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Biochemical Characterization of *Streptomyces* spp. Isolated from Rhizosphere Soils of Rice in Odisha

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ABSTRACT: *Streptomyces,* the predominant genus of Actinobacteria, is a group of bacteria that is most frequently isolated from soil. The isolation and biochemical characterization of *Streptomyces* spp. are the main objectives of this work. In the present study, rice rhizospheric soil samples were collected from different rice-growing areas of Odisha. A total of eight morphologically distinct actinomycetes were isolated and identified as *Streptomyces* spp. through a series of biochemical tests *viz*., gram staining, catalase test, gelatin hydrolysis, citrate utilization test, starch hydrolysis, indole test, HCN test, casein hydrolysis and cellulase test. Biochemical test results confirmed that all the eight isolates belong to the genus *Streptomyces*.

Keywords: *Streptomyces* spp., biochemical test, characterization, rice.

INTRODUCTION

Plant diseases caused by different types of microorganisms including fungi, bacteria, viruses, nematodes and protozoa seriously affect agricultural production, leading to substantial losses in crop yields (Chakraborty & Newton 2011). While there are several reasons for the decrease in crop yield, losses from pests and diseases account for a significant portion of the damages worldwide. Pathogenic infections alone are responsible for direct yield losses ranging from 20% to 40% of agricultural productivity worldwide (Savary *et al*., 2012). Different approaches have been used to mitigate these problems, including the use of insecticides, crop rotation and other control measures. However, because soil-borne diseases are persistent and resistant, the effectiveness of these strategies remains inadequate (Syed Ab Rahman *et al*., 2018). To increase agricultural production, modern agriculture depends on the application of certain chemical fertilizers. However long-term and excessive usage of these chemical fertilizers results in the accumulation of some chemical residues that may cause environmental damage, including groundwater contamination, soil structure alteration and ecological damage. Nowadays, research is focused on environmentally safe and acceptable alternatives as an essential part of an integrated crop management system to decrease plant diseases and boost agricultural production. There is increasing interest in the biological control of plant diseases and it can be long-lasting, gives few quick profits and non-lethal to life, but it is slow and inexpensive. Biological control by using potential actinomycetes is receiving greater attention all over the world.

Actinomycetes are a type of bacteria that are Gram-positive and have DNA containing a high percentage of G+C (>55%). The name 'Actinomycetes' comes from the Greek words 'aktis' meaning 'ray' and 'mykes' meaning 'fungus' (Pandey *et al*., 2004). Filamentous organisms known as actinomycetes are thought to constitute a transitional group between fungi and bacteria. These organisms are extensively found in nature and are highly valued for their advantageous characteristics. Actinobacteria are responsible for giving characteristic earthy odour in soil due to the production of the organic compound “geosmin”. Endophytic actinobacteria demonstrate the ability to inhibit various fungal pathogens both in laboratory conditions (in-vitro) and within living organisms (in-vivo) via activating crucial genes in the pathways for jasmonate/ethylene (JA/ET) or systemic acquired resistance (SAR) (Conn *et al*., 2008).

Streptomyces is the most well-known type of actinomycetes found in nature, with the largest variety of species and varieties (Takahashi and Omura 2003). *Streptomyces* produce a large amount of branching substrate and aerial mycelia, which form cross-walls in the multinucleate aerial filaments and eventually grow into chains of spores (Anderson & Wellington 2001). Soil *Streptomyces* play a significant part in the biological buffering of soils by aiding in the

decomposition of organic materials, which promotes crop production and the growth of agricultural plants (Keiser *et al*., 2000). These represent a few of the microbial antagonists that have been used for the biological control of plant diseases. The capacity of some *Streptomyces* species to produce a variety of secondary metabolites, such as vitamins, alkaloids, extracellular enzymes, antibiotics, and vitamins, has drawn attention to them (Chater *et al*. 2010; Shahidi *et al*. 2004). Numerous researchers have examined the production of extracellular hydrolytic enzymes and antifungal chemicals by different *Streptomyces* species in the context of the primary field of biocontrol of plant diseases (Prapagdee *et al*., 2008). Approximately 75% of antibiotics used in medicine and agriculture, as well as 60% of antibiotics used commercially, are produced by the species *Streptomyces* (Sanghvi *et al*., 2014). The aim of the study was to isolate the *Streptomyces* spp. from the rice rhizosphere of Odisha and characterize them by various biochemical methods.

