Production Of Low Molecular Weight Organic Acid Using Organic Kitchen Waste

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STUDENT DECLARATION

I, at this moment declare that the data presented in this Dissertation report entitled, "Production of low molecular weight organic acid using organic kitchen waste" is based on the results of investigations carried out by me in the Microbiology at the School of Biological Sciences and Biotechnology, Goa University under the supervision of Prof. 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 / College will not be responsible for the correctness of observations / experimental or other findings given the dissertation. I, at this moment authorize the University/college authorities to upload this dissertation to the dissertation repository or anywhere else as the UGC regulations demand and make it available to anyone as needed.

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CERTIFICATE

This is to certify that the dissertation report "Production of low molecular weight organic acid using organic kitchen waste" is a bonafide work carried out by Ms. Sanisha Vasudev Gaude under my supervision in partial fulfilment of the requirements for the award of the degree of Master of Sciences in the Discipline Microbiology at the school of Biological Sciences and Biotechnology, Goa University.

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PREFACE

This study focuses on the production of low molecular weight organic acids. Organic acids are traditionally produced through petroleum-based processes. use of petroleum for organic acid production is not sustainable and presents environmental and economic drawbacks. Research into alternative, bio-based sources like kitchen waste offers a more promising path for the future. This investigation aims to utilize kitchen waste as a substrate for organic acid production. reusing kitchen waste offers a win-win situation for the environment, economy, and society. It promotes sustainability, fosters innovation, and creates new opportunities while minimizing environmental burdens. Hence this approach offers a two-fold benefit: it utilizes waste as a resource for bio-based product development and contributes to sustainable waste management.

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ABBREVIATIONS USED

Abbreviation	Expansion
CMC	Carboxymethyl cellulose
GPP	Glucose phosphate broth
LCA	Life Cycle Assessment
NA	Nutrient agar
OKW	Organic kitchen waste
OPB	Oil phosphate broth
PPB	Peptone phosphate broth
SPB	Starch phosphate broth
TSI	Triple sugar iron

ABSTRACT

Waste generation growing exponentially with a growing population. Food waste is one of them and its management becomes tricky as it is produced in masses daily. Biomethanation and composting are some ways to transform this waste into valuable products. This study explores the potential of utilizing organic kitchen waste for its bioconversion into organic acids by acid-producing bacteria. The ability of bacterial isolated from local soil samples was investigated using carbohydrates, proteins, fats, and carboxymethyl cellulose (CMC) which are basic components of kitchen waste. The isolated strains were screened for their acid-producing capabilities, targeting organic acids. Methyl red indicator was employed as a preliminary screening tool for acid production. Promising isolates were biochemically characterized to identify their specific features. Finally, the production of organic acids by these isolates was quantified using a spectrophotometer. Successful outcomes could contribute to the development of sustainable waste management strategies and bio-based production of organic acids. These organic acids have diverse applications in various industries, including the production of bioplastics, pharmaceuticals, and food additives. Successful development of this technology could offer a sustainable solution for kitchen waste management while simultaneously promoting the production of bio-based chemicals.

Keywords

Organic acid

Kitchen waste

Methyl red

Starch phosphate broth

Spectrophotometer

INTRODUCTION

Food waste represents a significant and growing global challenge. Each year, an estimated one-third of all food produced is wasted, amounting to billions of tons (Gustavsson et al., 2011). This organic waste not only occupies valuable landfill space but also decomposes anaerobically, generating methane, a potent greenhouse gas (IPCC, 2019). Converting this waste into valuable resources is crucial for promoting sustainable waste management practices and mitigating environmental impacts.

The concept of a circular bioeconomy offers a framework for transforming organic waste into resources (Ghirelli et al., 2016). A circular bioeconomy emphasizes reducing waste generation, reusing resources, and recycling organic materials back into the production cycle. Organic kitchen waste, rich in carbohydrates, proteins, and fats, holds immense potential as a feedstock for the production of valuable bio-based products (Lee & Phan, 2014).

Organic acids are a diverse group of carboxylic acids with numerous applications in various industries. They are used in food and beverage production, pharmaceuticals, and as precursors for bioplastics and other bio-based products (Lee & Phan, 2014). Developing efficient methods for converting organic kitchen waste into organic acids offers a promising approach to waste valorization.

This thesis aims to explore the potential of utilizing organic kitchen waste as a feedstock for the production of organic acids. To achieve this aim, the following objectives will be addressed:

- Isolation of bacteria which can utilize carbohydrates, protein, and fats and convert them into organic acid.
- Application of these isolates on kitchen waste for production of organic acid.
- Characterization of bacteria that produce organic acid from kitchen waste.

- Optimizing process for the production of organic acid using kitchen waste.

If bacteria can catabolically process organic substrates such as carbohydrates, proteins, and lipids into organic acid, these bacteria can be utilized to convert kitchen waste for the production of organic acid.

This thesis will focus on the laboratory-scale investigation of fermenting organic kitchen waste for organic acid production. The research will isolate bacteria that can utilize carbohydrates, lipids, and fats and produce organic acid. optimize key parameters to enhance organic acid yield. While pilot-scale or industrial applications are not within the scope of this thesis, the findings will contribute valuable insights for future research and development in this field.

LITERATURE REVIEW

2.1 Organic kitchen waste and its management

Food waste generated in households poses a significant environmental challenge. However, this seemingly undesirable byproduct holds the potential to become a valuable resource. Organic kitchen waste comprises 52% of kitchen waste. This waste not only occupies valuable landfill space but also decomposes anaerobically, generating methane, a potent greenhouse gas (IPCC, 2019). Conventional waste management practices are increasingly unsustainable, necessitating innovative solutions for organic kitchen waste management. There are various strategies for managing organic kitchen waste, focusing on approaches that promote resource recovery and environmental sustainability.

Organic kitchen waste represents a significant portion of the municipal solid waste. Composting this waste offers environmental and economic benefits, but its effectiveness depends on understanding its composition. This review explores the composition of organic kitchen waste, focusing on key components and factors influencing variability.

2.1.1 Composition of organic kitchen waste

a) **Fruits and Vegetables (40-60%):** Forming the bulk of kitchen waste, fruits, and vegetables are rich in carbohydrates, fibers, vitamins, and minerals (Kader, 2002). These components readily decompose during composting, providing energy sources for microorganisms and contributing to the overall nutrient content of the finished compost. However, the high moisture content of fruits and vegetables necessitates proper bulking agents (like dry leaves or shredded paper) to maintain optimal moisture levels and aeration within the compost pile (The Cornell Waste Management Institute, 2023).

b) **Food Scraps:** Vegetable peels, eggshells, coffee grounds, and tea leaves fall under this category. They contribute a diverse range of organic materials, including carbohydrates, proteins, lipids, and minerals (Mussatto & Teixeira, 2010; Mussatto et al., 2011).

c) **Meat and Dairy Products (trace amounts):** While discouraged due to odor issues and potential pest attraction, meat and dairy products might be present in small quantities within kitchen waste. These materials are rich in proteins and fats (Yildiz et al., 2004). Although they can decompose during composting, their breakdown can be slower and attract unwanted pests. Additionally, excessive protein or fat content in the compost can lead to unpleasant odors.

2.1.2 Factors Influencing Variability in Kitchen Waste Composition:

a) **Diet and Consumption Habits:** Dietary preferences and consumption patterns significantly impact the composition of kitchen waste (Parfitt et al., 2010). Vegetarian diets tend to generate more fruit and vegetable scraps, while meat-based diets contribute more animal-derived products, such as meat, poultry, and dairy. This directly affects the nutrient profile of the resulting compost. Understanding these variations can help tailor composting practices to optimize outcomes.

b) **Seasonality:** The availability and consumption of fruits and vegetables fluctuate throughout the year, influencing the composition of kitchen waste (Garcia-Garcia et al., 2002). For instance, there might be a higher proportion of citrus peels and apple cores during winter months compared to summer, when seasonal fruits and vegetables dominate the waste stream. Composters can adjust bulking agents or additional amendments based on seasonal variations to maintain a balanced compost pile.

c) **Geographical Location:** Regional food preferences and agricultural practices influence the types of fruits and vegetables discarded, leading to compositional variations in kitchen waste (Jayasinghe et al., 2015). For example, regions with higher consumption of root vegetables might see a higher proportion of potato peels compared to areas where rice is a dietary staple. Understanding these geographical variations can inform strategies for large-scale composting initiatives.

2.1.3 Current Food Waste Valorisation Techniques

Waste generation cannot be entirely prevented, valorisation techniques offer an alternative to traditional disposal methods. These techniques aim to convert organic kitchen waste into valuable resources:

a) **Composting:** Composting transforms organic waste into nutrient-rich compost, a valuable soil amendment that improves soil health and fertility (Bernal et al., 2009). It offers a sustainable solution for diverting organic waste from landfills and generating a valuable resource for agriculture and horticulture (Singh & Ibrahim, 2018). Landfill diversion is a key benefit of composting. Organic waste decomposition in landfills generates methane, a potent greenhouse gas. Composting reduces landfill methane emissions and associated environmental burdens (Amlinger et al., 2017). Compost serves as a natural fertilizer, improving soil health by enhancing nutrient content, water-holding capacity, and overall soil structure (Li et al., 2014). Compost application can significantly reduce reliance on chemical fertilizers, promoting sustainable agricultural practices (Singh & Ibrahim, 2018). Landfill diversion through composting reduces greenhouse gas

emissions and conserves natural resources like water and land required for food production (Amlinger et al., 2017).

b) **Anaerobic Digestion (AD):** AD breaks down organic matter in an oxygen-depleted environment, producing biogas (methane) and digestate, a nutrient-rich biofertilizer (Mata-Alvarez et al., 2014). This process yields two valuable products: biogas, a methane-rich renewable energy source, and digestate, a nutrient-rich soil amendment (Angelidaki et al., 2018). AD is emerging as a sustainable solution for waste management and bioenergy production.

AD offers a multifaceted approach to environmental challenges. It effectively diverts organic waste from landfills, mitigating greenhouse gas emissions associated with landfill decomposition (Li et al., 2011). By converting waste into biogas, AD promotes renewable energy production and reduces dependence on fossil fuels (Scarlat et al., 2015). Additionally, the digestate produced from AD serves as a valuable organic fertilizer, improving soil quality and reducing reliance on chemical fertilizers (Yenigün & Demirer, 2013).

Research is ongoing to optimize AD processes for efficiency and broader applicability. Exploring factors like feedstock composition, digester design, and microbial communities is crucial for enhancing biogas yields and overall process stability (Mata-Alvarez et al., 2014). Additionally, advancements in pretreatment technologies can expand the range of suitable feedstocks for AD, making it an even more versatile waste management and bioenergy solution.

c) **Insect Composting:** This technique utilizes insects like black soldier fly larvae to decompose organic waste, resulting in insect biomass (protein source for animal feed) and frass (compost) (Makkar et al., 2014). This emerging technology offers a sustainable and efficient alternative to traditional composting methods, with potential environmental and economic benefits.

d) **Types of Insect Composting:**

- **Vermicomposting:** This method employs composting worms (Eisenia fetida) to break down organic waste (Butt et al., 2016). Vermicomposting systems are generally small-scale and often used for household or community food waste management.
- **Black Soldier Fly Composting:** Black soldier fly larvae (Hermetia illucens) are prolific decomposers that can efficiently convert various organic wastes into frass and insect biomass (Cai et al., 2017). BSF composting systems can be scaled for larger-scale organic waste processing facilities.

  While research is ongoing to optimize insect composting for larger-scale operations, broader adoption requires further economic feasibility studies and infrastructure development (Van et al., 2017). Social acceptance and overcoming potential negative perceptions surrounding insects are crucial for wider public adoption of insect composting technologies (Liu et al., 2018).

e) **In-vessel composting:** In-vessel composting (IVC) has emerged as a promising technology for efficient and controlled organic waste management. Unlike traditional open-air composting systems, IVC utilizes enclosed vessels to optimize the composting process, offering several advantages for large-scale waste processing facilities and urban environments. This review examines the principles of IVC, its benefits and limitations,

and its potential role in sustainable waste management. Maintaining optimal conditions within the vessels requires energy input, which can be a cost consideration (Amlinger et al., 2017). IVC systems typically have higher initial investment costs compared to open-air composting (Bernal et al., 2019). IVC systems may require specific feedstock characteristics for optimal performance, potentially limiting flexibility (Amlinger et al., 2017).

