African Journal of Microbiology Research

Volume 10 Number 6, 14 February 2016
ISSN 1996-0808

Academic Journals

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Pitout JDD, Church DL, Gregson DB, Chow BL, McCracken M, Mulvey M, Laupland KB (2007). Molecular epidemiology of CTXM-producing Escherichia coli in the Calgary Health Region: emergence of CTX-M-15-producing isolates. Antimicrob. Agents Chemother. 51: 1281-1286.

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African Journal of Microbiology Research

Table of Content: Volume 10 Number 6, 14 February, 2016

ARTICLES

academicJournals

Vol. 10(6), pp. 138-146, 14 February, 2016
DOI: 10.5897/AJMR2013.6453
Article Number: E5DDA1757103
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Review

Research progress in submerged mycelial culture of *Grifola frondosa*, a culinary-medicinal mushroom

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Received 27 October, 2013; Accepted 26 February, 2014

Grifola frondosa* or maitake, a kind of edible and medical fungus, has become a hotspot. This paper advances how to increase the mycelial growth and bioactive polysaccharide production of *G. frondosa* by submerged fermentation at home and abroad. It focus on two ways: one is improvement and optimization of *G. frondosa* self-strain, culture medium and culturing methods, and the other is the effects of adding some stimulators into submerged culture of *G. frondosa.* The last ten years have seen an unprecedented study on characteristics of bioactive polysaccharide from *G. frondosa* by submerged fermentation. Finally, a simple summary of the extracts and product of *G. frondosa* and some rare reports are given. The current review illustrates the role of *G. frondosa*, especially its polysaccharide, with the primary aim of illustrating the latest developments in research on *G. frondosa.

Key words: *Grifola frondosa*, maitake, submerged fermentation, culture, mycelial growth, biomass, polysaccharide.

INTRODUCTION

Grifola frondosa also known as maitake in Japan, a Basidiomycete fungus, belongs to the order *Aphyllophorales* and family Polyporeceae. It is not only an edible mushroom, but a type of medical fungus that is widely used as a culinary material and dietary supplement in Asia.

G. frondosa fruit body tastes tender and delicious. It is reported that *G. frondosa* is rich in various nutrients such as protein, amino acids, sugars, vitamins, minerals, etc. (Tao et al., 2007). Among them, *G. frondosa*'s active substances, especially its polysaccharide has the most biological and pharmacological activities, such as anti-tumor (Suzuki et al.,1989; Nanba, 1995; Lee et al., 2004; Shi et al., 2007; Cui et al., 2007b), hypoglycemic activities (Lee et al., 2004; Lei et al., 2007), enhancing immunity (Nanba, 1993; Adachi et al., 1987; Kodama et al., 2003; Deng et al., 2009), anti-HIV infections (Nanba et al., 2000), antioxidant and superoxide anion scavenging (Lin, 2011; Yeh et al., 2011; Chen et al., 2012), promoting longevity as a tonic and improving the quality of life (Kuo et al., 1996; Yang et al., 2000; Cui et al., 2007a). Among them, a polysaccharide called D-fraction from *G. frondosa* (Figure 1) seems to be a hotspot. Its various bioactivities were analyzed mostly in detail and systematically (Nanba, 1993, 1995; Nanba et al., 2000; Kodama et al., 2002, 2003, 2004, 2005).

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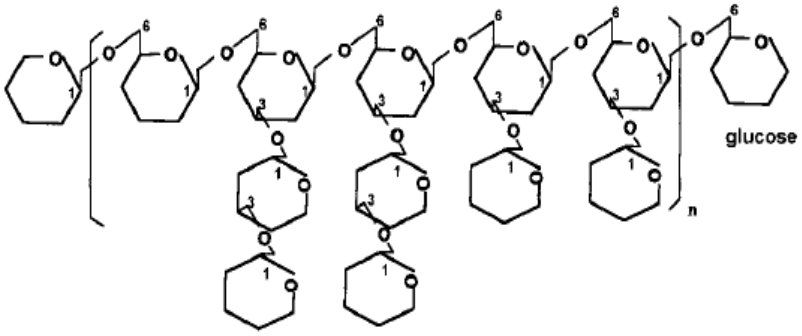

Figure 1. Chemical structure of D-fraction (Kodama et al., 2002).

Accordingly, based on these previous studies, the current review focuses on improving the production of biomass and bioactive polysaccharides of *G. frondosa*. Many investigators, including our groups, have spent time and effort in cultivating *G. frondosa* in submerged culture (Lee et al., 2004, 2008; Hsieh et al., 2006, 2008; Shih et al., 2008; Zhang et al., 2012; Xu et al., 2012; Wang et al., 2012, 2013; Wu et al., 2013).

Additionally, it is reported that as early as 2009, the total output of dry *G. frondosa* around the globe was up to about 10000 tons, of which Japanese, the country that first had development in maitake health food, has about 36000 tons of fresh mushroom every year (Xu et al., 2010). Accordingly, the industry of *G. frondosa* has formed an established downstream chain. Now there are maitake mushroom tea, capsule of maitake extract and its drops (D-fraction), compound capsule of maitake and gold maitake 404, etc. in the United States market. In China, *G. frondosa* health product is mainly capsule of maitake extract made by different producers. Consequently, *G. frondosa* health care products, as a new kind of anticancer health products have a broad prospect.

The purpose of this review was to stay up-to-date with the latest trends of *G. frondosa*, including its theoretical research and application. As great value is placed on our health; both theoretical and applied research on *G. frondosa* and its application will certainly be done in-depth and extensively.

RESEARCH ADVANCES ON MYCELIAL GROWTH AND BIOACTIVE POLYSACCHARIDE PRODUCTION OF GRIFOLA FRONDOSA BY SUBMERGED FERMENTATION

Because of medical functionality and health benefit from maitake polysaccharide, especially its extracellular polysaccharide (EPS), it has become important to know how to increase the mycelial growth and polysaccharide production of *G. frondosa* in maximum amount. We briefly summarize these reports at home and abroad, and two main aspects are included below:

1. Improvement and optimization of *G. frondosa* self-strain, culture medium and culturing methods (Table 1).
2. Effects of some stimulators added into submerged culture of *G. frondosa* (Table 2).

RESEARCH ADVANCES ON CHARACTERISTICS OF BIOACTIVE POLYSACCHARIDE FROM GRIFOLA FRONDOSA BY SUBMERGED FERMENTATION

It is reported that medical functionality and health benefits are mainly from maitake's polysaccharide; so now almost 70% of studies on *G. frondosa* are about characteristics of bioactive polysaccharide from *G. frondosa*. To illustrate the latest developments in research on *G. frondosa*, papers only in the last ten years are summed up in Table 3.

RESEARCH ADVANCES ON NON-POLYSACCHARIDE BASED ON EXTRACTS FROM GRIFOLA FRONDOSA IN SUBMERGED CULTURE

Compared with study on the activity of maitake polysaccharide extracts listed above, research on other extracts and product from *G. frondosa* in submerged culture is indeed scarce. However, we still sum up some studies on non-polysaccharide based extracts from *G. frondosa* in submerged culture (Table 4).

Additionally, Yang et al. (2013) analyzed fruiting bodies and mycelia of *G. frondosa*, respectively. The results showed that the fruiting bodies and mycelia contained 62 and 94 volatile compounds, respectively. Hereinto, 37 compounds in both fruiting bodies and mycelia accounted for 86.81 and 84.28% of total volatile substances and the content of isovaleraldehyde and lichen phenol reached 23.31 and 15.41%, respectively. Moreover, Chen et al. (2013) did a research on the speciation of iron in *G. frondosa* in details. After optimizing the extraction conditions, the soluble Fe was about 85% and the suspended Fe was about 15% in the water extracts. In

Table 1. Research progress in strategies to improve the *in vitro* growth of *G. frondosa.*

Procedure	Results	References
Comparison of mycelial growth and bioactive polysaccharide production in batch and fed-batch culture of *G. frondosa*	The fed-batch fermentation by glucose feeding greatly enhanced the accumulation of BIO and EPS; the BIO and EPS reached 8.23 g/L and 3.88 g/L at 13 d of cultivation after glucose feeding. In contrast, the BIO and EPS in the batch fermentation were 6.7 g/L and 3.3 g/L at 13 d of cultivation.	Shih et al., 2008
Two different series of experiments were carried out. In the first, the moisture in the solid substrate was maintained nearly constant while in the second the substrate was exposed to spontaneous drying by aeration.	Moistures in culture medium higher than 70% promote growth of *G. frondosa* mycelium and polysaccharide production. Four fractions of pure extracellular β-D-glucans with total mass 127.2 mg and four fractions of intracellular polysaccharides with total mass 47.2 mg were isolated. The extent of TNF-α induction was up to 322 pg mL^{-1} at a polysaccharide concentration of 200 µg mL^{-1} for the intracellular fraction.	Švagelj et al., 2008
Optimization of submerged culture conditions for mycelial biomass and exopolysaccharide (EPS) production by *G. frondosa* was studied.	Under optimal culture conditions, maximum biomass concentration and EPS production in a stirred-tank fermenter were 16.8 and 5.3 g/l, respectively.	Lee et al., 2004
A three-level Box-Behnken factorial design was employed combining with response surface methodology to optimize the medium composition.	A mathematical model estimated that a maximal yield of BIO (17.61 g/l) could be obtained when the concentrations of glucose, KH_2PO_4, peptone were set at 45.2 g/l, 2.97 g/l, 6.58 g/l, respectively; while a maximal exo-polymer yield (1.326 g/l) could be achieved when setting concentrations of glucose, KH_2PO_4, peptone at 58.6 g/l, 4.06 g/l and 3.79 g/l, respectively. Maximum BIO yield of 22.50 g/l was achieved in a 15-L fermenter using the optimized medium.	Cui et al., 2006
Different cereal grains for spawn production; and industrial by-products as substrates for mushroom production were evaluated.	1. The use of corn grains as substrate for spawn production results an important factor for reducing crop cycle time. A cold shock to 10°C was requisite for basidiome formation. 2. Coffee spent-ground was a good substrate for mycelial growth, but not for mushroom production. 3. When using oak sawdust plus corn bran as substrate, consistent yields with combined high biological efficiency (BE) (35.3%), best quality mushrooms, and a crop cycle of 12-14 weeks were obtained.	Barreto et al., 2008

the solution, the inorganic and organic Fe ratio was about 60 and 40%, respectively. Organic Fe was about 45 and 40% in protein combination and polysaccharide combination, respectively.

Interestingly, *G. frondosa* mycelium fermented liquid can be also used to produce its mycelium fermented wine. Zhu et al. (2012) developed a kind of maitake mycelium fermented wine in optimum fermentation conditions, and the produced wine was light yellow in color, clear and transparent without impurities. Furthermore, the wine tasted mellow and soft with its alcohol content as 11.75 % and its *G. frondosa* polysaccharide content as 1.56 g/L.

Finally, the fact that *G. frondosa* is a kind of medical and edible mushroom is not only proved by these evidences, but is also sustained by facts. In the year 2004, Tanaka et al. successfully treated a patient with occupational hypersensitivity pneumonitis (HP) caused by *G. frondosa* mushroom spore with an extra-fine aerosol corticosteroid; beclomethasone dipropionate (BDP) dissolved in hydrofluoroalkane-134a (HFA). A 49-year-old woman developed respiratory symptoms 3 months after beginning work on a mushroom farm. She was diagnosed as HP based on radiological and serological findings. Oral prednisolone therapy improved her HP and she returned to the same farm. Her HP relapsed after 5 months, and daily 400 µg of HFA-BDP was administered with gradual improvement. An extra-fine particle inhaled corticosteroid might reach appropriate alveoli to be effective therapy for mild HP (Tanaka et al., 2004).

Conclusion

Although, various strategies to improve the mycelial growth and bioactive polysaccharide production of *G. frondosa* have been proposed, practical application

Table 2. Research progress in effects of some stimulators' addition into submerged culture on *G. frondosa.*

Samples of stimulators	Results	References
Buckwheat	With addition of *Buckwheat* into the submerged culture of *G. frondosa*, and the EPS biosynthesis of *G. frondosa* increased by 0.5 g/L.	Zhao, 2008.
Rhizoma gastrodiae (*R. gastrodiae*)	1. With addition of *R. gastrodiae* into the submerged culture of *G. frondosa*, the EPS biosynthesis of maitake increased by 2.1 g/L (Zhao, 2008); 2. After screen and optimize maitake's medium with adding the ethanol extract of *R. gastrodiae* by Plackett-Burman, a further result showed that 3.91 g/L EPS could be obtained, which increased by 3.4% compared with the control (He and Wu, 2011); 3. With addition of 5%(v/v) ethanol extract of fresh *R. gastrodiae* into submerged culture of maitake, the BIO and EPS biosynthesis of maitake were both promoted from 0.564±0.09 to 1.324±0.25 g/L and from 71.69±0.53 to 107.08±0.85 mg/L, separately increased by 134.75 and 49.37%; However, intracellular polysaccharides (IPS) content declined from 60.38±0.87 to 45.71±0.66 mg/g, which decreased by 24.30% compared with the control group, respectively (Zhang et al., 2012); 4. From the perspective of fermentation kinetics and with addition of 7 %(v/v) alcohol extract of processed *R. gastrodiae* into the fermentation broth of *G. frondosa*, the biomass and EPS productions reached a maximum of 2.0630±0.0520 g/L and 89.3846±3.2422 mg/L, respectively after 10 and 8 of days' cultivation (Wang et al., 2012); 5. A maximum dry cell weight of 138.5 mg/L and the EPS at 0.606 g/L were obtained when the unprocessed *Gastrodia* tuber culture was added into submerged culture of *G. frondosa* (Xu et al., 2012); 6. *Rhizoma Gastrodiae* at 7 g/L significantly promoted the biosynthesis of EPS in *G. frondosa* when compared with blank control, increasing EPS yield from 3.72 g/L to 3.91 g/L (He et al., 2013).	Zhao, 2008; He and Wu, 2011; Zhang et al., 2012; Wang et al., 2012; Xu et al., 2012; He et al., 2013
Yam	With addition of *Yam* into the submerged culture of *G. frondosa*, and the EPS biosynthesis of *G. frondosa* increased by 1.2 g/L.	Zhao, 2008
Fructus arctii	(1) Some enzymes secreted from *G. frondosa*, such as β-glucosidase, would convert the glycosides (arctiin and caffeic acid derivatives) into aglycones (arctigenin and caffeic acid); (2) The fermented *Fructus arctii* extract with *G. frondosa* (G-FAE) had antioxidant and 5-lipoxygenase inhibitory activities.	Kim et al., 2010.
Olive oil	With 1% olive oil addition in 21% O_2 and 40% O_2, the production of BIO was enhanced and increased to 10.1 and 14.9 g/L, respectively, after 9 days' cultivation. And the EPS production increased from 0.7-0.9 g/L to 2.24 g/L and 3.00 g/L at day 13 with 21% O_2 and 40% O_2 aeration, respectively. In addition, the IPS increased rapidly and reached the maximum level of 28.2 mg/g at day 7 and this level remained till day 13 through the whole fermentation.	Hseih et al., 2006.
Plant oil and surfactant	(1) Olive, safflower seed, soy and sunflower oil were favorable plant oil sources to the mycelial growth of *G. frondosa*. The highest cell growth (~12.64 ± 0.47 g/L cell dry weight) could be obtained on day 13 of cultivation in the medium containing 1% all the plant oil sources. EPS production was slightly enhanced by olive oil but significantly inhibited by safflower seed oil and sunflower oil after 13 days of cultivation; (2) Amongst four plant oil sources examined, cell growth yielded relatively high BIO (11.22 ± 1.14 g/L) and that was achieved in 4% glucose medium with 0.5% soybean oil. The higher EPS production and slightly lower cell growth were found in 4% glucose media; the maximum EPS production was 2.248 ± 0.107 g/L found in 4% glucose media with olive oil addition; (3) Tween 80 and Span 80 addition had shown to increase cell growth and the maximal cell concentration of 9.10 ± 0.80 g/L was obtained with 1% Span 80 addition. Both EPS and IPS production were found to decrease with all the tested concentrations of Tween 80 and Span 80 addition. Span 80 added at the vegetative growth phase in 4% glucose media yielded the highest BIO of *G. frondosa* (8.95 ± 0.57 g/L); meanwhile Tween 80 added at the beginning cultivation had resulted in the highest EPS production (1.451 ± 0.098 g/L).	Hseih et al., 2008.
Olive oil press cakes	Olive oil press cakes reduced the mushroom yield, and the best biological efficiency was obtained on substrates supplemented with wheat bran and without olive oil press cakes. All extracts were capable of inducing splenocyte proliferation and were half as effective as the positive control (6.0 μg/mL phytohaemagglutinin). No correlation between substrate composition and bioactivity could be established. Extracts from wild-growing *G. frondosa* were superior to cultivated ones in respect to biological activity.	Gregori et al., 2009.

Table 3. Research progress in characteristics of bioactive polysaccharide from *G. frondosa* by submerged fermentation.

Samples of bioactive polysaccharide	Results	References
A polysaccharide of D-fraction	(1) The level of IL-10 as well as those of NO and IFN-γ were increased by D-fraction from *G. frondosa*. The result suggested that D-fraction induced a Th-2 dominant response through the activation of macrophages, resulting in the enhancement of humoral immunity rather than cell-mediated immunity. Furthermore, an increase in the percentage ratio of CD69 and CD89 expression on major histocompatibility complex II+ cells revealed activation of APCs 4 h after D-fraction administration. These results indicate that D-fraction enhances both the innate and adaptive arms of the immune response in normal mice. (2) D-fraction significantly enhanced the cytotoxicity against NK-sensitive YAC-1 cells and the expression of CD223 on NK cells. D-fraction also increased the expression of CD86 on macrophages. In addition, the levels of IL-12 in the culture supernatant of whole spleen cells and in serum increased, compared with the control corresponding to an increase in expression of IL-12 receptor βI on NK cells.	Kodama et al., 2004, 2005.
D-fraction from *G. frondosa* (GF-D)	D-fraction from *G. frondosa* (GF-D) or its combination with human interferon alpha-2b (IFN) alone could inhibit hepatitis B virus (HBV) DNA in HepG2 2.2.15 cells (2.2.15 cells) with the 50% inhibitory concentration (IC50) of 0.59 mg/ml and 1399 IU/mL, respectively. The combination of GF-D and IFN for anti-HBV activity synergistically inhibited HBV replication in 2.2.15 cells. In combination with 0.45 mg/ml GF-D, the apparent IC50 value for IFN was 154 IU/mL. This 9-fold increase in antiviral activity of IFN suggested that GF-D could synergize with IFN.	Gu et al., 2006.
Grifolan LE (GRN-LE)	The primary structure of Grifolan LE (GRN-LE), a purified β-D-glucan from liquid-cultured *G. frondosa*, comprised a 1, 3-β-D-glucan backbone with a single 1, 6-b-D-glucosyl side branching unit on every third residue.	Tada et al., 2009.
A chemically sulfated polysaccharide (S-GAP-P)	A chemically sulfated polysaccharide (S-GAP-P) from water-insoluble polysaccharide of *G. frondosa* mycelia significantly inhibited the tumor growth and enhanced the peritoneal macrophages phagocytosis in S180-bearing mice.	Nie et al., 2006.
Sulfated polysaccharide (S-GAP-P)	Chemically sulfated polysaccharide (S-GAP-P) from water-insoluble polysaccharide of maitake mycelia distinctly inhibited SGC-7901 cells growth in a dose-dependent manner and induced cell apoptosis evidenced by characteristic DNA ladder and sub-G_0/G_1 peak. Furthermore, the combination of S-GAP-P (10–50 μg/ml) with 1 μg/ml 5-FU resulted in a significant inhibition on SGC-7901 cells growth, meaning the beneficial interaction between the two drugs.	Shi et al., 2007.
Polysaccharide from *G. frondosa* (GFP-1, GFP-2 and GFP-3)	Three main fractions from the fruiting bodies of *G. frondosa* , GFP-1, GFP-2 and GFP-3, were obtained. Then the antioxidant activities of these three fractions showed that GFP-1, GFP-2 and GFP-3 possessed significant inhibitory effects on 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical, hydroxyl radical and superoxide radical; their reducing power, ferrous ions chelating effect and the inhibition ability of the rat liver lipid oxidation were also strong.	Chen et al., 2012.
Se-enriched *G. frondosa* polysaccharide (Se-GP)	A Se-enriched *G. frondosa* polysaccharide (Se-GP) was obtained from maitake enriched with Se by spraying a Na_2SeO_3 solution during fruit body growth. Under optimal conditions, polysaccharide yields and both the Se-GP and GP contents do not differ; however, the Se content of Se-GP (17.52μg/g) was 48.7 times that of GP. Three homogenous Se-GPs or GPs were obtained via DEAE-52 and Sephacryl S-400 purification. The antioxidant activity of Se-GP for the DPPH, ABTS and hydroxyl radicals was higher than that of GP and was highest for the hydroxylradical.	Mao et al., 2014.
Maitake liquid extract	No dose-limiting toxicity was encountered. Two patients withdrew prior to completion of the study due to grade I possibly related side effects: nausea and joint swelling in one patient; rash and pruritus in the second. There was a statistically significant association between maitake and immunologic function ($p < 0.0005$). Increasing doses of maitake increased some immunologic parameters and depressed others; the dose-response curves for many endpoints were non-monotonic with intermediate doses having either immune enhancing or immune suppressant effects compared with both high and low doses.	Deng et al., 2009.

among them is rare, and now the yield of its fruit body cannot meet the demand of people. From the above, all questions are put forward seemingly because of the same issue: *G. frondosa*'s bioactive polysaccharide.

Table 4. Research progress in non-polysaccharide based extracts from *G. frondosa* in submerged culture.

