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BIODEGRADATION POTENTIAL OF CYANIDE AND NITRILE USING BACTERIA OF THE GENUS RHODOCOCCUS

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Abstract

Cyanides and nitriles, characterized by their R-CN chains, are known for their toxicity, mutagenicity, and carcinogenicity, posing significant threats to environmental and human health. This study aims to explore the biodegradation capabilities of Rhodococcus sp. in breaking down cyanide and nitrile bonds. Rhodococcus pyridinivorans strain I-benzo was isolated from tanning waste and cultured in mineral media with a 20 mM benzonitrile substrate. The activity of this strain was tested using substrates such as benzonitrile, acetonitrile, acrylonitrile, benzamide, acetamide, and acrylamide, revealing positive reactions of nitrile hydratase and amidase enzymes through the Nessler measurement method, which indicated the production of ammonia and carboxylic acids. Furthermore, the degradation tests showed that the Vmax values for the biodegradation of potassium cyanide and sodium cyanide were 0.56 ppm/minute and 0.21 ppm/minute, respectively. These findings highlight the potential application of Rhodococcus pyridinivorans strain I-benzo in mitigating the environmental impact of cyanide and nitrile pollutants through efficient biodegradation.

Keywords: Biodegradation, Nitrile, Rhodococcus sp, Cyanide.

Introduction

Environmental pollution caused by industrial waste has become a major concern on a global scale (Vitolo, 2020). Approximately 6 million chemical compounds have been synthesized, with 1,000 new chemicals synthesized each year (Sharma & Dubey, 2022). Nearly 60,000 to 95,000 chemicals are used commercially as well as more than a billion pounds of toxins are released globally in the air and water (Singh SP, 2014; Diaz & Caizaguano, 2021). Cyanide and nitrile are toxic chemical components found in wastewater contamination. The presence of free cyanide is more toxic where the concentration of this free cyanide can damage 50% of the population in the ecosystem. The critical level of nitrile toxicity with acrylonitrile is produced as much as 4 million tons per year. Acrylonitrile plays a significant role in the rubber, plastics, and polymer industries (Eunike et al., 2018; Alvillo-Rivera et al., 2021). There are several methods to complete wastewater treatment, such as physical, chemical, and biological treatment, have begun to be developed by many researchers. One of them is the biological method of bioremediation which refers to all processes that use microorganisms such as bacteria, fungi, yeast, algae and enzymes produced by these microbes to clean or neutralize chemicals and waste safely and one of the alternatives in overcoming environmental problems. has the advantages of no or minimal interference, no secondary pollution, low-

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cost treatment, simple preparation, and in-situ remediation (Gomathi et al., 2020; Du et al., 2021). Different types of microbes have been used to degrade cyanide and nitrile. Some of them are *Rhodococcus sp. N-774, Pseudomonas sp. B23, Rhodococcus rhodochrous J-1. Like Rhodococcus UKMP-5M* has the ability to biodegrade cyanide from KCN. Whole cells in 1 g/L of biomass are able to degrade nearly 50% of 12 mM cyanide in a span of 10 hours. *Rhodococcus UKMP-5M* has the best degradability compared to other types of *Rhodococcus* (Nallapan et al., n.d; Ethica et al., 2021; Gerasinova et al., 2004). Degradation of cyanide can use autochthonous consortium bacteria (Deloya-Martínez, n.d.). Bacteria isolated from I-benzo can be used to degrade acetonitrile and benzonitrile in high concentrations. This type of bacteria is *Rhodococcus Pyridinovorans* (Sulistinah & Sunarko, 2020; Husain et al., 2021). Through this research, cyanide and nitrile biodegradation will be carried out. *Rhodococcus* as a genus of bacteria that has been studied is able to degrade cyanide and nitrile isolated from tanning waste. This study aims to explore the biodegradation capabilities of Rhodococcus sp. in breaking down cyanide and nitrile bonds.

