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12. A Study of Lactic Acid Fermentation by Lactobacillus bulgaricus NCIM-2359

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ABSTRACT

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.*

KEYWORDS

Lactic Acid, Fermentation, Lactobacillus bulgaricus, NCIM-2359.

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).

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. [1] 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%.[2] It is expected that by 2013, industrial applications will account for more than half of global lactic acid use.

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.[3] 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 electroneutrality. 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)

Fermentation technology is the oldest of all biotechnological processes. The term is derived from the Latin verb fevere, to boil-- the appearance of furit 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 fermentaiton 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.

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 soyfoods: 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.

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. It is a weak acid with low volatility (Casida [4], 1964). In solutions with roughly 20% or more lactic acid, self-estrification 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 [5], 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.

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

Thermochemistry:

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

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: Characteristics of Lactic Acid (Martin [6], 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.

Chemical Synthesis through Biotechnology:

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

(I) 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.

(II) 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 subcelluar 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 25-120m^3 fermenters at 45-50^oC 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 cacium. 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 stereospecifically reduced to L(+) or (D-) lactic acid

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 favoured high yields of lactic acid.

Forms of lactic acid

Lactic acid occurs in three forms:

(i) Levorotatory lactic acid

(ii) 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.

Homofermentative Species:

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

$$\underset{\text{Glucose}}{C_6H_{12}O_6} \longrightarrow \underset{\text{Lactic acid}}{2CH_3CHOH.COOH}$$

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:

COOH	COOH	
|	|	
HO—C* — H	H —C* —OH	*Chiral Carbon
|	|	
CH_3	CH_3	
L-Lactic acid	***D-Lactic acid***	

The isomer produced is characteristic of the species.

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.

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 CO_2 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:

$$\underset{\text{Pyruvic acid}}{CH_3.CO.COOH + H_2O} \longrightarrow \underset{\text{Acetic acid}}{CH_3.COOH} + \underset{\text{Formic acid}}{HCOOH}$$

The formic acid disappears as CO_2 and H_2 :

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

Neutralization of Acid:

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

$$\underset{\text{Lactic acid}}{2CH_3.CHOH.COOH} + \underset{\text{Calcium Hydroxide}}{Ca(OH)_2} \longrightarrow \underset{\text{Calcium Lactate}}{(CH_3CHOHCOO)_2Ca} + 2H_2O$$

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.

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 upto 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.

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: [7-25]

1. Glucose + ATP → Glucose-6 -phosphate + ADP
2. Glucose-6-phosphate ⇌ Fructose-6-phosphate
3. Fructose-6-phosphate+ ATP → fructose 1: 6-diphosphate + ADP
4. Fructose 1: 6-diphosphate

   3-phosphoglyceraldehyde + dihydroxyacetonephosphate

   (aldolase)

   (isomerase)
5. 3-phosphoglyceraldehyde+H_3PO_4 ⇌ (1: 3-diphosphoglyceraldehyde)
6. (1: 3-diphosphoglyceraldehyde) + DPN ⇌ 1: 3-diphosphoglyceric acid + DPN.2H
7. 1: 3-diphosphoglyceric acid + ADP ⇌ 3-phosphoglyceric acid+ ATP.
8. 3-phosphoglyceric acid ⇌ phosphoglyceric acid

9. 2-phosphoglyceric acid ⇌ ıosphoenol pyruvic acid

$+H_2O$

10. Phosphoenol pyruvic acid + ADP ⇌ pyruvic acid + ATP

Conclusion:

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.

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