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Biodegradation of 2,4 - Di-Tert-Butylphenol (2,4 DTBP) by Bacteria Isolates from Industrial Wastewater Treatment Plant

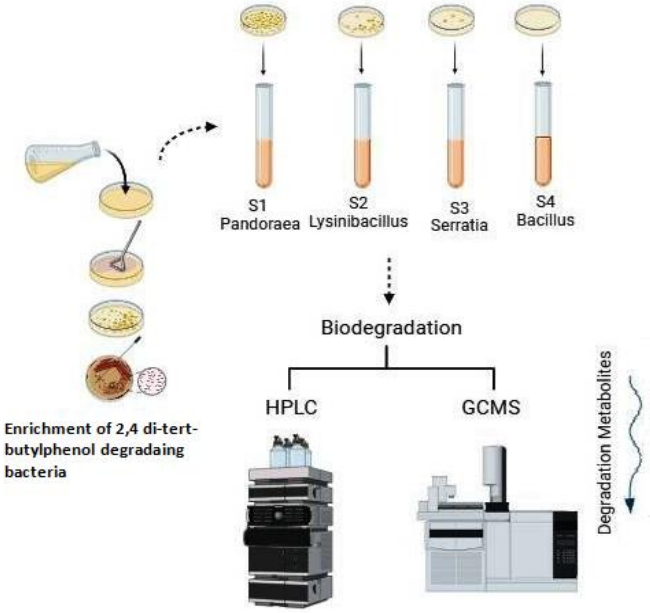
Malle Adolf[1], Farees Ud Din[2], Shuijing Wang[3], Anyi Sun[4], Zihao Zheng[5], Renjie Ni[6], Xiaoxing Liyan Song[7]

[1,2,3,4,5,6,7]School of resources and environmental engineering, Anhui University, Hefei 230601, China

Abstract:
2,4 Diterbutylphenol (2,4-DTBP) is a widely used antioxidant and stabilizer in various industrial applications including polymers, plastics, rubber, lubricants, adhesives, and fuels. 2,4-DTBP is used as an intermediate in the synthesis of other chemicals and as a component in the formulation of personal care products and pharmaceuticals. Despite its beneficial properties, 2,4-DTBP poses environmental and health risks due to its persistence, bioaccumulation potential, and toxicity to aquatic organisms and humans. The biodegradation of 2,4-ditertbutylphenol (2,4-DTBP) was investigated in this study. The degradation of 2,4-DTBP was carried out using bacteria isolated which were isolated from industrial waste water samples. Material and methods involved the isolation and characterization of bacteria isolates, optimization of degradation conditions, and analysis of degradation products. Identification was carried out through biochemical and morphological characteristics, 16S rRNA gene sequence analysis was used for bacteria isolate identification. Four bacteria isolates were successfully isolated and identified, this were isolate D2 (*Pandoraea sp.*), isolate D3 (*Lysinibacillus sp.*), isolate D5 (*Serratia sp.*.), isolate D7 (*Bacillus sp.*). HPLC test were conducted to determine the degradation rate of the bacteria isolates. Results showed significant degradation of 2,4-DTBP by the bacteria isolates, with bacteria isolate D3 (*Lysinibacillus sp.*) having the highest degradation rates reaching up to 89.31% after seven days of incubation. GC-MS analysis was performed to identify potential metabolites. Discussion of the results highlights the potential of microbial biodegradation as a promising approach for remediation of 2,4-DTBP-contaminated environments.

Keywords: 2,4 DTBP, Biodegradation, HPLC, 16S rRNA, GC-MS.

Graphical abstract.



1. Introduction:

2,4-Ditertbutylphenol (2,4-DTBP) is a chemical compound belonging to the family of phenols. It is commonly referred to as a phenolic antioxidant due to its ability to inhibit oxidative degradation processes in various materials. The chemical structure of 2,4-DTBP consists of a phenolic ring with two tert-butyl (tert-butyl groups) substituents at positions 2 and 4. The molecular formula of 2,4-DTBP is $C_{14}H_{22}O$. It is a colourless to pale yellow liquid or crystalline solid at room temperature, depending on its purity and form.

