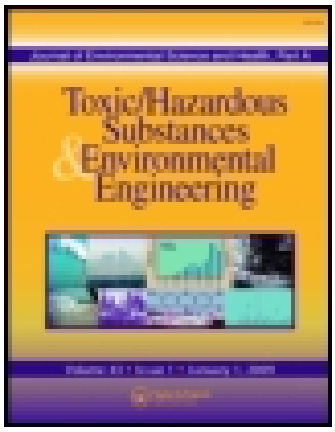

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The toxic effect of cadmium on pure microbes using a microcalorimetric method and a biosensor technique

HAIYAN CHEN[1], JUN YAO[1], YONG ZHOU[1], HUILUN CHEN[1], FEI WANG[1], NAN GAI[1], RENSHENG ZHUANG[1], BRUNELLO CECCANTI[2], THOMAS MASKOW[3] and GYULA ZARAY[4]

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A microcalorimetric technique based on microbes heat-output was explored to evaluate the effect of Cd (II) on *Bacillus subtilis* and *Candida humicola*. The power-time curves of the growth metabolism of *Bacillus subtilis* and *Candida humicola* and the effect of Cd (II) on it were studied by using a TAM III microcalorimeter, ampoules method at 28°C. For the evaluation of toxic effect on pure micro-organisms, the maximum peak-heat output power (P_{max}) in the growth phase, the growth rate constants (k), the log phase heat effects (Q_{log}), and the total heat effect (Q_T) for *Bacillus subtilis* and *Candida humicola* were determined. Dissolved oxygen and biochemical oxygen demand (BOD) were evaluated by a biosensor. Cadmium has been regarded as the essential biological trace element. Cd (II) solutions of different concentration have different effects on *Bacillus subtilis* and *Candida humicola* growth metabolism. The higher concentrations of Cd (II) inhibit the growth of *Candida humicola* (1600–3200 $\mu g \cdot mL^{-1}$), *Bacillus subtilis* (240–480 $\mu g \cdot mL^{-1}$); the lower concentrations can promote the growth of both micro-organism. The values of cell dry weight is also showed in conformity in the cell dry weight changes to the micro-organisms' growth time. Comparison of growth curves of two micro-organisms showed that both the trends of biochemical oxygen demand were exhibiting regressive changes with the passage of time during their generation times (t_G). Results from ultraviolet spectrophotometer and precision pH meter all showed that the control growth curves were visioning same trends with the thermodynamic curves of micro-organisms measured by microcalorimeter.

Keywords: *Bacillus subtilis*, *Candida humicola,* microcalorimetric technique, biosensor technology.

Introduction

Cadmium (Cd), a potentially toxic heavy metal with unknown biological function, exists widely in nature in small amounts, with an average content of 0.2 $mg.kg^{-1}$ in the geosphere. The Cadmium (Cd) concentration on rocks from 0.1–11 $mg.kg^{-1}$. Cadmium, in association with chlorides, hydroxyl, sulfhydryls and thiol groups, forms soluble complexes and these complexes largely control the biological activity of Cd. Industrial waste disposals, fertilizer application and sewage sludge disposals on land have also led to the accumulation of Cd in soil and its leaching under certain soil and environmental conditions, with eventual increase in its concentration in food crops. Its accumulation in the environment and its entry into the food chain are of great concern.[1–5] The accuracy of the heavy metal determinations based on well-defined extraction procedures was verified by using TAM(III), certified for *Bacillus subtilis* and *Candida humicola*, which were extracted from Cd(II) contaminated soil.[6–8] Significant quantities of cadmium have been added to the soils globally due to various anthropogenic activities, which raise concerns for environmental health, as a result, draw our attention to the global environmental problems.[1] At present, the toxic effects of heavy metals on soil microbiota are well-recognized, that is because of the differences in soil types and source of metal contamination (e.g., sewage sludge, soluble metal salts) that would have profound effects on Cd chemistry in soil and its impact on soil microbes.[1–6]

Heavy metals are widespread in nature (i.e., soil, water and sediments) because of several polluting anthropogenic activities. They have been recognized as a potential health risk due to their intrinsic chemical stability, high recalcitrance to different types of degradation and high toxicity to

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living organisms.[9] The toxic effects of Cd on plant growth, metabolism and enzyme activity are well documented: Cd inhibits plant growth and also disturbs photosynthesis, sugar metabolism, sulphate assimilation, and several enzyme activities even at low concentrations.[10] Most of the studies aimed at improving our understanding that toxic effects of Cd on a plant metabolism have been performed on hydroponic cultures for their easiness and suitability.[11,12] Levels of heavy metals in soil are increased by the deposition of fly ash from coal combustion plants, disposal of municipal waste, use of fertilizers or other soil additives, and indirectly by industrial activities.[13–16]

Microorganisms play a unique role in the soil ecosystem for their contributions to soil fertility. Contrasting trends, reported about the toxic effects of heavy metals including Cd on soil micro-organisms and their activities, which are attributable to short-term studies used to be limited to a single soil type and conducted under controlled laboratory conditions.[1] Soil serves as a habitat for diverse groups of microorganisms, comprised of algae, bacteria, fungi and other organisms. The major groups and their predominant functions related to soil health and fertility are highlighted here. *Bacillus subtilis* and *Candida humicola* which studied in our experiments are two kinds of essential soil microorganisms. In soil, fungi, although numerically much less abundant than bacteria, can account for twice the weight of bacteria and actinomycetes combined.[1–4] Changes in environmental conditions, whether natural or anthropogenic, can strongly influence the behaviour of both essential and toxic elements by altering the forms in which they exist.[8–9]

One chemical and microbiological evaluation of soils was done by using measures of routine chemical properties, bacterial counts and several enzyme activities. Soils with different pollution histories were preliminary characterized in terms of their chemical properties, enzymatic activity and culturable heterotrophic bacteria.[9]

The aim of this study was to determine the toxic effect of cadmium on pure microorganisms and to estimate simultaneous metabolic effective changes of the *Bacillus subtilis* and *Candida humicola* by constituents of the liquid phase of solution both in the closed and open experimental environment (combination of microcalorimetric method and biosensor technique). Every thermodynamic experiment is based on the gradual addition of heavy metal in two samples in order to push a certain reaction forward, from which to estimate the metabolic rule of *Bacillus subtilis* and *Candida humicola* in the contaminated environment.[17]

Materials and methods

Materials

Bacillus subtilis and *Candida humicola* were provided by the China Center for Type Culture Collection, Huazhong Agricultural University (Wuhan, P. R. China). Analytical-reagent grade $Cd(NO_3)_2{\cdot}4H_2O$ was obtained from Shanghai Tingxin Chemical Factory (Shanghai, P. R. China).

