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# Study the Optimal Condition of Fenpropathrin Degradation by Ochrobactrum Anthropi Based on Bacteria Microscopic Image Detection Method

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**Abstract:** In order to study fenpropathrin degrading more accurate than turbidimetry. We proposed Live Bacteria Detection method (LBD) based on high precision microscopic image processing and Support Vector Machine (SVM) identification to analyze the optimal condition of fenpropathrin degradation by Ochrobactrum anthropic. The optimal fenpropathrin degradation condition measured by LBD is pH 7.0 and 34°C. On the other hand, the optimal condition measured by turbidimetry is pH 8.0 and 35°C. The correlation coefficient of fenpropathrin concentration and Ochrobactrum anthropic concentration measured by both methods in this study indicate that Ochrobactrum anthropic concentration measured by LBD shows better decreasing linear relationship with fenpropathrin degradation concentration than turbidimetry.

**Keywords:** Fenpropathrin-degrading bacterium, microscopic image processing, ochrobactrum anthropi, optimal degradation condition, support vector machine identification

## INTRODUCTION

Fenpropathrin(a-cyano-3-phenoxybenzyl-2,2,3,3-tetrame-thylcyclopropanecarboxylate), as a typical pyrethroid insecticide developed in 1970s, is used to control a range of insects, especially mites, in fruits and vegetables in China, for its high toxicities to insects and low toxicities to mammals (Ross *et al*., 2006). Pyrethroid pesticides are extremely toxic to the aquatic environment and a concentration of them as little as 10.0 ng/L is enough to eradicate all of the invertebrate life in entire rivers and lakes (Schleier and Peterson, 2010). Furthermore, some of the pyrethroid pesticides have been classified as potential human carcinogens by the US Environmental Protection Agency (Holmes *et al*., 2008).

Several studies indicated that microbial degradation is a practical solution for detoxifying pyrethroid pesticides. Bacteria with the ability to degrade methyl parathion have been isolated worldwide (Cui *et al*., 2001; Fu *et al*., 2004; Hong *et al*., 2005).

The traditional method in bacterial concentration detection is the plate colony count method, but it usually takes 48 hours to detect the concentration of microbial. Several approaches were proposed to short the detecting time, such as Radiometry method (Durwood *et al*., 1978), impedance detection method (Allsoppt *et al*., 1999; Milner *et al*., 1998), Simplate TM full level detector count (TPC) (Vulindlu *et al*., 2004), microcolony technique (Asano *et al*., 2009) and petrifilm (Vail *et al*., 2003; McMahon *et al*., 2003). Although the accuracy, sensitivity and detection speed are improved greatly, these approaches still have the following problems. In the Radiometry method, $C_{14}$ is required to substrate to medium, which increases the difficulties in operating. The impedance detection method shortens the detection time, the equipment based on this method is expensive. TPC method still needs 24 h to detect and it costs high. Microcolony is rapid, economical and practical. But, as a shortage of adopting the count calculation of large area sample and fiber factor, its minimum detection is limited less than $10^3$cfu/mL. There is a phenomenon of colony proliferation and fusion when the bacteria produce too much gas and mucilage by petrifilm and it influences counting accuracy as well. In addition, the time required by petrifilm is in accordance with the plate colony count method. Microbial growth and reproduction may cause the increasing of medium turbidity. According to this feature, turbidimetry judges the growth and reproduction of microbes through the spectrophotometer's absorbance. Because this method has the characteristic of quick and simple to operate, it is widely applied in microbial concentration detection (Llosent, 1999). However, this method is not able to distinguish the live bacteria from the dead, so it cannot rule out the interference of microbial death in the process of degradation. The precision of turbidimetry will be influenced by the change of conditions like temperature, pH value and so on. At the same time, the dead bacteria were produced during the reproduction of microbes and the different temperature and pH will

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increase the amount of dead bacteria leading to the low detecting precision. While the study of optimal microbial degradation condition should detect the microbial concentration in different temperature and pH, so this method is not applicable to it.