MATERIALS AND METHODS

Soil samples were collected from different rice-cultivating areas of Odisha to isolate *Streptomyces* spp. Soil samples were collected from a depth of 10-20 cm below the soil surface. Briefly, 1 g of air-dried soil was suspended in 9 ml of sterile distilled water and successive serial dilutions were made by transferring 1 ml of aliquots to the second test tube with 9 ml of sterile distilled water, and subsequently diluted to 10^{-2},10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} (Kuester *et al*., 1964). An aliquot of 100 µl of 10^{-4}, 10^{-5} and 10^{-6} dilutions was spread evenly over the surface of SCA (Starch Casein Agar) media supplemented with rifampicin (2.5 µg/ml) and nystatin (50 µg/ml) in a petri dish. Following incubation at 30°C for 7 days, the morphologically distinct *Streptomyces* colonies were recovered and inoculated into International *Streptomyces* Project (ISP2) agar medium and incubated at 28°C for 7 days.

Maintenance of isolated *Streptomyces* spp. The colonies of *Streptomyces* spp. were picked up and transferred to ISP2 agar slants and stored at 4°C.

Biochemical characterization. To identify the *Streptomyces* isolates, a number of biochemical tests were carried out. A total of nine (9) biochemical tests were performed on all isolates, which included gram staining, catalase test, gelatin hydrolysis, citrate utilization test, starch hydrolysis, indole test, HCN test, casein hydrolysis and cellulase test.

Gram staining. This technique helps to differentiate and identify if the isolate is gram positive or gram negative. Using a clean glass slide, a thin smear was created for the staining process, which was then air-dried and flame-fixed. Gram's staining was then applied to the tiny smear. The smear was flooded with crystal violet solution for 1 min, followed by a brief decolorization with 95% ethanol and a counterstaining with safranine solution. Then, the slide was blot-dried and examined under microscope under oil immersion (Benson, 1994).

Catalase test. Catalase is an enzyme that catalyses the release of oxygen from hydrogen peroxide, and its presence is indicated by this test. A drop or two of 3% hydrogen peroxide was used. Subsequently, a loopful of a 24-48 hours old bacterial broth culture was added or mixed with the hydrogen peroxide. The observation was then conducted to detect the formation of bubbles, indicating the liberation of oxygen (Collee *et al*., 1996).

Gelatin hydrolysis test. According to Pickett *et al.* (1991), the isolates were tested for the formation of gelatinase on gelatin agar media. The culture medium plates were spot inoculated with 72 h old *Streptomyces* cultures and incubated at 28±0.1°C for a period of 5 days. When acidified mercuric chloride (6 g of HgCl2, 8 ml of concentrated hydrochloric acid and 40 ml of distilled water) was added, a transparent zone formed around the inoculation site. This showed gelatin hydrolysis.

Citrate Utilization test. This test evaluates an organism's capacity to use citrate as its only source of carbon and nitrogen for growth, and an ammonium salt as its only supply of nitrogen. For this assay, Simmon's citrate agar medium was used. A 24-48 hours old culture was added to the medium, and it was then incubated for 96 hours at 37°C. An observation was made that the medium's pH had changed, causing the colour to shift from green to blue (Collee *et al*., 1996).

Starch hydrolysis test. Starch agar medium was prepared, sterilized, and then transferred onto sterile petri dishes for this test. The test isolates were point-inoculated onto the plates, then plates were kept for a 3 days incubation period at 37ºC. Gram's iodine was added to the culture plates after incubation. Lastly, plates were examined to see if a distinct hydrolysis zone had formed surrounding the growth (Benson, 1994).

