BIOSYNTHESIS OF NANOPARTICLES USING RECOMBINANT BROMELAIN

ARINA NASRUDDIN [1], AZURA AMID [2]*, SARINA SULAIMAN [1], MUHD EZZA FAIEZ OTHMAN [3]

[1]Department of Biotechnology Engineering, Faculty of Engineering, International Islamic University Malaysia, Jalan Gombak, 53100 Kuala Lumpur, Malaysia.

[2]International Institute for Halal Research and Training, Level 3, KICT Building, International Islamic University. Malaysia (IIUM), Jalan Gombak, 53100 Kuala Lumpur, Malaysia.

[3]IIUM Advance Technology Sdn Bhd. International Islamic University. Malaysia (IIUM), Jalan Gombak, 53100 Kuala Lumpur, Malaysia.

**Corresponding author: azuraamid@iium.edu.my*

ABSTRACT: **Green chemical method was applied to synthesize nanoparticles using recombinant bromelain. Among the numerous applications of recombinant bromelain, there is still no research on nanoparticles synthesis which encourages its utilization in this study. Four chemicals which are copper (II) chloride dihydrate ($CuCl_2.2H_2O$), cerium nitrate hexahydrate ($Ce(NO_3)_3.6H_2O$), sodium selenite (Na_2SeO_3), and iron (III) chloride hexahydrate ($FeCl_3.6H_2O$) were selected to be screened for the suitability in nanoparticles biosynthesis by recombinant bromelain. The nanoparticles formed were characterized by using UV-visible absorption spectra. The biosynthesis process then was optimized by varying the centrifugation speed, temperature, and time to get the maximum absorption and weight of nanoparticles through central composite design (CCD) tool. Only $CuCl_2.2H_2O$ showed a positive result for the screening process which was represented by the formation of colloidal solution and a maximum absorption at 580 nm. Thus, optimization was carried out for this chemical. Based on the optimization model, maximum absorption and weight were predicted at 67.5°C, 2 hrs, and 9,600 rpm. These optimal conditions were validated by repeating the biosynthesis process. The absorption and weight of the nanoparticles depended on the reaction of the chemical with recombinant bromelain. 3D plots showed that the optimal condition for high responses mostly depends on temperature and time.**

KEYWORDS: *Nanoparticles; Biosynthesis; Recombinant bromelain; Optimization; and CCD.*

1. INTRODUCTION

Nanoparticles, as the name suggests, are nano-sized particles with at least one dimension between 1 to 100 nm. These particles have their unique physical properties which are depending on the particle size, inter-particle distance, nature of the protecting organic shell and shape, (Brust & Kiely, 2002). Currently, a wide variety of physical, chemical and biological methods to synthesize different types of nanoparticles are extensively studied. Widespread applications of metallic nanoparticles (gold, silver, iron, palladium, platinum, etc.) in biology, pharmaceuticals, and medicine encouraged the usage of biological process to produce nanoparticles because those applications require free of toxic contaminants. In medicine, nanoparticles are being used as antimicrobial agents such as in bandages as well as targeted drug delivery and clinical diagnostics.

Two decades ago, the field in green chemistry was formed by the Environmental Protection Agency (EPA) as a result of the rising concern about the environmental problems from the chemical industry. Anastas and Warner (1998), proposed twelve fundamental principles to elaborate the requirements of green chemistry and guide the implementation of chemical processes. Green chemical methods in nanoparticle synthesis are based on the three factors which are the utilization of non-toxic capping agents, less hazardous reducing agents, and selection of environmentally benign solvents. Several methods which follow these requirements as green chemical methods for biosynthesis of nanoparticles are microorganism-based, plant extract-based, and honey-mediated. The microorganism-based method is employed by adding metallic ions to the culture medium grown with microorganisms. For the plant extract-based method, the reducing and capping agents for nanoparticles synthesis can be obtained by soaking the plant materials in a solvent. Besides, honey which is rich in carbohydrates, enzymes and antioxidants are also used in the synthesis of platinum, gold, and silver nanoparticles, (Noruzi, 2015).

