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Full Length Article

Bacilysocin Encoding Gene (*YtpA*) Detection from *Bacillus subtilis* subsp. *subtilis* STTG 14 and Antifungal Compound Analysis using GC-MS and FTIR

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Abstract

Bacillus subtilis subsp. *subtilis* STGG 14 is isolated ricephyllosphere environment which are proved to have antifungal activity toward *Pyricularia oryzae* race 173. The antifungal genes owned by *B. subtilis* subsp. *subtilis* STGG 14 are assumed playing important role in interfering the growth of *P. oryzae.* Therefore, this research aimed to discover the antifungal gene from *B. subtilis* subsp. *subtilis* STGG 14 and analyse the properties of the crude extract. Antifungal genes from *B. subtilis* subsp. *subtilis* STGG 14 were amplified using PCR method with several primers which encode different antifungal compounds. In this study, we successfully amplified *ytpA* gene which encodes lysophospholipase like enzyme (lysophospholipase YtpA) that involved in bacilysocin synthesis. Furthermore, the intracellular crude phospholipid compounds of *B. subtilis* subsp. *subtilis* STGG 14 was obtained from bacterial culture in NG (nutrient growth) medium using butanol and methanol as solvent. The intracellular crude phospholipid compounds were then analysed using GC-MS (Gas Chromatography/Mass Spectrometry) and FTIR (Fourier-Transform Infrared Spectroscopy) to reveal its antifungal properties. Interestingly, methyl tetradecanoate, the fatty acid structure of bacilysocin have been detected using GC-MS. This finding is supported by FTIR analysis which revealed the existence of several functional group such as C=O, C-H_3, P=O and P-O-C. In addition, 15 chemical properties from crude extract are also detected by GC/MS and 5 of them displayed antifungal activities according to several references. According to the results, the crude extract produced by *Bacillus subtilis* subsp. *subtilis* STGG 14 contained phospholipid compound, but further study is needed to prove the obtained phospholipid compounds are bacilysocin as the existence of *ytpA* gene.

Keywords: Antifungal genes; *Bacillus subtilis*; Phyllosphere bacteria; Pyricularia oryzae; *ytpA* gene

Introduction

The sources of antifungal compounds are explored continuously to provide effective ways in controlling plant pathogenic fungi in agricultural field. One of the most promising antifungal compound resources is obtained from bacteria which have been proved by many studies. *Bacillus* species are the biggest bacterial group which are known for their ability to produce various antifungal compounds to inhibit the growth of fungal plant pathogens (Zhang *et al.* 2020a). Different from the other bacterial species, the production of antibiotic in *Bacillus* spp. is highly corelated to sporulation process especially at the initial stage and relied on nutrient availability, enzyme inactivation and bacterial growth phase (Nayak *et al.* 2017). Many secondary metabolites which belong to polyketides, terpenes, siderophores and ribosomally and nonribosomally synthesized peptides are usually produced enzymatically by *Bacillus* species and regulated by large biosynthetic gene cluster (BGC) (Harwood *et al.* 2018). Biosynthetic gene cluster are substantial part of *Bacillus* genome which allied to physiological functions. The level of conserved BGCs among *Bacillus* species correlated with the interactions between Bacillus and other microorganism (Cimermancic *et al.* 2014; Grubbs *et al.* 2017). BGCs also contributed in resistant mechanism of host cell against self-produced antimicrobial compounds (Xia *et al.* 2022).