In this study, We try to find a method based on high precision microscopic image processing and Support Vector Machine (SVM) identification to analyze the optimal condition of fenpropathrin degradation by Ochrobactrum anthropic more accurate than turbidimetry.

## MATERIALS AND METHODS

This LBD is formed by two major parts i.e. sample pretreatment, bacteria image identification. Then the LBD is used to measure the degradation condition.

**Sample pretreatment:** The fenpropathrin-degrading bacterium is isolated from activated sludge collected from wastewater-treating system of a pesticide manufacturer in April, 2011. Results DNA sequencing indicated that it is Ochrobactrum anthropi.

The samples of Ochrobactrum anthropi (a kind of bacteria for fenpropathrin degradation) that have been cultivated for less than 24 h, between 24 and 48 hand more than 48 h make 10-fold dilution, 20-fold dilution and 30-fold dilution, respectively. 0.1mL of methylene blue dye (0.25 g/L) is added to 0.1mL of the standard solution (1:1) above, then removing and fixing it after two minutes of staining. In order to keep the bacteria alive, staining step was done before smear the slides and the fixation step. Heat fixation will inactivate bacteria, so air-dried fixation was used to keep the bacteria alive. After staining, the live bacterium was circle (the cytoderm of the bacteria was thicker than the other parts) and the dead bacterium was solid shows in Fig. 1.

**Bacteria image identification:** The details of bacteria image identification show in the Fig. 2. We use the XSP-11C series biological microscope and optical CCD camera to obtain bacteria image.

The binary image of the bacteria is generated by the image segmentation technology. Image segmentation is a technology and process that divides the images into a number of specific and unique region and extracting the interested target. The images obtained from the HPCCD digital camera are true color image expressed by the R (red), G (green), B (blue) component, so we first converted them to grayscale images. Secondly select the average of the maximum grayscale and the minimum grayscale as the initial threshold value for interactive threshold segmentation.

After the binarization, the image may keep some noise points. Because the actual size of the majority of bacteria was between 0.5μm and 5μm, the method of

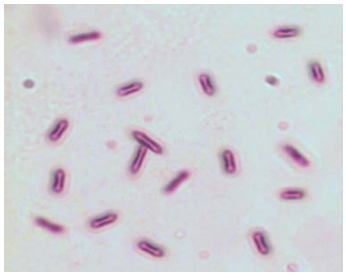


Fig. 1: One of the pictures after methylene blue staining captured by the bacteria image recognition system. The internal of the viable bacteria is colorless, on the other hand, the dead bacteria is full with color