The peptone culture medium where *Bacillus subtilis* grown was prepared by dissolving 5 g peptone, 3 g beef extracted and 5 g NaCl in 1000 mL of deionized water at pH 7.2. It was then sterilized with high-pressure steam at 120°C for 30 min. The potato culture medium where *Candida humicola* grown was prepared by dissolving 200 g potato in 1000 mL of deionized water boiled for 30 min, decanted through cotton-cloth, filtered through paper, volume added to 1000 mL, and then added 10 g glucose to the fluid. It was then sterilized with high-pressure steam at 120°C for 30 min.[18,19]

Instrumentation and methods

Microcalorimetric method

The microcalorimetric study was performed on a TAM III multi-channel thermal activity microcalorimeter. TAM III is modular, comprised of thermostats, calorimeters, sample handling systems and auxiliary equipment. It is designed to monitor a wide variety of processes and complex systems continuously such as the thermal activity of physical, chemical and biological processes in terms of heat, heat flow and heat capacity over a temperature ranging from 15–150°C. Microcalorimetry is used to test the thermodynamics curve and the toxic effect of heavy metal in soil microbes.[20]

In this experiment, Cd (II) compounds were dissolved in double distilled water. The Cd (II) solutions were diluted with the peptone and the potato culture mediums to obtain the final different concentration of Cd^{2+} in the culture medium. The 4-mL steel ampoules containing the *Bacillus subtilis* and *Candida humicola* were cleaned and sterilized. *Bacillus subtilis* were inoculated in 2 mL peptone culture medium and the initial density of *Bacillus subtilis* was 2×10^6 cells/mL. *Candida humicola* were inoculated in 2mL potato culture medium and the initial density of *Candida humicola* was 2×10^6 cells/mL.

Different concentrations of Cd^{2+} were added respectively to the peptone culture media and potato culture medium containing *Bacillus subtilis* and *Candida humicola* in the 4-mL steel ampoules. The ampoules with samples were put into the microcalorimetric system. The temperatures of the calorimeter system and the isothermal box were controlled at 28°C. At the same time, a computer was used to continuously to record the power-time curves of *Bacillus subtilis* and *Candida humicola* growth.[21,22]

The growth power-time curves of *Candida humicola* and *Bacillus subtilis* show that the log phases of growth obey the equation:

$$\ln P_t = \ln P_0 + kt \tag{1}$$

where t is the time, P the power output at time t, P_0 the power at time $t = 0$ and k is the growth rate constant.

Table 1. Effects of Cd^{2+} on *Candida humicola*, *Bacillus subtilis* and mixed micro-organisms.

Inhibitor	$C\ (\mu g/mL)$	$Q_T(J)$	$P_M(\mu W)$	$t_M(min)$	$k\ (\times 10^{-3} min^{-1})$	$t_G(min)$	$I\ (\%)$	$IC_{50}(\mu g/mL)$
Candida humicola	0	6.15	325.19	316.0	7.38	0.094	0.00	428.66
	200	10.06	702.04	365.0	5.77	0.120	21.82	
	400	10.01	421.64	689.5	3.80	0.182	48.51	
	800	11.86	230.92	1209.0	2.33	0.297	68.43	
	1600	—	—	—	—	—	100.00	
Bacillus subtilis	0	13.75	227.28	478.5	10.91	0.064	0.00	310.37
	30	13.56	242.19	967.5	7.33	0.095	32.81	
	120	7.84	253.30	525.5	7.47	0.093	31.53	
	240	13.59	157.85	792.5	14.01	0.049	29.79	
	480	—	—	—	—	—	100.00	
Mixed microorganisms	0	13.89	747.92	372.0	11.09	0.063	0.00	225.54
	200	24.92	908.81	528.5	5.69	0.122	48.69	
	400	15.91	430.85	742.5	4.54	0.153	59.06	
	800	16.54	305.9	1398.0	2.67	0.259	75.92	
	1600	—	—	—	—	—	100.00	

Values in brackets are standard errors of the estimate ($P < 0.05$).

Using the equation, the growth rate constant (k) of the log phase of the first peak obtained from the power-time curves was calculated. The generation times (t_G), which is $(\ln 2)/k$, were also obtained. The growth rate constants (k) and generation times (t_G) of *Candida humicola* and *Bacillus subtilis* growth are shown in Table 1. Comparing the results with a single organism to those of mixed organisms, we see that the inhibitory ratio (I) and the effect of restraint are different, because the concentrations of Cd^{2+} in *Bacillus subtilis* and the *Candida humicola* are different. I can be defined as:

$$I = [(k_0 - k_c)/k_0] \times 100\% \quad (2)$$

where k_0 is the rate constant of the controlled sample and k_c is the rate constant for *Bacillus subtilis* and *Candida humicola* inhibited by an inhibitor with a concentration of c. When the inhibitory ratio I is equal to 50%, the corresponding concentration of the inhibitor is called the half inhibitory concentration (IC_{50}). IC_{50} can be regarded as the inhibiting concentration of causing a 50% decrease of the *Candida humicola* and *Bacillus subtilis* growth rate constant. The values of I and IC_{50} are also depicted in Table 1.

