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MOLECULAR CHARACTERIZATION AND ANTIMICROBIAL SUSCEPTIBILITY OF MRSA ISOLATED FROM CHRONIC HEMODIALYSIS OUTPATIENTS AND THEIR CORRELATION TO MRSA COLONIZATION AMONG HEALTHCARE WORKERS

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ABSTRACT

The carriage of methicillin-resistant *Staphylococcus aureus* (MRSA) among dialysis patients is remarkable not only in terms of the risks of developing infections, but also in playing a principle part in transmission among dialysis unit staff. The aim of this study was to detect the colonization of Methicillin-sensitive *Staphylococcus aureus* and MRSA carriage. Also, our aim was to determine the relatedness of MRSA isolates and the potential routes of transmission using PCR- Restriction Fragment Length Polymorphism (PCR-RFLP) in Hemodialysis Unit of El Zagazig General Hospital, a tertiary medical center in Sharqia, Egypt. This study was conducted on 150 chronic hemodialysis outpatients and 200 non clinical control samples including environmental and healthcare workers (HCWs). Antibiotic susceptibility by VITEK-2 and disc diffusion, PCR amplification of mecA, pvl and *coa* genes and RFLP-PCR were conducted during the study period. In this study 3.3% of the patients and 3.2% of HCWs colonized with pvl positive MRSA. Fifty percent of MRSA isolates showed a single band PCR product amplification of 810bp fragment corresponding to *coa* gene. Ten distinct MRSA RFLP banding patterns designated as H1-H10 were obtained. The majority of strains belonged to RFLP banding pattern H1 (33.33%).The prevalence of MRSA carriage among hemodialysis patients was 14% and 9.7 % among HCWs with similar polymorphism patterns. The presence of one major *coa* gene type confirmed the occurrence of hospital acquired-associated MRSA.

Keywords: Hemodialysis patients; Healthcare workers; MRSA; *coa* gene; pvl gene; RFLP

INTRODUCTION

Colonization and infection with multi-drug resistant (MDR) strains such as MRSA occurs with increasing frequency in hospitals worldwide (**de Kraker *et al*., 2013)**. High mortality and morbidity rates are associated with MRSA infections, also prolonged length of hospitalization and higher healthcare costs are connected to MRSA infections (**Cosgrove *et al*., 2005)**. Plans to control the spread of MRSA in health care setting require standard data of the characteristics and prevalence of circulating MRSA strains and that can be obtained by active surveillance (**Cirkovic *et al*., 2015**). Hemodialysis (HD) patients may be at increased risk for MRSA infections and colonization (**Johnson *et al*., 2009**).
As HD patients have a multitude of risk factors, including repeated exposure to invasive medical devices and regular contact with healthcare workers, they are hundred-times more susceptible to invasive MRSA infections than the general population (**Lu *et al*., 2008**). Actually, MRSA infections are the subsequent leading cause of death among patients with end-stage renal disease (**National Institutes of Health, 2009**). Asymptomatic colonized patients and HCWs are the main sources of MRSA, in the health care settings. Transmission of MRSA among patients is concomitant to HCWs (**Malini *et al*., 2012**). HCWs are at the boundary between communities and hospitals, thus they act as a route of MRSA transmission (**Albrich *et al*., 2008**). Regular periodic testing, of MRSA positive colonization and decolonizing of the patients, is one of the possible policies to prevent MRSA infections among HD patients This is difficult to determine without strain typing whether a newly identified case is the result of health-care associated acquisition.
PCR-based typing methods, such as fragment length polymorphism (PCR-RFLP) analysis of the coagulase (*coa*) gene, play an important role in epidemiological studies and ideal typing method for MRSA analysis during MRSA outbreaks, because PCR-RFLP is rapid, simple, low in cost and applicable (**Godwin and Choyce 2001; Shittu and Lin 2006**). Typing of *coa* gene of *S. aureus* isolated is a simple, specific, discriminatory, and reproducible method, for that reason it has been used to identify and compare *S. aureus* genotypes (**Vimercati *et al*. 2006)**.
Panton Valentine leukocidin (PVL), is encoded by lukF-PV and lukS-PV genes, produced by some *S. aureus* strains, is one of the significant cytotoxins and responsible for dermal and soft tissue infections (**Genestier *et al*., 2005)**. Limited data available about the epidemiology of Egyptian PVL -positive MRSA strains (**Helal *et al*., 2012 and Hefzy *et al*., 2016)**

The present study aimed to assess the relatedness of MRSA isolates and the potential routes of transmission using PCR- RFLP by examining environmental samples and HCWs nasal swabs in relation to MRSA recovered from patients of Hemodialysis Unit of El Zagazig General Hospital, Sharqia, Egypt. In addition, the presence of PVL staphylococcal virulence factor gene was investigated.

MATERIALS AND METHODS

This study was conducted in the hospital-based outpatient Hemodialysis Unit of El Zagazig General Hospital, a tertiary medical center in Sharqia, Egypt for the period between January 2014 and September 2014. During the study period, 190 clinical specimens were collected from 150chronic hemodialysis outpatients. With regard to the clinical origin, the clinical specimens were isolated from blood(n=63),sputum(n=33),urine (n=30), nasal swabs (n=29), under nail swabs(n=20), purulent discharge(n=12) and skin swabs (n=3).A total of 200 non clinical control samples (Environmental cultures and health care personnel) were collected from the hemodialysis unit as follows; Water samples(n=30) (samples from incoming water tap before reverse osmosis, samples after storage tank), dialysate solution (n=30), effluent tubes of dialysis machines (n=30),beds, bed rails and doors (n=44), health care workers' skin swabs(n=31) , nasal swabs (n=27) and nail swabs (n=8).

Demographic and clinical data

Baseline information and medical data were collected from medical records of patients after signing informed consent form prior to their inclusion in the study. The study protocol was approved from the Ethics Committee of the Faculty of Pharmacy, Al-Azhar University. Descriptive information regarding participants' age, sex, and exposure to antimicrobial treatment and underlying chronic disease were collected.

Identification of isolates

Screening for Gram positive isolates was done using mannitol salt agar (Becton-Dickinson, Sparks, MD) and incubated for 24 h at 35°C and MRSA screen plates (Hardy Diagnostic, NY, USA) used to detect MRSA recovered from environmental samples and HCWs. Methicillin resistance was also detected using a cefoxitin disc (FOX, 30 mcg) on Mueller-Hinton agar plates supplemented with

4% NaCl according to Clinical and Laboratory Standards Institute guidelines (**Patel *et al*., 2014**).
Full identification and susceptibility testing of Gram positive isolates recovered from clinical isolates of patients was done using VITEK 2 (bioMerieux, Marcy l'E´toile, France) automated machine.

Susceptibility testing

Antibiotic susceptibility testing of all isolates was performed by VITEK-2 and modified Kirby Bauer disc diffusion method as recommended by CLSI guidelines (**Patel *et al*., 2014**) using Mueller Hinton agar. The antibiotics used in this study were amikacin (AN, 30 mcg), amoxicillin/clavulanic (AmC, 20/10 mcg), cefepime (FEP, 30 mcg), cotrimoxazole (SXT, 30 mcg),doxycycline (D, 30 mcg), erythromycin (E, 15 mcg), imipenem (IMP, 10 mcg), nitrofurantoin (F, 300 mcg), ofloxacin (OFX, 5 mcg), and rifampin (RA, 5 mcg) (Oxoid ltd., Basin Stoke, Hants, England).

PCR detection of themecA, pvl and*coa* genes

Genomic DNA was extracted from identified MRSA isolates, *S. aureus* ATCC 25923 and *S. epidermidis* ATCC 12228 using QiaAmp Mini DNA kit (Qiagen, Hilden, Germany).PCR was performed to detect mecA and pvl genes. The following specific primers were used; 5′-AAAATCGATGGTAAAGGTTGGC-3′, and 5′-AGTTCTGGAGTACCGGATTTGC-3′ (**Murakami *et al*., 1991)** for amplification of 533bp of mecA gene, whereas 5′-ACACACTATGGCAATAGTTATTT-3′ and 5′-AAAGCAATGCAATTGAT-3′ (**McDonald *et al*., 2005**) primers with amplicon size of 176bp were used for pvl genes detection. The amplification reaction consisted of an initial denaturation step at 94 ˚C for 5 min followed by 35 cycles of denaturation at 95˚C for 30 sec, annealing at 50˚C for 30 sec, extension at 72˚C for 30 min, followed by final extension at 72˚C for 7 min. PCR was performed for *coa* gene detection, using the following primers; 5'-CGAGACCAAGATTCAACAAG-3' and 5'-AAAGAAAACCACTCACATCA-3', **(Himabindu *et al*. 2009)** which were designed to amplify the 3'end hypervariable region containing 81bp tandem repeats of *coa* gene. The amplification reaction consisted of an initial denaturation step at 94 ˚C for 5min, followed by 30cycles of denaturation at 95˚C for 30 sec, annealing at 55˚C for 45sec, extension at 72˚C for 2 min, followed by final extension at 72˚C for 7 min.

PCR-RFLP of the coagulase gene

The 3' end region of the coagulase gene was amplified by PCR and RFLPs of the amplicons were determined by digestion with *HhaI* (New England Biolabs, MA, USA) as described by manufacturer.

Statistical analysis

Data are presented using absolute frequencies and percentages for categorical variables, and mean ± standard deviation for continuous variables. Chi-square test was performed. A p-value of <0.05 was considered indicative of a statistically significant difference.

RESULTS

Among 284 chronic hemodialysis outpatients admitted to the hemodialysis unit at El Zagazig General Hospital from January to September 2014, only 150 patients agreed to participate in the study. Out of 150 chronic hemodialysis patients 139 clinical isolates (60 Gram positive and 79 Gram Negative) were recovered from different 190 clinical specimens. Patients' ages ranged from 23–80 years old with mean of 52.4 ± 11.8 SD with male predominance 81/150 (54%). The patient's clinical history characteristics are summarized in table 1. A total of 60 (31.5.4%) *Staphylococcus* species were isolated from different patient samples of which 21 (35 %) were identified as *S. aureus* whereas 39 (65 %) isolates were *S. epidermidis*. All *S. aureus* isolates were shown to be MRSA by cefoxitin disc diffusion test, and by mecA PCR (Figure 1A). Thirty one Staphylococcus species were recovered from the nails and nasal swabs of HCWs (3 *S. aureus* and 28*S.epidermidis*). MRSA colonization was detected in 14% of the patients (n=150). Whereas, only 3(9.7%) MRSA isolates were identified among HCWs, recovered from nasal swabs (n=27) of HCWs (n=31). MRSA was not detected in any of environmental samples (n=134; water samples, dialysate solution, effluent tubes of dialysis machines, beds, bed rails and doors). The current study revealed that, species of bacteria (n=78) isolated from environmental samples were confined to four types, *Bacillus* species (68 %), S. epidermidis (17.9 %), K. pneumonia (7.7%), and K. oxytoca (6.4 %).
Twenty one MRSA isolates recovered from hemodialysis patients. The highest percentage of MRSA isolates fell in the age group from 40 to 60 years (42.8%) followed by (28.6%) in age group 20–40 years and (28.6%) in 60–80 years. Eleven (52.4%) of MRSA isolates were recovered from male patients (Table 1). The three health care workers age were between 40-60 years and all of them were females.

Table 1 Demographic characteristics and clinical data of 150 chronic hemodialysis patients

Demographic and clinical data	**Patients (n=150)**	**MRSA carrier (n=21)**	**Non MRSA carrier (n=129)**	**Chi^2/P value**
Female, no, (%)	69(46)	10(47.6)	59 (45.7)	0.026/0.872
Male, no, (%)	81(54)	11(42.4)	70 (54.3)	
Age group, no, (%)				
>20-40	36(24)	6 (28.6)	30 (23.3)	1.09 / 0.58
40-60	80(53.3)	9 (42.8)	71(55)	
≥61	34(22.7)	6 (28.6)	28 (21.7)	
Underlying diseases, no, (%)				
Hypertension	113(75.4)	19 (90.5)	104(80.6)	0.309/0.578
Hepatitis C	81(54)	11(42.4)	70 (54)	0.258/0.872
Heart diseases	27(18)	8 (38)	19(14.7)	6.68 /0.009
Diabetes mellitus	40(26.7)	6 (28.6)	34 (26.4)	0.045 /0.831
Respiratory tract infections*	60(40)	12 (57.1)	48 (37.2)	2.99/0.0837
Urinary tract infections	30(20)	0 (0)	30(23.3)	-
Artery and Vein shunt	70(46.7)	8 (38)	62 (48)	0.721/0.396
Underlying Surgery	150(100%)	21(100)	129(100)	-
Smokers	46(30.7)	4 (19)	42 (32.6)	1.55/0.213071
Non-smokers	104(69.3)	17(80.9)	87 (67.4)	1.550 /0.213
Antibiotic exposure*	150(100)	21(100)	129(100)	-
≥3 years undergoing hemodialysis	150(100)	21(100)	129(129)	-

Legend: * ≤3 months before sampling; P-value of <0.05 was defined as statistically significant.

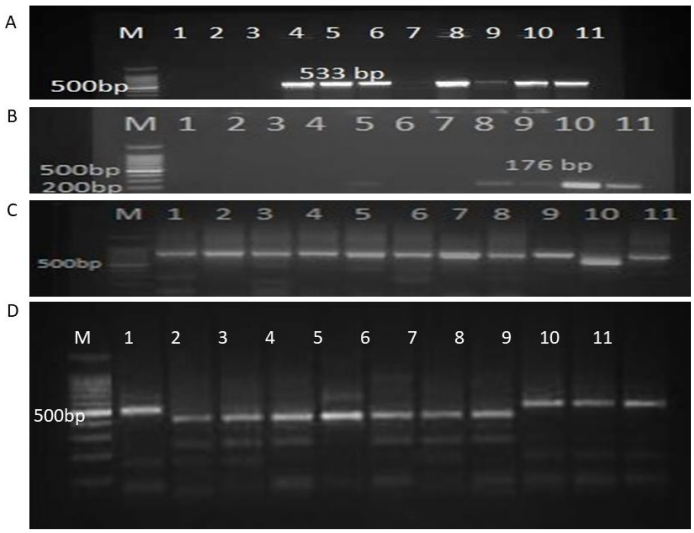

Figure 1 Gel electrophoresis of the PCR amplified products of mecA, PVL and coa genes and RFLP patterns of the coa amplicons after digestion**.** (A) Gel electrophoresis of the PCR amplified products of *mecA* gene with 533 bp amplification fragment. M: 100 bp DNA ladder; lane 1: *S. aureus* ATCC 25923; lane 2: *S. epidermidis* ATCC 12228; lane 3: negative control; lanes 4 to 11: different identified MRSA isolates. (B) Gel electrophoresis of the PCR amplified products of pvl gene with 176 bp amplification fragment. M: 100 bp DNA ladder; lane 1: *S. aureus* ATCC 25923; lane 2: *S. epidermidis* ATCC 12228; lane 3: negative control; lanes 4 to 11: different identified MRSA isolates. (C) Gel electrophoresis of the PCR amplified products of *coa* gene. M: 100 bp DNA ladder; lane 1: *S. aureus* ATCC 25923; lanes 2 to 11: different identified MRSA isolates. (D) *HhaI* restriction enzyme digests of the PCR *coa* gene products. M: 100bp DNA ladder; lanes 1 to 11: different identified MRSA isolates.

Antimicrobial susceptibility

MRSA isolates susceptibility to different antibiotics was tested. The results of antibiotic susceptibility are shown in Table 2. Susceptibility testing on one isolate from each MRSA carrier revealed that twenty (95%) out of 21 Staphylococcus isolates were resistant to both cefepime and erythromycin. Only one (4.8%) isolate showed resistance toward nitrofurantoin. The resistance rates to amoxicllin/clavulanic, doxycycline, amikacin, imipenem, ofloxacin, cotrimoxazole, and rifamycin were 80.8%, 61.9%, 47.6%, 28.6%, 28.6%, 14.3% and 9.5%, respectively. All the MRSA recovered from HCWs were resistant to amoxicillin/clavulanic acid, cefepime, and co- trimoxazole, while, all of them were sensitive to rifampin.

Table 2 Antibiotic susceptibility pattern of MRSA recovered from patients

Antibiotic	**(n=21) Susceptible, no (%)**	**Intermediate, no (%)**	**Resistant, no (%)**
Cefepime	1 (4.8%)	0 (0%)	20 (95.2%)
Co-trimoxazole	3 (14.3%)	15 (71.4%)	3 (14.3%)
Erythromycin	0 (0%)	1 (4.8%)	20 (95.2%)
Nitrofurantoin	16 (76.2%)	4 (19%)	1(4.8%)
Rifampin	16 (76.2%)	3 (14.3%)	2 (9.5%)
Imipenem	12 (57.1%)	3 (14.3%)	6 (28.6%)
Amoxicillin/Clavulanic acid	3 (14.3%)	1 (4.8%)	17 (80.8%)
Ofloxacin	12 (57.1%)	3 (14.3%)	6 (28.6%)
Doxycycline	6 (28.6%)	2 (9.5%)	13 (61.9%)
Amikacin	6 (28.6%)	5 (23.8%)	10 (47.6%)

Presence of PVL gene

Out of 21 MRSA isolates from patients, 5 (32.8%) were pvl gene positive, and out of the three MRSA isolates from HCWs one (33.33%) was pvl gene positive (Figure 1B).

Coagulase gene typing

The *coa* gene amplified products (figure 1 C) ranged from nearly 81bp to 810 bp, and these products showed seven different types of band patterns as shown in Table 3.PCR products of *coa* gene revealed that, most MRSA (11/21; 52.4%) isolated from patients produced a single band of 810 bp fragment followed by double amplicons of 810bp and 648bp (4/21; 19%) and a single pattern of 729 bp (2/21; 9.5%).On the other hand all MRSA recovered from health care worker produced three different banding patterns of *coa* gene that range approximately from 567bp – 810bp.

Table 3 PCR product of mecA, PVL and coa genes and RFLP patterns

Isolate number	Source	MecA gene	PVL gene	*Coa* PCR products (approximate bp)	RFLP pattern
1	Patients	+	-	810	H1
2	Patients	+	-	810	H1
3	Patients	+	-	729	H4
4	Patients	+	+	810	H2
5	Patients	+	-	405, 810	H5
6	Patients	+	+	810	H2
7	Patients	+	-	810	H1
8	Patients	+	-	810	H2
9	Patients	+	-	810	H1
10	Patients	+	-	810	H1
11	Patients	+	-	810	H1
12	Patients	+	-	810 , 81	H6
13	Patients	+	+	648, 810	H3
14	Patients	+	-	648, 810	H3
15	Patients	+	+	648, 810	H3
16	Patients	+	+	729	H4
17	Patients	+	-	810 , 567, 81	H7
18	Patients	+	-	810	H2
19	Patients	+	-	810	H1
20	Patients	+	-	648, 810	H8
21	Patients	+	-	810 ,324, 81	H9
22	HCWs	+	+	648,810	H3
23	HCWs	+	-	810	H1
24	HCWs	+	-	567, 810,	H10

Legend: There was not a significant correlation between PVL carriage MRSA isolated from 21 patients and 3HCWs at $p < 0.05$ (P-value is 0.72158).

