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Effects of Temperature and pH on the Morphology of Bio-polymeric Chitosan-alginate Nanoparticles Loaded with Coffee White Stem Borer Pheromone

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Abstract

Effects of temperature and pH on the morphology of bio-polymeric chitosan-alginate nanoparticles loaded with Coffee white stem borer (CWSB) pheromone were studied. The nanoparticles were prepared by the ionotropic pre-gelation of alginate with calcium chloride, followed by the complexation between alginate and chitosan. The influence of temperature, pH, and the stoichiometric relationships between the polyelectrolytes were assessed by photon correlation spectroscopy (PCS), using a Zeta PALS particle size analyzer. The data when subjected to analysis of variance (ANOVA) showed that at various pH readings (6, 9, 10, 11 and 12) and with temperatures over a range from 6 to 70 ^{0}C, there were no effects on nanoparticle size. Similarly, the zeta potential analysis results for the chitosan-alginate nanoparticles (CS-ALG NPs) loaded with the pheromone with a zeta potential at pH range 6 - 12 were not significantly correlated when subjected to ANOVA. The studies indicated that generally pH has a non-significant effect on zeta potential. However, at pH 8 the effect of temperature was found to be significantly correlated with particle size. This study helps in understanding the determination of the size of nanoparticles and their loading capacity for pheromones.

Keywords: alginate, chitosan, nanoparticles, zeta potential, coffee white stem borer

I. Introduction

Coffee white stem borer (CWSB), *Xylotrechus quadripes* chevrolat (Coleoptera: Cerambycidae) is the most serious pest in India of Arabica coffee (Figure 1). Host plants also include robusta coffee, tree coffee, teak, and others, but Arabica coffee is the principal host. Affected plants show ridges around their stem and plants may be affected during either of the two different pest flight periods, April-May and October-December. The female CWSB lay eggs in crevices of bark, and subsequently the larvae tunnel into the trunk. The burrowing larvae fill their tunnels with frass to exclude predators [Rhainds *et al*. 2001 and Rhainds *et al*. 2002]. Attempts at CWSB pest management can include tracing, scrubbing, uprooting, applying chemicals, and more recently using pheromone traps as a biological control [Venkatesha, M. G., Dinesh, A. S., 2012.].

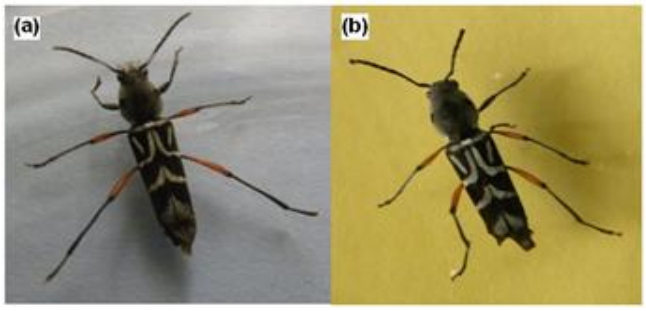


Figure 1. Adult *Xylotrechus quadripes* (a) male and (b) female.

Recently, nanotechnology in agriculture has become increasingly important [Bhagat *et al*. 2013a, 2013b, Santanu Bhattachraya 2014a, 2014b, 2014c, 2015]. For example, nanoparticles may enhance the properties of polymers to protect and control the release of bioactive substances as part of a a delivery system. Various materials can be used to synthesize nanoparticles but some polymers play a major role because of their biodegradability, biocompatibility, and their ability to function as inert carriers of biomaterials. Thus, chitosan-alginate nanoparticles (CS-ALG NPs) are used as nanocarriers for the controlled release of materials into a bio-system. These nanoparticles offer the advantage of effective loading due to their larger surface area, easy attachment and mass transfer of ingredients into a biological system [Ghormade *et al*. 2011].

The stabilization of biopolymers like chitosan and alginate, in the synthesis of CS-ALG with Pluronic F68 as the stabilizing agent, has been demonstrated. The composite has three biocompatible components, chitosan, alginate, and Pluronic F68 that are important for the delivery of bioactive molecules. The Pluronic F68 helps in the solvent polymer formation, segment interactions and hydration forces [Gigout *et al*. 2008].

The aqueous solubility of chitosan is pH dependant and in solution chitosan forms a polyelectrolyte with a positive charge [Florence, C., Christine, J., 2013]. The formation of "physical" hydrogels is based on the reversible interactions that can occur between polymer chains. The interactions have a non-covalent nature, such as electrostatic interactions, hydrophobic interactions or hydrogen bonds [Berger *et al*. 2004, Boucard *et al*. 2005]. These interactions can be dependent on various parameters like pH, concentration, and temperature, which make them unstable.