Research Methods

Tools: The equipment used in this study includes glass bottles, measuring cups, goblet glasses, measuring flasks, erlenmeyer, volume pipettes, measuring pipettes, drip pipettes, stirring rods, spatula, spray bottles, watch glasses, porcelain cups, hotplates, magnetic stirrers, magnetic bars, analytical balances, pH meters, centrifuge devices, centrifuge tubes, thermometers, rotary shaker incubators, incubators, microscopes, biofilm reactors, autoclaves, filter-sterilize. The instrumentation used for characterization is, optical density, UV-Vis Diffuse Reflectance spectrophotometer, UV-Vis spectrophotometer, Fourier Transform Infrared spectrophotometer (FTIR spectrophotometer).

Equipment : *Rhodococcus*, $Na_2HPO_4.2H_2O$, KH_2PO_4, $MgSO_4.7H_2O$ (1 M; Fisher Scientific), $CaCl_2.2H_2O$ (Fisher Scientific), $FeSO_4.7H_2O$, Yeast Extract (Himedia), Microelement, Aquadest, Granucult Nutrient Broth (NB) Merck, Granucult Nutrient Agar (NA) Merck, H_2O, NaCN (Merck), KCN (Merck), Benzonitrile (Sigma Aldrich), Benzamide (Sigma Aldrich), Acetonitrile (Sigma Aldrich), Acetamide (Sigma Aldrich), Methanol (Merck), Buffer phosphate (Merck), KOH (Merck), HCl (Merck).

Rhodococcus pyridinivorans strain I-benzo isolated from tanning waste is conditioned inside to be nutritious for further observation. The inoculum was prepared using a medium containing benzonitrile as a source of carbon and nitrogen energy for the growth of *Rhodococcus pyridinivorans strain I-benzo*. The bacterial culture was incubated in a shaker for 72 hours. The mineral manufacturing media for 1000 mL is as follows based on this ingredients $Na_2HPO_4.2H_2O$ 0,4475 gr, KH_2PO_4 0,1 gr, $MgSO_4.7H_2O$ 0,1 gr, $CaCl_2.2H_2O$ 0,01 gr, $FeSO_4.7H_2O$ 0,001 gr, Yeast Extract 0,01 gr, Microelement 1 mL, Aquadest 1 L. Pure cultures of the genus *Rhodococcus pyridinivorans strain I-benzo* were inoculated in a 100 mL erlenmeyer containing 50 mL of medium. Acetonitrile, benzonitrile, potassium cyanide, and sodium cyanide were

added to the culture medium as a source of carbon and nitrogen energy. The bacterial cultures were incubated in a shaker at 121 rpm with operating conditions of 28-30 ºC for 48-72 hours. The growth of bacteria is observed using Spektrofotometer Uv-Vis 1601 PC SHIMADZU (Shimadzu Biospec-1601 DNA/ Protein/ Enzyme Analyze) with optical density on wavelength 436 nm.

Biomass from the bacteria of the genus *Rhodococcus pyridinivorans strain I-benzo* is produced by being given nutrients from 25mM benzonitrile and 3% inoculum (v/v). The bacterial cultures were incubated in a rotating shaker at 121 rpm under operating conditions at 28ºC for 72 hours. Then the cells are harvested using a Kubota 6500 (Japan) type Centrifuge. The operating condition of the centrifuge is 9000 rpm at 4ºC for 6 minutes, then rinsed twice using 50mM phosphate buffer (KH_2PO_4) pH 7.2. The cell suspension is centrifuged and the cell granules are stored at -4ºC before being used for further testing.

The activity test of Rhodococcus pyridinivorans strain I-benzo was carried out using a variety of substrates of benzonitrile, acetonitrile, acrylonitrile, acetamide, benzamide, and acrylamide (Sigma Aldrich) using a concentration of 50 mM. The activity test was carried out using a phosphate buffer. The control is a phosphate buffer solution with susbrates. As for the mixed cell activity test, a phosphate buffer (10 mL) + substrate + 3% I-benzo cells (m/v) was made. The time variation in the activity test was carried out in a span of 10 minutes, namely at time 0; 10; 20; 30; 40; 50; 60. The sample was then centrifuged and tested for ammonia content with Nessler, the test was carried out with the Uv-Vis 1601 PC SHIMADZU Spectrophotometer at a wavelength of 420 nm.

