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Fermentative Synthesis of Lactic Acid

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Abstract: **Lactic acid, an organic acid is a product of glucose fermentation in certain cells of organisms. Lactic acid is a crucial metabolite that has gained immense industrial importance. Lactic acid application have a wide expanse including its use to synthesize various fermentation products in food industry, pharmaceutical solutions for drug delivery, preservative in foodstuffs, production of surfactants used in soaps and detergents, tanning leather, synthesis of bio composites and as a mordant in textile industries. Due to its wide applications, it is necessary to study and optimize fermentative synthesis of lactic acid. Lactic acid bacteria were isolated from sources of sauerkraut and cheese water. Sauerkraut is a fermentation product that has a number of lactic acid bacteria thriving on it including, *Lactobacillus plantarum, Leuconostoc argentinum, Lactobacillus brevis, Pediococcus pentosaceus*. Cheese being a major source of lactic acid bacteria such as *Lactobacillus helveticus, Lb. delbrueckii subsp. bulgaricus, Lactococcus lactis, Lb. casei* was investigated as a source for the synthesis of lactic acid. Fermentation parameters were optimized and fermentation was carried out for a period of 5 days. Extraction method was followed to separate and purify lactic acid produced. $CaCO_3$ precipitation was carried out to separate lactate from other impurities, followed by acidification with concentrated sulphuric acid. This method showed lesser concentration of lactic acid, so it was further optimized to concentrate the product. In the second method, the concentration of sulphuric acid was lowered (63%) as it outweighed the product and the main impurity water was removed by distillation. $CaCO_3$ precipitation, followed by acidification and distillation is an inexpensive technique in comparison to other techniques. The yield of lactic acid produced was checked by using buttermilk powder as a standard and it was found to be 42.78% and 61% respectively for sauerkraut and cheese mixed cultures.**

Keywords: Bioplastics, Lactic acid, Polylactic acid.

I. Introduction

Lactic acid was firstly discovered in sour milk in 1780 by a Swedish chemist, Carl Wilhelm Scheele, who initially considered it as a milk component. In 1789, Lavoisier named this milk component (acide lactique), which became the possible origin of the current terminology for lactic acid. However, it was in 1857 when Louis Pasteur discovered that it was not a milk component, but a fermentation metabolite generated by certain microorganisms [1].

Lactic acid can be manufactured by either chemical synthesis or fermentation. The commercial process for chemical synthesis is based on lactonitrile. Hydrogen cyanide is added to acetaldehyde in the presence of a base to produce lactonitrile. This reaction occurs in liquid phase at high atmospheric pressures. The crude lactonitrile is recovered, and purified by distillation. It is then hydrolysed to lactic acid, either by concentrated HCl or by H_2SO_4 to produce the corresponding ammonium salt and lactic acid.

On the other hand, an optically pure L(+)- or D(–)-lactic acid can be obtained by microbial fermentation when the appropriate microorganism is selected. The optical purity of lactic acid is crucial to the physical properties of poly (lactic acid) [2], and an optically pure L(+)- or D(–)-lactic acid, rather than racemic DL lactic acid. Lactic acid can be polymerized to produce a high crystalline PLA that is suitable for commercial uses. As a result, the biotechnological production of lactic acid has received a significant amount of interest recently, since it offers an alternative way to prevent environmental pollution caused by the petrochemical industry and the limited supply of petrochemical resources [3].

Lactic acid (2-hydroxypropionic acid CH_3-CHOHCOOH), is the most widely occurring carboxylic acid, having a prime position due to its versatile applications in food, pharmaceutical, textile, leather, and other chemical industries [4].

Lactic acid is used in parenteral/IV solutions, dialysis solutions, controlled drug delivery system, skin disorders, cosmetic and

other applications. Lactic acid occurs in food products as an acidulant, pH regulator and preservative. By being naturally present in foodstuffs it does not introduce a foreign element and provides great bactericidal activity. Lactic acid esters are used as green solvents.

