SUSTAINABLE PRODUCTION OF INDUSTRIALLY IMPORTANT CHEMICALS FROM AGRO-INDUSTRIAL WASTE

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by

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I Sonika Kag, hereby certify that the work which is presented in the thesis entitled "**Sustainable Production of Industrially Important Chemicals from Agro-industrial Waste**" in the partial fulfillment of the requirements for the award of the Degree of Doctor of Philosophy, submitted in the Department of Biotechnology, Delhi Technological University, is an authentic record of my own work carried out during the period from 14.08.2019 to 22.04.2024 under the supervision of Professor Pravir Kumar Department of Biotechnology and co-supervision of Dr. Rashmi Kataria, Department of BSBT, Vellore Institute of Technology, Vellore India.

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SUSTAINABLE PRODUCTION OF INDUSTRIALLY IMPORTANT CHEMICALS FROM AGRO-INDUSTRIAL WASTE

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ABSTRACT

The aim of the research is to provide a comprehensive study on potato peel waste, an agro - industrial waste. Thesis consists of three major parts, which distinguish detailed discussion of composition analysis and pretreatment, PHA production from extracted sugar from potato peel waste by two different bacterial species and characterization of extracted PHA biopolymer. Thesis summarizes the mass balance study of PHA from the both the species of bacteria which emphasizes the circular economy of a sustainable integrated waste management. Agro-Industrial waste is a source of renewable energy which comes from agricultural and industrial residues such as peels, husk, etc. and Chemical composition shows, these waste biomasses contain significant amounts of sugars, proteins, fats, minerals and complex nutrients. From different agro-industrial waste potato peel waste was selected for biopolymer (PHA) production due generation in huge amount, high amount of starch and low lignin content and wheat straw was selected for enzyme production due to easy availability. According to the world bank report, annually 2.01 billion tons of waste is generated as only industrial waste and by 2050 it will increase by 3.40 billion tons. According to “Food and Agricultural Organization of the United Nations,” globally, annual potato peel waste production exceeds 40 million tons, resulting in substantial quantities of potato peel waste. Approximately 40% of which is generated as byproducts in the food processing industry, including products such as fries, chips, and other packaged food. Commonly potato peel waste is utilized for generating low-value animal fodder, compost, or raw substrate for biogas production, resulting in the squandering of valuable nutritional resources within it.

Potato peel waste possess antioxidant, antibacterial, and anti-inflammatory and further more properties. However, the fundamental principle of the current study remains the efficient disposal of waste with the economic viability of processing advancement. After generation waste biomass is generally disposed of by landfilling, dumping and burning without any proper treatment that causes harmful impacts on the environment including emission of greenhouse gases, accumulation of plastic in the ocean, pollution caused by toxic gases. That is why it is essential to build up a sustainable management strategy for them. Utilization of renewable resources for bioenergy production can be a promising step towards waste management which fulfills the concept of biorefinery.

PHAs also known as carbonosomes, are polymers synthesized by a variety of bacteria inside their cell in the form of inclusion bodies and utilized as energy source in adverse condition. PHA are of three types on the basis of carbon chain length that are short chain length (3-5), medium chain length (6-10) and long chain length (10-16). PHA possess variety of industrial and medical applications such as packaging material, drug delivery and dental implantation etc. Bioplastics are gaining attention due to sustainability, biodegradability, biocompatibility, and lower carbon footprint. Nevertheless, the commercialization of PHA is predominantly hindered by the elevated production expenses arising primarily from the use of a pure sugar substrate. Our study has established a feasible method for bioplastic formation applying *Pseudomonas putida* MTCC (2475) and *Bacillus circulans* MTCC (8167), and potato peel waste as a carbon source. To optimize the sugar yield response surface methodology was used, which released 69.34 ± 0.25% reducing sugar. PHA production experiments using *Pseudomonas putida* were performed in hydrolysate containing media as well as commercial sugar containing mineral salt media. After 48 hours of fermentation of using this sugar, a biomass concentration of 2.19 g/L^{-1}, with a PHA production of 0.60 g/L (28.71 ± 0.55%) was obtained which was comparatively similar with synthetic media (2.56 g/L cell dry weight and 29.97 ± 0.45% PHA). Furthermore, the monomers of PHA produced by hydrolysate were characterized using Gas chromatography-mass spectrometry, Fourier transform infrared spectroscopy, differential scanning calorimetry, and nuclear magnetic resonance. This investigation has identified three distinct

monomers of medium-chain PHAs, namely, methyl 3-Hydroxydodecanoate, 3-Hydroxytetradecanoate, and Hexadecenoic acid 3-Hydroxy methyl esters. However, *Bacillus circulans* MTCC 8167 deposited hexadecenoic acid 3-hydroxy, methyl esters, pentadecanoic acid 14 methyl esters, and tetra decanoic acid 12- methyl esters. Crotonic acid assay was used for quantification of PHA and it was found highest (0.232 ± 0.04 g/L) at 37 °C and 36 h of incubation. Hence this study concludes a sustainable production of bioplastics from potato peel waste.

List of Publications

- Kag, S., Kumar, P. & Kataria, R. Potato Peel Waste as an Economic Feedstock for PHA Production by *Bacillus circulans*. *Appl Biochem Biotechnol* (2023). https://doi.org/10.1007/s12010-023-04741-1

- Kag S, Kumar P and Kataria R (2024), Acid hydrolysis of Solanum tuberosum periderm for accumulation of polyhydroxyalkanoates in Pseudomonas putida MTCC 2475. Front. Bioeng Biotechnol. 12:1343540.https://doi: 10.3389/fbioe.2024.1343540

- Neha Kukreti, Sonika Kag, Pravir Kumar, Rashmi Kataria, Potential of waste stream in conversion into sustainable metabolites: An overview and update, Bioresource Technology Reports, Volume 22, 2023101502, ISSN 2589-014X, https://doi.org/10.1016/j.biteb.2023.101502.

- Kag, S., Kukreti, N., Ruhal, R., Mann, S., Sharma, J., Kataria, R. (2022). Recent Technologies for Lignocellulose Biomass Conversion to Bioenergy and Biochemicals. In: Nandabalan, Y.K., Garg, V.K., Labhsetwar, N.K., Singh, A. (eds) Zero Waste Biorefinery. Energy, Environment, and Sustainability. Springer, Singapore. https://doi.org/10.1007/978-981-16-8682-5_2

- Kag, S., Kukreti, N., Kumar, P., and Kataria, R (2024) In: Garg.VK., and Kataria, N(eds). Bioeconomy for sustainable bioenergy and biofuels generartion Bioeconomy for Sustainability. Springer Nature group.

- Kukreti, N., Kag, S., Ruhal, R., Kataria, R. (2022). A Sustainable Biorefinery Approach to Valorize Corn Waste to Valuable Chemicals. In: Nandabalan, Y.K., Garg, V.K., Labhsetwar, N.K., Singh, A. (eds) Zero Waste Biorefinery. Energy, Environment, and Sustainability. Springer, Singapore. https://doi.org/10.1007/978-981-16-8682-5_10

- N., Rani, A., Kaushik, S., Kardam, S., Kag, V. S., Raj, R., K. Ambasta, P., Kumar, Reimagining old drugs with new tricks: Mechanisms, strategies and notable success stories in drug repurposing for neurological diseases, Progress in Molecular Biology and Translational Science, Academic Press, 2024,ISSN 1877-1173,https://doi.org/10.1016/bs.pmbts.2024.03.029.

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Symbol or abbreviations	Description
PHA	Polyhydroxyalkanoate
L.B	Luria Bertani Media
PHB	Polyhydroxy Butyrate
SCL	Short Chain Length
MCL	Medium Chain Length
RSM	Response Surface Methodology
DSC	Differential Scanning Calorimetry
Sp.	Species
FTIR	Fourier Transform Infrared Spectroscopy
TGA	Thermogravimetric Analysis
GCMS	Gas Chromatography Mass Spectrometry
NMR	Nuclear Magnetic Resonance
SEM	Scanning Electron Microscopy
CCD	Central Composite Design
DCW	Dry Cell Weight
DNS	Di Nitro Salicylic Acid
O. D	Optical Density

HCl	Hydrochloric Acid
NaCl	Sodium Chloride
H_2SO_4	Sulfuric Acid
$CHCl_3$	Chloroform
$CDCl_3$	Denatured Chloroform
CH_3OH	Methanol
Kbr	Potassium Bromide
NaOH	Sodium Hydroxide
$CaCO_3$	Calcium Carbonate
SmF	Submerged Fermentation
SSF	Solid State Fermentation
mM	Millimeter
MTCC	Microbial Type Culture Collection
TRS	Total Reducing Sugar
Pseudomonas putida	Pseudomonas Putida
U	Unit
Alpha	A
micron	M

ml	Milli Litter
g	Gram
mg	Milligram
RPM	Rotation Per Minute
%	Percentage
nM	Nanometer
DW	Dry Weight
cm	Center Meter
Uv	Ultraviolet
Na_2SO_3	Sodium Sulphite

Chapter 1. Introduction

1.1 Outline of Thesis

1. Biomass Collection and Processing

2. Composition Analysis

3. Optimization of Acid Pretreatment

4. Enzyme Production

5. Enzymatic Saccharification of Biomass

6. PHA Production by *Pseudomonas Putida* Using Acid Hydrolyzed Biomass

7. PHA Production by *Bacillus Cerculans* Using Acid Hydrolyzed Biomass

8. Characterization Of PHA

9. Technoeconomic and Mass Balance Analysis

1.2 Agro-Industrial Waste, Generation and Its Composition

The total world population has reached approximately eight billion by the end of the year 2020, and it will be more than 10 billion by 2057 (Leong et al., 2021). Demand for energy is increasing globally due to the enhancement in population, and it is estimated that by the end of 2040, it will further increase by 28% of its current value. Petrochemical energy resources, including crude oil, coal, and natural gas, are the classical energy sources and need to be replaced by alternatives. There is sharp depleting, high emission of toxic gases, and global warming due to continuous use of traditional energy sources (Bhatia et al., 2018). Energy security is not only limited to conventional sources but now renewable sources could be included. The annual production of terrestrial biomass can generate about four times higher energy than total energy demand globally (Meenakshisundaram et al., 2021). This lignocellulosic biomass (LCB) is known as the most prominent and cheapest source for energy and renewable chemicals synthesis (Kag et al., 2022). LCB, such as energy crops, agriculture residues, and forest leftovers, are the most abundant, non-conventional, renewable, cheap, and sustainable feedstocks for bio-based energy and chemicals generation (Meenakshisundaram et al. 2021). (Figure 1) Generally, agricultural leftovers such as crop residues and straw are used as cattle feed and fertilizer

applications environmental pollution results from unutilized biomass dumping and burning in many countries. Agricultural-based industries generate huge amounts of residues every year (Unuofin et al., 2019). When these remnants are released to the environment without any proper disposal procedure that may lead to environmental pollution and cause harmful effects on human health (Simair et al., 2018). Agro-industrial waste provides an enormous potential to produce sustainable bio products and bioenergy. Nowadays an integrated bio refinery is converted into an encouraging solution with multiple outputs such as biofuels, bioactive compounds, biomaterials and biopolymers (Donohoe et al., 2011). It is estimated that approximately 140 billion tons of agricultural biomass generated per year in the world, and a large part is recognized as waste and not conflicting with the food availability, e.g., leaves, roots, stalks, bark, bagasse residue, straw, seeds, peels and wood. Using alternative approaches to avoid additional losses and generate several high value-added biochemical could minimize the bulk of non-renewable materials which are used today enough to reduce greenhouse gas releases (Kumar et al., 2023). Therefore, considering their available volume and practically low costs locally and globally, associated with rich function, structure and chemical heterogeneity, all agro-industrial waste should also be considered for their chemical and material potential, as well as a source of energy (Thakkar et al., 2021).

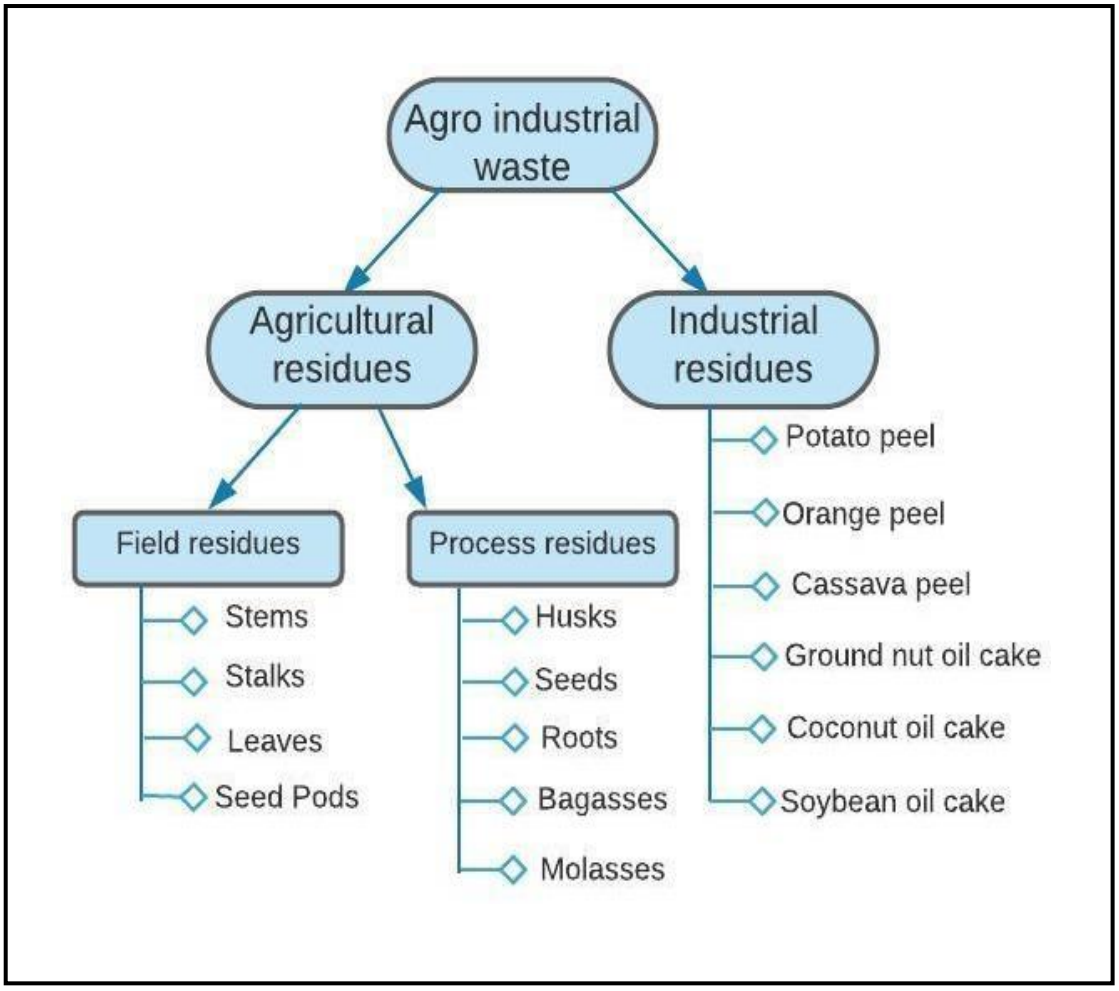


Figure 1 Types of Agro-industrial waste

1.3 Composition of Agro-Industrial Waste

Agro-industrial biomass is mainly composed of cellulose (homopolymer of hexoses), hemicellulose (heteropolymer of pentoses and hexoses), and lignin. It includes the whole plant body, such as roots, leaves, branches, stems, and rhizomes (Almeida et al., 2023; Empleton, 2010; Heerd et al., 2014). Agriculture, forest, industrial, animal, and municipal solid waste are some commonly known biomass types (Bazargan et al., 2020). (Figure 2) Approximately 80% of terrestrial plant biomass is forest plant-based biomass which is more attractive among researchers due to its complexity, diversity of habitats, and higher carbon present in dry weight (Bulkan et al., 2021). Since it is a heterogeneous organic matrix and universal availability is the reason for developing biomass-based biorefinery. Other than this, less emission of toxic greenhouse gases,

low cost, and easy processing make it as most prominent bioenergy alternative to classical energy resources (Mathew et al., 2016; Shen and Sun, 2021; Vasco-Correa et al., 2016). The selection of appropriate biomass sources, transportation, handling, storage, efficient pretreatment, and conversion technologies is challenging for biomass-derived bioenergy and biochemicals production (Bulkan et al., 2021). Terrestrial plant-based biomass (potato peel waste) is given more focus in this regarding composition, available pretreatment, conversion technologies, and bioproducts. Chemical composition can differ from plant species to species due to several factors like age, stress condition, and growth stage (Fatmawati et al., 2023; Ponce et al., 2021; Santos et al., 2014).

1.3.1 Cellulose

It is the most abundant natural polymer present on the earth's surface. Polymerization of 10,000 d-glucose units forms a linear, unbranched, and long-chain molecule. A β-1,4-glucoside linkage is present between two glucose monomers. Cellulose accounts for 40–50% of LCB composition (Kukreti et al., 2022). Cellulose is mainly distributed among the cell walls of plants and its crystalline (present in high amount) and amorphous nature (present in less amount). It gives structural support to the plant. Apart from plants, different microbes such as fungi, bacteria, and algae also produce a considerable amount of cellulose. Microfibrils are formed by assembling a few hundred of cellulose chains and covered by lignin and hemicellulose (Martín et al., 2019; S Lopes et al., 2020; X. M. Zhang et al., 2020).

1.3.2 Hemicellulose

In lignocellulosic biomass, hemicellulose contributes about 20–30% of total biomass. The branched heteropolysaccharide contains approximately 200–500 glucose units. The polymer consists of hexoses such as glucose, mannose, rhamnose galactose, and pentose sugars like arabinose xylose and uronic acids. In the backbone, two types of linkages, β-1,4 and β-1,3-glycosidic linkages are present (Pu et al., 2013). It is a heat-labile polymer, and the monomers recovery from plant biomass enhances the digestibility of cellulose. After thermochemical pretreatment, some types of byproducts are also generated, which lead to inhibit microbial fermentation during

biomass conversion to energy. Hydroxy-methylfurfural and furfural are known as potent inhibitors for microbial growth and eventually hinder the fermentation process if present in high amounts (Ortega et al., 2021).

1.3.3 Lignin

Aromatic phenolics are cross-linked together and forms lignin polymer. Monolignol molecules, P-coumaryl, sinapyl, and p-coumaryl, undergo oxidative polymerization and produce an end product known as lignin. Lignin provides structural and mechanical support to the plant (Vasco-Correa et al., 2016). Most significant part of lignin produces pollutants like aromatic and polycyclic aromatic hydrocarbons during burning. The presence of multi-functional groups like OH, OCH3, CO, and COR, seems to be an alternative substrate for producing renewable metabolites lignin is the one of the most abundant polymers in plant biomass, therefore gaining attention from researchers (Nasrullah et al., 2017). Due to its chemical constituents and water-insoluble nature, it acts as a strong barrier to biomass vaporization (Vasco-Correa et al., 2016).. Different pretreatment strategies including solubilization of lignin, pulping process, dilute acid hydrolysis are used to separate lignin from rest of the carbohydrate component (Greses et al., 2020). Table 1 indicates the percentage of cellulose, hemicellulose, and lignin in different lignocellulosic biomass.

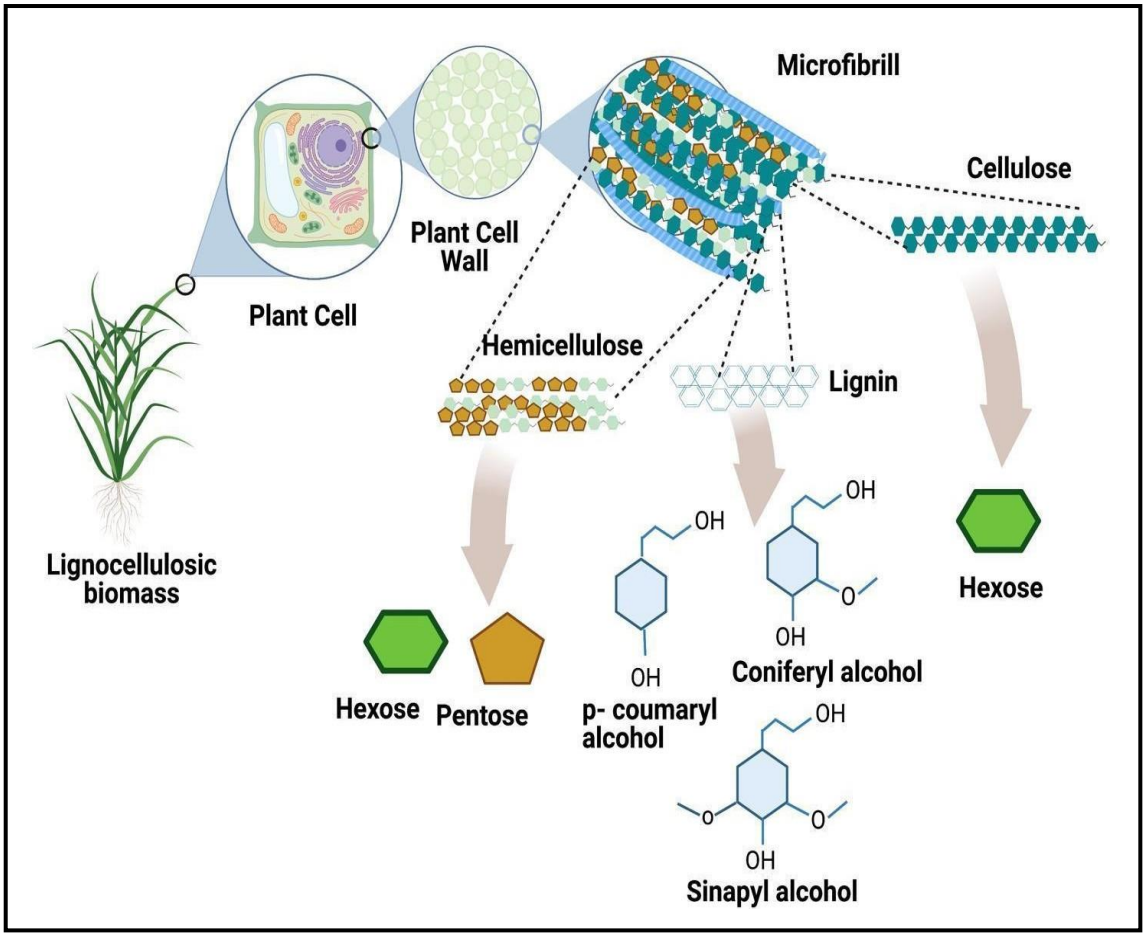


Figure 2 Components of lignocellulosic biomass

Chemical composition of agro-industrial waste is differed in type of waste. Agriculture residues majorly consist of cellulose, hemicellulose and lignin but their amount may vary (Ponce et al., 2021). Industrial leftovers differ in their constituents such as starch is major component in potato processing industry waste, amount of protein is high in meat processing industry, lipid is major part of dairy and refined oil industry. Due to reach in energy resources this waste can be transformed in to bioenergy (Mccarthy et al., 2020).

Table 1 Chemical Composition of Different Types of Agro-Industrial Waste

Name of biomass	Cellulose %	Hemicellulose %	Lignin%	Reference
Wheat straw	35.97	23.95	19.33	(Habets et al., 2013)
Corn cob	45.01	33.12	13.81	(Kumar et al., 2023)
Rice straw	36.1	27.0	13.7	(Millati et al., 2019)
Corn stalks	32.9	24.3	18	(Asgher et al., 2016)
Cotton stalks	39.85	ND	23.92	(L. Zhang et al., 2020)
Bamboo	37.6	30.4	23.6	(X. Gao et al., 2022)

1.4 Pretreatment / Hydrolysis of Agro-Industrial Waste

Agro-industrial waste composed of polymers of sugars and lignin. However, conversion of such substrates into energy rich products is often limited, due to their complex lignocellulosic structure (Becker et al., 2010). Cellulose, hemicellulose and lignin are the principal components of lignocellulosic biomass and among them, lignin is the most resistant to degradation, forming the barrier preventing access of the microbes to cellulose (Pu et al., 2013).

Apart from this cellulose crystallinity, biomass complexity, accessible surface area also behaves as an obstacle, which affect their degradability (Simair et al., 2018). he agro-industrial residues are rich in a variety of nutrients and require to be pretreated before their usage in the fermentation process. A lot of novel approaches in pretreatment of agro-industrial residues are used depending on their composition and type of waste produced. Each agro industrial biomass requires specific pretreatment strategies (Chundawat et al., 2020; Xu et al., 2020). A pretreatment strategy should be able to achieve an enhancement in the digestibility of the treated component l, while

minimizing pollution of environment, low energy requirements and limiting the production of inhibitors, such as organic acids, phenolic compounds and furan derivatives. From different pretreatment processes the chemical methods are considerably good and effective to depolymerize the complex lignocellulosic biomass (Mathew et al., 2016).

Physical Pretreatment Method of Lignocellulosic Biomass

1.4.1 Ball Milling

The physical pretreatment technique such as milling are known as effective method as there is no toxic product formation and hence washing and detoxification step is not required after this pretreatment (Y. M. Gu et al., 2018). Attrition type milling with chemical catalysts is a possible solution to maximize the conversion of feedstock (H. Gu et al., 2018). Milling of lignocellulosic feedstock increases the accessibility of cellulose for cellulase enzymes by reducing the particle size and enhancing the part of amorphous cellulose in biomass (Yanwen Wu et al., 2021). Loud noise during operation, and high energy conception and prolonged reaction time are major limitations of milling (Y. M. Gu et al., 2018; Yingji Wu et al., 2021). It was found that simultaneous physical and biological treatment such as Ball milling and enzymatic saccharification can enhance glucose conversion rate by 30% (Yanwen Wu et al., 2021). When corn stover was subjected to ball milling for 10–30 min and 80–100 °C, particle size reduction and disruption of the cell wall matrix of biomass was reported (H. Gu et al., 2018).

1.4.2 Microwave Irradiation

Microwave irradiation is one of the physical pretreatment methods for lignocellulose pretreatment (Vasco-Correa et al., 2016). This method has advantages like rapid heating, generates heat, uniform heating, and penetrates throughout the volume of the material. In ionic conditions, the sample oscillates towards the forward and backward direction of the dissolved charged particle due to an applied wave (Tsegaye et al., 2019). Microwaves are a substitute for traditional heating. It can rapidly generate intense direct heat and cause the change in the dipole moment of polar molecules (Li

et al., 2012). This physical pretreatment has sufficient heating capability and is simple to implement. It has proved that it changes the complex ultrastructure of cellulose molecules, depolymerizes lignin and hemicellulose fraction in biomass, and enhances the enzymatic hydrolysis of LCB (Lu et al., 2011). A high capital cost is required for this process, and moisture parameters should be regulated before pretreatment because it can affect the biomass structure (Dávila et al., 2019). Rice straw, switchgrass, and wheat straw are subjected to microwave pretreatment to enhance the bioethanol yield (Lu et al. 2011). When switchgrass was treated with microwave radiation and aqueous ammonia, the methane production was improved by 65% compared with untreated one (Li et al., 2012).

1.4.3 Ultrasonic Pretreatment

Ultrasonication pretreatment is based on the cavitation principle through the implementation of ultrasonic radiations. The cavitation generates the force that breaks the complex structure of lignocellulosic biomass and makes the easy extraction of cellulose, hemicellulose, and lignin (Q. Zhang et al., 2020). The Sonication pretreatment method has the advantage over other pretreatment methods. In this process a lower temperature is required to disrupt the cells, the extraction is faster, suitable for all cell types, no requirement of chemicals or beads, and a low production cost is observed. This method can compete with other procedures like dilute acid treatment as it has excellent performance for enzymatic hydrolysis using microalgal biomass (Alyousef et al., 2019). A study showed that the combination of different pretreatment methods (electrolysis and ultrasonication) is efficient for methane and organic matter production (Chauhan and Choudhury, 2020). It is was also reported a considerable delignification in wheat straw biomass when subjected to ultrasonic irradiation at the frequency of 40 kHz (Yan et al., 2021). However High energy requirement for operation is a significant drawback of this process (Yan et al., 2021).

1.4.4 Mechanical Extrusion

Extrusion is a thermo-mechanical type of pretreatment. It is a highly versatile and continuous process. It has good mixing and heat transfer capabilities. This process operates at mild temperature and requires lower chemicals and thus does not produce

inhibitory compounds which occur in severe conditions. This process works with the help of one or more screws, spins into a tight barrel, and temperature controlled. There are two different types of extrusion machines based on the extruders: single screw extruder and twin-screw extruder (Liu et al., 2020). There is an increase in accessibility for the enzyme after extrusion pretreatment. The extrusion processing helps in opening the woody material structures which enhance the access of cellulose to enzymes (H. Gu et al., 2018). Further study is required for the co-rotating twin-screw extrusion. Anaerobic digestion improved in five different samples of agricultural biomass with the help of extrusion. After 28 days, methane production increased by 18–70%, and after 90 days, methane production increased by 9 –28%. Extrusion plays a role in destroying slowly degrading compounds and otherwise non-degradable compounds (H. Gu et al., 2018). Due to extruders, energy yield and methane production increased in biogas plants (Yi et al., 2020). The high cost, this technique is still challenging for commercialization (Yang et al., 2021).

1.4.5 Acid Pretreatment

Different pretreatment methods are developed to enhance the bioconversion efficiency for enzymatic hydrolysis (Zhu et al., 2020). Two types of acid pretreatment are used in commercial biorefinery: concentrated acid at low temperature and dilute acid at high temperature. Out of them, the low acid concentration is a more promising method in lignocellulosic material due to its simplicity and cost-effectiveness (Nguyen et al., 2016; Shafiei Alavijeh et al., 2020). There is generation of various biproducts by like furfural, levulinic acid, hydroxymethylfurfural (HMF), and carboxylic acid during acid treatment. They possess an inhibitory effect on desired microbial growth and cell membrane functionality (Ortega et al., 2021). In the chemical pretreatment (acid/alkali) of lignocellulosic biomass, solubilization of generous amounts of fermentable sugars from the hemicellulose and cellulose part into a liquid phase of pretreated feedstock slurry occurs (Mathew et al., 2016). (Figure 3) Dilute hydrochloric acid and sulfuric acid are most commonly used for biomass pretreatment. Suitable neutralization methods are essential because pH, state, and chemical composition play a vital role in fermentation after e pretreatment (Li et al., 2017). Wheat straw was subjected to combination of phosphoric acid and hydrogen peroxide

with ratio of 79.6% and 1.9% at 40.2 ^{o}C for 2.9 h cellulose fractionation and lignin recovery reported (Wang et al., 2021).