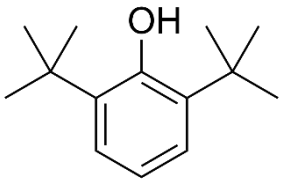


Figure 1. chemical structure of 2,4 – di-tert-butylphenol (2,4- DTBP)

The rapid industrialization and urbanization processes have led to the widespread release of various pollutants into the environment, posing significant risks to ecosystems and human health. Among these pollutants, phenolic compounds, such as 2,4-ditertbutylphenol (2,4-DTBP), have drawn considerable attention due to their toxic effects and persistence in the environment. (Zhang et al, 2020; Luo et al 2022). 2,4-DTBP is commonly used in industrial processes, including the production of antioxidants, stabilizers, and UV absorbers, leading to its presence in industrial effluents, wastewater, soil, and groundwater. Despite its widespread use, 2,4-DTBP poses environmental challenges due to its toxicity and persistence, necessitating effective remediation strategies to mitigate its adverse impacts on ecosystems and human health. Traditional remediation methods, such as chemical oxidation and adsorption, have limitations such as high cost, incomplete removal, and generation of toxic byproducts. Microbial biodegradation emerges as a promising alternative for the remediation of 2,4-DTBP-contaminated environments due to its cost-effectiveness, eco-friendliness, and potential for complete mineralization of pollutants (Toyama et al. 2009; Yang et al. 2016).

Other research have been done on the use of microbial isolates for the biodegradation of pollutants, using microorganism for the removal and degradation of the pollutants, for example Wang et al did a research on the use of newly isolated Pseudomonas sp. for the biodegradation of nicotin, where he achieved successful biodegradation of nicotin , (Wang et al. 2012), also Haddadi et al performed a research on Biodegradation of phenol in hypersaline conditions by Halomonas sp. strain PH2-2 isolated from saline soil (Haddadi et al, 2013). This goes to show the effectiveness of bacteria isolates in the degradation of phenol pollutants. Other researches have been conducted on the degradation of 2,4 DTBP (Klekner et al. 1992; Viggor et al. 2002), but no research has been done on the use of bacteria isolate for the biodegradation of 2,4 DTBP. In this study, we investigate the biodegradation of 2,4-DTBP using bacteria isolates isolated from waste water samples.

Isolation was accomplished via the enrichment technique, followed by the identification and characterization of the isolated bacteria. Microbial characterization was done using the 16S rRNA approach. Biodegradation investigations were conducted under controlled environmental conditions in a shaker incubator for 7 days. HPLC tests were performed to monitor the biodegradation rate and calculate the biodegradation percentage. The GC-MS analysis aimed to determine the biodegradation pathway and metabolites generated throughout the experiment.

2. Material and Methods

2.1. Sample collection

The effluent was collected from aerobic pool of the Dongzhi Chemical Industrial Park Wastewater Treatment Plant, they were then transferred to the laboratory in refrigerated containers. the samples were then frozen in a -80°C refrigerator and then kept in a 4°C refrigerator. After thawing, PBS buffer was added to the samples for mixing. The mixture was placed on vortex shaker machine and run at 3200 rpm for 5 minutes to get homogenized solution. Subsequently, high-speed centrifugation (12,000 rpm) for 5 minutes was done, and the supernatant was poured off. This process was repeated several times to remove organic material from the sample.

2.2. Isolation and characterization of 2,4 Ditert-butylphenol (2,4-DTBP) degrading bacteria:

The bacteria capable of degrading 2,4-DTBP were isolated using enrichment culture techniques. The isolation process involved inoculation of the waste water samples into selective media supplemented with 2,4-DTBP (10mg\L) as the sole carbon source. The mineral salt solution (Enrichment medium) was prepared by dissolving specific salts (KH_2PO_4, 2.5008 g/L; K_2HPO_4, 1.5003 g/L; NH_4Cl, 1.01 g/L; and $MgSO_4.7H_2O$, 0.5002g/L) in 1 liter of distilled water. the pH is then adjusted to 7.0 using NaOH, the solution was distributed into nine 250-ml Erlenmeyer flasks, 0.1 ml of microelements (micronutrients) was added into each flask, the flasks were then placed in shaker incubators at 30 °C for 7 days with shaking. After one weak in the shaker incubator repeated enrichment was then carried out to enrich the cultures, after the first enrichment, an aliquot of 4 ml of enriched culture was inoculated into another Erlenmeyer flask containing 100 ml of fresh MSM with the same concentrations of 2,4 DTBP for the second enrichment. Subsequent enrichments were carried out up till the 4^{th} enrichment, After the 4^{th} enrichment, cultures were inoculated on MSM agar plates. Isolation and purification of the bacterial isolates were carried out on nutrient agar plates through conventional spread plate techniques. Bacterial isolates were selected based on their morphology, they were then preserved and maintained at 4 ◦C on nutrient agar

slants and glycerol stocks at −20 ◦C for further investigation. The bacteria isolates were then labelled (bacteria isolate D1-D8)

2.3. Bacteria isolate growth and optimization

The bacteria isolates were tested for their ability of grow and use 2,4 DTBP as an energy and carbon source. 4ml of Each bacteria isolate was inoculated into 100ml MSM (pH adjusted to 7) supplemented with 2,4 DTBP as the sole carbon source. The samples were then placed in a shaker incubator shaking at 150 rpm at 30◦C. After one week in the shaker incubator the Bacterial growth was measured at 600 nm using a spectrophotometer, each bacteria isolate had 3 replicates, the measurements were taken and average calculated.