Indole test. The purpose of this experiment was to determine the ability of bacteria to convert the amino acid tryptophane to indole and cause its accumulation in the medium. A 24-48 hours old culture was inoculated to tryptone broth, and it was then incubated for 48 hours at 37°C. The coloured reaction was used to assess the synthesis of indole after adding Kovac's reagent. A red colouring suggested a positive reaction (Collee *et al*., 1996).

HCN test. The ability of the efficient test isolates to generate HCN was assessed following the procedure outlined by Wei *et al.* (1991). Tryptic soya agar medium (TSA) (Himedia, Mumbai) supplemented with glycine (4.4 g/l) was sterilized before being poured into sterile plates. Seven days old test isolates were streaked onto the medium. On the medium, seven days old test isolates were streaked. Each plate's filter paper (Whatman no. 1) was soaked in two millilitres of sterile picric acid solution (Picric acid, 2.5 g; Na_2Co_3, 12.5 g and distilled water, 1 litre) (Miller and Higgins 1970) and placed on top of each plate. The inoculated plates

were allowed to react chemically with picric acid on top and were sealed with parafilm to confine the gaseous metabolites generated by the antagonistic isolates. The emergence of an orange to red coloration signified the production of hydrogen cyanide (HCN) by the isolates (Ahmad *et al.,* 2008).

Bacterial isolates were cultured for 48 hours at 28±2°C in tryptic soya broth with saturated (10 cm long, 0.5 cm broad) strips of picric acid solution to quantify the production of HCN. The reddish substance generated from the sodium picrate in the filter paper was assessed by the amount of hydrocyanic acid that evolved. To elute the colour, filter paper was put in a clean test tube with 10 ml of distilled water, and absorbance was measured at 625 nm (Sadasivam and Manickam 1992).

Casein hydrolysis Test. This experiment involved inoculating 24-48 hours old cultures onto skimmed milk agar medium and incubating them for 48 hours at 37ºC. The plates were then examined to see for clear zone formation around the colony (Benson, 1994).

Cellulase test. A 72 hours old *Streptomyces* culture on actinomycete isolation agar (AIA) medium was inoculated on cellulase agar medium and incubated for five days at 28±0.1°C. Then, Gram's iodine was poured onto agar plates (Kasana *et al*., 2008). There was a colourless zone around the colonies, which indicated the hydrolysis of cellulase.

RESULTS AND DISCUSSION

A total of 85 actinomycetes were isolated from different rice-cultivating areas of Odisha. Isolated actinomycetes showed well-developed substrate mycelium, were filamentous branched and most aerial mycelium appeared floccose, granular and powdery. They were screened against rice blast disease causing agent, *Magnaporthe oryzae* (Data is not shown). Based on the screening results, only eight efficient *Streptomyces* spp. were selected and further used for biochemical characterization which included Gram staining, catalase test, gelatin hydrolysis, citrate utilization test, starch hydrolysis, indole test, HCN test, casein hydrolysis and cellulase test and Results are presented in Table 1 and interpreted as they belong to genus *Streptomyces*.

Table 1: Biochemical characterization of *Streptomyces* spp. collected from rice-growing areas of Odisha.

Sr. No.	Biochemical test	S-3	S-14	S-16	S-20	S-25	S-37	S-40	S-59	Control
1.	Gram staining	+	+	+	+	+	+	+	+	-
2.	Catalase test	+	+	+	+	+	+	+	+	-
3.	Gelatin hydrolysis	+	+	+	+	+	+	+	+	-
4.	Citrate utilization	-	+	-	-	+	-	-	-	-
5.	Starch hydrolysis	+	+	+	-	-	-	+	+	-
6.	Indole test	+	+	+	+	+	+	+	+	-
7.	HCN test	-	+	-	+	+	+	+	+	
8.	Casein hydrolysis	-	+	-	+	+	+	-	+	-
9.	Cellulase test	+	+	+	+	+	+	+	+	-

