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Evaluation of plant growth–promoting *Streptomyces* sp. SR13–2 immobilized with sugarcane bagasse and filter cake for promoting rice growth

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Abstract

The present study investigates the potential use of the sugarcane industrial by–products as carrier materials for immobilized with plant growth promoting (PGP) actinomycete and its effect on the growth of rice. The antagonistic actinomycete SR13–2 obtained from rice rhizospheric soils was selected and evaluated for PGP properties. Results showed that the isolate SR13–2 exhibited ability to produce 16.15 µg/mL of indole–3–acetic acid, solubilize inorganic phosphate on Pikovskaya's agar, but did not produce siderophore. Based on morphological and the 16S rDNA sequence analysis indicated the isolate SR13–2 belonged to the genus *Streptomyces*. The sugarcane bagasse and filter cake were evaluated as potential supports for immobilization of *Streptomyces* sp. SR13–2 by solid–state fermentation. On the seventh day of fermentation, the number of *Streptomyces* sp. SR13–2 cells in the bagasse and filter cake were 8.0×10^9 and 3.1×10^{10} CFU/g dry carrier, respectively. Pot experiments revealed that *Streptomyces* sp. SR13–2 immobilized with the mixture bagasse and filter cake treatment exhibited significant enhancement in shoot height, root lengths, and dry weight of shoot and root, compared with all treatments. These results suggest that *Streptomyces* sp. SR13–2 immobilized in the bagasse and filter cake could be a promising candidate for rice growth enhancement.

Keywords: Plant growth promoting rhizobacteria, actinomycete, filter cake, bagasse, rice

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1. Introduction

Rice (*Oryza sativa* L.) is one of the major crops cultivated in Thailand. Chemical fertilizers have enabled increased rice production. However, extensive use of chemical fertilizers not only causes resource waste and environmental pollution but also negatively affects soil quality including a decrease in soil organic matter content and repression of microbial activity. (Zhang *et al.,* 2017). Introduction of plant growth–promoting (PGP) rhizobacteria as biofertilizers is a more ecologically friendly and economical approach to enhance rice production and can be used as a sustainable option for improving nutrient availability, plant growth, and crop yields (Youseif, 2018). PGP rhizobacteria colonize the rhizosphere of plants, growing in, on or around plant tissues and stimulating plant growth by several mechanisms (Meena *et al.,* 2017), which can be useful in developing bio-inoculants for enhancement of growth and yield of crop plants. PGP rhizobacteria enhance plant growth both directly and indirectly. In the direct mechanism, they can synthesize some phytohormones, including indole–3–acetic acid (IAA), gibberellic acid, cytokines and ethylene, produce siderophores to scavenge ferric iron from the environment, solubilize mineral phosphates, fix nitrogen and break organic materials for the easy uptake by plants and for their own use. (Awla *et al.,* 2017; Meena *et al.,* 2015; Mukhtar *et al.,* 2017; Rungin *et al.,* 2012). Indirect mechanisms of growth promotion by PGP rhizobacteria occur against plant pathogens through several mechanisms, including induced systemic resistance, the production of antimicrobial compounds, and competition with pathogens for nutrients and colonization sites (Habibi *et al.,* 2019). PGP rhizobacteria that can promote plant growth, including *Bacillus*, *Burkholderia*, *Enterobacter*, *Serrratia*, *Azospirillum*, and *Pseudomonas*, have been isolated and characterized (Dias *et al.,* 2017).

Actinomycetes are aerobic, spore–forming gram-positive bacteria, belonging to the order Actinomycetales and characterized by substrate and aerial mycelium growth (Bhatti *et al.,* 2017). They are present in a high proportion of microbial biomass in the plant rhizosphere and are useful in soil nutrient cycling as well as PGP (Sreevidya *et al.,* 2016). Actinomycetes, particularly species of *Streptomyces* produce a variety of antibiotics and extracellular enzymes, protect plants against pathogens, and promote growth. PGP traits of *Streptomyces* have been reported on chickpea (Sreevidya *et al.,* 2016), rice (Awla *et al.,* 2017; Gopalakrishnan *et al.,* 2014), and sugarcane (Kruasuwan and Thamchaipenet, 2016).

