IMPROVEMENT OF THE *Ralstonia pickettii* STRAIN TT47 VIABILITY IN PASTE FORMULATION BY OSMOADAPTATION

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ABSTRACT

The limited survival of *Ralstonia pickettii* (*R. pickettii*) strain TT47 as an active ingredient under storage conditions with osmotic stress is an important limitation that can affect its efficacy in the field. This research sought to develop a technology to improve the viability of *R. pickettii* strain TT47 paste formulation while in storage using osmoadaptation treatments. Induction of osmoadaptation was done using two methods, namely direct induction (Ind-1) and indirect induction (Ind-2). Direct induction was achieved by growing *R. pickettii* strain TT47 directly in glucose minimum medium (GMM) with NaCl (0.3 M), while indirect induction involved growth in Luria-Bertani (LB) broth media then GMM until the stationary phase and subsequently 1 mL of grown *R. pickettii* strain TT47 was inoculated into 100 mL of the GMM + NaCl (0.3 M) media (1%, v/v) with the final density in the media of 10^7 CFU · mL^{-1}. The suspension of *R. pickettii* strain TT47 without induction (no induction, NI) was obtained by growing *R. pickettii* strain TT47 in LB broth media and used as control. The suspension of *R. pickettii* strain TT47 from direct induction (Ind-1), indirect induction (Ind-2), and no induction (NI) at a density of 10^9 CFU · mL^{-1} was formulated with a paste-based formulation. The formulation activities produced three biopesticides, namely FRP-Ind-1 (the active ingredient was osmoadapted with direct induction), FRP-Ind-2 (the active ingredient was osmoadapted with indirect induction), and FRP-NI (the active ingredient did not undergo induction). The decrease in viability of osmoadapted *R. pickettii* strain TT47 during storage at room temperature (26-30 ^oC), 16^oC, and 4^oC was found to be slower than that of control treatment (no induction). The viability of osmoadapted *R. pickettii* strain TT47 was in the range of 63.15 - 79.13% after 6 months at all storage temperature, while the viability of *R. pickettii* strain TT47 without osmoadaptation treatment decreased to 0% within 3-4 months, even at 4 ^oC storage temperature.

Key words: Biopesticide, sodium chloride, storage

INTRODUCTION

The use of biological pesticides is increasing due to public awareness regarding the safety of agricultural and environmental products. The magnitude of the use of biological pesticides is reflected by the trade value of these products. The largest market share of biological pesticides belongs to North America (44%), followed by Europe (20%), Asia (13%), Australia (11%), Latin America (9%), and Africa (3%) (Mishra et al. 2015). Unlike the case abroad, the use of biological pesticides and the number of biological pesticide products in Indonesia remains very low. The number of biological pesticide products registered and allowed to be commercialized in Indonesia during 2016 was 18 out

of a total of 3207 or less than 1% (Ditjen PSP 2016). Thus, there is significant opportunity for the development of biological pesticides in Indonesia.

The use of biological pesticides has increased, however, it is still lower than synthetic pesticides. The trade share of biological pesticide markets is around USD 1 billion, far less than synthetic pesticides which create USD 40 billion in trade value (Popp et al. 2013). One limiting factor for the low use is the low adaptability of biological agents to environmental factors, both biotic and abiotic, which causes an unstable level of efficacy (Bonaterra et al. 2005; Johnson et al. 2000). The retention of viability of biological agents is very important, both during storage and after being applied. Biological agents are generally very sensitive to osmotic stress due to the limited availability of water, especially for those products that include Gram-negative bacteria. Low water availability in formulation causes osmotic stress differences between the cytoplasm and outer environment of the cell so that the cell is easily dehydrated, thus reducing its viability. The decreased viability in the formulation during storage affects the effectiveness of the formulation of biological agents in the field. To overcome this problem, a strategy to increase the adaptability of biological agents to environmental conditions is an important key in biopesticide formulations development to support their effectiveness in the field.

Ralstonia pickettii strain TT47 is one of the potential biological control agents. This isolate is able to suppress the growth of several important rice pathogens such as *Xanthomonas oryzae* pv. *oryzae*, *Burkholderia glumae*, *Pyricularia oryzae*, *Rhizoctonia solani*, and *Drechslera oryzae*. The isolate has several mechanisms for suppressing these pathogens, namely antibiosis and lysis, induced plant resistance by increasing the activity of chitinase, peroxidase and phenylalanine ammonia lyase, and produce siderophores and phosphate solubilizing (Dewi et al. 2020). Based on its superior properties, *R. pickettii* strain TT47 has the potential to be developed as a biological pesticide, however as a Gram-negative bacterium, it faces challenges in terms of adaptability to unfavorable conditions as a formulation.