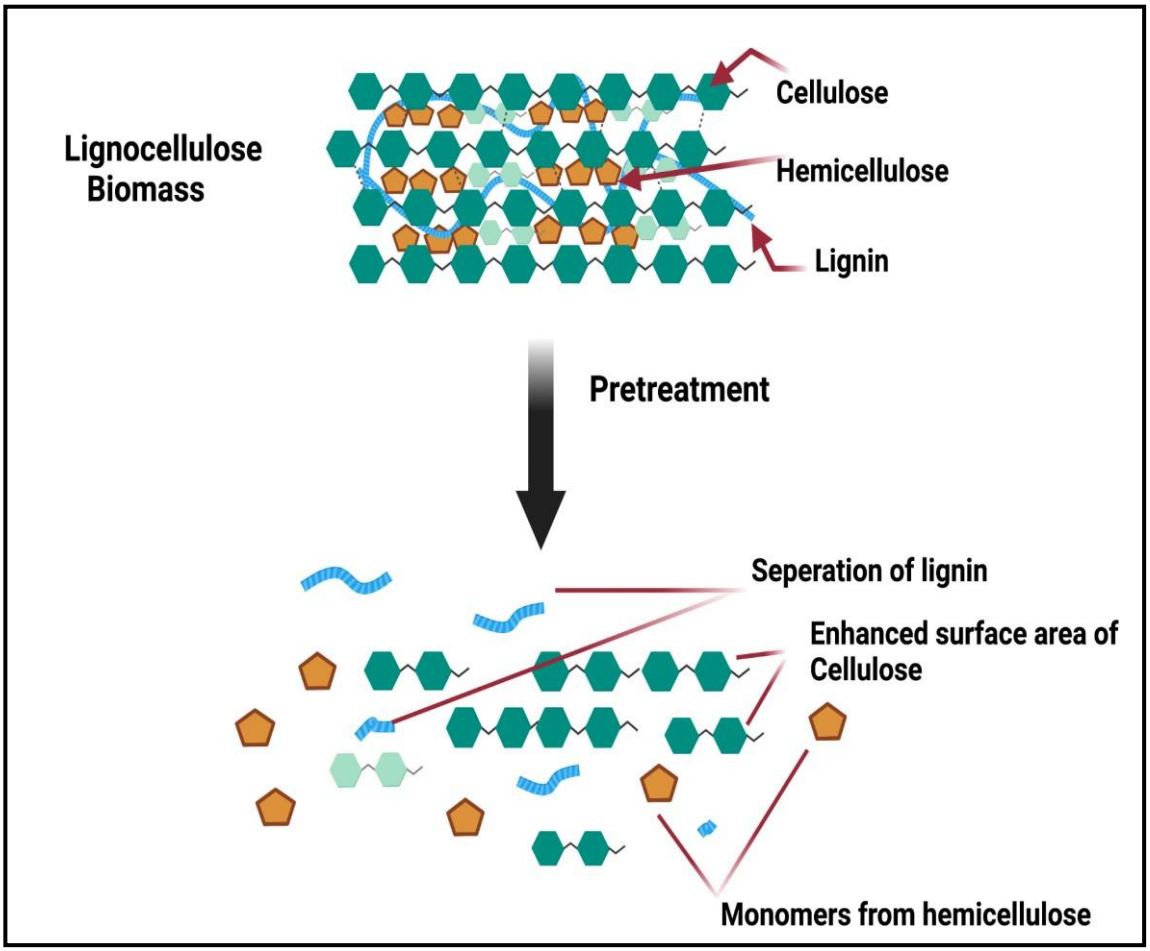


Figure 3 Effect of pretreatment on lignocellulosic biomass

1.4.6 Alkali Pretreatment

It is a lignin targeting pretreatment method performed in mild conditions (Yan et al., 2020). Various types of alkaline chemical, including sodium hydroxide, calcium hydroxide, sodium carbonate, etc., are used as active catalysts for the process of delignification (X. M. Zhang et al., 2020). The delignification process is positively correlated with the severity of treatment, that include alkali concentration and high temperature. High alkali strength leads to high reducing sugar yield after enzymatic hydrolysis. However, higher temperature conditions contribute to inhibitor generation, such as phenolics (Yan et al., 2020). Along with the removal of lignin, it maintains the

polymeric structure of cellulose. Sodium hydroxide pretreatment is one of the standard techniques used extensively in the bioconversion of recalcitrant lignocellulosic biomass. It is a very potent chemical that enhances agro-based residues and hardwood (Xu and Sun, 2016). This process is performed at comparatively low pressure and temperature or ambient conditions. When wheat straw was treated with 3% sodium hydroxide with a catalyst $CrCl_3$ at 120℃ for two hours, for delignification and it also yielded HMF significantly (Huy et al., 2021). The main limitation of this technique is time, as it can take several hours to days to complete the pretreatment process (Xu and Sun, 2016). Still, the use of concentrated alkali and disposal of waste alkaline liquid makes this classical pretreatment method to be challenging (Fan et al., 2012).

1.4.7 Organosolv Pretreatment

In the organosolv method, removal of hemicellulose and lignin is done by some organic liquids such as alcohols, phenols, ethers, etc. The mixture of organic liquids causes hydrolysis of the internal bonds between hemicellulose and lignin (Hesami et al., 2015). It helps to remove a considerable amount of lignin. Increased surface area and pore size make the enzymatic hydrolysis feasible and lignin, recovered by this process, is pure compared to other methods (Hesami et al., 2015). But high solvents requirement and washing step after pretreatment are the significant challenges of this technique (X. M. Zhang et al., 2020). Different pretreatment technologies were developed based on the type of catalyst used such as acid or alkali (Casper et al., 2021; Chen et al., 2015). It is reported an increased depolymerization of lignocellulose biomass at temperature of 180 °C/ 27.2 atm for 40 min with 50% ethanol and 1.7% sulfuric acid (Casper et al., 2021).

1.4.8 Ionic Liquid

Ionic liquids are a group of salts containing organic cations and inorganic anions. They are suitable solvents, non-volatile, chemically inert, and thermally stable, and possess industrial applications such as food, cosmetics, and pharmaceutic (X. M. Zhang et al., 2020). In the solubilization of lignocellulosic biomass towards lignin bridges, the negative ions play a crucial role. Anion makes a nucleophile attack on lignin, and β–O–4 linkage is targeted, which results in release of hydroxyl groups of phenolic

compounds at high temperatures (Nakasu et al., 2021). When lignocellulosic biomass is treated with ionic liquid, it produces amorphous cellulose and makes it accessible for enzymatic saccharification without formation of inhibitory products, however, ionic liquids are toxic in nature (Vasheghani Farahani et al., 2016). 1-butyl-3-methylimidazolium chloride ([Bmim] Cl) is used with combination of 0.75 g NaOH and H2O2 as catalyst for 120 min at 110 °C and reported considerable amount of lignin removal without significant loss of carbohydrate (Pang et al., 2021).

1.4.9 Ozonolysis

Ozonation is an effective technique for lignin degradation and it is used to oxidize carbohydrates in biomass. The reaction rate in this process is slower in comparison to other methods (Tomás-Pejó et al., 2011). Ozone gas is highly reactive. Double bonds containing lignin moieties are prone to oxidize in the process of ozonolysis (Tomás-Pejó et al., 2011).Variety of biomass such as crop straws, energy grasses, and bagasse are subjected to ozonolysis pretreatment due to considerable delignification, high reducing sugar yield, eco-friendly, and low operational charge (Li et al., 2021). When rice straw was subjected to ozonolysis (0.006 gO3/g RS) at 55 ℃ for 4 days, the crystallinity of cellulose reduced and significant amount of delignification reported (Ortega et al., 2021).

1.4.10 Liquid Hot Water Pretreatment

Above the boiling point, water is known as subcritical water or liquid hot water. At high temperatures and pressure, its physical and chemical properties change; due to this, it shows improved solvation properties (Yang et al., 2020). Liquid hot water pretreatment, simply known as hydrothermal pretreatment, water is used as a heating medium at a very high temperature (130–240 °C), and high pressure is also maintained without addition of any chemical agent (X. M. Zhang et al., 2020). At high temperatures, hemicellulose containing acetyl is converted into acetic acid, acts as a catalyst, and causes autohydrolysis (Yang et al., 2020). Depolymerization of complex cell walls occurs, and cellulose gets hydrolyzed to monomers. In the liquid hot water method, liquid state water is responsible for excellent solubilization efficiency compared to steam pretreatment (X. M. Zhang et al., 2020). It is a promising green

technology for sustainable biorefineries. It has been used in various feedstocks, including agro-industrial-based products, but high temperature and low yield are drawbacks of this technique (Soh et al., 2023). Wheat straw treatment with hot water at 80–95 °C for 50 min, leads to release 41.0–53.0% extractives from biomass (Ponce et al., 2021).

1.4.11 Steam Explosion

It is a physicochemical treatment in which water and crude biomass is used, cellulose and hemicellulose of biomass converted into monomer units that make cellulase more effective for enzymatic hydrolysis (Nasir et al., 2020). For the degradation of recalcitrance biomass, it is a promising alternative technique to less energy consumption and no addition of chemicals (Pereira et al., 2021). In this technique, a reactor is maintained at the optimum time, temperature, and pressure that holds the biomass for a fixed period. Sudden depressurization results in explosive decompression (Pereira Marques et al. 2021). Due to mechanical and chemical effects, the surface area of biomass increases, and depolymerization of cellulose and hemicellulose occurs due to the breaking of carbohydrate-lignin bonds. This technique is used to enhance enzymatic saccharification, second generation biofuels production and bioactive compounds extraction. This technique is eco-friendly and economical but less effective on lignin removal (Nasir et al., 2020). In a study of palm fibers and sugarcane bagasse subjected to steam explosion at 168 °C for 10 min lignin removal without structural change in biomass reported (Nasir et al. 2020).

1.4.12 Biological Pretreatment of Agro-Industrial Waste

Depolymerization of the complex lignocellulosic biomass (lignin and hemicellulose), with the help of specific microorganisms, known as biological pretreatment. Depolymerase enzymes such as lignin peroxidase, manganese peroxidase, and laccase are some potent lignin-degrading biocatalysts (Ummalyma et al., 2019). Biological pretreatment has attracted researchers as an advanced strategy due to the minimum generation of inhibitory compounds and environmentally friendly as no chemical or physical treatment is given (Akyol et al., 2019). Microbes that depolymerize the lignin can be bacteria or fungi. Still, due to less nitrogen requirement and ability to grow in

the presence of inhibitory toxic compounds, basidiomycetes (white rot) fungi gained interest (Vasco-Correa et al., 2016). Some fungal groups such as soft rot, white rot, and brown rot fungi are potential biological pretreatment agents due to their wood decaying efficiency of lignocellulosic biomass (Akyol et al., 2019). The presence of peroxidases and laccase, a particular dignifying enzyme in rot white-rot fungi including *Trametes Versicolor*, *Phanerochaete chrysosporium*, *Trichoderma reesei*, etc. make the biological pretreatment process feasible (Akyol et al., 2019). Peroxidases and laccase enzymes are a class of oxidoreductases secreted by microbes that degrade lignin by oxidation either directly or by mediators (Vasco-Correa et al. 2016). Single enzymes or cocktails of enzymes can be used for the delignification process. PH 3–8 is optimal pH, and 25–80 °C temperature is suitable for the procedure. Saccharification can be combined with physical and chemical methods (Moreno et al., 2019). High enzyme cost and long reaction time is a considerable obstacle to biological pretreatment in industrial scale. To overcome this problem, further research is required to develop an effective biological pretreatment (Moreno et al. 2019). In another study, laccase, β-glucosidase used as efficient conversion of perennial lignocellulosic biomass into bioethanol (Pu et al., 2013). When wheat straw treated with Phanerochaete chrysosporium at temperature 22 ℃ for 35 days with 70% moisture content, it was found that fibers of biomass became loose (Vasco-Correa et al., 2016).

1.5 Other Special Pretreatment Techniques

Hydrothermal Carbonization (HTC) It is a powerful thermochemical technology in which high moisture-containing biomass transforms into solid biofuels such as hydro-char at 180–250 °C. This treatment densifies the energy present in biomass (Soh et al., 2023). It is the possible alternative to another thermochemical technique, pyrolysis. In pyrolysis, the produced char has high potassium, sodium content and high heating value (Dávila et al., 2019; X. M. Zhang et al., 2020). Hydrothermal carbonization overcomes the heating value problem. Apart from this, it enhances the carbon percentage and decreases the ash content in feedstock, moisture-containing samples can be taken, low energy is required (Cheng et al., 2020). In the HTC technique, the breakdown of water occurs into hydronium and hydroxide ions. They are critical factors of hydrolysis of organic substrates. Several acids such as nitric acid/sulfuric

acid or hydrochloric acid can be used to lower down reaction time and temperature and help in the modification of hydro-char by adding functional groups to it (Cheng et al., 2020). Herbal tea waste is treated at 20–300 °C temperature with holding time at 5 °C/min, a structure change of biomass is reported (Soh et al., 2023).

1.5.1 Supercritical Fluid (SF)

Any material above the pressure and temperature (critical condition) expresses the property of liquid and gas, for instance, density and compressibility, known as a supercritical fluid. Most often used in SF is carbon dioxide, which is non-toxic, is economical, eco-friendly, with a critical temperature is 31.1 °C, and necessary pressure is 7.36 MPa (Li et al., 2021). This technique has been reported as an efficient pretreatment method to depolymerize the lignocellulosic composition of vegetal sources. Cellulose containing biomass is maintained in a reactor where carbon dioxide is pressurized at 35 °C, followed by a depressurization mechanism, giving rise to disruption of the cellulose (Millati et al., 2019). The surface area of lignocellulosic biomass is increased by this process, and biomass can be accessible to the enzymatic saccharification and enhance the sugar yield. This possible alternative technique is feasible as compared to the special techniques such as ammonia and steam explosion due to low operating temperature, and glucose does not degrade by the process (Millati et al., 2019).When yellow pine sawdust was subjected to super-critical CO_2 at 3100–4000 psi/112–165 °C for 60 min about 80% of cellulose hydrolyzed to monomers (Kang et al., 2004).

1.5.2 Ammonia Fiber Explosion

(AFEX) This is a thermochemical process, causes solubilization of lignin, depolymerization of cellulose into monomer reducing sugar, and hydrolysis of hemicellulose. During this process surface area of LCBs increase and promote the transformation of cellulose and hemicellulose to reducing sugar for fermentation (Chundawat et al., 2020). The process of ammonia fiber explosion works by rapid decompression with a combination of alkaline pretreatment that is similar to steam explosion catalyzed by sulfur dioxide. In this process, the feedstock is treated with liquid ammonia at a temperature (90 to 100 °C) and high pressure for 5 min and then

pressure is rapidly released (Donohoe et al., 2011). The ammonia used in this process can be recycled after the pretreatment is over. Coastal grass pretreatment with AFEX at 100 °C for 30 min cause relocalization of lignin. This process has been applied on corn stover, switchgrass, and rice straw but the high ammonia requirement and its recovery cost make the process inefficient for pilot scale use (Donohoe et al., 2011).

1.5.3 Enzymatic Hydrolysis

Hydrolysis of pretreated biomass for the production of fermentable sugars with a cellulase enzyme complex, known as enzymatic hydrolysis. Major steps involved in this process are: (a) transfer of biocatalyst, (b) binding of the enzyme with substrate, (c) cellulose hydrolysis (d) cellobiose hydrolysis in the form of glucose (Fanyin et al., 2023). Bioconversion of LCBs requires physicochemical pretreatment and enzymatic saccharification to transform polysaccharides into their monomer constituents (Jung et al., 2013; Soni et al., 2023a). Biological pretreatment or simply enzyme-mediated hydrolysis is carried out with the help of biological agents and complex biomass depolymerize into a range of monomer sugars namely glucose, mannose, galactose, xylose, etc. Carbohydrate-lignin bonds also break due to an effective pretreatment strategy (Jung et al., 2013; Soni et al., 2023a). In the first step of saccharification, cellulase and hemi-cellulase (potent enzymes for hydrolysis), depolymerize the cellulose and hemicellulose to sugar monomer. Cellulase, responsible for hydrolyzing cellulose, is of three types (a) exoglycanase (b) endoglucanase, and (c) beta-glucosidase. Xylans, glucomannan, and arabinoxylan are some commonly known hemi- cellulases (Julia et al., 2016). In a study seaweed is used as a biomass for enzymatic hydrolysis (19 AU cellulase, temperature. 40–50 °C for 12 h), reducing sugar increased up-to 7.937 mg/mL for bioethanol production (Julia et al., 2016).

1.5.4 Fermentation

In fermentation, substrates get converted into the valuable product by the enzymatic activity of microorganisms including bacteria yeast, and fungi (S Lopes et al., 2020). Bioprocessing is the potent alternative for the conversion of biomass to valuables. LCB generated from various industrial operations attract the formation of a variety of metabolites through the process of fermentation (Barrera et al., 2013). Food industry

biomass is chemically composed of a considerable amount of carbohydrates, proteins, and other micronutrients, which support the growth of desired microorganisms. The fermentation process is of two types: submerged fermentation and the second is solid-state fermentation (Barrera et al., 2013). Industrial production of enzymes can be done by solid-state fermentation of agro-industrial biomass. As improper disposal of agro-industrial biomass causes environmental issues, so the use of this waste as a substrate for microbial fermentation can be a possible alternative to high-cost feedstocks (Ntone et al., 2022). It has advantages over submerged due to the minimum requirement of pretreatment technique and less generation of wastewater after the process (Ntone et al., 2022). In submerged fermentation, the added nutrients and supplied oxygen can easily dissolve in liquid media and mix evenly all over in the bioreactor, and because of it, biomass mixing and heat transfer takes place evenly (Adhikari et al., 2018). Wheat straw was used for lactic acid production, after pretreatment biomass was subjected to fermentation with starter culture of Lactobacillus spp., at 25 °C for 120 h, lactic acid production reported.

1.5.5 Pyrolysis

It is a thermochemical conversion method of biomass. When biomass is treated at high temperature (300 –600 °C), and with a suitable atmospheric pressure in absence of oxygen, the biomass can be converted into three phases solid (char), liquid (bio-oil), and gaseous (syngas), the process known as pyrolysis (Javed et al., 2019). It has some advantages over other thermochemical methods that is pyrolysis plants can be established in remote locations so that transportation costs can be reduced. The complexity of this process is the biggest challenge of this technique (Cortazar et al., 2023; Osman et al., 2019). Many value-added products such as methane, biochar, paraffin ware produced from lignocellulosic biomass (Javed 2020). In one study wood residues for cadmium removal by pyrolysis performed at operating conditions 600 °C for 1 h using chemical such as NaOH and KOH (Gollakota et al., 2018; Habets et al., 2013).

1.5.6 Gasification

Gasification is a thermochemical process, where syngas commonly known as producer gas formed by the reaction of gasification of fuel. Few hydrocarbons such as methane, ethane, etc. are the main components of syngas (Kumar et al., 2023). It is a type of combustion. Heat, light, and chemical pollutants are generated as the result of combustion. During the conversion low-grade substrates such as coal, biomass into higher-grade fuel like methane, known as gasification. In this conversion technique, for energy generation, organic waste is processed at a very high temperature. LCB gasification can be achieved with oxygen, steam, carbon dioxide, and supercritical water (Kumar et al., 2023). The gasification takes place inside a closed chamber known as a gasifier. A variety of gasifiers such as fluidized bed, fixed beds and entrained flow gasifiers are often used for this process (Lee et al., 2019). Different techniques have been applied for lignin-based biorefinery and syngas is most commonly produced, it can be used as for microbial bio alcohol production (Cortazar et al., 2023; Rodrigues et al., 2022). Corn stover subjected to gasification at 350 °C for 30 min evolution of phenolic compound reported from biomass (Cortazar et al., 2023).

1.5.7 Torrefaction

Torrefaction is a thermal approach that is performed at higher temperatures in a range of 200 and 300 °C in an inert environment, which alters the chemical property of lignocellulosic biomass (Nunes et al., 2018).This process requires the energy supply and leads to the improvement of energy density, improved ignition, lowers the moisture, increases the C/O and C/H ratio, and requires lower grind ability. Hence this increases the combustion for LCB. The terrified material could be used for electricity generation and co-firing with coal. The co-firing lowers the coal utilization which leads to reduced carbon dioxide in the environment. This process gives better storage of biomass and could be used for energy production (Ong et al., 2020).

1.5.8 Anaerobic Digestion

Anaerobic digestion is an effective approach to convert organic components such as lignocellulosic biomass to Bioenergy. It is a process in which microorganisms break

down organic material in the absence of oxygen (Z. Gao et al., 2022). A large amount of energy can be generated from the wastes by taking into consideration the process of simultaneous anaerobic digestion and co-digestion. Biogas is the product of the bacteria feeding on biodegradable waste and releases methane, carbon dioxide, hydrogen, nitrogen, and hydrogen sulfide. The methane component in the biogas can produce heat and electricity. The agricultural residue which is lignocellulosic material is used to get higher added value (Colombo et al., 2017; Yadav et al., 2020). In a study forest residue such as mixture of spruce, pine, bark treated at 190 °C temperature with 50% of organic solvent for 60 min and an increased methane yield up-to 0.34 m3 CH4/kg was observed (Pan et al., 2021).

1.5.9 Transesterification

Transesterification is a chemical process in which lipids are esterified with acetylating agents and catalysts for biofuel production. Amid different types of biomasses, algal-oil biomass is known to be a potential feedstock for biofuel production by transesterification reaction, due to their fast-growing ability and lower nutritional requirement for cultivation (Gollakota et al., 2018). Algal biomass is composed of 7–22% of lipid, 5–50% protein, and 5–22% of carbohydrate. Chemical composition shows that it can be a perfect feedstock for biochemicals production (Bhatia et al., 2018; Casper et al., 2021). Two types of catalysts are used in transesterification, (1) homogenous (acid/alkali) (2) heterogeneous (lipase with solvents). Though base catalyzes processes are faster as compared to acid-based reactions but not suitable for fatty acids, as yield of the acid-based process is high but the reaction rate is very slow (Grosmann et al., 2022). In heterogeneous catalysts the separation is easy and high-grade purity is obtained but the process is slow and cost-effective (Uthman and Abdulkareem, 2014). When subjected to transesterification with lipase at 40 °C for 12 h, fatty acid methyl esters yield up to 97% (Elkady et al., 2015).

1.5.10 Photocatalytic Conversion

Photocatalytic conversion of Biomass In biorefinery, the production of platform molecules, from biomass conversion is the major step. Thermochemical and biochemical operations are commonly used techniques but the high cost and

generation of toxic compounds are considerable disadvantages of these techniques (Chen et al., 2021). Light-driven redox reactions with the use of some homogeneous/heterogeneous photocatalyst, known as photocatalytic conversion. In the process of photocatalytic conversion, the transformation of cellulose using solid catalysts such as TiO_2 and NiS shows considerable cellulose hydrolysis into sugar monomers (Chen et al., 2021). Oxidation of water for generation of OH radicals which strike hexose to form some intermediate. Biochemicals production from glucose oxidation is a promising technique nowadays (Shu et al., 2018). The use of heterogeneous photocatalysts such as TiO_2 is gaining attention by researchers due to less toxicity and economic feasibility. In study organic pollutants were subjected to photocatalytic conversion with $ZnIn_2S_4$ and thioacetamide at 160 °C for 6 h, an evolution of hydrogen by light irradiation enhanced the separation of pollutants (Wang et al. 2019).

1.6 Objectives of The Study

1.Composition Analysis and Pretreatment of Biomass

2.Saccharification and Sugar Analysis

3. Fermentation and Product Recovery

1.7 Scope and Significance of the Study

The purpose of the study is to use agro industrial waste such as Potato peel waste and wheat straw as a potential source of feedstock to produce important metabolites such as PHA and enzyme. One significant advantage using agro-industrial biomass as a feedstock in biorefinery processes is its abundance. It serves as a cost-effective source for producing platform chemicals. Successful utilization of these materials can help protect our delicate environment from degradation, promote energy diversity, and generate numerous rural employment opportunities. Variety of valuable chemicals can be produced from agro industrial waste such as food, feed, biochemical, bioenergy etc. The research covered optimization of thermochemical pretreatments and fermentation processes and resulting metabolites such as PHA and enzyme can serve as an alternative substrate to petrochemical derived nonrenewable chemicals such as plastic.

1.8 Literature Review

Over the last half-century, the expansion of agricultural, livestock farming, and industrial operations has led to a significant rise in the quantity of waste generated and accumulated. This surge, fueled by the application of chemical fertilizers and pesticides in farming, has disrupted the symbiotic connection between livestock and agricultural sectors. Consequently, the disposal of organic waste into the soil has emerged as a prominent issue contributing to environmental instability. Agro-industrial wastes are majorly made-up of complex biomolecules such as carbohydrates, protein and other constituents, etc. To address the current environmental challenges, researchers have been using agricultural and industrial wastes and their byproducts through recycling and clean technology by waste utilization. Till now a lot of valuable chemicals are produced from agro-industrial waste. Nazimuddin et al., 2021 extracted lignin from banana agro-waste. Lignin is recovered from the spent liquor produced during alkaline pre-treatment of agro-waste and precipitated by acidification and can be utilized to produce bioenergy. Bioethanol is a promising alternative resource to fossil-based fuels with increasing environmental awareness. Zhang et al., 2021 used combined technology where they used metal salts pretreatment to depolymerized the complex structure of cassava for bioethanol production using direct saccharification. Lactic acid has versatile applications in the food, pharmaceutical, and cosmetic industry. Chen et al., 2020 used waste lignocellulosic biomass (cassava bagasse) for lactic acid production by mixed culture of *Bacillus coagulans* and *Lactobacillus rhamnosus* (Fei et al., 2020).

Biodiesel is a potential alternative fuel for diesel engines. It is biodegradable, non-toxic, and reduces pollutant emissions, and matches the specifications of the European (EN) and American (ASTM) standards. Nano-sized sugar beet agro-industrial waste is used to make biodiesel. (Hvidsten and Marchetti, 2023). Apart from this lignocellulolytic enzymes and antioxidant compounds (Santos et al., 2014), carotenoid (Nanou et al., 2017), protease and lipase (S Lopes et al., 2020), xylose (Chen et al., 2021), pectin (Halambek et al., 2020), bacterial cellulose (Abdelraof et al., 2019) are produced from agro-industrial waste.

Potato peel biomass contains 40-80% sugar/ gram of dry weight in the form of starch, cellulose, and hemicellulose. According to the Food and Agricultural Organization of the United Nations, over 40 million tons of potato peel waste are produced globally each year. About 40% of this waste is generated as byproducts in the food processing industry, from products like fries, chips, and other packaged foods. Typically, potato peel waste is used for low-value purposes such as animal fodder, compost, or as a raw material for biogas production, leading to the underutilization of its valuable nutritional resources. Potato peel waste contains antioxidant, antibacterial, and anti-inflammatory properties. The current study focuses on the efficient disposal of this waste while ensuring the economic feasibility of processing advancements.

Worldwide production of Potato peel waste is consistently on the rise, reaching approximately 390 million tons in the year 2021 and its industrial processing generates large volumes of nearly 78-195 million tons (20-50%) of raw material. (Remedios and Domingues, 2023a). (Malakar et al., 2020). reported 76% moisture and 8% ash in S. tuberosum periderm, (Arapoglou et al., 2010) reported starch content at 52.14%, (Lima et al., 2021) reported cellulose and hemicellulose content ranging from 10% to 30%, and (Samarin et al., 2012) reported a total phenolic content of 522 µg GAE/g dry weight. However, it's worth noting that the composition of *S. tuberosum* periderm can vary among different *Solanum tuberosum* species (Sampaio et al., 2020).

Figure 4 Raw Biomass of Potato Peel Waste

It has been explored to produce various renewable metabolites, including bioethanol (Rodríguez-Martínez et al., 2023), biobutanol (Abedini et al., 2020), lactic acid (Liang et al., 2014), and bacterial cellulose (Abdelraof et al., 2019). Additionally, research has demonstrated the potential for bioplastic production from potato peel starch, both through chemical methods (BEZİRHAN ARIKAN and BİLGEN, 2019), and through microbial routes, such as SCL polymer production using *Bacillus megaterium* (Vu et al., 2021), and mcl PHA production using various strains of *Pseudomonas* (Mahato et al., 2021; Pan et al., 2021; Tanikkul et al., 2020a).

1.9 Value Added Products from Agro-Industrial Waste

Variety of value-added products such as bioethanol, biodiesel, bioactive compounds, enzyme, polymers can be produced from agro-industrial waste. PHA production is the main focus of this study.

1.9.1 PHA

The PHA are polyesters, produced intracellularly in their cytoplasm by variety of microbes (more than 150 genera of bacteria) in adverse condition such as nutrition deficiency, high temperature, UV radiation and high salinity conditions. And used as

a reserve food material in starvation condition.

1.9.2 History of PHA

PHAs are accumulated in the form of cytoplasmic granules inside bacterial and archaeal cells. Beijerinck seen the first microscopic view of PHA inside the bacteria in the year 1888 with the help of microscope. After few decades Lemoigne has explained the composition of PHA. The soil bacterium Bacillus megaterium was first seen to degrade the PHA in to its monomers in anaerobic condition. (Lithner et al., 2011). The 3HB was considered as intracellular reserve food material in the form of homopolymer and heteropolymer (Motavaf and Savage, 2021). At the start with 1951, the occurrence of PHA was confirmed in variety of bacterial species. After some time, the chemical properties of PHA including melting point, glass transition temperature, and crystallinity index were seen and industrial production of first PHA was done in 1970.

1.9.3 Characteristics of PHA

Polyhydroxyalkanoates (PHAs) are polyesters deposited by more than 250 different gram-negative and gram-positive bacterial species (Kacanski et al., 2023). Few genetically engineered plants species and recombinant yeast also reported for PHA production (Saranya et al., 2012). PHAs are accumulated in the form of lipid granules. The diameter of granules is 0.2–0.8 mm and about 2 mm thick protein and lipid membrane surrounds it. The inclusion bodies can take up to 90 % of the cell dry weight. (Motavaf and Savage, 2021; Zhou et al., 2022) The PHA deposition can visualized by phase contrast microscope and also by staining with Sudan Black B and Nile Blue A (Shah, 2012). The inclusion granules serve as reserve food materials in nutrition starvation condition (Porras et al., 2014). The biopolymer is water insoluble water and optically (Shah, 2012). The molecular weight of PHA may vary from 10,000 to 2,000,000 Da depending on the species of bacteria, the type of carbon source and the stress condition (Motavaf and Savage, 2021; Zhou et al., 2022) Monomeric unit can differ a in the form of saturated, unsaturated, straight or branched side-chains. The side-chains are usually aliphatic.

1.9.4 Types of PHA

On the basis of carbon chain length PHA are two major types such as short chain length (Scl) PHA contains 3-5 carbons, such as 3 Hydroxy butyrate, 3 hydroxy valerate and medium chain length (mcl) PHA contains 6-14 carbos such as poly (3 hydroxy hexadecanote), poly 3-(hydroxy heptadecanoate), poly 3- (hydroxy heptadecanoate) etc. and long chain length PHA contains more than 15 carbon in their chain. On the basis of polymer type (blending) it can be homopolymer and heteropolymer (Kacanski et al., 2023). Mcl-PHA are more thermoplastic, less crystalline, lower melting point and industrially important (Zhou et al., 2022).

1.9.5 PHA Synthesis Inside Bacterial Cell

PHA producing bacteria can be categorized into two class based on the composition of the culture media. In the first class, the bacteria accumulate PHA only in starvation of nutrient and synthesis of PHA occurs at late stationary where the bacteria the excess carbon source and start the polymer accumulation (Chanasit et al., 2016). The levels of essential nutrients can, of course, be monitored and optimized so the polymer synthesis starts only when sufficient amount of biomass is reached (Zhou et al., 2022). This group contains strains such as *Cupriavidus necator, Protomonas extorquens,* and *Pseudomonas oleovorans*. The second group does not require nutrient limitation and can also accumulate polymer during growth. These characteristics can be very beneficial for high polymer yields because there is no waiting time for the bacteria to reach accumulation phase.

1.9.6 PHA Metabolic Pathway

Variety of enzymes are involved in PHA synthesis and their degradation. Acetyl co enzyme A acetyl transferase (encoded by PHA A gene), acetyl Co A reductase (encoded by PHA B gene) and transacetylase (encoded by PHA C) are major enzymes, associated with Scl PHA biosynthesis inside bacterial species (Cerrone et al., 2023). PHA A, B and C gene organized in an operon known as class I operon. By the overexpression of thesis genes, PHA production can be enhanced. Mcl PHA synthesis occurs when bacteria enriched with fatty acid as a carbon source. However, synthesis of mcl PHA when sugar or glycerol used as a carbon source by de novo fatty acid

synthesis pathway. In the process malonyl - CoA is synthesized from the acetyl CoA by the action of enzyme acetyl CoA carboxylase. In the second step acetyl CoA and malonyl-CoA is combined form acetyl-ACP and malonyl-ACP (Cerrone et al., 2023). The reduction of Acetoacetyl-ACP to 3-hydroxybutyryl-ACP takes place. The second reduction takes place where krotonyl-ACP is reduced with NADPH to butyryl-ACP (Cerrone et al., 2023). Now the process repeats itself where butyryl-ACP condenses with the next malonyl-CoA creating acyl ACP with two more carbon atoms. The cycle can go up to C16 each time adding 2 carbon atoms. - 21 - To direct PHA accumulation from de novo fatty acid synthesis, PhaG uses (R)-3-hydroxyacylACP as a substrate turning it into (R)-3-hydroxyacyl-CoA (Sharma et al., 2012). Unlike in the first case with β-oxidation, this type of biosynthesis is considered to be non-substrate-related. However, both pathways share similarities because it is never 100% clear what kinds of monomeric units will be incorporated into the polymeric structure (Pereira et al., 2021). Another regulator protein known as phasin, a product of R gene, involve in elongation process. PHA Z responsible to code an enzyme PHA depolymerase. That is responsible for PHA degradation (Pereira et al., 2021) Figure 5.

