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Growth Inhibition Test of Glyphosate Herbicide For Glyphosate-Degrading-Bacteria Screening

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Abstract : Glyphosate is one of the most widely used herbicide for weed eradication. Excessive usage of glyphosate may lead to contamination of soil, water, and crops. Soil bioremediation using microorganisms to degrade glyphosate is an effective and cheap method when the level of glyphosate is higher than maximum permitted level. The resistance of the microorganism to glyphosate can be determined by observing IC_{50} parameter. The microorganisms which are resistant to high concentration of glyphosate can be selected as candidate for glyphosate biodegradation process. The objective of this study was to determine IC_{50} for consortium bacterial culture isolated from glyphosate-contaminated soil and uncontaminated soil. Generally IC_{50} value is determined by measuring optical density, but in this study IC_{50} value was determined using total number of cell to observe the real effect of glyphosate toward bacteria cell in the soil. Higher tolerance was observed for bacterial consortium culture isolated from uncontaminated soil (IC_{50} is 263.38mg/L) compared with the culture from glyphosate-contaminated soil (IC_{50} is 2.04 mg/L). Glyphosate at low concentration below 10 mg/L could increase bacterial growth. This study suggested that the bacteria could use low concentration glyphosate as nutrition source.
Keyword : Bioremediation, consortium culture, glyphosate tolerance, IC_{50}, natural consortium.

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1. Introduction

Glyphosate [N-phosphonomethyl)glycine, $C_3H_8NO_5P$] is the most widely used herbicide in the world, with total production of 620,000 tons in 2008[1]. Glyphosate is a herbicide which has the following characteristics: broad-spectrum, post-emergence, non selective, and systemic[2]. Glyphosate acts as inhibitor of 5-enolpyruvylshikimic acid-3-phosphatase synthase (EPSPS), a particular enzyme which plays a role on shikimate pathway. Glyphosate is a transition state analog of phosphoenylpyruvate, one of the substrates for EPSPS[3]and ensuing metabolic products, such as the aromatic acids phenylalanine, tyrosine and tryptophan that are required for protein synthesis[4]. Synthesis of aromatic amino acid in plants might be inhibited by glyphosate and leads to yellowing and decaying of leaves within 5-10 days [5].

Glyphosate demand increased 300-fold from 1974 to 2014 in the agricultural sector (from 0.36 to 113.4 million kgs). The main factor that increase of glyphosate utilization since commercial introduction in 1974 was commercialization of genetically engineered - herbicide tolerant crops. Genetic engineering glyphosate – tolerant (GT) had been performed in the maize, soybeans, canola, and cotton. Since glyphosate patent protection was ended in 2000, there were many companies began manufacturing technical glyphosate, and/or formulating glyphosate products[3][6].Growing uses of this herbicide leads to glyphosate contamination in the water [7][8]and crops[1]. It was also reported that glyphosate could increase plant pathogenic like *Fusarium spp.*[9]and *Phytium*, and also affected micronutrient availability[10]. Many studies also reported that glyphosate increased prevalence of rare liver and kidney tumors in chronic animal feeding studies, epidemiological studies reporting positive associations with non-Hodgkin lymphoma, and strong mechanistic evidence of genotoxicity and ability to trigger oxidative stress [6].

Recently, glyphosate biodegradation using glyphosate-degrading microorganisms have been studiedextensively to solve these glyphosate contamination problems.Many research on glyphosate biodegradation have been done using single bacterial culture like *Geobacillus caldoxylosilyticus*[11], *P. putida, R. aquatilis, Serratiam arcescens*[12], *Enterobacter cloacae* [13], *Bacillus subtilis*[14][15], *Trichoderma viride*[5]. On the other hand, many studies reported that biodegradation processes usually require the synergism of microbial consortium in the initial conversion, transformation phase, or mineralization phase of the toxic material. Consortium of many microbe species usually have the ability to do transformation which cannot be done by single species or accelerate the reaction rate compared to single reaction[16]. Glyphosate biodegradation process using bacterial consortium has not been reported extensively. In this study, the consortium culture was sourced from glyphosate-contaminated soil and uncontaminated soil.

