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Full Length Research Paper

Antimicrobial resistance patterns of *Klebsiella* isolates from clinical samples in a Saudi hospital

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Klebsiella **infection is often the leading cause of morbidity and mortality. Resistance pattern of antimicrobial susceptibility to commonly prescribed drugs were studied in *Klebsiella* isolates from a hospital in Madinah, Saudi Arabia. Clinical samples were collected from 6840 patients and screened for *Klebsiella* species over a period of 14 months. The strains were identified using microbiological and biochemical tests while the antibiotic susceptibility was performed by disk diffusion assay. Of all the positive samples isolated, only 275 samples were identified as *Klebsiella* species. Of these 66% were from males indicating that females are less vulnerable. Maximum isolates (66 to 70%) were collected from sputum and wound swabs of males. About 90% species were isolated from wound, sputum and catheter tips swabs. Antimicrobial sensitivity was studied using seventeen different antibiotics. Results revealed that antibiotic Imipenem has the highest sensitivity of 99.5% while Ampicillin revealed 100% resistance. The prescription of Imipenem antibiotic is recommended for the treatment of *Klebsiella* infections. In case of resistance to Imipenem, other antibiotics mainly Ceftazidime, Aztreonam, Ciprofloxacin, Cefoxitin and Piperacilline may be recommended. In acute cases, use of combined antimicrobial therapy may be required. Results also indicate that the intensity of *Klebsiella* infections is higher during summers (46.5%) than in winters (27.2%), while autumn (13.9%) and spring seasons (12.5%) showed the least recorded percentage. Our study will help in persistent and continuous monitoring of antimicrobial susceptibility and supplement the already available data on prevalence of antimicrobial resistance patterns of *Klebsiella*.**

Key words: *Klebsiella,* Enterobacteriaceae, antimicrobial susceptibility pattern, antibiotic resistance, multi-drug-resistance.

INTRODUCTION

Antibiotic resistance is a major problem world-wide including Saudi Arabia (Yezli et al., 2014). Several factors are responsible for the emergence of multidrug resistance, the major being wide-spread use of broad

spectrum antibiotics (Ventola, 2015). The resistant bacteria can be easily transmitted among patients and from healthcare workers to patients and *vice versa* (Chan, 2012; Aly and Balkhy, 2012). Besides, the resistance pattern to antimicrobial agents has changed dramatically. With time an increase in the rate of antimicrobial resistance has been observed in several species of Gram-negative bacteria (Mahmoud et al., 2016; Chastre, 2008). The resistance rates are higher in Intensive Care Units (ICUs). It is even more alarming to see that the antibiotics used to treat bacterial infections in humans are also used in animal industry (CDCP, 2013). There is hence a need for novel and more stringent antibiotic prescription guidelines to deal with this problem.

The *Klebsiella* species are non-motile rod shaped Gram-negative bacteria with a prominent capsule. These opportunistic human commensals belong to the family Enterobacteriaceae where *Klebsiella pneumoniae* and *Klebsiella oxytoca,* are two species that are responsible for the majority of infections (Nordamann et al., 2009; Algowaihia et al., 2016). These inherently opportunistic nosocomial pathogens are known to cause pneumonia specifically in chronic alcoholics. They also cause infections in urinary tracts, gastrointestinal tracts, surgical wound infections and blood (Kamal et al., 2017). *Klebsiella* species are often resistant to many antibiotics, including cephalosporins and aminoglycosides (Manikandan and Amsath, 2013).

K. pneumoniae has been identified as an important common pathogen for nosocomial pneumonia, septicaemia and wound infections (Jadhav et al., 2012). Epidemic and endemic nosocomial infections caused by *Klebsiella* species are leading causes of morbidity and mortality. In the United States of America, *Klebsiella* is the eighth most important infectious pathogens in hospitals (Manikandan and Amsath, 2013). In Saudi Arabia, the predominant organisms that cause UTIs are Gram negative bacteria which are highly resistant to commonly used oral antibiotics (Yezli et al., 2014).

Clinical isolates of *K. pneumoniae* are generally resistant to a wider range of antibiotics, and virtually always naturally resistant to ampicillin and amoxycillin. β-lactam antimicrobial agents are the most common treatment options for such infections (Jadhav et al., 2012). Extended-spectrum β-lactamases (ESBLs) are often found in the Enterobacteriaceae family of Gram-negative bacilli, particularly *Klebsiella* species, *Escherichia coli* and *Proteus mirabilis* (Al Johani et al., 2010). *Klebsiella* species that produce *Klebsiella pneumoniae carbapenemase* (KPC) are of serious concern as they have high-level resistance against most antibiotics. These KPC enzymes efficiently hydrolyze carbapenems, as well as other β-lactam antibiotics (Paterson et al., 2005). A study comparing ICU isolates from KSA and Kuwait showed that *E. coli* and *Klebsiella* species demonstrated multidrug resistance to monobactams, cephems, and aminoglycosides (Rotimi et al., 1998).

The risk of bacterial infection increases with long term antibiotic exposure, prolonged stay in ICUs, other severe illnesses, and instrumentation or catheterization (Jiao et al., 2015). Since there may be variations in antibiotic susceptibility depending on factors like gender, location, age, etc., it is essential to understand the antimicrobial susceptibility pattern of *Klebsiella* species so that relevant measures can be taken to control the rapid spread of multidrug resistance. Hence, a descriptive hospital based study was undertaken to isolate and identify *Klebsiella* species from various clinical samples using microbiological and biochemical methods and to study gender distribution of isolates and their antimicrobial sensitivity profile.

MATERIALS AND METHODS

Sample collection

Different clinical samples such as sputum, wound swab, cerebrospinal fluid (CSF), tracheal aspirate (Tr. asp.), throat aspirate (Th. asp.), catheter Tip, pus, abdominal abscess (Abd. ab.), ear swab, peritoneal wound swab (Peri. w.s.), pleural fluid (Pler. fluid), App: Appendix (App.), bile, Urethra (Ur), and semen were collected from 6840 patients suspected of bacterial infection at King Fahd Hospital at Madinah, Saudi Arabia. Clinical samples were cultured to isolate the organisms. Demographic data such as sex of the patients was recorded prior to sample collection.

Cultivation and Identification

The clinical samples were collected according to Centers for Disease Control and Prevention Specimen Collection Guidelines (CDCP, 2013), aseptically inoculated on plates of Blood agar, Chocolate agar, Cystine-Lactose-Electrolyte-Deficient (CLED) agar and MacConkey agar (Oxoid Cambridge, UK) and incubated at 37°C for 24 h.

Identification was done based on morphological characteristics of the colonies including size, shape, colour, pigmentation and haemolytic nature.

Biochemical characteristics

Suspected *Klebsiella* colonies isolated were further identified through biochemical tests (Barrow and Felthan, 2003) using standard procedures and Phoenix automated microbiology 100 ID/AST system (Becton Dickinson Company, Sparks, Md.).

Antimicrobial susceptibility test

Susceptibility to antimicrobial agents was determined by using the disk diffusion method (Oqunshe, 2006), and Phoenix automated

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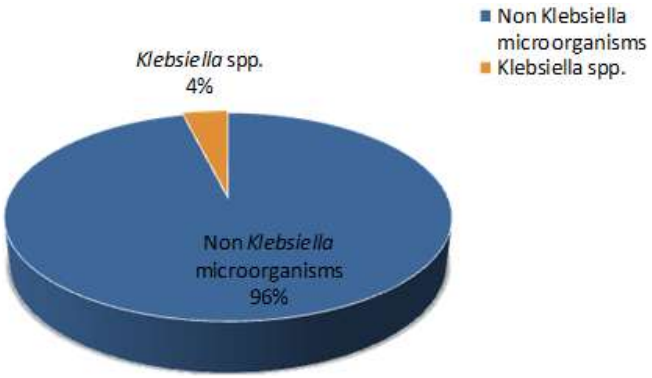

Figure 1. Percentage of *Klebsiella* spp. in comparison to other clinical isolates.

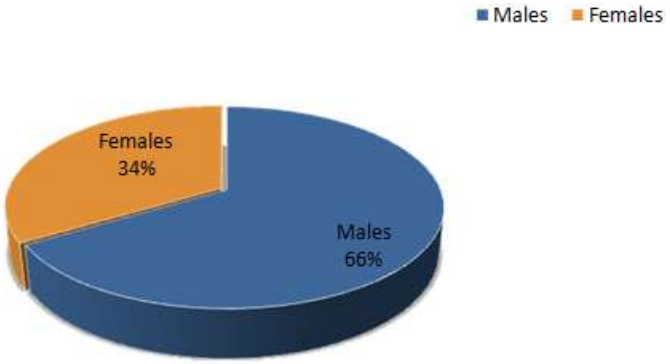

Figure 2. Percentage *Klebsiella* spp. isolates positive in males and females.

microbiology 100 ID/AST system (Becton Dickinson Company, Sparks, Md.). The following antimicrobial agents (obtained from BDH London, UK) were used: ampicillin (AP), augmentin (AUG), gentamycin (GM), cefoxitin (FOX), cephalothin (KF), cotrimoxazole (TS), amikacin (AK), ceftazidime (CAZ), aztreonam (AZT), piperacilline (PRL), imipenem (IMP), ciprofloxacin (CIP), cefpiramide (CPM), meropenem (Merop), tazobactam (Taz), colistin (Col), and nitrofurantoin (Nitro).

The inocula were prepared by growing the various *Klebsiella* strains on separate agar plates and colonies from the plates were transferred with a loop into 3 ml of normal saline. The density of these suspensions was adjusted to 0.5 McFarland standards. The surface of Muller-Hinton agar (Oxoid Cambridge, UK) plate was evenly inoculated with the organisms using a sterile swab. The swab was dipped into the suspension and pressed against the side of the test tube to remove excess fluid.

The wet swab was then used to inoculate the Muller-Hinton agar by evenly streaking across the surface. By means of a Disc Dispenser (Oxoid Cambridge, UK), the antibiotic discs were applied onto the surface of the inoculated agar and the plates were incubated overnight at 37°C. The diameter of zone of growth-inhibition observed was measured and compared to the chart provided by Clinical and Laboratory Standards Institute (CLSI, 2009).

RESULTS AND DISCUSSION

Patients, especially in ICUs are at risk of acquiring infections, most of which are associated with the use of invasive devices such as catheters and mechanical ventilators (Shulman and Ost, 2005). The ever increasing crisis of antibiotic resistance has been attributed to the overuse and misuse of antibiotics which needs to be regulated under stringent guidelines (Ventola, 2015).