The maintenance of PGP rhizobacteria survival and activity in soil is an important consideration for the success of any inoculation procedure. Methods for increasing the survival of bacterial inocula have been proposed and include using carrier materials to protect bacterial cells from biotic and abiotic stresses in soil (Abd El–Fattah *et al.,* 2013; Nimnoi *et al.,* 2017).

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Commonly used carriers are coal, peat and calcined clays, plant waste material (agricultural waste such as corn cobs and plant compost) and inert materials (vermiculite, perlite and other minerals) (Tripti *et al.,* 2017). In Thailand, filter cake and bagasse are waste material of the sugarcane industry that cause problems with storage and pollution in the environment around sugar mills when they accumulate. Filter cake, a residue from the treatment of sugar cane juice by filtration, is a rich source of organic matter and characterized as a soft, spongy, amorphous, and dark brown to brownish material (Dotaniya *et al.,* 2016; Prado *et al.,* 2013). Bagasse is a solid waste derived from the extraction of sugarcane juice. It is a dry pulpy residue and is fibrous (Janke *et al.,* 2016). In general, the major components of bagasse are cellulose, hemicellulose, and lignin (Dotaniya *et al.,* 2016). Both filter cake and bagasse are applied as organic fertilizer on the sugarcane fields. This study was carried out to evaluate the PGP properties of the selected isolate, antagonistic actinomycete isolate SR13–2, obtained from rice rhizospheric soils. This study also aimed to determine the effects of this isolate, when immobilized with sugarcane bagasse and filter cake as an inoculant, on rice production under pot experiment conditions.

2. Materials and Methods

2.1 Actinomycete isolate

In a previous study, 166 actinomycetes isolated from rice rhizospheric soil samples were screened for antagonistic properties against *Curvularia lunata* using the dual culture technique. Of these strains, the actinomycete isolate SR13–2 showed the highest inhibition of growth at 91.94% (Detraksa and Surawattanakij, 2018). In this study, the selected isolate, SR13–2, was further screened for PGP traits. The actinomycete isolate SR13–2 was grown on glucose yeast extract malt extract (GYM) (glucose 10 g/L, malt extract 4 g/L and yeast extract 4 g/L) agar for use in further studies.

2.2 Identification of the actinomycete isolate SR13–2

Genomic DNA was extracted from cultured cells following Kumar *et al*. (2010). The 16S rDNA was amplified using a standard PCR protocol with the universal primers 27F (AGAGTTTGATCCTGGCTCAG) and 1492R (TACGGYTACCTTGTTACGACTT) (Shen *et al*., 2016). The PCR products were subjected to DNA sequencing at Macrogen Inc. Seoul, Korea. The sequences of the 16S rDNA were compared with those from the GenBank database using BLAST. A phylogenetic tree was constructed using the neighbor-joining method. Bootstrap analysis with 1,000 replicates was performed to estimate the statistical stability of the branches in the cluster using MEGA version 6.0 program (Gopalakrishnan *et al*., 2011).

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2.3 Characterization of PGP traits

2.3.1 IAA production

IAA levels produced by actinomycete were carried out by using a colorimetric method (Himaman *et al.*, 2016). The actinomycete isolate was inoculated into glucose yeast extract broth (glucose 10 g/L and yeast extract 10 g/L) containing 0.2% (w/v) L–tryptophan and incubated at room temperature (28–30°C) with shaking at 180 rpm for 7 days. The culture was then centrifuged at 6,000 rpm for 10 min. The supernatant was mixed with Salkowski's reagent (50 mL, 35% of $HClO_4$, 1 mL 0.5 M $FeCl_3$ solution). The mixture was left at room temperature for 30 min in the dark. Optical density (OD) was read at 535 nm using a spectrophotometer and the amount of IAA produced was calculated by comparing to the standard IAA curve.

2.3.2 Siderophore production

Determination of siderophore production was done using the chrome azurol S (CAS) assay (Schwyn and Neilands, 1987). Actinomycete isolate was point inoculated onto a CAS agar plate and incubated at room temperature for 7 days. An orange halo indicated siderophore production.

