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ISOLATION AND IDENTIFICATION OF AN ACTINOMYCETE STRAIN WITH BIOCONTROL EFFECT AGAINST *Xanthomonas oryzae* pv*. oryzae* CAUSING BACTERIAL BLIGHT DISEASE IN RICE

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ABSTRACT

In this study, we performed experiments to screen and identify actinomycete strains that are antagonistic to *Xanthomonas oryzae* pv. *oryzae* causing rice bacterial blight disease. Among of 98 strains isolated, we obtained two strains capable of antagonizing *X. oryzae* pv. *oryzae* using agar diffusion plate method. The strain numbered 43 had a strong activity with a diameter of 22 mm clear zone of bacteria. The strain 43 showed white colonies after three days of incubation. Seven days of incubation the white colonies had grey borders, produced soluble pigments on the medium, grew well at 30°C and neutral pH, and adapted well to high salt concentration medium. The strain 43 was able toutilize different sources of carbon and nitrogen. Sequence analysis of 16S rRNA showed that strain 43 had a similarity of 100% compared to *Streptomyces diastaticus* subsp. *ardesiacus*. Based on morphology, culture, physiological and biochemical characteristics and molecular biological analyses the strain 43 was identified as *S. diastaticus* subsp. *ardesiacus*.

Keywords: 16S rRNA, *Streptomyces* sp., *Xanthomonas oryzae* pv*. oryzae*

Phân lập và định danh chủng xạ khuẩn có khả năng đối kháng với vi khuẩn *Xanthomonas oryzae* pv*. oryzae* gây bệnh bạc lá lúa

TÓM TẮT

Trong nghiên cứu này chúng tôi đã tiến hành tuyển chọn, nghiên cứu đặc điểm sinh học của chủng xạ khuẩn có khả năng đối kháng với vi khuẩn *Xanthomonas oryzae* pv. *oryzae* gây bệnh bạc lá lúa. Từ 98 chủng xạ khuẩn có nguồn gốc khác nhau, bằng phương pháp khuếch tán thỏi thạch chúng tôi đã thu được 2 chủng có khả năng đối kháng với vi khuẩn *X. oryzae* pv. *oryzae*. Trong hai chủng thu nhận được thì chủng số 43 thể hiện hoạt tính mạnh hơn với đường kính vòng kháng khuẩn là 22 mm. Chủng 43 có khuẩn lạc màu trắng, nuôi từ 7 ngày trở đi thì có màu trắng viền xám, sinh sắc tố tan trên môi trường, sinh trưởng tốt ở nhiệt độ 30°C, pH trung tính và chịu được nồng độ muối tương đối cao tới 7%. Chủng 43 có khả năng sử dụng nhiều nguồn đường và nitrogen khác nhau. Phân tích trình tự 16S rRNA cho thấy chủng 43 và chủng *Streptomyces aureofaciens* có độ tương đồng là 100%. Kết hợp các đặc điểm hình thái, nuôi cấy, sinh lý, sinh hóa và phân tích sinh học phân tử đã xác định chủng xạ khuẩn 43 thuộc vào loài *S. diastaticus* subsp. *ardesiacus.*

Từ khóa: 16S rRNA, *Streptomyces* sp., *Xanthomonas oryzae* pv. *oryzae*.

1. INTRODUCTION

Vietnam is one of the largest rice exporters in the world, however, the annual rice production is affected seriously by various diseases. The most serious disease is bacterial blight (BB) caused by *Xanthomonas oryzae* pv*. oryzae* (Xoo) (Gnanamanickam *et al.*, 1999). The bacterial blight is distributed worldwide in most of rice producing countries and yield loss can be as much as 70% when susceptible varieties are grown in environments favorable to the disease.

This disease may attack the rice plants at the young stage but devastated mainly in the flowering period. The bacterium can penetrate through roots and clog vascular system but often resides and attacks on the leaves. Under high humidity or in rainy season, the disease becomes very severe. The disease causes dried leaves and decreases photosynthesis and therefore plants die prematurely or reduce yield. However, *Xoo* survives in vascular system, making it difficult to radically destroy without affecting the crop. Therefore research and application of methods to prevent as well as elimilate pathogens prior to each rice cropping season are neccessary so that farmers can reduce the outbreak or spread of BB.

