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Growth Optimization of Naphthalene-Degrading Pseudomonas Stutzeri Strain BUK_BTEG1 Isolated from Petrochemical Contaminated Soil.

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Abstract

Bioremediation of PAHs such as naphthalene by the activity of microorganisms represents an eco-friendly, economical, and safe decontamination approach compared physical and chemical processes. In this study, the biodegradation conditions for naphthalene by Pseudomonas stutzeri strain BUK_BTEG1 was optimized through on-factor-at-a-time (OFAT). The Characterization was conducted by studying the effect of incubation time, nitrogen source, pH, inoculum size, substrate concentration and temperature in BH media containing naphthalene as sole source of carbon and energy. The growth and degradation of naphthalene by this strain was optimal at 1000 mg/L ammonium Nitrate, substrate concentration of 800mg/L, temperature of 35 °C, pH of 7.0, inoculum size of 4% (v/v) and 72 hours of incubation time. After 72 hours of incubation under optimal conditions, the degradation efficiency of this strain to naphthalene was 72%. The analysis using gas chromatography-mass spectrometry reveals 1,2-naphthalenediol, 1-hydroxy-napthoic acid, 1,4-Napthoquinone, salicylic acid, and catechol as metabolites of naphthalene degradation by comparing the fragmentation pattern and mass spectrum obtained with data available on NIST library. This strain demonstrates promising potential and could be handy for its application in the bioremediation of naphthalene contaminated sites.

Nomenclature and units

1.0 Introduction

A major problem for environmental contamination is a class of organic chemicals known as polycyclic aromatic hydrocarbons (PAHs), which are composed of two or more aromatic rings arranged in a cluster, an angular configuration, or both. They are extensively dispersed in the water, the soil, and the atmosphere. PAH production from fuels and incomplete combustion processes has recently increased (Honda and Suzuki, 2020). PAHs released into the environment by a variety of activities, including the combustion of fossil fuels, shipping, usage, and disposal of petroleum products, agricultural burning, coal, and wood-preserving goods, are chronically dangerous organic pollutants. Many of them have been shown to be toxic, mutagenic, and carcinogenic (Haritash& Kaushik, 2009; Varjani et al., 2017).

Due to their recalcitrant nature in natural environment, they pose harmful effects on microorganisms as as well as to higher forms of life including humans (Honda & Suzuki, 2020). Naphthalene is the most basic polycyclic aromatic hydrocarbon, with a fused pair of benzene rings as its structure. Naphthalene is primarily used in the synthesis of phthalic anhydride. Naphthalene is also used in carbamate insecticides, surface active agents, and resins; as a dye precursor, synthetic tanning agent, and moth repellant; and in a variety of organic compounds. Furthermore, naphthalene and phenanthrene are petroleum constituents. Naphthalene, like other PAHs, is a fairly hazardous chemical. Hemolytic anemia, liver damage, and brain damage are caused by inhalation, ingestion, and skin contact. Contact with naphthalene can result in cataracts and retinal damage. The Environmental Protection Agency (EPA) and the International Agency for Research on Cancer (IARC) have classified naphthalene as a Group C probable human carcinogen among the 16 priority PAHs due to its inherent toxicity (USEPA 2008; IARC, 2010).

Many conventional engineering-based physicochemical decontamination methods, such as soil washing, chemical inactivation (using potassium permanganate and/or hydrogen peroxide as a chemical oxidant to mineralize non-aqueous contaminants such as petroleum), and incineration, are costly due to the cost of excavating and transporting large quantities of contaminated materials for ex-situ treatment. Dispersion, dilution, sorption, volatilization, and abiotic transformations are some other physicochemical techniques used for the same purpose (Abo-state et al., 2018). The rising costs and limited efficacy of traditional physicochemical treatments have prompted the development of alternative technologies for in-situ applications, particularly those based on plant and microorganism biological remediation capabilities (Ghosal et al., 2016). Biodegradation, for example, is an environmentally acceptable, practical, and low-cost method for both ex situ and in situ cleanup of PAH-contaminated environments (Haritash& Kaushik, 2009; Ghosal., 2016; Gupta et al., 2022). Biodegradation is a promising and successful treatment approach for removing most organic pollutants such as PAH from polluted areas, in which hazardous organic chemicals are degraded by microbial enzymatic processes into simpler harmless molecules (Ghosal et al., 2016). PAH was utilized as a carbon source by microorganisms in natural degradation, which constitutes a significant portion of the indigenous microorganism populations found in polluted soils (Ghosal et al., 2016; Rabiu and Gimba, 2021).

