International Journal of
Advances in Scientific Research and Engineering (ijasre)

E-ISSN : 2454-8006

DOI: 10.31695/IJASRE.2020.33679

Volume 6, Issue 1
January - 2020

Saccharification of Banana Agro-waste by Isolated Cellulolytic Fungi

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ABSTRACT

Lignocellulose is the most abundant and renewable biomaterial on the earth. Conversion of lignocellulosic biomass into fermentable sugars by cellulase enzymes (Saccharification) has significant advantages over other alternative energy production strategies. Although both bacteria and fungi can produce cellulase enzymes, the latter has potential in producing more efficient cellulases. This study was initiated with isolation and screening of cellulolytic fungi from six places of banana growing fields. Six fungal isolates were obtained in this research and two isolates showed cellulolytic activity according to plate screening assay. By examining their microscopic morphology, these two isolates were belonging to two genera i.e. Penicillium and Trichoderma. Morphological observation was done on various media. The glucose production patterns of these two cellulolytic fungi were investigated in two broth cultures with different carbon sources. One culture medium contained cellulose and another one contained dried and ground banana psuedostem powder as sole carbon sources. A glucose standard curve was constructed to estimate the glucose concentration in the broth culture media of the two fungi. Along the 20-day period of incubation, Penicillium sp. produced the most amount of glucose,440.2651µg/mL, at 6th day in cellulose media. In banana powder media, glucose concentration in the culture of Trichoderma sp.,288.1893 µg/mL, at 8th day was more than that in the culture of Penicillium sp. during 20 days of investigation. Knowing the glucose production time and amount of cellulolytic fungi would be useful for the next upcoming research works.

Key Words: *Cellulolytic Fungi, Trichoderma sp., Penicillium sp.*

1. INTRODUCTION

This cellulases can be found in application of many fields, such as animal feeding, brewing and wine, food, textile and laundry, pulp and paper industries. Moreover, the growing interest toward the conversion of lignocellulosic biomass into fermentable sugars has generated an additional request for cellulases and their related enzymes. In fact, bioconversion of biomass has significant advantages over other alternative energy production strategies because lignocellulose is the most abundant and renewable biomaterial on our planet. The cellulase enzymes can be grouped into three different enzymes: β-1,4-endoglucanase (EC 3.2.1.4), β-1,4-exoglucanase (EC 3.2.1.91) and cellobiase (EC 3.2.1.21), that are produced by many microorganisms including bacteria and fungi [1].

The raw material needs to be cheap to produce cellulase economically. There are many types of low-cost carbon sources that could be used for cellulase production, such as sugar cane bagasse, banana agro-waste, sugar cane straw, wheat straw, wheat bran, corn cobs, etc., reducing the costs effects and being friendly environmentally [2]. The humus-rich soil is known to be a good source for isolating cellulolytic microbes. In this study, sample collection for isolation of cellulase producing fungi was done from banana growing fields in order to be sure that the isolated cellulolytic fungi might use banana agro-waste as sole carbon source in further investigation. After that, isolation, identification and screening of cellulolytic fungi were done. Cellulolytic fungi were morphologically characterized and their cellulase production activities were quantitatively determined.

2. MATERIALS AND METHODS

2.1 Collection of Soil Samples

Soil samples were collected from six fields at Patheingyi township, Mandalay, where banana has been grown for many years.

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2.2 Isolation of Fungi from Soil Samples

Firstly, six soil samples were serially diluted to 10^{-5} by using distilled water and then incubated on Shawky and Hickish enrichment medium at 28-30ºC for 4 days. To be sure the purification of the fungal cultures, morphologically different colonies were inoculated again on the same media and incubated for 7 days.

