Ulva fasciata-mediated preparation of zinc oxide nanocrystalline for one-pot multicomponent synthesis of 6-amino-3-methyl-4-phenyl-2,4-dihydropyrano[2,3-c]pyrazole-5-carbonitrile

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Abstract. ZnO has been successfully prepared by a simple co-precipitation method using sodium hydroxide, zinc acetate as precursor and Ulva fasciata polysaccharides as capping agent. Characterization using FTIR, XRD and SEM-EDS indicated the successful formation of ZnO nanocrystalline with crystallite size of 27.01 nm. In order to promote ZnO as a green catalyst, its application for one-pot multicomponent pyranopyrazole synthesis has been performed. Several reaction conditions have been investigated, and the highest yield (98%) was observed in the use of 10% mole of ZnO at room temperature for 90 min reaction time in aqueous medium to produce 6-amino-3-methyl-4-phenyl-2,4-dihydropyrano[2,3-c]pyrazole-5-carbonitrile.

1. Introduction
The study based on nanotechnology is growing rapidly, especially in the field of catalysts [1]. The principle of nanotechnology is to manipulate the properties and performance of materials in such a way that it becomes more effective, efficient and powerful. Nanoparticles have a very small particle size ranging from 1 to 100 nm. Through minimizing the size, nanoparticles will have unique physical and chemical characteristics, which are very different than the materials with larger particle sizes [1]. One of synthetic method of nanoparticles is biosynthesis. Biosynthesis of nanoparticles is performed using media from biological materials such as microorganisms, enzymes, or plant extracts. Biosynthesis has advantages over other methods, such as chemical and physical methods, which require a high cost, long time process, and produce a large amount of waste from the chemicals used. Thus, it can be an alternative and environment-friendly way to generate nanoparticles [1].

Recently, several studies have shown that seaweed can be used for the synthesis of silver and gold nanoparticles [2-4]. Azizi et al. (2014) [5] reported the use of brown seaweed in the biosynthesis of ZnO nanoparticles. The synthesized nanoparticles are known to have anti-bacterial activity, anti-fungal, wound dressings, photochemical and catalytic agent, so it can be applied in various fields, such as biomedical, pharmaceutical, and cosmetics [2]. One of the many synthesized nanoparticles is zinc oxide. This material has a number of potential applications in the fields of electronics and sensors due to its optical, acoustic and electrical properties. In addition, ZnO has several advantages including stable chemical structure, non-toxic, cheap, highly abundant in nature, and can be used as an additive in various materials [5].
Ulva fasciata is a type of non-economic seaweed that has not been used commercially. Consumers of this seaweed are still limited to the fishermen families or coastal communities, namely as seaweed chips. Green seaweed contains polysaccharides in bulk, especially cell wall polysaccharides such as ulvan. It is a water-soluble sulfate polysaccharides found in Ulva sp. [6]. The presence of sulfate groups in the polysaccharide is likely to become an active force in the formed nanoparticles.

Pyranopyrazole is widely used in biomedical and pharmaceutical applications. Its compounds have antibacterial, anti-inflammatory, insecticidal, antitumor and anticancer activity [7]. Synthesis of pyranopyrazole derivatives can be done through multicomponent reactions [7-9]. The advantages of using multicomponent reactions are short time, simple, and easy to purify. Pagore and co-workers reported that ammonium chloride can be utilized as an effective catalyst in the synthesis of pyranopyrazole with the use of a microwave [7]. In the other research, Vasuki & Kumaravel (2008) [10] have used piperidine as a catalyst. Although the reported methods are effective, but the methods have some disadvantages, such as the use of microwave is not suitable when used for large-scale synthesis. In addition, the use of toxic chemicals such as piperidine can also harm the environment. Therefore, in this research, we will investigate the performance of synthesized zinc oxide as solid catalyst for one-pot multicomponent pyranopyrazole synthesis.

2. Materials and methods

2.1. Materials

Green seaweed, Ulva fasciata, was obtained from Binuangeun, Banten. Seaweed was stored in plastic container and placed inside the cooling box, cooled with ice cubes and then brought to Research and Development Centre for Marine and Fisheries Product Competitiveness and Biotechnology, Jakarta. Furthermore seaweed was washed with fresh water and dried. The dried seaweed was milled into flour. All the chemicals required were purchased from Merck and used without further purification. Phase purity and particle size were determined by X-ray diffraction (XRD) analysis and recorded by diffractometer MiniFlex. The morphology and elemental composition of the sample were characterized by using Scanning Electron Microscope tandem Energy Dispersive X-Ray Spectroscopy (SEM-EDS) JEOL JCM-6000. The distribution and particle size were measured using Particle Size Analyser (PSA) Beckman Coulter DelsaMax Pro. Infrared spectra were recorded on PerkinElmer Spectrum One FTIR spectrophotometer using KBr pellets. $^{13}$C-NMR and $^{1}$H-NMR spectra were recorded using DMSO-d$_6$ solvent on JEOL 400 MHz with TMS as an internal standard. Mass spectra were scanned on LCMS-IT-TOF Shimadzu.

