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Potential Biological Deodorisation of Butyric Acid by Aerobic Bacterial Species of *Bacillus* Genus under Different pH Conditions

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Waste volatile compounds, such as butyric acid, generated from pit latrines may cause discomfort to pit latrine users and they have undesirable impacts on sanitation promotion efforts and public health. The purpose of this work was to isolate and identify highly efficient deodorant butyric acid bacteria and investigate their feasibility of biologically removing butyric acid from enriched liquid medium under different pH conditions. The determination of the ability of these bacterial strains on removal of butyric acid was at the concentration of 1000 mgL^{-1} in MSM. The experiment was designed in a batch reactor under aerobic condition by shaking in a temperature controlled incubator at orbital shaking speed of 110 rpm and of 30 ^{o}C and initial pH was adjusted to 7.0. The results showed that these bacterial strains were identified as *Bacillus cereus, Lysinibacillus fusiformis, Bacillus subtilis* and *Bacillus methylotrophicus.* The bacterial strains were capable of growth and degrading butyric acid at a wide range of initial pH values but the optimal growth and butyric acid degradation was achieved at neutral pH. Complete butyric acid degradation was achieved within 20-24 h. The bacterial strains have proved to be potential candidates for the development of biodeodorization systems for pit latrines.

1. Introduction

United Nations General Assembly and United Nations Human Rights Council recognised, alongside water, basic sanitation as a human right in 2010 (United Nations General Assembly, 2010). In 2015, adequate sanitation was unanimously recognised as a distinct and separate human right (Langford et al., 2017). This can be viewed as an affirmation of the fundamental significance of sanitation for human dignity (United Nations General Assembly 2015). However, in the same year, an estimated 2.4 billion people lacked access to improved sanitation facilities that hygienically separate human excreta from human contact, and 1.1 billion of the poorest people practiced open defecation as a result of lack of access to sanitation facilities worldwide (WHO and UNICEF, 2017). Indiscriminate disposal of human excreta has long-since been recognized as a major public health risk as it is responsible for transmission of bacterial, viral and parasitic infections such as diarrhoea, polio, schistosomiasis, hookworm, typhoid or cholera. In addition, this is further perpetuated by its intrinsic negative externalities. The most common route of transmission for enteric pathogens is via the faecal –oral route, therefore, access to adequate sanitation is a crucial barrier to faecal-oral transmission chain associated with poor sanitation (Duku et al., 2018). Improving sanitation is known to have a significant beneficial impact on health both in households and across communities in addition to offering a foundation for economic growth and social gains (Montgomery, et al., 2009)

Currently, decentralised sanitation systems, such as pit latrines, are the most common sanitation technology used in rural and low-income and highly dense informal or semi-formal communities throughout the developing countries (Njalam'mano et al., 2017). It is estimated that about 1.8 billion people use pit latrines as a primary means of sanitation (Graham and Polizzotto, 2013). In spite of their cheapness to construct and operate, and simplicity, pit latrines are often associated with performance difficulties such as offensive odour. Odour generation from pit latrines is one of the major nuisances that critically determine the consistent use of existing ones and adoption by some potential users (Obeng et al., 2018). These unpleasant odours compel

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people to prefer open defecation to foul-smelling pit latrines and in some cases even in the precincts of the latrines. This is due to many social, aesthetic and potential public health related concerns the malodours represent for the users and communities within the immediate vicinity. Thus, pit latrine odour abatement is an important issue and appropriate technologies are needed to increase its uptake, usage and for sanitation promotion to ultimately meet sustainable development goal (SDG) sanitation target.

Previous studies have demonstrated that butyric acid ($C_4H_8O_2$) is one of the key odorant compounds in a complex mixture of hundreds of volatile compounds proclaimed to be responsible for pit latrine malodorous emissions. It is usually emitted in significant concentrations much higher than its human odour detection threshold limit (Lin et al., 2013). Critical analysis of current abatement technologies and mitigation strategies of malodour gaseous emissions of pit latrines in the developing countries such as ventilated improved pit (VIP) latrines, addition of carboneous materials, urine separation etc., shows that they have socio-cultural, technological and economical limitations in their application. In recent years, bioremediation has received great attention. This is because the approach is more reliable and environmentally-friendly. Microorganisms including bacteria, fungi, yeast and algae with a high neutralising ability have been reported in literature. Biological deodorization is becoming popular, and can be designed for virtually complete removal of odours without causing secondary pollution. Bacteria play important roles in deodorization in other biological methods such as biocover and bio filter (Li et al., 2012). However, to design a full-scale biological odour abatement technology for pit latrines, the performance of butyric acid degrading bacteria should be evaluated. To the best of our knowledge, study related to deodorization of butyric acid by employing butyric acid-degrading bacteria from pit latrine faecal sludge in South Africa is very limited. Taking into consideration the significance of work, in this current work, indigenous bacterium capable of utilizing butyric acid as a sole source of carbon and energy was isolated from pit latrine faecal sludge. The performance of the isolated bacterium in the degradation of butyric acid was evaluated under different medium pH conditions.

