Potential of Organic acid bacteria to use wastewater to produce electricity

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I hereby declare that the data presented in this Dissertation report entitled, "**Potential of Organic acid bacteria to use wastewater to produce electricity**" is based on the results of investigations carried out by me in the Microbiology Programme at the School of Biological Sciences and Biotechnology, Goa University under the Supervision of Dr. Sandeep Garg and the same has not been submitted elsewhere for the award of a degree or diploma by me. Further, I understand that Goa University or its authorities will be not be responsible for the correctness of observations / experimental or other findings given the dissertation.

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PREFACE

Wastewater treatment, a critical component of environmental management, has traditionally depended on resource-intensive infrastructure. However, the discovery of organic acid bacteria as possible wastewater treatment agents indicates a move toward more cost-effective and ecologically acceptable alternatives.

This work looks into the various applications of microbial fuel cells (MFCs) and its potential in wastewater treatment and power generation. It travels through history, describing the development of microbial fuel cell technology from its initial stages to its current uses. From Potter's pioneering discoveries in the early twentieth century to recent developments based on enhanced bacterial strains.

This discussion isn't just looking back at history; it is also about how microbial fuel cells work. It covers from finding and studying lactic acid bacteria to developing these fuel cells.

Furthermore, this work attempted the diversity of microorganisms in the field of energy generation. Researchers conducted to experiment in order to obtain bacteria to extract energy from a variety of sources, including brewery wastewater and starch-derived syrups.

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TABLES & FIGURES

ABBREVIATIONS USED

Abbreviation	Expansion
°C	Degree celcius
CE	Columbic efficiency
COD	Chemical oxygen demand
g/l	Grams per litre
HCL	Hydrochloric acid
IMVIC	Indole, methyl red, voges proskauer & citrate
MFC	Microbial fuel cell
ml	mililitre
MRS agar	Deman Rogosa Sharpe agar
rpm	Revolutions per minute
SCMFC	Single chamber microbial fuel cell

ABSTRACT

Water is valuable resource that is being polluted in many ways. Wastewater treatment is an important component of environmental management. Traditional methods of wastewater treatment are dependent on expensive infrastructure. Therefore, there is need to find alternate methods for wastewater treatment, such as use of organic acid bacteria, can reduce operational cost. Organic acid bacteria also have the ability to break down organic materials present in wastewater and convert it into valuable byproducts. Bacteria will be utilizing organic molecule present in wastewater for acid production to create redox potential that can be used to generate electricity. The bacterial isolates were obtained from curd sample, idli and dhokla batter, and crushed fruits. The bacterial isolates were screened for acid production. The isolates were characterized by gram's staining, colony characterization and biochemical tests. The amount of acid produced by bacterial isolates were quantified. The MFC was prepared by connecting anode and cathode chamber by corn Starch Bridge. The maximum amount of current and voltage was generated on day 2.

INTRODUCTION

1.1 Background

Wastewater treatment is an important component of environmental management. Traditional methods of wastewater treatment are dependent on expensive infrastructure. Therefore, there is need to find alternate methods for wastewater treatment, such as use of organic acid bacteria, can reduce operational cost. Organic acid bacteria also have the ability to break down organic materials present in wastewater and convert it into valuable byproducts. Organic acid bacteria provide a more environmentally friendly approach as they produce nontoxic byproducts and can function in anaerobic conditions.

1.1.1 Lactic Acid Bacteria

Lactic acid bacteria are gram-positive, acid-tolerant, non-sporulating bacteria which generate lactic acid. Lactic acid bacteria consume sugars as their fundamental source of carbon. Normally rods or cocci, lactic acid bacteria are tolerant to low pH. Over 60 genera of lactic acid bacteria are known to exist, although the most common genera found in fermenting food include *Lactobacillus, Lactococcus, Leuconostoc, Pediococcus, Streptococcus, Enterococcus, Weissella*, etc (Wang et al., 2021).

These are employed as commercial starting cultures in the production of fermented products derived from meat products, milk products, vegetables, and bread. Lactic acid bacteria generally serve as antibacterial agents via lowering pH and generating lactic acid. Several metabolic products have been shown to be contributing to their antibacterial action, including bacteriocins, acetic acid, propionic acid, hydrogen peroxide, and diacetyls. It's possible that the lactic acid bacteria that reside in the gut environment as commensals have probiotic properties.

1.1.2 Isolation of Lactic Acid Bacteria

Lactic Acid bacteria are frequently found in fermented dairy products such as curd, buttermilk etc. Curd is a product made from milk that has been fermented and is rich in lactic acid bacteria. Curd bacteria, especially Latic acid bacteria, have been well accepted as GRAS (generally regarded as safe) (Shaikh & Shah, 2013).

In nature, lactic acid bacteria are found all over and frequently participate in several spontaneous food fermentations. The long-term storage of food and fermented products is a major function of Lactic acid bacteria. Most fermented foods, especially those found in Indian traditional cuisine, are made by Lactic acid bacteria fermentation Traditionally, idli batter is made using pre-soaked parboiled rice and dehulled black gram, which are then left to naturally ferment for 18 to 30 hours. The fermented batter is then steamed to make idlis (Agaliya et.al.,2013).

Even though dairy products are a common source of lactic acid bacteria, fresh vegetable and fruit sources have gained importance since they are also an endless source of Lactic acid bacteria. Various Lactic acid bacteria have been isolated from fruit as follows: *Lactobacillus rossiae* from pineapple; *L. plantarum* from tomato, pineapple, plum, kiwi, papaya, grape, strawberry, and cherries; *L. brevis* from tomato; and *Leuconostoc mesenteroides* subsp. *mesenteroides* and *Pediococcus pentosaceus* from cherries. Many fruits, including bananas, apples, grapes, kiwis, and oranges, contain Lactic acid bacteria (Garcia et al., 2016).

1.1.3 Organic acids produced by lactic acid bacteria

Lactic acid is one of the organic acids that can be produced by specific metabolic processes like lactic acid fermentation. Based on its optical rotation, lactic acid—is classified into two different forms: L-lactic acid and D-lactic acid. It finds extensive application in food, farming, healthcare, the pharmaceutical industry, and conserving the environment. There are two categories of lactic acid-producing bacteria: heterolactic fermentation and homolactic fermentation. *Leuconostoc*, and *Oenococcus* carry out heterolactic fermentation, whereas *Lactobacillus* and *Lactococcus* carry out homolactic fermentation. Lactic acid bacteria use glucose as their sole source of carbon. Once inside the cell, glucose undergoes glycolysis, a metabolic pathway that breaks glucose into pyruvate. In absence of oxygen the pyruvate produced during glycolysis is converted into lactic acid. This reaction is catalyzed by enzyme lactate dehydrogenase which reduces pyruvate to lactic acid. Thus, two moles of lactic acid are produced from one mole of glucose.

In heterolactic fermentation, lactic acid is formed as end products along with other co-products such as carbon dioxide, ethanol and acetic acid through phosphoketolase pathway. One mole of lactic acid is produced for every mole of glucose (Wang et al., 2021).

1.1.4 Potential of organic acid bacteria to produce electricity

The Microbial fuel cell (MFC) is a bioelectrochemical system that uses respiring microorganisms to convert organic material into electrical power without the use of external fuel sources. Fundamentally, the Microbial fuel cell (MFC) is a fuel cell that

produces energy from organic materials through the processes of oxidation and reduction. (Yaakop et al., 2023)

As a biocatalyst, microorganisms drive the breakdown of organic molecules to release electrons that pass via the electric circuit to the cathode side. The term "exoelectrogens" refers to these microorganisms. Hydrogen ions travel towards the cathode and combine with oxygen MFCs are environmentally beneficial devices that provide waste management and bioelectricity generation. This is because they employ organic materials, including wastewater. The two chambers of a microbial fuel cell are called the anode and cathode chambers, and these are divided structurally by a proton exchange membrane. Microorganisms that are electrochemically active are found on the anode side, whereas the cathode is abiotic. Within the internal circuit to generate water, while electrons travel via the exterior circuit to reach the cathode. Thus, this suggests that MFCs might be a viable option for "electricity" that is green (Obileke et al., 2021).

