African Journal of Microbiology Research

Volume 9 Number 14, 8 April, 2015
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

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African Journal of Microbiology Research

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academicJournals

Vol. 9(14), pp. 965-978, 8 April, 2015
DOI: 10.5897/AJMR2015.7195
Article Number: 3D94C2352245
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Review

Antibiotics and antibiotic resistant bacteria in wastewater: Impact on environment, soil microbial activity and human health

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Received 15 October, 2014; Accepted 12 January, 2015

In recent years, pharmaceutical compounds (PhCs) have aggravated increasing anxiety, particularly as no legitimate requirements have been set for discharge into surface water bodies of these ubiquitous, persistent and biologically active substances. Massive quantities of antibiotics are used in human and veterinary medicines in all parts of the globe to treat diseases with bacterial origins. After administration, antibiotics are excreted by the patient and transmitted in due course to the aquatic environment. These are also largely used in animal operations for growth promotion and for disease prophylaxis. These are often partially metabolized after administration and a significant fraction of the antibiotic can be excreted as the parent compound or in conjugated forms that can be converted back to the parent antibiotic. The residual antibiotics from human and animal use can enter the environment *via* various pathways, including wastewater effluent discharge, runoff from land to which agricultural or human waste has been applied, and leaching which deteriorate the whole ecosystem besides its deleterious impact on human health and aquatic organisms. Antibiotic resistance is a global phenomenon that has severe epidemiological ramifications worldwide and a major peril to public health. This article may give an idea about the sources and fate of commonly used antibiotics detected. More research is needed to quantify the risk of antibiotics in urban wastewater and effluents or surface water so that appropriate action can be taken prior to final discharge into surface water bodies to mitigate the harmful effects on aquatic environment and community health.

Key words: Antibiotics, antibiotics resistance, antibiotics resistance bacteria, health hazards, microbial activity, wastewater.

INTRODUCTION

In the last two decades, occurrence of antibiotics in water bodies and subsequent development of resistance in microorganisms have come into scientific and public focus as an issue of potential concern. Antibiotics can be defined as organic substances that are produced through the secondary metabolism of living microorganisms or synthesized artificially or semi-artificially that can kill other microorganisms or inhibit their growth or metabolic

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activity via biochemical actions (Lancini and Parenti, 1982; Thomashow and Weller, 1995; Thiele-Bruhn, 2003). Mass production and use of antibiotics in medicine and agriculture have existed and substantially benefited public health and agricultural productivity for over 60 years (Knapp et al., 2010). They have important uses in both human and veterinary medicine for their antibacterial properties and as growth promoters. Their consumption has increased worldwide, and their residues are frequently reported in aquatic environments. It is estimated that the total amounts of annual use of antibiotics had reached 100,000-200,000 tons worldwide (Wang and Tang, 2010), including veterinary antibiotics and medical antibiotics. Among them, veterinary antibiotics are in the majority of the total amount used. For example, they approximately accounted for 70% of the total consumption in the USA and about 70% of them are used for non-therapeutic purposes (Sassman and Lee, 2005; UCS, 2001). China is the largest country in the world for production and use of antibiotics. In 2003, China produced 28,000 and 10,000 tons of penicillin and oxytetracycline (OTC), occupying 60 and 65% of the global total output (Yang et al., 2010), respectively. The total production of major antibiotics in India was more than 2332 million tons in 2006-2007, with a growth rate of 10% (IDMA, 2009).

Medicines consumed for healthcare practices are often released into sewage, and after sewage treatment plant, it reaches the receiving water bodies of lakes or rivers. A huge amount of antibiotics were discharged in water bodies through effluents and get polluted (Dotaniya et al. 2014). Antibiotics used in human medicine are found at trace concen-trations in municipal wastewaters, and residual amounts reach ambient waters. Once administered, they are metabolized to varying degrees, and their excreted metabolites and unaltered parent compounds can also undergo further modification due to biological, chemical and physical processes in both sewage treatment facilities and receiving water bodies (Deblonde et al., 2011; Fatta-Kassinos et al., 2011; Miege et al., 2009; Monteiro and Boxall, 2010; Onesios et al., 2009). The majority of the compounds mentioned in the various studies are administrated orally, intramuscularly, endovenously or by inhalation, and in few cases on the skin. After administration to humans and animals, up to 90% of the antibiotics can be excreted unchanged via urine and/or feces (Hirsch et al., 1998). These sub-stances are only partially eliminated during sewage treatment (Ternes et al., 2003; Berset et al., 2004). Antibiotics from human sources can enter into the environment through discharge of treated effluents and sludge from domestic wastewater treatment plants (WWTPs) due to incomplete removal, and untreated wastewaters (Batt et al., 2006b; Watkinson et al., 2007; Xu et al., 2007). Therefore, WWTPs play a crucial role in the pollution control of antibiotics to the receiving environments. Antibiotic residues entering in sewage are directly proportional to their amount used for human and other uses in particular area. The β-lactam antibiotics, which include the penicillins and cephalosporins, represent the largest fraction of human antibiotics, accounting for approximately 18 tones in Switzerland. These were not frequently detected in wastewater because of easy hydrolytic cleavage of the β-lactam ring, despite the fact that β-lactams account for the highest proportion of consumption (Junker et al., 2006).

Antibiotic residues have been detected in different water matrices, including hospital wastewaters (Kummerer, 2001; Lindberg et al., 2004), sewage treatment plant (STP) effluents (Batt et al., 2006; Watkinson et al., 2007; Mutiyar and Mittal, 2013), STP biosolids (Kinney et al., 2006a), soil (Kinney et al., 2006b), surface waters (Kolpin et al., 2002, 2004; Batt et al., 2006), groundwater (Hirsch et al., 1999; Lindsey et al., 2001), sediments (Kerry et al., 1996; Kim and Carlson, 2006), and drinking water (Zuccato et al., 2000, 2005). The first reported case of surface water contamination by antibiotics was in England in 1980, when Watts et al. (1982) detected at least one compound from the macrolide, sulfonamide and tetracycline group of antibiotics in river water at concentrations of 1 µg L^{-1}. Wide range of pharmaceutical formulation is manufactured and used in India, which could lead to the release of more pharmaceuticals substances in the environment (Kurunthachalam, 2012). The PhCs in raw wastewaters are generally in the range of 10^{-3}-10^{-6} mg L^{-1} (Le Minh et al., 2010; Ziylan and Ince, 2011).

Livestock wastes containing antibiotic residues are directly or indirectly discharged into surface water or applied onto agricultural lands with little treatment. Chlortetracycline is less commonly used in humans, but it is widely used in animals to prevent and treat disease (Kemper, 2008). Similarly, the occurrence of lincomycin has been less reported in domestic wastewater (Watkinson et al., 2007), probably due to its main application in animals (Kemper, 2008). More importantly, significant amount of antibiotics and their bioactive metabolites or degradation products were introduced in agro-ecosystems through fertilization and irrigation with antibiotics-polluted manures, biosolids, sewage sludge, sediments and water. Subsequently, accumulation and transport of antibiotics in soil-crop systems, particularly soil-vegetable systems, for example, protected vegetable and organic vegetable production systems, poses great risks on crops, soil ecosystem and quality of groundwater and plant-based products (Ramana et al. 2002; Lianfeng and Wenke, 2012). These results are reported with a view to aiding scientists and administrators in planning measures aiming to reduce the impact of antibiotics in treated urban wastewater discharge into surface water bodies.

MOST COMMONLY USED ANTIBIOTICS

Antibiotics belonging to different classes (β-lactams,

Table 1. Most commonly used antibiotics with their class/subclass, molecular weight (MW) and chemical formula.

Antibiotic name	Class/subclass	Abbreviation	MW	Chemical formula
Amoxicillin	Penicillins	AMX	365	$C_{16}H_{19}N_3O_5S$
Azithromycin	Macrolide	AZM	749	$C_{38}H_{72}N_2O_{12}$
Cefaclor	Cephalosporins	CEC	368	$C_{15}H_{14}ClN_3O_4S$
Cefalexin	Cephalosporins	LEX	347	$C_{16}H_{17}N_3O_4S$
Cefotaxime	Cephalosporins	CTX	456	$C_{16}H_{17}N_5O_7S_2$
Chloramphenicol	Chloramphenicol derivatives	CAP	323	$C_{11}H_{12}Cl_2N_2O_5$
Chlortetracycline	Diaminopyrimidines	CTC	479	$C_{22}H_{23}ClN_2O_8$
Ciprofloxacin	Fluoroquinolones	CIP	331	$C_{17}H_{18}FN_3O_3$
Clarithromycin	Macrolide	CTM	748	$C_{38}H_{69}NO_{13}$
Clindamycin	Others	CLI	425	$C_{18}H_{33}ClN_2O_5S$
Cloxacillin	Penicillins	CXA	436	$C_{19}H_{18}ClN_3O_5S$
Doxycycline	Diaminopyrimidines	DC	463	$C_{22}H_{24}N_2O_8$
Enoxacin	Fluoroquinolones	ENX	320	$C_{15}H_{17}FN_4O_3$
Enrofloxacin	Fluoroquinolones	EFL	359	$C_{19}H_{22}FN_3O_3$
Erythromycin	Macrolide	ERY	734	$C_{37}H_{67}NO_{13}$
Lincomycin	Lincosamides	LIN	407	$C_{18}H_{34}N_2O_6S$
Lomefloxacin	Fluoroquinolones	LOM	351	$C_{17}H_{19}F_2N_3O_3$
Metronidazole	Nitroimidazoles	MTZ	171	$C_6H_9N_3O_3$
Norfloxacin	Fluoroquinolones	NOR	319	$C_{16}H_{18}FN_3O_3$
Ofloxacin	Fluoroquinolones	OFL	361	$C_{18}H_{20}FN_3O_4$
Oxytetracycline	Diaminopyrimidines	OTC	460	$C_{22}H_{24}N_2O_9$
Penicillin G	Penicillins	PEN G	334	$C_{16}H_{18}N_2O_4S$
Penicillin V	Penicillins	PEN V	350	$C_{16}H_{18}N_2O_5S$
Roxithromycin	Macrolide	RTM	837	$C_{41}H_{76}N_2O_{15}$
Spiramycin	Macrolide	SPM	843	$C_{43}H_{74}N_2O_{14}$
Sulfachloropyridazine	Sulfonamides	SCP	285	$C_{10}H_9ClN_4O_2S$
Sulfadiazine		SDZ	250	$C_{10}H_{10}N_4O_2S$
Sulfadimethoxine		SDM	310	$C_{12}H_{14}N_4O_4S$
Sulfamethazine	Sulfonamides	SMZ	278	$C_{12}H_{14}N_4O_2S$
Sulfamethoxazole		SMX	253	$C_{10}H_{11}N_3O_3S$
Sulfapyridine	Sulfonamides	SPD	249	$C_{11}H_{11}N_3O_2S$
Sulfasalazine	Sulfonamides	SSZ	398	$C_{18}H_{14}N_4O_5S$
Sulfathiazole	Sulfonamides	STZ	255	$C_9H_9N_3O_2S_2$
Tetracycline	Diaminopyrimidines	TC	444	$C_{22}H_{24}N_2O_8$
Trimethoprim	Diaminopyrimidines	TMP	290	$C_{14}H_{18}N_4O_3$
Tylosin	Macrolide	TYL	916	$C_{46}H_{77}NO_{17}$

sulfonamides, quinolones, tetracyclines, macrolides and others) have been frequently detected in sewage, activated sludge, digested sludge and effluents (Table 1). Among these antibiotics, tetracyclines were the most commonly used, followed by sulfonamides and macrolides that accounted for approximately 90% of the total antibiotics used in the United Kingdom and more than 50% in Korea and Denmark (Kim et al., 2011). Verlicchi et al. (2012) found the most commonly investigated compounds were trimethoprim, sulfamethoxazole, erythromycin and ciprofloxacin in Italy. The highest absolute concentrations were found for ofloxacin (32 µg L^{-1}), roxithromycin (17 µg L^{-1}) and ciprofloxacin (14 µg L^{-1}).

Other antibiotics exhibiting measured concentrations greater than 10 µg L^{-1} are: sulfapyridine (12.4 µg L^{-1}), trimethoprim (10.5 µg L^{-1}) and erythromycin (10.2 µg L^{-1}). The highest average antibiotic concentrations were found for ofloxacin and sulfadiazine (5.1 µg L^{-1}), followed by sulfapyridine (3.3 µg L^{-1}) and cefalexim (3.2 µg L^{-1}). No data were provided for enoxacin, lomefloxacin and spiramycin concentrations in the raw urban wastewater.

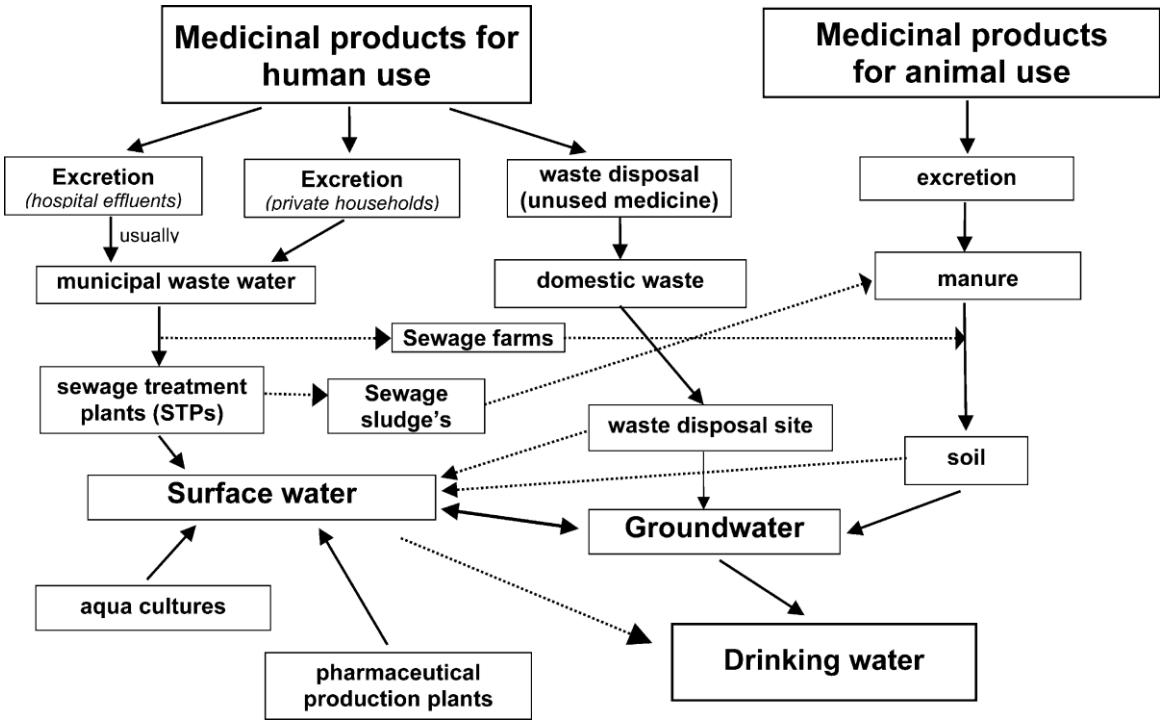

Figure 1. Scheme showing possible sources and pathways for the occurrence of pharmaceutical residues in the aquatic environment (Heberer, 2002).

OCCURRENCE OF ANTIBIOTICS

The occurrence of pharmaceuticals as contaminants in wastewater and in the aquatic environment has attracted increased attention. A high percentage of pharmaceuticals consumed by humans are excreted unchanged via urine and feces into the sewage. Ultimately, their residual amounts can reach ambient surface waters or groundwater. Occurrence of antibiotics in effluent from potential sources *viz.* hospitals, residential facilities, dairies and municipal wastewater are of prime concern (Figure 1). Most antibiotics from various sources will eventually enter in environmental compartments through different pathways. The excretion of incompletely metabolized antibiotics by humans and animals is the primary source of antibiotics in the environment. All kinds of manures, as another important carrier of veterinary antibiotics, their transfer and seepage from manures to soil, surface, and groundwater also contribute to the environmental release of antibiotics (Kim and Carlson, 2005; Kim et al., 2005; Burkhardt et al., 2005; Kay et al., 2005).

Other sources include the disposal of unused antibiotics and waste from pharmaceutical manufacturing processes. Residential (private residences, dormitories, hotels and residential care facilities) and commercial facilities (including hospitals) are known contributors of antibiotics to municipal wastewater (Guiliani et al., 1996; Guardabassi et al., 1998; Hartmann et al., 1998; Alder et al., 2003). Apart from these, further potential contributors of antibiotics to surface and ground water are effluent from wastewater treatment plants (WWTPs) (Golet et al., 2002; Hirsch et al., 1999; McQuillan et al., 2002; Alder et al., 2003) and industrial facilities (including pharmaceutical plants) (Guardabassi et al., 1998), and surface run-off from concentrated animal feeding operations (CAFOs) (Campagnolo and Rubin, 1998; Hirsch et al., 1999). Various antibiotics have been frequently detected in groundwater (Barnes et al., 2008; Batt et al., 2006a), drinking water (Focazio et al., 2008), surface water (Hirsch et al., 1999; Yang et al., 2011), sediment (Zhou et al., 2011) and agricultural lands (Hu et al., 2010; Karci and Balcioglu, 2009). In the municipal WWTPs, sulfamethoxazole, trimethoprim, norfloxacin, ofloxacin and anhydro erythromycin were the predominant antibiotics in the effluents, while in the sludge oxytetracycline, tetracycline, ofloxacin and norfloxacin were dominant with much higher concentrations than the others (Zhou et al., 2013).

Ye et al. (2004) found the largest number of antibiotics in its source water that is downstream of multiple wastewater plants and agricultural non-point discharge. Overall, fluoroquinolones were the most frequently detected antibiotics in source waters followed by sulfonamides, lincomycin, tetracyclines and macrolides (USP, 2003). Municipal wastewater had detections of sulfamethoxazole, trimethoprim, ciprofloxacin and ofloxacin, with four of six samples having at least one anti-

biotic present and 3 having 3 or more. The relatively high concentrations (up to 35,500 ng L^{-1}) of ofloxacin found in hospital and residential effluent may be of concern due to potential genotoxic effects and development of antibiotic resistance. The results from a study in South China showed the presence of sulfamethoxazole, norfloxacin, ofloxacin, anhydro erythromycin and trimethoprim were most frequently detected in the WWTPs wastewaters. Twenty-one antibiotics were found in the sewage from the two WWTPs at the concentrations up to 5800 ng g^{-1}, with tetracycline, oxytetracycline, norfloxacin and ofloxacin being the predominant antibiotics (Zhou et al., 2013).

The *β*-lactams, despite that they account for the highest proportion (50-70%) of the total human use antibiotic consumption, their occurrence was not detected frequently due to their unstable property (Kummerer, 2009). No *β*-lactams have been found in sludge samples all over the world despite their wide application in human and veterinary medicine (Minh et al., 2009), mainly due to their unstable characteristics and poor adsorption onto sludge. Golet et al. (2001) analyzed fluoroquinolone antibiotics in primary and tertiary wastewater effluents in Switzerland. In these samples, ciprofloxacin and norfloxacin occurred at concentrations between 249 and 405 ng L^{-1} and from 45 to 120 ng L^{-1}, respectively. Sacher et al. (2001) reported the occurrence of sulfamethoxazole (up to 410 ng L^{-1}) and hydroerythromycin (up to 49 ng L^{-1}) in groundwater samples in Germany. Holm et al. (1995) found residues of different sulfonamides at high concentrations in groundwater samples collected down gradient of a landfill in Grinsted, Denmark.

The concentration of antibiotics in the Yamuna River (India) varied from not detected to 13.75 µg L^{-1} (ampicillin) for the compounds investigated. Ampicillin had the maximum concentration in wastewater influents (104.2±98.11 µg L^{-1}) and effluents (12.68±8.38 µg L^{-1}). The fluoroquinolones and cephalosporins had lower concentrations (Mutiyar and Mittal, 2014). In another study from Patancheru, Hyderabad (India), it was reported that WWTP effluents have the highest levels of ciprofloxacin (CIP) antibiotics residues (up to 31,000 µg L^{-1}) (Larsson et al., 2007). Later on, Fick et al. (2009) reported that water bodies receiving WWTP effluents often have high pharmaceutical residues as high peak concentration of antibiotics residues (up to 14,000 µg L^{-1}) from surface, groundwater, and drinking water of Hyderabad area. Hirsch et al. (1999) in various sewage, surface and groundwater samples in Germany did not detect penicillins or tetracyclines because the penicillins are easily hydrolyzed and tetracyclines readily precipitate with cations such as calcium and accumulate in sewage sludge or sediments (Daughton and Ternes, 1999; Stuer-Lauridsen et al., 2000).

The hospitals are primary contributor of antibiotics to municipal wastewater. Antibiotic occurrence was less pronounced in residential wastewater than in hospital effluent. Hartmann et al. (1998) detected between 3 to 87 µg L^{-1} of the fluoroquinolone antibiotic ciprofloxacin in hospital effluents. Brown et al. (2006) reported that 58% of samples, out of 26 had at least one antibiotic present while 25% had three or more. Effluents discharged from hospitals showed the presence of sulfamethoxazole, trimethoprim, ciprofloxacin, ofloxacin, lincomycin and penicillin G. At the residential sampling sites, ofloxacin was found in effluent from assisted living and retirement facilities, while the student dormitory had no detection. Only lincomycin was detected in dairy effluent (in 2 out of 8 samples, at 700 and 6600 ng L^{-1}). Brown et al. (2006) also reported lincomycin antibiotic which is commonly used to treat foot warts and mastitis in lactating cows, was detected in two out of eight analyzed dairy effluent samples at concentrations of 700 and 6600 ng L^{-1}.

The ample occurrence of antibiotics in the environment could affect aquatic and terrestrial organisms (Costanzo et al., 2005; Kotzerke et al., 2008; Liu et al., 2009), alter microbial activity and community composition (Underwood et al., 2011), and lead to prevalence of bacterial resistance to antibiotics (LaPara et al., 2011; Su et al., 2012; Tao et al., 2010). Although, the occurrence level of individual antibiotics may be low, but the presence of a broad range of antibiotics in some source water suggests that the overall effect of the antibiotics as a contaminant group should not be underestimated. In order to reduce the negative impacts on the environment and human health, it is necessary to understand the input sources for various classes of antibiotics. Therefore, the fate of pharmaceutical compounds in wastewater and other water systems should be properly investigated, including monitoring ambient concentrations in STPs and receiving water bodies.

CONCENTRATION OF ANTIBIOTICS

Based on the literature reviewed, huge variability in the concentration of each compound in both raw urban influents as well as in secondary effluent was observed. Mutiyar and Mittal (2014) measured the mean residues concentration in influents was 104.2 µg L^{-1} for AMP, 20.1 µg L^{-1} for CIP, 2.7 µg L^{-1} for gatifloxacin (GAT), 22.5 µg L^{-1} for SPA and 3.4 µg L^{-1} for cefuroxime (CEF). For effluents, the mean antibiotics residues concentration was 12.68 µg L^{-1} for AMP, 8 µg L^{-1} for CIP, 1.22 µg L^{-1} for GAT, 0.14 µg L^{-1} for sparfloxacin SPA, and 0.22 µg L^{-1} for CEF. These are higher levels of antibiotics in wastewater than previously reported by others studies (Yang et al., 2005; Xu et al., 2007; Heidler and Halden, 2008; Logananthan et al., 2009) from different parts of the world, but lesser than those reported by Li et al. (2008). Xu et al. (2007) and Heidler and Halden (2008) reported similar levels of antibiotics to those reported here (10.8 µg L^{-1} in treated wastewater). Yang et al. (2005) reported concentrations of pharmaceutical resi-

dues around 1.1µg L^{-1}. Li et al. (2008) reported 389 mg L^{-1} of penilloic acid, a degradation product of penicillin G, with 153 µg L^{-1} of the parent compound also present. High concentrations of oxytetracycline (238-1680 ng g^{-1}) and tetracycline (117-1650 ng g^{-1}), ofloxacin (1500–5800 ng g^{-1}) and norfloxacin (1820-5610 ng g^{-1}) were found in sludge samples in China (Zhou et al., 2013; Gao et al., 2012; Jia et al., 2012) and doxycycline at a concentration of 1500 ng/g in Swedish sludge samples (Lindberg et al., 2005). CTC was found in 2.4% of the 84 surface water samples with the maximum concentration of 0.69 µg L^{-1} by Kolpin et al. (2002). Gulkowska et al. (2007) detected tetracycline, erythromycin, norfloxacin and trimethoprim in Hong Kong coastal waters. Managaki et al. (2007) reported that the veterinary antibiotics, *e.g.* erythromycin, trimethoprim and various sulfonamides, were detected in the Mekong Delta in Vietnam. The highest concentrations of penicillin V detected in influent and effluent were up to 13800 and 2000 ng L^{-1} (Watkinson et al., 2009), respectively.

Zhou et al. (2013) reported antibiotic concentration in two wastewater treatment plants in May and November and found that out of 50 target compounds, 19 and 15 antibiotics were quantified with concentrations ranging from 4.58 ± 0.03 to 942 ± 15.7 and 1.14 ± 0.32 to 695 ± 23.7 ng L^{-1} in the influent and final effluent of plant A, respectively. For plant B, 18, 16 and15 were detected at concentrations ranging from 3.22 ± 0.76 to 861 ± 30.6 ng L^{-1} in the influent, from 3.28 ± 0.53 to 277 ± 3.48 ng L^{-1} in the secondary effluent, and from 3.20 ± 0.06 to 101 ± 1.04 ng L^{-1} in the final effluent after disinfection, respectively. Trimethoprim was found in the concentrations ranging from 72.3 ± 0.83 (plant B in May) to 162 ± 9.09 ng L^{-1} (plant A in November) in the influents, and ranging from 31.1 ± 0.74 (plant B in May) to 64 ± 1.95 ng L^{-1} (plant A in November) in the effluents. Lincomycin was also detected in the two plants, with the concentrations ranging from 44.2 ± 0.10 (plant B in November) to 129 ± 2.96 ng L^{-1} (plant A in May) in the influents, and ranging from ND (not detected) (plant A in November and plant B in November) to 53.9 ± 4.31 ng L^{-1} (plant B in May) in the effluents. Chloramphenicol was only found in plant A with the concentrations below 22.1 ± 0.34 ng L^{-1} in the influents (in May) and below 5.8 ± 0.42 ng L^{-1} (in November) in the effluents. Whereas in the case of suspended solids and sludge, sulfonamides and trimethoprim were found with concentrations below 36.4±1.25 ng g^{-1} (sulfamethoxazole). Five tetracyclines and 7 fluoroquinolones were detected in concentrations ranging from 5.61 ± 1 (methacycline) to 1680 ± 16.7 ng g^{-1} (oxytetracycline) and from 1.72 (pefloxacin) to 5800 ± 157 ng g^{-1} (ofloxacin), respectively. Tetracyclines and fluoroquinolones were the predominant antibiotics detected in the sludge, and they contributed above 90% of the total loads of antibiotics in the dewatered sludge. Three macrolides found in the wastewater were quantified in the suspended solids and sludge, with the concentrations ranging from 4.64 ± 0.68 (roxithromycin) to 147 ± 6.01 ng g^{-1} (anhydroerythromycin) (Zhou et al., 2013).

Higher concentration levels of antibiotics have been reported in various rivers throughout the globe as per literature as follows: 11.92 µg L^{-1} of sulfamethoxazole in the Llobregat river in NE Spain (Munoz et al., 2009), 10 µg L^{-1} of CIP in the Arc river in France (Feitosa-Felizzola and Chiron, 2009), 1.3 µg L^{-1} of CIP in the Brisbane river Australia (Watkinson et al., 2009) and 80 µg L^{-1} of ERY in the Duhan river in Taiwan (Lin and Tsai, 2009). Yamuna River in India is one of the most contaminated river stretches in India. It receives around 3,000 million liter per day, that is, MLD of sewage from various drains. Concentration of amoxicillin in the untreated raw sewage at the STP located in Delhi varied from ND to 172.6 ng L^{-1} and after treatment; it varied from ND to 62.5 ng L^{-1} (Mutiyar and Mittal, 2013). The influent loadings varied from 301 to 11,462 g day^{-1} for the different antibiotics and 15.4-1,395 g day^{-1} were released in the effluents (Mutiyar and Mittal, 2014). The estimated loadings were 1,395 g day^{-1} for AMP, 880 g day^{-1} for CIP, 134 g day^{-1} for GAT, 15.4 g day^{-1} for SPA, and 24.2 g day^{-1} for CEF, and the total loading from these compounds is 2,428 g day^{-1}. Similarly, high concentrations of sulfonamides (20 x 10^{-3} ng/ml) have been found in pig farm wastewater, and detection of sulfamethazine has been suggested to serve as a marker for livestock-source contamination in Vietnam (Managaki et al., 2007).

FATE OF ANTIBIOTICS IN THE SOIL

After entering antibiotics in agricultural soil, several interrelated processes were involved, including degradation (Kreuzig and Holtge, 2005), adsorption (Rabolle and Spliid, 2000), transport (leaching and runoff) (Blackwell et al., 2007), and plant uptake (Migliore et al., 1996; Kumar et al., 2005a; Boxall et al., 2006; Dolliver et al., 2007). The active ingredients in the upper soil layer might either accumulate in soil or be absorbed by crops, or be readily available for transport into surface and groundwater through leaching and overland flow runoff (Jongbloed and Lenis, 1998). All dynamic processes of antibiotics in soils are closely interrelated and driven by crops, soil microorganisms, water and anthropogenic activities, which will ultimately determine the spatial-temporal distribution and environmental impacts of antibiotics. Tetracycline could persist in soil for over 1 year (Zuccato et al., 2000), and only a moderate degradation of various tetracycline occurred within 180 days (Hamscher et al., 2002). Kolpin et al. (2002) detected about 9.5 µg kg^{-1} CTC in the soil surface, two days before sampling manure with animal slurry. Many countries had also detected the antibiotic residues in soils (Boxall, 2004; Hamscher et al., 2005; Martinez-Carballo et al., 2007).

ANTIBIOTIC RESISTANCE

The emergence of resistance of bacteria to antibiotics is a common phenomenon. Antibiotic resistance is a form of drug resistance whereby sub-populations of a microorganism, usually strains of a bacterial species, are able to survive after exposure to one or more antibiotics; pathogens resistant to multiple antibiotics are considered multidrug resistant (MDR) or, more colloquially, superbugs. It is a serious and hasty growing phenomenon in contemporary medicine and has emerged as one of the pre-eminent public health concerns of the 21st century, in particular as it pertains to pathogenic organisms. A World Health Organization report released in April 30, 2014 states, "This serious threat is no longer a prediction for the future, it is happening right now in every region of the world and has the potential to affect anyone, of any age, in any country. Antibiotic resistance, when bacteria change, so antibiotics no longer work in people who need them to treat infections, is now a major threat to public health. Genes for resistance to antibiotics, like the antibiotics themselves, are ancient. However, the increasing prevalence of antibiotic-resistant bacterial infections seen in clinical practice stems from antibiotic use both within human medicine and veterinary medicine. Any use of antibiotics can increase selective pressure in a population of bacteria to allow the resistant bacteria to thrive and the susceptible bacteria to die off. As resistance towards antibiotics becomes more common, a greater need for alternative treatments arises.

In medicine, the major problem of the emergence of resistant bacteria is due to misuse and overuse of antibiotics (WHO, 2002). In some countries, antibiotics are sold over the counter without a prescription, which also leads to the creation of resistant strains. Wastewater from hospitals and intensive farming facilities is probably a major source of pathogenic and antibiotic-resistant organisms and antibiotic-resistance genes that are released into the environment. Other practices contributing to resistance include antibiotic use in livestock feed to promote faster growth (Ferber, 2002; Mathew et al., 2007). Household use of antibacterial in soaps and other products, although not clearly contributing to resistance, is also discouraged. Unsafe practices in the pharmaceutical manufacturing industry can also contribute towards the likelihood of creating antibiotic resistant strains (Larsson and Fick, 2009). The procedures and clinical practice during the period of drug treatment are frequently flawed; usually no steps are taken to isolate the patient to prevent re-infection or infection by a new pathogen. Resistance may take the form of biodegredation of pharmaceuticals, such as sulfamethazine degrading soil bacteria introduced to sulfamethazine through medicated pig feces (Topp et al., 2013). Along with antibiotic waste, resistant bacteria follow, thus introducing antibiotic resistant bacteria into the environment. As bacteria replicate quickly, the resistant bacteria that enter the environment replicate their resistance genes as they continue to divide. In addition, bacteria carrying resistance genes have the ability to spread those genes to other species via horizontal gene transfer. Therefore, even if the specific antibiotic is no longer introduced into the environment, antibiotic resistance genes will persist through the bacteria that have since replicated without continuous exposure.

Figure 2. Bacterial culture in the media for testing antibiotic resistance.

The antimicrobial resistance may be evidenced by dilution or disk diffusion methods. Figure 2 shows a test in which the strain on the right is resistant to most antibiotics tested.

Mechanisms of antibiotic resistance

Antibiotic resistance can be a result of mutation or horizontal gene transfer. The four main mechanisms by which microorganisms exhibit resistance to antimicrobials are:

1. Drug inactivation or modification: for example, enzymatic deactivation of penicillin G in some penicillin resistant bacteria through the production of β-lactamases.
2. Alteration of target site: for example, alteration of PBP (the binding target site of penicillins), or production of an additional PBP2a produced by strains of *S. aureus* methicillin resistant (MRSA).
3. Alteration of metabolic pathway: for example, some sulfonamide resistant bacteria do not require para-aminobenzoic acid (PABA), an important precursor for the synthesis of folic acid and nucleic acids in bacteria inhibited by sulfonamides, instead, like mammalian cells, they turn to using preformed folic acid.
4. Reduced drug accumulation: by decreasing drug permeability (for example quinolones or increasing active efflux (pumping out) of the drugs across the cell surface (for example tetracycline).

Antibiotic resistant bacteria

Antibiotics are of concern due to potential genotoxic

effects, disruption of aquatic ecology, promotion of antibiotic resistance, complications surrounding development of water reuse technologies, and possibly even increased human health risks (Daughton and Ternes, 1999). The widespread and often inappropriate administration of antibiotics in livestock, pets and humans has been shown to result in the development of antibiotic-resistant bacteria and is generally accepted to be a primary pathway for their proliferation in the environment. Although the concentration of antibiotics residue in the environment is very low, usually at the ng L^{-1} to µg L^{-1} level in water and the µg kg^{-1} to mg kg^{-1} level in soil, they still draw great attention all over the world because antibiotics and their transformation products may result in the development, maintenance, transfer or spread of antibiotic resistant bacteria and antibiotic resistance genes in the long term and have serious impacts on the ecosystem (Zhang et al., 2009).

Intense use of antibiotics in agricultural production may lead to the contamination of surface and groundwater by antibiotic-resistant bacteria. The spread of antibiotic-resistant bacteria in the environment may compromise the ability to treat microbial infections and has become an increasing challenge to public health. Today antibiotic resistance is a global health problem that involves all major pathogens and antimicrobial drugs (Capita and Alonso-Calleja, 2013) and is now classified as a global pandemic (EASAC, 2007). Discharge of antibiotics to the environment from the wastewater has been linked to the development of various resistant bacterial strains (Kummerer, 2004). Water discharged into water bodies from municipal sewage treatment plants may contain significant concentrations of the genes that make bacteria antibiotic-resistant. Timothy et al. (2011) explained that antibiotic resistant bacteria are abundant in the sewage that enters municipal wastewater treatment plants. Treatment is intended to kill the bacteria, and it removes many of the bacterial genes that cause antibiotic resistance. However, genes or bacteria may be released in the effluent from the plant.

The intensive use of antibiotics within primary production is the main reason for the spread of antimicrobial resistance throughout the food chain (Capita and Alonso-Calleja, 2013); with tetracycline being one of the most used antimicrobial agents in agricultural production (Kools et al., 2008). The use of antibiotics in agricultural production has resulted in a high level of antibiotic-resistant bacteria in animal waste (Parveen et al., 2006; Sapkota et al., 2007). Tetracycline resistance has been seen in 80% of *Escherichia coli* isolated from 90 Canadian farms (Varga et al., 2008) and in the range of 20–50% of *E. coli* isolated from farms in Florida (Parveen et al., 2006). As a consequence, the large-scale application of animal waste onto agricultural land releases large quantities of antibiotics, resistance genes, resistant bacteria and pathogenic bacteria into the soil environment (Kjaer et al., 2007; Chee-Sanford et al., 2009), counteracting the positive effects of spreading animal waste on agricultural land. The use of antibiotics in agricultural production has also led to the detection of resistant bacteria in surface water in the US and Mexico where it has been associated with agricultural activities (Lugo-Melchor et al., 2010; Sapkota et al., 2007). In a study, a total of 767 *E. coli* isolates were tested regarding their resistance to 24 different antibiotics. The highest resistance rates were found in *E. coli* strains of a sewage treatment plant which treats not only municipal sewage but also sewage from a hospital. Among the antimicrobial agents tested, the highest resistance rates in the penicillin group were found for ampicillin (up to 18%) and piperacillin (up to 12%); in the cephalosporin group for cefalothin (up to 35%) and cefuroxime-Axetil (up to 11%); in the group of quinolones for nalidixic acid (up to 15%); and for trimethoprime/sulfamethoxazole (up to 13%) and for tetracycline (57%) (Reinthaler et al., 2013). Similarly, developments of antibiotic-resistance bacteria (Middleton and Salierno, 2012; Shah et al., 2012) and risk to aquatic organisms (Zhang et al., 2012) have been correlated with pharmaceutical residues presence in wastewater from inadequate wastewater treatment systems. Samples from surface water sites near wastewater treatment plants in Australia had a significant increase of antibiotic resistant *E. coli* (Watkinson et al., 2007).

Kummerer (2004) reported the development of various resistant bacterial strains associated with discharges of antibiotics. Kim et al. (2008) and Oetken et al. (2004) reported death and decline in reproduction of standard test organisms like *V. fischeri, D. magna, M. macrocopa, O. latipes* and some invertebrates. Alighardashi et al. (2009) reported acute sensitivity of sludge bacteria to erythromycin causing floc disintegration and breakage of filaments. Genotoxic substances are often mutagenic and carcinogenic and are, therefore, potentially problematic in the development of antibiotic resistant organisms. The fluoroquinolone antibiotics were shown to display high genotoxicity at concentrations (3000 to 87,000 ng L^{-1}) detected in hospital effluent (Hartmann et al., 1998). Fluoroquinolone antibiotics have been shown to exert genotoxic effects on the genetically modified bacterial strain, *Salmonella Typhimurium*, at concentrations as low as 5000 ng/L for norfloxacin and 25,000 ng L^{-1} for ciprofloxacin (Hartmann et al., 1998). Fluoroquinolones are more toxic to prokaryotic than eukaryotic organisms and do not appear to pose an acute human genotoxic risk at concentrations found in hospital effluent. However, there may be concerns about their impact on aquatic microbial populations. The sewers downstream from a hospital increased prevalence of bacteria resistant to oxytetracycline, whereas sewers downstream from a pharmaceutical plant showed an increased pervasive-ness of bacteria resistant to multiple drugs, including sulfamethoxazole. These results lend credence to the concern that antibiotic-resistant bacteria might develop from long-term environmental exposure to relatively low

concentrations of antibiotics (Guardabassi et al., 1998).

Some examples of the resistant pathogens

1. The most serious concern with antibiotic resistance is that some bacteria have become resistant to almost all of the easily available antibiotics. These bacteria are able to cause serious disease and this is a major public health problem. Important examples are MRSA, vancomycin-resistant Enterococcus (VRE), multidrug resistant *Mycobacterium tuberculosis* (MDR-TB), *Acinetobacter baumannii*, KPC strains, *Pseudomonas aeruginosa* etc.
2. Antibiotics such as penicillin and erythromycin, which used to have a high efficacy against many bacterial species and strains, have become less effective, due to the increased resistance of many bacterial strains (Pearson, 2007).
3. *S. aureus* is one of the major resistant pathogens. It was one of the earlier bacteria in which penicillin resistance was found in 1947, just four years after the drug was mass produced. *S. aureus* infections in the US are resistant to penicillin, methicillin, tetracycline and erythromycin. The MRSA is the most frequently identified antimicrobial drug resistant pathogen in US hospitals. The United States in 2007 had reported seven cases of vancomycin-resistant *Staphylococcus aureus* (VRSA) infection, a serious development that has healthcare providers fearful of losing ground in their attempt to control the spread of *S. aureus*.
4. *Streptococcus pyogenes* infections can usually be treated with many different antibiotics. Strains of *S. pyogenes* resistant to macrolide antibiotics have emerged; however, all strains remain uniformly susceptible to penicillin (Albrich et al., 2004). Resistance of *Streptococcus pneumoniae* to penicillin and other *β*-lactams is increasing worldwide. *S. pneumoniae* is responsible for pneumonia, bacteremia, otitis media, meningitis, sinusitis, peritonitis and arthritis (Albrich et al., 2004).
5. *Clostridium difficile* is a nosocomial pathogen that causes diarrheal disease in hospitals worldwide (Gerding et al., 1995; McDonald, 2005). Clindamycin resistant *C. difficile* was reported as the causative agent of large outbreaks of diarrheal disease in hospitals in New York, Arizona, Florida and Massachusetts (Johnson et al. 1995). *C. difficile* strains resistant to fluoroquinolone antibiotics, such as ciprofloxacin and levofloxacin, were also reported in North America in 2005 (Loo et al., 2005).
6. Tuberculosis (TB) is increasing across the globe, especially in developing countries, over the past few years. Its resistant to antibiotics is called MDR TB (Multidrug Resistant TB - *M. tuberculosis* strain that is resistant to at least isoniazid and rifampin). Globally, MDR TB causes 150,000 deaths annually (Edward et al., 2013). It was considered one of the most prevalent diseases, and did not have a cure until the discovery of streptomycin by Selman Waksman in 1943 (Herzog, 1998). However, the bacteria soon developed resistance. Since then, drugs such as isoniazid and rifampin have been used. *M. tuberculosis* develops resistance to drugs by spontaneous mutations in its genomes. Resistance of *M. tuberculosis* to isoniazid, rifampin and other common treatments has become an increasingly relevant clinical challenge.

For a long time, it has been thought that, for a microorganism to become resistant to an antibiotic, it must be in a large population. However, recent findings show that there is no necessity of large populations of bacteria for the appearance of antibiotic resistance. The leaching of bacteria from agricultural soils is a problem in well-structured soils where preferential flow through soil macropores results in rapid downward transport.

IMPACTS ON SOIL MICROORGANISMS AND SOIL ENZYME ACTIVITY

The detection of antibiotic residues (even at subnanogram per liter) is alarming for ecosystem sustainability. These compounds are specially engineered to show their effect at trace levels. There are no reports on direct effect on human beings from contaminated water but the effects on other organisms have been documented. Long-term accumulation of persistent antibiotics and their metabolites in agro-ecosystems are bioactive and potentially hazardous to soil microorganisms and crops, particularly bacteria (Baguer et al., 2000). Degradation products of antibiotics exhibited toxicity to microorganisms (Ge et al., 2010). Residual antibiotics in the soils help in developing antibiotic resistant microbial populations (Witte, 1998; Morris and Masterton, 2002), microbial activity (Jjemba, 2002a, b), and alter soil microbial constitution and functions. There is limited information available on the direct effects of these drugs on soil biota, particularly those living in rhizosphere.

