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ASSESSMENT OF THE BIODEGRADATION OF 1-METHYL NAPHTHALENE USING IMMOBILIZED *PSEUDOMONAS MACERANS AND BACILLUS SUBTILIS* ON PERIWINKLE SNAIL SHELL

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ABSTRACT
The treatment of pollution using eco-friendly and sustainable methods is one of the bases of biotechnology. The work reports the use of periwinkle snail shell (PS) as carrier to immobilize Pseudomonas macerans and Bacillus subtilis for the biodegradation of 1-methyl naphthalene in aqueous medium. The biodegradation of 1-methyl naphthalene (500 mg L^{-1}) were monitored after 36 h and 72 h by determining the concentration of carbonic acid (by titrimetric method) following the release of carbon (IV) oxide. The pH as well as the Fourier Transform-infrared (FT-IR)of the metabolites from the bioreactors/reactors were also studied. The pH of all the supernatants in the bioreactors/reactors decreased with time. There was increase in the concentration of H_2CO_3 due to the biodegradation of 1-methyl naphthalene by immobilized Pseudomonas macerans and Bacillus subtilis on 1.0 g and 2.0 g of PS. However, the use of immobilized Pseudomonas macerans on PS resulted to significant biodegradation of 1-methylnaphthalene (range of 0.61 - 0.81 mg/L H_2CO_3) compared to degradation alone with PS after 72 h. FTIR of the metabolite at end products show new peaks within 3372- 3268 cm^{-1} and within 1643-1640cm^{-1}, these bands are attributed to overlapping of hydroxyl (OH) and carbonyl (C=O) stretching in carboxylic acid respectively, this implies that 1-methyl naphthalene got converted to carboxylic acid. Therefore, the use of carbonaceous wastes for localization in order to enhance biodegradation of hydrocarbons can be harnessed for mop-up of oil spills.

Keywords: *Biodegradation, Immobilized, Periwinkle snail shell, Pseudomonas macerans, Bacillus subtilis*

1. INTRODUCTION
Naphthalene is one of the polycyclic aromatic hydrocarbons (PAHs) considered to be among the dominant fraction of petroleum [1]. They are among the common pollutants found in areas where both oil exploration and transportation take place, and are of great concern due to their potency as carcinogens and mutants [2, 3]. These compounds are difficult to remove from contaminated water by some treatment methods suitable for compounds of the alkane group. United States, environmental protection agency (USEPA) has indicated some of these compounds as major sources of human cancer [4]. PAHs are common environmental pollutants that are found in soil, surface water and sediment [5]. Substitution in the aromatic rings of the compounds tend to cause substantial water solubility, hence alkyl aromatic compounds are usually bioaccumulative [4]. Several researches have established that, the rates of degradation decreases with increase in molecular weight and alkyl substituent [5, 6]. Low molecular weight PAHs (three or less benzene rings) tend to degrade with the aid of certain microorganisms, but the high molecular PAHs (four rings and above) are not capable of being degraded by bacteria. This is due to their hydrophobic nature, low solubility and the fact that they get absorbed in sediments [7, 8].
Various technologically complex and expensive procedures have been employed in the treatment of petroleum and petroleum products contamination [8]. But complete clean-up of contaminants is rarely achieved with the physical methods; while chemical

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processes could be toxic to living organisms within the area of operation [1].

Of recent, scientific researchers were able to find out the importance of establishing a biofilm community in the bioremediation technique. This is achieved via incorporation of microorganisms on natural solid support such as bagasse, chitin, kaolin, orange peels and pineapple peels for the biodegradation of pollutants. Several work have shown success in the degradation of hydrocarbon and heavy metals [9, 10, 11, 12].

Therefore, this work reports the use of periwinkle snail shell (PS) as a bio-support material to immobilized *Pseudomonas macerans* and *Bacillus subtilis* in the biodegradation of 1-methyl naphthalene. The use of this marine waste for pollution control of hydrocarbon will serve as a cost effective means.

