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Application of a Label-Free Immunosensor for White Spot Syndrome Virus (WSSV) in Shrimp Cultivation Water

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Abstract White spot syndrome virus (WSSV) is a major pathogen affecting the shrimp industry worldwide. In a preliminary study, WSSV binding protein (WBP) was specifically bound to the VP26 protein of WSSV. Therefore, we have developed the label-free affinity immunosensor using the WBP together with anti-GST-VP26 for quantitative detection of WSSV in shrimp pond water. When the biological molecules were immobilized on a gold electrode to form a self-assembled monolayer, it was then used to detect WSSV using a flow injection system with optimized conditions. Binding between the different copies of WSSV and the immobilized biological molecules was detected by an impedance change ($\Delta Z''$) in real time. The sensitivity of the developed immunosensor was in the linear range of 1.6×10^1–1.6×10^6 copies/μl. The system was highly sensitive for the analysis of WSSV as shown by the lack of impedance change when using yellow head virus (YHV). The developed immunosensor could be reused up to 37 times (relative standard deviation (RSD), 3.24 %) with a good reproducibility of residual activity (80–110 %). The immunosensor was simple to operate, reliable, reproducible, and could be applied for the detection and quantification of WSSV in water during shrimp cultivation.

Keywords White spot syndrome virus (WSSV) · WSSV binding protein (WBP) · Biosensor · Impedance · Label-free immunosensor · Shrimp cultivation

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Introduction

White spot syndrome virus (WSSV) is a major pathogen affecting the shrimp industry worldwide. It has caused severe economic losses to the shrimp farming industry and can result in mortality of up to 90 to 100 % within a 3- to 7-day period [1, 2]. Several diagnostic methods have been developed for detection of WSSV such as in situ hybridization [3], polymerase chain reaction (PCR) [4], immunological methods [5], and real-time PCR [6]. The molecular methods have been widely used for WSSV detection since they are highly sensitive and with good specificity and reliability, but they are not practicable for detecting the virus in pond sites. Moreover, these methods are quite expensive and also require many steps as well as an expert operator [7].

The infection may be caused by direct infection from the breeder or indirectly by crustaceans and water [8]. However, the water is a major pathway for WSSV to enter the cultivation cycle [9, 10]. In a previous work, it has been found that the WSSV binding protein (WBP) bound specifically to the VP26 protein of WSSV [11]. Thus, this protein has been used to develop the binding assay reported in this work. The WBP and the anti-GST-VP26 were further developed into an immune-based test assay for detecting the VP26 protein on the envelope of WSSV in shrimp pond water [12, 13]. Furthermore, a capacitive immunosensor has been developed for the quantitative detection of WSSV in shrimp pond water during shrimp cultivation [14]. In this study, the polyclonal antibody VP26 mixed WBP was immobilized on the immunosensor surface which both can bind specifically to the VP26 protein of WSSV to increase the sensitivity.

Therefore, a label-free affinity immunosensor for the quantitative detection of WSSV in shrimp pond water have been developed. The system is based on a flow injection technique using the WBP and the anti-GST-VP26 [12] as biological molecules immobilized on a gold electrode via a self-assembled monolayer (SAM). The binding between the biological molecules and WSSV was monitored by electrochemical impedance to detect the change ($\Delta Z''$) on the binding event [15]. The developed immunosensor was applied to quantify the WSSV in real water samples from shrimp cultivation ponds.

Materials and Methods

Materials

WSSV and yellow head virus (YHV) stock solutions contained 1.6×10^{10} copies and 10^7 copies, respectively. The stock solutions and recombinant phage pCANTAB-WBP were provided by the Center for Genomics and Bioinformatics Research, Faculty of Science, Prince of Songkla University, Hat Yai, Songkhla, Thailand. The viral titer was determined by real-time PCR. The polyclonal antibody VP26 was produced by the method of Loyprasert-Thananimit [12]. Thioctic acid and 1-dodecanethiol (98 %) were from Sigma-Aldrich (Milwaukee, USA). *N*-(3-dimethylaminopropyl)-*N*-ethylcarbodiimide hydrochloride (EDC) and *N*-hydroxysuccinimide (NHS) were from Sigma-Aldrich (Steinheim, Germany). All other chemicals were of analytical grade. Buffers were prepared with deionized water treated with a reverse osmosis-deionized system (Millipore, Bedford, USA). Before use, buffers were filtered through a nylon membrane filter (pore size, 0.20 μm; Albet, Spain) with subsequent degassing.

