

Isolation, Identification and Characterisation of Butyric Acid Degrading Bacterium from Pit Latrine Faecal Sludge

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Butyric acid is one of the volatile organic compounds that contribute significantly to pit latrine offensive smell. The objective of this work was to isolate, identify and characterize pure butyric acid-utilising bacteria from enriched mixed cultures. A bacterial strain capable of utilising butyric acid as a sole carbon source was isolated from the pit latrine faecal sludge. Based on the 16S Subunit ribosomal Ribo-Nucleic Acid (16S rRNA) analysis, the strain was identified as *Pseudomonas aeruginosa*. The strain has the capability to utilise 1000 mg/L and 5000 mg/L of butyric acid in 27 h and 42 h, respectively. Further experiments showed that its cell growth was optimal at pH 7 when incubated at 30 °C and 150 rpm. The bacterial strain utilises butyric acid even in the presence of paracresol. The isolation of the strain from indigenous pit latrine faecal sludge showed that there is significant potential for application of microbes in treating noxious smells from the pit latrines.

1. Introduction

Pit latrines are basic and cheapest forms of improved household on-site sanitation in rural and peri-urban low income settlements in the developing world (Thye et al., 2011). Malodorous smells that are associated with pit latrines, which elicit disgusting or repulsive response to pit latrine users, are one of the factors that affect their performance (Nakagiri et al., 2016). This is primarily due to the fact that some people have a miasmatic view that associates malodorous emissions with conditions of 'unhealthy' air that causes diseases (Jorgensen, 2013). In negative visceral reaction to these unpleasant odorous episodes, people are deterred from using the pit latrines thereby resorting to open defecation, if private and secluded spaces availability is not a constraint. Occasionally, this also takes place in the immediate precincts of the pit latrines. This 'open air' approach is considered healthier and more pleasant (Cavill, et al., 2015). This offers a vital but often unnoticed key barrier for adoption and usage of the pit latrines hereafter disturbing effective sanitation promotion (Rheinländer et al., 2013).

A study conducted by Chappuis et al. (2016) demonstrated that butyric acid (C₄H₈O₂) and paracresol (C₇H₈O) are some of the major malodorous compounds identified that contribute significantly to pit latrine stench. To the best of our knowledge, there are no reports in the literature on aerobic degradation of butyric acid in the presence of p-cresol by a pure culture of microorganisms. This could be due to the fact that sanitation related malodour is an intrinsically unappealing subject matter. Microorganisms capable of degrading malodorous compounds may be an attractive alternative to the existing odour control techniques currently used in low income settings in the developing world. These techniques include, inter alia: addition of carbonaceous materials (Awasiqhe et al., 2015), use of ventilation systems (Ryan and Mara, 1983) and urine separation (Niwigaba et al., 2009). Thus, bioremediation provides an alternative tool to remove or degrade the malodorous compounds through utilisation of odorous compounds as carbon and energy sources by the microorganisms in aqueous solution to innocuous state end products. The use of indigenous microorganisms has been reported to be good for the adaption, survival and degrading capacity of the microorganisms in the environment which is highly contaminated (Rodriguez-Mateus et al., 2016). Recently, interest in bioremediation is growing mainly due to these potent applications.

The objective of this work was to report the isolation, identification and characterisation of butyric acid-degrading bacterium from pit latrine faecal sludge in South Africa. The study specifically investigated the effect

of initial concentration of butyric acid on cell growth, effects of initial medium pH and the effect of paracresol concentration on the cell growth of butyric acid-utilising bacteria. The study goal is to elucidate a potential application for biodegradation of butyric acid in pit latrine faecal sludge in order to eliminate noxious smells.

2. Materials and Methods

2.1. Chemicals

All chemicals used in this study were of analytical grade. Butyric acid ($\geq 99\%$) was purchased from Sigma Aldrich Inc., St Louis, MO, USA. Sodium hydroxide (NaOH), paracresol (99%) and all chemicals used for preparation of growth medium were purchased from Merck Chemical (Pty) Ltd, Gauteng, South Africa. Distilled water was prepared by Water Still system (Daihan Labtec. Co. Ltd, Kyonggi-Do, Korea).