The resistance of the microorganism to glyphosate can be determined by observing IC_{50} parameter. Inhibitory concentration (IC_{50}) is usually used to estimate toxicity of some material to microorganisms, hence we can estimate the concentration of toxic material which start to inhibit bacterial growth [17]. $IC_{50}$is the concentration of glyphosate that inhibits the growth of half of tested viable bacteria.It is predicted that the microorganism which is resistant to high concentration of glyphosate can be selected as candidate for glyphosate biodegradation process. Microbial resistance towards glyphosate is related to the sensititivity of EPSP (5-enolpyruvylshikimate-3-phosphate synthase) which is encoded by the gene *aroA* and has the important role in shikimate pathway. EPSP synthases are divided into two classes: 1) Class I EPSPsynthases, found in all plants and in many Gram-negative bacteria, which are generallysensitive to glyphosate and 2) Class II EPSP synthases which are found in naturally glyphosate tolerant microbes.These two enzyme classes have amino acid identity less than 30% [18]. The objective of this study was to observe the effect of glyphosate herbicide towards consortium culture from glyphosate-contaminated soil and uncontaminated soil by determining IC_{50} parameter.

2. Material and method

2.1. Source of bacterial culture

Soil samples were taken from two differentareas. The first sample was taken from vegetable area in Hative Besar village, Ambon, Indonesia which has been exposed by glyphosate for more than 1 year with twice application in a year. The second sample was taken from the forest of Manglayang mountain, Cilengkrang, Bandung, Indonesiawhich has never been exposed by glyphosate. Samples were taken from depth of 0-15 cm. All samples were transported immediately to the laboratory and stored at 4^{o}C in refrigerator until use.

2.2. Chemicals and culture medium

Glyphosate herbicide Roundup® (containing 486 g/L isopropylamine salt of glyphosate or equal with 360 g/L glyphosate acid) was purchased from local store in Bandung, West Java, Indonesia. Medium composition for IC50 test was modified from Alsop *et al*[19],consists of: sodium acetate (CH_3COONa) 1,500 mg/L; potassium dihydrogen phosphate (KH_2PO_4) 3,300 mg/L; magnesium sulfate heptahydrate ($MgSO_4.7H_2O$) 12 mg/L;calcium chloride ($CaCl_2$) 14 mg/L;iron(III) chloride hexahydrate($FeCl_3.6H_2O$) 125 mg/L;and ammonium chloride (NH_4Cl) 850 mg/L.Nutrient agar is used as media for total plate count. All of these chemicals were analytical grade and purchased from Merck.

2.3. Bacterial consortium isolation and IC_{50} test of glyphosate

Isolation medium consists of:CH_3COONa 1,500 mg/L; KH_2PO_4 3,300 mg/L; $MgSO_4.7H_2O$ 12 mg/L; $CaCl_2$ 14 mg/L; $FeCl_3.6H_2O$ 125 mg/L; and NH_4Cl 850 mg/L. pH of the medium was set to 7.2 to supportthe bacterial growth. Neutral pH or slightly alkaline conditionraise the bacterial growth, while an acid pH induces fungal growth[20]. Bacterial consortium was isolated by adding 100 mg of each soil to 150 ml medium and incubatedat room temperature under on shaking condition of 150 rpm. Afterseven days, 3 ml volume of these soil suspension were transferred to medium containing various concentration of glyphosate(0.00 mg/L; 0.01 mg/L; 0.10 mg/L; 1.00 mg/L; 10.00 mg/L; 100.00 mg/L; and 1,000.00 mg/L) and stored at room temperature for 72 h. Control treatment received no soil suspension were kept in similar condition. Five ml samples were taken aseptically from each erlenmeyer every12hours for 72 hours.Optical density measurement and bacterial counting were performed on each sample.

