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Utilization of Benzoate and Phenol by *Bacillus pumilus* in Saline and Non-Saline Cultures

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ABSTRACT: Pollution by benzoate and phenol is currently a major global ecological challenge. Hence, utilization of benzoate and phenol by B*acillus pumilus* in saline and non-saline cultures were tested in this work. *Bacillus pumilus* was challenged with benzoate and phenol at various salt concentrations (0 M, 1 M, 2 M, 3 M, 4 M, and control (positive control, which had bacteria but no benzoate or phenol, and in negative control, which had benzoate and phenol but no bacteria respectively). The isolate (obtained from stock culture in our laboratory and originally isolated from spent engine oil polluted soil), was identified using biochemical and molecular tests. The reaction setup included mineral salts medium (MSM), benzoate or phenol, and the bacterium. *Bacillus pumilus* inoculum was incubated at room temperature (25ºC) in reaction tubes. Aseptically, 1.5 Eppendorf tubes were filled with 400µl of culture and centrifuged at 3,500 rpm for 10 minutes. 75µl supernatant was added to 1,425µl distilled water. Controls measured residual benzoate at 223 nm and phenol at 269nm. After 336 hours, reaction tubes were sacrificed after spectrophotometer readings. Readings began at hour 0 every two days. As the bacterium grew, residual benzoate decreased (from 0.779 nM to 0.417 nM for 0M NaCl, from 0.778 nM to 0.373 nM for 1M NaCl, from 0.754 nM to 0.583 nM in 2M NaCl, from 0.795 nM to 0.463 nM in 3M NaCl and from 0.803 nM to 0.368 nM in 4M NaCl). *Bacillus pumilus* used benzoate heavily. Increased salt concentrations decreased *Bacillus pumilus* phenol degradation (1M to 4M NaCl). Similar results were obtained for phenol. *Bacillus pumilus* can degrade phenol in halophilic and non-halophilic culture, but increasing the medium salt concentration inhibits cell growth. *Bacillus pumilus* degrades benzoate and phenol. *Bacillus pumillus* can remediate benzoate and phenol-polluted sites.

Keywords: Benzoate utilization, Phenol utilization, *Bacillus pumilus* Saline culture, Non-saline culture.

Introduction

Bacillus pumilus have been reported for its ability to tolerate high salt content in the soil (Kumar *et al*., 2021). Increased demand for irreplaceable and non-renewable energy sources like petroleum has increased petroleum exploration and exploitation and polluted both aquatic and terrestrial environments. Colonel Drake Edwin explored oil in 1859. (Beckwith, 2014). Its main causes are natural and anthropogenic. The natural cause is when oil seeps from the ocean floor and enters the marine environment due to hurricanes, heavy storms, and earthquakes.

The anthropogenic cause is due to human activities (Smila *et al*., 2020). Hydrocarbon pollution depletes plant nutrients, bioaccumulates and biomagnifies pollutants in plants and animals, and causes acute or chronic health effects, mutations, and death (Adetitun *et al*., 2019). Long-term oil exposure harms environmental organisms. It blocks air and sunlight, preventing photosynthesis and stunting plant growth. It prevents them from transpiring and kills most of them (Saadoun, 2015). On animals, it accumulates persistence and bio components of oil in tissue and bodies, causing liver or kidney diseases, bone marrow damage, and increased cancer risk (Mishra *et al*., 2001).

Acinetobacter species (Zhan *et al*., 2009), pseudomonads, and *Ralstonia eutropha* preferentially degrade mixtures of benzoate and phenol, which are converted into catechol and channeled into the β-ketoadipate pathway by ortho ring cleavage. This mixture inhibits Phenol consumption through an unknown molecular mechanism. Molecular studies on hierarchical aromatic compound utilization have mostly focused on benzoate and 4-hydroxybenzoate (4-Hb) mixtures, which are metabolized by different branches of the β-ketoadipate pathway (Harwood and Parales 1996). Benzoate inhibits 4-Hb degradation in *Pseudomonas putida* PRS2000 and *Acinetobacter baylyi* ADP1, demonstrating transcriptional repression in these gamma proteobacteria. In both species, transcriptional regulators of benzoate degradation and the pcaK gene, which encodes the 4-Hb permease, may repress catabolite (Brzostowicz *et al*., 2003).
Benzoate inhibits betaproteobacterium 4-Hb degradation. Cuadros-Orellana *et al*. (2012) isolated 10 Dead Sea halophilic archaea that degrade p-hydroxybenzoic acid as their sole carbon and energy source. *Euryarchaeota* strain L1 also converts benzoic acid to gentisate. Erdoğmuş *et al*. (2013) found that many *Halobacterium, Haloferax, Halorubrum,* and *Haloarcula* strains could degrade p-hydroxybenzoic acid in a 20% NaCl medium. These studies prove that p-hydroxybenzoic-metabolizing archaea are widespread. *Halomonas* spp. can degrade phenolics, benzoates, and non-oxygenated hydrocarbons. Thus, BTEX and PAH degradation studies are needed to maximize their remediation potential. This study investigated whether soil-isolated *Bacillus pumilus* could use phenol and benzoate.

