethanol, and is hygroscopic. Lactic acid is chiral and has two optical isomers. One is known as L-(+)-lactic acid or (S)-lactic acid and the other, its mirror image, is D-(-)-lactic acid or (R)-lactic acid.

In animals, L-lactate is constantly produced from pyruvate via the enzyme lactate dehydrogenase (LDH) in a process of fermentation during normal metabolism and exercise. It does not increase in concentration until the rate of lactate production exceeds the rate of lactate removal, which is governed by a number of factors, including monocarboxylate transporters, concentration and isoform of LDH, and oxidative capacity of tissues. The concentration of blood lactate is usually 1–2 mmol/L at rest, but can rise to over 20 mmol/L during intense exertion. In industry, lactic acid fermentation is performed by lactic acid bacteria. These bacteria can also grow in the mouth; the acid they produce is responsible for the tooth decay known as caries.[1-6]

In medicine, lactate is one of the main components of lactated Ringer's solution and Hartmann's solution. These intravenous fluids consist of sodium and potassium cations along with lactate and chloride anions in solution with distilled water, generally in concentrations isotonic with human blood. It is most commonly used for fluid resuscitation after blood loss due to trauma, surgery, or burn injury.

Lactic acid was refined for the first time by the Swedish chemist Carl Wilhelm Scheele in 1780 from sour milk. In 1808 Jöns Jacob Berzelius discovered that lactic acid (actually L-lactate) also is produced in muscles during exertion.[7] its structure was established by Johannes Wislicenus in 1873. In 1856, Louis Pasteur discovered Lactobacillus and its role in the making of lactic acid. Lactic acid started to be produced commercially by the German pharmacy Boehringer Ingelheim in 1895. In 2006, global production of lactic acid reached 275,000 tonnes with an average annual growth of 10%[8]. It is expected that by 2013, industrial applications will account for more than half of global lactic acid use.

During power exercises such as sprinting, when the rate of demand for energy is high, glucose is broken down and oxidized to pyruvate, and lactate is produced from the pyruvate faster than the tissues can remove it, so lactate concentration begins to rise. The production of lactate is a beneficial process because it regenerates NAD^+ which is used up in the creation of pyruvate from glucose, and this ensures that energy production is maintained and exercise can continue. The increased lactate produced can be removed in two ways:

Oxidation back to pyruvate by well-oxygenated muscle cells. Pyruvate is then directly used to fuel the Krebs cycle. Conversion to glucose via gluconeogenesis in the liver and release back into circulation; (Cori cycle.)[9]

If not released, the glucose can be used to build up the liver's glycogen if they are empty. Strenuous anaerobic exercise causes a lowering of pH and pain, called acidosis.

The effect of lactate production on acidosis has been the topic of many recent conferences in the field of exercise physiology. Robergs et al. have discussed the creation of H^+ ions that occurs during glycolysis.[10] and claim that the idea that acidosis is caused by the production of lactic acid is a myth (a "construct"), pointing out that part of the lowering of pH is due to the reaction $ATP^{-4}+H_2O=ADP^{-3}+HPO_4^{-2}+H^+$, and that reducing pyruvate to lactate pyruvate+NADH+H^+=lactate + NAD^+) actually consumes H^+.

However, a response by Lindinger et al.[11] has been written claiming that Robergs et al ignored the causative factors of the increase in concentration of hydrogen ions (denoted [H^+]). Specifically, lactate is an anion, and its production causes a reduction in the amount of cations such as Na+ minus anions, and thus causes an increase in [H^+] to maintain electro neutrality. Increasing partial pressure of CO_2, PCO_2, also causes an increase in [H^+]. During exercise, the intramuscular lactate concentration and PCO_2 increase, causing an increase in [H^+], and, thus, a decrease in pH. (Le Chatelier's principle)

During intense exercise, the respiratory chain cannot keep up with the amount of hydrogen atoms that join to form NADH. NAD^+ is required to oxidize 3-phosphoglyceraldehyde in order to maintain the production of anaerobic energy during glycolysis. During anaerobic glycolysis, NAD^+ is “freed up” when NADH combines with pyruvate to form lactate (as mentioned above). If this did not occur, glycolysis would come to a stop. However, lactate is continually formed even at rest and during moderate exercise. This occurs due to the metabolism of red blood cells that do not have mitochondria and limitations resulting from the enzyme activity that occurs in muscle fibers having a high glycolytic capacity[12]

Fermentation technology is the oldest of all biotechnological processes. The term is derived from the Latin verb fevered, to boil-- the appearance of fruit extracts or malted grain acted upon by yeast, during the production of alcohol.

Fermentation is a process of chemical change caused by organisms or their products, usually producing effervescence and heat. Microbiologists consider fermentation as ‘any process for the production of a product by means of mass culture of micro-organisms’. Biochemists consider fermentation as ‘an energy generating process in which organic compounds act both as electron donors and acceptors; hence fermentation is ‘an anaerobic process where energy is produced without the participation of oxygen or other inorganic electron acceptors.

In biotechnology, the microbiological concept is widely used. Lactic acid is also employed in pharmaceutical technology to produce water-soluble lactates from otherwise insoluble active ingredients. It finds further use in topical preparations and cosmetics to adjust acidity and for its disinfectant and keratolytic properties. Lactic acid is found primarily in sour milk products, such as koumiss, laban, yogurt, kefir, and some cottage cheeses. The casein in fermented milk is coagulated (curdled) by lactic acid. Lactic acid is also responsible for the sour flavor of sourdough breads. This acid is used in beer brewing to lower the wort pH in order to reduce some undesirable substances such as tannins without giving off-flavors such as citric acid and increase the body of the beer. Some brewers and breweries will use food grade lactic acid to lower the pH in finished beers.

In winemaking, a bacterial process, natural or controlled, is often used to convert the naturally present malic acid to lactic acid, to reduce the sharpness and for other flavor-related reasons. This malolactic fermentation is undertaken by the family of lactic acid bacteria[13-24]. As a food additive it is approved for use in the EU, [25,] USA[26] and Australia and New Zealand[,-27] it is listed by its INS number 270 or as E number E270.

Fermentation is the chemical transformation of organic substances into simpler compounds by the action of enzymes, complex organic catalysts, which are produced by microorganisms such as molds, yeasts, or bacteria. Enzymes act by hydrolysis, a process of breaking down or predigesting complex organic molecules to form smaller (and in the case of foods, more easily digestible) compounds and nutrients. For example, the enzyme protease (all enzyme names have the suffix - ase) breaks down huge protein molecules first into polypeptides and peptides, then into numerous amino acids, which are readily assimilated by the body. The enzyme amylase works on carbohydrates, reducing starches and complex sugars to simple sugars. And the enzyme lipase hydrolyzes complex fat molecules into simpler free fatty acids. These are but three of the more important enzymes. There are thousands more, both inside and outside of our bodies. In some fermentations, important by-products such as alcohol or various gases are also produced. The word "fermentation" is derived from the Latin meaning "to boil," since the bubbling and foaming of early fermenting beverages seemed closely akin to boiling.

Fermented foods often have numerous advantages over the raw materials from which they are made. As applied to soy foods, fermentation not only makes the end product more digestible, it can also create improved (in many cases meat like) flavor and texture, appearance and aroma, synthesize vitamins (including B-12, which is difficult to get in vegetarian diets), destroy or mask undesirable or beany flavors, reduce or eliminate carbohydrates believed to cause flatulence, decrease the required cooking time, increase storage life, transform what might otherwise be agricultural wastes (such as okara) into tasty and nutritious human foods (such as okara tempeh), and replenish intestinal microflora (as with miso or Acidophilus soymilk).

Most fermentations are activated by either molds, yeasts, or bacteria, working singularly or together. The great majority of these microorganisms come from a relatively small number of genera; roughly eight genera of molds, five of yeasts, and six of bacteria. An even smaller number are used to make fermented soy foods: the molds are Aspergillus, Rhizopus, Mucor, Actinomucor, and Neurospora species; the yeasts are Saccharomyces species; and the bacteria are Bacillus and Pediococcus species plus any or all of the species used to make fermented milk products. Molds and yeasts belong to the fungus kingdom, the study of which is called mycology. Fungi are as distinct from true plants as they are from animals.

The study of all microorganisms is called microbiology. While microorganisms are the most intimate friends of the food industry, they are also its ceaseless adversaries. They have long been used to make foods and beverages, yet they can also cause them to spoil. When used wisely and creatively, however, microorganisms are an unexploitable working class, whose very nature is to labor tirelessly day and night, never striking or complaining, ceaselessly providing human beings with new foods. Like human beings, but unlike plants, microorganisms cannot make carbohydrates from carbon dioxide, water, and sunlight. They need a substrate to feed and grow on. The fermented foods they make are created incidentally as they live and grow.

Human beings are known to have made fermented foods since Neolithic times. The earliest types were beer, wine, and leavened bread (made primarily by yeasts) and cheeses (made by bacteria and molds). These were soon followed by East Asian fermented foods, yogurt and other fermented milk products, pickles, sauerkraut, vinegar (soured wine), butter, and a host of traditional alcoholic beverages. More recently molds have been used in industrial fermentation to make vitamins B-2 (riboflavin) and B-12, textured protein products (from Fusarium and Rhizopus in Europe) antibiotics (such as penicillin), citric acid, and gluconic acid. Bacteria are now used to make the amino acids lysine and glutamic acid. Single-celled protein foods such as nutritional yeast and microalgae (spirulina, chlorella) are also made in modern industrial fermentations.

For early societies, the transformation of basic food materials into fermented foods was a mystery and a miracle, for they had no idea what caused the usually sudden, dramatic, and welcomed transformation. Some societies attributed this to divine intervention; the Egyptians praised Osiris for the brewing of beer and the Greeks established Bacchus as the god of wine. Likewise, at many early Japanese miso and shoyu breweries, a small shrine occupied a central place and was bowed to daily.

In ancient times fermentation joined smoking, drying, and freezing as basic and widely practiced food preservation techniques. Wang and Hesseltine (1979) note that "Probably the first fermentation were discovered accidentally when salt was incorporated with the food material, and the salt selected certain harmless microorganisms that fermented the product to give a nutritious and acceptable food." The process was taken a step further by the early Chinese who first inoculated with the basic foods with molds, which created enzymes; in salt-fermented soy foods such as miso, soy sauce, soy nuggets, and fermented tofu, these aided salt-tolerant yeasts and bacteria.

1.2 A Brief History of Fermentation in the West:

The origins of microbiology (other than the general knowledge of fermented foods which existed worldwide since ancient times) can be traced back to the invention of the compound microscope in the late 1500s. This relatively simple tool soon revolutionized man's knowledge of the heretofore invisible microbial world. In 1675 the Dutch merchant Anton van Leeuwenhoek, the greatest of the early microscopists, saw and reported one-celled organisms, which he called "animacules." (Today they are called "protozoa.") The discovery electrified the scientific world of the time. Then in 1680, using a microscope that magnified the diameter of each object 300-fold, he looked at yeast and found them to consist of tiny spheroids. While the protozoa were clearly alive, the yeast did not appear to be.

No connection was drawn between the existence of these tiny organisms and the well-known phenomenon of fermentation. So for 150 years after van Leeuwenhoek's pioneering observations, it was hardly thought that these minute organisms could be important enough to deserve serious study.

The early 1800s saw a great increase of interest in microbiology in Europe. The scientific period began with great advances in botany, increased interest in microscopy, and willingness to investigate individual organisms. The two major problems that would challenge the greatest researchers in the new field of microbiology concerned the basic nature of the fermentation process and the basic nature of enzymes. The scientific breakthroughs that would lead to the unraveling of the mysteries of fermentation starting in the 1830s were made primarily by French and German chemists.

In the late 1700s Lavoisier showed that in the process of transforming sugar to alcohol and carbon dioxide (as in wine), the weight of the former that was consumed in the process equaled the weight of the latter produced. In 1810 J.L. Guy-Lussac summarized the process with the famous equation $C_6H_{12}O_6$ yields $2CO_2$ + 2 C_2H_6O. The entire process was considered to be simply a chemical reaction and yeast (which was not yet even classified as a definite substance, much less a living organism instrumental to fermentation) was thought to play a physical rather than a chemical role, an idea dating back to the time of Georg Stahl in 1697. It was held that either the catalytic action at the yeast cell or the molecular vibrations from the decomposing organic matter arising from the death of the cells, sparked the chemical changes resulting in fermentation. Putrefaction, spoilage, and fermentation were all considered to be processes of death, not life.

The first solid evidence of the living nature of yeast appeared between 1837 and 1838 when three publications appeared by C. Cagniard de la Tour, T. Swann, and F. Kuetzing, each of whom independently concluded as a result of microscopic investigations that yeast was a living organism that reproduced by budding.

The word "yeast," it should be noted, traces its origins back to the Sanskrit word meaning "boiling." It was perhaps because wine, beer, and bread were each basic foods in Europe that most of the early studies on fermentation were done on yeasts, with which they were made. Soon bacteria were also discovered; the term was first used in English in the late 1840s, but it did not come into general use until the 1870s, and then largely in connection with the new germ theory of disease.

The view that fermentation was a process initiated by living organisms soon aroused fierce criticism from the finest chemists of the day, especially Justus von Liebig, J.J. Berzelius, and Friedrich Woehler. This view seemed to give new life to the waning mystical philosophy of vitalism, which they had worked so hard to defeat. Proponents of vitalism held that the functions of living organisms were due to a vital principal (life force, chi, ki, prana , etc.) distinct from physico-chemical forces, that the processes of life were not explicable by the laws of physics and chemistry alone, and that life was in some part self-determining. As we shall soon see, the vitalists played a key role in debate on the nature of fermentation. A long battle ensued, and while it was gradually recognized that yeast was a living organism, its exact function in fermentations remained a matter of controversy. The chemists still maintained that fermentation was due to catalytic action or molecular vibrations.

The debate was finally brought to an end by the great French chemist Louis Pasteur (1822-1895) who, during the 1850s and 1860s, in a series of classic investigations, proved conclusively that fermentation was initiated by living organisms. In 1857 Pasteur showed that lactic acid fermentation is caused by living organisms. In 1860 he demonstrated that bacteria cause souring in milk, a process formerly thought to be merely a chemical change, and his work in identifying the role of microorganisms in food spoilage led to the process of pasteurization. In 1877, working to improve the French brewing industry, Pasteur published his famous paper on fermentation, Etudes sur la Biere, which was translated into English in 1879 as Studies on Fermentation. He defined fermentation (incorrectly) as "Life without air," but correctly showed specific types of microorganisms because specific types of fermentations and specific end products. In 1877 the era of modern medical bacteriology began when Koch (a German physician; 1843-1910) and Pasteur showed that the anthrax bacillus caused the infectious disease anthrax. This epic discovery led in 1880 to Pasteur's general germ theory of infectious disease, which postulated for the first time that each such disease was caused by a specific microorganism. Koch also made the very significant discovery of a method for isolating microorganisms in pure culture.

Interestingly, until his death in 1873, the eminent German chemist J. von Liebig continued to attack Pasteur's work on fermentation, putrefaction, and infectious diseases. He recognized the similarity of these phenomena but refused to believe that living organisms were the main causative agents. Fermentation, he felt, was primarily a chemical rather than a biological process. History has shown, with the discovery of enzymes, that Pasteur was not entirely right, nor Liebig entirely wrong.

The work of Pasteur and his many colleagues and predecessors opened up vast new vistas in the fields of biochemistry, microbiology, and fermentation. The term "biochemistry" was first used in English in 1869, but this new science of the application of chemistry to biology was generally called "physiological chemistry" until the early 1900s. The two outstanding pioneers were Liebig and Pasteur. The term "microbiology" was first used in English in 1885, long after Pasteur's major discoveries. But basic knowledge of this new science of the study of minute living organisms closely related to human activity or welfare did not begin to enter the popular consciousness until the early 1900s. At about this time the scientific breakthroughs of the 1870s and 1880s had begun to produce a change in people's conception of the world around them as sweeping and profound as to be termed revolutionary. Food microbiology was finally set on a scientific foundation, based on the action of specific microorganisms. A rational theory of infectious diseases (which formerly were not differentiated from one another) set people's minds free from the age-old fear of vengeance from an unknowable and invisible disease-causing entity. And the ancient theory of spontaneous generation of lower life forms, which said they could arise de novo and fully formed from decomposing matter, was replaced by the verifiable theory of biogenesis. For the first time people began to accept the fact that they shared their environment with multitudes of minute organisms that exerted an ongoing powerful influence on human life. This new world view, among other things, provided a tremendous stimulus for new research on fermented foods.

Although showing that fermentation was generally the result of the action of living microorganisms was an epic breakthrough, it did not explain the basic nature of the fermentation process, or prove that it was caused by the microorganisms that were apparently always present. As early as the late 1700s it had been recognized that there was another type of chemical change that resembled the yeast fermentation in some respects.

This was the sort of changes that occur, for example, in the digestion of food. In 1752 Reamur, in studying the digestive processes of a falcon, showed that its digestive juices were able to dissolve meat. In 1785 William Irvine discovered that aqueous extracts of sprouted barley caused liquefaction of starch. The first clear recognition of what were later called "enzymes" came in 1833 when two French chemists, A. Payen and J.F. Persoz, made a more detailed investigation of the process of solubilizing starch with a malt extract to form a sugar that they called "maltose." They called the agent responsible for this transformation "diastase" and they showed that it was destroyed or inactivated by boiling, that without undergoing permanent change itself, a small amount of diastase could convert a large amount of starch to sugar, and that it could be concentrated and purified by precipitation with alcohol. In 1835 the German naturalist Swann, mentioned above for his early work with fermentation, isolated a substance from gastric juice which could bring about the dissolution of meat but which was not an acid. He called it "pepsin" from a Greek word meaning "digestion." It soon became fashionable to call organic catalysts such as diastase and pepsin "ferments," because digestion and fermentation, both allied with life, seemed to be somewhat similar processes. Under the influence of the vitalists, ferments were grouped into two types: those involved with life process were called "organized ferments" and those which were not (like pepsin) were merely "unorganized ferments." A relation between the two types of ferments was suspected by many, and in 1858 M. Traube put forward the theory that all fermentations were due to ferments, definite chemical substances he regarded as related to the proteins and produced in the cells by the organism. In 1876, to reduce confusion that existed concerning the two types of ferments, the German physiologist Wilhelm Kuehne suggested that an unorganized ferment, acting in the absence of life, be called an "enzyme," after the Greek words meaning "in yeast;' in 1881 this term was Anglicized to "enzyme" by William Roberts, and it had begun to catch on by the 1890s.

Many scientists, including Pasteur, had attempted unsuccessfully to extract the fermentation enzyme from yeast. Success came finally in 1897 when the German chemist Eduard Buechner ground up yeast, extracted a juice from them, then found to his amazement that this "dead" liquid would ferment a sugar solution, forming carbon dioxide and alcohol just like living yeasts. Clearly the so-called "unorganized ferments" behaved just the organized ones. From that time on the term "enzyme" came to be applied to all ferments. The term "ferment" dropped out of the scientific vocabulary altogether and the vitalist position collapsed, never to recover. Thereafter it was agreed that only one set of laws applied to all things, both animate and inanimate, and that there was no special vital force which characterized living things and acted under different laws. And it was finally understood that fermentation is caused by enzymes which are produced by microorganisms. In 1907 Buechner won the Nobel Prize in chemistry for his work, which opened a new era in enzyme and fermentation studies.

The sciences of microbiology, biochemistry, fermentation technology, mycology, and bacteriology all shared a deep interest in the nature and working of enzymes. Yet still by the early 1900s no one knew exactly what enzymes were or how they acted. As the agricultural microbiologist Conn asked in 1901, "How can they produce chemical actions without being acted upon or entering into the reactions? Are enzymes fully lifeless or semi-living? We still do not know the fundamental mystery of fermentation." Gradually an understanding of enzymes and catalysts developed. In 1905 Harden and Young discovered coenzymes, agents necessary for the action of enzymes. In 1926 the American biochemist J.B. Sumner first purified and crystallized an enzyme (urease) and showed that it was a protein, more precisely a protein catalyst.

Eventually enzymes came to be seen as the key catalysts in all the life processes, each highly specialized in its catalytic action and generally responsible for only one small step in complex, multi-step biochemical reactions. Enzymes are still produced only by living organisms, both animals and plants; they have never been synthesized.

Advances in microbiology and fermentation technology have continued steadily up until the present. For example, in the late 1930s it was discovered that microorganisms could be mutated with physical and chemical treatments to be higher yielding, faster growing, tolerant of less oxygen, and able to use a more concentrated medium. Strain selection and hybridization developed as well, affecting most modern food fermentations.

1.3 A Brief History of Fermentation in East Asia:

Traditional fermented foods play an unusually extensive role in East Asia food systems. These fermented foods have a number of important distinguishing characteristics: a number of the most important fermentations use molds; dairy products and other animal proteins (excepting fish) are not widely used, as they are in the West; and modern fermentation processes and technology are based largely on traditional processes, yet are extremely advanced and sophisticated. The main use of molds has been in the process of making koji (mold-fermented grains and/or soybeans), which serves as a source of more than 50 enzymes in a subsequent fermentation in much the same way that, in the West, the enzymes of malt (steeped and sprouted barley or other cereal grains) are used to make alcoholic beverages.

The nature of koji is embodied in the very characters with which the word is written. In the more traditional form--used with most miso koji and especially with barley koji--the ideographs for "barley:" and "chrysanthemum" are placed side by side. In the more recent form--used especially with ready-made rice koji--the ideographs for "rice" and "flower" are conjoined. The first form is said to have originated in China, whereas the latter was developed in Japan about 1,000 years ago. In both, the notion of grain covered with a bloom of mold is vividly expressed.

Since ancient times the koji making process has been unique to East Asia, where it has been used in the preparation of fermented foods such as miso, soy sauce, soy nuggets, sake, shochu (spirits), and rice vinegar (yonezu). The only traditional East Asian fermented soy food not prepared with molds is Japan's natto, and its relative’s thua-nao in Thailand and kinema in Nepal; these are bacterial fermentations. Some have suggested that molds are widely used since they grow well in areas having a humid climate and long rainy season during the warm months. In the West mold fermented foods are limited primarily to a number of cheeses characterized by their strong flavors and aromas:

Camembert, Blue, Brie, and related types. Because of the widespread use of mold-fermented foods in East Asia, the word "mold" there has a rather positive connotation, something East Asia, the word "mold" there has a rather positive connotation, something like "yeast" in the West. Most Westerners still have a deep-seated prejudice against moldy products, and they generally associate the word "mold" with food spoilage, as in "moldy bread." Surprisingly little has been published in English about the history of fermentation and knowledge of the fermentation process in East Asia, especially the history prior to the 1870s and 1880s, when the new science of microbiology was introduced from the West.

The earliest records of the koji-making process can be traced back to at least 300 BC in China and to the third century AD in Japan. Molds differ in one important respect from yeasts and bacteria in that they can be easily observed with the naked eye (without a microscope) and their growth, form, and color noted. In East Asia it was probably understood that fermentation was a life process long before it was in the West. By the sixth century AD, as recorded in the Ch'i-min yao-shu (the earliest encyclopedia of agriculture), the Chinese had distinct names for two types of molds used in fermented soy foods; what we now call Aspergillus was then called "yellow robe and Rhizopus was called "white robe." These cultures were carefully distinguished and propagated from year to year. By the 10th century a koji starter or inoculum was deliberately being used in the preparation of koji for fermented foods.

From these early times until the 1870s the traditional fermented foods industries in East Asia apparently advanced largely by an empirical, trial-and-error process without the benefit of general scientific research into the nature of microorganisms and of the fermentation process, and without any general theories in these areas.

From 1635 until 1854, Japan lived in relative seclusion and isolation under the Tokugawa shoguns. The great advances made in Western science during this 220 year period passed largely unnoticed. Then in 1854 the American Commodore Perry and two fully-armed steamships arrived in Japan and demanded that the country end its self-imposed isolation and open itself to trade with the West. Internal pressures and the intrusion of the Western powers helped topple the already declining Tokugawa Shogunate in 1868, and that year the emperor was restored as the political head of the nation, ushering in the Meiji Restoration. Openness, modernization, westernization, scientism, positivism, and the ideal of progress all formed the dominant ethos of the Meiji Period, which lasted until 1912. This new spirit corresponded with a golden age in the West of scientific and technological breakthroughs and empire building, which gave Westerners confidence in their ability to control the world and an optimistic faith in a bright new future. In Japan, Western knowledge, science, and technology were actively sought and cultivated, for it was generally thought that the very survival of the nation depended on their quick assimilation. By the 1870s governmental colleges had been established primarily for teaching Western science and technology. By paying princely salaries and offering high positions, the Japanese were able to attract top European scientists to staff the new universities, the foremost of which was Tokyo Imperial University, established in 1877. At the same time, for the first time in 220 years, Japanese were allowed to travel abroad; scientists and students were sent abroad for training. As Atkinson (1881b), one of the earlier exchange scientists to Japan, noted: The student of science in Japan has a wide field before him; that system of isolation which has prevented the introduction of Western knowledge till within the last quarter century has not been entirely fruitless, for it has resulted in the development of industrial processes which are as novel and interesting to the European as those of the latter are to the Japanese.

The imported European scientists and professors caused an almost immediate revolution in the field of East Asian food fermentations, for they brought both the powerful tools of the Western scientific method and a host of new discoveries in the fields of fermentation and microbiology. In Japan the effect of German (and to a lesser extent English) microbiologists and chemists was initially most pronounced. Some European countries sent their scientists out to their colonies; the Dutch (such as Went and Prinsen Geerligs) went to the Netherlands East Indies (Indonesia) and the French went to Indochina. In each area fermented soy foods were investigated.

It is interesting to note that no such studies of fermented foods were done in China by either Western or Chinese researchers during this vital pioneering period, for various reasons: China was slow to modernize and Westernize; the late 1900s were a period of rebellion and decline; and Western imperialism, so destructive to China, had made the Chinese closed to and suspicious of Western ideas. Nevertheless China, recognizing the rapid advance of Japanese science from its interaction from the West, eventually chose to get its information from the West indirectly via Japan.

Prior to 1870, makers of East Asian fermented foods were unaware of the basic nature of the fermentation process of microorganisms, enzymes, and their respective interactions. Makers of koji had no idea what caused the grains and/or soybeans to become covered with a fragrant white mycelium after several days of incubation in a warm koji room, or what later transformed the koji almost magically into delicious, savory seasonings such as miso, shoyu, or soy nuggets, or into heady beverages such as sake. The microscope was essentially unknown in East Asia prior to the 1880s.

The advances in food fermentations resulting from the exchange of people and ideas was most pronounced in Japan. The first generation of European scientists there plunged in to their investigations of the many fermented foods with great curiosity and enthusiasm. One of their first subjects of research was the koji mold, now known as Aspergillus oryzae, and the various foods in which it was used, especially sake and shoyu, which were major sources of tax revenue for the Meiji government. Tradition ascribed the introduction of sake brewing in Japan to some emigrants from Korea at about the end of the third century AD; they doubtless learned the process from China, where it had long been practiced. One of the earliest accounts of sake production by a Westerner appeared in 1874 when Dr. J.J. Hoffmann, a German professor in the medical school of today's Tokyo University, published a translation of an article on sake from a Japanese encyclopedia of 1714. The same year he wrote a detailed and scientific description of the process for making rice koji, based on his visits to the famous plant run by Mr. Sagamia Monjiro, which made sake, mirin, and shoyu in Nagareyama, 5 miles north of Edo (Tokyo). Although Hoffmann described accurately the process for making rice koji, he did not use the word "koji." In 1878 Korschelt, also a German, gave an extremely accurate and detailed (1600-word) description of exactly how koji and koji starter were made. He was the first Western scientist to use the words koji and tane koji (koji starter); he used them frequently and accurately. Actually the term kojiappeared slightly earlier in Hepburn's famous Japanese-English Dictionary, which translated it (inaccurately) as "barm or yeast." In a section of Korschelt's article, and in a Japanese article written with Matsubara in 1878, Ahlburg, another German, who taught natural history at Tokyo University, gave the first detailed description of the koji mold, which he called Eurotium Oryzae Ahlburg. In 1884, Ferdinand J. Cohn, a Polish botanist and microbiologist, first gave the koji mold its present name, Aspergillus oryzae. After 1884 the koji mold was referred to as Aspergillus oryzae (Ahlburg) Cohn, in recognition of Ahlburg's earliest accurate description. The mold's characteristics were subsequently clarified and elaborated by Buesgen.

Another pioneer in the field of koji research was Atkinson, who had a BS degree from London and was a professor of analytical and applied chemistry at Tokyo University. In 1878, after visiting sake factories, he wrote "On Sake Brewing," which contained a preliminary description of the koji-making process and mentioned the word "koji." In 1881, after extensive research with his assistant Mr. Nakazawa at the koji plant of Mr. J. Kameyama in Yushima near Tokyo, he published two major articles.

In "On the Chemistry of Sake Brewing," he gave a detailed account of koji making in underground caves in Tokyo and an analysis of its composition. His "On the Diastase of Koji" first demonstrated that the koji mold had strong diastatic (amylolytic) activity. In 1889 Dr. Oscar Kellner (a German Professor of Agricultural Chemistry at Tokyo University) and his Japanese co-workers published pioneering studies on koji, shoyu, and miso. Then in 1895 and 1901, C. Wehmer, who taught mycology at Hannover, described the koji mold in great detail. He also stated (1895) that koji was being made in America at a large Japanese sake brewery on U Street in Peoria, Illinois, the very area that would become America's leading center of research on koji and miso, starting in the 1960s! As Western researchers studied koji, they quickly realized that it has much the same relationship to shoyu and miso fermentation that malt has to Western alcoholic grain fermentations.

