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Spray-dried powder of *Bacillus amyloliquefaciens* strain C2-1 for control of rice diseases

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ABSTRACT

Rice cultivation in Thailand faces challenges in achieving high yields due to diseases. This research aims to develop a spray-dried powder containing *Bacillus amyloliquefaciens* strain C2-1 for control economically damaging rice diseases caused by *Xanthomonas oryzae*, *Fusarium moniliforme, Pyricularia grisea*, and *Bipolaris oryzae*. The *B. amyloliquefaciens* strain C2-1 was cultured in 3 different media, including 1) medium contained nutrient broth with glucose (NB), 2) medium contained beef extract, molasses, K_2HPO_4 and KH_2PO_4 (BM), and 3) medium contained yeast extract, molasses, K_2HPO_4, and KH_2PO_4 (YM). The results showed that YM medium gave the highest concentration of bacteria at $1.30x10^{16}$ cfu/ml, followed by BM medium at $9.88x10^{12}$ cfu/ml, and NB medium at $5.3x10^{12}$cfu/ml. Therefore, YM medium was selected as the optimal culture medium. Subsequently, the *B. amyloliquefaciens* strain C2-1 was produced into a spray-dried powder using the spray-drying technique. Tapioca starch and carboxymethyl cellulose were used as spray-drying carriers in a ratio of 10:1. The optimal spray-drying conditions included a hot air inlet temperature of 90°C, a hot air outlet temperature of 70°C, and a feed rate of 10-15 ml/min, resulting in the highest survival rate at 2.8×10^{12} CFU/g^{-1}. The efficacy of *B. amyloliquefacien* strain C2-1 powder was then tested against rice diseases. The inhibition of *X. oryzae* was assessed using the paper disc diffusion method, while the inhibitions of *F. moniliforme*, *P. grisae*, and *B. oryzea* were evaluated using dual culture assay. The results demonstrated that *B.amyloliquefacien* strain C2-1) powder exhibited strong inhibitory effects against *X. oryzae,* with clear zone measuring 5.5, 5.7, and 5.7 mm at 24, 48, and 72 h, respectively. Regarding inhibiting *F. moniliforme*, *P. grisae*, and *B. oryzea*, *B. amyloliquefaciens* strain C2-1 demonstrated effective control of these rice pathogenic fungi, resulting in inhibitions of 73.0%, 68.72%, and 48.42% at 7 days, respectively. These findings strongly suggest that the *B.amyloliquefacien* strain C2-1 obtained from spray drying serves as a promising biocontrol agent against bacterial leaf blight disease, bacterial blight, bakanae disease, rice blast disease, and brown spot disease caused by *F. moniliforme*, *P. grisae*, and *B. oryzea*, respectively.

Keywords: Antagonist bacteria, *B.amyloliquefacien*, biological control, rice disease, spray drying

INTRODUCTION

Rice (*Oryza sativa L.*) is the crucial main food crop belonging to the family Poaceae and is widely cultivated in most tropical and subtropical regions (Ezuka and Kaku, 2000). Nowadays, rice cultivation in Thailand still faces problems that affect the growth and yield of rice caused by weeds, soil, water, and environmental changes. One primary disease of rice is the bacterial leaf blight disease caused by *Xanthomonas oryzae* which can infect rice at any growth stage causing production losses depending on climate, growing season, rice variety, and growth; if the disease is severe, the yield can be reduced by 50 percent (Chintaganon et al., 2022). *Pyricularia grisea* is a fungus that causes blast disease that damages rice. Rice blast disease is favored by a number of factors such as high relative humidity (>80 percent), low temperature (15°C - 26°C), cloudy weather, more wet or rainy days, and excessive doses of nitrogen fertilizers (Saleh et al., 2020). *Bipolaris oryzae* causes brown spot disease that affects both quantity and quality of rice grains. This pathogen, under favorable epidemic conditions, can cause reduction in grain yield of up to 90 percent (Dorneles et al., 2020).

