NUSANTARA BIOSCIENCE
Vol. 7, No. 2, pp. 107-111
November 2015

ISSN: 2087-3948
E-ISSN: 2087-3956
DOI: 10.13057/nusbiosci/n070208

Isolation and screening of rhizobacteria from soil in Ngawi, East Java, as candidates of agent for liquid organic fertilizer production

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Manuscript was received: 15 August 2014. Revision was accepted: 15 October 2015.

Abstract. *Imamuddin H, Dewi TK, Antonius S. 2015. Isolation and screening of rhizobacteria from soil in Ngawi, East Java as candidates of agent for liquid organic fertilizer production. Nusantara Bioscience 7: 107-111*. A series of activities have been done to find indigenous microbes from Ngawi, East Java, as candidates for living organic fertilizers. The study was started with sampling of soils from 17 locations in Ngawi. Then, the samples were isolated to get population of soil microbes, including total, phosphate-solubilizing, N-fixing, and IAA-(Indole Acetic Acid)-producing bacteria. Five soil samples were used to test bacterial growth in several concentration of propoxur. Method of isolation used in this study was enrichment culture and the growth was determined using spectrophotometer at 436 nm wavelength. The results showed that the populations of total and IAA-producing bacteria in Ngawi soil were relatively high, namely 10^6 CFU/g soil, but the population N-fixing bacteria was only 10^{-5}, and no phosphate-solubilizing bacteria could grow. Twenty-one isolates of bacteria were found, 16 of which positively produced IAA and 5 isolates of which could grow in propoxur medium. The highest production of IAA was found in sample number 6.3 with a concentration of 123.535 ppm and isolate H-2-NG (sample number 2) could grow at concentration of 1000 ppm (MM medium)-3000 ppm (MSB medium). It is hoped that these isolates can be used as candidates of agent for liquid living organic fertilizers.

Keywords: Ngawi, IAA, propoxur, organic fertilizers

Abbreviation: IAA: indol-3-acetic acid, PGPR: Plant Growth Promoting Rhizobacteria, NFB: Nitrogen-fixing bacteria

INTRODUCTION

Rhizobacteria having beneficial effects on plant growth through direct and indirect mechanism are called Plant Growth Promoting Rhizobacteria (PGPR) (Juanda 2005). Interactions between plants and microbes in rhizosphere are responsible for the improvement of plant health and soil fertility (Khan 2006). These mechanisms can be active simultaneously or separately in several stages of plant growth (Ahmad et al. 2008). PGPR have been reported to increase growth directly through various mechanisms: nitrogen fixation, as phosphate solvent, siderophores production, and synthesis of plant growth hormone, namely indol-3-acetic acid (IAA), cytokinin and ethylene (Nelson 2004). Through indirect mechanism, PGPR act as agents of biological control for plant pathogens and harmful microbes.

Some soil bacteria, including the genera *Bacillus* and *Pseudomonas*, have the ability to transform insoluble materials into soluble ones, by secreting organic acid such as formiate, propionate, fumarate, lactate, glycolate and succinate acid (Vazquez et al. 2000). Organic fertilizers, mixtures of microbe inoculants, improve plant growth, productivity, and nutrient status. Organic fertilizers have been accepted internationally as alternatives to synthetic fertilizers (Vessey 2003). Significant increase of yield due to application of PGPR inoculants has been reported (Salamore 2000).

Propoxur (2-isopropoxyphenyl-N-methylcarbamate) is a widely used pesticide to control insect in houses. It is very effective in inhibiting cholinesterase and very toxic to human and animals (Kuhr dan Dorough 1976; Topp et al. 1993). Although propoxur has been used since 1959 (Thomson et al. 1985), only few people know its existence in soils. Propoxur belongs to carbamate pesticide, and it is assumed that the degradation path of it in soils starts with hydrolysis, the product of which is 2-isopropoxyphenol. It has been reported that most carbamate pesticides will be degraded in soils one year or longer after application on fields (Ou et al. 1992). The path of propoxur degradation showed in Figure 1 (Kamanavalli et al. 2000).