The antibiotic-resistant infections place a lot of burden on the economy of any country as health care costs rise (Golkar et al., 2014). The most serious Gram-negative infections occur in health care settings are most commonly caused by Enterobacteriaceae (mostly *Klebsiella pneumoniae*) (Ventola, 2015; Yezli et al., 2014). Untreatable or difficult-to-treat infections due to Carbapenem-resistant *Enterobacteriaceae* (CRE) bacteria are on the rise among patients in medical facilities. Each year around 1.5lakh, *Enterobacteriaceae* infections occur in the U.S. which is health care-associated and approximately 600 deaths result from infections caused by the two most common types of CRE, carbapenem-resistant *Klebsiella* species and carbapenem-resistant *E. coli* (CDCP, 2013).

There are relatively few studies that deal with antibiotic use in ICUs in Saudi Arabia (Rotimi et al., 1998; Al Johani et al., 2010). In the present work, we study the antimicrobial resistance patterns of *Klebsiella* isolates from patients of a big hospital in Madinah, a city visited by lakhs of pilgrims every year. Exactly 6840 clinical samples were collected from patients suspected of bacterial infection in King Fahad Hospital during a period of 14 months. Samples were screened and in total 275 isolates were identified as positive for *Klebsiella* species, representing about 4% of all the positive samples (Figure 1). Some samples like urine, blood, ascetic fluid, nasal swabs, axilla and perineum showed complete absence of isolates. It was observed that of the positive *Klebsiella* isolates, 66% were from males while 34% from females (Figure 2) indicating that males show greater vulnerability for these infections. *Klebsiella* species were positive for nitrate reduction test; urease test, citrate utilization test, Voges-Proskauer test, lactose fermentation; and negative for H_2S gas production, oxidase test, methyl-red test, indole test, and motility.

Table 1 gives an estimation of the number of male and female samples isolated from different sources. Majority of the *Klebsiella* species (66 to 70%) were isolated from the sputum and wound swabs of male patients. A higher isolation rate from sputum and wounds has been reported in earlier studies as well (Ali and Ali, 2014; Namratha et al., 2015). Of the 126 sputum samples that were positive for *Klebsiella*, 84 were obtained from males and 42 from females. In case of wound swabs, the male to female ratio was 66:29 indicating more than double cases in men. It has been reported earlier that adult males are more susceptible to infection with *Klebsiella* species than adult females (Janda and Abbott, 2006). A similar situation was seen with catheter tips where there were 16 male samples against 12 females.

Table 1. Number of male and female samples collected from different sources.

Sex	Source of samples														
	Sp	WS	CSF	Tr	Th	Cath	Pus	Abd	Ear	Peri	Pler	App	Bile	Ur	Semen
M	84 (66.6)	66 (69.5)	3 (100)	0 (0)	4 (50)	16 (57.1)	1 (50)	1 (33.3)	1 (100)	1 (50)	2 (100)	0 (0)	0 (0)	1 (100)	1 (100)
F	42 (33.4)	29 (30.5)	0 (0)	1 (100)	4 (50)	12 (42.9)	1 (50)	2 (66.7)	0 (0)	1 (50)	0 (0)	1 (100)	1 (100)	0 (0)	0 (0)
Total	126	95	3	1	8	28	2	3	1	2	2	1	1	1	1

M: Males, F: females, Sp: sputum, WS: wound swab, CSF: cerebrospinal fluid, Tr: tracheal aspirate, Th: throat aspirate, Cath: catheter Tip, Abd: abdominal abscess, Peri: peritoneal wound swab, Pler: pleural fluid, App: appendix, Ur: urethra. percentage (%) values are given in parentheses.

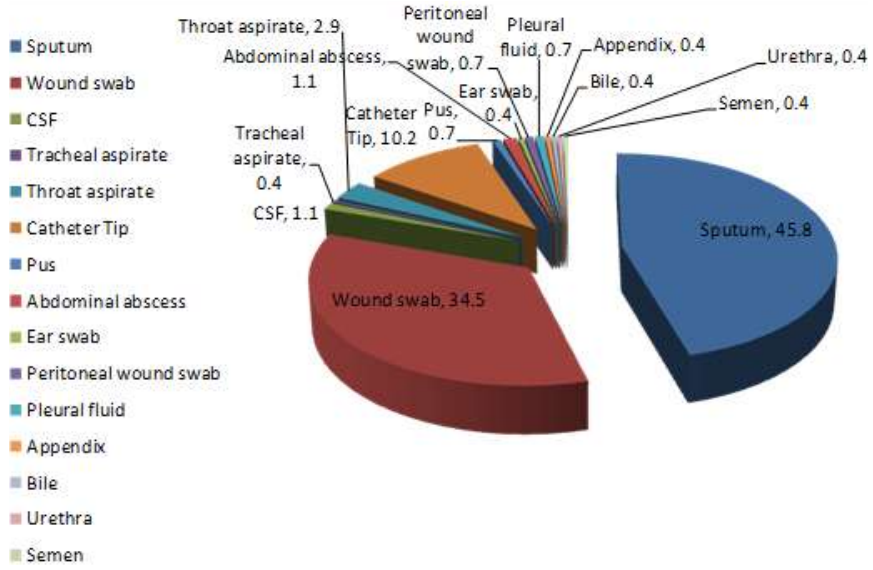

Figure 3. Percentage of *Klebsiella* samples isolated from different clinical samples.

Moreover, in a country like Saudi Arabia, where males represent a larger workforce, they are exposed more to infections, pollution, road accidents and hence more bone fractures and surgeries. All the 3 samples obtained from the cerebrospinal fluid were from males. In case of throat aspirates, the cases were from 50% males and 50% females. The numbers of the remaining samples were not enough to draw any substantial conclusion. We obtained only 1 sample each from tracheal aspirate, ear, appendix, bile, urine and semen; 2 to 3 samples from pus, abdominal abscess, peritoneal wound swab and pleural fluid.

Figure 3 shows the percentage of samples from various clinical sources that were positive for *Klebsiella* species. As mentioned earlier, the majority of isolates was from sputum and wound swabs, 45.8 and 34.5%, respectively followed by Catheter tips (10.2%). Very few strains were obtained from other clinical sources. The percentage of positive *Klebsiella* isolates from throat aspirates was 2.9% while from CSF and abdominal abscess it was 1.1%. Pus, peritoneal wound swabs and pleural fluid provided only 0.7%

Table 2. Percentage (%) of antimicrobial sensitivity pattern of *Klebsiella* samples to different antibiotics.

Antibiotic	Abbreviation	Sensitive	Resistant
Ampicillin	AP	0	100
Augmentin	AUG	66.1	33.9
Gentamycin	GM	87.3	12.4
Cefoxitin	FOX	93.7	6.3
Cephalothin	KF	49.1	50.9
Cotrimoxazole	TS	62.7	37.3
Amikacin	AK	86.6	13.4
Ceftazidime	CAZ	98.6	1.4
Aztreonam	AZT	96.6	3.4
Piperacilline	PRL	86.2	13.8
Imipenem	IMP	99.5	0.5
Ciprofloxacin	CIP	96.1	3.9
Cefpiramide	CPM	100	0
Meropenem	Merop	100	0
Tazobactam	Taz	100	0
Colistin	Col	100	0
Nitrofurantoin	Nitro	100	0

positive isolates. Tracheal aspirates, ear, appendix, bile, urethra, and semen were poor sources providing only 0.4% positive isolates each. The results here showed that the maximum number of *Klebsiella* species isolate were from sputum and wound swabs. They represented about 80% of all the clinical samples while the least number of isolates were from appendix, bile, urethra, tracheal aspirate, ear swabs and semen constituting a total of only 2.4%.

The positive samples were segregated on the basis of gender and the results have been summarized as Table 1. It was observed that in case of sputum and wound swab, around 66% and 69% samples, respectively were from males. Samples from catheter tips also included greater *Klebsiella* species from males (57%) and (42.9%) females. Another source throat aspirate revealed that male and female contribute equally, 50% each. The percentage of other samples was not enough to compare the male to female ratio (gender profile) and hence did not give reproducible results.

From the clinical samples considered here, results reveal that males are more vulnerable than females in acquiring *Klebsiella* infections which corroborates with previous reports (Shah et al., 2010; Namratha et al., 2015). As reported earlier (Janda and Abbott, 2006), this may be due to the fact that males are the major working class and being in the open, experience greater exposure to various infections.

Antimicrobial drug sensitivity was performed by disc diffusion assay using discs impregnated with seventeen antibiotics belonging to different families. These were ampicillin, augmentin, gentamycin, cefoxitin, cephalothin, cotrimoxazole, amikacin, ceftazidime, aztreonam, piperacilline, imipenem, ciprofloxacin, cefpiramide, meropenem, tazobactam, colistin, and nitrofurantoin. As shown in Table 2, Imipenem was the most effective antibiotic against *Klebsiella* species with 99.5% sensitivity followed by CAZ with 98.6% sensitivity for *Klebsiella*. The high sensitivity of Imipenem has been shown by others as well (Namratha et al., 2015; Gupta et al., 2012; Shiju et al., 2010). AZT was the third most sensitive antibiotic (96.6%) used in the present study showing high sensitivity against *Klebsiella* species. Amikacin is a fourth generation aminoglycoside which showed fairly good sensitivity, results being consistent with the previous studies (Ali and Ali, 2014; Namratha et al., 2015).

The percentage sensitivity with the remaining antibiotics in the present study was in this sequence: GM (87%) > AK (86.6%) > PRL (86.2%) > AUG (66.1%) > TS (62.7%) > KF (49.1%). The antibiotics CPM, Merop, Taz, Col and Nitro showed 100% sensitivity but as the number of strains was very low, significant conclusions could not be made. *Klebsiella* species showed 100% resistance to Ampicillin. Previous studies have shown similar resistance pattern with this drug (Jadhav et al., 2012). The chromosomally encoded β-lactamases could be responsible for this intrinsic resistance (Namratha et al., 2015). Nevertheless, it was encouraging to see that, most of the remaining antibiotics were showing high sensitivity towards *Klebsiella*. This was not the case with *Proteus* species as reported in a previous study conducted by the same group (Bahashwan and Shafey, 2013). The *Proteus* strains showed high resistance to most of the antibiotics except Imipenem which showed around 91% sensitivity. Imipenem was shown to be the most effective antimicrobial drug against Gram-negative bacteria by others (El-Tahawy, 2000).

