International Journal of Technical Research and Applications e-ISSN: 2320-8163,
www.ijtra.com Special Issue 12 (Jan-Feb 2015), PP. 39-43

STUDY ON ANTIFUNGAL ACTIVITY OF ALCALIGENES SPP. BY CULTURING IN RAW SUBSTRATES

Nann Miky Moh Moh, Khaing Nwe Nwe Oo, Win Min Than, Myo Myint
Department of Biotechnology,
Mandalay Technological University
Mandalay, Myanmar.
nannmikymohmoh7@gmail.com

***Abstract*— Two *Alcaligenes* spp. (A1 and A2) were isolated from acidic soil (pH 4-4.5) of the battery factory in Mandalay Industrial Zone. These two bacteria showed antifungal activity against three common plant pathogenic fungi (*Fusarium oxysporum, Rhizoctonia solani* and *Pythium* sp.). Peptone-based nutrient medium was used for culturing these two *Alcaligenes* spp. and it will not be suitable for large scale production in industry because of its high cost. So, two raw substrates such as groundnut meal cake and soy bean meal were used to study in the substitution of the peptone. According to the resulting data, the antifungal activities of two *Alcaligenes* spp. culturing in groundnut can compare with the activities in peptone although the activities in soy meal was not as good as that in peptone. So, the commercial production of biofungicide from *Alcaligenes* spp. can be considered by using substituted groundnut raw substrate.**

***Keywords*—*Alcaligenes* spp., antifungal activity, plant pathogenic fungi, raw substrates.**

I. INTRODUCTION

Our country, Myanmar, is an agricultural country. So, its economy is mainly dependent on the development of agriculture. But many agricultural losses are caused due to plant diseases [1]. Among them, about two-third of infectious plant diseases are caused by fungi [2].

Because of the harmful effects to human health, high cost and the environmental pollution problem of the chemical fungicides, the biological controls of the plant pathogenic fungi have become a tendency for agriculture [3], [4], [5]. The biological control is the reduction of inoculums density of disease producing activity of a pathogen or parasite in its active or dormant state by one or more organisms [6], [7], [8], [9], [10], [11], [12].

Alcaligenes is a genus of Gram-negative bacterium. They are rods or coccobacilli in shape and 0.5-1.2 x 1.0- 3.0 µm in size, usually occurring singly. They are motile with one to nine peritrichous flagella. *Alcaligenes* is obligately aerobic. Some strains are capable of anaerobic respiration in the presence of nitrate or nitrite. The optimum growth temperature is 20-37ºC. Colonies on nutrient agar are nonpigmented. Oxidase test and catalase test are positive. Indole is not produced. Cellulose, esculin, gelatin, and DNA usually are not hydrolyzed. *Alcaligenes* is chemoorganotrophic using a variety of organic acids and amino acids as carbon sources. Alkali is produced from several organic salts and amides. Carbohydrates usually are not utilized [13], [14].

The use of good, adequate and industrially usable medium is as important as the deployment of a suitable microorganism in industrial microbiology. The cheaper the raw materials the more competitive the selling price of the final product will be. Therefore, no matter how suitable a nutrient raw material is, it will not usually be employed in an industrial process if its cost is so high that the selling price of the final product is not economic. Due to these economic considerations the raw materials used in many industrial media are usually waste products from other processes [15], [16], [17], [18].

Myanmar is one of the major groundnut growing countries. In the decade of 2000-2009, the annual growth of groundnut area and yield in Myanmar was over 3.0% resulting in a 5.3% annual increase in production to reach 1.36 million tons in 2009. Groundnut cake is a by-product of oil extraction from groundnut. It is an excellent livestock feed because of its high protein content. The cake contains 45-60% protein, 22-30% carbohydrate, 3.8-7.5% crude fiber and 4-6% minerals [19], [20], [21].