Biodegradation of acetontril, benzonitrile, potassium cyanide, and sodium cyanide is carried out by adding 1.0 g of cells to 75 mL of 500 mM / 2% (v/v) of the sample to be degraded into 50 mM phosphate buffer (KH_2PO_4) with a pH of 7.2. After the mixing reaction is carried out, it is then incubated in a rotating shaker at a temperature of 28ºC for 180 minutes. A sample of 1.0 mL was taken periodically during a time interval of 15 minutes. The enzymatic reaction is stopped by adding 250µl of 4N HCl, and the sample is centrifuged. Residues from acetontril, benzonitrile, potassium cyanide and sodium cyanide samples in supernatants and degradation products are analyzed using a reaction with Nessler and will be analyzed by *Spectrophotometer Uv-Vis 1601 PC SHIMADZU (Shimadzu Biospec-1601 DNA/ Protein/ Enzyme Analyze*).

Immobilization is carried out by the entrapment method using zeolite or activated carbon. The activated carbon zeolite used is at a size of 80 – 100 mesh. Entrapment is carried out when activated carbon zeolite has been activated using HCl solution (5%) and heated in an oven at a temperature of 150ºC for 4 hours and 30 minutes. After that, the immobilization process takes place using a phosphate buffer solvent pH 7.0. The phosphate buffer used was 100 mL with activated carbon 10 grams and wet cells used 1 gram. Stirring with a magnetic stirrer lasts for 12 hours. Then *Rhodococcus pyridinivorans strain I-benzo* that had been immobilized with activated carbon was analyzed with *FTIR Shimadzu IRAffinity-1S*.

Results and Discussion

The growth of *Rhodococcus pyridinivorans microbacteria strain I-benzo* was carried out in a period of 0 hours, 24 hours, 48 hours, 72 hours, and 168 hours by the method of checking turbidity (*Optical Density*) using the *Uv-Vis 1601 PC SHIMADZU Spectrophotometer (Shimadzu Biospec-1601 DNA/ Protein/ Enzyme Analyze)* for the measurement of optical density was measured at a wavelength (λ) of 436 nm. The absorption was measured by *the photometry method*. Observation of the growth of *Rhodococcus pyridinivorans strain I-benzo* based on the variation in the concentration of benzonitrile substrate obtained the optimum substrate concentration in the 168-day growth observation at a benzonitrile substrate concentration of 20 mM with a maximum absorbance of 4.4950.

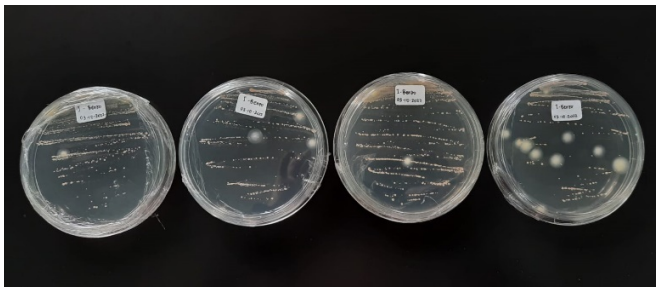


Figure 1. *Rhodococcus pyrinidovorans strain I-benzo* in agar media

Activity & growth test of *Rhodococcus pyridinivorans strain I-benzo* grown on benzonitrile isolate with a substrate concentration of 20 mM. *Rhodococcus pyridinivorans strain I-benzo* was grown in 500 mL mineral medium and a benzonitrile concentration of 20 mM. With an inoculum of 2% (v/v). Optical *density*, pH, and ammonia content tests were carried out using the Nessler method in the time range of 0, 6, 24, 30, 48, 54, 72, 78, 98, 168, and 192 hours.

