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NAME: Mr. Chaisit Preecha

THIS THESIS HAS BEEN ACCEPTED BY

THESIS ADVISOR

(Associate Professor Sutruedee Prathuangwong, Ph.D.)

COMMITTEE MEMBER

(Associate Professor Niphon Visarathanonth, M.S.)

COMMITTEE MEMBER

(Associate Professor Preparat Hormchan, Ph.D.)

PROGRAM CHAIRMAN

(Associate Professor Somnuk Wongtong, Ph.D.)

APPROVED BY THE GRADUATE SCHOOL ON _____

DEAN

(Associate Professor Gunjana Theeragool, D.Agr.)

THESIS

**FORMULATION DEVELOPMENT AND PARTIAL
CHARACTERIZATION OF MECHANISM CONFERRED BY
Bacillus amyloliquefaciens KPS46 AGAINST SOYBEAN DISEASE**

CHASIT PREECHA

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Protocols of *Bacillus amyloliquefaciens* KPS46 mass production in liquid fermentation of soybean meal and molasses or fish meal and molasses that cross linked matrix with several inexpensive organic carriers including dry cow dung, talcum, decomposed cow dung, or rice husk ash dust were developed. KPS46 survived in one selected formulation, a talcum-based product at $8.4 \log_{10}$ CFU/g for 360-day storage at room temperature ($28 \pm 4^{\circ}\text{C}$) that declined to approximately 31.5% of original cell population. Greenhouse and field experiments with KPS46 wettable powder-based talcum consistently enhanced biocontrol efficacy against important soybean diseases of seedling blight (caused by *Sclerotium rolfsii*), anthracnose (*Colletotrichum truncatum*), and bacterial pustule (*Xanthomonas axonopodis* pv. *glycines*) that were comparable to its fresh cells prepared. The developed formulation also increased marketable soybean yield in two-crop season field trials with 24.1 and 29.5% respectively compared to nontreated control. The formulation can develop a role of scale-up KPS46 production and stabilization of final effective biomass. The UV mutagenesis generated *srfAA* mutant strain M6 of KPS46 was unable to produce lipopeptide surfactin and cellulase. The M6 mutant also produced relatively low levels of extracellular enzymes, endoglucanase and protease compared to KPS46 wildtype. When soybean plant assays were employed under greenhouse conditions with these *srfAA* mutant and wildtype against *X. axonopodis* pv. *glycines*, strain M6 mutant significantly exhibited less effects on disease reduction compared with the parent wildtype. This result suggests that KPS46 reduced bacterial pustule severity on soybean is associated with its lipopeptide surfactin production that *srfAA* also effects the phenotypes of down regulated extracellular-enzyme production.

Student's signature

Thesis Advisor's signature

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Chaisit Preecha
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**FORMULATION DEVELOPMENT AND PARTIAL
CHARACTERIZATION OF MECHANISM CONFERRED BY
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INTRODUCTION

Soybean production in Thailand has grown steadily since 2002. The domestic consumption of soybean continues growing with the strong demand for animal feed, oil and food production. In 2002, Thailand imported 1,528,557 tons of soybeans (Norland, 2003). In 2004, the consumption demand grew up slightly to 1,868,721 tons. However domestic productivity of soybean was estimated only 270,890 tons, hence, 1,467,406 tons had to be imported. One important problem of low productivity is plant diseases such as anthracnose, rust, fungal leaf spot, downy mildew and bacterial pustule. Anthracnose, fungal leaf spots and bacterial pustule disease generally cause yield loss in susceptible cultivars of 11.32, 3.71 and 15-42 % respectively (Wrather *et al.*, 1997). Resistant cultivars and effective control measures are likely inadequate. In 2002, Kasem identified *Bacillus sp.*, strain KPS44 and KPS46 and *Lactobacillus sp.* strain SW01/4 as new effective bacterial antagonists against anthracnose and bacterial pustule. The control effect of the antagonist reduced disease severity of bacterial pustule by approximately 7.37-51.3% which raised productivity by 5.6-22.4 % (Prathuangwong and Kasem, 2003). Antagonists KPS46 and SW01/4 were later identified as *Bacillus amyloliquefaciens* and *Paenibacillus sp.* respectively (Prathuangwong *et al.*, 2005c).

Green soybean becomes the new importance economic crop imported to Japan and China of 9,233 and 8,950 tons in 2002/2003 and 2004 respectively (Pookpakdi, 2004). Green soybean needs growing system like vegetable cropping under intensive farming system most nutrition, irrigation and pest management. The more increasing of production seems the causal of raising pesticide application increasing product cost, environment and human health problem.

Bacillus amyloliquefaciens strain KPS46 originally isolated from a soybean plant at the Agricultural research station of central Thailand, has been reported to secrete antimicrobial metabolites (Prathuangwong and Kasem, 2004; Prathuangwong *et al.*, 2005b) and to induce systemic resistance to protect soybean and other crop plants against multiple plant pathogens. (Prathuangwong and Kasem, 2004; Prathuangwong *et al.*, 2004; Prathuangwong *et al.*, 2005a). The previous reports confirmed that *B. amyloliquefaciens* strain KPS46 was plant growth promoting rhizobia rhizobacteria/bacteria(PGPR/PGPB) and could promote plant growth. (Buensanteai *et al.*, 2007; Prathuangwong and Buensanteai, 2006; Buensanteai *et al.*, 2008). On soybean plant, this bacterium increased root, shoot length, and biomass by more than 20-40% compared with the non-treated control (Buensanteai, 2008). The bioassay data showed that the seedling growth phenotype increased by KPS46 was due to significant upregulation of elicited plant hormone, indole-3-acetic acid (IAA). *Bacillus amyloliquefaciens*, one of several strains belonging to the genus *Bacillus* in the same of subtilis group as the closely related *Bacillus subtilis* showed the effective biocontrol of multiple plant diseases and was mostly exploited as biopesticides phytohormone and lipopeptide to control plant diseases and enhance plant growth by inducing resistance and secretion of antimicrobial metabolites (Kloepper *et al.*, 2004; Araujo *et al.*, 2005).

Biological control of plant diseases has increased rapidly over the past 20 years. The market share report of biopesticides in 1993 was less than 1 million, whereas total sale of fungicides was \$ 5.5 billion. In response to environment and health concerns about extended use of fungicides and/or pesticides, these alternative control approaches are important for use in plant disease management. In 1998, it was reported that over half of commercial biological control product of total application in the greenhouse was around 300,700 ha, whereas total land outside was 1.5 billion ha (Paulitz and Belanger, 2001). Biological control is an important area in the discipline of plant pathology. Recently, every major university with a department of plant pathology has one or more members conducting basic and /or applied researches on organism biological control. Many biological control agents have been identified as potential antagonists, but only a few have been formulated for

commercial use. Stable formulations that are supported by some substrates have not been fully exploited. Additional formulations that have the advantages to withstand environmental stress and increase the survival rate of biocontrol bacteria are needed. To provide farmers more opportunity to use disease control effective formulation products are needed to be developed and introduced.

OBJECTIVES

To develop the effectively antagonistic formulation which provide more opportunities for disease control and motivation for farmers to use biological control agents emphasizing the following:

1. To develop the formulation of efficient *B. amyloliquefaciens* KPS46 which can survive at least 90 days?
2. To determine the mechanism in *B. amyloliquefaciens* KPS46 that influences the control efficacy.

LITERATURE REVIEW

Disease of soybean: bacterial pustule

The most prevalent bacterial disease on soybean in Thailand is bacterial pustule whose damage to plants is ranked number 4 after rust, anthracnose and downy mildew. Working on this on bacterial pustule disease will be closely related to a previous study that examined the antagonist KPS46 which has been firstly screened for suppressing the bacterial pustule pathogen (Kasem, 2002).

Bacterial pustule of soybean has been reported in most growing areas of the world, including Canada, North America, South America, Central America, Europe, Africa, Australia, and Asia. In Thailand, bacterial pustule was reported in all of soybean growing areas (Preecha, 1988). Symptoms in the initial stage of the disease are very small and include, pale, green spots or specks on both sides of leaf surfaces. The raised, light-colored pustules usually form in the center of the spots under the leaf and yellow hallows surrounding the spot form, which later pustules turn to brown. Spots vary from specks to large or irregular areas. Severe infected leaves early defoliate. Bacterial symptoms that include spots without pustules have also been reported (Sinclair, 1982).

Xanthomonas axonopodis pv. *glycines* (syn. *X. campestris* pv. *glycines* (Nakano), *X. campestrisc.* pv. *phaseoli* (Smith) and *X. phasioli*) is the causative agent of bacterial pustule disease. It is a monotrichous, Gram-negative rod, 0.5-0.9x1.4-2.3 micron in size. Colonies on beef-fusion agar and Wakimoto's agar are pale yellow, small, circular with smooth surfaces and entire margins. The bacterium can digest starch and gelatin, producing acid but not gas. The optimum temperature for growing is 30-33° C, but the bacterium can grow at 10 – 38° C.

X. axonopodis pv. *glycines* can survive in seeds (seedborn) for 30 months, on soybean debris in soil for 3 months, and on some Fabaceae (Leguminosae) hosts such

as *Phaseolus vulgaris*, *Pachyrhizus erosus*, *Glycine javanica* and *Arachis hypogaea* (Prathuangwong, 1979). It prefers the high humidity and temperature in the rainy season. The bacterium can be spreaded by rain or splashing, by wind, and is transmitted by bacterial infected seeds.

Resistance varieties and seed treatment with antibiotics (Aurimycin, Dumocycline and Agrimycin 100) can control bacterial pustule disease (Prathuangwong and Choochoa, 1990). Some chemicals, including copper oxychloride and streptomycin are applied by spraying against bacterial pustule, but long term antibiotic application can induce chemical resistance in pathogen. In 1993, Suwanto *et al.* screened *Pseudomonas fluorescens* to control bacterial pustule. Prathuangwong and Janekitiwong (1992) tested bacteriophage to control this disease. These new bacterial antagonists, *Bacillus* sp., KPS44 and KPS46 and *Lactobacillus* sp. SW10 strains isolated from soybean leaves and rhizospheres appeared to be a very effective control for *X. axonopodis* *pv. glycines*. (Kasem, 2002).

Biological control

Recently, sustainable agriculture and organic farming systems using biological control, including the use of microorganisms has become important. The mechanisms by which antagonistic microorganisms protect plant are generally attributed to parasitism, competition, and the production of secondary metabolites with toxic effects on pathogens. Many fungi and bacteria, including *Trichoderma* sp., *Chaetomium* sp., *Coniothyrium minitans*, *Gliocladium* sp., *Streptomyces* sp. *Pseudomonas* sp. and *Bacillus* .sp. have been screened and developed to control the soilborn pathogens, *Pythium* sp., *Phytophthora* sp., *Fusarium* sp., *Rhizoctonia* sp. and *Sclerotium* sp. Some antagonistic microorganisms, such as *Trichoderma* sp. *Ampilomyces quisqualis*, *Bacillus* sp., and *Ulocladium* sp. can also be developed for foliar disease control of powdery mildew and *Botrytis* rot. *Pseudomonas flocculosa* was recently developed for the control of powdery mildew (Paulitz and Belanger, 2001). The *B. amyloliquefaciens* KPS46 has been developed to control soilborn and foliar diseases, anthracnose and bacterial pustule of soybean (Prathuangwong *et al*,

2002; Prathuangwong and Kasem, 2003). It is now known that some bacteria, called plant growth-promoting rhizobacteria, can promote the growth of wide variety of crop plants. Also *Pseudomonas* sp. and *Bacillus* sp. strains show promise as biological control agents by suppressing infection by plant pathogen. The PGPR microorganism also can produce bacteriocin, that inhibits closely related bacterial species and induce systemic resistance (ISR) in plants. ISR mechanism of PGPR can inhibit pathogen infection or induce special physical barrier structures and biochemical barriers that induce defensive plant mechanism and systemic resistance against severe strains (Khan *et al.*, 2006; Kloepper *et al.*, 1989).

There have been several on the efficiency PGPR. For example *P. fluorescent* 2-79, from Crop Bioprotection Research, in liquid culture applied as seed treatment, is reported to control take-all disease caused by *Gaeumanomyces graminis* var *tritici*. Seed treatment with single and mixed strains of the PGPR *Bacillus pumilus* INR7, *Bacillus subtilis* GB03, and *Curtobacterium flaccumfaciens* MEI suppressed the cucumber pathogens *Colletotrichum orbiculare* causing antracnose, *P. syringae* pv. *lachyman* causing angular leaf spot, and *E. tracheiphilla* causing wilt both in the greenhouse and field (Georg and Kloepper 1998). The pioneering biological control products BioSave (*P. syringae* ESC-11) and Aspire (*Candida oleophila*) were registered by Environmental Protection Agency (EPA) in 1995 for control of post harvest rot of pome and citrus fruit, and is commercial available. (Janisiewicz and Korsten, 2002). The efficacy of two postharvest biocontrol products under commercial development was determined in semi-commercial tests on table grapes and sweet cherries. These products consist of the yeast *C. saitoana* combined with either chitosan (Bio-Coat) or an antifungal lytic enzyme (Biocure). On table grape (cv. 'Italia'), the field application of the two biocontrol products 21 days and 1 day before harvest significantly reduced the incidence of grey mould rots from 33 to 46%. (Schena *et al.*, 2005)

Antagonistic *Bacillus*

Robert Koch and Ferdinand Cohn recognized *Bacillus* sp. in 1872. When *Bacillus* is young, the cell wall stain shows Gram positive but changes to Gram negative in the stationary phase. Cells are 0.5-2.5x1.2-10 micron, straight, rod-shaped with a rounded or squared end. Cells are arranged in pairs or chains. *Bacillus* can move by peritrichous flagella. Endospore is oval or sometimes round or cylindrical, and is resistant to diverse extreme conditions of heat, draught, pH, and saline. The majority are mesophiles with the optimum temperature between 30-48°C, but some are thermophiles with the optimum temperature as high as 65°C. *B.cereus* can be induced to maximal acid tolerance at pH 5 (Jobin *et al*, 2002; Sonenshein *et al.*, 1993).

Most *Bacillus* species are versatile chemoheterotrophs or chemoorganotrophs using fermentation or respiration and a variety of simple organic compounds, sugars, amino acids and organic acids. Some *Bacillus* sp. causing agents of soft rot is capable of digesting pectin in plant cell wall or producing polysaccharides from sucrose. *Bacillus* use oxygen in respiration but some can grow as mild or strict anaerobes (Claus and Berkeley, 1986).

Bacillus species are easily isolated and grown in laboratory. The simple technique that enriches aerobic spore forming *Bacillus* is to pasteurize a diluted soil sample at 80°C for 15 minutes, then plate onto nutrient agar and incubate at 37°C for 24 hours up to several days. A 24-hour-colony is examined as catalase-positive for a typical *Bacillus* (O'Donnell *et al.*, 1980; Sonenshein *et al.*, 1993).

Bacillus sp. has been shown to produce a wide variety of antibacterial and broad spectrum antifungal compounds and other secondary metabolites. *Bacillus brevis* produces gramicidin and tyrocidin, *B. ceareus* produces cerexin and zwittermicin, *B. circulans* produces circulin, *B. licheniformis* produces bacitracin and *B. subtilis* produces difficidin, subtilin, and mycobacilin). The bacillus antibiotics,

bacitacin, pumilin, laterosporin, gramicidin, and tyrocidin are effective against Gram-positive bacteria which colistin and polymycin are anti Gram-negative while mycotbacilin and zwittermicin are anti fungal. Some *Bacillus* species, such as *B. larvae*, *B. lentimorbis*, *B. popillae* and *B. thurigiensis* are insect pathogens. *B. thurigiensis* forms paraporal crystal that is toxic to *Lipidoptera* (Parry *et al.*, 1983; Sonenshein *et al.*, 1993).

B. subtilis is a biological control agent used against fungal and bacterial plant diseases. The study by Ferreira *et al.* (1967) on the antibiotic produced by *B. subtilis* showed an inhibition effect on mycelium growth and ascospore germination of fungus, *Eutypa lata* that causes dieback in grape. Phae *et al.* (1992) reported on antagonistic effect of *B. subtilis* to control the fungus, *Pyricularia oryzae*, the cause of rice blast, *Cochliobolus miyabeanus*, the cause of rice brown spot and *Rhizoctonia solani*, and the cause of sheath blight of rice. Mektana (1993) detected a *B. subtilis* strain which could control *Phytophthora palmivora*, *X. campestris* pv. *citri*, *Fusarium roseum*, *Pythium* sp., *Sclerotium rolfsi*, and *Colletotricum truncantum*.

Bacillus normally is a versatile chemoorganotrophe, especially the *subtilis* group, including *B. megaterium*, *B. subtilis* and *B. amyloliquefaciens*. These bacteria can utilize nutrients from fermentation of complex molecules of organic matter, such as proteins and the disaccharide sucrose (Claus and Berkeley, 1986; Parry *et al.*, 1983; Sonenshein *et al.*, 1993). Soybean meal from an oil extraction process and fish meal, are mostly sold for animal feed as they are high in protein content. Both of them are rich in protein and amino acid. Amino acid contained in soybean meal includes Arginine 3.8 %, Lysine 3.4 %, Methionine 0.7 %, Cystine 0.8 %, Tryptophan 0.7 %, Histidine 1.4 %, Leucine 4.3 %, Isoleucine 2.8 %, Phenylalanine 2.2 %, Threonine 1.7 %, and Valine 2.8 %, and in those fish meal includes Arginine 4.5 %, Cystine 0.9 %, Histidine 1.6 %, Isoleucine 5.7 %, Lysine 6.2 %, Methionine 2.3 %, Leucine 4.3 %, Phenylalanine 3.1 %, Threonine 3.2 %, Tryptophan 0.9 %, and Valine 4.1 %.

Molasses is one sugar rich carbon source that contains not less than 46 percent sugar (Kellems, 2002).

Bacillus amyloliquefaciens was discovered in soil in 1943 by Fukumoto, a Japanese scientist, who gave the bacterium its name because it produced (*faciens*) a liquifying (*lique*) amylase (*amylo*) (Fukumoto, 1943). *B. amyloliquefaciens* has the ability to stimulate plant growth and to suppress plant pathogenic organisms. In 2002, Kasem reported that *B. amyloliquefaciens* KPS46 was an effective control agent against bacterial pustule disease that was the same as or greater than that observed by treatment with copper oxychloride and streptomycin. The rhizosphere competence and bio-control function of bacilli are partly caused by non-ribosomally produced cyclic lipopeptides acting against phytopathogenic viruses, bacteria, fungi, and nematodes. These lipopeptides are synthesized at modular multienzymatic templates (Stein *et al.*, 1996) and consist of a β -amino or β -hydroxy fatty acid component integrated into a peptide moiety. Some of these lipopeptides have been studied in greater detail, including surfactin, fengycins, and several iturins. Surfactin is a heptapeptide, via a lactone bond to a β -hydroxy fatty acid with 13 to 15 carbon atoms. Surfactin exerts its antimicrobial and antiviral effects by altering membrane integrity (Peypoux *et al.*, 1999). Fengycin, and the closely related plipastatin, are cyclic lipodecapeptides containing a β -hydroxy fatty acid with a side chain length of 16 to 19 carbon atoms. Four D-amino acids and ornithine (a non-proteinogenic residue) have been identified in the peptide portion of fengycin. It is specifically active against filamentous fungi and inhibits phospholipase A₂ (Nishikori *et al.*, 1986). Members of the iturin family, such as mycosubtilin, bacillomycin D, and iturin A, contain one β -amino fatty acid and seven amino acids. The peptide moiety of the iturin lipopeptides contains a tyrosine, in the D-configuration at the second amino acid position and two additional D-amino acids at positions 3 and 6. The members of the iturin family exhibit strong antifungal and hemolytic activities and limited antibacterial activity (Maget-Dana Peypoux., 1994).

Koumouts *et al.* (2004) sequenced the sampled genome of *B. amyloliquefaciens* FZB42 and identified 2,947 genes with >50% identity on the amino acid level to the corresponding genes of *B. subtilis* 168. Six large gene clusters encoding nonribosomal peptide synthetases (NRPS) and polyketide synthases (PKS) occupied 7.5% of the whole genome. Two of the PKS and one of the NRPS encoding

gene clusters were unique insertions in the FZB42 genome and were not present in *B. subtilis* 168. Matrix-assisted laser desorption ionization-time of flight mass spectrometry analysis revealed the expression of antibiotic lipopeptide products of surfactin, fengycin, and bacillomycin D. The FZB42 fengycin (*fen*) and the surfactin (*srf*) operons were organized and located in the genome as in *B. subtilis* 168.

A large 37.2-kb antibiotic DNA island containing the *bmy* gene cluster was attributed to the biosynthesis of bacillomycin D. The *bmy* island was found inserted close to the *fen* operon. The responsibility of the *bmy*, *fen*, and *srf* gene clusters for the production of the corresponding secondary metabolites was demonstrated by cassette mutagenesis, which led to the loss of the ability to produce these peptides. Although these single mutants still largely retained their ability to control fungal spread, a double mutant lacking both bacillomycin D and fengycin was heavily impaired in its ability to inhibit growth of phytopathogenic fungi, suggesting that both lipopeptides acted in a synergistic manner.

Chen *et al.*, (2006) identified the complete polyketide synthase (PKS) gene clusters for *Bacillus* antibiotics for the first time. Three giant modular PKS systems of the *trans*-acyltransferase type were identified in *B. amyloliquefaciens* FZB 42. One of them, *pks1*, is an ortholog of the *pksX* operon with a previously unknown function in the sequenced model strain *B. subtilis* 168, while the *pks2* and *pks3* clusters are novel gene clusters. Cassette mutagenesis combined with advanced mass spectrometric techniques, such as matrix-assisted laser desorption ionization-time of flight mass spectrometry and liquid chromatography-electrospray ionization mass spectrometry (MALAF) revealed that the *pks1* (*bae*) and *pks3* (*dif*) gene clusters encoded the biosynthesis of the polyene antibiotics bacillaene and difficidin or oxydifficidin, respectively. In addition, *B. subtilis* OKB105 (*pheA sfp*[°]), a transformant of the *B. subtilis* 168 derivative JH642, was shown to produce bacillaene, demonstrating that the *pksX* gene cluster directed the synthesis of that polyketide. The plant-growth-promoting activity of the rhizosphere colonizing *B. amyloliquefaciens* was investigated by Idriss *et al.* (2002). They found that *B. amyloliquefaciens* was able to degrade extracellular phytate (*myo*-inositol

hexakisphosphate). *B. amyloliquefaciens* strain FZB45 was found to have the greatest extracellular phytase activity and diluted culture filtrates of this strain stimulated growth of maize seedlings under phosphate limitation in the presence of phytate. The amino acid sequence deduced from the phytase *phyA* gene cloned from FZB45 displayed a high degree of similarity to known *Bacillus* phytases. The recombinant protein expressed by *B. subtilis* MU331 displayed 3(1) - phytase activity yielding D/L-Ins (1, 2, 4, 5, 6) P5 as the first product of phytate hydrolysis. A phytase-negative mutant strain, FZB45/M2, whose *phyA* gene was disrupted, was generated by replacing the entire wild-type gene on the chromosome of FZB45 with a *km: phyA* fragment. Culture filtrates obtained from FZB45/M2 did not stimulate plant growth. In addition, the growth of maize seedlings was promoted in the presence of purified phytase and the absence of culture filtrate. These genetic and biochemical experiments provide strong evidence that phytase activity of *B. amyloliquefaciens* FZB45 is important for plant growth stimulation under phosphate limitation.

