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Assessing the Susceptibility of *Bacillus Subtilis* to the Toxic effects of two lower Molecular Weight Phthalate Congeners in Pure Culture

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Abstract

Microbial degradation of phthalate esters has been proffered as one the most effective processes of remediating environmental media polluted by these ubiquitous compounds in the environment. A number of previous studies have shown Bacillus subtilis among a host of other soil bacteria to metabolize phthalates, however, the reported toxicity of phthalates to microbial life especially at certain levels of pollution could be a deterrent to this process. The nature of interaction between a pollutant and a bacterium depends on both the chemical properties of the pollutant and the metabolic characteristics of the bacterium. This work seeks to investigate the susceptibility of B. subtilis as a model soil microbe to the toxic effects of two lower molecular weight phthalate congeners (Dimethyl phthalate, DMP and Diethyl phthalate, DEP) using a combination of methods that examine the metabolic heat response and morphological changes of the bacterial cells in different dose of the phthalates both aqueous media. Microcalorimetry assessment suggests the phthalates stimulated growth and metabolic activities of the bacteria at doses between 50-100µg/mL but however produced inhibitory effects at higher doses. The half inhibitory dose index (ID_{50}) obtained from the metabolic heat response shows that DMP is relatively more toxic to the bacterium than DEP which is attributed to the slightly higher solubility of DMP permitting its easier mobility across cell membrane. Scanning electron microscopic images of cells incubated at different doses of the phthalates show that both DMP and DEP impeded the bacterial growth and reproductive process especially at doses ≥ 200µg/mL. Comparing these results to previous studies, lower molecular weight phthalates show relatively higher toxicity to B. subtilis than their higher molecular weight congeners. These evidences show that within certain low doses, phthalates can serve as carbon or energy sources to microbes by stimulating their metabolic activities but beyond certain limits, can exhibit their toxic effects by inhibiting microbial growth.

Keywords*: Bacillus subtilis*, phthalates, toxicity, microcalorimetry.

Introduction

Phthalate esters are anthropogenic organic compounds with wide ranging applications which have resulted to their environmental ubiquity over the years. They are mainly used as plasticizers[1] and additives in numerous products including insecticides, lubricants, dielectric fluids, synthetic fibres and personal care products[2]. As plasticizing agents, phthalates are loosely bound to parent polymer materials with a tendency of easily leaching or volatilizing from plastics products during use or after disposal, a potential risk for environmental and human exposure[3-5]. It is suggested that phthalate esters used as plasticizers in blood storage bags may lead to long term toxic exposure and the release of pro-inflammatory cytokine[6]. Phthalate esters mainly enter the environment through discharge of industrial effluent or waste water, disposal of plastic waste and the use of soil film in the agro-industry[7]. The sparing solubility in water and strong affinity of these phthalates for solid particles results to their easily precipitation from aqueous media and accumulation in sludge and sediment[8], indicating longer residence time in soils and possibly high toxicity impact on soil microbial community. Their toxicity to soil microbial community and implication as endocrine disrupting substances[9-12] have attracted enormous public health concerns and research interest.

Microbial degradation is considered the most viable option for removing phthalate esters from polluted environments. Previous research works have identified a number of microorganisms that degrade these compounds and have proposed bioremediation as a viable alternative for environmental cleanup and treatment of waste water or industrial effluents containing phthalates[13-17]. Nonetheless, assessing the biodegradation ability of phthalate degrading microbes against their susceptibility to phthalate toxicity has received very little attention. It is possible that a microbe which effectively metabolizes phthalate esters at certain low levels could be prone to their toxicity at higher levels. This scenario therefore creates the basis for a thorough investigation of phthalates toxicity to their degrading microbes as a way of optimizing their biodegradation. The outcomes of

such investigation will have far reaching implications on the effects of phthalate ester pollution on soil microbial activity and soil productivity.

