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Full Length Research Paper

Prevalence and antimicrobial susceptibility profile of *Salmonella* species from ready-to-eat foods from catering establishments in Jigjiga City, Ethiopia

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Food-borne pathogens are the leading cause of morbidity and mortality in developing countries. Changes in eating habits, mass catering, unsafe food storage conditions and poor hygiene practices are major contributing factors to food associated illnesses. In Ethiopia, the widespread habit of ready-to-eat food consumption is potential cause of food borne illnesses. The present study aimed at investigating the prevalence and antimicrobial susceptibility profile of *Salmonella* species from ready-to-eat foods from catering establishments in Jigjiga City and determining susceptibility pattern of bacterial isolates. This study aimed to assess the ready-to-eat foods *Salmonella* prevalence and antimicrobial resistance pattern of the isolates. In total, various food samples were collected from 120 hotels and processed to detect *Salmonella* presence. The ready-to-eat food samples were collected and assessed bacteriologically for *Salmonella* strains. A total of 120 food samples were bacteriologically evaluated. After confirmation, 25 samples (20.8%) were positive to *Salmonella*. A total of 5 drug resistance patterns were detected among *Salmonella* isolates. Out of the 25 isolates 10 (40%) were resistant to three antibiotics, 8 (28%) of the isolates were resistant to 4 of the antibiotics tested, whereas 5 (20%) were resistant to two antibiotics, only 1 (4%) isolate was resistant to 1 antibiotic but 1 (4%) isolate was resistant to five antibiotics. The lack of public sanitary facilities can be another hurdle to keep the desirable hands hygiene of the vendors. In this study, it is reported that severity of the current scenario among the hotel worker hygiene and they are the unknowingly playing role in spread of diseases like Salmonellosis.

Key words: Food Establishments, ready-to-eat foods, *Salmonella*.

INTRODUCTION

Foodborne outbreaks caused by *Salmonella* represent a major public health problem worldwide, and developing countries are affected by a wide range of foodborne diseases (Zeru and Kumie, 2007). These low-income countries face the highest burden of diarrheal and other

food-borne disease associated with the consumption of contaminated food (Zeru and Kumie, 2007). *Salmonella* are among the major disease bacteria in humans as well as in animals. *Salmonella* species are leading causes of acute gastroenteritis in several countries

and salmonellosis remains an important public health problem worldwide, particularly in the developing countries (Rotimi et al., 2008). Salmonellosis is the most common food borne disease in both developing and developed countries, although incidence rates vary according to the country (Stevens et al., 2006). The fecal wastes from infected animals and humans are important sources of bacterial contamination of the environment and the food chain (Ponce et al., 2008).

Antimicrobial-resistant *Salmonella* are increasing due to the use of antimicrobial agents in food at sub-therapeutic level or prophylactic doses which may promote on-farm selection of antimicrobial resistant strains and markedly increase the human health risks associated with consumption of contaminated food products (Zewdu and Cornelius, 2009). Food borne illness associated with the consumption of foods has been reported in several places in Ethiopia and elsewhere (Estrada-Garcia et al., 2004; Chumber et al., 2007; Ghosh et al., 2007). Antimicrobial use in animal production systems has long been suspected to be the cause of emergence and dissemination of antimicrobial resistant *Salmonella* (Alexander et al., 2009).

Different studies conducted in Ethiopia indicated considerable prevalence of *Salmonella* both in ready to eat foods and food handlers (Molla et al., 2003). So, the aim of this study was to determine the prevalence and antimicrobial susceptibility pattern of *Salmonella* isolates from ready-to-eat foods from Jigjiga City food establishments.

MATERIALS AND METHODS

Description of the study area and period

This study was conducted in Jigjiga City, located 650 km South East of Addis Ababa. The study was conducted from March to October, 2015 and laboratory activities were carried out at veterinary medicine microbiology laboratory, Jigjiga University. The study samples were collected from different food establishments of the city. Sample size was determined from 256 food establishments using prevalence rate of 50%, there was no other baseline to determine the prevalence at 5% level of significance and the following formula was employed (Yemane, 1967):

$$n = \frac{N Z_{\alpha/2}^2 P (1-P)}{d^2 (N-1) + Z_{\alpha/2}^2 P (1-P)}$$

Based on the above formula, the calculated sample size was 120. A total of 120 food establishments and 6 food types were randomly selected and included in this study. All the

samples including 20 kikil, 20 mahberawi, 20 beyayinet, 20 tibs, 20 minchet and 20 keywet were collected for the detection of *Salmonella*. The ready-to-eat food specimens were collected in a clean sterile aluminum foil directly from the kitchen. Approximately 25 g of ready-to-eat food was collected in a sterile aluminum foil. The samples were transported using an ice box and analyzed at veterinary medicine microbiology laboratory, Jigjiga University.

Isolation and identification of *Salmonella*

The isolation and identification of *Salmonella* were performed using techniques recommended by International Organizations for Standardization (ISO-6579, 2000), and those supported by the Global Salmonella Surveillance (GSS) and National Health Services for Wales (NHS) (HPA, 2008). The isolation and identification involves three steps; To test for the presence of *Salmonella*, 25 mm³ of each sample was aseptically transferred into sterile flask containing 225 ml buffered peptone water (BPW), homogenized for 5 min and then incubated at 37°C for 24 h for recovery and proliferation of cells which might be injured during processing or to make the number of target organisms grow to a detectable level. Following the BPW enrichment, the secondary enrichment broth namely the Rappaport Vassiliadis enrichment broth was used since the selective property of this broth lies in its ability to inhibit non-targeted microorganisms like Gram positive bacteria and coliforms and permits the rapid multiplication of *Salmonella*. After pre-enrichment in buffered peptone water, 1 ml of culture from the buffered peptone water was transferred into 10 ml of Rappaport Vassiliadis broth and was incubated at 43°C for 48 h. Solid media such as Salmonella–Shigella agar, xylose lysine desocholate (XLD) agar and Brilliant green modified agar were used for plating purpose. A loopful of culture from the Rappaport Vassiliadis broth were streaked onto each of the solid medium and incubated at 37°C for 18 h. Characteristic colonies from each selective agar were picked, further purified and tested biochemically.

All suspected non-lactose fermenting bacterial colonies, picked from Salmonella–Shigella (SS) agar, XLD agar or Brilliant green modified agar, were inoculated into the following biochemical tube for identification: triple sugar iron (TSI) agar, Simmon's citrate agar, sulphide indole motility (SIM) medium, lysine iron agar, urea agar, and fermentation of glucose, sucrose and mannitol.

Antimicrobial susceptibility testing

The antimicrobial susceptibility patterns of *Salmonella* spp. were carried out following the Kirby–Bauer disc diffusion method on Mueller-Hinton agar plates (Oxoid) as described by the Clinical and Laboratory Standards Institute (CLSI, formerly NCCLS) guidelines (NCCLS, 2002). Antimicrobial susceptibility testing for *Salmonella* spp was performed using the disk diffusion method at 37°C for 16–18 h and results were interpreted using the criteria of the National Committee for Clinical Laboratory Standards. *Escherichia coli* ATCC 25922 was used as a quality control organism for the criterion for selection of the antimicrobial agents was based on the availability and current use of these antibiotics for treating infectious antimicrobial susceptibility test (Hendriksen, 2002). The disease in health institution of Ethiopia. The following antibiotics were used with their respective concentration (in brackets) for

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Table 1. The prevalence of *Salmonella* from different food types at catering establishments.

Establishments type	Sample size	<i>Salmonella</i> positive	<i>Salmonella</i> positive (%)	P value
Beyayinet	20	6	30	p= 0.023
Kikil	20	5	25	
keywet	20	3	15	
Mahberawi	20	9	45	
Tibs	20	0	0	
Minchet	20	2	10	
Total	120	25	20.8	

Table 2. Antimicrobial resistance of *Salmonella* isolates by food establishment types (n=25).

Antimicrobial disc	Total number (%) isolates resistant from foods served at food establishments					
	Total isolate (n=25)	Beyayinet (n=6)	Kikil (n=5)	keywet (n=3)	Mahberawi (n=9)	Minchet (n=2)
AMP	25 (100)	6 (100)	5 (100)	3 (100)	9 (100)	2 (100)
STR	2 (8)	1 (16.7)	0 (0)	0 (0)	1 (11.1)	0 (0)
NOR	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
TET	7 (28)	2 (33.3)	1 (16.7)	1 (33.3)	3 (33.3)	0 (0)
KAN	16 (64)	4 (66.7)	3 (60)	2 (66.7)	6 (66.7)	1 (50)
GEN	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
CHL	6 (24)	2 (33.3)	1 (20)	0 (0)	2 (22.2)	0 (0)
CIP	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
NAL	22 (88)	5 (83.3)	4 (80)	3 (100)	8 (88.9)	2 (100)

Salmonella spp. isolates: chloramphenicol (30 µg), ciprofloxacin (15 µg), amoxicillin (15 µg), gentamicin (10 µg), erythromycin (20 µg), ampicillin (10 µg), co-trimaxazol (10 µg), doxacyclin (30 µg), ciprofloxacin (30 µg) (Micromaster, India).

Data analysis

Data was analyzed using SPSS, version 17.0 computer soft-ware (SPSS 17.0 Command Syntax Reference. SPSS Inc., Chicago, 2004) and presented in tables. A significant difference was taken as significant at a p ≤ 0.05.

Ethical consideration

The study was ethically approved by the Institutional Review Board of Jigjiga University, research and ethical clearance committee.

RESULTS

From a total of 120 samples examined, 25 (20.8%) meals from food establishments were found to be positive to *Salmonella* spp. (Table 1). Moreover, *Salmonella* spp. were isolated from 6 (30%) beyayinet, 3 (15%) keywet, 5 (25%) kikil and 9 (45%) of mahberawi, none of tibs and 2 (10%) of minchet. The frequencies of isolation of *Salmonella* spp. differed among the food types and it ranged from 0 (tibs) to 45% (mahberawi) (Table 1).

Significant variation was observed in prevalence of *Salmonella* among food types (p= 0.023).

A total of 25 isolates (n=25) were tested against nine commonly used antimicrobials viz. ampicillin (10 µg), chloramphenicol (30 µg), gentamycin (10 µg), streptomycin (10 µg), kanamycin (30 µg), Nalidixic Acid (30 µg), Ciprofloxacin (5 µg), tetracycline (25 µg) and norfloxacin (10 µg) following NCCLS 2000 guidelines. The results of the antimicrobial sensitivity test are shown in Table 2.

Among all the antimicrobials tested, Ampicillin (100%) and Nalidixic Acid (88%) were the most resistant drugs by *Salmonella* isolates followed by kanamycin (64%), tetracycline (28%) and chloramphenicol (24%). norfloxacin (0%), gentamycin (0%) and ciprofloxacin (0%) showed maximum activity.

A total of 5 drug resistance patterns were detected among *Salmonella* isolates (Table 3). Out of the 25 isolates 10 (40%) were resistant to 3 antibiotics, 8 (28%) of the isolates were resistant to 4 of the antibiotics tested, whereas 5 (20%) were resistant to 2 antibiotics, only 1 (4%) isolate was resistant to 1 antibiotic but 1 (4%) isolate was resistant to 5 antibiotics (Table 3).

DISCUSSION

Salmonella was found in 20.8% of food samples in

Table 3. Multi drug resistance pattern of *Salmonella* spp. isolated from different food types of food establishments.

MDR patter	Resistance patter	Number of isolates	Percent
One	Amp	1	4
Two	Amp, Nal	3	12
	Amp, Str	2	8
Three	Amp, Kan, Nal	7	28
	Amp, Tet, Nal	1	4
	Amp, Chl, Nal	1	4
	Amp, Str, Chl	1	4
Four	Amp, Tet, Kan, Nal	1	4
	Amp, Tet, Kan, Nal	4	16
	Amp, Kan, Chl, Nal	3	12
Five	Amp, Tet, Kan, Chl, Nal	1	4

Amp = Ampicillin, Str = streptomycin, Nor = norfloxacin, Tet = tetracycline, Kan = kanamycin, Gen = gentamycin, Chl = chloramphenicol, Cip = ciprofloxacin, Nal = nalidixic acid.

this study. This is a pathogenic micro-organism at very low doses of infection, and is transmitted from person to person but may also occur by consumption of contaminated water and foods including vegetables that have received little or no heat treatment. Food may become contaminated by infected food handlers who do not wash their hands with soap after using the toilet. Foods can also become contaminated if they are harvested from a field with sewage contamination in them and thus the need for caterers to ensure they buy the raw foods from reputable suppliers. In addition, frequent assessing of the raw food product supplier is encouraged.

Salmonella can also be transmitted by flies. Flies can breed in infected feces and then contaminate food and thus the need for caterers to have an elaborate pest control program. The prevalence of *Salmonella* in foods was too high, in contrast to the previous studies in Ethiopia. For instance, Akafete and Haileleul (2011) and Woldemariam et al. (2005) found that the prevalence of *Salmonella* from goat carcass swab was 8.3% at Modjo and 7.5% at Bishoftu, respectively. This difference could be due to differences in the hygienic and sanitary practices practiced in the food establishments at Jigjiga city.

Resistance to multiple antimicrobials (100%) which was observed in the current study was higher than other studies conducted in Ethiopia. There are reports which shows the multiple antibiotics resistance by *Salmonella*, for instance, Alemayehu et al. (2002), Endrias (2004) and Zelalem et al. (2011) reported 52, 23.5, 44.8 and 83.3%, respectively for the multidrug resistance of *Salmonella* isolated from food of animal sources, animals and humans, as well higher than reports from elsewhere (Stevens et al., 2006; Khaitsa et al., 2007; Al-Bahry et al.,

2007; Elgroud et al., 2009; Fadlalla et al., 2012) on multidrug resistance of *Salmonella*. This difference could be because, drug-resistant *Salmonella* are increasing due to the use of antimicrobial agents in food animals at prophylactic doses which may promote on-farm selection of antimicrobial resistant strains and markedly increase the human health risks associated with consumption of contaminated food products as stated by Molla et al. (2003, 2006) and Zewdu and Cornelius (2009).

Zewdu and Cornelius (2009) reported that the isolates of *Salmonella* from food items and workers from Addis Ababa were resistant to the commonly used antibiotics including streptomycin, ampicillin, and tetracycline. Furthermore, Zelalem et al. (2011) also indicated resistance of *Salmonella* isolates to commonly used antimicrobials including ampicillin, streptomycin, nitrofurantoin, kanamycin and tetracycline, with resistance rate of 100, 66.7, 58.3 and 33.3%, respectively. Similarly, previous reports from South India (Suresh et al., 2006), from Nigeria (Akinyemia et al., 2005) and from Cameroon (Akoachere et al., 2009) indicated a similar 100, over 90 and 100%, respectively resistance to ampicillin. The result of the current research also showed resistance of *Salmonella* isolates to commonly used antimicrobials including ampicillin, nalidixic acid and kanamycin with resistance rate of 100, 88 and 64%, respectively. However, higher resistance rate than previous reports with the exception of ampicillin and resistance to further drugs as well as to nalidixic acid with resistance rate of 56.8% was observed in this result. This difference could be due to the increasing rate of inappropriate utilization of antibiotics which favors selection pressure that increased the advantage of maintaining resistance genes in bacteria (McGeer, 1998;

Mathew et al., 2007). It is as well recognized that recent resistance additions include resistance to trimethoprim. The continuing development of antibiotic resistance may lead to sufficient pressure ultimately to restrict the antibiotics available to the veterinary profession for animal treatment (Gracey et al., 1999). Moreover, this increase antibiotic resistance, in addition to public health problems, may lead to economic loss in the countries due to loss of exporting meat and animal products and cost of drug of choice to treat human and animals due to resistance development.

Ciprofloxacin showed a good antimicrobial activity against these *Salmonella* isolates. It was found that all the 25 (100%) isolates were susceptible to ciprofloxacin. This result was comparable to previous reports by Molla et al. (2006) from central part of Ethiopia among isolates of sheep and goat meat, Akinyemia et al. (2005) from Nigeria, from human isolates and Zelalem et al. (2011), isolates of *Salmonella* from dairy farms in Addis Ababa. The effectiveness of drugs like ciprofloxacin could be because they are not widely used in countries like Ethiopia and other African countries (Zelalem et al., 2011). In addition to this, effectiveness of this drug could be because it is not well distributed in all societies and not simply prescribed rather it is used as drug of choice in antibiotic resistant person. In addition to this, ciprofloxacin is not commonly used to treat animals in Ethiopia.

The current study indicated the inevitability of a further research on the prevalence and antimicrobial susceptibility of *Salmonella*, by considering it as a potential food borne pathogen. Molecular characterization of the isolates with emphasis on resistant strains is also required to identify mechanisms of resistance. Moreover, careful and discreet use of antimicrobials in the health sectors is mandatory since high rate of antimicrobial resistant *Salmonella* isolates were identified.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Production and characteristics of bioflocculant TPT⁻¹ from a consortium of *Bacillus pumilus* JX860616 and *Alcaligenes faecalis* HCB2

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The combination of microorganisms in consortia enhances high bioflocculant yields. The study aimed at producing and characterising bioflocculant from *Bacillus pumilus* JX860616 and *Alcaligenes faecalis* HCB2. Bioflocculant TPT⁻¹ was obtained through ethanol extraction after optimum conditions were established. The characteristics of the bioflocculant TPT⁻¹ were obtained by scanning electron microscope equipped with elemental detector, Zetasizer nano, ultraviolet-visible spectrophotometry. Fourier transform infrared (IR) spectrophotometry and the thermal decomposition was used to conduct the thermal gravimetric analysis. The flocculation mechanism of TPT⁻¹ on Kaolin suspension was obtained by Zetasizer Nano. Glucose and yeast extract were the best energy sources, yielding 3.0 g/L of TPT⁻¹ in optimum conditions (30°C, 165 rpm, initial pH 6 and 72h). TPT⁻¹ revealed to be an anionic, heat stable glycoprotein, with the total carbohydrate content of 83.1% (w/w) and the total proteins content of 9.7% (w/w). The elemental analysis demonstrated the presence of N (1.3), C (15.0), O (44.8), P (0.8), Ca (9.0), Cl (2.8), Mg (0.4), S (12.1), K (11.4) and Na (1.9) in mass proportion (% w/w), while the IR spectrum showed the presence of hydroxyl, carbonyl and amine groups. Ba²⁺ mediated bridge flocculation mechanism between the bioflocculant TPT⁻¹ and Kaolin particles. The high flocculating capability (90%) and characteristics of TPT⁻¹ suggested its potentiality in industrial applications.