II. Materials and Methods

A. Sources for Isolation of Lactic Acid Bacteria

Sources used for isolation of lactic acid bacteria were cheese and sauerkraut liquid. Sauerkraut was prepared by finely shredding cabbage and storing it in an air tight container after salt addition. The natural flora of cabbage itself contains lactic acid bacteria which grow luxuriantly in the acidic environment created.

Cheese cube was incubated in distilled water at 37^0C for 24 hours to enhance bacterial growth. Both these sources were used as cultures.

B. Isolation of LAB

Isolation of LAB from the samples collected from two different sources was performed by spreading it on the media plates. The four streak plate method was used for streaking the samples on the MRS media (1% peptone, 1% beef extract, 0.4% yeast extract, 2% glucose, 0.5% sodium acetate trihydrate, 0.1% polysorbate 80, 0.2% dipotassium hydrogen phosphate, 0.2% triammonium citrate, 0.02% magnesium sulphate heptahydrate, 0.005% magnesium sulphate tetrahydrate, 1% agar, pH 6.2 at 20^0C; (Himedia Chemicals). These agar plates were incubated for 2 days at room temperature. Distinct colonies of bacteria were picked and sub cultured. These isolated bacteria were stored using MRS slants at 4^0C.

C. Phenotypic Characterization

The isolates from cheese and sauerkraut samples were identified phenotypically by observing their morphology under 40X microscope. Their genera were confirmed using tests such as Gram staining, catalase test, ability to produce CO_2 gas in MRS broth test tubes containing Durham's tube [5].

D. Fermentation and Optimization

Parameters for fermentation such as agitation, temperature were optimised by checking optical density. Fermentation was carried out using suitable media at 37^0C and pH of 6.5 for a period of 4-5 days using flasks of 100 ml. Agitation was kept at 120 rpm throughout fermentation. After fermentation, centrifugation was carried out at 2500 rpm to separate the cell debris and supernatant was collected [6].

E. Lactic Acid Identification Tests

- Titration to check lactic acid: Tests of titration were carried out for both; MRS and MacConkey's broth. Titration was carried out to determine concentration of acid produced. 1 M NaOH was titrated against the broth using phenolphthalein indicator. Colour change was observed from colourless to pink. Concentration of acid produced was determined. The results were as below: For MRS Broth: Concentration = 0.2M, For MacConkey's Broth: Concentration = 0.052M.
- Kelling's Test: Kelling's test is a chemical test used for detecting the presence of lactic acid. Based upon the colouring effect. Kelling's reagent causes a change in colour of the broth to yellow colour. This confirms presence of lactic acid.
- Sugar fermentation test: The test was performed to check presence of lactic acid. Bromophenol blue dye was added to the fermentation broth prior to fermentation. Fermentation was allowed to take place for 4-5 days. After fermentation, the colour of the dye changes from dark blue to light pink. This tests presence of lactic acid produced after breaking down glucose.

F. Extraction of Lactic Acid

Two methods were undertaken to extract and purify lactic acid synthesized during fermentation.

- Method 1

Centrifuge the fermentation broth at 2500 rpm for 15-20 minutes to separate cell debris and other contaminants. The supernatant is separated. Excess $CaCO_3$ was added to the supernatant and stirred on a magnetic stirrer for about 15 minutes. Calcium lactate is precipitated. Concentrated H_2SO_4 was added to regain the lactic acid. Similar procedure was followed using $Ca(OH)_2$ as a base instead of $CaCO_3$. However, there was much loss in lactic acid concentration in both the techniques. So process modification was necessary [7].

- Method 2

After fermentation, the broth was centrifuged and the cell debris was separated. The broth was heated under burner at 70^0C-80^0C so as to kill all the cells and remove odour. The broth was then filtered using Whatman filter paper to separate the cells and other impurities. Then the pH was adjusted to 11 using $CaCO_3$. It was allowed to agitate on a magnetic stirrer for an hour. The aqueous layer obtained contains lactic acid. Then the pH is now adjusted to 2.5-3 using 63% concentrated sulphuric acid. Later after the acidification step, centrifugation was done in order to remove the metal salt. Steam distillation of cheese and sauerkraut samples was done in order to concentrate the lactic acid produced. Also water, which is a major impurity in the sample, is removed by distillation.