During the 20th century, Japanese microbiologists have made many important contributions to the development of applied and industrial microbiology, including the manufacture of fermented soy foods, as well summarized by Tamiya (1958) and Sakaguchi (1972). Until quite recently, their strength was more in the area of application of scientific knowledge than in pioneering basic scientific and microbiological breakthroughs. From the early 1900s, important studies on the koji mold and its enzymes were done by Japanese scientists. Important advances in enzymology, with much of the work done on koji molds, began in the 1920s. In 1928 Miyazaki developed the combined Amylo-Koji process. By the 1950s Japanese scientists had isolated various protease and amylase enzymes, induced mutations, and used them commercially. They also developed the technology for the microbial production of L-glutamic acid and monosodium glutamate (MSG), lysine and other amino acids, flavor enhancing nucleotides such as inosinic acid, and organic acids. They used the koji mold Aspergillus oryzae in the commercial production of enzymes including proteases, amylases, amyloglucosidase, and lipase. They made microbial rennet and numerous other products. Indeed in the period following World War II, Japan became the world leader in the field of industrial fermentations. Wang and Hesseltine (1979) have suggested that this may have been "in large part due to the food fermentation base from which it launched its industrialization of microorganisms."

According to Tamiya, in 1958 food and drinks produced with koji retailed for $1,000 million a year, and the taxes from these foods amounted to more than $500 million, which was as much as 20% of the Japanese national budget! In 1970 in Japan, foods made from koji molds accounted for 1.5% of the nation's Gross National Product, or about $205,000 million. Prominent among these were miso and shoyu (Sakaguchi 1972). Production of fermented soy foods continues to be the most important of the fermented food industries of East Asia.

The many important developments in this field will be described in the following chapters. Starting in about the 1960s and increasing rapidly after the mid-1970s, East Asian fermented soy foods (especially soy sauce or shoyu, miso, and tempeh, in that order), began to be widely used in the West. Reasons for this include the growing general interest in soy foods, the cultural and religious movement toward meatless and vegetarian diets, the increasing interest in nutritious foods with less animal fats, the awareness these foods as a good vegetarian source of vitamin B-12, the growing worldwide travel stimulating interest in foreign foods, the increase of East Asian refugees to the West, and the increased interest in microbiology and enhanced image of fermented foods.

All of these developments indicate a bright future for fermented soy foods in the West.

Lactic acid is an organic hydroxyl acid whose occurrence in nature is widespread. It was discovered and isolated in 1780 by Swedish Chemist Carl Wilhem Scheele in sour milk (Datta and Tsai[28], 1995). It was the first organic acid to be commercially produced by fermentation, with production beginning in 1881 (Ruter[29], 1975 and Severson[30], 1998). It is present in many foods both naturally or as a product of microbial fermentation. It is also a principal metabolic intermediate in most living organisms from anaerobic prokaryotes to humans.

Lactic acid is considered as a very important chemical compound with significant applications in pharmaceutical, chemical industry and especially in the food industry. Worldwide demand for lactic acid is growing at a rate of approximately 12-15% a year. Lactic acid production from agricultural crops such as wheat, corn and beet has recently received much attention because of the increasing demands for polylactic acid, which is used in biodegradable plastics (Akerberg and Zacchi[31], 2000). The production of such biodegradable polymer can replace non-degradable plastics and thus solve the environmental pollution problem. The increasing use of chemical synthesis plastics, which takes about hundred years to degrade, has cause environmental deterioration, with these waste plastic clogging landfills, strangling wildlife and littering beaches. The production of PLA will increase if new economic production routes are developed to increase annual lactic acid consumption.

World demand for lactic acid is currently estimated at $150 million (100 000 tons). An annual growth of 8.6% of the lactic acid market is expected between 2010 and 2012. About 50% of the market is in food and beverage applications, which is a mature and stable market. For polylactic acid, the potential market has reached about 160 000 tons in 2003 and 390 000 tons in 2008 (Bogaert and Coszach[32], 2000). This type of fermentation could nevertheless be important because the carbon sources are waste product that would otherwise be difficult and expensive to discard, rather than agricultural crops that could be put to other uses in the production of human food and animal feed. Lactic acid can be produced by microbial fermentation or by chemical synthesis but in recent years fermentation process has become more industrially successful because of the increasing demand for naturally produced lactic acid. Lactic acid producing microorganisms are proprietary (Holten[33], 1971). However only homofermentative organism are of industrial importance for lactic acid manufacture. It is believed that most of the strains used in the industry belong to genus Lactobacillus, which usually produce one of the two kind isomers, L (+) or D (-), or a racemic mixture of both. However, ideal fermentation cultures need to produce exclusively L (+) lactic acid from an economic substrate (Buchta[34], 1983).

Currently, lactic acid production through free cell fermentation provides about 50% of the world supply, but productivity is very low in conventional batch processes. However employing cell immobilization method that provides high density can increase the productivity. Immobilization cell is one of the most attractive methods in maintaining high cell concentration in the bioreactor (Chang[35], 1996). Immobilized cell systems offer the advantages of high volumetric productivity than batch fermentation system, the possibility of continuous operation and higher stability (Goksungur and Guvenc[36], 1999). The immobilized preparation can then be reused either in batch or in a continuous system and hence diminished the cost of the process.

Stenroos et al[37]. (1982), immobilized Lactobacillus delbrueckii, Boyaval and Goulet (1988), immobilized Lactobacillus helveticus, Kurosawa et al[38]. (1988), co-immobilized Lactobacillus lactis and Aspergillus awamori, Guoqiang et al[39]. (1991), immobilized Lactobacillus Casei,

Roukas and Kotzekidou[40] (1991), co immobilized Lactobacillus Casei and Lactobacillus lactis, Abdel Naby et al[41]. (1992), immobilized Lactobacillus lactis and Kanwar et al[42]. (1995), immobilized Sporolactobacillus cellulosolvens in Ca-alginate gel for the production of lactic acid.

Lactic acid is one such product that has numerous applications in chemical compound pharmaceutical, cosmetic, technical and especially in food industry. New application such as biodegradable plastic made from poly (lactic) acid, have the potential to greatly expand the market for lactic acid if more economical processes could be developed[43].

1.4 Properties of Lactic Acid:

Pure anhydrous lactic acid is a white crystalline solid with a low melting point of 53^0C and appears generally in form of more or less concentrated aqueous solution, as syrupy liquid. Lactic acid is colorless, sour in taste, odorless and soluble in all proportions in water, alcohol and ether but insoluble in chloroform as shown in **Table-1.1.** It is a weak acid with low volatility (Casida[44], 1964).

In solutions with roughly 20% or more lactic acid, self-esterification occurs because of the hydroxyl and carboxyl functional groups and it may form a cyclic dimmer (lactide) or more linear polymers.

Lactic acid is very corrosive; therefore corrosion resistance material such as high molybdate stainless steel, ceramic, porcelain or glass lined vessel (Paturau[45], 1982) must be used for its production.

The presence of hydroxyl and carboxyl two functional groups permits a wide variety of chemical reactions for lactic acid. The primary classes of these reactions are oxidation, reduction, condensation and substitutions.

1.5 Properties:

Molecular formula	$C_3H_6O_3$
Molar mass	90.08 g mol^{-1}
Melting point	L: 53 °C D: 53 °C D/L: 16.8 °C
Boiling point	122 °C @ 12 mmHg
Acidity (pKa)	3.86

1.5.1 Thermochemistry:

Std enthalpy of combustion ΔCH^{e}_{298}	1361.9 kJ/mol, 325.5 kcal/mol, 15.1 kJ/g, 3.61 kcal/g

1.5.2 Related Compounds:

Other anions	Lactate
Related carboxylic acids	acetic acid
	glycolic acid
	propionic acid
	3-hydroxypropanoic acid
	malonic acid
	butyric acid
	hydroxybutyric acid
Related compounds	1-propanol
	2-propanol
	Propionaldehyde
	Acrolein
	sodium lactate

Table -1.1: Characteristics of Lactic Acid (Martin46, 1996):

Property	Characteristics
Optical activity	Exists as L (+), D (-) and recemic mixture
Crystallization	Forms crystals when highly pure
Color	None or yellowish
Odor	None
Solubility	Soluble in all proportions with water Insoluble in chloroform, carbon disulphide
Miscibility	Miscible with water, alcohol, glycerol and furfural.
Hygroscopicity	Hygroscopic
Volatility	Low
Self-esterification	In solutions of > 20%
Reactivity	Versatile; e.g. as organic acid or alcohol

Lactic acid is the simplest hydroxy acid having an asymmetric carbon atom and it therefore exists in a racemic form and in two optically active form with opposite rotations of polarized light L(+) and D(-)lactic acid.

The optically active form of lactic acid is simply an equimolecular mixture of both and may be denoted as DL-lactic acid or racemic mixture. The optical composition does not affect many of the physical properties with important exception of the melting point of the crystalline acid.

1.6 Glycolysis:

To extract chemical energy from glucose, the glucose molecule must be split into two molecules of pyruvate. This process also generates two molecules of adenosine triphosphate as an immediate energy yield and two molecules of NADH.

$$C_6H_{12}O_6 + 2\ ADP + 2Pi + 2\ NAD^+ \rightarrow 2CH_3COCOO^- + 2\ ATP + 2NADH + 2H_2O + 2H^+$$

The chemical formula of pyruvate is CH_3COCOO^-. Pi stands for the inorganic phosphate. As shown by the reaction equation, glycolysis causes the reduction of two molecules of NAD^+ to NADH. Two ADP molecules are also converted to two ATP and two water molecules via substrate-level phosphorylation.

1.7 Chemical Synthesis through Biotechnology:

It involves two distinct phases viz fermentation and recovery of product:

a. Fermentation in biotechnology means any process by which micro -organisms are grown in large quantities to produce any type of material. Thus, fermentation procedures must be developed for the cultivation of micro -organism under optimal conditions and for the desired production of enzymes and metabolites by the micro-organisms.
b. **Product Recovery**: It involves extraction and purification of desired products.

The word fermentation is derived from a Latin word ' fervere', which means to boil. The word was coined from the observation that during alcoholic fermentation the bubbles of carbon dioxide gas burst at the surface giving an appearance of boiling. Fermentation may be defined as the biochemical activity of a micro-organism in its growth, development, reproduction, and possibly even death. Yeasts and bacteria were the micro -organisms involved in fermentations in the traditional biotechnology. But now -a-days, a much border range of micro-organisms (such as cells of animals, plants, humans, algae, protozoa, insect, cellular organisms (i.e., viruses)) or subcellular organelles and enzyme complexes are used in fermentation. The fermentation medium must contain Vitamin-B in addition to glucose (12-13%) and $(NH_4)_2HPO_4$ (0.25%). Production is carried out in 25-120m3 fermenters at 45-50°C with an excess of $CaCO_3$ (added to maintain the pH between 5.5 and 6.5). It takes about 3-6-days for fermentation. When the fermentation completes, the broth is heated to dissolve the calcium lactate. The heated broth is filtered and sulfuric acid is added to precipitate calcium. After concentration of the lactic acid, it is further purified. The biosynthesis of lactic acid from glucose proceeds via glyceraldehyde-3-P; 1, 3-di-P-glycerate and pyruvate. During the oxidation of glyceraldehyde phosphate, the produced reducing power is transferred with an NAD-dependent lactate dehydrogenase to pyruvate. Pyruvate in turn, is stereo specifically reduced to L (+) or (D-) lactic acid.

Fermentation is often more of an art than a science[47]. After Pasteur’s researches, the word fermentation became more associated with micro-organisms and still later with enzymes[48-54].

Prescott and Dunn[55] have also reviewed and defined fermentation in a broad sense as: "A process in which chemical changes are brought about in an organic substrate, whether carbohydrate or protein or fat or some other type of organic material, through the action of biochemical catalysts known as 'enzymes' elaborated by specific types of living micro-organisms". A more restrictive definition of fermentation has also been proposed. According to Elsden[56]: "Fermentation may be defined as a biological process in which chemical energy is made available for growth by oxidative reactions, the ultimate hydrogen acceptors for which are substances other than oxygen".

The process of fermentation has been known since prehistoric days. Primitive man knew the method to prepare alcohol. The manufacture and consumption of alcoholic beverages were already established in the oldest civilization of which records are available. There is a mention of 'Soma-Rasa'produced by fermentation, in Rigved[57-59]. It has been described that juice of the ¬stem of sarcostema vaminalis soma-plant) was fermented to obtain 'Soma-Rasa'. According to Sir P.C. Ray[60] these products of fermentation were very well known to Indian during the period of 'MANU'.

1.8 Present Status of the Work:

In the past decades work has been done on different fermentations (e.g. Cheese[61-65], yeasts[66-78], acetone-butanol[79-100], pickles,[101-115], sauerkraut[116-125], glycerol[126-130], vinegar-acetic acid[131-138] 2,3-butanediol[139-161], gluconic acid[162-172], itaconic - itatartaric acid[173-183] kojic acid[184-190], hydrocarbon[191-197], antibiotics[198-211], Gallic acid[212-219] fumaric acid[220-224] propionic acid[225-227], enzymes[228-230], mushrooms[231-232], amino acids[236-236], vitamins[237-244] and ergot-alkaloids[245-254] has emerged as fermentation products and also in the processing of domestic and industrial wastes.

In these fermentations most of the workers have tried to get desired fermentation products on commercial scale from comparatively cheaper and economical source materials. Available literature reveals that most of the workers have added certain useful compounds e.g., vitamins, amino acids, trace-elements, mutagens etc. into the production medium which stimulates and accelerates fermentation processes and enhances the desired product to a great extent.

But still there are certain compounds e.g. chemical mutagens, active alkaloids, physiologically active organic compounds, micelles, coumarins sulphonamides, vitamins, amino acids, trace elements and cytokinine which has not been extensively used in fermentations especially lactic acid fermentation and may give alarming results when added to the production medium in their optimum concentrations.

1.9 Lactic Acid Fermentation:

Lactic acid, ($CH_3CHOH.COOH$) as an unnamed component of soured milk must have been known in human experience since the days when man first had his flocks and herds. Its true nature was discovered by Scheele, who isolated and identified it as the principal acid in sour milk in 1780.

It was investigated by Pasteur as one of his first microbiological problems. Schultze (1868) demonstrated the presence of lactic acid bacteria in yeast cultures of distilleries.

But it was not until the year 1877 that lactic acid bacteria were isolated in pure cultures, Dr. Lister having isolated Streptococcus lactis. During this same period Delbruck was endeavoring to determine the most favourable temperature for lactic acid fermentation in distilleries. He concluded that relatively high temperatures favored high yields of lactic acid.

1.10 Forms of Lactic Acid:

Lactic acid occurs in three forms:

a. Levorotatory lactic acid.
b. Dextrorotatory lactic acid (known also as "sarcolactic acid"), both of which are optically active acids, and i- lactic acid, an optically inactive acid.

Lactic acid of various forms is produced by the lactic acid bacteria. Lactobacillus delbureckii and S. lactis usually produce d - lactic acid, while L. leichmannii and Leuconostoc mesenteroides var. Sake commonly produce l - lactic acid. A few bacteria produce i-lactic acid, for example, Lactobacillus pentoaceticus (in "Bergey's Manual of Determinative Bacteriology" this organism is listed as a probable synonym of L. brevis). The lactic acid produced during fermentation is frequently inactive. In their action on glucose, the lactic acid organisms fall into two large groups: the homofermentative' species and the heterofermentative species.

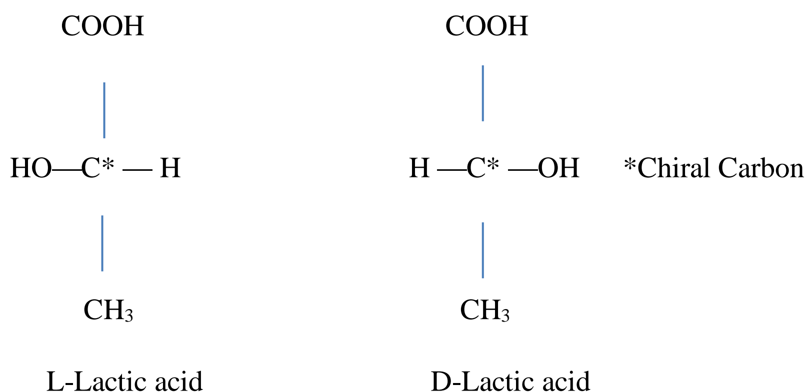
1.10.1 Homofermentative Species:

The members of this group convert about 95 per cent of fermentable hexoses to lactic acid:

$C_6H_{12}O_6$ → $2CH_3CHOH.COOH$

Glucose Lactic acid

Small amounts of volatile acids and carbon dioxide are also produced. Disaccharides are fermented in a similar manner; e.g., one mole of lactose yields four moles of lactic acid. The lactic acid may be dextrorotatory (D) or levorotatory (L), or a mixture of the two forms (DL) in equal quantities:



The isomer produced is characteristic of the species.

1.10.2 Heterofermentative Species:

These differ from the homofermentative species in that lactic acid is only one of several principal products formed from sugar, other compounds include ethyl alcohol, acetic and formic acids, and carbon dioxide. Mechanism of lactic acid formation. A large number of carbohydrates, notably glucose, lactose, and sucrose, are employed. Starches of various kinds may be first hydrolyzed to sugars by treatment with mineral acids or enzymes, then fermented to lactic acid. Molasses and whey are low-priced and serve as excellent sources of carbohydrates.

1.10.3 Homofermentative Species: are the main source of industrial lactic acid. It is generally believed that fermentation follows the same pathway as the alcohol production by yeasts. As pyruvate is formed, it is reduced to lactic acid

$C_6H_{12}O_6 \longrightarrow 2CH_3CHOH.COOH + C_2H_5OH + CO_2$

Glucose Lactic acid Ethyl alcohol

The heterofermentative species produce a lower concentration of lactic acid accompanied by a number of other products, the most important being ethyl alcohol, acetic and formic acids, and CO2¬ Some pyruvate follows the alcoholic path¬ way and some becomes reduced to lactic acid . The acetic and formic acids can be produced from pyruvate, according to the reaction:

$CH_3.CO.COOH + H_2O \longrightarrow CH_3.COOH + HCOOH$

Pyruvic acid Acetic acid Formic acid

The formic acid disappears as CO_2 and H_2:

$HCOOH \longrightarrow CO_2 + H_2 \rightleftharpoons 2H$

1.10.4 Neutralization of Acid:

Lactic acid is neutralized by calcium hydroxide during fermentation in the following manner:

$2CH_3.CHOH.COOH + Ca(OH)_2 \longrightarrow (CH_3CHOHCOO)_2Ca + 2H_2O$

Lactic acid Calcium Hydroxide Calcium Lactate

If the lactic acid were not neutralized, the lactic acid bacteria would not be able to tolerate the high acidity developed and the fermentation would not continue to completion.

Calcium (or zinc) hydroxide or carbonate may be added either at the beginning of the fermentation or intermittently as the fermentation progresses.

Peterson, Fred, and Davenport suggested that the preliminary introduction of a neutralizing agent was as efficacious as intermittent introduction from the point of view of the speed and completeness of the conversion of glucose to lactic acid. The advantage of adding the carbonate intermittently lies in the fact that an acid reaction helps to prevent contaminants from gaining ascendency during the fermentation.

1.11 The Lactic Acid Bacteria[227-238]:

At present it is well known that a great number of micro-organisms are capable of producing lactic acid in the substrate, to a greater or lesser extent. For example, the Cholera vibrio, the Diphtheria bacilli, the Colon group of bacteria and others produce lactic acid as a result of their life activities. But some organisms produce lactic acid as the principal product of their life activities, while others only as the by-products of their fermentation. The former group of organisms include the lactic acid bacteria. It was Pasteur, who first noted that the lactic acid fermentation is caused by living organisms. But it was Lister, who in 1877 isolated the pure culture of lactic acid bacteria. At present the microorganisms belonging to this group of lactic acid bacteria are characterized by the following general characteristics

a. On fermentation of sugars they give lactic acid as the principal product of fermentation.
b. Do not produce spores.
c. Non-motile.
d. In morphology they may be cocci or rods of different sizes.
e. Gram-positive.
f. Facultative anaerobes, though some of them grow better in absence of air.
g. Do not produce catalase.
h. Many of them do not grow in simple synthetic media. Even now there is much confusion on the detail classification of this group of organisms and the system of classification depends on the technique followed by the individual worker. The most prevalent system of classification of these Organisms is that of Orla-Jensen. In this system of classification the Physiology, the morphology and the habitat of these organisms are considered for their identification. Accordingly the following groups of Lactic acid bacteria may be considered.

1.12 The Mechanism of Lactic Acid Fermentation:

The mechanism of lactic acid fermentation has been less studied than that of ethyl alcohol. It may be said that in lactic acid fermentation all the steps up to the stage of pyruvic acid fermentation all the steps up to the stage of pyruvic acid formation is similar to those found in the fermentation of sugar in alcoholic fermentation. But after this step because of the absence of the enzyme carboxylase in lactic acid bacteria pyruvic acid is not decarboxylated to acetaldehyde and CO_2. Instead oxidation reduction reactions set in with phosphoglyceraldehyde and the pyruvic acid is reduced to lactic acid.

1.13 The Embden Meyerhoff Pathway:

In the 1930's the German biochemists G. Embden and O. Meyerhoff elucidated the sequence of reactions by which glycogen and glucose are degraded in the absence of oxygen (anaerobic conditions) to pyruvic acid.

The EMP constitutes generalization of great importance and occurs in both anaerobic acid aerobic conditions, consists of ten different enzymes and catalyses the conversion of glucose to pyruvic acid with the production of 2 moles of ATP per molecules of glucose.

The pathways may conveniently be divided into six parts and the various steps may be represented in the following schemes. The EMP Scheme shown earlier may also be represented in simple manner as follows:

The various steps may also be represented in the following schemes: [275-292]

Glucose + ATP → Glucose-6 -phosphate + ADP

Glucose-6-phosphate ⇌ Fructose-6-phosphate

Fructose-6-phosphate+ ATP → fructose 1: 6-diphosphate + ADP

Fructose 1: 6-diphosphate

3-phosphoglyceraldehyde + dihydroxyacetonephosphate

(Aldolase)

(Isomerase)

3-phosphoglyceraldehyde+H_3PO_4 ⇌ (1: 3-diphosphoglyceraldehyde)

(1: 3-diphosphoglyceraldehyde) + DPN ⇌ 1: 3-diphosphoglyceric acid + DPN.2H

1: 3-diphosphoglyceric acid + ADP ⇌ 3-phosphoglyceric acid+ ATP.

3-phosphoglyceric acid ⇌ 2-phosphoglyceric acid

2-phosphoglyceric acid ⇌ phosphoenol pyruvic acid +H_2O

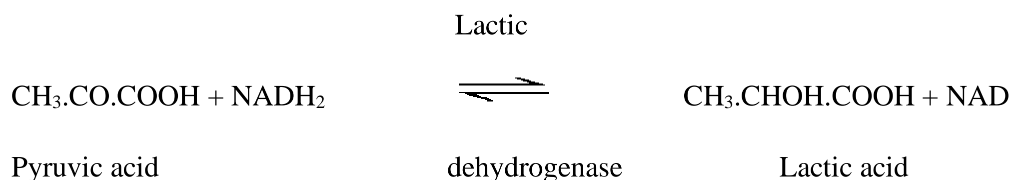
Phosphoenol pyruvic acid + ADP ⇌ pyruvic acid + ATP

1.14 Pyruvate obtained in the above Steps as A Very Important Intermediate:

Pyruvic acid is the crossroads compound; its metabolic fate is dependent upon the conditions (anaerobic or aerobic) and upon the organism under consideration.

In the presence of **NADH** the enzyme lactic dehydrogenase catalyses the reduction of pyruvic acid to lactic acid.

In the homofermentative fermentation, key pyruvic acid formed is acted upon by lactic dehydrogenase and **$NADH_2$** is reoxidised by a direct reduction of pyruvic acid to Lactic acid



All the above shown enzymes are present in a bacterial cells and sugar serves as a substrate for the fermentative production of lactic acid in the process at the end only two molecules of ATP are remained. The lactic acid is placed in barrels for marketing.

1.15 Industrial Applications of Lactic Acid:

Lactic acid is sold in food, pharmaceutical and technical grades. Since the lactic acid has gained increasing importance and has been used in a great variety of applications, its salt, ester and many derivatives have been developed. The uses of lactic acid can be broken down by grade and by lactic acid derivatives. Some of the important applications of lactic acid are detailed below.

Lactic acid is used in pharmaceutical industry as a very important ingredient. Pharmaceutical and food industries show presence for the L (+) lactic acid because the D (-) isomer is not metabolized by the human body. Lactic acid and its salts have been mentioned for various medical uses. They provide the energy and volume for blood besides regulation of pH. Calcium, sodium, ferrous and other salt of lactic acid are used in pharmaceutical industry in various formulations find use for their anti-tumor activity. Lactic acid finds medical applications as an intermediate for pharmaceutical manufacture, for adjusting the pH of preparations and in tropical wart medications293. In the last several years, lactic acid consumption for industrial applications has surpassed the food and beverages industry as the leading market for lactic acid. This shift is expected to continue as growth rates for industrial uses will be much higher than growth rates for other uses. This is a result of the continued high growth of PLA applications. It is expected that by 2013, industrial applications will account for more than half of global lactic acid use.

In recent years, Asia has become nearly equivalent to Western Europe as a consumer of lactic acid products. All three major regions—the United States, Western Europe and Asia (driven mainly by China)—will continue to show strong annual growth at 7%, 9% and 5.5%, respectively, in the next few years. Globally, lactic acid consumption will continue to increase significantly, at about 7% per year from 2008 to 2013.

Growth in demand for lactic acid, its salts and esters in industrial applications will be driven mainly by lactic acid–based biodegradable polymers and, to a lesser degree, lactate solvents. The use of polylactic acid, especially in the plastics packaging, container and cutlery markets, is being highly promoted because of its environmentally friendly characteristics. Environmental benefits include product biodegradability; composting of waste by-products from PLA production; growth in the use of plant-based materials, which reduces carbon dioxide in the atmosphere; and the potential energy saved versus conventional polymer production. In the United States, PLA demand for industrial applications such as fibers, containers and packaging is expected to continue to increase. Likewise, demand for PLA will increase significantly in Western Europe, mainly for packaging uses.

The main obstacles to large-scale use of biodegradable lactic acid–based polymers in packaging applications are cost, environmental legislation concerning waste disposal and composting, and consumer attitudes and behaviors concerning the environment. Also, there is ongoing debate about the true amount of energy (often in the form of fossil fuels) consumed to produce PLA from raw materials such as corn. With large-scale production, prices are expected to continue to decline; however, lactic acid–based biodegradable polymers are expected to remain more expensive than commodity polymers in the near future. The food and beverages market will also continue to drive lactic acid growth. In the United States, lactic acid will continue to be used mainly as an acidulant but will also continue to grow in the ready-to-eat meat industry. However, growth will depend on the prevailing economic situation. Likewise, growth in this market for Western Europe and Japan will be moderate. In China and Other Asia, growth in the food and beverages market will be stronger as lactic acid will continue to be used in local foods, as well as food fortifiers and pH adjusters. Pharmaceuticals and personal care products have become an important market for lactic acid, its salts and esters. This market will continue to increase steadily in the United States and Western Europe, while China will experience stronger growth in this area. Uses include intravenous solutions, shampoos, soaps, antiaging alpha-hydroxy skin creams and moisturizers.

The industrial applications that have been found for some microbial products especially for lactic acid are extremely varied, and have been reviewed by many workers.[294-329]. Lactic acid has a pleasant, sour taste and no odour, properties which make it a valuable acidulant in the food and beverage industries. Furthermore, since it is miscible with water in all proportions, there are no problems raised by crystallization, as there may be when using solid organic acids. An additional advantage is that in many foodstuffs it acts as a preservative. The acid is especially suitable for acidifying soups and jams and in preserving pickles and sauerkraut. The solubility of calcium lactate in water makes it possible to use technical grade lactic acid for de-liming hides in the leather industry. In the textile and laundry industries, it is used in 'finishing' silk rayon fabrics. When an aqueous solution of lactic acid is concentrated, anhydride formation and inter-esterification take place, to give poly lactic acids. These polymers are not themselves of any great commercial importance because of their relatively high residual acidity and poor resistance to hydrolysis, but, by heating them with vegetative oils, or with synthetic drying oils in the presence of a catalyst, valuable elastic resins are formed. Polylactic acids also give thermoplastic resins when heated with a mixture of furfural and a polyhydric alcohol such as glycerol.

Derivatives of lactic acid also find a wide application. Calcium lactate is used as a source of calcium in pharmaceutical preparations. This salt is also becoming increasingly important as a food supplement for farm animals and poultry 'salt-licks' containing calcium lactate help to prevent the customary fall-off in milk production by cows towards the end of the winter, and the use of calcium lactate in poultry feeds is reported to bring about a significant increase in egg production.