Both the aggregation and disaggregation tendencies of ZnO nanoparticles, over a wide range of pH values in the presence of Suwannee River humic acid (SRHA) at variable concentrations, have been studied to understand the stability of ZnO nanoparticles [Omar *et al*. 2014]. The zeta potential and the hydrodynamic size depend on pH and ionic strength [Suttiponparnit *et al*. 2011]. At lower pH levels a higher stability of complexes is observed and temperature effects also depend on pH [Bruno *et al*. 2006].

II. Materials and Methods

2.1. Materials

Medium molecular weight Chitosan (Sigma-Aldrich, Bangalore), sodium alginate (Titan Biotech Ltd, India), Calcium chloride and Pluronic F68 (Sigma-Aldrich, India), CWSB pheromone (Pest Control of India Pvt Ltd), ethanol and acetic acid (Merck Co, Mumbai) were obtained.

2.2. Nanoparticle preparation

2.2.1. Stock solution

A stock solution of calcium chloride was prepared by dissolving 0.554 g $CaCl_2$ in 5 ml of distilled water. Then, 0.2 g of sodium alginate was dissolved in 20 ml of distilled water; 0.1 g of chitosan was dissolved in 5 ml of 1% (V/V) acetic acid, 0.02 g of Pluronic F68 was then dissolved in 10 ml of distilled water.

2.2.2. Pre-gel preparation method

25 µl of pheromone was mixed with 3 ml of ethanol and 5 ml of 18 mM $CaCl_2$ standard solution and kept on a stirrer at 1000 rpm. Then 80 ml of sodium alginate solution was added while stirring. 15 ml of chitosan was added drop-wise to the above solution, while stirring, and then 2 ml of Pluronic F68 was added drop-wise. The whole solution was stirred for 2 hours at 1000 rpm. The particle suspension was centrifuged at 4 $^{\circ}$C in a centrifuge tube, using ultracentrifugation at 5000 rpm for 30 minutes to separate the free polymers from the nanoparticles. Nanoparticles in the centrifuge tube were collected and some of the nanoparticles suspended in the supernatant were collected by centrifuging the solution at 16000 rpm for 5 minutes at 4^0C.

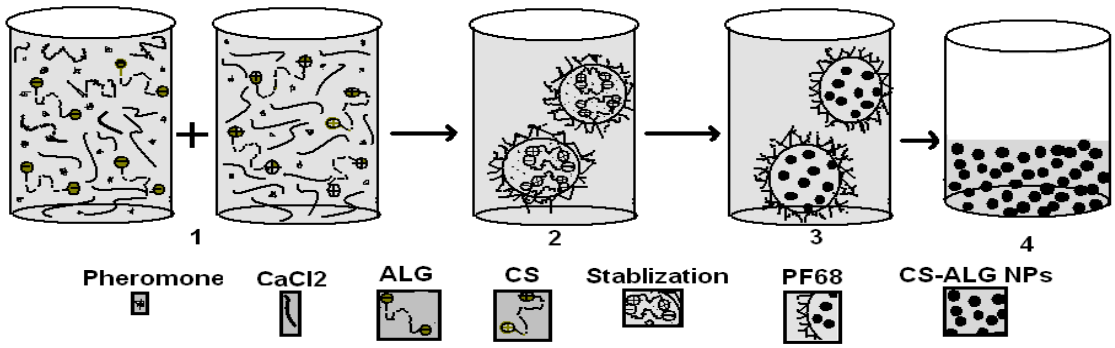


Figure 2. Schematic represenation of the pheromone CS-ALG NPs preparation by an ionic gelation method. **Step 1:** Aqueous solution having pheromone, CaCl2, ALG and CS. **Step 2:** Stabilized form of pheromone containing CS-ALG. **Step 3:** Pheromone CS-ALG particles in micelles (PF68). **Step 4:** Pheromone CS-ALG NPs separated in the aqueous phase.

