International Journal of Agricultural Technology 2022Vol. 18(6):2343-2352
Available online http://www.ijat-aatsea.com
ISSN 2630-0192 (Online)

Antioxidant activity of 2,4-di-tert-butylphenol isolated from plant growth promoting endophytic *Streptomyces* KCA-1

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Ayswarya, S., Radhakrishnan, M., Manigundan, K., Gopikrishnan, V. and Soytong, K. (2022). Antioxidant activity of 2,4-di-tert-butylphenol isolated from plant growth promoting endophytic *Streptomyces* KCA-1. International Journal of Agricultural Technology 18(6):2343-2352.

Abstract Endophytic *Streptomyces* produce vast amount of unique bioactive natural compounds, which they use to promote the growth and development of the host plant. In the current study, the putative endophytic *Streptomyces* strain KCA-1 was evaluated for its capacity to produce various hydrolytic enzymes and plant growth promoting (PGP) parameters. The five main antibiotics (Vancomycin (30µg), Gentamycin (10 mcg), Tetracycline (30 µg), Penicillin (6 µg), and Nalidixic acid (30 µg) were also tested for antibiotic sensitivity. Utilizing the DPPH, ABTS, and metal chelating assays, the antioxidant capacity of the bioactive compound 2,4-di-tert-butylphenol isolated from *Streptomyces* KCA-1 was assessed. *Streptomyces* KCA-1 produced ammonia and IAA at rates of 56.2 mg/ml and 47.7 µg/ml, respectively, according to quantitative analysis i01n UV-spectrophotometry. Additionally, by utilizing siderophore, amylase, protease, and cellulose, it was found to possess all additional PGP and enzyme characteristics. Tetracycline (30 µg) and penicillin (6 µg) were found to be resistant against the strain KCA-1, whereas vancomycin (30 µg), gentamycin (10 mcg), and nalidixic acid (30 µg) were found sensitive. The antioxidant activity of the pure bioactive compound 2,4-DTBP reached a maximum of 86.4%, 89.4%, and 85.0% of DPPH, ABTS scavenging, and metal chelating activity at 1000 µg/ml with an IC50 value of 60 µg/ml, 17 µg/ml, and 20 µg/ml, respectively. This work paints a comprehensive picture of endophytic *Streptomyces* KCA-1 as the possible source for 2,4-di-tert-butylphenol synthesis, which could be a promising potential source of drug in the pharmaceutical and agricultural sectors.

Keywords: Endophytic *Streptomyces*, Plant growth promotion, Antibiotic sensitivity, Antioxidant activity.

Introduction

Endosphere, or the plant's internal environment, is a highly developed micro-ecosystem that promotes the overall plant growth and development in a

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number of ways. Endophytes are microorganisms that inhabit the internal organs of plants and have no effect on either the host plant or the surrounding environment (Adeleke and Babalola, 2022; Santos and Olivares, 2021; Bhimba *et al*., 2010). Many of them act as a latent pathogen which, depending on the circumstances, could cause or contribute to infections on the host plant. Endophytes, which are microorganisms that dwell inside medicinal plants, have been related to the manufacture of a variety of secondary metabolites (Ogbe *et al*., 2020). These metabolites may increase a plant's resistance to stress and reduce the symptoms of disease caused by plant pathogens. The species composition of the whole endophytic population is controlled by the genotype of the host plant, environmental circumstances, soil type, and plant development stage (Wu *et al*., 2021). Endophytic microbial communities linked with medicinal plants have a significant potential to create new bioactive chemicals. The pharmaceutical industry, agriculture, and other industries could see a considerable range of growth and development with these compounds. In light of this, it is essential to distinguish endophytes from medicinal plants. The ability of endophytic actinobacteria to produce wide variety of bioactive metabolites, which may include therapeutic compounds such as antibacterial, antioxidant, anticancer, and immunosuppressive substances, is the characteristic for which they are most often recognized (Bahrami *et al*., 2022; Singh and Dubey, 2018). The majority of endophytic actinobacteria found in medicinal plants are members of the genus *Streptomyces* (Singh and Dubey, 2018).