Various *Bacillus* species which resulted antifungal compounds include *B. subtilis*, *B. brevis*, *B. licheniformis*, *B. circulans* and *B. cereus* (Yilmaz *et al.* 2006). Many studies prove that antifungal compounds from *Bacillus* species are potential to overcome plant pathogenic fungi in many crops. Antifungal lipopeptide compounds from two *Bacillus subtilis* GM5 and GM2 are able to inhibit the growth of *Fusarium solani* (Mardanova *et al.* 2017). Furthermore, the volatile

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organic compounds from rhizospheric *B. subtilis* ZD01 effectively inhibit several fungal pathogens i.e *Alternaria solani*, *Bipolaris sorokinianum*, *Botrytis cinerea*, *A. mali Roberts*, *F. solani*, *F. oxysporum* f.sp. *vasinfectum*, *Verticillium dahliae Kleb* and *F. graminearum* (Zhang *et al.* 2020b). The F2 protein resulted by *B. subtilis* strain Z-14 also capable to impede *Rizhoctonia cereals*, the fungal pathogen in wheat. This F2 protein displayed broad spectrum antifungal activity toward several fungal pathogens such as *F. oxysporum*, *V. dahliae*, *B. papendorfi* and *F. ploriferatum* (Zhang *et al.* 2020a,b).

Among Bacillus species, *B. subtilis* are widely use in agriculture field due to its important role in soil and rhizospheric environments through their capability to produce antimicrobial compounds with broad spectrum activity and survive in extreme environments (Allard-Massicotte *et al.* 2016). To impede the growth of pathogenic microbes, *B. subtilis* secrete various antimicrobial protein (Liu *et al.* 2014) include lipopeptide and phospholipid antifungal compounds like surfactin, iturin, fengycin, zwitermycin (Walia and Cameotra 2015) and bacilysocin (Tamehiro *et al.* 2002).

From our previous study, we successfully isolated *B. subtilis* subsp. *subtilis* STGG 14 from rice phyllosphere environment. *Bacillus subtilis* subsp. *subtilis* STGG 14 are capable to obstruct the growth of *Pyricularia oryzae* as well as decrease the rice blast symptoms of rice plant (Wiraswati *et al.* 2019). This research aims to detect the antifungal gene from *B. subtilis* subsp. s*ubtilis* STGG 14 and analyse the properties of intracellular crude extract using GCMS and FTIR.

Materials and Methods

Bacterial isolate preparation

B. subtilis subsp. *subtilis* STGG 14 was isolated from leave rice phyllosphere (Wiraswati *et al.* 2019) and collected in Microbiology laboratory, Biology Department IPB University. This bacterial isolate was regularly cultured on Luria Bertani Agar medium before used in this research.

Bacterial genome isolation

B. subtilis subsp. *subtilis* STGG 14 was cultivated in LB broth medium for 24 h and 1.5 mL of bacterial culture were transferred to 2 mL of microtube. Afterward, the bacterial genome was isolated using PrestoTM Mini gDNA Bacteria Kit (Geneaid Biotech Ltd) according to the manufacturer's procedures. The obtained bacterial DNA genome purity was quantified using Nanodrop 1000 (Thermo Scientific, Wilmington, DE, USA) and stored at -4°C before used for further analysis.

Antifungal genes amplification

Five different primers (Table 1) were used to amplify antifungal genes from *B. subtilis* subsp. *subtilis* STGG 14. The PCR mixture consist of 25 μL Go Taq Green Master Mix 1X (Promega ®, USA), 0.5 μL (10 pmol μL^{-1}) of each primer, 4 μL DNA genome and 20 µL *nuclease free water*. The PCR reaction of fengycin gene comprised predenaturation (95°C, 5 min) followed by 30 of denaturation (94°C, 1 min), annealing (55°C, 1 min), elongation (72°C 1 min) and post elongation (72°C, 10 min) (Gond *et al.* 2015). Meanwhile, the annealing temperature for *bacilomycin*, *bacilysocin*, *iturin* A and *zwittermycin* A are 50, 51, 58 and 50°C, respectively (Milner *et al.* 1996; Tamehiro *et al.* 2002; Tsuge *et al.* 2005; Mora *et al.* 2011). Each amplicon was then run on 1% agarose at 85 volt for 45 min and visualized under UV light to discover the obtained gene. The amplified antifungal gene was sequenced and compared to the database of NCBI using BLAST X program. Phylogenetic tree was constructed using MEGA 7 program.