2. MATERIALS AND METHODS

Sample Collection/Preparation

The Periwinkle snail shell (PS) was obtained in November 2015 from new Benin market in Benin City, Edo State, Nigeria. The state is located in south-southern Nigeria and is located at latitude 6° 30′ N, and longitude 6° 0′ E.

2.1 Preparation of PERIWINKLE Snail Shell [13]

The PS was removed from their shells and washed using detergent and distilled water. The shells were sun dried for a week and crushed into smaller sizes, and then gently ground into powder with an agate mortar and pestle. The sample was then passed through 2.0 mm, 0.5mm and 0.125 mm stainless steel sieve. The sample was autoclave at 20^0C in an oven for 21 min and finally stored in sterile sample bottles prior to analysis.

2.2 Preparation of Inoculums in Synthetics Waste Water [12]

The synthetic waste water used in this work composed of: K_2HPO_4 (1.0 g), $(NH_4)_2SO_4$ (0.5 g), NaCl (0.5 g), $CaCl_2$ (0.02 g), $MnSO_4$ (0.02 g), $CuSO_4.5H_2O$ (0.02 g), H_3BO_3 (0.01 g), $MgSO_4.7H_2O$ (0.5 g), $FeSO_4$ (0.02 g), Molybdenum powder (0.02 g), deionised water 1000 cm^3.

A primary culture was prepared by transferring two loops full of microorganism from an agar slant culture into 100 cm^3 of feed medium containing 20 cm^3 of minerals salt medium and 80 cm^3 of 500 mg/L of 1-methyl naphthalene (Sigma Aldrich, UK) solution in a 250 cm^3 Erlenmeyer conical flask. The flask was incubated in a rotary shaker for 48 hours at a temperature of 30^0C and agitated at a speed of 120 rpm. After the incubation period, about 10 cm^3 of the primary culture was transferred into another 100 cm^3 of feed medium in a conical flask and the incubation process was repeated as above. This secondary culture was used in the biodegradation process of 1-methyl Naphthalene.

2.3 Isolation of Hydrocarbon Degrading Bacteria

The microorganisms (*Pseudomonas macerans and Bacillus subtilis*) were isolated based on protocol of Collin and Lyne [14]. The bacterial isolates were considered hydrocarbon degraders and thus were used for the biodegradation studies. Pure cultures were prepared by inoculation on nutrients agar and subsequently sub-cultured to agar slant in bottles for identification.

2.4 Elemental and Proximate Analysis of Periwinkle Snail Shell

The elemental composition of the prepared to PS was carried out by using x-ray fluorescence spectrometry at the Multi-user Laboratory of Ahmadu Bello University, Zaria, Nigeria.

2.5 Determination of Organic Content [15]

$K_2Cr_2O_7$ solution (10 cm^3) was pipetted into a conical flask containing 1.0 g of periwinkle snail shell (PS) in triplicate, followed by immediate addition of 20 cm^3 of concentrated H_2SO_4, the mixture of each was the shaken vigorously. The flask contents were allowed to equilibrate for 30 min. Then distilled water (100 cm^3) was added to each of the mixture, alongside four drops of ferroin [Fe (O-phen)$_3$] SO_4 as indicator. The solution obtained was titrated against ferrous ammonium sulphate solution until the colour changed to maroon.

The percentage organic carbon was obtained as

$$\%\ organic\ carbon = (B - S) \times 0.4N \times 0.003 \times f \times mass\ of\ air\ dried\ sample \times 100$$

B= Constant called the blank=28.1; S=Titration Value; N=Normality; F = Correction factor =1.28, % organic matter of the sample = % organic carbon x 1.729.