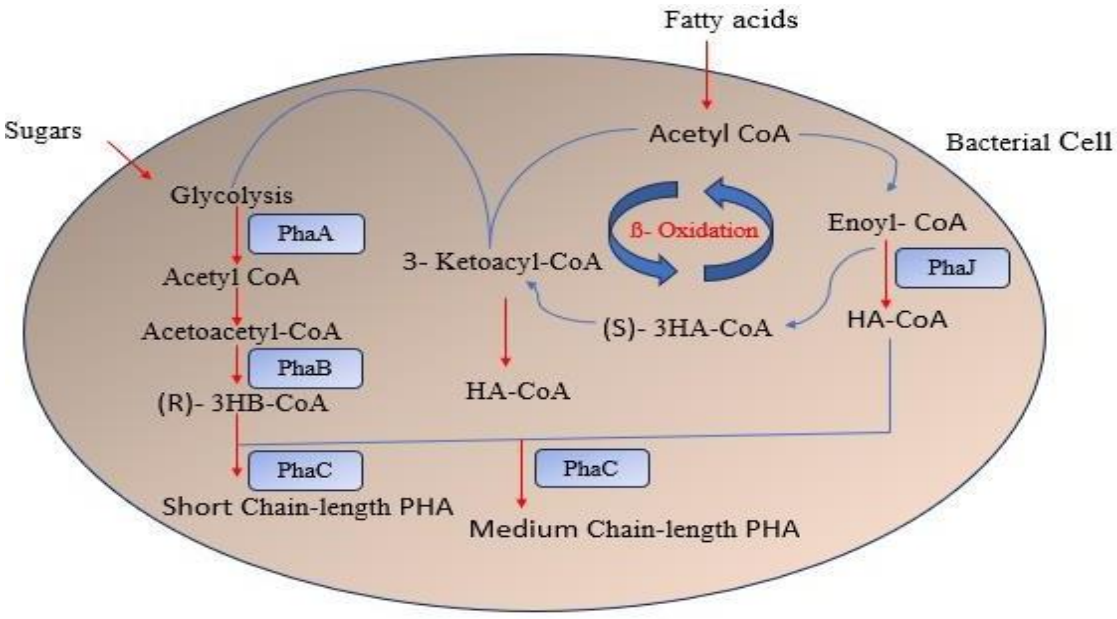


Figure 5 Metabolic Pathway of PHA Production Inside Microbial Cell

1.9.7 Criteria of Enhancement of PHA Production

For the high PHA, following factors are taken into consideration such fermentation strategy can be optimized whether fed-batch and continuous fermentation need be employed. Moreover, selection of potent PHA producing microorganism is also crucial step. Optimization of carbon, nitrogen and stress and section of inexpensive carbon sources are some major parameters that can be employed for PHA production (Kacanski et al., 2023).

1.9.8 Bacterial Species for PHA Production

More than 200 genera of Gram-positive and Gram-negative bacteria synthesize PHA in adverse condition but excess of carbon source. Some examples of PHA producing bacteria are given in Table 2

Table 2 Different Species of PHA Producing Bacteria and Their Accumulation Efficiency

S.no	PHA producing bacteria	Carbon source used	Amount	Reference
1	*Pseudomonas aeruginosa*	Palm oil	0.65g/L	(Tanikkul et al., 2020a).
2	*B. megaterium strain Ti3*	Lignocellulosic agro-wastes	-	(Israni & Shivakumar 2020).
3	*Pseudomonas putida*	Municipal solid waste	6g/L	(de Vrije et al., 2023)
4	*Salinivibrio sp. M318*	Aquaculture residues	51.7 wt%	(Thuoc et al., 2019)
5	*Burkholderia sacchari*	Paper waste	44%	(Battashi et al., 2022)
6	*P. aminovorans*	Agricultural wastes	55.4%	(Zhou et al., 2023).
7	Mixed Microbial culture	Sewage waste	92%	(Isern-Cazorla et al., 2023)

1.9.9 Applications of PHA

(a) Bioplastic

PHAs are biodegradable and sustainable bioplastic which reduce the dependency on the fossil based nonrenewable plastic. The application of PHA is based on its carbon chain length (polymer type). Scl PHA are more crystalline and amorphous in nature with low tensile strength which can be used to make disposable plastic items such as bags, straw, utensils etc. However, the medium chain length PHA are less crystalline and good tensile strength possess variety of industrial applications (Cooper et al., 2007; Czech et al., 2019). (Figure 4)

(b) Medical Applications

Many studies reveled that medium PHAs are biocompatible and nontoxic and can be used as a purpose of drug delivery, tissue engineering and skin regeneration purpose. Due to soft nature, it can be applied in heart valve. Due to water insoluble nature PHA can be used as a carrier for drug delivery and detection of cancer cells as PHB contains a specific target and help cancer cells to stick its surface while normal cells lack the ability to adhere at the surface of PHB (Hassan et al., 2016).

(c) Agricultural Application

Commercial plastic is utilized for mulching purpose in agriculture land but due to non-degradable nature is can deposited in soil and cause soil pollution. PHA is a good alternative of over commercial plastic it is suitable for soil structure, prevent contamination and increases soil fertility. PHA based growth bags are good for plant root as it not changes the root morphology and less toxic to plants (Kacanski et al., 2023).

(d) Fuel Additives

PHAs can be in biofuels as additives such as petroleum-based fuels, which provide a renewable and sustainable alternative to classical fuel additives (Hassan et al., 2016).

(e) Electronic Devices

PHAs are used in electronic devices and their components because of their electrical properties and biodegradability. PHA offers potential applications in flexible electronics and biodegradable sensors (Kacanski et al., 2023).

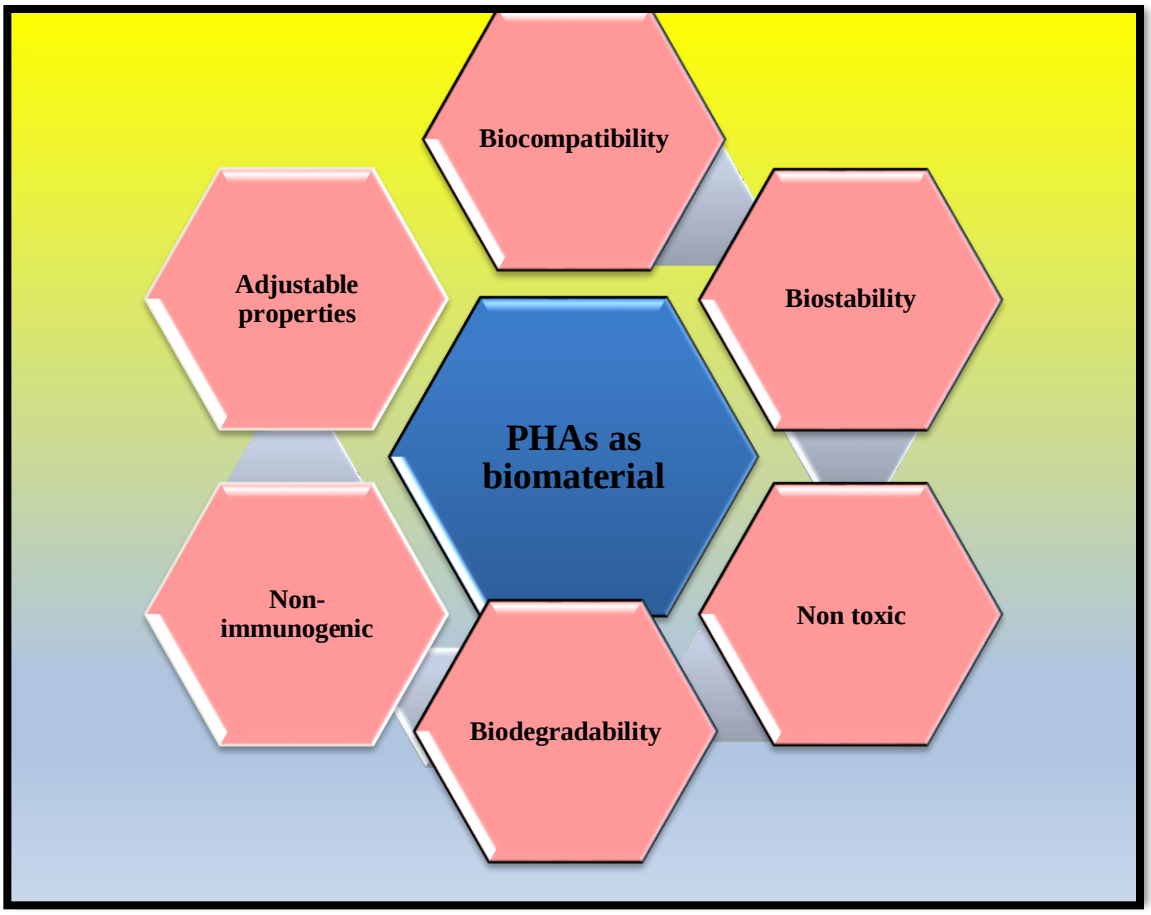


Figure 6 Properties of PHA

Chapter 2 Material and Methods

2.1 Selection of Waste

From different agro-industrial waste potato peel waste was selected for biopolymer (PHA,) production due generation in huge amount, high amount of starch and low lignin content and wheat straw was selected for enzyme production due to easy availability. (Figure 7a, 7b, 7c).

2.2 Sample Collection and Preparation

Potato peel was taken from the Delhi Technological University (DTU) canteen processing was done by washing followed by drying at 40℃ till constant weight. For particle size reduction physical pretreatment was performed.

Figure 7a Sample before drying

Figure 7b Sample after drying

Figure 7c Sample after grinding

2.3 Purchase of Chemicals and Glass wares

All the chemicals used in this study was taken from Thermo- fisher, sigma Aldrich, SRL, alfa Asar, and SD fine and glassware was purchased from Bororsil.

2.4 Different Instruments Used in the Analysis

instruments used in the analysis are: weighing balance (Sartorius Analytical Balance Model: BSA224S-CW), hot air oven (Matrix) centrifuge (laboratory grade microprocessor based), water bath (Khera instrument), spectrophotometer (GENESYS 50 UV- visible spectrophotometer Thermofisher), incubator shaker (Khera instruments) kjeldjal (Foss), GCMS (Shimadzu QP 2010), NMR (BRUKER Proton NMR 500MHz), FTIR (PEIR SUBTECH SPECTRUM ASCII PEDS 4.00).

2.5 Procurement of Microorganism

Microorganisms such as *Pseudomonas putida* (2475), *Bacillus circulans* (8167) and *Aspergillus niger* (10180) was purchased from Microbial Type Culture Collection Chandigarh, India

2.6 Maintenance of *Pseudomonas putida* (2475)

After procurement culture of p. putida was maintained on nutrient agar media containing beef extract 0.1g L^{-1}, yeast extract 2g L^{-1}, peptone 5g L^{-1}, NaCl 5g L^{-1} and agar 15 g L^{-1}. pH was 7. Culture was preserved in 50% glycerol stock at -20°C

2.7 Maintenance of *Bacillus circulans* (8167)

Bacteria *Bacillus circulans* MTCC 8167 purchased from microbial Type culture collection Chandigarh (India). And cultured on Luria Bertani media containing, yeast extract 5g/L, peptone 10g/L, NaCl 10g/L and agar 15g/L. Ph was adjusted to 7. Culture was preserved in 50% glycerol stock at -20 ℃

2.8 Maintenance of *Aspergillus niger* (10180)

Aspergillus niger was maintained on potato dextrose agar containing dextrose 20g/L, agar 15g/L and potato inclusion 200 g/L at 30 ℃

2.9 Chemical Composition of Potato Peel Waste

Dried powder of potato peel was subjected to composition analysis such as and Starch, Nitrogen, Protein, and Lignin, Total solid and Moisture analysis.

2.9.1 Total Solid and Moisture Analysis

Total solid and moisture analysis was done as suggested by (Amado et al., 2014). Briefly 1 gram biomass was kept in oven at 105°C till constant weight achieved. After 8 hours of incubation constant weight was achieved (Figure 8).

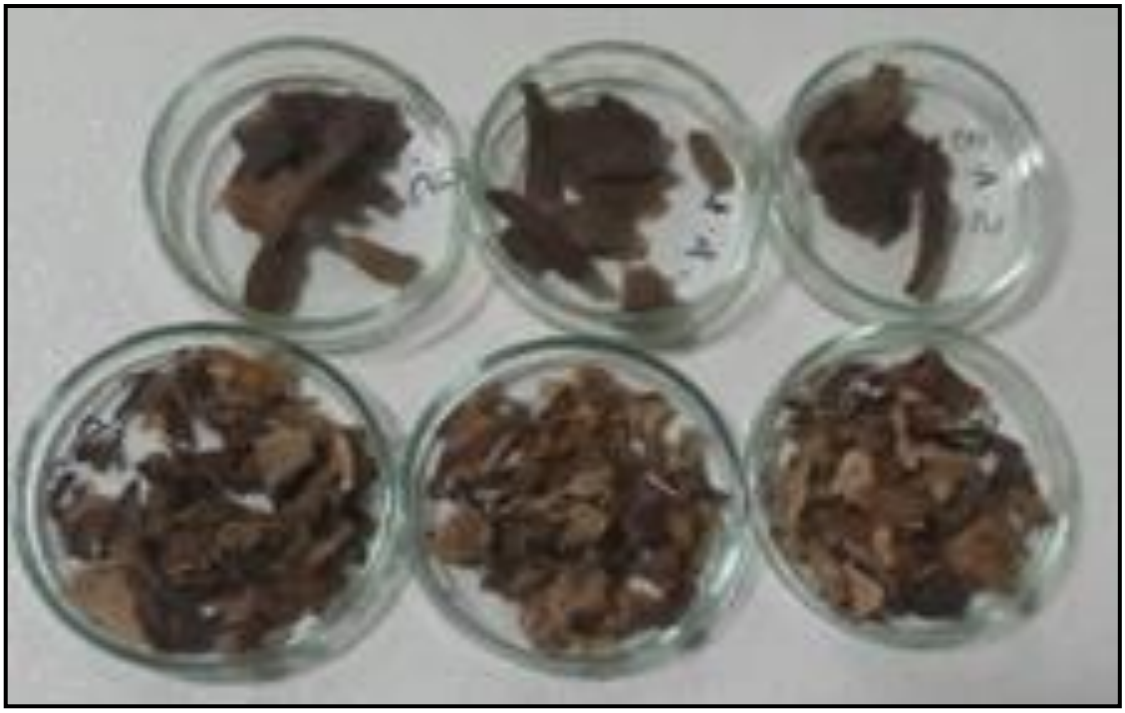


Figure 8 Total Solid and Moisture Analysis

2.9.2 Ash Content Analysis

Ash is inorganic residue, remains after combustion of any biomass at specific high temperature. Ash content was done using NREL (National Renewable Energy Laboratory) protocol. In which sample was placed in the muffle furnace at 575°C for a minimum of six hours. (Figure 9) After that crucibles were removed from the furnace directly into a desiccator and cooled for a specific amount of time, equal to the initial cool time of the crucibles. After weighing the crucibles and ash to the nearest 0.1 mg weight was recorded and analyzed gravimetrically by following formula:

$$ASH\% = weight\ of\ Ash \times 100 \div Weight\ of\ sample$$

Figure 9 Ash content in potato peel waste

2.9.3 Nitrogen and Protein Analysis

Nitrogen and protein content were estimated by method given by (kjeldahl 1883). The process was completed in three different steps: (Figure 10)

Digestion: in this step 1 gram sample (ppw), 7gm potassium sulphate, 0.8 gm cupric sulphate, 4 drop hydrogen peroxide and 12 mL sulfuric acid was taken in 170 mL test tube and heated at 420 °C for one hour.

Distillation: after digestion sample was subjected to distillation by 40 gm/ L boric acid and 400 g/L sodium hydroxide. Methyl red (7 mL) and bromo cynol green (10 mL) was used as an indicator dye (Foss).

Titration: titration was done with 0.1 N HCl.

After 3 steps the Nitrogen and protein content were calculated by the formula given below

$$Nitrogen\ \% = 14\ \times Normality\ of\ acid\ \times Titrant\ value\ \times 100 \div sample\ weight\ \times 100$$

$$Crude\ protein\ (\%) = Nitrogen\ \times 6.25$$

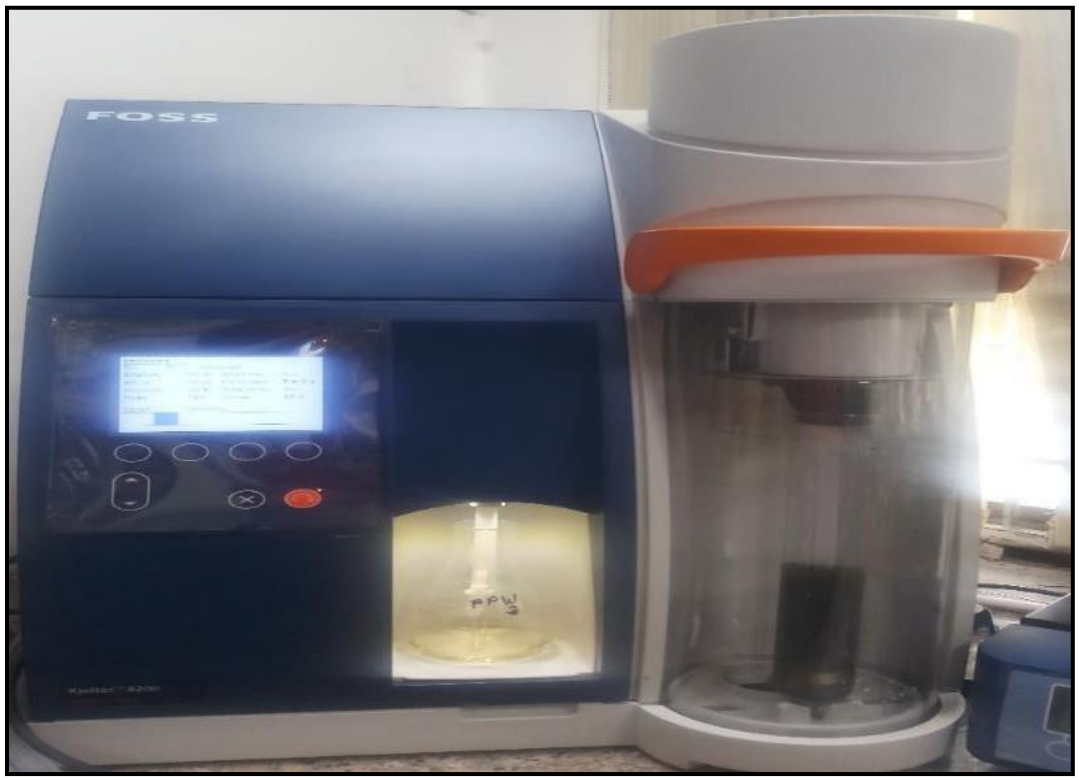


Figure 10 Kjeldhal distillation

2.9.4 Lignin Analysis

Acid soluble and insoluble lignin was done by protocol stablished by National renewable Energy resources (NREL). in which sample (100 mg) was treated with 72% sulfuric acid and after 2 hours of incubation at room temperature and after that dilution was done by adding 28ml of distilled water and subjected to autoclave for 1 hour. After that filtration was done in crucible. Acid soluble lignin was detected spectrophotometrically at 205nm and for acid insoluble lignin the solid residue was subjected to ash formation at 575 °C for 6 hr. Total lignin was calculated by following formula as given in a literature (Timung et al., 2016).

$$\text{Acid soluble lignin} = \frac{\text{UV absorbance x volume filtrate x dilution factor}}{\text{Absorbity X dry weight of sample x pathlength of cuvette}} \times 100$$

$$\text{Acid insoluble lignin} = \frac{\text{Final volume before Ash} - \text{volume after Ash}}{\text{Initial volume}} \times 100$$

2.9.5 Starch Analysis

Starch analysis was done by spectrophotometric method. In the starch analysis method, 1mL sample was taken in a test tube and 0.33 mL of potassium iodide (KI) solution was subsequently added. The blue color intensity was measured at 600 nm using a spectrophotometer against a KI blank. The amount of starch in the sample was determined by the standard curve (1mg/mL) as suggested in a literature (Landhäusser et al., 2018).

2.9.6 Total Phenolics

For total phenolics 1 gm sample was subjected to methanol extraction (1:10) and then subjected to incubation with Folin reagent (0.5 mL) and sodium carbonate (1 mL) for 2 h at room temperature and then subjected to spectrophotometric assay at 765 nm against the gallic acid standard (1 mg/mL) (Lima et al., 2021).

2.9.7 Cellulose and Hemicellulose Analysis

Cellulose and hemicellulose content was estimated using the chlorite method. Briefly, holocellulose content was determined using treatment with sodium chlorite. 17.5% NaOH was used to extract the cellulose compound from the holocellulose. Hemicellulose was determined by subtracting the value of cellulose from holocellulose (Ponce et al., 2021).

2.10 Acid Pretreatment of Potato Peel Waste

Potato peel biomass is composed of starch, lignin, protein, cellulose and Hemicellulose etc. To release fermentable sugar from polysaccharides, a proper treatment strategy is required before subjecting the biomass to microbial fermentation (Remedios and Domingues, 2023b). A Thermochemical treatment is done to convert the starch into glucose, this monomer sugar is further utilized for microbial conversion into PHA. For chemical hydrolysis, 1 L of 3.2 M HCl stock solution was prepared by mixing 275.86 mL of 10% HCl in 724.13 mL of distilled water. Different hydrochloric acid (HCl) concentrations (0.5, 0.75, 1, 1.25, 1.5, and 2%) were prepared from stock solution and pretreatment was done at temperatures (50, 100, and 121 ℃). Ten percent

solid loading (10 g potato peel in 100 mL of aqueous phase) was done in each combination. In the process of detoxification, dry calcium carbonate was added to acidic hydrolysate, and the pH of the sample was monitored throughout the time to reach 6.8-7 as described in the literature (Ahmed et al., 2019). After neutralization, centrifugation was done to get clear hydrolysate and followed by filtration to obtain the sugar-rich extract. Sugar analysis was performed by the DNS method (Miller, 1959).

2.11 Experimental Design by Response Surface Methodology

The highest reducing sugar yield is optimized using statistical software. The software (Minitab version 16.0) was used for regression analysis of the study. To achieve highest glucose yield, a total number of thirteen successive trials were outlined with Central composite design where two factors were Acid concentration and Time and five central points were deployed on the single response (reducing sugar yield). All 13 trials were done in triplicate and the standard deviation was reported. A regression equation was used to demonstrate the collective results of the two Variables Used in the resulting numerical data using response surface methodology. The regression equation is given below where, Y is the response of trial (% of reducing sugar) X_1 and X_2 are two independent factors such as acid % (X_1) and time in minute (X_2). The regression equation was used to translate the observed results. To mitigate the impact of unexpected mutability in the acquired findings parameters, the trials were randomized. Model unfitness, coefficient of determination (R^2) and F-test result was utilized to assess the effectiveness of the model using data from the ANOVA (analysis of variance). The interaction between two independent variables Table 3 and 4 was examined using counterplots and a pareto chart. By contrasting the experimental results with the model's projected values, the model's efficacy was assessed.

Table 3 Experimental Range of Levels Of Independent Process Variables And Coded Values in CCD

Variables	**Level**		
	-1	0	1
Time (min)	15	55	95
Acid concentration (%)	0.2	1.125	2

Table 4 Different Combination of Variables in Experimental Design

Run order	Concentration	Time
1	2 (+1)	55 (0)
2	0.25 (-1)	95 (+1)
3	0.25 (-1)	55 (0)
4	0.25 (-1)	15 (-1)
5	2 (+1)	95 (+1)
6	1.125 (0)	55 (0)
7	1.125 (0)	95 (+1)

8	1.125 (0)	55 (0)
9	1.125 (0)	15 (-1)
10	1.125 (0)	55 (0)
11	2 (+1)	15 (-1)
12	1.125	55 (0)
13	1.125	55 (0)

Table 5 Model Summery of The Experiment

Source	Degree of Freedom (DF)	Adjusted sum of square	Adjusted (mean square)	F-value	P-value
Model	3	7585.13	2528.38	40.71	0.000
Liner	2	6349.63	3174.81	51.12	0.000
Acid	1	6052.82	6052.82	97.47	0.000
Time	1	296.81	296.81	4.78	0.057
Square	1	1235.50	1235.50	19.90	0.002
Acid × Acid	1	1235.50	1235.50	19.90	0.002
Error	9	538.90	62.10		

2.12 Sugar Estimation

After dilute acid treatment, Hydrolysate was used for reducing sugar analysis by the Dinitro salicylic acid method briefly 1 ml of extracted hydrolysate with an equal amount of DNS solution (3,5 dinitro salicylic acid 1 gm, Na_2SO_3 0.05 g, sodium potassium tartrate 18.2 g, NaOH 1 gm, and phenol 0.2 gm per 100 mL of H_2O) was subjected to heating for 10 min at 100 °C and optical density at 540 nm was recorded upon cooling using standard glucose curve (1mg/mL). Total reducing sugars (glucose, fructose, and xylose) present in the biomass have been considered for estimation (Shangdiar et al., 2023). (Figure 11, 12, 13, and 14)

$$Sugar\ recovered\ \left(\frac{mg}{mL}\right) = Volume\ of liquid\ hydrolysate\ recovered \div Amount\ of\ sample\ taken\ for\ hydrolysis \times 100$$

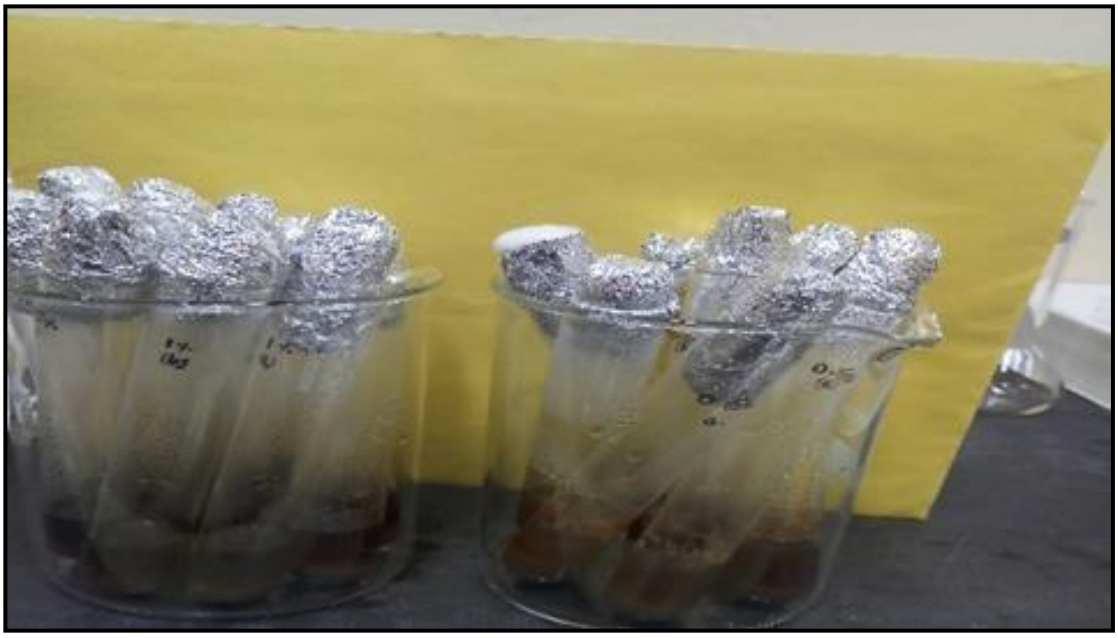


Figure 11 (A)

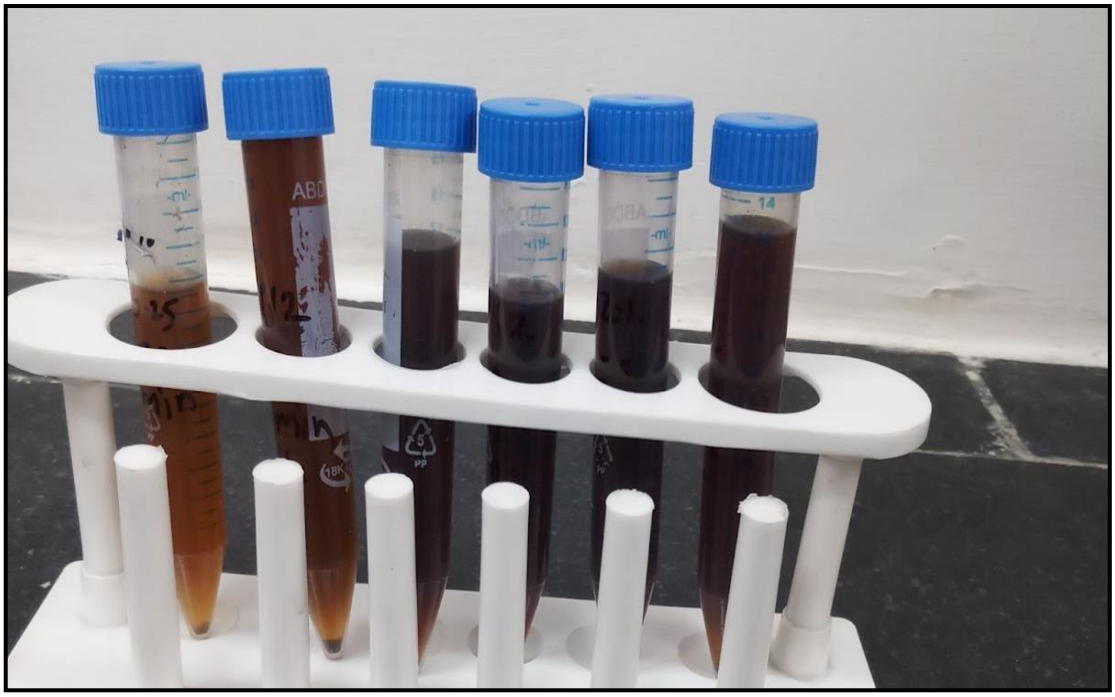


Figure 11 (B) Acid Hydrolysis of Potato Peel Waste

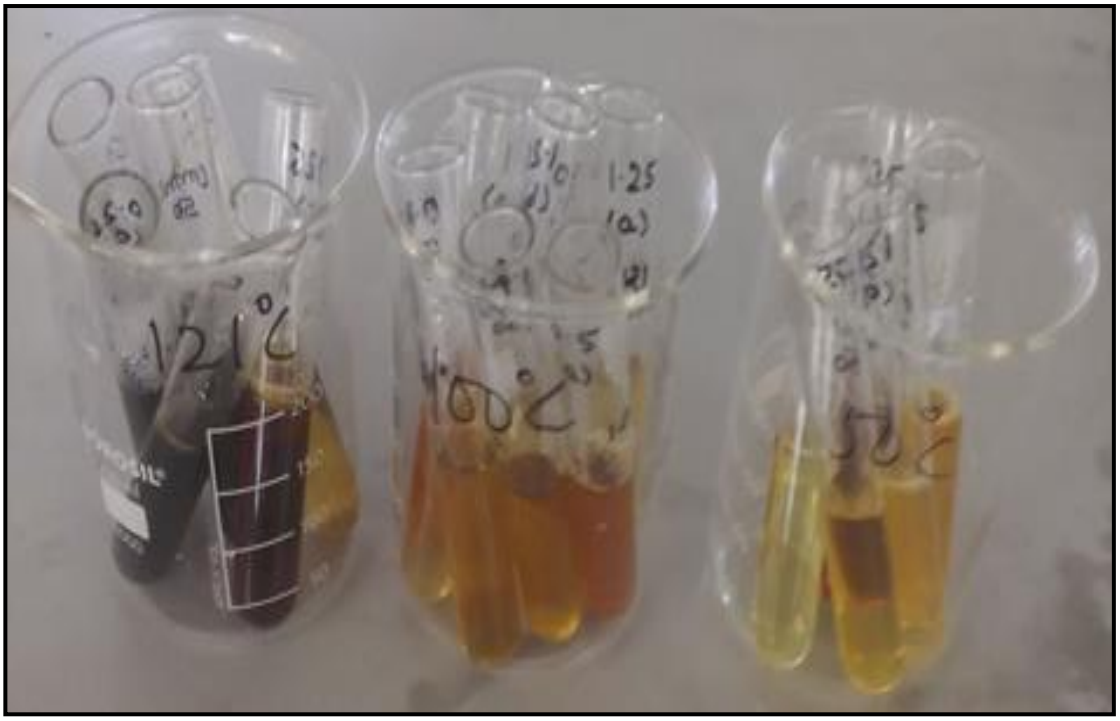


Figure 12 DNS Analysis of Potato Peel Waste

Figure 13 Obtimized Condition of Acid Hydrolyis (1.75% HCl)

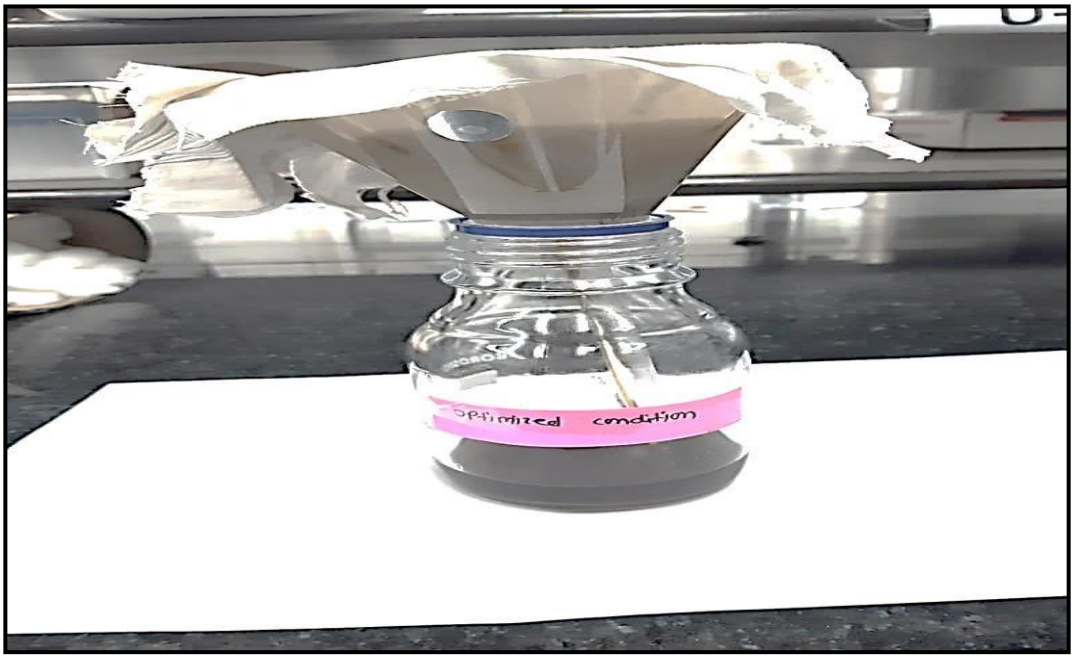


Figure 14 Filtration of Acid Hydrolyzed Biomass

2.13 Physicochemical Characterization of Biomass

Functional group analysis of both raw and acid-hydrolyzed *S. tuberosum* periderm waste was conducted using Fourier-transform infrared (FTIR) spectroscopy with KBr pelleting (PEIR SUBTECH SPECTRUM ASCII PEDS 4.00). Thermogravimetric analysis (TGA) of the *S. tuberosum* periderm was carried out to observe decomposition peaks at various heating rates under an inert nitrogen environment, using a PerkinElmer analyzer. The analysis covered temperatures ranging from 30 °C to 900 °C with a heating rate of 10 °C min^{-1}. The surface structure of both raw and treated *S. tuberosum* periderm biomass was observed using a Scanning Electron Microscope (ZEISS EVO 18) at a beam-accelerating voltage of 20 kV.