2.4. Molecular Identification of the Selected Bacterial Isolates

The single microbial colonies are then carefully picked up from the plate using a sterile loop and thereafter transferred to small tubes containing 2ml of glycerol. They are then cryopreserved in the glycerol. Following cryopreservation, the microorganisms (bacterial isolates) are then revived and delivered to the testing centre for the purpose of analysis and identification. The bacteria isolates were identified by 16S rRNA sequencing. The 16S rRNA gene was obtained by PCR amplification using universal bacterial primers 27F and 1492R. The PCR process commenced with an initial denaturation at 95℃ for 5 minutes, followed by 30 cycles denaturation at 95℃ for 45 seconds, annealing at 55℃ for 45 seconds, and extension at 72℃ for 90 seconds. The final extension step was performed at 72℃ for 5 minutes. The resulting sequences were compared against the available DNA sequences in the GenBank database using the Basic Local Alignment Search Tool (BLAST). The sequence alignment was generated using Cluster X and phylogenetic analysis was conducted using MEGA 7.0.

2.5 Biodegradation experiment (Shake Flask method)

2.5.1 Inoculum preparation

Pure cultures of bacterial strains were cryopreserved in 25% glycerol at -80°C to maintain their purity. The bacterial strains were revived and inoculated in a 250-ml autoclaved Erlenmeyer flask with 100 ml of sterile MSM supplemented with 2,4 DTBP at a concentration of 10 mg/L. Erlenmeyer flask were used 3 for each bacteria strain (replicates of 3s), A control flask containing 10 mg/L of 2,4 DTBP in a medium without any bacterial isolate was utilized to assess the non-biological degradation, including the abiotic losses of 2,4 DTBP during the tests. The experiment was carried out under controlled environmental conditions by continuously shaking at a speed of 150 rpm at 37 °C for duration of 7 days. One-millilitre samples were collected at 12-hour intervals over a period of 7 days for analysis using spectrometry, high-performance liquid chromatography (HPLC), and gas-chromatography mass spectrometry (GC-MS).

2.6. Quantification of 2,4 DTBP

The percentage degradation was calculated after measuring the concentration of 2,4 DTBP for each bacterial isolate using high-performance liquid chromatography (HPLC) according to a validated method previously described Higashi et al. (Higashi et al, 2017). 2,4 DTBP samples were centrifuged at 10,000 rpm for 10 minutes to separate cell mass from the supernatant. An inorganic solvent, specifically n-hexane, was used to extract the samples for examination. Chromatographic separation was performed using an HPLC system with a mobile phase consisting of methanol, a flow rate of 0.43 ml/min. A Varian C-18

column measuring 150 mm × 3.0 mm was utilized, with the column temperature set at 25 °C. Additionally, a UV detector was employed, specifically set at wavelength of 280nm. Using HPLC test, a standard calibration curve was constructed for 2,4 DTBP at different concentration (0.5ppm, 1ppm, 5ppm, 10ppm, 20ppm). The equation of curve was used as an accurate determination of the concentration of 2,4 DTBP.

2.7. Percentage degradation (%degradation)

The percentage degradation of 2,4 DTBP was calculated after measuring the final concentration for each bacterial isolate using high-performance liquid chromatography. The degradation percentage (%) was calculated for each of the four bacteria strains, the degradation percentage was calculated using the formula:

$$\% \text{ degradation} = \frac{\text{Initial concentration} - \text{final concentration}}{\text{Final concentration}} \times 100$$

Where the Initial concentration is the original concentration at the beginning, and the final concentration is the concentration after 7 days of incubation.

