

Original Research Article

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Optimization of Growth Conditions of *Bacillus megaterium* for Antifungal Activities against Cocoyam Phytopathogens

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ABSTRACT

Keywords

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The biocontrol potential of *Bacillus megaterium* isolated from rhizosphere of turmeric plants was investigated in vitro against cocoyam pathogenic fungi. Antagonistic activity was examined under optimized pH (3.0, 4.0, 5.0, 6.0, 7.0 and 8.0), carbon sources (glucose, xylose, sucrose and lactose), incubation period (24hrs, 48hrs, 72hrs, 96hrs, and 120hrs) and temperature (20⁰C, 30⁰C, 40⁰C, 50⁰C, 60⁰C and 70⁰C) for *B. megaterium*. *Bacillus megaterium* induced the presence of an inhibition halo, with values of 16.89±0.57mm when tested in vitro against *Fusarium* spp. it also produced a zone of clearing of 13.57±0.57mm against *Aspergillus* spp. The *B. megaterium* strain was greatly influenced by nutritional factors. Maximal antagonistic activity of the isolate was observed after 96h of incubation with over 18.0mm zone of inhibition against *Fusarium* and 15.2mm against *Aspergillus* species. Glucose and Lactose were found to be the ideal carbon source over xylose and sucrose for the growth of *B. megaterium* in the present work. In this present investigation, we have reported a soil-borne bacterium *Bacillus megaterium* which is antagonistic to cocoyam phytopathogens, and could make a substantial contribution to the prevention of spoilage of cocoyam.

Introduction

The human population has been predicted to rise to 9.2billion people in 2050 (Popp *et al.*, 2013). Such a vast increase will result in substantial increase in demand for food supply. Tuber crops and overall crop yield have always been affected by phytopathogenic fungi. Fungal plant pathogens are accountable

for large amounts of both pre- and post-harvest food losses and in the absence of appropriate control measures, these losses would be expected to double (Glare *et al.*, 2012).

In recent years, among the most important factors limiting production of different crops are soil-borne plant pathogens including fungi

from genera *Pythium*, *Rhizoctonia*, *Fusarium*, *Verticillium*, *Phytophthora* spp, *Sclerotinia*, *Sclerotium*, and *Rosellinia* (Sosa *et al.*, 2008). To contain this problem, several techniques have been devised as a means of controlling these pathogens (Parra and Ristaino, 2001). Among them include the use of cultural practices and chemical control using synthetic fungicides. Environmental pollution issues arising from the use of these synthetic chemicals with adverse consequences such as toxicity to humans as well as resistance of some pathogens to these fungicides has spurred the need for a better environmental friendly method of arresting these fungal pathogens (Hernández-Castillo *et al.*, 2005). An alternative to reduce the effect of these plant pathogens is the use of antagonistic microorganisms such as: some species of the genus *Bacillus* which is recognized as one of the most effective biological control agent because of their properties on pathogens growth inhibition (Schisler *et al.*, 2004; Sid *et al.*, 2003).

Soil-borne bacteria that are antagonistic to plant pathogens could make a substantial contribution to prevention of plant diseases, and therefore represent an alternative to the use of chemical pesticides in agriculture (Walsh *et al.*, 2001). Due to their role in plant health and soil fertility, soil and the rhizosphere have frequently been used as a model environment for screening of putative agents for use in biological control of soil-borne plant pathogens.

Cocoyam (*Colocasia esculentus*) is one of the important crops in Nigeria. Nigeria leads its production with 3.7 million tonnes (MT) per annum. Current yield levels of the cocoyam production are low on a worldwide basis.

An appraisal of the major constraints on cocoyam production indicated that it is not due to lack of demand but losses due to field

and especially post-harvest deterioration (Nwachukwu and Osuji 2008). Management of postharvest diseases using microbial antagonists, natural plant-derived products and compounds that are generally recognized as safe has been demonstrated to be most suitable to replace the synthetic fungicides, which are either being banned or recommended for limited use (Sharma *et al.*, 2009; Talibi *et al.*, 2014).

The bacteria of the genus *Bacillus* have a great potential as a biological control agent because they keep their viability with long-term storage (Nagorska *et al.*, 2007; Ongena and Jacques 2008).

