CHAPTER 5
BIOLOGICAL CONTROL PRODUCT OF *STREPTOMYCES* SP. STRAIN S4 FOR CONTROLLING PLANT SEEDLING DAMPING OFF DISEASE

5.1 Abstract

Streptomyces sp. strain S4, antagonistic actinomycetes against seedling damping off fungi: *P. infestans* and *P. aphanidermatum*. For producing the antagonist cells to be used as biological control product, antagonist cell propagation by solid state fermentation of the appropriately available agroindustrial wastes was researched. *Streptomyces* sp. strain S4 produces both cellulase and chitinase. Therefore, the rice bran from rice milling industry and shrimp shell waste from a frozen shrimp industry were chosen for being raw materials in the fermentation. The fermentation was optimized by using the response surface methodology (RSM) based on the central composite design (CCD). The relationship between three variables, ratio of shrimp shell and rice bran at 100:0; 75:25, 50:50, 25:75, and 0:100; moisture content at 40, 45, 50, 55, and 60%; and inoculum sizes at 0.5, 1.0, 1.5, 2.0, and 2.5 % which affected the antagonistic cell growth was monitored. A second-order polynomial was determined by multiple regression analysis. The optimum values of the tested factors were obtained as follows: 50:50 ratio of shrimp shell and rice bran, 60% moisture, 1.5% inoculum with a maximum antagonistic cell production at 6.94 logCFU/g substrate. The fermented product was processed into powder and packed in aluminum foil bags. The viable cell counts of the *Streptomyces* sp. strain S4 culture powder keeping at 4°C and room temperature were determined using serial dilution plate count on nutrient agar every month for 6 months. The product had the initial antagonist cell at 7.3 logCFU/g. The batch keeping at 4°C could maintain the cell numbers at this level up to 6 months, while the batch keeping at room temperature could retain the same cell numbers for only 4 months and followed with gradually decline. The efficacy of the *Streptomyces* sp. strain S4 biological control product in controlling the seedling damping off disease was existed even after 6 months storage.

Keywords : Solid state fermentation / Formulation / Biological control product

5.2. Introduction

The solid state fermentation (SSF) is generally used for microbial cell cultivation in solid substrates (Pandey, 1992; Robinson et al., 2001; Pang and Ibrahim, 2005; Gassara et al., 2010). The products and/or by-products from agro-industry were reported as the suitable substrates providing solid matrix, carbon sources, nutrients, and moisture in solid state fermentation (Pandey, 2003). In the fermentation process, the important parameters that need to be considered are pre-treatment process, particle-size of fermenting materials, moisture content, pH, aeration, temperature, inoculum density, and microorganisms (Nigam & Pandey, 2009). Several of value-added compounds were obtained from fermentation processes of microorganisms such as enzymes, organic acids, flavor and aroma compounds, fructooligosaccharides, bioactive compounds, bioinsecticides, bioethanol, and mycelium or conidiospores (Rodriguez et al., 2010; Mussatto et al., 2009; Santa et al., 2005; Holker et al., 2004; Pandey et al., 2003; Medeiros et al., 2000;).

The biological control is environmental friendly method for pests' protection by using the living organisms. Several antagonistic bacteria and fungi were reported as the biological control agents for controlling plant diseases. Various biocontrol microbes usually prepared as cells and/or spores by culturing in solid state fermentation. Then, the commercial biological control agent is usually formulated from the propagules or spores of biocontrol microbes. The product efficacy, shelf life, transportation, and application of biocontrol microbes have been affected by the formulation process (Fravel et al., 1998; Burges and Jones, 1998.; Melin et al., 2011).The formulation method is become the important method for keeping and improving a high efficacy of biological control agents (BCAs). The stability of viable cells or spores in the formulated BCAs is difficult to control. The types of BCAs formulation (powders, beats, granules and liquid forms) was depended on the intended use**.** Combining with soil or root dipping or spraying are generally application method followed by the formulation of BCAs. In the recent study formulating of a biological control agent as a powder or lowering the water content was observed to be the most suitable for transporting, handling, storing, reforming and using than others formulation.

In this study, the cell mass production of *Streptomyces* sp. strain S4 in solid state fermentation medium was investigated. The formulation of *Streptomyces* sp. strain S4 was generated and monitored as biocontrol product. The efficacy of the biocontrol product was testified for the ability to protect chinese spinach seedlings from damping off disease caused by *P. aphanidermatum*.