Table 1. Observation data *Rhodococcus pyridinivorans strain I-benzo* with benzonitrile substrate 20 mM (2% v/v)

Sampel (I-benzo)	pH	OD (abs)	Nessler (abs)
0hr	7,46	0,1012	0,0436
6hr	7,27	0,2184	0,1265
24hr	6,82	0,6330	0,7168
30hr	6,81	1,0128	1,1780
48hr	6,77	1,6650	1,3336
54hr	6,87	2,2680	1,7670
72hr	6,89	2,5900	1,2490
78hr	6,77	3,0800	1,1760
98hr	6,98	4,0000	0,8810
168hr	7,94	5,2110	0,8860
192hr	8,67	5,1220	0,8870

Qualitative measurements by the ammonia colorimetric method from various sources have been carried out in general with the Nessler method. In the Nessler method, a strong base of potassium hydroxide is added as a catalyst for Nessler reagents. This is to give a

yellowish color to the solution which indicates the presence of ammonia in the solution (Muramatsu, 1967). Nessler's reaction is as follows:

$$2K_2HgI_4 + NH_3 + 3\ KOH \longrightarrow HgO.Hg(NH_2)I + 7KI + 2H_2O$$

Figure 2. Ammonia Identification Reaction with Nessler Reagent

Based on the results of activity tests using benzonitrile, acetonitrile, acrylonitrile, acetamide, benzamide, and acrylamide substrates, positive results were obtained in both nitrile and amide substrates. Qualitative analysis can be seen that all the substrates tested react positively in producing ammonia. Because it was used analysis with Nessler and produced a yellow color until the 60th minute.

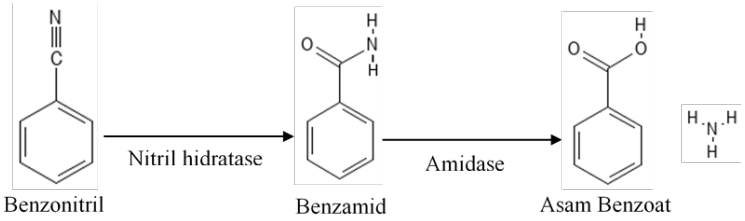


Figure 3. Enzymatic reaction kinetics of benzonitrile and benzamide substrates

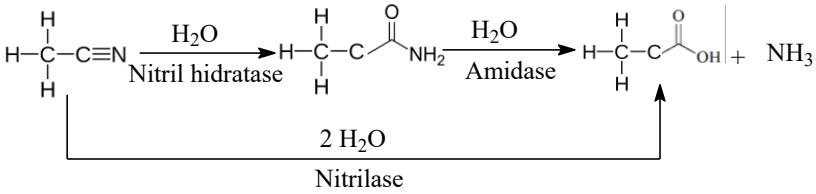


Figure 4. Enzymatic reaction kinetics of Asetonitrile and Acetamide substrates

The equation for calculating ammonia levels produced from the biodegradation pathway with *Rhodococcus pyridinivorans strain I-benzo,* is as follows:

$$y = 0{,}0287\ x + 0{,}0506$$

$$x = \frac{y - 0{,}0506}{0{,}0287}$$

Information:

$$y = absorbant$$

Table 2. Ammonia content in Nessler's solution (mg/L)

Solution	Time (minutes)						
	0	10	20	30	40	50	60
Benzonitrile Control (mg/L)	3,0767	2,7735	2,8397	3,0209	3,4042	3,6899	3,4634
Benzamide Control (mg/L)	1,7178	3,6655	3,8467	3,9338	4,2997	4,0244	4,1951
Whole Cell with Benzonitrile (mg/L)	4,3136	5,7526	5,8084	7,0244	7,2683	9,3136	10,8746
Whole Cell with Benzamide (mg/L)	3,9791	6,1812	5,4495	8,1672	10,3659	10,9164	10,6655
Acetonitrile (mg/L)	1,9512	1,9233	1,8153	1,9686	1,5470	1,4599	1,9686
Acetamide Control (mg/L)	5,7596	9,1707	7,4042	7,2300	6,8502	4,3589	6,7143