2.3.3 Phosphate solubilization

The actinomycete isolates were cultured on GYM agar, incubated at room temperature for 7 days and the culture was then spot inoculated on Pikovskaya's agar plates (Pikovskaya, 1948). The plates were incubated at room temperature for 2 weeks. The solubilization index (SI) was determined by measuring the halo (clear zone) diameter and the colony diameter, using the following formula (Meena *et al.*, 2015):

$$SI = (\text{Colony diameter} + \text{Halo zone diameter}) / \text{Colony diameter}$$

2.4 Carrier materials

The carrier materials used in this study were sugarcane bagasse and filter cake. These were collected from the Saraburi Sugar Company Limited, Wang–Muang, Saraburi province, Thailand. Each carrier was dried at 60ºC for 48 h, finely ground in a blender, sieved to particle size 150–300 µm, and stored at room temperature.

2.5 Preparation of inoculants

Batches of 100 g each of the treated carrier materials were added to 50 mL of distilled water in Erlenmeyer flask, followed by sterilization at 121°C for 30 min. Actinomycete isolate were inoculated into glucose yeast extract broth incubated at room temperature with shaking at 180 rpm for 3 days. The culture was then centrifuged at 6,000 rpm for 10 min and the cell pellet was suspended in 0.85% NaCl solution to form the seed suspensions of actinomycete

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(4.8×10^{10} CFU/mL as determined by the plate count method with GYM agar). The seed suspensions of actinomycete (20 mL) were inoculated into 100 g of each of the carrier materials and grown at room temperature for 7 days. The population of actinomycete in each carrier materials was determined by the plate count method using GYM agar.

2.6 Pot experiments

Rice seeds (Pathum Thani 1) were surface sterilized using 1% sodium hypochlorite followed by three rinses with sterile distilled water. The seeds were then stored in flasks at room temperature for 3 days to allow germination. The germinated seeds were planted in plastic pots (30 cm in diameter) containing 3,000 g of soil. The soil used in this study was collected from the rice plantation in Lopburi province. Each treatment included three blocks of 15 pots, with three seedlings planted in each pot. Therefore, a total of 45 seedlings was used for each treatment. The design of the pot experiments was: (1) un-inoculated control soil; (2) soil inoculated with bagasse; (3) soil inoculated with actinomycete immobilized with bagasse; (4) soil inoculated with filter cake; (5) soil inoculated with actinomycete immobilized with filter cake; (6) soil inoculated with a 1:1 (w/w) mixture of bagasse and filter cake; (7) soil inoculated with actinomycete immobilized with the a 1:1 (w/w) mixture of bagasse and filter cake. All inoculants were 10% of the final total weight. Pots were watered on alternate days with non-sterilized water. The plants were harvested after 45 days and selected parameters such as root length, shoot length, and dry weight of shoot and root were measured.

2.7 Data analysis

The means and standard deviations of the data were calculated and analyzed using analysis of variance (ANOVA) and Duncan's multiple range tests ($P \leq 0.05$). Statistical analyses were performed using SPSS 22.0.

3. Results and Discussion

3.1 Strain characterization

The actinomycete SR13-2 isolated from rice rhizospheric soil samples was selected as it had previously shown maximum growth inhibition against *Curvularia lunata* (Detraksa and Surawattanakij, 2018). The selected isolate could grow on GYM agar plates and produced water- soluble brown pigment. The aerial mycelium was white and the reverse side color was dark brown. The bearing spore chains were spiral type when observed under the light microscope. Phylogenetic analyses based on the 16S rDNA sequences showed that the isolate SR13-2 was most closely related to the species *Streptomyces rimosus* AC44 and *Streptomyces cellulosae* VJDS (Fig 1). The isolate SR13-2 showed 99.79% similarity with the two species of *Streptomyces*. This result confirmed that the isolate SR13-2 belonged to the

genus *Streptomyces*, but the 16S rRNA was not diverse enough to identify the species level in this genus. However, as the similarity percentage of the 16S rDNA between isolate SR13–2 and these species is >97%, DNA–DNA hybridization is necessary in this case for a final decision on their taxonomy (Yekkour *et al*., 2012).