Many bacteria that are capable of degrading PAHs have now been identified and described. Few of these bacteria have been linked to Proteus species; most of them are from the genera *Pseudomonas*, *Rhodococcus*, *Paenibacillus*, *Acinetobacter*, *Bacillus*, and *Mycobacterium* (Suzuki and Takizawa, 2019; Bibi et al., 2019; Shehu et al., 2021; Muhammed et al., 2022). The rate and extent of PAH biodegradation, on the other hand, are affected by a variety of parameters such as pH, nutrient availability, temperature, and bacterial species. *Pseudomonas stutzeri* strain BUK BTEG1 (NCBI accession No. OMO39162) was first isolated and identified from our laboratory based on its capacity to degrade anthracene and phenanthrene (Muhammed et al., 2022). As a result, our primary aims are to discover the ideal conditions for naphthalene biodegradation by this strain, as well as to investigate the effects of several process parameters (pH, nitrogen source, substrate concentration, inoculum size, and temperature) on biodegradation. The metabolite formed during the breakdown process was also studied.

2.0 Materials and Methods

2.1 Media and chemicals

All of the chemicals utilized in this project were of the highest purity possible and came from commercial sources (Sigma-Aldrich, Fisher Scientific Pvt. Ltd and HiMedia).

Sigma Aldrich at Bellefonte, United States, provided the naphthalene (Nap). Acetone was purchased from Fisher Scientific Pvt. Ltd. in Mumbai, India as the solvent for the study. Other chemicals were procured from HiMedia Laboratories Pvt. Ltd., Mumbai, India. Stock solutions 1000mgL^{-1} was prepared in acetone and kept in brown bottles at 4 °C.

2.2 Methods

2.2.1 Microorganism and media preparation

Previously isolated *Pseudomonas stutzeri strain BUK_BTEG1* (Muhammed *et al.*, 2022)initially isolated from petrochemical contaminated soil of Kwakwachi in Kano, Nigeria was culture in Bushnell-Haas (BH) liquid medium at 37^{0}C. The medium is made up of 0.2 g $MgSO_4$, 1g KH_2PO_4, 1g K_2HPO_4, 1gNH_4NO_3, 0.02 g $CaCl_2$ and 0.05 g of $FeCl_3$ in 100 cm^3 of distilled water. In the case of Bushnell Hass solidd, 15 g of agar-agar (Cole-Palmer® product) was added and sterilized in Astell® autoclave. The isolate was maintained and sub-cultured every two weeks in Laurie-Bertanni agar medium.

2.2.2 Optimisation of Naphthalene Degradation Using One-Factor-at-a-Time (OFAT)

The OFAT method was used to investigate naphthalene breakdown by optimizing the bacterium's growing conditions. Nitrogen source and concentration, pH, substrate (naphthalene) concentration, inoculum size, and temperature were the parameters optimized. Each parameter was tested progressively using previously optimized values in 100 cm3 BH medium on an orbital shaker (150 rpm) at 37 C for 72 hours of incubation period. The nitrogen sources used were ammonium chloride, ammonium nitrate, ammonium sulphate and sodium nitrate. Ammonium nitrate was the best and subsequently tested at concentration ranging from 200mg/l-1400mg/l. media without the addition of any nitrogen source was run as control. The effect of initial pH was examined across a range of 5.5 to 8.5. The effect of inoculum size was also examined across the range of 1 to 10%. (1- 10 cm^3) The influence of initial concentration of the carbon source (naphthalene) ranges from 100 mg/L to 1000mg/L were also examined. Likewise, the effect of temperature across the range of 25^{0}C to 50^{0}C was also examined. The ability of the bacteria to utilize and degrade naphthalene from the BH broth was supervised by measuring the increase in turbidity of the broth at 24 hours interval at OD $_{600\ nm}$ with a UV-VIS spectrophotometer (Spectrum-Lab 7525).

2.2.3 Quantification of Naphthalene Degradation

At the end of the incubation period (72 h), the residual amount of naphthalene in each triplicate flask was extracted with 20cm^3 of ethyl acetate. The extraction yielded two layers and the upper organic layer was removed and measured using a spectrophotometer by the optical density (OD) at λmax 255nm and the formula below (1) (Rabani*et al.*, 2020) was used to compute the percentage degradation:

