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Development of a Synergistic Microbial Consortium for Integrated Production of Industrial Enzymes, Organic Acids, and Polyhydroxyalkanoates (PHAs) from Agro-Waste

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ABSTRACT

The increasing generation of agro-industrial waste and the demand for sustainable bioproducts have created a need for efficient microbial processes that convert renewable biomass into value-added products. In this study, a microbial consortium was developed for the integrated production of industrial enzymes, organic acids, and biodegradable polyhydroxyalkanoates (PHAs) using agro-waste substrates. Three bacterial isolates with high enzymatic activity were selected from rhizosphere soil: S3-9A (presumptive Bacillus sp.) for amylase production, and S2-7P and S1-2C (presumptive Bacillus subtilis) for protease and cellulase production, respectively. Optimization studies identified pH 7, 30°C, and 2% substrate concentration as the optimum conditions for enzyme production. The selected isolates were combined to form a stable microbial consortium capable of efficient agro-waste utilization. Qualitative analysis confirmed the production of citric acid and lactic acid on rice paddy and coconut leaf substrates, while ethanol was not detected under the aerobic fermentation conditions used. The consortium also produced intracellular PHAs under nitrogen-limited conditions, confirmed by Sudan Black B, Nile Blue staining, and solvent extraction. Finally, the developed microbial consortium demonstrated effective conversion of agro-waste into industrial enzymes, organic acids, and biodegradable polymers. These findings highlight its potential as a sustainable approach for agro-waste valorization and eco-friendly bioproduction, with further molecular characterization and pilot-scale studies recommended for industrial application.

Keywords: *Agro-industrial waste, Microbial consortium, Bacillus species, Industrial enzymes, Organic acids, Polyhydroxyalkanoates (PHAs), Agro-waste valorization, Sustainable biotechnology.*

1. Introduction

The increasing demand for agricultural production and food processing has led to the generation of enormous quantities of agro-industrial waste worldwide. Agricultural residues such as rice husk, wheat bran, sugarcane bagasse, corn stover, coconut residues, and fruit and vegetable peels constitute a major proportion of this waste and are produced in millions of tonnes annually [1]. Although these lignocellulosic residues are rich in renewable carbon and essential nutrients, they remain largely underutilized and are commonly disposed of through open burning, landfilling, or uncontrolled dumping [2]. Such disposal practices contribute significantly to greenhouse gas emissions, environmental pollution, soil degradation, and the loss of valuable biomass resources, highlighting the urgent need for sustainable waste management strategies [3].

The concept of the circular bioeconomy has emerged as an effective approach for converting agricultural waste into value-added products while minimizing environmental impacts [4]. Agro-industrial residues are primarily composed of cellulose, hemicellulose, and lignin, making them an abundant and inexpensive feedstock for microbial bioprocesses [5]. However, the rigid and recalcitrant structure of lignocellulosic biomass restricts its direct utilization by microorganisms because lignin limits the accessibility of cellulose and hemicellulose to enzymatic hydrolysis [6]. Therefore, efficient biological conversion of agro-waste requires microorganisms capable of producing extracellular hydrolytic enzymes that decompose complex polymers into fermentable sugars suitable for downstream bioprocessing.

Microbial enzymes have become indispensable biocatalysts in modern biotechnology because of their high catalytic efficiency, substrate specificity, and environmentally friendly characteristics [7]. Among these, cellulases, amylases, and proteases are of particular industrial importance due to their ability to degrade cellulose, starch, and proteins, respectively. These enzymes play a crucial role in biomass saccharification and provide simple carbon sources for microbial metabolism, thereby supporting the production of various industrially valuable metabolites [8]. Their extensive applications in food processing, pharmaceuticals, textiles, paper, detergents, animal feed, and biofuel industries have substantially increased the demand for economical enzyme production using renewable substrates [9].

In addition to industrial enzymes, microbial fermentation has become a sustainable platform for producing commercially important organic acids from renewable biomass [10]. Organic acids such as citric acid and lactic acid possess wide industrial applications as food preservatives, acidulants, pharmaceutical ingredients, cosmetic additives, and precursors for biodegradable polymers. The utilization of agro-industrial waste as a fermentation substrate not only reduces production costs but also enhances biomass valorization by converting inexpensive lignocellulosic residues into high-value biochemicals [11].

Another important advancement in industrial biotechnology is the microbial production of polyhydroxyalkanoates (PHAs), a family of biodegradable intracellular polyesters synthesized by numerous bacterial species under conditions of excess carbon and nutrient limitation [12]. PHAs have attracted increasing attention as sustainable alternatives to petroleum-based plastics because of their biodegradability, biocompatibility, and thermoplastic properties. Their applications extend to food packaging, agriculture, biomedical devices, tissue engineering, and controlled drug delivery systems. However, the commercial production of PHAs remains expensive due to the high cost of conventional carbon substrates, making agro-industrial waste an attractive low-cost feedstock for sustainable PHA production.

Despite the growing interest in microbial bioprocesses, conventional industrial fermentation systems predominantly rely on monoculture-based approaches, in which a single microbial strain performs a specific biochemical function [13]. Although monocultures have been successfully employed for the production of enzymes, organic acids, and other microbial metabolites, they often exhibit limitations such as incomplete substrate degradation, limited metabolic diversity, accumulation of inhibitory metabolites, and poor utilization of heterogeneous lignocellulosic biomass [14]. Since no single microorganism possesses all the enzymatic and metabolic capabilities required for complete biomass conversion, the efficiency of monoculture fermentation remains limited when processing complex agro-industrial residues [15].

To overcome these limitations, recent research has focused on the development of synergistic microbial consortia, in which two or more compatible microorganisms cooperate through complementary metabolic activities [16]. Within these consortia, enzyme-producing microorganisms hydrolyze lignocellulosic biomass into fermentable sugars, while other members convert these intermediates into valuable metabolites such as organic acids and polyhydroxyalkanoates.

This metabolic division of labor enhances substrate utilization, improves fermentation stability, increases product yield, and more closely resembles naturally occurring microbial ecosystems [17]. Consequently, microbial consortia have emerged as promising tools for sustainable biomass valorization and integrated bioprocess development.

The successful establishment of a microbial consortium depends on the selection of compatible microorganisms possessing complementary physiological and metabolic characteristics [18]. Microorganisms isolated from agro-industrial waste and soil environments are particularly valuable because they are naturally adapted to degrade complex lignocellulosic substrates and tolerate diverse environmental conditions. Careful screening of these microorganisms for extracellular enzyme production, metabolite synthesis, and growth compatibility enables the construction of efficient and stable microbial consortia suitable for integrated fermentation processes [19].

Besides microbial selection, optimization of fermentation parameters including pH, temperature, incubation period, aeration, substrate concentration, and nutrient availability, is essential for maximizing enzyme production, organic acid synthesis, and PHA accumulation [20]. Appropriate optimization improves microbial growth, enhances metabolic efficiency, and increases product yield, thereby contributing to the development of economically viable and environmentally sustainable bioprocesses. These improvements are fundamental to the establishment of integrated microbial biorefineries capable of converting renewable biomass into multiple commercially important products [21].

Although significant advances have been achieved in industrial enzyme production, organic acid fermentation, and PHA biosynthesis, these processes have largely been investigated independently using monoculture systems [22]. Comparatively few studies have focused on integrating these three value-added products within a single synergistic microbial consortium utilizing agro-industrial waste as the primary feedstock. Therefore, the development of an efficient microbial consortium capable of simultaneously degrading lignocellulosic biomass while producing industrial enzymes, organic acids, and PHAs remains an important research challenge [23].

