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Isolation and characterization of cellulose-degrading *actinomycetes* isolates

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Abstract

A total of twenty-one strains were isolated from various soil sources under wood decaying matter, manure and vegetative fields in the campus of Mandalay Technological University, Mandalay, Myanmar. Among them, eight strains were confirmed as Actinomycetes according to the biochemical examinations, cultural morphology, microscopic morphology and colonial morphology. Their cellulolytic activity was screened by using different types of cellulose substrates such as cellulose powder, CMC powder, acid treated rice straw and base treated rice straw. Quantitative determination was done by DNS reducing sugar analysis method and strains M_2, V_2 and W_1 showed best results in reducing sugar productivity of 0.0504mg/ml using 1% CMC, 0.2100mg/ml using 0.5% cellulose and 0.1596mg/ml using 2% rice straw treated with 2M NaOH respectively.

Keywords: *Actinomycetes,* cellulolytic activity, cellulose substrates, CMC, reducing sugar.

Introduction

The plant cell wall mainly consists of cellulose which is the most abundant renewable energy source with the maximum annual output[1]. Dry weight of the plant cell wall includes cellulose (35–50%), hemicellulose (20–35%), and lignin (5–30%)[2]. The utilization of plant cellulose was popular in fuel producing, animal feed and manure, and in the paper industry. Although the plant cellulose was utilized in many purposes, enormous amount of agricultural cellulosic wastes still remained and that lead to the environmental pollution. Some of the microorganisms and fungi play a key role in the production of cellulose and related enzyme because of their cellulolytic activity[3]. Cellulolytic bacterial have been isolated from various environments, such as the bovine rumen[4], soil organic waste[5] and ruminant animal waste[6].

Among the cellulolytic bacteria, *Actinomycetes* become popular for cellulose degradation[7]. *Actinomycetes* can be widely isolated from natural environment, such as soil under wood decaying, vegetative fields and compost. The ability of *Actinomycetes* for decomposition of the organic matters is practically boundless owing to synthesis of the various enzymes destroying wood, lignin, cellulose, chitin, wax, pitch and microbial cells[8]. *Actinomycetes* were potentially used in plant biomass-degrading because of their cellulolytic activity of against the plant cellulose[9].

Rice straw, one of the most abundant agricultural cellulosic waste in the world, is a by-product of rice cultivation and potentially used in biomass resource. The annually production of the rice straw was about 731 million tons and 667.6 million tons was produced from Asia. The chemical composition of rice straw was 32-47% cellulose, 19-27% hemicellulose, 5-24% lignin and 18.8% ashes[9].

Pretreatment is necessary for the bioconversion of lignocellulosic materials to fuels and other chemicals[10]. The main purpose of pretreatment is to break down the lignin and to alter the crystalline structure of cellulose[11]. By using the pretreatment method, cellulose become more easily to break down into enzymes that convert the carbohydrate polymers into sugars. Many different pretreatment methods have been reported in the past and they can be classified into physical pretreatment method such as milling, grinding and irradiation, chemical pretreatment method like alkali, dilute acid, oxidizing agents and organic solvents, physicochemical pretreatment method such as steam pretreatment, hydrothermolysis and wet oxidation and the last one was biological pretreatment methods[12].

The aim of this research is to isolate the *Actinomycetes* strains from various soil sources for the selection of the best strains for cellulose-rich agricultural waste degradation.

Materials and methods

Samples collection: Samples of the soil were collected from various sources under wood decaying matter, manure and vegetative fields in the campus of Mandalay Technological University, Mandalay Region, Myanmar.

Isolation and Identification of *Actinomycetes*: Collected soil samples were air-dried for 7 days. 1g of each soil sample was treated with calcium carbonate for 24hr and then subjected to serial dilution up to 10^{-6}. One milliliter of the diluted sample was then cultured on starch casein agar media and incubated at 30ºC for one week to get pure colonies. Pure *Actinomycetes* colonies were maintained in starch casein agar media at 4ºC. The morphological and physiological characteristics of the

isolate strains were determined by the methods described in the International Streptomyces project[13] and Bergey's Manual of Systematic Bacteriology[14].