Two lower molecular weight phthalate esters congeners, dimethylphthalate (DMP) and diethylphthalates (DEP) mostly used as solvents and as plasticizers in soft plastics are of interest in this work. Relative to other phthalates, they have higher solubility in aqueous media and higher aquatic toxicity potential than do the transitional and higher molecular weight phthalates. Their widespread use has resulted to their significant detection in natural environments[18]. Their presence in both aqueous and soil media could influence microbial activities with the potential of altering soil ecological balance and productivity. Although the toxic effects of phthalates to aquatic animals[19,20] and humans[21,22] have been widely studied, not much work has been done to completely understand their influence on soil microbial activity. An assessment of phthalate toxicity to *Bacillus subtilis*, a ubiquitous soil bacteria and known to degrade a variety of phthalate esters[23-25] would shed light on the overall effects on these pollutants on microbial life and furnish new information relevant to environmental bioremediation of phthalates and in waste water treatment. This work will separately investigate the impact of the DMP and DEP on the microbial metabolic activity of *B. subtilis* incubated in pure culture and by extension examine the mechanism(s) by which phthalates impart their toxicity to microorganisms.

Material and Methods

Bacterium and growth medium: *B. subtilis* (G010, CCAM020074) strain was obtained from the State Key Laboratory of Agricultural Microbiology, Huazhong Agricultural University (Wuhan, P.R. China). DMP and DEP (99%) were purchased from Acros Organics (Geel, Belgium). Stock solutions were prepared by dissolving each phthalate in ethanol to make a concentration of 5000µg/mL. Appropriate dilutions to obtain the desired concentrations (0, 50, 100, 200, and 400µg/mL) were done in the growth medium.

Peptone medium was used to incubate the bacterium and was prepared by dissolving 10.0gpeptone, 5.0g beef extract (Beijing Shuangxuan Microbe Culture Medium Products Factory, Beijing, P.R. China) and 5.0g NaCl (Sinopharm Group Chemical Reagent Co., Ltd., Beijing, P.R. China) in 1.0L of deionized water at pH 7.2. Solid growth medium was prepared by adding 1.0% w/v agar to the peptone medium and sterilized by autoclaving at 120°C for 30min. The bacterium was grown aerobically at 37°C between 12 and 16h with constant shaking at 200rpm. All reagents of analytical grade or above were used as obtained from vendors.

Growth measurement in pure culture: The toxic effect of each phthalate on the bacteria growth in pure culture was monitored by measuring the optical density (OD) of the growth medium and viable (bacteria) count of cell colonies over time in triplicates. Both measurements were done simultaneously using a set of three controls (A, B and C) aimed at eliminating the effects of growth medium or solvent on the growth pattern of the bacterium. Controls 'A' contained the growth medium and solvent (ethanol) without bacteria; 'B' contained the growth medium, solvent and bacterium; and 'C' contained the growth medium and the phthalate solution (of the same doses as in the main experiment) without inoculation. In the main experiment, bacterial cells were inoculated into 150mL growth media containing various doses of the phthalates in 250-mL Erlenmeyer flasks and incubated at 37°C with constant agitation at 150rpm. The bacteria growth was monitored by quenching small aliquots of the medium for viable count and OD measurement at 600nm and 2-h interval over a 24-h period. In each case, a portion of the quenched medium was appropriately diluted serially using ultra-pure water and OD measured by pouring the diluted medium (after vortex mixing) into a 2.0-mL quartz cuvette in a Spectrum lab 752s UV–visible absorption photometer (Lingguang Co., Ltd., Shanghai, P.R. China). The OD of the bacterium growth was recorded by the subtracting the background absorption of the third control containing the medium and the respective concentration of each phthalate without bacterium. For the viable count measurement, 100µL of various serial dilutions of the growth medium was spread on LB agar plates and incubated overnight at 37°C. The number of colonies formed was counted and expressed as colony forming units per mL (CFU/mL).

Microcalorimetric Method: A six-channel (five sampling and one reference) thermal activity monitor (TAM III, Järfälla, Sweden) was used to record the heat flow rate of the bacterium metabolic activity in growth media containing various doses of the phthalates. Each channel comprises two calorimetric units (one vertically placed on top of the other) that are separated by a small 'primary' heat sink, which is in thermal contact with a surrounding steel tube[26]. The channels are inserted into a precise liquid thermostat which serves as the main heat sink for all the calorimetric units. Calibration is achieved by the release of electrical energy in a resistor while the thermal effects of the sample ampoules are adjusted by an electrical calibration[27]. The precision of the devise is achieved by its control over the isothermal conditions in the thermostatic bath and sensitivity in detecting the thermal events in the system[28].