2.3 Primary Screening for Cellulolytic Activity by Plate Assay

Pure fungal cultures were inoculated and incubated on Shawky and Hickish enrichment medium containing 0.5% cellulose as carbon source at 28-30ºC for 7 days. The ingredients per liter are: cellulose 5g; NaCl 3g; $(NH_4)_2SO_4$ 1g; KH_2PO_4 0.5g; K_2HPO_4 0.5g; $MgSO_4$ 0.1g; $CaCl_2$ 0.1g; yeast extract 0.25 g; agar 20g and pH 5.5 [3]. After incubation period, the cultures are flooded with 1% congo red solution for 30 minutes and then destained with 1M NaCl for 20 minutes. Clear zone formation was identified as having the cellulolytic activity.

2.4 Identification of Purified Cellulolytic Fungi

Fungal isolates that showed cellulolytic activity were incubated on different media and they were identified based on colony morphology on media and microscopic characteristics according to [4] and [5].

2.5 Preparation of Banana Powder

Firstly, psuedostem of banana from local farm after harvesting was chop into pieces, washed, air-dried and ground. After that, the resulted powder was sieved through different mesh sizes using sieve shaker. The particles with the size of -75+50 µm were used along the experiment.

2.6 Construction of the Glucose Standard Curve

Six test tubes were taken and labeled as blank and 1 to 5. Glucose standard solution of 1mg/mL was prepared and dilution was done by transferring respective amount of glucose from the standard glucose solution and adjusting it to a total volume of 2000 mL by adding distilled water as mentioned in Table 1. After that, 2 mL of dinitrosalicylic acid (DNS) reagent solution (DNS 1g, sodium metabisulfite 0.05g, NaOH 1g, distilled water 100mL) was added to all the six test tubes and mixed well. The test tubes were kept in heating block at 100°C for 10 minutes. And then, the test tubes were cool to room temperature in cold water and each were added 1 mL of 40% potassium sodium tartrate solution and mixed well. Finally, absorbance of the blank test tube was measured by spectrophotometer at 540 nm and made it zero, and measuring the absorbance of other 5 test tubes was followed [6] [7]. And then, the glucose standard curve was constructed.

Table1. Dinitrosalycylic Acid (DNS) Method

Concentration of standard glucose solution	1mg/mL					
Tube No.	Blank	1	2	3	4	5
Volume of standard glucose taken (µL)	0.0	200	400	600	800	1000
Volume of distilled water added (µL)	2000	1800	1600	1400	1200	1000
Vol. of DNS added (mL)	2	2	2	2	2	2
Keep in heating block at 100°C for 10 minutes						
Vol. of 40% Rochell's salt added (mL)	1	1	1	1	1	1
Observed UV absorbance at 540 nm						

DOI: 10.31695/IJASRE.2020.

2.7 Qualitative Examination for Cellulase Production of the Two Fungi

The two fungi were incubated on the basal media containing: CMC 10g/L, NaNo3 10g/L, K2HPO4 6.5g/L, yeast extract 0.3g/L, KCl 6.5g/L, MgSO4 2g/L, agar 17.5g/L [8]. After incubation of 5 days, the plates were flooded with 1% Congo red solution for 30 minutes and rinsed with distilled water. And then, they were added with 1 M NaCl 5mL and left for 5 minutes. After discarding NaCl solution and rinsing the plates with distilled water, 5% acetic acid was added and left for 5 minutes again and then halo zone formation was observed [9].

2.8 Preparation of Starter Culture

The basal medium was also used as starter culture to which cellulose 10g/L was added for carbon source. MnSO4 2g/L, ZnSO4 3g/L and NaMo4 0.2g/L were supplemented to the media. The pH was adjusted to 5 before autoclaving at 121°C and 15 psi for 15 minutes and then Streptomycin sulphate 30mg/L was added to prevent contamination. After inoculation of the two cellulolytic fungi to two flasks each containing basal media, they were incubated in waterbath shaker at 30±2°C for seven days to use as starter cultures.