Microbial growth was monitored by optical density at 540 nm using T80+ UV/Vis Spectrophotometer, PG Instruments Ltd.and by bacterial countingusingtotal plate countmethod on nutrient agar media. All tests were prepared in quadruplicate. Medium without inoculum was used as blank for spectrophotometric reading.

The relative response are calculated as a percentage by this equation:

$$E[Y] = \frac{TA}{CA}\ x\ 100\% \quad (1)$$

E[Y] = relative response, CA = the absorbance produced in the control without toxicant, TA = absorbance produced in the test with different concentrations of toxicant [21].

For IC50 determining, relative responsewas calculated by dividing the total number of cell in the medium with certain glyphosate concentration with the total number of cell from medium without glyphosate at 24 hours incubation. The total number of cellswas determined by total plate count method using pour plate method with nutrient agar as medium. Bacteria colonies were counted after incubating at 37°C for 48 hours.The relative responsepercentages are plotted against the test sample concentration, as shown in Fig. 3. The test concentration corresponding to 50% of the control is termed the 50% inhibition concentration (IC50)[19]. IC_{50} was calculated from equation resulting from regression analysis.

2.4. Generation time and specific growth rate

The number of generation per unit time is expressed as the generation per hour.

$$k = \frac{\log Nt - \log No}{0.301\ t} \quad (1)$$

Where N_0 = the initial population number, Nt = the population at time t

Specific growth rate (μ) was determined according equation (2)

$$\mu = \frac{\ln Nt - \ln No}{t - to} \quad (2)$$

Where μ is specific growth rate (h^{-1}); Nt is number of cells (cfu/mL) at the end of exponential phase; N_0 is number of cells (cfu/mL) at the beginning of exponential phase; t is time point (h) for Nt and t_0 is time point for N_0[17]. Lag phase time, generation time, and specific growth rate of consortium culture from glyphosate exposed soil and unexposed soil at various glyphosate concentration are shown in Table 1.

3. Result and Discussion

3.1. Growth response at various glyphosate concentrations

Turbidity measurement and total number of cell at variousglyphosate concentrationsare shown in Fig. 1 and Fig. 2.Growth response were not only observed by turbidity measurement, but also confirmed by total number of living bacterial cell.