These valorization techniques provide sustainable solutions for managing organic kitchen waste while generating valuable products that benefit agriculture and potentially other sectors.

2.1.4 Organic Acids from Kitchen Scraps: A Bacterial Feast

Bacteria, nature's tiny alchemists, can transform kitchen scraps rich in carbohydrates, proteins, and lipids into a diverse range of organic acids. These acids have numerous applications, making this a win-win scenario for waste reduction and bioproduct creation.

Acidogenesis, a crucial stage in anaerobic digestion (AD), orchestrates the breakdown of complex organic matter into organic acids. Acidogenesis forms the bridge between hydrolysis and the subsequent stages of AD, acetogenesis. Acidogenic bacteria break down complex organic polymers like carbohydrates, proteins, and fats into simpler acids (Alshawabkeh et al., 2017). Some products produced by acidogenesis may serve as primary substrates that can be utilized for other processes and may lead to biogas production (Appels et al., 2011). Efficient acidogenesis ensures a steady and controlled breakdown of complex organic material, preventing overloading of subsequent stages in AD. If acidogenesis is slow or inefficient, complex organic matter accumulates, leading to an imbalanced system. This can cause a buildup of intermediate products and hinder the activity of bacteria in later stages.

A diverse consortium of fermentative bacteria thrives in the acidogenic stage:

a) **Hydrolytic and fermentative bacteria:** Are essential players in decomposing organic matter, a crucial role in nutrient cycling within ecosystems. They work in a complementary fashion to break down complex organic materials into simpler compounds usable by other organisms. These bacteria degrade complex organic matter, producing various VFAs like acetate, propionate, and butyrate (Zhao et al., 2017). Hydrolytic bacteria act as the initial decomposers, equipped with specialized enzymes that break down complex organic polymers like carbohydrates, proteins, and lipids. Here's how they function:

- **Enzyme Secretion:** Hydrolytic bacteria secrete extracellular enzymes that break down large organic molecules outside the cell (Rahman et al., 2012).
- **Specificity:** Different hydrolytic bacteria produce enzymes specific to particular substrates. For example, cellulases break down cellulose (a complex carbohydrate) in plant material (Lynd et al., 2002).
- **Products:** Hydrolysis by these bacteria results in simpler molecules like sugars, amino acids, and fatty acids.

Fermentative bacteria come into play after hydrolytic bacteria have done their job. They utilize the simpler molecules produced by hydrolysis and convert them through fermentation pathways:

- **Substrate Utilization:** Fermentative bacteria can utilize various simple sugars, amino acids, and fatty acids as energy sources.
- **Fermentation Products:** Depending on the specific type of fermentative bacteria and the fermentation pathway used, various products can be formed, including:

- **Volatile Fatty Acids (VFAs):** Acetate, propionate, and butyrate are common VFAs produced by fermentative bacteria.
- **Alcohols:** Ethanol and lactic acid are examples of alcohols produced during fermentation.
- **Hydrogen Gas:** Some fermentative bacteria produce hydrogen gas as a byproduct.

Hydrolytic and fermentative bacteria work together in a synergistic relationship. Hydrolytic bacteria break down complex organic matter, creating simpler molecules readily utilized by fermentative bacteria. The products of fermentation, like VFAs, can be purified and utilized in various industrial applications.

c) Homoacetogens: Specialised bacteria convert certain fermentation products like lactate and ethanol into acetate, a key VFA for methanogenesis (Liu & Angelidaki, 2018). These bacteria play a crucial role in anaerobic digestion (AD), a process that converts organic waste into valuable products. This review delves into their unique metabolic capabilities, ecological significance in AD, and potential applications. Homoacetogens stand out from other fermentative bacteria due to their ability to utilize various substrates and convert them primarily into acetate, a key precursor for methane production in AD (Liu & Angelidaki, 2018). Their distinct metabolic pathway, the Wood-Ljungdahl pathway, allows them to:

- **Substrate Versatility:** Homoacetogens can utilize a wide range of carbon sources, including simple sugars, amino acids, and even secondary fermentation products like lactate and ethanol produced by other bacteria in the AD process (Srinivasan et al., 2016).
- **Acetate Formation:** Through the Wood-Ljungdahl pathway, homoacetogens convert these substrates into acetate as the primary product, alongside hydrogen and carbon dioxide

(Lee et al., 2019). Their ability to utilize diverse organic substrates makes them valuable for efficient waste degradation in AD systems.

Understanding and potentially manipulating the homoacetogen population within the AD microbiome can improve acetate production and process stability (Liu & Angelidaki, 2018). Strategies need to be explored that will enhance the ability of Homoacetogens to utilize complex substrates and improve overall waste biodegradation efficiency in AD. homoacetogens have potential in biorefinery processes for the production of valuable bioproducts like acetate, a platform chemical for various industrial applications.

Several factors impact the efficiency of acidogenesis in AD:

- **Substrate Composition:** The type and complexity of organic matter fed to the digester influence the types and activities of acidogenic bacteria (Mata-Alvarez et al., 2014).
- **pH:** Acidogenic bacteria generally thrive in a slightly acidic environment (pH 5.5-6.0). Significant pH fluctuations can disrupt their activity (Chen et al., 2018).
- **Temperature:** Optimal temperature ranges vary for different acidogenic bacteria. Maintaining a suitable temperature within the digester is crucial (Appels et al., 2011).
- **Nutrient Availability:** Essential nutrients like nitrogen, phosphorus, and trace elements are required for optimal bacterial growth and activity (Zhao et al., 2017).

Organic kitchen waste is a complex buffet for bacteria. Sugars like glucose and fructose, abundant in fruits and vegetables, are readily fermentable. While bacteria can also utilize proteins and fats for energy, carbohydrates are the primary targets for organic acid production. Several studies have demonstrated the potential of this approach. For instance, Tan et al. (2016) successfully employed a mixed bacterial consortium, a diverse community of bacteria working together, to ferment

kitchen waste and produce significant quantities of lactic acid, a common food additive and building block for bioplastics.

The specific organic acid produced depends on the type of bacteria employed. Here are some key players:

- **Lactic Acid Bacteria (LAB):** These bacteria, commonly found in yogurt and fermented foods, excel at producing lactic acid. Strains like *Lactobacillus casei* and *Lactobacillus rhamnosus* are frequently used in kitchen waste fermentation for this purpose (Wang et al., 2017; Mussatto et al., 2015).
- **Volatile Fatty Acid (VFA) Producers:** Anaerobic bacteria, those that thrive in oxygen-depleted environments, can be harnessed for VFA production. *Clostridia* species and *Propionibacterium acidipropionici* are examples of such bacteria, converting kitchen waste into VFAs like acetic acid and propionic acid (Zhang et al., 2010; Chen et al., 2018). VFAs have applications in biofuel production and agriculture.

Different fermentation techniques can be employed, some are mentioned below:

- **Solid-state fermentation (SSF):** This technique involves fermenting the waste in a solid state with minimal liquid. It is simple, requires minimal setup, and generates less wastewater (Mussatto et al., 2014). Wang et al. (2017) successfully used SSF for lactic acid production from kitchen scraps using *Lactobacillus casei.*
- **Submerged liquid fermentation (SLF):** Here, the waste is fermented in a liquid medium. This technique offers greater control over parameters like temperature and pH but requires additional steps for pre-treating the waste (Mussatto et al., 2014). Chen et al.

(2018) demonstrated propionic acid production from kitchen waste using SLF with *Propionibacterium acidipropionici*.

- **Consolidated bioprocessing (CBP):** This approach combines enzymatic breakdown of complex carbohydrates with subsequent bacterial fermentation. CBP can potentially enhance efficiency by making more sugars readily available for bacteria (Mussatto et al., 2014). Mussatto et al. (2015) reported successful CBP of kitchen waste for lactic acid production using *Lactobacillus rhamnosus*.

Several key factors influence the amount of organic acid produced:

- **Substrate pre-treatment:** Breaking down the waste into smaller particles or using enzymes to pre-digest complex molecules can improve bacterial access to fermentable sugars (Mussatto et al., 2014).
- **Bacterial strain selection:** Choosing the right bacterial strain for the desired acid and optimizing its growth conditions are crucial (Jayasinghe et al., 2010).
- **Environmental parameters:** Maintaining optimal temperature, and pH, and providing essential nutrients for bacterial growth are essential for efficient fermentation (Li et al., 2014).

By optimizing these parameters, researchers are continuously improving the efficiency of converting kitchen scraps into valuable organic acids. This glimpse into the world of bacterial fermentation using kitchen waste reveals the immense potential for transforming waste into resources.

Organic acids are a diverse group of carboxylic acids with numerous applications in various industries. Traditionally, they have been derived from petroleum-based sources, but a growing focus on sustainability has led to increased interest in bio-based production using bacteria.

This approach offers several advantages:

- **Renewable feedstocks:** Bacteria can utilize various renewable substrates, including agricultural waste, industrial byproducts, and even greenhouse gasses like carbon dioxide, for organic acid production.
- **Environmentally friendly:** Bacterial fermentation generally has a lower environmental footprint compared to traditional chemical synthesis methods.
- **Product diversity:** Different bacterial strains can be employed to produce a wide range of organic acids, catering to specific industrial needs.

2.1.5 Bacterial Fermentation for Organic Acid Production

Bacteria play a crucial role in the bio-based production of organic acids. The general process involves fermentation, a metabolic pathway where bacteria convert organic substrates into various products, including organic acids, under anaerobic (oxygen-depleted) or aerobic(oxygen-rich) conditions. The specific type of organic acid produced depends on the bacterial strain, fermentation conditions, and the substrate used. Here are some examples:

- **Lactic Acid:** Lactic acid bacteria (LAB) are commonly used for fermenting sugars derived from carbohydrates to produce lactic acid. This acid has applications in the food and beverage industry, pharmaceuticals, and bioplastics production (Jayasinghe et al., 2010).

- **Acetic Acid:** Acetic acid bacteria, such as Acetobacter aceti, can convert various carbohydrates and alcohols into acetic acid through aerobic fermentation. This acid is widely used in the food industry as a vinegar component and has applications in chemical synthesis (Zhang et al., 2014).
- **Citric Acid:** Aspergillus niger, a fungal mold, is traditionally used for citric acid production. However, some bacterial strains, like Corynebacterium glutamicum, can also be employed for this purpose (Ahn et al., 2016). Citric acid finds applications in food and beverages, pharmaceuticals, and as a chelating agent.
- **Propionic Acid:** Propionic acid bacteria, such as Propionibacterium acidipropionici, can ferment various organic substrates to produce propionic acid. This acid has applications in food preservation, animal feed production, and as a platform chemical for biofuel production (Chen et al., 2018).

Factors Affecting Organic Acid Production

Several factors influence the efficiency and yield of organic acid production using bacteria:

- **Bacterial strain selection:** Choosing the right bacterial strain with the desired metabolic pathway for the target organic acid is crucial.
- **Substrate composition:** The type and availability of fermentable sugars and other nutrients in the substrate significantly impact bacterial growth and acid production.
- **Fermentation conditions:** Maintaining optimal parameters like temperature, pH, and oxygen availability is essential for efficient fermentation.
- **Downstream processing:** Recovering and purifying the produced organic acid from the fermentation broth is an important step for industrial application.

Bio-based production of organic acids using bacteria holds immense promise for a more sustainable future. Continuous research efforts are focused on:

- **Engineering bacterial strains:** Optimizing existing strains or developing new ones for enhanced acid production efficiency and broader substrate utilization.
- **Developing efficient fermentation processes:** Exploring novel fermentation techniques that are cost-effective and scalable for industrial applications.
- **Utilizing diverse feedstocks:** Identifying and utilizing a wider range of renewable and waste-derived substrates for sustainable organic acid production.

Enhancing Efficiency and Substrate Utilization:

- **Strain Engineering:** Researchers are engineering bacterial strains for improved performance. For instance, a study by Li et al. (2020) genetically modified Lactobacillus casei to utilize various sugars present in OKW, leading to increased lactic acid production (Li et al., 2020).
- **Co-culturing:** Utilizing mixed bacterial consortia is another approach. Wang et al. (2021) demonstrated enhanced propionic acid production from OKW by co-culturing Propionibacterium acidipropionici with cellulose-degrading bacteria, improving complex carbohydrate utilization (Wang et al., 2021).