Key words: Bioflocculant TPT⁻¹, flocculating activity, flocculation mechanism and bacterial consortium.

INTRODUCTION

Flocculation is the natural process whereby flocculants are used to flocculate, settle and remove particles, suspended solids and colour in solutions (Cong-Liang et al., 2012). Flocculating agents are widely used in industrial fields such as dredging, textile dyeing, mining, pharmacology, cosmetology, wastewater treatment, food

and fermentation processes (Zhang et al., 1999; Salehizadeh and Shojaosadati, 2001). Flocculants are grouped as inorganic (aluminium salts), synthetic (polyacrylamide) and natural occurring flocculants (bioflocculants and chitosan) (Okaiyeto et al., 2015). Inorganic and synthetic organic flocculants are

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extensively used in vast biotechnological applications due to their cost effectiveness. However, they are both reported to impose health risks and environmental hazards (Salehizadeh and Shojaosadati, 2001). Synthetic flocculants have shown adverse health effects such as neurotoxicity, carcinogenicity and cause Alzheimer's diseases (Serdar et al., 2011). Moreover, chemical flocculants are non-degradable and their by-products are often toxic (Okaiyeto et al., 2015). Owing to the shortcomings, the need for eco-friendly flocculants that impose less health threats is of high demand. Bioflocculants are macromolecules secreted by microorganisms due to substrate metabolism, microbial growth, cell lysis and degradation of microorganisms or microbial components (Carlos et al., 2011). Bioflocculants lack secondary pollution and thus considered as environmental friendly substances. This is due to the special bioflocculant components (carbohydrates, proteins, nucleic acid and lipid), microbial flocculant matrix that show biodegradability adsorption abilities and hydrophilicity or hydrophobicity (More et al., 2014). However, production costs and low flocculant yields have been restrictive factors in the industrial applicability of bioflocculants. Moreover, the lack of knowledge of the active ingredients of most bioflocculants and the mechanism involved during flocculation also decreases their market potential (Barredo, 2005). In nature, microorganisms do not live in isolation; they coexist with many other microorganisms forming synergism and symbiosis relationships (Chapelle, 2001). Zhu et al. (2004) and Zhang et al. (2007) have reported that the combination of strains of microorganisms in consortia produce bioflocculants that have better flocculating activity and higher bioflocculant yields than pure strains. *Bacillus pumilus* JX860616 and *Alcaligenes faecalis* HCB2 produced bioflocculants (2.4 and 2.7 g/L, respectively), with high flocculating activities of 93.3 and 92.1%, respectively. Thus, in attempt to improve the quantity and quality of the bioflocculants, a consortium of these bacterial strains was constructed and the characteristics and flocculation mechanism of the produced bioflocculant was determined.

MATERIALS AND METHODS

Bacterial strains

Bacterial strains were previously isolated from Sodwana Bay sediment in the north of KwaZulu-Natal, (RSA). The bacterial strains were identified by 16S rRNA nucleotide sequencing genes with subsequent BLAST analyses. Nucleotide sequences were placed in GenBank and the repository accession numbers were *Bacillus pumilus* JX860616 and *Alcaligenes faecalis* HCB2. The bacterial strains were then preserved in 20% glycerol stock at -80°C in the Department of Biochemistry and Microbiology, University of Zululand, (RSA).

Production media

The standard fermentation medium by Zhang et al. (2007) was

used. The medium composed of glucose (20.0 g), KH₂PO₄ (2.0 g), K₂HPO₄ (5.0 g), (NH₄)₂SO₄ (0.2 g), NaCl (0.1 g), CH₄N₂O (0.5 g), MgSO₄ (0.2 g) and yeast extract (0.5 g) in a litre of filtered seawater and was autoclaved at 121°C for 15min.

Bacterial consortium

B. pumilus JX860616 and *A. faecalis* HCB2 were used in combination to construct bacterial consortium. A loopful of each strain colony was inoculated separately into 50 ml of the fermentation medium and incubated for 72 h at 30°C with the shaking speed of 165 rpm. The preculture was then used as the standard inoculum. For construction of the bacterial consortium, 1ml of the standard inoculums of bacterial strains were both inoculated into the same 50ml of the fresh fermentation medium and incubated at 30°C at the shaking speed of 165 rpm. After 72h, the culture broth was centrifuged at 8000 × g for 30 minutes at 4°C and the supernatant was used for determination of flocculating activity (FA) (Zhang et al., 2007).

Determination of flocculating activity

The method by Kurane et al. (1986) was adapted to access the flocculating activity. 100 ml of Kaolin clay solution (4 g/1000 ml) was mixed with 3 ml of 1% w/v CaCl₂ and 2 ml of obtained supernatant. The mixture was vigorously agitated, poured into measuring cylinder and left to stand at room temperature. After 5min, the optical density (OD₅₅₀) was read and the flocculating activity determined using the formula:

$$FA (\%) = [(A - B/A)] \times 100$$

Where A was the optical density of Kaolin suspension at 550nm and B was optical density of a sample at 550 nm.

Optimisation of flocculation conditions

The effect of the different inoculum sizes (1 to 5%) of the constructed consortium on flocculating activity were assessed (Zhang et al., 2007). Bacterial species were cultivated in various organic carbon sources (glucose, fructose, sucrose, lactose, maltose, xylose, starch and molasses) and nitrogen sources (casein, beef extract, yeast extract, ammonium sulphate urea and peptone) for determination of suitable carbon and nitrogen sources for bioflocculant production (Okaiyeto et al., 2016). The concentration of 20 g/L of culture medium was used for carbon sources and 1.2 g/L for nitrogen sources. The effect of initial pH on flocculating activity was determined using a pH meter (Eutech instruments-pH 700) (Liu et al., 2010). The pH of the culture medium was adjusted by 0.1 M HCl and 0.1 M KOH in the range of 3 to 12. The effect of cultivating temperatures (20 to 45°C) on the flocculating activity were assessed and various metal cations (NaCl, KCl, LiCl, MnCl₂, BaCl₂ and FeCl₃) were also used to evaluate their effect on flocculating activity by replacing the CaCl₂ used in an original medium (Zhao et al., 2013).

Time course assay

The effect of fermentation time on flocculating activity was conducted in accordance with a previous study by Cosa et al. (2013). Bacterial cultures in consortium were incubated under obtained optimal growth conditions. From the fermentation broth, samples were drawn every 12h for 120h and the final pH and

Table 1. Effect of inoculum size, carbon sources, nitrogen sources and cations on flocculation.

Inoculum size (ml)	FA percentage (%) ± SD	Carbon source	FA (%)±SD	Nitrogen source	FA percentage (%) ± SD	Cations	FA percentage (%) ± SD
0.5	76.4 ± 6.34 ^a	Glucose	80.5 ± 9.07 ^a	Yeast extract	97.4 ± 0.57 ^a	Control	31.5 ± 9.37 ^a
1	93.5 ± 3.39 ^a	Molasses	76.6 ± 1.90 ^{a,b}	(NH ₄)SO ₄	92.8 ± 1.71 ^{a,b}	Li ⁺	87.3 ± 1.11 ^b
1.5	85.1 ± 2.25 ^a	Lactose	72.3 ± 4.07 ^a	Peptone	84.9 ± 8.87 ^a	K ⁺	89.4 ± 3.82 ^b
2	84.3 ± 6.52 ^a	Starch	70.1 ± 5.13 ^a	Urea	84.7 ± 0.61 ^{a,b}	Na ⁺	93.3 ± 5.0 ^b
2.5	82.4 ± 14.93 ^a	Maltose	64.4 ± 8.92 ^a	Casein	79.8 ± 7.53 ^b	Ba ²⁺	96.2 ± 1.25 ^b
-	-	Xylose	64.2 ± 11.89 ^a	-	-	Mn ²⁺	89.9 ± 3.59 ^b
-	-	Sucrose	63.1 ± 9.54 ^a	-	-	Fe ³⁺	56.0 ± 1.91 ^c
-	-	Fructose	43.1 ± 17.65 ^b	-	-	-	-

Percentage flocculating activity with different letters (a, b, c, and d) are significantly ($p < 0.05$) different.

optical density (OD₅₅₀) of the broth were determined. The fermentation broth was then centrifuged (8000 × g, 30 min) including the supernatant used for evaluation of FA.

Extraction and purification bioflocculant TPT⁻¹

The extraction and purification of bioflocculant TPT⁻¹ was done in accordance with the standard method by Chang et al. (1998). After 72h of incubation, the culture broth was centrifuged (8000 × g, 4°C, 30min). The distilled water (1000 ml) was added to the supernatant phase and centrifuged (4000 × g, 30 min at 4°C) to remove the insoluble substances. Ethanol (2000 ml) was added to the supernatant, agitated and then left at 4°C to precipitate overnight. The precipitate was vacuum-dried and the crude product dissolved in 100ml of distilled water. Hundred millilitre of the mixture of chloroform and butanol (5:2 v/v) was then added, agitated and left to settle overnight at room temperature. The supernatant was again centrifuged (4000 × g, 4°C, 30 min) and vacuumed-dried.

Physicochemical characterisation of bioflocculant TPT⁻¹

The morphological structure of the bioflocculant, Kaolin floc and Kaolin particles were determined by scanning electron microscope (SEM) (SEM-Sipma-VP-03-67) and the electrical charge of the bioflocculant was analysed by Malvern Zetasizer Nano. The total sugar content was evaluated by phenol-sulphuric acid method (Chaplin and Kennedy, 1994). Ultraviolet-visible spectrophotometry was used to qualitatively determine the protein content (Harrington and Raper, 1968), and the total protein concentration was determined by Bradford, (1976) method. Ninhydrin method was quantitatively used to determine the presence of amino acid (Kay et al., 1956). The elemental analysis was carried out with SEM equipped with elemental analyser. Prior to SEM analysis, 5mg of bioflocculant, was added on slides coated with silicon and fixed by spin coater at rpm, 60s. The functional groups of the produced TPT⁻¹ were assessed by a Fourier transform infrared (IR) spectroscopy (Okaiyeto et al., 2013). The solubility test was done in different solvents (water, benzene, acetone, ethyl acetate, chloroform, dichloromethane, ethanol, hexane, methanol and butanol) (Zaki et al., 2011). Determination of optimum dosage of the produced bioflocculant was done in different dosages (0.2 to 1.0 mg/ml) (Okoh and Ugbenyen, 2014)

Effect of cations, salinity, pH and thermal on the flocculating activity of TPT⁻¹

The synergistic effect of cations (KCl, NaCl, MnCl₂, CaCl₂, BaCl₂

and FeCl₃) on the flocculating activity of the bioflocculant was evaluated (Okaiyeto et al., 2013). The effect of salinity on bioflocculant activity was determined by varying NaCl concentrations (5 to 35 g/L) in Kaolin solution (4g/L). The effect of pH on the flocculating activity of the purified bioflocculant was assayed according to Okaiyeto et al. (2013), whereby the pH of Kaolin solutions in separate flasks were adjusted using KOH and HCl in a range of 3 to 12 prior to the assessment of the flocculating activity of the bioflocculant. Thermal stability of the purified bioflocculant was determined at different temperatures (50 to 100°C) (Wang et al., 2011). The pyrolysis analysis of the purified bioflocculant was assessed at the range of 22 to 900°C at a rate constant of 10°C min⁻¹ under constant flow of nitrogen gas using thermo-gravimetric instrument (Okaiyeto et al., 2013).

Zeta potential

The zeta potential of the bioflocculant TPT⁻¹, Kaolin solution, Kaolin solution with BaCl₂, Kaolin solution with both BaCl₂ and bioflocculant TPT⁻¹ were measured by a ZetaSizer Nano (Malvern, UK) to determine and propose the flocculation mechanism involved during flocculating of Kaolin particles in suspension by of bioflocculant TPT⁻¹ (Aljuboori et al., 2015). The pH of the suspensions were adjusted to pH 3 using HCl or KOH. The zeta potential of each sample was determined using a Malvern Zetasizer Nano with clear disposable cuvettes at 25°C. The cuvettes were thoroughly washed and 2.5 ml of samples were injected carefully whilst avoiding any air bubbles in the cuvette.

Statistical analysis

The data was obtained in triplicate experimentation and subjected to one-way analysis of variance (ANOVA) using the MINITAB Student Release 12 statistical package. A significance level of $p < 0.05$ was used.

RESULTS

Inoculum size

Table 1 displayed the effect of inoculum size of mixed bacterial isolates on bioflocculant TPT⁻¹ production. The inoculum size of 2% (v/v) gave the highest flocculating activity (93%) compared to the other four sizes. The

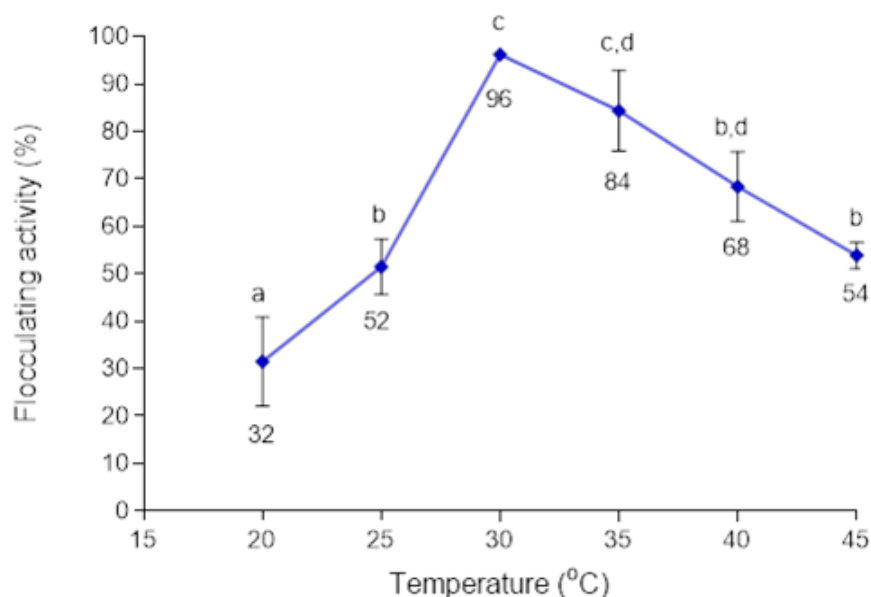


Figure 1. Effect of temperature on flocculation activity.

increase or decrease in inoculum size led to a slight decrease in the flocculating activities. Thus, an inoculum size of 2% was used in all experiments.

Effect of carbon and nitrogen sources on flocculating activity

Bacteria in consortium utilized various carbon and nitrogen sources in the culture medium. Glucose was the most preferred carbon source with the highest flocculating activity of 80.5%, followed by lactose with 70.5% (Table 1). Fructose, sucrose, maltose, xylose, starch and molasses were poorly utilised by the bacteria for bioflocculant production as shown by the low flocculating activities which were generally less than 70%. The effect of different nitrogen sources on bioflocculant production was studied and the results were illustrated in Table 1. All the nitrogen sources showed a potential to be used in bioflocculant production as they resulted in flocculating activities above 80%. Ammonia sulphate was the most preferred nitrogen source with the highest flocculating activity of 97% while casein had the least flocculating activity (80%).

Cations effect on flocculating activity

Table 1 demonstrated the effect of metal cations on flocculating activity. All the cations used, including the control (without cation), stimulated flocculation process and resulted in over 70% of flocculating activities. Ba^{2+} was the most active and preferred metal cation, resulting with the highest flocculating activity of 96%. Fe^{3+} showed

the lowest flocculating activity of 77%.

Effect of temperature on flocculating activity

Fermentation temperature has an impact on bioflocculant production. Figure 1 illustrated the effect of fermentation temperatures on flocculation activity. When the fermentation temperature was 30°C, the flocculating activity reached the maximum of 96%. The increase or decrease in fermentation temperature above or below 30°C resulted in the decrease in flocculating activity. Thus, 30°C was used as the optimal temperature for the bioflocculant production.

Effect of Initial pH on flocculating activity

Figure 2 shows the effect of initial pH of the fermentation medium on the flocculating activity. The bacteria in consortium maintained over 80% of flocculating activity over the weak acidic (pH 5 to 6) and alkaline (pH 8), with the highest flocculating activity (97%) at pH of 6. It was observed that highly alkaline pH 10 to 12 did not favour bioflocculant production and resulted in poor flocculating activity of less than 70%; pH 12 gave the least flocculating activity of 44%.