One of the strategies to increase or maintain the viability of biological agents is osmoadaptation. Several studies of induction technology to environmental stress adaptation have been conducted (Bonaterra et al. 2005; Cabrefiga et al. 2011). Adaptation to environmental stress on biological agents were used to manipulate physiological functions so that these agents are able to adapt in extreme or unfavorable conditions. Induction is generally carried out through the cultivation of biological agents under sub-optimal media and supplemented with induction agents. The use of sodium chloride (NaCl) and the addition of betaine glycine during inoculum preparation kept the viability of *Pantoea agglomerans* EPS125 on the peel surface of apples under different storage conditions (Bonaterra et al. 2005). The availability of formulation technology that is able to maintain the viability of biological agents in the formulation is expected to contribute to the development of biological control. This study sought to develop the technology to enhance viability of *R. pickettii* strain TT47 in paste formulations during storage by osmoadaptation.

MATERIALS AND METHODS

Microorganism and culture preparation. *Ralstonia pickettii* strain TT47 was obtained from the Plant Bacteriology Laboratory, Department of Plant Protection at IPB University. The *R. pickettii* strain TT47 was isolated from soil samples collected from dry fields with grass vegetation in Indragiri Hilir Regency, Riau, Indonesia (Rustam 2012). The isolate was stored at room temperature (26-30 ^{o}C) in sterile water as a stock culture. A pure isolate was recultured in TSA (tryptone soya agar) media.

Induction of osmotic stress adaptation. Osmoadaptation was performed using methods described by Bonaterra et al. (2005). Osmoadaptation was done using two methods, direct induction (Ind-1) and indirect induction (Ind-2).

Direct induction. The method used was a modified osmoadaptation method (Bonaterra et al. 2005). Induction was completed by growing *R. pickettii* strain TT47 directly on glucose minimum medium (GMM) with NaCl added to make 0.3 M. GMM media composition was: 5 g glucose, 1 g NH_4Cl, 3 g KH_2PO_4, 2.4 g Na_2HPO_4, 0.5 g NaCl, and 0.2 g $MgSO_4$ with a pH of 7. An agar block of *R. pickettii* strain TT47 culture was inoculated directly in 50 mL GMM + NaCl 0.3 M media, then grown in an orbital shaker at 150 rpm at room temperature (26-30 ^oC) for 48 hours or until reaching a density of 10^9 CFU $\cdot$ mL^{-1} (OD = 1.2).

Indirect induction. Induction was carried out using the methods described in Bonaterra et al. (2005). The *R. pickettii* strain TT47 was grown on Luria-Bertani broth media and incubated in an orbital shaker at 100 rpm at room temperature (26-30 ^oC) for 24 hours. A total of 1 mL of *R. pickettii* strain TT47 culture (density 10^9 CFU $\cdot$ mL^{-1}) was added into 100 mL of GMM media, then grown in an orbital shaker at 100 rpm at room temperature until the stationary phase was reached. A total of 1% (v/v) *R. pickettii* strain TT47 culture in the stationary phase in GMM media was inoculated into GMM + NaCl 0.3 M, then grown in an orbital shaker at 150 rpm at room temperature for 48 hours or until a density of 10^9 CFU $\cdot$ mL^{-1} 1 (OD = 1.2) was obtained.

In the induction process, the growth of *R. pickettii* strain TT47 on induction media (GMM + NaCl 0.3 M) was observed. The growth of *R. pickettii* strain TT47 on LB media as control was also observed. The LB media used to compare the growth of the bacteria on optimal media with induction media. The population of *R. pickettii* strain TT47 was counted using the plate count method.