Non-polysaccharide based extracts	Results	References
A low-molecular-weight protein fraction (MLP-fraction)	A low-molecular-weight protein fraction (MLP-fraction) was obtained from the fruiting body of the maitake mushroom. The effect of the MLP-Fraction on the immune system resulted in a simultaneous increase in splenocyte proliferation and production of cytokines such as interleukin (IL)-1α, tumor necrosis factor-α, IL-10, IL-12, and interferon (IFN)-γ. The possibility was confirmed that the MLP-fraction acts as a BRM using colon-26 carcinoma-bearing mice. This fraction enhanced the production of IL-12 and IFN-γ by splenocytes in tumor-bearing mice and clearly showed an inhibitory effect on tumor cell growth.	Kodama et al., 2010.
A new protein (GFP)	It is the first to reveal the critical role of GFP, a new *G. frondosa* protein from maitake fruiting bodies, in modulating the immune response and to link the immune-enhancing effects of maitake to its antitumor activities. GFP is a nonglucan heterodimeric 83 kDa protein that consists of two 41 kDa subunits. GFP induced interferon-γ secretion by murine splenocytes and natural killer cells and activated the maturation of bone marrow-derived dendritic cells (BMDCs) via a TLR4-dependent mechanism. GFP-treated BMDCs promoted a Th1 response and exhibited significant antitumor activity when transferred into tumor-bearing mice.	Tsao et al., 2013.
Methanolic extracts	Methanolic extracts from maitake mycelium showed high antioxidant activities (85.4-94.7%) at 25 mg ml^{-1}. Reducing powers of the methanolic extracts were 0.97-1.02 at 25 mg ml^{-1}, and scavenging effects on 1, 1- diphenol-2-picrylhydrazyl radicals were 78.8-94.1% at 10 mg ml^{-1}. However, there was no scavenging effect on hydroxyl radicals. Chelating effects on ferrous ions were high (90.3-94.4%) at 10 mg ml^{-1}. Total phenols were the major naturally occurring antioxidant components found in methanolic extracts. EC50 value below 10 mg ml^{-1} indicated that the mycelium had a good antioxidant property except for the scavenging effect on hydroxyl radicals.	Mau et al., 2004.
Exo-biopolymer (EX-GF)	The exo-biopolymer (EX-GF) was fractionated into EX-GF-Fr. I, II, and III by Sephadex G-100 gel chromatography. Anti-complementary activity of EX-GF-Fr.III was highest (71.1%), and its activation system occurred through both classical and alternative pathways. Lysosomal enzyme activity and nitric oxide production ability of macrophage were also found to be mediated by EX-GF-Fr.III. The molecular weight of the three fractions was estimated to be about 163, 40, and 2.8 kDa, respectively. Total sugar and protein contents of the three fractions were 80.3, 61.9 and 89.3%, and 17.3, 35.2, and 10.7%, respectively.	Yang et al., 2007.
Anti-HSV-1 protein (GFAHP)	This antiviral protein from *G. frondosa* fruiting bodies (GFAHP), a molecular weight of 29.5 kDa, could inhibit herpes simplex virus type 1 (HSV-1) replication. Higher concentrations of GFAHP (125 and 500 µg/ml) also significantly reduced the severity of HSV-1 induced blepharitis, neovascularization, and stromal keratitis in a murine model. Topical administration of GFAHP to the mouse cornea resulted in a significant decrease in virus production.	Gu et al., 2007.
Proteolytic enzymes (ProGF)	1. Highly active proteolytic enzymes were found in the fruiting bodies of *G. frondosa* (ProGF). The optimal pH for ProGF activity was pH 3 or 7 using hemoglobin or Hammersten casein as a substrate, respectively. The optimal temperature were 55 °C; 2. These proteases were substrate-specific, mainly cleaving at Ala14-Leu15, Tyr16-Leu17, and Pro28-Lys29 bonds, with occasional cleavage of Phe24-Phe25 bonds in the oxidized insulin B-chain; 3. The ProGF also liberated hydrophobic amino acids, using the oxidized insulin B-chain as a substrate; 4. When soy protein was used as a substrate, valine, leucine, phenylalanine, and tyrosine were selectively released from the hydrolysate.	Nishiwaki et al., 2009.

Table 4. Contd.

Water extract (GFW)	GFW, a water extract of the fruiting body of *G. frondosa*, (1-100 µg/mL) dose-dependently inhibited vascular endothelial growth factor (VEGF)-induced angiogenesis. In addition, GFW inhibited VEGF-induced proliferation, chemotactic migration, and capillary-like tube formation of human umbilical vein endothelial cells (HUVECs). Upon stimulation by VEGF, HUVECs rapidly increased reactive oxygen species production, which was significantly blocked by the treatment with GFW. Moreover, phosphorylation of extracellular signal-regulated kinase 1/2, a downstream signaling molecule following VEGF receptor activation, was also inhibited by GFW.	Lee et al., 2008.
Ethanolic, cold-water and hot-water extracts	At 1 mg/mL, *G. frondosa* T1 and T2 cold-water extracts showed high reducing powers of 1.02 and 0.50, respectively. Chelating abilities on ferrous ions of *G. frondosa* T1 and T2 were higher for cold-water extracts than for ethanolic and hot-water extracts. For the scavenging ability on 1,1-diphenyl-2-picrylhydrazyl radical, *G. frondosa* T1 and T2 extracts were effective in the following order: ethanolic > hot-water > cold-water. The *G. frondosa* hot-water extract showed high scavenging ability on superoxide anions. Total phenols, flavonoids, ascorbic acid and α-tocopherol were the major antioxidant components found in the various *G. frondosa* extracts.	Yeh et al., 2011.
A class I hydrophobin HGFI	A novel hydrophobin from *G. frondosa*, which was named HGFI and belongs to class I. The purified HGFI was found to have 83 amino acids. The protein sequence deduced from the cDNA sequence had 107 amino acids, from which a 24 aa signal sequence had been cleaved off in the mature protein. This signal sequence was 5 aa longer than had been predicted on the basis of signal peptide analysis of the cDNA.	Yu et al., 2008.
Lysophosphatidylethanolamine (LPE)	Lysophosphatidylethanolamine (LPE) from *G. frondosa* (GLPE) was confirmed to induce the activation of MAPK of cultured PC12 cells and was found to suppress cell condensation and DNA ladder generation evoked by serum deprivation, suggesting that the GLPE had antiapoptotic effects. Moreover, GLPE could induce the MAPK cascade [EGFR-MEK1/2-extracellular signal-regulated protein kinases (ERK1/2)] of PC12 cells, the activation of which induced neuronal differentiation and suppressed serum deprivation-induced apoptosis.	Nishina et al., 2006.
Lignocellulolytic enzyme	*G. frondosa* degraded both substrates (oak-sawdust plus corn bran, and oak/corn bran supplemented with coffee spent-ground) decreasing 67 and 50% of their lignin content, along with 44 and 37% of the polysaccharides (hemicellulose and cellulose) respectively. 35.3% biological efficiency was obtained when using oak sawdust plus corn bran as substrate. Coffee spent-ground addition inhibited mushroom production, decreased growth, xylanase and cellulase activities. Enzyme highest activities during colonization achieved were: endoglucanase 12.3, exoglucanase 16.2, β-glucosidase 2.3, endoxylanase 20.3, amylase 0.26, laccase 14.8 and Mn-peroxidase 7.4 U/g dry substrate.	Montoya et al., 2012.
Extracellular laccase	The optimal temperature and pH value for laccase activity were 65 ℃ and pH 2.2, respectively. Enzyme activity was also affected by buffer composition. *G. frondosa* laccase was relatively heat stable. Halide ions could strongly inhibit laccase activity.	Zhao et al., 2012.
Polysaccharide-peptide (GFPPS1b)	1. GFPPS1b had anti-tumor activity and could signigcantly inhibit the proliferation of SGC-7901 cells, whereas slightly influences the growth of human normal liver cell line L-02. When treated with GFPS1b, SGC-7901 cells showed typical apoptotic morphological features. The results showed that GFPS1b could reduce cell survival via arresting cell cycle and inducing apoptosis of tumor cells (2007a); 2. A 21-kDa heteropolysaccharide, coded as GFPS1b from the cultured mycelia of *G. frondosa* GF9801 by hot-water extraction, exhibited more potent anti-proliferative activity on MCF-7 cells than other polysaccharide fractions. GFPS1b was composed of D-glucose, D-galactose, and L-arabinose with a molar ratio of 4:2:1. A analysis revealed that GFPS1b had a backbone consisting of α-(1→4)-linked D-galacopyranosyl and α-(1→3)-linked D-glucopyranosyl residues substituted at O-6 with glycosyl residues composed of α-L-arabinose-(1→4)- α-D-glucose (1→ linked residues (2007b).	Cui et al., 2007a, 2007b.

However, it is the medical functionality and health benefit of bioactive polysaccharide from *G. frondosa* that have illustrated the important role of *G. frondosa*. Accordingly, further theoretical study on *G. frondosa*'s bioactive polysaccharide will play an important role in promoting maitake production.

Conflict of Interests

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

The experimental and characterization works were performed at the Food Science Engineering Research Center in Guizhou University, China. The financial support of this research by a grant from the Natural Science Foundation of China (No. 31060272) and Guizhou Province Natural Science Foundation of China (No.2009106) is gratefully acknowledged.

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academicJournals

Vol. 10(6), pp. 147-155, 14 February, 2016
DOI: 10.5897/AJMR2015. 7744
Article Number: 3F0BC6157104
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Rheological characteristics of *Ganoderma applanatum* exoploysaccharides

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Received 1 September, 2015; Accepted 15 November, 2015

Glucose yeast extract peptone media (GYP) proved to be the most suitable medium for organism growth and exopolysaccharides (EPS) production which recorded 0.320 + 0.01 and 0.1+0.01 (mg/ml) dry weight undershaken and static conditions respectively. Rheological study of *Ganoderma applanatum* exopolysaccharides indicated that viscosity of each EPS concentrations increased with increased shear rate (S.R) values which confirm the dillatent behavior of the different concentrations of EPS. pH value has a valuable effect on the rheological pattern of EPS, with best effect at pH5.5. Furthermore, it was found from the results that sucrose addition as carbon source enhanced the (consistency index cP) viscosity of EPS solution while arabinose decreased EPS viscosity. Therefore, EPS of *G. applanatum* isolated from AlBaha area, Saudi Arabia might have promising applications in field of food and textile industry.

Key words: Growing media, exopolysaccharides, rheology, pH and *Ganoderma applanatum.*

INTRODUCTION

Carbohydrate macromolecules (Glycoproteins, proteoglycans/ glycosaminoglycans, lipoglycans, oligosaccharides and polysaccharides) are the most abundant natural products (Lamari et al., 2003).

Microbial polysaccharides are important since they have many applications in industry and medicine fields. It has been shown that basidiomycete mushrooms contain active antitumor and immunostimulative polysaccharides. Xu et al. (2012) noticed that polysaccharides- protein complex isolated from *Pleurotus pulmonarius* had anticancer activity against liver cancer cells. Also, Cao et al. (2015) reported that polysaccharides extracted from *Pleurotus ostreatus* mycelia markedly reduced both volume and weight of gastric cancer .However, recently there is an evidence from a small number of randomized controlled trials does not support the use of *Glucidum* for treatment of cardiovascular risk factors in people with type 2 diabetes mellitus (Klupp et al., 2015). Current state-to-art researches in this area indicated that published market studies predict that the future of biomass-based polymers is bright, with polysaccharides and polysaccharide-derived polymers being in the forefront. New insights in the structure and functions of polysaccharides in nature open new ways for application developments though polysaccharide engineering. So, it is expecting to see the surge of a deeper understanding of the complexity of biomass, of its bioformation. There is a belief that a new era for polysaccharide science is starting(https://hal.inria.fr/docs/00/57/24/26/PDF/Persin_etal_CarbohydratePolymers2011.pdf).

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Furthermore, may be over 700 species from higher heterobasidiomycetes and homobasidiomycetes fungi have been discovered to contain active polysaccharides that can be derived from fruit-bodies, culture mycelium and culture broths (Reshetnikov et al. 2001). Zhang and Peng (2003) demonstrated the presence of polysaccharide-protein complex in *Ganoderma tsugae* mycelium. The current research designed to study the production and rheology exopolysaccharides from *G.applanatum* for future applications in different aspects. So, the question is what is the behavior of *G. applanatum* EPS rheological behavior?

Polysaccharides are used as thickeners in the printing and textile industry. The rheological properties of the polysaccharide are important in restricting the flow of the dye. Kim et al. (2003b) studied the influence of pH on reheology during exo-polysaccharides production by *Cordyceps militaris* C738. Yun et al. (2002a) studied the effect of carbon source and aeration rate on carbohydrate rheology during red pigment production by *Ganoderma lucidum* in a batch bioreactor. Fazenda et al. (2010) mentioned that controlling the dissolved oxygen (DO) in the fed-batch culture of the medicinal mushroom *G. lucidum* led to a two-fold increase of the maximum biomass productivity compared to uncontrolled DO conditions. Bae et al. (2000) reported that broth rheology changed during mycelial growth and exo-polysaccharide production through *Cordyceps militaris* fermentation. Morris (1990) clarified that the use of polysaccharides changes by studying the rheological properties of water within foods to modify the texture of foods. Cheen et al. (2002) showed that the viscosity of the expolysaccharides produced from *Cordyceps militaris* gave non-significant high values (100-170cP) and its viscosity is lower than the polysaccharide from other mushroom.

The aim of the current work was to study the production of exopolysaccharide (EPS) from *G. applanatum* culture and study its rheological behavior followed by Brockfield rheometer. Rheological results of the isolated polysaccharides will be manipulated for different applications in food, medicine or textile industry in the near future.

MATERIALS AND METHODS

Studied fungal strains

The studied fungal strain was *Ganoderma applanatum* which was collected from Salix stem trees located in different parts in Alhashas and Khaira villages , Albaha, Saudi Arabia (Figure 1).

Isolation and preservation of fungal strains

Isolation of Ganoderma applanatum:

Fresh collected *Ganoderma applanatum* fruiting body was used to isolate its mycelia from the hymenium layer that was picking up with sterilized forceps. The fresh basidospore part of hymenia was inoculated with sterilized malt agar media (Malt 20 g and yeast extract 1 g. It was made up to 1 L of distilled water and inoculated at 28°C for 9 days.

Figure 1. Fruiting body of *Ganoderma applanatum* collected from Albaha area, Saudi Arabia.

Preservation of fungal strains

For maintenance, *G. applanatum* mycelia was collected from a well grown culture and inoculated in potato dextrose agar slants (Peeled Potato, 250 g; Dextrose, 20 g made up to 1 L with distilled water). Monthly subcultures were made on PDA agar slants with dextrose as carbon source.

Media effect on EPS-producers

A screening experiment was carried out using 5 liquid media in order to determine the most suitable medium for EPS production; [Potato dextrose media (PDA), Malt dextrose media (M). Glucose yeast extract peptone media (GYP) as designed by Wang et al. (2001) consists glucose (40 gm), yeast extract (10 gm) and peptone (5 gm). It is made up to 1 Liter in distilled water. Czapek's media consists of glucose 20 gm, $NaNO_3$ 2 gm, KH_2PO_4 1 gm, MgSO4. $5H_20$ 0.5gm, KCl 0.5 gm and $FeSO_4$. $7H_2O$ 0.001 gm. Both static and shaken cultures were tried in order to select the best for EPS production.

Culture type

Shaken culture

Erlenmeyer flasks (250 ml) were filled with 50 ml of 5 types of media which were Potato dextrose media (PDA), Malt dextrose media (M), Glucose yeast extract peptone media (GYP), and Czapek's media.

The media was inoculated with the fungal disc by using sterilized cork borer of 1 cm diameter. It was obtained from solid parent fresh culture of tested fungi subcultured on solid PDA. The fungal strain was *G. applanatum*. The cultivated flasks with initial pH 6.2 were then incubated at 25°C under shaking condition in a rotary shaker (150 rpm) for 5 days for all tested media (Yun et al., 2002a).

Table 1. Effect of different media on exopolysaccharide production and mycelial growth in *Ganoderma applanatum* under shaken conditions at (150 rpm).

Media	Mycelial drywt g/50 ml	EPS drywt mg/ml	Final pH
Czapek's medium	0.1532±0.01	0.096±0.013	5.8±0.05
Potato dextrose medium.	0.299±0.001	0.196±0.02	5.9±0.1
Malt medium	0.1189±0.008	0.116±0.02	6.1±0.1
Glucose yeast extract peptone	0.4662±0.057	0.320±0.01	5±0.1

At the end of incubation period, EPS was precipitated from the culture filtrate, then lyophilized and its weight was estimated. Also, the dry weight of mycelium was measured after repeated washing of the mycelial pellet with distilled water and drying at 70^{o}C for overnight to a constant weight.

Static culture

The same previous procedure was carried out without shaking in rotary shaker and incubated for 15 days.

Isolation and purification of *Ganoderma applanatum* exopolysaccharides

Ethanol precipitation

The culture filtrate from shaken flasks was centrifuged at 10000 g for 20 min and the resulting supernatant was filtered (0.45 μm, Millipore). The resulting culture filtrate was mixed with 4 times the volume of absolute ethanol, stirred vigorously and kept overnight at 4^{o}C. The precipitated EPS was centrifuged at 10000 g for 20 min discarding the supernatant (Bae et al., 2000). Details for EPS purification and chemical structure will be presented elsewhere.

Rheology of EPS

The theological behavior of different EPS-treatments was studied using cup and Bob viscometer Brookfield DV-III Rheometer, Rheocale 1.4 software, using USA spindle: ULA, model: RV). The sample was sheared in the space between the outer wall of the bob and the inner wall of the cup into which the bob fits. The rotation ranged between 100-250 rpm. The rheograms and viscosity curves were platted using the supplied software. This program needed to be supplied with two readings namely upper and lower ones of torque and rotation (rpm) to detect the shear thinning index (ST). The EPS behavior was deduced according to (ST) values as follows: "1" means that the system behaves as newtonian, lower than "1" indicates that the system is dilatant and more than "1" means that the system is pseudoplastic (Martin et al., 1983).

The following formulas were used to calculate and display the Rheometer data after each packet of data was obtained from the DV-III.

Viscosity (cP) = 100 x TK x SMC x Torque RPM
Shear Rate (1/Sec) = RPM x SRC
Shear Stress (Dynes/Cm2) = TK x SMC x SRC x Torque

Where:

RPM = Current Rheometer spindle speed in RPM
TK = Model spring constant
SMC = Current spindle multiplier constant
SRC = Current spindle shear rate
Torque = Current Rheometer torque in percent expressed as a number between 0 and 100.
*TK, SMC and SRC are obtaining from standard known tables.

Viscosity-concentration relationships

The viscosity of purified EPS at concentrations of 0.25, 0.5, 1.5 and 2.5% was determined at a constant temperature of 25°C.

The effect of different levels of pH on viscosity of EPS

The effect of different pH values at 4.5, 5, 5.5 6.0 and 6.5 on EPS at concentration of 0.25% (W/V) was tested by measuring the viscosity at constant temperature of 25°C (Bae et al., 2000).

Why is pH measured against 0.25% EPS of *G. applanatum*? Because, it has been mentioned by many researchers that pH influences the stability of foams with protein–polysaccharide complexes at their interfaces and plays a vital role in ionization process of solutions, so it is kept as minimum as possible.

Effect of different carbon sources on viscosity

The effect of different carbon sources (arabinose, galactose, glucose, sucrose and carboxymethyl cellulose) on the viscosity of EPS at constant concentration of 0.25% (W/V) was tested by measuring the viscosity at constant temperature of 25°C (Yun et al., 2002b).

RESULTS

Glucose yeast extract peptone media (GYP) proved to be the more suitable for organism growth and exopolysaccharides production which recorded 0.320 + 0.01 and 0.1+0.01 dry weight mg/ml under shaken and static conditions respectively (Tables 1 and 2).

Rheological characterization of EPS

Effect of EPS concentration on its rheology (flow behavior)

The rheological characters of different concentrations of EPS (0.25, 0.5, 1.5 and 2.5%) (w/v) in distilled water were studied using a brook field digital DV III rheometer linked to a computer for processing, recording and storing of data.

Table 2. Effect of different media on exopolysaccharide production and mycelial growth in *Ganoderma applanatum* under static conditions.

Media	Mycelial dry wt. g/50 ml	EPS dry wt. mg/ml	Final pH
Czapek's medium	0.584±0.04	0.064±0.001	5.8±0.05
Potato dextrose medium.	0.595±0.09	0.082±0.01	5.9±0.1
Malt medium	0.386±0.02	0.05±0.005	6.1±0.1
Glucose yeast extract peptone	1.183±0.2	0.12±0.01	5±0.1

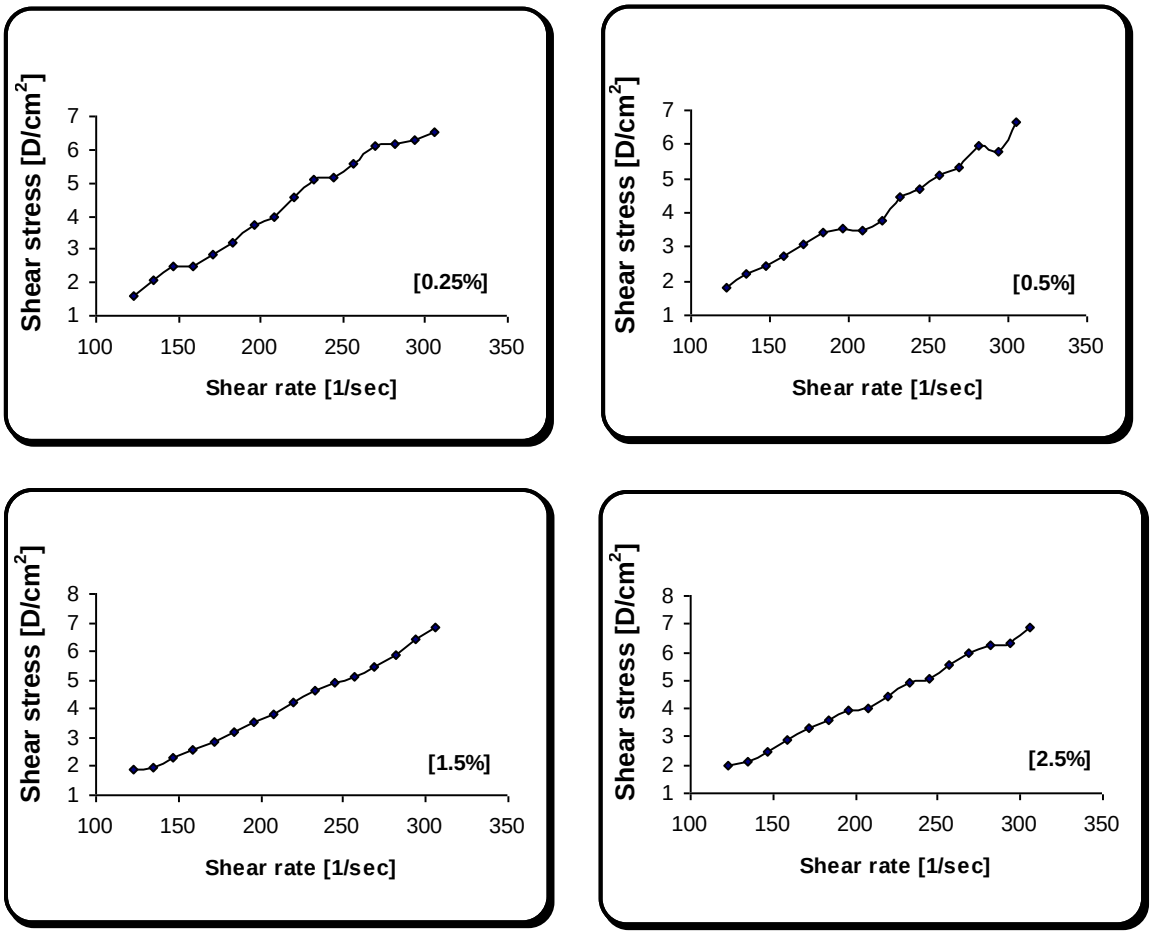

Figure 2a. Shear rate-shear stress relationships.