1.1.5 Microbial Fuel Cell

Sustainable, renewable, and alternative sources of energy are something that our society is always looking for. Frequently, solar power cells or windmills come to mind when people consider these energy sources. Additionally, microbial fuel cells (MFCs) might be included in the image. A bio-electrochemical device known as a microbial fuel cell (MFC) uses the organic metabolism of microorganisms to generate energy. Microbes in the MFC use the nutrients in their surroundings as food, releasing some of the food's energy as electricity. Over the course of the remaining 20th century, there was occasional research on this topic and the development of MFCs. Recently, there has been a lot of interest in improving MFC technology to meet the demands of humans for both wastewater treatment and renewable clean energy sources (Li, 2013).

MFC technology is a promising approach to producing electric current from a variety of materials, including complicated organic waste and natural organic matter. It may be combined with advantageous uses in wastewater treatment. MFCs are built with a variety of materials and designs(table 1.1.5.1). These systems can be run under a variety of settings, including low or high temperatures, basic or acidic pH, various electron acceptors, etc., but they are often operated at optimal parameters to draw more power from the system (Kumar et al., 2017).

Table 1.1.5.1 list of materials used in MFCs

Components	Materials
Anode	Carbon paper electrode, carbon cloth, graphite felt, stainless steel, gold
cathode	Graphite, carbon paper, zinc, copper
Anolyte	Glucose, acetate, butyrate, glycerol, citrate
Catholyte	Ferricyanide, oxygen, potassium permanganate, bicarbonate
Membrane	Nafion , ceramic membrane, cassava starch, clay Manihot starch, corn starch
Electrical components	Voltmeter, ammeter, copper wires, aluminum wires

1.1.6 Design of Microbial Fuel cell

The following four components comprise the MFC:

Anode chamber, cathode chamber, proton exchange membrane and electrodes.

The anode chamber is where organic material and bacteria are kept in an anaerobic atmosphere. Cathode chamber containing a saltwater solution. Proton-exchange membrane, commonly referred to as the salt bridge, which permits protons to flow between the two chambers and divides the anode and cathode.

The external circuit serves as a pathway for electrons to move through when they are extracted from the solution that is in the anode and permits electrons to get into the cathode. When bacteria oxidize as a part of their digestion process, protons and electrons are produced in the anode chamber.

Upon reaching an electrode, the electrons are extracted from the anode's solution. The cathode's electrode then conducts the electrons via the external circuit to enter the cathode chamber. The proton-exchange membrane allows the protons from the anode's solution to meet the electrons that are at the cathode (Li, 2013).

1.2 Aims and Objectives

- To check whether organic acid bacteria can produce acid for electricity production.
- To develop wastewater treatment process.

1.3 Hypothesis

Bacteria will be utilizing organic molecule present in wastewater for acid production to create redox potential that can be used to generate electricity.

1.4 Scope

Microbial fuel cells that use wastewater and organic acid producing bacteria have a great deal of potential for producing power. Research has demonstrated that a range of bacterial species may efficiently generate electricity from organic substrates found in wastewater. Several bacterial species may use domestic organic waste as a substrate, which can lead to the generation of energy. These research highlight how organic acid bacteria may effectively transform wastewater into renewable energy.

LITERATURE REVIEW

2.1 History of microbial fuel cell

The idea that microbes may generate energy was initially put out more than a century ago. Potter, a professor in the University of Durham's Botany Department, in 1911 made the first demonstration of microbes producing electricity. To investigate the potential of microorganisms to produce electricity, he carried out an experiment employing yeast and several additional bacteria in a glass jar with a porous cylinder inside. He noticed that when glucose was utilized as a substrate, *Saccharomyces cerevisiae* and *Escherichia coli* generated electric current. Up until 1966, there was no further research on microbial fuel cells (MFC). But in their trials, the researchers employed synthetic electrochemical mediators to help bacteria and electrodes transport electrons more easily. A breakthrough in MFCs was described in 1999 when Kim and colleagues used a Fe (III)-reducing bacterium to create the first mediator-less MFC, demonstrating that external mediators were not required to be introduced to transport electrons from the cells of bacteria to electrodes. The cell suspension of *Shewanella putrefaciens* IR-1 was able to generate current without redox mediator in presence of lactate as main carbon source. Another important bacterium *Geobacter sulfurreducens* can transfer electrons to electrode in the absence of mediators with high current generation and has become an important issue in MFC research. Since the emergence of mediator-less MFCs, researchers have been more interested in studying MFCs, particularly in the treatment of wastewater, as mediator-less MFCs offer a more viable and practical method of using microbial systems to recover power from organic material and wastewater. Many research labs are now working to improve MFC technologies to increase energy output and effectively remove wastewater by creating a variety of MFC designs, including membrane-less MFC, stacked MFC, single chamber MFC, and tubular MFC (Feng et al., 2015).

2.2 Organisms used in microbial fuel cell

D'Souza et al. (2013) used a pure culture of *E.coli* k 805 as inoculum in the anodic chamber of microbial fuel cell. Substrate used in microbial fuel cell was glucose and brewery waste. It was reported that 4-6% of glucose concentration was best for bacterial growth. When glucose was utilized as substrate, the first day's measurement was recorded as 351mv. Remarkably 534 mv was the measurement that was recorded when brewery wastewater was used as substrate. Bioelectricity generation by *E.coli* has been determined using whey as substrate in two chamber microbial fuel cell (Nasirahmadi et al., 2011). Masih et al. (2012) investigated optimizing power generation in a dual chambered aerated membrane MFC utilizing sodium acetate as the substrate and *E. coli* as the biocatalyst at two distinct culture densities (0.5 and 1.0 OD). The voltage of 783 mV, the power density of 222.84 mW/cm2, and the coulombic efficiency (CE) of 86.56% were determined to be the ideal values. Compared to fermentable substrate, Coulombic efficiency was greater when sodium acetate was used as the substrate.

Borole et al. (2008) examine the utilization of iron-reducing acidophilic heterotroph *Acidiphilum cryptum* as biocatalyst in MFC anode chamber using glucose as electron source. Important factors in the selection of *A. cryptum* included its capacity to operate in a pH range where Fe (II) is soluble (pH 4) and utilize glucose, a renewable resource, as the electron donor. It was shown by experiment that *A. cryptum* could catalyze the generation of electricity using Fe (III) as an electron mediator. With FeCl3 acting as the mediator, an MFC's highest open circuit voltage was 0.295 V.

Juliano et al. (2020) have developed a relatively inexpensive microbial fuel cell that treats household wastewater while producing low voltage energy. By utilizing different carbon sources in solution, variations in voltages were noted. The MFC produced a voltage of 830

mV when *P. fluorescens* was added to the anode that contained the substrates and fermented rice.

Darmawan et al. (2018) quantify the potential current and voltage produced when whey cheese was used as a substrate for the metabolism by bacteria. *Streptococcus thermophillus, Lactobacillus bulgaricus* and *Lactobacillus casei* were the species of bacteria employed in this investigation. Operational time of 100 hours was tested. The *Lactobacillus bulgaricus* bacteria generated the highest voltage of 529.3 mV and the maximum current of 74.6 μA. current strength *Lactobacillus casei* produced the second-highest during this treatment, with an average maximum electrical strength of 69 μA. *Streptococcus thermophillus* produced the lowest electric strength throughout this treatment, with an average maximum current strength of 62.3 μA.

Febriawan et al. (2023) studied development of MFC using palm oil waste as a substrate. Carbon rods were used as electrodes in MFC and KMnO4 (0.2M) was used as electrolyte solution. The study examined the potential for producing electrical energy from palm oil liquid waste substrate by using *Lactobacillus burglarious* bacteria. The highest value of current obtained was 0.9640 mA and voltage generated was 0.6760 V.

Mane et al. (2017) demonstrated that bioelectricity may be produced using yeast cells from pH 6 to 8 at room temperature utilizing the MFC approach. Bioelectricity was generated in anaerobic conditions for a whole day using the single dual chamber and multi chamber MFC methods. The 2.26V were created after 12 hours of use of multichambered technique use. This voltage produced a noticeable light in the LED bulb. According to this study, baker's yeast is a viable option for producing bioelectricity using an MFC method.