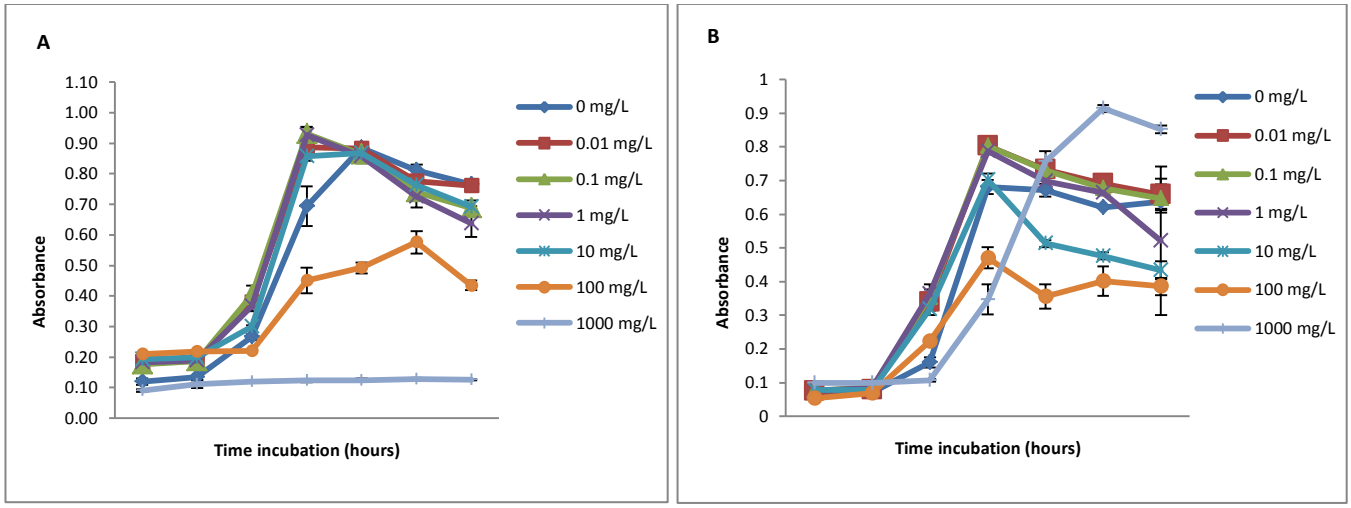

Figure 1. Growth response (turbidity of cell) of consortium culture from (A) glyphosate exposed soil and (B) unexposed soil at various glyphosate concentration

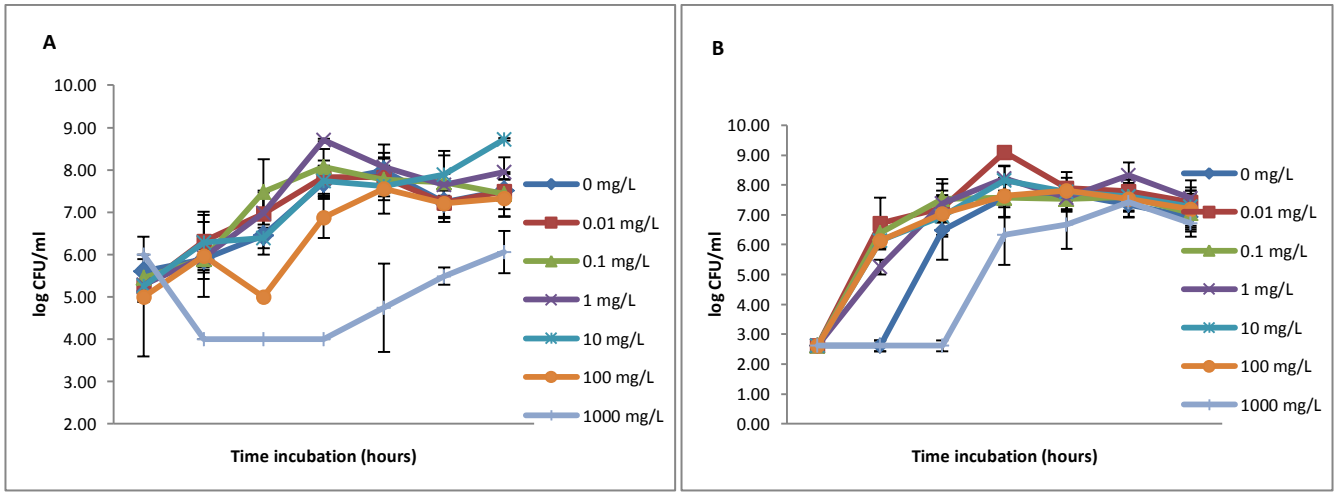

Figure 2. Growth response (number of cell) of consortium culture from (A) glyphosate exposed soil and (B) unexposed soil at various glyphosate concentration

Many previous studies regarding glyphosate biodegradation reported that bacterial growth were decrease along with the increasing of glyphosate concentration. But all of the previous studies were done in high concentration of glyphosate, like for *Bacillus cereus* CB4 at glyphosate concentration of 4 - 12 g/L [22], *E. cloacae* K7 at glyphosate concentration of 0 – 5 mM[13], *Acetobacter sp*. and *Pseudomonas fluorescens* at glyphosate concentration of 7.2 – 250 mg/mL, *Bacillus subtilis* at glyphosate concentration of 7.2 – 200 mg/mL[14]. The result of this study was in contrary with the previous studies. At low concentration of glyphosate (0.01 mg/L to 1 mg/L), the bacterial population from both soil sampleswere increased (Fig.3). At glyphosate concentration of 10 – 100 mg/L, bacterial population from unexposed soil was still higher than those in control, whereas those taken from glyphosate-contaminated soil were lower.