2.2. Culture Media

Nutrient broth (NB) was composed of 1.0g meat extract, 2.0g yeast, 5.0g peptone and 8.0g sodium chloride in 1 L of distilled water. Nutrient agar (NA) was composed of 1.0g meat extract, 2.0g yeast, 5.0g peptone, 8.0g sodium chloride and 15.0g agar in 1 L of distilled water.

Mineral salt medium (MSM) contained the following (in grams per litre): 0.535g NH_4Cl ; 4.259g Na_2HPO_4 ; 2.722g KH_2PO_4 ; 0.114g Na_2SO_4 ; 0.0493 MgSO_4 and 1 mL of trace element solution also in 1 L of MSM solution. The trace element solution contained the following (in grams per litre): 5.549g CaCl_2 ; 6.950g FeSO_4 ; 0.0136g ZnCl_2 ; 0.0341g CuCl_2 ; 0.0103g NaBr ; 0.0121g NaMoO_2 ; 0.0198g MnCl_2 ; 0.0166g KI ; 0.0124g H_3BO_3 ; 0.0238g CoCl_2 and 0.0128g NiCl_2 in distilled water (Roslev et al., 1998). The media were then autoclaved at 121°C at 2 atm for 15 min. Thereafter, the pH was adjusted to 7.0 by titration with 6M. NaOH before use.

2.3. Isolation and identification of butyric acid degrading bacteria

Faecal samples were collected from the pit latrines in the semi-rural area of Kendal in Mpumalanga, South Africa. MSM was used as the enrichment medium amended with butyric acid (500 mg/L), as a sole carbon source to isolate butyric acid degrading bacteria. 100 g of faecal sludge sample was mixed with 1L of sterile distilled water and filtered. 1 mL of supernatant obtained from this was aseptically inoculated into each sterile 250mL Erlenmeyer volumetric flask with 100 mL of the 500 mg/L butyric acid amended MSM. The flasks were incubated at 30°C for 24-48 h at 150 rpm in the dark. From then on, a series of four consecutive enrichments in the fresh medium were carried out under the same conditions as above to enrich a butyric acid-degrading microbial consortium.

The single colonies were streaked onto nutrient agar plates, incubated at 30°C for 24 h. The process was done successively for at least three times in order to realise near pure culture of each identified colony. All the pure isolates were aseptically cultivated in nutrients broth at 30°C for 24 h. The bacterial cells were harvested at the speed of 6000 rpm at 4°C for 10 min. using Sorvall Lynx 600 centrifuge (ThermoFisher Scientific, Osterode am Harz, German). The cells were preserved in 2 mL of MSM with 30 % glycerol at -80°C until use. The butyric acid degrading isolates were identified by 16 Subunit ribosomal Ribo-Nucleic Acid (16S rRNA) genotype fingerprinting. Partially sequenced amplified 16 rDNA fragment was compared with other gene sequences in Gen Bank using a basic BLAST of the National Center for Biotechnology Information (NCBI) gene library. This was aligned with gene sequence of the isolates.

2.4. Characterisation of the butyric acid utilisation potential and its growth pattern

Prior to inoculation of the cultures in the flask for bacterial cell growth studies, the culture was acclimatised by cultivating the cultures at 30°C in 150 mL MSM amended with 500 mg/L of butyric acid in 250 mL Erlenmeyer flasks in an aseptic condition. Sub-culturing of the cells was done by transferring the cells at exponential phase to fresh MSM amended with 500 mg/L butyric acid. This was to ensure that the glycerol used for the preservation of the pure culture was completely eliminated prior to cell growth studies. Eventually the cells were harvested. Cell suspension for the experiments was prepared by mixing 100mg of the harvested cells with 10mL of sterile MSM to ensure that the flasks are inoculated with well homogenised cell suspensions. In each of 250 mL Erlenmeyer flasks containing 150 mL sterile MSM, 100 μL of the aliquot was transferred aseptically. The flasks were plugged with sterile cotton wool and incubated in the dark at 30°C at agitation rate of 150 rpm until the cell growth reached the death phase. .