Biosensor method

A biosensor for determination of glucosinolates has been fabricated by employing a bienzyme system composed of myrosinase and glucose oxidase immobilized onto an eggshell membrane and a dissolved oxygen electrode as the transducer.[23] A low-cost, simple biosensing method for screening homocysteine in human plasma has been developed. The biosensor was fabricated from a D-amino acid oxidase immobilized eggshell membrane and an oxygen electrode. The effects of enzyme loading, glutaraldehyde concentration, pH, ionic strength, and temperature on the response of the biosensor have been investigated in detail. The sensing method is based on monitoring the decrease in the dissolved oxygen level of the sample solution. The proposed biosensor shows fast response time (<1 min), good repeatability (n = 6, relative standard deviation = 2.38%), and long-term stability (85% of its initial sensitivity after 2 months of storage).[24]

This experiments use the model PASCO CI-6542 dissolved oxygen sensor to monitor and explore factors that affect the concentrations of dissolved oxygen molecules (O_2) in aqueous solutions. The studies also monitor dissolved oxygenlevels in the field as a part of ecological surveys of aqueous habitats, including BOD (biochemical oxygen demand) studies. This experiment carried through as the same time as the power-time curves measured by TAM III microcalorimeter.

The dissolved oxygen sensor is designed for use in aqueous media at temperatures ranging from 10°C to 40°C, it was calibrated at approximately the same temperature as the test solution. Because this experiment needs a long period of equilibration requirement, the test solution has been flowed constantly by the membrane of experimenter.[25]

The dissolved oxygen sensor has a polarographic probe composed of a platinum (Pt) cathode and a silver (Ag) anode surrounded by a potassium chloride (K^+CL^-) electrolyte solution. The sensor functions by measuring the electric current produced in a chemical reaction in the probe. When the dissolved O_2 probe is placed in an aqueous medium such as deionized water that contains dissolved O_2, the dissolved O_2 molecules diffuse across a thin silicon membrane into the electrolyte that surrounds the electrodes of the probe. As soon as the dissolved O_2 molecules pass through the silicon membrane into the electrolyte solution, they come into close proximity to the platinum cathode. The negative charge (excess electrons) of the cathode induces the reduction of the dissolved O_2 forming hydroxide

ions (OH^-):

$$O_2 + 2H_2O + 4e^- \longrightarrow 4OH^- \text{ (reduction potential} = 0.40 \text{ V)} \quad (3)$$

The negative charge hydroxide ions diffuse into the proximity of the silver-coated anode. There they combine with silver (Ag) atoms from the silver coating of the anode, forming silver oxide and releasing electrons that join the current in the electrode in the following chemical reaction:

$$2Ag + 2OH^- \text{ —> } Ag_2O + H_2O + 2\,e^- \text{ (reduction potential} = 0.343 \text{ V)} \quad (4)$$

The released electrons produce a current that passes from the electrodes and is amplified.

Ultraviolet spectrophotometer method

All fluorescence measurements were done on a Hitachi F-4500 spectrofluorometer (Tokyo, Japan) with computer control and data recording. The excitation and emission slits were set at 5.0 nm, respectively. The emission intensity of the fluorescent material, $[Ru(dpp)_3][(4\text{-}Clph)_4B]_2$, at 610 nm was collected at an excitated wave length of 470 nm. All measurements were performed in air-saturated buffer solutions at 25 ± 1°C. The temperatures of the testing solutions were monitored by a temperature probe (Model 17B, Fluke, Shanghai, China).[26]

Ultraviolet spectrophotometer method is a common method applied in the microbial experiment to evaluate the absorbance and transmissivity of solution, and this experiments has monitored the OD_{600} (the absorbance of liquid under a wavelength of 600 nanometer) growth curves by using the model Spectrumlab 752s ultraviolet spectrophotometer method.

For off-line experiments the cell dry weight was determined by separating the cells from the culture medium (1.0 mL) by centrifugation at 10,000 *g* for 5 minutes. In the case of centrifugation, we carefully discarded the clear culture medium from the centrifuge tube (1.5 mL). The centrifuge tube was washed with double-distilled water and centrifuged it. Those processes were carried out a 3 times. Then the cell paste was dried in an oven at 105°C. The weight of the centrifuge tube with the cell paste was measured periodically until there was no further decrease in the dry weight. It will take 4–6 hours to dry the sample completely. The difference in the weight was calculated and the dry weight was expressed in $mg.mL^{-1}$.

The pH meter method

This experiment also measured the pH value using Model pHS-3C a precise pH meter made by Shanghai Precision and Scientific Instrument Co.

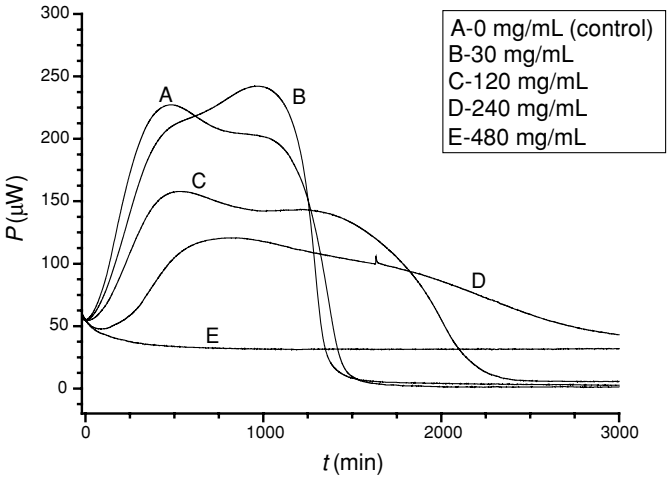

Fig. 1. The thermogenic curves of the growth of Bacillus subtilis for the different concentrations of Cd^{2+} at 28°C–30°C.

Results

Thermogenic and biomass curves of Candida humicola *and* Bacillus subtilis *and their mixture growth at 28°C*

A typical thermal power (=heat production rate)-time curve of germination at 28°C is presented in Figures 1–3, for *Candida humicola*, *Bacillus subtilis* and their mixture gave similar curves and the growth thermogenic curves were recorded. Turbidity curves in the absence of Cd^{2+} and the cell dry weight were also obtained. The curves were shown in Figures 1–3. It is shown in the curves that they can all be divided into four phases, that is, lag phase, log phase, stationary phase and decline phase. During the lag phase and the log phase for *Candida humicola* and *Bacillus subtilis*, Cd^{2+} inhibits their growth for different extents, the inhibitory effect increasing with the concentrations of Cd^{2+}.