Pre-treatment of rice straw: Agricultural rice straw waste was collected in plastic bag and dried at room temperature. Then 25 of the agricultural cellulosic residuce was ground into powder using a blender. The sterilization of agricultural residuce powder was taken at 121˚C for 15 minutes and then dried at 40˚C in oven. The dried rice straw was treated with 2M, 3M, 5M (HCl and NaOH) for overnight. After overnight treatment, the rice straw powders were washed with H_2O for many times to neutralize the pH 6.5-7.5 and then dried at 40˚C in the oven.

Screening of cellulose production activity: The screening for the cellulolytic activity was conducted by using Congo red dye[15]. The isolated *Actinomycetes* strains were screened for the presence of cellulase enzymes with various substrates such as CMC, cellulose and pretreated rice straw. In screening process, all the isolated strains were inoculated on specific media by spot inoculation method to screen the cellulolytic activities with different substrates. The culture plates were incubated at 30ºC for 7 days. After incubation, the isolated *Actinomycetes* culture were flooded with Congo red solution (0.1w/v) for 15 minutes. After then the Congo red solution was discarded and de-stained with 1M sodium chloride solution for 10–15 minutes. The ratio of the clear zone diameter to colony dimeter was measured and calculated the cellulolytic index to select for the highest cellulolytic activity producer. The formula for the cellulolytic index was as follow:

$$\text{Cellulolytic index} = \frac{Clear\ zone\ diameter - Bacterial\ colony\ diameter}{Bactrial\ colony\ diameter}$$

Detection of Cellulase assay: Cellulase activity was assayed spectrophotometrically with 3,5-dinitrosalicylic acid through the determination of the amount of reducing sugar liberated from cellulose[16]. Bacterial cultures were centrifuged at 8000 rpm for 10 minutes. The culture supernatants were used for the assay of the extracellular enzyme. 1ml of citrate buffer (0.05M) containing was added to 0.5ml of culture supernatant at pH 4.8 in test tube. The mixture was incubated for reaction at 50℃ for 60 minutes in a water bath at 80-85rpm for taking reaction. After reaction period 3ml of DNS reagent containing 40% potassium sodium tartrate (Rochelle salt) solution was added and mixed. Then, all tubes were heated at 90^0C for 5-10 minutes in a vigorously boiling water bath to develop the red-brown color. After cooling at room temperature in a cold water bath, 0.2ml of mixture is added with 2.5ml of water and then the reducing sugar was measured by optical density method at 540nm.

Results and discussion

In the present study, twenty-one bacterial strains were cultured and isolated from various sources of soil under wood decaying matter, cow dung and vegetative fields around Mandalay Technological University campus. Among them, only eight selected strains were characterized as *Actinomycetes* according to their morphology and cultural characteristic as mentioned in Table-2. The sampling location and origin of the selected eight strains were shown in Table-1.

Table-1: Sampling location and origin of the bacterial isolates.

Isolates	Soil Sources	Sampling Location
W_1	Wood decaying matter	Mandalay Technological University (MTU)
W_2	Wood decaying matter	
M_1	Cow dung	
M_2	Cow dung	
V_1	Vegetable field	
V_2	Vegetable field	
V_3	Vegetable field	
V_4	Vegetable field	

According to the cultural characters, the selected eight strains processed different characteristics. The two isolates from wood decaying matter (W1, W2) were differed in form and color of the cultural characteristics. The isolates from cow dung were not differed in cultural form but differed in color. Although V3 isolated from vegetable field was differed in the form of culture from other strains, the remaining three isolates were not quite difference. But in the culture color, all four isolates from vegetative field were differed from each other.

The selected eight *Actinomycetes* were biochemically characterized for some biochemical test including various sugar fermentation patterns and the results were shown in Table-3. Positive reactions were shown in Nitrate reduction, Catalase, Starch hydrolysis and Citrate utilization tests by all bacterial isolates. In Gelatin liquefaction test, they all shown negative reaction. In carbohydrate fermentation tests, all isolates showed positive reaction to Glucose, Mannitol, Sucrose, Dextrose and lactose. However, V1 and V2 isolates showed negative reaction to Sorbitol, the remaining isolates showed positive reaction.