Plant-mediated synthesis has gained great significance because plants are economically viable, eco-friendly and does not require elaborate processes. Thus, the use of plant extracts for making nanoparticles is inexpensive, easily scaled up and environmentally benign. This method can provide nanoparticles of a controlled size and morphology. Fruit extracts of the pineapple plant, *Ananas comosus* (L.), has been used as one of the studies to synthesize gold nanoparticles, (Basavegowda *et al.*, 2013). In this study, recombinant bromelain is directly used to synthesize four types of nanoparticles which are copper (Cu), cerium oxide (CeO_2), selenium (Se), and iron oxide (Fe_3O_4) nanoparticles.

2. MATERIALS AND METHODS

2.1 Materials

Recombinant bromelain enzyme in powder form with 4000 GDU activity was provided by IIUM Advanced Technology Sdn. Bhd. It was stored in 4°C freezer and the enzyme solution used in this study must be freshly prepared for each sample. Copper (II) chloride dihydrate ($CuCl_2.2H_2O$), cerium nitrate hexahydrate ($Ce(NO_3)_3.6H_2O$), sodium selenite (Na_2SeO_3), and iron (III) chloride hexahydrate ($FeCl_3.6H_2O$) were provided by IIUM.

2.2 Method of Screening for Suitable Chemicals

The chemical solution was mixed with recombinant bromelain, and heated in a water bath. The temperature was varied starting at room temperature and then increased until there is a formation of nanoparticles. Then, the nanoparticles were collected by centrifugation at room temperature, 8,500 rpm and washed with distilled water. The nanoparticles obtained were dried at room temperature, weighed and stored in an air-tight jar. The suitable chemical for nanoparticle biosynthesis using recombinant bromelain was screened by measuring the maximum absorption of the nanoparticles dissolved in distilled water using UV-visible absorption spectra. The maximum absorption for each type of nanoparticle enables the identification of nanoparticle formation.

2.3 Optimization of the Biosynthesis Process

The biosynthesis process was optimized by varying the centrifugation speed, temperature, and time. The optimized process was determined by measuring the absorption and weight of the nanoparticles formed. Optimization studies were conducted using Face Centered Composite Design (FCCD) of Design-Expert Software (version 6.0.8) Stat Ease Inc., USA with 3 replicates at the center point making a total of 17 runs. Factors selected for the study are presented in Table 1. The first response, the absorption of the nanoparticles, were recorded with a Libra S12 Biochrom UV-visible spectrophotometer (UK) to determine the maximum absorption for the formation of nanoparticles. The weight of the nanoparticles as the second response was measured with a weighing balance and recorded to get the optimized parameters for the highest weight measured.

Table 1: Design of Experiment.

Factors			
	Name	**Low**	**High**
A:	Temperature (°C)	27	99
B:	Time (h)	2	10
C:	Speed (rpm)	6,500	10,500

2.4 Validation

The methods for biosynthesis of nanoparticles were repeated using the optimized conditions generated by CCD tool to validate the results.

3. RESULTS AND DISCUSSION

3.1 Screening of the Chemicals

In this study, the screening process involved the reaction with recombinant bromelain with four selected chemicals which were $CuCl_2$, $Ce(NO_3)_3$, Na_2SeO_3, and $FeCl_3.6H_2O$ to form nanoparticles using UV-visible absorption spectra. The identification of the nanoparticles can be achieved because the color exhibit by nanoparticles is due to surface plasmon resonance (SPR) effect that shows maximum absorption at a specific wavelength, (Noruzi, 2015).

This screening process aims to identify the suitable chemicals in the production of nanoparticles after the reaction with recombinant bromelain. UV-visible absorption spectra analysis was used to show the maximum absorption at a specific wavelength. The formation of nanoparticles was verified by obtaining the maximum absorption at the specific wavelength of the nanoparticles as shown in Table 2.