B. amyloliquefaciens KPS46 has been reported to be endophytic colonizer and induced resistance strain. The presence of phenolic accumulation as one of the resistant contributors was detected 96 hours after KPS46 inoculation compared with soybean seedling inoculated with bacterial pustule pathogen and distilled water showing less phenols. All KPS46-inoculated seedlings were symptomless with the ability to establish endophytic association detected in all plant parts of root, hypocotyl and epicotyl of soybean of 6.8×10^4 , 3.7×10^3 and 0.9×10^3 CFU/ml respectively. Seedling growth promotion was shown for root length, shoot weight and fresh weight when KPS46 was allowed endophytic colonization (Dechmanee et al., 2005).

Prathuangwong et al. (2005a) reported that the biocontrol bacterium KPS46 used seed treatment and SW01/4 as a foliar spray could induce systemic resistance and promote plant growth in the experiment initiated at Sankampheang, ChiangMai under farm production of vegetable soybean. The KPS46 seed treatment and SW01/4 foliar spray showed an increase in yield of 42 and 17% and better quality of standard pods required than those of the nontreated and conventional controls (application only chemical bactericide and fertilizer). Over the duration of the trial, KPS46 and SW01/4

had the highest decrease fungal stem rot and wilt, and effectively induced systemic resistance against foliar diseases included anthracnose, bacterial pustule, and viruses.

B. myloliquefaciens strain KPS46 also showed another evidence in inducing systemic resistance against bacterial pustule pathogen with increased phenols, phenylalanine ammonia lyase, peroxidases and 1, 3- β -glucanases in soybean plants (Prathuangwong and Buensanteai, 2006). Soybean seeds treated with strain KPS46 at sowing and challenged 14 days later with a bacterial pustule pathogen showed few necrotic lesions and has reduced disease severity as compared with soybean plants not bacterized with KPS46. Production of total phenols, phenylalanine ammonia lyase, peroxidases and 1,3- β -glucanases were expressed at higher levels in treatment with KPS46 when challenged with inoculation of the pathogen, as compared to the diseased and control plants. Almost all the defense-related enzymes detected were found to accumulate in soybean leaf tissues of the co-inoculation at one day and reached maximum the 4th day after pathogen challenge, respectively. These products appeared to be one mechanism of biological control by strain KPS46 and may play a role in plant defense against pathogen infection.

Antagonistic formulation

Liquid formulation: Liquid formulations use antagonist suspended in water or oil emulsion with some additive agents. The formulations can be easily applied, but cannot be stored for a long time. Zhang *et al.* (2003) studied the effect of surfactant and adjuvant and determined that Tween 20 (surfactant) reduced germination and mycelium growth in some *Phoma* and all *Colletotricum* isolates, whereas Tween 40 and Tween 80 stimulated germination and growth of all isolates. Fungi were not responsive to sorbital (adjuvant) but *Colletotricum* was highly viable in gelatin. Ditchstaporn (1995) developed a *B. subtilis* liquid formulation to paste on stem or branch rot of durian caused by *P. palmivora*. These data are essential for producing effective formulation of antagonists.

Powder formulation: Powder formulations are very useful, easily developed, stored, and applied. Many spore forming antagonists are developed in powder or wettable powder formulations to be used as biological control agents. Procedures began with antagonist powder preparation and protectant and carrier mixing. In 1995, Ditchstaporn developed *Trichoderma* sp. powder with 1:1 (v/w) diatomite carrier which could be stored for 7 months at room temperature. Viability only dropped from 10^{10} to 10^7 CFU/g. Chamswarng (2000) reported that the mixture of *T. hazianum* and diatomite 1:3 (w/w), reduced viability from 4.7×10^5 to 1.1×10^4 CFU at room temperature during the 334 day-storage.

Vidhyasekaran and Muthamilan (1995) developed a *P. fluorescens* powder formulation with mixture of talcum and carboxy methyl cellulose that after storage for 240 days at room temperature, dropped viability from 3.75×10^8 to 1.3×10^8 CFU. Tahnontin (1998) reported that *Bacillus* spp., KK-T03 and CH6 isolates incorporated with diatomite, reduced viability from 8.8×10^8 and 7.1×10^8 CFU to 10^4 and 10^6 after storage at room temperature for 180 days and decreased viability to 8.8×10^7 and 6.4×10^7 CFU, respectively after storage at 4 C. The powder formulation developed by Jiamwijit (2002) demonstrated that the carrier enhanced viability of *Bacillus* sp. MK007. Mixing *Bacillus* with rice husk ash dust, cerite, carboxy methyl cellulose, bentonites and elastic gel, after drying at 55°C for 2 hours, showed the slight reduction in CFU from 1.9×10^9 to 3.95×10^8 , 2.71×10^8 , 2.71×10^8 , 1.73×10^8 and 1.6×10^8 , respectively compared with the CFU of crude cells which dropped to 8.5×10^5 . Further investigations also showed that a completed powder formulation of gelatinous cassava starch, gelatinous wheat starch, Arabic gum, and carboxy methylcellulose, were effective binding agents (Jiamwijit, 2002).

Pellet or granular formulation: Jiamwijit (2002) developed granular formation of *Bacillus* sp. against sheath blight of rice caused by *R. solani*. The formulation was made by incorporating 1.95×10^9 CFU *Bacillus* with rice husk ash dust at a ratio of 1:1, one hundred gram of 25 % gelatinous gel and 50 g of rice husk ash dust were added. They were then mixed with 200 g of coarse rice meal and formed into granules

and dried at 55°C for 3 hours. *Bacillus* viability in this formulation showed a little change from 1.95×10^9 to 1.1×10^9 CFU.

Johnson *et al.* (2001) developed a system for stabilizing the bacterium *Serratia entomophilar* for biological control of grass grub (beetle lava). The formulation was developed by using a biopolymer matrix to protect cells and mixing with clay before exuding to form clay pellets. After storage in plastic containers at ambient temperature for 6 months, cell viability was 1.8×10^9 CFU.

Shabana *et al.* (2003) revealed the formulation of fungal propagules (*Fusarium oxysporum f.sp. orthoceras*) encapsulated in a wheat-gluten matrix that was suitable for controlling broomrape (*Orobanchy cumana*) parasitic weed of sunflowers. Yeast extract, sodium alginate, glycerol and sucrose were effective to the protect propagule at the developing processes.

Dehydration: Dehydration involves the simultaneous application and removal of moisture from the products under controlled conditions in order to remove the majority of water content by evaporation. The main purpose of dehydration is to extend the shelf life of products to maintain viability of antagonists. Unlike hydration, dehydration inhibits microbial growth and enzyme activity, reduces weight and bulk and reduces transport and storage cost. Dehydration also provides convenient products for more easily handle ingredients in processing.

In drying using heated surfaces moisture is evaporated from the exposed surfaces. Equipment of drying using heated surfaces is classified as heated-surface or contact driers and hot air driers. In hot air driers, hot air passes over the product to initial drying. Hot air driers are bin driers, cabinet or tray driers, tunnel driers, conveyers or belt driers, fluidised-bed driers, pneumatic driers, rotary driers, spray driers and sun and solar drying. In spray dryers, the product is atomated to form fine droplets and then sprayed into the current flow of hot air (Fellow, 2000).

Packaging: Packaging provides a barrier between the product and environment by protecting against light, heat, gas, moisture transmission, microorganisms and insect contamination. These factors cause deterioration of products. Moreover, packages provide easy transportation, storage and handling for consumers. Types of materials used for packaging include metal, aluminum packaging, glass, flexible film, coated film, laminated film etc. (Fellow, 2000).

Mutagenesis

To study the functions of some bacterial genes and or genetic metrics involved in their antagonist activities requires mutagenesis. The genetic material of a cell includes not only DNA molecules and chromosomes, but also the structures that play a role in the transmission of the genetic information of the cell to its daughter cells. Among these structures are the proteins and other elements of the spindle apparatus. Important mutagenic mechanisms include substitution of a base pair in the DNA molecule (point mutation), loss of one or more base pairs (deletion), transfer of a gene to another site on the genome (translocation), and identical replication of DNA segments (amplification). Typical mutagens generally contain electrophilic substructures that act on the enzymes of DNA metabolism. That inhibits the spindle apparatus, or produce reactive components such as oxidative radicals. This latter category of mutagen includes UV light and ionizing radiation. Mutagen-induced damage to DNA can be corrected by means of various DNA repair mechanisms. Identification and quantification of the generally small number of mutant cells within the large number of nonmutant cells is achieved by selection of the functionally altered cells. In most cases this is done on the basis of mutation-induced resistance to toxic substances that bring about the death of nonmutant cells. In untraet whereas the resistant mutant cells survive, multiply, and form colonies. The tools to generate mutants include using randomize transposon mutagenesis, mutagenic chemicals, and UV and ionizing radiation gamma ray (Sonenshein *et al.*, 1993).

In vivo, random mutagenesis of *B. subtilis* by use of TnYLB-1, a *mariner*-based transposon was done by Le Breton *et al.* (2006). Two pUC19-derived plasmids

were created containing the *mariner-HimarI* transposase gene, modified for expression in *B. subtilis*, under the control of either A- or B-dependent promoters. Both plasmids also contained a transposable element (TnYLB-1) consisting of a KanR cassette bracketed by the *HimarI*-recognized inverse terminal repeats, as well as the temperature-sensitive replicon and *Ermr* gene of pE194ts. The TnYLB-1 transposed into the *B. subtilis* chromosome with high frequency (10^2) from either plasmid. Southern hybridization analyses of 15 transposants and sequence analyses of the insertion sites of 10 of these were consistent with random transposition, requiring only a “TA” dinucleotide as the essential target in the recipient DNA. Two hundred transposants screened for sporulation proficiency and auxotrophy yielded five Spo clones, three with insertions in known sporulation genes (*kinA*, *spoVT*, and *yqfD*) and two in genes (*ybaN* and *yubB*) with unknown functions. Two auxotrophic mutants were identified among the 200 transposants, one with an insertion in *lysA* and another in a gene (*yjzB*) whose function was unknown.

X. axonopodis pv. *glycines* (Xag) mutant strain KUMNTP2 obtained from Tn5 mutagenesis in phosphoenol pyruvate synthase (*ppsA*) genes showed deficient disease symptoms and hypersensitive response (HR) development on susceptible soybean (*Glycine max* cv. SJ4) and tobacco (*Nicotiana tabacum* cv. Xanthi) respectively. The mutant also gave alteration in the level of virulence factors affecting cellulase, a cell wall-degrading enzyme from the type II secretion systems. In this study, they demonstrated that *ppsA*, a mutant deficient in cellulase and pathogenicity had the ability to trigger induced systemic resistance of soybean linking to the production of defense related-enzyme expression, against virulent Xag wildtype infection (Kasem, 2007). Rukayadi *et al.* (2000) reported that a nonpathogenic mutant of Xag strain M715 could reduce colonization virulent strain in the soybean phyllosphere. Additionally, they found that the epiphytic fitness was similar to the virulent wildtype strain even though the density of the mutant was slightly less than that of its parent. The mutant was able to survive for 16 days after inoculation on soybean leaves and maintained population densities of approximately 10^4 to 10^5 CFU/g (fresh weight) of leaves.

UV radiation has been a driving force for the evolution of life. It acts as a mutagen by its DNA-damaging capacity and as a selective agent at the same time. In 1991, Mulligan and Chow used UV radiation to mutate a *B. subtilis* prototroph strain ATCC 21332 to enhance production of biosurfactant. The obtained strain allowed for higher yields in the production of surfactin, a lipopeptidic biosurfactant possessing exceptional surface activity. In order to obtain a mutant *B. subtilis* producing increased amounts of surfactin, bacterial strain ATCC 21332 was grown to logarithmic phase and then approximately 3,000 cells were plated on nutrient agar plates. The plates were then UV radiated for 35 seconds with short wave.

MATERIALS AND METHODS

1. Developing modified medium for KPS46 multiplication using molasses, soybean meal and fish meal

The utilization of soybean meal, fish meal and molasses were tested for enhancing multiplication of the antagonistic *B. amyloliquefaciens* strain KPS46 for mass production to replace peptone, beef extract and dextrose, the nutrient source of general standard media, nutrient glucose agar (NGB). The ratio of varied soybean meal: constant molasses were 0: 10, 1.25: 10, 2.5: 10, 5: 10, and 10: 10 g/l; the ratio of varied fish meal: constant molasses were 0: 10, 1.25: 10, 2.5: 10, 5: 10, and 10: 10 g/l; the ratio of constant soybean meal: varied molasses were 10: 0, 10: 1.25, 10: 2.5, 10: 5, and 10: 10 g/l; and the ratio of constant fish meal: varied molasses were 10:0, 10: 1.25, 10: 2.5, 10: 5, and 10: 10 g/l. Soybean meal and fish meal were blended and mixed with molasses up the ratio mention above and added 100 ml distilled water in 250 ml flask.

Starter culture of the antagonistic *B. amyloliquefaciens* strain KPS46, isolated from soybean phyllosphere collected from Kampong Sean Nakhon Phatom were prepared. *B. amyloliquefaciens* strain KPS46 starter was cultured on nutrient glucose agar for 24 hours, one loop was taken into 100 ml nutrient glucose agar then incubated on the 150 rounds/minute rotary shaker set at 150 rpm at room temperature (28±2 °C). After 24 hours, one percentage (10 ml/l) of it was transferred into the modified media, and later incubated on rotary shaker. Productivity was evaluated by taking 1-ml suspension after 24, 48 and 72 hours of incubation. Sample make serial diluted, and 100µl was spread plated and the colonies counted after incubation for 48 hour.

The experimental design was a completely randomized design with 3 replications. The CFU number was converted to log number before statistically analysis. The productivity of cells in the modified media investigated was compared

with nutrient glucose agar. The cost of ingredients was evaluated to determine reasonable input of production.

Cost of all ingredient material used in modified media and nutrient glucose agar calculated based on retail price. For the productivity calculated from log (CFU)/ liter/ingredient cost of medium modified from benefit cost rate (Pipathsithee, 2001) and least cost combination theory (Jamornkul and Pupongsakorn, 1985)

2. Investigation on optimal culture period for growth and endospore production of KPS46

Molasses-soybean modified medium (MS) at previous used of 10 g soybean meal and 5 g molasses and nutrient glucose agar were prepared. One percentage of KPS46 starter cultured in nutrient glucose agar for 24 hours was added into nutrient glucose agar and MS modified medium then incubated on rotary checker at 150 rpm at room temperature (28 ± 2 °C). The bacterial suspension was taken in periodic sampling after incubation for 24, 36, 48, 60, and 72 hours for growth investigation by plate count technique. For endospore forming was evaluated by incubating bacterial suspension in water bath at 80 °C for 10 minutes before making plate count.

3. Development of wettable powder formulation

The wettable powder formulation of *B. amyloliquefaciens* KPS46 was developed as a biological control agent against bacterial pustule of soybean. The n KPS46 was cultured on MS modified medium developed previously for 24 hours, one loop was taken into MS then incubated on the 150 rounds/minute rotary shaker at room temperature (28 ± 2 °C). After 48 hours, one percentage (10 ml/l) of it was transferred into the modified media, later incubated on rotary shaker. After 48 hours incubation, the culture suspension was centrifuged with 13,000 x g for 10 minutes to collect bacterial cells. Total CFU was adjusted to desire log CFU evaluated by taking 1-ml suspension to make serial dilution, and brought 100 μ l to spread plate and the colonies were counted after incubation for 48 hour. The carriers including dry cow

dung, talcum, decomposed cow dung, and rice husk ash dust were milled to fine particle before dried in dry tray to eliminate moisture, and sterilized later. The KPS46 cells suspension were incorporated with carriers at ratio 15: 100 (v/w). The moisture content in dry formula was adjusted to 15 %.

4. Shelf life of *B. amyloliquefaciens* strain KPS46 in wettable powder formula

Shelf lives of KPS46 maintained in various carriers including dry cow dung, talcum, decomposed cow dung, and rice husk ash dust dry cow dung, talcum, decomposed cow dung, and rice husk ash dust were investigated. Developed wettable powder formulations packed in aluminum foil laminate storage at room temperature (28 ± 2 °C) were evaluated for viability. Bacterial viability within the formulation was evaluated throughout storage by taking each triplicate of 1 g sub-sample dissolving in 9 ml distill water making serial dilution for plating and counting the colony forming unit of KPS46. The viability was evaluated at the beginning and after stored for 30, 60, 180 and 360 days.

5. Screening for carrier used in development of wettable powder formulation

The good trend of carrier used in dry formulation with high viability and disease control efficacy was investigated. The physical properties of wettable powder formulation were studied including wet ability, suspense ability, deposition ability and formulation stability. Wet ability was assessed through the ability to absorb water. The suspense ability and deposition were assessed by the time that the carrier could suspense in water and the precipitate measured after leaving the suspension for 5 minutes. For formulation stability was evaluated by observing the compact granule forming. The cost of carriers used in formulation was calculated to compare for the reasonable price in producing commercial product. It included the price of material and cost of fine particle milling.

6. Efficacy of wettable formulation

6.1 Greenhouse experiment

Enhanced seedling growth: KPS46 maintained in talcum as wettable formula previously after 30 and 180 days storages were brought to test for efficacy. Ten seeds of green soybean, S292 c.v were bacterilized with suspension of KPS46 in dry wettable formula and fresh culture. The KPS46 from stock culture was streaked on nutrient agar; after incubation for 24 hours, it was transferred on to MS modified medium, and put on the 150 rpm rotary shaker for 48 hours at room temperature. KPS46 suspension was adjusted to 0.2 OD at the 600 nanometer wavelength (10^8 CFU/ml) and added 0.1 % wetting agent (Tension T-7) as fresh culture, and 1ml of fresh culture was added to 100 g of green soybean seeds. After shaking in order to mule bacterilized seeds, seeds were sow on sand in 4 inches plastic pot. Germination rate seedling vigor, stem high, root length and fresh weight were observed 7 days after planting. The experimental design and analysis was a Comletly Randomized Design with 5 replications.

Bacterial pustule control: Green soybean S292 c.v. plants were grown in 6-inch plastic pots with 3 standing plants in each pot. The KPS46 in dry a wettable powder was sprayed to R1 growth stage soybean plants. Tap water was sprayed earlier on soybean plants and allowed to dry before inoculation. A fresh cultured of KPS46 cultured on MS, in wettable powder and bactericide (copper oxychloride) were compared for control efficacy against *X. a. pv. glycines* (Xag). Xag cultured on nutrient broth (NB) was put on the 150 rounds/minute rotary shaker for 24 hours, then suspension adjusted to 0.2 OD at the 600 nanometer wavelength (10^8 CFU/ml) and adds 0.1 % wetting agent (Tension T-7) before inoculating to soybeans at R1 growth stage. The Xag was inoculated onto soybean plant after applying antagonist KPS46 for 24 hours. The bacterial pustule severity was estimated by the method of Preecha (1988) after pathogen inoculation for 14 days. Experiments were arranged in Comletly Randomized Design with 5 replications.

5.2 Field experiment I-cropI

The field experiment was conducted to evaluate the developed wettable dry formula of *B. amyloliquefaciens* strain KPS46 at the 30-day shelf life along with various bioproducts of both commercial and natural-made formulations. The ability to inhibit damping-off caused by *Sclerotium* sp., foliar bacterial pustule and to promote plant growth of green soybean was investigated under field condition at Lopburi Field Crop Research Center. The first crop was carried out in rainy season, during July - September, 2005 and the second crop during June - August, 2006. Disease incidence of damping-off and disease severity of bacterial pustule, growth, and yield components including, height, number of nods and branches, total yield, and the marketable yield were assessed.

The efficacy of ISR bacterial *B. amyloliquefaciens* strain KPS46 in controlling against bacterial pustule caused by *Xanthomonas axonopodis* pv. *glycines* and damping-off caused by *Sclerotium* sp. and promoting plant growth of green soybean undertaken under field condition was also investigated. The experiment was conducted at Lopburi Field Crop Research Center during July- September, 2005 for the first crop. The RCBD with 3 replications was employed in the the experimental design. Each plot was 5x5 m²; spacing between row and plant was 50x20 cm². The selected, green soybean AGS292 var was sown and 3 plants/hill were left after emergence.

The screened agents of natural products and bioproduct were employed in the experiment. ISR bacteria KPS46 alone and directly combined with several screened agents of natural products and bioproducts were applied. Dry formulation of KPS46 (KPS 46 – WP) was mixed with the decomposed cow dung (cow manure in local area) and applied by mixing with soil before seed sowing and again after weeding 30 days after planting. The fresh liquid formulation of KPS46 cultured on MS modified medium (MSI) developed by Preech and Prathuangwong (2005), and MS modified medium II (MSII) developed by Kasem *et al.* (2005) and nutrient broth

were applied alone directly and in combination with several screened natural products and bioproducts. KPS46 cultured on MSI modified medium was directly mixed with natural products and bioproducts, natural- made liquid decomposed from vegetable and shrimp head, and commercial products, such as amino acid (AMINO-L), algae-extract (GOEMAR BM 86), and CaB (SORBA-SPRAY). The application methods of liquid formulation with seed treatment followed up by 3 times -14 days interval foliar spray, started 14 days after seed sowing. Treatment of control, including fungicide, seed treated with carbendazim and 3 times -14 days interval spray with copper oxychloride, started 14 days after seed sowing.

Non-treated control and chemical fertilizer (15-15-15) plus 25 kg/rai were set for comparison. All treatments were added with Rhizobium before seeding. Xag was cultured on nutrient broth and adjusted to 10^8 CFU/ml before artificial inoculated by foliar spray on soybean plants 21 days after planting. Disease severity of damping-off and bacterial pustule was investigated. Growth component with height, number of nods and branches as well as yield component with total and marketable yields. Marketable yield was defined based on the good quality of 2 and 3 seed-pod including the pod size not smaller than $1.3 \times 4.5 \times 0.7$ cm³ and total number of pod not more than 175 pods/ 500 g were collected (Nguyen, 1998).

5.3 Field experiment I-crop II

For the second crop, an experiment was conducted at Lopburi Field Crop Research Center during June - August, 2006. Statistical design and experiment unit was also set up similarly to the first crop. The effective treatment of local material from the first crop was elected for the second crop compared with additional treatment of commercial decomposed and biological control agent. Decomposed cow dung (cow manure in local area: Decomposed I) and decomposed commercial pellet (Decomposed II) were mixed with dry formulation of KPS46 and commercial wettable powder of *Bacillus subtilis* (LAMINA), then applied by mixing with soil before seed sowing and again after weeding 30 days after planting. Both decomposed manure were co-applied by mixing with soil before sowing seed treatment with fresh

culture of KPS46 cultured on MS I and MSII, *Penibacillus pumili* strain SW01/4 (Prathuangwong *et al.*, 2005b) and LPT09 (the new indigenous bacterium obtained in this study) cultured on nutrient broth. Biological control agent, bioproducts applied alone, and also treatment of control, including fungicide, seed treated with rhizobium and non-treated control the same as the previous were conducted for comparison. Disease severity of damping-off and bacterial pustule was investigated. Growth component with height, number of nods and branches as well as yield component with total yield, the marketable yield were checked.