The objective of this study was to isolate indigenous microbes in various locations in Ngawi, East Java, Indonesia. It is hoped that those microbes can be used as agent for organic fertilizer production.

MATERIALS AND METHODS

Soil samples

Soil samples were taken from 17 locations (collection numbers 1-17) in Ngawi District, East Java, Indonesia. The samples were collected by some farmer groups in the district. The samples were contained in plastic bags and kept in storage at 4ºC for testing.

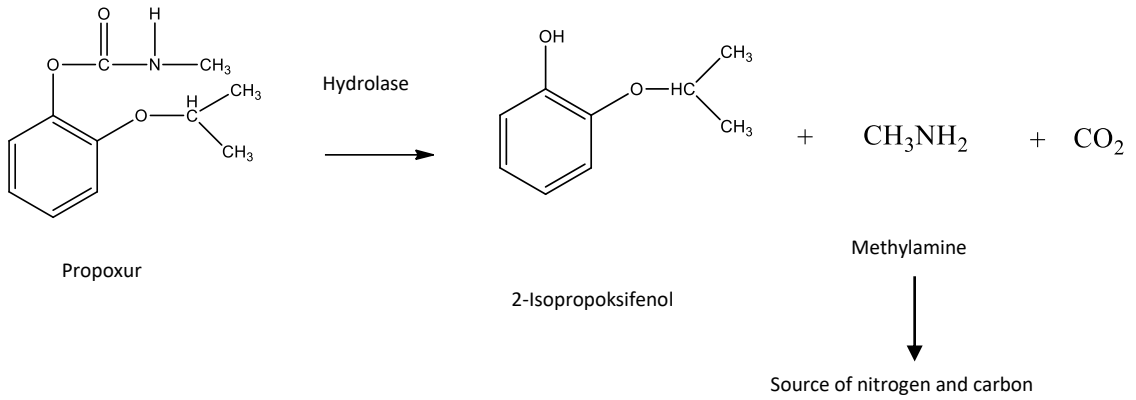

Figure 1. The path of propoxur degradation (Kamavalli et al. 2000)

Isolation of total bacteria

Total bacteria were isolated from soil rhizosphere samples using serial dilution technique with NA (Nutrient Broth) as the medium. Then they were incubated at 30±0°C for 24-120 hours. The colony of bacteria growing on the medium was the total bacteria population.

Isolation of IAA-producing bacteria

The isolation of IAA-producing bacteria was conducted using serial dilution technique with TSB (Trypticase Soy Broth) as the medium. The colony growing on TSB was moved to other TSB media in *Duplo* (double sets). After growing for 24-48 hours, one set of it was added with 1 ml of Salkowsky solution for each petri dish (1 set); the other set was used for further test. When the colony turned pink, it meant that the isolates positively produced IAA. Subsequently, quantitative tests were done.

Detection of IAA using colorimetry (quantitative test)

The Production of IAA was measured using colorimetry method (Gravel et al. 2007). The medium used for analysis was TSB 50% (half strength), with the following composition: 10 g of Peptone 10 g, 2.5 g of NaCl, 20 g of Agar, and 1000 mL of distilled water. After the medium was sterilized, precursor L-Tryptophan 200 ppm was added to the médium. Before the analysis, standard curve of IAA (0-50 ppm) was made.

The making of Salkowski reagent

As much as1 mL of 0.5 M $FeCl_3$ was pipetted and added with 50 mL of $HClO_4$ 50% (vol/vol). Then it was kept in opaque bottles or covered with aluminum foil. (0.5M of $FeCl_3$= 1.35 g/10 mL) ($HCl0_4$ 50% = 25 mL of $HClO_4$ + 25 mL of distilled water). For analyses of IAA, 1 mL of supernatant was taken from the sample after centrifugation for 10 minutes at 8000 rpm, added with 2 mL of Salkowski reagent, and then it was incubated in a dark room for 30 minutes. Measurement was taken using spectrophotometer at 530 nm wavelength.