The medium for cellulase production usually contains cellulose-rich substrates as a carbon source because cellulases are inducible enzymes by the bacteria[17]. In this investigation, various carbon sources with different concentrations were determined their cellulolytic activity using plate screening assay and the results were shown in Table-4. By screening the cellulolytic activity using Congo-red medium, all strains were found to have cellulolytic activity. Among these eight strains, strain M_2 showed the highest cellulolytic index of 5.4 and 5 on 1% CMC and 0.5% cellulose substrates respectively. Moreover,

strains W_1, V_2 and V_3 also showed good result of 3.8, 4.2 and 4.2 on 1% CMC, and 5 and 4.6, 4.6 on 0.5% cellulose substrates. In the cellulolytic activity measurement on cellulose substrate, M2 shown the highest cellulolytic index 5.4 and it was nearly the same as the study by Gupta P. *et. al*[18] and higher than the study by Andri F. *et. al*[19].

Table-2: Cultural characteristics of *Actinomycetes* isolates.

Character	Isolates							
	W_1	W_2	M_1	M_2	V_1	V_2	V_3	V_4
Form								
Surface	Smooth	Rough	Rough	Rough	Smooth	Smooth	Rough	Smooth
Texture	Leathery	Leathery	Powdery	Powdery	Leathery	Powdery	Leathery	Powdery
Elevation	Flat	Raised	Raised	Raised	Raised	Raised	Flat	Raised
Colour								
Aerial Mycelium	Yellow	White	Gray	Cream-white green	Gray	White	Cream	Cream
Reverse side colour	Transparent	Yellowish brown	Yellow	Greenish gray	Gray	Yellow	Light yellow	Light gray
Diffusible	Transparent	Yellowish brown	Yellow	No pigment	No pigment	Yellow	No pigment	No pigment
Odour	Earthy odour	Earthy odour	Earthy odour	Earthy odour	Earthy odour	Earthy odour	Earthy odour	Earthy odour

Table-3: Biochemical characterization and carbohydrate fermentation of *Actinomycetes* isolates.

Biochemical Test	Isolates							
	W1	W2	M1	M2	V1	V2	V3	V4
Nitrate reduction	+	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+	+
Starch hydrolysis	+	+	+	+	+	+	+	+
Citrate utilization	+	+	+	+	+	+	+	+
Gelatin liquefaction	-	-	-	-	-	-	-	-
Mannitol	+	+	+	+	+	+	+	+
Glucose	+	+	+	+	+	+	+	+
Sucrose	+	+	+	+	+	+	+	+
Dextrose	+	+	+	+	+	+	+	+
Lactose	+	+	+	+	+	+	+	+
Fructose	+	+	+	+	+	+	+	+
Sorbitol	+	+	+	+	-	-	+	+

Table-4: Cellulolytic index of the selected *Acetomyces* strains on various substsrates.

Substrate	Cellulolytic Index of the isolated bacerial strains							
	M_1	M_2	V_1	V_2	V_3	V_4	W_1	W_2
0.5%Cellulose	3.8	5.4	3.8	4.6	4.6	4.2	5	3.8
1% CMC	3.8	5	3.4	4.2	4.2	4.2	3.8	3
2% Rice Straw (2MHCl)	1.4	2.4	1.2	1.6	1.8	2.6	1	1.6
2% Rice Straw (3MHCl)	1.4	1.2	1	2	1.2	1.6	0.8	1.4
2% Rice Straw (5MHCl)	1	1.2	0.6	0.8	0.6	1	1.2	0.6
2% Rice Straw (2MNaOH)	2.2	3	2.6	2.8	2.2	2.2	3.4	3
2% Rice Straw (3MNaOH)	1.2	1.8	0.8	0.8	2	0	1.2	0
2% Rice Straw (5MNaOH)	0.8	0.8	2.6	0.8	1.4	1.2	1.4	0.8

Soni et al. reported that cultivation of *A. fumigatus* produced the maximal amount of endoglucanase in rice straw when comparing different carbon sources[20]. Rice straw powders pretreated with various concentrations of hydrochloric acid and sodium hydroxide solvents were used as substrates to screen the cellulolytic activity of all eight *Actinomycetes* strains. M_2 and W_1 strains showed good activity of 1.2 cellulolytic index on 2% rice straw treated with 5M HCl, W_1 showed 3.4 and strains M_2 showed the cellulolytic index of 3 on 2% rice straw (2M NaOH), strains V_3 showed 2 cellulolytic index on 2% rice straw (3M NaOH), strains W_1, V_1 and V_3 showed 1.4, 2.6 and 1.4 on 2% rice straw (5M NaOH). From this result, 2M NaOH treatment of rice straw showed the higher cellulolytic index than the other concentration of NaOH treatment and HCL treatment.

