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Isolation of pathogenic *Streptomyces* spp. and determination of variability among the strains collected from potato

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ABSTRACT

A study was undertaken during 2008-2009, 2009-2010 and 2010-11 cropping seasons to isolate the variability(s) of Streptomyces spp. from lesion of common scab affected potato tubers in the Northern parts of Bangladesh (Rangpur division). There were twelve different pathogenic species isolated, which were different for colony colour, spore colour, spore ornamentation, spore chain morphology, other physiological characteristics (temperature, pH, melanin production, colour secret in oatmeal broth medium, carbon utilization and growth response in NaCl solution) and pathogenic reaction. Overall all collected isolates grow well within temperature range of 25°C to 30°C. In the present investigation, it was observed that isolates S_2 and S_{11} could grow at pH 4.50. It was found that the growth of isolates increased as the pH value of medium increased. Considering all the characteristics of different isolates, isolate S_5 agreed with the description of S. scabies having smooth, grey spores borne in spiral chains, producing melanin and utilizing eight recommended ISP diagnostic sugars with raffinose as a sole source of carbon. Rest eleven isolates could not identify.

Key words*: Common scab, pH, OMB medium, Melanin and Pathogenicity.*

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I. Introduction

In Bangladesh, potato (*Solanum tuberosum* L.) is a crucial and productive winter crop grown from October to March. Bangladesh has the second-highest rice production and growing area for potatoes. The primary use of potatoes is as a fresh vegetable. It thrives everywhere in the nation, particularly in the northwest, north and center. A total of 9.254 million tons are produced annually for the 2014–15 crop year from 0.471 million hectares of land (Anon, 2016). Bangladesh is currently a potential exporter of potatoes. Common scab is a significant risk to producing ware and seed potatoes. A significant potato disease, common scab, impairs tubers' quality, sometimes to the point where they can no longer be sold

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(Wanner, 2004). A serious infection may cause potato plants to emerge later and produce less potatoes (Hiltunen et al., 2005). As its name suggests, common scab is one of the most prevalent and pervasive diseases affecting the production of potatoes in almost every region of the world where the crop is grown (Gouws, 2006). Since the causative organism is widespread, it poses a threat in almost all soils (Stalham et al., 2010). Streptomycetes are the culprits behind common scab diseases that affect potatoes (*Solanum tuberosum* L.) and a few other root crops. Aerobic, filamentous, Gram-positive prokaryotes of Actinomycetales, suborder Streptomycineae, family Streptomycetaceae, and genus Streptomyces are known as *Streptomyces* spp. (Gouws, 2006). Streptomycetaceae was described by Kutzner (1981) as having a highly branched, unbroken vegetative mycelium that gave rise to chains of 5–50 conidia, or "arthrospores," with a thin fibrous sheath on each end. Cell walls contained L,L-diaminopimelic acid with glycine in interpeptide bridges and G + C content ranging from 69–73 mol%. The majority of Streptomyces species are saprophytes found in soil. The four species that can cause disease to spread to underground parts of economically significant tuber crops have been identified. They are *Streptomyces scabiei* (also known as *S. scabies*), *Streptomyces acidiscabies*, *Streptomyces turgidiscabies*, and *Streptomyces ipomea* and they are unrelated to one another based on a number of factors, such as 16S ribosomal DNA sequence, DNA-DNA relatedness, morphological characteristics and biochemical characteristics (Healy and Lambert, 1991; Takeuchi et al., 1996; Miyajima et al., 1998). The pathogenic species *S. acidiscabiei, S. caviscabiei, S. turgidiscabiei, S. europaeiaescabiei, S. stelliscabiei, S. reticuliscabiei, S. luridiscabiei, S. puniciscabiei*, and *S. niveiscabiei* have all recently been described (Stead and Wale, 2004).

According to Lambert and Loria (1989) *S. scabiei* is probably the most prevalent pathogen worldwide. Typical common scab is also brought on by *S. acidiscabiei and S. europeaiscabiei* (Lambert and Loria, 1989). The majority of the other species produce various symptoms. These include *S. reticuliscbiei, S. stelliscbiei, S. turgidiscabies* (Miyajima et al., 1998) and *S. caviscabies* (Bouchek-Mechiche et al., 2000). Recently, three new species from Korea were identified and all three exhibit symptoms of common scab that are more severe than those caused by other species. These include *S. niveiscabiei*, which has snow-white spores, *S. puniciscabiei*, which has white to purple spores and *S. luridiscabiei*, which has pale yellow spores (Park et al., 2003). In India, scabs are a typical symptom of *S. setonii*. Despite having been first identified in North America, *S. acidiscabiei* is now widespread in Japan and Korea (Song et al., 2004). *S. caviscabiei* was initially discovered in Canada, but it is also thought to exist elsewhere (Stead and Wale, 2004). No information was provided regarding the number of Streptomyces plant pathogenic species responsible for common potato scab in Bangladesh. So, an effort was made to identify the pathogenic species of Streptomyces from tubers in the Rangpur division affected by common scab (northern part of Bangladesh).