Thermal effects of microbial activity are recorded from 4.0mL stainless steel ampoules that are hermetically sealed with Teflon discs to prevent evaporation. Into each ampoule, 2.0 mL of sterilized soil of growth medium was added and amended with different doses of a particular phthalate (0, 50, 100, 200, and 400µg/mL), equilibrated to 37°C and amended with 50µL bacterial suspension with cell concentration of 10^6 cells mL^{-1} (microscopically counted using a bacteria chamber). The ampoules are firmly sealed and inserted into the top units of the channels. The thermal effects associated with the microbial activity in the different doses of the phthalates at constant temperature (37°C) were recorded as a function of time on an

interfaced computer. Thermal response data were processed and analyzed using the statistical software Origin Lab 8.0 software (Origin Lab Corporation, Northampton, MA, USA). Various metabolic parameters as microbial response indicators at different doses of each phthalate were calculated from the power–time curves[29, 30].

Scanning electron microscopy: In order to assess the morphological effects or otherwise of the phthalates to the bacterium, a JEOL JSM-6510 LV Scanning Electron Microscope (SEM, Tokyo, Japan) was used. Three doses (0, 200 and 400µg/mL) of each phthalate were separately impregnated in solid agar growth media and inoculated with freshly activated cells of the bacterium and incubated at 37°C for 12h (an estimated mid-exponential phase period of normal bacterial growth from the viable count measurements). Bacterial cell colonies were harvested and gently washed in phosphate buffer saline and centrifuged three times. The cell pellet was transferred into 0.25% fixative glutaraldehyde solution and incubated overnight. The cells were centrifuged and washed three times with ultra-pure water followed by serial dehydration using 30, 50, 70 and 90% ethanol solutions successively. In each case, the bacteria suspensions were centrifuged and the supernatant liquid decanted. The cells were finally freeze-dried for 6h and stubs prepared for observation under the SEM.

Statistical Analyses: Metabolic heat response measurement of the bacteria in sterilized soil, pure culture optical density measurements and colony forming units (CFU) counts were carried out in three parallel experiments. Analysis of variance (ANOVA) method was used for comparison of data obtained for the two phthalates at a significance level of $P \leq 0.05$. Where appropriate, data are presented as mean ± standard deviation (SD).

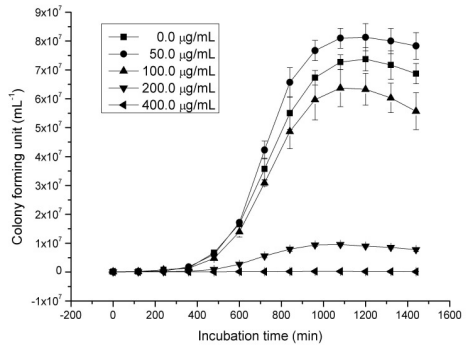
Results and Discussion

Optical density and viable count: Figure 1 shows the effects of DMP and DEP on the growth activity of *B. subtilis* monitored by viable count method with trends that are more or less typical of those obtained from measurement of growth media turbidity. The calculated growth rate constants obtained from both measurements are comparable and show similar trends as shown in table 1.

Table-1
Growth Rate Constants of Bacillus subtilis at different doses of DMP and DEP obtained from Optical Density (OD) measurements

Dose (µg/mL)	*Growth rate Constants ($k \times 10^{-3}$), (min^{-1})*	
	DMP	**DEP**
0	11.54 ± 0.11	12.19 ± 0.04
50	15.83 ± 0.05	14.06 ± 0.10
100	8.81 ± 0.09	17.37 ± 0.08
200	2.35 ± 0.03	5.93 ± 0.07
400	nil	0.81 ± 0.02

DMP: Dimethylphthalate; DEP: Diethylphthalate. Growth rate constants calculated from colony forming units (cfu) are similar to these obtained from (OD) and follow similar trends. Values are given as mean ± 1 SD, n=3