Biosynthesis of antibiotics from microorganisms is often regulated by nutritional and environmental factors. El-Banna (2006) reported that antimicrobial substances produced by bacterial species were greatly influenced by variation of carbon sources.

Several abiotic factors, such as pH and temperature, have been identified as having an influence on antibiotic production from bacteria. Antifungal peptides produced by *Bacillus* species include mycobacillins, surfactins, mycosubtilins, and fungistatins (Sadfi *et al.*, 2001). It can produce a wide range of other metabolites, including chitinases and other cell wall-degrading enzymes, volatiles, and compounds that elicit plant resistance mechanisms (Sadfi *et al.*, 2001). Volatile metabolites produced from *Bacillus* sp. have been reported to inhibit mycelia growth of *Fusarium oxysporum*.

This study was therefore aimed at isolating, characterizing and identifying *Bacillus* species from the rhizosphere soil of turmeric plant with antifungal potentials against cocoyam phytopathogens as well as to carry out optimization studies on the best conditions

necessary for antifungal activities of *Bacillus* species with very high antifungal potentials.

Materials and Methods

Soil samples were randomly collected from the rhizospheric portions of turmeric plants. All samples were carefully collected by scraping the soil surface with a sterile scoop and were transferred to the laboratory in sterilized polyethylene bags.

One gram of each soil sample was suspended in 9 ml of sterile distilled water to obtain an appropriate dilution and plated on nutrient agar (NA) modified with 3% glycerol to become glycerol modified nutrient agar (GMNA) at 30°C for 48hours. Once there was establishment of growth, subcultures were made from different distinct colonies based on morphological differences to obtain pure cultures of the different isolates. The isolated bacterial strains were stored in agar slants for further study.

Pathogenicity Test

The deliberate infestation Techniques (DIT) described by Alimi *et al.*, (2012) was adopted. Healthy cocoyam corms were surface disinfected and with the aid of a flamed 5mm cork borer, holes were bored on the corm flesh and discs cuts of each isolate taken from 48hours old culture were put inside the bored hole and covered with the removed flesh. The point of infection was sealed with sterile paraffin. The inoculated corms were incubated for 10-14days. They were observed for signs of rot including softening, dry-up, discoloration, exudates and offensive odours.

After incubation, the corms were cut open along the line of inoculation and isolation was made again. Organisms which caused rots measuring 7 to 10mm were considered as pathogenic.

Screening on fungicidal activities of *Bacillus* species on cocoyam phytopathogens

This was done according to the methods stated by Aboy-Aly (2008). Each *Bacillus* isolates was cultured in nutrient broth for 48hours at room temperature. The culture broths were centrifuged at 3000rpm for 10 to 15 minutes. The residue (bacterial cells) were then diluted to the 4th diluent to give a suspension of about 1×10^8 /ml with optical density of 0.45 at 610nm wavelength as described by Haripras and Niranjana (2008). The suspensions were used in the agar well diffusion techniques. Shallow narrow wells were bored at distance of 2cm from the edge of the Petri dish and opposite sides of the plates.

One ml of the bacterial suspension was poured into the wells bored on the surface of sterile nutrient agar plates. After 24 hours, the plates were flooded with 1ml of a 48 hours broth culture of test organisms (cocoyam phytopathogens) and incubated at 30°C for 5 days.

The presence of clear zone around the wells containing *Bacillus* isolates was indicative of a positive antifungal activity against the cocoyam pathogen.

Optimization of the *Bacillus* isolate for antifungal activity

In order to investigate the best conditions for antifungal activities of the selected *Bacillus* isolate, the role of different environmental factors such as carbon source, pH, temperature, incubation time were determined. This method below follows the early findings of Awais (2007).

Nutrient media adjusted to varying pH (3.0, 4.0, 5.0, 6.0, 7.0 and 8.0) using different buffers were inoculated with 0.1ml of overnight broth culture of test organism and

incubated at 30°C and the antifungal activity was determined using the agar well diffusion method. Similarly, test tubes containing 10ml of nutrient broth were each inoculated with 0.1ml of overnight culture of the test organism. Incubation was done at 20°C, 30°C, 40°C, 50°C, 60°C and 70°C in order to determine the optimal temperature for the antifungal activity of *Bacillus* isolate. After 24hours, the antifungal activity was determined using agar well diffusion method. Antifungal activities were also evaluated after 24hrs, 48hrs, 72hrs, 96hrs, and 120hrs of incubation.