Strains of *B. amyloliquefaciens* strain C2-1 have been reported as a potential biological agent for controlling other crop diseases (Shrestha et al., 2016). This is accompanied by Srivastava et al. (2016), who reported that *B. amyloliquefaciens* (SN13) acts as a biocontrol agent and enhance the immune response against *R. solani*,

a necrotrophic fungus causing sheath blight in rice, by modulating various physiological, metabolic, and molecular functions. Moreover, Prabhukarthikeyan et al. (2019) also reported that *Bacillus amyloliquefaciens* strain BS5 effectively reduced the brown spot disease in rice under *in vitro*, glasshouse, and field conditions.

The spray-drying technique has been routinely used for the preservation and concentration of microorganisms due to its gentle protocols of drying by exposing substances to only a short burst of extreme temperature, then providing a cooling effect via the evaporation process, which is able to maintain a high survival rate of microorganisms and low production cost (Desmond et al., 2001). In order to prolong shelf life, and increase efficacy, it is important to keep the bacterium in a dormant state, while maintaining its viability. The efficacy of bio-product was tested in a greenhouse. The result showed that the disease incidence was reduced by 100 and 61.54% when treated the rhizome with bio-products MTR13 and PS6, respectively, before planting and application of their suspension was done one hour after the pathogen inoculation when compared with the control. (Thano and Akarapisan, 2018). The aim of this study was to develop bio-based products based on *B. amyloliquefaciens* strain C2-1 under optimal spray drying conditions for controlling rice disease. Therefore, the information on efficacy testing may be useful and able to apply or recommended to farmers or those who are interested in controlling diseases in rice fields.

MATERIALS AND METHODS

Microorganism and inoculum preparation

The antagonist bacterium *B. amyloliquefacien* strain C2-1 was isolated from food waste compost obtained from the Center for Genomics and Bioinformatics Research, Faculty of Science, Prince of Songkla University, Songkhla, Thailand. The rice pathogen, namely *X. oryzae, F. moniliforme, P. grisea, B. oryzae.* The mass losses may be attributed to the effect of the different parameters of spray drying, such as inlet temperature and airflow, as well as to the low Tg of carrying agents obtained from Phattalung Rice Research Center, Phattalung, Thailand. The *B. amyloliquefaciens strain C2-1 and X. oryzae* were cultured in nutrient broth (NB) (nutrient broth 13 g/L; glucose 10 g/L) and glucose broth medium (NGB) (13 g of nutrient broth and 12.5 g of glucose anhydrous per 1 L of distilled water), respectively at 37°C for 24 h on 200 rpm of the rotary shaker to prepare the inoculum. The *F. moniliforme, P. grisea, B. oryzae* were grown on potato dextrose agar (PDA) at 28°C for 7 days to prepare the inoculum.

Substrates and chemicals

All chemicals were of analytical grade, including nutrient broth (NB) (Himedia, Nashik, India) and KH_2PO_4 (anhydrous, Loba Chemie, Mumbai, India). Yeast extract powder was obtained from TM Mida (Rajasthan, India), beef extract from Srichem (Mumbai, India); KH_2PO_4 was obtained from Cloisters (Cherrybrook, Australia); glucose was purchased from Utopian Co., Ltd., Samuthprakarn, Thailand; and molasses was purchased from local markets (Songkla, Thailand).

Effect of culture media on antagonist bacterium growth

The antagonist bacterium *B. amyloliquefaciens strain C2-1* was cultured in different culture media, including nutrient broth (NB), beef extract molasses broth (BM) (beef extract 3 g/L; molasses 20 g/L; K_2HPO_4 0.05 g/L; KH_2PO_4 0.5 g/L) and yeast extract molasses broth (YM) (Yeast extract 0.5 g/L; Molasses 20 g/L; K_2HPO_4 0.05 g/L; KH_2PO_4 0.5 g/L) were prepared in each 250 mL flask with 100 mL medium containing. Each flask was cultured at 37°C for 24 h on 200 rpm of the rotary shaker before checking the colony forming unit (CFU/mL) using the serial dilution method on the NA agar medium. This experiment was maintained with three replications.

