# Effect of Temperature and benzoate concentration(s) upon the growth of *P. Putida* and *P. aeruginosa* using agar and broth of Tryptone Soya and Basal Salt as growth media(s).

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## Abstract

As emerging technology utilises various micro- organism (Bioremediation) including the *Pseudomonas* strains to remediate environmental contaminants. This study utilises various temperature(s) ranging from 25- 50 °C to investigate the ability of *P. putida* and *P. aeruginosa* to thrive on both minimal and nutritional media including Basal Salt and Tryptone Soya in broth and agar while utilising benzoate as carbon source. The results indicated that all the organisms grow between 25- 40$^{o}$C on TSA only with a slow growth observed at 50$^{o}$C. While no cell growth was observed on BSA only with the exception of *P. putida* 347 after 120hrs. However, the results indicated that all the organism grew between 25- 37$^{o}$C on BSA containing 0.5%w/v benzoate with slow or no growth observed between 40- 50$^{o}$C. Using variable benzoate concentrations, the results indicate that all the organisms grow well between 0.5- 1.0%w/v of benzoate on TSA with slow growth observed between 2- 3%w/v of benzoate. Furthermore, growth analysis using anaerobic (absence of $O_2$) in plates BSA containing 0.5% benzoate at 37$^{o}$C and results showed absence of growth for all the organisms within 72hrs. Further analysis of *P. putida* strains (347, 459, 616) in liquid media of TSB containing 0.5%w/v benzoate at same temperature indicated an optimum growth for 3 *P. putida* between 30- 37 °C, low growth at 25 °C and growth decline between 40-50$^{o}$C. Thus, results using the broth media of Basal Salt containing variable benzoate concentration reflect *P. putida 347* to have an optimum growth temperature at 30$^{o}$C which confirms that obtained using the solid media (BSA) with a mean generation time of 8hrs. In contrary to the solid media results, however, *P. putida 616* grows faster in combined BSB + benzoate media at 37$^{o}$C with a mean generation time of 11hrs and *P. putida 459* having the least growth rate based on its cell density at both 30$^{o}$C and 37$^{o}$C in the combined BSB and benzoate media.

***Keywords:*** *Pseudomona*s, Temperature, Tryptone Soya, Basal Salt, Benzoate, Agar, Broth.

## Introduction:

Several remediation techniques encompassing pollution control and environmental restoration including physical, thermal and chemical treatments have been reported, however, costly nature, time consumption and health risk associated with some of the processes, such as the production of secondary pollutants, citing methane for instance, is a major drawback regarding the precision of this processes (Chikere, 2013: Yergeau *et al.,* 2012). As such, much preference is given to biological treatment as an emerging and an eco- friendly method of environmental remediation technology via the use of microbes or their enzymes (bioremediation), to facilitate environmental restoration to its original or less toxic state. This is due to cost effective nature of the practise, absent of secondary pollutants as well as good process control (Adam, 2016: Elkhir, 2015). According to Kristein *et al.* (2012), microbes primarily decompose pollutants via bio-degradation process which involve a complete mineralisation of enormous amount of organic compounds including hydrocarbons and related compounds to carbon dioxide and water ($CO_2$ and $H_2O$).

Such microbes may function as a single entity or multiple entity (microbial consortium) depending upon the level and type of pollutants in question. While some fungi and micro-fauna including protozoans, worms and insects are active participants in bioremediation, yet, bacteria are reported to be the primary microbes involved in bioremediation (Chhatre, 2006). Examples of these microbes include *Actinobacter, Arthrobacter, Bacillins, Flavobacterium, Mycrobacterium, Nitrosomonas, Phanerochaete and Pseudomonas* to name a few (Adam, 2016: Kuppusamy *et al*., 2016). This bacteria remediate pollutants in either soil or groundwater via the biodegradation method using specific and non-specific processes in form of single unit microbe or consortium (multiple) respectively (Tomei, 2013). However, Cezairliyan *et al.,* (2013) reported that for a particular bioremediation process to be effective, certain conditions need to be put into consideration including the physiological versatility of the microbe, environmental conditions as well as the nature of the pollutant to be remediated. Taking for instance, the *Pseudomonas* specie that present high ability for bioremediation of recalcitrant hydrocarbons due to their abundance in microbial community, metabolic diversity and ability to withstand high concentrations of recalcitrant pollutants (Fu *et al.,* 2014: Gulsap *et al.,* 2006). In addition, Das *et al.,* (2015): Elkhir, (2015) reported that *Pseudomonas* species including *P. putida and P. aeruginosa* are considered to be the most efficient strains involved in the degradation of recalcitrant hydrocarbons (HC) due to their biodiversity and metabolic capabilities.

*P. putida*s often regarded as a superbug due its amenability to genetic manipulation as it contains 16S rRNA nucleotides, this gives it a diverse applications in bioremediation, recycling and in bio-control (Simon *et al.,* 2002). In addition, as a gram negative (–ve) soil bacterium, *P. putida* also function as a soil inoculum in the restoration of naphthalene-contaminated soils (Hicks, 2016). Although *P. aeruginosa* is regarded more of an opportunistic microbe responsible for various infections in humans, yet, its ability to biodegrade recalcitrant pollutants has been reported (Das *et al.,* 2015). According to Gulsap *et al.* (2006), *P. putida* and *P. aeruginosa* can undergo bioremediation process by covering a wide range of temperature from 4$^{o}$C to 50$^{o}$C, making them suitable for adaptation regarding

fluctuating environmental temperature conditions. However, Di *et al.* (2012) reported that despite *P. putida and P. aeruginosa* been versatile and adaptable to certain environmental conditions due to their genetic make- up, their growth and biodegrading capability could be influenced by factors including specific/selective nutritional supplements. For instance, De Rosa *et al.,* (2015): Curry, (1971) reported that *P. putida* and *P. aeruginosa*are capable of utilising selective media such as Phenanthroline and Tryptone Soya (TS) as growth media via laboratory controlled culturing. In addition, Whyte, (2007) reported the ability of the *Pseudomonas* specie in utilising different salts of different composition as their carbon source.However, effect of environmental condition including varying temperature as well as varying concentrations of carbon sourceon *P. putida* and *P. aeruginosa* ability to metabolise both enriched and basic nutritional including Tryptone Soya and Basal salts, is yet to be determined.