The rheogram and viscosity curves of different EPS concentrations in distilled water were plotted using Rheocale 1.4 software computer program. The rheograms are presented in Figure 2a and b. It can be noticed that the increment of shear rates (S.R) was accompanied with an increase in the shear stress (S.S), indicating all concentrations of EPS followed dillatent behavior. For further confirmation of these results, the values of ST index (shear thinning index) as well as the solution behavior were determined. This program (Rheocale 1.4 software program) needs to be supplied with two readings namely upper and lower ones deduced from the computer program according to ST values as follows: "1" means that system behaves as Newtonian; less than "1" indicates that the system is dillatent and more than "1" means that the system is pseudo plastic.

This method, which was convenient in identifying the flow behavior at upper and lower shearing rates, was applied for every solution in this work and it was found that the ST index for all solution was less than "1";

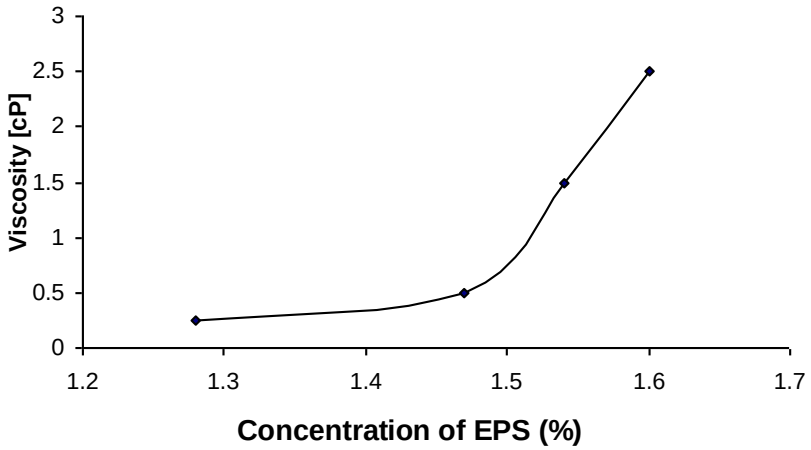

Figure 2b. The flow behavior of EPS from *G. applanatum* at concentration (0.25-0.5%) as computed from Rheocale V.1.4.

Table 3. Detection of ST index of different EPS concentrations.

EPS conc. (w/v)	* RPM	Torque %	** ST Index
0.25	100	2 ± 0.13	0.8 ± 0.04
	110	2.6 ± 0.2	
0.5	100	2.4 ± 0.15	0.87 ± 0.06
	110	2.8 ± 0.20	
1.5	100	2.5 ± 0.10	0.92 ± 0.08
	110	2.9 ± 0.30	
2.5	100	2.7 ± 0.20	0.96 ± 0.09
	110	3.2 ± 0.20	

* ST index is shear thinning index. ** RPM means round per minutes.

Table 4. Relation between EPS concentration and viscosity.

EPS conc. (w/v)	Viscosity (cP)**
0.25	1.30 ± 0.10
0.5	1.47 ± 0.07
1.5	1.54 ± 0.15
2.5	1.60 ± 0.13

* Data at constant (122.3) shear rate. ** cP index is viscosity index.

indicating dillatent behavior of the system (Table 3).

Viscosity

Table 4 Indicates that viscosity of each EPS concentration increased with increasing S.R values which confirm the dillatent behavior of the different concentrations of EPS. It is clear that, the viscosity of EPS increased with increased EPS concentrations.

Effect of different level of pH on the rheology of EPS

The effect of different level of pH on the flow behavior of EPS was studied using Rheocale 1.4 software computer program. The parameters of the previous experiment were studied and the results are illustrated in Table 5. It was found that the viscosity increased with increasing S.R values until reaching pH value 5.5 after which it decreased. So, pH value affects the rheological pattern of EPS. Concerning the viscosity of EPS, pH=5.5 increased the viscosity of EPS solutions as illustrated in Table 6.

Effect of different carbon sources on the rheology EPS

The results of the current experiment are shown in Table 7. It was noticed that different carbon sources affect the rheology of EPS and all solutions had dillatent behavior.

Addition of sucrose increased the viscosity of EPS solutions (Table 8 and Figure 3).

As summarized in Table 8, it could be concluded that

Table 5. Detection of ST index of different level of pH at constant concentrations of EPS at 0.25% (%, w/v).

pH values	*RPM	Torque %	** ST Index
4.5	100	2.0 ± 0.1	0.74 ± 0.06
	125	2.8 ± 0.2	
5	100	2.1 ± 0. 2	0.84 ± 0.07
	125	3.1 ± 0. 1	
5.5	100	2.4 ± 0.2	0.95 ± 0.09
	125	3.2 ± 0.3	
6	100	1.15 ± 0.1	0.72 ± 0.06
	125	1.43 ± 0.2	
6.5	100	1.09 ± 0.1	0.62 ± 0.05
	125	1.42 ± 0.1	

Table 6. Effect of different pH values on viscosity of EPS at constant concentrations of EPS 0.25% (%, w/v).

pH values	* Viscosity (cP)
4.5	1.22 ± 0.10
5	1.34 ± 0.09
5.5	1.54 ± 0.14
6	1.20 ± 0.11
6.5	1.00 ± 0.10

Data at constant (122.3 1/Sec.).

Table 7. Detection of ST index of different carbon sources at constant concentrations of EPS at 0.25% (%, w/v).

Carbon sources (%, w/v)	*RPM	Torque %	** ST Index	*** cP index
Arabinose	100	2.4 ± 0.20	0.74 ± 0.06	0.17 ± 0.01
	110	2.5 ± 0.15		
Galactose	100	2.2 ± 0.20	0.94 ± 0.08	0.13 ± 0.01
	110	2.5 ± 0.16		
Carboxy methyl cellulose (CM)	100	2.4 ± 0.21	0.88 ± 0.07	0.18 ± 0.01
	110	3.0 ± 0.25		
Glucose	100	2.3 ± 0.14	0.97 ± 0.08	0.22 ± 0.02
	110	2.7 ± 0.25		
Sucrose	100	2.3 ± 0.20	0.82 ± 0.07	0.26 ± .016
	110	3.1 ± 0.30		

*** cP index is viscosity index.

sucrose addition enhanced the viscosity of EPS solutions, while arabinose addition decreased EPS viscosity.

DISCUSSION

Exopolysaccharide and endo polysaccharide produced by different fungi, bacteria, algae and endopolysaccharides are found also in plants. Their importance came from their applications as anti-inflammatory and immunoactive (Lull, 2005). Also, as antifibrotic agent on liver fibrosis in rats (Nan et al., 2001). Enopdysaccharides also have a hypoglycemic activity (Kiho, et al., 1999) and exopolysaccharides have

Table 8. Effect of different C. sources on viscosity of EPS at constant concentrations of EPS 0.25% (%, w/v).

Carbon sources 0.25 (%, w/v)	*Viscosity (CP)
Arabinose	1.27 ± 0.1
Galactose	1.44 ± 0.13
Carboxy methyl cellulose	1.40 ± 0.13
Glucose	1.41 ± 0.1
Sucrose	1.47 ± 0.10

*Data at constant (122.3 1/sec.).

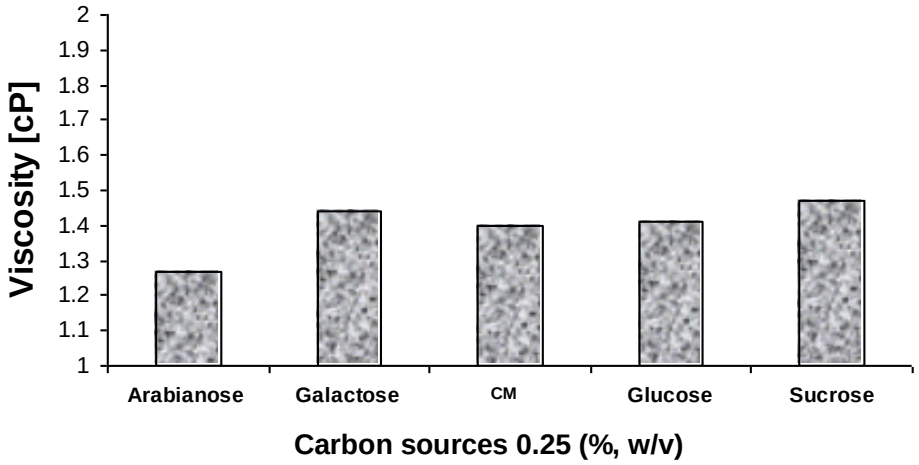

Figure 3. Effect of different C. sources on viscosity of EPS at constant concentrations of EPS 0.25% (%, w/v), (CM): Carboxy methyl cellulose.

a hypoglycemic activity (Jeong et al., 2001). Exopolysaccharides have a protective effect from experimental infections (Nagumo, 1973). They are important as thickening agents (Clementi et al., 1998).

The obtained data indicate that, the shaken culture was favorable for maximum EPS production more than static culture. Our current data indicate that glucose yeast extract peptone media (GYP) is the suitable medium for organism growth and exopolysaccharides production which recorded 0.320 + 0.01 and 0.1+0.01 dry weight mg/ml under-shaken and static conditions respectively (Tables 1 and 2). Osman et al. (2014) have found that yeast extract and peptone led to high production of EPS and mycelial biomass. However, Zapata et al. (2012) studied the effect of different non-conventional carbon sources on mycelial biomass and polysaccharides production in the submerged culture of Lingzhi or Reishi medicinal mushroom, *Ganoderma lucidum*, in less time, using non-conventional carbon sources to minimize the high costs of current culture media. The optimal medium composition was defined as (g/L): 50 of barley flour, 0.2 of KH_2PO_4, 0.1 of $MgSO_4.7H_2O$, and 1 NH_4Cl. Cultivated under this complex culture medium, the mycelial biomass production was 23.49 ± 0.37 g/L; the extracellular polysaccharides production was 2.72 ± 0.11 g/L; the intracellular polysaccharides production was 2.22 ± 0.06 g/L. Fraga et al. (2014) have stated that a low peptone level (1.65 g L^{-1}) favored mycelium biomass, EPS purity, but a higher supply of peptone (4.80 g L^{-1}) is needed for maximum EPS production. Concerning the carbon source, Shen et al. (2013) reported that xylose is the suitable carbon source for maximum production of EPS from *P.pulmonarius.*

This is almost similar to that concluded by Kim et al. (2003a) who studied the effect of aeration on the production of mycelial biomass and exopolysaccharides in fungus *Paecilamycess sinclairii*. They concluded that aeration is associated with higher hyphal density and increased EPS.

Due to the importance of polysaccharide, a lot off researchers employed their work to study the production of polysaccharides for their medical importance (Mizuno, 1996). Also polysaccharides have many industrial importance like those of *Cordyceps militaris* C738 and *Paecilomyces japonica* (Sinha et al., 2001).

In the present investigation, the effects of different media under shaken conditions at velocity (150 rpm) on exopolysacchrides production and mycelial growth were

studied and also under static conditions. The results for shaken conditions confirmed the maximum yield of exopolysaccharide (0.316 mg/ml) for *Ganoderma applanatum* on GYP medium for five days, but was less under static conditions; the EPS was 0.1 mg/ml on GYP media static conditions. These results were similar with those obtained from Martinez et al. (1996) who found that the exopalysaccharid produced from *Hericium erinaceus* and *Hericium laciniatum*d yield more EPS on GYP medium in shaken condition (100 rpm) at 25°C for 25 days.Yun et al. (2002a) studied the EPS production using different media (MCM=mushroom complete medium, Ym= yeast extract malt medium and PMP= malt extract potato dextrose broth medium) to grow different edible mushrooms *Agrocybe cylindracea*, *Ganoderma lucidum* No. 1., *Lentinus edodes*, *Pleurotus ostreatus* No. 1*, P. ostreatus* No. 2 and *P. sajor-caju*. From their result, the EPS production (1158 mgL^{-1}) was achieved in *G. lucidum* under shaken conditions (150 rpm) on MCM medium more than YM medium. The results of mycelial growth of various mushrooms were in the order Cordyceps militaris > *P. ostreatus* No. 1 > *P. trametes*.

In the present study, the rheological property of EPS was described whereas, factors affecting the viscosity such as concentrations of EPS solutions pH level and carbon sources were studied.

The flow property of different concentrations of experimental *G. applanatum* indicated that the system had a dillatent behavior, that is the viscosity of the system increased by increasing shearing rate.

In the present work, shear thinning index measurement was done using Rheocale 1.4 software program at two readings namely high and low ones of torque and rotation (rpm). The experimental EPS of *G. applanatum* has also shear thinning that is, ST index <1 because its viscosity increased with increasing shear rate. This confirms the dilatants behavior of EPS.

So, with increasing EPS concentrations the viscosity increased and EPS increased due to increasing biomass. Relatively few studies have been aimed at quantifying the influence of biomass concentration on rheology. *Penicillium chrysogenum* is a good example in that viscosity is higher when the cell is highly grown (Warren et al., 1995). Yun et al. (2003) concluded that viscosity of EPS of *Cordyceps militaris* was much influenced by EPS conc. than by mycelial biomass. In the present investigation, the effect of different levels of pH on the flow behavior of EPS was studied using Rheocale 1.4 software computer program. It was found that it increased with increased pH value 5.5 after which it decreased. These results are similar to that obtained by Yun et al. 2003), who study the influence of pH on broth rheology and EPS production. The EPS production and viscosity increased at pH value 6. Also, similar results were concluded by Wang and McNeil (1995), who reported that EPS production and EPS viscosity are affected by pH culture.

In present study, it was found from the results that sucrose addition as carbon source enhanced the (consistency index cP) viscosity of EPS solution while arabinose decreased EPS viscosity.

Yun et al. (2002b) studied the influence of carbon sucrose on EPS rheology of *Ganoderma lucidum*. They found that the consistency index (cP) in sucrose medium was markedly higher than that in starch medium while the higher value of flow behavior index (ST) was indicated at the late stationary phase in starch medium. The non-Newtonian behavior (dilatent) is relatively predominant at lower shear rate and low (ST) index (Kim et al., 1983).

Conclusion

In the present study, it was found that glucose yeast extract peptone medium is the most suitable for *G. applanatum* growth and exopolysaccharides production. EPS rheology has a dillatent behavior. The viscosity of each EPS concentration increased with increasing S.R values which confirm the dillatent behavior of different concentrations of EPS. pH value affects the rheological pattern of EPS where pH 5.5 increased the viscosity of EPS. Sucrose addition as carbon source enhanced the (consistency index cP) viscosity of EPS solution while arabinose decreased EPS viscosity. These data give a promising application field of food application and textile industry.

Conflict of Interests

The authors have not declared any conflict of interests.

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Vol. 10(6), pp. 156-171, 14 February, 2016
DOI: 10.5897/AJMR2013.7765
Article Number: DF77D1F57110
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Evaluation of antifungal activity of novel marine actinomycete, *Streptomyces* sp. AA13 isolated from sediments of Lake Oubeira (Algeria) against *Candida albicans*

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Received 21 September, 2015; Accepted December 11, 2015

A new actinomycete strain, designated AA13 was isolated from a marine sediment sample obtained from Lake Oubeira, in the North-East of Algeria and selected for its antifungal activities against *Candida albicans*. Morphological, physiological and biochemical properties and 16S rRNA gene sequencing strongly suggested that this strain was a new species, which belonged to the genus *Streptomyces*. Study of the influence of different nutritional compounds and culture conditions on growth and production of compounds with antifungal activity by the *Streptomyces* sp. strain AA13 indicated that the highest biomass and biological activities were obtained by utilizing the glycerol and peptone as carbon and nitrogen sources, respectively, with pH 7.0 and incubation temperature of 30°C. Two bioactive spots were detected by analysis of the ethyl acetate extract by thin-layer chromatography (TLC) and bioautography analyses. Among these bioactive compounds (antibiotics), a complex AA13-B that showed the interesting antifungal activity, was selected and purified by high performance liquid chromatography (HPLC), which indicated the presence of three peaks. Interestingly, the infrared spectroscopy (IR) studies showed that the molecule AA13-B2 contain an aromatic ring substituted by aliphatic chains. However, the investigations which determine the structure of the antifungal molecule are in progress.

Key words: *Streptomyces*, antifungal activity, *Candida albicans*, Lake Oubeira, sediments.

INTRODUCTION

The incidence of invasive fungal disease (IFDs), have been widely studied in recent years, largely because of the increasing population at risk. The FDs, are a significant cause of morbidity and mortality in

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immunocompromised patients, and are associated with increased healthcare costs. In fact, FDs, which is nevertheless a serious international health problem, has dramatically increased over the past few decades in both the hospital and community settings paralleling the rising number of immunocompromised patients (Joshua, 2006), and immunodeficiency diseases (Kwon-Chung and Bennett, 1992; Zaehner and Fiedler, 1995; McGinnis et al., 1999; Van Burik and Magee, 2001; Watve et al., 2001; Bennett and Klich, 2003), as well as the changing spectrum of pathogens and antibiotic resistance (Tanaka and Mura, 1993; Culotta, 1994; Cassell, 1997).

Candida albicans is an opportunistic human fungal pathogen that causes candidiasis. It is found in the oral and gut mucosae in approximately 50-60% of healthy humans. Nevertheless, *C. albicans* can also be the agent of different types of infections, reaching from relatively harmless superficial infections like vaginal candidiasis or oral thrush of newborns to life-threatening blood stream infections (Glick and Siegel, 1999).

With the exponential emergence of *C. albicans* becoming resistant to antifungal antibiotics, the problems of drug resistance, patient sensitivity and inability to control infectious diseases have given real impetus for continuous search for new antibiotics all over the world (Chopra et al., 1997). This situation highlights the need for advent of safe, novel, and effective antifungal compounds. Microbial natural products still appear as the most promising source of the future antibiotics that the society is expecting. It is well known that the actinobacteria are the potential products of antibiotics, which could profitably be developed in the pharmaceutical industries. Among the so far reported actinobacteria organisms, is the genus *Streptomyces*, which are widely recognized as industrially important microorganisms because of their ability to produce many kinds of novel secondary metabolites including antibiotics, seem to offer a wide range of advantages, including accelerated accumulation of biomass (Williams et al., 1983; 1989; Crandall and Hamil, 1986; Korn-Wendisch and Kutzner, 1992; Ozgur et al., 2008). Furthermore, the importance of streptomycetes to medicine results from their production of over two-thirds of naturally derived antibiotics in current use (and many other pharmaceuticals such as anti-tumor agents and immunosuppressants), by means of complex secondary metabolic pathways (Miyadoh, 1993; Tanaka and Mura, 1993).

In the course of screening for new antibiotics, several studies are oriented towards isolation of streptomycetes from different habitats. Marine sediments constitute a large carbon reservoir and an untapped source for many useful drugs and an assessment of this potential is imperative. It is an environment with numerous microorganisms (Whitman et al., 1998; Biddle et al., 2005). Recent investigations indicate the tremendous potential of marine actinomycetes, particularly *Streptomyces* sp. as a useful and sustainable source of new bioactive natural products (Benouagueni et al., 2015).

Of particular relevance to the present study, the Lake Oubeira located in the North-East of Algeria, being an unexplored area in this field, with unique ecological niches and rich in biodiversity. The microbiology of sediment has to be further explored in order to get benefit out of the precious bio-wealth, because any new antibiotics and its producing organisms have been a great demand from the health care point of view to combat against the existing and emerging drug resistant pathogens. Accordingly, the present study reports, for the first time, on the effects of various nutritional and environmental factors on cell growth and antimicrobial metabolite production from *Streptomyces* sp. strain AA13, newly isolated from the Lake Oubeira, against *C. albicans*. It also provides basic information on the partial characterization of the bioactive molecules from the strain AA13.

MATERIALS AND METHODS

Substrates and chemicals

Unless specified, all substrates, chemicals, and reagents were of the analytical grade or highest available purity and purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Sample collection

Sediment samples were collected from the sediments of the Lake Oubeira in the North East of Algeria, using a sediment grab sampler and transferred to one liter sterile plastic containers. Next, the container volume was filled with 60% sediment and 40% seawater from the sampling site. This was done in order to ensure aerobic conditions under storage upon processing. All the samples were kept at 4°C until use.

Strain isolation

One gram of sediments was taken in 9 ml of distilled water and shaken vigorously for 1 min. Different aqueous serial dilutions (10^{-1} to 10^{-3}) of the suspension were carried out, and were decanted for 30 min. The supernatant (100 µl) was spread on medium casein-starch recommended by Shirling and Gottlieb (1966). The medium was supplemented with 10 µg/ml of Gentamicin and 25 µg/ml of Nystatin. Plates were incubated at 30°C for 21 days and colonies were purified by streaking on medium called International *Streptomyces* Project (ISP)-2 agar.

Cultural and microscopic characteristics

Cultural features of strain AA13 were characterized following the directions given by the ISP media Viz., ISP-1, ISP-2, ISP-3, ISP-4, and ISP-5. The production of melanoides pigments was carried out on ISP-6 and ISP-7 at 30°C for 7-14 days (Shirling and Gottlieb, 1966), and the Bergey's Manual of Systematic Bacteriology (Cross, 1989).

Morphology of spore bearing hyphae with the entire spore chain was observed with a light microscope (Model SE; Nikon) using cover-slip method in ISP-2 media after Gram staining (You et al., 2005).

Biochemical and physiological characteristics

These characteristics included the ability of the isolate to utilize different carbon. It was determined on plates containing ISP basal medium 9 to which carbon sources were added to a final concentration of 1% (Pridham and Gottlieb, 1948), using glucose as positive control and using carbon source free medium as negative control (for comparison more suitable). The plates were incubated at 30°C for 7 to 21 days.

Hydrolysis of gelatin, starch and nitrate reduction was examined as described by Williams et al. (1983), Gordon et al. (1974) and Boudjella et al. (2006), respectively. The degradation of casein was given according to method of Gordon et al. (1974).

Analytical profiling index (API) strip tests were carried out to identify the genus to which the AA13 strain belonged. The nature of Gram staining, motility in hanging drop preparations, and physiological and biochemical characteristics of the strain were investigated using API 50 CH and API ZYM strips in accordance with the manufacturer's instructions (bioMérieux, SA, Marcy-l'Etoile, France). The reading was carried out after incubation at 30°C for 24 h and then 48 h (Humble et al., 1977). The APILAB software (bioMérieux) was then used to obtain the percentage admitted, the identity of the bacteria sought by comparison with standard profiles. The other physiological and biochemical characteristics were determined using the method described by Williams et al. (1983). All tests were performed at 30°C.

Antifungal bioassay

C. albicans ATCC 10231 strain resistant to antibiotics like Flconazole, Nystatin and Amphotericin B was grown at 30°C on Sabouraud dextrose medium. The culture was stored at 4°C. The antifungal activities of the isolates were determined by using the double layer agar method as described by You et al. (2005). The actinomycetes were inoculated on Petri dishes containing 15 ml ISP-2 agar and incubated at 30°C for 5 days, then Sabouraud dextrose medium was poured onto the basal layer containing strain AA13 colonies, and the *C. albicans* were plated onto the top layer. The inhibition zones were measured after incubation at 30°C for 48 h.