5.3. Materials and Methods

5.3.1 Selection and Composition Analysis of Raw Materials for Solid State Fermentation

Streptomyces sp. strain S4 produces chitinase or cellulase to release reducing sugars from chitin or cellulose for using as their carbon source for growth. The enzymes are inducible enzyme which will be produced only in the presence of substrate chitin or cellulose. For this reason, the raw materials used in the fermentation should be selected from the agro-industrial wastes that having chitin or cellulose as the major components. Shells or exoskeletons of shrimp contain chitin and calcium carbonate. Heads and shells of shrimp are major solid waste of frozen seafood industries. Shrimp head was reported to have high nutritional value especially protein and lipid (Limam et al., 2008). Rice bran is a by-product generated from rice mill factory. Rice bran is a rich source of carbohydrates, proteins, fats, minerals and micronutrients (Moongngarm et al., 2012). Therefore, shrimp head and rice bran were analyzed for their nutritional values and formulated to be carbon and nitrogen sources in the fermentation medium. The analysis was performed using standard methods of AOAC; the Kjedahl method for protein ŧ analysis, dry ashing by a high temperature muffle furnace (550°C) for ash analysis, the soxhlet and hot extraction for crude fat analysis, (Appendix A). The chitin was analyzed by modified methods of Percot (2003) (Appendix A).

5.3.2 The Experimental Design for Cell Mass Production in Solid State Fermentation Medium

The cell mass production of *Streptomyces* sp. strain S4 was optimized by using the response surface methodology (RSM) based on the central composite design (CCD). The important factors were selected including three variables; ratio of shrimp shell and rice bran (X_1), initial moisture content (X_2) and inoculum size (X_3). Each variables were coded at five different levels (−2, −1, 0, +1, and +2) in Table 5.1. Coding forms of

all values, 20 runs were listed as the full experimental plan (2^3 CCD) in Table 5.2. Following the experimental design, 10g of solid state fermenting medium was prepared in 250-ml Erlenmeyer flasks and autoclaved at 121 °C 15 lbs/in^2 for 15 min. The *Streptomyces* sp. strain S4 was cultured in 1% shrimp shell broth (pH 6) for 2 days at 37°C under shaking conditions (200 rpm) and used as starter ($1x10^6$ CFU/ml). The starter was transferred into 10g of solid mediums and completely mixed. The experimental flasks were incubated at room temperature. The solid state fermenting media were mixed by manual stirring every day. The serial dilution standard plate-count technique was used to monitor the growth of *Streptomyces* sp. strain S4 (Jorgensen et al., 1979). Design Expert 7.0.0 (STATEASE INC; Minneapolis, MN, USA) software was used to analyse the variance (ANOVA) and fit a second order quadratic polynomial equation (Eq.1). Three-dimensional response surface of the experimental results was finished up with the model graphs.

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{1,2} X_1 X_2 + \beta_{1,3} X_1 X_3 + \beta_{2,3} X_2 X_3 + \beta_{1,1} X_1^2 + \beta_{2,2} X_2^2 + \beta_{3,3} X_3^2 \quad (1)$$

Where Y was response variable, β_0 was intercept, β_1, β_2 and β_3 were linear coefficients, $\beta_{1,1}$, $\beta_{2,2}$ and $\beta_{3,3}$ were squared coefficient, $\beta_{1,2}$, $\beta_{1,3}$, and $\beta_{2,3}$ were interaction coefficient and X_1, X_2, X_3, X_1X_2, X_1X_3, X_2X_3, X_1^2, X_2^2, and X_3^2 were the level of independent variables.

Table 5.1 Range of the independent variables for response surface methodology.

Variables	Parameters	Code factor levels				
		-2	**-1**	**0**	**+1**	**+2**
X_1	Shrimp shell/Rice bran	100/0	75/25	50/50	25/75	0/100
X_2	Initial Moisture	40	45	50	55	60
X_3	Inoculum size	0.5	1.0	1.5	2.0	2.5

Table 5.2 The experimental design of central composite design of response surface methodology (2^3 CCD).

