

Full Length Research Paper

Dimethoate degradation and calcium phosphate formation induced by *Aspergillus niger*

Xingjia Yin¹ and Bin Lian^{2*}

¹Key Laboratory of Karst Environment and Geological Hazard Prevention, Ministry of Education, Guizhou University, Guiyang 550003, China.

²State Key Laboratory of Environmental Geochemistry, Institute of Geochemistry, Chinese Academy of Sciences, Guiyang 550002, China.

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A novel method of dimethoate degradation using the fungus *Aspergillus niger* was proposed. In addition, calcium phosphate was obtained as a byproduct from the organophosphate pesticide by inductive synthesis. The method involves culturing *A. niger* in a modified Czapek liquid medium with glass powder, CaCl₂, and dimethoate. Gas chromatography was subsequently used to analyze the degradation effect of *A. niger* on the dimethoate and X-ray diffraction was used to identify the components of the mineral particles wrapped in mycelial pellets. The results indicate that *A. niger* can effectively degrade the pesticide in the modified Czapek medium and, under the conditions used, induce the formation of calcium phosphate from the phosphorus released in the degradation products and the calcium ions available in the liquid medium. The research highlights the microbial degradation of organophosphate pesticides and the inductive synthesis of bio-mineralization. Thus, a new technology for environmental bio-remediation was proposed.

Key words: *Aspergillus niger*, organophosphate pesticides, dimethoate, degradation, calcium phosphate, bio-mineralization.

INTRODUCTION

Organophosphate pesticides are a widely used and effective group of insecticides developed shortly after organochlorine pesticides. There are more than 150 different types and they play an important role in agricultural production (Dai, 2008). However, their use will contaminate soil, underground water, and surface water following prolonged or excessive use. Moreover, cholinesterase is inhibited *in vivo* in living creatures upon absorbing organophosphate pesticides, and can cause serious nervous disorders (Ragnarsdottir, 2000). Therefore, it is essential that an effective way of eliminating organophosphate contamination is found. Detoxification and degradation of residual pesticide using

microbes is becoming an effective method of controlling and treating environmental contamination especially in the case of pesticides (Aislabie and Lloyd-Jones, 1995). Eliminating pesticide contamination using microbes has several advantages, such as the large variety of microbes available, high mutation speeds, and convenience in culturing.

Thus, the method shows good potential in the field of bio-remediation technology development (Wu and Zheng, 2007). For instance, Ai et al. (2006) isolated a fungal strain which was capable of degrading dimethoate through co-metabolism more effectively compared to other methods.

The strain was identified as a kind of *Aspergillus* (*Aspergillus* sp.). Liu and Zhong (1999) isolated a strain of *A. orantus* from soil long contaminated by methamidophos organophosphate pesticide. Their research showed that the fungi possess a relatively high

*Corresponding author. E-mail: bin2368@vip.163.com. Tel: +86-851-5895148.

activity in degrading methamidophos. Xu et al. (2004) isolated a strain of aerobic fungi from the sludge from a pesticide factory, identified as *A. niger*, which could be cultured by dimethoate as the only source of phosphorus and was effective in degrading dimethoate activity.

Due to its massive population, steady genetic status, and the strong survivability of spore-bearing fungi in the natural environment, fungi screened with a broad spectrum in organophosphate degradation is an effective way of dealing with large areas of land contaminated with low concentrations of organophosphates (Liu et al., 2004).

He et al. (2011) discovered that in a liquid potato dextrose agar (PDA) medium with Na_2HPO_4 and CaCO_3 , *A. niger* is able to degrade CaCO_3 and induce the formation of hydroxyapatite.

