EFFECT OF CARBON AND NITROGEN SOURCES ON ANTIBACTERIAL METABOLITE PRODUCTION BY ENDOPHYTIC FUNGUS *ASPERGILLUS CANDIDUS* AGAINST *AGROBACTERIUM TUMEFACIENS*

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Abstract

In present study, endophytic fungus was isolated from the petiole of *Vigna mungo* (L.) Hepper in Thae Phyu Village, Hinthada Township, Ayeyawady Region during June in 2018. The endophytic fungus was isolated by surface sterilization method. The effect of various carbon and nitrogen sources were observed for the colony characters, growth condition and maximum antibacterial metabolite production of isolated fungus. The isolated fungus was identified as *Aspergillus candidus* based on morphological-microscopical characters and references key. The antibacterial activities of *Aspergillus candidus* were studied by agar well diffusion assay method. The result showed that maximum production of antibacterial metabolite (20.69 mm) was obtained when medium was supplemented with rice powder as carbon source. Fermentation medium containing yeast extract as nitrogen source showed the highest antibacterial metabolite (22.80 mm) production. This study reveals that endophytic fungus serves as a potential source for the production of an effective compound.

Keywords: Endophytes, *Aspergillus candidus*, Antibacterial activities

Introduction

Plants are a tremendous source for the discovery of new products of medicinal value for drug development. Today several distinct chemicals derived from plants are important drugs currently used in one or more countries in the world. The evolving commercial importance of secondary metabolites has in recent years resulted in a great interest in secondary metabolism, particularly in the possibility of altering the production of bioactive plant metabolites (Alexopoulos *et al*., 1996).

Carbon is the most important medium component as it is an energy source for the microorganisms and plays an important role in the growth as well as in the production of primary and secondary metabolite. The rate at which the carbon source is metabolized can often influence the formation of biomass and /or the production of primary or secondary metabolites. Like carbon, the selection of nitrogen source and its concentration in the medium

also play a crucial role in metabolite production. The microorganism can utilize both inorganic and/or organic sources of nitrogen. Use of specific amino acids can increase the productivity in some cases and conversely, unsuitable amino acids may inhibit the synthesis of secondary metabolites (Marwick *et al*., 1999).

For medium formulation, carbon and nitrogen sources showing enhancement effect on the desired metabolite production in supplementation experiments are generally tried to be used as a whole carbon and nitrogen source (Niwas *et al*., 2013).

Given this in view, nutrients type and their concentrations in the medium play an important role in commencing the production of primary and secondary metabolites as limited supply of an essential nutrient can restrict the growth of microbial cells or product formation. Generally, carbon

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and nitrogen sources present in the medium can influence the metabolite production (Singh *et al*., 2017).

In present study, the influence of different carbon and nitrogen sources on the colony morphology, growth conditions and antibacterial metabolite production by *Aspergillus candidus*. The aim and objectives of the study are to take a comprehensive look at most recent research on antibacterial metabolite produced by endophytic fungus *Aspergillus candidus.*

Materials and Methods

Collection of plant specimen

Vigna mungo (L.) Hepper plant sample was collected at Thae Phyu Village, Hinthada Township, Ayeyawady Region during June in 2018. Healthy plant sample was placed in plastic bags and labelled with date and site of collection until isolation procedure was completed. The identification of these plant was referred by Dassanayake, 1991; Wu *et al*., 2010 and Heuze´ *et al*., 2016.

Isolation of antibiotic producing fungus

The endophytic fungus was isolated from petiole of *Vigna mungo* (L.) Hepper by using surface sterilization method (NITE, 2004). Plant sample was washed thoroughly in running tap water and air dried before it was processed. The material was then surface sterilized by immersing them sequentially in 70% ethanol for 1 minute and then, also immerse 10% sodium hypochloride for 1 minute and rinsed thoroughly with sterile distilled water. Then, with a sterile scalpel, outer tissues were removed and the inner tissues of 0.5 cm size were carefully dissected and placed on petri-disc containing Czapek–Doz Agar (CZA) medium. The medium was supplemented with chloramphenicol to suppress bacterial growth. The Petri-disc was incubated at room temperature for three to seven days until fungal growth appeared.