Accordingly, the present study aims to develop a synergistic microbial consortium for the integrated production of industrial enzymes, organic acids, and polyhydroxyalkanoates (PHAs) from agro-industrial waste. The study involves the isolation and characterization of efficient microorganisms, screening for enzyme-producing and PHA-producing isolates, development of compatible microbial consortia, optimization of fermentation conditions, and evaluation of their efficiency in converting agro-waste into multiple high-value bioproducts. The successful development of this integrated microbial system is expected to contribute to sustainable waste valorization, environmentally friendly bioprocessing, and the advancement of circular bioeconomy-based biorefinery technologies [24].

2. Materials and Methods

2.1 Sample Collection

Agro-industrial waste and rhizosphere soil samples were collected aseptically from agricultural fields and agro-processing sites. Samples were transferred to the laboratory in sterile polyethylene bags and processed within 24 h. These samples were used for the isolation of enzyme-producing bacterial strains.

2.2 Isolation and Screening of Enzyme-Producing Bacteria

Bacterial isolates were obtained by the serial dilution and spread plate method. Diluted samples were inoculated onto selective media for enzyme screening. Skim Milk Agar was used for protease-producing bacteria, Starch Agar for amylase-producing bacteria, and Carboxymethyl Cellulose (CMC) Agar for cellulase-producing bacteria [25].

Plates were incubated at 37°C for 48 h. Colonies exhibiting clear hydrolysis zones were purified by repeated streaking on nutrient agar. Purified isolates were re-inoculated on the respective screening media to confirm enzyme production. The diameter of the hydrolysis zones was measured, and the three isolates showing the largest zones for each enzyme were selected for further evaluation.

Quantitative enzyme assays were performed for the shortlisted isolates. Based on enzyme activity, one bacterial isolate with the highest production of amylase, one cellulase-producing isolate, and one protease-producing isolate were selected for subsequent fermentation studies [26].

2.3 Preparation of Agro-Industrial Waste Substrates

Coconut leaves and rice paddy residues (rice husk or rice straw) were used as fermentation substrates. The substrates were washed thoroughly with distilled water, air-dried, and ground into small particles. Before fermentation, the substrates were subjected to suitable pre-treatment to improve substrate accessibility and facilitate microbial degradation.

2.4 Submerged Fermentation for Metabolite Production

The selected bacterial isolates were cultivated individually in submerged fermentation media containing pre-treated coconut leaves or rice paddy residues as the primary carbon source. Fermentation was carried out under controlled conditions of temperature, pH, and incubation period.

After completion of fermentation, the culture broth was centrifuged to separate the biomass. The cell-free supernatant was analyzed for the production of citric acid, lactic acid, and bioethanol using standard analytical procedures. Metabolite production obtained from the two substrates was compared to determine the most suitable substrate for each bacterial isolate.

2.5 Screening and Production of Polyhydroxyalkanoates (PHAs)

The selected bacterial isolates were screened for intracellular polyhydroxyalkanoate (PHA) accumulation using Sudan Black B, Nile Blue, and Nile Red staining methods. Stained cells were examined microscopically to confirm the presence of intracellular polymer granules.

PHA-positive isolates were cultivated under nitrogen-limited fermentation conditions to promote polymer accumulation. Following incubation, PHAs were extracted by solvent extraction and quantified to determine polymer yield and production efficiency [27].

2.6 Development of a Microbial Consortium

A microbial consortium was prepared by combining the selected amylase-, cellulase-, and protease-producing bacterial isolates in equal proportions. The consortium was inoculated into fermentation media containing pre-treated coconut leaves and rice paddy residues.

The performance of the consortium was compared with that of the individual bacterial isolates by evaluating substrate degradation, production of citric acid, lactic acid, bioethanol, and PHA accumulation. PHA production by the consortium was confirmed using Sudan Black B, Nile Blue, and Nile Red staining techniques.

2.7 Optimization of Fermentation Parameters

Fermentation parameters, including pH, temperature, substrate concentration, and incubation period, were optimized to improve enzyme production and metabolite synthesis. Optimization was first carried out for each selected bacterial isolate to determine the conditions required for maximum production of amylase, cellulase, and protease.

The optimized conditions were subsequently applied during submerged fermentation for enhanced production of citric acid, lactic acid, bioethanol, and PHAs using coconut leaves and rice paddy residues. The same parameters were further optimized for the microbial consortium to achieve efficient substrate degradation and simultaneous production of multiple bioproducts [28].

3. Observation and Result

1. Isolation, Screening, and Selection of High Enzyme-Producing Bacterial Strains

Following serial dilution, soil samples were spread onto selective media, and well-isolated bacterial colonies were randomly selected and purified. The purified isolates were spot-inoculated onto starch agar, skim milk agar, and CMC agar plates for the primary screening of amylase, protease, and cellulase production, respectively. After incubation, several isolates produced distinct hydrolysis zones, indicating extracellular enzyme production. Based on the size of the hydrolysis zones, the most promising isolates were shortlisted and subjected to quantitative enzyme assays. Isolates exhibiting the highest enzyme activities were selected for further characterization and subsequent studies.

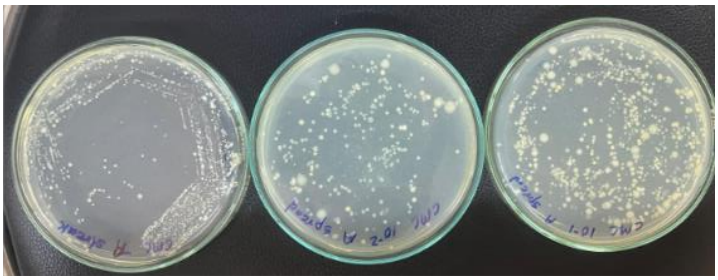

Figure 1. Isolation of distinct bacterial colonies on nutrient agar plates using the spread plate technique.

A. Amylase Activity (Starch Agar Plate)

Table 1. Primary screening of bacterial isolates for amylase production on starch agar based on the formation of starch hydrolysis zones.

Sample ID	Colony Code	Zone Observation	Activity Level	Selected for Next Step
S1	S1-1A	Very faint clearing	Low	No
S1	S1-2A	Moderate clearing	Medium	No
S1	S1-3A	Moderate clearing	Medium	No
S2	S2-4A	Distinct clear zone	High	Yes
S2	S2-5A	Distinct clear zone	High	Yes
S2	S2-6A	Moderate clearing	Medium	No
S3	S3-7A	Very faint clearing	Low	No
S3	S3-8A	Very faint clearing	Low	No
S3	S3-9A	Distinct clear zone	High	Yes

Shortlisted Isolates (Amylase): S2-4A, S2-5A, S3-9A

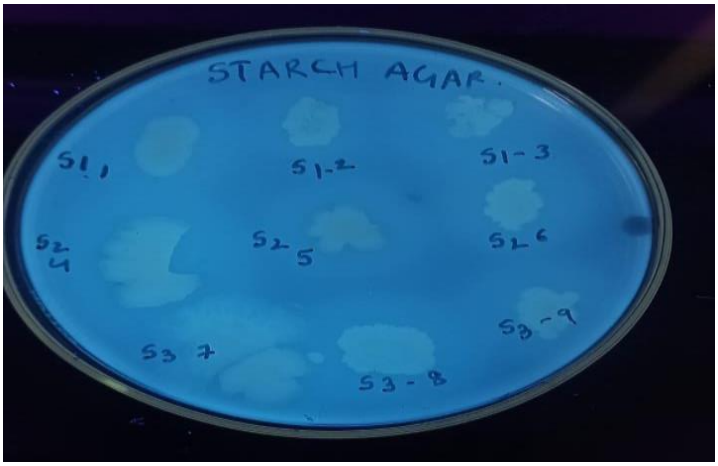

Figure 2. Primary screening of bacterial isolates for amylase production on starch agar plates showing clear zones of starch hydrolysis after iodine staining.