**Hypothesis:**

The hypothesis for this study is that *P. putida* and *P. aeruginosa* can grow well at variable controlled environmental temperature(s) using both solid and liquid media of Tryptone Soya (TS) and Basal Salt (BS) as sole growth and selective media respectively due to their versatility.

**Aims and objectives:**

Isolation and screening of different *Pseudomonas* strains obtained from the University of Wolverhampton (UK) bacterial stock culture collection. To evaluate growth rate of *P. putida* and *P. aeruginosa* in a fortified solid and liquid media of Tryptone Soya (TSA & TSB) as well as basic solid and liquid media of Basal Salt (BSA & BSB) using variable concentrations (0.5- 3%w/v) of benzoate as carbon source. In addition, to analyse the effect of varying temperature (25- 50$^{o}$C) and impact on growth rate of *P. putida* and *P. aeruginosa* using both solid and liquid media of Tryptone Soya (TSA & TSB) and Basal Salts (BSA & BSB) alongside the benzoate carbon source. Furthermore, the study aim at detecting the optimum temperature(s) required for growth of *P. putida* and *P. aeruginosa* with respect to time (hrs.) and to also demonstrate that *Pseudomonas specie* are generally non-fastidious microbes capable of utilising simple salt in both solid and liquid in the presence of carbon source(s) due to their versatility. The study also aims to single out the most suitable hydrocarbon degrader which include among the experimental Pseudomonas strains used.

**Materials and Methods:**

**Bacterial Strains;** Bacterial cultures engaged for screening in this study consist of four strains of *Pseudomonas putida* (347, 457, 559 616) and one strain of *Pseudomonas aeruginosa* (67), obtained from the University of Wolverhampton (UK) bacterial stock collection. The bacterial samples were prepared from the freeze dried bacterial ampoules on TSA plates. Furthermore, the bacterial/organisms were aseptically sub- cultured by streaking a single colony on prepared TSA plates followed by 24hrs incubation at 30$^{o}$C. Note that the cultures were routinely streaked ahead of each experiment so as to obtain viable, fresh and pure cells.

**Gram stain (Identification test for bacterial cultures):**

Gram staining test was used to verify the identity of the provided bacterial cultures by heating fixed deposit of suspended bacterial cells in saline solution followed by crystal violet flooding for 1 minute, then Grams iodine for another 1 minute. De-staining with alcohol and subsequent washing with distilled water after which the slides were drained with Safranin for 2mins followed by rinsing with distilled water. The slide were finally air-dried and examined under oil immersion objective of a Zeiss light microscope and the different shapes/reaction were identified.

**Culture Media and Reagents:**

**Preparation of Tryptone Soya Agar (solid) and Broth (liquid) media;** 30g of Tryptone Soya Agar (TSA) was dissolved in 1 litre of distilled water in a 2000ml container and brought to dissolution using a magnetic stirrer. Accompanied by plate pouring of the prepared media and cooling at 45$^{o}$C in a sterile laminar air flow cabinet before storage of the agar plates at 8-15$^{o}$C until further use. Note that TSA plates media containing different Na- benzoate concentrations (0.5%w/v, 1%w/v, 2%w/v and 3%w/v) were also prepared by dissolving 5g, 10g, 20g and 30g of Na-benzoate in the described solid TSA media. For liquid media preparation, 30g of Tryptone Soya Broth (TSB) was prepared by dissolving in 1 litre of distilled water in a 2000ml container and stored between 2-8$^{o}$C until ready for inoculation.

**Preparation of Basal salt (BS) media (solid and liquid);** Basal salt agar was prepared from chemical stock including trace elements using the recipe of Rowe *et al.,* (2008) and Mishra *et al.,* (2012), while liquid broth was prepared using same chemical stock as the solid media with the exception of Agar No.2. The prepared medium was stored between 2-8$^{o}$C until ready for use. In addition, separate solid and liquid BS media containing different concentrations of Na- benzoate (0.5%w/v, 1%w/v, 2%w/v and 3%w/v) as described earlier was also prepared and stored in sterile condition for further use.

**Note:** All culture media (TS & BS) were sterilised by autoclaving at 121$^{o}$C for 15mins before final storage. In addition, all incubations were conducted alongside control plates containing either TS or BS without inoculation.

**Screening on different Solid (TSA & BSA) media at different temperature using both aerobic and anaerobic process:**

Initial stage involved partitioning and labelling of the 6 bacterial cells on the prepared TSA and BSA plates only as well as TSA and BSA plates containing different benzoate concentration (0.5%w/v, 1%w/v, 2%w/v and 3%w/v). A single colony from the appropriate TSA bacterial cells plates was aseptically transferred using sterile plastic loops to TSA only plates and TSA plates containing different benzoate concentrations (0.5%w/v, 1%w/v, 2%w/v and 3%w/v) via streak spread method, followed by 24hrs incubation at 25$^{o}$C, 30$^{o}$C, 37$^{o}$C, 40$^{o}$C and 50$^{o}$C for TSA only plates and 48hrs incubation at 30$^{o}$C for TSA- benzoate media, alongside control plates that were not inoculated. The second stage involved streak plating BSA only and BSA with 0.5% benzoate plates by aseptic colony transfer as described earlier. The cultures were then incubated at 25$^{o}$C, 30$^{o}$C, 37$^{o}$C, 40$^{o}$C and 50$^{o}$C for 5 days alongside control plates without inoculation. Anaerobic incubation was also carried out for all strains in BSA containing 0.5%w/v benzoate at 37$^{0}$C for 5 days alongside control plates

without inoculation and growth was recorded for all media and their respective temperatures visually using either + or – sign. Furthermore, all the bacterial cells were streaked on prepared BSA plates containing 0.5% benzoate which were then placed in an anaerobic glass jar followed by 7days incubation at 37$^{o}$C.