2.8. Determination of metabolites

The samples were further examined using gas chromatography-mass spectrometry (GC-MS) to identify metabolites (by- products) formed during degradation. A gas chromatography method described by Aravinth et al (Aravinth A et al, 2023) was used in carrying out GC-MS. A gas chromatograph STG model GC 101B (Sanayeh Teif Gostar, Tehran, Iran) equipped with a flame ionization detector (FID) was used. The injection port system functioned as a split less injector. The capillary column and GC conditions employed in this study possessed the following characteristics: The column has length of 30 meters and an inner diameter of 0.25 mm. It is coated with CARBOWAX 20M; with a stationary phase of thickness of 0.5 mm. The carrier gas nitrogen, flows at rate of 30 cm/sec at a temperature of 80 °C. For the FID flame, the flow rates of air and hydrogen are 300 and 35 millilitres per minute, respectively. The injection port temperature was set to 200 °C in split less mode; after 4 minutes, the split valve was opened at a split ratio of 1:50. The detector temperature was set to 250 °C; oven temperature was set to start at 80 °C for 5 minutes, then ramped up to 220 °C with a rate of 10 °C per minute and was held at 220 °C for 5 minutes.

2.9 Statistical Analysis

Statistical analysis was done using Excel 2021 which was used to calculate the mean and standard deviation (M±SD). Plots were generated using the Origin2022b software package and Microsoft excel 2021.

3. Results

3.1. Isolation and screening of 2,4 DTBP degrading bacteria.

In order to isolate bacteria capable of degrading 2,4 DTBP, the enrichment technique was used to culture bacteria isolates. Mineral salt medium was prepared and supplemented with 2,4 DTBP (10mg/L). To determine the ability of the bacteria, isolate to use 2,4 DTBP as a carbon and energy source, the growth of the bacteria isolate was measured using the spectrometer (OD600). Among the 8 successfully isolated bacteria isolate, 4 was able to grow and utilise 2,4 DTBP as a carbon and energy source. These bacteria isolate include bacteria isolate D2, bacteria isolate D3, D5, D7. The 4 bacteria isolates were then selected for characterization.

3.2 Identification of microbial isolates (Bacterial strains)

The bacteria isolates were identified by sequencing the 16S rRNA gene to determine the species. Using the universal bacterial primers 27F and 1492R, PCR amplification produced the 16S rRNA gene. In PCR, reaction was set at 95℃ denaturation for 5 minutes, followed by 30 cycles of 95℃ denaturation for 45s, annealing at 55℃ for 45s, and elongation 72℃ for 90s, and a final step of 72℃ for 5 minutes extension. The resulting sequences were compared against the available RNA sequences in GeneBank using the Basic Local Alignment Search Tool (BLAST). The sequence alignment was constructed using Cluster X, and phylogenetic analysis was conducted using MEGA 7.0. Bacteria isolate D2 16S rRNA amplicons exhibited a 99.72% identity with *Pandoraea sp.* with accession number MT393046, Bacteria isolate D3 exhibited a 99.65% identity with *Lysinibacillus sp.,* accession number KC041973 ， Bacteria isolate D5 16S rRNA amplicons exhibited 99.79% identity with *Serratia sp.* strain with accession MK645672, bacteria isolate D7 exhibited a 99.65% identity with Bacillus sp. sp. strain with accession number KC819803

The genomic sequences are available in GenBank, access No.: MT365654, MT365854, MT365485, MT365954.

Table 1. Identification of microbial isolates

Bacteria sample	Proposed bacteria specie	Identity (%)	GenBank sequence ID
Isolate D2	*Pandoraea sp.*	99.72%	MT393046
Isolate D3	*Lysinibacillus sp.*	99.65%	KC041973
Isolate D5	*Serratia sp.*	99.79%	MK645672
Isolate D7	*Bacillus sp.*	99.65%	OC819803

3.3 Degradation kinetics

3.3.1 Standard calibration curve

A standard calibration curve was constructed from the HPLC at different concentration to precisely quantify the amounts of pollutants and accurately identify the concentrations of 2,4 DTBP as shown in figure 3. The total antioxidant capacity was determined by employing the linear regression equation (y= 575.90x + 1940.52, R^2= 0.9989 where x is the concentration of 2,4 DTBP and y is the peak area of the HPLC spectra.

3.3.2 Biodegradation of 2,4 DTBP (Degradation Studies)

The bacterium isolate D2 (*Pandoraea sp.*) exhibited the most rapid and substantial development rate in comparison to the other bacterial isolates, whereas the bacterium isolate D5(*Serratia sp.)* had the slowest growth rate. Below table 2 is an exclusive illustration of mean absorbance of the three replicates of each bacterial isolate following three and seven days of incubation highlighting individual growth of bacteria isolate D2, D3, D5, D7 in mineral salt medium (MSM) using 2,4 DTBP as sole carbon and energy source.