II. Materials and Methods

Collection of samples: Samples (scabby tubers) were collected in 2009, 2010 and 2011 from different potato growing areas of greater Rangpur. The samples were preserved in a cool room at 4°C for isolation.

Isolation: *Streptomyces spp*. was isolated following the dilution method (Lindholm et al., 1997). Tubers were washed with sterile distilled water (SDW) and then surfaced sterilized with 5% sodium hypochlorite (NaOCl) solution for 1 minute and rinsed well with SDW. The corky layer of each lesion was lifted aseptically and 1g of straw-diseased tissue (underneath the layer) for each sample was removed by a sterile scalpel and homogenized in sterile water (1g/10ml) in a mortar with pestle. One milliliter of the homogenate was mixed with 9 ml of phenol-water suspension (1:40). Three times diluted homogenate was plated on yeast malt extract (YME –ISP medium 2) agar medium (pH 7.0). Plates were incubated at 30°C for 10 days. A single colony was transferred to fresh yeast malt extract agar for pure culture. Isolates were maintained on YME slants at 4°C for further use.

Morphological and Physiological tests

Isolates were identified according to the ISP method (Shirling and Gottlieb, 1966) comprising the following characteristics:

A. Morphological characteristics

Aerial mass colour (spore colour): Each isolate was cultured for ten days in the dark at 30°C on YME and oatmeal broth (OMB) medium. In this medium pH was adjusted at 7.0. The colour of the

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mycelium and spores was recorded and isolates were classified according to the Tresner-Backus colour wheel (Tresner-Backus, 1963).

Reverse pigmentation (colony colour): Production of characteristic diffusible pigments in the reverse of colonies on YME was observed.

Spore chain morphology: The isolates were inoculated and grown around a round microscope cover slip obliquely inserted in YME plates to observe sporulation. The coverslips were removed after sporulation and the adhering mycelium and spore chains were observed under a light microscope at 100x magnification.

B. Physiological characteristics

Carbon utilization: Inoculum was prepared from 14 day-old cultures on oatmeal agar. Spores and mycelium of each isolate were scraped from the medium and suspended in a test tube containing 5 ml SDW. The contents of each tube were transferred to 25 ml tryptone yeast extract broth (TYB - ISP medium 1) in 125 ml Erlenmeyer flasks and incubated for 48 hours in the dark at 28°C on a rotary shaker. Sterile glass beads were added and the cultures were vortexed to disperse aggregated growth. These served as inocula. D-fructose, raffinose, D-manntiol, rhamnose, D-xylose, sucrose, L-aribonose and D-glucose were dissolved in distilled water at 2% (m/v). Each solution was filter-sterilized through a 0.22 µm Millipore filter and added to cooled autoclaved basal mineral salt agar (BSM - ISP medium 9) at 100 ml l^{-1}. Plates poured with the various sugar media were streak-inoculated in triplicate with each isolate. BSM without any sugar served as control. Plates were incubated for 10-14 days at 30°C and growth was recorded as positive or negative. No growth was evident on the BSM control plates.

Melanin production: Peptone-yeast extract-iron agar (PYI - ISP medium 6) and tyrosine agar (TA - ISP medium 7) plates were inoculated in triplicate with each *Streptomyces* isolates. Uninoculated PYI and TA slants were included as a control. The plates were incubated at 28°C in the dark and observed after 7 and 10 days. Isolates that produced a greenish-brown to brownish-black pigment on both media were considered melanin producers.

pH: Citrate-phosphate buffer solutions with a pH of 4.5, 5.0, 5.5, 6.0, 6.5, 7.5 and 8.0 were prepared as described by Cruickshank (1965). D-glucose (10 g), L-asparagine (0.5 g) and agar (15 g) were added per litre of each buffer and the various buffer solutions were autoclaved for 10 minutes at 120°C. After confirming the pH of each solution it was dispensed into Petridishes and allowed to solidify. The plates were inoculated similarly as in the carbon utilization test. Inoculated plates were incubated at 30°C in the dark for 10-14 days and growth was recorded as positive or negative. Minimum and maximum growth rates of each isolate were also recorded.

