Indonesian Journal of Biotechnology, June, 2007 Vol. 12, No. 1, pp. 973-979

Superoxide Dismutase of *Micrococcus* sp. S2 and Its Involve in Paraquat Detoxification

Sebastian Margino [1*], Erni Martani [1] and Medhina Magdalena [2]

[1]Dept. of Agricultural Microbiology, Fac. of Agriculture, Gadjah Mada University
[2]Post Graduate School in Biotechnology, Gadjah Mada University

Abstract

As an active ingredient of herbicide, paraquat will induce formation of superoxide radicals. The previous research succeeded in isolating paraquat degrading bacteria from peat soil, *Micrococcus* sp. S2, that tolerant to high concentration of paraquat. An anti-oxidative enzyme, namely superoxide dismutase (SOD, EC.1.15.1.1), was believed to be responsible for the paraquat tolerance. This research was conducted to study the characteristic of the SOD synthesize by *Micrococcus* sp. S2 and its ability on neutralize superoxide which arise from paraquat reoxidation.

To observe the effect of paraquat on *Micrococcus* sp. S2, the bacteria was grown in 10% Luria Bertani broth medium amended with several concentrations of paraquat, from 0 (control) up to 100 mg/ml. Within incubation time of 72 hours, bacterial growth, activity of superoxide dismutase and paraquat residue were analyzed. The isozymes of superoxide dismutase were distinguished using two kinds of specific inhibitor, namely H_2O_2 and KCN.

The results showed that paraquat significantly inhibit the growth of *Micrococcus* sp. S2. The higher paraquat cocentration in the medium caused the higher growth inhibition. However, the bacteria is still survive in the medium containing toxic herbicide, and this ability was suggested related to superoxide dismutase activity in removing the superoxide radicals. Analysis using gel electrophoresis indicated that at least three types of SOD isozyme were synthesized by *Micrococcus* sp. S2; they were Ferri-SOD (Fe-SOD), Mangani-SOD (Mn-SOD), and the last one was suspected to be the Cupro Zinc-SOD (CuZn-SOD). The Mangani-SOD was suspected to play an important roles on detoxifying superoxide which arise from paraquat oxidation.

Keywords : *Micrococcus sp.*S2, paraquat, superoxide dismutase, isozymes

Introduction

Several kinds of herbicide with paraquat as active agent were used to control the growth of weeds, one of them is Gramoxone®. Paraquat is an bipiridillium herbicide with chemical structure 1,1'-dimethyl-4,4'-bipiridinium dichloride. Due to the contact mechanism, this herbicide have to apply routinely during planting time (Husny *et al.*, 1989). Repeated application of this herbicide caused accumulation of paraquat residues in peat soils which affects the dinamics of soil microbial population (Martani *et al*, 2001; Martani *et al*, 2002).

Paraquat herbicide is toxic to microbial cell due to free radicals superoxide which released from reaction of paraquat free radicals with oxygen molecule, such as O_2^- and OH^- (Carr *et al.*, 1986). Although it is not the most reactive toxic radical, reaction of O_2^- with other free radicals can stimulate synthesis of more toxic substances. The O_2^- causes induction of protein degradation, lipid peroxidation and polysaccharide depolimeration which damage the cell (Donelly and Robinson, 1991). Superoxide radicals were formed in procaryotic and eucaryotic cells. In case of procaryote cells, O_2^- was formed through electron transport, and highly affected by growth condition and enzymatic activity (Halliwell and Gutteridge, 1999).

*corresponding author : Sebastian Margino, Department of Agricultural Microbiology, Faculty of Agriculture, Gadjah Mada University, Yogyakarta, Indonesia

Yanti *et al.* (2003) isolated paraquat degrading bacteria, one of the isolates was coded as *Micrococcus* sp. S2. This bacteria was isolated from Central Kalimantan peat soil, it still survive in high concentration of paraquat. This Gram positive – coccus bacteria, forms yellow colony in agar medium and show catalase activity. Molecular characterization of this bacteria in Biotechnology Research Center, Tokyo University, Japan showed that it had 98% similarity with *Micrococcus luteus* strain Ballarat (Wijayanto, 2003).