Table 2. mean absorbance of bacteria isolates after 3 days and 7 days of incubation

Bacteria isolates	Absorbance (Mean ± SD) 3rd Day	Absorbance (Mean ± SD) 7th Day
Pandoraea sp. (D2)	0.521 ±0.004	0.673 ±0.005
Lysinibacillus sp. (D3)	0.301±0.005	0.492±0.008
Serratia sp. (D5)	0.051 ±0.001	0.21 ±0.003

Bacillus sp. (D7)	0.221 ±0.005	0.359±0.008

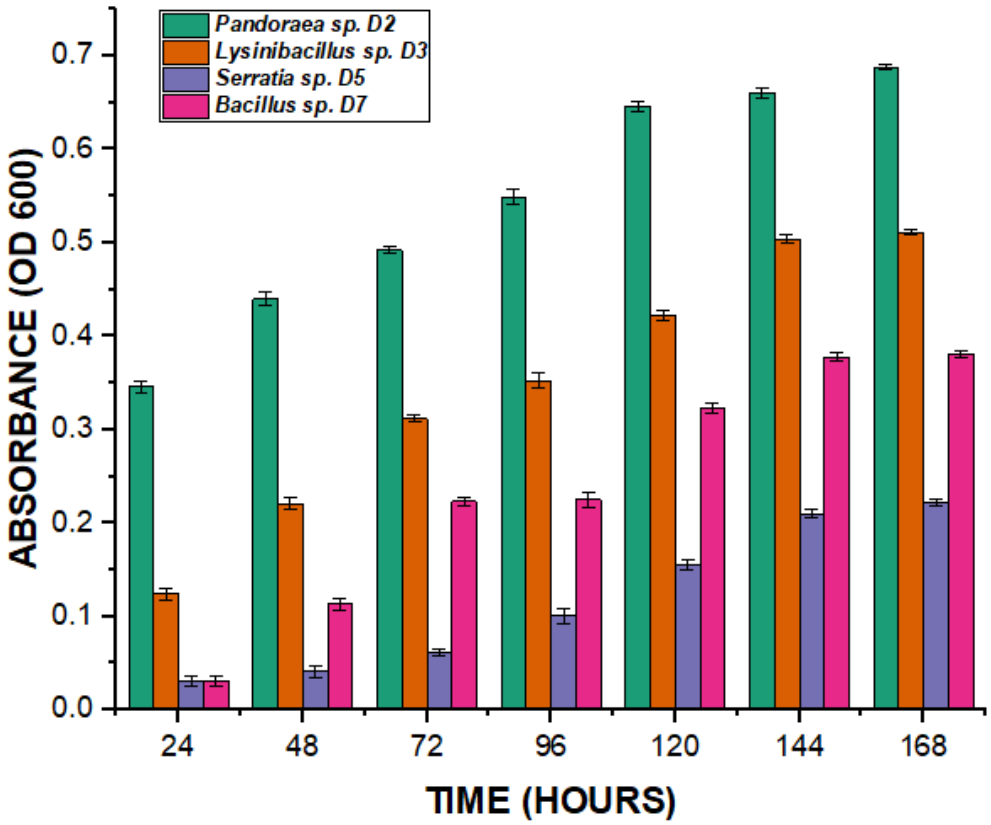


Figure 2. Schematic diagram showing the mean absorbance of bacteria isolates after seven days of incubation.

In addition, the degradation of 2,4 DTBP was verified by HPLC and GC-MS analysis. Samples were extracted at regular intervals, with a frequency of one sample per 12 hours over a span of 7 days. The biodegraded amount and the quantity of intermediates formed during biodegradation were analyzed by HPLC and GC-MS, respectively. Bacterial strains D2, D3, D5, and D7 exhibited the capability to break down 2,4 DTBP at a concentration of 10ppm (parts per million). The degradation percentage was calculated from the HPLC data using the linear equation derived from the standard calibration curve. The bacteria isolate D3 demonstrated the highest calculated degradation percentage, reaching up to 89.31±1.19%, for the degradation of 2,4 DTBP. The isolate D2 followed with a degradation percentage of 82.24±1.15%. Among the isolates, the lowest degradation percentage of 74.31±0.91% was observed for isolate D7. Following a 7-day of incubation period, isolate D5 exhibited a degradation rate of 80.42 ± 0.89%.

Table 3. An illustration of HPLC generated data percentages of 2,4 DTBP degradation by bacterial isolates after 3rd day and 7th day of incubation.

Bacteria isolates	**Degradation % (Mean±SD) 3rd Day**	**Degradation % (Mean±SD) 7th Day**
Pandoraea sp. (D2)	53.43 ±0.51	82.24 ±0.79

Lysinibacillus sp. (D3)	59.68 ±0.49	89.31 ±1.19
Serratia sp. (D5)	57.33 ±1.11	80.42 ±1.10
Bacillus sp. (D7)	52.11 ±1.41	74.13 ±0.91

The control group, which was devoid of microorganisms, exhibited minimal alteration over a 7-day incubation period under identical experimental circumstances. The bacterial strains D2, D3, D5, and D7 exhibited the ability to breakdown 2,4 DTBP at a concentration of 10 ppm, resulting in degradation rates of 82.24%, 89.31%, 80.42%, and 74.13%, respectively as shown table 3.

