academicJournals

Vol. 8(18), pp. 1841-1849, 30 April, 2014
DOI: 10.5897/AJMR2013.6162
Article Number: 5EE92E444385
ISSN 1996-0808
Copyright © 2014
Author(s) retain the copyright of this article
http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Antifungal activities of *Bacillus subtilis* isolated from some condiments and soil

Oyedele, Adedayo Omowumi[1]* and Ogunbanwo, Temitope Samuel[2]

[1]Institute of Agricultural Research and Training, Obafemi Awolowo University, Moor Plantation, Ibadan, Nigeria.
[2]Department of Microbiology, University of Ibadan, Ibadan, Nigeria.

Received 8 July, 2013; Accepted 4 April, 2014

Bacillus subtilis **are beneficial organisms that can be used as biological control agents. A total of 62 species of *Bacillus* were isolated from four condiments (Ogiri, Iru, Okpehe and Dawadawa) and soil samples. The isolates were identified as *Bacillus subtilis* (44%), *Bacillus megaterium* (16%), *Bacillus licheniformis* (14%), *Bacillus pumilus* (23%) *and Bacillus cereus* (3%). Nine strains of *Bacillus subtilis* were selected based on their antifungal activity against some indicator fungi namely; *Aspergillus niger, A. flavus, Fusarium oxysporum* and *Rhizopus stolonifer*. As potential bio-control agents, a hazard assessment was conducted on potato tubers and garlic cloves. Only two strains; *B. subtilis* DB4 and *B. subtilis* B6 did not cause soft rot disease in the potato tubers and garlic cloves. The optimal conditions for the production of antifungal compounds by *B. subtilis* DB4 was at pH 6.5, 37°C at 24 h while that of *B. subtilis* B6 was at neutral pH, 37°C and at 36-48 h. The antifungal activity was optimal in both strains when sucrose was the carbon source. The antifungal activity of *B. subtilis* DB4 was optimal when casein was the nitrogen source, while that of *B. subtilis* B6 was optimal when urea was used.**

Key words: *Bacillus subtilis*, antifungal, hazard assessment, optimization.

INTRODUCTION

The use of biological control in the management of agriculture pests and diseases is an effective alternative to the use of pesticides, which are often accumulated in plants and are lethal to beneficial organisms present in the soil (Nogórska et al., 2007). Antifungal agents produced by microorganisms may be used as bio-control agents. Some soil borne fungi, bacteria and action-mycetes have been identified and used as antagonistic microbes (Sivanantham et al., 2013). *Bacillus* strains with broad anti-fungal activity have been reported by Rossall (1994), Awais et al. *(*2007) also reported the activities of *B. subtilis* and *B. pumilus* against *Micrococcus luteus* and *Staphylococcus aureus,* Tabbene et al. (2009), reported the use of a *Bacillus subtilis* strain against some pathogenic and spoilage organisms, while Vijayalakshmi et al. (2011) reported the use of a *Bacillus amyloliquefaciens* strain against *Staphylococcus aureus.*

Considering the amount of money spent on chemical pesticides, the toxic effects of the pesticides to human and other non-target species and the environmental effects,

*Corresponding author. E-mail: adeedii@yahoo.com. Tel: +234-8034958469.

Author(s) agree that this article remain permanently open access under the terms of the Creative Commons Attribution License 4.0 International License

biological control offers an attractive alternative to synthetic chemical fungicides. Bio-pesticides can be safer, more biodegradable, and less expensive to develop. Bio-pesticides developed from microorganisms are highly desired for integrated pest management programs in agri-culture, public health and urban settings. (Whipps (2000) stated that the key to achieving successful, reproducible biological control is the gradual appreciation that knowledge of the ecological interactions taking place in soil and root environments is required to predict the conditions under which bio-control can be achieved (Deacon, 1994; Whipps, 1997). Utkhede and Smith (1992) reported that *Bacillus* has the potential for biological control of several tree diseases.

An instance is seen with Fusarium wilt, which causes huge economic losses in a wide variety of crops (Inoue et al., 2002). The pathogen, *Fusarium oxysporum*, infects plants through the roots by direct penetration or wounds, colonizes the vascular tissue and causes plant death (Simons et al., 1998).

Chemical soil fumigation is the main treatment of Fusarium wilt. Broad-spectrum biocides, particularly methyl bromide, can be used to fumigate the soil, but they cause serious environmental damage (Fravel et al., 2003). Recently, scientists have paid attention to biological methods of defense against plant diseases. Control of pathogens by antagonistic microorganisms or their antibiotic products is now considered a viable disease control technology (Klich et al., 1994; Cook et al., 1995; Han et al., 2005).

USEPA (1997) reported earlier works on *B*. *subtilis*; Claus and Berkeley (1986) considered *B. subtilis* not to be a plant pathogen, but there are several reports in the literature that associate *B. subtilis* with certain plant diseases. Kararah et al. (1985) produced soft rot of garlic cloves by injecting *B. subtilis* into them, Yakovleva et al. (1990) reported that *B. subtilis* was the cause of a broad open cancer ulcers on Norway maples in forests in the Urals, while Stanghellini and Rasmussen (1989) stated another report that an organism identified as *B. subtilis* was consistently isolated from glasswort (*Salicornia*) plants suffering from a soft-rot disease. Therefore, to be an effective bio-control agent, the organism must not exhibit any form of hazard effect by causing diseases in crops.

This present study was undertaken to determine the antagonistic activities of the isolated and identified *B. subtilis* strains on some fungal pathogens followed by a hazard assessment of the strains with the potential to be used in the biological control of these fungi. This work also carried out an optimization of antifungal activity of the *B. subtilis* strains.