2.6 Determination of moisture content [16]

Two crucibles were washed and rinsed with distilled water; this was then dried in an oven at 105^oC and cooled in a desiccator. The weights of the crucible were recorded. One gramme of (1.0 g) of PS was weighed into each of the crucibles. These were dried to a constant weight for 3 h in an air-circulation oven and allowed to cool in a desiccator then weighed.

$$Moisture\ (\%) = \frac{loss\ in\ weight\ on\ drying\ (g)}{initial\ sample\ weight\ (g)} \quad (1)$$

2.7 Determination of Loss on Ignition [16]

Three crucibles were washed and oven dried at 105°C for an hour. The crucibles were weighed and 1.0 g PS sample was poured into each. Each crucible was then placed in a muffle furnace (RHF15/3, United Kingdom) and the temperature allowed to rise slowly to 500°C and maintained for 3 h. After burning, the sample was cooled to room temperature in desiccators. The crucibles and their content were then reweighed.

$$Lost\ on\ iginition\ (L.O.I) = \frac{final\ weight}{initial\ weight} \times 100 \quad (2)$$

2.8 Determination of Phosphorus Content [15]

About 1.0 g of PS sample was weighed and mixed with the extracting solution (15 cm^3 of NH_4F and 25 cm^3 of HCl to 460 cm^3 of distilled water) in a beaker. The mixture was shaken vigorously for 15 min, then 2 cm^3 of the clear supernant was pipetted into a 20 cm^3 test-tube, followed by the addition of 5 cm^3 of distilled water, 2 cm^3 of ammonium molybdate solution $[(NH_4)_6MO_7O_{24}.4H_2O]$ and 1 cm^3 of stannous chloride ($SnCl_2{\cdot}2H_2O$). The absorbance was measured after 5 min, using an electro- spectrophotometer (Specto20D, USA) at 660 nm.

The content of the extractable phosphorous in the sample was calculated using the equation obtained from the graph passing through the origin of a calibration curve that was previously obtained.

$$y = mE \quad (3)$$

Phosphorous content = Average m X E; where E = electro-spectrophotometer reading

2.9 Biodegradation Study [12]

A set of experiments (n = 9) were conducted for the biodegradation of 1-methyl naphthalene by *Pseudomonas macerans* and *Bacillus subtilis* immobilized on PS. This was done by adding 100 cm^3 of the organisms inoculums into six (three for *Pseudomonas* inoculums and Bacillus inoculums) sets of 250 cm^3 Erlenmeyer conical flasks each containing varied masses of PS (1.0 g, 2.0 g, 3.0 g), followed by the addition of 150 cm^3 of synthetic waste water containing initial concentration of 500 mg/L of 1-methyl Naphthalene into each of the flask and then sealed. The flaks were placed in a rotary shaker for 72 h at a speed of 180 rpm and temperature of 30°C so as to reach equilibrium. For the degradation without the microorganisms, 150 cm^3 of synthetic water containing 500 mg/L of 1-methyl naphthalene was added into three sets of 250 cm^3 Erlenmeyer conical flask containing varied masses of PS (1.0 g, 2.0g, 3.0 g). The flasks were equally placed in a rotary shaker for 72 h at a speed of 180 rpm at temperature 30°C. The maximum amount of degraded and biodegraded 1-methyl naphthalene at equilibrium, Qe was calculated according to the equation:

$$Qe = \frac{(Co - Ce)V}{W} \quad (4)$$

Where Q_e is the amount of hydrocarbon degraded and biodegraded respectively at equilibrium (mg/g),C_o is the initial concentration(mg/L) of the hydrocarbon and C_e is the equilibrium concentration (mg/L) of the hydrocarbon

The ability of the microorganisms to mineralize the hydrocarbon was monitored by determining the pH level and the evolution of CO_2 quantified by the concentration of carbonic acid in the mixture titrimetrically.

Then FTIR analysis was carried out on the metabolites in the reactors/bioreactors, in order to determine the functional group of the end products of the experiments.