+: Positive, –: Negative

Plant growth-promoting (PGP) rhizobacteria are part of the beneficial rhizobacterial community, which is essential for growth and development of the plant host (Morgan *et al*., 2005). These bacteria play a role in regulating plant developmental processes, suppressing the growth of phytopathogens, enhancing nutrient availability, and facilitating nitrogen fixation (Lugtenberg and Kamilova 2009). A well-known PGPR is *Streptomyces*, which has a wide range of plant species due to its antibacterial properties against phytopathogens and characteristics that promote plant growth in wheat and rice (Jog *et al*., 2014; Gopalakrishnan *et al*., 2014). Sawant *et al*. (2022) isolated bacteria from the rhizosphere region and used biochemical characterization, such as gram's staining, KOH test, starch hydrolysis, IAA formation, siderophore synthesis, HCN test, and protease activity, to validate the identification of the bacterial isolates as *Bacillus* spp. Akshatha *et al*. (2022) conducted a series of biochemical tests such as; IAA, starch hydrolysis, catalase, casein hydrolysis, nitrate reduction, urease, voges proskauer, methyl red, gelatin hydrolysis, sodium chloride tolerance, cellulose degradation, citrate utilization and hydrogen sulphide production in order to identify the genus *Streptomyces* spp.

Our present study is in concurrence with the findings of Shrivastava *et al.* (2015), who isolated 66 actinomycetes, Out of these, 8 isolates with the potential to promote plant growth were studied further and identified through physiological and biochemical traits. The results indicated that the majority of these isolates belong to the genus *Streptomyces* spp. *Streptomyces* has the capacity to be used as an alternative to traditional chemical treatments for biocontrol purposes as they are widely distributed in soil and have been demonstrated to inhibit a variety of phytopathogenic organisms *in vivo* and *in vitro* (Viaene *et al*., 2016).

Catalase test. All the *Streptomyces* isolates produced a positive reaction when 3% Hydrogen peroxide (H_2O_2) was added to the broth containing *Streptomyces* isolates. Formation of gas bubbles was observed (Fig. 1). This result was supported by Malviya *et al*. (2013).

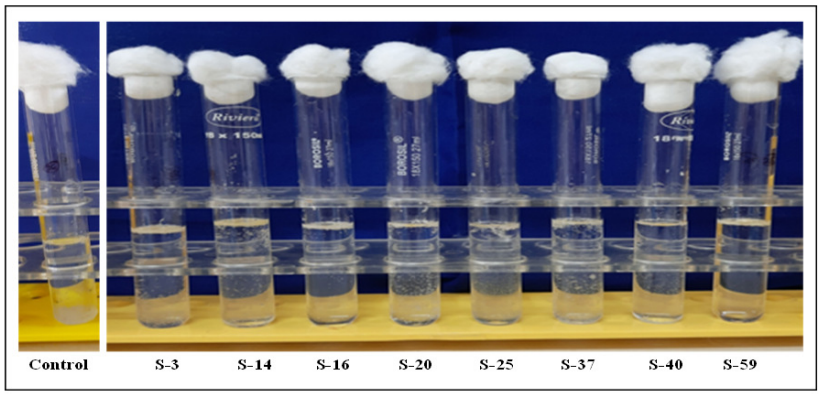

Fig. 1. Catalase test by effective *Streptomyces* isolates.

Gelatin hydrolysis. All the *Streptomyces* isolates showed a positive reaction to gelatin hydrolysis. The formation of a transparent zone was shown by all the isolates. S-14 has shown highest reaction to the gelatin hydrolysis (Fig. 2). Our findings are in concurrence with Sowmya *et al*. (2022); Islam *et al*. (2014).

Citrate utilization test. Two *Streptomyces* isolates S-14 and S-25 produced a positive reaction to the citrate utilization test. Due to the change in pH by effective utilization of citrate by S-14 and S-25 isolates has resulted in the alteration of media colour from green to blue (Fig. 3). Our findings are in agreement with Malviya *et al*. (2013).