Understanding seasonal trends in the incidence of nosocomial infections will help in improving surveillance and evaluation of infection prevention guidelines. Several reports claim that bacterial infections always peak during summers and winters (Eber et al., 2011; Anderson et al., 2008). Table 3 depicts the percentage of *Klebsiella* infections during the four different seasons. It was observed that the percentage of infections was the highest during summers (46.4%) followed by winters (27.2%). During autumn and spring, the percentages are not too high being 13.9% during autumn and 12.5% during spring. Winters are known for the outbreaks of bacterial infections especially related to the respiratory systems (Anderson et al., 2008; Eber et al., 2011). The autumn season incidentally coincides with the major pilgrimage period when a large number of pilgrims visit Madinah during Haj. But interestingly, the percentage of *Klebsiella* infections was not very high in this period in comparison to summers and winters. This can be attributed to the efforts of the health authorities who take special care to control the outbreak of bacterial and other infections. We have reported a similar pattern in case of

Table 3. Percentage (%) of *Klebsiella* infections pattern during different seasons.

Season	Percentage (%) of *Klebsiella* infections
Summer (22 June -22 September)	46.4
Autumn (23 September -21 December): Pilgrimage season	13.9
Winter (22 December -30 Mars)	27.2
Spring (21 Mars-21 June)	12.5

Proteus infection during the same period of study (Bahashwan and Shafey, 2013). Although Madinah, just like Makkah, expects a large population all year around, it is during the Haj season that there are dangers of an outbreak of epidemic.

To avoid epidemic like situation, special care is taken which could be the reason for such low percentage of infection during the pilgrimage season. The introduction and implementation of the World Health Organization (WHO) hand hygiene program by Saudi Arabia, and the conception of the Gulf Cooperation Council (GCC) Infection Control Program can be seen as a good initiative in reducing spread of resistant pathogens in healthcare units (Yezli et al., 2014).

Conclusion

Males are more vulnerable to *Klebsiella* infections than females. The most effective antibiotic with the highest sensitivity and lowest resistance was Imipenem which can hence be prescribed to patients with least reservations. All *Klebsiella* strains were resistant to Ampicillin indicating that this antibiotic should be prescribed with care.

The intensity of infections was highest during summers followed by winters. Lack of awareness, self-medication and misuse of antibiotics has aggravated multidrug resistance in microbes. There is an ardent need to formulate and adhere to new guidelines for drugs based on their sensitivity profiles.

By studying the antimicrobial resistance pattern of pathogens, we can formulate and implement better infection control policies. Developing nationwide, healthcare guidelines is essential nowadays due to increasing resistance patterns.

Furthermore, by developing a local antibiogram database we can improve the knowledge of antimicrobial resistance patterns in a particular area and will help in improving treatment strategies in Saudi Arabia. Compliance to infection prevention guidelines is essential to eliminate major outbreaks in the future.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Effectiveness of *Pseudomonas* species in the management of tomato early blight pathogen *Alternaria solani*

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Management of tomato early blight pathogen (*Alternaria solani*) has relied principally on application of synthetic fungicides. The use of biological control agents has been recognized as a viable option to synthetic chemicals in plant disease control. The present study evaluated the effects of *Pseudomonas fluorescens, P. aeruginosa, P. putida,* and *P. cepacia* on tomato early blight pathogen and investigated their efficacy on the yield components and yield of tomato plants infected with *A. solani.* Tomato seeds raised in the nursery beds were transplanted to the main field after three weeks. The experiment was laid in a randomized complete block. Treatment application was done by immersion of roots of three-week-old seedlings of tomato in *Pseudomonas* solution prior to transplanting. The treatments consisted of plots treated with *P. fluorescens, P. aeruginosa, P. putida, P. cepacia* and the control. Data were collected on disease severity, plant height, stem girth, number of fruits per plant, fruit length and fruit weight. Tomato plants in the control plot had significantly ($p \leq 0.05$) higher disease severity (52.0) There was no significant difference in the severities of early blight on tomato plants treated with *P. fluorescens*, *P. putida* and *P. cepacia*. Tomato plants treated with *P. aeruginosa* had significantly ($p \leq 0.05$) higher height (39.0 cm) than all other treatments. There was no significance difference in the fruit weights of tomato plants treated with *P. fluorescens*, *P. aeruginosa*, *P. putida,* and *P. cepacia*. Tomato plants in the control plots had a significantly lower fruit weight (69.5 g). Findings of the present study demonstrated a promising approach of biological control of early blight pathogen with *Pseudomonas* species. Results of this work could be used as bedrock for formulation of an effective and eco-friendly strategy for the management of early blight disease.

Key words: Tomato, *Alternaria solani*, *Pseudomonas*, biological control, early blight.

INTRODUCTION

Tomato *(Solanum lycopersicum)* is the most important vegetable crop grown in Nigeria (Usman and Bakari, 2013; Nwosu et al., 2014). The vegetable plays an important role in human nutrition, providing essential amino acids, vitamins and minerals (Sainju et al., 2003). Tomato contains lycopene, a very potent antioxidant that prevents cancers (Agarwal and Rao, 2000). Nigeria is the thirteenth largest producer of tomatoes in the world and

the leading country in Sub-Saharan Africa, with a production estimate of 1.7 million tons per annum (Donkoh et al., 2013). In spite of this high ranking, the country is the largest importer of tomato paste in the world (Nwosu et al., 2014).

Optimum productivity and economic value of tomato is hindered by an array of abiotic and biotic factors. The main constraints include low level of technology, declining soil fertility, socio-economic factors, as well as pests and diseases (Tanzubil and Boatbil, 2014).

Pests and diseases have been identified as the major biotic factor limiting tomato production in Nigeria. Early blight, incited by the fungus, *Alternaria solani* is the most destructive disease of tomato, causing significant reduction in the quantity and quality of fruit yields (Tewari and Vishunavat, 2012). The disease is characterized by the appearance of brown to dark brown necrotic spots with concentric rings on foliage, stem and fruits (Chohan et al., 2015). Yield losses of about 80% have been attributed to early blight disease.

The variability in pathogenic isolates of *A. solani*, prolonged disease cycle phase and broad host range have made the control of early blight very difficult (Chohan et al., 2015). Management of the disease has relied principally on the application of synthetic fungicides. However, continuous use of agrochemicals for controlling the disease may pose several problems like toxicity to non-target organisms, development of resistance in the populations of the pathogen and environmental pollution (Varma et al., 2008). The use of environmentally safer microbial bioagents (antagonists) is considered as an effective alternative tool which can be exploited for the management of crop diseases (Varma et al., 2008; Singh et al., 2015). The use of plant growth promoting rhizobacteria, *Pseudomonas fluorescens* (Migula) is one of the promising biological control modules, and its commercial formulations have been tested against several crop diseases caused by pathogens (Kaur et al., 2016).

The use of biological control agents has been recognized as a viable option to synthetic chemicals in plant disease control and is currently being advocated worldwide (Ganeshan and Kumar, 2005; Salaheddin et al., 2010). Bioagents possess antagonistic effects against pathogen of plants (Muriungi et al., 2013). Inducing crops with *Pseudomonas* species to improve disease resistance has showcased the potential of developing plant protection products from biological control agents. Biocontrol treatments of plant diseases with *P. fluorescens* have proved to be eco-friendly and effective against many plant pathogens and have been considered as a long term solution to management of plant diseases (Varma et al., 2008; Kaur et al., 2016). Kaur et al. (2016) reported 39 to 46% reduction of tomato early blight disease with talc based formulation of *P. fluorescens*.

The present study was therefore carried out to evaluate the effects of *P. fluorescens, P. aeruginosa, P. putida,* and *P. cepacia* on tomato early blight pathogen and investigated their efficacy on the yield components and yield of tomato plants infected with early blight disease.

MATERIALS AND METHODS

Site description

The study was carried out at the Teaching and Research Farm of Landmark University, Omu-Aran, Kwara State, Nigeria. Omu-Aran is located in the North central part of Nigeria in the South-Eastern direction of Ilorin and has a latitude 8.9`N and longitude 50`61 E. The annual rainfall pattern of the area is 600 to 1,500 mm between the months of April and October with peaks in June. The humidity ranges from 50% in the dry season to about 85% during wet season.

Source of materials

The seeds of tomato cultivar used in this study were obtained from the Teaching and Research Farm of Landmark University, Omu-Aran, Nigeria while the four *Pseudomonas* species, that is, *P. fluorescens, P. aeruginosa, P. putida* and *P. cepacia*, were obtained from the Federal Institute of Industrial Research Oshodi (FIIRO), Lagos, Nigeria.

Experimental design and treatment application

A total land area of 270 m^2, partitioned into forty- five beds was used for the experiment. Each bed measured 2 m × 3 m. Each plot was separated from the adjacent bed by 0.5 m alley. The experiment was laid in a randomized complete block design with five treatments replicated three times. The seeds were raised in nursery beds for three weeks and the seedlings transplanted to the main field at 0.3 m and 0.5 m intra- and inter-row spacings, respectively.

Inoculation of the biocontrol agent was done by immersion of roots of three-week-old seedlings of tomato in *Pseudomonas* solution (8 g *Psf* powder L^{-1} of water) for 5 min prior to transplanting while roots of seedlings of plants in the control plots were dipped in water. The treatments consisted of plots treated with *P. fluorescens, P. aeruginosa, P. putida, P. cepacia* and the control. The tomato plants were monitored for symptoms of *Alternaria solani* infection, that is, brown to dark brown necrotic spots with concentric rings on foliage, stem and fruits.

Data collection and analysis

Data were collected on disease severity, plant height, stem girth, number of fruits per plant, fruit length and fruit weight. Disease severity assessment was carried out on a scale of 0 to 9 according to Latha et al. (2009) where: 0 = healthy; 1 = 1-5%; 2 = 6-10%; 3 =

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11-25%; 5 = 26-50%, 7 = 51-75%, and 9 = > 76% of the leaf area infected with early blight symptoms.

Fruit length was measured with a vernier caliper. For yield determination, tomato fruits were harvested and weighed as and when ripe until the experiment was terminated at 24 weeks after transplanting.

The data collected were subjected to analysis of variance (ANOVA) and the means were separated using Duncan multiple range test (DMRT) at 5% probability level.

RESULTS

Disease severity

Tomato plants in the control plot had significantly ($p \leq 0.05$) higher disease severity (52.0) There was no significant difference in the severities of early blight on tomato plants treated with *P. fluorescens*, *P. putida* and *P. cepacia*. Tomato plants in plots treated with *P. aeruginosa* had significantly lower early blight severity (11.0).

Stem girth

There was no significant difference in the stem girths of tomato plants treated with *P. aeruginosa*, *P. putida*, *P. cepacia* and the control.

Plant height

Tomato plants treated with *P. aeruginosa* had significantly ($p \leq 0.05$) higher height (39.0 cm) than all other treatments. This was followed by tomato plants in plots treated with *P. putida* which has a height of 35.7cm. There was no significant difference in the heights of tomato plants treated with *P. fluorescens* and the control, which have heights of 30.0 and 31.7 cm, respectively. Tomato plants treated with *P. cepacia* had significantly lower plant height (24.0 cm).