2.10 Determination of Carbonic Acid (H_2CO_3) and pH [17]

An aliquot of the mixture in each bioreactor (1.0 cm^3) was collected with a sunction pipette after 36 and 72 hours of treatment and titrated against 0.05 M NaOH solution. Phenolphthalein was used as the indicator; appearance of stable pink colour indicates the end point. The amount of CO_2 (mm/Hg) in form of carbonic acid was obtained using the equation

$$Free\ CO_2 = \frac{Titre\ x\ Normality\ of\ NaOH}{Volume\ of\ the\ bioreactor} \times 44 \quad (5)$$

The pH was determined by the use of pH meter (HY003120).

2.11 Statistical Analysis

Students't-test with significant difference taken at $P < 0.05$ was used to assess the effects of the type of microorganism on the degradation of 1-methyl naphthalene.

3. RESULTS AND DISCUSSION

3.1 Elemental and Proximate Composition of Periwinkle Snail Shell

The XRF analysis of the periwinkle snail shell indicated that CaO (50.80%) was found to be major constituent, followed by Al_2O_3 (1.75 5%), alongside SiO_2, TiO_2, CaO, MgO, Fe_2O_3, Na_2O, Ca_2O, K_2O, MnO in traces amount (Table 1). The high percentage of CaO in the PS sample

indicates that calcium carbonate is the dominant mineral in the sample. This is in line with the result obtained by Jatto *et al*. (2010) [18].

From the study PS has pH 7.76, this can be attributed to apatite and calcium carbonate present in the shell; according to Aboua (1995) [19]. The loss on ignition was 45.60%, moisture content 2.04%, organic content 3.54%, percentage nitrogen was 0.01% and phosphorus content was 0.124 mg/kg (Table 2).

Table 1: Proximate composition of periwinkle snail shell (PS)

Parameters	PS
pH	7.76
LOI	45.60
Moisture Content (%)	2.04
Organic Content/Organic Matter (%)	3.54
Nitrogen Content (%)	0.01
Phosphorus Content (ppm).	0.124

Table 2: Characterization of periwinkle snail shell (PS) using XRF

% Oxide Composition	PS
SiO_2	0.26
TiO_2	0.11
Al_2O_3	1.75
Fe_2O_3	0.630
CaO	50.80
MgO	0.02
Na_2O	0.001
K_2O	0.002
MnO	0.15
V_2O_5	-
Cr_2O_3	-
CuO	0.011
ZnO	0.010
BaO	0.11
Ta_2O_5	-
Nb_2O_5	-
SrO	0.559

3.2 Biodegradation of 1-Methyl Naphthalene

The isolates were identified to be *Pseudomonas macerans* and *Bacillus subtilis* on the basis of colour change in the identification test kit media.

From the result obtained for the biodegradation by the immobilized *Pseudomonas macerans* on PS (Table 3), the concentration of H_2CO_3obtained after 36 hours of incubation in the sealed reactors at dosage of 1.0 g, 2.0 g, and 3.0 g was 0.55 mg/L, 0.65 mg/L and 0.71 mg/L. After 72 hours the concentration increased to be 0.61 mg/L, 0.72mg/L and 0.81 mg/L respectively. Also, as the reaction period increased from 36 to 72 h, the pH value of the systems decrease from 8.23-8.12, 8.16-8.11, 8.00-7.98 for 1.0 g, 2.0 g and 3.0 g dosage respectively.

The recorded increase in the release of CO_2 (measured as H_2CO_3) with increased PS and duration of incubation confirms that the immobilized *Pseudomonas macerans* on PS is capable of breaking down the hydrocarbon.

Also the decrease in pH is due to the neutralizing effect of carboxylic acid formed during degradation [18].