Production of WBP

The procedure of Youtong [1] was followed. Briefly, one colony of *Escherichia coli* HB2151 containing recombinant phage pCANTAB-WBP was cultured in Luria-Bertani (LB) containing 2 % (*w*/*v*) glucose at 37 °C for 16–18 h. Then, 1 ml of culture was transferred into 10 ml of LB broth containing 2 % (*w*/*v*) glucose and 80 μg/ml of ampicillin and incubated at 30 °C for 1.15 h. The culture was centrifuged at 1500 rpm for 20 min at room temperature. The pellet was resuspended in 10 ml of LB broth containing 1 mM IPTG and 80 μg/ml of ampicillin and incubated at 30 °C for 26 h. The mixture was centrifuged at 1500 rpm for 20 min. The protein was purified from the supernatant using a Sephadex™ G-25 column (GE Healthcare Bio-Sciences AB, Sweden) connected to an AKTAprime plus (GE Healthcare Bio-Sciences AB, Sweden) and analyzed using electrophoresis on a 15 % sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Protein concentrations were determined by the Lowry protein assay [16]. The prepared protein solution was then divided into small aliquots and stored at −80 °C.

Immunosensor for Detecting WSSV

Immobilization of Biological Molecules on the Electrode

The procedure followed that of Loyprasert-Thananimit [12]. Briefly, a gold electrode was polished using alumina slurries and cleaned through sonication in deionized water, followed by electrochemical etching in 0.5 M H_2SO_4 using a potential from 0 to 1.5 V versus an Ag/AgCl reference electrode with a scan rate of 0.1 V/s for 25 scans. The cleaned gold electrode was then immersed in 250 mM thioctic acid solution at room temperature for 12 h. During this step, a self-assembled monolayer was obtained on the electrode surface [17]. The gold electrode was then immersed in an EDC/NHS solution (1 % (*v*/*v*) EDC, 2.5 % (*v*/*v*) NHS in 0.05 M phosphate buffer at pH 5.00 with 0.05 M KCl) for 5 h. Then 20 μl containing 200 μg/ml of each biological molecules (the optimal ratio of WBP and the polyclonal antibody VP26 was 70 and 30 % (*v*/*v*), respectively (data not shown)) in phosphate buffer at pH 7.4 was immediately placed to cover the electrode surface and left overnight at 4 °C. Finally, the electrode was immersed in 10 mM 1-dodecanethiol ethanolic solution for 20 min to block any pinholes on the electrode surface, before being connected to the immunosensor system.

Impedance Measurement

A flow injection technique was set up as shown in Fig. 1. The modified gold electrode (working electrode), a custom-made Ag/AgCl (reference electrode), and a platinum wire auxiliary electrode (auxiliary electrode) were placed in the flow cell and connected to the Autolab PGSTAT30 electrochemical impedance analyzer and potentiostat/galvanostat (Eco Chemie B.V., Netherlands). Impedance was measured at a +200 mV dc potential with an ac amplitude of ±10 mV. The Eco Chemie software, Frequency Response Analyzer (FRA; 4.9.005), was used to monitor the impedance. To investigate the change of impedance, the imaginary part of the impedance (Z'') was monitored [18]. When the WSSV was injected and bound to the immobilized