Run	Substrate (Shrimp head:Rice bran)	Initial Moisture (%)	Inoculum size (%)
1	25:75	55	1.0
2	50:50	50	1.5
3	50:50	50	1.5
4	25:75	45	2.0
5	75:25	45	1.0
6	75:25	55	2.0
7	50:50	50	1.5
8	25:75	45	1.0
9	25:75	55	2.0
10	75:25	45	2.0
11	75:25	55	1.0
12	50:50	50	1.5
13	50:50	50	1.5
14	0:100	50	1.5
15	50:50	50	1.5
16	50:50	50	2.5
17	50:50	60	1.5
18	50:50	50	0.5
19	50:50	40	1.5
20	100:0	50	1.5

5.3.3 Production of the Antagonistic Cell Powder for making Biological Control Product

The two days culture of *Streptomyces* sp. strain S4 in shrimp head basal mineral salts broth was used as starter ($1x10^6$ CFU/ml) in the selected solid state medium. The 2% starter of *Streptomyces* sp. strain S4 was inoculated into the solid medium and mixed well using stainless steel spatula. The fermentation was done at room temperature with manually shaking by hand every day. The fermented medium was spread on a sterilized stainless steel tray and dried at 37°C for 36-48 hours. The dried solid culture of *Streptomyces* sp. strain S4 was crushed into powder in a blender. 5g of the biological control powder was packed into a small aluminum foil bag with vacuum seal. Six bags of the product were stored at 4°C and another six bags were stored at room temperature.

The viable cell counts of the *Streptomyces* sp. strain S4 culture powder was determined using serial dilution plate count on nutrient agar every month for 6 months.

5.3.4 Shelf Life and Efficacy of the *Streptomyces* sp. strain S4 Biological Control Product

The shelf life of the biological control product (BCP) was estimated from the duration that the stored product could maintain the antagonistic cell viability not lower than the initial product. The efficacies of the biological control *Streptomyces* sp. strain S4 product in protecting seedlings from damping off disease caused by *P. aphanidermatum* were evaluated. Peat moss was used as planting material. The experiment was designed with six treatments of planting material preparation; 1) presence of *P. aphanidermatum* alone, 2) presence of *P. aphanidermatum* and BCP keeping at 4°C, 3) presence of *P. aphanidermatum* and BCP keeping at RT, 4) presence of *P. aphanidermatum* and methlaxyl, 5) presence of BCP alone, and 6) absence of both *P. aphanidermatum* and BCP as a control. One gram of six month old of BCP was mixed to 250 g sterilized peat moss. 100 Chinese spinach seeds were planted for 10 days in each planting material treatment with 10 replicates. The efficacy of seedling protection of BCP storing at 4°C and RT were determined from the percentage of non-infested seedlings compared to other treatments.

5.4. Results and Discussion

5.4.1 Chemical Composition of Shrimp Head and Rice Bran

Shrimp heads obtained from a shrimp frozen industry in Samutsakhon province were dried at 80°C in a hot air oven for 36 hours. The dried shrimp heads were changed into powder by grinding in a blender. The proximate composition was analyzed by following standard methods of AOAC (Appendix A). The shrimp head powder had high protein and chitin (Table 5.3), therefore, it could be a good nitrogen and carbon sources for the antagonistic *Streptomyces* strain S4. The results was confirmed by the work of Chen and Yang, 1994; and Ruth Hagen et al, 2007 which reported that shrimp heads had 17.0-23.6% chitin, 30-40% protein, 7-8% fat, and 23-42% ash. However, many studies have suggested that the chemical composition was depended on the species of shrimp, farm location, and environmental factors. The nutritional analysis of the rice bran (Table 5.3) demonstrated that rice bran used in the experiment had high carbohydrate content

and moderate amount of protein. Rice bran is a by-product from rice milling process. The price of rice bran is not so high and easily to find in Thailand, therefore, rice bran could be used to combine with shrimp head to formulate solid state fermentation media for cultivating the antagonistic *Streptomyces* strain S4.

Table 5.3 Chemical Composition of Shrimp head powder and Rice bran

Composition	Dry weight basis (%)	
	Shrimp head	**Rice bran**
Crude protein	37.07	12.5
fat	5.685	15.5
Ash	26.825	9.5
Chitin	21.14	-

5.4.2 The Growth of *Streptomyces* sp. strain S4 in Solid State Fermentation Media

The responses of cell growth optimized by RSM design following CCD are presented in Table 5.4. The results analyzed by using the analysis of variance (ANOVA) demonstrates that the model is statistically valid (F = 11.31, and $P < 0.05$). The coefficient of determination value (R^2) was 0.9271 which agreed with the adjusted R^2 of 0.8451. From the "Prob > F" values of model terms; X_2, X_3, X_1^2, X_2^2, and X_3^2 less than 0.05 indicated that their were significant terms for cell production in Table 5.5. After calculating, the following regression equation was performed in terms of coded factors to estimate the predicted response in Eq. (2).

$$\begin{aligned} Y \quad &= 6.93+0.066X_1+0.40X_2+0.19X_3 \\ &\quad -0.18X_1X_2+ 0.11X_1X_3 – 0.16X_2X_3 \\ &\quad -0.36X_1^2 - 0.20X_2^2 - 0.27X_3^2 \end{aligned} \qquad (2)$$

where Y is the predicted response, X_1 is ratio of shrimp head and rice bran, X_2 is initial moisture content and X_3 is inoculum size.