G. Lactic Acid Standardization

Buttermilk was taken in plastic beakers and allowed to lyophilise for 7-8 hrs. It was stored in refrigerator for further use. This sample was used as a basis for standard graph as buttermilk contains almost 90% lactic acid.

For the standard graph of lactic acid, 1mg/ml and 10 mg/ml of buttermilk powder solution was prepared. However, the samples were too dilute, hence the readings of absorbance were not in the required range. Hence, standard graph of lactic acid was prepared using 100 mg/ml buttermilk powder.

Standard graph of lactic acid was prepared by using varying concentrations of 100 mg/ml of sample. The reagent used for colorimetric assay was Kelling's reagent and absorbance was measured at 410 nm.

TABLE I: STANDARD LACTIC ACID DETERMINATION ASSAY

Sr. No.	Sample (100 mg/ml) (ml)	Conc. (mg/ml)	Distilled Water (ml)	Kelling's Reagent (ml)	Absorbance At 410 nm
Blank	0	0	4.0	1	0
1	0.2	5	3.8	1	0.15
2	0.4	10	3.6	1	0.33
3	0.6	15	3.4	1	0.54
4	0.8	20	3.2	1	0.76
5	1.0	25	3.0	1	0.87

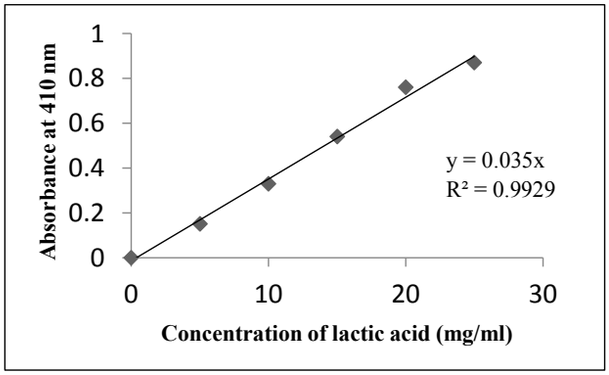

Fig. 1: Standard Graph of Lactic Acid

III. RESULTS

A. Isolation

The colonies were selected and stored on MRS agar slants.

B. Phenotypic Characterization

Gram staining of bacteria isolated from sauerkraut showed mostly pink coloured colonies, whereas that of cheese showed mostly purple coloured colonies.

The isolates were tested for catalase test. When a loopful of culture is added to hydrogen peroxide, the bubbles of oxygen were observed. Durham's tubes were placed in test tubes containing the media. The broth was allowed to ferment for 4-5 days. It was observed that no gas bubbles were seen in the tubes which are a characteristic of lactic acid fermenters.

C. Optimization of Fermentation Parameters

- Temperature: The temperature were monitored and controlled during the fermentation process at elevated temperatures of 30, 37, 40, 45, 50, and 55^{0}C. A graph of absorbance versus temperature was plotted. It was found that growth was optimum at 37^{0}C.

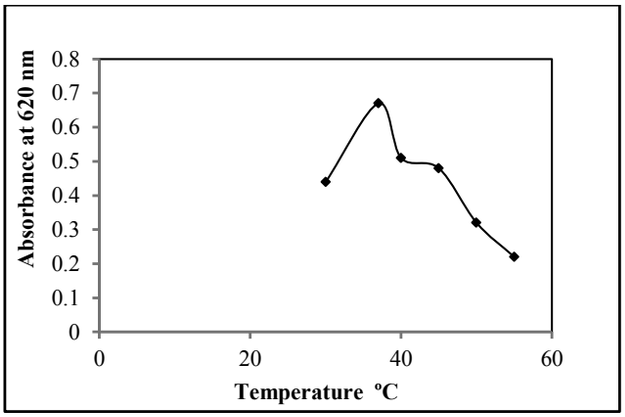

Fig. 2: Effect of Temperature on Cell Growth

- pH: The effect of pH on cell growth was performed. The initial pH of the medium was maintained at 4.0, 4.4, 4.8, 5.2, 5.6 and 6.0. A graph of Absorbance versus pH was plotted. High growth rate occurred at a pH of 5.6.