2. Materials and methods

2.1 Culture medium

The mineral salt medium (MSM) used in this study comprised of (in gL^{-1}); K_2HPO_4 2.72, Na_2HPO_4 4.26, NH_4Cl 0.535, $MgSO_4$ 0.049, Na_2SO_4 0.114 and 1 mlL^{-1} of trace element solution. The trace element solution contained (in gL^{-1}); $CaCl_2$ 5.55, $FeSO_4$ 6.95, $ZnCl_2$ 0.014, $CuCl_2$ 0.035, NaBr 0.011, $NaMoO_2$ 0.012, $MnCl_2$ 0.020, KI 0.017, H_3BO_3 0.012, $CoCl_2$ 0.024 and $NiCl_2$ 0.013 (Roslev et al., 1998). The medium was autoclaved for 15 min at 121° C. Sterilised analytical grade butyric acid with ≥99% purity was added into the MSM to a final concentration of 1000 mgL^{-1} unless otherwise indicated. The pH of the media was adjusted by 6 M NaOH by titration unless otherwise indicated.

2.2 Enrichment, isolation and molecular identification of bacterial strain procedure

Faecal sludge was collected from pit latrines in Mpumalanga Province, South Africa. One hundred grams of the thoroughly homogenised faecal sludge sample were aseptically added to a sterilised flask containing 1000 mL of sterile deionised water. The mixture was vigorously vortexed for 5 min and allowed to settled for 10 min. The supernatant was preserved at 4 °C prior to use for bacterial isolation. One hundred millilitres of the supernatant were aseptically added to a 250 mL shaking flask containing 150 ml of the MSM supplemented with 500 mgL^{-1} butyric acid with its pH adjusted to 7.0 and incubated in a temperature controlled rotary shaker at 110 rpm at 30 ^oC for 24 h in the dark. This procedure was repeated thrice, and the 100 µL of the final culture broth was serially diluted and spread on nutrient agar plate. This was incubated at 30 ^oC in the dark. The morphologically distinct colonies appearing on the nutrient agar plates after 24-48 h incubation at 30 °C in the dark were picked and subcultured on fresh nutrient agar plates to obtain pure cultures. Microscopic examination was carried out for bacterial strain purity confirmation. The purified cultures were preserved in the MSM supplemented with 20% sterilized glycerol at - 80 °C. Genomic DNA of the isolates was extracted using the boiling method incubating the cell suspension for 10 min at 100 °C in water bath. The 16S rRNA encoding genes of isolates were amplified by Polymerase chain reaction (PCR) PCR using the forward primer (27F: 5' GAG TTT GAT CCT GGC TCA G 3') and reverse primer (1492R: 5' GGT TAC CTT GTT ACG ACT 3'). The amplification was done by initial denaturation performed at 94 °C for 10 min followed by 30 cycles of denaturation at the same temperature of 94 °C for 1 min, 63 °C for 1 min, 71 °C for 1.5 min; 20 cycles of 93 °C for 1 min, annealing at 58 °C for 1 min and extension at 72 °C for 1 min and then a final extension step at 72 °C for 5 min. The purified PCR product was sequenced in both directions using using ChromasLite v2.01 (Technelysium, Queensland, Australia) and BioEdit v7.05. The phylogenic relationship of the isolates was determined by comparing the sequencing data with sequences of known 16s rRNA gene sequences in the

GenBank nucleotide database maintained by National Centre for Biotechnology Information (NCBI) using BLAST searches at http://www.ncbi.nlm.nih.gov/BLAST.