This indicated that the microbe can promote the combination of phosphate radicals and calcium ions to form a kind of hard-dissolved phosphate mineral. Hence, it can be deduced that organophosphate pesticides can, theoretically, be degraded by microbes to generate phosphate radicals which can then combine with calcium ions. Formation of such phosphate minerals is promoted by biological induction. Also, the absorption properties of the formed minerals, especially towards heavy metal ions in the fluid (Takeuchi and Arai, 1990; Li et al., 1993, 2001; Mavropoulos et al., 2002; Zheng et al., 2006), can be utilized to achieve further elimination of contamination in the water. In order to achieve such an objective, it is critical to determine whether the microbes can use the phosphorus generated from the organophosphate to form other phosphate compounds.

Up till now, most researchers have focused on the use of microbes to induce the formation of minerals (Che et al., 2000; Lei et al., 2000; Wei et al., 2008; Xia 1993; Yin et al., 1994; Dongye et al., 2003). However, the work has been mostly directed towards the research and practical stages of mineral prospecting and mineral processing (Hao et al., 2000; Jiang and Wang, 2005; Sadowski et al., 2008). Consequently, research on microbe-induced formation of minerals geared towards environmental bio-remediation remains the weak aspect of the technology.

In this research, a widely used phosphorus-bearing pesticide, dimethoate, was used as an experimental material to test fungal degradation of an organophosphate pesticide. More specifically, we chose to study the effectiveness of *A. niger* towards bio-degradation of the dimethoate. Furthermore, we consider if the phosphorus generated from the degraded pesticide, and added calcium ions, are combined to form phosphate minerals.

Some novel ideas are generated by the process mentioned above with regards eliminating environmental contamination caused by organophosphate pesticides and constructing new environmental bio-remediation technologies.

MATERIALS AND METHODS

Fungi strain and culture

The *A. niger* was provided by the Centre of Environmental Bio-science and Technology Research, Institute of Geochemistry, Chinese Academy of Sciences and Czapek medium was selected as the growth medium. Inoculating activation was first performed on the slope of the solid medium (cultured for 2 days at 28°C). Then, two rings of activated, tube cultured material on the slope were injected into 250 ml of Czapek liquid medium. The medium was cultivated for 2 days at 28°C with shaking at 120 r/min. Mycelial pellets of uniform size were formed (Figure 1). Each mycelial pellet, which was about 5mm in diameter, were then used as inoculums for further experimentation.

Dimethoate degradation and formation of calcium phosphate

The fungal degradation experiment was carried out in modified Czapek medium (3 g NaNO_3 , 0.5 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1.0 g KCl, 0.01 g Fe_2SO_4 , 30 g sucrose, and 1 L distilled water) with glass powder, CaCl_2 , and dimethoate. In the medium, the calcium resource was provided by CaCl_2 and the phosphorus resource was provided by the dimethoate (40% EC, manufactured by Chongqing Chemical Pesticides Group Co. Ltd).

Modified Czapek liquid (100 ml) was placed into a 250 ml conical flask and CaCl_2 (0.6 g) and glass powder (1 g) was added. After sterilization, 10 mycelial pellets (Figure 1) and dimethoate solution was also added to the experimental group. In each group, 3 parallels sets were performed. The cultures were shaken at 120 r/min for 12 days at 28°C. Measured aliquots (2 ml) of the supernatant were extracted from the culture liquid on the initial inoculation, 2nd, 4th, 6th, 8th, 10th, and 12th days. The pH values of the samples were measured using an accurate pH meter (PHS-3, manufactured by Shanghai Shengci Instrument, Inc.). The overall phosphorus concentration in the solution was measured using an ammonium molybdate spectrophotometric method (spectrophotometer 721, manufactured by Shanghai Longyu Instrument, Inc.). Finally, the biomass of the mycelial pellets was determined using an electronic balance (MP2002, Shanghai Hengping Science Instrument, Inc.) by following a previously described method of weighing (Yuan, 2009).