Extraction of the genomic DNA

The total preparation of DNA from the strain AA13 was carried out according to Hopwood et al. (1985). Preparations of small size of plasmids from *Escherichia coli* were given according to Sambrook et al. (1989) and Fourati-Ben Fguira et al. (2005). Digestion with ribonucleases of restriction, the separation of the fragments of DNA by the electrophoresis on agarose gel, the dephosphorylation with the alkaline phosphatase of calf intestine, the ligation of the fragments of DNA and the transformation of *E. coli* were all performed in accordance with the method of Sambrook et al. (1989).

Amplification by polymerase chain reaction (PCR)

The 16S rRNA gene of the strain AA13 was amplified by PCR using two universal primers: forward primer, called pA: AGA GTT TGA TCC TGG CTC AG (8-28), and reverse primer, called pH: AAG GAG GTG ATC CAG CCG CA (1542-1522), designed from base positions 8 to 27 and 1541 to 1525, respectively, which were the conserved zones within the rRNA operon of *E. coli* (Edwards et al., 1989; Mellouli et al., 2003). Roughly, 50 ng of the DNA matrix is employed with 30 pmol each primer by a final volume of the reaction mixture of 50 µl. To improve the denaturation of the DNA, 50% (v/v) DMSO was added with the mixture to the reaction. Amplification was carried out with a thermocyclor automated (Perkin-Elmer) by using one unit of DNA Tag polymerase (Stratagene, Agilent Technologies, Santa Clara, CA, USA) according to the following amplification profile: a stage of denaturation of the matrix: 94°C (3 min), followed by 40 cycles of which each one include: a stage of denaturation: 94°C (30 s), a stage of hybridization of the oligonucleotides to the matrix: 50°C (1 min), a stage of elongation: 72°C (10 min). The product of PCR was analyzed by electrophoresis on agarose gel 0.8%.

Sequencing of the 16S rRNA gene

The nucleotide sequences of both strands of the cloned 16S rRNA gene sequence were determined using BigDye Terminator Cycle Sequencing Ready Reaction kits and the automated DNA sequencer ABI PRISM® 3100-Avant Genetic Analyser (Applied Biosystems, Foster City, CA, USA). Multiple nucleotide sequence alignment was performed using the BioEdit version 7.0.2 software program and CLUSTALW program at the European Bioinformatics Institute server (http://www.ebi.ac.uk/clustalw). Phylogenetic and molecular evolutionary genetic analyses were performed using the molecular evolutionary genetics analysis (MEGA) software version 4.1. Distances and clustering were calculated using the neighbor-joining method. The tree topology of the neighbor-joining data was evaluated by Bootstrap analysis with 100 re-samplings.

Optimization of nutritional and cultural conditions

Culture medium on growth and bioactive metabolite production of the strain were optimized by using different parameters such as carbon and nitrogen sources, incubation temperature and pH as follows:

Effect of carbon and nitrogen sources

Different carbon and nitrogen sources were used to replace the carbon and nitrogen sources in basal medium, starch nitrate broth and all other components were kept constant. The sources were sterilized separately and added just prior to inoculation. Glucose, maltose, lactose, sucrose and glycerol were added separately as carbon sources into the basal medium at 1% concentration. Different nitrogen sources such as KNO_3, tryptone, peptone and meat extract were provided separately into the basal medium at 1% concentrations. The respective biomass and antifungal metabolites production were also recorded.

Effect of incubation temperature

The optimum temperature was assayed by incubating the production medium at temperature ranges varying from 25 to 37°C, and maintaining all other conditions at optimum levels at original concentration.

Effect of initial pH of the culture medium

To determine the influence of initial pH value of culture medium on growth and bioactive metabolite production, the strain AA13 was

cultivated in basal medium with different initial pH values (5-11). The pH was adjusted using hydrochloric acid or sodium hydroxide at 0.1 M.

Biomass determination

Samples (10 ml) were centrifuged at 5000 rpm for 5 min. The supernatants were discarded and the cell pellet washed twice with distilled water and then dried at 70°C. Dry weight was measured and recorded.

Disk diffusion method

A paper disk, was impregnated with the supernatant and then placed on the surface of Muller Hinton agar pre-inoculated with the pathogenic strain test and incubated at 30°C after 48 h. Plates were examined for evidence of antimicrobial activities represented by a zone of inhibition of growth around the paper disk.

Extraction and purification of active compounds

The cultivation medium ISP-2 from 1000 ml of shake flask culture of the *Streptomyces* sp. strain AA13 at 30°C for 7 days was harvested to remove the biomass. The cell-free supernatant was extracted with an equal volume of organic solvent. Four extraction solvents were tested for effectiveness, including n-hexane, ethyl acetate, acetic acid 5% and n-butanol. Each organic extract was evaporated to dryness using a Rotavapor. The resulting dry extract was recuperated in 1 ml of methanol (Zitouni et al., 2005; Boudjella et al., 2006).

For the TLC analysis, the crude extract was loaded using a capillary tube on silica-coated plate (Merck, Darmstadt, Germany) on the line drawn around 1.5 cm from one of the plate. The plate was immersed in the solvent just below the line where samples were loaded. The solvents used were n-butanol-acetic acid-water (3:1:1). After the solvent front reached about half of the plate, the plate was removed and dried. Pigment spots were detected by bioautography (Betina, 1973) on silica gel plates seeded with *C. albicans*. The active spots were visualized under UV irradiation at 254 (absorbance) and 365 nm (fluorescence) and Rf values were calculated (Palanichamy et al., 2011). The fraction that showed antifungal activity was purified by HPLC under the following conditions: C18 column (7.8 × 300 mm), mobile phase gradient elution system of methanol-water, flow rate, 1 ml/1 min. InfraRed spectrum was obtained by dispersing 2 mg of the analyzed molecule in potassium bromide (KBr) with IR (Nicolet 470 FTIR) spectrometer.

Statistical analysis

All determinations were performed in three independent replicates, and the control experiment without xylanase was carried out under the same conditions. The experimental results were expressed as the mean of the replicate determinations and standard deviation (mean ± SD). Statistical significance was evaluated using t-tests for two-sample comparison and one-way analysis of variance (ANOVA) followed by t-test. The results were considered statistically significant for P values of less than or equal to 0.05. Statistical analysis was performed using the R package Version 3.1.1 (Vanderbilt University, USA).

Nucleotide sequence accession number

The 16S rRNA gene sequence (1507 bp) of strain AA13 has been deposited into publicly available databases (DDBJ/EMBL/ GenBank) under the accession number JQ965757.

RESULTS

Cultural and microscopic characteristics

Actinomycetes have been intensively studied in several underexplored environments; niche and extreme habitats in various parts of the world in the last few years. However, their presence in marine sediments has not been extensively investigated, although their ubiquitous presence in the marine sediments has been well-documented (Jensen et al., 1991; Takizawa et al., 1993; Moran et al., 1995). The characterization of *Streptomyces* sp. was studied by following methods recommended by ISP.

The morphology of strain AA13 in different ISP media showed filamentous bacterium with extensively branched aerial mycelia and grew well on ISP medium, which include both synthetic and organic media described and the colonies were spreading. The aerial mycelium of strain appeared grayish with white outline on casein starch (Figure 1A) and ISP-2 media, greyish white on ISP-1 and ISP-5, grayish on ISP-3, 4, 6, and 7 media and the substrate mycelium was light and dark brown, light and dark yellow (Table 1). The strain produced brown diffusible pigments in ISP-6 and ISP-7.

The observation with light microscopy (100× magnification), on the ISP-2 to ISP-5, showed that the spore-bearing hypha were Spiral chain (S), Rectus-Flexibilis (RF), and Retinaculum-Apertum (RA) (Figure 1B). The number of the spores was higher than ten, which made them to be referred to as the long chains of spores. The vegetative hyphae was branched but not fragmented.

The characteristics of AA13 strains were compared with those of the known species of actinomycetes described in Bergey's manual of systematic bacteriology (Whitman et al., 1998), and obtained morphological properties suggested strongly that strain AA13 belonged to the genus *Streptomyces*.

Physiological and biochemical characteristics

The physiological and biochemical tests were performed according to standard methods described for actinomycetes. The strain showed an ability to assimilate 21 carbon sources but it could not utilize 11 other sugar (Table 2). It produces seven enzymes such as phosphatases, lipase, proteases, urease and osidases (Table 3). This strain also peptonized milk, liquefied gelatin and reduced nitrate, but it did not produce H_2S.

Molecular identification of the strain AA13

A rapid method for the identification of filamentous

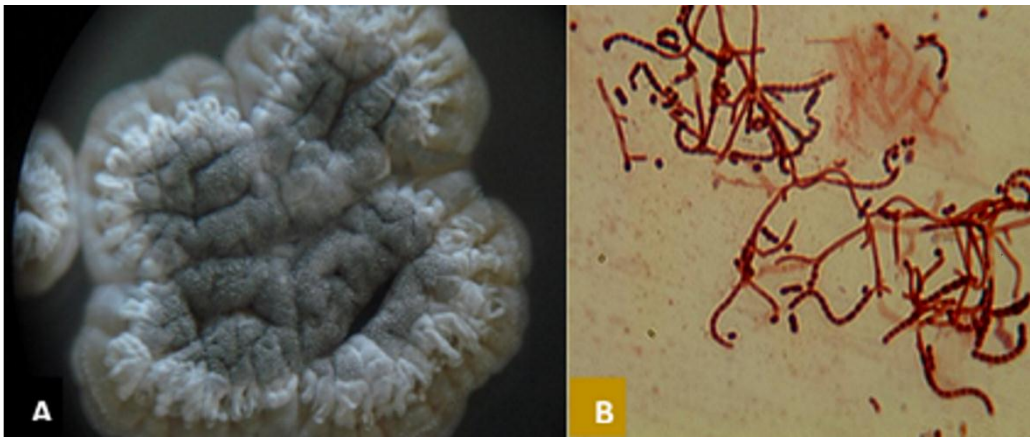

Figure 1. Cultural and micro-morphological characteristics of *Streptomyces* sp. AA13. (A) Colonies in casein-starch medium. (B) Micromorphology of aerial hyphae and spore chain structure (light microscope 100x).

Table 1. Culture characteristics of isolate AA13 in different media after 7 days of incubation at 30°C.

Medium	Growth	Aerial mycelium	Substrate mycelium	Soluble pigment
Casein-starch	Good	Grey with white outline	Light brown	-
ISP-1	Moderate	White-Grey	Darkbrown	-
ISP-2	Good	Grey with white outline	Light brown	Brown
ISP-3	Good	Grey	Light brown	-
ISP-4	Good	Grey	Light brown	-
ISP-5	Good	White-Grey	Darkyellow	-
ISP-6	Good	Grey	Light yellow	Brown
ISP-7	Moderate	Grey	Light yellow	Brown

Table 2. Biochemical characteristics of isolate AA13.

Carbon source	Growth characteristic	Carbon source	Growth characteristic
Esculin	+	L-Arabinose	+
Cellobiose	+	Ribose	+
Maltose	+	D-Xylose	+
Lactose	+	L-Xylose	+
Sucrose	+	Adonitol	-
Trehalose	+	Galactose	+
Melibiose	-	Glucose	+
Raffinose	-	Fructose	+
Melezitose	+	Mannose	+
Starch	+	L-Sorbose	-
Glycogen	-	Rhamnose	-
Inulin	-	Dulcitol	-
Xylitol	+	Inositol	-
Glycerol	+	Sorbitol	+
Erythritol	-	Mannitol	-
D-Arabinose	+	N-Acetylglucosamine	+

actinomycete was based on the analysis of the 16S rRNA gene restriction fragment patterns. This method begins

Table 3. Enzyme production of isolate AA13.

Enzyme production		Growth characteristic
Phosphatases	Alkaline phosphatase	+
	Acid phosphatase	-
	Naphtol-AS-BI-phosphohydrolase	±
Lipases	Esterase (C4)	-
	Esterase-lipase (C8)	+
	Lipase (C14)	-
Proteases	Leucine arylamidase	+
	Valine arylamidase	+
	Cystine arylamidase	-
	Trypsin	-
	Alpha-Chymotrypsin	-
Osidases	Alpha-galactosidase	-
	Beta-galactosidase (lactase)	+
	Beta-glucuronidase (hyaluronidase)	-
	Alpha-glucosidase (maltase)	+
	Beta-glucosidase (cellulase)	-
	N-acetyl-beta-glucosaminidase (chitinase)	-
	Alpha-mannosidase	-
	Alpha-fucosidase	-

by isolating DNA and amplifying the gene coding for 16S rRNA using the polymerase chain reaction. The total nucleotide sequence of 1507 pb was determined in both strands (accession No. JQ965757). The alignment of this sequence through matching with the 16S rRNA reported genes sequences in GeneBank indicated that it belonged to the genus *Streptomyces* and represented a novel species that was readily distinguished from all recognized *Streptomyces* species (Figure 2).

Antifungal bioassay

According the tests of antifungal activity on ISP-2 medium by using the technique of double-layer, the *Streptomyces* sp. AA13 showed a significant antifungal activity against *C. albicans* with zone of inhibition of 42 mm.

Optimization of nutritional and cultural conditions

A number of carbohydrates were investigated for their effect on growth of AA13 and on its antibiotic production. The strain AA13 is able to grow and produce antifungal activities with the five kinds of carbon sources. Kinetics of growth and active molecules production studies, showed that the secretion of biological activities was closely correlated with the biomass production. Maximum biological activities and biomass production were obtained after 5 days of incubation for all tested carbon sources. Glycerol, lactose, maltose and sucrose were propitious to growth and antifungal antibiotic production by the strain AA13. The medium including glycerol gave the highest biomass (0.173 g/l) and antifungal activity (DIZ = 59 mm) (Figure 3). Other carbon sources such lactose, maltose, sucrose and glucose also favored growth but their intensity was less when compared with glycerol.

The findings of utilization of nitrogen source indicated that the medium including peptone gave the highest biomass (0.121 g/l) and antifungal activity (DIZ = 35 mm), followed by cultures containing meat extract, tryptone, and KNO_3 but did not show significant effect (Figure 4).

In order to investigate the effect of incubating temperature on growth and antifungal production, the strain AA13 was cultivated at 25-37°C. The results indicated in the Figure 5 showed maximum antifungal activity at 30°C with the highest biomass of 0.116 g/l and the maximum diameter of inhibition zone of 48 mm. The strain AA13 was cultivated in the above improved medium with different initial pH values (5-11). The results showed that the best pH for antifungal antibiotic production was 7.0, and the corresponding maximum diameter of inhibition zones was 57 mm with the highest biomass of 0.118 g/l (Figure 6).

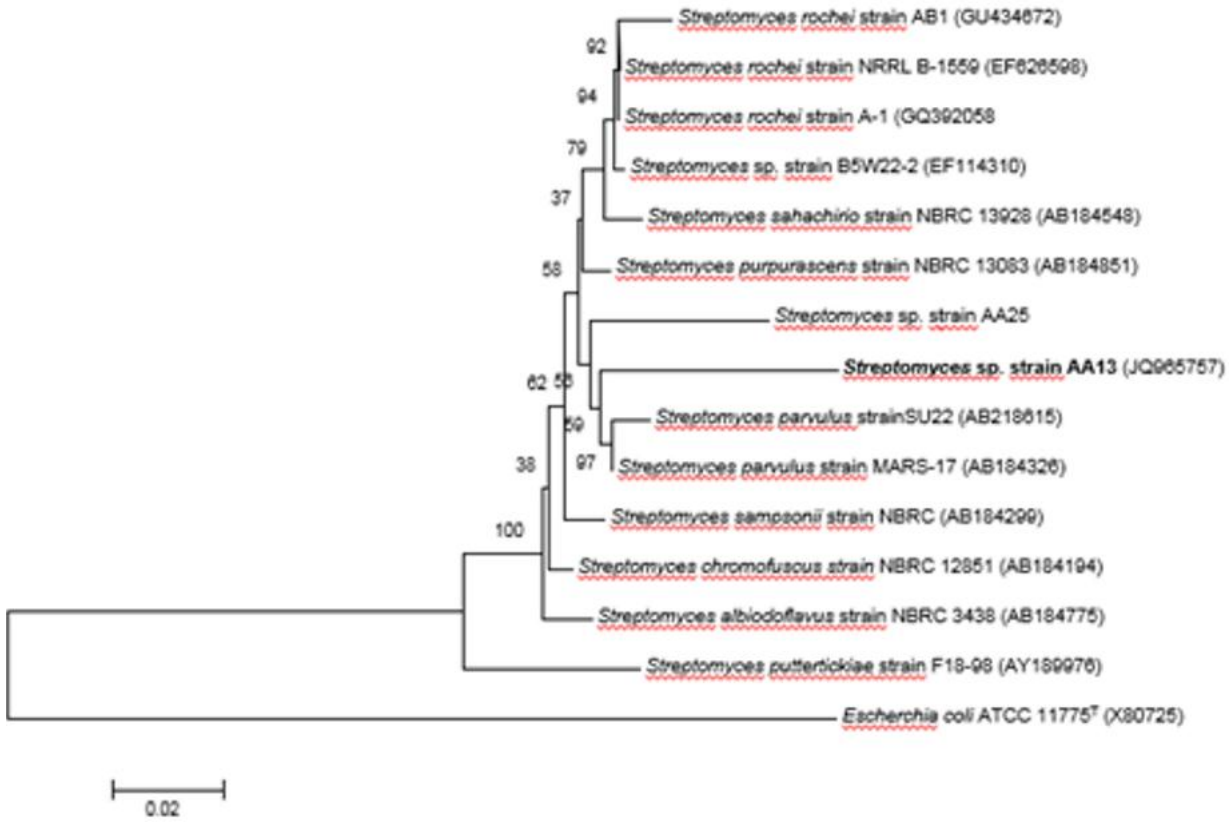

Figure 2. Phylogenic tree based on 16S rRNA gene sequences showing relationships among *Streptomyces* sp. AA13 and the most close type strain species of *Streptomyces*.

Extraction and purification of active compounds

Antifungal compounds were extraction from the supernatant with equal volumes of four organic solvents, including n-hexane, ethyl acetate, acetic acid and n-butanol. The crude extract obtained by ethyl acetate showed maximal zonation of 31 mm against *C. albicans*. The residues were analyzed by TLC and bioautography. Two bioactive regions were detected and the Rf values are 0.31 to 0.48. HPLC was used in an attempt to purify the antimicrobial compounds from the crude solvent extract of isolate AA13. Among, two pure molecules, AA13-B complex that made the strongest antifungal activity were purified by HPLC. The chromatogram showed three peaks. Only, AA13-B2 had the highest antifungal activity against *C. albicans*.

The partial characterization by infrared spectrum of AA13-B2 (Figure 7) showed hydroxyl group (bands at 3540 and 3460 cm^{-1}), alkyl groups (bands between 3000 and 1800 cm^{-1}) and carbonyl group (band at 1645 cm^{-1}). The band 1560 cm^{-1} strongly indicates the presence of aromatic ring, and the bands at 1500 to 1300 cm^{-1} showed presence of C-H and C-O bonds as secondary bands. The aromatic ring is supported by secondary bands at 760 and 610 cm^{-1}.

DISCUSSION

The emergence of fungal resistance threatens to return us to the era before the development of antifungal antibiotics (Smith et al., 1999; Shantikumar et al., 2006). The need for the investigation of new, safe and effective antimicrobials for replacement with invalidated anti-microbials or use in antibiotic rotation programs is necessary (Gerding et al., 1991; Quale et al., 1996; Niedreman, 1997).

As already mentioned, *C. albicans* is the agent of different types of infections, reaching from relatively harmless superficial infections like vaginal candidiasis or oral thrush of newborns to life-threatening blood stream infections. The human immune system is normally able to limit the abundance of *C. albicans*, keeping a healthy equilibrium in the commensally flora of the human mucosa (Glick and Siegel, 1999). Intriguingly, when the immune system is seriously weakened, *C. albicans* is able to become dominant in the mucosa, colonize different zones of the human body and cause severe infections. From a natural products perspective, marine bacteria remain a relatively unexplored resource for novel secondary metabolites. However, recent data suggest that actinomycetes, in particular genus *Streptomyces* are

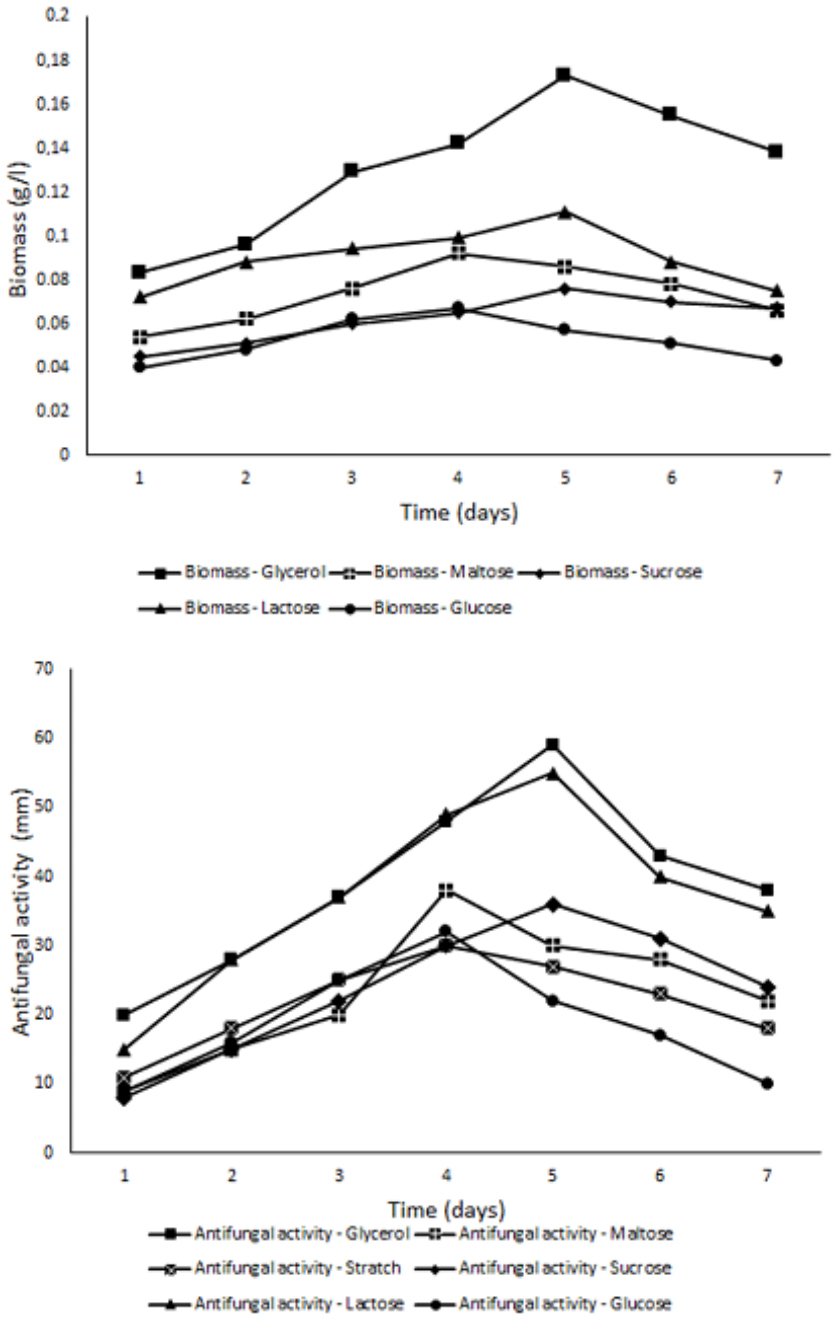

Figure 3. Kinetics of carbon source, growth and antifungal activities in starch nitrate medium, against *Candida albicans*.

widely distributed in marine environments (Sergey, 2012).