Integrated cultivation methods include field sanitation, eradication of weeds, adhering to the principles of intensive farming, proper fertilization, reasonable water level adjustment, and use of resistant varieties have been employed. In addition, seed and field chemical treatment before planting to eliminate the pathogen was highly effective but this may lead to the abuse of chemicals and environmental pollution and formation of chemical resistance of the pathogen. Therefore, it is essential to find out new compounds which are effectively in managing the disease but less harmful to environment and ecosystem as well.

The *Actinomycetes* are known as a special group of bacteria which have the potential to generate a great deal of compounds that are able to kill bacteria and fungi. Scientists estimated that around 23,000 compounds, which have biological activity, were produced from microorganisms, of which more than 10,000 compounds were isolated from *actinomycetes* (Watve *et al.*, 2001). Therefore, *actinomycetes* are promising sources of bioactive substance production (Mitra *et al.*, 2008). This study was carried out to screen and identify *actinomycetes* resistant to *Xanthomonas oryzae* pv. *oryzae*, since the search for new alternatives which are safe, efficient and environmental friendly in plant protection is of crucial significance.

2. MATERIALS AND METHODS

2.1. Materials

The *Xanthomonas oryzae* pv. *oryzae* strain was isolated and stored at the Department of Molecular Biology and Applied Biotechnology, Faculty of Biotechnology, Vietnam National University of Agriculture.

Around one hundred of actinomycete strains were isolated from various soil samples in Vietnam and stored at the Department of Microbial Biotechonology, Faculty of Biotechnology, Vietnam National University of Agriculture.

2.2. Selection of *actinomycete* strains resistant to *Xanthomonas oryzae* pv. *oryzae*

The *actinomycete strains* were plated on the Gause-1 medium (soluble starch 20 g/l; K_2HPO_4 0.5 g/l; $MgSO_4.7H_2O$ 0.5 g/l, NaCl 0.5 g/l; KNO_3 0.5 g/l; $FeSO_4$ 0:01 g/l; Agar 20 g/l; pH = 7 to 7.4) at 30°C for five days. Cultured lumps of agar containing actinomycetes with 7 mm in diameter were placed onto Wakimoto medium (300g potatoes, $Ca(NO_3)_2.4H_2O$ 0.5 g, $Na_2HPO_4.12H_2O$ 2 g, sucrose 15 g, Peptone 5 g, agar 20 g, water 1l, pH 7.0) containing *Xanthomonas oryzae* pv. *oryzae*, then incubated at 4°C for one hour to diffuse the active ingredients to the medium. The sample was transferred to 30°C incubator and observed after 12 hours of incubation, then the diameter of clear zone (if any) was measured.

2.3. Identification the biological characteristics of the selected *actinomycete* (strain 43)

The *actinomycete* strain numbered 43 was cultured on the Gause-1 medium at 30°C for five days and the morphology, color and size of the colonies were recorded.

To identify spore chain morphology and spore surface the strain 43 was grown on the Gause-1 medium which was pinned by lamella with an angle of 45ºC. After 3 days of incubation at 30ºC, we drew out the lamella with aerial mycellium and observed spore chain morphology

under an optical microscope. The morphology surface of actinomycete spores were observed under a scanning electron microscope (SEM).

To check melanin pigmentation the strain 43 was cultured on ISP-6 medium (Peptone 10 g/l; yeast extract 1 g/l; iron citrate 0.5 g/l; Agar 20 g/l; pH = 7.0 to 7.2) at 30^0C. The color of medium was observed for 21 days based onthe color change from yellow to brown or black.

Checking the ability to assimilate carbon sources: the strain 43 was cultured on ISP-9 medium ($(NH4)_2SO_4$ 2, 64 g/l; KH2PO4 2.38 g/l; $K_2HPO_4.3H_2O$ 5.65 g/l ; $MgSO_4.7H_2O$ 1 g/l; 1.0 ml of solution B; Agar 20 g/l; pH = 6.8 to 7.0) supplemented with 1% by weight of the different sugar sources include D- glucose, D-fructose, D-manotol, sucrose, rhamnose, Inositol, L-arabinose, cellulose, D-xylose, raffinose. The ability to assimilate carbon sources was assessed by the viability and growth of actinomycetes on the medium.