Time course

The effect of time course on flocculating activity (FA), bacterial growth (shown as optical density (OD)) and pH are shown in Figure 3. The flocculating activity increased

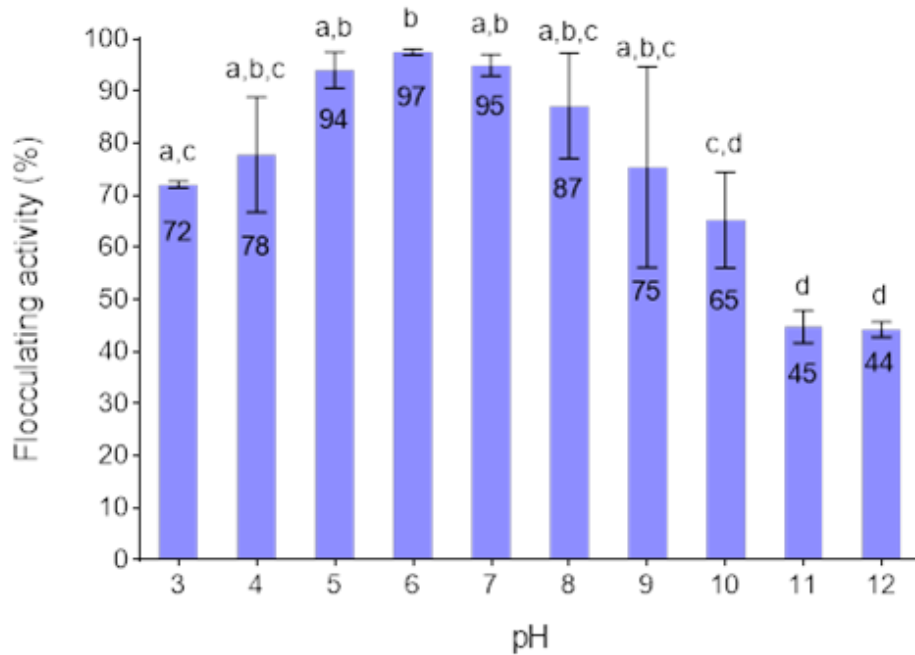


Figure 2. Effect of initial pH on flocculating activity.

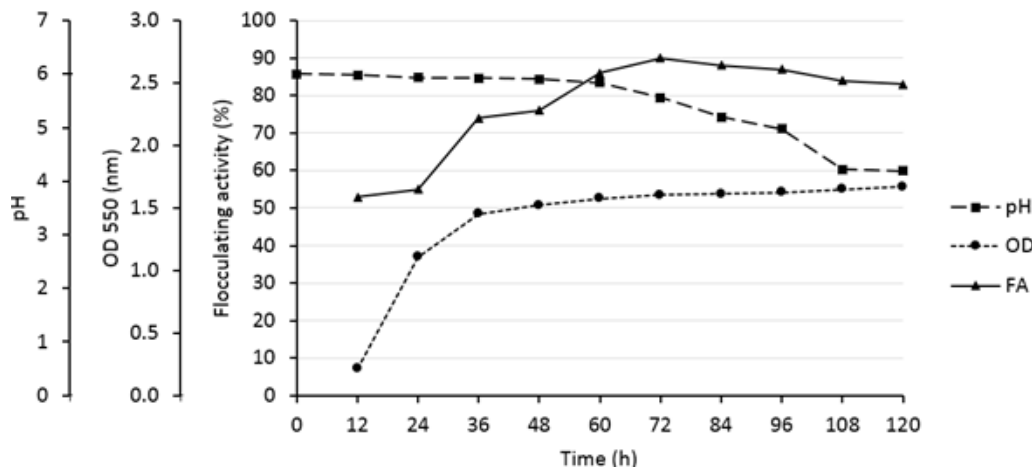


Figure 3. Effect of time on FA, initial pH and optimum density (OD).

relatively to the bacterial growth until 72 h of fermentation. The highest flocculating activity (90%) was obtained after 72 h. A slight decrease in flocculating activity was observed after 72 h. The initial pH of the medium dropped constantly from the initial pH of 6.0 to the final pH of 4.3.

Bioflocculant yield and solubility

The purified bioflocculant TPT⁻¹ of 3.0 g was obtained from 1 L of fermentation broth of consortium of *B. pumilus*

JX860616 and *A. faecalis* HCB2. The extracted and purified bioflocculant was insoluble in all solvents, with the exception of water as a solvent (Table 2).

Effect of bioflocculant dosage size on flocculating activity

Table 3 shows the results obtained during dosage size assay. The high flocculating activity of 84% was obtained at the low bioflocculant concentration of 0.2 mg/ml. Although 0.2 mg/ml is half of 0.8 mg/ml in concentration,

Table 2. Solubility assay of bioflocculant TPT⁻¹.

Solvent	Solubility
Water	+
Acetone	-
Methanol	-
Hexane	-
Benzene	-
Ethyl-acetate	-
Chloroform	-
Dichloromethane	-

Key: Soluble + and insoluble –

it gave significantly high flocculation activity (84%) and was thus the more preferred over 0.8 mg/ml that had 86% of flocculating activity.

Effect of metal cations on flocculating activity of the bioflocculant

Cations enhance flocculating rate by neutralizing and stabilizing the residual negative surface charge of the functional groups on the bioflocculant. The flocculating activity of bioflocculant was greatly stimulated by the addition of Li⁺, Mn²⁺ and Ba²⁺ at concentrations of about 1% (Table 1). These cations showed the flocculating activity above 80%. Ba²⁺ was the most preferred metal cation with the flocculating activity of 89%, while Fe³⁺ was the least cation with the flocculating activity of 56%.

Physicochemical composition of the bioflocculant TPT⁻¹

Figure 4 shows SEM surface images. TPT⁻¹ had a porous and crystal-like morphology (Figure 4a). Kaolin particles revealed a fine and smooth structure (Figure 4b) and the clump like structure was observed in flocculated Kaolin particles (Figure 4c).

Electrical charge of the bioflocculant

The surface charge of TPT⁻¹ was determined and the results were illustrated in Figure 5. The bioflocculant had a surface negative charge of -15.1 mV, as illustrated by the ZetaSizer Nano. The zeta potential of TPT⁻¹ revealed the bioflocculant as an anionic biomolecule.

Chemical composition of bioflocculant TPT⁻¹

TPT⁻¹ was Ninhydrin positive. The UV-vis spectrum of the

bioflocculant demonstrated a sharp absorption peak at 289 nm, which was a characteristic of proteins (Figure 6). Thus, the results suggested that the bioflocculant had protein content.

Chemical composition of bioflocculant TPT⁻¹

Table 4 shows the quantitative chemical composition of TPT⁻¹. The bioflocculant was predominantly composed of the total carbohydrates (83.1%) and trace protein content (9.7%).

Elemental and functional group analysis

Figure 7 illustrates the elemental analysis of the bioflocculant. The bioflocculant composed of; N (1.3), C (15.0), O (44.8), P (0.8), Ca (9.0), Cl (2.8), Mg (0.4), S (12.1), K (11.4) and Na (1.9), in mass proportion (% w/t).

IR spectrophotometry analysis

The functional groups of TPT⁻¹ were determined and the results shown in Figure 8. The IR spectrum revealed the presence of different functional groups of the bioflocculant. Hydroxyl (3313 cm⁻¹), carbonyl (1616 cm⁻¹) and amino groups (1080 cm⁻¹) were the main functional groups observed.

Thermal stability of bioflocculant TPT⁻¹

The effect of temperature on flocculating activity of bioflocculant TPT⁻¹ is shown in Figure 9. There was a slight, insignificant drop in flocculating activity with the increase in temperature. TPT⁻¹ also showed high flocculating activity of 86% even at 100°C after an hour of heat exposure.

Pyrolysis property of TPT⁻¹

Figure 10 shows the pyrolysis properties of bioflocculant TPT⁻¹. The first degradation was observed at 150°C. The two other degradation temperatures were observed at 270.03 and 402.58°C.

pH stability of bioflocculant TPT⁻¹

Figure 11 presented the effect of pH on flocculating activity of the bioflocculant. The flocculating activity of TPT⁻¹ was more than 80% in the acidic conditions (pH 3 to 6) and the maximum flocculating activity (89%) was at pH 3.0. pH 12 demonstrated the least flocculating activity of 58%.

Table 3. Effect of dosage size and cations on flocculating activity.

Dosage (mg/ml)	FA percentage (%) \pm SD	Cations	FA percentage (%) \pm SD
0.2	78.6 \pm 3.33 ^a	Na ⁺	59.8 \pm 0.96 ^{a,e}
0.4	84.1 \pm 1.96 ^a	K ⁺	68.5 \pm 3.39 ^{a,b}
0.6	82.9 \pm 3.08 ^a	Ca ²⁺	77.9 \pm 0.23 ^{b,c}
0.8	85.03 \pm 2.63 ^a	Mn ²⁺	82.5 \pm 2.93 ^{c,d}
1	84.3 \pm 1.60 ^a	Ba ²⁺	88.6 \pm 4.94 ^d
-	-	Fe ³⁺	56.8 \pm 6.44 ^e

Percentage flocculating activity with different letters (a, b, c, and d) are significantly ($p < 0.05$) different.

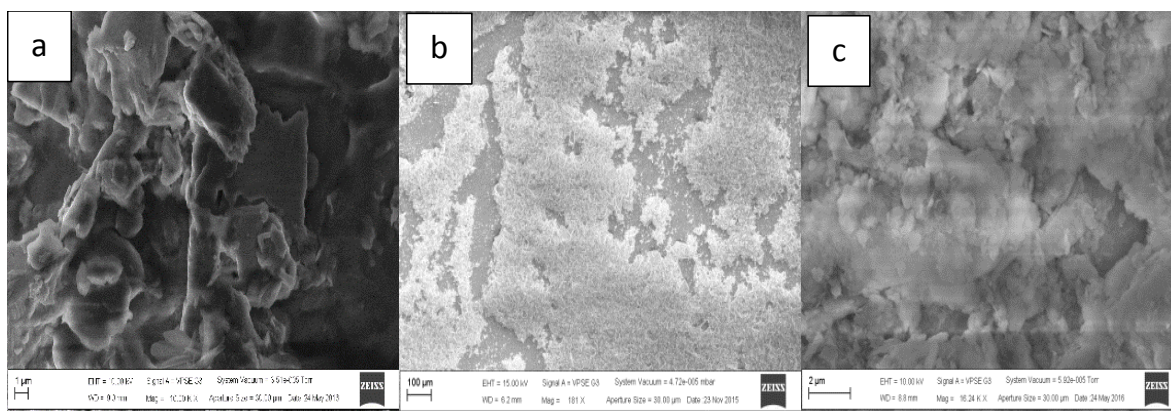


Figure 4. SEM surface images of flocculated Kaolin suspension; bioflocculant TPT⁻¹ (4a), Kaolin particles (4b) and flocculated Kaolin particles (4c).

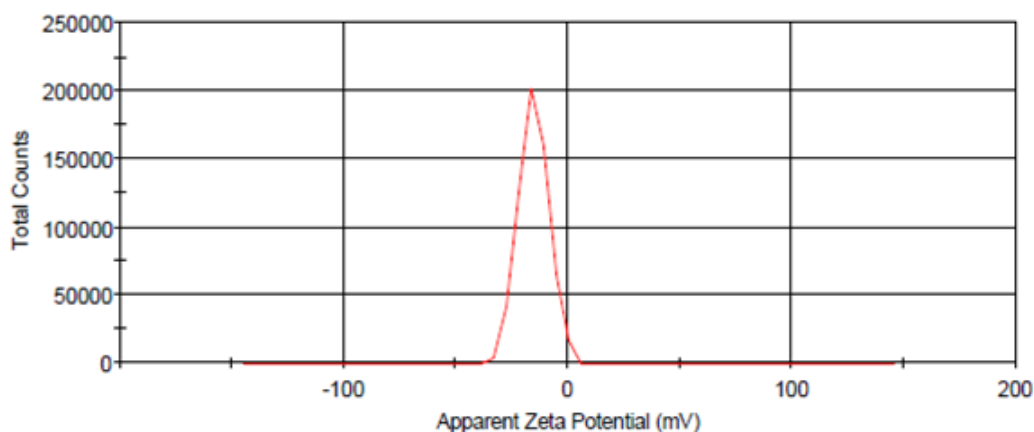


Figure 5. Zeta potential of the purified bioflocculant TPT⁻¹.

Salinity stability of bioflocculant TPT⁻¹

The effect of Na⁺ concentration on the flocculating activity of bioflocculant TPT⁻¹ is illustrated in Table 5. The flocculating activity of the bioflocculant decreased proportionally with the increase in Na⁺ concentration.

However, TPT⁻¹ maintained high flocculation activity (79.4%) even at the high salinity (35 g/L).

Proposed flocculation mechanism of TPT⁻¹

Table 6 illustrates the zeta potential values of the

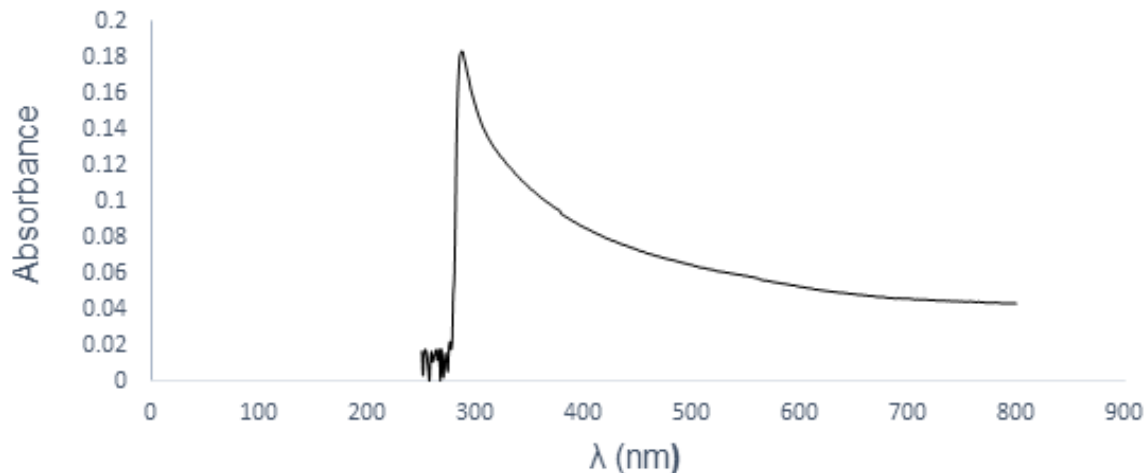


Figure 6. UV-vis spectrum of the purified TPT¹.

Table 4. Chemical components of the purified bioflocculant TPT¹.

Component	Concentration (% w/w)
Proteins	9.7
Carbohydrates	83

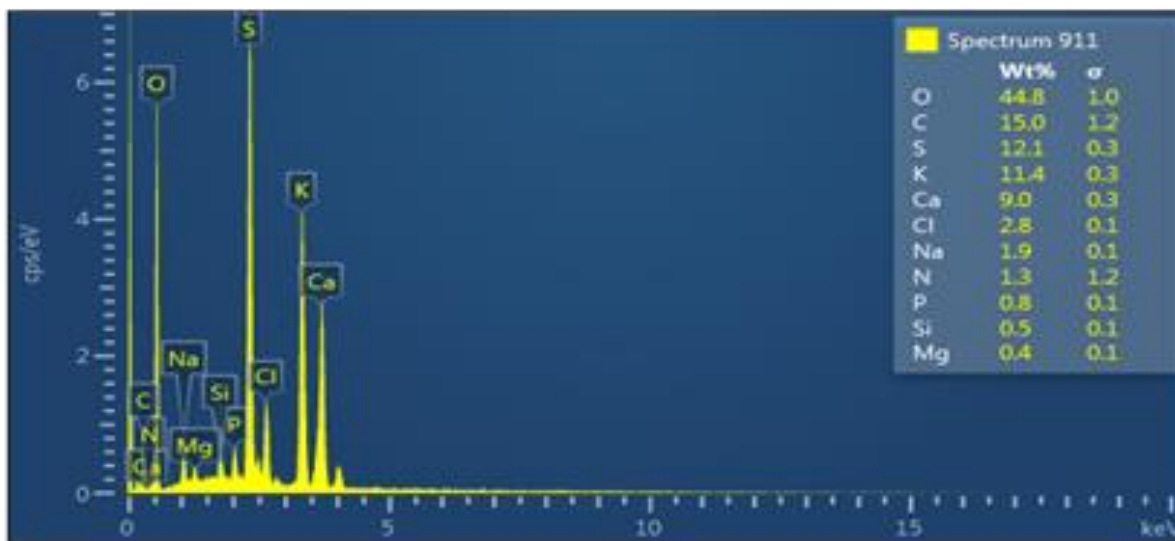


Figure 7. Elemental analysis of bioflocculant TPT.¹

bioflocculant, Kaolin suspension, Kaolin plus Ba²⁺ suspension and Kaolin suspension flocculated with Ba²⁺ and TPT¹. There was an increase in the zeta potential with the addition of Ba²⁺. Thus, it was suggested that Ba²⁺ mediated bridge flocculation mechanism between the bioflocculant and Kaolin particles in solution.

