OPTIMIZATION OF REACTION PARAMETERS FOR THE BIOSYNTHESIS OF SELENIUM NANOPARTICLES MEDIATED BY *BACKHOUSIA CITRIODORA* AQUEOUS EXTRACT

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(Received 27th Jun 2024; accepted 22nd Oct 2024)

Abstract. The biosynthesis of metallic nanoparticles (NPs) has drawn increasing interest due to its simple, non-toxic, cost-effective, and environmentally friendly approach. Compared with other biological methods in NPs production, plant-mediated synthesis of NPs is favored because it is faster and more stable. This study aimed to optimize the reaction conditions for the biosynthesis of selenium nanoparticles (SeNPs) using *Backhousia citriodora* leaf aqueous extract (BCAE). Four reaction conditions were optimized for synthesizing *B. citriodora* SeNPs (BC-SeNPs), including precursor sodium selenite (Na_2SeO_3) concentration, reaction temperature, time, and pH. The biosynthesis of BC-SeNPs was confirmed via the color change and UV-Vis spectrophotometric analysis. The phytochemical screening of BCAE revealed the presence of phenols and phenolics, flavonoids, alkaloids, tannins, carbohydrates, and steroids. The change from the yellow color of the reaction mixture to red confirmed the successful synthesis of BC-SeNPs. UV-Vis spectra analysis revealed a single surface plasmon resonance (SPR band) in all optimization groups at 380-450 nm and assigned to Se^0. The findings of the study indicated that the optimal condition for the biosynthesis of BC-SeNPs was 50 mM of Na_2SeO_3, 50˚C, pH 8, and 24 hours of reaction time, validated by the position of the SPR bands and intensity using UV-Vis spectrophotometer. The short-term stability test results demonstrated that the synthesized BC-SeNPs were stable in water at room temperature. The current study led to the biosynthesis of eco-friendly SeNPs employing BCAE as stabilizing and reducing agents.
Keywords: *SeO_3^{2-} ions, plant extract, green synthesis, UV-visible spectrometry, Surface plasmon resonance*

Introduction

In recent years, there has been an increasing attention on selenium (Se) owing to its significant contribution to human well-being. However, excessive intake or prolonged supplementation may pose potential risks to humans, as indicated by Rayman (2020). Therefore, compared to its organic and inorganic equivalents, nanoscale Se has become popular globally due to its superior absorption capacity, notable bioactivity, minimal toxicity, and remarkable efficacy in reducing oxidative damage (Zambonino et al., 2023). Unlike bulk materials, nanoparticles (NPs) have unique qualities such as small size, low polydispersity, large surface area, high stability in colloidal form, capacity to modify their surface chemistry and surface charge rapidly, and multifunctionalization (Zambonino et al., 2023). To the best of our knowledge, NPs can be synthesized by chemical, physical, and biological processes. Nevertheless, the traditional methods, which include the chemical and physical formation of NPs, have several drawbacks,

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2301_12031221

including the need for expensive, specialized equipment, toxic, and in many cases, excessive expenses.

The "green synthesis" approaches have emerged as a prominent trend in nanotechnology and materials science, gaining great attention in recent years. Unlike conventional methods, often involving high-energy processes and hazardous chemicals, green synthesis approaches employ environmentally friendly and sustainable resources, such as plant extracts and microbes, to produce NPs. Utilizing biological materials, including enzymes, bacteria, algae, yeast, fungi, and plants, allows for the clean, safe, affordable, and accessible synthesis of NPs through biological processes (Ghaderi et al., 2022). Furthermore, biogenic synthesis employing plant extracts has several advantages compared to other biological entities like bacteria. These include quick single-step synthesis, high plant metabolites that improve reduction and NPs stability, and biosafety compared to some microorganisms (Fouda et al., 2022). Additionally, green SeNPs synthesis has significant promise for biomedical applications due to its remarkable biological features such as antioxidant, antibacterial, antiparasitic, anti-inflammatory, anticancer, and antidiabetic effects (Mikhailova, 2023).

Recently, the production of NPs has emphasized green synthesis, implementing extracts from medicinal plants that have increased in popularity. In particular, phytochemicals are the foundation of plants, which contribute to the stability of NPs with less toxicity (Alagesan and Venugopal, 2019). These phytochemical compounds, such as flavonoids, terpenoids, vitamins, polysaccharides, and others, may contribute to the reduction process and the capping of SeNPs. These properties prevent SeNPs from clumping together in an aqueous solution during the green synthesis process and encourage the production of smaller SeNPs (Mikhailova, 2023).

Backhousia citriodora (BC), commonly known as lemon myrtle, is an aromatic type of plant high in phytochemicals such as gallic acid, myricitrin, hyperin, and quercitrin (Yamamoto et al., 2022). *B. citriodora,* from the Myrtaceae family, is gaining increasing interest in Malaysia due to its various biological functions. The solvent extracts and essential oil of BC have been shown to exhibit high antioxidant, antimicrobial and antibiofilm activities (Lim et al., 2022). Several studies indicated that plant-based SeNPs offer promising potential as a sensory probe, therapeutic drug delivery with precision, anticancer agents, heavy metal detectors, and strong antibacterial capabilities. Additionally, the potential for biosynthesis of SeNPs from BC remains unexplored. Therefore, this study aimed to develop a sustainable approach for biosynthesizing SeNPs mediated by *B. citriodora* leaf aqueous extract (BCAE) by optimizing various reaction conditions.

Materials and methods

Plant specimen collection and authentication

The leaves of *Backhousia citriodora* were collected from an organic lemon myrtle plantation located in Kuala Linggi (2°23'17.6"N 101°59'01.1"E), Malacca, Malaysia. The plant specimen was taxonomically verified by a botanist from the Institute of Bioscience (IBS), Universiti Putra Malaysia (UPM). The voucher specimen with the number MFI 0145/19 has been deposited at IBS, UPM.

