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PRODUCTION OF POLYHYDROXYBUTYRATE FROM *BACILLUS MEGATERIUM* BY USING LIGNIN FROM COIR PITH

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Abstract
One of the serious environmental issues is the outbreak of petroleum based plastics which are non-biodegradable and toxic resulting in global warming. To overcome the environmental issue the present study focuses on replacing the harmful plastics by innovative strategy, the bioconversion of Kraft lignin (KL) into polyhydroxybutyrate (PHB) a bioplastic produced by bacterium *Bacillus megaterium* strain MTCC 8944 using KL as the sole carbon source. The strain was able to accumulate endogenously 73% of PHB with culture optimization of 35^0 C at 7.0 pH under constant aeration of 120rpm for 48 hours. Further the PHB extracted was characterized by Fourier Transform Infrared (FTIR) and Gas Chromatography Mass Spectrometric (GC-MS) analysis. These optimization results proved the bacteria to be best strain capable of converting KL by lignin depolymerisation and producing PHB as alternate strategy of sustainable renewable resource utilization in industrial era.

Key words – Polyhydroxybutyrate, Bioplastic, Bacillus megaterium, coirpith, lignin extraction

Highlights of present research work

1. Present work focuses on the Utilization of Renewable Resources: The creation of bioplastic from coirpith, a byproduct of processing coconut husk, makes use of a renewable resource that would otherwise be thrown away or allowed to decay, adding to the volume of waste produced. We lessen the burden on conventional petroleum-based plastic feedstocks, which are limited and greatly contribute to environmental deterioration during extraction and processing, by turning this agricultural waste into bioplastics.

2. Diminished Carbon impact: When compared to traditional plastics made from fossil fuels, the manufacture of bioplastics from coirpith has the potential to have a substantially smaller carbon impact. Because coirpith is a naturally occurring substance, the process of turning it into bioplastics produces fewer greenhouse emissions, which helps to slow down global warming.

3. Biodegradability and Compostability: Bioplastics derived from coirpith are generally biodegradable and compostable, which means that, given the correct circumstances, they will eventually decompose spontaneously into non-toxic components. Bioplastics made from coirpith provide a more environmentally friendly end-of-life option by lowering pollution and damage to the ecosystem, in contrast to traditional plastics that linger in the environment for hundreds of years.

1. INTRODUCTION

The constant reminder of environmental pollution as a result global warming is one of the causes of excessive use of petroleum based plastics, a non-biodegradable product persists in the environment for a prolonged periods causing ecological reduction by unbalancing the environmental conditions [1]. The solution to the present scenario is by developing innovative strategy to replace plastics into sustainable by product such as bioplastics of biological origin, these bioplastics are synthesized by some microorganisms and can be easily degradable, bioplastic polymers are accumulated in the cells during the growth of microorganisms are considered to be natural and easily biodegradable and can be catabolized to carbon-di-oxide and water [2]. There are numerous bacterial species known to produce PHB such as *Bacillus, Pseudomonas, Staphylococcus, Alcaligenes*,[3] *Cupriavidus, Aeromonas spp., Actinobacillus, Azotobacter, Agrobacterium, Rhodobacter* and *Pseudochrobactrum asaccharolyticum* [4]. Although a few kinds of marine bacteria have been investigated for Polyhydroxyalkanoate production under some marine conditions, some haloarchaea species belonging to genera like *Haloferax, Haloarcula, Natrialba, Haloterrigena, Halococcus, Pseudodonghicola xiamenensis* [5] have been reported as most significant PHB producers synthesizing and storing PHB endogenously. PHB accumulates due to presence of excess carbon as energy sources under stress of limited oxygen nitrogen and phosphorous which can be restored by addition of limited nutrients [6].

Polyhydroxybutyrate (PHB) is a type of biopolymer synthesized by microorganisms at different stress conditions by limiting the essential nutrient supplementation to the growing media. As PHB are biocompatible 100% natural and accumulate in the cells of bacteria by metabolizing the energy from carbon sources provide in absence of oxygen, nitrogen and sulphur [3]. At present, there are many bioplastics on the market, but they have not been able to phase out conventional petroleum-based plastics for the simple reason that they are economically non-accessible due to high production cost. Innovative methods to reduce the cost of extraction involved in the production of commercially available lignin-based bioplastic such as klason lignin, kraft lignin have also been explored along with redirecting food waste and industrial waste into bioplastic production thereby reducing the cost in terms of raw material [7].