Lactic acid was first produced on a large scale in order to make calcium lactate for use in baking and this salt is still added to baking powders to help regulate the rate of gas evolution. The copper salt of lactic acid finds a use in electroplating. Several lactic acid derivatives are used in the manufacture of plastics; alkyl lactates are especially useful in this respect. Acrylic acid esters can be used to make valuable polymers, which are then employed in the preparation of lacquers and varnishes. Methyl acrylate can be made from lactic acid by pyrolizing the acetyl derivative of methyl lactate (methyl acetoxypropionate).

Although lactic acid is used in a variety of ways in industry, these are not as numerous as was at once thought likely. This is almost certainly a result of the comparatively high market price of the acid as produced by the fermentation process, and in particular of the very heavy cost of recovering and purifying the lactic acid from the fermented medium. Lactic acid that is to be used in manufacturing plastics needs to be of a fairly high purity. In addition, the equipment used in processing concentrated lactic acid is expensive, since it needs to be made of non-corrosive materials. Consequently, attempts have been made to manufacture lactic acid by purely chemical means. In particular, processes based on the action of alkalies on sugars to give lactic acid have been actively studied. Haworth. Gregory and Wiggings have described a process for producing lactic acid by the action of calcium hydroxide on sucrose, and have reported yields of over 70 percent. Starch and cellulose have also been considered as raw materials in this process. To date, however, these chemical methods have met with only limited success commercially. During the last few years the very remarkable observation has been made for lactic acid fermentation exposed to some organic molecules and some other fermentative products330-360 that has got much attention and importance. Therefore, lactic acid fermentation by using the bacterial strain of Lactobacillus bulgaricus NCIM–2359. Has been undertaken for the study. It would be easy to stress too much the practical applications of the lactic acid which is of much interest to organic chemists, for instance in revealing new types of ideas and technology of natural origin.

The difficult problems of the mode of for lactic acid fermentation on susceptible organism's offers the biochemist a fascinating field of study and the knowledge acquired may help towards the unravelling of process of microbial metabolism.

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Chapter 2

General Experimental Methods

2.1 Introduction:

Experimentation is the step in the scientific method that helps people decide between two or more competing explanations – or hypotheses. These hypotheses suggest reasons to explain a phenomenon, or predict the results of an action. An example might be the hypothesis that "if I release this ball, it will fall to the floor": this suggestion can then be tested by carrying out the experiment of letting go of the ball, and observing the results. Formally, a hypothesis is compared against its opposite or null hypothesis ("if I release this ball, it will not fall to the floor") the null hypothesis is that there is no explanation or predictive power of the phenomenon through the reasoning that is being investigated. Once hypotheses are defined, an experiment can be carried out - and the results analysed - in order to confirm, refute, or define the accuracy of the hypotheses.

An experiment is a method of testing - with the goal of explaining - the nature of reality. Experiments can vary from personal and informal (e.g. tasting a range of chocolates to find a favorite), to highly controlled (e.g. tests requiring complex apparatus overseen by many scientists hoping to discover information about subatomic particles). More formally, an experiment is a methodical procedure carried out with the goal of verifying, falsifying, or establishing the accuracy of a hypothesis. Experiments vary greatly in their goal and scale, but always rely on repeatable procedure and logical analysis of the results. A child may carry out basic experiments to understand the nature of gravity, while teams of scientists may take years of systematic investigation to advance the understanding of a phenomenon.

The experimental method involves manipulating one variable to determine if changes in one variable cause changes in another variable. This method relies on controlled methods, random assignment and the manipulation of variables to test a hypothesis. When most people think of scientific experimentation, research on cause and effect is most often brought to mind.

Experiments on causal relationships investigate the effect of one or more variables on one or more outcome variables. This type of research also determines if one variable causes another variable to occur or change. An example of this type of research would be altering the amount of a treatment and measuring the effect on study participants. A simple experiment is used to establish cause and effect, so this type of study is often used to determine the effect of a treatment. In a simple experiment, study participants are randomly assigned to one of two groups. Generally, one group is the control group and receives no treatment, while the other group is the experimental group and receives the treatment.

Experiment is the step in the scientific method that arbitrates between competing models or hypotheses.1, 2 Experimentation is also used to test existing theories or new hypotheses in order to support them or disprove them.3, 4 an experiment or test can be carried out using the scientific method to answer a question or investigate a problem.

First an observation is made. Then a question is asked, or a problem arises. Next, a hypothesis is formed. Then experiment is used to test that hypothesis. The results are analyzed, a conclusion is drawn, sometimes a theory is formed, and results are communicated through research papers. Francis Bacon was an English philosopher and scientist in the 17th century and an early and influential supporter of experimental science. He disagreed with the method of answering scientific questions by deduction and described it as follows: “Having first determined the question according to his will, man then resorts to experience, and bending her to conformity with his placets, leads her about like a captive in a procession” Bacon5 wanted a method that relied on repeatable observations, or experiments. He was notably the first to order the scientific method as we understand it today.

In this chapter the general experimental methods used in the study of lactic acid fermentation by Lactobacillus bulgaricus NCIM – 2359 has been discussed. It includes chemical cleaning and steam sterilization of glasswares (fermentor flask, petri-dishes, platinum needle, pipettes and micro-pipettes), preparation and sterilization of different media such as culture medium, inoculum medium and production medium. Seeding of culture tubes. Inoculation of inoculum medium and production medium. Preparation of buffer solution, incubation of culture tubes, inoculum medium, production medium. Colorimetric determination of lactic acid formed and molasses (substrate) left unfermented during the course of present investigation.

2.2 Cleaning of Glasswares:

Cleaning laboratory glassware is not as simple as washing the dishes. Here’s how to wash our glassware so that we won’t ruin our chemical solution or laboratory experiment.

2.2.1 Cleaning Basics:

It’s generally easier to clean glassware if we do it right away. When detergent is used, it’s usually one designed for lab glassware, such as Liquinox or Alconox. These detergents are preferable to any dishwashing detergent you might use on dishes at home.

Bacteria are widely distributed in nature being universally present everywhere and if given the opportunity they may contaminate everything, every one of the equipment and medium used for fermentative investigation. While employing bacteria for detailed study it is necessary to take utmost care to avoid the contaminants. It is therefore, necessary to remove or kill all bacteria from fermentative equipment used for facile biotransformation of sugars to lactic acid or to eliminate as well reduce the possibility of unwarranted contaminants entering subsequently in them. Therefore, the physico chemical composition of the medium, substrate composition concentration, the length of contact and temperature, chemical substance have different effect on microbes and microbial processes.

When carrying out an experiment it is essential to use a cleaning mixture to clean the glasswares to be used. Although biologist and microbiologist employ a variety of approaches in conducting research, the experimentally oriented scientists often use general cleaning mixture of cheap and economic importance. The role of some chemicals are lethal to microbes and microbial processes. Hypochlorite solutions and phenolic are used as general laboratory infectants. However, these chemicals may not cause complete sterilization under mild conditions.

2.3 Chemical Sterilization:

Sterilization is a term referring to any process that eliminates (removes) or kills all forms of microbial life, including transmissible agents (such as fungi, bacteria, viruses, spore forms, etc.) present on a surface, contained in a fluid, in medication, or in a compound such as biological culture media. Sterilization can be achieved by applying the proper combinations of heat, chemicals, irradiation, high pressure, and filtration.

The microbial investigations require a good deal of complete killing and removal of microbes from different medium and glasswares. For this purpose a common cleaning mixture for glasswares cleaning has been employed by author which has been prepared as under:

$K_2Cr_2O_7$	:150 g,
Conc. H_2SO_4	:150 ml,
Water	: 200 ml

The chromic mixture for cleaning purposes were prepared by thorough mixing and a gentle heating and then used to clean the culture tubes, fermentor flasks, petri dishes, micro-pipettes and other glasswares employed during the course of present investigation, i.e., lactic acid fermentation by Lactobacillus bulgaricus NCIM – 2359. The glasswares were finally washed thoroughly with running tap water for about 5 minutes.

2.3.1 Sterilization and Disinfection:

The process of complete elimination or killing of all microbes is called "sterilization". Pasteur was perhaps the first to show that heat can eliminate microorganisms. He showed that if urine was heated to a high temperature and kept covered, it would remain free of microbial growth. The word "disinfection" refers to the removal of those organisms which cause infection, and this can be accomplished either by the use of chemicals, by Pasteurization, by controlled irradiation or by filtration. Agents which disinfect are called disinfectants. In addition to heat, one of the earliest chemicals used as a disinfectant was phenol (carbolic acid).

2.3.2 Sterilization by Heat:

Moist or dry heat is used to sterilize media and materials. The use of boiling for preservation of food has been known since long. Boiling for short periods of 5-15 minutes is sufficient to destroy most vegetative forms of microorganisms. However, boiling in water will not cause complete sterilization since endospores remain viable even after boiling. Boiling repeatedly with intervals of cooling, may permit growth of the endospores into vegetative cells and their subsequent destruction. Boiling in water is a simple method of sterilization and needs no special apparatus. However, one disadvantage is that it is time consuming and this may also bring about changes in the chemical composition of the materials. An alternate to boiling water is to heat with steam under pressure. Since water boils at a higher temperature under pressure it is possible to raise the boiling point of water above 100^0C and this can be accomplished in an autoclave (Pressure Cooker). The relationship of steam pressure to temperature is shown in Table-2.1.

Hot steam is a more efficient sterilizing agent since it first hydrates the cells and then coagulates the proteins while dry heat cannot do this. For this reason, while autoclaving the exhaust valve of the autoclave or cooker is left open till all the air escapes before the steam pressure is allowed to increase.

After the appropriate temperature is reached, the materials is held at that temperature for a short period (10-30 minutes for normal growth media). The pressure is then allowed to return to normal slowly to avoid damage to the material. The time required for sterilization by autoclaving will depend on the material to be sterilized and the initial microbial load. Solid materials must be heated for a longer time (1-2 hours) so as to allow heat penetration while liquid media can be efficiently sterilized in 15-30 minutes. Also, acidic materials require shorter periods of sterilization.

Sometimes dry heat is also used in sterilization although it is not as efficient as moist heat. The time period required is however, long and the temperature for complete sterilization required is high (above 165^0C for several hours). Hot air sterilization can be accomplished in a hot air oven. Glassware, mineral oils, soil etc. which are not affected by high temperature can be sterilized by this method.

The autoclave is usually operated at 15 lb. steam pressure for 30 min., which, as seen from the above table, corresponds to 121.5^0C. This temperature for a period of 30 min. is sufficient to kill all the spores and vegetative cells of microorganisms. The autoclave is used to sterilize usual non-carbohydrate media, broths and agar media, distilled water, normal saline solutions, discarded cultures, contaminated media, aprons, rubber tubing, rubber gloves, etc. This type of sterilization is also used in the commercial canning of fruits and vegetables and also in order to manufacture sterilized milk.

2.3.3 The Purity of Chemicals Used:

Only AR grade chemicals of high quality has been used throughout the present investigation carried out by the author for lactic acid fermentation by Lactobacillus bulgaricus NCIM – 2359.

2.4 Cotton Wool Plugs:

In fermentation, microbial experiment claimed to prove spontaneous generation, a cork has been often used to prevent the entry of contaminants in the fermenter flasks from outside. But this proved ineffective as bacteria could enter round the side of the cork the vessel cooled after sterilization. Sealing of the fermentor flasks were not proper as air (oxygen) known to be essential for many bacterial forms of life, could no longer enter the fermentor flasks.

It was necessary, therefore, to include some kind of special filter to check the entry of any type of bacteria but not the air. This led to the necessity and development of cotton wool plug that was soon adopted universally by bacteriologists.

In the present investigation, i. e., lactic acid fermentation by Lactobacillus bulgaricus NCIM – 2359 the cotton wool plugs have been used frequently for the sealing of fermentor flasks and culture tubes etc.

2.4.1 Plugging of Fermentor Flasks and Culture Tubes:

Bacteria constitutes a very antique group of living organisms and are universally present everywhere in nature. Therefore, in order to avoid entry of such undesired bacteria into fermentor flasks and culture tubes, cotton wool plugs has been employed throughout during the course of present investigation, i. e., biosynthesis of lactic acid by Lactobacillus bulgaricus NCIM – 2359. To meet the purpose the mouth of the fermentor flasks and culture tubes were tightly plugged with non-absorbent cotton wool plugs to avoid their undesired entry into the fermentor flasks. Thus, for fermentor flasks three inch square and for culture tubes (standard size) one inch square cotton wool pieces were prepared by folding it through hard rolling and by hand pressing.

Thus, cotton wool plugs tightly fitting with the mouth of the culture tubes and fermentor flasks were prepared as required during the course of present investigation, i. e., and lactic acid fermentation by Lactobacillus bulgaricus NCIM – 2359. In the present investigation, i.e., lactic acid fermentation by Lactobacillus bulgaricus NCIM – 2359 the author has used an autoclave maintained at 121°C (15 lb./in2) for the sterilization of glasswares and other apparatus as well culture and production medium.

Table – 2.1: Relationship between pressure and temperature of steam.

Pressure Above Atmospheric (lb./sq. inch)	Temperature ^{0}C
0	100
5	109
10	115
15	121
20	126
25	130
30	134

2.5 The Lactic Acid Bacteria (LAB):

The lactic acid bacteria (LAB) are a group of Gram positive bacteria united by a constellation of morphological and physiological characteristics. The major genera, and physiological characteristics. The major genera, Lactobacillus, Leuconostoc, Lactococcus, Pediococcus and Enterococcus form the core of the group. The LAB are amongst the bacteria with ancient and broad applications because of their diverse metabolic capabilities. They can be used in several fermentative processes for the production and preservation of food. The LAB are safe both from human and environmental point of view as they have GRAS status[6, 7].

It is well known that rich nutrition, like carbohydrates, minerals, nitrogen compounds or other substances are necessary for the growth of LAB. Their isolation is reported from vegetables[8-9], aerial plant surfaces[10], pickled cabbage[11] grass silage[12], malted cereals[13] and also from soil[14]. They are used as bio preservative organisms in several foods. The bio preservative ability of LAB is due to the production of antibacterial[16-18] and antifungal substances [15-21].

The Microorganism Used:

Classification	
Kingdom	Bacteria
Division	Firmicutes
Class	Bacilli
Order	Lactobacillales
Family	Lactobacillaceae
Genus	Lactobacillus
Species	L. delbrueckii
Subspecies	L. d. bulgaricus

Lactobacillus bulgaricus is a species of genus Lactobacillus found in the human intestine and mouth. This particular species of Lactobacillus is documented to have a wide pH and temperature range, and complements the growth of L. Bulgaricus, a producer of the enzyme amylase (a carbohydrate-digesting enzyme). The most common application of L. Bulgaricus is industrial, specifically for dairy production. However, a team of scientists from Simón Bolívar University in Caracas, Venezuela found that, by using L. Bulgaricus bacteria in the natural fermentation of beans, the beans contained lower amounts of the compounds causing flatulence upon digestion.[22] Lactobacillus bulgaricus is typically the dominant species of nonstarter lactic acid bacteria (NSLAB) present in ripening Cheddar cheese, and, recently, the complete genome sequence of L. Bulgaricus has become available. L. bulgaricus is also the dominant species in naturally fermented Sicilian green olives.[23]

A commercial beverage containing L. Bulgaricus strain Shiroma has been shown to inhibit the growth of Helicobacter pylori in vivo. But, when the same beverage was consumed by humans in a small trial, H. pylori colonization decreased only slightly, and the trend was not statistically significant.[24] Some L. Bulgaricus strains are considered as probiotic, and may be effective in alleviation of gastrointestinal pathogenic bacterial diseases. According to World Health Organization, those properties have to be demonstrated on each specific strain—including human clinical studies—to be valid.[25] in the past few years, there have been many studies in the decolonization of azo dyes by lactic acid bacteria such as L. Casei TISTR 1500, L. paracasei, Oenococcus oeni. With the azoreductase activity, mono-, di- azo bonds are degraded completely, and generate other aromatic compounds as intermediates.[26]

Lactobacillus bulgaricus NCIM – 2359 has been used throughout the investigation and it has been procured from NCL Poona, India. It is notable that a proper, specific and efficient strain of selected microorganism is the heart of fermentation. The strain under trial must have the following qualities: Efficiency of the culture for the synthesis of a desirable metabolite. Synthesis of related undesired metabolites in proportion to the desirable one. Large number of species of bacteria and some other species of moulds produces lactic propionic acid in significant quantities from fermentable carbohydrate materials.

Lactic acid fermentation is a process of anaerobic fermentation, though the lactic acid bacteria usually grow well in the presence of oxygen. Lactic acid fermentation breaks down one molecules of glucose into two molecules of lactic acid and releases 18 calories of heat.

In the process of lactic acid fermentation also very little of the potential energy of the sugar is given out and the greater portion of the energy is left unreleased in the lactic acid.

Certain microorganisms producing lactic acid are classed as being heterofermentative. Thus, they produce some lactic acid but at the same and probably by way of the pentose phosphate metabolic pathway, they produce CO_2, C_2H_5OH, and CH_3COOH and trace amounts of a few other products. These organisms are of little use for industrial lactic acid fermentations, because too much of the substrate carbon is directed towards products other than lactic acid.

Lactic acid production utilizes 'homofermentative strains of lactic acid bacteria which produces only trace amounts of end product other than lactic acid. Thus, these bacteria utilize the EMP scheme to produce crossroads compound, i.e.; pyruvic acid which is then reduced by their lactic dehydrogenase enzyme to lactic acid. In the present investigation the following fermentative lactic acid bacteria has been employed by the author for biosynthesis of lactic acid

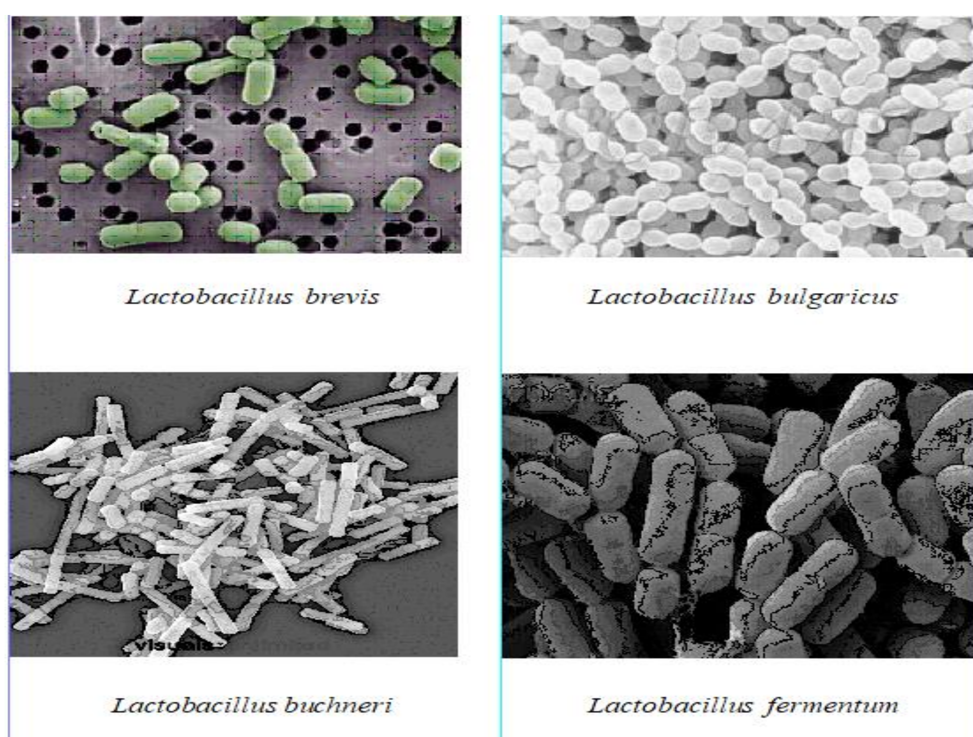


Table- 2.2

1.	Lactobacillus brevis	NCIM – 2090
2.	Lactobacillus bulgaricus	NCIM – 2359
3.	Lactobacillus buchneri	NCIM – 2357
4.	Lactobacillus fermentum	NCIM –2165

Used in the present investigation.

All the above lactic acid producing bacteria were employed for biosynthesis of lactic acid and the results so obtained are recorded in the **Table -2.3** as given below:

Table – 2.3

Sr. No.	Isolate		Yield of lactic acid * in g/100ml
(K)	Lactobacillus brevis	NCIM – 2090	5.31253
(L)	Lactobacillus bulgaricus	NCIM – 2359	8.15796**
(M)	Lactobacillus buchneri	NCIM – 2357	7.23464
(N)	Lactobacillus fermentum	NCIM –2165	6.50363

* Each value represents mean of three trials, (from 20% molasses).
** Maximum yield of lactic acid in comparison to others.

The data recorded in the T**able -2.3** shows that the microbial strain designated as (L), i.e., Lactobacillus bulgaricus NCIM – 2359 has valuable and significant yield of lactic acid , i.e., 8.15796g/100 ml in comparison to rest isolate taken into trial, i.e., (K) (M) and (N), i.e., Lactobacillus brevis NCIM – 2090, Lactobacillus buchneri NCIM – 2357 and Lactobacillus fermentum NCIM –2165 respectively.

It is thus concluded that all the bacterial isolate taken for study are not equally suitable for maximum production of lactic acid, though they are similar in many ways in nature. It is thus obvious that Lactobacillus bulgaricus NCIM – 2359 is comparatively more productive and competent so for as biosynthesis of lactic acid is concerned.

It is on the basis of high production of lactic acid, the bacterial strain of Lactobacillus bulgaricus NCIM – 2359 has been selected and employed throughout the present investigation and has been maintained by its periodical transfer into the freshly prepared culture tubes containing enriched nutrition medium. The culture medium for Lactobacillus bulgaricus NCIM– 2359 has been prepared as follows:

2.6 Preparation of the Culture Medium[27]:

In order that the homofermentative lactic acid bacteria may retain its metabolic process, it was carefully periodically cultured. The fresh enriched culture were prepared after every fifteen days (fortnightly) as follows:

2.6.1 Formulation of Culture:

Formation of enriched culture medium for Lactobacillus bulgaricus NCIM – 2359 the following bio-ingredients has been employed:

Glucose	0.25%
Lactose	0.25%

Sodium-Acetate	300.00 mgs
Liver-Extract	300.00 mgs
Peptone	300.00 mgs
Salt Solution A	0.10 ml
Salt solution B	.10 ml
pH	6.0-6.2
Sterilization	15lbs for 25-30 minutes
Sub-culture	Once a month
Distilled water	To make up 100 ml

Requisite amount of distilled water was added to make the total volume 100 ml.

2.7 Preparation of Salt Solution A:

It was prepared by mixing the following with water:

KH_2PO_4 : 25.00 g

K_2HPO_4 : 25.00 g

Requisite amount of distilled water was added to make the volume 250ml.

2.7.1 Preparation of Salt Solution B:

It was prepared by mixing the following with water:

$MgSO_4.7H_2O$: 10.00 g

NaCI : 500.00 mgs

$MnSO_4.5H_2O$: 500.00 mgs

$FeSO_4.7H_2O$: 500.00 mgs

The combination of above amount were dissolved in requisite amount of distilled water to make up the volume up to 250 ml.

The volume of the culture medium was taken in a dry and clean flask and was plugged with non-absorbent cotton wool plugs. About 12 clean and dry culture tubes were similarly plugged with non-absorbent cotton. These culture tubes and culture medium were sterilized in an autoclave at 1.71 Kg/ Cm^2 steam pressure for 20 minutes. For solid growth medium 5 % agar-agar was added in this solution before sterilization.

After cooling, 5.0 ml of the culture medium from the conical flask was transferred to each culture tube. The culture tubes were then allowed to stand in slant position overnight.

2.8 Seeding:

The seeding of the culture tubes were done with the help of a sterilized inoculating platinum needle with its straight end. A small quantity of Lactobacillus bulgaricus NCIM – 2359 from the previous culture tube was transferred to the freshly prepared culture tubes with the help of sterilized platinum needle.

2.9 Preparation of the Fermentation Medium:

In the present investigation the fermentation medium used by the author has the following ingredient composition:

Molasses	:	20% (w/v)
Malt Extract	:	0.60 %
Yeast Extract	:	0.60 %
Peptone	:	0.60%
$(NH_4)_2HPO_4$	:	0.60%
$CaCO_3$	:	8.0 %
pH	:	6.1
Distilled water	:	To make up 100 ml.

The pH of the medium was adjusted to 6.1 by adding requisite amount of phosphate¬ buffer solution which was prepared as follows:

During microbial growth, pH changes can occur for one of several reasons. Obviously, an acidic or alkaline fermentation product can alter the pH value.

Calcium carbonate often is incorporated in fermentation media to provide neutralization of acidic fermentation products, although it is relatively poor buffer; its poor solubility in water allows only slow reaction with acidic products.

Additional buffering capacity in this pH range also is provided by phosphates such as the system of monobasic sodium phosphate dibasic sodium phosphate which provide buffering capacity.

In contrast to the foregoing discussion, at times it may not be desirable to employ a medium to be buffered at the pH values initially provided at medium make-up. Thus, increase or decrease in pH during the fermentation can allow increased yield of certain fermentation products because of specific effects of acidity or alkalinity on the metabolism of the microorganism.

2.9.1 Preparation of Phosphate-Buffer Solution:

It includes stock solution A and stock solution B as follows.

a. **Stock Solution (A):**
   0.2 M solution of monobasic sodium phosphate
b. **Stock Solution (B):**
   0.2 M solution of dibasic sodium phosphate.

2.10 Molasses:

Molasses is a thick, brown to deep black, honey-like substance made when cane or beet sugar is processed. It is enjoyed as a sweetener in many countries, and most particularly in England where it is called treacle. For hundreds of years, molasses and sulfur, or treacle and brimstone were thought to have healthful benefits, and children were frequently given doses of the product.

The constipating or sometimes laxative effect of brimstone and treacle could be misused to keep appetites down. Charles Dickens makes mention of its application in Nicholas Nickle by, where the starving students of Mr. Wackford Squeers' school are frequently dosed with such to cut down on their porridge consumption. Molasses had a somewhat unsavory history during prohibition in the US.

It is the primary base for the manufacture of rum. Molasses importation became synonymous with the bootlegging industry and with organized crime.

Today, uses for molasses are quite benign. It is used primarily in baking. No gingerbread would be quite the same without the addition of molasses. Some people enjoy using it on hot cereals like cream of wheat or cornmeal mush.

Molasses is also a necessary ingredient in the Thanksgiving holiday traditional pumpkin pie. In England, treacle tart, is not, however, made with molasses, but it is enjoyed as a sweetener on porridge. Homemade caramel corn is especially good with a dollop of molasses added to the sugar mixture.

Molasses are by-products of the sugar industry. Of these, blackstrap molasses prepared from sugarcane normally is the cheapest, economical and the most used sugar source for industrial fermentations. Molasses contains approximately 52 percent total sugars calculated as sucrose (30 percent sucrose, and 22 percent invert sugars) and is known as black-strap molasses.

When this molasses is used as a fermentation medium component, it is considered to contain 50 percent fermentable sugars. Refinery blackstrap molasses is a similar product that differs from black-strap molasses only in that it is the residual mother liquor that has accumulated in the recrystallization refining of the crude sucrose.

In the present investigation the author has used cane molasses as a sugar substrate for lactic acid fermentation by Lactobacillus bulgaricus NCIM – 2359. The following medium has been used in the commercial production of lactic acid.

2.11 Brietzke's Medium[28]:

Corn sugar	:	10% + 2%
Malt sprout	:	8% + 0.75%
Diammonium phosphate	:	1.5% + 0.25
Distilled water	:	To make up 100 ml
$CaCO_3$	:	8%

2.12 Inoculation of the Fermenting Medium:

0.05 ml bacterial suspension of Lactobacillus bulgaricus NCIM – 2359 has been transferred for inoculation. First of all highly enriched culture broth medium was prepared in conical flask which was then sterilized and cooled to room temperature as usual methods descried earlier. Then a small quantity of Lactobacillus bulgaricus NCIM – 2359 from culture tubes were transferred to a culture broth medium with the help of a sterilized platinum needle and it was incubated for 48 hours at 38°C. With the help of a sterilized glass dropper, this 48 hours old lactic acid culture was added in a definite amount (0.05 ml bacterial suspension of Lactobacillus bulgaricus NCIM–2359 to the fermenting basal medium employed for experimental trials. The same amount, i.e., 0.05 ml. Lactobacillus bulgaricus NCIM – 2359 bacterial suspension incoculum was employed in all the other sets of experiments throughout the present investigation to obtain comparable results.

The fermentor flasks were kept in an incubator maintained at a constant temperature of 38°C after inoculation of the fermenting media. The fermented mass was analysed after 3, 6 and 9 days of incubation period for lactic acid formed and molasses left unfermented.