Preparation of antagonist bacterium in the spray-dried powder using spray-drying technique

The antagonist bacterium was cultured in YM, NB, and BM at 37 °C for 24 h on 200 rpm of the rotary shaker. The cell suspension was adjusted to 0.5 optical density (O.D. 600 nm) using the spectrophotometer (Thermo Fisher Scientific, USA) before mixing with tapioca starch and carboxymethyl cellulose (CMC). The mixing solution consisted of 30 mL cell suspension, 18.18 g tapioca starch, and 1.82 g CMC (Teera-Arunsiri et al., 2003) Spray drying was carried out in a Mini Spray Dryer B290 (BÜCHI, Labortechnik AG, Flawil, Switzerland) at the following operation conditions: feed pump of 5 ml/min °C, aspiration of 100% and pressure of 1.5 bar. The inlet and outlet temperatures were set as 90 and 70 °C, respectively. The obtained spray-dried powder was calculated as a percentage yield as following Eq. (1) that those reported by (Chumthong et al., 2016). Three replicates were performed for each formulation.

$$\% \text{ Yield} = [\text{outlet weight/inlet weight}] \times 100 \quad (1)$$

Viable bacterial counts in the formulations were tested against the bacterium before drying using the drop-on plate method (Zuberer, 1994) with bef. 0.1g/mL suspension of spray-dried powder was prepared in sterile distilled water and was serially diluted from 1×10^{-1} to 1×10^{-12}. The viable bacteria were cultured on NA medium at 37 °C for 24 h, after which the CFU was counted. The number of colony-forming unit per gram (CFU/g) was the average of six drops per dilution. The cell concentrations exhibited between 30 and 300 CFU (1×10^{-4} and 1×10^{-5}) were selected (Daniel et al., 2020). Equation (2) was employed for the quantification of culturability. All experiments were conducted in triplicate, and the reported values represented the average of the calculated values. Formulations with the concentrations of highest were chosen for further studies.

$$\text{CFU/g} = \frac{\text{[N°plate colonies x dilution factor]}}{\text{mL sample seeded}} \quad (2)$$

When N° is the initial population of *B. amyloliquefaciens* strain C2-1 (cfumL^{-1})

Antagonistic activities of B. amyloliquefacien strain C2-1 for pathogenic bacteria inhibition assays

Rice pathogenic bacteria, *X. oryzae* was cultivated in an NGB medium. 100 mL working volume of the NGB medium was conducted in each 250 ml Erlenmeyer flask for cultivation. The optical density of cell suspension was measured at 600 nm (OD600) with a UV-vis spectrophotometer (Thermo Fisher Scientific, USA). The culture flasks were inoculated with 10% (v/v) inoculums (initial cell suspension equal to 0.2 at OD600) at 25°C for 24 h on 200 rpm of the rotary shaker. The inhibition assays of spray-dried powder of *B. amyloliquefacien*s strain C2-1 testing were done using the agar well diffusion method (Yang et al., 2012). Glucose agar medium was prepared by pouring 10 mL glucose agar (NGA) medium into a petri dish, and let and let it set in a laminar flow hood as a basal layer. After that, use a cork borer, a diameter of 5 mm, drill 5 holes, and then using a cotton swab sterile dip the pathogens that have been prepared and make a swab over the entire surface of the culture medium. In this method, the agar plate was inoculated with a powder of *B. amyloliquefaciens strain C2-1*- dissolved in water by dropping each hole 10 ul of and placed on the four corners of the seed layer. At the same time,10 uL of sterile water was dropped into one hole in the center of the same petri dish (control). The petri dishes were then incubated at 37 °C for 24, 48, 72 h, and 1 week, and then measured the diameters of inhibition growth zones. The inhibition growth zones can be calculated from Equation (3).