MATERIALS AND METHODS

Isolation and identification of bacterial isolates

Samples of four different condiments namely: Dawadawa, Iru, Ogiri and Okpe used for the purpose of isolation were obtained from three different markets in Ibadan, Nigeria, while the soil samples used were obtained from several locations at the University of Ibadan, Nigeria. The samples were conveyed to the Department of Botany and Microbiology University of Ibadan Laboratory in sterile containers for further analyses. Identification was done using cultural, morphological and biochemical characteristics using the methods described by Holt et al. (1994). Elevation, color and shape were studied on the agar. The cells were Gram stained, tested for motility, sugar fermentation and other biochemical tests including starch hydrolysis tests, indole tests, methyl red tests, Voges-Proskauer tests, citrate tests, urease tests, gelatinase tests, DNase tests, catalase tests, oxidase tests, and a nitrate tests. A total number of twenty seven *B. subtilis* strains were identified and these were used for further work.

Screening for antifungal producing *B. subtilis* strains

Preparation of fungal and bacterial inocula

Four different fungi namely; *Aspergillus flavus, A. niger*, *Fusarium oxysporum*, and *Rhizopus stolonifer* were used as indicator organisms, they were obtained from the Department of Botany and Microbiology, University of Ibadan, Nigeria. The moulds were grown on potato dextrose agar (PDA) slants at 25°C for 7 days and stored at 5°C. Inoculums containing the spores or conidia were prepared by growing the moulds on PDA slants and were grown for 7-10 days in the dark to induce sporulation. The spores were harvested by pouring 10 ml of sterile distilled water on the slants and the spores suspended by vigorous shaking. Spore suspensions were filtered using a Whatman No 2 filter to remove hypha fragment (Magnusson and Schnurer, 2001). The spore concentrations were then determined using a Buckner heamocytometer and adjusted to 10^4 spores per ml by adding distilled water (Krauss et al., 1988). For the preparation of the bacterial inocula, the different *B. subtilis* strains were grown at 37°C for 24 h in nutrient broth. The number of Colony Forming Units (CFU) was determined by plating of dilution steps of the culture broth onto nutrient agar. Suspensions were then diluted to a concentration of approximately 4×10^8 CFU/ml.

Agar well diffusion test

PDA agar plates containing 10^4 fungi conidia per ml agar were prepared, and wells with a diameter of 5 mm were cut in the agar prepared using a sterile cork borer. A droplet of the agar was added to each well in order to avoid leakage. From the bacterial inocula of *B. subtilis* strain, 70 µl was taken and added to the well and allowed to diffuse into the agar during a 5 h pre-incubation period at room temperature. This was followed by aerobic incubation at 37°C for 3 to 5 days. The antimicrobial effects were recorded measuring the zone of inhibition around the well (Schillinger and Lucke, 1989).

Hazard assessment

The bacterial inoculum was prepared by growing the *B. subtilis* strains at 37°C for 24 h in nutrient broth. Cells were harvested by centrifugation at 3000 revolutions per minute for 20 min and the cell pellet was washed twice with sterile saline water (0.85% NaCl). Vegetative cell suspensions were then diluted to a concentration of approximately 4×10^8 CFU/ml. In order to ascertain the influence of the *B. subtilis* strains on garlic cloves and potato tubers, healthy potato tubers and garlic cloves were cleaned and washed with sterilized distilled water. The experimental garlic cloves and potato tubers were each injected with 0.1 ml of the bacterial suspension while 0.1 ml of sterile saline water was injected into the controls. The experiment was performed in duplicates.

Table 1. Antifungal activity of *B. subtilis* against some indicator fungi.

Isolate code	Indicator fungi			
	Aspergillus flavus	*Aspergillus niger*	*Rhizopus stolonifer*	*Fusarium oxysporum*
DB3	+(13.5 mm)	+(10.0 mm)	+(10.0 mm)	+(18.0 mm)
Db4	+(13.0 mm)	+(8.5 mm)	+(10.0 mm)	+(9.0 mm)
Il3	+(13.5 mm)	-	+(12.5 mm)	+(19.0 mm)
If7	+(12.0 mm)	+(11.0 mm)	-	+(13.0 mm)
Og7	+(15.0 mm)	+(22.5 mm)	(+14.0 mm)	+(17.5 mm)
A6	+(24.5 mm)	+(9.0 mm)	-	+(17.5 mm)
B5	+(10.0 mm)	+(10.0 mm)	-	-
B6	+(21.0 mm)	+(19.5 mm)	-	+(18.5 mm)
B11	+(18.0 mm)	+(11.0 mm)	-	-

+ = sensitive, - = resistance, 10 - 15 mm = very strong inhibition, 5 - 9 mm strong inhibition, 0.5 - 4 mm = weak inhibition.

Effect of temperature on the production of antifungal compound

The effect of incubation temperature on the production of antifungal compound was carried out. Test tubes containing 10 mls of nutrient broth were each inoculated with 0.1 ml of an overnight culture (4.0×10^8 CFU/ml) of the test organisms. Incubation was done at 4, 30, 37, 40 and 45°C in order to determine the appropriate temperature for the production of antifungal compound. After 24 h the antifungal activity was determined using the agar well diffusion method as described earlier.

Effect of pH on the production of antifungal compound

The effect of initial pH on production of antifungal activity was investigated by employing media with varying initial pH (5.5, 6.5, 7.0, 7.5, 8.5 and 9.5) adjusted by using different buffers (Na-acetate buffer for pH 5.5; potassium phosphate buffer for pH 6.5 to 7.5; Tris-Cl buffer for pH 8.5; Glycine-NaOH for pH 9.5). Each medium was inoculated with 0.1 ml of an overnight broth culture (4.0×10^8 CFU/ml) of the test organisms and incubated at 37°C for 24 h and the antifungal activity was determined using the agar well diffusion method.

Effect of incubation period on the production of antifungal compound

The effect of incubation time on the production of antifungal compound was carried out. Test tubes containing 10 ml of nutrient broth with an initial pH of 6.5 were each inoculated with 0.1 ml of an overnight broth culture (4.0×10^8 CFU/ml) of the test organisms, and incubated at 37°C. The antifungal activity was observed after 12, 24, 36, 48, 56 and 72 h using the agar well diffusion method.