Table 5.4 The experimental responses of dependent variable growth.

Run	Substrate (Shrimp head:Rice bran)	Initial Moisture (%)	Inoculum size (%)	Growth (logCFU/g)	
				Actual value	Predicted value
1	25:75	55	1.0	6.5	6.34
2	50:50	50	1.5	7.25	7.04
3	50:50	50	1.5	6.71	7.04
4	25:75	45	2.0	6.635	6.52
5	75:25	45	1.0	5.3	5.33
6	75:25	55	2.0	6.5	6.63
7	50:50	50	1.5	6.56	6.81
8	25:75	45	1.0	5.55	5.36
9	25:75	55	2.0	6.5	6.41
10	75:25	45	2.0	5.47	5.57
11	75:25	55	1.0	6.5	6.56
12	50:50	50	1.5	6.94	6.81
13	50:50	50	1.5	6.78	6.95
14	0:100	50	1.5	5.4	5.65
15	50:50	50	1.5	7.3	6.95
16	50:50	50	2.5	6.3	6.26
17	50:50	60	1.5	6.94	6.94
18	50:50	50	0.5	5.385	5.46
19	50:50	40	1.5	5.3	5.36
20	100:0	50	1.5	5.58	5.39

Table 5.5 Analysis of variance (ANOVA) for the fitted quadratic polynomial model for cell mass production.

Source	Sum of Squares	Degree of freedom	Mean Square	F Value	p-value Prob > F	
Model	7.80	9	0.87	11.31	0.0012	significant
X_1	0.07	1	0.07	0.91	0.3687	
X_2	2.50	1	2.50	32.61	0.0004	
X_3	0.59	1	0.59	7.76	0.0237	
$X_1 X_2$	0.25	1	0.25	3.26	0.1084	
$X_1 X_3$	0.10	1	0.10	1.37	0.2763	
$X_2 X_3$	0.20	1	0.20	2.57	0.1477	
X_1^2	3.09	1	3.09	40.34	0.0002	
X_2^2	0.97	1	0.97	12.71	0.0074	
X_3^2	1.76	1	1.76	22.96	0.0014	
Residual	0.61	8	0.077			
Lack of Fit	0.26	5	0.052	0.44	0.8011	not
Pure Error	0.35	3	0.12			significant
Total	8.86	19				

The interaction of three responses was created as three-dimensional response surface plots (Figure 5.1-5.3). The results indicated that increasing and decreasing the carbon's

ratio from the optimum value at the ratio of 50/50 shrimp shell and rice bran could decrease growth production similar with the inoculum size response. Moreover, the growth production could increase when the initial moisture value increasing up to 60% at the optimum value of substrate and inoculum size. From the result using shrimp shell and rice bran at the ratio of 50/50, initial moisture content at 60%, and 1.5% inoculum size could produce maximum growth production at 6.94 logCFU/g of substrate. Therefore, these solid-state fermentation medium was selected as the appropriate medium for cell mass production.