Table 2: Nanoparticles wavelength for maximum absorption.

Nanoparticles	**Wavelength for maximum absorption (nm)**	**Ref.**
Cu	580	[16]
CeO_2	315	[14]
Se	395	[18]
Fe_3O_4	415	[13]

Each of the chemicals was mixed with recombinant bromelain solution and then heated in a water bath. Any color change was observed as an indication for the formation of nanoparticles because of the ability of nanoparticles to produce a particular color due to their specific properties. Small metal nanoparticles exhibit the absorption of visible electromagnetic waves by the collective oscillation of conduction electrons at the surface, (Link & El-Sayed, 2000). This is known as the surface plasmon resonance effect which enables the identification of the nanoparticles by showing maximum absorption at a specific wavelength.

Among the four chemicals investigated, only one chemical had shown the positive result which was $CuCl_2$. The plasmon resonance of the copper nanoparticles (CuNPs) appeared at 580 nm with maximum absorption of 0.4083. The reduction process begins with color change and forming a pale brown colloidal solution at the bottom of the tube. Other chemicals showed negative results which indicated that there was no reaction with recombinant bromelain to form nanoparticles. Thus, only $CuCl_2$ was utilized in the optimization process.

The mechanism for the nanoparticle formation is not yet clear since there are only some studies that predicted the mechanism for nanoparticles formation even for synthesis by living organisms. The studies claim that the formation of nanoparticles is because of the enzymes produced by bacteria and fungi or biomolecules especially phenolic compounds in plants, (Bharde *et al.*, 2008; Moon *et al.*; 2010 and Elcey *et al.*; 2014). Tetgure *et al.*, (2015), proposed a possible mechanistic pathway for synthesis of nanoparticles with aspartic protease, enzymes found in *Ficus racemosa* latex, as the reducing agent. This enzyme undergoes oxidative cleavage in the biosynthesis process which leads to the release of free electrons to be utilized for the reduction of ions of the chemical solution to their metallic states resulting in the formation of nanoparticles, (Tetgure *et al.*, 2015).

3.2 Analysis

For the optimization process, factors of temperature, time and centrifugation speed were varied. Central Composite Design (CCD) of Design-Expert Software (version 6.0.8) Stat Ease Inc., USA was used to determine the optimized conditions for the best response which were absorbance and weight of the nanoparticles. The results obtained depend on the reaction of the chemicals with recombinant bromelain. This process aimed to optimize the nanoparticles synthesis by recombinant bromelain reaction with the identified chemicals by measuring its absorbance and weight.

Table 3 shows the result of CCD of three parameters and the two responses. For absorbance, the highest response was found at the center points with an average value of 0.4907, whereas for weight, the highest response, 0.251 g was found at run 6. The lowest reading for both responses was found at run 15.

Table 3: CCD design of three variables and two responses.

Run	A: Temperature (°C)	B: Time (h)	C: Speed (rpm)	Response 1: Absorbance	Response 2: Weight (g)
1	99	10	6500	0.2820	0.141
2	27	10	6500	0.0740	0.059
3	99	10	10500	0.2875	0.148
4	27	2	6500	0.1920	0.121
5	63	6	8500	0.4730	0.230
6	63	2	8500	0.4410	0.251
7	63	6	8500	0.4925	0.241
8	99	2	10500	0.3170	0.159
9	99	6	8500	0.3205	0.166
10	27	2	10500	0.2170	0.145
11	63	10	8500	0.3950	0.182
12	27	6	8500	0.0785	0.063
13	63	6	10500	0.4110	0.195
14	63	6	6500	0.4325	0.223
15	27	10	10500	0.0715	0.051
16	99	2	6500	0.2990	0.153
17	63	6	8500	0.5065	0.244

Table 4: ANOVA for CCD quadratic model of Response 1 (Absorbance).