Sample processing and analyses

Gas chromatography analysis

Chloroform (AR) was used to extract organic components from the supernatant on the 0th, 4th, and 12th days. The extract was dried over anhydrous sodium sulfate (AR) and analyzed using gas chromatography (Agilent 6890 GC, Agilent Technologies Ltd., USA). A chromatographic column Hp-5 19091J-413 (30 m × 0.132 mm × 0.25 μm) was employed. The column temperature was 140°C for 2 min, followed by raising to 260°C at a speed of 10°C/min for 2 min. The injection port temperature was 330°C (splitless injection) and the NDP detector temperature was 350°C.

A fixed amount (100 ml) of sample was put into a 250 ml flask and its pH value adjusted to 6.5. The sample was transferred to a 250 ml separating funnel and extracted 3 times with chloroform (5 ml). For each extraction, the liquids were shaken for 5 min and then allowed to stand until separated. Finally, the chloroform layers were combined. The chloroform solution was then dried using anhydrous sodium sulfate. A 1 μL sample was then introduced into the gas chromatograph (GC).



Figure 1. Mycelial pellets cultured and formed by *A. niger*.

The national standard liquid (developed by Agro-Environmental Protection Institute, Ministry of Agriculture) tested for dimethoate is acetone with concentration of 100 µg/ml.

X-ray diffraction (XRD) analysis

Mycelial pellets that were cultured on the 12th day were taken and washed using deionized water. Then, their insides were washed after they were opened with forceps. The white powder in the wash water was collected and dried overnight at 60°C. A little of the dried sample was ground in an agate mortar for passing through a 200 mesh sieve. The mineral composition was then analyzed using an X-ray diffractometer (D/Max-2200, Rigaku Corporation Japan). The control samples are obtained from the residuals in the bottom of the same medium flask (note: glass powder presence mainly) with no inoculation and analysis by XRD.

RESULTS AND DISCUSSION

Testing of the Biomass

Mycelial pellets were collected on the 0th, 2nd, 4th, 6th, 8th, 10th, and 12th days. They were opened with forceps and the white powder inside was washed out using distilled water. Thalli were collected (thalli and white powders were collected respectively for 12th day' sample, the powder is only for XRD analysis, see the section of XRD analysis) and dried at 50°C for 12 h, for weighing. The change in biomass of the thalli with culture time is shown in Figure 2. Figure 2 shows that the *A. niger* biomass gradually increases with time. However, the growth slowed down on the 10th day and became stable,

that is, until the 12th day there was no significant change in biomass. Furthermore, the mycelial pellets were found to have a villous state at that stage. For convenient comparison, the sampling time adopted for dimethoate degradation and induction of mineral formation was chosen to be the 12th day of *A. niger* growth.

Gas chromatography analysis

The GC analysis results showing the dimethoate content are shown in Figure 3 (the retention time is on the abscissa and peak height is on the ordinate).

By comparing the peak areas with those from standard dimethoate solution, the concentrations of dimethoate in the culture liquid at the 3 different times were calculated to be: $a = 326.6$ mg/l, $b = 282.8$ mg/l, and $c = 217.8$ mg/l. The results clearly show that the concentration of dimethoate decreases as the fungi interaction time progresses, thus indicating that the fungi can degrade dimethoate and use the phosphorus within it for the growth.

Total phosphorus concentration

Figure 4 shows the change in the overall phosphorus concentration as a function of time during *A. niger* growth.

The figure shows that the overall phosphorus concentration increased on the second day. This is because the initial phosphorus concentration test (day 0)