Biffinger et al. (2011) investigated the viability of *Shewanella japonica* for power applications by using a variety of carbon sources. The study reported that sucrose was utilized by *S.japonica* for power production.

2.3 Substrates

2.3.1 Acetate

Acetate is a compound with the molecular weight of 59.004 g/mol and the formula CH_3COOH. It can serve as a carbon source for the development of bacteria and the production of energy. Liu et al. (2005) investigated the production of electricity with a single-chambered MFC utilizing acetate. It is evident that acetate is the preferred aqueous substrate for electricity production in MFCs since power generated with acetate (506 mW/m^2) was up to 66% greater than that fed with butyrate (305 mW/m^2).

2.3.2 Other carbon sources

Numerous other substances, including butyrate, propionate, and glucose, were also employed as sources of carbon in addition to acetate. With a maximum Coulombic efficiency of 72.3%, the acetate fed MFC was superior to butyrate (43.0%), propionate (36.0%), and glucose (15.0%). Chae et.al (2009) used glucose as a fermentable substrate. It is likely used by a variety of competing metabolisms, including methanogenesis and fermentation, which are unable to create energy. This is why glucose exhibited the lowest Coloumbic Efficiency (CE).

2.3.3 Monosaccharides

With the chemical formula $C_6H_{12}O_6$ having molecular weight of 180.156 g/mo, glucose is a monosaccharide and a widely utilized substrate. Catel et al. (2008) investigated electricity generation utilizing 12 carbon sources including six hexoses, three pentoses, two uronic

acids, and one aldonic acid. The maximum power density was obtained using glucose as substrate and the lowest power densities was obtained using mannose. Rabaey et al. (2005) reported that a glucose fed batch MFC produced a maximum power density of 216 W/m^3 using 100 mM ferric cyanide as the cathode oxidant.

2.3.4 Starch processed wastewater

The most prevalent type of storage polysaccharide in plants, starch provides a cheap source for making syrups with glucose, fructose, or maltose that are utilized extensively in the food industry. Further, starch-derived sugars may be fermented to create organic acids, amino acids, and bioethanol, among other products (Zhao et al., 2009). Kim et al. (2004) used wastewater from starch manufacturing plants to create a microbial fuel cell. After being enriched over 30 days inside a fuel cell, microbes almost totally eliminated organic pollutants from wastewater while also producing energy. It was noted that the chemical oxygen demand (COD) decreased from 1.7 g/L to 0.05 g/L in six weeks.

2.3.5 Brewery industries Wastewater

Beer brewery wastewater is ideal for production of electricity as it has a high amount of organic matter and low amounts of inhibitory substances. Feng et al. (2008) looked at the efficacy of treating wastewater from brewery with an air-cathode MFC. The study compared power generation between two different types of wastewaters, that is domestic and beer brewery wastewater. The beer brewery wastewater showed maximum power generation as compared to domestic wastewater. The maximum power density observed with brewery wastewater was 63 mW/m^2.

2.3.6 Synthetic wastewater

Rodrigo et al., (2009) investigated the use of a microbial fuel cell (MFC) to produce power by using synthetic wastewater supplied with glucose and soybean peptone as carbon sources. Two different compositions of wastewater were examined. One had 50% glucose and 50% soybean peptone, while the other had 80% peptone and 20% glucose. The performance of microbial fuel cell containing 50% of glucose was low as compared to the performance of MFC containing 20% of glucose. This indicates that peptone degradation results in intermediate that favor electricity.

2.3.7 Paper recycling wastewater

Huang and Logan (2008) investigated the efficiency of employing microbial fuel cells to treat wastewater from a paper recycling factory and produce energy. When 50 mM of phosphate buffer was put in wastewater the power density of 501 mW/m2 and columbic efficiency (CE) of 16 mW/m^2 was achieved. Effectively, 73 % of organic load and 96% of cellulose was eliminated from effluent.

2.4 Electrodes

Liu et al., (2014) tested a single chamber microbial fuel cell (SCMFC) with a single air cathode and eight graphite electrodes (anodes). The single chambered Microbial Fuel Cell reactor removed up to 80% of the COD from the wastewater while producing electricity (up to 26 mW m^{-2}). Logan and Ahn, (2010) constructed Air-cathode, single-chamber MFCs with ammonia-treated graphite fiber brush anodes. Two titanium wires served as the brush anode's core, while graphite fibers were cut to a length of 2.5 cm and an outside diameter of 2.5 cm. The continuous flow produced the maximum power density of 422 mW/m^2.

A prototype constructed by Dumas et al. (2007) had a stainless-steel anode submerged in marine sediments and a cathode buried in seawater. The maximum power density generated, 4 mW m^{-2}. The results of the studies indicated that stainless steel cathodes were promising alternatives for use in marine MFC

Richter et al., (2008). studied using *Geobacter sulfurreducens* which was able to grow on gold anodes and can produce equal current same as using graphite anode. This discovered that gold may be used as a viable anode material for microbial fuel cells.

METHODOLOGY

3.1 Isolation of lactic acid bacteria

Five curd samples were collected. Lactic acid bacteria were isolated from curd samples on Deman Rogosa Sharpe agar (MRS agar) (appendix1). Curd samples were diluted using sterile saline and were plated on MRS agar plates. Plates were incubated at room temperature in anaerobic chamber for 24-48 hours.

Idli batter was prepared by soaking black gram and rice separately. Rice and black gram were ground separately. Both the batters were combined with addition of little salt (Ghosh & Chattopadhyay, 2010).

Dhokla batter was prepared by soaking bengal gram and rice separately. Rice and bengal gram were ground separately. Both the batters were mixed with addition of salt. The batter was allowed to ferment overnight in warm temperature (Sharma et al., 2018).

Lactic acid bacteria were isolated from fermented batter. Fermented idli and dhokla batter was serially diluted using sterile saline. The dilutions were spread plated on MRS agar. Plates were incubated anaerobically in anaerobic chamber at room temperature for 24-48 hours. Plates were checked for growth after 24-48 hours.

Fruits namely Bananas, guava, apples, strawberries and grapes were purchased at the market. Fruits were cleaned by washing. Washed fruits were crushed and ten to twenty grams of crushed fruits were weighed and put in a 50 ml falcon tube. One to five grams of soil was weighed and placed in a tube and mixed with water. The tubes were capped tightly and kept under anaerobic conditions for 2 to 4 days. After 2-4 days this mixture was serially diluted and spread plated on MRS agar. Plates were incubated anaerobically in an anaerobic chamber at room temperature for 24-48 hours. The colonies were obtained and were again streaked on MRS agar plate for purification.

3.2 Screening of isolates for acid production

Glucose phosphate broth (appendix1) was prepared. The bacterial isolates were inoculated in broth. The tubes were incubated anaerobically in an anaerobic chamber for 24-48 hours. After 24 hours the isolates were checked for acid production by adding methyl red indicator (appendix II) to culture broth. If the pH of culture broth is less than 4.4 the indicator will turn red.

3.3 Characterization of the isolates

The isolates showing acid production were characterised by Gram's staining, colony characterisation and biochemical tests.

3.3.1 Gram's staining

Bacterial smear was prepared on a clean grease free slide. Smear was air dried, and heat fixed. Primary stain (crystal violet) was added to the smear. Stain was allowed to remain for 1 minute. Smear was rinsed with water. The smear was flooded with Gram's iodine for 1 min and the slide was washed. The smear was decolorized with 95% alcohol for 1 min. The slide was washed. The slide was counter stained with saffranine for 30 seconds, washed, air dried, and observed under oil immersion objective. The gram-positive organism will appear purple and gram-negative organism will appear pink.

3.3.2 Colony characteristics

Morphological characteristics of such as shape, size, colour, elevation, margin, opacity and surface texture of isolated bacterial colonies were observed and noted.

3.3.3 Biochemical tests

3.3.3.1 IMVIC Test

Indole test:

Tryptone broth (appendix I) was prepared and sterilized. Tryptone broth was added to sterile test tubes. The bacterial isolates were aseptically inoculated in tryptone broth. The tubes were incubated for 24 hours at room temperature. After 24 hours, 0.5 ml of Kovac's reagent (appendix II) was added to culture broth. The rapid development of red colour after addition of Kovac's indicates positive indole test. Formation of orange colour on addition of Kovac's reagent indicates negative indole result.