Equally, different carbon sources (1% glucose, 1% xylose, 1% sucrose and 1% lactose) were separately added into a basal medium containing 5% NaCl, 2% tryptone, 0.15% MgSO₄, 0.15% K₂HPO₄ and 3% glycerol. They were inoculated with 0.1ml of an overnight broth culture of the test organisms and incubated for 24hrs at 30°C with an initial pH of 6.5. The antifungal activity was determined using agar well diffusion method described earlier.

Results and Discussion

The results of antagonistic potentials of *Bacillus megaterium* strains on the growth (in vitro) of different fungal pathogens of cocoyam are shown in table 1. *Bacillus megaterium* and *B. subtilis* induced an inhibition halo of 15.66mm and 13.33mm respectively on *Aspergillus* species; 16.89mm and 16.26mm on *Fusarium* species. *Bacillus megaterium* had a higher antagonistic activity than the other species with a diameter zone of inhibition of 13.57mm against *Penicillium* species. The highest inhibition halos produced by *B. megaterium* and *B. subtilis* against *Fusarium* species were observed to be significantly different from each other (p<0.05). The findings of this study were also in agreement with the reports of Madhaiyan *et*

al., (2010) and Zhang *et al.*, (2012), who found that strains of *B. methylotrophicus* have a high antagonistic activity against a wide diversity of phytopathogens fungi. Kumar *et al.*, (2012) reported the antagonistic activity of *Bacillus* strain, which strongly inhibited the growth of several phytopathogens such as *Macrophomina phaseolina*, *Fusarium oxysporum*, *F. solani*, *Sclerotinia sclerotiorum*, *Rhizoctonia solani* and *Colletotricum sp. in vitro*.

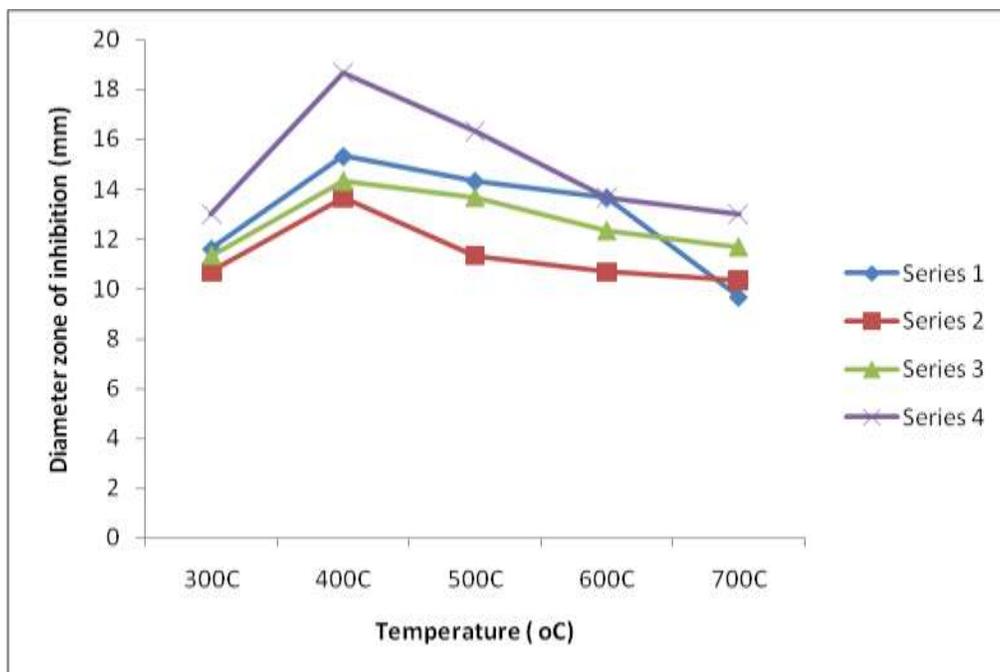
In this study, the *in vitro* inhibition of the growth of the phytopathogens by *B. megaterium* seems to indicate that cell wall hydrolytic enzymes might be responsible for the inhibitory activity (cell lysis). Production of extracellular enzymes by biocontrol bacteria is a well-documented phenomenon that is thought to be involved in lysis of the cell wall of phytopathogenic fungi (Kumar *et al.*, 2012; Kuddus and Ahmad, 2013). Among *Bacillus* spp., *B. subtilis* and occasionally, *B. megaterium*, *B. cereus*, *B. pumilus* and *B. polymixa* have been studied as biocontrol agents. In this respect, microbial bio-control agents have shown a great potential as an environmentally friendly alternative to the use of synthetic pesticides (Kotan *et al.*, 2009). The degradation of fungal cell walls with the production of hydrolytic enzymes of bacterial isolates has been described as one of the most important mechanisms for biocontrol of phytopathogenic fungi (Weller 2007; Elshafie *et al.*, 2012). An optimum pH (5–7 as was observed in this study) promoted cell growth and it can thus be seen that pH plays a key role in enzyme production for enhanced antagonistic activity. Earlier studies reported that near-neutral pH is suitable for the production of antagonistic substances (Shanmugaiah *et al.*, 2008). The *B. megaterium* strain was greatly influenced by nutritional factors.