2.12.1 Procedure of the Experiment:

General procedure for biosynthesis of lactic acid by Lactobacillus bulgaricus NCIM – 2359. The fermenting medium was first prepared by dissolving the following ingredients in 100 ml. of distilled water:

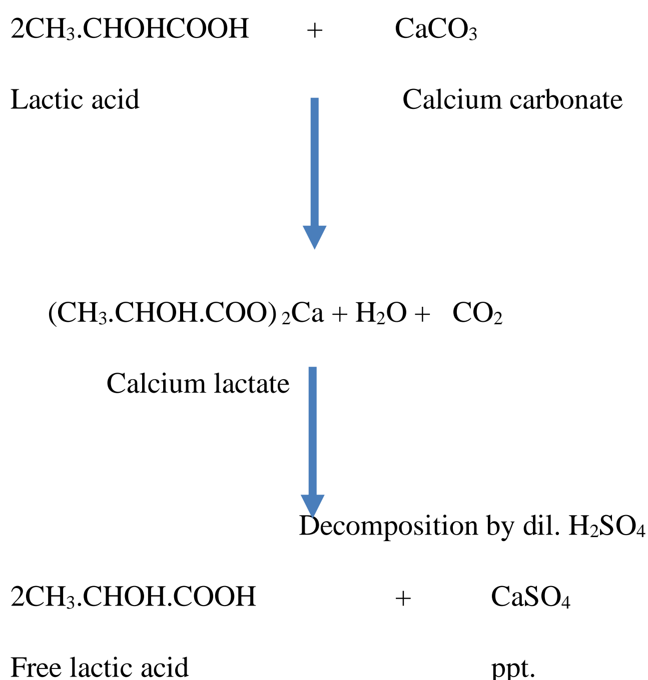
Molasses	:	20%
Malt extract	:	0.60%
Yeast extract	:	0.60%
Peptone	:	0.60%
Diammonium hydrogen phosphate	:	0.60%
$CaCO_3$	:	8%

pH	:	6.1
Distilled water	:	To make up 100 ml

The pH of the medium was adjusted to 6.1 by adding requisite amount of phosphate-buffer solution. The total volume of the medium was well mixed and its volume was set to be 100ml by adding requisite amount of distilled water and transferred in 250ml conicial flask. Nine more sets of the above composition was prepared in 250 ml conical flask.

The above flasks were then plugged with non-absorbent cotton wool plugs and kept for 20 minutes for sterilization in an autoclave maintained at 15 lbs steam pressure. Then the flasks were removed from autoclave and allowed to cool at room temperature. These flasks were then inoculated with 0.05 ml inoculum of Lactobacillus bulgaricus NCIM – 2359 from 38 hours old culture broth. The flasks were then kept in an incubator maintained at 38°C for a required period of incubation.

After completion of the incubation period, the calcium lactate thus formed is separated. The calcium lactate separated above is decomposed by dil H_2SO_4 to liberate free lactic acid and calcium-sulphate precipitates out:



A slight excess of dil. H_2SO_4 is used to precipitate the last traces of calcium as calcium sulphate. After removal of calcium sulphate, the filtered acid does not give a precipitate when treated with ammonium oxalate.

Barium hydroxide is now added to remove excess amount of dil. H_2SO_4 although a very a slight excess of dil. H_2SO_4 is desired in view of the fact that water used for diluting the lactic acid may contain a slight amount of calcium.

Thus, a very slight excess of sulphuric acid will balance calcium added by the dilution water. Final filtration of the solution removes all precipitates of barium sulphate and calcium sulphate.

The free lactic acid thus obtained and the molasses sugars left unfermented are determined colourimetrically[29, 30] as follows:

2.12.2 Colorimetric Estimation of Lactic Acid Formed and Molasses Left Unfermented:

The substrate molasses sugars and other interfering materials of the filtrate obtained were removed by the treatment with copper sulphate or copper hydroxide solution. Now, an aliquot of the resulting solution containing free lactic acid is heated with AR-grade conc. H_2SO_4 to convert the lactic acid into acetaldehyde which is then estimated colorimetrically by measurement of the developed purple colour due to the action of p-hydroxydiphenyl in the presence of Cu-ions[31-34].

2.13 Reagents:

For the photo-colorimetric determination of lactic acid formed the following reagents were used:

a. **Sulphuric Acid:**
   Only AR grade conc. H_2SO_4
b. **Calcium Hydroxide:**
   Calcium hydroxide powder
c. **Copper Sulphate:**
   4% and 20% Solution of $CuSO_4.5H_2O$
d. **Sodium Hydroxide:**
   5% solution of NaOH solution
e. **p – Hydroxydiphenyl:**

p - hydroxydiphenyl solution was prepared by dissolving 1.5 g of it in 10 ml of 5% NaOH solution followed by mild heating, constant stirring and finally diluting it to 100 ml with distilled water.

The above solution thus prepared was stored in a brown bottle fitted with a tight stopper. The reagent is stable for many months.

f. **Preparation of Lactate Standard:**

It is prepared by dissolving 0.213g of AR-grade pure and dry lithium lactate in 100 ml of distilled water in one-littered volumetric flask. Now, 1.0ml of conc. sulphuric acid solution was poured into it and diluted to one-liter and thoroughly shacked to mix well.

Thus, reagent prepared is stable for many months if kept in a refrigerator and contains 1.0 mg of lactic acid per 5.0 ml of the solution.

2.14 Working Standard:

Working standard of the solution formed above is prepared by diluting 5.0 ml of standard solution to 100 ml with requisite amount of distilled water. Now, this working lactate standard solution contains approximately 0.01, mg of lactic acid per ml and is preferably prepared fresh daily.

2.15 Procedure:

The procedure for the estimation of lactic acid formed during lactic acid fermentation by Lactobacillus bulgaricus NCIM – 2359 has been described in the following steps:

Step First (Copper-Calcium Treatment):

The molasses sugars and other interfering substances are removed by treatment with copper sulphate and copper hydroxide solution. Now, it test tubes 5.0 ml of protein free filtrate containing lactic acid is added to 1.0 ml of 20% copper sulphate solution and 10.00 ml distilled water along with 1.0g of powdered calcium hydroxide.

The above test tubes are shaken thoroughly and allowed to stand at room temperature for 30 minutes with occasional shaking and finally centrifuged.

Step. Second (Acetaldehyde Formation):

Duplicate aliquot of 1.0 ml supernatant fluid is carefully withdrawn and transferred into two test tubes followed by addition of 0.05 ml of 4% copper sulphate solution to each test tube and placement of these tubes on a water bath

Now, 6.0 ml of AR-grade conc. sulphuric acid is poured to each test tubes gently followed by shaking. Finally these test tubes are placed on a boiling water bath for approximately 5 minutes and then allowed to cool at low 20°C.

Step. Third (Development of Purple Colour):

0.1 ml (two drops) of the p-hydroxydiphenyl solutions are now added to the contents of the tube from a pipette known to deliver 0.05ml of this solution per drop.

The precipitated reagents should be dispersed throughout the acid as quickly and uniformly possible. The shaking of the tubes should be lateral. The tubes are placed in a beaker of water at 30^{0}C and allowed to stand for at least 30 minutes.

The precipitated reagent should be dispersed by shaking at least once during the incubation period. Excess of reagents are dissolved by heating the tube in boiling water bath for 90 seconds followed by cooling in ice cold water.

Step. Fourth: (Colorimetric Measurement of Purple Colour Developed due to Lactic Acid)

Now, the intensity of the purple colour developed in the above test tubes (step third) are measured colorimetrically using a green filter with peak transmission at about 560 nm. For the initial (zero) setting of colorimeter a reagent blank (distilled water) is used.

2.16 Observation:

The klett readings for lactic acid determination formed during biosynthesis of lactic acid by Lactobacillus bulgaricus NCIM – 2359 are shown in the table-4 given below:

Table – 2.4

Sr. No.	Volume of lactate solution in ml	Lactate in (mg)	Volume of distilled water in (ml)	4% $CuSO_45H_2O$ solution (in ml)	Conc. H_2SO_4in (ml)	p-hydroxy diphenyl Solution in (ml)	Klett Readings*
1	0.0 ml	00 mg	1.0 ml	0.05 ml	6.0 ml	0.1 ml	00.00
2	0.1 ml	10 mg	0.9 ml	0.05 ml	6.0 ml	0.1 ml	20.00
3	0.2 ml	20 mg	0.8 ml	0.05 ml	6.0 ml	0.1 ml	40.00
4	0.3 ml	30 mg	0.7 ml	0.05 ml	6.0 ml	0.1 ml	60.00
5	0.4 ml	40 mg	1.6 ml	0.05 ml	6.0 ml	0.1 ml	80.00
6	0.5 ml	50 mg	0.5 ml	0.05 ml	6.0 ml	0.1ml	100.00

*Each value represents mean of three observations.
Experimental deviation +1.5 to 3.0%.

2.16.1 Calculation of the Results:

The average of the duplicate Klett readings just obtained in above observation is used to calculate the lactate contents of the aliquot by reference to the calibration curve relating colorimetric Klett readings and known concentration of standard lactic acid.

2.17 Estimation of the Molasses Left Unfermented:

All the carbohydrates (sugars) are converted into furfural by its dehydration in the presence of conc. H_2SO_4 Phenol when reacts with furfural gives a brown colored complex which is estimated colorimetrically.

2.17.1 Reagents:

For the photo colorimetric determination of molasses left unfermented after lactic acid fermentation is over the following reagents have been used.

a. **Sulphuric acid[35]:**
   Only AR-Grade conc. H_2SO_4 (specific gravity 1.84 and 95.5% pure) has been employed.

b. Phenol:

AR-Grade phenol of 80% by weight has been employed. The above phenol was prepared by adding 20g of glass distilled water to 80g of redistilled AR-Grade phenol.

2.17.2 Procedure:

The procedure for the estimation of molasses sugars left unfermented during lactic acid fermentation by Lactobacillus bulgaricus NCIM-2359 has been described as follows. 2.0 ml molasses solution left unfermented after lactic acid fermentation by Lactobacillus bulgaricus NCIM-2359 fermentation is over is pipetted into Klett tubes followed by addition of 0.5 ml AR-Grade 80% phenol to it. Now, 50 ml of conc. sulphuric acid is carefully; poured side by into the above Klett tubes containing unfermented molasses and phenol.

The above Klett tubes were allowed to stand for around 10 minutes and after that the Klett tubes were shaken thoroughly and allowed to stand for 10-15 minute in a water bath maintained at 25-30^{0}C and thus brown colour is developed. The intensity of the brown colour developed due to reaction of reagents with molasses is measured calorimetrically using a filter with peak transmission of about 540 nm For initial (zero) setting of colorimeter a reagent blank (distilled) water is used.

2.17.3 Calculation of the Results:

For the calculation of molasses sugars left unfermented after the lactic acid fermentation is over, the average of the duplicate colorimetric Klett readings thus obtained is employed to calculate the molasses sugars contents of the aliquot by reference to a calibration curve relating colorimetric readings.

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Chapter 3

Parametric Determination of Lactic Acid Fermentation by Lactobacillus Bulgaricus NCIM-2359

3.1 Introduction:

Organic acids, widely used in the food, pharmaceutical and chemical industries, are important chemicals. Fermentation technology for the production of organic acids in particular has been known for more than a century and acids have been produced in aqueous solutions. The development of lactic acid fermentation, with environmental impacts (production of polylactate biodegradable plastics) is welcome in our days. Many studies are focused on how to obtain pure lactic acid. Lactic acid fermentation has been gaining increased attention in the recent years primarily due to its importance as a building block in the manufacture of biodegradable plastics. Lactic acid can be produced from various substrates such as whey permeate, starch hydrolysates which are sources of lactose and glucose respectively. Lactic acid can be manufactured by either chemical synthesis or renewable carbohydrate fermentation. An optimization of parameter (or a decision variable, in the terms of optimization) is a model parameter to be optimized. During the optimization process, the parameter's value is changed in accordance to its type within an interval, specified by lower and upper bounds. The goal of the optimization process is to find the parameter values that result in a maximum or minimum of a function called the objective function Objective function is a mathematical expression describing a relationship of the optimization parameters or the result of an operation (such as simulation) that uses the optimization parameters as inputs. The optimization objective is the objective function plus optimization criterion. The latter determines whether the goal of the optimization is to minimize or maximize the value of the objective function. Parametric studies on production of lactic acid from molasses fermentation by Lactobacillus bulgaricus NCIM-2359 is virtually as important to the success of an industrial fermentation as is the selection of an organism to carry out the fermentation.

A poor selection of medium components can effect cellular growth and little if any yield of fermentation products.[1-11] The optimization of parameters like concentration of selected raw material, hydrogen ion concentration, temperature and incubation period of the fermentation medium can partially or fully influence the types and ratios of products from among those for which a microorganism has biosynthetic capability[12-15]. Thus, optimization of parameters for production of lactic acid from molasses fermentation by Lactobacillus bulgaricus NCIM-2359 is very important and critical. All organisms require source of energy for their metabolism. Some organisms can use reduced inorganic compounds as electron donors while other organisms use organic compounds as electron donor.

From this brief excursion into the nutritional requirement of bacteria, it is apparent that to grow bacteria successfully the laboratory worker must provide the proper and appropriate kind of medium and also an appropriate set of physical condition such as temperature, incubation period, and pH etc.

Thus, by understanding the various physico-chemical parameters controlling enzyme catalysed activities of different microbes, especially lactic acid bacteria Lactobacillus bulgaricus NCIM-2359, the biological activity may be increased, decreased, or destroyed partially or completely. Among the significant physico-chemical parameters for submerged fermentative production of lactic acid by Lactobacillus bulgaricus NCIM-2359 are the selection of substrate raw material and its percent dilution concentration, H^+ ion concentration (pH) of the medium, temperature and incubation period.

Indeed, enzymes are very sensitive to elevated temperatures and other fermentation parameters. Because of the protein nature of an enzyme thermal denaturation of the enzyme protein with increasing temperatures will decrease the effective concentration of an enzyme and consequently decrease the reaction rate. Thus, on increasing the temperature enzyme activity gradually increases, but at certain stages temperature inactivates the rate of reaction and finally enzyme is denatured (at high temperature inactivates the rate of reaction and finally enzyme is denatured (at high temperature) as it is proteinaceous in nature[16-19].

Generally speaking, there is an optimum relation between the concentrations of enzyme and substrate for maximum activity. But in order to study the effect of increasing enzyme concentration upon the reaction rate, the substrate concentration must be in excess.

This means that the reaction must be independent of the substrate concentration so that any variation in the amount of product formed is a function only of the enzyme concentration present. If the amount of enzyme is kept constant and the substrate concentration is then gradually increased, the velocity of reaction will increase until it reaches a maximum. Any further increase in substrate concentrations will not increase the reaction velocity. Each enzyme functions optimally at a particular pH and temperature.

There is a high chemical affinity of the substrate for certain areas of the enzyme surface called the active site. The active site on the enzyme surface is actually a very small area, which means the large regions of the enzyme protein (which has hundreds of amino acids) do not contribute to enzyme specificity or enzyme action. It should also be emphasized that the "fit" between an active site of the enzyme surface and the substrate is not a static one; rather it is dynamic interaction in which the substrate induces a structural change in the enzyme molecule, as a hand changes the shape of a glove. Among the conditions affecting the activity of enzymes[27-32] are the following:

- Concentration of enzymes
- Concentration of substrate
- pH and
- Temperature

It is obvious that the deviations from the optimal conditions result in significant reduction of enzyme activity. This is characteristic of all enzymes. Extreme variations in pH can even destroy, as can high temperatures; boiling for a few minutes will denature (destroy) most enzymes. Extremely low temperature for all practical purposes, stop enzyme activity but do not destroy the enzymes. Many enzymes can be preserved by holding them at temperatures around 0^0C or lower. Optimum conditions must be estimated in terms of what is based for the entire cell.

The activity of an enzyme can be inhibited[33-35] (slowed down or stopped) by chemical agents in several different ways. It has been reported that high sugar concentration is generally not used in the fermentation processes because the Calcium-lactate salt produced at higher sugar concentration tends to crystallize from the fermentation medium late in the fermentation process, thereby slowing down the rate of fermentation processes.[36-42]

Generally carbohydrates are utilized by bacterial cells mainly in the form of simple sugar glucose. Following have been among such raw materials employed for the various fermentation process to produce many valuable fermentative products with the help of selected strains of microorganisms, viz. Beet molasses[43] cane molasses[44-49] unrefined sugar sucrose[50] gur[51] cassava root and starch cake[52-55] guava Juice[56], potato-starch[57] tapioca[58] and whey[59–78] a resource for meeting energy demands. Singh et al.[79] and Singh[80-81] found that simple sugar glucose and fructose and some other sugars like maltose and glactose was better than the rhamnose, arabinose, xylose, lactose, mannitol and starch for the fermentative production of lactic acid using different strains of thermophlic lactic acid bacteria.

The production of lactic acid through fermentation from other sources like maize-sugar, have been described by Inskeep, Taylor and Brietzem82 in United of America while Michell[83] has given an account of production methods in Great Britain. On a continuous basis from whey, fermentative production of lactic acid has been described by Wittier and Rogers[84]. Leonard, Peterson and Johnson[85] used sulphite waste liquor for the production of lactic acid. Napierula[86] reported leg beet sugar as the most useful raw material for the production of lactic acid.

The sugar concentration in the fermenting broth solution is also a very important deciding factor for the production of lactic acid by submerged fermentation. The substrate (sugars) in mashes is normally ranged to a concentration of 5 to 30% depending upon the quality of substrate and formulation, composition of the submerged fermentation medium. Krumphanzal ET al[87] suggested sucrose medium of 5-25% for the fermentation process. Beek and Gross[88] employed only 2% molasses solution for varied microbial nutrition. A 10% sugar solution for lactic acid fermentation by L. delbrueckii has been suggested by Zagrodzki et al[89]. Encouraging yield of lactic acid using 13% glucose solution has been reported by Antonio[90]. Singh et al[91-93] also employed 5% sugar solution for the maximum production of lactic acid. The life activity and functioning of the microorganisms are influenced by H^+ ion concentration of the medium. It has been established that lactic acid fermentation favours the pH towards acid side of neutrality. Leonard etal[94] reported a pH value of 5.8 optimum for lactic acid production using sulphite waste liquor. A pH range of 5 to 6 has been reported for maximum production of lactic acid[95] by L. delbrueckii. A number of group workers[96-102] have also reported maximum yield of lactic acid employing different sugar sources and lactic acid bacteria at pH ranging between 5.0 to 6.5.

Thus from above literature it is obvious and may be concluded that lactic acid fermentation process approaches best when pH of the fermentation medium is maintained on the acid side of neutrality. Like hydrogen ion concentration, temperature also play an important and vital role for production of lactic acid by Lactobacillus bulgaricus NCIM-2359.

Temperature of the fermentation medium is the most important factor that effects the growth and metabolism of the microbe involved, Week[103] reported an optimum temperature of 38°C for lactic acid fermentation of whey using the lactic acid bacteria L. bulgaricus.

On the other hand Speck and Robert[104] observed a range 37°C-50°C temperature as most effective for growth and activity of some bacteria that is L. bulgaricus. Tiwari and co-workers[105-108] found 47°C, temperature as optimum temperature for production of lactic acid from different strain of lactic acid bacteria.

Again a number of group workers[109-112] have also suggested a temperature range between 45°C-50°C for maximum production of lactic acid using varied strains of lactic acid bacteria.

Like temperature, incubation period also play an important and vital role for the economic control and turnover of lactic acid formed by Lactobacillus bulgaricus NCIM-2359. Incubation period of fermentation medium is an important factor and also play an interesting and important role in the completion of microbial enzymatic reactions and consequently in the formation of desired metabolites.

An optimum incubation period of 7 days has been reported by Tiwari, Vyas[113] and Vratislav et al[114] for lactic acid fermentation by the strain L. delbrueckii S-25. A group of workers[115-143] have suggested the normal incubation period of lactic acid fermentation between 4 to 6 days.

Singh et al[144] studied different parameters for in vitro production of lactic acid by L. Casei NCIM - 2056 and found that it proceeds best when a 20% molasses solution is allowed to ferment at pH 6.3 and temperature 43^0C for 140 hours incubation period.Singh[145] optimized the parameters for submerged lactic acid fermentation by L. bulgaricus BS - 18 and found that lactic acid fermentation attains its best activity when a 10.5% surcorse solution is allowed to ferment for 5 days at 48^0C temperature by maintaining the pH value of fermentation medium to 6.2. Khursheed et al[146] obtained maximum lactic acid from enzymatic hydrolysis of molasses containing fermentable sugars equivalent to 12.5% (W/V) by Lactobacillus casei-21.

Some worker[147-199] reported fermentation process at temperature in between 30^0C to 48^0C pH 2.2 to 6.2 incubation period 2 days to 12 days and concentration of substrates in between 10 to 25%. Among the factors that affect growth and activity of Lactobacillus bulgaricus NCIM-2359 different raw material concentration of raw material pH temperature and incubalation period raw material concentration of raw material pH temperature and incubalation period is known to play and important role so far as production of lactic acid from molasses fermentation by Lactobacillus bulgaricus NCIM-2359 is concerned.

Keeping in view the wide difference in culture conditions of various strains of microbes and for fermentative lactic acid production, the author thought it necessary to monitor and investigate the optimization of parameters, that is; molasses substrate and its percent dilution H^+ ion concentration, temperature and incubation period for production of lactic acid by Lactobacillus bulgaricus NCIM-2359.

3.2 Experimental:

3.2.1 Medium:

The composition of the production medium for each fermentor flask containing 100 mL production medium is as below:

Molasses : 20%, Malt Extract: 0.60% $(NH_4)_2HPO_4$: 0.60%,

Yeast extract : 0.60%, $CaCO_3$: 8%, pH: 6.1

3.2.3 Culture Medium:

Glucose : 0.6%, Lactose: 0.6%

Sodium-Acetate : 500.00 mgs

Liver-Extract : 500.00 mgs

Peptone : 500.00 mgs Salt Solution A: 0.5ml

Salt solution B : 0.5ml, pH: 5.5-6.1

Sterilization : 15lbs for 30 minutes

Sub-culture : Once a month

Requisite amount of distilled water was added to make the total volume 100 ml.

3.2.4 Preparation of Salt Solution A:

It was prepared by mixing the following with water: KH_2PO_4: 25.00 g; K_2HPO_4: 25.00 g

Requisite amount of distilled water was added to make the volume 250 ml.

3.2.5 Preparation of Salt solution B:

It was prepared by mixing the following with water:

$MgSO_4 \cdot 7H_2O$: 10.00 g, NaCl: 500.00 mgs

$MnSO_4 \cdot 5H_2O$:500.00 mgs $FeSO_4 \cdot 7H_2O$:500.00 mgs

The above amount were dissolved in requisite amount of distilled water to make up the volume up to 250 ml.

The volume of the culture medium was taken in a dry and clean flask and was plugged with non-absorbent cotton pieces. About 12 clean and dry culture tubes were similarly plugged with non-absorbent cotton. These culture tubes and culture medium were sterilized in an autoclave at 1.71 Kg/I Cm^2 steam pressure for 30 minutes.

For solid growth medium 1.5 to 2.0 % agar-agar was added in this solution before sterilization. After cooling, 5.0 ml of the culture medium from the conical flask was transferred to each culture tube. The culture tubes were then allowed to stand in slant position overnight.

3.3 Sterilization:

The growth and production media were sterilized in an autoclave maintained at 15 lbs steam pressure for 30 min.

3.4 Strain:

Lactobacillus bulgaricus NCIM-2359 was used in the present study. The strain was procured from NCL, Pune, India.

3.5 Assay Methods:

Evaluation of lactic acid formed and molasses left unfermented was made colorimetrically[200-201]

3.6 Age of the Inoculum: 50 hours old.

Quantum of the inoculum: 0.5 ml bacterial suspension of Lactobacillus bulgaricus NCIM-2359.

Molasses Concentration: 2%, 4%, 6%, 8%, 10%, 15%, 20%*, 25%, 30% and 35%

Temperature (in^{0}C): 10, 20, 30, 32, 33, 35, 38*, 40, 45, 50, 52, 54, 56, 58 and 600C

Incubation period: 1, 2, 3, 6*, 9, 11, 13, 15, 16, 17, 18, 19, 20, 21and 22 days

pH: 5.2, 5.4, 5.8, 6.0, 6.1*, 6.3, 6.4, 6.5, 6.6, 6.7 and 6.8 and 6.9

Results and Discussion:

The results of colorimetric analysis are given in the Table 1-6. The value reported are mean of three trials in each case.

Table –3.1: Study of the Effect of Different Carbohydrates on Lactic Acid Fermentation by Lactobacillus Bulgaricus NCIM-2359

Sr. No.	Carbohydrates Substrates used	Yield of lactic acid* in g/100ml	Sugar left unfermented g/100ml
1	Arabinose	1.4186472	-
2	Rhamnose	0.3942169	-
3	Xylose	0.9067528	-
4	Glucose	8.8170593	-
5	Fructose	6.9360413	-
6	Galactose	4.9817162	-
7	Sorbose	0.5531321	-

Sr. No.	Carbohydrates Substrates used	Yield of lactic acid* in g/100ml	Sugar left unfermented g/100ml
8	Lactose	4.9986254	-
9	Sucrose	7.9830156	-
10	Maltose	2.2162190	-
11	Starch	0.2968514	-
12	Inuline	0.4878350	-
13	Dextrine	0.44113977	-
14	Raffinose	2.2016188	-
15	Mannitol	1.5968274	-
16	Molasses**	7.9481130	-
	20% (w/v)		

- Each value represents mean of three observation; (1-7) monosaccharides; (8-10)-disaccharides; (11-14) poly saccarides; (15)- Polyalcohol; S. No. (16) Molasses contains approximately 52% fermentable sugars.
- Molasses was employed as raw material due to economic cost.

Table -3.2: Study of the Effect of Concentration of Molasses on Lactic Acid Fermentation by Lactobacillus Bulgaricus NCIM-2359 in 6 Days of Incubation Period at pH 6.1 and Temperature 38^{0}C

Sr. No.	% Concentration of molasses (in g) (W/V)	Yield of lactic acid* in g/100ml	Sugar left* unfermented in g/100ml
1	2%	0.710952	0.1281695
2	4%	1.4160386	0.3068953
3	6%	2.131487	0.6597280
4	8%	2.8861365	1.3097563
5	10%	3.6501386	1.5986204
6	15%	5.6254136	1.9835912
7	20% **	7.8926957***	2.1065714
8	25%	8.7430267	3.6958362
9	30%	10.0811367	4.5678069
10	35%	****	-

* Each value represents mean of three observations.
** Optimum concentration of molasses
*** Optimum yield of lactic acid
**** Insignificant value

Table -3.3: Study of the Effect of Different pH on Lactic Acid Fermentation by Lactobacillus Bulgaricus NCIM-2359 from Molasses (20%) in 6 Days of Incubation Period at Temperature 38^{0}C

Sr. No. of Sets of Flasks	pH	Yield of Lactic Acid* in g/100 ml.	Molasses Left Unfermented* in g/100 ml.
1	5.2	4.5498263	5.4491737
2	5.4	4.8459011	5.1439873
3	5.8	6.1819757	3.8079245
4	6.0	7.3841362	2.6086387
5	6.1**	8.2336951***	1.7574215
6	6.3	7.1469590	2.3543298
7	6.4	6.9036182	2.3326210
8	6.5	5.4536518	2.3081422
9	6.6	****	—
10	6.7	****	—
11	6.8	****	—
12	6.9	****	—

* Each value represents mean of three observations.
** Optimum pH value
** Optimum yield of lactic acid.
**** Insignificant yield of lactic acid.

Table – 3.4: Study of the Effect of Different Temperature on Lactic Acid Fermentation by Lactobacillus Bulgaricus NCIM-2359 from (Molasses 20%) in 6 Days of Incubation Period at pH 6.1

Sr. No. of sets of flasks	pH	Yield of lactic acid* in g/100ml	Molasses left unfermented* in g/100 ml.
1	10	2.1563112	7.8136814
2	20	3.6569310	6.3271480
3	30	4.9018976	5.0761138
4	32	5.4591301	4.5411395
5	33	5.7968562	4.1985795
6	35	6.9828950	3.0148138
7	38**	8.1864325***	1.7932015
8	40	7.9535975	1.7803952
9	45	7.6536310	1.7786106
10	50	****	-
11	52	****	-

Sr. No. of sets of flasks	pH	Yield of lactic acid* in g/100ml	Molasses left unfermented* in g/100 ml.
12	54	****	-
13	56	****	-
14	58	****	-
15	60	****	-

* Each value represents mean of three observations.
** Optimum temperature
*** Optimum yield of lactic acid.
**** Insignificant yield of lactic acid.

Table -3.5: Study of the Effect of Different Incubation Period on Lactic Acid Fermentation by Lactobacillus Bulgaricus NCIM-2359 from (Molasses 20%) at pH 6.1

Sr. No. of Sets of Flasks	pH	Yield of Lactic Acid* in g/100ml	Molasses Left Unfermented* in g/100 Ml.
1	1	1.1563952	8.8170391
2	2	2.9513263	7.0431958
3	3	5.8241302	4.1708123
4	6**	7.9126123***	2.0861150
5	9	7.45711630	2.5338131
6	11	6.2535759	3.7456241
7	13	****	-
8	15	****	-
9	16	****	-
10	17	****	-
11	18	****	-
12	19	****	-
13	20	****	-
14	21	****	-
15	22	****	-

* Each value represents mean of three observations.
** Optimum Incubation period
*** Optimum yield of lactic acid.
**** Insignificant yield of lactic acid.

3.7 Discussion:

The data recorded in the **Table-3.1** shows the study of effect of different carbohydrate material on production of lactic acid by Lactobacillus bulgaricus NCIM-2359.