Checking the possibility of using nitrogen sources: the strain 43 was cultured on nitrate starch medium (Starch 20 g/l; $NaNO_3$ 2 g/l; K_2HPO_4 1 g/l; $MgSO_4.7H_2O$ 0.5 g/l; KCl 0.5 g/l; $FeSO_4.5H_2O$ 0:01 g/l; pH 6.8 - 7) as a control. The nitrogen sources include beef extract, KNO_3, NH_4Cl, peptone, $(NH_4)_2SO_4$, NH_4NO_3 can be replaced with $NaNO_3$.

The effects of temperature, pH, and NaCl concentration on the growth and development of the strain 43 were determined by was culturing the strain on Gause-1 medium with the different culture temperatures (4, 20, 30, 40, 45, 50°C), pH (4 - 12) and NACl concentration (0 - 9%).

2.4. Identification of the actinomycete strain 43

Based on morphological characteristics and culture: the strain 43 was cultured on the medium and the morphology of colonies, substrate mycelium, aerial mycelium, conidiophore and surface of spore. The features identified were compared with known actinomycete strains in the international classification system (ISP) (Shirling and Gottlieb, 1966).

Based on sequence analysis of 16S rRNA: DNA from the strain 43 was extracted by method described by Marmur (1961). PCR reaction amplified conservative regions of 16S rRNA with primers: 5'-AGAGTTTGATCCTGGCTCAG-3' and 5'- ACGGCTACCTTGTTACGACTT-3'. PCR products were checked on agarose gel 1%, and sent to company 1tsBASE (Singapore) for sequencing. The degree of similarity in the sequence of 16S rRNA of the strain 43 was compared with strains published in GenBank using Blast search tool (http://blast.ncbi.nlm.nih.gov/Blast .cgi). MEGA6 software was used to determine genetic relationships. Maximum Parsimony selection method with reliability was calculated by bootstrap algorithm with 1000 repetitions. Based on the phylogentic tree and bootstrap values genetic relationship of the strain was determined.

3. RESULTS AND DISCUSSION

3.1. Screening of actinomycete strains antagonistic against *Xanthomonas oryzae* pv. *oryzae*

The *actinomycetes* is supposed to be antagonistic against other microorganisms because of compounds with biological activities, especially many types of antibiotics. These substances are secreted to medium by *actinomycetes* during cultivation, so we used diffusion method on agar plates for selection and evaluation for antibacterial activity of *actinomycetes*. Gause-1 medium was used to grow the *actinomycetes*, Wakimoto medium was used to grow *X. oryzae* pv. *oryzae*. Through the screening, two of the 98 studied actinomycete strains were identified to be resistent to *X. oryzae* pv. *oryzae*. Among them, the strain numbered 43 was able to antagonize strongly with 22 mm of inhibited zone in diameter (Figure 1 A), the strain numbered 978 had smaller inhibited zone with 16 mm in diameter (Figure 1 B). In recent years, there have been some publications in the world in search for the actinomycetes capable of being antagonistic to *X. oryzae* pv. *oryzae*. These studies focused on the

discovery and extraction of active ingredients against *Xoo* as well as some other plant pathogens (Jiang *et al.*, 2013; Kim *et al.*, 2015; Park *et al.*, 2011). Compared to the previous results, the strain 43 had relatively high activity. This result showed that the actinomycete strain 43 had potential for application.

3.2. Biological characteristics of the actinomycete strain 43

3.2.1. Morphological characteristics

One of the first criteria to study biological characteristics and classification of actinomycetes is based on morphological characteristics (Miyadoh *et al.,* 2016). The actinomycete strains were grown on Gause-1 medium at 30°C for 7 days to observe the color, size, shape of the colony. After three days of culture, the colony of strain 43 showed round shape, 0.2 - 0.4 mm in size with off-white color. The color of colony changed after some days of incubation and colonies were almost dark brown at day 5.

