International Journal of Water Resources and Arid Environments 11(1): 01-12, 2022
ISSN 2079-7079

Development of Nanoformulation to Encapsulate Fungicides to Minimize Water Pollution in Agriculture Lands

W.M.W.W. Kandegama

Faculty of Agriculture and Plantation Management,
Wayamba University of Sri Lanka (WUSL), Sri Lanka

Abstract: Conventional pesticides cause water pollution due to high toxic nature. Alternatively, scientific evidence revealed that nanotechnology has a potential to fix this drawback and minimize environmental pollution. Pesticide nanoformulation played a dominant role developing controlled releasing, target releasing and smart releasing delivery systems. However, most of them were not commercialized due to cost and poor efficacy. In this study, we synthesis a nanocarrier with an average size of 300nm – 600nm, using Sodium Alginate metrics and encapsulate broad spectrum, highly effective fungicide of Strobilurin family combining with spray drying technique. Benzothiostrobin was encapsulated for the first time and achieved 14% encapsulation efficiency (EE), novel nanoformulation was characterized according the standard methods. The highest EE obtained from ALG-S 40 treatment. Toxicity evaluation demonstrated, ALG-S 40 and ALG-S 45 treatments can protect live fish with 7 times higher concentration than the lethal dosage. Further, sample ALG-S 40 has inhibited pathogen [*Colletotrichum gleoesporioides*] with the same efficacy as pure Benzothiostrobin. Further, our nanoformulation shown comparatively very low toxicity on fish and did not release to environment. Therefore, novel nanoformulation shown high potential to minimize water pollution and protect biodiversity.

Key words: Nanoformulation · Encapsulation · Fish toxicity · Environmental pollution

INTRODUCTION

Scientists attention was now drowning dramatically towards pesticide encapsulation. Many new findings have been published in this area and their applications in the broad range of Agriculture. various type of materials were employed such as polymer-based materials, solid lipid particles, inorganic porous materials, clays, layered double hydroxides (LDHs), to achieve this target [1-5]. Based on the particle sizes, they were categorized as micro or nanoformulation. In nanoformulation, various polymer materials such as Alginate, Chitosan, Xanthum gum, CMC and Pectin, were dominantly used [6-8]. However, the major drawback of some polymer based pesticide formulations is residues accumulation in soil, hence selecting a polymer for encapsulation pesticide is very important. Among various polymers, alginate has become one of the most popular polymers in many industries such as food, agriculture and pharmaceutical. Alginate was given special attention due to its unique physicochemical nature [9, 10], such as nontoxic, naturally abundant and biodegradable [11]. It is composed of two different Alginic acid monomers commonly known as uronic acids, Alginate is extracted from brown algae or bacteria [12, 13]. Alginate is anionic [4] and forms gel by complexation with polyvalent cations or poly amides [14] and makes three dimensional network [15]. The cavities can bind with cations [7, 16]. An image of Alginate bond with divalent cations (Ca^{+2}) obtained from N.E. Simpson *et al.* [15]. The proportion of M units and G units in Alginate products dependent on the source of Alginate [17] and residue based order and molecular weight [15].

Number cross-linking varies with reaction duration and cations concentration, by adjusting these conditions number of ross link can be changed which help to optimize drug release [5, 18, 19]. Alginate shows high cations selectivity [20]. Commercially Alginate commonly known as Sodium Alginate [21] and forms a thick gel with Na^{+2} [22]. Furthermore, Fuat Topuz *et al.,* [23] shows that alginate gelatinized with Mg^{+2}.

Corresponding Author: W.M.W.W. Kandegama, Faculty of Agriculture and Plantation Management, Wayamba University of Sri Lanka (WUSL), Sri Lanka.

Micro-encapsulation with Alginate were studies predominantly and many patents have been deposited [24]. Moreover, Alginate matrices have been used for controlled release of pesticides and drugs delivery systems in various researches recently [5, 25-27]. In 1987, Alginate digestibility in fish gut was studied by Trond Storebakken *et al.* [28] and also it has been confirmed that same like fish, other monogastric animal cannot digest Alginate due to the structure of Uronic acids chain [29]. Apart from the encapsulation due to crosslink with polyvalent cations, Alginate can be incorporated with inorganic materials such as Silica which enhanced encapsulation properties, controlled releasing properties.

Silica well-known inorganic material and has been used to develop various micro and nano delivery systems [30]. T. Coradin *et al*, in 2003, [31] explained, that Silica is a tough, thermostable, non-swelling component and it can be associated with many bio-polymers, leading to promising route for new bio-composites. Therefore silica has become a potential candidate for pesticide encapsulation and controlled releasing systems in agriculture [32]. Mitra *et al*. in 2011 [33] found that commercial pesticide fabricated with silica nanoparticles shows higher insect mortality.

Optimize Alginate capsulation properties and releasing efficiency can be alter when incorporate Silica in to the matrix was reported in several researches [30, 34, 35,]. Moreover, Alginate-Silica composites exhibit enhanced mechanical and thermal stability [31]. After incorporation of SiO_2, binding possibilities improve the wall strength of the capsule and regulated controlled releasing of pesticide. Further it will provide better protection for active ingredient from adverse conditions such as sun light [17].

So far, spray dying technique is not a popular in pesticide encapsulation, however, it is well known mechanism to transform liquids into solid dry powder. Spray dying is widely used in food, chemical and material industries [36] and pharmaceuticals drugs formulation [36-38]. Spray drying systems has also improved simultaneously to produce nanoparticles with acceptable particle sizes and a satisfactory yield [37].

Fungicides commercially available in the market have a specific mechanism to control fungi in selected crops. In general broad spectrum fungicides are not common as discovering is very complicated. Therefore, Scientists pay more attention to discover new candidates for crops protection.

Benzothiostrobin ($C_{20}H_{19}NO_4S_2$, Molecular Weight 401.50, (CAS: 070975-53-9) [(E)-2-[2-(5-methoxy-benzothiazol-2-methylthio) phenyl]-3- methoxyacrylate] is a novel fungicide belong to Strobilurin family, with the development code; Y5247 by Key Laboratory of Pesticide & Chemical Biology of Ministry of Education, Central China Normal University (CCNU), China and patented in 2010 [39]. Benzothiostrobin is a broad spectrum fungicide which can be used for many crops [39]. A few researches have been conducted to determine the conjugated effect [40], competitive and noncompetitive phage immunoassays [41] and residue detection method was developed [42]. it appeared with work against *Blumeria graminis* on wheat leaf [43]. It has also been tested activity against *Sclerotinia sclerotiorum* [44] and degradation, adsorption and leaching characteristics [45]. According to the "Safety Evaluation of Chemical Pesticide Quality Supervision and Inspection Center China", Benzothiostrobin shows high toxicity for fish, with the concentration of 0.1mg/L active ingredient, all fish died in 24 hours, which may restrict application in the field. Benzothiostrobin also shown a poor solubility in water [46],

In this study, we have developed a smart controlled releasing nanoformulation for Benzothiostrobin fungicide combining polymer suspension with spry drying technique, having an objectives to minimize Benzothiostrobin fungicide toxicity on aquatic fauna, while maintaining the higher activity against pathogen. In addition, formulation leads to improve Benzothiostrobin dispersion in water without solvent. Sodium Alginate and Silica nano particles were used to fabricate pesticide delivery system.