2.9 Evaluation of the Growth of the Two fungi

The two cellulolytic fungi were incubated on the two supplemented basal media, one with cellulose 10g/L and another with banana powder 10g/L, with agar 17.5g/L at 30±2°C for six days.

2.10Incubation of the Two Cellulolytic Fungi

Total four Erlenmeyer flasks each containing 300 mL of supplemented basal media were prepared. The two flasks contained cellulose 10g/L and another two flasks contained banana psuedostem powder 10g/L as carbon sources respectively. The media at pH 5 were autoclaved and added with Streptomycin sulphate 30mg/L. After that, each 10 mL of media broth from four flasks were transferred into autoclaved test tubes under aseptic condition. There were two groups of sixty test tubes, totally 120, with two different carbon sources. Each 0.2 mL of the two starter cultures were separately inoculated into the test tubes of the two media groups and incubated in a waterbath shaker at 30±2°C. There were four groups of 30 test tubes made by two carbon sources and two fungal cultures.

2.11Investigation of Glucose Production by DNS Method

Starting from the 4th day, each three test tubes from four groups were collected and these cultures were centrifuged at 5000 rpm for 15 minutes. The media without any inoculation was autoclaved at 121°C, 15psi for 15 minutes and used as blank after being centrifuged. After that, each 2 mL of supernatants were mixed with 2 mL of DNS solution and placed in heating block at 100° C for 10 minutes. And then, the mixtures were cool in cold water and added 1mL of 40% sodium potassium solution. Their UV absorbance was recorded with spectrophotometer at 540 nm. Calculation of UV absorbance to glucose concentration was done by the use of glucose standard curve. The means of triplicate results were recorded along the 20-day period.

3. RESULTS AND DISCUSSIONS

Six fungal growths were detected on the isolated media and only 2 colonies showed cellulolytic activity. Although the growth of these two cellulolytic fungi were obvious on every media, morphological appearances were different based on the media on which they were incubated. Examining the morphology of the growth on various media and investigating under light microscope revealed that the two fungi that showed cellulolytic activities were *Penicillium* sp. and *Trichoderma* sp. respectively. *Penicillium* sp. colonies were dark greenish with white boundary on media. In microscopic view, *Penicillium* sp. have the vegetative body or mycelium that gives rise to simple and long conidiophores which branch at about two thirds of the way to the apex, in a fashion that's typical of a broom. The conidiophores branches terminate in a cluster of conidiogenous cells as shown in figure 7. *Trichoderma* sp. colonies were white in early and later changed to light green. In microscopic view, this species has branched conidiophores that cluster into fascicles, broad and straight/flexuous branches as shown in figure 8. Evaluation of DNS method resulted a glucose standard curve and that was used in calculation of glucose concentration from UV absorbance of fungal cultures. Although there were many other assays to determine the glucose concentration in a certain solution, DNS method was one of the most reliable and rapid methods and therefore it was selected to estimate the glucose concentration in the fungal cultures. In evaluation of the growth, after 6-day incubation period, the two fungi showed the growth on the media with two different carbon sources. But the growth of both fungi on the media with cellulose as carbon source was lesser than the one with banana powder as carbon source (figure 10 and figure 11). Both fungi showed halo zones around the fungal growth for qualitative cellulase production. However, morphological observation could not estimate the release of glucose units in the cultures. During the 20-day period of incubation in cellulose broth culture media, glucose concentration in the culture of Penicillium sp. was more than that in the culture of Trichoderma sp. The highest glucose concentration, 440 µg/mL, was detected at 6th day in the culture of

DOI: 10.31695/IJASRE.2020.

Penicillium sp. along the experiment while Trichoderma sp. could produce 327 µg/mL at 18th day (figure 12.). The former did not only take less time but also could produce more amount of glucose than the latter. In banana powder (Bp) media, 288 µg/mL of glucose was observed in the culture of Trichoderma sp. at 8th day and it was the most amount of glucose produced by both fungi throughout the 20 days (figure 13). For both cellulolytic fungi, in comparison of cellulose media and banana powder media, the glucose concentration in cellulose media was more than that in the banana powder media along the experiment (figure 14 and figure 15). One of the reasons for less glucose concentration in banana powder media might be the lignocelluloses contained in banana pseudostem that could not easily be degraded by cellulolytic fungi.