Growth response at different temperatures: Yeast malt extract (ISP-2) agar plates were inoculated in triplicate with each *Streptomyces* isolate for each temperature. The isolates were incubated at different temperatures (15, 20, 25, 30, 35 and 40°C) for 14 days. Minimum and maximum growth responses of each isolate were recorded.

Growth response at different concentrations of salt (NaCl): Growth response of different isolated Streptomyces spp. was determined by using oatmeal agar with different concentrations of NaCl. The concentration of NaCl was 4%, 5% and 6% and without NaCl served as control. The pH was adjusted to 7.0 by the addition of KOH drop by drop. Plates poured with the various NaCl media were inoculated in triplicate with each isolate and incubated at 30°C for 10-14 days. The growth response was recorded as positive or negative. The constituents of the medium were as follows:

Modified oat meal agar medium
Oat ---- 20 g
NaCl ---- 4 g – 6 g depending on the concentration of NaCl
Agar ---- 18 g
Distilled water ---- 100 ml

Pathogencity tests

Pathogencity was tested according to the procedure provided by Lambert and Loria (1989) with slight modification. Healthy mini tubes of potato cv. Diamant susceptible to common scab (provided by TCRC,

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BARI, Bangladesh) were tested for this experiment.

Inoculum preparation
Selected 12 isolates were inoculated separately in 250 ml Erlenmeyer flask containing 50 ml OMB medium and incubated at 28°C on Shaker (150 rpm) for seven days. Thus requisite amount of spore suspension of each isolate was prepared in OMB medium.

The constituents of oat meal broth (OMB) medium were as follows:
Oatmeal 20 g, Distilled water 1000 ml and Trace salt solution 1ml, pH adjustment at 7.
Trace salt solution was composed of $FeSO_4$. $7H_2O$ 0.1 g, $MnCl_2$. $4H_2O$ 0.1 g, $ZnSO_4$. $7H_2O$ 0.1 g and Distilled water 100 ml.

Inoculation and observation
Four mini tubers were soaked in the spore suspension ($2x10^5$ cfu/ml) of each isolate and incubated in a sterile moist chamber using sterilized moist filter paper at 28°C in the dark site. Mini tubers were also soaked into non-inoculated OMB as control. With pathogenic isolates, symptoms were noticeable within 7-10 days. The tubers that exhibited common scab lesions were recorded. Isolations were made from all lesions to confirm Koch's postulates.

III. Results

12 isolates of *Streptomyces* spp. were isolated from scabby lesions of scabby tubers collected from different surveyed areas in greater Rangpur. White and grey coloured spores and different coloured (light brown to dark brown, creamy to brown, dark brown and brown) colonies were observed in YME medium. Spore ornamentation of isolates was rough and smooth and spore chain morphology was spiral and flexuous (Table 01). White spores were observed in isolates S_2, S_3, S_7, S_8, S_9 and S_{12} while grey spores were in S_1, S_4, S_5, S_6, S_{10} and S_{11} isolates. Smooth spore ornamentation was recorded in S_1, S_5, S_9, S_{11} and S_{12} isolates and the rest were rough (Plate A and Table 01). Spiral spore chain morphology was found in S_1, S_4, S_5, S_8 and S_{10} and rest were flexuous.

Table 01. Morphological character of 12 isolates of *Streptomyces* spp.

Isolate no.	Colony characters in YME			
	Spore colour	Colony colour	Spore ornamentation	Spore chain morphology
S_1	Grey	Brown	Smooth	Spiral
S_2	White	Light brown to dark brown	Rough	Flexuous
S_3	White	Creamy to brown	Rough	Flexuous
S_4	Grey	Dark brown	Rough	Spiral
S_5	Grey	Dark brown	Smooth	Spiral
S_6	Grey	Dark brown	Rough	Flexuous
S_7	White	Brown	Rough	Flexuous
S_8	White	Light brown to dark brown	Rough	Spiral
S_9	White	Brown surrounded by light brown	Smooth	Flexuous
S_{10}	Grey	Creamy to brown	Rough	Spiral
S_{11}	Grey	Brown	Smooth	Flexuous
S_{12}	White	Brown	Smooth	Flexuous

Physiological characteristics
Temperature: Out of twelve isolates, S_8 and S_9 had no growth in 15°C conditions, but it was clear that overall all isolates grew well within 25°C to 30°C. At 40°C, growth was observed in all isolates except S_2, but the isolates viz. S_4 and S_{11} could grow well at 40°C (Table 02).

pH: Results showed that only two isolates S_2 and S_{11} could grow in the pH range of 4.50 to 8.00. The only isolate only, S_3 could grow at pH range of 6.00 to 8.00. Colony growth of the rest of all isolates was observed in pH range of 5.00 to 8.00. But most of the isolates grew well between pH 6.00 and 8.00 (Table 03).