Materials and methods

Sample collection, isolation and identification of bacterium isolate: Various aspects of *Bacillus pumilus* (obtained from stock culture in our laboratory and originally isolated from spent engine oil polluted soil), including its colonial, cellular, biochemical, and molecular properties, were studied to better understand it. Molecular characterization of *Bacillus pumilus* was outsourced to Inqaba Biotech West Africa Limited in Ibadan, Nigeria. The process of DNA isolation, amplification by polymerase chain reaction (PCR), and sequencing was performed. The BLAST procedure then took place.
Sterilization of equipment: To prevent contamination, all materials were properly sterilized before and after use. Materials used were sterilised in the autoclave at 121ºC for 15 minutes. All glassware was washed in detergent and sterilized for 60 minutes at 170 degrees Celsius. Both the inoculating loops and the straight wire loops were sterilized by being heated to red hot before and after use. Cotton swabs doused in 70% ethanol were used to clean the laboratory bench. Sterilized disposable pipette tips were used Fawole and Oso, 1997).
Preparation of culture media: Nutrient agar, peptone water, and mineral salts medium were prepared using standard procedures (Fawole and Oso, 1997; Adetitun *et al*., 2019).
Subculturing: The work bench was swabbed with 70% ethanol, the plates were labeled appropriately and the inoculating loop was sterilized by heating to redness and allowed to cool. Strains were isolated from the stock culture and streaked onto nutrient agar plates using a sterile inoculating loop. The inoculating loop was sterilized after use and the plates were incubated in an inverted position at 37 ^{0}C for 24 h.
Preparation of Bacillus pumilus suspension: *Bacillus pumilus* added to the reaction mixture was prepared in such a way that about n*10^3 was the starting inoculum added to the reaction mixture. The reason for this is to give room for multiplication of the bacterium in the reaction mixture. Serial dilution of the original bacterial suspension was done and plated until n*10^3 cells were obtained. The dilution that gave us n*10^3 that was used for adding the organism to the reaction mixture. The necessary plate counts were done on nutrient agar (Fawole and Oso, 1997; Adetitun *et al*., 2019).
Characterization and identification of Bacillus pumilus: Various aspects of *Bacillus pumilus* (obtained from stock culture in our laboratory and originally isolated from spent engine oil polluted soil), including its colonial, cellular, biochemical, and molecular properties, were studied to better understand it. Molecular characterization of *Bacillus pumilus* was outsourced to Inqaba Biotech West Africa Limited in Ibadan, Nigeria. The process of DNA isolation, amplification by polymerase chain reaction (PCR), and sequencing was performed. The BLAST procedure then took place.
Experimental setup: Forty milliliters of sterile MSM had benzoate added to it. The medium was spiked with a 100µl suspension of *Bacillus pumilus*. Spectrophotometer readings were taken at a wavelength of 223 nm from samples taken at 48-hour intervals. Same procedure was followed for phenol. The spectrophotometer readings for phenol were taken every 48 hours, and the samples were analyzed at a wavelength of 269 nm. NaCl at -0M, 1M, 2M, 3M, and 4M concentrations were used separately in separate reaction flasks.. *Bacillus pumilus* was used in the positive control, which had bacteria but no benzoate or phenol, and in negative control, which had benzoate and phenol but no bacteria.

Determination of benzoate and phenol utilization by isolated bacteria: From the culture flask, exactly 400µL was aseptically transferred to 1.5 ml Eppendorf tubes. There was a 10-minute centrifuge run at 3500 rpm. To 1425µL of distilled water, 75µL of the supernatant was added. We used distilled water to "blank" the spectrophotometer (Model SP-UV52). The remaining benzoate was measured at a wavelength of 223 nm. Data was collected and recorded from the controls. With a wavelength of 269 nm, the phenol residue was measured and recorded. Readings from the controls were recorded too.