Effect of Carbon sources on the production of antifungal compound

To test the influence of the type of carbon source, the test organisms were grown in modified nutrient broth containing different carbon sources. The basal medium contained 5% NaCl, 2% tryptone, 0.15% $MgSO_4$, 0.15% K_2HPO_4 and 1% of carbon sources such as glucose, sucrose, mannitol and starch as the organic carbon source, while $CaCO_3$ was the inorganic carbon source. They were inoculated with 0.1ml of an overnight broth culture (4.0×10^8 CFU/ml) of the test organisms and incubated for 24 h at 37°C with an initial pH of 6.5. The antifungal activity was determined using the agar well diffusion method.

Effect of nitrogen sources on the production of antifungal compound by *B. subtilis* strains

To determine the influence of different nitrogen sources, the test organisms were grown in the basal medium containing 2% glucose, 5% NaCl, 0.15% $MgSO_4$, 0.15% K_2HPO_4 and1% of nitrogen sources such as urea, casein and peptone as the organic nitrogen source while ammonium sulphate was the inorganic nitrogen source. They were inoculated with 0.1 ml of an overnight culture (4.0×10^8 CFU/ml) of the test organisms and incubated for 48 h at 37°C with an initial pH of 6.5. The antifungal activity was determined using the agar well diffusion.

RESULTS

A total of 62 strains of *Bacillus* species were isolated from the condiments (Ogiri, Iru, Okpehe and Dawadawa) and soil samples. The isolates were identified as *Bacillus subtilis, B. megaterium, B. licheniformis, B. pumilus and B. cereus. B. subtilis* had the highest frequency of occurrence of 44% followed by *B. pumilus, B. megaterium* and *B. licheniformis* with frequency of occurrence of 23, 16 and 14%, respectively, while *B. cereus* had the lowest occurrence of 3%. The isolated Bacillus subtilis were used for further work.

As shown in Table 1, *Aspergillus flavus* was the most sensitive organism with 100% sensitivity, followed by *A. niger* which had 89% sensitivity while *R. stolonifer and F. oxysporum* had 44 and 78% sensitivity respectively. *B. subtilis* DB3 and Og7 were the only two isolates that showed antifungal activity against all the indicator organisms.

The result of the hazard assessment of the *B. subtilis* strains on garlic cloves and potato tubers are shown in Table 2. Only *B. subtilis* Db4 and B6 did not cause any

Table 2. Degree of soft rot of garlic cloves and potato tubers caused by *B. subtilis* strains.

Isolate code	Effect on garlic cloves		Effect on potato tubers	
	7 days	14 days	7 days	14 days
DB3	+++	++++	-	-
Db4	-	-	-	-
Il3	-	+	-	-
If7	-	-	-	+
Og7	-	-	+	++
A6	+	+	+++	++++
B5	++	+++	+++	++++
B6	-	-	-	-
B11	-	+	-	-

- = no effect; + = Very little; ++= little; +++= strong; ++++= very strong

Plate 1. Soft rot of potato caused by *B. subtilis* A6.

rot in both the garlic cloves and the potato tubers after the 14 day trial period. *B. subtilis* A6 and B5 caused a degree of rot in both the garlic cloves and the potato tubers. While all other organisms showed some degree of rot in either the potato tubers or garlic cloves after the fourteen day trial period. The effect of the *B. subtilis* strains on the potato tubers is shown on Plate 1.

The results of the optimization of the antifungal activity carried out on the two strains that did not exhibit any hazard effect are shown in Figures 1 to 5. Figure 1 shows the effect of temperature on the antifungal activities of *B. subtilis* DB4 and *B. subtilis* B6. Antifungal activity was recorded between 30 and 37°C, and the activity was maximum at 37°C. However *B. subtilis* B6 did not have any antifungal effect on *R. stolonifer* irrespective of the culture temperature. At 40°C very little activity was shown by *B. subtilis* DB4 against *A. flavus* only and activity was completely lost at 45°C. No antifungal activity was recorded at 4°C.

The inhibitory effect of the culture broth of these two strains was influenced by variations of pH. They both had antifungal effect over a wide range of pH, the antifungal

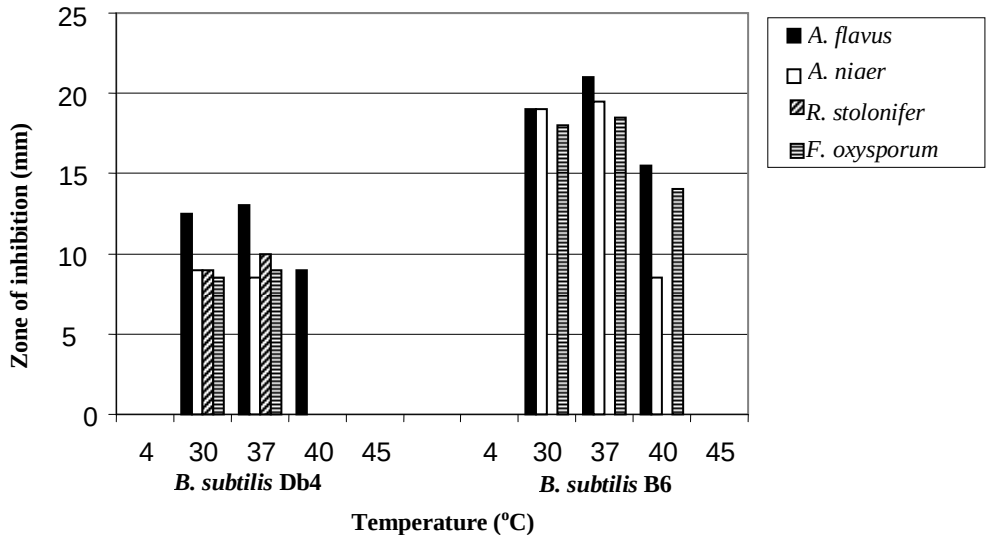

Figure 1. Effect of temperature on antifungal activity.