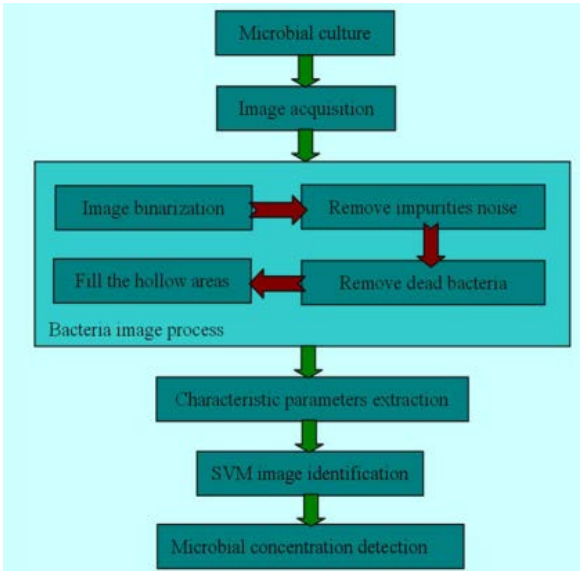



Fig. 2: The flow chart of processing the bacteria image

eliminating small areas and large areas can be used to eliminate the objects with too large or too small black connected region. The size of the bacteria image amplified by the detection system should be between 1.25 mm and 12.5 mm. As a result, the calculation speed of the following steps can be improved by removing the impurity particles whose connected black region is either larger than $\pi \times 12.5 \times 12.5$ mm$^2$ or less than $\pi \times 1.25 \times 1.25$ mm$^2$. In order to remove the impact of the dead bacteria by detecting the hollow, we calculated the center coordinates by the formula $[(X_{max}-X_{min})/2, (Y_{max}-Y_{min})/2]$ and judged whether or not the connected region is hollow by the sum of center coordinates and the pixel value of surrounding 8 points. If the sum of the pixel value of the nine points is not zero, there are at least one of the nine points are white and the connected area can be defined as hollow area. The opposite situation is defined as the solid area. The pixel value of the solid area sets to 255 (white), eliminating the solid areas, removing the impact of the dead bacteria and the elimination.

The hollow should be filled to extract the feature vectors such as circularity. This filling method is scanning the binary image from left to right and top to bottom until the first target point (the pixel value is 0) is found. Setting the target point as the starting point do the contour tracking and follow the "right look" criteria to do the clockwise track. When the extracting trace of the feature information is back to the starting point and the direction chain code is in the same direction with the starting point, end the tracking. To prevent a duplicate track and putting the program into an infinite loop, we filled the tracing area into gray area (the pixel value is 128). In other words, we finished the filling hole of single cell as well.

Seven morphological parameters were extracted; area, perimeter, circularity, ellipiticity length of major axis, length of minor axis and type to describe morphology of the object. When we take pixel as the unit of measurement, area is the number of pixels of the target object within the boundary (including the boundary) and perimeter is the number of pixels of the target object on the boundary. Circularity is the measure of the sharpness of a particle's edges and corners. Ellipticity is the degree of divergence of an ellipse from a circle. We used morphological algorithms to extract the target object boundary. The algorithm is described as follows:

$$\beta(\mathrm{A}) = A - (A \Theta B) \tag{1}$$

$\beta(A)$ is the boundary of target object $A$ and $A$ is an suitable structuring element. In the morphology, B eroding A is written as $A\Theta B$. It is defined as:

$$A\Theta B = \{z \mid (B)_z \subseteq A\} \quad A\Theta B = \{z \mid (B)_z \subseteq A\} \tag{2}$$

The equation indicates that the erosion of A by B is the set of all points z such that B, translated by z, is contained in A.

The SVM is a set of related supervised learning methods that analyze data and recognize patterns, used for classification and regression analysis. It is programmed by C++ language to implement LBD, which count live bacteria automatically.

Totally, 60 images of single bacteria, 20 images of connected bacteria and 20 image of non-bacterial were taken as disruptors form obtained images to be the training samples. The area, perimeter, roundness, major axis and ellipticity of samples were obtained as the training parameters to access the training of them. After the training was completed, take the shape parameters of each target object in the bacterial images to be identified as the inputs of SVM to carry out the classification and recognition.

**The method to determine the optimal condition of fenpropathrin degradation:** The kinetics of degradation of fenpropathrin and growth of Ochrobactrum anthropi were investigated simultaneously in the basic medium (1L) with fenpropathrin (20 mg) as the carbon source and at pH of 3.0, 4.0, 5.0, 6.0, 7.0, 8.0 and 9.0. Bacterial suspensions Ochrobactrum anthropi (10mL) were inoculated in basic medium (990 mL) under the condition of 30 °C. Samples were taken once per 1day. After the processing, the concentration of Ochrobactrum anthropi was detected by both turbidimetry and LBD.

(a) (b) (c)

Fig. 3: The results of bacteria image processing, (a) Binary images, (b) Image after removing noise and dead bacteria, (c) Image after filling hollow

**The method to determine the temperature condition:** The kinetics of degradation of fenpropathrin and growth of Ochrobactrum anthropi were investigated simultaneously in the basic medium (1L) with fenpropathrin (20 mg) as the carbon source and at temperature of 20, 25, 30, 35 and 40 °C, respectively. Bacterial suspensions with Ochrobactrum anthropi (10mL) were inoculated in basic medium (990 mL) under the condition of pH 7. The concentration of bacteria was detected by turbidimetry and LBD, respectively. The concentration of fenpropathrin was detected by GC.