Crude phospholipid compounds extraction

The extraction of intracellular crude phospholipid compounds was conducted according to Tamehiro *et al.* (2002) with modification in bacterial cell disruption and evaporation. The bacterial isolate (*B. subtilis* subsp. *subtilis* STGG 14) was cultured on 10 mL of NG medium for 72 h in shaker incubation (110 rpm) and used as inoculum for antifungal compounds production. Furthermore, 4 mL of inoculum were inoculated to 400 mL of NG medium and incubated at 110 rpm of shaker incubation for 72 h. The bacterial cells were harvested by centrifugation at 10,000 rpm for 10 min and intracellular antifungal phospholipid compounds were further extracted by shaking the mixture of pellet cells, 4 mL butanol and glass bead for 30 min. The mentioned step was repeated for 3 times and the collected butanol extracts were evaporated at 40°C for 24 h. Evaporated crude extract was dissolved 3 times using 4 mL of ethyl acetate and the liquid layer was then transferred to Erlenmeyer flask aseptically. pH of liquid layer was adjusted to 2 by adding HCl and re-extracted using ethyl acetate for 3 times. For final step, the ethyl acetate extract was evaporated at room temperature until all solvent was vaporized.

GC-MS analysis of crude phospholipid compounds

The extracted crude phospholipid compounds from previous step were analysed using Agilent/HP 6890/5973 GC-MS system (Agilent Technologies, California). The crude antifungal compounds were firstly dissolved on butanol and manually injected into GC 6890 machine. The GC machine use agilent 19091S-433 capillary column with 5% phenyl methyl siloxane as stationary phase and helium gas as mobile phase. The capillary column was 325°C initial temperature, 9.35 psi of pressure, 1 mL/min of constant stream with 37 cm/min of flow speed. The detector was set at 250°C of temperature, 40 mL/min of hydrogen flow and 450 mL/min of air flow. Resulted molecules from GC machine will be detected using mass spectrometer (MS).

FTIR analysis of crude phospholipid compounds

The crude extract content was also detected by Fourier Transform Infrared (FTIR) spectrometer OPUS (version 5.0) windows software (Bruker, Germany). The antifungal crude extract which are dissolved using butanol was dropped between NaCl plats and placed in the FTIR machine in order to be analysed. Previously, FTIR machine had been set to detect infrared spectrum range 400 – 4000 cm^{-1} using 100 µm of CaF_2 cell transmission. The detected infrared spectrum was then visualized in graph shape between absorbance length and density of each infrared peak. Each absorbance value described the functional groups of sample content.

Results

Antifungal genes from *B. subtilis* subsp. *subtilis* STGG 14

From antifungal genes amplification, we obtained *YtpA* gene (812 bp) from isolate STGG 14 which encoded lysophospholipase like enzyme that involved in bacilysocin production. Meanwhile, the other antifungal gene *i.e*. iturin A, fengycin, bacilomycin and zwitermycin are unsuccessfully amplified from the isolate STGG 14 genome (Table 2). Bacilysocin is antifungal gene from *B. subtilis* with antifungal activity which belong to phospholipid compound group.

Phylogenetic tree of antifungal gene from *B. subtilis* subsp. *subtilis* STGG 14

The phylogenetic tree of amplified antifungal gene from isolate STGG14 were constructed by comparing with the phospholipase enzyme from database. According to the phylogenetic tree analysis, the *ytpA* gene from isolate STGG 14 showed highest similarity with lysophospholipase enzyme from *B. subtilis* strain 168. Furthermore, the nucleotide and protein sequences of *ytpA* from isolate STGG 14 are similar to *alpha/beta hydrolase* and *phospholipase YtpA* genes (Fig. 1).

