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Influence of Palmitic Acid and Amino Acids Addition on Iturin A Productivity by *Bacillus subtilis* RB14-CS

Y U L I A R ♥
Microbiology Division, Research Center for Biology, The Indonesian Institute of Science, Bogor 16002.

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ABSTRACT

The aim of this study was to observe the influence of palmitic acid and amino acids on enhancement of iturin A productivity using *Bacillus subtilis* RB14-CS. The concentrations of palmitic acid examined were 0.8%, 1.6%, and 3.2%. A good yield was observed when 1.6% palmitic acid was added to Polypepton S medium. The production of iturin A increased about 18% than the control. Addition of 3.2% palmitic acid was not effective on iturin A production. It gave lower pH and slightly higher viable cell number of RB14-CS than control and the others addition concentration. Whereas the addition of 0.8% of each of the following amino acids; L-arg, L-asn , L-gln , L-glu, L-gly, L-leu, L-lys, L-trp, L-tyr, and L-val could not increase iturin A productivity, but changed the proportion of iturin A peaks. L-leu, L-val, and L-asn addition produced the highest proportion of peak 3, 4, and 1 respectively.

Key words: iturin A, *Bacillus subtilis* RB14-CS, palmitic acid, and L-amino acids.

INTRODUCTION

The use of microorganisms for biological purposes has become an effect to control plant pathogens. Various strains of *Bacillus subtilis* (NB22, YB8, UB24, and SB4) suppressed phytopatogenic fungi or bacteria *in vitro* and greenhouse test (Phae *et al*., 1990). Berna *et al*., (2002) demonstrated that mutant strain of *Bacillus* sp. has a higher antagonistic activity against the plant pathogen *Botrytys cinera* (grey mould), *Rastonia solanacearum* (bacterial wilt) and *Erwinia carotovora* (bacterial soft rot).

As producer of antibiotics, *Bacillus* spp. secrete three groups of the following lipopeptide antibiotics; surfactin group, iturin group, and plipastatin- fengycin group (Tsuge *et al.*, 2001). Among of these antibiotics, iturin has the strongest antifungal activity on large variety of yeasts and fungi. As iturin A is one of the most powerful antifungal substance, it has good prospect on agriculture and medical application. Since *Bacillus* produce the lipopeptide antibiotics, therefore why this bacteria suppres various plant pathogens. Iturin families that were found are; iturin A, iturin C, iturin D, iturin E, baccilomycin D, bacillomycin F, bacillomycin L. Iturin A consist of five homologues and the five peaks pattern of iturin A as shown in Figure1.

The highest productivity of iturin A was 138 mgl^{-1}, produced by *Bacillus subtilis* S499 (Hbid *et al.*, 1996). Beside carbon and nitrogen source, palmitic acid and L-amino acid may also influence the antibiotics production. Palmitic acid was incorporated into lipid moiety of iturin A by *B. subtilis* on Landy medium (Besson *et al*., 1990). The metabolism of amino acids, purine, and pyrimidines provides the nitrogenous precursor for antibiotics biosynthesis. Specific amino acids are direct precursors for many peptide antibiotics (Aharonowitz, 1980). In order to know whether palmitic acid or L- amino acid can enhance iturin A productivity, the addition effect of them to the medium cultivation of *Bacillus subtilis* RB14-CS was studied.

MATERIALS AND METHODS

Microorganism. *Bacillus subtilis* RB14-CS as mono producer of iturin A was used in this study. This microorganism is belong to Prof. Makoto Shoda laboratory (Chemical Resources Lab., Tokyo Institute of technology).

LB medium. This media were used as seed media and consist of 10 g/l polypepton, 5 g/l yeast extract, and 5 g/l NaCl. The pH was adjusted to 7.00 with NaOH. Five milliliters of this media were transferred into a test tube using micropipette and sterilized at 121^{o}C for 20 minutes. For LB agar media, 2% of agar was added to the media.