Novel Fermentation Techniques:

- **Solid-State Fermentation (SSF) with Optimization:** SSF offers a simple and low-cost approach. Recent studies focus on optimizing parameters like moisture content and particle size for improved efficiency. A study by Sun et al. (2018) reported successful lactic acid production from OKW using SSF with optimized parameters (Sun et al., 2018).

- **Consolidated Bioprocessing (CBP):** CBP combines enzymatic breakdown of complex molecules with subsequent fermentation. Research by Zhang et al. (2019) explored CBP for succinic acid production from OKW, demonstrating its potential for high-value bioproducts (Zhang et al., 2019).

Expanding Substrate Range and Valorization:

- **Mixed Waste Streams:** Researchers are investigating the use of mixed OKW streams, including fruits, vegetables, and even coffee grounds. This approach maximizes waste utilization, as reported in a study by Tan et al. (2020) on lactic acid production from a mixed OKW substrate (Tan et al., 2020).
- **Waste Pretreatment Techniques:** Advanced pre-treatment methods like microwave irradiation or ultrasound are being explored to enhance the breakdown of complex molecules in OKW, improving accessibility for bacteria. A study by Zhao et al. (2018) demonstrated the effectiveness of microwave pre-treatment for increased volatile fatty acid production from OKW (Zhao et al., 2018).

Integration with Waste Management Systems:

- **Decentralized Fermentation Systems:** Research is exploring the development of small-scale fermentation units suitable for household or community-level OKW processing. This could promote localized waste management and resource recovery (Jayasinghe et al., 2020).
- **Life Cycle Assessment (LCA):** Researchers are conducting LCA studies to assess the environmental impact of OKW-based organic acid production. This helps identify areas for improvement and ensures the overall sustainability of the process (Caulfield et al., 2020).

These advancements highlight the growing potential of OKW as a sustainable feedstock for organic acid production. By continuing to optimize fermentation techniques, engineering bacterial strains, and exploring novel applications, researchers are paving the way for a more circular bioeconomy that utilizes waste resources for valuable products.

2.1.6 Applications of Bacterial-Produced Organic Acids

Organic acids produced by bacteria have a wide range of applications in various industries:

- **Food and Beverage Industry:** has long capitalized on the unique properties of bacterial organic acids. Lactic acid, a cornerstone of fermentation produced by lactic acid bacteria (LAB), is not only responsible for the tangy flavors in yogurts, buttermilk, and fermented vegetables like kimchi but also acts as a natural preservative [Yáñez et al., 2015]. By lowering the pH, lactic acid creates an inhospitable environment for spoilage microbes, thus extending shelf life [Yáñez et al., 2015]. Citric acid, another champion produced by bacterial fermentation, adds a citrusy zing to soft drinks, candies, and juices. Its functionality extends beyond taste; citric acid acts as a chelating agent, binding to metal ions and improving the stability and shelf life of these products [Behera et al., 2017]. This chelating property also makes citric acid a valuable food additive, enhancing the vibrant colors of fruits and vegetables [Behera et al., 2017].

- **Pharmaceuticals:** Organic acids can be used in the production of pharmaceuticals and as starting materials for drug synthesis. citric acid's chelating properties come into play as a blood anticoagulant during transfusions, preventing blood clots from forming [Behera et al., 2017]. Lactic acid, with its mild acidity, is used in topical creams for its exfoliating and antimicrobial effects [Yáñez et al., 2015]. The chemical industry is another beneficiary of

this diverse group of molecules. Lactic acid serves as a green platform chemical – a renewable starting material for the synthesis of various industrial chemicals. One such example is polylactic acid (PLA), a biodegradable plastic gaining significant traction as a sustainable alternative to traditional plastics derived from fossil fuels [Yáñez et al., 2015]. cosmetic industry utilizes citric acid as a pH adjuster and mild chelating agent in lotions and cleansers [Behera et al., 2017]. Agriculture can benefit from the antimicrobial properties of organic acids like acetic acid, a component of vinegar, which has potential as a natural herbicide and fungicide [Yáñez et al., 2015].

- **Bioplastics:** Lactic acid is a critical building block for bioplastics, offering a more sustainable alternative to petroleum-based plastics.
- **Biofuels:** Propionic acid can be used as a platform chemical for biofuel production
- **Agriculture:** Some organic acids have applications in animal feed production and preservation. Organic acids like propionic acid are used as preservatives in animal feed to prevent spoilage caused by mold and bacteria [Yáñez et al., 2015].

METHODOLOGY

3.1. SAMPLING

Samples were taken from several locations with high organic content to isolate bacteria capable of producing organic acids from a common kitchen waste substrate. Soil samples collected were as follows:

1. Industrial site soil:

    1.1. Corlim: near the vicinity of the Vico industry.
    1.2. Madkaim: near Third Eye Distillery, Madkaim
2. Akar-Mardol, Ponda, Goa:

    2.1. Soil with composted kitchen waste: kitchen waste composed of fruit peels, vegetable remnants, and cooked rice was buried in the soil. Samples were collected:

        a) After 8 days of inoculation when partial degradation was observed.
        b) After one month when the kitchen waste was fully degraded.

    2.2. Fish waste-amended soil: uncooked fish scraps were buried in the soil, and a sample was collected after 10 days when the fish waste was fully degraded except for the scales.

    2.3. Garden soil: Topsoil was collected from the garden for bacterial isolation.

    2.4. Sewage sample: A wet sewage sample was collected to isolate acid-producing bacteria.

    2.5. Kitchen sink drain: A sample was collected from the drainage area of the built-in concrete sink where small food particles accumulated.

    2.6. Rotten tomato sample: The tomato was rotting aerobically on the soil. Samples were collected along with the soil on which it was degrading.

3. Soil from Block E, SBSB Goa University, where dried wood was decaying naturally.

4. Three soil samples were collected from Banstari, Goa:

    4.1. Soil near the pond: Soil was collected from the bank of a small pond.

    4.2. Soil from the sewage area: Soil samples were collected from areas where kitchen waste was accumulated and degraded aerobically in an open environment.

    4.3. Fallow land soil: Samples were collected from the land where no cultivation was practiced anymore.

5. Mandavi River water: A river sample was collected from the shore of the Mandavi River.

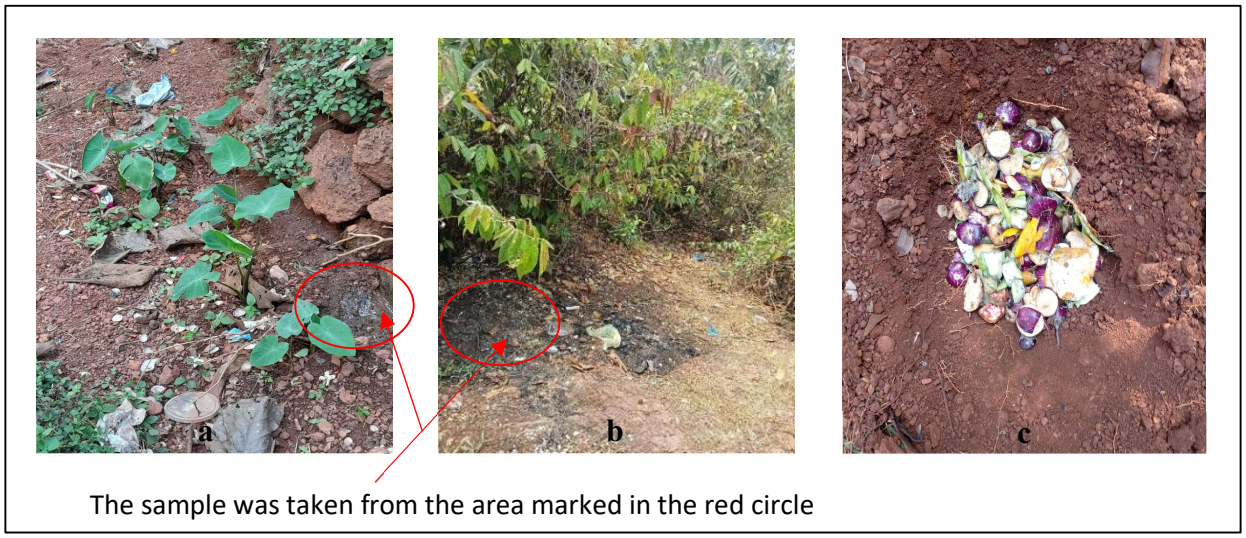


Figure 3.1.1. Sampling sites: a) wet sewage sample site, Akar-Mardol, Goa; b) sewage soil sample site, Banstari, Goa; c) kitchen waste infested into the soil, Akar-Mardol

3.2. ISOLATING BACTERIA FOR ACID PRODUCTION

Samples underwent processing within 24 hours of collection. No pretreatment was given to the samples. They were directly inoculated into a medium for isolation of acid-producing bacteria under sterile conditions.

3.2.1. Preparation of medium

Four modified versions of Glucose Phosphate Broth were prepared and named Starch Phosphate Broth, Peptone Phosphate Broth, CMC Phosphate Broth, and Oil Phosphate Broth (Appendix 1). These modifications aimed to investigate whether collected samples had bacteria that could utilize different substrates for acidic product formation. Substrates were selected based on the composition of kitchen waste, such as carbohydrates (starch), proteins (peptone), complex polymers (CMC), and fats (oil).

3.2.2. Enrichment of acid producers

Five milliliters of each broth were dispensed, and 1 gram of soil sample and 1 milliliter of river water sample were inoculated into each tube containing different substrates. A 1ml culture broth was taken and centrifuge to seprate bacterial cells from the broth.The centrifugation conditions were 10,000 rpm for 10 minutes. The supernatants were collected, and a few drops of methyl red were added. Broths showing a positive acid production were selected for bacterial isolation. These broths were mixed thoroughly and serially diluted tenfold up to 10^{-6}. The last three serial dilutions were spread evenly onto separate nutrient agar plates using a sterile spreader. Following inoculation, the plates were incubated at room temperature for 24 hours to facilitate the development of visible bacterial colonies.

3.2.3. Purification of isolate:

Based on colony colour, shape, opacity, surface texture, edge morphology, and elevation, bacteria were restreaked from the master plate onto fresh nutrient agar plates. These plates were incubated at room temperature. This process was repeated until pure isolated colonies were obtained. The isolates were then maintained in the refrigerator and restreaked onto fresh NA plates every four days.

3.3. SCREENING FOR ORGANIC ACID-PRODUCING BACTERIA

Purified isolates were inoculated into 5ml starch phosphate broth for an incubation period of 24 hours at room temperature. The 1ml broth was taken and centrifuged at 10000 rpm for 10 minutes. 400microliter supernatant was poured into two wells of 48 well titer plates. A few drops of methyl red and thymol blue indicators were put into these wells separately. This procedure was repeated two times, after incubation of 24 hours and 48 hours.

3.3.1. Screening for organic acid production from Peptone, CMC and Oil

The isolates that showed a positive methyl red test with starch were further inoculated into three versions of phosphate broth (as mentioned in section 3.2.1) to check whether the bacteria could produce acid utilizing peptone, CMC, or coconut oil as a substrate, in addition to starch. The broths were inoculated and incubated at room temperature for 24 hours. Acid production was then tested by adding methyl red as an indicator. If broth turn red in colour than broth contain acidic products while Yellow colour consider as negative test.

3.4 SCREENING OF ENZYME ACTIVITY

MR-positive isolates were screened to test for their production of hydrolytic enzymes. Nutrient agar was used as the medium to study the hydrolytic enzyme activity of purified isolates.

3.4.1 Amylase Activity

90 milliliters of nutrient agar (appendix I) was prepared. One gram of starch was dissolved in 10 mL of distilled water and autoclaved separately. The starch solution was then added to the autoclaved nutrient agar and mixed thoroughly. The cultures were spot-inoculated onto nutrient agar plates, which were incubated for 24 hours at room temperature. To detect the presence of

amylase activity, the plates were flooded with 1% Lugol's iodine solution (Appendix 2) after incubation. Amylase activity was observed as a zone of clearance around the colonies against a blue-black background.