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Preparation of Backhousia citriodora leaf aqueous extract (BCAE)

The leaves were cleaned thoroughly with tap water to remove dust and contaminants. The leaves were then air-dried at room temperature for one week. After drying, the leaves were ground into powder with a mixer grinder. To prepare the BCAE, 20 g of the grounded leaf was added to 500 ml of sterile boiled distilled water. The mixture was left on a hot plate for 10 minutes (Meenambigai et al., 2022). The leaf BCAE was cooled and filtered through Whatman filter paper No.1. The resulting BCAE was collected and stored at 4°C until further use.

Qualitative phytochemical analysis

The qualitative phytochemical screening tests for BCAE were performed using standard methods to detect the presence of phytoconstituents, including phenols and phenolics, flavonoids, alkaloids, tannins, carbohydrates, and steroids (Shaikh and Patil, 2020). All qualitative phytochemical screening tests were conducted in triplicates in three independent experiments.

Biosynthesis of Backhousia citriodora selenium nanoparticles (BC-SeNPs)

The biosynthesis of BC-SeNPs was conducted according to the protocol described by Sani-e-Zahra et al. (2022) with some modifications. To biosynthesis BC-SeNPs, solutions with 4:1 (BCAE: Na_2SeO_3) reaction mixtures were prepared for 10 mM, 25 mM, 50 mM, and 100 mM Na_2SeO_3, respectively. The reaction mixtures were vortexed homogenously and incubated at 37˚C for 24 hours. The color of the reaction mixtures was observed and recorded after overnight incubation.

Optimization at different reaction conditions for BC-SeNPs synthesis

Parameters such as different (i) concentrations of Na_2SeO_3 (10 mM, 25 mM, 50 mM, and 100 mM), (ii) reaction temperature (4°C, 25°C, 37°C, 50°C, and 60°C), reaction time (24 h and 48 h) and pH (pH 6, 8 and 10) were adjusted to find out the ideal conditions for biosynthesis of BC-SeNPs. In order to determine the effect of pH on the BC-SeNPs synthesis, 1 M of sodium hydroxide (NaOH) and hydrochloric acid (HCl) were used to maintain the desired pH. The optimization was carried out in three replicates at each reaction parameter across three independent experiments.

Characterization by UV-Vis spectrophotometer analysis

The initial bio-reduction of Na_2SeO_3 by BCAE was monitored by observing the color change. The absorbance of the synthesized NPs was measured using UV-Vis spectroscopy (Spectroquant Pharo 300, Merck) at wavelengths ranging from 200 to 800 nm with 2 nm intervals to detect intense absorption peaks associated with surface plasmon excitation. The blank was represented by BCAE without the addition of Na_2SeO_3.

Stability test

The optimized BC-SeNPs reaction solution was centrifuged at 10,000 rpm for 10 min to remove unwanted components. The supernatant was decanted to collect the pellets. Then, the pellet containing BC-SeNPs was washed several times using sterile distilled water, and the purified pellets were resuspended in sterile distilled water for a stability

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test. The resuspended purified BC-SeNPs were kept in darkness for 10 days at room temperature, and their stability was determined by measuring the absorbance in the wavelength range of 200 to 800 nm using a UV-Vis spectrophotometer (Kokila et al., 2017).

Statistical analysis

The findings were analyzed using IBM SPSS for Windows version 23.0 software. The absorbance intensities for optimization of BC-SeNPs synthesis using different Na_2SeO_3 concentrations and reaction temperatures were subjected to One-Way Between Groups Analysis of Variance (ANOVA). Duncan's Multiple Range test (DMRT) was applied as a post-hoc comparison test to determine their differences. An Independent Sample T-test was performed to determine the differences in the absorbance intensities for reaction time (24 and 48 hours). All differences were considered significant at $P < 0.05$.

Results

Qualitative phytochemical screening tests for BCAE

The results of the qualitative phytochemical tests for BCAE detected the presence of phenols and phenolic compounds, flavonoids, tannins, carbohydrates, steroids, and alkaloids, as indicated in *Table 1*.

Table 1. *Qualitative phytochemical screening of BCAE*

Test	Phytochemicals	Observations	*Inference
Ferric chloride test	Phenols and phenolic compounds	Bluish black color	+
Alkaline test	Flavonoids	Loss of intense yellow color after addition of diluted HCl	+
10% NaOH test	Tannins	Formation of emulsion	+
Molish's test	Carbohydrates	Appearance of violet ring	+
H_2SO_4 test	Steroids	Formation of an interface with a reddish-brown coloration	+
Mayer's test	Alkaloids	Formation of a cream precipitate	+

*+ means the presence of the phytochemical

Green synthesis of BC-SeNPs

In the present study, the BC-SeNPs were successfully synthesized using BCAE. The BCAE was mixed up with Na_2SeO_3 solution in 4:1 ratios. The BCAE reduced the Se salt; hence, BC-SeNPs were produced after incubation. The color of the reaction mixture changed immediately after mixing up the BCAE and Na_2SeO_3 solutions. *Figure 1* shows the clear Na_2SeO_3 solution (*Figure 1A*), yellowish solution of Na_2SeO_3 with BCAE mixture (*Figure 1B*) to red synthesized BC-SeNPs solution (*Figure 1C*) after 24 h incubation. The red color solution was formed after incubation, which indicated the biosynthesis of BC-SeNPs. *Figure 2* presents the UV-Vis absorption spectra of the biosynthesized BC-SeNPs, alongside the absorption spectra of the pure BCAE and Na_2SeO_3 solution. The results indicated that the Na_2SeO_3 solution did not give any absorption peak, while BCAE showed a sharp absorption peak at 232 nm and

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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BC-SeNPs displayed a sharp absorption peak at 388 nm. The biosynthesis of SeNPs was optimized by adjusting several reaction parameters and confirmed by measuring the surface plasmon resonance (SPR) band and absorbance peak using a UV-Vis-spectrophotometer. The UV-Vis spectra demonstrated the SPR wavelength in the 380-450 nm range, with a single SPR band observed for all optimization parameters.