Solution	Time (minutes)						
	0	10	20	30	40	50	60
Whole Cell with Acetonitrile (mg/L)	1,7840	3,7247	3,4530	6,4634	6,2962	7,4878	7,8606
Whole Cell with Acetamide (mg/L)	5,8885	9,3240	12,7875	16,3136	19,0836	19,6272	23,6167
Acrylonitrile Control (mg/L)	3,4425	3,5052	3,2648	3,8711	4,3693	3,5122	3,5192
Acrylamide Control (mg/L)	3,0627	3,4704	3,8467	3,2648	3,2718	3,5122	3,5679
Whole Cell with Acrylonitrile (mg/L)	3,9233	5,6132	7,4321	10,6411	10,7247	12,3206	14,3902
Whole Cell with Acrylamide (mg/L)	4,0557	6,0488	9,5366	9,6063	8,9059	9,4146	14,9303

Table 3. Nessler analysis results

Solution	Results	Enzyme
Whole Cell with Benzontrile (ammonia)	++	Nitrile Hydratase and Amidase
Whole Cell with Benzamide (ammonia)	++	Nitrile Hydratase and Amidase
Whole Cell with Asetonitrile (ammonia)	++	Nitrile Hydratase and Amidase
Whole Cell with Acetamide (ammonia)	++	Nitrile Hydratase and Amidase
Whole Cell with Acrylonitrile (ammonia)	++	Nitrile Hydratase and Amidase
Whole Cell with Acrylamide (ammonia)	++	Nitrile Hydratase and Amidase

In addition to determining the enzymatic activity of nitrile hydratase and amide from the presence of ammonia products, the calculation of cell unit activity is carried out. One unit of cell activity is the activity needed to release 1 mmol of ammonia per gram of cells per minute. The cell mass used is 3% of the solution, which is 0.3 grams of 10 mL of phosphate solution and substrate. While the time (t) used is the one shown on the graph, namely at the 0 time; 10; 20; 30; 40; 50; 60 minutes.