Endophytic microbes, namely actinobacteria of the genus *Streptomyces*, isolated from medicinal plants have reduced the prevalence of penicillin-resistant *Staphylococcus aureus* (Singh and Dubey, 2018). The finding was the result of the hunt for novel drugs to treat infections with multiple drug resistance. In addition to their antibacterial characteristics, the endophytic actinobacteria isolated from medicinal plants were extraordinarily potent as larvicidal, antimalarial, antioxidant, anticancer, anti-diabetic, and plant growth-stimulating substances (Golinska *et al*., 2015; Palanisamy *et al*., 2017). As a result, we evaluated the multifunctional characteristics of endophytic *Streptomyces* by screening for a variety of properties that promote plant growth, produce enzymes, and are antibiotic-resistant. In addition, the bioactive compound 2,4-di-tert-butylphenol was extracted from endophytic *Streptomyces* KCA-1 and are subjected to a series of antioxidant activity such as DPPH, ABTS, and metal chelating characteristics.

Methods and materials

Streptomyces strain KCA-1

Streptomyces KCA-1 is an endophytic actinobacterial strain that was isolated from the plant *Phyllanthus niruri*, previously described by Ayswarya *et*

al., 2022. The bacterium KCA-1 viability was sustained at 4℃ and -80℃, respectively, on yeast extract malt extract (YEME) agar medium slants and 20% glycerol broth.

In-vitro plant growth and enzyme production

IAA production

Streptomyces KCA-1 was inoculated into 250 ml Erlenmeyer flasks with 50 ml of YEME broth that had 50 ml of L-tryptophan added to it (5 mg ml^{-1}). After that, the flasks were placed on a rotating incubator shaker and incubated for five days at 30℃ and 200 rpm. The synthesis of IAA was then measured by combining 2 ml of the supernatant with 4 ml of Salkowski reagent, which was made consisting of 1 ml of 0.5 M $FeCl_3$ solution in 49 ml of a 35% (w/v) solution of $HClO_4$. The combination was then incubated for 30 min in the dark and centrifuged at 4000g for 10 min. The amount of IAA production was determined based on how pink the solution turned out to be after incubation. The optical density was determined using an UV-spectrophotometer set to 530 nm, and the amount of IAA was calculated using a graph of pure IAA (Passari *et al*., 2015).

Siderophore production

On chrome azurol agar (CAS) plates, the siderophore production was evaluated for quality control (Hu and Xu, 2011). The CAS agar medium was incubated for a week at 30℃ after being inoculated with a *Streptomyces* KCA-1. *Streptomyces* colonies developing pink or orange haloes indicates the production of siderophore.

Ammonia production

The ability of an endophytic strain *Streptomyces* KCA-1 to produce ammonia according to Cappuccino and Sherman, 2002. *Streptomyces* KCA-1 seed culture inoculated into 10ml of peptone water, and incubated at 30 ℃ for 15 days at a rate of 120 rpm. After adding 0.5 ml of Nesseler's reagent to the culture mixture, the ammonia production test was deemed successful when the mixture color changed from brown to yellow. The absorbance was determined using a spectrophotometry reading at 530nm. Then, a standard curve for ammonium sulphate $(NH_4)2SO_4$ that was expressed in mg/ml was used to compare this production.

Hydrolytic enzymes production

On mineral salt agar, the cellulose activity was analyzed and assessed using carboxy methyl cellulose (CMC) as a carbon source. The production of amylases and proteases was measured using media comprised of starch agar

and skim milk agar, respectively (Cattelan *et al*., 1999; Saadoun and Muhana, 2008).

Antibiotic resistance profile

Antibiotic resistance to a total of five different antibiotics was assessed in *Streptomyces* KCA-1 endophytes. The utilized antibiotics and their concentrations are vancomycin (30 g/ml), gentamicin (10 g/ml), penicillin (10 g/ml), tetracycline (30 g/ml), and nalidixic acid (30 g/ml). According to Williams *et al*. (1989), the antibiotic concentrations employed were the greatest inhibitory concentrations practical for the antibiotics utilized. Both narrow- and broad-spectrum antibiotics were used. On Muller-Hinton agar medium, the growth of isolates was observed after antibiotic inoculation and the results were classified as antibiotic-sensitive (S), antibiotic-intermediate (I), or antibiotic-resistant (R).