DISCUSSION

Biologically, synthesised active bioflocculants are produced in extremely low quantities naturally. This is due to the fact that they are none essential for microbial survival or are present in sufficient amounts to satisfy

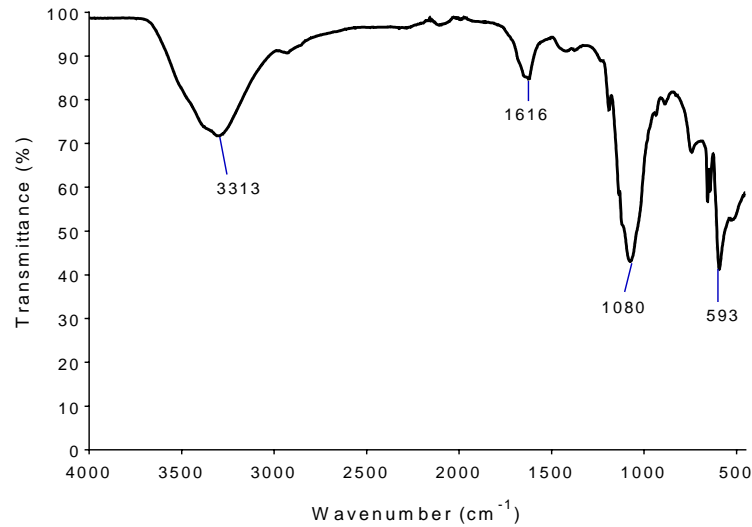


Figure 8. IR spectrophotometric analysis of the bioflocculant TPT.¹

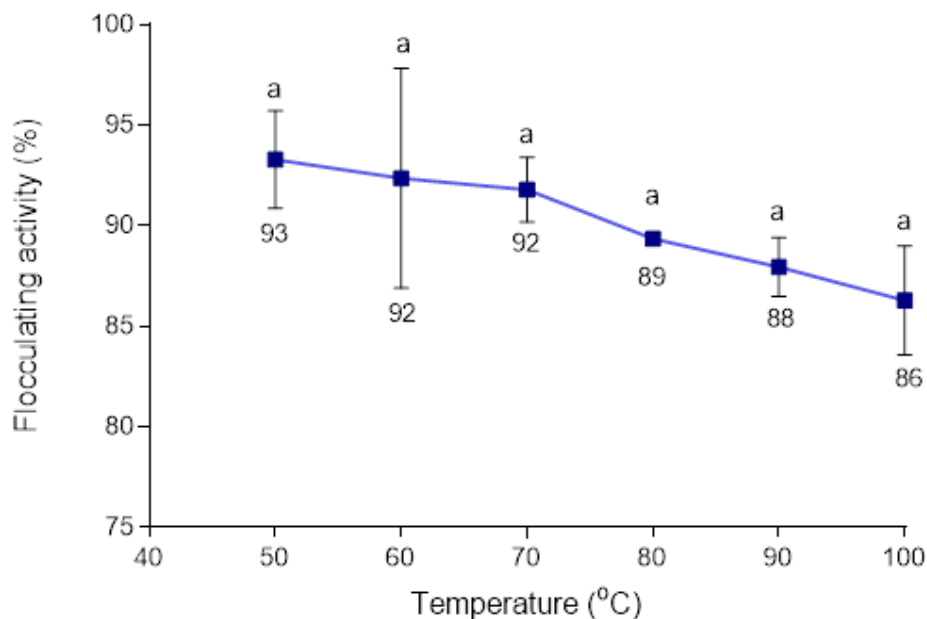


Figure 9. Effect of temperature on flocculating activity of bioflocculant TPT.¹

their primary needs. Low productivity often translates economical unfriendliness especially at industrial level (Barredo, 2005). Thus, to increase bioflocculant production and improve flocculating activity, optimization of cultivation conditions are done.

Naturally, microorganisms coexist in ecological niches and have relationships that have all positive effect on biological adequacy of all interacting species. Microbial species interact with one another and with physico-chemical surroundings in diverse ways, forming convoluted relationships which include symbiosis and

synergism (Manahan, 2005). Thus, it have been reported that mixed microbial strains in consortia produce bioflocculants that have better flocculating activity and higher yields than pure strains (Zhu et al., 2004).

The growth kinetics of cultured bacteria and the production of bioflocculant synthesis greatly depended on inoculum size. Small inoculum sizes lengthen the stagnant phase during bioflocculant synthesis while large inoculum sizes cause a niche overlap and thus tend to hinder bioflocculant production (Aljuboori et al., 2014). The optimum inoculum size was 2% (1 ml (v/v)) and gave

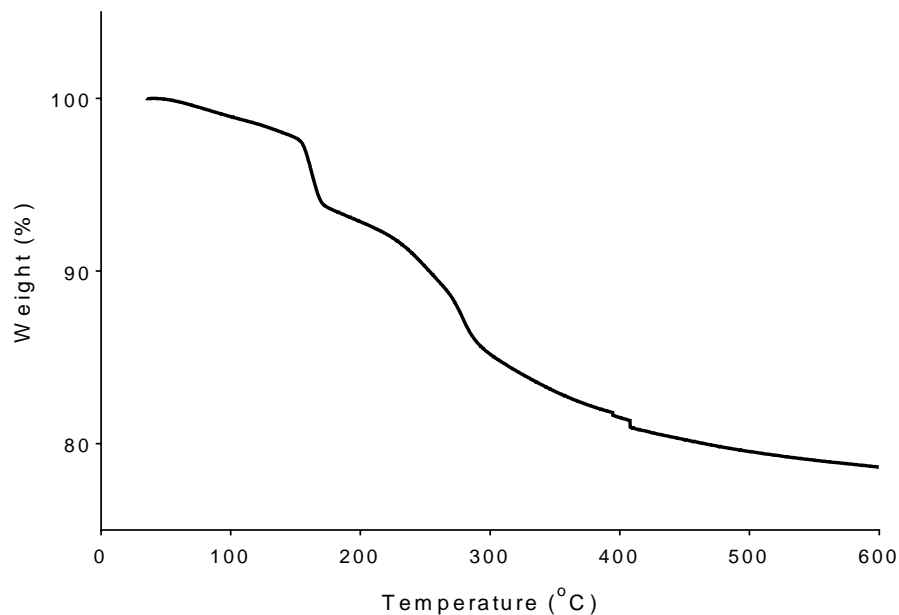


Figure 10. Thermo-gravimetric analysis of the bioflocculant TPT.⁻¹

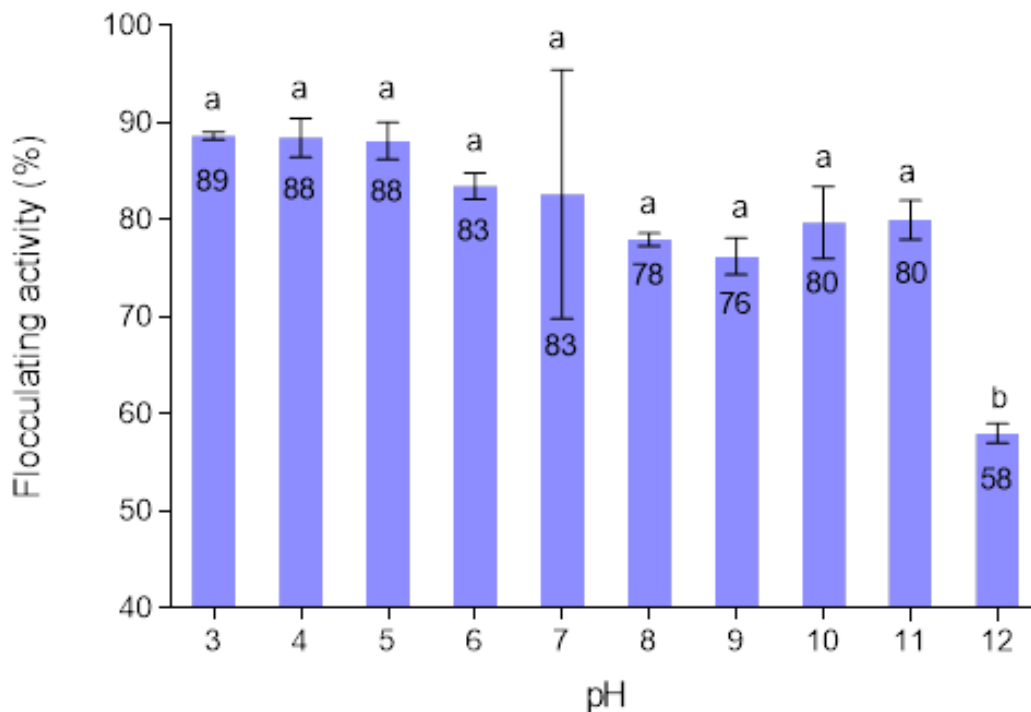


Figure 11. Effect of pH on flocculating activity of TPT.⁻¹

the highest flocculating activity (93%) (Table1). The desirable inoculum sizes range from 1 to 5% (v/v) (Okoh et al., 2012), and 2% inoculum size was within the preferred range.

Bacterial strains acquire their energy for metabolic

processes and reproduction by mediating chemical reactions. The bulk of net energy-yielding or energy consuming metabolic reactions in microorganisms involves change in the oxidation state of carbon or nitrogen elements in substrates (Mahan, 2005). Table 1

Table 5. Effect on salinity on flocculating activity of a purified bioflocculant.

NaCl (g/L)	FA percentage (%) \pm SD
5	94.7 \pm 2.23 ^a
10	93.5 \pm 2.15 ^{a,b}
15	92.2 \pm 1.87 ^{a,b}
20	92.1 \pm 1.04 ^{a,b}
25	91.2 \pm 1.60 ^{a,b}
30	88.9 \pm 1.66 ^b
35	79.4 \pm 1.59 ^c

Percentage flocculating activity with different letters (a, b, c, and d) are significantly ($p < 0.05$) different.

Table 6. Zeta potential.

Sample	Zeta potential (mV)
Bioflocculant TPT ¹	-15.1
Kaolin particles	-9.24
Kaolin particles with Ba ²⁺	-6.96
Kaolin particles flocculated with Ba ²⁺ and TPT ¹	-9.08

showed the effects of various carbon and nitrogen sources on bioflocculant production. Glucose was the most preferred carbon source, yielding flocculation activity of 80.5 \pm 9.07%.

Okoh et al, (2012), found identical observations whereby glucose enhanced production of bioflocculant by mixed culture of *Streptomyces* sp. Gansen and *Cellulomonas* sp. Okoh. Yeast extract revealed to be the most preferred nitrogen source, resulting in flocculating activity of 97.4 \pm 0.57%. Cations enhance flocculation by accelerating bridge formation between colloids and bioflocculants (Lu et al., 2005). Cations increase the initial adsorption of bioflocculants onto colloids by decreasing the negative charges on functional groups of bioflocculants and colloids. Table 1 showed that all divalent cations used, significantly improved flocculation giving the flocculation activities above 80%. Ba²⁺ was the most profound cation with the highest flocculation activity of 96.2 \pm 1.25%.

The cultivating temperature of microorganisms has direct relationship with their metabolism. Maximum enzymatic reactions for bioflocculant production are obtained at the optimum temperature. A lower culture temperature may cause bacteria to hibernate and delay activation of bioflocculant production while the high temperature can denature enzymes and thus limit bioflocculant production. Bacteria in consortium had the maximum flocculating activity (96%) at 30°C (Figure 1). The results resembled those by Zhang et al. (2007), whereby strain BAFRT4, HXCS2, HXTD2, CYGS1 and CYGS4 in consortia had the highest flocculating activity

at 30°C.

The initial pH of the culture medium affects bioflocculant production. Initial pH determines the electric charge of the microbial cells and the oxidation-reduction potential, which in turn can affect biochemical reactions and adsorption of nutrients. Summarily, initial pH affects the biosynthesis of bioflocculants and bacterial growth (Zhang et al., 2007). The initial pH of 6 was the optimum pH for the fermentation medium, yielding 97% of the flocculating activity (Figure 2). *B. pumilus* JX860616 and *A. faecalis* HCB2B might have had symbiosis or synergism relationship, which enabled them to survive and produce bioflocculant within a wide range of pH (Manahan, 2005). The findings were similar to those reported by Zhang et al. (2007), whereby the initial pH of 6 was the best pH for bioflocculant production by strain BAFRT4, HXCS2, HXTD2, CYGS1 and CYGS4 in consortia.

Figure 3 shows that the flocculating activity increased in parallel with cell growth for the first 72h. This suggested that the bioflocculant accretion in the medium during exponential growth phase was produced by biosynthesis and not cell autolysis (Okoh et al., 2012). The flocculating activity decreased slowly after 72h, and it was assumed to be due to cell autolysis and partial decrease in enzymatic reactions. Okoh et al. (2013) also found that the flocculant production by consortium of *Methylobacterium* sp. and *Actinobacterium* sp. Mayor was at its peak after 72 h.

Moreover, the initial pH of the medium dropped constantly from the initial pH of 6.0 after 12h to the final

pH of 4.3 (Figure 3). The decrease in the initial pH value might be due to the acidic components of the produced bioflocculant (Gomaa, 2012).

Three grams of the purified bioflocculant TPT⁻¹ was obtained from 1 L of fermentation broth of consortium of *B. pumilus* JX860616 and *A. faecalis* HCB2. Zhang et al. (2007) reported the highest bioflocculant production (15 g/L) ever, from multiple-microorganisms consortium in a litter. The obtained yield was five times lower in quantity. Nevertheless, it was higher than the yields mostly produced by single bacterial isolates (Lin and Harrichud, 2011).

Solubility is as the result of solvation of polar and charged groups on the surface of bioflocculant molecule (Walker and Wilson, 2005). TPT⁻¹ was soluble in water but insoluble in all other solvents used (Table 2). The presence of hydroxyl groups might have built up strong forces of attraction between bioflocculant TPT⁻¹ make-ups and resulted in relatively rigid crystalline solids – whereby hydrogen bonding could occur. Since these strong forces were not dissociable by organic solvents, the bioflocculant TPT⁻¹ was insoluble in all organic solvents (Patil et al., 2011). The solubility of TPT⁻¹ in aqueous solution was due to the principle that “like dissolve like” (Boyd, 2015). Thus, polar and charged functional groups of TPT⁻¹ were solvated by aqueous molecules. The hydroxyl groups of the bioflocculant have the probability of forming hydrogen bonds with water, thus making TPT⁻¹ soluble and hydrophilic (Patil et al., 2011).

Low dosage or over-dosage of bioflocculant often turns to lower flocculation activity (Cosa et al., 2013). Table 3 shows that the highest flocculating activity (85.03 ± 2.63%) was at the high concentration of 0.8 mg/ml. However, there was no significant difference between 0.2 mg/ml and 0.8 mg/l in flocculating activity. Thus, 0.2 mg/ml have the most preferred dosage size with the flocculating activity of 78.6 ± 3.33%. At a very low concentration of 0.2 mg/ml, the bridging flocculation mechanism of the bioflocculant was effectively enhanced as most Kaolin particles in solution which have enough bioflocculant molecules to bind to. The low concentration implied that the bioflocculant TPT⁻¹ is potentially economical (Okaiyeto et al., 2016). Lu et al. (2005) reported that cations have significant effect on flocculation. Cations are used as coagulant aids in achieving high flocculation activities by neutralizing the negatively charged functional groups of bioflocculants and suspended particles, thereby increasing the adsorption of bioflocculants to the suspended particles (He et al., 2010). The stimulating effect of cations in flocculation is dependent on the concentration of the cations as well as the valence ions (Nwodo et al., 2016). The obtained results affirmed the hypothesis (Table 3). All the divalent cations used (Ca²⁺, Mn²⁺ and Ba²⁺) significantly enhanced flocculation activity, with Ba²⁺ being the best cation with the flocculation activity of 88.6±4.94%. The results are in agreement with those of Okoh and Ugbenyen. (2014),

whereby the flocculating activity of the bioflocculant from the consortium of *Cobetia* sp. and *Bacillus* sp. was significantly stimulated by all divalent cations used (Mg²⁺, Mn²⁺ and Ca²⁺) but reduced slightly by monovalent cations (K⁺ and Li⁺). The chemical functionalities and structures of bioflocculants affect the flocculating efficiency and flocculating mechanisms (Badireddy et al., 2010; Li-Fan and Cheng, 2010). The SEM images in Figure 4, revealed bioflocculant TPT⁻¹ to be crystal-like in structure (Figure 4a).

The configuration of TPT⁻¹ might be accountable for its flocculation activity. The Kaolin clay particles (Figure 4b) appeared to be fine and evenly scattered and the floc seemed to be clustered together (Figure 4c). The observations were in consistent with the findings of Zhang et al. (2007), whereby the images of the flocculated Kaolin particles appeared aggregated. TPT⁻¹ was Ninhydrin positive, implying that amino acids were one of the components of the bioflocculant (Gomaa, 2012). The zeta potential spectrum showed bioflocculant TPT⁻¹ to be negatively charged (-15.1 mV) (Figure 5). This implied that the nature of bioflocculant TPT⁻¹ is anionic. The net negative charge of the bioflocculant TPT⁻¹ was concluded to be from the carbohydrates and proteins contents, which normally have the negatively charged functional groups (carboxyl groups) (Liu et al., 2015). Proteins usually show absorption peaks in the range of 275 to 280 nm and between 265 to 295 nm, the absorbance often originate from exposure of the tryptophan residue (Lucas et al., 2006).

The UV-vis spectrum in Figure 6, displayed a clear absorption peak at 289 nm, which have distinctive characteristic of protein. There was no characteristic absorption peak observed at 260 nm, implying that bioflocculant TPT⁻¹ had no nucleic acid composition. The total carbohydrates concentration of 83.1% w/w of the TPT⁻¹ was much higher than the total protein concentration (9.7% w/w) (Table 4). The results confirmed the hypothesis that the predominant components of bioflocculants are carbohydrates (More et al., 2014). Thus, it was assumed that carbohydrates were most active components in flocculation process. The elemental state of biomolecules, often consist of carbon, hydrogen, oxygen, nitrogen, sulphur, phosphorus, iron, sodium, calcium and magnesium (Singh and Kapoor, 2010). Figure 7 shows the results obtained from quantitative analysis of different elements in TPT⁻¹. The presence of carbon, oxygen and nitrogen elements further confirmed TPT⁻¹ as a glycoprotein bioflocculant (Devi et al., 2015). The non-sugar components were little, although they may be responsible for flexibility and stability of TPT⁻¹ (Cosa et al., 2013).