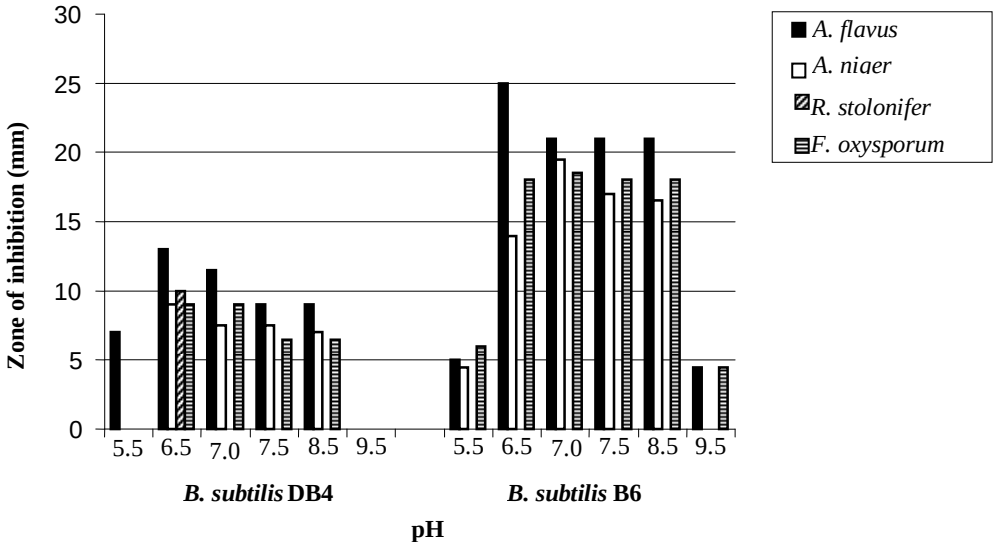

Figure 2. Effect of pH on antifungal activity.

activity of *B. subtilis* DB4 was recorded at pH 5.5 to 8.5; while that of *B. subtilis* B6 was recorded at pH 5.5 to 9.5. *B. subtilis* DB4 showed maximum antifungal activity against all the indicator fungi at pH 6.5 and still exhibited antifungal activity at pH 7to 8.5 with moderate reduction, however, activity was lost against *R. stolonifer* when the pH was adjusted from 6.5 to 7.0. At pH 5.5, it had effect only against *A. flavus* and no activity was recorded at pH 9.5. *B. subtilis* B6 did not show any antifungal activity against *R. stolonifer* at all the pH condition, it had a very high activity against *A. flavus* at pH 6.5 and the optimum activity was almost stable between neutral conditions to 8.5. It showed very little activity when the pH was adjusted to 5.5 and 9.5 (Figure 2).

Figure 3 shows the antifungal activities of the two strains according to the culture time in nutrient broth for 72 h. Antifungal activity was observed at every 12 h interval but was optimum and almost stable between 24 - 48 h with reduced and low activity at 12 and 72 h. While *B. subtilis* B6 did not show antifungal activity against *R. stolonifer* irrespective of the time, *B. subtilis* DB4 showed antifungal activity against it only at 24 h.

In the investigation of carbon sources on the antifungal activity of the strains, the basic medium was supplemented with varying carbon sources. There was a high degree of variation in the level of antifungal activity when

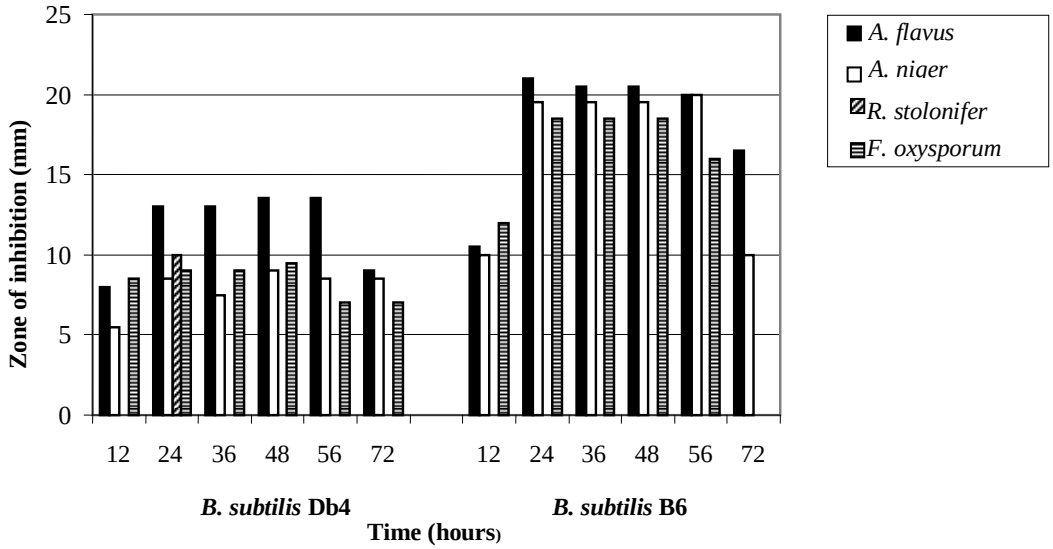

Figure 3. Effect of incubation period on antifungal activity.