2.2. Methods

2.2.1. Extraction of water soluble polysaccharide. Dry U. fasciata powder (5 g) was extracted in ultrapure water (1:15 w/v) for 3 h at 80°C. The extract was filtered and centrifuged at 8,000 rpm for 10 min. The hot water soluble polysaccharide was precipitated by adding ethanol 95% (v/v). The precipitated polysaccharide was filtered, washed with ethanol, and dried for 12 h at 40°C in an oven [2].

2.2.2. Preparation of ZnO nanocrystalline. Synthesis of ZnO nanocrystalline refers to a method performed by Pandimurugan and Thambidurai (2014) [11]. Polysaccharide (0.5 g) was mixed with 25 mL of 25% zinc acetate solution. The mixture was heated at 70°C for 2 h while stirring on magnetic stirrer. Subsequently, the aqueous NaOH solution (25% b/b) was slowly added to the mixture until the mixture reached pH 10. The precipitate was separated with centrifuge at 8,000 rpm for 10 min. Furthermore, the precipitate was dried at 105°C for 20 h.

2.2.3. Catalytic activity test of ZnO on pyranopyrazole synthesis. Benzaldehyde (1 mmol), ethyl acetoacetate (1 mmol), hydrazine hydrate (1.5 mmol), malononitrile (1 mmol), various amount of ZnO (2.5, 5, 7.5 and 10 % mol catalyst) and ultrapure water were mixed in a 250 beaker glass. The suspension was stirred using a magnetic stirrer at various temperatures and times (reaction time: 30, 60, 90, 120, and 180 min; temperature: 30, 70 and 100°C). Once the reaction was completed, the reaction product was cooled, filtered and washed with hot ethanol (5 mL) to separate the product from the catalyst.
Ethanol from the filtrate was allowed to evaporate at room temperature in order to obtain the product. The product was purified by recrystallization using warm ethanol. The crystal formed was separated from the solution and characterized.

3. Results and discussion

3.1. Catalyst characterization

One type of green seaweed, *Ulva fasciata* is still not widely known and commercially exploited, so it can be regarded as a non-economic seaweed. Seaweed is rich in polysaccharide consisting of ulvan, cellulose, xyloglucan and glucurunan [12]. Based on this research, it was known that the yield of hot water soluble polysaccharide from *U. fasciata* is equal to 17.32 ± 0.09% of the dry weight of the seaweed. Siddhanta *et al.* (2001) [13] reported that water-soluble polysaccharides were divided into two categories, soluble in cold water and hot water with a yield value of 1.2 to 6.5% and from 5.2 to 16.9%, respectively.

As a sulfate polysaccharide, the sulfate content of *U. fasciata* polysaccharides was 15.05 ± 0.15 %. These polysaccharides has higher sulfate content than *Ulva armoricana* (10.3 to 13.8 %) and *Ulva rotundata* (9.2 to 12.5 % ) [14-15], but lower than *Ulva conglobata* (23.04 to 35.2 %) [16]. With the sulfate polysaccharide, Zn ions would be covered by the sulfate groups in the polysaccharide to prevent the formation of the large-sized particles. This is consistent with the research of Mahdavi *et al.* (2013) [17], who reported that the polysaccharide acts as a capping agent in the production of metal oxide nanoparticles.

The FTIR spectra of *U. fasciata* (a), polysaccharides (b), and ZnO (c) can be seen in figure 1. Figure 1 shows that the algae and polysaccharide has a similar spectrum. It means that the main component of algae is polysaccharides. The characteristic frequencies of *U. fasciata* were observed at 3429 cm\(^{-1}\) for vibration mode of O-H group, 2924 cm\(^{-1}\) due to asymmetric C-H. While the peaks at 1648 and 1427 cm\(^{-1}\) due to the characteristics of the symmetric stretching vibration of C=O. Whereas the peak at 1031 cm\(^{-1}\) shows guluronic acid groups [11]. Sulfate band is appeared at 850 cm\(^{-1}\) (C-O-S). In ZnO sample, wavenumbers below 450 cm\(^{-1}\) is attributed to Zn-O stretching vibration. This is consistent with the study results of Pandimurugan & Thambidurai (2014) [11], who stated that the Zn-O band is observed at 423 cm\(^{-1}\).
Figure 2. SEM analysis of ZnO with magnification (a) 1500 × and (b) 3400 ×.

Figure 3. The result of EDS analysis of ZnO.