***Coa*-RFLP Typing using *HhaI* Restriction Enzyme**

RFLP patterns and genotype frequency are shown in Table 4.Restrictiondigestionwasperformedonthe amplified coagulase PCR products with *HhaI*. Ten distinct RFLP banding patterns (designatedasH1, H2, H3, H4, H5, H6, H7, H8, H9 and H10) were obtained among the 24 MRSA isolates. The produced fragments of amplicon products of *coa* gene generated different quantities of bands, varying from 2 to 5, and their sizes were approximately 81 - 648 bp (figure 1 D). Eight out of 24 (33.3%) isolates studied belongedtoRFLPbandingpatternH1 (one of them isolated from HCWs and the remaining 7 were isolated from patients). Four MRSA isolates belonged to pattern H3; 3 of them were recovered from patients' samples and 1 from HCWs. Only one MRSA isolate belonged to pattern H10 which was recovered from HCWs. All MRSA isolates that belonged to patterns H2, H4, H5, H6, H7, H8 and H9 were isolated from patients.

Table 4 Restricted fragment size patterns of *coa* gene of the MRSA isolates

RFLP pattern (10)	**Size of *HhaI* fragments (approximate bp)**	**24 MRSA isolates (%)**		
		Patient isolates	HCWs isolates	
H1 (3 bands)	162-243-405	7	1	(12.5%)
H2 (3 bands)	81, 243, 486	4	0	(29%)
H3 (4 bands)	81,162-567-648	3	1	(4.2%)
H4 (3 bands)	81, 162, 486	2	0	(20.8%)
H5 (3 bands)	81,405 , 648	1	0	(4.2%)
H6 (4 bands)	81, 162, 243, 405	1	0	(4.2%)
H7 (5 bands)	81, 162, 243, 405, 567	1	0	(4.2%)
H8 (5 bands)	81, 162,243, 324, 648	1	0	(4.2%)
H9 (5 bands)	81, 162, 243,324, 405	1	0	(4. 2%)
H10 (4 bands)	162, 243,405, 567	0	1	(12.5%)

DISCUSSION

The distribution of circulating MRSA among hemodialysis patients admitted to outpatient hemodialysis unit (El Zagazig General Hospital, Sharqia, Egypt) as well as among healthcare workers were studied here. MRSA represented 100% of total *S. aureus* isolates in our study. Nasal swabs were the most common source of recovered MRSA (61.9%). In other Egyptian study by Omar *et al*., MRSA represented 75% of total *S. aureus* isolates and 35 (46.7%) were isolated from respiratory system**(Omar *et al*., 2014)**.

The most common causative agents of peritonitis concomitant with peritoneal dialysis are *S. aureus* and coagulase-negative Staphylococcus, worldwide **(Piraino *et al*., 2005)**. Although, *S. aureus* is coupled with increased chances of hospitalization, the most severe episodes, catheter removal and mortality, the main etiological agent of peritonitis is coagulase-negative Staphylococcus in the world **(Barrett*et al*., 2007; Grothe*et al.*2010)**.

The present study showed that 21 (14%) *S. aureus* and 39(26%) *S. epidermidis* was isolated from the hemodialysis patients (n=150). The antimicrobial resistance rates in the case of *S. epidermidis* isolates are higher than that of MRSA except for Amoxicillin/Clavulanic acid (Table 2).

Close contact between patients and HCWs carrying MRSA (on their skin or in nasal cavity), is the reason of the dissemination of infection in hospitals. Screening of MRSA among HCWs in the hospital is mandatory. HCWs act as the source of infection to their immune-compromised patients, leading to prolog hospitalization **(Malini et al., 2014)**, since HCWs are at the border between communities and hospitals. In our study, nasal and skin swabs were collected from 31 health care personnel who are working at the hemodialysis unit in El Zagazig General Hospital. The estimated prevalence of MRSA (11.1%) recovered from HCWs nasal swabs (n=27) in our study was much lower than that reported in other countries; 12.4% in Libya **(Al-haddad *et al*., 2014)**, 17.5% in India **(Radhakrishna *et al*., 2013)**, 20.8% in West Bank of Palestine **(Kaibni *et al*., 2009)** and 21% in Kuwait **(Dimitrov *et al*., 2003)**. Lower rates (3.4%) were detected in Nepal **(Khanal *et al*., 2015)**.these differences in MRSA carriage could be explained by differences in adherence to infection control measures in each hospital.

Out of 134 environmental samples there were 56 negative bacterial cultures and 87 positive bacterial cultures. Out of 78 positive bacterial cultures, 53 (68%) Gram positive bacterial rods, 14 (17.9 %) Gram positive cocci and 11(14.1 %) Gram negative bacterial rods were isolated Four types of bacterial species were isolated from environmental samples which including *Bacillus* species, *S. epidermidis*, *K. pneumonia*, and K. *oxytoca*. In the current study MRSA was not detected in any of environmental samples. Variants in the detection percentage of MRSA in hospital environment have been described in different studies maybe due to differences in hospital's cleaning/disinfection procedures, patient colonization load, laboratory techniques used and study strategy (**Boye et al., 1997; Boyce et al., 2007; Freeman et al., 2014; Russotto et al., 2015)**.

MRSA typing is required to outline epidemiological trends and infection control policies. The *coa* gene is one of the principal criterion for the characterization of *S. aureus* isolates **(Montesinos *et al*., 2002)**. The 3′ end of *coa* gene contains an 81 bp tandem short sequence repeat series, the number of repeats varies between strains (**Shopsin *et al*., 2000**). The *coa* gene was identified in all MRSA isolates in this study, indicating 100% type ability. In the present study, amplified *coa* gene presented seven PCR types and a single amplified product of 810bp fragment was corresponding to *coa* gene in most of MRSA isolates. Similarly Himabindu and his colleges reported that the majority of isolates belonged to the band class of 810bp **(Himabindu *et al*. 2009)**.Whereas; they showed that the sizes of *coa* gene amplified products were classified into 3 band classes which was different from our findings. A 2014 Egyptian study reported9 different amplicons of the *coa* gene which was quite similar to our findings **(Omar *et al*., 2014)**.There was variability in *coa* gene PCR sizes and bands detected in this study. This may be attributed to the presence of various allelic arrangements of *coa* gene in MRSA, permitting a single strain to induce multiple amplicons **(Goh *et al*., 1992)**.

Our findings showed that after digesting of PCR products with *HhaI*, 2 to 5 bands for each isolate were observed. The size of the bands produced by enzyme digestion was divided into 8 different band classes (81, 162, 243, 324, 405, 486, 567, 648bp) each containing multiples of 81bp tandem repeat units. Ten distinct RFLP patterns were observed based on the number and size of the produced bands (Table 4). The majority of isolates (8/24=33.33%) belonged to RFLP banding pattern H1. Although MRSA colonization was detected in 3 HCWs only, the transmission among HCWs and patients was high. This is evident in our findings as in case of patterns H1 and H3.

PVL is the major virulence factor of *S. aureus*, responsible for necrosis, apoptosis and destruction of white blood cells. .Currently, we reported pvl gene carriage rate of 25% (6/24) among MRSA isolates. The prevalence of pvl gene positive MRSA varies between countries ; 2.7% in Algiers, 4.9 % in UK, 5 % in France, 7% in Taiwan, 8.1 % in Saudi Arabia, 14.3 % in Bangladesh, 17% in Egypt; 64% in India and 100% in Tunisia (**Lina *et al*., 1999; Souza *et al*., 2010;Holmes *et al*., 2005; Moussa *et al*., 2008; Afroz *et al*., 2008; Antri *et al*., 2011; Helal *et al*., 2012; Ben Nejma *et al.*, 2013)**, indicating that the prevalence of PVL genes differs greatly between strains in different geographic areas and inhabitants.

In this study, pvl gene-positive MRSA was clustered under three restriction types (H2, H3 and H4). Only the H3 restriction pvl gene-positive MRSA type was identified between patients and HCWS.

CONCLUSION

There was a low prevalence of MRSA colonization among hemodialysis out patients (14%) and HCWs (9.7%). We found a correlation between MRSA isolated from the out patients and HCWs. We observed heterogeneity among patients and HCWs associated MRSA isolates using coa gene polymorphisms, but only two types were dominant between both patients and HCWs.The molecular typing of MRSA from patients, hospital atmosphere and HCWs, as a routine practice, will help to detect nosocomial spread in a hemodialysis centers, and opens the possibility of a rapid response. The presence of the PVL carriage MRSA possibly reflected the existence of community acquired MRSA which serve as a reservoir for MRSA transmission in hospitals. It is important to stick to infection control measures in order to reduce the spread of infection by MRSA among susceptible individuals.

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REGULAR ARTICLE

EXTRACTION & PURIFICATION OF HEMOLYSIN FROM TOXIN PRODUCING MICROORGANISMS AND ITS EFFECT ON FORENSICALLY IMPORTANT BLOOD AND SALIVA SAMPLES

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ABSTRACT

Examination of Blood and saliva samples in forensic investigation is now become crucial part of judiciary as it can be useful for the identification of an individual. Individual can be identified from such biological samples by blood grouping as well as DNA analysis. In the study isolation of microorganisms was done from different surfaces having highest possibility of presence of biological evidence, Gram staining and morphological characterization of microorganisms was done, the extraction of toxin from toxin producing microorganism which was determined by the zone of hemolysis on blood agar media was done by ammonium sulphate and sodium azide method. Purification was done and confirmation of toxin was done by ditch method, TLC & SDS-PAGE. Effect of toxin on blood was examined by Drabkin's method for determining hemolytic unit. Effect of hemolysin on saliva was examined by starch hydrolysis test as well as loss of an antimicrobial Property of saliva. As the toxin isolated is hemolysin toxin means it degrades the blood, but in the study we observed the effect of the hemolysin toxin on saliva as well. As mentioned in results blood samples loses its ABO blood grouping activity as it got affected by toxin. Saliva was also highly affected by toxin isolated, as the saliva loses the amylase activity and antimicrobial activity as well. The study is significant in cases where blood and saliva may found as crucial evidence. As RBCs and surface antigens got degraded by hemolysin blood as evidence may be undetectable by presumptive test and may give false results. Saliva samples also become untraceable during the presumptive test as loses its amylase activity.

Keywords: Hemolysin, toxin, blood, saliva

INTRODUCTION

Hemolysin toxins are mainly of four types, α-hemolysin, β-hemolysin, γ-hemolysin and δ-Hemolysin. α-Hemolysin is the most characterized virulence factor of S. aureus. Upon binding to the cell surface, α-hemolysin monomers assemble into a homo-heptamer, forming a pre-pore. The pre-pore subsequently transitions to a mature β-barrel trans-membrane pore (**Bhakdi S and Tranum-Jensen J, 1991**), thereby leading to the formation of a 14-Å diameter aqueous channel (**Song L *et al.*, 1996**). This pore allows the transport of molecules smaller than 2 kD (**Menestrina, 1986**). β-hemolysin does not form pores in the plasma cell membrane but instead is a neutral sphingomyelinase hydrolyzing sphingomyelin, which is a plasma membrane lipid. β-hemolysin's enzymatic activity is required for its hemolytic activity (**Huseby *et al.*, 2007; Ira J and Johnston L J, 2008**). The mechanism leading to cytotoxicity is still poorly understood. Sphingomyelin is enriched in lipid-ordered membrane microdomains with high content in cholesterol. Sphingomyelinase treatment of synthetic lipid bilayers leads to aggregation of cholesterol-rich microdomains (**Ira J and Johnston L J, 2008**), suggesting that cell death may result from the modification of host cell plasma membrane fluidity and destabilization of the bilayer structure. Alternatively, cell death might result from the formation of large ceramide-rich signaling platforms. This class of membrane-damaging peptides was identified over 60 years ago with the purification of δ-hemolysin (**Wiseman, 1975**). Three different mechanisms as reviewed by **Verdon *et al.* (2009)** have been proposed to explain its hemolytic activity. Briefly, δ-hemolysin could (i) bind to the cell surface and aggregate to form trans-membrane pores; (ii) bind to the cell surface and affect the membrane curvature, thereby destabilizing the plasma membrane; or (iii) at high concentration, act as a detergent to solubilize the membrane. γ-Hemolysin and, to a lesser extent, LukED are hemolytic to rabbit erythrocytes (**Morinaga N, Kaihou Y, Noda M, 2003**).

Interest in rapid and less invasive diagnostic tests has grown exponentially in the past decade, which has led to extensive research on saliva as a biological fluid for clinical diagnosis (**Sun F and Reichenberger E J, 2014**). Saliva has some advantages compared to blood and urine, two of the most used diagnostic fluids in laboratory setting. Saliva collection is easy and non-invasive requiring relatively simple instructions for collection and it possesses lower protein content, less complexity and varying composition than serum (**Nunes *et al.*, 2011**). The main functions of saliva include protection and integrity maintenance of oral mucosal health through lubrication, buffering action, antibacterial and antiviral activity, and food digestion (**Humphrey S P, Williamson R T, 2001**). Recent proteomic studies have identified and characterized more than 1000 salivary proteins and peptides. Most of these are commonly found in plasma and some are solely produced and secreted by salivary glands having no correlation with blood levels (**Denny *et al.*, 2008**). In Forensic examination blood and saliva may be found as important evidence. Blood and saliva found at crime scenes may be present on the victim, suspect, or any surfaces can be considered significant and treated as such when documenting, collecting, and preserving. Traditionally blood at crime scenes has been documented and collected for identification of suspect or victim through Blood grouping and DNA, at a crime laboratory. Saliva can be useful for identification of an individual as DNA analysis can be done as well as can determine the presence of any poisonous material or drugs. As hemolysin toxin producing microorganisms may be present in any habitat and are of diverse range may be present on crime scene. As blood and saliva are biological fluids, there are chances of contaminations of microorganisms. Study is done to determine the effect of hemolysin toxin on blood and saliva samples and the extent of effect. Once the effect of hemolysin on forensically important blood and saliva samples will be known then false results and wrong identification of an individual can be overcome.

MATERIALS AND METHODS

As we know when saliva and blood found as evidence most of the time found in dried form. As it was not possible to collect samples from real crime scene so as here in study the mimic of crime scene was created in laboratory. Blood and saliva samples are the major requirements for the study. Human blood sample and saliva samples were collected from the volunteers of the Department of Life Science, Gujarat University, Ahmedabad with consent. 20ml of ABO blood was collected by vein blood collection method by expert from four volunteer. Saliva was collected by maintaining aseptic condition and collected 5 ml from each volunteer. To create simulated crime scene collected 20 ml blood and saliva samples were poured on different surfaces, for each surface 2ml blood and 2ml saliva was used. Surfaces used here in study are metal, aluminium, glass & wood which may useful as weapon, apart from these ceramic, tile, stone, soil, paper and fabric materials surfaces used from which possible presence of evidence to be found. Then poured saliva and blood were allowed to dry on different surfaces as to provide same condition as real crime scene.

Blood agar media was prepared for isolation of hemolysin producing microorganisms by using 3 gms of HiMedia™ blood agar powder in 100 ml of sterile distilled water. For the isolation of microbes cotton swabs were dipped in sterile double distilled water and the wet cotton swab was rubbed on different surfaces by following the procedure as followed by forensic investigator from

crime scene and collected in sterile plastic bag and was allowed to dry for further preparation of culture suspension. For swab preparation sterile double distilled water used instead of normal saline as sterile water is also suitable for preservation of bacteria pathogenic to plants or humans (**Liao C H and Shollenberger L M, 2003**). A few instances of much more prolonged survival in water have been recorded but **Ballantyne (1930)** have been unable to find any record of prolonged survival of bacteria in NaCl solution.

Culture suspension was spread on blood agar plates for isolation. The plates were incubated at 37°C. After growth of desired colony on the blood agar plates; these different zone forming colonies were isolated on the blood agar slants (having same composition of blood agar plates) and on the basis of morphological study and nomenclature of individual colony and an activity was determined on the basis of their characteristics of giving hemolytic zone and comparing the diameter of the zone giving colony and the diameter of zone that surrounds a bacterial colony growing on blood agar, which represents partial or complete discoloration or breakdown of Hb. Sterilized Glucose broth flasks were inoculated with selected highly hemolytic five colonies out of fifty five colonies named as: C-SN-1 (ɣ-hemolytic), G-SN-1 (α-hemolytic), P-SN-1 (β-hemolytic), CON-2 (β-hemolytic), and CON-3 (α-hemolytic). Inoculation had been done by adding a loopful of each colony to its respected flasks. All five flasks were incubated at 37°C for 48-72 hours in orbital shaker at 120 rpm.

25ml of inoculated Glucose broths from each flask were collected in REMI centrifuged tubes. Each tube was contained 8ml of inoculated Glucose broths. 0.02% of Sodium azide was added were Centrifuged at 10,000 rpm and at 4°C for 20 minutes. 1/20 volume of TrisHCl buffer was added to supernatant. 60% of solid ammonium sulphate was added to the treated supernatants and incubated at 4°C for overnight. Next day, centrifuged at 10,000 rpm for 30 minutes. Precipitates were treated with 25 ml of 50 mM TrisHCl buffer (pH: 8.0), 1 ml of 1mM EDTA and 1 ml of 3mM sodium azide. Dialyzed all the treated precipitates against the dialyzing buffer and centrifuged at 10,000rpm for 10 minutes. Supernatant was collected which contained crude hemolysin. Folin Lowry estimation was done at 750nm to confirm the presence of protein (**Lowry *et al.*, 1951**).

Figure 1 Precipitations of toxin in centrifugal tubes after treated with ammonium sulfate

For Ammonium sulphate precipitation 6 ml of broth cultures were collected from each five flasks in five centrifuge tubes. 3gm of Solid Ammonium sulphate was added in each tubeand centrifuged at 2500-3000rpm for 5 minutes at room temperature. Then, precipitates were collected as shown in fig.1 and treated with 10 ml Tris HCL buffer (pH-8.0). Then, precipitates were incubated at 4˚C for 24 hours.

For Blood agar ditch method wells were prepared on blood agar plates having 6mm diameter. Then serial dilutions for P-SN-1, C-SN1, and CON-2, CON-3, G-SN-1 were prepared such as, 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4} were added. The plates were incubated at 37°C for 48 hours and examined for zone of hemolysin toxin.

TLC was performed using: 20ml of methanol:water solvent system (70:30) And Butanol:acetic acid:water solvent system (8:2:2). Ninhydrin solution was used as spraying reagent.