3.4.2 Protease activity

To test the protease activity of bacterial isolates, nutrient agar (appendix I) medium was supplemented with 1% (w/v) skimmed milk. The skimmed milk was autoclaved separately and then aseptically incorporated into the autoclaved agar medium. The isolates were spot-inoculated onto the medium plates and incubated for 24 hours at room temperature. Protease activity was observed as a zone of clearance around the colonies against the opaque background of the medium.

3.4.3 Lipase Activity

To screen for lipase activity, nutrient agar (appendix I) plates were supplemented with 1% Tween 80 as a substrate. The Tween 80 solution was autoclaved separately and then aseptically added to the autoclaved agar medium. The plates were then spot-inoculated with bacterial isolates and incubated for 24 hours at room temperature. The presence of a clear zone around the colonies would indicate Tween 80 degradation and potential lipase activity.

3.4.4 Cellulase Activity

One gram of cellulose was suspended in 10 mL of water. The mixture was lightly heated to disperse any clumps and then autoclaved separately. The autoclaved CMC solution was then added to 190 mL of autoclaved nutrient agar (appendix I) medium. Bacterial isolates were spot-inoculated onto the medium plates. These plates were incubated at room temperature for 24 hours. After incubation, the plates were flooded with 1% Congo red (appendix II) solution for 15 minutes and

destained with 1 M NaCl solution (appendix II). A clear zone around the colonies, against the red background, indicated cellulase activity.

3.5 INVESTIGATING SUGAR UTILIZATION FOR ACID PRODUCTION BY BACTERIAL ISOLATES

Glucose Phosphate Broth (appendix I) was modified. This modification involved replacing the original sugar, glucose, with four different test sugars: lactose, sucrose, xylose, and fructose. Each test sugar was added at a concentration of 0.5%. Separate tubes containing 5 ml of the modified broths were inoculated with bacterial isolates. These isolates were then incubated for 24 hours. After incubation, a few drops of a methyl red indicator were added to each sample. A red colour change in the broth after adding methyl red indicates a positive test. This suggests that the bacteria were able to utilize the sugar and produce acid. Conversely, a yellow colour after adding methyl red signifies a negative test. This suggests the bacteria were unable to utilize the sugar effectively or produce enough acid to alter the pH significantly.

3.6 CHARACTERIZATION OF ISOLATED MICROORGANISMS

3.6.1 Gram's staining

A smear of the bacterial isolate was prepared under sterile conditions using 0.85% saline solution. The suspension was allowed to air dry and was heat-fixed. After heat fixing slide were taken out and the smear was stained with crystal violet for 1 minute, excess stains were removed. A few drops of Iodine were then put onto the smear. After 1 minute drops of 70% ethanol were poured to decolourise excess Crystal Violet-Iodine complex. To counterstained, a few drops of safranine were put onto slides, and after 30 seconds distilled water was poured dropwise from the edge of the tilted slide to remove excess stain. Following Gram staining and observation under a

microscope at 100x magnification with oil immersion, Gram-positive bacteria appeared violet, while Gram-negative bacteria appeared pink.

3.6.2. Catalase Test

A loopful of inoculum from a 24-hour culture plate was transferred to a clean, grease-free slide. A few drops of 0.85% saline were added to create a homogenous suspension of the inoculum. Then, two drops of hydrogen peroxide solution were added to the suspension. The presence of gas bubbles following the addition of hydrogen peroxide signifies a positive test for catalase activity in the bacteria.

3.6.3. Methyl Red test

Five milliliters of Glucose Phosphate Broth (appendix I) was inoculated with a loopful of a 24-hour bacterial culture. The inoculated broths were then incubated for 24 hours at room temperature. Following incubation, a few drops of methyl red indicator were added to each broth, and the colour change was observed.

Result Interpretation:

Red colouration: A red colour change after adding methyl red indicates a positive test for acid production by the bacteria utilizing the sugar provided in the medium.

Yellow colouration: No change in colour (remaining yellow) indicates a negative test for acid production.

3.6.4 Kligler's Iron Test

Triple Sugar Iron (TSI) agar (appendix I) slants (HiMedia) were inoculated using both streaking and stabbing techniques to assess various fermentation and gas production patterns of the bacterial cultures. The inoculated slants were then incubated for 24 hours at room temperature. Following incubation, the colour changes in the agar medium were observed and interpreted as follows:

Acid Production: A yellow colour change in the medium throughout the slant and butt (deep portion) indicates fermentation of one or more sugars present (glucose, lactose, and sucrose).

Sugar Fermentation Patterns:

Yellow slant/yellow butt: Fermentation of all sugars (glucose, lactose, and sucrose).

Yellow slant/red butt: Fermentation of only glucose.

No Fermentation: The red colour of the medium remains unchanged, indicating no sugar fermentation.

Gas Production: The presence of bubbles or cracks in the agar medium signifies gas production during fermentation.

Hydrogen Sulfide (H_2S) Production: The blackening of butt of the medium indicates the production of hydrogen sulfide gas (H_2S) by the bacteria, resulting in the formation of iron sulfide precipitates.

3.6.5 Voges-Proskauer (VP) Test

A bacterial isolate was inoculated into Glucose Phosphate Broth (appendix I) and incubated for 24 hours at room temperature. Following incubation, the O'Mera reagent (appendix II) was added to the culture. A red colour change after adding the reagent indicates a positive VP test, bacteria can

metabolize the pyruvate into a neutral intermediate product called ‘acetylmethylcarbinol’ or ‘acetoin’. while a yellow colour signifies a negative test.

3.6.6 Indole Test

Bacterial isolates were inoculated into tryptone broth (appendix I) for 24 hours. After 24 hours, a few drops of Kovac’s reagent (apeendix II) was added. Formation of a pink ring at interphase is a positive result for the indole test. suggesting bacteria can metabolized tryptophane to indole. while no colour ring formation is negative result for indole.

3.6.6 Urease Test

Urease agar slant was prepared from readymade urease agar media(Christensen’s urea medium) (appendix I), and 40% urea stock solution was prepared and filter sterilized before adding to autoclave agar. 24-hour-old bacterial cultures were inoculated in medium. After a 24-hour incubation period, the slants were visually inspected for any alterations in colour. If medium turns pink from yellow it's positive. Indicates test isolate can split urea, through the production of the enzyme urease. No change in colour, negative for urease.

3.6.7 Huge Leifson

This test was carried out to find out whether bacteria ferment sugar, aerobically or anaerobically. Huge Leifson agar(appendix I) was prepared, and 5 ml of medium was distributed into test tubes. A bacterial isolate was stab-inoculated in duplicate. One tube was overlaid with paraffin oil. Both tubes were incubated at 37°C for 24 hours.

Test results were interpreted as follows:

- The presence of a yellow colour after incubation signifies a positive test for fermentation. When both tubes turn yellow, it indicates the bacteria are fermentative. This means they can generate energy by fermenting glucose even in the presence of oxygen (aerobically) or in its absence (anaerobically).
- If aerobic tube turns yellow while anaerobic tube remains green than bacterial isolate have oxidative metabolic pathway.
- If both tubes show no colour change than bacteria is non sacchrolytic, HF test is negative

3.6.8 Citrate test

A Simmon citrate agar (HiMedia) slant was prepared. Bacterial isolate 24-hour-old cultures were inoculated in streaked and stab method on a slant at room temperature for 24 hours. The citrate test is considered positive if the colour of the agar slant changes to blue after incubation. Conversely, no observable colour change indicates a negative test result.

3.6.9 Colony morphological characteristics

On nutrient agar plates, colony characteristics of each bacterial isolate were documented. This included size, colour, shape, opacity, consistency, margin, and elevation.

3.7 SPECTROPHOTOMETRIC ANALYSIS OF ACID PRODUCED BY BACTERIAL ISOLATES

Bacterial isolates were inoculated into starch phosphate broth and incubated for 24 hours at room temperature. Before quantifying the acid produced by the bacteria, the peak absorbance of methyl red with an organic acid was determined. To achieve this, 100 microliters of 0.5M acetic acid were added to 1 ml of uncultured starch phosphate broth. The mixture was gently swirled, and then 100

microliters of methyl red indicator were added. The absorbance of this mixture was measured between 430 and 530 nm. Peak absorbance were found at 510nm.

The 1 ml of the inoculated broth was taken. Before determining the acid concentration, the OD at 600 nm of the broth was measured to estimate the cell count present at that time. The broth was then centrifuged at 12,000 RPM for 10 minutes. The supernatant (cell-free broth) was collected, and 100 microliters of methyl red were added. The mixture was gently mixed, and the OD was measured at 510 nm.

The amount of acid produced was determined using a standard curve generated with various concentrations of acetic acid.

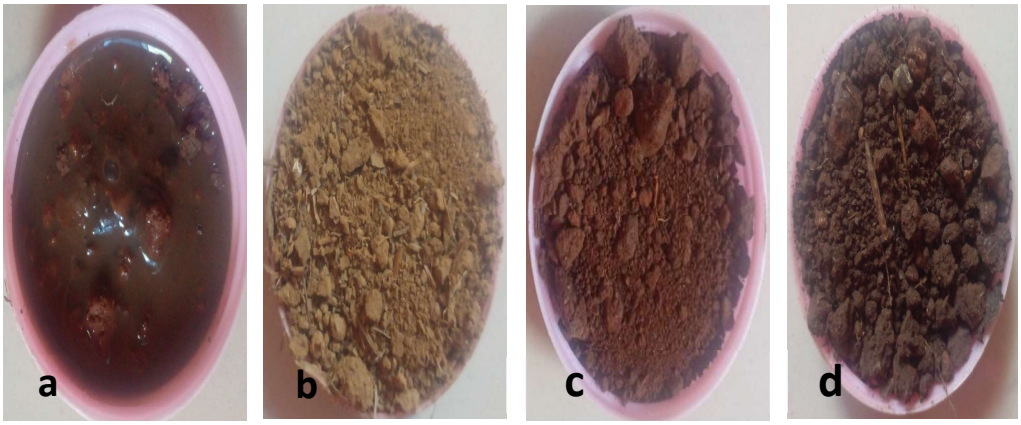
To obtain this standard curve, acetic acid solutions were prepared ranging from 0.1M to 1M. For absorbance of each concentration, 100 microliters of acetic acid were mixed with 100 microliters of methyl red indicator. The absorbance was measured at 510 nm for each mixture.

The results obtained from this experiment will be discussed in the following sections.

ANALYSIS AND CONCLUSION

4.1. SAMPLING SITES

Sampling was conducted at various locations to explore acid-producing bacteria in different habitats. The samples collected were mostly organic matter-rich soil.



4.1.1 Representative sample collected for isolation : a)wet sludge sample b)soil sample from bank of pond; c) Fallow soil sample; d) sewage soil sample

Two industrial sites with ongoing construction and material handling were chosen for soil sampling.

At one site, the soil was inoculated with kitchen waste (primarily fruit and vegetable scraps) buried for natural degradation. To monitor changes over time, samples were obtained periodically: the first collection occurred on day eight, followed by additional collection at one-month intervals.

At another site, located more than one kilometer away from the previously mentioned site, uncooked fish scraps were buried. Soil samples were taken after ten days from this location.

Additional samples included fresh garden soil, wet sewage samples, kitchen sink waste accumulations, and aerobically degrading rotten tomato on soil.

To further explore acid producers, soil samples were collected from:

- Block E, Goa University, SBSB
- Fallow land

A water sample was taken from the Mandavi River shoreline due to suspicion of high organic content based on the strong odor.

4.2 ISOLATION AND PURIFICATION OF ISOLATE

The principle of methyl red test was employed to isolate bacteria capable of producing acid from broth cultures. Four modified versions of glucose phosphate broth were prepared named Starch Phosphate Broth, Peptone Phosphate Broth, CMC Phosphate Broth, And Oil Phosphate Broth (Appendix 1). This modification was done to check whether bacterial isolates can utilize this substrate to produce stable acidic products. Substrates were selected based on composition of kitchen waste i.e. carbohydrates(starch), proteins(peptone), complex polymer(CMC), and fats(oil).