Soybean meal also known as Soybean Oil Cake is a flour made by grinding the solid residue of soybean oil production. It is widely used as a filler and source of protein in animal diets, including pig, chicken, cattle, horse, sheep, and fish feed. Soybean meal is the product remaining after extracting most of the oil from whole soybeans. Protein content of soy bean meal is 48% [22], [23], [24].

The purpose of this study is to control the plant pathogenic fungi with *Alcaligenes* spp. by culturing in cost-effective raw substrate. The objectives of this study are to isolate and identify the antifungal bacteria, to examine their antifungal activity and to substitute raw substrate in peptone medium.

II. MATERIALS AND METHODS

A. Isolation and Identification of Alcaligenes spp.

The soil sample was collected from the battery factory in Mandalay Industrial Zone. The serial dilutions of soil sample (1 gram of the sample is diluted in 9 ml of sterile distilled water, then 1 ml of this dilution is added to another 9 ml of distilled water and the this is repeated until the dilution of 10^{-6}) were done and 0.1 ml of the dilutions 10^{-4}, 10^{-5} and 10^{-6} were cultured on peptone based medium (peptone, yeast and KCl) at 37±2°C for 24 hours. The antagonastic antivity of each different single colony was tested against three common plant pathogenic fungi such as *Fusarium oxysporum, Pythium* sp. and *Rhozoctonia solani*.

For the identification of two antifungal bacterial isolates, the phenotypic characterization and some biochemical characterizations were carried out according to the Bergey's Manual of Determinative Bacteriology. The 16s rDNA genome sequencing analysis was also carried out for the identification of bacteria. To evaluate the analysis of 16s rRNA sequences, the resulting sequences (800 bp) were compared with the known reference sequences using the BLAST (Basic Local Alignment Tools) function of GreenGene and the National Centre for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov)

B. Study on Antibiotic Sensitivity of Alcaligenes spp.

The antibiotic sensitivities of two *Alcaligenes* spp. (A1 and A2) were examined with five antibiotics such as tetracycline, ampicillin, amoxillin, streptomycin and chloramphenicol. These two bacteria were inoculated into Muller-Hinton broth and incubated at 37±2°C and 120 rpm for 6 hours. Each bacterial broth was swabbed onto Muller-Hinton agar plates and 7 mm-diameter wells were punched out. Tetracycline, ampicillin and streptomycin were dissolved in water. Amoxillin was dissolved in methanol (70%) and chloramphenicol in ethanol (70%). Each well was filled with 30µg of tetracycline, 10µg of ampicillin, 20µg of amoxillin, 10µg of streptomycin and 30µg of chloramphenicol and incubated at 37±2°C for 24 hours. The inhibition zone diameters were measured in the next day and compared with the standard chart to examine their sensitivity. [25]

C. Collection of Plant Pathogenic Fungi

Three common plant pathogenic fungi; *Fusarium oxysporum*, *Rhizoctonia solani* and *Pythium* sp. were obtained from the Department of Biotechnology, Mandalay Technological University. These fungi were cultured in Potato Dextrose Agar (PDA) at 28±2˚C for 5 days and examined their morphological characteristics.

D. Examination of Antifungal Activity of Alcaligenes spp.

1) Dual culture Method: The antagonistic activity of the bacteria against the plant pathogenic fungi was screened by the dual culture method. Three common plant pathogenic fungi were inoculated in each test tube containing 10ml of Potato Dextrose Broth (PDB) and then incubated on the shaker at 28±2˚C and 120 rpm for three days. Then two *Alcaligenes* spp. were cultured on the peptone based medium to get the fresh cultures. After that, each fungal culture broth was swabbed onto each Potato Dextrose Agar (PDA) plate. The culture colonies of each bacterium were streaked onto these plates. Then, the dual culture plates were incubated at 28±2˚C for five days. After incubation, the biological control activity of each bacterium was studied.