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Table 02. Response of 12 isolates in different temperature

Isolate no.	Different temperature					
	15 °C	20 °C	25 °C	30 °C	35 °C	40 °C
S_1	A	A^+	A^+	A^{++}	A^+	A
S_2	A	A^+	A^+	A^{++}	A^+	A^-
S_3	A	A^+	A^+	A^{++}	A^+	A^+
S_4	A	A^+	A^{++}	A^{++}	A^{++}	A^{++}
S_5	A	A^+	A^{++}	A^{+++}	A^{++}	A^+
S_6	A	A^+	A^{++}	A^{+++}	A^{++}	A
S_7	A	A^+	A^{++}	A^{++}	A^+	A
S_8	A^-	A	A^{++}	A^{++}	A^+	A
S_9	A^-	A	A^+	A^{++}	A^+	A
S_{10}	A	A^+	A^{++}	A^{+++}	A^+	A
S_{11}	A	A^+	A^{++}	A^{+++}	A^{++}	A^{++}
S_{12}	A	A^+	A^{++}	A^{++}	A^+	A

Visual estimation: A^-, A, A^+, A^{++}, A^{+++} represent degree of growth where +++ is maximum and A just colony growth is initiated while A^- means no growth.

Melanin Production: The isolates S_5, S_6 and S_8 produced melanin pigments in both ISP-6 and ISP-7 medium and the isolates S_2, S_3, S_4, S_{10} and S_{12} could produce melanin pigment only in ISP 7 (Table 04). The isolates S_2, S_5, S_6, S_{10} and S_{12} developed deep brown colour in oatmeal broth (OMB) medium, S_3 and S_4 produced medium brown colour. Light brown colour was observed in S_1, S_7 and S_9 added OMB medium, while isolate S_8 secreted bottle green colour and S_{11} developed white (Table 04).

Table 03. Response of 12 isolates in different pH

Isolate no.	Different pH							
	4.5	5.0	5.5	6.0	6.5	7.0	7.5	8.0
S_1	-	++	++	++	+++	+++	+++	+++
S_2	+	+	+	++	+++	+++	+++	+++
S_3	-	-	-	+	+++	+++	+++	+++
S_4	-	+	+	++	+++	+++	+++	+++
S_5	-	+	++	++	+++	+++	+++	+++
S_6	-	++	++	+++	+++	+++	+++	+++
S_7	-	+	++	++	+++	+++	+++	+++
S_8	-	+	+	+++	+++	+++	+++	+++
S_9	-	+	++	++	+++	+++	+++	+++
S_{10}	-	+	+	++	+++	+++	+++	+++
S_{11}	++	+++	+++	+++	+++	+++	+++	+++
S_{12}	-	+	+	++	+++	+++	+++	+++

+, ++ and +++ represent degree of growth where +++ is maximum and + just colony growth is initiated, while - is no growth.

Table 04. Physiological characters of 12 isolates of *Streptomyces* spp.

Isolate no.	Melanin production		Colour secret in OMB
	ISP-6	ISP-7	
S_1	-	-	Light brown
S_2	-	+	Deep brown
S_3	-	+	Medium brown
S_4	-	+	Medium brown
S_5	+	+	Deep brown
S_6	+	+	Deep brown
S_7	-	-	Light brown
S_8	+	+	Bottle green
S_9	-	-	Light brown
S_{10}	-	+	Deep brown
S_{11}	-	-	White
S_{12}	-	+	Deep brown

+ = Growth and - = No Growth

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Carbon Utilization: The isolates viz. S_3 and S_{11} could not catabolize raffinose sugar when rest isolates could utilize eight ISP sugars (Table 05).

Table 05. Carbon utilization of 12 isolates of *Streptomyces* spp.

Carbon source utilization	Isolate no.											
	S_1	S_2	S_3	S_4	S_5	S_6	S_7	S_8	S_9	S_{10}	S_{11}	S_{12}
D-Glucose	+	+	+	+	+	+	+	+	+	+	+	+
D-Fructose	+	+	+	+	+	+	+	+	+	+	+	+
Sucrose	+	+	+	+	+	+	+	+	+	+	+	+
Raffinose	+	+	-	+	+	+	+	+	+	+	-	+
D-xylose	+	+	+	+	+	+	+	+	+	+	+	+
L-Arbinose	+	+	+	+	+	+	+	+	+	+	+	+
Rhamnose	+	+	+	+	+	+	+	+	+	+	+	+
D-mannitol	+	+	+	+	+	+	+	+	+	+	+	+

+ = Growth and - = No Growth

Growth response in NaCl solution: All the isolates grew well irrespective of concentration of NaCl, so no variation among the isolates was evident (Table 06).