Lignocellulose biomass is one the most abundant form of renewable resource of untapped energy source. As lignin based biomass is one among the best to produce bioplastic, shows resistance to natural elements [8]. Due to their tensile strength and rigid structure they are reinforced into the biopolymers in production of bioplastics such as PHB. One among this abundantly available biomass in nature is the renewable, non-degradable raw material coir pith. Coir pith is husk material obtained after retting process of coconut fibre processed in the back waters of coastal areas [9, 10]. The coir pith obtained is dumped in hillocks pose huge environmental pollution by releasing toxic phenols. The carbon and nitrogen ratio in coir pith is highly variable i.e 93:1 which proves to be suitable for the acclimatization of bacteria and bio conversion [11].

Lignocellulose is mainly composed of cellulose, hemicellulose and lignin, while hemicellulose and cellulose are utilized into various bioproducts but lignin cannot be utilized efficiently. Hence lignin is discarded as waste byproduct from paper industries as kraft lignin soda lignin and lignosulphonates [12]. Because of its macromolecular structure, the utilization of lignin in polymer applications has proven to be rather challenging, despite its attractiveness. The structure of individual molecules within the sample itself may differ greatly, according to spectroscopic investigations. It doesn't seem feasible to use lignin directly as a polymer because of its difficult melt processing and low heat stability. Rather, because lignin's phenol groups may scavenge free radicals, it is combined with other polymers to give resistance against UV degradation and thermo-oxidation [13].

Bacillus megaterium is a Gram-positive, rod-shaped bacterium renowned for its versatile metabolic capabilities and its potential in various biotechnological applications. One such application is the production of polyhydroxybutyrate (PHB), a biodegradable polymer with immense potential in the sustainable plastics industry [15]. The biosynthesis of PHB in *Bacillus megaterium* involves several metabolic pathways. The key precursor for PHB synthesis is acetyl-CoA, which is derived from the

metabolism of carbohydrates via glycolysis or fatty acids via β-oxidation. Acetyl-CoA is then converted to (R)-3-hydroxybutyryl-CoA by the action of β-ketothiolase and acetoacetyl-CoA reductase enzymes. Finally, (R)-3-hydroxybutyryl-CoA is polymerized to form PHB, typically catalyzed by the enzyme PHB synthase [22].

The current objective uses the ubiquitous soil bacterium *Bacillus megaterium* cultured in low nutrition medium to bioconvert lignin recovered from pretreatment of coir pith to a biopolymer PHB. *Bacillus megaterium* can grow using any carbon source in the minimum media. It has accumulated up to 32% of PHB's dry cell weight rate. They have evolved to be highly tolerant to changes in pH and temperature. In light of the effectiveness of producing PHB from lignocellulosic waste, an effort was therefore undertaken to extract PHB using temporal growth factors, thereby minimizing environmental effects, supporting a less expensive alternative to PHB's industrial operations, and effectively converting waste into valuable byproducts in order to contribute to the sustainable use of energy.

2. Materials and Methods

2.1 Bacterial culture maintainennce and coirpith pretreatment

Bacterial strain *Bacillus megaterium* was collected from Microbiological Culture Collection Center (MTCC 8944). Cells were grown in Luria Broth medium with agitation in shaker incubator at 120 rpm until the optical density of 600 nm reached ~1. The culture was transferred into MSML media with kraft lignin as sole carbon source.

Coir pith was pre-treated to obtain industrial waste alkaline lignin, kraft lignin (KL) of the composition [14]. 10g of powdered coir pith, 5ml of 1% sulphuric acid was added and heated in a hot air oven at 80°C for 20min then the solution was cooled, followed by adding 100mL of 4% sodium hydroxide then boiled for 30min**.** Dark colour lignin obtained from boiling was filtered and filtrate was autoclaved at 15lbs for 10min. 1% Alkaline lignin was further added to Minimal Salt Medium Lignin solution (MSM-L) was made by mixing (g/L of deionized water) 4.44g K_2HPO_4, 0.533g KH_2PO_4, 0.5g $MgSO_4$, 5g NH_4NO_3 components and autoclaved.

The bacteria was enriched in 250 ml conical flask containing MSML media incubated at 120rpm for 7days at 35°C. The culture from MSML medium were further transferred to Minimal Davis Medium (MDMKL) for the production of PHB with kraft lignin and the standardised media composition is as follows [1]- Dipotassium phosphate- 7g/L, Monopotassium diphosphate – 2g/L, Sodium citrate – 0.5g/L, Magnesium sulphate - 0.1g/L, Ammonium sulphate – 1g/L, Calcium chloride - 0.02g/L.