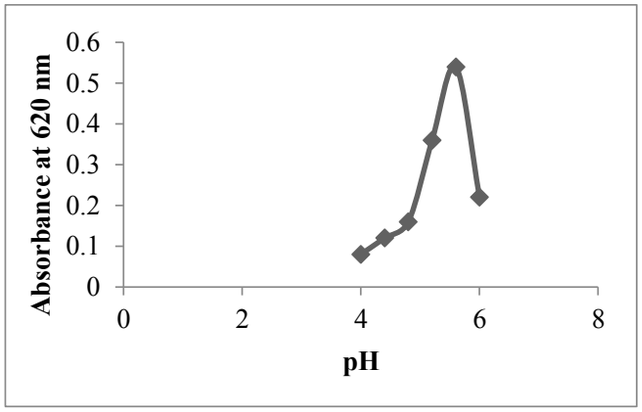

Fig. 3: Effect of pH on Cell Growth

- Agitation: Effect of agitation was checked by comparing two flasks; one with agitation of about 120 rpm and other without agitation. It was observed that there was no much difference in growth with agitation.

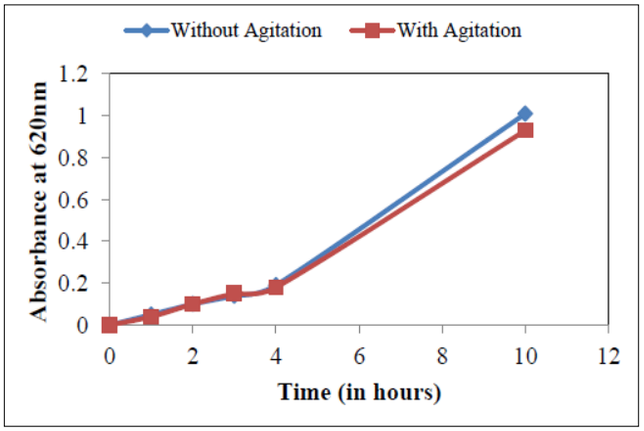

Fig. 4: Effect of Agitation on Cell Growth

D. Extraction and Purification

Extraction was performed using both the methods and samples obtained from cheese and sauerkraut cultures were concentrated by distillation. Further, the concentration of lactic acid produced was calculated from the standard graph.

E. Standardization and Quantification of Lactic Acid

The standard assay was performed for the samples obtained using cheese and sauerkraut mixed cultures. Absorbance readings were noted at 410 nm.

TABLE II: LACTIC ACID QUANTIFICATION AND ANALYSIS

Sr. no	Sample (ml)	Distilled water (ml)	Kelling's reagent (ml)	Absorbance at 410 nm	Concentration (mg/ml)	% yield
Cheese	0.5	3.5	1	0.89	25	61%
Sauerkraut	0.5	3.5	1	0.59	16.6	42.78%

IV. CONCLUSION

Lactic acid bacteria were isolated from cheese and sauerkraut sources. Fermentation was optimized for factors such as pH, temperature and agitation. Batch fermentation was carried out using these optimized parameters. Extraction method involved precipitation using basic salts followed by acidification. Further to concentrate the lactic acid, distillation method was followed. Biosynthesis of lactic acid using lactic acid bacteria was successfully performed. The yield obtained from cheese bacteria was 61% and that from sauerkraut bacteria was 42.78%. Hence it can be concluded that the strains from cheese samples are more effective for the production of lactic acid under favourable conditions.

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