Antibiotics act as an ecological factor in the environment that could potentially influence microbial communities. Ding and He (2010) summarized the effect of antibiotics in the environment on microbial populations. The effects include phylogenetic structure alteration, resistance expansion, and ecological function disturbance in the micro-ecosystem and changes of microbial community structure upon addition of antibiotics in soil and water environment. Effects of antibiotics on ecological functions also include nitrogen transformation, methanogenesis, and sulfate reduction. Diao et al. (2004) reported that apramycin inhibited soil bacteria growth significantly. Tetracyclines, a group of natural and semisynthetic antibiotics inhibited bacterial protein synthesis. Hernando et al. (2006) showed adverse effects of pharmaceutical residues on bacteria, invertebrates and

algal populations in soils. The impacts of antibiotics on soil organisms will certainly modify enzyme activities and soil biochemical processes. The presence of tetracycline disturbed the structure of microbial communities and inhibited soil microbial activities in terms of urease, acid phosphatase, and dehydrogenase (Wei et al., 2009). The antibiotics, including CTC, TC, TYL, SMX, SMZ and trimethoprim, inhibited soil phosphatase activity whereas sulfonamides (SMX and SMZ) and trimethoprim inhibited the soil respiration (Liu et al., 2009). The OTC decreased the urease, sucrase, phosphatase, hydrogen peroxidase and microbial biomass nitrogen in rhizosphere of wheat, and increased the microbial biomass carbon (Yao et al., 2010). Besides deleterious effects on soil microflora and crop growth, antibiotics can provoke resistant pathogens through long-time exposure to the soil environment due to genetic variation and transfer. As novel persistent pollutants, antibiotics were included in limitations issued by the Food and Agriculture Organization (FAO) and the USA. High concentrations of antibiotics in the sludge would have negative impacts on the environment if the sludge were applied on agricultural land, including effects on soil microbial diversity, respiration and denitrification due their antimicrobial activities (Chander et al., 2005; Kotzerke et al., 2011; Zielezny et al., 2006).

ENVIRONMENTAL RISK AND HEALTH HAZARDS

Antibiotics present in the sewage water could possibly have harmful ecotoxicological effects on aquatic organisms. Aquatic lives in the water bodies receiving treated sewage are exposed to a mixture of drug residues, not just single compounds. The presence of antibiotic residue in sewage, activated sludge, digested sludge and urban biosolids has become an increasingly recognized environmental risk (Zhang and Li, 2011). Residues of the antibiotics in the farm dust present a new source of health hazard for farmers (Hamscher et al., 2003). Hamscher et al. (2003) in Germany detected five different antibiotics in 90% of dust samples collected from the piggery of which total amounts were up to 12.5 mg kg^{-1} dust.

The results suggested that the risks may arise from the inhalation of dust contaminated with antibiotics. Crops absorb antibiotics from polluted soils accumulated due to sewage irrigation or manure amended soils through fertilization (Dotaniya et al. 2014). High level accumulation of antibiotics in food crops may raise potential human health concerns through the food chain. Uptake varies for different antibiotics and it also depends on plant and their species. Some antibiotics, for example, TYL, could not be absorbed by corn, onion and cabbage because of their high molecular weight (Kumar et al., 2005a). However, since little is known regarding environmentally safe levels for antibiotics in wastewater or surface waters (Golet et al., 2002), more research is needed to quantify the risk so that appropriate action can be taken to mitigate harmful effects or alternately, redirect efforts and limited resources.

CONCLUSION

Environmental contamination by antibiotics has become an increasingly serious problem worldwide, which poses great risks to ecosystems and human health. Most of the antibiotics detected in source water are not detected in finished water or are present at a much lower level, if detected. It indicates their partial removal during conventional treatment processes or chemical trans-formation during disinfection. The incomplete removal of some antibiotics, such as ciprofloxacin, is of concern due to their unknown health effects if they do persist in finished waters even at ng L^{-1} levels. Currently, there is lack of understanding on ecotoxic impacts of antibiotics on soil ecology, particularly growth, community structure, function and diversity. In addition, some beneficial rhizosphere microorganisms, like *Arbuscular mycorrhizal*, fungus and *Rhizobium* have not been investigated till now. So, future research will be focused on how these antibiotics persist and transform during water treatment processes. More research is needed to evaluate the potential impacts of these antibiotics with high mass loads on the receiving environment following the discharge of effluents and disposal of sludge.

Conflict of interest

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

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academicJournals

Vol. 9(14), pp. 979-987, 8 April, 2015
DOI: 10.5897/AJMR2014.7046
Article Number: 279E54652251
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Bacterial degradation and decolorization of textile dyes by newly isolated *Lysobacter* sp.

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Received 2 August, 2014; Accepted 20 March, 2015

A bacterial strain, P28, with significant capability to decolorize the textile dye, Congo Red and Yellow HEGR was isolated from the effluent collected from a textile industry. Phenotypic characterization and phylogenetic analysis of the 16S rRNA sequence indicated that the bacterial strain belonged to the genus Lysobacter. Bacterial isolate P28 showed a remarkable ability to decolorize azo dyes, including Congo Red and Yellow HEGR. *Lysobacter* sp. P28 grew well in a concentration of dye 50 mg L^{-1} (Congo Red and Yellow HEGR) resulting in approximately 80.4 and 80.1% decolorization extent in 72 h, and could tolerate up to 200 mg L^{-1} of dye with 60.4 and 50.8% decolorization extent in 84 h. High decolorization extent and facile conditions show the potential for this bacterial strain to be exploited in the biological treatment of dyeing mill effluents.

Key words: Azo dyes, bacterial degradation, *Lysobacter*, decolorization.

INTRODUCTION

Dyes and dyestuffs are extensively used within the food, pharmaceutical, cosmetic, textile and leather processing industries. From the existing literature, it can be estimated that approximately 75% of the dyes discharged by textile processing industries belong to the classes of reactive (~36%), acid (~25%) and direct (~15%) dyes. Throughout the dyeing progression, about 10-15% of the dyes used are released into the wastewater. Textile dyes in the aqueous ecosystem are the cause of serious environmental and health concerns (Chen et al., 2015; Khan and Malik, 2014; Saratale et al., 2013; Sarayu and Sandhya, 2012; Fang et al., 2004; Asad et al., 2007; Clarke and Anliker, 1980; Pinheiro et al., 2004). Among these dyes, azo dyes are the most widely used; these account for over 60% of the total number of dye structures known to be produced (Zollinger, 1991). Azo dyes can be distributed in monoazo, diazo and triazo classes, and are available in six application categories: acid, basic, direct, disperse, azoic, and pigments. Pollution problems due to textile industry effluents have increased in recent years. Because color in wastewater is highly visible and affects esthetics, water transparency and gas solubility in water bodies, and particularly because many dyes are made from identified carcinogens, such as benzidine and other aromatic compounds, dye wastewaters have to be treated (Banat et al., 1996). Moreover, it is very difficult to treat textile industry effluents because of their high biological oxygen demand (BOD), chemical oxygen demand (COD), heat, color, pH and the presence of metal ions. Numerous methods

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are used to treat textile effluents to attain decolorization. A number of physical and chemical methods have been recommended for the treatment of dye-contaminated wastewater, but such methods are not widely used due to the high cost of input and secondary pollution that can be generated by the excessive use of chemicals (Jadhav et al., 2007). Unconventionally, biodegradation systems of color removal through the use of bacteria have been shown to be highly effective (Pearce et al., 2003). Micro-organisms are nature's original recyclers, converting toxic organic compounds to harmless products, often carbon dioxide and water. Ever since it was discovered that microbes have the ability to transform and/or degrade xenobiotics, scientists have been exploring the microbial diversity, particularly of contaminated areas in search for organisms that can degrade a wide range of pollutants (Jain et al., 2005). Although numerous microorganisms can decolorize such dyes, only a few are able to mineralize these compounds into CO_2 and H_2O (Junghanns et al., 2008). These include bacteria, fungi and algae, capable of decolorizing a wide range of dyes with high efficiency (Chen et al., 2015; Khan and Malik, 2014; Saratale et al., 2013; Sarayu and Sandhya, 2012; Fu and Viraraghavan, 2001).

In particular, systems based on biological processes using a large variety of bacterial strains, allow for degradation and mineralization with a low environmental impact and without the use of potentially toxic chemical substances, under mild pH and temperature conditions (Pandey et al., 2007). Microbial decolorization and degradation has appeared as an environmentally pleasant and cost-competitive alternative to chemical decomposition processes (Ozdemir et al., 2008). Bacterial strains that are able to decolorize azo dyes under aerobic (*Xenphylusazovorans* KF46F, *Bacillus* strain, *Kerstersia* sp. strain VKY1 and *Staphylococcus* sp.) and anaerobic conditions (*Sphingomonasxenophaga* BN6, *Eubacterium* sp., *Clostridium* sp., *Butyrvibrio* sp. or *Bacteroides* sp.) have been extensively reported (Olukanni et al., 2006; Dos Santos et al., 2007; Lin and Leu, 2008). Thus, the main objective of this study was to observe the degradation of azo dyes in a successive process using exclusively a novel bacterium isolated from textile dye effluents, process optimization and molecular characterization of the isolate.

In the current study, a bacterial strain, P28, with remarkable ability to decolorize the Congo Red and Yellow HEGR, was isolated from the textile dye contamination sites collected from a textile mill. Our results also showed that *Lysobacter* sp. P28 exhibited decolorizing activity through biodegradation.

MATERIALS AND METHODS

Dyestuff, chemicals and microbiological media

In order to obtain a high-performance bacterial decolorization, Congo red and yellow HEGR, commonly used azo dyes were chosen for the screening of dye degradative bacteria. All the chemicals were of analytical grade. Textile dyes and textile effluent were obtained from local textile industries (Shivalik Polymer Ltd.) Faridabad (Haryana), India. Reagents and other fine chemicals were obtained from Hi-media Laboratory, India.

Revival of reference culture

The reference culture (*Pseudomonas putida* MTCC 2445) was obtained from Institute of Microbial Technology (IMTECH), Chandigarh. The seal packaged ampoules containing reference culture was broken under aseptic conditions and the culture was revived in nutrient broth.

Isolation and screening of the microorganism

The soil and effluent samples were collected from various sites of textile industry. Samples were collected in pre-sterilized plastic and polyethylene bags and stored at 4°C. From serially diluted samples, 1 ml of diluted sample was spread on nutrient agar plates incorporated with azo dyes and then incubated at 37°C for 4-5 days. For primary screening of isolates, test tube containing 25 ml of mineral salt broth with azo dyes at 10 mgL^{-1} concentrations were prepared. Autoclaved broth was inoculated with 1% inoculum and then incubated at 37°C for five days. Reduction in color was observed after every 12 h.

During secondary screening, selected isolates from primary screening were further checked against various concentrations of textile dyes (10-200 mgL^{-1}). Decolorization of dye was measured as the decrease in optical density of the supernatant obtained upon centrifugation (10,000 rpm for 20 min) at their respective peak maxima (Y. HEGR-400 nm, C.R.-530 nm). Isolate with the maximum decolorizing extent, designated as P28, was preserved at -20°C in LB medium with 10% glycerol.

Measurement of decolorization extent

Samples (500 µL) were collected after every 12 h and centrifuged at 5000 g for 5 min. Decolorization extent was determined by measuring the absorbance of the culture supernatant at 560 nm using a double beam spectrophotometer (Systronics). Decolorization extent was calculated using the following equation:

$$\text{Decolorization extent (\%)} = (OD1 - Odt)/OD1 \times 100$$

Where, OD1 refers to the initial absorbance, ODt refers to the absorbance after incubation; and t refers to the incubation time.

Optimization of cultural conditions for textile dye decolorization

The bacterial isolate showing efficient decolorization activity in secondary screening was selected for the optimization of cultural conditions. Azo dye decolorization process was optimized by the conventional strategy; varying one factor at a time. Effect of temperature on decolorization of textile dyes was observed. The inoculated media containing flasks were incubated at different temperatures (25 to 45°C) for 4 days. The effect of pH on decolorization was observed by incubating flasks containing dye media and appropriate inoculums at different pH values at optimum temperature for 4 days. The effect of aeration on decolorization of textile dyes was observed by incubating media flasks containing dye in both static and shaking conditions at optimum temperature and optimum pH. The effect of non-living culture (Adsorption effect)

on decolorization of textile dyes was observed by inoculating the media flasks containing dye with both living and autoclaved cells (dead cells).

Effect of textile effluent concentrations on decolorization

Various concentrations of textile effluent (25, 50, 75 and 100%, v/v) were used. Each flask (250 ml) containing 100 ml textile effluent (pH-7.0) of respective concentration was autoclaved and inoculated with 1% inoculums and incubated at temperature 37°C for five days. Decolorization activity was observed.

Effect of inoculum size on decolorization activity and dry weight of cells

The effect of inoculum size on textile effluent (100%) decolorization was observed by inoculating the isolates at various concentrations in the textile effluent. Different concentrations (1.0, 2.0, 3.0, 4.0 5.0, 6.0, 7.0, 8.0, 9.0, 10.0%, v/v) of 24 h old cultures were inoculated in textile effluent and then incubated for 3 days. After interval of 12 h, 4 ml of sample was used for decolorization extent measurement. Simultaneously dry weight of each sample was also recorded. After centrifugation, pellets were suspended in saline and dried under 45°C temperature.

Dry weight of cells = weight of dried pellets with china dish - weight of empty china dish

Effect of different concentration of textile effluent on seed germination and toxicity test

Toxicity of raw textile effluent and treated textile effluent was observed by seed germination experiments (Rehman et al., 2009). Mung bean (*Vigna radiata*) seeds were surface sterilized with 0.1% $HgCl_2$ for 2 min and then washed repeatedly with sterilized distilled water. Initially, the effect of different concentrations of untreated (25, 50, 75 and 100%, v/v) textile effluent was observed on seeds of mung beans. The surface sterilized seeds were spread on sterilized Petri dishes lined with sterilized filter paper. Then, the seeds were treated with equal volume (10 ml) of raw and treated textile effluent. The Petri dishes were kept at room temperature (25 ± 2°C) and the seed germination percentage was recorded after every 24 h. The emergence of radical of 2 mm length was taken as criterion for germination. After 7 days, 3 ml of textile effluent was added to the Petri plate and for the seedling growth, 7 days old seedlings were measured. After 15 days, seedlings were taken out from each Petri dish and their respective root and shoot lengths were measured. Seedling components were separated into root and shoot and measured separately for their fresh weight. These seedling parts were dried in an oven for 12 h at 45°C and their dry weight was taken. The plant growth measurements included shoot length, root length, wet shoot weight, wet root weight, dry shoot weight, dry root weight and vigor index as noted in 15^{th} day old seedlings with various seed germination parameters.

Comparison of live and autoclaved cell for decolorization

Two fresh culture broth of each strain was prepared, half of them were autoclaved. Both the autoclaved (inactive) and living cells were centrifuged at 5000 g for 4 min. To determine if extracellular byproducts or bacterial cells are involved in decolorization, the supernatant and pellets of the living and nonliving cells were incubated with the dye, absorption was used as a measure of their decolorization activity (Khehra et al., 2005).

Morphological and biochemical identification

The isolated organism was further identified on the basis of morphological and biochemical characteristics from MTCC, Institute of Microbial Technology (IMTECH), Chandigarh.

Molecular identification

Total genomic DNA of bacteria was isolated by using method of Charles and Nester (1993) with slight modifications. Pure cultures of bacteria were raised in 10 ml of nutrient broth medium for 18 - 24 h to obtain cell O.D of 0.6 at 600 nm and 12,000 rpm. The bacterial pellet was washed in 1.5 ml of 0.85% NaCl, centrifuged for 2 min at 12,000 rpm and was resuspended in 0.4 ml Tris-EDTA buffer (T_{10} E_{25}). Cell lysis was done by adding 20 µl of 25 % SDS, 50 µl of 1% lysozyme and 50 µl of 5 M NaCl followed by incubation at 68°C for 30 min in a circulatory water bath. For protein precipitation, 260 µl of 7.5 M ammonium acetate solution was added to the micro centrifuge tubes and tubes were kept in ice for 20 min followed by centrifugation at 13,000 rpm for 15 min at 20°C. Supernatant was carefully pipette out in another fresh sterile micro centrifuge tube in which 1 µl RNase (4 mg ml^{-1}) was added followed by incubation at 37°C for 20 min. Equal volume of chloroform was added in the tubes and proper mixing was done by inverting the tube up and down several times. RNA was precipitated by centrifuging for 1 min at 12,000 rpm. The top layer containing total cell DNA was pipette out in fresh microfuge tube and used for the next step. DNA was precipitated by adding 0.8 volume of iso-propanol followed by incubation on ice for 30 min and pelleted by centrifuging at 10,000 rpm for 15 min. DNA was further washed with 0.5 ml of 70% ethanol and spun down at 10,000 rpm for 1 min. Traces of ethanol were removed by air drying the tubes in inverted position. Pure DNA sample was then suspended in 20 µl Tris-EDTA buffer ($T_{10}E_1$) or deionized water and stored at 4°C for further use.

Quantification through agarose gel electrophoresis

The genomic DNA samples of bacteria were quantified through agarose gel electrophoresis by analyzing their migration on 0.8% agarose gels prepared in 0.5 M Tris-borate-EDTA (TBE) buffer and run in an electrophoresis tank filled with the same concentration of TBE buffer. The genomic DNA was diluted with Tris-EDTA buffer so as to achieve a concentration of 50 ng in 10 µl to be used as a template DNA in PCR amplification reaction.

16S rRNA PCR-amplification of isolates

Primers

The forward and reverse primers were custom synthesized from “Ocimum link biotech” Hyderabad, India. The sequences of the oligonucleotide primers used for amplification of 16S rRNA genes are:
Forward primer 41f (5'-GCTCAAGATTGAACGCTGGCG-3'); Reverse primer 1488r (5'-GTTACCTTGTTACGACTTCACC-3') (Maatallah et al., 2002). The stock solution (100 ng ml^{-1}) of primers was prepared by reconstituting lyophilized primers in sterilized deionized (milliQ) water and stored at 20°C.

Amplification of 16S r-DNA was carried out by polymerase chain reaction using a thermal cycler (BioRed). The amplifications were carried out with 50-90 ng of pure genomic DNA. The amplification reactions were performed in a 25 µl mixture containing 0.6 U of Tag DNA Polymerase (Genei from 3U µl of 10X Tag Polymerase buffer, 0.4 µl of d NTP mix and 0.3 µl each of the two primers described above). For every PCR reaction, a negative control (no template

DNA) and a positive control (template DNA giving amplified product) were invariably maintained. The amplified product was run on a 1.2% agarose gel along with 1 kb MW marker (Genei), at a constant voltage, and visualize under uv light.

Bacterial identification by 16S rRNA sequences and phylogenetic relationship

Phylogenetic identity of bacteria was determined by BLAST result, sequences were aligned by using alignment software, that is, Clustal W. Phylogeny calculations and dendrogram was constructed by Mega 4.0 software package using neighbor joining (NJ) methods (Saitou and Nie, 1987). Bootstrap analysis (Felsenstein, 1985) was conducted using 1000 replicates samplings of data.

RESULTS AND DISCUSSION

Isolation of decolorizing bacteria

A strain of bacterium, P28, with strong decolorizing ability on Congo red and yellow HEGR was isolated. A significant decolorization extent (73.5%) was observed after 24 h incubation and a maximum value (80.4%) was achieved after incubation for 72 h at 50 mgL^{-1} w/v concentration. Further, a significant decolorization extent (60.4%) was achieved after incubation for 84 h at 200 mgL^{-1} w/v concentration. In case of Yellow HEGR, a significant decolorization extent (80.1%) was observed after 24 h incubation and a maximum value (80.6%) was achieved after incubation for 84 h at 50 mgL^{-1} w/v concentration. Further, a significant decolorization extent (50.8%) was achieved after incubation for 84 h at 200 mgL^{-1} w/v concentration. Whereas, *P. putida* MTCC 2445 (reference culture) showed decolorization extent (29.8%) after 24 h at 50 mgL^{-1} w/v and a maximum value (56.8%) was achieved after incubation for 84 h at 50 mgL^{-1} w/v concentration. Further, the extent of decolorization was decreased (30.8%) after incubation for 84 h at 200 mgL^{-1} w/v concentration (Figure 1 and Table 1).

The colony of bacterial isolate P28 showed swarming colony morphology on agar plates. The colony of bacterial isolate P28 was highly mucoid and cream colored. P28 was observed to be a non-spore forming Gram negative rod. Sequence analysis of 16S rRNA showed that strain P28 had highest similarity with the species *Lysobacter* sp. (99%) which has been proved to have decolorizing ability against azo dyes.

Decolorization of Cong Red and Yellow HEGR under shaking and non-shaking conditions

Decolorization of Congo red was more than 71% in static conditions and only 66.7% in shaking conditions (150 rpm). In the case of Yellow HEGR dye, decolorization extent was more than 62% in static conditions and only 55% in shaking condition. Whereas, reference culture also showed maximum decolorization extent (41.5, 45.6%) in static conditions and only (32.8%, 40.6%) in shaking conditions for Congo red and yellow HEGR, respectively (Figure 2). This result suggested that *Lysobacter* sp. P28 was shown to have slightly higher decolorization activity in conditions as compared to shaking conditions.

Effects of initial dye concentration, pH and temperature on decolorization

With increase of the initial dye concentration, the decolorization extent over the same time interval decreased. When the effect of different initial dye concentrations of Congo Red and Yellow HEGR on decolorization was observed using 25, 50, 100 and 200 mgL^{-1}, the required times to reach a maximum decolorization extent were 12, 24, 36, 48, 60, 72, 84, 96, 108 and 120 h, respectively. It was reported that dye decolorization can be strongly inhibited when a high concentration dyestuff was used to examine the poisonous effect of the dye on the degrading microorganisms (Khalid et al., 2008). To accurately appraise the decolorizing ability of *Lysobacter* sp. P28, decolorization was investigated by assessing the amount of dyes decolorized. The decolorization extent was only 58.3% (Congo Red) at 200 mg l^{-1} dye and 49.5% (Yellow HEGR) after 120 h incubation. This result indicated that *Lysobacter* sp. P28 showed high decolorizing performance even in high initial dyestuff concentrations. The best decolorization was achieved at pH 7.0-8.0, with ~81% decolorization in 72 h and 84 h (Figure 3). This could be due to the optimum pH for the growth of *Lysobacter* sp. P28. pH has a major effect on the efficiency of dye decolorization, and the optimal pH for color removal is often between 7.0 and 9.0 (Guo et al., 2007). The pH tolerance of decolorizing bacteria is quite important because reactive azo dyes bind to cotton fibers by addition or substitution mechanisms under alkaline conditions and at high temperatures (Aksu, 2003). It should be mentioned that the pH of the wastewater samples used for isolation of *Lysobacter* sp. P28 was about 8-9.

The fact that *Lysobacter* sp. P28 could decolorize reactive dyes in a relatively different range of pH, make it suitable for practical bio-treatment of dyeing mill effluents. *Lysobacter* sp. P28 showed strong decolorizing activity from 37°C. Although a lag phase was observed and the decolorization rate was comparatively low at 25°C, the decolorization extent increased to a similar level from 25 to 37°C (Figure 4). Decolorizing activity was significantly suppressed at 45°C, which might be due to the loss of cell viability or deactivation of the enzymes responsible for decolorization at 45°C.

Effect of living and autoclaved cells and size of inoculum of isolates on decolorization of Congo Red and Yellow HEGR dye

It was observed that autoclaved cells exhibited no

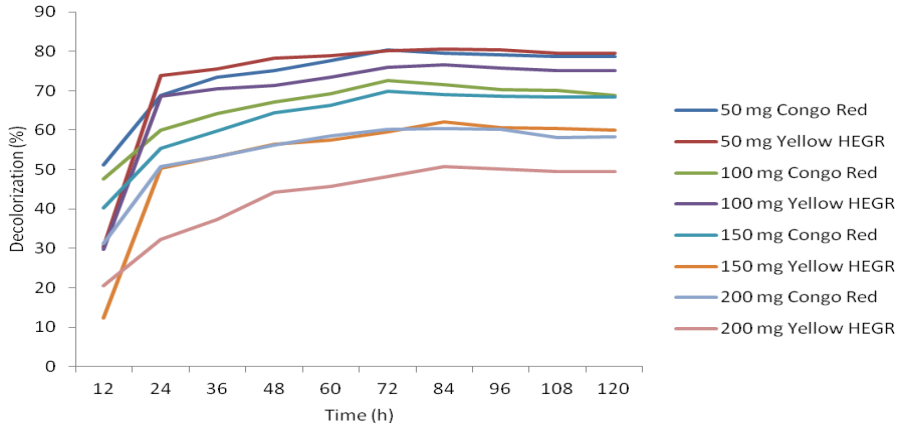

Figure 1. Growth of *Lysobacter* sp. P28 cultures and dye decolorization.

Table 1. Decolorization of textile dyes at different time intervals.

Time	Decolorization (%)			
	P28	P28	*P. putida* MTCC 2445	*P. putida* MTCC 2445
	Congo Red	Yellow HEGR	Congo Red	Yellow HEGR
12 h	47.6	29.7	10.6	22.5
24 h	60.1	68.7	19.8	45.6
36 h	64.2	70.5	28.5	56.4
48 h	67.1	71.4	32.4	62.1
60 h	69.3	73.4	38.2	64.8
72 h	72.6	76.0	44.3	65.3
84 h	71.5	76.6	50.5	65.7
96 h	70.2	75.7	50.6	64.8
108 h	70.1	75.2	50.9	64.5
120 h	68.9	75.2	50.3	64.5

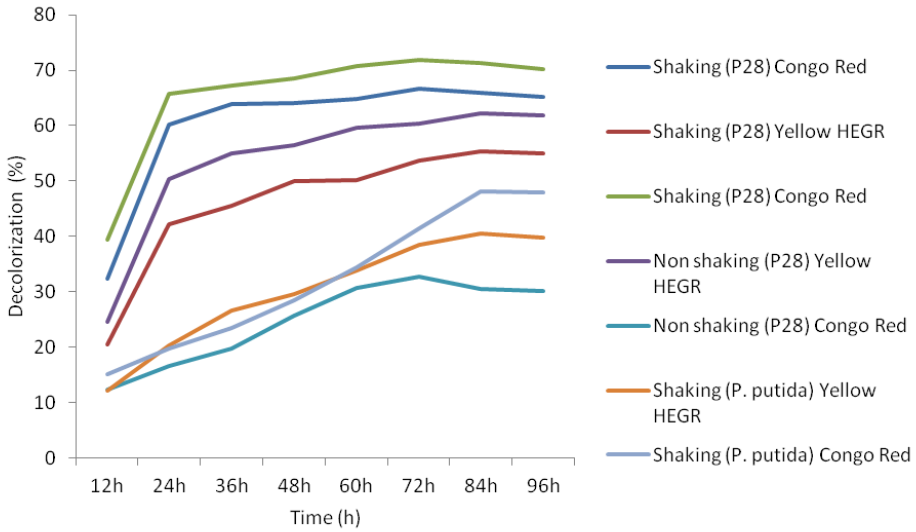

Figure 2. Growth of Lysobacter sp. P28 culture and dye decolorization in shaking and static conditions.

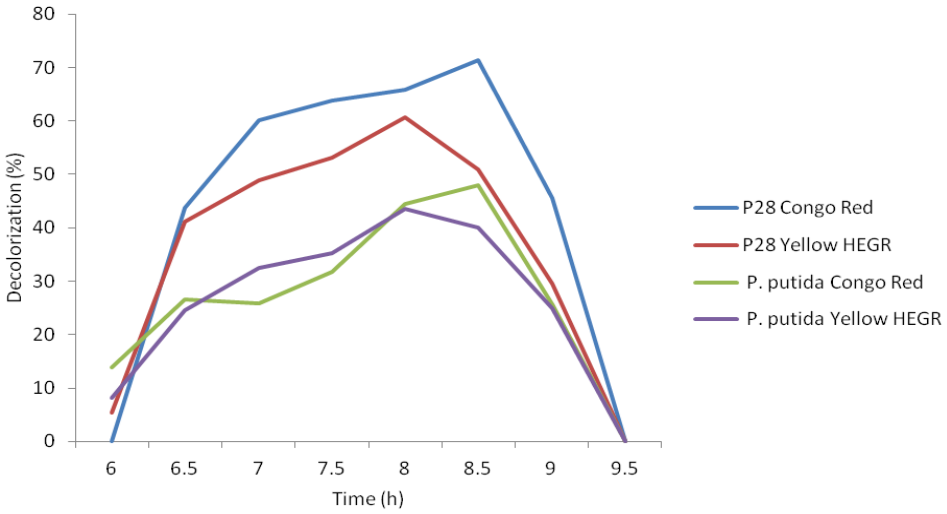

Figure 3. Effect of pH on decolorization of Congo red and yellow HEGR.

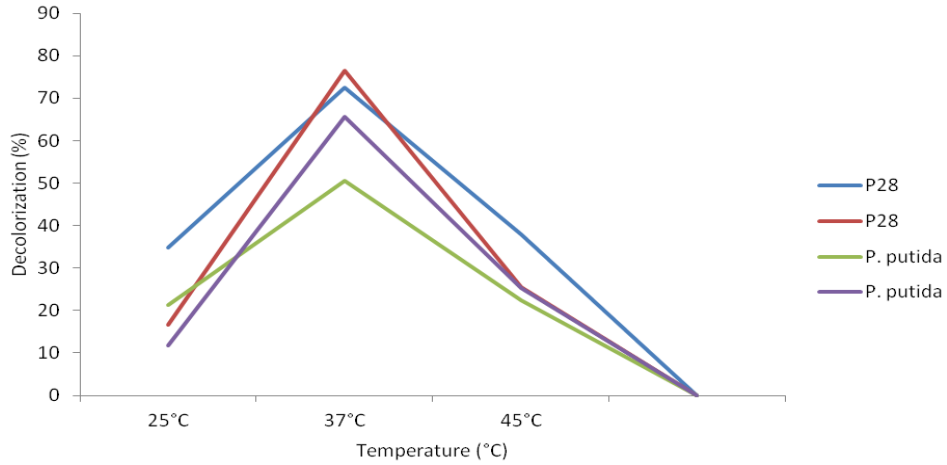

Figure 4. Effect of temperature on decolorization of Congo red and yellow HEGR.

decolorization activity, while decolorization of Congo Red dye by living cells appeared within 12 h of incubation. As seen in Figure 5, living cells of isolate *Lysobacter* sp. P28 showed 39.4 and 65.3% decolorization activity after 12 and 24 h of incubation (Figure 5). Maximum activity of 71.5% was observed in 72 h of incubation. Activity decreased to 70.6 and 70.2% in 84 and 96 h of incubation. In comparison with this, no activity was shown by autoclaved cells of *Lysobacter* sp. P28. Living cells of isolate *Lysobacter* sp. P28 showed 50.1% activity within 24 h of incubation and increased to 54.8, 56.3, 59.5 and 60.5% in 36, 48, 60 and 72 h, respectively. It showed maximum activity of 62.4% in 84 h of incubation. *P. putida* (MTCC 2445) showed maximum activity of 48.8% in 84 h of incubation with inoculums of living culture only. No activity was shown by autoclaved cells of *P. putida* (MTCC 2445). Maximal decolourisation was observed with the 9% inoculum size (Figure 7).

Effect of varying textile effluent concentrations

Effect of various concentrations of textile effluent (25, 50, 75 and 100%, v/v) on decolorization was studied. As shown in Figure 6, the decolorization percentage of selected isolates at 25% (v/v) concentration of textile effluent increased with increase in incubation period for each isolate. All the isolates showed decolorization and it increased variably till 96 h of incubation and after that slight decrease in decolorization was observed. It was found that in 24 h, isolate P28 showed 10.8% activity in 12 h, it increased to 28.2% in 48 h and maximum

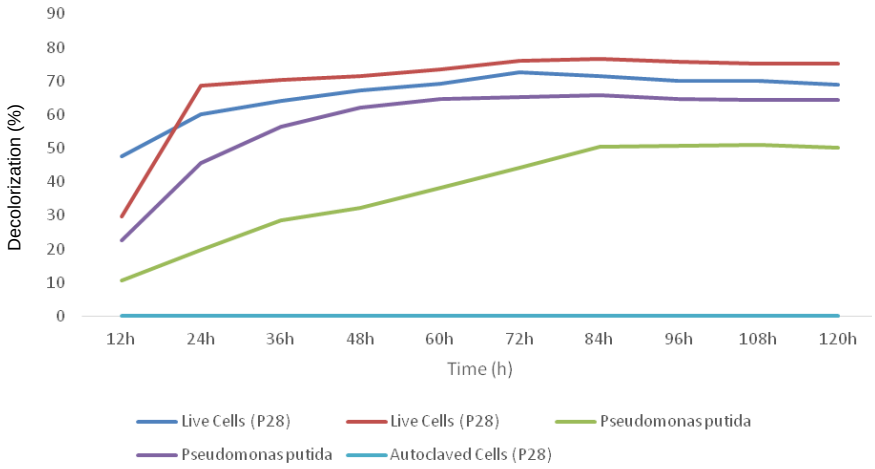

Figure 5. Decolorization of Congo Red and Yellow HEGR by live cells and dead cells (Autoclaved-killed) of Lysobacter sp. P28.

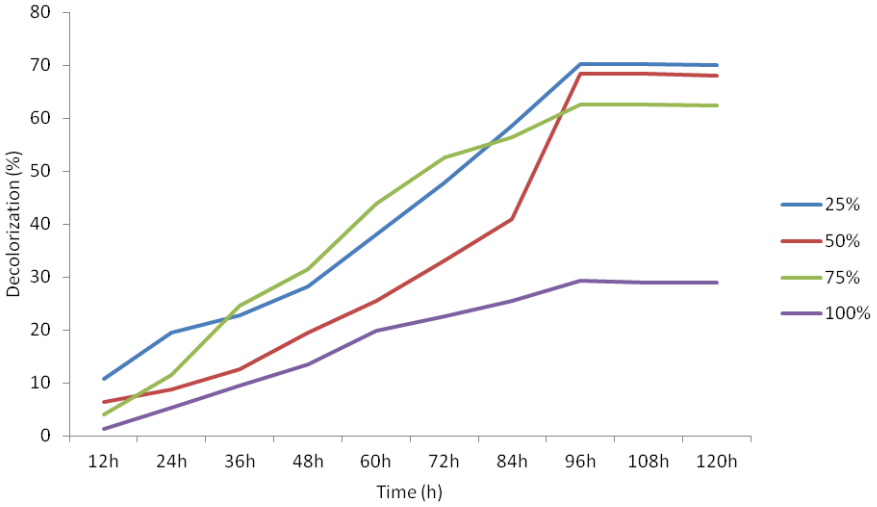

Figure 6. Effect of varying textile effluent concentrations on decolorization.

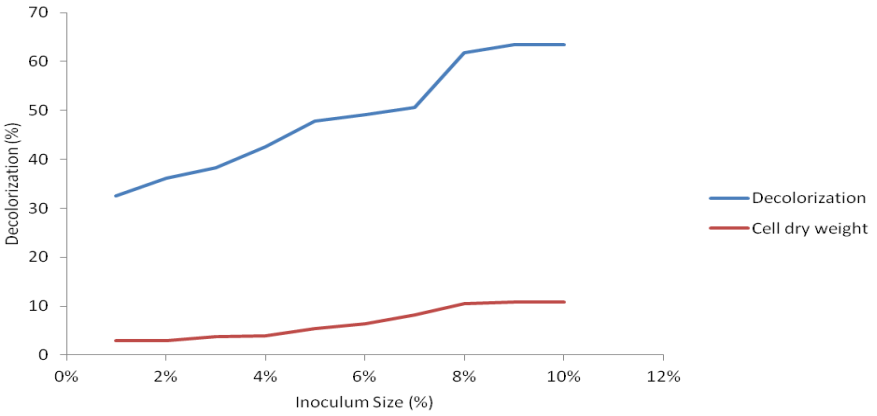

Figure 7. Effect of inoculum size on decolorization extent and dry weight of cells.

Table 2. Different parameters studied for germination treated with raw and bioremediated effluent.

Parameter	Raw effluent	Treated effluent	
		P28	*P. putida*
Germination (%)	30.3±0.67	83.1±0.77	70.9±0.61
Germination speed	3.03±0.51	7.30±0.48	5.16±0.44
Emergency index	2.0±0.40	8.3±0.32	7.10±0.51
Peak value	3.03±0.35	7.30±0.41	5.16±0.29
Vigor index	0.582±0.42	308.8±0.44	274.5±0.46
Shoot length (cm/seed)	0.067±0.32	3.710±0.55	3.110±0.44
Root length (cm/seed)	0.160±0.54	3.01±0.51	3.13±0.45
Wet root weight (mg/seed)	2.5±0.38	47.10±0.51	40.50±0.50
Wet shoot weight (mg/seed)	18.0±0.47	85.42±0.73	76.43±0.56
Dry root weight (mg/seed)	0.503±0.31	2.51±0.40	1.94±0.33
Dry shoot weight (mg/seed)	5.01±0.44	10.0±0.38	7.84±0.46

decolorization of 70.2% was observed in 96 h of incubation. Decolorization remained unchanged on 108 (70.2%) and 120 h (70.1%).

Effect of effluent/decolorized effluent on seed germination

Effect of raw effluent and treated effluent was studied and it was found that the seed germination rate was high (83.1 and 70.9) in the case of treated effluent as compared to the raw effluent. This is possibly due to the reduction in dyes concentration as degraded dye test organism (Table 2). Furthermore, vigor index, shoot length and root length were also observed better in the case of treated effluent.

Identification

Based on the phenotypic characteristics and phylogenetic analysis, strain P28 was identified as *Lysobacter*. Physiological characteristics were determined according to the procedures outlined in Bergey's Manual of Determinative Bacteriology.

Gene bank accession number

Phylogenetic position was carried out by searching the National Center for Biotechnology Information NCBI (BLAST). The 16S rRNA sequence of *Lysobacter* sp. P28 is availed under the GenBank accession number HQ316115.

Conclusions

In this study, a decolorizing bacterial strain, *Lysobacter* sp. P28, was isolated from textile industry effluent contaminated sites. *Lysobacter* sp. P28 showed decolorizing activity through a degradation mechanism rather than adsorption, and it could tolerate high concentrations (up to 200 mgL^{-1}) of Congo Red and Yellow HEGR. With high degradative and decolorizing activity against various azo dyes commonly used in the textile industries, it is proposed that *Lysobacter* sp. P28 has a practical application potential in the biotransformation of various dye effluents.

Conflict of interests

The authors did not declare any conflict of interest.

ACKNOWLEDGEMENTS

The University Grant Commission is highly acknowledged for providing financial support in the form of "Rajiv Gandhi National Fellowship" to Poonam Ranga.

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academicJournals

Vol. 9(14), pp. 988-1000, 8 April, 2015
DOI: 10.5897/AJMR2015.7416
Article Number: C4127A452286
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Resilience and relative virulence of strains of entomopathogenic fungi under interactions of abiotic stress

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Received 10 February, 2015; Accepted 25 March, 2015

The objective of this study was to examine the effect of interacting conditions of water stress (0.995-0.96 water activity; a_w), elevated temperature (25-37°C) and CO_2 (350, 1000 ppm) on growth and sporulation of strains of three entomogenous fungi, *Beauveria bassiana, Metarhizium anisopliae* and *Isaria farinosa*. Subsequently, using bioassay systems with locust (*Schistocerca gregaria*), we examined the effect of elevated CO_2 (control, 350; 650; 1000 ppm CO_2) on efficacy of strains of all three species and used crickets (*Acheta domesticus*) to examine interacting conditions of elevated temperature and CO_2 at two relative humidities (25-35°C; 350, 1000 ppm CO_2; 96, 98 and >99% RH) on efficacy of a strain of *B. bassiana* for the first time. The 3-way interacting factors had a significant effect on growth of the strains of all three species, especially at 35-37°C and 0.96-0.98 a_w and 1000 ppm CO_2. Under these conditions, only one strain of *B. bassiana* and *M. anisopliae* was able to grow at a reduced rate as compared to the controls. No strain of *I. farinosa* was able to grow at 35-37°C either in normal air or in elevated CO_2 at 0.995-0.96 a_w showing a high level of sensitivity to these interacting factors. Sporulation of the three strains of each species was also significantly affected by these three-way environmental interactions. There were some intra-strain differences and in most cases for the three species, water stress (0.98-0.96 a_w) at 35-37°C and 1000 ppm CO_2 resulting in either no sporulation or no growth. One strain of *M. anisopliae* (Ma 29) was particularly tolerant at 0.96 a_w at 37°C and 1000 ppm CO_2. Bioassays with the *S. gregaria* showed when CO_2 was elevated from 350 to 650 and to 1000 ppm, the relative virulence of two strains of each species was reduced over a 6-day temporal study. Further studies with *B. bassiana* in a detailed bioassay using crickets under three way abiotic interactions (25-35°C, 99-96% RH and 350 or 1000 ppm CO_2) showed that virulence was decreased with no efficacy occurring at 30-35°C and 1000 ppm CO_2 at 96% RH. This study suggests that climate change factors could have a profound impact on the efficacy of such biocontrol agents and thus have major implications for pest control using such approaches.

Key words: Water stress, temperature, elevated CO_2, growth, sporulation, entomopathogenic fungi, pest control.

INTRODUCTION

There has been significant interest in the impact that abiotic change scenarios may have on economically important crops and the associated pests and diseases. Indeed most climate change models suggest that there

will be a marked decrease in summer precipitation and increases in temperature, which will result in related drought stress episodes interspersed with periods of unusually high precipitation depending on the part of the world (European Commission, 2007; Solomon et al., 2009; Chalcraft, 2009). The environment in which crops will be grown in the next 10-20 years may change markedly with atmospheric CO_2 concentrations expected to double or triple. Due to this increase and that of other greenhouse gases, the global temperature is expected to increase by between +2 to 5°C (Dawson and Spannagle, 2008; Gray, 2009). The effects have been predicted to be detrimental or advantageous depending on the region. For example, in northern Europe, a mean temperature increase of 3 to 4.5°C, with a significant increase in precipitation of 30-40% was predicted (IPCC, 2007). Southern Europe is expected to be a hot spot for extreme temperature and drought stress which may have impacts on crop yield and pests and fungal diseases (Maistrello et al., 2006). Similar hot spots have been predicted to occur in parts of sub-Saharan Africa, South America and parts of Asia (IPCC, 2007). A recent study has predicted that, on a global scale, pests and diseases are moving to the poles at the rate of 3-4 km/year (Bebber et al., 2013). The possible implications this may have for the development of strategies to minimize pest and fungal pathogens of staple crops, especially the use of biological control agents has not been addressed.

The use of entomopathogenic fungi for pest management, especially as part of an Integrated Pest Management (IPM) strategy, combined with cultural and other methods has increased because of the reduction in available chemical control measures (Ansari et al., 2011; Beris et al., 2012; Pelizza et al., 2012; Svedese et al., 2012). It has however been previously shown that both temperature and water availability (water activity, a_w) are bottlenecks in the efficacy of entomopathogenic fungi against pests (Magan, 2007). High environmental temperatures and drought may reduce the growth rates of entomopathogens. For example, a recent study by Borisade and Magan (2014) screened the environmental tolerance of strains of *Beauveria bassiana*, *Metarhizium anisopliae* and *Isaria farinosa* strains (5-6 for each), it showed that very few strains were able to tolerate elevated temperatures (35°C) and water stress (0.96-0.94 a_w). Strains of *M. anisopliae* were the most tolerant, regardless of the region of isolation. The study shows that sporulation of the strains of these fungi was significantly affected, with implications for secondary infection under interacting environmental conditions. The study, however, took no account of the influence of elevated CO_2. Thus, information is required on the impact that interactions of a_w, temperature and elevated CO_2 may have on growth, sporulation and insect infection by strains of these fungi.