SDS PAGE was performed by preparing 12% separating gel and 5% stacking gel. 20 µl of the samples was loaded in sample well. Electrophoresis was done at 200V & 110A. After sufficient run gel was stained overnight and distained next day and observed for protein bands.

Hemolysis assay had been done on the bases of checking hemoglobin concentration released from the lysed RBCs. Released hemoglobin can be measured by Drabkin's method (**Drabkin 1949**). Spectroscopic analysis was done at 540nm and Hemolytic unit was measured.

To determine the effect of toxin on saliva Starch hydrolysis test was performed. Saliva was mixed with different concentrations of toxins as shown in table 1. For standard 300µl saliva was taken into the microfuge tubes. 1000 µl 0.5% starch solution was added to the microfuge tubes. 0 min reading was taken as control by testing it with iodine solution in pitted tiles. Color change was observed at every five minutes interval.

Antibiotic activity loss of saliva was measured by preparing 5 N-agar plates using HiMedia N-agar powder Suspension was made from the PA-14 colony on which saliva was giving antimicrobial activity and was spreadonto each of the 5 plates. Four wells were ditched on each of the plates and werelabeled as 1,2,3,4. Different concentration of the mixture containing saliva and toxin which were incubated at different time were added to the wells as follows:

Table 1 Concentration of Saliva and toxin mixture with respect to time

Well 1 (Toxin/saliva)	Well 2 (Toxin/saliva)	Well 3 (Toxin/saliva)	Well4 (Toxin/saliva)	Incubation period(mins)
1/1 ml	0.5/1 ml	0.5/1.5 ml	0.2/1.5 ml	10
1/1 ml	0.5/1 ml	0.5/1.5 ml	0.2/1.5 ml	20
1/1 ml	0.5/1 ml	0.5/1.5 ml	0.2/1.5 ml	40
1/1 ml	0.5/1 ml	0.5/1.5 ml	0.2/1.5 ml	60
1/1 ml	0.5/1 ml	0.5/1.5 ml	0.2/1.5 ml	75

RESULTS

Sodium azide method has been performed for the extraction of pure hemolysin toxin from the hemolytic microorganisms. Toxin was obtained in a form of precipitation.

TLC plates which gives positive amino acid bands under the U.V illuminations which have different Rf values from each other which separates hemolysin toxin such as P-SN-1 and CON-2 β-hemolysin toxin on the basis of its relative affinity towards stationary and mobile phase.

Table 2TLC results for the toxins show following results:

Distance travelled by Solvent	Culture Extract	Rf Value (cm)
7.0 cm	P-SN-1	3.5
	CON-2	3.6
	CON-3	3.7

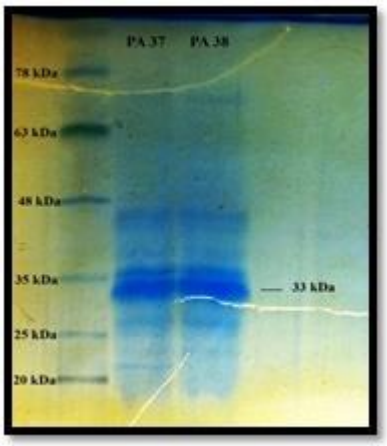

Figure 2 SDS-PAGE analysis for the extracted toxins. Lane: 1 Protein marker, Lane:2 Hemolysin toxin with molecular weight 33 kDa.

As we can see in the figure 2, the bands of the toxin that are toxin 3 and toxin 5 obtained from the different strains in the wells of the SDS-PAGE gel, which are clearly visible after distaining process. And comparing the sample bands with protein ladder gives the bands at 33 kDa size. These bands confirm the presence of the protein that is our crude toxin protein as it shows some light bands above the original band. SDS PAGE analysis confirms the toxin is hemolysin toxin.

As we can see table: 3 Hemoglobinconcentrations is increasing with respect to time interval of 1 hr. So it clearly confirms the hemolytic activity of crude protein collected and gives hemolytic unit 1.

Table 3 Hemoglobin concentrations after reacting with toxin

Tubes	Absorbance at 540nm	Hb. Conc.(mg)
Blank	0.000	00.00
Standard	0.009	11.19
C-SN-1	0.042	24.86
G-SN-1	0.025	31.08
P-SN-1	0.020	52.22
CON-2	0.043	53.46
CON-3	0.034	42.27

Starch hydrolysis of saliva

The salivary amylase enzyme present in saliva digests the starch. On adding starch solution to the saliva as control, it gives color change at the interval of every five minutes as shown in in Fig. 3(A). But on adding starch solution to the mixture of saliva and toxin as sample, the toxin starts reacting with saliva, it degrades the salivary amylase and therefore when it was reacted with iodine solution it gives violet color at the interval of every five minutes. No color change was observed with respect to time interval of five minutes up to 5 hours as shown in Fig. 3(B). Which clearly indicates that Salivary amylase lose its activity after reacting with toxin.

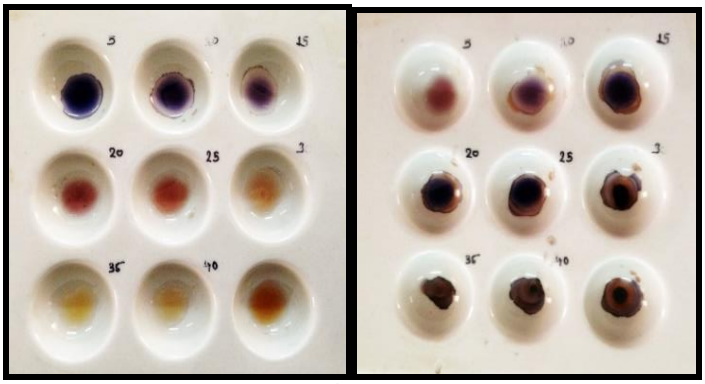

Figure 3 Salivary amylase activity of Control untreated saliva with starch (A) and Sample saliva mixed with toxin and treated with starch solution (B)

Loss of antibiotic activity of saliva

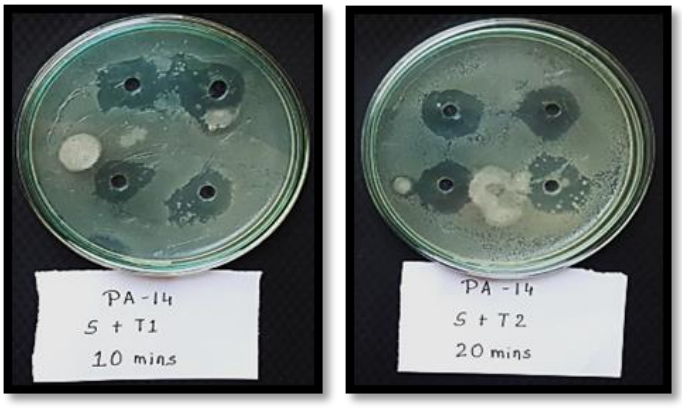

Figure 4 Loss of antibiotic activity of saliva reacting with toxin checked on N-agr media plated with bacterial strain PA14 isolated from biological samples.

As shown in figure 4, when saliva was mixed with toxin and reacted with strain PA-14 on which saliva was giving antimicrobial effect and giving clear zone, after the incubation of 10 minutes, the lysozyme enzyme present in the saliva do not get digested at all by the activity of toxin, therefore strain PA-14 shows the antimicrobial activity against the toxin after 10, 20 and 40mins of incubation. After 60mins of incubation saliva started losing its antimicrobial activity on same strain PA-14 as uneven zones can be seen. , after the incubation of 75 minutes, the lysozyme enzyme present in saliva get digested by the activity of toxin and therefore it does not act on the strain PA-14.

DISSCUSSION

As mentioned above **Bhakdi S and Tranum-Jensen J (1991)** stated that α-hemolysin is pore forming toxin secreted by bacteria which degrade the blood cells by binding on cell surface. **Huseby et al. (2007)** and **Ira J and Johnston L J (2008)** have reported that β- hemolysin is not pore forming but degrades blood cells. None of the study have been reported the effect of hemolysin toxins on saliva samples. The study is unique in a way that it specifies the effect of hemolysin toxin for forensically important blood and saliva samples and how it may interfere during forensic investigation and identification procedure of biological samples.

CONCLUSION

Forensic investigation of blood and saliva has now become a significant part of any crime investigation. Blood and Saliva samples may be very useful evidence to identify an individual. Most of the time these samples found at the crime scene in very small quantity through its potency is very high. If such evidence may be affected by any circumstances then it is difficult to identify individual or person involved in crime. As the blood and saliva are biological fluid and there are chances that it may get affected by any external factors such as environmental conditions or any microbial contamination. The present study was carried out on how toxin producing microorganism can affect the blood and saliva samples. As the mimic of the real crime scene was created, toxin-producing microorganisms

were isolated from surfaces from which saliva may be found from the real crime scene. As the toxin isolated is hemolysin toxin means it degrades the blood, but in the study, we observed the effect of the hemolysin toxin on saliva as well. As mentioned in results blood sample lose its ABO blood grouping activity as it got affected by the toxin. Saliva was also highly affected by toxin isolated, as the saliva loses the amylase activity and antimicrobial activity as well. The study is significant in cases where blood and saliva may found as crucial evidence. As the stains found on the crime scene, looked like saliva, a forensic scientist performs primary identification by amylase activity of starch digestion to recognize that the stain is of saliva. But as per the study, if saliva is affected by the toxin it cannot be recognized by the primary identification method.

ABBREVIATIONS: Not Applicable

Competing interests: The authors declare that they have no competing interests.

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REGULAR ARTICLE

COMPARATIVE STUDY OF THE ANTIBACTERIAL ACTIVITY OF SEAWEED *(Sargassum muticm)* AND FRESHWATER WEED (*Spirodela polyrrhiza)*

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ABSTRACT

Development of new drugs is needed to resist the situation of diseases caused by drug resistant bacteria for public health safety. Natural resource is a big source to find candidates having antibacterial activity and aquatic weed is such a natural resource possessing such activity. The current study was aimed to determine the effectiveness of sea weed (*Sargassum muticum*) and fresh water weed/duckweed (*Spirodela polyrrhiza)* against six bacterial isolates *Klebsiella pneumonia*, *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Pseudomonas luteola* and *Bacillus subtilis.* The potency of methanol and ethanol extracts of these weeds was compared to determine the best candidate of weeds in inhibiting bacteria. Both agar well diffusion method and micro dilution was done to observe the antibacterial activity. Ethanol extract of *Sargassum muticum* worked best against *Pseudomans aeruginosa* (30mm zone of inhibition) and no activity against *Bacillus subtilis*. Methanol extract of the same *Sargassum muticum* showed less activity compared to ethanol extract except for *Bacillus subtilis* where it showed 21mm zone of inhibition. Ethanol and methanol extracts of *Spirodela polyrrhiza* showed less antibacterial activity against the bacteria compared to *Sargassum muticum.* They showed no antibacterial activity against *Klebsiella pneumonia* and *Staphylococcus aureus*. On average, the extracts impart a significant antibacterial activity against these six bacteria which are resistant to several antibiotics. Even one of them (*Escherichia coli*) is resistant to 4th generation cephalosporin but still fairly susceptible for extracts. The antibacterial properties of these marine and freshwater weeds can be subjected to develop new therapeutics to inhibit the resistant bacteria.

Keywords: Seaweed; freshwater weed; resistance; antibacterial activity

INTRODUCTION

Scientific community is searching for alternative components which can fight against antibiotic resistant infectious microorganisms **(Das etal., 2012; Jeyasree et al., 2012).** Their interest is now rising towards the medicinal plants and herbs again to seek for bioactive components for treatment of infections. Plant originated medicines are safer for patients, better bioavailability,no significant side effects with minimal toxicity **(Pradhan et al., 2009; Singh et al., 2010; Ekpo et al., 2011; Thanigaivel et al., 2015;).** Water originated plants contain antimicrobial and phytochemical components like phenols, phycobiline, phenolic compounds, phlorotannins, acrylic acid, halo-genated ketones and alkanes, fatty acids,steroids, flavonoids, terpenoids, cyclic polysulphides, polysaccharide etc. **(Abd El-Baky et al., 2008; Khan et al., 2014; Kavita et al., 2014; Hossain et al., 2018).** Freshwater plants often are used for treating skin diseases and inflammation with potent antioxidant activity. Marine weeds (seaweeds) often impart antibacterial, antiviral, antitumor and antioxidant properties according to different studies **(Abulude et al., 2007; Patra et al; 2008; De Fallcio et al., 2010; Kim et al., 2011; Devi et al., 2011).**They also contain vitamins like A, B1, B12, C, D, E, panthothanic acid, riboflavin, folic acid niacin, including minerals like P, Ca, K, Na **(Prakash et al., 2018).** In the current study, both seaweed (*Sargassum muticum*)and fresh water weed (*Spirodela polyrrhiza*) was subjected to the determination of antibacterial efficacy against several pathogenic bacteria and then their result was compared to understand which weed possesses the better antibacterial activity. *Sargassum muticum*, a brown algae contains higher phlorotannin contents among the marine phenolic compounds which have been found during a study **(Kostić et al., 2012).** Fresh water weed, *Spirodela polyrrhiza* as already been known in the scientific community for its bioremediating ability by removing heavy metal, arsenic as well excess nutrients **(Rahman et al., 2007; Devaleena et al., 2011; Loveson et al., 2013).** So this study was aimed to find out antibacterial activity of both *Sargassum muticum* and *Spirodela polyrrhiza* from marine and freshwater origin and compare their results accordingly Similar studies with *Spirodela polyrrhiza* had also been conducted in India and China **(Das et al., 2010; Qiao et al., 2011; Daboor et al., 2012).**

MATERIAL AND METHODS

Collection of samples

Brown algae (*Sargassum muticum*) was selected to study in the current experiment and was collected from the Saint Martin Island of Bangladesh in South Asia. And *Spirodela polyrrhiza* was selected as a candidate from the freshwater weed which is commonly known as duckweed. Both of these samples were collected in the month of January, 2019 in sterile bags along with some water to make them alive and fresh and taken back to the laboratory as soon as possible. The name of these water weeds were confirmed after close observation of their physical features.

Sample processing

After taking to the laboratory, the samples were washed thoroughly first with tap water and then with distilled water several times to wash out the salt, mud, dirt or any other impurities. After that the samples were shed dried for three to four days to make it all dry followed by blending to get fine powder of these samples. These dried powder samples were then stored in airtight jars until further processing for the assessment of antibacterial activity.

Preparation of extracts

20g of each seaweed and duckweed powdered samples were taken and mixed with 80ml of 95% ethanol and methanol separately in sterilized glass bottles and incubated at 37^{o}C in shaking condition for 48 hours. After 48 hours shaking, the ethanol and methanol extracts of both samples were filtered through sterilized cheesecloth and then through Whatman filter paper respectively. Extracts were then kept in evaporator for evaporation of the alcohol and the concentrates were then collected as stock solution and kept at 4^{o}C until use.

Test organisms

Six different bacterial isolates were collected from different sources to analyze the antibacterial activity of the seaweed and freshwater weed. The bacteria isolates include *Escherichia coli*, *Pseudomonas aeruginosa*, *Pseudomonas luteola*, Klebsiella pheumoniae (collected from clinical laboratory), *Staphylococcus aureus* (collected from the collection of clinical freeze dried laboratory isolates from Department of Microbiology, Stamford University Bangladesh), *Bacillus subtilis* (collected from environmental soil sample). All the microorganisms were biochemically identified by standard biochemical tests.

Antibiotic susceptibility of the tested organisms

Susceptibility of the bacterial isolates to the antibiotics was determined by agar disc-diffusion method called the Kirby Bauer method. Antibiotics used in this study included 25 antibiotics like Amikacin 30 µg.disk^{-1}, Cefepime 30 µg.disk^{-1},

Gentamycin 10 µg.disk^{-1}, Colistin 10 µg.disk^{-1}, Nitrofurantoin 100 µg.disk^{-1}, Cephradine 30 µg.disk^{-1}, Ceftriaxone 30 µg.disk^{-1}, Rifampin 5 µg.disk^{-1}, Novobiocin 30 µg.disk^{-1}, Nalidixic Acid 30 µg.disk^{-1}, Amoxicillin 30 µg.disk^{-1}, Ampicillin 10 µg.disk^{-1}, Cefepime 30 µg.disk^{-1}, Cefoperazone, Tigecycline, Piperacillin/Tazobactam 100/10 µg.disk^{-1}, Meropenem 10 µg.disk^{-1}, Imipenem 10 µg.disk^{-1}, Ciprofloxacin 5 µg.disk^{-1}, Trimethoprim/Sulfamethoxazole, Entrapenem 10 µg.disk^{-1}, Cefpodoxime 30 µg.disk^{-1}, Neomycin 30 µg.disk^{-1}, Erythromycin 15 µg.disk^{-1}, Tetracycline 30 µg.disk^{-1}. A suspension of *Escherichia coli*, *Pseudomonas aeruginosa*, *Pseudomonas luteola*, Klebsiella pheumoniae, *Staphylococcus aureus*, *Bacillus subtilis* were prepared after standardizing with 0.5 McFarland solution for the study. Lawn of the bacterial suspension was prepared using sterile cotton swab evenly over the entire surface of Mueller-Hinton agar plates separately for each bacteria. Using sterile forceps antibiotic discs were placed aseptically over the surface of the inoculated plates and incubated at 37ºC for 8 hours. After incubation the plates were examined for the presence of the zones of inhibition and measured in mm.

Antibacterial activity of the extracts

Bacterial suspensions were prepared by inoculating the isolates into normal saline and incubated at 37ºC. The cultures were ready when they matched with the McFarland turbidity standard (10^8 CFU/ml) (Jorgensen et al.,1999). Bacterial lawn was prepared on the Muller Hinton agar media using sterile cotton swab separately for each kind of bacteria. Ethanol and methanol extracts of *Sargassum muticum* and Spirodelapolyrrhiza placed over the media. 10 µl, 30 µl extracts (impregnated in sterile discs), 50 µl and 100 µl extracts (in well on the media) were used for antibacterial study. Plates were then kept in refrigerator for better absorption for 20 to 30 minutes in upright position and then incubated at 37ºC for 24 hours. After incubation plates were observed for the presence of zone of inhibition and measured in mm.

Determination of MIC (Minimum Inhibitory Concentration)

Minimum inhibitory test was done using 96 well plates. Nutrient broth was taken as base for dilution. At first 100 µl broth was added equally in wells from 1 to 11. From well number 2 to 11, extracts were added sequentially from 10 µl to 100 µl. Number 1 well was kept free of any extract solution to compare the growth of bacteria without any extracts as positive control. After that, 100 µl of bacterial suspension was added equally in all the wells from 1 to 11. Ethanol and methanol extracts were applied in two separate 96 well plates and for these two extracts five bacterial suspensions (*Escherichia coli*, *Pseudomonas aeruginosa*, *Pseudomonas luteola*, Klebsiella pheumoniae, *Staphylococcus aureus*, *Bacillus subtilis*) were inoculated into the wells of separate rows. The plates were then covered and incubated at 37ºC for 24 hours. In each plate different concentrations (10 µl to 100 µl) of the extracts were used for all of the five bacteria. After incubation our aim was to determine the well where no visible growth of bacteria was found after comparing with the growth of bacteria in well 1.