**Screening in different liquid media for growth at different temperature:**

On the basis of the solid media screening results, 3 *Pseudomonas* strains 347, 459 and 616 were selected and further studied on different liquid media which include TSB containing 0.5%w/v of benzoate at different temperatures. Initially, overnight culture of the 3 *Pseudomonas* strains were grown in 10ml TSB and 2%w/v of the culture was inoculated into test tubes containing 8ml TSB followed by incubation at 25$^{o}$C, 30$^{o}$C, 37$^{o}$C, 40$^{o}$C and 50$^{o}$C and reading were recorded at 6hrs, 24hrs, 48hrs and 72hrs. Growth was evaluated via spectrophotometric analysis at 600nm wavelength using TSB blank control to measure optical density (OD). The *Pseudomonas* strains 347, 459 and 616 were further tested in BS broth media containing 0.5%w/v benzoate as sole carbon source by inoculating 2% of overnight BSB culture of the strains in the benzoate growth medium followed by static incubation at 30$^{o}$C and 37$^{o}$C for 3 days and growth was analysed via spectrophotometric analysis at 600nm.

**Growth method:**

**Growth by viable cell count (V/C);** Re- evaluation of viable cell count on solid media was carried out by adapting serial dilution method as described by Maclowry *et al.*, (1970). After which 100µl of sample were transferred using 200µl finn- pipette from each tube and plated on a petri dish (plates) containing either TSA, BSA or BS- Benzoate agar (0.5%w/v, 1%w/v, 2%w/v and 3%w/v) followed by gentle spreading in a rotating manner (120 degrees) using a plastic disposable spreader. After spreading, plates were inverted and incubated at desired temperature for a specified period followed by colony counting (30 – 300 colonies) in colony forming units per ml *(Cfu/ml)* using either black tile or colony counter.

**Growth by optical density (OD);** Growth (biomass) analysis using optical density (OD) was conducted via Spectrophotometer by placing samples in either 1ml, 2ml or 3ml cuvette which were then placed within the absorbance region of the machine (photometer) and transmission was measured as a function of wavelength (600nm). Note that calibrations using blank control (growth media TSB or BSB) was carried out before measurements and at regular intervals while taking measurements. In the case of cells that produces pigments *(Pseudomonas strains)*, after measurements samples were placed in 1ml capacity centrifuge tubes and subjected to centrifugation (Eppendorf centrifuge) at 6000rpm, 4$^{o}$C for 1min. OD of the supernatant (pigments and growth media) were measured again and the actual biomass of cells were obtained by subtracting OD of the supernatant from the initial OD readings of the culture.

**Growth rate analysis via Mean generation time (Mgt);**

Under standard nutritional conditions, growth multiplication rate of the *Pseudomonas* bacterial strains during exponential phase in either TS or BS media were measured using the equation:

$$\textbf{Doubling} = \frac{\textit{Duration or time x log2}}{\textit{Log (final concentration) – Log (initial concentration)}} = \frac{\textit{Log2 x T}}{\textit{Log b – log a}}$$

**Results and Discussion:**

Gram stain reaction was used to confirm the identity of organisms obtained from bacterial stock collection of the University of Wolverhampton (UK), and the results indicated that all the bacterial strains provided were of the *Pseudomonas*species due to the formation of reddish or pink colour specifying negative Safranin retention as shown in figure/table **1**.

***Table 1: Gram reaction and shape of bacterial strains under light microscope.***

| | *P. putida (347)* | *P. putida (459)* | *P. putida (559)* | *P. putida (616)* | *P. aeruginosa (67)* |
|---|---|---|---|---|---|
| **Gram reaction** | - | - | - | - | - |
| **Shapes** | Pink-red Rods | Pink-red Rods | Pink-red Rods | Pink-red Rods | Pink-red Rods |

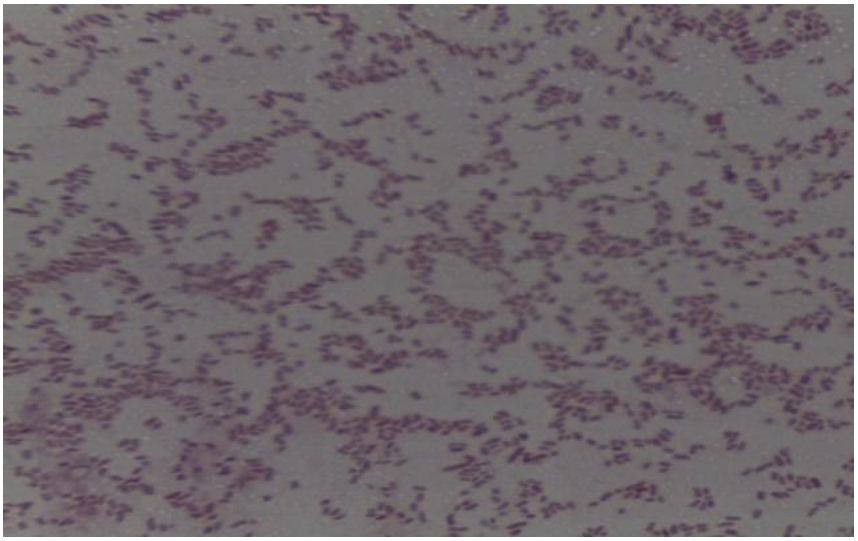

***Figure 1: Pseudomonas strains (pink)***

**Effect of Temperature upon the growth of bacterial cells:**

Since bioremediation depends upon environmental temperature, then temperature effect upon growth of cells was evaluated in TSA and BSA only and BSA containing 0.5%w/v benzoate. The results (Table 2) indicated that all the organisms grow between 25- 40$^{o}$C on TSA only