2) *Well Diffusion Method:* To examine the secondary metabolite activity of bacteria, the well diffusion method was carried out. Two *Alcaligenes* spp. were inoculated in each conical flask containing the culture broth and incubated on the shaker at 37±2˚C with 120 rpm for 7 days. Each culture broth was centrifuged at 6000 rpm for 15 mins. Two plant pathogenic fungi (*Rhizoctonia solani* and *Pythium* sp.) were used as the indicator fungi in agar well diffusion method. Then, each fungal broth was swabbed on PDA plates as previously and 7 mm diameter wells were made on PDA plates then 50 µl of the supernatants of two bacteria were put into the wells and incubated at 28±2˚C for 5 days. And then the inhibition clear zones formations were studied.

E. Determination of Optimum Incubation Period for Antifungal Activity of Alcaligenes spp.

The optimum incubation period for the antifungal activities of two *Alcaligenes* spp. was determined by using agar well-diffusion method. Each bacterium was inoculated into each conical flask containing the peptone-based liquid media and incubated at 37±2°C and 120 rpm for ten days. Each culture sample was pulled out daily and centrifuged at 6,000 rpm for 15 minutes to obtain the supernatant. Each fungal broth of *Rhizoctonia solani* and *Pythium* sp. was swabbed onto each PDA medium and 7 mm diameter wells were punched out. Each well was filled with 50 µl of supernatant and incubated for five days. After 5 days incubation, the antifungal activities of *Alcaligenes* spp. were studied and then measured the inhibition zone diameters (mm). To confirm the resulting data, the procedure was repeated for triplicate.

F. Preparation of Raw Substrates

Two types of raw substrates (groundnut meal cake and soy bean meal cake) were used to study for the substitution of peptone. Firstly, the groundnut meal cake and soy bean meal cake were crushed to obtain the crude powder. Then, these two resulting powders were soaked in ethanol for about 24 hours to dissolve the oil from powders. The oil-dissolving ethanol was discarded and the crude powders were dried at room temperature. Finally, these powders were used as the substitute of peptone. The peptone-based media of bacteria contained peptone, yeast and potassium chloride. The resulting groundnut meal cake powder and soy meal cake powder were used in the place of peptone.

G. Comparison of Antifungal Activity of Alcaligenes spp. Culturing in Raw Substrates and Peptone Media

To determine the antifungal activities of two bacteria cultured in the raw substrate media, the agar well-diffusion method was used. Each bacterium was inoculated into each conical flask containing peptone, groundnut or soy meal-based broth and incubated at 37±2°C and 120 rpm for ten days. Then, each bacterial culture broth was taken out daily and centrifuged at 6000 rpm for 15 minutes to obtain the supernatant. Each fungal broth (*Rhizoctonia solani* and *Pythium* sp.) was swabbed onto each PDA medium and 7 mm diameter wells were punched out. Then, each well was filled with 50 µl of each bacterial supernatant and incubated for three days. Then, the antifungal activities of two *Alcaligenes* spp. culturing in two raw substrates and peptone were examined and measured the inhibition zone diameters.

To examine whether the groundnut powder and soy bean powder have the antifungal activity or not, the raw substrates containing liquid medium without bacterium were used to test the antagonistic activity against common plant pathogenic fungi such as *Pythium* sp. and *Rhizoctonia solani.*

III. Results and Discussions

A. Isolation and Identification of Alcaligenes spp.

According to resulting data from physiological and biochemical characterization, two bacterial isolates were gram-negative bacteria and showed positive results in motility, oxidase and catalase tests and negative results in indole test and spore staining. These bacteria had the ability to utilize citrate as the sole carbon and energy sources for their growth. They could also hydrolyze starch. Comparison of sequences by BLAST with 16s rRNA sequences in the database exhibited 99% homology with *Alcaligenes* spp. (Table I).