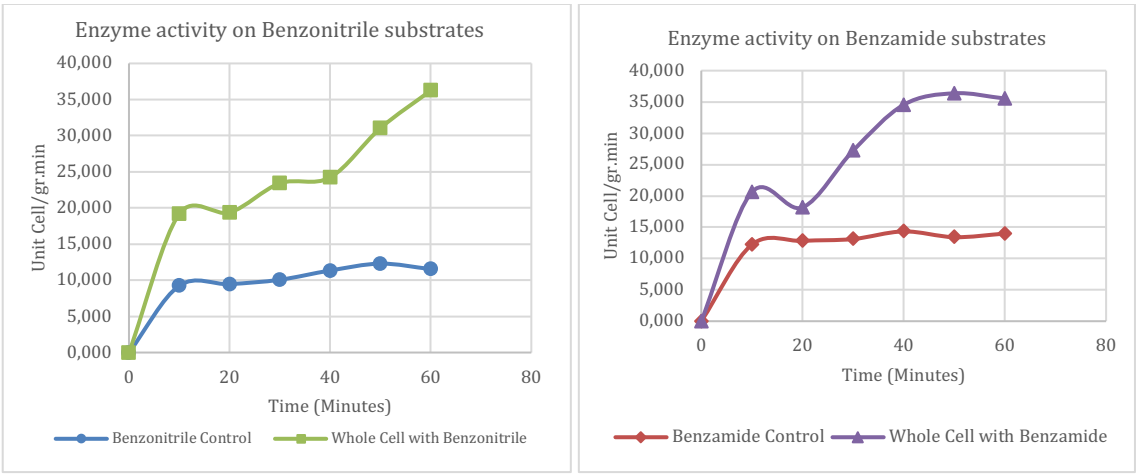


Figure 5. Enzyme activity on Benzonitrile and Benzamide substrates

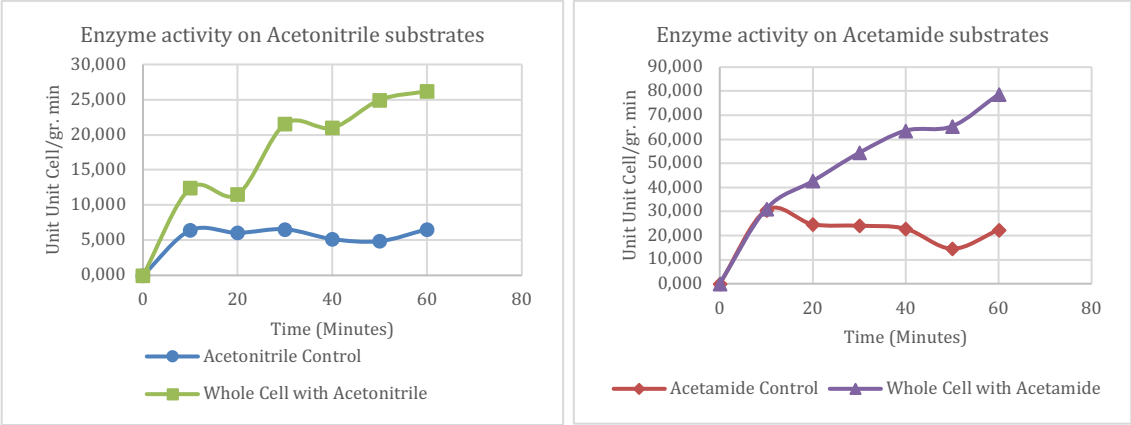


Figure 6. Enzyme activity on Acetonitrile dan Acetamide

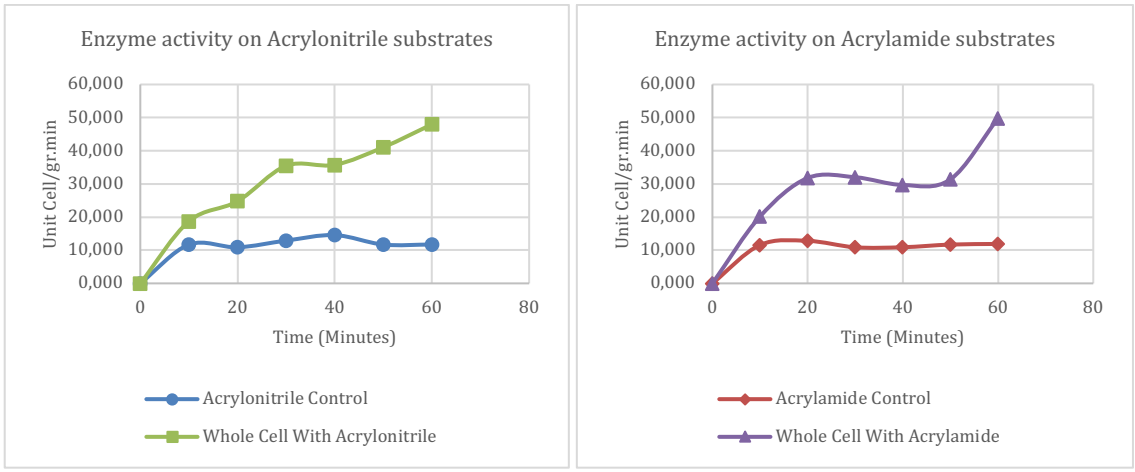


Figure 7. Enzyme activity on Acrylonitrile dan Acrylamide

Enzyme immobilization of *Rhodococcus pyridinivorans strain I-benzo* bacteria was carried out by the entrapment method. Activated carbon zeolite is activated using HCl solution (5%) and heated in an oven at a temperature of 150ºC for 4 hours and 30 minutes. From the FTIR results, it can be seen that there are 11 peaks. In the observation results, the absorption band at the wave number of 3244.80 cm^{-1} shows the absorption of the O – H stretching bond. Then, the absorption band at wave number 2974.76 cm^{-1} is the absorption from the Aliphatic saturated bond C – H streching, as well as the absorption from the O = H bending bond at wave number 1653.27 cm^{-1}. In addition, there is absorption at wave numbers 1393.2 and 1428.27 cm-1 which is the O – H vibration of the alcoholic chain. The absorption bands at wave numbers 959.08 and 900.679 cm^{-1} are absorption from C-C vibrations and can indicate the presence of aromatic chains of enzymes previously grown in benzonitrile substrates that are immobilized into activated carbon. The absorption band at the wave number 861.11 cm^{-1} is the C – H vibration. The absorption band at the wave number 731.04 cm^{-1} is the C – H out of plane bending vibration. The absorption band at wave numbers 659.39 cm^{-1} and 685.33 is the detection of the presence of Si – O silica bonds contained in zeolite.