2.5. Determination of cell growth

The pattern of cell growth was spectrophotometrically determined at single wavelength $\lambda = 600\text{ nm}$ using a UV Lightwave II spectrophotometer (Labotec, South Africa). The cuvette of 10 cm path length was used to carry the aliquot samples in the sample chamber of the spectrophotometer. The measurements were blanked to zero using sterile MSM as a reference. All the tests were conducted in triplicates and all were done in comparison to a control.

3. Results and Discussion

3.1. Isolation and identification of butyric acid degrading bacteria

A total of nine bacterial strains were successfully isolated from the enrichment cultures as described earlier in section 2.3 and showed to utilise butyric acid. The butyric acid degradation activity was not confirmed by the measurement of butyric acid degradation. However, the cell growth kinetic studies were done in which butyric acid was utilised as the sole source of carbon and energy. All isolates were established to possess the ability to utilise the odorous compound (butyric acid) as their source of carbon and energy. Among all the strains tested, the strain designated as B1b, exhibited the ability to utilise butyric acid and its relatively higher observed growth on butyric as a sole carbon source (data not shown). This characteristic is the one that impelled the researchers to subject it for further study.

The molecular identification of the strain was performed by amplifying and sequencing the 16S rRNA gene of strain B1b. This was submitted and compared with other genes in GenBank using a basic BLAST of the National Center for Biotechnology Information (NCBI) (<http://www.ncbi.nlm.nih.gov>). The phylogenetic tree generated based on the \pm 700 bp partial sequence of the 16S rRNA gene sequences of the strain B1b showed that the bacterial strain had the highest sequence homology (100 %) that clearly marched with *Pseudomonas aeruginosa* LMG 1224 (Gen- Bank Accession No.: Z76651) as shown in Figure 1. Henceforth, on the basis of this method the bacterial strain was identified as *Pseudomonas aeruginosa*.

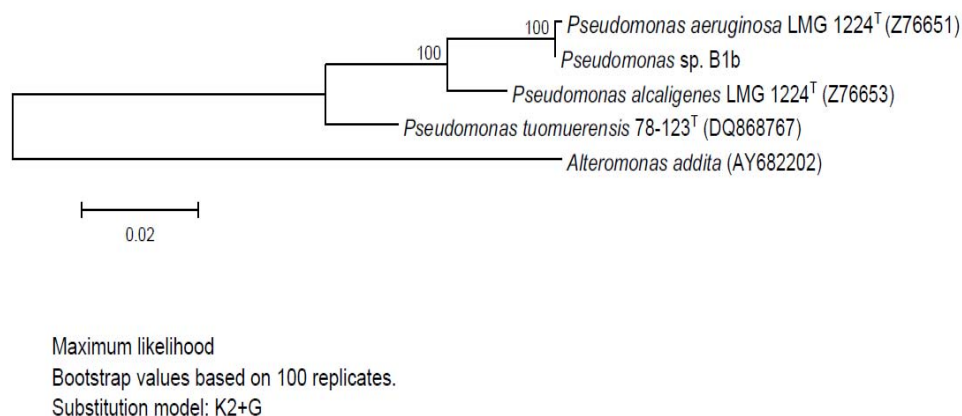


Figure 1: Phylogenetic tree based on 16S rRNA gene sequences showing genetic relationship of strain B1b with taxonomically related strains. GeneBank access number of each strain is given in parentheses

3.2. Effect of initial concentration on *Pseudomonas aeruginosa* cell growth

The experiments were conducted to explore the effect of initial concentration of butyric acid as a sole carbon and energy source on the cell growth of *Pseudomonas aeruginosa*. This was done at four different initial concentrations of 1000 mg/L, 5000 mg/L, 9000 mg/L and 12000 mg/L. The MSM without amended with butyric acid was a control. Based on the preliminary studies (data not shown) that were performed the investigation was done at optimal growth conditions of pH 7, temperature of 30 °C. Thus the strain is mesophilic bacteria. The laboratory experiment was designed in a batch reactor under aerobic condition by shaking on orbital shaker at rotation speed of 150 rpm. The pattern of microbial growth was analysed. It was observed that aerobic growth of the strain on butyric acid, butyric acid as its source of carbon was different dependent on the initial butyric acid concentration. Fig. 2 shows the utilization of different concentrations of butyric acid by *Pseudomonas aeruginosa* in 250 mL flask. *Pseudomonas aeruginosa* could not continue growing after 27 h when the initial concentration of butyric acid was 1,000 mg/L. This could be attributed to complete utilization of butyric acid. Thus, there was no more carbon for cell growth.