## RESULTS AND DISCUSSION

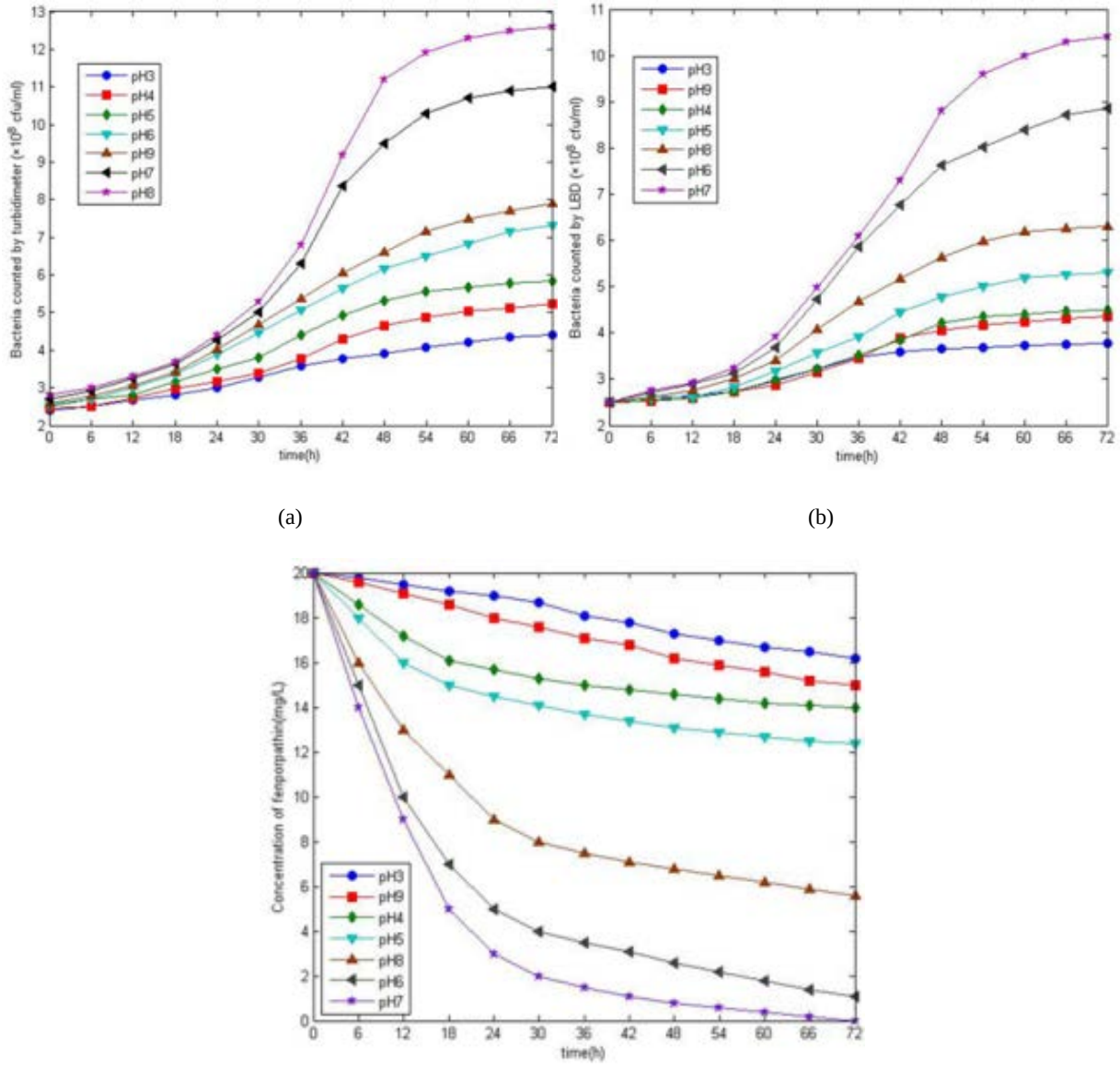
**The results of bacteria image processing:** The Fig. 3a shows the binarized images of the color bacteria image. From Fig. 3a, we can conclude that the dead bacteria segmented present a solid connected region while the live bacteria present a hollow connected region and the latter generate less noise than the former one. The results of removing impurities noise and dead bacteria are shown in Fig. 3b. It is clear that the impurities noise and dead bacteria in Fig. 3a has been removed. After the scanning of the entire image is finished, filling all the gray areas into black area (the pixel value is 0), thus the hole filling of the entire image is finished and the background is still keep white, the target is black. Figure 3c shows the image after filling.