From the results it is clear that sugar substrate becomes very smooth and easy as the molecular size and structure configuration of the carbohydrate substrate molecules becomes simple. The monosaccharides, especially glucose and fructose sugars both have been found much fermentable amongst monosaccharides due to the presence of active carbonyl group being common in glucose and fructose (aldehydic and ketonic groups) are easily phosphorelated due to energy conversation of living cells which is fundamental properties of microbes.

Galactose exists in both open-chain and cyclic form. The open-chain form has a carbonyl at the end of the chain. Four isomers are cyclic, two of them with a pyranose (six-membered) ring, two with a furanose (five-membered) ring.

Galactofuranose occurs in bacteria, fungi and protozoa.[192] Galactose is a monosaccharide. When combined with glucose (monosacccharide), through a dehydration reaction, the result is the disaccharide lactose. The hydrolysis of lactose to glucose and galactose is catalyzed by the enzymes lactase and ß-galactosidase.

Lactose is found primarily in milk and milk products. Galactose metabolism, which converts galactose into glucose, is carried out by the three principal enzymes in a mechanism known as the Leloir pathway. In the human body, glucose is changed into galactose via hexoneogenesis to enable the mammary glands to secrete lactose. However, most galactose in breast milk is synthesized from galactose taken up from the blood, and only 35±6% is made by de novo synthesis.[193] Glycerol also contributes some to the mammary galactose production.[194]

Galactose is a simple sugar, without a doubt. However, it is also of considerable importance to the human organism, more than one would expect of a "simple" sugar. Galactose is close to glucose and differs in one hydrogen and one hydroxyl group at position C–4. The degree of ferment ability of galactose is very much near to glucose and fructose. Arabinose is a five-carbon monosaccharide that naturally occurs as a constituent in various plant and bacterial polysaccharides Arabinose occurs in Dextro- and Levo- configurations. The L-arabinose is abundant in nature and can be obtained from hemicelluloses and gums in many plants. Synthetically, it is produced to be used mainly as culture medium for certain bacteria. Rhamnose is a crystalline sugar $C_6H_{12}O_5$ that occurs usually in the form of a glycoside in many plants and is obtained in the common dextrorotatory L form. Sorbose is a ketose belonging to the group of sugars known as monosaccharides. It has a sweetness that is equivalent to sucrose. The commercial production of vitamin C (ascorbic acid) often begins with sorbose. L-Sorbose is the configuration of the naturally occurring sugar.

The fermentation value of arabinose, rhamnose, and Sorbose sugars were almost in traces. Living cells produce useful currency of energy-ATP, which is regarded as the cells's energy currency. Microbes have the property of maintaining a stock of ATP, which is possible due to consumption of sugars like glucose and fructose. The world produced about 168 million tonnes of table sugar in 2011.[195]

Sucrose is a molecule with five stereo centers and many sites that are reactive or can be reactive. The molecule exists as a single isomer. In sucrose, the components glucose and fructose are linked via an ether bond between C1 on the glucosyl subunit and C_2 on the fructosyl unit. The bond is called a glycosidic linkage. Glucose exists predominantly as two isomeric "pyranoses" (α and β), but only one of these forms the links to the fructose.

Fructose itself exists as a mixture of "furanoses", each of which having α and β isomers, but only one particular isomer links to the glucosyl unit. What is notable about sucrose is that, unlike most disaccharides, the glycosidic bond is formed between the reducing ends of both glucose and fructose, and not between the reducing end of one and the nonreducing end of the other.

This linkage inhibits further bonding to other saccharide units. Since it contains no anomeric hydroxyl groups, it is classified as a nonreducing sugar. Sucrose is the organic compound commonly known as table sugar and sometimes called saccharose. A white, odorless, crystalline powder with a sweet taste, it is best known for its role in human nutrition. The molecule is a disaccharide composed of glucose and fructose with the molecular formula $C_{12}H_{22}O_{11}$.

Sucrose is the main component of sugarcane juice and each molecule of sucrose consists of one glucose molecule attached to one molecule of fructose. The first step of microbe’s activity is to break a part the glucose and fructose units which enter the energy metabolisation machinery to provide energy. If microbes grows in oxygenated medium, the sugar will be broken down step-by-step, into smaller and smaller molecules and at the end simple invert sugars are liberated. In the present investigation amongst disaccharides sucrose has been observed only useful and most suitable substrate for its maximum conversion into the lactic acid. Lactose is hydrolysed to glucose and galactose, isomerised in alkaline solution to lactulose, and catalytically hydrogenated to the corresponding polyhydric alcohol, lactitol.[196]

Lactose is a disaccharide sugar that is found most notably in milk and is formed from galactose and glucose. Lactose makes up around 2.8% of milk (by weight), although the amount varies among species and individuals. It is extracted from sweet or sour whey. The name comes from lac or lactis, the Latin word for milk, plus the -ose ending used to name sugars. It has a formula of $C_{12}H_{22}O_{11}$. Lactose did not give significant yield of lactic acid during production of lactic acid by Lactobacillus bulgaricus NCIM-2359.

Mannitol has also been found much less fermentable to produce lactic acid. In case of polysaccharides, starch, inulin and dextrin were found very much unsuitable and undesirable for production of lactic acid by Lactobacillus bulgaricus NCIM-2359. The microorganisms associated with fermentation utilize various metabolic processes, depending on the substrate. Thus, it may be concluded that glucose amongst the substrate monosaccharides and sucrose amongst disaccharide are of having high ferment ability values and are most useful for the production of lactic acid by Lactobacillus bulgaricus NCIM-2359.

Molasses is a viscous by-product of the beating of sugarcane, grapes or sugar beets into sugar. The word molasses comes from the Portuguese word melaço, which ultimately comes from Mel, the Latin word for "honey".

The quality of molasses depends on the maturity of the sugarcane or sugar beet, the amount of sugar extracted, and the method of extraction. Sweet sorghum is known in some parts of the United States as molasses, though it is not considered true molasses.

In Nepal it is called chaku and is used in the preparation of various Newari condiments like the yomari. It is also a popular ingredient in 'ghya-chaku'.

Molasses

Molasses is a thick, brown to deep black, honey-like substance made when cane or beet sugar is processed. It is enjoyed as a sweetener in many countries, and most particularly in England where it is called treacle. For hundreds of years, molasses and sulfur, or treacle and brimstone were thought to have healthful benefits, and children were frequently given doses of the product. Molasses had a somewhat unsavory history during Prohibition in the US. It is the primary base for the manufacture of rum. Molasses importation became synonymous with the bootlegging industry and with organized crime. Today, uses for molasses are quite benign. It is used primarily in baking. No gingerbread would be quite the same without the addition of molasses. Some people enjoy using it on hot cereals like cream of wheat or cornmeal mush.

Molasses is also a necessary ingredient in the Thanksgiving holiday traditional pumpkin pie. In England, treacle tart, is not, however, made with molasses, but it is enjoyed as a sweetener on porridge. Homemade caramel corn is especially good with a dollop of molasses added to the sugar mixture. Molasses has somewhat more nutritional value than does white or brown sugar. The process by which it is extracted and treated with sulfur results in fortification of iron, calcium and magnesium. Some natural health food experts still advocate its use for ailments of the stomach. Concerns about sulfur, however, have led to many brands of molasses that are sulfur-free. These are widely available in both natural food and chain grocery stores. Calories in molasses are approximately the same as sugar, about 16 calories per teaspoon (5 ml). However it only contains about half the sucrose as sugar. It is also made up of both glucose and fructose. Though it is high in iron, it is also high in calcium, which tends to prevent iron from being absorbed by the body. Thus its benefits as a mineral supplement may be a bit overrated. Molasses is a viscous by-product of the processing of sugar cane, grapes or sugar beets into sugar. The word molasses comes from the Portuguese word melaço, which is a superlative from Mel, the Latin (and Portuguese) word for “honey”. The quality of molasses depends on the maturity of the sugar cane or sugar beet, the amount of sugar extracted, and the method of extraction. Sweet sorghum syrup is known in some parts of the United States as molasses, though it is not true molasses. The molasses can also be utilized in lactic acid production. Molasses are the by – products of sugarcane processing and consists of 52% total sugars calculate as sucrose (30 percent sucrose, and 22% invert sugars) and is also known as black-strap molasses. When this molasses is used as a fermentation medium component, it is considered to contain approximately 50% fermentable sugars.

Since molasses is rich in sugar contents and economical, it has been selected as a carbohydrate source during the course of present investigation, i.e., production of lactic acid by Lactobacillus bulgaricus NCIM-2359.

The data recorded in T**able-3.2** and 6 shows the effect of different concentration of sugary raw material, i.e., molasses substrate. The best result has been noted when 20% (w/v) molasses substrate solution has been allowed for production of lactic acid by Lactobacillus bulgaricus NCIM-2359. It has been found that lower concentration of molasses did not give significant yield of lactic acid while higher molasses solution has been found to interfere with the bacterial enzyme activity and thereby retards the production of lactic acid by Lactobacillus bulgaricus NCIM-2359.

The data recorded in **Table-3.3** and 6 shows the influence of different pH on fermentative production of lactic acid by Lactobacillus bulgaricus NCIM-2359. Acidic and basic are two extremes that describe a chemical property. Mixing acids and bases can cancel out or neutralize their extreme effects. A substance that is neither acidic nor basic is neutral. This is a rough measure the acidity of a solution. The "p" stands for "potenz" (this means the potential to be) and the "H" stands for Hydrogen.

The pH scale measures how acidic or basic a substance is. The pH scale ranges from 0 to 14. A pH of 7 is neutral. A pH less than 7 is acidic. A pH greater than 7 is basic. The pH scale is logarithmic and as a result, each whole pH value below 7 is ten times more acidic than the next higher value. For example, pH 4 is ten times more acidic than pH 5 and 100 times (10 times 10) more acidic than pH 6.0 The same holds true for pH values above 7, each of which is ten times more alkaline (another way to say basic) than the next lower whole value. For example, pH 10 is ten times more alkaline than pH 9 and 100 times (10 times 10) more alkaline than pH 8. Pure water is neutral. But when chemicals are mixed with water, the mixture can become either acidic or basic. Examples of acidic substances are vinegar and lemon juice. Lye, milk of magnesia, and ammonia are examples of basic substances.

It has been found that production of lactic acid increases with the increase of pH towards acidic side of neutrality. The fermentative production of lactic acid is inhibited in the strong acidic medium solution. It has been found that on advancing the pH values from 5.2 to 6.1, lactic acid production also advances, i.e., 4.5498263g/100 ml to 8.2336951g/100 ml and further increase of pH from 6.3 and onwards the fermentative production of lactic acid by Lactobacillus bulgaricus NCIM-2359 decreases. It was thus concluded that production of lactic acid by Lactobacillus bulgaricus NCIM-2359 does not proceed smoothly in strong acidic as well as neutral pH medium. The optimum pH of the fermentation was thus found to be 6.1 which was most suitable for production of lactic acid by Lactobacillus bulgaricus NCIM-2359 and thus all the experiment conducted by the author for production of lactic acid by Lactobacillus bulgaricus NCIM-2359 has been maintained at optimum pH value of 6.1.

The data recorded in **Table3.4** and 6 shows the influence of different temperature on production of lactic acid by Lactobacillus bulgaricus NCIM-2359. Temperature changes have profound effects upon living things. Enzyme-catalyzed reactions are especially sensitive to small changes in temperature. It has been found that production of lactic acid increases with increase of temperature from 10°C to 38°C. At lower temperatures, i.e., 10^0C, 20^0C and 30^0C the yield of lactic acid was found to be discourasing.

While the yield of lactic acid gradually falls with the rise of temperature, i.e., 40^0C and onwards. The 38^0C temperature has been found most significant, suitable, and effective for maximum production of lactic acid, i.e., 8.1864325 g/100ml by Lactobacillus bulgaricus NCIM-2359 and therefore, this temperature, i.e., 38°C was selected and maintained throughout the experiments described in the thesis.

The data recorded in the **Table-3.5** & 6 reveals the influence of different incubation period on production of lactic acid by Lactobacillus bulgaricus NCIM-2359. It has been found that conversion of molasses to lactic acid increases with the increase in incubation period from 1 to 6 days and then normally falls.

It was also found that usually consumption of molasses corresponded with the yield of lactic acid and in 6 days, 7.9126123g/100 ml of lactic acid has been obtained. No further increase in the yield of lactic acid has been observed with the further increase in the incubation period.

It may, therefore, be concluded that production of lactic acid by Lactobacillus bulgaricus NCIM-2359 proceeds best when a molasses solution of 20% (W/V) is allowed to ferment for 6 days of incubation period at 38°C temperature by maintaining the pH values of fermenting medium at 6.1 along with other bio ingredients supplements required by Lactobacillus bulgaricus NCIM-2359.

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Chapter 4

Lactic Acid Fermentation by Lactobacillus Bulgaricus NCIM- 2359 Exposed to Some Active Organic Molecules

4.1 Introduction:

Organic molecules are the chemicals of life, compounds composed of more than one type of element that are found in, and produced by, living organisms. The feature that distinguishes an organic from inorganic molecule is that organic contain carbon-hydrogen bonds, whereas inorganic molecules do not. The four major classes of organic molecules include carbohydrates, proteins, lipids and nucleic acids. It has been found that a few physiologically and pharmacologically active organic molecules are very active and play biological properties of vital importance in the biosynthesis of some useful micro and macro organic molecules. Though biologically active organic molecules are not essentially growth promoter for some or all microbes yet a few organic molecules are utilized by some or all microbes for their nutritional requirements[1-17].

A biologically active compound is defined as one that has a direct physiological effect on a plant, animal, or another microorganism. Many known compounds with biological activity are found only in trace amounts in soil. Research has shown that there are essential, highly active organic molecules that can, even in extremely small quantities, vastly influence the fermentative actions and interactions. A number of organic molecules and their derivatives are well known to show physiological and pharmacological property.

Information regarding their role in biological system is very much limited and still unsettled. Although a group of workers have tried to explore the effect of some organic molecules and their derivatives on microbial enzymes systems, yet there is no definite opinion regarding its influence on submerged fermentation processes.

There are large group of some organic molecules which when introduced to the submerged fermentation medium can affect the enzyme responsible for the biosynthesis of micro and macro molecules in the microbial cells as well bioconversion of raw substrate into desired products and such organic compounds may be referred to as physiologically active organic compounds. A group of workers[18-26] have studied some organic compounds having barbiturate nucleus in their structure and found them most significant and effective for different industrial fermentation processes.

Zahid and Baxter[27] also reported barbitone as a growth promoter and enzyme stimulant for many biological fermentative enzymes induction[28-29]. Elizabeth[30] studied about glycolates and reported it stimulant for growth of L. gibba Singh et al[31] found fumaric acid stimulant for L. delbrueckii 9646. The stimulant action of fumaric acid were also supported by Krasil[32] and Kanji ET al[33]. On the other hand Pilone et al[34] reported inhibitory action of fumaric acid.

Tandon and Mishra[35-36] studied on different organic compounds for N. agilis and noted that up to a certain concentrations of citric acid and furmaric acid rate of nitrate formation was significantly enhanced.

Friend[37] has reported stimulating effect of some physiologically active organic compounds on citric acid fermentation process[38-39].

Tiwari[40-41] worked on nitro groups containing organic compound and reported that compounds having nitro groups in aromatic ring are very much toxic and inhibitory for various enzymatic systems for microbes. Singh[42-43] in his investigation also supported the fact that nitro groups present in aromatic compounds retard different fermentation processes. Singh et al[44] also found that xanthine creatinine and creatine has been stimulant for alcoholic fermentation.

Henis et al[45] observed that a slight variation in fat composition of L. delbruecki is stimulated by the addition of fatty acids. L. acidophillus also needs malvalic acid[46-49] having highly strained cyclopropene ring as a growth factor. Davis[50] reported p-hydroxybenzoic acid useful for E. coli Oleic acid requirements by lactic acid bacteria has been reported by a number of workers[51-53].

Many questions are still open concerning the mode of action of these physiologically and pharmacologically active organic molecules on the enzyme system involved in the pathways leading to the biogenesis of the rifomycin antibiotics[54-69]. Whatever their function may be these compounds should be incorporated into class of secondary factors in the fermentations.[70-72]

Secondary factors, which must be still gathered under this rather unfortunate heading, are those that presumably are contained in various raw materials employed widely in the fermentation industry.

The only thing we know about them is that they very favourably affect certain process as view from the industrial yield obtained. In this respect they have much in common with the non-identifiable growth factors from nutrition studies[73-74].

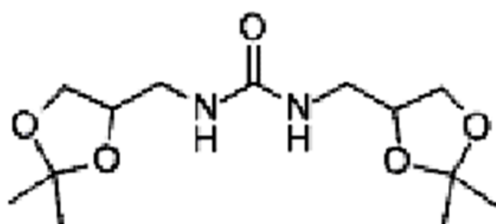
Singh et al.[75] further studied some other physiologically active compounds and found that 2, 6 dihydroxy purine enhances fermentative production of lactic acid.

Singh et al[76] also reported ortho and meta amino benzoic acid stimulant for lactic acid fermentation.

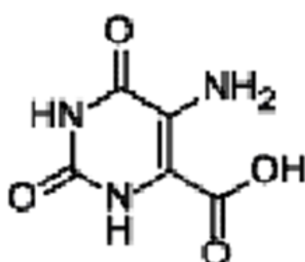
The influence of aniline, acetanilide, and toluene and phenyl acetic acid has been studied on lactic acid fermentation and has been found that acetanilide and phenyl acetic acid enhances the production of lactic acid. Shamim[77] observed that 5, 5-dimethyl hydantoine and 1, 3 diethyl 2-thiobarbituric acid enhances the production of lactic acid. As a result of such interesting and conflicting observations it is obvious that much work has been done on different active organic molecule of importance and their requirements by different bacteria, fungi and yeasts but no substantiate work has been done on lactic acid fermentation by Lactobacillus bulgaricus NCIM-2359 exposed to some active organic molecule. An attempts, therefore, has been made to study the influence of different active organic molecule on lactic acid fermentation by Lactobacillus bulgaricus NCIM-2359.

These includes the following:

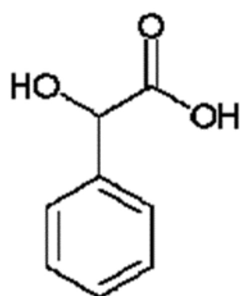
a. 1, 3-Bis [(2, 2-Dimethyl-1, 3-Dioxolan-4-Yl) Methyl] Urea:



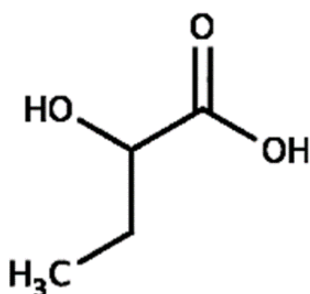
b. 5-Aminoaorotic Acid:



c. Mandelic Acid:



d. 2-Hydroxybutyric Acid:



4.2 Experimental:

The influence of 1, 3-bis [(2, 2-dimethyl-1, 3-dioxolan-4-yl) methyl] urea on lactic acid fermentation by Lactobacillus bulgaricus NCIM-2359

The composition of the production medium for the production of lactic acid by Lactobacillus bulgaricus NCIM-2359 was prepared as follows:

Molasses	:	20% (w/v)
Malt Extract	:	0.60 %
Yeast Extract	:	0.60 %
Peptone	:	0.60%
$(NH_4)_2HPO_4$	:	0.60%
$CaCO_3$	:	8.0%
pH	:	6.1
Distilled water	:	To make up 100 ml.

The pH of the medium was adjusted to 6.1 by adding requisite amount of phosphate-buffer solution, and the pH was also ascertained by a pH meter. The above composition medium represents volume of a fermentor flask, i. e., "100ml" production medium for lactic acid fermentation. Now, the same production medium for lactic acid fermentation by Lactobacillus bulgaricus NCIM-2359 was prepared for 99 fermentor flasks, i. e., each fermentor flask containg '100 ml' of production medium.

The above fermentor flasks were then arranged in ten sets, each comprising 9 fermentor flask. Each set was again rearranged in three subsets, each comprising of 3 fermentor flasks. The remaining nine fermentor flasks out of 99 fermentor flasks were kept as control and these were also rearranged in three subsets each consisting of three fermentor flasks.

Now M/1000 solution/suspension of 1,3-bis[(2,2-dimethyl-1,3-dioxolan-4-yl) methyl]urea was prepared and 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0 ml of this solution was added to the fermentor flasks of 1st to 10th sets respectively. The control fermentor flasks containing no active organic molecule. Now the total volume in each fermentor flask were made up to 100ml by adding requisite amount of distil water. Thus, the concentration of 1,3-bis[(2,2-dimethyl-1,3-dioxolan-4-yl) methyl]urea in 1st, 2nd, 3rd, 4th, 5th, 6th, 7th, 8th, 9th and 10th subsets were approximately as given below:

	$A \times 10^{-x}M$	$6.0 \times 10^{-5}M$	
i. e.,	$1.0 \times 10^{-5}M$	$7.0 \times 10^{-5}M$	Where A = amount of

2.0 x 10^{-5}M	8.0 x10^{-5}M	active organic molecule in ml,
3.0 x 10^{-5}M	9.0 x 10^{-5}M	i.e., 1.0 ml to 10ml.
4.0 x 10^{-5}M	10.0 x 10^{-5}M	x = molarity of the
5.0 x 10^{-5}M		solution containing AOM

The fermentor flasks were then sterilized, cooled, inoculated, incubated and analysed after 3, 6 and 9 days for lactic acid formed and molasses sugars left unfermented as described in the experimental portion, i. e., chapter II of this thesis. The experimental procedure for the study of influence of other active organic molecules were exactly the same as described above with the only difference that in place of M/1000 solution of 1,3-bis[(2,2-dimethyl-1,3-dioxolan-4-yl) methyl]urea other active organic molecule under trials were added to the lactic acid fermentation medium respectively.

4.3 Results and Discussion:

The results obtained in the study of the influence of some active organic molecules on lactic acid fermentation by Lactobacillus bulgaricus NCIM-2359 are tabulated in the tables from 1 to 4.

Table -4.1: Lactic Acid Fermentation by Lactobacillus Bulgaricus NCIM-2359 Exposed to 1, 3-Bis [(2, 2-Dimethyl-1, 3-Dioxolan-4-Yl) Methyl] Urea

Concentration of AOM Used A x 10^{-X} M	Incubation Period in Hours	Yield of Lactic Acid* in g/100 ml	Molasses Substrate* Left Unfermented in g/100 ml	% of Lactic Acid Increase in 3, 6, 9 Days of Incubation Pd.
Control (–AOM)	3	5.8367560	4.1632443	—
	6	7.9115967	2.1864033	—
	9	7.4181653	2.0173518	—
1.0 x 10^{-5} M (+ AOM)	3	5.8993879	4.1006129	(+) 1.07306010
	6	8.0457850	2.1453130	(+) 1.6960963
	9	7.4948316	1.9985373	(+)1.0334940
2.0 x 10^{-5} M (+ AOM)	3	5.9139596	4.0861370	(+) 1.3227141
	6	8.1356740	2.1341618	(+) 2.8322639
	9	7.5623459	1.9761815	(+) 1.9437390
3.0 x 10^{-5} M (+ AOM)	3	3.0426261	3.9573812	(+) 3.5271321
	6	8.3675937	2.1251719	(+) 5.1636532
	9	7.7419296	1.95303907	(+) 4.3644794
4.0 x 10^{-5} M (+AOM)	3	6.1169896	3.8831573	(+) 4.8011875
	6	8.5050567	2.1031316	(+) 7.5011406
	9	7.8752418	1.9374262	(+) 6.16158418

Concentration of AOM Used A x 10^{-X} M	Incubation Period in Hours	Yield of Lactic Acid* in g/100 ml	Molasses Substrate* Left Unfermented in g/100 ml	% of Lactic Acid Increase in 3, 6, 9 Days of Incubation Pd.
5.0 x 10^{-5} M (+AOM)	3	6.1817193	3.8182413	(+) 5.9101888
	6	8.6078985	2.0927563	(+) 8.8010274
	9	7.9530261	1.9261819	(+) 7.2101493
Control (– AOM)	3	5.8367560	4.1632443	—
	6	7.9115967	2.1864033	—
	9	7.4181653	2.0173518	—
6.0 x 10^{-5} M** (+ AOM)	3	6.3276381	3.6723610	(+) 8.4101870
	6	8.8380566***	2.0031182	(+) 11.7101507
	9	8.1674852	1.8931375	(+) 10.1011485
7.0 x 10^{-5} M (+ AOM)	3	6.2634339	3.8516542	(+) 7.3101890
	6	8.7193818	2.1137063	(+) 10.2101400
	9	8.0420441	1.9141853	(+) 8.4101496

Table – 4.1.1: Lactic Acid Fermentation by Lactobacillus Bulgaricus NCIM-2359 Exposed to 1, 3-Bis [(2, 2-Dimethyl-1, 3-Dioxolan-4-Yl) Methyl] Urea

Concentration of AOM Used A x 10^{-X} M	Incubation Period in Hours	Yield of Lactic Acid* In g/100 ml	Molasses Substrate* Left Unfermented in g/100 ml	% of Lactic Acid Increase In 3, 6, 9 Days of Incubation Pd.
8.0 x 10^{-5} M (+ AOM)	3	6.1466988	3.8971656	(+) 5.3101894
	6	8.5540293	2.1573642	(+) 8.1201383
	9	7.8936897	1.9474253	(+) 6.4102696
9.0 x 10^{-5} M (+ AOM)	3	****	–	–
	6	****	–	–
	9	****	–	–
10.0 x 10^{-5} M (+AOM)	3	****	–	–
	6	****	–	–
	9	****	–	–

* Each value represents mean of three trials.
** Optimum concentration of AOM.
*** Optimum yield of lactic acid
**** Insignificant value

(+) Values indicate % increases in the yield of lactic acid

Experimental deviation + 2.5 – 3.5%

Table – 4.2: Lactic Acid Fermentation by Lactobacillus Bulgaricus NCIM-2359 Exposed to 5-Aminoaorotic Acid

Concentration of AOM Used A x 10^{-X} M	Incubation Period in Hours	Yield of Lactic Acid* in g/100 ml	Molasses Substrate* Left Unfermented in g/100 ml	% of Lactic Acid Increase In 3, 6, 9 Days of Incubation Pd.
Control (–AOM)	3	5.8545703	4.1463012	—
	6	7.9347015	2.0658130	—
	9	7.4368268	2.0057311	—
1.0 x 10^{-5} M (+AOM)	3	5.8786842	4.1221869	+ 0.4118816
	6	7.9921379	2.0627129	+0.7238633
	9	7.4758560	2.0046638	+ 0.5248098
2.0 x 10^{-5} M (+AOM)	3	5.8898986	4.1036368	+ 0.6034311
	6	8.0389525	2.0605697	+1.3138616
	9	7.5048593	2.0039879	+ 0.9148054
3.0 x 10^{-5} M (+ AOM)	3	5.9025069	4.0813694	+ 0.8187893
	6	8.0855709	2.0583167	+ 1.9013872
	9	7.5427875	2.0031610	+1.4248106
4.0 x 10^{-5}M (+AOM)	3	5.9078579	4.0798137	+ 0.9101880
	6	8.1497429***	2.0517160	+ 2.7101384
	9	7.5941205	2.0029320	+2.1150647
5.0 x 10^{-5}M (+AOM)	3	5.8855290	4.0829157	+ 0.5287954
	6	8.1269378	2.0598762	+ 2.4227288
	9	7.5643538	2.0032613	+ 1.7148039
Control (– AOM)	3	5.8545703	4.1463012	—
	6	7.9347015	2.0658130	—
	9	7.4368268	2.0057311	—
6.0 x 10^{-5} M** (+ AOM)	3	5.8732925	4.0886931	+ 0.3197877
	6	8.0317231	2.0627010	+1.2227504
	9	7.4825476	2.0036813	+0.6147890
7.0 x 10^{-5} M (+ AOM)	3	5.8615250	4.0923000	+ 0.1187909
	6	7.9986892	2.0696588	+ 0.8064285
	9	7.4749112	2.0039982	+0.5121055

Table –4.2.1: Lactic Acid Fermentation by Lactobacillus Bulgaricus NCIM-2359 Exposed to Mandelic Acid

Concentration of AOM Used A x 10^{-X} M	Incubation Period in Hours	Yield of Lactic Acid* in g/100 ml	Molasses Substrate* Left Unfermented in g/100 ml	% of Lactic Acid Increase in 3, 6, 9 Days of Incubation Pd.
8.0 x 10^{-5} M (+ AOM)	3	****	–	–
	6	****	–	–
	9	****	–	–
9.0 x 10^{-5} M (+ AOM)	3	****	–	–
	6	****	–	–
	9	****	–	–
10.0 x 10^{-5} M (+AOM)	3	****	–	–
	6	****	–	–
	9	****	–	–

* Each value represents mean of three trials.
** Optimum concentration of AOM.
*** Optimum yield of lactic acid
**** Insignificant value

(+) Values indicate % increases in the yield of lactic acid

Experimental deviation + 2.5 – 3.5%

Table -4.3: Lactic Acid Fermentation by Lactobacillus Bulgaricus NCIM-2359 Exposed to 2-Hydroxybutyric Acid

Concentration of AOM Used A x 10^{-X} M	Incubation Period in Hours	Yield of Lactic Acid* in g/100 ml	Molasses Substrate* Left Unfermented in g/100 ml	% of Lactic Acid Increase in 3, 6, 9 Days of Incubation Pd.
Control (–AOM)	3	5.7946637	4.1953363	—
	6	7.8869820	2.1053769	—
	9	7.3860495	2.0148635	—
1.0 x 10^{-5} M (+ AOM)	3	5.8235269	4.1664833	+ 0.4980996
	6	7.9501178	2.0864224	+ 0.8005064
	9	7.4312145	2.0125139	+ 0.6114906
2.0 x 10^{-5} M (+ AOM)	3	5.8311225	4.1593015	+ 0.6291788
	6	7.9748176	2.0837150	+1.1136782
	9	7.4459868	2.0104131	+ 0.8114933
3.0 x 10^{-5} M (+ AOM)	3	5.8478256	4.1381632	+ 0.9174285
	6	7.9826562	2.0816182	+ 1.2130647
	9	7.4617486	2.0093131	+ 1.0248929

Concentration of AOM Used A x 10^{-X} M	Incubation Period in Hours	Yield of Lactic Acid* in g/100 ml	Molasses Substrate* Left Unfermented in g/100 ml	% of Lactic Acid Increase in 3, 6, 9 Days of Incubation Pd.
4.0 x 10^{-5} M (+AOM)	3	5.8496320	4.1071618	+ 0.9486020
	6	8.0062844***	2.0785357	+ 1.5126495
	9	7.4698735	2.0082416	+ 1.1348962
5.0 x 10^{-5} M (+AOM)	3	5.6903590	4.1181396	- 1.8000130
	6	7.6721435	2.0799836	- 2.7239633
	9	7.2301926	2.0089716	- 2.1101523
Control (– AOM)	3	5.7946637	4.1953363	—
	6	7.8869820	2.1053769	—
	9	7.3860495	2.0148635	—
6.0 x 10^{-5} M** (+ AOM)	3	5.6149287	4.1359782	- 3.1017330
	6	7.4453100	2.0825163	- 5.6000127
	9	7.0673482	2.0093116	- 4.3149088
7.0 x 10^{-5} M (+ AOM)	3	5.5380193	4.1505693	- 4.4289783
	6	7.2864703	2.0956012	- 7.6139605
	9	6.9122423	2.0098363	- 6.4148933

Table –4.3: Lactic Acid Fermentation by Lactobacillus Bulgaricus NCIM-2359 Exposed to 2-Hydroxybutyric Acid

Concentration of AOM used A x 10^{-X} M	Incubation period in hours	Yield of lactic acid* in g/100 ml	Molasses substrate* left unfermented in g/100 ml	% of lactic acid increase in 3, 6, 9 days of incubation pd.
8.0 x 10^{-5} M (+ AOM)	3	****	–	–
	6	****	–	–
	9	****	–	–
9.0 x 10^{-5} M (+ AOM)	3	****	–	–
	6	****	–	–
	9	****	–	–
10.0 x 10^{-5} M (+AOM)	3	****	–	–
	6	****	–	–
	9	****	–	–

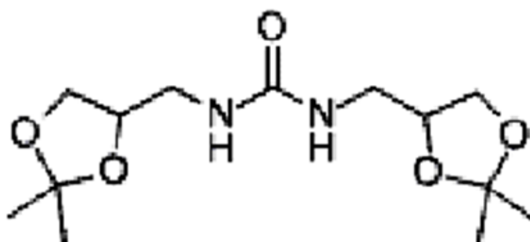
* Each value represents mean of three trials.
** Optimum concentration of AOM.
*** Optimum yield of lactic acid
**** Insignificant value

(+) Values indicate % increases in the yield of lactic acid

Experimental deviation + 2.5 – 3.5%

4.4 Discussion:

The influence of 1, 3-bis [(2, 2-dimethyl-1, 3-dioxolan-4-yl) methyl] urea



1, 3-bis [(2, 2-dimethyl-1, 3-dioxolan-4-yl) methyl] urea Compound – I

The addition of 1,3-bis[(2,2-dimethyl-1,3-dioxolan-4-yl) methyl]urea vide table -1 in the production medium for lactic acid fermentation by Lactobacillus bulgaricus NCIM-2359 has been found significant.