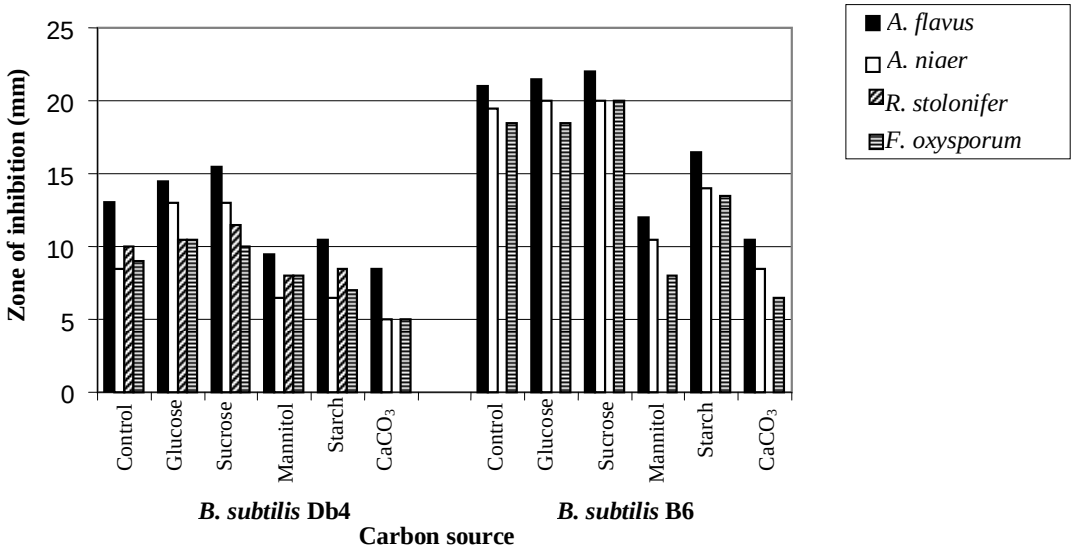

Figure 4. Effect of carbon sources on antifungal activity.

the different carbon sources were tested in the medium. Figure 4 described the results of the antifungal activity. The best activity was observed when the medium was supplemented with sucrose and glucose also enhanced the antifungal activity of *B. subtilis* DB4 but had little effect on the activity of *B. subtilis* B6 while less activity was observed with mannitol and starch. The inorganic carbon source ($CaCO_3$) did not enhance the antifungal activity but repressed it. To examine the effect of nitrogen sources on the antifungal activity of the strains, the effect of different organic and inorganic nitrogen sources on the antifungal activity was studied as shown in Figure 5, organic nitrogen sources including casein and peptone fairly supported the antifungal activity of *B. subtilis* DB4 while urea had no effect on the antifungal activity. In the case of *B. subtilis* B6, the organic sources including urea and peptone fairly supported the antifungal activity with the activity being optimal with the use of peptone while casein did not enhance the antifungal activity. It was observed also that *B. subtilis* B6 did not have any antifungal effect on *R. stolonifer* irrespective of the carbon source. The inorganic nitrogen source $(NH_4)_2SO_4$ had no effect on the antifungal activity of the strains, it greatly repressed it.

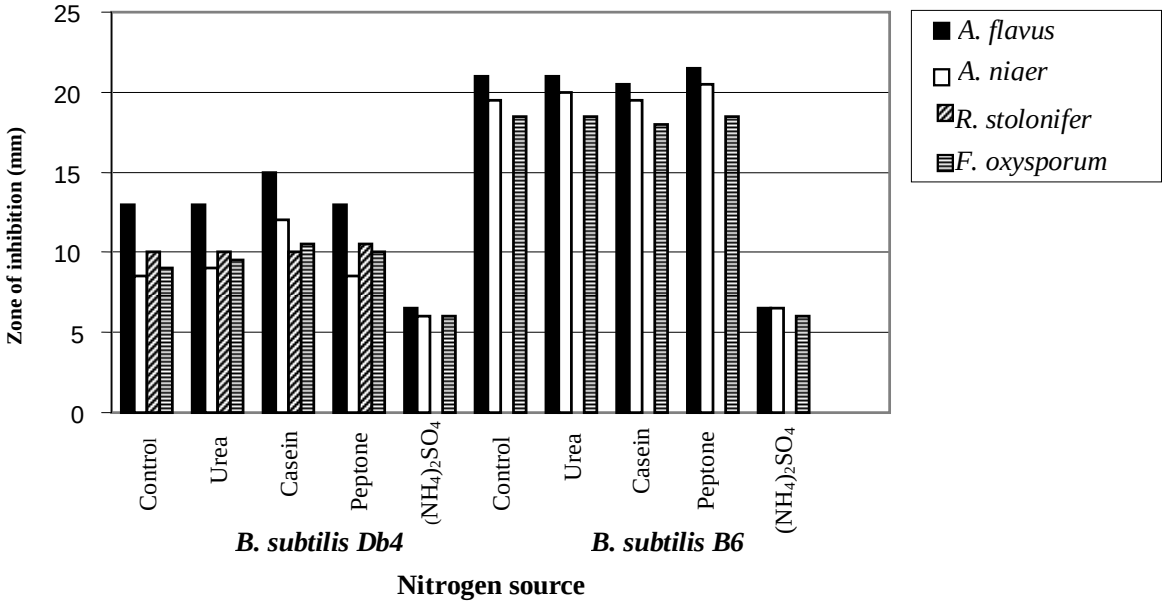

Figure 5. Effect of nitrogen sources on antifungal activity.

DISCUSSION

In this study, *Bacillus* species were isolated from soil and four condiments namely; Iru, Dawadawa, Ogiri and Okpehe, and were characterized. The predominant organisms isolated from the condiments were *Bacillus* species namely *B. subtilis, B. licheniformis, B. megaterium, B. pumilus* and *B. cereus*. The predominant species isolated from Iru, Dawadawa and Ogiri was identified as *B. subtilis* which confirmed earlier works on the dominance of *Bacillus* species especially *B. subtilis* during the production of indigenous condiments (O' Mahony et al., 2001; Okanlawon et al., 2010; Oguntoyinbo et al., 2010). However in Okpehe, *B. licheniformis* was the dominant species isolated. Azokpota et al. (2006) also reported the predominance of *Bacillus* species in three different types of condiments namely *Afitin*, *Iru* and *Sonru* from Benin, which were produced from African locust bean (*Parkia biglobosa*). *Bacillus* species was also the predominant organisms isolated from soil and *B. subtilis* had the highest frequency. Aslim et al. (2002), Awais et al. (2007) and Gajbhiye et al. (2010) have also reported the predominance of *Bacillus* species in soil samples.