Results

The Gram reaction and molecular test confirmed that the isolate used in this experiment was *Bacillus pumilus* (Table 1 and the DNA sequences that follow). A graphical representation of the absorbance of benzoate in saline and non-saline cultures is depicted in Figure 1.

It was observed that the decrease in benzoate started immediately from Day 1 to Day 10 with a sharp increase in residual benzoate from *Day 10 to Day 13:* The highest quantity of residual benzoate was observed to be 0.78 on Day 1 and the lowest value recorded was 0.14 on Day 10. Meanwhile, an increase from 0.14 to 0.42 was observed on Day 13. In the halophilic culture of 1 M of NaCl, a great utilization rate was measured from day 1 to 4 with absorbance from 0.79 to 0.42, a little increase was measured from day 4 to 6 with benzoate absorbance from 0.42 to 0.45, after which utilization was observed in the culture sample till day 10 measured with 0.12 absorbance. Then comes a drop in utilization as the absorbance of benzoate increased on the last day of observation which measured 0.37 absorbance.

Table 1: Results obtained for biochemical tests.

Biochemical tests	**Result**
Gram staining	Positive
Endospore staining	Vegetative cell
Sugar fermentation test	Positive
Coagulase test	Negative
Catalase test	Positive
Motility test	Positive
Capsule staining	Positive (purple)
Oxidase test	Positive
Oxygen relationship test	Strict aerobe
Voges-Proskauer-Methyl Red Test	Negative
Indole test	Positive
Citrate utilization	Negative

Confirmation of *Bacillus pumilus*

The sequences below are those obtained for *Bacillus pumilus*

ACCGGAAATGCAAGCGAGCGGACAAGGGAGTGTCCCGGATGTTAGGGGGACGGGGAGTAGCACT
GGGTAACCTGGCTGAGGACTGGGATAACTCCGGGAAACCGGAGCTAATACCGGATAGTCCTTGAA
CCGATGGTCAAGGATGAAAACGTTCGCTGACTTACATGGACCCGGGGATTAGTATGGTGGGGTAA
TGGTCACCAAGGGACGATGCTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACG
CCCAACTCCTACGGGAGGAGCAGAGGAATCTTCCGCAATGGACAAACTGACGAGCAACCCCGGGA
GTGATGAAGGTTTTCGGATCGTAAAGCTCTGTGTAGGAAGAACAAGAAGTAACTGCTCCCCTTGAC
GGTACCTAACCAAAACACGGCTAACTACGTGCCACACCGCGGTAATACAGTGGCAAGGTTGGCCG
GAATTATTGGGGAAAGGGTCAGGGTTCTTAATCTGATGTGAAACCCCCGGTCACCGGGGAGGCAT
TGAAACTGAAACTTGAGTGCAGAAAGAAGTGGAATTCCACGAGCGGGAATGCTAAATGGAGGAA
CACCAGCAGGCACTCTCTGCTGAACTGACCTGATGAGCAAACGGGAGCAACGATTAGATACCCTG
GTAGCCACGCCGACGATGAGTGTATGTAGGTTCGCCCCTTATGCTGCAGCTAACCATGCACTCCGC
CTGGGGAGTACGGCCAAGATGAACTCAAAGGATTGACGGGGCCCGCACAAGCGGTGGAATGGGTT
AATTTGAAGCACCGAAGACCACCCGGCTTGACATCCTTGACACCCTAGAATAGGGCTCTCCGTTCC
GGGGAAGTACGAAGGTGTGCAATGGCTGTTCGTCAGCATCGTGTCGTTGAGATGCTGGACTAAGG
TCCCAGCCAGATCGAGCTCACTCGAATCCTAGATGCTAGGCCATTCCAATGGGACTGCTAGTGCA

The result obtained for benzoate (Figure 1) shows a high rate of hydrocarbon degradation by *Bacillus pumilus* until day 13 of observation which shows rather a high decrease in utilization as well as an increase in residual benzoate. This sharp increase was observed to be the highest across all concentrations with an increase in

absorbance from 0.17 to 0.58 on days 10 to 13 respectively. However, the highest rate of degradation occurred starting from day 8 to 10 with absorbance from 0.55 to 0.17. Appreciable utilization was observed in 3 M of NaCl which is high halophilic culture. The residual benzoate from day 1 to 10 measured 0.80 to 0.13 absorbance. With an increase in residual benzoate, observation on day 13 was measured at 0.46 absorbance. Rapid utilization of benzoate at a high level of salinity was observed as shown in 4M NaCl. *Bacillus pumilus* was able to utilize benzoate from day 1 to day 10 measuring 0.80, 0.46, 0.41, 0.29, and 0.12 on days 1, 4, 6, 8, and 10 respectively.