Source	Sum of Squares	DF	Mean Square	F Value	Prob > F	
Model	0.33	9	0.036	19.66	0.0004	Significant
A	*0.076*	*1*	*0.076*	*41.44*	*0.0004*	
B	*0.013*	*1*	*0.013*	*6.89*	*0.0342*	
C	*6.002E-005*	*1*	*6.002E-005*	*0.033*	*0.8618*	
A^2	*0.14*	*1*	*0.14*	*75.88*	*< 0.0001*	

B^2	*2.538E-004*	*1*	*2.538E-004*	*0.14*	*0.7213*	
C^2	*9.589E-005*	*1*	*9.589E-005*	*0.052*	*0.8259*	
AB	*5.886E-003*	*1*	*5.886E-003*	*3.20*	*0.1168*	
AC	*1.250E-007*	*1*	*1.250E-007*	*6.796E-005*	*0.9937*	
BC	*2.000E-004*	*1*	*2.000E-004*	*0.11*	*0.7512*	
Residual	0.013	7	1.839E-003			
Lack of Fit	*0.012*	*5*	*2.462E-003*	*8.70*	*0.1063*	*not significant*
Pure Error	*5.662E-004*	*2*	*2.831E-004*			
Cor Total	0.34	16				

Table 5: ANOVA for CCD quadratic model of Response 2 (Weight).

Source	**Sum of Squares**	**DF**	**Mean Square**	**F Value**	**Prob > F**	
Model	0.063	9	7.014E-003	16.26	0.0007	significant
A	*0.011*	*1*	*0.011*	*24.94*	*0.0016*	
B	*6.150E-003*	*1*	*6.150E-003*	*14.26*	*0.0069*	
C	*1.000E-007*	*1*	*1.000E-007*	*2.318E-004*	*0.9883*	
A^2	*0.027*	*1*	*0.027*	*62.92*	*< 0.0001*	
B^2	*4.898E-006*	*1*	*4.898E-006*	*0.011*	*0.9181*	
C^2	*1.013E-004*	*1*	*1.013E-004*	*0.23*	*0.6428*	
AB	*2.211E-003*	*1*	*2.211E-003*	*5.13*	*0.0580*	
AC	*1.125E-006*	*1*	*1.125E-006*	*2.608E-003*	*0.9607*	
BC	*1.201E-004*	*1*	*1.201E-004*	*0.28*	*0.6140*	
Residual	3.020E-003	7	4.314E-004			
Lack of Fit	*2.911E-003*	*5*	*5.822E-004*	*10.72*	*0.0876*	*not significant*
Pure Error	*1.087E-004*	*2*	*5.433E-005*			
Cor Total	0.066	16				

According to the analysis of variance (ANOVA), the quadratic model was found to be significant at a *p*-value less than 0.05. The values for response 1 and 2 are presented in Table 4 and Table 5 respectively.

Values of Prob > F for both responses were less than 0.05 indicating that the model terms were significant, where a lower probability value represents a higher significance for the regression model. For the first response *p*-value < 0.0004 and for the second response p-value < 0.0007. The F value presents how the mean square of the model compared to the mean square of the residuals. For the first response, F value of 19.66 implies that the model is significant and suggesting that there is only a 0.04% chance for the model F value to occur due to noise while for the second response, F value of 13.26 implies that the model is significant and there is only a 0.07% chance for the model F value to occur due to noise. For both responses, linear term A (temperature) and B (time), and one of the quadratic term A^2 were statistically significant model terms.