Fruit length

There was no significant ($p \leq 0.05$) difference in the fruit lengths of the tomato plants treated with *Pseudomonas* species and the control. However, tomato plants in plots treated with *P. cepacia* had a numerically higher fruit length (3.1 cm).

Number of fruits

There was no significant ($p \leq 0.05$) difference in the number of fruits on tomato plants treated with *P. aeruginosa, P. putida* and *P. cepacia*. Similarly, no significance difference was observed in the number of fruits on tomato plants treated with *P. fluorescens* and the control, although tomato plants in the control plots had the least number of fruits (6.33).

Fruit weight

There was no significance ($p \leq 0.05$) difference in the fruit weights of tomato plants treated with *P. fluorescens*, *P. aeruginosa*, *P. putida* and *P. cepacia*. However, tomato plants treated with *P. aeruginosa* had a numerically higher fruit weight (202.82 g). Tomato plants in the control plots had a significantly lower fruit weight (69.53 g).

DISCUSSION

The present study investigated the effect of *P. fluorescens, P. aeruginosa, P. putida* and *P. cepacia* on early blight pathogen and assessed their effects on plant height, stem girth, fruit length, number of fruits and fruit weight of tomato plants infected with early blight disease. The results showed that the tomato plants treated with the *Pseudomonas* species had lower disease severities at the different weeks of assessment compared with the control.

Pseudomonas species influence plant growth through inhibition of fungal plant pathogens and by their effects on the roots of plants (Moore et al., 2006). Certain strains of *Pseudomonas* secrete metabolites that inhibit plant pathogens and stimulate plant growth. Thus, the lower disease severities recorded on tomato plants treated with *Pseudomonas* species in the current study might be attributed to the production of metabolites that inhibited the growth of *A. solani*.

The rhizosphere (zone around the roots of plants) is noted for intense microbial activity, arising from secretion of organic and amino acids by plants. *Pseudomonas* species are among the most effective rhizosphere colonizers of soil (Lugtenberg et al., 2001). They possess traits that enable them to exploit the resources in the rhizosphere. Species of this biocontrol agent also effectively inhibit the colonization of plants by other microorganisms (Brianciotto et al., 1996). According to Moore et al. (2006), *P. putida*, *P. fluorescens*, as well as other *Pseudomonas* species colonize and proliferate quickly in the rhizosphere of a number of agriculturally important plants. Furthermore, *P. fluorescens*, *P. putida* and *P. cepacia* produce antibiotics like pyrol, nitrin, oomycin-A and hormone such as indole acetic acid, gibberellic acid and siderophores that inhibit the growth of pathogens (Bhattacharjee and Dey, 2014). These attributes might have assisted the *Pseudomonas* species used in the current study to inhibit *A. solani* establishment in the rhizosphere of the tomato plants.

P. putida is a versatile, aggressive colonizer that establishes itself and persists within the rhizosphere and in bulk soils at high cell densities (Molina et al., 2000).

Table 1. Effect of four *Pseudomonas* species on disease severity, stem girth, plant height, fruit length, number of fruits and fruit weight of tomato plants infected with early blight pathogen (*A. solani*).

Treatment	Parameters					
	Disease severity	Stem girth (cm)	Plant height (cm)	Fruit length (cm)	Number of fruits	Fruit weight (g)
P. fluorescens	16.0^{b}	2.3^{b}	30.0^{c}	3.0^{a}	8.4^{b}	113.1^{ab}
P. aeruginosa	11.0^{c}	2.5^{ab}	39.0^{a}	2.5^{a}	25.4^{a}	202.8^{a}
P. putida	15.3^{b}	2.4^{ab}	35.7^{b}	2.1^{a}	14.6^{ab}	130.6^{ab}
P. cepacia	15.7^{b}	2.4^{ab}	24.0^{d}	3.1^{a}	13.6^{ab}	103.4^{ab}
Control	52.0^{a}	2.5^{a}	31.7^{c}	2.9^{a}	6.3^{b}	69.5^{c}

Values are means of three replicates. Means in the same column followed by the same alphabet are not significantly different according to Duncan's multiple range test ($p \leq 0.05$).

This trait was demonstrated by this strain in the current study as the lowest early blight severity was recorded at maturity stage of the tomato plants at 9 weeks after transplanting (Table 1). Features that contribute to rhizosphere and soil fitness of *P. putida* include adhesion and colonization abilities, antibiotic production, resistance to multiple antibiotics, capability to utilize seed and root exudates, production and utilization of siderophores, and ability to cope with oxidative stresses (Jataraf et al., 2005; Jorjani et al., 2011).

Biological control of crop diseases by antagonistic microorganisms has become the most effective alternative to synthetic chemical pesticides (Alemayehu, 2014). Among the antagonistic bacteria, several strains of *P. fluorescens*, *P. putida, P. cepacia* and *P. aeruginosa* have been widely used for biological control of fungal, viral and bacterial pathogens (Raupach and Kloepper, 1998; Vidhyasekaran and Muthamilan 1999; Salaheddin et al., 2010). The mechanisms employed by bacterial antagonists for plant disease control include antibiosis, siderophore production, enzyme secretion, hormone production, competition for nutrients and space, activation of plant defenses and inducing systemic resistance in host plants (Duffy et al., 2003; Jataraf et al., 2005; Jorjani et al., 2011).

Conclusion

Findings of the present study demonstrated a promising approach of biological control of early blight pathogen with *Pseudomonas* species. Results of this work could be used as bedrock for formulation of an effective and eco-friendly strategy for the management of early blight disease.

CONFLICT OF INTERESTS

The authors of this manuscript declare that there is no conflict of interest.

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Full Length Research Paper

Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) using a novel dilution tube method

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A novel 'dilution tube method' (DTM) which is a modification of the 'dilution method' (DM) is hereby described for the determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). This new DTM uses only broth medium in tubes and the required antibiotic. MIC and MBC for *Escherichia coli*, *Klebsiella pneumoniae* and *Staphylococcus aureus* were determined in tubes by double diluting (or higher dilutions), broths containing gentamicin concentrations that inhibit bacterial growth, and incubating at 37°C for 18 to 24 h. The tube for MIC showed growth and appeared turbid after incubation while that for MBC remained clear. The results obtained using DTM agrees completely with those obtained with DM. The advantages of this novel DTM include the elimination of extra stress, time and costs associated with preparing and inoculating agar medium as done in DM.

Key words: Antibiotics, minimum inhibitory concentration, minimum bactericidal concentration, dilution tube method, dilution method.

INTRODUCTION

Antibiotics are antimicrobial agents produced by microorganisms that inhibit the growth or kill other microorganisms while being harmless to the host cells. The determination of the susceptibility of pathogens to antibiotics is necessary for the selection of the most appropriate one for treating microbial infections. Antibiotics which kill bacteria are said to be bactericidal, while those that only prevent their multiplication are referred to as bacteriostatic. However, some antibiotics can act as both bacteriostatic and bactericidal depending on their concentration. Antibiotics are evaluated for their inhibitory potentials. A few methods used for evaluating antibiotics include the filter paper disc (Kirby-Bauer) method (Bauer et al., 1966), agar and broth dilution method (Wiegand et al., 2008), and the dilution method (Brown and Young, 1947; Bradshaw, 1979; Owuama, 2015). The dilution method is mainly useful in determining minimum inhibitory concentration (MIC),

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which is the least concentration of antimicrobial agent that prevents microbial growth, as well as the determination of minimum bactericidal concentration (MBC), which is the least concentration of antimicrobial agent required to kill microorganisms (Andrews, 2001). Microdilution and macrodilution methods have been used in the determination of MIC and MBC (Lambert and Pearson, 2000; Eucast, 2003). Usually, the determination of MIC and MBC by the Dilution Method (DM), involves the inoculation of an indicator bacterium into various concentrations of an antibiotic, incubating for 18 to 24 h and thereafter testing for bacterial viability by sub-culturing on agar media prepared without the antibiotic (Andrews, 2001; CLSI, 1998). Usually, for MIC and MBC determinations, agar medium free of antibiotic is prepared and inoculated with samples from tubes which show no turbidity or growth. Preparation of agar medium involves extra time, additional stress and use of Petri dish. Hence, an improved new method, dilution tube method (DTM) which is easier and cheaper as it does not require agar medium but uses only broth medium in tubes for determining MIC and MBC is hereby described.

MATERIALS AND METHODS

Bacterial species, *Klebsiella pneumonia*, *Escherichia coli* and *Staphylococcus aureus* were obtained from Microbiology Department, Modibbo Adama University of Technology, Yola.

A modification of the dilution method for the determination of MIC and MBC was used. Briefly, gentamicin was diluted into various concentrations, 5, 6, 7, 7.5, 9.0, 9.5, 9.8, 10 and 15 µg/ml, in sterile nutrient broth in test tubes. Using standard wire loop (Merck), a loopful (10 µl) of *E. coli* culture, 0.5 McFarland standard (Eucast, 2003), was inoculated into test tubes containing 1 ml of the various concentrations of gentamicin in nutrient broth. Similarly, this was repeated for *K. pneumoniae* and *S. aureus*. The tubes were incubated at 37°C for 18 to 24 h and thereafter observed for growth or turbidity. Subsequently, a loopful of broth from each test tube not showing growth, was inoculated into nutrient agar plate. Thereafter, equal volumes of sterile nutrient broth were added into the test tube cultures and incubated further for 24 h at 37°C. Then, the tubes and agar plates were examined for growth or turbidity using unaided eye (CLSI, 2012). A repeat of the DTM with higher dilution (1:10 dilution, that is, 9 vol. of nutrient broth to 1 vol. of broth culture) instead of the double dilution with nutrient broth was done. These experiments were repeated three times.

RESULTS

Similar results were obtained using the DM of determining the MIC and MBC (which requires inoculation onto agar plates devoid of antibiotic), and the new DTM described in the work. Tubes containing concentrations of antibiotic that showed no bacterial growth or turbidity after the first 24 h incubation but showed growth (on agar plate) or turbidity (in tube) after the addition of equal volumes of sterile nutrient broth and further 24 h incubation, were said to have minimum inhibitory concentration, while tubes with least antibiotic concentration that showed no growth or turbidity after the first 24 h and still no growth (on agar plate) or turbidity (in tubes) after further 24 h (that is, after the addition of equal volume of sterile nutrient broth), were regarded as minimum bactericidal concentration, MBC. In Table 1, both *E. coli* and *K. pneumonia* showed turbidity after incubation in nutrient broth with different gentamicin concentrations at 5 and 6 µg/ml, but not at higher concentrations, while *Staphylococcus aureus* showed turbidity at 9.5 µg/ml, but not at 9.8 µg/ml or higher gentamicin concentrations.

