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Nanoencapsulated Formulation of Antibacterial Metabolites by Soil Actinomycete, *Nocardia sp.* TP5 from Tangkuban Perahu Mountain, West Java, Indonesia, with The Ionic Gelation Technique Using Na Alginate

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ABSTRACT

Formulation of nanoencapsulation of antibacterial metabolites from the fermentation of actinomycete strain designed as TP5 has been carried out. Nanoencapsulated formulations performed with Na alginate polysaccharides obtained from brown seaweed can maintain the antibacterial metabolite activity. The aim of this study was to enhance the antibacterial activity of *N. niigatensis* TP5 against *Escherichia coli* and *Staphylococcus aureus* in the nanoencapsulated formulation by ionic gelation technique using Na alginate and $CaCl_2$. The nanocapsules were prepared by combining the extracellular secondary metabolite *Nocardia sp.* TP5 with the encapsulation sourced from Na alginate and $CaCl_2$ by ionic gelation technique. The manufacturing methods include fermentation of *Nocardia sp.* TP5, nanoencapsulated formulation by varying the concentration and ratio of Na alginate, $CaCl_2$, antibacterial metabolites, as well as analysis of nanocapsules. The analysis and characterization of nanoencapsulation using SEM-EDS and PSA included: surface morphology, particle size, chemical constituents, and zeta potential, as well as antibacterial testing against *E. coli* and *S. aureus*. The results showed that the best-nanoencapsulated formula contains the composition of Na alginate 0.3%, $CaCl_2$ 0.06% with a ratio of Na alginate: $CaCl_2$: antibacterial metabolite is 2: 4: 1. The capsule particles formed are evenly distributed over the entire surface with a particle size of 425 nm, zeta potential of -27 mV, and antibacterial activity inhibited the growth of *E. coli* and *S. aureus* by 20 and 21 mm, respectively. The variation of the appropriate concentration ratio of Na alginate and $CaCl_2$ greatly affects the uniform nanocapsules size and increases the antibacterial activity.

Keywords: antibacterial nanoencapsulation, soil actinomycete *Nocardia sp.* TP5, Na alginate, ionic gelation, $CaCl_2$.

INTRODUCTION

Today drug technology has developed rapidly. Most types of drugs that prevent various diseases, including antibiotics, have been successfully developed according to the demands of the pharmaceutical industry, which requires improving system performance, such as by enhancing penetration to the intended target.

An actinomycete is a group of microorganisms whose populations are widely distributed in nature (Oskay *et al*., 2004). Seventy percent of the total antibiotics produced by microorganisms come from the Actinomycete family, of which 75% have been used in the medical field (Berdy, 2005). The genus Nocardia belongs to the actinomycete family, with a broad spectrum against pathogenic Gram-positive and Gram-negative bacteria (Wardani *et al*., 2013; Sharma *et al*., 2016; Mishra *et al*., 2019; Dhakal *et al*., 2019).

Fermented antibacterial metabolites are very susceptible to oxidation, heat, humidity, which will result in decreased activity, so they need to be protected using an encapsulated matrix. Na alginate is a food-grade polysaccharide matrix

derived from brown seaweed, biodegradable, easily absorbed, and decomposes naturally, so it is safe for living things.

Alginate is a biocompatible, biodegradable and non-toxic polysaccharide extracted from brown marine algae and formed by alternatingly linked -L-gluronic (G residue) blocks 1→4 (G residue) and -D-mannuronic acid (G residue) M). Sodium alginate is a water-soluble biopolymer that forms a gel structure in the presence of divalent cations such as calcium. The interaction of Ca^{2+} ions with the residue block G, causing cross-links that will strengthen the mechanical strength of the particles formed. Through ionic gelation technique positively and negatively charged biopolymers (coacervation complexes) trap bioactive particles formed by electrostatic complexation. Non-polar and polar bioactive molecules are applied in this technique (Rehm, 2009; Vos *et al*., 2010).

The nanoencapsulated formulation will increase the bioavailability of antibacterial compounds against pathogens. The nano-sized particles allow for a better distribution of the product and can expand the contact surface of the particles with the material. Utilization of antibacterial metabolites in the form of nanoencapsulation can open opportunities for the production of practical and stable antibacterial nanoencapsulation by heat, light, and oxygen (Ezhilarasi *et al*., 2012; Andayani *et al.,* 2019).