It was also observed that *Pseudomonas aeruginosa* ceased to show any cell growth with an initial concentration of butyric acid of 5000 mg/L after 42 h. This indicates that the metabolic activity of the bacteria was dependent on the availability of carbon source in the MSM. As seen in Fig. 2 it was observed that there was a corresponding increase in OD value of the culture with increase in butyric acid concentration in MSM from 1,000 mg/L to 5,000 mg/L. However, the lag phase was longer at higher initial concentrations of butyric acid. This showed that butyric acid could be used as a source of carbon by *Pseudomonas aeruginosa*, but might be detrimental to the bacterial strain at a higher concentration. This observation was confirmed at initial

concentrations of 9,000 mg/L and 12,000 mg/L. It was observed that the detrimental effects of increased concentration of butyric acid were much pronounced with negligible or no cell growth.

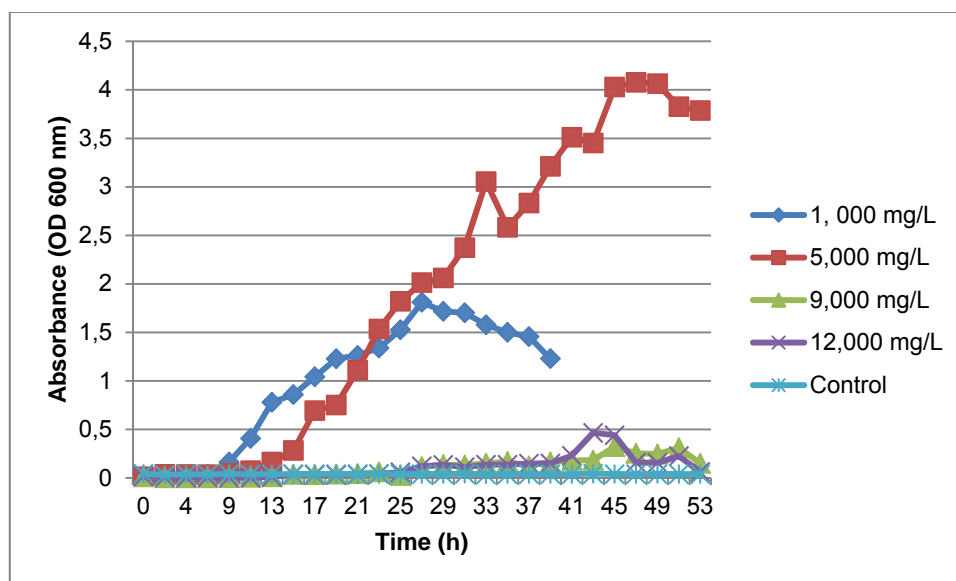


Figure 2: Growth of *Pseudomonas aeruginosa* in butyric acid of different initial concentrations at temperature of 30 °C and pH 7

The results of the effect of initial concentration on the growth of the bacterial strain showed that there was an increase in pH from a neutral initial medium pH to weakly basic and basic levels by the end of the experiment. The medium pH increased to 7.46 and 8.51 for the concentration of 1,000 mg/L and 5,000 mg/L respectively while the pH for the concentrations of 9,000 mg/L, 12,000 mg/L and control remained nearly neutral. The increase in medium is presumed to be as a result of the production and accumulation of different metabolites during the course of growth of the bacteria (Mueller et al., 1990), and suggests significant utilization of the substrates.