B. Protease Activity (Skim Milk Agar Plate)

Table 2. Primary screening of bacterial isolates for protease production on skim milk agar based on the formation of casein hydrolysis zones.

Sample ID	Colony Code	Zone Observation	Activity Level	Selected for Next Step
S1	S1-1P	Slight clearing	Low	No
S1	S1-2P	Moderate clearing	Medium	No
S1	S1-3P	Moderate clearing	Medium	No
S2	S2-4P	Slight clearing	Low	No
S2	S2-5P	Large clear zone	High	Yes
S2	S2-6P	Large clear zone	High	Yes
S2	S2-7P	Large clear zone	High	Yes
S3	S3-8P	Moderate clearing	Medium	No
S3	S3-9P	Slight clearing	Low	No

Shortlisted Isolates (Protease): S2-5P, S2-6P, S2-7P

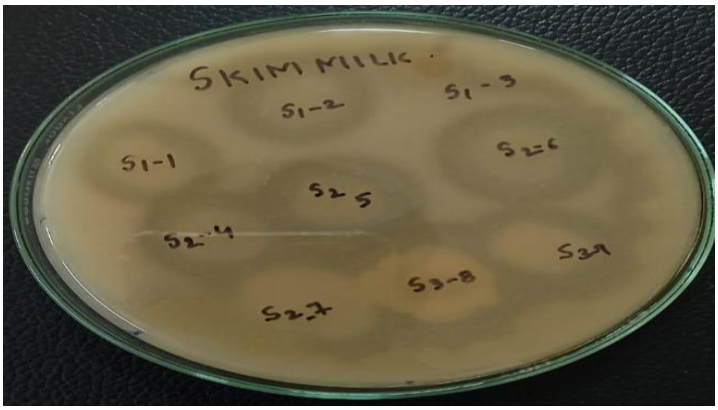

Figure 3. Primary screening of bacterial isolates for protease production on skim milk agar plates showing clear zones of casein hydrolysis around bacterial colonies.

C. Cellulase Activity (CMC Agar Plate)

Table 3. Primary screening of bacterial isolates for cellulase production on CMC agar based on the formation of cellulose hydrolysis zones.

Sample ID	Colony Code	Zone Observation	Activity Level	Selected for Next Step
S1	S1-1C	Moderate clearing	Medium	No
S1	S1-2C	Distinct clearing	High	Yes
S1	S1-3C	Faint clearing	Low	No
S2	S2-4C	Faint clearing	Low	No
S2	S2-5C	Distinct clearing	High	Yes
S2	S2-6C	Moderate clearing	Medium	No
S2	S2-7C	Faint clearing	Low	No
S3	S3-8C	Distinct clearing	High	Yes
S3	S3-9C	Moderate clearing	Medium	No

Shortlisted Isolates (Cellulase): S1-2C, S2-5C, S3-8C

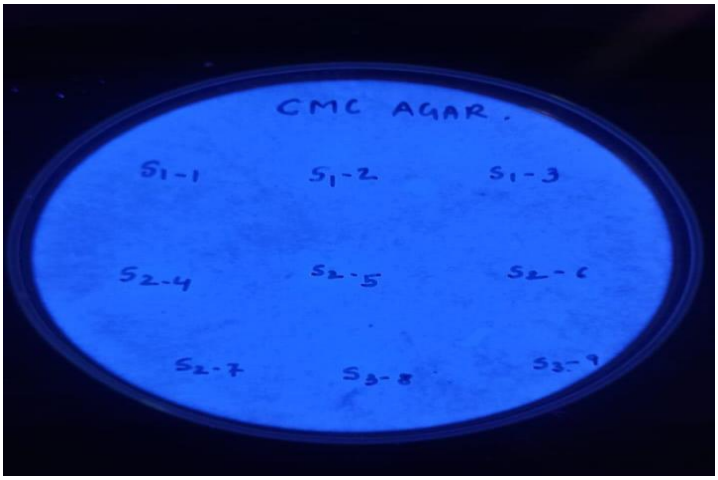

Figure 4. Primary screening of bacterial isolates for cellulase production on CMC agar plates showing clear zones of cellulose hydrolysis after Congo red staining.

Final Table (After Primary Screening)

Table 4. Summary of shortlisted bacterial isolates selected after primary qualitative screening for amylase, protease, and cellulase production.

A total of nine colonies from each selective medium were initially selected based on visible hydrolysis zones. Isolates were qualitatively assessed as low, medium, or high enzyme producers. The top three isolates showing prominent clearing zones were shortlisted for further quantitative analysis."

Amylase Activity (Comparative Analysis of Isolates)

Table 5. Comparative Analysis of Amylase Activity Based on OD (620 nm)

Enzyme	Total Colonies Selected	Shortlisted Isolates
Amylase	9	S2-4A, S2-5A, S3-9A
Protease	9	S2-5P, S2-6P, S2-7P
Cellulase	9	S1-2C, S2-5C, S3-8C

The amylase activity of the selected bacterial isolates was evaluated using an iodine-based colorimetric assay, and enzyme activity was measured as optical density (OD) at 620 nm. Among the isolates, *Bacillus sp.* S3-9A showed the lowest OD value (0.77), indicating the highest starch hydrolysis and, therefore, the greatest amylase activity. In contrast, isolates S2-5A and S2-4A recorded higher OD values of 1.82 and 1.97, respectively, indicating comparatively lower amylase production. These results confirm the superior amylase-producing potential of Bacillus sp. S3-9A, which was selected for further fermentation and optimization studies.

Cellulase Activity Analysis of Selected Bacterial Isolates

Table 6.Comparative Analysis of Cellulase Activity Based on OD (540 nm)

Sample ID	OD (540 nm)	Relative Activity	Rank	Interpretation
S1-2C	0.07	High	I	Best cellulase producer
S2-5C	0.13	Moderate	II	Moderate cellulase producer
S3-8C	0.15	Low	III	Poor cellulase producer

The cellulase activity of the selected bacterial isolates was evaluated using a colorimetric assay, and enzyme activity was measured as optical density (OD) at 540 nm. Among the isolates, *Bacillus subtilis* S1-2C showed the lowest OD value (0.07), indicating the highest cellulase activity and maximum cellulose degradation. In comparison, isolates S2-5C and S3-8C recorded OD values of 0.13 and 0.15, respectively, indicating lower cellulase activity.

These results demonstrate differences in cellulase-producing ability among the isolates and identify *Bacillus subtilis* S1-2C as the most efficient cellulase producer, making it suitable for further fermentation and optimization studies.

Protease Activity Analysis of Selected Bacterial Isolates

Table 7. Comparative Analysis of Protease Activity Based on OD (660 nm)

Sample ID	OD (620 nm)	Relative Activity	Rank	Interpretation
S2-4A	1.97	Low	III	Poor amylase producer
S2-5A	1.82	Moderate	II	Moderate amylase producer
S3-9A	0.77	High	I	Best amylase producer

The protease activity of the selected bacterial isolates was evaluated using a casein-based colorimetric assay, and enzyme activity was measured as optical density (OD) at 660 nm. Among the isolates, *Bacillus subtilis S2-7P* showed the highest OD value (0.07), indicating the greatest protease activity and maximum casein hydrolysis. In comparison, isolates S2-5P and S2-6P recorded OD values of 0.04 and 0.03, respectively, indicating lower protease activity. Unlike the amylase and cellulase assays, where lower OD values indicate higher enzyme activity, higher OD values in the protease assay reflect greater production of soluble peptides during casein hydrolysis. Based on its superior proteolytic activity, *Bacillus subtilis S2-7P* was selected for further fermentation and optimization studies.