After determining the characteristics of colonies, we determined the formation of conidiophore, spore chain and surface of spore from the strain 43. The results observed under an optical microscope at a magnification of 1000 showed that after 48 h of incubation the actinomycetes strain 43 started sporulation. The spores arranged in long, branched and twisted chains. After 60 hrs of culture, the spores began to leave the series and released to the culture medium. To determine more accurately the morphology of the strain 43, we observed the morphology of the spore chain and the surface of spore under a scanning electron microscope (SEM). Specimen handling, observation and analysis of the image were carried out in the National Institute of Hygiene and Epidemiology. Using SEM with 5000 times magnification, it showed that the spore chain of the strain 43 was typical with a white spiral spring form, each chain bearing 10 - 20 spores (Figure 2 A). Spores of the strain 43 had short oval shape with size of 0.6 - 0.8 x 0.9 - 1.1 µm and smooth surface (Figure 2 B).

3.2.2. Ability of melanin pigmentation

According to the International Streptomyces Project (ISP), melanin formation was determined on the ISP6 medium at 30°C for at least 21 days. Melanin production changed the color of medium from pale yellow to brown and black (Shirling and Gottlieb, 1966).

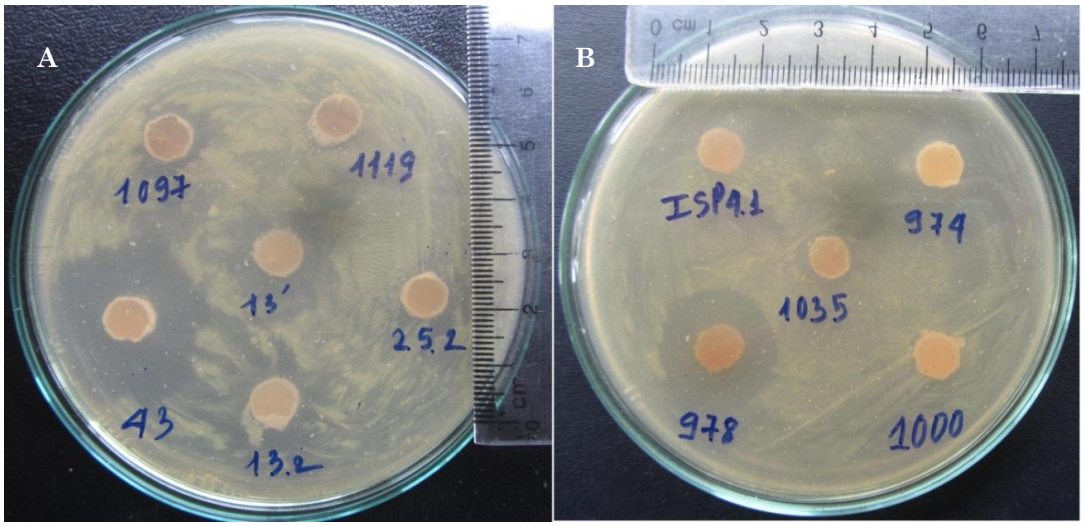
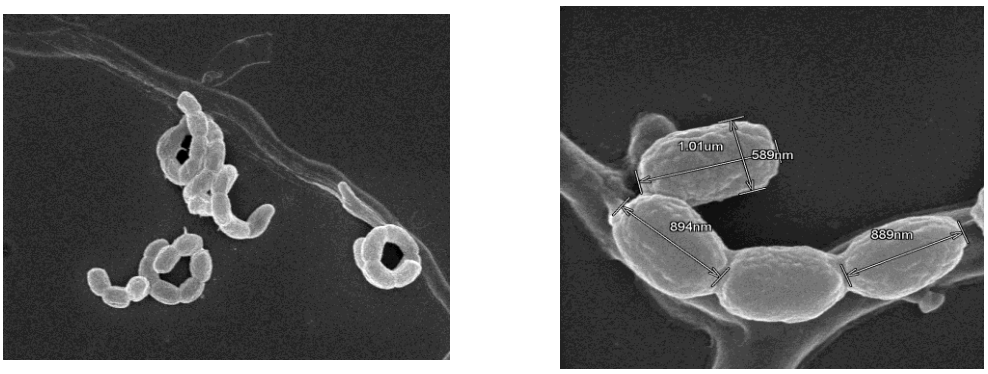
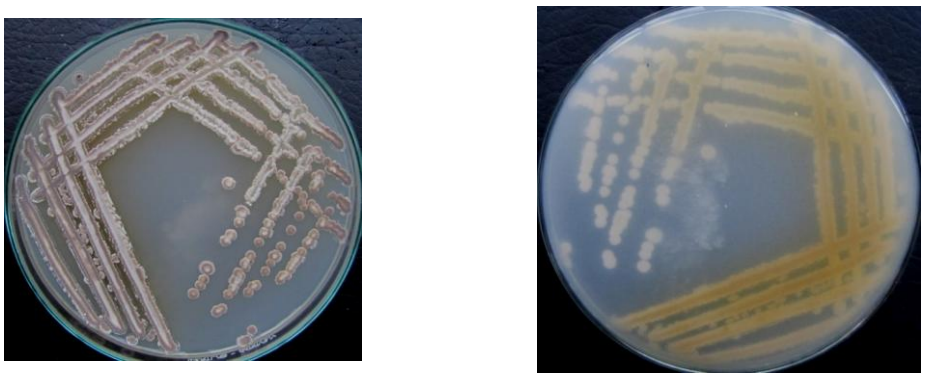