$$R_a = (D_c - D_s) / 2 \quad (3)$$

where R_a is the inhibition radius (mm); D_c is the diameter of the clear area (mm), and D_s is the diameter of the specimen (mm)

Antagonistic activities of B. amyloliquefaciens strain C2-1 for pathogen fungi inhibition assays

Three fungal pathogens, *F. moniliforme, P. grisea*, and *B. oryzae* were cultured on PDA at 28°C for 7 days to test the inhibition of pathogen mycelial growth. The pathogen fungi inhibition assays of spray-dried powder of *B. amyloliquefaciens strain C2-1 spray-dried* was dissolved in 1 mL of sterile water. Each treatment was conducted in 3 replications. Mycelial inhibition of pathogen fungi was assessed as a percentage of mycelial inhibition at 3, 5, and 7 days after culturing pathogen fungi. The inhibition growth zones can be calculated from Equation (4) (Morton and Stroube, 1995).

$$\text{Percentage of pathogen mycelial inhibition} = [(R_1 - R_2) / R_1] \times 100 \quad (4)$$

Where R_1 is the colony radius of pathogen fungi on PDA incorporated powder *B. amyloliquefaciens* strain C2-1 solution of bacterial formulation, and R_2 is the colony radius of pathogen fungi control on PDA.

RESULTS AND DISCUSSION

Cultural media basic suitable for growth

The antagonist bacterium *B. amyloliquefaciens* strain C2-1 grown in YM medium performs the highest cell viability, followed by BM and NB, respectively (Table 1). As a result, YM and BM media contained a higher nutrient composition than the NB medium. It also can be seen that YM used molasses as a major ingredient which is a cheap carbon source, and yeast extract is a good source of nitrogen for growth. The buffered K_2HPO_4 and KH_2PO_4 can also help the infection grow better. It may help to adjust the pH condition so that it does not change according to the metabolites produced by the germ during growth.

Table 1. Viability of *B. amyloliquefaciens* C2-1 grown in different media

Formulation	cfu/g*		
	1st	2nd	3rd
NB	$1.8\pm0.0 \times 10^{12}$	$2.8\pm0.1 \times 10^{12}$	$2.6\pm0.0 \times 10^{12}$
BM	$3.6\pm0.1 \times 10^{12}$	$3.8\pm0.2 \times 10^{12}$	$5.5\pm0.2 \times 10^{12}$
YM	$4.9\pm 0.0 \times 10^{15}$	$5.2\pm 0.0 \times 10^{15}$	$4.7\pm0.1 \times 10^{15}$

Antagonist bacterium in the spray-dried powder using spray-drying

The initial amounts of powder dry matter of *B. amyloliquefaciens* strain C2-1 that grew in all three media after spray drying at a constant 90 °C inlet temperature were similar. From the initial amount of dry matter, 20 grams, after drying, the remaining 13 grams, thus the calculated average yield was 65%. The mass losses may be attributed to the effect of the different parameters of spray drying, such as inlet temperature and airflow, as well as to the low Tg of carrying agents (León-Martínez et al., 2010). Although the spray-dried product of *Bacillus* sp., especially *B*. subtilis (Yánez et al., 2012), has been devised and tested to control insect pests and plant disease, in this study, a spray-dried product of *B. amyloliquefaciens* strain C2-1 had been formulated and tested for the control of rice disease in the laboratory. The characteristic of spray-dried powder can be seen in Fig.1. The powder is fine and slightly yellowish-white. The viability of spray-dried powder of *B. amyloliquefaciens* strain C2-1 grown in different media is shown in Table 2. The result found that *B. amyloliquefaciens* strain C2-1 grown in YM medium performed the highest cell viability followed by BM and NB, respectively, which gave the same experiment result as the effect of culture media on antagonist bacterium growth. Therefore, the spray-dry powder of *B. amyloliquefaciens* strain C2-1 with YM medium was further studied for the pathogen inhibition assays.