2.14 Alkali Pretreatment of Wheat Straw

Wheat straw was subjected to 3% NaOH pretreatment at 121°C/15min/15lbs. After pretreatment solid part of the biomass was recovered and subjected to neutralization with distilled water. (Figure 15)

Figure 15 Alkali Pretreatment of Wheat Straw

2.15 PHA Production by *Pseudomonas putida* MTCC 2475

Pseudomonas putida (2475) procured from MTCC Chandigarh India. The culture was revived once in a month using the Luria Bertani media (yeast extract 5g L^{-1}, NaCl 10g L^{-1}, agar 15g L^{-1} and tryptone 10 g L^{-1}), maintained at low temperature (4 °C). For inoculum development, loopful bacterial colonies from pure culture, transferred to 150 mL in the nutrient broth medium and incubated at 30 ℃ at 180 rpm for 24h. Initially Growth pattern of *P. putida* in relation to optical density 600 nm using the (GENESYS 50 UV- visible spectrophotometer Thermofisher) sugar consumption and cell biomass, was observed in L.B media, mineral salt media containing synthetic glucose as carbon source as well as in hydrolysate containing modified media. Nitrogen limited medium was used as a growth medium with 0.1% tryptone. 1% Inoculum (O.D 0.8 and $312x10^6$ cfu) was used from freshly prepared seed culture. A total number of four media was selected for PHA production study was conducted in 2 L conical flasks containing 1L modified media (Media A) having defined volume of acid pretreated ppw hydrolysate 10g L^{-1} (after neutralization with calcium carbonate), 5g L^{-1} NaCl, tryptone1g L^{-1} in shaking incubator (180rpm) at 30 °C for 24h, 36, 48 and 72h respectively. Similar experiments were performed with (Media B) pure hydrolysate media (10g L^{-1}), (Media C) Mineral salt media containing $Na_2HPO_4.7H_2O$ (30g L^{-1}), KH_2PO_4 (15g L^{-1}), NaCl (5g L^{-1}), NH_4Cl (1g L^{-1}), $MgSO_4$ (2g L^{-1}), $CaCl_2$ (0.1g L^{-1}) and synthetic glucose (10g L^{-1}) as suggested by (Sriyapai et al., 2022) and (Media D) Luria-Bertani broth media at the same conditions. (Figure 16, 17, 18, and 19) Average PHA production values were taken into account after each experiment was run in duplicate.

2.16 Cell Dry Weight Measurement and Extraction of PHA

After incubation, the culture broth was subjected to centrifugation at 10,000 rpm using an Eppendorf 5810 centrifuge for 15 minutes at 4 °C. Following centrifugation, the supernatant was carefully drained, and the cell pellet was recovered. (Figure 20, 21, 22, and 23) The recovered cell pellet was subsequently washed sequentially with 25 mL deionized water, 25 mL acetone, and 25 mL ethanol, and then dried at 40 °C until a constant weight was achieved. This recovered cell biomass, referred to as cell dry mass, was determined gravimetrically, as per the method outlined in the literature

(Tanikkul et al., 2020b). Polymer extraction was carried out using the chloroform extraction method with slight modifications, following the procedure described by (Filippi et al., 2021). (Table 5) 2.1 grams cell pellet was crushed with the help of a mortar pestle and then incubated in 20-fold chloroform (43.8 mL) at 60 °C in a water bath for 2 hours. The resulting mixture was filtered using Whatman filter paper (1), and the liquid was concentrated through evaporation. PHA was obtained by precipitation of the whole recovered solution obtained after heating with 22 mL ice-chilled methanol and subsequently analyzed using GC-MS, following the previously established protocol (de Souza Reis et al., 2020). Briefly, For the analysis of monomer composition, we performed GC MS-MS. First, 20 mg of dry cell biomass was subjected to acidic methanolysis. Methyl esters were prepared by combining 15% sulfuric acid in 85% methanol (2 mL) with chloroform (2 mL) in a screw-capped culture tube and heating it at 100 °C for 2 hours and 20 minutes (de Souza Reis et al., 2020).

Table 6 Pattern of cell dry weight and PHA accumulation by *P. putida* in each type of production media

Parameters	Media A				Media B			
Time	24	36	48	72	24	36	48	72
Cell dry weight (g/L)	1.05 ± 0.07	1.28 ± 0.22	2.19 ± 0.74	1.74 ± 0.36	1.01 ± 0.01	1.24 ± 0.10	1.95 ± 0.11	1.10 ± 0.14
PHA%	6.25 ± 0.77	12 ± 0.56	28.71 ± 0.55	18.04 ± 0.26	0.35 ± 0.21	1.7 ± 0.56	3.25 ± 0.63	2.21 ± 0.48
Residual sugar (g/L)	1.87 ± 0.08	1.76 ± 0.02	1.09 ± 0.07	0.21 ± 0.02	3.03 ± 0.25	2.08 ± 0.25	1.43 ± 0.65	0.44 ± 0.25

Parameters	Media C				Media D			
Time	24	36	48	72	24	36	48	72
Cell dry weight (g/L)	1.26 ± 0.19	1.62 ± 0.12	2.95 ± 0.10	1.96 ± 0.07	1.06 ± 0.21	1.16 ± 0.09	1.56 ± 0.15	1.10 ± 0.06
PHA%	7.24 ± 0.82	14.2 ± 0.84	29.97 ± 0.45	20.51 ± 0.78	0.65 ± 0.21	2.65 ± 0.49	3.75 ± 0.21	2.61 ± 0.26
Residual sugar (g/L)	3.48 ± 0.51	2.55 ± 0.13	1.5 ± 0.11	0.28 ± 0.05	1.67 ± 0.21	1.31 ± 0.24	1.10 ± 0.17	0.21 ± 0.08

2.17 Monomer Identification GCMS-MS Analysis

After cooling, 2 mL of water was added to separate the organic layer from the aqueous layer. The GC-MS-MS analysis was conducted using the Triple quadrupole 7000D GC/TQ Agilent, equipped with a triple-axis detector. For the analysis, PHA was used in the form of hydroxy alkanoic acid methyl esters, following the method reported by (Mahato et al., 2021). A sample of 2 µL at a split ratio of 1:50 was automatically injected into the GC and the injection temperature was 200 °C. Helium served as the carrier gas at 48 mL^{-1}min and 0.42 bar pressure (Hierro-iglesias et al., 2023). Monomer identification was performed using the NIST 17 library.

2.18 Preparation and Characterization of PHA Film

The extracted PHA film was prepared in a fume hood by dissolving 200 mg of extracted PHA in 20 mL of chloroform at room temperature and subsequently pouring the solution into a Petri dish till complete evaporation of the solvent. To determine the melting temperature of the PHA film, we conducted Differential Scanning Calorimetry (DSC) using a temperature-regulated system (DSC 8000, Perkin Elmer). We took 5 mg of extracted PHA in an aluminum pan and subjected it to a nitrogen flux rate of 10

ml/min, heating it from 30 to 250 °C, following the procedure outlined in the literature (Kacanski et al., 2023) with some modifications.

For the analysis of functional groups, we recorded Infrared (IR) spectra in the range of 4,000 to 450 cm^{-1}. This was done by preparing KBr pellets and using a (Perkin Elmer Frontier Shelton CT08484). In brief potassium bromide (KBr) was subjected to drying at 110 °C for 2 h for moisture removal. A 5 mg sample was mixed with 200 mg of powdered KBr. After pulverization, the mixture (sample and KBr) was placed into a pellet-forming machine, and the resulting pellet was taken for IR analysis (Cerrone et al., 2023). To analyze the chemical shift of the PHA, we utilized 1H Nuclear Magnetic Resonance (NMR) by dissolving a 5 mg sample in 600 microliters of $CDCl_3$, employing a (BRUKER Proton NMR 500MHz), as described by (Kacanski et al., 2023). The chemical shift of PHA was recorded in parts per million (ppm).

Figure 16 Media A containing Tryptone, hydrolysate and NaCl at 24, 36, 48, and 72h respectively

Figure 17 Media B containing pure sugar hydrolysate at 24, 36, 48, and 72h respectively

Figure 18 Media C Mineral salt media at 24, 36, 48, and 72h respectively

Figure 19 Media D containing Luria Bertani broth at 24, 36, 48, and 72h respectively

Figure 20 Dry cell weight of media (A) at 24, 36, 48, and 72 h respectively

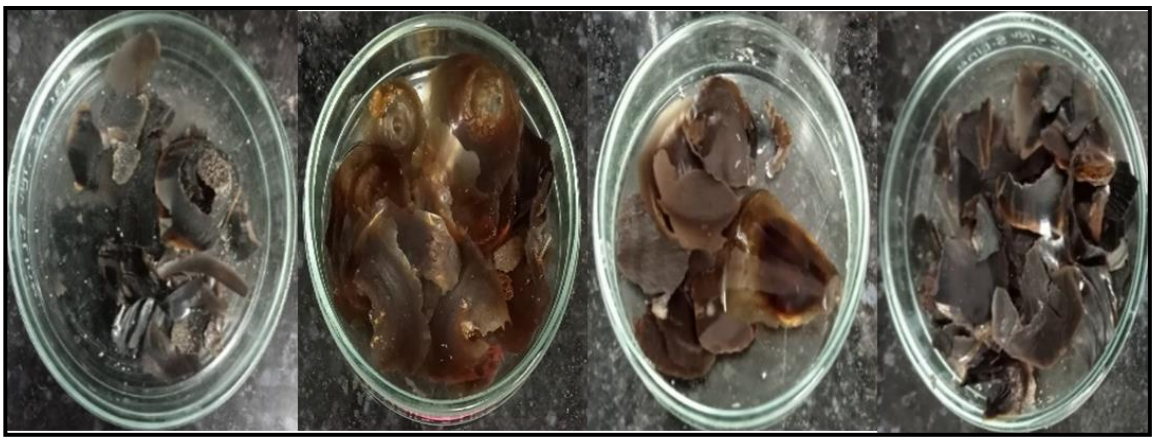


Figure 21 Dry cell weight of media (B) at 24, 36, 48, and 72 h respectively

Figure 22 Dry cell weight of media (C) at 24, 36, 48, and 72 h respectively

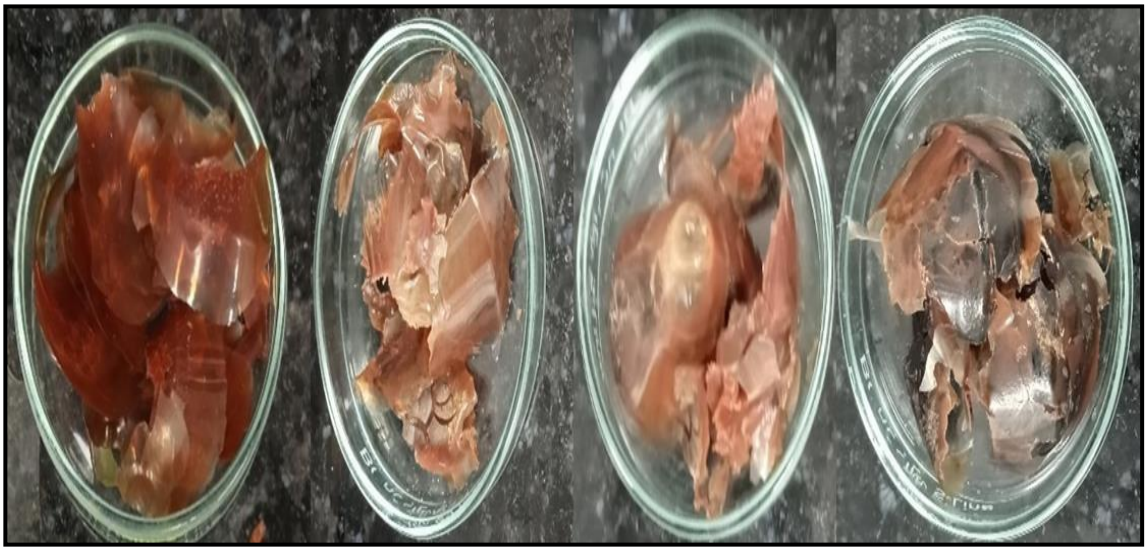


Figure 23 Dry cell weight of media (D) at 24, 36, 48, and 72 h respectively

2.19 PHA production by *Bacillus circulans* MTCC 8167

2.19.1 Fermentation of Potato Peel Waste for PHA Production

After optimization of acid hydrolysis conditions, the total reducing sugar concentration the liquid hydrolysate (10g/L), was applied as a carbon source for the cultivation of *Bacillus circulans.* In the hydrolysate, before the autoclave, sodium chloride (5 g/L)

and organic nitrogen source tryptone (1g/L) was also added. Bacterial culture was prepared by inoculation of a single colony of bacteria in a culture medium in a 250 ml flask at 37 °C in a shaking incubator at 180 rpm. The culture was taken from the mid-exponential phase for 1% inoculum for the hydrolysate fermentation. The growth pattern analysis in terms of cell dry weight, optical density (O.D at 600 nm), and sugar consumption was analyzed using a thermo-scientific UV visible spectrophotometer (GENESYS 50). Cell dry weight was determined in different time intervals such as 24, 36, and 48h (Ciesielska et al., 2017).

2.19.2 Cell Dry Weight Measurement and Extraction of PHA

After fermentation, the culture suspensions were subjected to centrifugation (microprocessor-based laboratory centrifuge) at 6000 RPM for 15 minutes at room temperature. Cell pellets were washed with deionized water and then dried in the oven (Matrix Scientific) at 55 ℃ till the constant weight of cell dry weight in each condition was recorded. The extraction of PHA was done with 1:20 ratios of sample and chloroform by heating at 60 ºC for 120 minutes in a water bath. After cooling the extractives, precipitation was done with a ten-fold volume of ice-chilled methanol as described in the literature (Shah, 2012).

2.19.3 Quantification of PHA By Crotonic Acid Assay

The polymer obtained from the method mentioned above was estimated by the Crotonic acid method. PHA is heated with H_2SO_4, it is converted into crotonic acid, and its concentration is determined by spectrophotometric assay. The stock solution of crotonic acid was prepared by dissolving 0.1 mg of crotonic acid into 1 ml of sulphuric acid. Different concentrations of 2, 5, 7, 8, 9, and 10 µg /ml for crotonic acid were prepared from the stock solution with a working volume of 1 ml. Sulfuric acid was used as blank, and absorbance was recorded at 235 nm. The standard graph was plotted for concentration v/s absorbance. From the standard curve concentration of the test sample (PHA) was determined (Hiremath and Sura, 2015).

2.19.4 GC-MS of Polyhydroxyalkanoates

Biopolymer (PHA) was analyzed by acidic methanolysis. The 20 mg dried biomass was subjected to methanolysis. In which 2 ml $CHCl_3$, 1.7ml CH_3OH, and 0.3ml H_2SO_4 heated at 100°C for 2 h and 20min. A 2µl sample from the bottom organic layer was used for GCMS analysis (Shimadzu QP 2010) and the injection temperature of the column was 260°C. Monomers were analyzed by the NIST library as previously done by (Mahato et al., 2021).

2.19.5 Characterization of PHA By FTIR, DSC, and NMR

The functional group of PHA from *Bacillus circulans* was analyzed by FT-IR with a spectral range of 4000-400 cm^{-1} (Perkin Elmer (frontier Shelton CT08484) as described in the literature (Maheshwari et al., 2018). The thermal degradation behavior of extracted PHA powder was checked using a Differential Scanning Calorimeter (DSC) with an instrument make of Perkin Elmer model 8000). Nitrogen gas was used for purging with a flow rate of 10 ml/min. 10 mg PHA was subjected to a thermal profile from -10°C to 300°C and the heating rate was 10°C/min (Dinh et al., 2022). To check chemical properties ^1H NMR was performed. Briefly, 6 mg of pure PHA was dissolved in 1 ml $CDCl_3$ (Denatured chloroform) and spectra were recorded on (500 MHz Bruker spectrophotometer) (Copolymer and Cfr-, 2012).

2.19.6 Crude Enzyme Production from *Aspergillus niger*

wheat straw Delignified (3% sodium hydroxide) was utilized as a feedstock for crude enzyme production by *Aspergillus niger*. The fermentation experiment was done in 250 mL flasks in which treated wheat straw was blended with some fermentation nutrients such as (1 g) yeast extract, (0.4 g) KH_2PO_4, and (0.2) g NH_4Cl for every 100g of substrate. The components were mixed and then subjected to sterilization at 121 ˚C in the autoclave for 20 mins. Media was inoculated with 3.25*106 spore count and media was incubated at 28 ºC for 7 days. (Figure 24)

Figure 24 Solid state crude enzyme production by *Aspergillus niger*

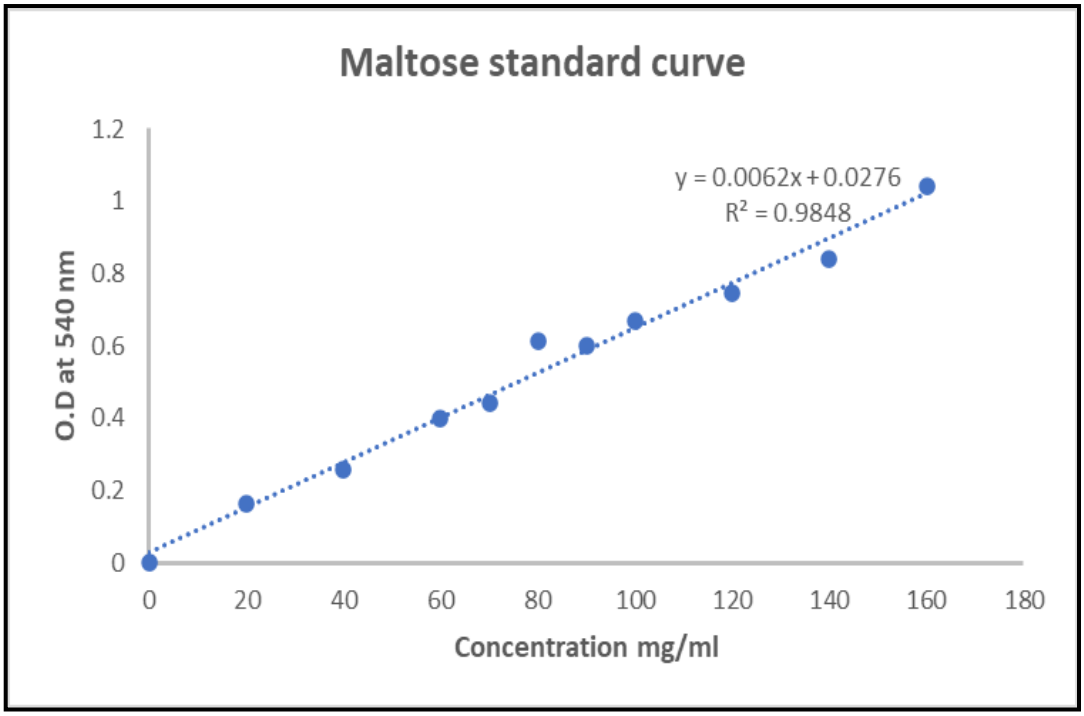


Figure 25 Solid state crude enzyme production by *Aspergillus niger*

2.19.7 Enzyme Activity

Obtained enzyme activity was measured at 60ºC in a tube containing 0.5 mL crude enzyme extract and 0.5 mL of 1% starch (substrate) solution in 0.25 molar CH_3COONa buffer pH 5.0. The amount of glucose released was estimated by the glucose oxidase assay as suggested by (Aliyah et al., 2017). One enzyme unit (U) was explained as the quantity of enzyme that releases 1µM of glucose/min/ mL of the reaction mixture. (Figure 25)

2.19.8 Hydrolysis of Potato Peel Waste with Commercial Enzyme and Crude Enzyme

Starch-containing potato peels were subjected to enzymatic hydrolysis with commercial amylase obtained from SRL (product code- 46504) as well as in-house crude enzyme (containing Amylase, Glucoamylase, Cellulase) produced from *Aspergillus niger*. The process was optimized through Response surface

methodology (Minitab). Two parameter substrate loading (X_1) and enzyme unit (X_2) shown in Table 6 was taken for study at 60°C for 24h.

Table 7 Variables and Coded Values in Central Composite Design

Variables	Level		
	-1	0	1
Enzyme unit (U)	20	60	100
Substrate loading (%)	0.5	1.25	2

Table 8 Coded Coefficient of Commercial Enzyme

Term	Coef SE	SE Coef	T-Value	P-Value	VIF
Constant	3.485	0.103	33.93	0.000	
Substrate%	1.664	0.117	14.28	0.000	1.00
Enzyme U	1.357	0.115	11.81	0.000	1.00
Substrate%*Substrate%	-2.134	0.179	-11.92	0.000	1.02
Enzyme U*Enzyme U	-0.201	0.174	-1.16	0.285	1.02
Substrate%*Enzyme U	0.861	0.233	3.70	0.008	1.00

Table 9 Model Summery of Commercial Enzyme

S	R-sq	R-sq (adj)	R-sq (pred)
0.229675	98.59%	97.58%	89.96%

Table 10 Analysis of Variance of Commercial Enzyme

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	5	25.7494	5.1499	97.63	0.000
Liner	2	18.1099	9.0550	171.66	0.00
Substrate%	1	10.7534	10.7534	203.85	0.00
Enzyme U	1	7.3567	7.3567	139.46	0.00
Square	2	7.5089	3.7544	71.17	0.00
Substrate%*Substrate%	1	7.5005	7.5005	142.19	0.00
Enzyme U*Enzyme U	1	0.0705	0.0705	1.34	0.285
2-Way Interaction	1	0.7225	0.7225	13.70	0.008
Substrate%*Enzyme U	1	0.7225	0.7225	13.70	0.008
Error	7	0.3693	0.0528	-	-
Lack-of-Fit	3	0.3688	0.1229	1024.38	0.000
Pure Error	4	0.005	0.001	-	-

Total	12	26.1187	-	-	-

Table 11 Analysis of Variance of Crude Enzyme

Source	Degree of Freedom (DF)	Adjusted sum of square	Adjusted (mean square)	F-value	P-value
Model	5	61.0554	12.211	83.89	0.000
Liner	2	28.2812	14.1406	97.15	0.000
Substrate %	1	19.5943	19.5943	134.62	0.000
Enzyme U	1	8.6869	8.6869	59.69	0.000
Square	2	32.7728	16.3864	112.58	0.0000
Substrate % × Substrate %	1	31.0847	31.0847	213.56	0.000
Enzyme U × Enzyme U	1	4.0560	4.0560	27.87	0.000

Table 12 Model Summary of Crude Enzyme

S	R-sq	R-sq (adj)	R-sq(pred)
0.381516	98.36%	97.19%	88.40

Table 13 Coded Coefficients of Crude Enzyme

Term	Coef	SE Coef	T- Value	P- Value
Constant	6.533	0.171	38.29	0.000
Substrate %	2.213	0.191	11.60	0.000
Enzyme U	1.474	0.191	7.73	0.000
Substrate% × Substrate %	-4.227	0.289	-14.61	0.000
Enzyme U × Enzyme U	-1.527	0.289	-5.28	0.001
substrates % × Enzyme U	-0.135	0.381	-0.35	1.00

2.19.9 Optimized Condition for Commercial and Crude Enzyme

After thirteen runs of trial, the potato peel waste is further subjected to an optimized condition which was found 1.8% substrate loading and 116U of enzyme and the predicted response was 5.3g/L sugar in the case of commercial amylase while in the case of crude enzyme 1.51% substrate loading and 86.84U of the enzyme was found for 7.16g/L.

Chapter 3 Results and Discussion

3.1 Composition Analysis

The composition analysis of *S. tuberosum* periderm (potato peel), collected during winter was conducted, and the findings are presented in Table 13. One gram of the sample was used for composition analysis, and the results were reported in triplicate.

The composition of the *S. tuberosum* periderm was as follows: starch at 64.44%, ash at 14%, total lignin at 6.5%, phenolics at 0.6% of *S. tuberosum* periderm, cellulose at 9.01%, hemicellulose 1.89%, and the total solid content was found to be 90% (w/w).

In comparison, (Malakar et al., 2020). reported 76% moisture and 8% ash in S. tuberosum periderm, (Arapoglou et al., 2010) reported starch content at 52.14%, (Lima et al., 2021) reported cellulose and hemicellulose content ranging from 10% to 30%, and (Samarin et al., 2012) reported a total phenolic content of 522 µg GAE/g dry weight. These findings support the results of our study. However, it's worth noting that the composition of *S. tuberosum* periderm can vary among different *Solanum tuberosum* species (Sampaio et al., 2020).

Table 14 Composition Analysis of Potato Peel Waste

Parameters	% (W/W)
Total solid	90.366 ± 0.40
Moisture	09.633 ± 0.40
Ash	14.033 ± 0.05
Total lignin	06.564 ± 0.20
Starch	64.44 ± 0.26
Total phenolics	0.6 ± 0.09
Cellulose	9.01 ± 0.48

Hemicellulose	1.89 ± 0.48

3.2 Acid Hydrolysis

As a result of acid hydrolysis, the complexity of peel biomass decreases, and the amount of fermentable sugar increases. Different concentrations of dilute acid (HCl) and time variation for hydrolysate development were applied in this study which resulted in a varied amount of reducing sugar. Based on the sugar yields comparative results are shown in Table 14, 15, and 16. In the acid hydrolysis process, a water molecule is added through a nucleophilic reaction due to the breakdown of chemical bonds of starch, and a change in porosity occurs which leads to the release of sugar upon hydrolysis (Celikci et al., 2020). Experimental conditions used in this study such as 50℃ and 100℃ at different acid ranges were found insufficient for maximum reducing sugar extraction because sugar yield is directly proportional to hydrolysis temperature at a certain limit. (Tesfaw and Tizazu, 2021). In the case of 121℃, out of six concentrations (0.5, 0.75, 1, 1.25,1.5 and 2% acid), 2% acid concentration has yielded the best result in this study is 71.23 ± 0.46% reducing sugar because of a suitable combination of thermal-acidic treatment. (Figure 26). The reason behind this observation is that soft biomass liberates high sugar at mild acidic conditions and high temperatures which is previously observed in the literature (Timung et al., 2016). Similarly, in a report, 23g/L of sugars using 1% sulfuric acid pretreatment in autoclave condition was obtained. (Malakar et al., 2020). In another study (Deshmukh and Pande, 2022), 1-5% HCl was used to hydrolyze potato peel and they reported 62g/L glucose from 5% acid concentration at 90℃ for 120 min reaction time. (Martín et al., 2019) reported almost similar amount (72%) conversion of glucose from cassava stems where in the first step cassava stems are subjected to acid pretreatment with 0.6% H_2SO_4 and then enzymatic hydrolysis was with a combination of amylase and glucoamylase. Though high temperatures of more than 121℃ and increased acid concentration lead to the accumulation of toxic material such as phenolics due to the degradation of sugars (Tesfaw and Tizazu, 2021). Though the goal of this study was

to extract maximum sugar for PHA production and therefore 2% acid was used which leads to liberating 71.23 ± 0.46% reducing sugar.

Table 15 Reducing Sugar Yield (%) After Dilute Acid Pretreatment

Acid %	Sugar % at 121 °C	Sugar % at 100 °C	Sugar % at 50 °C
0.5	12.47 ± 0.40	7.61 ± 0.52	2.58 ± 0.15
0.75	13.71 ± 0.54	9.69 ± 0.30	4.55 ± 0.46
1	23.58 ± 0.17	16.44 ± 0.29	9.28 ± 0.51
1.25	32.77 ± 0.36	19.06 ± 0.38	12.41 ± 0.53
1.5	43.66 ± 0.34	26.59 ± 0.14	16.22 ± 0.46
2	71.23 ± 0.46	51.84 ± 0.29	35.50 ± 0.20

Table 16 Reducing Sugar Yield (mg/gram of potato peel waste) After Acid Hydrolysis

Acid %	Sugar mg/g of potato peel at 121 °C	Sugar mg/g of potato peel at 100 °C	Sugar mg/g of potato peel at 50 °C
0.5	125.55 ± 0.31	76.11 ± 0.22	25.84 ± 0.16
0.75	137.15 ± 0.18	96.94 ± 0.13	45.57 ± 0.40
1	235.84 ± 0.2	164.48 ± 0.29	92.89 ± 0.15
1.25	327.77 ± 0.23	190.64 ± 0.17	124.11± 0.21
1.5	436.61 ± 0.11	265.97 ± 0.30	162.28 ± 0.35
2	712.33 ± 0.30	518.46 ± 0.22	355.01 ± 0.20

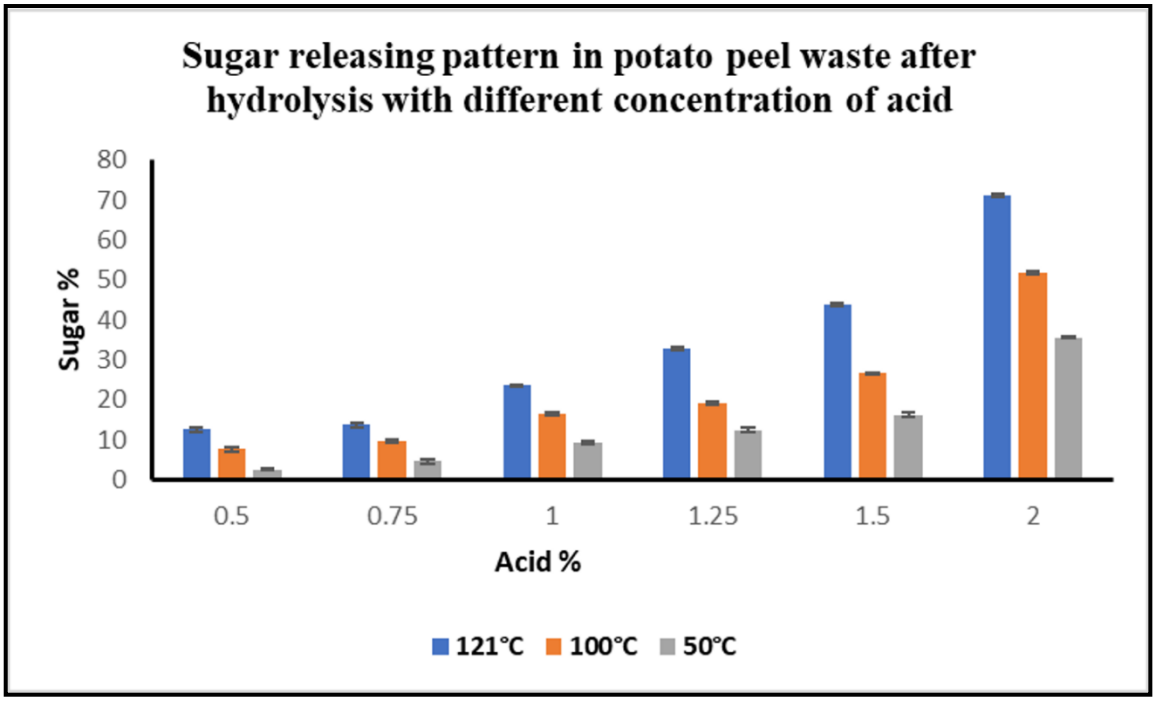


Figure 26 Sugar Releasing Pattern in Potato Peel after Hydrolysis With Different Acid Concentrations

3.3 Optimization of Hydrolysis Condition Through RSM

Different concentrations of dilute acid and time variation for hydrolysate development were used in this study which resulted in a varied amount of reducing sugar and their combined severity factor ($LogR_o$). Based on the above parameters, results are presented in (Figure 30, 31). From the obtained results it can be concluded that a very low acid concentration (0.25), is unable to liberate high sugar at any time. Briefly 0.25 % acid at 15 min extracted 0.05% reducing sugar. However, at 55 min and 95 min sugar yield was 5.49% and 16.45% respectively. The center point (1.12 % acid and 55 min time) was obtained through the software and the sugar yield in all five runs was between 59- 63%. While High acid concentration (2%) releases the highest amount of sugar such as 76% at 15 min, and 70% at 95 min. From the above observations, it can be said that sugar yield increases with the increase in acid concentration. However, time is not a very significant factor in terms of sugar removal as 2% acid at 15 min can extract more sugar as compared to 95 min. The reason behind the diversity in sugar

removal is high acid concentration and longtime combinedly generate toxic metabolites that lead to degraded sugar molecules. Therefore, a comparatively low acid concentration was optimized from the response surface methodology for maximum sugar with low toxicity. Further, the optimized condition was used for acid hydrolysis.