$$\% \text{ Degradation efficiency} = \frac{Co-Cf}{Co} \times 100$$

C0= Initial concentration in control

Cf= final concentration in test

2.2.4 Biodegradation experiment

Isolate was grown on nutrient agar plates for 24 hours before being inoculated and cultured in LB broth for 10 minutes at 4000 rpm before being collected as pellets. The pellets were standardized BH medium with a spectrophotometer to form a concentrated bacterial solution (1.000.02). Naphthalene degradation by the bacteria strain was performed in autoclaved 250-cm3 conical flasks. Naphthalene was dissolved in acetone at an 800 mg/L concentration. 1 cm3 of the solution was pipetted into a sterilized empty flask and filled to the 99-cm3 mark with BH liquid. The resulting solution was left in shaker overnight to get rid of the acetone solvent. Then, 4% (v/v) inoculum The isolate solution was added to all flasks except the control flasks, and they were incubated for 72 hours at 35 °C in a shaker at 150 rpm. After incubation, the medium was passed through a UV light to stop microbial activity and release the cytoplasmic content, and ethyl acetate was used as a solvent to remove the leftover naphthalene and its breakdown products residue from each medium. 20 cm3 of ethyl acetate was added to the medium and sonnicated for 10 minutes before separation with a separating funnel. The medium was divided into two layers, the upper layer holding the residual naphthalene and its breakdown product and the lower layer containing the remaining naphthalene and its breakdown product. The naphthalene from the top layer was recovered. Allowing the extract to dry, the volume of

each extract was increased to 100 cm3 by adding more ethyl acetate. Before it was used, the residue was stored in a refrigerator at 4^0C.

2.2.5 Gas Chromatography- Mass Spectrometry (GC-MS) analysis

The efficiency of degradation of naphthalene was measured using an Agilent Gas chromatograph (GC 7890B, MSD 5977A, Agilent Tech) fitted with a DB 35-MS Capillary Standard Non-polar column (30 m0.25 mm0.25 M) film thickness. GC-MS was used to examine 1 microliter of the organic phase. For the GC-MS analysis, a gas chromatograph equipped with a split-split less injector (split ratios of 50:1) was employed. Helium was the carrier gas, with a constant flow rate of 1 cm3/min. For the first 5 minutes, the oven temperature was set at 40 degrees Celsius. The injection temperatures, transfer line, and ionization source were all 270 ºC with a pressure of 37.1 Kpa. The mass spectrometer was run in electron impact (EI) mode at 70 electron volts (EV) in full scan mode from 85 to 450 m/z. The temperatures of the injector and detector were 270°C and 280°C, respectively. To confirm and identify the metabolites of naphthalene degradation, NIST GC–MS library program search was used to relate m/z fragmentation pattern of the detected metabolite to the ones in the reference Database (NIST, 2017).

$$\% \text{ Degradation efficiency} = \left[\frac{\text{Standard AUC} - \text{sample AUC}}{\text{standard AUC}}\right] \text{X } 100$$

AUC = Area under the curve/peak area

Statistical Analysis:

All tests were carried out in triplicate. The data shown were the mean and standard deviation (SD) of three replicates (n = 3). The one-way ANOVA test was performed to examine significant differences between more than two sets of data, with the Tukey post-test utilized to compare each separate group with p 0.05 using (SPSS v20) for windows.

3.0 Results and Discussion

In the present study, the bacteria strain *Pseudomonas stutzeri* strain BUK_BTEG1 was tested for its ability to degrade naphthalene. Optimisation of bacteria growth and Naphthalene degradation was carried out using OFAT approach.

3.1 Optimization of naphthalene-degrading bacterium

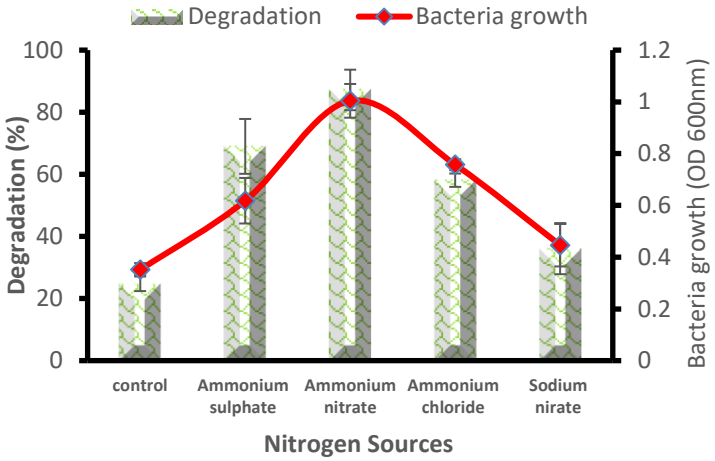

Figure 1: The effect of nitrogen sources on the growth and degradation naphthalene by *P.stutzeri* incubated for 72 h at 37 °C in BH media. Error bars represent mean ± standard deviation, *n*= 3