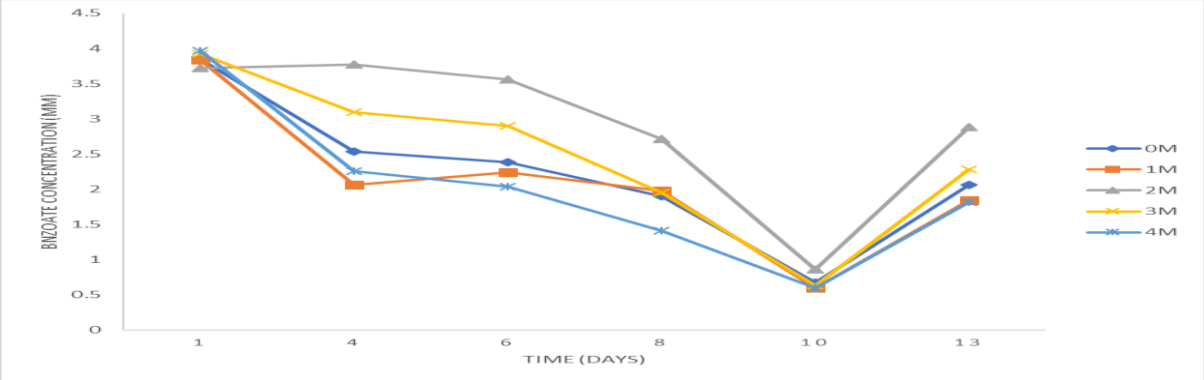

Figure 1: Utilization of benzoate by *Bacillus pumilus* in the presence of difference concentrations of NaCl

Bacillus pumilus consistently utilized phenol as the sole carbon and energy source in the presence of 0M NaCl (Figure 2). On day 1, the phenol reading was 0.6695 nm. At day 4, it decreased to 0.295nM. At day 6, it was 0.5nm. By day 10, it was 0.0855nM and finally at day 13, it was 0.1285nM. The result showed a decrease in the concentration of phenol from day 1 to 13. *Bacillus pumilus* was also evaluated for it degradation of phenol at 1M, 2M, 3M and 4M NaCl (Figure 2). The result showed that degradation of phenol occurred at all concentrations of NaCl. However, degradation was best at 0M NaCl as only 19% of the initial concentration of phenol remained at the end of the experiment. At 1M NaCl, 26% of the initial concentration remained. At 2M NaCl, 26.7% of the initial concentration of phenol remained. At 3M NaCl, 31.4% of the initial concentration of phenol remained while at 4M NaCl, 46.3% of phenol remained on the 13th day. This shows a decrease in the amount of phenol degraded as NaCl concentration increases.

