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10. Analysis of Lactic Acid Fermentation by *Lactobacillus Bulgaricus* NCIM – 2359

Dr. Vinod Mandal
Associate Professor,
Department of Chemistry,
A. N. College Patna, Patliputra University,
Patna, Bihar.

ABSTRACT:

In this paper 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 sterilisation 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.

KEYWORD:

Lactobacillus bulgaricus, lactic acid fermentation, fermentation, NCIM – 2359.

Introduction:

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 favourite), 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. Experimentation is also used to test existing theories or new hypotheses in order to support them or disprove them. 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" Bacon 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.[1-5]

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.

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, everyone of the equipments 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 equipments 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 economical importance. The role of some chemicals are lethal to microbes and microbial processes. Hypochlorite solutions and phenolics are used as general laboratory infectants. However, these chemicals may not cause complete sterilization under mild conditions.

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.

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).

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).

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 an 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 sterilise 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 sterilisation is also used in the commercial canning of fruits and vegetables and also in order to manufacture sterilised milk.

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.

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.

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., lactic acid fermentation by *Lactobacillus* ulgaricus 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 – 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

The Lactic Acid Bacteria (LAB):

The lactic acid bacteria (LAB) are a group of Grampositive bacteria united by a constellation of morphological and physicological 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 biopreservative organisms in several foods. The biopreservative ability of LAB is due to the production of antibacterial[16-18] and antifungal substances [15-21].

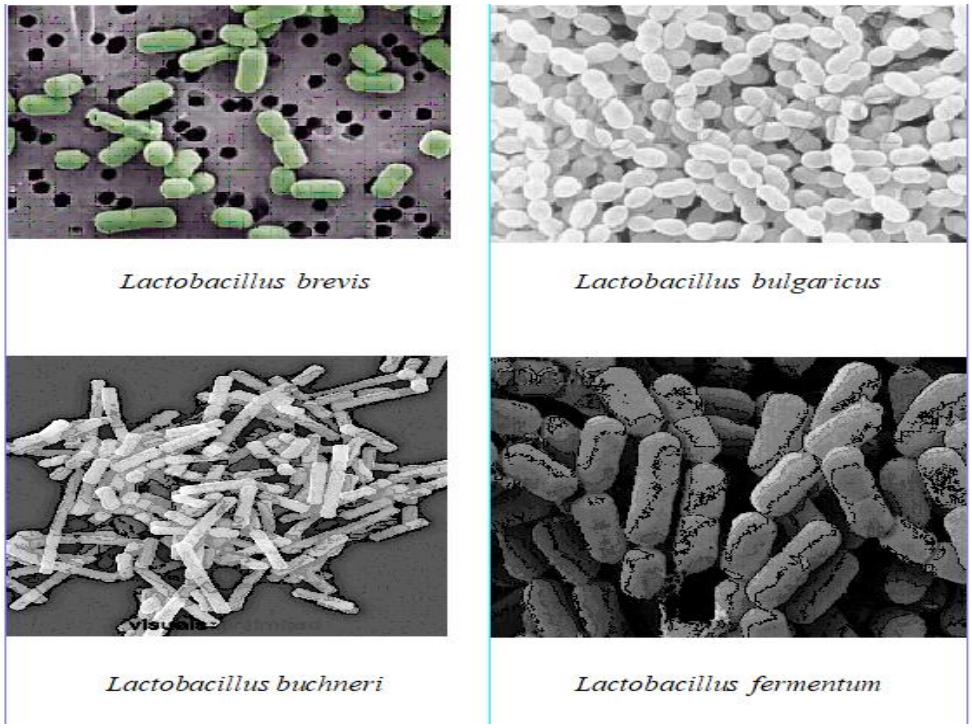
The Microorganism Used:

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).

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.



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

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:

$2CH_3.CHOHCOOH$ + $CaCO_3$

Lactic acid — Calcium carbonate

$(CH_3.CHOH.COO)_2Ca + H_2O + CO_2$

Calcium lactate

Decomposition by dil. H_2SO_4

$2CH_3.CHOH.COOH$ + $CaSO_4$

Free lactic acid — ppt.

An 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[22,23] as follows:

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[24,27].

Reagents:

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

(i) **Sulphuric acid:**
Only AR grade conc. H_2SO_4

(ii) **Calcium hydroxide:**
Calcium hydroxide powder

(iii) **Copper sulphate:**
4% and 20% Solution of $CuSO_4.5H_2O$

(iv) **Sodium hydroxide:**
5% solution of NaOH solution

(v) ***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.

(vi) **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-litre 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.

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.

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 ar 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 through out 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 30^0C 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.

Observation:

The klett readings for lactic acid determination formed during biosynthesis of lactic acid by *Lactobacillus bulgaricus* NCIM – 2359 are shown in the table-2 given below :

Table – 2

Sr. No.	Volume of lactate solution in ml	Lactate in (mg)	Volume of distilled water in (ml)	4% $CuSO_4.5H_2O$ solution (in ml)	Conc. H_2SO_4 in (ml)	p-hydroxy diphenyl Soution 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 $\pm$1.5 to 3.0%.

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.

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 coloured complex which is estimated colorimetrically.

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

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

(ii) Phenol:

Only 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.

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.

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.

Conclusion:

"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 colourimetric 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.

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