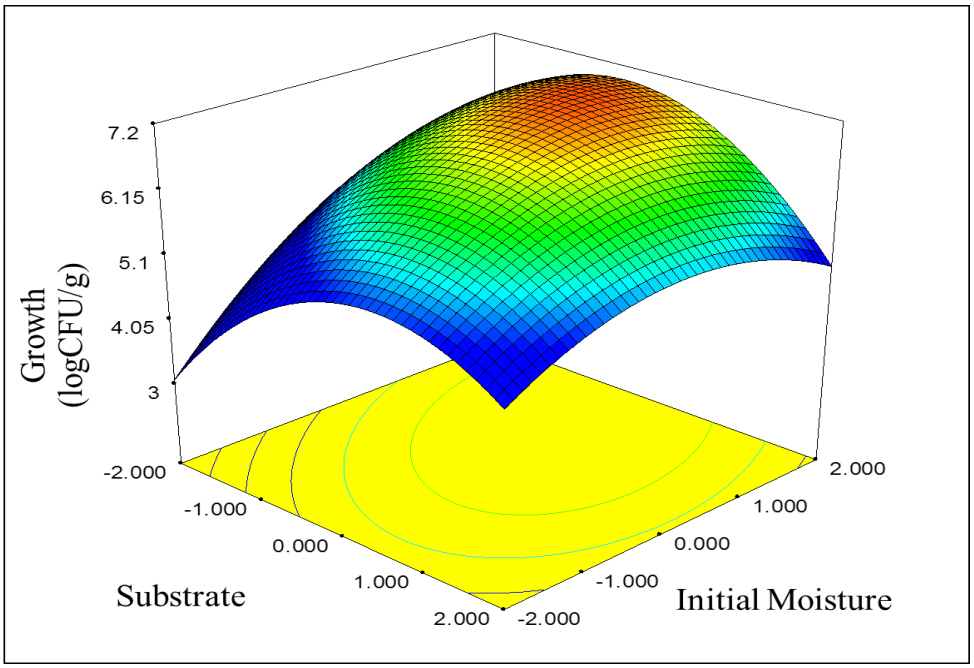

Figure 5.1 Contour plot to study the effect of substrate as ratio of shrimp head and rice bran and initial moisture (%) on the growth production (logCFU/g) at inoculum size coded level of zero.

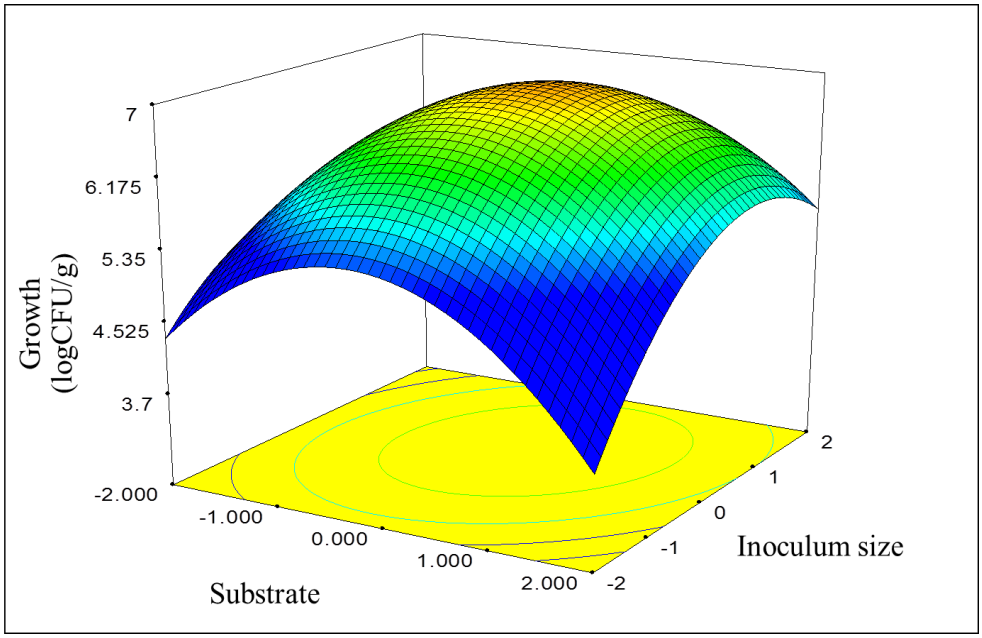

Figure 5.2 Contour plot to study the effect of substrate as ratio of shrimp head and rice bran and inoculum size on the growth production (logCFU/g) at initial moisture (%) coded level of zero.