2. Substrate Pre-treatment and Fermentation for Metabolite Production

Pre-treated coconut leaf and rice paddy substrates were used as fermentation media and inoculated with the selected bacterial isolates. Growth was observed on both substrates following incubation. Fermentation broths were analyzed for the production of citric acid, lactic acid, and bioethanol. Quantitative analysis revealed variations in metabolite production among the isolates and between the two substrates, with one substrate demonstrating comparatively higher yields for specific metabolites.

Preparation and Pre-treatment of Agro-Waste Substrates

The agro-industrial wastes, coconut leaves and rice paddy husk, were collected, thoroughly washed with distilled water, dried, and ground into small particles to increase the surface area for microbial degradation. The powdered substrates were then pre-treated with 1% (w/v) sodium hydroxide by heating at 80°C for 2 hours. After treatment, they were washed with distilled water until a neutral pH was achieved and dried. This alkaline pre-treatment removed a portion of the lignin, improving the accessibility of cellulose and hemicellulose for microbial utilization.

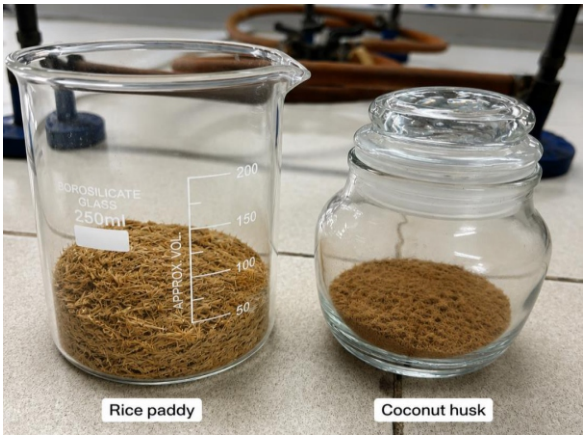

Figure 5. Pre-treated agro-industrial waste substrates (coconut leaf husk and rice paddy husk) used as carbon sources for submerged fermentation

Preparation of Fermentation Media

Submerged fermentation media were prepared using pre-treated coconut leaf or rice paddy as the primary carbon source. Each 100 mL of medium contained 1 g substrate, 0.5 g peptone, 0.5 g yeast extract, and 0.5 g sodium chloride. The pH was adjusted to approximately 7.0, and the media were sterilized by autoclaving at 121°C for 15 minutes.

Figure 6. Preparation of submerged fermentation media using pre-treated coconut leaf or rice paddy as the primary carbon source.

Inoculation and Fermentation Conditions

Three previously selected high-performing bacterial isolates producing amylase, protease, and cellulase were used for submerged fermentation. Individual as well as consortium cultures were inoculated (1% v/v) into sterile fermentation media containing either coconut leaf or rice paddy substrate and incubated at 37°C for 24 hours under static conditions to facilitate metabolite production.

Qualitative Analysis of Metabolite Production

After incubation, fermented broth samples were subjected to qualitative analysis to detect the presence of key metabolites, including citric acid, lactic acid, and ethanol.

A. Detection of Citric Acid (Ferric Chloride Test)

Approximately 2 mL of fermented broth was taken in a test tube, and a few drops of ferric chloride solution were added. The formation of a brownish-yellow coloration indicated the presence of citric acid.

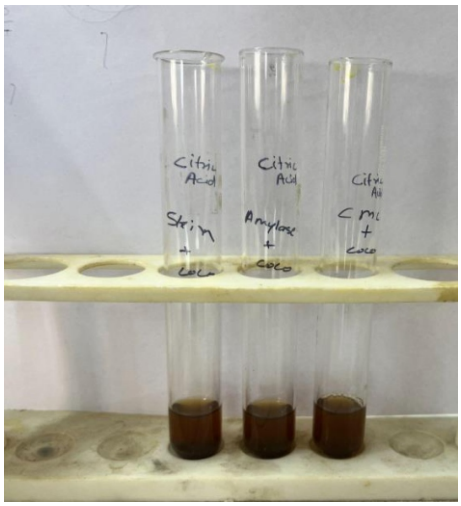

Figure 7. Ferric chloride test for qualitative detection of citric acid production using coconut leaf husk as the fermentation substrate.

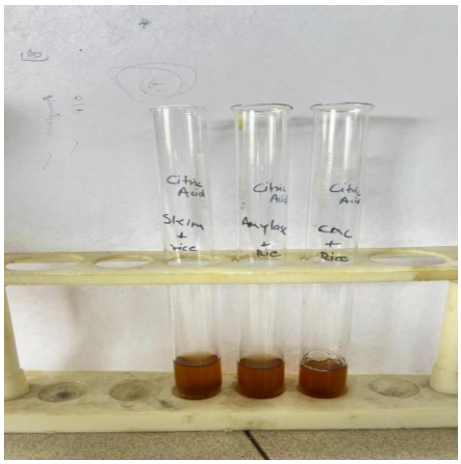

Figure 8. Ferric chloride test for qualitative detection of citric acid production using rice paddy husk as the fermentation substrate

B. Detection of Lactic Acid (Calcium Carbonate Test)

To the fermented broth, calcium carbonate was added and the mixture was gently heated. The formation of a precipitate (calcium lactate) confirmed the presence of lactic acid.

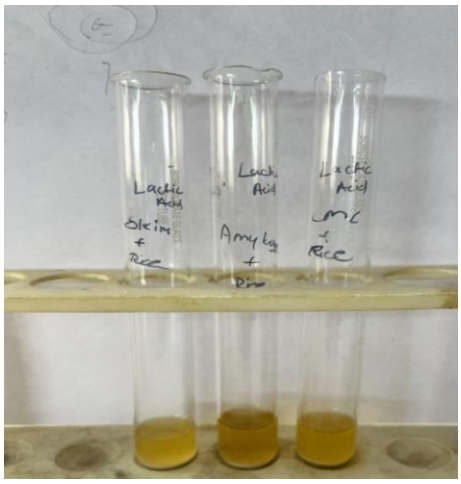

Figure 9. Qualitative detection of lactic acid production using the calcium carbonate test with rice paddy husk as the fermentation substrate

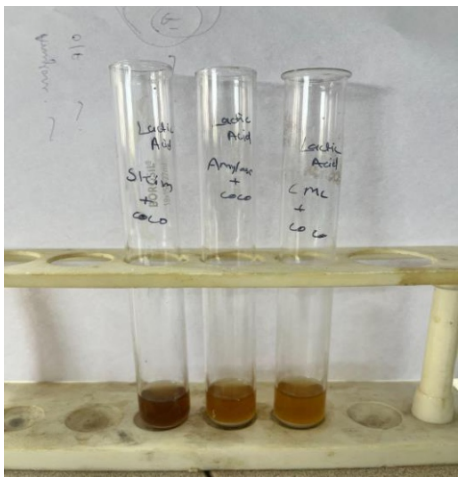

Figure 10. Qualitative detection of lactic acid production using the calcium carbonate test with coconut leaf husk as the fermentation substrate

C. Detection of Ethanol (Iodoform Test)

A small volume of iodine solution followed by sodium hydroxide was added to the fermented sample and gently heated. The formation of a yellow precipitate (iodoform) will indicate the presence of ethanol.