Figure 1. Characteristic of powder of *B. amyloliquefaciens* strain C2-1 after spray drying at constant 90 °C inlet temperature.

Table 2. Viability of spray-dried powder of *B. amyloliquefaciens* strain C2-1 grown in different media

Formulation	cfu/g*		
	1st	2nd	3rd
NB	$3.4\pm0.0 \times 10^{8}$	$1.8\pm0.1 \times 10^{7}$	$1.7\pm0.1 \times 10^{7}$
BM	$4.2\pm0.2 \times 10^{10}$	$5.7\pm0.1 \times 10^{10}$	$5.8\pm0.0 \times 10^{10}$
YM	$2.6\pm0.1 \times 10^{12}$	$3.4\pm0.1 \times 10^{12}$	$2.8\pm0.1 \times 10^{12}$

Biocontrol activities of antagonistic B. amyloliquefacien strain C2-1

The test results of the efficacy in inhibiting the rice pathogen *X. oryzae* by the spray-dried powder of *B. amyloliquefaciens* strain C2-1 can be seen in Figure 2. The inhibition zone showed that the spray-dried powder was still effective against *X. oryzae* rice pathogens at 24, having clear zones of 1.65. This result shows that *B. amyloliquefaciens* strain C2-1 gave the inhibition of the causative agent of rice leaf blight. This inhibition may be caused by volatile substances from *B. amyloliquefaciens* strain C2-1, according to Wu et al. (2015), who reported that *B. amyloliquefacien* FZB42 had antibacterial activity against *Xoo* rice pathogens.

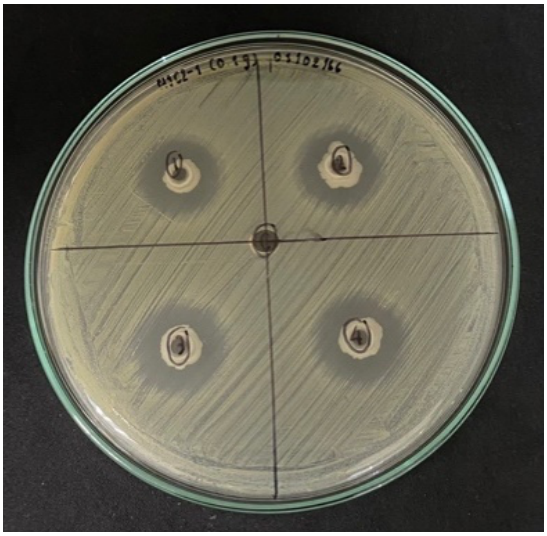

Figure 2. Reaction of the *X. oryzae* in agar well diffusion assay after inoculation for 24 hr. at 37 °C temperature, when inhibited by Powder *B. amyloliquefacien* strain C2-1.

The spray-dried powder of *B. amyloliquefacien* strain C2-1 showed antifungal activity against all 3 rice pathogenic fungi in dual culture tests incubated for 7 days. The spray-dried powder showed the strongest inhibition, as seen in Fig. 3, mycelial growth was reduced compared to control plate 3, 5, and 7 days. The inhibition percentages *F. moniliforme* were 0.78, 52.51, and 73.0%, respectively. For *B. oryzae*, the inhibition percentages by powder *B. amyloliquefaciens* strain C2-1 were 0.65, 22.86, and 48.42%, respectively. The mycelial growth of the pathogen *P. grisea*, and the inhibition levels by powder *B. amyloliquefaciens* strain C2-1 were 0.76, 56.99, and 68.72 %, respectively. Thus, powder *B. amyloliquefaciens* strain C2-1 had comparatively high effectiveness in suppressing the pathogens, after incubation for 3, 5, and 7 days.