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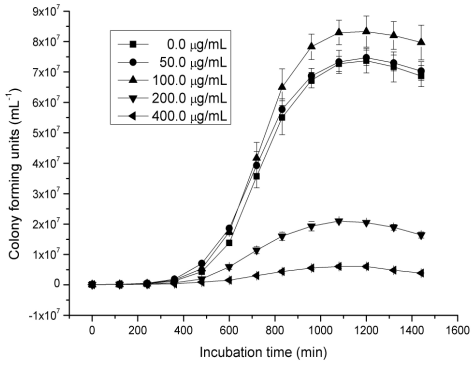


(b)

Figure-1
Trends in colony forming units (CFU) obtained by the viable count method showing the effects of various doses of (a) DMP (b) DEP on the growth of *B. subtilis*

From the growth trends and calculated rate constants both phthalates manifest dual effects on the growth pattern of the bacterium; stimulating its growth at lower doses (≥50µg/mL for DMP and ≥100µg/mL for DEP) relative to the control but subsequently inhibiting growth at higher doses climaxed by an infinitesimal growth at 400µg/mL of DMP. In other words, *B. subtilis* shows the ability to degrade both compounds at lower doses but was prone to their toxicity at higher doses. Comparing the growth rate constants of the bacterium at various doses of the two phthalates (table 1), the relatively low molecular weight DMP stimulated the bacterium growth at 50µg/mL compared to DEP. However, while DMP inhibited the bacterium growth at doses >50µg/mL, DEP effected enhanced growth stimulation at doses ≤100µg/mL. This result highlights the fact that lower molecular weight phthalate congeners could be more readily targeted by extra-cellular metabolic enzymes produced by the bacterium or mobilized across the cell membrane; general making them more degradable at lower doses but producing

more toxic effect at higher doses. This could be attributed to DMP's slightly higher solubility in aqueous media.

Metabolic Response analysis: Figure 2 shows the metabolic heat responses of the bacteria at different doses of the two phthalates. Single peaks were recorded for each dose with the respective thermal curves showing distinct lag, exponential, stationary and decline phases. Table 2 summarizes the quantitative indices obtained from the thermal response data: total metabolic heat (Q_T), metabolic rate constant (k), maximum power (P_{max}) and the corresponding time (T_{max}), generation time (T_G), inhibitory ratio (I) and the half inhibitory dose (ID_{50}), characterizing the bacterium metabolic activities at various doses of the phthalates.

The total heat produced Q_T, which relates to the bacterium's ability to metabolize the phthalates as either carbon or energy source, was calculated by integrating each power-time curve from the outset to the end. The associated maximum heat output and corresponding time were obtained and given as P_{max} and T_{max} respectively. The metabolic rate constant (k), which indicates the chemical stress impacted by the phthalates on the metabolic activity of the cells was calculated as the slope of the plot of time against the natural logarithm of the heat response (power) from the power-time curves (figure2) for the different doses considered according to the relationship illustrated in equation 1:

$$\ln P_t = \ln P_o + kt \quad (1)$$

Where P_t and P_o are the powers at time t and t=0, respectively.

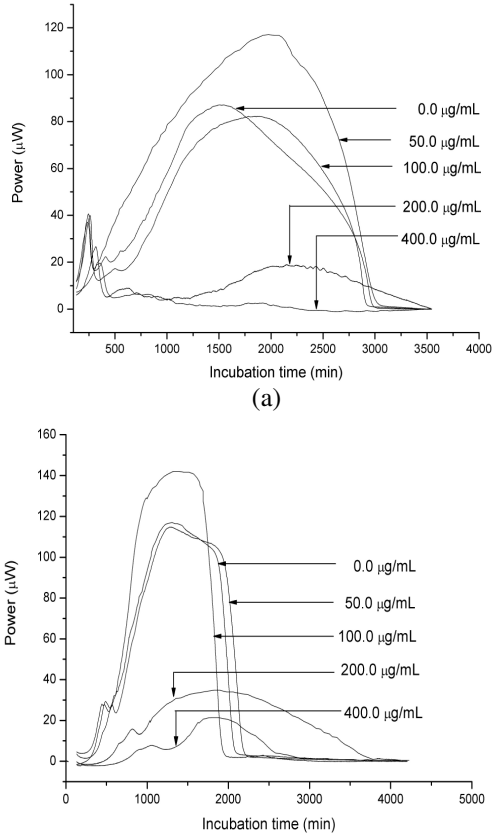


Figure-2
Power–time curves of themicrobial metabolic activities of *B. subtilis* in different doses of (a) DMP (b) *DEP*