Following from Table 2, a similar trend was observed for the concentration of CO_2 generated as well as the pH change of the digest during the biodegradation of 1-methyl naphthalene by *Bacillus subtilis* immobilized on PS as a function of incubation time and mass of PS. The pH change is within the optimum recorded for other biodegradation studies. Biodegradation can occur under a wide-range of pH; however, a pH of 6.5 to 8.5 is generally optimal for biodegradation in most aquatic and terrestrial systems [20]. The concentrations of CO_2 generated after 36 hours by using 1.0 g to 3.0 g PS ranged from 0.48 - 0.65 mg/L; at 72 hours the CO_2 generated ranged from 0.55 - 0.76 mg/L. This indicated that localization of *Pseudomonas macerans* on PS generally resulted to enhanced biodegradation than the use of *Bacillus subtilis* immobilized on PS. However, from the study, there was no significant difference in the biodegradation of 1-methyl naphthalene by *Pseudomonas macerans* compared to *Bacillus subtilis*immobilized on PS. The result is similar to the report by using species of Bacillus, Citrobacter, Flavobacterium, Micrococcus, Pseudomonas, Acinetobacter and Staphylococcus for hydrocarbon degradation, analysis of the results revealed that among the isolates from water sources, *Pseudomonas aeruginosa* had the greatest ability to degrade diesel while *Staphylococccus aureus* had the least capacity [21].

On the other hand, the degradation of 1-methyl naphthalene by PS alone had the concentration of H_2CO_3 obtained after 36 hours of incubation in the sealed reactors at PS dosage of 1.0 g, 2.0 g, and 3.0 g being 0.42 mg/L, 0.49 mg/L, 0.52 mg/L. After 72 hours the concentration of H_2CO_3 increased to 0.46 mg/L, 0.55 mg/L and 0.59 mg/L respectively by using 1.0 g, 2.0 g, and 3.0 g of PS (Table 5). Also, as the reaction period increased from 36 to 72 h, the pH value of the mixtures decrease from 9.12-8.73, 8.77-8.57, 8.35-8.24 for 1.0 g, 2.0 g and 3.0 g PS dosage respectively. The study indicated that he use of immobilized *Pseudomonas macerans* on PS resulted to significant

biodegradation of 1-methylnaphthalene (range of 0.61 - 0.81 mg/L H_2CO_3) compared to degradation alone with PS (0.46 -0.59 mg/L) after 72 h.

Generally, the use of immobilized *Pseudomonas macerans* on PS resulted to significant biodegradation of 1-methylnaphthalene (range of 0.61 - 0.81 mg/L H_2CO_3) compared to degradation alone with PS after 72 h. The concentration of CO_2 produced by immobilized *Pseudomonas macerans* and *Bacillus subtilis* on PS and during degradation alone with PS increased with mass of PS (Table 3 - 5). This is because the solid support has enough active sites for adsorption and biodegradation. So increase in the dosage brings about aggregation of the adsorbent, and consequently increased biodegradation, since optimization has not resulted [8, 22]. Also, the immobilized cells have advantage of mineralizing the hydrocarbon compared to solid support alone, this is because, the immobilized cells is a combination of both adsorption and biodegradation processes [23].

Table 3: Biodegradation of 1-methylnaphthalene by immobilized Pseudomonas macerans on periwinkle snail shell (PS)

Mass of immobilized organism (g)	pH after 36 hours	pH after 72 hours	Concentration of CO_2(mg/L) after 36 hours	Concentration of CO_2(mg/L) after 72 hours
1.0	8.23	8.12	0.55	0.61
2.0	8.16	8.11	0.65	0.72
3.0	8.00	7.98	0.71	0.81

Table 4: Biodegradation of 1-methylnaphthalene by immobilized Bacillus subtilis on periwinkle snail shell (PS)

Mass of immobilized organism (g)	pH after 36 hours	pH after 72 hours	Concentration of CO_2(mg/L) after 36 hours	Concentration of CO_2(mg) after 72 h
1.0	8.33	8..15	0.48	0.55
2.0	8.21	8.19	0.60	0.68
3.0	8..10	8..00	0.65	0.76

3.3 Effect of dosage of the immobilized microbes on periwinkle snail shell (ps) on the percentage removal of 1-methyl naphthalene

The percentage removal of 1-methylnaphthalene by using PS alone was observed to increase with dosage (72 - 77%).*Pseudomonas macerans* immobilized on PS had the highest percentage removal of 78 - 84% 1-methylnaphthalene (Table 6). This result presents the viability of periwinkle snail shell alone as a good medium for degradation of hydrocarbons in petroleum products spillage.