Methyl red test:

Glucose phosphate (appendix1) broth was prepared according to the composition. The bacterial isolates (18-24 hours old culture) were inoculated in broth. The tubes were incubated anaerobically in an anaerobic chamber for 24-48 hours. After 24 hours the isolates were checked for acid production by adding methyl red indicator (appendix II) to culture broth. If the pH of culture broth is less than 4.4 the indicator will turn red. yellow colouration of the reagent indicates negative results.

Voges-Proskauer test:

The test organisms were inoculated in glucose phosphate broth (appendix 1) and incubated for 48 hours. After 48 hours, O'Meara's reagent (appendix II) was added. A Voges Proskauer positive result is indicated by cherry red colour at the surface and negative result is indicated by yellow-brown colour.

Citrate test:

Simmons citrate agar (appendix1) slants were prepared. Aseptically, the test organisms were streaked on citrate agar slants. The slants were incubated at room temperature for 24 hours. After 24 hours, the slants were observed for blue coloration. The change in colour

of citrate slant from green to blue indicates citrate positive result. Negative result is indicated by no colour change.

3.3.3.2 Kligler's iron test :

Triple sugar iron slants (appendix1) were prepared. The bacterial isolates were streaked on TSI agar slants and stabbed in the butt. The tubes were incubated for 24 hours at room temperature. The slants were observed after 24 hours. A red slant and yellow butt indicate fermentation of dextrose only. The yellow slant and yellow butt indicate fermentation of lactose, sucrose and dextrose. Red slant and red butt indicate no fermentation. Blackening of the medium indicates presence of H_2S gas. Bubbles and cracks in the agar indicates CO_2 production.

3.3.3.3 Urease test:

Urea Agar Base (Christensen agar) (appendix1) slants were prepared. The surface of the slants were streaked with a test organism and incubated at room temperature for 24 hours. Urease positive cultures will produce pink colour. No colour change in slant indicates urease negative test.

3.3.3.4 Hugh-leifson's test:

Hugh-leifson's medium (appendix1) was prepared. Using sterile inoculation loop tubes were inoculated with test organism in two sets. In one set of tubes paraffin oil was poured making a layer of 1ml (this will create anaerobic conditions). The tubes were examined for carbohydrates utilization after incubating at room temperature for 24 hours. Acid production is detected by change in colour of medium from green to yellow. Acid production in both the tubes (aerobic and anaerobic) indicates, organism is fermentative. Acid production in aerobic tube only indicates organism is oxidative. No acid production in both tubes indicates organism is non-saccharolytic.

3.3.4.5 Catalase test

Clean grease free slide was taken. Using a sterile inoculation loop, a smear was prepared on clean grease free slide. A drop of hydrogen peroxide was placed on the top of the smear. Appearance of bubbles indicates catalase positive result. No appearance of bubbles indicates negative result.

3.4 Screening of isolates for acid production

3.4.1 Screening of isolates for acid production using different sugars

Glucose phosphate broth, Medium 1, medium 2 and medium 3 (appendix1) was prepared. The bacterial isolates (18-24 hours old culture) were inoculated in broth. The tubes were incubated anaerobically in an anaerobic chamber for 24-48 hours. After 24 hours the isolates were checked for acid production by adding methyl red indicator to culture broth. If the pH of culture broth is less than 4.4 the indicator will turn red. Yellow colouration of the reagent indicates negative results.

3.4.1 Screening of isolates for acid production using different amino acids

Medium 4 (appendix I) was prepared by using amino acid glycine. Medium 5 (appendix I) was prepared by using amino acid arginine. The bacterial isolates (18-24 hours old culture) were inoculated in broth. The tubes were incubated anaerobically in an anaerobic chamber for 24-48 hours. After 24 hours the isolates were checked for acid production by adding methyl red indicator to culture broth.

If the pH of culture broth is less than 4.4 the indicator will turn red. Yellow colouration of the reagent indicates negative results.

3.5 Screening of isolates for enzyme activity

3.5.1 Amylase Activity

MRS agar supplemented with 0.5% of starch was prepared, poured in sterile Petri plates and allowed to solidify. Isolates were spot inoculated on solidified medium. The plates were incubated in anaerobic conditions and observed after 24 hours. The plates were flooded with iodine solution (appendix II) to observe degradation of starch. Zone of clearance around the colony indicates starch degradation.

3.5.2 Protease activity

MRS agar supplemented with 0.5% of skim milk was prepared, poured in sterile Petri plates and allowed to solidify. Isolates were spot inoculated on solidified medium. The plates were incubated in anaerobic conditions and observed after 24 hours. Zone of clearance around the colony indicates proteolytic activity

3.6 Quantification of Acid produced by organic acid bacteria

Test organism was inoculated in 10 ml of medium 1 (appendix I) broth and incubated in anaerobic conditions for 24 hours. After 24 hours, 5 ml of culture broth was centrifuged and 1ml of supernatant was transferred to a sterile falcon tube. To this, 4 ml of ice-cold ethanol was added and was incubated for 15 mins on ice. The falcon tube was centrifuged for 10 mins at 10000 rpm, at 4°C.The supernatant was collected in falcon tube and 860 microlitres of NaOH was added. The tube was centrifuged at 10000 rpm at 4°C for 10 mins and supernatant was collected. To this, 1.2 ml of HCL was added. 3.5 ml of solvent phase was collected in lyophilization flask. The sample was then layered on the flask by placing in ultra deep freezer at -80°C and rotating it periodically. The layered sample flasks were kept overnight in the freezer at -80°C. The Ilshin freeze dry series was put on and allowed

to reach a temperature of -77°C.The vacuum pump was started.After 8 hours the lyophilized sample was collected in empty falcon tube. The weight of lyophilized product was noted.

3.7 Preparation of Microbial fuel cell

3.7.1 Microbial Fuel cell setup

Two Plastic containers of 2000 ml capacity were used as anode and cathode chambers. The cathode and anode were the zinc plates. The zinc plates were joint to aluminium rods which were fitted to the lid of the containers. PVC pipe was used as proton exchange membrane casing. PVC pipe with valve were fitted on each side of both the containers. Voltage and current was measured using multimeter

3.7.2 Preparation of catholyte

The catholyte was 1.5 g/l of potassium permanganate solution. The solution was added to aerated cathode chamber

3.7.3 Preparation of the anolyte

The anode chamber contained medium 1 (appendix I) with lactose as a carbon source. Ten ml of bacterial culture was used as inoculum. The anode chamber was covered with a lid and sealed using Teflon tape to prevent atmospheric oxygen from entering the chamber.

3.7.4 Proton exchange membrane

One fifty grams of dry corn starch was mixed with 150 ml of distilled water and the mixture was heated at the temperature of 80-100°C for 30-45 minutes with constant stirring. Ten grams of sodium chloride was added and mixed well. The hot gel was put into PVC pipe. The gel was allowed to cool and solidify. Both the chambers were connected by a starch salt bridge. The pipe was secured to chambers with a PVC pipe through a valve.

ANALYSIS AND CONCLUSIONS

4.1 Isolation of Lactic Acid Bacteria

A total of twelve isolates were obtained from five curd samples. One isolate was obtained from the idli batter sample. Two isolates were obtained from dhokla batter samples. One isolate was obtained from banana and guava each. Two isolate were obtained from grapes and one isolate was obtained from strawberry.

4.2 Screening of isolates for acid production

In total twenty one isolates were screened for acid production. Eight isolates showed acid production within 24 hours and continued to do so after 48 hours.

4.2.1 Acid production by the isolates obtained from curd sample

Out of 12 isolates obtained from curd sample,Only 3 isolates showed acid production within 24 hours and continued to do so after 48 hours.