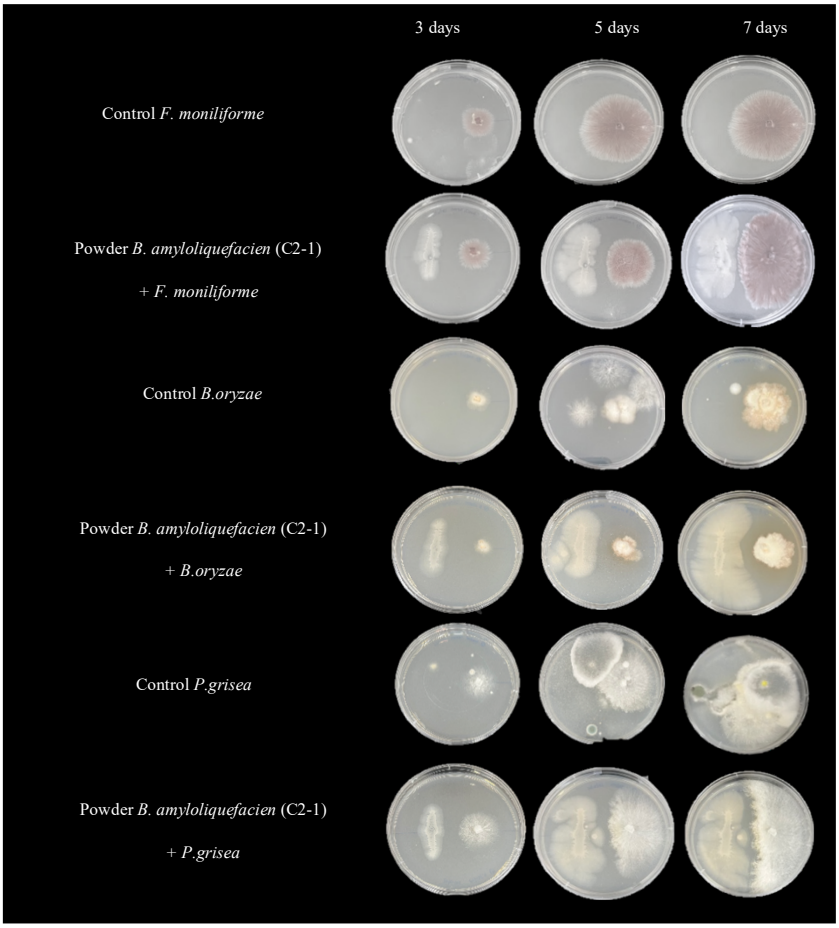

Figure 3. Effects of spray-dried powder of *B. amyloliquefaciens* strain C2-1 against *F. moniliforme, P. grisea,* and *B. oryzae* on dual culture plates incubated for 3, 5, and 7 days at room temperature.

CONCLUSIONS

In this study, *B. amyloliquefacien* strain C2-1 isolated from food waste compost, effectively inhibiting the growth of *X. oryzae* as well as the mycelial growth of *F. moniliforme, P. grisae,* and *B. oryzea* strain. Among the three general media tested, YM was selected as the optimum culture media for cell growth and viability after spray drying due to its cost-effectiveness and highest cell concentration.-The inhibition of *Xoo B. amyloliquefacien*s strain C2-1 powder against *X. oryzae* gave the widest clear zones. Regarding inhibiting *F. moniliforme*, *P. grisea*, and *B. oryzae*, it was found that *B. amyloliquefacien* strain C2-1 was able to control the growth of all these pathogenic rice fungi with 73.0%, 68.72 and 48.42 % inhibitions, at 7 days respectively. Thus, our results highlight the potential of spray-dried powder of *B. amyloliquefacien*s strain C2-1 as a promising biocontrol agent against the prevalent rice diseases currently affecting crops.

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