Figure 1. *(A) Na_2SeO_3 solution, Colour change from the yellow mixture of BCAE with Na_2SeO_3 (B) to red BC-SeNPs solution (C) after 24 h incubation*

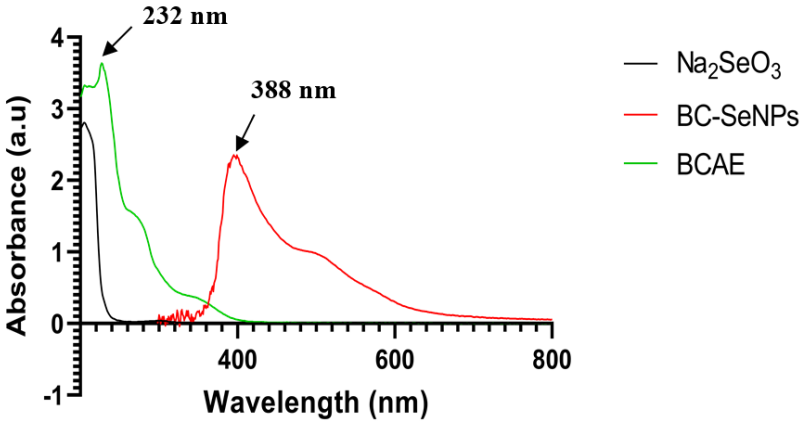

Figure 2. *UV-Vis spectra analysis of BCAE, BC-SeNPs, and Na_2SeO_3 solution*

Effects of concentration of Na_2SeO_3 solution on the biosynthesis of BC-SeNPs

To obtain the maximum amount of BC-SeNPs synthesis, the concentration of Na_2SeO_3 solution was optimized at 10 mM, 25 mM, 50 mM, and 100 mM in this study (*Figure 3*). The results showed that the absorbances increased in accordance with the concentrations of Na_2SeO_3 solutions. The UV-Vis spectrophotometric analysis demonstrated that the absorbance of the 100 mM Na_2SeO_3 solution was the highest, with a prominent SPR band at 404 nm, followed by 50 mM, 25 mM, and 10 mM. However, the SPR band produced by 100 mM Na_2SeO_3 solution exhibited a longer wavelength, broader peak and more noise than 50 mM and 25 mM Na_2SeO_3 solutions. Therefore, 50 mM Na_2SeO_3 solution was selected as an optimum concentration for BC-SeNPs synthesis since it exhibited higher SPR intensity than 25 mM Na_2SeO_3 in this study.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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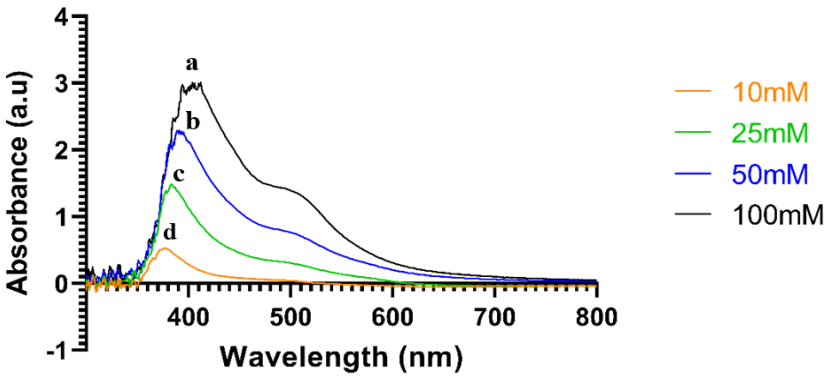

Figure 3. *UV-Vis spectra analysis of BC-SeNPs for different concentrations of Na_2SeO_3. Reaction pH (pH 8), temperature (37˚C), and time (24 h) remained constant. The optimum Na_2SeO_3 concentration was 50 mM. a-d, the mean of absorbance readings with different letters differs significantly at $P < 0.05$*

Effects of reaction temperature on the biosynthesis of BC-SeNPs

The UV-Vis spectra analysis of five different reaction temperatures (4°C, 25°C, 37°C, 50°C, and 60°C) on the biosynthesis of BC-SeNPs with 50 mM Na_2SeO_3 were presented in *Figure 4*. The increase in the reaction temperature from 4 to 50°C increased the SPR intensity, showing an increase in BC-SeNPs synthesis rate. At 4˚C, the BC-SeNPs displayed the lowest ($P < 0.05$) SPR intensity, with the SPR wavelength at 359 nm. It was noted that the biosynthesis of BC-SeNPs was diminished at 60°C, as shown by a reduction in the SPR intensity compared to that at 50°C. In this study, the SPR intensity of BC-SeNPs on the five tested reaction temperatures at 50 mM Na_2SeO_3 is indicated in the following decreasing order: 50°C > 37°C > 60°C > 25°C > 4°C as shown in *Figure 4*. The findings revealed that the highest ($P < 0.05$) absorbance of BC-SeNP synthesis was observed at 50°C, which had a strong SPR at 395 nm. According to these observations, the optimized reaction temperature for the biosynthesis of BC-SeNPs with 50 mM Na_2SeO_3 was 50˚C.

