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PRODUCTION & CHARACTERIZATION OF BIOPLASTICS FROM VEGETABLE PEELS-AN IDEAL STRATEGY FOR FOOD WASTE DISPOSAL

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AUTHORS' CONTRIBUTIONS

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

The use of bioplastics has gained momentum as it is biodegradable. Use of cost effective and easily available substrates in the production of polyhydroxy butyrate (PHB) enhances its application and hence PHB can be successfully used as a substitute for plastics. The present work was performed to study the optimum production of polyhydroxy butyrate (PHB) by bacteria isolated from soil and effluent samples using vegetable peels as carbon source and its efficiency was checked by PHB production at different time intervals. The maximum PHB production was observed with potato peel as carbon source. Thus, waste food as peels could be utilized as alternate sources of substrates for PHB production. Further investigations are undertaken.

Keywords: Potato peel; polyhydroxy butyrate; soil; bacteria.

1. INTRODUCTION

Food waste management has now become a great challenge globally. Statistics report that 33-50% of all food produced is not consumed and when food waste is used as landfill, methane is generated, which is more deadly than carbon dioxide. Thus, food wastage increases the carbon pollution of the environment.

Hence food waste management has gained significance and food waste has valuable biomass which could be used as efficient carbon sources to create eco-friendly industrial products. Thus, biotransformation of vegetable and fruit wastes could aid in production of useful industrial products. The conversion of food waste to biodegradable eco-friendly plastics could be a good alternative to replace synthetic plastics. Synthetic plastics were favoured for their good mechanical and thermal properties [1]. But its persistence in the environment has raised several problems in the ecosystem. Hence, replacement of non-biodegradable plastics by poly hydroxy butyrate (PHB), a biodegradable polymer has gained momentum [2]. The increased cost in production of PHB has discouraged the use of the polymer [3]. However, the use of inexpensive and renewable carbon substrates as agrowastes and by products can contribute towards the reduction of cost by 50% [4,5]. Hence production of vegetable and fruit peels can provide cost effective and environment friendly biodegradable polymer [6-9].

There are very few reports on the utilization of starch by bacteria to produce PHB [3]. Hence, the aim of the current study was to explore and evaluate the potential of the isolate to produce PHB from potato peels.

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2. MATERIALS AND METHODS

2.1 Sample Collection

The bacteria used in this study was collected from Soil samples and effluent for screening of best PHB producing bacteria.

2.2 Serial Dilution

Soil and effluent samples were collected in clean bags. One gram of soil sample is dispensed in 10ml of sterile distilled water. This is mixed vigorously and 1ml from this is taken and added to another tube with 9ml sterile distilled water to get a dilution of 10^{-1}. This serial dilution is repeated to get dilutions of 10-2, 10-3, 10-4, 10-5, 10-6and 10-7. For the isolation of organisms.0.1ml of each dilution was plated onto a nutrient rich medium by spread plate method for the propagation of microbial growth. The plates were incubated at 300 C for 48 hours. Colonies with different characteristic features were maintained as pure cultures.

2.3 Microscopic Observation-Gram Staining & Motility

The unknown bacteria were subject to Gram staining technique and motility test by hanging drop technique.

2.4 Antibiotic Sensitivity Assay (Disc Diffusion Assay)

The test organisms were inoculated in peptone water and incubated for 3-4 hours at 35^0C. The turbidity of the suspension was adjusted to match 0.5 ml of the standard and used for antimicrobial (antibacterial) sensitivity assay.

Muller Hinton agar was prepared and poured into the petri plates. 0.1mL of turbid bacterial culture was inoculated on the surface of culture plates and spread by using L- rod. The bacterial culture plates were allowed to dry for 15minutes. The Antibiotic discs were placed over the surface of previous petri plates and was incubated at 37^0C for 18-24 hours. The plates were examined for zone of inhibition and was measured in mm and the results, were interpreted as per standard. The results were expressed in the form of sensitive, moderate and resistant.