Table 4.2.1.1Acid production by the isolates obtained from curd sample

Isolates	After 24 hours	After 48 hours
A1	–	–
A2	–	–
A3	–	–
A4	–	–
A5	–	–
B1	+	+
B2	–	–
C1	–	–
C2	–	–
D1	–	–
D2	+	+
E1	+	+

Positive: +	Negative : -

4.2.2 Acid production by the isolates obtained from dhokla and idli batters

In total three isolates were obtained from dhokla and idli batter, and all showed acid production within 24 hours and continued to do so after 48 hours.

Table 4.2.2.1 Acid production by the isolates obtained from dhokla and idli batter

Isolates	Source	After 24 hours	After 48 hours
I	Idli batter	+	+
DKL1	Dhokla batter	+	+
DKL2	Dhokla batter	+	+

Positive: +	Negative : -

4.2.3 Acid production by the isolates obtained from fruits

Out of six isolates obtained from fruits, Only two isolates showed acid production within 24 hours and continued to do so after 48 hours.

Table 4.2.3.1 Acid production by the isolates obtained from crushed fruits

Isolates	Source	After 24 hours	After 48 hours
BA1	Banana	+	+
GU	Guava	–	–
S	Strawberry	–	–
AP	Apple	–	–
G1	Grapes	–	–
G2	Grapes	+	+

Positive: +	Negative : -

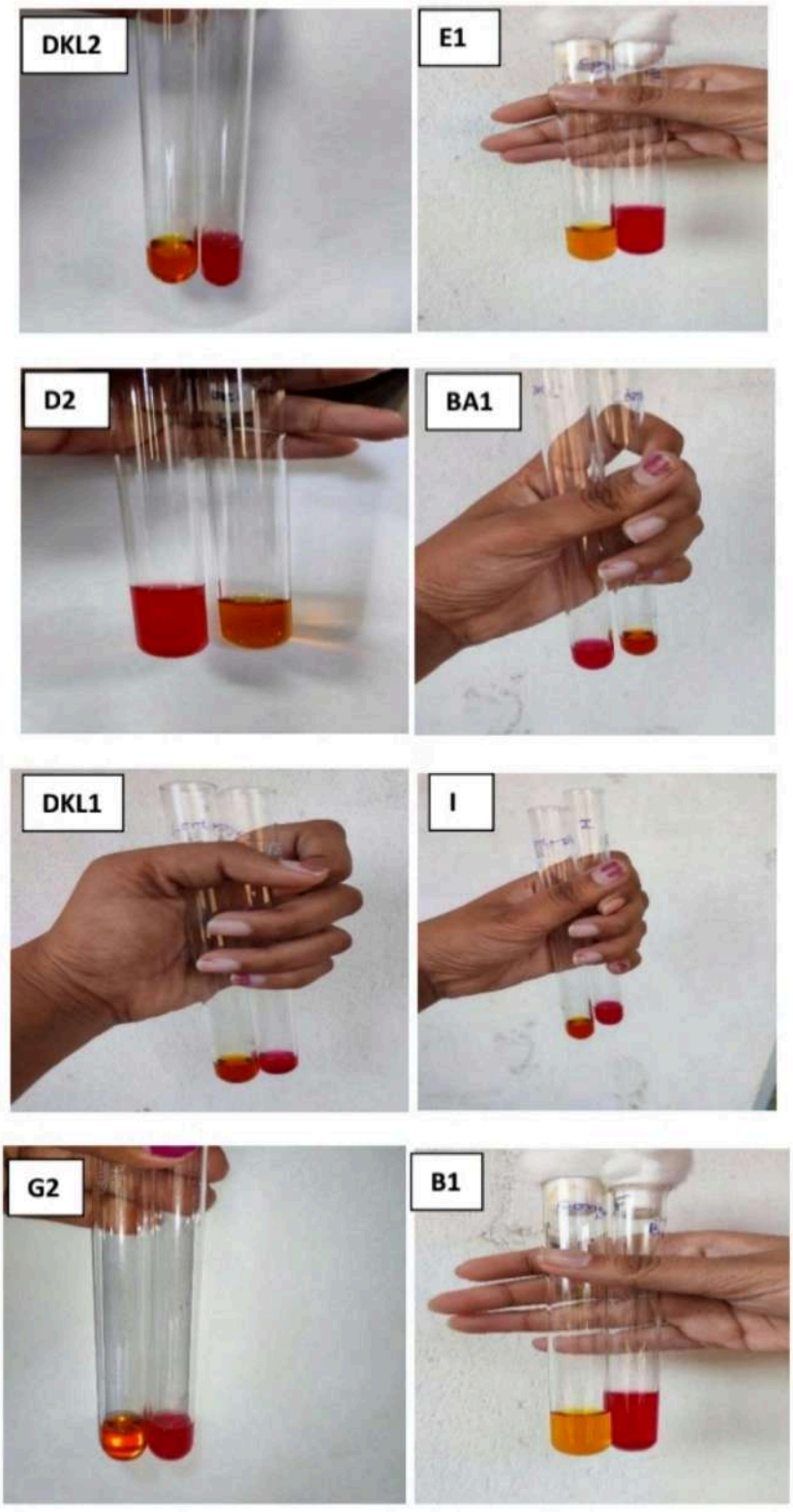


Fig 4.2.1 Acid production by isolates (E1, D2, B1-isolates obtained from curd ; DKL1, DKL2-isolate obtained from dhokla batter; I-isolate obtained from idli batter; BA1, G2-isolate obtained from fruits

4.3.1 Gram staining

All the isolates were found as gram positive bacteria. The isolate D2, DKL1, DKL2, I, B1 and BA1 formed short rods. The isolate E1 formed coccobacilli. The isolate G2 formed cocci.