Strain Code	Method Used	Database Used	Max Identity %	Identity
A1	MicroSeq and BigDye v.3.1	Green Gene	99.72%	*Alcaligenes* sp.str.IS-18
		NCBI	99%	
A2	MicroSeq and BigDye v.3.1	Green Gene	99.86%	*Alcaligenes* sp.str. PGBS001
		NCBI	99%	

TABLE I. Identification of two Bacteria by Genome Sequencing Analysis

B. Study on Antibiotic Sensitivity of Alcaligenes spp.

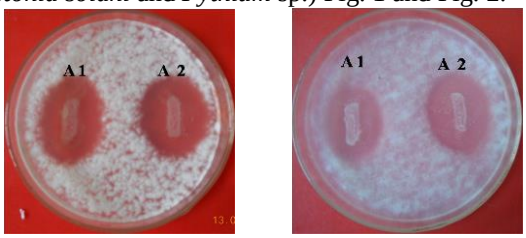
The antibiotics sensitivities of two *Alcaligenes* spp. (A1 and A2) were examined with five antibiotics such as tetracycline, ampicillin, amoxillin, streptomycin and chloramphenicol by well diffusion method. Both bacteria were sensitive to four antibiotics such as ampicillin, amoxillin, streptomycin and chloramphenicol. However, the antibiotic sensitivity of bacteria against tetracycline was intermediate by comparing with the standard chart. Two bacteria were more sensitive to ampicillin and amoxillin than the other antibiotics (Table II).

TABLE II. ANTIBIOTIC SENSITIVITY OF TWO *ALCALIGENES* SPP.

Antibiotics		Inhibition Zone Diameter (mm)	
		A1	*A2*
Tetracycline	30µg/well	14.5 (I)	18.5 (I)
Ampicillin	10µg/well	27.5 (S)	29 (S)
Amoxillin	20µg/well	26.5 (S)	29 (S)
Streptomycin	10µg/well	16.5 (S)	19.5 (S)
Chloramphenicol	30µg/well	20.5 (S)	21.5 (S)

C. Examination of Antifungal Activity of Alcaligenes spp.

Two *Alcaligenes* spp. (A1 and A2) showed strong antigonastic activity against two plant pathogenic fungi (*Rhizoctonia solani* and *Pythium* sp.) Fig. 1 and Fig. 2.



(a) (b)

Fig. 1. Antifungal Activities of two *Alcaligenes* spp. against (a) *Fusarium oxysporum*. and (b) *Pythium* sp on PDA Media after 5-days Incubation

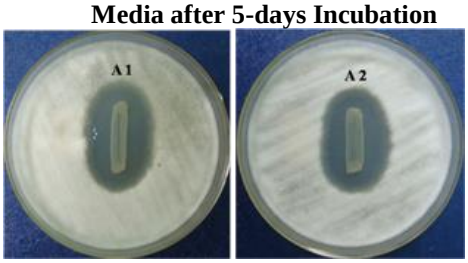


Fig. 2. Antifungal Activities of two *Alcaligenes* spp. against *Rhizoctonia solani* on PDA Media after 5-days Incubation

D. Determination of Optimum Incubation Period for Anntifungal Activity of Alcaligenes spp.

The agar well diffusion method was used to determine the optimum incubation period of two bacteria for their antifungal activities. Each bacterium was cultivated in each peptone-based liquid medium for 10 days and daily tested their activities against two common plant pathogenic fungi; *Rhizoctonia solani*, and *Pythium* sp.. To confirm the data, the procedure was repeated for triplicate. According to the resulting data, the antifungal activities of two bacteria at 7th day incubation period were optimum. Although the antifungal activities of two bacteria decreased after 7th day incubation period, two bacteria showed their antifungal activities till 10-day incubation period. The resulting data were shown in Table III and Table IV.