2,4-di-tert-butyl phenol (2,4-DTBP)

The pure compound 2,4-DTBP was isolated from endophytic *Streptomyces* strain KCA-1 using standard column chromatography and HPLC analysis. Utilizing spectral techniques such as UV, FT-IR, LC-MS, and NMR, the structure of the pure molecule 2,4-di-tert-butylphenol was also identified (Ayswarya *et al*., 2022).

Antioxidant activity

DPPH radical scavenging assay

Methanol was used to make a 0.1 mM solution of DPPH (2,2-Diphenyl-1-picrylhydrazyl), which was then kept in a dark place. Five distinct test tubes each had 2.5 ml of 2,4-DTBP at concentrations of 10, 50, 100, 500, and 1000 µg/ml. The DPPH solution was then added to each test tube in amounts of 500 µl. At room temperature, the reaction mixture was vigorously agitated for 30 minutes. After the incubation process, the absorbance at 517 nm was measured using a UV- spectrophotometer. Value of ascorbic acid is utilized as the control. The proportion of DPPH removed from the sample was calculated using the formula below: The formula for calculating percent inhibition is

$$\text{Percent inhibition} = (A0 - A\,1 / A0) \times 100$$

where A0 stands for the absorbance of the control reaction and A1 for the absorbance of the test or standard sample.

ABTS assay

The procedure followed by Re *et al*. (1999), is used to evaluate the 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) test of 2,4-DTBP. An aqueous solution of 7mM ABTS was combined with a solution of 2.4mM potassium persulfate to create the ABTS solution. The reaction was then

allowed to run its course for 12 to 16 hours at room temperature and in the dark. This solution was previously diluted in ethanol (at a ratio of about 1:89 v/v) and kept at a temperature of 30 ℃ to attain an absorbance at 734 nm. In a nutshell, 20µl of 2,4-DTBP were taken in five distinct test tubes at various concentrations (10, 50, 100, 500, and 1000 µg/ml), and 2 ml of the ABTS solution was added to each reaction tube. The tubes were then kept in a dark incubation for 30 minutes prior to the measurement of the absorbance at 734 nm. Results were analyzed using the aforementioned equation.

Metal chelating assay

To produce an effect, different doses of the pure chemical 2,4-DTBP were spiked into a solution containing 0.1 mM $FeSO_4$. The samples had concentrations of 10, 50, 100, 500, and 1000 µg/ml. 0.25 mM ferrozine (0.4 ml) was added to start the reaction, which was then vigorously stirred for 10 minutes at room temperature. A 562 nm wavelength was used to compute absorbance. The typical treatment, EDTA, was used. The equation previously presented allowed for the interpretation of the proportion of metal chelation.

Results

In-vitro PGP and enzymatic attributes of the actinobacteria

In-vitro plant growth-stimulating traits of a potential endophytic *Streptomyces* KCA-1 were examined, including IAA, siderophore, and ammonia. Their enzymatic properties, including cellulase, amylase, and protease, were also examined. While the UV-spectrophotometry analysis showed that it produced 47.7 mg/ml of ammonia, and 56.2 µg/ml of IAA. The strain KCA-1 was also capable of producing all other PGP and enzyme traits, including as siderophore, amylase, protease, and cellulose (Figure 1 and Table 1).

Antibiotic sensitivity profiling

The antibiotic resistance of the strain KCA-1 against penicillin (P), tetracycline (T), nalidixic acid (Na), vancomycin (V), and gentamicin (G) were determined and found to be susceptible to vancomycin, gentamycin, and nalidixic acid but resistant to tetracycline and penicillin (Figure 1 and Table 1).