The SVM classification and recognition results of the objects in Fig. 3c are shown in Table 1. Table 1 shows that the output results of SVM are just infinitely close to the desired results. In order to get more specific classification, the classification results of SVM were rounded to integer in this study.

Table 1: Recognition results of objects in example image

| Mark | Identify by human eye | Identify by SVM | After rounding |
|---|---|---|---|
| 1 | 1 | 1.1465 | 1 |
| 2 | 1 | 0.9189 | 1 |
| 3 | 1 | 0.9979 | 1 |
| 4 | 1 | 0.9899 | 1 |
| 5 | 1 | 0.9959 | 1 |
| 6 | 2 | 1.9919 | 2 |
| 7 | 1 | 1.0090 | 1 |
| 8 | 1 | 1.1223 | 1 |
| 9 | 1 | 1.0883 | 1 |
| 10 | 1 | 0.9261 | 1 |
| 11 | 1 | 1.0388 | 1 |
| 12 | 1 | 0.9275 | 1 |

The processed results are exacted the same as the human eyes recognition results. In conclusion, the counting by equipment can replace the human eyes counting to monitor the concentration of bacteria in the experiment. The stained area is much larger than the "field of view", so the stained area needs several sample images to represent. The "field of view" is the area that is visible to you when you look through the microscope eyepieces. Thus the number of the sample image is stained area divided by the area of "field of view". After calculation, we find 0.1 mL bacterial suspension needs about 350-sample image to cover the whole stained area. The bacterial concentration




(a) (b)

(c)

Fig. 4: Effect of pH on growth of Ochrobactrum anthropi and degradation of fenpropathrin; (a) Measured by LBD; (b) Measured by turbidimeter; (c) The fenpropathrin degradation curve at different pH

measured by LBD is calculated by the following equation:

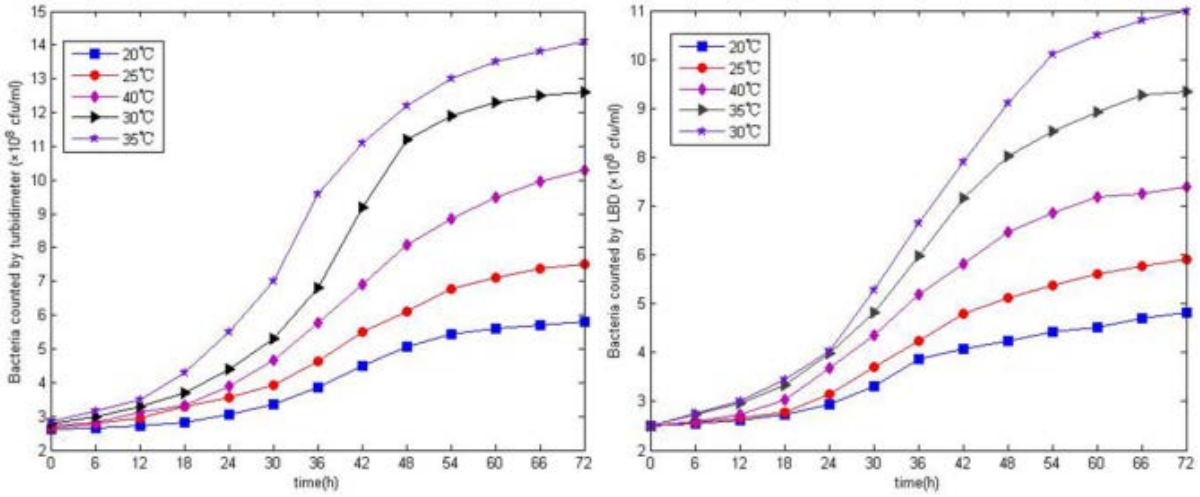
$$\lambda = \tau \times 3500 \times \sum_{i=1}^{20} x_i \div v \quad (3)$$