5.4 Field experiment II

Field tests in using KPS46 maintained in wettable formula at 180-day shelf life was put on trial in late rainy season, during September to October, 2007 at Corn and Sorghum National Research, Pakchong, Nakhon Ratchasima. The selected, S292 cultivar of green soybean was used in this experiment. The plot size was 3x5.5 m² with each plot containing 6 rows, 1 m plot path (hoop edge) wide. Spacing between rows and plants were 50x20 cm² with 6 rows per plot. Seeds were planted at a rate of 3-4 seeds/ hill and 3 plants/hill were left after emergence. Soybean plant springer irrigated 24 h before applying antagonist and pathogen inoculation was conducted. Preparation of KPS46 in fresh culture and wettable powder including pathogen inoculant was performed the same as the greenhouse experiment. There was a total of 4 treatments arranged in Completely Randomized Design with three replicate plots. They were adjusted to 10⁸CFU/ml which KPS46 was cultured in MS and put on the 150 rounds/second rotary shaker for 48 hours. The KPS46 suspension was adjusted to 0.2 OD at the 600 nanometer wavelength (10⁸CFU/ml) and 0.1 % wetting agent (Tension T-7) was added to the test inoculants. The pathogen was inoculated after applying antagonist for 24 hours. The bacterial pustule severity was estimated by the method described by Preecha (1988) after pathogen inoculation for 14 days. Disease severity of damping-off naturally occurred was also investigated. Growth and yield component were checked. The data was subjected to analyse of variance with general model procedure of SAS. Treatment means were assessed using Duncan's New Multiple Rang Test (DNMRT) and all testing of significant conduct at $p \leq 0.05$.

7. Cost of production and return on investment

The cost of production, including material cost, employed labor wages, irrigation, rent of production area (indirect cost), and rate were calculated. The return on investment (ROI) was calculated using the following equation below (Pipathsithee, 2001; Jamornkul and Pupongsakorn, 1985). ROI was compared between conventionally fungicide application and biological control application of KPS46 developed as wettable formula.

$$\text{ROI} = \text{Benefit/Total cost} \times 100$$

8. Antagonistic mechanism of *B. amyloliquifaciens* KPS46 against bacterial pustule

8.1 Generating of UV mutants

UV mutagenesis minus secondary metabolite was carried out to determine the mechanism biological control efficacy involving secondary metabolite production by *B. amyloliquifaciens* KPS46. KPS46 growing to logarithmic phase approximately 1,000 cells was plated on nutrient agar plates. The plates were then radiated with a 65 watt UV for 0, 1, 2, 5, 10, and 15 minutes to select the dosage of UV light that allowed 10 to 20% colony survival rate. The UV-radiated plates were then incubated at room temperature (28° C) in the dark until the colonies were visible. In order to detect whether the obtained colonies produced secondary metabolites, mutants derived from UV mutagenesis was screened. The survival colonies were taken into 5 ml nutrient broth and incubated at room temperature for 24 hours.

Xanthomonas axonopodis pv. *glycines* was cultured in nutrient broth and incubated on 150 rpm at room temperature for 24 hours. Cell suspension was adjusted to 0.2 OD at 600 nm, 0.5 ml mixed with 10 ml of nutrient agar melted and incubated in water for 50° C before pouring plate for anti bacterial secondary metabolite

evaluation. Paper disk method was used to evaluate that colony to predict secondary metabolite of UV mutants. Sterilized 0.5 centimeter paper disks (Whatman Filter Paper Set number No. 1001 110) were plated on NA mixed with Xag, then dropped 5 μ l of culture of KPS46 24 hours grown after adjusted to 0.2 OD at 600 nm. Secondary metabolite inhibition clear zone of UV mutants were evaluated after incubation for 48 hours.

8.2 PCR identification

The selected UV mutagenesis colonies were run using PCR to confirm identified UV mutant from KPS46 using specific conserve region 16S rDNA. The sequencing primers using were 16s-F 5' -TAATACGACTCACTA TAG GG- 3' and 16s-W 5' -CGATTTAG GTGACACTATAG-3'. The master mix of PCR reaction was made; each 24 μ l reaction included: distilled H₂O 15.4 μ l, 5X Buffer(con 1X) 5 μ l, 25 mM MgCl₂ (1.5 mM)1.5 μ l, 10ug/ μ l SA(0.16 μ M) 0.4 μ l, 10 μ M kpsa (0.2 μ M) 0.5 μ l, 10 μ M kpsb (0.2 μ M) 0.5 μ l, 10 μ M dNTPmix (0.2 mM) 0.5 μ l, 5 units/ μ l and Taq(1 u/25 μ l) 0.2 μ l and then adding 1 μ l of DNA fragment of KPS46. PCR reaction condition included: initial denaruration at 98 °C for 5 min 35 cycles, denaruration at 95 °C for 1 min, annealing at 58 C for 1 mins, extension at 72 °C for 1.5 mins, final extension at 72 °C for 5 mins and cooling for 4 °C. A 10 μ l of PCR product used to run standard DNA agarose gel electrophoresis using 1 % agarose gel at 75 V for 2 hours.

9. Detection of surfactin

Surfactin is one of antibiotic lipopeptide including: surfactin, fengycin, and iturin family such as bacillomycin D, mycosubtilin and iturin. The various homologous compounds and isoforms were characterized by liquid chromatography (HPLC) as described by Akpa *et al.* (2001) using specific elution gradients for each lipopeptide family. The lipopeptide, surfactin-producing bacterial KPS46 and UV mutant were cultured in nutrient broth containing 0.2% glucose and incubated at room temperature on 150 rpm shaker for 48 hours at room temperature. The culture

suspension was centrifuged with 13,000 x g at 4 °C for 15 minutes to remove bacterial cells and the supernatant was acidified to pH 2.0 with concentrated HCl then left refrigerated overnight for lipoproteins precipitation. The precipitates were collected by centrifugation (13,000xg, 4 °C, 15 minutes) and washed three times with distilled water. The crude lipopeptide was dissolved in butanol and was loaded onto a column of silica gel previously equilibrated with butanol. The column was then washed with the same solvent. The lipopeptide was eluted with a linear gradient of 100% butanol. HPLC spectra were detected by a UV monitor at 210 nm to compare with standard lipopeptide surfactin

10. PCR confirm identification of the *surfAA*

PCR was used to confirm the identification of the *surfAA*, gene for secondary metabolite surfactin production. Genes amplifying surfactin of *B. amyloliquefaciens* FZB42 was described by Koumouts *et al.* (2004). To confirm the random UV mutant deficient producing lipopeptide surfactin was investigated by PCR followed by Koumouts *et al.* (2004). A 1.75-kb PCR product from the *surfAA* gene region was amplified with the primers Srfkn-1 (5'-AGCCGTCCTGTCTG ACGACG) and Srfkn-2 (5'-TCTGCTGCCATA CCGCATAGTC). The master mix of PCR reaction was made; each 24 ml reaction included: ddH₂O 15.4 µl, 5X Buffer (con 1X) 5 µl, 25mM MgCl₂(1.5 mM) 1.5 µl, 10ug/µl SA(0.16 µM) 0.4 µl, 10 µM *srfI* (0.2 µM) 0.5 µl, 10uM *srf2* (0.2 µM) 0.5 µl, 10 µM dNTPmix (0.2 mM) 0.5 µl, 5units/µl and *Taq*(1u/25 µl) 0.2 µl and then adding 1 µl of DNA fragment of KPS46 and UV mutant. PCR reaction condition included: initial denaruration at 98 °C for 5 min 35 cycles, denaruration at 95 °C for 1 min, annealing at 58 °C for 1 min, extension at 72 °C for 1.5 min, final extension at 72 °C for 5 min and cooling for 4 °C. A 10 µl of *bmya* PCR product was run standard DNA agarose gel electrophoresis using 1 % agarose gel at 75 V for 2 hours.

11. Extracellular enzyme assay

The production of extracellular enzymes including amylase, cellulases, chitinase, endoglucanase, and proteases were investigated using a plate assay method. A method providing simultaneous detection of polysaccharide and protein degrading microorganisms was described for detection of micro-organisms exhibiting both degrading activities and special combinations.

Alpha-amylase plate assay: Amylase is one of extracellular enzyme secreted by degrading microorganism. Amylase secretion of wildtype and UV mutants of KPS 46 was investigated in vitro on alpha-amylase assay medium using hydrolysis starch containing of 0.5% yeast extract, 1% tryptone, 0.25% NaCl, 0.02 % soluble starch and 0.8 agarose (Ray *et al.*, 2002; Lory, 1998). Solid culture of starch medium was cut using a 0.5 centimeter diameter cork borer and cut agar was taken out. Wildtype and UV mutant of KPS46 were cultured on nutrient broth for 24 hours and adjusted to 0.02 OD at 600nm, then 10 µl of culture was dropped onto well of starch agar and incubated for 24 hours. After incubation, iodine solution was added to the surface of the plate; positive demonstrating a zone of clearing around the colony was observed.

Endoglucanase: Endoglucanase (CMCase) activity was determined by measuring the amount of glucose released from carboxymethyl cellulose (CMC). The investigation of endoglucanase was followed using the method of Gough *et al.* (1988) and a plate assay using NGYA medium adding 0.125 % CMC. A 10 µl aliquot of KPS46 and UV mutant culture in nutrient broth for 24 hours were dropped into wells on agar plate. After inoculation for 24 hours, 0.1 % Congo red was flooded over the agar plate surface and left for 30 minutes, then the excess Congo red was washed two times with tap water for 15 minutes and destaining with 1 M NaCl solution. Endoglucanase enzyme activity was detected from a yellow light halo clear zone surrounding the colonies against the orange background.

Chitinase plate assay: Chitinase secretion by KPS46 and the UV mutant were evaluated on chitin plate assay. This assay was prepared according to the method of

Sampson and Gooday (1998). The chitinase inducing medium contained 10.0% colloidal chitin (wet weight), 0.1% $(\text{NH}_4)_2\text{SO}_4$, 0.3% KH_2PO_4 , 0.01% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.7% K_2HPO_4 , 0.05% sodium citrate, 0.02% yeast extract, adjusting the pH to 7 and 2% agar. KPS46 and UV mutant culture in nutrient broth for 24 hours were inoculated on solid chitinase-inducing plates and incubated at 30°C for 72 h. Chitin hydrolysis was detected by formation of a whitish, opaque halo around a translucent area (totally hydrolysed chitin) surrounding the growing colony.

Protease plate assay: A plate diffusion assay for the detection of proteases of microorganism utilizes a simple substrate that can be performed in plate assay using a skim milk as substrate cleaved by the protease. Nutrient yeast glucose agar (NYGA) added with skim milk medium using as protease plates assay containing 0.5% peptone, 0.35% yeast extract and 2% glycerol, 0.8% agar (Difco Laboratories) (Daniels *et al.*, 1984) and adding 0.5% skim milk (Barber *et al.*, 1997) was prepared. Wells were cut into the plates with a sterile 0.5 centimeter diameter cork borer after the agar solidified. KPS46 and UV mutant culture in nutrient broth for 24 hours were dropped in well on agar plate and inoculated for 24 hours. Protease enzymatic activity secretion by KPS46 and UV mutant was assessed by diffused clear zone of degradation of skim milk.

12. Growth rate comparison

The growth rate between mutant and wildtype were compared by growing both of them on nutrient broth. The starters of wildtype and UV mutant were cultured on nutrient agar for 24 hours, then one loop was added into 20 ml nutrient broth and incubated on the 150 rounds/minute rotary shaker at ambient. After 24 hours, 1% of each bacterial strain suspension was transferred into 100 ml of nutrient broth in 350 ml flask. Medium suspension was kept incubating on 150 rpm rotary shaker at room temperature (28°C). Growth rates of both wildtype and UV mutant were evaluated at periodic intervals of growth thorough CFU counting by taking 1-ml suspension cultured at 12, 24, 36, 48, and 56 hours for dilution plate and colony counting was done.

13. Swarm motility

Phenotypic swarming motility assays to characterize in KPS46 and UV mutant were initiated at different concentrations of 0.5, 1 and 1.5 % agar on differential media between nutrient broth and Luria agar (LA) medium the procedure previously described by Calvio *et al.*(2005) and Senesi *et al.* (2002). KPS46 and UV mutant cultures were grown in nutrient broth for 24 hours at room temperature, culture suspensions were adjusted to 0.02 OD at 600 nm before 10 µl was spotted onto the center of agar plate and culture at room temperature for 48 hours, then diameters of halos due to bacterial migration was measured and growing pattern was also observed.

14. Enhancing seedling growth

Ten seeds of green soybean, cv S292 bacterilized with KPS46 and its UV mutant was cultured on nutrient broth for 24 hours on rotary checker set at 150 rpm at room temperature. Cell suspension was adjusted to 0.2 OD at the 600 nanometer wavelength (10^8 CFU/ml). A 0.1 % of wetting agent (Tension T-7) was added to the suspension before adding to 100 g of green soybean seeds. After shaking the bacterilized seed, ten seeds were sown in sand in 4 inches plastic pot. Germination rate seedling vigor, stem height, root length and fresh weight were observed after 7 day planting. Statistic experiment design and analysis was Completely Randomized Design with 5 replications.

15. Bacterial pustule control

Soybean plants, green soybean cv S292 were grown in 6-inch plastic pots with 3 standing plants in each pot. KPS46 and its UV mutant were sprayed to R1 growth stage soybean plants. Tap water was sprayed earlier on soybean plants and dried before inoculation. Cell suspension cultured on nutrient broth for 24 hours was

adjusted to 0.2 OD at the 600 nanometer wave length (10^8 CFU/ml) and added 0.1 % wetting agent (Tension T-7). 60 ml of suspension was sprayed onto each pot of soybean plant. Xag cultured on nutrient broth was put on the 150 rounds/minute rotary shaker for 24 hours, then adjusted suspension to 0.2 OD at the 600 nanometer wave lengths (10^8 CFU/ml) and added 0.1 % wetting agent (Tension T-7) before inoculating to soybeans at R1 growth stage. The Xag was inoculated onto soybean plant after applying antagonist KPS46 for 24 hours. The bacterial pustule severity was estimated used the method of Preecha (1988) after pathogen inoculation for 14 days. The experiment was arranged in Completely Randomized Design with 5 replications.

16. Statistical analysis

Mean comparison of swarm motility and extracellular enzyme assay were Students t-test at $p = .05$. Experimental design and statistical analysis of germination rate seedling vigor, stem height, root length, fresh weight and disease severity were Completely Randomized Design (CRD) with 5 replications. The data most in this research was finally submit to analysis by using SAS program.

RESULTS

1. Developing modified medium for KPS46 multiplication using molasses, soybean meal and fish meal

The utilization of soybean meal, fish meal and molasses was tested for enhancing multiplication of antagonistic *B. amyloliquefaciens* strain KPS46 for mass production. The result revealed that the ratio of soybean meal: molasses at 10: 10 g/l enhanced antagonistic *B. amyloliquefaciens* strain KPS46 multiplication with 9.0, 9.3 and 9.4 log CFU/ml after incubation for 24, 48, and 72 hours respectively. This was better compared with nutrient glucose broth that yielded 8.7, 9.2 and 9.2 log CFU/ml incubated at the same period (Fig. 1). When modifying constant soybean meal at 10 g with molasses varied at 0, 1.25, 2.5, 5, and 10 g/l the result showed the ratio of soybean meal: molasses at 10: 5 of 8.6, 9.6 and 9.6 log CFU/ml and 10: 10 of 8.7, 9.2 and 9.3 log CFU/ml after incubation for 24, 48 and 72 hours respectively respectively compared with NGB of 9.1, 9.3 and 9.2 log CFU/ml (Fig. 2). They gave the growth equal to or better than nutrient glucose broth. The costs of the preferred ratio of soybean meal: molasses at 10: 10 and 10: 5 g per liter were 0.32 and 0.46 Baht per liter, respectively, compared to nutrient glucose broth with the highest cost of 48.52 Baht per 10 liters (Table 1). For the productivity of the preferred ratio of soybean meal: molasses at above ratio above, were 34,450 and 24,090 log (CFU) /Baht, where nutrient glucose agar was the smallest of 250 log (CFU) /Baht (Table 2).

When fish meal was used as a source of nitrogen in modified media, it varied at 0, 1.25, 2.5, 5, and 10 g/l with constant molasses at 10 g; and constant fish meal at 10 g with molasses varied at 0, 1.25, 2.5, 5, and 10 g/l. The results were similar to soybean meal: molasses whose ratio at 10: 10 g/l enhanced antagonistic KPS46 multiplication to 9.2, 9.5 and 9.6 log CFU/ml after incubation for 24, 48, and 72 hours respectively, better than yielded of 9.1, 9.3 and 9.2 log CFU/ml incubated at the same period (Fig 3). At 10 g constant fish meal of with varied molasses at 0, 1.25, 2.5, 5, and 10 g/l, the result showed the ratio of fish meal: molasses 10: 5 and 10: 10 enhancing multiplication to be better than nutrient glucose broth. The CFU of KPS46

culture in modified medium of fish meal and molasses at ratio of 10: 5 and 10: 10 g/l were 8.4, 9.7 and 9.8 log CFU/ml and 9.2, 9.4 and 9.6 log CFU/ml respectively (Fig 4). The cost per liter of fish meal: molasses at the ratio of 10:5 and 10: 10 g/ml were 0.40 and 0.50 Baht respectively (Table 1). The productivity of that ratio of modified media mention above and NGB were 30,220, 22,470, 19,820, and 250 log CFU/Baht respectively (Table 2).

2. Investigation optimal culture period for growth and endospore production of KPS46 culture on MS for producing wettable formula

The investigation of optimal growth and endospore production was conducted to determine the culture harvesting period to collect cells/endospores using in wettable formulation. The result showed that MS modified medium (MS) and nutrient glucose broth (NGB) gave the high cell growth from 24-48 hours with the highest at 48 hours. In this incubation period, cell growth of KPS46 cultured on MS and NGB were 9.6 and 9.3 log CFU/ml respectively. Endospore forming was similar to cell growth. They gave the highest endospores at 48 hour incubation period of 7.4 and 7.3 log CFU/ml (Fig 5). This result indicated that the 48-hour culture period gave the high yield to harvest cell and endospore in both MS modified medium and NGB.

3. Shelf life of *B. amyloliquefaciens* strain KPS46 in wettable powder formula

The development and test for a dry formulation of *B. amyloliquefaciens* KPS46 was investigated. The new formula was developed by using several local materials as carrier to reduce cost and to enhance value of production. The local material, dry cow dung, talcum, decomposed cow dung, and rice husk ash dust showed good approach of carrier using in dry formulation developed with high viability after stored at room temperature. The developed formula had high viability of KPS46 enhancement of 10.2, 10.7, 10.5, and 10.6 log CFU/g after storage at room temperature for 30 days, a little drop down from the beginning (Fig 6).

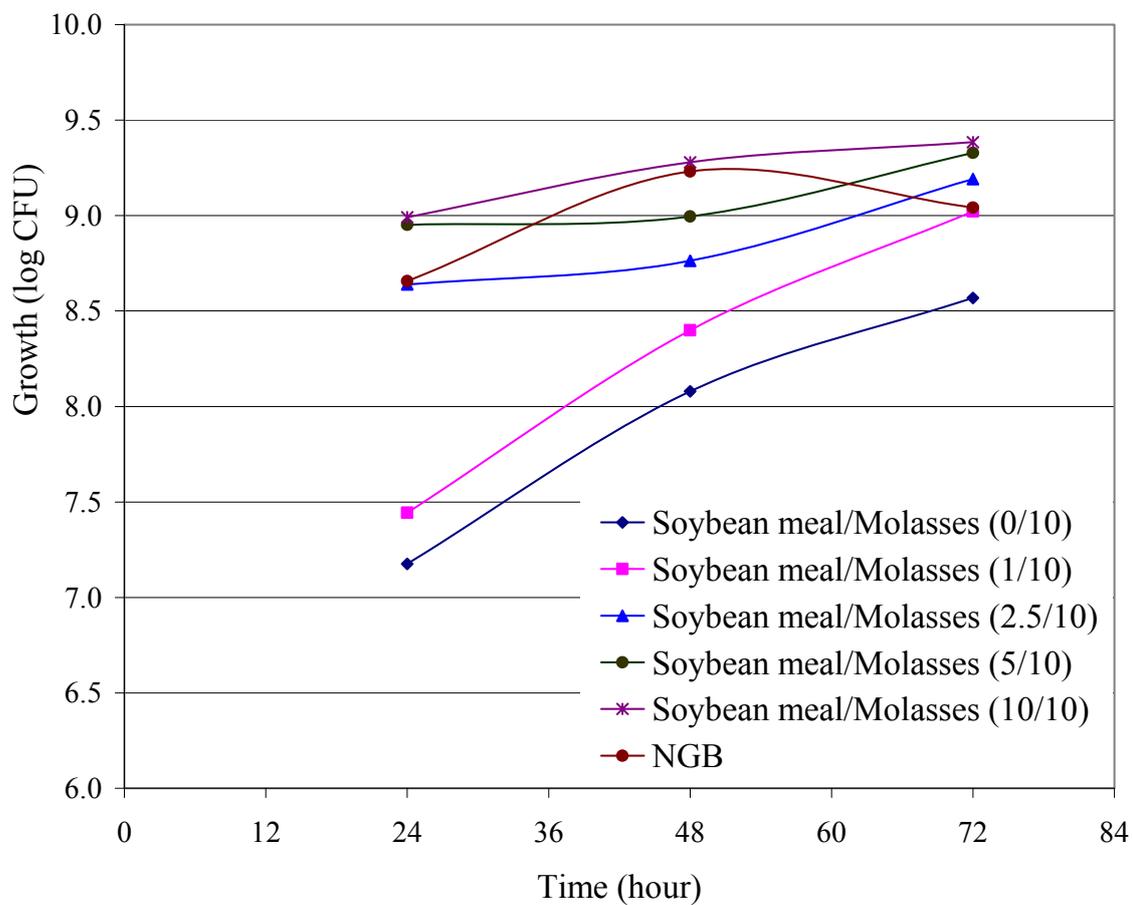


Figure 1 Colony forming unit (CFU) of antagonistic *Bacillus amyloliquefaciens* KPS46 cultured on modified media at constant molasses with varied soybean.