The final product was characterized by using FTIR, SEM, TEM and DLS techniques. Encapsulation Efficiency (EE) was determined by HPLC. Leaf contact angle was compared with commercial fungicide. Efficacy was evaluated *in vitro* and toxicity on fish was tested. Environmental fate was observed according to previously published methods. According to our knowledge, Benzothiostrobin fungicide was never encapsulated by any researcher before.

MATERIALS AND METHODS

Materials: Benzothiostrobin fungicide and Fluorescence Probe [HPLC purity >99.5 %, provided by key laboratory of Pesticides & Chemical Biology, School of Chemistry,

CCNU, China]. Sodium Alginate, Magnesium Chloride Hexahydrate, Acetone, Anhydrous Methanol, Acetonitrile (HPLC grade) and Methanol (HPLC grade), Ethanol (95%), were purchased from [Sinopharm Chemical Reagent Co.ltd, Chin]. Silicon Dioxide [99.8% Metal Basis, 7-40nm - Aladdin Industrial Corporation, China], Butyl Glycidyl Ether were purchased from [Tianjin Heowns Biochem LLC, China]. Tween™ 80, Potassium Bromide, Dextrose, Glucose and Dialysis bag (less than 3500 MW), Fungi Colony, Zebra Fish (*Danio rerio)* and glass fish were purchased from [Aladdin Industrial Corporation, China],

Experimental Steps

Preparation of Alginate-Silica Suspension with Fungicide: (i) Silicon dioxide (SiO_2) nano particles (10nm) were weighed according to the sampling plan and add in to 100ml of DI water. The mixture was sonicated using (KQ 100DA, China) at 40 KHz and 60W for 4 minutes under room temperature. Then the mixture was stirred on a magnetic stirrer (MR Hel-Tec, Germany) for 30 minutes with 600RPM at 60°C. (ii) 100mg of Benzothiostrobin was added in to 2ml of standard solution (Methanol: Acetone: Tween 80: Butyl glycidyl ether) and sonicated in for 3mins gently add in to silicon dioxide mixture, continue stirring for 30 minutes. (iii) Sodium Alginate was weighed according to the sample plane and added to the above mixture and continued stirring for 3hrs and formed a viscous thick dispersion. (iv) 10ml of $MgCl_2$ solution (300mg of $MgCl_2$ in 10ml of DI H_2O) was prepared and added in to Alginate Silica Suspension drop wise, continue stirring for 1 hour at 800RPM, at 60°C.

Preparation of Dry Nano Particles: Spray Dryer (DC1500, Shanghai Attainpak Co. Ltd) was used to transform liquid polymer suspension in to free floating powder, drying temperature; inlet air 200°C, outlet 100°C. Nanopowder was obtained with the yield around 70%.

Characterization

Determine Encapsulation Efficiency: The encapsulation efficiency of Benzothiostrobin was determined by High Performance Liquid Chromatography (HPLC) Method (Agilent, USA) using an Eclipse XDB C_8 column (150 × 4.6 mm, 5ìm; ZORBAX, USA). The flow rate of mobile phase was 1mL min^{-1} using Acetonitrile and Water (65:35, v/v), injecting volume 0.5ìL. The absorbance was taken at 210nm. analysis duration per sample was 10 min with an elution at 5.7 min. Benzothiostrobin concatenation determined as; 10 mg of each samples were taken in to micro tube and 1mL of (HPLC grade) Methanol was added. The mixture well and left for 24 hours, then the micro tubes were centrifuged for 8 minutes at 6000RPM in (Dragon Lab AC220, China). Finally, supernatant was obtained and sent through 0.22ìL filter and inject in to HPLC.

Determine the Presence of Active Ingredient (AI) in the Nanoformulation by Fourier Transform Infrared Spectroscopy (FTIR) Method: The IR analysis of Benzothiostrobin, Sodium Alginate, Silica nano particles, Sodium Alginate & Silica particles (intermediate) and final nanoformulation were individually performed. Functional group of each compound and final product were characterized and recorded by using FTIR (Perkin Elmer PE 983, USA) Spectrophotometer, Samples were prepared as KBr pellets and scanned against a blank KBr pellet background at wave numbers ranging from 500-4000 cm^{-1} with a resolution of 4.0 cm^{-1}. All the samples were analyzed as dry powder and pellet was made by grinding 1% w/w sample.

Determine the Morphology and the Size of the Particles Through Scanning Electronic Microscope (SEM): SEM (JEOL, JSM 6700F Japan) was used to study the morphology and size of sample and examined under 10000X and 2500X magnification. All samples were observed as solid powder. Images were captured in the various places of the same sample and observed particle distribution.

Confirm the Morphology and the Size of the Particles Using Transmission Electronic Microscope (TEM): The morphology and the structure of the sample were characterized through TEM images. The sample was diluted in ethanol and a drop was deposited on to a standard copper grid and dried at ambient temperature until evaporating methanol, the samples were visualized under transmission electron microscopy model H-7000FA (Hitachi, Japan).

Determine the Particle Size Distribution (PSD): Zeta sizer nano ZS (Malvern Instruments, Malvern, UK) was used to determine the size distribution of Benzothiostrobin loaded nanoformulation. The sample was diluted well in DI water, then transferred into 1 cm^2 cuvettes in a zita analyzer. Each sample was tested 3 times and taken the average values under 25°C temperature.

Confirm the Particle Size Distribution Employing Dynamic Light Scattering Technique (DLS): Dynamic Light Scattering (DLS) technique, DynaPro Nano star, equipment from Wyatt technology, UK was used to determine polydispersity index by measuring the average size of nano capsule. The size distribution data was analyzed by Dynamics V7 software. Data were obtained under the wavelength of 659.930nm, with 5 second aquiasition time, total 10 aquiasition were obtained under temperature 25°C.