Identification of antibiotic producing fungus

The morphological and microscopical characters of antibiotic producing fungus was observed by the methods of Ando and Inaba 2004, Ando 2006, Domsch *et.al.*, 1993 and Watanabe 2002 and 2010. Microscopical characters were studied by microscope (Olympus; Sato Shouji Microscope Attached Camera). Comparison of these characters with reference keys was undertaken to identify according to Ando and Inaba, 2004;Ando, 2006; Domsch *et al.*, 1993; Watanabe 2002 and 2010.

Carbon sources (each 1.0 g) such as rice powder, potato, tapioca powder, fructose, glucose, sucrose, maltose, oat, corn, lactose, glycerol, soluble starch, carrot were used with agar 1.8 g and DW 100 mL for studying the colony characters and growth conditions of selected fungus.

Nitrogen sources (each 1.0g) such as yeast extract, poly peptone, meat extract, peptone, malt extract, casein, peanut cake, soybean, gelatin, fish cake, potassium nitrate, ammonium sulphate and ammonium chloride were used with agar 1.8 g and DW 100 mL for studying the colony characters and growth conditions of selected fungus.

Test bacterial strain

The bacterial strain such as *Agrobacterium tumefaciens* (NITE 09678) was received from B.R.B.D.C (Biological Resources and Biotechnology Development Centre, Pathein University).

Screening for antibacterial activities (NITE, 2005)

The isolated fungus was grown on CZA medium at room temperature for 4 days. After incubation period, these fungus inoculated into the seed medium (glucose 2.5g, yeast extract 0.8g, $MgSO_4 7H_2O$ 0.01g, K_2HPO_4 0.01 g and DW 100 mL at pH 6.5) for 3 days at room temperature. After three days, the seed medium (2%) was transferred into the basal fermentation medium (glucose 1.0 g, yeast extract 0.5 g, Soluble starch 0.3 g, $MgSO_4 7H_2O$ 0.02 g, K_2HPO_4 0.01 g and DW 100 mL at pH 6.5) and carried out for 4 - 10 days and evaluated the antibacterial activities by agar well diffusion method.

Screening of antibacterial activities by agar well method (Collins,1965)

The antibacterial activities of *Aspergillus candidus* isolate was tested by using agar well diffusion method. Two days old culture test broth (0.1 mL) was added to 25 mL of assay medium and thoroughly mixed and poured into plate. After solidification, a well of about 8 mm with sterile cork borer was aseptically punched on each agar plate. The fermentation broth of *Aspergillus candidus* (20 µL) was carefully added into the wells and incubated at room temperature for 24 - 48 hours. The diameter of the zones of inhibition around each well was measured and recorded after 24 - 48 hours incubation.

Effect of different carbon utilization on fermentation

To determine the effect of carbon sources on antibacterial production in *Aspergillus candidus,* different carbon sources such as rice powder, potato, tapioca powder, fructose, glucose, sucrose, maltose, oat, corn, lactose, glycerol, soluble starch and carrot were employed. 100 mL of basal fermentation broth medium with different carbon sources (1%) in 250 mL conical flask that autoclaved at 121 ºC for 45 minutes. The inoculated flasks were incubated at room temperature for 4 to10 days under stationary condition. The fermented broth was tested by using agar well diffusion method for antibacterial activities against *Agro. tumefaciens*.

Effect of different nitrogen utilization on fermentation

The optimization of nitrogen source used in fermentation broth during antibacterial metabolite production was carried out by employing various nitrogen sources such as yeast extract, poly peptone, meat extract, peptone, malt extract, casein, peanut cake, soybean, gelatin, fish cake, potassium nitrate, ammonium sulphate and ammonium chloride. 100 mL of basal fermentation broth medium with different nitrogen sources (1 %) in 250 mL conical flask that autoclaved at 121 ºC for 45 minutes. The inoculated flasks were incubated at room temperature for 4 to10 days under stationary condition. The fermented broth was tested by using agar well diffusion method for antibacterial activities against *Agro. tumefaciens.*

Results

Collection of plant specimen

In present endeavor, plant sample of the *Vigna mungo* (L.) Hepper was collected from Thae Phyu Village (Hinthada Township), Ayeyawady Region.