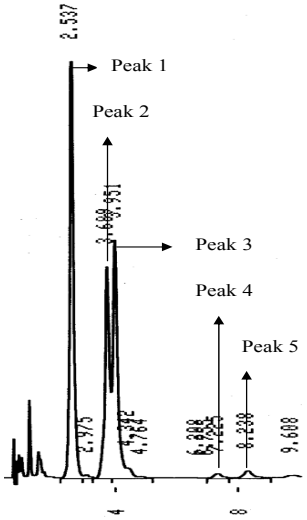
Polypepton S medium. Polypepton S (3.2 g) were dissolved in 23 ml distilled water in 200 ml flasks and sterilized at 121^{o}C for 20 min. After sterilization, 6.7% of maltose, 0.5% K_2HPO_4, 0.05% $MgSO_4.7H_2O$, 25 ppm $FeSO_4.7H_2O$, 22 ppm $MnSO_4.5H_2O$, and 184 ppm $CaCl_2.2H_2O$ were added into flasks.

Pre-cultivation. Five milliliters sterilized LB media in a test tube were added with 5µl of streptomycin (20 mgl^{-1}) and inoculated with 10µl of RB14-CS stock. Inoculated media was incubated at 37^{o}C and shake 124 spm (horizontal shaker) for about 16 hours.

Cultivation of RB14-CS on Polypepton S media contains palmitic acid. Three concentrations of palmitic acid i.e 0.8%, 1.6%, and 3.2% were each added into polyppeton S media and inoculated with 400µl RB14-CS, then incubated at 30^{o}C, 120 spm for 7 days.

Cultivation of RB14-CS on Polypepton S that contains L-amino acids. 0.8% of each of L-amino acid i.e L-arg, L-asn , L-gln , L-glu, L-gly, L-leu, L-lys, L-trp, L-tyr,

♥ **Alamat korespondensi:**
Jl. Ir. H. Juanda 18 Bogor 16002.
Tel.: +62-251-321038, Fax.: +62-251-325854.
e-mail: novikur@yahoo.com



R—CHCH$_2$CO→L-Asn→D-tyr→D-Asn
NH←L-ser←D-Asn←L-pro←L -Gln

	R	β-amino acid group
Peak 1	CH_3-CH_2-CH_2-*n*-C14-	β-amino acid
Peak 2	CH_3-CH_2-CH(CH_3)-*anteiso*-C15-	β-amino acid
Peak 3	CH_3-CH(CH_3)-CH_2-*iso*-C15-	β-amino acid
Peak 4	CH_3-CH(CH_3)-CH_2-CH_2-*iso*-C16-	β-amino acid
Peak 5	CH_3-CH_2-CH_2-CH_2- -C16-	β-amino acid

Figure 1. Structure of iturin A and HPLC elution pattern of iturin A homologues.

and L-val were added into Polypepton S medium before autoclaving. Sterilized media were inoculated with 400µl RB14-CS, then incubated at 30°C, 120 spm for 7 days.

Extraction and measurement of iturin A. The culture (100µl) were transferred into one milliliter Eppendorf tube, and diluted with 900µl of the buffer that composed of CH_3CN: 10mM CH_3COONH_4 (35:65; v/v). The mixture were rotated for about 30 minutes at room temperature, and it were centrifuged at 15,000x *g* for 10 minutes at 4°C. The supernatant were filtrated through 0.20µm PTFE membrane filter (Advantec *020*). This filtrate (20 µl) were injected into ODS RP-18C (Merck) column, and monitored by the UV detector at 205 nm (LC-800 system, JASCO). HPLC was performed using acetonitrile: 10mM CH_3COONH_4= (35:65, v/v) solvent, and flow rate was 2ml/min, and absorbance were measured at 30°C. Iturin A production was observed on day 2, day 5, and day 7.

Viable cell number. The culture was diluted 10^7 times in small sterile test tubes, then 100µl of the dilution sample was spread onto the LB agar medium. After the plates were incubated in incubator at 37°C overnight, the cell number was counted. The viable cell number was observed on day 2, day 5, and day 7.

RESULTS AND DISCUSSION

As we see in figure2, the addition of 1.6% palmitic acid on Polypepton S medium enhanced iturin A production 18%. Without an addition the production was about 2200 mgl^{-1} and the addition of 1.6% of palmitic acid yielded iturin A about 2600 mgl^{-1}. This is in agreement with Hourdou *et al.* (1988) who reported that by 0.05% of palmitic acid addition to the culture medium (LANDY medium), iturin A β-amino acids by GC analysis showed an increase of straight chain β-amino acids: 33% instead of 24% in the control for *n*-C_{14} and 11% instead of 6% in the control for *n*-C_{16}. Besson *et al*. (1990) also reported that palmitic acid is the precursor of β–amino acids of iturin A.