Table 06. Growth response of 12 isolates of *Streptomyces* spp. in different concentration of NaCl

Isolate no.	Control	4% NaCl	5% NaCl	6% NaCl
S_1	+	+	+	+
S_2	+	+	+	+
S_3	+	+	+	+
S_4	+	+	+	+
S_5	+	+	+	+
S_6	+	+	+	+
S_7	+	+	+	+
S_8	+	+	+	+
S_9	+	+	+	+
S_{10}	+	+	+	+
S_{11}	+	+	+	+
S_{12}	+	+	+	+

+ = Growth and - = No Growth; Control = Media with no salt

Pathogenecity of the isolates: Results revealed that all the isolates of *Streptomyces* were pathogenic, but among them, there were distinct variations in degree of pathogenicity (Table 07). Isolate S_1 and S_{11} appeared less virulent compared to rest isolates. Five isolates (S_4, S_6, S_8, S_9 and S_{12}) exhibited more virulence than all isolates of *Streptomyces.* The isolate only S_5 having the characters smooth, grey spores borne in spiral chains, producing melanin and utilizing eight recommended ISP diagnostic sugars with raffinose as a sole carbon source similar to *Streptomyces scabies*. The rest eleven isolates were unknown though they were all plant pathogenic.

Table 07. Degree of pathogenecity

Isolate no.	Severity
S_1	+
S_2	+++
S_3	+++
S_4	++++
S_5	++
S_6	++++
S_7	++
S_8	++++
S_9	+++++
S_{10}	++
S_{11}	+
S_{12}	++++

+, ++, +++, ++++ and +++++ represent degree of produced common scab symptom while +++++ maximum

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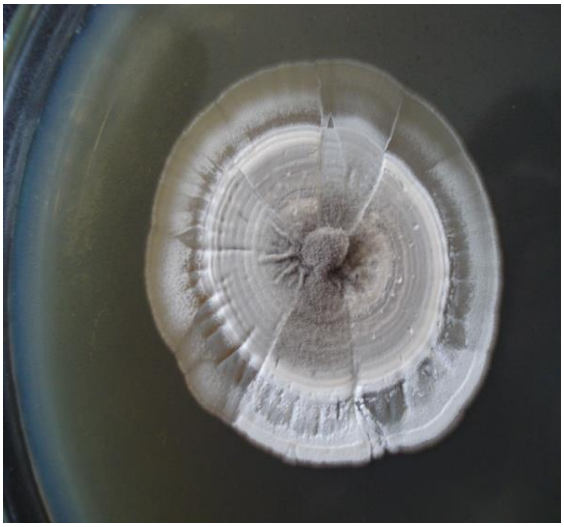
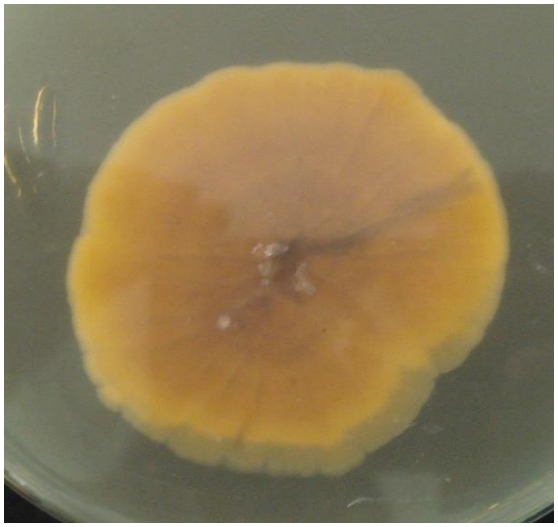
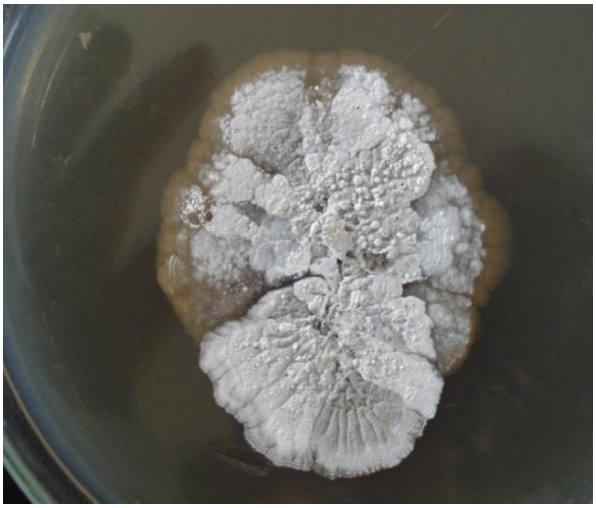