2.3 Effect of initial medium pH on biodegradation of butyric acid and bacterial growth

To evaluate the effect of initial growth medium pH biodegradation of butyric acid by and bacterial growth of the bacterial strains at different initial medium pH values, 150 ml sterilised MSM in 250 ml Erlenmeyer flasks was adjusted to pH values of 5, 6, 7, 8, 9 and 10 by titration with 32% HCl and 6.0 M NaOH. The MSM was supplemented with butyric acid to a concentration of 1000 mg l^{-1}. The MSM in the flasks was inoculated by adding 1 ml of bacterial inoculum (OD_{600} = 2.0). These inoculated flasks were incubated at 30 ^{o}C in a rotary shaking incubator at 110 rpm for 24 h in darkness. Controls of un-inoculated flasks were used to check abiotic degradation. The experiments were conducted in triplicates The percent degradation of butyric acid (A) was determined according to Eq 1:

$$A = (C_k - C_o)/C_k x\ 100\% \quad (1)$$

wherein C_k is the concentration of residual butyric acid in the biotic bioreactor in which k is the incubation time in h and C_0 is the concentration of residual butyric acid in the abiotic reactor (control).

2.4 Analytical procedures

The concentrations of residual butyric acid in the medium were measured by Waters Alliance 2695 Separation Module HPLC system (Waters Corporation, Milford, MA, USA), equipped with a Waters 2998 Photodiode array detector (PAD) (Waters Corporation, Milford, MA, USA). For all separations an Aminex HPX-87H ion-exclusion organic acid, 300 mm × 7.8 mm, 9 µm particle size column (Bio-Rad Laboratories, Berkeley, CA, USA) was used. The solvent was 0.02 M sulphuric acid (H_2SO_4). The separation temperature was kept constant at 60 °C, isocratic flow rate and sample injection volume were set to 1 mLmin^{-1} and 10 µL respectively. All separations were monitored at 210 nm. Retention time for butyric acid was 12.2 min and the total run time was set at 15 min. Bacterial growth was measured spectrophotometrically at 600 nm with a UV Lightwave II spectrophotometer (Labotec, Gauteng, South Africa).

2.5 Statistical analysis

The results of all the analysed samples for butyric acid degradation were expressed as means ± standard error (SE). The statistical significant differences between the means of the analysed samples were determined by using one-way analysis of variance (ANOVA) and Tukey's post hoc analysis at a p-value ≤ 0.05 using SPSS 25.0 for Windows (SPSS Inc., Chicago, USA).

3. Results and discussion

3.1 Isolation and identification of butyric acid utilizing bacterial strains

Nine strains of butyric acid-utilizing bacteria were isolated fecal sludge obtained from pit latrines in Mpumalanga, South Africa. The determination of the ability of these bacterial strains on biodegradation of butyric acid was at the concentration of 1000 mgL^{-1} in MSM. The experiment was designed in a batch reactor under aerobic condition by shaking in a temperature controlled incubator at orbital shaking speed of 110 rpm and room temperature of 30 ^{o}C and initial pH was adjusted to 7.0. The results of HPLC analyses of residual butyric acid showed that all bacteria strains were capable of degrading butyric acid completely within 20 and 24 h (data not shown) compared to the sample that was not inoculated with any bacteria strain. The phylogenetic analyses inferred from 16S rRNA gene sequence (phylogenetic tree based on the 16S rRNA gene sequences not shown) of the isolated strains demonstrated that out of nine bacterial strains four bacterial strains were closely related to species of the genus *Bacillus* shown in Table 1.

Table 1: Closest relatives of the 16S rRNA gene sequences of bacterial strains

	Isolate designation	Closest hit	Accession No.	Homology (%)
1	B7a	*Bacillus cereus* ATCC14579	AE016877	100
2	C4c	*Lysinibacillus fusiformis* NRS-350^{T}	AF169537	100
3	CrNb	*Bacillus methylotrophicus* CBMB205^{T}	EU194897	100
4	CrNc	*Bacillus subtilis* DSM10^{T}	AJ276351	100

Highly diverse nature of Bacillus genus established in this work is in accordance with the observations made by Osibote et al. (2016) based upon microbiological analyses conducted on faecal sludge samples collected from pit latrines. The predominance of bacterial strains of *Bacillus* genus among other genera in the bioremediation processes, indicates that these bacterial strains are the main agents responsible for the degradation of butyric acid

3.2 Effects of initial medium pH on butyric acid degradation and bacterial growth

Environmental pH has a strong effect on their cell metabolism and growth. The effect of initial medium pH on butyric acid deodorisation and bacterial growth in a batch system with 1000 mgL^{-1} of butyric acid as sole organic source at under different pH conditions in the range of 5 to 10 at 30 ^{o}C and 110 rpm are shown in Fig. 1 and Fig. 2, respectively. The pH range was carefully selected to mimic the range of pH values found in pit latrine faecal sludge according to previous studies by Zuma et al (2015). The butyric acid deodorisation as well as the bacterial growth depend upon the medium pH. The butyric acid degradation efficiency of all the bacterial strains were identically influenced by the medium pH value. The butyric acid deodorisation was concomitant with bacterial growth as shown by Figure 1 and Figure 2