Table.1 Antifungal activities of *Bacillus* isolates on the cocoyam pathogens (zone of inhibition (mm))

<i>Bacillus</i> Isolates	Cocoyam pathogens		
	<i>Aspergillus</i> species	<i>Penicillium</i> Species	<i>Fusarium</i> Species
<i>Bacillus subtilis</i>	13.33 ± 0.57 ^c	8.66 ± 0.57 ^a	16.26 ± 0.57 ^c
<i>Bacillus licheniformis</i>	11.33 ± 0.57 ^b	11.33 ± 0.57 ^{bc}	12.33 ± 0.57 ^b
<i>Bacillus megaterium</i>	15.66 ± 0.57 ^d	13.57 ± 0.57 ^c	16.89 ± 0.57 ^b
<i>Bacillus thuringensis</i>	7.33 ± 0.57 ^a	7.66 ± 0.57 ^a	9.66 ± 0.57 ^a
<i>Bacillus cereus</i>	10.33 ± 0.57 ^b	10.66 ± 0.57 ^b	13.66 ± 0.57 ^b
Ketoconazole (Control)	24.00 ± 0.57 ^e	22.66 ± 0.57 ^d	25.66 ± 0.57 ^d

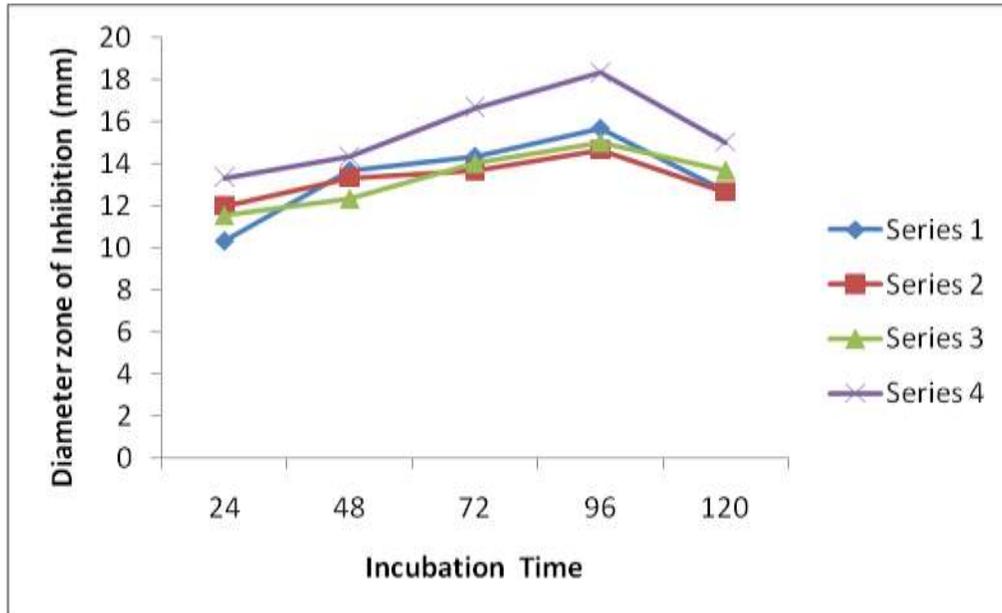
Values are the mean ± standard deviation of two replication of each parameter. Values with different superscript down a column are significantly different from each other.