The present study focused on antifungal properties of *B. subtilis,* therefore only the 27 isolated *B. subtilis* strains were used for further work. *Bacillus* species are known to produce antimicrobial substances against various organisms *(*Perez et al., 1992; Ouoba et al., 2007; Xie et al., 2009; Demirkan and Usta, 2013), they also worked on the antimicrobial activities of *Bacillus* species. The antimicrobial activities of *B. subtilis* have also been documented by the earlier works of Zheng and Slavik (1999), Aslim et al. (2002) and Fernandes et al. (2007) against different bacteria while Liu and Yao (2004) and Moita et al. (2005) worked on the production of broad-spectrum antifungal metabolites against selected fungi. Aslim et al. (2002) showed that the *B. subtilis* strains isolated from soil samples did not have antimicrobial effects, however the present study showed that *B. subtilis* strains isolated from soil samples were the predominant organisms showing antifungal properties.

Several works have been done on *Bacillus* species and also *B. subtilis* as effective biotechnological control agents against bacteria and some fungi but little has been done to further assess the hazard effect of these organisms against non-target plants and plant products. To be an effective bio-control agent, no incidents of damage to crops or non-target plants should be reported, hence a need for the hazard assessment. Claus and Berkeley (1986), stated that B*. subtilis* is not considered to be a plant pathogen, but noted that pectin and polysaccharides of plant tissues can be decomposed *by B. subtilis* and that it can cause soft rot of potato tubers. Kararah et al. (1985) also produced soft rot of garlic cloves by injecting *B. subtilis* into them. Although, USEPA (2006) in a more recent research, reported that a review of scientific literature does not indicate that *B. subtilis* is a common pathogen or phytotoxic agent , this work carried out a hazard assessment of the *B. subtilis* strains showing antifungal activities on potato tubers and garlic cloves for a trial period of 14 days. Based on the result of the trial assessment, only two strains; *B. subtilis* DB4 and *B. subtilis* B6 showed potentials as bio-control agents by not showing any hazard to the potato tubers and garlic cloves during the 14 days trial period.

An assessment of the physicochemical factors showed that the optimal conditions for antifungal activity of *B. subtilis* DB4 was at 37°C, pH 6.5 and at 24 h while that of *B. subtilis* B6 was at 37°C, neutral pH, and at 36-48 h. Earlier works have also reported similar optimal temperature with lower levels of activity observed at all other temperatures investigated (Teo and Tan, 2005; Kumar et al., 2009). Chiba et al. (1999) reported that antimicrobial substances in bacteria, in general, are produced over a pH range of 6.0 - 7.5. Similar results of optimal pH at 7.0 have been reported by other researchers (Gong et al., 2006; Kalpana et al., 2010) however, Awais et al. (2007), and Demirkan and Usta (2013) reported higher pH of 7.5 and 8, respectively. Generally, in *Bacillus*, the time of antibiotic activity is between 24-72 h of incubation. The optimal time depends on the particular species of *Bacillus* because different species have different metabolic pathways (Demirkan and Usta (2013). The range recorded in this work is supported by earlier findings of Bushra et al. (2007), Muazz et al. (2007) and Awais et al. (2007).

Antifungal activities of *B. subtilis* DB4 and B6 were greatly influenced by nutritional factors. The carbon source needed for maximal yield of the antifungal activity seems to be the same among the bacterial strains while the nitrogen sources were different. The antifungal activity was optimal in both strains when sucrose was used as the carbon source and this was followed by glucose. This agrees with the earlier work of Al-Ajlani et al. (2007) who observed that sucrose supplementation caused a marked enhancement in surfactin production by *B. subtilis* MZ-7 and also Joshi et al. (2008) who noted that glucose was found to enhance the production of lichenysin by *B. licheniformis.* However, El-Banna (2005) noted that other carbon sources which include fructose, ribose, starch, maltose, glycerol and arabinose increased the antimicrobial activity of various *Bacillus* species. The other carbon sources used in this work; mannitol, starch and the inorganic carbon source ($CaCO_3$) did not enhance the antifungal activities. While the antifungal activity of *B. subtilis* DB4 was optimal when casein was the nitrogen source, the antifungal activity of *B. subtilis* B6 was optimal when urea was used as the nitrogen source. Depending on the biosynthetic pathways involved, nitrogen sources may significantly affect antibiotic formation (Gesheva et al., 2005). The inorganic nitrogen source used in this work did not increase the activity but repressed it. This is in accordance to an earlier work by Yu et al. (2008) which noted that, compared with inorganic nitrogen sources, organic nitrogen sources were associated with relatively higher antifungal antibiotic production from microorganisms.

The research work has demonstrated the antimicrobial properties of *B. subtilis* isolated from condiments and soil against some pathogenic fungi. The trial hazard assessment carried out suggests two strains *B. subtilis* DB4 and *B. subtilis* B6 as promising agents in the bio-control of fungal diseases in agriculture. Further hazard assessment and application of biotechnology and genetic engineering may make these two organisms valuable potential bio-control agents.

Conflict of Interests

The author(s) have not declared any conflict of interests.

REFERENCES

Al-Ajlani MM, Sheikh MA, Ahmad Z, Hasnain S (2007). Production of surfactin from *Bacillus subtilis* MZ-7 grown on pharmamedia commercial medium.Microb. Cell Fact. 6:17.

Aslim B, Saglam N, Beyatli Y (2002). Determination of some properties of *Bacillus* isolated from soil. Turk. J. Biol. 26:41-48.