Efficacy Evaluation

***In vivo* Fish Toxicity Determination:** Fish Toxicity Determination under Natural Condition: Fish toxicity evaluation was conducted using adult zebra fish (*Danio rerio)* according to the method of "Safety Evaluation of Chemical Pesticide Quality Supervision and Inspection Center China", with minor modifications. Water quality parameters; Temperature and pH, were monitored daily and recorded. Fish death count was observed in 4hrs, 24hrs, 48hrs, 72hrs and 96hrs and extended up to 14 days when necessary. 10 fish were introduced in to experiment tanks and added nano formulated Benzothiostrobin with the concentration of 0.3mg/L, 0.5mg/L, 0.7mg/L and 0.9mg/L. Observation was continued daily. Simultaneously, a series of control experiments were scheduled to determine; (a) the influence of coating materials for fish death and (b) compare fish toxicity against pure Benzothiostrobin fungicide.

Fish Toxicity Determination Using Nano Probe: Fish toxicity evaluation was conducted as maintained as 2.4.1.1. Single molecular, fluorescence sensor, which was discovered by Yufeng Zhanga *et al.,* 2016 [47] to detect Mercury in the living cells. we used this fluorescence to detect nano particles inside the fish body. First, fluorescence sensor (10^{-5}mol/ L) was incorporated in to Alginate-Silica nanoparticles and introduced to fish tank, after 2hrs, Mercury (10^{-4} mol/L) was added in to fish tank. 30 minutes later, fish body was visualized through microscope (COIC BA 2500 Microscope, China) and captured the image by camera (Canon FA-ON ESO 4D, Japan) under UV light. Fluorescence probe toxicity for fish was tested prior to the experiment.

***In vivo* Fungal Toxicity Determination:** Fungal toxicity experiment was carried out on Potato Dextrose Agar Medium (PDA) according to Growth Rate Method described by Kun Qian *et al.* 2011 [21], under controlled environment at 25°C. Fungal growth was observed after 7 days. IC_{50} value; 10.47 ± 1.174ppm was calculated in excel to determine the fungicide concentration required. Fungicidal activity was evaluated against *Colletotrichum glecosporioides* with two concentration as; 5ppm and 15ppm. The experiment was performed in triplicates and measurement of average colony growth.

Environmental Fate Evaluation

Controlled Release in Water: Releasing of Benzothiostrobin from the new nanoformulation was determined according to the earlier reported method [8], with minor modification. In this study, we used dialysis bag (3500 MW) to avoid free dispersion of particles in to air. 10mg of Benzothiostrobin was taken in small pouch made from dialysis bag and added to 25mL water in a beaker and kept in room with controlled temperature. The experiment was carried out for two months and collected sample in 5 days interval and tested to quantify the amount of Benzothiostrobin released. 1mL of water was removed for HPLC test and replaced with 1ml of DI water to keep the volume as original.

Controlled Release in Different Temperature: Release of Benzothiostrobin from the new nanoformulation was determined as per the method reported earlier by Choudhary *et al.* [8] with minor modification. 10mg of sample was weighted accurately and taken in to a dialysis bag and added to 25 mL water in a beaker. Finally the beakers were kept on the magnetic stirrer under four different temperatures conditions as; 4°C, 20°C, 40°C and 60°C. Thermometer was fixed for continuous temperature monitoring. Water in the beaker was gently stirred to facilitate passing fungicide though dialysis bag. 1 mL of sample were removed from each experiment at different time intervals as; 5 days, 10 days, 15 days, 20 days, 25 days, 30 days, 35 days, 40 days, 45 days 50 days, 55 days and 60 days to determine released Benzothiostrobin in water. Same amount was replaced with DI water to maintain the volume constant.

Controlled Release in Various pH Conditions: Three different buffer solutions; pH3.2, pH5.9 and pH10 were used for release analysis of Benzothiostrobin from nanoformulation. it was taken in dialysis bag in three replicates and added to 25mL. Then beaker was kept at 30°C. Samples were collected in the time intervals of 3 days, 6 days, 9 days, 12 days, 15 days, 18 days, 21 days,

24 days, 27 days and 30 days. 1mL each solution was obtained for HPLC test and replaced with 1 mL of respective buffer to keep the volume unchanged [8].

Controlled Release in Soil: We followed previously reported method by G. Choudhary *el al*. 2006 [8], with minor modification. A soil sample was obtained from the forestry to make sure that no added chemical in the soil due to agriculture or disposals waste. Soil sample was dried, crushed and sieved through 2mm mesh. 25g of soil was taken in to a beaker and added water until soil gets wet. 10mg of nanoformulation was dispersed in water and transferred into a dialysis bag and closed both ends tightly to avoid leakage. Then dialysis bag was placed in the soil and the beaker was kept in to Biological Oxygen Demand (BOD) incubator and samples were obtained in 5 days, time interval.

Determination of Benzothiostrobin in soil: in the given time intervals, dialysis pouch were carefully removed from the soil container and the soil sample was washed three times with methanol. Then methanol was removed from the Benzothiostrobin using rotary evaporator. Finally the dry Benzothiostrobin was dissolved in 1ml of HPLC grade methanol, filtered and measured in to HPLC.

RESULT AND DISCUSSION

Proposed Mechanism of Cross Link Binding of the Nanoformulation and its Structure: In the new nanoformulation, Silica particle acted as a base material facilitating polymer chain to wrap around and create individual capsules. There is a possibility to interact COO- and OH groups of Alginate with Silica and Alginate polymer which make the capsule stable [17]. Those consecutive polymer layers are linked by Mg^{+2} and make the structure more stable. Benzothiostrobin fungicide was wrapped by polymer and hold around the Silica particle with the help of Mg^{+2} divalent bonds. Moreover, Silica is also taking part in wall strengthening, Size and the shape of the dried powder particle was determined by the nozzle pressure and Inlet temperature of the spry dryer, Fig. 3.1.

When polymer concentration is higher, rapid releasing occurs due to poor strength of the walls, whereas high concentration of silica occupies more space in the matrix resulting less fungicide encapsulation. On the other hand, Magnesium iron concentration is also important as less cross link leads rapid erosion where as higher number of cross link cause gelation of the Alginate-Silica suspension.

Characterization

HPLC Analysis: In chromatographic procedures a linear relation is observed between the detector response (Y) and analytic concentration (x). The linearity of a method measures of range within which the results are directly, or by a mathematical calculation, [48]. The second extraction was performed to ensure that there is no left fungicide in the sample after the first extraction. Nano formulated sample was kept in methanol for 24 hour to facilitate dissolve all fungicide in solvent. Then the sample was sent for HPLC test.

10 different nano formulations were synthesized using various combinations of Alginate and Silica as shown Table 3.2. Fungicide and other materials concentrations were maintained as constant. All samples were prepared maintaining the same conditions. Samples were transferred in clean containers with lid and stored dry ambient temperature for further characterization and efficacy evaluation.

Various combination of Polymer-Silica have demonstrated different capacities of encapsulating of Fungicide. The highest encapsulation rate is 14.02 which observed at sample ALG-S 40. EE variation of the Alginate –Silica Matrixes were given in Fig. 3.3.