with a slow growth observed at 50°C. However, *P. putida* 459 and 347 grows better and faster at 30°C while *P. putida* 616, 559 and *P. aeruginosa* grows better at 37°C. In addition, no growth was observed on TSA control plates without inoculation. While table 3 indicated that all the organism grew between 25- 37°C on BSA containing 0.5%w/v benzoate with slow or no growth observed between 40- 50°C, although a few colonies were observed for *P. aeruginosa 67* at 40°C. However, heavy growth was observed for *P. putida 459, 616 and 347* at 30°C and *P. aeruginosa 67*. No growth was observed on all control plates and BSA only, although at 120hrs (5days) slow growth was observed for *P. Putida 347* at 30°C using BSA only.

| Organisms | 25°C (24hrs) | 30°C (24hrs) | 37°C (24hrs) | 40°C (24hrs) | 50°C (24hrs) |
|---|---|---|---|---|---|
| ***P. putida 459*** | ++ | +++ | ++ | ++ | +/- |
| ***P. putida 616*** | ++ | ++ | +++ | ++ | +/- |
| ***P. putida 347*** | ++ | +++ | ++ | ++ | +/- |
| ***P. putida 559*** | ++ | ++ | +++ | ++ | +/- |
| ***P. aeruginosa 67*** | ++ | ++ | +++ | ++ | +/- |

***Table 2: Effect of Temperature upon the growth of bacteria on TSA. (+++) heavy growth, (++) normal growth, (+) moderate growth, (+/-) slow and (-) no growth.***

| Organisms | 25°C 24hrs | 25°C 96hrs | 25°C 120hrs | 30°C 24hrs | 30°C 48hrs | 30°C 120hrs | 37°C 24hrs | 37°C 96hrs | 37°C 120hrrs | 40°C 24hrs | 40°C 48hrs | 40°C 120hrs | 50°C 24hrs | 50°C 96hrs |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *P. putida 347* | +/- | ++ | ++ | +/- | ++ | +++ | + | ++ | ++ | +/- | +/- | +/- | – | – |
| *P. putida 459* | +/- | ++ | ++ | + | ++ | +++ | +/- | + | + | +/- | +/- | +/- | – | +/- |
| *P. putida 559* | +/- | ++ | +++ | + | ++ | +++ | +/- | ++ | +++ | +/- | +/- | +/- | – | +/- |
| *P. putida 616* | +/- | +/- | ++ | +/- | + | ++ | +/- | +/- | +/- | – | +/- | +/- | – | – |
| *P. aeruginosa 67* | +/- | ++ | ++ | – | + | +++ | +/- | ++ | +++ | +/- | +/- | +/- | – | – |

***Table 3: Effect of temperature on bacterial growth on BSA containing 0.5%w/v benzoate as sole carbon source (+++) heavy growth, (++) normal growth, (+) moderate growth, (+/-) slow and (-) no growth.***

**Effect of temperature (30$^{o}$C) and benzoate concentrations upon growth of bacteria growing on TSA:**

Effect of benzoate concentration was studied on the bacterial cells so as to evaluate toxicity/inhibitory effects upon bacterial growth. The results (Table 4) indicate that all the organisms grow well between 0.5- 1.0%w/v of benzoate on TSA with slow growth observed between 2- 3%w/v of benzoate. However, heavy growth was observed at 0.5%w/v for *P. putida 459*, 616, 347 and *P. aeruginosa 67*, although heavy growth was also observed for *P. aeruginosa 67* at 1.0%w/v benzoate. However, no growth was observed on all benzoate plates without inoculation (control).

| Organisms | 0.5% 24hrs | 0.5% 48hrs | 1% 24hrs | 1% 48hrs | 2% 24hrs | 2% 48hrs | 3% 24hrs | 3% 48hrs |
|---|---|---|---|---|---|---|---|---|
| *P. putida 459* | +++ | +++ | ++ | ++ | +/- | +/- | _ | +/- |
| *P. putida 616* | +++ | +++ | ++ | ++ | +/- | +/- | _ | +/- |
| *P. putida 347* | +++ | +++ | ++ | ++ | +/- | +/- | +/- | +/- |
| *P. putida 559* | ++ | ++ | ++ | ++ | +/- | +/- | +/- | +/- |
| *P. aeruginosa 67* | +++ | +++ | ++ | +++ | +/- | +/- | _ | _ |

***Table 4: Effect of Benzoate concentration upon growth of bacteria growing on TSA at 30$^{o}$C. (+++) heavy growth, (++) normal growth, (+) moderate growth, (+/-) slow and (-) no growth.***

**Effect of temperature (37$^{o}$C) and anaerobic incubation upon the growth of bacterial cells using 0.5% benzoate as carbon source:**

Anaerobic (absence of $O_2$) effect upon bacterial growth was studied on BSA containing 0.5% benzoate at 37$^{o}$C and results (table 5) showed absence of growth for all the organisms within 72hrs. However, after 72hrs slow growth was observed for *P. aeruginosa 67* and no growth for *P. putida 616, 457, 559 and 347.*

| Organisms | 24hrs | 48hrs | 72hrs | 96hrs | 120hrs | 144hrs | 168hrs |
|---|---|---|---|---|---|---|---|
| *P. putida 459* | - | - | - | - | - | - | - |
| *P. putida 616* | - | - | - | - | - | - | - |
| *P. putida 347* | - | - | - | - | - | - | - |
| *P. putida 559* | - | - | - | - | - | - | - |
| *P. aeruginosa 67* | - | - | - | - | +/- | +/- | +/- |