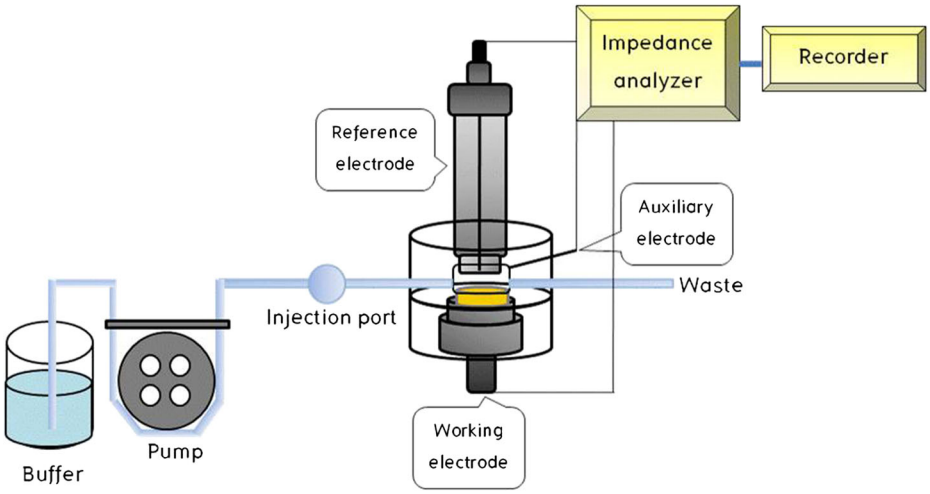

Fig. 1 Schematic diagram showing the label-free impedimetric immunosensor

biological molecules on the working electrode, the impedance increased. The impedance change ($\Delta Z''$) was measured to determine the signal. The experiment was repeated with three replicates for each concentration using HCl at a pH of 2.50 as the regenerating agent. The conditions used in the flow injection immunosensor followed the procedure of Loyprasert-Thananimit [12] (carrier buffer 10 mM phosphate buffer at pH 7.0 containing 2.7 mM KCl and 137 mM NaCl, flow rate 100 μl/min, and sample volume 400 μl).

Determination of WSSV by PCR

The same samples were also tested with a PCR to confirm the amount of WSSV to confirm the performance of the developed immunosensor. The PCR in a 25-μl reaction volume contained 400 ng of extracted DNA, 1 μM of each primer, 4 mM of dNTP, 2.5 mM of $MgCl_2$, 1× PCR buffer, and 2.5 U tag DNA polymerase. The primer was designed from the shrimp white spot syndrome virus isolate Japan 98 VP19 gene, GenBank accession number AY249447.1. The forward and reverse primers were VP19-FB: 5′CGGGATCCATGGCCACCACGACTAA3′, VP19-RX: 5′GCCTCGAGCCTGATGTTGTGTTTTCTATA3′, respectively. The PCR was performed according to Youtong [11]. PCR was conducted with an initial denaturation step at 94 °C for 1 min, followed by 30 cycles of 94 °C for 1 min, 59 °C for 1 min, and 72 °C for 1 min. The PCR product was analyzed by electrophoresis on a 1.5 % agarose gel.

Real Sample Analysis

Seawater used for shrimp cultivation was collected from Songkhla province, Thailand. The water samples were analyzed by the immunosensor. A standard curve and seawater, spiked with a series of WSSV concentration, were studied to test for a matrix effect.

Results and Discussions

Production of WBP from pCANTAB-WBP *E. coli* (HB2151)

WBP was produced from pCANTAB-WBP in *E. coli* (HB2151) and purified by a Sephadex™ G-25 column (GE Healthcare Bio-Sciences AB, Sweden). The WBP size was 3 kDa as determined from the 15 % SDS-PAGE (Fig. 2).

Immunosensor for Detecting WSSV

Linear Dynamic Range and Detection Limit

Since impedance depends on frequency, the optimum frequency to monitor the immunoreaction was determined. The single frequency was obtained by a Bode plot where the system exhibited a near-ideal capacitor behavior [19–21]. This frequency was found to be 117 Hz, and the impedance signals in a flow system were then obtained for real-time monitoring of Z'' at this frequency. When the WSSV was injected, it bound to biological molecules on the electrode surface causing Z'' to increase (Fig. 3, inset) which corresponded with the concentration of WSSV. The change of impedance ($\Delta Z''$) was described by Eq. (1)

$$Z'' = Z''_{(\text{biological molecules}-\text{WSSV})} - Z''_{(\text{biological molecules})} \quad (1)$$

Where $Z''_{(\text{biological molecules} - \text{WSSV})}$ was the value of the imagined part of the impedance when the WSSV was bound to the biological molecules and $Z''_{(\text{biological molecules})}$ was the value of the immobilized biological molecules.