TABLE III. ANTIFUNGAL ACTIVITY OF TWO *ALCALIGENES* SPP. (SUPERNATANT) AGAINST *RHIZOCTONIA SOLANI*

Days	Inhibition Zone Diameters (mm)	
	A 1	*A 2*
1 day	12	9
2 days	12.5	9.5
3 days	14.5	12
4 days	20.5	16.5
5 days	21	18
6 days	22.5	18
7 days	23	23.5
8 days	19.5	23
9 days	18.5	22
10 days	18	19

TABLE IV. ANTIFUNGAL ACTIVITY OF TWO *ALCALIGENES* SPP. (SUPERNATANT) AGAINST *PYTHIUM* SP.

Days	Inhibition Zone Diameters (mm)	
	A 1	*A 2*
1 day	11	9
2 days	12.5	10.5
3 days	14.5	16.5
4 days	15.5	13
5 days	16.5	18
6 days	18.5	18
7 days	19.5	20.75
8 days	17.5	17
9 days	17.5	17.5
10 days	16	16.5

E. Preparation of Raw Substrates

Protein-rich raw substrates were considered to use as a substitute of peptone that contained in the bacterial culture medium. The oil content in culture medium could affect the growth pattern of some bacteria. So, the by-products of oil extraction such as groundnut meal cake and soy meal cake were considered to study in this research (Fig. 3).

(a) (b)

Fig. 3. Raw Substrates after Air Dry at Room Temperature
(a) Groundnut Meal Powder
(b) Soy Bean Meal Powder

F. Comparison of Antifungal Activity of Alcaligenes spp. Culturing in Raw Substrates and Peptone Media

According to the data, each bacterium cultivated in groundnut, soy meal and peptone-based liquid media showed its antifungal activities against the tested fungi. Two bacteria showed their highest antifungal activities at 7th day incubation period when they were culturing in peptone-based medium. When two bacteria were culturing in groundnut-containing medium, they showed their highest activities at 6th day, 7th day and 8th day incubation periods. Moreover, the inhibition zone diameters of two bacteria culturing in groundnut-containing

medium were wider than that of two bacteria in peptone-based medium. The resulting data were showed in Table V, Table VI, Table VII and Table VIII.

In order to examine whether the groundnut or soy meal powder had the antifungal activities themselves or not, bacteria-free groundnut or soy meal-containing liquid media was used to test the antagonistic activity against two common plant pathogenic fungi (*R. solani*, and *Pythium* sp.). From the resulting data, the groundnut or soy meal medium did not show the antagonistic activity without containing the effective bacterium. Therefore, only two bacteria showed the antifungal activities but not the groundnut or soybean meal.

TABLE V. ANTIFUNGAL ACTIVITY OF *ALCALIGENES* SPP. A1 (SUPERNATANT) IN RAW SUBSTRATES AND PEPTONE AGAINST *PYTHIUM* SP.

Days	Inhibition Zone Diameters (mm)		
	Peptone	*Groundnut Powder*	*Soy Bean Powder*
1 day	11	9	8.5
2 days	12.5	14.5	9
3 days	14.5	15.5	9
4 days	15.5	15	9.5
5 days	16.5	15.5	12.5
6 days	18.5	17.5	13.5
7 days	19.5	17.5	11.5
8 days	17.5	16.5	13
9 days	17.5	16.5	9.5
10 days	16	12.5	9

TABLE VI. ANTIFUNGAL ACTIVITY OF *ALCALIGENES* SPP. A2 (SUPERNATANT) IN RAW SUBSTRATES AND PEPTONE AGAINST *PYTHIUM* SP.