In Table 2, both bacterial species showed turbidity at 5 and 6 µg/ml, but not at 7.0 µg/ml concentrations suggesting that 7.0 µg/ml was the MIC for both *E. coli* and *K. pneumonia*, while *S. aureus* showed turbidity at 9.0 and 9.5 µg/ml, but not at 9.8 µg/ml suggesting that 9.8 µg/ml was the MIC for *S. aureus*. Inoculation of loopful of samples of the *E. coli* and *K. pneumoniae* from 7.0 µg/ml tubes onto nutrient agar without antibiotic, and addition of equal volume of sterile nutrient broth without antibiotic (that is, double dilution) into 7.0 µg/ml tube sample, showed growth in both the tubes and agar plates (after 24 h incubation at 37°C). Thus, confirming that 7.0 µg/ml is the minimum inhibitory concentration, MIC. For *S. aureus*, inoculation of a loopful of sample from 9.8 µg/ml tube onto nutrient agar without antibiotic, and addition of equal volume of sterile nutrient broth without antibiotic into 9.8 µg/ml tube sample, showed growth in both the tube and agar plate after 24 h incubation at 37°C. Thus, confirming that 9.8 µg/ml was the MIC for *S. aureus*.

In Table 3, MBC is also the same for both bacterial species, that is, 7.5 µg/ml, using both the DM and the new DTM, as there were no growths in *E. coli* and *K. pneumoniae* tubes after 24 h incubation at 37°C, as well as in the tubes after double dilution and inoculation onto agar plates, following further 18 to 24 h incubation. However, for *S. aureus*, the MBC was 10 µg/ml using both the DM and new DTM, as there were no growths in their tubes after 24 h incubation at 37°C, as well as in the tubes after double dilution and inoculation onto agar plates after further 24 h.

In addition, adding sterile nutrient broth (to make 1:10 dilution) to tubes which were not turbid, that is, showed no growth, revealed similar results with those of double dilution, indicating that dilutions of the tube broth higher than double dilution will give same results.

DISCUSSION

Application of the DM (Bradshaw, 1979; CLSI, 1998) and the novel DTM yielded similar results for MIC for *E. coli* and *K. pneumoniae* (Table 2) and MBC (Table 3) of

Table 1. Inhibition pattern of *E. coli, K. pneumoniae* and *S. aureus* using different concentrations of gentamicin in broth after 24 h incubation at 37°C.

Gentamicin conc. (µg/ml)	Turbidity in broth								
	5 0	6.0	7.0	7.5	9.0	9.5	9.8	10	15
Escherichia coli	+	+	-	-	-	-	-	-	-
Klebsiella pneumoniae	+	+	-	-	-	-	-	-	-
Staphylococcus aureus	+	+	+	+	+	+	-	-	-

+ = Growth; - = no growth.

Table 2. Growth pattern of *E. coli, K. pneumoniae* and *S. aureus* 24 h after double and 1:10 dilutions of concentrations of gentamicin broth in tubes that inhibited bacteria with nutrient broth.

Gentamicin conc. (µg/ml)	Turbidity in broth								
	5 0	6.0	7.0	7.5	9.0	9.5	9.8	10	15
Escherichia coli	+	+	+	-	-	-	-	-	-
Klebsiella pneumoniae	+	+	+	-	-	-	-	-	-
Staphylococcus aureus	+	+	+	+	+	+	+	-	-

+ = Growth; - = no growth.

Table 3. Growth pattern of *E. coli, K. pneumoniae* and *S. aureus* 24 h after inoculation of broth from different concentrations of gentamicin that inhibited bacteria onto nutrient agar plates.

Gentamicin conc. (µg/ml)	Growth on agar								
	5 0	6.0	7.0	7.5	9.0	9.5	9.8	10	15
Escherichia coli	+	+	+	-	-	-	-	-	-
Klebsiella pneumoniae	+	+	+	-	-	-	-	-	-
Staphylococcus aureus	+	+	+	+	+	+	+	-	-

+ = Growth; - = no growth.

gentamicin. Observation of same results clearly indicated that both methods are interchangeable in the determination of MIC and MBC. The DM as described by Eucast (2003) and Bradshaw (1979) shared similarity at the first stage with DTM, but differed in the later stages particularly because of the use of agar in DM unlike the new DTM. It is known that the determination of MBC using DM, macrodilution or microdilution requires sub-culturing a sample from wells or tubes, yielding a negative microbial growth after incubation on the surface of non-selective agar plates to determine the number of surviving cells after 24 h of incubation (CLSI, 1998; Eucast 2003). In DTM, it is understandable that the addition of equal volume or higher volume of sterile nutrient broth to the broth in non-turbid tubes, invariably reduced the antibiotic strength below the MIC. Thus, for the gentamicin concentration (7.0 µg/ml) which only inhibited but not kill the bacteria, adding equal volume of broth devoid of antibiotic will doubly dilute and reduce the concentration medium to 3.5 µg/ml which is less than the MIC, and consequently permit the growth of the 'dormant' bacteria in the tube. Similarly, for 7.5 µg/ml concentration which killed the bacteria, that is, the MBC, killed bacteria will not grow even after reducing the antibiotic concentration by double dilution or even 1:10 dilution in the tube with sterile nutrient broth, to 3.75 or 0.75 µg/ml, because the bacteria were already dead and will no longer revive. Similarly, the interpretations for MIC and MBC of *E. coli* and *K. pneumoniae* explain the MIC and MBC of gentamicin for *S. aureus*.

In conclusion, the principle underlying the development of DTM is that bacteria made dormant (but not killed) by a particular concentration of antibiotic can be revived if that antibiotic concentration is remarkably reduced by dilution, while bacteria killed by antibiotic at a given concentration cannot be revived no matter the dilution. Interestingly, the new DTM and DM give same results, however, DTM has the advantage that it is less expensive, less time

consuming and less stressful as no nutrient agar plates are required. Thus, the new method is recommended for MIC and MBC determination.

CONFLICT OF INTERESTS

The author has not declared any conflict of interests.

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Full Length Research Paper

Promising biosurfactant produced by a new *Candida tropicalis* UCP 1613 strain using substrates from renewable-resources

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The current work aimed to use agro-industrial wastes as a strategy to obtain biosurfactant from a new *Candida tropicalis* UCP 1613 isolated from mangrove sediments of Rio Formoso, Pernambuco State, Brazil. The yeast was identified based on 18S rRNA sequencing method and blast homology search. The promising strain showed the ability to use an optimal composition of a cheap medium containing whey (3%), cassava wastewater (7%) and soybean post frying oil (10%) during 96h which reduced significantly the surface tension from 70 to 28.8 mN/m. The yield of the biosurfactant obtained was 4.9 g/L and the minimum value of critical micelle concentration was 1.5%. In addition, this was isolated and characterized as an anionic polymeric molecule, composed of proteins (51%), lipids (37%) and carbohydrates (11%), and confirmed by Fourier transform infrared spectroscopy (FTIR) analysis. Also, biosurfactant was capable in forming stable emulsions at different ranges of temperature, pH and salinity. Alternatively, the biosurfactant displayed no toxicity against the different vegetable evaluated seeds *Brassica oleracea, Lactuca sativa* L. and *Solanum lycopersicum.* The antimicrobial activity of this biosurfactant was verified showing the main efficacy against Gram-positive bacteria besides inhibiting the antifungal activity against the yeast tested. The results obtained suggest its potential application to pharmaceutical, as well as environmental area.

Key words: *Candida tropicalis,* mangrove sediment, oil dispersion, biosurfactant.

INTRODUCTION

Nowadays the presence of surfactants in the daily activities of man makes life easier. These compounds are mainly obtained through chemical synthesis from oil and possess in their composition both hydrophilic and hydrophobic structural moieties. Thus, this duality gives them the ability to lower the surface tension, critical

micelle concentration, and interfacial tension between liquid–liquid/liquid–solid systems (Marchant et al., 2012; Sharma et al., 2014). In contrast, biosurfactants are the main classes of natural surfactants produced by fungi, yeast and bacteria.

According to their chemical structures, they are grouped into five major classes of lipopeptides, glycolipids, phospholipids, neutral lipids, and polymeric compounds (Desai and Banat, 1997; Khopade et al., 2012). Microbial surfactants as also known, have numerous advantages when compared to their chemically synthesized counterparts due to their lower toxicity, higher biodegradability, and better environmental compatibility, ability to be synthesized from renewable resources, higher foaming, higher selectivity and specific activity at extreme temperature, pH and salinity (Zheng et al., 2012; Rufino et al., 2014). These properties confer them commercial importance evidenced through the biotechnological applications in pharmaceutical, biomedical, cosmetic, petroleum, and food industries (Nitschke and Costa, 2007; Banat et al., 2010).

However, the benefit of these molecules contrasts with its high costs of the production associated with inefficient methods of recovery turning with distant reality for industry (Makkar et al., 2011). In this context, biocircular economy brings an innovative approach as a demand for global sustainability. Thus, efforts toward the recovery and reuse of waste from agro industrial origin through bioprocesses allow in valorizing the microbial synthesis of biobased products as biosurfactants (Mohan et al., 2016).

In addition, other significant aspects to be explored are the absence of toxicity and antimicrobial activity. These properties are suitable for the biotechnological applications of biomolecules such as biosurfactants (Sobrinho et al., 2013). With this in mind, the present paper was focused on the characterization of the biosurfactant produced by *Candida tropicalis* UCP 1613 isolated from mangrove sediment in Northeast of Brazil. The biosurfactant was extracted and characterized through compositional analyze, ion charge and Fourier transform infrared spectroscopy (FTIR). The stability of the biosurfactant under different conditions of pH, temperature and salinity was also studied and verified as its efficacy in the phytotoxicity assay and antimicrobial activity.

MATERIALS AND METHODS

Mangrove sediments collection

The samples isolated from mangrove sediments of Rio Formoso (localized at 08°39' 50" S 35°09' 32"), Pernambuco state, Brazil, were collected in July, 2015. The samples collected from mangrove ecosystem were previously marked in the area, collected in sterilized bottle, and immediately conducted to the Nucleus of Research in Environmental Sciences and Biotechnology (NPCIAMB), of the Catholic University of Pernambuco.

Agro-industrial substrates

The production medium was composed by agro-industrial wastes: cassava wastewater, a waste from cassava (*Manihot esculenta* Crantz) processing, if released directly into the environment before proper treatment, which could be a source of pollution. The whey was kindly supplied from the dairy industry of São Bento do Una, PE, Brazil, and waste cooking oil from informal commerce in Recife, PE, Brazil.

Yeast Isolation

The fresh mangrove sediment 1g was added to Erlenmeyer flasks of 250 mL containing 100 mL of Yeast Mold Broth (YMB) with the following composition (w/v): yeast extracts (0.3%), malt extract (0.3%), tryptone (0.5%), d-glucose (1.0%).