Determination of MBC (Minimum Bactericidal Concentration)

For minimum bactericidal concentration, loop full sample from the wells starting from the MIC concentration to the last well (well no. 11) were taken and streaked over nutrient agar media. After 24 hours incubation at 37ºC, the presence of the growth of bacteria on the streaking line was observed. The concentration of extract where no growth bacteria was first appeared can be determined as the minimum bactericidal concentration.

RESULTS

Bacteria from different origin were subjected to know the effects of the extracts on both clinical pathogenic bacteria and environmental bacteria (Table 01).

Table 01 Biochemical identification of bacteria collected from different sources.

Test	*Pseudomonas aeruginosa*	*Klebsiella pneumoniae*	*Pseudomonas luteola*	*Escherichia coli*
Gram negative bacteria				
APPA	-	-	-	-
H_2S	-	-	-	-
BGLU	(-)	+	-	-
ProA	+	+	+	-
SAC	-	+	-	-
ILATk	+	+	+	+
GlyA	+	+	-	-
O129R	+	+	+	ND
ADO	-	+	-	-
BNAG	-	-	-	-
dMAL	-	+	-	+
LIP	-	-	-	-
dTAG	-	-	-	-
AGLU	-	-	-	-
ODC	-	-	-	+
GGAA	+	-	-	ND
PyrA	+	+	-	-
AGLTp	-	(+)	-	-
dMAN	-	+	-	+
PLE	-	+	-	-
dTRE	-	+	-	+
SUCT	+	+	+	+
LDC	-	+	-	+
IMLTa	+	+	+	ND
IARL	-	-	-	-
dGLU	+	+	+	+
dMNE	+	+	+	+
TyrA	+	+	+	-

Test	*Bacillus subtilis*	*Staphylococcus aureus*
Gram positive bacteria		
APPA	-	-
H_2S	-	-
BGLU	-	-
ProA		+
SAC	+	+
ILATk	+	+
NAG	ND	+
O129R	ND	+
NOVO	ND	-
LAC	-	-
dMAL	ND	+
BGURr	ND	-
AGLU	-	-
dGAL	ND	+
dRIB	+	-
PyrA	-	+
dRAF	-	-
dMAN	-	+
dXYL	-	ND
dTRE	+	+
dMNE	-	+
TyrA	ND	-
URE	+	-
AGAL	-	-
BGAL	+	-
dSOR	-	-
PHOS	+	+
BGUR	+	-

CIT	+	+	+	-
NAGA	-	+	-	-
IHISa	-	+	+	-
ELLM	-	-	-	ND
dCEL	-	+	-	-
GGT	+	+	+	-
BXYL	-	+	-	-
URE	-	+	-	-
MNT	+	+	+	-
AGAL	-	+	-	+
CMT	+	-	+	+
ILATa	+	+	+	ND
BGAL	-	+	-	+
OFF	-	+	-	+
BAlap	+	ND	ND	-
dSOR	-		-	+
5KG	-	+	-	+
PHOS	-	-	-	-
BGUR	**(-)**	-	-	-

ADONITOL=ADO, L-Pyrrolydonyl-ARYLAMIDASE=PyrA, L-ARABITOL=IARL, D-CELLOBIOSE=dCEL, BETA-GALACTOSIDASE=BGAL, H_2S production=H_2S, BETA-N-ACETYL-GLUCOSAMINIDASE=BNAG, GlutamylArylamidasepNA=AGLTp, D-GLUCOSE=dGLU, GAMMA-Glutamyl-TRANSFERASE=GGT, FERMENTATION/GLUCOSE=OFF, BETA-GLUCOSIDASE=BGLU, D-MALTOSE=dMAL, D-MANNITOL=dMAN, D-MANNOSE_dMNE, BETA-XYLOSIDASE=BXYL, BETA-Alanine arylamidasepNA=BALap, L-Proline ARYLAMIDASE=ProA, LIPASE=LIP, PALATINOSE=PLE, Tyrosine ARYLAMIDASE=TyrA, UREASE=URE, D-SORBITOL=dSOR, D-TAGATOSE=dTAG, D-TREHALOSE=dTRE, CITRATE(SODIUM)=CIT, MALONATE=MNT, 5-KETO-D-GLUCONATE=5KG, L-LACTATE alkanization=ILATk, ALPHA-GLUCOSIDASE=AGLU, SUCCINATE alkanization=SUCT, Beta-N-ACETYL-GALACTOSEAMINIDASE=NAGA, ALPHA-GALACTOSIDASE=AGAL, PHOSPHATASE=PHOS, Glycine ARYLAMIDASE=GlyA, ORNITHINE DECARBOXYLASE=ODC, LYSINE DECARBOXYLASE=LDC, L-HISTIDINE assimilation=lHISa, COUMARATE=CMT, BETA-GLUCORONIDASE=BGUR, O/129 RESISTANCE (comp. vibrio.)=O129R, Glu-Gly-Arg-ARYLAMIDASE=GGAA, L-MALATE assimilation=lMLTa, L-LACTATE assimilation=lLATa, D_XYLOSE=dXYL, BETA GLUCORONIDASE=BGURr, D-GALACTOSE=dGAL, LACTOSE=LAC, N-ACETYL-D-GLUCOSAMINE=NAG, NOVOBIOCIN RESISTANCE=NOVO, D-RAFFINOSE=dRAF, D-TREHALOSE=dTRE, D-RIBOSE=dRIB.
ND= Not done

To know the antibiotic susceptibility towards the commonly prescribed antibiotics, Kirby-Bauer antibiotic susceptibility test was performed. 25 antibiotics from different groups were selected for antibiotic susceptibility test of the six selected bacterial isolates. For each bacterium separate antibiotics were used upon the availability of antibiotics. Amikacin, Cefoperazone/Sulbactam, Imipenem, Piperacillin/Tazobactam, Meropenem antibiotics were tested for four isolates among six and showed to be effective against all of the four isolates. Gentamicin was effective for all of the isolates. Cefpodoxime, Neomycin, Tetracycline, Erythromycin were used only for environmental and laboratory isolates *Bacillus subtilis* and *Staphylococcus aureus* and found to be positive in producing clear zone of inhibition. *Bacillus subtilis* and *Staphylococcus aureus* were also susceptible for Cephradine, Rifampicin, Ampicillin. The pathogenic isolates *Pseudomonas aeruginosa* (Tigecycline), *Pseudomonas luteola* (Colistin), *Klebsiella pneumonia*(Ampicillin), *Escherichia coli* (Cefepime, Nalidixic acid, Ceftriaxone, Ciprofloxacine, Cefuroxime) showed resistance to various antibiotics. Among them only *Escherichia coli* showed resistance against five antibiotics which include 3rd and 4th generation cephalosporins (Table 02).

Table 02 Antibiotic susceptibility test of the bacterial isolates.

Antibiotics	**Group of antibiotic**	***Escherichia coli***	***Bacillus subtilis***	***Pseudomonas aeruginosa***	***Pseudomonas luteola***	***Klebsiella pneumoniae***	***Staphylococcus aureus***
Nitrofurantoin (100 µg)	Macrobid	S	-	-	-	S	-
Cefepime (30µg)	Cephalosporins (4th)	R	-	S	S	S	-
Gentamycin (10µg)	Aminoglycosides	S	S	S	S	S	S
Piperacillin/Tazobactam (100/10µg)	Piperacillin/βlactamase inhibitor	S	-	S	S	S	-
Cefuroxime (30µg)	Cephalosporins (2nd)	R	-	-	-	S	-
Cephradine (30µg)	Cephalosporins	-	S	-	-	-	S
Colistin (10µg)	Polymixins	S	-	S	R	S	-
Amoxicillin (30µg)	Aminobenzyl penicillin	S	-	-	-	S	-
Amikacin (30µg)	Aminoglycosides	S	-	S	S	S	S
Ampicillin (10µg)	Aminobenzyl penicillin	-	S	-	-	R	S
Meropenem (10µg)	Carbapenems	S	-	S	S	S	-
Ertapenem (10µg)	Carbapenems	S	-	-	-	S	-
Cefoperazone/Sulbactam (75/30µg)	βlactamase inhibitor	S	-	S	S	S	-
Trimethoprim/Sulfamethoxazole	Trimethoprim/Sulfonamide	S	-	-	S	S	-
Ciprofloxacin (5µg)	Quinolones (2nd)	R	-	S	S	S	-
Imipenem (10µg)	Carbapenems	S	-	S	S	S	-
Neomycin (30µg)	Amynoglycoside	-	S	-	-	-	S

Tetracycline (30µg)	Tetracyclines	-	S	-		-	S
Rifampicin (5µg)	Ansamycins	-	S	-	-	-	S
Ceftriaxone (30µg)	Cephalosporins (3rd& 4th)	R	-	-	S	S	-
Erythromycin (15µg)	Macrolides	-	S	-	-	-	S
Cefpodoxime (30µg)	Cephalosporins (3rd& 4th)	-	S	-	-	-	S
Tigecycline (15µg)	Glycylcyclines	S	-	R	S	S	-
Nalidixic Acid (30µg)	Fluoroquinolones (1st)	R	-	-	-	S	-
Novobiocin (30µg)	Aminocoumarin	-	S	-	-	-	-

Antibacterial activity of seaweed (*Sargassum muticum*) against *Escherichia coli*, *Pseudomonas aeruginosa*, *Pseudomonas luteola*, Klebsiella pheumoniae, *Staphylococcus aureus*, *Bacillus subtilis* were determined. Four different concentrations were used to find visible clear zone. 10µl, 20 µl suspension was absorbed into sterile filter paper and soaked on media. 50 µl, 100 µl suspension was added inside the well made on the media. For *Sargassum muticum*ethanol extracts showed better effectiveness than methanol extraction (Figure 01). For ethanol extraction, 10µl extract showed no activity and 20 µl extract showed a little activity against *Pseudomonas luteola*but the activity increased as the concentration rose to 50 µl, 100 µl. *Klebsiella pneumonia* was showed very little zone of inhibition with 100 µl extract whereas *Pseudomonas aeruginosa*and Staphylococcus aureu shoed moderate activity at 100 µl extract. On the other hand, for methanol extraction, *Pseudomonas luteola* showed no inhibition at all and slightly inhibition occurred for *Escherichia coli* and *Staphylococcus aureus*. *Pseudomonas aeruginosa* and *Bacillus subtilis* showed moderate inhibition and *Klebsiella pneumonia* showed the best results for highest inhibition among all the bacteria against methanol extraction (Table 03).

Table 03 Antibacterial activity of *Sargassum muticum* against selected bacterial isolates.

Bacterial isolates	**Ethanol extract**				**Methanol extract**			
	10 µl	**20 µl**	**50 µl**	**100 µl**	**10 µl**	**20 µl**	**50 µl**	**100 µl**
Pseudomonas luteola	-	+	++	+++	-	-	-	-
Pseudomonas aeruginosa	-	-	-	++	-	-	-	++
Escherichia coli	-	-	-	-	-	-	-	+
Staphylococcus aureus	-	-	+	++	-	-	-	+
*Klebsiella pneumonia*e	-	-	-	+	-	-	-	+++
Bacillus subtilis	-	-	-	-	-	-	-	++

Antibacterial activity of freshwater weed (*Spirodela polyrrhiza*) against thee similar bacterial isolates were determined. For *Spirodela polyrrhiza* ethanol and methanol extracts showed similar effectiveness like *Sargassum muticum*. *Pseudomonas luteola* showed no zone of inhibition against any extracts. Both extracts had only slight and moderate antibacterial activity against *Bacillus subtilis* and *Escherichia coli* respectively with 100 µl concentration. Ethanol and methanol extract showed moderate and low antibacterial activity against *Pseudomonas aeruginosa* at 100µl concentration (Table 04).

Table 04 Antibacterial activity of *Spirodela polyrrhiza* against selected bacterial isolates by agar well diffusion.

Bacterial isolates	**Ethanol extract**				**Methanol extract**			
	10 µl	**20 µl**	**50 µl**	**100 µl**	**10 µl**	**20 µl**	**50 µl**	**100 µl**
Pseudomonas luteola	-	-	-	-	-	-	-	-
Pseudomonas aeruginosa	-	-	-	++	-	-	-	+
Escherichia coli	-	-	-	++	-	-	-	++
Staphylococcus aureus	-	-	-	-	-	-	-	-
Klebsiella pneumonia	-	-	-	-	-	-	-	-
Bacillus subtilis	-	-	-	+	-	-	-	+

After identifying the antibacterial activity of *Sargassum muticum*and *Spirodela polyrrhiza* against some pathogenic and environmental bacteria, the study aimed to determine the MIC and MBC of both ethanol and ethanol extracts of the aquatic weeds from both marine and freshwater origin. Apparently it can be seen from the table that *Sargassum muticum* has the ability to lower the growth of bacteria to stop visible growth both with ethanol and methanol extraction. *Pseudomonas luteola* (ethanol- 30 µl, methanol- 80 µl) showed the MIC in lowest concentration for ethanol extracts. Other isolates showed MIC at higher concentrations. MBC was found with both extracts only for *Escherichia coli* (methanol- 100 µl) and *Pseudomonas aeruginosa* (ethanol-90 µl, methanol-100µl). *Staphylococcus aureus* was unable to be killed with any of these extracts. *Bacillus subtilis* and *Klebsiella pneumonia* were killed with methnol extract at 70 µl and 100 µl respectively. In case of *Spirodela polyrrhiza*, *Staphylococcus aureus* showed no MIC and MBC within the range of concentrations of the extracts used in the study (10 µl- 100 µl). Ethanol and methanol extracts showed MIC and MBC for *Pseudomonas aeruginosa*, *Pseudomonas luteola* and *Escherichia coli*only. They were observed only to inhibit (90 µl) the growth of *Klebsiella pneumonia* and *Bacillus subtilis* (Table 05).

Table 05 MIC and MBC of *Sargassum muticum* and *Spirodela polyrrhiza* against different bacteria.

Bacteria	Solvents	*Sargassum muticum* extracts		Spirodelapolyrrhiza Extracts	
		MIC	MBC	MIC	MBC
Pseudomonas luteola	Ethanol	30 µl	100 µl	70 µl	90 µl
	Methanol	80 µl	-	90 µl	100 µl
Pseudomonas aeruginosa	Ethanol	70 µl	90 µl	60 µl	80 µl
	Methanol	90 µl	100 µl	40 µl	60 µl
Escherichia coli	Ethanol	-	-	60 µl	90 µl
	Methanol	100 µl	-	50 µl	80 µl
Staphylococcus aureus	Ethanol	80 µl	-	-	-
	Methanol	90 µl	-	-	-
Klebsiella pneumonia	Ethanol	90 µl	-	-	-
	Methanol	70 µl	100 µl	90 µl	-
Bacillus subtilis	Ethanol	90 µl	-	-	-
	Methanol	60 µl	70 µl	90 µl	-

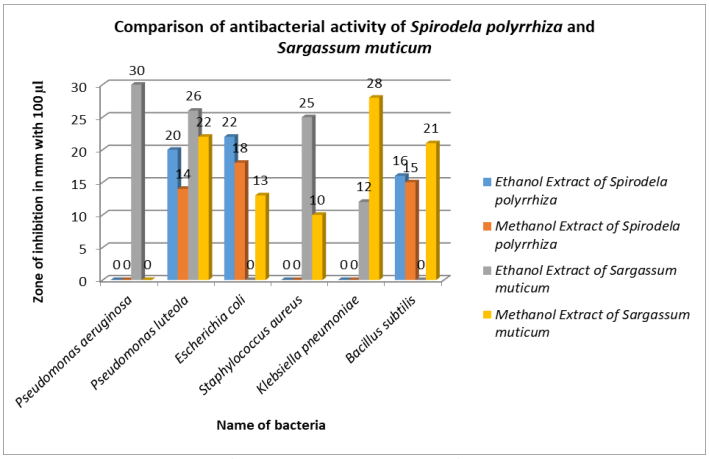

Figure 01 Comparison of antibacterial activity of *Spirodela polyrrhiza* and *Sargassum muticum*

DISCUSSION

As antibiotic drugs are getting to a situation where many pathogenic bacteria have become resistant, alternative resource is needed to combat such infectious pathogens with abundant, cost effective and consumer safe antibacterial products. With the same point of view, candidates of aquatic weeds (because of their high availability) from marine and fresh water region were chosen to investigate such properties.

Firstly, we biochemically confirmed the collected bacteria (*Escherichia coli*, *Pseudomonas aeruginosa*, *Pseudomonas luteola*, Klebsiella pheumoniae, *Staphylococcus aureus*, *Bacillus subtilis*) and detected the antibiotic susceptibility of them toward different antibiotics. Here we have observed *Escherichia coli*was resistant to few 2nd, 3rd and even 4th generation cephalosporin drug with resistance to 1st generation Fluoroquinolones. Other bacterial isolates were somewhat sensitive towards the antibiotics we used for them with resistance towards a few antibiotics. From this part of study of understood the risk with infectious *Escherichia coli* which showed higher degree of resistance, and there is a need to discover newer agents to inhibit them. Environmental laboratory freeze dried isolates showed sensitivity toward the antibiotics we used for them. As they have not been encountered with antibiotics before, they have not started to get the resistance from other drug resistant isolates yet.

After studying the antibiotic resistance, we further attempted to determine the antibacterial activity of ethanol and methanol extracts of *Sargassum muticum* and *Spirodela polyrrhiza* towards those bacteria. *Pseudomonas aeruginosa*, Pseudomonas lutiola, *Staphylococcus aureus* showed satisfactory results with ethanol extracts of *Sargassum muticum* and *Pseudomonas aeruginosa*, *Klebsiella pneumonia*, *Bacillus subtilis* showed good results with methanol extracts of *Sargassum muticum*. As like *Escherichia coli* was most resistant to the advanced antibiotics, it showed little sensitivity to methanol extract of *Sargassum muticum*. Aqueous extract as well as raw extracts can be further studied to get the complete picture of its activity to *Escherichia coli* as it is of utmost importance to get an a alternative drug. Other isolates are satisfactorily sensitive to the extracts though they impart some resistance towards few antibiotics. So *Sargassum muticum* can also be an alternative drug of choice against *Pseudomonas aeruginosa*, Pseudomonas lutiola, *Staphylococcus aureus*, *Klebsiella pneumonia*, *Bacillus subtilis*.