This study was designed to establish a linear equation based on thirteen experimental runs, including five center points, all subjected to analysis of variance (ANOVA). Two factors, time and acid concentration, were optimized through Central Composite Design (CCD) and further analyzed using regression analysis and analysis of variance. The independent variables and their respective values are presented in Table 3 and 4.

Following the completion of thirteen trial runs, the optimal conditions for achieving the highest yield of reducing sugars were determined using Minitab software and graphically represented in (Figure 27, 28, and 29). The Pareto chart illustrates the standardized effects of individual factors: (A) acid concentration, (B) time, and the combined effects of both factors. The Pareto chart indicates that factor (A) exerts the most significant influence on sugar extraction, while the impact of time is relatively negligible. Furthermore, the combined influence of acid and time explains a smaller portion of sugar extraction.

The contour plot displays the region of maximum sugar yield, falling within the ranges of 1.5-2% for acid concentration and 50-90 minutes for time. Based on the above observations, this study aimed to exact the maximum sugar at mild acid concentration. At 2% acid concentration and after 95 minutes of hydrolysis time, sugar was observed to be 70.80 ± 0.27, however, the acid concentration and time was a bit higher. While at 2% acid and 15 mins of time sugar yield was maximum (76%) but acid concentration was higher. Hence, the maximum sugar liberation and milder conditions were established to obtain optimized conditions. The predicted optimal conditions from the model were determined for 1.75% acid concentration and 50 minutes of reaction time, resulting in a predicted sugar yield of 70.47%. To validate the accuracy of the predicted response, a hydrolysis experiment was conducted under the specified parameters, and the trials were conducted in triplicate. The average sugar yield obtained was 69.34 ±

0.25%, which closely matched the predicted value. Further, this extracted sugar was used as a carbon source for PHA production by *P. putida* in this study.

The combined severity factor ($LogR_o$) under the optimized conditions was calculated to be 1.99. In a study, 5% hydrochloric acid, yielded 62 g L^{-1} of sugars (Khawla et al., 2014). In a two-stage hydrolysis study, 32 g of potato peel in 400 mL of 1% w/w H_2SO_4 at 180 °C for 60 min was performed, For second-stage hydrolysis with cellulase, α-amylase, and glucoamylase 36 g L^{-1} glucose was obtained (Abedini et al., 2020). In another two-step hydrolysis process of potato peels, 141 g L^{-1} of sugar was obtained with 3% w/w H_2SO_4 in an autoclave condition for 15 min, The second stage enzymatic hydrolysis was done with the crude enzyme complex produced by *Aspergillus sp.* For 72 h at 50 °C and 150 rpm (Soni et al., 2023b). In an experiment, potato peel waste underwent pretreatment with 1% H_2SO_4 at 121 °C for 30 minutes, with a substrate loading of 10%. In the subsequent hydrolysis step, 10 grams of the pretreated substrate were exposed to enzymatic hydrolysis using cellulase, hemicellulose, and amylase (at concentrations of 30 U, 5 U, and 70 U/g of substrate respectively). This hydrolysis process occurred at 50 °C in a rotatory shaker incubator, with agitation at 700 rpm for 48 h. The outcome of this process revealed the presence of 77 g/L of reducing sugar (Kalafat et al., 2018).

In the current study, we achieved a considerably high yield of reducing sugars at 1.75% HCl, which is milder in terms of the generation of toxic byproducts such as furfural and hydroxymethylfurfural. Details regarding the responses after the trial runs and their Coefficient of Standard Deviation (CSD) are provided. Table 3 and 4.

RSM (Response Surface Methodology) was employed to assess the combined impact of two independent variables: Acid concentration (X_1) and Time (X_2), From the numerical data obtained in this study, the simplified linear regression model for sugar yield from S. tuberosum periderm can be expressed as follow

$$-24.19 + 93.8\ X_1 + 0.1758\ X_2 - 25.54\ X_1 \times X_1$$

Where X_1 = Acid concentration (%) and X_2 = Time (min)

The regression analysis of the trial runs yielded a statistically significant result, with an R-squared value of 93.14%, indicating a strong fit for the model. Additionally, the lack of fit for the model was found to be negligible. The output of the regression analysis for glucose yield is presented in Table 16, where a p-value greater than 0.05 indicates the significance of the model.

To create the best-fit model and eliminate irrelevant conditions (those with p-values greater than 0.05), a linear equation for regression was applied. So, a model for the optimization of reducing sugar yield from *S. tuberosum* periderm was successfully generated. Based on the obtained values, the sequence of relevance for the two factors on acidic hydrolysis was acid concentration >time.

The counterplots and Pareto chart were used to visually depict the effects of individual variables and the combined interactions of acid concentration (X_1) and Time (X_2) on sugar yield.

These statistical analyses and graphical representations are valuable tools for understanding the impact of the variables on the hydrolysis process and optimizing the yield of reducing sugars from *S. tuberosum* periderm waste.

Table 17 Sugar release Pattern by Central Composite Design

Std Order	Run Order	Pt Type	Blocks	Time (X_1)	Conc. (X_2)	Sugar (%)	Combined severity factor Log (R_0)
6	1	-1	1	55	2	65.12 ± 0.17	0.18
3	2	1	1	95	0.25	16.45 ± 0.30	1.26
5	3	-1	1	55	0.25	5.49 ± 0.16	1.52
1	4	1	1	15	0.25	0.05 ± 0.04	0.75

4	5	1	1	95	2	70.80 ± 0.27	1.83
10	6	0	1	55	1.125	61.39±0.23	1.83
8	7	-1	1	95	1.125	67.36 ± 0.41	1.83
12	8	0	1	55	1.125	60.99 ±0.11	1.83
7	9	-1	1	15	1.125	35.72 ± 0.26	1.83
9	10	0	1	55	1.125	61.58 ±0.06	2.09
2	11	1	1	15	2	76.64 ±0.17	0.99
13	12	0	1	55	1.125	59.82 ±0.13	2.07
11	13	0	1	55	1.125	63.67 ± 0.28	2.33

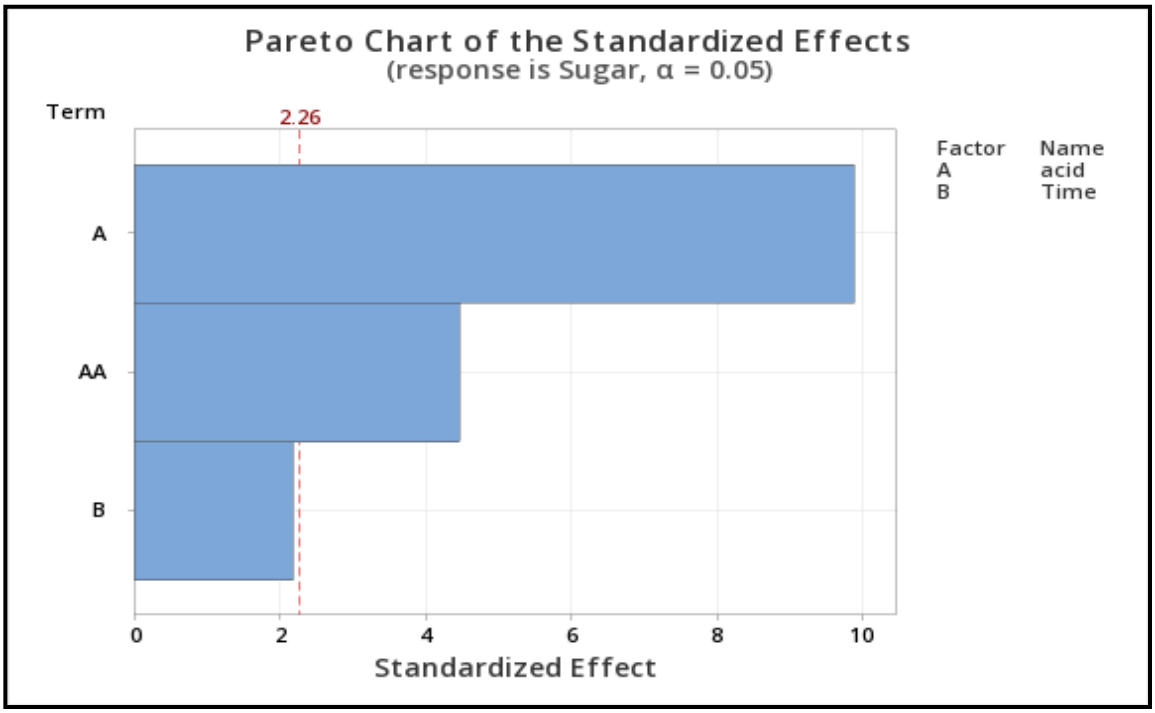


Figure 27 Pareto Chart of Standardized Effect

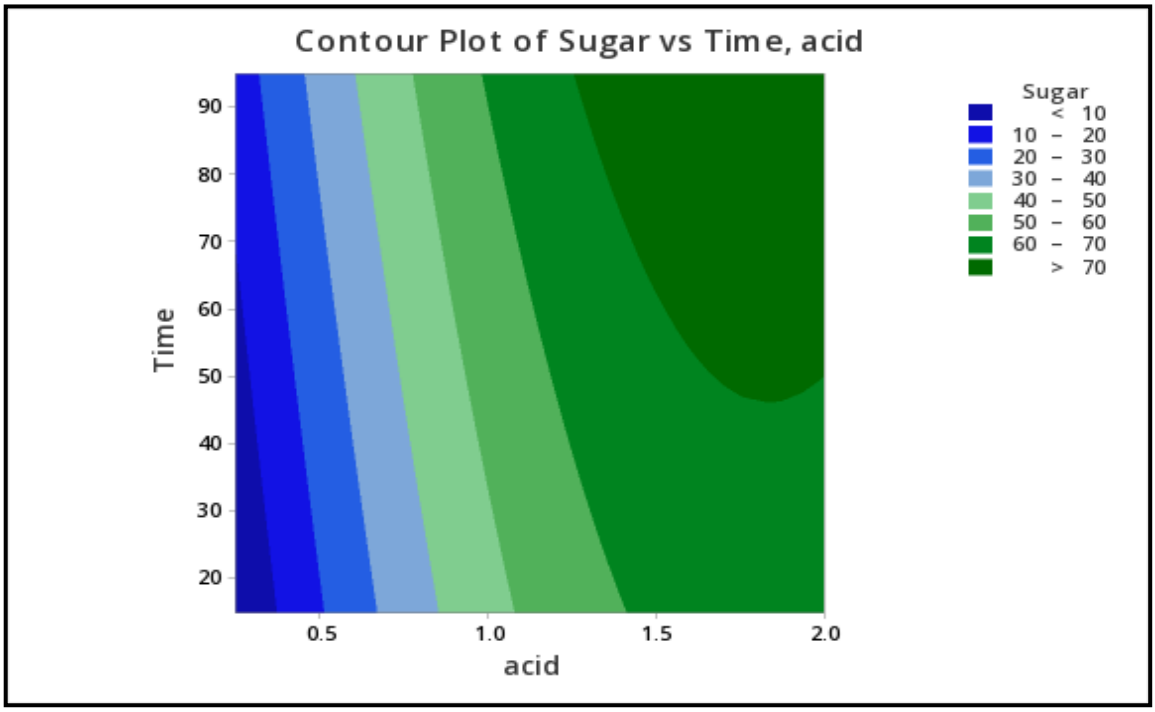


Figure 28 Counter Plot of Sugar Vs Time and Acid by Central Composite Design

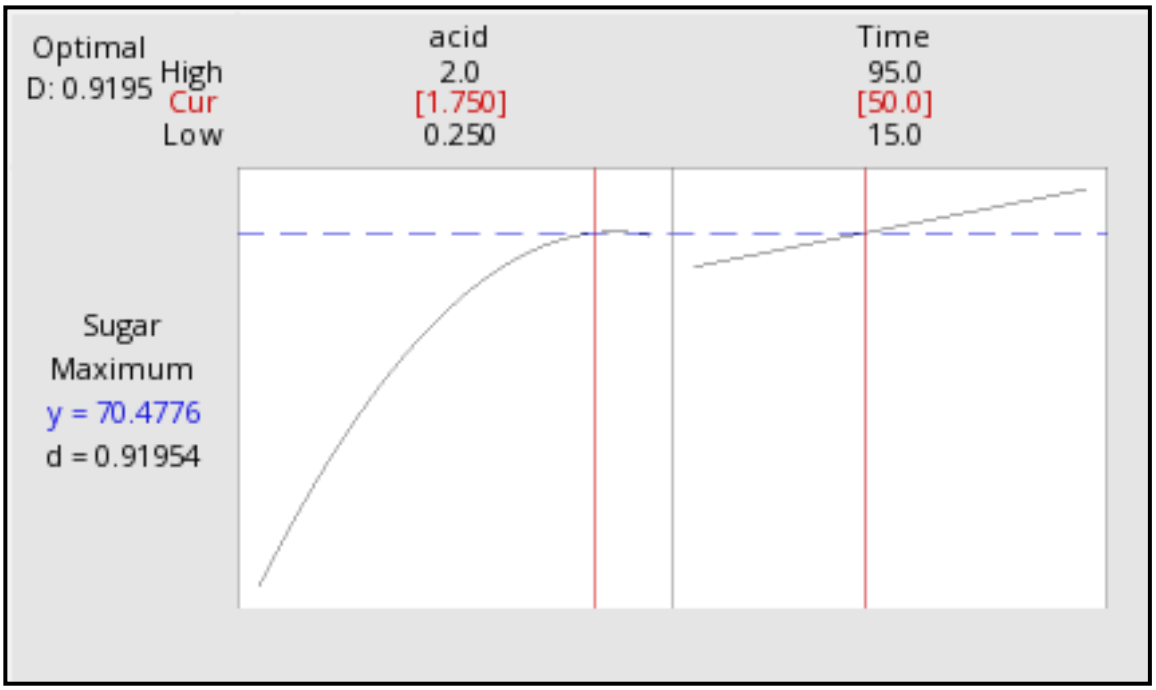


Figure 29 Optimum Condition of Sugar Extraction by Central Composite Design

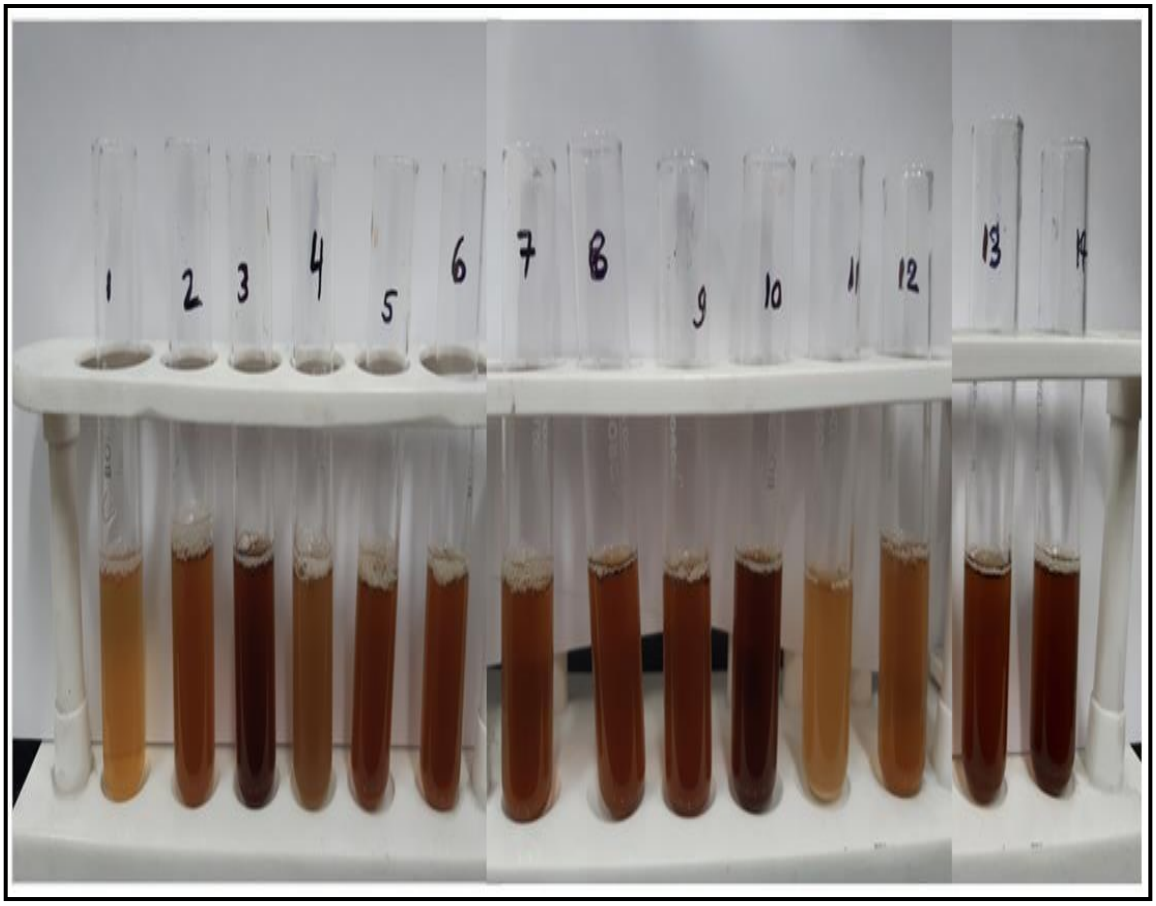


Figure 30 Recovered Liquid after Acid Hydrolysis Using Response Surface Methodology

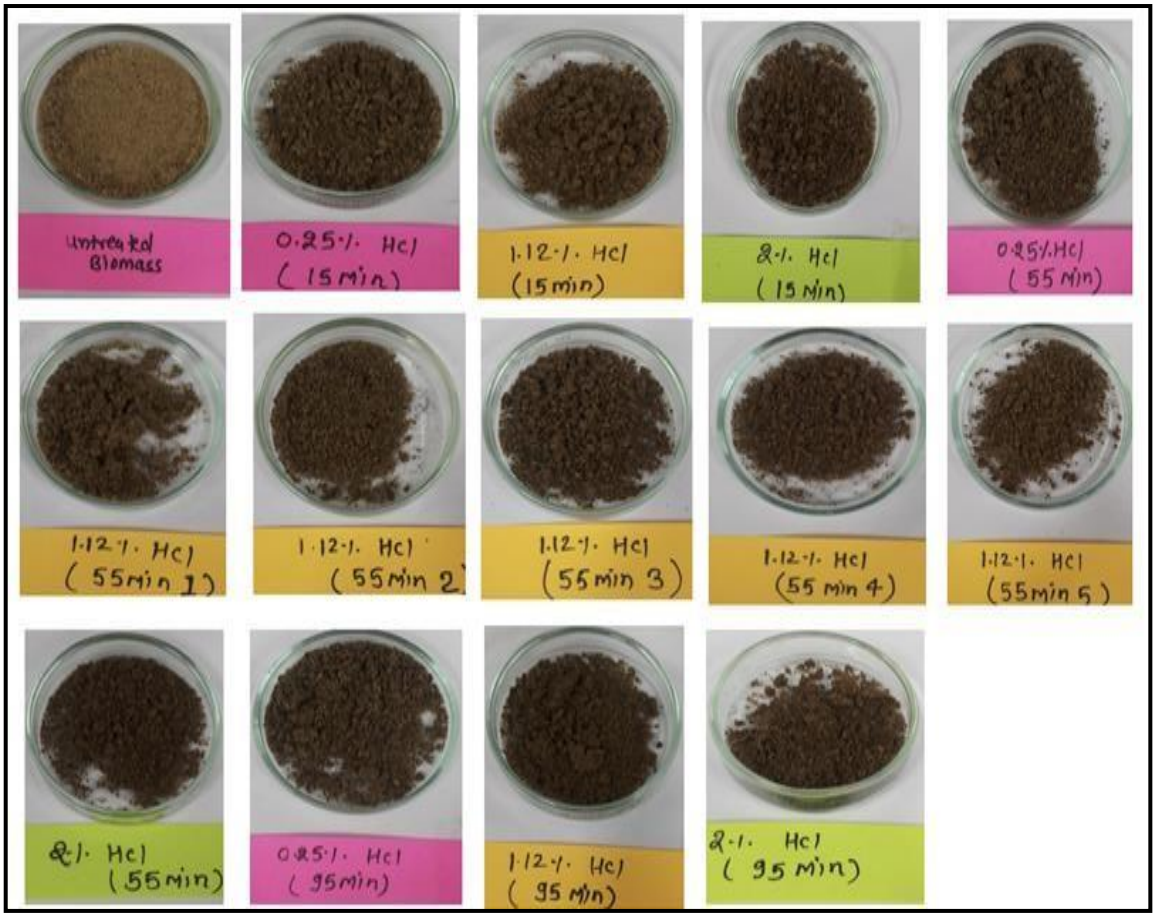


Figure 31 Recovered Solid after Acid Hydrolysis Using Response Surface Methodology

3.4 Thermochemical Characterization of Biomass

To check the efficiency of acid pretreatment characterization of pretreated and untreated biomass was done by FTIR, TGA and SEM analysis

3.4.1 FTIR

Infrared (IR) studies were conducted to assess the chemical modifications in *S. tuberosum* periderm waste before and after acid hydrolysis treatment. Key peaks in the FTIR spectra of treated and untreated biomass are depicted in Figure 32. Treated samples exhibited lower peak intensities compared to untreated ones, indicating the efficient removal of various biomass constituents.

In the untreated sample, a peak at 3480 cm^{-1}, corresponding to the free and intermolecular O-H stretch, suggests the presence of starch. However, in the case of

treated biomass, a reduction in peak intensity and a shift in the band to 3369 cm^{-1} indicate the conversion of starch into glucose.

In the untreated sample, the peak at 3000 cm^{-1} corresponds to the free hydrogen-bonded OH stretching of cellulose. The band at 2947 cm^{-1} represents the stretching of functional groups, methyl and methylene of cellulose. Post-treatment, there is a significant reduction in cellulose content, as evidenced by the low-intensity peak at 2850 cm^{-1} as compared to untreated sample (3000 cm^{-1})

Sharp peaks observed between 1657-1463 cm^{-1} in treated *S. tuberosum* periderm are attributed to the C=C stretching of the aromatic structure of suberin. This peak distribution pattern is consistent with findings from previous studies, such as the work of (Malakar et al., 2020) on acid-treated *S. tuberosum* periderm waste and the research conducted by (Liang and McDonald, 2014), which compared raw biomass with its residue after fermentation. Both studies reported similar patterns in O-H stretching, C-H (indicative of carbohydrate presence), C=O, and C-O-C (related to hydroxy fatty acid and suberin).

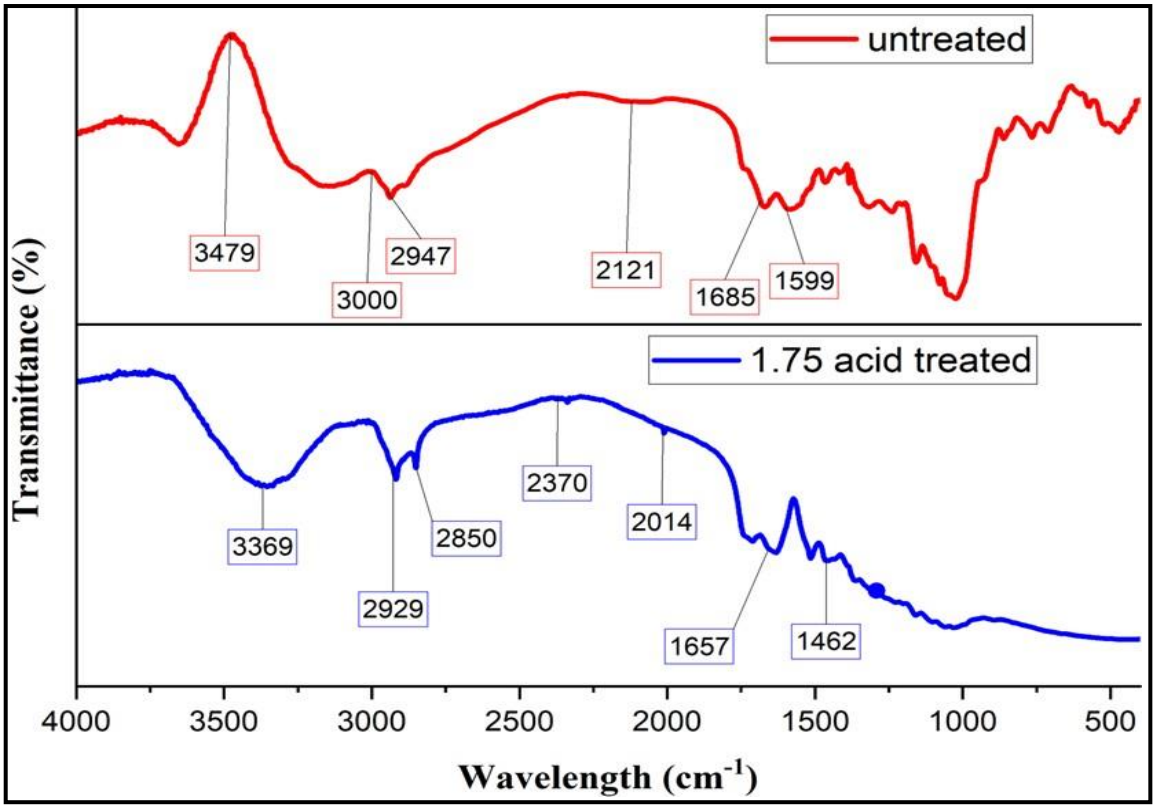


Figure 32 Thermochemical Characterization of Potato Peel Waste Before and After Acid hydrolysis . Above Figure Represents the FTIR of the Untreated and Treated Sample

3.4.2 TGA

Pre-treated (1.75%), and untreated potato peels were thermogravimetrically analyzed to compare their thermal decomposition rate (Figure 33, 34) Considering DTG of Peel biomass has two weight fall regions in all the samples, the first weight loss peak was noted at 100 °C, which was mainly due to dewatering, Weight loss between 200-300°C shows the depolymerization of cellulose and hemicellulose. The peak at 350-380 °C shows the breakage of the glycosidic bond of starch. TGA graph of untreated showed peak at 354 °C, loss of 59.941% weight loss and in case of optimum condition that is 1.75% acid and 50 minutes time approx. 60% weight loss observe at 429 °C which shows the removal of maximum starch from potato peel due to acidic hydrolysis. Thermal decomposition behavior in terms of TGA and DTG of potato peel sample was found similar in study by (Liang and McDonald, 2014). Presence of distinct peak areas of starch, cellulose and hemicellulose at (220-400 °C) while in case of lignin and

suberin, notable peaks found at (250 -290 °C). Behavior of similar pattern of thermal degradation of potato peel sample, as described in mentioned literature proves the presence of distinct biomolecules in the sample.