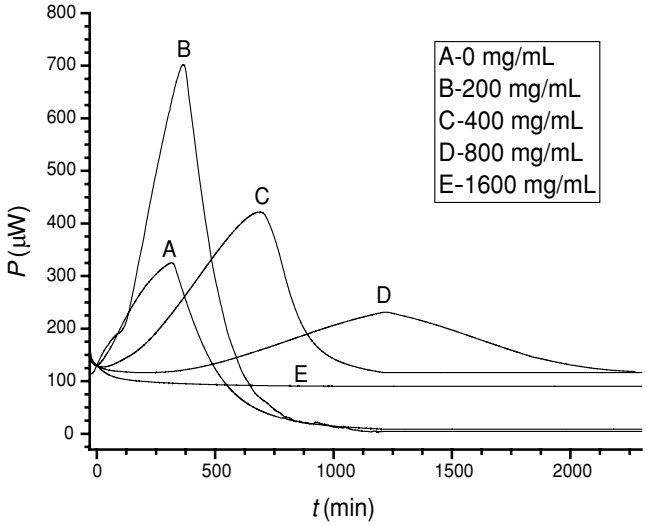

Fig. 2. The thermogenic curves of the growth of Candida humicola for the different concentrations of Cd^{2+}at 28°C–30°C.

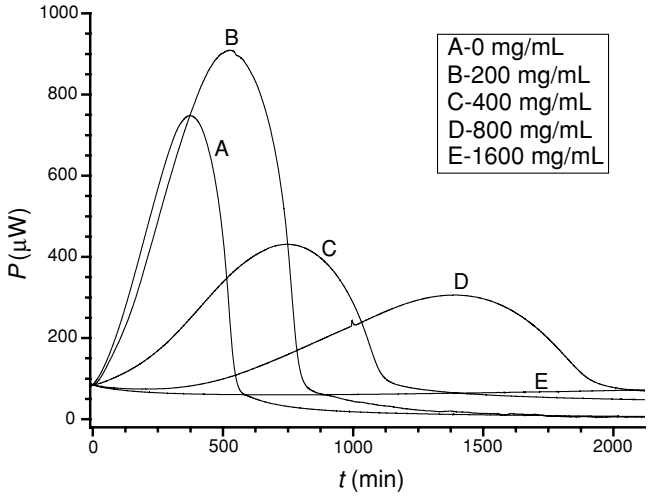

Fig. 3. The thermogenic curves of the growth of mixed microorganisms for the different concentrations of Cd^{2+} at 28°C–30°C.

According to the data and curves, the Cd^{2+} has a positive effect on the *Candida humicola* when the concentration is less than 200 μg/mL, and when the dosage is 60 μg/mL, this positive effect is also reflected on *Bacillus subtilis*. With increasing dosage, experimental results showed that Cd^{2+} inhibited in both microorganisms.

Determination of cell dry weight

Microbial biomass serves as a pool of nutrients and is a sensitive indicator of microbial changes in soil. Generally, microbial biomass in soils, measured by the fumigation extraction method, is not adversely affected by Cd even at extremely high concentrations of 500–1000 mg.kg^{-1}. However, Cd, applied at 500 mg.kg^{-1}, albeit innocuous to microbial biomass as estimated by the fumigation extraction method, affected a decrease in ATP content. Evidently, microbial biomass may not always be a reliable indicator of metal stress. Cd caused a distinct decrease in the ATP

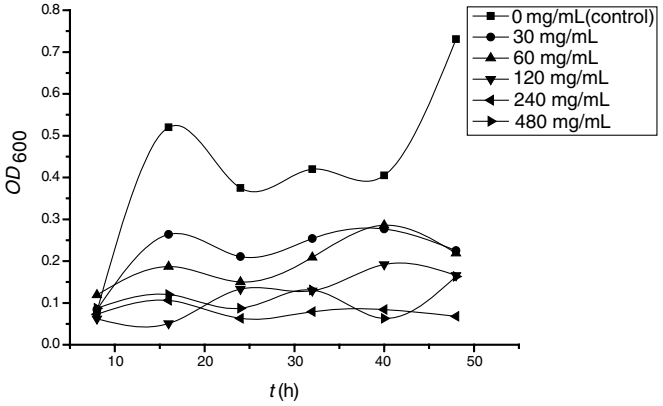

Fig. 4. The OD_{600} growth curves of *Bacillus subtilis* influenced by different concentration of Cd^{2+} at 28°C, measured by spectrophotometer.

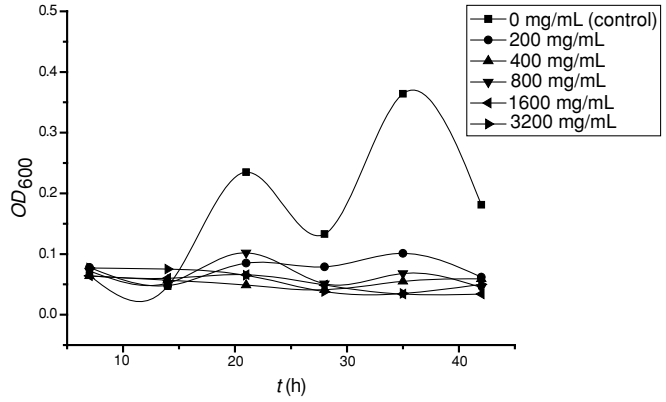

Fig. 5. The OD_{600} growth curves of *Candida humicola* influenced by different concentration of Cd^{2+} at 28°C, measured by spectrophotometer.

content whereas Hattori reported an increase in ATP content following increased Cd addition to soil. In a recent study, Cd, applied both separately and in combination, at 12 mg.kg^{-1}, was the least toxic among the 3 metals Cu, Cd and Zn. But the concentrations of metals used in this study were different (Cd, 12 mg.kg^{-1} soil; Cu, 140 mg.kg^{-1}; Zn, 300 mg.kg^{-1} soil).[1] The biomass curves were measured by the high speed centrifugal machine. The values of cell dry weight is shown in Table 4, from which it was see that the cell dry weight of microorganisms have changed with the microorganisms' growth time.