Aerial mass colour-Grey

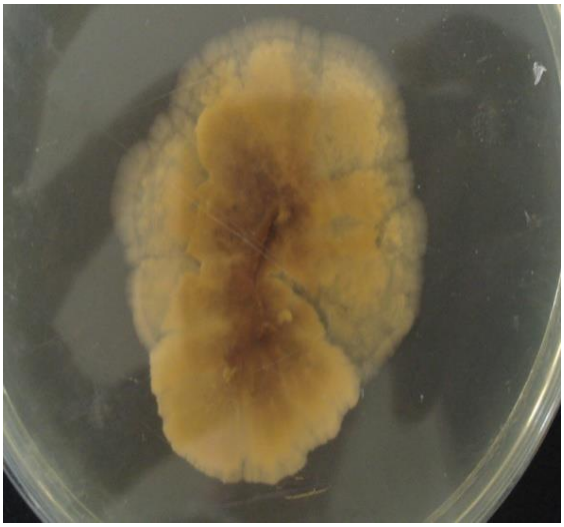


Colony colour- Brown

S_1



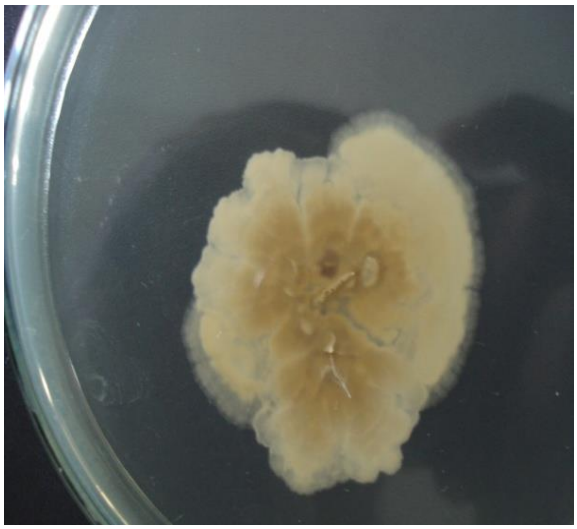
Aerial mass colour-White



Colony colour- Light brown to dark brown

S_2

Aerial mass colour-White



Colony colour- Creamy to brown

S_3

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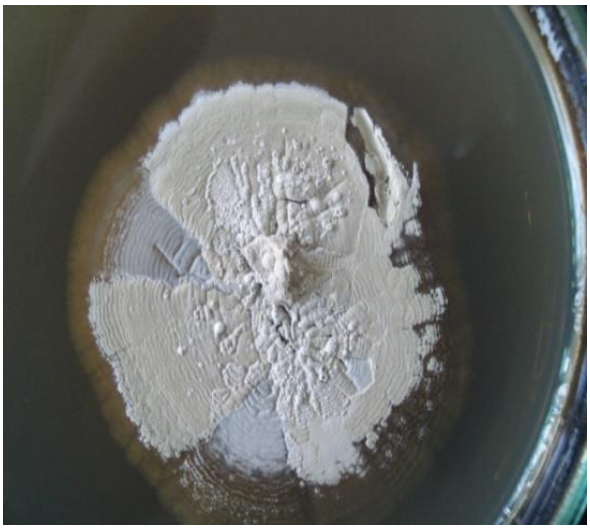
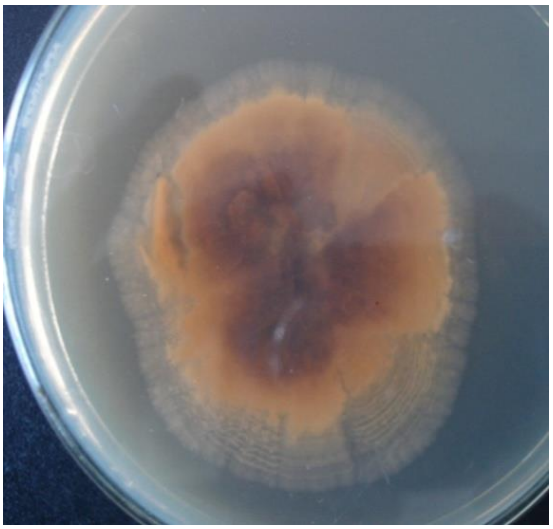
Aerial mass colour-Grey