After a 24-hour incubation period, each inoculated broth was examined to determine if acid had been produced. A methyl red indicator solution was added to facilitate this assessment. This indicator changes colour based on the acidity of the solution:

- Yellow: pH 4.4 and above
- Red: pH 4.0 and below

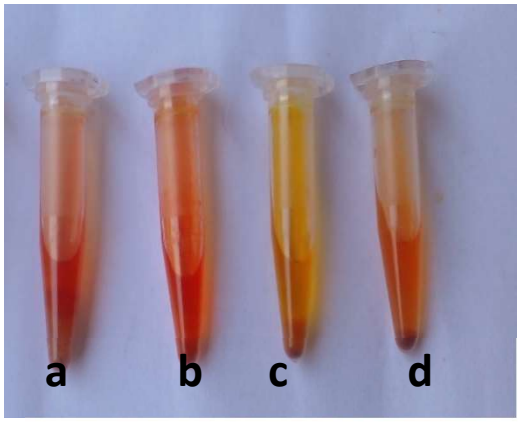
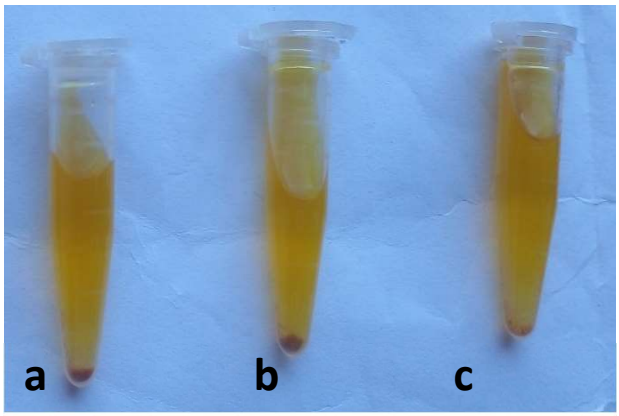


Figure 4.2.1.soil analysis for presence of acid producers using SBP: a) soil from pond bank side; b) vegetable infested soil; c) soil from university site; d) fallow farmland soil



4.2.2. soil analysis for presence of acid producers using PPB: a) soil from pond; b) corlim soil sample; c) tomato soil sample

Broths that exhibited a positive acid production after adding indicator methyl red, were selected for further isolation of acid-producing bacteria. Images of master plates of some sample dilutions are presented below.

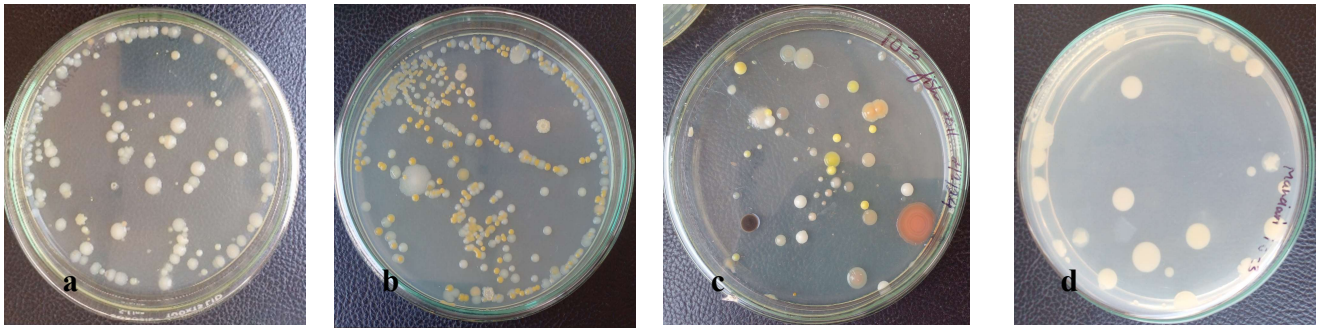


Figure 4.2.3. Representative master plates of: a) vegetable soil sample; b) Madkaim soil sample ; c)fish soil sample; d) Mandavi river sample.

4.3 SELECTION OF ORGANIC ACID PRODUCERS

The purified bacterial isolates were inoculated into fresh starch agar broth and allowed to grow for 24 hours at room temperature. Following incubation, culture broths from each tube were analyzed for acid production using two indicator dyes. Thymol blue indicator was used to provide a wider

range of pH detection alongside methyl red. Bacterial isolates categorized as 'weak positive' in Table 4.3.1 exhibited a mildly acidic pH range, falling between 6 and 4. Their broth turned orange-yellow for thymol blue and yellow for methyl red. Strong acid producers displayed a red color for methyl red and an orange color for thymol blue. Strong acid producers were selected for further testing. The number of positive isolates obtained from each sample is shown below:

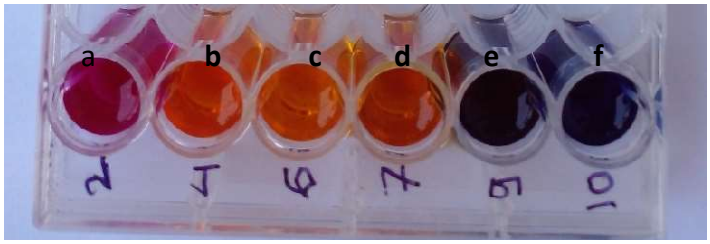


Figure 4.3.1. Thymol blue color response at various ph levels. a) At pH 2; b) At pH 4; c) At pH 6; d) At pH 7; e) At pH 9; f) At pH 10

4.3.1 Evaluating Bacterial Acid Production on Diverse Substrates

Isolates were further tested to determine their ability to utilize peptone, oil, and CMC (carboxymethyl cellulose) for organic acid production. All strong acid producers were inoculated into four modified versions of GPB. Acid production was tested using a methyl red indicator. All isolates tested negative for acid production when peptone and oil were used as substrates. Whereas Isolate RS3(2) exhibited cellulase activity along with slight acidification of broth when CMC was used as a substrate for the Methyl Red test, which can be particularly beneficial for degrading cellulose, another major component of vegetable waste. All other isolates were negative for acid production with CMC.

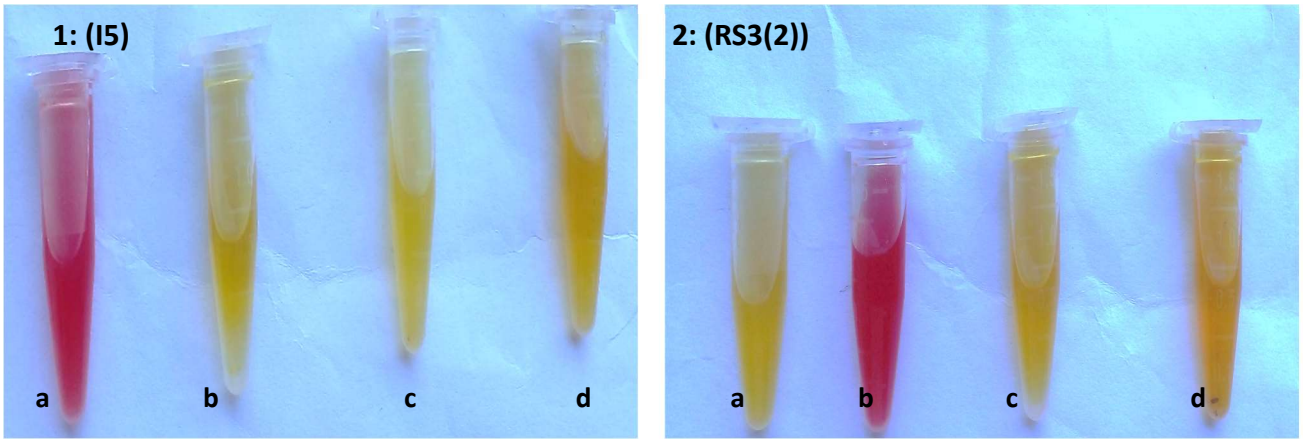


Figure 4.3.2. Representative images of Methyl Red test results for acid production from various substrates:

1:(isolate:I5): a) Starch; b) Peptone; c) Oil; d) Cellulose;

4.4 HYDROLYTIC ENZYMES PRODUCE BY BACTERIAL ISOLATES:

Eighteen isolates were tested, to check for hydrolytic enzyme production using nutrient agar medium supplemented with different substrates.

4.4.1 Amylase

Starch was used as a substrate to check for amylase activity. All 18 isolates showed amylase enzyme production. Results of their amylase activity are presented in tabulated form in the table.

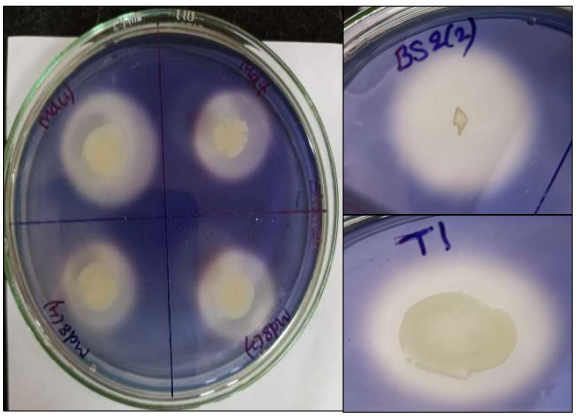


Figure 4.4.1.1. Visualization of amylase activity in representative bacterial isolates on starch agar plates

Table 4.4.1 Amylase activity in bacterial isolates (48-hour incubation)

Isolate name	Diameter of zone of clearance(mm)	Diameter of colony(mm)	Amylase index
BS2(2)	23	4	5.75
Md5(1)	22	10	2.2
Sludge8	25	10	2.5
BS12(2)	28	3	9.33
Md7	20	4	5
Md8(3)	20	8	2.5
Md8(2)	20	10	2
T1	20	5	4
I5	21	5	4.2
BS8	3	2	1.5
Md8(1)	23	13	1.76
Md8(4)	20	8	2.5
Md8(5)	22	5	4.4
RS3(2)	33	8	4.12
Md5(2)	58	15	3.86
BS7	5	10	0.5
Md1	20	5	4
Sludge5	18	10	1.8

All the bacteria studied were able to break down starch (amylase activity) and produce acid. Among the bacteria tested, isolate BS12(2) showed the highest level of amylase activity, while isolate BS7 showed the least. This indicates that isolate BS12(2) was most efficient at breaking down starch. Importantly, even though all the bacteria produced acid and broke down starch, the amount of acid produced wasn't necessarily linked to how well they broke down starch (amylase activity). This suggests that these two processes might be independent of each other in these bacteria.

4.4.2 Protease

All 18 isolates were showing protease activity on nutrient agar supplemented with skimmed milk—representative pictures of some bacterial isolates are given below.

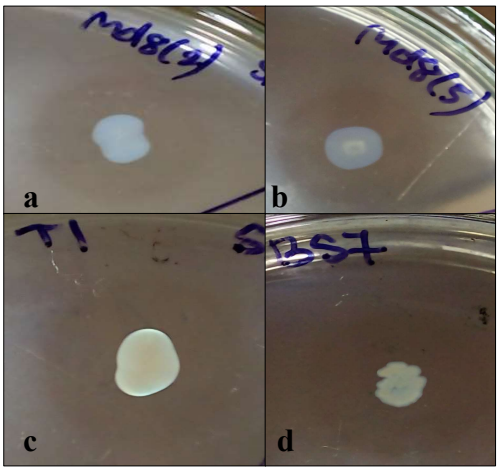


Figure 4.4.2.1. Representative images of agar plate assay for protease activity in bacterial isolates:

a) Md8(2); b) Md8(5); c) T1; d) BS7

Table 4.4.2.1 Proteolytic potential of bacterial isolates

Isolate name	Diameter of zone of clearance(mm)	Diameter of colony(mm)	Proteolytic index
BS2(2)	10	3	3.33
Md5(1)	18	4	4.5
sludge8	19	5	3.8
BS12(2)	10	3	3.33
Md7	7	5	1.4
Md8(3)	16	5	3.2
Md8(2)	15	4	3.75
T1	15	5	3
I5	15	5	3
BS8	16	4	4
Md8(1)	15	5	3
Md8(4)	15	7	2.14
Md8(5)	15	5	3
RS3(2)	18	6	3
Md5(2)	18	4	4.5
BS7	16	5	3.2
Md1	10	4	2.5
sludge5	18	5	3.6

All the bacteria studied (isolates) were able to degrade proteins because they showed protease activity on skimmed milk agar. This indicates they have enzymes that can break down proteins in kitchen waste into simpler molecules like amino acids. Isolate Md5(1) was most efficient (highest index) at protein breakdown, while Md7 was least efficient (lowest index). Other isolates had a moderate range of proteolytic potential (2-4). Interestingly, none of the bacteria directly produced acid from protein itself. However, the isolates with moderate proteolytic activity (range 2-4) also showed good acid production. This suggests that while they don't directly convert protein to acid, they might be breaking down proteins into other substances that can be further converted to acid by these bacteria or other microbes present.