2.5 Screening for PHB Producing Bacteria

All the bacterial isolates were qualitatively tested for PHB production following the viable colony method of screening using Sudan Black Dye. For this screening of PHB producers, nutrient agar media supplied with 1% of glucose was autoclaved at 1210c for 20 min at 15lbs pressure. This media was poured into sterile Petri plates and allowed for solidification. The plates were divided into 5 equal parts and bacterial isolates were spotted. These plates were incubated for 24 hours. Then ethanolic solution of 0.02% Sudan Black B was spread over the Petri plates containing colonies and was kept undisturbed for 30 min. They were washed with 96% ethanol to remove excess stain from colonies.

2.6 Standard Sudan Black Staining

Staining of cells with Sudan black B smears of cells deposited on a glass slide were heat fixed and stained with a 3% (w/v in 70%ethanol) solution of Sudan Black B (Sigma) for 10 min, followed by immersion of the slide in xylene until it was completely decolorized. The sample was counter stained with safranin (Sigma 5% w/v in deionized water) for 10 s, washed with water and dried. A few drops of immersion oil were added directly on the completely dry slide, and the cells were examined by contrast microscopy. In this staining, lipid inclusion granules are stained blue black or blue grey, while the bacterial cytoplasm is stained light pink.

2.7 Extraction of PHB from Bacterial Cells

All the Sudan Black B positive isolates were subjected to quantification of PHB production as per the method of Jhon and Ralph [10]. The PHB produced by the bacterial cells were extracted by mass culturing. Fresh culture of the selected bacterial strain (16 h) of the selected potential bacterial isolate was inoculated (3-5% inoculum) in different media, such as 1 litre of nutrient broth with vegetable peel extract, water with vegetable peel extract and incubated at 370C for 96 h in a rotary shaker. Potato and pumpkin peels used in the study were weighed and crushed using a mixer and were mixed with the media. After growth, the biomass obtained in the culture flask was subjected to PHB extraction using boiling chloroform as solvent since it is considered to be efficient for extracting polymer.

2.8 Disruption of Cells by Chemical Methods and PHB Estimation

Nutrient broth was prepared in test tubes and inoculated with cultures. The medium was incubated at room temperature for 24-96 hours. PHB was estimated at every 24 hours interval.

Fig. 1. Photo showing vegetable peels as substrates

About 5 ml of culture was taken and centrifuged at 10,000rpm for 10 minutes. Supernatant was discarded and the pellet was suspended in 2.5 ml of sodium hypochlorite and 2.5 ml of chloroform and it was incubated at 30ºC for 1 hour. The above content was centrifuged at 1500 rpm for 10 minutes at room temperature. The upper hypochlorite phase, the middle chloroform containing undisturbed cells and the bottom chloroform phase with PHB were obtained. The upper and middle phases were separated the contents were again centrifuged at 1500 rpm for 10 minutes at room temperature and the phase other than chloroform with PHB was removed carefully. Concentrated sulphuric acid was added to the chloroform phase containing PHB. It was then boiled at 100ºC in a water bath for 10 minutes. The absorbance of the samples was read at 230 nm using UV Spectrophotometer. The readings were plotted in standard graph of crotonic acid and the concentration of PHB was determined.