Similarly, the antibacterial activity of both ethanol and methanol extracts of *Spirodela polyrrhiza* was quite satisfactory for *Pseudomonas aeruginosa*, *Bacillus subtilis* and *Escherichia coli*. It is great to know the extracts of the freshwater weed (duckweed) possess antibacterial activity to three of the common bacterial pathogens including one bacteria (*Escherichia coli*) with resistance to 4th generation cephalosporins. This finding definitely could be a great opportunity to the pharmaceutical industries to take initiatives for production of new drug of choice for resistant pathogenic bacteria (*Escherichia coli*) enabling to keep the public health safe by lessening mortality and morbidity rate by the infection of multi drug resistant *Escherichia coli*.

The MIC and MBC test for the isolates with ethanol and methanol extracts of *Sargassum muticum* and *Spirodela polyrrhiza* represents similar results like agar well diffusion test. During this part of study we determined the MIC and MBC to determine the dosage of the extracts to inihibit the visisble growth as well as to kill the bacteria. For ethanol and methanol extracts of those two aquatic weeds, higher concentrations were observed (80µl-100µl). *Bacillus subtilis* and *Pseudomonas aeruginosa* ware inhibited at 60µl and 70µl respectively, comparatively lower concentrations than others.

CONCLUSION

Modern age is facing problem to combat diseases using antibiotics as many pathogenic bacteria have become multidrug resistant to the most advanced antibiotics. As a consequence, the pathogens are able to cause life threatening conditions which were before very easy to treat with antibiotic use. So alternatives or new drugs are necessary to treat infected people with such resistant bacteria. In our current study we observed that *Sargassum muticum* and *Spirodela polyrrhiza* have the ability kill some of such bacteria which are pathogenic and also have some resistance to some antibiotics. The most significant result was found for *Escherichia coli* (having resistance to 4th generation antibiotics) towards *Spirodela polyrrhiza* extracts and little activity towards *Sargassum muticum*. This breakthrough information can be aimed to develop new drugs for treating 4th generation cephalosporin resistant bacteria.

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REGULAR ARTICLE

CORRELATION BETWEEN CLASSROOM POPULATION, VENTILATION BACTERIAL LOADS AND THIER ANTIMICROBIAL PATTERNS IN SCHOOLS WITHIN IKOT EKPENE, NIGERIA

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ABSTRACT

Indoor air of classroom in eight schools (4 nursery; NS1, NS2, NS3 and NS4, and 4 secondaries; SS1, SS2, SS3 and SS4) within Ikot Ekpene, Akwa Ibom State, Nigeria, were analyzed at ambient and populated sampling conditions using natural sedimentation on nutrient agar medium. The results revealed varying ventilation patterns in each of the classrooms, and the following airborne bacterial counts; NS1 (16.6 cfu/m^3), NS2 (13.3 cfu/m^3), NS3 (23.3 cfu/m^3), NS4 (33.3 cfu/m^3), SS1 (6.6 cfu/m^3), SS2 (6.6 cfu/m^3), SS3(28.3 cfu/m^3) and SS4 (15 cfu/m^3) at ambient sampling and 40 cfu/m^3, 41.6 cfu/m^3, 58.3 cfu/m^3, 68.3 cfu/m^3, 6.6 cfu/m^3, 31.6 cfu/m^3, 56.6 cfu/m^3 and 25 cfu/m^3 respectively at populated sampling. Bacterial isolates identified were *Lactobacillus, Staphylococcus, Bacillus, Rothia, Kurthia, Corynebacterium, Pseudomonas, Brevibacterium,* and *Flavobacterium.* Statistical analysis of the results revealed negative relationships between class area and aerobic plate counts ($p>0.05$), class population and aerobic plate count ($p>0.805$), and significant increase in aerobic plate counts at populated conditions over that at ambient conditions ($p<0.05$). The results therefore point to the dimensions of classrooms, ventilation and population of the classrooms as important factors in determining the bacterial air quality, and invariably affecting the health condition of students.

Keywords: indoor air, bacteria, contamination, health, antibiotic susceptibility

INTRODUCTION

Air serves as a key route and major source of human microbial exposure **(Jones, 1999)**. The knowledge of microbial air contamination is therefore an important criterion for the assessment of hygiene conditions of indoor air quality **(Nandasena *et al.*, (2010)**. In modern societies, people spend over ninety per cent of their time indoors such as in schools and classrooms, constantly exposing them to contents present in this primary habitat which include inhalable microbes **(Flynn *et al.*, 2000).** Microbial causative agents of adverse health conditions have been documented in aerosols of different indoor built environment such as schools and such agents can be transmitted between individuals in close proximity **(Wargocki and Wyon, 2006)**, including methicillin-resistant *Staphylococcus aureus* (MRSA) **(Gehring *et al.*, 2010).**
Researchers such as **Dunn *et al.* (2013)** have observed that microbial communities are vastly different between different types of indoor environments such as schools, houses and hospitals, even different rooms within the same building (e.g. bedroom vs. bathroom) exhibit distinct microbiomes. Air quality in classrooms is of special concern, since students spend a lot of time indoors, which potentially exposes them to many contaminants present in the air **(Jones, 1999).** Studies done on school indoor environments when compared to that of other building, show heightened health risks due to low funding for operation and maintenance of facilities **(Wargocki and Wyon, 2006; Zhao *et al.*, 2008)**. Environmental problems may be more pronounced in school buildings in poor and developing countries due to the perennial problems of overcrowding, low funding, age of buildings, materials employed in building construction (e.g. asbestos and lead), as well as ventilation patterns **(Espejord, 2000).**
The main concern about microbial growth in classroom indoor environments is related to the strong link to the adverse health effects on the occupants **(Douwes *et al.*, 2003)**. Indoor air pollutants might increase the chance of both long and short-term health problems among pupils and staff, reduce the productivity of teachers and degrade the pupils learning environment and comfort **(Shaughnessy *et al.*, 2006).** Hence there is also need to further characterize microbes in classroom indoor environment, and assess their antimicrobial sensitivity. This work is focused on studying the correlation between bacteriological air quality in schools within Ikot Ekpene and classroom population and ventilation pattern.

MATERIALS AND METHODS

Sampling location

Classroom atmosphere in four (4) nursery schools (labeled NS1, NS2, NS3 and NS4) and four (4) secondary schools (labeled SS1, SS2, SS3 and SS4) within Ikot Ekpene metropolis were sampled. A single classroom was sampled in all schools.
Table 1 and Figure 1 show the characteristics of the eight school buildings. All buildings were single floor buildings except NS2 and SS2 which were located in a four-storey building with classrooms sampled located on the ground floor. Classroom sampled in NS1 was located on the upper floor of a two-storey building, and classroom at NS3 was located on the ground floor of a two-storey building
All schools were brick with mortar-surfaced walls. Most schools had wooden windows which could be opened; NS2 and SS2 were in a building installed with sliding aluminum windows. SS1 and SS3 had no windows in place, although window frames had been installed. Additionally, SS1 classroom had no ceiling boards in place. None of the schools had any form of air handling system.

Sample collection

The air of classroom atmosphere of seven selected schools was sampled at different time intervals; ambient samples were obtained between 6-7.30 am, while samples under populated conditions were taken between 10 and 12 pm (while students were in class). Plates were set up at a height of 1.5 m above floor level, representing the normal human breathing zone **(Obbard and Fang, 2003).** Sampling was done by natural sedimentation; nutrient agar plates prepared and preserved overnight in the refrigerator at 4 ^{o}C were exposed in air in each of the classroom for 5 minutes (to avoid drying of the agar surface and overloading of the collection plate **(Stetzenbach *et al.*, 2004)** and then covered, labeled.

Bacteriological analysis of classroom atmosphere

Replica plates of nutrient agar plates were incubated at 37 °C for 48 hours to allow the growth of aerobic bacteria. Emerging visible discrete colonies of bacteria were enumerated and subcultured in fresh nutrient agar medium and incubated. Pure colonies so obtained were stocked on nutrient agar slants and incubated at 30 ^{o}C for 24 hours and were then preserved in the refrigerator at 4 ^{o}C for further tests. Bacterial colonies were initially characterized by morphology and microscopic appearance, and identified further by biochemical tests. Isolates were identified based on comparison of biochemical and physiological characteristics with that of known taxa in the Bergey's Manual of Systematic Bacteriology **(Holt *et al.*, 1994).**

Statistical analysis

The total number of colony forming units (cfu) enumerated was converted to organisms per cubic meter of air (cfu/m^3). The data were processed with SPSS and statistically significant differences were determined by one-way and two-way analysis of variance (ANOVA). P-value less than 0.05 was considered statistically significant.

Antibiotic susceptibility testing

The antibiotic susceptibility profile of the bacterial isolates was determined using the disk diffusion method on Mueller-Hinton agar according to the methods of Chessbrough, (1984). Bacterial isolates were tested against seven OPTUN disc antibiotics comprising Ceporex (CPX 10 µg), Norbactin (NB 10 µg), Gentamycin (GN 10 µg), Amoxil (AML 20 µg), Streptomycin (S 30 µg), Augmentin (AUG 30 µg), Rifampicin (R 20µg), Erythromycin (E 30 µg), Chloramphenicol (CH 30 µg), Ampiclox (APX 20 µg), and Levofloxacin (LEV 20 µg). The inoculum was standardized by adjusting its density to 0.5 McFarland turbidity standard (equal the turbidity of a barium sulphate ($BaSO_4$)). Examination of the cultures for zones of clearing was done after 24 hours of incubation at 37 ºC. Diameters of zones of inhibition observed were measured in millimeters (mm), and interpreted according to CLSI (2017) standards.

RESULTS

Table 1 shows the measurements of classrooms sampled., SS4 classroom had the largest space 19200 m^2 with 3 doors and eight windows, followed by SS3 classroom with 6750 m^2 with one (1) door and 7 windows, SS1 had an area of 6732 m^2 with one (1) door and 4 windows, NS1 had the smallest space: 712.5 m^2 with 2 doors and 4 windows.

Airborne bacterial counts of classroom atmosphere were as follows; NS4 (33.3 cfu/m^3), SS3 (28.3 cfu/m^3) and SS1 and SS2 were both observed to have lower plate counts of 6.6 cfu/m^3 at ambient sampling. Under populated conditions, NS4 had the highest plate count 68.3 cfu/m^3, NS3 (58.3 cfu/m^3), and SS4 classroom had the lowest count of 25 cfu/m^3.

Correlation coefficient between area and aerobic plate count was -0.417. Relationship between class size and aerobic plate count was negative (r = 0.015), and p-value of 0.805 (p>0.805). A t-calculated value of 5.16, and a t-critical of 2.36 at 0.05 level of significance and p-value of 0.001 (p<0.05) was obtained.

A total of 9 bacterial genera were identified from the classroom atmosphere and identified as *Rothia* sp, *Bacillus* sp, *Corynebacterium* sp, *Lactobacillus* sp, *Flavobacterium* sp, *Staphylococcus* sp, *Brevibacterium* sp, *Pseudomonas* sp, *Kurthia* sp and both in ambient and populated sampling respectively.

According to the bacterial distribution in the air, 28 % were *Lactobacillus* sp, 21 % were *Pseudomonas* sp, 18 % were *Staphylococcus* sp. *Bacillus* sp, *Flavobacterium* sp *and Brevibacterium* sp were 4% respectively, *Corynebacterium* sp, *Rothia* sp *and Kurthia* sp 7 % respectively.

The pattern of resistance of the bacterial isolates were Ceporex (11.1 %), Norbactin (66.7 %), Gentamicin (22.2 %), Amoxicillin (44.4 %), Streptomycin (22.2 %), Rifampicin (33.3 %), Erythromycin (33.3 %), Chloramphenicol (22.2 %), Ampiclox (44.4 %), Levofloxacin (33.3 %).

Table 1 Classroom area and populations in selected schools in Ikot Ekpene

Schools	Class sampled	Classroom Area	Number of Students	Number of staff
NS1	Nursery 2	712.5m^2	15	2
NS2	Nursery 1	7225 m^2	20	2
NS3	Primary 4	2500 m^2	26	1
NS4	Nursery 1	2400 m^2	26	2
SS1	JSS 3	6732 m^2	17	1
SS2	SS 3	4800 m^2	10	1
SS3	JSS 2	6750 m^2	36	1
SS4	SS 3	19200 m^2	95	0

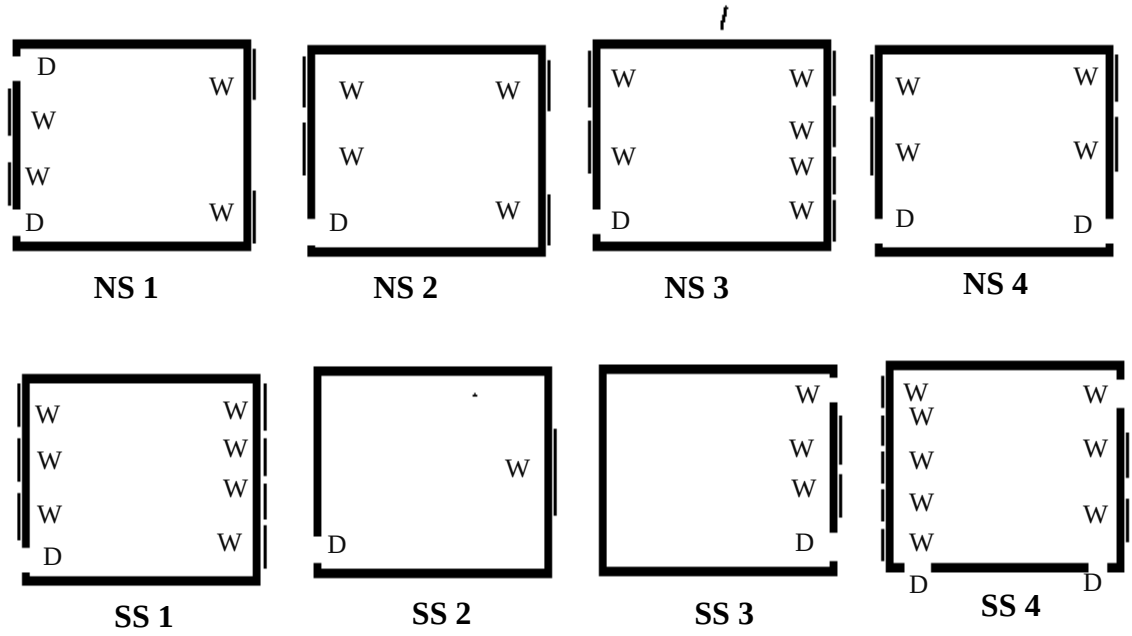

Figure 1 Ventilation patterns in school classrooms Key; W=Window, D = Door

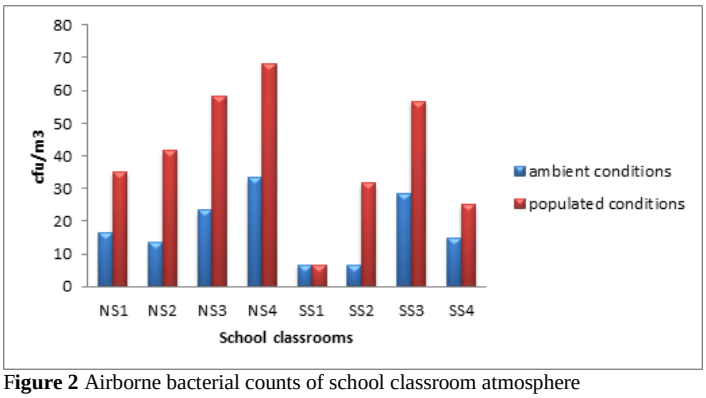

Figure 2 Airborne bacterial counts of school classroom atmosphere

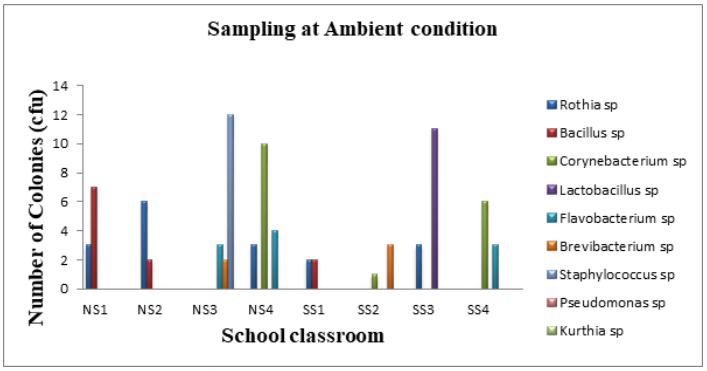

Figure 3 Distribution of bacteria in classroom atmosphere at ambient condition

Table 2 Biochemical characteristics of bacterial isolates from classroom atmosphere

Isolates	1	2	3	4	5	6	7	8	9
Spore	-	+	-	-	-	-	-	-	-
Motility	-	+	-	+	-	-	-	+	-
Coagulase	+	+	-	+	-	-	+	+	+
Oxidase	+	-	+	+	+	+	-	+	-
Urease	+	+	+	+	+	+	+	+	+
Citrate	+	+	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+	+	+
Sucrose	OO	AO	OO	AG	AO	OO	AO	OO	AO
Glucose	AO	AO	AG	AG	AO	OO	AO	OO	AO
Lactose	AO	AG	AO	AO	AO	AO	AO	OO	AO
Mannitol	OO	AO	OO	AO	AO	OO	AO	OO	AO

Legend: AG = acid and gas production; A O = acid production only; OO = no reaction; + = positive reaction; - = negative

Table 3 Phenotypic dentification of bacterial isolates from classroom atmosphere

Isolates	Cultural features	Gram/ shape	Probable isolate
1	Milky, rhizoid, viscous, translucent	+ rods	*Rothia* sp
2	Milky, circular, curled, viscous, opaque	+ rods in chains	*Bacillus* sp
3	Milky, circular, butyric, dull, opaque	+ rods in pairs	*Corynebacterium* sp
4	Brown, circular, viscous, shiny, translucent	+ rods in pairs	*Lactobacillus* sp
5	Yellow, circular, viscous, raised, translucent	+ rods in pairs	*Flavobacterium* sp
6	Pink, circular, viscous, raised, opaque	+ single short rods	*Brevibacterium* sp
7	Milky, circular, viscous, shiny, opaque	+ cocci in clusters	*Staphylococcus* sp
8	Milky, circular, viscous, shiny, opaque	- single rods	*Pseudomonas* sp
9	Milky, filamentous, viscous, shiny	+ cocci	*Kurthia* sp

Table 4 Antibiotic susceptibility patterns of bacterial isolates from classroom atmosphere

	(CPX)	(NB)	(GN)	(AML)	(S)	(R)	(E)	(CH)	(APX)	(LEV)
***Rothia* spp**	S	S	R	S	S	S	I	R	S	I
***Bacillus* spp**	S	R	I	R	R	I	I	I	I	R
***Corynebacterium* spp**	S	I	S	R	S	S	R	S	R	S
***Lactobacillus* spp**	S	R	R	I	S	R	R	S	S	R
***Flavobacterium* spp**	S	R	S	S	S	S	S	I	S	S
***Brevibacterium* spp**	R	R	I	R	I	R	R	R	R	R
***Staphylococcus* spp**	S	R	S	R	R	I	S	S	R	S
***Pseudomonas* spp**	S	S	S	I	S	S	S	I	R	S
***Kurthia* spp**	S	R	S	I	S	R	S	S	I	S
Percentage of resistance	11.1%	66.7%	22.2%	44.4%	22.2%	33.3%	33.3%	22.2%	44.4%	33.3%

Legend: CPX=Ceporex, GN= Gentamycin, S= Streptomycin, E=Erythromycin, APX= Ampiclox, NB=Norbactin, AML=Amoxicillin, R=Rifampicin, CH=Chloramphenicol, LEV=Levofloxacin

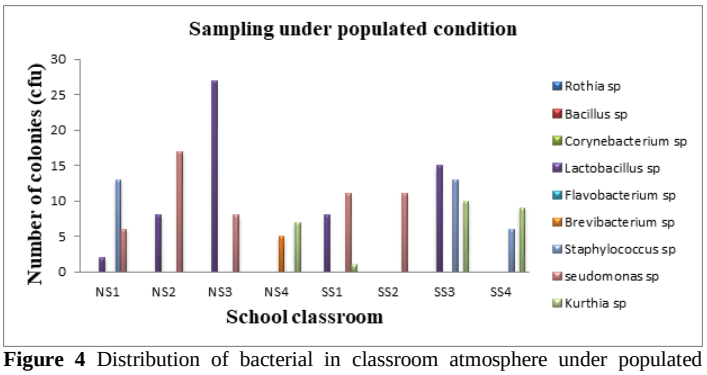

Figure 4 Distribution of bacterial in classroom atmosphere under populated condition

Table 5 Correlation between class area and airborne bacterial count

	Area	Populated aerobic plate count
Area	1	
Populated aerobic plate count	-0.417	1
	(0.304)	

Table 6 Correlation between class size and airborne bacterial count

	Class size	Aerobic plate count
Class size	1	
Aerobic plate count	-0.105	1
	(0.805)	

Table 7 Correlation between class size and airborne bacterial coun

	Class size	Aerobic plate count
Class size	1	
Aerobic plate count	-0.105	1
	(0.805)	

DISCUSSION

Microbial concentration of indoor air of schools is affected by many factors including human activity, the age of the school building, ventilation conditions, outdoor air and season (primarily temperature and humidity). In this study, classroom atmosphere of seven schools within Ikot Ekpene metropolis were sampled using natural sedimentation techniques.