Of particular interest of this study, the Lake Oubeira is rich in biodiversity of flora, fauna, and microbial diversity (Morakchi et al., 2009; Benouagueni et al., 2015). The microbiology of its sediments has to be further explored in order to get benefit out of the precious bio-wealth. In our screening program for bioactive compounds, an antifungal activity of *Streptomyces* sp. strain AA13 isolated from the sediments of Lake Oubeira highlights its importance as candidate for further investigation in biological control of *C. albicans*. There is little published information describing the ecology of actinomycetes in marine habitats, because actinomycetes represent a small component of the total bacterial population in marine sediments, and their role in the marine environment is difficult to assess.

The actinomycetes have been isolated from Neuston sediments, as well as from marine sponges and sea

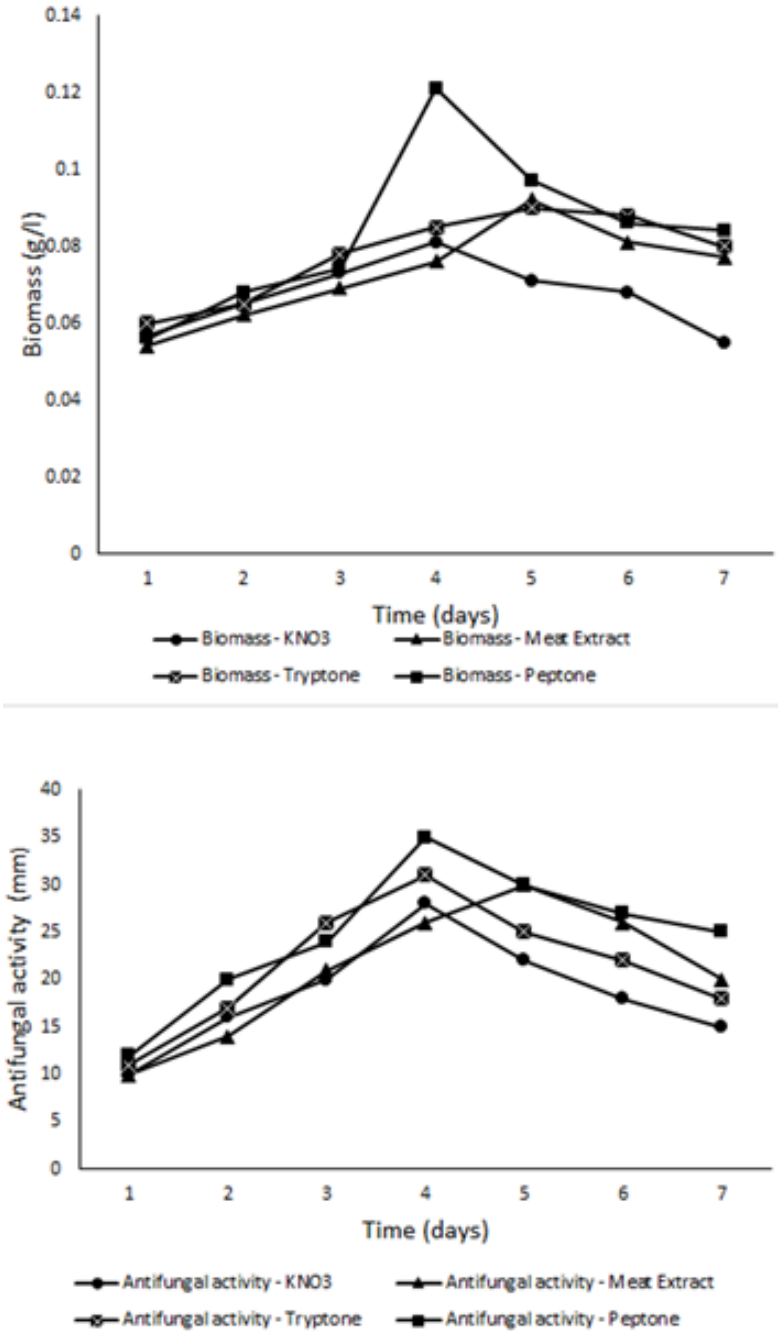

Figure 4. Kinetics of nitrogen source, growth and antifungal activities in starch nitrate medium, against *Candida albicans*.

weeds (Bull and Stach, 2007; Goodfellow and Fiedler, 2010; Sergey, 2012). Jensen et al. (2005) isolated five actinomycetes phylotypes from marine sediments collected around the island of Guam. Marine actinomycetes *Kocuriaery thromyxa*, *Rhodococcus erythropolis* and *Dietzia maris* were isolated from a sub-seafloor sediment core collected at a depth of 1225 m of Hokkaido (Inagaki et al., 2003). However, recent studies have shown that the distribution of actinomycetes in marine sediments and the requirements of seawater for growth give conclusive evidence that actinomycetes adapted to the marine environment represent a physiologically unique class of microorganisms (Morakchi et al., 2009). On the other hand, the presence of indigenous marine actinomycetes indicate the wide distribution in different marine environments and habitats

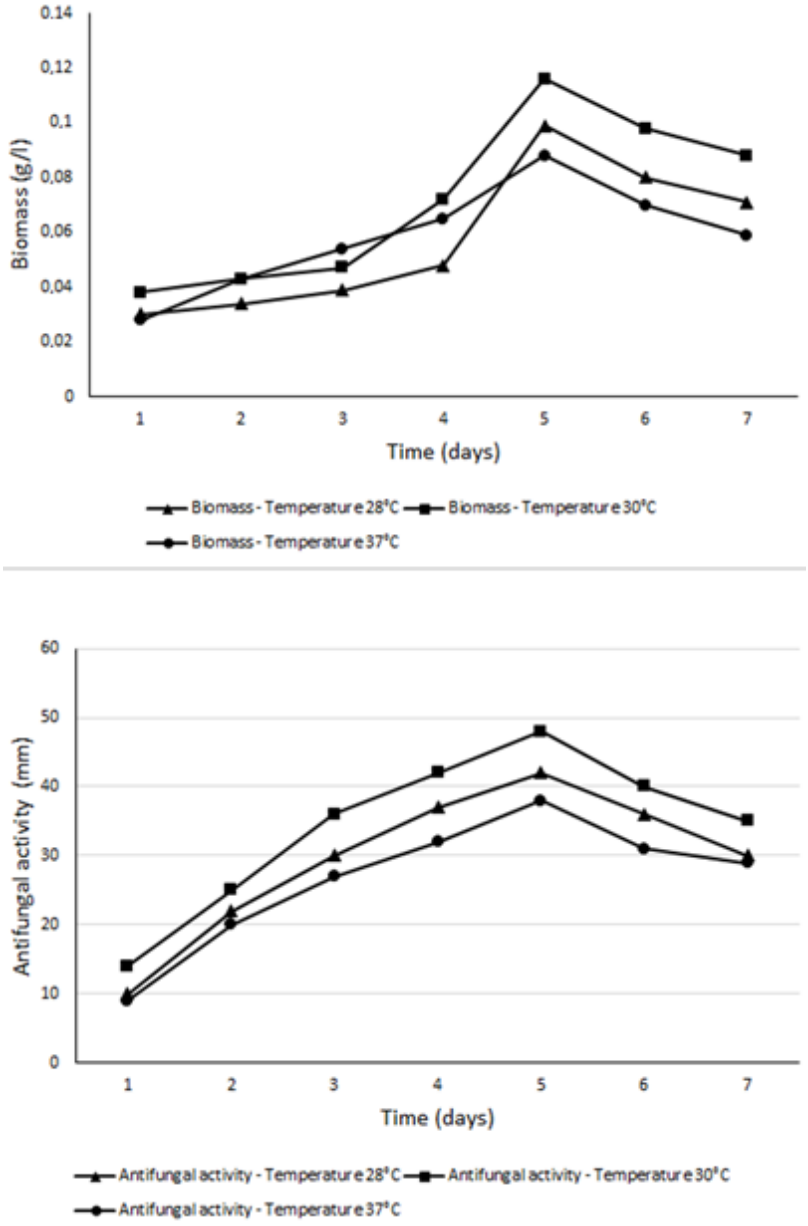

Figure 5. Kinetics of incubation temperature, growth and antifungal activities in starch nitrate medium, against *Candida albicans*.

(Küster, 1976; Al-Diwany and Cross, 1978; Collins and Jones, 1980; Goodfellow and Williams, 1983; Lam, 2006). Whereas other studies indicate that the *Streptomyces* are not part of the indigenous microflora, as the possibility of wash-in from surrounding terrestrial habitats must always be considered (Goodfellow and Williams, 1983).

This latter view was supported by the observation that the number of actinomycetes in marine habitats decrease with increasing distance from land (Collins and Jones, 1980; Goodfellow and Williams, 1983). In this study, the sediment samples were collected from distance of 4 m from land by inserting a grab sampler 20 cm into the sediments.

Several reviews describing biologically active molecules isolated from marine actinomycetes have recently been published (Fenical and Jensen, 2006; Bull and Stach, 2007; Goodfellow and Fiedler, 2010; Mayer et al., 2011; Sergey, 2012), and this article provides a rather brief and general overview of this subject.

A recent publication describes the isolation of four macrodiolide antibiotics, marinomycins A-D from *Marinispora* strain CNQ-140 that exhibit impressive cancer cell toxicities against eight melanoma lines in the

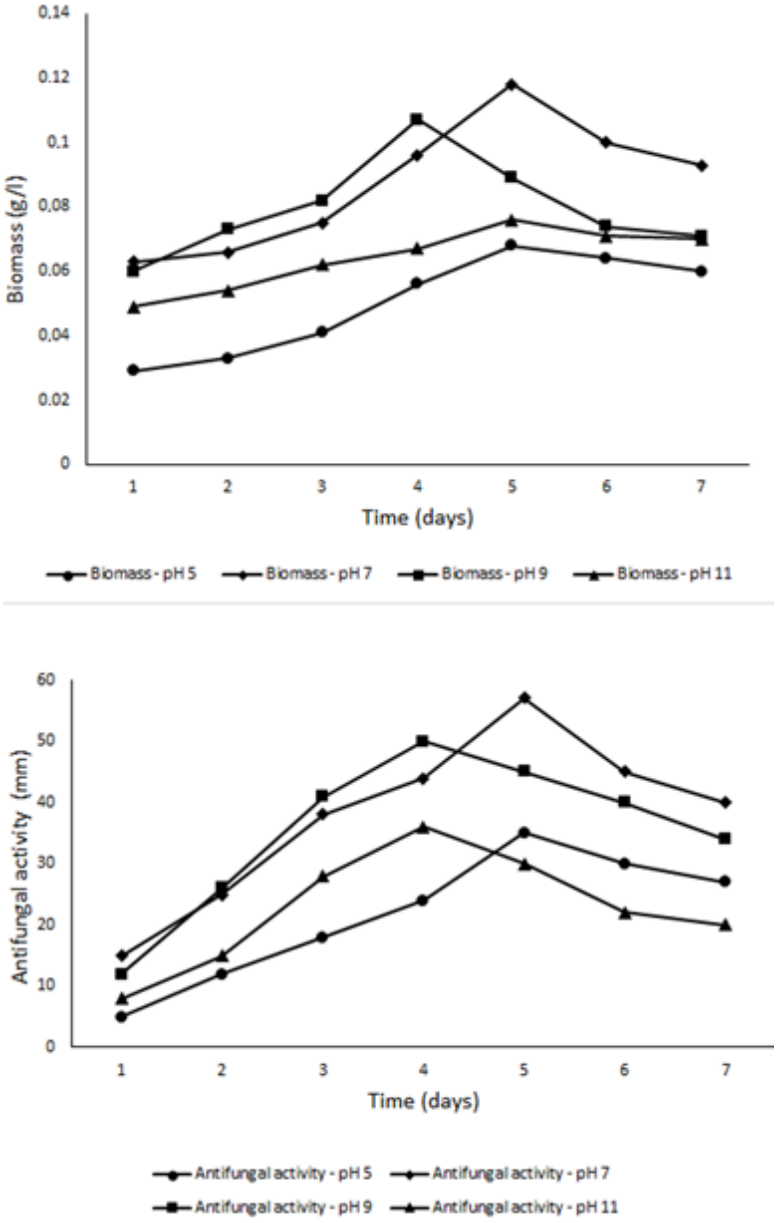

Figure 6. Kinetics of pH, growth and antifungal activities in starch nitrate medium, against *Candida albicans*.

NCI's 60-cell line panel (Kwon et al., 2006).

Based on its morphological properties, the isolate AA13 was classified in the genus *Streptomyces*. Methods described by Shirling and Gottlieb (1966) have been used in the ISP. Those characteristics were considered important and are now commonly used in the key for classification of *Streptomcyes* species. The taxonomy of *Streptomyces* species was mainly based on, the color of aerial and substrate mycelia and of soluble pigment, the shape and ornamentation of spore surface because of its stability (Forar Laidi et al., 2007). The aerial mycelium, substrate mycelium growth and pigmentation showed distinct variation based on the culture media in which the isolates were grown. Among the culture media used, the isolate growth was excellent in starch casein agar and this may be due to sufficient amount of nutrient included in this media (Ghanem et al., 2000; Gebreselema et al., 2013). Furthermore, some additional physiological characteristics (such as degradation of starch, gelatin, casein and reduction of nitrates) were carried out for adequate identification. Some additional tests relative to the use of some carbon source are also considered as certain species classification of new isolates strains are recommended by Shirling and Gottlieb (1972) and Holt et

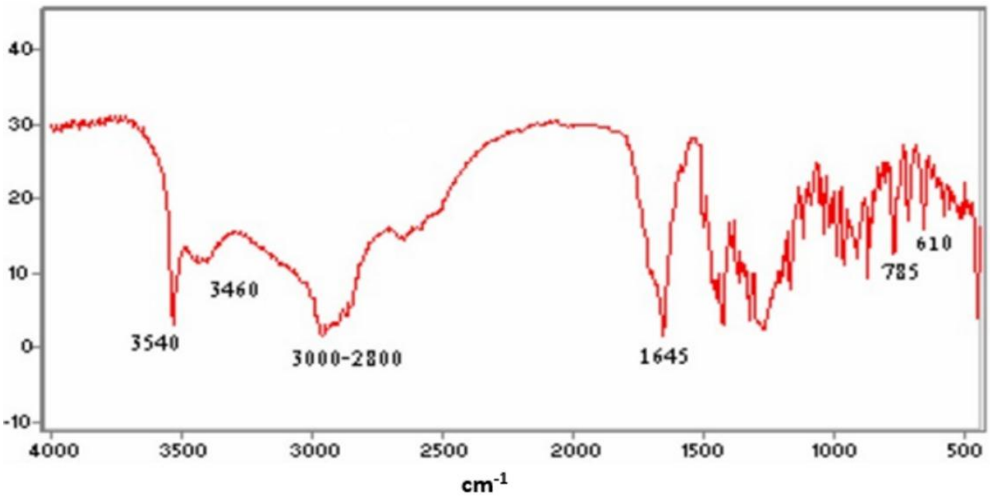

Figure 7. Infrared spectrum of antibiotic AA13-B2.

al. (1989).

The *Streptomyces* sp. strain AA13 contained various baggage of enzymes. This important producing actinomycetes have been reported (Ramesh and Mathivanan, 2009; Ayari et al., 2012; Ramesh and Aalbersberg, 2012). Such cellulolytic activity of marine actinomycetes was described by Chandramohan et al. (1972), and chitinolytic activity was reported by Pisano et al. (1992). Actinomycetes are also reported to contribute to recycling of organic compounds (Goodfellow and Haynes, 1984). In addition, they play a significant role in mineralization of organic matter and fixation of nitrogen (Valli et al., 2012).

Boudemagh et al. (2005) mentioned that molecular approaches for identification were often used due to their speed and efficiency. For this, the 16S rRNA gene sequence of the strain AA13 (1507 pb) was PCR-amplified, sequenced and submitted to GenBank (accession number is JQ965757). A neighbour-joining tree based on 16S rRNA sequences showed that the isolate occupies a distinct phylogenetic position within the radiation including representatives of the *Streptomyces* family.

In the current study, strain AA13 that seem to be strong fungal inhibitor, showed antibiosis against *C. albicans*. Although, the exact mechanisms by which this actinomycete isolate operate to reduce disease incidence is not elucidated, one possibility is that these biocontrol agents exert a direct inhibitory effect on structure of yeast pathogens (Zakalyukina and Zenova, 2007; Loqman et al., 2009; Oskay, 2009). Several hypotheses were formulated on the possible natural role of these molecules. Most probably, they were produced under stress conditions, such as nutrient starvation, to protect and preserve the producer from the other competitors present in the same environment (Marinelli, 2009). Antibiotic production usually occurs late in growth, during late stages of the development of the aerial mycelium on solid medium and just before entry into stationary phase in liquid cultures (Chouayekh and Virolle, 2002). The genes responsible for the biosynthesis of an antibiotic were usually gathered on the chromosome, and their growth phase-dependent coordinated expression was under the control of one or several specific pathways as well as pleiotropic regulators (Hopwood et al., 1995).

In *Streptomyces*, several physiological studies have demonstrated that antibiotic biosynthesis, was elicited by phosphate limitation and conversely, strongly repressed by exogenous phosphate (Chouayekh and Virolle, 2002). Nevertheless, it has been reported that nutritional requirements of *Streptomyces* play an important role during metabolite synthesis process. Amongst various nutritional requirements, antifungal substance production has been known to be influenced by media components and cultural conditions, such as aeration, agitation (Bode et al., 2002), pH, temperature (Sujatha et al., 2005), carbon and nitrogen source, which vary from organism to organism (Stanbury et al., 1997; Dahiya et al., 2006; Asha Devi et al., 2008; Yu et al., 2008; Oskay, 2009).

From the results it was evident that maximum growth and antifungal metabolite production was obtained at 30°C, which clearly indicates the mesophilic nature of the isolate. Previous reports, illustrate that optimal temperature range was between 26 and 35°C for antibacterial metabolites by *Streptomyces* strains (Macedo et al., 2007; Madan and SingaraCharya, 2013).

Maximum zone of inhibition was observed when the glycerol was used as carbon source. This indicated the presence of an active uptake system for these compounds in the isolate. Similar results have been reported by many investigators (Mansour et al., 1996; Vahidi et al., 2004; Madan and SingaraCharya, 2013). Maltose, sucrose and glucose are poor carbon source for antibiotic production. It is possible that these carbon

sources were utilized rapidly for the synthesis of cellular material so that little would be available as carbon and energy source for antibiotic synthesis. Therefore, carbon source plays a critical role as sources of precursors and energies for synthesis of biomass building blocks and secondary metabolite production (Jia et al., 2009; Oskay, 2011). However, Tanaka et al. (1986) observed that in certain cases, some excessive nutritional components such as glucose, amino acids and other carbon and nitrogen sources affected antibiotic production in fermentation broth.

However, controversially, there was no production of antimicrobial compound by *Nocardiopsis* sp. strain MAD08 when the medium was supplemented with different carbon sources at a concentration of 1% (w/v) (Selvin et al., 2009). The present study results also indicated that peptone served as ideal nitrogen source. The change in initial pH of the culture medium that affected maximum growth and antifungal production was obtained at 7.0.

Augustine et al. (2005) showed that cultural conditions affected antifungal metabolite production by *Streptomyces rochei*strain AK 39. The change in pH of the culture medium induces production of new substances that affect antibiotic production. However, El-Mehalawy et al. (2005) reported the effects of factors on fungal production *Streptomyces lydicus*, *Streptomyces ederensis*, *Streptomyces erumpens* and *Streptomyces antimycoticus*. Glycerol had positive effects on antifungal production followed by starch. In addition, it was observed that the optimum temperature for antifungal production by *S. lydicus* and *S. ederensis* was 24°C, while for *S. erumpens* and *S. antimycoticus*, it was 28°C. The optimum pH value for antifungal production by these species was 7.0.

The crude extracts showed wide range of inhibition zone against tested *C. albicans*. Similar findings were reported earlier by Gebreselema et al. (2013). In general, the antifungal activity of crude extracts fluctuated widely. Therefore, crude extracts could be a potent source for antibiotic production, which leads to the development of novel drugs for the treatment of infectious diseases. It has been reported that the antimicrobial activity of the compounds from different strains of actinomycetes vary depending on the strains, from which the compound was obtained, the solvent used for the extraction and the nature of the pathogens tested against such compound (Pridham and Gottlieb, 1948; Saadoun and Al-Momani, 2000; Sahin and Ugur, 2003; Narayana et al., 2005). It is evident that the antimicrobial efficacy of the bioactive compound is the expression of the genetic potentiality of the organisms, and the sensitivity of the test organisms is one of the genetic properties of the organism. Hence, studies on the genetic relationship between the organisms involved in the microbial interaction could throw more light on the underlying mechanisms. In addition, some studies were done in relation to the industrial enzymes production by microorganisms, eg: Sharma and Pant (2001) isolated aquatic actinomycetes in Bengal gulf and their results showed that the aquatic actinomycetes could be a source for production of bioactive compounds (Ward and Bora, 2006). Istamycins, Aplasmomycins and Altemicidin, isolated by researchers at the Institute of Microbial Chemistry in Tokyo, were produced by various actionomycetes isolated from marine sediment samples collected from Sagami Bay, Japan (Okami et al., 1979; Fenical and Jensen, 1993).