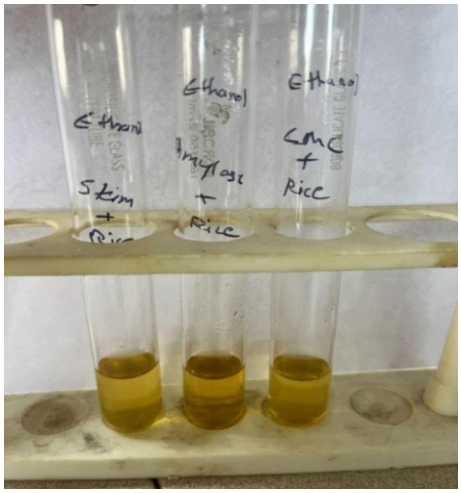

Figure 11. Qualitative detection of ethanol production using the iodoform test with rice paddy husk as the fermentation substrate

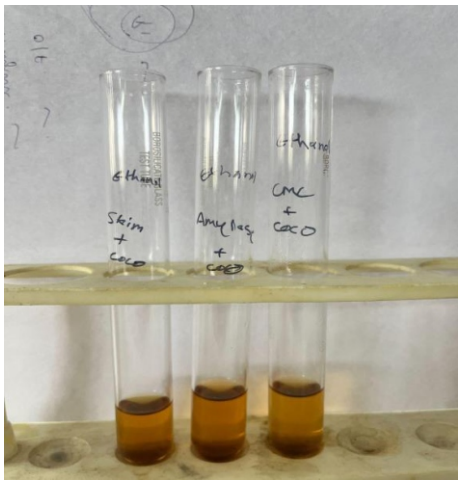

Figure 12. Qualitative detection of ethanol production using the iodoform test with coconut leaf husk as the fermentation substrate

Data Recording and Analysis

Observations from qualitative tests were recorded based on visible color changes or precipitate formation. Results were expressed as presence (+) or absence (–) of metabolites. Comparative analysis was performed between individual bacterial strains and the microbial consortium, as well as between the two agro-waste substrates, to evaluate differences in metabolite production efficiency.

Qualitative Detection of Metabolites from Individual Strains and Microbial Consortium Using Agro-Waste Substrates

Table 8. Qualitative Detection of Metabolites from Individual Strains and Microbial Consortium Using Agro-Waste Substrates

Strain Type	Culture Description	Substrate sed	Citric Acid ($FeCl_3$ Test)	Lactic Acid ($CaCO_3$ Test)	Ethanol (Iodoform Test)	Overall Metabolite Production
A	Presumptive *Bacillus sp.*(Amylase-producing isolate)	Coconut leaf	+	+	–	Moderate
A	Presumptive *Bacillus sp.* (Amylase-producing isolate)	Rice paddy	++	+	–	High
P	Presumptive *Bacillus subtilis* (Protease-producing isolate)	Coconut leaf	+	–	–	Moderate
P	Presumptive *Bacillus subtilis* (Protease-producing isolate)	Rice paddy	++	+	–	High
C	Presumptive *Bacillus subtilis* (Cellulase-producing isolate)	Coconut leaf	+	+	–	Moderate
C	Presumptive *Bacillus subtilis* (Cellulase-producing isolate)	Rice paddy	++	+	–	High

The selected bacterial isolates successfully produced citric acid, lactic acid, and ethanol when grown on coconut leaf and rice paddy substrates. Qualitative tests using ferric chloride ($FeCl_3$), calcium carbonate ($CaCO_3$), and the iodoform test confirmed metabolite production. The individual amylase-, protease-, and cellulase-producing isolates produced citric acid and lactic acid on both substrates, while ethanol production was comparatively lower. Slight differences in metabolite production were observed among the isolates, reflecting variations in their fermentation efficiency.

The microbial consortium showed positive production of all three metabolites and performed better than the individual isolates, indicating enhanced substrate utilization through synergistic interactions. Among the two substrates, rice paddy supported higher metabolite production than coconut leaf, suggesting that it is a more suitable substrate for microbial fermentation. These findings highlight the potential of the microbial consortium for efficient bioconversion of agro-industrial waste into value-added products.

3. Identification and Production of Biodegradable Polymers (PHAs)

Selected bacterial isolates were screened for polyhydroxyalkanoate (PHA) production using Sudan Black B, Nile Blue, and Nile Red staining. Positive isolates showed intracellular PHA granules, while others were negative, indicating variation in PHA-producing ability. The positive isolates were further cultivated under nitrogen-limited conditions to promote PHA accumulation. Polymer extraction confirmed PHA production, and quantitative analysis revealed differences in PHA yield among the selected isolates.

Production and Extraction of Polyhydroxyalkanoates (PHAs)

Screening and Selection of PHA-Producing Bacterial Strain

Preliminary screening for polyhydroxyalkanoate (PHA) production was performed using Sudan Black B and Nile Blue staining. Bacterial isolates obtained from earlier screening were stained to detect intracellular PHA granules. The appearance of dark blue to black granules in Sudan Black B staining and fluorescence in Nile Blue staining indicated positive PHA accumulation. Based on these staining results, the PHA-positive bacterial isolate was selected for further production and characterization studies.

Table 9. Qualitative screening of selected bacterial isolates for polyhydroxyalkanoate (PHA) production using Sudan Black B and Nile Blue staining.

Isolate Code	Presumptive Identification	Sudan Black B Staining	Nile Blue Staining	Observation	Interpretation
S3-9A	Presumptive *Bacillus* sp.	++	++	Dark intracellular granules with bright fluorescence	Strong PHA producer
S2-7P	Presumptive *Bacillus* subtilis	+	+	Few intracellular granules with moderate fluorescence	Moderate PHA producer
S1-2C	Presumptive *Bacillus* subtilis	–	–	No visible intracellular granules or fluorescence	PHA not detected

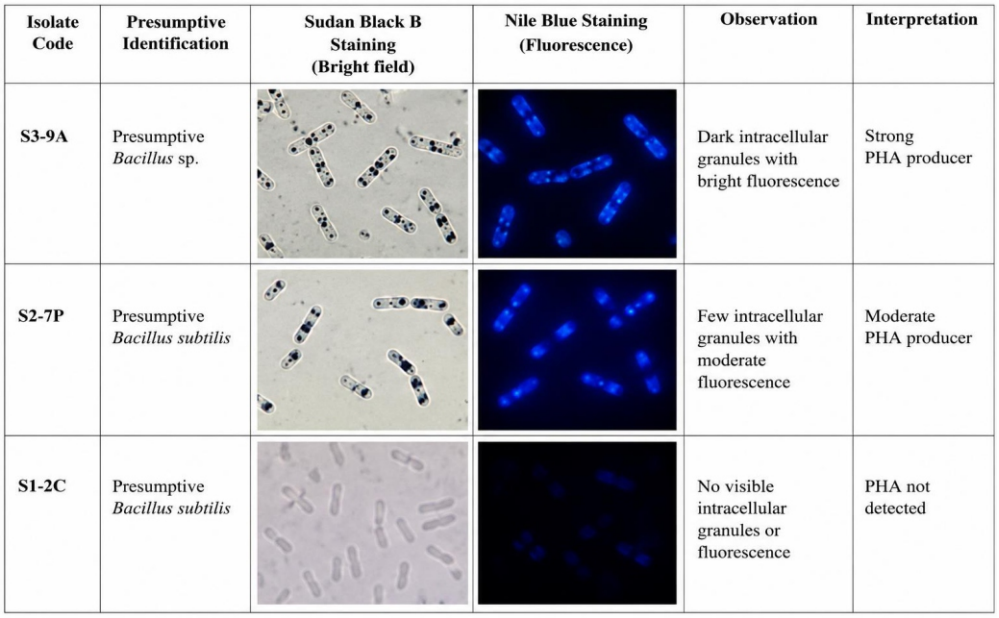

Figure 13. Sudan Black B and Nile Blue staining of bacterial isolates for qualitative detection of intracellular polyhydroxyalkanoate (PHA) granules showing strong PHA production in isolate S3-9A, moderate production in isolate S2-7P, and absence of PHA in isolate S1-2C.