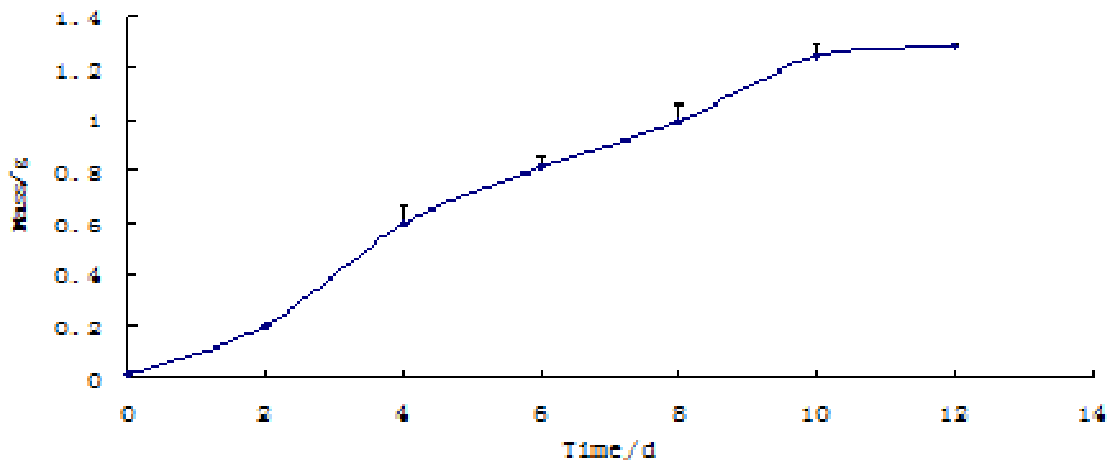


Figure 2. Biomass of *A. niger*.

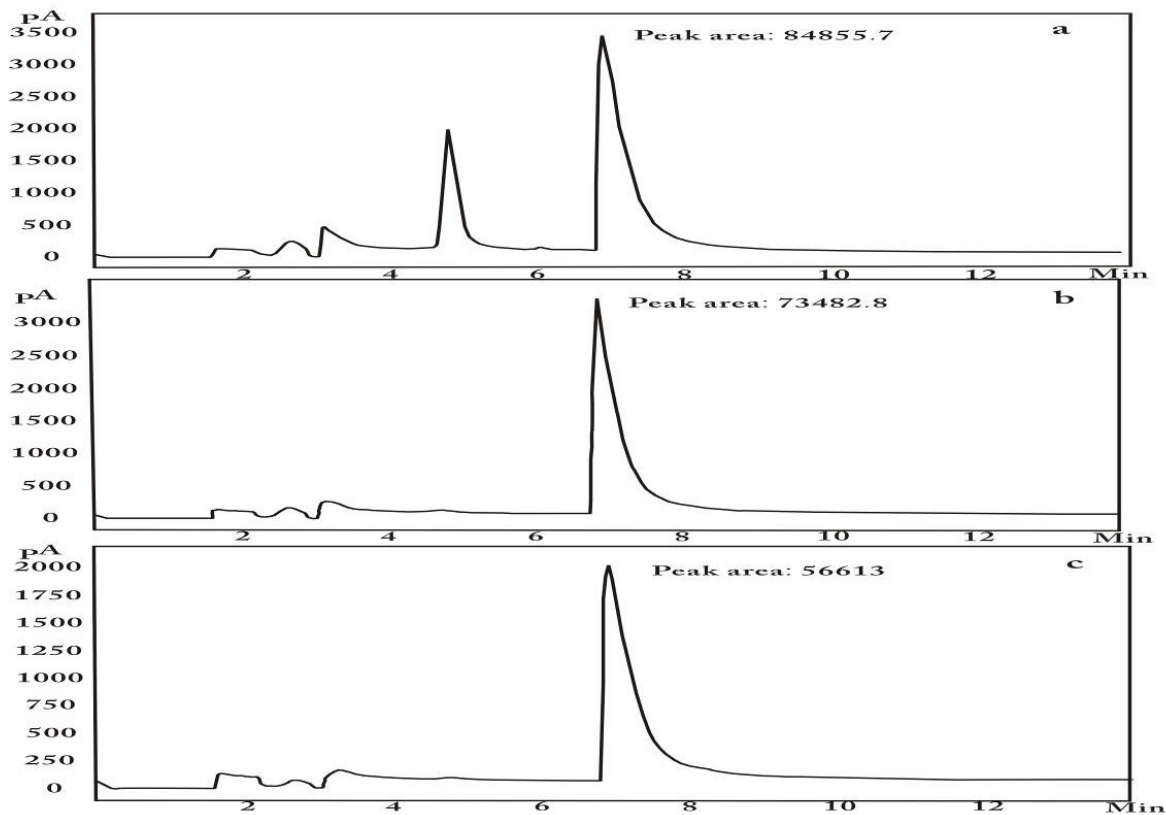


Figure 3. Gas chromatograms obtained from the supernatant liquid on the (a) 0th, (b) 4th, and (c) 12th day.