Many organisms were able to neutralize oxidative damage by certain mechanisms such as enzymatic activity which cause the decreasing of free radicals or detoxification of free radical toxicity. Superoxides formed inside the cell will be dismutated to form H_2O_2 and O_2 (Berg *et al.*, 2002). Dismutation reaction was catalyzed by superoxide dismutase metalloenzyme (EC.1.15.1.1).

This enzyme was devided into several isoenzymes based on their metal cofactor. In eucariotic and procariotic cells, at least there are five kinds of superoxide dismutase isozymes (SOD), namely Cupro Zincum-SOD (CuZn-SOD), Mangani SOD (Mn-SOD), Ferri-SOD (Fe-SOD), extracellular SOD (Ec-SOD) and Nickel-SOD (Ni-SOD) (Halliwell dan Gutteridge, 1999). In procarryotic cells, CuZn-SOD ussually located in periplasmic space; but in cases of Mn-SOD and Fe-SOD are located in cell membrane and cytoplasmic, respectively. Several microorganisms only have one or several types of those kinds of SOD. The research concerning Ec-SOD and Ni-SOD is still limited (Fridovich, 1997); but Ec-SOD was detected in several rodentia (Halliwel and Gutteridge, 1999).

This research was conducted to investigate the superoxide dismutase (SOD) synthezised by *Micrococcus* sp. S2, characteristics this enzyme, and its responsibility to paraquat detoxicafication.

Materials and Method

Bacterial culture

Micrococcus sp. S2, was isolated from saphric peat soil of Pangkoh, Central Kalimantan (Yanti *et al.*, 2003). It tolerant to high concentration of paraquat and able to degrade this herbicide.

The Growth f of Micrococcus sp. S2.

Micrococcus sp. S2 was grown in modified Luria Bertani (LB) 10 % (v/v) broth medium, then its growth was measured by turbidimetry method (Optical Density, OD, wave length 356 nm). The composition of normal LB broth medium is (g/1000ml) Bacterial Pepton 10.0; Yeast Extract 5.0; NaCl 5.0. The wave length was investigated in previous research and decided based on maximum OD of this bacterial culture, namely 356 nm. The resulted growth curve was used to determine the time for addition of paraquat during the growth of *Micrococcus* sp. S2.

Effect of paraquat on the growth of Micrococcus sp. S2.

This experiment was done by growing *Micrococcus* sp. S2 in modified LB medium (10% LB, v/v), added with Gramoxone® as paraquat source at concentration ranging between 0 (control) to 100 ppm (w/v). This herbicide was added at early logarithmic phase (sub 2). Bacterial growth was determined periodically based on Optical Density at 356 nm. Paraquat residue (Pack, 1967), protein concentration (Bio Rad), SOD activity (spectrophotometrically, Crapo, 1978), and visualization of SOD activity (Tanksley and Orton, 1983) were also analyzed periodically.

Determination of SOD Isozyme (Halliwell and Gutteridge, 1999; Santos, 1999)

The non-denaturant gel resulted in electrophoresis was soaked in nitroblue tetrazolium (NBT) added with 10 mM of

H_2O_2 or KCN as specific inhibitors. The peroxide (H_2O_2) only inhibit the activity of Fe-SOD, but KCN inhibits CuZn-SOD activity. Both of them can not inhibit Mn-SOD. Based on the specificity of these inhibitors, we can identify the type of SOD isozyme synthesized by *Microccocus* sp. S2.

Results and Discussion

Micrococcus sp. S2 was grown in modified LB broth medium (10%) and measured periodically its population based on the OD (356 nm). The resulted data was depicted in Figure 1. This bacteria needs 2 hours (lag-phase) and enter the early logarithmic phase, then after 15 hours it reach stationary phase. Based on this data, in the experiment of effect of paraquat on *Micrococcus* sp. S2, this herbicide was added after incubation time for 4 hours, namely in early logarithmic phase.