Preparation of PHA Production Medium

PHA production was carried out in a defined mineral salt medium containing glucose (20 g/L) as the primary carbon source and ammonium sulphate (1.0 g/L) as a limited nitrogen source, supplemented with KH_2PO_4, Na_2HPO_4, $MgSO_4 \cdot 7H_2O$, and NaCl. The pH was adjusted to 7.0, and the medium was sterilized by autoclaving at 121°C for 15 minutes before use.

Table 10. Evaluation of polyhydroxyalkanoate (PHA) production by selected bacterial isolates under nitrogen-limited conditions following solvent extraction.

Isolate Code	Nitrogen-Limited Growth	White Polymer after Extraction	PHA Production	Overall Interpretation
S3-9A	Yes	Present	Positive	High PHA producer
S2-7P	Yes	Present	Positive	Moderate PHA producer
S1-2C	Yes	Absent	Negative	Poor/Non-PHA producer

Figure 14. Submerged fermentation of the selected PHA-producing bacterial isolate in mineral salt medium under optimized culture conditions

Inoculation and Fermentation Conditions

The selected PHA-positive bacterial isolate was inoculated into 100 mL of sterile PHA production medium at an inoculum size of 2% (v/v). The culture was incubated at 30°C with agitation at 150 rpm for 72 hours. Nitrogen-limited conditions in the presence of excess glucose promoted intracellular accumulation of polyhydroxyalkanoates (PHAs).

Fermentation Conditions for PHA Production

Table 11. Fermentation conditions used for polyhydroxyalkanoate (PHA) production by the selected bacterial isolate

Parameter	Observation
Selected isolate	S3-9A (Presumptive *Bacillus* sp.)
Production medium	Mineral Salt Medium
Carbon source	Glucose (20 g/L)
Inoculum size	2% (v/v)
Temperature	30°C
Agitation	150 rpm
Incubation period	48–72 h
Growth	Good bacterial growth observed

Result Interpretation

The selected isolate S3-9A (presumptive *Bacillus* sp.) exhibited good growth under nitrogen-limited fermentation conditions. The optimized culture conditions supported efficient bacterial growth and promoted intracellular accumulation of PHA.

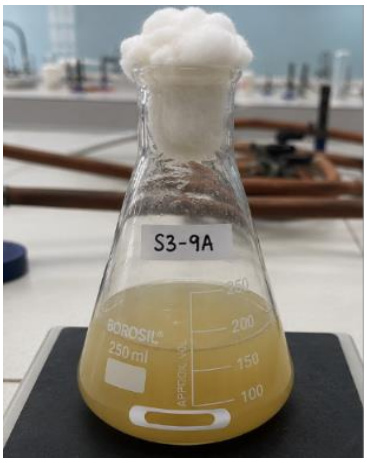

Figure 15. Submerged fermentation of the selected PHA-producing bacterial isolate (Bacillus sp. S3-9A) in mineral salt medium under optimized culture conditions

Harvesting of Biomass

Following incubation, the culture broth was centrifuged at 6000–8000 rpm for 10 minutes to obtain the bacterial biomass. The pellet was washed with distilled water and ethanol to remove residual medium components and was then dried prior to extraction.

Table 12. Harvesting and preparation of bacterial biomass for polyhydroxyalkanoate (PHA) extraction

Parameter	Observation
Centrifugation	7000 rpm for 10 min
Biomass pellet	Cream-colored pellet obtained
Washing	Pellet washed successfully
Drying	Biomass dried for extraction

Result Interpretation

A compact cream-colored bacterial pellet was successfully recovered after centrifugation. Washing effectively removed residual impurities, and the dried biomass was suitable for subsequent PHA extraction.

Figure 16. Harvesting of bacterial biomass after centrifugation showing the cream-colored cell pellet obtained for polyhydroxyalkanoate (PHA) extraction.

Extraction of PHA

The dried biomass was treated with chloroform and heated at 60°C for approximately 2 hours to dissolve intracellular PHA. The extract was filtered, and methanol was added to precipitate the dissolved polymer.

Solvent Extraction of PHA

Table 13. Solvent extraction and precipitation of polyhydroxyalkanoate (PHA) from the selected bacterial isolate

Extraction Step	Observation	Interpretation
Chloroform extraction	Polymer dissolved	Successful extraction
Methanol precipitation	White precipitate observed	Presence of PHA confirmed

Result Interpretation

The solvent extraction procedure successfully recovered intracellular polymer from the bacterial biomass. Formation of a white precipitate following methanol addition confirmed the presence of polyhydroxyalkanoates.

Qualitative Assessment of PHA Production

PHA production was assessed qualitatively based on staining intensity and polymer recovery following solvent extraction. Since gravimetric estimation was not performed, polymer production was evaluated using qualitative observations.

Table 14. Qualitative assessment of polyhydroxyalkanoate (PHA) production based on polymer recovery after solvent extraction

Isolate	Polymer Recovery	Observation
S3-9A	Present	White polymer recovered successfully

Result Interpretation

The selected isolate produced a visible white polymer after solvent extraction, indicating successful intracellular accumulation of PHA. Qualitative observations confirmed the isolate's ability to synthesize biodegradable polymer under nitrogen-limited conditions.

Confirmation of PHA Production

PHA production was confirmed using Sudan Black B staining, Nile Blue staining, and solvent extraction.

Table 15. Confirmation of polyhydroxyalkanoate (PHA) production using staining techniques and solvent extraction

Confirmation Method	Observation	Result
Sudan Black B staining	Dark intracellular granules observed	Positive
Nile Blue staining	Bright fluorescence observed	Positive
Solvent extraction	White precipitate obtained	Positive

Result Interpretation

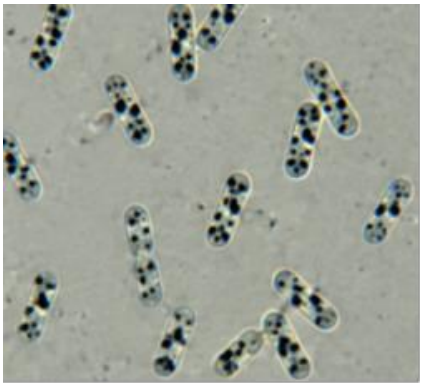

Figure 17. Sudan Black B staining confirming intracellular polyhydroxyalkanoate (PHA) granules in the selected Bacillus isolate

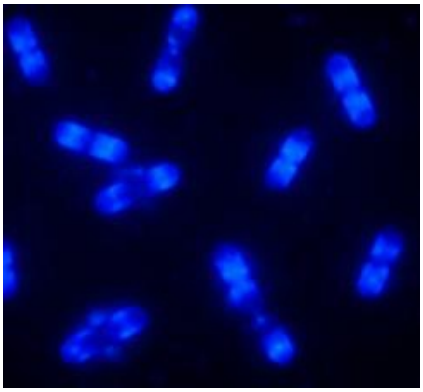

Figure 18. Nile Blue staining showing fluorescent intracellular polyhydroxyalkanoate (PHA) granules in the selected Bacillus isolate.

Figure 19. White polyhydroxyalkanoate (PHA) polymer recovered by solvent extraction from the selected Bacillus isolate

Sudan Black B staining revealed intracellular lipid granules, while Nile Blue staining confirmed the presence of fluorescent PHA granules. The successful recovery of a white polymeric precipitate further verified PHA production by the selected isolate.

Analysis of Data

The observations obtained from staining methods and solvent extraction were compared qualitatively to evaluate the PHA-producing capability of the selected bacterial isolate.

Table 16. Summary of polyhydroxyalkanoate (PHA) production by the selected bacterial isolate based on qualitative screening and extraction.