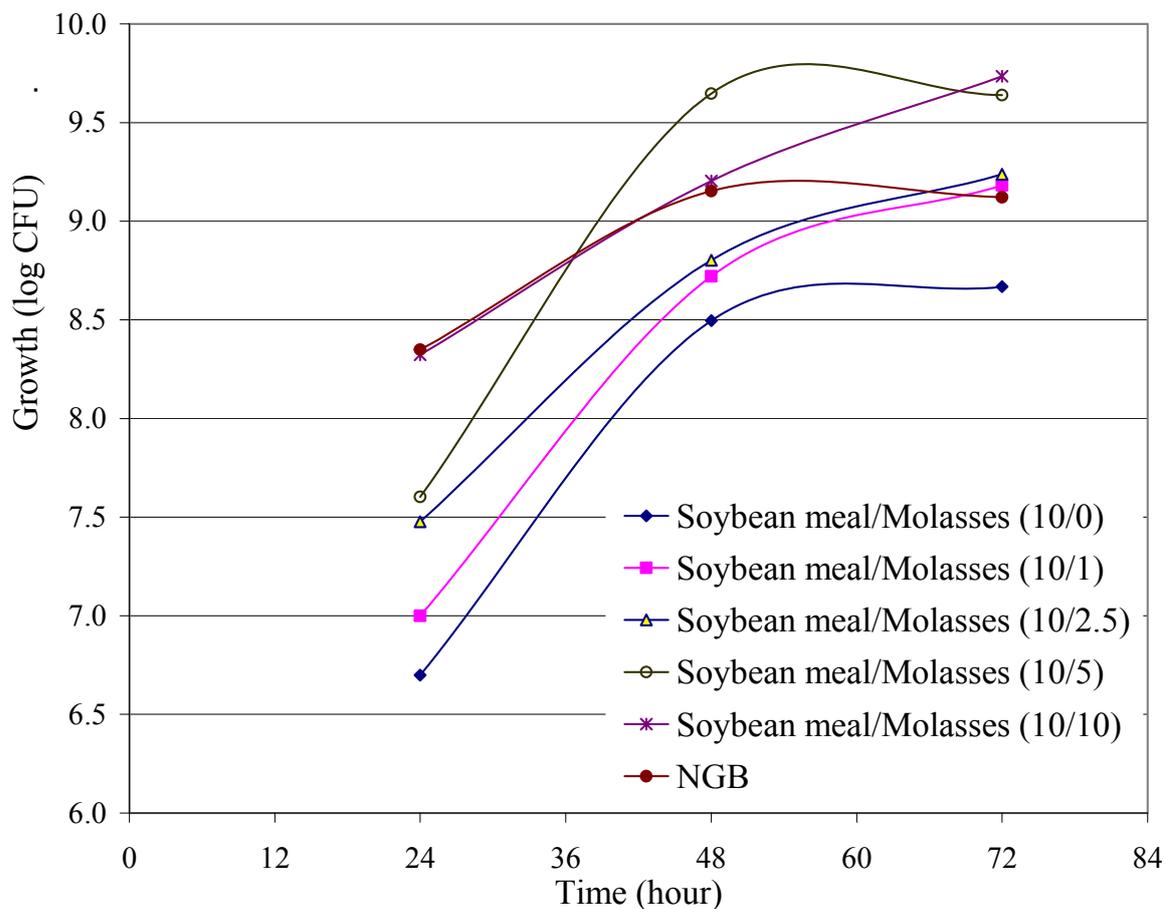


Figure 2 Colony forming unit (CFU) of antagonistic *Bacillus amyloliquefaciens* KPS46 cultured on modified media at constant soybean meal with varied molasses.

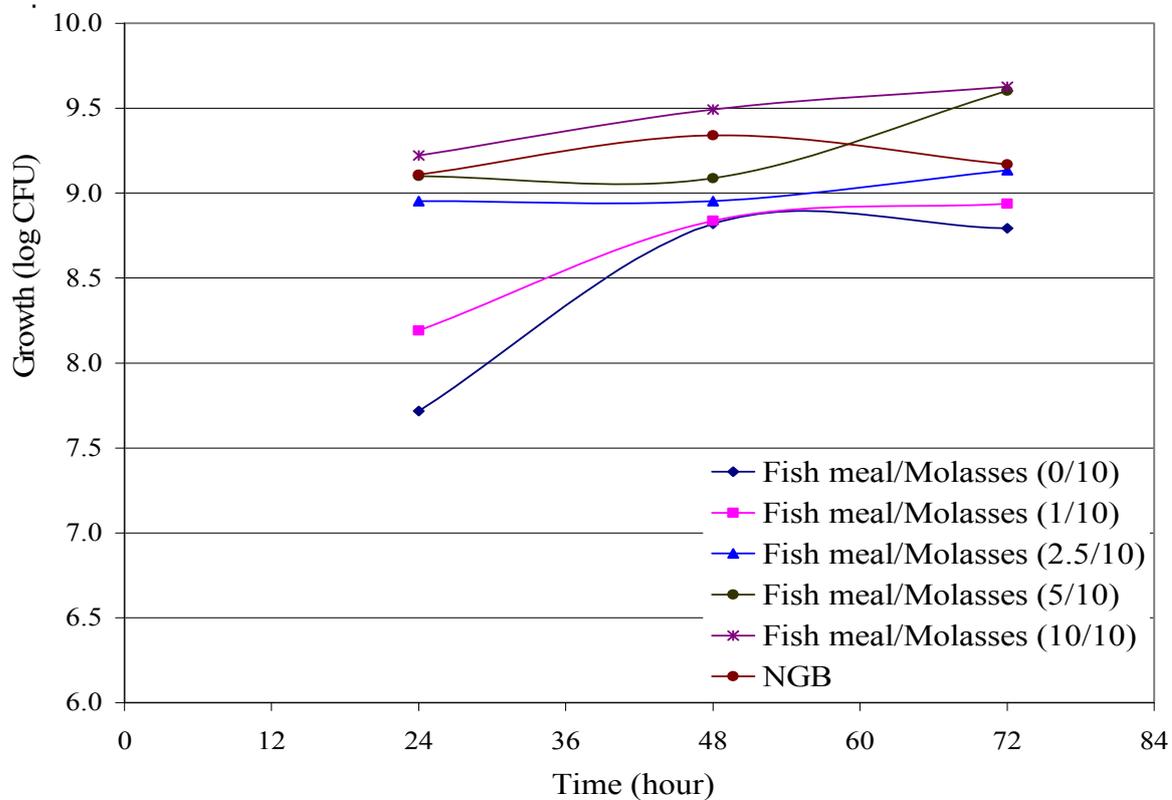


Figure 3 Colony forming unit (CFU) of antagonistic *Bacillus amyloliquefaciens* KPS46 cultured on modified media at constant molasses with varied fish meal.

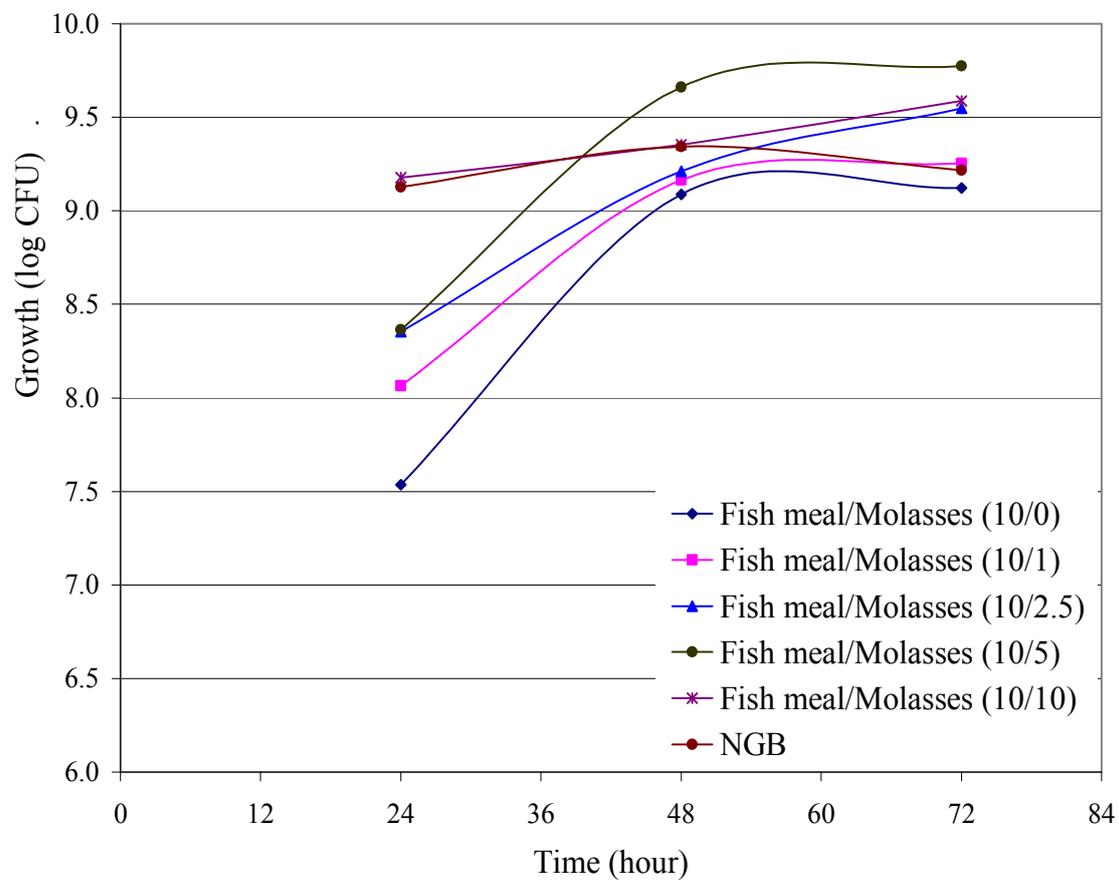


Figure 4 Colony forming unit (CFU) of antagonistic *Bacillus amyloliquefaciens* KPS46 cultured on modified media at constant fish meal with varied molasses (B).

Table 1 Cost of all different ratio of ingredient material used in modified media and NGB per litter calculated base on retail price.

Ingredient material	Cost/Kg (Baht)	Cost in Baht of each ratio				
		Ratio of soybean meal(g): molasses(g)				
		0: 10	1.25: 10	2.50: 10	5: 10	10: 10
Soybean meal	11.85	0.00	0.01	0.03	0.06	0.12
Molasses	40	0.40	0.40	0.40	0.40	0.40
Total cost/litter		0.40	0.41	0.43	0.46	0.52

		Ratio of soybean meal(g): molasses(g)				
		10: 0	10: 1.25	10: 1.25	10: 2.50	10: 10
Soybean meal		0.12	0.12	0.12	0.12	0.12
Molasses		0.00	0.05	0.10	0.20	0.40
Total cost/litter		0.12	0.17	0.22	0.32	0.52

		Ratio of fish meal(g): molasses(g)				
		0: 10	1.25: 10	2.50: 10	5: 10	10: 10
Fish meal	20.38	0.00	0.03	0.05	0.10	0.20
Molasses	40	0.40	0.40	0.40	0.40	0.40
Total cost/litter		0.40	0.43	0.45	0.50	0.60

		Ratio of fish meal(g): molasses(g)				
		10: 0	10: 1.25	10: 1.25	10: 2.50	10: 10
Fish meal	20.38	0.20	0.20	0.20	0.20	0.20
Molasses	40	0.00	0.05	0.10	0.20	0.40
Total cost/litter		0.20	0.25	0.30	0.40	0.60

		Ratio of peptone(g):beef extract(g):dextrose (g)		
		5: 3: 2.5		
Peptone	4,670.60			23.35
Beef extract	5,928.00			17.78
Dextrose	1,478.00			3.69
Total cost/litter				44.83

Table 2 The *Bacillus amyloliquefaciens* KPS46 productivity calculated from log (CFU) /liter / ingredient cost of medium compared with nutrient glucose Agar (NGB).

Ratio of ingredient material	Log (CFU) /Baht
Soybean meal: Molasses	
0: 10	24,800
1.25: 10	25,040
2.5: 10	25,080
5: 10	24,090
10: 10	21,680
Soybean meal: Molasses	
10: 0	82,670
10: 1.25	60,590
10: 2.5	47,760
10: 5	34,450
10: 10	21,380
Fish meal: Molasses	
0: 10	26,030
1.25: 10	24,710
2.5: 10	24,390
5: 10	22,470
10: 10	19,190
Fish meal: Molasses	
10: 0	52,850
10: .25	43,250
10: .5	38,030
10: 5	30,220
10: 10	19,820
NGB	250

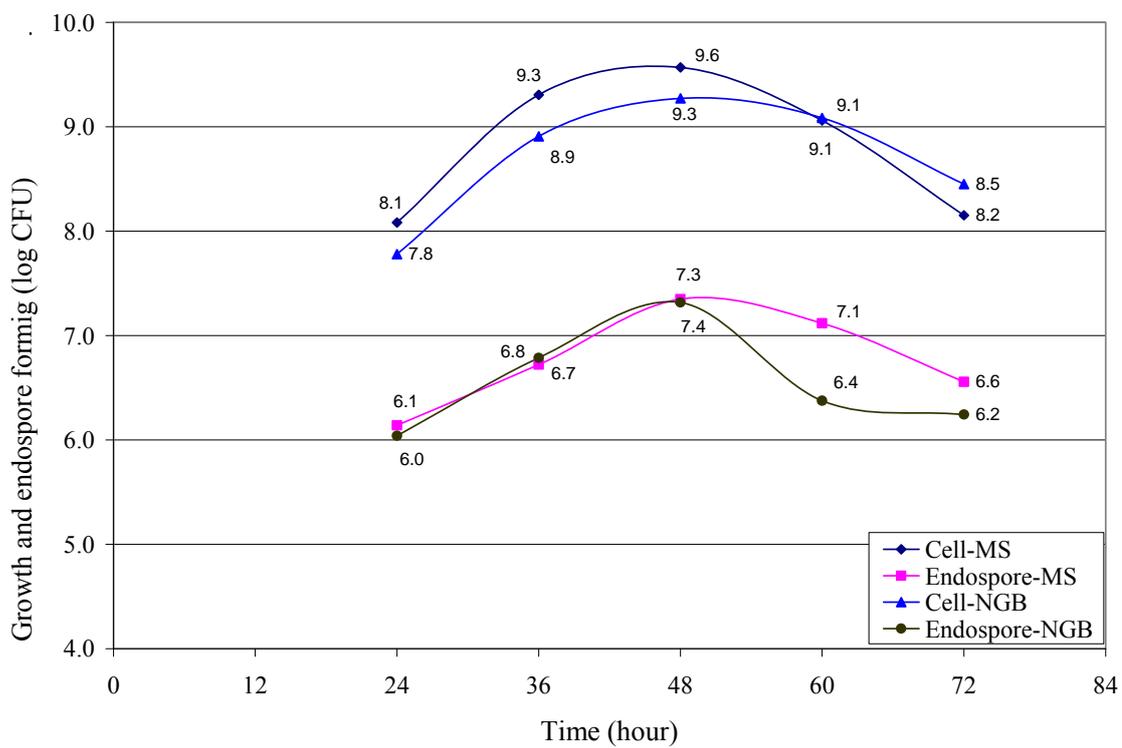


Figure 5 Bacterial growth and endospore forming cultured on nutrient glucose broth (NGB) and molasses-soybean modified medium (MS) of *Bacillus amyloliquefaciens* KPS46.

After 180-day shelf life of KPS46 maintained in all local materials, rice husk ash dust, talcum, dry cow dung, and decomposed cow dung, as carrier in dry formula showed viability with high log CFU of 9.6, 9.6, 9.4, and 9.3 log CFU/g respectively. When investigated after 360-day shelf life of KPS46, it still gave high CFU enough to apply in field. The CFU of KPS46 maintained in wettable formula used rice husk ash dust, dry cow dung, decomposed cow dung, and talcum with 8.8, 8.7, 8.5, and 8.4 log CFU/g respectively (Fig 6).

4. Screening for carrier used in development of wettable powder formulation

Some properties of formula were investigated to select materials for the best performance and possibility to develop a commercial product. The investigation showed that pH of material, decomposed cow dung, dry cow dung, talcum, and rice husk ash dust using in this research were 6.6, 7.2, 8.2, and 9.6 respectively. The wetting ability, one of the important factors to enhance formula efficacy indicated that all of these materials had good stability, after storage for 360 days. They did not form compact granule or particle. Talcum and rice husk ash dust were the better material than the storage others used in developing formula. They could absorb water suddenly while both dry cow dung and decomposed cow dung absorbed water very slow (1 g of material could absorb 1 ml water for more than 5 minutes). They also slowly sedimented and could disperse in water better than both dry cow dung and decomposed cow dung (Table 3).

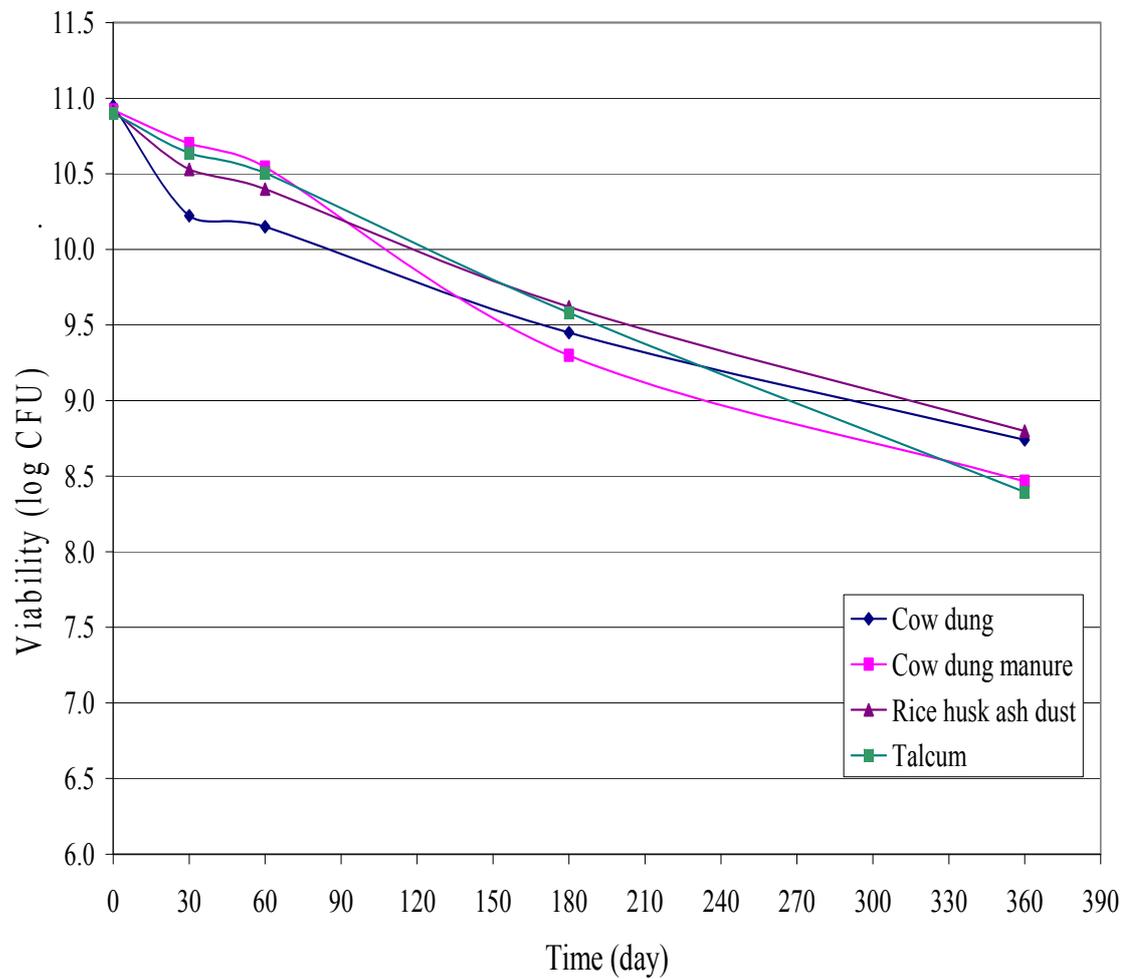


Figure 6 The available of *Bacillus amyloliquefaciens* KPS46 obtained in dry formula developed from various local materials.

Table 3 Some physical and chemical property screening for carrier using to develop wetttable fomula of *Bacillus amyloliquefaciens* KPS46.

Carrier	pH	Wett ability	Suspend ability ^{1/}	Formula stability ^{2/} (360 days)
Cow dung	7.2	very slow	-	+
Decomposed cow dung	6.6	very slow	-	+
Rice husk ash dust	9.6	fast	+	+
Talcum	8.2	fast	+	+

^{1/} + = well suspend ability.

- = not well suspend ability.

^{2/} + = no compact granule or particle forming.

5. Efficacy of wettable formulation

5.1 Green house experiment

Enhanced seedling growth: The investigation of the wettable powder formula to maintain shelf life of KPS46 for 30 days was compared to fresh culture and fungicide (copper oxychloride) in the greenhouse. Seeds were treated with KPS46 and fungicide before planting. Germination rate observed after 7 day planting showed that KPS46 maintained in talcum developed as wettable powder formula initiated germination rate of 74.00 %. It was not different from fresh culture and copper oxychloride of 78.00 and 76.00 % respectively ($p=.05$), but higher than non-treated control with germination rate of 62.99%. For growth stimulating, most stem length, root length and fresh weight were significantly differed from the non-treated control, but they did not significantly differ from copper oxychloride. The fresh weight of seedling, the indicating biomass produced of soybean plants was enhanced by KPS46 increased higher than the biomass in the treatment of copper oxychloride and non-treated control. The fresh weight of soybean seedling (weigh of 10 seedlings) enhanced by KPS46 in fresh culture and dry formula were 22.64 and 23.02 g respectively, while copper oxychloride and non-treated control were 19.79 and 18.63g respectively (Table 4).

The investigation of wettable powder formula for 180-day shelf life of KPS46, germination rate observed after 7 day planting showed that KPS46 maintained in talcum developed as wettable powder formula initiated germination rate of 64.00 %. It was not different from fresh culture of 68.00 %, but was higher than copper oxychloride and non-treated control with germination of 57.33 and 54.00 % respectively ($p=.05$). For growth stimulating, even though stem length and root length were not different, the adventitious root number was significant different between seed treatment and non-treated control. The data indicated that KPS fresh cell and maintained in dry formula could stimulate the increasing adventitious root number with root number of 42.69 and 42.42 respectively. They were higher than non-treated control with root number of 38.60 but did not differ from copper oxychloride with

root number of 41.44. The fresh weight of seedling enhanced by KPS46 increased higher than copper oxychloride and non-treated control. The fresh weight of soybean seedling (weighting from 10 seedlings) enhanced by KPS46 in fresh culture (MS) and dry formula, copper oxychloride and non-treated control were 20.72, 19.79, 18.17 and 17.97 g respectively (Table 5).

Bacterial pustule control: The control efficacy of KPS46 maintained in wettable formula at 30-day and 180-day shelf lives against bacterial pustule was investigated in the greenhouse condition. The bacterial pustule severity was estimated by the method of Preecha (1988) 14 days of inoculation *Xag* onto soybean plant at R1 (growth stage) after applying antagonist KPS46 for 24 hours. The result showed that KPS46 maintained in wettable formula at both 30-day and 180-day shelf lives still suppressed bacterial pustule on green soybean, S292 cultivar similar to KPS46 fresh culture and equal to copper oxychloride. Bacterial pustule severity investigation of 30-day shelf life, KPS46 fresh cell and maintained in dry formula, and copper oxychloride were 25.05, 21.19 and 22.96 %. Those treatments could control bacterial pustule better than non-treated control with disease severity of 48.15 %. The control efficacy of KPS46 180-day shelf life did not decline. It could suppress disease severity of 37.54 % and no difference was found between from fresh culture and copper oxychloride with disease severities of 34.14 and 38.12 % respectively. The control efficacy of KPS46 180-day shelf life was also better than non-treated control with disease severity as high as 85.79 % (Table 6).

Table 4 Potential of developed wettable formula of *Bacillus amyloliquefaciens* KPS46 30-day shelf life stored at room temperature to enhance seedling growth in greenhouse condition.