The metabolic regularity was externalized in the growth curves measured by ultraviolet spectrophotometer method at 28° C

The OD_{600} of microorganisms with different concentration of Cd^{2+} added was measured by ultraviolet spectrophotometer. The results are shown in Figure 4 and Figure 5, the parameters are displayed in Table 2. The value of the result (Table 2) showed that the control growth curves were viewing a gradually increase trend in the *Bacillus subtilis*. The low concentration of Cd^{2+} on *Bacillus subtilis* has a slight promoting influence at 30–120 μg/mL. When the concentration is up to 240–480μg/mL, the growth curves present a dropping trend. The curves' changes of *Candida humicola* were significantly different. The low concentration of Cd^{2+} on *Candida humicola* has a slight promoting influence at 0–800 μg/mL, when the time is at 22 hours, the curves of low concentration get their first peak value. Then at 35 hours, the curves of low concentration get their second peak value. When the high concentration of Cd^{2+} on *Candida humicola* gets to 1600 μg/mL and 3200 μg/mL, the curves are having a regression.

The metabolic regularity was externalized in the growth curves measured by biosensor at 28°

The biochemical oxygen demand (BOD) curves of *Bacillus subtilis* and *Candida humicola* influenced by different

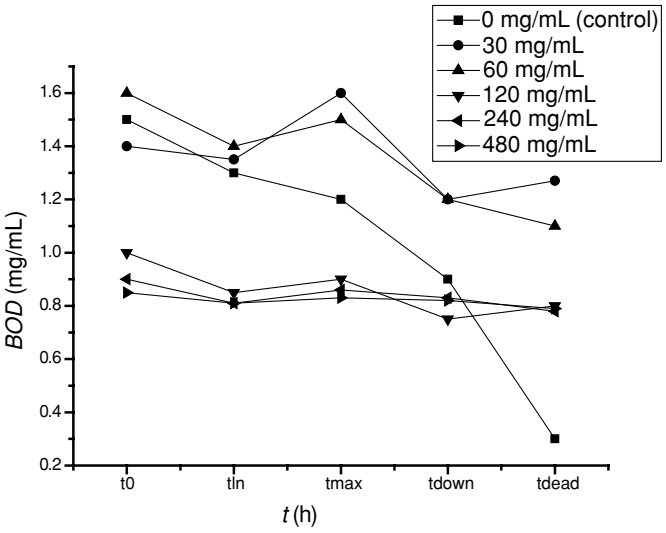

Fig. 6. The biochemical oxygen demand (BOD) curves of *Bacillus subtilis* influenced by different concentration of Cd^{2+} at 28°C, measured by biosensor.

concentration of Cd^{2+} were measured and recorded by biosensor at 28°C, shown in Figure 6 and Figure 7. Comparison of growth curves of two microorganisms' show that both the trends of biochemical oxygen demand exhibited regressive change with the passage of time during their generation times (t_G). The low concentration of Cd^{2+} has a promoting effect on the *Bacillus subtilis* at 30 μg/mL and 60 μg/mL. When the concentration is intensifying, it has an inhibitory effect. The growth curves of *Candida humicola* incarnated a distinct consistency of regressive trend, the biochemical oxygen demand (BOD) is 6.52 on average when their life cycle is in the decline phase.

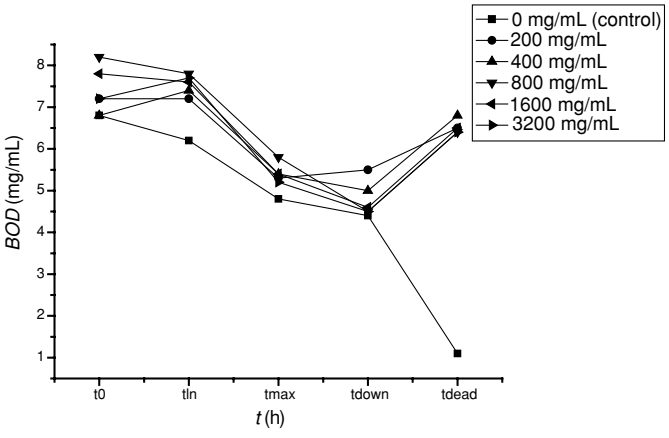

Fig. 7. The biochemical oxygen demand (BOD) curves of *Candida humicola* influenced by different concentration of Cd^{2+} at 28°C, measured by biosensor.

Table 2. The OD_{600} values of *Bacillus subtilis* and *Candida humicola* influenced by different concentrations of Cd^{2+} at 28°C, measured by spectrophotometer.

t	OD_{600} $\mu g/mL$					
	Bacillus subtilis					
	0	30	60	120	240	480
1	0.08	0.084	0.119	0.062	0.073	0.087
2	0.52	0.264	0.187	0.051	0.106	0.12
3	0.375	0.211	0.15	0.133	0.063	0.081
4	0.42	0.254	0.209	0.129	0.079	0.131
5	0.405	0.277	0.286	0.192	0.084	0.063
6	0.731	0.225	0.219	0.166	0.068	0.163
	Candida humicola					
	0	200	400	800	1600	3200
1	0.065	0.078	0.064	0.072	0.064	0.077
2	0.047	0.048	0.057	0.052	0.06	0.075
3	0.235	0.085	0.049	0.102	0.066	0.065
4	0.133	0.079	0.041	0.051	0.049	0.038
5	0.664	0.101	0.055	0.068	0.034	0.035
6	0.181	0.062	0.059	0.045	0.034	0.05

Values in brackets are standard errors of the estimate ($P < 0.05$).