The objective of the present study was to examine the effect of interactions between a_w, temperature and elevated CO_2 on (a) growth and (b) sporulation of three strains each of *B. bassiana*, *M. anisopliae* and *I. farinosa*. Bioassays were subsequently carried out with two strains of each entomogenous fungal species in bioassays with *S. gregaria* to compare the effect of temperature and equilibrium relative humidity (ERH) changes on virulence of the spores. Further efficacy of three way interactions of elevated CO_2 (1000 ppm), temperature (25-35°C) and ERH (>99%-96%) on virulence was assessed using *A. domestica*. The cricket was chosen for this bioassay because it survived the degree of abiotic stress factors being examined in the absence of fungal inoculum and is a useful bioassay system to examine the impact of the abiotic change scenarios on efficacy of such entomopathogenic biocontrol agents.

EXPERIMENTAL PROCEDURES

Source of fungal strains

Table 1 lists the species and strains examined in this study. The *B. bassiana* and *M. anisopliae* strains were kindly provided by the International Institute of Tropical Agriculture (IITA), Republic du Benin, West Africa, Prof T. Butt (University of Swansea, U.K.) and Dr D. Chandler (Warwick University, U.K.). The *I. farinosa* isolates with previously reported potential virulence against insects were kindly provided by the USDA-ARS Collection of Entomopathogenic Fungal Cultures (Richard A. Humber, Insect Mycologist and Curator/ARSEF; USDA-ARS Biological Integrated Pest Management Research Unit, Robert W. Holley Centre for Agriculture and Health, Ithaca, NY 14853, USA).

Media preparation, inoculation and incubation

Sabouraud Dextrose Agar (Oxoid Ltd; 0.995 a_w) was modified with glycerol to 0.98, 0.96 and 0.94 a_w (Chen and Mujumdar, 2009). The accuracy of the modifications was confirmed using an Aqualab 3TE instrument (Decagon, Pullman, WA, USA) and found to be within ± 0.005 of the target a_w.

The agar media in 9 cm Petri plates were centrally inoculated with a 5 µl spore suspension containing about 1.4×10^7 spores ml^{-1} (Hallsworth and Magan, 1999). The replicates of each treatment strain and species were placed in separate polyethylene chambers (25 L capacity) together with 2 x 500 ml glycerol/water solution of the same a_w as the treatment plates. These were incubated at 25, 30, 35 and 37°C.

Elevated CO_2 exposure system

The polyethylene chambers containing the treatments and replicates were flushed with the required CO_2 concentrations of atmospheric

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Table 1. Origin of the fungal isolates used in this study.

Species	Isolate/strain	Host/Source	Country
B. bassiana	BB 315	Soil	Benin Republic
B. bassiana	BB 432.99	Unknown	United Kingdom
B. bassiana	BB 776.05	Coleoptera	United Kingdom
M. anisopliae	Ma 29	Soil	Benin Republic
M. anisopliae	Ma 27	Coleoptera	Benin Republic
M. anisopliae	V275	Unknown	United Kingdom
I. farinosa	IF 5081	Aleyrodidae	Pakistan
I. farinosa	IF 5676	Scutelleridae	Turkey
I. fumosorosea	PF 790.05	Unknown	United Kingdom

air (350 ppm), synthetic air with the CO_2 concentration specification of 1000 ppm provided by the British Oxygen Company (BOC). The chambers were flushed every two days with the required CO_2 concentrations at 2 $Lmin^{-1}$ to replace three times the volume of the incubation chamber. This was done immediately after growth measurements were made. The synthetic air moisture was controlled by inserting a bubbling device, containing a_w controlled solution of water/glycerol, just prior to the inlet valve when flushing each treatment chamber. The valves were sealed and the chambers incubated at the target treatment temperatures.

Growth and sporulation quantification

The diameter of the colonies was measured in two directions perpendicular to each other every two days for a period of up to 14 days. The diametric growth rate (mm day^{-1}) of the colonies of each strain and species under the different sets of interacting environmental conditions were computed by plotting the diameter of the colonies against time. Regression lines were made of the time points which represented the linear phase of the growth curves. These were used to determine the relative growth rates under different interacting conditions (Borisade and Magan, 2014). Experiments were carried out with three replicates per treatment.

The 14 day old cultures of all treatments were harvested by flooding the surface of the agar plate with 2 x 10 ml sterile water containing 0.05% Tween 80 and agitating with a sterile glass rod. Spore suspensions were poured into 25 ml universal bottles and centrifuged at 2500 rpm for 15 min. The supernatant was decanted and the spore concentrate made up to 1 ml. The spores were counted using a Neubauer haemocytometer (Abdel-Hadi et al., 2009). The sporulation data is presented as numbers of spores per ml per cm^2 of colony.

Bioassays with *S. gregaria* and *A. domesticus*

Nymphs of desert locust, *S. gregaria* and house crickets, *A. domesticus* (age of nymphs not known) were purchased from a commercial insectary in the UK. The locusts were fed with green leaves while *A. domesticus* was fed with rice bran in insect rearing cages at 25°C and 85% relative humidity for 24 h before being used for bioassays.

Fungal culture and inoculum preparation for bioassays

Two strains each of *B. bassiana* (BB 315 and BB 776.05), *I. farinosa* or *I. fumosorosea* (ARSEF 5081, IF 790.05) and *M. anisopliae* (Ma 275.86 DC, and V275) were cultured on Sabouraud Dextrose Agar (SDA) plates, sealed with parafilm and kept in sealable nylon bags. The plates were incubated at 25°C in the dark. Spores from 14 days old SDA plates were harvested by flooding the plate with 10 ml sterile reverse osmosis water and the spores gently dislodged with a glass rod. No surfactant was used. The spore suspension was transferred into 15 ml disposable Eppendorf centrifuge tubes and centrifuged at 3500 rpm for 20 min to concentrate the spores. The spores were thereafter made up to 1 ml, serial dilutions were made and the spore concentration was determined with a haemocytometer. A single concentration bioassay was done with 1.0 x 10^5 conidia ml^{-1} of each isolate as the inoculum.

All experiments were performed using 15 cm Petri-dishes with a 1 cm^2 cut lid and the cut area lined with muslin cloth held in place with masking tape to prevent the insects escaping. A Whatman No. 1 filter paper was placed flat in the bottom of each Petri dish to serve as an absorbent material for excess water in the inoculum preparation. Ten (10) nymphs of either *A. domestica* or *S. gregaria* were separately placed in each Petri-dish. Each set-up consisted of triplicate plates and a control. 1 ml of spore suspension containing about 1 x10^5 spores per ml was sprayed directly on the nymphs in each plate. The bio-assay was a single dose experiment. The dishes were arranged inside a plastic box measuring 30 x 30 x 25 cm^3 and a tight fitting lid. 500 ml of either deionized water or saturated salt solutions in glass beakers were placed in each box to modify the ERH (99-96% ± 1% ERH) at different temperatures (25-35°C) (Lewis, 1976; Charles and David, 1992). The mortality of the insects was observed daily for 6 days.

Effect of elevated CO_2 and relative humidity on fungal virulence using *A. domesticus*

The house cricket was used in a model study to evaluate the effect of interacting climate change factors on efficacy of fungi for management of insect pests of agricultural crops.

Two strains each of *B. bassiana* (BB 315, BB 776.05), *I. farinosa* or *I. fumosorosea* (IF 5081, PF 790) and *M. anisopliae* (Ma 275.86, V275) were used to investigate the effect of elevated CO_2 (650 and 1000 ppm carbon dioxide-air mixture) on spore virulence at 100% relative humidity and 25°C. The experimental set-up consisted of 3 replicates of 10 insects per jar covered with muslin. The inoculum (0.5 ml of 1.0 x 10^5 conidia ml^{-1}) was sprayed on the insects through a slit cut on the muslin. Thereafter, the slit was covered with a masking tape. The treatment for each isolate consisted of insects + inoculum + CO_2 (air, 650 or 1000 ppm). The outlet tap of the box was kept open after the jars were arranged in the box and the lid tightly placed while the inlet tap was connected to a CO_2-air mixture

ANOVA Table 1. Effect of abiotic interactions on sporulation of 3 strains of *Beauveria bassiana.*

Tests of between-subjects effects					
Dependent variable: Log_{10} (spore cm^{-2} + 1)					
Source of variations	**SS**	**DF**	**MS**	**F**	**Sig.**
CO_2 atmosphere	228.544	1	228.544	91750.655	sig
Temperature	320.821	3	106.940	42931.988	sig
Water activity (a_w)	143.901	2	71.950	28885.005	sig
Isolates x CO_2 atmosphere	122.546	2	61.273	24598.513	sig
Isolates x Temperature	44.692	6	7.449	2990.305	sig
Isolates x a_w	27.782	4	6.946	2788.345	sig
Isolates x CO_2 atmosphere x Temperature	38.782	6	6.464	2594.897	sig
Isolates x CO_2 atmosphere x a_w	5.689	4	1.422	570.931	sig
Isolates x Temperature x a_w	107.828	12	8.986	3607.362	sig
Isolates x CO_2 atmosphere x Temperature x a_w	62.207	12	5.184	2081.133	sig
Total	3134.049	216			
Corrected Total	1325.433	215			
a. R Squared = 1.000 (Adjusted R Squared = 1.000)					

MS= Mean square, SS= sum of squares, DF= degree of freedom, Sig. = significant (P<0.05).

ANOVA Table 2: Effect of abiotic interactions on sporulation of 3 strains of *M. anisopliae*.

Tests of between-subjects effects					
Dependent variable: Log_{10} (Spore cm^{-2} + 1)					
Source of variations	**SS**	**DF**	**MS**	**F**	**Sig.**
Isolates	277.665	2	138.832	103472.858	sig
CO_2 atmosphere	324.579	1	324.579	241911.345	sig
Temperature	291.516	3	97.172	72423.180	sig
a_w	44.113	2	22.057	16438.977	sig
Isolates x CO_2 atmosphere	35.044	2	17.522	13059.280	sig
Isolates x Temperature	123.604	6	20.601	15353.856	sig
Isolates x a_w	98.132	4	24.533	18284.575	sig
Isolates x CO_2 atmosphere x Temperature	221.603	6	36.934	27527.048	sig
Isolates x CO_2 atmosphere x a_w	28.613	4	7.153	5331.411	sig
Isolates x Temperature x a_w	75.212	12	6.268	4671.325	sig
CO_2 atmosphere x Temperature x a_w	8.974	6	1.496	1114.719	sig
Isolates x CO_2 atmosphere x Temperature x a_w	59.107	12	4.926	3671.081	sig
Total	4139.254	216			
Corrected total	1813.954	215			
a. R Squared = 1.000 (Adjusted R Squared = 1.000)					

source. The gas flow rate was adjusted to 5 L min^{-1} for 15 min after which the inlet tap was closed followed by the outlet. The set up was maintained at 25°C and daily cumulative insect mortality was recorded for 6 days. The box was flushed with the treatment CO_2 level every 24-h period.

The experiments included controls which consisted of (a) Insects + normal air, (b) Insects + CO_2 (650 ppm) and (c) Insects + CO_2 (1000 ppm). The controls (Insects + normal air) were compared with the 2 different CO_2 levels (650 and 1000 ppm) to evaluate toxicity of elevated CO_2 alone to the insects.

The 2 different elevated CO_2 was found to not be lethal to the insects and mortality was comparable with that in normal air. Overall, there was less than 10% mortality in normal air and elevated CO_2 conditions during the period of the study. During the six days observation period, there was no mortality in the controls at 650 ppm while <10% mortality was recorded both in the control with normal air and elevated CO_2 at 1000 ppm.

Data analysis

Growth and sporulation data were log transformed and checked for normality. Equality of error variances was confirmed with Levene's test and thereafter analysed using ANOVA procedure and different means were compared using the Tukeys test at P = 0.05. The data was back transformed for graphical presentation. All analyses were done using the statistical package, IBM SPSS Statistics 20 (ANOVA Tables 1 to 3).

ANOVA Table 3. Effect of abiotic interactions on sporulation of 3 strains of *Isaria farinose.*

Tests of between-subjects effects					
Dependent variable: Log_{10} (Spore cm^{-2} + 1)					
Source of variations	**SS**	**DF**	**MS**	**F**	**Sig.**
Isolates	194.000	2	97.000	97.925	sig
CO_2 atmosphere	19.548	1	19.548	19.734	sig
Temperature	283.209	3	94.403	95.303	sig
a_w	150.225	2	75.113	75.829	sig
Isolates x CO_2 atmosphere	11.645	2	5.822	5.878	sig
Isolates x Temperature	111.009	6	18.501	18.678	sig
Isolates x a_w	26.785	4	6.696	6.760	sig
Isolates x CO_2 atmosphere x Temperature	16.476	6	2.746	2.772	sig
Isolates x CO_2 atmosphere x a_w	2.147	2	1.073	1.084	sig
Isolates x Temperature x a_w	33.654	6	5.609	5.663	sig
Isolates x CO_2 atmosphere x Temperature x a_w	37.596	4	9.399	9.489	sig
Error	104.008	105	.991		
Total	2016.216	153			
Corrected total	1028.612	152			
a. R squared = 0.899 (Adjusted R squared = 0.854)					

For *S. gregaria*, a daily cumulative mortality was recorded and the mean percentage survival was calculated (Keyser et al., 2014). The daily cumulative mortality was similarly recorded for *A. domesticus* and a graph of the values of corrected mean percentage mortality was plotted against time. LT_{50} was calculated from the regression equation of the mortality against time.

RESULTS

Effect of a_w x temperature x elevated CO_2 on growth of the strains of entomopathogens

Figures 1, 2 and 3 compare the effect of these interacting factors on the relative growth rates of the three strains of each species examined (*B. bassiana*, *M. anisopliae* and *I. farinose*, respectively). For *B. bassiana*, with freely available water (0.995 a_w) all the strains could grow over the 25-37°C range in air. As temperature was increased to 35°C, there was a significant decrease in growth rate, especially at 0.98 a_w. With drier conditions (0.96 a_w), BB 432.99 could only grow at 25°C. Interactions between a_w, temperature and elevated CO_2 affected the growth rate of the strains, especially at 0.995 a_w and 25-30°C. While growth was often slower than in atmospheric air, one *B. bassiana* strain (BB 315) was able to grow effectively at 0.98 and 0.96 a_w at 35°C in the presence of elevated CO_2. None of the strains could grow at 37°C and 0.96 a_w, regardless of CO_2 treatments.

For *M. anisopliae* there was a marked difference in growth between one of the strains (Ma29) and the others in both normal atmospheric air and elevated CO_2, regardless of a_w and temperature treatment. Interestingly, in elevated CO_2, the *M. anisopliae* strain Ma29 was able to grow better than in air at both 0.995 and 0.98 a_w across the temperature range tested. The other two strains were more sensitive to the three way interacting conditions and unable to grow at 37°C regardless of a_w regime imposed.

I. farinosa strains on the other hand were less tolerant; only ARSEF 5676 and IF 790.05 could grow at 25°C and three treatment a_w levels (0.995, 0.98 and 0.96) under elevated CO_2 whereas, under normal air conditions the temperature windows for growth of the 2 strains was 25-30°C at 0.995 and 0.98 a_w, respectively. In contrast, ARSEF 5081 could grow at 35°C and 0.96 a_w under elevated CO_2 while no growth occurred under such conditions in the normal air.

Effect of a_w, temperature and elevated CO_2 on sporulation of the entomopathogens

The effect of the interactions of temperature and a_w on sporulation of the strains of *B. bassiana*, *M. anisopliae* and *Isaria* strains are shown in Tables 2, 3 and 4. The optimum temperature for sporulation varied with a_w and was also strain dependent. The *B. bassiana* strain BB 315 produced significantly higher numbers of spores than BB 432.99 and BB 776.05 at higher temperatures (35 and 37°C) and 0.995 a_w under normal atmospheric conditions. In contrast, the strain BB 432.99 produced significantly higher spore numbers under elevated CO_2 at 0.995 a_w and 25, 30 and 35°C. The *B. bassiana* strain BB 315 was unusual as it could grow under elevated CO_2 at 35°C and 0.96 a_w but not in normal air. However, it could not sporulate at 0.98 a_w and 35°C under elevated CO_2.

One of the three *M. anisopliae* strains (V275) could not

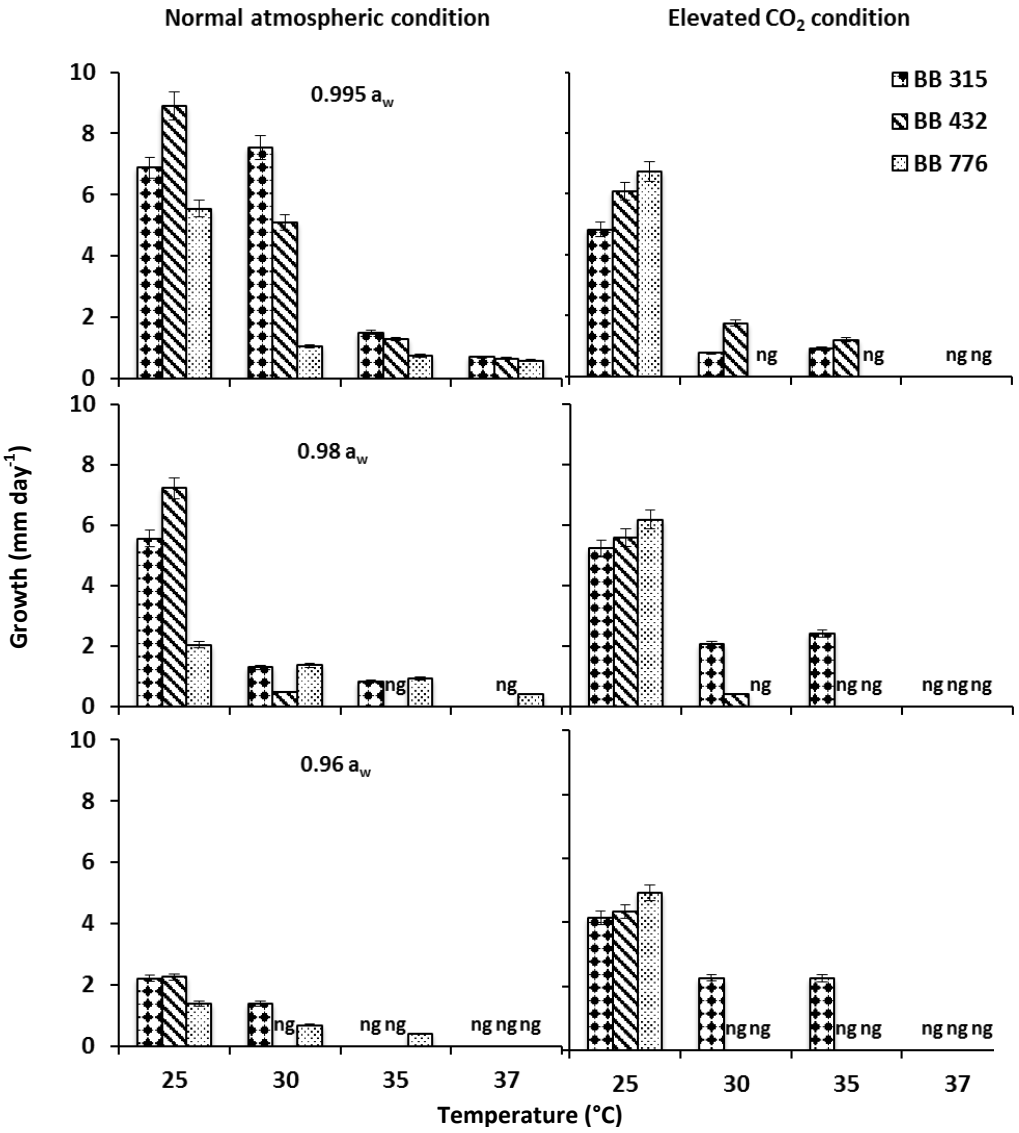

Figure 1. Comparisons of effect of temperature and a_w on growth of *B. bassiana* under normal atmospheric air composition and elevated CO_2 conditions. Ng = no growth.

sporulate under interacting abiotic stress conditions (0.995 a_w, 30 and 35°C; 0.98 a_w and 30°C) in elevated CO_2 atmosphere conditions. In some cases, no sporulation occurred under elevated CO_2 even at high water activity (0.995 a_w) and 30°C in *M. anisopliae* strains. Thus, In elevated CO_2, the combined stresses appeared to have a detrimental effect on sporulation with significantly less or no spore production occurring, even when water was freely available (0.995 a_w) and moderate temperature of 30°C.

Overall, the *Isaria* strains were very sensitive to changes in the three interacting factors with sporulation significantly affected by elevated CO_2, especially at elevated temperatures. The *Isaria* strains IF 5081 and IF 5676 could not sporulate at 0.98 a_w x 37°C and 0.96 a_w x 25°C, respectively under elevated CO_2 whereas sporulation was possible under such conditions in the normal air.

Relative virulence of the entomopathogens in normal air and elevated CO_2 (650 and 1000 ppm) against *S. gregaria*

Figure 4 compares the effect of normal air, 650 and 1000 ppm CO_2 on the survival of the desert locust, *S. gregaria* under 100% RH conditions on the relative virulence of 2 strains each of *B. bassiana* (BB 315, BB 776.05), *M. anisopliae* (275.86DC, V275), and one strain each of *I. farinosa* (ARSEF 5081) and *I. fumosorosea* (790.05) over

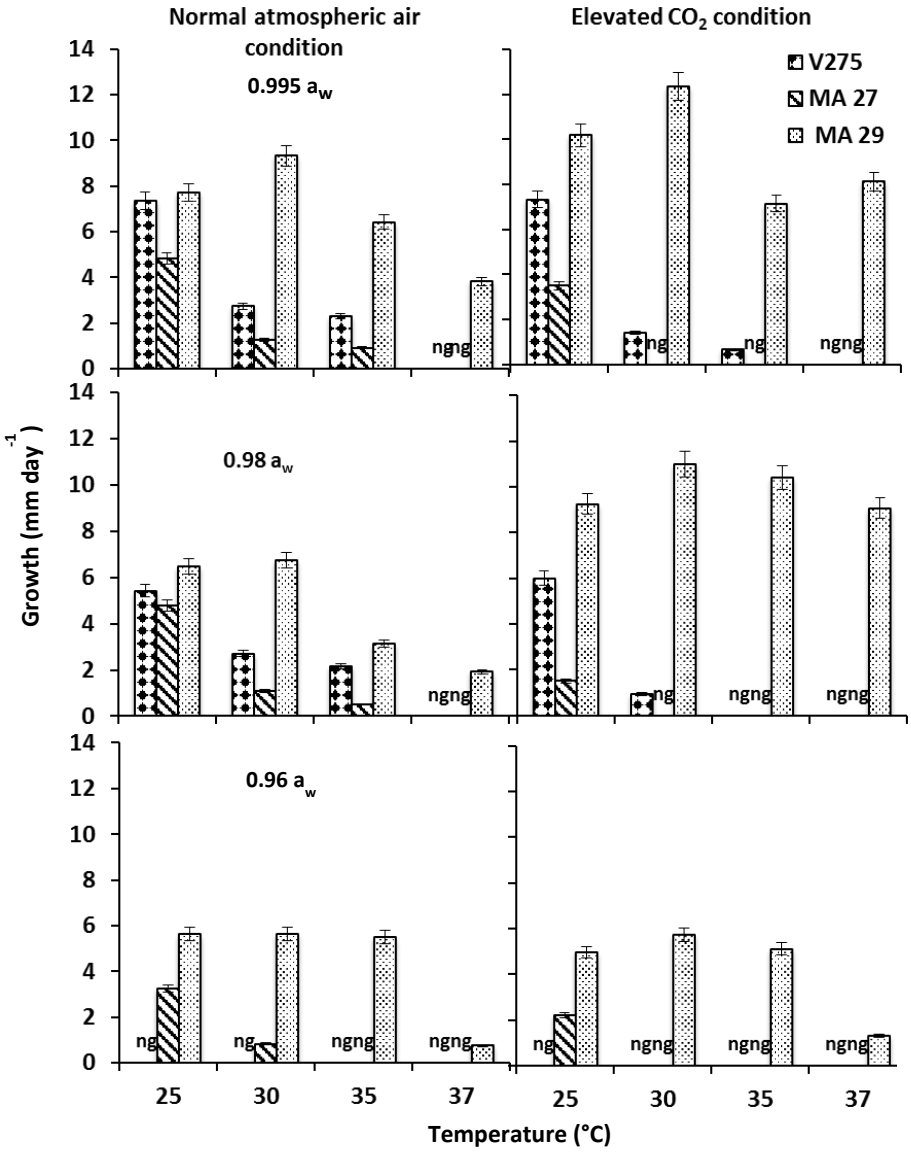

Figure 2. Comparisons of effect of temperature x a_w on growth of *M. anisopliae* under normal atmospheric air composition and elevated CO_2 conditions.

six days.

Under ambient atmospheric air composition, a strain of *B. bassiana* (776.05) showed relatively weak pathogenicity to *S. gregaria.* After 5 days of application of the spores to *S. gregaria*, the data showed 27% of the insects survived the treatment whereas, 93-100% mortality was recorded in the treatment with other strains. *I. fumosorosea* caused 100% mortality after 4 days and this was the most virulent of all the tested strains.

Under elevated CO_2 (650, 1000 ppm CO_2) more of the insects survived the treatments. For example, the virulence of the weakly pathogenic strain (776.05) was further affected such that 60 and 93% of the inoculated insects survived after 5 days at 650 and 1000 ppm, respectively. A similar reduction in virulence by elevated CO_2 was observed in all the strains. 20% of the inoculated insects survived inoculation with spores of the most pathogenic isolate, *I. fumosorosea* 790.05 after 4 days with 7% surviving after 5 days. With the exception of the *Isaria* strain (ARSEF 5081) where 100% mortality of the inoculated insects was recorded after 6 days, about 6-13% of the insects survived after inoculation with the other strains at 1000 ppm.

Relative virulence of *B. bassiana* (BB 315) to *A. domesticus* under different elevated temperature x CO_2 x relative humidity conditions

Figure 5 shows the changes in virulence of *B. bassiana*

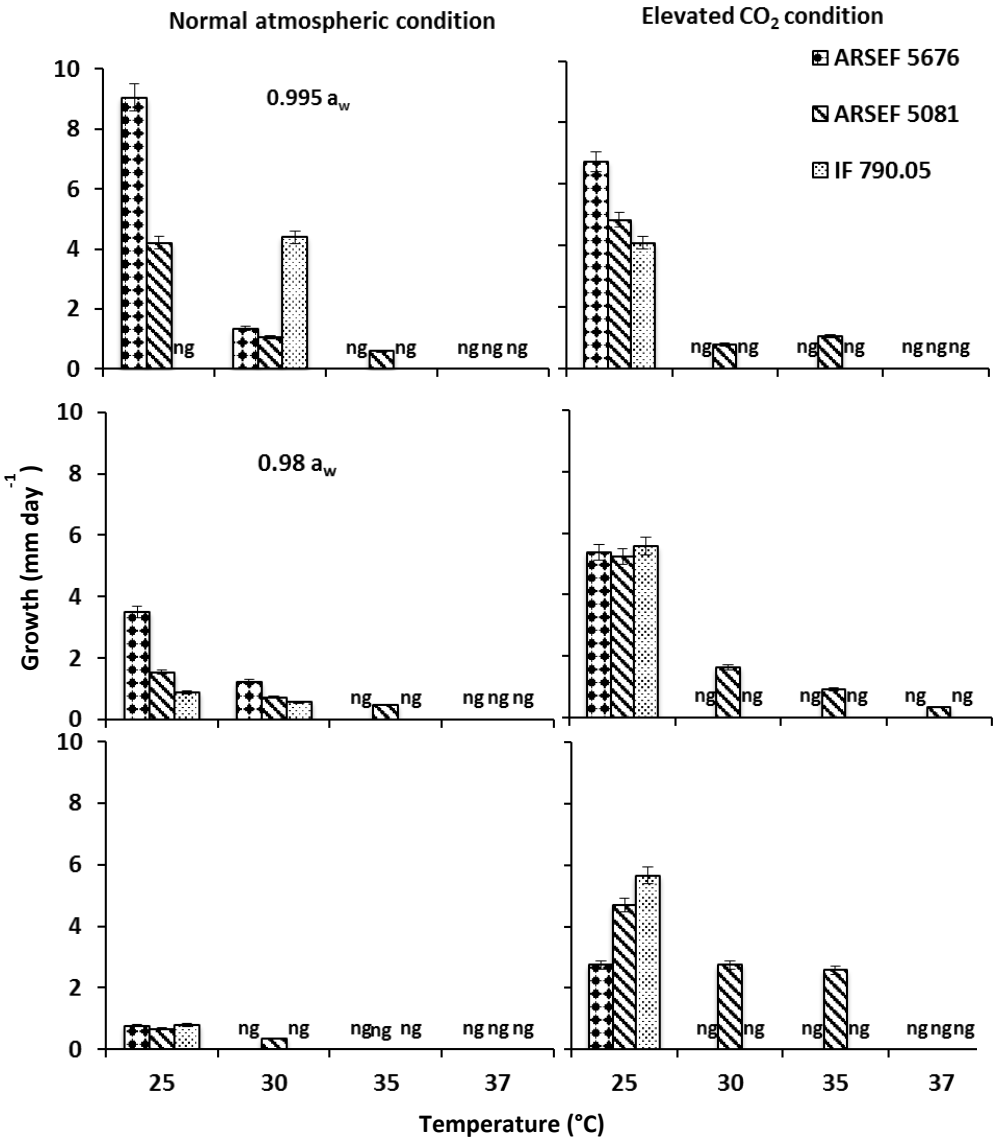

Figure 3. Comparisons of effect of temperature x water activity (a_w) on growth of *I. farinosa* under normal atmospheric air composition and elevated CO_2 conditions.

(BB 315) under normal air and 1000 ppm CO_2 and elevated temperatures and different relative humidities. There was an extension in the time (LT_{50}) required for this entomopathogen to cause mortality of the insect populations. At 25°C and >99% ERH, the LT_{50} of the strain was 5.9 days. Under a stress condition (96% ERH and 25°C), this increased to 6.5 days. Higher LT_{50} values were recorded as the interacting climate change factors became increasingly severe. The LT_{50} of the fungi to *A. domesticus* at 35°C and 96% ERH (9.3 days) was more than double of that at 35°C and >99% ERH.

Elevation of CO_2 concentration to 1000 ppm significantly increased the LT_{50} regardless of temperature and ERH. For instance, 30°C and >99% RH was optimum for virulence of the strain (lowest LT_{50}= 4.4 days) in ambient air. In the presence of 1000 ppm CO_2 and >99% RH, the LT_{50} at the same temperature increased to 13.1 days. Under drier conditions (96% RH) and 30-35°C, all the inoculated insects survived showing that under these conditions, the biocontrol entomopathogen was ineffective over the experimental period.

DISCUSSION

This is the first study that examine the impact of interacting climate change conditions (temperature, a_w and elevated CO_2) on the growth and sporulation of entomopathogens. This study has shown that these

Table 2. Comparison of the effect of interactions of temperature (25-37°C) and water activity (0.995 - 0.96 a_w) on sporulation (log_{10} spore cm^{-2}) of three strains of *B. bassiana* under ambient CO_2 (350 ppm CO_2) and elevated CO_2 (1000 ppm).

Fungal strain	0.995 a_w			0.98 a_w			0.96 a_w		
	BB315	BB 432.99	BB 776.05	BB315	BB 432.99	BB 776.05	BB315	BB 432.99	BB 776.05
25°C									
Ambient	4.77[b]	3.37[b]	5.07[a]	6.25[a]	3.76[b]	4.61[a]	5.77[a]	4.75[a]	4.75[a]
1000 ppm CO_2	5.62[a]	5.76[a]	3.63[b]	3.37[b]	4.76[a]	3.88[b]	3.17[b]	4.05[b]	3.57[b]
30°C									
Ambient	5.09[a]	3.86[b]	6.03[a]	5.91[a]	5.92[a]	5.08[a]	4.95[a]	NG[c]	5.38[a]
1000 ppm CO_2	3.93[b]	4.80[a]	NG[c]	4.58[b]	3.89[b]	NG[c]	3.48[b]	NG[c]	NG[c]
35°C									
Ambient	6.23[a]	4.69[a]	6.11[a]	5.43[a]	NG[c]	5.18[a]	NG[c]	NG[c]	5.14[a]
1000 ppm CO_2	3.16[b]	4.17[b]	NG[c]	NS[c]	NG[c]	NG[c]	NS[c]	NG[c]	NG[c]
37°C									
Ambient	5.67[a]	4.98[a]	5.25[a]	NG[c]	NG[c]	5.13[a]	NG[c]	NG[c]	NG[c]
1000 ppm CO_2	NG[c]	NG[c]	NG[c]	NG[c]	NG[c]	NG[c]	NG[c]	NG[c]	NG[c]

The table compares effects of atmospheric CO_2 within each level of combination of the other factors (temperature and water activity) shown. These tests are based on the linearly independent pairwise comparisons among the estimated marginal means. For each strain and within the same level of interaction (temperature and a_w), values followed by different letters are significantly different ($P<0.05$) at different a_w and temperature levels for ambient vs. 1000 ppm CO_2. NG = No growth, NS = there was growth but no sporulation

interacting abiotic factors profoundly influenced the growth and sporulation of the fungal species and strains examined. Elevated CO_2 influenced the temperature range for growth and changed the set of conditions for optimum growth and sporulation in all the strains. Optimal growth conditions for all the strains were between 25-35°C and 0.995-0.98 a_w under unmodified CO_2 and elevated CO_2 conditions.

No comparisons can be made with previous data except where a_w and temperature conditions on growth and sporulation were considered (Hallsworth and Magan, 1996; Borisade and Magan, 2014). Indeed, Borisade and Magan (2014) showed that elevated temperatures to simulate those under climate change conditions when interacting with drought stress significantly influenced the ability of strains of *B. bassiana*, *M. anisopliae* and *I. farinosa* to grow. In these studies, only a few strains were able to tolerate 35-37°C and drought stress (0.94-0.96 a_w) and these were one strain each of latter two species. The source of the strains did not influence tolerance to elevated temperatures and drought stress.

In the present study, the interaction with elevated CO_2 suggests that further modulation of growth may occur. It may be that some strains are able to change their growth morphology to tolerate such conditions. For example, *I. farinosa* (ARSEF 5081) was able to tolerate elevated atmospheric CO_2 by changing its growth morphology under the specific temperature and a_w conditions in the present study. In earlier studies (Alves et al., 2002), *B. bassiana* was found to develop yeast like cells on SDA in the presence of the imposed ionic solute stress using NaCl. Yeast-like cell formation in entomopathogenic fungi is an adaptation for survival in the insect haemolymph (which could be rich in CO_2 and solutes). Yeast-like cells of *B. bassiana* have been reported to occur on media when the condition of the media mimics the haemolymph of insects (Alves et al., 2002). This may be responsible for the higher growth at lower a_w and elevated CO_2 in some strains of the species examined in the present study.

Overall, the strain Ma 29 of *M. anisopliae* showed a relatively higher resilience to the interactions of the extremes of the abiotic stress factors, as the temperature profile for growth (25-37°C) was not altered under elevated CO_2 and when combined with water stress.

It appeared that elevated CO_2 significantly influenced the ability for conidial production and

Table 3. Comparison of the effect of interactions of temperature (25-37°C) and water activity (0.995 - 0.96 a_w) on sporulation (log_{10} spore cm^{-2}) of three strains of *M. anisopliae* under ambient (350 ppm CO_2) and elevated CO_2 (1000 ppm CO_2).

Fungal strain	0.995 a_w			0.98 a_w			0.96 a_w		
	Ma 29	Ma 27	V275	Ma 29	Ma 27	V275	Ma 29	Ma 27	Ma V275
25°C									
Ambient	4.62[a]	7.05[a]	6.03[a]	4.12[a]	7.04[a]	5.25[a]	4.23[a]	7.42[a]	NG[c]
1000 ppm CO_2	3.98[b]	6.18[b]	5.54[b]	3.46[b]	5.97[b]	5.76[b]	4.14[b]	6.15[b]	NG[c]
30°C									
Ambient	4.29[a]	8.06[a]	5.78[a]	4.82[a]	8.38[a]	6.90[a]	7.19[a]	7.93[a]	NG[c]
1000 ppm CO_2	4.34[a]	NG[c]	NS[c]	4.71[b]	NG[c]	NS[c]	3.20[b]	NG[c]	NG[c]
35°C									
Ambient	4.09[a]	8.40[a]	4.73[a]	4.61[a]	8.90[a]	6.24[a]	4.59[a]	NG[c]	NG[c]
1000 ppm CO_2	3.37[b]	NG[c]	NS[c]	3.71[b]	NG[c]	NG[c]	3.64[c]	NG[c]	NG[c]
37°C									
Ambient	4.28[a]	NG[c]	NG[c]	6.25[a]	NG[c]	NG[c]	5.60[a]	NG[c]	NG[c]
1000 ppm CO_2	3.39[b]	NG[c]	NG[c]	3.67[b]	NG[c]	NG[c]	4.70[b]	NG[c]	NG[c]

The table compares effects of atmospheric CO_2 within each level of combination of the other factors (temperature and water activity) shown. These tests are based on the linearly independent pairwise comparisons among the estimated marginal means. For each strain and within the same level of interaction (temperature and a_w), values followed by different letters are significantly different (P<0.05) at different a_w and temperature levels for ambient vs. 1000 ppm CO_2. NG = No growth, NS = there was growth but no sporulation.

Table 4. Comparison of the effect of interactions of temperature (25-37°C) and water activity (0.995 - 0.96 a_w) on sporulation (log_{10} spore cm^{-2}) of three strains of *I. farinosa* in ambient (350 ppm CO_2) and under elevated CO_2 (1000 ppm).

Fungal strain	0.995 a_w			0.98 a_w			0.96 a_w		
	ARSEF 5081	ARSEF 5676	IF 790.05	ARSEF 5081	ARSEF 5676	IF 790.05	ARSEF 5081	ARSEF 5676	IF 790.05
25°C									
Ambient	4.17[a]	4.97[a]	3.71[b]	4.61[a]	4.73[b]	5.89[a]	4.75[a]	7.53[a]	6.32[a]
1000 ppm CO_2	3.55[b]	2.73[b]	3.44[b]	3.88[b]	7.01[a]	4.38[b]	3.57[b]	3.88[b]	NS[c]
30°C									
Ambient	5.72[a]	6.08[a]	4.40[a]	5.08[a]	6.21[a]	6.55[a]	7.33[a]	NG[c]	NG[c]
1000 ppm CO_2	4.80[b]	NG[c]	NG[c]	NG[c]	6.94[a]	NG[c]	4.22[b]	NG[c]	NG[c]
35°C									
Ambient	6.08[a]	NG[c]	4.93[a]	6.61[a]	NG[c]	NG[c]	NG[c]	NG[c]	NG[c]
1000 ppm CO_2	4.46[b]	NG[c]	NG[c]	6.59[a]	NG[c]	NG[c]	3.86[b]	NG[c]	NG[c]
37°C									
Ambient	NG[c]	NG[c]	NG[c]	NG[c]	NG[c]	NG[c]	NG[c]	NG[c]	NG[c]
1000 ppm CO_2	NG[c]	NG[c]	NG[c]	**NS**[c]	NG[c]	NG[c]	NG[c]	NG[c]	NG[c]

The table compares effects of atmospheric CO_2 within each level of combination of the other factors (temperature and water activity) shown. These tests are based on the linearly independent pairwise comparisons among the estimated marginal means. For each strain and within the same level of interaction (temperature and a_w), values followed by different letters are significantly different (P<0.05) at different a_w and temperature levels for ambient vs. 1000 ppm CO_2. NG = No growth, NS = there was growth but no sporulation.

reduced the range of a_w and temperature over which this occurred. Previous studies have examined effects of temperature or a_w and temperature on sporulation indices but have not included the climate change factor and the concentrations used here of 1000 ppm CO_2 (Alves et al., 2002; Lord, 2009; Garza-Lopez et al., 2011; Borisade and Magan, 2014).

Overall, at least one strain of each entomogenous species showed considerable resilience to the effect of elevated CO_2 interactions at various temperatures and a_w

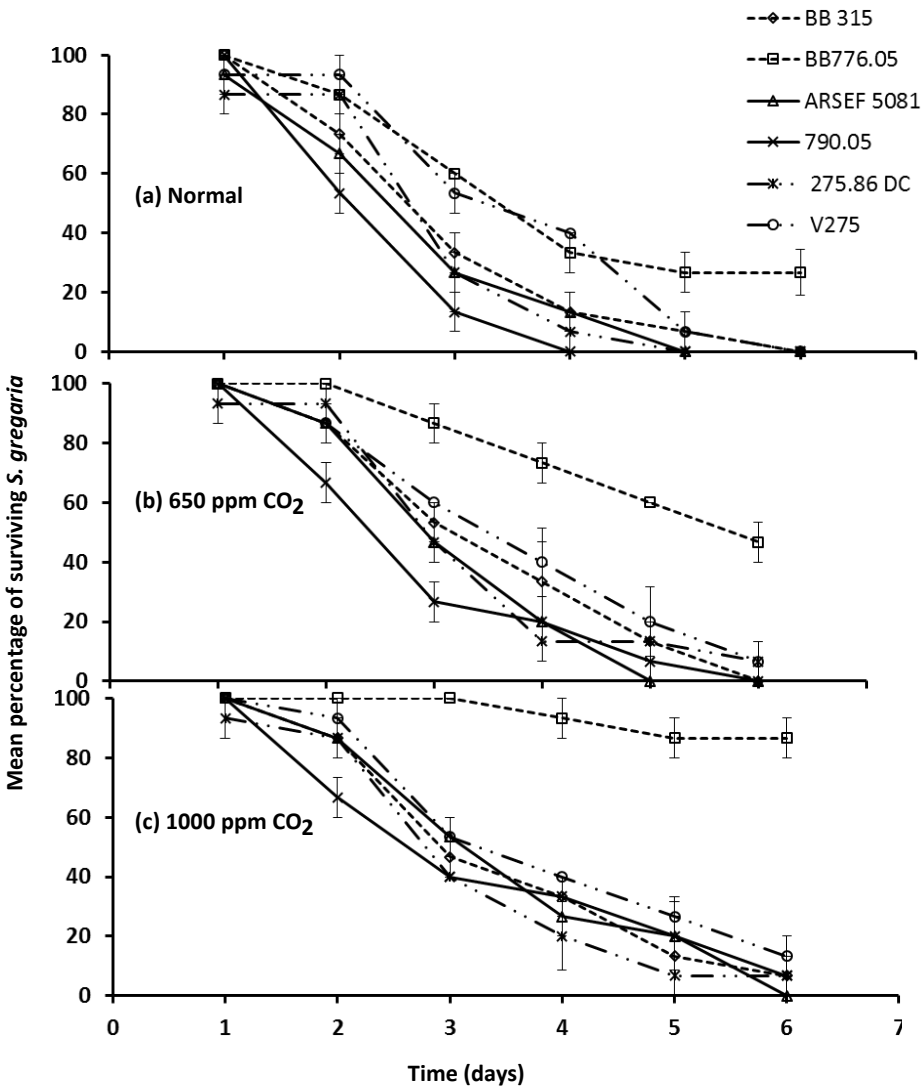

Figure 4. Mean percentage survival of desert locust (*Schistocerca gregaria*) separately exposed to entomogenous *Beauveria bassiana* (BB 315, BB 776.05), *Isaria farinosa* (ARSEF 5081), *Isaria fumosorosea* (790.05) and *Metarrhizium anisopliae* (275.86DC, V275) conidia at 3 different concentrations of CO_2 : (a) Normal air, 350 ppm, (b) 650 ppm, (c) 1000 ppm. Bars indicate standard error of the mean.

levels. However, elevated CO_2 significantly reduced sporulation potential. Currently, there is little information on the effect of CO_2 on sporulation of entomopathogenic fungi. The simultaneous effect of the interactions of the three abiotic stress factors under consideration in this study has not been previously reported for these strains.