***Table 5: Effect of anaerobic incubation upon the growth of bacteria cells on BSA containing 0.5% benzoate as sole carbon source at 37oC. . (+++) heavy growth, (++) normal growth, (+) moderate growth, (+/-) slow and (-) no growth.***

**Effect of temperature on bacterial growth in liquid culture (TSB and BSB containing 0.5%w/v benzoate):**

Based on the solid media screening results, 3 *Pseudomonas* strains 347, 459 and 616 were selected and studied in TSB containing 0.5%w/v of benzoate at different temperatures. Figure 2 indicated that the *P. putida* strains grow well between 25-37$^{o}$C, while minimal or no cells growth was found when incubated at 40- 50$^{o}$C. However the graph indicated *P. putida* 459 to have optimum growth temperature of 25$^{o}$C, while *P. putida 347 and 616* have optimum growth temperature of 30$^{o}$C and 37$^{o}$C respectively. In addition, mean generation time (mgt) for the 3 strains was observed at 2hrs at 30$^{o}$C and 37$^{o}$C with slightly faster and higher yield for *P. putida 347* as compared to *P. putida 457 and 616.* While a growth decline in cells were observed in between 48- 72hrs (fig 3).

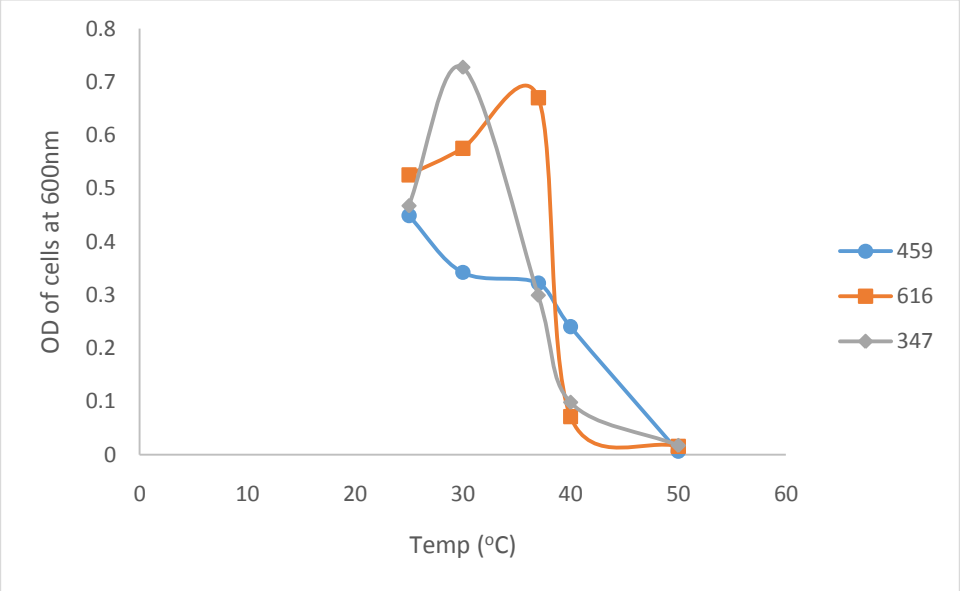


***Figure 2: Effect of temperature upon growth of the P. putida strains in TSB for 72hrs.***

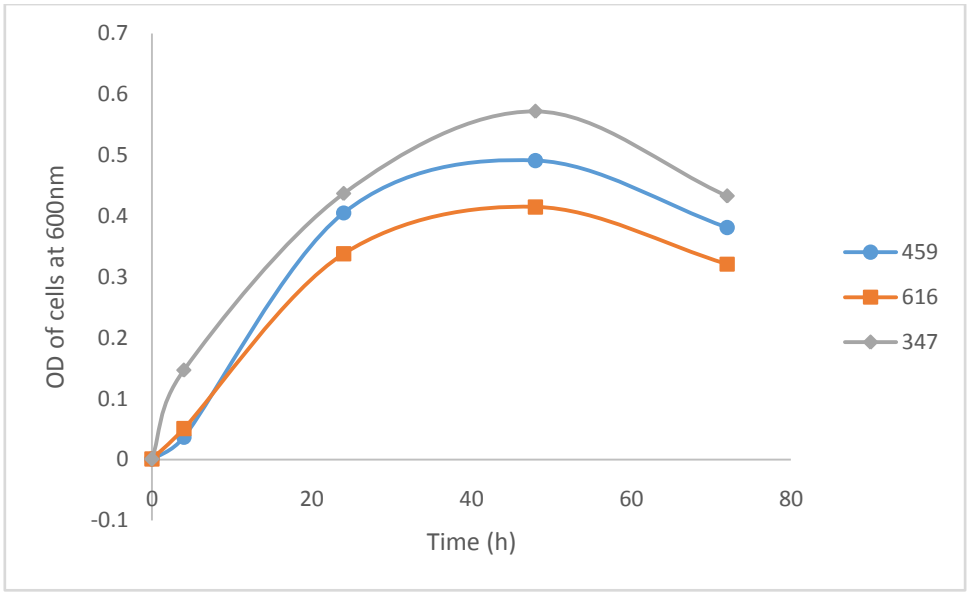


***Figure 3: Time effect upon growth of the P. putida strains in TSB at 30°C and 37°C.***

Effect of temperature on bacterial growth in BSB media containing 0.5%w/v benzoate was studied and growth of the *P. Putida* strains as presented in figures 4 & 5 confirms *P. Putida 347* grows faster at 30°C with an mgt of 8hrs. While figure *P. Putida 616* grows faster at 37°C with an mgt of 11hrs and *P. Putida 459* having the least growth rate based on its cell density at both 30°C and 37°C.