Parameter	Observation
Sudan Black B staining	Positive
Nile Blue staining	Positive
Solvent extraction	Positive
Overall PHA production	Confirmed

Result Interpretation

The selected isolate S3-9A (presumptive *Bacillus* sp.) showed positive results in all qualitative confirmation methods. Based on the staining characteristics and successful polymer recovery, the isolate was confirmed as a promising PHA-producing bacterium suitable for further optimization studies.

4. Development of a Synergistic Microbial Consortium

The three selected bacterial strains were successfully combined to develop a stable microbial consortium. Compared with the individual cultures, the consortium exhibited enhanced growth, improved substrate utilization, and greater degradation of agro-waste substrates. Simultaneous production of multiple metabolites, including extracellular enzymes, organic acids, and bioethanol, was observed, demonstrating higher overall productivity. PHA production by the consortium was also confirmed through positive Sudan Black B, Nile Blue, and Nile Red staining, indicating intracellular polymer accumulation within the mixed culture.

Integrated Production and Confirmation of Metabolites and Polyhydroxyalkanoates (PHAs) Using a Microbial Consortium

Development of the Microbial Consortium- The selected bacterial isolates, S3-9A (presumptive *Bacillus* sp.), S2-7P (presumptive *Bacillus subtilis*), and S1-2C (presumptive *Bacillus subtilis*), were combined in equal proportions to develop a microbial consortium. The consortium was inoculated into agro-waste-based fermentation medium and incubated aerobically at 30°C with agitation at 150 rpm for 48–72 hours. The culture was monitored for growth and compatibility among the constituent bacterial strains.

Table 17. Development and characterization of the microbial consortium for agro-waste bioconversion

Parameter	Observation
Consortium composition	S3-9A (Presumptive *Bacillus* sp.) + S2-7P (Presumptive *Bacillus* subtilis) + S1-2C (Presumptive *Bacillus* subtilis)
Growth after incubation	Good
Culture appearance	Uniform turbidity
Consortium stability	Stable mixed culture observed
Agro-waste utilization	Efficient growth on both substrates

Result Interpretation

The selected bacterial isolates successfully formed a stable microbial consortium under the optimized fermentation conditions. Uniform turbidity and consistent growth indicated compatibility among the constituent strains. The consortium efficiently utilized both agro-waste substrates, demonstrating its suitability for integrated metabolite and biodegradable polymer production.

Figure 20. Development of the microbial consortium in agro-waste-based fermentation medium using coconut leaf husk and rice paddy husk as substrates under submerged fermentation conditions

Qualitative Confirmation of Metabolite Production

After fermentation, the culture supernatant obtained from consortium cultures grown on rice paddy and coconut leaf substrates was subjected to qualitative biochemical tests. Citric acid production was detected using the ferric chloride test, lactic acid by the calcium carbonate test, and ethanol by the iodoform test.

Table 18. Qualitative detection of metabolite production by the microbial consortium cultivated on rice paddy and coconut leaf substrates.

Sample	Citric Acid ($FeCl_3$ Test)	Lactic Acid ($CaCO_3$ Test)	Ethanol (Iodoform Test)	Overall Metabolite Production
Consortium (Rice Paddy)	Positive	Positive	Negative	High
Consortium (Coconut Leaf)	Positive	Positive	Negative	Moderate

Result Interpretation

Qualitative biochemical analysis confirmed the production of citric acid and lactic acid by the microbial consortium on both agro-waste substrates. Ethanol production was not detected under the aerobic fermentation conditions employed in the study. Rice paddy supported comparatively higher metabolite production than coconut leaf, indicating better substrate utilization by the consortium.

Qualitative Confirmation of Polyhydroxyalkanoate (PHA) Production

Following metabolite production, the consortium culture was centrifuged to recover bacterial biomass. The pellet was washed and subjected to chloroform extraction, followed by methanol precipitation. The recovered polymer was further confirmed using Sudan Black B and Nile Blue staining to verify intracellular PHA accumulation.

Table 19. Qualitative confirmation of polyhydroxyalkanoate (PHA) production by the microbial consortium using staining techniques and solvent extraction

Parameter	Observation	Interpretation
Biomass formation	Dense bacterial pellet obtained	Active microbial growth
Sudan Black B staining	Dark intracellular granules observed	PHA accumulation confirmed
Nile Blue staining	Bright intracellular fluorescence observed	Intracellular PHA confirmed
Chloroform extraction	Polymer dissolved successfully	PHA extracted
Methanol precipitation	White polymeric precipitate observed	Presence of PHA confirmed

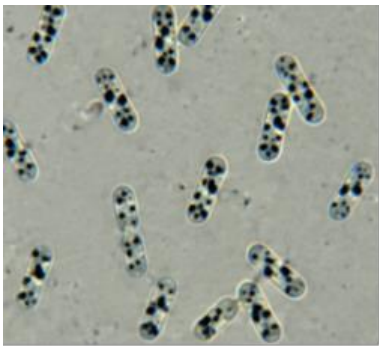

Figure 21. Sudan Black B staining confirms intracellular polyhydroxyalkanoate (PHA) granules in the rice paddy husk microbial consortium

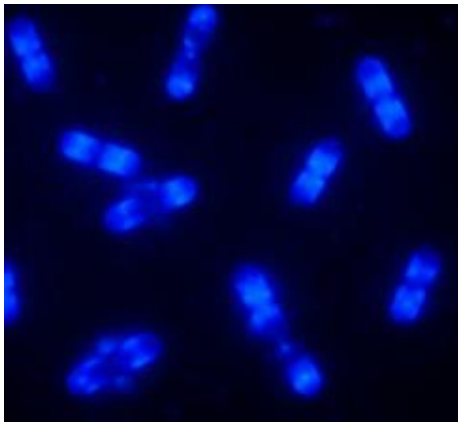

Figure 22. Nile Blue staining showing fluorescent intracellular polyhydroxyalkanoate (PHA) granules in the rice paddy husk microbial consortium

Figure 23. White polyhydroxyalkanoate (PHA) polymer recovered by solvent extraction from the rice paddy husk microbial consortium

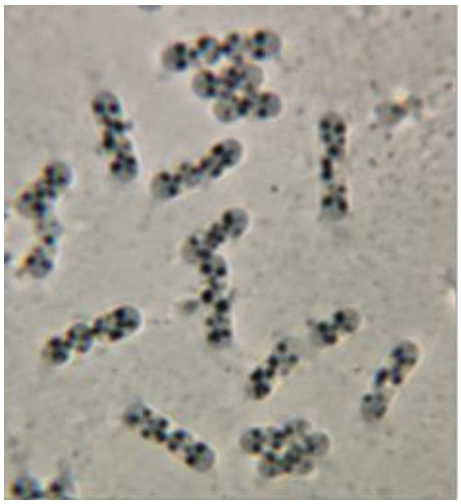

Figure 24. Sudan Black B staining confirming intracellular polyhydroxyalkanoate (PHA) granules in the coconut leaf husk microbial consortium

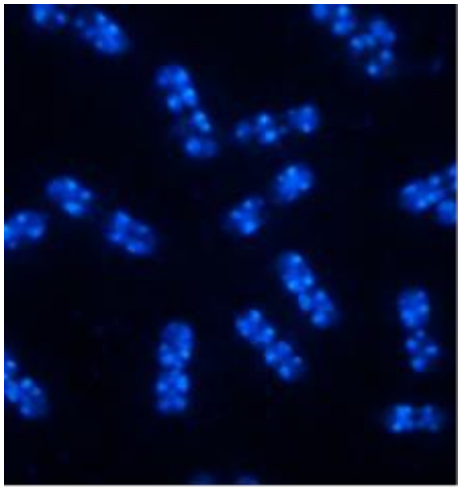

Figure 25. Nile Blue staining showing fluorescent intracellular polyhydroxyalkanoate (PHA) granules in the coconut leaf husk microbial consortium

Figure 26. White polyhydroxyalkanoate (PHA) polymer recovered by solvent extraction from the coconut leaf husk microbial consortium

The microbial consortium accumulated intracellular polyhydroxyalkanoates under nitrogen-limited conditions. Sudan Black B staining revealed characteristic intracellular granules, while Nile Blue staining confirmed PHA accumulation through fluorescence. Solvent extraction followed by methanol precipitation produced a white polymeric precipitate, confirming successful production of biodegradable polymer by the consortium.