2.10 DNA Isolation and 16s rRNA Amplification

The selected culture was inoculated in to 50 ml of culture medium and incubated until it reached the OD_{600} of 1 to 2. The cells were harvested by centrifuging at 1250 g at ambient temperature. The cell pellet was re-suspended in 467 µl TE buffer and further incubated with 33 µl of lysing buffer (30 µ1 of 10% SDS and 3 µl of 20 mg/ml proteinase K) for 1 hr at 37°C. The lysate was extracted with an equal volume of 25:24:1 phenol: chloroform: isoamyl alcohol. The aqueous phase was transferred to a 1.5 micro centrifuge tube and added with 1/10 volume of 3M sodium acetate and incubated at -20ºC for 30 min. After the incubation 0.6 volumes of isopropanol was added and mixed gently and centrifuged at 14000 g for 20 min at 4°C. The DNA pellet was washed with 1 ml of 70% ethanol twice by spinning at 14000 g for 20 min at 4°C. The DNA pellet was resuspended in 100 µl TE buffer and stored at 4°, overnight. The quality and quantity of the DNA was estimated by OD ratio of 260/280, 260/230 nm through 8 port Nanodrop (Thermo Fisher Scientifics, USA) and 1% agarose gel respectively. The 16S rRNA gene sequence of the bacteria was amplified with the extracted DNA using universal primers 8 F 5'-AGA GTT TGA TCC TGG CTC A and 1492 R 5'-GGT TAC CTT GTT ACG ACT T (Edwards et al. 1989). The PCR reaction mix consisted 50 mM KCl 10 mM Tris, 1.5 mM $MgCl_2$, 0.2 mM of each deoxynucleoside triphosphate, 1 µM of each primer, 50 ng of extracted DNA and 1 U of Taq polymerase (NEB, UK) in a volume of 50 µl. Amplification was carried out in a thermal cycler (GeneAmp 2700, Applied Biosystems, USA) using following temperature program: initial denaturation at 94ºC for 5 min, 30 cycles of 94ºC for 30 s, 55ºC for 30 s and 72ºC for 1.5 min, and final extension of 72ºC for 15 min. The amplicons were resolved in 0.8% agarose gel containing 10% of 5 mg/l ethidium bromide and the amplicons compared with standard 1kb marker ladder (NEB, UK).

Sequencing was carried out with same universal primers on ABI 3100 automated DNA sequencer by standard BigDye® Terminator v3.1 Cycle Sequencing Kit (Life technologies, USA) cyclic amplification method at PAR Life Sciences and Research Pvt Ltd, Trichy.

2.11 GC-MS Analysis

GC-MS analysis was carried out on a GC clarus 500 Perkin Elmer system comprising a AOC-20i autosampler and gas chromatograph interfaced to a mass spectrometer (GC-MS) instrument employing the following conditions: column Elite-1 fused silica capillary column (30 × 0.25 mm ID ×1EM df,composed of 100% Dimethyl poly siloxane), operating in electron impact mode at 70 eV; helium

(99.999%) was used as carrier gas at a constant flow of 1ml/min and an injection volume of 0.5 EI was employed (split ratio of 10:1) injector temperature 250°C; ion-source temperature 280°C. The oven temperature was programmed from 110°C (isothermal for 2 min), with an increase of 10°C/min, to 200°C/min, then 5°C/min to 280°C/min, ending with a 9 min isothermal at 280°C. Mass spectra were taken at 70 eV; a scan interval of 0.5 s and fragments from 40 to 550 Da.

3. RESULTS AND DISCUSSION

3.1 Selection of PHB Producing Bacterial Isolates from Soil and Effluent Samples

Among the several bacterial isolates from soil, two showed positive result and from effluent, one showed positive result for PHB production by Sudan black staining. Hence they were chosen for the study.

3.2 Antibacterial Activity

Their sensitivity to various antibiotics was evaluated by disc diffusion assay. Results from Table 1 revealed that isolates 1-8 were sensitive to two antibiotics as gentamycin and tetracycline used in the study. All the eight isolates were resistant to cefixime, whereas, isolates 1,2,5 and 6 were resistant to ampicillin.

3.3 PHB Production

Poly-3-hydroxy butyrate production of the 8 isolates were determined in both minimal broth and also in minimal broth supplemented with 2% glucose. The PHB production after 24hr incubation was estimated by determining the OD at 235nm as shown in Table 2. Among the two bacteria chosen for the study, isolate 1 was the predominant producer of PHB. The increase in PHB production is depicted in graph. Among the two peels, potato peel favored PHB production, indicating the increased hydrolysis of carbohydrates in potato peel, whose yield was 100 µg/L.