A WSSV standard solution between 1.6 and 1.6×10^7 copies/μl were tested under optimum conditions, with three replications for each concentration. Figure 3 ashows the linear relationship between the impedance change and the logarithm of the WSSV

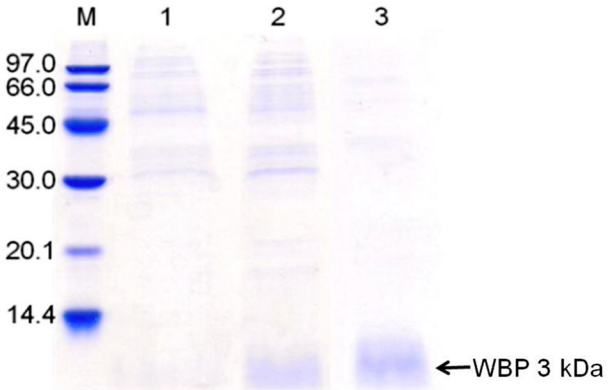

Fig. 2 Expression in *E. coli* (BL21) of WBP protein on 15 % SDS-PAGE. *Lane M*, low molecular weight standard marker; *1*, lysate of bacteria containing pCANTAB-WBP plasmid before induction with 1 mM IPTG; *2*, lysate of bacteria containing pCANTAB-WBP plasmid after induction with 1 mM IPTG; and *3*, WBP purified protein (3 kDa)

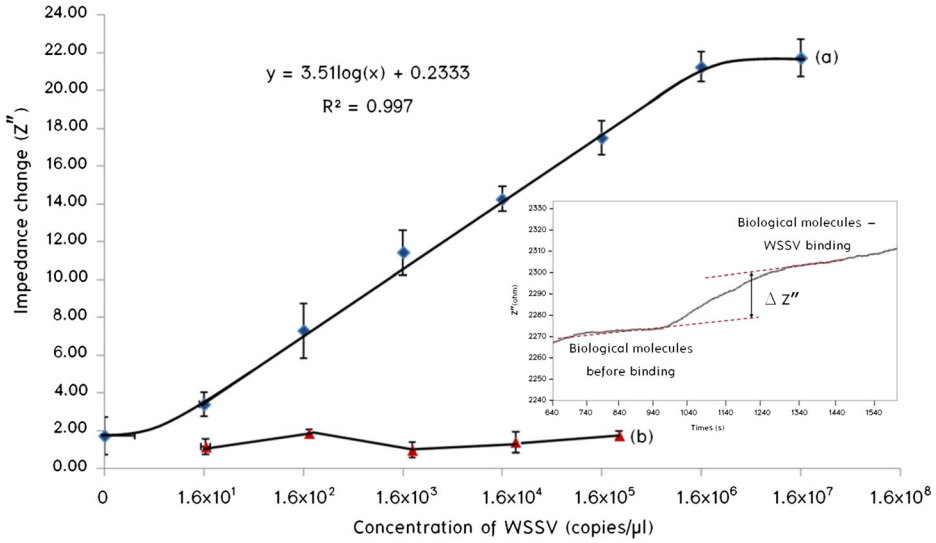

Fig. 3 Calibration plot of the white spot syndrome virus (*WSSV*) was obtained from the flow injection from the label-free impedance immunosensor (**a**) and impedance change obtained from the yellow head virus (*YHV*) (**b**). *Inset*, after the WSSV was injected, it bound to biological molecules on the electrode surface causing *Z*″ to increase

concentration that have been demonstrated for the WSSV amount in the range of 1.6×10^1–1.6×10^6 copies/µl, with an *r* value of 0.997, and the detection limit was 1.6×10^1 copies/µl. In terms of sensitivity, this immunosensor has less sensitivity compared with a previous work [14]. However, it is good enough since the amount of WSSV in water that is infective and lethal in shrimp was more than 1.6×10^4 copies/µl [13].

Selectivity

YHV is one of the most serious pathogens to infect shrimp [22] so YHV was used to test the selectivity of the immunosensor system. For the additional study, the *Vibrio harveyi* and *E. coli* were tested using this polyclonal antibody for WSSV, indicating that there was no significant cross-reaction of the immunosensor [12]. A standard YHV solution in the range of 1.6×10^1–1.6×10^5 copies/µlwas tested using the same conditions as for the WSSV. The result showed that YHV gave a very low signal response compared with the WSSV as shown in Fig. 3b.