2.2 Synthesis and separation of PHB

The enriched bacterial culture from MSML media was transferred to MDMKL medium for the production PHB. PHB extraction was done by modified solvent extraction method [3] 40mL of bacteria which was cultured in the production media was taken in the centrifuge tube and centrifuged at 5000 rpm for 15 min at 4°C, after centrifugation supernatant was discarded then pellet was dried and DCW (dry cell weight) was noted. For the recovery of PHB 10ml of 4% sodium hypochlorite was added to the pellet incubated for 60 min and centrifuged at 5000 rpm for 15min, after centrifugation pellet was collected and washed with 10ml acetone and ethanol mixture (1:1) followed by 10ml hot chloroform to precipitate granules. Then precipitate was allowed to evaporate at 50°C and weight of the extracted PHB was noted.

The percentage of PHB accumulation was calculated using the formulae: **Dry weight of extracted PHB × 100/Dry weight of biomass.**

2.5 PHB molecular characterisation

Spectroscopic characterization of polyhydroxybutyrate was done by the method - Fourier transform Infrared Spectroscopy (FTIR). The instrument used for this analysis was FTIR spectrophotometer Model No-HV 5500 (Manasa Gangothri, Mysore University). FTIR analysis was performed for characterization PHB that extracted from the selected isolate in order to know the functional groups

present in PHB extract, and used for recording IR spectra in the range 4000–600 cm−1. The results from present study depicted the presence of PHB granules by the presence of functional groups such as CH3, CH2, C=O, C-O, CH and OH when which compared with standard PHB curve confirmed the presence of PHB.

Spectroscopic characterization of polyhydroxybutyrate was done by the method - Gas chromatography Mass Spectroscopy (GC-MS). The sample was subjected to methanolysis and further loaded for GC-MS analysis. PHB was heated at 100°C for two hours after being suspended in 1.0 mL of chloroform and 1.0 mL of H_2SO_4/methanol (15:85) in a screw-capped tube. Upon cooling, the mixture was vortexed for one minute with the addition of 0.5 mL of demineralized water. Using a GCMS equipped with a quadrupole ion trap mass detector connected with a CP–Sil 5 CB (0.25 mm i.d × 30 m length) capillary column, the organic phase containing the monomers of the resultant methyl esters. The mass range was 50–600 amu, the ionization energy was 70 eV, and the scan interval was 1.5 s. The oven temperature was set to 280°C for ten minutes after being scheduled to start at 50 °C for 1.0 minute with increments of 10°C. The detector and injector temperature was kept at 280°C, and helium was used as the carrier gas [15].

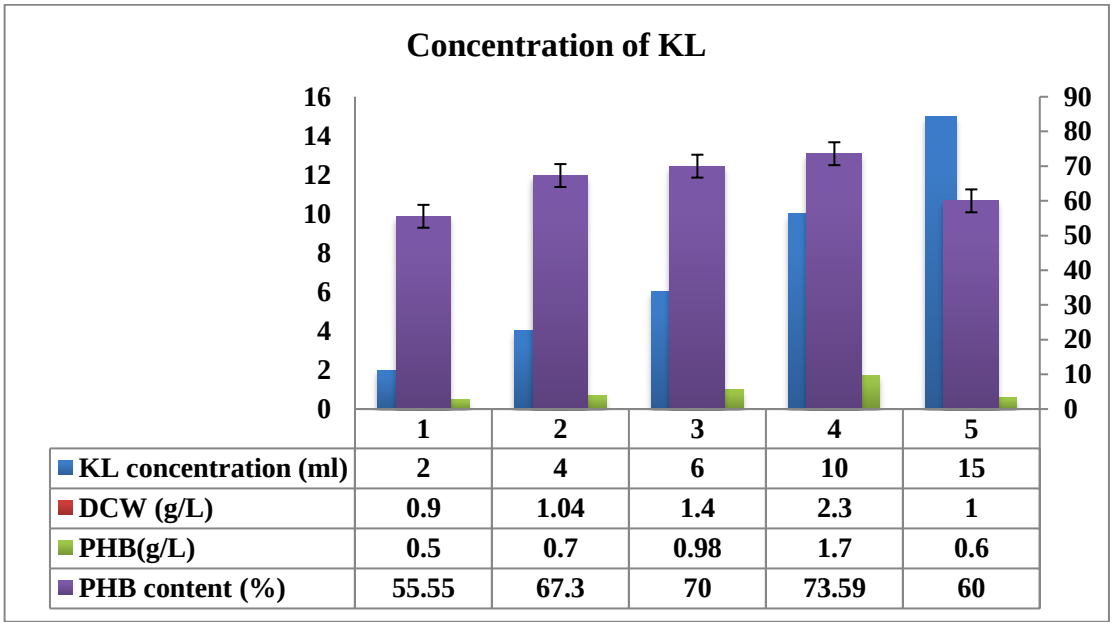
2.6 Standardisation of culture conditions by various parameters

Using Minimal Davis Media (MDM) media, the effects of varied time courses, pH, temperature, and various sources of carbon were studied in relation to PHB formation by taking 200ml of sterile production medium and inoculating 5% of bacterium. Incubation parameter was assessed with 24-96 hour basis, temperature parameters were from 20,25,30, 35,40 and 45 ~C, pH was from 4, 6, 7, 8, and 10 using 0.1 N NaOH and 0.1 N HCl in order to find the ideal (pH) and Kraft lignin concentrtation were triplicates with concentration of 2ml, 4ml, 6ml, 10ml, 15ml.