4.4.3 Lipase

The lipase activity of 18 isolates was tested. The result is presented in tabulated form as follows.

Table 4.4.3.1 Lipase activity of bacterial isolates.

Isolate name	Lipase activity of bacterial isolates
BS2(2)	-
Md5(1)	+
sludge8	+
BS12(2)	-
Md7	+
Md8(3)	+
Md8(2)	+
T1	+
I5	+
BS8	-
Md8(1)	+
Md8(4)	+
Md8(5)	+
RS3(2)	-
Md5(2)	+
BS7	-
Md1	+
sludge5	+

Key: (+): Positive; (-): Negative

Lipase activity were shown by bacterial isolates I5, Md1, Md5(1), Md5(2), Md7, Md8(1), Md8(2), Md8(3), Md8(4), Md8(5), sludge5, sludge8 and T1.

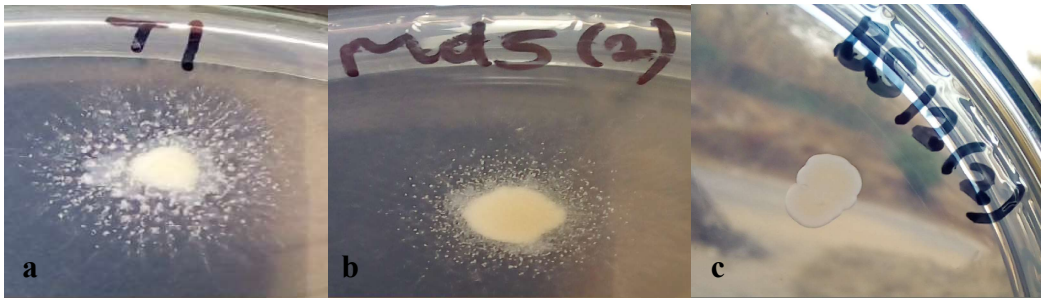


Figure 4.4.3.1. Lipase activity of bacterial isolates visualization of high activity: a) T1; b)Md5(2) and no activity: c) BS12(2)

None of the bacteria studied were able to directly convert oil into acid. This means they lack the specific enzymes needed for this particular process.

However, some isolates were able to degrade TWEEN 80. TWEEN 80 is a synthetic compound, but it's often used as a substitute for oil in tests because it can be broken down by enzymes called lipases. So, the ability to degrade TWEEN 80 indicates that these isolates likely produce lipases, which are enzymes that break down fats and oils. Due to their combined abilities:

- Degrading starch (amylase activity)
- Degrading proteins (protease activity)
- Degrading fats/oils (lipase activity, based on TWEEN 80 degradation)

These isolates become strong candidates for forming a consortium (a mixed group of microbes). Each isolate brings a specific enzymatic capability to the table, making the consortium more versatile in breaking down different components of kitchen waste.

While the isolates can't directly convert oil to acid, their ability to break down fats and oils (through lipases) could be a valuable step in the overall process of waste breakdown within a consortium. Perhaps other members of the consortium could convert the breakdown products of fats and oils into acids.Overall, the analysis highlights the potential of these isolates, particularly in a consortium setting, for developing a system that can effectively break down various organic components present in kitchen waste.

4.4.4 Cellulase

In addition to starch, protein, and fats, the ability of the isolates to degrade other, more complex carbohydrates, cellulose, was also investigated. Cellulose is a major component of plant cell walls and contributes significantly to the composition of kitchen waste. Except for isolate RS3(2), none of the isolates exhibited cellulase activity, making it the only isolate with cellulase activity in this group.

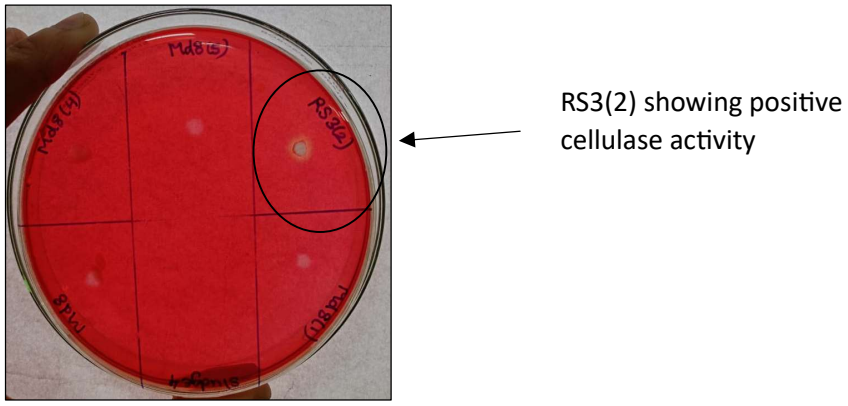


Figure 4.4.4.1 Agar plate assay for cellulase production in bacterial isolates

4.5 Morphological characteristics of isolated bacteria:

Isolates were differentiated based on their morphological differences, results were tabulated as below:

4.5.1.colony characteristics:

Isolate name	Bs2(2)	Bs12(2)	Bs7	Bs8	Sludge 5	Sludge 8	T1	I5	Rs3(2)
Source	Brown soil	Brown soil	Brown soil	Brown soil	Wet sludge	Wet sludge	Rotten Tomato	Industrial soil (Corlim)	Fallow land soil
Media	Nutrient agar	Nutrient agar	Nutrient agar	Nutrient agar	Nutrient agar	Nutrient agar	Nutrient agar	Nutrient agar	Nutrient agar
Time	24 hours	24 hours	24 hours	24 hours	24 hours	24 hours	24 hours	24 hours	24 hours
Temperature	Room temperature	Room temperature	Room temperature	Room temperature	Room temperature	Room temperature	Room temperature	Room temperature	Room temperature
Size	2mm	3mm	4mm	3mm	4mm	4mm	4mm	4mm	6mm
Shape	Circular	Circular	Circular	Irregular	Circular	Circular	Circular	Circular	Circular
Colour	Yellow	Yellow	Yellow	Orange yellow	Colourless	Cream	White	Cream	White
Margine	Entire	Entire	Entire	Entire	Entire	Entire	Entire	Entire	Entire
Elevation	Raised	Flat	Flat	Flat	Raised	Flat	Flat	Flat	Flat
Surface texture	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth	Dry
Opacity	Opaque	Opaque	Transparent	Transparent	Transparent	Opaque	Opaque	Transparent	Opaque
Consistency	Butryous	Butyrous	Butyrous	Mucoid	Mucoid	Butyrous	Butyrous	Butyrous	Butyrous

Table 4.5.1.b. Colony characteristics of bacterial isolates

Isolate name	Md5(1)	Md5(2)	Md1	Md7	Md8(1)	Md8(2)	Md8(3)	Md8(4)	Md8(5)
Source	Mandavi River	Mandavi River	Industrial soil (Madkai)	Industrial soil (Madkai)	Mandavi River	Mandavi River	Mandavi River	Mandavi river	Mandavi River
Media	Nutrient agar	Nutrient agar	Nutrient agar	Nutrient agar	Nutrient agar	Nutrient agar	Nutrient agar	Nutrient agar	Nutrient agar
Time	24 hours	24 hours	24 hours	24 hours	24 hours	24 hours	24 hours	24 hours	24 hours
Temperature	Room temperature	Room temperature	Room temperature	Room temperature	Room temperature	Room temperature	Room temperature	Room temperature	Room temperature
Size	3mm	4mm	4mm	3mm	5mm	3mm	6mm	4mm	3mm
Shape	Irregular	Irregular	Circular	Circular	Circular	Circular	Irregular	Circular	Circular
Colour	Cream	Cream	Cream	Cream	Cream	Cream	Cream	Cream	Cream
Margine	Entire	Entire	Entire	Entire	Entire	Entire	Entire	Entire	Entire
Elevation	Flat	Flat	Flat	Flat	Flat	Flat	Flat	Raised	Flat
Surface texture	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth
Opacity	Transparent	Transparent	Transparent	Transparent	Transparent	Transparent	Opaque	Opaque	Opaque

4.6 ACID PRODUCTION FROM DIFFERENT SUGARS

Bacterial isolates were inoculated into Glucose Phosphate Broth. Acid production was also analyzed using modified Glucose Phosphate Broth where glucose was replaced by lactose, sucrose, xylose, and fructose (Appendix 1). These tests were performed to determine if the isolates could utilize these sugars to form stable acidic products(Table 4.6.1.). All these sugars are majorly found in Organic kitchen waste composition, All isolates showed positive results for glucose and sucrose. Notably, isolate Md8(2) utilized xylose, a component of vegetable cell walls, further expanding the range of usable substrates. Additionally, all isolates except Md5(1), showed the positive methyl red test with fructose suggesting the potential for acid production from fruit-derived sugars. None of the isolates were able to utilize lactose.

Table 4.6.1. Acid production from various sugars in bacteria.

Sr. No.	Test	Bs2(2)	Bs12(2)	Bs7	Bs8	Sludge 5	Sludge 8	T1	I5	Rs3(2)	Md5(1)	Md5(2)	Md1	Md7	Md8(1)	Md8(2)	Md8(3)	Md8(4)	Md8(5)
1	Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
2	Lactose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3	Sucrose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
4	Xylose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-
5	Fructose	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+

Key: + : positive; - : Neagative

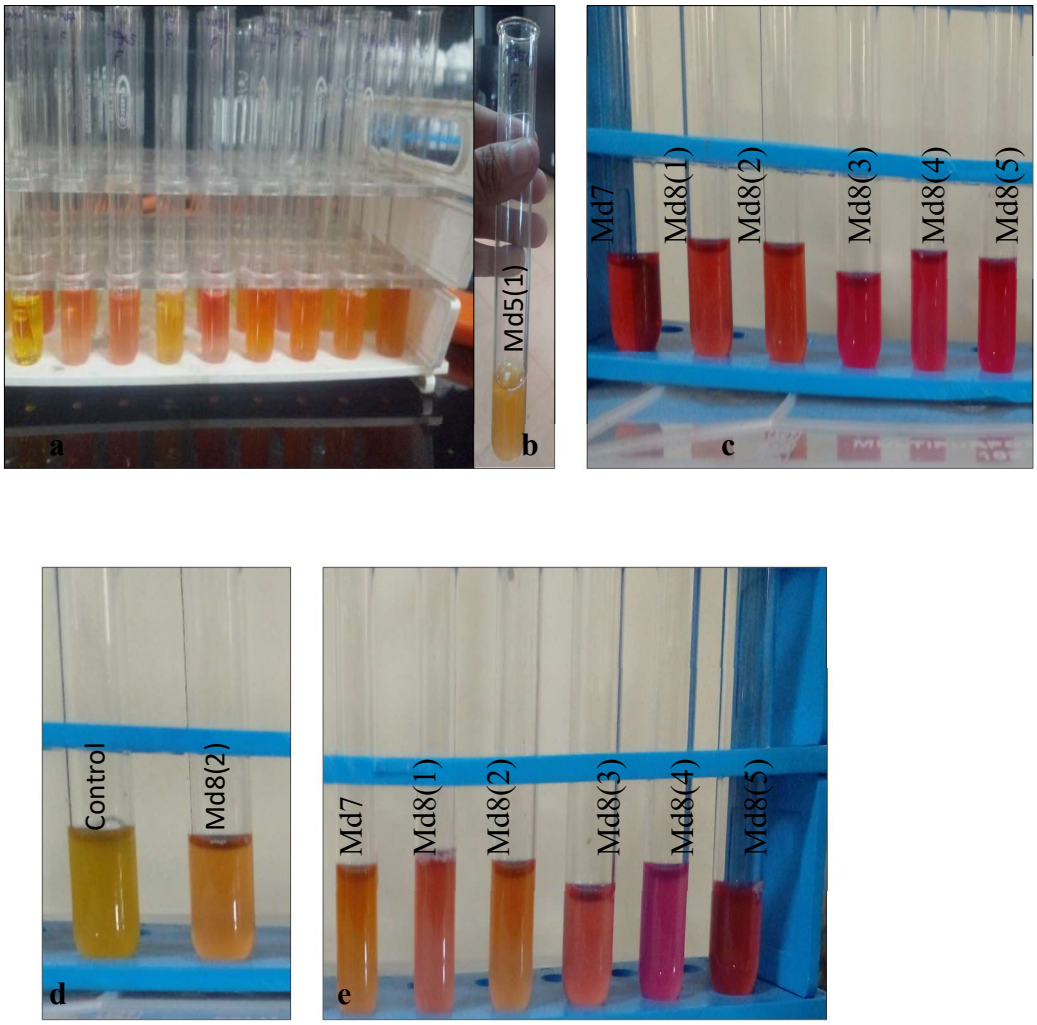


Figure 4.6.1 visual representation of methyl red test: a) Fructose MR test; b) Md5(1) showing negative MR test for fructose; c) Positive test for Glucose; d) Md8(2) showing positive for xylose along with negative control; e) Isolates showing positive test for sucrose.