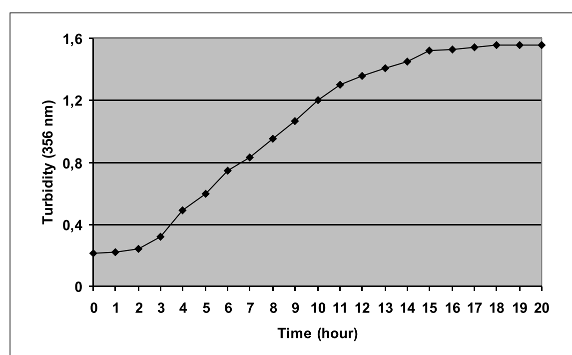

Figure 1. Growth curve of *Micrococcus* sp. S2.

Paraquat concentrations and incubation time affected the growth of *Micrococcus* sp. S2 (Fig. 2). The increase of paraquat concentrations would be followed by the derease of bacterial growth, as shown by the decrease of culture turbidity detected by OD at 356 nm. Within 72 hours incubation at paraquat 0 ppm, the turbidity value increased up to 1.2; but at paraquat 20 and 40 ppm the maximal OD were 1.0; and at 100 ppm was less that 0.6. At the concentrations of 60 to 100 ppm, the biomass decreased until 50% compared with control (no paraquat addition). Statistically, there was a significant interaction between those factors, paraquat concentration and incubation time. Namely, the higher paraquat concentration and longer incubation time resulted in the reduction of growth of the bacteria.

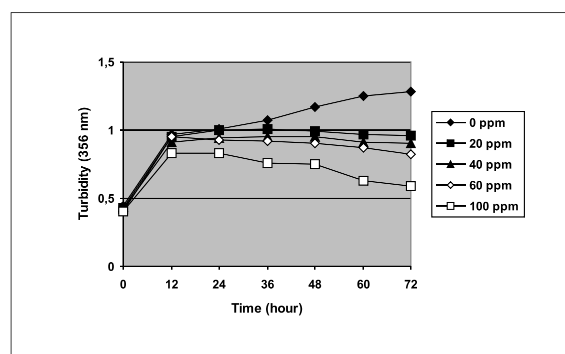

Figure 2. Effect of paraquat (0-100 ppm) on the growth of *Micrococcus* sp. S2

Those data indicated that paraquat was toxic to *Micrococcus*, especially at high concentrations. Paraquat induced the release of toxic anionic such as O_2 causing cell damage. At concentration of 0,1 – 1 mM (equal to 25-250 ppm), paraquat is bacteriostatic towards *Escherichia coli*, and this bactaria will die if paraquat concentration is increased to more than 100 mM (Halliwell and Gutteridge, 1999).

The decrease of bacterial population was accompanied by the decreasing of *Micrococcus* sp. S2 activity to degrade paraquat (Fig. 3). During the incubation time of 72 hours, there was a decrease of paraquat concentration, especially at relatively low initial concentration (lower than 60 ppm); indicating that *Micrococcus* sp. S2 did degrade paraquat. Within 48 h, around 20% of paraquat was degraded if its initial concentration was 20 and 40 ppm. However, at 60 ppm or higher, paraquat degradation

was not significant; namely only 5 – 10% was degraded within 72 hours. These data showed that degradation activity of *Microccocus* was affected by initial concentration of paraquat.

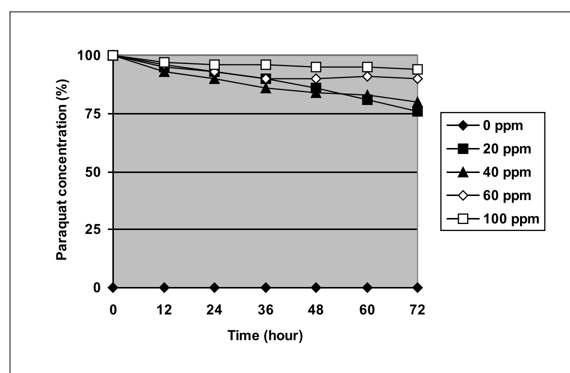

Figure 3. Paraquat residue during the incubation time of 72 hours

The data were accomplized by previous research that Micrococcus sp. S2 was able to degrade paraquat in a minimal medium and also in several peat soils (Martani *et al.*, 2001; Yanti *et al.*, 2003). A yeast *Lipomyces starkeyi*, was able to degrade paraquat in minimal medium and used this chemical as N source (Carr *et al.*, 1985).