Treatment	Germination rate (%)	Stem length (cm)	Root length (cm)	Root number	Fresh weight (g)
KPS46 in dry formula	74.00a	14.68a	14.68a	14.68ab	22.64a
KPS46 fresh culture	78.00a	14.8a	14.80a 13.56a	14.80b 13.56bc	23.02a
Copper oxychloride	76.00a	13.56ab	b		19.79b
Non-treated control	62.00b	13.32b	13.32b	13.32c	18.63b
CV. (%)	11.94	6.35	6.35	5.48	9.06

Means followed by the same letter in the same column were not significantly different at $p = 0.05$.

Table 5 Potential of developed wettable formula of *Bacillus amyloliquefaciens* KPS46 180-day shelf life stored at room temperature to enhance seedling growth in greenhouse condition.

Treatment	Germination rate (%)	Stem length (cm)	Root length (cm)	Root number	Fresh weight (g)
KPS46 in dry formula	64.00ab	12.74	13.08	42.96a	19.79a
KPS46 fresh culture	68.00a	13.09	13.58	42.42a	20.72a
Copper oxychloride	57.33bc	13.02	12.52	41.44ab	18.17b
Non-treated control	54.00c	12.74	12.66	38.60b	17.97b
CV. (%)	19.71	8.97	9.77	5.85	5.57

Means followed by the same letter in the same column were not significantly different at $p = 0.05$.

Table 6 Potential of developed wettable formula of *Bacillus amyloliquefaciens*

KPS46 30-day and 180-day shelf life stored at room temperature to suppress bacterial pustule of green soybean in greenhouse condition.

Treatment	Bacterial pustule severity (%)	
	30- day shelf life	180-day shelf life
KPS46 in dry formula	25.05b	37.54b
KPS46 fresh culture	21.19b	34.14b
Copper oxychloride	22.96b	38.12b
Non-treated control	48.15a	85.79a
CV.	13.35	20.46

Means followed by the same letter in the same column were not significantly different at $p = 0.05$.

5.2 Field experiment I -crop I

Disease control efficacy: Disease incidence of damping-off caused by *Sclerotium* sp. was assessed 14 days after planting at the field experiment. It showed that seed treated with KPS46 alone and directly combined with Liquid decomposed treatment expressed efficacy to suppress the damping-off. KPS46 maintained in talcum developed as wettable dry formula could suppress damping-off which was not different from fresh culture applied alone, combined application and fungicides. Its disease incidence was 9.94 % compared with non-treat control of disease incidence of 14.46%, while decomposed liquid combined with KPS46 cultured on MSI; KPS46 cultured on MSI, and KPS46 cultured on MSII applied alone showed the highest control efficacy against the damping-off with lowest disease incidence of 6.88, 7.23 and 7.64 % respectively. Disease incidence was significantly ($p=.05$) lower than treatment of fungicide (seed treated with carbendazim and foliar spray with copper oxychloride) of 12.09 %. They was also lower than natural products and bioproducts applied alone, including Liquid decomposed, Decomposed manure, amino acid, CaB, and algae-extract with disease incidence of 14.85, 13.20, 13.20, and 11.68 % respectively ($P=.05$). They were very distinguished disease control efficacy from non-treated with disease incidence of 14.46 %. The result in this experiment indicated that most application of KPS46 in wettable formula, in fresh culture of MSI and MSII, natural product, and bioproduct alone or by combination could suppress damping-off better or equal with fungicides application (Table 7).

The control efficacy against bacterial pustule evaluated 35 days after seeding, KPS46 applied alone and combined with natural products and bioproducts was applied by seed treatment and 14 day-interval 3 time foliar spray showed the good trend to control bacterial pustule. The control efficacy of KPS46 in talcum wettable formula (KPS46 – WP) was not different from treatment of KPS46 combined application treatment including, algae-extract + KPS46, Decomposed manure + KPS46, amino acid + KPS46, Liquid decomposed+KPS46, and CaB + KPS46 with 9.72, 9.76, 10.23, 10.37, and 10.39 % of disease incidence respectively, did not differ from KPS46 applied alone, including KPS46 – NGB, KPS46 – MSII,

and KPS46 – MSI with disease severities 9.90, 10.83, and 10.65 % respectively. The control efficacy level between application KPS46 alone and directly combined with natural products and bioproducts to fungicide with disease severity 9.16 % was found not to be different, but higher than non-treated control and applied alone with severity of 15.13 and 16.07 % respectively ($P=.05$), whereas natural products and bioproducts applied alone including, CaB, and algae-extract with severity 12.59 and 12.67 % respectively did not reduce severity compared with non-treated control (Table 7).

Consequences of heavy rain, disease severity of bacterial pustule were evaluated at 50 days after planting seriously damaged soybean. Disease severity assessed from soybean treated with KPS46 alone including fresh culture of KPS46 cultured on NGB, KPS46 in wettable powder formula, fresh culture of KPS46 cultured on MSI, and culture on MSII with disease severities of 67.76, 69.12, 69.24, and 73.33 % respectively and combined application with natural product and bioproduct including, CaB + KPS46, algae-extract + KPS46, Liquid decomposed + KPS46, amino acid + KPS46, and Decomposed manure + KPS46 with disease severities of 70.00, 70.37, 73.32, 77.39, and 80.00 % respectively. The application of KPS46 alone and directly combined with natural product and bioproduct did not show different efficacy in bacterial pustule control, and also not different from disease control by fungicide. All those treatments showed effective control of bacterial pustule better than non-treated control ($P=.05$). The experiment indicated that directly combined applied KPS46 with natural products and bioproducts did not reduce control efficacy of KPS46 (Table 7)

Growth promoting: Soybean plants were randomly sampled to select 10 plants/ plot sample size in order to evaluate growth promotion of both KPS46 applied alone and combined with natural products and bioproducts. The assessment of growth component revealed that KPS46 applied alone and directly combined with natural products and bioproducts, algae-extract, CaB + KPS46, amino acid, CaB+ KPS46 and algae-extract + KPS46 enhanced plant growth and showed the highest stem with 27.37, 27.07, 27.07, 26.87, and 26.73 centimeters respectively. They were significant

different from non-treated control with stem height of 24.87 centimeters, but did not significantly differ from another treatment of natural products, bioproducts, KPS46 applied alone and chemical treatment, whereas number of node and branches were not different for all treatment (Table 8).

Quantitative and qualitative of yield: Ten hills of soybean green at harvesting stage were randomly sampled to evaluate yield component, total yield, and marketable quality yield. The statistical analysis of collected data concluded that the total yield was no significant different, but the marketable quality yield was. The result indicated that KPS46 and natural products and bioproducts applied alone or combination enhanced marketable quality yield of vegetable soybean in field trial. All of KPS46 directly combined application with natural products and bioproducts stimulating plant growth and highly increased yield of green soybean. KPS46 combined application, Decomposed manure + KPS46, amino acid + KPS46, CaB + KPS46, algae-extract + KPS46, and KPS 46 in wettable powder formula applied alone and Chemical fertilizer gave the highest marketable quality yields of 1,907.97, 1,904.82, 1,901.87, 1,900.67, 1,891.97 and 1,888.00 kg/rai respectively, but were not significant different from treatment of Liquid decomposed +KPS46 of 1,845.25 kg/rai and treatment of application alone, KPS46 – MSII, KPS46 – MSI, algae – extract, Liquid decomposed, KPS46 – NGB, amino acid, and Decomposed manure with marketable quality yield 1,873.20, 1,820.93, 1,798.40, 1,798.27, 1,754.40 and 1.728.00 kg/rai respectively and were also not different from treatment of Chemical fertilizer Decomposed manure I + KPS46 (Table 9).

When calculated the marketable quality yield based on total yield into percentage, it indicated that all treatments of KPS46 application alone and combine application with natural products and bioproducts raised the ratio of marketable quality yield were higher than 50%, whereas the ratio of application of natural products and bioproducts alone, fungicides and non-treated control were equal or lower than 50 % (Table 9). For marketable quality yield grading using standard quality, the soybeans of 2 and 3 seed-pod including the pod size were not smaller than $1.3 \times 4.5 \times 0.7 \text{ cm}^3$ (Nguyen, 1998). The pod size of marketable quality from this

experiment showed equal or above standard, pod- size: 1.29 – 1.36 cm wide, 5.32 – 5.73 cm long and 0.75 – 0.83 cm thick, except 1.29 centimeters pod width of non-treated control being under standard of 1.3x4.5x0.7 cm³ (Table 10). From the result it could be concluded that KPS46 maintained in talcum as wettable formula could keep cell and exhibit growth promotion and control efficacy as well as fresh cell.

Table 7 Efficacy of combine application *Bacillus amyloliquefaciens* KPS46 with natural products and bioproducts to suppress disease of green soybean in field trial.

Treatment	Disease severity (%)			
	Damping – off (14 DAP)	Anthrax- nose (60 DAP)	Bacterial pustule (35 DAP) (50 DAP)	
Algae-extract + KPS46 – MSI	8.34cde	8.20cd	9.72cd	70.37bc
Amino acid + KPS46 – MSI	8.75cde	11.07bcd	10.23cd	77.39abc
CaB + KPS46 – MSI	9.10cde	12.27bcd	10.39cd	70.00bc
Decomposed manure + KPS46 – MSI	9.86bcde	7.73d	9.76cd	80.00abc
Liquid decomposed + KPS46 – MSI	6.88e	9.33bcd	10.37cd	73.32bc
KPS46 – MSI	7.23e	9.20bcd	10.65cd	69.24bc
KPS46 – WP	9.94bcde	9.54bcd	10.83bcd	69.12bc
KPS46 – MSII	7.64de	9.33bcd	10.83bcd	73.33bc
KPS46 – NGB	8.34cde	9.33bcd	9.90cd	67.76c
Chemical fertilizer	12.64abc	13.07b	13.14abc	81.10ab
Algae – extract	11.33abcd	12.40bc	12.59abc	77.40abc
Amino acid	13.20ab	10.73bcd	11.62bcd	80.75ab
CaB	11.68abc	12.33bc	12.67abc	74.44bc
Decomposed manure	13.20ab	10.60bcd	11.37bcd	78.14abc
Liquid decomposed	14.85a	13.33b	11.37bcd	76.26abc
Fungicide	12.09abc	13.13b	9.16d	80.73ab
Non-treated control	14.46a	17.87a	15.13a	86.68a

Means followed by the same letter in the same column were not significantly different at $p = 0.05$.

KPS46–MSI = KPS46 fresh culture growing on MS modified medium.

KPS46–MSII = KPS46 fresh culture growing on MS modified medium.

KPS46 – NGB = KPS46 fresh culture growing on nutrient broth.

Table 8 Potential of combination *Bacillus amyloliquefaciens* KPS46 with natural products and bioproducts to enhance growth of green soybean in field trial.

Treatment	Stem height (cm)	No. of Node	No. of Branch
Algae-extract + KPS46 – MSI	26.73ab	6.93a	6.37a
Amino acid + KPS46 – MSI	26.17abc	7.23a	6.97a
CaB + KPS46- MSI	27.07ab	7.57a	7.16a
Decomposed manure + KPS46 – MSI	25.27bc	7.33a	7.00a
Liquid decomposed+KPS46 – MSI	25.50bc	7.91a	7.07a
KPS46 – MSI	26.13abc	7.60a	6.83a
KPS46 – wetable powder	26.17abc	8.23a	7.77a
KPS46 – MSII	25.27bc	7.43a	7.57a
KPS46 – NGB	25.67abc	7.47a	7.03a
Chemical fertilizer	26.00abc	7.03a	7.10a
Algae-extract	27.37a	8.17a	7.93a
Amino acid	26.87ba	7.77a	7.40a
CaB	26.87ab	7.70a	7.73a
Decomposed manure	25.33bc	7.53a	6.57a
Liquid decomposed	26.87ab	7.77a	6.87a
Fungicide	25.07abc	7.97a	7.17a
Non-treated control	24.87c	7.57a	6.77a

Means followed by the same letter in the same column were not significantly different at $p = 0.05$.

KPS46–MSI = KPS46 fresh culture growing on MS modified medium.

KPS46–MSII = KPS46 fresh culture growing on MS modified medium.

KPS46 – NGB = KPS46 fresh culture growing on nutrient broth.

Table 9 Potential of combination *Bacillus amyloliquefaciens* KPS46 with natural products and bioproducts to enhance and gain yield of green soybean in field trial.

Treatment	Total yield (kg/rai)	Good pod weight (kg/rai)	Percentage of good pod weight
Algae-extract + KPS46- MSI	3,366.64	1,900.67a	58.49
Amino acid + KPS46- MSI	3,620.00	1,904.82a	52.83
CaB + KPS46- MSI	3,646.64	1,901.87a	53.71
Decomposed manure + KPS46- MSI	3,260.00	1,907.97a	59.64
Liquid decomposed+KPS46- MSI	3,333.36	1,845.25ab	58.28
KPS46 - MSI	3,546.64	1,837.80ab	56.21
KPS46 – wetable powder	3,682.24	1,891.97a	51.68
KPS46 - MSII	3,600.00	1,873.20ab	67.81
KPS46 - NGB	3,106.64	1,798.27ab	59.30
Chemical fertilizer	3,540.00	1,888.00a	53.88
Algae-extract	3,580.00	1,820.93ab	50.88
Amino acid	3,746.64	1,734.40ab	47.38
CaB	3,453.36	1,659.65bc	49.20
Decomposed manure	3,906.64	1,728.00ab	46.07
Liquid decomposed	4,073.36	1,798.40ab	45.46
Fungicide	3,493.36	1,555.07cd	47.76
Non-treated control	3,166.64	1,480.45d	47.07

Means followed by the same letter in the same column were not significantly different at $p = 0.05$.

KPS46–MSI = KPS46 fresh culture growing on MS modified medium.

KPS46–MSII = KPS46 fresh culture growing on MS modified medium.

KPS46 – NGB = KPS46 fresh culture growing on nutrient broth.

Table 10 Potential of combination *Bacillus amyloliquefaciens* KPS46 with natural products and bioproducts to improve yield quality of green soybean in field trial.

Treatment	Pod width (cm)	Pod length (cm)	Pod thickness (cm)
Algae-extract + KPS46- MSI	1.30	5.54	0.79
Amino acid + KPS46- MSI	1.32	5.41	0.78
CaB + KPS46- MSI	1.32	5.45	0.77
Decomposed manure + KPS46- MSI	1.33	5.49	0.80
Liquid decomposed+KPS46- MSI	1.33	5.63	0.78
KPS46 - MSI	1.32	5.42	0.78
KPS 46 – wetable powder	1.32	5.54	0.83
KPS46 - MSII	1.31	5.37	0.83
KPS46- NGB	1.30	5.73	0.83
Chemical fertilizer	1.31	5.33	0.77
Algae-extract	1.30	5.51	0.78
Amino acid	1.34	5.56	0.75
CaB	1.30	5.68	0.85
Decomposed manure	1.31	5.61	0.82
Liquid decomposed	1.32	5.51	0.82
Fungicide	1.30	5.37	0.79
Non-treated control	1.29	5.52	0.76

Means comparison were not significantly different at $p = 0.05$.

KPS46–MSI = KPS46 fresh culture growing on MS modified medium.

KPS46–MSII = KPS46 fresh culture growing on MS modified medium.

KPS46 – NGB = KPS46 fresh culture growing on nutrient broth.

5.3 Field experiment I -crop II

Disease control efficacy: Treatments from the first crop (Decomposed manure I) were selected. Seeds were treated with fresh ISR bacterial SW01/14 cultured on nutrient broth and sowed after mixed local bioproduct decompose cow dung. The application was followed with soil and following up by 3 times -14 day interval foliar spray, started 14 days after seed sowing. Decomposed commercial pellet (Decomposed manure II) was combination applied with fresh KPS46 (culture on MSI) the as same as treatment above expressed. They gave the best effective control damping-off seedling with less disease incidence of 6.19 and 6.25 % respectively. The control efficacy of those treatments did not differ from decomposed manure and KPS46 alone and combine application, chemical control (seed treated with carbendazin and foliar spray with copper oxychloride) and *B. subtilis*, commercial product, but significantly differ from non-treated control ($P>F=.05$) with 14.38 % disease incidence (Table 11). Although KPS46 maintained in talcum as wettable formula did not show the highest control efficacy to decrease damping-off seedling, most of treatments of wettable formula included combined application with Decomposed manure II, Decomposed manure I, and applied alone could suppress damping-off were non-significant different from the highest treatment mentioned above with disease severities of 6.75, 7.50 and 8.63% respectively. Disease incidence of damping-off caused by *Sclerotium* sp. was assessed 14 days after planting of second crop seemed to be less severity than the first cropping season, the reason of lately rainy season.

The disease severity of bacterial pustule from natural infection was not recorded in this cropping season. The assessment of anthracnose incidence infected on pod showed that Decomposed manure II combined applied with *B. subtilis* gave the highest control with 3.50 % disease severity, followed by Decomposed manure I + KPS 46, *Bacillus subtilis*, and SW01/4 with similar 3.50 % disease severity. The severity of all those treatment above did not significantly differ, but differed from non-treated control with 7.25 % disease severity (Table 11). KPS46 maintained in talcum as wettable formula of most treatment including treatments of combined

application with Decomposed manure I, Decomposed manure II and applied alone could decrease disease severities of 4.25, 4.75 and 4.75 % respectively. The results in the second crop were similar to the first crop. The KPS46 maintained in talcum as wettable formula of most treatment including treatments of combined application and applied alone did not show significant difference from treatment of KPS46 and tested antagonistic bacteria SW01/4, indigenous bacteria isolated from this area, LPT09 in fresh culture; and commercial wettable formula of *B. subtilis* but it showed distinguishably higher significant than non-treated control

Growth promoting: The evaluation growth promotion by both KPS46 applied alone and combine applied with natural products and bioproducts compared with LPT09, SW01/14, and commercial product *B. subtilis* was under taken. Decomposed commercial pellet (Decomposed manure II) applied alone, Local bioproduct of decomposed cow dung, selected from the first cropping season (Decomposed manure I) combine applied with KPS46 in treatment of Decomposed manure I+ LPT09, Decomposed manure I+ KPS 46, Decomposed manure I. + SW01/4 and Chemical fertilizer+ fungicides promoted the highest soybean plant growth with stem heights of 36.79, 36.78, 36.75, 36.70, and 36.68 cm respectively were significant different from non-treated control and KPS46 applied alone in treatment of KPS46 WP, SW01/4 nutrient broth with stem heights of 34.56, 35.20 and 34.81cm respectively. Several formulas of KPS46 applied alone, *Bacillus subtilis*, KPS46-MSI and LPT09 nutrient broth stimulated plant growth did not differ from combine applied with bioproduct. The numbers of nod and numbers of branch were 6.43 - 7.80 nods and 3.85 – 5.53 branches. The data did not show statistical difference in most of those treatments (Table 12).

Quantitative and qualitative yields: The result from the second crop showed that the total yields of all treatments were not significant different from one another. KPS46 combine application with natural products and bioproducts also enhancing the marketable quality yield were not significant different in this field trial experiment similar to the first crop. Combine application treatment of Decomposed

manure II + *B. subtilis* and Decomposed manure II. + KPS 46 gave the significantly (P=.05) highest marketable quality yields of 2,384.00 and 2,294.00 kg/rai respectively and significantly higher than Chemical fertilizer + fungicides treatment with yield of 1,721.60 kg/rai. The other treatment of KPS 46 combine application with natural product and bioproduct such as Decomposed manure II + SW01/4, Decomposed manure I + LPT09, Decomposed manure I + KPS46, and Decomposed manure II + KPS46 (WP); gave yields of 1,939.20, 1,856.00, 1,840.00, and 1,792.00 kg/rai respectively, equaling to chemical fertilizer + fungicides, but higher than non-treated control of 1,424.00 kg/rai (Table 13). The pod size of marketable quality from this experiment showed equal or above standard (Table 14).

Table 11 Efficacy of combination *Bacillus amyloliquefaciens* KPS46 with bioproduct and natural product on disease severity of vegetable soybean.

Treatment	Disease severity (%)	
	Damping-off ^{1/} (14 DAP)	Anthrachnose (45 DAP)
Decomposed manure I + <i>B. subtilis</i> ^{1/}	8.06bc	4.25cde
Decomposed manure I + KPS 46	8.13bc	3.50de
Decomposed manure I. + SW01/4	6.19c	4.75bcde
Decomposed manure I + LPT09	8.75bc	5.75abc
Decomposed manure I + KPS 46 (WP) ^{1/}	7.50bc	4.25cde
Decomposed manure II + <i>B. subtilis</i>	8.75bc	3.00e
Decomposed manure II. + KPS 46	6.25c	5.50abc
Decomposed manure II + SW01/4	9.38b	4.25cde
Decomposed manure II + LPT09	6.94bc	6.50ab
Decomposed manure II + KPS 46 (WP)	6.75bc	4.75bcde
Decomposed manure I	7.50bc	4.50bcde
Decomposed manure II	9.13bc	4.50bcde
Chemical fertilizer + fungicides	8.13bc	4.25cde
Non-treated control	14.38a	7.25a
<i>B. subtilis</i>	8.13bc	3.50de
KPS46-MSI	7.88bc	4.25cde
SW01/4-NB	7.94bc	3.50de
LPT09-NB	6.88bc	4.50bcde
KPS46-WP	8.63bc	4.75bcde

Means followed by the same letter in the same column were not significantly different at $p = 0.05$.

^{1/}Disease severity of damping-off = Number of damping-off seedling X 100 / Total of sampling seedling.

Table 12 Potential of combination *Bacillus amyloliquefaciens* KPS46 with natural products and bioproducts to enhance growth of green soybean in field trial.

Treatment	Stem height (cm)	No. of node	No. of branch
Decomposed manure I + <i>B. subtilis</i>	36.58ab	7.20a	4.03a
Decomposed manure I + KPS46	36.75a	7.03a	4.13a
Decomposed manure I. + SW01/4	36.70a	6.70a	4.53a
Decomposed manure I + LPT09	36.78a	6.63a	4.35a
Decomposed manure I + KPS46 (WP)	36.31abc	7.00a	4.40a
Decomposed manure II + <i>B. subtilis</i>	36.50ab	6.75a	4.65a
Decomposed manure II. + KPS46	36.16abc	6.60a	4.05a
Decomposed manure II + SW01/4	35.80abcd	6.85a	4.05a
Decomposed manure II + LPT09	35.69abcd	7.10a	5.03a
Decomposed manure II + KPS46 (WP)	35.75abcd	6.30a	5.53a
Decomposed manure I	35.4abcd	7.43a	4.25a
Decomposed manure II	36.79a	7.80a	4.63a
Chemical fertilizer + fungicides	36.68a	7.58a	4.05a
Non-treated control	34.56de	6.70a	3.85a
<i>B. subtilis</i>	35.73abcd	7.20a	4.75a
KPS46-MSI	35.53abcd	7.00a	4.70a
SW01/4-NB	34.81cde	6.43a	4.07a
LPT09-NB	35.48abcd	6.68a	4.88a
KPS46-WP	35.20bcd	6.83a	4.60a

Means followed by the same letter in the same column were not significantly different at $p = 0.05$.

Table 13 Potential of combination *Bacillus amyloliquefaciens* KPS46 with natural products and bioproducts to enhance and gain yield of green soybean in field trial.