Results obtained revealed high aerobic plate counts in classroom atmosphere of two schools during ambient sampling; NS4 and SS3 with (33.3 cfu/m^3) and (28.3 cfu/m^3) respectively. The absence of proper ventilation in SS3 classroom (the windows and doors were on only one side of the room, with no windows or doors on the three adjoining walls) is suggested to be a major factor. During populated sampling under population, four schools; NS4, NS3, SS3 and NS2 and SS2 section were revealed as having high aerobic plate counts (63.3 cfu/m^3, 58.3 cfu/m^3, 56.6 cfu/$m^3$and 41.6 cfu/m^3) respectively. Sampling under populated conditions revealed higher aerobic plate counts compared to sampling at ambient conditions in all the schools, correlating the findings of **Tham and Zurami, (2005)** who observed that human activities including movement, rafting, desquamated skin scales, sneezing, and coughing are main contributors of elevated viable microbial concentration in indoor air. The lower aerobic plate counts in ambient samples are suggested to be due to particle settling at night when the classrooms are empty.

Organisms that are capable of causing airborne diseases must be airborne, this means that environmental conditions of the sites as related to building design must be conducive for microorganisms to be airborne and cause pollution **(Wemedo *et al*., 2012)**. In this study, NS4 recorded the highest bacteria count suggested as due to overcrowding; 26 students occupying a small space of (92 cfu/m^2 per student). In contrast, SS2 classroom had a lower bacteria count than most classrooms in this study due to adequate ventilation and low occupancy (only 10 students occupied the classroom with an average space of 480 m^2 per student), as well as the use of quality building materials. This may have assisted in minimization of potential dispersion of dusts, mites or spores resulting in low bacterial counts. Classroom of NS3 may have encouraged the high aerobic plate count reported (58.3 cfu/m^3) due to poor ventilation, lack of windows and doors and also large number of students; 26 occupying a space of 96 m^2 per student.

Stryjakowska *et al*. (2007) and **Soto *et al.* (2009)** in their work have also observed a significant increase in concentration of bacteria in afternoon air sampling during lesson hours compared to ambient sampling at morning hours in various classes of schools, and concluded that bacterial contamination in indoor air derives from human presence. **Karwowsk, (2003)** also reported higher levels of bacterial contamination in schools during school hours (50-100 cfu/m^3 and 200 cfu/m^3). **Nandasena *et al.* (2010)** concluded in their study that as the number of people living in a space rises, so does the counts of organisms in the air.

Building design has been shown to determine the microbial quality of various schools **(Badir *et al*., 2016).** The building conditions of other schools like NS1, NS2, and SS4 though with higher number of students had larger spaces or well positioned windows and doors for proper ventilation and free flow of air. Schools where building design did not allow well positioned windows and doors, and gave high bacteria counts. This include; NS3, NS4 and SS3.

Prevalent bacterial genera isolated from the classroom atmosphere were; gram positive including: *Rothia, Bacillus, Corynebacterium,, Lactobacillus, Brevibacterium, Staphylococcus* and *Kurthia*, and gram negative bacteria including; *Pseudomonas* and *Flavobacterium*. This is also similar to previous reports by **Badir *et al.* (2016)** who isolated *Staphylococcus*, *Bacillus* and *E. coli* as main Gram-positive bacteria belonging to saprophytic microflora generally correlating to human skin and mucosa which can be dispersed through droplets or skin peeling and maintained in the air.

Correlation between area and aerobic plate count implies a negative relationship between the two variables, indicating that as the area increases, aerobic plate count decreases. The p-value of 0.304 ($p>0.05$) implies that this relationship is not significant ($p>0.05$). A negative relationship also exists between class size and aerobic plate count.

A significant difference however exists between aerobic plate count at ambient and population condition. Based on the means, it is clear that aerobic plate count at population condition is significantly higher than aerobic plate count at ambient. The levels of bacteria in classroom sample did not exceed standards (<500 cfu/m^3) **(EPA, 2000)**, but show the presence of potential bacterial pathogens in air.

Bacterial isolates obtained from these classrooms atmosphere are implicated in infectious disease commonly transmitted through air, e.g. *Staphylococcus aureus* which is an airborne bacteria dispersed into the air from human skin, oral and nasal surfaces and hair, and able to cause impetigo which is commonly seen among children. *Pseudomonas* causes pneumonia which affects the lungs. *Bacillus* especially *Bacillus cereus* causes conjunctivitis or orbital abscess which can be transmitted among children in the classroom through air. *Corynebacterium* are bacteria widely distributed in nature including air, causing diphtheria and transmitted through respiratory droplets part of human flora.

CONCLUSION

This study concludes that bacteria are present in, and can be isolated from the atmosphere such as in classroom atmosphere. Airborne bacterial counts of classroom atmosphere can be affected by major factors including classroom population, classroom dimensions, as well as the ventilation patterns. Since poor air quality can pose great risk to the health of students in a classroom, it is therefore important to manage and design classrooms, the class population size, and make adequate provisions for ventilation to maximize the air quality of such environments which will enhance learning, eliminate the risks of airborne transmissions of pathogens and infections among student and staff populations.

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REGULAR ARTICLE

IDENTIFICATION, PRODUCTION AND CHARACTERIZATION OF NATTOKINASE, BACTERIOCIN FROM BACTERIAL SPECIES

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ABSTRACT

The study was aimed at identification, production and characterization of nattokinase, bacteriocin from bacterial species. Nattokinase and bacteriocins finds a wide range of applications in Pharmaceutical industry, health care and medicine. Nattokinase is a highly active fibrinolytic enzyme secreted by *Bacillus subtilis* and bacteriocins are proteinaceous toxins produced by *Lactobacillus* to inhibit the growth of closely related bacterial strains. *Bacillus subtilis* and *Lactobacillus* isolates shown positive results to microscopic, biochemical analysis. The nattokinase and bacteriocins were produced by optimizing the media. The enzymes were purified by ammonium sulfate precipitation and HPLC. The enzyme activity for nattokinase was found at 7 mg/ml, pH 8.0 and temperature 48 ºC and the enzyme activity for bacteriocin was found at 3.9 mg/ml, pH 6.5 and temperature 30 °C. Bacteriocins from *Lactobacillus* showed good antagonistic activity against pathogenic bacteria. Nattokinase from *Bacillus subtilis* played a significant role in thrombolytic and anti-coagulation at *in vitro*. The results indicated that the pure enzyme has a potential in dissolving blood clot.

Keywords: Fibrinolytic, Thrombolytic, Anti-coagulation, Bacteriocins, Nattokinase, Ammonium sulfate precipitation

INTRODUCTION

Nattokinase is a fibrinolytic enzyme that belongs to the second wide family of serine protease. It hydrolyses fibrin and is the major protein constituent of blood clots, an insoluble white protein formed by the conversion of fibrinogen through thrombin. Nattokinases was initially discovered and extracted from Japanese conventional fermented soybean foods that were of certain importance due to their efficient biological thrombolysis of fibrin and clots of blood in blood vessels **(Sumaya Ali Hmood *et al.*, 2016)**. Previous studies observed that these enzymes could also be purified from brewing rice wine and Indonesian fermented soybean **(Liu *et al.*, 2005)**. Most of the nattokinases are secreted by different *Bacillus* spp. including *B. subtilis, B. amyloliquefaciens, B. amylosacchariticus* and *B. licheniformis* **(Wang *et al.*, 2006)**. A mild and frequent enhancement of the fibrinolytic activity in the plasma is seen by oral administration and stability in the gastrointestinal tract of Nattokinase **(Tai *et al.*, 2006).** The fibrinolytic activity of Nattokinase can be retained in the blood for more than 3 hours, which makes it probable for clinical use and now it is widely considered as a promising oral medicine for thrombolytic therapy **(Vignesh H *et al.*, 2014).** It has been reported that rich foods is very much useful for prevention of disease as well as aging process. Enzymes are in use as digestion aids but mostly ignored as drug **(Patil R *et al.*, 2018).** Bacteriocins from lactic acid bacteria (LAB) are natural antimicrobial peptides or proteins with interesting potential applications in food preservation and health care **(Savita Jandaik *et al.*, 2013).** Bacteriocins are small proteins with bactericidal or bacteriostatic activity. This organism prevents the growth of pathogenic bacteria in different ecosystems by production of antimicrobial substance such as organic acids and hydrogen peroxide **(Arokiyamary *et al.*, 2011).** The health benefits offered by LAB can be therapeutic including production of vitamins, immunomodulation, reduction in the risk of diarrhoea and mutagenic activity and a decrease in serum cholesterol (**Anwar A. Abdulla *et al.*, 2014**). Several bacteriocins from *Lactobacillus spp*. has been characterized with respect to their protein sequence, molecular mass, biochemical properties and antimicrobial activity spectrum. Bacteriocins that have all D-amino acids have antibacterial activity but exhibit more resistance to proteolytic enzymes and are less cytotoxic compared with bacteriocins that have all L-amino acids **(Pangsomboon *et al.*, 2006).** Genes that encode bacteriocins are found exclusively on the chromosome, and it is bacterial ribosomal synthesized peptides or proteins with antimicrobial activity **(Sano *et al.*, 2002).** Depending on the producer organism bacteriocins can be classified into several groups as Class I, II and III **(Ashok kumar *et al.*, 2011).** LAB bacteriocins are divided into three main groups, based on their amino acid sequence, mode of action, heat tolerance, biological activity, presence of modified amino acids, and secretion mechanism **(Hoda Mahrous *et al.*, 2013).**

MATERIALS AND METHODS

Collection of samples

The milk and fermented curd were collected from local dairy, Hosur, Tamil Nadu, India. The *Bacillus subtilis* MTCC 5981 was obtained from MTCC (Microbial Type Culture Collection), Chandigarh, India.

Isolation of bacteria

Lactobacillus was isolated by streak plate method from the collected milk and curd samples were streaked on MRS (De Man Rogosa & Sharpe) agar plate and incubated at 37 °C anaerobically for 48 hrs. At the end of 48 hrs, when the colonies became predominant, morphologically distinct and well developed colonies were picked and transferred to MRS broth. *Bacillus subtilis* was isolated by streak plate method from the obtained sample into nutrient agar plates and the plates were incubated at 37 °C for 24 hrs. The isolated colonies were picked and transferred to nutrient broth.

Identification of bacteria

The isolates were identified on the basis of Morphological and Biochemical Characteristics as given in the Bergey's Manual of Systematic Bacteriology **(Holt *et al.*, 1994)**. The tests were performed for identification of bacteria by Grams staining, Motility, Catalase, Methyl red (MR), Voges proskaur (VP), Urease, Citrate utilization, Fermentation test- lactose, glucose, sucrose.

NaCl tolerance test

The media was adjusted with different concentration (5, 7 & 9%) of NaCl with composition of nutrient agar, phenol red and manitol (1%). After sterilization, each plate was inoculated with 1% fresh overnight culture of *Lactobacillus and Bacillus subtilis* and incubated at 37 °C for 24 – 48 hr. The culture plates were observed for the presence or absence of growth.

pH tolerance test

The media of MRS agar and nutrient agar was adjusted with different pH (3, 4, 5 for *Lactobacillus* & 5.5, 6.5, 7.5, 8.5 for *Bacillus subtilis*). pH was determined by a digital electrode pH meter. After sterilization, each plate was inoculated with 1% fresh overnight culture of *Lactobacillus and Bacillus subtilis* and incubated at 37 ℃ for 24 – 48 hr. The culture plates were observed for the presence or absence of growth.

Enzyme production

The media used for the optimal production of nattokinase was composed of Soy peptone- 0.5%, NaCl- 0.25%, Na_2HPO_4- 0.1%, $MgSO_4$- 0.02, $MnCl_2$- 0.05%, Glucose- 1% and Casein- 1%. The pure culture was inoculated into a media and was incubated at 37 ºC in an incubator for 72 hrs and inoculum used for the production of the nattokinase enzyme. The media was prepared on MRS broth for the optimal production of bacteriocins. The pure culture was inoculated into a media and was incubated at 37 ºC in an incubator for 72 hrs and inoculum used for the production of the Bacteriocin.

Partial purification

Ammonium sulfate precipitation

Solid ammonium sulfate was added to the culture filtrate at 70% saturation for NK and Bacteriocins and stirred for 24 hrs at 4 ºC **(Deepika *et al.*, 2017).**The precipitate obtained was separated by centrifugation at 10000 rpm for 20 mins at 4 ºC. The resultant supernatant was solubilized in 1N NaOH.

Enzyme assay

The protein is estimated by Lowry's method for both enzymes. 0.5 ml of sample was mixed with 1.125 ml of Lowry's reagent. The tubes were incubated at room temperature for 10mins. 0.5 ml of Folin's - Phenol reagent was added and incubated at dark in room temperature for 30 mins and the OD was observed at 560 nm.

Blood clot analysis

Human and chicken blood drawn for analyzing nattokinase and were transferred to different pre-weighed sterile micro-centrifuge tubes and incubated at 37 ºC for 45 mins **(Deepika *et al.*, 2017).** After clot formation, serum was completely removed and each tube with clot was again weighed to determine the clot weight.

Clot weight = [Weight of clot containing tube] - [Weight of tube alone]

Each microfuge tube, containing clot and 100 µl of crude enzyme and ammonium sulfate precipitated enzyme was added to the clots. All the tubes were incubated at 37 ºC for 90 mins and observed for clot lysis. After incubation, the obtained fluid was removed and the tubes were again weighed to observe the difference in weight after clot disruption. The obtained difference in weight taken before and after clot lysis was expressed as percentage of clot lysis.

Antagonistic activity

The antagonistic activity of bacteriocins for *Lactobacillus* spp. isolates was measured by the agar well diffusion assay and tested against *Klebsiella pneumoniae, Salmonella typhi, Escherichia coli, Verticillum lecanii.* It was measured by the diameter of zone of inhibition.

HPLC analysis

The crude sample of nattokinase and bacteriocins was analyzed by high pressure liquid chromatography (HPLC) using C 18 column containing 5 mm sized particles (250 mm and 4.5 mm ID Lichrosper m 100) and a 20 µl loop injector. Mobile phase was used as a mixture of acetonitirile (A) and water acidified to pH 3 with o- phosphoric acid and methanol (B) in the ratio of 1.1 with flow rate of 1.2 ml/min. Detection was carried out by UV detector at 248 nm.

RESULTS AND DISCUSSION

Isolation of Bacteria

MRS Agar was reported to be the best suitable selective media for the isolation of *Lactobacillus spp.* by (**Gad G.F.M *et al.*, 2014)**. Selective media is used to cultivate specific type of microorganisms. Thus, the ability of bacterial species to be cultured on specific media is regarded as an important characteristic in identification of the microorganisms. For *Bacillus subtilis* bacterial colonies were produced as the result of pour plate method which was done on nutrient agar plates which were incubated at 37 ˚C overnight.

Morphological Characteristics

Lactobacillus was identified on the basis of characteristic morphology, gram positive, rod shaped bacteria. However, another report showed that *Lactobacilli* were physiologically diverse group of rod-shaped, gram-positive **(Hoque *et al.*, 1994)**. *Bacillus subtilis* identified on the basis of morphology and showed gram positive, rod shaped bacteria. Previously, *Bacillus subtilis* were identified and confirmed by gram positive **(Debajitborah *et al.*, 2012)**.

Biochemical Characteristics

Catalase Test

Enzymes that decompose hydrogen peroxide into water and oxygen. Hydrogen peroxide forms as one of the oxidative end products of aerobic carbohydrate metabolism. If this is allowed to accumulate in the bacterial cells it becomes lethal to the bacteria. *Lactobacillus* showed negative and *Bacillus subtilis* showed positive results.

Methyl Red Test

This test is the ability of the organism to produce acid end product from glucose fermentation, this is a qualitative test for acid production. Both *Lactobacillus* and *Bacillus subtilis* showed negative results as absence of red colour was observed.

Voges proskaur (VP) Test

This test is used to determine the ability of the organisms to produce neutral end red colour product from glucose fermentation. Both *Lactobacillus* and *Bacillus subtilis* showed negative results.

Citrate utilization Test

This test based on the ability of an organism to use citrate as its only sole source of carbon and ammonia as its only source of nitrogen. *Lactobacillus* showed negative and *Bacillus subtilis* showed positive results thus growth on the plate and colour changes from green to blue.