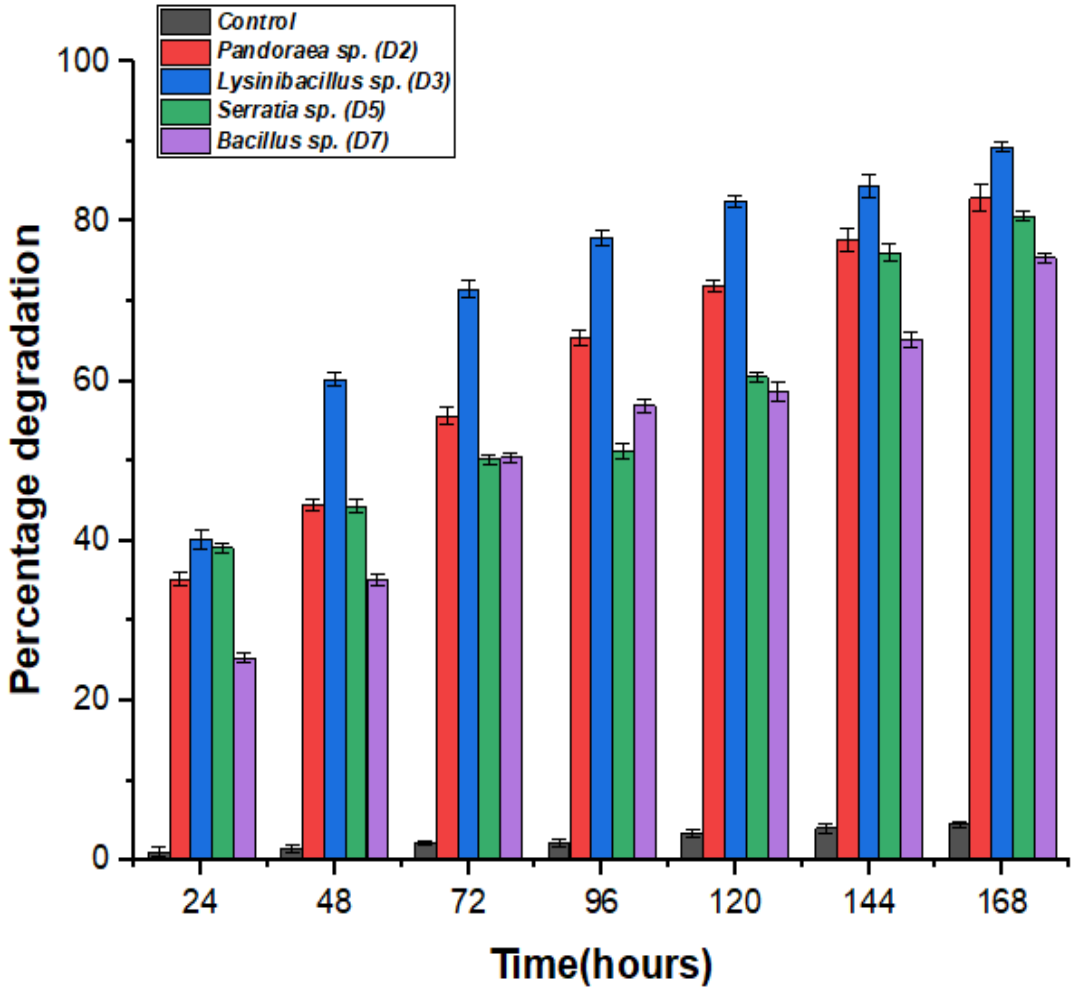


Figure 3. percentage degradation of 2,4 DTBP by bacteria isolate after 7 days of incubation 3.3.3. GC- MS (metabolite determination)

Gas chromatography-mass spectrometry (GC-MS) were used to examine the samples in order to determine the metabolites formed during degradation process. GC-MS analysis of 2,4-DTBP revealed the presence of several metabolites. These included hydroxylated derivatives, di-tert-butyl-catechol, glucuronide and oxidized derivatives. These metabolites suggest the involvement of various metabolic pathways, including hydroxylation, oxidation, methylation, and conjugation.