Treatment	Total yield (kg)	Good pod weight (kg.)	Percentage of good pod weight
Decomposed manure I + <i>B. subtilis</i>	3,027.59	1,635.20bcd	54.01
Decomposed manure I + KPS 46	3,066.67	1,840.00bc	60.00
Decomposed manure I. + SW01/4	3,135.46	1,648.00bcd	52.56
Decomposed manure I + LPT09	3,071.32	1,856.00bc	60.43
Decomposed manure I + KPS 46 (WP)	3,088.13	1,696.00bcd	54.92
Decomposed manure II + <i>B. subtilis</i>	3,984.62	2,384.00a	59.83
Decomposed manure II. + KPS 46	3,813.83	2,294.40a	60.16
Decomposed manure II + SW01/4	3,746.52	1,939.20b	51.76
Decomposed manure II + LPT09	3,363.18	1,728.00bcd	51.38
Decomposed manure II+KPS 46 (WP)	3,189.18	1,792.00bcd	56.19
Decomposed manure I	3,490.57	1,776.00bc	50.88
Decomposed manure II	3,013.29	1,632.00bcd	54.16
Chemical fertilizer + fungicides	3,151.38	1,721.60bcd	54.63
Non-treated control	2,982.82	1,424.00d	47.74
<i>B. subtilis</i>	2,905.91	1,593.60bcd	54.84
KPS46-MSI	3,347.02	1,712.00bcd	51.15
SW01/4-NB	2,830.62	1,616.00bcd	57.09
LPT09-NB	3,052.31	1,488.00cd	48.75
KPS46-WP	3,102.40	1,536.00cd	49.51

Means followed by the same letter in the same column were not significantly different at $p = 0.05$.

Table 14 Potential of combination *Bacillus amyloliquefaciens* KPS46 with natural products and bioproducts to improve yield quality of green soybean in field trial.

Treatment	Pod width (cm)	Pod length (cm)	Pod thickness (cm)
Decomposed manure I + <i>B. subtilis</i>	1.32	5.31	0.73
Decomposed manure I + KPS 46	1.32	5.61	0.74
Decomposed manure I. + SW01/4	1.33	5.45	0.75
Decomposed manure I + LPT09	1.31	5.11	0.72
Decomposed manure I + KPS 46 (WP)	1.31	5.45	0.73
Decomposed manure II + <i>B. subtilis</i>	1.30	5.55	0.72
Decomposed manure II. + KPS 46	1.32	5.37	0.72
Decomposed manure II + SW01/4	1.34	5.48	0.73
Decomposed manure II + LPT09	1.36	5.45	0.75
Decomposed manure II+KPS 46(WP)	1.30	5.37	0.72
Decomposed manure I	1.32	5.51	0.74
Decomposed manure II	1.34	5.10	0.74
Chemical fertilizer + fungicides	1.31	5.57	0.72
Non-treated control	1.28	5.19	0.73
<i>B. subtilis</i>	1.31	5.16	0.89
KPS46-MSI	1.32	5.28	0.72
SW01/4-NB	1.29	5.31	0.72
LPT09-NB	1.29	5.60	0.72
KPS46-WP	1.31	5.27	0.73

5.4 Field experiment II

The field experiment to evaluate the potential of the developed dry wettable formula to enhance germination rate and also to suppress seedling damping-off caused by *S. rolfsii* was carried out. The result showed that dry formulation of KPS46 using talcum as carrier, application by seed bacterilization and 3 times foliar spray 14, 28 and 35 day after planting enhanced 83.29 % seed germination rate. It was similar to chemical treatment application by seed treatment with carbendazim and foliar spray again 14 days after planting and 2 times foliar spray 28 and 35 days after planting with copper oxychloride with germination rate of 85.17 %. It was not different from commercial produce application of *B. subtilis* in wettable formula (Larminar) of 84.37 % germination rate. The result revealed that KPS46 in wettable formula enhanced better than fresh culture and non-treated control with 75.34 and 74.00 % germination rates respectively. (Table 15)

The field trial of KPS46 in wettable formula to control seedling damping-off green soybean was assessed to evaluate the formula potential. The disease incidence of damping-off assessed 14 day after planting indicated that wettable formula of KPS46 of 4.18 % was similar to KPS46 fresh culture of 4.02 %. It suppressed disease incidence better than non-treated control with 8.82 %. It showed control efficacy equaling to chemical control of seed treatment with carbendazim with 3.03 % disease incidence (Table 15).

The potential of developed wettable formula of KPS46 to control anthracnose assessed 45 and 65 days after planting and control bacterial pustule 45 days after planting was compared with fresh culture of KPS46, commercial product of *Bacillus subtilis* and chemical fungicides (carbendazim and copper oxychloride). The anthracnose incidence assessed 45 days after planting of soybean plant treated with wettable formula of KPS46 was 7.18 %. The control efficacy was similar to that treated with fresh culture of KPS46, fungicides and commercial biological control agent product with disease incidences of 6.02, 6.87 and 8.02 % respectively. All those treatments are better at suppressing anthracnose than the 11.96 % highest

disease incidence of non-treated control (Table 16). Likewise the incidence on plant part, the incidence on pod assessed 65 days after planting (harvest time) and all treatment showed the better control efficacy than non-treated control. Disease incidence controlled by chemical fungicides, fresh culture of KPS46, wettable formula of KPS46, and commercial product were 9.08, 9.30, 9.47, and 9.66% respectively compared with non-treated control of 15.19 % (Table 16). The efficacy to control bacterial pustule assessed 45 days after planting resulting from field trial exhibited the control efficacy of applied wettable formula of KPS46 to reduce disease incidence of 12.08 % which was not different from fresh culture of KPS46, fungicides and commercial biological agent products of 11.28, 13.18 and 15.57 % respectively, but better than 17.42 % non-treated control (Table 16).

Growth enhancement of green soybean in field trial of developed wettable formula of KPS46 was investigated comparing with fresh culture, commercial biological control agent product, fungicides, and non-treated control at Corn and Sorghum National Research Center. The investigation in field showed that each growth component was not different, only total fresh weight 65 days after planting expressed significantly different from non-treated control. The fresh weight of green soybean from application with wettable formula of KPS46 was 4,586.67 kg/rai, which was not significant different from fresh culture and fungicides of 4,533.33 and 4,486.67 kg/rai respectively. However, it showed contrast higher weight than commercial biological control agent product and non-treated control of 4,266.67 and 3,804.33 kg/rai respectively (Table 17).

Quantitative and qualitative yields: Yield component, total yield, and marketable quality yield were assessed for the final evaluation of developed wettable formula of KPS46. Ten hills of green soybean at harvesting stage were randomly sampled to evaluation. The collected data subjected to analysis and was concluded that both total yield and marketable quality yield were significantly different. The data in Table 18 show the highest total yields from fungicides treatment, wettable formula of KPS46, and fresh culture of 2,776.32, 2,758.40 and 2,726.40 kg/rai respectively, which were higher than commercial biological agent product and non-treated control

of 2,625.28 and 2,295.04 kg/rai respectively. The total yield compared between treatment of fresh culture of KPS46 was found not be different from the commercial biological agent product (Table 18). When focused on marketable yield, the result show that the treatment of wettable formula treatment of fungicides and fresh culture of KPS46 gave highest yield of 1,779.20, 1,740.80 and 1,721.60 kg/rai respectively, but better than commercial biological agent product and non-treated control of 1,632.00 and 1,373.44 kg/rai respectively (Table 18). When the ratios of marketable yield based on total yield were change into percentage, wettable formula of KPS46 gave the highest ratio of 64 %, followed by fresh culture of KPS46, commercial biological agent product, fungicides and non-treated control of 62.40, 62.02, 60.81 and 54.86 % respectively (Table 18).

The quality of marketable yield graded using standard quality must be 2 and 3 seed-pod which the pod size including wide, length and thickness were not smaller than $1.3 \times 4.5 \times 0.7 \text{ cm}^3$ (Nguyen, 1998). The pod size of marketable quality from the treatment of developed wettable formula, and fresh culture of KPS46, commercial biological control agent product, fungicides, and non-treated control were $1.35 \times 5.37 \times 0.83$, $1.35 \times 5.32 \times 0.85$, $1.34 \times 5.63 \times 0.91$, $1.33 \times 5.20 \times 0.83$, and $1.32 \times 5.15 \times 0.82 \text{ centimetre}^3$ respectively. Most treatment showed all pod size parameters above standard (Table 19).

6. Cost of production and return on investment

The most cost in green soybean production, return, benefit and rate of return on investment in this experiment to compare between conventional applications of fungicide with biological control application KPS46 developed at wettable formula were done. Cost/rai of conventional was 13,030.82 Baht higher than 12,415.57 Baht, cost of biolocal control. The return, rai of conventional and biological control were 17,408.00 and 17,792.00 Baht respectively, which benefit of 4,377.18 and 5,376.43 Baht respectively. When calculated ROI it showed the high ROI in both case but the biological control gave ROI of 43.30% higher than conventional of 33.59% (Table 20). The result revealed that biological control application KPS46 in wettable

developed in this research spent lower loan to investment while gave the higher return on investment than conventional.

Table 15 Potential of developed wettable formula of *Bacillus amyloliquefaciens* KPS46 to enhance germination rate and suppress damping-off of green soybean in field trial.

Treatment ^{1/}	Germination rate (%)	Damping-off incidence (%)
KPS46 (WP)	83.29a	4.18c
KPS46 (MS)	75.34b	4.02c
Carbendazim + Copper oxychloride	85.17a	3.03c
Comercial BCA	84.37a	5.88b
Non-treated	74.00b	8.82a
CV.	4.99	9.76

Means followed by the same letter in the same column were not significantly different at $p = 0.05$.

- 1/ - KPS46 (WP) = KPS46 maintain in wettable formula application by seed bacterilization and 3 times foliar spray at 14, 28 and 35 day after planting.
- KPS46 (MS) = KPS46 fresh culture growing on MS modified medium application by seed bacterilization and 3 times foliar spray at 14, 28 and 35 day after planting.
 - Carbendazim + Copper oxychloride = chemical treatment application by seed treatment with carbendazim and foliar spray again at 14 days after planting follow with 2 times foliar spray at 28 and 35 days after planting with copper oxychloride.
 - Comercial BCA = commercial produce of *Bacillus subtilis* in wettable formula (Larminar) application by seed bacterilization and 3 times foliar spray at 14, 28 and 35 day after planting.

Table 16 Potential of developed wettable formula of *Bacillus amyloliquefaciens* KPS46 control anthracnose and bacterial pustule of green soybean in field trial.

Treatment ^{1/}	Anthracnose incidence on plant (%)	Anthracnose incidence on pod(%)	Bacterial pustule severity (%)
KPS46 (WP)	7.18b	9.47b	12.08bc
KPS46 (MS)	6.02b	9.30b	11.28c
Carbendazim + Copper oxychloride	6.87b	9.08b	13.18bc
Comercial BCA	8.02b	9.66b	15.57ab
Non-treated	11.96a	15.19a	17.42a
CV.	20.06	7.62	14.98

Means followed by the same letter in the same column were not significantly different at $p = 0.05$.

- 1/ - KPS46 (WP) = KPS46 maintain in wettable formula application by seed bacterilization and 3 times foliar spray at 14, 28 and 35 day after planting.
- KPS46 (MS) = KPS46 fresh culture growing on MS modified medium application by seed bacterilization and 3 times foliar spray at 14, 28 and 35 day after planting.
 - Carbendazim + Copper oxychloride = chemical treatment application by seed treatment with carbendazim and foliar spray again at 14 days after planting follow with 2 times foliar spray at 28 and 35 days after planting with copper oxychloride.
 - Comercial BCA = commercial produce of *Bacillus subtilis* in wettable formula (Larminar) application by seed bacterilization and 3 times foliar spray at 14, 28 and 35 day after planting.

Table 17 Potential of developed wettable formula *Bacillus amyloliquefaciens* KPS46 to enhance growth of green soybean in field trial.

Treatment ^{1/}	Stem height (cm)	No. of node	No. of branch	Fresh weight (kg)
KPS46 (WP)	35.87	7.33	2.47	4586.67a
KPS46 (MS)	38.53	5.97	2.73	4533.33a
Carbendazim + Copper oxychloride	37.60	6.10	2.90	4486.67a
Comercial BCA	36.37	6.97	2.33	4266.67b
Non-treated	34.70	6.90	2.50	3804.33c
CV.	6.39	20.46	20.53	2.16

Means followed by the same letter in the same column were not significantly different at $p = 0.05$.

- ^{1/} - KPS46 (WP) = KPS46 maintain in wettable formula application by seed bacterilization and 3 times foliar spray at 14, 28 and 35 day after planting.
- KPS46 (MS) = KPS46 fresh culture growing on MS modified medium application by seed bacterilization and 3 times foliar spray at 14, 28 and 35 day after planting.
 - Carbendazim + Copper oxychloride = chemical treatment application by seed treatment with carbendazim and foliar spray again at 14 days after planting follow with 2 times foliar spray at 28 and 35 days after planting with copper oxychloride.
 - Comercial BCA = commercial produce of *Bacillus subtilis* in wettable formula (Larminar) application by seed bacterilization and 3 times foliar spray at 14, 28 and 35 day after planting.

Table 18 Potential of developed wettable formula *Bacillus amyloliquefaciens* KPS46 to enhance and gain yield of green soybean in field trial.

Treatment ^{1/}	Total yield (kg/rai)	Good pod weight (kg/rai)	Percentage of good pod weight
KPS46 (WP)	2,758.40a	1,779.20a	64.54
KPS46 (MS)	2,726.40ab	1,721.60a	62.40
Carbendazim + Copper oxychloride	2,776.32a	1,740.80a	60.81
Comercial BCA	2,625.28b	1,632.00b	62.02
Non-treated	2,295.04c	1,373.44c	54.86
CV.	3.87	2,86	7.47

Means followed by the same letter in the same column were not significantly different at $p = 0.05$.

- ^{1/} - KPS46 (WP) = KPS46 maintain in wettable formula application by seed bacterilization and 3 times foliar spray at 14, 28 and 35 day after planting.
- KPS46 (MS) = KPS46 fresh culture growing on MS modified medium application by seed bacterilization and 3 times foliar spray at 14, 28 and 35 day after planting.
- Carbendazim + Copper oxychloride = chemical treatment application by seed treatment with carbendazim and foliar spray again at 14 days after planting follow with 2 times foliar spray at 28 and 35 days after planting with copper oxychloride.
- Comercial BCA = commercial produce of *Bacillus subtilis* in wettable formula (Larminar) application by seed bacterilization and 3 times foliar spray at 14, 28 and 35 day after planting.

Table 19 Potential of developed wettable formula of *Bacillus amyloliquefaciens* KPS46 to improve yield quality of green soybean in field trial.

Treatment ^{1/}	Pod width (cm)	Pod length (cm)	Pod thickness (cm)
KPS46 (WP)	1.35	5.37	0.86
KPS46 (MS)	1.35	5.32	0.85
Carbendazim + Copper oxychloride	1.34	5.63	0.91
Comercial BCA	1.33	5.20	0.83
Non-treated	1.32	5.15	0.82
CV.	6.62	7.99	6.03

- ^{1/} - KPS46 (WP) = KPS46 maintain in wettable formula application by seed bacterilization and 3 times foliar spray at 14, 28 and 35 day after planting.
- KPS46 (MS) = KPS46 fresh culture growing on MS modified medium application by seed bacterilization and 3 times foliar spray at 14, 28 and 35 day after planting.
- Carbendazim + Copper oxychloride = chemical treatment application by seed treatment with carbendazim and foliar spray again at 14 days after planting follow with 2 times foliar spray at 28 and 35 days after planting with copper oxychloride.
- Comercial BCA = commercial produce of *Bacillus subtilis* in wettable formula (Larminar) application by seed bacterilization and 3 times foliar spray at 14, 28 and 35 day after planting.

Table 20 Cost and return on investment per rai compared between conventional cultural practice application chemical fungicides with biocontrol application antagonistic bacterial *Bacillus amyloliquefaciens* KPS46 conducted at Sorghum and Corn National Research Center , Pack Chong, Nakhon Ratchasrima.

List of cost	Conventional	Biocontrol
Material		
Seed	800.00	800.00
Chemical fertilizer	1,125.00	1,125.00
Biological antagonist KPS46	-	28.81
Carbendazim	185.60	-
Copper oxychloride	400.00	-
Insecticides	432.00	432.00
Herbicides	650.00	650.00
Employed labor wages		
Planting preparation	600.00	600.00
Herbicides spraying	150.00	150.00
Seed sowing	300.00	300.00
Insecticides and disease control spraying	800.00	800.00
Weeding and fertilizer application	1,050.00	1,050.00
Harvesting	1,200.00	1,200.00
Irrigation	600.00	600.00
Rent of production area (indirect cost)	3,500.00	3,500.00
Total	11,792.60	11,235.81
Rate (10.5 %)	1,238.22	1,179.76
Total cost	<u>13,030.82</u>	<u>12,415.57</u>
Return (฿10/ Kg)	<u>17,408.00</u>	<u>17,792.00</u>
Benefit	<u>4,377.18</u>	<u>5,376.43</u>
Return on investment (ROI)	<u>33.59</u>	<u>43.30</u>

7. Antagonistic mechanism of *B. amyloliquefaciens* KPS46 against bacterial pustule

7.1 Generating of UV mutants

The UV-radiated plates incubated at room temperature (28 °C) in the dark until the colonies to be visible in order to detect colonies produce minus secondary metabolite mutants derived from UV mutagenesis was evaluated. Bacterial Xag inhibition clear zone was observed to determine and select the colony. The selected anti bacterial secondary metabolite of UV mutagenesis colonies by evaluated inhibition clear zone on Xag agar found that 10 colonies did not still produce secondary metabolite to inhibit Xag. The 22 out of 32 sampling colonies showed various inhibition clear zones, two of them were similar as KPS46 wildtype, the 12 colonies were shown decreasing inhibition clear zone and 8 colonies increasing production of secondary metabolite showed the larger clear zone (data did not show).

7.2 PCR confirm identification

16S-rDNA was better at confirming colonies of *B. amyloliquefaciens* strains. The 16S-rDNA conserve region was confirmed by PCR analysis with primers that described in method above. KPS46 wildtype and six UV mutant strains were grown on NGB. The wildtype KPS46 and six UV mutant strains are M6, M16, M17, M20, M24 and M33 were amplified the target product (Fig.7). The wildtype and all UV mutant strain were used in the next step.

8. Detection of surfactin

When the surfactin lipopeptide extract from the culture fluid of KPS46 was analyzed by HPLC, high concentrations of bioactive non-polar antibiotics were detected (Fig. 8). Surfactin-type lipopeptides were identified in the extract on the basis of their retention times being similar to those of purified surfactin standards, such as surfactin produced by *B. subtilis* ATCC21332 (Fig 8). C18 homologues represented together more than 50% of the total amount of surfactin lipopeptides present in the

extract. Based on HPLC peak areas of the surfactin lipopeptide extract compared with values obtained for standards, the total amount of surfactins produced by strain KPS46 was $550 \text{ mg l}^{-1} \pm 20.267 \text{ mg l}^{-1}$ (mean and standard deviation calculated from three independent cultures). The strains of KPS46 was detected the surfactin product but four UV mutant M6 was unable to detect the product (Fig. 8).

9. PCR confirm identification of the *urfAA*.

The *urfAA* was detected in KPS46 wildtype and three strains of *B. amyloliquefaciens* UV mutant strains following PCR analysis. Primers complementary to conserved regions for *urfAA* in *B. amyloliquefaciens* was used. An amplification product of 650-bp was observed for all KPS46 and its UV mutant strains that produced surfactin lipopeptide (Fig 9). The result indicated that KPS46 and three UV mutant strains of *B. amyloliquefaciens* have the *urfAA* gene.

10. Extracellular enzyme assay

Alpha-amylase plate assay: The production of extracellular enzymes including amylase, cellulases, chitinase β -glucanase and proteases were investigated using plate assay method to compare differentiated characteristic between UV mutagenesis and wildtype strains. UV mutants of KPS 46 were investigated in vitro on alpha-amylase assay medium using hydrolysis starch after incubating for 24 hours and flooding plates with iodine solution. Both UV mutagenesis and KPS46 wildtype showed the positive a zone of clearing around the colony. In this test, the results indicated that both UV mutagenesis and KPS46 could produce amylase in vitro but different ability to produce amount of amylase/high activity index from various sizes of clear zone. The amylase production in order to clear zone size convinced that UV mutant M6 produced higher activity of amylase than wildtype (Table 21).

Endoglucanase plate assay: Endoglucanase, one of enzyme complex breaks down polysaccharides such as cellulose and xyloglucan to be nonspecific, releasing reducing sugar. The investigation of endoglucanase producing plate assay using NGYA medium adding CMC enzyme activity detected from a yellow light halo clear

zone surrounding the colonies against the orange background appeared both wildtype and UV mutant. The result showed wildtype of KPS46 produced the high activity of this enzyme with clear zone of 16.00 centimeters diameter It was bigger than UV mutant M6 of 10.00 millimeters (Table 21)

Chitinase plate assay: Chitinase secretion enzyme of KPS46 and UV mutant were evaluated on chitin plate assay. This assay was prepared according to the method of Sampson and Gooday (1998). Chitin hydrolysis was detected by formation of a whitish, opaque halo around a translucent area (totally hydrolysed chitin) surrounding the growing colony. The results revealed that most of KPS46 and UV mutagenesis did not produce chitinase (Table 21).

Protease plate assay: Chemoorganotrophs microorganism survival on absorbing smaller molecules, amino acid by producing protease to catalyze from large protein to smaller form. The ability to produce extracellular proteases degradation enzymes of wildtype and UV mutant of KPS46 were detected by plate diffusion assay using NYGA added skim milk medium. Protease enzymatic activity secretion by KPS46 and UV mutant assessed by diffuse clear zone of degradation skim milk was shown ability to produce protease most of wildtype and UV mutant. Wildtype of KPS46 showed the highest protease productivity of 15.00 millimeters clear zone diameter over UV mutant M6 of 13.00 millimeters (Table 21).

Table 21 Plate assays of extracellular enzymes included amylase, cellulases, chitinase endoglucanase and proteases compared between UV mutant M6 and wildtype of *Bacillus amyloliquefaciens* KPS46.

UV radiation mutagenesis	Clear zone diameter (mm)			
	amylase	Chitinase	Endoglucanase	Protease
Mutant M6	18.33a	-	10.00a	13.00a
KPS46	12.50b	-	16.00b	15.00a

Means followed by the same letter in the same column were not significantly different at $p = 0.05$.