Fermentation Test

Glucose

This test was done to help differentiate species of family *Enterobacteriaceae* and for the bacteria's ability to ferment glucose, produce gas/an acid end product. *Lactobacillus* and *Bacillus subtilis* showed positive result by appearance of yellow colour and indicating the glucose fermentation.

Sucrose

This test was done to help differentiate species of family *Enterobacteriaceae* and for the bacteria's ability to ferment glucose, produce gas/an acid end product. *Lactobacillus* and *Bacillus subtilis* showed positive result by appearance of yellow colour and indicating the sucrose fermentation.

Lactose

This test for the bacteria ability to ferment lactose. *Lactobacillus* and *Bacillus subtilis* showed positive result by appearance of yellow colour and indicating the lactose fermentation.

Urease Test

This test is performed to determine the bacteria's ability to hydrolyze urea to make ammonia using the enzyme urease. Formation of pink colour showed the presence of urease in *Bacillus subtilis* and absence in *Lactobacillus.*

Motility Test

This test used to help differentiate the species of bacteria that are motile from non-motile. Both *Lactobacillus* and *Bacillus subtilis* showed non-motile, there will only be growth along the stab line.

NaCl tolerance Test

The isolated *Lactobacillus* and *Bacillus subtilis* were able to tolerate 1-10% NaCl. The growth of *Lactobacillus* was observed at a 7 % NaCl while *Bacillus subtilis* showed a growth at a 9 % NaCl. Thus *Lactobacillus* and *Bacillus subtilis* are salt tolerant bacteria. The other physiological parameter for growth of a cell is the requirement of sodium chloride as the physiological saline prevents the cell from osmotic shock **(Kavitha *et al.*, 2016).**

Table 1 Biochemical test results

Tests	*Bacillus subtilis*	*Lactobacillus*
Catalase	+	-
Urease	+	-
MR	-	-
VP	-	-
Citrate	+	-
Lactose	+	+
Sucrose	+	+
Glucose	+	+

Where**,** + positive, - negative

pH tolerance Test

The isolated *Lactobacillus* and *Bacillus subtilis* were able to tolerate pH. There was a growth in the *Lactobacillus* at pH 4 while *Bacillus subtilis* showed growth at pH 7.5**. Marwa A *et al.*, 2015** reported to the effect of pH values on the activity of the bacteriocins extracted from L. acidophilus, the activity of bacteriocins were very stable over a wide range of pH (2, 4, 6 and 8).

Enzyme Production

The composition of medium influencing the production of bacteriocin by *Lactobacillus* isolates. It showed that maximum activity was noted at pH 6.5, temperature 30 °C. Bacteriocin production was frequently regulated by pH and growth temperature, as has been shown in several studies involving the pediocin **(Ibrahim Khalil *et al.*, 2017).** Thus variation in the concentration of constituents of cultivation media might have an influence on the amount of bacteriocin produced by microorganisms. Nattokinase media optimization revealed that addition of soy peptone enhanced the production of enzyme. And maximum enzyme activity was observed at pH 8.0 and temperature 48 ºC in the optimized media. **Peng *et al.*, 2003** showed that the fibrinolytic enzyme produced by *Bacillus amyloliquefaciens* had an optimal activity at 48 °C and pH 9.0.Nattokinase yield varied in different optimized fermentation media. However, comparison of these fibrinolytic activities is difficult due to different activity assay methods and the absence of specific activity.

Ammonium sulfate precipitation

The crude extract produced was initially subjected to precipitation with ammonium sulfate. In the present study, 70% ammonium sulfate saturation ratio was selected to determine the best ratio for nattokinase and bacteriocins precipitation. Solid ammonium sulfate was selected to precipitate the nattokinase and bacteriocin due to its high solubility, availability, low cost and proteins stabilization that occurs **(Deepika *et al.*, 2017).**

Estimation of protein

The nattokinase and bacteriocins showed the presence of protein content. Previously, **Debajitborah *et al.*, 2012** found that amount of protein in nattokinase was to be 5.4 mg/ml and **Ibrahim Khalil *et al.*, 2017** showed the protein concentration in bacteriocins was found to be 1.32 mg/ml. Proteins are compared in the collected sample extracts to know the amount of proteins present using BSA (Bovine Serum albumin) as standard. The amount of protein present in the nattokinase and bacteriocins was found to be 7 and 3.9 mg/ml.

Table 2 Protein Estimation

Samples	Absorbance at 560 nm	Amount of protein (mg/ml)
Nattokinase (BS-1)	1.907	7
Bacteriocins (LB-C)	1.201	3.9

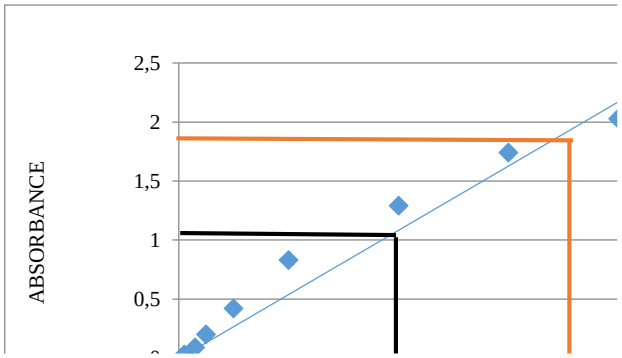

Figure 1 Graph shows protein content with absorbance at 560 nm

Antagonistic activity

The bacteriocin produced by Lactic Acid Bacteria was checked for their Antagonistic activity against *Klebsiella pneumoniae, Salmonella typhi, E. coli, Verticillum lecanii*. From this it was found that bacteriocins from probiotic seem to be most active against bacterial species. The supernatant of *L. acidophilus* strains showed variable degree of antagonistic activity with respect to inhibition zones. It was found that isolates from curd was most inhibitory against the tested bacterial pathogens. *Lactobacillus fermentum* showed inhibitory activity against *Escherichia coli, Salmonella typhi, Bacillus cereus, Staphylococcus aureus, Pseudomonas mirabilis, Klebsiella pneumonia* and *Pseudomonas aeuroginosa*. **(Udhayashree *et al.*, 2012)**. **Chumchalova *et al.*, 2004** observed the antagonistic activity of four strains of *Lactobacillus plantarum* against *Salmonella typhi, E. coli, Pseudomonas aeruginosa, Staphylococcus aureus, Salmonella paratyphi, Bacillus cerus, Bacillus subtilis, and Bacillus megaterium.* The probiotic bacteria may also have competed for nutrients, simultaneously produced hydrogen peroxide and bacteriocins that acted as antibiotic agents **(Ibrahim Khalil *et al.*, 2017).** Thus bacteriocin showed the antimicrobial activities against the pathogens and the results showed in table 3.

Table 3 Antagonistic activity results

Organisms	25 (µl)	50 (µl)	75 (µl)	100 (µl)
Klebsiella pneumonia	6 mm	8 mm	7 mm	10 mm
Salmonella typhi	7 mm	6 mm	11 mm	9 mm
Escherichia coli	0 mm	0 mm	8 mm	10 mm
Verticillum leccani	6 mm	5 mm	6 mm	8 mm

Blood clot analysis

The blood clot lysis was also assayed in the microfuge tubes. Human and chick blood clot was incubated with the enzyme, and clot degradation was analyzed. Previously, **Sumaya Ali Hmood, *et al.*, 2016** concluded that, the nattokinase purified from *Bacillus spp.* B24 using wheat bran as substrate displays excellent fibrinolytic activities *in vitro*. **Subathra *et al.*, 2015** showed 94% clot lysis was visually observed after 10 minutes in the tube that received 70% precipitate of mutant UV60 strain. The clot was weighed to determine the clot weight and results showed in table 4.

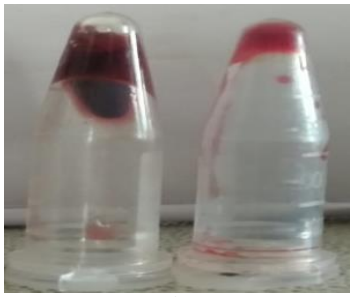

Figure 2 Before treatment

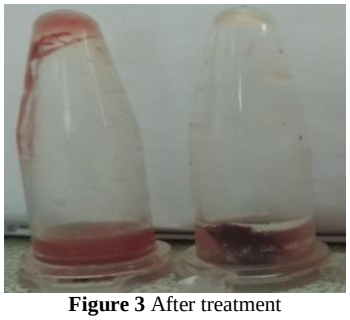

Figure 3 After treatment

Table 4 Blood clot analysis results

Samples	Weight of the tube alone	Before Treatment	After Treatment	% of clot lysis
Human blood	1.025	1.288	1.118	26.3 %
Chicken blood	1.026	1.428	1.332	30.6%

HPLC analysis

The high performance liquid chromatography was performed for nattokinase and bacteriocins. The sample was made to run for 10 min and the volume of sample taken for analysis is 20 µl and the analysis was made at 260 nm. Methanol was used as the solvent system for the study. After the analysis, peak was obtained as shown in table 5.

Table 5 HPLC Results

Samples	RT (min)	Area (m)	Height (mAU)	Width (min)
Nattokinase	10.847	1054.504	0.097	0.505
Bacteriocin	7.455	5386.817	0.518	0.535

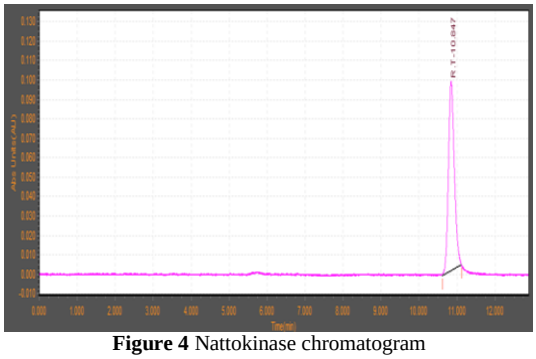

Figure 4 Nattokinase chromatogram

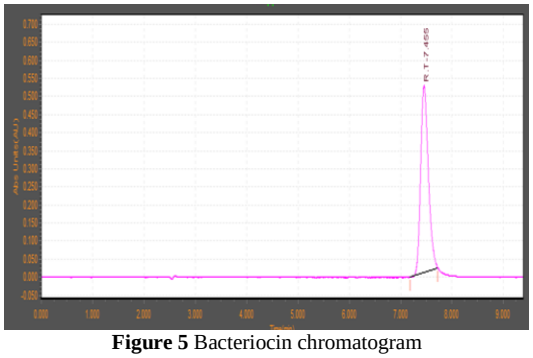

Figure 5 Bacteriocin chromatogram

CONCLUSION

The isolates *Bacillus subtilis and Lactobacillus* showed positive results to microscopic, biochemical analysis. For Nattokinase, enzyme activity was found at pH 8.0 and temperature 48 ºC, for bacteriocin, enzyme activity was found at pH 6.5, temperature 30 °C. Bacteriocins from *Lactobacillus* showed good activity against pathogenic bacteria. This makes a good antagonistic agent. Nattokinase from *Bacillus subtilis* played a significant role in thrombolytic and anti-coagulation in vitro. The results indicated that the pure enzyme has a potential in dissolving blood clot.

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REGULAR ARTICLE

ANTIBACTERIAL ACTIVITY AND PHYTOCHEMICAL OF *Caulerpa sertularoides* AND *Padina australis* FROM NAIN ISLAND, NORTH SULAWESI, INDONESIA

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ABSTRACT

Methanol extract of *Caulerpa sertularoides* and *Padina australis* were screened against Gram positive (*Staphylococcus aureus* ATCC 6538) and Gram negative (*Escherichia coli* TCC 25922) bacteria. The methanol extract were further extracted by multiple phase partitioning method using water, ethyl acetate and hexane and also screened against those bacteria. The hexane fraction of *P.australis* showed high antibacterial activity against *S.aureus* (inhibitory zone diameter > 20 mm). Phytochemistry result showed that ethyl acetate fraction from *C. sertularoides* extract has phenolic, flavonoid, steroid, triterpenoid, saponin and tanin. Overall, it can be concluded that the methanol and its fractions extract from both types of marine algae have antibacterial potency against *S. aureus* and *E.coli*.

Keywords: *Caulerpa sertularoides, Padina australis,* Antibacterial, Phytochemicals

INTRODUCTION

Nain Island is protected by barrier reefs which restrains streams from the sea. This water movement in this area facilitates marine algae to grow properly due to even distribution of the the nutrients carried by the water current (**Mudeng , 2007**). *Caulerpa sertularoides* and *Padina australis* are two types of marine algae that thrive in this area. Algae contain secondary metabolites (phytochemicals) utilized in the medical, cosmetics as well as in other industries (**Subtijah, 2002**). Previous study reported that marine algae have antimicrobial activity, including those from Morocco waters, namely *Ulva rigida*, *Ulva lactuca, U olivascens*, *Enteromorpha compressa*, *E.linza*, *E. intestinalis*, *Chaetomorpha linum*, *Caulerpa prolifera*, *Codium dichotomum*. They were reported to have antibacterial activities against *E. coli* ATCC 25922 *S. aureus* ATCC 25923 and *E. faecalis* ATCC 29212 (**Zbakh *et al*., 2012**). The aqueous extract of *Caulerpa active rasemosa* has been shown to inhibit *Pseudomonas pavanaceae* and *Pseudomonas syntata* while its methanol extract showed activity against *Pseudomonas denitrificans* and *Pseudomonas syntata* (Izzati, 2007). Previous study demonstrated green marine algae (*Caulerpa sertularoides)* and brown marine algae (*Padina australis)* isolated from Nain Island, North Sulawesi to have antioxidant activity **(Dotulong *et al.*, 2013)**. The purpose of this study was to explore the antibacterial potency of marine algae *Caulerpa sertularoides* and *Padina Australia* from Nain Island, North Sulawesi, Indonesia.

MATERIAL AND METHODS

Materials

Raw materials, *Caulerpa sertularoides* and *Padina australis* algae, were collected from Nain Island, North Minahasa, North Sulawesi, Indonesia. The bacterial strains used in this study were *Staphylococcus aureus* ATCC 6538, and *Esherichia coli* ATCC 25922 was a Licroprep RP-18 (Merck), hexane, ethyl acetate (Merck Co. Ltd) and methanol (Sigma Aldrich Co. Ltd), Muller Hinton Agar from (Merck Co. Ltd), Broth Agar from (Merck Co. Ltd). Eyela brand ovens, NDO-410, for sample drying, Buchi rotary evaporators for evaporation of solvents, autoclave machines HVE-50 Hirayama and Incemator Memmert.

Making Seaweed Flour

The sample was dried in the room for 3 days and in an oven at 40 °C until the weight was reduced 10 times. The dried samples were powdered by blender and further sifted.

Methanol Extraction

Samples were extracted with methanol as described by **Dotulong (2014)**. 200 g samples was macerated with 2 L of technical methanol for 48 h, maserat was separated from the pulp by filtering with whatman filter paper No. 1, pulp was macerated again in the same way as previously described twice, so that ± 6 L of maserat is obtained. Maserat was evaporated with a vaccum rotary evaporator at a temperature of 40 ºC.

Fractionation of methanol extract with hexane, ethyl acetate and water (Harborne, 2006 and Tamat *et al*., 2007).

Methanol extract was partitioned with 200 mL of n-hexane-water mixture (1:1), n-hexane fraction separated and collected in an evaporative flask. The same treatment was repeated 3 times, the n-hexane fraction was collected and evaporated in the rotary evaporator vacuum temperature of 40 ºC until dry. The water fraction was partitioned with ethylacetate three times each of 200 mL, ethyl acetate fraction was separated from the water fraction, then all ethyl acetate fractions were collected and then evaporated in the rotary evaporator vacuum at low temperature and pressure as above until dry. The remaining filtrate is the water fraction, then evaporated in a rotary evaporator vacum at a low temperature and pressure as above until a dry extract (water fraction) is obtained. The final results of this extraction process are each n-hexane fraction (non polar fraction), etil fraction lasetate (semi polar fraction) and and water fraction (polar fraction).

Calculation of Rendement

The yield was calculated by the following formula: $\frac{w_2}{w_1} \times 100\%$ (1)

w_1: initial weight of fresh sample
w_2: final weight of extract/fraction

Phytochemical Analysis

Phytochemical analysis were conducted according to **Tamat *et al.* (2007)**. Analysis was carried out qualitatively on methanol extract and hexane, ethylacetate and water fractions from marine algae *C. sertularoides* dan *Padina australis*. The secondary metabolites analyzed were phenolic, flavonoids, tannins, triterpenoids, saponins and steroids and alkaloids which are responsible for antibacterial activity.

Antibacterial Testing (Stephen, 2005).

A sterile cotton sticks was used to inoculate a microbial culture to the agar evenly. Then filter paper disc (about 6 mm in diameter) containing 50 µL of test

compound at a desired concentration, are paced on the agar surface. The petri dishes are incubated at 37 ℃ for a day. The final step is to make observations by measuring the diameter of inhibition zone.

RESULTS AND DISCUSSION

Yield

Table 1 showed the yield data of seaweed extracts and fractions. This data showed that the yield of *C. sertularoides* methanol extract was 0.52% and 1.015% in *P. Australis* methanol extract. The partitioned fractions of methanol extract showed that the water fraction had the highest yield of. 0.255% in *C.sertularoides* and 0.96% in *P.australis*. This data indicated that more polar compounds are contained in this sample of marine algae. This result is supported by the report of **Santoso *et al*., (2004)** on green marine algae (*Ulva reticulata).* In their work, water extracts had a higher yield (7.24%) in comparison to ethyl acetate extract (2.20%) and chloroform extract (0.20%). They also reported in the same study that green marine algae *Caulerpa lentilifera* methanol (polar) extract had a higher yield compared to ethylacetate (semi polar) and hexane (non polar) extracts.

Table 1 Rendement of *C.sertularoides* and *P.australis* Extract and Fraction

Samples	Average Rendement (%)	
	C.sertularoides	***P.australis***
Methanol Extract	0,520	1,015
Hexane Fraction	0,035	0,020
Ethyl Acetate Fraction	0.235	0,025
Water Fraction	0,255	0,965

Antibacterial Activity

Antibacterial activity of methanol extract, hexane, ethylacetate and water fractions from marine algae *C. sertularoides* and *P. australis* can be seen in Table 2.