For the pH, particle size, and zeta potential studies, the nanoparticles (1 g) were dispersed in 10 ml of distilled water using a Sonicator. Then, 18 aliquots were prepared with different pHs like natural pH (6.02), or regulated pH (8 to 12) in 3 replications, respectively, which were maintained by using 0.1N NaOH. After completing the reaction, the pH of the 25 µl CWSB sample was 6.02. After addition of the base (0.1N NaOH), the particles dispersed in the form of coagulated matter and became mostly invisible. On further addition of base when the pH reached 10, the coagulation completely disappeared, but on further addition of base when the pH reached 11 and 12, the coagulation of the particle reappeared in the form of a precipitation, which clearly indicated that at pH-10 the solution was stable.

2.3. Particle characterization

Nanoparticle size and zeta potential were assessed by photon correlation spectroscopy (PCS) using a Zeta PALS particle size analyzer (Brookhaven Instruments). For this procedure, a 2 ml sample was sonicated for 2 minutes in the Sonicator and measured immediately. All measurements were performed at room temperature.

III. Results and Discussion

Role of temperature and pH on size of nanoparticles is studied more in pharmaceutical field. To the best of our knowledge, first time we are reporting the effect of pH and temperature on pheromone loaded chitosan-alginate nanoparticles. Ghorashi *et al*. 2012 demonstrated controllable synthesis of silver nanoparticles using citric acid as complexing agent and studied the role of citrate ion species on size control of nanoparticles. The size of nanoparticles obtained at different pH was characterized using Scanning electron microscopy, Transmission electron microscopy, X-Ray Diffraction. The results showed that with increase in pH, citrate ion species concentration increases which inturn reduces the size of particles without any change in geometry of nanoparticles. Jayalakshmi *et al.* 2014 studied the role of pH on the size of gold nanoparticles using ascorbic acid as reducing and gum arabic as stabilizing agent. The study revealed that size of particle reduces when pH is reduced. The difference in results of Ghorashi *et al*. which shows that particle size decreases with increase in pH, and Jayalakshmi *et al.* which revealed that particle size decreases with decrease in pH, may be because of weaker reducing ability of citrate ions when compared to ascorbic acid as explained by Jiang *et al*. 2010. Kumar *et al*., 2015 studied the control release behaviour of nanocapsules prepared by the polyelectrolyte complexation of alginate and chitosan loaded with acetamiprid (neonicotinoid insecticide) at three different pH. It was reported that at pH 10 maximum release of acetamiprid occurs from nanocapsules followed by pH 7 and pH 4. The study concluded that acidic pH is best suited for controlled release of acetamiprid from nanocapsules. Grassian *et al*. 2011 focused on the dissolution and aggregation of well

characterized ZnO nanoparticles of 4nm in aqueous environment as a function of pH, ionic strength and natural organic matter. At neutral pH, the water properties including ionic strength and natural organic matter have an effect on stability of commercial ZnO nanoparticles. The study shows that dissolution rate is directly proportional to particle surface area and small nanoparticles dissolve faster than bulk materials. In an aqueous environment, natural organic matter adsorbs onto the surface of nanoparticles and such nanoparticles have enhanced stability at high ionic strength. Liu *et al*. 2012 investigated and exhibited controlled release from drug vessels AL/IBU/Fe_3O_4 nanoparticles and (AL/IBU/Fe_3O_4) @ $(CS\text{-}CMCS)_3$ nanoparticles at pH 1.8 and 7.4, which indicates that the drugs are pH responsive and at pH 7.4 drug release is more as compared to pH 1.8. Gallego Urrea *et al*. 2014 investigated the effect of pH on the stability of titania nanoparticles. They studied the stability of titania nanoparticles using sodium alginate, fulvic acid and humic acid at pH 2.5 and pH 12. They found that the stability increases with an increase in pH followed by a decrease in the surface charge of particles. Prabhakar Vattikuti *et al*. 2015 studied the effect of temperature on structural, morphological and magnetic properties of $Cd_{0.7}Co_{0.3}Fe_2O_4$ nanoparticles. They reported that the average particle size increased from 25 to 35nm as the calcination temperature increased from 300^0C to 600^0C, respectively, which indicates that with increase in temperature particle size increases. Y. Qu *et al.* 2006 worked on the optimum synthesis process of uniform $CoFe_2O_4$ nanoparticles with a coprecipitation technique and examined the effect of reaction temperature on particle size, crystal structure and magnetic properties of $CoFe_2O_4$ nanoparticles. Results showed that with an increase in temperature, particle size increases. In comparison to our study at pH 8 with an increse in temperature the diameter of nanoparticle remained constant from 40^0C-55^0C and gradually decreased from 60^0C-70^0C.
John Philip *et al*. 2007 investigated the effect of initial pH and temperature of iron salt solutions on the formation of magnetite nanoparticles. At the initial stages of pH 5 the size of nanoparticles remained the same. As the temperature and pH increased, the size of particle increased, which shows that initial pH and temperature are the important parameters for the control of size of nanoparticles. Guiying Li *et al*. 2014 studied the effect of pH and temperature for the controlled release of 5-fluorouracil from chitosan-graftpoly(N-isopropylacrylamide) (CS-g-PNIPAM) and sodium alginate-graft-poly(N-isopropylacrylamide) (SA-g-PNIPAM) polyelectrolyte complex. They found that the release of drug increases with increase in temperature and decrease in pH.