3. Results and Discussion

3.1 Effect of pre-treated coir pith at various concentration of KL

The production of PHB by bacterium *Bacillus megaterium* under the stress induced environment when subjected to growth under different concentration of extracted and diluted kraft lignin with agitation speed of 120 rpm for a duration of 48hours at 35^0c and 7.0 pH from figure 3 showed increased % yield of PHB from 55% for 2ml to maximum of 73% at 10ml concentration, while the sudden drop in the yield was seen in 15ml concentration of 60% yield when compared with control of 50% yield showed a gradual increase in the PHB concentration than control with no kraft lignin. Similar reports are also seen using various substrates like corn steep liquor produced 43% [23], date syrup 38.85% [13] finger millet straw was 8.31g/l [24]. The other research findings have utilized various substrates for production of PHB to overcome an existing issue of resolving the high cost of substrates, while we approached an alternate method of utilising coir pith as a substrate rich in polyphenols and carbon. Coir pith is one such substrate that is economically cheap and considered as waste extract after retting process. Present study co relates that for 10% concentration of KL acclimatized for *B. megaterium* yielded 73% PHB.

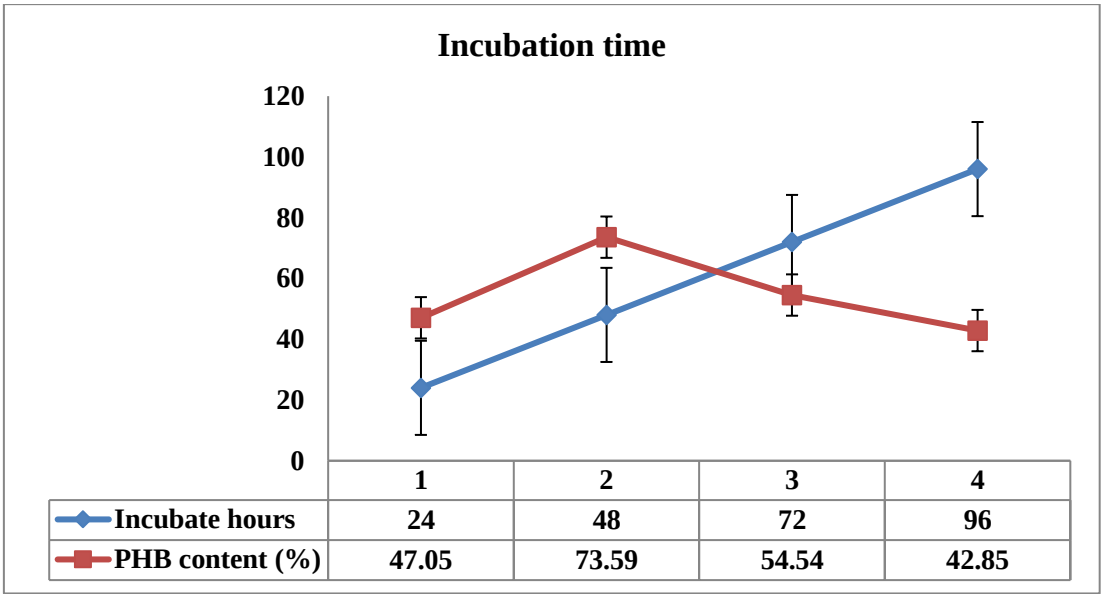


	1	2	3	4	5
KL concentration (ml)	2	4	6	10	15
DCW (g/L)	0.9	1.04	1.4	2.3	1
PHB(g/L)	0.5	0.7	0.98	1.7	0.6
PHB content (%)	55.55	67.3	70	73.59	60

Figure 1 Effect of different concentration of KL incubated culture medium in PHB production (significance at *P*< 0.05 level)