The pH curves of Candida Humicola *influenced by different concentrations of Cd^{2+} at 28° C, measured by precision pH meter*

pH value measurement of this experiment was involved in the estimation. The pH values were measured by precise pH meter using the same values of concentrations as thermogenic curves of two different microorganisms. From the *pH-t* curves (Fig. 8) of *Bacillus subtilis* influenced by different concentration of Cd^{2+}, the low concentration of Cd^{2+} on *Bacillus subtilis* has a slight promoting influence at 30–120 μg/mL, when the concentration gets to the 240 μg/mL and 480 μg/mL, it behaves decreased. The last value of pH with high concentration all gets to the 7.93, putting up

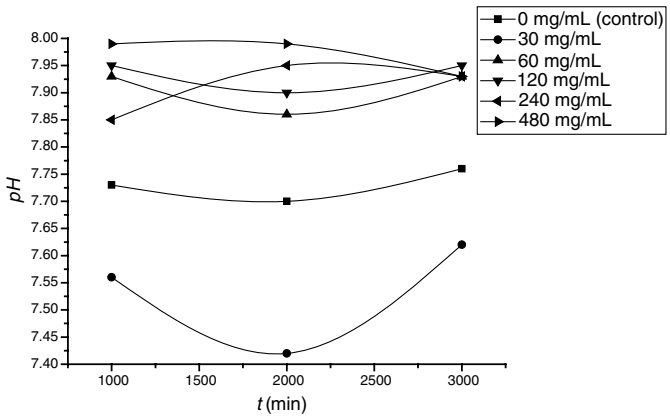

Fig. 8. The pH curves of *Bacillus subtilis* influenced by different concentration of Cd^{2+} at 28°C, measured by precision pH meter.

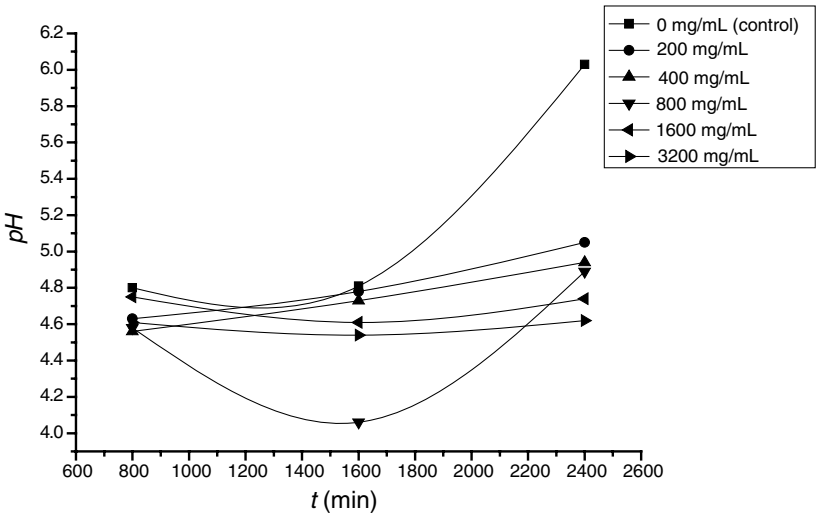

Fig. 9. The pH curves of *Candida humicola* influenced by different concentration of Cd^{2+} at 28°C, measured by precision pH meter.

about neutrality. The tide of the curves (Fig. 9) *Candida humicola* influenced by different concentration of Cd^{2+} behaving approximately the same, they all showed they trend upwards, the values of pH present the decline with the increase of concentration. The average value gets 5.05, it is an appreciably acidic property.

Relationship between the total thermal effect Q_T and the concentration of Cd (II) at 28°C

To show the results in a more quantitative way, the total thermal effects (Q_T) from the power–time curves of *Bacillus subtilis* and*Candida humicola* under different concentrations of Cd (II) have been calculated and are displayed in Table 1. In certain concentration ranges of Cd (II), some approximate linear relationships between the total thermal effects (Q_T) and concentration of Cd (II) are observed. In general, with the increase of the concentration of Cd (II), the total thermal effects (Q_T) of two microorganisms decrease were depicted in Figure 10 and Figure 11. The difference between the two curves is that the first peak value appears at the concentration 70 μg/mL for *Bacillus subtilis*, for *Candida humicola* the first peak value appears at 200 μg/mL.

Table 3. The biochemical oxygen demand (BOD) values of *Bacillus subtilis* and *Candida humicola* influenced by different concentrations of Cd^{2+} at 28°C, measured by biosensor.

t	*BOD* $\mu g/mL$					
	Bacillus subtilis					
	0	30	60	120	240	480
t0	1.5	1.4	1.6	1	0.9	0.85
tln	1.3	1.35	1.4	0.85	0.81	0.81
tmax	1.2	1.6	1.5	0.9	0.86	0.83
tdown	0.9	1.2	1.2	0.75	0.83	0.82
tdead	0.3	1.27	1.1	0.8	0.78	0.79
	Candida humicola					
	0	200	400	800	1600	3200
t0	6.8	7.2	6.8	8.2	7.8	7.2
tln	6.2	7.2	7.4	7.8	7.6	7.7
tmax	4.8	5.3	5.4	5.8	5.4	5.2
tdown	4.4	5.5	5	4.5	4.6	4.5
tdead	1.1	6.5	6.8	6.4	6.5	6.4

Values in brackets are standard errors of the estimate ($P < 0.05$).

Discussion

Thermogenic and biomass curves of Candida humicola *and* Bacillus subtilis *and their mixture growth at 28°C*

The experimental values of the temperatures, biomass from each experiment are given in Table 1 and Table 4. The mean values and standard deviation of the mean are included as the last entry for each microorganism study. The purity of the two compounds was high enough to achieve reliable data for enthalpies of transitions and heat capacities.[27] This enhancement in the heat capacity curve was reproducible and all the experiments done with different samples showed the same behavior.