Table 1. Antibiogram pattern of the isolates from soil and effluent

Isolate \ Antibiotics	Ampicillin (10 mcg)	Tetracycline (30 mcg)	Cefixime (5 mcg)	Gentamicine (10 mcg)
1	-	10 mm	-	10 mm
2	-	20 mm	-	20 mm
3	9 mm	21 mm	-	22 mm
4	10 mm	23 mm	-	23 mm
5	-	18 mm	-	28 mm
6	-	21 mm	-	20 mm
7	6 mm	16 mm	-	17 mm
8	10 mm	25 mm	-	24 mm

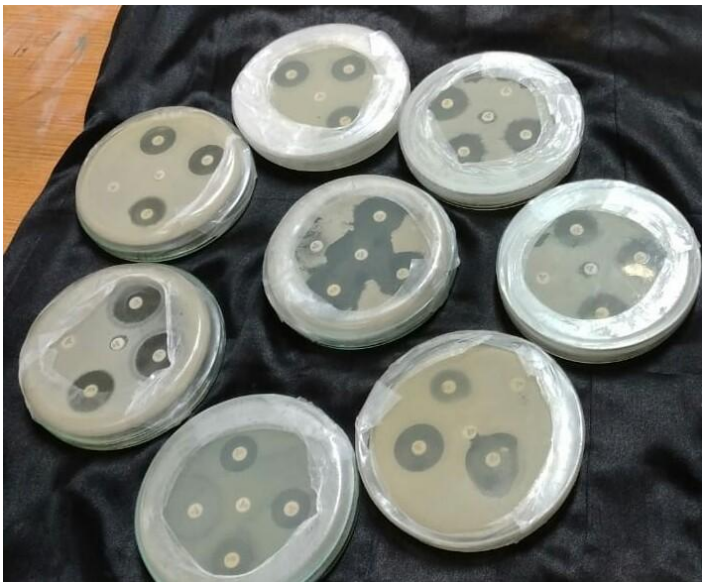

Fig. 2. Plates showing zones of inhibition against a few antibiotics

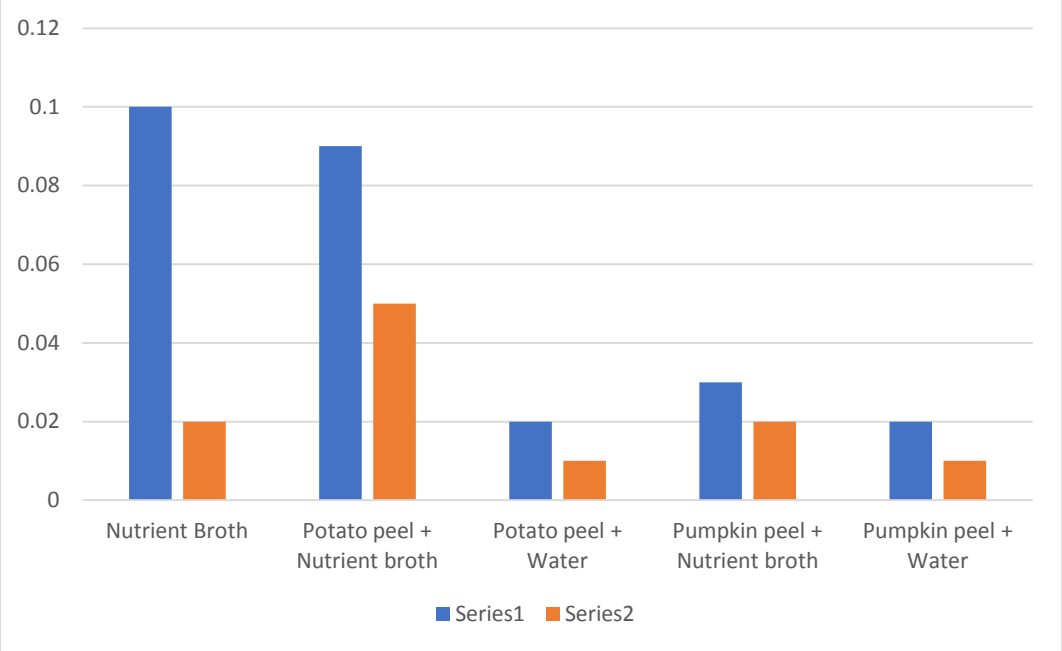

Fig. 3. Graph showing the interaction of different Nutrient

Table 2. Effect of different peels as carbon sources on PHB production (µg/ml)