4.7 Biochemical characterization of bacterial isolates:

To differentiate the bacterial isolates, biochemical tests were performed. The results are presented in the table 4.7.1 a and 4.7.1 b

Table 4.7.1.a. Biochemical characterization of bacterial isolates.

Sr. No.	Test	Bs2(2)	Bs12(2)	Bs7	Bs8	Sludge 5	Sludge 8	T1	I5	Rs3(2)
1	Citrate	-	-	-	-	-	-	-	-	-
2	Klingler's Iron									
2.1	Slant/butt	Yellow/yellow	Yellow/yellow	Yellow/yellow	Yellow/yellow	Yellow/yellow	Yellow/yellow	Yellow/yellow	Yellow/yellow	Pink/pink
2.2	Acid production	+	+	+	+	+	+	+	+	-
2.3	H2S gas production	-	-	-	-	-	-	-	-	-
3	Urease	-	-	-	-	-	-	-	-	-
4	Huge leifson:									
4.1	Aerobic	-	-	+	+	+	+	+	+	+
4.2	Anaerobic	-	-	-	-	+	+	+	+	+
5	Catalase	+	+	+	+	+	+	-	+	+
6	Gram character	Gram negative	Gram negative	Gram positive	Gram negative	Gram positive	Gram positive	Gram positive	Gram negative	Gram positive

Table 4.7.1.b. Biochemical characterization of bacterial isolates.

Sr. No.	Test	Md5(1)	Md5(2)	Md1	Md7	Md8(1)	Md8(2)	Md8(3)	Md8(4)	Md8(5)
1	Citrate	+	+	-	+	+	+	+	+	+
2	Klingler's Iron									
2.1	Slant/butt	Yellow/yellow	Yellow/yellow	Pink/pink	Yellow/yellow	Yellow/yellow	Yellow/yellow	Yellow/yellow	Yellow/yellow	Yellow/yellow
2.2	Acid production	+	+	-	+	+	+	+	+	+
2.3	H2S gas production	-	-	-	-	-	-	-	-	-
3	Urease	-	-	-	-	+	+	+	+	+
4	Huge leifson:									
4.1	Aerobic	+	+	+	+	+	+	+	+	+
4.2	Anaerobic	+	+	+	+	+	+	+	+	+
5	Catalase	-	+	+	-	+	+	+	+	+
6	Gram character	Gram negative	Gram negative	Gram positive	Gram negative	Gram negative	Gram positive	Gram positive	Gram positive	Gram negative

key:- (+): postive; (-): negative

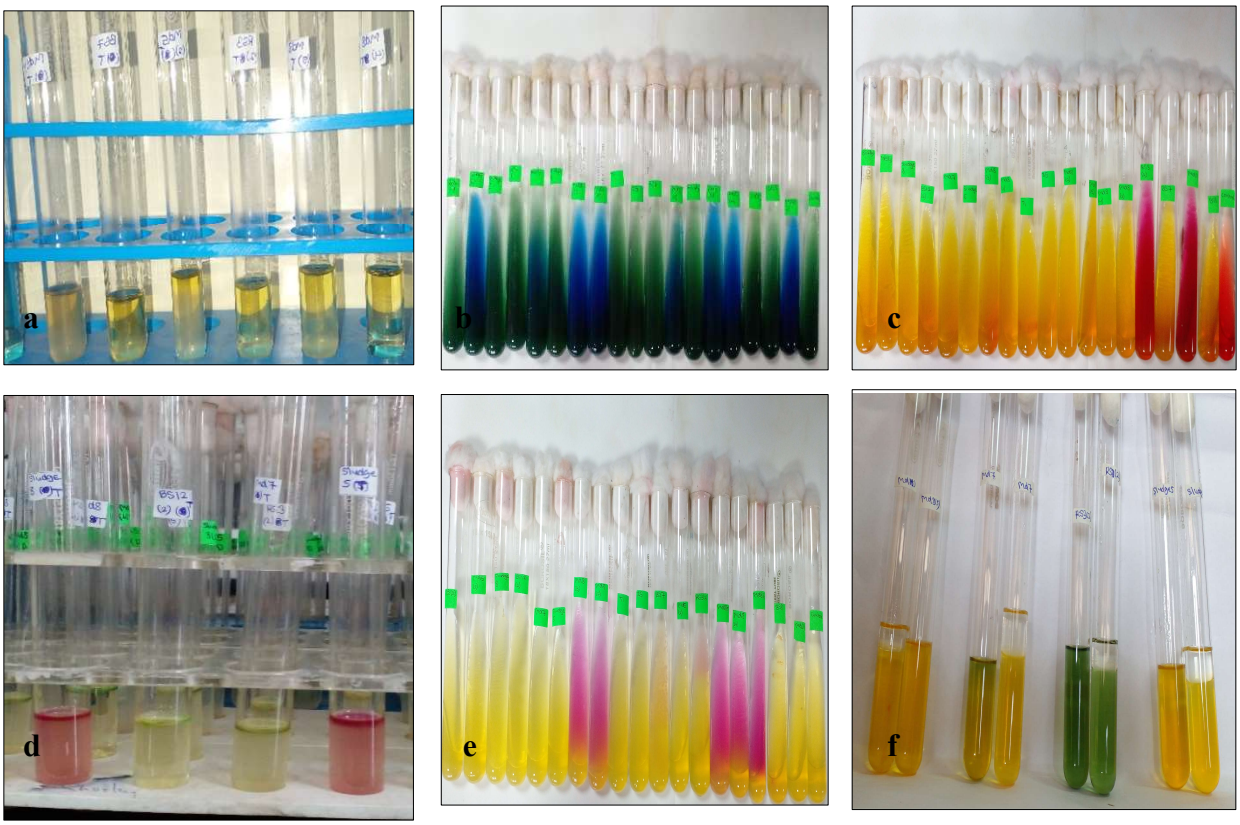


Figure 4.7.1 Visual representation of biochemical test. a) Vogues Prosker test; b)Citrate test; c) Kligler's Iron Test d) Indole test e) Urease test f) Huge Leifson test.

Figure 4.7.2 Representation of catalase activity of RS3(2), Md5(2), BS7, Md5(1), Md7, BS12(2)

4.8 Spectrophotometric analysis of Acid production:

Isolates exhibiting acid production were selected for quantification. To quantify the amount of acid produced by each bacterial isolate, a spectrophotometer was employed. This instrument measures the absorbance of light at specific wavelengths, which can be used to determine the concentration of a particular molecule in solution.

Acetic acid was chosen as a reference organic acid to determine the absorbance peak of methyl red with organic acid. By comparing the absorbance of the bacterial culture supernatants with methyl red to the absorbance of known concentrations of acetic acid, concentration of acid produced by each bacterial isolate were calculated.

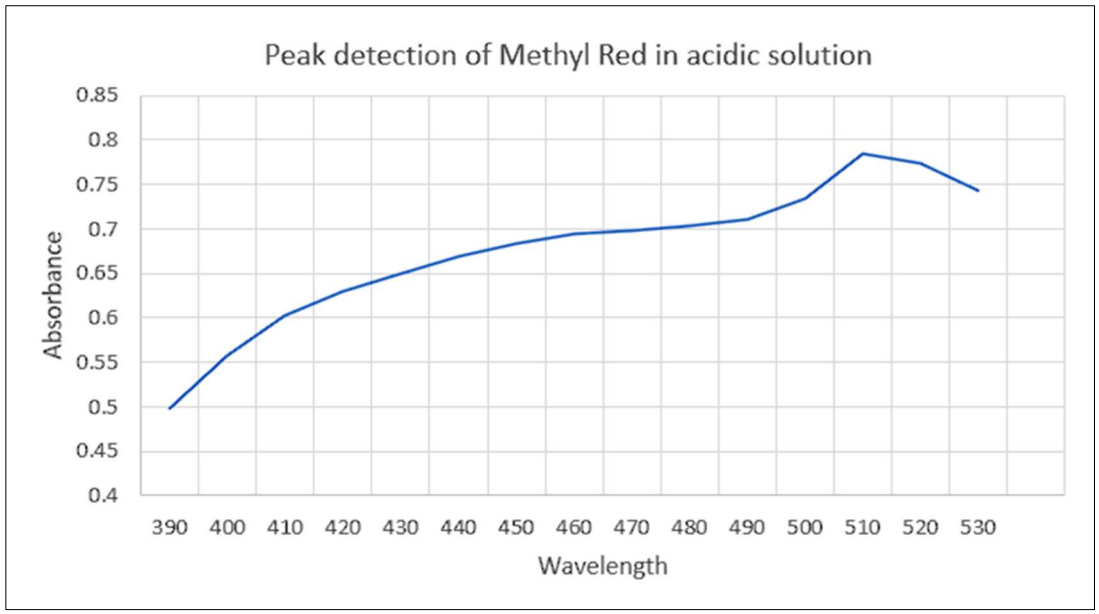


Figure:4.8.1 Peak detection of Methyl Red in acidic solution

As shown above, the peak value was observed at an OD (optical density) of 510 nm. This peak was then used to generate a standard calibration curve for various concentrations of acetic acid in the culture broth. The resulting graph is presented below:

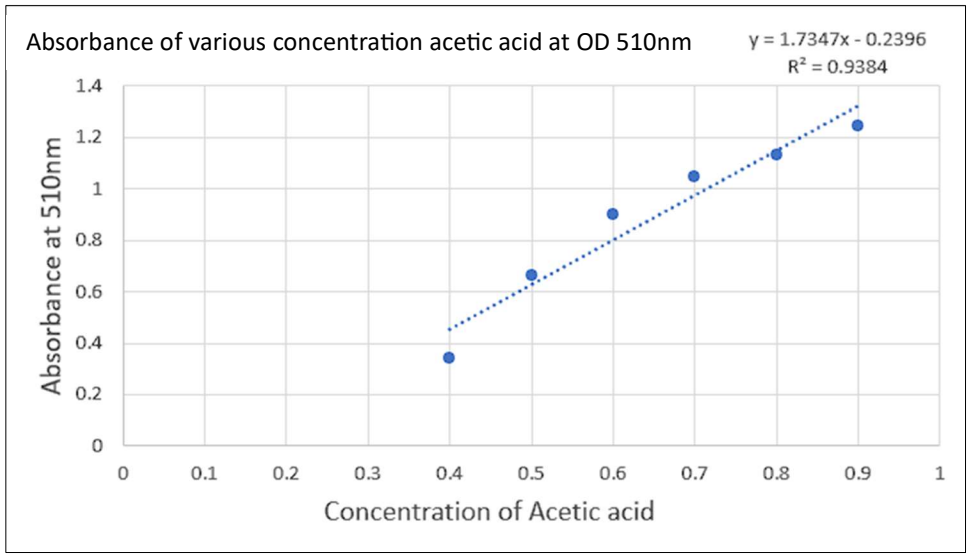


Figure 4.8.2 Absorbance of various concentration Acetic Acid at 510nm

All isolates demonstrated the ability to produce acid from starch, a readily available carbohydrate source in kitchen waste. Data of organic acid production by bacterial isolates from starch.The results for each isolate are presented below (**refer to Table 4.8.1**).