In this study, *B. bassiana* (BB 315) was able to grow at 0.96 a_w x 35°C under elevated CO_2 but unable to sporulate. Similarly, *I. farinosa* (ARSEF 5676) could grow at 0.96 a_w x 25°C and *M. anisopliae* (V275) could grow at 0.995 a_w x 30/35°C under elevated CO_2, but no sporulation occurred. The observed loss of sporulation capabilities under a combination of abiotic stress factors can have a significant impact on the success of entomopathogenic strains in pest management system under climate change scenarios.

Previous studies have reported a decrease in conidial production in the presence of elevated CO_2 at concentrations significantly higher (10% CO_2) in *Aspergillus niger* and *Trichoderma viridis* (Desgranges and Durand, 1990). Similarly, Garza-Lopez et al. (2011) reported 85% decrease in conidial production in *B. bassiana* under 5% CO_2 enriched atmosphere while Lord (2009) reported increased germination lag times and subsequently, a decrease in both mycelial growth and sporulation.

The studies carried out with both *S. gregaria* and *A. domesticus* represent the first attempt to try and examine

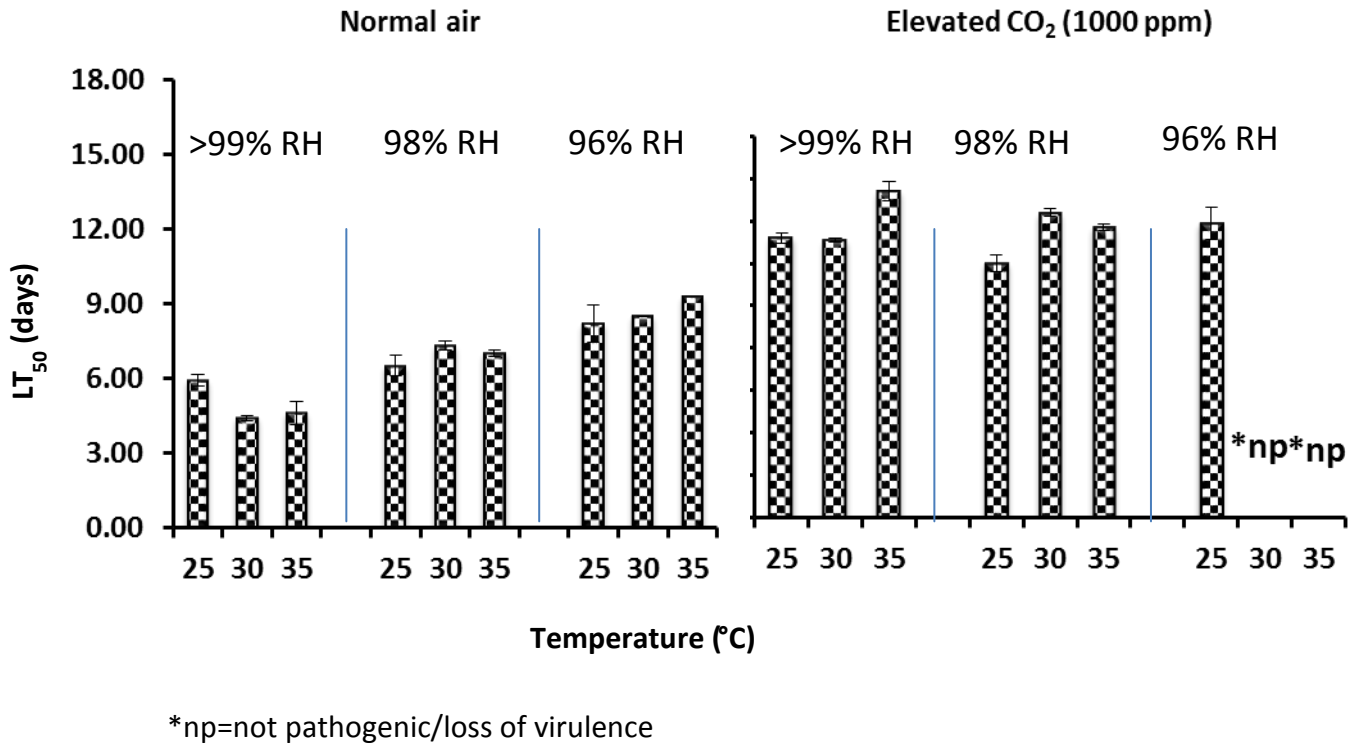

Figure 5. Relative virulence of *B. bassiana* (BB 315) to crickets (*Acheta domesticus,* Orthoptera: Gryllidae) under the influence of different interacting climate change factors (temperature x relative humidity x CO_2 concentrations). Bars indicate standard error of the mean.

the potential impact of temperature x elevated CO_2 on relative virulence of strains of these species in relation to factors which may simulate climate change scenarios. The results with two strains of each entomogenous species (*S. gregaria*) and that with *B. bassiana* in relation to the mortality of crickets have demonstrated that the virulence may be reduced, impacting on the level of control achieved.

Under a climate change scenario, the temperature is expected to rise by 2-4°C, the CO_2 to increase by up to three times existing levels (700-1000 ppm) under drought conditions. These combined factors may have very different impacts than one or two together and influence both pest and disease epidemics (Magan et al., 2007, 2011; Bebber et al., 2013). Thus, it is critical that this type of data are obtained and utilised to help in the selection of strains for use under the marginal conditions that may be present in a climate change scenario. This also suggests that IPM strategies may need to be significantly modified or formulations of entomogenous biopesticides need to be modified to facilitate consistent efficacy under such pressures. It may be that strains of entomogenous fungi need to be isolated from native agro-ecological zones or the formulation may require modification to enable efficacy to be relied upon under such abiotic change pressures. Indeed, less effective pest control in an IPM strategy may have a significant impact on food security of staple commodities, their quality and availability.

Conflict of interests

The authors did not declare any conflict of interest.

ACKNOWLEDGEMENTS

Dr. O. A. Borisade is very grateful to the Tertiary Education Trust Fund (TETFUND) Nigeria for funding this study and The Faculty of Agricultural Sciences, Ekiti State University, for moral supports.

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academicJournals

Vol. 9(14), pp. 1001-1011, 8 April, 2015
DOI: 10.5897/AJMR2014.6828
Article Number: 58FF2B852289
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Achromobacter sp. and *Virgibacillus pantothenticus* as models of thermo-tolerant lipase-producing marine bacteria from North Delta sediments (Egypt)

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Received 12 April, 2014; Accepted 20 March, 2015

Sediments of North Delta (Egypt) are a rich source of many thermo-tolerant bacteria, which could be used as a good source of many enzymes. Sediment samples were collected from six different sites in North Delta region. The counts of thermo-tolerant marine bacteria (at 55°C) in sediment samples ranged from $9.8x10^2$ to $6.8x10^3$ CFU g^{-1}. It was found that the occurrence of thermo-tolerant bacteria in North Delta sediments at 55°C reached 11.1% with respect to the total viable count at 30°C. Five of seven selected thermo-tolerant bacterial isolates showed optimum growth at pH 9 and NaCl concentration of 4% (w/v) after 24 h of incubation at 55°C. Two thermo-tolerant marine bacteria were selected and identified as *Achromobacter* sp. HEGN 014 and *Virgibacillus pantothenticus* HEGN 114 using 16S rDNA analysis. Their sequence similarities were 96 and 97%, respectively. Their optimum growth was at pH 9 and NaCl concentration of 4% (w/v) with tolerance to higher concentration of 7 and 9% (w/v), respectively. Moreover, both strains were investigated to produce an extracellular lipase, while they showed no productivity for cellulase, chitinase or protease at 55°C, however, gelatinase was produced only by *V. pantothenticus* HEGN 114. Antibiotics resistance was observed for *Achromobacter* sp. HEGN 014 and *V. pantothenticus* HEGN 114. The optimum reaction temperatures for the purified lipases from both strains were the same at 35 and 55°C. The amino acid analysis showed that arginine represented 89.982 mmole of total detected amino acids of lipase produced from *Achromobacter* sp. HEGN 014 with tolerance to higher concentration of 7 and 9% (w/v) NaCl.

Key words: *Achromobacter* sp., *Virgibacillus pantothenticus*, thermo-tolerant, lipase.

INTRODUCTION

Life exists almost everywhere on earth. Presence of liquid water is a prerequisite for life (Oren, 2008). Moderate environments are important to sustain life. Any environmental condition that can be perceived as beyond the normal acceptable range is an extreme condition. A variety of microbes, however, survives and grows in such environments. These organisms, known as extremophiles, not only tolerate specific extreme

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condition(s), but also usually require these for survival and growth. Most extremophiles are found in the microbial world. The range of environmental extremes tolerated by microbes is much broader than other life forms (Satyanarayana et al., 2005). In these habitats, environmental conditions such as pH, temperature and salinity concentrations are extremely high or low. Extreme environments are populated by groups of organisms that are specifically adapted to these particular conditions (Mahmoud, 2006).

As a result of adaptation to extreme environments, extremophiles have evolved unique properties, which can be of biotechnological and commercial significance (Margesin and Schinner, 2001). Life in extreme environments has been studied intensively focusing attention on the diversity of organisms, molecular and regulatory mechanisms involved. The products obtainable from extremophiles such as proteins, enzymes (extremozymes) and compatible solutes are of great interest to biotechnologists (Satyanarayana et al., 2005).

Despite the fact that, to date more than 3000 different enzymes have been identified and many of these have found their way into biotechnological and industrial applications, the present enzymes toolbox are still not sufficient to meet all demands.

A major cause for this is the fact that many available enzymes do not withstand industrial reaction conditions (Madigan and Marrs, 1997). The major share of the industrial enzyme market has been occupied by hydrolytic enzymes such as lipases, esterases, proteases and amylases (Gupta et al., 2004).

Lipids constitute a large part of the earth's biomass and lipolytic enzymes play an important role. Lipases and esterase have been recognized as very useful biocatalyst (Mohan et al., 2008). Lipases are a class of hydrolases that catalyze a variety of reactions, such as the hydrolysis of fatty acid ester, trans-esterification and ester synthesis at the interface between the insoluble substrate and water.

Microbial lipases are currently receiving much attention because of their biotechnological potential applications (Aly et al., 2012; Wang et al., 2014). They are the major industrial enzymes extensively used in pharmaceuticals, textiles, food, medical, detergent manufacturing and other chemical industries (Mohan et al., 2008; Sangeetha et al., 2011; Aly et al., 2012; Nerurkar et al., 2013; Chatterjee et al., 2014).

The major cause of limiting industrial usage of known lipases or esterases is their limited thermo-stability, mainly at high temperatures, pH and inorganic salts. Therefore, the search for new microbial enzyme sources is important for the development of new thermo-stable enzymes for industrial applications (Gupta et al., 2004; Faiz et al., 2007).

This study aims to isolate and characterize thermo-tolerant marine bacteria from North Delta sediments. Moreover, the study extended to screen their abilities to produce industrially valuable enzymes.

MATERIALS AND METHODS

Sampling and isolation of bacteria

Sediment samples were collected from six different sites (Demitta, Gamasa, Baltim, El-Brolus, Abou-Kashaba and Rashid) in North Delta region. For estimation of total viable count (TVC), sediments samples were suspended in 100 ml sterilized aged seawater to dissociate the adhered bacterial population, then diluted up to 10^{-6}. Each dilution was plated by pour plat method onto seawater nutrient agar (SWNA) with the following composition g 1^{-1}: peptone, 5.0; yeast extract, 3.0; agar, 15; aged seawater. Plates were incubated at 30 and 55°C for 24-48 h. Triplicates were used for each sample.

Isolates selection

Seven bacterial isolates have been isolated from growing colonies at 55°C. These colonies were selected according to morphological characters to represent the most dominant colonies. These seven thermo-tolerant bacterial isolates were selected for further studies: Demitta (A1), Gamasa (A2), Baltim (A4), El-Brolus (A9 and A10), Abou-Kashaba (A22) and Rashid (A31).

Testing for thermotolerance, alkalitolerance and halotolerance

All growth experiments were conducted in triplicate in seawater nutrient broth medium, unless otherwise stated. Growth was determined by measuring optical density at 550 nm ($O.D._{550}$) with a spectrophotometer (U–1500, Hitachi). Effect of temperatures on growth was determined in the range of 35 - 85°C. Effect of pH on growth was determined by adjusting pH of the medium from 5 to11. NaCl requirements were determined in the same medium containing 4-13% (w/v) NaCl.

Molecular identification

The identification was carried out at City for Scientific Research and Technology Applications, Arid Land Institute, Molecular Plant Pathology Department, New Borg El Arab City, 21934, Alexandria, Egypt.

Characterization of the selected strains

The characters of the selected organisms were studied following the standard microbiological methods as described in Bergy's manual (Holt et al., 1994). Colony morphology, Gram reaction and spore characteristics were observed. The physiological and biochemical characters included production of β- galactosidase, arginine dehydrolase, lysine decarboxylase, ornithine decarboxylase, sulphide, urease, tryptophane deaminase, indole, acetoin and gelatinase, oxidase, catalase and citritase tested. Also, the utilization of different sugars such as D-glucose, D-mannitol, inositol, D-sorbitol, rhamnose, D-sucrose, D-melibiose, amygdalin and L- arabinose were determined.

Resistance to antibiotics

Resistance of thermo-tolerant strains were tested against: ampicillin-sulbactam, 20 µg; ciprocin, 5 µg; gentamycin, 10 µg;

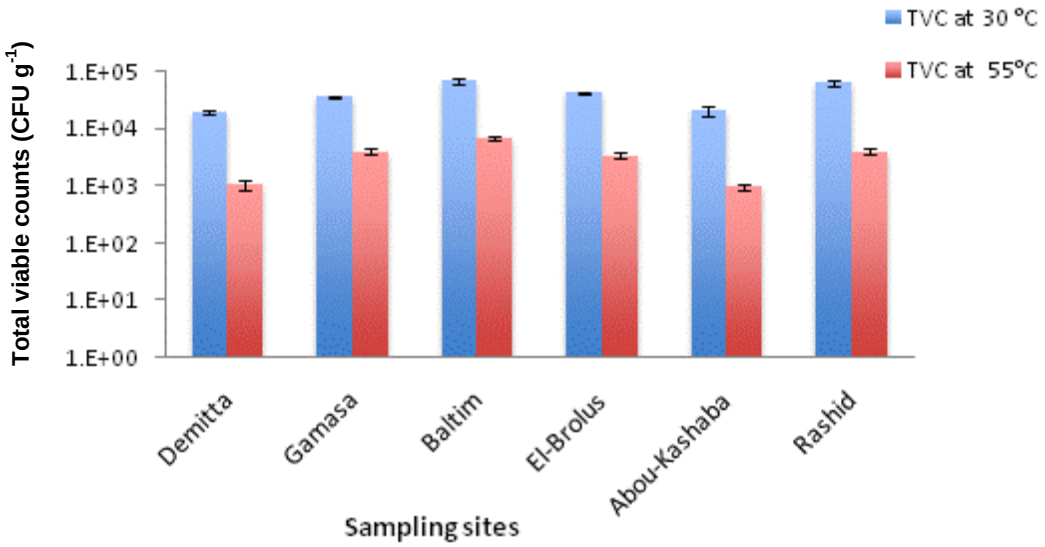

Figure 1. The viable counts of thermotolerant bacteria (CFU g^{-1}) in North Delta sediments (values are average ± SD).

ampicillin, 10 µg; imipenem, 10 µg; norfloxacin, 10 µg; cephalexin, 30 µg; cefadroxil, 30 µg; ceftazidine, 30 µg; erythromycin, 15 µg; and flucloxacillin, 5 µg by disk diffusion method (Bauer et al., 1966).

Enzymes productivity

The selected isolates were tested for production of gelatinase (Ventosa et al., 1982), protease, lipase, chitinase (Hankin and Anagnostakis, 1975) and cellulase (Ariffin et al., 2006) enzymes. After incubation at 37°C, the appearance of clear zones around the colonies was scored as a positive result.

Lipase production

Mineral based broth contained (g l^{-1}): $NaNO_3$, 0.30; K_2HPO_4, 0.01; $MgSO_4.7H_2O$, 0.05; KCl, 0.05; $FeSO_4.7H_2O$, 0.001; yeast extract, 0.50 and sucrose, 0.5. The cotton seeds oil (100 ml) were warmed to 70°C, then emulsified with Arabic gum (0.5 g) with continuous stirring, the pH of the medium was adjusted to 7.5. For lipase production, mineral bases broth was supplemented by 1% (v/v) cotton seeds oil, and production media (50 ml each in 200 ml capacity Elementary flasks) inoculated by 48 h old culture. The cells free broth was harvested by centrifugation at 10,000g and 4°C (Kanwar et al., 2006).

Lipase assay

Lipase activity was measured by titrimetric method using cottonseeds oil as a substrate. Cottonseeds oil (10% v/v) was emulsified with Arabic gum (5% w/v) in 0.05 mM Tris buffer pH 7.0. 100 µl of enzyme was added to the emulsion and incubated for 30 min at 37°C. The reaction was stopped and fatty acids were extracted by addition of 1.0 ml of acetone : ethanol solution (1:1). The amount of the fatty acids liberated was estimated by titrating with 0.05 M NaOH until pH was 10.5 using a phenophathelin indicator (Jensen, 1983). One unit of enzyme is defined as the amount of enzyme required to hydrolyse µmol of fatty acids from triglycerides per minute.

Effect of different temperatures on enzyme activity

To examine the effect of temperature of the reaction on the activity of the purified enzyme, the enzymatic reaction was carried out for 30 min at different temperature: 35, 40, 45, 50, 55 and 60°C using an enzyme protein and substrate concentration of 4 mg and 0.1 ml cottonseeds oil, respectively, per 1200 µl reaction mixture. A control was made using previously heated enzyme solution in the reaction.

Amino acids analysis

Analysis of amino acids was conducted in central laboratories unit, National Institute of Oceanography and Fisheries (NIOF) using AAA-Direct, Dionex Amino Analyzer and Amino Pac PA10 according to Irvine (1997).

RESULTS

Viable count of thermotolerant bacteria

The occurrence of thermo-tolerant bacteria in North Delta sediments reached 11.1% of the total isolated bacteria in Gamasa. The highest count of thermo-tolerant marine bacteria (at 55°C) in sediment samples was detected in Baltim ($6.8x10^3$ ± 4.9 $x10^2$ CFU g^{-1}) which represented 10.13% of the total viable count at 30°C. The lowest count was estimated in Abou-Kashaba ($9.8x10^2$ ± 1.1 $x10^2$ CFU g^{-1}) representing 4.67% of the total viable count at 30°C (Figure 1).

Growth of isolates at different temperatures

The growth (OD_{550}) of the seven selected isolates at different temperatures was determined after 24 h of incubation in the standard medium. All isolates were able to grow over a wide range of temperature from 35 to

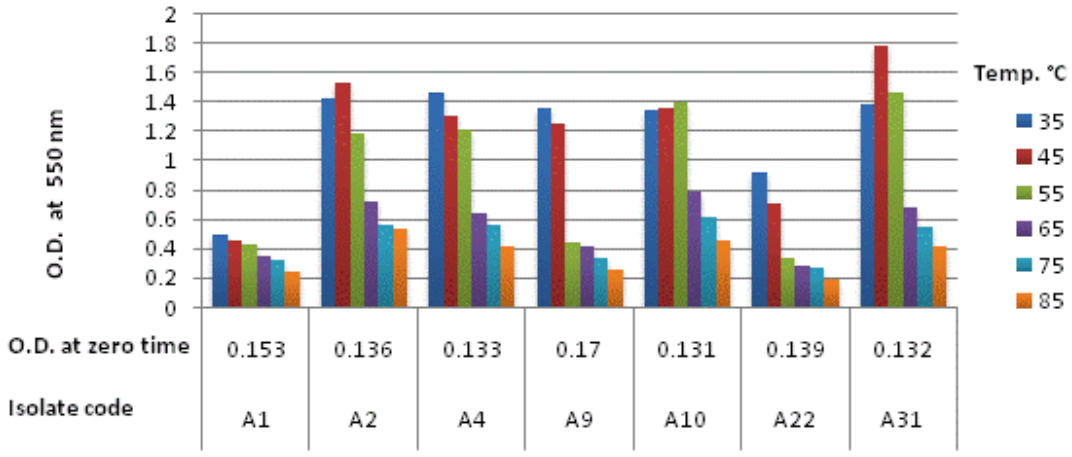

Figure 2. The growth ($O.D._{550}$) of the selected isolates at different temperatures after 24 h incubation.

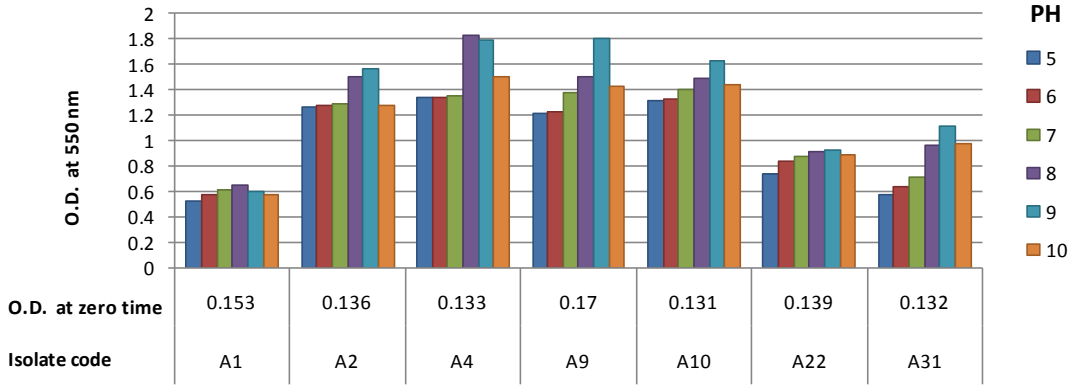

Figure 3. The growth ($O.D._{550}$) of the selected isolates at different pH after 24 h incubation.

85°C (Figure 2). Optimum growth of isolates A_1, A_4, A_9 and A_{22} were at 35°C, while the optimum growth of isolates A_2 and A_{31} were at 45°C. The optimum growth of isolate A_{10} was at 55°C. Isolates A_{10} and A_{31} showed the maximum tolerance to the elevated temperature.

Growth of the isolates at different pH

In order to test the effect of pH on the growth, the interested isolates were subjected to different pH. All isolates were able to grow at wide range of pH after 24 h of incubation at 55°C (Figure 3). Optimum growth of isolates A_1 and A_4 were at pH 8, but the optimum growth of isolates A_2, A_9, A_{10}, A_{22} and A_{31} were estimated at pH 9.

Growth of the isolates at different concentrations of sodium chloride

Increasing the NaCl concentration in the medium showed halotolerance. The isolates showed wide range of salinity tolerance (Figure 4). All isolates gave the maximum growth at salinity 4% (w/v) after 24 h of incubation at 55°C. Considerable growth was determined at 7% NaCl for isolate A_{10}, and at 9% NaCl for isolate A_{31}. Isolate A_{31} showed considerable growth at higher ranges. Isolate A_{31} showed the maximum tolerance to 13% NaCl.

Molecular identification of the selected isolates

DNA of the selected isolates was extracted and the 16S rDNA gene fragment was amplified for partial sequence. The produced amplicons were analyzed using agarose gel electrophoresis. The GenBank accession number for the 16S rDNA sequences were KP212417 and KP212418 for isolates A_{10} and A_{31} respectively. They were identified as *Achromobacter* sp. HEGN 014 and *Virgibacillus pantothenticus* HEGN 114 with similarity percentage 94 and 96% respectively. Figures 5 and 6 represents the phylogenetic relationships among representative

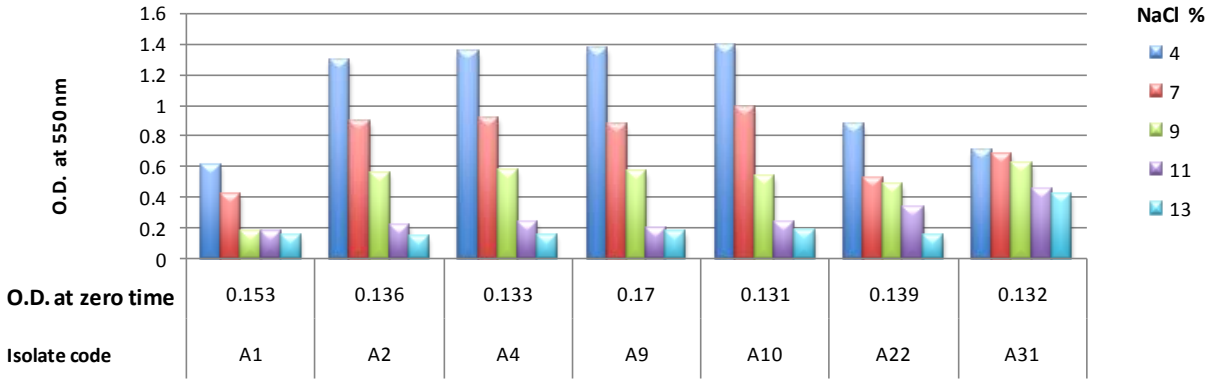

Figure 4. The growth ($O.D._{550}$) of the selected isolates at different salinities (NaCl % w/v) after 24 h incubation.

Figure 5. Phylogenetic relationships among representative experimental strain and the most closely related *Achromobacter* sp. HEGN 014.

experimental strains and the most closely related species using FAST MINIMUM EVOLUTION TREE METHOD, NCBI web server.

Characterization of the strains

The physiological and biochemical characters of *Achromobacter* sp. HEGN 014 (the isolate code A10) and *Virgibacillus pantothenticus* HEGN 114 (the isolate code A31) are represented in Table 1.

Resistance to antibiotics

The thermo-tolerant strains were characterized by resistance to most of the tested antibiotics (Table 2). It

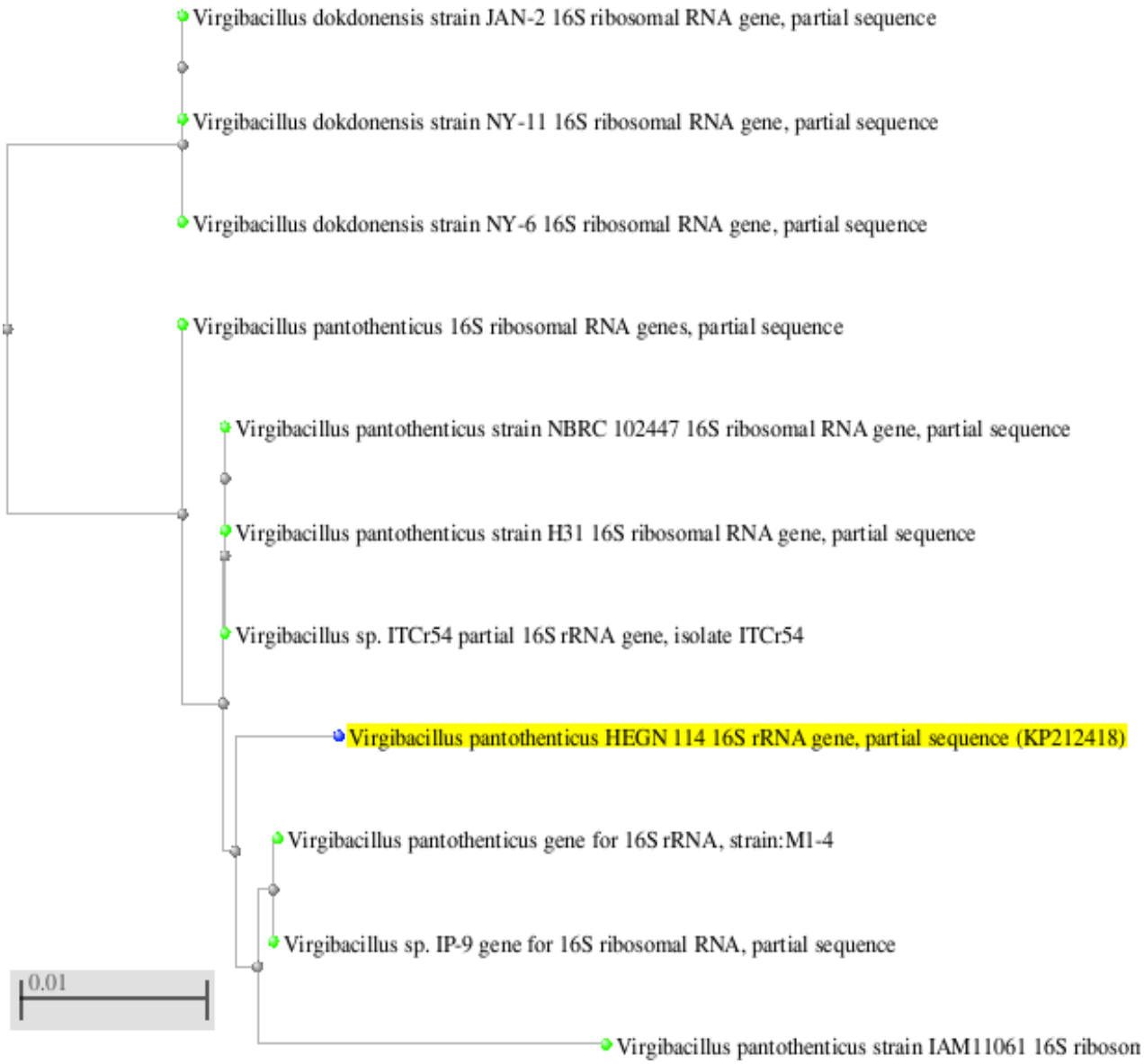

Figure 6. Phylogenetic relationships among representative experimental strain and the most closely related *V. pantothenticus* HEGN 114.

was noticed that *Achromobacter* sp. HEGN 014 resisted most tested antibiotics, gentamycin, 10 µg; ampicillin, 10 µg; imipenem,10 µg; norfloxacin,10 µg; cephalexin, 30 µg; cefadroxil, 30 µg; ceftazidine, 30 µg; erythromycin, 15 µg; and flucloxacillin, 5 µg except ampicillin-sulbactam (20 µg) and ciprocin (5 µg) where the detected inhibition zones were 20 and 30 mm, respectively. On the other side, *V. pantothenticus* HEGN 114 resisted all tested antibiotics.

Enzymes productivity

Thermo-tolerant *Achromobacter* sp. HEGN 014 and *V. pantothenticus* HEGN 114 showed good activities for lipase production, but gelatinase was produced only by *V. pantothenticus* HEGN 114. Both strains showed no activity for protease, chitinase, or cellulase productivity at 55°C (Table 3). The more width of the clearing zone around the strain growth indicates more activity. This indicate that the degradation zone which resulted from the growth of *Achromobacter* sp. HEGN 014 (++) was larger than that produced by *V. pantothenticus* HEGN 114 (+).

Effect of different temperatures on lipase activity

Lipase enzyme produced from selected strains was partially purified using ammonium sulphate precipitation

Table 1. The characteristics of the interested strains.

Characteristic	*Achromobacter* Sp*	*Virgibacillus pantothenticus***	Characteristic	*Achromobacter* Sp*	*Virgibacillus pantothenticus***
Morphological characters			Indole production	-	-
Colony color	White	Yellow	Acetoin production	+	+
Colony margin	Smooth	irregular	Gelatinase production	-	+
Colony elevation	Flat	Low convex	Oxidase production	+	+
Colony configuration	Rod shape	rod shape	Catalase production	+	+
Gram reaction	-	+	Nitrate production	+	+
Presence of spores	-	+	Utilization of		
Physiological characters			D-glucose	-	+
β-galactosidase production	-	+	D-mannitol	-	-
Arginine dihydrolase	+	-	Inositol	-	-
Lysine decarboxylase	-	-	D-sorbitol	-	-
Ornithine decarboxylase	+	-	D-rhamnose	-	-
Citrate utilization	+	+	D-sucrose	-	+
H2S production	-	-	D-melibiose	-	-
Urease production	-	+	Amygdalin	+	-
Tryptophane deaminase	-	-	L-arabinose	-	-

*Achromobacter sp. HEGN 014, **_Virgibacillus pantothenticus_ HEGN 114.

Table 2. Resistance of thermo-tolerant strains to the different antibiotics.

Tested antibiotics	*Achromobacter* sp. HEGN 014	*Virgibacillus pantothenticus* HEGN 114
Ampicillin/sulbactam, 20 µg	-*	+
Ciprocin, 5 µg	-*	+
Gentamycin, 10 µg	+	+
Ampicillin,10 µg	+	+
Imipenem, 10 µg	+	+
Norfloxacin, 10 µg	+	+
Cephalexin, 30 µg	+	+
Cefadroxil, 30 µg	+	+
Ceftazidine,30 µg	+	+
Erythromycin, 15 µg	+	+
Flucloxacillin, 5 µg	+	+

*Detection of inhibition zones, 20 and 30 mm in the presence of Ampicillin/sulbactam, 20 µg and Ciprocin, 5 µg.

Table 3. Production of different enzymes by the selected strains.

Tested enzymes	*Achromobacter* sp HEGN 014	*Virgibacillus pantothenticus* HEGN 114
Gelatinase	-	+
protease	-	-
Lipase	++	+
Chitinase	-	-
Cellulase	-	-

followed by anion exchanger chromatography step for getting the purified lipase and the purity of the enzyme was confirmed by Gel filtration using Sephadex G-100 (data not shown).

For selection of optimum temperature for the highest activity of purified lipase, the reaction temperatures

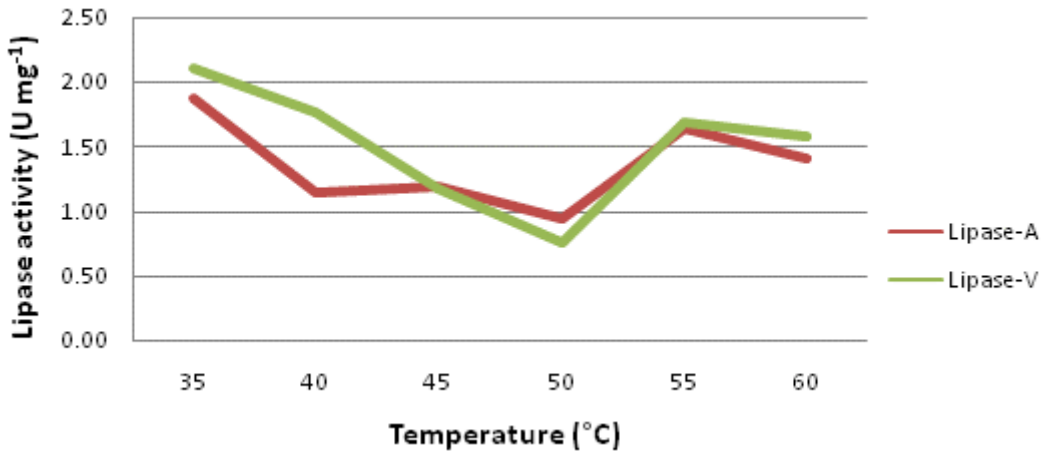

Figure 7. Effect of different temperatures on lipase activity. Lipase-A = lipase produced by *Achromobacter* sp. HEGN 014, Lipase-V = lipase produced by *V. pantothenticus* HEGN 114.

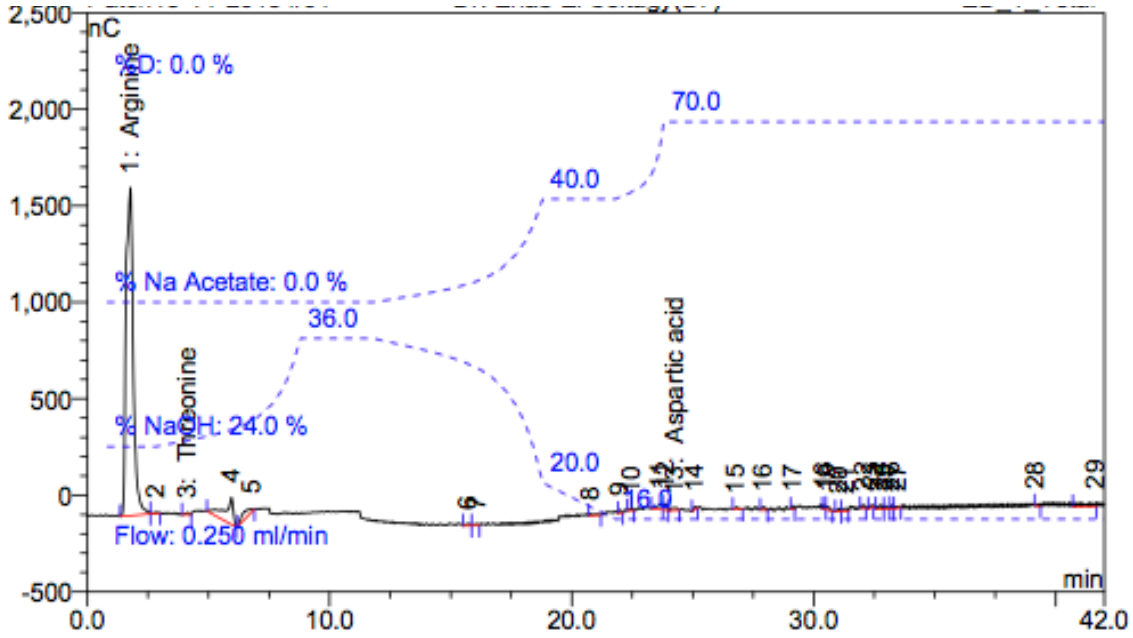

Figure 8. Amino acids analysis of purified lipase from *Achromobacter* sp. HEGN 014.

varying from 35 to 60°C were selected and examined for both purified lipase of *Achromobacter* sp. HEGN 014 and *V. pantothenticus* HEGN 114. The data in Figure 7 shows that, the purified lipases (lipase-A and lipase-V) produced from *Achromobacter* sp. HEGN 014 and *V. pantothenticus* HEGN 114 had the same pattern with slight difference in lipases activity. The optimum reaction temperature giving the highest activity for lipase-A and lipase-V was 35°C recording 1.88 and 2.11 U mg^{-1}, respectively. The enzymes activities of lipase-A and lipase-V at 50°C decreased to remain 52 (1.64 U mg^{-1}) and 36% (1.69 U mg^{-1}), respectively, of initial activity at 35°C. Yet, both lipase-A and lipase-V moved upward to increase the lipases activity to reach temperature of 55°C recording 91 and 80% of initial lipases activity at 35°C.

Amino acid analysis

The amino acid analysis (Figure 8) of the purified lipase produced from strain *Achromobacter* sp. HEGN 014 showed the presence of 3 detectable peaks of amino acids. These amino acids were detected as arginine, threonine and aspartic acid at peak no. 1, 3 and 13.

Arginine was the major one representing about 99.26% of the total amount of detected amino acids (90.651 mmole) as the amount of arginine was estimated to represent about 89.982 mmole. Unlike arginine, threonine and aspartic acid showed the lowest percentages (0.21 and 0.53%), respectively, of total detected amino acids in the purified lipase. The amount of threonine and aspartic acid were 0.198 and 0.480 mmole, respectively.

DISCUSSION

A major impetus that has driven extensive and intensive research efforts on extremophiles during the last decades is the potential biotechnological applications associated with these microbes and their products. The likely potential has been increasing exponentially with the isolation of new microbial strains, the identification of novel compounds and pathways, and the molecular and biochemical characterization of cellular components (Satyanarayana et al., 2005).

Thermo-tolerant microorganisms are the organisms belonging to the mesophilis, but which adapt to live in a higher temperature environment (Suntornsuk et al., 2005). Although thermophilic microorganisms usually produce thermostable enzymes, limited mesophilic microorganisms can also produce thermostable or thermotolerant lipases. Microbial extracellular enzymes are of considerable commercial interest for biotechnological applications as they can be produced at low cost (Khoramnia et al., 2011).

The results of this work showed that North Delta sediments in Egypt are a rich source of thermo-tolerant bacteria, which could be a good source of many interested enzymes from the industrial point of view and further studies are needed on this area including study of microbial biodiversity and the biotechnological applications of the isolated strains. It was found that the occurrence of thermo-tolerant bacteria in North Delta sediments at 55°C reached 11.1% with respect to the total viable count at 30°C.

The isolates were either alkalitolerant (showed very good to excellent growth from pH 7.0 to 9.0, but no growth at pH 11) or alkalophilic (showed very good and excellent growth between pH 7.0 and 11.0), while they show less cellular yield at pH.6.0 (Khalil, 2011). In North Delta sediments, five from the seven (71.4%) selected bacterial isolates showed optimum growth at pH 9.

According to the definition of halotolerant microorganisms, they are identified as microorganisms that have no specific requirement for salt other than the usual NaCl needed by all (non-halotolerant) organisms (Khalil, 2011). Halophiles can be classified into three groups on the basis of their response to NaCl, slightly halophiles which grow optimally at 2-5% (0.2-0.85 M), moderate 20% NaCl (0.85-3.4 M) and the extreme halophiles which optimally grow at 20-30% NaCl (3.4-5.1 M) (Jayachandra et al., 2012). In the present study, all bacterial isolates gave the maximum growth at 4% (w/v) NaCl. Two thermo-tolerant bacterial isolates from North Delta sediments (Egypt) were identified as *Achromobacter* sp. HEGN 014 and *Virgibacillus pantothenticus* HEGN 114.

The species *V. pantothenticus* HEGN 114 and related organisms comprising this new genus can be distinguished from members of *Bacillus* rRNA group 1 (*Bacillus sensustricto),* and from members of *paenibacillus* and other aerobic endospore-forming bacteria by routine phenotypic tests (Heyndrickx et al., 1998). Subsequently, four further species, *Virgibacillus proomii*, *Virgibacillus carmonensis*, *Virgibacillus necropolis* and *Virgibacillus picturae*, were described. *Virgibacillu*s are very abundant in the coastal regions of Karwar and Mangalore, due to its potentiality in producing the extracellular hydrolytic enzymes; it has gained importance in industries for their commercial usage (Rohban et al., 2009; Jayachandra et al., 2012).

Some extremophilic microorganisms are able to overcome more than one type of extreme conditions in their environment (Oren, 2008). The optimum growth of thermo-tolerant *Achromobacter* sp. HEGN 014 and *V. pantothenticus* HEGN 114 isolated from North Delta sediments were at pH 9, so it is considered as alkalophilic, and also tolerate concentrations of NaCl reached 7 and 9% (w/v), respectively.

An extremely halophilic bacterium *Virgibacillus* sp. strain JS5 was isolated from the Arabal soil of west coast of Karnataka, India. The strain was Gram positive, motile rod shaped cells and spore forming. It was strictly aerobic, ferments several carbohydrates. Catalase and oxidase test were found to be positive. Also, the strain grew in the presence of 0-25% (w/v) NaCl, with optimum growth at 10% (w/v) NaCl, pH of 6-10 and temperature range of 20-45°C, with an optimum growth temperature of 35°C, showing that the halophilic bacterium belong to the extremophilic group. It has potential to produce the extracellular enzymes such as amylase, protease, inulinase and gelatinase (Jayachandra et al., 2012, 2013).