The Rf values from TLC showed the presence of two active compounds from *Streptomyces* sp. strain AA13 in the finding of Hongjuan et al. (2006). The sizes of the inhibition zones varied with the most antagonistic isolate producing bigger zones in the bioautograms. This corroborates reports by Asha Devi et al. (2008) that the size of the inhibition zone positively correlates with the amount of antibiotics produced. The various separation and purification steps led to the isolation of two pure bioactive molecules. The infrared spectrum indicated that the antifungal AA13-B2 belongs to the group, which contain aromatic ring substituted by aliphatic chains. Boudjella et al. (2006) mentioned that the antifungal B extracted from *Streptosporangium* Sg 10, purified via HPLC and characterized by infrared spectroscopy, belonged to the group of glycosylated aromatics. Badji et al. (2006) indicated that four antibiotics produced by *Actinomadura* sp. strain AC104 isolated from Algerian Saharian soil, belonged to the same chemical family containing a benzenic ring di-substituted by aliphatic chains. Narayana et al. (2008) mentioned that the bioactive compounds isolated from *Streptomyces* sp. strain ANU 6277 were identified as benzyl alcohol, phenylethyl alcohol and 2H-1, 4-benzoxazin-3 (4H)-one. Although, the two active molecules were characterized from the new isolated *Streptomyces* sp. strain AA13 strain, we think that our strain is very interesting because it produces simultaneously two active molecules, which can be used in human therapy.

Study on the influence of different nutritional compounds and culture conditions on growth and production of compounds with antifungal activity by the *Streptomyces* sp. strain AA13 strain isolated from the sediments of Lake Oubeira, showed that the high biomass and biological activities were obtained when glycerol or peptone were added in basal medium with pH 7.0 and incubation temperature at 30°C.

The antimicrobial substance produced by *Streptomyces* sp. strain AA13 was purified and its antifungal characteristics were investigated in this study. The crude culture supernatant showed antifungal activity against *C. albicans*. The extraction and purification steps led to isolation of two pure molecules having biological activities. The partial characterization of the compound AA13-B2, indicated that the presence of aromatic ring was substituted by aliphatic chains.

These findings indicated that the produced substance

might be the alternative antimicrobial substance which is a tool for controlling fungi diseases.

Conflict of Interests

The authors have not declared any conflict of interests.

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Vol. 10(6), pp. 172-184, 14 February, 2016
DOI: 10.5897/AJMR2015.7645
Article Number: 741DC9357109
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Diversity of putatively toxigenic *Aspergillus* species in maize and soil samples in an aflatoxicosis hotspot in Eastern Kenya

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Received 27 June, 2015; Accepted 12 November, 2015

Aflatoxin contamination impinges on grain quality worldwide. The causative agent, *Aspergillus* spp. colonizes grain in the field down to postharvest stages in storage where they may produce toxins. Kenya has experienced recurring cases of aflatoxicosis in Eastern region especially during periods of maize grain deficit. The risk of chronic exposure has not been widely studied. Therefore, seasonal variation in abundance and species composition of toxigenic *Aspergillus* in maize and soils of Eastern Kenya was investigated. Samples were obtained from farmers, two months after the first (May) and second (December) harvest seasons. *Aspergillus* spp. were isolated from maize and soil samples by direct and dilution plate techniques respectively on Czapek Dox Agar (CZ) and thereafter sub-cultured on potato dextrose agar (PDA). Positive identification was done using culture-morphological and microscopic characteristics in PDA media. The ammonium vapour test was used to screen for the putative toxigenic strains. A total of 229 *Aspergillus* spp. cultures were obtained (55% -maize, 45% -soil). Eleven *Aspergillus* sp. were identified: *Aspergillus niger, Aspergillus flavus, Aspergillus clavatus, Aspergillus awamori, Aspergillus parasiticus, Aspergillus ochraceus*, *Aspergillus candidus, Aspergillus ustus*, *Aspergillus niveus*, *Aspergillus terreus* and *Aspergillus wentii*. Of these 41 (18 %) were potentially toxigenic while the rest were putatively atoxigenic. Out of the 41 toxigenic isolates, 22 were from maize. The first season had 15 (68.2%) toxigenic maize isolates while 7 (31.8%) were from the second season. Generally, there were more fungal isolates in the first season (54.1%) than the second one (45.9%) while *Aspergillus niger* was the most abundant in both seasons. Such variation in fungal abundance supports the hypothesis that aflatoxin contamination of grain may vary seasonally but that remains to be unravelled and herein, a contrary opinion was presented.

Key words: Aflatoxin, mycotoxin, *Aspergillus*, maize, soil.

INTRODUCTION

Fungi belonging to the genus *Aspergillus* produce various toxins, which are of importance to human health. In particular, *Aspergillus flavus* and *Aspergillus parasiticus* mainly produce aflatoxins (Abbas et al., 2005) while other congeneric species: *Aspergillus* sections *Nidulantes, Versicolores, Usti, Circumdati* and *Nigri* produce various

metabolites related to aflatoxins which cause food poisoning (Blumenthal, 2004; Do and Choi, 2007). These fungi colonize grain in the field during planting and continue to do so during storage when they may produce toxins (Waliyar et al., 2015). Apart from aflatoxins, these fungi produce other metabolites such as ochratoxins and oxalic acid, which are hazardous to humans (Palencia et al., 2010). The effect of mycotoxin ingestion is dose-dependent, it may result in either death (acute) or other clinical complications (chronic). Chronic intake may lead to immunosuppression (Jiang et al., 2008), cancers, poor growth and abnormal foetal development (Gong et al., 2004; Probst et al., 2011).The United States Food and Drug Administration (FDA) has referred to aflatoxins as inevitable food contaminants, hence it has set the maximum allowable limit in human food products at 20 parts per billion. However, in the US, the levels vary between 10 parts per billion for humans and 20 parts per billion for livestock (Williams et al., 2004), while in Europe the limit is 4 ppb (Commission Regulation (EC) No 1881/2006, 2006). In Kenya, the limits are 10 parts per billion (Mutiga et al., 2014).

Over the last four decades, Kenya has experienced repeated cases of aflatoxicosis. In the year 2004, 317 people were hospitalised due to acute aflatoxin exposure 125 cases were fatal (CDC, 2004). It was hypothesised that there could be an underestimation of human aflatoxicosisburden in the country for four reasons: 1. Many cases may not be reported due to lack of infrastructure of capturing episodes in real time. 2. There is scanty clinical data to account for chronic sub-lethal exposure, 3. There is a systemic difficulty in enforcing residual limits policies among cereal traders and this ensures the circulation of cereals with high toxin levels. 4. There is a belief that aflatoxin contamination is a problem in only one region of the country yet it is more widespread covering other maize producing areas including Western and Rift Valley. Most of the human fatalities have been recorded in the Eastern region of Kenya, which is now considered a hot spot for aflatoxicosis (Probst et al., 2007; Muthomi et al., 2009). Fatalities common in the Eastern region is perhaps due to acute grain shortage, which force the populace to consume any available grain that is probably considered unfit for consumption. However, a survey by Mutiga et al. (2014) reported on high incidences of aflatoxin contamination in the Eastern region of Kenya following a bumper harvest in 2010. Preharvest drought and postharvest moisture are considered the most critical drivers of aflatoxin accumulation, which are factors that are largely influenced by cropping seasons. In Eastern Kenya, acute aflatoxicosis has been reported following periods of moisture stress during maize crop development, when rainfall occurs after the crop has attained physiological maturity and before it is harvested, and when harvests are stored incorrectly. For this reason, there has been renewed effort to study various aspects of the causal fungi and its interactions, which include intraspecific variations and competition, climatic and seasonal influence, toxigenicity (Probst et al., 2011), distribution in maize and soil (Muthomi et al., 2009).

Perhaps one of the most promising control efforts is in biological control whose success story has been reported in other countries like Nigeria (Fapohunda, 2009) and is undergoing initial stages of quarantined field trials (IITA, 2014). This approach uses non-toxigenic fungi to “competitively exclude” toxigenic ones from accessing maize kernels, thereby preventing toxin build-up (Cotty et al., 2007). As such, an inoculum of non-toxin producing fungi is introduced into the soil surface beneath the crop canopy 40-45 days after planting (two-to-three weeks before flowering) (Atehnkeng et al., 2014). However, considering the variations in both quantities and types of toxins produced by fungi of the genus *Aspergillus*, it is imperative to consider these variations alongside species composition, abundance and geographical distribution as would be affected by seasonal changes. This information would be important in understanding the possible toxin risks the population faces with each harvest and enable early preparedness. This study was conducted to determine the seasonal variation in the abundance and composition of putatively toxigenic *Aspergillus* species in maize and soil in Eastern Kenya. This has not been previously established in this region.

MATERIALS AND METHODS

Sampling

Maize and soil samples were collected in May and December 2013 two months after respective harvest seasons. The Eastern region of Kenya is a semi-arid region that experiences annual rainfall of between 250 and 500 mm (Freeman and Coe, 2002). The long rains start at the end of March and last until May while the short rains start in October to December. The minimum and maximum temperatures in this region range from 23 to 34°C (Funk, 2010). A transect was selected (along the main road cutting across the counties) from which sampling points were set every 5 km. At every sampling point, farmers were selected randomly on both sides of the road from whom half a kilogram of shelled maize was collected. Soil was collected from the open area where farmers dried their grain as well as under the storage facility. Samples were separately put in khaki bags, transported in a cool box to the laboratory, and stored at 4°C until analysis was done. During the second season, repeated sampling was undertaken in the same areas as the first one. In total, about 200 maize samples were collected during both seasons, but were segregated depending on whether the farmer had planted or purchased their grain. All samples obtained from farmers who had purchased maize during either of the seasons

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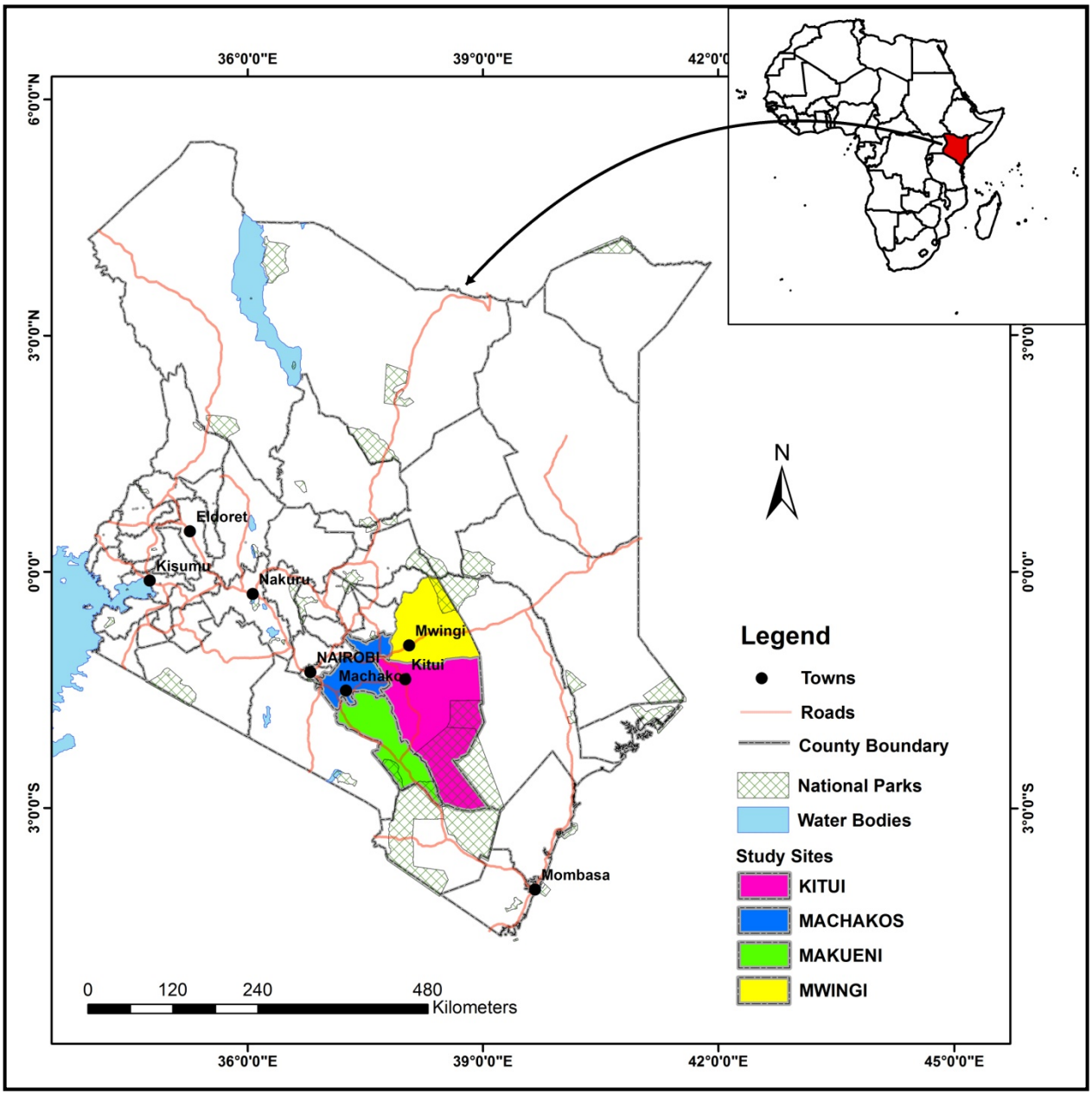


Figure 1. Map of the study area: Kitui, Machakos, Makueni and Mwingi. Source: Cartographer, Department of Geography, Egerton University (2015).

were excluded from the study. This procedure was guided by findings reported by Daniel et al. (2011) that home-grown maize was the major source of contamination compared to purchased maize. Owing to poor harvest that affected most of the region during 2013, most farmers sampled had bought maize for their daily use. Thus, only a total of 50 (maize with their respective soil) samples, which had been stored for two months were analysed further. These are as follows: location [Total samples = Season I + Season II]: Machakos [4=4+0], Makueni [16=16+0], Kitui [17=4+13] and Mwingi [13=1+12] (Figure 1).

Fungi isolation and identification

Fungi isolation from maize was carried out following the procedure described by Muthomi et al. (2009) after surface sterilisation of kernels in 2% NaOCl. Fifteen kernels were randomly picked from the khaki bags and introduced into a conical flask containing NaOCl and swirled gently for one minute. Afterwards, they were removed and rinsed thrice in sterile distilled water. Five kernels were plated about 2 cm apart on Petri dishes (90 × 15 mm) containing Czapek Dox Agar (HiMedia Laboratories Pvt. Ltd) amended with 50 mg each of streptomycin sulphate and penicillin (Zhonghuo Pharmaceutical Shijazhuang Co. Ltd., China) per litre of medium. The set up was replicated thrice for each sample. As for soil samples, dilution plate technique was used. Briefly, 1 g of soil was suspended in 9 ml sterile distilled water and serially diluted to 1 x 10^{-4}. One ml of 10^{-3} and 10^{-4} respectively were uniformly spread in duplicates in Czapek Dox Agar (amended as above). Afterwards, all

culture plates were incubated in a growth chamber at 28°C for 7 days. *Aspergillus* species were isolated from colonies in Petri dishes and dilution plates then sub-cultured in Potato Dextrose Agar (HiMedia Laboratories Pvt. Ltd, India) and incubated as above. Upon maturation, fungi were classified based on cultural and morphological features such as colony diameter, colony colour on agar, front and reverse and colony texture (Klich, 2002a; Rodrigues et al., 2007). This was followed by the preparation of slide cultures and incubation in moist chambers at 28°C for 5 days before observation under a light microscope. For microscopic characterisation, microscopic features studied included conidiophores, conidial shape, phialides and metulae, presence and shape of vesicles. These are the common features used to identify *Aspergillus* fungi to species level (Diba et al., 2007; Klich, 2002a).

Screening for toxigenic *Aspergillus* species

Toxin producing ability of the fungi was tested following the ammonium hydroxide (NH_4OH) vapour test (Kumar et al., 2007). A single fungal colony was grown in the centre of a Petri dish containing yeast extract-sucrose medium for 5 days at 28°C. Then 2 drops of concentrated (27%) NH_4OH solution were added to the inverted lid of the Petri dish and allowed (30 min) to react. Toxin production (positive test) was evidenced by formation of a pink to plum-red colour on the underside of the fungal colony while negative tests had no observable colour changes (Zrari, 2013).

Data analysis

The data on the abundance of the fungi in each region were represented as a percentage of score total count. Analysis of variance (ANOVA) was performed to determine whether the distribution of the *Aspergillus* isolates in the four sites was significantly different. Student t-test was performed to determine whether the distribution of the species between the two seasons was statistically significant. SPSS (version 20.0) was used in the analysis of data.

RESULTS

Aspergillus species isolates from maize and soil

The morphological and cultural features of the *Aspergillus* isolates are presented in Table 1. In total (from both seasons), 229 isolates were obtained from maize and soil samples. The identification process resulted into 11 *Aspergillus* species (Plate 1 and 2a – k).

The species and their order of abundance (%) were as follows: *A. niger* (47.6), *Aspergillus flavus* (22.3), *Aspergillus clavatus* (12.2), *Aspergillus awamori* (4.4), *Aspergillus parasiticus* (3.9), *Aspergillus ochraceus* (2.2), *Aspergillus candidus* (1.7), *Aspergillus ustus* (1.7), *Aspergillus niveus* (1.7), *Aspergillus terreus* (1.3) and *Aspergillus wentii* (0.9). There was consistency in species identity as individual isolates (across the four study sites) belonging to a single species showed same morphological traits on PDA. Conversely, *A. parasiticus* showed similar cultural and morphological traits to *A. flavus* but were segregated on the basis of conidia colour since *A. parasiticus* (conifer green) differed from *A. flavus* (dark green) (Plate 2 d(i) and h(i) respectively).

There were some species with somewhat similar cultural traits, but were segregated microscopically. For example, *A. candidus* was distinguished from *A. niveus* by a fertile region of the vesicle and colony diameter. Isolates of *A. niveus* were fertile on the top one-to-two thirds of the vesicle while *A. candidus* were fertile on the entire vesicle with colony diameters not exceeding 35 mm in PDA (Klich, 2002a).

Aspergillus species abundance and composition

Due to the sampling procedure and the choice of only analysing the farmer planted maize samples and excluding the bought maize samples from this study, it was not possible to reliably compare the study sites in Eastern region and respective seasons but rather consider the entire region as a block. This could partly explain why there were some observable differences in distribution of fungi in the two sampling seasons but they did not appear significant (Table 2).

In general, there were more *Aspergillus* species isolated from maize (54.6%) than from soil samples (45.4%). This was consistent in each location except Makueni where there were more isolates from soil than from maize (Table 3).

The percentage proportion of fungal isolates of maize to soil in each location was as follows: Kitui (57:43), Makueni (47:53), Mwingi (62:38) and Machakos (52:48). In addition, the abundance (%) of fungi appeared to correlate positively with the number of samples collected from each location as follows: Kitui (34.9), Makueni (31.9), Mwingi (23.1) and Machakos (10).

In terms of species composition, three species (*A. clavatus, A. niveus*and *A. wentii*) were isolated strictly from soil, but not from maize (Figure 2). All species obtained from maize were present in soil, which was consistent with the expectations. Five species (*A. flavus, A. niger, A. parasiticus, A. ochraceus* and *A. ustus*) were more abundant in maize than in soil samples. In contrast, two species (*A. terreus* and *A. awamori*) were more abundant in soil than maize samples (Figure 3).

Toxigenic *Aspergillus* species from maize and soil

The colony reverse of the toxigenic species changed from pale yellow to pink or plum-red (Plate 3). In general, of the 229 *Aspergillus* isolates, 41 (18%) were toxigenic while the rest were non-toxigenic, 22 (53.7%) of the toxigenic isolates were from maize while 19 (46.3%) from soil. 15 (68.2%) of the isolates from maize were from first season while 7 (31.8%) were from the second season. For the soil isolates, 11 (57.9%) were from season one while 8 (42.1%) were from the second season (Table 4).

Table 1. Cultural, morphological and microscopic features of the identified *Aspergillus* species.

Species name	Cultural and morphological features on PDA			Microscopic features			
	Colony diameter (mm)	Surface colour	Reverse colour	Shape of vesicle	Conidial head	Seriation	Conidiophore
A. awamori	58±2	Dark brown to black conidia with white to yellow mycelia arranged in alternating concentric rings	Cream to dull yellow reverse with a wrinkled centre	Globose	Radiate	Biseriate	Colourless, long and smooth
A. candidus	26±2	Pure white conidia with dense white mycelia	Light yellow wrinkled reverse	Globose	Radiate	Biseriate	Colourless, short and finely roughened
A. clavatus	32±2	Bluish green conidia, white mycelia with a white margin	Brown centre with alternating yellow and brown concentric rings	Clavate	Radiate	Uniseriate	Brownish, short and finely roughened
A. flavus	40±2	Deep green conidia or olive green conidia with white margin Presence of white mycelia	Cream to light brown reverse with a smooth texture	Globose	Columnar	Biseriate	Colourless, relatively short roughened conidiophores
A. niger	62±2	Dark brown to black densely packed conidia, inconspicuous white mycelia, thin white to cream margin	Yellow to dull brown reverse with a wrinkled texture	Globose	Radiate	Biseriate	Brownish, relatively long conidiophores with smooth surfaces
A. niveus	22±2	Dull orange-white conidia with white mycelia	Yellow-gold reverse with star-shaped striations and concentric ring patterns	Columnar	Radiate	Biseriate	Colourless, short and finely roughened
A. ochraceus	45±2	Wheat-coloured conidia, purplish sclerotia with yellowish exudates	Yellow to light brown wrinkled reverse	Globose	Radiate	Biseriate	Colourless relatively long roughened conidiophores
A. parasiticus	Full plate	Conifer green conidia with white mycelia and white margin	Cream reverse with slightly wrinkled centre	Globose	Columnar	Uniseriate	Colourless short and finely roughened
A. terreus	29±2	Brownish orange conidia with white mycelia	Yellow to gold reverse with star-shaped striations and concentric ring patterns	Sub-globose	Columnar	Biseriate	Colourless short smooth-walled conidiophores

Table 1. Contd.