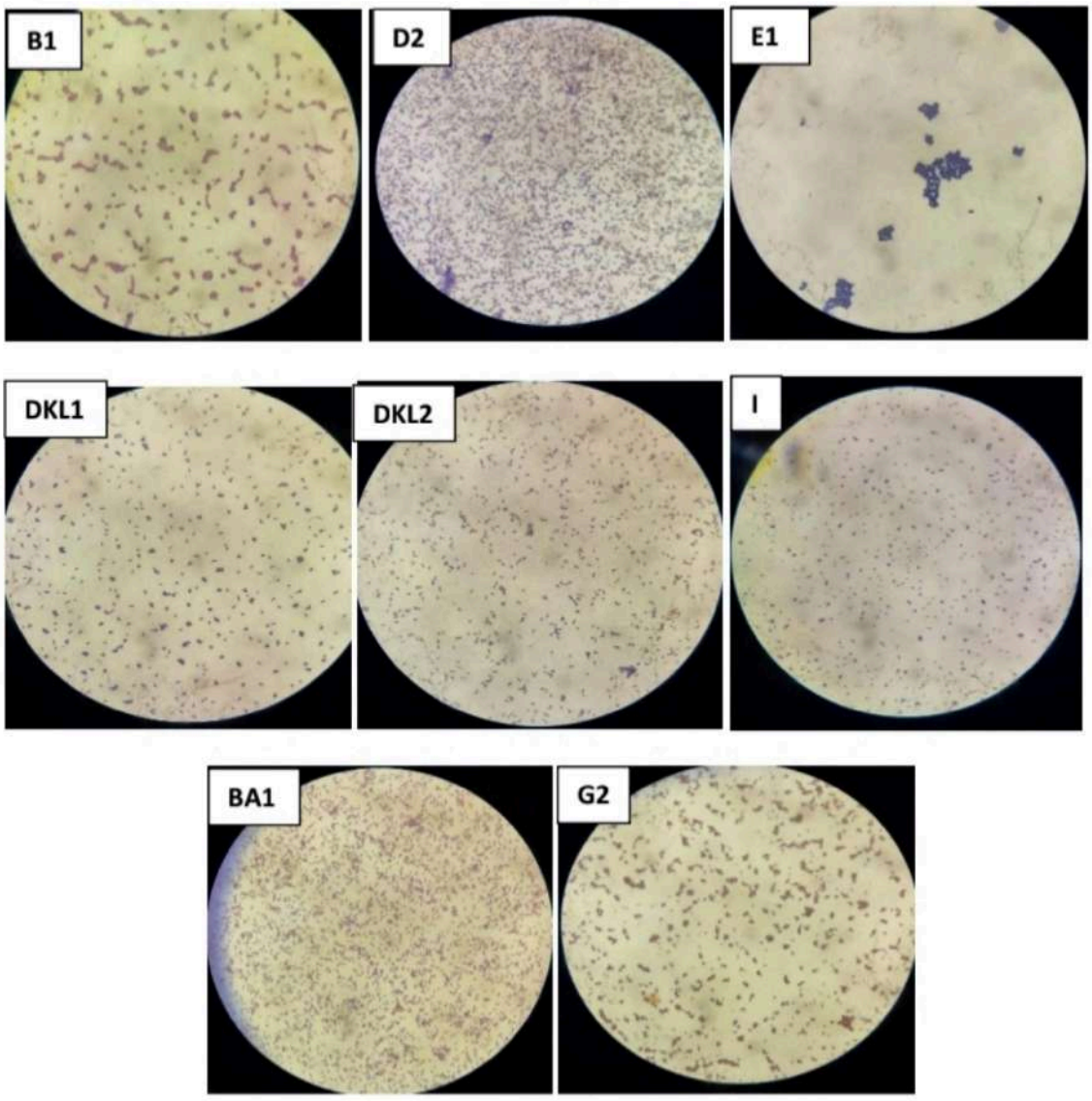


Fig 4.3.1.1 : Microscopic examination of the gram stained isolates (E1, D2, B1- isolates obtained from curd; DKL1, DKL2-isolate obtained from dhokla batter; I-isolate obtained from idli batter; BA1, G2- isolate obtained from fruits

4.3.2 Colony characteristics

The colonies of the isolates E1, D2, DKL1, DKL2, I, G2, and BA1 were circular in shape. The colony of the isolate B1 was irregular in shape. All the isolates were colourless and opaque.Colonies of the isolates B1, E1, D2, I, G2 and BA1 were flat in elevation. The colony of isolate DKL1 was convex and colony DKL2 was raised in elevation. The margin of the colony formed by the isolates E1, D2, DKL1, DKL2, I, G2 and BA1 was entire and the margin of the colony formed by isolate B1 was wavy. Surface texture of colonies of the isolates E1 and G2 was smooth and isolate B1, D2, DKL1, DKL2, I, and BA1 was mucoid.

Table 4.3.2.1 Colony characteristics of the isolates B1, E1, D2 and DKL1 (BI, D2, E1-isolate obtained from curd; DKL1-isolate obtained from dhokla batter)

	B1	**E1**	**D2**	**DKL1**
Shape	Irregular	Circular	Circular	Circular
Size	4mm	Pinpoint	3mm	4mm
Colour	Colourless	Colourless	Colourless	Colourless
Opacity	Opaque	Opaque	Opaque	Opaque
Elevation	Flat	Flat	Flat	Convex
Margin	Wavy	Entire	Entire	Entire
Surface texture	Mucoid	Smooth	Mucoid	Mucoid

Table 4.3.2.2 Colony characteristics of the isolates DKL2, I, G2, BA1 (DKL2-isolate obtained from dhokla batter; I-isolate obtained from idli batter; G2-isolated obtained from grapes; BA1-isolate obtained from banana)

	DKL2	**I**	**G2**	**BA1**
Shape	Circular	Circular	Circular	Circular
Size	4mm	2mm	1mm	5mm
Colour	Colourless	Colourless	Colourless	Colourless
Opacity	Opaque	Opaque	Opaque	Opaque
Elevation	Raised	Flat	Flat	Flat
Margin	Entire	Entire	Entire	Entire
Surface texture	Mucoid	Mucoid	Smooth	Mucoid

4.3.3 Biochemical Test

All the isolates were Indole and Voges proskauer negative (Table 4.3.3.1) . All the isolates showed positive result for Methyl red test. Isolates B1, D2, DKL1, DKL2, I, G2 and BA1 were citrate negative. Isolate E1 was citrate positive.

For Kliger's iron test, isolate B1, D2, DKL2, I, G2 and BA1 showed acidic slant and acid butt.. This indicates that isolates B1, D2, DKL2, I, G2 and BA1 use more than one sugar. Isolate E1 and DKL1 showed alkaline slant and acidic butt. This indicates that isolates E1 and DKL1 utilizes only glucose.

No isolate showed production of H_2S gas. There was no production of CO_2 gas by any of the isolate.

Hugh-Leifson's test indicated that all the isolates were fermentative.

Table 4.3.3.1 Characterization of isolates by Biochemical tests

Biochemical test		**B1**	**D2**	**E1**	**DKL1**
Indole		–	–	–	–
Methyl red		+	+	+	+
Voges proskauer		–	–	–	–
Citrate		–	–	+	–
Kligler's iron test	**Slant**	Acidic	Acidic	Alkaline	Alkaline
	Butt	Acidic	Acidic	Acidic	Acidic
	H2S	–	–	–	–
	Gas	–	–	–	–
Urease test		–	–	+	–
Hugh-Leifson's test	**Aerobic**	+	+	+	+
	Anaerobic	+	+	+	+
Catalase		–	–	–	–

Positive: +	Negative : -

Table 4.3.3.2 Characterization of isolates by Biochemical tests

Biochemical test		**DKL2**	**I**	**G2**	**BA1**
Indole		–	–	–	–
Methyl red		+	+	+	+
Voges proskauer		–	–	–	–
Citrate		–	–	–	–
Kligler's iron test	**Slant**	Acidic	Acidic	Acidic	Acidic
	Butt	Acidic	Acidic	Acidic	Acidic
	H2S	–	–	–	–
	Gas	–	–	–	–
Urease test		–	–	–	–
Hugh-Leifson's test	**Aerobic**	+	+	+	+
	Anaerobic	+	+	+	+
Catalase		–	–	–	–