The properties of crude phospholipid compounds

GC-MS analysis of crude antifungal phospholipid compounds from isolate STGG 14 resulted 16 dominant peaks which identified as various chemical compounds (Fig. 2). The identified compounds have various biological activity according to several references. Among the identified compounds, 5 compounds were displayed antifungal activity toward various plant pathogenic fungi (Table 3). In addition, fatty acid compounds, *methyl tetradecanoat* which composed bacilysocin was detected at 8.953 of retention time with 1.36% of abundance (Fig. 2).

The properties of crude phospholipid compounds

The infrared wavelength spectrum of FTIR results indicates certain functional group compounds which contained in crude antifungal phospholipid extract. Spectrum 3340 cm^{-1} and 1072 cm^{-1} indicated OH and CO functional group from butanol solvent and crude extract of bacilysocin which composed of phospholipid compounds. Carbonyl functional group (C=O) from methyl tetradecanoat were detected at 1657 cm^{-1} spectrum, while alkyl carbon functional group (CH) were detected at 2872 cm^{-1}, 2933 cm^{-1} and 2960 cm^{-1} spectrums. In addition, CH3 and CH2 functional groups were detected at 1433 cm^{-1} and 1463 cm^{-1}. The phospholipid compounds contained in intracellular crude extract were indicated by P=O and P-O-C functional groups which are detected at 1251 cm^{-1} and 1113 cm^{-1} (Fig. 3).

Table 1: Specific primers to amplify antifungal genes from *B. subtilis* subsp. *subtilis* STGG 14

Antifungal compounds	Primers	Amplicon size (bp)	References
Iturin A	ITU-P f ITU-P r	2000	Tsuge *et al.* (2005)
Fengycin	FenD1f FenD1r	964	Gond *et al.* (2015)
Bacillomycin	BACF BACR	370	Mora *et al.* (2011)
Zwittermycin A	Swt 678F Swt 667R	951	Milner *et al.* (1996)
Bacilysocin	ytpA F1 ytpA R2	812	Tamehiro *et al.* (2002)

Table 2: Amplified antifungal gene from *B. subtilis* subsp. *subtilis* STGG 14

Antifungal compounds	Primers	Amplification results
Iturin A	ITU-P f ITU-P r	negative
Fengycin	FenD1f FenD1r	negative
Bacillomycin	BACF BACR	negative
Zwittermycin A	Swt 678F Swt 667R	negative
Bacilysocin	ytpA F1 ytpA R2	positive

Discussion

From the previous study, *Bacillus subtilis* subsp. *subtilis* STGG 14 was proved having antifungal activities against *P. oryzae* race 173 in vitro and in vivo experiments. This isolates also capable to produce extracellular antifungal compounds from coumarins group (Wiraswati *et al.* 2020). Moreover, *B. subtilis* species are also able to produce several antifungal compounds belong to lipopeptide as well as phospholipid compounds like surfactin, iturin, fengycin, zwittermycin (Walia and Caemotra 2015) and bacilysocin (Tamehiro *et al.* 2002). In this study, several antifungal genes detection were conducted as preliminary step to reveal antifungal compounds involved in decreasing the blast diseases which have been conducted in the previous research.

Among 5 antifungal genes, *YtpA* gene which encoded

Table 3: Chemical composition of crude intracellular compounds from isolate STGG 14 and their biological activity (Similarity Index >90%)

No	Chemical composition	Molecular structure	Retention time	Peak area (%)	Similarity (%)	Bioactivity (references)
1	Hexadecane	C16H34	8.215	9.46	98	antimicrobial (Yogeswari *et al.* 2012)
2	Methyl tetradecanoate	C15H30O2	8.953	1.69	94	Antibacterial
3	1-Octadecen	C18H36	9.285	3.59	99	N/A
4	Hexadecanoic acid, methyl ester	C17H34O2	9.995	1.51	97	antibacterial (Shaaban *et al.* 2021)
5	7,9-di-tert-butyl-1-oxaspiro eca-6,9-diene-2,8-dione	C17H24O3	10.103	3.31	95	antioxidant
6	Methyl-3-(3,5-Ditertbutyl-4-Hydroxy phenyl) Propionate	C18H28O3	10.178	2.03	92	antioxidant (Li *et al.* 2014)
7	1,2-Benzenedicarboxylic acid	C8H6O4	10.292	39.89	95	antibacterial, antifungal (Shoge *et al.* 2016)