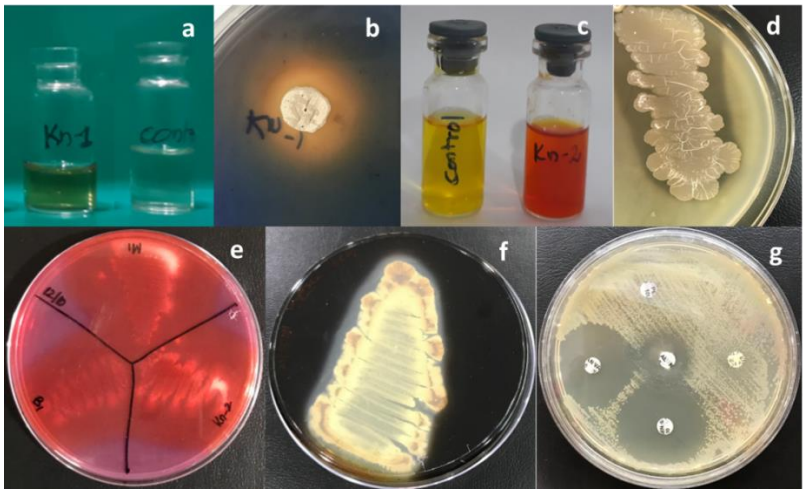

Figure 1. Screening of endophytic *Streptomyces* KCA-1 for *in-vitro* plant growth promoting, enzyme producing and antibiotic sensitivity test

Table 1. *In-vitro* plan growth promoting and enzymatic properties of endophytic *Streptomyces* KCA-1

S. No	Properties	Endophytic *Streptomyces* KCA-1
1.	IAA production	56.2
2.	Siderophore production	2
3.	Ammonia production	47.7
4.	Amylase production	1
5.	Cellulase production	3
6.	Protease production	1
7.	Vancomycin (30 µg)	S
8.	Gentamycin (10mcg)	S
9.	Tetracycline (30 µg)	R
10.	Penicillin (6 µg)	R
11.	Nalidixic acid (30 µg)	S

Degree of antibiotic susceptibility: S: sensitive (>10 mm); I: intermediate (5.0-9.9 mm); R: resistant (0.0-4.9 mm).

The rating scales for siderophore, cellulase, amylase and protease were as follows: 0 = no halo zone, 1 = halo zone of 1–10 mm, 2 = halo zone of 11–20 mm, 3 = halo zone of 21–30 mm

Antioxidant activity

In a DPPH radical scavenging assay, the pure compound 2,4-DTBP showed a maximum of 86.4% and 70.6% scavenging activity at 1000 µg/ml and 500 µg/ml concentrations, respectively, with an IC50 value of 60 µg/ml. Additionally, at dosages of 10 and 30 µg/ml, the substance 2,4-DTBP showed the least inhibition of DPPH activity such as 34 and 35.4%, respectively. In the ABTS test, it is shown that at 1000 µg/ml, the highest level of radical

scavenging activity was seen, while at 500 µg/ml, 78.5% was seen, and the IC50 value was 17 µg/ml. With a 48.6% inhibition rate, the concentration of 10µg/ml shown the least level of ABTS activity, while the concentration of 30 µg/ml showed a 51.6% inhibition rate. The pure substance 2,4-DTBP showed a peak inhibition of 85% in the metal chelating assay, with an IC50 value of 20 µg/ml. At 500 µg/ml, this proportion dropped to 80.6% (Table 2).

Table 2. Antioxidant activity of 2,4-DTBP isolated from endophytic *Streptomyces* KCA-1

2,4-DTBP Concentration (µg/ml)	DPPH	ABTS	Metal chelating
	% Inhibition		
10	34	48.6	46
30	35.4	51.2	53.5
100	58.3	61.5	70.8
500	70.6	78.5	80.6
1000	86.4	89.4	85

Discussion

Endophytic microorganisms, including endophytic actinobacteria, are discovered residing within the tissues of plants. They have evolved certain characteristic properties that enable them to flourish without obviously infecting their host. They have gained global attention for their capacity to produce naturally occurring bioactive chemicals. Nowadays, endophytic actinobacteria obtained from medicinal plants are of the highest importance. This is due to the increasing likelihood of finding novel natural compounds and the increased bioactivity of these substances (Xia *et al*., 2022; Singh *et al*., 2017).