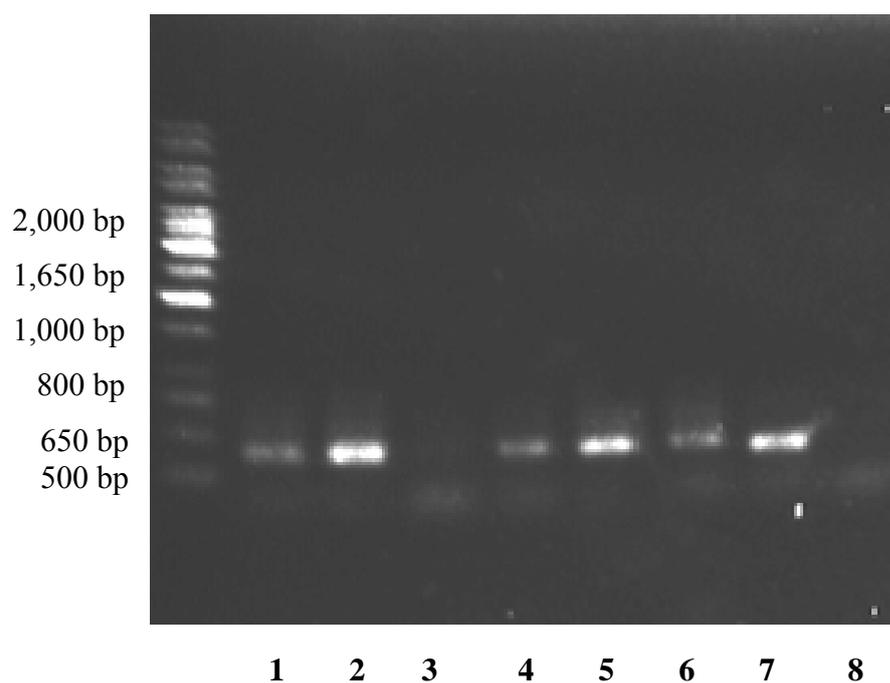
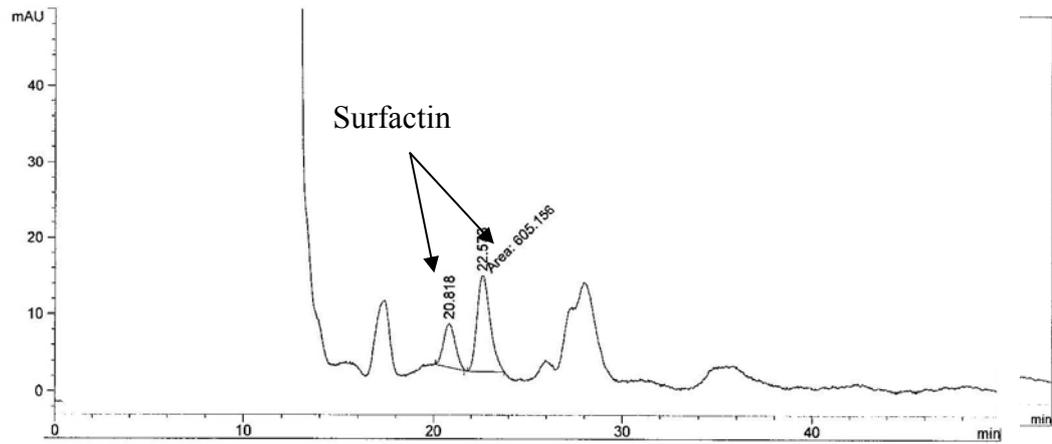
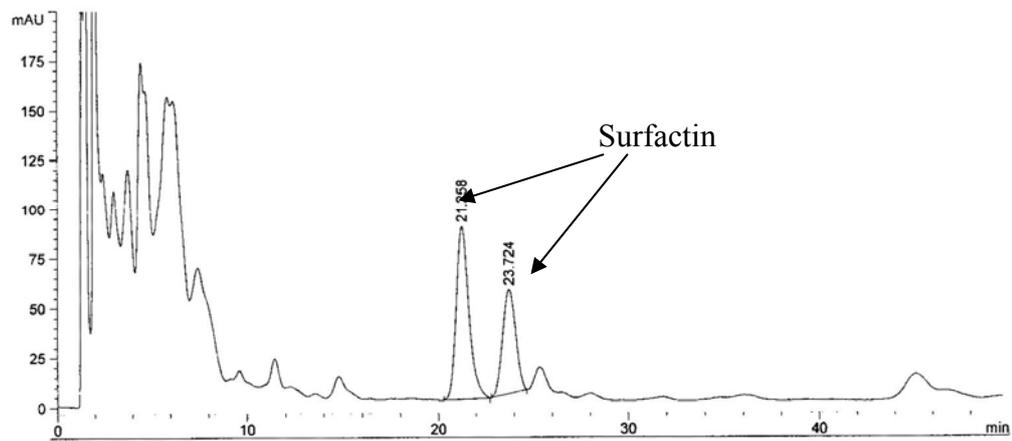


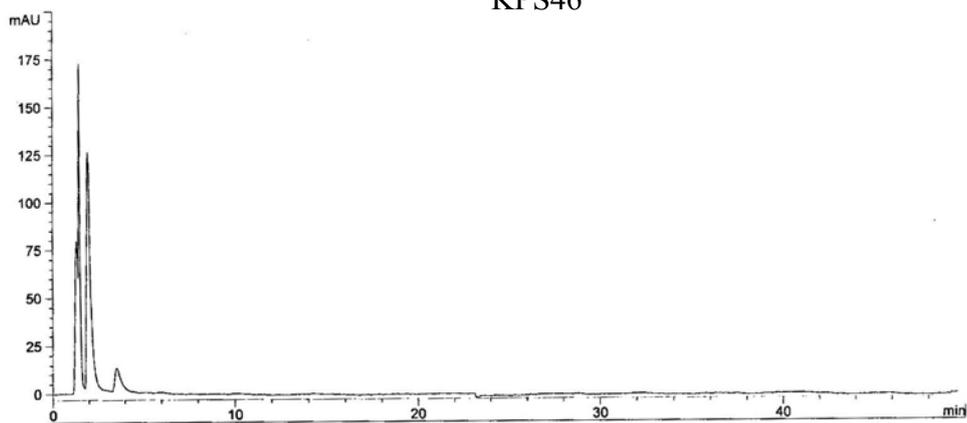
Figure 7 Polymerase chain reaction (PCR) amplified the 16S-rDNA of *Bacillus amyloliquefaciens* KPS46 UV mutant by 16S-F and 16S-R primers. The PCR fingerprinting pattern of mutants, M6 (1), M16 (2), M17 (3), M20 (4), M24 (5), M33 (6), wildtype KPS46 (7) and no template (8).



Standard surfactin



KPS46



UV mutant M6

Figure 8 HPLC profiles of surfactin produced by *Bacillus amyloliquefaciences* KPS46 and UV mutant M6 cultured in nutrient glucose broth compared with standards surfactin produced by *B. subtilis* ATCC21332.

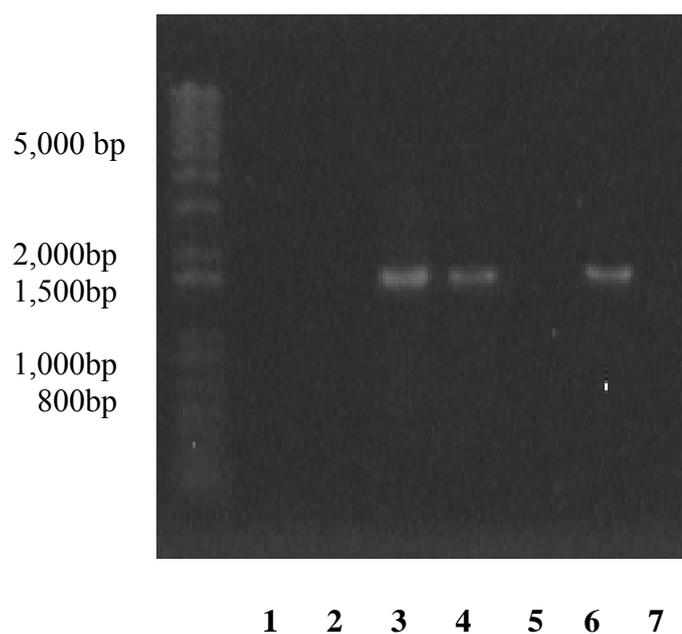


Figure 9 Polymerase chain reaction (PCR) amplified the *srfAA* gene of *Bacillus amyloliquefaciens* KPS46 UV mutant by *srfAA*-F and *srfAA*-R primers. The PCR fingerprinting pattern of mutants, M6 (1), M16 (2), M17 (3), M24 (4), M33 (5), wildtype KPS46 (6) and no template (7).

11. Growth rate comparison

The growth rate between mutant and wildtype compared by culture both of them on nutrient broth and growth rate of both wildtype and UV mutant were evaluated periodic intervals of growth through CFU counting by taking 1-ml suspension cultured at 12, 24, 36, 48, and 60 hours for dilution plate and colony counting was done. The growth curve from this study showed growth rate of UV mutant M6 higher than wildtype (Fig 10). The maximal growth of KPS46 wildtype was 6.92×10^{12} CFU whereas UV mutant M6 was 1.70×10^{13} CFU at 36 hours incubation.

12. Swarm motility

Phenotypic swarming motility assays for characterize in KPS46 and UV mutant M6 were initiated at various concentrate of 0.5, 1 and 1.5 % agar on differential media between NA and Luria Bertani agar (LBA) medium. Colony growing pattern and diameters of halos due to bacterial migration have been observed. The investigation indicated that most of UV mutant and wildtype show differential ability of migration on surface agar in both different medium and various concentrate of agar from 0.5, 1 and 1.5 %. The result show that UV mutant M6 migrated on LBA slower than wildtype with 32.50, 23.50 and 22.00 centimeters respectively, while wildtype migrated at 48.00, 32.00 and 17.00 centimeters respectively at 24 hours incubation. After 48 hours incubation, the motility migration was similar to 24 hours. KPS46 wildtype continue fast motile with colony growth of 51.67, 38.33 and 27.67 on LBA and 90.00, 59.00 and 53.00 on NA respectively, when compared with slow motile of UV mutant M6 of 46.35, 35.00 and 31.50 on LBA and 51.80, 46.00 and 28.33 on NA respectively (Table 22). The pattern of migration observed from colony form of KPS46 on LBA and UV M6 on NA and LB were regular or seemly round colony while pattern of KPS46 on NA were spreading / irregular radius branching motile colony (Fig 11).

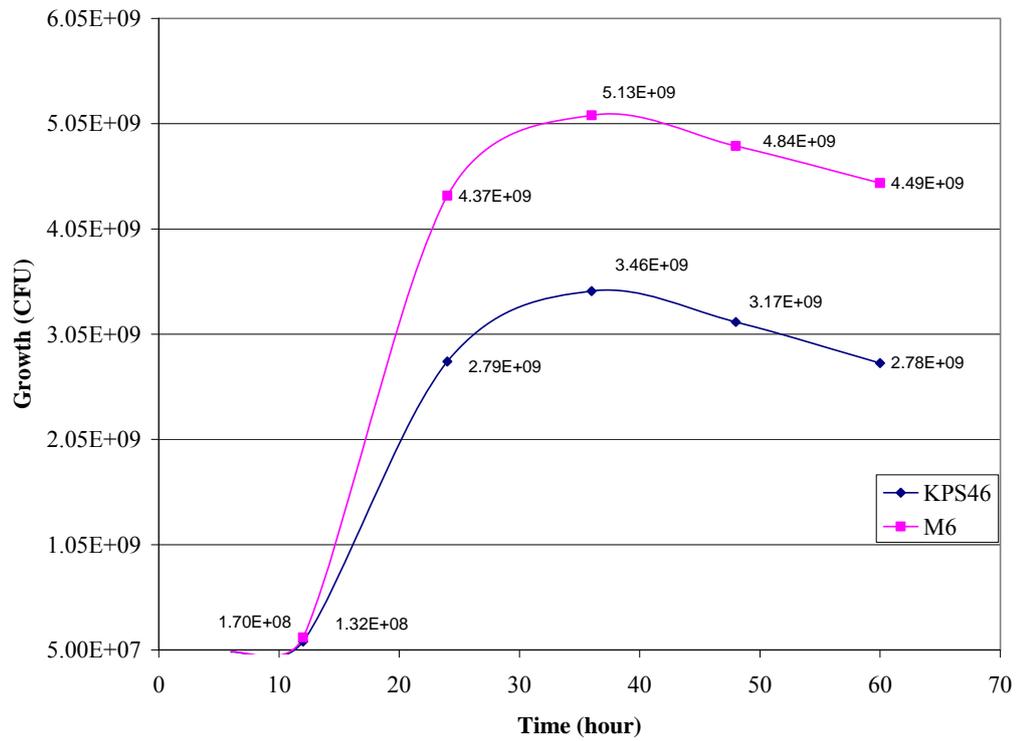


Figure 10 Growth rate of wildtype and UV mutagenesis of *Bacillus amyloliquefaciens* KPS46 on nutrient broth sampling evaluated periodic intervals of growth through CFU plate counting at 6, 12, 24, 36, 48 and 60 hours.

Table 22 Swarm motility evaluated through diameters of growing colony due to bacterial migration of UV mutant and wildtype of *Bacillus amyloliquefaciens* KPS46 investigated on Luria-Bertani agar (LBA) and nutrient agar (NA) at various concentrate of 0.5, 1 and 1.5 % agar.

UV radiation Bacterial Bacterial strain	LBA (cm)			NA(cm)		
	0.5 % agar	1 % agar	1.5 % agar	0.5 % agar	1 % agar	1.5 % agar
24 hours						
UV mutant M6 KPS46	32.5b	23.5b	22.0b		29.0b	26.0a
	48.0a	32.0a	17.0a		82.5a	21.5a
48 hours						
UV mutant M6 KPS46	46.6b	35.0b	31.5a		51.8b	46.0b
	51.6a	38.3a	21.6b		90.0a	59.0a

Means followed by the same letter in the same column were not significantly different at $p = 0.05$.

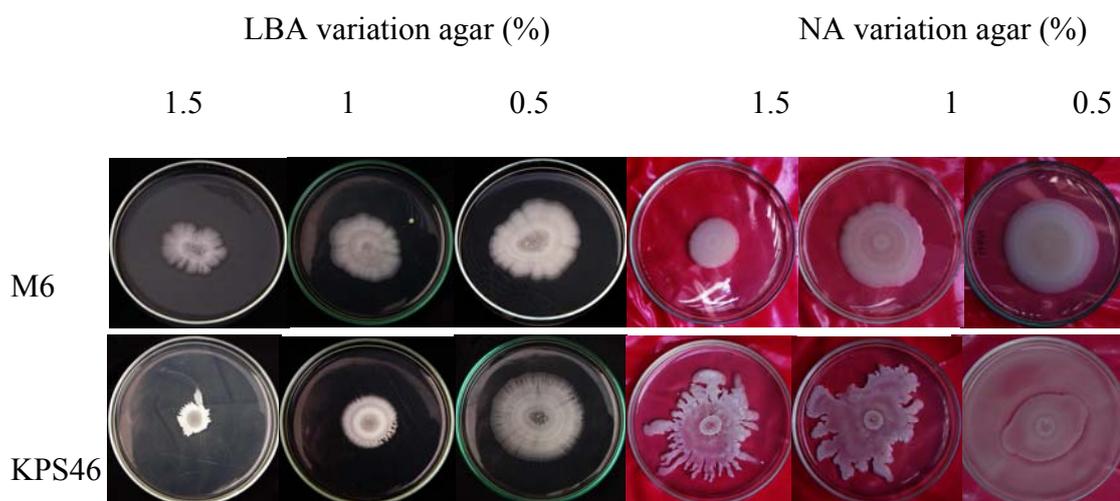


Figure 11 The pattern of motility migration observed from colony forming of the regular round colony and spreading/radial branching of *Bacillus amyloliquefaciens* KPS46 wildtype and UV mutant M6 on Luria-Bertani agar (LBA) and nutrient agar (NA) plate various concentrate of 0.5, 1 and 1.5 % agar (photo at 48 hours incubation).

13. Enhance seedling growth

Germination rate observed after 7 days planting showed that wildtype stimulated seed germination similar UV mutant M6 of 83.00 % while germinate rate of seed treated with copper oxychloride and non-treated control gave the germination rate at 77.00 and 73,00 % respectively. Not only well germination rate stimulation but also could enhance growth of seedling better or equal with wildtype and showed distinguish higher growth than non-treated control. Eventhough stem high, root length, and adventitious root number were not significant different , but fresh weight of 10 seedlings was 25.12 g equal with wildtype of 24.10 g. It showed significant higher than seed treatment with copper oxychloride and non-treated control which germination rate were 22.41 and 22.77 % respectively ($p=.05$) (Table 25).

14. Bacterial pustule control

Control efficacy against bacterial pustule of soybean caused by Xag test in greenhouse was conducted to compare control efficacy between wildtype and UV mutant M6. The bacterial pustule severity was estimated after pathogen inoculation for 14 days and subjected to statistical analysis. The UV mutant M6 which did not produce secondary metabolite in vitro showed decreasing efficacy to control bacterial pustule in the greenhouse compared with wildtype. Disease severity of bacterial pustule treated with UV mutant M6 was 41.58 % higher than treated with wildtype KPS46 of 34.97 % and copper oxychloride of 36.60 %. However, it showed the control efficacy significantly higher than non-treated control of 49.73 % (Table 24).

Table 23 The investigation of secondary metabolites minus product UV mutant M6 to enhance seed germination rate and seedling vigour compared with wildtype of *Bacillus amyloliquefaciens* KPS46, copper oxychloride and non-treated control.

Treatment	Germination rate (%)	Stem length (cm)	Root length (cm)	Root number	Fresh weight (g)
KPS46 in fresh culture	83.00a	12.86ab	13.58	35.04a	24.10a
UV mutant M6	83.00a	13.36a	13.72	33.64ab	25.12a
Copper oxychloride	77.00b	12.78ab	13.72	32.48ab	22.41b
Non-treated control	73.00b	12.18b	13.42	31.80b	22.77b
CV. (%)	7.42	4.11	2.88	5.55	3.56

Means followed by the same letter in the same column were not significantly different at $p = 0.05$.

Table 24 Potential of developed wettable formula of *Bacillus amyloliquefaciens* KPS46 to suppress bacterial pustule of green soybean in greenhouse condition.

Treatment	Bacterial pustule severity (%)
KPS46 in fresh culture	34.97c
UV mutant M6	41.58b
Copper oxychloride	36.60c
Non-treated control	49.73a
CV.	12.19

Means followed by the same letter in the same column were not significantly different at $p = 0.05$.

Discussion

Developing formulations of *B. amyloliquefaciens* KPS46

The utilization of soybean meal, fish meal and molasses were tested for enhancing multiplication of antagonistic *B. amyloliquefaciens* strain KPS46 for mass production to replace peptone, beef extract and dextrose, the nutrient source of general standard media, NGB. The result showed that, the antagonistic *B. amyloliquefaciens* strain KPS46 could utilize soybean, fish meal and molasses as source of nutrient in modified culture media. Soybean and fish meal enrich protein and amino acid, source of nitrogen. Crude protein contain in soybean meal varied from 44 -50 % depend on source and method of oil extract process. Similar as soybean meal, fish meal contain high protein as 50-77% and also high phosphorus containing at 0.6 -0.7 % in soybean meal and 1.07-2.67 % in fish meal (Kellems and Church, 2002; Sethapukdee, 1985). The component of them consist at least 11 amino acids and phosphorus which necessary for growth of antagonistic bacteria especial phosphate can be initiated microorganism metabolism. Phosphate is also an organophosphorus can form many polymeric ions found as formula of many compounds such as glucose 6-phosphate to be reactant in pentose phosphate pathway of living organism including bacillus. The response of *B. subtilis* to the availability of sugars is well understood utilization sugars or organic acids as sources of carbon and energy (Schilling *et al.*, 2007). These nutrients are metabolited by glycolysis, the pentose phosphate pathway, and the Krebs citric acid cycle.

Molasses (black strap) consist of 50-75% which 60 % total sugar was found to be sucrose and 30 % of fructose and glucose that bacillus can use as normal source of carbon for growth and metabolism. Moreover, crude protein was found 4.1-6.5 % from molasses (Kellems and Church, 2002). Bacillus normally is versatile chemoorganotrophe especial subtilis group including *B. megaterium* *B. subtilis* and *B. amyloliquefacience* can utilize nutrient from fermentation of complex molecule of organic mater such as protein and disaccharide as sucrose (Claus and Berkeley, 1986; Parry, 1983; Sonenshein *et al.*, 1993). The utilization of optimal ratio of soybean meal,

fish meal and molasses at ratio 10:5 g/l preferred to enhance antagonistic *B. amyloliquefacience* strain KPS46 multiplication of 8.6, 9.6 and, 9.6 log CFU/ml, and 8.4, 9.7 and 9.8 log CFU/ml after incubation for 24, 48, and 72 hour respectively compared with NGB that yielded of 9.1, 9.3 and 9.2 log CFU/ml incubated the same period respectively. Those ratios can be enhanced multiplication of antagonistic *B. amyloliquefaciens* strain KPS46 for mass production. The costs of those per liter are very low of 0.32 and 0.42 Baht compared with NGB that showed highest cost of 48.52 Baht per liter. The result showed that we can use fish meal soybean meal and molasses as a modified medium to replace the high cost of peptone, beef extract, and dextrose, the nutrient source of general standard media (NGB). The low cost of tested materials in modified medium and high potential possible lead to use soybean meal, fish meal and molasses in formulation production at commercial scale.

The new formula was developed by using several local materials as carrier to reduce cost and enhance valuable of production. Most of local material using as a carrier in formula, dry cow dung, talcum, decomposed cow dung, and rice husk ash dust were appropriate material to develop dry formulation which high viability enhancement after stored for 30, 60, 180, and 360 days respectively at room temperature. The developed formula using local material from this research facilitate transfer its application from laboratory to field. The carrier in formulation maintained long shelf life by optimizing storage condition, keep formula stability and protect from harmful environment.

The survival of antagonist and its efficacy in disease control depends largely on the type of material used in the formula development. The carrier is the key of successful development antagonistic formulation. Most of materials used in this research are both form of organic and inorganic from local resource. Vermiculite, clay calcium sulfate and talcum are the inorganic material most popular used in dry formulation for antagonistic bacteria and fungi. Talcum is one of the easy formulation developments. It gave the high proportion to maintain shelf life and also enhance control efficacy both in greenhouse condition and field trial. It has previous support report from Vidhyasekaran (1995) by mixed of talcum and carboxy methyl cellulose for developing powder formulation to enhance shelf life of *P. fluorescens* for 240 days

at room temperature. EL Hassan *et al.* (2006) developed different formulations of *Bacillus subtilis* using glucose and talcum powders. *Bacillus subtilis* survived in this formula at 8.6 and 7.8 log₁₀ CFU/g, respectively, for 1 year of storage at room temperature compared with 3.5 log₁₀ CFU/g on a peat formulation. Greenhouse experiments using soil and seed treatments showed that lentis seed treatments with formulations of *B. subtilis* on glucose, talcum and peat significantly enhanced its biocontrol activity against *Fusarium*.

Dry cow dung, decomposed cow dung, and rice husk ash dust, organic compound used carrier not only maintained shelf life with polymer component as a cellulose to protect bacterial cell, but KPS46 can use cellulose as a source of carbon to survive. However, Fravel *et al.* (1995) report that different C: N ratio of corn straw, soybean meal, wheat dust with C: N ratio 96.6, 25.4 and 21.1 respectively effected to enhance shelf life of *Talaromyces flavus*. The viability of *Talaromyces flavus* maintained in formula developed of wheat straw which high C: N ratio was higher than soybean meal that it contained low C: N ratio. The carrier, decomposed cow dung, dry cow dung, talcum, and rice husk ash dust also prepared the optimum pH at 6.6, 7.2, 8.2 and 9.6 respectively compared with optimal growth of *B. amyloliquefaciens* at pH ranging between 6-9, only rice husk ash dust was over optimal growth pH (Teodoro and. Martins, 2000). While liquid formulations are unlikely maintained antagonists in water or oil emulsion added with some additive agents. Although the liquid formulations can be easily applied, it cannot be stored for a long time. Wherear wettable power formulation is more convenient all of shelf life maintaining, transporting, low cost and also practical field application than liquid formula. The result showed that after 180-day shelf life of KPS46 maintained in all local materials, rice husk ash dust, talcum, dry cow dung, and decomposed cow dung, as carrier in dry formula showed viability with high log CFU/g of 9.6, 9.6, 9.4, and 9.3 log CFU/g. After 360 days shelf life of KPS46, it still gave high CFU enough to application in field with 8.8, 8.7, 8.5, and 8.4 log CFU/g respectively.