Formulation of biological pesticide of *Ralstonia pickettii* strain TT47. The formulation contained active ingredients (*R. pickettii* strain TT47) and additive agents. The additives consisted of talc, palm oil, molasses, Tween 80, and CMC (carboxymethyl cellulose). The concentration of the additive agents used did not have a negative effect on the growth of *R. pickettii* strain TT47. The concentration of each additive agents was determined by toxicity test, carried out using the paper disc diffusion assay method (Ng and Amsaveni 2012) with three replications. The concentrations tested consisted of several concentration levels. Talc was tested at 22-50%, 5-80% palm oil, while Tween 80, molasses, and CMC were tested at 0.1-5%. The inhibition zones formed around the filter paper were assessed and determined to be negative (-) if no inhibition zone was formed and positive (+) if an inhibition zone was formed.

Biological pesticide formulations produced in this study were pastes. The concentration of the formulation component was determined based on the results of the toxicity test. The composition of the formulation that showed the best characters of paste homogeneity, viscosity, and solubility was used in the trials. The formulation contained 45% of talc and 38% palm oil, 3% molasses, 3% Tween 80, 1% CMC (w/w), and 10% (v/w) of *R. pickettii* strain TT47. The *R. pickettii* strain TT47 suspension was treated by direct induction (Ind-1), indirect induction (Ind-2), and control or without induction (non-induction, NI). The *R. pickettii* strain TT47 used as control was grown in LB broth and incubated in an orbital shaker at 100 rpm. Bacterial suspension of each treatment, containing 10^9 CFU $\cdot$ mL^{-1} population density, was harvested and used to make the formulation.

Formulation analysis. Formulation analysis was performed on the physical characteristics and stability of the formulation, which included: shape, color, and solution form. The stability test was carried out by dissolving the formulations in distilled water and ionic water and observing for the formation of sediment and foam. The volume of sediment or foam were assessed at 30, 60, and 120 minutes of observation and expressed as a percentage (Dobart 1998).

Viability of *R. pickettii* strain TT47 formulation while in storage. The storage temperature treatments consisted of 4 ^oC, 16 ^oC, and room temperature (26-30 ^oC). Viability was observed using

the serial dilution method and was done once a month for six months. Bacterial viability in the formulation was calculated per gram and expressed as a percent (Lian et al. 2002).

RESULTS AND DISCUSSION

Effect of osmoadaptation on growth of *Ralstonia pickettii* strain TT47. Adaptation of osmotic stress or osmoadaptation is a physiological adaptation mechanism for different osmotic stresses (Galinski 1995). This occurs in cells through the accumulation of certain compounds that are able to maintain the balance of osmotic pressure between the environment inside the cytoplasm and outside the cell. The accumulation of these compounds may occur because of induction. This induction can be done using compounds such as NaCl (Bonaterra et al. 2005). The induced adaptation process will certainly affect the physiology of the bacteria. Osmoadaptation using induction media supplemented with NaCl had a significant effect on the growth of *R. pickettii* strain TT47. Growth patterns of *R. pickettii* strain TT47 in induction media were found to be significantly different from growth on LB media. The growth of *R. pickettii* strain TT47 in the induction media by the indirect method was slower than in LB broth media. The growth duration to reach an optimum population in induction media was longer than in LB media. The optimal population in LB media was achieved after 16 hours of incubation, while in induction media, the optimal population was reached after 42 hours of incubation (data not shown).

The growth of *R. pickettii* strain TT47 in LB media followed the pattern of bacterial growth in general, which consisted of lag, log, stationary, and death phases, which was indicated by population decline. This is different from the growth in induction media (GMM + NaCl 0.3 M). The population of *R. pickettii* strain TT47 at the beginning of growth decreased for 18 hours of incubation, then increased into the log phase, which was followed by stationary and the death phases. Increased populations of *R. pickettii* strain TT47 is an indication that is able to adapt or that it is able to survive on induction media, i.e. the cells can osmoadapt. The effect of osmoadaptation on bacterial growth has also been reported by Bonaterra et al. (2005) in *P. agglomerans*. Growth of *P. agglomerans* on GMM media amended with NaCl was slower compared to media without NaCl.

Toxicity of the formulation material against *Ralstonia pickettii* strain TT47. The results of the toxicity test to the additive agents, which consisted of talc, palm oil, Tween 80, molasses, and CMC indicate that most of the results showed negative toxicity reactions, except Tween 80 at a 5% concentration, which had a positive reaction. A negative reaction means that the component did not have a negative effect on the growth of *R. pickettii* strain TT47. Based on the results of the toxicity test, all components were safe and can be used as additive agents in the formulation, except for Tween 80, which can be used at a 3% concentration.