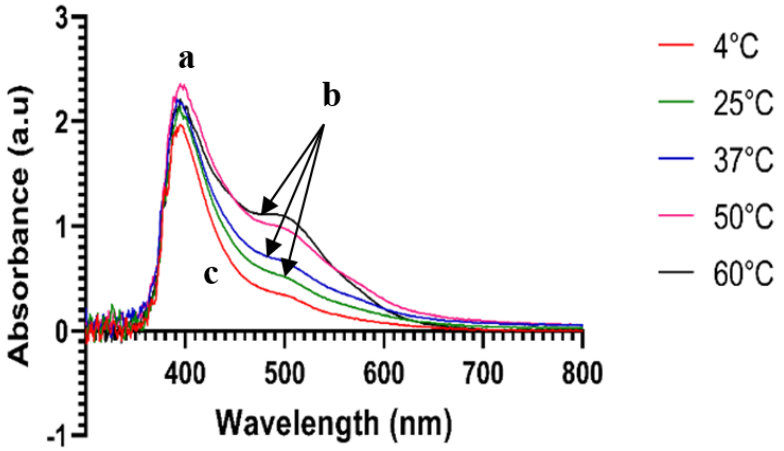

Figure 4. *UV-Vis spectra analysis of BC-SeNPs at different reaction temperatures with 50 mM Na_2SeO_3. The pH level (pH 8), 50 mM concentration of Na_2SeO_3 and reaction time (24 h) remained constant. The optimum reaction temperature was 50 ˚C. a-c, the mean of absorbance readings with different letters differs significantly at $P < 0.05$*

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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Effects of reaction time on the biosynthesis of BC-SeNPs

As shown in *Figure 5*, the UV-Vis spectrophotometric analysis of BC-SeNPs indicated that a 24-h reaction time yielded significantly ($P < 0.05$) higher SPR intensity at 395 nm, compared to a 48-h reaction time (394 nm). Based on the findings, the optimized time of incubation for BC-SeNPs synthesis using 50 mM Na_2SeO_3 was 24 h.

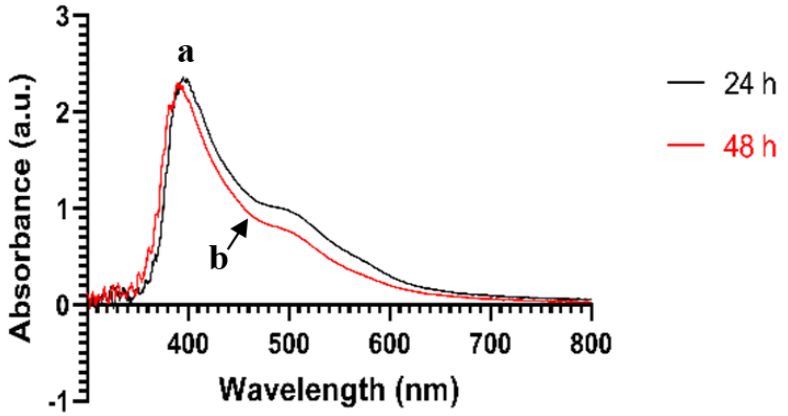

Figure 5. *UV-Vis spectra analysis of BC-SeNPs at 24 h and 48 h reaction time. The pH level (pH 8), concentration of Na_2SeO_3 (50 mM) and reaction temperature (50˚C) remained constant. The optimum reaction time was 24 hours. a,b, the mean of absorbance readings with different letters differs significantly at $P < 0.05$*

Effects of pH on the biosynthesis of BC-SeNPs

The present study investigated the green synthesis of BC-SeNPs at pH 6, 8 and 10 for 50 mM Na_2SeO_3 (*Figure 6*). As indicated in *Figure 6*, there was an initial BC-SeNPs synthesis, but no characteristic SPR peak was shown at the reaction mixture of pH 6. This observation suggested that the synthesis of BC-SeNPs was not favorable in acidic conditions. The best SPR absorption for BC-SeNPs synthesized with 50 mM Na_2SeO_3 was observed at pH 8, with a strong SPR band centered at 389 nm. The current study revealed that the BC-SeNPs synthesis was compromised or inhibited under high alkaline conditions, specifically at pH 10, indicated by the absence of the characteristic SPR peak and broader band. Therefore, the optimized pH to biosynthesis BC-SeNPs at 50 mM Na_2SeO_3 was pH 8.

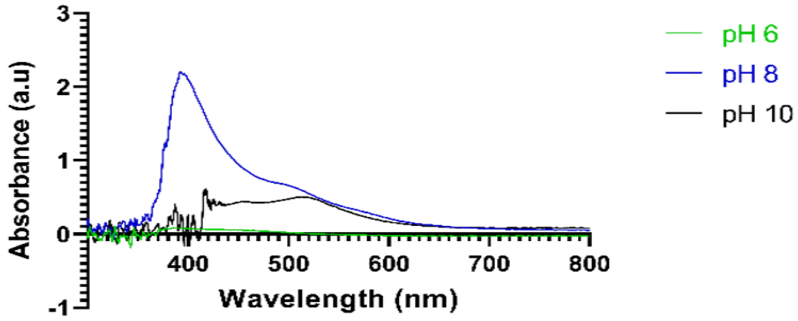

Figure 6*. UV-Vis spectra analysis of BC-SeNPs at different pH. The concentration of Na_2SeO_3 (50 mM), reaction time (24 h) and incubation temperature (50˚C) remained constant. The optimum reaction pH was pH 8*

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DOI: http://dx.doi.org/10.15666/aeer/2301_12031221

Stability test

Figure 7 illustrates the UV-Vis spectra analysis of the optimized BC-SeNPs in water for 10 days at room temperature. The study results indicated that the peak positions of the absorption spectra for the optimized BC-SeNPs were consistent, and no significant changes were observed during the 10 days. Optimized BC-SeNPs were not agglomerated over the 10-day stability testing period.