Figure 3 shows the pH range and viable cell number of RB14-CS on iturin A productivity. A high concentration of palmitic acid addition (3.2%) was not effective in iturin A production. It gave lower the pH and slightly higher of the viable cell number than control and the others concentration addition. The pH change in the sample with the addition of 0.8% and 1.6% palmitic acid was almost similar with the control. The range of viable cell number change of the strain was 10^{10} to 10^8 CFU/ml.

Figure 4 below illustrates iturin A productivity in the presence of 0.8% of each of the following amino acids; L-arg, L-asn , L-gln , L-glu, L-gly, L-leu, L-lys, L-trp, L-tyr, and L-val on Polypepton S medium. The addition of these L-amino acids did not improve the iturin A productivity, but changed the proportion of iturin A peaks (Figure 5). Whereas suplementation of L-amino acids changed the proportion of iturin A peaks. HPLC separation pattern of iturin A homologous is denoted as peak 1 to peak 5 according to elution time order of HPLC. Each of them (peaks 1 to 5) corresponds to the following components respectively; *n*-C14-β-amino acid , *anteiso*-C15-β-amino acid, iso-C15-β-amino acid, iso-C16-β-amino acid, and *n*-C16-β-amino acids (Fig. 1). When L-leu was supplemented to the cultivation of RB14-CS the highest proportion of peaks 3 (*iso*-C15-β-amino acid) of iturin A was observed. Hourdou *et al*. (1988) observed that leucine increased the production of *iso*-C15-β-amino acid and *iso*-C15 fatty acid of iturin A. While Besson and Hourdou (1987) observed the influence of leucine on bacillomycin F production, and they found that leucine increased both odd *iso* fatty acid and odd *iso* β amino acids to about 55%. By addition of L-val to the cultivation, the strain produced the highest proportion of peak 4 (*iso*-C16-βamino acid). Hourdou *et al*. (1988) reported that valine was found to increase *iso*-C16 and *n*-C14-β-amino acid of iturin A. Besson and Hourdou (1987) found that valine increased *iso* C-14 and C-16, but did not induce synthesis of *iso*-C14-β-amino acid on bacillomycin production. Akpa *et al*. (2001) also stated that valine is a precursor of even fatty acids, and the use of leucine increased the rate C15-β-amino acid (44%). Theobald *et al.* (2000) observed that in a chemically defined medium, L-val was better nitrogen source than L-glu and L-leu for the simocyclinone D8 production. Whereas Kempf *et al*., (1999) increased gallidermin production to about 25% by *Staphylococcus gallinarum* by the addition of glutamic acid (final concentration 20 gl^{-1}) at fermentation time of 30 hours.

The viable cell number and the pattern of pH change of L-amino acids supplementation was almost similar to the control one (Table 1). The addition of L-asn gave the smallest cell number on day five, after that all of cell number decreased to about 10^8CFU/ml.

Table 1. pH and viable cell number in amino acids experiment shown in figure 4.

Treatment	Viable cell number (x10^9 CFU/ml)			pH		
	Day 2	Day 5	Day 7	Day 2	Day 5	Day 7
Control	13.9	10.1	0.15	7,62	8.75	9.18
L-arg	16.0	3.4	0.15	7,95	9,06	9.06
L-asn	9.1	1.0	0.11	7,71	9.09	9.14
L-gln	11.6	10.4	0.01	7.94	8.92	9.16
L-glu	9.9	1.13	0.10	8.01	8.20	9.23
L-gly	9.1	8.4	0.12	7.76	8.60	9.16
L-leu	14.8	13.2	0.20	7.73	8.02	8.87
L-lys	14.1	14.4	0.20	7.54	8.49	8.85
L-trp	14.4	5.60	0.27	7.43	8.78	9.04
L-tyr	14.9	9.20	0.22	7.47	8.83	9.04
L-val	13.6	12.80	0.01	7.59	7.46	8.76

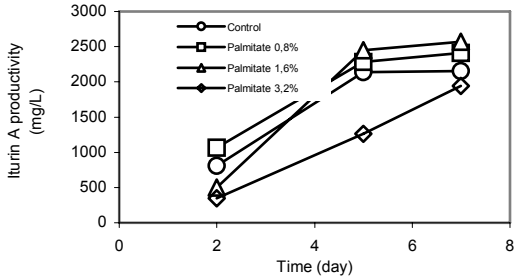


Figure 2. Influence of palmitic acid on iturin A productivity.