Determination of WSSV by PCR

To verify the result of the analysis from the immunosensor system, the same sample amounts of WSSV in the range of 1.6×10^2–1.6×10^6 copies/µl were also tested by PCR. The PCR product of the WSSV (400 bp) was serially increased to correspond to the copies of WSSV as shown in Fig. 4. It confirmed the performance of the immunosensor that could be used for the quantification of WSSV.

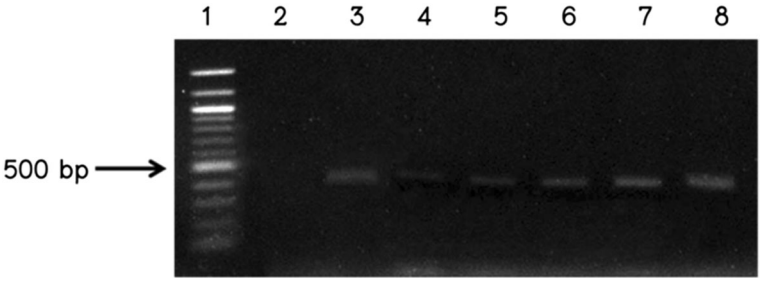

Fig. 4 The PCR products of different copies of WSSV using the 5′FB and 3′RX primer after electrophoresis on a 1.5 % agarose gel. *Lane 1*, 100 bp DNA marker; *2*, negative control; *3*, positive control; *4–8*, PCR product of WSSV at 1.6×10^2–1.6×10^6 copies/μl, respectively

Reproducibility

In this work, HCl at pH 2.50 was chosen to undo the binding between the WSSV and biological molecules. WSSV was detected by regenerating the electrode 15 times/day for about 4 days. The reproducibility of the immunosensor responses was investigated using the standard WSSV (160 copies/μl) with optimum conditions. The impedance change between before and after the regeneration was used to calculate the percentage residual activity of biological molecules. The successive analyzes showed relative standard deviation (RSD) of 3.24 % with 97.58 % residual activity of the original impedance change (ΔZ″) signal obtained when the sensing surface on the electrode was repeated for up to 37 times of consecutive measurement as shown in Fig. 5. After that, the percentage of residual activity dropped. This showed that the modified electrode could be reused with good reproducibility for up to 37 times with a RSD of 3.24 %.

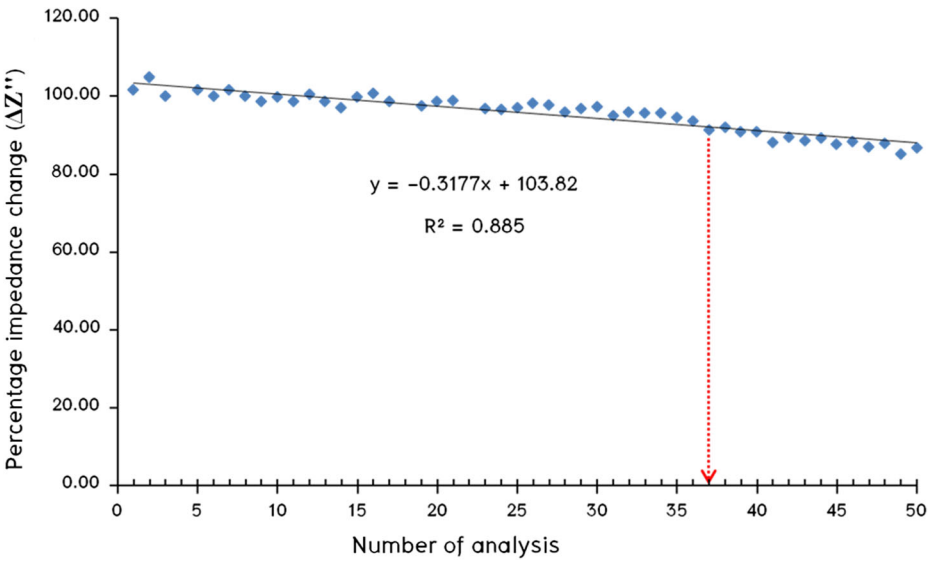

Fig. 5 Reproducibility of the label-free immunosensor to detect 1.6×10^2 copies of WSSV after regeneration (HCl, pH 2.5) between each individual assay

Determination of WSSV in Culturing Water in Shrimp Pond

High amounts of salt in seawater samples used for the shrimp culture site could affect the impedance response. To reduce this matrix influence, the dilution is one of the alternatives and could be easy to apply in the case of high level of WSSV in the real sample. However, if WSSV level in real water samples was very low, the dilution method is not a practical option. The effect of salt can also be then reduced by a desalting process using desalt columns (BIO-RAD, Hercules, CA, USA). Therefore, the seawater samples were treated with two methods before analysis: desalting and dilution to study the influence of matrix effect on the immunosensor response.