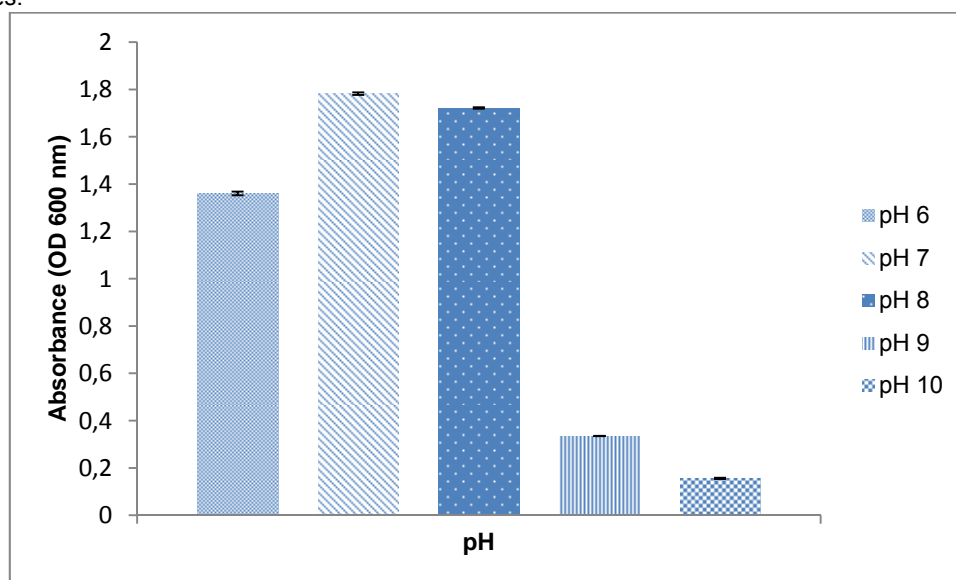


Figure 3: Effect of initial pH on growth of *Pseudomonas aeruginosa*

This probably accounts for the slight increase in the medium pH during the growth course, signifying the probable production of acidic basic metabolites. With certainty, this is due to the bioavailability of substrates (butyric acid), and the capacity of the bacteria to metabolise them to simpler utilisable materials. These results are consistent with the study on aerobic degradation of various volatile fatty acids other than butyric acids by

Acinetobacter calcoaceticus C6. There was also an increase in the medium pH by the end of the experiment (Chin et al. 2010).

3.3. Effects of pH on *Pseudomonas aeruginosa* cell growth

The results on the final medium pH by the end of the experiment compelled the researchers to investigate the effect of initial medium pH on the cell growth. The MSM solution amended with 1,000 mg/L were inoculated with 0.1mL of cell suspension and cultivated in the shaking incubator at 30 °C and agitation rate of 150 rpm. The initial pH was adjusted to 6.0, 7.0, 8.0, 9.0 and 10.0.

As seen in Fig.3 bacterial cell growth increased with the increase in pH and reached the maximum at pH 7 and thereafter the bacterial cell growth drastically decreased with increase in pH. This implies that the bacterial strain has its growth optimum between pH 6 and pH 8. Hence, the bacterial strain B1b can be classified as neutrophile, thus, the butyric-degrading enzymes have their optimum enzymatic activity in neutral environments. This consequentially suggests that when the pH of fecal sludge or wastewater becomes too basic or acidic the bacterial strain B1b growth and metabolic processes would be hindered. This also suggests that it is not always that the slowdown in cell growth is as a result of the substrate being completely utilised as was indicated in section 3.2. The change in environmental conditions such as increase or decrease in medium pH might also affect the enzymatic activities of the bacteria which consequently inhibit the cell growth.

3.4. Effects of paracresol on *Pseudomonas aeruginosa* cell growth

Paracresol, indole and dimethyl trisulfide are some of the key odorous compounds which frequently and significantly contribute to pit latrine stench along with butyric acid (Chappius et al., 2016). In this study, paracresol was used as a representative to check the effect of the presence of another odorous compound in the utilization of butyric acid for growth of *Pseudomonas aeruginosa*. The butyric acid degrading capacity in the presence of paracresol was investigated in the batch process. The results of the utilisation of butyric acid in the presence of paracresol are presented in Fig.3. From the observations it is evident that *Pseudomonas aeruginosa* utilised butyric acid even in the presence of paracresol. It was investigated at five different paracresol initial concentrations of 0 mg/L, 10 mg/L, 20 mg/L, 50 mg/L and 100 mg/L designated as BA, BA+10, BA+20, BA+50 and BA+100 respectively in Fig.4 while the initial concentration of butyric acid was kept constant at 1,000 mg/L. The maximum was chosen based on previous study in which paracresol was one of the odorous compounds that was analysed in the pit latrine in India and Africa. The highest recorded concentration was 89 mg/L (Lin et al. 2013).