Fig.1 7-day-old culture of *Penicillium* sp. on PDA medium

Fig. 2 7-day-old culture of *Trichoderma* sp. on PDA medium

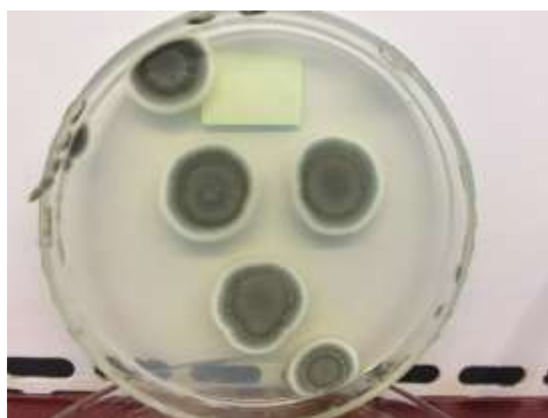

Fig.3 7-day-old culture of *Penicillium* sp. on Czapek-Dox medium.

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Fig.4 7-day-old culture of *Trichoderma* sp. on Czapek-Dox medium

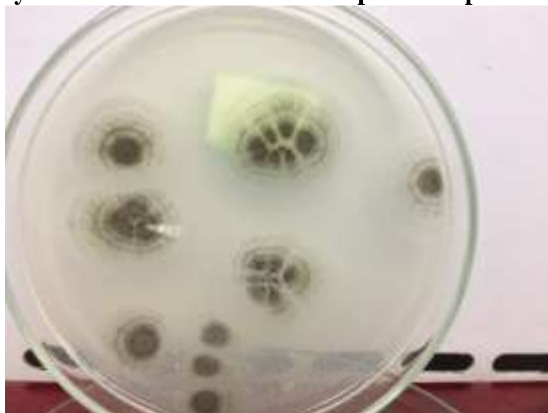

Fig.5 7-day-old culture of *Penicillium* sp. on Shawky and Hickish medium

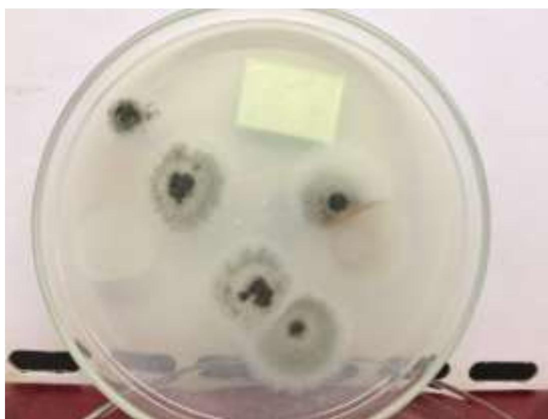

Fig.6 7-day-old culture of *Trichoderma* sp. on Shawky and Hickish medium

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Fig.7 Microscopic view of *Penicillium* sp. that showed cellulolytic activity