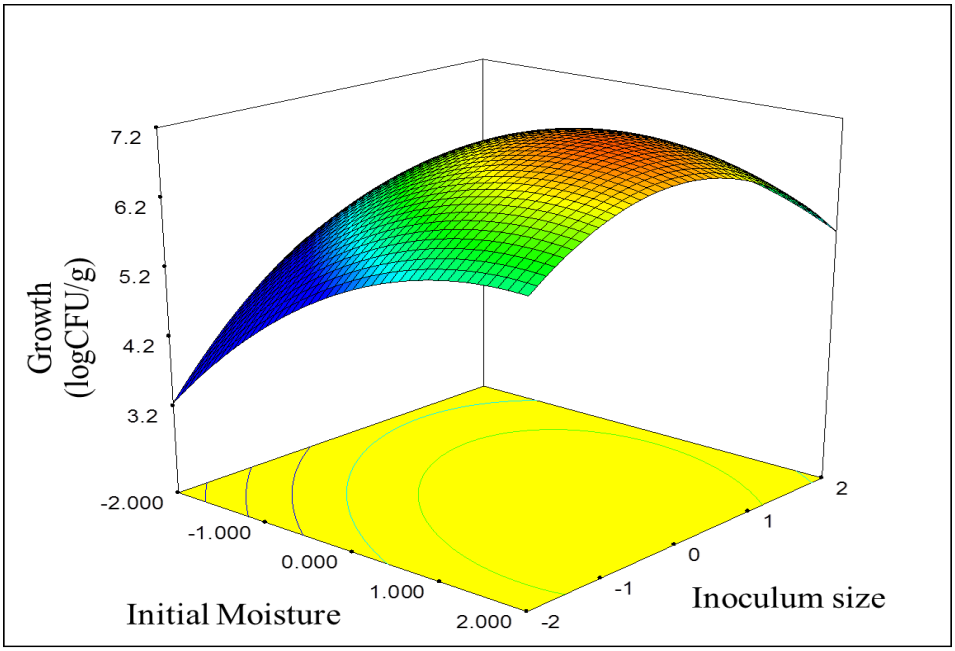

Figure 5.3 Contour plot to study the effect of initial moisture (%) and inoculum site on the growth production (logCFU/g) at substrate as ratio of shrimp head and rice bran coded level of zero.

5.4.3 The Cell Mass Production of *Streptomyces* sp. Strain S4

Streptomyces sp. strain S4 cells were cultured in selected solid-state fermentation medium. The fermentation was done at room temperature with manually shaking by hand every day for 3 days. The fermented medium was spread on a sterilized stainless steel tray and dried at 37°C for 48 hours. The dried solid culture of *Streptomyces* sp. strain S4 was crushed into powder in a blender and sifted through a sifter. The dried *Streptomyces* sp. strain S4 powder had an initial viable cell at $2.0x10^{7}$CFU/g dry powder (Figure 5.2). In the large-scale production, the solid-state fermentation method was easy for handing, shipping, and formulating (Durand et al., 1993). The drying step is often important step in cell mass production which can be affected to shelf life or biocontrol efficacy**.** The powder of *Streptomyces* sp. strain S4 was contained the fragmented *Streptomyces* filaments. The formulation of BCAs are usually used the dormant spores more than vegetative propagules (Chamberlain and Crawford, 1999; Lewis et al., 1995; Lumsden et al., 1995; Tahvonen and Avikainen, 1987; Trejo-Estrada et al., 1998).