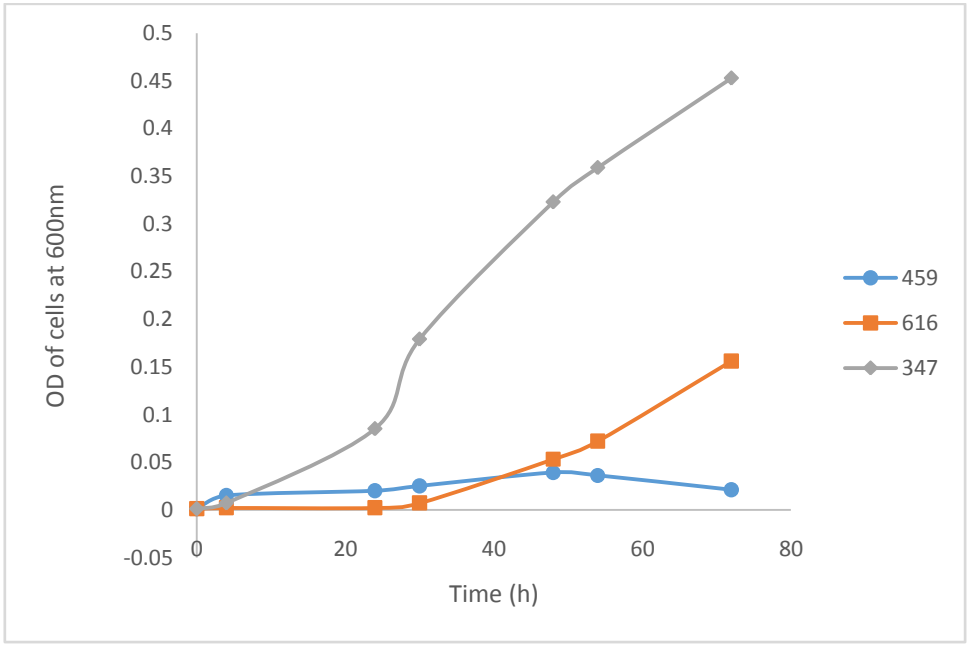


***Figure 4: Growth of P. putida 347, 457and 616 in BSB (0.5% benzoate) with time at 30°C.***

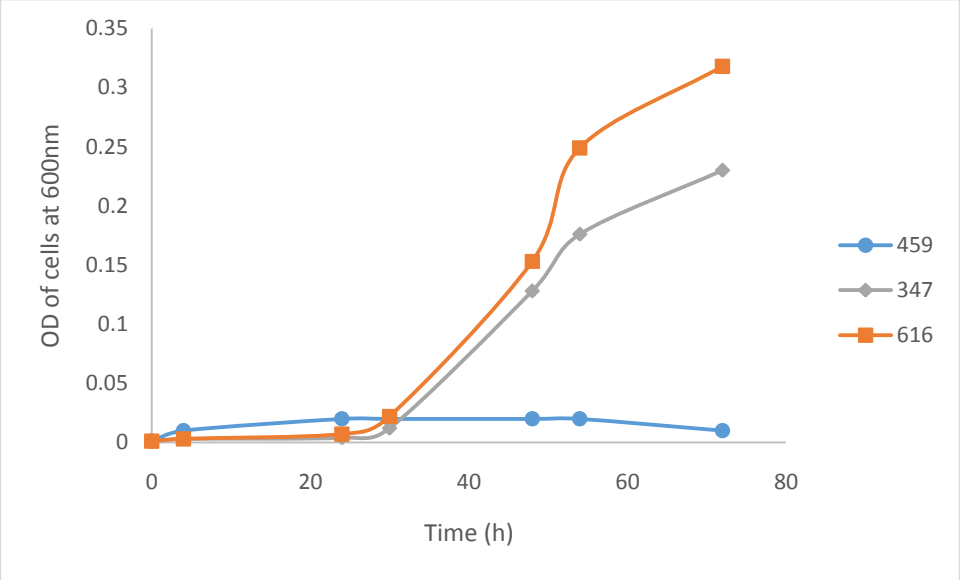


***Figure 1: Growth of P. putida 347, 457and 616 in BSB (0.5%w/w benzoate) with time at 37$^{o}$C.***

**3.1 Effect of benzoate concentrations on *P. putida* 347 in BSB (solid and liquid) media:**

On the basis of the previous data, *P. putida 347* was selected as the potentially fastest benzoate degrader and was tested for subsequent experiments to ascertain the effect of benzoate concentrations (0.5%w/v, 1%w/v, 2%w/v and 3%w/v) in BSB solid media upon cell viability via serial dilution after 72hrs incubation at 30$^{o}$C. Results (fig 14) indicated heavy growth (+++) of *P. putida 347* on solid media with a viable count of 3.9x10$^{8}$cfu/ml at 0.5%w/v benzoate concentration after 72hrs of incubation. While slow growth (+/-) was observed at 1%w/v benzoate concentration and a viable count of 7x10$^{5}$ after 72hrs of incubation, with no growth observed at 2-3%w/v after 72hrs of incubation. Furthermore, figure 14 shows growth of *P. putida 347* at different benzoate concentrations in liquid BSB media incubated at 30$^{o}$C for 72hrs and results indicated that the strain grows faster at 0.5%w/v benzoate concentration with a lag phase of 2hrs and mgt of 11hrs while slow growth was observed at 1%w/v concentration with a lag phase of 7hrs and mgt of 24hrs, thus no growth was observed at 2-3%w/v benzoate concentration.