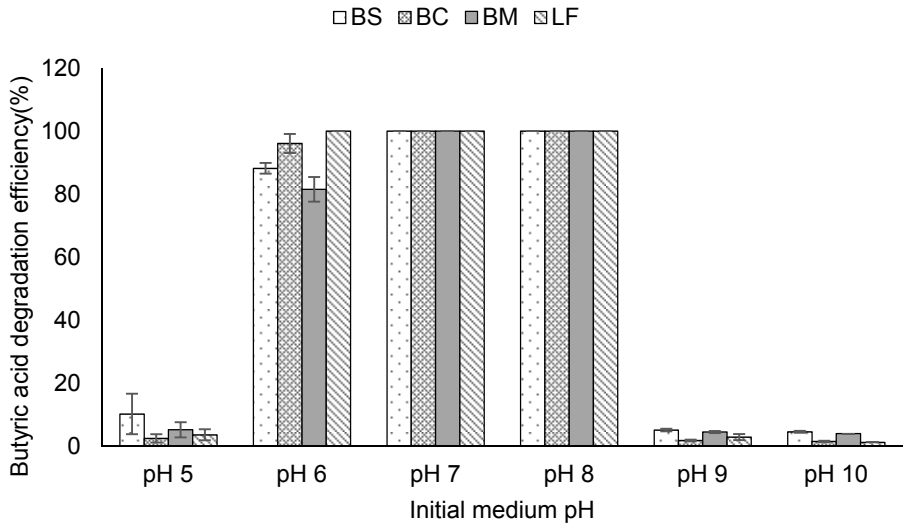

Figure 1: Butyric acid degradation efficiencies by different bacterial strains; Bacillus cereus (BC), Lysinibacillus fusiformis(LF), Bacillus methylotrophicus(BM) and Bacillus subtilis(BS) under different initial medium pH conditions after 24 h incubation

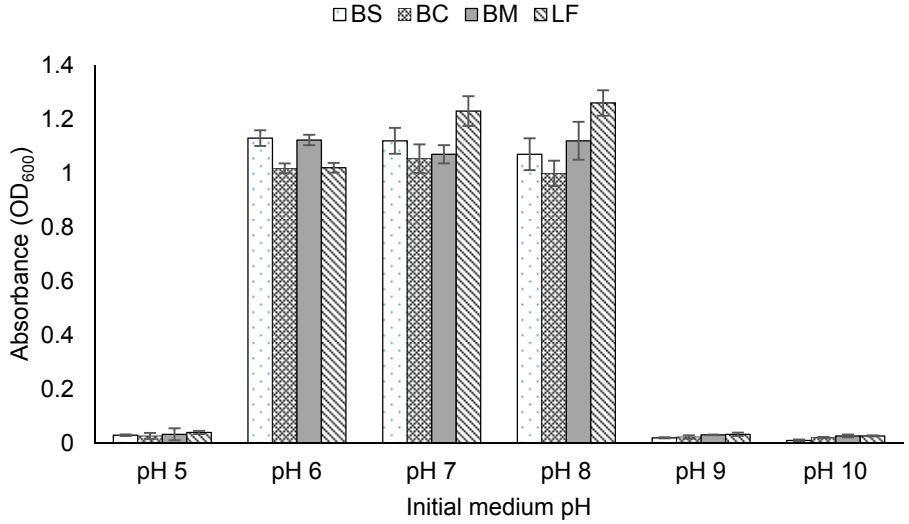

Figure 2: Bacterial growth of different bacterial strains; Bacillus cereus(BC), Lysinibacillus fusiformis(LF), Bacillus methylotrophicus(BM) and Bacillus subtilis(BS) under different initial medium pH conditions after 24 h incubation