There islimited study observing the effect of low concentration glyphosate on bacterial growth. This study were in line with the previous study that observed the growth enhancement of *Pseudomonas sp.*along with

the increasing of glyphosate concentration from 7.2 mg/mL up to 40 mg/mL [14], but it was not compared with the one in the medium without glyphosate.Another previous study also reported a significant growth of *Aeromonas sp.* in sample containing glyphosate of 50 mg/L and 100mg/L[23]. In this study, the growth of natural consortium in low concentration of glyphosate (0.01 mg/L to 1 mg/L) were even higher than those of control without glyphosate. Their growth was inhibited at glyphosate concentration more than 10 mg/L. Bacterial culture can use glyphosate at low concentration as nutrition source. Glyphosate can be utilized by some microorganisms as carbon source[24][25] or phosphorus source[11][26].

At high concentration of glyphosate,enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) involved in Shikimate pathway was inhibited [27].This is an essential enzyme for synthesis of aromatic amino acid and many aromatic metabolites in plant, fungi, and microorganisms. Shikimate-3-phosphate (S3P) and phosphoenolpyruvate (PEP) will be converted by this enzyme into 5-enolpyruvylshikimate-3-phosphate (EPSP). Glyphosate acts as competitive inhibitor with PEP and binds to S3P in the active site of this enzyme and imitate the enzyme-substrate complex[28].

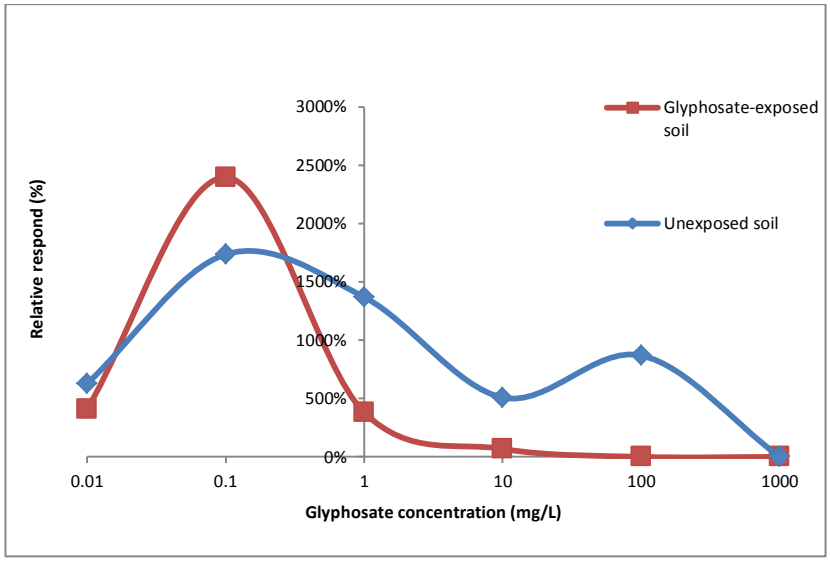

Figure 3. Relative response of consortium culture from glyphosate exposed soil and unexposed soil at 24 hours time incubation.

3.2. IC_{50} determination

Plotting of inhibition percentage at various glyphosate concentration fit the curve using non linear regression as shown in Fig.4 and Table 1. IC_{50} for consortium culture from glyphosate-contaminated soil was 2.04 mg/L and from unexposed soil was 263.38 mg/L. In glyphosate-contaminated soil, only the glyphosate-resistant bacteria willsurvive, hence the bacterialdiversity is lower than unexposed soil. Biodegradation of toxic materials are usually done by consortium culture with various roles. The consortium of many species usually have the ability to do transformation process which is cannot be done by single species or speed up the reaction rate compared with the single species[16]. The study regarding consortium of microorganisms for glyphosate biodegradation has been still limited. Previous study found that some bacteria could use AMPA as phosphorus source but they could not degrade glyphosate themselves[29]. Another previous study reported that the consortium of *Pseudomonas putida*, *Pseudomonas aeruginosa*, and *Acetobacter faecalis* did not accelerate the biodegradation process, but they could reduce lag time from 24 hours to 12 hours [30].The higher IC_{50} value of consortium culture from unexposed soil could be caused by high diversity of bacteria in unexposed soil.