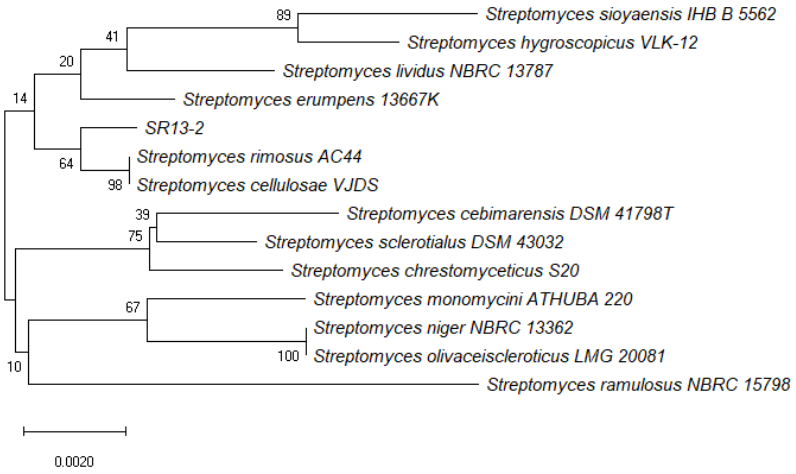

Fig 1 Phylogenetic relationship between the actinomycete SR13–2 and representative species based on 16S rDNA. The phylogenetic tree was constructed using the neighbor-joining method. The number at each branch is the percentages of times the group of strains in that branch occurred, based on 1000 cycles in the bootstrap analysis

3.2 Plant growth promoting activities of *Streptomyces* sp. SR13-2

When screened for PGP traits, *Streptomyces* sp. SR13–2 showed IAA production and solubilization of phosphate, but no siderophore production. IAA is the main auxin in plants, controlling physiological processes including cell enlargement and division, tissue differentiation, and responses to light (Ji *et al*., 2014). In the rhizosphere soils, root exudates are the natural source of tryptophan for microorganisms, which may enhance auxin biosynthesis in the rhizosphere. (Khamna *et al*., 2010). In this study, *Streptomyces* sp. SR13–2 produced 16.15 µg/mL of IAA at 7 days of incubation. IAA–producing microorganisms are receiving attention as good sources of biofertilizer (Nutaratat *et al*., 2014). Several reports have shown that *Streptomyces* from many crop rhizosphere soils found capable to produce IAA, such as *Streptomyces ramulosus* EUSKR2S82 (28.03 µg/mL) and *Streptomyces malaysiensis* EURKR1S17 (10.59 µg/mL) isolated the *Eucalyptus* roots (Himaman *et al*., 2016), *Streptomyces corchorusii* UCR3–16 (30.5 µg/mL) from rice rhizosphere soils (Tamreihao *et al*., 2016), and *Streptomyces atrovirens* ASU14 (22 µg/mL) isolated from wheat rhizospheric soil (Abd–Alla *et al*., 2013).

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Streptomyces sp. SR13–2 could solubilize inorganic phosphate on Pikovskaya's plates after 7 days of incubation and was showing 2.22 phosphate solubilization index (SI). Singh *et al*. (2014) screened rhizobial isolates from root nodules of different legumes and found that 70 strain showed phosphate SI in a range of 2.2 to 4.1 on Pikovskaya's agar media. Wahyudi *et al*. (2019) reported that the phosphate SI of the actinomycetes isolated from soybean rhizosphere were various ranging from 2.05–2.72. PGP rhizobacteria have ability to convert mineral phosphates into soluble phosphates by the production of organic acids such as malic acid, acetic acid, oxalic acid, citric acid and gluconic acid (Mukhtar *et al*., 2017). *Streptomyces* sp. SR13–2 could not produce siderophore as assessed by the CAS agar assay. Siderophores are low molecular weight ferric ion–specific chelating agents synthesized by rhizobacteria and plants growing under low ironic stress (Kumar *et al*., 2018). However, this isolate did show the IAA production and solubilization of phosphate that are necessary to enhance plant growth. In addition, a previous study reported *Streptomyces* sp. SR13–2 was able to produce both hydrolytic enzyme protease and cellulose (Detraksa and Surawattanakij, 2018), which is an indirect PGP activity that helps to promote antagonistic activities against plant pathogens. Therefore, *Streptomyces* sp. SR13–2 was also investigated for its ability to promote rice growth in pot conditions.