Table-2
Effects of various doses of DMP and DEP on the metabolic activity of *B. subtilis* obtained by microcalorimetry

Dose (μg/mL)	Q_T *(J mL^{-1})*	$k \times 10^{-3}$ *(min^{-1})*	P_{max} *(μW)*	T_{max} *(min)*	T_G *(min)*	*I (%)*	ID_{50} *(μg/mL)*
DMP							141.39
0.0	8.932	9.98 ± 0.05	87.11	1502	69.49	nil	
50	13.187	12.53 ± 0.07	171.11	1974	55.33	–25.6	
100	8.341	9.13 ± 0.03	82.24	1890	75.93	27.1	
200	1.574	1.81 ± 0.01	18.94	2156	382.11	81.8	
400	0.252	0.03 ± 0.005	6.44	689	23819.5	99.7	
DEP							
0.0	8.170	10.13 ± 0.03	114.74	1259	68.44	nil	184.85
50	8.319	11.03 ± 0.05	116.95	1305	62.84	–8.9	
100	9.237	14.18 ± 0.07	142.01	1324	48.90	–40.0	
200	4.245	3.42 ± 0.03	34.88	1825	202.56	66.2	
400	1.436	1.12 ± 0.01	21.44	1815	619.99	89.0	

Q_T: Total metabolic thermal effect; k: Metabolic rate constant; P_{max}: Maximum metabolic heat. T_{max}: Time at P_{max}; T_G: Generation time; I: Inhibitory ratio; ID_{50}: Half inhibitory dose. DMP: Dimethy phthalate; DEP: Diethylphthalate. k values are given as mean ± 1 S.D. (n = 3 and P < 0.0001).

The corresponding generation time T_G was calculated as seen in equation (2):

$$T_G = \frac{ln2}{k} \quad (2)$$

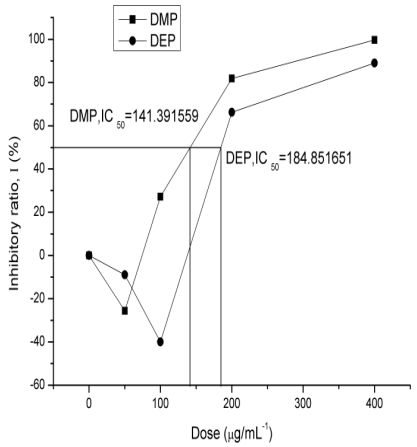
The inhibitory ratio, *I*, a quantitative estimation of the relative inhibitory effect of each dose of phthalate on the metabolic activity of the bacterium, was calculated from equation (3):

$$I = \frac{k_o - k_c}{k_o} X\ 100\ \% \quad (3)$$

Where k_0 is the rate constant of the control and k_c is the rate constant for microbial activity at various phthalate doses. From a plot of *I* against dose, the corresponding half inhibitory dose (ID_{50}) was estimated as the dose at *I* equals 50%.

Generally, the trend of Q_T, k, P_{max} and T_{max} values suggest that lower phthalate doses stimulated the bacterium growth, thus implying that the bacterium conveniently utilized the phthalates as energy sources at such doses. The lowest doses that effected maximum growth stimulation for DMP and DEP are 50 and 100µg/mL respectively as depicted by both the total heat output and growth rate constant values (table 2). Comparatively, 50µg/mL of DMP resulted inenhanced growth stimulation than the same dose of DEP but while DEP continued to effect more stimulation at doses up to100µg/mL DMP showed inhibitory effects. Invariably, beyond doses of 50µg/mL for DMP and 100µg/mL for DEP, the phthalates inhibited the growth and metabolic activities of the bacterium as evidenced by the reverse trend in values of the growth parameters (Q_T, k), and the doses of the phthalates as seen in the relationship between phthalate dose and bacterium growth rate constant in figure 3b. This observation is re-echoed by inhibitory ratios, *I*, with negative values for lower doses of the phthalates indicating growth stimulation of the bacterium but increasingly positive values for higher doses depicting a reverse effect. The same trend holds true for the calculated generation time, T_G, with lower values for lower doses of phthalates relative to the control indicating that the cells required less time to double their population, thus reinforcing the fact that the low doses of the phthalates stimulated cell growth. However, higher doses (>50 µg/mL for DMP and >100 µg/mL for DEP), T_G continued to increase with increase in phthalate dose depicting greater constraint to cell growth and metabolism. It is therefore evident from the foregoing, that the phthalates had biphasic effects on the growth and metabolic activity of *B. subtilis*, stimulating its growth at low doses but inhibiting growth at higher doses[31].