Different Carbon Sources	Isolate 1	Isolate 5
Nutrient Broth	0.10	0.02
Potato peel + Nutrient broth	0.09	0.05
Potato peel + Water	0.02	0.01
Pumpkin peel + Nutrient broth	0.03	0.02
Pumpkin peel + Water	0.02	0.01

Several agro-industrial residues including potato starch [11] have been reported for PHB production. Several other carbon sources as various oils were also tried for PHB production (Fukui and Doi,1998). PHB production utilizing potato starch was reported by Ramadas et al. [7]. The tremendous amount of food waste from diverse sources is an environmental burden when there are no proper disposal methods. Hence, conversion of food wastes to value-added products is expected to reduce the production cost of biodegradable plastics. Though several reports emphasize on the conversion of several food wastes to valuable products by microbial consortium [12,13], only a limited number of studies reported the potential production of PHB from a single type of food waste, such as waste cooking oil [14] and cheese whey [15]. Therefore, due to comparable properties of PHB and conventional polymer, bioplastic is an ideal alternative in the context of environmental sustainability. Over the past decade, enormous efforts have been put into converting FW into PHB as a practical option for FW management. Direct fermentation of potato residues, in combination with potato starch, into lactic acid was carried out in septic systems [16]. Though several vegetable and fruit wastes have been used in the production of PHB after hydrolysis, yet the utilization of potato peels without hydrolysis have not been reported.

GC-MS results of the ethanol extract of potato peels revealed the presence of 25 compounds, where the predominant compounds were phenols and flavonoids (Table 3), whereas only 15 compounds were found in spent potato peel medium (Table 4), suggesting the hydrolysis of starch in potato peels. The peels were hydrolysed by the bacterial isolates and used as carbon source for PHB production. The hydrolysis of potato peels and utilization of the sugars was confirmed by the presence of only 15 compounds in the hydrolysed potato peel extract (Table 4).