where,

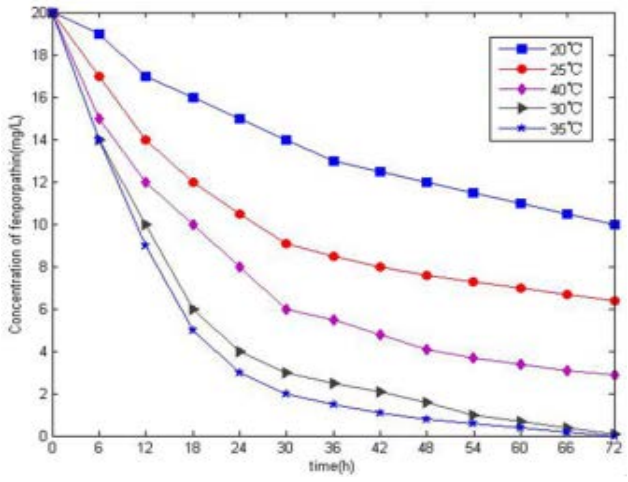
$\lambda$ = The concentration of bacteria
$\tau$ = The times of dilution
$x_i$ = The number of bacteria that identified in each figure
$v$ = Sample volume

The absolute concentration of bacteria is determined by the flow cytometry. The relative error is determined by the absolute concentration of bacteria and concentration of bacteria measured by LBD and turbidimetry. The relative error of LBD is 5% and the turbidimetry is 15.6%.

**Effect of pH on fenpropathrin degradation and growth of Ochrobactrum anthropi:** The Fig. 4a and b illustrates the reproduction curve of Ochrobactrum anthropi at different pH value by LBDand turbidimetry. The best reproduction condition of Ochrobactrum anthropi detecting by turbidimetry is at pH 8.0, while by LBD is at pH 7.0. The reason is that breaking the culture medium pH changes the chemical balance, which makes the content of inorganic salt precipitation changes and results in the medium turbidity changed greatly. Figure 4c shows the degradation of




(a) (b)




(c)

Fig. 5: Effect of temperature on growth of Ochrobactrum anthropi and fenpropathrin degradation of fenpropathrin; (a) The concentration of bacteria measured by LBD; (b) The concentration of bacteria measured by turbidimeter; (c) The fenpropathrin degradation curve at different temperature

fenpropathrin at different pH. The results indicate that the degradation of fenpropathrin and growth of Ochrobactrum anthropi reach best at the pH of 7.0 which is in accordance with the pH value of best reproduction of Ochrobactrum anthropi detected by LBD. This phenomenon indicates that turbidimetry caused a large error in the degradation of Ochrobactrum anthropi pH value. So pH 7.0 is the best for fenpropathrin degradation by Ochrobactrum anthropi.

**Effect of temperature on fenpropathrin degradation and growth of Ochrobactrum anthropi:** Figure 5a and b shows the concentration of bacteria measured by LBD and turbidimetry. The results indicate that the best temperature for growth of Ochrobactrum anthropi is about 30°C detected by LBD, however the best temperature for growth of Ochrobactrum anthropi is about 35°C detected by turbidimetry. This is because that with the increasing of temperature, the metabolism of bacteria accelerating leads to the increase number of dead bacterial cells, thus affecting the turbidity. Therefore, turbidimetry also had a larger error in detection of Ochrobactrum anthropi. Figure 5c shows the degradation of fenpropathrin at different temperature. The Fig. 5a and c indicate that the best temperature for growth of Ochrobactrum anthropi is about 35°C, while the best temperature for Ochrobactrum anthropi is about 35°C, while the best temperature for fenpropathrin degradation by Ochrobactrum anthropi is about 30°C, which is not the best temperature for the growth of bacteria. Because of the difference in best temperature, we should analyze the best temperature through weighted average evaluation.