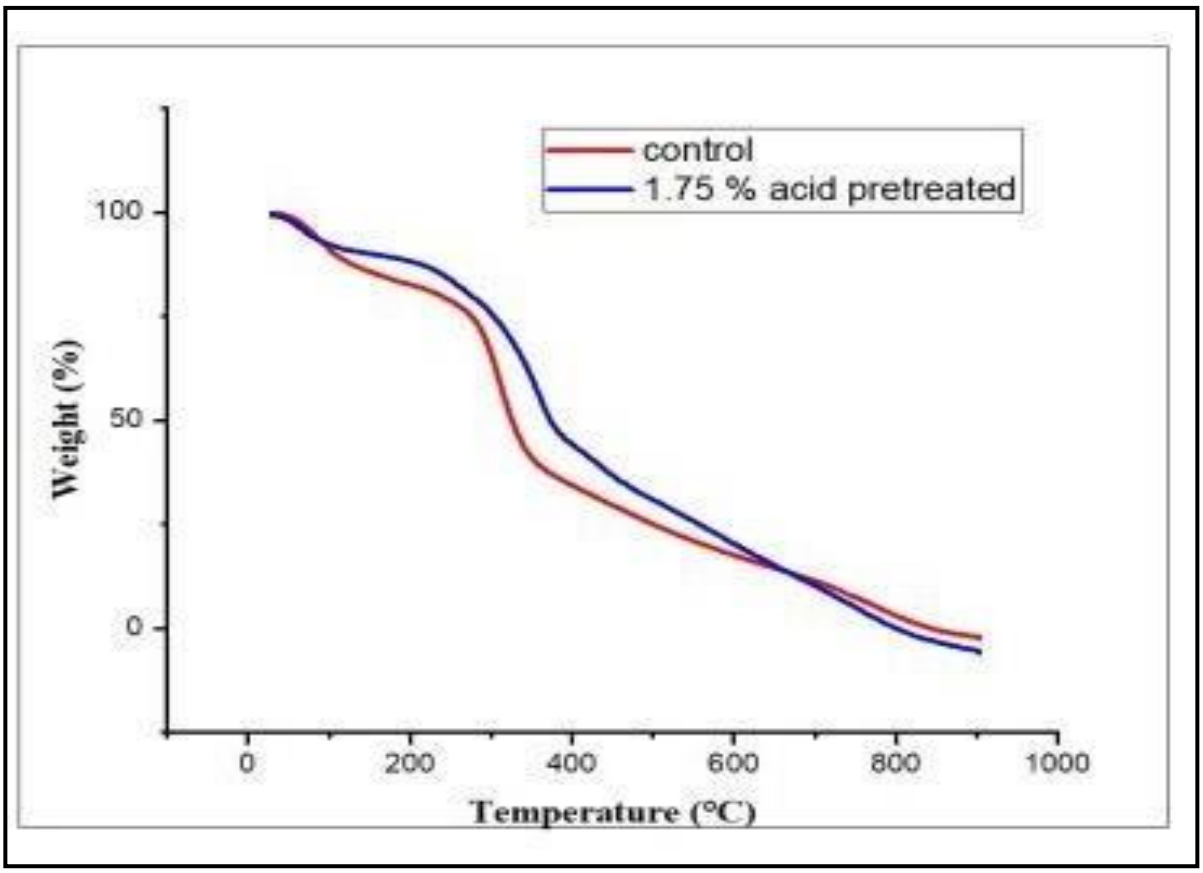


Figure 33 Thermochemical Characterization of Potato Peel Waste Before and After Acid Hydrolysis Represents the TGA

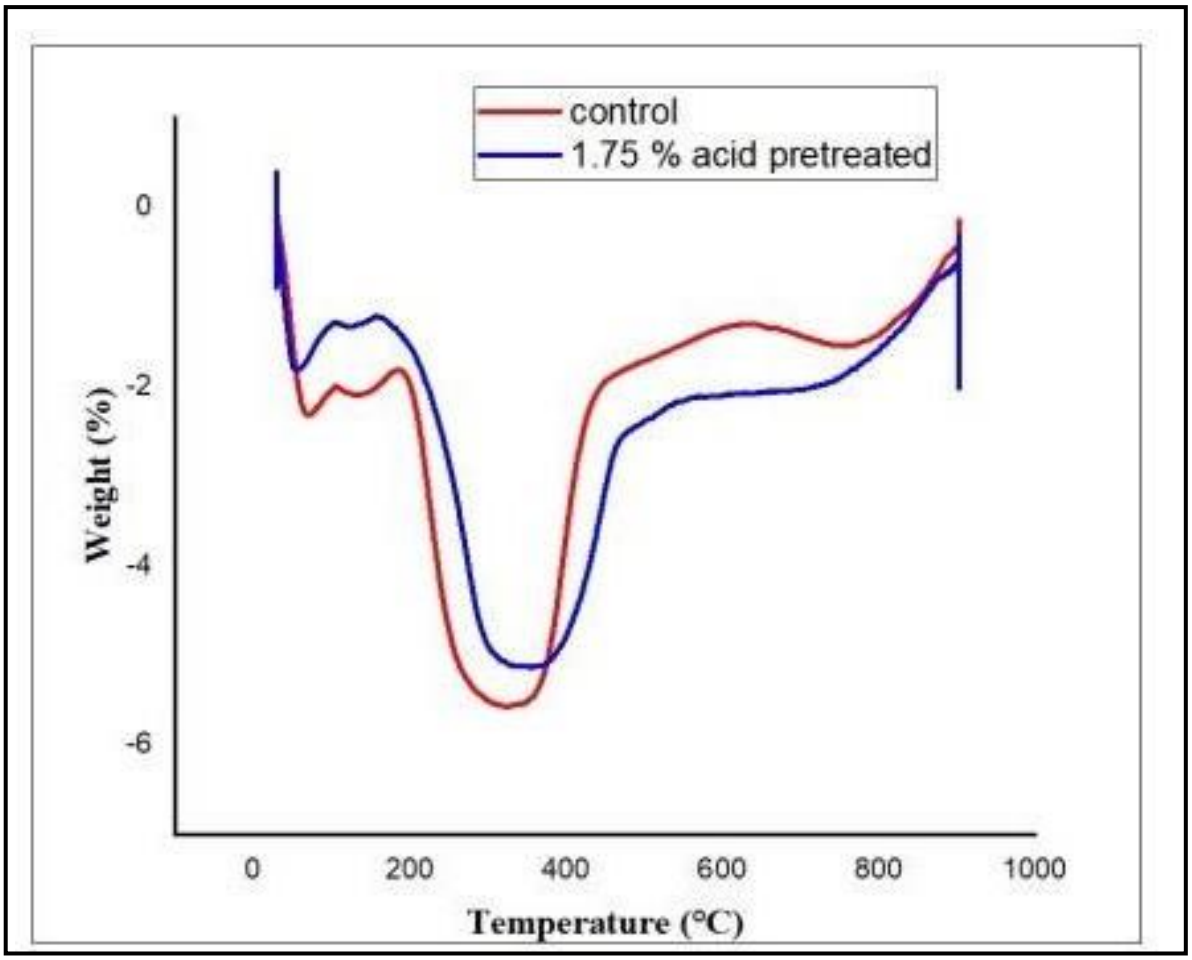
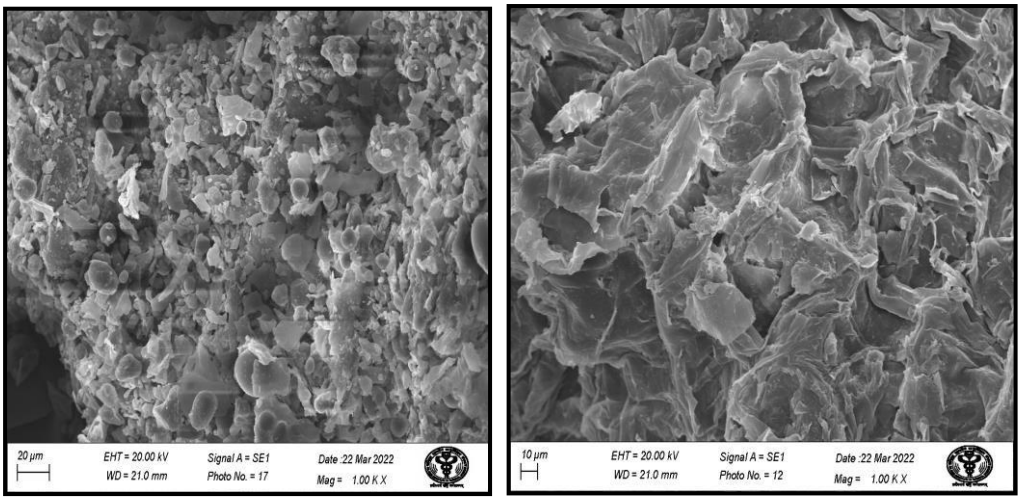


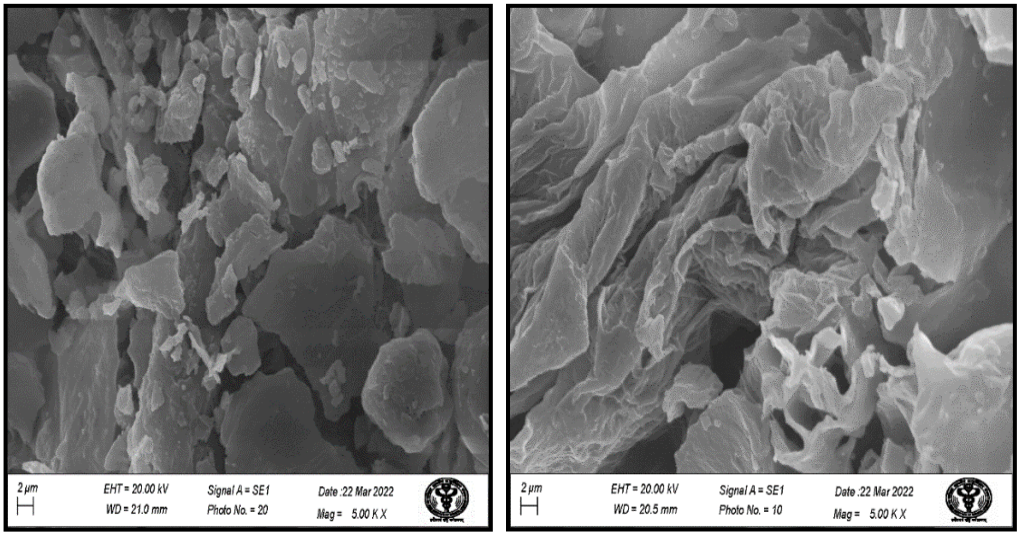
Figure 34 Thermochemical Characterization Of Potato Peel Waste Before and After Acid Hydrolysis Represents The DGA

3.4.3 SEM

Surface morphology of pre-treated and untreated potato peel waste was shown with the help of scanning electron microscopy. The images clearly show the difference between pre-treated and untreated samples. The untreated sample is compact, and smooth whereas structural alteration shown in case of pretreated sample. Suggested that due to acidic treatment, release of maximum sugars observed as the bond between lignin and carbohydrate broke down. (Figure 35).



(A) (B)



(C) (D)

Figure 35 Thermochemical Characterization of Potato Pee*l* Waste Before and After Acid Hydrolysis (A & C) Represents the Untreated Sample And (B & D) Represents the Treated Sample

3.5 PHA Production by *Pseudomonas Putida* and Extraction in Different Production Media

Growth pattern of *Pseudomonas putida* was observed in different types of production media (A, B, C and D) shown in Figure 36, 37, 38, and 39. Cell biomass was collected at different time intervals such as 24, 36, 48 and 72h in Four different media mentioned above. Biopolymer accumulation decreases inside the bacterial cell after 48h of fermentation in each type of media due to depletion of carbon source because deposited PHA supplies energy for the persistence of Cells in carbon exhausting condition (Maheshwari et al., 2018). CDW was found highest in the case of 48h in each production media. (Figure 40) contains detailed information in terms of type of media, time (h) cell dry weight (g), PHA% and residual sugar after each fermentation experiment. Mineral salt media containing synthetic glucose was found to be the best PHA accumulator (29.97± 0.45%) due to presence desired salt concentration for optimum growth of *P .putida* but the ultimate goal of this study is to use a sustainable carbon source (ppw) to minimize the production cost of PHA as carbon source cost approximately 50% of entire process (Jiang et al., 2016). So, the mineral salt media (C) was taken just to compare the potato peel hydrolysate (extracted sugar) in terms of PHA accumulation. Other than mineral salt media cell dry weight was slightly higher in case of modified media (A) containing ppw hydrolysate, tryptone and NaCl as compared to media (B) only hydrolysate (1%) and media (D) L.B media. Considerable difference observed in PHA accumulation indicates that PHA producing bacteria require nutritional stress (N) and carbon excess condition to harbor PHA which was not suitable in case of L.B media. In the case of only potato peel hydrolysate carbon was in the desired amount (1%) but NaCl, to maintain osmotic balance and nitrogen was completely absent which shows that biomass obtained from only ppw hydrolysate lacked biopolyesters components (polyhydroxyalkanoates) as reported by (Passanha et al., 2014). However, % of extracted PHA is calculated gravimetrically in each media which is shown in (Figure 36, 37, 38, and 39) but for GCMS MS analysis only media (A) is selected due highest extracted PHA%. However, PHA production from potato peel waste is reported for the first time in this study with 28.71±0.55 yield at 48h with media (A) which is close to PHA accumulation in mineral salt media (C) Although, it

is assumed that acid pretreated sugar has its own stress factors (phenolics) which are responsible for accumulation of high percentage of PHA. (Sharma et al., 2012) reported 22.6% mcl PHA of CDW from an isolated strain of *Pseudomonas putida* LS46 which is closely related to KT2440 (a potent PHA producing recombinant strain) by using synthetic glucose (2%) as a carbon source but This study shows a satisfactory amount of PHA production using only sugar hydrolysate (1%) NaCl (0.5%) and negligible amount of tryptone (0.1%) Also at the economic point of view, high amount of sugar (70%) can be extracted from potato peel biomass which is quite higher in case of other lignin and complex sugars containing organic waste. The comparative study with *P. putida* with other carbon sources is discussed in Table. However, extracted sugar % can vary in different species of potato peels and applied peeling method but considerable amount of starch is present in potato peel, could be used for biodegradable bioplastic (PHA) production which is discarded otherwise.

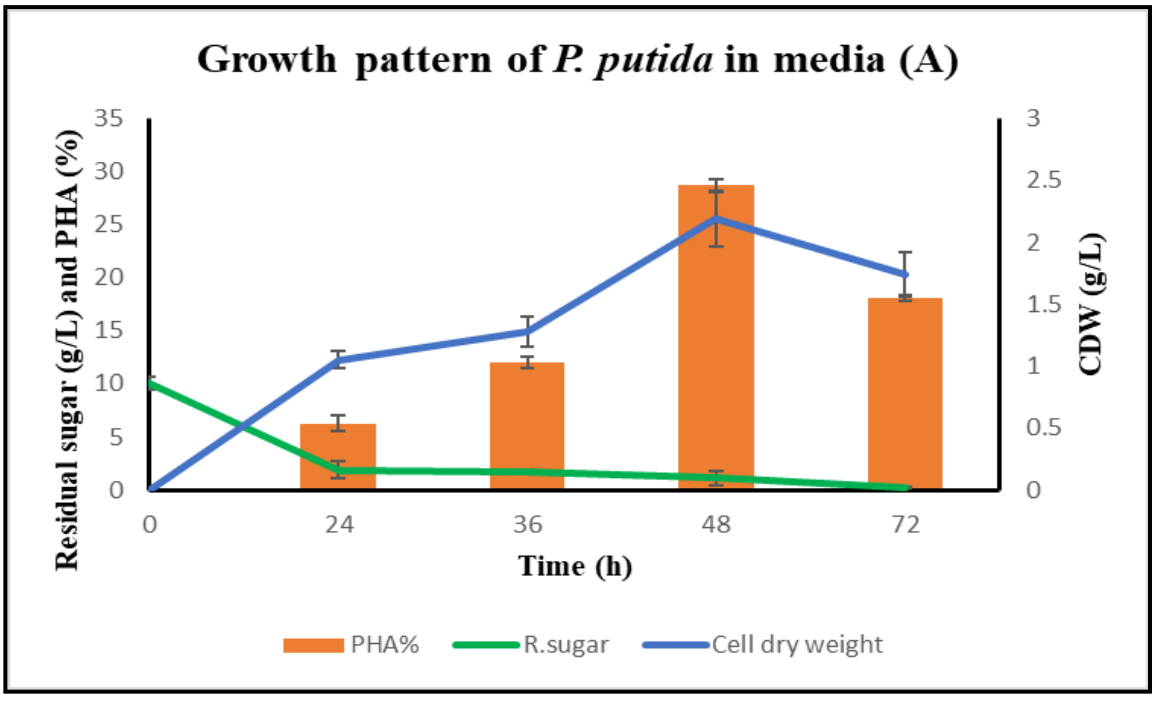


Figure 36 Growth Pattern of *P. putida* in Media A Containing Tryptone, Sodium Chloride, and Potato Peel Hydrolysate

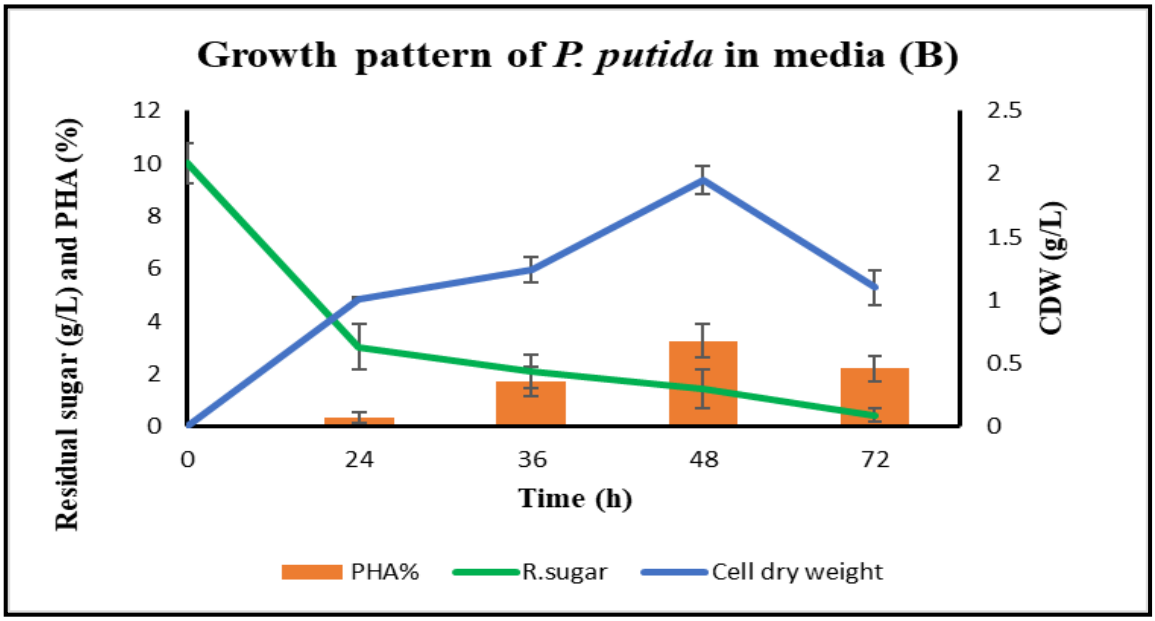


Figure 37 Growth Pattern of *P. putida* in Media B Containing Only Potato Peel Hydrolysate

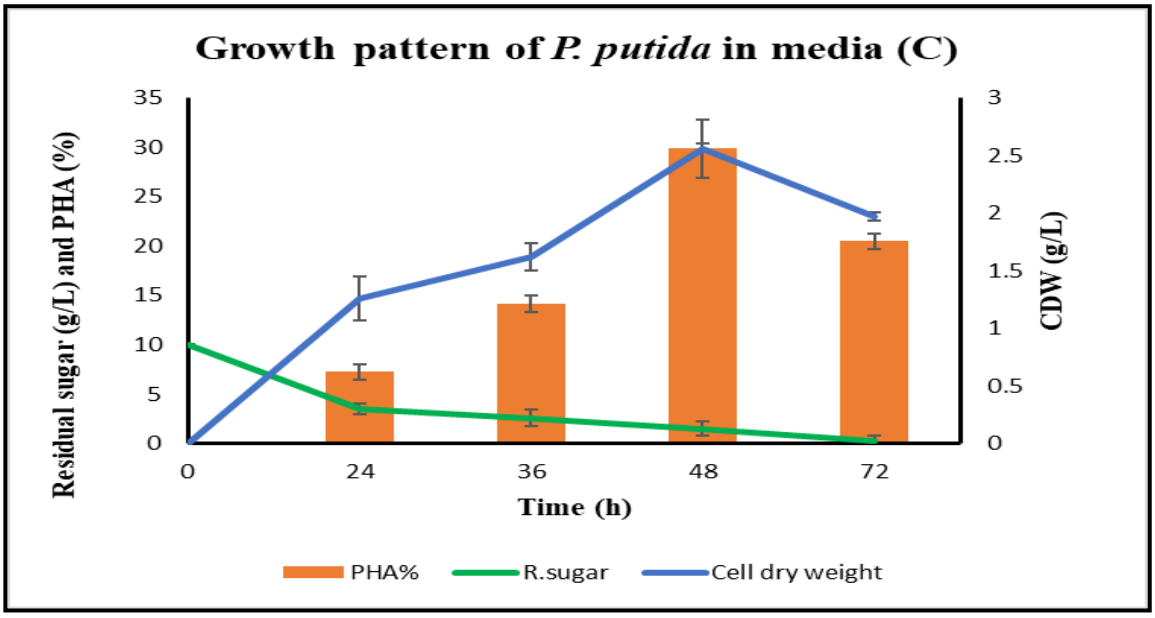


Figure 38 Growth Pattern of *P. putida* in Media C Mineral Salt media Containing $Na_2HPO_4 \cdot 7H_2O$ (30 g/L), KH_2PO_4 (15 g/L), NaCl (5 g/L), NH_4Cl (1 g/L), $MgSO_4$ (2 g/L), $CaCl_2$ (0.1 g/L), and synthetic glucose (10 g/L)

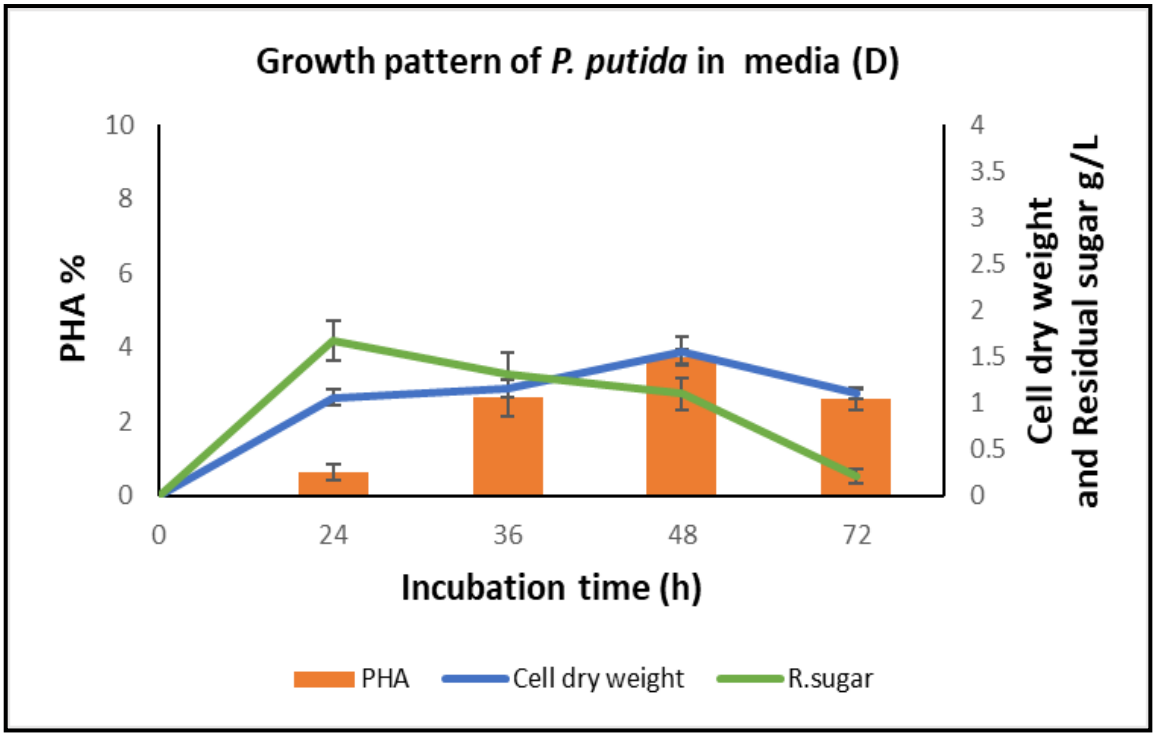


Figure 39 Growth Pattern of *P. putida* in Luria Bertani Media

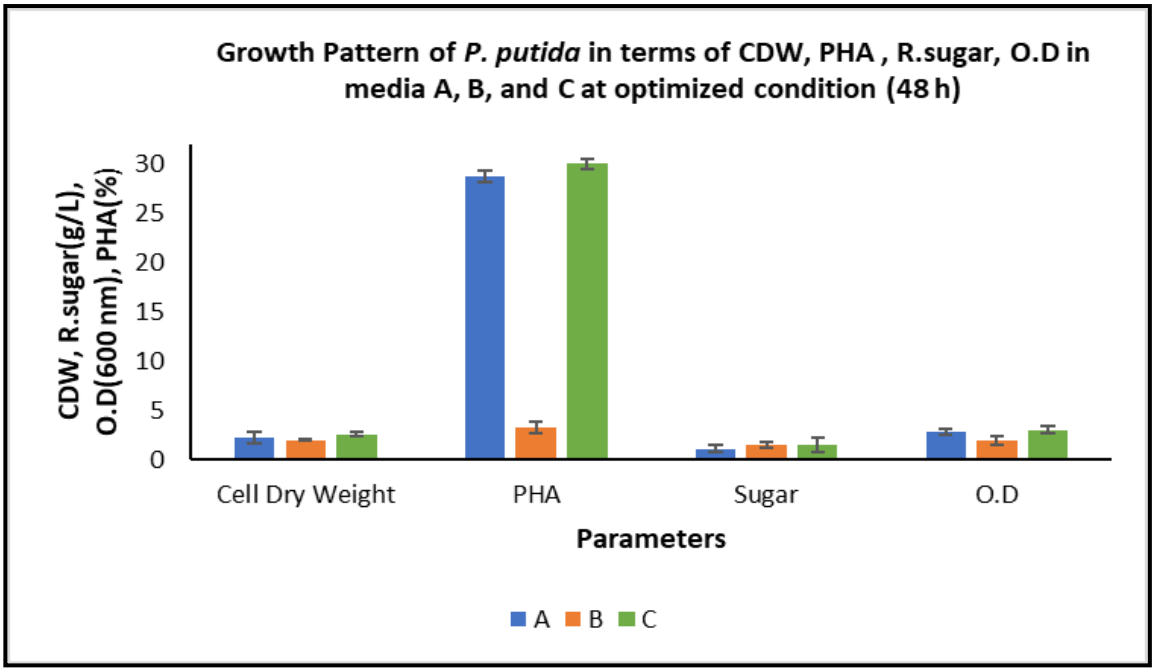


Figure 40 Growth pattern of *P. putida* in terms of Cell Dry Weight, PHA, Residual

Sugar and Optical Density at Optimized Condition (48 h).

3.6 PHA Monomer Conformation

Based on the GC-MS-MS analysis, using *S. tuberosum* periderm waste hydrolysate as the sole carbon source resulted in the formation of medium-chain length polyhydroxyalkanoates (mcl PHA) containing three distinct monomers: 3-hydroxydodecanoate (3HDD) with a retention time (RT) of 6.99 minutes and a peak area of 1.75%., 3-hydroxydecanoate (3HD) with an RT of 10.34 minutes and a peak area of 1.72% and 3-hydroxytetradecanoate (3HTD) with an RT of 8.52 minutes and a peak area of 19.2%. (Figure 41).

These identified monomers demonstrate the feasibility of producing industrially important mcl PHA with carbon sources of low cost, such as *S. tuberosum* periderm hydrolysate. This approach enables the construction of mcl PHA with chain lengths ranging from 5 to 14 carbons, making it an economically attractive option.

This finding aligns with the work of (Mahato et al., 2021), who reported the presence of beta-3-hydroxybutyric acid (7.41%), 3-hydroxytetradecanoic acid methyl esters, and 3-hydroxyhexadecanoate (5.03%) in PHA produced by *Pseudomonas aeruginosa*. However, it's important to note that their PHA production was observed in mineral salt media containing different oils as carbon sources, which may not be as sustainable and could potentially incur higher costs compared to the approach used in the present study. In a study (Ciesielska et al., 2017) reported mcl PHA monomers such as 3-hydroxyhexadecanoate and 3- hydroxyoctadecanoate from *Pseudomonas putida* KT2440 using oleic acid as a carbon source.

By comparing PHA monomers produced by *Pseudomonas* from different studies it is evident that PHA extracted from *P. putida* MTCC 2475 (present study) is mcl PHA.

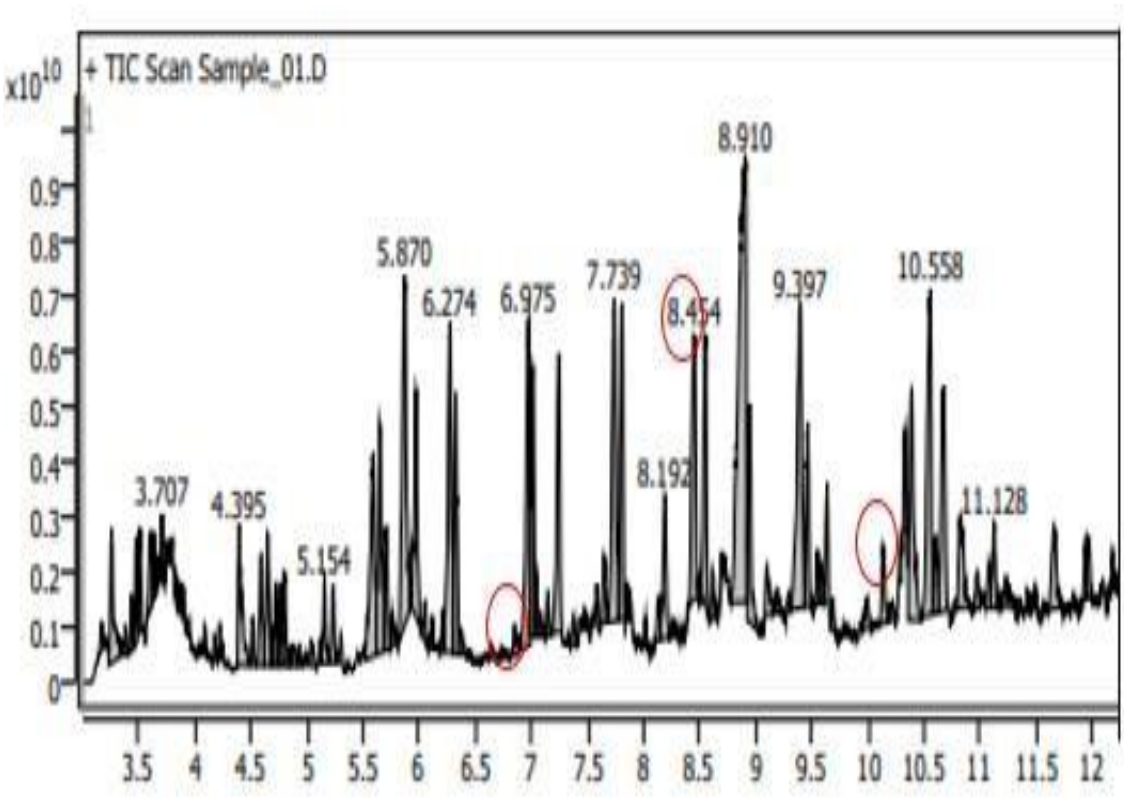


Figure 41 GCMS-MS Spectra of Extracted PHA Monomers Using *Pseudomonas putida* in Acid Hydrolyzed Potato Peel Media

3.7 Characterization of Extracted PHA Film

The PHA film was created by adding extracted PHA in solvent (Chloroform) and stored securely under aseptic conditions at room temperature. (Figure 45). The Differential Scanning Calorimetry (DSC) thermogram presented in Figure 44 reveals the melting temperature (T_m) of the produced Polyhydroxyalkanoates (PHA), which was found to be approximately 170 °C. This temperature closely aligns with the melting temperature of 3-hydroxydecanoic acid (166 °C), as reported in the literature (Sharma et al., 2017).

The identification of functional groups within the extracted PHA was accomplished through Fourier Transform Infrared Spectroscopy (FTIR), as illustrated in Figure 42. The spectral pattern of functional groups closely resembled findings from (Mahato et al., 2021), who identified different forms of decanoic acid methyl esters, including

Methyl 3-hydroxytetradecanoate and hexadecanoic acid methyl ester, from *Pseudomonas aeruginosa*. Notable absorption bands included: (i) A band at 3437 cm^{-1}, attributed to the O-H stretching of the hydroxyl group in PHA.

(ii) The band at 2929 cm^{-1}, assigned to the CH_2 group. (iii) An absorption band at 1733 cm^{-1}, indicating C=O stretching vibration, confirming the presence of an ester bond in the PHA monomer. (iv) A band at 1277 cm^{-1}, associated with the asymmetric C–O–C stretching vibration. Furthermore, the Proton Nuclear Magnetic Resonance (NMR) spectrum of the PHA film is depicted in Figure 43. The complex multiple resonance bands between 1.26 and 1.33 ppm (peak no. 1) suggest the presence of methyl groups ($–CH_3$) in the PHA film. Multiple resonance spectra observed between 2.17 and 2.60 ppm (peak no. 2) confirm the existence of methylene groups (–CH2). The band position ranging from 5.22 to 5.27 ppm (peak no. 3) indicates the presence of methane (–CH) in the PHA polymer. Peak no. 4 corresponds to the solvent $CDCl_3$. These observed peaks align with prior studies on medium-chain length PHA produced by *Pseudomonas* strains, as reported by (Gumel et al., 2014). Comparing the results of the present study with findings from various previous investigations, it can be confidently affirmed that the extracted polymer is indeed medium-chain length Polyhydroxyalkanoates (PHA).