A 200 mg/L of chloramphenicol was supplemented after sterilization to minimize bacterial growth. The flasks were incubated at 150 rpm and 28°C for 48 h. After this period, serial dilutions from 10^{-1} to 10^{-5} were prepared and aliquots of 0.1 mL of each dilution were added to Petri dishes containing Yeast Mold Agar (YMA) with the following composition (w/v): yeast extract (0.3%), malt extract (0.3%), tryptone (0.5%), d-glucose (1.0%) and agar (5%). These plates were then incubated at 30°C for 72 h.

Identification of the isolated yeast

The colonial morphology of yeast was identified according to Accorsini et al. (2012) by color, borders and size of the colonies, texture, surface appearance and elevation. The molecular characterization was modified from the methodology performed by White (1990). DNA extraction was performed using Wizard ®Genomic DNA Purification Kit (Promega).

From the internal transcribed spacer region (ITS), which separates the genes 18, 5.8 and 28S rDNA, each sample were amplified using the pair of oligonucleotide primers ITS1 (5' TCC GTA GGT GAA CCT GCT GG 3') and ITS4 (5' TCC TCC GCT TAT TGA TAT GC 3'). The polymerase chain reactions (PCR) were performed with a final volume of 25 uL containing: 50 ng DNA, 10pm of each primer, 0.2 mM dNTPs, 1U Taq DNA polymerase, 1.5 mM $MgCl_2$ and buffer 1X (20 mM Tris-HCl pH 8.4; 50 mM KCl). The following parameters were used in the thermocycler (Eppendorf Mastercycler pro, model 6325): initial denaturation 95°C for 4 min, 40 cycles of denaturation, annealing and extension (92°C/1 min, 55°C/1 min and 72°C/2 min, respectively) and finally, the extension at 72°C for 5 min.

Then, amplicons were purified and sequenced by capillary electrophoresis on the ABI3130 platform (Life Technologies) which allows sequencing to 900 pb quality by the company Myleus Biotechnology. The chromatograms generated were subjected to

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Phred-Phrap software to read quality check of the base, during the sequencing step. The consensus sequences were submitted for analysis of similarity with sequences deposited in Gene Bank (Genbank) from the National Center for Biotechnology Information (NCBI), using the online tool basic local alignment search nucleotides (Blastn). The hits that had the highest percentage of similarity with the sequence under study, considering the best combination of "score" and "e-value" were regarded as identical.

Culture conditions

Cell grown of yeast on a slant were transferred to 50 mL of YMB. The culture was incubated in an orbital shaker at 150 rpm and 28°C for 24 h. Erlenmeyer's flask containing medium with the following composition: $MgSO_4$ 0.2 g/L, K_2HPO_4 1.0 g/L, KH_2PO_4 1.0 g/L, $CaCl_2$ 0.02 g/L, $FeCl_3$ 0.05 g/L and NH_4NO_3 1.0 g/L, supplemented with whey (3%), cassava wastewater (7%) and soybean post frying oil (10%) were adjusted to pH 5.3. The medium was sterilized by autoclaving at 121°C for 20 min.

After this period, 0.5% of YMB culture containing 10^4 cells/mL was used to initiate the growth. The culture was incubated at 28°C in an orbital shaker at 150 rpm for 96 h in triplicate and at regular intervals, every 24 h samples was taken to determinate surface tension and biomass.

Biomass determination

For biomass determination, 5 mL samples were mixed in pre-weighed tubes with chilled distilled water and centrifuged at 5000 rpm for 20 min. After two washing cycles, the cell pellet was dried in an oven 90°C for 24 h. All the assays were carried out in triplicate (Rufino et al., 2014).

Determination of surface tension

The determination of surface tension was carried out in the cell-free broth, obtained by centrifugation of the cultures at 5000 rpm for 20 min at room temperature, using a Sigma 700 digital surface tensiometer (KSV Instruments LTD, Finland), working on the principle of the Du Nuoy ring method (Gilanyi et al., 1976).

Isolation of biosurfactant

The biosurfactant was isolated by the precipitation method using the cell-free metabolic liquid with acetone 1:1 (v/v). The precipitate was allowed to stand for 24 h at 4°C, and after this period it was centrifuged at 5000 rpm for 20 h, at 5°C.

The supernatant was discarded and the isolated biosurfactant was submitted to dialysis against deionized water, which was changed every 3 h, for 96 h at 5°C. The yield of the isolated product was calculated as g/L and the analyses were performed in triplicate. The biosurfactant was collected and freeze-dried (Shavandi et al., 2011).

Critical micelle concentration (CMC)

The concentration at which micelles began to form was defined Critical Micelle Concentration (CMC). From known amounts of crude precipitate resuspended in distilled water, these determine the critical micelle concentration (CMC).

These measurements were realized using DuNouy Tensiometer model Sigma 70 (KSV Instruments LTD, Finland) at room temperature. The CMC was reached by measuring the surface tension until observing a constant value of the surface tension. All experiments were performed in triplicate (Rufino et al., 2014).

Compositional analysis

The protein content in the isolated biosurfactant was determined using the total protein test kit from Labtest Diagnóstica S.A., Brazil. The phenol-sulphuric acid method allowed in determinating the total carbohydrate content (Dubois et al., 1956). In the case of the lipid content 0.5 g of the isolated material was extracted with chloroform: methanol in different proportions (1:1 and 1:2, v/v). The organic extracts were then evaporated under vacuum and the lipid content was determined by gravimetric estimation (Manocha et al., 1980).

Ionic charge

The ionic charge of the biosurfactant was determined by using a Zeta potentiometer model ZM3-D-G, Zeta Meter System 3.0+, with direct images to the video of the Zeta Meter, San Francisco, CA, USA.

The top row was filled with a pure compound of a known ionic charge. The substance is known as anionic sodium dodecyl sulfate (SDS) at a concentration of (0.02 M) cationic substance and barium chloride (0.05 M). Petri dish was kept at room temperature for 48 h. The result was calculated when the precipitation lines appear as per Meylheuc et al. (2001) (Silva et al., 2014).

Fourier transform infra-red (FTIR) analysis

The identification of functional groups in the isolated biosurfactant was determined using a FTIR spectrophotometer (Bruker IFS 66) with KBr in the pestle. The FTIR spectrum was obtained at a frequency range of 4000 - 400 cm^{-1} (Smith, 1996).

Stability assay

In order to demonstrate biosurfactant stability under different conditions, the superficial tension of its partially purified solution (1 g/L) was examined. Samples were heated at 0, 5, 28, 70, and 100°C and cooled to room temperature, after which the surface tension was measured.

In the case of pH, this was adjusted to various pH values (2 to 12) by adding HCl or NaOH to a solution at room temperature and the surface was measured. The effect of NaCl concentrations (2 to 12%) was also a determinate. The tests were performed in triplicate (Tados, 2005).

Phytotoxicity assay

The phytotoxicity of the biosurfactant was evaluated based on seed germination and root elongation of cabbage (*Brassica oleracea)*, lettuce *(Lactuca sativa* L.) and tomato (*Solanum lycopersicum*), according to Tiquia et al. (1996).

Solutions of the isolated biosurfactant were prepared with distilled water at concentrations of 1, 1.5 and 2%. Toxicity was determined in sterilized Petri dishes (1 × 10 cm) containing Whatman N°1 filter paper. The seeds were pre-treated with sodium hypochlorite. Ten (10) seeds were inoculated in each Petri dish with 5 mL of the test solution at 27°C. After five days of incubation in the dark, seed germination, root elongation (≥5 mm) and the

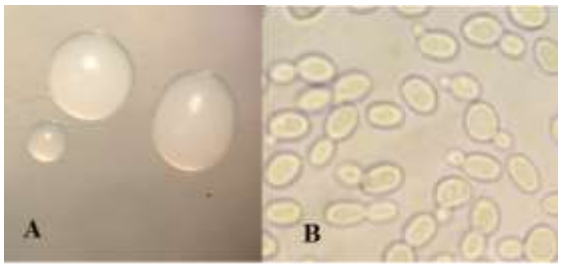

Figure 1. Colonies of yeast strain RF8 on yeast mold agar medium (A) and observation at optic microscopy (40X) (B).

germination index (a factor of relative seed germination and relative root elongation) were determined as follows:

Relative seed germination (%) = (number of seeds germinated in the extract/number of seeds germinated in the control) x 100

Relative root length (%) = (mean root length in the extract/mean root length in the control) x 100

Germination index = [(% of seed germination) x (% of root growth)]/100%.

Controls were prepared with distilled water to replace the biosurfactant solutions. Mean and standard deviation values of triplicate samples were calculated for each concentration.

Antimicrobial assay

The isolated biosurfactant was evaluated by the agar disc diffusion method (Bauer et al., 1996). Sterile discs (0.6 cm) soaked with the biosurfactant solution in methanol was assayed on the surface of and nutrient agar and malt extract medium for bacteria and yeast, respectively inoculated with the tested microorganism.

After incubation period for 24 h at 37± 2°C and 48 h at 25 ± 2°C for bacteria and yeast, respectively, the diameter of inhibition zones was measured (Bradshaw, 1992). Negative controls were prepared using the same solvents as employed to obtain the extract. Positive controls, ofloxacin (5 µg, Oxoid) was used for Gram-positive bacteria, cefaperazone–sulbactam (10 µg, Oxoid) for Gram-negative bacteria and amphotericin B (30 µg, Sigma) for *Candida albicans*. All the experiments were conducted in triplicates.

RESULTS

Morphological and molecular identification of the yeast producer of biosurfactant isolated from mangrove sediment

According to morphological observations of strain, yeast showed a characteristic of cream, smooth, glossy colonies whereas microscopic analysis revealed cells with small and medium sized with budding and no filaments characteristic. These traits allow presumptively the classifying of the isolate as *Candida* sp. (Figure 1).

Additionally, the molecular analyses of the sequences of ITS region were compared with the nucleotide database using the NCBI- blast tool. *Candida* sp. showed highest genetic agreement with *C. tropicalis* with a similarity of 99%. Thus the isolated was identified as *C. tropicalis* UCP 1613.

Kinetics of growth, biosurfactant production and biosurfactant yield

In this study, *C. tropicalis* UCP 1613 was cultivated in medium containing the agro-industrial substrates whey, cassava wastewater and soybean post frying oil during 96 h at 28°C. As shown in Figure 2, the growth of microorganism started rapidly after inoculation and remained increasing to 96 h of cultivation, when biomass reached 7.7 g/L. The surface tension dropped rapidly from 70 to 32.9 mN/m in the first 24 h and continued decreasing to 28.5 mN/m at 48 h of growth, indicating excellent surface-active properties.