Fig.8 Microscopic view of *Trichoderma* sp. that showed cellulolytic activity

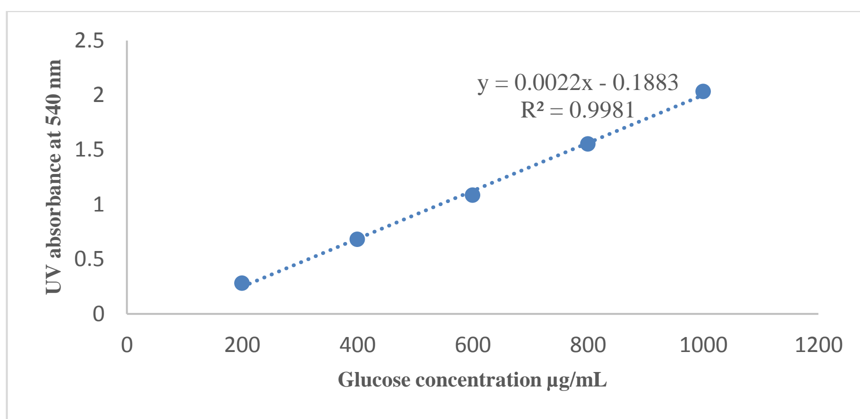

Fig.9 Glucose standard curve by DNS method to estimate the glucose concentration of fungal cultures

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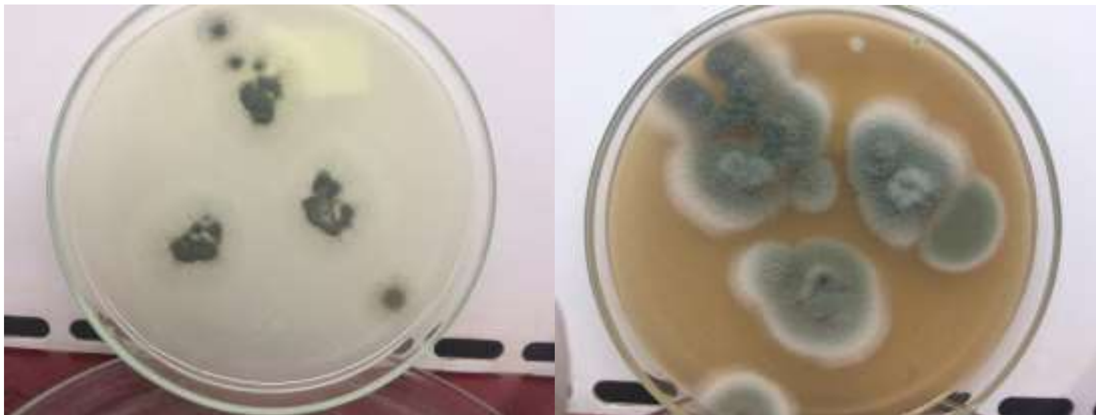

Fig.10 The growth of *Trichoderma* sp. on the media with cellulose and banana powder as carbon sources

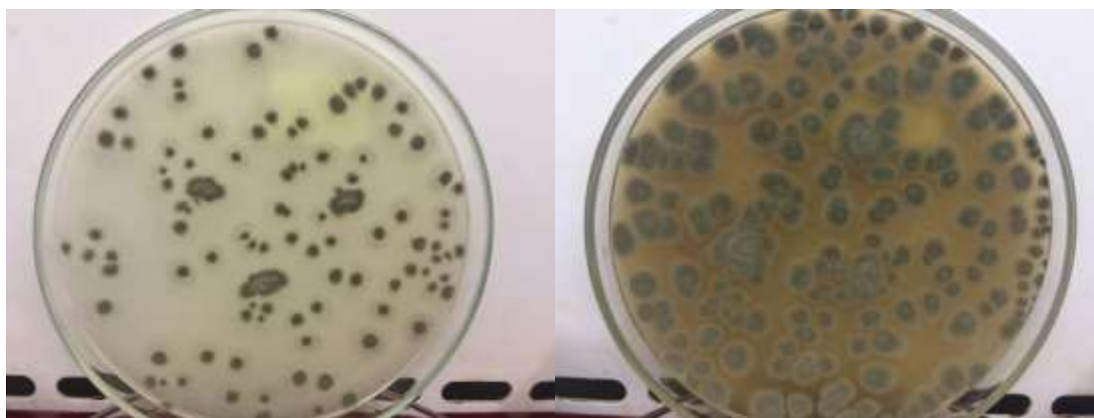

Fig.11 The growth of *Penicillium* sp. on the media with cellulose and banana powder as carbon sources