Starch hydrolysis. All the *Streptomyces* isolates except S-20, S-25 and S-37 produced positive reactions by hydrolysing starch and the clear zone around the colony. S-3 and S-14 have shown the highest clear zone (Fig. 4). Our findings are in concurrence with Shrivastava *et al*. (2015) who reported that starch utilization was shown by *Streptomyces* isolates.

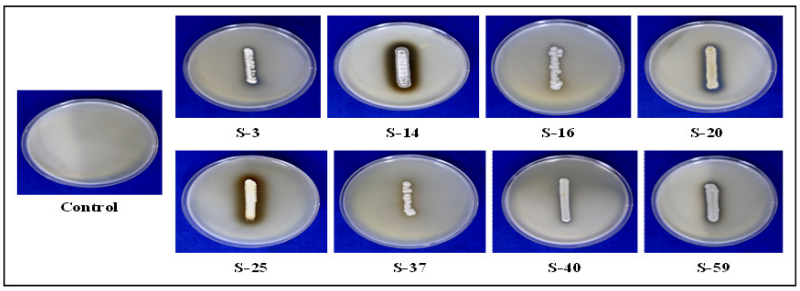

Fig. 2. Gelatin hydrolysis by effective *Streptomyces* isolates.

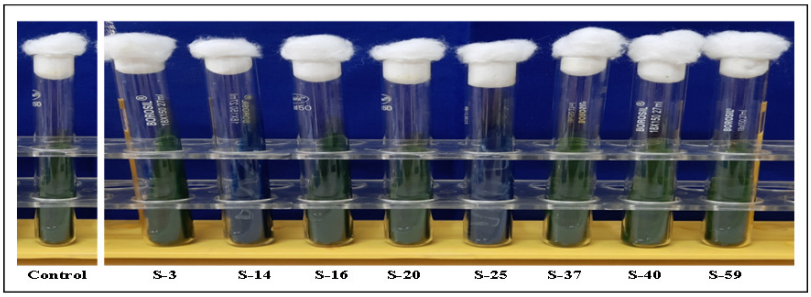

Fig. 3. Citrate utilization by effective *Streptomyces* isolates.

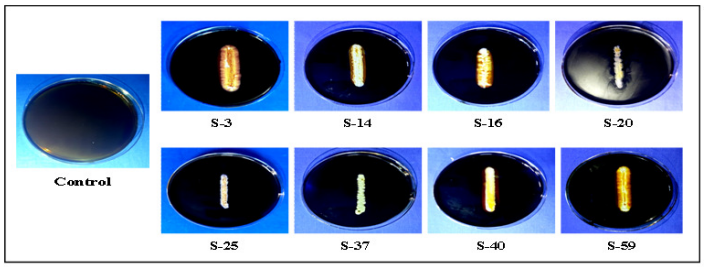

Fig. 4. Starch hydrolysis by effective *Streptomyces* isolates.

Indole test. In our studies all the *Streptomyces* isolates exhibited a positive reaction when reacted with Kovac's reagent, developing red colour, indicating a positive result for IAA synthesis (Fig. 5). Several studies have shown that endophytic and rhizospheric soil *Streptomyces* can produce indole acetic acid, which in turn stimulates plant development (Khamna *et al*., 2010; Yandigeri *et al*., 2012). In the rhizosphere soils, root exudates serve as the natural source of tryptophan for rhizosphere micro-organisms, potentially enhancing auxin (IAA) biosynthesis in the rhizosphere. More than 80% of rhizosphere bacteria have the ability to secrete IAA, according to Bhavdish *et al.* (2003). *Streptomyces* spp., which are found in the rhizospheres of several plants, are a great source of IAA. The possibility for the *Streptomyces* to synthesize and release IAA is created by the large supply of substrates found in root exudates (Kravchenko *et al*., 1991; Martens and Franken Berger 1994). Indole-3-acetic acid (IAA) is the auxin produced by PGPR that has been studied most extensively. It participates in interactions between microbes and plants (Afzal *et al*., 2015). *Streptomyces* are known for their PGP attributes and antagonistic activities. They have the capability to produce IAA (Salla *et al*., 2014). Our findings are in agreement with the other researchers.