Gray et al. (2010) mentioned that *Achromobacter* is a genus containing members that are Gram negative and oxidase positive. They are rod shaped, have flagella and grow well at 37-42°C with a pH 6.5-8.5. Also, *Achromobacter* sp*.* is widespread in aquatic habitats (Coenye et al., 2003).

The thermo-tolerant *Achromobacter* sp. HEGN 014 and *V. pantothenticus* HEGN 114 isolated from North delta sediments were antibiotics resistance. Duggan et al. (1996) reported that most *Achromobacter* species isolates have been found to be resistant to first- and second-generation cephalosporins, aminoglycosides and narrow-spectrum penicillins; susceptible to sulfonamides, carbapenems, broad-spectrum penicillins and third-generation cephalosporins; and variably susceptible to fluoroquinolones, ceftazidime, piperacillin, imipenem,

ticarcillin/clavulanic acid and varying degrees of resistance to ciprofloxacin and of loxacin.

The extracellular bacterial lipases are of considerable commercial importance, as their bulk production is much easier. Although, a number of lipase-producing bacterial sources are available, only a few are commercially exploited as wild or recombinant strains. Of these, the important ones are: *Achromobacter, Alcaligenes, Arthrobacter, Bacillus, Chromobacterium and Pseudomonas* (Gupta et al., 2004)

In fact, it is generally true that the enzymes of an organism are adapted to function optimally at or near its growth conditions, accordingly, the range of extremes at which life is found defines the range of conditions at which enzyme activity might be detected. In particular, it is believed that the outstanding stability of extremophilic enzymes will contribute to filling the gap between chemical and biological processes (Khalil, 2011).

Potential enzymes such as amylase, protease, DNA polymerase, xylanases and chitinases have been identified in thermophilic microorganisms including the lipase enzyme (Zuridah et al., 2011; Tayyab et al., 2011). The lipases used are usually of fungal or bacterial origin (Kanwar et al., 2006). Bacterial lipases are mostly extracellular and are greatly influenced by nutritional and physico-chemical factors, such as temperature, pH, nitrogen and carbon sources, inorganic salts, agitation and dissolved oxygen concentration (Gupta et al., 2004; Mobarak-Qamsari et al., 2011; Padhiar et al., 2012).

In the present study, the thermo-tolerant *Achromobacter* sp. HEGN 014 and *V. pantothenticus* HEGN 114 produced an extracellular lipase, presumably because the activity was associated with the cell (Hande-lsman and Shoham, 1994). Some thermophilic microbial strains are able to produce thermostable lipases (Stathopoulou et al., 2013).

The optimum reaction temperatures giving highest activity of the purified lipases from *Achromobacter* sp. HEGN 014 and *V. pantothenticus* HEGN 114 were obtained at 35°C followed by a few lost in activity at 55°C, and the enzyme showed a relatively low activity in a temperature ranging from 40 to 50°C. The most likely explanation for this result is that the lipases could exist in isozyme form as multiform of lipase with the same function in terms of lipase activity.

Chahinian et al. (2000) found that lipase enzyme produced from *Pennicllium cyclopoium* exists in several glycosylated forms (40-43 KDa). This result shows that one form of this isozyme is a thermo-tolerant lipase which tolerate the reaction temperature of 55°C and the other one is non-thermo-tolerant lipase where the optimum temperature was 35°C. This temperature range agreed with other lipases purified from other microorganisms. Lee et al. (1999) found that the optimum temperature for thermophilic *Bacillus thermoleovorans* ID-1 was between 70-75°C. Also, Kaminishi et al. (1999) found that the optimum temperature for *Eurotrium hebariorum* NU-2 was 37°C. In addition, the optimum temperature for *Pichiaburtonii* was found to be 45°C (Sugihara et al., 1995) and for *Pseudomonas sp*. was between 45-60°C (Dong et al., 1999).

Amino acids analysis of lipase from *Achromobacter* sp. HEGN 014 proved to be rich in arginine. This result indicates that arginine plays a vital role in the mode of action of lipases with the substrate especially at higher temperature. The most likely explanation for the other undetected amino acids is that the concentrations of other amino acids were very low due to low concentration of the sample as compared to the higher concentrations of arginine, threonine and aspartic acid in *Achromobacter* sp. HEGN 014. A few research papers have discussed the role of arginine in simulating the release of lipase (Morita et al., 2008).

Conflict of interests

The authors did not declare any conflict of interest.

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academicJournals

Vol. 9(14), pp. 1012-1119, 8 April, 2015
DOI: 10.5897/AJMR2014.7282
Article Number: 0D3223152303
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Potential bacterial pathogens of external ocular infections and their antibiotic susceptibility pattern at Hawassa University Teaching and Referral Hospital, Southern Ethiopia

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Received 17 November, 2014; Accepted 25 March, 2015

Bacterial external ocular infection is a common health problem along with increase and spread of drug resistance in Ethiopia. The objective of this study was to identify potential bacterial isolate of external ocular infections and their antimicrobial susceptibility patterns in patients attending eye clinic of the Hawassa University Teaching and Referral Hospital, from December 2012 to April 2013. A total of 281 consecutive, non-repetitive ocular specimens were collected among conjunctivitis cases (n=140), blepharitis cases (n=55), keratitis cases (n=31), dacryocystitis cases (n=19), and other cases (n=36). All samples were processed for culture and identification by standard methods. Susceptibility testing was done by Kirby-Bauer method as per Clinical and Laboratory Standards Institute (CLSI) guideline. Out of 281 ocular specimens submitted to culture, 137 (48.8%) specimen were positive. The most common bacterial isolates were Gram positive cocci (n=88; 61.5%). The predominant bacterial species isolated was *Staphylococcus aureus* (n=30; 21.0%) followed by coagulase negative Staphylococci (CoNS) (n=26; 18.2%) and *Streptococcus pneumoniae* (n=20; 14.0%). *In vitro* ciprofloxacin was effective against 86% of isolated pathogen. Multi-drug resistance was observed in 69.9% of the bacterial isolates. Our study confirmed that *S. aureus* was the overall predominant isolated pathogen followed by CoNS, *S. pneumoniae and Klebsiella* spp. Gram positive isolates were more susceptible to amoxicillin-clavulanic acid and vancomycin, whereas Gram negative isolates were more susceptible to ciprofloxacin and gentamicin. Relatively, ciprofloxacin is effective against most isolated pathogen.

Key words: External ocular infections, conjunctivitis, blepharitis, keratitis, dacryocystitis, susceptibility.

INTRODUCTION

Ocular infections can cause damage to structures of the eye, which can lead to reduced vision and even blindness if left untreated. The cause of ocular infections can be bacteria, fungi, viruses and parasites (Joseph, 2009).

Bacteria are the most common pathogens and involved in infections of all the tissues of the eye. The most frequently affected part of the eye is conjunctiva, lid and cornea (Ubani, 2009) which are external part of the eye. External bacterial infections of the eye are usually localized but may frequently spread to adjacent tissue due to some predisposing factors such as during trauma, previous surgery, ocular surface disease, contact lens wear, ocular adnexal dysfunction (including tear deficiencies) and other exogenous factors, systemic diseases (Bharathi et al., 2003) and immunosuppression may alter the defense mechanisms of the outer eye and permit bacteria to spread (AOA, 1995; Seal and Uwe, 2007).

Bacterial agents are known to cause external ocular infections such as conjunctivitis, keratitis, blepharitis, hordeolum, dacryocystitis, etc. which are responsible for increased incidence of morbidity and blindness worldwide (Modarrres et al., 1998; Sharma, 2011). Clinical presentations are not diagnostic of the cause, so microbiological isolation and identification of bacterial pathogens along with antibiotic susceptibility pattern is essential (Finegold et al., 1990)

Even though, the clinical importance of external eye infections has been reported in some studies in Ethiopia (Alene and Abebe, 2000; Tiliksew, 2002), by clinical observation only, there are no much microbiologic studies with culture and drug sensitivity test which showed the magnitude of the problem. Due to lack of access to microbiology laboratory, high cost and long time for diagnosis, most clinicians advocate the use of broad-spectrum, empirical therapy and reserve culture for hyper acute conditions or those that fail to respond to initial therapy which in turn leads to emergence of antibiotic resistant bacteria and increased cost for proper management of infection.

In Ethiopia, it is a common practice that antibiotics can be purchased without prescription, which leads to misuse of antibiotics. This may contribute to the emergence and spread of antimicrobial resistance (Anagaw et al., 2011; Teweldeet al., 2013). Moreover, poor hygienic and infection control practice in the area may play a major role in an increased prevalence of resistant bacteria in a community. Thus, periodic monitoring of etiology and re-evaluation of antimicrobial agents is important to make a rational choice of initial antimicrobial therapy.

As far as our knowledge is concerned, no study has been conducted on identification of potential bacterial isolate and its distribution in the case of external ocular infection in different clinical features and their antibiotic susceptibility pattern in study area. Therefore, the aim of this study was to identify potential bacterial isolate and its distribution in the case of external ocular infection in different clinical features and their antibiotic susceptibility pattern of bacterial isolate at Hawassa University Teaching and Referral Hospital.

MATERIALS AND METHODS

A cross-sectional study was conducted to identify potential bacterial isolate and their drug susceptibility pattern among 281 patients who were diagnosed as having external ocular infections at Hawassa University Teaching and Referral Hospital from December, 2012 to April, 2013. The hospital is a tertiary level teaching hospital that provides health service to over six million inhabitants in southern Ethiopia and is located 273 km south of Addis Ababa.

All patients examined and diagnosed on the slit-lamp bio-microscope by ophthalmologist as external ocular infections and willing to give written consent were included in this study. Patients on antibiotics for the past 1week were excluded. Demographic data was collected from patients using structured and predesigned questionnaire. Sample from external parts of the eye (eyelid, conjunctiva, lacrimal sac and cornea) was collected using either swabbing or scraping as per the routine clinical management of the patients (Tabbara and Robert, 1995). Specimens were immediately delivered to the bacteriology section for culture and other bacteriological analysis.

Specimens were cultured by the streak plate methods using wire loop into chocolate agar, MacConkey agar and two blood agar plates (Oxoid Basingstoke, UK). MacConkey agar and one blood agar plates were incubated at 37°C aerobically and the other blood agar and chocolate agar plates were incubated at 37°C with in a candle jar to enhance the growth of bacterial pathogens that needs 5-10% CO_2.

After overnight incubation, plates were examined for the growth of bacteria. Specimens taken from the eyelid, conjunctiva or lacrimal sac were considered as culture positive according to microbiological procedure for diagnosis of ocular infection (Therese and Madhavan, 2004). In the case of microbial keratitis, a culture was considered positive when there was growth of the same organism on two or more media or confluent growth of a known ocular pathogen at the site of inoculation on one solid medium (Pinna et al., 1999). Plates which did not show any growth were further incubated for additional 24 h. All positive cultures were identified by their characteristic appearance on their respective media and Gram stain reaction. Furthermore, it was confirmed by the pattern of biochemical reactions using the standard method (Cheesbrough, 2006).

Antimicrobial susceptibility testing was performed for bacterial isolates using disc diffusion method on Mueller-Hinton agar (Oxoid Basingstoke, UK) according to the direction of the Clinical and Laboratory Standards Institute (CLSI) (Bauer et al., 1966; CLSI, 2007*)*. The antimicrobials for disc diffusion testing were obtained from Oxoid Basingstoke, UK in the following concentrations: trimethoprim-sulphamethoxazole (SXT, 25 µg), gentamicin (CN, 10 µg), penicillin (P, 10 IU), vancomycin (VA, 30 µg), ceftriaxone (CRO, 30 µg), erythromycin (E, 15 µg), ciprofloxacin (5 µg), tetracycline (30 µg), chloramphenicol (C, 30 µg), amoxicillin-clavulanicacid (AMC, 30 µg), ampicillin (AMP, 10 µg) and oxacillin (OX, 1 µg). *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 25923) and *Pseudomonas aeruginosa* (ATCC 27853) were used as reference strains for culture and sensitivity testing. Data entry and analysis was performed using SPSS version-16. Descriptive summaries were presented and Chi-square test (x^2)

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Table 1. External ocular infections and different demographic characteristics among patients attending Hawassa University Teaching and Referral Hospital, 2013.

Characteristics	Total tested (%)	Number positive (%)	Chi-square value	P-value
Sex				
Male	167(59.4)	77(46.1)	1.154	0.283
Female	114(40.6)	60(52.6)		
Age in years				
≤ 5	40(14.2)	20(50.0)	5.392	0.249
6-15	42(14.9)	17(40.5)		
16-34	86(30.6)	42(48.8)		
35-55	57(20.3)	24(42.1)		
>55	56(19.9)	34(60.7)		
Residence				
Urban	101(35.9)	48(47.5)	0.095	0.757
Rural	180(64.1)	89(49.4)		
Occupation				
Farmer	84(29.9)	41(49.4)	6.401	0.380
House wife	44(15.7)	25(55.6)		
Student	49(17.4)	23(46.9)		
Employee	31(11.0)	10(32.3)		
Merchant	16(5.7)	7(43.8)		
Pre-school	42(14.9)	21(50.0)		
Others*	15(5.3)	10(66.7)		
Education				
Pre-school	43(15.3)	22(51.2)	1.242	0.537
Illiterate	124(44.1)	64(51.6)		
Literate	114(40.6)	51(44.7)		

Others*- daily laborer, driver and retired

was used to assess difference between proportions. P-value less than 0.05 were considered as statistically significant.

The study was approved by the Research and Ethical Review Committee of College of Medicine and Health Sciences, Hawassa University. Written consent was sought from all adult patients, while for the children and infants; written consent was obtained from their parents/guardians. All the data obtained were kept confidential by using only code numbers and locking the data. Participation of the study subjects was purely voluntary. Culture positive patients were treated accordingly.

RESULTS

Socio-demography of study population

A total of 281 patients clinically diagnosed as external ocular infection gave specimens for microbiological evaluation during the study period. Of these, 167 (59.4%) were males and 114 (40.6%) were females. The median age of the study subjects was 30 years (range, 1 day to 100 years). Most of the study participants were rural, 180 (64.1%) in residence, farmer 83 (29.5%) in occupation and illiterate 124 (44.1%) in educational status (Table 1).

Bacterial isolate and clinical feature

Of the total processed external ocular specimens, 137 (48.8%) were found culture positive. The majority, 131 (95.6%) of the infected participants had single infection; while 6 (4.4%) were mixed infection, which makes the total number of bacterial isolates 143 (50.9%). *S. aureus* were the most predominant bacteria in mixed growth. The rate of isolation was higher among the age group > 55 years (60.7%) followed by age group ≤5 years (50.0%), among female (52.6%), those who were rural residence (49.4%) and among house wife (55.6%). Bacterial isolation in both sexes (P-value = 0.283) and various age

Table 2. Frequency of bacterial isolate from different clinical futures of ocular infections among patients attending Hawassa University Teaching and Referral Hospital, 2013.

Clinical features	Total cases N (%)	Culture positive cases N (%)	Single bacterial isolates N (%)	Mixed bacterial isolates (%)	Total bacterial isolates N (%)
Blepharitis	55(19.6)	27(49.1)	26(96.3)	1(3.7) ***	28 (19.6)
Conjunctivitis	140(49.8)	63(45.0)	59(93.7)	4(6.3) ****	67 (46.9)
Blepharo-conjunctivitis	7(2.5)	2(28.6)	2(100)	0	2(1.4)
Keratitis	31(11.0)	14(45.2)	13(92.9)	1(7.1) *****	15(10.5)
Ex.Hordeolum*	11(3.9)	3(27.3)	3(100)	0	3(2.1)
Dacryocystitis	19(6.8)	16(84.2)	16(100)	0	16(11.2)
Lid abscess	6(2.1)	4(66.7)	4(100)	0	4(2.8)
Others **	12(4.3)	8(66.7)	8(100)	0	8(5.6)
Total	281	137(48.8)	131(95.6)	6(4.4)	143(100)

*Ex.hordeolum= external hordeolum, **orbital and preseptal cellulitis, post traumatic suppurativescleritis and lid laceration, ***1 case (*S.aureus + S. marcesens*), ****4 cases (S*. aureus + H.influenzae; S. pneumoniae + P. mirabilis, S. pyogenes; S. Viridians + E. coli*) ***** 1 case (*S. Aureus + E. coli*).

Table 3. Distribution of bacterial isolates from different clinical features of external ocular infections at Hawassa University Teaching and Referral Hospital, 2013.

Bacterial isolate No. (%)	Blepharitis (55)	Conjunctivitis (140)	B. Conjunctivitis (=7)	Keratitis (=31)	Ex.hordeolum (=11)	Dacryocystitis (=19)	Lidabsces (=6)	Other (=12)	Total (=281)
S. aureus	9(32)	11(16.4)	0	3(20)	2(66.7)	2(12.5)	0	3(37.5)	30(21)
CoNS*	10(35.6)	9(13.4)	1(50)	1(6.7)	1(33.3)	3(18.8)	0	1(12.5)	26(18.2)
S. pneumoniae	1(3.6)	8(11.9)	0	4(26.6)	0	5(31.3)	1(25)	1(12.5)	20(14)
S .pyogenes	1(3.6)	4(6)	0	1(6.7)	0	0	0	0	6(4.2)
S. viridians	1(3.6)	3(4.5)	0	1(6.7)	0	0	0	1(12.5)	6(4.2)
Moraxella spp.	0	1(1.5)	0	1(6.7)	0	2(12.5)	0	0	4(2.8)
H. influenzae	0	6(8.9)	0	0	0	0	0	0	6(4.2)
Pseudomonas spp.	1(3.4)	4(6)	0	2(13.2)	0	0	0	0	7(4.9)
E. coli	1(3.6)	3(4.5)	1(50)	1(25)	0	0	0	0	7(4.9)
Klebsiella spp.	0	5(7.5)	0	1(6.7)	0	2(12.5)	0	0	9(6.3)
Citrobacter spp.	0	2(3)	0	0	0	1(6.2)	0	0	3(2.1)
Entrobacter spp.	1(3.6)	3(4.5)	0	0	0	0	0	1(12.5)	5(3.4)
S.marcescens	1(3.6)	1(1.5)	0	0	0	0	2(50)	0	4(2.80
P.mirabilis	1(3.6)	3(4.5)	0		0	1(6.2)	0	0	5(3.4)
Other NLF**	1(3.6)	4(6)	0	0	0	0	0	0	5(3.4)
Total (%)	28(19.6)	67(46.8)	2(1.4)	15(10.5)	3(2.1)	16(11.2)	4(2.8)	8(5.6)	143(100)

*Ex.hordeol um = external hordeolum, *CoNS: Coagulase negative *Staphylococci,* **other NLF: non lactose fermenter Gram negative rods (*Salmonella, Shigella* and *Providencia* spp.)

groups (P-value = 0.249) were not statistically significant.

Out of 281 cases of eye infections studied, conjunctivitis accounted for 140 (49.8%) followed by blepharitis 55 (19.6%), keratitis 31 (11.0%), dacryocystitis 19 (6. 8%), external hordeolum 11 (3.8%), blepharo-conjunctivitis 7 (2.5%), lid abscess 6 (2.1%) and others like orbital and preseptal cellulitis, post traumatic suppurative scleritis and lid laceration accounted for 12 (4.3%). The rate of culture-positivity was found to be significantly higher among study subjects with dacryocystitis 84.2% (AOR = 7.876 (95% CI: 1.80-34.29) (p=0.006), than lid abscess 66.7% (4 of 6), blepharitis 49.1% (27 of 55), keratitis 45.2% (14 of 31), conjunctivitis 45% (63 of 140), blepharoconjunctivitis 28.6% (2 of 7), external hordeolum 27.3% (3 of 11) and other infections 66.7% (8 of 12) (Table 2).

In this study, the predominant isolate observed in blepharitis cases was CoNS (35.6%) followed by *S. aureus* (32%); in conjunctivitis was *S. aureus* (16.4%); in keratitis and dacryocystitis were *S. pneumoniae* (26.6 and 31.3%), respectively. All the isolated *Haemophilus influenzae* were recovered from conjunctivitis cases (Table 3).

The overall predominant isolated pathogen was *S. aureus* (30; 21%) followed by CoNS (26; 18.2%), *S. pneumoniae* (20; 14.0%), *Klebsiella* spp. (9; 6.3%),

Table 4. Antibiotics sensitivity pattern of bacterial isolates at Hawassa University Teaching and Referral Hospital, 2013.

Bacterial isolates	No. of strains sensitive to Antibiotics (%)												
	No.	AMP	AMC	CRO	C	CIP	CN	TE	SXT	OX	E	P	VA
Gram positive													
S. aureus	30	9(30)	27(90.0)	28(93.3)	28(93.3)	28(93.3)	28(93.3)	12(40.0)	23(76.7)	28(93.3)	25(83.3)	5(16.7)	29(96.7)
CoNS*	26	18(69.2)	25(96.2)	22(73.3)	19(73.1)	21(81.8)	20(76.9)	9(34.6)	16(61.5)	21(80.8)	23(88.5)	2(7.7)	24(92.3)
S. pneumoniae	20	16(80.0)	20(100)	20(100)	18(90.0)	18(90.0)	6(30.0)	14(70.0)	11(55.0)	15(75.0)	19(95.0)	13(65.0)	20(100)
S. pyogenes	6	6(100)	6(100)	6(100)	5(83.3)	5(83.3)	3(50.0)	4(66.7)	2(33.3)	6(100)	5(83.3)	6(100)	6(100)
S. viridians	6	6(100)	6(100)	6(100)	5(83.3)	5(83.3)	4(66.7)	5(83.3)	3(50.0)	4(66.7)	6(100)	4(66.7)	6(100)
Total Gram positive	88	55(62.5)	84(95.5)	82(93.1)	75(85.2)	75(85.2)	59(67)	43(48.9)	55(62.5)	73(83.0)	75(85.2)	28(31.8)	85(96.6)
Moraxella spp.	4	4(100)	4(100)	4(100)	4(100)	4(100)	3(75.0)	2(50.00	3(75.0)	ND	ND	ND	ND
H. influenzae	6	2(33.3)	6(100)	6(100)	6(100)	5(83.3)	5(83.3)	3(50.0)	2(33.3)	ND	ND	ND	ND
Pseudomonas spp.	7	0	1(14.3)	1(14.3)	5(71.4)	7(100)	7(100)	5(71.4)	7(100)	ND	ND	ND	ND
E. coli	7	4(57.1)	7(100)	5(71.4)	6(85.7)	5(71.4)	5(71.4)	6(85.7)	6(85.7)	ND	ND	ND	ND
Klebsiella spp.	9	1(11.1)	7(77.8)	8(88.9)	8(88.9)	8(88.9)	8(88.9)	8(88.9)	8(88.9)	ND	ND	ND	ND
Citrobacter spp.	3	1(33.3)	3(100)	2(66.7)	1(33.3)	3(100)	1(33.3)	0(0.0)	2(66.7)	ND	ND	ND	ND
S. marcescens	4	1(25.0)	1(25.0)	1(25.0)	3(75.0)	4(100)	4(100)	3(75.0)	4(100)	ND	ND	ND	ND
P. mirabilis	5	0	3(60.0)	3(60.0)	3(60.0)	5(100)	5(100)	3(60.0)	3(60.0)	ND	ND	ND	ND
Other NLF	5	0	1(20.0)	3(60.0)	3(60.0)	5(100)	5(100)	3(60.0)	5(100)	ND	ND	ND	ND
Total Gram negative	55	16(29.1)	37(67.3)	38(69.1)	38(69.1)	49(89.1)	48(87.3)	36(65.5)	44(80.0)				
Overall total	143	68(47.5)	117(81.8)	115(80.4)	114(79.7)	123(86)	101(70.6)	77(53.8)	95(66.4)	74(84.1)	78(88.6)	30(34.1)	85(96.5)

*CoNS: Coagulase negative *Staphylococci*, Amp- Ampicillin, AMC-Amoxicillin-clavulanic acid, CRO- Ceftriaxone, C- Chloramaphenicol , CIP- Ciprofloxacin, CN- Gentamycin, TE- Tetracycline, SXT- Co-trimoxazole , OX, oxacillin, E- Erythromycin, P- penicillin, VA- Vancomycin, ND-not done.

Pseudomonas spp. and *E. coli* (7; 4.9% each), *Streptococcus pyogenes, Streptococcus viridians and H. influenzae* (6; 4.2% each), *Entrobacter* spp., *P. mirabilis*, *Moraxella* spp. and *S. marcescens* (4; 2. 8% each), *Citrobacter* spp. (3; 2.1%) and non-lactose fermenting (NLF) Gram negative rods (5; 3.4%) (Table 3).

Antibiotic resistance profile of bacterial isolate

Antimicrobial susceptibility of isolated bacteria is as follows: Vancomycin (96.5%), Erythromycin (88.6%), Ciprofloxacin (86%), Oxacillin (84.1%), Amoxicillin-clavulanic acid (81.8%), Ceftriaxone (80.4%), Chloramaphenicol (79.7%), Gentamycin (70.6%), Trimethoprim-sulphamethoxazole (66.4%), Tetracycline (53.8%), Ampicillin (47.5%), Penicillin (34.1%) (Table 4).

Multidrug resistance

In this study, the overall multi-drug resistance (resistance two or more) antimicrobials were 100 (69.9%) and only 14 (9.8%) were sensitive to all antimicrobials tested (Table 5).

DISCUSSION

An external ocular infection is a major public health problem in Ethiopia. In this study, the overall prevalence of bacterial external ocular infections was 48.8%, which is similar to previous result in central Ethiopia (Addis Ababa) (47.4%) (Nigatu, 2004) and northwest Ethiopia (Gondar) (54.2%) (Anagaw et al., 2011). However, it is lower than result from other study in Gondar (60.8%) (Dagnachew et al., 2014), southwest Ethiopia (Jimma) (74.7%) (Tewelde et al., 2013) and other countries such as: India (58.8%)

Table 5. Multiple antibiotic resistance pattern of bacterial isolate from external ocular infection, Hawassa University Teaching and Referral Hospital, 2013.

Organism	Antibiogram pattern							
	No. (%)	R0	R1	R2	R3	R4	R5	R6
S. aureus	30(21.0)	2(6.7)	3(10.0)	6(20.0)	13(43.3)	2(6.7)	1(3.3)	3(10.0)
CoNS*	26(18.2)	0	7(27.0)	1(3.8)	7(27.0)	3(11.5)	3(11.5)	5(19.2)
S. pneumoniae	20(14.0)	2(10.0)	6(30.0)	5(25.0)	2(10.0)	1(5.0)	2(10.0)	2(10.0)
S. pyogenes	6(4.2)	0	1(16.7)	3(50.0)	1(16.7)	1(16.6)	0	0
S. viridians	6(4.2)	1(16.7)	1(16.7)	2(33.2)	1(16.7)	1(16.7)	0	0
Moraxella spp	4(2.8)	2(50.0)	1(25.0)	0	1(25.0)	0	0	0
H. influenzae	6(4.2)	1(16.7)	1(16.7)	1(16.7)	2(33.2)	1(16.7)	0	0
Pseudomonas spp.	7(4.9)	0	1(14.2)	0	2(28.7)	4(57.1)	0	0
E. coli	7(4.9)	2(28.6)	2(28.6)	2(28.6)	0	0	0	1(14.2)
Klebsiella spp.	9(6.3)	0	5(55.6)	1(11.1)	3(33.3)	0	0	0
Citrobacter spp.	3(2.1)	1(33.3)	0	0	0	2(66.7)	0	0
Entrobacter spp.	5(3.4)	3(60.0)	0	0	1(20.0)	1(20.0)	0	0
S. marcesens	4(2.8)	0	1(25.0)	0	1(25.0)	2(50.0)	0	0
P. mirabilis	5(3.5)	0	0	1(20.0)	2((40.0)	2(40.0)	0	0
NLF Gram negative rods*[2]	5(3.5)	0	0	2(40.0)	1(20.)	2(40.)	0	0
Total	143(50.9)	14(9.8)	29(20.3)	24(16.8)	37(25.9)	22(15.4)	6(4.1)	11(7.7)

R_0 - sensitive to all antibiotics; R_1 - resistant to 1 antibiotic; R_2 - resistant to 2 antibiotics; R_3- resistant to 3 antibiotics; R_4- resistant to 4 antibiotics, R_5 - resistant to 5 antibiotics R_6 - resistant to 6 and more antibiotics, *CoNS: coagulase negative Staphylococci.

(Bharathi et al., 2010). The varying rate of isolation from one place to another might be due to varying distribution of bacterial aetiology with geographic variation, study period, variation with the study population and infection prevention practice in diverse settings.

In this study, Gram positive cocci are still the most common isolates (61.5%). Several other studies in Ethiopia (Nigatu, 2004; Anagaw et al., 2011; Tewelde et al., 2013 and Dagnachew et al., 2014); in India (Sherwal and Verma, 2008; Bharathi et al., 2010; and Ramesh et al., 2010), in Nigeria (Ubani, 2009); in USA (Adebukola et al., 2011) and other parts of world have shown similar results inferring Gram positive cocci as a primary cause of bacterial ocular infection. The predominant bacterial isolates were *S. aureus* (21.0%) followed by CoNS (18.2%) and *S. pneumoniae* (14.0%). This finding is in agreement with previous works elsewhere (Modarrres et al., 1998; Nigatu, 2004; Ubani, 2009; Bharathi et al., 2010 and Anagaw et al., 2011). However, in other studies by Dagnachew et al. (2014) and Summaiya et al. (2012), the predominant isolates were CoNS. The increased prevalence of Gram positive cocci may be due to contamination of the eye from skin normal flora as a result of touching eyes with hands, cataract extraction, lens implantation, and use of contact lens.

The rate of isolation was higher among the age group >55 years (60.7%) followed by age group ≤5 years (50.0%). The prevalence of ocular infection was not significantly associated with age. However, statistically significant association was observed in the age group ≤ 2 years in study conducted in Gondar (Dagnachew et al., 2014) and Iran (Modarrres et al., 1998). The reason for increased susceptibility to infection in babies may be that they are at a greater risk after their maternal immunity has disappeared and before their own immunity system had matured (Ubani, 2009), while in old age it may be due to dry eye and weaning immunity. Moreover, similar to previous study conducted in Ethiopia (Anagaw et al., 2011) and Iran (Modarrres et al., 1998*)* the prevalence of ocular infection has no significant association with sex.

Among the clinical features, significant association of culture-positivity was observed among study subjects with dacryocystitis which is in agreement with the study done in India (Bharathi et al., 2010). *S. pneumoniae* was found to be the predominant isolate in the cases of microbial dacryocystitis (31.3%) and keratitis (26.6%). This is in agreement with study conducted in Addis Ababa (Nigatu, 2004) and India (Bharathi et al., 2003, 2007; Sherwal and Verma, 2008; Prakash et al., 2012). However, studies conducted in Jimma (Tewelde et al., 2013), China (Zhang et al., 2008) and Malaysia (Hooi, 2005) showed *P. aeruginosa,* was found to be the predominant isolate in cases of microbial keratitis. This may be due to difference in study population, study period, health of cornea and geographic location. While, the predominant isolate observed in blepharitis cases were CoNS (35.6%) followed by *S. aureus (*32%). This is in agreement with the study conducted in Iran (Modarrres et al., 1998), i India (Sherwal and Verma, 2008), Nigeria (Ubani, 2009) and Jimma (Tewelde et al., 2013). The reason for high rate of CoNS and *S. aureus* among blepharitis cases may be virulence factor such as exo-

enzymes and a surface slime that may play a role in the pathogenesis (Abdalla et al., 2014).

Rapid use of antibiotics for severe ocular infections is routine in ophthalmic practice resulting in increased drug resistance. In our study, among the commonly used topical antibiotics 20.3% of all strains were chloramphenicol resistant; 29.4% of all strains and 70% of *S. pneumoniae* were resistant to gentamicin. Moreover, 46.8% of all strains were tetracycline resistant. While, ciprofloxacin were susceptible in 80-100% of all strains except for *E. coli* (71.4%). This is in agreement with the study conducted in Gondar (Anagaw et al., 2011) and Jimma (Tewelde et al., 2013). The reason for increased resistance for chloramphenicol, gentamicin and tetracycline may be earlier exposure of the isolates to these drugs (allocated as first line drug). Moreover, these drugs are very common due to low cost and often purchased without prescription in different areas while, ciprofloxacin were reserved for refractory cases (DACA, 2010).

In this study, most of bacterial isolates have shown high resistance to penicillin (65.9%), ampicilin (52.5%) and trimethoprim-sulphamethoxazole (33.6%). Similar findings have been reported in Gondar (Anagaw et al., 2011), Jimma (Tewelde et al., 2013) and Iran (Modarrres et al., 1998). However, ceftriaxone (80.4%), amoxicillin-clavulanic acid (81.8%), oxacillin (84.1%), erythromycin (88.8%) and vancomycin (95.6%) showed susceptibility. This is in agreement with study conducted in Gondar (Ferede et al., 2012) and Gujarat (Summaiya et al., 2012).

Prevalence of multidrug resistance (MDR) to two or more of bacterial isolates to the commonly prescribed antimicrobials was observed in 69.9% of the isolates. This is in agreement with the previous studies (Anagaw et al., 2011; Ferede et al., 2012). However, high prevalence of multidrug resistance was previously reported in Gondar (Dagnachew et al., 2014). This may be due to the difference in type and generation of antibiotic that we use for susceptibility testing. In this study, the limitations were due to lack of facility anaerobic bacteria and *Chlamydia trachomatis* were not isolated.

Conclusion

S. aureus was the overall predominant isolated pathogen followed by CoNS*, S. pneumoniae and Klebsiella* spp. High rate of culture-positivity was observed among study subjects with dacryocystitis. Gram positive isolates were more susceptible to amoxicillin-clavulanic acid and vancomycin, whereas Gram negative isolates were more susceptible to ciprofloxacin and gentamicin. Relatively ciprofloxacin is effective against most isolated pathogen.

Conflict of interest

The authors declare that no potential competing interest exists.

ACKNOWLEDGEMENTS

The authors would like to thank to study participants and the staff of Microbiology Laboratory and Ophthalmology Department of Hawassa University Hospital, for their technical assistance during laboratory work and sample collection. They also extend their gratitude to Addis Ababa University and Hawassa University for funding this research.

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academicJournals

Vol. 9(14), pp. 1020-1025, 8 April, 2015
DOI: 10.5897/AJMR2015.7390
Article Number: 9EAEA0252309
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

In vitro antibacterial activity of two plant extracts against *Enterococcus faecalis*

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Received 19 January, 2015; Accepted 13 March, 2015

The antimicrobial activity of two crude plant extracts– *Solanum paniculatum* L. (jurubeba) and *Bixa orellana* L. (annatto) against *Enterococcus faecalis* was tested *in vitro*, and their efficacy was compared with that of a chemical agent with known antibacterial activity – chlorhexidine digluconate at 0.12% concentration. Extracts were prepared from jurubeba roots and annatto seeds after selection by phytochemical screening. Microorganisms were divided into two groups: *E. faecalis* (ATCC 292012) and *E. faecalis* (44 - AB). The minimum inhibitory concentration (MIC) and the minimum inhibitory concentration of adherence (MICA) of the extract and the control were determined, and the cytotoxic potential and lethal dose (LD) of jurubeba extract were assessed because it was the only extract to exhibit inhibitory activity against bacteria. Only the *S. paniculatum* Linn (jurubeba) extract exhibited activity against the two bacterial strains– MIC: up to 1:64 against *E. faecalis* ATCC 292012, up to 1:32 against *E. faecalis* of the oral environment (44-AB); MICA: up to 1:512 against the two strains, exhibiting cytotoxicity at the 1:2 dilution (250 mg/mL) and LD = 0. The *B. orellana* Linn extract exhibited no inhibitory activity in any of the experiments; thus, it was not assayed in the cytotoxicity experiments, nor was its LD determined. The low toxicity of *S. paniculatum* L. extract (cytotoxicity only at the 1:2 dilution, or 250 mg/mL) in addition to its LD = 0 and good antibacterial performance in the tests suggest the potential use of this product for the treatment of endodontic and periodontal oral infections; however, further experiments strengthening these results should be performed. The *B. orellana* L. extract exhibited no antimicrobial activity.

Key words: Antimicrobial activity, plant extracts, phytotherapeutics, *Enterococcus faecalis,* endodontic infections, *Bixa orellana* L., *Solanum paniculatum* L.

INTRODUCTION

Brazilian studies on phytotherapeutics, especially with regard to the application of these compounds in clinical dentistry, are currently limited in number and results (Barbosa et al., 2012). Some of these studies have aimed to determine the antimicrobial activity of ethanolic and hydroethanolic plant extracts on oral microorganisms,

including *Enterococcus faecalis* strains, which are considered invasive bacteria of root canals and are frequently associated with persistent infections and lesions linked to unsuccessful endodontic treatments (Peciuliene et al., 2008; Rôças and Siqueira, 2008; Skucaite et al., 2010; Santos et al., 2011; Rôças and Siqueira, 2012).

According to Fisher and Phillips (2009), *Enterococcus* species were initially not considered important in clinical pathology until their association with nosocomial infections was demonstrated, which turned them into a major bacterial pathogen. As stated by those authors, these bacteria are resistant to a wide range of temperatures (from 5 to 50°C) and show great ability to survive in adverse environments. In root canal microbiota, they are present at low amounts in untreated infected canals (Peciuliene et al., 2008). However, the possibility of root canal system (RCS) invasion by *E. faecalis* is high considering their ability to survive in harsh environments as compared to other bacteria, making their presence common in persistent infections (Peciuliene et al., 2008; Skucaite et al., 2010; Rôças and Siqueira, 2012).

To treat these infections, irrigation solutions are used in combination with mechanical cleaning methods, and intracanal medication is used in the intervals between treatment sessions (Siqueira, 2011). Thus, phytotherapy may represent an interesting alternative as an antimicrobial treatment against this microorganism (Pereira et al., 2010).

Bixa orellana L., a shrub of the family Bixaceae, also known as annatto, occurs from northern to southern Brazil. The most commonly used parts are the seeds, although a few phytochemical and antimicrobial activity studies have also used the leaves, stems and roots (Tamil et al., 2011; Almeida et al., 2012). The roots and stems of *Solanum paniculatum* Linn. (jurubeba), a member of the family *Solanaceae*, are also commonly used by humans to disinfect wounds (Garcia et al., 2008). However, the healing and antibacterial activities of the plant have not yet been confirmed (Lobo et al., 2010).

This study was aimed at a preliminary research to investigate the potential antimicrobial effects of two Brazilian plant extracts against *E. faecalis* strains (standard and from the oral environment) and, according to the results, broaden the number of microorganisms to be tested.

MATERIALS AND METHODS

Extract preparation

After phytochemical screening by thin-layer chromatography to evaluate the phytocompounds of several Brazilian plants, the seeds of *B. orellana* L. (annatto) and the peels of *S. paniculatum* L. (jurubeba) were selected for this study due to the following components present in good concentrations: terpenes, tannins, saponins and essencial oils (found in both extracts – annatto and jurubeba) and flavonoids (found in jurubeba extract).

An ethanolic extract or vegetable tincture of *B. orellana* L. seeds harvested from shrubs registered in the Federal University of Maranhão (Universidade Federal do Maranhão - UFMA) Ático Seabra Herbarium (São Luís, Maranhão, Brazil) under voucher specimen no. 00815 was prepared in the laboratory of the UFMA School of Pharmacy. Seeds from mature fruits were collected in April 2014 by a collector wearing rubber gloves. The seeds were placed in paper bags and kept dry and ventilated until being sent to the laboratory two days later. In the laboratory, the annatto seeds were spread, dried at room temperature and pulverised in an electric mill to obtain powder. Next, the powder underwent extraction (maceration) for 48 h with 70% ethanol at a 1:3 ratio v/v and was filtered. This procedure was repeated three times, and the filtrate of the three extractions was pooled, concentrated in a rotary evaporator and named hydroethanolic extract (HE). To determine the dry weight and yield, three 1 mL aliquots of the HE were used, separated into three flasks. The aliquots were evaporated under a hot air stream, and the flasks were then cooled and weighed in a digital analytical balance. This procedure was also repeated three times until constant weight was obtained. The extraction yield was based on the powder weight, the final volume of the concentrated HE and the residue weight.

S. paniculatum L. (jurubeba) peels were collected in September 2010 in the city of Natal, Rio Grande do Norte, Brazil. Botanical identification was performed by Prof. Dr. Maria Iracema Bezerra Loyola (Department of Biology/UFRN), where one voucher specimen was deposited (no. 5468). After collection, stem samples were desiccated in a forced air oven at a mean temperature of 45°C for three to four days and then pulverised in a mechanical mill, turning them into powder. The dried plant powder underwent thorough maceration with two litres of 95% ethanol for 72 h, and this process was repeated three times to obtain the maximum extraction of the chemical compounds. The resulting extraction solution was filtered and concentrated in a rotary evaporator under reduced pressure at a temperature not higher than 40°C.

Microbial strains

Samples of *E. faecalis* (ATCC 292012) and *E. faecalis* (44 – AB, isolated from endodontic infections) were obtained upon request from the School of Dentistry of the Estácio de Sá University (Universidade Estácio de Sá) (Rio de Janeiro/RJ) and the School of Dentistry of the Federal University of Rio Grande do Norte (Universidade Federal do Rio Grande do Norte – UFRN) (Natal/RN). The samples were subsequently reactivated in the Laboratory of Microbiology of UFRN, Natal/RN.

Determination of the minimum inhibitory concentration of *S. paniculatum* linn and *B. orellana* linn extracts

The minimum inhibitory concentration (MIC) of the *S. paniculatum* L. and *B. orellana* L. extracts was determined using the method described by Bauer et al. (1966), with modifications, based on the lowest concentration of the extract able to inhibit bacterial growth as compared to the control (0.12% chlorhexidine digluconate), which was based on the presence of a zone of inhibition measured in

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millimetres with a calliper.

Determination of the minimum inhibitory concentration of adherence of *S. paniculatum* l. and *B. orellana* l. extracts

The minimum inhibitory concentration of adherence (MICA) of the extracts was determined from the crude extract (CE) up to the maximum dilution obtained– 1:512. Bacterial inocula were obtained and distributed as subcultures into haemolysis tubes with 0.2 mL of the solution corresponding to the respective extract dilution. The tubes were then incubated at 37°C under anaerobic conditions at a 30° inclination. Microorganism adherence to the tube wall after shaking was visually determined and compared with the controls (0.12% chlorhexidine). The MICA was determined based on the lowest concentration yielding adhered bacteria.

Toxicity tests

After determining the MIC and MICA, the cytotoxic potential of the *S. paniculatum* Linn (jurubeba) extract was assayed because it was the only one to exhibit an inhibitory effect against the *E. faecalis* strains. After approval from the Human Research Ethics Committee, protocol registration no. 743/10, cover sheet no. 390665, Certificate of Presentation for Ethical Appreciation (Certificado de Apresentação para Apreciação Ética - CAAE) no. 6396.0.000.126-10 of March 3, 2011, the extract was evaluated at different concentrations using the method of human erythrocytes - A, B, O and AB - (Rangel et al., 1997; Prokofeva et al., 2004), derived from blood that could not be used for transfusion (to be discarded). The material was obtained from the Transfusion Unit of the Lauro Wanderley University Hospital/Federal University of Paraíba (Hospital Universitário Lauro Wanderley/Universidade Federal da Paraíba - UFPB) and handled and disposed of in accordance with the Safety Guidelines followed by this unit.

Determination of the lethal dose

The LD of the *S. paniculatum* L. extract was also determined. The animal observation method was based on the experimental protocol developed by the Psychopharmacology unit of UFRN and performed using Swiss albino mice. The mice were provided by the UFRN vivarium, grouped in cages and kept under a mean temperature of 27°C without any medication and had free access to food (Purina® feed pellets) and potable water in graduated polyethylene bottles. The animals were maintained under a 12-h light/dark cycle prior to the experiment and transferred to the experimental room 30 min before the experiment. They were then divided into groups and treated with *S paniculatum* L. extract (at different concentrations), intraperitoneally (IP) with a single dose - 0.1 mL/animal. Distilled water was given to the control group.