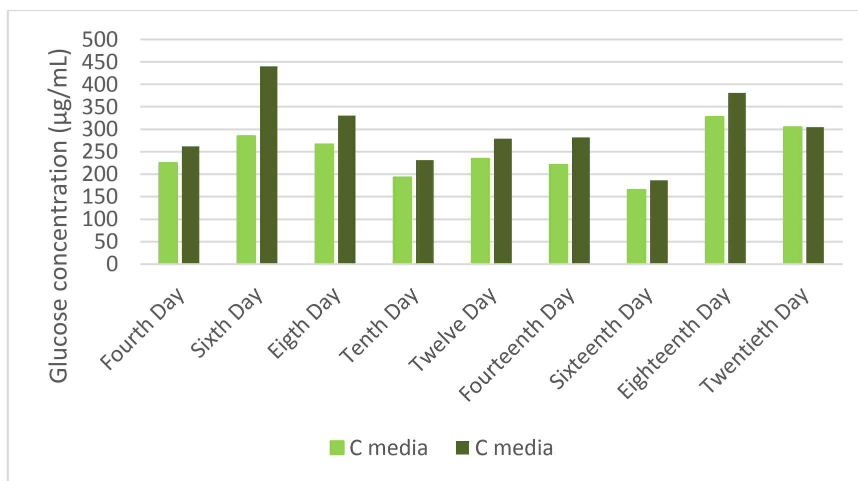

Fig.12 Glucose production pattern of *Trichoderma* sp. and *Penicillium* sp. during 20-day incubation period in cellulose(C) media

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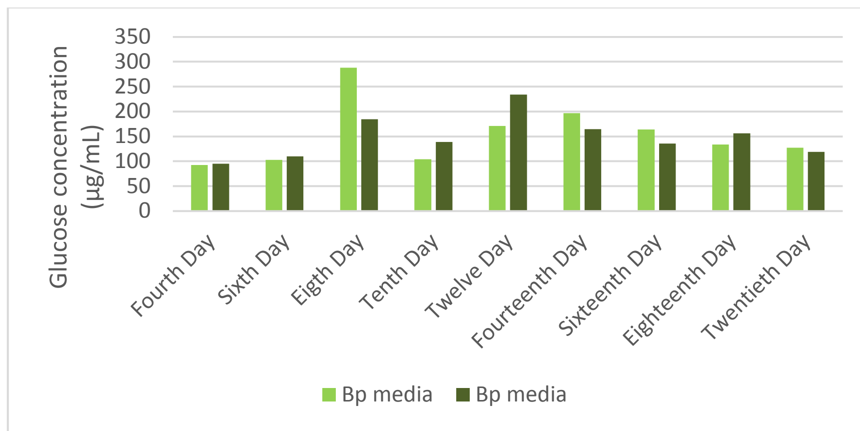

Fig.13 Glucose production pattern of Trichoderma sp. and Penicillium sp. during 20-day incubation period in banana powder (Bp) media