Figure 1. Antagonistic activity of some actinomycetes against *Xanthomonas oryzae* pv. *oryzae* by diffusion method on agar plates. Two strains of actinomycete strains identified as active strains are strain 43 (A) and strain 978 (B)



(A) **(B)**

Figure 2. Mophology of spore chain and spore surface from the strain 43 under scanning electron microscope at magnification of 5,000 times (A) and 3,0000 times (B)



(A) **(B)**

Figure 3. Melanin formation of the strain 43 when cultured on the ISP-6 medium after 21 days. Photographs of the up-side (A) and bottom-side (B) of cultured dish

Table 1. Ability of using various carbon and nitrogen sources of strain 43

Carbon source	Development of the strain 43 after 5 days of culture	Nitrogen source	Development of the strain 43 after 5 days of culture
Fructose	+	$NaNO_3$	+
Mantose	+	KNO_3	+
Xylose	+	Beef extract	++
Arabinose	+	NH_4Cl	-
Rhanbinose	++	Pepton	+
Sucrose	-	$(NH_4)_2SO_4$	-
Lactose	+	NH_4NO_3	-
Manitol	++	Yeast extract	++
Sobitol	-		
Dextrose	++		
Ribose	-		
Galactose	++		

Note: (+) the strain 43 could grow; (++) the strain 43 grow develop well; (-) the strain 43 cannot grow

The strain 43 was cultured on the ISP6 medium and observed after 21 days of culture. The result showed that areas around colonies appeared yellowish, indicating that the strain 43 was capable of generating melanin, however, this ability was relatively weak (Figure 3).

3.2.3. The ability to use the carbon and nitrogen sources of strain 43

We performed this study to investigate the possibility of using carbon and nitrogen sources of the strain 43. This result served as one of the bases to classify actinomycetes according ISP system, and, at the same time, to provide information on nutrition of the strain 43 for the future fermentation process. The strain 43 was cultured on the ISP-9 medium added with different sugar sources and on the starch nitrate medium where NaNO3 sources were replaced by different nitrogen sources as described in materials and methods section. Results showed that the strain 43 could utilize carbon from different sources such as fructose, L-arabinose, raffinose, D-xylose, inositol, D-mannitol, cellulose, sucrose, etc., in which rhanbinose, mannitol, dextrose and galactose showing highest efficiency (Table 1). This result was consistent with the published studies (Mohana and Radhakrishnayn, 2014; Miyadoh *et al.,* 2016). The strain 43 was capable of using nitrogen from various sources such as beef extract, peptone, yeast extract, and KNO_3 (Table 1). In particular, the strain 43 grew persistent in medium adding nitrogen from beef extract or yeast extract.

Table 2. The influence of some environmental conditions on the development of strain 43

Factors	Optimum value	Endurance value
Temperature (^{0}C)	30 - 35	20 - 45
NaCl (%)	< 3	< 7
pH	6 - 9	5 - 12

3.2.4. The ability to adapt to culture medium conditions of the strain 43

Investigation of medium culture factors on the growth and development of the actinomycete strain 43 provides useful information about culture conditions for further research. The actinomycete strain 43 was cultured on Gause medium at different temperatures, pH and salt concentrations. Observations of the growth and development of strain 43 after five days of culture are summarized in table 2. Results showed that the strain 43 was capable of adapting relatively high to the test conditions. However, the strain 43 developed best at 30 - 35°C and neutral or slightly alkaline media with pH 6 - 9 and had ability to withstand concentrations of NaCl up to 7%. (Table 2, Figure 4).