Decreasing of degradation activity and the growth of *Micrococcus sp. S2* at high concentration of paraquat, showed the toxicity of this chemical on the bacteria. Carr *et al* (1985) reported that degradative activity of *Lipomyces starkeyi* toward paraquat was related to integrity of cell membran and cel wall. If there was cell wall desruption caused by peroxidation reaction by free radicals, this yeast will lost its activity to degrade paraquat.

Oxidation pressure in media or natural environment will induce specific reaction of microbial cells in order to survive in those pressures. In relation with paraquat pressure to microorganisms, oxidative pressure occur when paraquat was re-oxidated, in which during this time free radicals superoxides were released into environment. The release of these free radicals will induce cell reaction to reduce the superoxides from their metabolic pathway through dismutation reaction catalysed by SOD enzymes. It means that free radical superoxides released during oxidation of paraquat will increase the specific activity of SOD.

In this study, it was shown that at 0 ppm paraquat addition (control) resulted in very low specific activity of SOD, (Fig. 4). These data indicated that in case of *Micrococcus* sp. S2, the SOD was constitutive and their specific activity was low. Although not significant, an increase of paraquat concentrations from 20 to 60 ppm resulted in the increase of specific activity. It should be caused that in relative low concentrations, the toxicity of paraquat was als low. So that in those low concentrations SOD activity were low.

Significant increase of spcific activity of the SOD was found at 80 and 100 ppm of paraquat. Within 72 h, specific activity of the SOD in 80 or 100 ppm paraquat increased to 150 or 355 U/mg, respectively (Fig. 4). These results indicated that SOD activity of the *Micrococcus* sp.S2 was induced by the paraquat which was mediated by the release of O_2. Higher concentration of paraquat resulted in higher activity of this enzyme.

Experimental toxicity on several microorganisms showed that toxicity mechanism of paraquat is not anly caused by the synthesis of O_2, and cellular response to paraquat by showing the increase of SOD activity. These phenomena were shown at least by strains of *Bacillus subtilis* and *Pseudomonas* sp., in which their SOD activity did not corelated with the paraquat induction, (Carr *et al.* 1986)

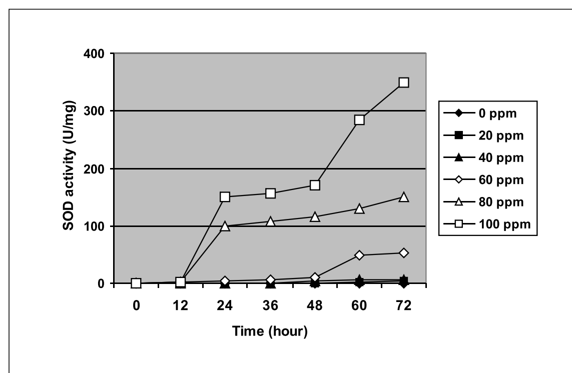

Figure 4. Effect of paraquat concentration on the SOD specific activity

To prove that O_2 free radical detoxification of *Micrococcus* sp.S2 occured due to SOD activity, in this study activity visualization was conducted on polyacrylamide gel electrophoresis with negative color process. Visualization of SOD activity was depicted in Figure 5, in case *Micrococcus* sp.S2 showed at least three types of SOD isozymes, namely band *a*, band *b* and band *c*. Additionally, triple bands *a*, *b* and *c* only shown at 0 ppm of paraquat; but band *b* is still detected in all paraquat concentrations. Therefore, it was suggested that only band *b* has responsibility to the paraquat detoxification.