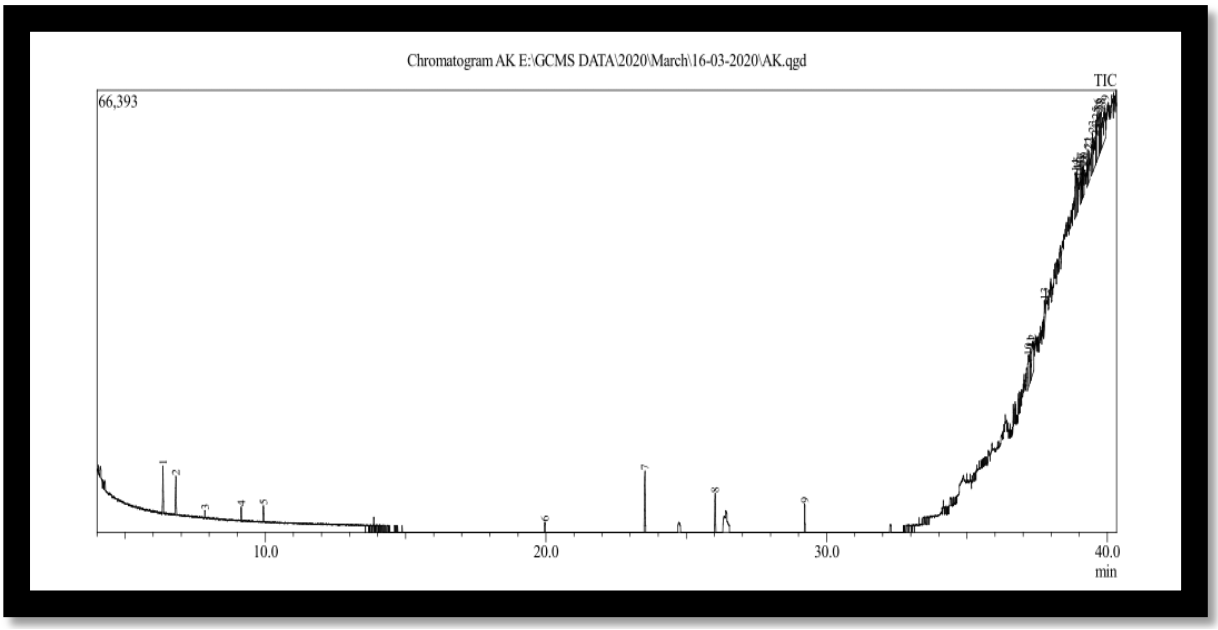

Fig. 4. Chromatogram of potato peel extract

Table 3. Compounds present in Potato peel ethanol extract

S.NO	AREA	NAME
1	73266	BUTANOIC ACID, 3-METHYL-
2	114546	BUTANOIC ACID, 3-METHYL-
3	405525	Butanoic acid, 2-methyl-
4	243030	Butanoic acid, 2-methyl-
5	106986	Pentanoic acid
6	62420	1H-PHENALEN-1-ONE,9-(5-BUTYL-2,3,DIMETHOXYPHENYL)-2,4-DIMETHOXY-
7	437940	3',4',5,6,7,8-Hexamethoxyflavone
8	2211250	3',4',5,6,7,8-Hexamethoxyflavone
9	1476065	3',4',5,6,7,8-Hexamethoxyflavone
10	2817365	3',4',5,6,7,8-Hexamethoxyflavone
11	91073	7-OXABICYCLO[4.1.0]HEPTAN-2-OL,1,2,5,5-TETRAMETHYL-6-(3-METHYL-1,3-BUTADIENYL)-, [1.ALPHA.,2.ALPHA.,6.ALPHA.(E)]-(.+-.)-
12	21396	1H-AZEPINE, HEXAHYDRO-
13	15652	TRICYCLO[4.1.0.0(2,7)]HEPTANE-1,7-DIMETHANOL
14	21723	(2E,6,7-ANTI)-1-BENZOYLOXY-6,7-DIHYDRO-3,7-DIMETHYL-2-OCTENE
15	33648	Cyclohexanamine, N,N'-1,2-ethanediylidenebis-
16	19255	2-Iodo-3-pyridinol
17	17566	1-PHENYL-4,4-DI-P-TOLYL-3-BUTEN-1-ONE
18	40732	2-[(2E)-3,7-DIMETHYL-2,6-OCTADIENYL]-3,7-DIMETHYL-6-OCTENE-1,3-DIOL #
19	6154	2,5,5,8A-TETRAMETHYL-3,4,4A,5,6,7,8,8A-OCTAHYDRO-1-NAPHTHALENYL ACETATE
20	19570	1,2-BIS(ALLYLOXY)-4-TERT-BUTYLBENZENE
21	14307	2-OXAZOLEMETHANOL,4-METHYL-.ALPHA.-PHENYL-, (.+-.)-
22	15251	Fumaric acid, 2-methoxyphenyl tridec-2-yn-1-yl ester
23	21993	Acetate ester, pentafluorophenyl dimethyl sylyil
24	6665	1H,1H,2H-Perfluoro-1-decene
25	33561	Pimelic acid, di(2-(2-methoxyethyl)hexyl) ester