In the pharmaceutical industry, the application of nanoencapsulation technology will provide several advantages, including in terms of increased activity, product consistency, absorption, and the availability of bioactive components from the encapsulated core compound. This process provides material protection to the core material compound from direct contact with the environment and provides a controlled release. However, nanoencapsulation of dispersion systems for water-soluble hydrophilic bioactive components is still rarely done.

The aim of this study was to enhance the antibacterial activity of *N. niigatensis* TP5 against *E.coli* and *S. aureus* in the nanoencapsulated formulation by ionic gelation technique using Na alginate and $CaCl_2$.

In nanoencapsulation research, several studies oriented towards encapsulation from different sources continue to be carried out. There are still a few researches on the role of carbohydrate nanocarriers on the metabolism of encapsulated bioactive components, and there is no study that mentions antibacterial metabolites of *Nocardia sp*. TP5, isolated from the volcanic soil of Mount Tangkuban Perahu, West Java Province, Indonesia, was able to fight pathogenic bacteria, especially in the form of nanoencapsulation.

MATERIAL AND METHODS

Identification of TP5 Isolate

Morphological identification of TP5 Isolates was carried out using the Gram stain method and analyzed under light and electron microscopy (William *et al*., 1994; Holt *et al*., 1994; Cappuccino and Sherman, 1999). Molecular identification of TP5 isolates was carried out based on partial genetic analysis on bacterial 16S ribosomal RNA through DNA isolation and DNA extraction processes, electrophoresis and PCR reactions, purification of PCR products, and BLAST at the online DNA database center at DDBJ (http://www.ddbj.nig.ac.jp) or (http://www.ncbi.nlm.nlh.gov/). (Pitchers *et al*.,1989; Hiraishi *et al*.,1995; Thompson *et al*.,1997).

Phylogenetic analysis of the sequence data was carried out using the neighbor-joining method (NJ method) with the program Phylogenetic Analyses Using Parsimony (PAUP) version 4.0b10 (Felsenstein *et al*.,1985; Saitou *et al*.,1987).

Fermentation process

Fermentation of TP5 Isolate was carried out on media containing (g/L): glucose 10, soluble starch 10, tryptone 5, yeast extract 2.5, and calcium carbonate 1. The fermentation process was conducted for 36 hours, 120 rpm, one vvm, 30 ˚C, pH 9. During the fermentation process, observations were made on microbial growth, pH, glucose and protein concentration, and antibacterial activity against *E. coli* and *S. aureus* (Stanbury *et al*., 2016).

Synthesis of secondary metabolite nanoencapsulant *Nocardia sp* TP5

The manufacture of nanoencapsulants was carried out by combining fermented extracellular metabolites with Na alginate and $CaCl_2$ solution (Table I). Then stirred to form an emulsion for 4 h at a speed of 600 rpm at a temperature of 25 ˚C. The filtrate was centrifuged at 3000 rpm for 20 minutes, and the precipitate was dried by the freeze-drying method for 48 h (Mishra, 2016).

Nanoencapsulant characterization and antibacterial activity test

The characterization of nanoencapsulants was carried out on surface morphology, particle

size, and zeta potential using SEM-EDS and PSA instruments and antibacterial activity tests against *E. coli* and *S. aureus* by agar diffusion. Antibacterial activity was analyzed by measuring the clear zone formed around the well in mm after incubation for 24h.

$$\text{Encapsulation efficiency \%} = \frac{\text{MMBE - MEAT}}{\text{MMBE}} x\ 100$$

MMBE = Mass of the molecule used before encapsulation
MEAT = Mass of encapsule after treatment

RESULT AND DISCUSSION

Morphology and molecular identification

The analysis results showed that TP5 isolate is a white colony in PDA media, aerial spore and hyphae, and a hairy surface in light and electron microscope (Figure 1. a, b, c). It is a new species from the genus Nocardia with 95% similarity to *Nocardia niigatensis*, so the antibacterial metabolites it produces may also be different. It will opportunity to obtain antibacterial metabolites with new properties as well as overcome resistant antibacterial (Figure 2 and Table I).