In this study, the strain 43 could grow in medium up to 7% of salt concentration. Therefore, it could be classified as moderate salt endurance group, but it thrived best at concentration of 1 - 3%. The results found in this study were similar to those published by Mohana and Radhakrishnan (2014).

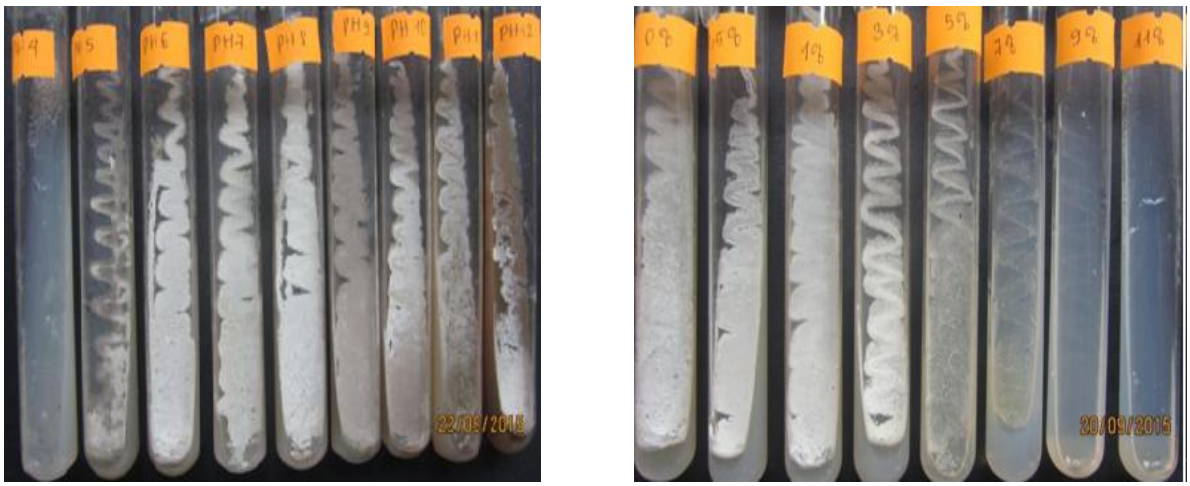
3.3. Identification of the actinomycete strain 43

The identification of the actinomycete strain 43 was based on the similarity of the 16S rRNA gene fragment with actinomycete strains published in the gene bank. We conducted DNA extraction following method described by Marmur (1961). The primers 27F and 1492R were used to amplify 16S rRNA gene fragment of strain 43. Electrophoresis resulted in one single DNA band with about 1500 bp in size, consistent with the theoretical sizes when amplifying with this primer (Figure 5).

PCR products were purified and sequenced at the company 1stBASE (Singapore). We compared the obtained sequence with other sequences in the gene bank by blast tool and built phylogentic trees for strain 43 (Figure 6).

The resulting phylogenetic tree based on 16S rRNA gene sequence showed that the

actinomycete 43 located in the same branch with *Streptomyces diastaticus* subsp. *ardesiacus* with 100 of bootstrap value. The bootstrap point is a value of checking the compatibility level of the data with the model of evolution and sorting classification branches in the phylogenetic tree. Bootstrap value greater than 70% of repetitions is considered to have more than 95% of statistical value. The 16S rRNA bootstrap value of the strain 43 with the *S. diastaticus* subsp. *ardesiacus* was 100%, within the confidence interval. Combined with information of nucleotide sequence, the compatibility in the 16S rRNA sequence of actinomycete 43 with *Streptomyces diastaticus* subsp. *ardesiacus* was 100% and comparison of the similarity in the characteristics studied guarantees the reliability of the species relationship (Miyadoh *et al.*, 2016). Therefore, based on biological characteristics and molecular identification, we concluded that the actinomycete strain 43 is *Streptomyces diastaticus* subsp. *ardesiacus.*