It has been found that there is a gradual increase in the production of lactic acid with stepping up of the compound 1, 3-bis [(2, 2-dimethyl-1, 3-dioxolan-4-yl) methyl] urea till the maximum yield of lactic acid, i. e., 8.8380566 g/100 ml was obtained at its molar concentration of 6.0 x 10-5M which is 11.7101507% higher in comparison to control fermentor flasks in 6 days of optimum incubation period.

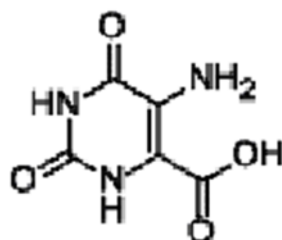
The enzymes activities of the compound I, i. e., 1,3-bis[(2,2-dimethyl-1,3-dioxolan-4-yl) methyl]urea may be attributed to the presence of >C=O groups of the nucleus and -NH-CO-NH-linkage, i.e., peptide linkage, present in the molecule. It has been found that many organic molecules having the -NH-CO-NH-linkage have been found of great biological significant for the maximum growth and activity of different microbes. The compound taken under trial, i.e., 1, 3-bis [(2, 2-dimethyl-1, 3-dioxolan-4-yl) methyl] urea possesses active unsaturated >C=O groups which may serve as a more efficient source of energy and influences the growth and activity of the enzyme system associated with Lactobacillus bulgaricus NCIM-2359.

Margalith and Pagani[66] during their industrial investigations successfully studied and compared different derivatives of barbituric acid, i.e., barbiturates and reported that the organic molecules, i.e., barbiturates has been found to be most effective and useful for various industrial fermentations process.[78-80] Barbiturates in general has been found most effective and useful in different biological processes and a lot of questions are still unsettled and open concerning the mode of action of these barbiturate molecules on the enzymes catalysed systems involved in the pathways leading to the mode of enzyme functions. However, whatever their biological functions may be, these organic molecules should be incorporated in to the fermentation medium for the better functioning of the process and improved yield of the desired products.

It is a secondary factor that influences the fermentation technique associated with enzymes of Lactobacillus bulgaricus NCIM-2359 Further, a group of researchers[81-88] have reported stimulatory effect of barbituric acid and its derivatives possessing barbiturate nucleus. Since the organic molecule, i. e., 1, 3-bis [(2, 2-dimethyl-1, 3-dioxolan-4-yl) methyl] urea also possess part structure combination of barbiturate nucleus, it may influence critically the outcome of lactic acid by the bacterial strain of Lactobacillus bulgaricus NCIM-2359. Rizvi[89] also studied effect of such type of compound on biosynthesis of citric acid by LSCF process and found it very significant for higher production of citric acid. Poonam[90] also studied the effect of 5, 5-diethyl hydantoin and 1, 3-dimethyl-2-thiobarbituric acid (both having barbiturate nucleus in their structure) on lactic acid fermentation and observed that both the compounds are very effective and stimulating for lactic acid fermentation process. Singh et al[91-92] also found 5, 5'-diphenylhydantoin and 5-phenyl hydantoin stimulatory for lactic acid and citric acid fermentation respectively. Mishra et al[93] found 3-ethyl-3-phenyl piperidine-2, 6-dione stimulatory for lactic acid fermentation by L. delbrueckii NCIM-1663.

Biosynthesis of citric acid by the fungal strain of Aspergillus niger NCIM-501 has been carried out by Suraiya et al.[94] using 26% (w/v) molasses substrate solution at 30^0C temperature, 2.0 pH and 12 days of optimum incubation period exposed to DL-5-(4-hydroxyphenyl) -5-phenylhydantoin. The results show that the incorporation of DL-5-(4-hydroxyphenyl) -5-phenylhydantoin to the production medium stimulates the citric acid fermentation process and thereby enhances the yield of citric acid to an extent of 12.47718% higher in comparison to control. Singh et al[95] studied influence of phenobarbital on facile biosynthesis of homolactic acid by L. Casei NCIM-1159 and found that it is stimulatory for lactic acid fermentation and enhances the production of lactic acid to a great extent in comparison to control.

4.5 The Influence of 5-Aminoorotic Acid:



5-aminoorotic acid [Compound II,]

The data recorded in the table-4.5 shows that the addition of 5-aminoorotic acid into the lactic acid fermentation medium enhances the production of lactic acid significantly. It has been observed that there is also a gradual increase in the yield of lactic acid with gradual stepping up of the compound II, i. e., 5-aminoorotic acid till the maximum yield of lactic acid is reached which is 8.6267162% higher in comparison to control fermentor flasks, i. e., 9.2999988g/100 ml at 5.0 x 10^{-5}M molar concentration of the compound 5-aminoorotic acid in 6 days of optimum incubation period.

It has been observed that the compound 5-aminoorotic acid is a very important active organic molecule and its biological activities may be attributed to the active >C = O groups associated with six membered hetero organic molecule and -CO-NH-CO-linkage.

Since no clear evidence could be put forward regarding its activity and stimulating properties of lactic acid fermentation process the compound 5-aminoorotic acid is considered to influence critically some metabolic enzymatic pathways intimately concerned with the lactic acid fermentation by using the bacterial strain of Lactobacillus bulgaricus NCIM-2359.

It has been discussed earlier that barbiturates and most of its derivative compounds possesses most effective >C=O groups and-NH-CO-NH-linkage. The compound I & II bears more than two >C=O groups and -NH-CO-NH-linkage which serves as a most effective energy source and influences significantly the growth and activity of the lactic acid bacteria Lactobacillus lactic acid.

It was also interesting to note that almost at all the concentrations of compounds I & II the % of lactic acid produced was higher than control.

The favourable and significant response of the compound I & II may also be attributed to the fact that in compound I & II, at least at the bonds where oxygen is attached with carbon of compound I & II there is a chance and probability of accepting protons given by the different enzyme surface area itself thereby getting increased electronegativity.

The bacterial activity under the above circumstances is expected to go more exogenously because the increase of electronegativity more and more of related enzymes are expected to participate and to take the position for reaction with active sites of the compound I & II and because of this stream of mobility population of the cell enzymes, most of them are expected to occur at the surroundings area of bacterial cells or enzymes elaborated by the strain Lactobacillus bulgaricus NCIM-2359 in the fermentation medium.

Although the exact mechanism of the role of compound I & II is still uncertain and at present it is very difficult to predict the real reason of the significant response of the compound I & II on lactic acid fermentation by Lactobacillus bulgaricus NCIM-2359 because there may be wide spread probable possibilities in this regard as follows:

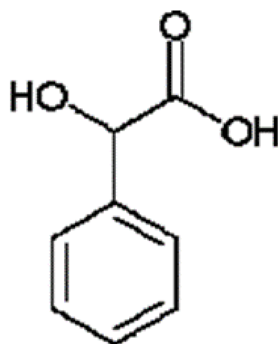
Incorporation of compounds I & II in fermentation medium may cause an alternation in the structure and behavior of enzymes that geometrically fits with the molasses substratum which is vital force as well source of lactic acid production by Lactobacillus bulgaricus NCIM-2359.

It may also cause an enhancement of the quantum of the enzymes which may thus increase the efficiency per unit of the elaborated enzyme and hence lactic acid fermentation by utilizing a major percent of molasses substrate, i. e., and molasses in the fermentation medium.

It may, therefore, be concluded that addition of 5-aminoorotic acid to the production medium has stimulatory effect at all its concentrations used, i.e., from 1.0×10^{-5} M to 70.0×10^{-5}M and the yield of lactic acid has been found greater in each case in comparison to control fermentor flasks.

However, incorporation of 5-aminoorotic acid at higher concentration level, i.e. 8.0×10^{-5}M and onwards is not encouraging for lactic acid production by Lactobacillus bulgaricus NCIM-2359.

4.5.1 The Influence of Mandelic Acid:



Mandelic acid

The data given in the table-3 shows that the compound mandelic acid has inhibitory effect on lactic acid fermentation by Lactobacillus bulgaricus NCIM-2359.

It has been observed that there is a gradual increase in the yield of lactic acid with the stepping up of mandelic acid up to the molar concentration of 5.0 x 10^{-5}M but after this concentration a big fall in the production of lactic acid has been noticed.

It has been observed that at concentrations in between 5.0 x 10^{-5}M, 6.0 x 10^{-5}M and 7.0 x 10^{-5}M the yield of lactic acid has been recorded to be 2.4227289% and 1.2227504% and 0.8064285% higher respectively in comparison to control fermentor flasks, i. e., and 7.9347015 g/100ml in 6 days of optimum incubation period.

It is interesting to note that the higher concentrations of mandelic acid, i.e., 8.0 x 10^{-5} M and onwards for the production of lactic acid has been found much inhibitory and yield of lactic acid on these concentrations has been found insignificant in comparison to control.

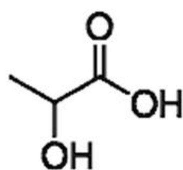
The inhibitory action of mandelic acid may be explained from the fact that mandelic acid is partially structurally similar to lactic acid

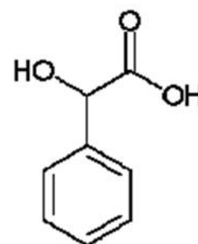
Therefore, mandelic acid happens to be biological metabolite structural antagonist and the enzyme catalysed activity of Lactobacillus bulgaricus NCIM-2359 is expected and supposed to be inhibited in the fermentation process.

The mandelic acid which happens to be structural metabolite antagonist has one end similar to the lactic acid.

Thus, the compound mandelic acid a structural biological metabolite antagonist may perhaps prevent the proper functioning of enzyme catalysed reactions of Lactobacillus bulgaricus NCIM-2359 but probably remains firmly attached to it by its one end.

The probable structurally similar combination of mandelic acid with lactic acid may be illustrated as given under:

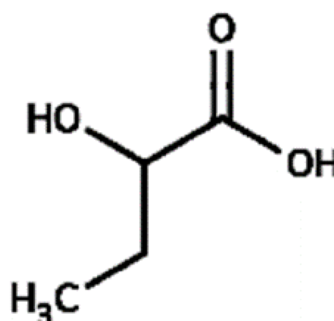




Lactic acid (LA) **Mandelic acid**

Therefore, mandelic acid antagonizes with one upper end of the lactic acid and thus retards the lactic acid fermentation by Lactobacillus bulgaricus NCIM-2359.

4.5.2 The Influence of 2-Hydroxybutyric Acid:



2-hydroxybutyric acid

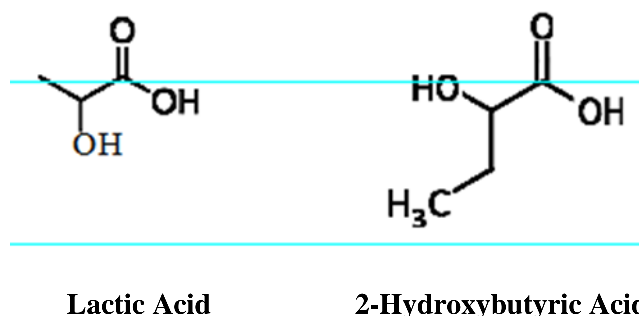
The data recorded in the table-4 shows that 2-hydroxybutyric acid is not beneficial instead much inhibitory for lactic acid fermentation by Lactobacillus bulgaricus NCIM-2359

It has been observed that initial concentration of 2-hydroxybutyric acid, i.e., from 1.0 x 10^{-5} M to 4.0 x 10^{-5} M has been slight stimulatory for lactic acid fermentation by Lactobacillus bulgaricus NCIM-2359 as the yield of lactic acid at these molar concentrations has been slightly more than that of control fermentor flasks.

It has been further observed that at concentration 4.0 x 10^{-5} M the yield of lactic acid has been found maximum, i.e., 8.0062844g/100 ml which is 1.5126495 % higher in comparison to control fermentor flasks, i.e., 7.8869820 g/ 100 ml in 6 days of optimum incubation period.

It has been observed that at concentration 5.0×10^{-5}M to 7.0×10^{-5}M there is a gradual fall in the yield of lactic acid and the yield of lactic acid has been found to be -2.7239633%, -5.6000127% and -7.6139605% less in comparison to control fermentor flasks.

However, at concentrations 8.0 x 10^{-5} M and onwards the yield of lactic acid has been found insignificant.



Lactic Acid **2-Hydroxybutyric Acid**

***Structural Antagonist Combination Have One Common Shaded Upper End:**

Therefore, like mandelic acid the compound 2-hydroxybutyric acid also antagonizes with the lactic acid fermentation by Lactobacillus bulgaricus NCIM-2359 thereby retarding the yield of lactic acid.

4.6 Summary:

A comparative assessment of the different active organic molecules on lactic acid fermentation by Lactobacillus bulgaricus NCIM-2359 can be had from the **Table-4.4** given below.

Table – 4.4: Study of the Influence of 1, 3-Bis [(2, 2-Dimethyl-1, 3-Dioxolan-4-Yl) Methyl] Urea, 5-Aminoaorotic Acid, Mandelic Acid 2-Hydroxybutyric Acid on Lactic Acid Fermentation by Lactobacillus Bulgaricus NCIM-2359 in 6 Days of Optimum Incubation Period

AOM Used	Optimum Concentration of The AOM Used.	Max. Yield of Lactic Acid* in Control Flasks in g/100ml	Max. Yield of Lactic Acid* in the Presence of AOM in g/100ml	% of Lactic Acid Increase or Decrease in 6 Days of Incubation Pd.
1	6.0×10^{-5}M	7.9115967	8.8380566	(+) 11.7101507
2	5.0×10^{-5}M	7.8926956	8.6267162	(+) 9.2999988
3	4.0×10^{-5}M	7.9347015	8.1497429	(+) 2.7101384
4	4.0×10^{-5}M	7.8869820	8.0062844	(+) 1.5126495

* Each value represents mean of three observations

(+) Values indicates % increase in the yield of lactic acid.

Experimental deviation (+) 2.5 to 3.5%.

a) 1, 3-bis [(2, 2-dimethyl-1, 3-dioxolan-4-yl) methyl] urea
b) 5-aminoaorotic acid

c) Mandelic acid
d) 2-Hydroxybutyric acid

Thus, it may be summarized that 1,3-bis[(2,2-dimethyl-1,3-dioxolan-4-yl) methyl]urea and 5-aminoaorotic acid enhances the lactic acid fermentation by Lactobacillus bulgaricus NCIM-2359 at all concentrations used; while mandelic acid and 2-hydroxybutyric acid antagonizes the course of lactic acid fermentation by Lactobacillus bulgaricus NCIM-2359 at their higher concentrations used, i.e., 4.0×10^{-5}M and 4.0×10^{-5} respectively retarding thereby the yield of lactic acid. However, it was interesting to point out that 1, 3-bis [(2, 2-dimethyl-1, 3-dioxolan-4-yl) methyl] urea was very effective amongst the active organic molecule used which could increase significantly the yield of lactic acid to an extent of 11.7101507% in comparison to control fermentor flasks, on the other hand 5-aminoaorotic acid was also found effective for the lactic acid fermentation by Lactobacillus bulgaricus NCIM-2359 but it could enhance the yield of lactic acid only to an extent of 9.2999988% in the same experimental conditions.

4.7 References:

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Chapter 5

Lactic Acid Fermentation by Lactobacillus Bulgaricus Ncim- 2359 Exposed to Some Chemical Mutagens

5.1 Introduction:

Mutations can involve large sections of DNA becoming duplicated, usually through genetic recombination[1-5]. These duplications are a major source of raw material for evolving new genes, with tens to hundreds of genes duplicated in animal genomes every million years[6]. Most genes belong to larger families of genes of shared ancestry[7].

Novel genes are produced by several methods, commonly through the duplication and mutation of an ancestral gene, or by recombining parts of different genes to form new combinations with new functions[8-9]. Here, domains act as modules, each with a particular and independent function, that can be mixed together to produce genes encoding new proteins with novel properties.[10] For example, the human eye uses four genes to make structures that sense light: three for color vision and one for night vision; all four arose from a single ancestral gene.[11] Another advantage of duplicating a gene (or even an entire genome) is that this increases redundancy; this allows one gene in the pair to acquire a new function while the other copy performs the original function.[12-13] Other types of mutation occasionally create new genes from previously noncoding DNA.[14-15]

Changes in chromosome number may involve even larger mutations, where segments of the DNA within chromosomes break and then rearrange. For example, in the Homininae, two chromosomes fused to produce human chromosome 2; this fusion did not occur in the lineage of the other apes, and they retain these separate chromosomes.[16] In evolution, the most important role of such chromosomal rearrangements may be to accelerate the divergence of a population into new species by making populations less likely to interbreed, and thereby preserving genetic differences between these populations.[17]

Sequences of DNA that can move about the genome, such as transposons, make up a major fraction of the genetic material of plants and animals, and may have been important in the evolution of genomes.[18] For example, more than a million copies of the Alu sequence are present in the human genome, and these sequences have now been recruited to perform functions such as regulating gene expression.[19] Another effect of these mobile DNA sequences is that when they move within a genome, they can mutate or delete existing genes and thereby produce genetic diversity.[2] Nonlethal mutations accumulate within the gene pool and increase the amount of genetic variation.[20] The abundance of some genetic changes within the gene pool can be reduced by natural selection, while other "more favorable" mutations may accumulate and result in adaptive changes. Neutral mutations are defined as mutations whose effects do not influence the fitness of an individual. These can accumulate over time due to genetic drift. It is believed that the overwhelming majority of mutations have no significant effect on an organism's fitness.

Also, DNA repair mechanisms are able to mend most changes before they become permanent mutations, and many organisms have mechanisms for eliminating otherwise permanently mutated somatic cells. Beneficial mutations can improve reproductive success.

5.2 Spontaneous Mutation:

Spontaneous mutations on the molecular level can be caused by: [21]

Tautomerism: A base is changed by the repositioning of a hydrogen atom, altering the hydrogen bonding pattern of that base resulting in incorrect base pairing during replication.

Depurination: Loss of a purine base (A or G) to form an apurinic site (AP site).

Deamination: Hydrolysis changes a normal base to an atypical base containing a keto group in place of the original amine group. Examples include C → U and A → HX (hypoxanthine), which can be corrected by DNA repair mechanisms; and 5MeC (5-methylcytosine) → T, which is less likely to be detected as a mutation because thymine is a normal DNA base.

Slipped Strand Mispairing: Denaturation of the new strand from the template during replication, followed by renaturation in a different spot ("slipping"). This can lead to insertions or deletions.

5.2.1 Induced Mutation:

Induced mutations on the molecular level can be caused by:-
Chemicals: Hydroxylamine NH_2OH
Base analogs (e.g. BrdU)

5.3 Alkylating agents (e.g. N-ethyl-N-nitrosourea):

These agents can mutate both replicating and non-replicating DNA. In contrast, a base analog can only mutate the DNA when the analog is incorporated in replicating the DNA. Each of these classes of chemical mutagens has certain effects that then lead to transitions, transversions, or deletions. Agents that form DNA adducts (e.g. ochratoxin A metabolites) [21, 22]

DNA intercalating agents (e.g. ethidium bromide)
DNA crosslinkers
Oxidative damage

Nitrous acid converts amine groups on A and C to diazo groups, altering their hydrogen bonding patterns which leads to incorrect base pairing during replication.

5.4 Radiation:

Ultraviolet radiation (nonionizing radiation). Two nucleotide bases in DNA – cytosine and thymine – are most vulnerable to radiation that can change their properties.

UV light can induce adjacent pyrimidine bases in a DNA strand to become covalently joined as a pyrimidine dimer. UV radiation, particularly longer-wave UVA, can also cause oxidative damage to DNA[23] Mutation rates also vary across species. Evolutionary biologists [citation needed] have theorized that higher mutation rates are beneficial in some situations, because they allow organisms to evolve and therefore adapt more quickly to their environments. For example, repeated exposure of bacteria to antibiotics, and selection of resistant mutants, can result in the selection of bacteria that have a much higher mutation rate than the original population (mutator strains).

5.5 By Effect on Structure:

The sequence of a gene can be altered in a number of ways. Gene mutations have varying effects on health depending on where they occur and whether they alter the function of essential proteins. Mutations in the structure of genes can be classified as:

Small-scale mutations, such as those affecting a small gene in one or a few nucleotides, including:

Point mutations, often caused by chemicals or malfunction of DNA replication, exchange a single nucleotide for another.[24] These changes are classified as transitions or transversions.[25] Most common is the transition that exchanges a purine for a purine (A → G) or a pyrimidine for a pyrimidine, (C →T). A transition can be caused by nitrous acid, base mis-pairing, or mutagenic base analogs such as 5-bromo-2-deoxyuridine (BrdU).

Less common is a transversion, which exchanges a purine for a pyrimidine or a pyrimidine for a purine (C/T→ A/G). An example of a transversion is adenine (A) being converted into a cytosine (C). A point mutation can be reversed by another point mutation, in which the nucleotide is changed back to its original state (true reversion) or by second-site reversion (a complementary mutation elsewhere that results in regained gene functionality). Point mutations that occur within the protein coding region of a gene may be classified into three kinds, depending upon what the erroneous codon codes for:

Silent Mutations: which code for the same (or a sufficiently similar) amino acid.

Missense Mutations: which code for a different amino acid.

Nonsense Mutations: which code for a stop and can truncate the protein.

Insertions add one or more extra nucleotides into the DNA. They are usually caused by transposable elements, or errors during replication of repeating elements (e.g. AT repeats [citation needed]). Insertions in the coding region of a gene may alter splicing of the mRNA (splice site mutation), or cause a shift in the reading frame (frame shift), both of which can significantly alter the gene product. Insertions can be reverted by excision of the transposable element. Deletions remove one or more nucleotides from the DNA. Like insertions, these mutations can alter the reading frame of the gene. They are generally irreversible: though exactly the same sequence might theoretically be restored by an insertion, transposable elements able to revert a very short deletion (say 1–2 bases) in any location are either highly unlikely to exist or do not exist at all.

Note that a deletion is not the exact opposite of an insertion: the former is quite random while the latter consists of a specific sequence inserting at locations that are not entirely random or even quite narrowly defined. Large-scale mutations in chromosomal structure, including: Amplifications (or gene duplications) leading to multiple copies of all chromosomal regions, increasing the dosage of the genes located within them. Deletions of large chromosomal regions, leading to loss of the genes within those regions.

Mutations whose effect is to juxtapose previously separate pieces of DNA, potentially bringing together separate genes to form functionally distinct fusion genes (e.g. bcr-abl). These include:

Chromosomal Translocations: interchange of genetic parts from nonhomologous chromosomes.

Interstitial Deletions: an intra-chromosomal deletion that removes a segment of DNA from a single chromosome, thereby apposing previously distant genes. For example, cells isolated from a human astrocytoma, a type of brain tumor, were found to have a chromosomal deletion removing sequences between the "fused in glioblastoma" (fig) gene and the receptor tyrosine kinase "ros", producing a fusion protein (FIG-ROS). The abnormal FIG-ROS fusion protein has constitutively active kinase activity that causes oncogenic transformation (a transformation from normal cells to cancer cells).

Chromosomal Inversions: reversing the orientation of a chromosomal segment.

Loss of Heterozygosity: loss of one allele, either by a deletion or recombination event, in an organism that previously had two different alleles.

Loss-of-function mutations are the result of gene product having less or no function. When the allele has a complete loss of function (null allele) it is often called an amorphic mutation. Phenotypes associated with such mutations are most often recessive. Exceptions are when the organism is haploid, or when the reduced dosage of a normal gene product is not enough for a normal phenotype (this is called haploinsufficiency).

Gain-of-function mutations change the gene product such that it gains a new and abnormal function. These mutations usually have dominant phenotypes. Often called a neomorphic mutation.

Dominant negative mutations (also called antimorphic mutations) have an altered gene product that acts antagonistically to the wild-type allele. These mutations usually result in an altered molecular function (often inactive) and are characterized by a dominant or semi-dominant phenotype.

It was once thought that, Marfan syndrome is an example of a dominant negative mutation occurring in an autosomal dominant disease where the defective glycoprotein product of the fibrillin gene (FBN1) antagonizes the product of the normal allele. However, this has since been deemed false and it has been shown that Marfan's is really a result of Haploinsufficiency because the absence of one normal allele causes the disease not the presence of an abnormal allele.

Lethal mutations are mutations that lead to the death of the organisms which carry the mutations. A back mutation or reversion is a point mutation that restores the original sequence and hence the original phenotype.[26]

In applied genetics it is usual to speak of mutations as either harmful or beneficial. A harmful mutation is a mutation that decreases the fitness of the organism. A beneficial mutation is a mutation that increases fitness of the organism, or which promotes traits that are desirable. In theoretical population genetics, it is more usual to speak of such mutations as deleterious or advantageous. In the neutral theory of molecular evolution, genetic drift is the basis for most variation at the molecular level.

A neutral mutation has no harmful or beneficial effect on the organism. Such mutations occur at a steady rate, forming the basis for the molecular clock. A deleterious mutation has a negative effect on the phenotype, and thus decreases the fitness of the organism. An advantageous mutation has a positive effect on the phenotype, and thus increases the fitness of the organism. A nearly neutral mutation is a mutation that may be slightly deleterious or advantageous, although most nearly neutral mutations are slightly deleterious.