Awais M, Shah AA, Hameed A, Hasan F (2007).Isolation, Identification and Optimizationof bacitracin produced by *Bacillus*sp. Pak. J. Bot. 39(4):1303-1312.

Azokpota P, Hounhouigan DJ, Nago MC, Jakobsen M (2006). Esterase and protease activities of *Bacillus* spp. from afitin, iru and sonru; three African locust bean (*Parkia biglobosa*) condiments from Benin. Afr. J. Biotechnol. 5(3):265-272.

Bushra J, Hasan F, Hameed A, Ahmed S (2007). Isolation of *Bacillus subtilis* MH-4 from soil and its potential of polypeptidic antibiotic production. Pak. J. Pharm. Sci. 20(1):26-31.

Chiba H, Agematu H, Kanito R, Terasawa T, Sakal K, Dobashi K, Yoshioka T (1999). Rhodopeptins (Mer-N 1033), Novel cyclic tetra peptides with antifungal activity from rhodococcus species. J. Antibiot. 52 (8):695-699.

Claus D, Berkeley RCW (1986).Genus Bacillus Cohn 1872, 174AL. In: Sneath PHA, Mair NS, Sharpe ME, Holt JG, editor. Bergey's Manual of Systematic Bacteriology. Vol. 2. Baltimore: Williams & Wilkins; 1986. pp. 1105-1139.

Cook RJ, Thomashow LS, Weller DM, Fujimoto D, Mazzola M, Bangera G, Kim, DS (1995). Molecular mechanisms of defense by rhizobacteria against root disease. Proc. Natl. Acad. Sci. USA. 92: 4197-4201.

Deacon JW (1994).Rhizosphere constraints affecting biocontrol organisms applied to seeds. In: Seed treatment: prospects and progress. BCPC Monograph 57. Thornton Heath: British Crop Protection Council, 315-327.

Demirkan E, Usta A (2013). The effect of growth parameters on the antibiotic activity and sporulation in *Bacillus* Spp. isolated from soil. J. Microbiol. Biotechnol. Food Sci. 2 (5) 2310-2313.

El-Banna N (2005). A study on the antimicrobial activity of *Corynebacteriumkutscheri*isolated from Jordanian soil. Umm Al-Qura Univ. J. Sci. Med. Eng. 17:127-135.

Fernandes PAV, Renata de Arruda I, Botelho dos Santos AFA, Albertin de Araújo A, Maior AMS, Ximenes EA (2007). Antimicrobial activity of surfactants produced by *Bacillus subtilis* R14 against multidrug-resistant bacteria. Braz. J. Microbiol. 38:4

Fravel DR, Olivain C, Alabouvette C (2003). *Fusariumoxysporum* and its biocontrol. New Phytol.157:493-502.

Gajbhiye A, Rai AR, Meshram SU, Dongre AB (2010).Isolation, evaluation and characterization of*Bacillus subtilis* from cotton rhizosperic soil with biocontrol activity against *Fusariumoxysporum.* World J. Microbiol. Biotechnol. 26:1187-1194.

Gesheva V, Ivanova V, Gesheva R (2005). Effects of nutrients on the production of AK-111-81 macrolide antibiotic by *Streptomyces hygroscopicus*. Microbiol. Res. 160 (3):243-248.

Gong M, Wang J, Zhang J, Yang H, Lu X, Pei Y, Cheng J (2006).Study of theantifungal ability of *Bacillus subtilis* strain PY-1 *in Vitro* and identification of its antifungal substance (Iturin A). Acta. Biochim. Biophys. 38:233-240.

Han JS, Cheng JH, Yoon TM, Song J, Rajkarnikar A, Kim WG, Yoo ID (2005). Biological control agent of common scab disease by antago-