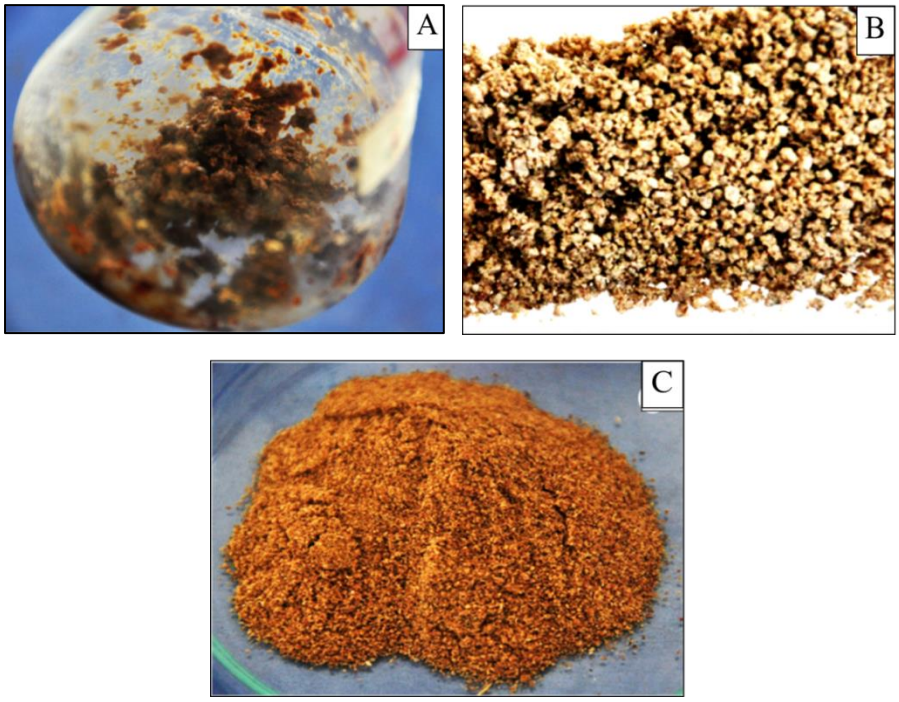

Figure 5.4 Selected solid state fermentation of *Streptomyces* sp. strain S4, Three-day-old fermentation in selected medium (A), dried *Streptomyces* sp. strain S4 cells (B), *Streptomyces* sp. strain S4 powders (C).

5.4.4 Shelf Life of Biological Control Product of *Streptomyces* sp. Strain S4

At 4°C, the initial viable cell of strain S4 was stilled constantly at 7.47 logCFU/g powder (100% viability) until the end of 6 months storage (Figure 5.3). The product stored at room temperature had a slowly decline after 4 months and the number of cell was 5.81 logCFU/g dry powder (95% viability) after six month storage. At low temperature, the viable cells minimized their metabolism in order to reduce the accumulation of toxic substances for extending the cell viability (Kirsop and Doyle, 1991).

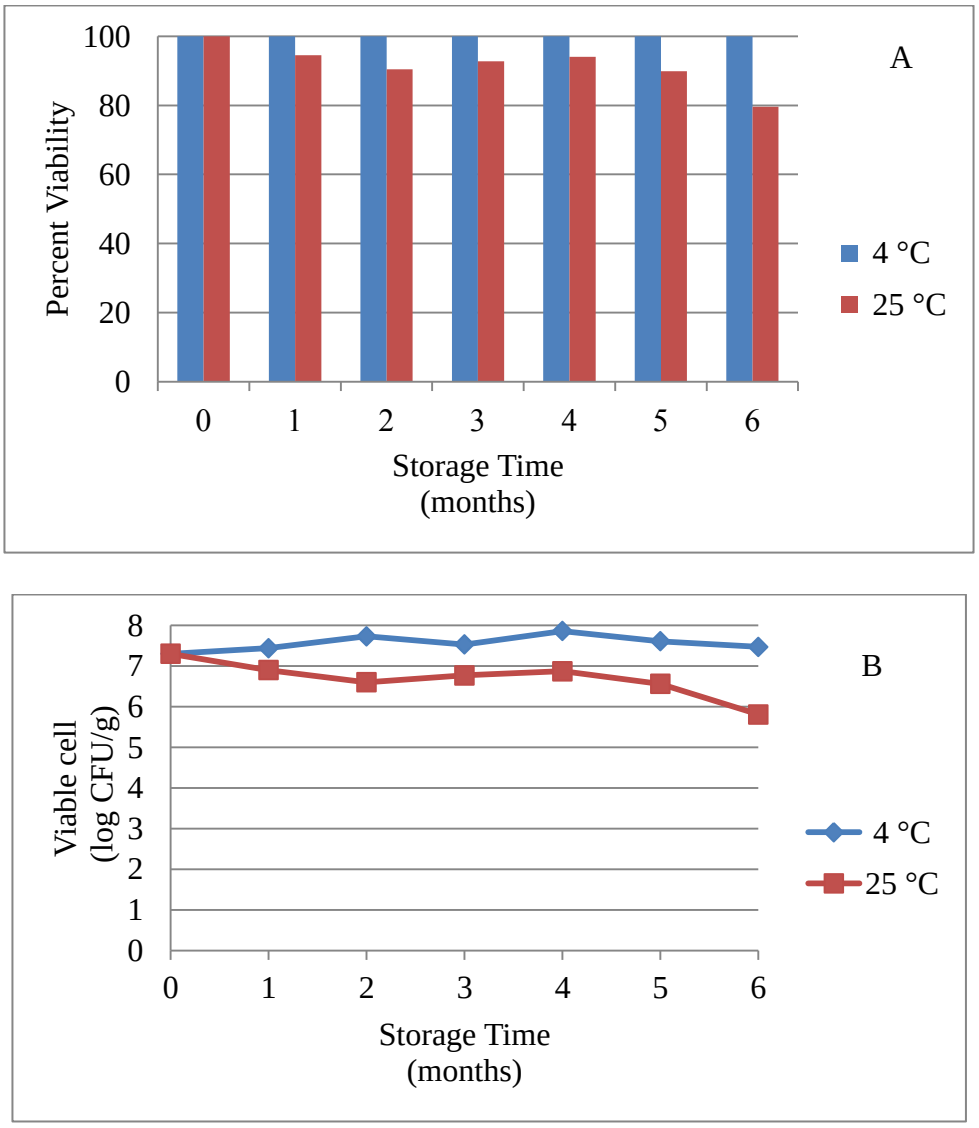

Figure 5.5 *Streptomyces* sp. strain S4 powder stored at 4°C and room temperature (RT). Percent viability of strain S4 powder (A), the viable cell counts of strain S4 (B).