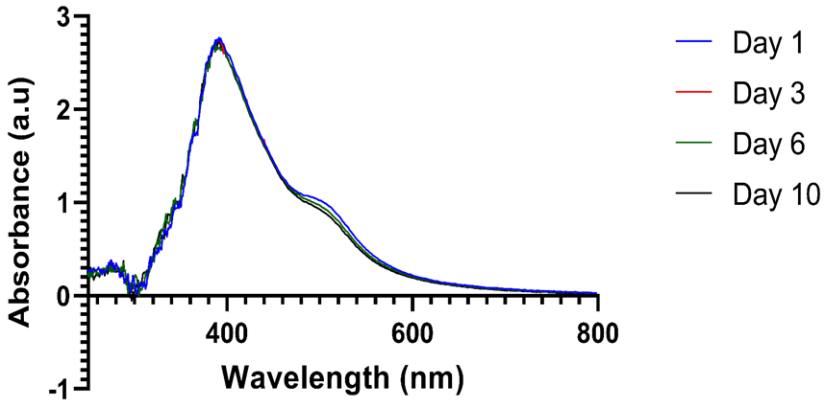

Figure 7. *UV-Vis spectra analysis of optimized BC-SeNPs 10 days for stability testing. The optimized BC-SeNPs were synthesized using 50 mM Na_2SeO_3 with pH 8 at 50˚C for 24 h*

Discussion

Green synthesis of BC-SeNPs

The green synthesis of metallic NPs using plants has been reported in several studies (Alagesan and Venugopal, 2019; Sheikhlou et al., 2020; Ghaderi et al., 2022), which is the most environment-friendly route for NP synthesis. According to the previous paper (Shnoudeh et al., 2019), the metal salt solution and plant extract are combined at room temperature in a simple synthesis procedure that produces a change in solution color that indicates the presence of NPs in the reaction mixture. The reaction pH, temperature, time, concentration of metal salts, and additional features of the plant extract affect the size, shape, and quality of the synthesized NPs.

In the present study, the BCAE was chosen and presented as the mediator for the synthesis of SeNPs. The observed color change from light yellow to red confirmed the rapid formation and nucleation of BC-SeNPs. The appearance of red color can be attributed to the SPR effect and the conversion of selenium oxyanion (SeO_3^{2-}) to elemental selenium (Se^0) by BCAE. The results of the qualitative phytochemical screening revealed the presence of secondary metabolites such as phenols and phenolic compounds, flavonoids, tannins, carbohydrates, steroids, and alkaloids in BCAE, which play a vital role in the biosynthesis of BC-SeNPs. These phytochemicals may act as reducing and capping agents during the biosynthesis of SeNPs (Chetehouna et al., 2024).

In the current study, the absorption spectrum of the BCAE was recorded to examine its interaction with and reduction of Se ions by the BCAE. UV-Vis spectra analysis was useful in identifying aromatic rings, chromophoric groups, π-bonds, σ-bonds, and lone pairs of electrons in the phytochemicals. It has been documented that the presence of

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heteroatoms and unsaturated groups in the phytochemicals, including nitrogen (N), oxygen (O) and sulfur (S), is indicated by the appearance of one or more peaks in the UV-Vis spectra, especially in the range of 200-400 nm (Singh et al., 2024). These signals highlight the electronic transitions occurring within these molecular structures. Therefore, the sharp and narrow peak at 232 nm observed in the BCAE was mostly attributed to the UV absorption of polyphenols (Mabasa et al., 2021). Several studies have indicated that the distinct peaks detected in the wavelength ranging from 230 to 350 nm corresponded to the presence of flavonoids, phenolic compounds and their derivatives (Renuka et al., 2016; Patle et al., 2020; Mabasa et al., 2021). As indicated in *Figure 2*, the absorption band of BC-SeNPs was recorded at 388 nm, which was the characteristic band of SeNPs due to SPR. In the present study, the peak at 232 nm from the BCAE was found to have completely disappeared after the biosynthesis of BC-SeNPs. This indicated that the BCAE consisting of polyphenolic compounds such as flavonoids and phenolic acids could potentially reduce and stabilize the Se ions and lead to the biosynthesis of BC-SeNPs at 388 nm. Therefore, this demonstrated that the interaction between the BCAE phytochemicals and the Se ions in the solution influences the optical transition corresponding to this peak in the study.

The biosynthesis of BC-SeNPs in this study contributed by the rich content of phytochemicals such as phenolic acids and flavonoids in BCAE (Yamamoto et al., 2022; Cáceres-Vélez et al., 2024), which play important roles in reducing Se ions and stabilizing synthesized BC-SeNPs. The availability and high amount of flavonoids in BC leaves, such as quercitrin (38.8%), hyperin (19.1%) and myricitrin (12.6%) (Yamamoto et al., 2022), function as reducing agents by donating electrons or hydrogen atoms, especially those located on the catechol B-ring and pyran C-ring, to the Se ions. This process is linked to their oxygen-scavenging and antioxidant properties, which reduce Se ions to elemental selenium (Se^0), as evidenced by a color change from light yellow to red. Quercitrin, a glycoside form of quercetin, is notably effective due to its ability to chelate metal ions with their carbonyl groups or electrons. This similar mechanism is also observed in other plant-derived NP syntheses (Jain and Mehata, 2017; Pandian et al., 2021). Similarly, phenolic compounds found in the BCAE, including gallic acid, catechin and epicatechin (Cáceres-Vélez et al., 2024), facilitate the reduction of Se ions via mechanisms such as (1) hydrogen atom transfer (HAT), (2) single-electron transfer—proton transfer (SET-PT), and (3) sequential proton-loss-electron transfer (SPLET). These mechanisms are similar to their antioxidant action (Rodríguez-Félix et al., 2022).

Regarding stabilization, phytochemicals in BC play a vital role in inhibiting the aggregation of NPs. Phenolic compounds such as gallic acid in BC could stabilize NPs by adsorbing onto their surfaces via steric hindrance and electrostatic interactions. A similar mechanism has been indicated in *Terminalia cuneata* bark extract, which is rich in tannins, polyphenols, and gallic acids, and contains a high density of hydroxyl groups. In this case, the phenolic compounds of *T. cuneata* bark extract oxidize into quinone forms, driving NP stabilization via the quinone structures' lone pair and pi electrons (Edison et al., 2016). This stabilization mechanism through quinone structures helps maintain NP stability in the solution. Furthermore, flavonoids of plant extract stabilize the synthesized SeNPs via resonance, where the structure of hydroxyl groups distributes the electron density, resulting in relatively stable flavonoid radicals. This stabilization process can prevent the aggregation of SeNPs, leading to the synthesis of uniform NPs and enhancing their stability (Dias et al., 2021; Rodríguez-Félix et al.,

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2022). Generally, the synergistic action of flavonoids and phenolic compounds in reducing and stabilizing makes BC an eco-friendly and effective agent for SeNP synthesis.