The relationship among the parameters of microorganisms, which are from the growth curves measured by microcalorimeter at 28°C

The raw data of the calorimetric experiment are displayed in Figures 1–3.[17] Heat capacity changes and temperature dependence of the thermodynamic parameters, the calculation of thermodynamic functions imply the usual approximation of setting standard enthalpies equal to those

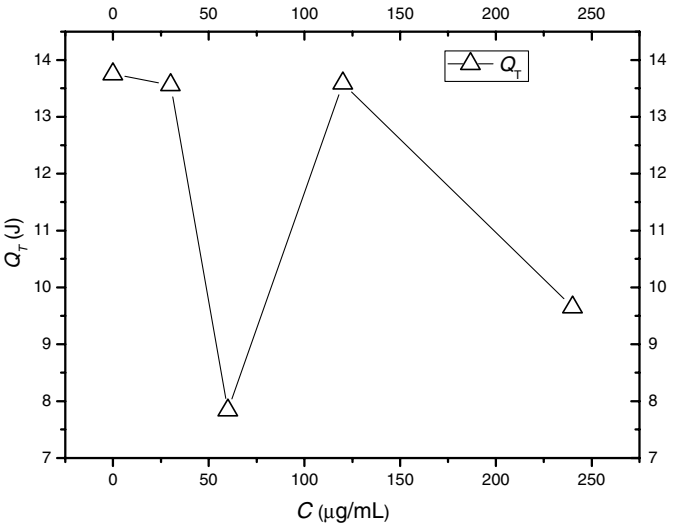

Fig. 10. The curve of relationship between total thermal effect (Q_T) and concentration (C) of *Bacillus subtilis* at 28°C.

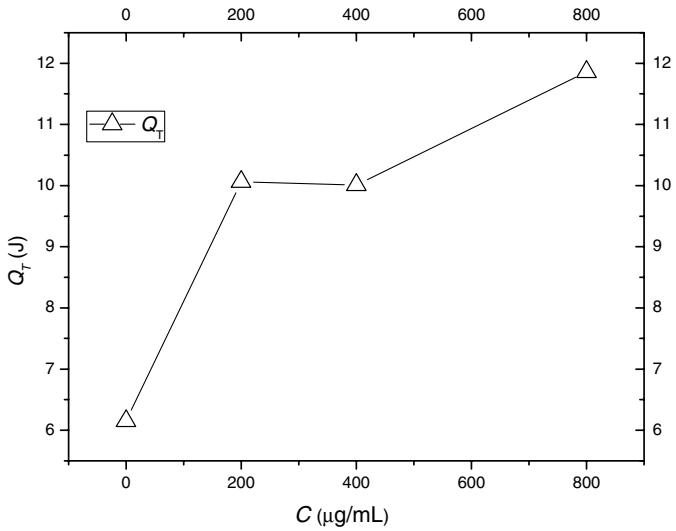

Fig. 11. The curve of relationship between total thermal effect (Q_T) and concentration (C) of *Candida humicola* at 28°C.

observed. On the other hand, the sign of the entropic change on binding provides some clues to the kinds of physical processes involved. The values obtained are different for the three inhibitors studied.[28] The emblematical curves described in the relationship between the Total Thermal Effect Q_T and the Concentration of Cd (II). From the values it has been shown that curves decreased along with the increase of concentration decreas ing. The total thermal effects (Q_T) from the power–time curves of *Bacillus subtilis* and *Candida humicola* under different concentrations of Cd (II) were detached in 4 phases. Because the life cycle of microorganisms was also detached into 4 phases, which can be seen on the control curves sought out individually. When the concentration is down to the low concentration at the same time with the controlled, we can find that the peak value is higher than the control, so it can be explained that the heavy metal Cd (II) has a small stimulative effect on the microorganisms.

The analysis of metabolic regularity was externalized in the growth curves measured by biosensor at 28° C

The comparison of growth curves measured by biosensor and the thermodynamics curves measured by the microcalorimetric method offered two kinds of high correlation models of life process and metabolic process of two microorganisms. The aim of biosensor is to test the biochemical oxygen demand (BOD) based on the open environment. Despite the experimental environment is closing or opening, the growth cycles can be remarkably described in the concentration chosen in the experiments. Before the initiation of experiment for biosensor, the confirmation of time as well as thermodynamics curves is very important. Choose the certainty equivalents of time value at the certain moment, respectively, the same as curves measured by the microcalorimetric method: t_0 (means time=0), $t_{\ln}$ (the time of logarithmic growth phase), $t_{\max}$ (values of peak time), t_{down} (the time of death phase), t_{dead} (the time of totally inhibition).

Where the $t_{\ln}$and t_{down} is the value we seek out at the certain time from the exponential phase and death phase. The BOD curves of *Bacillus subtilis* influenced by different concentration of Cd (II) showed that the growth metabolism of *Bacillus subtilis* were inhibited with the different concentration of Cd (II), it will explain that this kind of microorganism is susceptible for the heavy metal, especially Cd (II), which is reflected in aspects of concentration, the total inhibition is just 480 μg/mL. The pH demand is also an important factor that affects the growth of *Bacillus subtilis*, the mean value of pH is neutrality. It is a little different in the case of *Candida humicola*.

When the time is in the death phase, the BOD curves change in a slightly increased trend, it is because the pH values were decreasing with the extension of time, which means the number of hydrogen ion in the liquor is increasing, and the biochemical oxygen demand was increased correspondingly. The temperature is another factor affecting the result of BOD values. The empirical value of micro-organisms is 28°C, so temperature changes will affect the BOD value.

The metabolic regularity was externalized in the growth curves measured by ultraviolet spectrophotometer method at 28° C

A simple, rapid ultraviolet spectrophotometric method for the determination of optical density and transmittivity has been used in this experiment. This permits batch analysis and the routine handling of large numbers of samples. Varieties in substrate concentration, buffer composition,

Table 4. The Biomass values of *Bacillus subtilis* and *Candida* influenced by different concentrations of Cd^{2+} at 28°C.