The identification of metabolites provides insights into the metabolic pathways of 2,4-DTBP in biological systems. Hydroxylation and oxidation appear to be prominent pathways, leading to the formation of hydroxylated and oxidized derivatives（Olubusoye et al. 2023; Zerhusen et al. 2023）

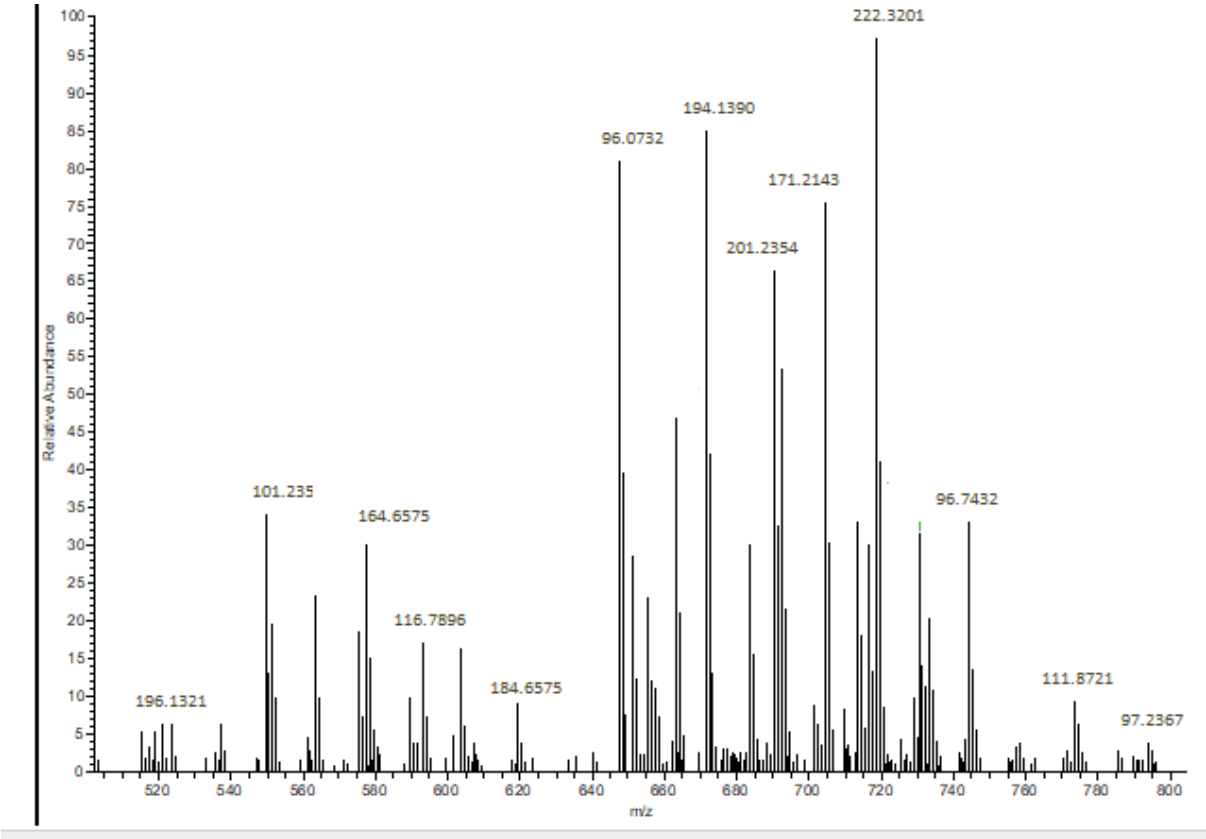


Figure 4. GC-MS results of 2,4 ditertbutylphenol showing possible metabolites formed after degradation

4. Discussion:

This research focuses on the capability of bacteria isolates to degrade 2,4 ditert-butylphenol (2,4-DTBP). The results of this study demonstrate the varying degrees of 2,4-DTBP degradation efficiency among the four bacterial isolates. The bacterial strains were isolated using the enrichment technique; a total of four bacterial were successfully isolated. The 16S rRNA gene sequence analysis was used confirmed the identification of the bacteria isolates. According to sequence analysis the bacteria were identified as *Pandoraea sp.* (bacteria isolate D2), *Lysinibacillus sp.* (bacteria isolate D3), *Serratia sp.* (bacteria isolate D5), *Bacillus sp.* (bacteria isolate D7).

Growth analysis were carried out on the bacteria isolate using a spectrometer to measure their ability to grow and utilise 2,4 DTBP as an energy and carbon source. Results showed that all four bacteria isolates were able to grow over a period of seven days as seen in figure, this goes to show that the bacteria isolates were capable of utilizing 2,4 DTBP as an energy and carbon source observed in the increase of absorbance value (OD600) over time as seen in figure 2. The rise in absorbance value over time indicates microbial growth and increase in microbial cells which in turns absorbs more light.