The animals were observed for 24 h to map possible behavioural changes suggestive of effects on the central nervous system (CNS) and/or the autonomic nervous system (ANS). At the end of the experiment, the number of dead animals was recorded to determine the dose causing death of 50% of the experimental animals (lethal dose = LD_{50}).

RESULTS AND DISCUSSION

Phytochemical screening

Phytochemical screening tests showed the strong presence of toluene and formic acid in the seeds of *B. orellana* L., as well as low but evident concentrations of alkaloids and flavonoids. In the roots of *S. paniculatum* L., several chemical compounds were found, such as tannic acid, flavonoids and tannins. These findings were considered to be an important indication of antimicrobial activity due to the biological functions and biotechnological applications of these phytocompounds (Alves et al., 2009; Hammer et al., 1999; Chatuverdi et al., 2010).

Determination of the minimum inhibitory concentration and minimum inhibitory concentration of adherence of the extracts

Our results corroborate those from other studies reporting relative and limited microbicidal and antiseptic activity for annatto extract against several types of bacteria (Table 2 and 4). This limitation is associated with the different parts of the plant used and seasonal and geographical characteristics (Bertini et al., 2005; Agra et al., 2008).

Majolo et al. (2013) reported that different plant accessions harbour distinct levels of antimicrobial activity, perhaps due to different characteristics of the soil, climate, genetics and availability of phytonutrients, which affect the chemical content associated with this activity. Additionally, differences in soil site, climatic region, humidity level and sampling period may promote different concentrations of saponins, flavonoids, alkaloids and steroids. Assuming that these variables, combined or individually, may have influenced the results presented here, we suggest that further experiments be conducted in the near future using other seed samples as well as leaves collected from the same voucher specimen, though in periods different from when the seeds used in the present study were extracted (April 2014 – no rain, medium humidity level, very hot sun).

Giridhar and Venugopalan (2012) have observed antibacterial activity of annatto leaves only, corroborating the data reported by Coelho et al. (2003) and Fleischer et al. (2003). Silva et al. (2010), after testing the inhibitory activity against bacteria of *B. orellana* L. hydroethanolic extracts obtained from the fruit, stem, root and leaves of the plant, found that the products from the leaves and stems exhibited bacteriostatic activity against several bacteria. However, Majolo et al. (2013) and Mital et al. (2013) used seeds from multiple accessions and concluded that the hydroethanolic preparation exhibited the best inhibitory activity, with Gram-positive bacteria being the most sensitive, resulting in a moderate zone of inhibition. Chaturvedi et al. (2010) and Almeida et al. (2012), after testing extracts of *B. orellana* L. seeds on bacteria and fungi with no success, suggested further testing with the product.

The results obtained here with the jurubeba extract confirm the inhibitory potential of the plant on microorganisms (Tables 1 and 3), a result also found by Lobo et al. (2010), Rodrigues et al. (2013) and Costa (2011).

Table 1. Minimum inhibitory concentration (mean zone of inhibition in mm) in solid medium of *S. paniculatum* Linn extract and chlorhexidine digluconate.

Microbial Strains	***Solanum paniculatum* Linn extract**									
	CE	**1:2**	**1:4**	**1:8**	**1:16**	**1:32**	**1:64**	**1:128**	**1:256**	**1:512**
	500 mg/mL	**250 mg/mL**	**125 mg/mL**	**62.5 mg/mL**	**31.25 mg/mL**	**15.65 mg/mL**	**7.81 mg/mL**	**3.90 mg/mL**	**1.95 mg/mL**	**0.97 mg/mL**
E. faecalis 29212 ATCC	16	14	16	14	14	12	12	0	0	0
E. faecalis 44 AB	15	15	15	13	13	12	0	0	0	0
Microbial	0.12% Chlorhexidine digluconate									
Strains	PS	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512
E. faecalis 29212 ATCC	20	17	17	16	15	15	15	15	13	10
E. faecalis 44 AB	21	18	18	17	16	14	0	0	0	0

*Crude extract; *PS– pure substance.

Table 2. Minimum inhibitory concentration (mean zone of inhibition in mm) in solid medium of the annatto extract and chlorhexidine digluconate.

Microbial	***Bixa orellana* L. extract**									
Strains	**CE**	**1:2**	**1:4**	**1:8**	**1:16**	**1:32**	**1:64**	**1:128**	**1:256**	**1:512**
E. faecalis ATCC	0	0	0	0	0	0	0	0	0	0
E. faecalis AB	0	0	0	0	0	0	0	0	0	0
Microbial	**0.12 and 2% Chlorhexidine digluconate**									
Strains	**PS**	**1:2**	**1:4**	**1:8**	**1:16**	**1:32**	**1:64**	**1:128**	**1:256**	**1:512**
E. faecalis ATCC										
E. faecalis AB	21	18	18	17	16	14	0	0	0	0

*CE– Crude extract; *PS– pure substance.

Table 3. Minimum inhibitory concentration of adherence (MICA) of *S. paniculatum* Linn extract for *E. faecalis* when compared with MICA of the control.

Microbial Strains	***Solanum paniculatum* Linn extract (MICA)**	**0.12% CLX**
E. faecalis ATCC	1:512	1:512
E. faecalis AB	1:512	1:512

*CLX– chlorhexidine

However, few studies have demonstrated the antimicrobial effects of the plant *S. paniculatum* Linn.

The MIC of 250 mg/mL (1:2) was a good result as compared to the zone of inhibition of the controls with chlorhexidine (Table 1). The inhibition of microbial growth was homogeneous, according to the degree of concentration of the extract, showing a progressive decrease in the diameter of the halos with increasing dilutions. The *S. paniculatum* Linn extract exhibited inhibitory effects on *E. faecalis* up to a concentration of 7.81 mg/mL (1:64). Moreover, it also affected microbial adhesion up to the maximum dilution (1:512 and 0.97 mg/mL) (Table 3). Out of the two groups of microorganisms, *E. faecalis* 29212 ATCC was the strain most sensitive to jurubeba. It could not establish a statistical analysis due to the small number of strains tested (two only).

Table 4. Minimum inhibitory concentration of adherence of *B. orellana* L. extract (annatto) for *E. faecalis* when compared with MICA of the control.

Microbial strains	*Bixa orellana* Linn extract (MICA)	0.12% CLX
E. faecalis ATCC	0	1:512
E. faecalis AB	0	1:512

*CLX – chlorhexidine.

Jurubeba cytotoxicity

The *S. paniculatum* (jurubeba) extract haemolysed 41.2% of human erythrocytes type A, 45.1% of type B, 16.4% of type O and 24.8% of type AB. However, cytotoxicity was observed at the 1:2 dilution (250 mg/mL). Since this is a preliminary study, these results demonstrate a promising non-toxic behavior of jurubeba extract.

Determination of jurubeba lethal dose

No mortality was observed in the animals treated intraperitoneally with the *S. paniculatum* Linn extract at any dilution tested (from non-diluted up to 1:512) at 24, 72 h or 15 days after the experiment. Thus, LD = 0. Moreover, no significant behavioural changes were observed.

Conclusions

The low toxicity of *S. paniculatum* L. extract (cytotoxicity only at the 1:2 dilution or 250 mg/mL), its LD = 0, and its good antifungal and antibacterial performance revealed in the *in vitro* tests suggest the potential use of this plant in the treatments of endodontic and periodontal oral infections; however, further experiments providing more evidence should be performed. The *B. orellana* L. extract exhibited no antimicrobial activity, and further studies with the product are also recommended.

Conflict of interests

The authors did not declare any conflict of interest.

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Vol. 9(14), pp. 1026-1036, 8 April, 2015
DOI: 10.5897/AJMR2014.6835
Article Number: D631A0352314
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Biofilm production by clinical isolates of *Staphylococcus epidermidis* and its relationship with genotypic profile, presence of virulence-related genes and antibiotic resistance

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Received 19 April, 2014; Accepted 23 February, 2015

***Staphylococcus epidermidis* is considered as the main infectious agent associated with implanted medical devices. This study determined biofilm production and composition, pulsed-field gel electrophoresis (PFGE) profile, antimicrobial susceptibility and presence of virulence-related genes (*ica* operon, *aap, bhp, embp, capB* and IS*256* transposase) in 49 clinical isolates of *S. epidermidis*. Twenty-five isolates (51%) were classified as biofilm producers in microtiter plate (MTP) assay. In Congo red assay (CRA) test, 14 (29%) showed positive reaction and three (6%) had indeterminate reaction, all were biofilm-producers in MTP assay. Fourteen isolates with positive reaction in CRA test had the chemical nature of biofilm determined as polysaccharide, had the *ica* operon (PIA-dependent producers) and the majority was strong biofilm producer. Eight biofilm producer isolates showed negative reaction in CRA test and the chemical nature of their biofilm was proteinaceous (PIA-independent producers). Antimicrobial resistance rates were generally higher in biofilm producers and resistance to beta-lactams ranged from 82-96%, while 61% of the isolates were multidrug resistant (≥ 10 drugs). Resistance to daptomycin, quinupristin/dalfopristin, rifampin and trimethoprim/sulfamethoxazole was observed only in PIA-dependent isolates, while the resistance to gentamicin was present in all PIA-independent isolates and in just 53% of PIA-dependent of isolates. The most prevalent virulence-related genes were *capB* (80%) and *embp* (67%); the other genes were less frequent: *ica* operon (41%), *aap* (31%), IS*256* transposase (22%) and *bhp* (10%). The presence of *ica* operon and IS*256* transposase gene showed significant association with biofilm production and strong biofilm production. Moreover, these isolates presented significant higher resistance to levofloxacin, moxifloxacin, rifampin and trimethoprim/sulfamethoxazole. PFGE analysis showed 23 profiles, having the prevalent type 15 isolates. Of these, seven were PIA-independent biofilm producers and just one was PIA-dependent producer, unlike what was observed in other studies, where isolates of prevalent profiles were PIA-dependent biofilm producers.**

Key words: *Staphylococcus epidermidis*, biofilm, *ica* operon, virulence-related genes, IS*256,* PFGE, multi resistant staphylococci.

INTRODUCTION

Staphylococcus epidermidis is primarily, a normal inhabitant of the healthy human skin and mucosal microbiota. In recent decades, this species has emerged as a common cause of numerous healthcare associated infections, preferentially affecting immunocompromised, long-term hospitalized and critically ill patients (Mack et al., 2013; Ziebuhr et al., 2006). These infections are often linked to the use of implanted medical devices and take a chronic or persistent course. Moreover, antimicrobial therapy is frequently unsuccessful and removal of the implanted device is often required (Mack et al., 2013). Unlike other pathogens, *S. epidermidis* has a limited set of virulence factors. Biofilm formation was the first one recognized and provides protection against mechanisms of immune defense and antimicrobials (Fey and Olson, 2010).

The most well-studied and prevalent molecule involved in *S. epidermidis* biofilm formation is polysaccharide intercellular adhesin (PIA), synthesized by enzymes encoded by the *icaADBC* operon. PIA is a homoglycan (Rohde et al., 2010) that can be specifically degraded by hexosaminidase dispersin B (DspB) (Kaplan et al., 2004) or undergo oxidation at linkages between glucosamine residues in the presence of metaperiodate (Wang et al., 2004). Although infrequent and poorly studied, biofilms constituted of proteins may be produced by clinically significant isolates of *S. epidermidis*. Accumulation associated protein (Aap) (Rohde et al., 2005), Bap homologue protein (Bhp) (Tormo et al., 2005) and extracellular matrix-binding protein (Embp) (Christner et al., 2010) have been described as involved in the formation of these PIA-independent biofilms. Microtiter plate (MTP) biofilm assay (Christensen et al., 1985) and Congo red assay (CRA) test (Freeman et al., 1989) are the most commonly used methods for detection of *in vitro* biofilm formation. MTP biofilm assay is used to quantitatively measure the optical density of stained bacterial films adhered to wells of a tissue culture plate, and CRA test employs a culture medium that qualitatively differentiates exopolysaccharide-forming isolates.

Another possible virulence factor in *S. epidermidis* is poly-γ-glutamic acid (PGA), an extracellular anionic polymer renowned for being responsible for the pathogenicity of *B. anthracis*. PGA contributes to the resistance of *S. epidermidis* to cationic antibacterial peptides and inhibits phagocytosis, playing a key role in the persistence of *S. epidermidis* during device-related infections (Kocianova et al., 2005).

Some studies have found a high frequency of the insertion sequence IS*256* in clinically significant *S. epidermidis,* in comparison with commensal isolates, suggesting that this genetic element may be used to discriminate these isolates (Gu et al., 2005; Koskela et al., 2009; Kozitskaya et al., 2004). The reversible transposition of IS*256* into genes of *ica* operon or their global regulators (*rsbU* and *sarA*) determines phase variation in the production of PIA-dependent biofilm (Ziebuhr et al., 1999). This possibility of variation in the expression of virulence associated surface factors is an effective strategy already observed in many pathogens. In addition, IS*256* has the capacity to influence antibiotic resistance (Conlon et al., 2004; Hennig and Ziebuhr, 2010).

Pulsed-field gel electrophoresis (PFGE) analysis is a typing method known to provide reliable information on the short-term epidemiology of healthcare associated *S. epidermidis*, mainly when associated with resistance to antimicrobials and virulence markers (Nunes et al., 2005; Cherifi et al., 2014).

The aim of this study was to investigate in clinical isolates of *S. Epidermidis*, the biofilm production, the presence of the major virulence encoding genes (*aap, bhp, embp* and *capB)* and *IS256* element*,* resistance to antimicrobials and genotypic profiles, and establish possible interrelationships between them*.*

MATERIALS AND METHODS

Bacterial isolates

Forty-nine clinical isolates of *S. epidermidis* (45 from blood cultures and four from central venous catheters) were obtained from infected patients, hospitalized between November 2011 and April 2012 at Hospital Federal dos Servidores do Estado (HFSE) in Rio de Janeiro city, Brazil. Blood cultures were processed using the BacT/Alert™ system (bioMérieux) for aerobic and anaerobic bacteria, and central venous catheters were processed by the semi-quantitative roll plate method (Maki et al., 1977). Biofilm non-producing *S. epidermidis* ATCC 12228 and the biofilm producer *S. epidermidis* ATCC 35984 were used as reference strains in phenotypic and genotypic methods. The Human Research Ethics Committee from HFSE approved this study with reference number 000.417.

Species identification and antimicrobial susceptibility analysis

The identification and determination of minimum inhibitory concentrations (MIC) of isolates was performed with MicroScan WalkAway-96 System (Dade Behring Inc.). The drugs tested were: amoxicillin/clavulanic acid (AMC), ampicillin/sulbactan (SAM), ampicillin (AMP), ceftriaxone (CRO), clindamycin (DA), daptomycin (DAP), erythromycin (E), gentamicin (GM), levofloxacin (LVX), linezolid (LZD), moxifloxacin (MXF), oxacillin (OX), penicillin (P), quinupristin/dalfopristin (SIN), rifampin (RD), tetracycline (TE), trimethoprim/sulfamethoxazole (SXT) and vancomycin (VA).

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Microtiter plate biofilm assay

Biofilm production was quantified using the MTP assay as previously described (Stepanović et al., 2000). Briefly, an overnight culture grown in tryptone soya broth (TSB - HiMedia) was diluted to 1:100 in fresh medium and 200 µL per well were seeded in 96-well flat-bottomed polystyrene microtiter plate (Nunclon; Nunc A/S). These plates were incubated for 24 h at 35°C. After three washes with distilled water, wells were treated with 200 µL of methanol for 15 min, emptied and air-dried, then stained for 5 min with 200 µL of 2% Hucker's crystal violet solution. Plates were washed under running distilled water, air-dried and optical density (OD) at 570 nm was determined in a microplate reader (TP-Reader - Thermo Plate Devices). After extraction with 200 µL of 95% ethanol for 30 min, the OD of biofilm extract was measured. The average of OD values was determined for all isolates and negative control (*S. epidermidis* ATCC 12228), all tests being performed in triplicate and repeated three times. The OD cut-off value (ODc) was defined as three standard deviations (SD) above the mean OD of the negative control: Odc = OD average of negative control + (3 x SD of negative control). The OD average of biofilm extract of isolates was used to interpretation of results: OD≤ODc = no producer; ODc<OD≤2xODc = weak producer; 2xODc<OD≤4.Odc = moderate producer; 4xODc<OD = strong producer (Stepanović et al., 2007).

Congo red agar test

Congo red agar (CRA) medium was composed of brain heart infusion broth (BHI – Oxoid) 37 g/L, sucrose (Merck) 50 g/L and agar (Agar No 1 – Oxoid) 10 g/L, and prepared as previously described by Freeman et al. (1989). The CRA plates were seeded with 10 µL of overnight TSB cultures by spot plate technique, incubated for 24 h at 35°C and overnight at room temperature. The tests were performed in triplicate and repeated three times. A positive result was considered when the spot was black, with a dry crystalline consistency and/or metallic sheen, negative if its color was light red and an indeterminate result was indicated by a darkening of the spot, but with the absence of a dry crystalline consistency (Freeman et al., 1989).

Biofilm detachment assay

The chemical nature of the biofilm matrix was determined by degradation in a test resembling MTP biofilm assay (Wang et al., 2004). Solutions of two degradation agents in 0.1 M PBS (pH 7.0) were used, 40 mM sodium metaperiodate (Vetec) for polysaccharide-dependent biofilm and 1 mg/mL proteinase K (Sigma) solutions for protein-dependent biofilm. The isolates were grown in a microtiter plate and the wells washed once with distilled water. Then, each well was treated with 200 µL of degrading agents or PBS (control) and the plates were incubated for 2 h at 35°C. The wells were washed twice with distilled water, and next steps follow as described in MTP biofilm assay. Tests were performed in triplicate and repeated three times. A reduction of over 50% in OD average, when compared to the control, of wells treated with degrading agents, indicated the chemical nature of the biofilm.

Detection of virulence-related genes

The DNA of all clinical isolates and reference strains was extracted using a boiling method (Ninin et al., 2006). Bacterial suspensions in distilled water, with a turbidity equivalent to 1.0 McFarland standard, were prepared from grown isolates in tryptone soya agar (TSA - HiMedia). Suspensions were boiled for 5 min, centrifuged at 12,000 xg for 5 min and supernatants were removed to be used as DNA template in polymerase chain reaction (PCR).

Identification of *S. epidermidis* was carried out using a PCR method to detect species-specific genomic fragment of 705-bp (Martineau et al., 1996). Others genes detected were: *icaA*, *icaB* and *icaC* (Ziebuhr et al., 1999); *icaD* (de Silva et al., 2002); *icaR* (Arciola et al., 2004); *aap*, *bhp* and *embp* (Rohde et al., 2004); *capB* (Kocianova et al., 2005) and IS*256* transposase (Gu et al., 2005). PCR reactions were performed with Ampliqon Taq DNA Polymerase 2x Master Mix (1.5 mM $MgCl_2$) (Ampliqon A/S, Denmark), according to the manufacturer's directions, in a LifePro Thermal Cycler (Hangzhou Bioer Technology Co.). The amplified products were analyzed by agarose (1.5%) gel electrophoresis with GelRed™ and visualized using UV light. Their sizes were estimated by comparison with 100 bp DNA Ladder (Invitrogen - Life Technologies, Canada).

Pulsed field gel electrophoresis (PFGE) analysis

The preparation of agarose disks, containing chromosomal DNA for PFGE and SmaI DNA restriction fragments separation, was performed as previously described (Chung et al., 2000). The interpretation of the band patterns obtained was based on the criteria described by Tenover et al. (1995) and the analyses were performed using BioNumerics® software version 5.0 (Applied Maths, Kortrijk, Belgium). A dendrogram of similarity was built using the unweighted pair-group method with arithmetic averages (UPGMA).

Statistical analysis

The degree of association between genes was measured by the significance of the Phi correlation coefficient. Results of antimicrobial susceptibility were compared using Chi-square or Fisher's exact test. All tests were performed using BioEstat 5.3 (Instituto Mamirauá) with confidence level of 95% ($\alpha = 0.05$).

RESULTS

Species identification

All isolates were identified as *S. epidermidis* by using MicroScan System and these results were confirmed by PCR analysis of species-specific 705-bp genomic fragment.

Microtiter plate biofilm assay

In the MTP biofilm assay, 21 (51%) of isolates were sorted as producers. Regarding the level of biofilm of these isolates, 14 were strong, five were moderate and six were weak biofilm producers (Table 1). In the same procedure, the OD before extraction of the dye bound to the biofilm with alcohol was also determined. This method is widely used in various studies to determine the biofilm production and the cut-off point, generally, fixed in 0.1 a 0.12 (Stepanović et al., 2007). With this method, only 35% of isolates were classified as biofilm producers. This difference was due to eight isolates classified as weak (5) and moderate (3) when the OD_{be} was used (data not show).

Table 1. Results of biofilm production in MTP biofilm assay, CRA test reaction and detection by PCR of virulence related genes in clinical isolates of *S. epidermidis*.

Biofilm production in MTP assay [a]			Number (%) of clinical isolates								
			CRA test [b]			Detection by PCR of virulence related genes					
			positive	negative	indeterminate	operon *ica*	*aap*	*bhp*	*embp*	*capB*	IS*256* [c]
Producer		25 (51)	14 (56)	8 (32)	3 (12)	17 (68) *	11 (44)	0 (0)	18 (72)	21 (84)	9 (36) *
Level	Weak	6	0	5	1	1	1	0	4	6	1
	Moderate	5	1	3	1	2	1	0	4	5	0
	Strong	14	13	0	1	14 *	9	0	10	10	8 *
Non-producer		24 (49)	0	24 (100)	0 (0)	3 (12)	4 (17)	5 (21)	15 (63)	18 (75)	2 (8)
Total		49 (100)	14 (29)	32 (65)	3 (6)	20 (41)	15 (31)	5 (10)	33 (67)	39 (80)	11 (22)

*, Significant association in Phi correlation coefficient ($p < 0.05$) between the gene detected and biofilm production or biofilm production level [a]= Microtiter Plate biofilm assay; [b]= Congo Red Agar Test; [c] = IS256 transposase gene

Congo red agar test

In the CRA test, 14 (29%) of isolates showed positive reaction, 32 (65%) were negative and three (6%) were indeterminate (Table 1). Of the 25 isolates classified as biofilm producers in MTP assay, 14 showed positive reaction in CRA test, eight were negative and three were indeterminate. Of these 14 isolates that had positive reaction in CRA test, 13 were strong biofilm producers and one was a moderate producer. The eight isolates with negative reaction in CRA test that were classified as producers in MTP assay, showed biofilm production levels weak (5) and moderate (3). In turn, each one of the three isolates that had indeterminate reaction in CRA test presented a different level of production in MTP assay.

Biofilm detachment assay

The determination of the chemical nature of the 25 biofilm producer isolates showed that 14 (56%) were polysaccharide-dependent and eight (32%) were protein-dependent. In three isolates, the treatment with the two degrading agents did not reach the minimal standard of OD reduction (50%). However, the results of degradation with sodium metaperiodate were very close to the standard. These results, supported by detection of transcription of *ica*A gene in real time PCR experiments (data not show), led us to assume that the biofilm of these isolates would be polysaccharide-dependent. Therewith, the number of isolates that produced this type of biofilm increased to 17 (68%). In MTP biofilm assay, 14 of these isolates were ranked as strong, two as moderate and one as weak. Furthermore, in CRA test, 14 of these isolates showed positive reaction and three were indeterminate. Regarding the eight protein-dependent biofilm producers, in MTP assay five were weak and three moderate producers, and all presented negative reaction in CRA test.

Detection of virulence-related genes

The most prevalent genes were *capB* (80%) and *embp* (67%) (Table 1). The *icaA*, *icaB*, *icaC* and *icaD* genes that form the *ica* operon, and the regulatory *icaR* gene were always detected together in 41% of isolates studied. Regarding the other genes searched, the *aap* gene was detected in 31% of isolates, IS*256* transposase gene in 22% isolates and the *bhp* gene in only 10% of the isolates. The percentage of detection of all genes was higher in producer than in non-producer isolates, except *bhp* gene that was detect only in biofilm non-producer isolates.

The *ica* operon genes (*ica*+) showed a significant association with the presence of *aap* gene ($p= 0.0058$), biofilm production ($p = 0.0003$) and strong biofilm production ($p= 0.0006$). Likewise, the detection of IS*256* transposase gene presented a significant correlation with biofilm production ($p = 0.0479$) and strong biofilm production ($p = 0.0389$). Furthermore, the IS*256* transposase gene showed full association ($p< 0.0001$) with *ica*+ isolates.

Seventeen of the 20 isolates *ica*+ were biofilm producers including all that were strong (14), two moderate and one weak producers. All of these *ica*+/biofilm producer isolates had polysaccharide-dependent biofilms (PIA-dependent biofilm).

The eight biofilm producer isolates that did not have the *ica* operon, presented protein-dependent biofilm (PIA-independent). The search for other genes related to biofilm-production in these isolates detected *embp* (7) and *aap* (1) genes.

Antimicrobial susceptibility analysis

All isolates were susceptible to linezolid and vancomycin, and just one was resistant to daptomycin. The percentage of antimicrobial resistance was slightly higher in the biofilm producer isolates than in non-producers, except for quinupristin/dalfopristin and tetracycline where the resistance percentage for both antimicrobials was slight

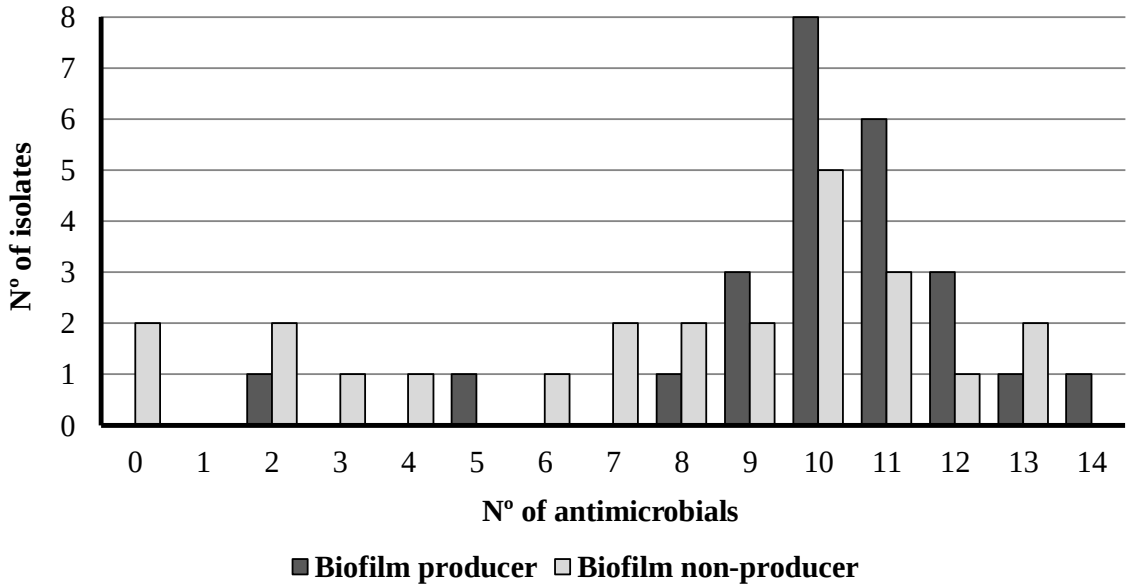

Figure 1. Number of antimicrobials to which biofilm producer and non-producer clinical isolates of *S. epidermidis* exhibited resistance.

higher in the biofilm non-producers (13 versus 12% and 17 versus 12%, respectively).

The resistance to beta-lactam antibiotics, including their combination with beta-lactamase inhibitors, ranged from 82 to 96% for all isolates, however being always higher in biofilm producers. Specifically in relation to oxacillin, resistance was observed in 84% of all isolates, and higher in biofilm producers (92%) than in non-producers (75%).

In PIA-dependent and independent biofilm producer isolates, the results of resistance to most antimicrobials tested were very close. However, resistance to daptomycin (6%), quinupristin/dalfopristin (18%), rifampin (35%) and trimethoprim/sulfamethoxazole (76%) was observed only in PIA-dependent isolates. On the other hand, resistance to gentamicin was present in all PIA-independent isolates, while resistance to this drug in PIA-dependent isolates was observed in 53% of isolates.

Resistance to 10 or more antimicrobials (multidrug resistant– MDR) was detected in 61% of isolates (Figure 1). Of these, 63% were biofilm producers and 37% of non-producers. The MDR isolates were resistant to all beta-lactam antibiotics. Resistance to erythromycin, gentamicin and levofloxacin was seen in all MDR biofilm non-producer isolates, while in MDR biofilm producers the frequencies were 95, 74 and 95%, respectively. The most expressive difference between these isolates was the resistance to gentamicin, which was lower (74%) in biofilm producers due to the small number of resistant isolates among PIA-dependent isolates (64%) in comparison with PIA-independent biofilm producers (100%). Another expressive difference between MDR biofilm producer isolates was the resistance to rifampin (43%) and trimethoprim/sulfamethoxazole (86%) only expressed by PIA-dependent.

All isolates that carried the IS256 transposase gene were resistant to beta-lactam antibiotics tested and levofloxacin (Figure 2), as well as 82% of them were MDR. Moreover, the resistance in isolates that had this genetic element was significantly higher than the quinolones tested, levofloxacin (p= 0.0068), moxifloxacin (p= 0.0327), as well as rifampin (p= 0.0364) and to the combination of trimethoprim/sulfamethoxazole (p= 0.0029).

PFGE analysis

The 49 isolates produced a broad range of restriction patterns, which were distributed into 23 different PFGE types. PFGE type A was the prevalent with 15 (31%) isolates. The other PFGE types, considered sporadic types, were B (4); H and O (3); C, D, E, I and V (all with 2), while the other types had a single isolate.

The PFGE type A profile was detected in almost equal proportions in biofilm producer (32%) and non-producer (29%) isolates. Among the eight biofilm producer isolates with this profile, seven were PIA-independent and just one was a PIA-dependent producer. In turn, sporadic PFGE types group was composed of equal numbers of biofilm producer (68%) and non-producer (71%) isolates. However, these represented 16 of 17 PIA-dependent biofilm producer isolates, and was detected in only one PIA-independent biofilm producer.

Regarding the virulence-related genes, none of PFGE type A isolates showed *aap* or *bhp* genes, and the percentages of detection of the *embp* (80%) and *capB* (87%) (Table 2) were slightly higher than sporadic PFGEtypes group (62% and 76%, respectively). isolates studied (67 and 80%, respectively). Moreover, the *ica*

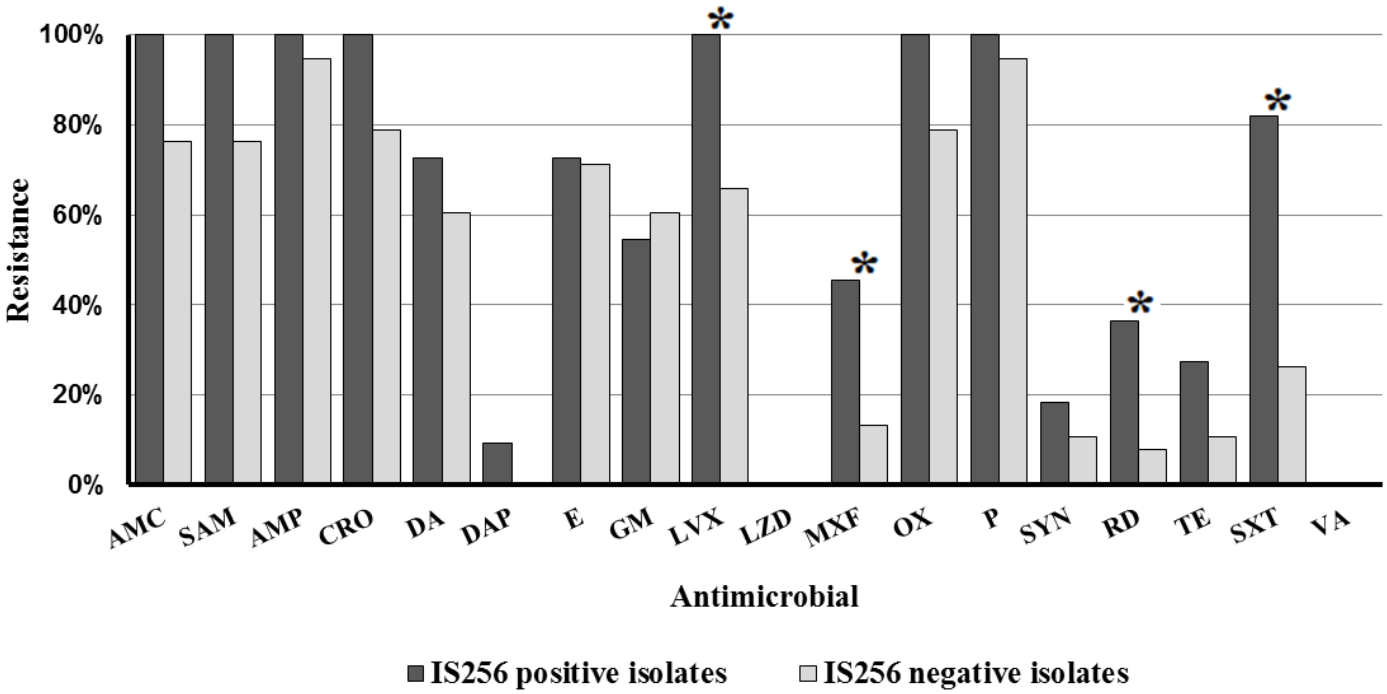

Figure 2. Antimicrobial resistance patterns of clinical isolates of S. epidermidis according to the detection by PCR of the IS256 transposase gene. *, Significant differences ($p < 0.05$) between PFGE type A and sporadic PFGE types; amoxicillin/clavulanic acid (AMC), ampicillin/sulbactan (SAM), ampicillin (AMP), ceftriaxone (CRO), clindamycin (DA), daptomycin (DAP), erythromycin (E), gentamicin (GM), levofloxacin (LVX), linezolid (LZD), moxifloxacin (MXF), oxacillin (OX), penicillin (P), quinupristin/dalfopristin (SYN), rifampin (RD), tetracycline (TE), trimethoprim/sulfamethoxazole (SXT), vancomycin (VA). (Resistance data are represented as percentages of IS256 positive and IS256 negative isolates).

Table 2. Detection by PCR of virulence encoding genes and IS256 transposase gene in prevalent and sporadic PFGE types of clinical isolates of *S. Epidermidis.*

PFGE analysis	Presence of virulence-related genes - n°(%) of clinical isolates					
	***ica* operon**	***aap***	***bhp***	***embp***	***capB***	**IS*256*[a]**
PFGE type A (n= 15)	1 (7)	0 (0)	0 (0)	12 (80)	13 (87)	1 (7)
Sporadic PFGE types (n= 34)	19 (56)	15 (44)	5 (15)	21 (62)	26 (76)	10 (29)

[a] = IS*256* transposase gene

operon and the IS*256* transposase genes were detected in just one isolate of PFGE type A (coincidentally the same isolate).

In the isolates of sporadic PFGE types group, the percentages of detection of *ica* operon, *aap* and IS*256* genes were higher than those in the set of isolates studied. However, except for a single isolate, all others that had *ica* operon and IS*256* transposase genes belonged to sporadic PFGE types group.

The prevalent PFGE type showed higher resistance to beta-lactam antibiotics, clindamycin, erythromycin, gentamicin ($p=0.0121$), levofloxacin and moxifloxacin than isolates of sporadic PFGE types group (Figure 3). On the other hand, in the sporadic PFGE types group, the percentage of resistance to quinupristin/dalfopristin, rifampin and trimethoprim/sulfamethoxazole ($p=0.0247$) was higher than in isolates of the prevalent PFGE type. Moreover, resistance to daptomycin and tetracycline was present only in isolates of the sporadic PFGE types group.

DISCUSSION

S. epidermidis has great importance as the causative agent of healthcare associated infections, in particular, those linked to the use of implanted medical devices. Biofilm production has a known role in the pathogenesis of these infections, explaining its chronic or persistent course and also antimicrobial treatment failures (Mack et al., 2013).

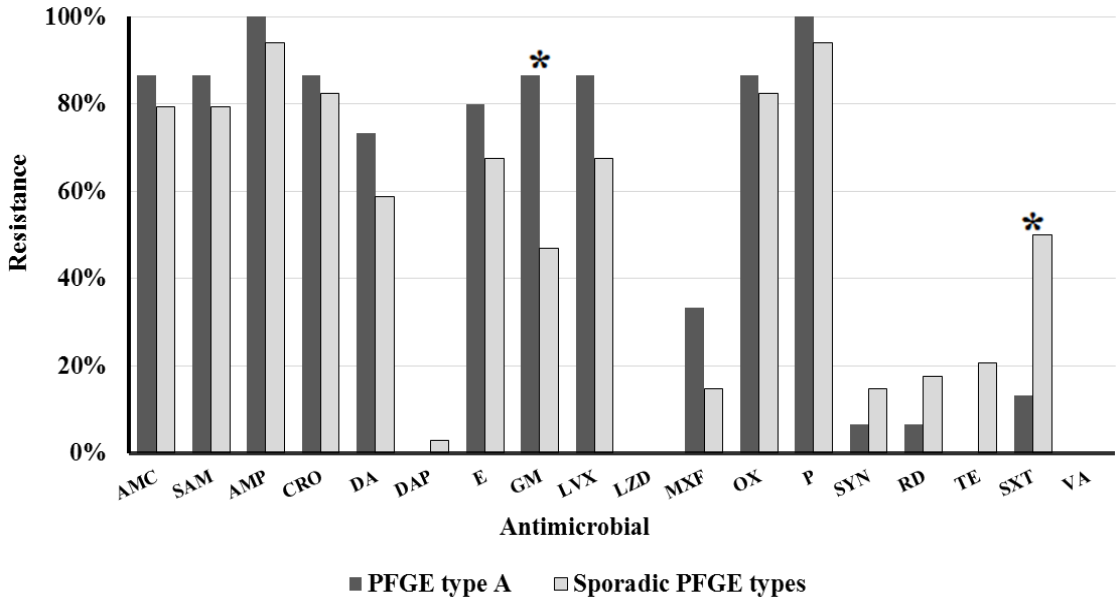

Figure 3. Antimicrobial resistance patterns of clinical isolates of *S. epidermidis* clustered according to predominant and sporadic PFGE types. *, Significant differences ($p < 0.05$) between PFGE type A and sporadic PFGE types; amoxicillin/clavulanic acid (AMC), ampicillin/sulbactan (SAM), ampicillin (AMP), ceftriaxone (CRO), clindamycin (DA), daptomycin (DAP), erythromycin (E), gentamicin (GM), levofloxacin (LVX), linezolid (LZD), moxifloxacin (MXF), oxacillin (OX), penicillin (P), quinupristin/dalfopristin (SYN), rifampin (RD), tetracycline (TE), trimethoprim/sulfamethoxazole (SXT), vancomycin (VA). (Resistance data are represented as percentages of PFGE type A and sporadic PFGE types).

As reported in other similar studies (Arciola et al., 2006; García et al., 2004; Jain and Agarwal, 2009; Oliveira and Cunha, 2010), a higher positivity in the MTP biofilm assay than in CRA test was observed. The differences in these results may be explained by the mechanism of positive reactions in CRA medium, which depends on polysaccharide biofilm production (Freeman et al., 1989), while the MTP assay would be able to detect biofilm production, regardless of its composition.

In fact, all our isolates whose composition of biofilm matrix was identified as polysaccharide by degradation with sodium metaperiodate in detachment assay showed a positive CRA reaction. Conversely, the isolates that presented biofilm of proteinaceous composition had negative reaction in CRA test and isolates with reaction indeterminate did not have the chemical nature of their biofilm determined in detachment assay.

The originally described MTP biofilm assay technique measures directly the OD of stained bacterial films adherent to the bottom of microtiter plates (Christensen et al., 1985). Nevertheless, if the biofilm produced on bottom of the well is not homogeneous, an incorrect measure will occur, since the microplate reader measures the OD only at one point in the middle of the well. Therefore, to avoid this problem, solubilization of the dye attached to the biofilm cells with ethanol can be done, followed by measuring the OD of the biofilm extract (Stepanović et al., 2007).

In our study, the alcoholic biofilm extract OD value was used to evaluate and quantify biofilm production, and also the direct reading of the OD before alcoholic extraction was determined. Comparing the OD values before alcoholic extraction with the alcoholic biofilm extract, the evaluation of biofilm production of the isolates was concordant, except for the isolates characterized in biofilm detachment assay as protein-dependent biofilm producers. These isolates, classified as weak or moderate biofilm producers according to the alcoholic biofilm extract OD value, presented results of OD before alcoholic extraction very close or below the predefined values for the cut-off point. Therefore, if the results of OD before alcoholic extraction had been used to classify these isolates, they would have been misclassified as non-producers. These low levels of biofilm production in protein-dependent biofilm isolates, considerably smaller than that observed in the polysaccharide-dependent biofilm, also reported in others studies (Rohde et al., 2007), contribute to their low detection.

Another difficulty related to the detection of protein-dependent biofilm is that its formation can be impaired by the test conditions routinely employed in the MTP biofilm assay. This has already been described for the Aap protein that mediates biofilm formation after limited proteolysis by addition of trypsin, elastase or cathepsin G to the culture medium (Rohde et al., 2005). Similarly, the Embp protein is required for biofilm production, the

growth of the isolate in the presence of goat serum (50%) and prolonged (48 h) incubation (Christner et al., 2010).

The prevalence of detection of *ica* operon genes in our isolates was close to other studies with clinical *S. epidermidis* (Cafiso et al., 2004; Koskela et al., 2009; Li et al., 2009; Mekni et al., 2012). All genes (*icaA, icaD, icaB* and *icaC*) of the operon were always present, as well as accompanied by the regulatory gene *icaR*. The entire *ica* operon was also observed in other investigations (de Silva et al., 2002; Koskela et al., 2009; Ninin et al., 2006), together with the *icaR* when it was investigated (Arciola et al., 2005; Cafiso et al., 2004). However, some studies have reported the detection of isolated *ica* genes (Diemond-Hernández et al., 2010; Oliveira; Cunha, 2010; Paluch-Oleś et al., 2011), sometimes in the absence of *icaR* gene (Esteban et al., 2010).

Biofilm producer isolates that had *ica* operon genes (biofilm+/*ica*+) showed positive CRA reaction and had a polysaccharide-dependent biofilm, suggesting the PIA involvement in their biofilm. Almost all of these isolates expressed strong production, and a single isolate have a moderate production of biofilm. Therefore, the detection of IS*256* transposase gene in this isolate could be one of the reasons for this decreased expression of *ica* operon (Conlon et al., 2004). Unlike these, three biofilm+/*ica*+ isolates could not be precisely characterized in CRA test and detachment assay. They were indeterminate in CRA test and the result of biofilm degradation with sodium metaperiodate was close to the 50% of degradation. Thus, the classification of these isolates as PIA-dependent was supported by the detection of the *ica*A gene transcription.