Isolation of antibiotic producing fungus

In this research work, endophytic fungus was isolated from petiole of *Vigna mungo* (L.) Hepper. The endophytic fungus was isolated by surface sterilization method. The surface view is pale yellow in the center and edge white colour and reverse view is pale orange colour Figure 1.

Surface view Reverse view

Figure1 Colony morphology of isolated fungus on Czapek–Doz Agar (CZA) medium

Carbon sources utilization for growth

The effect of different carbon sources on morphological growth by isolated fungus is presented in figure 2. The results obtained in carbon sources study indicated that rice powder, potato, oat and carrot were excellent growth and tapioca powder was moderate growth and then fructose, glucose, sucrose, maltose, corn, lactose, glycerol and soluble starch showed poor growth Figure 2 and Table 1.

Culture on Rice powder Culture on Potato Culture on Tapioca powder

Culture on Fructose Culture on Glucose Culture on Sucrose

Culture on Maltose Culture on Oat Culture on Corn

Culture on Lactose Culture on Glycerol Culture on Soluble Starch Culture on Carrot

Figure 2 Morphological growth of isolated fungus on different carbon sources

Table 1 Growth of isolated fungus on different carbon sources

Sr. No.	Carbon sources	Growth
1.	Rice powder	6.1 cm (Excellent)
2.	Potato	6.2 cm (Excellent)
3.	Tapioca powder	3.6 cm (Moderate)
4.	Fructose	1.5 cm (Poor)
5.	Glucose	1.2 cm (Poor)
6.	Sucrose	1.0 cm (Poor)
7.	Maltose	2.0 cm (Poor)
8.	Oat	7.0 cm (Excellent)
9.	Corn	1.5 cm (Poor)
10.	Lactose	1.9 cm (Poor)
11.	Glycerol	1.0 cm (Poor)
12.	Soluble Starch	1.0 cm (Poor)
13.	Carrot	8.6 cm (Excellent)

1.0 to 3.0 cm - Poor 3.1 to 6.0 cm - Moderate 6.1 cm above – Excellent

Nitrogen sources utilization for growth

In the study on the utilization of nitrogen sources, moderate growth on yeast extract, poly peptone, meat extract, peptone, casein, peanut cake, soy bean, gelatin, fish cake and potassium nitrate; malt extract, ammonia sulphate and ammonia chloride indicated poor growth Figure 3 and Table 2.