3.1.1 Effect of nitrogen source

The availability of appropriate nitrogen sources is one of the most important elements in biodegradation processes (Ibrahim *et al.*, 2020). The effect of nitrogen sources on the growth and degradation of naphthalene by the isolate in BH broth is presented in **Figure 1**. The strain exhibits significant increase ($p<0.05$) in growth and degradation on various nitrogen sources (Ammonium chloride, ammonium nitrate, ammonium sulfate, and sodium nitrate). Out of four nitrogen sources used, the best performance was observed when using ammonium nitrate leading to about 87.18% naphthalene degraded. The results of a one-way ANOVA revealed significant ($p<0.05$) differences between ammonium nitrate and the other nitrogen sources evaluated, including the control ($p<0.05$). Nitrogen is necessary for the metabolism and development of all microorganisms, as well as the production of RNA, DNA, and proteins. In the absence of a nitrogen supply, far less growth and degradation were seen, demonstrating the need of providing a suitable nitrogen source. The results of the effects of different nitrogen sources obtained here agree with the studies reported by (Kalantary*et al.*, 2014). However, the result contradicted the works of (Al-dossary*et al.*, 2021; Rani *et al.*, 2021) who reported

sodium nitrate and pepton as the best nitrogen for the degradation of naphthalene.

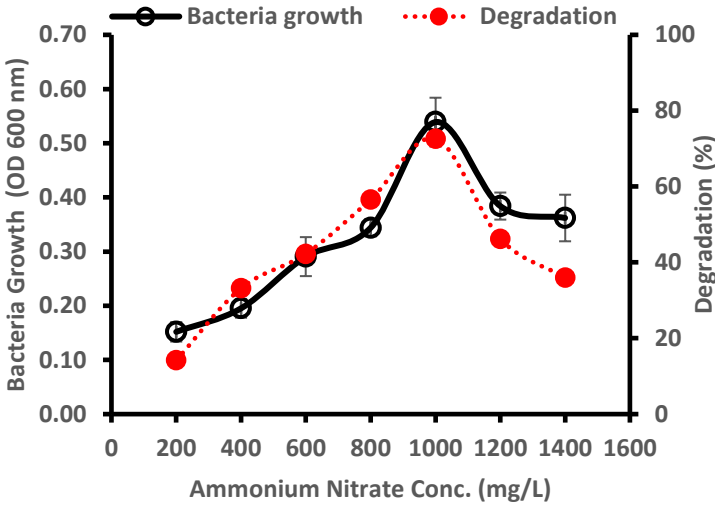

Figure 2. Effect of Ammonium nitrate concentration on the growth and degradation naphthalene by *P.stutzeri* incubated for 72 h at 37 °C in BH media. Error bars represent mean ± standard deviation, *n*= 3

3.1.2 Effect of ammonium nitrate concentration

Having access to an appropriate nitrogen source is crucial, however concentration is also important (Fareez*et al.*, 2021). **Figure 2** present the effect of ammonium nitrate concentration on growth and degradation of naphthalene. There was a steady and progressive increase ($p<0.05$) in growth and degradation of the contaminant by the isolate as the concentration of the ammonium nitrate increases with the isolate attaining the optimum at 1000 mg/L concentration with about 72.64.06% of the naphthalene degraded. At a concentration beyond the optimum, the rate degradation decreases. This might be owing to a lack of ammonium nitrate available to support the bacterium's replication rate, or it could be due to a rise in pH produced by the ammonia content in the medium. Excess ammonia causes the environment to become more alkaline, thereby affecting the rate of degradation. Similarly, the results agree with Singh and Tiwary (2017), who found that 1000mg/L ammonium nitrate was optimal for degradation by bacteria consortium and Bacillus species, but differ from Amani et al., (2020) who found that 3000 mg/L ammonium nitrate was optimal for degradation by bacteria consortium and Bacillus species.

3.1.3 Effect of initial pH

The pH value of the surrounding environment plays a crucial role in microbial degradation of PAH as a sole source of carbon and energy (Abatenh*et al.*, 2017). The effect of initial pH on the growth and degradation of naphthalene by the isolate in standardized BH broth was presented in **Figure 3.** Both growth and degradation were found to be optimum at pH 7 and 87.06 % of the naphthalene degraded. A significant increase ($p<0.05$) both in growth and degradation was observed at pH higher than 5.5. The percentage degradation for at the optimum pH was about 86%, following 72 h incubation at 37 ºC. The pH differences in terms of bacterial growth and naphthalene breakdown were found to be significant ($p<0.05$) using one-way ANOVA. According to other investigations, the optimal pH for hydrocarbon breakdown for individuals and bacteria consortiums was pH 7. (Al-dossary et al., 2021 ; Shehu et al., 2021; Elufisan et al., 2020). Furthermore, Patel et al. (2012) reported 100% naphthalene breakdown at alkaline pH 8.0-10.0, and Wang et al. (2019) confirmed a favorable pH range of 6.0-8.0 for the development of microorganisms for biodegradation, which is consistent with the current investigation.