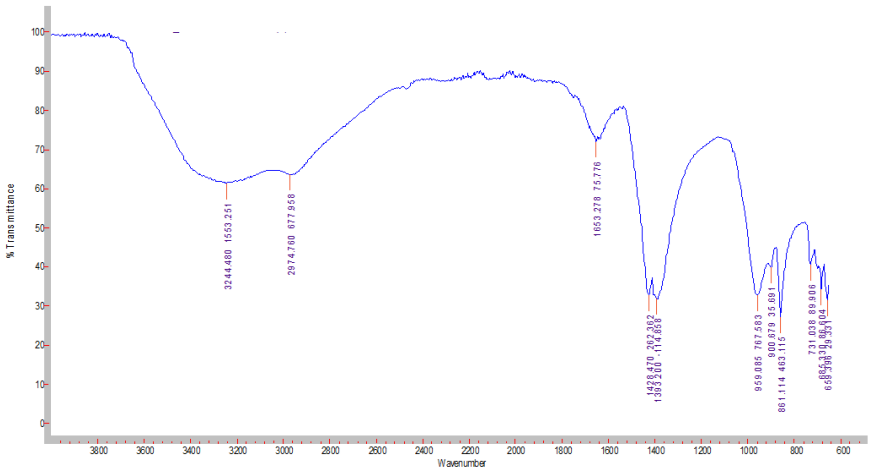
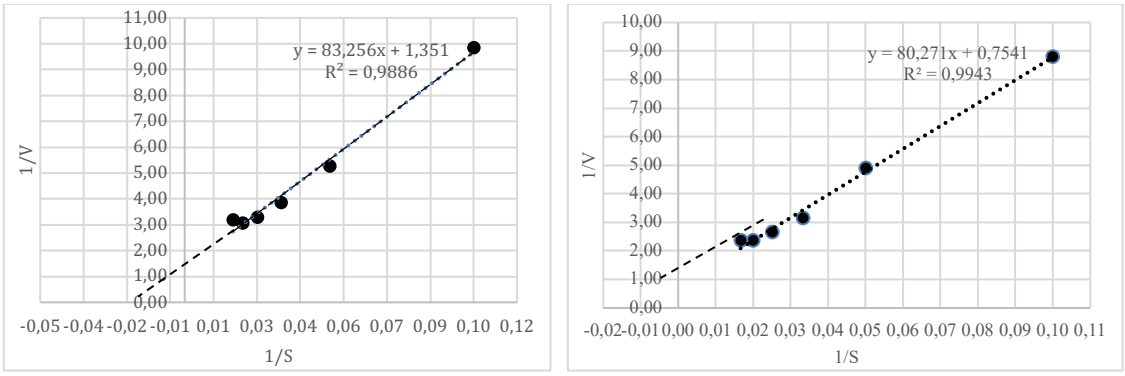


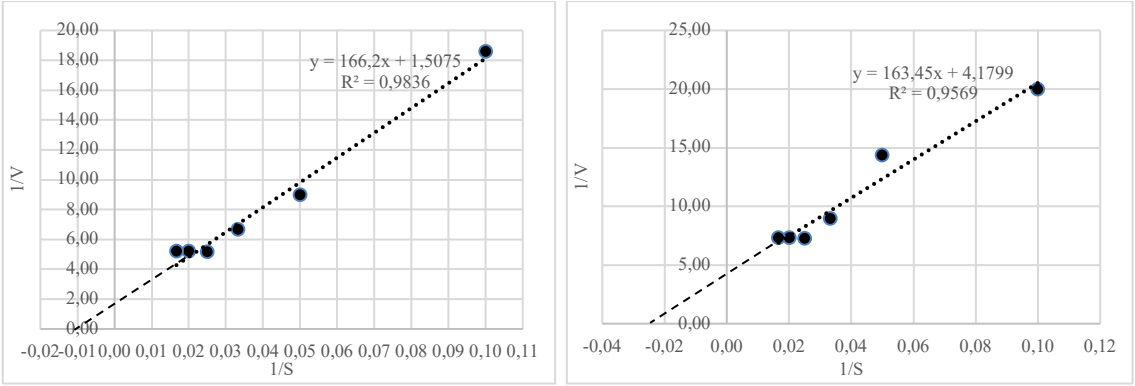
Figure 8. FTIR testing of activated activated carbon is immobilized