It can be seen from Fig. 5a clearly that the speed of Ochrobactrum anthropi growth is very slow in 24 h, but the speed of fenpropathrin degradation is fast. This phenomenon indicates that the correlations between the fenpropathrin degradation and Ochrobactrum anthropi growth are less significant. The average slopes $k_1$, $k_2$ of the best Ochrobactrum anthropi growth curve and the best fenpropathrin degradation curve within 24 h are analyzed as weighting values. We can reach the best optimum degradation temperature by weighted average algorithm as follows:

$$T_O = 30 \times \frac{k_1}{k_1 + k_2} + 35 \times \frac{k_2}{k_1 + k_2} \quad (4)$$

The 30 and 35 in the formula represent 30 and 35°C, respectively. It can be reached by calculation that the average slopes k1 = 0.09, k2 = 0.67 and the optimum degradation temperature TO is 34°C.

**The comparison of LBD and turbidimetry at optimal condition:** Several experiments of fenpropathrin degradation by Ochrobactrum anthropi are applied based on the optimal condition mentioned on the former context. The Ochrobactrum anthropic concentration measured by LBD and turbidimetry is sampled every 6 hours. The same sampling time interval is applied on fenpropathrin concentration measured by GC. Then draw the concentration curves by all the measurement points are shown in Fig. 6. The measurement points during 24-48 h (logarithmic phase) is chosen to calculate the correlation coefficient of Ochrobactrum anthropi concentration and fenpropathrin concentration. The reason is that in this period the cells are dividing rapidly, which cause a huge difference between LBD and turbidimetry. The results of experiment show that the correlation coefficient measured by turbidimetry and LBD is -0.9214 and -0.9495, respectively. The correlation coefficients indicate that LBD is more correlated with the fenpropathrin concentration curve, so LBD is better. In theory, only the live bacteria contribute to the degradation of fenpropathrin. The turbidimetry is a total bacteria detection method, while LBD is live bacteria detection method. So detecting live bacteria is more precise than detecting the whole bacteria. Form this point of view, it also prove that LBD is better than turbidimetry.

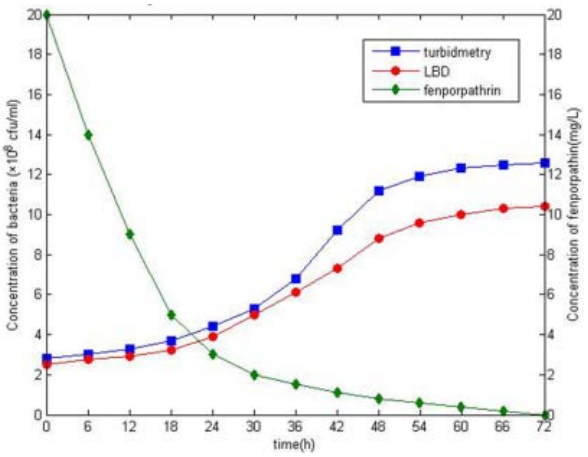



Fig. 6: Relationship between fenpropathrin degradation and Ochrobactrum anthropi growth detected by LBD and turbidimetry

## CONCLUSION

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A Live Bacteria Detection (LBD) method based on high precision microscopic image processing and SVM identification to analyze the optimal condition of fenpropathrin degradation by Ochrobactrum anthropic is introduced in this study. The methylene blue dyeing technology helped us to distinguish live bacteria from the dead, the high precision microscopic image processing technology helped us to remove the impurities in the microscopic image and the SVM identification technology helped us to identify and count the number of live bacterial cells. Through

comparing the correlate coefficients, we conclude that the dead bacteria largely affect the precision on detection. So LBD is a good method to analyze the bacteria degradation condition.

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