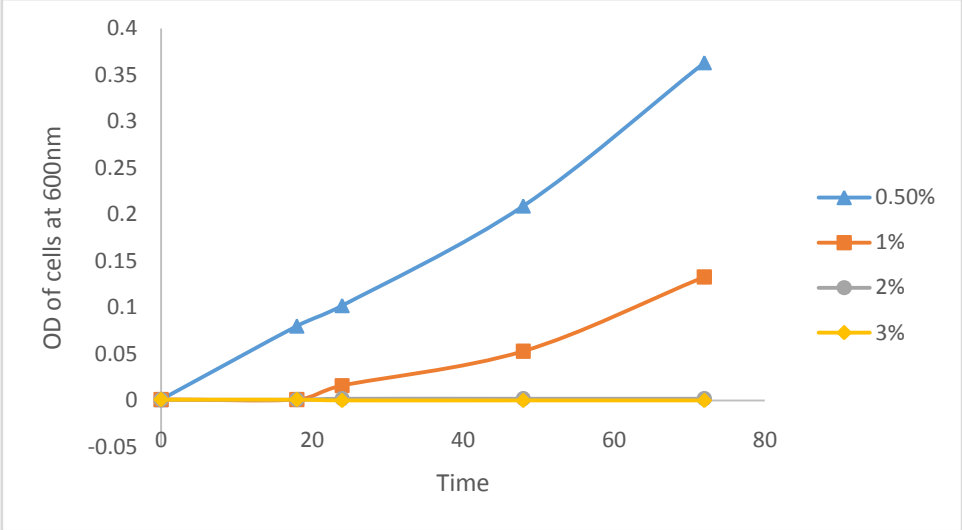


***Figure 2: Effect of different benzoate concentrations in BSB on growth of P. putida 347 at 30$^{o}$C.***

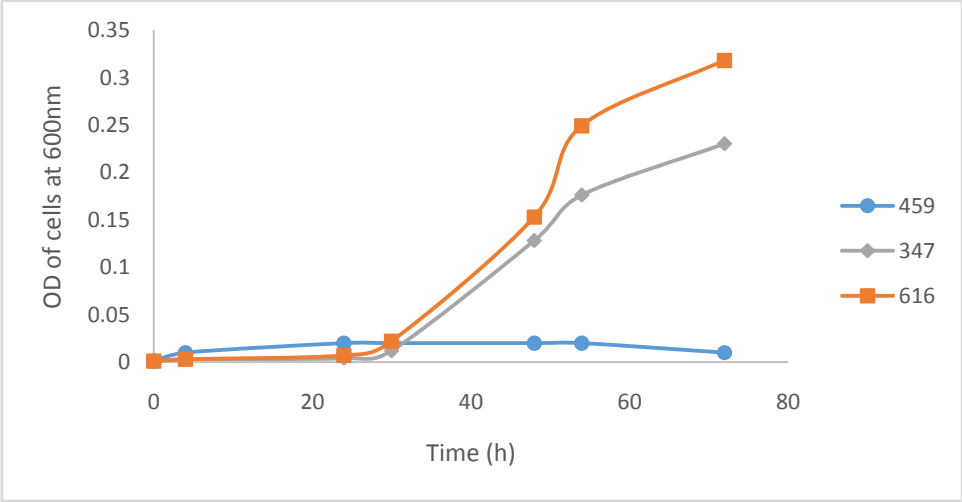


***Figure 5: Growth of P. putida 347,457and 616 in BSB (0.5%w/w benzoate) with time at 37$^{o}$C.***

In this study several experimental procedures were designed to evaluate the growth of *P. putida and P. aeruginosa* in different nutritional source (TS & BS) and carbon source (benzoate) concentrations, variable temperatureas well as in anaerobic conditions. TS and BS media were used as the main growth media and the results indicated faster growth rate in TS media than in BS media as the strains growth was observed within 12hrs while that of BS could take up to 48hrs. Thus, the mean generation time obtained in this study for the *Pseudomonas strains* ranges from 4- 6 hrs in TS media and 7- 24hrs in BS media.This could be due to additional nutrients such as vitamins, amino- acids and high nitrogen content which are present in TS media but absent in BS media (Flemming *et al.,* 2011). This was demonstrated by Williams *et al.,* (2005), where high benzoate removal efficacy (89%) by *P. Putida* strain was observed within 42hrs alongside loss of toluene- xylene catabolic genes

when using TS broth as growth media in a bio- trickle filter industrial wastewater treatment. However, only 64% removal efficacy was observed in same studies after 120hrs when the *P. putida* strain was grown in BS media. Jiang *et al.,* (2016) also reported high concentration of biomass and PHA using TSB after 48hrs fermentation with *R. eutropha* while no production of PHA was observed in BSB media. Benzoate (0.5%w/v, 1%w/v, 2%w/v, 3%w/v) in combination with either TS or BS media was used as the sole carbon source and the results showed 0.5%w/v growth confidence by all the organisms and slow or no growth at greater than 0.5%w/v with an mgt of 8- 18 hrs. Slow growth at higher concentrations could be attributed to lower utilisation at higher concentration, toxic inhibition or the formation of toxic intermediates by non- specific enzymes as reported by (Nweke *et al.,* 2014). In agreement, Piotrowska *et al.,* (2016) reported that lack of growth may be due to inhibitory effect by high concentrations that results in the formation of acidic intermediates (hydroxyl radical) resulting in changes at both cellular and genetic (molecular) levels. Tarik *et al.,* (2004) reported 6hrs mgt of *P. putida F1 ATCC* growing in BSB using benzene (90g/L) as sole carbon source. While Sudarsan *et al.,* (2016) reported 5hrs mgt of *P. putida KT2440* growing in TSB using benzoate as sole carbon source under optimal laboratory conditions. *E. coli, S. lactis and C. jejeni* under optimal laboratory conditions have mgt ranging from 12- 17mins and 26- 48mins respectively, this indicated slower growth on benzoate generally irrespective of the concentration (Diaz *et al.,* 2001). In this study, the optimum growth temperature for the and the *Pseudomonas* strains to be between 30- 37$^{o}$C, while a growth decline was found for all the bacterial cells at 40- 50$^{o}$C. As reported by Munna *et al.,* (2016): Fonseca, *et al.,* (2012) most microorganism have optimum growth temperature at 25- 37$^{o}$C and could be because most microbial enzymes responsible for all metabolic activities have optimum temperature of 25- 37.5$^{o}$C. At higher temperature (>40$^{o}$C) the active site of enzymes where specific molecules tends to bind may change shape (Bowman's theory), this might lead to enzyme denaturation or consequently death of the organism due to heat shock (Srivastava *et al.,* 2008). From a genetic point of view, rapid increase in temperature hampers the translational capacity of the cell due to the formation of increasingly unstable mRNA secondary structures and diminished ribosomal activities that might result into slower protein folding and impaired cytoplasmic membrane fluidity, thereby negatively affecting transport processes (Sabath *et al.,* 2013).