Formulation characteristics. The results of the formulation obtained three formulation products of biological pesticides, namely: 1) formulation of induced *R. pickettii* strain TT47 with direct induction method (FRP-Ind-1), 2) formulation of induced *R. pickettii* strain TT47 with indirect induction method (FRP-Ind-2), and 3) formulation of non-induced *R. pickettii* strain TT47 (FRP-NI). All formulations were formulated used the same additives and composition. The total population of *R. pickettii* strain TT47 in the formulation was 10^8 CFU $\cdot$ g^{-1} formulation.

Physical performance of formulation and solution characteristics are shown in Figure 1. The obtained formulation was a thick paste (*slurry*) with brownish color. Solution of the formulation in distilled water formed a dirty brown suspension.

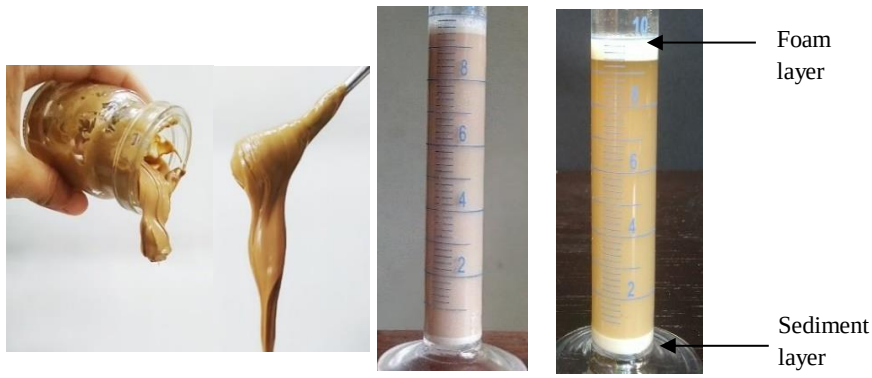

Fig. 1. Characteristic of *R. pickettii* strain TT47 paste formulation (a), paste formulation of *R. pickettii* strain TT47, (b) suspension of formulation, and c) formation of a foam and sediment layer at formulation stability test

The stability test result of the formulations can be seen in Table 1. All the formulations formed sediments and foam layers when dissolved in distilled and ionic water. The sediment layers began to appear after 30 minutes of observation and increased continuously for up to 60 minutes of observation. Sediment volume was stable for up to 2 hours of observation. The percentage of sediment was higher in FRP-Ind-1 and FRP-Ind-2 than FRP-NI in both solvents. The presence of a sediment layer will determine the application technique, which requires good and constant agitation to be completed mechanically by an operator (farmer), and will quickly settle down if the agitation ceases. Thicker sediment layers were produced in ionic water. This is because of the nature of ionic water, which is known to trigger mineral precipitation and reduce foam formation in soap solutions. The percentage of the foam layer in the FRP-Ind-1 and FRP-Ind-2 were lower than the FRP-NI in both distilled and ionic water. The foam layer on the FRP-NI after 2 hours of observation was 6% and thicker than the others (3%).

Table 1. Stability test of FRP-NI, FRP-Ind-1, and FRP-Ind-2 according to foam and sediment formed in distilled and ionic water.

Solvent	Formulation	Sediment layer (%) at the time of observation (minute)			Foam layer (%) at the time of observation (minute)		
		30	60	120	30	60	120
Distilled water	FRP-NI	3	4	6	3	4	4
	FRP Ind-1	3	4	6	3	4	4
	FRP Ind-2	4	5	5	6	6	6
Ionic water	FRP-NI	4	6	7	4	3	3
	FRP Ind-1	4	6	7	3	3	3
	FRP Ind-2	4	5	5	6	6	6

Viability of *Ralstonia pickettii* strain TT47 in storage. The viability of three *R. pickettii* strain TT47 formulations at three storage temperatures declined due to storage duration (Table 2). Observational results of bacterial viability, which was done once a month for six months, indicated that osmoadaptation to *R. pickettii* strain TT47 by NaCl during preparation was able to improve the viability of bacteria in induction formulation better than without osmoadaptation treatment.