was carried out before adding mycelial pellets, which were cultured in Czapek medium bearing phosphorus, and because the mycelial pellets themselves bear some phosphorus. On the 4th day, the phosphorus concentration decreased significantly. This indicates that at this stage the degradation effect of the microbes had

occurred and that part of the degraded phosphorus had been absorbed by the fungi. The volatility of the phosphorus concentration after the 4th day is due to changes in fungi growth, the variation in ability to absorb phosphorus, and, possibly, the effect of mineral formation. After the 12th day, the surface of the mycelial

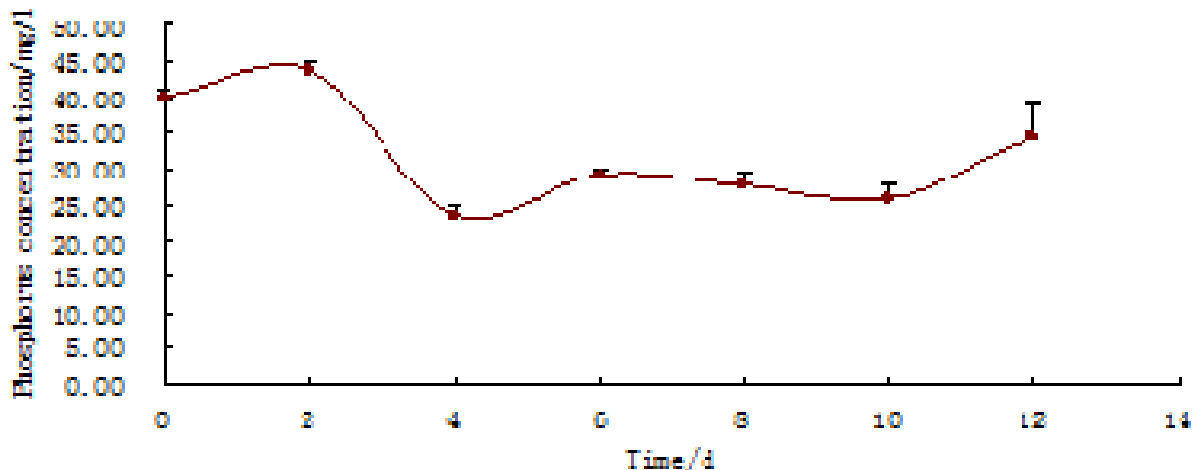


Figure 4. Graph showing the overall phosphorus concentration.

pellets became villous and they started to autolyze. Autolysis of the fungi releases some phosphorus causing an increase in phosphorus concentration. As the overall phosphorus concentration decreased it can be deduced that some of the phosphorus was transformed into calcium phosphate and was wrapped inside the mycelial pellets. To prove this assertion, XRD analysis was carried out.

XRD analysis

Mycelial pellet samples on the 12th day were collected and the white powder wrapped in the pellets analyzed using XRD. The results were then compared with the precipitation in the bottom of the medium with no inoculation. The results are shown in Figure 5.

Figure 5 shows that as the white powder collected contains more glass powder, more amorphous components and multiple peaks are found. It is apparent from Figure 5a, however, that the white powder contains CaCO_3 and calcium phosphate because of the peaks due to CaCO_3 (3.0355) and calcium phosphate (2.7810). On the other hand, while CaCO_3 peaks are found in the control sample b, but no calcium phosphate is seen. The results prove that calcium phosphate is generated by the fungi, and additionally it may also generate CaCO_3 . The CaCO_3 that resulted may be formed by biological induction or by some other means. For example, it may be due to the reaction of calcium ions with CO_2 in the air (this is the most likely cause).