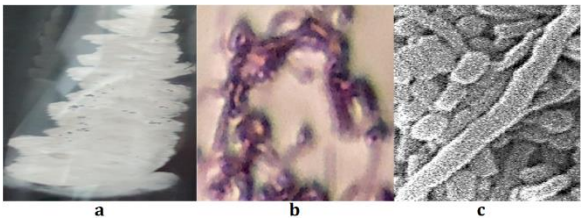

Figure 1. Colony morphology of TP5 isolate on PDA media (a), hyphae (b), on light microscopy (100x) and spores (c) on electron microscope (15,000x)

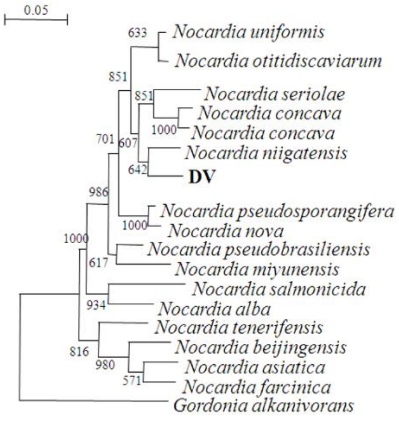

Figure 2. Phylogeny tree of TP5 Isolate (DV)

Table I. Homology of 16srRNA of TP5 Isolate with *Nocardia sp.*

Region	***Nocardia sp.***	**Homology (%)**
16S rRNA	*Nocardia niigatensis*	95

Fermentation of TP5 Isolate

The fermentation of TP5 Isolate lasted for 36 hours. The growth of TP5 isolate started from the lag and log phases in which TP5 Isolate produced primary metabolites. From the 20th to the 34th hour, the fermentation process enters a stationary phase, namely the formation of secondary metabolites at pH 7 and the use of carbon and protein sources from 0.8 mg/mL to 0.2 mg/mL. The fermentation process of TP5 Isolate stopped at 36th h, called the death phase (Figure 3. a, b, c, d).

Synthesize and characterization of nanoencapsulated antibacterial of *Nocardia sp.*TP5

Nanoencapsulation of an antibacterial agent of TP5 was successfully obtained by ionic gelation method with a combination of positive charges from Na alginate and negative charges from $CaCl_2$ (Table II).

Process conditions and the formulation of Na alginate and $CaCl_2$ are very influential on morphology, average particle size, and the potential of nanoencapsulated synthesis. In nano size, the surface volume ratio will be increased, which results in increased effectiveness (Rahnemoon *et al*., 2021).

N. niigatensis TP5 produces metabolic products called metabolites. The compounds produced by *N. niigatensis* during the stationary phase are called secondary metabolites. The secondary metabolite of *N. niigatensis* TP5 has antibacterial activity inhibiting the growth of Gram-positive bacteria more strongly than against Gram-negative bacteria (Table II and 3). This is because the cell walls of Gram-negative bacteria are covered by lipopolysaccharides (Cappuccino and Sherman, 2005). Apart from this, the antibacterial activity was influenced by the concentration of the Na alginate as a matrix and the $CaCl_2$ as a crosslinker. Increasing the concentration of the matrix can increase the density between the matrices so that the efficiency of the microspheres increases, but the antibacterial metabolite of *N. niigatensis* TP5 will be difficult to separate from the matrix.