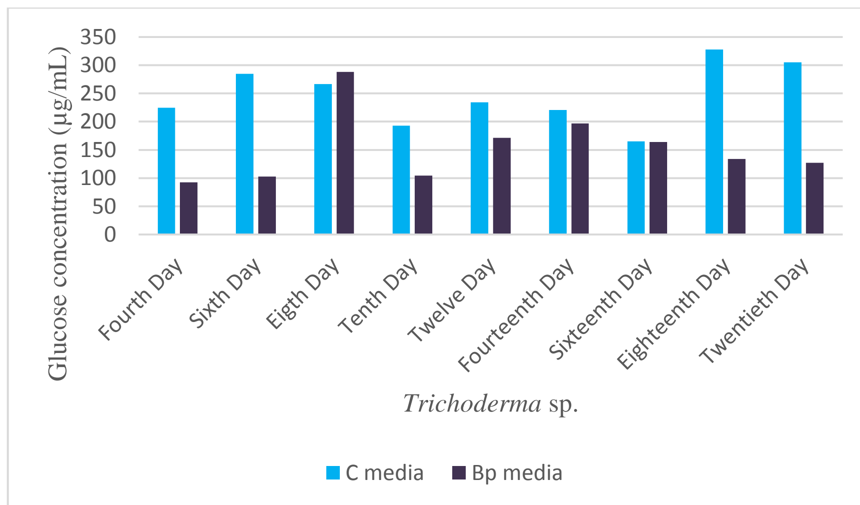

Fig.14 Glucose production pattern of *Trichoderma* sp. during 20-day incubation period in cellulose(C) media and banana powder (Bp) media

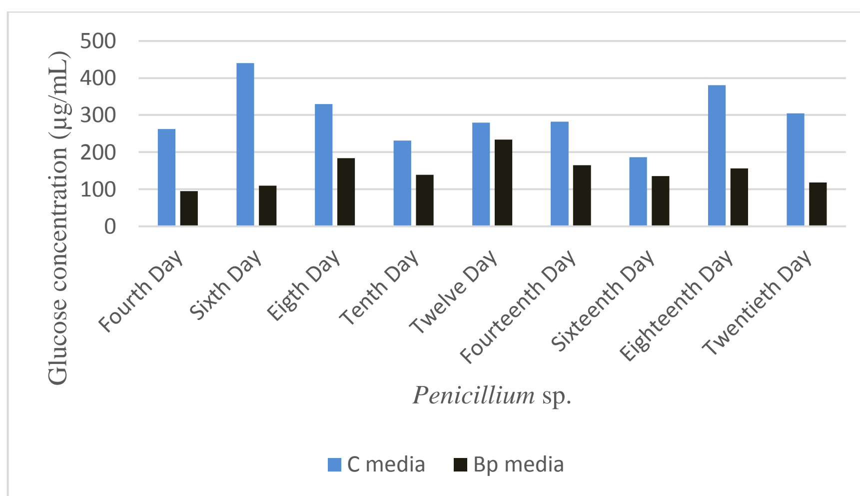

Fig.15 Glucose production pattern of *Penicillium* sp. during 20-day incubation period in cellulose media and banana powder media

4. CONCLUSION

Among the six isolated soil samples, two isolates showed cellulolytic activities. Morphological appearance of these two isolates were varied depend on different media. The glucose standard curve constructed by DNS method was reliable to estimate the glucose production in fungal cultures. Both of the two cellulolytic fungi produced more amount of glucose in broth cultures

DOI: 10.31695/IJASRE.2020.

with cellulose as carbon source than the ones with banana powder as sole carbon source. Banana powder was not given any pretreatment such as acid hydrolysis and therefore the lignocelluloses contained in the banana powder could not be easily degraded by cellulase produced from the two fungi. The maximum glucose concentration was observed in the culture of *Penicillium* sp. in cellulose media at 6th day while the *Trichoderma* sp. could produce the most glucose concentration in banana powder media at 8th day along the 20-day experiment. Therefore, in this research, *Trichoderma* sp. showed better performance to produce glucose by using banana agro waste than *Penicillium* sp. although the latter was better at glucose production using cellulose as carbon source than the former.

ACKNOWLEDGMENT

First and foremost, the author would like to express the highest gratitude to Prof. Dr. Myo Myint, professor and Head, Department of Biotechnology, Mandalay Technological University, for his precious permission and guidance.

The author would like to express special thanks to Dr. Tin Tin Hla, professor, Department of Biotechnology, Mandalay Technological University for her invaluable help and support.

Many thanks are due to the colleagues from the Department of Biotechnology, Mandalay Technological University for their help and encouragement to finish this work.

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