3.2 Incubation Duration's Impact on PHB Production

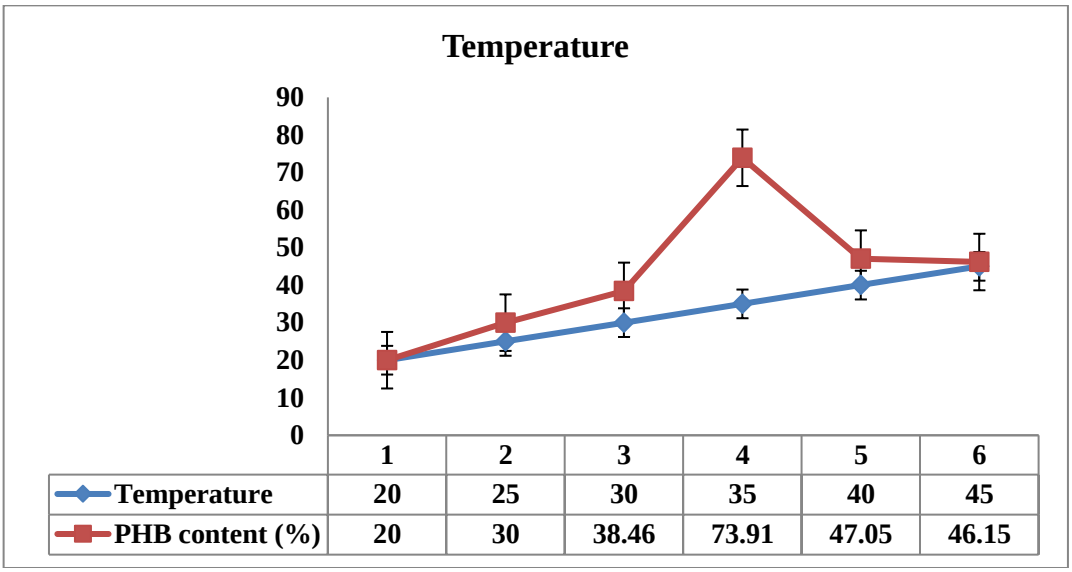
Production medium was prepared and inoculated with inoculum containing bacteria which were kept at 30^0c for different incubation periods (24, 48, 72, 96) hours. The yield of PHB with kraft lignin as a sole carbon source was grown under different incubation time periods to measure the maximum yield. It showed the yield was maximum 73% when culture attained 48 hours of incubation while 24 hours still showed yield fluctuation and 72 hours and 96 hours showed the decreaseof PHB yield. The similar results correlated with previous reports [25 ,15, 24] study, they found *B.subtilis* recorded the highest production of PHB after 48 hours. reported that PHB production and its accumulation significantly increased when the growth reached the logarithmic phase after 18 hours until stationary phase [1].



	1	2	3	4
Incubate hours	24	48	72	96
PHB content (%)	47.05	73.59	54.54	42.85

Figure 2 Effect of different incubation time period for culture medium in PHB production (significance at *P*< 0.05 level)

3.3 Impact of different temperature on production of PHB



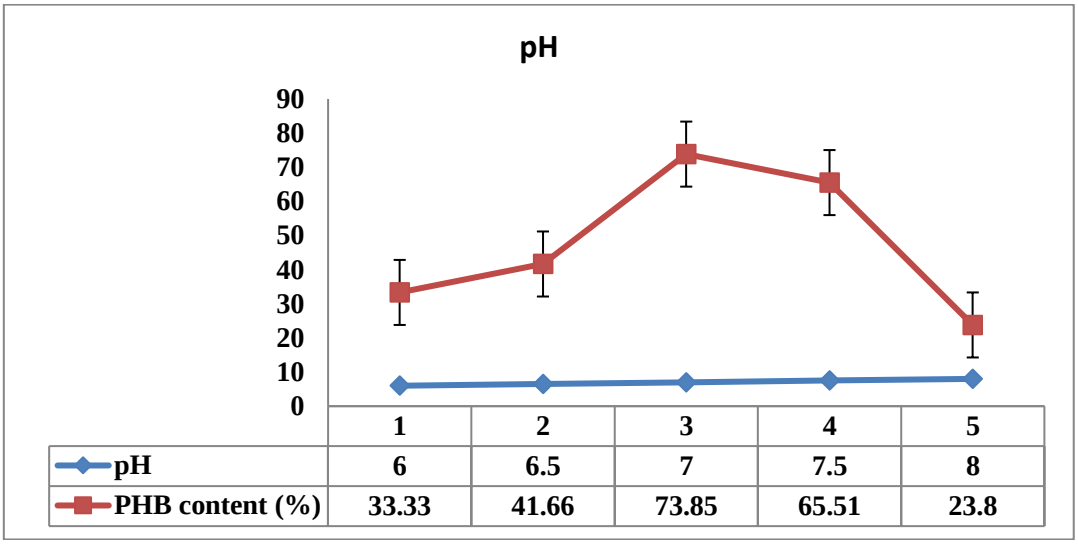
	1	2	3	4	5	6
Temperature	20	25	30	35	40	45
PHB content (%)	20	30	38.46	73.91	47.05	46.15