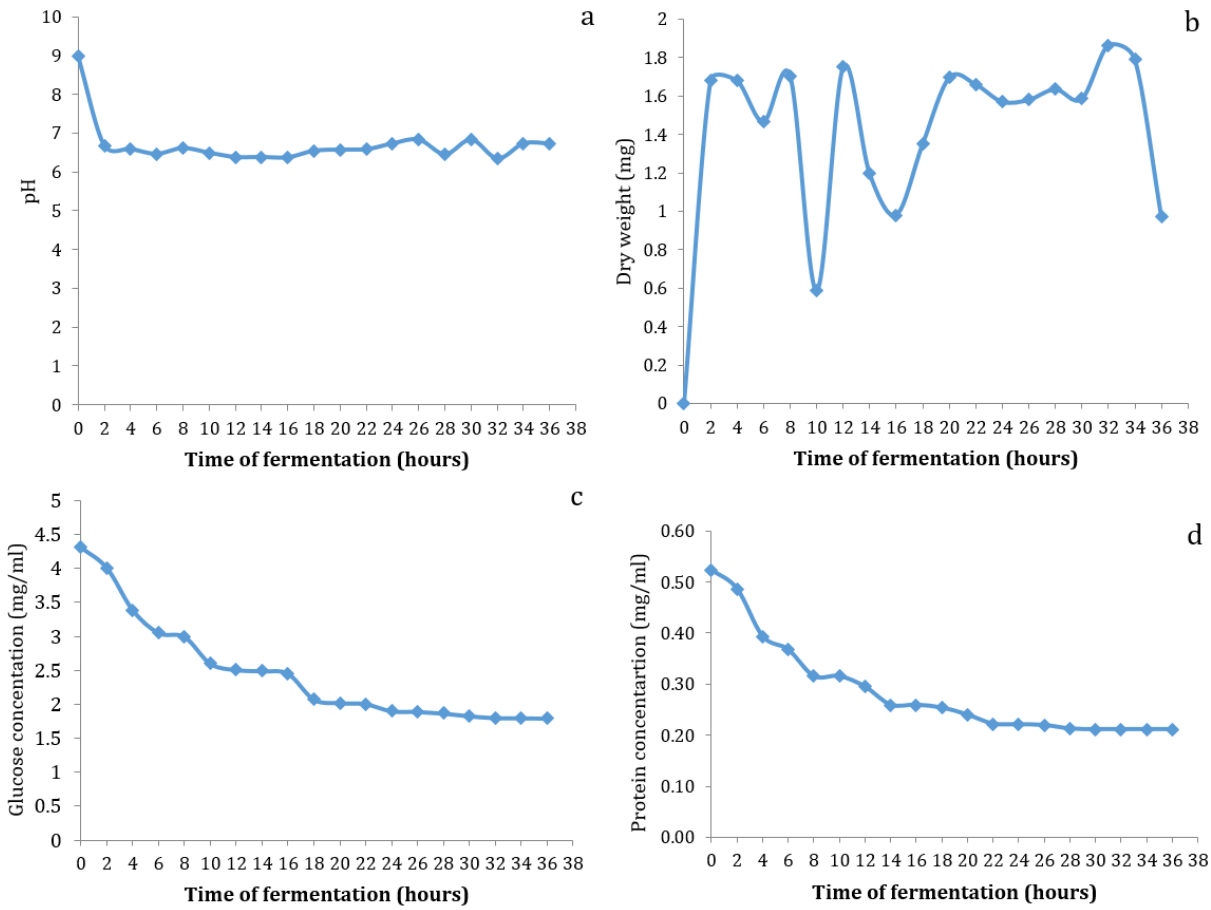

Figure 3. Characterization of secondary metabolite formation of TP5 Isolate a. pH, b. dry weight (mg), c. Glucose concentration (mg/ml), d. Protein concentration (mg/ml)

Table II. Characterization of antibacterial nanoencapsulation of *Nocardia sp*. TP5

Experiments	Alginate (%)	$CaCl_2$ (%)	Alginate ratio	$CaCl_2$ ratio	Anti bacterial metabolite ratio	Alginate (g)	Powder weight (g)	Particle size (nm)	Zone of inhibition (mm): *E. coli/S. aureus*	Efficiency (%)
1	0.3	0.06	2	2	1	0.12	2.8	475	0/18	95.7
2	0.1	0.02	3	3	1	0.06	2	465	14/19	97
3	0.1	0.1	3	1	1	0.02	4.7	735	0/20	99.6
4	0.3	0.06	2	2	1	0.12	7.6	630	0/20	98.4
5	0.5	0.1	1	1	1	0.1	8.7	680	0/18	98.9
6	0.3	0.06	4	2	1	0.24	5.6	730	0/14	95.7
7	0.3	0.06	2	2	1	0.12	5.8	700	0/16	97.9
8	0.1	0.1	1	1	1	0.02	6.6	600	0/25	99.7
9	0.3	0.14	2	2	1	0.12	11.4	455	19/15	99
10	0.3	0.06	2	4	1	0.02	33.7	425	20/21	99.9
11	0.1	0.1	3	1	1	0.06	9.7	700	0/13	99.4
12	0.7	0.06	2	2	1	0.28	9.1	500	0/11	96.9
13	0.1	0.02	1	1	1	0.02	9.3	575	0/11	99.8
14	0.5	0.02	1	3	1	0.1	8.1	655	0/13	97.5