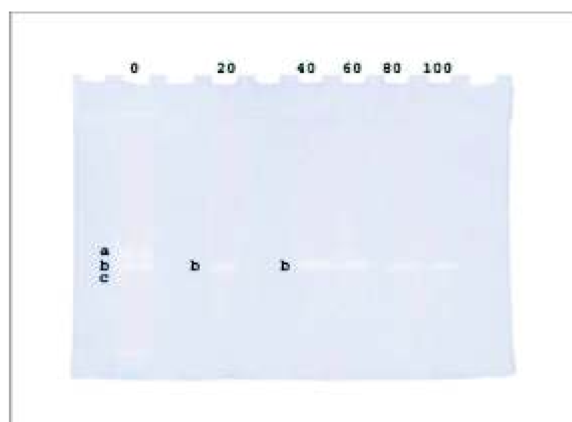

Figure 5.Visualization of intraselular SOD activity of *Micrococcus* sp. S2 on polyacrylamide gel on 0-100 ppm of paraquat. The numbers show the paraquat concentrations, and notations of *a*, *b* and *c* are the locations and SOD activities. The same notation means same type of SOD.

Peroxide (H_2O_2) and KCN can be used as specific inhibitors for the activity of SOD. The H_2O_2 only inhibit the activity of Fe-SOD, but KCN inhibits CuZn-SOD activity (Halliwell and Gutteridge, 1999; Santos, 1999). However, both of them can not inhibit Mn-SOD. Based on the specificity of these inhibitors, we can identify the type of SOD isozymes found in the *Microccocus* cell. The results were shown in Figure 6, in which inhibition of KCN and H_2O_2 were shown in Fig. 6A and Fig. 6B, respectively. *Escherichia coli* was used as culture reference because this bacteria synthesizes several kinds of SOD, for instances Mn-SOD, Fe-SOD, CuZn-SOD, and Hy-SOD ((Halliwell and Gutteridge, 1999; Santos, *et al.*, 1999). Halliwell and Guteridge, 1999 reported that result of De Rosa experiment found that extract cow liver containing 7 acromatic bands of SOD isozymes which illustrated of CuZn-SOD.

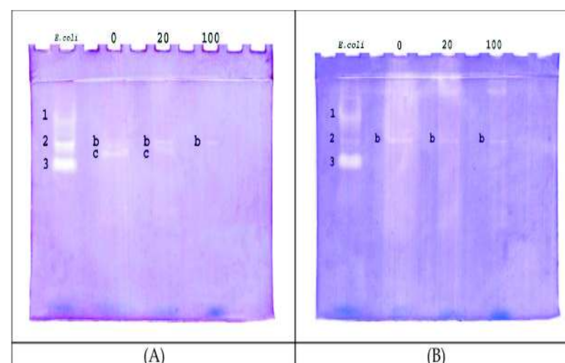

Figure 6.Activity of SOD in the occurance of KCN (A) and H_2O_2 (B) as inhibitors.Line A is crude extract of *E.coli* DH5a. Lines 4, 6 and 8 are the crude extract of *Micrococcus* sp.S2 grown in paraquat 0 ppm, 20 ppm and 100 ppm, respectively.

This figure showed that acromatic band b was synthezised by this bacteria which was grown in 0 to 100 ppm paraquat, and this enzyme still showed the activity in the gel electrophoresis although KCN or H_2O_2 was added. It means that both spesific inhibitors could not inhibit the activity of SOD

isonzymes of the *Micrococcus* sp. S2. Therefore, it was suggested that the dominant SOD isozymes (band *b*) in preventing *Micrococcus* sp. S2 from toxicity of O_2 released from paraquat oxidation, was MnSOD.

Different data was found in case of band *c*. This band was detected in gel which treated by addition of KCN inhibitor, but addition of H_2O_2 could not find (Fig. 5). We assumed that this band was Fe-SOD, but this enzyme was not important and had no responsibility to the paraquat detoxification, because it was only detected when *Micrococcus* sp. S2 was grown in 0 or 20 ppm of paraquat.