3.1. The role of pH at different temperatures on the mean effective diameter of coffee stem borer pheromone encapsulated in CS-ALG

The role of pH at different temperatures on the mean effective diameter of coffee stem borer pheromone encapsulated in CS-ALG is shown in Graph 1. At pH-6, the SEd value was 0.213, CD (5%) was 0.439 and CV% was 23.48, at pH-8, the SEd value was 0.117, CD (5%) was 0.242 and CV% was 13.13 which shows a significant effect of temperature and pH on the diameter of nanoparticles, at pH-9, the SEd value was 0.426, CD (5%) was 0.876 and CV% was 45.33, at pH-10, the SEd value was 1.47, CD (5%) was 3.02 and CV% was 98.43, at pH-11, the SEd value was 0.54, CD (5%) was 1.11 and CV% was 52, at pH-12, the SEd value was 2.81, CD (5%) was 5.78 and CV% was 94.17 which in all cases shows a non-significant effect of temperature and pH on the diameter of nanoparticles (Supplementary file: Table 1-6, Statistical analysis 1-6).

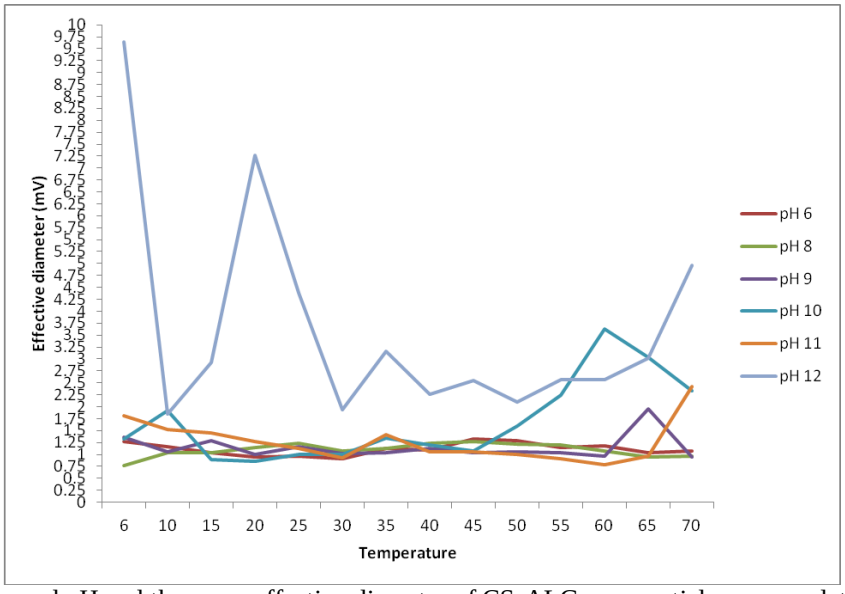
3.2. Determination of zeta potential at different pH

The stability of colloidal systems is directly related to the magnitude of their zeta potential. In general, if the zeta potential is large, the colloidal system will be stable; conversely, if the zeta potential is small, the colloidal system will more easily agglomerate. The surface charge of the particles is of substantial importance in all the production steps of the particles because the efficiency of the different steps is directly related to the establishment of electrostatic interactions. The effect of pH 6, 8, 9, 10,11