Table 4.8.1 Data of organic acid production by bacterial isolates from starch at 510nm.

Day	BS2(2)	BS12(2)	BS7	BS8	sludge 5	sludge 8	T1	I5	RS3(2)	Md5(1)	Md5(2)	Md1	Md7	Md8(1)	Md8(2)	Md8(3)	Md8(4)	Md8(5)
1	0.785	0.918	0.446	0.591	1.244	1.141	1.594	1.217	1.217	1.224	1.045	1.19	0.897	0.886	1.123	1.3	1.206	1.019
2	1.178	1.15	1.45	1.466	1.689	1.432	1.773	1.491	1.614	1.546	1.288	1.459	1.058	1.265	1.365	1.555	1.443	0.598
3	1.056	1.115	1.354	1.238	1.831	1.471	1.823	1.488	1.112	1.549	1.622	1.53	1.498	1.263	1.377	1.526	1.462	1.357
8	0.861	1.647	0.742	0.883	1.688	1.574	1.776	1.332	1.358	0.903	1.168	0.976	1.024	1.261	0.716	0.861	1.054	1.009

Absorbance of inoculated broths with methyl red was measured at 510 nm on days 1, 2, 3, and 8. A standard curve of acetic acid showed an increase in absorbance with increasing concentration. For acetic acid, the absorbance ranged from 0.6 to 1.2. After 48 hours, isolates Md8(1), Sludge 5, Sludge 8, T1, I5, RS3(2), Md5(1), Md5(2), Md1, Md8(2), Md8(3), Md8(4), and Md8(2)showed absorbance values between 1.2 and 1.8. A 1M solution of acetic acid showed an absorbance of 1.279. Isolates mentioned above had absorbance values equal to or exceeding this value. This suggests that the bacterial isolates might be producing organic acids with longer carbon chains.On day 8, BS2(2), BS12(2), BS7, BS8, Md5(1), and Md8(2) showed a decrease in absorbance, indicating that they might be utilizing the acids produced in the medium.

The concentration of acid produced by the bacteria was determined using a standard calibration curve for acetic acid. This approach allows us to convert the measured optical density (OD) of the culture broth at a 510nm wavelength into a quantifiable amount of acid. The results of this analysis are presented in the below:

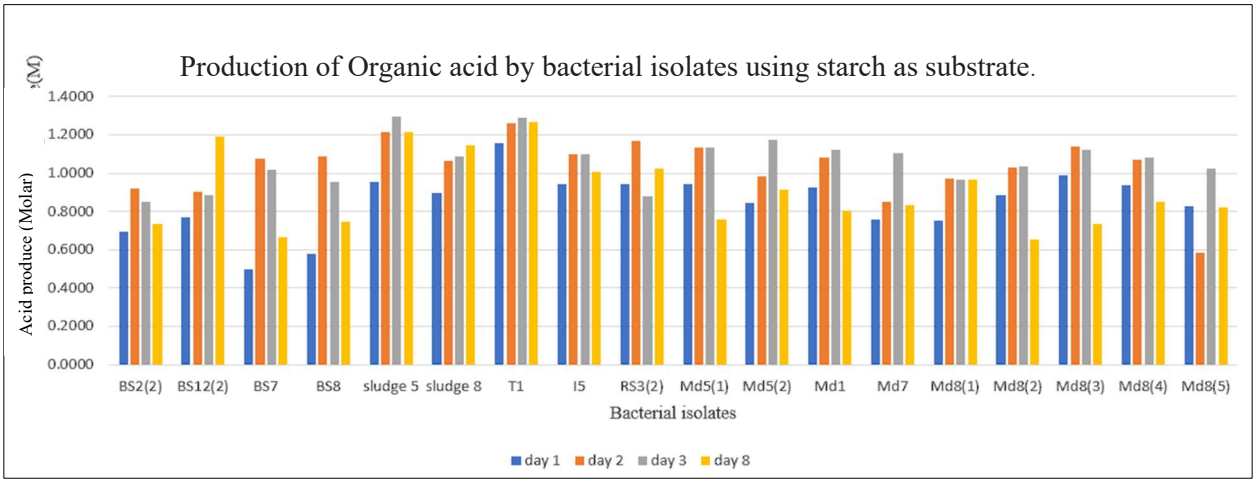


Figure 6.2. Production of organic acid by bacterial isolates using starch as substrate.

All isolates exhibited acid production exceeding 0.8 M in terms of the acetic acid standard graph After 48 hours of incubation, making them potential candidates for consortium development to be tested on organic kitchen waste.

In addition to quantifying acid production, the growth of the bacteria at the time of acid measurement was also analyzed. This analysis helps to understand the relationship between bacterial growth and acid production. this data will be useful while contructing consortium. Those isolates whose OD were at 0.8 and above had showned good acid production. The data related to bacterial growth at the time of acid measurement is presented in a separate table below:

Table 4.8.2 Data of Absorbance taken at 600nm, to estimate cell present during acid production

Day	BS2(2)	BS12(2)	BS7	BS8	sludge 5	sludge 8	T1	I5	RS3(2)	Md5(1)	Md5(2)	Md1	Md7	Md8(1)	Md8(2)	Md8(3)	Md8(4)	Md8(5)
1	0.312	0.335	0.016	0.098	0.617	0.428	0.71	0.549	0.86	0.659	0.481	0.753	0.384	0.494	0.692	0.827	0.781	0.634
2	0.626	0.586	0.812	0.829	0.981	0.707	1.009	0.829	1.182	0.966	0.683	1.005	1.52	0.955	0.912	1.061	1.009	0.203
3	0.521	0.557	0.676	0.588	0.958	0.689	0.967	0.758	1.645	0.948	1.012	1.049	0.911	0.783	0.865	0.98	0.983	0.898
8	0.301	1.282	0.242	0.284	0.828	0.649	0.849	0.545	0.988	0.476	0.526	0.524	0.495	0.754	0.352	0.301	0.544	0.562

This study investigated the potential of various bacterial isolates to produce organic acids and degrade complex molecules present in kitchen waste.

These findings suggest that the studied bacterial isolates possess a versatile metabolic capacity, enabling them to utilize various components of kitchen waste for growth and acid production.

This research paves the way for the development of a sustainable approach to waste management using these bacteria. Further investigation is needed to optimize the process for efficient acid production and explore potential applications of the produced organic acid.

FUTURE PROSPECTUS

The immense problem of food waste, especially leftovers from kitchens, squanders valuable resources, chokes landfills, and pollutes the atmosphere with greenhouse gases. Converting this waste into valuable products like organic acids offers a sustainable solution. This study investigated the possibility of converting kitchen waste into organic acids by harnessing bacteria found in soil rich in organic matter.

This study successfully isolated bacteria from organic-rich environments with the ability to utilize key components of kitchen waste, including starch, protein, fats, and carboxymethyl cellulose (CMC). All isolates demonstrated the potential to produce organic acids from starch, a major component of kitchen waste. This versatility highlights their suitability for processing diverse kitchen waste streams.

The next phase of research will focus on developing a potent bacterial consortium by combining the most promising isolates. This consortium will be designed to synergistically degrade a wider range of kitchen waste components for enhanced organic acid production.

The consortium will be applied to actual kitchen waste to assess its efficacy in producing organic acids. The quantity and specific composition of the produced organic acids will be rigorously analyzed to determine the most valuable products generated.

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APPENDIX I

MEDIA COMPOSITION

1) **Starch phosphate broth**

Ingredients	g/litre
Peptone	7
Dipotassium phosphate	5
Starch	5
Sodium chloride	1.4
pH	7.2+/- 0.2

2) **Peptone phosphate broth**

Ingredients	g/litre
Peptone	7
Dipotassium phosphate	5
Sodium chloride	1.4
pH	7.2+/- 0.2

3) **CMC phosphate broth**

Ingredients	g/litre
Peptone	7
Dipotassium phosphate	5
Carboxymethylcellulose	5
Sodium chloride	1.4
pH	7.2+/- 0.2

4) **Oil phosphate broth**

Ingredients	g/litre
Peptone	7
Dipotassium phosphate	5
Coconut oil	5
Sodium chloride	1.4
pH	7.2+/- 0.2

5) Nutrient agar

Ingredients	g/liter
Peptone	5
Yeast extract	2.5
NaCl	5
Agar	15
Distilled water	1 liter
pH	7.0

6) Hydrolytic Enzyme Screening:

Nutrient agar was supplemented with 1% substrate as follows:

a) Skimmed Milk (Protease Activity)

- One milliliter of skimmed milk was separately autoclaved and aseptically added to 99 ml of autoclaved nutrient agar (NA).

c) Soluble Starch (Amylase Activity)

- One gram of soluble starch was dissolved in 10 ml of distilled water. This solution was then separately autoclaved and aseptically added to 90 ml of autoclaved NA.

b) Carboxymethylcellulose (CMC) (Protease Activity)

- One gram of CMC was added to 10 ml of distilled water to form a suspension. The suspension was heated and gently swirled to disperse any clumps. This suspension was then separately autoclaved and aseptically added to 90 ml of autoclaved NA.

BIOCHEMICAL MEDIUM COMPOSITION

1) Glucose Phosphate Broth (MR-VP medium)

Ingredients	g/liter
Peptone	7.0
Dipotassium phosphate	5.0
Glucose	5.0
Sodium chloride	1.4
Ph	7.2+/- 0.2

2) Sugar stock solution (0.5%)

Ingredients	g/liter
Sugar (Lactose/Sucrose/Maltose/Fructose)	5.0
Distilled water	1L

Sugars were prepared as 0.5% (w/v) 10 ml stock solutions, one for each sugar. These solutionswere intended for use in the methyl red test. Each solution was autoclaved separately for 10 minutes at 121°C.

3) Tryptone broth

Ingredients	g/liter
Tryptone	10.0
NaCl	5.0
Distilled water	1 liter

4) Christensen's Urea medium

Ingredients	g/liter
Peptone	1.0
KH_2PO_4	2.0
NaCl	5.0
Urea	20.0
Phenol red	0.1
Distilled water	1 liter
pH	6.8

Urea was added separately to the medium, as it is prone to degradation during autoclaving. 20% urea stock solution was prepared and sterilized by filter sterilization method. The appropriate volume of urea stock was added to the above medium broth, after it has been sterilized by autoclaving at 121°C for 20 minutes.

5) Simmons Citrate agar

Ingredients	g/liter
NaCl	1.0
$MgSO_4.7H_2O$	0.2
$NaHPO_4$	1.0
KH_2PO_4	1.0
Sodium citrate	1.0
Bromothymol blue	9.0
Agar	8.0
Distilled water	1liter
pH	6.8

6) Huge Leifson medium

Ingredients	g/liter
Peptone	2.0
Sodium chloride	5.0
Dipotassium phosphate	0.3
Glucose	10.0
Bromothymol blue	0.03
Agar	3.0
pH	7.1

APPENDIX I I

REAGENTS AND STAINS

1) 1% Lugol's iodine solution

Ingredients	g/100ml
Lugol's Iodine	1ml
Distilled water	100ml

2) 0.1% Congo Red solution

Ingredients	g/100ml
Congo red	0.1
Distilled water	100ml

3) 1M NaCl solution (for destaining)

Ingredients	g/100ml
NaCl	24.72
Distilled water	100ml

4) 2% Saline

Ingredients	g/100ml
NaCl	2
Distilled water	100ml

5) Methyl red reagent (for MR test)

Ingredients	g/400ml
Methyl red	6.2
Ethyl alcohol	600ml
Distilled water	400ml

6) Kovac's reagent (Indole test)

Ingredients	g/liter
Isoamyl alcohol	150ml
p-dimethyl amino benzaldehyde	10.0
Concentrated HCl	50ml
Distilled water	1000ml

7) Omeara's reagent (Vogues-Proskauer test)

Ingredients	g/40ml
Creatine	0.15
KOH	20.0
Distilled water	40ml

8) Gram's Crystal Violet

Ingredients	g/80ml
Crystal Violet	2.0
Ammonium oxalate	0.8
Ethyl alcohol	20ml
Distilled water	80ml

9) Safranine

Ingredients	g/50ml
Safranine-O	0.5
Ethyl alcohol	50ml

10) Gram's Iodine

Ingredients	g/300ml
Iodine	1.0
Potassium iodide	2.0
Distilled water	300ml