Table III. Antibacterial activity against *E.coli* and *S. aureus*

Pathogenic bacteria	Zone of inhibition before nanoencapsulation (mm)	Zone of inhibition after nanoencapsulation (mm)
E. coli	9	20
S. aureus	12	21

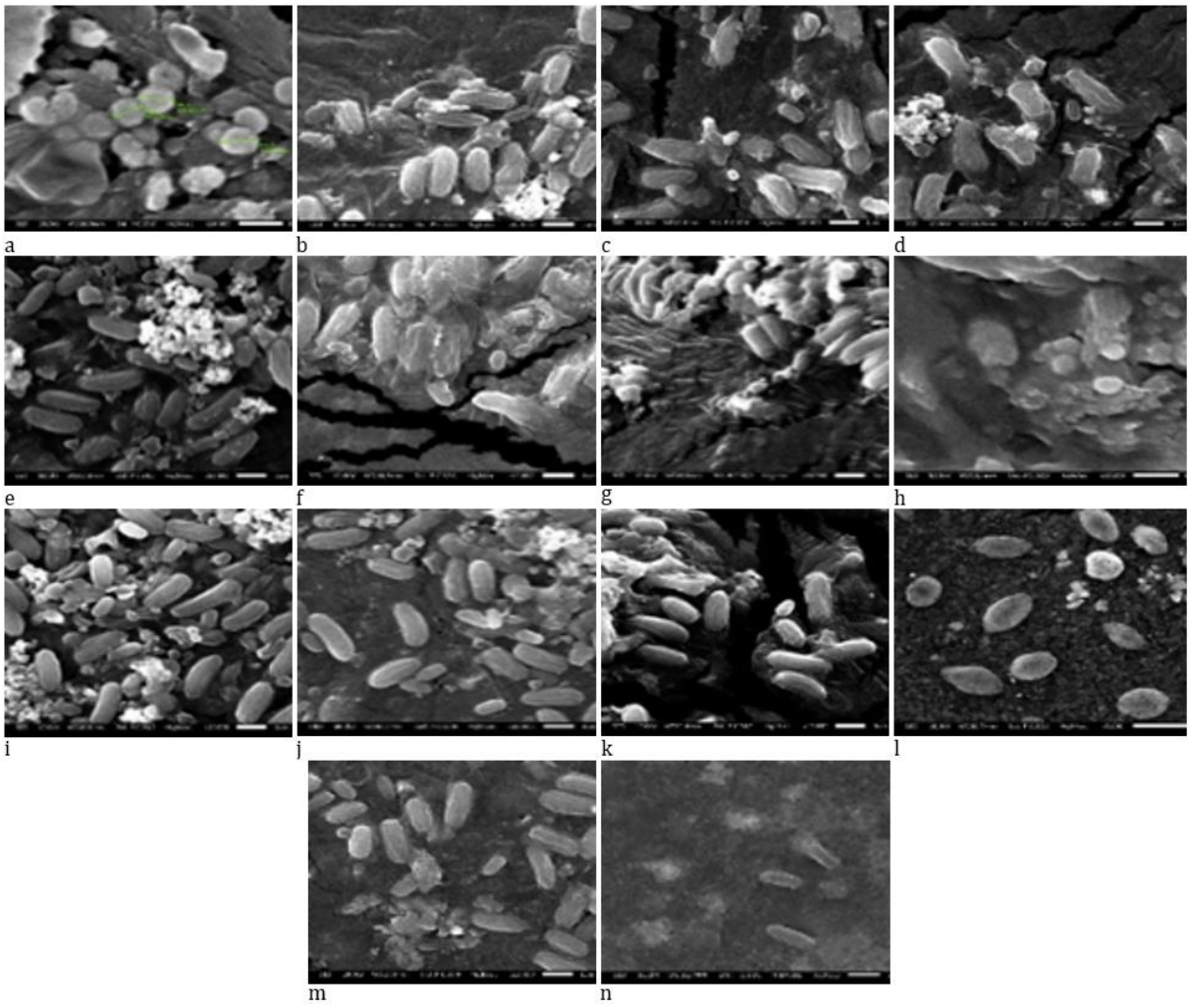

Figure 4. The morphology of the nanoencapsulated antibacterial metabolite of *Nocardia sp.* TP5 (a-n) on experiments 1 - 14 in SEM (10,000 x)