Biosurfactant production started in the early stages of the exponential growth phase, simultaneously to the surface tension reduction which increased significantly until the end of cultivation, which attained the maximum biomass yield. The yield of the crude biosurfactant produced by *C. tropicalis* UCP 1613 was 4.9 g/L after 96 h of cultivation in medium containing 3% whey, 7% cassava wastewater and 10% soybean post frying oil (Figure 3).

Critical micelle concentration (CMC)

Thus in this study the biosurfactant obtained from the use of agro-industrial wastes showed a great surface tension reduction capacity, since the water surface tension was reduced from 70 to 28.5 mN/m with the increase of the biosurfactant concentration up to CMC of 1.5%, and then remained constant (Figure 4).

Preliminary characterization of biosurfactant

The determination of biochemical composition of the biosurfactant analyzed revealed the presence of 51% proteins, 37% lipids and 11% carbohydrates, suggesting its polymeric nature. Furthermore, an anionic profile with −57.4 ZPmv, 3, 4 µS/cm at 25.3°C was detected. In addition, the FTIR analysis was also used as complementary in this study.

According to the spectrum showed in Figure 4, stretching bonds of functional groups N–H between 3,514-3,257cm^{-1} indicated the presence of a peptide component. Also, a complex sequence of peaks due to the stretching vibration mainly C-C and C-O-P of oligo and polysaccharides (starches) were detected at an interval of 1200 to 1000 cm^{-1}. In addition, functional

Figure 2. Phylogenetic tree based on the ITS1 primer used for the identification of *Candida tropicalis* UCP 1613.

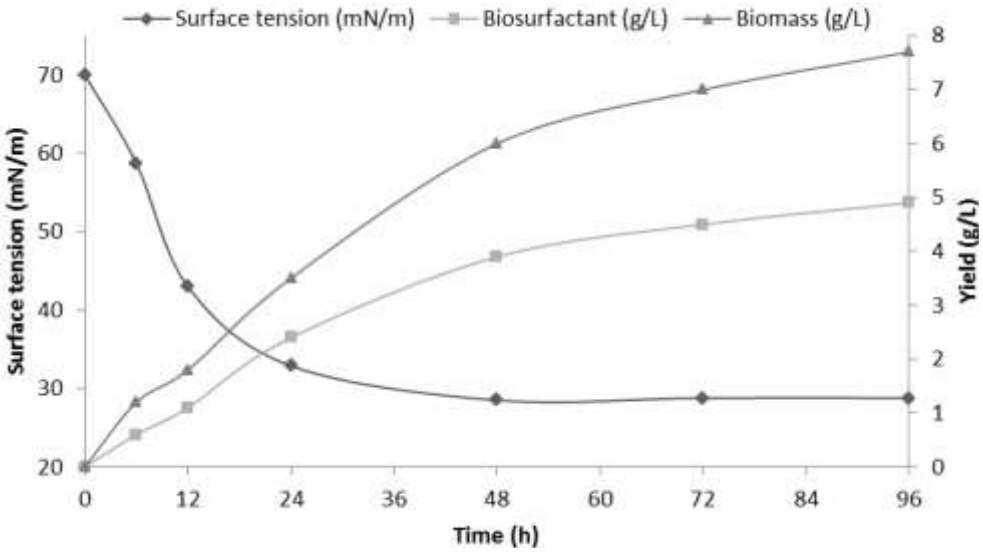

Figure 3. Growth, surface tension and yield of biosurfactant isolated from *C. tropicalis* UCP 1613 cultured in mineral medium supplemented with 3% whey, 7% cassava wastewater and 10% soybean post frying oil.

groups -CH=CH_2 and CH_3 were identified at 1448.27 cm^{-1} corresponding to fatty acids (Figure 5). Bands at approximately 1756.31 cm^{-1} showed the presence C=O consistent with ester functional group in lipids (Verma et al., 2015).

Biosurfactant stability

The behavior of the biosurfactant produced by *C. tropicalis* UCP 1613 was tested in different conditions of temperature, pH and NaCl concentrations. As it is shown in Figure 6, it was noticeable how the surface tension of the biosurfactant remained stable over a wide range of temperature, pH and NaCl concentrations.

Biosurfactant phytotoxicity

The phytotoxicity effect was evaluated through the germination index (GI) which combines measures of relative seed germination and relative root elongation. Seeds of vegetables *Brassica oleracea*, *Lactuca sativa* L. and *Solanum gilo* were used with different concentrations

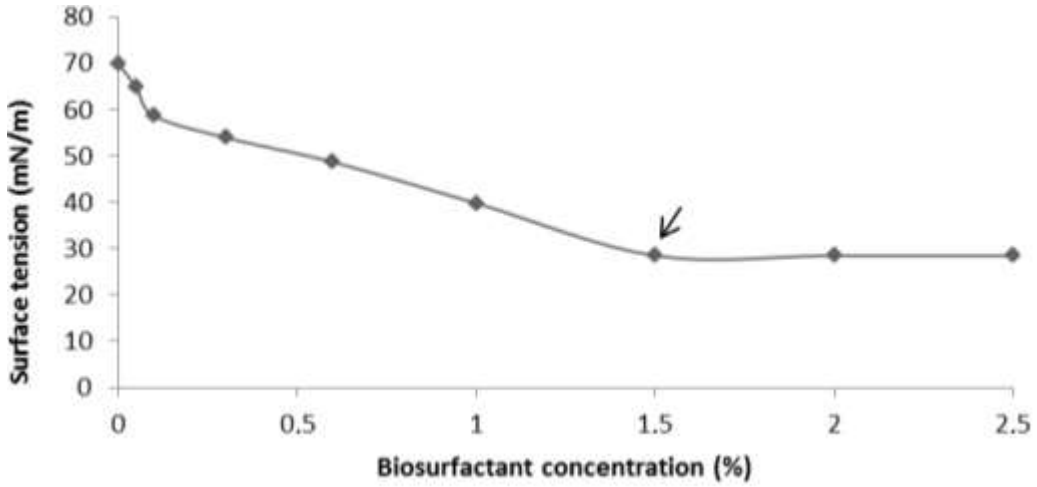

Figure 4. Surface tension versus concentration of the biosurfactant isolated from *C. tropicalis* UCP 1613 cultured in mineral medium supplemented with 3% whey, 7% cassava wastewater and 10% soybean post frying oil.

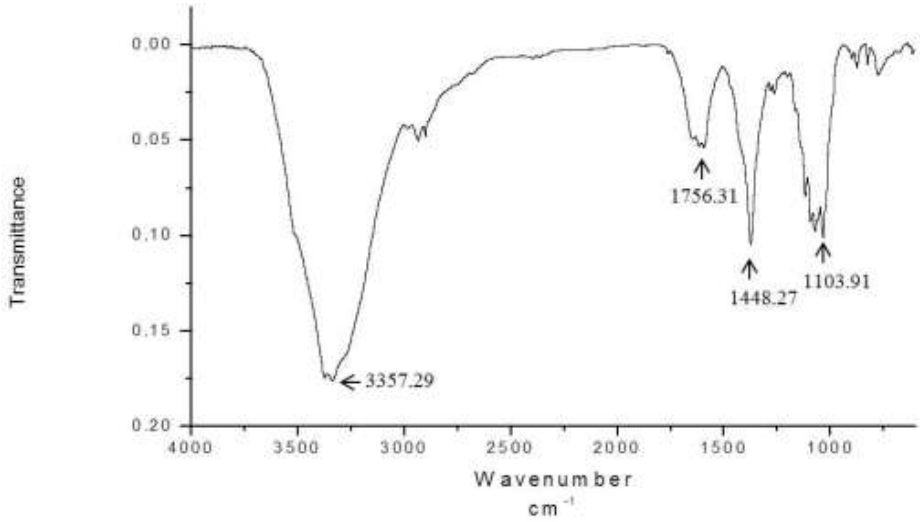

Figure 5. FTIR spectrum of extracted biosurfactant produced from *C. tropicalis* UCP 1613 in mineral medium supplemented with 3% whey, 7% cassava wastewater and 10% soybean post frying oil.

of the biosurfactant isolated from *C. tropicalis* UCP 1613.

In this regard, the Table 1 which displays that this metabolite did not show a phytotoxic effect against seeds tested. Also, it was interesting, the proportional relation was observed between the different germination index (GI) and the concentrations of biosurfactant.

Antimicrobial activity

In the present study, the polymeric biosurfactant produced by *C. tropicalis* UCP 1613 exhibited interesting antimicrobial activities. Table 2 shows that, Gram-positive bacteria tested mainly *Lactobacillus* sp. displayed susceptibility to the biosurfactant, albeit to different degrees. In contrast, Gram-negative bacteria were less sensitive as well as yeast.

DISCUSSION

The interest on *Candida* species has been increased in recent years due to its diverse biotechnological role. Specifically, *C. tropicalis* has been recovered from

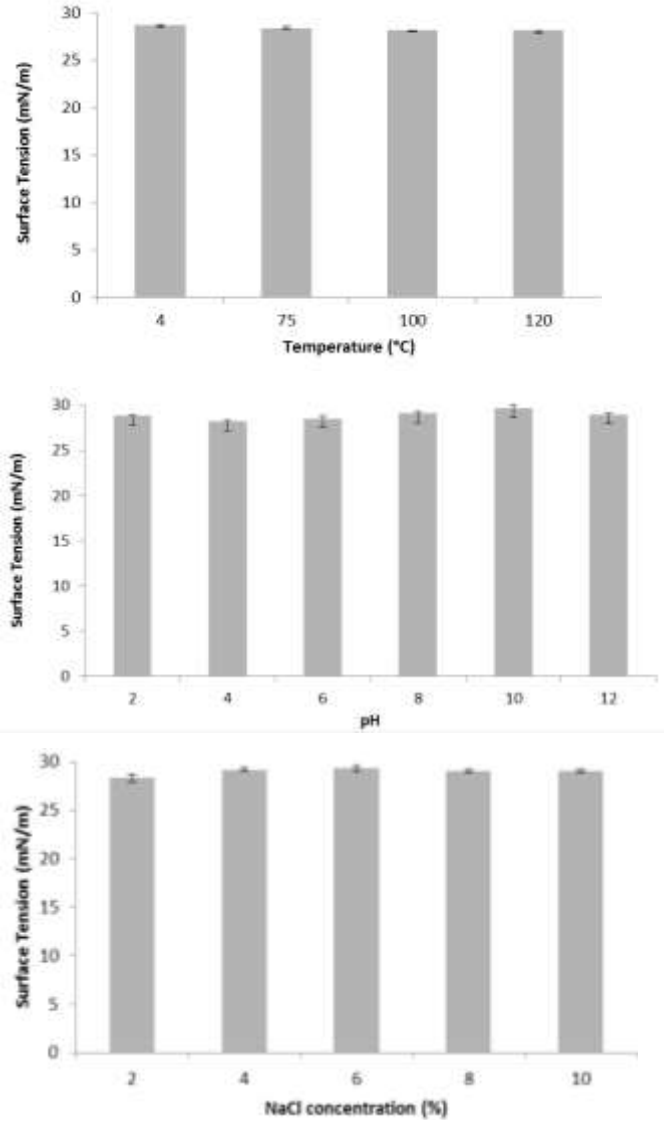

Figure 6. Stability of surface tension of biosurfactant produced by *Candida tropicalis* UCP 1613 in mineral medium supplemented with 3% whey, 7% cassava wastewater and 10% soybean post frying oil. Influence of temperature (A), pH (B) and sodium chloride concentrations (C) on surface tension stability.

seawater, sediments, mangrove plants, mud flats, marine algae and shrimp, indicating its wide distribution in tropical and subtropical marine environment (Luna et al., 2011; Yadav et al., 2012). The use of agro-industrial residues for the production of biosurfactant by members of the genus *Candida* have been previously described by several researchers (Luna et al., 2011; Rufino et al., 2014; Brasileiro et al., 2015). The ability of these microorganisms to grow and produce biosurfactants in wastes-based medium has been used to reduce the high costs of production of surface active compounds of biotechnological interest.