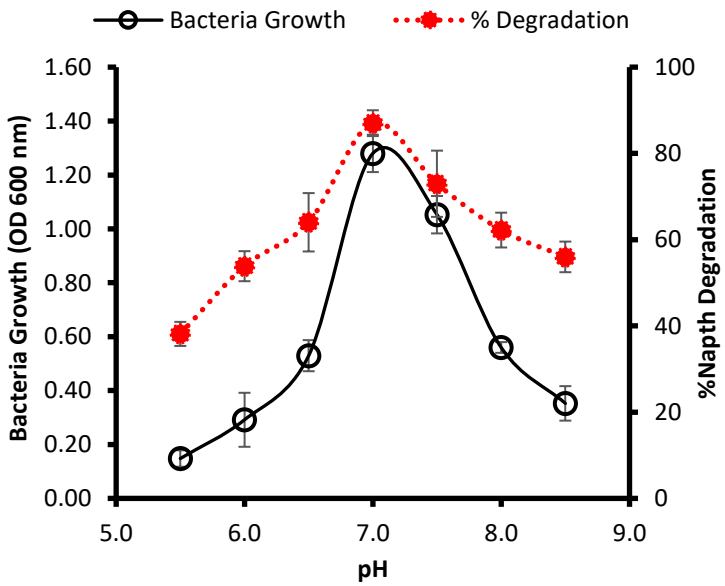

Figure 3. The effect of initial pH on the growth and degradation naphthalene by *P.stutzeri* incubated for 72 h at 37 °C in BH media. Error bars represent mean ± standard deviation, *n*= 3

3.1.4 Effect of inoculum size

The amount of biomass used also had a significant influence in the PAH biodegradation process, and the number of bacteria present in the medium has an impact on how well cells adapt and how much enzyme is produced for cell metabolism (Koutsoumanis and Sofos, 2015). The effect of inoculum size was studied over a range of 1–10% *(v/v)* ***(Figure 4)***. Maximum naphthalene degradation (90.93%) was observed at 4% (v/v) inoculum size. The naphthalene degradation was significantly ($p<0.05$) higher than other inoculum size after 72 h of incubation. There was significant decline in growth when higher inoculum more than optimum was used. In a similar study, Patel et al. (2012) found that strain DMVP2 inoculum levels more than 4 percent (v/v) caused a dramatic decrease in phenanthrene degradation. The decline in growth and degradation seen at higher inoculum beyond the optimum may be caused by the rapid increase in cell density that competes for the scarce nutrients and kills off the less competent cells, or it may likely be caused by insufficient total dissolved oxygen combined with nutrient depletion. (Kyazze, 2014'). Contrary to the results of this study, Rani et al. (2021) showed a fast rise in naphthalene breakdown at 1% inoculum size.

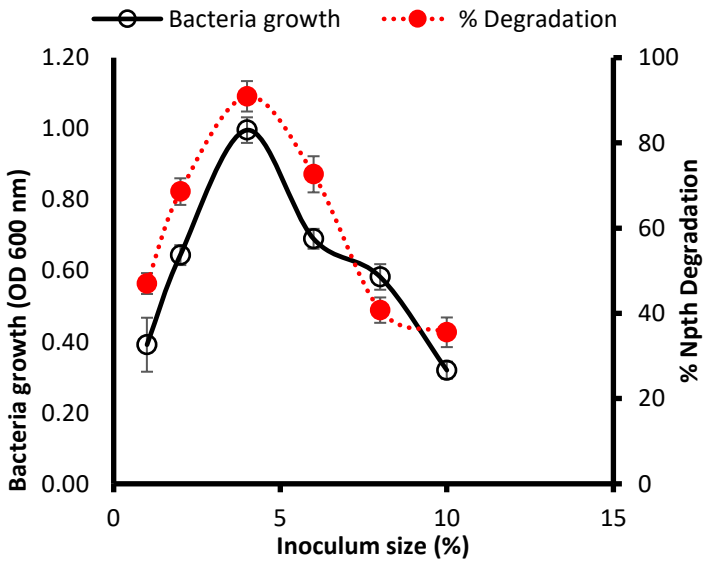

Figure 4: Effect of inoculum size on the growth and degradation of naphthalene by *P.stutzeri* incubated for 72 h at 37 °C in BH broth. Error bars represent mean ± standard deviation, *n*= 3