(A) **(B)**

Figure 4. Examination of the ability of strain 43 to adapt to different pH condition (A) and salt concentration (B) on the Gause medium after seven days

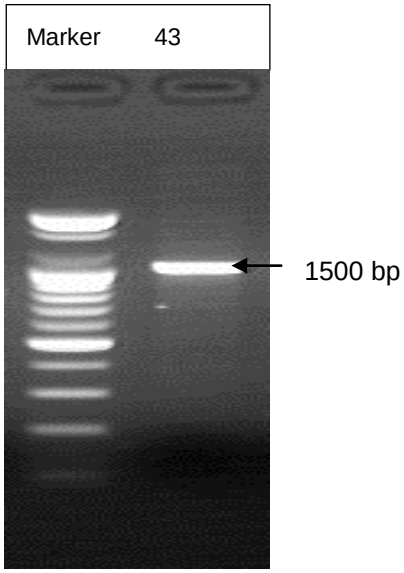


Figure 5. Electrophoresis of PCR product on 1.2% agarose gel

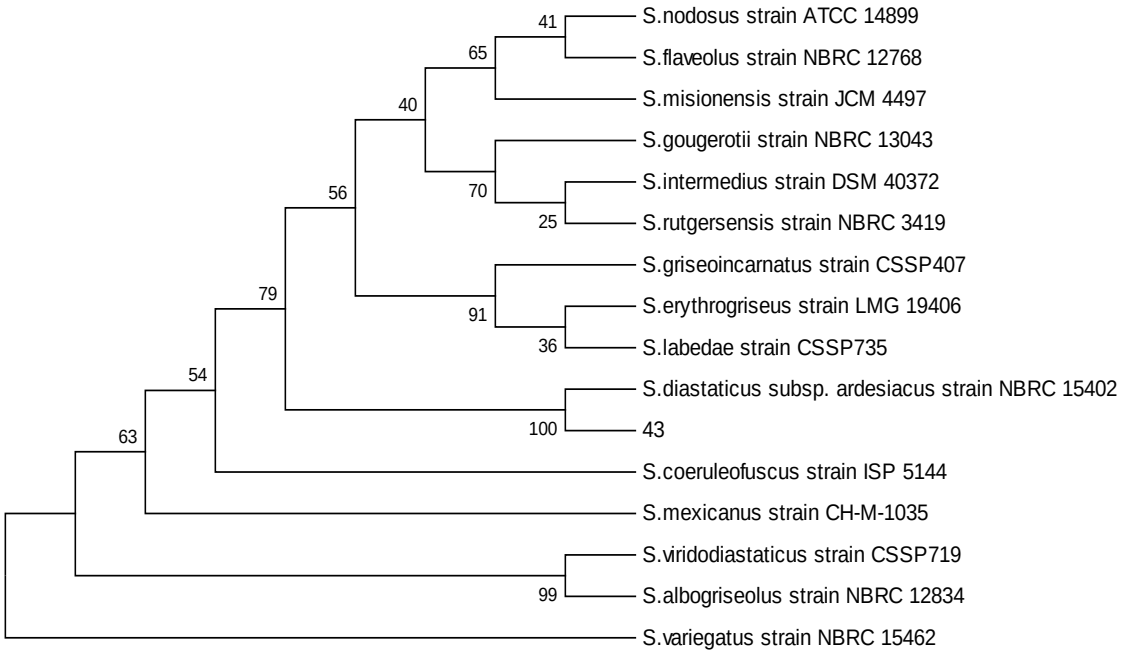


Figure 6. Phylogenetic tree of the actinomycete strain 43 based on 16S rRNA sequence

4. CONCLUSION

Two actinomycete strains which expressed antagonistic activity against *Xanthomonas oryzae* pv. *oryzae* causing bacterial leaf blight were selected. Among them, the strain 43 showed stronger activity with 22 mm in diameter of clear zone.

The biological characteristics of the actinomycete strain 43, such as morphology, culture, physiology and biochemistry were studied.

By the molecular biological methods combined with traditional classification key, the strain 43 was identified as *Streptomyces diastaticus* subsp. *ardesiacus.*

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