5.4.5 Efficacy of Biological Control Product of *Streptomyces* sp. Strain S4 in Controlling of Seedling Damping Off Disease

Biological control product of *Streptomyces* sp. strain S4 stored for 6 months at 4°C and room temperature were tested for seedling damping off disease protecting efficacy. There were six treatments of planting material preparation; 1) presence of *P. aphanidermatum* alone, 2) presence of *P. aphanidermatum* and BCP keeping at 4°C, 3) presence of *P. aphanidermatum* and BCP keeping at RT, 4) presence of *P. aphanidermatum* and methlaxyl, 5) presence of BCP alone, and 6) absence of both *P. aphanidermatum* and BCP as a control. One gram of six month old of BCP was mixed to 250 g sterilized peat moss. 100 Chinese spinach seeds were planted for 10 days in each planting material treatment with 10 replicates. The quality of the seeds used in the experiment could be demonstrated in the treatment 6. When seeds were planted in the planting material without pathogen and BCP, the percentage of healthy seedling was 90%. For treatment 1, planting material contaminated with fungal pathogen alone, the percentage of non-infested seedlings was only 35%. The BCP storing at 4°C and RT could protect the seedlings from the disease, resulted in 100% protection when comparing to the control. At the same time, the BCP had a better protection than chemical fungicide. Therefore, the biological control product of *Streptomyces* sp. strain S4 could be used to control seedling damping off disease effectively even the product had been stored at 4°C or RT for 6 months.

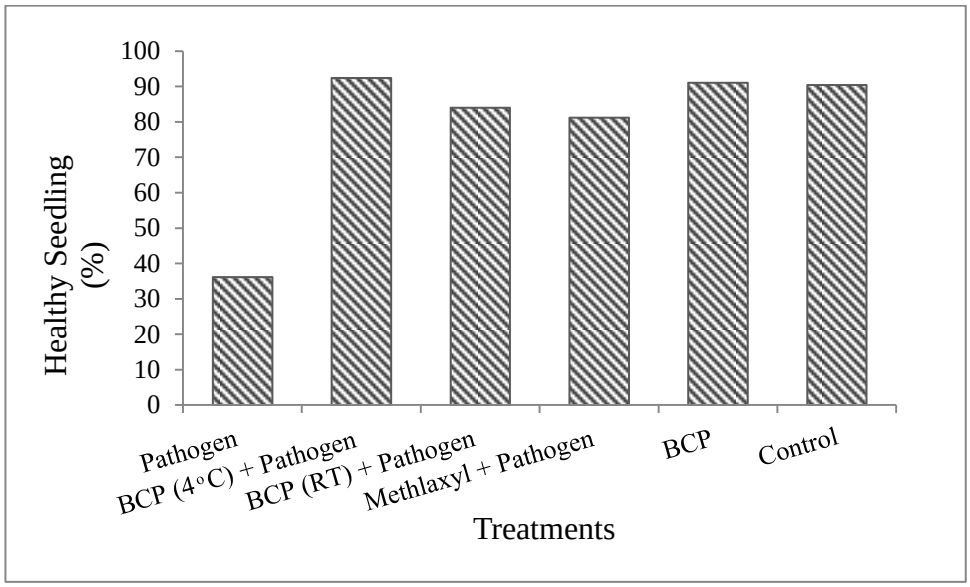

Figure 5.6 Efficacy of Biological Control Product of *Streptomyces* sp. strain S4 in controlling of *P. aphanidermatum*.

5.5 Conclusion

The cell mass of *Streptomyces* sp. strain S4 could be produced on solid state fermentation medium containing the mixture of shrimp head powder and rice bran at the ratio of 50/50 with 60% initial moisture content. The biological control product (BCP) has long shelf life for more than six months. The seedling damping off disease protection efficacy of BCP was still valid even though the product had been stored at 4°C or room temperature for 6 months.