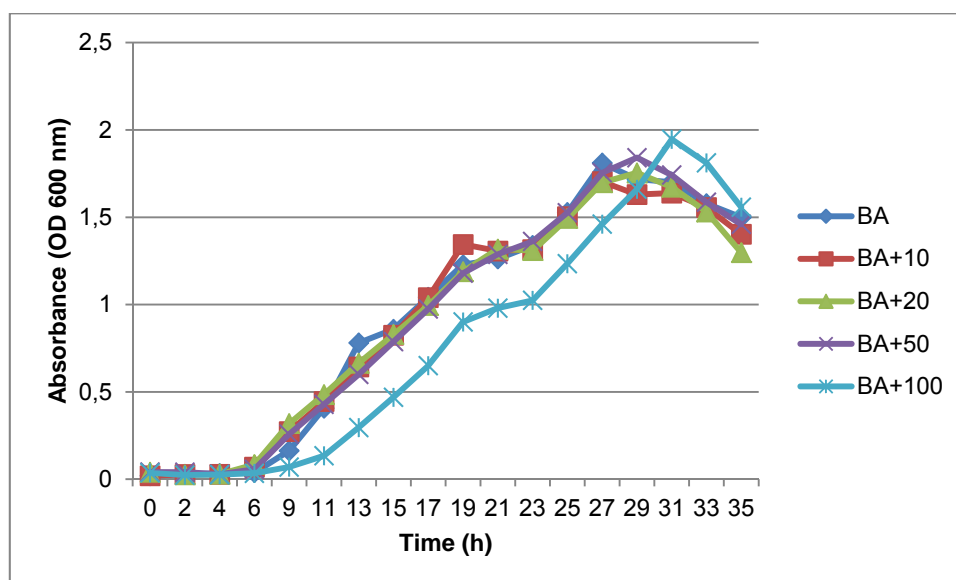


Figure 4: Effect of paracresol on growth of *Pseudomonas aeruginosa* with butyric acid initial concentrations of 1000mg/L

Further Fig. 4 indicates that paracresol up to concentration of 50 mg/L did not inhibit the butyric utilisation of butyric acid by *Pseudomonas aeruginosa*, whereas, for higher concentration of 100 mg/L of paracresol rate of cell growth capacity was considerably reduced and the lag phase was extended. However, investigation at higher concentration of paracresol than 100 mg/L should be carried out to confirm if the reduction in cell growth capacity was virtuously due to increase in paracresol concentration.

4. Conclusions

In this study, bacterial strain capable of utilising butyric acid as a source of carbon was isolated from pit latrine faecal sludge in South Africa. The bacterial strain was identified as *Pseudomonas aeruginosa*. The strain showed that it can effectively utilise butyric acid in the presence of paracresol. The results of the present study therefore, indicates that *Pseudomonas aeruginosa* is a potential candidate for application in the elimination of butyric acid in the presence other odorous compounds in pit latrines at the source of emission. The use of indigenous bacterial strain with butyric acid utilizing capabilities as inoculum onto butyric acid contaminated environment could prove a more environmentally-friendly approach to bioremediation. This can enhance sustainable development rather than the use of alien bacterial strains and the current existing odour control approaches in the low income communities. Further studies are needed to determine the butyric acid degradation efficiency of the strain to confirm if the cell growth of *Pseudomonas aeruginosa* is positively related to degradation of butyric acid.

Acknowledgments

The University of Pretoria is greatly acknowledged for providing UP Commonwealth Doctoral Scholarship to John to undertake this study.

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