A. ustus	50±2	Light brown to greyish conidia, with white to greyish mycelia	Cream reverse with yellowish wrinkled centres	Pyriform	Columnar	Biseriate	Brownish, long, smooth-walled conidiophores
A. wentii	28±2	Greyish-yellow to olive-brown conidia	Yellow to pale brown reverse	Globose	Radiate	Biseriate	Colourless and relatively long conidiophores with smooth walls

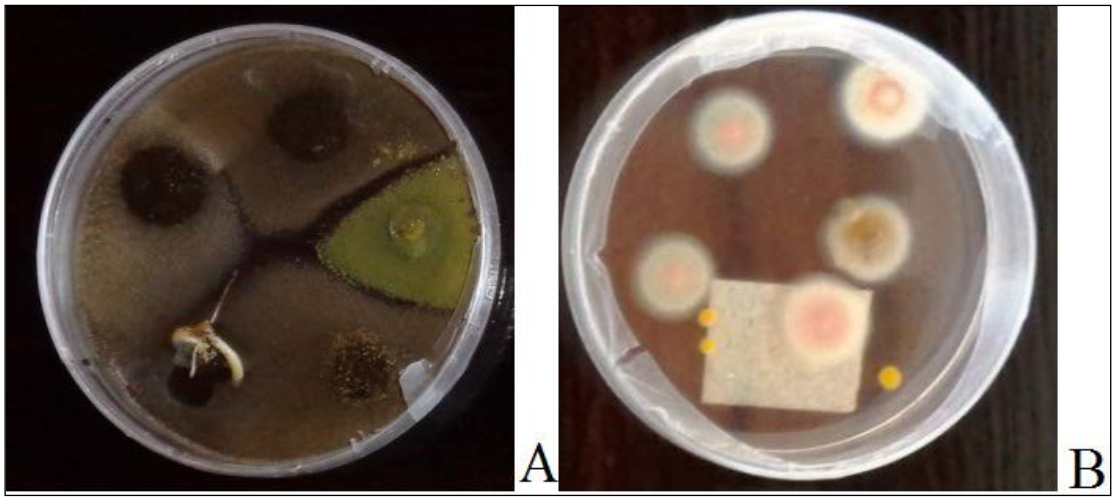
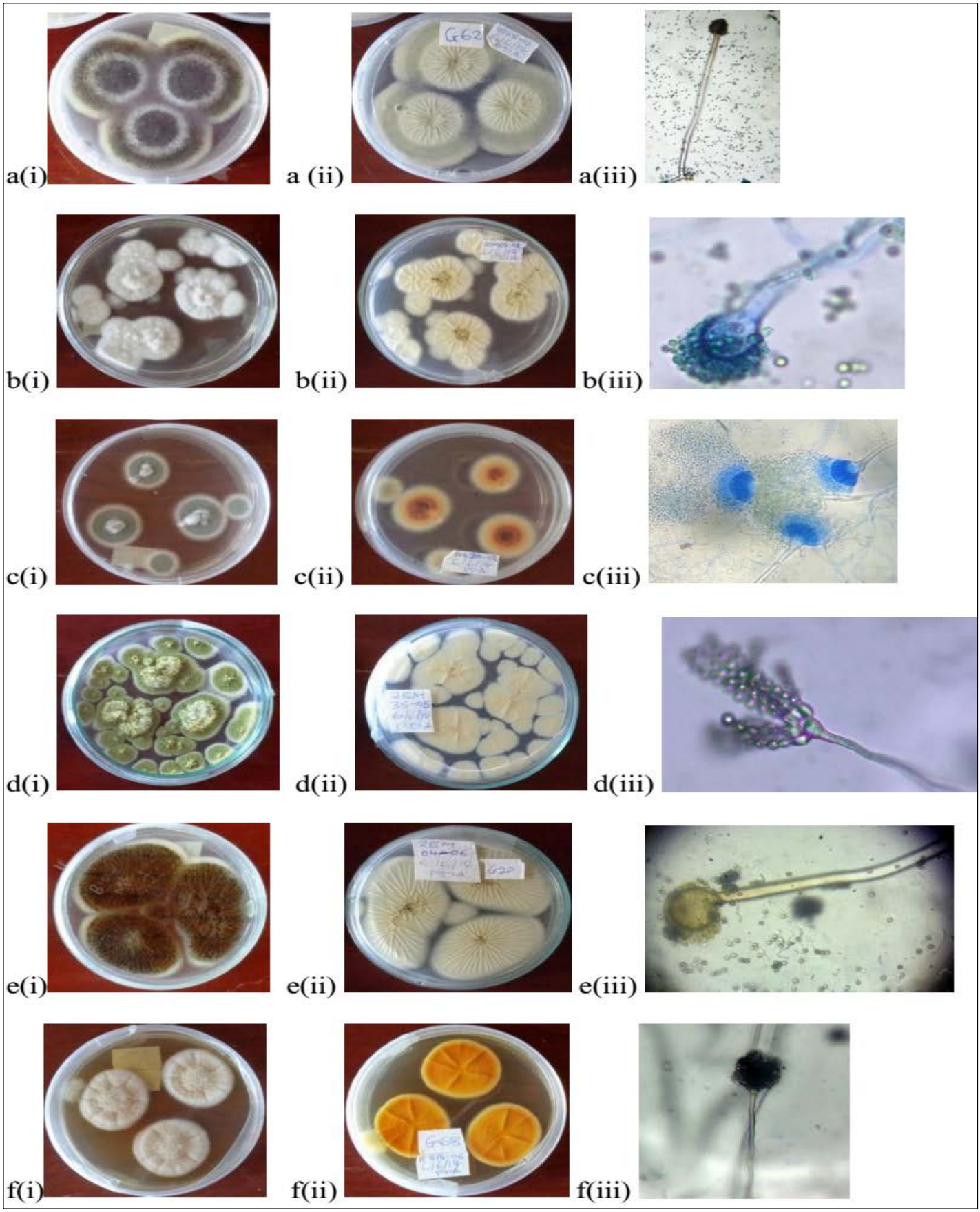


Plate 1. (A) Growth of *Aspergillus niger* and *Aspergillus flavus* on maize kernels in CZ media after 7 days of incubation at 28°C and (B) colonies of Aspergillus species from soil serial dilutions on CZ media after 7 days of incubation at 28°C.

The toxigenic *Aspergillus* isolates were of the species *A. flavus* (24), *A. parasiticus* (3), *A. ochraceous* (3), *A. clavatus* (8), *A. ustus* (1), *A. niveus* (1) and *A. wentii* (1).

DISCUSSION

It is important to determine whether the abundance and composition of putatively toxigenic *Aspergillus* species in maize and soil of Eastern Kenya are affected by the climatic changes in the two planting seasons. In the current study, *A. niger* was the most abundant amongst the eleven species isolated in both seasons while *A. flavus* was the second most abundant. *A. niger* was formerly believed to be harmless and non-toxigenic (Blumenthal, 2004), but recent studies present it as potentially being toxigenic, producing fumonisins (Palencia et al., 2010). *A. flavus* is known to produce aflatoxins (Probst et al., 2007; Rodrigues et al., 2007). The current findings of the abundance of *A. niger*in semi- arid soils contradicts the propositions of Klich (2002b) that this black *Aspergillus* species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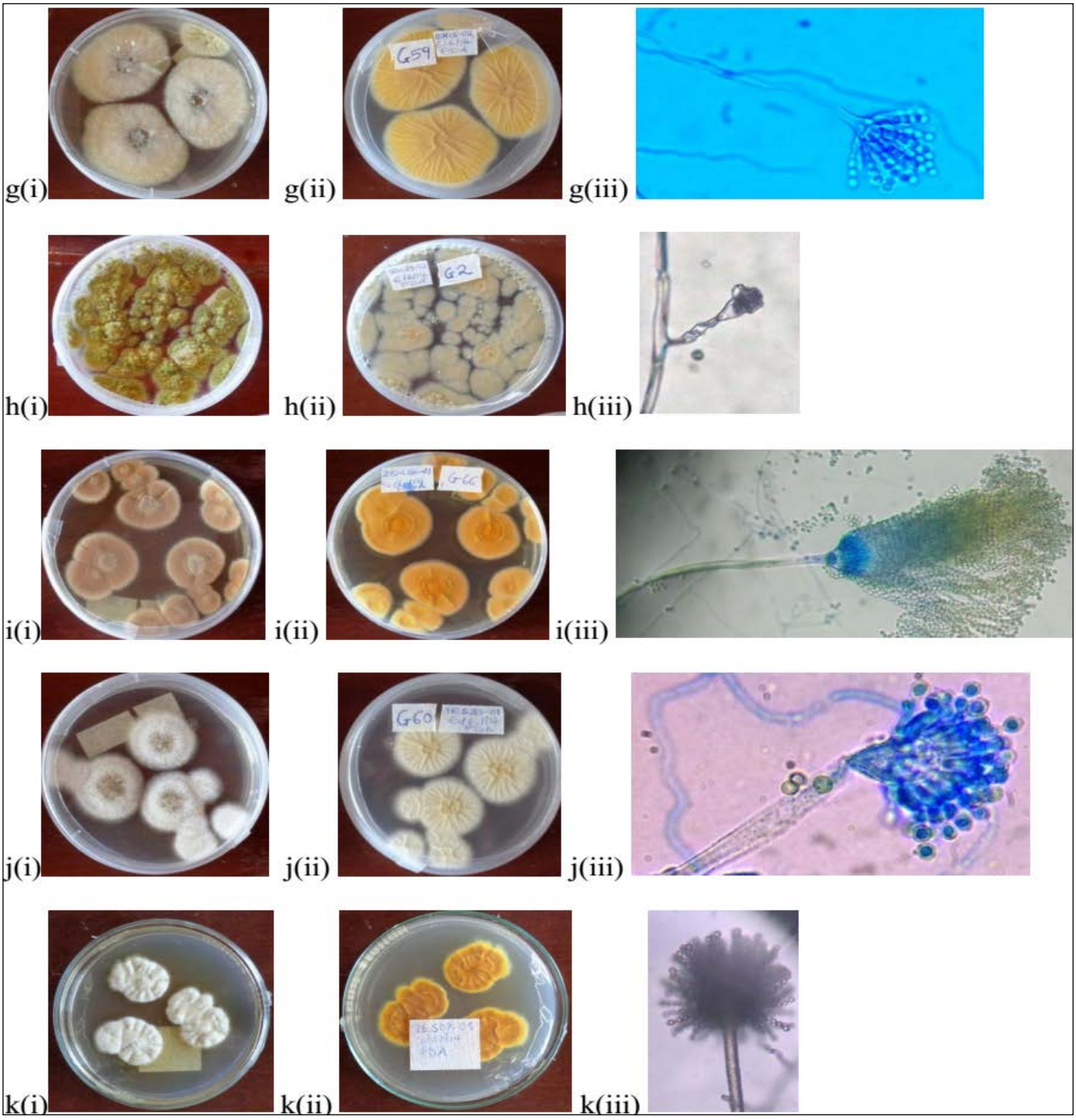

Plate 2. The cultural and morphological traits of the 11 *Aspergillus* species growing in PDA after 7 days of incubation. a(i) *A. awamori* surface, a(ii) *A. awamori* reverse and a(iii) colourless, long, and smooth conidiophore of *A. awamori* (Mg=400×); b(i) *A. candidus* surface, b(ii) *A. candidus* reverse and b(iii) globose vesicle in *A. candidus* (Mg=400×); c(i) *A. clavatus* surface, c(ii) *A. clavatus* reverse and c(iii) clavate vesicle in *A. clavatus* (Mg=1000×); d(i) *A. flavus* surface, d(ii) *A. flavus* reverse and d(iii) a biseriate conidial head with a globose vesicle of *A. flavus* (Mg400×); e(i) *A. niger* surface, e(ii) *A. niger* reverse and e(iii) brownish, relatively long and smooth conidiophore of *A. niger* (Mg=400×); f(i) *A. niveus* surface, f(ii) *A. niveus* reverse and f(iii) short, finely roughened stipe in *A. niveus* (Mg=400×); g(i) *A. ochraceus* surface, g(ii) *A. ochraceus* reverse and g(iii) globose vesicle, colourless relatively long conidiophore of *A. ochraceus* as observed under the microscope (Mg=400×). h(i) *A. parasiticus* surface, h(ii) *A. parasiticus* reverse and h(iii) short stipe of *A. parasiticus* as observed under the microscope (Mg=400×); i(i) *A. terreus* surface, i(ii) *A. terreus* reverse and i(iii) columnar conidial ornamentation in *A. terreus* (Mg=1000×) j(i) *A. ustus* surface and j(ii) *A. ustus* reverse and j(iii) biseriate conidial head in *A. ustus* (Mg=×1000); k(i) *A. wentii* surface, k(ii) *A. wentii* reverse and k(iii) radiate conidial head with roughened stipe of *A. wentii* (Mg=400×).

Table 2. The distribution of *Aspergillus* sp. in the two planting seasons.

Aspergillus sp.	Season				
	I		II		Total
	Count	%	Count	%	Count
A. awamori	5	50.0	5	50.0	10
A. candidus	1	25.0	3	75.0	4
A. clavatus	23	82.1	5	17.9	28
A. flavus	26	51.0	25	49.0	51
A. niger	55	50.5	54	49.5	109
A. niveus	2	50.0	2	50.0	4
A. ochraceous	3	60.0	2	40.0	5
A. parasiticus	5	55.6	4	44.4	9
A. terreus	1	33.3	2	66.7	3
A. ustus	3	75.0	1	25.0	4

Table 3. The incidence (%) and distribution of *Aspergillus* species across the four study sites.

Aspergillus sp.	Site			
	Kitui (N=17)	Machakos (N=4)	Makueni (N=16)	Mwingi (N=13)
A. awamori	50.0	0.0	40.0%	10.0
A. candidus	50.0	0.0	0.0	50.0
A. clavatus	32.1	17.9	35.7	14.3
A. flavus	37.3	11.8	25.5	25.5
A. niger	33.9	7.3	35.8	22.9
A. niveus	25.0	0.0	50.0	25.0
A. ochraceus	40.0	20.0	40.0	0.0
A. parasiticus	11.1	22.2	11.1	55.6
A. terreus	66.7	33.3	0.0	0.0
A. ustus	50.0	0.0	50.0	0.0
A. wentii	0.0	0.0	0.0	100.0
Total	**34.9%**	**10.0%**	**31.9%**	**23.1%**

is more dominant in forests and well-cultivated soils than in dry regions. The sampled region in this study is largely characterized by semi-arid dry climatic conditions. However, they postulated that the black *Aspergillus* species (Section *Nigri*) and *A. flavus* are highly likely to be found in areas of latitudes ranging (26 - 35°), which is close to our sampled areas in the latitude range (Mwingi 38.05°- Machakos 37.26°). The fact that *A. flavus* was the second most abundant species in both seasons implies that the risk of exposure to aflatoxin occurs all year round and not during any particular cropping season.

The findings of this study differ from those of Muthomi et al. (2009), who reported *A. flavus* as being the most abundant in the same region in two consecutive years (2008-2009). While there seem to be some differences, they could be explained by differences in collection times, sampling strategy and even the season of collection. In addition, Muthomi et al. (2009) reported seven species (*A. flavus, A. niger, A. terreus, A. ochraceus, A. fumigatus, A. clavatus* and *A. versicolor*). While the current study reported eleven, five of which matched the earlier study except for *A. versicolor* and *A. fumigatus,* which were not isolated in this study. We report five *Aspergillus* species that were not reported by Muthomi et al. (2009) including *A. parasiticus, A. ustus, A. candidus, A. niveus, A. awamori* and *A. wentii*, out of which *A. parasiticus* is known to be toxigenic.

Recently, Odhiambo et al. (2013) while prospecting for candidates for biological control in the same region reported the dominance of *A. flavus* but absence of *A. niger* from Makueni. In addition, four other species (*A. glaucus, A. sydowii, A. nidulans* and *A. fumigatus*) reported in the same study were not reported in the current one. Here, the reverse was reported, *A. niger* dominating (39 isolates) followed by *A. flavus* (13 isolates). Such variations can be due to differences in the sampling strategies (McHugh et al., 2014) or even species overlap as seasons change, hence the time of sampling is critical (Kennedy et al., 2006). In their work, Odhiambo et al. (2013) sampled 2 weeks after harvest as opposed to 2 months in this study. This could explain changes in fungal community structure in the course of storage with selection forces favouring more adapted species. The choice of sampling time in this study was informed by the observation that toxin build up approaches peak in store at 6-8 weeks post-harvest. The findings of this study could inform the development of a sampling protocol towards assurance of food safety as knowledge of abundance and composition of toxigenic species helps in early warning systems. This is more important, considering that the maize sampled is what was being consumed. A striking finding corroborated in the two studies is the low density of *A. parasiticus* isolated from Makueni County. This is attributable to geographic factors such as the latitudinal position of the area, besides competition and other limiting factors like water and nutrients Klich (2002b).

In India, Venkataramana et al. (2013) determined the mould incidence and mycotoxin contamination in 150 freshly harvested maize samples and obtained 288 fungal isolates consisting of *Fusarium*, *Aspergillus* and *Penicillium* species. *A. flavus* was the predominant *Aspergillus* species as opposed to *A. niger* in the current study. In this study, 229 *Aspergillus* isolates were obtained from 50 maize and soil samples thus implying a higher fungal burden in the Kenyan samples. One possibly contributing factor could be the climatic differences during sampling as well as the samples. Venkataramana et al. (2013) collected freshly harvested samples in winter, which is non-existent in Kenya. In this study, the samples were collected 2 months postharvest. The hot climatic conditions of Eastern Kenya as well as the postharvest (storage) conditions of the samples in

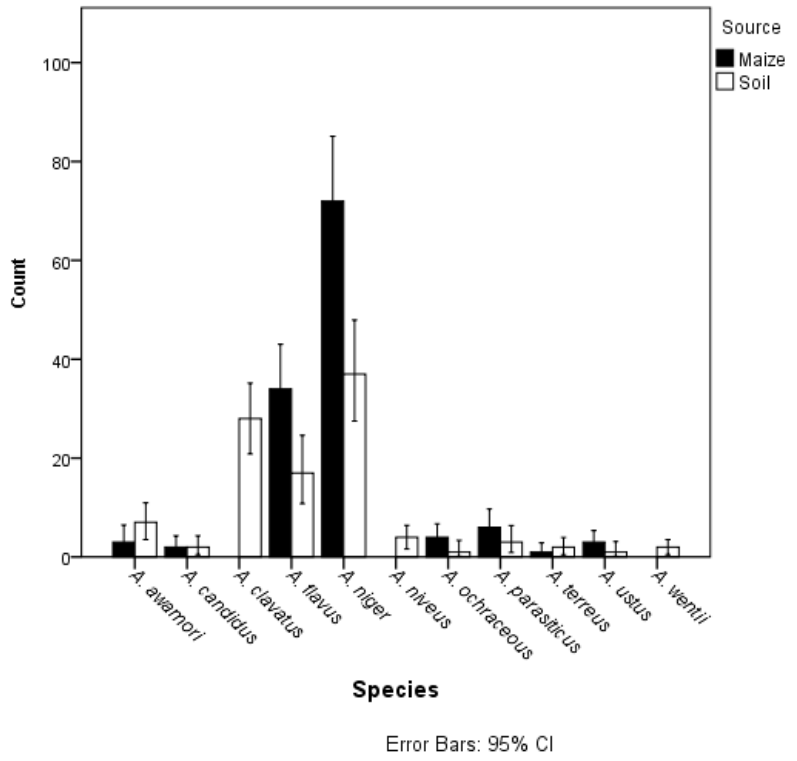

Figure 2. The incidence of the eleven *Aspergillus* species in maize and soil from the Eastern region of Kenya.

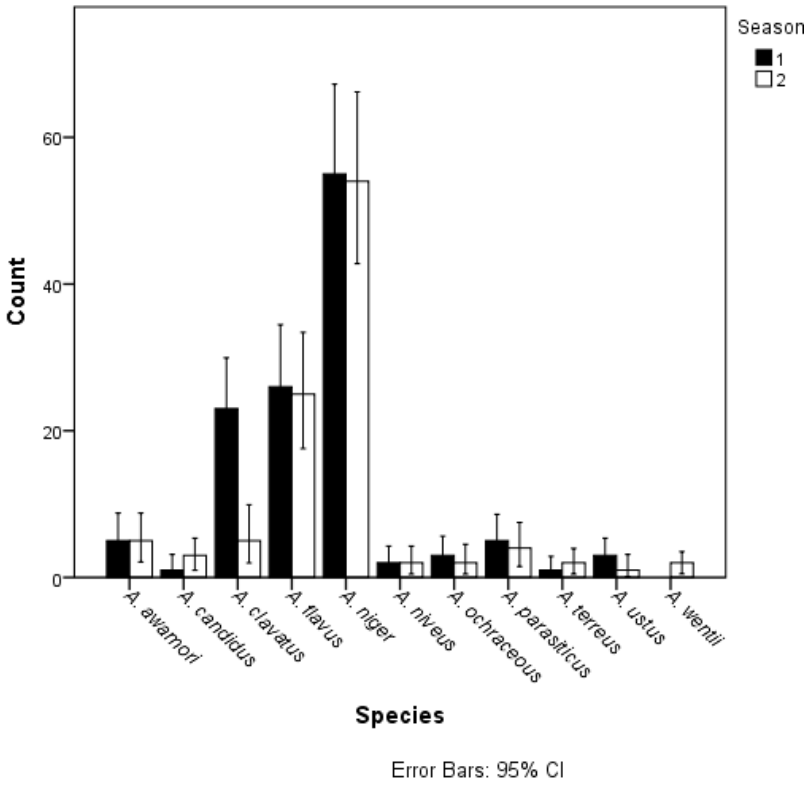

Figure 3. The distribution of *Aspergillus* spp. in the two planting seasons.

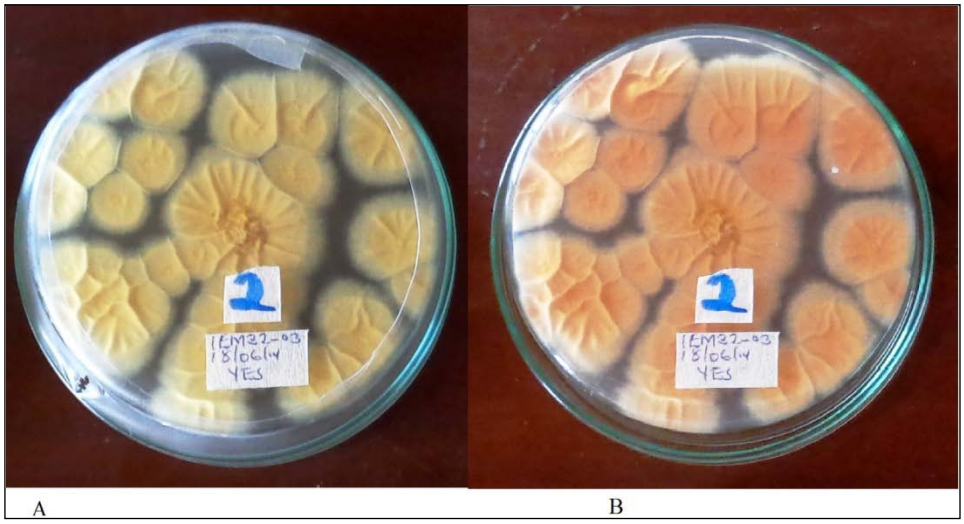

Plate 3. Toxigenic *Aspergillus* species. A and B show the colony reverse of *Aspergillus flavus* isolate from maize in yeast extract sucrose media before and after exposure to NH_4OH. The colony reverse turned from pale yellow to pink to indicate the production of toxins.

Table 4. Abundance of toxigenic and non-toxigenic *Aspergillus* species from maize and soil.