In reality, viewing the fitness effects of mutations in these discrete categories is an oversimplification. Attempts have been made to infer the distribution of fitness effects (DFE) using mutagenesis experiments and theoretical models applied to molecular sequence data. Distribution of fitness effects, as used to determine the relative abundance of different types of mutations (i.e. strongly deleterious, nearly neutral or advantageous), is relevant to many evolutionary questions, such as the maintenance of genetic variation,[27] the rate of genomic decay[28] and the evolution of sex and recombination.[29] In summary, DFE plays an important role in predicting evolutionary dynamics.[30, 31]. A variety of approaches have been used to study the distribution of fitness effects, including theoretical, experimental and analytical methods.

Mutagenesis Experiment: The direct method to investigate DFE is to induce mutations and then measure the mutational fitness effects, which has already been done in viruses, bacteria, yeast, and Drosophila. For example, most studies of DFE in viruses used site-directed mutagenesis to create point mutations and measure relative fitness of each mutant.[32-35] In Escherichia coli, one study used transposon mutagenesis to directly measure the fitness of a random insertion of a derivative of Tn10.[36]

In yeast, a combined mutagenesis and deep sequencing approach has been developed to generate high-quality systematic mutant libraries and measure fitness in high throughput.[37] However, given that many mutations have effects too small to be detected[38] and that mutagenesis experiments can only detect mutations of moderately large effect, DNA sequence data analysis can provide valuable information about these mutations.

Molecular sequence analysis: With rapid development of DNA sequencing technology, an enormous amount of DNA sequence data is available and even more is forthcoming in the future. Various methods have been developed to infer DFE from DNA sequence data.[39-42.] By examining DNA sequence differences within and between species, we are able to infer various characteristics of the DFE for neutral, deleterious and advantageous mutations.[43]. Specifically, the DNA sequence analysis approach allows us to estimate the effects of mutations with very small effects, which are hardly detectable through mutagenesis experiments.

One of the earliest theoretical studies of the distribution of fitness effects was done by Motoo Kimura, an influential theoretical population geneticist. His neutral theory of molecular evolution proposes that most novel mutations will be highly deleterious, with a small fraction being neutral.[44-45]. Hiroshi Akashi more recently proposed a bimodal model for DFE, with modes centered around highly deleterious and neutral mutations.[46] Both theories agree that the vast majority of novel mutations are neutral or deleterious and that advantageous mutations are rare, which has been supported by experimental results. One example is a study done on the distribution of fitness effects of random mutations in vesicular stomatitis virus.[32] Out of all mutations, 39.6% were lethal, 31.2% were non-lethal deleterious, and 27.1% were neutral. Another example comes from a high throughput mutagenesis experiment with yeast.[37]. In this experiment it was shown that the overall distribution of fitness effects is bimodal, with a cluster of neutral mutations, and a broad distribution of deleterious mutations.

Though relatively few mutations are advantageous, those that are play an important role in evolutionary changes.[47]. Like neutral mutations, weakly selected advantageous mutations can be lost due to random genetic drift, but strongly selected advantageous mutations are more likely to be fixed. Knowing the distribution of fitness effects of advantageous mutations may lead to increased ability to predict the evolutionary dynamics. Theoretical work on the DFE for advantageous mutations has been done by John H. Gillespie[48] and H. Allen Orr.[49] They proposed that the distribution for advantageous mutations should be exponential under a wide range of conditions, which has generally been supported by experimental studies, at least for strongly selected advantageous mutations.[50-52]

In summary, it is generally accepted that the majority of mutations are neutral or deleterious, with rare mutations being advantageous; however, the proportion of types of mutations varies between species. This indicates two important points: first, the proportion of effectively neutral mutations is likely to vary between species, resulting from dependence on effective population size; second, the average effect of deleterious mutations varies dramatically between species.[43]. In addition, the DFE also differs between coding regions and non-coding regions, with the DFE of non-coding DNA containing more weakly selected mutations[43].

A frameshift mutation is a mutation caused by insertion or deletion of a number of nucleotides that is not evenly divisible by three from a DNA sequence. Due to the triplet nature of gene expression by codons, the insertion or deletion can disrupt the reading frame, or the grouping of the codons, resulting in a completely different translation from the original.[53] The earlier in the sequence the deletion or insertion occurs, the more altered the protein produced is.

In contrast, any insertion or deletion that is evenly divisible by three is termed an in-frame mutation

A nonsense mutation is a point mutation in a sequence of DNA that results in a premature stop codon, or a nonsense codon in the transcribed mRNA, and possibly a truncated, and often nonfunctional protein product.

Missense mutations or nonsynonymous mutations are types of point mutations where a single nucleotide is changed to cause substitution of a different amino acid. This in turn can render the resulting protein nonfunctional. Such mutations are responsible for diseases such as Epidermolysis bullosa, sickle-cell disease, and SOD1 mediated ALS.

A neutral mutation is a mutation that occurs in an amino acid codon which results in the use of a different, but chemically similar, amino acid. The similarity between the two is enough that little or no change is often rendered in the protein. For example, a change from AAA to AGA will encode arginine, a chemically similar molecule to the intended lysine.

Silent mutations are mutations that do not result in a change to the amino acid sequence of a protein, unless the changed amino acid is sufficiently similar to the original. They may occur in a region that does not code for a protein, or they may occur within a codon in a manner that does not alter the final amino acid sequence. The phrase silent mutation is often used interchangeably with the phrase synonymous mutation; however, synonymous mutations are a subcategory of the former, occurring only within exons (and necessarily exactly preserving the amino acid sequence of the protein). Synonymous mutations occur due to the degenerate nature of the genetic code.

In multicellular organisms with dedicated reproductive cells, mutations can be subdivided into germ line mutations, which can be passed on to descendants through their reproductive cells, and somatic mutations (also called acquired mutations), which involve cells outside the dedicated reproductive group and which are not usually transmitted to descendants.

A germline mutation gives rise to a constitutional mutation in the offspring, that is, a mutation that is present in every cell. A constitutional mutation can also occur very soon after fertilization, or continue from a previous constitutional mutation in a parent.

The distinction between germline and somatic mutations is important in animals that have a dedicated germ line to produce reproductive cells. However, it is of little value in understanding the effects of mutations in plants, which lack dedicated germ line. The distinction is also blurred in those animals that reproduce asexually through mechanisms such as budding, because the cells that give rise to the daughter organisms also give rise to that organism’s germ line. A new mutation that was not inherited from either parent is called a de novo mutation.

Diploid organisms (e.g. human) contain two copies of each gene – a paternal and a maternal allele. Based on the occurrence of mutation on each chromosome, we may classify mutations into three types. A heterozygous mutation is a mutation of only one allele. A homozygous mutation is an identical mutation of both the paternal and maternal alleles. Compound heterozygous mutations or a genetic compound comprises two different mutations in the paternal and maternal alleles. A wild type or homozygous non-mutated organism is one in which neither allele is mutated.

Conditional mutation is a mutation that has wild-type (or less severe) phenotype under certain "permissive" environmental conditions and a mutant phenotype under certain "restrictive" conditions. For example, a temperature-sensitive mutation can cause cell death at high temperature (restrictive condition), but might have no deleterious consequences at a lower temperature (permissive condition).

Changes in DNA caused by mutation can cause errors in protein sequence, creating partially or completely non-functional proteins. Each cell, in order to function correctly, depends on thousands of proteins to function in the right places at the right times.

When a mutation alters a protein that plays a critical role in the body, a medical condition can result. A condition caused by mutations in one or more genes is called a genetic disorder. Some mutations alter a gene's DNA base sequence but do not change the function of the protein made by the gene. One study on the comparison of genes between different species of Drosophila suggests that if a mutation does change a protein, this will probably be harmful, with an estimated 70 percent of amino acid polymorphisms having damaging effects, and the remainder being either neutral or weakly beneficial. However, studies in yeast have shown that only 7% of mutations that are not in genes are harmful.[54]

If a mutation is present in a germ cell, it can give rise to offspring that carries the mutation in all of its cells. This is the case in hereditary diseases. In particular, if there is a mutation in a DNA repair gene within a germ cell, humans carrying such germ-line mutations may have in increased risk of cancer, such as the list on Wikipedia of inherited human DNA repair gene mutations that increase cancer risk. On the other hand, a mutation may occur in a somatic cell of an organism. Such mutations will be present in all descendants of this cell within the same organism, and certain mutations can cause the cell to become malignant, and thus cause cancer.[55, 56].

A DNA damage can cause an error when the DNA is replicated, and this error of replication can cause a gene mutation that, in turn, could cause a genetic disorder. DNA damages are repaired by the DNA repair system of the cell. Each cell has a number of pathways through which enzymes recognize and repair damages in DNA. Because DNA can be damaged in many ways, the process of DNA repair is an important way in which the body protects itself from disease.

Once a DNA damage has given rise to a mutation, the mutation cannot be repaired. DNA repair pathways can only recognize and act on "abnormal" structures in the DNA. Once a mutation occurs in a gene sequence it then has normal DNA structure and cannot be repaired. Although mutations that change in protein sequences can be harmful to an organism; on occasions, the effect may be positive in a given environment. In this case, the mutation may enable the mutant organism to withstand particular environmental stresses better than wild-type organisms, or reproduce more quickly. In these cases a mutation will tend to become more common in a population through natural selection[57, 58].Point mutations may arise from spontaneous mutations that occur during DNA replication. The rate of mutation may be increased by mutagens. Mutagens can be physical, such as radiation from UV rays, X-rays or extreme heat, or chemical (molecules that misplace base pairs or disrupt the helical shape of DNA). Mutagens associated with cancers are often studied to learn about cancer and its prevention.

Mutation can result in several different types of change in DNA sequences; these can either have no effect, alter the product of a gene, or prevent the gene from functioning properly or completely[59-61]. Studies in the fly Drosophila melanogaster suggest that if a mutation changes a protein produced by a gene, this will probably be harmful, with about 70 percent of these mutations having damaging effects, and the remainder being either neutral or weakly beneficial.[62] Due to the damaging effects that mutations can have on cells, organisms have evolved mechanisms such as DNA repair to remove mutations. Therefore, the optimal mutation rate for a species is a trade-off between costs of a high mutation rate, such as deleterious mutations, and the metabolic costs of maintaining systems to reduce the mutation rate, such as DNA repair enzymes.[63]

Viruses that use RNA as their genetic material have rapid mutation rates,[64] which can be an advantage since these viruses will evolve constantly and rapidly, and thus evade the defensive responses of e.g. the human immune system.[65] Mutations can involve large sections of DNA becoming duplicated, usually through genetic recombination.[66] These duplications are a major source of raw material for evolving new genes, with tens to hundreds of genes duplicated in animal genomes every million years.[67] Most genes belong to larger families of genes of shared ancestry.[68] Novel genes are produced by several methods, commonly through the duplication and mutation of an ancestral gene, or by recombining parts of different genes to form new combinations with new functions.[69,70] Here, domains act as modules, each with a particular and independent function, that can be mixed together to produce genes encoding new proteins with novel properties.[71] For example, the human eye uses four genes to make structures that sense light: three for color vision and one for night vision; all four arose from a single ancestral gene.[72] Another advantage of duplicating a gene (or even an entire genome) is that this increases redundancy; this allows one gene in the pair to acquire a new function while the other copy performs the original function[73,74] Other types of mutation occasionally create new genes from previously noncoding DNA.[75,76] Changes in chromosome number may involve even larger mutations, where segments of the DNA within chromosome involve even larger mutations, where segments of the DNA within chromosomes break and then rearrange. For example, two chromosomes in the Homo genus fused to produce human chromosome 2; this fusion did not occur in the lineage of the other apes, and they retain these separate chromosomes.[77] In evolution, the most important role of such chromosomal rearrangements may be to accelerate the divergence of a population into new species by making populations less likely to interbreed, and thereby preserving genetic differences between these populations.[78] Sequences of DNA that can move about the genome, such as transposons, make up a major fraction of the genetic material of plants and animals, and may have been important in the evolution of genomes[79].

For example, more than a million copies of the Alu sequence are present in the human genome, and these sequences have now been recruited to perform functions such as regulating gene expression[80]. Another effect of these mobile DNA sequences is that when they move within a genome, they can mutate or delete existing genes and thereby produce genetic diversity.

A mutation has caused this garden moss rose to produce flowers of different colors. This is a somatic mutation that may also be passed on in the germ line. In multicellular organisms with dedicated reproductive cells, mutations can be subdivided into germ line mutations, which can be passed on to descendants through their reproductive cells, and somatic mutations (also called acquired mutations)[81], which involve cells outside the dedicated reproductive group and which are not usually transmitted to descendants. If the organism can reproduce asexually through mechanisms such as cuttings or budding the distinction can become blurred.

Mutation is generally accepted by biologists as the mechanism by which natural selection acts, generating advantageous new traits that survive and multiply in offspring as well as disadvantageous traits, in less fit offspring, that tend to die out. Mutation rates also vary across species. Evolutionary biologists have theorized that higher mutation rates are beneficial in some situations, because they allow organisms to evolve and therefore adapt more quickly to their environments. For example, repeated exposure of bacteria to antibiotics, and selection of resistant mutants, can result in the selection of bacteria that have a much higher mutation rate than the original population (mutator strains).erminus of the amino acid were it to be present as in the wild type.[82-92]

Changes in DNA caused by mutation can cause errors in protein sequence, creating partially or completely non-functional proteins. To function correctly, each cell depends on thousands of proteins to function in the right places at the right times. When a mutation alters a protein that plays a critical role in the body, a medical condition can result. A condition caused by mutations in one or more genes is called a genetic disorder. Some mutations alter a gene's DNA base sequence but do not change the function of the protein made by the gene.[93, 34]

If a mutation is present in a germ cell, it can give rise to offspring that carries the mutation in all of its cells. This is the case in hereditary diseases. On the other hand, a mutation may occur in a somatic cell of an organism. Such mutations will be present in all descendants of this cell within the same organism, and certain mutations can cause the cell to become malignant, and thus cause cancer[95]. Often, gene mutations that could cause a genetic disorder are repaired by the DNA repair system of the cell. Each cell has a number of pathways through which enzymes recognize and repair mistakes in DNA. Because DNA can be damaged or mutated in many ways, the process of DNA repair is an important way in which the body protects itself from disease. Beneficial mutations although most mutations that change protein sequences are neutral or harmful, some mutations have a positive effect on an organism. In this case, the mutation may enable the mutant organism to withstand particular environmental stresses better than wild-type organisms, or reproduce more quickly. In these cases a mutation will tend to become more common in a population through natural selection[96-98].

Mutation is the ultimate source of all genetic variation; it provides the raw materials for evolution. Recombination (independent assortment plus recombination of genetic variability present in individual chromosomes; merely rearranges this genetic variability into new combinations and natural (or artificial) selection simply preserves the combinations best adapted to the existing (or desired) environmental conditions[99-100]. Without mutation, all genes would exist in only one form alleles would not exist, and thus genetic analysis would not be possible. Most important, organisms would not be able to evolve and adapt to environmental changes.[101-107] Treatment with mutagenic agents can increase mutation frequencies by order of magnitude[108-109.] The mutation frequency per gene in bacteria and viruses, for example, can easily be increased to over 1 percent by treatment with potent chemical mutagens. That is over 1 percent of the genes of the treated organisms will contain a mutation or stated differently over 1 percent of the individual phase of bacteria in the population will have a mutation in a given gene. This simple relationship between mutation and radiation has been interpreted by Timofeeff-Ressovsky[110], Lea[111], and Catchaside[112]. And others to mean that the gene is a "target" and its mutation is caused by a single "hit" of radiation.

The search for genes that respond differently to mutagenic agents had some success (Shukla[113]) but no mutagen was found that confined its effects to only one particular gene on a chromosome and affected no others at all. Thus the wide array of possible chemical reactions caused by most compounds and the wide array of compounds that cause mutations made it difficult for a long period to assign any specific reaction in the cell to a particular mutagen acridine dyes such as proflavin and acridine orange and other mutagens that seems to produce direct effects on the DNA molecule. According to Leman[114] acridine dyes act by inserting themselves between two neighbouring purine bases in a single DNA strand. The consequence of such incorporation. According to Brenner and co-workers[115.] Is to cause either the insertion or deletion of a single nucleotide. Thus acridine mutations would not be expected to cause transitions as do base analogues, nitrous acid, hydroxylamine, and alkylating agents. To distinguish differences in the mutation-inducing processes of these agents.

Freese[116-117] performed a classical experiment on r II mutations in phase T_4. His procedure was to induce and collect r II mutations caused by all these agents and then test whether reverse mutation to normal (r+) could be induced by the 2-aminopurine and 5-bromouracil base analogues[118-130]. The base analogues were effective in reverting r II mutations that they themselves proceed. Similarly they could revert high percentages of rII mutations that had been caused by hydroxylamine, nitrous acid, and alkylating agents. General review [131-137] reveals that there is a rather widespread agreement as to the best strategy for a programme of strain development of screening designed to improve best and potent mutant of micro-organism. Singh and others[138] in their investigation on microbial synthesis of lactic acid by Lactobacillus acidophilus found that only hydrazine sulphate enhances the microbial synthesis of lactic acid while *p*-nitro-phenylhydrazine; 2, 4-dinitrophenylhydarzine and lithium fluoride has been found almost detrimental or valueless for the microbial synthesis of lactic acid. Reeta Rani[139] also worked on a few chemical mutagens and found that only methoxy caffeine enhances the fungal synthesis of ergot-alkaloids while sodium azide, hydrazine hydrochloride, and lithium fluoride has been found almost detrimental and valueless for the fungal synthesis of ergot-alkaloids by Claviceps purpurea.

A large variety of compounds[140-143] like peroxides, caffeine, gaseous butadiene, ethylene and thiourea causes mutation in different microbes. Peroxides and epoxides as mutagenic chemicals were also reported by a group of workers[144-145] as a very specific mutagens, Nishi ET al[146] and others[147-151] have worked on microorganisms and fermentation process such as N-methyl-N-nitrosourea, EMS, or X-rays to induce the microbial process and achieve the improved yields. Mahana[152] and Khan[153] have also reported the mutagenic properties of ethyl methane sulphonate. Tiwari et al [154] found that lactic acid fermentation process was inhibited with increasing concentration of camphor. Several phenolics[155-157] flavonoids[158] and aldehydes were also found mutagenic in variety of strain of different fungi and bacteria[159-161]. Freese and Freese[162-163] found appreciable mutagenic action of hydrazine at 1.0M optimum concentration. The alkylated hydrazine has also been found to be specific mutagen like hydrazine[164 but] was not as effective as hydrazine.

Hydroxylamine[165-168] has been found to be most specific mutagenic chemical for a number of microbes. Singh and Roy[169] in their investigations reported chloralhydrate as a growth inhibitor chemical mutagen. Jiang et al[170] also reported chloralhydrate as a strong mutagenic chemical for *S.* typhimurian but it has been found weak mutagenic[171] in many other cases. Singh ET al[172] reported chloralhydrate as a retarding mutagen for production of lactic acid by L delbrueckii. Colchi-mutation has also been reported by some workers[173-178]. Proflavin and acridine orange are two important mutagenic[179] dyes. The mechanism of dyes action is not fully understood and it may take place during recombination[180]. The influence of various dyes on activity of different microbes have been reported by a group of workers[181-185].

Ziemmermann[186] demonstrated the influence of the ionizing radiations to induce mitosis gene conversion by using deploid strain of *S.* cerevisiae. Gammarays[187] and ultrasonic waves[188-189] were found as effective mutagens for lactic culture and the fungus A. Niger. Singh ET al[190-191] reported N-sulphonic urethane as an effective mutagen for L. Casei NCIM-2125. Faizi et al[192] has also studied some chemical mutagens on fermentation processes and has reported only benzyl carbamate has significant influence while 2, 4-dinitrophenyl hydrazine, acetone phenyl hydrazone and acetaldehyde phenyl hydrazone insignificant for fermentative process, especially for citric acid fermentation.

On the other hand Singh ET al.[193] studied efficacy of p-toluenesulphonyl azide and p-toluene sulphonyl hydrazine on microbial synthesis of bioalcohol and found that p-toluene sulphonyl azide retards the yield of bioalcohol while p-toluene sulphonyl hydrazine enhances the yield significantly. Narayan and Sharma[194] found nitrosoguanidine and ethylene imine mutagens useful and significant for higher yields of alcohol. Singh[195] studied the influence of 2-aminofluorine and dimethyl nitrosamine on lactic acid fermentation by L.bulgaricus NCIM-2056 and found that both the chemical mutagens are beneficial for higher production of lactic acid. Narayan and Sharma[196] studied the influence of ethylene oxide and butyl carbamate mutagens on alcoholic fermentation and they found that ethylene oxide and butyl carbamate enhances the production of alcohol to an extent of 3.89% and 14.7% respectively in comparison to control. Singh and Srivastava[197] studied efficacy of ethidium bromide on SmF biosynthesis of homolactic acid by Lactobacillus helveticus-2733 and found that EtBr in lower concentrations enhances production of lactic acid significantly. Savita ET al.[198] studied homolactic acid fermentation exposed to benzopyrone mutagen and reported that benzopyrene has influenced the production of homolactic acid significantly and have enhanced the yield of homolactic acid to an extent of 11.32% in comparison to control. Chanchal et al[199] studied mutagenic action of iodine azide and 2-bromophenyl hydrazine hydrochloride on fermentative production of bioalcohol and found that 2-bromo chemical mutagen enhance the yield of lactic acid while iodine azide was inhibitory and reduced the yield of bioalcohol. Savita et al[200]in their observation found that aflatoxine chemical mutagen under trial has influenced the production of homolactic acid insignificantly and have decreased the yield of homolactic acid to a great extent in comparison to control. Singh and Srivastava[201] studied efficacy of nitrofurazone on SmF biosynthesis of homolactic acid by Lactobacillus helveticus-2733 and found that nitrofurazone in lower concentrations is slightly stimulatory for SmF biosynthesis of homolactic acid while at higher concentration it has been found quite insignificant. Ranveer[202] has studied nimorazole and diepoxybutane mutagens on SmF transformation of molasses pollutant to alcohol by Saccharomyces cerevisiae - 1255 and have observed both the chemical mutagens effective for alcoholic fermentation and enhanced the production of alcohol to an extent of 5.88% and2.96% respectively in comparison to control. The efficacy of sodium diethyldithiocarbamate on SmF biosynthesis of homolactic acid by Lactobacillus helveticus-2733 has been reported and has been found that sodium diethyldithiocarbamate in all concentration has detrimental effect on production of homolactic acid and retards thereby the yield of homolactic acid.[203] The efficacy of d,l-diepoxybutane on SmF biosynthesis of homolactic acid by Lactobacillus helveticus - 2733 was also studied and has been reported that d,l-diepoxybutane enhance the production of lactic acid[204] significantly to an extent of 11.39985% higher in comparison to control fermentor flasks. Mutagenic action of nitrofurantoin was studied on microbial biodegradation of molasses to ethanol by Saccharomyces cerevisiae -3078. It has been reported that nitrofurantoin enhances the yield of ethanol[205] significantly to an extent of 5.45722% higher in comparison to control.

The efficacy of furylfuramide on biosynthesis of homolactic acid by Lactobacillus helveticus - 2733 has been reported by Singh and Singh[206] assessed. It has been found that furylfuramide is active at the concentration of 5.0 μg/100 mL and enhances the production of lactic acid to an extent of 1.24990 % higher in comparison to control. However, higher concentration of furylfuramide has been found inhibitory and retards the yield of lactic acid. Mutagenic action of diphenylnitrosamine was also studied by Geeta Kumari et al.[207]on biotransformation of molasses to ethanol by Saccharomyces cerevisiae S- 1605. It has been reported that diphenylnitrosamine enhances the yield of ethanol significantly to an extent of 11.02941% higher in comparison to control.

The influence of diepoxybutane (DB) on bioenergetics transformation of sucrose to citric acid by A. oryzae NCIM-963 has been studied. It has been reported[208] that DB has stimulatory effect on citric acid fermentation and the yield of citric acid is enhanced to an extent of 6.21374 g/100 ml when 11% sucrose solution is allowed to ferment for 11 days at the pH 2.1 and temperature 26^0C in comparison to control.

The efficacy of furoxone on biotransformation of molasses to ethanol by Saccharomyces cerevisiae NCIM-3044 has been assessed. It was reported[209] that furoxone enhances the production of ethanol significantly to an extent of 16.87116% higher in comparison to control fermentor flasks.

The influence of 4-nitroquinoline-1-oxide on bioenergetics transformation of sucrose to citric acid by A. clavatus NCIM-1885 has been studied. It has been reported[210] that 4-nitroquinoline-1-oxide has stimulatory effect on citric acid fermentation and the yield of citric acid is enhanced to an extent of 6.01273 g/100 ml when 12% sucrose solution is allowed to ferment for 11 days at the pH 2.2 and temperature 29^0C in comparison to control.

The efficacy of 2-naphthylhydroxylamine on bioenergetic dissimilation of molasses to alcohol by Saccharomyces cerevisiae J-54 has been assessed. It has been observed[211] that the mutagenic chemical 2-naphthyl hydroxylamine is most effective at the molar concentration 6.0×10^{-5}M and enhances the production of alcohol to an extent of 17.73049% higher in comparison to control. However, 2-naphthyl hydroxylamine under trial is stimulatory at all its molar concentration used, i.e., from 1.0×10^{-5}M, to 10×10^{-5}M.

The efficacy of di (2-chloroethyl) sulphide on LSF production of lactic acid by Lactobacillus bulgaricus NCIM 2159 has been assessed. It has been reported[212] that di (2-chloroethyl) sulphide is very active at the concentration 8.0×10^{-5}M and enhanced the production of lactic acid to an extent of 10.709993668% higher in comparison to control. However, higher concentration of di (2-chloroethyl) sulphide has been found insignificant are retards the yield of lactic acid.

The influence of N-acetyl-4-biphenylhydroxylamine on LSF biosynthesis of citric acid by Aspergillus Niger NCIM-1269 has been studied. It has been reported[213] that N-acetyl-4-biphenylhydroxylamine has stimulatory effect on citric acid fermentation and the yield of citric acid is enhanced to an extent of 8.801057276 % higher in comparison to control fermentor flask when 25% molasses solution is allowed to ferment for 10 days at the pH 2.2 and temperature 29^0C. The efficacy of 2-aminoethanethiol on alcoholic fermentation by SmF technique has been assessed. It has been reported[214] that the mutagen 2-aminoethanethiol under trial has stimulatory effect on alcoholic fermentation and enhances the yield of ethanol to an extent of 7.183098591% higher in comparison to control fermentor flasks, i.e., 7.10ml/100mL in 50 hours of optimum incubation period, 5.1 pH and 27^0C temperature with 24% molasses solution.

The efficacy of 2-hydroxyethylhydrazine[215] on facile biosynthesis of homolactic acid by L.casei NCIM-1159 has been reported. It has been assessed that 2-hydroxyethyl hydrazine is very active at the concentration 6.0×10^{-5}M and enhances the yield of homolactic acid to an extent of 8.6127218% higher in comparison to control. However, higher concentration of 2-hydroxy ethylhydrazine has been reported insignificant and retards the yield of lactic acid.

The efficacy of 1-allyl-2, 2-dimethoxy-3, 4-methylenedioxybenzene[216] on production of citric acid by LSCF process has been assessed. It has been reported that 1-allyl-2, 2-dimethoxy-3, 4-methylenedi -oxybenzene has stimulatory effect on the production of citric acid and enhances the yield of citric acid to an extent of 8.1950085% higher in comparison to control fermentor flasks, i.e., 7.05896320 g/100 ml in the optimized conditions. It is obvious from above review of literature that various chemical mutagens and some other mutagenic agents are used to produce mutants.

Thus, it is concluded that a large number of mutagens have been employed to generate the mutatnts of different microbes but still there are some chemical mutagens whose influence on lactic acid fermentation by species of Lactobacillus have not been well studied and established. Moreover, survey of the literature reveals that there has been not enough mention to study the lactic acid fermentation exposed to mutagens especially chemical mutagens. Therefore, in the present investigation the author has made an attempt to study the lactic acid fermentation by Lactobacillus bulgaricus NCIM-2359 exposed to the following chemical mutagens mentioned below:

a) Benzidine
b) 3, 3'-dichlorobenzidine
c) 1-methyl-3-nitro-1-nitrosoguanidine
d) N-methyl-N-nitrosoethyl carbamate

5.6 Experimental:

The influence of benzidine on lactic acid fermentation by using the bacterial strain of Lactobacillus bulgaricus NCIM-2359.

The composition of the production medium for the production of lactic acid by fermentation was prepared as follows:

Molasses	:	20% (w/v)
Malt Extract	:	0.60 %
Yeast Extract	:	0.60 %
Peptone	:	0.60%
$(NH_4)_2HPO_4$	:	0.60%
$CaCO_3$	:	8.0%
pH	:	6.1
Distilled Water	:	To make up 100 ml.

The pH of the medium was adjusted to 6.1 by adding requisite amount of phosphate-buffer solution, and the pH was also ascertained by a pH meter. The above composition medium represents volume of a fermentor flask, i. e., "100ml" production medium for lactic acid fermentation.

Now, the same production medium for lactic acid fermentation by Lactobacillus bulgaricus NCIM-2359 was prepared for 99 fermentor flasks, i. e., each fermentor flask containg '100 ml' of production medium. The above fermentor flasks were then arranged in ten sets, each comprising 9 fermentor flask.