When all the bacterial strains were subjected to anaerobic conditions using a glass jar at 37$^{o}$C in BSA- 0.5% benzoate media, the results indicated better growth of the bacterial strains under aerobic condition as no visible growth was observed under anaerobic conditions even after 168hrs for all strains in agreement with Rowe *et al.,* (2008). Nevertheless, *P. aeruginosa 67 a*lthough an aerobic organism demonstrated weak growth after 120hrs as it is also capable of being a facultative anaerobe due to the possession of CioAB enzyme that enables it to cope with low oxygen content (Franjipani *et al.,* 2008). A faulty jar might also attribute to low oxygen diffusion into the jar.

At different temperatures, pigment production by the *Pseudomonas strains* especially *P. aeruginosa* was observed in both solid and liquid media. Similar results were found by El Foul*yet al.,* (2015) that the strain secretes a variety of pigments including pyocyanin (blue-

green), pyoverdine (yellow- green and fluorescent), pyorubin (red-brown) and aeruginosin A. Pyocyanin is the virulence factor of the bacteria that leads to the production of exotoxin A which causes the inactivation of eukaryotic elongation factor 2 that subsequently leads to inability to synthesise proteins and necrotise by the host cell (Cheluvappa *et al.,* 2014). The strain also produces Phenazine which is a redox- active pigment responsible for quorum sensing, virulence and iron acquisition in host cells (Dietrich *et al.,* 2006). Hence, *P. aeruginosa* is identified as an opportunistic (affects immunocompromised) pathogen responsible for infection of ear, burn injuries, urinary tract and gastro-intestinal tract as well as cystic fibrosis, redial keratotomy and community- acquired pneumonia in rare cases (Lau *et al.,* 2004). While high concentration of such pigments in a medium could lead to the death of the organism responsible for its synthesis it can also be found in distilled water in laboratories as well as medical devices such as catheters (Cezairliyan *et al.,* 2013). Thus, on this basis *P. aeruginosa* was excluded from the study while. Another interesting observation made was the presence of few specific colonies (8) of *P. aeruginosa 67* which were assumed to be mutants based on their pigment coloration (brown). According to Chiang *et al.,* (2003) a single colony of mutant is found in every 1 million colonies as well as their ability to grow on benzene, biphenyl and benzoate. As reported by Alzahrani *et al.,* (2016) however, the pigments produced by *Pseudomonas spp* could be removed by Seitz filter using Dextran gel as column material or extracted from cell- free filtrate using chloroform, purified by column chromatography and characterized using UV-vis absorption.

*P. putida* strains (347, 459, 616) were selected for further screening while *P. putida 559* was excluded due to the production of a yellowish pigment that contains 1-Hydroxyphenazine and 5-methylphenazine-1-carboxylic acid betaine (Wang *et al.,* 2016) that makes it responsible for skin lesion, hence dangerous to work with. Further testing at temperatures 30$^{o}$C and 37$^{o}$C using the selected growth media BSB- 0.5%w/v benzoate was carried out on the three *P. putida* strains and *P. putida 347* proved to grow faster with 8hrs mean generation time followed by *P. putida 616 and P. putida 457* with 11hrs and 18hrs mgt respectively.

**Conclusion:**

Temperature plays an important role in growth and enzymes activities of the *Pseudomonas* strains in general and *P. putida* and *P. aeruginosa* microbes in particular as indicated by findings of this study. The results proved the ability of *P. putida* and *P. aeruginosa* to thrive on simple media including Basal Salt to a certain level in the presence of a specific carbon source which proves to their versatility and capability in bioremediation processes involving of recalcitrant pollutants or contaminants. However, higher concentrations of contaminants or recalcitrant pollutants might hinder the growth capabilities of the bacterial strains as indicated when the carbon source concentrations was increased to 3%w/v of benzoate. Furthermore, the results proved that *P. putida* and *P. aeruginosa* as non- fastidious microbes and adaptable, although results obtained from both media indicated more adaptability of *P. putida* strains to simple media thanthe *P. aeruginosa* strain. In addition, the studies indicated that moderate temperature of 30 and 37$^{o}$C serves as optimum temperature for various *Pseudomonas* species.

**Recommendation:**

The possibility of biofilms formation via immobilisation method of the *P. putida* strain so as to facilitate the utilisation of higher concentrations of carbon source(s) should be investigated. Furthermore, effect of pH, salinity as well as bio-stimulation on the growth level the *P. putida* strain should be investigated. This will shed more light on the versatility of the said microbe and its usefulness in bioremediation practice.

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