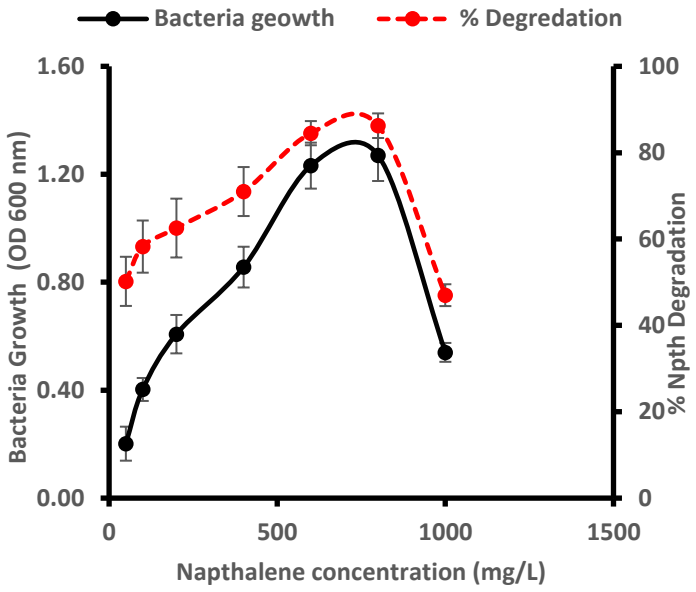

Figure 5: The effect of substrate (naphthalene) concentration on the growth and degradation by *P.stutzeri* incubated for 72 h at 37 °C in BH media. Error bars represent mean ± standard deviation, *n*= 3

3.1.5 Effect of Substrate (Naphthalene) Concentration

Some substrates can be hazardous to microbes at high concentrations (Ibrahim *et al.*, 2015). However, certain microbial species have been shown to withstand high levels of PAH (Bibi *et al.*, 2018; Mohapatra and Phale, 2021). Any prospective biodegradation strain should be able to tolerate and breakdown naphthalene at high concentrations. The effect of different naphthalene concentration (100-1000mg/L) was studied. Optimum growth, and naphthalene degradation of about 86.25% was obtained at initial naphthalene concentration of 800mg/L (**Figure 5**) after 72 h of incubation. The was significant ($p<0.05$) decline in growth and degradation when higher initial concentration of naphthalene was used beyond the optimum. However, there was no significant difference in terms of the strain growth and naphthalene degradation between the 600mg/L and 800mg/L, but 800mg/L significantly ($p<0.05$) differed from other concentrations significantly. Patel. *et al.*, (2018) and Shehu *et al.*, (2022), similarly reported high PAH degradation at initial concentrations of 50 mg/L-1000 mg/L, which then drastically reduced at an initial concentration of 500 mg/L 400mg/L respectively. Additionally, Bibi et al. (2018) reported a similar outcome, with the optimal concentration at 1000 mg/l and a proportionate drop in growth and

degradation at concentrations higher than the optimum. This shows that larger concentrations of PAH may be harmful to metabolic activities, affecting the bacteria's growth and degradation efficiency. In contrast this study, several other studies have demonstrated optimal breakdown at low PAH concentrations (Singh and Tiwari, 2017; Abarian*et al.,* 2018; Rabani*et al.,* 2021; Rani *et al.,* 2021).

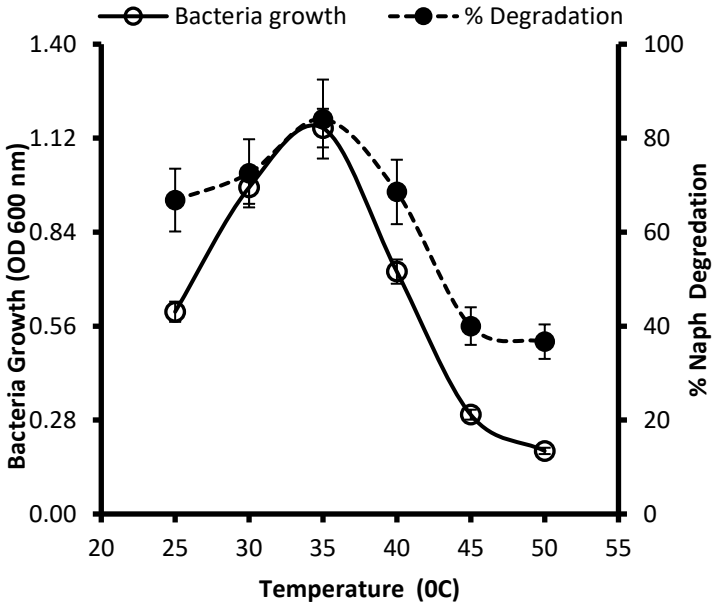

Figure 6: Effect of temperature on the growth and degradation naphthalene by *P.stutzeri* incubated for 72 h at 37 °C in BH media. Error bars represent mean ± standard deviation, *n*= 3