FTIR Analysis: FTIR analysis was performed to identify the specific functional groups of the compound. The results confirm that Benzothiostrobin has not interacted with Alginate or Silica. Pure Benzothiostrobin was characterized with some specific peaks located at 1706.33cm^{-1} and 1431cm^{-1} in the nanoformulation. 1706.33cm^{-1} stretching vibration is corresponding to ester group of Benzothiostrobin and other peaks responsible for benzene ring were overlapped with other peaks of polymer and Silica. The sodium alginate showed a broad band between 3000 cm^{-1} - 3600 cm^{-1}, assigned to complex stretching vibration of intra-molecular hydrogen bond and inter-molecular hydrogen bonds.

SEM Analysis: All sample shown relatively smooth texture in white colored, fine powder with no bigger particles observable by the naked eye. However, particle uniformity and surface texture morphology were captured and extensively examined as shown in the Fig. 3.5. The particles were found as individual, well dispersed and irregular shape and size as displayed in Fig. 3.5(b). Sizes of the particles were ranged from 200nm to 1200nm. Most of the particles were arranged with hollow structures in side as shown in Fig. 3.5(a).

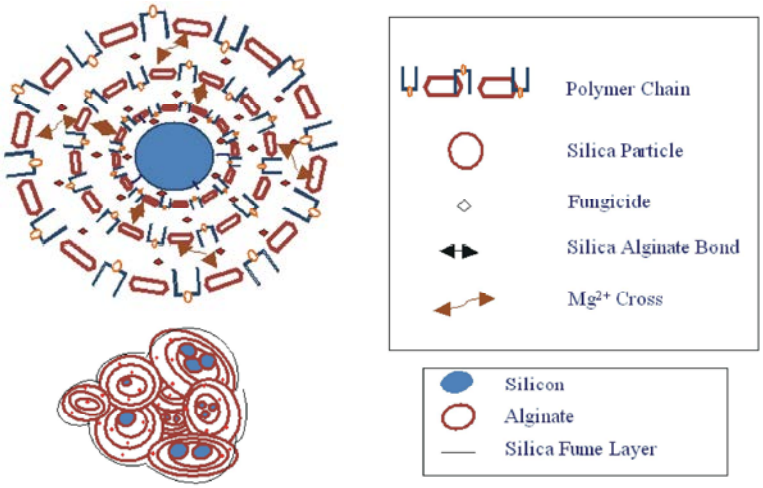


Fig. 3.1: Mechanism of cross liked Alginate Silica nano particles

Table 3.2: Average encapsulation percentage of Benzothiostrobin

Sample Name	Alginate wt. %	Silica wt. %	Fungicide wt.%	EE %
ALG-S 20	80	20	20	6.08
ALG-S 25	75	25	20	8.70
ALG-S 30	70	30	20	10.50
ALG-S 35	65	35	20	12.39
ALG-S 40	60	40	20	14.02
ALG-S 45	55	45	20	13.40
ALG-S 50	50	50	20	12.60

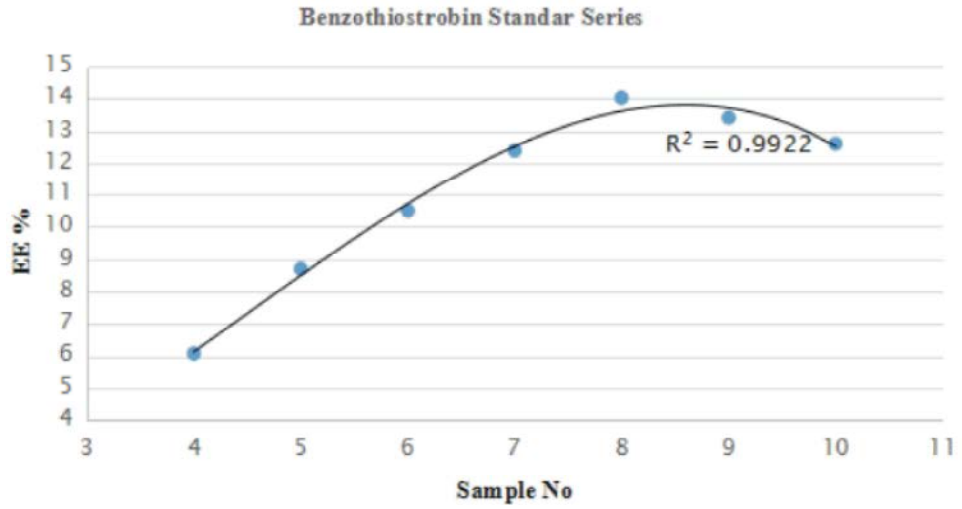


Fig. 3.3: Benzothiostrobin encapsulation efficiency

TEM Analysis: As shown in images, the particles were evenly distributed, spherical and individual as shown in Fig. 3.6 (c) and (d) and the diameters of particles were estimated as 200nm to 1200nm.

These pictures also confirmed that Alginate polymer has been arranged, around Silica particles and built individual matrix with holding fungicide in size. In addition, magnifying particle size up to 150000X, we have observed that the surface of the particle is irregular and gained higher surface area Fig. 3.6 (a) and (b).

PSD Analysis by Zita Sizer: Average particle size distribution of the Benzothiostrobin nanoformulation was determined, the data revealed, particle size has been