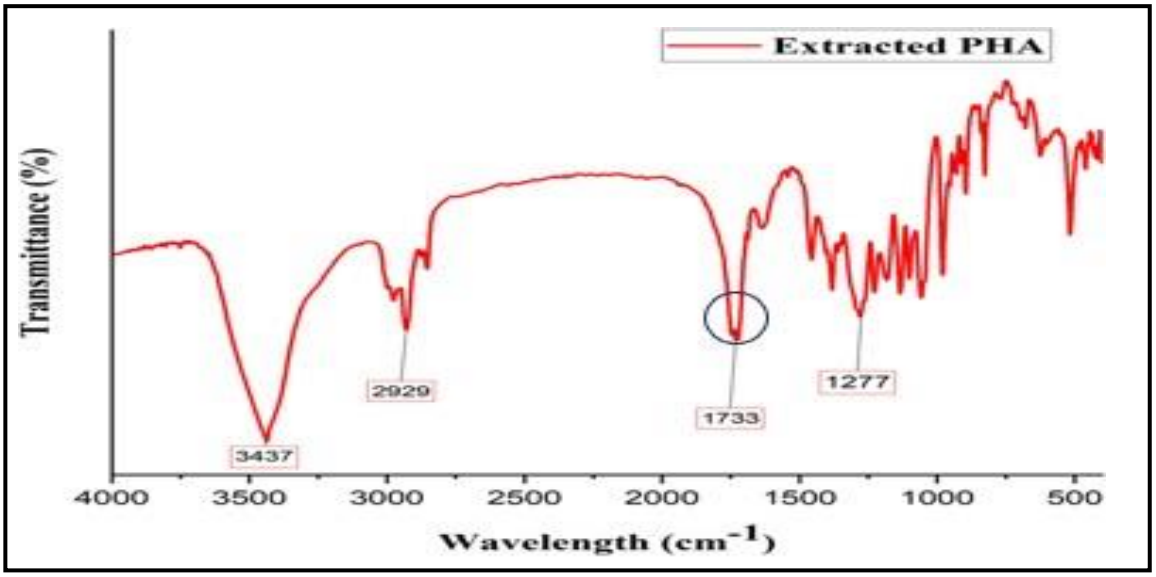


Figure 42 FTIR Spectra of Extracted PHA Monomers Using *Pseudomonas putida* in Acid Hydrolyzed Potato Peel Media

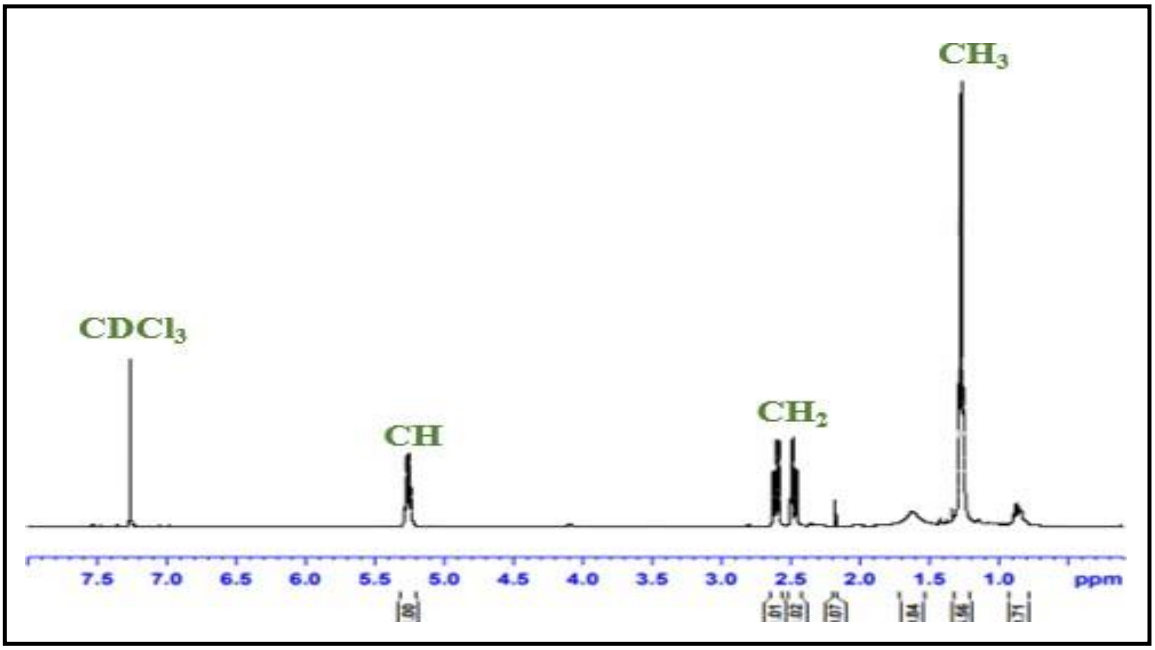


Figure 43 NMR Spectra of Extracted PHA Monomers Using *Pseudomonas Putida* in Acid Hydrolyzed Potato Peel Media

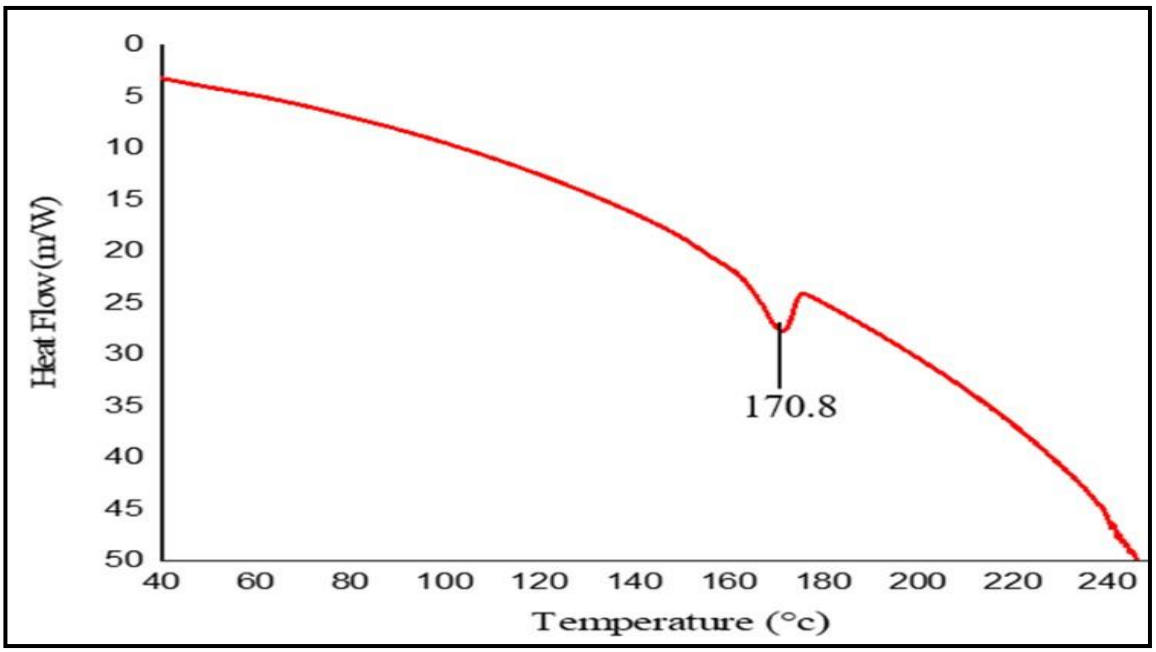


Figure 44 Thermal Degradation Analysis of Extracted PHA Monomers Using *Pseudomonas Putida* in Acid Hydrolyzed Potato Peel Media

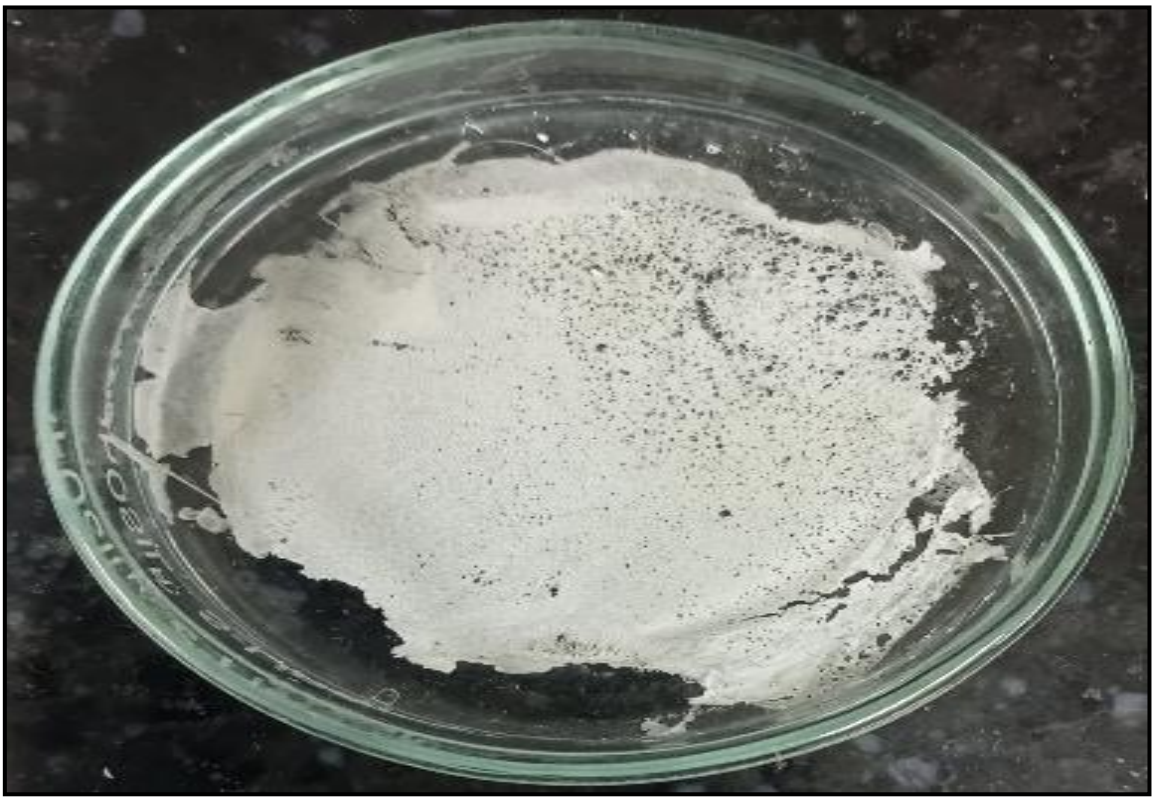


Figure 45 PHA Film

3.8 Mass Balance and Economic Analysis of The Study

The experiment commenced with 100 grams of *S. tuberosum* periderm waste used for sugar extraction, with a solid loading of 10%. This extraction process yielded 69.34 ± 0.25%, of sugar. Out of the total sugar obtained, 10 grams were used for the fermentation process per liter, resulting in the production of 2.19 grams of dry cell weight and 0.60 g/L PHA. Conducting an economic assessment revealed that the production of 1-liter media with a concentration of 0.60 g/L of PHA incurred a cost of 239 Rs. In contrast, the acid hydrolysis method utilized a total of 100 grams of biomass, yielding approximately 70 grams of sugar. For the subsequent fermentation, only 10 grams of this sugar were utilized, allowing the preparation of an additional 6 L fermentation media. Scaling down the acid hydrolysis process for a single-liter production media has the potential to decrease cost. Moreover, the cost of PHA cannot

be compared with conventional plastic because along with packaging material it possesses biodegradable, sustainable, eco-friendly, medical application properties.

Based on these results, it can be estimated that approximately 4.35 grams of PHA can be produced from 100 grams of *S. tuberosum* periderm waste. Figure 46 provides an operational flow diagram of mass balance and economic analysis, illustrating the process of PHA production from *S. tuberosum* periderm waste.

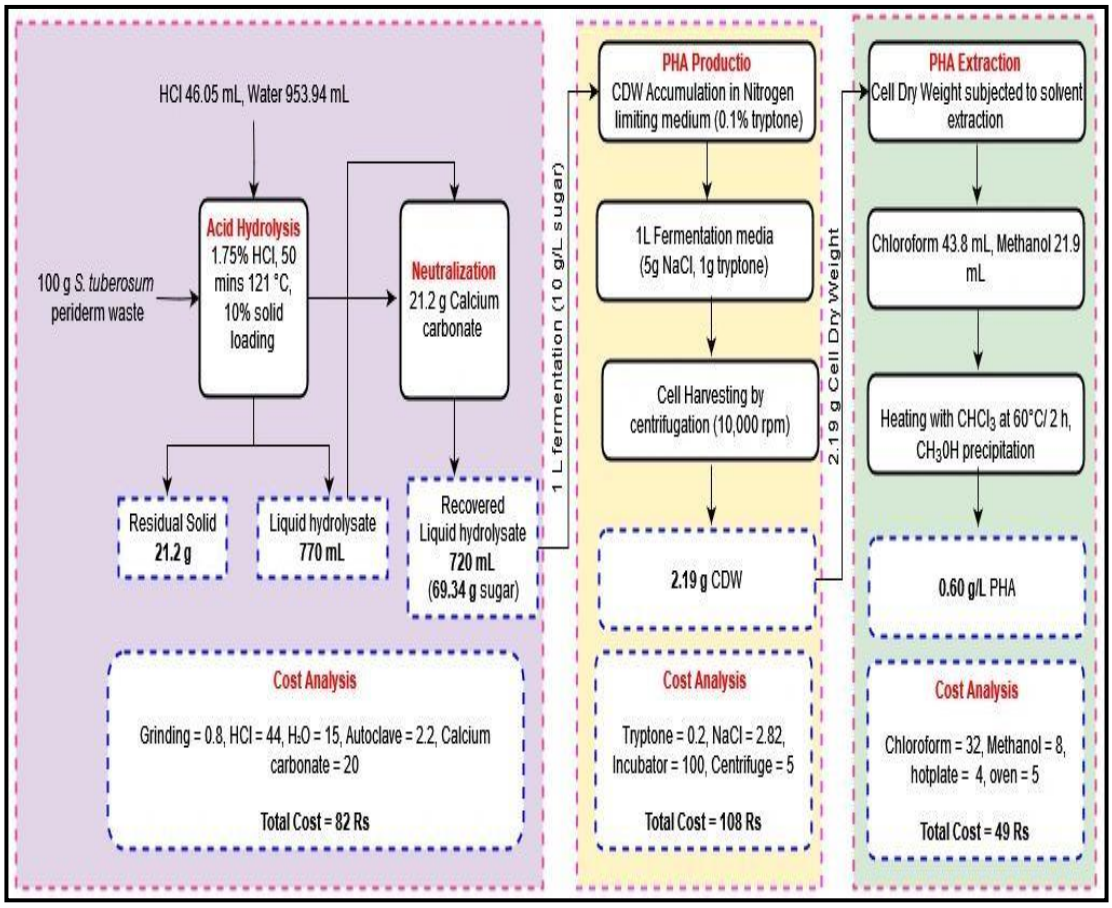


Figure 46 Mass Balance Study of PHA Production from Potato Peel Waste Using *P. putida*

Comparison of PHA production by different strains of *Pseudomonas putida* based on fermentation parameters such as carbon source, incubation time, agitation speed, and results were obtained in the form of cell dry mass, PHA%, and monomer composition. of fermentation parameters such as carbon source, incubation time, agitation speed,

and results were obtained in the form of cell dry mass, PHA%, and monomer composition are present in Table 17

Table 18 PHA Accumulation by Different Species of *Pseudomonas*

S. No	Bacteria used	Carbon source	Cell DW	PHA	Monomer	Reference
1	*P. putida* KT2440	Glycerol (1%	2.6 g L−1	20.70%	Methyl 3-Hydroxyhexadecanoic acid, methyl 3-hydroxyoctadecanoic acid, methyl 3-hydroxydecanoic acid, methyl 3-hydroxydodecanoic acid, methyl 3-hydroxytetradecanoic acid	(Xu et al., 2021).
2	*P. putida* GO19	Terephthalic acid	-	23%	3-hydroxyhexanoic acid, 3-hydroxyoctan-oic acid, 3-hydroxy-dodecanoic acid, 3-hydroxydodecenoic acid	(Kenny et al., 2008).
3	*P. putida* (MTCC 2475)	*Solanum tuberosum* periderm	2.19	28.71 ± 0.55% (0.60 g/L)	Methyl 3-hydroxydodecanote methyl 3 hydroxytetradecanote hexadecanoic 3 hydroxy methyl esters	Present study

3.9 PHA Accumulation by Bacillus circulans

3.9.1 PHA Production

The PHA production by Bacillus circulans in potato peel hydrolysate-containing media was observed in three different time intervals (24, 36, and 48 h). The physical growth parameters play a vital role in cell dry weight accumulation and PHA production. At 24 h of incubation, biomass was minimum (0.926±0.01), as actively dividing cells utilize sugar only for growth purposes. Cell biomass was found at maximum (1.36±0.02) up to 36 h as secondary metabolites (PHA) produced during the late stationary phase of the growth cycle when nutrients are in the limit. The stationary phase is signal when PHA synthesis enzymes such as 3-keto thiolase, acetoacetyl-CoA reductase, and PHA synthase start converting PHA from acetyl-CoA which is commonly knowns as inclusion bodies (Cooper et al., 2007; Czech et al., 2019; Motavaf and Savage, 2021; Raghu and Divyashree, 2021). A depletion of cell biomass (1.09 ± 0.06) until 48 h was observed as storage granules are utilized as a source of energy by the bacteria. Table 4 and Figure 47 show representation of cell dry weight and PHA accumulation pattern in different time intervals. All three-production media (24, 36, and 48 h) are further subjected to extraction by solvent extraction method. Different studies have shown the PHA accumulation ability of a variety of Bacillus species by utilizing different organic wastes as carbon sources (Maheshwari et al., 2018; Muangwong et al., 2016). Subsequently, these carbon sources were applied in discrete combinations of other nutrient components, which showed the high PHA accumulation in the bacterial cytoplasm. For instance, in an optimization study [40], high yield of PHA (46.57%) was observed in *Bacillus endophyticus*; however, bacteria were cultivated in media containing salts such as Na_2HPO_4, KH_2PO_4, and sucrose 40 g/L was used as a carbon source. As several studies observed, PHA enhancement by optimizing the desirable C:N ratio [4, 48, 52] or by use of genetically engineered microbes. But in the present study, wild-type Bacillus circulans was taken and carbon was 10 g/L used from waste potato peels, and other growth-promoting micronutrients were not added in production media. A comparison of PHA production by different strains of Bacillus based on different parameters including polymer type, yield, and utilized carbon source is shown in Table 18. From the comparative studies, explained

in above-mentioned table, it is observed that potato peel waste can be a good carbon source for an adequate amount of PHA production by Bacillus circulans.

Table 19 PHA Production by Different Species of *Bacillus*

Bacteria	Type of polymer	Carbon source	Yield (%)	Reference
Bacillus megaterium	PHB	DSMZ media containing glycerol as a carbon source	14.11	(Hiremath and Sura, 2015).
Bacillus cereus	PHB	Grape residue	18.79	(Andler et al., 2021).
Bacillus sp.	PHB	Mineral salt media with 2% glucose	20	(Hassan et al., 2016).
Bacillus megaterium	PHA	Food waste-derived volatile fatty acid	10	(Vu et al., 2021).
Bacillus circulans	PHA	Potato peel waste	23	Present study

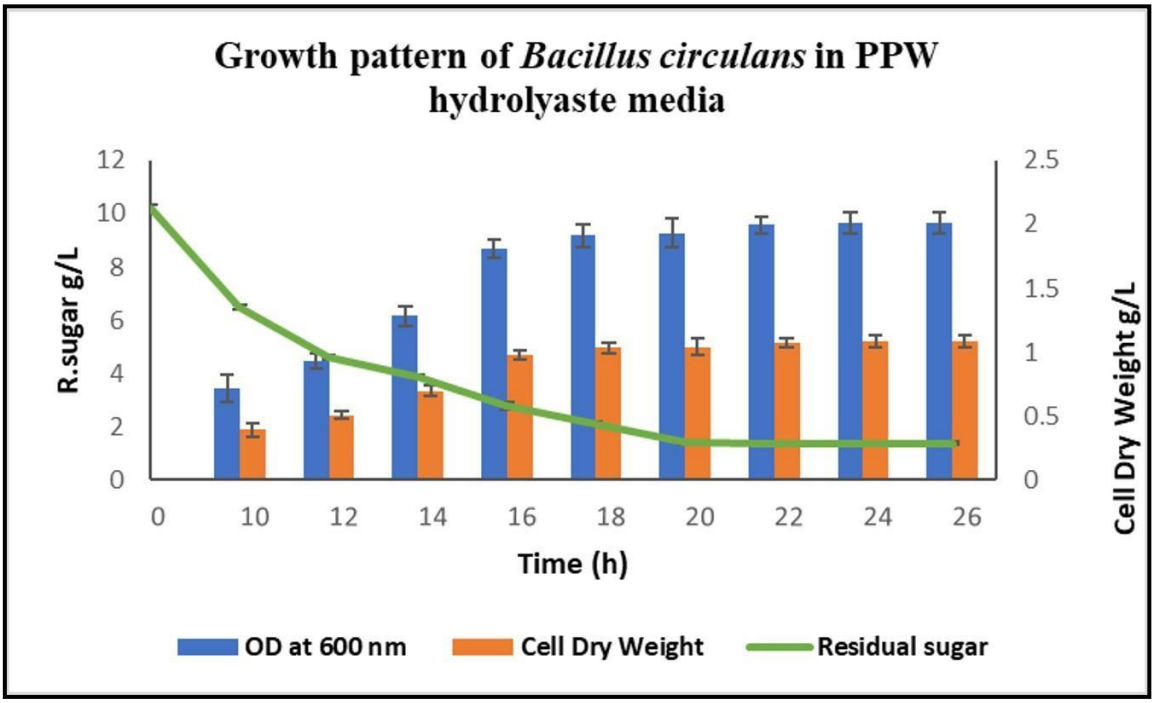


Figure 47 Growth Pattern of *Bacillus circulans* in Potato Peel Hydrolysate Media

3.9.2 PHA Production

PHA production by *Bacillus circulans* was done in potato peel hydrolysate media. Table 4 Representation of cell dry weight accumulation pattern in different time intervals (Table 19). As cell dry weight deposition was highest (1.36±0.02) at 36 h which is further subjected to extraction by solvent extraction method. Different studies have shown the PHA accumulation ability of a variety of *Bacillus species* by utilizing different organic wastes as carbon sources (Andler et al., 2021; Hiremath and Sura, 2015; Vu et al., 2021). Subsequently, these carbon sources were applied in discrete combinations, of other nutrient components, which showed the high PHA accumulation in the bacterial cytoplasm. For instance, in an optimization study (Raghu and Divyashree, 2021) high yield of PHA (46.57%) was observed in *Bacillus endophyticus*, however, bacteria were cultivated in media containing salts such as Na_2HPO_4, KH_2PO_4, and sucrose 40g/L was used as a carbon source. As several studies observed PHA enhancement by optimizing the desirable C: N ratio (Ahn et al., 2015; Rojas and Fajardo, 2021; Zhou et al., 2022) or by use of genetically engineered microbes (Gao et al., 2015). But in the present study wild type, *Bacillus circulans* was

taken and carbon was 10g/L used from waste potato peels, and other growth-promoting micronutrients were not added in production media. A comparison of PHA production by different strains of *Bacillus* based on different parameters including polymer type, yield, and utilized carbon source is shown in Table 18. From the comparative studies, explained in above mentioned Table, it is observed that potato peel waste can be a good carbon source for an adequate amount of PHA production by *Bacillus circulans.*

Table 20 Cell Dry Weight (g/L), PHA (g/L), and Residual Sugar (g/L) at Different Time Intervals in Potato Peel Hydrolysate Media

Time (h)	Cell dry weight (g/L)	PHA (g/L)	Residual sugar (g/L)
24	0.926 ± 0.01	0.1 ± 0.031	1.38 ± 0.05
36	1.36 ± 0.02	0.232 ± 0.041	0.8 ± 0.21
48	1.09 ± 0.06	0.184 ± 0.015	0.11 ± 0.14

3.9.3 GC-MS Analysis

By GC-MS analysis medium chain length, PHA monomers such as hexa decanoic acid 3-hydroxy, methyl ester, pentadecanoic acid 14- methyl -esters, and tetra decanoic acid, 12- methyl esters were identified with area % 4.25, 1.94, and 7.43% respectively using potato peel waste as a carbon source. However, Scl PHA was reported by B. cereus SS105 (Maheshwari et al., 2018). Moreover (Choonut et al., 2020) reported five different types of mcl PHA monomers reported by *Bacillus theroaylovorans*-related strains. The type of monomer accumulation inside the bacterial cell depends on the type of carbon source (Catherine et al., 2022).

3.9.4 Quantification of PHA

Extracted polymer (1 mg) was transformed into crotonic acid by adding 10 ml H_2SO_4 and absorbance was recorded at 235 nm against H_2SO_4 blank. (Figure 48). Obtained PHA was found at 0.232 ± 0.04 g/1000ml of production media. In the study 0.5g/L of

PHA was extracted from the *Bacillus marcorestinctum* and mineral salt media containing NH_2PO_4, KH_2PO_4, $MgSO_4$,$(NH_4)_2SO_4$, $CaCl_2$, NaCl, NH_4Fe, Citrate, mixture of trace elements, 2% cane molasses along with 10g/L glucose was used for PHA production (College, 2019). In literature (Choonut et al., 2020) reported 0.41± 0.01g/L of PHA by thermotolerant bacteria *Bacillus thermoamylovorans*, isolated from palm mill effluent where the MS media (mineral salt) enriched with $Na_2HPO_4.12H_2O$ (9g/L), KH_2PO_4 (1.5g/L), nitrogen stress in the form of 0.1g/L NH_4Cl and $MgSO.7H_2O$ was taken as the culture media. However, in the present study, 1% sugar from potato peel waste, NaCl, and 0.1g/L tryptone was used in production media which is very economic as compared to above mentioned study.

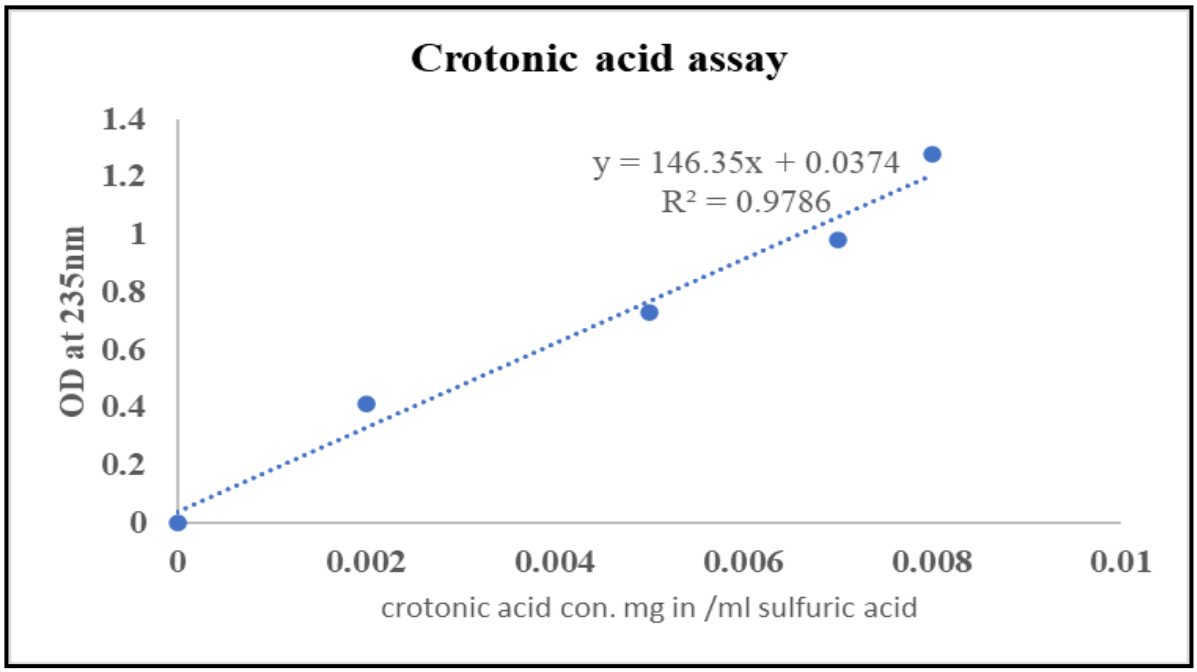


Figure 48 Standard Curve of Crotonic Acid Assay

3.9.5 Characterization of PHA

3.9.5.1 FTIR

Characterization of extracted PHA from *Bacillus circulans* was done with FTIR analysis. The distribution of functional groups present in FTIR spectra was performed based on existing literature (Mahato et al., 2021; Shah, 2012). PHA shows the band at

3437.13 cm^{-1} represents the hydroxyl group, the band at 2925.31 cm^{-1} represents C-H which shows the presence of methane and methylene groups, sharp band at position 1724.25 cm^{-1} indicates the presence of ester bond (C=O) which is a characteristic feature of polyhydroxyalkanoates (Joyline, 2019). 1460.02cm^{-1} which represents CH2, and band position at 1280.21 corresponds to C-O and 1184.10cm-1 corresponds to C-O-C (Figure 49). similar pattern of FTIR spectroscopy was obtained by *Bacillus megaterium BBST4* in a study done by (Porras et al., 2014). Almost similar pattern of FTIR peaks was found in PHA accumulated by *Bacillus cereus SS105* in which the spectral peak at 3264cm^{-1} represents the O-H group of polymer, for CH_3 group peak, found at 2973cm^{-1}, an outcome of changes of crystalline structure and 1736cm^{-1} band observed due to the presence of ester bond (Maheshwari et al., 2018). Hence it is confirmed that the deposited polymer in *Bacillus circulans* MTCC 8167 is PHA.

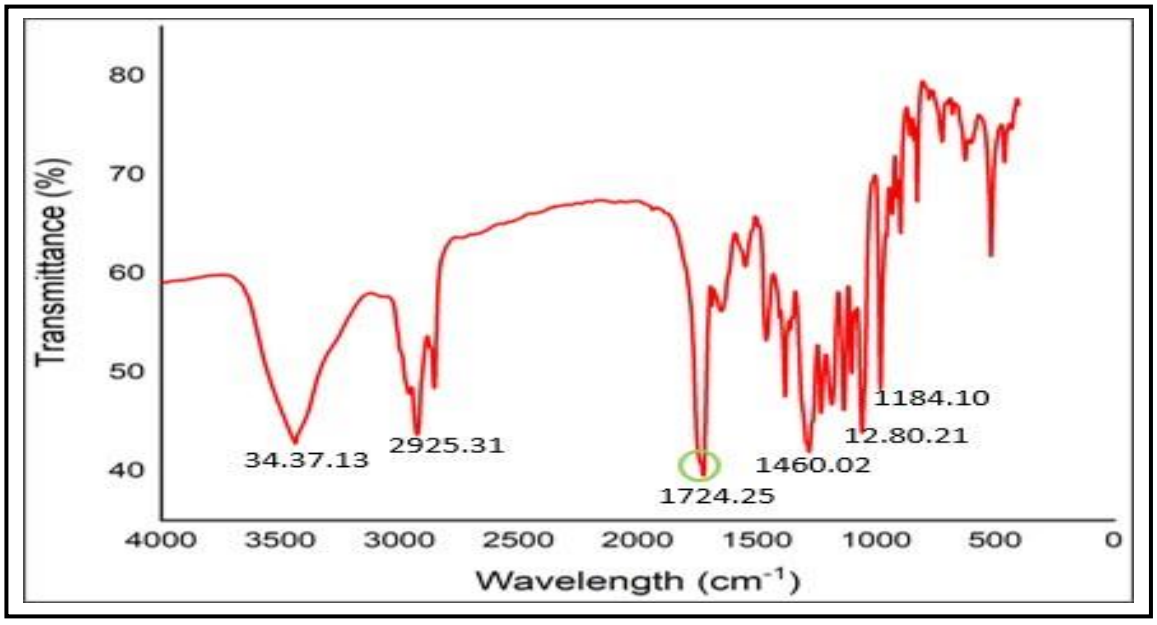


Figure 49 FTIR Spectra of Extracted PHA Monomers Using *Bacillus circulans* in Acid Hydrolyzed Potato Peel Media

3.9.5.2 DSC

A differential scanning calorimetry technique is done to check the melting temperature (T_m) of extracted PHA. The peak obtained in the DSC curve at 165.24°C indicates the

crystalization of the PHA polymer (Figure 50) Thermal characterization of PHA provides detail about the self-life of PHA and its stability. DSC of standard PHA ranges between 165- 170°C. Polymer degradation at low temperatures may be because of impurities. Although the rate of degradation of polymer is species-specific and may change upon using different extraction methods (Joyline, 2019). (Cueva-almendras, 2022) checked the DSC curve of PHA from isolated *Bacillus thuringiensis* and they found T_m at 166.92°C close to the curve observed in this study. (Vu et al., 2021) reported 145.43°C T_m which is lower than the present report from polymer accumulated by Bacillus megaterium using volatile fatty acid as a carbon source (Hassan et al., 2016) observed the Tm at 175.9°C of a polymer obtained from the *Bacillus sp*. which is higher from the present study.