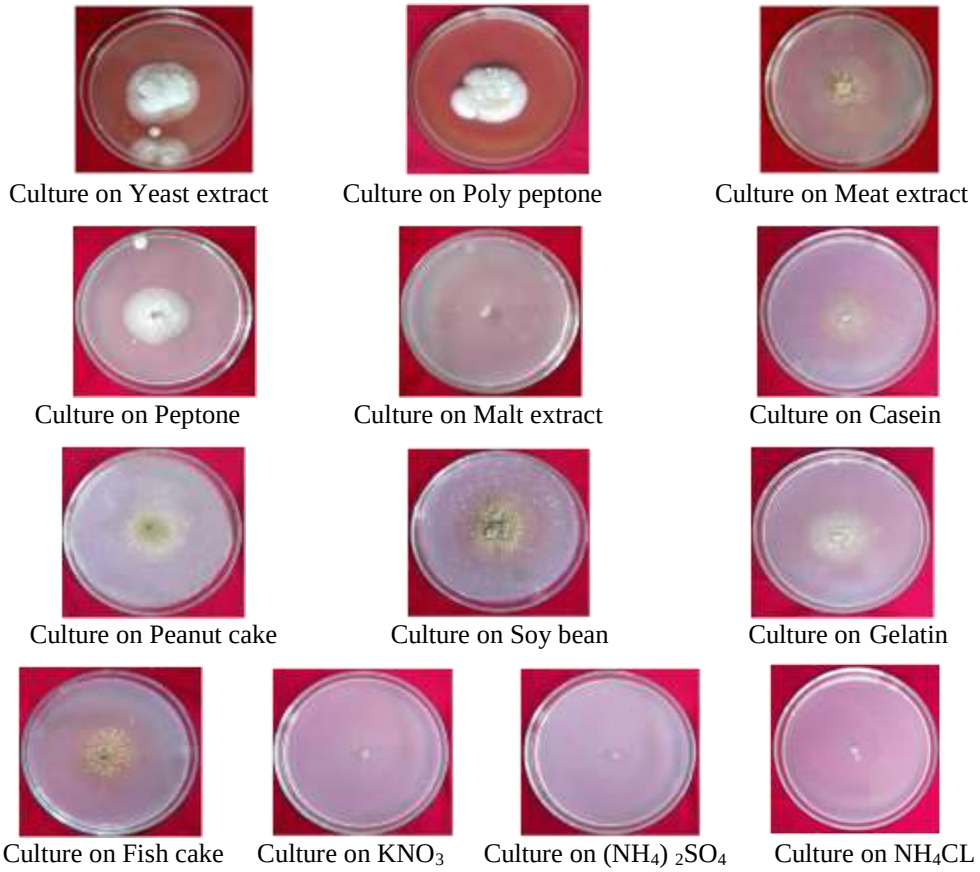

Figure 3 Morphological growth of isolated fungus on different nitrogen sources

Table 2 Growth of isolated fungus on different nitrogen sources

Sr. No.	Nitrogen sources	Growth
1.	Yeast extract	4.2 cm (Moderate)
2.	Poly peptone	4.3 cm (Moderate)
3.	Meat extract	4.1 cm (Moderate)
4.	Peptone	3.7 cm (Moderate)
5.	Malt extract	1.2 cm (Poor)
6.	Casein	3.4 cm (Moderate)
7.	Peanut cake	3.5 cm (Moderate)
8.	Soy bean	4.9 cm (Moderate)
9.	Gelatin	3.4 cm (Moderate)
10.	Fish cake	4.2 cm (Moderate)
11.	KNO_3	3.5 cm (Moderate)
12.	$(NH_4)_2SO_4$	1.2 cm (Poor)
13.	NH_4CL	1.0 cm (Poor)

1.0 to 3.0 cm - Poor 3.1 to 6.0 cm - Moderate 6.1 cm above – Excellent

Identification of antibiotic producing fungus

The colony morphologies of endophytic fungus from petiole of *Vigna mungo* (L.) Hepper, macroscopic and microscopic observations were identified by using references keys of Ando and Inaba, 2004; Ando, 2006; Domsch *et al*., 1993; Watanabe, 2002 and 2010. The isolated fungus was identified as *Aspergillus candidus* using colony morphology, colour of colony and the sporulating structure Figure 4.

Figure 4 Macroscopic and microscopic characters of isolated fungus

Effect of different carbon utilization on fermentation

Rice powder as a carbon sources resulted in the maximum antibacterial activities showed the highest inhibition zone reached (20.69 mm), followed by potato (20.35 mm), tapioca powder (19.75 mm), fructose (19.52 mm), glucose (19.05 mm), sucrose (19.04 mm), maltose (18.84 mm), oat (18.62 mm), corn (17.72 mm), lactose (17.36 mm), glycerol (16.66 mm), soluble starch (16.03 mm) and carrot (16.02 mm) Figure 5 and Table 3.

Rice powder Potato Tapioca powder

Fructose Glucose Sucrose

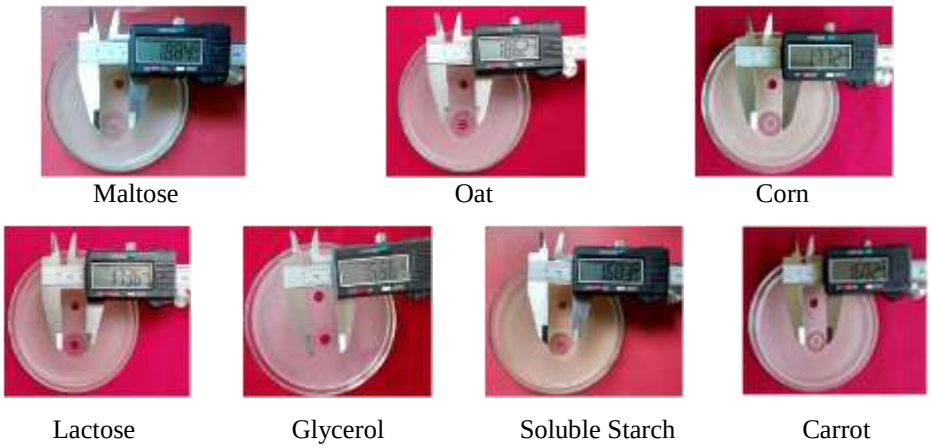

Figure 5 Anitibacterial activities of *Asp.candidus* on different carbon sources fermentation against *Agro.tumefaciens*