3.3 Inoculant preparation

The bagasse and filter cake were suitable carriers to support populations of *Streptomyces* sp. SR13–2, containing a colony count of 8.0×10^9 and 3.1×10^{10} CFU/g dry carrier, respectively after 7 days of fermentation. In many countries, inoculants containing 10^7 CFU/g or more are considered bioorganic fertilizer production standard (Wei *et al*., 2015). In this study, high viable cell counts were observed in all carriers, which supports probability of the survival of the strain in stressful environments.

3.4 Pot experiment

Rice plants treated with *Streptomyces* sp. SR13–2 immobilized with bagasse and filter cake under pot trial conditions showed a significant difference in rice growth–promoting parameters, including root length, shoot height, and dry weight of shoot and root, when compared with the un–inoculated control treatment ($P \leq 0.05$) (Table 1). Carrier treatments alone also increased rice growth. Importantly, all treatment containing SR13–2 immobilized with the carrier material also showed significant increases in rice growth-promoting parameters when compared to the carrier material alone treatments. These results indicated *Streptomyces* sp. SR13–2 could be used to facilitate an effective plant growth promotion in rice plants. The inoculant containing a mixture of bagasse and filter cake enhanced root development (including root length and shoot and root dry weight) and showed 63.69% increase in shoot

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and root dry weight compared to results with the carrier materials alone (Fig 2). Production of IAA is one of the direct PGP effect of rhizobacteria on plants. IAA is generally considered the most important phytohormone that coordinates different developmental processes in plants; a low concentration of IAA promotes primary root elongation, whereas a high IAA concentration stimulates lateral and adventitious root formation (Youseif, 2018). Overall, the greatest increases in growth-promoting parameters compared to control were found in rice inoculated with SR13–2 immobilized with a 1:1 mixture of bagasse and filter cake.

This study has demonstrated the available carrier material will improve the survival of inoculants as well as improve plant growth upon delivery of bacterial inoculum into the soil. Bagasse is a valuable by–product, as a lignocellulosic feedstock for second-generation biofuels. In general, bagasse contains cellulose (47–52%), hemicellulose (25–28%), and lignin (20–21%) (Dotaniya *et al*., 2016). Filter cake consists of 80% water and 0.9–1.5% sugar, organic matter, nitrogen, phosphorus, potassium, calcium, sulfur, coagulated colloids, and other materials in varying amounts. Filter cake has been used as a complete or partial substitute for mineral fertilizers in sugarcane cultivation (Prado *et al*., 2013). The significant increase in shoot and root dry weight upon inoculation of a mixture of bagasse and filter cake indicated that *Streptomyces* sp. SR13–2 have ability to provide better nutrient flux to the plant host which resulted in the increase of plant biomass.

Table 1 Effect of different inoculants on the growth of rice at 45 days after inoculation

Treatments	Plant height (cm)*	Root length (cm)*	Dry weight of shoot and root (g/plant)*
Control (un–inoculated)	44.9±0.40^{e}**	19.10±1.85^{e}	0.20±0.02^{g}
Bagasse	53.07±0.81^{d}	21.17±1.04^{de}	0.34±0.07^{f}
SR13–2 with bagasse	61.40±0.79^{c}	22.93±1.50^{cd}	0.67±0.03^{e}
Filter cake	71.17±0.93^{b}	24.27±0.87^{c}	2.15±0.10^{d}
SR13–2 with filter cake	77.23±0.68^{a}	34.73±2.00^{a}	3.52±0.10^{b}
Bagasse and filter cake (1:1)	72.93±1.68^{b}	28.47±0.85^{b}	2.79±0.11^{c}
SR13–2 with bagasse and filter cake (1:1)	77.13±1.33^{a}	36.37±0.65^{a}	4.38±0.09^{a}