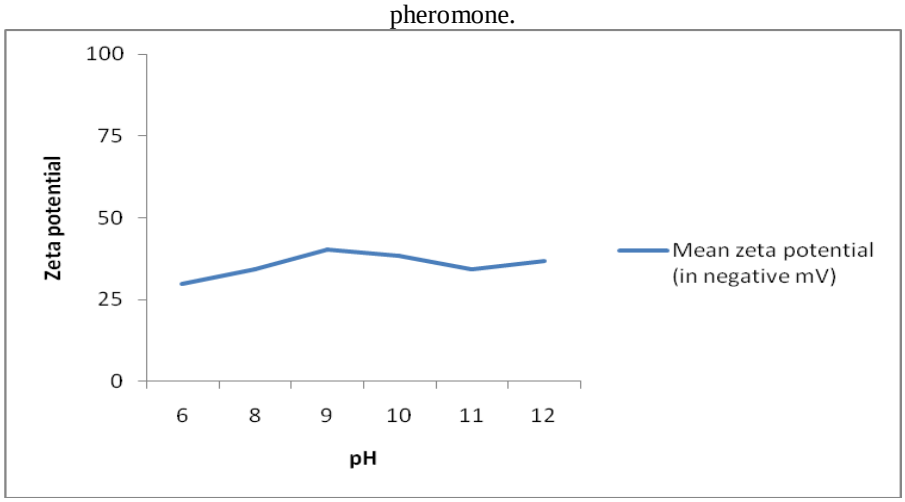
and 12 on the zeta potential of CS-ALG NPs loaded with pheromone at temperature 25°C was studied as shown in Graph 2. At pH 6, 8, 9, 10,11 and 12, the zeta potential in negative mV are 29.97, 34.41, 40.32, 38.39, 34.31 and 36.69. The SEd was 5.195, the CD (5%) was 11.56 and the CV% was 17.82. Results showed that pH has a non-significant role on zeta potential (Supplementary file: Table 8-11, Statistical analysis 7).