Table 1. Phytotoxicity of biosurfactant isolated from *Candida tropicalis* UCP 1613 cultured in mineral medium supplemented with 3% whey, 7% cassava wastewater and 10% soybean post frying oil against *Brassica oleracea*, *Lactuca sativa* L, *Solanum lycopersicum*.

Germination index (%)	Biosurfactant concentration (%)		
	1	1.5	2
Brassica oleracea	100±0.5	121±0.4	135±0.2
Lactuca sativa L.	100±0.2	102±0.2	118±0.1
Solanum lycopersicum	132±0.2	145±0.3	157±0.4

Experiments were performed in triplicate and the results represent means ± standard deviations of the three independent experiments.

In this study, the biosurfactant showed a growth-associate production, similar to the ones produced for *Candida sphaerica* cultivated in distilled water supplemented with ground-nut oil refinery residue and corn steep liquor as substrates (Sobrinho et al., 2008) and for *Candida lipolytica* cultivated in soybean oil refinery residue and glutamic acid (Rufino et al., 2014).

Although the biosurfactants produced by bacteria are more effective in reducing the surface tension, with values up to 25 to 26 mN/m, several yeasts biosurfactants described in the last few decades have shown similar values to those obtained by bacteria (Luna et al., 2011). One example is the biosurfactant produced by *C. tropicalis* in this study, which reduced the surface tension of medium to 28.5 mN/m after 48 h of cultivation. This value is lower than other biosurfactants produced by *C. tropicalis*: 35 mN/m (Haba et al., 2000); 35.44 mN/m (Coimbra et al., 2009), 37.86 mN/m (Campos et al., 2014) and 29.98 mN/m (Almeida et al., 2017).

By other side, the biosurfactant yield of *C. tropicalis* UCP 1613 was 4.9 g/L, which was better than others, previously reported for *C. tropicalis* strains: 3.0 g/L by *C. tropicalis* UCP 0996 cultured for 120 h in medium containing molasses, corn steep liquor and waste canola oil (Almeida et al., 2015) and 3.61 g/L by *C. tropicalis* cultivated in waste frying oil (Batista et al., 2010). However, other *Candida* members showed highest biosurfactant yields, for example: *C. lipolytica* (8.0 g/L) cultivated during 72 h in optimized medium containing soybean oil refinery residue and glutamic acid (Rufino et al., 2014) and *C. sphaerica* (9.0 g/L) cultured during 144 h in medium composed by ground-nut oil refinery residue and corn steep liquor (Luna et al., 2016).

In addition, *Candida utilis* achieved 12.52 g/L after 88 h of cultivation in waste canola frying oil and ammonium nitrate (Campos et al., 2014). These results demonstrate that the medium composition and culture conditions influence on yields of biosurfactants is produced by *Candida* strains. Also, the suitability of whey, cassava wastewater and soybean post frying oil was confirmed as alternative substrates for biosurfactant production (Silva

Table 2. Antimicrobial activity of the biosurfactant produced by *Candida tropicalis* UCP 1613.

Microorganism	Inhibition diameter (mm)		
	Biosurfactant concentration (%)		
	1	1.5	2
Pseudomonas sp.	10±0.3	12±0.3	14±0.4
Salmonella typhimurium UCP 6017	11±0.4	13±0.3	15±0.2
Serratia marcescens UCP 1549	12±0.2	14±0.4	15±0.1
Escherichia coli	11±0.3	13±0.2	14±0.3
Lactobacillus sp.	18±0.3	21±0.2	23±0.3
Staphylococcus aureus	16±0.2	18±0.2	19±0.5
Staphylococcus epidermidis	14±0.3	17±0.4	18±0.2
Bacillus licheniformis UCP 1013	15±0.4	16±0.2	18±0.5
Candida albicans	10±0.1	11±0.5	13±0.2
Candida lipolytica	11±0.2	13±0.4	14±0.5

et al., 2014; Andrade et al., 2015). Another important characteristic of biosurfactants is the critical micelle concentration (CMC) which is defined as the minimal concentration of the compound, required to yield maximal surface tension reduction of water which initiate micelle formation (Santos et al., 2013). The biosurfactant produced showed a greater capacity to reduce surface tension in comparison to biosurfactants from *Candida glabrata* (31 mN/m) (Luna et al., 2009), and *C. lipolytica* (32 mN/m) (Verma et al., 2015) (33 mN/m) (Santos et al., 2013). But also, this displayed a lower value of CMC than other biosurfactants reported, considering rates of 2.5% such as the biosurfactant from *C. glabrata* (Luna et al., 2009) and *C. lipolytica* (Sarubbo et al., 2007).

The compositional information of microbial surfactants allows the determining of the heterogeneous nature of these compounds. Thus, these are classified according to their molecular structure into glycolipids, lipopeptides, phospholipids, fatty acids, lipopolysaccharides, protein complexes, neutral lipids and polymers (Aparna et al., 2012). The analytical evidence confirmed the presence of carbohydrates and proteins in hydrophilic region of the molecule and also, the existence of lipids in the region hydrophobic of the biosurfactant. Similarly to the biosurfactant obtained in this study from *C. tropicalis* UCP 1613, *C. lipolytica* UCP 1002 has been also reported as producer of a heteropolymer composed 45% protein, 20% lipid and 10% carbohydrate (Sarubbo et al., 2006). However, surfactants produced by this genus can differ widely from one species to another (Rufino et al., 2014). In addition, the zeta potential determines the function of the surface charge of the particle which serves to predict and control the stability of colloidal suspensions and emulsions. The higher values obtained indicate good stability by repulsion between hydrophilic particles, as per the literature (Pornsunthorntawee et al., 2008; Satpute et al., 2010). Other biosurfactants produced by *Candida* species also show an anionic character (Sobrinho et al., 2008; Andrade et al., 2015; Luna et al., 2013).

Different environmental factors such as pH, salinity and temperature affect biosurfactants activity and stability. Therefore, it is important to study the influence of these parameters when considering specific applications for these compounds (Velmurugan et al., 2015). These results obtained suggest the feasibility of application in industries which works under extreme conditions of salinity, temperature and pH, as it was informed for other biosurfactants from *Candida* species (Gusmão et al., 2010; Luna et al., 2014) In this regard, the absence of toxicity is also fundamental for possible application of biosurfactant in the environment. Eco-toxicity bioassays are analytical methods which evaluate toxic effect of the chemical substances.

The exposure of living organisms employed as bio-indicators to these substances constitutes a valuable tool (Fletcher, 1991). The use of plants in toxicity tests offers several advantages among them is low maintenance cost and rapid results, with a special benefit assessment of the potential eco-toxic compounds in terrestrial environments (Farré and Barceló, 2003). In general, in this study was observed that from the different solutions of biosurfactant tested there was a positive effect on the growth seeds (Table 1). Also from the observation of leaves and the elongation of secondary, it was suggested a growth stimulating effect which was verified considering that GI higher than 80%, indicate the absence of phytotoxicity (Tiquia et al., 1996). Similar results were observed by Luna et al. (2013) and Rufino et al. (2014), who investigated the phytotoxic potential of the biosurfactant produced by *C. sphaerica* UCP 0995 and *C. lipolytica* UCP 0988, respectively. The same observations were confirmed by Krawczyńska et al. (2012), Alsohim et

al. (2014) and Silva et al. (2015) which detected the positive effect of biosurfactants in the seedling development.

Alternatively one useful property of many biosurfactants that has been reviewed recently is their antimicrobial activity (antibacterial, antifungal and antiviral) (Banat et al., 2014; Rienzo et al., 2015; Borsanyiova et al., 2016). In this study, the yeasts were less sensitive than Gram-negative bacteria. Some studies confirm that biosurfactants, even in low concentrations, may destabilize the microorganism's membranes and finally inhibit their growth (Carrillo et al., 2003; Calvo et al., 2009). In this regard, it has been observed that Gram-positive bacteria are more susceptible to biosurfactants than Gram-negative bacteria (Elving et al., 2000), which are not inhibited at all or present a slight inhibition. This evidence is in agreement with the results obtained in this study. Similarly, biosurfactants produced by *C. sphaerica* and C. *lipolytica* have also demonstrated antimicrobial activity against different species of fungi and bacteria, suggesting the use of these biomolecules as an alternative antimicrobial agents in the medical field (Luna et al., 2011; Rufino et al., 2011).

Conclusions

In the present study with the new yeast isolated from mangrove, sediments were identified as *C. tropicalis* UCP 1613. The optimal carbon and nitrogen sources was a combination of whey (3%), cassava wastewater (7%) and soybean post frying oil (10%) resulting significantly reduction of the surface tension.

The biosurfactant produced was non-cytotoxic which showed stable surface tension to high temperature, acid to alkaline pH, and all concentrations of electrolyte (up to 12%). Besides, the new biosurfactant is characterized as anionic and polymeric molecule. The tension-active molecule showed properties to effective antimicrobial activity, Gram-positive and negative bacteria, and yeasts. This study strongly suggested the biosurfactant produced by *C. tropicalis* UCP 1613 which play a promising role in biomedical application due to its non-toxicity, stability and higher antimicrobial activity. The use of renewable resources as agro-industrial waste from circular bio-economy approach allowed the obtaining of biosurfactant as a value added product.

CONFLICT OF INTERESTS

The authors declared no conflict of interest.

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