	Season									
	I					II				
	Negative		Positive		Total	Negative		Positive		Total
Maize	45	75.0	15	25.0	60	58	89.2	7	10.8	65
Soil	53	82.8	11	17.2	64	32	80.0	8	20.0	40
Total	98	79.0	26	21.0	124	90	85.7	15	14.3	105

this study were likely to have influenced the abundance and diversity of fungal isolates observed. This is in line with the observation that successful colonization postharvest as influenced by storage conditions may influence the growth of mycotoxigenic fungi and the subsequent production of toxins (Chulze, 2010). Priyanka et al. (2014) studied the molecular diversity of toxigenic *Aspergillus* species from food samples grown in high-rainfall regions in India where 200 isolates were recovered from 320 grain samples. This study also points to a higher fungal burden in Kenya as compared to India. The areas sampled in this study have temperate climatic conditions and experience high rainfall, which is a contrast to eastern Kenya. Even though the authors do not compare the incidence of the fungi by seasons, it is evident that the predominant *Aspergillus* species in both continents are *A. flavus* and *A. parasiticus*.

In the current study, seven putatively toxigenic species (*A. flavus*, *A. parasiticus*, *A. ochraceous*, *A. clavatus,A. ustus*, *A. niveus* and *A. wentii*) were reported. This corroborates earlier reports of toxin production by *Aspergillus* species other than *A. flavus* for example; *A. parasiticus* produces aflatoxins (Abbas et al., 2005); *A. ochraceous* produce ochratoxin A (Montessinos et al., 2015) while *A. wentii* produces the mycotoxins emodin (Pitt and Hocking, 2009). A polyisoprenoid toxin referred to as Austin was reported to be produced by *A. ustus* (Chexal et al., 1976) whereas *A. niveus* has been reported to produce fumonisins (Storari et al., 2012) and aspochalasin Z (Gebhardt et al., 2004). *Aspergillus clavatus* was isolated from soil only and was not isolated from maize, it was classified as toxigenic in this study. Since it was not isolated from maize, human risks appear to be reduced. Earlier studies have reported this species to produce a variety of secondary metabolites (SM) such as patulin, pseurotin A and cytochalasin E (Zutz et al., 2013). On the other hand, other species which did not produce toxin in this study have been reported to be toxigenic in earlier studies for example; *A. candidus* was reported to produce a mycotoxin known as AcT1 (Chattopadhyay et al., 1987), whereas *A. awamori* has been reported to produce fumonisins (Storari et al., 2012) and aspochalamins A-D (Gebhardt et al., 2004). *Aspergillus terreus* has been reported to produce territrems in bakery products and grains (El-Sayed Abdalla et al., 1998). The ammonium test was used to

test the production of toxin in YES medium. It is a reliable method that has been published by Kumar et al. (2007). Another method is to include beta cyclodextrins in the culture medium, which would enhance the natural fluorescence of aflatoxins under ultra violet light (Fente, 2001; Yazdani, 2010). So far, three species of *Aspergillus* namely *A. flavus*, *A. parasiticus* and *A. nomius* have been reported to secrete aflatoxins (Varga et al., 2011). In this paper, additional putatively toxigenic species that may also be producing aflatoxins or its precursors are reported. For these reasons, there is need to confirm the toxigenicity of these fungi through analytical procedures such as HPLC, or through the use of molecular markers to confirm presence of toxin coding sequences in the various *Aspergillus* isolates' genome. Follow-up studies such as molecular studies to confirm the species of the fungi reported herein is also recommended.

The findings of this study are vital in understanding the possible toxin risks the population faces with each harvest and enable early preparedness. There is very little information on the seasonal variations of toxigenic *Aspergillus* species in Kenya, particularly in the Eastern region where repeated cases of aflatoxicosis have been reported. Therefore, our findings contribute to new knowledge with respect to the Eastern region of Kenya.

The wide variety of toxigenic fungi reported in this study points out that the burden of mycotoxin exposure in Eastern Kenya is likely higher than previously thought. This is supported by the fact that the sampled maize grain was what was being consumed at the local household. The situation is further aggravated by lack of regulatory testing at that level. It is also possible that fatalities reported in Kenya could be due to cases of acute exposure to a cocktail of toxins.

In Kenya, the routine tests of toxins by the regulatory agencies (The Government Chemist and The Kenya Bureau of Standards) mainly aimed at aflatoxins (B1, B2, G1 and G2). While, this is proper due to the abundance of aflatoxins (Probst, 2007), we hold the view that consideration to include Ochratoxins and Fumonisins should be made since isolates of *Aspergillus* in this and previous studies point to higher risks of exposure (Bayman et al., 2002).

Conflict of Interests

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

The authors acknowledge funding from the Bill and Melinda Gates Foundation (Grant ID# OPP1060246) from which reagents to carry out the analysis were purchased. Sampling was carried out with support from The National Commission for Science and Technology Foundation (NACOSTI/RCD/ST&I 5th Call MSc 085). The authors express their heartfelt gratitude to the farmers in Eastern Kenya for their co-operation in this work. They are grateful to Collins Omondi (Egerton University) for technical support in microbiology work.

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Vol. 10(6), pp. 185-190, 14 February, 2016
DOI: 10.5897/AJMR2015.7688
Article Number: 05AB94857114
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Distribution of *Malassezia* species in Mexican seborrheic dermatitis patients

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Received 31 July, 2015; Accepted 29 November, 2015

Yeasts of the *Malassezia* genus are linked to seborrheic dermatitis (SD) in humans; however, etiological species causing this disease can vary according to their geographical location. *M. globosa* and *M. restricta* are the most often isolated microorganisms and can be found in the skin of patients with SD. Nevertheless, species identification by molecular methods and the relationship among etiological agents and the clinical severity of the disease have not been determined in Mexican patients. The goal of this study was to analyze the prevalence of *Malassezia* species in the skin of SD patients by molecular methods in order to establish their distribution according to the severity of the disease in Monterrey, Nuevo Leon, Mexico. Skin samples from patients with SD (n = 60) were obtained by scraping and were cultured on modified Dixon agar. The *Malassezia* colonies were identified by amplification of the D1/D2 regions of 26S rDNA by polymerase chain reaction (PCR) and subsequent sequencing and BLAST analysis in GenBank. The positive *Malassezia* culture rate was 48.3%. The most commonly isolated species were *Malassezia furfur* (20%), *Malassezia globosa* (16.7%), *Malassezia sympodialis* (6.7%), *Malassezia restricta* (3.3%) and *Malassezia slooffiae* (1.7%). No significant difference was found in the distribution of *Malassezia* species according to disease severity.

Key words: Seborrheic dermatitis, *Malassezia*, LSU rDNA D1/D2, yeasts, *Malassezia furfur*; *M. globosa*.

INTRODUCTION

Malassezia species are lipophilic yeasts that are part of the flora of human skin and skin of warm blooded animals (Crespo et al., 1999; Matousek and Campbell, 2002; Scott et al., 2001). These yeast have been associated

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with diseases, such as seborrheic dermatitis, atopic dermatitis (Hirai et al., 2004; Sugita et al., 2002, 2004), folliculitis (Cabello and Cermeño-Vivas, 2004), catheter-associated neonatal sepsis (Falk et al., 2005; Van Belkum et al., 1994) pityriasis versicolor (Erchiga et al., 2000; Gaitanis et al., 2006) and external otitis (Cafarchia and Otranto, 2004; Crespo et al., 2000). *Malassezia* genus taxonomy has been recently restructured based on molecular DNA analysis and lipid requirements; *Malassezia* genus has been divided into seven species, including *Malassezia furfur* (Robin) [Baillon, 1889], *Malassezia pachydermatis* (Weidman) [Dodge, 1935], *Malassezia sympodialis* [Simmons and Gueho, 1990], *Malassezia globosa* Midgley (Guého et al., 1996), *Malassezia obtusa*, *Malassezia restricta* and *Malassezia slooffiae*. *M. pachydermatis* is the only non-lipid-dependent species. Currently, the genus *Malassezia* is composed by 14 species just accepted: *Malassezia dermatis*, *Malassezia japonica, Malassezia yamatoensis, Malassezia nana*, *Malassezia caprae*, *Malassezia equine* and *Malassezia cuniculi*. SD is an inflammatory dermatosis characterized by chronic eczema with erythematous plaques and dry or greasy scales (Gerd and Thomas, 2008). Usually this disease occurs in young adults in areas rich in sebaceous glands such as the face, scalp and upper trunk (Del Rosso, 2011; Tajima et al., 2008). SD is more frequent in patients with HIV than in the general population with a prevalence of 30-55% (Tajima et al., 2008; Gandra et al., 2006; Gupta and Bluhm, 2004; Rincón et al., 2005; Schechtman et al., 1995) as compared to 1-3% of the general population, being more prevalent in men than in women (Johnson and Roberts, 1977; Naldi and Rebora, 2009). Recently, published studies have demonstrated that *Malassezia* spp. may be a normal host in human skin (Fredricks, 2001; Mastrolonardo et al., 2003; Pierard, 2003; Schwartz et al., 2006). In contrast, the therapeutic response of SD to antifungal treatment suggests the role of the yeast as an etiological factor (Sugita et al., 2002). Some studies have investigated the relationship between SD and the presence of *Malassezia* yeast in human scales and observed that *M. restricta* and *M. globosa* are the most commonly associated species (Devlin, 2006; Zarei-Mahmoudabadi et al., 2013; Zhang et al., 2013). The association between clinical severity and each species remains unclear (Picardo and Camelli, 2008).

In the present study, the prevalence of *Malassezia* spp. in patients with SD lesions on the face and scalp was analyzed by molecular techniques and correlated the etiologic agents with clinical severity.

MATERIALS AND METHODS

Study subjects

Sixty patients with SD with active facial and scalp involvement (32 women and 28 men, with a mean age of 37 years) were selected. In each case, the extent and severity of the lesions were assessed. Only patients who had not used any topical or oral treatment during the previous month were included. A survey to assess disease onset, first degree relatives affected, stress-related outbreaks and history of other dermatological diseases was performed. Informed consent from all participants was obtained according to the requirements of the institutional ethics committee. Sampling was performed from the facial and scalp lesions, and the skin scales removed with a sterile blade transferred to the laboratory in a sterile Petri dish, were processed at the microbiology laboratory of the Faculty of Veterinary Medicine. The samples were cultured on modified Dixon's medium containing chloramphenicol (0.5%) and cycloheximide (0.5%). Plates were incubated at 32°C for 7 -14 days, and identification of *Malassezia* species was performed according to the methods described by Guého et al. (1996) and Guillot et al. (1996). The presence of the yeast *Malassezia* was microscopically observed using Gram stain from the colonies grown on modified Dixon´s medium, and the results were confirmed by amplified PCR of the variable D1 and D2 regions of the 26S rRNA gene, using the conserved fungal oligonucleotide primers NL1 and NL4 (O'Donnell, 1993).

Ethical approval

All procedures involving human participants were performed in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Samples

DNA extraction

For DNA isolation, cells were harvested from 4-5 day old cultures in modified Dixon'smedium, and the DNA was prepared as described previously by Ferrer et al. (2001). Briefly, the yeasts cells were incubated for 1 h at 65°C in 500 µL extraction buffer (Tris-HCl 50 mM, EDTA 50 mM, SDS 3% and 2-mercaptoethanol 1%). The lysate was extracted using phenol : chloroform : isoamyl alcohol (25:24:1). Then, 65 µL of 3 M sodium acetate and 75 µL of 1 M NaCl were added to 350 µL of the supernatant, and the resulting volume was incubated at 4°C for 30 min. The DNA was recovered by isopropanol precipitation; washed with 70% (v/v) ethanol; dried under a vacuum; and resuspended in TE buffer (Tris-HCl 10 mM, EDTA 1 mM, pH 8). The Ultrospec 3000 pro (Amersham Bioscience, GE Healthcare, DF, México) was used to determine the DNA concentration.

PCR and sequencing of isolates

Genomic DNA (100 ng) obtained from samples that were suspected to have *Malassezia* spp. was used as a template in a PCR reaction with a final concentration of 10 mM buffer 10X (BioTherm), 0.1 mM dNTP´s, 1.5 mM MgCl, 0.198 µM each primer (NL1 5´ GCATATCAATAAGCGG AGGAAAAG-´3; NL4 5´-GGTCCGTGTTTCAAGACGG-´3) (O'Donnell, 1993) and 1 U Taq DNA polymerase (Bio Therm Piscataway, NJ 08854 USA). PCR conditions consisted of a pre-denaturing step at 94°C for 5 min and 30 amplification cycles: denaturation at 94°C for 45 s; annealing for 1 min at 51°C; extension at 72°C for 3 min; and a final extension at 72°C for 10 min. The PCR reactions were performed in a MaxyGene Gradient (Foster City CA, USA). The PCR products were run on a 2% agarose gel containing 0.5 mg/ml ethidium bromide. Analyses and imaging of the gel were performed with the TransilluminatorMultiDoc-Lt. Digital Imaging System (Upland CA, USA). The PCR products were sequenced in the ABI Prism 3130

Table 1. DNA samples from patients analyzed by PCR and sequencing.

Species	Samples (n=60)	Samples analyzed (%)
*M. furfur**	12	12/60 (20)
M. globosa^{+}	10	10/60 (16.6)
M. sympodialis$^{\#}$	4	4/60 (6.6)
M. restricta$^{\&}$	2	2/60 (3.3)
M. slooffiae$^{\%}$	1	1/60 (1.6)
Positive	29	29/60 (48.3)
Negative	31	31/60 (51.6)
Total	60	60/60 (100)

*M. furfur** 26S rRNA gene (GenBank accession No. KF733799); *M. globosa*$^{+}$ 26S rRNA gene (GenBank accession No. KF733801.1); *M. sympodialis*$^{\#}$ 26S rRNA gene (GenBank accession No. KF733798); *M. restricta*$^{\&}$ 26S rRNA gene (GenBank accession No. KF733797); *M. slooffiae*$^{\%}$ 26S rRNA gene (GenBank accession No. KF733800.1).

system (Applied Biosystems, Foster City CA). The positive strand sequences were analyzed with BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to compare with available *Malassezia* spp. sequences. The sequences obtained from five samples were submitted to Genbank.

Statistical analysis

A Chi-squared test was performed to evaluate the distribution differences of *Malassezia* species using SPSS v. 17.0 (Chicago, IL USA). *P* values of < 0.05 were considered significant.

RESULTS

Observation with the microscope allowed the detection of small cells, oval or ellipsoid, with the shape of a bottle or bowling pins, which is characteristic of this yeast.

Malassezia species identification

Of the 60 patient samples analyzed, 29 were positive for *Malassezia*, including 12 for *M. furfur* (20%), 10 for *M*. *globosa* (16.7%), 4 for *M. sympodialis* (6.7%). The lowest frequency values were for *M. restricta* and *M. slooffiae* with 2 (3.3%) and 1 (1.7%), respectively. Of the total samples, 51.6% (31/60) were negative for yeast (Table 1). Sequence alignments indicated that *M. furfur, M. sympodialis, M. restricta* and *M. slooffiae* isolates had 99% homology with the type strains. Only the *M. globosa* sequence had 100% homology with *M. globosa* (GenBank accession No KF733801.1) (Table 1).

Species distribution according to disease severity

The patients were divided into three groups according to the severity scale published by Peyri and Lleonart (2007) which evaluates the signs and symptoms of the disease, such as erythema, scaling, itching and infiltration. Moderate disease was the predominant clinical form in our population (47.6%), followed by severe (33.0%) and mild forms (18.3%). There was no significant difference in the species distribution among these groups ($P > 0.05$) (Figure 1). In the survey, 56.7% of the immediate family members were affected by SD (Figure 2). The father was the most affected family member (18.3%) (Figure 3). Outbreaks with emotional stress were experienced by 65.0% of patients.

DISCUSSION

SD is a chronic inflammatory skin condition and is a frequent cause of dermatology consults with an important impact on patients' quality of life (Smith, 2001; Fivenson et al., 2002). The cause of seborrheic dermatitis is not completely understood. SD has an increased prevalence in patients using psychotropic medications and patients with Parkinson's disease, other dermatoses (such asacne and rosacea), stress and AIDS (Jensen et al., 2000). One of the most studied causes is the association of colonization by the lipophilic yeast of the genus *Malassezia*. However, this association remains unclear. Yeast colonization in skin lesions varies from 45-100%. In our study, *Malasssezia* was isolated in 48.3%, a value similar to that reported by Byung et al. (2010) in Korean patients. The controversy of the association of yeast with SD pathogenesis remains under discussion because *Malassezia* is not isolated in all cases. In recent years, there has been special interest in determining the distribution of *Malassezia* species in SD, and several studies have demonstrated important variations in the species isolated depending on geographic location. Some researchers have found predominantly *M. globosa* (Van Belkum et al., 1994; Rincón et al., 2005; Byung et al., 2010; Gaitanis et al., 2006; Gupta et al., 2001; Nakabayashi et al., 2000) while others *M. restricta* (Gemmer et al., 2002; Lee et al., 2001) or *M. sympodialis* (Falk et al., 2005). None of the studies reported *M. furfur* as the most common agent. To our knowledge, only Hernandez et al. (2003) has previously studied Mexican patients. In their study, of 15 cases, the most commonly

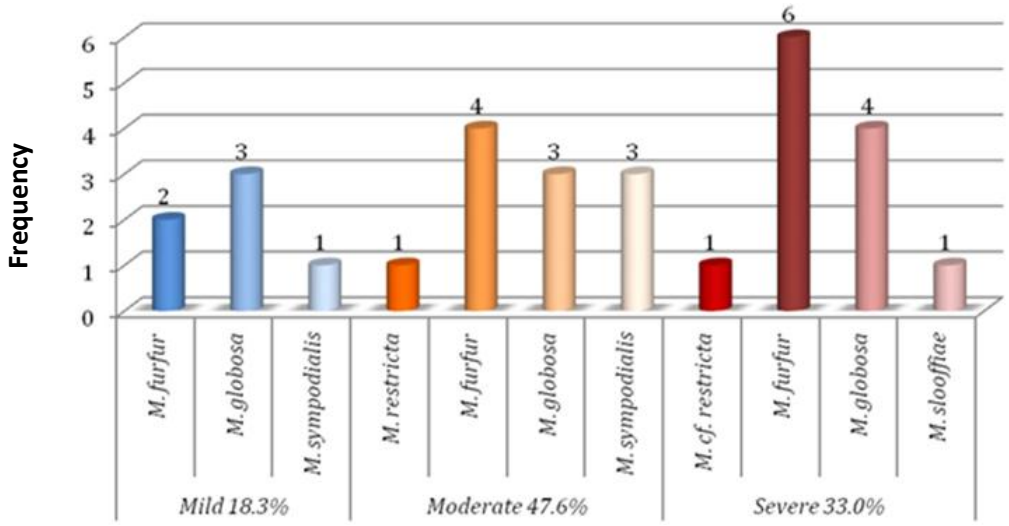

Figure 1. Distribution of Malassezia species according to severity of the disease. Moderate disease was most common in our population and *M. fur* was found in all severities.

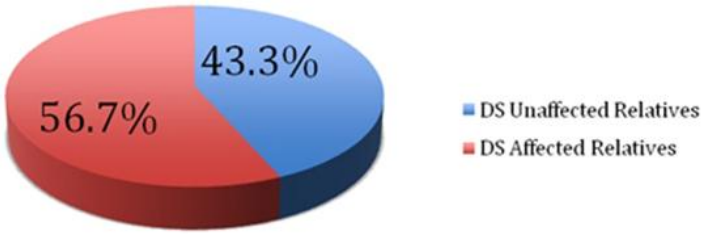

Figure 2. Percentage of patients reporting any immediate family member affected or unaffected with SD. Most patients had some affected relatives (56.7%).

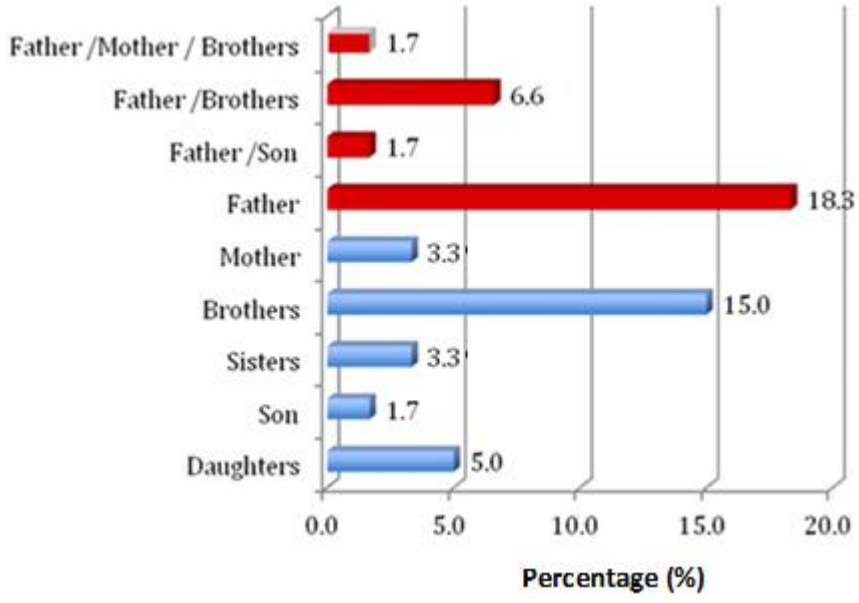

Figure 3. Detailed graph of family members reported with SD. The farthest was the most affected (28.3%).

isolated species was *M. sympodialis* (38.2%) followed by *M. furfur* (26.5%). These findings contrast with our study where the most frequently isolated species was *M. furfur* (20.0%) followed by *M. globosa* (16.7%) and *M. sympodialis* (6.7%). a correlation was not found between the severity of SD and species distribution. This result is similar to the data obtained by Prohic (2010), in a study of 40 patients where the species identification was performed by chemical methods. The most commonly isolated fungi were *M. restricta* (27.5%), followed by *M. globosa* (17.5%) and *M. slooffiae* (15%). However, the authors did not find a significant difference in the distribution of isolated *Malassezia* species according to the severity of scalp involvement. A study by Misery et al. (2007) found that 100% of the SD cases were associated with stressful conditions. In another study, the clinical characteristics of SD, therapeutic strategies employed in current clinical practice, and impact on quality of life were studied in a Spanish population. Two thousand-one-hundred and fifty nine patients were included. The mean age was 43.6 years; and 55% were men. In total, 42% had a family history of seborrheic dermatitis. Ninety-eight percent of patients reported trigger factors, such as stress/depression/fatigue (76%) and seasonal variation (44%) (Prohic, 2010; Dawson, 2007). In the present study, the majority (65%) of patients reported that stress was related to outbreaks. Another finding (not previously described) was the disparity between the prevalence of an affected father (20%) as compared to the mother (5%). This finding could suggest direct contact transmission that could be studied using subtyping methods or whole genome sequence comparisons.

Conclusion

This is the first study in Mexico that determined (by molecular methods) the presence and species distribution of *Malassezia* in the skin of patients with SD. *M. furfur* was the dominant species in our population and is in contrast with other studies. There was no significant association between the species isolated and disease severity. It was found that most patients mentioned a paternal relative with the same dermatosis. There are probably genetic or environmental factors that alter the ecosystem of saprophyte microorganisms in human skin, which could make individuals susceptible to this condition. Studies with a larger numbers of patients are necessary to confirm these findings.

Conflict of interests

The authors have not declared any conflict of interest.

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