To check the fitness of the model, the coefficient of determination (R^2) was used. An R^2 value close to 1 implies the better correlation between experimental and predicted responses. Thus, it is important for a good model R^2 to be within the range of 0 to 1, and the closer it is to 1, the more fit the model is deemed to be. For response 1 and 2, the correlation coefficient (R^2) value of 0.9620 and 0.9543 respectively are at a reasonable agreement with the adjusted determination coefficient (R^2_{Adj}) value of 0.9130 and 0.8957 respectively regarding a high significance of the model. The adequate precision measures the signal to noise ratio and values less than 4 is considered appropriate for the desired model. In the developed model, the adequate precision value of 12.845 and 13.134 for response 1 and 2 respectively indicate the model can be used to navigate the design space.

The second order regression equation presented the dependence of absorbance and weight on temperature, time and centrifugation speed. The relation between the parameters and each response was established using a second order polynomial equation regarding coded factors:

$$\begin{aligned}\text{Absorbance} &= +0.45 + 0.087\ \text{A} - 0.036\ \text{B} \\ &+ 2.450E\text{-}0.03\ \text{C} - 0.23\ \text{A}^2 \\ &- 9.732E\text{-}003\ \text{B}^2 - 5.982E\text{-}003\ \text{C}^2 \\ &+ 0.027\ \text{AB} + 1.250E\text{-}004\ \text{AC} \\ &- 5.000E\text{-}003\ \text{BC}\end{aligned}$$

$$\begin{aligned}\text{Weight} &= +0.23 + 0.033\ \text{A} - 0.025\ \text{B} \\ &+ 1.000E\text{-}0.04\ \text{C} - 0.10\ \text{A}^2 \\ &+ 1.352E\text{-}003\ \text{B}^2 - 6.148E\text{-}003\ \text{C}^2 \\ &+ 0.017\ \text{AB} - 3.750E\text{-}004\ \text{AC} \\ &- 3.875E\text{-}003\ \text{BC}\end{aligned}$$

The statistical Eq. (1) and (2) indicate that the positive values have a synergistic effect on the response and the negative values represent an antagonistic effect on the response, where

A = Temperature (°C)

B = Time (h)

C = Centrifugation speed (rpm)

In these equations, the coefficient of one factor presented the effectiveness of this particular factor. To analyze the absorbance and weight through the coefficient values from the equations, it is clear that temperature gives a higher positive effect for both responses as compared to other parameters.

A 3D surface response plot is the graphical representation of the regression equation obtained from the established model, which is used to study the interaction between the parameters and to define the optimum condition of each parameter for maximum absorption and weight of nanoparticles. Furthermore, the plot is based on the function of two variables while the third variable is in its optimum condition. Additionally, the elliptical or saddle shape of the contour plot specifies the level of the interaction significance and an elliptical or saddle plot will be obtained when there is a perfect interaction between independent variables.