The presence of other substance(s) associated with the matrix of the biofilm, affecting the presentation of a typical positive reaction in these tests, could explain these results. Due to the relation of *ica* operon to PIA production, it would be expected that all *ica*+ isolates were biofilm producers, as observed in the majority of our *ica*+ isolates. However, three *ica*+ isolates were biofilm non-producers in the MTP assay, and in two of them, the presence of IS*256* transposase gene was detected. Biofilm-/*ica*+ isolates are not uncommon and have been reported in other studies (Arciola et al., 2006; Koskela et al., 2009; Līduma et al., 2012; Oliveira; Cunha, 2010; Rohde et al., 2007; Stevens et al., 2008). The presence of IS*256* element has been identified as a possible reason for diminished or lack of *ica* operon expression in isolates with biofilm-/*ica*+ profile, since it can impair biofilm production when inserted in *ica* operon (Ziebuhr et al., 1999) or in the global regulatory genes, σB activator *rsbU* and *sarA* (Conlon et al., 2004).

The surface protein Aap has distinct roles in *S. epidermidis* biofilm, being able to determine the formation of PIA-independent biofilm when alone. In turn, it can increase the adhesion of PIA in PIA-dependent biofilm producers, by linking it to the Aap G5 domain (Otto, 2009). The prevalence of *aap* gene detection in our isolates was low as compared to other studies with clinical or commensal *S. epidermidis* (de Araujo et al., 2006; Petrelli et al., 2006; Pourmand et al., 2011; Rohde et al., 2004, 2007; Stevens et al., 2008; Vandecasteele et al., 2003). Although this gene has been most common in biofilm producer isolates, this finding may not be associated with the formation of a biofilm having Aap as a main structural component, since this gene was only detected in a single isolated protein-dependent biofilm producer. Nevertheless, the presence of *aap* gene showed significant correlation with the presence of *ica* operon genes, determining its prevalence in PIA-dependent isolates and in strong biofilm producers. Carriage of *ica* operon cannot serve as a general virulence marker in clinically relevant isolates (Līduma et al., 2012; Rohde et al., 2004, 2007). However, its association with strong biofilm (Mateo et al., 2008; Mekni et al., 2012) or *aap* gene (Stevens et al., 2008) has a better correlation with pathogenicity than these markers individually.

In relation to *capB* and *embp* genes, although they have been detected at high frequency, they cannot be considered as key determinants in biofilm formation, because they were found in equal proportions in biofilm producer and non-producer isolates. Conversely, the *bhp* gene was detected in only 10% of the isolates, being all biofilm non-producers.

Many authors have pointed out the IS*256* as one of the best genetic markers correlated with *S. epidermidis* virulence. This statement was based in the high frequency of IS*256* detection in clinical relevant isolates, which contrasts with its very low occurrence in commensal isolates (Gu et al., 2005; Koskela et al., 2009; Kozitskaya et al., 2004; Mekni et al., 2012). In our study, however, the prevalence of IS*256* transposase gene was low. Despite this, its presence showed a significant correlation with biofilm production. Moreover, the *ica* operon was detected in all IS*256*-positive isolates. This association of IS*256* with *ica* operon has already been observed and is pointed as a characteristic that, along with antimicrobial resistance, may indicate specific clones of *S. epidermidis* which are highly adapted to the hospital environment (Kozitskaya et al., 2005; Li et al., 2009).

High rates of resistance to beta-lactam antibiotics, and absence or low rates of resistance to linezolid, vancomycin or daptomycin were also observed in other studies (Cabrera-Contreras et al., 2013; Sader et al., 2009). Likewise, biofilm producing isolates were, generally, slightly more resistant than non-producing (Cabrera-Contreras et al., 2013). Among PIA-dependent and independent biofilm producer isolates, resistance to daptomycin, quinupristin/dalfopristin, rifampin and trimethoprim/sulfamethoxazole was observed only in PIA-dependent isolates and resistance to gentamicin was present in all PIA-independent isolates. The number of MDR isolates was higher in biofilm producing than in non-producing isolates, and the most remarkable differences were in gentamicin, rifampin and trimethoprim/sulfame-

thoxazole resistance. These differences were a result of higher resistance percentages to rifampin and trimethoprim/sulfamethoxazole presented by PIA-dependent isolates, and gentamicin by PIA-independent.

Our results show that all IS*256*-positive isolates were resistant to the beta-lactams tested and to levofloxacin. Resistance to gentamicin in IS*256*-positive isolates would be expected due to the association of this element with Tn*4001* transposon, implicated in the mediation of aminoglycoside resistance. Contrarily to this, resistance to gentamicin in IS*256*-positive isolates was minor than in IS*256*-negative isolates, and low if compared with that reported in other studies (Kozitskaya et al., 2004; Montanaro et al., 2007). A significant correlation of resistance to trimethoprim/sulfamethoxazole with the presence of IS*256* was observed in our study, and all isolates that had this genetic element and were resistant to gentamicin also showed resistance to trimethoprim/sulfamethoxazole. The association of resistance to trimethoprim and gentamicin was described in *S. epidermidis* resistant to sulfamethoxazole by Totake et al. (1998) due to the formation of a composite transposon of IS256 with Tn*4001* and Tn*4003* transposons.

Such as was observed in our results, a large genetic variability in PFGE analysis of healthcare associated *S. epidermidis* has been reported in many studies (Nunes et al., 2005; Cherifi et al., 2013; Sani et al., 2014). In spite of the diversity observed in the PFGE analysis, multilocus sequence typing (MLST) studies of these isolates showed that the prevalent PFGE types generally belonged to few sequence types (ST) of clonal complex 2 (CC2); among them, ST2 and ST23 in Brazil (Iorio et al., 2012), ST2 and ST54 in Belgium (Cherifi et al., 2014) and ST2 in China (Li et al., 2009; Du et al., 2014). Isolates of these ST, most or all, had *ica* operon genes, and *aap* and/or *bhp* gene.

Conversely, in our study, just one isolate of the prevalent PFGE type had the *ica* operon, and none of them had *aap* or *bhp* genes. Despite the absence of major genes related to biofilm formation in the other isolates, seven isolates of this PFGE type produced protein-dependent biofilm. The frequency of detection (32%) of PIA-independent isolates and the fact that most of them belong to the prevalent PFGE type (A) gives them a highlight rarely observed in similar studies.

The second largest PFGE type (B) was included in sporadic PFGE type group because it was composed of just four isolates. All of them harbored the *ica* operon and *aap* gene, being also oxacillin resistant and MDR, features present in the most frequent ST of the worldwide healthcare associated with *S. epidermidis* of CC2 (Cherifi et al., 2014; Du et al., 2014; Iorio et al., 2012; Li et al., 2009).

Conflict of interests

The authors declare that they have no competing interests.

ACKNOWLEDGEMENTS

The authors are grateful to the Hospital Federal dos Servidores do Estado do Rio de Janeiro for providing the clinical isolates and Universidade Federal do Estado do Rio de Janeiro for supplying materials and laboratory equipment.

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academicJournals

Vol. 9(14), pp. 1037-1043, 8 April, 2015
DOI: 10.5897/AJMR2015.7441
Article Number: FF2AA0F52318
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Isolation and characterization of antibiotic producing *Bacillus* species in Lake Bogoria, Kenya

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Received 20 February, 2015; Accepted 25 March, 2015

Bacilli are a large homogeneous group of bacteria that survive in a wide range of environmental conditions. Formation of resistant spores allows them to survive in high temperature zones where other organisms cannot. Eighty samples were collected and inoculated directly into nutrient broth. Of the eighty samples collected, thirty three exhibited growth. Nine of these were Gram positive rods, twenty were Gram negative rods, and four were Gram positive cocci. Only cultures that yielded Gram positive rods were processed further. Antimicrobial profiling was performed using standard organisms: *Staphylococcus aureus* ATCC 29213, *Escherichia coli* ATCC 25922, *E. coli* 35218 and *Pseudomonas aeruginosa* ATCC 27853. No inhibition was noted against *S. aureus* ATCC 29213 and *E. coli* 35218. Five of the nine Gram positive isolates revealed inhibitory properties against the standard organisms. Phylogenetic analysis of amplified 16S rDNA gene confirmed that all the six antagonistic isolates formed close phylogenetic clusters with known members of *Bacillus* species with a 88-99% sequence identity. The current study shows the presence of thermophilic *Bacillus* species, which are potential biomolecule producers within the hot springs of Lake Bogoria, Kenya. However, further investigation will be useful for the discovery of novel bioactive substances effective against wide range of pathogens.

Key words: *Bacillus*, Lake Bogoria, secondary metabolites, antibiotics.

INTRODUCTION

The major impetus behind extremophile research is the biotechnological potential associated with extremophilic organism. Indeed, applying extremophiles in industries has opened a new era in biotechnology. In the last decade, the number of antibiotic multi-resistant bacterial strains as etiological agents of infectious diseases has increased at an alarming rate; challenging physicians to find an anti-infective therapy that ensures an effective result (Bansidhar et al., 2013). Extermophile micro-organisms produce molecules adapted to unusual life conditions and have been recognized as an important source of new biological products (Sanchez et al., 2009). Until recently, majority of antimicrobial compounds were isolated from terrestrial microorganisms. In the last two

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decades, however, the rate of discovery of novel compounds from this source has significantly declined. This phenolmenon has been exemplified by the fact that extracts from soil-derived actinomycetes have yielded high numbers of clinically unacceptable metabolites (Mincer et al., 2002). While these microorganisms continue to be studied extensively, the rate of discovery of novel metabolites from terrestrial microorganisms is decreasing (Sarker et al., 2010). Discovery and identification of new sources of natural products, therefore, plays an important role in the uncovering of novel drug candidates and drug development (Jorgensen and Turnidge, 2003). Saline and hot water lakes are now becoming increasingly appreciated as rich and untapped reservoirs of useful novel natural products.

Antibiotics have been recognized as the most significant means of effective microbial growth control (Bertrand, 2004) after the discovery of penicillin and other antimicrobial agents by Alexander Fleming in 1928. Since then, there has been ongoing search for effective antibiotics that can withstand emergence of drug resistance among microorganisms (Song, 2008). In Sub Saharan Africa for example, resistance to most available antibiotics has resulted in morbidity and mortality from treatment failures and the ever-increasing costs of antibiotics (Bertrand, 2004).The main approach in which new antibiotics have been discovered has been by screening groups of microorganisms such as *Bacillus, Penicillium, Streptomyces* and other microorganisms (Demain and Fang, 2000; Oluoch et al., 2010). Currently, standard reference strains such as the America Type Culture Collection (ATCC) organisms are used to standardize screening tests. Isolates that demonstrate evidence of antibiotic production are then subjected to further studies to determine if the antibiotic they produce is new. When an organism producing a new antibiotic is discovered, it is produced in large quantities, purified and tested for cytotoxicity and therapeutic activity in infected animals (Talaro and Talaro, 2006). Most of new antibiotics will fail the *in vivo* testing, but a few of the new antibiotics that prove medically useful are then produced commercially (Yarborough et al., 2009).

Bacilli, which consists of a group of Gram-positive, facultative anaerobic, sporulating rods, is known to produce more than 45 antimicrobial molecules some of which are of clinical significance (Stein, 2005). *Bacillus* species produce antibiotics in a soluble protein form which they synthesize and secret into the growing medium. Therefore, the antibiotics they produce have been found to be cheaper and effective, hence preferred in commercial production. The potent antimicrobial activities of *Bacillus* lantibiotics against pathogenic microorganisms such as *Bacillus cereus*, *L. monocytogenes* and *Staphylococcus* spp. make them good candidates for application in the food and medical industry (Stein, 2005).

Bacillus has for a longtime been regarded as a phylogenetic heterogenous group (Ash et al., 1991). Conventionally, *Bacillus* has been identified in the laboratory through biochemical tests and fatty acid methyl ester profiling (Bobbie and White, 1980; Vaerewijck et al., 2001). Since 1990 however, 16S rDNA has been successfully applied in determining phylogenetic relationships of the aerobic, endospore forming bacteria which played an important role in the creation of several families and genera Bacillales (Garrity et al., 2007). To date, 16S rDNA forms a vital standard of taxonomy not only for *Bacilli* species but bacteria in general.

In the present research study, we investigated the presence of thermophilic Bacilli in the various hot springs of Lake Bogoria and evaluated their antibiotic producing capabilities by performing antibacterial studies.

MATERIALS AND METHODS

Collection of water samples

Samples were collected randomly from each of the four hot springs of Lake Bogoria. Ten sediment and ten surface water samples were collected from four sites each from Chemurkei, Loburu, Koiobei and Losaramat hot spring to make a total of 80. The samples were obtained within a temperature range of 45-60°C. Samples were randomly collected and inoculated directly into nutrient broth in universal bottles. In every hot spring, each of ten universal bottles containing 15 ml nutrient broth was directly inoculated with 5 ml sediment sample. Equally, 5 ml of surface water sample was inoculated into ten 15 ml of nutrient broth in universal bottles from each of the four hot springs. The inoculated bottles were put into insulated boxes and immediately transported to the laboratory.

Culture and isolation of *Bacillus* species from samples

Samples in nutrient broth were incubated without shaking for two days (48 h) at 45°C. Growth was determined visually by checking for turbidity. All tubes that showed growth were sub-cultured in nutrient agar plates using streak plate method. The plates were then incubated at 40°C for 24 h. Different colonies obtained after incubation were used to make smears on clean slides which were subsequently Gram-stained. Colonies with Gram-positive rods (nine) were sub-cultured in fresh nutrient agar as pure cultures. Since *Bacilli* are Gram-positive rods, all Gram-positive cocci and Gram-negative rods were not processed further. Stock cultures of each selected strain were prepared and preserved in nutrient agar slants at 4°C.

Biochemical identification

Bacilli isolates were characterized using glucose and lactose fermentation, gas production, catalase production, hydrogen sulfide production, indole production and motility tests according to the methods described by Sneath (1984).

Sensitivity testing

The selected isolates were inoculated into nutrient broth in conical flasks enriched with 1% glucose and incubated in a shaker incubator (100 rpm) at 37°C for 4 days. Five ml of the broth from each of the flasks was taken and put in labeled sterile tubes and

Table 1. Morphological characterization of Gram positive rods isolated from Lake Bogoria.

Isolate	Colony characterization				Cell characterization	
	Colour	Form	Elevation	Margin	Cell arrangement	Gram reaction
S7	White	Irregular	Flat	Undulate	Large rods	Positive
D8	Cream	Irregular	Flat	Undulate	Small rods	Positive
S17	White	Irregular	Flat	Undulate	Large rods	Positive
D22	White	Irregular	Flat	Undulate	Large rods	Positive
D5	White	Irregular	Flat	Undulate	Large rods	Positive
D18	Cream	Irregular	Raised	Even	Large rods	Positive
D10	Cream	Spreading	Flat	Undulate	Small rods	Positive
S13	Cream	Spreading	Flat	Undulate	Small rods	Positive
D1	White	Irregular	Raised	Undulate	Large rods	Positive

centrifuged at 10,000 rpm for 15 min. The cell free supernatant was sterilized using 0.2 µl filter paper and put into sterile Eppendorf tubes. Six millimeter sterile filter paper disks were dipped in each of the sterile supernatant in the Eppendorf tubes and dried in a vacuum for 10 min.

Four standard ATCC organisms were used to test for production of antibiotics: *Staphylococcus aureus* ATCC 29213, *Escherichia coli* ATCC 25922, *E. coli* ATCC 35218, and *Pseudomonas aeruginosa* ATCC 27853. Agar disc diffusion assay was performed on 90 mm Petri-plates containing 20 ml of Mueller Hinton agar. A lawn of the test organism standardized using Mac Farland 0.5 turbidity standard was prepared and the extract impregnated disks placed on top. The Petri-dishes were incubated overnight at 37°C for 24 h. Zones of inhibition were noted as clear areas around the disks.

Phylogenetic analysis of 16S rDNA of the isolates

ZR Fungal/Bacterial DNA Miniprep™(Zymo Research, USA) was used to extract genomic DNA from the bacterial cells according to the manufacturer's specification based on the method of James et al. (2003). The DNA product was semi-quantified on 1% agarose gel at 80 V for one hour and visualized by ethidium bromide staining (Sambrook et al., 1989). Target 16S rDNA sequences were amplified using universal bacterial primers 27f AGAGTTTGATCCTGGCTCAG and 1525r AAGGAGGTGATCCAGCCGCA (Bioneer, USA) in relation to *E. coli* gene sequence (Lane, 1991; Embley and Stackebrandt, 1994). Amplification was performed using an advanced Eppendorf 96 AG, model 22331 thermal cycler (Hamburg, Germany) according to the procedure described by Roux (1995). The PCR products were separated on agarose gel electrophoresis (FisherBiotech Electrophoresis system Australia) at 80 V for one hour and visualized by ethidium bromide staining (Sambrook et al., 1989).

Sequencing of purified product was done in both directions without cloning using a commercial service provider (BECA, ILRI). Sequencing reactions were carried out with ABI PRISM Dye terminator Cycle Sequence Ready Reaction Kit (Applied Biosystems Inc., USA) on an ABI 310 DNA Sequencer according to manufacturer's instructions. Sequence data was analyzed with CLC Main Workbench version 7.0.3 software package (http://www.clcbio.com). Alignments were checked and corrected manually where necessary based on conserved regions. The 16S rDNA gene sequences obtained were compared with sequences in the public database using basic local alignment search tool (BLAST) on the National Centre for Biotechnology Information (NCBI) website (http://www.ncbi.nih.gov) in order to determine similarity with sequences in the GeneBank database (Altschul et al., 1990; Shyne et al., 2003). The 16S rDNA sequence identity was confirmed using the RDP Naive Bayesian rRNA Classifier Version 2.6 (Wang et al., 2007), Michigan State University, (https://rdp.cme.msu.edu). The 16S rDNA gene sequences with high similarities to those determined in the study were retrieved and used in the construction of a phylogenetic tree. Sequences were aligned using Molecular Evolutionary Genetics Analysis version six (MEGA6) (Tamura et al., 2013) software and the aligned sequences used to construct a maximum likelihood phylogenetic tree.

RESULTS

Isolation

There was high diversity of bacteria in hot waters of Lake Bogoria. Isolates grew well producing different colonies on nutrient agar. Colonial morphology description and Gram stain reaction (Table 1) of the isolates were the initial identification criteria used. Micro-morphological observation of the isolates revealed the organisms had typical characteristics of the group Bacilli including formation of spores and presence of branched rods. Of the 33 isolates that grew on nutrient media, 27.3% were Gram positive rods.

Characterization of isolates

All the nine isolates were subjected to baseline biochemical tests as suggested by Brock (2006). Physiological tests showed that all the isolates were catalase positive, all except D22, D5, D18 and D1 were indole positive. Most of the organisms utilized glucose as an energy source whereas none produced gas from sugar fermentation. S13 and D10 were lactose and/or sucrose fermenters as indicated by the butt and slant turning yellow. D22, S7 and D8 fermented glucose and produced enough acid to turn the butt yellow. D5, S17 and D18 did not change the color of the medium. Isolates S13, D10 and D8 produced hydrogen sulfide which was shown by blackening of the butt. The details of physiological and biochemical characteristics of the isolates are given in Table 2.

Table 2. Biochemical results for all nine Gram positive isolates.

Isolate	Biochemical test		Indole	Lactose fermentation	Glucose fermentation	H_2S production	Gas production
	Catalase	Motility					
S13	+	+	+	+	+	+	-
D10	+	+	+	+	+	+	-
S7	+	+	+	+	+	-	-
D22	+	+	+	+	+	-	-
D8	+	+	+	+	+	+	-
S17	+	-	-	-	-	-	-
D5	+	-	-	-	-	-	-
D18	+	-	-	-	-	-	-
D1	+	-	-	-	-	-	-

(+) Positive, (-) Negative.

Table 3. Antimicrobial assay of extracts obtained from the Gram positive isolates against *P. aeroginosa, S. aureus* and two strains of *E. coli* using disc diffusion method.

Organism	Zones of inhibition			
	***P. aeruginosa* ATCC 27853**	***S. aureus* ATCC 29213**	***E. coli* ATCC 35218**	***E. coli* ATCC 25922**
D10	R	R	R	R
S17	R	R	R	R
D18	R	R	R	R
S7	R	R	R	R
D22	+++	R	R	R
D1	+	R	R	R
D8	+	R	R	R
S13	+	R	R	+
D5	++	R	R	++
Control	+++	R	R	++

Inhibition was regarded as clear zones around the discs; (R) resistance, (+) weak inhibition, (++) strong inhibition, (+++) very strong inhibition. Control (Trimethoprim/sulfamethoxazole).

Bioassay against test bacteria

Antimicrobial screening in the present study was carried out at 37°C against four standard ATCC organisms. Five of the nine Gram positive isolates screened showed antagonistic activity against one or more of the standard test organisms. The four isolates that did not show any antagonistic activity against the test organisms were not investigated further. The isolates which were active against the standard organisms were identified with the following codes: D1, D22, D8 and D5 from sediment samples; S13 for surface sample. Antimicrobial activity of the five isolates ranged from weak to strong antagonism using the paper disc assay (Table 3).

16S rDNA gene sequences of the isolates

BLAST analysis of partial 16S rDNA gene sequences showed that the five isolates were closely affiliated with members of the genus *Bacillus.* A phylogenetic tree was constructed using nearest neighbor obtained from the GeneBank to show the phylogenetic position of each of the isolates studied (Figure 1). The isolates shared identities of between 88 and 99% with known *Bacillus* species. D1 and D5 clustered together with a bootstrap value of 63% and supported by a sequence identity of 96%. These isolates also clustered very closely with *Bacillus halodurans* (Acc. NR_025446.1) with isolate D5 having a sequence identity of 99% while isolate D1 had a sequence identity of 95% with the organism. Isolate D8 clustered with isolate S15 with a high bootsrap value of 99% and supported by a sequence identity of 88%. They also clustered with *Paenibacillus thiaminolyticus* (Acc. NR_113266 and NR_040887) and *Paenibacillus dentritiformis* (Acc. NR_042861) supported by a bootstrap value of 81% and sequence identities of 89, 89 and 90%, respectively for D8 and 88, 92 and 94% for S8,

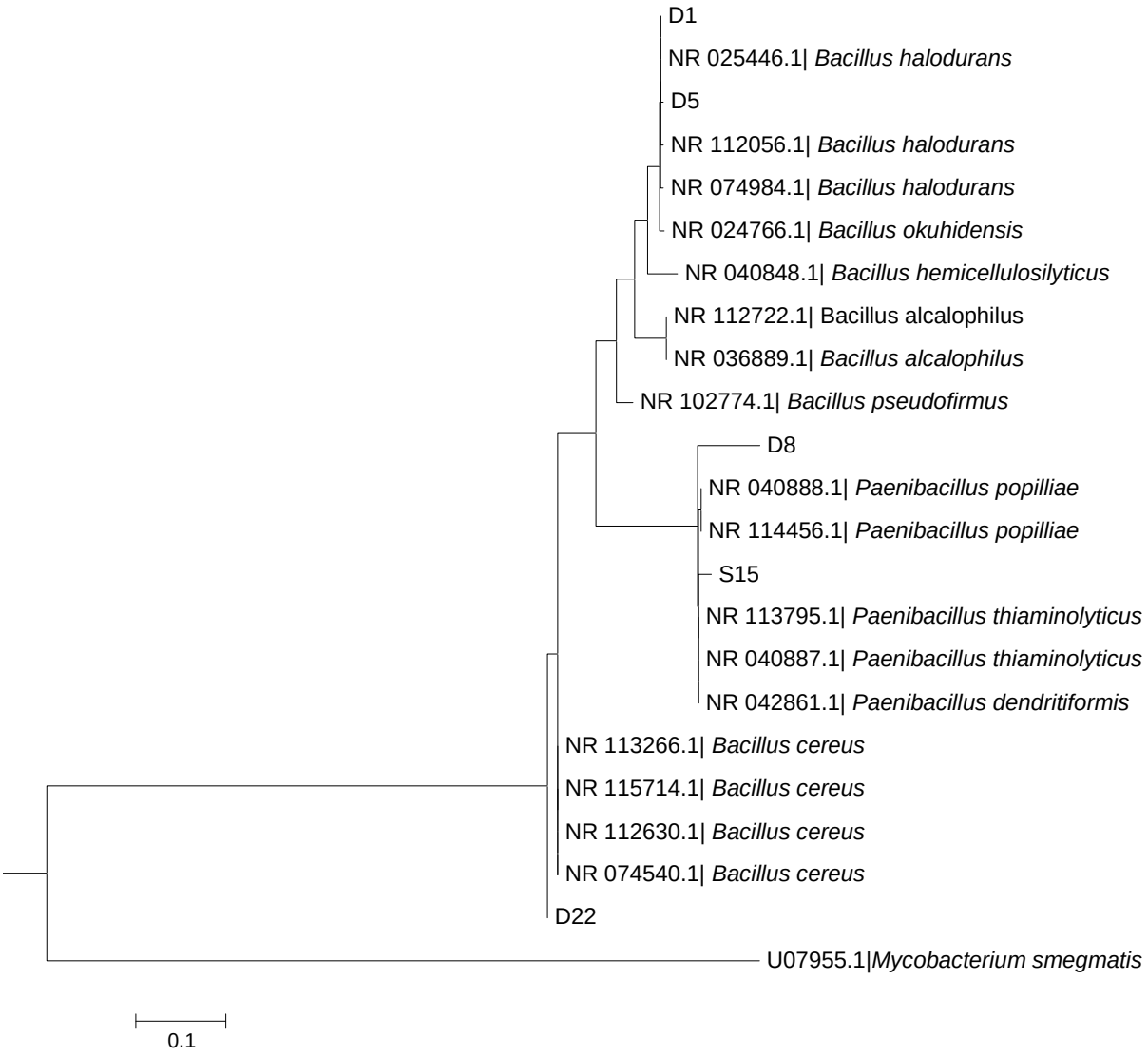

Figure 1. A maximum likelihood tree based on partial 16S rDNA gene sequences showing the phylogenetic relationships between the isolates with antibacterial activity against standard ATCC bacteria and related to *Bacillus* species. The tree is drawn to scale, with the branch lengths measured in the number of substitutions per site. The 16S rDNA sequence of *Mycobacterium smegmatis* was used as an outgroup.

respectively. D22 clustered with *B. cereus* group with a bootstrap value of 11% and sequence identities of 93%.

DISCUSSION

The main goal in this research was to bioprospect for thermophilic Bacilli from selected hotsprings in Lake Bogoria and to characterize those with antibacterial activity using morphological, biochemical and molecular methods. The results obtained showed the presence of a wide variety of bacteria in these environments predominantly Gram-negative rods. However, our main interest was focused on Gram-positive rods as they represent the group of interest, the Bacilli. Sampling was done on both surface (denoted as S) and sediment (denoted as D) samples. We found that more growth was obtained from samples obtained from below the surface. This region was warmer than the surface. However, we could only speculate that Bacilli preferred living in the warmer waters below the surface, since extensive literature search did not reveal any theory to support this.

Different scientists have reported inhibition of various organisms by microbial products produced by *Bacillus* species. Marahiel et al. (1997) isolated a strain of *B. subtilis* C126 from sugarcane fermentation that produced a polypeptide antibiotic, bacitracin, which inhibited the growth of *Micrococcus flavus.* A *Bacillus licheniformis* strain, 189,

isolated from a hot spring environment in the Azores, Portugal, was found to inhibit strongly growth of Gram-positive bacteria by producing peptide antibiotic (Mendo et al., 2004), further confirming the significance of extreme environments as sources of antimicrobials.

Initial screening in the present study was carried out at 37°C against four standard ATCC organisms. Inhibition was achieved against *P. aeruginosa* ATCC 27853 and *E. coli* ATCC 25922. No inhibition was noted against *S. aureus* ATCC 29213 and *E. coli* ATCC 35218 using the disk diffusion method. Diameters of zones of growth inhibition were approximated and taken to be related to susceptibility of isolates to the extracts (Wayne, 2009).

The crude extract antimicrobial profile we obtained against the standard ATCC organisms agrees with the findings of another study by researchers Maria et al. (2006), who also found strain ATCC 35218 to be resistant and strains ATCC 25922 and ATCC 27853 to be susceptible to phenolic extracts obtained from apple fruits. *S. aureus* ATCC 29213 was resistant to all extracts. In a different study, the effect of a “non-antibiotic” trimebutine was investigated against reference ATCC organisms (Kountouras et al., 2012). It was noted that the MIC and MBC were similar for *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 whereas for *S. aureus* ATCC 29213 and *E. coli* ATCC 35218 the MBC was higher. Five of the nine isolates showed inhibition against the standard organisms used indicating that the remaining isolates were not able to produce antimicrobial substances effective against any of the tested organisms.

Phylogenetic analysis showed all isolated organisms lied within the Bacilli group. Isolate D1 and D5 were closely related with 96% sequence identity. The two organisms closely grouped with *B. halodurans* (Acc. NR_025446.1) with D1 having a sequence identity of 99% while D5 had a 96% sequence identity showing that the two organisms could be different strains with the organism. The two could morphologically be separated from each other by D1 having raised colonies whereas D5 had flat colonies. *B. halodurans* has been found to contain unique genes and sigma factors that have aided its adaptation to more alkaline conditions (Takami, 2000) and strains of it have been known to produce a two-peptide lantibiotics, haloduracin, with activity against a wide range of Gram-positive bacteria (Lawton et al., 2007). Trent and Wilfred (2009) reported that though Haloduracin was active against Gram-positive bacteria, it had no potency against Gram-negative bacteria. Extracts from isolate D1 and D5 were active against Gram-negative bacteria indicating that they are different from *B. halodurans.*

Isolate D22 clustered with a group of different strains of *B. cereus* with a sequence identity of 93%. Typical *B. cereus* colonies are large, raised and opaque with undulate margins and lack the ability to split indole from the amino acid tryptophan (Wong et al., 1988). The colonies of isolate D22 were flat and the organism degraded tryptophan showing it to be different from typical colonies of *B. cerus.* Strains of *B. cereus* have been known to produce a few antibiotics such as zwittermicin A (Laura et al., 1994) that has a wide spectrum of activity (Laura et al., 1998) including against diverse Gram-negative bacteria and certain Gram-positive bacteria.

Isolate S15 was the only isolate obtained from surface water that had any inhibitory effect against the standard organisms. The isolate clustered with D8 with a sequence identity of 88% showing that their relation is significant. Isolate D8 clustered with *P. thiaminolyticus* (Acc. NR_113266 and NR_040887 and *P. dentritiformis* (Acc. NR_042861) with sequence identities of 89, 89 and 90% respectively, whereas S15 clustered with the same organisms with sequence identities of 88, 92 and 94% respectively. Both D8 and S15 produced cream colored colonies with undulate margins and the Gram appearance presented small Gram-positive rods. D8 had irregular colonies whereas S15 had spreading colonies. D8 and S15 are able to produce the enzyme catalase, hydrogen sufide and indole indicating that they could be different trains of the same organism. *Paenibacillus*, a genus initially included in the genus *Bacillus* was reclassified as a separate genus in 1993 (Priest and Collins, 1993). Like D8 and S15, *Paenibacillus* are Gram-positive endorespore forming bacteria (Ash et al., 1991) that develop complex colonies with intricate architectures (Ingham and Jacob, 2008).

The five isolates that showed antimicrobial activity are likely to be potential candidates for discovery of novel bioactive molecules for bio-control and biotechnological applications. The isolates present novel strains that should be investigated further to determine their identity, biochemical and biotechnological properties. Indeed the isolates that have active antimicrobial properties could be developed as good biological control agents.

Conflict of interests

The author(s) did not declare any conflict of interest.

ACKNOWLEDGEMENTS

The authors are grateful to Kenya Wildlife Service for allowing them to collect samples in Lake Bogoria, which is a protected site in Kenya and for providing technical assistance. They are also indebted to the National Council of Science, Technology and Innovation for their technical and financial support. This work was supported by Kenyatta University Departments’ of Biochemistry and Biotechnology and Medical Laboratory Sciences.

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Vol. 9(14), pp. 1044-1052, 8 April, 2015
DOI: 10.5897/AJMR2015.7447
Article Number: FB9934152324
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Effect of pH and temperature on the production and activity of *Schwanniomyces polymorphus* extracellular proteases in fermentation medium

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Received 24 February, 2015; Accepted 20 March, 2015

Proteases are among the most studied enzymes due to the great interest with regard to their cleavage of peptide bonds in industrial applications. With the goal of maximizing the advantages of these biocatalysts, new producers of proteolytic enzymes are being prospected from various microhabitats. This study aimed to select a yeast strain with high yield of extracellular proteolytic enzyme and characterize the yield and activity of theses enzymes. Among the 521 yeast isolates tested for proteolytic activity on solid medium, 20 isolates were selected for the determination of proteolytic activity in liquid medium. Enzymatic assay was performed using azocasein as substrate, and one unit of protease activity was defined as the amount of enzyme able to produce an increase in absorbance of 0.001/min and expressed in U/mL. A yeast extracted from Jabuticaba fruit, identified as *Schwanniomyces polymorphus* through the analysis of its rDNA sequence, showed the highest proteolytic activity. The optimal conditions for protease production, with a maximum value of 289.9 U.mL^{-1}, were pH 7.8, 28ºC and 72 h of fermentation. The activity of extracellular proteases contained in the culture supernatant was subjected to enzymatic characterization: the optimal pH was 8.0 and the optimal temperature was 35ºC. Therefore, among the yeasts isolated from tropical fruits, *S.* polymorphus presented the highest proteolytic enzyme activity under conditions of minimal fermentation, at temperature of 28ºC and pH of 7.8.

Key words: Proteolysis, fermentation, biochemical characterization, tropical fruits.

INTRODUCTION

The increasing body of knowledge about enzymes has motivated advances in enzymology (Lee and Huang, 2008). With this development of enzyme technology, the interest in industrial enzymes has increased greatly with the goal of maximizing the advantages of catalysis and minimizing the disadvantages to enable the use of these

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catalysts on a large scale (Buchholz et al., 2012). Enzymes like proteases, lipases and pectinases are one of the most important biomolecules with a wide range of industrial applications, having a great impact in the production of textiles, detergents, food, processing of fruit drinks and alcoholic beverages (Gurung et al., 2013).

Proteases, which catalyze the cleavage of peptide bonds in proteins and peptides, are among the most studied, encompassing a class of enzymes with multiple applications in biology and various other fields. Microbial proteases represent one of the three largest groups of industrial enzymes, corresponding to approximately 60% of the total sale of industrial enzymes worldwide (Rai and Mukherjee, 2010).

Due to its wide application in industry, proteases form the most studied group of hydrolases, prompting the exploration of new sources of these enzymes. The industrial demand for proteolytic enzymes with appropriate specificities and stabilities with regard to pH, temperature, metal ions, compatibility with such detergent compounds as surfactants and organic solvents continues to stimulate the search for new supply sources of these enzymes for research. Proteases with high activity and stability in certain ranges of pH and temperature are interesting for applications in bioengineering and biotechnology. In general, microbial proteases are extracellular in nature and are secreted directly into the fermentation broth during industrial production, thus simplifying the processing of the enzyme compared to the proteases obtained from plants and animals (Zavala et al., 2004; Lageiro et al., 2007).

Yeasts are another attractive host strains for cell-surface display systems due to their safety, ease of high-cell density cultivation and the capability of eukaryotic proteins folding and glycosylation (Gai, 2007).These properties make enzyme displayed yeast be of great value in biocatalytic process, especially in the industrial production using displaying yeast as whole-cell biocatalysts (Huang et al., 2012; Tanino, 2009). Yeasts are abundant in habitats in which carbohydrates are present, such as fruits, flowers and tree bark (Kurtzman and Fell, 1998). Yeast species have a great potential for the production of microbial enzymes for industry, and these microrganisms offer an alternative source of these enzymes.

The enzymes from these microrganisms are well distributed in nature, making these microrganisms preferable sources for bioprocess fermentation because they have a rapid growth rate and can be genetically constructed to produce enzymes with desired capabilities or simply for enzyme overproduction (Lucena et al., 2007).

The use of specific yeast strains with high enzyme activity is an important factor in industrial production, and the identification of yeasts with these characteristics is based on various molecular techniques, including the sequencing of D1/D2 domains of the 26S subunit of the rRNA gene (Gildemacher et al., 2006). These techniques are rapid, simple and accurate, making them suitable for the rapid screening and identification of yeast isolates (Kurtzman and Fell, 1998; Lachance et al., 2000; González et al., 2004; Fell et al., 2000).

Northeast Brazil presents a great diversity of tropical fruits that can harbor several species of yeast (Souza Filho et al., 2002), representing an important micro-habitat for a wide variety of yeast species due to the high sugar concentration and various pH values of the fruit (Lachance et al., 1982). The main goal of this study was to select and identify yeast isolates from the surface of tropical fruits that are able to secrete high amounts of proteases and to optimize the culture conditions for the production and activity of protease enzymes.

MATERIALS AND METHODS

Source of organisms

For this work, we used yeast strains belonging to the bank of the Applied Microbiology Laboratory at Universidade Federal de Sergipe, Sergipe, Brazil. The strains had been isolated from the fruits mangaba (*Hancornia speciosa* Gom.), pitanga (*Eugenia uniflora* L.), acerola (*Malpighia punicifolia* L.), umbu (*Spondias tuberosa* Avr. Cam) and jabuticaba (*Myciaria cauliflora* Berg) collected from the Sergipe State, Brazil.

Protease activity selection on plates

For the selection of yeast isolates showing the production of proteolytic enzymes, the different yeasts were grown on plates of milk-gelatin agar medium (5.0 g/L meat peptone, 3.0 g/L yeast extract, 12.0 g/L agar, 10.0 g/L skim milk and 10.0 g/L gelatin powder, pH adjusted to 7.2 with 1 M HCl) sterilized under conditions of 121°C and 15 lb pressure for 15 min. The plates were incubated at 30°C ± 3°C for 48 h. The presence of the proteolytic activity was indicated by formation of clear halos around the colony after the addition of 10% glacial acetic acid to the plates. The enzymatic activity was measured according to the modified method of Price (1982) by the value of the precipitation zone (PZ). Therefore, the enzyme activities were measured by dividing the diameter of the colony by the diameter of the colony plus the precipitation zone. The results were presented in code: the value 1 when PZ = 1.0 (no enzyme activity), value 2 when 0.63 <PZ <1.0 (moderate enzyme activity) and 3 when PZ value <0.63 (strong enzyme activity).

Production medium

For protease production, 1 mL of each isolate selected for extracellular protease activity on the solid medium was inoculated into 250 mL Erlenmeyer flasks containing 100 mL of liquid minimal medium for fermentation (MMF), (20.0 g/L glucose, 7.5 g/L meat peptone and 4.5 g/L yeast extract, 0,1% gelatine powder, pH adjusted to 7.2 with 1 M HCl) sterilized under conditions of 121°C, 15 lb pressure for 15 min. The incubation was performed in an incubator with shaking at 150 rpm and a temperature 28°C for 72 h.

Estimation of total protein

The total protein determination was performed using the Bradford

Method (1976), with bovine serum albumin (BSA) as the standard. The standard curve was prepared from concentrations of 0-100 $\mu g.mL^{-1}$ of BSA stock solution (1 mg/ml).

Enzymatic assay

The proteolytic activity in the culture supernatant was determined using the method of Weckenmann and Martin (1984), with modifications. The reaction mixture contained 100 µL supernatant and 1.0% azocasein (w/v) in 0.2 M Tris-HCl buffer (pH 7.2) containing 1.0 mM $CaCl_2$. The reaction mixture was incubated in a water bath at 37°C for 1 h and reaction was stopped by the addition of 1.0 mL of 20% trichloroacetic acid (TCA). The solution was centrifuged 23000 x *g* for 15 min at 4°C, and 200 µL of 3 M NaOH was added to 1.0 mL of the supernatant (Dosoretz et al., 1990; Leighton et al., 1973; Porto et al., 1996). The absorbance was spectrophotometrically (FEMTO 480) measured at 440 nm. One unit of proteolytic activity was defined as the amount of enzyme capable of producing an increase in absorbance of 0.001/ min and was expressed as U/mL. The specific activity was calculated by the ratio between the total protease activity and total protein concentration (U/mL). The experiments were performed in triplicate. Enzymatic activity was presented as mean and standard deviation of three replicates.

The effects of pH and incubation temperature on protease production

The yeast that was selected based on its high production of protease was propagated in the minimum liquid medium for fermentation (MMF) at pH values ranging from 6.5 to 7.8 and temperatures between 26 to 35°C in an incubator with shaking at 150 rpm. The production of protease by the yeast was determined as described above.

The effects of pH and incubation temperature on protease activity

To determine the optimal incubation temperature, we evaluated the activity of the enzyme at temperatures between 25 to 45°C in a water bath for 1 h. To determine the optimal pH, the protease was incubated at different pH values. Buffers used were: phosphate (pH 6.2 to 6.8) and Tris-HCl (pH 7.2 to 8.0) and reactions were incubated in a water bath at 37°C. The protease activity was determined as described above.

Electrophoretic procedures

Electrophoresis was performed by SDS-PAGE (polyacrylamide gel in sodium dodecyl sulfate) to identify the protease isoenzymes produced by the selected strain. The analysis followed the method of Laemmli (1970). The gel system consisted of a resolving gel (12%) plus 1% (w/v) gelatin and a stacking gel (5%). The proteolytic activity was detected according to Li et al. (1997). Briefly, after electrophoresis, the gel was washed twice in 50 mM Tris-HCl (pH 9.0) containing 5% (v/v) Triton X-100 for 15 min at 4°C. The gel was then incubated in 50 mM Tris-HCl (pH 9.0) for 12 hat 56°C to allow the degradation of the gelatin. The gel was stained with 0.1% Coomassie Brilliant Blue G250 (w/v) in 45% (v/v) methanol and 10% (v/v) acetic acid and destained by 30% (v/v) methanol and 10% (v/v) acetic acid. The band with proteolytic activity was observed as a clear colorless area.

Taxonomy of selected of yeast

The strain of yeast selected due to protease activity was identified based on the sequence of the conserved D1 and D2 domains of the large subunit 26S rRNA gene (Lachance and Starmer, 1998; Kurtzman and Suzuki, 2010). The code yeast sequence was analyzed using the ABI 3730 DNA Analyzer (Applied Biosystems), with the BigDye® Terminator Cycle Sequencing v3.1 kit, as a service provided by Centro de Estudos do Genoma Humano - USP, São Paulo, Brazil. BLAST (Basic Alignment Search Tool Locus) and nucleotide-nucleotide (BLASTn), which are available at the NCBI website (http://www.ncbi.nlm.nih.gov/blast/), and was used for a comparison with the sequences deposited in GenBank. The matrix of the sequence was determined by a similarity greater than or equal to 99%.

RESULTS

Screening for the enzymatic production of extracellular proteases on solid medium

The screening was performed using the collection of strains isolated from tropical fruits and was composed of 521 isolates. Of these, several yeast isolates were identified by their morphology and physiology as belonging to the following genera: *Candida*, *Cryptococcus*, *Pichia*, *Rhodotorula*, *Schizosaccharomyces*, *Saccharomyces*, *Trichosporon*, *Geotrichum*, *Pseudozyma*, *Leucosporidium*, *Kluyveromyces*, *Issatchenkia*, *Kloeckera*, *Myxozyma*, *Metschnikowia*, *Torulaspora*, *Zygosaccharomyces* and *Black Yeast*.

Through the production of extracellular proteases in plate containing milk-gelatin agar were halos of degradation that were detected and measured by testing PZ. Out of 521 isolates, only 20 presented Pz classified as moderate proteolytic activity (Table 1).

Identification of the selected isolates

The D1/D2 domains of the 26S subunit of the isolated 8Cb6 code yeast were sequenced. The nucleotide sequence obtained was compared with sequences deposited in the GenBank database using the BLASTn program 2.2.26 + (Altschul et al., 1997). According to the results, the selected microrganism in this study showed 100% similarity with *S. polymorphus* (accession number 319439558|FR774544.1).