Differences in butyric acid degradation efficiencies were statistically insignificant between pH 5 and 9, pH 9 and 10, and pH 5 and 10 for *Bacillus Subtilis, Bacillus methylotrophicus*, and *Bacillus cereus*. At pH 7 and 8, the butyric degradation efficiencies were not statistically different for all the bacterial strains.
The butyric acid degradation increased from pH 6 to pH 8 in correspondence to the bacterial growth. The maximal butyric acid deodorisation was observed when the initial medium pH was set at 7 to 8. This indicates that neutral or weak alkaline pH are favourable for butyric acid deodorisation. However, the bacterium, *Lysinibacillus fusiformis* achieved 100% removal of butyric acid after 24 h incubation at pH 6 to 8. All the bacterial strains can be regarded as neutralophilic with respect to the range of pH for growth. These results suggest that all the bacterial strains have a similar mechanism in enzymatic butyric acid oxidation. Also, these results are in perfect accordance with Chin et al (2010) observation that rapid degradation of butyric acid as a sole source of carbon and energy when it was inoculated with *Acinetobacter calcoaceticus*, *Burkholdeira cepacia* and *Wautersia paucula* was accomplished at neutral pH. It is possible that some key enzymes responsible for butyric acid degradation have their optimal enzymatic activity at neutral pH. Further increase in pH from 8 to 10 or decrease from 6 to 5 drastically lowered butyric acid deodorisation efficiency and bacterial growth for all the bacterial strains. This could be due to change in structure of macromolecules, disruption of enzymatic activities and membrane potential of the bacterial strains at those pH levels (Jeong et al., 2008). However, it was noted that the pH of the culture medium of initial pH values in acidic condition was increasing with incubation time shifting towards the optimal neutral pH whereas the pH of the culture medium of initial pH values of extreme alkaline condition was decreasing with incubation time shifting towards the optimal pH. The variations in pH of the liquid culture after 24 h of incubation are shown in Table 2.

Table 2: Variations in medium pH after 24 h incubation for different bacterial strains

Initial pH	Final pH				
	Bacillus subtilis	*Bacillus cereus*	*Bacillus methylotrophicus*	*Lysinibacillus fusiformis*	Control
5.00	5.64 ± 0.12	5.26 ± 0.08	5.20 ± 0.06	5.42 ± 0.07	5.00 ± 0.02
6.00	6.18 ± 0.11	6.45 ± 0.05	6.30 ± 0.21	6.27 ± 0.09	6.00 ± 0.04
7.00	7.13 ± 0.05	7.38 ± 0.03	7.32 ± 0.06	7.26 ± 0.08	7.00 ± 0.05
8.00	8.20 ± 0.07	8.11 ± 0.04	8.14 ± 0.05	8.11 ± 0.08	8.00 ± 0.02
9.00	8.34 ± 0.07	8.25 ± 0.09	8.27 ± 0.10	8.32 ± 0.08	9.00 ± 0.01
10.00	9.14 ± 0.04	9.23 ± 0.08	9.28 ± 0.05	9.21 ± 0.02	10.00 ± 0.04

This is a manifestation that the bacterial strains have the capacity to modify and react to environmental pH variations. According to Jain and Sinha (2008), this is one of the several coping mechanisms the bacteria develop to alleviate the effects of lowered or risen cytoplasmic pH and maintain cellular pH homeostasis that is compatible with optimal functional and structural integrity. The culture pH (i.e. with initial medium pH of 5-6) increase towards neutral pH could be due to indirect involvement of decarboxylase activity of glutamate, arginine and lysine (Hommais et al., 2001). This is accomplished via sequestering of the intracellular protons through biochemical reactions that either use up protons (i.e. amino acid decarboxylation) or produce ammonia (i.e. deiminase, deaminase and urease) (Lund et al., 2014). The culture pH (i.e. with initial medium pH of 9-10) decrease towards neutral pH could be as a result of metabolic activities that lead to increased acid production through amino acid deaminase (Padan et al., 2005).

4. Conclusions

Four out of the nine bacterial strains that were capable of degrading butyric acid isolated from pit latrine faecal sludge were of Bacillus genus. These efficiently butyric acid degrading strains were identified as *Bacillus cereus, Lysinibacillus fusiformis, Bacillus subtilis* and *Bacillus methylotrophicus* with nucleotides homology of 100%. The optimum pH for growth and butyric acid degradation for all the bacterial strains was nearly neutral. To the best of our knowledge this is the first time that these bacterial species of *Bacillus* genus has been reported for biological deodorization application. The use of indigenous bacterial strains with butyric acid degrading capabilities as seed onto pit latrine contaminated with butyric acid could prove a more environmentally-friendly approach to bioremediation which would have enhanced sustainable development rather than the use of alien bacterial strains. The results suggested that the bacterial strains could be potential candidates for pit latrine biological deodorization.

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