Fig.1 Effects of different temperature on the antifungal activities of *Bacillus megaterium*



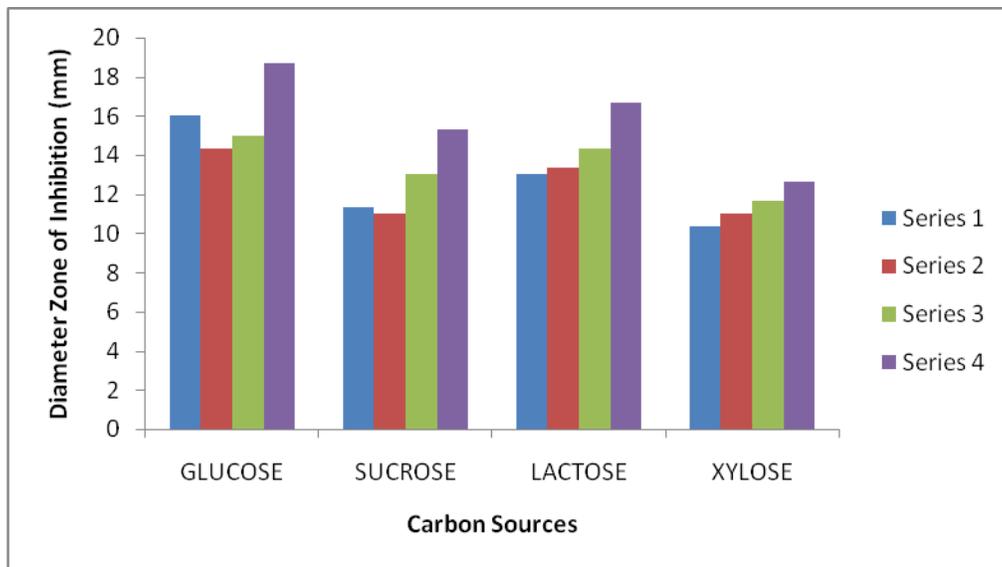
Keys: Series 1 = *Aspergillus* species; Series 2 = *Penicillium* species
 Series 3 = *Rhizopus* species; Series 4 = *Fusarium* species

Fig.2 Effects of Incubation time on the antifungal activities of *Bacillus megaterium*



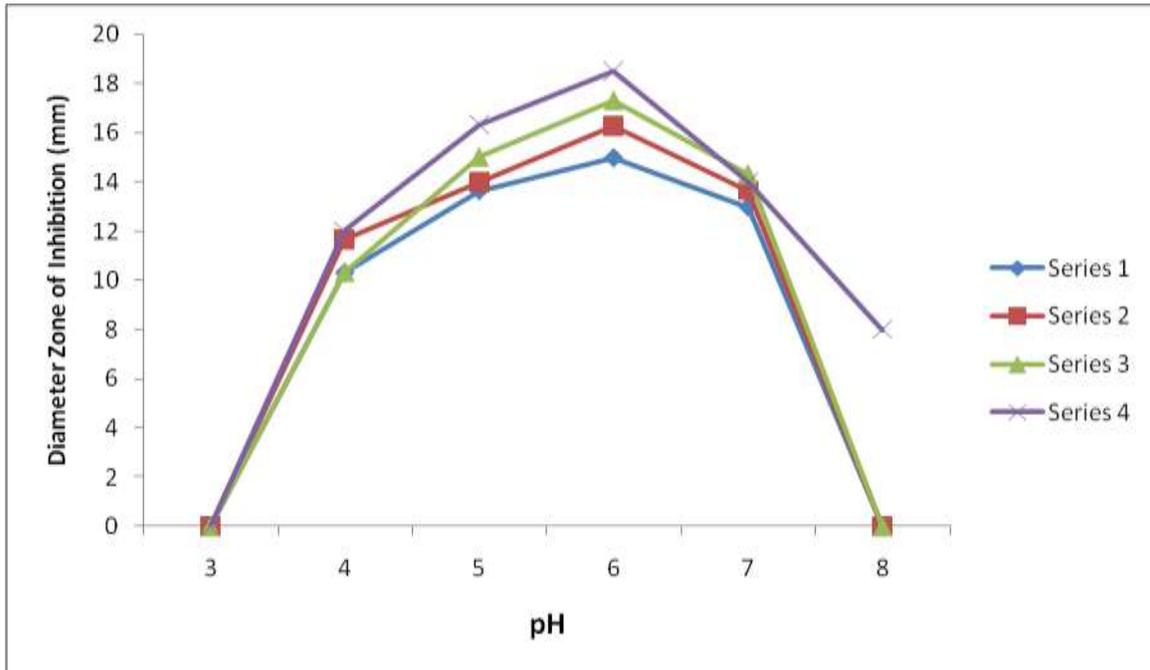
Keys :-Series 1 = *Aspergillus* species; Series 2 = *Penicillium* species; Series 3 = *Rhizopus* species; Series 4 = *Fusarium* species