Table 3 Anitibacterial activities of *Asp.candidus* on different carbon sources fermentation against *Agro.tumefaciens*

Sr. No.	Carbon sources	Antibacterial Activities Inhibitory zone (mm)
1.	**Rice powder**	**20.69**
2.	Potato	20.35
3.	Tapioca powder	19.75
4.	Fructose	19.52
5.	Glucose	19.05
6.	Sucrose	19.04
7.	Maltose	18.84
8.	Oat	18.62
9.	Corn	17.72
10.	Lactose	17.36
11.	Glycerol	16.66
12.	Soluble Starch	16.03
13.	Carrot	16.02

Effect of different nitrogen utilization on fermentation

Furthermore, in the study of nitrogen sources, maximum antibacterial activities of yeast extract (22.80 mm), poly peptone (21.14 mm), meat extract reached (20.45 mm) followed by peptone (20.09 mm), malt extract (19.08 mm), casein (19.05 mm), peanut cake (18.94 mm), Soy bean (18.88 mm), gelatin (18.75 mm), fish cake (18.94 mm), potassium nitrate (18.74 mm), ammonia sulphate (17.50 mm), ammonia chloride (15.98 mm) were respectively Figure 6 and Table 4.

The maximum production of antibacterial metabolite as nitrogen sources (22.80 mm to 15.98 mm). There was a reduction in antibacterial activities when ammonia sulphate and ammonia chloride were used as nitrogen sources.

Figure 6 Anitibacterial activities of *Asp.candidus* on different nitrogen sources fermentation against *Agro.tumefaciens*

Table 4 Anitibacterial activities of *Asp.candidus* on different nitrogen sources fermentation against *Agro.tumefaciens*

Sr. No.	Nitrogen sources	Antibacterial Activities Inhibitory zone (mm)
1.	Yeast extract	22.80
2.	Poly peptone	21.14
3.	Meat extract	20.45
4.	Peptone	20.09
5.	Malt extract	19.08
6.	Casein	19.05
7.	Peanut cake	18.94
8.	Soy bean	18.88
9.	Gelatin	18.75
10.	Fish cake	18.94
11.	KNO_3	18.74
12.	$(NH_4)_2SO_4$	17.50
13.	NH_4CL	15.98

Discussion and Conclusion

Culture medium components such as carbon and nitrogen sources may effect metabolites production in isolated fungus. Carbon or nitrogen sources which are quickly metabolized will increase the mycelium growth but it potentially inhibits secondary metabolite production (Kumar *et al*., 2012).

In present research work isolated endophytic fungus *Aspergillus candidus* from the *Vigna mungo* (L.) Hepper plant showing considerable antibacterial activity against *Agrobacterium tumefaciens.* The antibacterial spectrum of the *Aspergillus candidus* was tested against *Agrobacterium tumefaciens* through agar well diffusion assay method.

In this study, different carbon and nitrogen sources supplement to the culture broth strongly influenced the growth and biosynthesis of active metabolite by *Aspergillus candidus.* On studying the effect of different carbon source, the results indicated that rice powder affected the antibacterial production by *Aspergillus candidus.* As Moe Moe Aye 2019 reports that rice power is the best other than carbon sources.

Other than the carbon, the source of nitrogen is important for the production of antibiotic substances. Nitrogen is known as one of major component of complex macromolecule within living organism. It is an essential component of amino acid required for biosynthesis of various bioactive compounds. In this study, medium with yeast extract as nitrogen source showed the highest activity followed by poly peptone. S Aung Myo Htay 2017 has reported similar results that promoted the biosynthesis of secondary metabolite.

The results of antibacterial susceptibility tests indicated that maximum antibacterial production was obtained in culture supplemented with rice powder as carbon source and yeast extract and poly peptone as nitrogen source. Studies using one single fungus cultivated under different culture conditions are not only suitable to produce different compounds, but also provide conditions to guide the production of a specific compound and become easier for the purification process. In conclusion, carbon and nitrogen sources play an important role for the production of various bioactive metabolites.

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