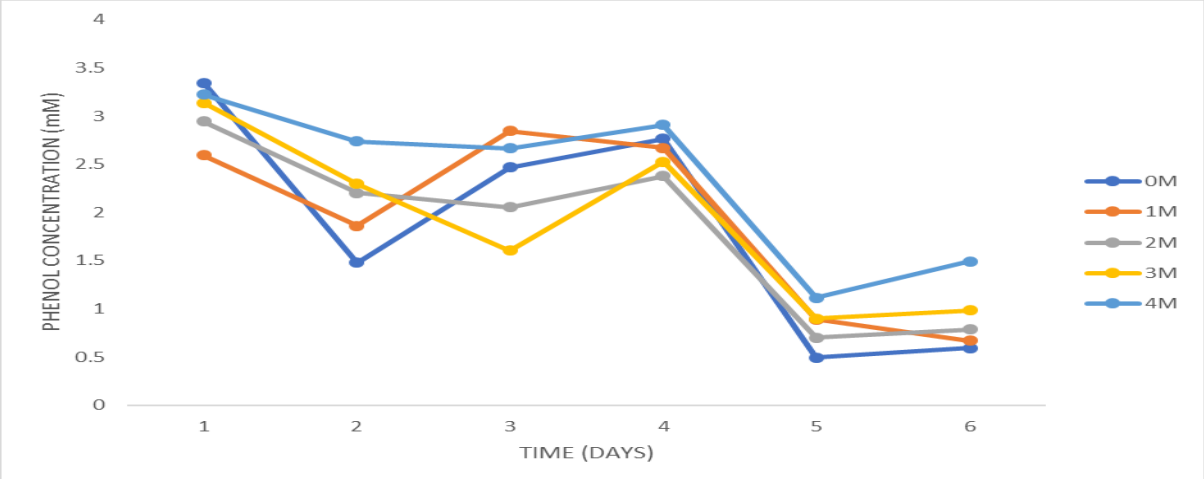

Figure 2: Utilization of Phenol by *Bacillus pumilus* in the presence of difference concentrations of NaCl

Discussion

The results obtained from this experiment clearly indicate a significant level of biodegradation of phenol and benzoate in both saline and freshwater cultures. The initial decrease in residual benzoate in this study has shown that the identified *Bacillus pumilus* possess the ability to degrade benzoate while utilizing it as its sole carbon

and energy source across a variety of NaCl concentration. This agrees with Esikova *et al.* (2021). These workers characterized soil bacteria that could degrade benzoate. They reported that *Bacillus* was capable of benzoate degradation.

However, the sharp fall in the degradation on the last day of observation (day 13) across all concentrations could be as a result of the inability of *Bacillus pumilus* to produce enzymes that can completely degrade benzoate thereby resulting in the increase of benzoate in the experiment. It could also be because of the inability of a single strain (*B. Pumilus)* used in this research, to effectively degrade benzoate. Tian *et al*. (2018) observed that single bacterial strains were not effective to ensure the biodegradation of hydrocarbons and this was supported by Ghorbannezhad *et al*., 2018; Khan *et al*., 2018, who stated that an association of bacterial strains is able to achieve biodegradation of petroleum hydrocarbons at higher rates than single bacterial strains. Another research by Adetitun *et al.* (2020) stated that efficient biodegradation of hydrocarbons depends on optimal microbial functionality and the size of biomass.

The degradation of phenol by *Bacillus pumilus* decreased with increasing NaCl concentration. It indicated that increasing NaCl concentration was detrimental to cell growth and this affected phenol degradation negatively. This idea is consistent with the report that high salt concentration led to hypertonic osmotic imbalance and loss of metabolic activity of degraders (Jiang *et al.*, 2015; Su *et al.*, 2019). *Bacillus pumilis* was able to degrade 81% of phenol at 0M NaCl concentration. The rate of degradation decreased with increasing phenol concentration too. The lowest degradation was seen at 4M NaCl where only 53.72% of phenol was degraded. Non-halophilic microorganisms show optimal growth at concentrations below 2% NaCl, and halotolerant and halo-dependent microorganisms are able to grow in the presence of up to 30% NaCl (Margesin and Schinner, 2001; Castillo-Carvajal *et al.*, 2014).

Deng *et al.* (2018), reported that a decrease in the degradation of phenol associated with increased salinity is attributed to strong inhibition of cell growth and phenol biodegradation in high salinity. Patil (2014), reported the biodegradation of phenol by a strain of B*acillus pumilus* under minimal salt concentration. It showed complete phenol degradation up to 1250mg/by *Bacillus pumilis* at low salt concentrations. It further explained that the phenol degradation rate can be reduced when the pH and salinity of the solution are increased.

Nowak *et al.* (2022) reported the degradation of phenol by *S. maltophilia* at different salinity. It was observed that the degradation of phenol of *S. maltophilia* was reduced at higher salinity. It explained that the reduction in phenol degradation by the bacteria at high salt concentration is attributed to the fact that the enzymes catechol 1,2-D and catechol 2,3-D need specific catalytic groups in the ionized or non-ionized state to interact with the substrate without changing its active conformation and enzyme stability and that the *ortho* and *meta* pathway induction depends on the culture conditions. Studies have shown that *Bacillus pumilus* can degrade phenol under non-halophilic cultures.

It is concluded in this study that *Bacillus pumilus* is a hydrocarbon degrader and has the ability to degrade benzoate and phenol at the appropriate salinity levels. Our next work will focus on the enzymes involved in the biodegradation of benzoate and phenol under different salinity levels.

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