3.1.6 Effect of temperature

Temperature is one of the most important physical elements influencing microorganism growth and multiplication. Temperature influences the physical and chemical constitution, composition, and rate of metabolism of the microbial population during hydrocarbon biodegradation (Al-Hawash *et al.,* 2018). For every enzyme-mediated breakdown process, there is an optimal temperature (Abatenh et al, 2017). Naphthalene degradation at 25 ºC, 30 ºC, 35 ºC and 40ºC were found to be 66.82%, 72.51%, 84.06 %, 68.55 %. After 72h of incubation respectively (Figure 6). Optimum naphthalene degradation which was significantly ($p<0.05$) higher was achieved at a temperature of 35ºC as compared with other initial temperatures. This may be due to an increase in PAHs solubility and bioavailability of naphthalene with an increase in temperature (Ghosal *et al.,* 2016). Columbia *et al.,* (2013) and Nzila*et al.,* (2018) also reported an optimum degradation temperature of 35 °C for *Pseudomonas citronellolis* strain PHC3Z1A and a mixed culture of*Pseudomonadales*, *Actinobacteria*, *Caulobacterales*, *Rhizobiales*, and *Xanthomonadales* (Kumar *et al.,* 2018). The optimal growth conditions can allow microorganisms to release a wide range of enzymes in the environment, which can breakdown the dangerous substances in concern. Unsuitable temperatures can thwart enzyme function by preventing access to substrate due to low temperature (insolubility) or by interfering with enzyme conformation (high temperature). In bacteria, metabolic activity involves enzymes that are prone to irreversible protein structure breakdown at high temperatures Similar to this, numerous investigations have found that mesophilic bacteria studied at temperatures between 30 and 40 °C exhibit enhanced hydrocarbon metabolism (Abo-state et al., 2018; Rani et al., 2021).

3.2 Biodegradation study

Environmental parameter improvement might significantly increase naphthalene biodegradation. Biostimulation is the modification of environmental condition to promote the growth and enzymatic activity of bacteria present (Bibi *et al.,* 2018). The degradation efficiency (%) was determined by subjecting the isolate to optimum conditions achieved through one-factor-at-a-time optimization which include, 1000 mg/L ammonium nitrate pH value of 7.0, , inoculum size 4% (v/v), naphthalene concentration 800 mg/L, temperature 35 ºC and incubated for 72 hours at 120 rpm.

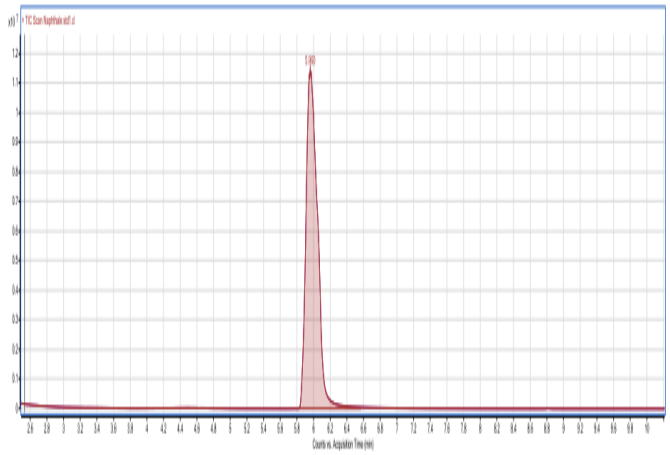

Figure 7A. GC-MS chromatogram of standard naphthalene (uncultured medium) (800mg/L at pH 7.0 ± 0.2 shaken at 120 rpm incubated at 35 ºC for 72h.