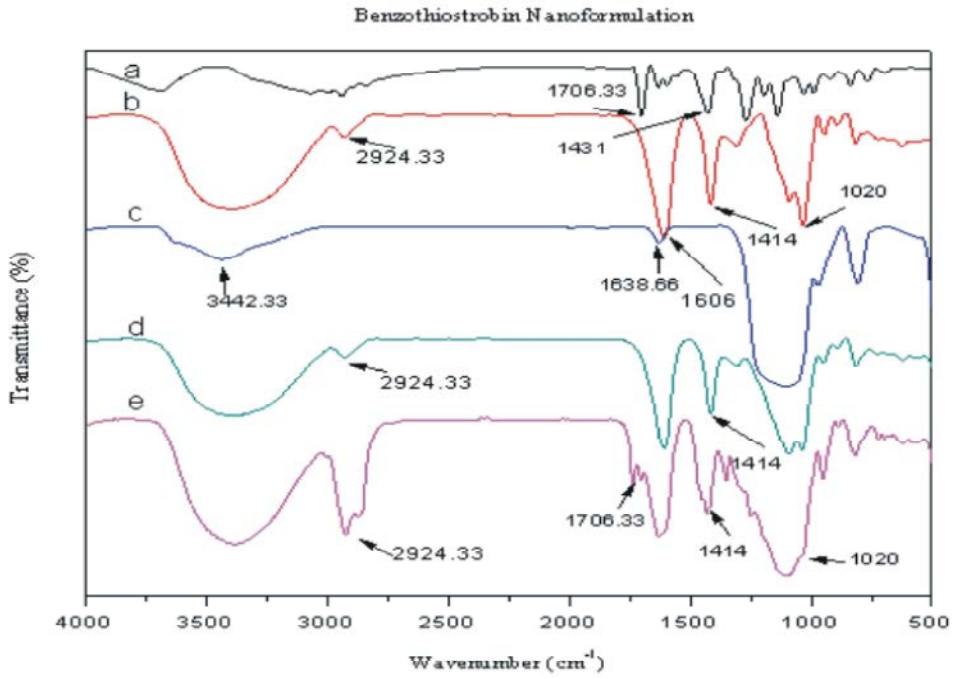
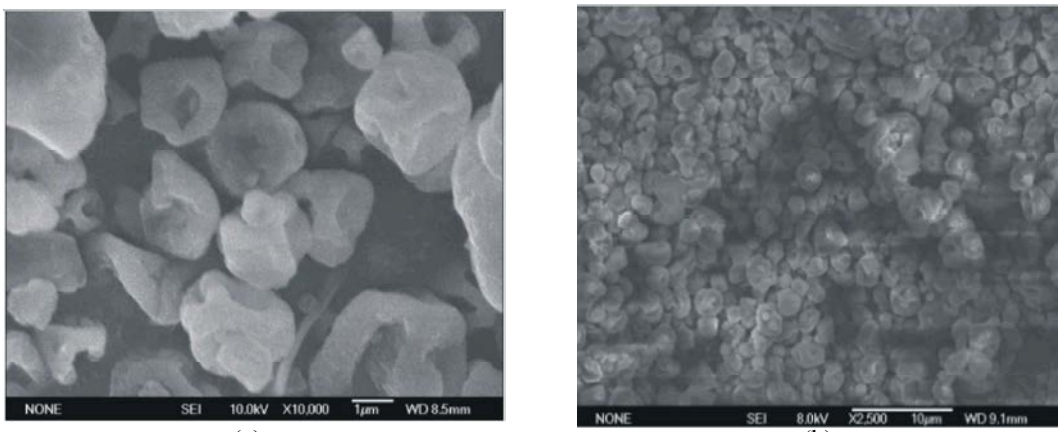
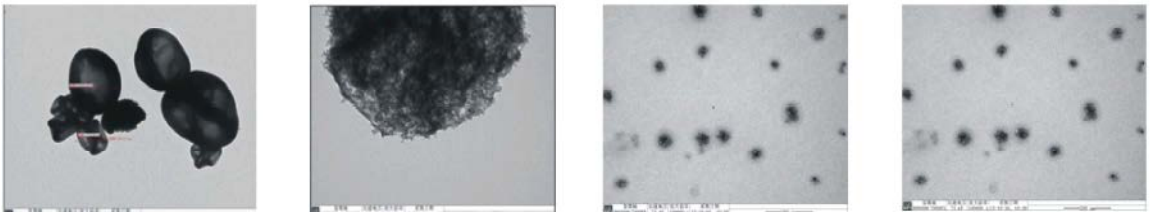


Fig. 3.4: FTIR spectra for (a) Benzothiostrobin (b) Alginate (c) Silica (d) Alginate + Silica (e) Nanoformulation



(a) (b)

Fig. 3.5: Scanning Electronic Microscope images of fungicide nano formulation; (a) Particle morphology and (b) Particle distribution



(a) (b) (c) (d)

Fig. 3.6: TEM image of fungicide nanoformulation (a) Particle size, (b) Surface morphology, (c) and (d) Particles distribution

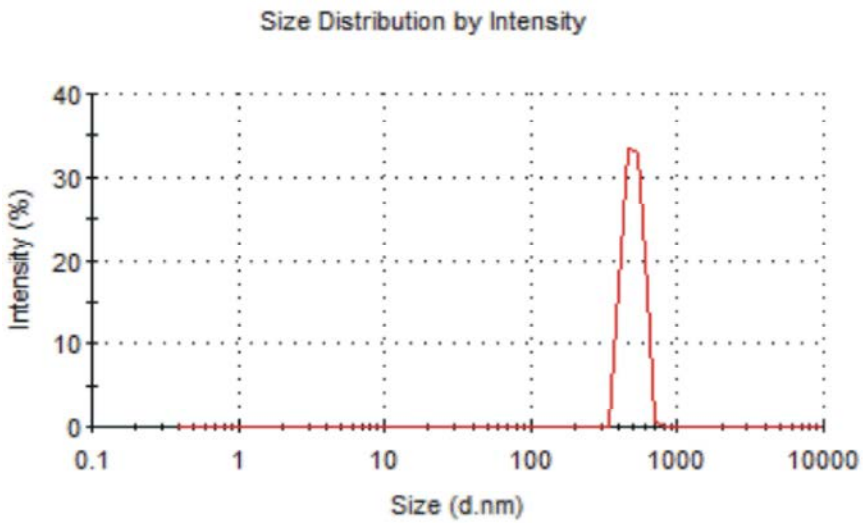


Fig. 3.7: Particle distribution of the Benzothiostrobin nanoformulation obtained from Zita sizer.