Osmoadaptation treatment was also able to induce the biological agents to be more tolerant to storage temperatures. The viability of osmoadapted *R. pickettii* strain TT47 (FRP-Ind-1 and FRP-Ind-2) that were stored at room temperature (26-30 ^{o}C) decreased at a slower rate and was not significantly different from its viability at 16 °C and 4 °C storage temperature. The decrease in viability was faster for the non-osmoadapted treatment.

Generally, formulation stored in lower than room temperature was able to maintain the viability. The high temperatures can encourage faster bacterial growth, stimulating toxic metabolite production and pH changes that negatively affect bacteria and even cause cell death (Dearmon et al. 1962). However, this research showed that storage at 16 ^{o}C was able to maintain the viability better than at 4 ^{o}C and room temperatures (26-30 ^{o}C). The FRP-Ind-1 and FRP-Ind-2 stored at 16 ^{o}C were able to maintain the viability at 79.13% and 76.84%, respectively.

The stable viability of *R. pickettii* strain TT47 in induced formulation was suspected to be caused by the physiological function changes in bacterial cells. Osmoadapted cells accumulate trehalose and glycine betaine and showed a higher tolerance to desiccation compared to non-osmoadapted cells (Bonaterra et al. 2005). There is a relationship between trehalose accumulation with increased osmotic tolerance in *Escherichia coli* and *Trichoderma harzianum* (Welsh and Herbert 1999; Harman 1991). The mechanism of adaptation is acquired through protection of the phospholipid membrane by hydrogen and phospholipids binding directly, which allows the liquid composition in the cell to remain balanced (Csonka 1989). The possibility of trehalose and glycine betaine or other solutes accumulating in *R. pickettii* strain TT47 needs further research.

According to viability observation during storage, osmoadaptation affects significantly the viability of *R. pickettii* strain TT47 in formulation. Osmoadaptation is able to maintain or improve *R. pickettii* strain TT47 viability and it was significantly different from non-osmoadapted formulation after two months of storage. The viability of *R. pickettii* strain TT47 induced with the direct induction method (induction-1) and indirect induction (ind-2) showed no significant difference, thus both methods could be used. However, in the induction process, the direct induction method (Ind-1) is better i.e. easier and faster than the indirect method (Ind-2).

CONCLUSION

Induction of osmotic stress adaptation for *R. pickettii* strain TT47 using GMM with additional NaCl (0.3 M) was found to influence the growth of *R. pickettii* strain TT47. This was slower than on regular media (LB) (non-osmoadaptation treatment) and maintained or improved the viability of *R. pickettii* strain TT47 in a paste formulation. The direct induction method can be used as an induction technology to increase viability of the formulation. It is best to store the formulation at 16 ^{o}C as the *R. pickettii* strain TT47 viability declined slowly.

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Table 2. Viability of bacterial biological agents in the formulation during storage

Storage Temperature	Formulation	Viability of bacteria in formulation (%)[a] during storage (months)						
		0	**1**	**2**	**3**	**4**	**5**	**6**
Room Temperature (26 - 30°C)	FRP-NI	100.00[a]	97.73[a]	57.58[b]	43.24[c]	0.00[c]	0.00[b]	0.00[d]
	FRP Ind-1	100.00[a]	98.69[a]	98.73[a]	96.39[ab]	94.86[a]	85.80[a]	65.79[c]
	FRP Ind-2	100.00[a]	99.32[a]	98.01[a]	95.50[b]	93.72[a]	87.17[a]	63.15[c]
Temperature: 16°C	FRP-NI	100.00[a]	97.73[a]	57.58[b]	43.24[c]	0.00[c]	0.00[b]	0.00[d]
	FRP Ind-1	100.00[a]	99.49[a]	99.32[a]	98.41[a]	96.64[a]	89.79[a]	79.13[a]
	FRP Ind-2	100.00[a]	99.75[a]	98.43[a]	97.64[ab]	97.01[a]	88.48[a]	76.84[ab]
Temperature: 4°C	FRP-NI	100.00[a]	91.58[a]	45.41[c]	0.00[d]	0.00[e]	0.00[b]	0.00[d]
	FRP Ind-1	100.00[a]	99.41[a]	98.78[a]	97.26[ab]	96.12[a]	90.78[a]	72.51[b]
	FRP Ind-2	100.00[a]	99.37[a]	97.94[a]	96.87[ab]	94.86[a]	86.94[a]	66.94[c]

[a] Means in the same column followed by same letter are not significantly different according to a Tukey test at α=5%.

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