Table 5: Result of the degradation of 1-methylnaphthalene by periwinkle snail shell (PS)

Mass of immobilized organism (g)	pH after 36 hours	pH after 72 hours	Concentration of CO_2(mg/L) after 36 hours	Concentration of CO_2(mg/L) after 72 h
1.0	9.12	8.73	0.42	0.46
2.0	8.77	8.57	0.49	0.55
3.0	8.35	8.24	0.52	0.59

Table 6: Effect of dosage of immobilized microbes on periwinkle snail shell (PS) on the percentage removal of 1-methylnaphthalene

Treatment	Dosage	% Removal
PS	1.0	72
	2.0	74
	3.0	77
Pseudomonas macerans immobilized on PS	1.0g	78
Pseudomonas macerans immobilized on PS	2.0	82
Pseudomonas macerans immobilized on PS	3.0	84
Bacillus subtilis immobilized on PS	1.0	75
Bacillus subtilis immobilized on PS	2.0	79
Bacillus subtilis immobilized on PS	3.0	81

3.4 Fourier- Transform Infrared (FTIR) Result of the Degradation End Products of 1-methylnaphthalene

The FTIR spectrum of 1-methylnaphthalene (Figure 1) and that of the extracts of the biodegradation metabolites of the bioreactors/reactors (Figure 2) are presented. The metabolite at the end products show new peaks within 3372- 3268 cm^{-1} and within 1643-1640cm^{-1} (Figure 2) when compared to Figure 1. These bands are attributed to overlapping of hydroxyl (OH) and carbonyl(C=O) stretching in carboxylic acid respectively, this implies the 1-methyl naphthalene got converted to carboxylic acid.