The half inhibitory dose ID_{50}obtained by plotting inhibitory ratio, *I,* against dose at I=50%, as shown in figure 3a is the minimal dose of an inhibitor that produces half the total inhibition/toxicity to the target organism. Thus, with respect to *B. subtilis*, DMP with a lower ID_{50} (141.4µg/mL) is comparatively more toxic than DEP with a higher ID_{50} (184.9µg/mL). In other words, although DMP stimulates the growth and metabolic activity of *B. subtilis* more than DEP below certain minimal doses (≤50 µg/mL), the bacterium is nonetheless more susceptible to its inhibitory effects at higher doses compared to DEP. In addition, DEP has a wider dose range (≤100 µg/mL) than DMP (≤50 µg/mL) for stimulating the growth and metabolic activity of *B. subtilis*. Comparing the relatively higher ID_{50} values previously reported for higher molecular weight phthalates to *B. subtilis*[31], it is indicative that the lower molecular weight phthalates (DMP and DEP) have more toxic effects on the metabolic activities of *B. subtilis*.



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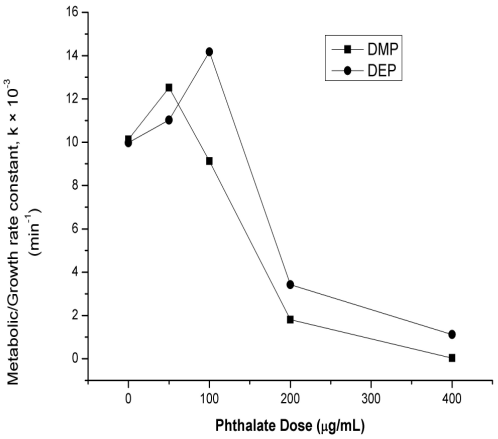
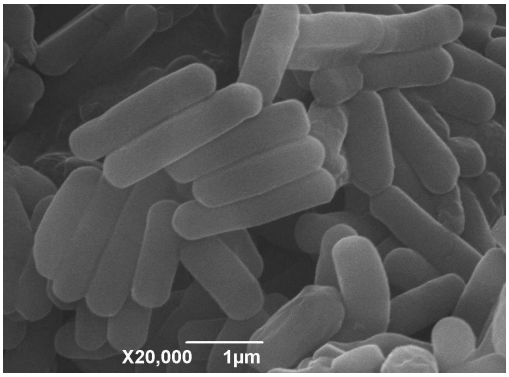


(b)

Figure-3
Relationship between (a) Phthalate dose and inhibitory ratio (b) Metabolic rate constant and Phthalate dose

Scanning electron microscopy: SEM images of *B. subtilis* obtained of cells treated with three doses selected within the range of phthalate doses used in this work are shown in figure 4. Figure 4a shows cells grown in a pure medium without any phthalate dose. In this image, the cells appear normal without

any form of morphological deformity. The cells are clearly seen as either single cells or in the process of cell division. By stark contrast; however, figures 4b-e showing cells incubated on agar media impregnated with different doses of the two phthalates illustrates visible morphological deformities with shrunk or elongated strands depicting growth inhibition and interferences with the bacterial reproductive processes. The extent of morphological deformity from mere visual inspection seems to have a positive correlation with phthalate dose and appeared to be more pronounced for DMP. It is likely that the phthalates known for mutagenicity could have caused bacterial DNA damage characterized by cell circle arrest and cell division disruption, conditions that could arise through what is known as global response by the cells in attempts to repair such damages.