UV-Vis spectrophotometric analysis

UV-Vis spectroscopy is a simple and reliable method commonly used to monitor the stability of NP solutions. The formation of SeNPs is primarily verified by applying the UV-Vis spectroscopy technique (Desai et al., 2012), with absorption values in the wavelength range of 300-600 nm (Alvi et al., 2021). Hence, the UV-Vis spectra analysis was carried out to confirm the biosynthesis of SeNPs from Na_2SeO_3 mediated by BCAE in the current study. This was done because of a time-dependent visual change in which the color of BC-SeNPs turned red, indicating that the Na_2SeO_3 solution could be biologically reduced to the Se^0. SeNPs demonstrate the features of the SPR vibration effects, as shown by the present study, displaying the absorption peak within the 380-450 nm range, with a single prominent peak across all optimization parameters. A similar finding was reported by Alvi et al. (2021), where the synthesized SeNPs from *Citrus paradisi* and *Citrus limon* showed absorbance peaks between 300–550 nm. Another recent study also reported that biosynthesis of SeNPs study using *Moringa oleifera* aqueous extract provided an absorption peak between 400-520 nm (Ahamad Tarmizi et al., 2023).

Effects of reaction parameters for biosynthesis of BC-SeNPs

Various studies have been conducted on the green synthesis of NPs using different plant extracts; however, very few investigations have been reported on the optimization of NP fabrication (Fattah, 2021). Hence, this study evaluated the effects of several reaction parameters on the biosynthesis of SeNPs mediated by BCAE. These reaction parameters were optimized to obtain the highest quantity of BC-SeNPs in a constant reaction volume. Studies have shown that reaction parameters play a significant role in the physicochemical characteristics of green synthesis NPs (Ahamad Tarmizi et al., 2023; Azad et al., 2023).

In the current study, four reaction parameters in the biosynthesis of BC-SeNPs were optimized: (i) concentration of Na_2SeO_3 solution, (ii) reaction temperature, (iii) reaction time, and (iv) pH. These reaction parameters were optimized in this study according to the earlier studies on the biosynthesis of SeNPs using plant extracts (Alam et al., 2019; Ahamad Tarmizi et al., 2023; Shah et al., 2023). It has been reported that the SPR bands are particularly useful in the characterization of NPs, attributing to the sensitivity of the UV-Vis optical characteristics to variations in shape, size, aggregation, composition, and dielectric environment of the synthesized NPs (Hu et al., 2017; Philip and Kumar, 2022; Negri et al., 2024). The variations in NP's shape, such as spherical, triangular and rod-like, can lead to the formation of distinct SPR peaks due to variations in electron density distribution. Mie's theory states that isotropic NPs (such as spheres) usually display a single SPR peak, while anisotropic (such as rods) typically exhibit multiple absorption peaks corresponding to different orientations (Mie, 1908; Marhaba, 2018; Ezealisiji et al., 2019). In the present study, a single SPR peak was observed throughout the optimization process for all reaction parameters, and it is proposed that the synthesized BC-SeNPs were considered spherical in shape.

The size of SeNPs can be controlled by varying the concentration of the precursor used, which in this case is Na_2SeO_3. The present study observed that as the

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DOI: http://dx.doi.org/10.15666/aeer/2301_12031221

concentrations of Na_2SeO_3 increased (10-100 mM), the single SPR band shifted to a longer peak wavelength, the peak became broader, and the SPR intensity increased while keeping other reaction parameters (pH, reaction time, temperature, and ratio of extract:precursor) constant. It has been documented that the peak position of the SPR band in the absorption spectrum is strongly associated with the size of the NPs. Generally, as the NP size increases, the peak wavelength at which the SPR appears also increases. This demonstrates that larger NPs will exhibit SPR bands with a longer peak wavelength (called red-shifting), while smaller NPs will show SPR bands at a shorter peak wavelength (Sharma et al., 2024). This relationship arises due to the quantum confinement effect, where the electronic properties of the NPs are influenced by their size. The larger NPs displayed increased scattering and required less energy for their electron oscillations (plasmon resonances), resulting in longer SPR wavelengths of absorbed or scattered light (Li et al., 2015), while the smaller NPs work in the opposite way. Additionally, the larger NPs scatter more light due to their larger optical cross sections and albedo (ratio of scattering to total extinction), which increases with NP size (Shafiqa et al., 2018). The peak broadening exhibited by larger NPs due to the greater variations in the light path lengths within the NPs, increased scattering effects, and multipolar plasmon modes (higher-order plasmon resonance). All these effects lead to a broader distribution of resonance frequencies, resulting in broadening absorption or scattering peaks in larger NPs (Sharma et al., 2024).

The increase in the intensity of the SPR band demonstrates an increase in the concentration of NPs synthesized via the green synthesis approach (Wen et al., 2021). This occurs as a higher concentration of NPs in a solution can lead to stronger collective electron oscillations; more light is absorbed and scattered within the same path length, increasing the observed SPR intensity (Su et al., 2012). Thus, in this study, 10 mM and 100 mM Na_2SeO_3 were not selected for further optimization process as 10 mM produced the lowest SPR intensity (the least concentration of synthesized BC-SeNPs), while 100 mM provided the longest SPR wavelength (largest size of synthesized BC-SeNPs) with more noise than 25 and 50 mM Na_2SeO_3. The widening SPR band indicated that the synthesized BC-SeNPs exhibited polydispersity at higher concentration (100 mM). Additionally, a recent study has shown that higher precursor concentrations could lead to instability and aggregation of metal NPs (Khan et al., 2020). Therefore, 50 mM Na_2SeO_3 was selected for further optimization using different reaction parameters (temperature, reaction time and pH) as it produced the optimum characteristics in terms of SPR peak, intensity and wavelength compared to other concentrations of Na_2SeO_3 tested in the present study. These findings were corroborated with the results of Boroumand et al. (2019) and Ahamad Tarmizi et al. (2023).