* Data are means ± standard deviations (SD) of five replicates

** Values with different letters within the same column are significantly different at P≤0.05 according to Duncan's test

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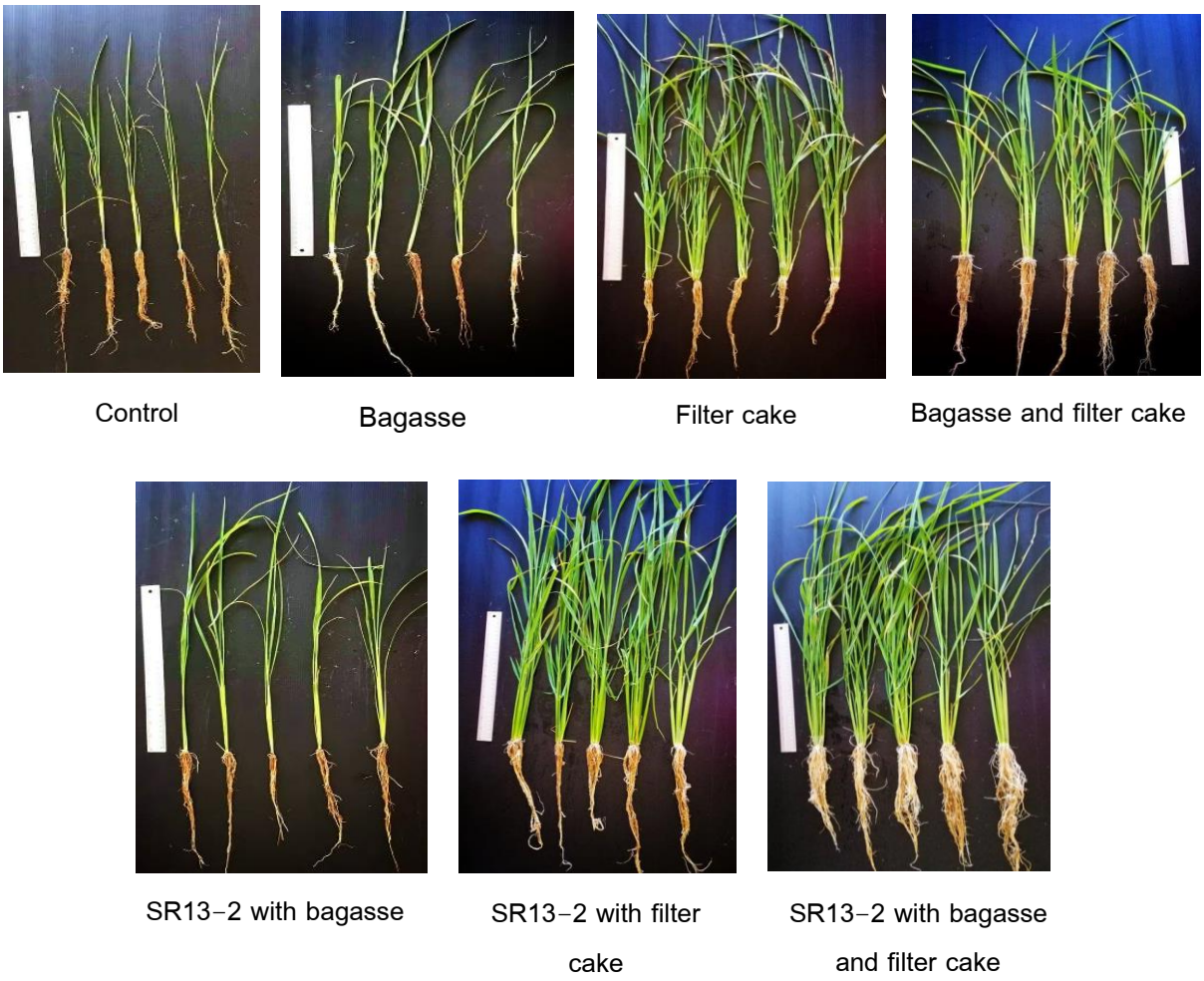

Fig 2 Effect of *Streptomyces* sp. SR13–2 immobilized with bagasse and filter cake under pot trial conditions at 45 days after inoculation

4. Conclusion

The antagonistic actinomycete, *Streptomyces* sp. SR13–2, was obtained from rice rhizospheric soils and exhibited plant growth–promoting activities included IAA production and solubilization of phosphate. This study showed that sugarcane bagasse and filter cake were suitable carrier materials for *Streptomyces* sp. SR13–2 inoculants to enhanced rice growth. These results support ongoing prospecting of actinomycete isolates for inclusion into commercial PGP inoculants for sustainable agriculture. The use of PGP inoculants as a replacement for chemical fertilizers in agricultural fields could enable the recovery of the natural ecosystem environment.

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