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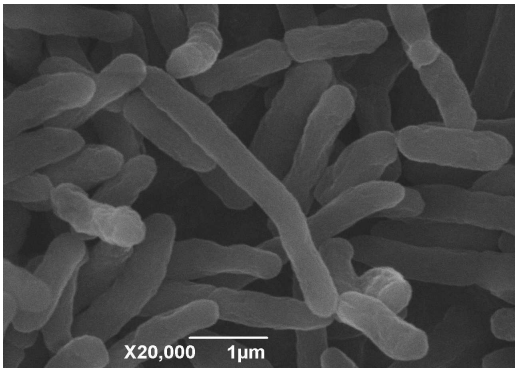

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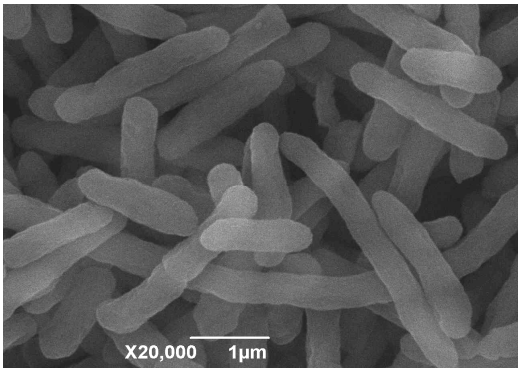

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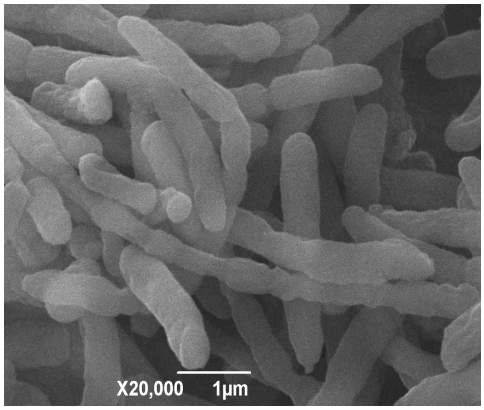

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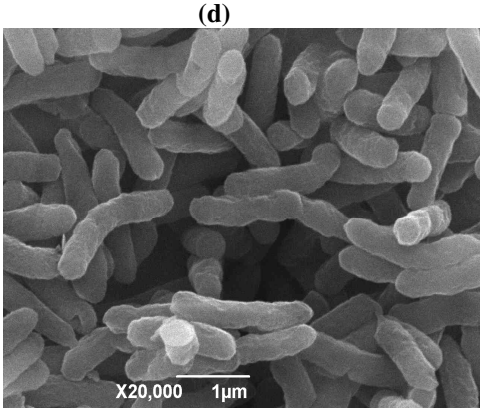

(e)

Figure-4
SEM images of *B.subtilis* cells grown in different doses of DMP and DEP (a) cells in pure culture medium without any phthalate (b & c) cells grown in 200 µg/mL of DMP and DEP respectively (d & e) cells grown in 400 µg/mL of DMP and DEP respectively

Conclusion

Results obtained from both tradition microbial methods (CFU and growth medium optical density measurements) and microcalorimetry suggest that *B. subtilis* metabolizes phthalate esters at low doses as either a carbon or energy source but it is however susceptible to phthalate toxicity at higher doses. Doses of ≤50 µg/mL for DMP and ≤100 µg/mL for DEP stimulated the bacterium's growth while higher doses were inhibitory. The relative toxicity of alkyl phthalates depends on nature of the alkyl group attached to the phthalate ring. The results support the notion that lower molecular weight phthalates are relatively more toxic to microbial growth and metabolic activities compared to higher molecular weight phthalates. This is evident by the lower ID_{50} (a parameter with inverse relations to toxicity) obtained for two lower molecular weight phthalates (141.4µg/mL and 184.9 µg/mL for DMP and DEP respectively) compared to the values reported for the higher molecular weight octyl congeners DEHP and DOP[31]. Generally, in spite of the varying toxicity levels of different molecular weight phthalates, they all tend to affect bacteria via similar mechanisms, possibly acting as exogenous agents that cause bacterial DNA damage.

The susceptibility of *B. subtilis* as a ubiquitous component of soil microbial community [32]to phthalates toxicity should give valuable information about the response of soil microbial community to phthalate pollution. Although phthalates are biodegradable in aquatic media especially at low concentrations, their strong affinity for soil particles and humic materials coupled with their relatively low degradability in anaerobic conditions[33] would result in the disruption of soil microbial activity and a tendency to accumulate in polluted soils.

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