Positive: +	Negative : -

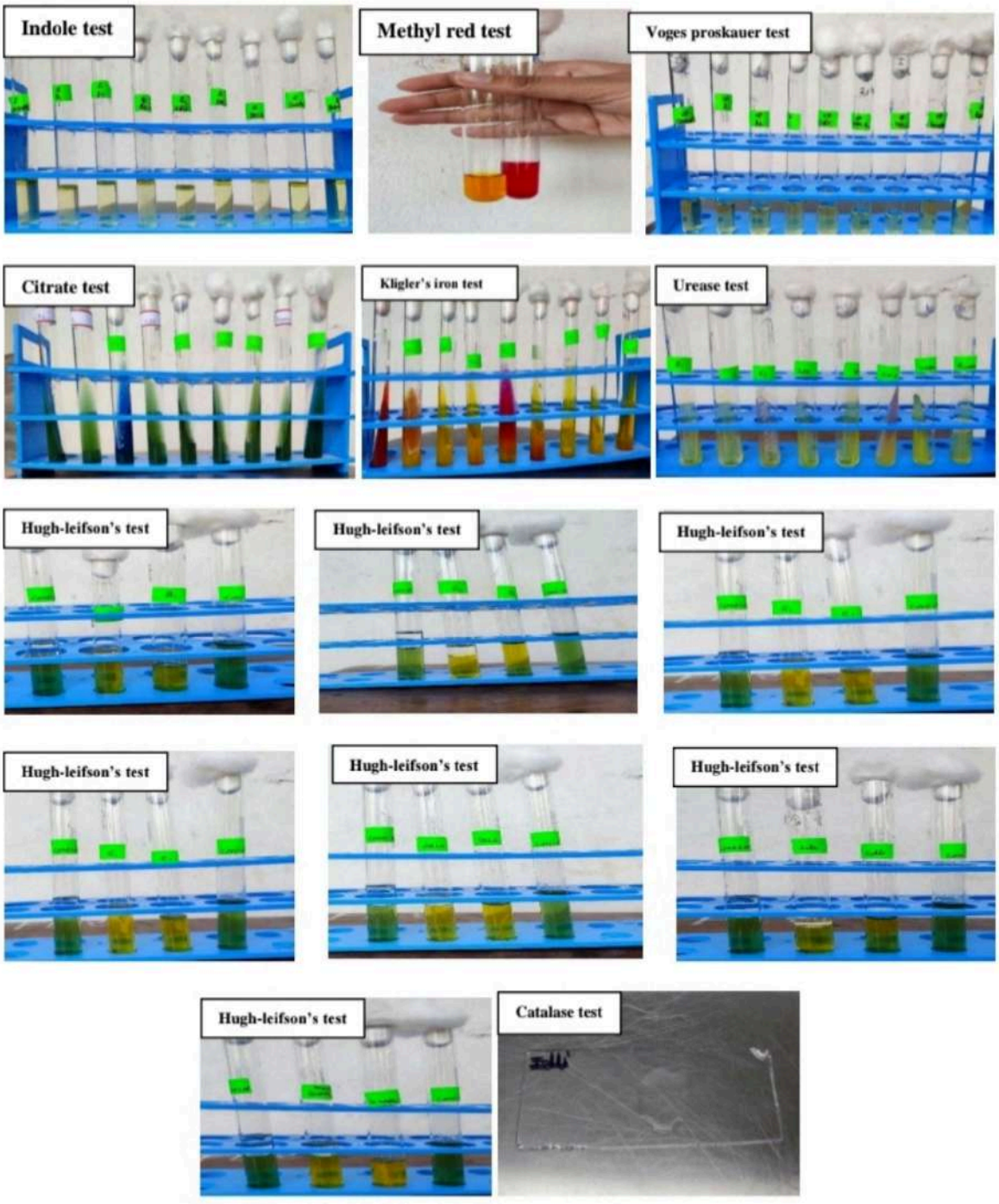


Fig 4.3.3.1 Characterization of isolates by Biochemical tests

4.4 Screening of isolates for acid production

4.4.1 Screening of isolates for acid production using sugars

Isolate B1, D2, I and G2 showed acid production using sucrose as carbon source (table 4.4.1.1). Isolates B1, D2, E1, DKL1, DKL2, I and G2 showed acid production using maltose as carbon source. Isolates D2, I and G2 showed acid production using lactose as carbon source. All the isolates showed acid production using glucose as carbon source.

Table 4.4.1.1 Screening of isolates for acid production using sugars

Isolates	Sucrose	Maltose	Lactose	Glucose
B1	+	+	_	+
D2	+	+	+	+
E1	_	+	_	+
DKL1	_	+	_	+
DKL2	_	+	_	+
I	+	+	+	+
BA1	_	_	_	+
G2	+	+	+	+
Control	_	_	_	_

Positive: +	Negative : -

Positive: **red**	Negative : **yellow**

Fig.4.4.1.1 Testing for Acid production using sugars glucose, maltose, lactose and sucrose

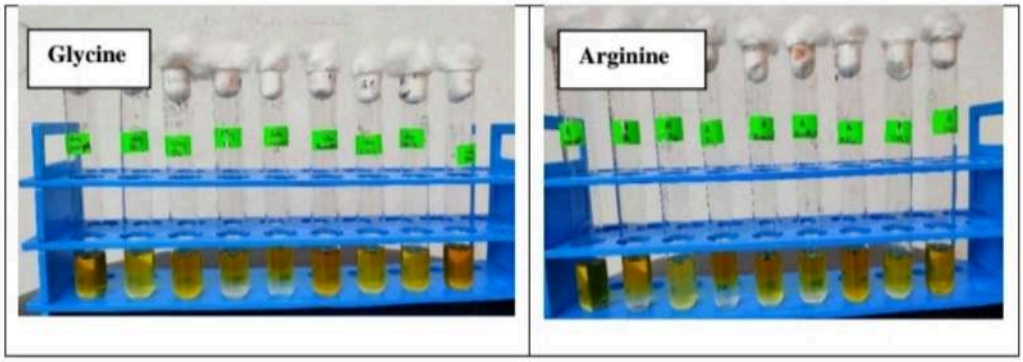
4.4.2 Screening of isolates for acid production using amino acids

No isolates showed acid production using glycine as carbon source(table 4.4.2.1). No isolates showed acid production using arginine as carbon source.

Table 4.4.2.1 Screening of isolates for acid production using amino acids

Isolates	Glycine	Arginine
B1	-	-
D2	-	-
E1	-	-
DKL1	-	-
DKL2	-	-
I	-	-
BA1	-	-
G2	-	-
Control	-	-

Positive: +	Negative : -



Positive: **red**	Negative : **yellow**

Fig 4.4.2.1 Testing for Acid production using amino acids glycine and arginine

4.5 Screening of isolates for enzyme activity

The isolates did not show zone of clearance around the colony after flooding with iodine solution. This indicated that there was no amylase activity.

The isolates did not show zone of clearance around the colony. This indicated that there was no protease activity.

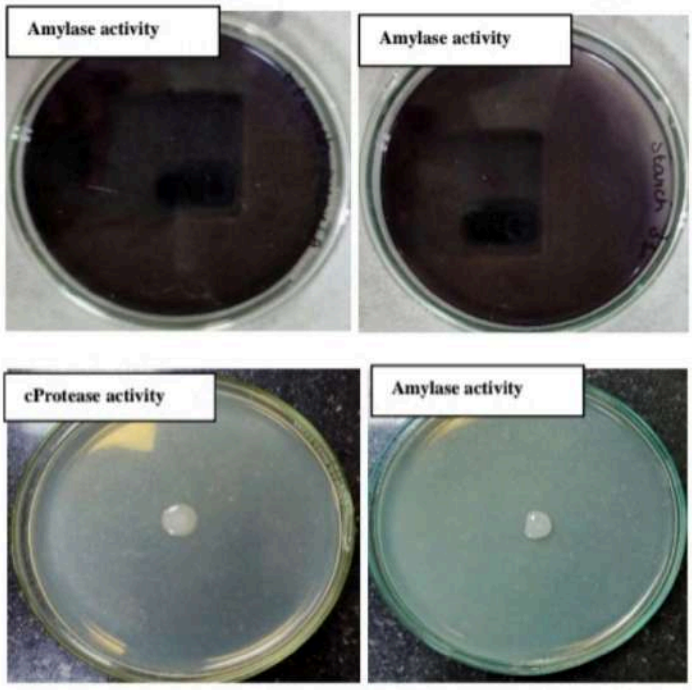


Fig 4.5.1 Screening of isolates for amylase and protease activity

4.6 Quantification of Acid produced

The amount of acid was quantified by lyophilisation and weight of lyophilized product was observed as follow:

$$Ac = \frac{w}{y} \times \frac{Ace}{x}$$

Particular	Isolate G2	Isolate I
Volume of culture broth (x) (ml)	1	1
Volume of acetone used for extraction (Ace) (ml)	4	4
Volume of acetone extracted acid used for lyophilisation (y) (ml)	3.5	3.5
Weight of lyophilised extract (w) (mg)	14.9	11
Amount of acid in culture broth (Ac) (mg/ml)	17	12

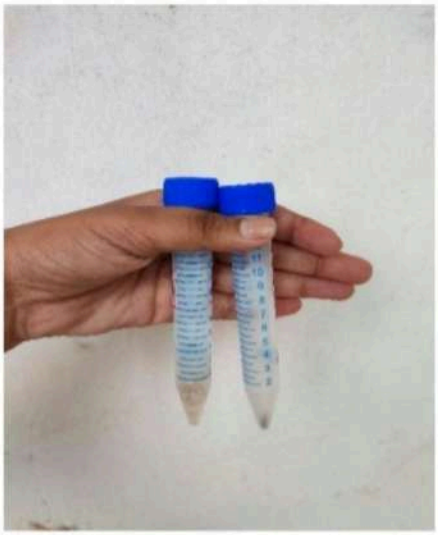


Fig 4.6.1 Lyophilized acetone extracted acid

4.7 Microbial fuel cell

Microbial fuel cell was prepared as shown in fig 4.7.1.The anode and cathode chamber was connected by salt bridge. The isolate I and G2 showed acid production in all the four sugars (lactose, glucose, maltose and sucrose). The amount of acid produced by isolate I and G2 were quantified. Since amount of acid produced by isolate G2 was maximum, it was used as inoculum in microbial fuel cell. The voltage and current was recorded by multimeter.

As seen in table 4.7.1, the maximum voltage was obtained on day 2. The maximum amount of current was generated on day 2.