The functional moieties of bioflocculant TPT⁻¹ revealed by IR spectrum in Figure 8, showed the presence of strong broad O-H stretch (3313 cm⁻¹), which indicated the presence of alcohol (Cosa et al., 2013). The carbonyl stretching absorption was observed between 1616 cm⁻¹

and the C-N stretch (1080 cm^{-1}), representing aliphatic amine. There was also a strong absorption peak at 593 cm^{-1} , representing presence of halo compound. The revealed functional groups were the major adsorptive forces of TPT⁻¹ and were perceived to have been involved in flocculating activity of the bioflocculant (Wang et al., 2011). The results also suggest that the functional groups were the binding sites for Ba²⁺, permitting an enhanced flocculation process (Zheng et al., 2008).

Bioflocculant TPT⁻¹ was heat stable and retained high flocculating activity of 86% at 100°C (Figure 9). The thermal stability of TPT⁻¹ was consistent with the fact that bioflocculants rich in polysaccharides are more thermally stable than those that are mainly proteinous in nature, which are thermally labile (Zaki et al., 2013). The carbonyl and hydroxyl groups of TPT⁻¹, as shown by IR spectrum (Figure 8), might have permitted the formation of hydrogen bonds which might be responsible for thermal stability of TPT⁻¹ (Okoh and Ugbenyen, 2014). Figure 10 illustrated the pyrolysis property of bioflocculant TPT⁻¹.

The first degradation temperature (*T_d*) was observed at 150°C. The higher the carboxyl content the greater the affinity of the bioflocculant to interact with water molecules. The observed initial weight loss between 35 and 150°C was due to loss of moisture content as a result of the levels of carboxyl groups present in bioflocculant TPT⁻¹. The decrease in weight after 150°C was credited to the first degradation of the bioflocculant TPT⁻¹ and the onset decomposition further occurred at 270.03 and 402.58°C, which led to the drastic weight loss. However, the results suggested that bioflocculant TPT⁻¹ is thermostable.

The change in pH may affect the charge status of the functional groups of bioflocculants and the stability of colloidal materials and formation of bigger flocs, consequently affecting the flocculating activity (Zhang and Lin, 1999). TPT⁻¹ was found to be effective at a wide range of pH (pH 3 to 9), with the highest flocculation activity (89%) at pH 3 (Figure 11). However, the decrease in flocculating activity at pH 12, suggested the alkaline degradation of the bioflocculant TPT⁻¹, which could have resulted from changes that include molecular rearrangements of the polysaccharide chain fragmentation of the bioflocculant (Patil et al., 2011). It was, thus assumed that the slight decrease in flocculation activity was due to OH⁻ ions which might have inhibited the formation of flocs of TPT⁻¹ and Kaolin particles in solution. At acidic pH, TPT⁻¹ and the Kaolin particles were able to bind together, forming settleable flocs. The pH stability of the bioflocculant TPT⁻¹ at low pH might be due to its chemical composition that is mainly polysaccharides (Li et al., 2008).

The flocculating activity of TPT⁻¹ decreased slightly in proportion to the increase in NaCl concentration (Figure 12). This maybe due to the fact that high concentrations of Na⁺ did interfere with the establishment of the flocs of bioflocculant TPT⁻¹ and Kaolin particles. Moreover, high

concentrations of Na⁺ may also have denatured some functional moieties of TPT⁻¹ that might be responsible for flocculation process (Aljuboori et al., 2015). However, TPT⁻¹ maintained high flocculation activity (79.4%), even at high Na⁺ concentration (35 g/L). The saline stability of TPT⁻¹ maybe due to the fact that the bioflocculant was produced by marine bacterial isolates, which survive in marine environment and is characterised by high NaCl concentrations (35 g/L), of which 61.2% is the salt and sodium chloride. Thus, TPT⁻¹ has potential effectiveness in high salinity water bodies. The results were similar to the findings by Li et al. (2008), whereby EPS SM9913 showed high flocculating performance at high salinity of 5 to 100% (Li et al., 2008).

Zeta potential shows the strength of the repulsion force between colloidal particles and the distance which must be overlapped to allow particles to agglomerate together and is measured in millivolts (mV) (Hadgson et al., 2004). The zeta potential values of TPT⁻¹, Kaolin suspension, Kaolin particles plus BaCl₂ and flocculated Kaolin particles with BaCl₂ and TPT⁻¹ were all negative (Table 6). In Kaolin suspension, the net zeta potential significantly increased with an addition of BaCl₂ and bioflocculant. The flocculation process of the negatively charged Kaolin particles by the anionic TPT⁻¹ might also be activated by cationic bridge formation between Kaolin particles and the functional groups of the bioflocculant molecule. Based on these results, it was concluded that Ba²⁺ stimulated flocculation process by neutralization and stabilization of residual functional groups of TPT⁻¹, forming bridges that aggregated Kaolin particles together. Ba²⁺ compacted the double layer of Kaolin particles, deteriorated the static repulsive force between Kaolin particles and reduced the distance between Kaolin particles and the functional groups of TPT⁻¹ (He et al., 2010). The observations were similar to those of Song et al. (2014), whereby the bridging mechanism was concluded as the main flocculation mechanism of bioflocculant ETH-2.

Conclusion

Bioflocculant TPT⁻¹ is an anionic, heat stable glycoprotein molecule which is best produced when glucose and yeast extract are used as energy sources in optimum fermentation conditions (30°C, 165 rpm, initial pH 6 and 72 h). The produced bioflocculant TPT⁻¹ (3.0 g/L) had good flocculating activity at low concentration (0.2 mg/ml) and performed effectively in a wide range of pH and salinity. The bridging mechanism was involved during flocculation of Kaolin particles. Ba²⁺ effectively mediated flocculation by neutralizing and stabilizing the TPT⁻¹ residual functional groups. The revealed properties of TPT⁻¹ suggested its potential in industrial applicability. For further studies, the bioflocculant TPT⁻¹ will be applied on wastewater treatment and the molecular methods will also be done so to increase the bioflocculant yield.

Conflicts of interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Occurrence and susceptibility patterns of *Campylobacter* isolated from environmental water sources

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Environmental waters are established sources of *Campylobacter* infections in humans. The aim of this study was to assess the distribution and susceptibility profiles of *Campylobacter* species isolated from irrigation and domestic water sources in Ghana. Samples were pre-enriched with CCDA broth and isolated on mCCDA agar. Isolates were confirmed on API CAMPY kit and with the Kirby-Bauer disk diffusion method to determine the susceptibility patterns of species. Of the 188 water samples analyzed, 42 isolates were confirmed to be contaminated with *Campylobacter* species, giving a prevalence rate of 22.3%. Prevalence of *Campylobacter* in the various water sources were 35.7% in rivers, 26.2% in streams, 21.4% in wells, 9.5% in ponds and 7.1% in boreholes. Sixty four percent (64%) of *Campylobacter* species were *Campylobacter jejuni*, followed by *Campylobacter coli* (19%) and *Campylobacter lari* (14%). Resistance was 100% to the β -lactams, 98% to erythromycin, 48-69% to the quinolones, 45-55% to the aminoglycosides, 71% to trimethoprim sulphamethoxazole, 76% to tetracycline and 90% to chloramphenicol. All the isolates (100%) were multidrug resistant. The presence of multidrug resistant *Campylobacter* species in the different water sources sampled in this study may indicate a significant health risk to humans and animals.

Key words: *Campylobacter*, antibiotic resistance, water sources, Ghana.

INTRODUCTION

Worldwide, *Campylobacter* species are known to be common agents of enteritis in humans, generally regarded as foodborne pathogens. They are frequent residents of the intestinal tract of all food-producing animals and

humans (Duncan et al., 2013). *Campylobacter* are also widespread in the environment, for instance in drinking water, effluents, livestock farms, rivers, streams and wild birds (Jones, 2001; Abulreesh et al., 2005). Water

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sources were previously not recognized to be a major transmission route for campylobacteriosis due to the dormant state of species in water, commonly referred to as viable and non-culturable (VBNC) (Murphy et al., 2006). Nevertheless, *Campylobacter* spp. have been isolated from different sources of environmental water, which include rivers (Ugboma et al., 2012), streams (Carter et al., 1987), ponds (Abulreesh et al., 2005), ground water (Ugboma et al., 2013) and coastal waters (Obiri-Danso and Jones, 1999a). The main source of large *Campylobacter*-associated outbreaks around the world has been linked to untreated or contaminated drinking water (Leclerc et al., 2004). In many tropical developing countries, waterborne associated campylobacteriosis are underreported due to the occasional nature as well as challenges in source attribution of *Campylobacter* infections (Miller and Mandrell, 2005).

In Ghana, a significant urban poor population depends on wells, rivers, ponds and streams as domestic water sources due to inadequate supply of potable water. These water sources may also function as drinking water sources for both domestic and farm animals. Contamination of water with enteric pathogens such as *Campylobacter* poses direct threat to public health as increasing resistance has been reported globally in this organism from different sources of infection. The purpose of this study was therefore to establish whether *Campylobacter* species were present in the various water sources of Ghana, and to evaluate the resistance levels of species to clinically relevant antibiotics.

MATERIALS AND METHODS

The study was carried out in Kumasi, Capital of the Ashanti Region and the second largest city in Ghana. The city is traversed by major rivers and streams which include the Subin, Wiwi, Sisai, Owabi, Aboabo and Nsuben among others. One hundred and eighty eight (188) water samples were obtained during early mornings from major rivers, ponds, wells, streams and boreholes beginning from May 2013 to May 2014. The sampling sites were selected based on the extent of use of the water for domestic and recreational activities. Approximately, 500 mL of surface water was collected into sterile bottles and transported on ice packs to the laboratory in an insulated opaque box.

Sample processing, isolation and identification

About 500 ml of water was filtered through Durapore 0.45 µm filters, diameter 47 mm, with the aid of a suction pump (Edwards RV3). Filters were aseptically transferred using sterile forceps into 30 ml universal bottles enriched with 30 ml of blood-free *Campylobacter* broth (Oxoid CM0963) supplemented with CCDA supplement (Oxoid, SRO 155E) and incubated overnight at 37°C. Enrichment broth was gently shaken and cultured on mCCDA agar (Oxoid CM0689) using a sterile swab stick and incubated in a microaerophilic atmosphere at 42°C for 48 h using Campy Gen (Oxoid CN0025A). Biochemical tests including Gram stain, oxidase and catalase were performed on colonies showing typical morphology of *Campylobacter* spp. Isolates which were small,

curved Gram negative bacilli, catalase and oxidase positive, were further subjected to standard phenotypic tests using API CAMPY (bioMerieux, France) to confirm the species.

Antibiotic susceptibility testing

The Kirby-Bauer disk diffusion method was done using Mueller-Hinton agar (Liofilchem-Italy) supplemented with 5% sheep blood; inoculated with 0.5 McFarland suspension and incubated microaerophilically at 48°C for 24 h. Assayed antibiotics obtained from ROSCO (Denmark) included; Ampicillin (10 µg/disc), chloramphenicol (30 µg/disc), ciprofloxacin (5 µg/disc), kanamycin (30 µg/disc), erythromycin (15 µg/disc), gentamicin (10 µg/disc), nalidixic acid (30 µg/disc), tetracycline (30 µg/disc), cephalexin (30 µg/disc), trimethoprim sulfamethoxazole (25 µg/disc), norfloxacin (10 µg/disc), cefotaxime (30 µg/disc) and imipenem (10 µg/disc). The inhibition zones were measured and interpreted according to EUCAST- CLSI 2013 breakpoints for *Campylobacter*. Breakpoints established by EUCAST and CLSI 2013, for enterobacteriaceae were used to interpret the results of norfloxacin, trimethoprim sulfamethoxazole, cefotaxime and kanamycin as CLSI breakpoints for these antibiotics has not yet been established for *Campylobacter*. Quality control was achieved using *Escherichia coli* (ATCC 25922) and *Staphylococcus aureus* (ATCC 25923) strains.

Statistical analysis

Percentages were used for the descriptive analysis. Associations were determined using the Chi-square test at a significance level of < 0.05 Stata 14.0 software was used for statistical analysis.

RESULTS AND DISCUSSION

This study recorded an overall *Campylobacter* prevalence of 22.3% from different water sources, with the highest, 35.7% in rivers, 26.2% in streams, 21.4% in wells, 9.5% in ponds and 7.1% in boreholes (Table 1). These levels were lower as compared to the 40.4 and 40.5% reported in environmental water samples from Denmark and England, respectively (Kemp et al., 2005; Ramonaite et al., 2014). Groundwater is however perceived to be free of microorganisms hence the improved quality of water from boreholes, wells and springs (Stanley et al., 1998a). The recorded levels in this study were low as compared to the 52.7% in Nigeria (Ugboma et al., 2013).

The presence of *Campylobacter* in surface waters often relates to recent fecal contamination by avian birds, livestock, farm animal manure runoffs and non-disinfected sewage effluent or septic leakage (Jones, 2002; Abulreesh et al., 2006). Additionally, the absence of piped sewerage systems in most developing countries has resulted in large volumes of untreated or partially treated wastewater ending up in nearby water sources (Danquah, 2010). Higher *Campylobacter* contamination of surface waters have been reported in India (41.5%), in Norway (53.3%), in Nigeria (68.7%) and in Poland (70%) (Popowski et al., 1997; Rosef et al., 2001; Baserisalehi et al., 2005; Ugboma et al., 2012). The observed differences in the contamination levels from the different studies

Table 1. Prevalence of *Campylobacter* species isolated from water sources in Kumasi.

Water source	No. of samples	No. of <i>Campylobacter</i> spp. identified (%)	p-Value
Streams	25	11(26.2)	(Chi-square, df)
Wells	39	9 (21.4)	< 0.0001
Boreholes	17	3 (7.1)	(54.74, 4)
Ponds	87	4 (9.5)	
Rivers	20	15(35.7)	
Total	188	42(22.3)	

Table 2. Distribution of *Campylobacter* species from various water sources in Kumasi.

Water source	No. of isolates	<i>C. jejuni</i>	<i>C. doylei</i>	<i>C. coli</i>	<i>C. lari</i>
Streams	11	7	0	1	3
Wells	9	3	0	6	0
Boreholes	3	2	0	1	0
Ponds	4	4	0	0	0
Rivers	15	11	1	0	3
Total	42	27(64)	1(3)	8(19)	6(14)

C. doylei= *C. jejuni* sub sp. *Doylei*.

could be attributed to isolation by culture methods (Percival et al., 2004) or the use of PCR techniques (Van-Dyke et al., 2010).

The type of *Campylobacter* species found in environmental water sources often relates to the source(s) of contamination (Abulreesh et al., 2006). *Campylobacter jejuni* is the most described from surface waters although *C. coli* and *C. lari* have also been isolated from these sources (Hokajärvi et al., 2013), and its presence is associated with sewage discharges (Obiri-Danso and Jones, 1999a, Jones, 2001).

In this study, over 64% of the isolates from the water sources were *C. jejuni* (Table 2); thus, affirming reports that *C. jejuni* is the most dominant species in surface waters followed by *C. coli* (19%) (Popowski et al., 1997; Rosef et al., 2001; Kemp et al., 2005; Ugboma et al., 2013).

Sources of *Campylobacter* in streams relates to input sources: streams running through pasture lands contain mainly *C. jejuni* with some *C. coli* shed by grazing cattle; agricultural run-offs and effluents from water treatment plants (Popowski et al., 1997; Obiri-Danso and Jones 1999a); or from wild birds, (Jones, 2001). In this study, most of the river and stream water samples were from sources influenced by open defecation (human faeces), untreated sewage, agricultural run-offs; and wild birds (Obiri-Danso and Jones, 1999a). *Campylobacter jejuni* sub. sp. *doylei* has no animal host and it is only humans that serve as reservoirs of this sub species (On, 2005), giving an indication that the *C. jejuni* species and sub species (*doylei*) isolated from the studied rivers may have

come from human origin. Moreover, the 0% recovery of *C. coli* from the sampled ponds and rivers could mean that *C. jejuni* has the ability to thrive in the environment regardless of their fastidious nature, since they have demonstrated aerotolerance, starvation and longer survival in culturable forms than *C. coli* in water (Bronowski et al., 2014). This assertion of *C. coli* susceptibility to environmental stresses was supported by findings where 75% of the *C. coli* were recovered from wells while 12.5% each were obtained from boreholes and streams.

Campylobacter species from the various water sources exhibited high levels of resistance to almost all the antibiotics except imipenem which proved to be effective, 0% resistance (Table 4). Again, 100% resistance was recorded against cephalixin, cefotaxime and Ampicillin which agrees with the work of Baserisalehi et al. (2005) who documented 100% resistance in environmental samples. Resistance against trimethoprim sulfa-methoxazole and tetracycline was 71 and 76%, respectively; however, a higher resistance rate of 96% each has been reported in Malaysia (Adzitey et al., 2012). The 98% erythromycin resistance recorded in this study was far higher than the 1 and 40.8% reported in Malaysia and India. Similarly, the 69% resistance also recorded in this study against ciprofloxacin was higher as compared to the 0% resistance documented by Baserisalehi et al. (2005) and Adzitey et al. (2012). Ciprofloxacin resistance in some European countries especially in the Scandinavian countries was previously reported to be low (0%) or less than 10%; however, rates

Table 3. Multidrug resistance exhibited by *Campylobacter* species from the various water sources.