Each set was again rearranged in three subsets, each comprising of 3 fermentor flasks. The remaining nine fermentor flasks out of 99 fermentor flasks were kept as control and these were also rearranged in three subsets each consisting of three fermentor flasks.

Now M/1000 solution/suspension of benzidine was prepared and 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0 ml of this solution was added to the fermentor flasks of 1st to 10th sets respectively.

The control fermentor flasks contain no chemical mutagens. Now the total volume in each fermentor flask were made up to 100ml by adding requisite amount of distil water. Thus, the concentration of-benzidine in 1st, 2nd, 3rd, 4th, 5th, 6th, 7th, 8th, 9th and 10th subsets were approximately as given below:

	$A \times 10^{-x}M$	$5.0 \times 10^{-5}M$	
i. e.,	$1.0 \times 10^{-5}M$	$6.0 \times 10^{-5}M$	Where A = amount of
	$2.0 \times 10^{-5}M$	$7.0 \times 10^{-5}M$	chemical mutagen
	$3.0 \times 10^{-5}M$	$8.0 \times 10^{-5}M$	in ml,
	$4.0 \times 10^{-5}M$	$9.0 \times 10^{-5}M$	i.e., 1.0 ml to 10ml.
	$10.0 \times 10^{-4}M$		x = molarity of the
			Solution containing Chemical mutagen

The fermentor flasks were then sterilized, cooled, inoculated, incubated and analysed after 3, 6 and 9 days for lactic acid formed and molasses sugars left unfermented as described in the experimental portion, i. e., chapter II of this thesis.

The experimental procedure for the study of influence of other chemical mutagen were exactly the same as described above with the only difference that in place of M/1000 solution of benzidine other chemical mutagen under trials were added to the lactic acid fermentation medium respectively.

5.7 Results and Discussion:

The results obtained in the study of the influence of some chemical mutagens on lactic acid fermentation by Lactobacillus bulgaricus NCIM-2359 ¬¬¬are tabulated in the tables from 1 to 4.

Table – 5.1: Lactic Acid Fermentation by Lactobacillus Bulgaricus NCIM-2359 Exposed To Benzidine

Concentration of AOM Used A x 10^{-X} M	Incubation Period in Hours	Yield of Lactic Acid*in g/100 ml	Molasses Substrate* Left Unfermented in g/100 ml	% of Lactic Acid Increase in 3, 6, 9 Days of Incubation Pd.
Control (– Mutagen)	3	6.1088532	3.8912463	—
	6	8.1579630	1.8420567	—
	9	7.9541847	1.6319838	—
1.0 x 10^{-5}M (+Mutagen)	3	6.1462275	3.8538720	(+) 0.6118055
	6	8.2852753	1.8141675	(+) 1.5605893
	9	8.0433815	1.6148695	(+) 1.1213820
2.0 x 10^{-5} M (+Mutagen)	3	6.1899791	3.8362261	(+) 1.3280053
	6	8.3784390	1.8098679	(+) 2.7025864
	9	8.1293862	1.6081658	(+)2.2026330
3.0 x 10^{-5}M (+Mutagen)	3	6.2253416	3.8172266	(+)1.9068783
	6	8.4364398	1.7861342	(+) 3.4135580
	9	8.1859560	1.5735960	(+) 2.9138284
4.0 x 10^{-5}M (+Mutagen)	3	6.4276139	3.7638252	(+) 5.2180121
	6	8.7138145	1.6986013	(+) 6.8136065
	9	8.4395978	1.5045131	(+) 6.1026128
5.0 x 10^{-5}M (+Mutagen)	3	6.4693856	3.7281810	(+) 5.9018016
	6	8.8598589	1.6235910	(+) 8.6038132
	9	8.5359412	1.4175654	(+)7.3138419
Control (– Mutagen)	3	6.1088532	3.8912463	—
	6	8.1579630	1.8420567	—
	9	7.9541847	1.6319838	—
6.0 x 10^{-5}M** (+Mutagen)	3	6.4948119	3.5051869	(+) 6.3180221
	6	8.9259125***	1.3286950	(+) 9.4134957
	9	8.6393446	1.2842671	(+) 8.6138293
7.0 x 10^{-5}M (+Mutagen)	3	6.4398312	3.5618635	(+) 5.4180054
	6	8.8198581	1.3694263	(+) 8.1134849
	9	8.5518485	1.3073741	(+) 7.5138285

Table -5.1.1: Lactic Acid Fermentation by Lactobacillus Bulgaricus NCIM-2359 Exposed To Benzidine

Concentration of AOM Used A x 10^{-x} M	Incubation Period in Hours	Yield of Lactic Acid* in g/100 ml	Molasses Substrate* Left Unfermented in g/100 ml	% of Lactic acid Increase in 3, 6, 9 Days of Incubation pd.
8.0×10^{-5} M (+Mutagen)	3	6.3665250	3.6364817	(+) 4.2180060
	6	8.6720246	1.3941366	(+) 6.3013475
	9	8.3599582	1.3294110	(+) 5.1013839
9.0×10^{-5} M (+Mutagen)	3	****	–	–
	6	****	–	–
	9	****	–	–
10.0×10^{-5} M (+Mutagen)	3	****	–	–
	6	****	–	–
	9	****	–	–

* Each value represents mean of three trials.
** Optimum concentration of AOM.
*** Optimum yield of lactic acid
**** Insignificant value

(+) Values indicate % increases in the yield of lactic acid

Experimental deviation + 2.5 – 3.5%

Table – 5.2: Lactic Acid Fermentation by Lactobacillus Bulgaricus NCIM-2359 Exposed To 3, 3'-Dichlorobenzidine

Concentration of AOM Used A x 10^{-x} M	Incubation Period in Hours	Yield of Lactic Acid* in g/100 ml	Molasses Substrate* Left Unfermented in g/100 ml	% of Lactic Acid Increase in 3, 6, 9 Days of Incubation pd.
Control (–Mutagen)	3	6.1133671	3.8765326	—
	6	8.2346156	1.7656313	—
	9	7.9762581	1.5862016	—
1.0×10^{-5} M (+Mutagen)	3	6.2183945	3.7715049	(+)1.7179959
	6	8.4579612	1.6764853	(+) 2.7122771
	9	8.1448594	1.4794268	(+)2.1137894
2.0×10^{-5} M (+Mutagen)	3	6.2428479	3.7434256	(+) 2.1179948
	6	8.5486319	1.6191370	(+)3.8133692
	9	8.2246223	1.4272163	(+)3.1137934

Concentration of AOM Used A x 10^{-x} M	Incubation Period in Hours	Yield of Lactic Acid* in g/100 ml	Molasses Substrate* Left Unfermented in g/100 ml	% of Lactic Acid Increase in 3, 6, 9 Days of Incubation pd.
3.0 x 10^{-5} M (+Mutagen)	3	6.3651250	3.6348690	(+) 4.1181544
	6	8.8039142	1.1946537	(+) 6.9134811
	9	8.4639198	1.1035361	(+) 6.1139157
4.0 x 10^{-5} M (+Mutagen)	3	6.4751568	3.5248430	(+) 5.9180103
	6	8.9685928***	1.0314071	(+) 8.9133146
	9	8.6162564	1.0259364	(+) 8.0237912
5.0 x 10^{-5} M (+Mutagen)	3	6.4323620	3.5575624	(+) 5.2179902
	6	8.8368426	1.0398675	(+) 7.3133589
	9	8.4958149	1.0298843	(+) 6.5137912
Control (– Mutagen)	3	6.1133671	3.8765326	—
	6	8.2346156	1.7656313	—
	9	7.9762581	1.5862016	—
6.0 x 10^{-5} M** (+Mutagen)	3	6.3956829	3.6043169	(+) 4.6180082
	6	8.1215678	1.2784330	(+) 5.9134782
	9	8.3921245	1.2594263	(+) 5.2138031
7.0 x 10^{-5} M (+Mutagen)	3	6.3396716	3.6385461	(+) 3.7017979
	6	8.6299873	1.3064627	(+) 4.8013376
	9	8.3043946	1.2894160	(+) 4.1139152

Table – 5.2.1: Lactic Acid Fermentation by Lactobacillus Bulgaricus NCIM-2359 Exposed To 3, 3'-Dichlorobenzidine

Concentration of AOM Used A x 10^{-x} M	Incubation Period in Hours	Yield of Lactic Acid* in g/100 ml	Molasses Substrate* Left Unfermented in g/100 ml	% of Lactic Acid Increase in 3, 6, 9 Days of Incubation pd.
8.0 x 10^{-5} M (+Mutagen)	3	6.2611986	3.7387915	(+) 2.418682
	6	8.5568659	1.3984163	(+) 3.9133617
	9	8.2325983	1.3670139	(+) 3.2137901
9.0 x 10^{-5} M (+Mutagen)	3	****	–	–
	6	****	–	–
	9	****	–	–
10.0 x 10^{-5} M (+Mutagen)	3	****	–	–
	6	****	–	–
	9	****	–	–

* Each value represents mean of three trials.
** Optimum concentration of AOM.
*** Optimum yield of lactic acid
**** Insignificant value

(+) Values indicate % increases in the yield of lactic acid

Experimental deviation + 2.5 – 3.5%

Table -5.3: Lactic Acid Fermentation by Lactobacillus Bulgaricus NCIM-2359 Exposed To 1-Methyl-3-Nitro-1-Nitrogoguanidine

Concentration of AOM Used A x 10^{-x} M	Incubation Period in Hours	Yield of Lactic Acid* in g/100 ml	Molasses Substrate* Left Unfermented in g/100 ml	% of Lactic Acid Increase in 3, 6, 9 days of Incubation pd.
Control (–Mutagen)	3	6.1241869	3.8658131	—
	6	8.3540970	1.6458714	—
	9	7.9936560	1.4371863	—
1.0×10^{-5} M (+Mutagen)	3	6.1619431	3.8281426	(+) 0.6165095
	6	8.4638112	1.5364260	(+) 1.3132981
	9	8.0587053	1.3681056	(+) 0.8137615
2.0×10^{-5} M (+Mutagen)	3	6.2355321	3.7643810	(+) 1.8181221
	6	8.6799598	1.3200801	(+) 3.9006346
	9	8.2095958	1.2081634	(+) 2.7013897
3.0×10^{-5} M (+Mutagen)	3	6.2845253	3.7154760	(+) 2.6181173
	6	8.8397356	1.1698126	(+) 5.8131788
	9	8.3994679	1.1354929	(+) 4.7013769
4.0×10^{-5} M (+Mutagen)	3	6.3753799	3.6260127	(+) 4.1016547
	6	9.0068271	1.1280480	(+) 7.8132932
	9	8.4983764	1.1041457	(+) 6.3140120
5.0×10^{-5} M (+Mutagen)	3	6.3953645	3.6046145	(+) 4.4279772
	6	9.0318789	1.1079371	(+) 8.1131677
	9	8.5383462	1.0841826	(+) 6.8140310
Control (–Mutagen)	3	6.1241869	3.8658131	—
	6	8.3540970	1.6458714	—
	9	7.9936560	1.4371863	—
6.0×10^{-5} M** (+Mutagen)	3	6.5591256	3.4418073	(+) 7.1019827
	6	9.1321390	1.1008136	(+) 9.3132986
	9	8.6822312	1.0183692	(+) 8.6140209

Concentration of AOM Used A x 10^{-x} M	Incubation Period in Hours	Yield of Lactic Acid* in g/100 ml	Molasses Substrate* Left Unfermented in g/100 ml	% of Lactic Acid Increase in 3, 6, 9 days of Incubation pd.
7.0×10^{-5} M (+Mutagen)	3	6.6825889	3.3117400	(+) 9.1179777
	6	9.4245315***	1.1006312	(+) 12.8132878
	9	8.8181266	1.0135296	(+) 10.3140615

Table – 5.3.1: Lactic Acid Fermentation by Lactobacillus Bulgaricus NCIM-2359 Exposed To 1-Methyl-3-Nitro-1-Nitrogoguanidine

Concentration of AOM Used A x 10^{-x} M	Incubation Period in Hours	Yield of Lactic Acid* in g/100 ml	Molasses Substrate* Left Unfermented in g/100 ml	% of Lactic Acid Increase in 3, 6, 9 days of Incubation pd.
8.0×10^{-5} M (+Mutagen)	3	6.5233691	3.4765308	(+) 6.5181256
	6	9.2081612	1.1013620	(+) 10.2232976
	9	8.6582395	1.0156311	(+) 8.3138866
9.0×10^{-5} M (+Mutagen)	3	6.4376315	3.5623612	(+) 5.1181422
	6	9.0911939	1.1018602	(+) 8.8231786
	9	8.4983583	1.0194756	(+) 6.3137855
10.0×10^{-5} M (+Mutagen)	3	****	–	–
	6	****	–	–
	9	****	–	–

* Each value represents mean of three trials.
** Optimum concentration of AOM.
*** Optimum yield of lactic acid
**** Insignificant value

(+) Values indicate % increases in the yield of lactic acid

Experimental deviation + 2.5 – 3.5%

Table – 5.4: Lactic Acid Fermentation by Lactobacillus Bulgaricus NCIM-2359 Exposed To Exposed To N-Methyl-N-Nitrosoethyl Carbamate

Concentration of AOM Used A x 10^{-x} M	Incubation Period in Hours	Yield of Lactic Acid* in g/100 ml	Molasses Substrate* Left Unfermented in g/100 ml	% of Lactic Acid Increase in 3, 6, 9 Days of Incubation pd.
Control (– Mutagen)	3	6.0816379	3.9163692	—
	6	8.1273413	1.8726590	—
	9	7.9381629	1.6342065	—
1.0 x 10^{-5} M (+Mutagen)	3	6.1253194	3.8727269	(+) 0.7182522
	6	8.2259786	1.77402210	(+) 1.2136478
	9	8.0097163	1.5418279	(+) 0.9013848
2.0 x 10^{-5} M (+Mutagen)	3	6.1374736	3.8625259	(+) 0.9181029
	6	8.3062629	1.6937313	(+) 2.2014776
	9	8.0424691	1.5303681	(+) 1.3139841
3.0 x 10^{-5} M (+Mutagen)	3	6.2378983	3.7621054	(+) 2.5693801
	6	8.3829346	1.6169543	(+) 3.1448574
	9	8.1694769	1.5084927	(+) 2.9139827
4.0 x 10^{-5} M (+Mutagen)	3	6.2399853	3.7501398	(+) 2.6036966
	6	8.4657369***	1.5342529	(+) 4.1636691
	9	8.2399240	1.4794266	(+) 3.8016971
5.0 x 10^{-5} M (+Mutagen)	3	6.2095639	3.8011260	(+) 2.1034793
	6	8.3794898	1.5968261	(+) 3.1024721
	9	8.1535942	1.4998254	(+) 2.7138684
Control (– Mutagen)	3	6.0816379	3.9163692	—
	6	8.1273413	1.8726590	—
	9	7.9381629	1.6342065	—
6.0 x 10^{-5} M** (+Mutagen)	3	6.1923185	3.8076851	+ 1.819943
	6	8.3641352	1.6358639	+ 2.9135468
	9	8.0899886	1.5276195	+ 1.9126049
7.0 x 10^{-5} M (+Mutagen)	3	6.1799562	3.8201398	+ 1.6166417
	6	8.3072438	1.6978501	+ 2.2135467
	9	8.0582453	1.5764261	+ 1.5127227

Table – 5.4.1: Lactic Acid Fermentation by Lactobacillus Bulgaricus NCIM-2359 Exposed To Exposed To N-Methyl-N-Nitrosoethyl Carbamate

Concentration of AOM Used A x 10^{-x} M	Incubation Period in Hours	Yield of Lactic Acid* in g/100 ml	Molasses Substrate* Left Unfermented in g/100 ml	% of Lactic Acid Increase in 3, 6,9 Days of Incubation pd.
	3	****	–	–

Concentration of AOM Used A x 10^{-x} M	Incubation Period in Hours	Yield of Lactic Acid* in g/100 ml	Molasses Substrate* Left Unfermented in g/100 ml	% of Lactic Acid Increase in 3, 6,9 Days of Incubation pd.
8.0×10^{-5} M (+Mutagen)	6	****	–	–
	9	****	–	–
9.0×10^{-5} M (+Mutagen)	3	****	–	–
	6	****	–	–
	9	****	–	–
10.0×10^{-5} M (+Mutagen)	3	****	–	–
	6	****	–	–
	9	****	–	–

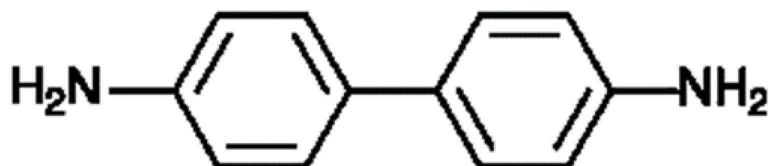
* Each value represents mean of three trials.
** Optimum concentration of AOM.
*** Optimum yield of lactic acid
**** Insignificant value

(+) Values indicate % increases in the yield of lactic acid

Experimental deviation + 2.5 – 3.5%

5.8 Discussion:

The data recorded in the table 1 shows that benzidine has stimulatory effect on lactic acid fermentation by Lactobacillus bulgaricus NCIM-2359



Benzidine

The maximum yield of lactic acid, i.e.; 8.9259125g/100jml in the presence of benzidine was observed at 6.0×10^{-5}M molar concentration in 9 days of optimum incubation period which is 9.4134957% higher in comparison to control fermentor flasks, i.e.; 8.1579630/100 ml in the same times course and other same experimental parameters.

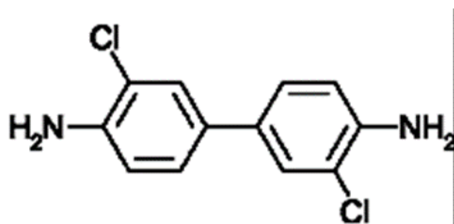
The higher molar concentrations of benzidine were not much favourable for the lactic acid by Lactobacillus bulgaricus NCIM-2359. So the gradual addition of the mutagen benzidine after certain concentrations were not beneficial for the lactic acid fermentation process.

It has been observed that molar concentration of the mutagen, i.e., benzidine from 1.0 x 10^{-5} M to 6.0 x 10^{-5} M enhances the yield of lactic acid to a certain order being 1.5605893%, 2.7025864%, 3.4135580%, 6.8136065%, 8.6038132 and 9.4134957% higher in comparison to control flasks but at 7.0 x 10^{-5} M and 8.0 x10^{-5} M the yield of lactic acid shifted to be 8.1134849% and 6.3013475% higher in comparison to previous concentrations of benzidine taken into experimental trials.

It has been observed further that after optimum concentration, i.e., 6.0 x 10^{-5} M, the addition of the same mutagen to the production medium causes fall in the yield of lactic acid gradually and at 9.0 x 10^{-5}M and 10.0 x 10^{-5}M.; the production of lactic acid has been found insignificant. However, at all the experimental concentrations of mutagen used the yield of lactic acid by submerged fermentation has been found higher in comparison to control fermentor flasks.

5.9 The Influence of 3, 3'-Dichlorobenzidine:

The data recorded in the **table-5.2** shows that 3, 3'-dichlorobenzidine **also** has stimulatory effect on lactic acid fermentation by Lactobacillus bulgaricus NCIM-2359



3, 3'-Dichlorobenzidine

The data (vide **Table-5.2**) reveals that the chemical mutagen 3,3'-dichlorobenzidine stimulates the lactic acid fermentation process and enhances the yield of lactic acid up to its 3,3'-dichlorobenzidine concentrations from 1.0 x 10^{-5} to 4.0 x 10^{-5} M.

The effect of 3,3'-dichlorobenzidine on the productivity (yield) of lactic acid was gradually in increasing order and attains its best role at 4.0 x 10^{-5}M where maximum yield of lactic acid, i.e., 8.9685928 g/100 ml is given in 9 days of optimum incubation period which is 8.9133146 % higher in comparison to control fermentor flask, i.e., 8.2346156 g/100 ml.

In the second phase of mutagenic chemical's effect the molar concentration, i.e., from 5.0 x 10^{-5}M to 10 x 10^{-5}M the production of lactic acid has been enhanced but the order of lactic acid productivity is reverse in respect to increasing molar concentrations of 3,3'-dichlorobenzidine.

However lactic acid fermentation by Lactobacillus bulgaricus NCIM-2359 under the influence of each concentration of 3, 3'-dichlorobenzidine used has been stimulating and the yield of lactic acid has been found greater than that obtained in the control fermentor flasks. In both the phases the order of productivity and % of lactic acid formed is as below:

Phase- I:

Concentration of 3, 3'-dichlorobenzidine from 1.0 x 10-5M to 4.0 x 10-5M.

Productivity of Lactic Acid:

2.7122771%, 3.8133692%, 6.9134811% and 8.9133146%.

Phase – II:

Concentration of 3, 3'-dichlorobenzidine from 5.0 x 10-5 M to 10.0 x 10-5M.

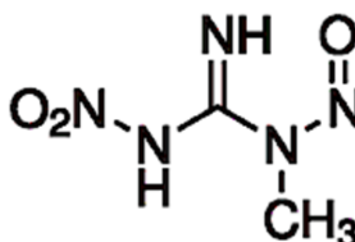
Productivity of Lactic Acid:

7.3133589%, 5.9134782%, 4.8013376%, 3.9133617%, insignificant and insignificant.

Exposure of bacterial strain to 3, 3'-dichlorobenzidine may produce a variety of effects. Depending upon the concentration of 3, 3'-dichlorobenzidine to which bacterial strain Lactobacillus bulgaricus NCIM-2359 were exposed may influence disruption of cells, precipitation of cell protein, and inactivation of enzymes and leakage of amino acids from the cells. Although the special mode of action is not very clear, there is a consensus that the lethal effect is associated with physical damage of the membrane structure of the cell surface, which initiates further deterioration. Thus, it is concluded that Lactobacillus bulgaricus NCIM-2359 at lower concentrations is stimulatory and at higher concentrations is deterioratory for the lactic acid fermentation by Lactobacillus bulgaricus NCIM-2359

5.9.1 The Influence of 1-Methyl-3-Nitro-1-Nitrosoguanidine:

The data given in the table 3 shows that the mutagen 1-methyl-3-nitro-1-nitrosoguanidine has been found stimulatory for lactic acid fermentation by Lactobacillus bulgaricus NCIM-2359. From the data given in the table it is obvious that 1-methyl-3-nitro-1-nitrosoguanidine influences the lactic acid fermentation process in different phases. The main characteristics of the 1-methyl-3-nitro-1-nitrosoguanidine is as follows:



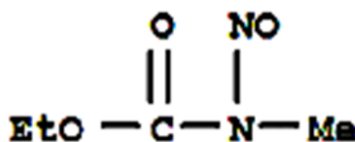
1-Methyl-3-Nitro-1-Nitrosoguanidine

a. 1-methyl-3-nitro-1-nitrosoguanidine is stimulatory at its all molar Concentrations used during course of the lactic acid fermentation, i.e. from 1.0 x10^{-5}M to 10.0 x 10^{-5}M.
b. The molar concentration 1.0x10^{-5}M, 2.0 x10^{-5}M and 3.0 x 10^{-5} of 1-methyl-3-nitro-1-nitrosoguanidine influence the yield of lactic acid in an approximately regular doubling order after each state i.e. 1.3132981%, 3.9006346% and 5.8131788%.
c. The molor concentration 4.0 x 10^{-5}M, 5.0 x 10^{-5}M and 6.0 x10^{-5} of 1-methyl-3-nitro-1 nitrosoguanidine now influence the productivity of lactic acid in a regular manner enhancing the yield from X to 1 + X and 2+ X approx. respectively where X is the % increase in the yield of lactic acid in comparison to control. The % increase in the yield of lactic acid at respective molar concentration of 1-methyl-3-nitro-1-nitrosoguanidine has been found to be as follows: 7.8132932%, 8.01131677%, and 9.3132986 (X, 1 + X, and 2 + X)
d. The molar concentrations, i.e., 8.0 x 10^{-5}M and 9.0 x 10^{-5} of 1-methyl-3-nitro-1-nitrosoguanidine influences the yield of lactic acid in decreasing order and therefore, the % difference in the yield of lactic acid has been found to be in the order as mentioned below: 10.2232976%, 8.8231786% and insignificant respectively.
e. The higher molar concentrations, i.e. 10 x 10^{-5}M of 1-methyl-3-nitro-1-nitrosoguanidine decreases the yield of lactic acid and the result is very much insignificant.

5.9.2 The Influence of N-Methyl-N-Nitrosoethyl Carbamate:

The data recorded in the table-4 shows that the chemical mutagen N-methyl-N-nitrosoethyl carbamate at higher concentration has insignificant effect on lactic acid fermentation by Lactobacillus bulgaricus NCIM-2359.

The yields of lactic acid obtained at concentration of mutagens i.e. 1.0×10^{-5} to 4.0×10^{-5}M has been found in increasing order and slight better in comparison to control fermenter flasks.



N-Methyl-N-Nitrosoethyl Carbamate

The maximum yield of lactic acid, i.e., 8.4657369 g/100 ml. in the presence of N-methyl-N-nitrosoethyl carbamate , i.e., at 4.0 x 10^{-5} M was found in 6 days of optimum incubation period and this optimum yield has been found to be 4.1636691% higher in comparison to control fermentor flasks, i.e., 8.1273413g/100ml.

However, at higher concentrations, i.e., 8.0×10^{-5} M and onwards of the chemical mutagen, i.e., N-methyl-N-nitrosoethyl carbamate the production of lactic acid was found almost negligible and insignificant.

Thus, it is obvious from the results that the chemical mutagen N-methyl-N-nitrosoethyl carbamate under trial at higher concentrations is much detrimental and inhibitory for lactic acid fermentation by Lactobacillus bulgaricus NCIM-2359.

It is interesting to note that benzidine, 3, 3'-dichlorobenzidine, and 1-methyl-3-nitro-1-nitrosoguanidine stimulates and enhances the lactic acid fermentation by Lactobacillus bulgaricus NCIM-2359 significantly. It has been observed that 1-methyl-3-nitro-1-nitrosoguanidine has influenced the production of lactic acid significantly to a great extent while benzidine and 3,3'-dichlorobenzidine were equally effective for lactic acid fermentation by Lactobacillus bulgaricus NCIM-2359 and could increase the lactic acid production nearly in the range of 9.4134957 and 8.9133146 in comparison to control.

However, the N-methyl-N-nitrosoethyl carbamate was found to be detrimental and inhibitory at higher concentration which deactivates lactic acid by Lactobacillus bulgaricus NCIM-2359.

From the present investigation it is obvious that the chemical mutagens used e.g. benzidine, 3, 3'-dichlorobenzidine, 1-methyl-3-nitro-1-nitrosoguanidine and N-methyl-N-nitrosoethyl carbamate at their however concentrations are all useful for lactic acid production by Lactobacillus bulgaricus NCIM-2359 and therefore, can be employed for the improved yield of lactic acid.

5.10 Summary:

A comparative assessment of the different chemical mutagens on lactic acid fermentation by Lactobacillus bulgaricus NCIM-2359 can be had from the table-5 given below.

Table – 5.5: Study of the Influence of Benzidine; 3, 3'-Dichlorobenzidine; 1-Methyl-3-Nitro-1-Nitrosoguanidine and N-Methyl-N-Nitrosoethyl Carbamate on Lactic Acid Fermentation by Lactobacillus Bulgaricus NCIM-2359 in 6 Days of Optimum Incubation Period

Chemical Mutagens Used	Optimum Concentration of the Mutagens Used.	Max. Yield of Lactic Acid* in Control Flasks in g/100ml	Max. Yield of Lactic Acid* in the Presence of Mutagens	% of Lactic Acid Increase in 6 Days Of Incubation Pd.
1	6.0×10^{-5} M	8.1579630	8.9259145	(+) 9.4134957
2	4.0×10^{-5} M	8.2346156	8.9685928	(+) 8.9133146
3	7.0×10^{-5} M	8.3540970	9.4245315	(+)12.813283
4	4.0×10^{-5} M	8.1273413	8.4657369	(+) 4.1636691

* Each value represents mean of three observations (+) Values indicates % increase in the yield of lactic acid.
Experimental deviation (+) 2.5 to 3.5%.

a. Benzidine
b. 3, 3’-dichlorobenzidine
c. 1-methyl-3-nitro-1-nitrosoguanidine
d. N-methyl-N-nitrosoethyl carbamate

Thus, it may be summarized that benzidine; 3, 3’-dichlorobenzidine and 1-methyl-3-nitro-1-nitrosoguanidine stimulates and enhances the lactic acid fermentation by Lactobacillus bulgaricus NCIM-2359.

It has been observed that 1-methyl-3-nitro-1-nitrosoguanidine has influenced the production of lactic acid significantly to a great extent while benzidine and 3, 3’-dichlorobenzidine were approximately equally effective for lactic acid fermentation by Lactobacillus bulgaricus NCIM-2359 and could increase the lactic acid production nearly in the range of 9.4143957 and 8.9133146 in comparison to control.

However, the N-methyl-N-nitrosoethyl carbamate was found to be inhibitory at higher concentration which deactivates lactic acid fermentation by Lactobacillus bulgaricus NCIM-2359.

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