Based on the results of this present study, it can be concluded that the potential endophytic Streptomyces KCA-1 produces a quantity of IAA comparable to that which Verma *et al*. (2012) and Khamna *et al*. (2009). Approximately 80% of the microorganisms that have been isolated from the rhizosphere soil have the ability to create and release IAA as secondary metabolites, which has also been shown. These substances have a history of promoting plant growth and root expansion (Patten and Glick, 2002). Through the production of plant hormones, which in turn stimulates the production of metabolites beneficial to the microorganism's own growth, aid in the development of plants. The halotolerant *Streptomyces aureofaciens* produced IAA, phosphate solubilization, siderophore, ammonia, and the hydrolytic enzymes chitinase, amylase, and urease, according to research published by Shrivastava *et al*. (2017). The production of ammonia is a procedure that indirectly promotes plant growth and has the potential to significantly reduce

phytopathogens. Similar results discovered by Nimnoi *et al*. (2010) who reported that an isolated strain of *Streptomyces hainanensis* named S4303 displayed an ammonia production of 60.0mg/ml, supported our present finding. The production of ammonia by bacteria, according to Marques *et al*. (2010) may also collect and provide the plant with nitrogen, promoting the development of the plant's shoots and roots and eventually raising plant biomass. Tetracycline and penicillin were resistance against the *Streptomyces* strain KCA-1 that we examined in this study, while vancomycin, gentamycin, and nalidixic acid were susceptible against it. *Streptomyces thermocarboxydus* strain BPSAC147, *Streptomyces olivaceus* strain BPSAC77, *Streptomyces* sp. strain BPSAC101, and *Streptomyces* sp. strain BPSAC121 all shown resistance to seven of the twelve tested antibiotics (Passari *et al*., 2017). Additionally, it has been shown that the endophytic actinobacterial strain *Micrococcus* is resistant to antibiotics (Pal *et al*., 2012).

It has been extensively studied how to measure an organism potential to scavenge DPPH radicals in order to determine its antioxidant capacity. Antioxidants have the ability to donate hydrogen, which allows them to change into stable diamagnetic molecules, which aids in the scavenging of DPPH radicals. The chemicals convert the purple-colored DPPH radical's yellow product into a new compound; the extent to which the color changes relate to the tested sample's ability to donate hydrogen. It was evident that the extracts possessed hydrogen donating ability and the potential to function as free radical scavengers, even if the radical scavenging activity of the extracts that were studied was not as high as the radical scavenging activity of ascorbic acid (Munteanu and Apetrei, 2021). This result supports other findings regarding the actinobacterial extracts that have a mild amount of scavenging activity. Free oxygen has been linked to the emergence of many diseases, including cancer, autoimmune disorders, cardiovascular disease, and neurological diseases, according to recent study (Pham-Huy *et al*., 2008; Uttara *et al*., 2009). Our present study antioxidant results were also correlated with others (Passari *et al*., 2017; Kawahara *et al*., 2012). The scavenging action of *Streptomyces* was also shown to be improved with increasing extract concentrations, according to the results of Kekuda *et al*., 2010. Additionally, the marine *Streptomyces* sp. VITTK3 was used to produce a compound known as 5-(2, 4-dimethylbenzyl) pyrrolidin-2-one, which shown potent antioxidant activity (Tenmozhi *et al*., 2010). A fresh strain of *Streptomyces* Eri12 that was discovered in the rhizosphere sample also showed strong DPPH radical scavenging abilities (Zhong *et al*., 2011). The actinobacteria isolated from traditional medicinal plants had substantial antioxidant capacity in 66.6% of the cases, according to Tanvir *et al.* (2014). This present study concluded that endophytic *Streptomyces* strains are rich microbial resource for bioactive natural products, and the

secondary metabolite 2,4-di-tert-butylphenol isolated from endophytic *Streptomyces* KCA-1 has a great potential in agriculture and pharmaceutical applications.

Acknowledgements

Authors acknowledge the management of Sathyabama Institute of Science and Technology (SIST), Chennai Tamil Nadu for the research facilities provided.

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(Received: 5 August 2022, accepted: 30 October 2022)