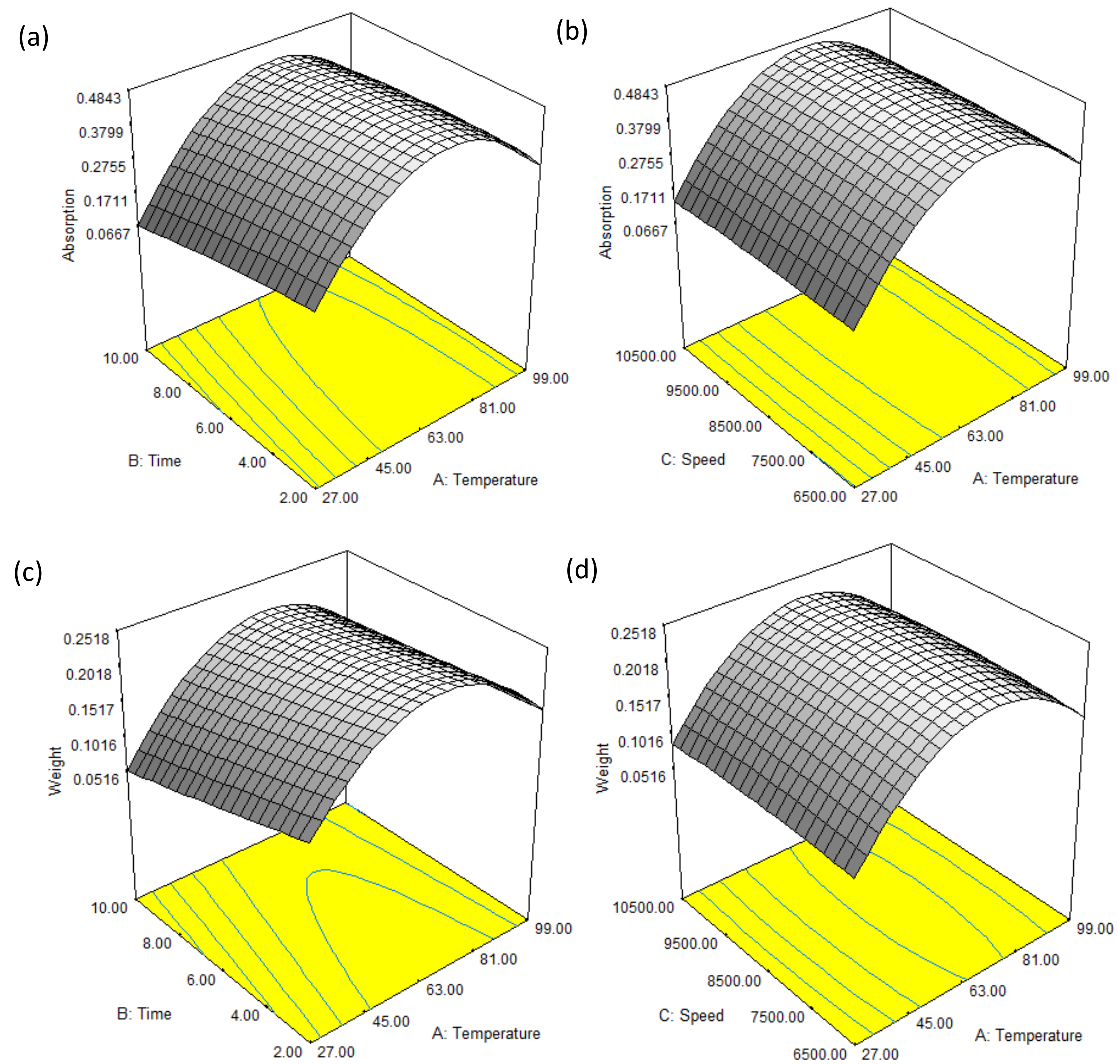

Fig. 1 (a-d) 3D surface plots of CuNPs biosynthesis for the two responses.

Fig. 1 (a-d) demonstrate the 3D plot of absorbance and weight using the interactions of all three variables used. All the 3D response surface curve were u-shaped by default suggesting that there were optimized conditions of temperature (A) effect on absorption and weight of the nanoparticles. The time (B) is increasing with absorption and weight while the speed (C) effect on both responses is almost constant. The surface plot indicates that the optimal condition of high responses mostly depends on temperature (A) and time (B). This is because an enzyme works best at its optimum temperature and suitable time requires to ensure complete reduction process is obtained.

According to Haverkamp and Marshall (2009), plants have a limited capacity for reducing metal ions to form nanoparticles. The total capacity of the plant to carry out reduction may depend mostly on the immediately available reducing agents. Another minor factor is the ability of the plant to manufacture additional reducing agents. This has two consequences for metal nanoparticle formation which are the limitation of the metal ions that are reduced to form nanoparticles and the dependence solely on the reduction potential of the ion to be reduced. The effective reducing capacity of the plant is larger for an easily reduced ion compared to a more difficult to reduce ion, (Haverkamp & Marshall, 2009). These limitations can be overcome by the utilization of recombinant bromelain instead of pineapple extract as the source of bromelain for biosynthesis of nanoparticles, (Basavegowda *et al.*, 2013).