Figure 3 Effect of different temperature for culture medium in PHB production (significance at *P*< 0.05 level)

The PHB yield obtained when the bacterium *B. megaterium* was cultivated under different temperature conditions with kraft lignin as their sole carbon source as in figure 3yielded the highest percentage of bioplastic at the temperature of 32 - 35^0 C yielded 73% while the increase in the temperature as well as decrease lead to the decrease in the yield of PHB produce. Similar results have been shown in previous studies [15, 4]. Hence the increase in the temperature affects the bacterial growth in the production of PHB by causing the degradation of metabolites and intermediate by-product during carbon metabolism.

3.4 Impact of different pH values on production of PHB

The results showed that the pH values were tested such as (6, 6.5, 7, 7.5, 8) using 0.1N NaOH and 0.1N HCl.The maximal PHB production measured at pH 7 with 63.6% is displayed in figure 4. The synthesis of PHB is reduced as pH levels rise. This conclusion is consistent with that of[25], who discovered that pH 7 had the highest PHB production, at 43.04 percent. Additionally, [23] suggested that the pH range of 6.0 to 7.5 is favorable for microbial growth and PHB formation.



	1	2	3	4	5
pH	6	6.5	7	7.5	8
PHB content (%)	33.33	41.66	73.85	65.51	23.8

Figure 4 Effect of different pH for culture medium in PHB production (significance at *P*< 0.05 level)

3.5 Impact of various culture media on PHB production

B. megaterium was inoculated in different production medium such as, (Nutrient broth, Luria broth, MDM+KL, MDM+glucose, MDM). The PHB yield was confirmed by subjecting the bacterium *Bacillus megaterium* to different media composition as well by altering the carbon sources such as glucose, kraft lignin, Luria broth, nutrient broth and in absence of carbon source. The experimental growth showed the increase in PHB yield when grown under the stress of carbon source such as kraft lignin produced 73% and with glucose as carbon source yielded 66% but with Luria broth it was 64% and nutrient broth was 61% indicating the increase of PHB yield with the changes in carbon sources. Highest yield was recorded with respect to Kraft lignin.

The effects of several carbon sources (glucose, kraft lignin, Luria broth, nutrient broth) on PHB synthesis are depicted in the data in Figure 7. The findings demonstrate that the bacteria's capacity to use various carbon sources varies and is influenced by a number of variables, including the kind of substrate they use and the kind of enzyme they create.

The best carbon source for PHB synthesis was discovered to be MDM with KL, which is followed by glucose. A readily utilizable carbon source, glucose stimulates the production of PHB by bacteria. The study's findings indicate that while complex compounds like lignin are absorbed by bacteria, simple carbohydrates like glucose are simply used by them and increase the PHB. These findings are consistent with previous research on *Bacillus species* conducted by [26] and *Pseudochrobactrum asaccharlyticum* by [4]This result showed that the isolated *Bacillus wiedmannii* [15] isolate has a maximal PHB production from glucose of 144mg/l. According to [2] *Bacillus cereus's* PHB content achieved its maximum level (1.19 g/L) in a solution containing glucose (5 g/L) as a carbon source.