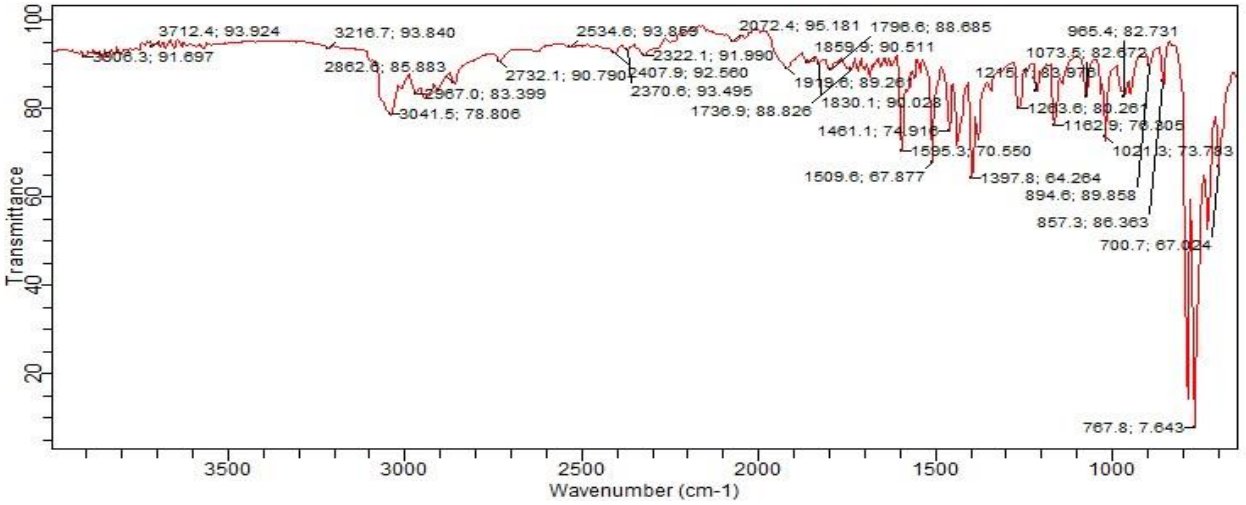

Figure 1: FTIR Spectrum of 1-methylnaphthalene

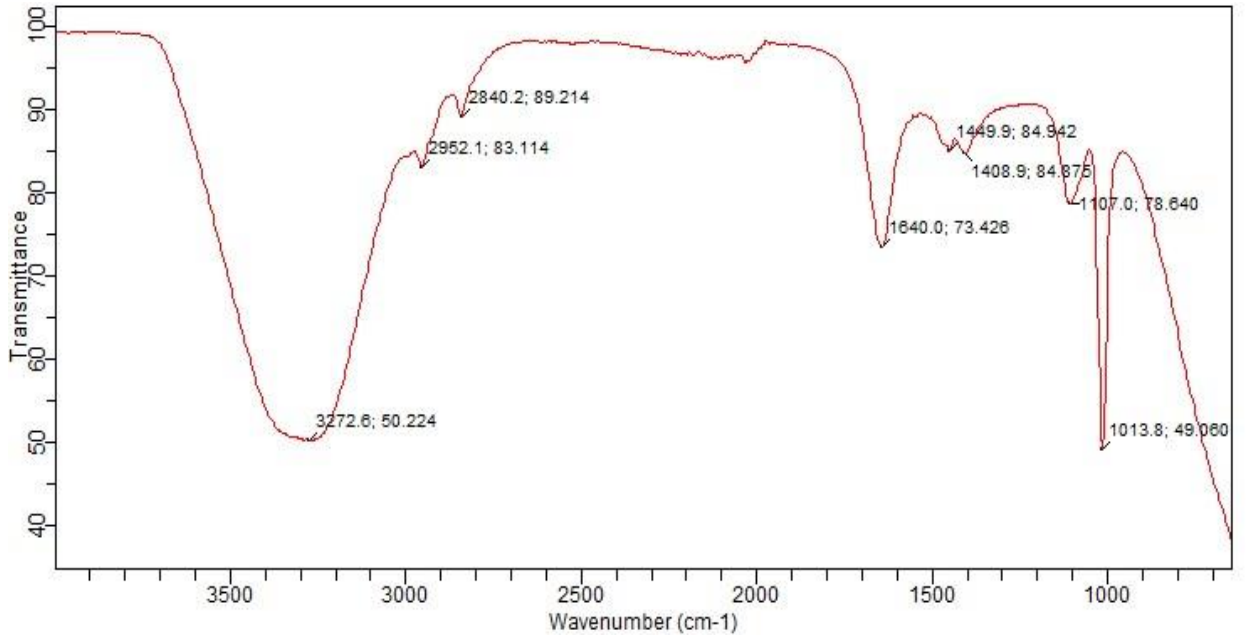

Figure 2: FTIR Spectrum of the Degradation metabolites of 1-methylnaphthalene

4. CONCLUSION

From the study the amount of carbon (IV) oxide produced in the biodegradation of 1-methyl naphthalene by the immobilized *Pseudomonas macerans* and *Bacillus subtilis* on periwinkle snail shell was greater than by the periwinkle snail shell alone. This implies that the immobilized cells have more advantage in the mineralization of the hydrocarbon to the basic end product which is carbon dioxide. Also, it can be concluded that 1-methyl naphthalene got converted to carboxylic acid, as such that, some of the hydrocarbons mineralized to oxygenated compound and some proceeded to more basic products.

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