Temperature is another crucial factor in the biosynthesis of SeNPs. Temperature will affect the shape and size of SeNPs, as well as the rate at which they are synthesized. In this study, the influence of temperature on the biosynthesis of BC-SeNPs was evaluated by monitoring the UV-Vis spectra for reactions exposed to different temperatures ranging from 4˚, 25˚, 37˚, 50˚ and 60˚C. The results demonstrated that the SPR intensity rose with increasing the temperature of the reaction mixture, implying a continuous synthesis of BC-SeNPs. The maximum SPR intensity for the reaction mixture of 50 mM Na_2SeO_3 (395 nm), was shown at 50˚C, indicating the completion of the biosynthesis of BC-SeNPs. These findings demonstrated that 50˚C was the optimum temperature for the completion of biosynthesis of BC-SeNPs; thereafter, at 60˚C, the unstable BC-SeNPs were formed. Ahamad Tarmizi et al. (2023) reported that 50˚C was the optimum

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reaction temperature for the biosynthesis of SeNPs with *M. oleifera* aqueous extract, which supports our observation. It has been suggested that the low reaction temperatures delay the biosynthesis of SeNPs. Studies have revealed that raising the temperature of the reactions reduces the rate of SeO_3^{2-} ions, resulting in the homogenous nucleation of selenium nuclei. This facilitates the synthesis of SeNPs in smaller sizes (Long et al., 2022). It was reported that increasing the reaction temperatures resulted in a decrease in the rate of NP synthesis but an enhancement in the stability of NP (Nurdin et al., 2016). Interestingly, the SPR intensity at 60˚C was lower than those recorded at 37˚C and 50˚C in the current study. It could be inferred that the bioactive compounds of the reaction mixture decreased at 60˚C due to the degradation of flavonoids and quercitrin resulting from the increased temperature (Stephenus et al., 2023; Zemour et al., 2024). Furthermore, a recent study also revealed that when the NPs are unstable, the original SPR peak will decrease in intensity due to the depletion of stable NPs, resulting in aggregation of NPs. Aggregated NPs exhibit different optical properties than individual NPs; these aggregated NPs usually lead to broader and less intense SPR peaks (Mirzaei et al., 2017).

For the biosynthesis of NPs, reaction time is another important factor that needs to be optimized, as it will ensure the appropriate interaction between the salt and the reducing compounds in the testing extracts (Barzinjy and Azeez, 2020). In this study, 24- and 48-hour reaction times were chosen to optimize the biosynthesis of BC-SeNPs. Generally, as the reaction time increases, the quantity of NPs increases, but only up to a specific duration. Following that, the agglomeration of SeNPs may occur due to their instability (Mohandass et al., 2013). The UV-Vis spectra analysis showed that the maximum or optimum BC-SeNPs synthesis occurred at 24 hours compared to 48 hours of reaction time. The high SPR intensity at 24 hours indicated that a higher amount of BC-SeNPs had been produced than at 48 hours, where agglomeration was observed. This finding suggested that the synthesis of BC-SeNPs was completed at 24 hours rather than 48 hours, which could be attributed to a few factors. Firstly, the reaction kinetics might have attained saturation or equilibrium at 24 hours, indicating that all available reactants had already been used to produce SeNPs throughout the synthesis process. Furthermore, the longer reaction time of more than 24 hours could lead to the degradation or aggregation SeNPs, thereby decreasing their yield and quality. Additionally, the activity of the biological compounds and the stability of the reaction conditions may gradually decrease with time, deteriorating the efficiency of NPs formation. Several recent studies have also reported that the optimum and complete biosynthesis of SeNPs using plant extracts was achieved at 24 hours of reaction time (Alagesan and Venugopal, 2019; Garza-García et al., 2023). Therefore, the reaction time of 24 hours emerged as the optimal duration for reaching maximum BC-SeNPs synthesis in the present study.

The proper control of the pH is vital for optimizing the BC-SeNPs synthesis in the current study, as pH plays a significant role in determining NP size, quantity and shape. Thus, pH levels of 6, 8, and 10 were investigated in this study to optimize BC-SeNP synthesis. The study's results showed that the SPR intensity of BC-SeNPs increased as the pH increased. However, the UV-Vis spectra analysis demonstrated that the BC-SeNPs synthesis at pH 8 is the most optimum compared to pH 6 and 10. At pH 10, the characteristic SPR peak was absent, and the SPR band was broader and shifted towards a longer wavelength (red shift). These observations suggested the formation of larger and polydispersity of synthesized BC-SeNPs, and the agglomeration occurred at pH 10. Recently, Fouda et al. (2022) and Ahamad Tarmizi et al. (2023) also revealed that the

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optimum pH for the synthesis of *Portulaca oleracea* and *M. oleifera* SeNPs, respectively, was pH 8. They suggested that this pH level enhanced the functional groups in the aqueous plant extract, resulting to increased synthesis efficiency.

In contrast, at pH 6, the BC-SeNP synthesis was not prominent, and the UV-Vis spectra analysis did not show a sharp SPR peak at the range of 300 to 500 nm, corresponding to those documented in previous SeNP studies (Sarkar and Kalita, 2022; Ahamad Tarmizi et al., 2023). This observation confirmed that the biosynthesis of BC-SeNP is a pH-dependent process. The reaction pH could alter the electrical charges of the biomolecules, which may influence their capping and stabilizing capacity, subsequently affecting the biosynthesis of NPs. In acidic conditions, the high concentration of protons can result in the protonation of the functional groups of plant biomolecules. This protonation will inhibit the capacity of these functional groups to bind and reduce metal ions, which is a critical step in the biosynthesis of SeNPs. Hence, the BC-SeNPs are thus unstable and prone to agglomeration, even if they were synthesized under an acidic condition (Sumi Maria et al., 2015). Under alkaline conditions, the bioavailability of functional groups increases, promoting the synthesis of SeNPs. Nevertheless, at higher pH values, such as pH 10, SeNPs become unstable, leading to agglomeration, as indicated in this study. Habeeb Rahuman et al. (2022) demonstrated that a lower pH, around pH 8, was favored for promoting the reduction of metal ions through ionic bonding in the green synthesis approach, which is attributable to the positively charged functional groups of biomolecules. Therefore, pH 8 was determined as the optimum pH in BC-SeNPs synthesis.