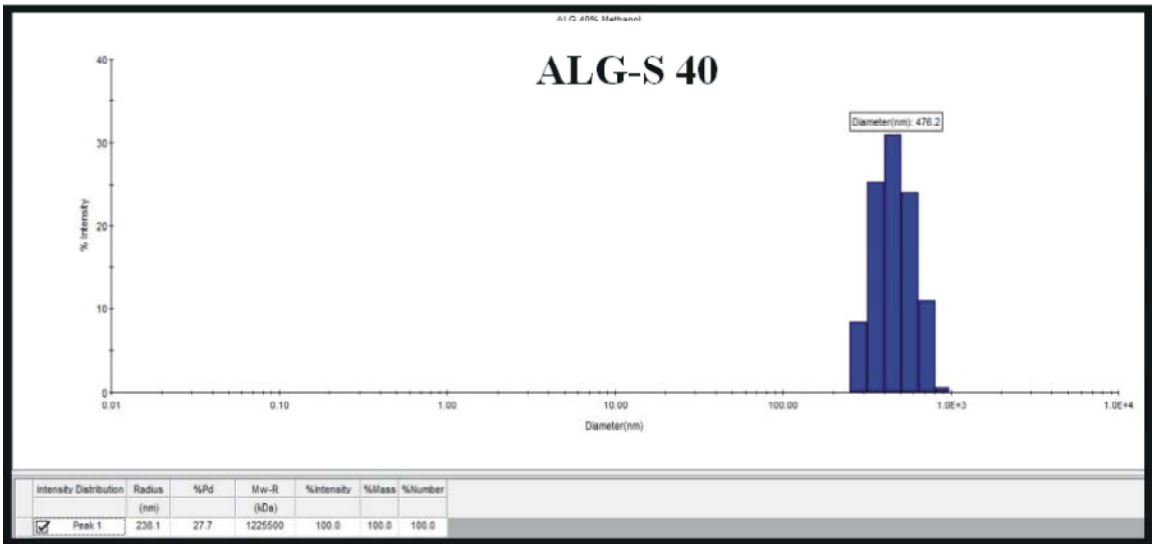


Fig. 3.8: Particle size distribution of the nano formulated Benzothiostrobin

speeded between 200nm to 900nm range and the most particles are around 510nm in size. However, very small quantities of particles have reached beyond the nanorange; it may course due to aggregation of particles. According to the result we can confirm that the scope of the study was achieved. Fig 3.7 depicted the graphical image if the particle distribution obtained from Zita sizer equipment.

Dynamic Light Scattering Technique (DLS): DLS result verified that the majority of particles in the sample was possessed 476.2nm diameter. Moreover small proportion of the particle population has reached higher than nano size. Both techniques Zita sizer and DLS have given almost similar result. In Fig. 3.8 has displayed particle size distribution range in the sample.

Leaf Contact Angle Determination: Initial observation was determined with 5µL of water on the rice leave and obtained the angle around 150 and used the similar volume for all tests with the concentration of 10ppm. In this study we identified that new nanoformulation has a better contact than pure Benzothiostrobin on crop leaves, as shown in Fig. 3.9.

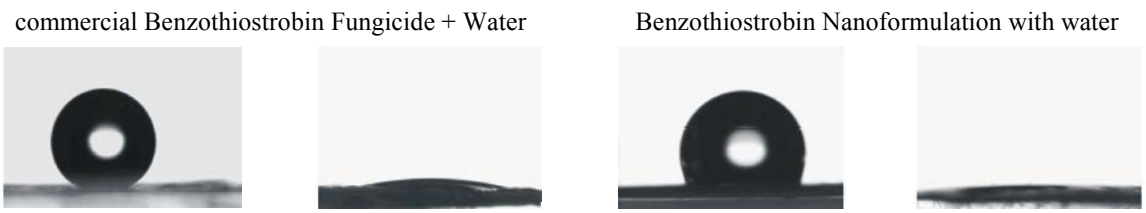


Fig. 3.9: Leaf surface contact angle determination (a) Pure Benzothiostrobin with water on rice leaf (b) Pure Benzothiostrobin with water on cotton leaf (c) Nano Formulated Benzothiostrobin fungicide on rice leaf (d) Nano Formulated Benzothiostrobin fungicide on cotton leaf

Furthermore Benzothiostrobin nanoformulation droplet stick in the rice leaves (Supper hydrophobic surface) with a 122.8 angle, whereas pure Benzothiostrobin attached to rice leaf with an angle of 147.5. Both products were shown better results on cotton leaf (hydrophilic surface) with less than 90 degrees angle. This study confirmed that new Benzothiostrobin nanoformulation has performed higher potential of retention on crop leaves.

Evaluation of Efficacy

***In vivo* Fish Toxicity Determination**

Fish Toxicity Determination of under Natural Water Condition: Fish toxicity experiment was continuously monitored for 96 hours. Different samples showed various toxicity levels for fish as demonstrated in the Fig. 3.10. In the control experiment with pure Benzothiostrobin, all fish were dead at the concentration of 0.1mg /L. within one day. However nano formulated Benzothiostrobin can be increased the concentration up to 0.7mg/L (7 times higher than the lethal dose), specially sample no ALG-S35, ALG-S40, ALG-S45 showed extremely better characteristics than other.

Fish Toxicity Determine by the Nano Probe: The images Fig. 3.11 of fish proved that nano formulated fungicide was ingested in to fish body. According to literature fish cannot digest Alginate [28]. However, surface of the particles can be digested due to microbial activity inside the fish gut. Therefore, very high concentration of nano formulated fungicide may course fish death.

***In vivo* Fungicide Efficacy Determination**

Colletotrichum Glecosporioidea: Fungal growth in plates were measured from three different angles and taken the average to calculate inhibition ratio. Inhibition ratio was tested under two concentration; 15ppm and 5 ppm after 7 days. The results revealed that new pesticide formulation can inhibit fungal growth same as pure Benzothiostrobin. Pure Benzothiostrobin has inhibited growth the highest with 15 ppm concentration, 54.4% whereas 5 ppm concentration, 39.5%, fungal colony growth were given in Fig. 3.12.

On the other hand, nano formulated Benzothiostrobin sample no. ALG-S40 gained the highest inhibition ratio as 56.5% at the concentration of 15ppm and 42.1% at 5ppm concentration. This evaluation confirmed that the new nano formulation has demonstrated the similar capacities of controlling fungal growth as pure active ingredient.

Environmental Fate

Fungicide Releasing in Water: Benzothiostrobin releasing from the nano formulation in water was observed for 60 days with 5 days interval. Benzothiostrobin concentrations of collected samples were tested through HPLC. We have noticed that Benzothiostrobin was not released from the nano formulation during the testing period. It confirmed that the nano formulation can hold fungicide effectively.

Fungicide Releasing in Different Temperature Conditions: Effects of temperature on the release rate of Benzothiostrobin nanoformulation was observed in different temperatures as; 4°C, 20°C, 40°C, 60°C for two weeks in aqueous condition. pH was maintained approximately 7. The cumulative release rate was observed. Fungicide was not releasing at 4°C, 20°C and 40°C. Further, at 60°C fungicide releasing was appeared only in the first week according to the HPLC report.

Fungicide Releasing in Various pH Conditions: Effects of pH on the release rate of Benzothiostrobin from nanoformulation were observed for one month with various pH conditions as pH 3.2, pH 5.9 and pH 10. We observed that Benzothiostrobin was not releasing from the formulation.

(a). No fluorescence probe (b). With fluorescence probe (c). With fluorescence probe $+Hg^{+2}$

Fig. 3.11: Images of fish with fluorescence probe detector (a) control experiment with no fluorescence probe, (b) with fluorescence probe (c) with fluorescence probe and Hg^{+2} iron

Name / Concentration	Control	Benzohiostrobin	ALG-S30	ALG-S35	ALG-S40	ALG-S45	ALG-S50
15ppm							
Inhibition Ratio	0	54.4%	41.5%	42.4%	56.5%	37.5%	46.9%
5ppm							
Inhibition Ratio	0	39.5%	39.7%	34.5%	42.1%	30.2%	36.6%

Fig. 3.12: Fungal inhibition ratio of nano formulated Benzothiostrobin

Fungicide Releasing in Soil: Wu Ping *et al*. [45] has successfully completed a research on degradation and adsorption of Benzothiostrobin in soil. Following the same method, here we have scheduled experiment to determine the releasing efficiency of Benzothiostrobin in to soil for two days. Fungicide releasing began at 2 hours and increased releasing amount till 6 hours. After 6 hour, releasing was disappeared. Hence. The reason for the scenario is unknown.