3.3 Experimental Model Validation

To verify the optimization results and validate the developed model, a triplicate experiment was performed according to the optimum conditions for absorption and weight of nanoparticles. Three solutions were found from the optimization design, and the optimal value of each variable is shown in Table 6. The model predicted by the suggested solution for absorption and weight as 0.4868 and 0.2519 g, respectively. The experimental verification using the same optimal value found that the absorption of 0.4335 and weight of 0.2241 g with 10.9% and 11.0% error, respectively. Since the error is not big, therefore the model is acceptable to predict the amount of absorption and weight.

Nanoparticles formation was optimized at 67.5°C reaction temperature which was lower compared to plant-mediated synthesis. More components available in the plant extract may require a higher temperature to break down into suitable reducing agents for nanoparticles synthesis. Recombinant bromelain enzyme did not require a high temperature and was proven to be stable at 60°C and 75°C, (Amid *et at.*, 2011).

The reduction of copper ions and the formation of stable nanoparticles occurred rapidly within 2 hours of the reaction indicated by the highest absorption and weight achieved, making it one of the fastest biosynthesis methods to produce copper nanoparticles, (Gawande *et al.*, 2016). Other biosynthesis method using plant extract that has been reported mostly require 24 hours to several days of reaction for copper nanoparticles formation, (Abboud *et al.*, 2014; Jang *et al.*, 2015 and Cuevas *et al.*, 2015). As there was reported copper nanoparticles formation within 20 minutes, (Nasrollahzadeh *et al.*, 2014), other parameters such as pH, chemical concentration, and molar ratios should be introduced in the future research to further optimize the current parameters especially reaction time and temperature.

Table 6: Model validation.

Optimal conditions	**Predicted responses**		**Experimental responses**	
	Absorption	**Weight (g)**	**Average Absorption**	**Average Weight (g)**
Temperature (67.5°C) Time (2 hrs) Speed (9,600 rpm)	0.4868	0.2519	0.4335	0.2241

4. CONCLUSION

$CuCl_2$ was a suitable chemical for biosynthesis of nanoparticles using recombinant bromelain. From the experimental design and the model, it was shown that the highest absorption was found at the center points with an average value of 0.4907, whereas for weight, the highest response, 0.251 g was found at run 6. The surface plot indicates that the optimal condition of high responses mostly depends on temperature and time. This biosynthesis method should be implemented because it follows green chemistry principles to ensure the biocompatibility for further application.

ACKNOWLEDGEMENTS

My greatest gratitude to International Islamic University Malaysia for providing me with the related chemicals for this project.

REFERENCES

[1] Abboud Y., Saffaj T., Chagraoui A., El Bouari A., Brouzi K., Tanane O., Ihssane B. (2014) Biosynthesis, Characterization and Antimicrobial Activity of Copper Oxide Nanoparticles (CONPs) Produced using Brown Alga Extract (Bifurcaria bifurcata). Applied Nanoscience, 4(5): 571−576.

[2] Amid A., Ismail N. A., Yusof F., Mohd Salleh H. (2011) Expression, Purification, and Characterization of a Recombinant Stem Bromelain from Ananas comosus. Process Biochemistry, 46(12): 2232–2239.

[3] Anastas P.T., Warner J. C. (1998) Green Chemistry : Theory and Practice. Oxford University Press.

[4] Basavegowda N., Sobczak-Kupiec A, Malina D., Yathirajan H. S., Keerthi V., Chandrashekar N., Dinkaar S., Liny P, (2013) Plant Mediated Synthesis of Gold Nanoparticles Using Fruit Extracts of Ananas comosus (L.)(Pineapple) and Evaluation of Biological Activities. Adv. Mater. Lett., 4(5): 332–337.

[5] Bharde A. A., Parikh R. Y., Baidakova M., Jouen S., Hannoyer B., Enoki T. (2008) Bacteria-mediated precursor-dependent biosynthesis of superparamagnetic iron oxide and iron sulfide nanoparticles. Langmuir, 24(11): 5787–5794.