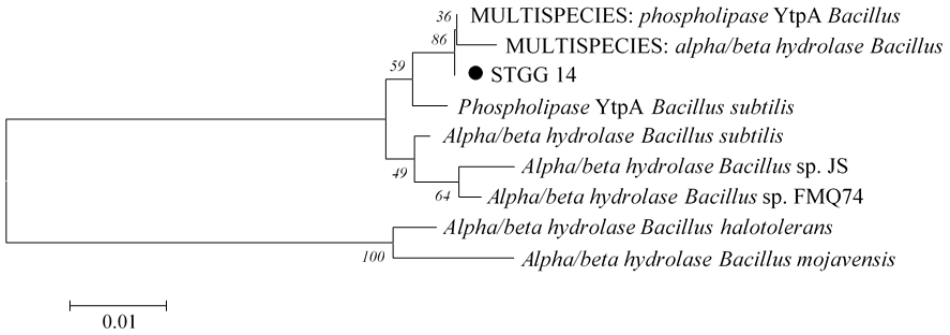

Fig. 1: The phylogenetic tree showing the similarity of *YtpA* gene from isolate STGG 14 and phospholipase enzymes from database. The homology percentage is displayed by bootstarp testing (1,000 replicates)

homologues protein of lysophospholipase (LPL) enzyme (lysophospholipase YtpA) is successfully amplified from isolate STGG 14. LPL enzymes have a role in monoacyllysophospholipd and diacyllysophospholid hydrolysis which is difficult enough to distinguish between LPL and phospholipase B enzymes. The activity of LPL enzyme is similar to phospholipase A and B which hydrolyse acyl ester bond. The LPL enzyme is also expected having a role in the final step of bacylisocin synthesis by hydrolysing phosphatidylglycerol into bacylisocin or lysophosphatidylglycerol (Tamehiro *et al.* 2002).

Bacylisocin is intracellular phospholipid antibiotic which are produced shortly before endospores forming by *B. subtilis* 168. The structure of bacylisocin, *1-(12-methyltetradecanoil)-3-phosphoglyceroglycerol* are resulted from hydrolysis of acyl ester on phosphatidylglycerol by lysophospholipase enzyme (YtpA). Naturally, the phosphatidylglycerol composed phospholipid component of *B. subtilis* up to 75% from total phospholipid (Tamehiro *et al.* 2002). The existence of *YtpA* gene in *B. subtilis* species also plays a role in endospores forming. Tamehiro *et al*. (2002) engineered *B. subtilis* 168 by deleting the *YtpA* gene which resulted in reduction of endospores forming activity.

The specific investigation about bacylisocin is still limited. Another study by Kazim and Alden (2014) discovered that phospholipid compounds have antifungal activity against *Candida albicans* and *Aspergillus niger*. Phospholipid compounds can inhibit the fungal growth through lipid membrane devastation in fungal cell wall. This antifungal mechanism permits the damage of membrane stability and pores formation in fungal cell wall.

To prove the expression of *YtpA* gene in *B. subtilis* STGG 14, we extracted intracellular crude phospholipid compounds from STGG 14 using butanol as solvent. The crude phospholipid compounds were further analysed using GC-MS and FTIR methods to investigate the composition compounds of the extract. From GC-MS analysis, we successfully detected *methyl tetradecanoat*, a fatty acid compounds which compose bacilysocin. Methyl tetradecanoat is methyl ester fatty acid with long fatty acid chain which have been esterified with methyl. Fully phospholipid compounds cannot be detected using GC-MS because this phospholipid compound was degraded by high temperature along GC-MS instrument before the GC-MS pressure is high enough to push the sample to pass GC column. Therefore, this study also analysed crude phospholipid compounds using FTIR spectroscopy instrument.