The seawater samples were then spiked with WSSV from 10 to 100 copies/μl. Both calibration curves were performed in triplicate for both spiked water samples used as "spiked curve" and running buffer used as "standard curve." The slopes of standard solution curve and the spiked sample curves were compared by two-way analysis of variance (ANOVA), calculated by R software [23] to test whether they differ significantly. If there is no difference, it indicates that the matrix has no effect on the response of the immunosensor. For the spiked curve of the desalting method, the results showed that there was no matrix effect. Therefore, water samples can be directly analyzed after desalting. For alternative study, seawater samples were diluted before being analyzed to reduce the matrix effect. Several dilution factors were tested and found that there was no significant difference between the slopes of the spiked diluted samples and standard solutions when seawater samples were diluted 1000 times. That is, the matrix effect can be eliminated by dilution. These results also indicated that there is negligible effect due to nonspecific adsorption from the constituents of seawater. The recovery of the spiked samples was investigated with both methods. The obtained response from immunosensor was used to calculate the concentration from the calibration curve obtained prior to analysis. The recovery of analyte was calculated by Eq. (2).

$$\text{Recovery}\ (\%) = \frac{C_1 - C_2}{C_3} \times 100 \tag{2}$$

where C_1 = the concentration determined in the fortified sample, C_2 = the concentration determined in the unfortified sample, and C_3 = the concentration of fortification [24]. The results showed average recoveries between 80 and 110 % (Table 1); these are acceptable values for these concentrations [25]. This demonstrated that the developed immunosensor was well adapted for analysis for WSSV in real water samples.

Conclusions

In conclusion, a WBP that bound specifically to the VP26 protein was produced and purified for use to develop a label-free affinity immunosensor for the quantitative detection of WSSV in seawater for shrimp cultivation. Biological molecules were immobilized on the gold electrode surface through a SAM to detect WSSV under a flow injection system under an optimal condition. Binding between different copies of WSSV and immobilized biological molecules

Table 1 Comparison of recovery of spiked shrimp pond water samples treated with desalting columns and treated with dilution ($n = 3$)

Spiked concentration (copies/μl)	Desalting columns		Dilution 1:1000	
	Detected concentration (copies/μl)	%Recovery	Detected concentration (copies/μl)	%Recovery
10	8.65 ± 0.22	86.48 ± 22	8.30 ± 0.06	82.96 ± 6
20	19.15 ± 0.16	95.74 ± 16	19.43 ± 0.09	97.15 ± 9
30	31.48 ± 0.25	104.94 ± 25	30.49 ± 0.19	101.65 ± 19
40	44.17 ± 0.31	110.42 ± 31	41.28 ± 0.07	103.19 ± 7
50	50.51 ± 0.14	101.02 ± 14	53.82 ± 0.12	107.64 ± 12
60	53.89 ± 0.15	89.82 ± 15	55.37 ± 0.10	92.29 ± 10
70	68.48 ± 0.13	97.83 ± 13	68.48 ± 0.13	97.83 ± 13
80	83.21 ± 0.26	104.01 ± 26	78.63 ± 0.16	98.28 ± 16
90	92.30 ± 0.13	102.55 ± 13	93.21 ± 0.10	103.57 ± 10
100	97.73 ± 0.14	97.73 ± 14	99.84 ± 0.13	99.84 ± 13

was monitored by an impedance change ($\Delta Z''$) that increased with the virus concentration. The immunosensor could detect WSSV in the linear range of 1.6×10^1–1.6×10^6 copies/μl which is a very low limit for detecting the system and gave good selectivity due to the absence of impedance change when using YHV. Furthermore, this immunosensor was simple to operate, reduced the cost of analysis (reused up to 37 times with good residual activity), and reliable with a good recovery percentage (80–110 %) in the real sample. Therefore, the developed system could be readily applied at the shrimp culture site.

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