CONCLUSION

We have successfully developed a nanoformulation for Benzothiostrobin and encapsulated into smart controlled releasing delivery system. The new nanoformulation demonstrated a specific characteristics including efficient control of common pathogen and low aquatic toxicity. It has also shown that nanformlation was not released fungicide in water under natural environmental conditions. Hence, the toxicity for aquatic fauna is very low. The materials we used are highly abundant, economically viable and the synthetic rout is short and simple.

Further study should be carried out to optimize the spray drying by employing a purpose built advance nano spray drying system.

Fungicidal toxicity experiment can be expanded to determine the activity against other pathogenic organisms as pure Benzothiostrobin fungicide shown a broad spectrum fungicide activity in a range of crops.

REFERENCES

1. Ghormade, V., M.V. Deshpande and K.M. Paknikar, 2011. Perspectives for Nano-Biotechnology Enabled Protection and Nutrition of Plants. Biotechnol. Adv. 29(6): 792-803.
2. Yin, Y., Q. Guo, Y. Han, L. Wang and S. Wan, 2012. Preparation, Characterization and Nematicidal Activity of Lansiumamide B Nano-Capsules. J. Integr. Agric., 11(7): 1151-1158.
3. Nussinovitch, A., 2010. Bead Formation, Strengthening and Modification. In Polymer Macro- and Micro-Gel Beads: Fundamentals and Applications; Springer New York: New York, NY, pp: 27-52.
4. Krishna, A., P. Sailaja and P.C. Amareshwar, 2011. Different Techniques Used for the Preparation of Nanoparticles Using Natural Polymers and Their Application. Int. J. Pharm. Pharm. Sci., 3(2): 45-50.

5. Dalaty, A., A. Al-Karam, M. Najlah, R.G. Alany and M. Khoder, 2016. Effect of Non-Cross-Linked Calcium on Characteristics, Swelling Behaviour, Drug Release and Mucoadhesiveness of Calcium Alginate Beads. Carbohydr. Polym., 140: 163-170.
6. Vijaya, Y., S.R. Popuri, V.M. Boddu and A. Krishnaiah, 2008. A. Modified Chitosan and Calcium Alginate Biopolymer Sorbents for Removal of Nickel (II) through Adsorption. Carbohydr. Polym., 72(2): 261-271.
7. Sankalia, M.G., R.C. Mashru, J.M. Sankalia and V.B. Sutariya, 2007. Reversed Chitosan-alginate Polyelectrolyte Complex for Stability Improvement of Alpha-Amylase: Optimization and Physicochemical Characterization. Eur. J. Pharm. Biopharm., 65(2): 215-232.
8. Choudhary, G., J. Kumar, S. Walia, R. Parsad and b.s. Parmar, 2006. Controlled Release of Carbofuran in Water from Some Polymeric Matrices. Pestic. Res. J., 18(1): 65.
9. Saini, P., M. Gopal, R. Kumar and C. Srivastava, Development of Pyridalyl Nanocapsule Suspension for Efficient Management of Tomato Fruit and Shoot Borer (Helicoverpa Armigera). J. Environ. Sci. Heal. Part B, 49(5): 344-351.
10. Barreca, S., S. Orecchio and A. Pace, 2014. The Effect of Montmorillonite Clay in Alginate Gel Beads for Polychlorinated Biphenyl Adsorption: Isothermal and Kinetic Studies. Appl. Clay Sci., 99: 220-228.
11. Tønnesen, H.H. and J. Karlsen, 2002. Alginate in Drug Delivery Systems. Drug Dev. Ind. Pharm., 28(6): 621-630.
12. Sabra, W.A.P. and W.D.D. Zeng, 2001. Bacterial Alginate: Physiology, Product Quality and Process Aspects. Appl. Microbiol. Biotechnol., 56: 315-325.
13. Nishinari, K., M. Takemasa, H. Zhang and R. Takahashi, 2007. Storage Plant Polysaccharides: Xyloglucans, Galactomannans, Glucomannans. In Comprehensive Glycoscience; Elsevier, pp: 613-652.
14. Kenawy, E.R. and M.A. Sakran, 1996. Controlled Release Formulations of Agrochemicals from Calcium Alginate. Ind. Eng. Chem. Res., 35(10): 3726-3729.
15. Simpson, N.E., C.L. Stabler, C.P. Simpson, A. Sambanis and I. Constantinidis, 2004. The Role of the CaCl2-guluronic Acid Interaction on Alginate Encapsulated βTC3 Cells. Biomaterials, 25(13): 2603-2610.
16. George, M. and T.E. Abraham, 2006. Polyionic Hydrocolloids for the Intestinal Delivery of Protein Drugs: Alginate and Chitosan—a Review. J. Control. Release, 114(1): 1-14.
1.7. Yang, M., Y. Xia, Y. Wang, X. Zhao, Z. Xue, F. Quan, C. Geng and Z. Zhao, 2016. Preparation and Property Investigation of Crosslinked Alginate/silicon Dioxide Nanocomposite Films. J. Appl. Polym. Sci., 133(22): 43489.
18. Li, W.T.; D.R. Hwang, C.P. Chen, C.W. Shen, C.L. Huang, T.W. Chen, C.H. Lin, Y.L. Chang, Y.Y. Chang, Y.K. Lo, H.Y. Tseng, C.C. Lin, J.S. Song, H.C. Chen, S.J. Chen, S. Wu and C.T. Chen, 2003. Synthesis and Biological Evaluation of N-Heterocyclic Indolyl Glyoxylamides as Orally Active Anticancer Agents. J. Med. Chem., 46(9): 1706-1715.
19. Daemi, H. and M. Barikani, 2012. Synthesis and Characterization of Calcium Alginate Nanoparticles, Sodium Homopolymannuronate Salt and Its Calcium Nanoparticles. Sci. Iran., 19(6): 2023-2028.
20. Fenoradosoa, T.A., G. Ali, C. Delattre, C. Laroche, E. Petit, A Wadouachi and P. Michaud, 2010. Extraction and Characterization of an Alginate from the Brown Seaweed Sargassum Turbinarioides Grunow. J. Appl. Phycol., 22(2): 131-137.
21. Qian, K., T. Shi, S. He, L. Luo, X. Liu and Y. Cao, 2013. Release Kinetics of Tebuconazole from Porous Hollow Silica Nanospheres Prepared by Miniemulsion Method. Microporous Mesoporous Mater., 169: 1-6.
22. Heng, P.W.S., L.W. Chan and T.W. Wong, 2003. Formation of Alginate Microspheres Produced Using Emulsification Technique. J. Microencapsul., 20(3): 401-413.
23. Topuz, F., A. Henke, W. Richtering and J. Groll, 2012. Magnesium Ions and Alginate Do Form Hydrogels: A Rheological Study. Soft Matter, 8(18): 4877.
24. Safaríková, M., I. Roy, M.N. Gupta and I. Safarík, 2003. Magnetic Alginate Microparticles for Purification of α-Amylases. J. Biotechnol., 105(3): 255-260.
25. Kumar, S., N. Chauhan, M. Gopal, R. Kumar and N. Dilbaghi, 2015. Development and Evaluation of Alginate-chitosan Nanocapsules for Controlled Release of Acetamiprid. Int. J. Biol. Macromol., 81: 631-637.
26. McHugh, D.J., Production, Properties and Uses of Alginates. In Chapter 2; FAO.
27. Aston, R., M. Wimalaratne, A. Brock, G. Lawrie and L. Grøndahl, 2015. Interactions between Chitosan and Alginate Dialdehyde Biopolymers and Their Layer-by-Layer Assemblies. Biomacromolecules, 16(6): 1807-1817.
28. Storebakken, T. and E. Austreng, 1987. Binders in Fish Feeds. Aquaculture, 60(2): 121-131.