Fig.3 Effects of different carbon sources on antifungal activities of *Bacillus megaterium*



Keys: Series 1=*Aspergillus* species; Series 2=*Penicillium* species
Series 3 = *Rhizopus* species; Series 4=*Fusarium* species

Fig.4 Effects of pH on the antifungal activities of *Bacillus megaterium*



Keys: Series 1 = *Aspergillus* species; Series 2 = *Penicillium* species
Series 3 = *Rhizopus* species; Series 4 = *Fusarium* species

This finding is consistent with earlier reports for *B. megaterium*, *B. subtilis*, *B. circulans* and *B. cepacia* strains showing that the production of antibacterial and antifungal substances and secondary metabolites in potent organisms was greatly influenced by carbon source (El-Banna, 2006; El-Banna and Qaddoumi, 2016).

It was observed that maximal antagonistic activity of the isolate was after 96h of incubation (Fig 4.4) with over 18.0mm zone of inhibition against *Fusarium* and 15.2mm against *Aspergillus* species. The incubation period seemed to be ideal for industrial production of biocidal product.

The present study was comparable with that of Nalisha *et al.*, (2006) who observed maximum growth of *B. subtilis* at 36 hrs of incubation as in the present study. Okanlawon *et al.*, (2010) found highest growth at 48 hrs for most of the isolates in their study. Prescott *et al.*, (2005)

and Ynte *et al.*, (2004) observed *B. cereus* was able to grow between 18 to 48 hrs.

Glucose and Lactose were found to be the ideal carbon source over xylose and sucrose for the growth of *B. megaterium* in the present work. Results of this study are consistent with those of previous studies, where different carbon source had a significant influence on the growth of *B. subtilis* and the highest levels of growth inhibition occurred in the presence of (2%) glucose (De Sarrau *et al.*, 2012, Singh *et al.*, 2013). Usama (2003) observed lactose as the ideal carbon source in a previous study. Mizumoto *et al.*, 2007 showed addition of glucose as carbon source in minimal salt medium containing Okra enhanced the bioactive iturin A production in solid state fermentation (SSF) by *B. subtilis* RB14-CS. Joshi *et al.*, (2008) observed glucose in minimal salt media enhanced the production of lichenysin by *B. licheniformis*. Usama (2003) tested several carbon sources reported

that the maximum growth of *B. subtilis* and β -glucanase production was obtained with lactose as sole carbon source.

In the present investigation, 38°C was found to be ideal for the growth of *B. megaterium*. Hence these bacteria and their products seem to be ideal for the prevailing conditions in most part of the soil. Okanlawon *et al.*, 2010 observed optimum growth of *B. cereus* at 37°C. Another *B. subtilis* strain, showed optimum temperature for the production of antifungal substance at 30°C in liquid cultivation, but at below 25°C in solid state cultivation (Ohno *et al.*, 1995).

The use of *B. megaterium* as a biocontrol agent against cocoyam pathogens may be an economically viable way of suppressing postharvest rot. The spore forming ability of this organism and the vast array of antimicrobial compounds it can produce make it a valid candidate for biocontrol.

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