Water isolates	Total	Multi-drug resistance				
		Stream	Well	Borehole	Pond	River
<i>C. coli</i>	8	1(9.0)	6(66.7)	1(33.3)	-	-
<i>C. lari</i>	6	3(27.3)	-	-	-	3(20.0)
<i>C. jejuni</i>	28	7(63.6)	3(33.3)	2(66.7)	4(100.0)	12(80.0)
Total	42	11	9	3	4	15

Table 4. Resistance and susceptibility profile of *Campylobacter* obtained from water bodies in Kumasi.

Antibiotic	Sensitive (%)	Intermediate (%)	Resistant (%)
	N=42		
Nalidixic acid	40	NA	60
Tetracycline	19	5	76
Erythromycin	2	NA	98
Ciprofloxacin	14	17	69
Chloramphenicol	10	0	90
Ampicillin	0	0	100
Cefotaxime	0	0	100
Kanamycin	36	19	45
Gentamicin	40	5	55
Norfloxacin	28	24	48
Trimethoprim sulfamethoxazole	29	0	71
Cephalexin	0	NA	100
Imipenem	64	36	0

are presently increasing between 50 and 100% in some European member countries (Mackiw et al., 2012).

Fluoroquinolones and macrolides are recognized drugs of choice for *Campylobacter* infections (Zhao et al., 2010) but this recommendation cannot be admitted in sub-Saharan countries where resistance against these drugs is high.

The high MDR (100%) as shown in Table 3 is a reflection of the poor management of antibiotics in agriculture, human and veterinary medicine which has a rippling effect on environmental water sources. Most hospitals and pharmaceutical industries dispose their improperly treated effluents and refuse into the environment which ultimately ends up in water bodies. Secondly, open defecation, poor sewage systems and the use of some water sources as sewer lines are significant contributors to the high levels of resistance in the environment.

Thirdly, poultry and livestock farmers and crop farmers dispose their expended drug containers and wastes primarily by dropping them into drains and other environmental water sources (Sekyere, 2014).

Conclusion

The widespread distribution of multidrug resistant *Campylobacter* species in the different water sources sampled in Ghana suggest these sources are an important reservoir of antibiotic resistance which has implications in the health of people living in the rural and peri-urban areas of the study region where rivers and streams are their main sources of water supply, including domestic and farm animals exposed to them.

Conflict of interest

The authors declare that there is no conflict of interest.

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Full Length Research Paper

Fungi toxic efficiency of some plant volatile essential oils against plant pathogenic fungi

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Plant essential oils have the potential to replace the synthetic fungicides in the management of different fungal diseases. Four different essential oils of eucalyptus (*Eucalyptus globulus*), citronella (*Cymbopogon citrate*), karanj (*Pongamia pinnata*) and neem (*Azadirachta indica*) were selected because of their high inhibitory activities against ten phytopathogenic fungi. The antifungal screening for all the four Eos clearly indicates the effective lowest concentration to control the fungal growth. The more lower the MIC value, the better antifungal potency of the relative plant volatile *E. globulus*. Among the plant volatile essential oils, eucalyptus oil showed the lowest minimum inhibitory concentration (MIC), i.e. 0.5 mg/disc. Higher MIC values were registered, in the order, for citronella, karanj and neem oils, singly used to control all the ten fungal pathogens. Highest zone of inhibition (ZI) values followed the same pattern. These results indicated that plant volatile essential oils after suitable formulation could be used to control of different fungal pathogens. This may encourage the farmers to produce organic commodities to generate more revenue.

Key words Essential oils, minimum inhibitory concentration (MIC), zone of inhibition, fungal disease.

INTRODUCTION

Essential oils (Eos) may be defined as volatile oils that may be obtained from plant materials by steam distillation (Guenther, 1949). In the last few years there has been an increasing interest in Eos as substitutes for conventional synthetic pesticides. This has been due, in part, to concerns over pollution, the development of resistance to conventional pesticides (Holmes and Eckert, 2009), and to the needs of producers of organic agricultural products.

Certain parts of various plant species harbour secondary metabolites, which show a variety of chemical structures (Bell and Charlwood, 1980). Their roles are mostly unknown though many of them have been found to exhibit anti-fungal properties (Patel and Jasrai, 2009; Sujatha, 2010). Various types of anti-fungal chemicals such as saponins, unsaturated lactones, cyanogenic glycosides, oils and phenolic compounds are found to be present in relatively large quantities in tissues of some

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Table 1. CFU count of ten different fungal pathogen under experiment.

Fungus	Host plant	Plant parts	Incubation (days)	CFU
<i>Fusarium equiseti</i> (Fe)	brinjal	Root	3	1.36×10 ⁶
<i>Colletotrichum gloeosporioides</i> (Cg)	brinjal	Leaf	6	2.64×10 ⁶
<i>Alternaria alternata</i> (Aa)	tomato	Leaf	4	2.11×10 ⁶
<i>Pestalotiopsis theae</i> (Pt)	tea	Leaf	7	3.45×10 ⁶
<i>Aspergillus flavus</i> (Af)	ground nut	Pod	5	1.54×10 ⁶
<i>Fusarium solani</i> (Fs)	potato	Root	3	1.67×10 ⁶
<i>Alternaria solani</i> (Ac)	mustard	Leaf	5	2.82×10 ⁶
<i>Bipolaris oryzae</i> (Bo)	rice	Leaf	7	0.97×10 ⁶
<i>Erysiphe pisi</i> (Ep)	pea	Leaf	4	1.98×10 ⁶
<i>Cercospora nicotianae</i> (Cn)	tobacco	Leaf	8	2.65×10 ⁶

Table 2. Chemical compound present in the selected plant volatile essential oils.

Volatile oil source	Chemical compound
Eucalyptus leaves	1,8-cineole, eucalyptol, ursolic acid, globulol, geranyl acetate, terpinolene, limonene, epiglobulol, p-cymene, camphene, pinocarveol. A and β pinene, α-phellandrene, β-sabinene, α-terpineol, α-terpineol acetate
Citronella leaves	Myrcene, citral, citronellal, cymbopogonol, limonene, citronellic acid, citronellal, citronellol and geraniol
Karanj leaves	Saponins, hydroxyl furanoflavones, beta sitosterol glucoside and aurantiamide acetate, triterpenes, isoflavonoid diglycosides
Neem leaves	Linoleic acid, Oleic acid, 9-Hexadecenoic acid, Alpha-linolenic acid, Methyl isoheptadecanoate, 2,6,10,14-Tetramethylheptadecane, Hexadecanoic acid, Octadecanoic acid

*source: Pawar and Thaker (2007).

plant species. Their occurrence, distribution and possible functions have been reviewed by Schlosser (1988). Antifungal action of Eos and other chemical plant components has been reported by several scientists (Mann and Markhan, 2006; Deena and Thopil, 2008; Demirci et al., 2009; Mathpal et al., 2005 and Simic et al., 2004).

Considering the environmental pollution, we should exploit different plant volatile Eos for disease management in organic system (Barman et al., 2015). Keeping the value of synthetic pesticide free organic commodity, *in vitro* study was carried out. In this study, the antifungal potency of four different plant volatile Eos from eucalyptus (*Eucalyptus globulus*), citronella (*Cymbopogon citrate*), karanj (*Pongamia pinnata*) and neem (*Azadirachta indica*) has been tested against ten selected plant pathogenic fungal isolates and results achieved are presented.

MATERIALS AND METHODS

This experiment was conducted during the year 2012 to 2013. Distilled Eos (Upshaw Aromatics Private Ltd. Hyderabad) of four plant species, namely eucalyptus, citronella, karanj and neem were purchased from the local market, and were screened for *in-vitro* antifungal activity against the following 10 phytopathogenic fungi (Table 1) isolated from infected material: *Fusarium equiseti*, *Colletotrichum gloeosporioides*, *Alternaria alternata*, *Pestalotiopsis theae*, *Aspergillus flavus*, *Fusarium solani*, *Alternaria solani*,

Bipolaris oryzae, *Erysiphe pisi*, and *Cercospora nicotianae* grown in Sabouraud Dextrose Agar (SDA) medium and maintained in the laboratory at 25±1°C.

The pathogens were subjected to Koch's postulates for verification of the diseases. Thereafter, they were incubated at 4°C. After seven days of incubation, the hyphal tip of the fungus radiating from the infected tissue was transferred onto SDA slants. Freshly prepared sterile SDA slants were used for the maintenance of the fungal cultures by sub-culturing periodically. Pathogens grown on sterile SDA media were stored in two different conditions, viz. at low temperature in refrigerator (at 4°C) and in incubator at 27±1°C. At the interval of one week, subculture was done taking sample from incubator at 27±1°C for preparation of inoculums for different experiments.

To avoid loss of virulence, fresh isolations were made when required. The chemical compounds present in the selected plant volatile Eos are listed in Table 2. The antifungal screening was performed through Disk diffusion assay method (Patel and Jasrai, 2011).

Paper disc preparation for the assay

Sterilized Whatman paper (No.1) discs (6.5 mm in diameter) were impregnated with the known quantity of plant volatile EO at 0.5 to 8 mg/disc, and then air dried. The impregnated discs were used to conduct bioefficacy study against the above mentioned fungal isolates.

Inoculum preparation of test fungi

50 µl of each fungal culture from SDA slant was transferred and established into a 150 ml conical flask containing 25 ml of SDA

Table 3. Essential oil minimum inhibitory concentration (MIC) values determined through Disc-diffusion assay method towards the selected fungi.

Volatile oil source	Inhibition of fungi at MIC value (mg/disc)					
	0.5	1.0	2.0	3.5	5.0	8.0
Eucalyptus	Af, Fs	Fe, Aa	Cg, Ac	Ep, Bo, Pt,	Cn	-
Citronella	-	Ac, Aa	Bo, Ep	Fe, Fs, Af	Cg, Cn	-
karanj	-	Cn, Cg	Pt, Aa	Af, Bo, Ac	Ep, Fe, Fs	-
Neem	-	Aa	Af, Cg	Ep, Pt	Ac, Bo, Fe, Fs	Cn

*Fusarium equiseti (Fe), Colletotrichum gloeosporioides (Cg), Alternaria alternata (Aa), Pestalotiopsis theae (Pt), Aspergillus flavus (Af), Fusarium solani (Fs), Alternaria solani (Ac), Bipolaris oryzae (Bo), Erysiphe pisi (Ep), Cercospora nicotianae (Cn).

Table 4. Zone of inhibition (ZI) against fungi exhibited by eucalyptus (*Eucalyptus globulus*) volatile oil at various concentrations in Disc-diffusion assay method.

Fungal phytopathogen	Zone of inhibition (mm) at different essential oil concentration (mg/disc)					
	0.5	1.0	2.0	3.5	5.0	8.0
<i>Fusarium equiseti</i>	-	3.44	4.67	8.48	10.45	12.37
<i>Colletotrichum gloeosporioides</i>	-	-	5.14	7.59	11.23	12.64
<i>Alternaria alternata</i>	-	4.12	5.39	6.78	9.78	11.34
<i>Pestalotiopsis theae</i>	-	-	-	6.45	8.68	10.98
<i>Aspergillus flavus</i>	-	4.31	6.54	8.45	10.28	13.88
<i>Fusarium solani</i>	3.89	4.37	6.98	8.59	11.67	14.45
<i>Alternaria solani</i>	-	-	4.69	5.67	9.58	13.54
<i>Bipolaris oryzae</i>	-	-	-	5.98	9.57	11.25
<i>Erysiphe pisi</i>	-	-	-	5.78	7.78	9.56
<i>Cercospora nicotianae</i>	-	-	-	-	4.12	6.48

medium for bioassay study. The inoculated flasks were successively incubated for a specific time period at room temperature (25±1°C). The number of all fungal colonies/flask was recorded by means of a haemocytometer spore count (Table 1). Then the fungal colonies formed in each single flask were homogenized in sterile conditions and the relative suspensions were used in the bio-assay study (Patel and Jasrai, 2011).

Evaluation of antifungal activity

Fungitoxic spectrum of the selected plant volatile Eos was determined at various concentrations (0.5 to 8 mg/disc) using the standardized protocol of the Disk diffusion assay (Patel and Jasrai, 2011) under axenic conditions. For this, an aliquot 0.1 ml fungal culture of known (spore count) unit forming colonies (UFC) was aseptically transferred with micropipette in each Petri plate containing SDA medium (15 ml in a 3.5 cm thick layer), and uniformly seeded on its surface with sterilized cotton swabs (Himedia). At the same time, extract loaded single Whatman paper discs were placed and slightly pressed on the media surface with sterile forceps to ascertain a firm contact. Then the plates were incubated in upside-down position for 72 h at 25±1°C. The experiment was performed in triplicate with untreated controls. The Zone of inhibition (ZI) indicating the EO antifungal effectiveness was measured (in mm) by the antibiotic Zone reader (Labfine) (Patel and Jasrai, 2011). The experiment was carried at five different concentration viz. 0.5, 1.0, 2.0, 3.5, 5.0 and 8.0 mg/disc. Three replications were maintained for each pathogen with CRD design. The data were subjected to statistical analysis using

INDOSTAT package developed by Indostat service Hyderabad, India.

RESULTS AND DISCUSSION

Results of this study showed that the four tested plant volatile Eos had excellent broad- spectrum antifungal activity against the ten selected plant fungal pathogens. The inhibition of fungal phytopathogens by the tested Eos can be due to presence of complex mixture of secondary metabolites containing different volatile compounds such as phenylpropanes, various terpenoids and their oxygenated derivatives. The fungi toxic spectrum or MIC (minimum inhibitory concentration) values of the tested Eos, determined in terms of zone of inhibition (ZI), is presented in Tables 4, 5, 6 and 7. The antifungal screening for all the four Eos clearly indicates the effective lowest concentration to control the fungal growth. The more lower the MIC value, the better antifungal potency of the relative plant volatile EO. The lowest MIC value, 0.5 mg/disc, was recorded for EO from *C. citrate*. *P. pinnata* and *A. indica* EOs showed the maximum MIC values. *A. indica* EO showed minimum inhibition of fungal pathogen at certain MIC level (Table 3).

Table 5. Zone of inhibition (ZI) against fungi exhibited by citronella (*Cymbopogon citrate*) volatile oil at various concentrations in Disc diffusion assay method.

Fungal pathogen	Zone of Inhibition (mm) at different volatile oil concentration (mg/disc)					
	0.5	1.0	2.0	3.5	5.0	8.0
<i>Fusarium equiseti</i>	-	-	-	4.45	6.47	8.84
<i>Colletotrichum gloeosporioides</i>	-	-	-	-	4.25	6.58
<i>Alternaria alternata</i>	-	5.23	7.54	9.25	11.02	13.84
<i>Pestalotiopsis theae</i>	-	4.25	6.34	8.45	9.45	12.08
<i>Aspergillus flavus</i>	-	-	-	4.87	5.27	8.58
<i>Fusarium solani</i>	-	-	-	6.78	8.57	10.22
<i>Alternaria solani</i>	-	4.57	5.65	6.89	7.98	9.65
<i>Bipolaris oryzae</i>	-	-	5.97	7.35	9.56	12.35
<i>Erysiphe pisi</i>	-	-	4.89	6.58	8.69	10.91
<i>Cercospora nicotianae</i>	-	-	-	-	4.34	6.23

Table 6. Zone of inhibition (ZI) against fungi exhibited by karanj (*Pongamia pinnata*) volatile oil at various concentrations in Disc-diffusion assay method.

Fungal pathogen	Zone of inhibition (mm) at different volatile oil concentration (mg/disc)					
	0.5	1.0	2.0	3.5	5.0	8.0
<i>Fusarium equiseti</i>	-	-	-	-	4.87	6.78
<i>Colletotrichum gloeosporioides</i>	-	5.17	6.12	8.27	9.27	11.67
<i>Alternaria alternata</i>	-	-	6.24	7.98	8.94	11.28
<i>Pestalotiopsis theae</i>	-	-	6.78	8.37	10.45	12.98
<i>Aspergillus flavus</i>	-	-	-	4.65	6.58	7.98
<i>Fusarium solani</i>	-	-	-	-	4.78	6.25
<i>Alternaria solani</i>	-	-	-	4.15	6.78	11.28
<i>Bipolaris oryzae</i>	-	-	-	4.57	6.78	8.49
<i>Erysiphe pisi</i>	-	-	-	-	4.87	6.87
<i>Cercospora nicotianae</i>	-	4.26	5.67	7.89	9.45	11.34

Table 7. Zone of inhibition (ZI) against fungi exhibited by neem (*Azadirachta indica*) oil at various concentrations in Disc-diffusion assay method.