Stability test for optimized BC-SeNPs using UV-Vis method

In the current study, the stability of the biosynthesized BC-SeNPs using the optimized reaction conditions (50 mM of Na_2SeO_3, pH 8, 4:1 ratio of BCAE:Na_2SeO_3, 24 hours, and 50˚C of reaction temperature) was measured by recording the UV-Vis spectra at intervals of 1, 3, 6 and 10 days after storage. This method has been described for monitoring the synthesis and testing the stability of the biosynthesized NPs, proving effective in synthesizing highly potent and stable NPs (Habibullah et al., 2022; Kokila et al., 2024). The stability results showed that the biosynthesized BC-SeNPs were considered stable over 10 days in water without notable changes in the peak position. The stable position of the absorbance peak demonstrated that the biosynthesized BC-SeNPs did not undergo aggregation, indicating that the integrity of the optical characteristics of the BC-SeNPs was maintained over time. By using BCAE as both reducing and capping agents, the functional groups of the phytochemicals in the BCAE contributed to the synthesis of highly stable BC-SeNPs by forming an organic protective layer, thus enhancing colloidal stability (Bélteky et al., 2019; Franco et al., 2024). Conversely, NPs synthesized using physical or chemical approaches typically lack an intrinsic biocompatible layer and require the inclusion of excess stabilizers. Most NPs synthesized through these approaches tend to exhibit complete agglomeration, despite the existence of these stabilizers (Deshmukh et al., 2019).

Generally, optimizing reaction parameters for biosynthesis of SeNPs plays an important role in determining the size, stability and functionality of the biosynthesized SeNPs, making them ideal for various applications. Biosynthesized SeNPs have gained significant attention in numerous fields, especially medicine, environmental science and agriculture. Plant-based SeNPs have shown promising antioxidant, anticancer, and antibacterial activities, making them potential therapeutic agents for drug delivery,

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cancer treatment or other therapeutic applications in medicine. For example, *Carica papaya* latex-mediated SeNPs have demonstrated apoptotic effects on breast cancer cells, resulting in chromatin condensation and apoptotic body formation, key factors in their efficacy against cancer (Ikram et al., 2021). Furthermore, biosynthesized SeNPs using fenugreek seed extract have been applied to deliver the FDA-approved anticancer medication doxorubicin, showing enhanced anticancer activity and reduced toxicity compared to doxorubicin alone (Ramamurthy et al., 2013). Recently, Satpathy et al. (2024) reported the SeNPs synthesized from *Nyctanthes arbor-tristis* L. displayed strong antimicrobial and anti-biofilm activities against pathogenic bacteria, including *Escherichia coli*, *Klebsiella pneumoniae* and *Staphylococcus epidermidis*. In environmental sensing, plant-derived SeNPs have not yet been widely investigated. The current methods that are mostly used for synthesizing NPs on environmental sensing mainly include chemical and physical methods (Wang et al., 2010). However, a recent study by Jebril et al. (2021) revealed that AgNPs biosynthesized from *Melia azedarach* demonstrated potential in developing highly sensitive electrochemical sensors for phenol detection in water samples, indicating strong sensing capabilities and long-term stability. Besides, in agriculture, plant diseases are one of the main problems impacting crop productivity. A recent study also found that the SeNPs biosynthesized using *Melia azedarach* leaf extract can be used as antifungal agents and super-growth promoters in mango plants. These SeNPs have improved the physiological, biochemical and antioxidant defense system of the mango (*Mangifera indica*) infected with mango malformation disease at an optimal concentration of 30 μg/mL, while also inhibiting *Fusarium mangiferae*, the fungus responsible for the disease (Shahbaz et al., 2023). These examples demonstrated the versatility of green synthesized SeNPs in various applications, highlighting their potential for wider applications in medicine, environmental monitoring, and agriculture.

Conclusion

Green synthesis of metal NPs using plant extracts garnered high interest due to its simple technique, cost-effectiveness, environmental friendliness, readily available raw materials, and feasibility for large-scale production. The optimization of various variables, such as pH, plant extract and metal ion concentration, reaction time, and temperature, play a vital role in the biosynthesis of NPs. The present study demonstrated that the green synthesis of SeNPs was optimized using an aqueous leaf extract of *B. citriodora*. This is the first study conducted to synthesize SeNPs using *B. citriodora* aqueous extract as the reducing and stabilizing agents. The findings of this study demonstrated that the maximum and stable BC-SeNPs were successfully synthesized at pH 8, 50 mM of Na_2SeO_3 using 4:1 ratio of BCAE:Na_2SeO_3 for 24 hours at 50˚C of reaction temperature, with good stability in water. The biosynthesis of BC-SeNPs was confirmed by the color change from light yellow to red and the UV-spectrophotometric analysis, where a single SPR band showed within the 380-450 nm range. Therefore, the current study supports the approach of plant-mediated green synthesis of SeNPs, indicating its promising potential to be utilized in various industry applications for different plant species. However, further characterization methods can be conducted in the future to provide more information on the quality and functionality of the biosynthesized BC-SeNPs.

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Acknowledgements. The authors extend their appreciation to Universiti Kebangsaan Malaysia (UKM) for funding this work under the Young Researcher Encouragement Grant (*Geran Galakan Penyelidik Muda)* with the grant reference number GGPM-2022-042.

Conflict of interests. The authors declare that they have no conflict of interests.

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