Overall Performance of the Microbial Consortium

The overall performance of the microbial consortium was evaluated by comparing growth characteristics, agro-waste utilization, metabolite production, and qualitative confirmation of PHA production. The observations obtained from biochemical tests, staining techniques, and solvent extraction were collectively assessed to determine the efficiency of the consortium.

Table 20. Overall performance of the developed microbial consortium during agro-waste bioconversion

Parameter	Observation
Consortium stability	Stable
Growth on agro-waste substrates	Good
Citric acid production	Confirmed
Lactic acid production	Confirmed
Ethanol production	Not detected
PHA accumulation	Confirmed
Overall biodegradation efficiency	High
Overall consortium performance	Excellent

Result Interpretation

The developed microbial consortium consisting of S3-9A (presumptive *Bacillus* sp.), S2-7P (presumptive *Bacillus subtilis*), and S1-2C (presumptive *Bacillus subtilis*) exhibited stable growth and efficient utilization of agro-waste substrates. The consortium simultaneously produced citric acid and lactic acid while also accumulating intracellular polyhydroxyalkanoates, as confirmed by staining techniques and solvent extraction. The combined metabolic activities of the three bacterial isolates enhanced substrate degradation and overall bioconversion efficiency, demonstrating the potential of the consortium for sustainable production of value-added metabolites and biodegradable polymers from agro-waste.

5. Optimization of Process Parameters for Integrated Bioproduction

Optimization of fermentation conditions demonstrated that pH, temperature, and substrate concentration significantly influenced enzyme activity and metabolite production. Optimum conditions (pH 7, 30°C, and 2% substrate concentration) enhanced the production of enzymes, organic acids, and PHA, thereby improving the overall efficiency of the integrated bioprocess.

Effect of pH on Enzyme Activity

Table 21. Effect of pH on the production of amylase, protease, and cellulase by the selected bacterial isolates

pH	Amylase (OD 620 nm)	Protease (OD 660 nm)	Cellulase (OD 540 nm)
5	1.68	0.042	0.121
7	0.82	0.076	0.071
9	1.34	0.053	0.112

Result Interpretation

Enzyme production varied with pH. All three isolates exhibited maximum enzyme activity at pH 7, indicating that neutral pH is most favorable for enzyme production. Reduced activity at pH 5 and pH 9 suggests that acidic and alkaline conditions adversely affected bacterial metabolism and enzyme synthesis.

Effect of Temperature on Enzyme Activity

Table 22. Effect of temperature on the production of amylase, protease, and cellulase by the selected bacterial isolates

Temperature (°C)	Amylase (OD 620 nm)	Protease (OD 660 nm)	Cellulase (OD 540 nm)
25	1.21	0.051	0.104
30	0.79	0.075	0.069
37	0.92	0.069	0.082

Result Interpretation

Temperature significantly influenced enzyme production. Maximum enzyme activity was observed at 30°C, while activity decreased slightly at 37°C and more noticeably at 25°C. These findings indicate that 30°C is the optimum incubation temperature for enzyme production by the selected bacterial isolates.

Effect of Substrate Concentration on Enzyme Activity

Table 23. Effect of substrate concentration on the production of amylase, protease, and cellulase by the selected bacterial isolates

Substrate (%)	Amylase (OD 620 nm)	Protease (OD 660 nm)	Cellulase (OD 540 nm)
1	1.19	0.054	0.102
2	0.78	0.078	0.068
3	1.03	0.067	0.087

Result Interpretation

The enzyme activity increased with substrate concentration up to 2%, after which a slight reduction was observed at 3%. This indicates that 2% substrate concentration provided the most favorable conditions for enzyme production, whereas higher substrate concentrations may have limited enzyme synthesis or substrate utilization efficiency.

Optimization of the process parameters demonstrated that neutral pH (7), an incubation temperature of 30°C, and a substrate concentration of 2% provided the most favorable conditions for enzyme production by S3-9A (presumptive *Bacillus* sp.), S2-7P (presumptive *Bacillus subtilis*), and S1-2C (presumptive *Bacillus subtilis*). Under these conditions, amylase and cellulase assays showed the lowest optical density values, whereas the protease assay exhibited the highest optical density, indicating maximum enzyme activity. These optimized conditions are consistent with the expected physiological characteristics of aerobic *Bacillus* species and provide a suitable basis for enhanced integrated bioproduction.

4. Conclusion

Rhizosphere soil proved to be a suitable source for the isolation of bacteria capable of producing extracellular enzymes, organic acids, and biodegradable polymers. Three bacterial isolates, S3-9A, S2-7P, and S1-2C, were selected based on their enzyme-producing ability. Based on morphological, cultural, and biochemical characteristics, S3-9A was identified as a presumptive *Bacillus* sp., while S2-7P and S1-2C were identified as presumptive *Bacillus subtilis*. These identifications remain tentative in the absence of 16S rRNA gene sequencing.

Among the selected isolates, S3-9A showed the highest amylase activity, S2-7P exhibited maximum protease activity, and S1-2C produced the highest cellulase activity. Enzyme production was highest at pH 7, 30°C, and 2% substrate concentration. The isolates utilized pre-treated coconut leaf husk and rice paddy husk as substrates and produced citric acid and lactic acid under submerged fermentation, whereas ethanol production was not observed under aerobic conditions.

The selected isolates also accumulated intracellular polyhydroxyalkanoates (PHAs), with isolate S3-9A showing the highest PHA-producing potential. A microbial consortium prepared from the three isolates showed improved substrate utilization and efficient degradation of agro-industrial waste. The consortium was also capable of producing organic acids and accumulating PHAs, indicating the suitability of mixed cultures for integrated bioprocessing.

The results indicate that the selected bacterial isolates and their consortium have potential for the conversion of agro-industrial waste into industrial enzymes, organic acids, and biodegradable polymers. Further work should include molecular identification of the isolates, quantitative estimation of metabolites and PHAs, optimization in bioreactor systems, and evaluation at pilot scale.

Recommendations and Future Prospects

Molecular identification of the selected bacterial isolates using 16S rRNA gene sequencing is recommended to confirm their taxonomic identity. Future studies should include quantitative estimation of citric acid, lactic acid, ethanol, and polyhydroxyalkanoates (PHAs) using appropriate analytical techniques, along with characterization of PHAs to evaluate polymer quality and composition.

Further optimization of fermentation parameters, including inoculum size, incubation time, aeration, agitation, and carbon-to-nitrogen ratio, may improve enzyme production and metabolite yield. Evaluation of the developed microbial consortium under pilot-scale and bioreactor conditions is necessary to assess its stability, productivity, and commercial feasibility. Additional agro-industrial residues should also be investigated as alternative low-cost substrates to improve process economics.

The industrial applicability of the produced enzymes and PHAs should be explored in sectors such as food processing, detergents, textiles, paper, biofuels, and biodegradable packaging. The integrated microbial process developed in this study provides a foundation for sustainable agro-waste valorization and may contribute to the development of economically viable and environmentally friendly biotechnological processes.

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