Changes of the pH value in the culture medium

Figure 6 shows the changes occurring in the pH values of

the experiment and control samples. Chemical synthesis of apatite and calcium phosphate is favored by alkaline conditions (Wang et al., 2009). However, the pH of the culture medium of the experimental sample decreases remarkably as degradation proceeds, which is unfavorable to the formation of apatite. Despite this, *A. niger* can produce mycelial pellets with mineral powder inside during the shaken culturing process. As the micro environment in the mycelial pellets is relatively sealed this may provide a sufficiently buffered environment to adjust the pH value to more favorable values. Thus, the conditions inside the mycelial pellets are completely different from those outside and are favorable to the formation of calcium phosphate (Lian et al., 2008; Hu et al., 2011; Rosling et al., 2007).

The XRD analysis results prove that the mineral powder in the pellets contains calcium phosphate. However, the generated calcium phosphate content was relatively small. Typical calcium phosphate crystals were difficult to find using an electron microscope. Therefore, future research should focus on increasing the amount of calcium phosphate formed.

CONCLUSIONS

Our main conclusions are:

The organophosphate pesticide dimethoate can be degraded by adding *A. niger* in modified Czapek liquid medium in the presence of glass powder and CaCl_2 .

During the degradation of dimethoate by *A. niger*, phosphorus released can be used to induce the formation of calcium phosphate.

Using *A. niger* to degrade organophosphate pesticides and induce bio-mineralization is a new way to effectively eliminate organophosphate pesticide contamination. It is