Incubation time (h)	*Biomass μg/mL*					
			Bacillus Subtilis			
	0	30	60	120	240	480
0	0.005	0.005	0.005	0.005	0.005	0.005
5	0.04 ± 0.01	0.04 ± 0.02	0.06 ± 0.01	0.05 ± 0.01	0.08 ± 0.01	0.05 ± 0.01
10	0.22 ± 0.04	0.16 ± 0.05	0.13 ± 0.03	0.13 ± 0.03	0.13 ± 0.03	0.13±0.03
15	0.34 ± 0.08	0.24 ± 0.08	0.24 ± 0.09	0.24 ± 0.08	0.24 ± 0.08	0.24 ± 0.08
20	0.30 ± 0.02	0.35 ± 0.02	0.30 ± 0.02	0.42 ± 0.02	0.30 ± 0.02	0.35 ± 0.02
25	0.35 ± 0.05	0.36 ± 0.04	0.40 ± 0.04	0.38 ± 0.04	0.36 ± 0.04	0.36 ± 0.04
30	0.42 ± 0.06	0.42 ± 0.07	0.42 ± 0.06	0.42 ± 0.06	0.42 ± 0.06	0.42 ± 0.06
35	0.40 ± 0.05	0.41 ± 0.04	0.39 ± 0.04	0.41 ± 0.05	0.39 ± 0.04	0.38 ± 0.04
40	0.40 ± 0.09	0.46 ± 0.09	0.41 ± 0.05	0.46 ± 0.08	0.41 ± 0.09	0.41 ± 0.09
45	0.38 ± 0.06	0.39 ± 0.05	0.42 ± 0.04	0.39 ± 0.04	0.39 ± 0.04	0.40 ± 0.04
50	0.49 ± 0.08	0.35 ± 0.08	0.39 ± 0.05	0.42 ± 0.08	0.39 ± 0.08	0.43 ± 0.08
			Candida humicola			
	0	200	400	800	1600	3200
0	0.005	0.005	0.005	0.005	0.005	0.005
90	0.06 ± 0.03	0.05 ± 0.01	0.06 ± 0.02	0.05 ± 0.05	0.06 ± 0.03	0.07 ± 0.01
180	0.22 ± 0.01	0.15 ± 0.03	0.12 ± 0.01	0.16 ± 0.01	0.33 ± 0.01	0.14 ± 0.01
270	0.20 ± 0.06	0.23 ± 0.04	0.20 ± 0.06	0.20 ± 0.03	0.28 ± 0.03	0.20 ± 0.05
360	0.33 ± 0.05	0.31 ± 0.05	0.31 ± 0.05	0.31 ± 0.06	0.36 ± 0.05	0.32 ± 0.05
450	0.40 ± 0.02	0.43 ± 0.02	0.40 ± 0.02	0.45 ± 0.02	0.43 ± 0.05	0.43 ± 0.05
540	0.42 ± 0.04	0.46 ± 0.04	0.43 ± 0.04	0.43 ± 0.04	0.43 ± 0.07	0.43 ± 0.09
630	0.39 ± 0.03	0.42 ± 0.01	0.39 ± 0.03	0.42 ± 0.04	0.40 ± 0.03	0.37 ± 0.03
720	0.42 ± 0.07	0.40 ± 0.06	0.38 ± 0.06	0.42 ± 0.05	0.42 ± 0.06	0.38 ± 0.08
810	0.39 ± 0.05	0.40 ± 0.03	0.40 ± 0.03	0.43 ± 0.03	0.42 ± 0.03	0.39 ± 0.01
900	0.41 ± 0.06	0.36 ± 0.05	0.39 ± 0.04	0.41 ± 0.04	0.39 ± 0.06	0.42 ± 0.05
990	0.42 ± 0.06	0.38 ± 0.04	0.39 ± 0.05	0.40 ± 0.04	0.38 ± 0.04	0.40 ± 0.04

Values in brackets are standard errors of the estimate ($P < 0.05$).

incubation time, and microorganisms' concentration have been investigated systematically. The effects of varying proportions of micro-organisms and inhibitors in the solution have been studied. A method is recommended for calibration of the performance parameters of the spectrophotometers, thus permitting the use of unsophisticated spectrophotometers.[29]

This experiment tested the OD_{600} of the *Bacillus subtilis* and *Candida humicola* then gave the data and the OD_{600}-time curves in Figure 4 and Figure 5. OD_{600} is the absorbance of liquid under a wavelength of 600 nm.

In order to evaluate the suitable wavelength to monitor the concentrations of micro-organisms in water, solutions of microorganisms with various concentrations of Cd (II) were spiked and their UV-Vis spectra were measured. Parts of the spectra are given in Figure 4 and Figure 5. From the figures, the absorbance of the microorganisms increases as the concentrations increase can be seen.[30]

The curves' changes of *Candida humicola* were significantly different. The low concentration of Cd^{2+}on *Candida humicola* has a slight promoting influence at 0–800 μg/mL, when the time is at 22 hours, the curves of low concentration get their first peak value. Then it is at about 35 hours when the curves of low concentration get their second peak value.

The pH curves of microorganisms influenced by different concentrations of Cd^{2+} at 28°C, measured by precision pH meter

Since the increase growth micro-organism is caused by a more favourable contribution, the reason why Candida humicola is higher for the acidic derivative can be explained from the phenomena hydrogen ion in a more humid. The cause of this phenomena lies in the following: one is of *Bacillus subtilis* is more susceptible to the Cd (II) than *Candida humicola*, when the heavy metal and microorganisms are mixed together, it will trigger a complex biochemical reaction. The Cd (II) put in the *Bacillus subtilis* will consume during the biochemical reaction so that it will not surplus, which made the solution exhibit neutrality; but in the mixed solution of Cd (II) and *Candida humicola*, it was not the same case. There will be some residual Cd (II) in the solution because of the resisitance to the heavy metal, so

this part of Cd (II) will hydrolyzatic dissociation in the solution, it makes the mixed solution of Cd (II) and Candida humicola exhibit weak acidity.

Conclusion

A thermophysical study of the behaviour as a function of the temperature was carried out for *Bacillus subtilis* and *Candida humicola* and a comparative analysis with analogous biosensor, ultraviolet spectrophotometer and pH value was made. An enhancive change occurs with the increasing of concentration of Cd (II). The changes of the measuring apparatus all showed the same life regularity of microorganisms regardless of the environment in the open or closed environment. The regularity depicted in the low concentration will have an accelerated effect on the two micro-organisms, the high concentration will show the inhibitory effect of two micro-organisms. The different impact is that the two organisms have different growth environmental demands. For instance, the *Bacillus subtilis* mainly grows in the mediosilicic environment, and the *Candida humicola* mainly grows in the acidic environment, which are depicted in Figures 8 and 9 calculated by the pH meter. The data analysed from the experiments will give referenced value to the study of environmental toxicology and interrelated knowledge.

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