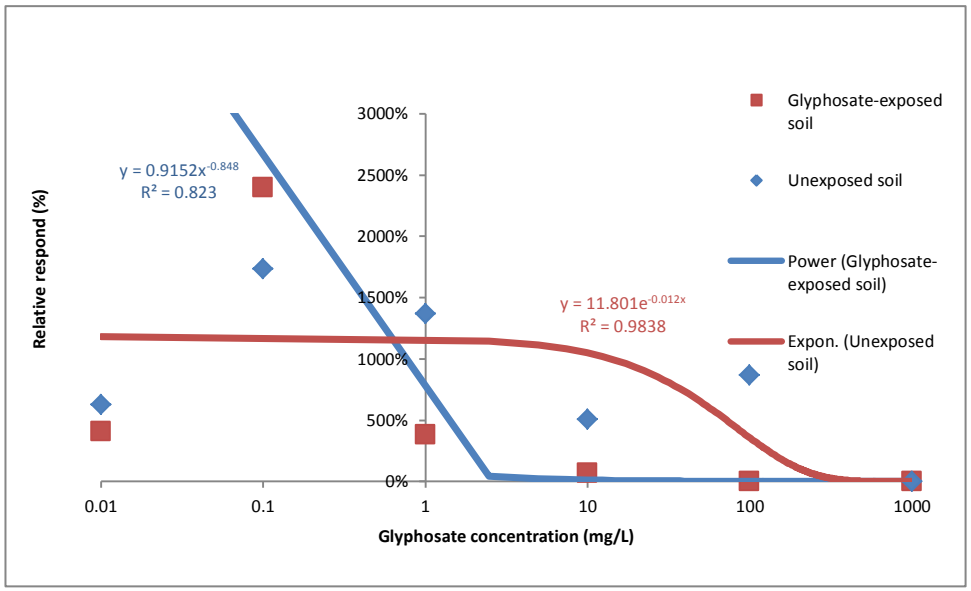

Figure 4. : Non linear regression of relative response of consortium culture at 24 hours time incubation

Table 1. IC50 value for consortium culture from glyphosate-contaminated soil and unexposed soil

Soil Sample	Regression equation	R^2	IC50 based on total number of cell (mg/L)
Glyphosate-exposed soil	$y = 0.9152x^{-0.848}$	0.823	2.04
Unexposed soil	$y = 11.801e^{-0.012x}$	0.984	263.38

Based on previous studies, 50% growth inhibition of *P. fluorescens* AFT36, *Escherichia coli, Bacillus subtilis*, *B. jabonicum*, and *P. aeruginosa*, *P. putida* W1616 were caused by glyphosate concentration of 3.0 µM, 75 µM, 174 µM, 1.1 mM, 1.1 mM, and 3 mM, respectively[31][4].Several previous studies have also been carried out to determine IC50 value of the EPSPS enzyme of some bacteria like *E. coli*(0,055 mM), *Ochrobacterium anthropi*(0,61 mM)[28]; *Rhizobium leguminosarum*(1,025 mM)[18],*S. aureus*(1,6 mM)[32]; *Agrobacterium tumefaciens* CP4 (11 mM)[33]. Molecular mechanism of EPSP synthase in natural glyphosate-tolerant microorganisms had not been studied intensively [33].

3.3. Generation time and specific growth rate

Table 2 present the effect of glyphosate at various concentration to consortium culture in terms of initial lag phase, generation time, and specific growth rate.