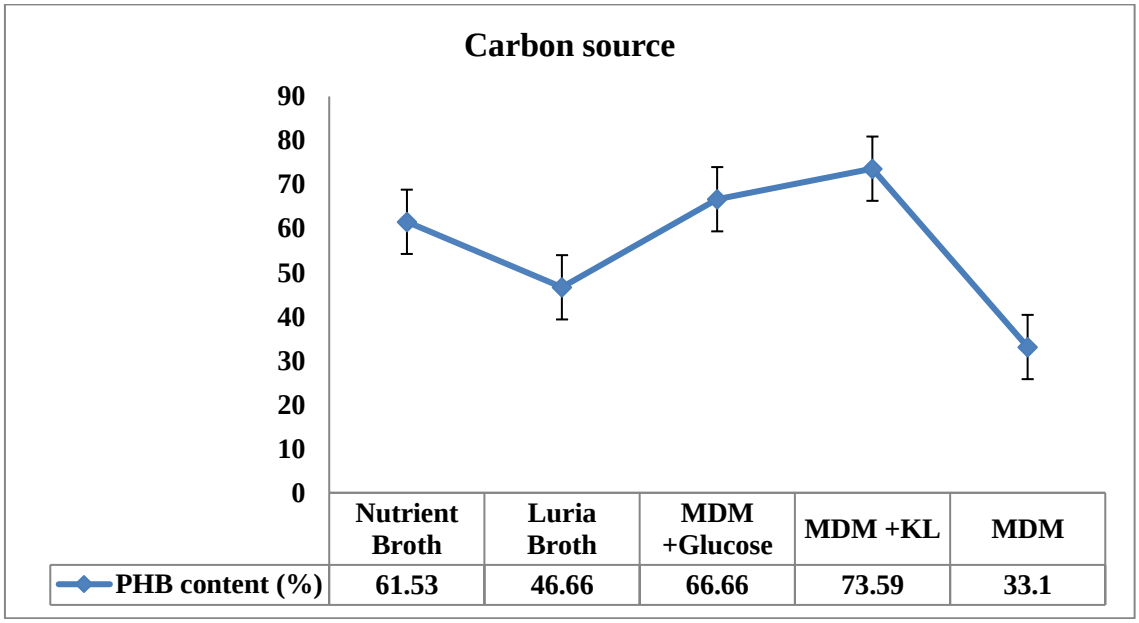


Figure 5 shows the various carbon sources for culture medium in PHB production (significance at *P*< 0.05 level)

3.6 FTIR analysis

FTIR analysis was performed for characterization PHB that extracted from *B. megaterium* in order to know the functional groups present in PHB extract, and used for recording IR spectra in the range 4000–600 cm−1. The results of IR spectra showed two intense absorption bands specific for C=O and C–O stretching vibrations at 1730.21 and 1274.99 cm−1, respectively, and C–H stretching vibrations of methyl, methylene groups at 2954 and 2976 while OH stretch at 3405 and 3430. These absorption bands confirm the structure of PHB with inclusion of Kraft lignin as well as without it. These absorption bands confirm the structure of PHB which co related with the results recorded in the other studies[16-19].

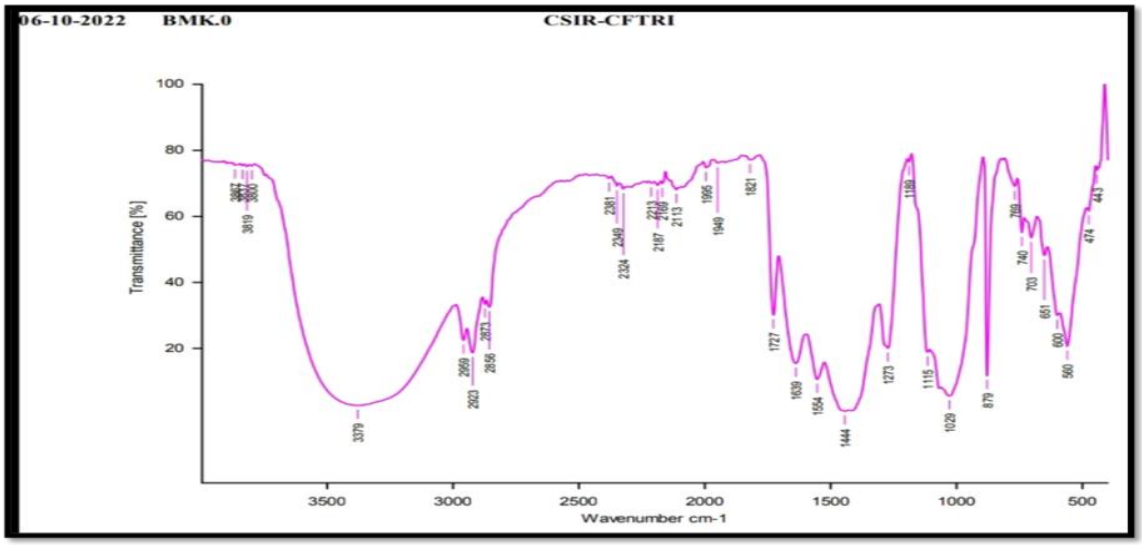


Figure 6 FTIR results showing the presence of C=O and C–O stretching vibrations

3.7 Molecular result of GC-MS analysis

The PHB polymer isolated from *B. megaterium* was analyzed by GC-MS to determine the monomeric composition of PHB. Several peaks with RT values of 2.7, 3.7, 5.0, and 5.58 min were detected corresponding to butanoic acid, whereas another peak at RT of 7.70 is related to 2-Butenoic acid and 1-methyl ethyl ester. Additionally, a peak with RT of 11.577 correspond to propanoic acid and 2, 2-dimethyl. Multiple peaks at different RT correspond to 1-Hexadecanol, 2-methyl-, Octadecanoic acid, 3-hydroxy-, methyl ester, 1-Hexadecanol, 2-methyl-, and 9-Hexadecenoic acid, confirming the presence of PHB. These results are compatible with the previous results of Brinda devi *et al.*, 2015, Mostafa *et al.*, 2020.