29. Humphreys, E.R. and J.T. Triffitt, 1968. Absorption by the Rat of Alginate Labelled with Carbon-14. Nature, 219(5159): 1172-1173.
30. Sakai, S., T. Ono, H. Ijima and K. Kawakami, 2001. Synthesis and Transport Characterization of Alginate/aminopropyl-Silicate/alginate Microcapsule: Application to Bioartificial Pancreas. Biomaterials, 22(21): 2827-2834.
31. Coradin, T., N. Nassif and J. Livage, 2003. Silica-alginate Composites for Microencapsulation. Appl. Microbiol. Biotechnol., 61(5): 429-434.
32. Barik, T.K., B. Sahu and V. Swain, 2008. Nanosilica-from Medicine to Pest Control. Parasitol. Res., 103(2): 253-258.
33. Mitra, S., B. Subia, P. Patra, S. Chandra, N. Debnath, S. Das, R. Banerjee, S.C. Kundu, P. Pramanik and A. Goswami, 2012. Porous ZnO Nanorod for Targeted Delivery of Doxorubicin: In Vitro and in Vivo Response for Therapeutic Applications. J. Mater. Chem., 22(45): 24145-24154.
34, Boninsegna, S., R. Dal Toso, R. Dal Monte and G. Carturan, 2003. Alginate Microspheres Loaded with Animal Cells and Coated by a Siliceous Layer. J. Sol-Gel Sci. Technol., 26(1): 1151-1157.
35. Coradin, T., E. Mercey, L. Lisnard and J. Livage, 2001. Design of Silica-Coated Microcapsules for Bioencapsulation. Chem. Commun., 23: 2496-2497.
36. Li, X., N. Anton, C. Arpagaus, F. Belleteix, T.F. Vandamme, 2010. Nanoparticles by Spray Drying Using Innovative New Technology: The Büchi Nano Spray Dryer B-90. J. Control. Release, 147(2): 304-310.
37. Oliveira, A.M., K.L. Guimarães, N.N.P. Cerize, A.S. Tunussi, J.G.R. Poço, 2013. Nano Spray Drying as an Innovative Technology for Encapsulating Hydrophilic Active Pharmaceutical Ingredients (API). J. Nanomed Nanotechnol., 4(186): 2.
38. Li, H.Y. and F. Zhang, 2012. Preparation of Nanoparticles by Spray-Drying and Their Use for Efficient Pulmonary Drug Delivery. In Nanoparticles in Biology and Medicine; Humana Press: Totowa, NJ, pp: 295-301.
39. Yang, G., P. Zhao, W. Huang and Z. Liu, 2008. Methoxy Acrylate Fungicide, Its Preparation Process and Application for Prevention and Control of Plant Fungal Diseases., September 24, 2008.
40. Sun, G., T. Yao, C. Feng, L. Chen, J. Li and L. Wang, 2017. Identification and Biocontrol Potential of Antagonistic Bacteria Strains against Sclerotinia Sclerotiorum and Their Growth-Promoting Effects on Brassica Napus. Biol. Control, 104: 35-43.
41. Hua, X., L. Zhou, L. Feng, Y. Ding, H. Shi, L. Wang, S.J. Gee, B.D. Hammock and M. Wang, 2015. Competitive and Noncompetitive Phage Immunoassays for the Determination of Benzothiostrobin. Anal. Chim. Acta, 890: 150-156.
42. Yuan, Y., X. Hua, M. Li, W. Yin, H. Shi and M. Wang, 2014. Development of a Sensitive Indirect Competitive Enzyme-Linked Immunosorbent Assay Based on the Monoclonal Antibody for the Detection of Benzothiostrobin Residue. RSC Adv., 4(46): 24406.
43. Anke, T., 1995. The Antifungal Strobilurins and Their Possible Ecological Role. Can. J. Bot., 73(S1): 940-945.
44. Xu, C., Y. Hou, J. Wang, G. Yang, X. Liang and M. Zhou, 2014. Activity of a Novel Strobilurin Fungicide Benzothiostrobin against Sclerotinia Sclerotiorum. Pestic. Biochem. Physiol., 115: 32-38.
45. Wu, W.Z., Z.H. Han and H. Yang, 2016. Desorption and Mobilization of Three Strobilurin Fungicides in Three Types of Soil. Environ. Monit. Assess., 188(6): 1-11.
46. Yang, G., P. Zhao, W. Huang and Z. Liu, 2008. Methoxy Acrylate Fungicide, Its Preparation Process and Application for Prevention and Control of Plant Fungal Diseases. CN101268780A, September 24, 2008.
47. Zhang, Y., H. Chen, D. Chen, D. Wu, Z. Chen, J. Zhang, X. Chen, S. Liu and J. Yin, 2016. A Colorimetric and Ratiometric Fluorescent Probe for Mercury (II) in Lysosome. Sensors Actuators B Chem., 224: 907-914.
48. Francotte, E., A. Davatz and P. Richert, 1996. Development and Validation of Chiral High-Performance Liquid Chromatographic Methods for the Quantitation of Valsartan and of the Tosylate of Valinebenzyl Ester. J. Chromatogr. B Biomed. Sci. Appl., 686(1): 77-83.