[6] Brust M., Kiely C.J. (2002) Some recent advances in nanostructure preparation from gold and silver particles: A short topical review. Colloids Surfaces A Physicochem. Eng. Asp., 202(2): 175–186.

[7] Cuevas R., Duran N., Diez M. C., Tortella G. R., Rubilar O. (2015) Extracellular Biosynthesis of Copper and Copper Oxide Nanoparticles by Stereum Hirsutum, A Native White-Rot Fungus from Chilean Forests. Journal of Nanomaterials, 16(1): 1–7.

[8] Elcey C., Kuruvilla A. T., Thomas D. (2014) Synthesis of magnetite nanoparticles from optimized iron reducing bacteria isolated from iron ore mining sites. Int. J. Curr. Microbiol. Appl. Sci., 3: 408–417.

[9] Gawande M. B., Goswami A., Felpin F. X., Asefa T., Huang X., Silva R., Varma R. S. (2016) Cu and Cu-Based Nanoparticles: Synthesis and Applications in Catalysis. Chemical Reviews, 116(6): 3722–3811.

[10] Haverkamp R. G., Marshall A. T. (2009) The mechanism of metal nanoparticle formation in plants: Limits on accumulation. Journal of Nanoparticle Research, 11(6): 1453–1463.

[11] Jang G. G., Jacobs C. B., Gresback R. G., Ivanov I. N., Meyer H. M., Kidder M., Moon J. W. (2015) Size Tunable Elemental Copper Nanoparticles: Extracellular Synthesis by Thermoanaerobic Bacteria and Capping Molecules. Journals of Materials Chemistry C, 3: 644–650.

[12] Link S., El-Sayed M. A. (2000) Shape and size dependence of radiative, non-radiative and photothermal properties of gold nanocrystals. International Reviews in Physical Chemistry, 19(3): 409–453.

[13] Mahdavi M., Namvar F., Ahmad M., Mohamad R. (2013) Green biosynthesis and characterization of magnetic iron oxide (Fe_3O_4) nanoparticles using seaweed (Sargassum muticum) aqueous extract. Molecules, 18: 5954–5964.

[14] Maqbool Q., Nazar M., Naz S., Hussain T., Jabeen N., Kausar R., Jan T. (2016) Antimicrobial potential of green synthesized CeO_2 nanoparticles from Olea europaea leaf extract. International journal of nanomedicine, 11: 5015.

[15] Moon J. W., Rawn C. J., Rondinone A. J., Love L. J., Roh Y., Everett S. M., Phelps T. J. (2010) Large-scale production of magnetic nanoparticles using bacterial fermentation. Journal of

[16] Industrial Microbiology & Biotechnology, 37(10): 1023–1031.

[17] Nasrollahzadeh M., Sajadi S. M., Khalaj M. (2014) Green synthesis of copper nanoparticles using aqueous extract of the leaves of Euphorbia esula L and their catalytic activity for ligand-free Ullmann-coupling reaction and reduction of 4-nitrophenol. RSC Advances, 4(88): 47313–47318.

[18] Noruzi M. (2015) Biosynthesis of gold nanoparticles using plant extracts. Bioprocess Biosyst. Eng., 38(1): 1–14.

[19] Prasad K. S., Patel H., Patel T., Patel K., Selvaraj K. (2013) Biosynthesis of Se nanoparticles and its effect on UV-induced DNA damage. Colloids and Surfaces B: Biointerfaces, 103: 261-266.

[20] Tetgure S. R., Borse A. U., Sankapal B. R., Garole V. J., Garole D. J. (2015) Green biochemistry approach for the synthesis of silver and gold nanoparticles using Ficus racemosa latex and their pH-dependent binding study with different amino acids using UV/Vis absorption spectroscopy. Amino Acids, 47(4): 757–765.