Isolation of Total phosphate-solubilizing bacteria

Phosphate-solubilizing bacteria were isolated from soil rhizosphere samples using serial dilution method. The medium used was Pikovskaya, incubated at 30±0°C for 24-120 hours. The colony showing hollow zone was considered positively as P-solubilizing bacteria. Solubilization zone was determined by subtracting the diameter of total zone with the diameter of bacteria colony zone (Gupta et al. 1994).

Isolation of N-fixing bacteria (NFB)

The medium used was NFB and the method was plate count with serial dilution. If the colony turned blue, it could be concluded that the isolate was positively N-fixing bacteria.

Isolation and growth of bacteria in propoxur

Ten gram of soil sample was taken from sample numbers 1a, 2, 6, 8 and 10, and then they were put into 250 ml-Erlenmeyer containing 100 mL liquid NB medium + 1000 ppm propoxur. The solution was shaken for 10 days. Then, isolation was done by pipetting 1 mL of suspension from each Erlenmeyer, and then they were inoculated in solid NA medum + 1000 ppm propoxur. The growing colony was moved to another petri dish for purification. After purification, 5 isolates were found capable of growing in 1000 ppm propoxur, but after growth test was done, only isolate H-2-Ng was able to grow at 1000 ppm MM medium. The isolated test was subsequently conducted on MSB medium, using 1000-3000 ppm propoxur.

RESULTS AND DISCUSSION

Isolation has been done to soil samples using plate count method to determine the population of total, IAA-producing, P-solubilizing, N-fixing, and pesticide-degrading bacteria. The results of isolation are presented in Figure 2.

From the isolation of total bacteria, it was found that sample number 13 showed the biggest population and sample number 9 had the smallest 9 population. Population of NFB was observed only in sample number 7, while other samples had population fewer than 10^6. The biggest population of IAA-producing bacteria was observed in sample 3 and the smallest was found in sample number 1b. Quantitative analyses were done for IAA-producing bacteria to determine the amount of IAA produced by isolates which had been qualitatively determined as IAA producers. The results of this study were in accordance with the report by Kumar et al. (2012) that PGPR colonies in plant roots could induce growth through various mechanisms. The definite mechanism with which PGPR colonies can stimulate plant growth is not known, although some mechanisms such as phytohormone production, pathogenic organism suppression, activation of phosphate solubilizing, improvement of nutrient uptake are known to play roles in plant growth (Glick 1995; Lalande et al. 1989; Kumar et al. 2012).

Quantitative tests to determine the concentration of IAA were conducted for isolates growing on TSB medium. The growing isolates were added with 1 mL of Salkowsky solution. Then, they were incubated for 24 hours in a dark room. The pink colonies positively contained IAA. The test results showed that 16 isolates were capable of producing IAA.

Table 1 shows that out of 16 isolates tested, isolate 6.3 had the highest concentration of IAA, namely 123.535 ppm. From the data in table, it can be concluded that 3 isolates were the best, namely numbers 6.3, 5.5, and 6.5 (printed in red) because they could produce relatively high concentration of IAA.

Screening of propoxur degrader

Five soil samples (numbers 1a, 2, 6, 8 and 10) which had relatively small population of bacteria were tested. Culture enrichment produced 10 consortia, each of which was purified and grown in MM medium. Only isolate H-2-Ng could grow until 2000 ppm propoxur in MM medium (the results were not presented). However, after it was tested again, it could grow only until 1000 ppm propoxur and in MSB medium until 1000-3000 ppm propoxur (Figures 3A and B). Figure 3A shows that isolate H-2-NG utilize the propoxur degradation products, as it could not grow well without propoxur. At 500 ppm propoxur, it grew well after 6 hours of incubation and then, it declined until 72nd hour, whereas at higher concentration, it grew until the third day (72nd hour) and reached its peak at 96th hour.