When investigated after 360-day shelf life of KPS46, it still gave high CFU enough to apply in field. The CFU of KPS46 maintained in wettable formula used rice husk ash dust, dry cow dung, decomposed cow dung, and talcum with (Fig 6).

The precautions to enhance the survival of microbial antagonist in severe environmental conditions may drastically limit to establish on a host target site. The greenhouse and field trial investigation for efficacy of dry developed formula were done to evaluate formulation efficacy. Both greenhouse and field experiment revealed that dry formula of KPS46 maintained in talcum application by seed treatment before planting enhance germination rate observed after 7 days planting was not different from fresh culture. The fresh weight of seedling (weighting from 10 seedlings) from seed treated with KPS46 in wettable formulation likely initiation biomass increasing with KPS46 from fresh culture. The non significant difference of fresh weight between KPS46 maintain in talcum of wettable formula and fresh culture indicated that developed wettable formulation enhance viability and still activate to enhance plant growth which can explain by seedling fresh weight. Similar to fresh weight, bacterial pustule control efficacy at green house condition, the result showed that KPS 46 from wettable formula showed non-different suppressing bacterial pustule on green soybean with fresh culture of disease severity 37.54 and 34.14% respectively. The control efficacy of KPs46 was similar to application copper oxychloride with disease severity 38.12 % and control efficacy better than non-treated control with disease severity high as 85.79 %. From greenhouse investigated, the result supported that dry formulation maintained the optimum condition for enough cell forming unit and kept KPS46 in activating condition to give efficacy similar with fresh culture when compared application on plant in greenhouse condition.

The research results of wettable formulation show that the formulation is important to facilitate the development of antagonistic microbial on transfer its application from laboratory research to farmer field application. This may involve the optimization of factors such as stability upon storage, increasing persistence, protection from harmful environmental factors, and enhancing the activity of microbial antagonist apply on plant. The survival of a potential biological antagonist and its control efficacy depends on the type of material used in the development of the formulation. Normally, polymers such as cellulose and alginates (1,4-linked β -D-mannuronic and L-galuronic acid in different proportion) and inorganic material including vermiculite, clay, calcium sulfate and talcum has been extensively explored for formulating biocontrol agents. Those included talcum (used in this research) were

used to form formula not only for the purpose to increase shelf life but they can protect cell from directly expose to sunlight. UV light and temperature are important abiotic factors that can affect the viability and consequently to efficacy of a microbial antagonist living on leaf surface. Balley *et al.* (1996) observed that the commercial *Bacillus thurigiensis* product used to control apple moth caused by *Epiphyas postvittana* lost more than half of its activity within a day on vine plants when the leaf surface was fully exposed to light. The same bacterium applied to shaded leaves showed survival of more than 60 % after 2 days' exposure to sunlight. Nicholson *et al.* (1997) concluded that the spore coats and DPA both offer significant protection to inactivation of spores by solar UV. In UV mutagenesis of KPS46 by direct exposed to 254 nm UV for 15 -20 minutes, the result revealed that UV radiation killed 100 % of population, when exposed for 2.5 minutes, KPS46 population survival only 11.47 % (data not shown).

Field experiment of KPS46 to evaluate formula efficacy, the result in the first crop was likely higher diseases incidence and severity than in the second crop in both application KPS46/natural and bio-product alone since the first crop was affected from heavy rain. It revealed that combination application suppressed either damping-off and bacterial pustule better than or equal fungicide. KPS46 could be reduced disease incidence of damping-off and disease severity of bacterial pustule as showed in table 1 and table 5. Although disease severity of bacterial pustule assessed at 50 days after planting showed the very high from heavy rain, it was control better than in treatment of chemical fungicide application and non-treated control.

The previous report could be confirmed that KPS46 product secondary metabolite to inhibit competitor and also attacked pathogen for available food and colonized root plant. KPS46. *Bacillus* sp. has been shown to produce a wide variety of antibacterial and broad spectrum antifungal compounds or secondary metabolites (Parry, 1983; Sonenshein *et al.*, 1993). Kasem (2002) has reported that *B. amyloliquefaciens* KPS46 was an effective control agent against bacterial pustule the same as or greater than those observed in copper oxychloride and streptomycin. *B. myloliuefaciens* KPS46 also has been reported to induce systemic resistance against bacterial pustule pathogen with increased phenols, phenylalanine ammonia lyase,

peroxidases and 1, 3- β -glucanases in soybean plants by Prathuangwong and Buensanteai (2006). For growth promoting and yield enhancing, in both applications KPS46/natural and bio-product alone and in combination applied showing did not contrast in most growth component, but could increased marketable yield in both, the first and second crop. The result could be supported by previous experiments. KPS46 was now known as plant growth-promoting rhizobacteria (PGPR) could promote the growth of widely crop plants. (Prathuangwong *et al.*, 2002; Prathuangwong and Kasem, 2003).

Investigation by Idriss *et al.* (2002), identified that *B. amyloliquefaciens* was able to degrade extracellular phytate. *B. amyloliquefaciens* strain FZB45 was detected the highest extracellular phytase activity and diluted culture filtrates of this strain stimulated growth of maize seedlings under phosphate limitation in the presence of phytate. More report released by Buensanteai (2008) confirmed the control efficacy, plant growth promotion and induced systemic resistance by the KPS46 strains. Her research reports clarified that KPS 46 produced high amount of elicitors including IAA, surfactin and extracellular proteome at stationary phase. The strain KPS46 and its elicitors as IAA, surfactin and extracellular protein are able to corroboratively elicit with growth development of soybean by increasing the shoot length, root length, root dry weight, shoot dry weight, number of lateral root and root surface area under gnotobiotic and greenhouse assay. It could be induced resistance on soybean and *Arabidopsis* plant against bacterial disease by pattern of induced systemic resistance (ISR) associated with the accumulation of phenolic content and defense related enzymes of increased β -1,3-glucanase and peroxidase activity levels in plants. The strain KPS46 was able to activate rapid salicylic acid (SA) and delayed jasmonic acid/ethylene (JA/ET) dependent pathways of induced systemic resistance in soybean plants cv. Spencer with high and low production levels respectively.

Natural products and bioproducts also showed the good trend for combined application with KPS46. The most effective treatment from 2 crops to suppress disease, stimulate growth and enhance yield was the combined application of KPS46. The combined application with Decompose manure, amino acid, CaB, and algae-extract gave the highest marketable yield in the first crop; while Decompose manure II

gave the highest marketable yield similar to commercial biological agent *B. subtilis* combined application with Decomposed manure II in the second crop.

Naturally organic amendments and inorganic compounds are used as additives to improve soil physical conditions. Soil moisture distribution was governed by the amount of water conserved favor for growth and activity of biocontrol agent (Rahman *et al.*, 1996). The composed organic matter was not just a great soil amendment, but could actually suppress harmful pathogens in the soil and control fungal diseases more effectively and at less cost than toxic fumigants. Several beneficial strains of soil microbes, was apparent that some of them could induce systemic resistance to diseases in plants themselves (Logsdon, 2004). In fresh organic matter, free nutrients are plentiful and both pathogens and biocontrol agents proliferate. The biocontrol agents, had plenty to resort to their survival tactic of producing antibiotics that would kill or suppress their pathogen competitors. Vinarov *et al.* (2005) have described in their patent that using biological additions to the organic-mineral fertilizers accelerate the release of nutrients from the fertilizers and the assimilation of the released substances by the plants. Moreover, these biological additions favor the accumulation of the nutrients in the soil in a form that can be easily assimilated by plants. These two experiment crops could be confirmed that combined application of natural products and bioproducts set up the favor condition for KPS46 to express the disease suppressing ability, growth promoting and potential to induce systemic resistance of green soybean in field experiment.

When application KPS46 in wettable formulation and fresh culture alone at field condition was carried out in experiment II, germination rate was higher than KPS46 neck cell in fresh culture without protectant barrier. The efficacy in field trial of KPS46 in wettable formula to control seedling damping-off green soybean assessed at 14 days after planting indicated that wettable formula of KPS46 was similar with KPS46 in fresh culture of 4.18 and 4.03 % respectively and did not differ from chemical control of seed treatment with carbendazim. The disease incidence of bacterial pustule anthracnose was also reduced by KPS46 from both wettable formulation and fresh culture. They enhanced highest marketable yield similar the results in experiment I. The previous supporting result reported by Prathuangwong et

al. (2005b) showed that fresh culture of KPS46 application by seed treatment and SW01/4 as foliar spray at commercial production farm showed an increase yield of 42 % and better quality of the standard pods compared with non-treated. Biological control bacterial KPS46 and SW01/4 were highly decreased fungal stem rot and wilt and effectively induced systemic resistance against foliar diseases included anthracnose, bacterial pustule, and viruses. This indicated that wettable formula maintained viability for long time storage and kept cell of KPS46 in the activation condition to mediate for disease reducing in the expose of enduse environment. The cell activity to control disease from this experiment due to formulating of talcum in wettable powder kept KPS46 in the appropriate condition. It could express the antagonism mode as same as cell from fresh culture.

The total cost in green soybean production, return, benefit and rate of return on investment (ROI) in applications of fungicide and biological control application KPS46 developed at wettable formula were calculated to compare. Cost/rai and the return/ rai of conventional were not contrast different from biological control which benefit of 5,376.43 Baht and 4,377.18 respectively. When calculated ROI it showed the high ROI in both cases, however the biological control gave ROI of 43.30% higher than conventional of 33.59%. The biological control application KPS46 of wettable developed was not only increasing ROI, but it could reduce chemical application and also gave high and more environmental friendly.

Antagonistic mechanism of *B. amyloliquefaciens* KPS46 against bacterial pustule.

The plate assay to investigate anti-microbial secondary metabolites produced by KPS46 showed ability to inhibit bacterial *X. axonopodis* pv. *glycines*. Those colonies picked from UV radiating plate showed that UV mutagenesis of KPS46, 10 colonies did not produce secondary metabolite to inhibit *X. a. pv. glycines*. The 22 out of 32 sampling colonies showed various inhibition clear zones against *X. a. pv. glycines*, 2 of them were similar to KPS46 wildtype. Twelve colonies showed decreasing inhibition clear zone and 8 colonies increasing larger clear zone.

UV mutant M6 was completely non-inhibition clear zone against bacterial *X. a. pv. glycines* in vitro. When detected surfactin by HPLC, we found that antimicrobial surfactin was detected from wildtype but it absented in UV mutant M6. The PCR of *srffAA* genes was amplified from wildtype. It was not amplified from UV mutant M6. When investigated control efficacy of the UV mutant M6 to determine antagonism, the result showed that disease severity of bacterial pustule treated with UV mutant M6 was higher than treated with wildtype KPS46 and copper oxychloride. This *srffAA* mutant strain M6 significantly exhibited less effect on disease reduction compared with the parent wildtype strain.

Surfactin is one of several secondary metabolites that related to control efficacy. This investigation indicated that secondary metabolite surfactin was the one directed mechanism control efficacy of KPS46, when it defected to produce secondary metabolites, the control efficacy directly decreased. Previous investigation revealed that *Bacillus* spp. associated with biological control mechanisms include production of antibiotics and extracellular hydrolytic enzymes such as chitinase, laminarinase, lipase, and protease contribute to degradation cell wall (Helistö *et al.*, 2001; Paulitz and Belanger, 2001). The secondary metabolite involved in antimicroorganism produced by *B. amyloliquefaciens* FZB42, were studied, including surfactin, fengycins, and several iturins (Koumouts *et al.*, 2004). Polyketides, bacillaene and difficidin were reported that they were the main antibacterial secondary metabolites (Chen *et al.*, 2006). Surfactin was reported on effective surface hydrophobicity, bacterial adhesion and movement on surfaces, and antimicrobial activity (Ahimou *et al.*, 2000; Bonmatin *et al.*, 2003; Kinsinger *et al.*, 2003).

Surfactin exerted its antimicrobial and antiviral effect by altering membrane integrity (Peypoux *et al.*, 1999). It related to control efficacy of UV mutant M6 against bacterial pustule in greenhouse was reduced when extracellular secretion secondary metabolite surfactin, one of the directly control effect against pathogen did not produce. The absence of antimicrobial secondary metabolite both non-inhibition effect to fungi and bacteria in vitro test related to directly decrease of control efficacy of UV mutant M6 at greenhouse conditions. However, it still showed the control efficacy significant higher than non-treated control. This result revealed that KPS46 could

suppress disease not only by antimicrobial secondary metabolite but it could do another mechanism such as elicitor to induce systemic resistance and enhance growth. These were supported by several reports from our lab. The previous experiment confirmed that it could enhance defense mechanism of host plant through ISR mechanism against bacterial pustule pathogen with increased phenols, phenylalanine ammonia lyase, peroxidases and 1, 3- β -glucanases in soybean plants (Prathuangwong *et al.*, 2004; Prathuangwong *et al.*, 2005a; Buensanteia 2006; Prathuangwong and Buensanteai; 2006).

The first report by Buensanteai (2008) to examine soybean responses to a biological control agent in connection with induced resistance using analysis of phenolic concentration and 1,3- β -glucanase and peroxidase activities, which are common plant responses to pathogens. While her investigation found that treatment of soybean seed with KPS46 to cause measurable increases in these resistance markers in the leaves, inoculation with *Xag* was essential for maximum expression of the markers in KPS46-treated plants. The differential expression of the markers after pathogen challenge was similar to the induction of phenolic deposition in soybean cotyledons by wounding that was potentiated by exposure to a cell wall glucan preparation from *Phytophthora sojae* (Graham and Graham, 1996) and is indicative of priming as defined by Conrath *et al.* (2006).

Her investigation found increases in both salicylic acid and jasmonic acid levels in KPS46-treated plants compared to the controls, with the elevation of these signaling compounds being more pronounced in KPS46-treated plants that were inoculated with *X. a. pv. glycines*. These results support the conclusion that KPS46 primes soybean plants. They are also evidence that KPS46-induced resistance in soybean involves both salicylic acid -dependent and jasmonic acid -dependent pathways. The latter is more like the pathogen-induced jasmonic acid -dependent pathway because this pathway requires jasmonic acid production (Penninx *et al.*, 1996), whereas the rhizobacteria-induced jasmonic acid -dependent pathway is not associated with increased production of jasmonic acid (Pieterse *et al.*, 2000).

Our bioassay for extracellular indicated that although UV mutant M6 grew fast than wildtype in vitro test, some survival support factors such as extracellular enzymes, included endoglucanase and protease were not similar to wildtype. It produced only amylase higher than wildtype. It produced endoglucanase and protease lower than wildtype with statistical significant. The ability to utilize complex nutrient source such as cellulose chitin and peptide should reduced and possibility decreased competition/epiphyte fitness. Extracellular enzymes secreted by microorganisms do not only enroll in degrading activities and special combinations of nutrient but they act as hydrolytic enzymes to degrade cell wall of plant pathogens. The microorganisms exhibiting degrading can be used to degrade cell wall components of plant pathogenic fungi, to treat in agriculture and food wastes, to prepare animal feeds and other materials (Lorito *et al.*, 1993; Kumar and Takagi, 1999; Yang *et al.*, 2000; Koh *et al.*, 2002).

The growth rate between mutant and wildtype was compared by culture both of them on Nutrient Broth. UV mutant M6 grew faster than wildtype and amylase activity were also higher. For amylase production is typical characteristic by name of *B. amyloliquefaciens* whose produce liquid amylase. Several evidences supported that amylases were produced by a variety of living organisms, ranging from bacteria to plants and humans. Bacteria and fungi secrete amylases to the outside of their cells to carry out extra-cellular digestion. *B. amyloliquefaciens* and *B. subtilis*, *B. licheniformis*, and *Aspergillus niger* are one of the most commonly used for amylase industrial production (Bernfeld, 1951; Abe *et al.*, 1988; Burgess-Cassler *et al.*, 1991; Gupta *et al.*, 2003). Amylase was confirmed to be the growth index of bacillus. Hill *et al.* (1996) demonstrated that the pattern of growth curve of *B. amyloliquefaciens* was similar to amylase production. The several species such as *B. stearothermophilus* (Welker and Campbell, 1963) and *B. flavothenus* (Kelly *et al.*, 1977) were also reported to the similar pattern of cell growth and amylase production.

Phenotypic swarming motility assays for characterize UV mutant M6 and wildtype showed differential ability of migration on surface agar in both different medium and various concentrate of agar. The motility of UV mutant M6 was slower than wildtype. The swarm motility is a rapid and coordinated translocation of a

bacterial population across solid or semi-solid surfaces. Swarming bacteria undergo morphological differentiation in migration front of typically hyperelongated, hyperflagellated and grouped in multicellular raft structures (Henrichsen, 1972). In some species, swarming motility requires the self-production of biosurfactant and it is usually under the control of an intercellular quorum sensing communication system (Eberl *et al.* 1996). From bioassay of surfactin, swarming motility requires, was detected by HCLP from wildtype only due to faster motile than UV mutant M6.

The investigation of secondary metabolite minus product UV mutant M6 enhanced seed germination rate and enhanced growth of seedling equal with wildtype. It showed higher growth than seed treated with copper oxychloride and non-treated control. Eventhough stem height, root length, and adventitious root number were not significant different but fresh weight or biomass of seedling was not different from wildtype. It was significantly higher than seed treatment with copper oxychloride and non-treated control. The investigation of Buensanteai (2008) reported that KPS46 promoted plant growth on soybean to increased root, shoot length, and biomass by more than 20-40% compared with non-treated control by produced elicited IAA.

The first analysis of metabolite production by *B. amyloliquefacians* KPS46 in relations to plant growth promotion was done by Buensantia (2008). Her study in laboratory found that KPS46 culture fluid extracts containing secreted indoles, lipopeptides and proteins individually can influence the growth of soybean to the same degree as cells of KPS46 washed free of pre-formed exoproducts. In this result investigation in greenhouse conditions revealed that although UV mutant M6 did not produce surfactin, it could induce soybean growth similar to parental KPS46. This indicated that in absence of surfactin it could stimulate plant growth throught another such as indoles and proteins which were previosly reported (Buensuntia, 2008). Whether or not the same type of compounds are secreted by KPS46 cells while existing in the spermosphere or rhizosphere remains to be determined, but nevertheless, the results are consistent with the hypothesis that strain KPS46 promotes the growth of soybean by secretion of several types of compounds.

Buensanteai surmise that components within each extract had direct effects on soybean, perhaps acting as signaling compounds. Analysis of the constituents of the extracts revealed some compounds that potentially could play dual roles. Its role in causing the growth promotional effects of that extract needs to be confirmed. There is evidence that certain synthetic surfactants can stimulate plant growth by synergizing auxin action, activating certain plant enzyme systems, or affecting plant cell membrane permeability, thereby increasing water or nutrient uptake or excretion of plant factors such as riboflavin (Ernst et al., 1971; Parr and Norman, 1965). But other than a report that surfactin can induce resistance in bean (Ongena et al., 2007), there is no precedence for surfactin having a direct effect on plants leading to elevated growth.

CONCLUSIONS

The utilization of soybean meal, fish meal and molasses used in this tested enhancing multiplication of antagonistic *Bacillus amyloliquefaciens* strain KPS46 for mass production that could be restored peptone, beef extract and dextrose, the nutrient source of general standard media, NGB were the soybean meal: molasses and fish meal: molasses at 10:5 g/l.

The costs of those per liter calculated from all ingredient material used in modified media and NGB calculated base on retail price are 0.32 and 0.42 Baht compared with NGB that showed highest cost of 48.52 Baht.

The local material, dry cow dung, talcum, decomposed cow dung, and rice husk ash dust showed good approach of carrier using in dry formulation development which high viability enhancement after stored for 30, 60, 180, and 360 days respectively at room temperature. After 180 days shelf life of KPS46 maintained in all local materials, dry cow dung, talcum, decomposed cow dung, and rice husk ash dust as carrier in dry formula showed a high viability with high log /g of 12.96, 12.65, 12.52, and 12.46 respectively and after 360 days shelf life of KPS46, it gave high CFU with 12.30, 12.27, .25, and 11.99 log CFU/g respectively.

The investigation of the wettable powder formula to maintain 30-day and 180-day shelf life of KPS46 from previous compared with fresh culture and fungicide (copper oxychloride) conducted in greenhouse by seed treatment before planting and germination rate observed after 7 days planting showed that KPS46 maintained in talcum developed as wettable powder formula initiated germination and growth component did not differ from fresh culture. The control efficacy of KPS 46, both in wettable power formula and fresh culture suppressed bacterial pustule on green soybean, S292 cultivar similar to application copper oxychloride.

Field tests for using antagonist against bacterial pustule was put on trial at Corn and Sorghum National Research, Pakchong, Nakhon Ratchasima and was evaluated along with various bioproducts of both commercial and natural-made formulations

under field experiment at Lopburi Field Crop Research Center during Most of field trial test confirmed that KPS46 in developed dry wettable formula could reduce both root disease (*Sclerotium damping-off*) or foliar diseases (bacterial pustule caused by *Xanthomonas axonopodis* pv. *glycines*) and promote plant growth of green soybean under field experiment gave the highest quality and quantity yield equal/ better convention chemical application.

The cost of production including material cost, employed labor wages, irrigation, rent of production area (indirect cost) and rate were calculated. The return on investment of biological control application of KPS46 developed as wettable formula was 43.30% higher than conventional of 33.59%.

The one independently-generated *srfAA* mutant strain M6 of *B. amyloliquefaciens* KPS46 was unable to produce lipopeptide surfactin and extracellular enzyme cellulase. The M6 mutant also produced relatively low levels of extracellular enzymes, endoglucanase and protease compared to the parental strain KPS46 wildtype. The *srfAA* mutant was greater α -amylase production and growth rate in Nutrient Broth than the parental strain.

This *srfAA* mutant strain M6 significantly exhibited less effects on disease reduction compared with the parent wildtype strain. This result suggests that *B. amyloliquefaciens* KPS46 reduced bacterial pustule severity on soybean is associated with its lipopeptide surfactin production that *srfAA* also effects the phenotypes of down- or up-regulated extracellular-enzyme production.

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CIRRICULUM VITAE

NAME : Mr. Chaist Preecha

DATE OF BIRTH : 12 February 1961

PLACE OF BIRTH : Nakhorn Si Thammarat, THAILAND

EDUCATION	:YEAR	INSTITUTE	DEGREE/DIPLOMA
	1982	Kasetsart University	B.Sc. (Agriculture).
	1988	Kasetsart University	M.S. (Agriculture).
	2008.	Kasetsart University	Ph.D. (Tropical Agriculture)

POSITION/TITLE : Assistant Professor

WORK PLACE : Faculty of Agriculture, Rajamangala University of
Technology Srivijaya

SCHOLARSHIP/AWARD : DSE Federal Republic of Germany. 1994-1995,
2000.