Colony colour-Dark brown

S_4



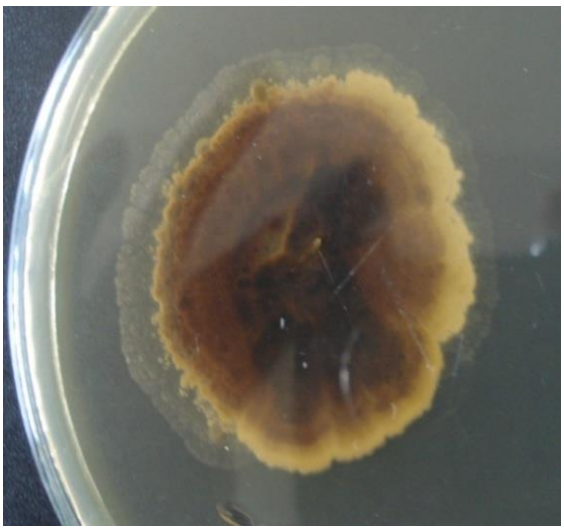
Aerial mass colour-Grey



Colony colour-Dark brown

S_5

Aerial mass colour-Grey



Colony colour-Dark brown

S_6

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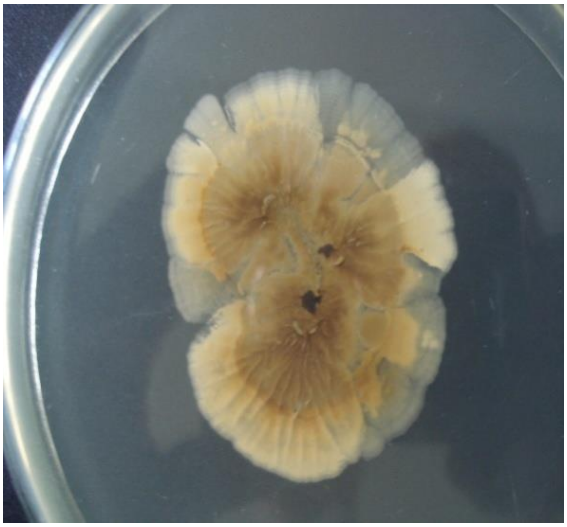
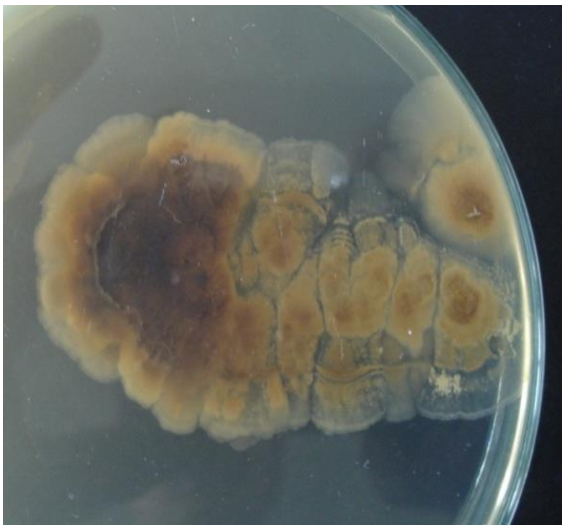
Aerial mass colour-White