Days	Inhibition Zone Diameters (mm)		
	Peptone	*Groundnut Powder*	*Soy Bean Powder*
1 day	9	10	9
2 days	10.5	12.5	9
3 days	16.5	15.5	9
4 days	13	18.5	9.5
5 days	18	18.75	15.5
6 days	18	18.5	13.5
7 days	20.75	21.75	18
8 days	17	18.5	14.5
9 days	17.5	16	15.5
10 days	16.5	16	15

TABLE VII. ANTIFUNGAL ACTIVITY OF *ALCALIGENES* SPP. A1 (SUPERNATANT) IN RAW SUBSTRATES AND PEPTONE AGAINST *RHIZOCTONIA SOLANI*

Days	Inhibition Zone Diameters (mm)		
	Peptone	*Groundnut Powder*	*Soy Bean Powder*
1 day	12	13	12.5
2 days	12.5	19.5	17
3 days	14.5	20	21.5
4 days	20.5	20.5	22
5 days	21	21	19
6 days	22.5	27	23
7 days	23	27	23
8 days	19.5	27	27.5
9 days	18.5	16.5	16
10 days	18	17	12

TABLE VIII. ANTIFUNGAL ACTIVITY OF *ALCALIGENES* SPP. A2 (SUPERNATANT) IN RAW SUBSTRATES AND PEPTONE AGAINST *RHIZOCTONIA SOLANI*

Days	Inhibition Zone Diameters (mm)		
	Peptone	*Groundnut Me Powder*	*Soy Bean Powder*
1 day	9	9	10.5
2 days	9.5	11.5	12.5
3 days	12	19	15.5
4 days	16.5	20	12.5
5 days	18	24.5	15.5
6 days	18	29	20
7 days	23.5	27	21
8 days	23	28	25.5
9 days	22	22	16
10 days	19	10.5	11.5

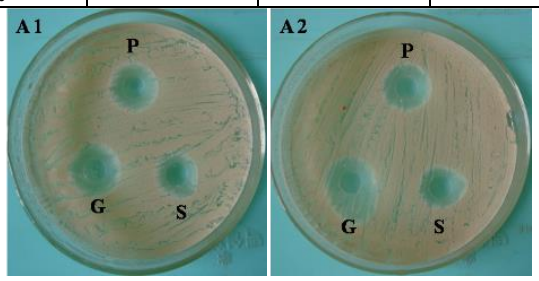


Fig. 4. Antifungal Activities of two *Alcaligenes* spp. (Supernatant) after 7 days incubation period in Peptone and Raw Substrates against *Pythium* sp

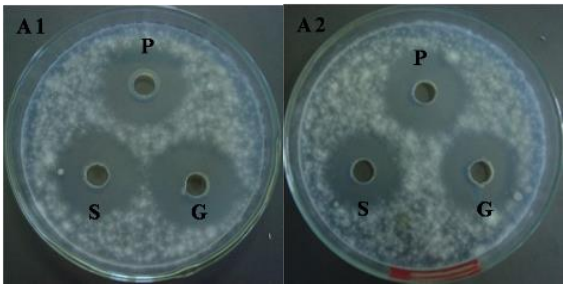


Fig. 5. Antifungal Activities of two *Alcaligenes* spp. (Supernatant) after 7 days incubation period in Peptone and Raw Substrates against *Rhizoctonia solani*

IV. CONCLUSIONS

Two *Alcaligenes* spp. had antifungal activities against three common plant pathogenic fungi; *Pythium* sp., *Rhizoctonia solani* and *Fusarium oxysporum*. The optimum incubation period for antifungal activities of these two *Alcaligenes* spp. was at 7th day incubation in peptone-based medium. When two bacteria were culturing in groundnut-containing medium, they showed their highest activities at 6th day, 7th day and 8th day incubation periods and their activities in groundnut were as good as that in peptone. Moreover, the antifungal activities of two *Alcaligenes* spp. cultivated in groundnut powder were better than their activities in soybean powder. So, groundnut meal cake powder was selected to use as a substituent of peptone.

Therefore, *Alcaligenes* spp. can be considered to use in the production of potential effective biofungicide. These bacteria could also grow and showed the antifungal activity while culturing in the raw substrate media. This result could be used for the commercial production of biofungicide.

ACKNOWLEDGMENT

We would like to thank Ministry of Science and Technology for financial support. We also would like to thank Dr. Aye Aye Khai for her kind advice and suggestion.

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