HPLC analysis was used to determine the concentration of 2,4 DTBP over time. The results from the HPLC test demonstrate a reduction in the concentration of 2,4 DTBP, this underscores the capability of the bacteria isolates to breakdown and degrade 2,4 DTBP over time. The results from the HPLC tests were used to calculate the degradation percentage of each of the bacteria isolates, the results which are illustrated in figure 3 shows an increase in the percentage degradation of 2,4 DTBP by the bacteria isolates

further confirming the ability of the bacteria isolate to use 2,4 DTBP as a carbon and energy source thereby leading to its breakdown and degradation.

Our findings revealed considerable variability in the degradation efficiencies among these isolates, with Bacteria D3 (*Lysinibacillus sp.)* exhibiting the highest degradation rate of 89.31%, followed by Bacteria D2 with 82.24%, Bacteria isolate D5 had an 80.42 % degradation rate while bacteria isolate D7 had the lowest degradation rate of 74.13%. Over the period of 7 days of incubation, the control sample with no bacteria isolate (abiotic loss) had a very low degradation rate of 4% after 7 days of incubation.

Bacteria isolate D3 (*Lysinibacillus sp.)* showed the highest degradation rate, suggesting its potential for effective bioremediation of 2,4-DTBP-contaminated environments. The high degradation efficiency of Bacteria isolate D2 could be attributed to its metabolic capabilities and enzymatic machinery tailored for aromatic compound degradation. Conversely, Bacteria D2, D5, D7 exhibited relatively lower degradation efficiency, indicating differences in substrate specificity or metabolic pathways among the isolates. The differences in degradation efficiency among the bacterial isolates could be attributed to several factors, including the presence of specific degradation genes, enzyme kinetics, this finding aligns with previous studies indicating the presence of specific genes encoding enzymes involved in the degradation of phenolic compounds, such as 2,4-DTBP, in certain bacterial species (Sun et al, 2021; Iwaki et al, 2015). The observed differences in degradation efficiencies among the bacterial isolates could also be attributed to differences in metabolic pathways, Bacteria isolate D3(*Lysinibacillus sp.)*, characterized by its superior degradation efficiency, likely possesses specialized catabolic pathways or enzyme systems optimized for aromatic compound degradation. (Li et al, 2017; Chen et al, 2023)

Furthermore, gas chromatography-mass spectrometry (GC-MS) analysis was done to show the metabolites formed during the degradation of 2,4 Ditert butylphenol.

GC-MS analysis revealed the presence of several intermediate metabolites generated during the degradation of 2,4-DTBP by the bacterial isolates. Commonly observed degradation products of 2,4-DTBP include tert-butylphenols, hydroquinone, catechol, and aliphatic acids. Tert-butylphenols are typically formed through initial hydroxylation of the aromatic ring, followed by subsequent oxidation and cleavage reactions, leading to the formation of smaller aromatic and aliphatic compounds (Ďurišová et al,2022; Li et al, 2017) Hydroquinone and catechol has also been identified as central intermediates in the degradation of aromatic compounds (Manabe et al. 1999; Yan et al. 2018)

CONCLUSION

The isolation of microorganisms for biodegradation is a highly successful approach for breaking down contaminants and environmental pollution. This research study investigates the potential of microorganisms isolated from waste water to breakdown 2,4 DTBP. The wastewater consisted of microorganisms that could degrade 2,4 DTBP. These microorganisms (bacteria isolates) were isolated and used for the biodegradation of 2,4 DTBP. The research investigation demonstrated the efficient degradation of 2,4 DTBP by bacterial isolates. Four bacterial strains were identified and isolated: D2-Pandoraea sp., D3-Lysinibacillus sp., D5-Serratia sp., and D7-Bacillus sp. The bacterium isolate D3（Lysinibacillus sp) exhibited superior degradation ability for 2,4 DTBP compared to other bacterial isolates. It achieved a degradation efficiency of up to 89.31% for 2,4 DTBP within a period of 7 days. The degradation pathway was examined using GC-MS. In the GC-MS test, we noticed that 2,4 DTBP was broken down into Various metabolites. Our current study demonstrates that bacteria isolates are a highly successful approach for the biodegradation of contaminants.

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Declaration of Interest

We, the authors of the "Biodegradation of 2,4 Di-tert-butylphenol by Bacterial Isolates from Industrial Wastewater Treatment Plant" declare the following potential conflicts of interest:

The authors have no financial or proprietary interests in any material discussed in this article.

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