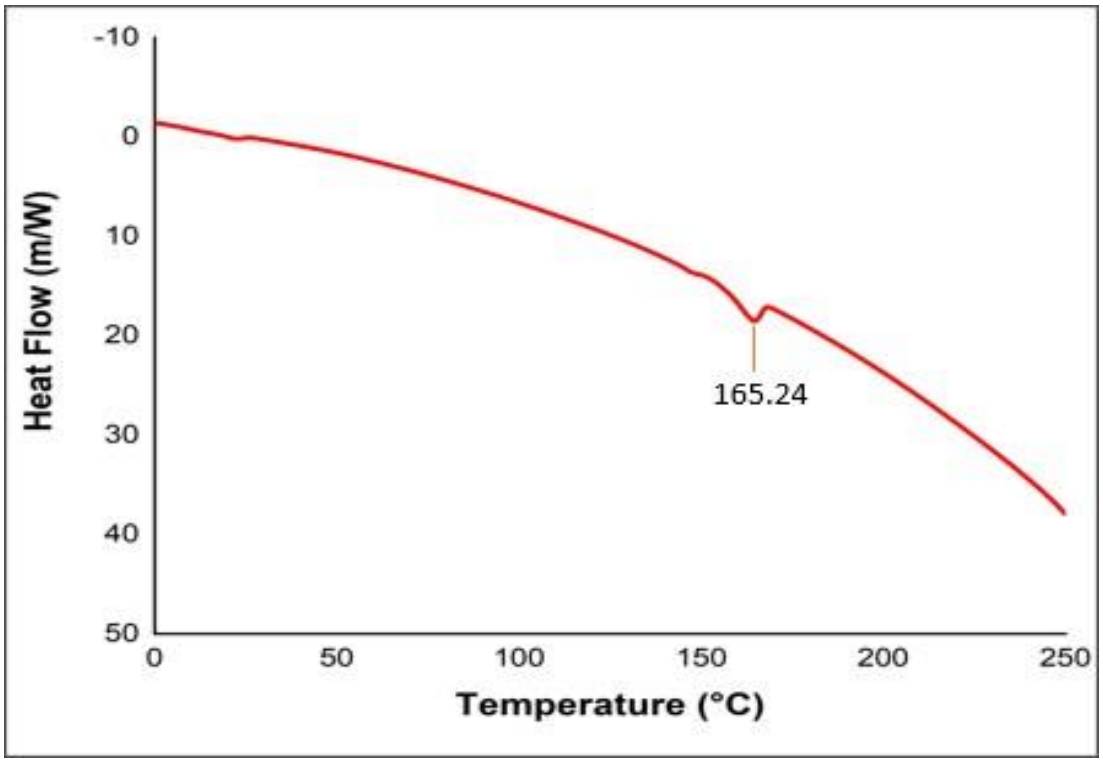


Figure 50 DSC Spectra of Extracted PHA Monomers Using *Bacillus Circulans* In Acid Hydrolyzed Potato Peel Media

3.9.5.3 NMR

The Presence of methyl and methylene groups are characteristic features of PHA monomer. The intensity of signals obtained from proton NMR spectra of extracted biopolymer indicated the presence of PHA polymer. The doublet peak at 1.37 is an indication of the presence of the CH_3 group while the doublet quadruplet at the signal at 2.5 ppm is a characteristic feature of the presence of the CH_2 group in the sample moreover peak intensity between 5.28- 5.3 ppm is an indication of CH group. The peak at 7.2 ppm is attributed to solvents (denatured chloroform) (Figure 51). By comparing the peak intensities with previous work, a similar pattern of proton NMR spectra was reported by (Raghu and Divyashree, 2021) in PHA accumulated inside *Bacillus flexus* when it is grown on castor oil as a carbon source. (Ensifer, 2022) identified the same type of peak pattern of proton NMR of PHA polymer accumulated by a soil isolate *Ensifer sp.* Moreover, the pattern of NMR obtained by the studies (Hassan et al., 2016; Sriyapai et al., 2022) confirms the polymer obtained from *Bacillus circulans* using potato peel waste in this study is PHA

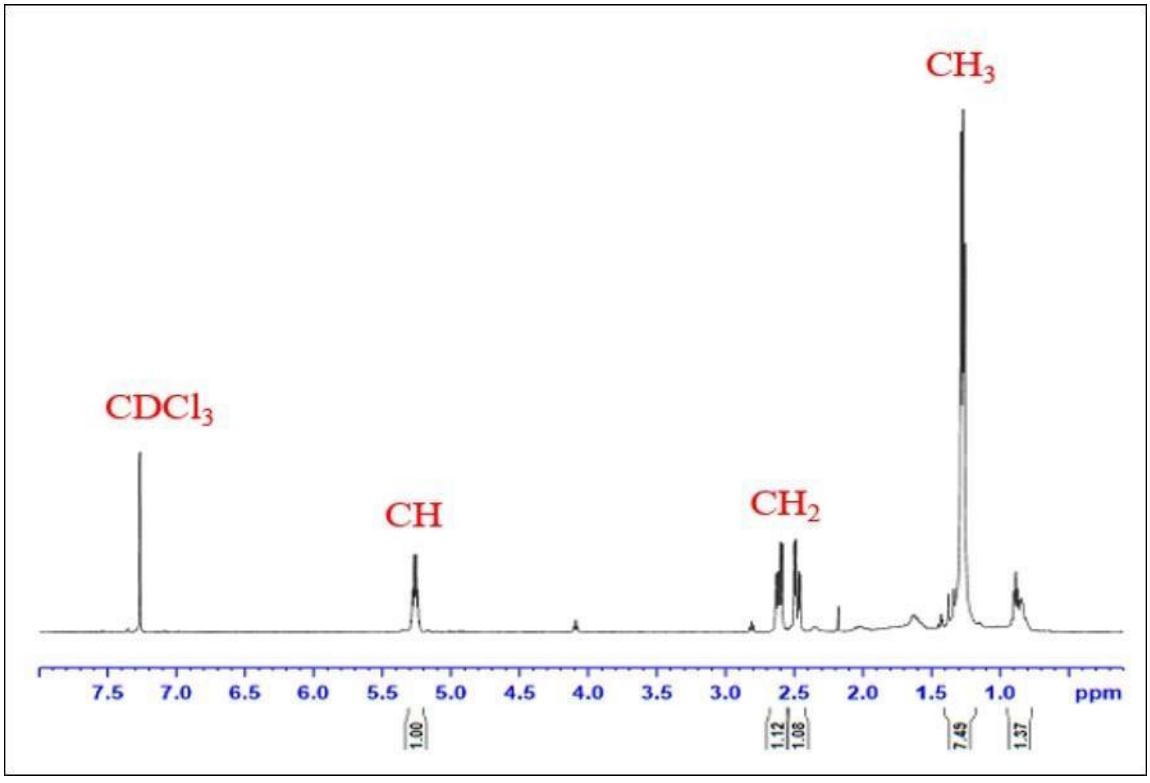


Figure 51 NMR Spectra of Extracted PHA Monomers Using *Bacillus circulans* in

Acid Hydrolyzed Potato Peel Media

3.10 Crude Enzyme Production by *Aspergillus niger*

After seven days 10 ml Milli Q water was added to the flasks and the mixture was subjected to centrifugation at 6000 RPM for 10 minutes (microprocessor-based laboratory-grade centrifuge). The liquid portion of the mixture was used to check the amylase enzyme activity by spectrophotometric method at 540 nm using spectrophotometer made up of Thermo scientific model number GENESYS 50.

3.11 Enzyme Activity

Amylase enzyme activity was checked against the maltose standard curve (1mg/ml) the R^2 value was 0.98 and enzyme activity was calculated with the formula mol of product formed/Total volume * incubation time and it was found 32.94 unit /ml of crude enzyme.

3.12 Hydrolysis of Potato Peel Waste with the Commercial and In-house Crude Enzyme.

The one-step hydrolysis process was used to check the efficiency of the commercial enzyme where a total of 13 experiments were designed with two parameters substrate loading and enzyme unit (Table 7, 8, and 9). The two independent variables are shown in Table 7 and alpha values for substrate loading were 0.18 and 2.31% while in the case of enzyme unit 116.55 and 3.43 (U) respectively. Detail related to the combinational experiment is given in Table 20. After 13 trial runs the final run was performed with the optimized conditions given by software based on experimental values with both the enzymes which are shown in (Figure 52-54 and 55-57) respectively. From the surface and counterplot obtained for the crude enzyme, it can be explained that the maximum reducing sugar yield can be achieved by 1.5% substrate loading and 60 U of the enzyme and in the case of commercial enzyme more than 2% substrate loading and 100U unit required for extractions maximum reducing sugar which is comparatively low from the crude enzyme. The regression equation in the case of crude amylase enzyme was found

-5.397+ 11.621 substrate % + 0.0861 Enzyme U- 3.760 Substrate %* substrate % - 0.000477 Enzyme U* Enzyme unit * Enzyme U – 0.00225 Substrate % * Enzyme U.

while in the case of commercial enzyme equation was:

-1.89+ 5.260 substrate % + 0.01404 Enzyme U- 1.847 Substrate %* substrate % - 0.000063 Enzyme U* Enzyme unit * Enzyme U + 0.01417 Substrate % * Enzyme U.

The observed sugar extraction value by commercial enzyme was 5.01 ± 0.1 g/L However 7.34 ± 0.08g/L sugar liberation was observed in the case of crude enzyme. (Table 10,11, 12) The difference is due to crude enzymes produced from Aspergillus niger containing a cocktail of enzymes including amylase, glucoamylase, and cellulase enzymes. (Izmirlioglu and Demirci, 2012). Reported Low sugar yield in the case of commercial amylase indicated incomplete hydrolysis because the saccharification step is required by the glucoamylase enzyme as previously observed by (Khawla et al., 2014). This study aimed to check the economic feasibility between two enzymes. From the experimental data, it can be concluded that the crude enzyme cocktail is more effective than the commercial amylase as potato peel contains other components such as lignin, pectin, and suberin, etc. compounds that are major hinder enzymatic saccharification. Moreover, amylase alone is not sufficient for the enzymatic hydrolysis of starch.

Table 21 Experimental Conditions, Sugar Recovery (%) by Commercial Enzyme Pretreated PPW by Central Composite Design.

Std Order	Run Order	Pt Type	Blocks	Substrate loading (X_1)	Enzyme unit (X_2)	Reducing sugar (g/L) by commercial enzyme	Reducing sugar (g/L) by crude enzyme
9	1	0	1	1.25	60	3.53 ± 0.2	6.6 3 ± 0.2
5	2	-1	1	1.25	3.43	2 ± 0.1	3.57± 0.28

11	3	0	1	1.25	60	3.54 ± 0.1	6.5 ± 0.21
3	4	1	1	2	20	2 ± 0.2	4.03 ± 0.04
2	5	1	1	0.5	100	1.51 ± 0.08	2.91 ± 0.05
13	6	0	1	1.25	60	3.54 ± 0.04	6.52 ± 0.21
6	7	-1	1	1.25	116.55	5.2 ± 0.03	7.3 ± 0.31
1	8	1	1	0.5	20	0.60 ± 0.1	1.08 ± 0.2
12	9	0	1	1.25	60	3.58 ± 0.02	6.67 ± 0.21
7	10	-1	1	0.18	60	0.06 ± 0.03	0.18 ± 0.03
8	11	-1	1	2.31	60	3.23 ± 0.04	5.1 ± 0.23
10	12	0	1	1.25	60	3.53 ± 0.02	6.56 ± 0.06
4	13	1	1	2	100	4.62 ± 0.03	5.68 ± 0.02

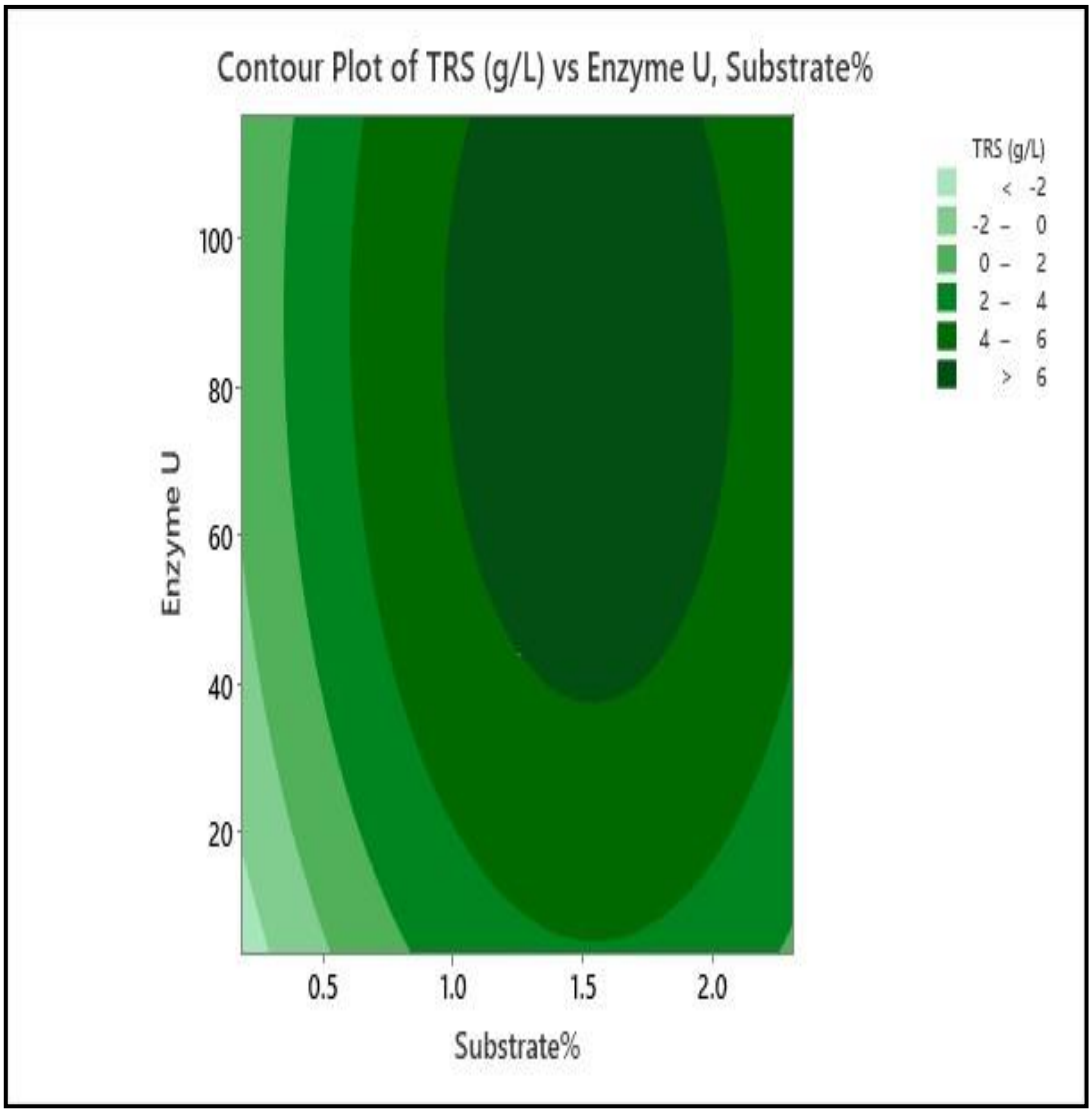


Figure 52 Conter Plot of Enzymatic Hydrolysis of Potato Peel Waste by Inhouse Crude Enzyme

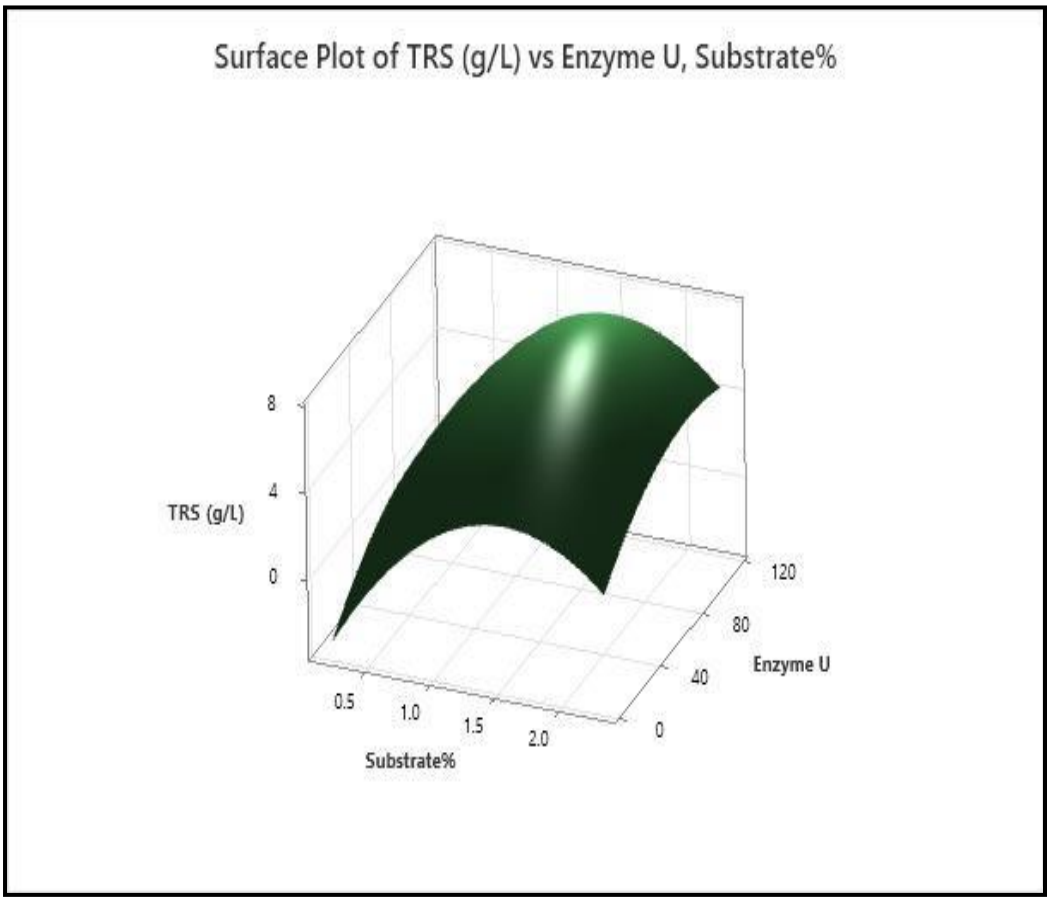


Figure 53 Surface Plot of Enzymatic Hydrolysis of Potato Peel Waste by Inhouse Crude Enzyme

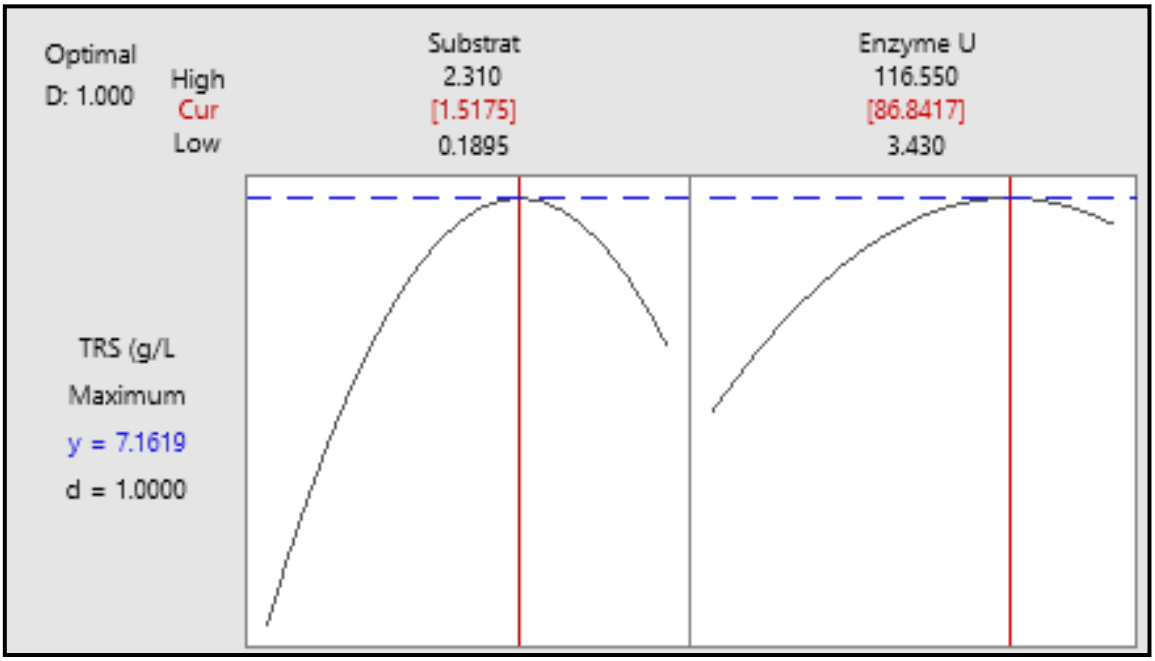


Figure 54 Optimized Condition of Enzymatic Hydrolysis of Potato Peel Waste by Inhouse Crude enzyme

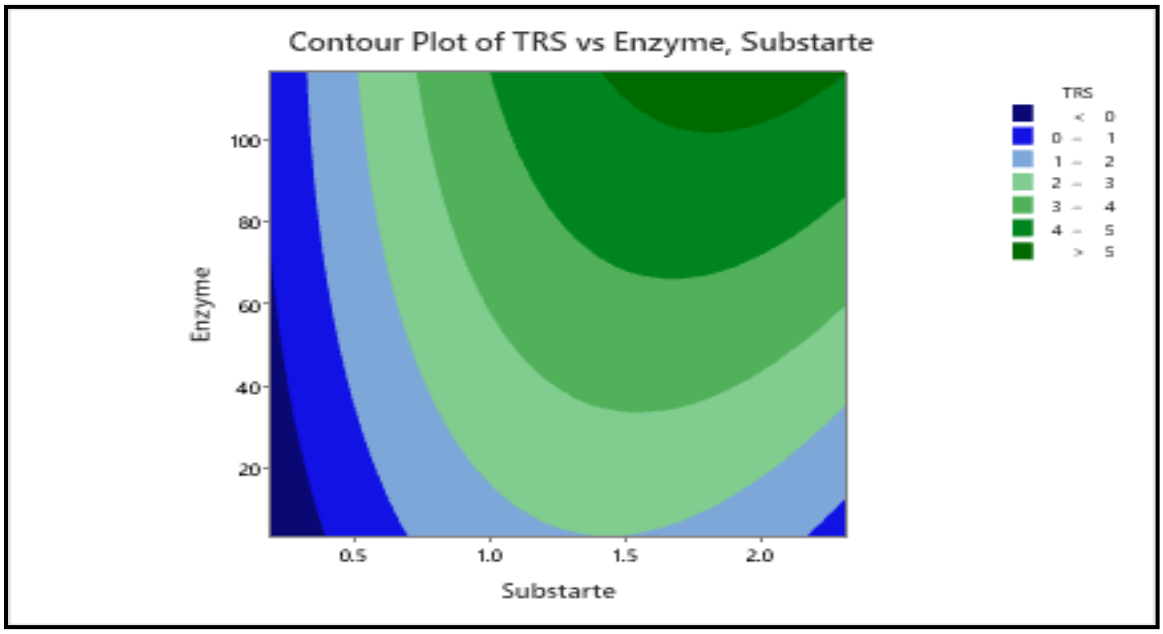


Figure 55 Conter Plot of Enzymatic Hydrolysis of Potato Peel Waste by Commercial Amylase Enzyme

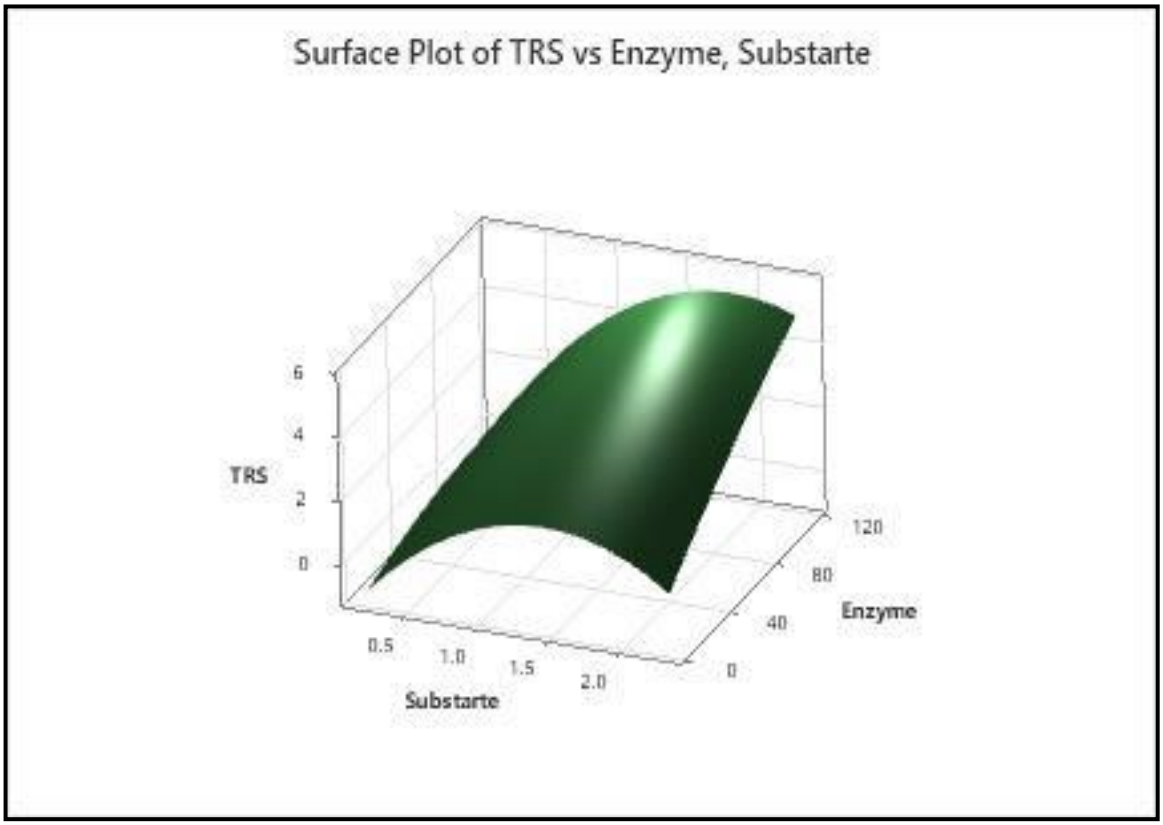


Figure 56 Surface plot of Enzymatic Hydrolysis of Potato Peel Waste by Commercial Amylase Enzyme

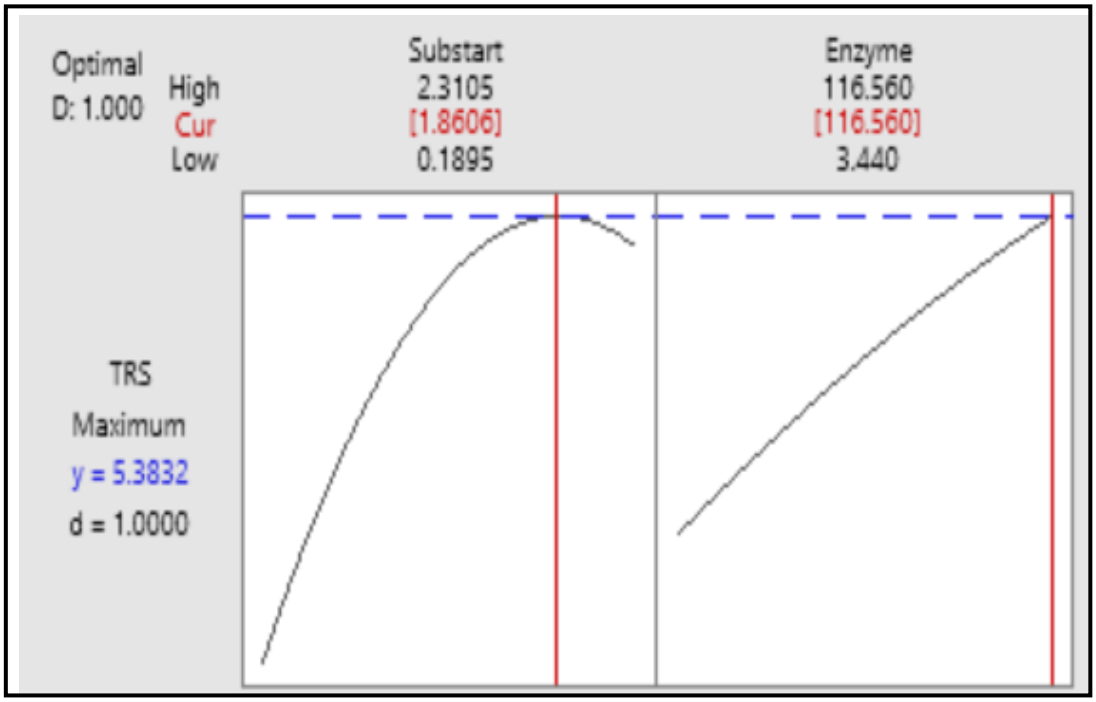


Figure 57 Optimized Condition of Enzymatic Hydrolysis of Potato Peel Waste by Commercial Amylase Enzyme

Chapter 4 Conclusion and Future Prospectives

Potato peel waste was containing a high amount of sugar in form of starch (~64%), cellulose (~9%), and Hemicellulose (~2%). In First step for sugar extraction different concentration of HCl concentration (0.25, 0.5, 0.75, 1, 1.25, 1.5, and 2%) and temperature 50, 100 and 121 °C, were used. And in the second step the sugar extraction study was optimized using response surface methodology which leads to generate (~70%) reducing sugar. Biomass was further subjected to enzymatic saccharification using commercial enzyme (amylase and glucoamylase) and inhouse produced crude enzyme by *Aspergillus niger*, which leads to generate 5.4 g/L sugar (32%) and 7.34g/ L (54%) respectively.

PHA is a promising biopolymer for replacing traditional plastics. The extent of PHA deposition by microbes depends on several factors, including the microbial species' potency and its ability to utilize available carbon sources. The data obtained from this study holds significant potential for enhancing the economic sustainability of Polyhydroxyalkanoates production from potato peel waste using *Pseudomonas putida* and *Bacillus circulans.* The optimization of sugar concentration, achieved through RSM represents a critical step in PHA production, enabling higher sugar yields.

Potato peel waste is utilized to liberate sugars, and the resulting reducing sugars serve as a carbon source for PHA accumulation by *Bacillus circulans* and *Pseudomonas putida*. During a 36-h incubation period substantial amount of PHA (0.232 ± 0.04 g/L) was extracted from Bacillus circulans while intracellular deposition of PHA in Pseudomonas putida was found (0.28 ± 0.55 g/L) by acid hydrolyzed biomass and using economic fermentation media (tryptone 0.1%, NaCl 0.5% and hydrolysate 1%) and their characteristics were further analyzed using FTIR, DSC, and NMR. While achieving a desirable amount of CDW (cell dry weight) remains a challenge, this obstacle can potentially be overcome by optimizing growth parameters, such as the ideal quantity of carbon and nitrogen ratio, use of genetically engineered bacterial strain and use of industrial grade bioreactor to achieve a higher dry cell weight. Hence,

agro-industrial waste (potato peel waste) shows promise as a cost-effective alternative carbon source for PHA production.

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Conference Attended

S. No	Title of Paper	Title of the conference	Organizers conference	Date	National/International	Venue of conference
1	Extraction of Glucose from Potato Peel Waste by Thermochemical Pretreatments.	First International conference on innovations in biotechnology and life sciences (ICIBLS 2020)	Department of Biotechnology, DTU	18.12.2020 to 20.12.2020	International	Online
2.	Chemical hydrolysis of potato peel waste for sugars and value-added metabolites production	Bioengineering Conference 2020	NIT Rourkela	10.12.2020 to 11.12.2020	National	Online

Workshops Attended

- On 21st May, 2020 Attended online scientific writing program on the topics structuring manuscript to impress journal editors and selecting a journal and preparing a great submission package organized by Editage.
- On 29th May,2020 Attended Milli-Q webinar for research.
- From 10-14th June, 2020 participated in the TEQIP-III Sponsored online short-term course on "Trends and Prospects in Biorefinery" organized by Department of Biotechnology, D.r B.R. Ambedkar National Institute of Technology Jalandhar, Punjab, India
- From 2nd-6th September2020 Attended workshop on computational tools for analysis of biological system by Dr. B.R. Ambedkar National Institute of Technology, Jalandhar.
- From 7th - 11th September2020 Attended workshop on Intellectual Property Rights by Dr. B.R. Ambedkar National Institute of Technology, Jalandhar.
- From 19th – 21st September 2020, attended workshop on waste to energy organized by center of Environment, Institute of science and technology and Jawaharlal Nehru Technological University, Hyderabad.
- On 09/12/2020, attended webinar on NIR in feed and, grain milling oil and laboratories related applications along with best practices for NIR networking and management by FOSS India Pvt. Ltd.
- Attended International workshop on Bioinformatics from 14/12/2020 - 18/12/2020 by Department of Biotechnology, Delhi Technological Universit

ANNEXURE-IV

DELHI TECHNOLOGICAL UNIVERSITY

(Formerly Delhi College of Engineering)
Shahbad Daulatpur, Main Bawana Road, Delhi-42

PLAGIARISM VERIFICATION

Title of the Thesis: Sustainable Production of Industrially Important Chemicals from Agro-Industrial Waste.

Total Pages: 161 Name of the Scholar: Sonika Kag

Supervisor (s)

(1) Prof. Pravin Kumar

(2) Dr. Rashmi Kataria

(3)

Department: Biotechnology

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Summary

Brief Profile of Candidate

Name	Sonika Kag
Nationality	Indian
Email	Sonika09kag@gmail.com
Master`s Degree	M.sc Microbiology
University	Devi Ahilya Vishwavidyalaya Indore
Ph.D.	Biotechnology
University	Delhi Technological University
Supervisor	Prof. Pravir Kumar
Co-Supervisor	Dr. Rashmi Kataria
Research Area	Bioprocess engineering
Title of the Thesis	Sustainable Production of Industrially Important Chemicals from agro-industrial waste
Awards Received	Awarded CSIR-JRF NET fellowship in 2017 with all India rank 88. Qualified ARS-NET in October 2016 in Agricultural Microbiology. Qualified GATE in 2016 in Life Science with all India rank 1475.