SEM-EDS analysis was conducted to determine the surface morphology and elemental composition of a solid sample. Figure 2 shows the results of the SEM analysis with 2 magnifications, 1500 and 3400 times magnification. Measurement with smaller magnification (1500 times) was conducted to determine the morphology of the whole sample. SEM analysis showed the shaped of particles like blobs, although there were also the small particles. Meanwhile, further analysis with EDS (figure 3) shows that ZnO metal oxide is formed, with Zn atoms and O at 39.54% by 60.56%, respectively.

XRD analysis was conducted to determine the structure and phase of ZnO samples. In figure 4, the XRD diffractogram of the ZnO has a dominant 2θ at 36.3216; 31.8360; and 34.4914. The crystallite size of ZnO was calculated using Debye-Scherrer equation (D = Kλ/βcosθ), based on the most dominant peak intensity in the XRD analysis, where K = factor Crystals (0.9 for a sphere); λ = wavelength used (0.154 nm Cu-Kα); β = the value of FWHM (full width at half maximum) and θ = diffraction angle Brag. From the calculations, the average crystallite size for ZnO is 27.01 nm.

3.2. Synthesis of pyranopyrazole using ZnO
Synthesis of pyranopyrazole was performed using 4 components: ethyl acetoacetate, hydrazine hydrate, malononitrile and benzaldehyde. To investigate the effect of catalyst amount, zinc oxide was varied from 2.5-10 mol%. The reaction was performed for 1.5 hours at 70°C with a solvent of 10 mL ultrapure
Figure 4. XRD patterns of ZnO nanocrystalline.

Table 1. Several optimizations in pyranopyrazole synthesis*

| Temperature (°C) | Catalyst (% mol) | Time (min) | Yield (%) |
|------------------|------------------|------------|-----------|
| 30               | 10               | 90         | 98        |
| 70               | 10               | 90         | 88        |
| 100              | 10               | 90         | 73        |
| 70               | 7.5              | 90         | 65        |
| 70               | 5                | 90         | 45        |
| 70               | 2.5              | 90         | 41        |
| 70               | 10               | 30         | 67        |
| 70               | 10               | 60         | 75        |
| 70               | 10               | 120        | 86        |
| 70               | 10               | 180        | 85        |

*Reaction conditions: benzaldehyde (1 mmol), ethyl acetoacetate (1 mmol), hydrazine hydrate (1.5 mmol) and malononitrile (1 mmol).

Stability of ZnO as a solid catalyst was determined experimentally under the optimum conditions. In each reaction cycle, the catalyst which has been separated from the reaction mixture was washed with ethanol, then dried at 50°C under vacuum to remove the residual solvent, and then reused for the next cycle. The catalyst can be reused up to five times without significant loss of catalytic activity. The result of recoverability showed that the catalyst can be well-recovered (figure 5). It is an advantage for the large scale and sustainable synthesis of pyranopyrazole using ZnO nanocrystalline.
The product was successfully characterized as 6-amino-3-methyl-4-phenyl-2,4-dihydropyrano [2,3-c] pyrazole-5-carbonitrile. Yellowish crystals; IR absorption FT-IR $\nu$ (cm$^{-1}$): 3372 and 3310 (NH$_2$), 2192 (C≡N), 1649 (C=C), 1276 (C-C, pyrazole), 1184 (C-O), and 1073 (N-N). $^1$H-NMR (400 MHz, DMSO-d$_6$), $\delta$H (ppm): 12.11 (s, 1H, -NH); 7.3-7.1 (m, -ArH); 6.86 (s, 2H, -NH$_2$); 4.58 (s, 1H, -CH-); 1.06 (s, 1H, -CH$_3$). $^{13}$C-NMR, $\delta$C (ppm): 160.95; 154.21; 144.52; 135.71; 128.55; 127.56; 126.85; 120.93; 97.73; 57.19; 36.27; 9.82.

In the synthesis of pyranopyrazole, ZnO can serve as Lewis acidic and base sites (figure 6). The Lewis acid site of ZnO (Zn$^{2+}$) will receive a pair of electrons on oxygen atom of the carbonyl group of the pyrazolone, which is the result of the reaction of ethyl acetoacetate with hydrazine hydrate. Meanwhile, the Lewis base of ZnO (O$_2^-$) will enable malononitrile resulting in deprotonated C-H bond to form an active methylene group. The existence of an active methylene group will initiate the Knoevenagel condensation reaction between benzaldehyde and malononitrile to form arylidene
malononitrile. Furthermore, Michael addition reaction occurs between pyrazolone and arylidene malononitrile followed by cyclization and tautomeration to form pyranopyrazole.

4. Conclusions
ZnO was successfully synthesized using *Ulva fasciata* as capping agent. The optimum conditions of the pyranopyrazole synthesis was obtained in aqueous solvent, 10 mol% of catalyst with reaction time of 90 min at 70°C, and the name of the pyranopyrazole compound is 6-amino-3-methyl-4-phenyl-2,4-dihydropyrano [2,3-c] pyrazole-5-carbonitrile.

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