On the other hand, a decrease in the matrix concentration can cause a reduction in the efficiency of the microspheres because the matrix cannot trap the active ingredients optimally, and the active ingredients are quickly released (Rehm, 2009). The concentration and ratio of matrix Na alginate and crosslinker $CaCl_2$, which gave the highest activity in this research, were 0.3 %, 0.06 %, 2, and 4, respectively (Table II).

The SEM image of nanoencapsulated antibacterial of *Nocardia sp*. TP5 SEM displayed a homogeneous distribution and stable formulation with spherical and rod shape and coated with transparent layer from ionic gelation between Na alginate and $CaCl_2$. The image of Nanoencapsulation of an antibacterial metabolite of *Nocardia sp*. TP5 morphology on 14 variations (Figure 4).

Antibacterial metabolites produced through the fermentation process can be protected from damage by environmental influences such as oxidation, heat, humidity, and pH by encapsulation. Ionic gelation and freeze-drying methods using Na alginate are mutually supportive methods to encapsulate antibacterial metabolites in nano size. Encapsulation in nano-size will increase the penetration of the active ingredient through the target wall deeper, resulting in increased antibacterial activity. In the form of nanoencapsulated, the lipophilic antibacterial properties of TP5 will increase through the cell membrane of pathogenic bacteria, resulting in increased activity.

At a balanced concentration, the higher the ratio of Na alginate: $CaCl_2$, the larger the particle size obtained was directly proportional to the antibacterial activity and the efficient use of raw materials. Of the 14 experiments that have carried out, the best formula obtained in the 10th experiment with the composition of alginate and $CaCl_2$: 0.3% and 0.06%, the ratio of alginate: $CaCl_2$: antibacterial metabolite was 2:4:1, the zone of inhibition against *E. coli* and *S. aureus* was 20 and 21 mm respectively, along with particle size of 425 nm evenly distributed with round and rod-shaped surfaces, and the efficiency reached 99.9% (Table II).

Research conducted by Wardani *et al*. (2013), Sharma *et al*. (2016), and Mishra *et al*. (2019) showed *Nocardia sp*. TP1, *Nocardia sp*. PB-52, and *Nocardia sp*. CS682 produces antibacterial metabolites that are active against the pathogenic Gram-positive *S. aureus* but not against Gram-negative *E. coli*. Likewise, the secondary metabolite *Nocardia sp.* TP5 showed weak activity against *S. aureus* and *E. coli* (Table III), but the antibacterial activity increased significantly after being formulated in nanoencapsulated form (Table IIi and Figure 4.j).

The composition of the nanoencapsulated antibacterial *Nocardia sp.* TP5 is dominated by carbon elements as much as 74.84%, followed oxygen 22.12%. Na 2.66%, Ca 0.17%, K 0.12%, and Mg 0.08%, and zeta potential, was negative charges with a value of -27 mV, indicating the nanoencapsulated antibacterial of *Nocardia sp.* TP5 formed has a fairly strong bond, high stability, and prevents particle aggregation,

This study proves that alginate as a carbohydrate nanocarrier can protect the antibacterial nanoencapsulation with high activity against Gram-positive and negative. Nanoencapsulation with the ionic gelation method is one way to preserve fermented metabolites from damage because it is carried out at low temperatures and is hydrophilic.

CONCLUSION

Encapsulated formulation of antibacterial metabolite by Na alginate and $CaCl_2$ is crucial in the synthesis of nanoencapsulants. The preparation of the encapsulation composition of Na alginate, $CaCl_2$, and antibacterial metabolite of *Nocardia sp*. TP5 are beneficial to obtain uniform nanocapsule size with high bioactivity.

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