nistic strain *Bacillus* sp. sunhua. J. Appl. Microbiol. 99:213-221.
Holt J, Krieg NR,Sneath PHA, Staley JTA, Williams ST (1994*)*.Bergey's manual of determinative bacteriology. Williams & Wilkins*,* Baltimore, MD*.*
Inoue I, Namiki F, Tsuge T (2002). Plant colonization by the vascular wilt fungus *Fusarium oxysporum* requires FOW1, a gene encoding a mitochondrial protein. Plant Cell 14:1869-1883.
Joshi S, Yadav S, Desai AJ (2008). Application of response surface methodology to evaluate the optimum components for the enhance production of lichenysin by *Bacillus licheniformis* R2. Biochem. Eng. J. 41:122-127.
Kalpana S, Bagudo AI, Aliero AA (2010). Effect of inhibitory spectrum and physical conditions on the production of antibiotic substance from *Bacillus laterosporus* ST-1. Niger. J. Microbiol. 24(1):2134-2139.
Kararah MA, Barakat FM, Mikhail MS, Fouly HM (1985). Pathophysiology in garlic cloves inoculated with *Bacillus subtilis* and *Bacillus pumilus*, and *Erwiniacarotovora*. Egypt. J. Phytopathol. 17 (2):131-140.
Klich MA, Arthur KS, Lax AR, Bland JM (1994).Iturin A: A potential new fungicide for stored grains. Mycopathologia 127:123-127.
Krauss UI, BidwellR, Ince J (1988). Isolation and preliminary evaluation of mycoparasites as biocontrol agents of crown rot of banana. Biol. Control 13:111-119.
Kumar A, Saini P, Shrivastava JY (2009). Production of peptide antifungal antibiotic and biocontrol activity of *Bacillus subtilis*. Indian J. Exp. Biol. 47:57-62.
Liu J, Yao J (2004). Study on mutagenic breeding of *Bacillus subtilis* and properties of its antifungal substances. Plasma Sci. Technol. 6(4):2433-2436.
Magnusson J, Schnurer J (2001). *Lactobacillus coryniformis* subsp. *Coryniformis* strains $S_1$3 produce a broad-spectrum proteinaceous antifungal compound. Appl. Environ. Microbiol. 67:1-5.
Moita C, Feio SS, Nunes L, Curto MJM, Roseiro JC (2005).Optimisation of physical factors on the production of active metabolites by *Bacillus subtilis* 355 against wood surface contaminant fungi. Int. Biodeterior. Biodegrad. 55: 261-269.
Muaaz MA, Sheikh MA, Ahmad Z, Hasnain S (2007). Production of surfactin from *Bacillus subtilis* MZ-7 grown on pharmamedia commercial medium. Microb. Cell Fact.10:6-17.
Nogórska K, Bikowski M, Obuchowski M (2007). Multicellular behaviour and production of a wide variety of toxic substances support usage of *Bacillus subtilis* as a powerful biocontrol agent. Acta. Biochim. Pol. 54:495-508.
O'mahony T, Rechif N, Cavadini C, Fitzgerald GF (2001). The application of a fermented food ingredients containing Variacin, a novel antimicrobial produced by *Kocuriavarians* to control the growth of *Bacillus cereus* in chilled dairy products. J. Appl. Microbiol. 85:17-24.
Oguntoyinbo FA, Huch M, Cho GS, Schillinger U,.Holzapfel WH, Sanni AI, Franz CM (2010). Diversity of *Bacillus* species isolated from Okpehe, a traditional fermented soup condiment from Nigeria. J. Food Prot. 73:870-878.
Okanlawon BM, Ogunbanwo ST, Okunlola AO (2010). Growth of *Bacillus cereus* isolated from some traditional condiments under different regimens. Afr. J. Biotechnol. 8(14):2129-2135.
Ouoba LI, Diawara B, Jespersen L, Jakobsen M (2007). Antimicrobial activity of *Bacillus subtilis*and*Bacillus pumilus* during the fermentation of African locust bean (Parkiabiglobosa) for Soumbala production. J. Appl. Microbiol. 102(4):963-70.
Perez C, Suarez C, Castro GR (1992). Production of antimicrobials by *Bacillus subtilis* MIR 15. Biotecnology 26:331-336.
Rossall S (1994). U.S. Patent No. 5344647. http://www.google.com/patents/US5344647
Schillinger U, Lucke FK (1989). Antimicrobial activity of *Lactobacillus sake* isolated from meat. Appl. Environ. Microbiol. 55:1901 - 1906.
Simons G, Groenendijk J, Wijbrandi J, Reijans M, Groenen J, Diergaarde P, Van der Lee T(1998). Dissection of the *Fusarium* I2 gene cluster in tomato reveals six homologs and one active gene copy. Plant Cell 10:1055-1068.
Sivanantham T, Rasaiyah V, Satkunanathan N, Thavaranjit AC (2013). *In vitro* screening of antagonistic effect of soil borne bacteria on some selected phytopathogenic fungi. Arch. Appl. Sci. Res. 5(1):1-4.
Stanghellini ME, Rasmussen SL (1989). Two new diseases of *Salicornia* sp. caused by *Bacillus subtilis* and *Macrophominaphaseolina*. Annual Meeting of the American Phytopathological Society, Pacific Division, June 20-21, 1989. Phytopathology 79(8):912.
Tabbene O, Slimene IB, Djebali K, Mangoni ML, Urdaci MC, Limam F (2009). Optimization of medium composition for the production of antimicrobial activity by *Bacillus subtilis* B38. Biotechnol. Prog. 25(5):1267-1274.
Teo AY, Tan H (2005). Inhibition of *Clostridium perfringens* by a Novel Strain of *Bacillus subtilis* Isolated from the Gastrointestinal Tracts of Healthy Chicken. Appl. Environ. Microbiol. 71(8):4185.
U.S. Environmental Protection Agency (USEPA) (1997).*Bacillus subtilis* final risk assessment. Biotechnology program under the toxic substance control act (TSCA). http://www.epa.gov/biotech_rule/pubs/fra/fra009.htm.
U.S. Environmental Protection Agency (USEPA) (2006). Biopesticide Registration action Document.*Bacillus subtilis*Strain QST 713 (PC Code006479).http://www.epa.gov/pesticides/chem_search/reg_actions/registration/decision_PC-006479_9-Aug-06.pdf
Utkhede RS, Smith EM (1992). Promotion of apple tree growth and fruit production by the EBW- strain of *Bacillus subtilis* in apple replant disease soil. Can. J. Microbiol. 38:1270- 1273.
Vijayalakshmi K, Premalatha A, Suseela Rajakumar G (2011). Antimicrobial protein production by *Bacillus amyloliquefaciens* MBL27: Optimization of culture conditions using Taguchi's experimental design. Indian J. Sci. Technol. (4)8:931-937.
Whipps JM (1997). Developments in the biological control of soil-borne plant pathogens. Adv. Bot. Res. 26**:**1-134.
Whipps JM (2000). Microbial interactions and biocontrol in the rhizosphere. J. Exp. Bot. (52):487-511.
Xie J, Zhang R, Shang C, Guo Y (2009).Isolation and characterization of a bacteriocin produced by an isolated *Bacillus subtilis* LFB112 that exhibits antimicrobial activity against domestic animal pathogens. Afr. J. Biotechnol. 8 (20):5611-5619.
Yakovleva LM, Derevyankin PV, Gvozdyak RI (1990). Bacteriosis in the Norway maple. Mikrobioloicheskii Zhurnal (Kiev) 52(4):60-64.
Yu J, Liu Q, Liu Q, Liu X, Sun Q, Yan J, Qi X, Fan S (2008). Effect of liquid culture requirements on antifungal antibiotic production by *Streptomyces rimosus* MY02. Bioresour. Technol. 99:2087-2091.
Zheng G, Slavik MF (1999). Isolation, partial purification and characterization of a bacteriocin produced by a newly isolated *Bacillus subtilis* strain. Lett. Appl. Microbiol. 199 (28):363- 367.