As reported by Fridovich (1975), Mn-SOD was one of SOD isozymes located in cell membrane and had an important roles in the respiration process. Therefore this isoenzyme was suggested to be responsible to the paraquat detoxification by neutralizing the free radicals released during oxidation of this herbicide. In case of the Fe-SOD which is located in the cytoplasmic cell, was important to prevent cytosolic enzymes from the toxicity of superoxide radicals.

Conclussions

From the results of this study, it can be concluded that Paraquat inhibits the growth of *Micrococcus sp.S2* and higher the concentration of paraquat, higher the effect of toxicity that was observed. Survival of *Micrococcus sp.S2* in high paraquat concentration was due to its ability to synthetize three kinds of SOD isozymes, which are CuZn-SOD, Mn-SOD, and Fe-SOD, that prevent cells from free radical superoxides of paraquat oxidation. Mn-SOD of *Micrococcus sp.S2* roled in paraquat detoxification.

References

Berg, J.M., J.L. Tymoczko, and L.Stryer. 2002. *Biochemistry*. 5th ed. W.H.Freeman and Company.

Carr, R.J.G., R.F Bilton and T. Atkinson. 1985. Mechanism of Biodegradation of Paraquat by *Lipomyces starkeyi*. *Appl. Environ.Microbiol.* 49:1290-1294.

Carr, R.J.G., R.F Bilton and T. Atkinson. 1986. Toxicity of Paraquat to Microorganisms. *Appl. Environ.Microbiol.* 52:1112-1116.

Crapo, J.D., J.M. McCord and I. Fridovich. 1978. Preparation and Assay of Superoxide Dismutase. *Methods. Enzymol.* 53:382-393.

Donelly, J.K and D.S. Robinson. 1991. Superoxide Dismutase. In *Oxidative Enzymes in Food.* D.S Robinson and N.A.M Eskin (eds). Elsevier Applied Science.

Husny, Z., I. Anas dan P. Bangun. 1989. Pengaruh Persiapan Tanam dan Sistem Pengendalian Gulma terhadap Aktivitas Mikroorganisme Tanah. *In Prosiding Kongres Nasional V, Himpunan Ilmu Tanah Indonesia.* Buku I. Medan, Indonesia.

Martani, E., K. Wibowo, B. Radjagukguk and S.Margino. 2001. Influence of Paraquat on a Soil Bacteria, *Rhizobium* sp. *Jurnal Manusia dan Lingkungan.* VIII: 82-90.

Martani, E., B.H. Sunarminto, L.F. Yeni and M. Magdalena. 2002. The Impact of Paraquat Repeated Application in Peat Soil on Microbial Population. *Biologi.* 2:845-859

Martani, E., N.A. Yanti and S. Margino. 2001b. Acceleration of paraquat degradation by isolated soil bacteria. *J.Plant.Protect.* 8 : 35-43.

Fridovich, I. 1975. Superoxide Dismutases. *Annual Review of Biochemistry.* E.S. Snell, P.D. Boyer, A.Meister and C.C.Richardson (eds). Annual

Reviews Inc.

Halliwell, B and J.M.C. Gutteridge. 1999. *Free Radicals in Biology and Medicine.* Oxford University Press, UK

Pack, D.E. 1967. Paraquat. In *Analytical Methods of Pesticides: Plant Growth Regulators and Food Additives.* 5th vol. G.Zweig (ed). Academic Press, USA

Santos, R,. S. Bocquet, A. Puppo and D.Touati. 1999. Characterization of an Atypical Superoxide Dismutase from *Sinorrhizobium meliloti. J. Bacteriol.* 181:4509-4516

Tanksley, S.D and T.J.Orton. 1983. *Isozymes in Plant Genetics and Breeding.* Part A. Elsevier.

Wijayanto, D. 2003. Kajian Viabilitas dan Stabilitas Bakteri Perombak Paraquat di Tanah Gambut, vertisol dan Karier. *Thesis* (un-published). Post Graduate School in Biotechnology, Gadjah Mada Uiversity, Yogyakarta

Yanti, N. A, E. Martani and S. Margino. 2003. Isolasi Bakteri Pendegradasi Paraquat dari Tanah Gambut. *Gama Sains.* V:7-14.