Table 4. Compounds present in spent Potato peel ethanol extract

S. NO	AREA	NAME
1	7.75	Solanidan-3.beta.-ol
2	9.4	2H-PYRROL-2-ONE,4-(1,1 DIMETHYLETHYL)-1,5-DIHYDRO-5-METHOXY-
3	6.66	1,5-DIOXASPIRO[5.5]UNDEC-3-EN-2-ON,7-ISOPROPYL-4,10-DIMETHYL-3-(2-PROPENYL)-
4	7.13	Cyclohexanecarboxylic acid, 4-butyl-, 4-methoxyphenyl ester
5	3.03	MIXTURE OF 2-HYDROXY-1,4,6,7-TETRAMETHYLENEOCTANE AND 2-HYDROXY-1,4,5,7-TETRAMETHYLENEOCTANE
6	6.36	3-(2-Methylpiperidino)propionitrile
7	15.24	1,5-Dioxaspiro[5.5]undec-3-en-2-one, 7-isopropyl-4,10-dimethyl-3-(2-propenyl)-
8	7.42	Glutaricacid,2-fluorophenyl 2-methoxyphenyl ester
9	5.31	1H-INDOLE-2-ETHANOL,BETA.-(3-ETHYLIDENE-1-METHYL-4-PIPERIDINYL)-3-METHYL-
10	3.52	Glutaric acid, 2-chloro-6-fluorophenyl 2-methoxyphenyl ester
11	3.06	2,4-BIS(METHYLTHIO)-2,4-(DIMETHYLMETHANO)ADAMANTANE
12	1.5	2-BUTYL-2-(4-CHLOROBUTYL)-1,3-DIOXOLANE
13	10.33	Heptanoic acid, docosyl ester
14	9.66	.alpha.-Methyl-D-mannopyranoside
15	3.62	1-Bromo-2-chloro-1,1,2-trifluoroethane

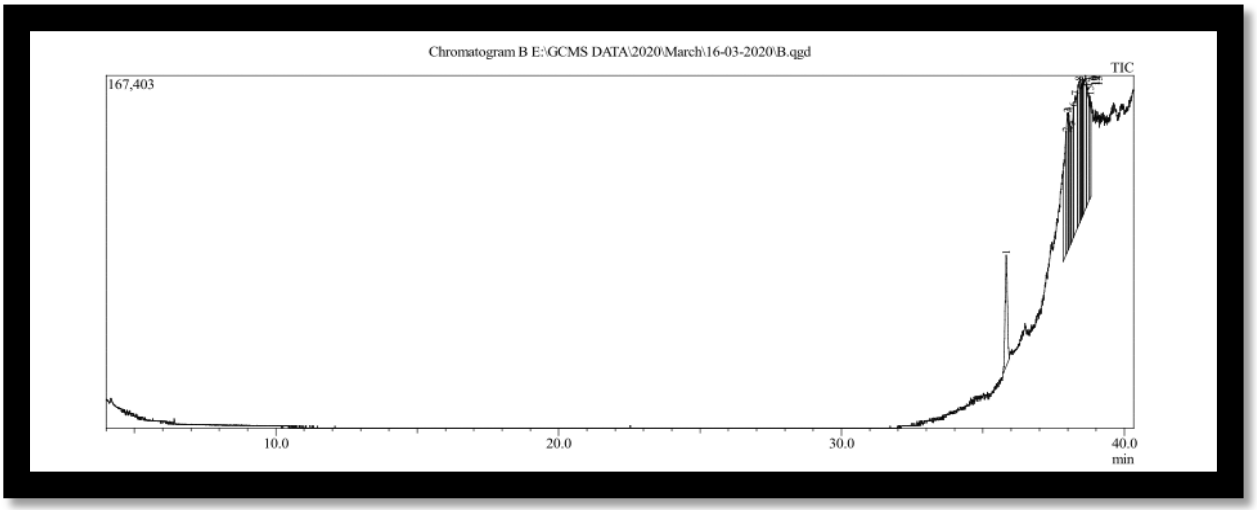

Fig. 5. Chromatogram of spent potato peel extract

4. CONCLUSION

The potato peel, a zero value material of potato processing industries, is augmented day by day with the ever-escalating consumption of processed potato products. However, it is concluded that the utilization of potato peel waste is the same as converting iron into gold hence boosting economic sector for developing countries.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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