On the first day, isolate H-2-NG did not grow well; only in 1000 ppm propoxur it started growing although it was slow with OD < 0.4. On the 48th hour, isolate H-2-NG started to grow rapidly with OD > 1.5 at 2000 ppm concentration. At the 72nd hour, it grew at the same rate between 2000 ppm and 3000 ppm with OD 2.8-2.9.

Table 1. Concentration of IAA from selected isolates from Ngawi soil samples

Isolates	IAA (ppm)			
	0 hour	24 hours	48 hours	72 hours
6.1	0,000	4,936	3,866	3,474
6.2	0,000	3,254	3,592	3,445
6.3	0,000	34,655	113,658	**123,535**
6.5	0,000	0,000	2,352	**15,499**
6.6	0,000	0,183	0,496	6,115
5.1	0,000	5,210	1,633	9,020
5.2	0,000	3,311	9,743	3,240
5.3	0,000	7,022	9,046	2,472
5.4	0,000	4,005	6,403	9,482
5.5	0,000	17,443	17,379	**18,988**
5.6	0,000	4,020	3,555	5,142
4.1	0,000	0,489	0,548	2,132
4.3	0,000	2,567	2,570	2,868
4.4	0,000	**10,479**	9,995	8,567
4.5	0,000	1,164	1,090	2,699
4.6	0,000	5,032	3,511	3,782