Table 2. Generation time and specific growth rate of consortium culture from glyphosate exposed soil and unexposed soil

Glyphosate concentration (mg/L)	Glyphosate-exposed soil				Unexposed soil			
	Lag phase (h)	Exponential phase (h)	µ (h^{-1})	Generation time (h)	Lag phase h)	Exponential phase (h)	µ (h^{-1})	Generation time (h)
0.00	24	24 – 36	0.103	2.934	12	12 – 24	0.227	0.735
0.01	No lag phase	0 – 12	0.113	2.671	No lag phase	0 – 12	0.410	0.734
0.10	12	12 – 24	0.158	1.908	No lag phase	0 – 12	0.310	0.973
1.00	24	24 – 36	0.126	2.396	No lag phase	0 – 24	0.220	1.368
10.00	No lag phase	0 – 12	0.133	2.261	No lag phase	0 – 36	0.163	1.849
100.00	No lag phase	0 – 12	0.130	2.315	No lag phase	0 – 24	0.212	1.421
1,000.00	36	36 - 72	0.072	4.154	24	24 - 60	0.143	2.102

It was observed that specific growth rate of consortium culture sourced from unexposed soil were higher than the ones from glyphosate-exposed soil. It can be caused as glyphosate degradation was predicted as a cometabolic process. Previous study had been carried out to find the glyphosate degradation pathway by bacteria. Two major glyphosate degradation pathways are sarcosine pathway and aminomethylphosphonic acid (AMPA) pathway. Most of the bacteria have the ability to transform glyphosate to AMPA, and this ability exists in any bacteria even that have never been exposed to glyphosate, but there are only a few strains that can metabolize AMPA[29]. In highly-exposed soil, glyphosate may be toxic to microorganisms that have a particular important role in glyphosate biodegradation process, such as AMPA metabolism or other roles.

The highest specific growth rate of consortium culture from glyphosate-exposed soil was 0.158 h^{-1}at glyphosate concentration of 0.1 mg/L. At this point, consortium culture need the shortest time to doublé, the culture need time of 1.908 hours per generation. At glyphosate concentration below 10 mg/L, consortium culture was still survive and specific growth rate were even higher than control without glyphosate. It was predicted that consortium culture has the ability to use glyphosate as either carbon, phosphorus, or nitrogen source.However, at glyphosate concentration of 100 mg/L, there were two growth peaks, at 12 hours and 36 hours respectively. It was estimated that glyphosate toxicity affected culture growth thus causing a decrease in cell numbers after 12 hours. At 24 hours, the bacterial growth was increased. It was predicted that bacteriawhich were resistant to glyphosate could grow because they were able to use glyphosate as nutrient source.At glyphosate concentration of 1,000 mg/L, the culture need 36 hours of lag phase, longer than the one sourced from unexposed soil.

The highest growth of consortium culture from glyphosate-exposed soil was at glyphosate concentration of 0.01 mg/L with specific growth rate and generation time were 0.410 h^{-1} and 0.734 h per generation, respectively. At concentration of 0.01 – 100 mg/L, the culture did not have lag phase. The consortium culture could adapt to the new environments with glyphosate exposure, even the culture were sourced from unexposed soil. The culture need 24 hours of lag phase at glyphosate concentration of 1,000 mg/L.

4. Conclusion

IC50 parameter can be used as the first parameter to screen microorganisms that have potency to be glyphosate biodegrader. Consortium culture from unexposed soil can be isolated and tested further to observe its ability to degrade glyphosate. Consortium growth is not always decrease along with the increasing of glyphosate concentration. Low concentration of glyphosate (0.01 mg/L to 1 mg/L) can increase bacterial growth as it is potential to be utilized as nutrition source either carbon, phosphorus, or nitrogen source. Bacterial growth started to be inhibited at glyphosate concentration above 10 mg/L. Residual glyphosate contamination in soil under 10 mg/L still can be tolerated by natural soil bacterial consortium and even increase their growth.

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