The kinetics of the enzymatic reactions of cyanide and nitrile biodegradation were calculated based on the equations *of Michaelis Menten* and *Lineweaver Burk*. Cyanide and nitrile biodegradation testing using concentration variations at 0; 10; 20; 30; 40; 50; 60 mM with a contact time of 60 minutes. The substrate used for the cyanide type is potassium cyanide (KCN). The substrates used for nitrile are benzonitrile and acetonitrile.



(i) (ii)

Figure 9. Lineweaver-Burk Chart Benzonitrile Substrate (i) and Acetonitrile (ii)



(i) (ii)

Figure 10. Lineweaver-Burk Chart Kalium Cyanide Substrate (i) and Potassium Cyanide (ii)

In the degradation test with benzonitrile substrate, the value of Km = 50 mM was obtained and in the acetonitrile substrate, the value of Km = 100 mM was obtained. The maximum rate or Vmax for benzonitrile biodegradation is 0.714 ppm/min and for acetonitrile is 1.25 ppm/minutes. At a substrate concentration of 50 mM, the peak rate of kinetic reactions takes place on acetonitrile and benzonitrile substrates. The percentage of degradation of benzonitrile substrate at a concentration of 50 mM is 21.6% during one hour will increase with the length of contact time of the degrading enzyme with the substrate, this is characterized by the formation of 10.8 mM ammonia as a product of nitrile degradation by the enzyme nitrile hydratase. The percentage of degradation of acetonitrile substrate at a concentration of 50 mM is 28.3% over an hour will increase with the length of contact time of the degrading enzyme with the substrate, this is characterized by the formation of 14.6 mM ammonia as a product of nitrile degrading by the nitrile hydratase enzyme. The degradation test with potassium cyanide substrate obtained a value of K_m = 100 mM and on the sodium cyanide substrate obtained a value of K_m = 41.6 mM. The maximum rate or Vmax for potassium cyanide biodegradation is 0.56 ppm/min and for sodium cyanide is 0.21 ppm/min. The percentage degradation of potassium cyanide was 16.1 % over one hour with ammonia formed at a V_{max} substrate concentration of 40 mM was 6.4 mM. Meanwhile, the percentage of sodium cyanide degradation was 11.5% over one hour with ammonia formed at a concentration of 40 mM V_{max} substrate was 4.6 mM. This percentage of degradation will continue to increase with the length of contact and will be stationary until it decreases if the enzyme reaches its peak of activity.

Conclusion

The bacteria *Rhodococcus pyridinivorans strain I-benzo* can grow well at pH 6-7 and can grow well degrading aliphatic and aromatic substrates such as acetonitrile and benzonitrile. *Rhodococcus pyridinivorans bacteria strain I-benzo* grows optimally in mineral media using a benzonitrile substrate at a concentration of 20 mM. The bacteria *Rhodococcus pyridinivorans strain I-benzo* has enzymatic activity from nitrile hydratase and amidase. *Rhodococcus pyridinivorans I-benzo strain* bacteria can be immobilized by the entrapment method using activated carbon zeolite. The enzymatic reaction kinetics of *Rhodococcus pyridinivorans strain I-benzo* which has been immobilized with activated carbon has been proven to degrade cyanide at an optimum concentration of 40 mL and nitrile at an optimum concentration of 50 mL with a degradation percentage for one hour in the range of 11% to 28%. In the next study, to be able to test the image of Rhodococcus pyridinivorans I-benzo strain bacteria and zeolite that have been mobilized by Rhodococcus pyridinivorans I-benzo strain. Tests are carried out for other toxicity parameters and also for the metal content in the wastewater.

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