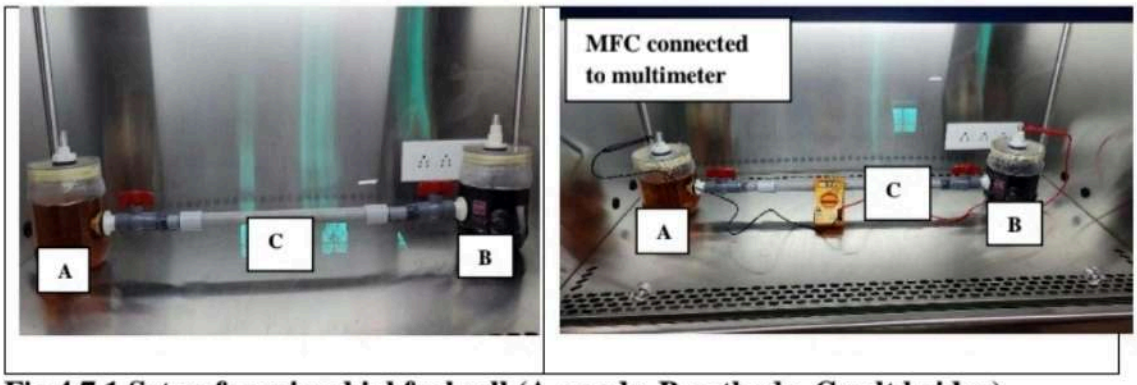


Fig 4.7.1 Setup for microbial fuel cell (A-anode, B-cathode, C-salt bridge)

Table 4.7.1 Measurement of voltage and current generated in microbial fuel cell

Incubation period	Current	Voltage
0 hours	0.0	065
3 hours	001	169
24 hours	058	645
72 hours	027	369
96 hours	015	261
144 hours	042	500
168 hours	028	375

4.8 Discussion

A total of twelve isolates were obtained from curd sample. One isolate was obtained from idli batter. Two isolate were obtained from dhokla batter. . One isolate was obtained from banana and guvava each. Two isolate were obtained from grapes and one isolate was obtained from strawberry.

The isolates were screened for acid production by methyl red test. Out of twenty one isolates, eight isolates showed acid production within 24 hours and continued to do so after 48 hours.

Characterisation of isolates was carried by gram's staining, colony characterisation and biochemical test. Gram staining of isolates revealed that all the isolates were gram positive in nature.

Isolates were screened for acid production using sugars and amino acids. Isolate B1, D2, I and G2 showed acid production using sucrose as carbon source. Isolates B1, D2, E1, DKL1, DKL2, I and G2 showed acid production using maltose as carbon source. Isolates D2, I and G2 showed acid production using lactose as carbon source. All the isolates showed acid production using glucose as carbon source. No isolates showed acid production using glycine and arginine as carbon source.

Isolates were then screened for enzyme activity. The isolates did not show zone of clearance around the colony after flooding with iodine solution. This indicated that there was no amylase activity.

The isolates did not show zone of clearance around the colony. This indicated that there was no protease activity.

Acid produced by the isolate I and isolate G2 was quantified. Acid produced by Isolate G2 was 17 mg/ml of culture broth. Amount of acid produced by isolate I was 12 mg/ml of the culture broth.

The MFC was prepared by connecting anode chamber and cathode chamber by salt bridge made from corn starch and sodium chloride. Amount of acid produced by isolate G2 was maximum, it was used as inoculum in microbial fuel cell. The multimeter was connected to anode and cathode chamber to measure current and voltage.

Obasi et al. (2012) prepared MFC using proton exchange membrane made up of cassava starch and salt. The current and voltage was monitored for 10 days. The maximum amount of current was observed on day 5. There current decreased on day 6. On first day, the voltage was 1.09 volts which increased on day 3. The voltage decreased from day 4.

The voltage and current was monitored for 168 hours. On day 2, the maximum amount of current produced by the isolate G2 was observed and maximum amount of voltage was also observed.

CONCLUSION

The Microbial fuel cell technology has potential for energy generation. MFC technology also highlights its utility in wastewater treatment. The utilization of corn starch as a salt bridge presents a cost effective alternative to energy recovery within MFC system. These outcomes demonstrate MFC's potential for addressing both environmental and economic challenges associated with waste management.

FURTHER SCOPE

MFC can provide a efficient solution for treating wastewater from various sources such as agriculture waste, industrial wastewater and brewery effluent. A variety of microbes, including yeast, fungus, and bacteria, can be for production of energy in MFC systems. Proton exchange membrane research also shows potential for improving MFC installations' ability to produce power.

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APPENDIX I

1.MRS Agar

Ingredients	g/l
Protease peptone	10
Beef extract	10
Yeast extract	5
Dextrose	20
Polysorbate 80	1
Ammonium citrate	2
Sodium acetate	5
Magnesium sulphate	0.100
Manganese sulphate	0.050
Dipotassium phosphate	2.00
Agar	12.000
Final pH	6.5

2.Glucose Phosphate broth

Ingredients	g/l
Peptone	7
Dipotassium phosphate	5
Glucose	5
Sodium chloride	3.5
Distilled water	1

3. Tryptone broth

Ingredients	g/l
Casein enzymatic hydrolysate	10
Sodium chloride	5
Final pH	7.5

4.Simmons citrate agar

Ingredients	g/l
Magnesium sulphate	0.200
Ammonium dihydrogen phosphate	1.000
Dipotassium phosphate	1.000
Sodium citrate	2.000
Sodium chloride	5.000
Bromothymol blue	0.080
Agar	15.000
Final pH	6.8

5. Triple sugar iron agar

Ingredients	g/l
peptone	10.000
Tryptone	10.000
Yeast extract	3.000
HM peptone B	3.000
Lactose	10.000
Sucrose	10.000
Dextrose	1..000
Sodium chloride	5.000
Ferrous sulphate	0.200
Sodium thiosulphate	0.300
Phenol red	0.024
Agar	12.000
Final pH	7.4

6.Urease test

Ingredients	g/l
Dextrose	1.000
Peptic digest of animal tissue	1.500
Sodium chloride	5.000
Monopotassium phosphate	2.000
Phenol red	0.012
Agar	15.000
Final pH	6.8

7. Hugh leifson medium

Ingredients	g/l
Peptic digest of animal tissue	2.000
Sodium chloride	5.000
Dipotassium phosphate	0.300
Glucose	10.000
Bromothymol blue	0.050
Agar	2.000
Final pH	6.8

8. Medium 1

Ingredients	g/l
Peptone	7
Dipotassium phosphate	5
Lactose	5
Sodium chloride	3.5
Distilled water	1

9. Medium 2

Ingredients	g/l
Peptone	7
Dipotassium phosphate	5
Sucrose	5
Sodium chloride	3.5
Distilled water	1

10. Medium 3

Ingredients	g/l
Peptone	7
Dipotassium phosphate	5
Maltose	5
Sodium chloride	3.5
Distilled water	1

11. Medium 4

Ingredients	g/l
Peptone	7
Dipotassium phosphate	5
Glycine	5
Sodium chloride	3.5
Distilled water	1

12. Medium 4

Ingredients	g/l
Peptone	7
Dipotassium phosphate	5
Arginine	5
Sodium chloride	3.5
Distilled water	1

APPENDIX II

1. Methyl red indicator

Methyl red	0.2g
Ethano (95%)l	60.0 ml
Distilled water	40.0 ml

2. Crystal violet

Crystal violet	20 g
Ethyl alcohol	20 ml
Ammonium oxalate	80 ml

3. Gram's iodine

Potassium iodine	1 g
Distilled water	300 ml

4. Counter stain safranin

Safranin	100 ml
Ethyl alcohol	100 ml
Distilled water	100 ml
Xylol	5 ml

5. Kovac's reagent

p-dimethyl amino benzaldehyde	5.0 g
Amyl alcohol	75 ml
Hydrochloric Acid	25 ml

6. O'Meara Reagent

Potassium hydroxide	40 g
Creatine	0.3 g
Distiiled water	100 ml

7. Iodine solution

Iodine	0.34 g
Potassium iodide	0.66 g
Distilled water	100 ml