HCN test. All the *Streptomyces* isolates except S-3 and S-16 produced positive reactions (Fig. 6).

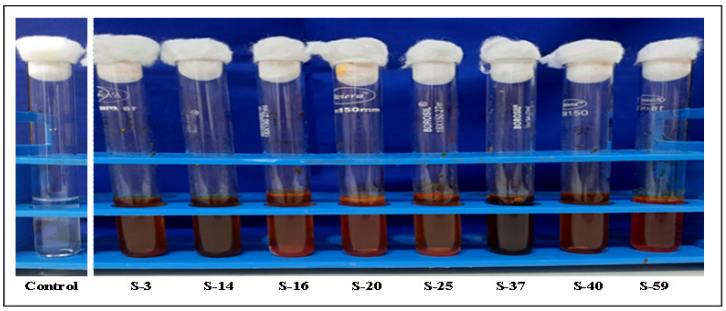

Fig. 5. Indole test by effective *Streptomyces* isolates.

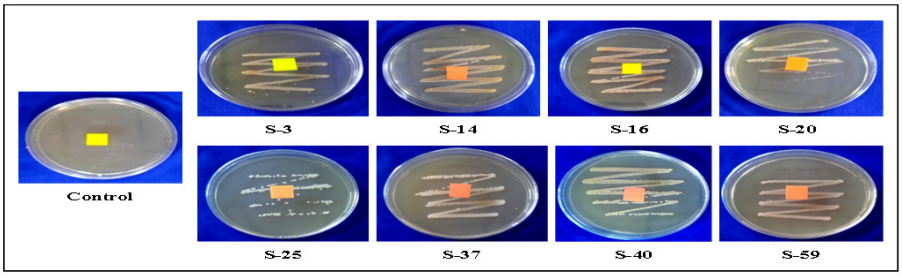

Fig. 6. HCN test by effective *Streptomyces* isolates.

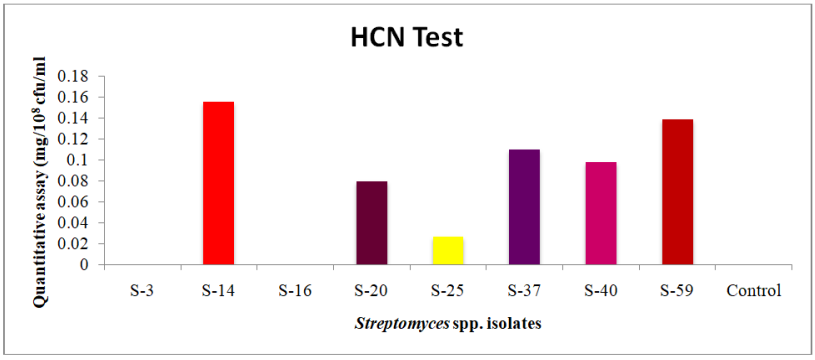

Fig. 7. Quantitative assay for Production of HCN production.

The isolates were also subjected to a quantitative approach to HCN production. The isolate S-14 showed more production of HCN *i.e.* 0.156 mg/ml followed by S-59 and S-37 isolates. S-25 produced the least production of HCN *i.e.* 0.02 mg/ml (Fig. 7). HCN is generated by numerous rhizobacteria and is postulated to be involved in the biological control of pathogens (Defago *et al*., 1990). Rhizobacteria that promote plant growth produce chemical compounds with diverse benefits for the plants. HCN enhanced phosphate availability for plant hosts and rhizobacteria, hence indirectly promoting plant growth (Rijavec, 2016). Similarly, Passari *et al*. (2015) reported positive results for HCN production, with isolate *Streptomyces* sp. 34 exhibiting the maximum production. Blom *et al*. (2011) stated that while rhizobacteria did not directly affect

pathogenic microbes and produced phytotoxic effects in the majority of *in vitro* trials, it is believed that HCN production by rhizobacteria stimulates plant development through an indirect mechanism. HCN plays a role in plant development as well as in the antagonistic activity of bacteria. As 6 out of 8 *Streptomyces* isolates produced HCN it can be utilized for disease management in plants. Similarly, Sreevidya *et al.* (2016) studied the role of microbial metabolites, such as HCN, in potentially enhancing plant establishment in the rhizosphere. Our findings are in concurrence with all the previous studies.