Enzyme assay for extracellular proteolytic enzyme production into fermentation media

To analyze the production capacity of extracellular proteolytic enzymes in fermentation, 20 isolates were selected and their production capacities of total protein and proteolytic activities were measured. However, according to the results, the production of total proteins was not correlated with proteolytic activity, because not all isolates that produced high protein concentration also showed high activity proteolytic (Table 2). The largest production of proteolytic enzymes was observed in the

Table 1. Screening of yeast with extracellular proteolytic activity by Pz Method in plate containing solid medium Agar-gelatine-milk under a temperature of 30ºC ± 3° C for 48 h.

Species	Order code	Source substrate isolates	Precipitation zone (halo) (mm)	Enzyme activity	Pz
Candida valida	R03	Umbu	10	0.75	2
Kloeckera apis	R37	Acerola	05	0.75	2
Candida sergipensis	R78	Acerola	02	0.83	2
Black yeast	R474	Acerola	05	0.83	2
Cryptococcus humicolus	R301	Pitanga	05	0.86	2
Cryptococcus yarrowii	R354	Pitanga	05	0.87	2
Candida vartiovaarae	R374	Pitanga	05	0.75	2
N.I	3C1-4	Jabuticaba	05	0.83	2
N.I	4Ab2	Jabuticaba	04	0.90	2
N.I	4Ab7	Jabuticaba	02	0.91	2
N.I	6Ca3	Jabuticaba	02	0.83	2
N.I	8Aa4	Jabuticaba	02	0.91	2
N.I	8Ab4	Jabuticaba	02	0.84	2
N.I	8Ab12	Jabuticaba	02	0.79	2
N.I	8Ab13	Jabuticaba	04	0.83	2
N.I	8Ca1	Jabuticaba	02	0.80	2
N.I	8Ca3-4	Jabuticaba	02	0.83	2
N.I	8Cb4	Jabuticaba	03	0.79	2
N.I	8Cb6	Jabuticaba	05	0.75	2
N.I	10Aa12	Jabuticaba	05	0.75	2

NI = Not identified. The results were presented in the code value 1 determining PZ=1.0 (No enzyme activity), value 2 when 0.63 <PZ<1,0 (moderate activity) e value 3 when PZ≤0,63 (strong enzymatic activity).

Table 2. Proteolytic activity of yeast isolates in minimal medium for fermentation (MMF) under a temperature of 28ºC and pH 7.2 for 72 h.

Order code isolates	Source substrate isolates	Total protein ($\mu g.mL^{-1}$)	Proteolytic activity ($U.mL^{-1}$)	Specific activity ($U.\mu g^{-1}$)
R03	Umbu	11.7	4.77 ±4.80	0.41
R37	Acerola	10.4	8.0 ±2.50	0.77
R78	Acerola	49.4	ND	ND
R301	Pitanga	71.7	5.3 ±2.39	0.10
R354	Pitanga	54.3	23.3 ±7.72	0.43
R374	Pitanga	124.5	4.8 ±4.22	0.04
R474	Acerola	65.7	16.6 ±1.98	0.25
3C1-4	Jabuticaba	17.2	8.30 ±1.21	0.48
4Ab2	Jabuticaba	37.2	ND	ND
4Ab7	Jabuticaba	-	ND	ND
6Ca3	Jabuticaba	-	8.58 ±1.62	ND
8Aa4	Jabuticaba	181.9	76.1 ±3.18	0.42
8Ab4	Jabuticaba	158.0	77.2 ±5.46	0.49
8Ab12	Jabuticaba	3.7	6.86 ±3.23	1.85
8Ab13	Jabuticaba	21.6	15.6 ±3.72	0.73
8Ca1	Jabuticaba	-	9.0 ±0.33	ND
8Ca3-4	Jabuticaba	10.5	2.1 ±3.63	0.20
8Cb4	Jabuticaba	-	15.7 ±0.40	ND
8Cb6	Jabuticaba	139.0	257.4 ±9.4	1.85
10Aa12	Jabuticaba	58.6	ND	ND

Total proteins concentration below 3.0 $\mu g.mL^{-1}$; ND = no proteolytic activity detected.

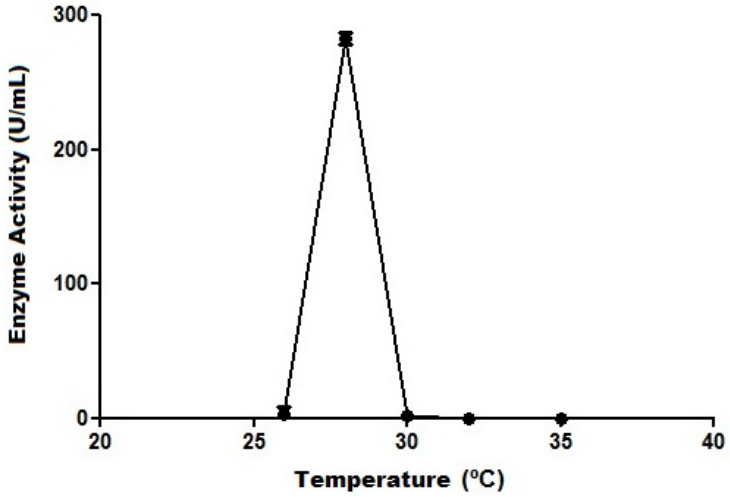

Figure 1. Influence of temperature on the production of extracellular proteolytic enzymes by *Schwanniomyces polymorphus* with minimal medium for fermentation (MMF) at different temperatures for 72 h.

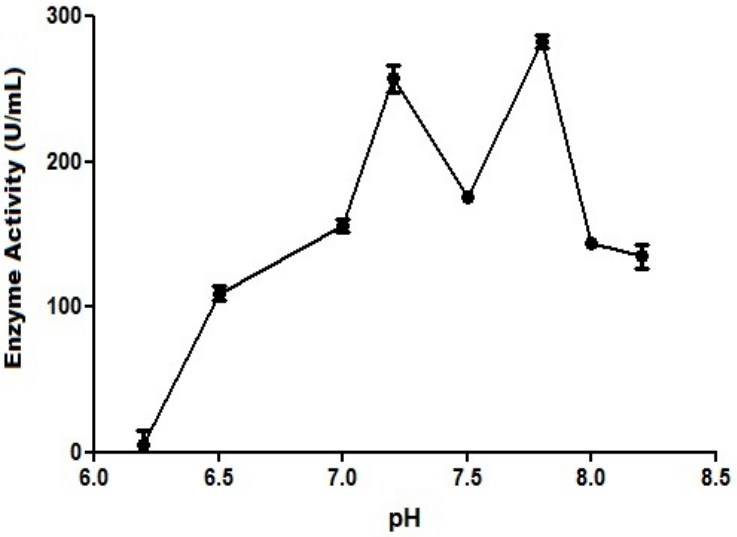

Figure 2. Influence of pH on the production of extracellular proteolytic enzymes by species *S. polymorphus* in different pH of incubation with minimal medium for fermentation (MMF).

isolate extracted from jabuticaba with order code 8Cb6 and was, therefore, selected for enzyme characterization and species identification.

Effects of temperature and pH on protease production in the selected isolate *S. polymorphus*

Effect of temperature

The optimum temperature for the maximum production of proteolytic enzymes was 28°C after 72 h of incubation with agitation (Figure 1). Other temperatures presented low or no production, with a temperature of 28ºC being ideal for growth and the secretion of proteolytic enzymes in the fermentation media.

Effect of pH

Using the optimum temperature of 28°C for extracellular enzyme production, the optimum pH for the maximum production of proteolytic enzymes was found to be pH 7.8 after 72 h of incubation with shaking (Figure 2). The proteolytic activity increased from pH 6.2, reaching a

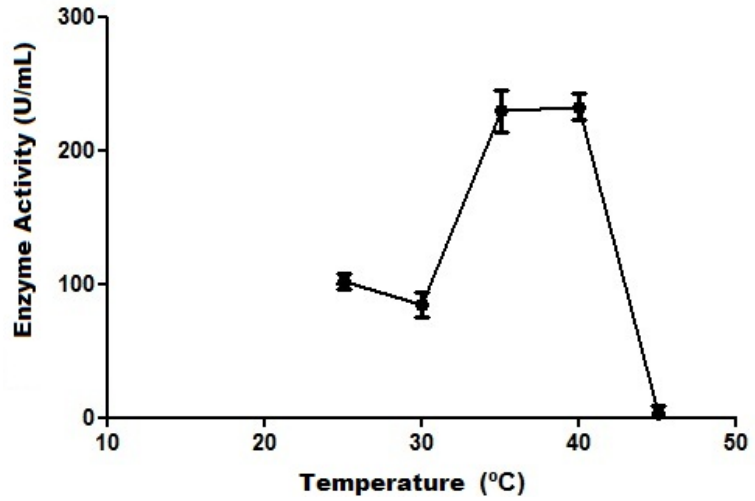

Figure 3. Effect of temperature on the activity of extracellular proteolytic enzymes produced by species *Schwanniomyces polymorphus* in incubation. The optimum temperature was determined by assay of protease activity in different incubation temperatures in a water bath.

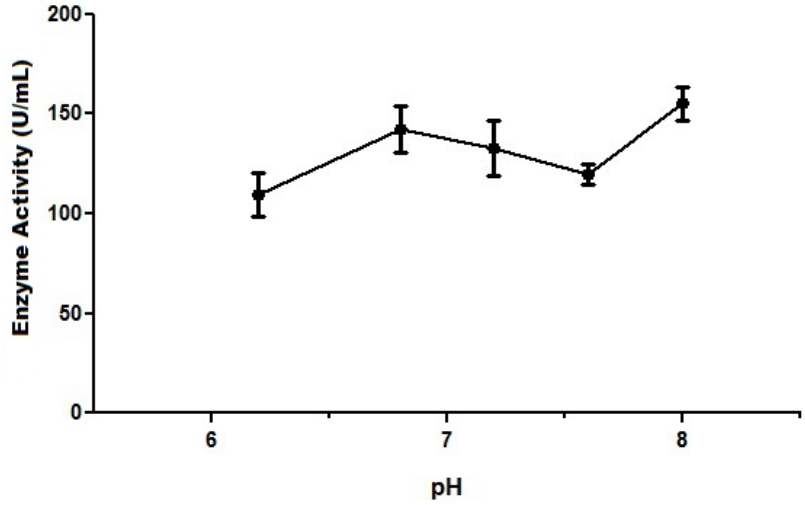

Figure 4. Effect of pH on the activity of extracellular proteolytic enzymes produced by species *Schwanniomyces polymorphus* in incubation. The optimum pH was determined by assay of protease activity in different pHs on incubation at 37° C in a water bath.

peak at pH 7.2 which was followed by a decrease at pH 7.5. Increase in pH values above 7.8 resulted in decreased proteolytic activity.

Enzymatic characterization

The activity of the proteolytic enzymes in the culture supernatant was evaluated regarding the optimum temperature. Reaction mixture was incubated in temperatures between 25 and 45ºC and the optimum activity was observed at temperatures between 35 and 40ºC with 229.9 and 232.8 U/mL, respectively (Figure 3). Above this temperature, the enzymes may denature, losing their catalytic activity. The enzymatic activity of the extracellular proteases was also evaluated at pH values from 6.2 to 8.0. Very similar activities were observed between pH 6.8 and 8.0, with enzymatic activity varying from 142.4 to 155.0 U/mL, respectively (Figure 4). These data allow us to conclude that the extracellular proteolytic enzymes remain active in environments in which the temperature is maintained at approximately 37.5 ± 2.5°C and near-neutral pH.

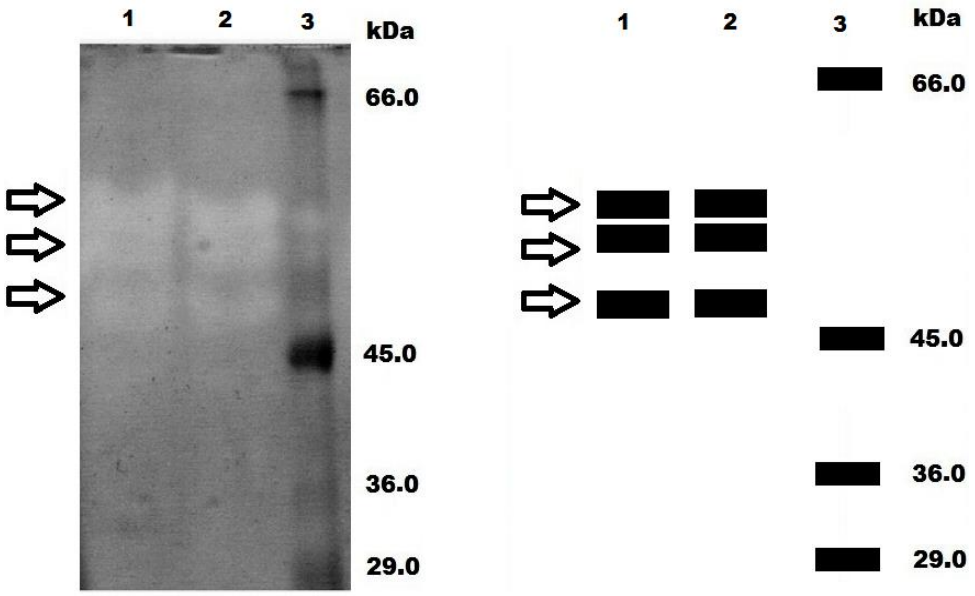

Figure 5. Polyacrylamide gel electrophoresis (12%) containing 1% gelatine for determination of the proteolytic activity of *Schwanniomyces polymorphus*. The bands with proteolytic activity present apperars clear and colorless in the gel. Line 1 and 2, supernatant from fermented; Line 3, Molecular marker.

Determination of the profile of proteolytic enzymes from *S. polymorphus*

The use of SDS-PAGE containing gelatin to determine the profile of *S. polymorphus* extracellular proteolytic enzyme activity presented a protease profile of three well-defined bands between 45 and 60 kDa (Figure 5). The bands suggest the presence of at least three proteases in the crude extract because the transparent gel bands indicate the enzymatic action of proteases on the substrate in the gel.

DISCUSSION

This study describes the production of extracellular proteases from isolates of yeast extracted from tropical fruits. Through the method described by Riffel and Brandelli (2002), we selected 20 of 521 isolated microorganisms based on activity of PZ, through the formation of clear halos in solid culture medium. Selected microorganisms demonstrated moderate production of extracellular proteases.

After this selection, the isolates that showed extracellular proteolytic activity were subjected to fermentation in nutrient broth for growth and the production of proteolytic enzymes (Porto et al., 1996; Alves et al., 2005). The effectiveness of protease production in fermentation medium was determined by an enzyme spectrophotometric assay using azocasein. The data obtained in this step were critical for the screening of the isolate with the highest production of extracellular proteolytic activity. To obtain optimal performance with respect to enzyme production during the development of microrganisms, different types of media are utilized to increase the amount of cells and also to increase the production of enzymes of industrial interest (Brumano et al., 1993; Papagianni and Moo-Young 2002). In this study, a medium with minimum requirements for fermentation was used to evaluate the potential of the isolates under conditions similar to their natural habitat. Under these culture conditions, an isolate with code 8Cb6 provided the largest activity of proteolytic enzymes extracellularly.

The use of yeast strains with high enzyme activity is an important factor in the industrial production of enzymes, as they are responsible for the transformation of raw materials into products for use in food, medicine or pharmacology. Therefore, the identification of yeast strains suitable for the fermentation and production of extracellular enzymes in certain industrial processes is critical (Fernández-González et al., 2004). After the screening of extracellular proteolytic activity, the identifycation of this strain was determined by sequencing the conserved D1/D2 region of the large 26S rDNA subunit, as described ((Fell et al., 2000; Lachance et al., 2000; Kurtzman and Suzuki, 2010), with 100% similarity with order Saccharomycetales and 100% similarity with *S. polymorphus*. This species was first reported in 2010 by Kurtzman and Suzuki (2010) in his work on the phylogenetic analysis of ascomycetes. Studies focused on the characterization of extracellular proteolytic enzymes

produced by this isolate which have not been reported to date.

The optimal growth conditions to protease activity and production of this isolate were determined. Temperature is one of the most important factors influencing the growth and survival of organisms, and temperatures of approximately 30 to 40ºC are generally used in the cultivation of fermenting microrganisms. The temperature is a critical parameter that influences relevant enzyme production and requires control (Chaloupka, 1985). The amount of total protein in the strains may rather demon-strate the ability to form zymogens, non-proteolytic enzymes or enzymes that degrade other substrates, in addition to enzymes that hydrolyze protein substances. The largest rates of enzyme production in this study occurred in the range of 28ºC. For the results presented for proteolytic activity in the media of the fermentation temperature of 28ºC (Table 2), it may be noted that some of the isolates showed low rates of total protein production, but with proteolytic activity, it was positive but without specific activity detection. However, the isolated 8Ab12 presented low rate of total protein, and proteolytic activity, but a low specific activity similar to the selected 8Cb6. However, this work prioritize the selection of yeast that has the highest proteolytic enzymes production considering a crucial factor in industrial production on a large scale and by the benefits considered in its specific activity. In similar studies, it was reported that some species of *Candida* sp. showed maximum proteolytic activity in a range of temperature close to 30°C (Chantawannakul et al., 2002; Kanekar et al., 2002; Neves et al., 2006). Another important factor is the pH of the fermentation culture medium. We found that the optimal range of enzyme production for the isolated strain was near pH 7.8, similar to that of the medium, pH 7.2. The pH affects the ionization of amino acids, which dictate the primary and secondary structure of enzymes and, therefore, control enzyme activity (Savitha et al., 2011). Proteases with distinct optimal values of pH have been reported, and the value presented in this paper describes proteases that are active at a near-neutral pH. The data presented are similar to those of the alkaline proteases from *Bacillus subtilis* CN2 reported in the pH range of 7-11 (Uchida et al., 2004). Bolumar et al. (2005) showed that neutral-alkaline proteases ranging in pH 6.0 to 12, showed beat around pH 8.0 with stability at temperatures above 75ºC, while there was decrease of stability at 37°C. It is possible that in this study, alkaline neutral isozymes exhibited optimum pH around 7.2 and 7.8 approaching that that has been reported in other studies.

It was observed that the optimal pH for most fungal proteases was in the pH range from 7.0 to 9.0. These observations showed that the optimum pH for most alkaline proteases was between pH 7.0 and 10.0. Typically, the proteases in this pH range generate a lower bitterness in protein-hydrolyzed food when compared to proteases of animal origin and are, therefore, quite valuable for use in the food industry (Rao et al., 1998). Focusing only on the characteristics of the enzymes produced by incubation at different pH and temperatures, our results indicate proteases belonging to the alkaline or neutral classes. Thus, the number of proteases produced in fermentation was determined by zymography and three proteases were observed with apparent molecular weights between 45 - 60 kDa.

Conclusions

Therefore, it is possible to conclude from this study that *S. polymorphus*, extracted from jabuticaba, showed the highest productivity of extracellular proteases among the yeast isolates used in this study. Optimum conditions to protease production in fermentation medium minimum were 28ºC, pH 7.8 and 72 h of growth. Biochemical characterization of supernatant proteases showed that the enzymes have optimum catalytic activity around 35°C and pH 8.0.

Conflict of interests

The authors did not declare any conflict of interest.

ACKNOWLEDGMENTS

The author Alexander França Santos is thankful for the opportunity granted by Universidade Federal de Sergipe (UFS), Aracaju, Brazil, and to Capes for providing the master's scholarship and Cleiton F. F. Melo Junior had a undergraduate scholarship from de program PIBIT/UFS.

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Vol. 9(14), pp. 1053-1059, 8 April, 2015
DOI: 10.5897/AJMR2015.7448
Article Number: A7E7E7152332
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Serotyping and antimicrobial resistance of enteropathogenic *Escherichia coli* and enterohemorrhagic *E. coli* O157 isolated from children under five years of age with diarrhea in rural Burkina Faso

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Received 24 February, 2015; Accepted 25 March, 2015

Diarrheagenic *Escherichia coli* (DEC) is one of the main causes of acute gastroenteritis in infants and young children in sub-Saharan Africa. This study investigated the prevalence, serotypes and antibiotic resistance patterns of enteropathogenic *E. coli* (EPEC) and enterohemorrhagic *E. coli* (EHEC) among diarrheal children in rural Burkina Faso. Stool specimens were collected from 280 children under 5 years of age visiting hospital due to acute diarrhea between July 2009 and June 2010. *E. coli* isolates were subjected to serotyping and their susceptibility to 9 antimicrobial agents was determined by the disc diffusion method. Sixty patients (21.4%) had DEC of which 54 strains (19.3%) belonged to EPEC O serogroups and six (2.1%) to EHEC O157 serotype. A higher rate of EPEC (91.6%) was found in patients aged less than 2 years. More than 70% of *E. coli* isolates were resistant to ampicillin, amoxicillin/clavulanic acid, tetracycline, trimethoprim-sulfamethoxazole and 28.3% to nalidixic acid. Twenty three (38.3%) of the isolated *E. coli* were ESBL producers with higher prevalence (56.5%) in children under 12 months of age. This study stresses the public health importance of diarrheal *E. coli* with serotypes of EPEC and EHEC O157 and their resistance to antibiotics in Burkina Faso.

Key words: *Escherichia coli* serotypes, antibiotics resistance, diarrheal children, Burkina Faso.

INTRODUCTION

Acute diarrheal disease is a major public health problem throughout the world; with over two million deaths occurring each year, and affecting mostly children under 5 years of age in developing countries (Kosek et al.,

2003; Bryce et al., 2005). The aetiological agents include a wide range of viruses, bacteria and parasites. Among bacterial pathogens, diarrheagenic *Escherichia coli* (DEC) are important agents of endemic and epidemic diarrhea worldwide (Nataro and Kaper, 1998). DEC are also reported to be a common cause of childhood diarrhea in developing countries with a higher incidence during the first 2 years of life (Rodrigues et al., 2002; Presterl et al., 2003). DEC strains can be divided into six main categories on the basis of distinct molecular, clinical and pathological features (Kaper et al., 2004): enteroaggregative *E. coli* (EAEC), enterohemorrhagic/Shiga-toxin producing *E. coli* (EHEC/STEC), enteroinvasive *E. coli* (EIEC), enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC) and diffusely adherent *E. coli* (DAEC).

Each pathotype possesses specific virulence genes associated with the disease symptoms. Moreover, the prevalence and other epidemiological features of these pathogens as causative agents of diarrhea vary in different regions, even within the same country (Kaper et al., 2004; Nguyen et al., 2005a; Nataro et al., 2006; Bonkoungou et al., 2012).

However, antimicrobial resistance in enteric pathogens as DEC complicates the situation in developing countries, where acute diarrhea is endemic and indiscriminate use of antimicrobial agents is common (Ahmed et al., 2009). Determination of the serotype and the antimicrobial susceptibility pattern is usually adequate for defining outbreak strains of DEC in order to determine appropriate therapies for patients with suspected *E. coli* infections (Nataro et al., 2007).

In Burkina Faso, studies using molecular techniques were conducted in urban area to determine the occurrence of DEC (Bonkoungou et al., 2012, 2013). These studies have shown that EPEC and ETEC are an important, though still unrecognized, cause of childhood diarrhea in Burkina Faso while EAEC was not associated with diarrhea. The present study was initiated to determine the prevalence of *E. coli* (EPEC and EHEC O157) in rural settings of Burkina Faso, using conventional methods. Also, *E. coli* serotypes, their antimicrobial susceptibility and the production of extended spectrum β-lactamases were investigated.

MATERIALS AND METHODS

Study design, population and settings

A prospective cross sectional study was conducted to determine the serotypes and antimicrobial susceptibility of diarrheagenic *E. coli* among children visiting hospitals in rural settings of Burkina Faso. This study was conducted between July 2009 and June 2010 (during one year) in two remote rural areas, in north (Gourcy, distance 140 km) and western (Boromo, distance 185 km) of the capital Ouagadougou, Burkina Faso (Figure 1). The main sources of income in these rural settings are subsistence farming, animal husbandry and small scale trade.

Stool samples were collected from 280 children (150 from Gourcy and 130 from Boromo) under 5 years of age who had acute diarrhea (as described by WHO, 2005) and/or were admitted to medical district hospital of Gourcy and Boromo.

Specimen collection

Fecal samples were taken by trained healthcare personnel using a swab transport system (M40 transystem Amies Agar Gel Without Charcoal; Copan Italia Spa, Brescia, Italy) and transported to the laboratory within 24 h of their collection for analysis. Information regarding the age and sex were recorded for each child using a questionnaire.

Isolation and serotyping of EPEC

Isolation of *E. coli* was carried out onto eosin methylene blue (Liofilchem, Italy) and plates were incubated at 37°C for 18-24 h. Additional tests such as urea, indole and API 20E were used to confirm the isolates. Following incubation, presumptive colonies (green color with a metallic sheen) were streaked onto Muller Hinton agar (Liofilchem, Italy). Serotyping was performed using serum nonavalent, trivalents I, II, III, IV and corresponding monovalent antisera according to the instructions of manufacturer (Bio-Rad, France).

Isolation and serotyping of EHEC O157

Isolation of *E. coli* was carried out onto Mac Conkey agar sorbitol (Liofilchem, Italy) and plates were incubated at 37°C for 18–24 h. Additional tests such as urea, indole and API 20E were used to confirm the isolates. Suspect colonies (Sorbitol-negative) were streaked for purity and confirmed as *E. coli* O157 using the dry spots test, specific for presumptive *E. coli* O157 according to the instructions of the manufacturer (Oxoid, England).

Antimicrobial susceptibility testing of the bacterial isolates

The susceptibility of *E. coli* strains to 9 antimicrobials was evaluated by the disc diffusion method on Mueller-Hinton agar (Liofilchem, Italy) at 37°C for 24 h. The antimicrobials evaluated were ampicillin (10 µg), amoxicillin/clavulanic acid (30 µg), chloramphenicol (30 µg), tetracycline (30 µg), trimethoprim-sulfamethoxazole (25 µg), norfloxacin (10 µg), nalidixic acid (30 µg), gentamicin (10 µg) and ceftriaxone (30 µg) (Liofilchem, Italy). The recommendations of "Comité de l'Antibiogramme de la Société Française de Microbiologie" (CASFM) were used for validating the antimicrobial test results. The inhibition zone were measured and tested isolates were classified as "resistant", "intermediate sensitive" or "sensitive" (CASFM, 2014). Extended spectrum β-lactamases (ESBL) activity was screened using the double-synergy test (CLSI, 2010). Multi-drug resistance was defined as resistance to at least three families of antibiotics.

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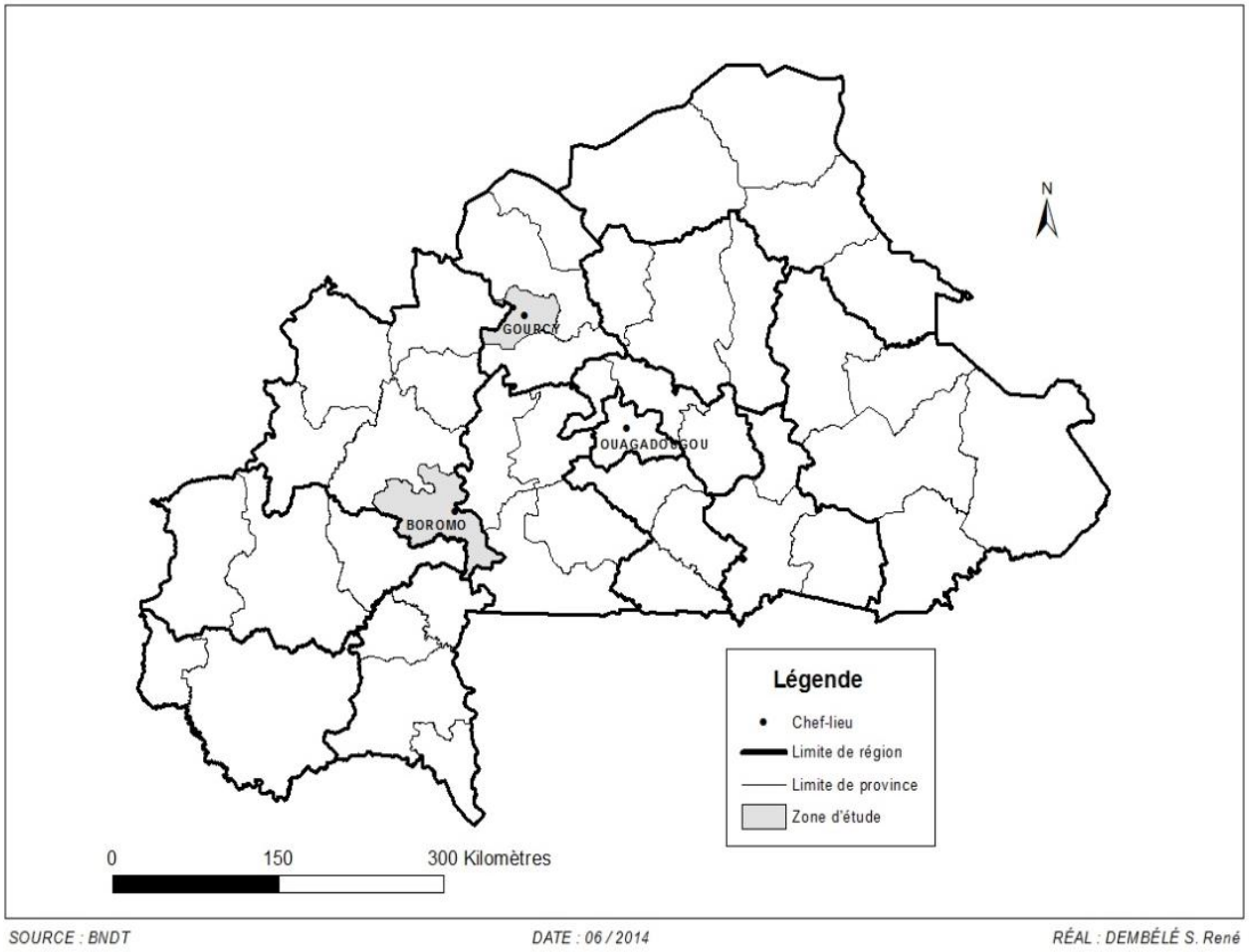

Figure 1. Map of Burkina Faso: colored parts = the study areas, Gourcy and Boromo.

Ethical considerations

The study protocol was approved by the Ethical Committee(s) of Burkina Faso. An informed verbal consent was obtained from the parents/guardians of every child before taking the stool samples.

Statistical analysis

The Fisher's exact test with two tailed *p* of Open Epi version 7.1.2.0 was used to determine the statistical significance of the data. A *p* value of ≤ 0.05 was considered statistically significant.

RESULTS

Prevalence of diarrheagenic *E. coli*

Of the 280 isolates analyzed, 60 (21.4%) DEC serotypes (both EPEC and EHEC O157 serotypes) were isolated. EPEC serotypes (O26, O55, O86, O111, O114, O119, O124, O125, O126, O127, O128 and O142) represented 19.3% of the total DEC isolated while EHEC O157 represents 2.1%.

Prevalence of DEC was high in children under 12 months of age

Our results showed that 38 (63.3%) of the DEC serotypes were isolated from patients under 12 months of age ($p < 0.05$). Seventeen (28.3%) of the DEC serotypes were isolated from patients aged 13-24 months of age and five (8.4%) from patients aged 25-59 months (Table 1). The sex distribution of *E. coli* showed 58.3% for females versus 41.7% of males ($p > 0.05$).

Large diversity of diarrheagenic *E. coli* serotypes

The 60 DEC isolated belonged to thirteen different serotypes (Table 2). The most commonly isolated serotypes were EPEC O127 (n = 11; 18.4%), EPEC O114 (n = 7; 11.7%), EPEC O26, EPEC O86 and EHEC O157 (n = 6; 10% each), EPEC O124 (n = 5; 8.3%). Several uncommon serotypes were EPEC O125 (n = 4; 6.7%), EPEC O128, EPEC O142, EPEC O55 (n = 3; 5% each), EPEC O111, EPEC O119, EPEC O126 (n = 2; 3.3% each) (Table 2). Thirty nine (65%) of the DEC were

Table 1. Age distribution of DEC serotypes in patients with diarrhea.

Serotypes	Age groups (months)			Total No. (%)
	0-12	13-24	25-59	
EPEC O26	5 (83.3)	1 (16.7)	0 (0)	6 (10)
EPEC O55	1 (33.3)	2 (66.7)	0 (0)	3 (5)
EPEC O86	3 (50)	3 (50)	0 (0)	6 (10)
EPEC O111	2 (100)	0 (0)	0 (0)	2 (3.3)
EPEC O114	5 (71.4)	2 (28.6)	0 (0)	7 (11.7)
EPEC O119	1 (50)	0 (0)	1 (50)	2 (3.3)
EPEC O124	4 (80)	1 (20)	0 (0)	5 (8.3)
EPEC O125	2 (50)	1 (25)	1 (25)	4 (6.7)
EPEC O126	1 (50)	1 (50)	0 (0)	2 (3.3)
EPEC O127	8 (72.7)	3 (27.3)	0 (0)	11 (18.4)
EPEC O128	1 (33.3)	2 (66.7)	0 (0)	3 (5)
EPEC O142	1 (33.3)	0 (0)	2 (66.7)	3 (5)
EHEC O157	4 (66.6)	1 (16.7)	1 (16.7)	6 (10)
Total No. (%)	38 (63.3)	17 (28.3)	5 (8.4)	60 (100)

Table 2. Prevalence of *E. coli* serotypes in the two regions.

Serotypes	Boromo No. (%)	Gourcy No. (%)	Total No. (%)
EPEC O26	3 (5)	3 (5)	6 (10)
EPEC O55	2 (3.3)	1 (1.7)	3 (5)
EPEC O86	3 (5)	3 (5)	6 (10)
EPEC O111	1 (1.7)	1 (1.7)	2 (3.4)
EPEC O114	6 (10)	1 (1.7)	7 (11.7)
EPEC O119	2 (3.3)	0 (0)	2 (3.3)
EPEC O124	3 (5)	2 (3.3)	5 (8.3)
EPEC O125	3 (5)	1 (1.7)	4 (6.7)
EPEC O126	2 (3.3)	0 (0)	2 (3.3)
EPEC O127	5 (8.3)	6 (10)	11 (18.3)
EPEC O128	2 (3,3)	1 (1.7)	3 (5)
EPEC O142	3 (5)	0 (0)	3 (5)
EHEC O157	4 (6.7)	2 (3.3)	6 (10)
Total No. (%)	39 (65)	21 (35)	60 (100)

isolated from Boromo compared with twenty one (35%) from Gourcy (p = 0.001).

Antimicrobial susceptibility testing

Out of 60 DEC isolates, 53 isolates (88.3%) were resistant to at least one of the 9 antibiotics. Eighty-five percent (85%) were resistant to tetracycline (TE) followed by 76.7% to ampicillin (AMP), 73.3% to amoxicillin/clavulanic acid (AMC), 71.7% to trimethoprim-sulfamethoxazole (SXT), 28.3% to nalidixic acid (NA), 20% to norfloxacine (NOR), 11.7% to gentamicin (CN), 10% to chloramphenicol (C) and 8.3% to ceftriaxone (CRO) (Figure 2). It was also observed that the percentage of isolates resistant to any of the antibiotics tested were higher in young children (0-12 months) than in others (13-24 and 25-59 months age). Multiple drug resistance (resistance to three and more families of antibiotics) was observed in this study for 44 isolates (73.3%).

Extended-spectrum β-lactamases (ESBLs) producing *E. coli*

In the present study, 23 out of 60 isolates (38.3%) were found to be ESBL producers. This ESBL producing *E. coli* were mainly EPEC (n=22/23; 95.6%). The ESBL production varies by age groups as follows: 13 (56.5%) from patients under 12 months of age, 9 (39.1%) from

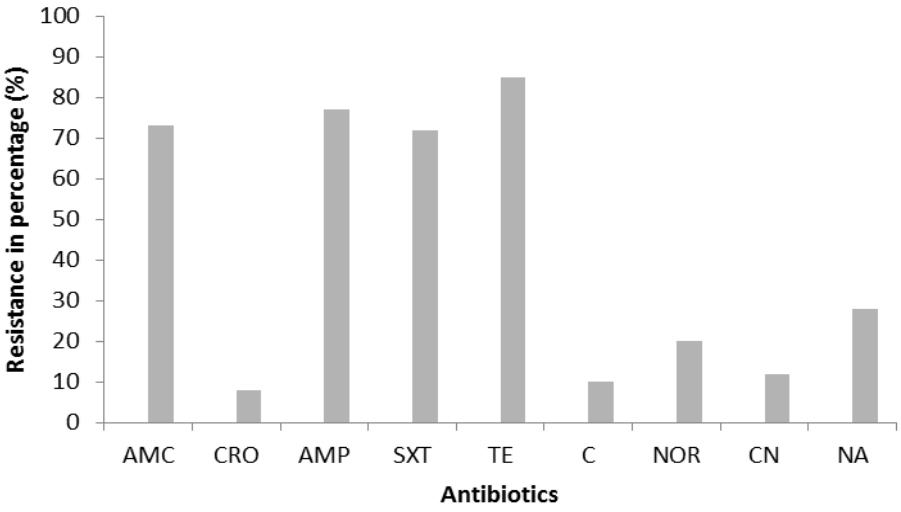

Figure 2. Global antibiotic resistance. AMC = amoxicillin/clavulanic acid, TE = tetracycline, AMP = ampicillin, SXT = trimethoprim-sulfamethoxazole, NA = nalidixic acid, NOR = norfloxacin, CN = gentamycin, C = chloramphenicol, CRO = ceftriaxone.

Table 3. Age distribution of ESBLs producing *E. coli* in patients with diarrhea.

Age groups (months)	ESBLs producing *E. coli* No. (%)
0-12	13 (56.5)
13-24	9 (39.1)
25-59	1 (4.4)
Total No. (%)	23 (100)

patients aged 13-24 months of age, 1 (4.4%) from patients aged 25-59 months (Table 3).

DISCUSSION

In this study, we investigated the occurrence of two DEC pathogroups by serotyping in 280 stool samples from children with diarrhea. The overall prevalence of DEC isolated was 21.4% and the positive rate of EPEC was 19.3%. Similar observations have been made previously in Burkina Faso (16%) (Bonkoungou et al., 2012) and China (22.2%) (Chen et al., 2014). A slightly higher percentage of EPEC (26.5%) was reported in South Africa (Galane and Roux, 2001) using the serotyping by slide agglutination method. Although large outbreaks of infant diarrhea due to EPEC have largely disappeared from industrialized countries, EPEC remains an important cause of potentially fatal infant diarrhea in developing countries (Trabulsi et al., 2002).

The prevalence of EHEC O157 (2.1%) observed in our study is similar to those previously reported in Burkina Faso (Bonkoungou et al., 2012) and other parts of the world (Moyo et al, 2007; Vilchez et al, 2009; Chen et al., 2014). Researchers from South Africa (Galane and Roux, 2001) found a relatively higher rate of EHEC O157 (7.7%) in their study. This difference may be understood as serotype O157 isolation frequency varies depending on the country (Germani, 1995; Tarr, 1995; Cobejic et al., 1996). This way, somewhere else in Africa and in non-epidemic settings, EHEC appears to be more frequent in adults rather than in children (Okeke et al., 2003).

Considering the different age groups (Table 1), DEC serotypes were detected in all of them, but most cases (88.3%) occurred among children <2 years old which is in agreement with what has been previously reported in Burkina Faso (Bonkoungou et al., 2012). EPEC was mainly detected among children aged younger than 2 years (80%) and EHEC mainly in children under the age of 1 year (80%), as previously reported in Burkina Faso (Bonkoungou et al., 2012) and Tunisia (Al-Gallas et al., 2007). EPEC can cause infection in humans through ingestion of contaminated food and animals feces (Lee et al., 2009; Alonso et al., 2011; Kagambèga et al., 2012). According to Norazah et al. (1998) in Malaysia, EPEC presence in food indicated fecal contamination related to unhygienic practices. A previous study conducted in Burkina Faso, further showed that the flies landing on food or the surface of equipment can spread bacteria, since they have contact with dirty matters (Barro et al., 2006). Contamination of children can also occur through the feces of the animals because of the high presence of these animals in rural settings. Moreover, it is possible that the freely roaming pigs in Burkina Faso get infected through cattle feces and then serve as an additional possible reservoir for human infections and pigs may therefore be a noteworthy reservoir for several DEC pathogroups in this country (Kagambèga et al., 2012).

Our results showed high resistance rates against the

commonly used antimicrobial agents; ampicillin, amoxicillin/clavulanic acid, tetracycline, cotrimoxazole and nalidixic acid which is in agreement with results from Sudan (Atif et al., 2000) and Chad (Bessimbaye et al., 2013). Similarly, a study in Vietnam reported 77.2% resistance to chloramphenicol and 19.1% resistant to nalidixic acid (Nguyen et al., 2005b). *E. coli* is an important opportunistic pathogen that has shown an increasing antimicrobial resistance to most antibiotics (Miranda et al., 2004). There are several reports from different parts of the world about antimicrobial sensitivity with varying results showing the bacterial resistance to antimicrobials differs widely in different areas (Nguyen et al., 2005b; Moyo et al., 2010; Al-Hussaini et al., 2014). Antimicrobial resistance has become a major medical and public health problem and the reason for high levels of resistance of antibiotics in the study areas here might be the overuse and misuse of antibiotics. This argument is supported by several authors showing that the main factor responsible for development and spread of bacterial resistance is injudicious use of antimicrobial agents (Urassa et al., 1997; Al-Hussaini et al., 2014).

Of the 60 diarrheagenic *E. coli* tested for ESBL production, 23 (38.3%) were found to be ESBL producers. This shows that the prevalence of ESBL producers in rural area of Burkina Faso is higher than those found in Tanzania and Democratic Republic of the Congo (29%) (Moyo et al., 2010; Mshana et al., 2011; De Boeck et al., 2012) and in Libya (13.4%) (Ahmed et al., 2014). Most of the ESBL producers were EPEC (95.6%) which is similar to what is reported from Iraq (96.4%) (Shamki et al., 2012). Investigating the ESBL production is important for understanding the epidemiology of DEC serotypes and may provide information on emerging serotypes. The emergence of extended-spectrum-β-lactamase (ESBL)-producing *E. coli* in the community has been one of the most significant epidemiologic changes in infectious disease in recent years (Ahmed et al., 2014). During the last 2 decades, extended-spectrum β-lactamases (ESBLs) found in Gram-negative bacilli have emerged as a significant mechanism of resistance to oxyimino-cephalosporin antibiotics (Paterson et al., 2003). Organisms producing ESBLs are clinically relevant and remain an important cause for treatment failure with cephalosporins (Pitout et al., 2005). However, further investigations, including the use of DNA probes and serotyping (specific for a particular group of *E. coli*), are necessary to allow precise identification and epidemiological studies of these pathogens.

This study demonstrates the high occurrence of diarrheal EPEC and EHEC O157 in the rural area of Burkina Faso. These pathogenic bacteria were also shown to be highly resistant to antimicrobials, indicative of public health risks. Therefore, increased awareness in the rural community about food and environmental hygiene and stopping indiscriminate use of antimicrobials are useful to preserve population health.

Conflict of interests

The author(s) did not declare any conflict of interest.

ACKNOWLEDGEMENTS

The authors express their gratitude to the “Centre de Recherches en Sciences Biologiques, Alimentaires et Nutritionnelles (CRSBAN)” for the fund provided for the study. They also thank the parents and guardians of the study children and the Boromo and Gourcy Health District Authorities for their cooperation.

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