Fungal pathogen	Zone of inhibition (mm) at different volatile oil concentration (mg/disc)					
	0.5	1.0	2.0	3.5	5.0	8.0
<i>Fusarium equiseti</i>	-	-	-	-	4.35	6.98
<i>Colletotrichum gloeosporioides</i>	-	-	5.18	7.45	9.45	11.85
<i>Alternaria alternata</i>	-	4.25	6.57	8.48	10.78	12.98
<i>Pestalotiopsis theae</i>	-	-	-	4.75	6.45	9.48
<i>Aspergillus flavus</i>	-	-	4.57	6.25	8.49	10.95
<i>Fusarium solani</i>	-	-	-	-	4.08	6.94
<i>Alternaria solani</i>	-	-	-	-	4.02	7.03
<i>Bipolaris oryzae</i>	-	-	-	-	4.67	7.15
<i>Erysiphe pisi</i>	-	-	-	4.91	6.45	8.47
<i>Cercospora nicotianae</i>	-	-	-	-	-	4.83

In the present study, *E. globulus* EO exhibited highest zone of inhibition against *F. solani* (14.45 mm) followed

by *A. flavus* (13.88 mm). Its lowest MIC, 0.5 mg/disc, was recorded against *F. solani* followed by inhibition of *Aspergillus flavus*. The same EO at only 0.5 mg/disc was able to control *F. solani*. *C. citrate* EO showed maximum ZI against *A. alternata* (13.84 mm). *P. theae* was inhibited only at concentration of 1 mg/disc (Table 5). In contrast, *P. pinnata* EO successfully inhibited all tested fungi and exhibited its maximum ZI against *A. alternata* (12.98 mm) followed by *C. gloeosporioides* (11.67 mm).

In addition, the assay revealed that *P. pinnata* restricted the growth of all tested fungi at MIC of 5.0 mg/disc (Table 6). *A. indica* EO demonstrated highest ZI against *A. alternata* (12.98 mm) followed by *C. gloeosporioides* (11.85 mm), *A. flavus* (10.95 mm). But *A. indica* EO did not perform well in comparison to the other Eos (Table 7). All the selected fungi were inhibited with all the Eos even if at different performance levels. Fungal growth inhibition by Eos often involves prevention of hyphal growth and sporulation, interruption in nutrient uptake and metabolism, plasma membrane disruption, mitochondrial structure disorganization and interference with respiratory enzymatic reactions of the mitochondrial membrane (Patel and Jasrai, 2011).

Conclusions

Locally available plant volatile Eos may play a great role in controlling major plant disease. This may encourage the farmers to produce organic commodities to generate more revenue. As EOs antifungal activity is very probably due to the synergistic action of chemical compound mixtures, there would be a negligible chance of resistance development in fungal pathogens. Disease control through such natural available volatile substances would also be an important tool for integrated disease management in organic farming.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Bacteria associated with sugarcane in Northeastern Brazil

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Sugarcane has a high demand in nitrogen, increasing costs and causing damages to the environment. It is necessary to find alternatives to reduce nitrogen fertilizers use. Diazotrophic bacteria have capacity to promote growth in grass with potential to fix N₂ and solubilize inorganic phosphate. This study aimed to evaluate bacterial community associated with different sugarcane varieties in Northeastern Brazil, select bacteria with plant growth-promoting characteristics, and identify endophytic and epiphytic bacterial lineages in sugarcane. Endophytic bacteria of leaves and roots and epiphytic bacteria of rhizoplane were isolated from three sugarcane commercial varieties and selected for their capacity to fix N₂ and solubilize inorganic phosphate. Bacterial strains from different morphological groups were isolated and a sample of 27 strains with potential for the simultaneous development of these characteristics were selected and identified. The bacterial community that interacted with sugarcane was more associated with rhizoplane and roots regions than with leaves, and had a high potential to fix N₂ and solubilize inorganic phosphate. Bacterial lineages were mainly from genera *Pantoea* sp. and *Burkholderia* sp., but there were also genera *Enterobacter* sp., *Klebsiella* sp. and *Pseudomonas* sp. and two lineages at the species level: *Pantoea stewartii* and *Burkholderia cenocepacia*.

Key words: Plant/bacteria interaction, plant growth-promoting bacteria, N₂ fixing bacteria, bacteria identification.

INTRODUCTION

In Northeastern Brazil, sugarcane cultivation is traditional and its management is performed in the conventional

way, using a large amount of chemical inputs, especially nitrogen and phosphate fertilizers, increasing production

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costs and contaminating the environment by hydric resources eutrophication. Studies associating microorganisms with plant growth promotion are very important for sugarcane cultivation in this region and represent technological innovation.

There are various bacterial genera associated with plants that have the capacity to fix N₂ biologically, among which *Azoarcus* sp., *Azospirillum* sp., *Azotobacter* sp., *Beijerinckia* sp., *Burkholderia* sp., *Enterobacter* sp., *Erwinia* sp., *Herbaspirillum* sp., *Gluconoacetobacter* sp., *Klebsiella* sp., *Pseudomonas* sp. and *Pantoea* sp. (Carvalho et al., 2014).

Biological N₂ fixation (BNF) has been one of the mechanisms explored in the interaction between plant and microorganisms in many studies and in different crops, such as wheat (Didonet et al., 1996), rice (Verma et al., 2001), maize, sorghum and wheat (Roncador-Maccari et al., 2003), soybean (Souza et al., 2008) and common bean (Brito et al., 2011). In sugarcane, the studies have focused on varieties cultivated in Southeast region of the country and have found important bacterial groups that perform BNF (Rennie et al., 1982; Oliveira et al., 2002; Loiret et al., 2004; Perin et al., 2004; Reis et al., 2004; Luvizotto et al., 2010; Costa et al., 2014).

Resende et al. (2006) reported that biological N₂ fixation (BNF) performed by bacteria associated with sugarcane can be responsible for up to 60% of the total N accumulated in the plants. This means that BNF can contribute to reducing the use of N fertilizers, especially for the production of sugarcane in the first crop cycle (Polidoro et al., 2006; Resende et al., 2006).

Moreover, microorganisms perform essential functions for increasing P availability in the soil to plants, through mechanisms that affect structure, chemistry, biochemistry and physiology of the root environment. These mechanisms include capacity to solubilize insoluble inorganic phosphates, increasing P soluble content in the soil solution and, consequently, its availability to plants (Goldstein et al., 1999; Chen et al., 2006).

Microorganisms use mechanisms like production and release of low-molecular-weight organic acids, which solubilize P precipitated forms such as Fe and Al phosphates in acid soils and Ca phosphates in alkaline soils, acting as sources of protons or chelating accompanying element of the phosphate ion (Marra et al., 2012).

Association between diazotrophic bacteria and sugarcane involves singular mechanisms, which are still little understood (Carvalho et al., 2014). The main sugarcane productive region in Brazil is Southeast region, where only some genera of endophytic bacteria were isolated in this crop and a few were studied, such as: *Enterobacter* sp., *Erwinia* sp., *Klebsiella* sp. (Rennie et al., 1982), *Herbaspirillum* sp. (Oliveira et al., 2002) and *Burkholderia* sp. (Reis et al., 2004; Luvizotto et al., 2010). Some species were also found and described, such as: *Bacilluspolimixa* (Rennie et al., 1982), *Pantoeaagglomerans*

(Loiret et al., 2004) and *Gluconoacetobacter diazotrophicus* (Perin et al., 2004).

Some studies in South region (Magnani et al., 2010; Beneduzi et al., 2013) identified bacteria in sugarcane from Parana state and Rio Grande do Sul State, but sugarcane is not expressive crop in this region.

For Northeast region, there are no published studies that have isolated and identified genera of endophytic bacteria in sugarcane. The study of Resende et al. (2006) performed a N balance in the soil/plant system in an experiment in Pernambuco state and concluded that N entry through BNF was considerable and consistent with the low responses to N application in fertilizer form and also with low efficiency of use of N fertilizer.

Some studies have recently been conducted in Northeast region (Lira-Cadete et al., 2012; Santos et al., 2012; Silva et al., 2012; Barros et al., 2014); however, they only emphasize bacteria association with sugarcane in aspects related to BNF, production of growth phytohormones and solubilization of inorganic phosphate. It is important that these studies continue to be conducted, but they should also associate these benefits of plant growth with the identification of endophytic bacterial genera in sugarcane in Brazil Northeast region.

Knowledge on the bacterial community associated with sugarcane and study on their beneficial effects can increment agricultural yield, reduce chemical inputs use, reduce production costs and minimize contamination of the production environment.

Therefore, this study aimed to evaluate cultivable bacterial community associated with different varieties of sugarcane in the first crop cycle at four months of age in Northeastern Brazil, select bacteria with plant growth-promoting characteristics and identify endophytic and epiphytic bacterial lineages in different plant/bacteria association regions.

MATERIALS AND METHODS

Site description

Bacterial community study was conducted in plant samples obtained from first-cycle commercial plantations of three sugarcane varieties at Sugarcane Experimental Station of Carpina (EECAC), which is located at 7°51'04" S and 35°14'27" W, in sugarcane region of Zona da Mata in Pernambuco state, Northeast Brazil. Soil cultivated with the varieties was classified as dystrocohesive Yellow Argisol, according to Santos et al. (2013), corresponding to Ultisol (Soil Survey Staff, 1998). This soil is common in Northeast Brazil, predominantly in Pernambuco, and normally used for sugarcane cultivation.

Experimental area was prepared with a disk harrow, a leveling harrow and a two-row furrower. The leveling harrow also served to incorporate 1.5 mg ha⁻¹ of dolomitic limestone with relative neutralizing value (RNV) of 95%, 30 days before planting in the areas cultivated with RB 867515 and RB 863129 varieties. Acidity correction was not necessary in the area cultivated with the variety RB 92579. Fertilization at planting corresponded to the application of 60, 120 and 90 kg ha⁻¹ of N, P₂O₅ and K₂O, respectively, at the bottom of the planting furrows. The fertilizer sources used were

ammonium sulfate, triple superphosphate and potassium chloride, respectively. The amounts of correctives and fertilizers at planting were based on the Manual of Recommendation of Fertilization for Pernambuco State (Cavalcanti et al., 2008). The area cultivated with RB 867515 variety received pre-emergence herbicide at the dose of 2.5 L ha⁻¹, because it was vulnerable to emergence, growth and development of spontaneous plants.

Leaves, roots and soil sampling

Sugarcane plant varieties used for sampling were RB 863129, RB 92579 and RB 867515. These are commercial varieties used in the Brazil Northeast region, and Simões Neto and Melo (2005) reported that RB 863129 has early maturation and can be used in any production environment, while RB 92579 and RB 867515 have medium and late maturation, respectively. These authors also point out that RB 867515 can be used in soil with low fertility, due to its high efficiency in nutrients use. Endophytic bacteria of leaves and roots and epiphytic bacteria of the rhizoplane (region where the soil is adhered to the roots) were isolated from these three commercial varieties of sugarcane. At for months of age, ten plants of each sugarcane variety were selected, totaling 30 samples. Plants with higher physiological vigor were selected for a better representation of the variety. Each plant, its root system and the soil from the rhizosphere were placed in 100 L plastic bags, which were identified and taken to Laboratory of Genetics and Microbial Biotechnology (LGBM), at Academic Unit of Garanhuns (UAG/UFRPE), for the procedures of isolation, plant growth promotion tests and identification.

Bacteria isolation

Endophytic bacteria isolation

The procedure was performed in four plants of each variety, using 12 samples of leaf mass and 12 samples of roots. Endophytic bacteria were isolated in cubes of tissues weighing from 1 to 3 g of fresh matter of leaves and roots. Leaves and roots were washed in running water.

Epiphytic bacteria isolation

The soil of the rhizosphere was used for epiphytic bacteria isolation. These bacteria were isolated using a phosphate-buffered saline (PBS) solution in 500 mL Erlenmeyer flask for each root sample (12 samples). Each Erlenmeyer received 50 mL of PBS buffer solution and glass beads to facilitate soil removal from rhizoplane. Epiphytic bacteria from rhizoplane, and endophytic bacteria from leaves and roots were isolated according to Kuklinsky-Sobral et al. (2004) and Mendes et al. (2007). TSA (Trypticase Soy Agar) medium at 10% supplemented with the fungicide Cercobyn 700 (50 µg mL⁻¹) was used for samples incubation. The samples were maintained at 28°C during incubation and evaluated after 3, 8 and 14 days. Bacterial population (colony-forming units – CFU) per gram of fresh plant tissue (CFU/g FPT) was estimated by counting colonies cultivated in 10% TSA medium. Characteristic colonies of each morphological type were subcultured from isolation plates, purified and maintained in liquid 10% TSA supplemented with 20% of glycerol at -20°C for formation and organization of a collection of bacterial cultures associated with sugarcane varieties cultivated in Brazil Northeast region.

Selection N₂-fixing bacteria

The bacteria were inoculated in semi-solid NFb medium [5 g L⁻¹

malate; 0.5 g L⁻¹ K₂HPO₄; 0.2 g L⁻¹ MgSO₄·7H₂O; 0.1 g L⁻¹ NaCl; 0.01 g L⁻¹ CaCl₂·2H₂O; 4 mL L⁻¹ Fe.EDTA (1.64% solution); 2 mL L⁻¹ bromothymol blue (0.5%); 2 mL L⁻¹ solution of micronutrients (0.2 g L⁻¹ Na₂MoO₄·2H₂O; 0.235 g L⁻¹ MnSO₄·H₂O; 0.28 g L⁻¹ H₃BO₃; 0.008 g L⁻¹ CuSO₄·5H₂O); 1.75 g L⁻¹ agar], buffered at pH 6.8 and maintained incubated at 28 °C for 8 days (Dobereiner et al., 1995).

The tests were performed in triplicate and the positive result for N fixation was characterized by the presence of a growth halo inside the vials, which grew until the surface of the culture medium. The bacteria were subcultured five times more in new semi-solid NFb media for the confirmation of atmospheric N₂ fixer characteristic.

Selection inorganic phosphate-solubilizing bacteria

Inorganic phosphate-solubilizing bacteria were selected according to the procedure of Rodriguez et al. (2000), with a few modifications. This methodology recommends the use of 0.7 g L⁻¹ of tri-calcium phosphate [Ca₃(PO₄)₂]. Since it was not possible to obtain this compound, 4.0 g L⁻¹ of mono-calcium phosphate (CaHPO₄) were used. The solubility of Ca₃(PO₄)₂ is five times higher than that of CaHPO₄; thus, it was used an amount five times higher of the latter.

Bacteria were inoculated in solid culture medium (10 g L⁻¹ glucose; 5 g L⁻¹ NH₄Cl; 1 g L⁻¹ NaCl; 1 g L⁻¹ MgSO₄·7H₂O; 15 g L⁻¹ agar), containing insoluble phosphate (4 g L⁻¹ CaHPO₄) buffered at pH 7.2 and maintained incubated at 28°C for 72 h. The experiments were performed in triplicate and the presence of a clear halo around the colony indicated phosphate solubilization.

Lineages identification

The bacterial lineages were cultivated in 5 mL of TSA for 24 h at 28 °C under agitation at 166 g. Then, 4 mL of the culture were centrifuged for 5 min at 15,500 g and the cells were re-suspended in 500 µL of TE (10 mM Tris-HCl buffered at pH 8.0), centrifuged and re-suspended again in 500 µL of TE, with the adding of 0.5 g of glass beads (0.1 mm diameter) and 15 µL of 20% sodium dodecyl sulfate (SDS).

Cells were agitated in a homogenizer for 30 s at 3,875 g. Then, 500 µL of phenol were added to the lysed cells, homogenized through inversion and centrifuged for 5 min at 15,500 g. Aqueous phase was extracted once with chloroform-phenol (1:1) and once with chloroform. Later, DNA was precipitated with 1/10 volume of 5 mol L⁻¹ NaCl and 6/10 volume of isopropanol, and collected through centrifugation (10 min).

DNA precipitate was washed with 70% ethanol, dried at 37°C and re-suspended in 50 µL of TE. Total DNA was analyzed through electrophoresis in agarose gel (0.8% m/v) in 1x TAE buffer (40 mM Tris-acetate; 1 mM EDTA) and colored with ethidium bromide (0.5 µg mL⁻¹), according to the procedures described by Sambrook et al. (1989).

16S rDNA was amplified through PCR technique using the universal primers for bacteria P027F (5'-GAGAGTTTGATCCTGGCTCAG-3') and 1378R (5'-CGGTGTGACAAGGCCCGGAACG-3'), according to Heuer et al. (1997). The reactions were performed in 50 µL final volume containing 0.5 ng of total DNA (1.0 µL); 0.2 µM of each primer (0.1 µL); 0.2 mM of each dNTPs (4.0 µL); 3.75 mM MgCl₂ (7.5 µL); 0.05 U of enzyme Taq DNA polymerase (*Fermentas*) (0.5 µL); 5.0 µL of 10x buffer Taq (750 mM Tris-HCl pH 8,8, 200 mM (NH₄)₂SO₄ and 0,1% Tween). Final volume was completed with autoclaved pure water.

Amplification reaction was performed in a thermocycler programmed for initial denaturation at 94°C for 4 min, 25 cycles denaturation at 94°C for 30 s, annealing temperature at 63°C for 1 min and extension of primers at 72°C for 1 min, followed by final

Table 1. Total population density of endophytic bacterial community in leaves and roots and epiphytic bacterial community in the rhizoplane of different 4-month-old sugarcane varieties in the first crop cycle.

Variety	Plant/bacteria association region			Average
	Leaf	Root	Rhizoplane	
CFU g ⁻¹ FPT (Log ₁₀)				
RB 92579	3.02	6.09	8.65	6.08 ^a
RB 867515	3.92	5.43	8.39	5.43 ^b
RB 863129	4.18	6.88	8.66	6.88 ^a
Average	3.92 ^C	6.08 ^B	8.64 ^A	
Variation coefficient (%)			11.08	
Overall average			6.13	

Averages followed by same letter, lowercase in the column and uppercase in the row, do not differ by Scott-Knott test ($p > 0.05$).

extension at 72°C for 7 min. After amplification, 10 µL of PCR reaction were evaluated through electrophoresis in agarose gel (1.2% m/v) in 1x TAE buffer and colored with ethidium bromide (0.5 µg mL⁻¹).