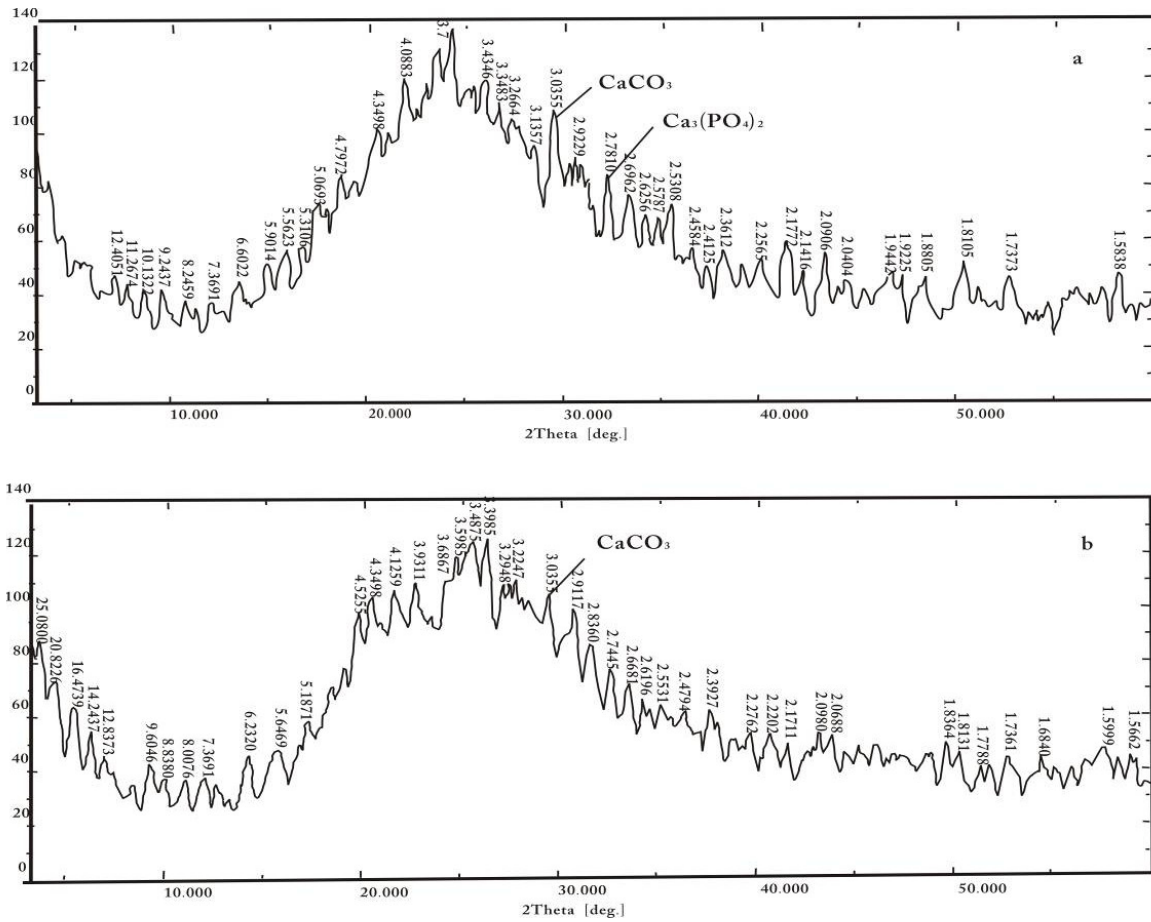


Figure 5. XRD maps showing (a) the mineral composition of white powder inside the mycelial pellets on the 12th day, and (b) the mineral composition of the precipitation in the control medium with no inoculation.

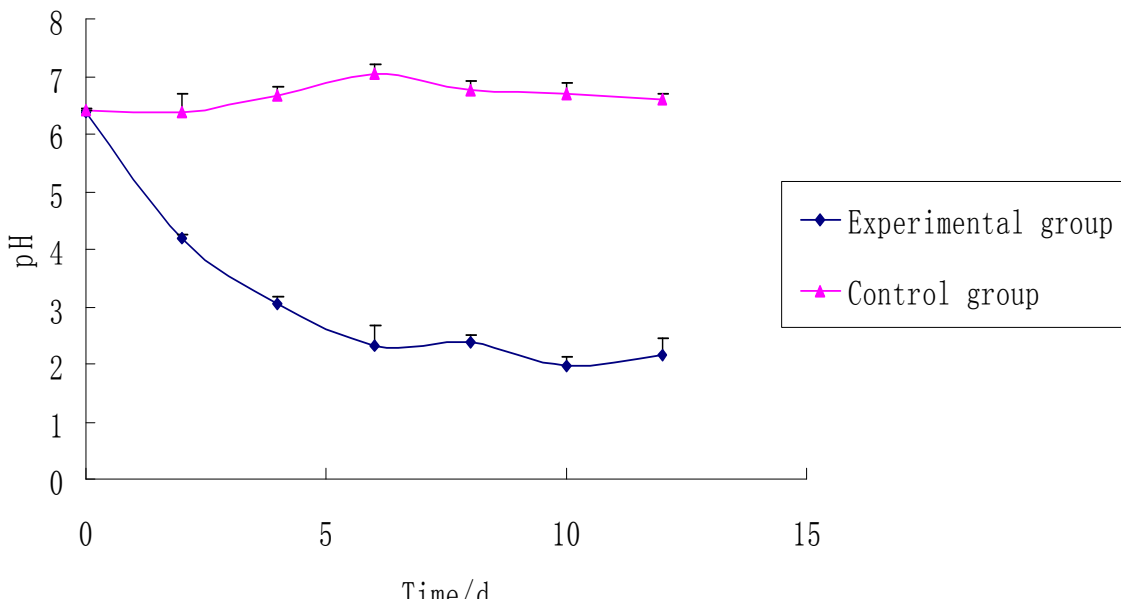


Figure 6. Curves showing the change in pH of the control and experimental samples.

certainly a technique worthy of further discussion and research.

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