FTIR analysis is usually used to detect functional group from sample according to absorption of infrared frequency. Each functional group have certain vibration frequency and will absorb identical frequency of infrared. This wave frequency will be detected by spectrophotometer each detected frequency represent different functional group. The existence of butanol as solvent was indicated by hydroxyl (OH) and carboxyl (CO) which strongly detected at 3340 cm^{-1} and 1072 cm^{-1} of spectrum. The spectrum of hydroxyl and carboxyl functional group indicates the presence of alcohol in certain sample. Meanwhile, the presence of phospholipid compounds in crude phospholipid extract was indicated by carbonyl (C=O) and methyl (CH_3) which detected from fatty acid compounds as well as P=O

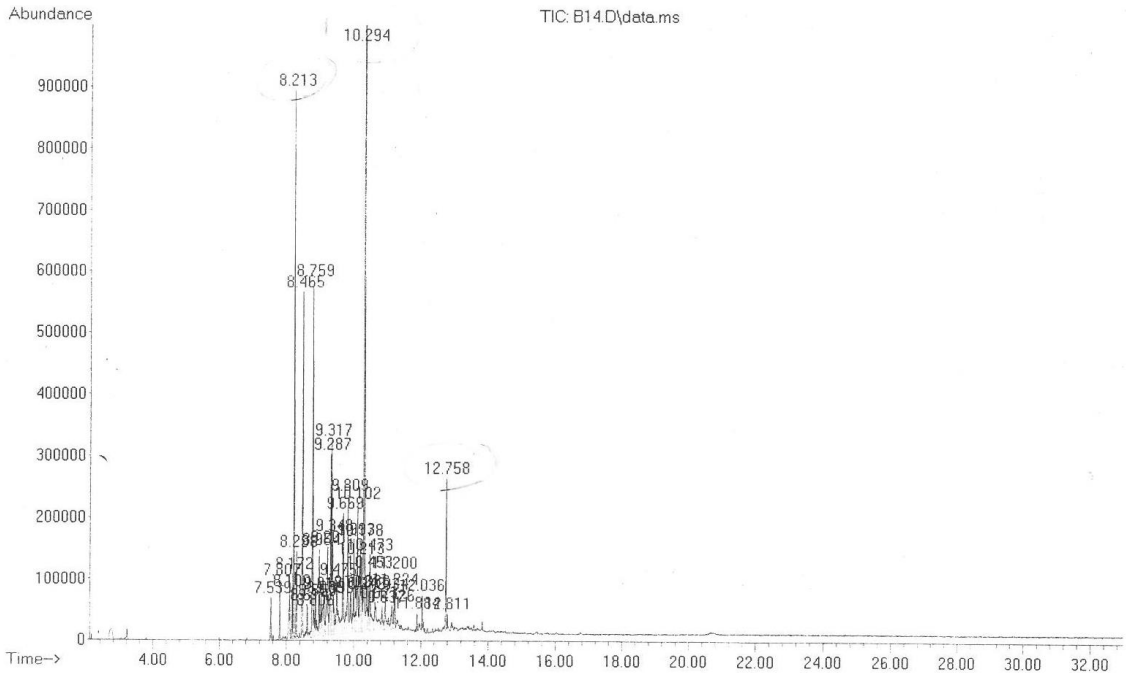

Fig. 2: Mass spectrum of crude antifungal compounds from isolate STGG 14 analysed using GC-MS