Coir pith chemical composition includes cellulose, hemicellulose [20, 21] and lignin was of 53% and it is constituent of polyphenolic substituents for high physical stability and forms a great carbon source for the bacterium to degrade and accumulate PHB endogenously [21]. Pretreatment of coir pith with alkaline medium (NaoH) results in release of black coloured solution known as kraft lignin (KL) which is rich in polyphenols and disaccharides. This pretreatment method allows the easy removal of lignin by hydrolysing the covalent linkage between the lignin and hemicellulose to provide access to holocellulose content as the bacterial carbon source [22]. By hydrolyzing the covalent bonds between lignin and hemicellulose, the pretreatment of lignocellulosic biomass with alkaline (NaOH) is thought to be a viable technique for removing lignin. This increases the surface area of holocellulose and the biomass's enzymatic accessibility.It was shown that the delignification and hydrolysis yields of coirpith lignin increased by 38–51% and 47–63%, respectively, with a considerable increase in NaOH concentrations. With an increase in alkali concentration, a higher rate of delignification may be linked to the hydroxyl ion catalyzing the breaking of ester and ether linkages in the lignin-carbohydrate structure. During the pretreatment step, the NaOH is split into hydroxide ions (OH–) and sodium ions (Na+). As a result, the pace of the hydrolysis reaction increases in tandem with the rise in OH– concentration [24].

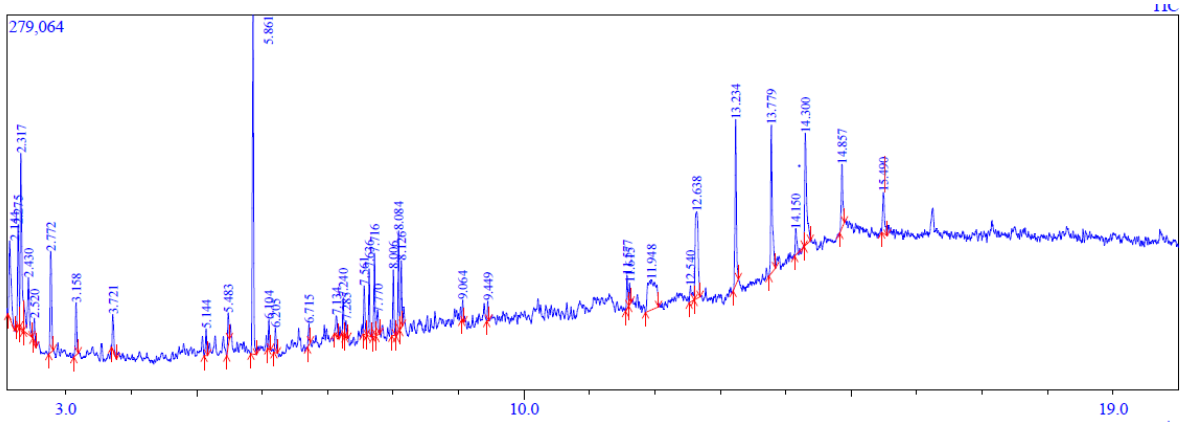


Figure 7 GC-MS analysis of PHB and their monomeric units

This a novel approach as none of them have incorporated various concentration kraft lignin extracted from Coir pith to investigate the growth of bacteria accumulating PHB endogenously as the sole carbon source. From the figure 1 it is evident that Kraft lignin at the concentration of 10 ml for 200ml of nutritive media it is able to produce PHB of 73.59 % by the bacterium *B. megaterium*.

4. Conclusion

Bacillus megaterium holds significant promise for PHB production due to its metabolic versatility and robustness. Understanding the metabolic pathways involved in PHB biosynthesis and employing biotechnological strategies for strain improvement and process optimization are essential for enhancing PHB production yields. With on-going research and development efforts, *Bacillus megaterium*-based PHB production has the potential to contribute to a more sustainable and environmentally friendly plastics industry.

The alternative method of bio plastic production by lignin from coir pith, a cheap lignocellulose biomass obtained from alkaline pretreatment method in which bacteria cultured under stress induced condition with lignin as a sole carbon source yields maximum concentration of 8.5g/l proves to be an effeciant yet alternate approach to a sustainable utilization of secondary raw material. The eco – friendly aspect provides a future platform for developing various novel strategies to culture microbes of native soil and degrade the waste generated. Hence further investigation of these indigenous microbes to degrade biomass with the assist of high throughput technology serves a promising idea towards green earth.

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