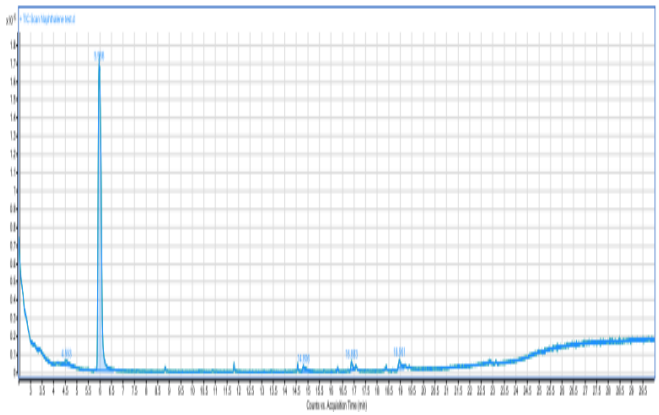

Figure 7B: GC-MS chromatogram of naphthalene degradation product (cultured medium) by *pseudomonas stutzeri* at pH 7.0 ± 0.2, 1.0 g/L $(NH_4)_2NO_3$, 4% (v/v) inoculum size, 800 mg/L and shaken at 120 rpm incubated at 35 ºC for 72 h.

3.2.1 GC-MS analysis

Figures 7A and 7B show the GC-MS chromatograms for naphthalene standard and degraded samples, respectively. The naphthalene standard had an area under the curve (AUC) of 109481798.83 with a retention time of 5.962 (Figures 7A), whereas the degraded naphthalene sample had an AUC of 15894855.37 with a retention time of 5.961 (Figures 7B), showing a degradation efficiency of 85.58% percent within 72 hours of incubation under optimal conditions.

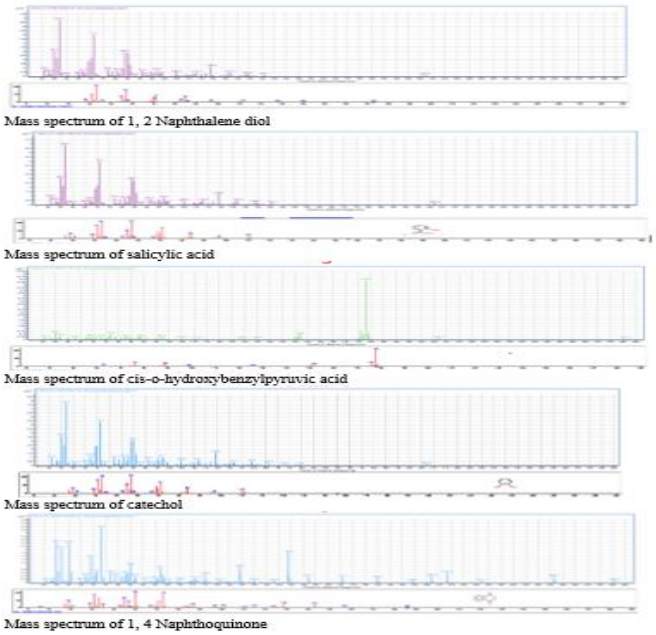

Figure 8 Mass spectrum of naphthalene biodegradation metabolites.

The GC–MS techniques are of great importance in order to highlight some structures, which may constitute intermediates of naphthalene biodegradation. **Figure 8** depicts the metabolites of naphthalene breakdown by this isolate. When the data from the NIST library based on the mass spectrum and fragmentation pattern were compared to the metabolites profile of naphthalene breakdown in this study, compounds such as 1,2-naphthalenediol, Salicylic acid, 1-hydroxy-napthoic acid, 1,4-Napthoquinone and Catechol were identified as naphthalene degradation byproducts. These findings suggested that the PAHs degradation pathway by the study goes through the salicylate pathways, which is similar to the pathways reported for *Pseudomonas sp*. strain Jpyr-1 (Ma *et al.*, 2013), strain CECT 930 (Moscoso*et al.*, 2015), *Pseudomonas otitidis* strain and *Pseudomonas.Stutzeri*P4 (Singh and Tiwary, 2017),

4.0 Conclusion

The degradation of naphthalene, a PAH, by *Pseudomonas stutzeri* BUKBTEG1 was studied to determine the ideal conditions for the degradation process via OFAT. The following are the ideal conditions for the deterioration process: 800 mg/L naphthalene concentration, pH 7, 400 percent (v/v) inoculum size, and a temperature of 35 °C. 1000 cmg/L $(NH_4)_2NO_3$. After 72 hours of incubation, the isolate was able to degrade up to 85.58 %of naphthalene under optimal conditions. The metabolic product/intermediate such as 1,2-naphthalenediol, salicylic acid, and catechol amongst others were found during degradation process through GC–MS. These metabolites were shown to be less toxic to humans and pollute the environment less than the parent substance. As a result, this isolate may be a promising option for future bioremediation of naphthalene-polluted environments.

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Declaration of conflict of interest

The authors declare that there is no conflict of interest.

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