PCR products were purified (*PCR purification Power Clean-Up kit, MoBio Laboratories*) and subjected to sequencing with primer 1378R, in an automated sequencer. Sequences were analyzed using BLASTn against database from NCBI (National Center for Biotechnology Information website [<http://www.ncbi.nlm.nih.gov>]).

Statistical analyses

Total population density data of bacterial community were subjected to analysis of variance and effects of variety, plant/bacteria association region and interaction were evaluated by F test ($p < 0.05$). When main effects and/or interaction were significant, Scott Knott test ($p < 0.05$) was applied. For relative frequency of bacterial strains number per variety and plant/bacteria association region, Chi-square test ($p < 0.01$) was applied.

RESULTS

Total population density of bacterial community associated with sugarcane varied from 10³ to 10⁸ CFU g⁻¹ FPT (Table 1). And there was preferential colonization of endophytic and epiphytic bacteria in the root zone for the sugarcane varieties evaluated.

Population density of epiphytic bacteria was significantly higher (2.2 times) than that of endophytic bacteria in the leaves and significantly higher (1.4 times) than that of endophytic bacteria in the roots (Table 1). Endophytic bacteria population density was significantly higher (1.6 times) in the roots compared with the leaves (Table 1). The root environment, whether of endophytic or epiphytic bacteria, was more favorable for the bacterial community associated with sugarcane.

Total population densities of the bacterial community associated with the sugarcane varieties RB 92579 and RB 863129 were similar, and higher compared with RB 867515 (Table 1). Herbicide application was

necessary in the area cultivated with RB 867515, which may have compromised the bacterial colonization in this variety.

In these three sugarcane varieties evaluated were isolated 142 strains, being 88 strains of endophytic bacteria from leaves and roots and 54 strains of epiphytic bacteria from rhizoplane. Different morphological groups appeared and strains representing each group were selected.

The 142 isolated bacterial strains were evaluated regarding their potential to fix N₂ and to solubilize inorganic phosphate. According to the results, 79 strains were able to grow in NFb medium (Table 2), which represented 56% of the group of the evaluated bacterial strains. Therefore, the sugarcane varieties showed potential to fix N₂.

Between 142 bacterial strains evaluated for their potential to solubilize inorganic phosphate, a solubilization halo was observed in 51 strains, which represented 36% of the group of evaluated bacterial strains. From these 51 strains, more than half (53%) were found in the rhizoplane of the varieties (Table 2), as also observed in the strains with potential to fix N₂.

On the other hand, bacterial strains with potential to solubilize inorganic phosphate were found in all sugarcane varieties, with no difference between regions of the plants (Table 2).

When the strains in leaves, roots and rhizoplane were evaluated in each variety, only RB 867515 did not show higher number of bacterial strains with potential to solubilize inorganic phosphate in the rhizoplane (Table 2).

Depending on region of bacterial colonization, varieties showed different amounts of strains with these characteristics. RB 863129 variety differed from the others in rhizoplane region and showed the highest number of strains with potential to fix N₂ and solubilize inorganic phosphate (Table 3).

In bacterial strains evaluation per region from each

Table 2. Number and relative frequency of endophytic and epiphytic bacterial strains with capacity to fix N₂ or solubilize inorganic phosphate in different regions of three 4-month-old sugarcane varieties in the first crop cycle.

Factor			Factor		
Region	Variety	Relative frequency unit (%)	Variety	Region	Relative frequency unit (%)
Nitrogen-fixing strains					
Leaf	RB 92579	4 (25)	RB 92579	Leaf	4 (16)
	RB 867515	7 (44)		Root	5 (20)
	RB 863129	5 (31)		Rhizoplane	16 (64)
χ^2		0.000 ^{n.s}	χ^2		1.385 ^{n.s}
Root	RB 92579	5 (29)	RB 867515	Leaf	7 (32)
	RB 867515	3 (18)		Root	3 (14)
	RB 863129	9 (53)		Rhizoplane	12 (54)
χ^2		0.059 ^{n.s}	χ^2		0.000 ^{n.s}
Rhizoplane	RB 92579	16 (35)	RB 863129	Leaf	5 (16)
	RB 867515	12 (26)		Root	9 (28)
	RB 863129	18 (39)		Rhizoplane	18 (56)
χ^2		4.840*	χ^2		4.840*
Inorganic phosphate-solubilizing strains					
Leaf	RB 92579	4 (33)	RB 92579	Leaf	4 (25)
	RB 867515	7 (59)		Root	5 (31)
	RB 863129	1 (8)		Rhizoplane	7 (44)
χ^2		1.000 ^{n.s}	χ^2		0.529 ^{n.s}
Root	RB 92579	5 (42)	RB 867515	Leaf	7 (47)
	RB 867515	2 (16)		Root	2 (13)
	RB 863129	5 (42)		Rhizoplane	6 (40)
χ^2		0.111 ^{n.s}	χ^2		2.579 ^o
Rhizoplane	RB 92579	7 (26)	RB 863129	Leaf	1 (5)
	RB 867515	6 (22)		Root	5 (25)
	RB 863129	14 (52)		Rhizoplane	14 (70)
χ^2		1.087 ^{n.s}	χ^2		1.087 ^{n.s}

χ^2 Calculated; ^{n.s}Not significant; ^oSignificant (p>0.10); *Significant (p>0.05).

variety, RB 863129 had the strains concentrated in the rhizoplane, which differed from leaves and roots. For the other varieties, there was no difference in the number of strains between leaves, roots and rhizoplane (Table 3).

Among 43 strains identified with potential to fix N₂, a sample of 27 lineages with higher potential to solubilize inorganic phosphate was selected and subjected to identification through the analysis of partial 16S rDNA gene sequencing. Sequences were subjected to the database of the NCBI (*National Center for Biotechnology Information* [http://www.ncbi.nlm.nih.gov]) and analyzed using BLASTn (*Basic Local Alignment Search Tool*), being aligned and compared with existing sequences and identified at the level of genus and species when they showed an index of similarity higher than 90% (Table 4).

In these selected lineages, eight are from genus *Pantoea* sp., eight from *Burkholderia* sp., four from *Pseudomonas* sp., three from *Enterobacter* sp., two from *Klebsiella* sp. and two lineages were identified with similarity at the species level: *Pantoea stewartii* and

Burkholderia cenocepacia (Table 4). Identified bacterial lineages were mainly from the genera *Pantoea* sp. and *Burkholderia* sp. Genus *Burkholderia* sp. is widely found and studied due to the great capacity of its species to produce pentachlorophenols (PCPs), fix N₂ and solubilize inorganic phosphate in many agricultural crops of economic interest and under various environmental conditions. It is a genus that has a considerable physiological versatility and occupies a great variety of ecological niches (Perin et al., 2006a). Perin et al. (2006b) report that the genus *Burkholderia* includes more than 30 species, but not all of them are able to fix N₂. However, in the present study, all the lineages from this genus are N₂ fixing and most of them solubilize inorganic phosphate (Table 4).

DISCUSSION

Variation observed in total population density of bacterial

Table 3. Bacterial strains with capacity to fix N₂ and solubilize inorganic phosphate from sugarcane varieties in the first crop cycle.

Factor			Factor		
Region	Variety	Relative frequency unit (%)	Variety	Region	Relative frequency unit (%)
Nitrogen-fixing and inorganic phosphate-solubilizing strains					
Leaf	RB 92579	4 (33)	RB 92579	Leaf	4 (29)
	RB 867515	7 (58)		Root	3 (21)
	RB 863129	1 (9)		Rhizoplane	7 (50)
χ^2		0.333 ^{n.s}	χ^2		0.077 ^{n.s}
Root	RB 92579	3 (60)	RB 867515	Leaf	7 (58)
	RB 867515	0 (0)		Root	0 (0)
	RB 863129	2 (40)		Rhizoplane	5 (42)
χ^2		0.200 ^{n.s}	χ^2		1.667 ^{n.s}
Rhizoplane	RB 92579	7 (27)	RB 863129	Leaf	1 (6)
	RB 867515	5 (19)		Root	2 (12)
	RB 863129	14 (54)		Rhizoplane	14 (82)
χ^2		4.263*	χ^2		4.263*

Variation observed in total population density of bacterial community with sugarcane was also obtained in other studies on bacteria associated with other host plants (Kuklinsky-Sobral et al., 2004; Barreto et al., 2008). Preferential colonization of endophytic and epiphytic bacteria in the root zone, regardless of type of sugarcane variety, reflected the presence of greater amount of nutrients located in the rhizosphere and rhizoplane, compared with the leaves (Table 1), because these nutrients are responsible for higher bacterial growth and development (Cocking, 2003; Medeiros et al., 2006).

Rhizoplane of the sugarcane varieties was the most populated region by bacteria. In the traditional fertilization management of this crop, all the fertilizers are locally applied at the bottom of the planting furrow and in a single application, as performed in the present study. Therefore, this type of management may have favored the bacterial colonization for making the micro-environment of the rhizoplane more fertile.

Concerning differences between studied varieties, possibly the herbicide application in the RB 867515 area had promoted the lowest result in total population density in this variety area. This application was performed in the soil immediately after planting, which may have negatively affected bacterial colonization, because there were reductions in population densities of endophytic bacteria from roots and epiphytic bacteria from rhizoplane, and not in that of endophytic bacteria from leaves (Table 1), for RB 867515 variety. Malkones (2000), working with effects of different herbicide formulations on soil microorganisms activity, observed a reduction in bacterial colonization and warned for the deleterious effects of certain cultural practices on plant/microorganism association.

Bacterial strains with potential to fix N₂ found in leaves and roots did not differ between the sugarcane varieties.

However, in rhizoplane, there was a significant difference in number and percentage of strains found in these varieties (Table 2). 39% of bacterial strains were isolated in rhizoplane of RB 863129, suggesting that the N fertilization management of this variety may significantly differ from that of RB 92579 and RB 867515. In RB 863129 variety, rhizoplane was the region with the highest number of bacterial strains with potential to fix N₂ (56%), in comparison to leaves and roots. In the other varieties, there were no differences between leaves, roots and rhizoplane (Table 2).

In this work, most bacterial strains (46) with potential to fix N₂ were found in rhizoplane region (58%). This was a region of high bacterial colonization (Table 1) and with characteristics highly favorable for sugarcane nutrition, such as the potential to supply N (Table 2).

Beyond N, P is another important element to plant nutrition. And P fixation in weathered tropical soils is high, due to both adsorption to Fe and Al oxides and precipitation (Simões Neto et al., 2015). These processes limit P availability to plants. When a group of bacteria is able to solubilize phosphate, which occurs preferentially in the rhizoplane, it significantly favors plant phosphate nutrition, especially in sugarcane, because the usual P fertilization management corresponds to one single application at planting.

In modern agriculture, it is common to apply large amounts of P from soluble sources. In tropical soils, this P is rapidly fixed. Hence, one way of minimizing these losses is the application of inoculants containing bacteria that can solubilize this P and transform it into forms assimilable by plants, resulting in better use and crop yield (Wakelin et al., 2004; Canbolat et al., 2006; Dias et al., 2009), and contributing to sustainability.

Since endophytic bacteria colonize inside of host plant and inorganic phosphate is in the soil, it is possible to

Table 4. Identification of bacterial genera and species found in the NCBI by the BLAST database in different regions of three 4-month-old sugarcane varieties in the first crop cycle, index of similarity and their potential to promote plant growth.

Strains	Genus	Similarity (%)	Variety	Region	Growth promotion	
					N ₂ -fixing	Phosphate-solubilizing
UAGC 5	<i>Pantoea stenwartii</i>	91	BR 92579	Leaf	+	+
UAGC 7	<i>Pantoea sp.</i>	99	BR 92579	Leaf	+	+
UAGC 8	<i>Pantoea sp.</i>	99	BR 92579	Leaf	+	+
UAGC 9	<i>Pantoea sp.</i>	99	BR 92579	Leaf	+	+
UAGC 16	<i>Pantoea sp.</i>	99	BR 867515	Leaf	+	+
UAGC 17	<i>Klebsiella/Enterobacter</i>	99	BR 867515	Leaf	+	+
UAGC 18	<i>Pseudomonas sp.</i>	100	BR 867515	Leaf	+	+
UAGC 19	<i>Klebsiella/Enterobacter</i>	99	BR 867515	Leaf	+	+
UAGC 26	<i>Pantoea sp.</i>	97	BR 867515	Leaf	+	+
UAGC 46	<i>Pantoea sp.</i>	100	BR 92579	Root	+	+
UAGC 62	<i>Pantoea sp.</i>	98	BR 867515	Root	+	-
UAGC 70	<i>Enterobacter sp.</i>	99	BR 863129	Root	+	-
UAGC 76	<i>Burkholderia sp.</i>	99	BR 863129	Root	+	-
UAGC 78	<i>Burkholderia sp.</i>	99	BR 863129	Root	+	-
UAGC 86	<i>Pseudomonas sp.</i>	99	BR 92579	Rhizoplane	+	+
UAGC 87	<i>Pseudomonas sp.</i>	99	BR 92579	Rhizoplane	+	-
UAGC 97	<i>Pseudomonas sp.</i>	100	BR 92579	Rhizoplane	+	-
UAGC 102	<i>Pantoea sp.</i>	99	BR 92579	Rhizoplane	+	+
UAGC 103	<i>Enterobacter sp.</i>	96	BR 867515	Rhizoplane	+	-
UAGC 105	<i>Burkholderia cenocepacia</i>	99	BR 867515	Rhizoplane	+	-
UAGC 114	<i>Burkholderia sp.</i>	99	BR 867515	Rhizoplane	+	+
UAGC 122	<i>Enterobacter sp.</i>	94	BR 863129	Rhizoplane	+	+
UAGC 125	<i>Burkholderia sp.</i>	99	BR 863129	Rhizoplane	+	+
UAGC 127	<i>Burkholderia sp.</i>	99	BR 863129	Rhizoplane	+	+
UAGC 130	<i>Burkholderia sp.</i>	99	BR 863129	Rhizoplane	+	+
UAGC 131	<i>Burkholderia sp.</i>	99	BR 863129	Rhizoplane	+	+
UAGC 140	<i>Burkholderia sp.</i>	98	BR 863129	Rhizoplane	+	+

+ and + : N₂-fixing and phosphate-solubilizing; + and - : N₂-fixing and not confirmed phosphate-solubilizing in later tests.

speculate that endophytic bacteria, during their process of plant colonization, are able to solubilize phosphate and increase its availability to the host plant (Massensini et al., 2008).

Regarding plant growth promoted by endophytic or epiphytic bacteria, it is important that these bacteria have more than one characteristic that promotes such growth. Bacterial strains that are able to fix N₂ and solubilize inorganic phosphate are more efficient than those that have only one of these characteristics. In the present study, from 142 isolated bacterial strains, 43 were identified with potential to fix N₂ and solubilize inorganic phosphate, representing 30% of the group of evaluated strains.

In general, bacterial strains were concentrated in rhizoplane, which is the region where the interaction between plant and soil is more intense. This observation suggests that localized fertilization management in sugarcane cultivation must be further intensified, creating micro-environments favorable for these microorganisms

development. Managements that promote local application of fertilizers, irrigation and minimum tillage favor the creation of micro-environments and soil/plant/microorganism relationships.

Identification of bacterial genera found in the Northeast indicated that the bacterial niches are different for each region of the country, because we found one genus (*Pseudomonas sp.*) and two lineages at the species level (*P. stewartii* and *B. cenocepacia*). Beneduzi et al. (2013), working with diversity and plant growth promoting evaluation abilities of bacteria isolated from sugarcane cultivated in the South of Brazil, found the genus *Pseudomonas sp.* that yet there was not been described in the country. Review by Carvalho et al. (2014) on nitrogen signaling in plant interactions with associative and endophytic diazotrophic bacteria not related the genus *Pseudomonas sp.* associate with any crop. However, Kuklinsky-Sobral et al. (2004) identified the species *Pseudomonas citronellolis* in soybean. Genus *Pantoe sp.* was described in rice and soybean (Kuklinsky-

Sobral et al., 2004; Verma et al., 2001) and species *Pantoea aglomerans* in sugarcane (Loiret et al., 2004) different from species found this study (*P. stewartii*). Genus *Burkholderia* sp. was identified in rice (Baldani et al., 2000; Oliveira et al., 2002), maize (Riggs et al., 2001) and sugarcane (Reis et al., 2004; Luvizotto et al., 2010; Beneduzi et al., 2013). In South of Brazil, Beneduzi et al. (2013) identified two species: *Burkholderia phenazinum* and *Burkholderia cepacia*, but in our study we found a different species (*B. cenocepacia*). Evidencing that each bacterial group changes its presence in the habitat according to its demands and the environment where they are inserted.

However, even at Northeastern Region there are different soils under sugarcane growth, and the micro-organisms population may change because soil and climate conditions. It would be necessary deeper studies in other sugarcane areas to find and identify them. Moreover, after strains identification, we need to apply these micro-organisms and test them in their capacity to improve sugarcane development and productivity. This would allow a more sustainable tillage, decreasing chemical fertilizers use, and improving environmental quality of soils.

Conclusions

In this work, bacterial community that interacted with sugarcane at four months of age in the first crop cycle in Northeast Brazil was more associated with the regions of rhizoplane and roots than with leaves. Bacterial community that interacted with sugarcane had a high potential to fix N₂ and solubilize inorganic phosphate in vitro. And identified bacterial lineages were mainly from *Pantoea* sp. and *Burkholderia* sp., but there were also the genera *Enterobacter* sp., *Klebsiella* sp. and *Pseudomonas* sp. and two lineages at species level: *P. stewartii* and *B. cenocepacia*.

Conflicts of Interests

The authors have not declared any conflict of interests.

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