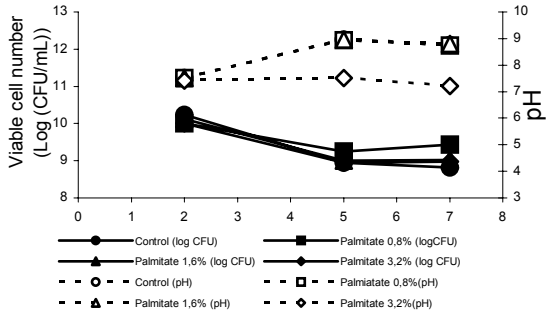


Figure 3. Viable cell number and pH changes in the palmitic acid addition experiment.

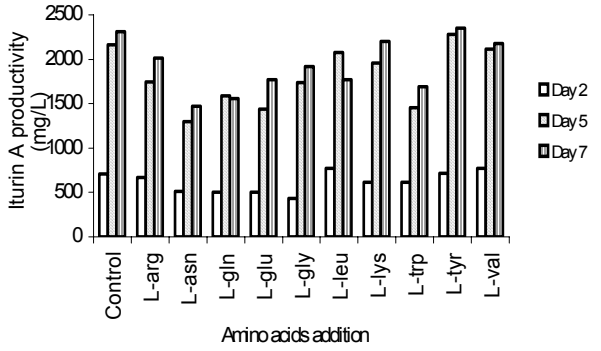


Figure 4. The influence of amino acids on iturin A productivity.

CONCLUSION

This research can be concluded as follows: (i) supplementation of 1.6% palmitic acid to the Polypepton S medium increased the production of iturin A about 18 %, (ii) the addition of 0.8% of each the following amino acids L-arg, L-asn, L-gln, L-glu, L-gly, L-leu, L-lys, L-trp, l-tyr, and L-val could not increase iturin A production, but change the peaks proportion of iturin A, (iii) L-leu addition produced the highest proportion of peak 3, L-val addition gave the highest proportion of peak 4, and L-asn addition resulted the highest proportion of peak 1.

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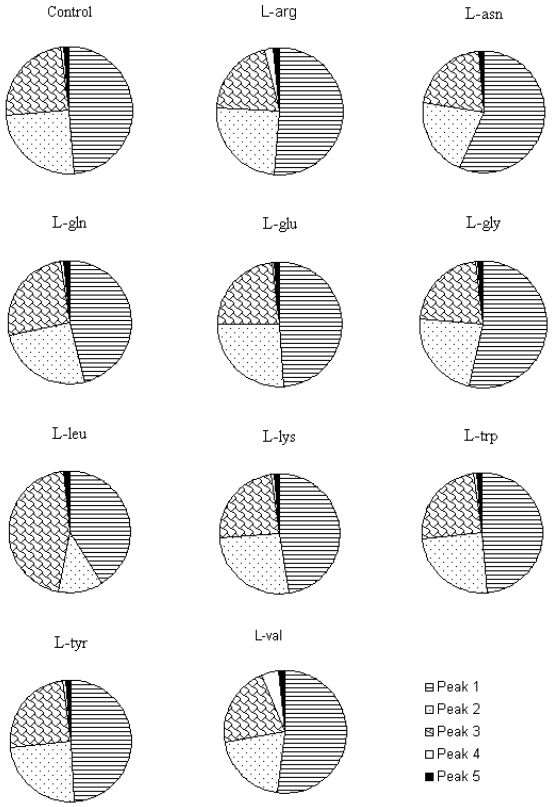

Figure 5. The influence of amino acids addition on the peaks proportion of iturin A.

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