Based on the results of qualitative screening of cellulolytic activity, W_1, V_1, V_3 and M_2 strains were selected for quantitative determination of reducing sugar analysis by DNS method. Results from pretreated rice powder informed that 2M NaOH should be used for the pretreatment of rice straw. Quantitative determination using DNS reducing sugar analysis was shown in Figure-1 and revealed that the strains W_1 and V_2 showed good results with reducing sugar productivity of 0.2100 mg/ml and 0.0672l mg/ml in degrading 1%CMC. However, M_2 showed the best result of 0.2857l mg/ml reducing sugar content 0.5% cellulose degradation. Strains V_2 produced best reducing sugar content 0.3781 mg/ml using 2% pretreated rice straw. In addition, strains M_2 and V_3 also showed the good cellulolytic activity to produce reducing sugar content of 0.2857 and 0.2941 mg/ml respectively on 2% rice straw pretreated with 2M NaOH.

Conclusion

In this experiment, cellulose enzyme activity was higher in carboxymethyl cellulose than in both cellulose and pretreated rice straw. According to the DNS method, V_2 was the best strain for producing cellulose activity after 7 days incubation in the substrate of rice straw pretreated with 2M NaOH. So, V_2 found to have the potential of cellulolytic activity to utilize the biofertilizer production in the future.

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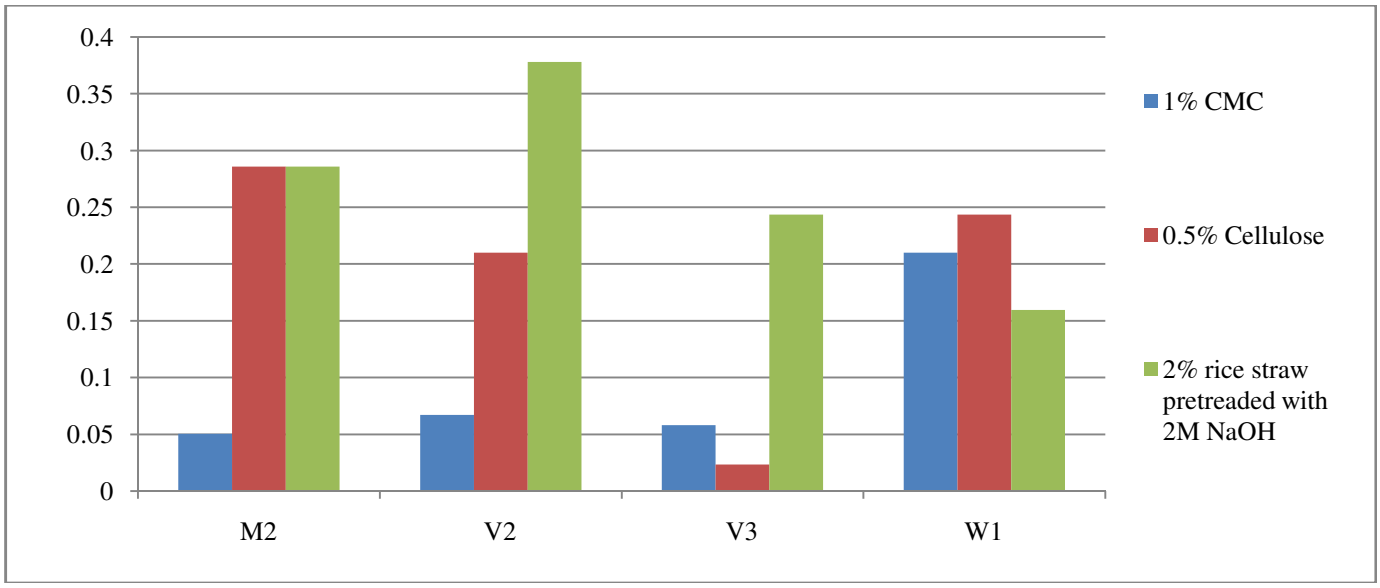

Figure-1: Reducing sugar content of selected four isolated strains after 7 days incubation using cellulose, CMC, and NaOH pretreated rice straw substrates.

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