Casein hydrolysis. All the *Streptomyces* isolates except S-3, S-16 and S-40 produced positive reactions and clear zones around the colony. The highest clear zone formation was shown by S-14 and S-25 (Fig. 8). In this present study 5 *Streptomyces* isolates showed casein hydrolysis. Similarly, Perez-Corral *et al.* (2022) performed various biochemical tests and obtained that most of the *Streptomyces* isolates showed positive to casein hydrolysis.

Cellulase test. All the *Streptomyces* isolates produced a positive reaction to the cellulase test. The highest colourless zone surrounding the colonies was shown by S-14 and S-59 (Fig. 9). Six actinomycetes isolates from rice rhizosphere belonging to the genus *Streptomyces* produced cellulase as reported by Gopalakrishnan *et al.* (2013).

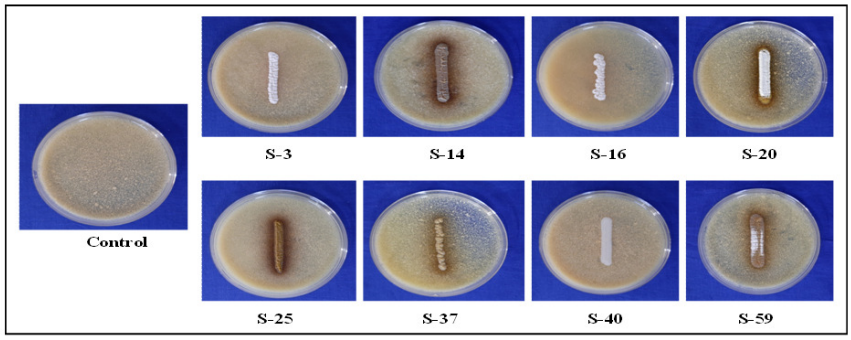

Fig. 8. Casein hydrolysis by effective *Streptomyces* isolates.

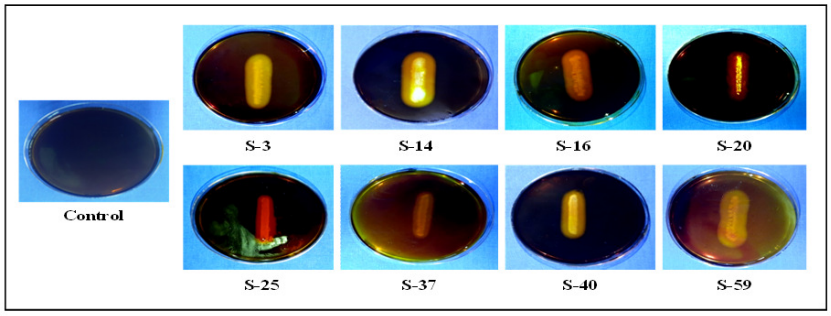

Fig. 9. Cellulase test by effective *Streptomyces* isolates.

CONCLUSIONS

This study aimed to investigate the potential of *Streptomyces* species associated with rice as rhizobacteria that promote plant growth for enhancing agricultural productivity and plant disease resistance. The isolates were subjected to biochemical characterization. The isolates were also tested for the production of indole acetic acid and other biocontrol properties such as hydrogen cyanide production. The findings revealed that all eight isolates were found to belong to the genus *Streptomyces*. At the field level, the potential of these isolates could be thoroughly examined.

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Conflict of Interest. None.

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