Colony colour-Brown

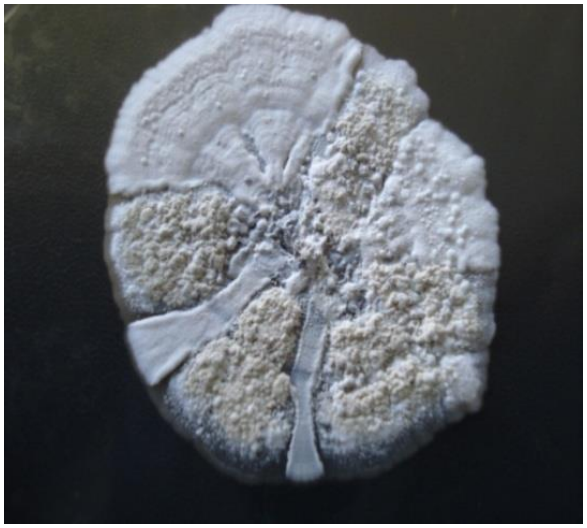
S_7

Aerial mass colour-White



Colony colour-Light brown to dark brown

S_8



Aerial mass colour-White

Colony colour-Brown surrounded by light brown

S_9

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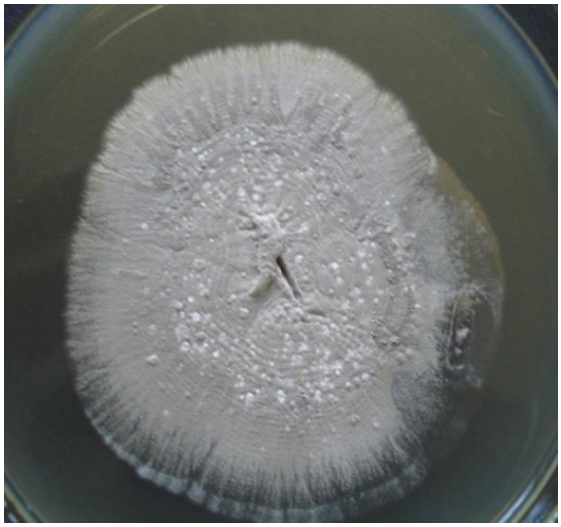
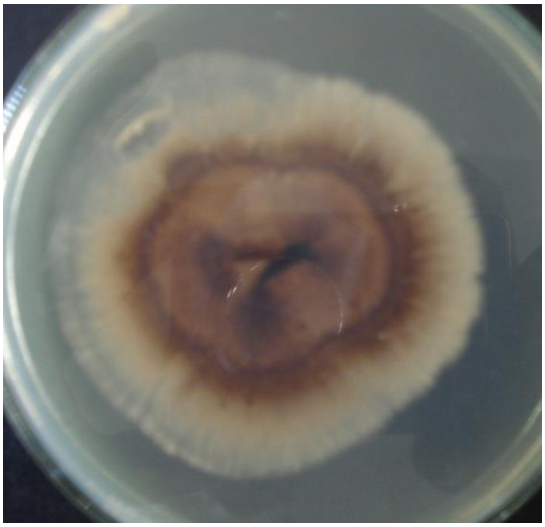
Aerial mass colour-Grey

Colony colour-Creamy to brown

S_{10}



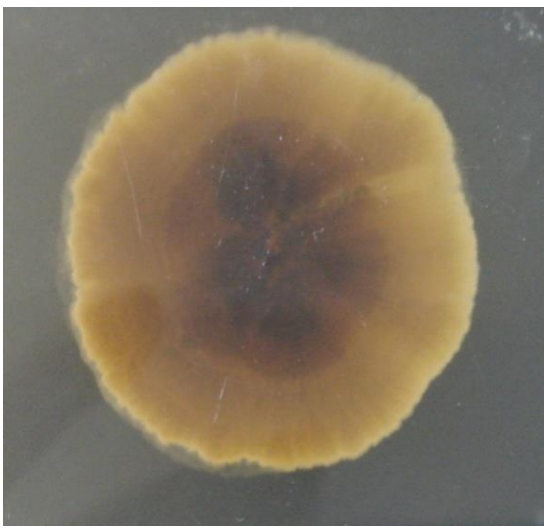
Aerial mass colour-Grey



Colony colour-Brown

S_{11}

Aerial mass colour-White



Colony colour-Brown

S_{12}

Plate A. View of twelve different isolates of *Streptomyces* spp.

IV. Discussion

Overall all collected isolates grow well within temperature range of 25°C to 30°C. This result is supported by the observations of Hooker (1981) and Loria et al. (1997). This study tested the ability of

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collected pathogenic Streptomyces isolates to grow between pH 4.50 to 8.0 at 0.50 unit intervals. Earlier, it was thought that the causal agent of common scab of potato does not grow at a pH below 5.0 (Lambert and Loria, 1989). But in the present investigation, isolates S_2 and S_{11} can grow at pH 4.50. Studies by Lambert and Loria (1989) revealed that common scab causing agent *S. acidiscabies* can cause typical symptoms at pH down to 4.50. However, isolates S_2 and S_{11} could not be identified up to species. The present investigation found that the growth of isolates increased as the pH value of medium increased. This observation is similar to the findings of Osborn (1995). Considering all characteristics of different isolates, the isolate S_5 agreed with the description of *S. scabies* by Lambert and Loria (1989), having smooth, grey spores borne in spiral chains, producing melanin and utilizing eight recommended ISP diagnostic sugars with raffinose as the sole source of carbon. It is challenging to say the species of rest eleven isolates. DNA-based studies will be required to elucidate the identity of rest eleven isolates.

V. Conclusion

Among twelve isolates, S_5 is suitable as per description of *S. scabies,* having smooth, grey spores borne in spiral chains, producing melanin and utilizing eight recommended ISP diagnostic sugars with raffinose as sole source of carbon which was based on only morphological identification. DNA-based studies will be needed for more confirmation.

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