3.3. Determination of Effective diameters of CS-ALG NPs at different temperatures and pH

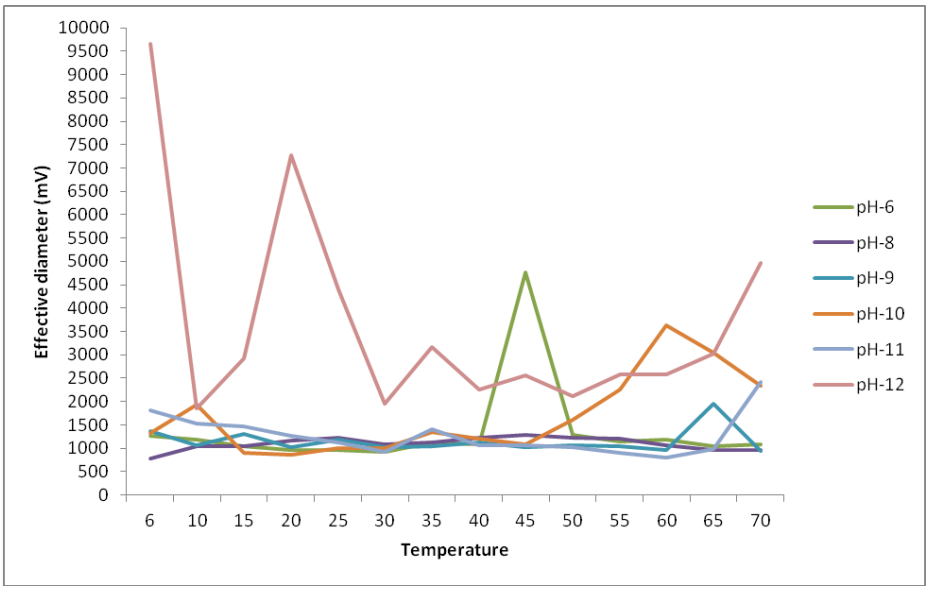
Effective diameters of CS-ALG NPs were studied over a range of temperatures from 6 to 70 ^{0}C and at different pH 6, 8-12 as shown in Graph 3. At temperature 40 ^{0}C, effective diameter in mV at pH 6, 8, 9, 10,11 and 12 are 1090.3, 1232.7, 1124, 1207.8, 1067.8 and 2263.4, the SEd was 342.89, the CD (5%) was 764.02 and the CV% was 31.55 and at temperature 45 ^{0}C, effective diameter values in mV at pH 6, 8, 9, 10,11 and 12 are 4756, 1289.8, 1032.5, 1080.1, 1056.4 and 2555.8, SEd value was 403.86, CD (5%) value was 899.86 and CV% value was 35.55 showed a significant role with respect to temperature and pH (Supplementary file: Table 12, Graph 4 & 5). At temperature 6 ^{0}C, effective diameter in mV at pH 6, 8, 9, 10,11 and 12 are 1272,773.66, 1375, 1334, 1821.5 and 9658.2, the SEd was 3136.39, the CD (5%) was 6988.36 and the CV% was 144.97, at temperature 10 ^{0}C, effective diameter in mV at pH 6, 8, 9, 10,11 and 12 are 1177.2, 1041.9, 1064.01, 1929, 1525.3 and 1847.8, SEd value was 649.47, CD (5%) value was 1447.12 and CV% value was 55.59, at temperature 15 ^{0}C, effective diameter in mV at pH 6, 8, 9, 10,11 and 12 are 1039.3, 1038.2, 1299.4, 892.33, 1462.5 and 2923.6, the SEd was 377.81, the CD (5%) was 841.82 and the CV% was 32.08, at temperature 20 ^{0}C, effective diameter values in mV at pH 6, 8, 9, 10,11 and 12 are 955.03, 1156.7, 1014.6, 853.2, 1271.4 and 7271.7, the SEd was 2891.41, the CD (5%) was 6442.51and the CV% was 169.69, at temperature 25 ^{0}C, effective diameter in mV at pH 6, 8, 9, 10,11 and 12 are 1039.3, 1038.2, 1299.4, 892.33, 1462.5 and 2923.6, the SEd was 377.81, the CD (5%) was 841.82 and the CV% was 32.08, at temperature 30 ^{0}C, effective diameter in mV at pH 6, 8, 9, 10,11 and 12 are 913.9, 1084.7, 1026.7, 1000.8, 930.1 and 1949.2, the SEd was 316.41, the CD (5%) was 705.01 and the CV% was 33.67, at temperature 35 ^{0}C, effective diameter in mV at pH 6, 8, 9, 10,11 and 12 are1126.9, 1127.5, 1036.2, 1348.6, 1415.9 and 3167.8, the SEd was 1000.52, the CD (5%) was 2229.3 and the CV% was 79.72, at temperature 50 ^{0}C, effective diameter in mV at pH 6, 8, 9, 10,11 and 12 are 1291.7, 1229.7, 1055.3, 1606.9, 1019.7 and 2105.6, the SEd was 641.87, the CD (5%) was 1430.18 and the CV% was 56.77, at temperature 55 ^{0}C, effective diameter in mV at pH 6, 8, 9, 10,11 and 12 are 1147, 1201.3, 1049.2, 2259.4, 910.7 and 2577.3, the SEd was 802.43, the CD (5%) was 1787.94 and the CV% was 64.48, at temperature 60 ^{0}C, effective diameter in mV at pH 6, 8, 9, 10,11 and 12 are 1188.4, 1071.9, 970.9, 3634.7, 792.7 and 2576.9, the SEd was 1604.74, the CD (5%) was 3575.61 and the CV% was 115.21, at temperature 65 ^{0}C, effective diameter in mV at pH 6, 8, 9, 10,11 and 12 are 1033.6, 956.9, 1956.2, 3044.4, 978.7 and 3018.4, the SEd was 1433.15, the CD (5%) was 3193.29 and the CV% was 95.84, at temperature 70 ^{0}C, effective diameter in mV at pH 6, 8, 9, 10,11 and 12 are 1083.5, 958.4, 940.8, 2338.8, 2424 and 4966.5, the SEd was 1462.3, the CD (5%) was 3258.24 and the CV% value was 84.53 which in all cases showed a non-significant role with respect to temperature and pH (Supplementary file: Table 12).



Graph 1. Temperature and pH and the mean effective diameter of CS-ALG nanoparticles encapsulating coffee stem borer pheromone.



Graph 2. Zeta Potential of chitosan-alginate nanoparticles loaded with coffee white stem borer pheromone at various pH



Graph 3. Effective Diameters (mV) at various temperatures and pH.

IV. Conclusion

Effects of temperature and pH were studied on the morphology of bio-polymeric chitosan-alginate nanoparticles loaded with Coffee white stem borer (CWSB) pheromone. The data when subjected to ANOVA showed that at various pH readings (6, 9, 10, 11 and 12) and with temperatures over a range from 6 to 70 °C, showed there were no effects on nanoparticle size. Similarly, the zeta potential analysis results for the chitosan-alginate nanoparticles (CS-ALG NPs) loaded with the pheromone and zeta potential at pH range 6 - 12 was not significantly correlated when subjected to ANOVA. The present study indicated that generally pH has a non-significant effect on zeta potential. However, at pH 8 the effect of temperature was found to be significantly correlated with particle size. This study helps in understanding the determination of the size of nanoparticles and their loading capacity for pheromones.

V. Acknowledgement

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