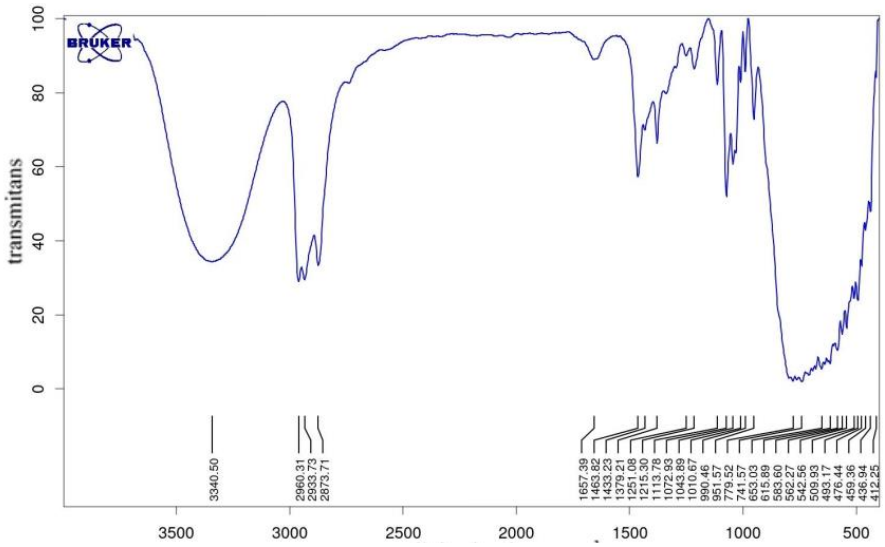

Fig. 3: FTIR spectrum transmission of crude phospholipid compounds from *B. subtilis* subsp. *subtilis* STGG 14

and P-O-C functional group. The C=O, CH3, P=O and P-O-C functional group were detected at spectrum 1657 cm^{-1}, 1433 cm^{-1}, 1251 cm^{-1} and 1113 cm^{-1} respectively. P=O was derived from phosphate group of phospholipid compounds, while P-O-C was derived from phosphate and fatty acid which relate to glycerol. This result showed that the bacterial crude extract contained phospholipid compound which predicted to have antimicrobial activity. According to the FTIR result, the similarity of detected phospholipid compounds and bacilysocin are unconfirmed due to the limited of control bacilysocin analysis result from the previous study.

In addition, the similarity of lysophospholipase YtpA with phospholipase A and B affected toward the specificity of enzyme activity. The activity of phospholipase and lysophospholipase toward acyl bonds 1 and 2 are triggered by glycerophospholipid as substrate (Tamehiro *et al.* 2002). According to the polarity of molecule which bond to glycerol, glycerophospholipid are divided into phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI) and phosphatidylserine (PS). The mentioned derivates compounds of

glycerophospholipid can be recognised by lysophospholipase YtpA, hence this enzyme hydrolysed both phosphatidylglycerol, a bacylisocin precursor and other glycerophospholipid derivatives. The lysophospholipase YtpA is capable to hydrolyse phosphatidylcholine into lysophosphatidylcholine when phosphatidylcholine is only available as substrate (Tamehiro *et al*. 2002). According to this phenomenon, *YtpA* gene from isolate STGG 14 are possible to produce but its activity is dependent to available substrate in the environment. The similarity structure of phospholipid compounds from isolate STGG 14 and bacilysocin should be further studied due to LPL YtpA enzyme from isolate STGG 14 not only hydrolyse phosphatidylglycerol.

Conclusion

The *YtpA* gene which encode lysophospholipase like enzyme was successfully amplified from *B. subtilis* STGG 14. This enzyme is predicted to have important role in phospholipid compounds synthesis by isolate STGG 14 which have antifungal activity toward *P. oryzae* race 173. Moreover, functional groups of phospholipid compounds and methyl tetradecanoate which composed bacilysocin were detected from intracellular crude extract of *B. subtilis* STGG 14 according to GCMS and FTIR analysis. Further study is needed to confirm that phospholipid compounds from isolate STGG 14 have similar structure and activity with bacilysocin compound.

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Author Contributions

SMW and ATW planned the research and wrote up the manuscript, AAN and IR interpreted the data, SMW conducted and analysed the results.

Conflict of Interest

All authors declared no conflict of interest

Data Availability

Data presented in this study will be available on a fair request to the corresponding author.

Ethics Approvals

This study did not involved animals or human participants as well as conducted on any protected areas

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