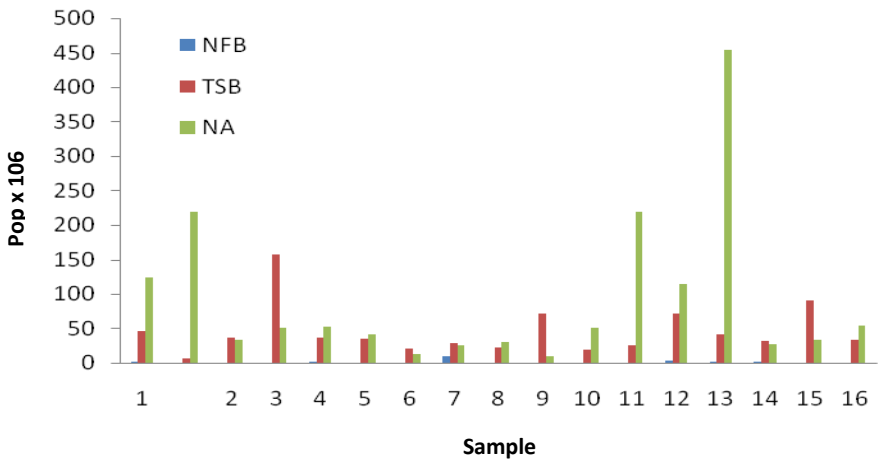

Figure 2. Population of total, N-fixing and total phospate-solubilizing bacteria

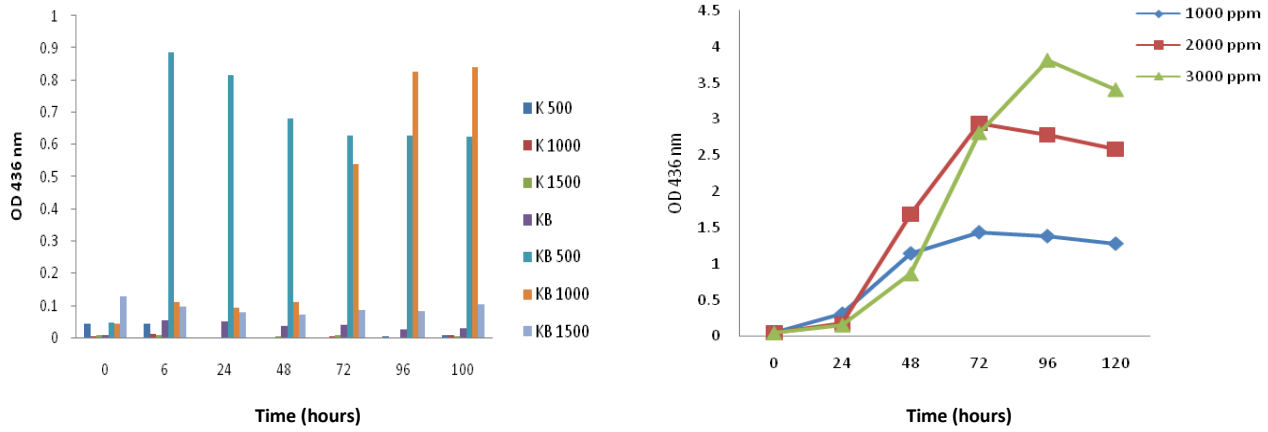

Figure 3. The growth of isolate H-2-NG in MM (A) and MSB (B) media

Discussion

The isolation of total bacteria showed that sample number 13 had the biggest population (45 x 10^7) and sample number 9 had the smallest one (0.30x10^6). The total bacteria population in this study was bigger than that of Bahig et al. (2008) in various locations and seasons. In Bahig's study, the total bacteria population was only about 10^2, while in this study it was 10^6. The population of total bacteria in samples from polluted area was usually small (soil samples numbers 1a, 2, 6, 8 and 10). This finding was different from that of Bahig et al. which showed that the total bacteria population in polluted location was higher.

The population of NFB was observed only in sample number 7. Other samples had small population, fewer than 10^6. Sample number 3 had the biggest population of IAA-producing bacteria, while sample number 1b had the smallest one. Quantitative analyses were done for IAA-producing bacteria to determine the amount of IAA produced by isolates which had been qualitatively determined as IAA producers. The results of this study were in accordance with the report by Kumar et al. (2012) that PGPR colonies in plant roots could induce growth through various mechanisms. The definite mechanism with which PGPR colonies can stimulate plant growth is not known, although some mechanisms such as phytohormone production, pathogenic organism suppression, activation of phosphate solver, improvement of nutrient uptake are known to play roles in plant growth (Glick 1995; Lalande et al. 1989; Kumar et al. 2012).

Concentration of IAA in this study was lower than that reported by Kumar et al. (2012), but the tryptophan added in their study was also higher, namely 500 µL, while in this study it was only 200 µL. The results of Kumar et al. (2012) was slightly different from that by Egamberdieva (2008) which showed that the IAA produced was 1.6-3.3 ppm, despite the addition of 500 µL tryptophan. So, the results in this study were much higher, ranging from 2.132 ppm to 123.3 ppm, despite the addition of the same amount of tryptophan (200 µL). The production of IAA in this study was also higher than that by Reetha et al. (2014) whose study only produced 12.67 ppm IAA from *Bacillus* sp. and 15.38 ppm from *Pseudomonas* sp. Whether the addition of tryptophan can influence the production of IAA needs to be studied further because tryptophan is a precursor of IAA (Xie et al. 1996; Kumar et al. 2012). More research needs to be done to determine precisely the most appropriate concentration of tryptophan which produces higher concentration of IAA, because the production of IAA varied among species and strain and the IAA production was also influenced by isolate, growth rate and the availability of substrate (Mirza et al. 2001). It has been revealed that the addition of tryptophan precursor increases production IAA in various media and species of bacteria (Ahmad et al. 2008).

The result of screening of propoxur-degrading bacteria was better than that in the study of Kamavalli et al. (2000) and Anusha et al. (2009). In this study, some isolates were capable of growing at 3000 ppm propoxur, while in the previous two studies the isolates were able to grow only at 200-2000 ppm propoxur. The good growth in high concentration of propoxur indicates that isolate H-2-NG can use propoxur as source of N and C from secondary metabolite with the end results are carbon and nitrogen, and not from 2-isopropoxyphenol which is the product of propoxur hydrolysis (Kamanavalli et al. 2000). However, this claim must be proved with further analyses to determine whether or not the isolates degrade propoxur into isopropoxyphenol or methylamine.

ACKNOWLEDGEMENTS

The authors are grateful to DIPA PN5 which provided fund for this research. We also thank Nani Mulyani, Astri Anggraeni and Arie Rosmalia who have helped us in this study.

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