Table 2 *C. sertularoides* and *P.australis* Antibacterial Activity

No	Samples	Concentration	*C. sertularoides*		*P.australis*	
			E. coli	***S. aureus***	***E. coli***	***S. aureus***
1	Methanol extract	10	7.5	9.5	7,0	10,0
		20	8.5	10,0	9,0	14,0
		30	9,0	11.5	10.5	17,0
		40	10,0	11,5	12,0	18,0
		Control	0	0	0	0
2	Fraction hexane	10	10.5	10,0	7,0	23,0
		20	12,0	12.5	7.5	24.5
		30	12.5	14,0	7.5	30,0
		40	13,0	14,5	9,0	27.5
		Control	0	0	0	0
3	Ethyl Acetate Fraction	10	8,0	6,0	9.5	7,0
		20	10,0	13.5	10,0	9,0
		30	14,0	13,0	10.5	11,0
		40	14,0	16,0	11.5	10,0
		Control	0	0	0	0
4	Water Fraction	10	8.5	7,0,	9,0	9.5
		20	9,0	8,0	8,0	9.5
		30	10,0	9,0	8.5	10,0
		40	11,0	9,0	10,0	8.5
		Control	0	0	0	0

The data in Tables 2 show that the antibacterial activity increased as we increase the sample concentration. Our study revealed that the higher antibacterial activity was indicated by the increased in the diameter of inhibitory zone. Our study is in line with previous study by **Pelczar and Chan (2005).** They reported that at the concentration of brown algae extract *Sargassum* sp 20; 30; 40; 50; 60; 70; 80; 90 and 100 % had antibacterial activity (inhibitory zone) of 0.8; 1,2; 4; 8; 9; 13; 15.7 and 18.6 mm. The results of our study also showed that *S. aureus* were more sensitive to *C.sertularoides* extract with methanol, *C.sertularoides* hexane fraction and *C.sertularoides* ethylacetate fraction and *P.australis* hexane fraction compared to *E. coli.* On the other hand *E. coli* were more sensitive to *C.sertularoides* water fraction, *P australis* water fraction and *P.australis* ethylacetate fraction compared to *S.aureus* (the difference in the sensitivity of bacteria to a bioactive compound is caused by differences in the structure of the bacterial cell wall. Based on differences in the composition and structure of the cell wall, bacteria are divided into Gram positive bacteria and Gram negative bacteria. It was concluded that this difference in cell wall composition can cause differences in bacterial sensitivity to certain compounds (**Bachtiar *et al*., 2012**). The difference in antibacterial activity of a sample against test bacteria is also due to other factors, namely habitat, sampling time, seaweed growth stage, extraction method, extraction solvent and others (**Adaikalaraj *et al.*, 2012**).

The results also showed that *P. australis* hexane fraction had a strong antibacterial activity against *S. aureus* bacteria with a diameter of inhibitory zone greater than 20 mm at a sample concentration of 10 to 40% (Figure 1). A sample is classified as having strong antibacterial activity if it has a zone of inhibition greater than 20 mm (**Lorian, 1980**). The results of this study also indicated that the potential antibacterial compounds against *S. aureus* from *P.australis* marine algae are non-polar compound since they are found in the n-hexane fraction. This data was supported by the results of previous study by Dotulong et al. (2016) They reported that *Laurencia tronoi*, at sample concentration of 2% of n-hexane fraction had a 9.55 mm inhibitory zone diameter against *S.aureus*

The phytochemical analysis shows that *C.sertularoides* ethyl acetate fraction contains almost all phytochemical components, while hexane fractions of *P. australis* contains triterpenoids and saponins. Both components (triterpenoids and saponins) found in the *P. australi*s hexane fraction have the potential as antibacterials. Triterpenoid compounds in plants function as protectors to resist insects and microbial attacks (**Riyanto *et al*., 2013**). Saponins are compounds that are easily crystallized through acetylation so that they can be purified and further studied, potentially hard or toxic saponins are often called sapotoxins (**Harborne, 2006**).

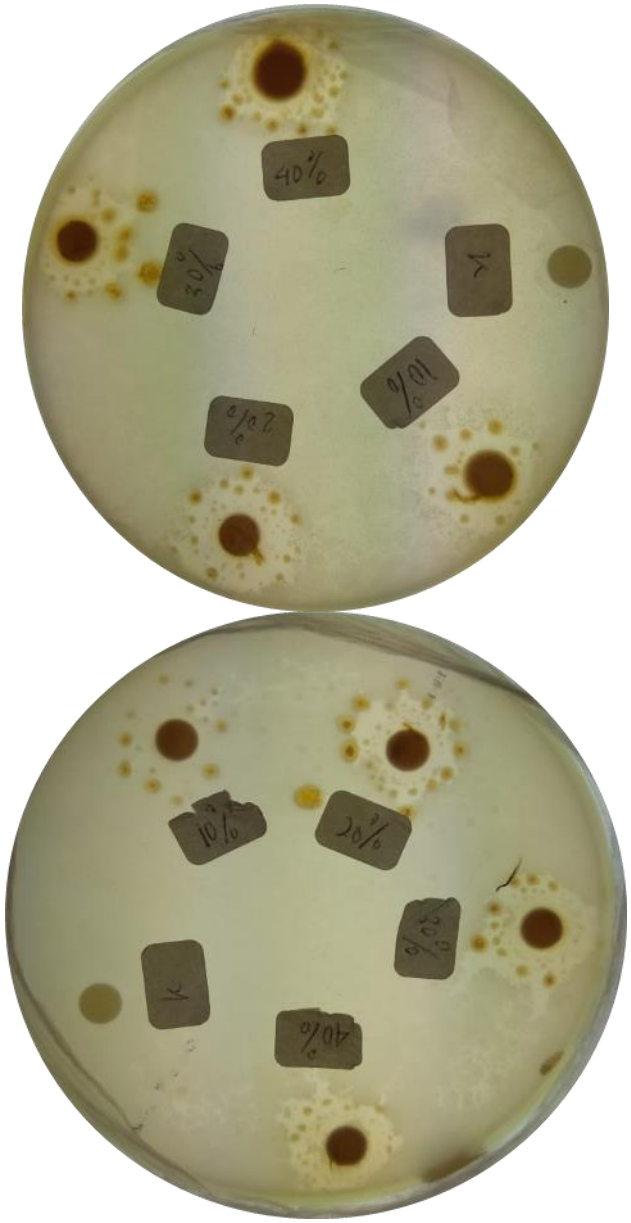

Figure 1 Inhibition zone of n-hexane fraction of *P. australis against S.aureus* at concentration 10, 20, 30 dan 40%

CONCLUSION

S. aureus (gram-positive bacteria) is more sensitive to *C.sertularoides* methanol extract, *C.sertularoides* hexane fraction, *C.sertularoides* ethylacetate fraction and *P.australis* methanol extract compared to *E. coli* (gram-negative bacteria). *E.coli* (gram negative bacteria) is more sensitive to *C.setularoides* water fraction, *P.australis* hexane fraction, *P.australis* ethylacetate fraction, and *P.autralis* water fraction compared to *S.aureus* (gram positive bacteria). Phytochemical analysis results showed that *C.sertularoides* ethylacetate fraction containing almost all phytochemical components, while extracts and other fractions were only a few detected component types. Overall it can be concluded that methanol and fraction-fraction extracts from both types of marine algae have antibacterial activity; the fraction that has the best antibacterial activity is the hexane fraction of *P.australis* against *S.aureus* bacteria because it has a inhibitory zone diameter greater than 20 mm.

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REGULAR ARTICLE

ANTIBIOTICS PRODUCING BACTERIA ISOLATED FROM FARMLANDS

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ABSTRACT

The need for new antibiotics has been highlighted recently with the increasing pace of emergence of drug resistance pathogens. Emerging strains of bacteria resistant to most advanced antibiotics have become issues of very important public health concern. Modification of existing antibiotics with the addition of side chains or other chemical group and genomics based drug targeting have been the preferred method of drug development at the corporate level in recent years. In this regard, soil samples were collected from farmlands located in Ibadan in Oyo state, Ago – Iwoye, and Ikenne in Ogun state, Nigeria. Two putative *Streptomyces* strains and *Bacillus* strains isolated from the 16 selected farmland soils were characterized and assessed for antibiotic production and activity against a wide range of bacteria including *Klebsiella pneumonia*, *Serratia marscens*, *Proteus vulgaris*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus aureus*, and *Enterococcus faecalis*.
The extracts of the putative obtained from *Streptomyces somaliensis*, *Streptomyces anulatus*, *Bacillus megaterium*, and *Bacillus subtilis* showed activities against minimum of 3 and maximum of the 4 of the 7 tested bacteria. Inhibition zones were found to range between 2.0 - 25.0 mm diameters at a concentration of 1ml. The minimum inhibitory concentrations (MICs) of the crude extracts against the tested organisms ranged from 50% and above.
Bacillus megaterium, and *Streptomyces somaliensis* were found to inhibit all the pathogenic bacteria, while *S. anulatus* was unable to inhibit *Proteus vulgaris* and *Staphylococcus aureus*, and *B. Subtilis* was unable to inhibit *Enterococcus faecalis.*

Keywords: Antibiotics, *Bacillus*, Bacteria, Soil, *Streptomyces*

INTRODUCTION

The term soil refers to the outer loose material of the earth crust (**Certini and Ugolini, 2013**). Living portion of the soil body includes small animals and microorganisms such as fungi and bacteria, but it is generally considered that it's microorganisms that play the important role in soil **(Arifuzzaman *et al*., 2010**). Actinomycetic bacteria are best known for their ability to produce antibiotics and the *Streptomyces* are the dominant group (**Pidot *et al*., 2014**). The genus *Streptomyces* is responsible for the formation of more than 60% of known antibiotics (**Gad et al., 2015; Rashad et al., 2015**). Another important species of bacteria known to produce antibiotics is the *Bacillus* spp. Antibiotics are low molecular-weight (non-protein) molecules produced as secondary metabolites, its production is mainly by microorganisms that live in the soil (**Jamil et al., 2007**). Resistance by pathogenic bacteria has become a major health concern, many Gram positive bacteria and Gram negative opportunistic pathogens were becoming resistant to virtually every clinically available drugs **(Redgrave *et al*., 2014**). The use of antimicrobial drugs for prophylactic or therapeutic purposes in human and veterinary or for agricultural purposes, have provided the selective pressure favouring the survival and spread of resistant organisms. Selective pressure favouring the survival and spread of vancomycin-resistant enterococci (VRE) was the consequence of the use of antibiotics in food and agricultural practices. Vancomycin-resistance is often associated with multiple-drug resistance (**Chang *et al*., 2015**).
Another cause of great concern is the Gram-negative antibiotic-resistant opportunistic pathogens. These bacteria, like *Pseudomonas aeruginosa*, are common environmental organisms, which act as opportunistic pathogens in clinical cases where the defense system for patient is compromised (**Sanjar *et al*., 2016**). For instance, over 80 % of cystic fibrosis (CF) patients become chronically infected with *P. aeruginosa*. In addition, other intrinsically antibiotic resistant organisms such as *Burkholderia cepacia* and *Stenotrophomonas maltophilia* are emerging as opportunistic pathogens (**Chung *et al*., 2013**). The appearance of multi resistant pathogenic strains have caused a therapeutic problem of enormous proportions (**Barsby *et al*., 2001**). For instance, they cause substantial morbidity and mortality especially among the elderly and immunocompromised patients. With increasing misuses of antibiotics, the serious problems of antibiotic resistance are developing at an alarming rate. Thus, new therapeutic drugs and approaches are needed to improve the management of these diseases and overcome these problems (**Taylor *et al*., 2002**).
Hence, intensive search for new antibiotics has become imperative worldwide especially from natural sources such as soil which is known as the greatest source of antibiotics. New microbial metabolites are permanently needed due not only to the increase in resistant pathogens, but also to the evolution of novel diseases and toxicity of currently used compounds **(Parugao *et al*., 2008**).
This study aims at exploring the antibiotics production potentials of some indigenous soil bacteria isolated from farmlands in some selected locations in Oyo and Ogun state, South Western part of Nigeria. The objectives include: (i) to isolate antibiotic producing bacteria from farmland (ii) to characterize to species level the isolated bacteria (iii) to determine and estimate the antimicrobial efficiency of each of the antibiotics produced by the bacteria.

MATERIALS AND METHOD

Study area

The soil samples were collected from farmlands located in Ibadan, Ago – Iwoye, and Ikenne. Seven samples were collected from individual farmlands in Ibadan which is the capital of Oyo state, while five samples were collected from government farmland located in Ago – Iwoye, and four other samples were collected from IITA research station located in Ikenne, both located in Ogun state.

Media used

The media used were nutrient agar, potato starch casein agar, starch casein agar, and Mac Conkey agar. They were prepared according to the manufacturer's instruction. The media were left to cool to about 45^0c after sterilization before being poured aseptically into the Petri dish.
Serial dilution of soil samples: The soil samples were labeled according to the area of collection. Then 1g was weighed from each soil samples for serial dilution where the10^{-2} to 10^{-4} dilutions were plated.

Plating of the samples

The diluted soil samples were inoculated onto the appropriate agar medium using the streaking method, and they were labeled accordingly. Nutrient agar was used for isolating bacteria, while potato starch casein agar was used for isolating actinomycetes. After inoculation, the plates were incubated at 37^0C over night for bacteria, and while in the case of actinomycetes incubation lasted for 4 days at a temperature of 30^oC before morphological changes can be seen.

Sub culturing

After the completion of the incubation period, the culture media were checked for the growth of organisms, colonies were seen on nutrient agar indicating the presence of bacteria, while potato starch casein agar also show the presence of colonies. Upon this observation, Mac-Conkey agar, and starch casein agar were both prepared aseptically by autoclaving and following the manufacturer's instruction. Using a sterile inoculating loop, morphologically different colonies were picked from the mixed culture of bacteria in the nutrient agar into a new medium (Mac-Conkey agar) under an aseptic condition with proper labeling and it was incubated overnight at 37^0 C, while morphologically different colonies were also picked from potato starch casein agar using a sterile inoculating loop under an aseptic condition into a medium of starch casein agar and it was incubated at 30ºC for 4 days.

Pure culturing

Nutrient agar medium was prepared in a conical flask and sterilized. The nutrient agar medium is poured into sterile vials and allowed to solidify by placing in a slanting manner in the laminar air flow. Pure isolates were carefully collected and streaked on the slant aseptically. The vials were properly closed by cotton plugs and kept in the refrigerator as stock culture.

Biochemical tests

Gram reaction, spore staining, catalase test, gelatin hydrolysis test, casein test, tyrosine test, xanthine test, urease test, starch hydrolysis test, growth in lysozyme, lactose fermentation, cellobiose test, xylose test, mannitol fermentation test, lipid hydrolysis test, glucose fermentation test, and voges proskauer (vp) test were conducted on the isolated organisms and the results were recorded.

Preparation of suspension

Colonies of the pure stocked culture were carefully picked and aseptically suspended into nutrient broth and they were incubated at 35ºC for 24 hours and 4 days for bacteria and *Streptomyces* respectively according to **Biradar *et al***. (2016).

Primary screening for antibiotic production

After the incubation period, the broth cultures were centrifuged for 15 minutes at 4000 rpm to obtain the supernatant of their metabolites which will be used to test some organisms. The supernatants were filtered and were aseptically transferred to a Mac-Cartney bottle and they were labeled and stored at a temperature of -4ºC following the method of **Rai *et al*.** (2016).

Preparation of test organisms

The test organisms used for the screening were clinical samples, and they include; *Klebsiella pneumonia*, *Serratia marscens*, *Proteus vulgaris*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus aureus*, and *Enterococcus faecalis*. All the test bacteria were grown in 4.50 ml nutrient broth for 12 hours, and then standardized to Mac farland standard of 0.1 at optical density (OD) 600nm before use as described by **Othman *et al***. (2011).

Screening for antibacterial activity

The supernatants gotten were screened for antibacterial activity using the cup well agar method as described by **Valan *et al***. (2012). For this purpose sterilized molten Mueller Hinton agar (MHA) was poured into a Petri dish and allowed to solidify under the laminar air flow, then 50µl of the standardized bacteria was swirl gently and aseptically spread on the medium. Sterile cork borer (6mm diameter) was used to bore a hole in the plate. Then 100 µl of the antibiotic extract was carefully dispensed into the bored holes. This was done without allowing the extract to overflow the well. Extracts were allowed to diffuse for about 2 hrs before incubating. Plates were incubated at 37ºC for 24 hours. The presence of zone of inhibition around each well was indicative of antibacterial activity.

RESULTS

A total number of 85 organisms belonging to six different species of bacteria were isolated from the soil samples. *S. Somaliensis*, *B. subtilis*, *Proteus vulgaris*, and *Pseudomonas aeruginosa* were found to be the most predominant of the isolated microorganisms with prevalence rate of 18.82%, this was closely followed by *B. megaterium* and *S. anulatus* with prevalence rate of 12.94% and 11.77% respectively as shown in (Table 1).

Table 1 Prevalence of microorganisms in the examined soil samples

Organisms	N	%
S. somaliensis	16	18.82
S. anulatus	10	11.77
B. subtilis	16	18.82
B. megaterium	11	12.94
Proteus. vulgaris	16	18.82
Pseudomonas. Aeruginosa	16	18.82
TOTAL	**85**	**100**

The Gram reaction and the biochemical characteristics of the organisms isolated from the farmlands examined indicated that *Streptomyces* spp was the only isolated actinomycetes. The isolated *Streptomyces* were *S. somaliensis*, and *S. anulatus*. These two organisms were differentiated based on their cell morphology on plates or cultural characteristics (casein utilization, tyrosine utilization, xanthine utilization, urea utilization, xylose utilization, cellubiose utilization, gelatin hydrolysis, starch hydrolysis, growth in lysozyme, and lactose fermentation). *S. somaliensis* produces cream to brown colour on starch casein agar, while *S. anulatus* was found to be white, chalky, velvety colour on the same medium. *S. anulatus* was positive to almost all the tests except gelatin hydrolysis test, while *S. somaliensis* was unable to utilize xanthine, urea, xylose, and cellubiose. Also it didn't hydrolyse gelatin, and was found to be a non lactose fermenter as depicted by (Table 2).

Table 2 Gram reaction and biochemical characteristics of actinomycetes isolated from different farmlands

Gr	Cm	Ca	Ty	Xa	Ur	Ge	St	Gi	La	Xy	Ce	Probable Organism
+	Cream to brown	+	+	-	-	-	+	+	-	-	-	*S.somaliensis*
+	Chalky, white, or velvety	+	+	+	+	-	+	+	+	+	+	*S.anulatus*

Key: Xa = Xanthine Utilization, Ur = Urea Utilization, Gr = Gram Reaction, Ge = Gelatin Hydrolysis, Cm = Cell Morphology, St = Starch Hydrolysis, Ca = Casein Hydrolysis, Gi = Growth in Lysozyme, Ty = Tyrosine Utilization, La = Lactose Fermentation, Xy = Xylose Utilization, Ce = Cellobiose Utilization.

The other bacteria isolated from this study apart from *streptomyces* were *Bacillus subtilus*, *Bacillus megaterium*, *Proteus vulgaris* and *Pseudomonas aeruginosa*. *Bacillus. subtilus* and *B. megaterium* were differentiated based on the size of their spore, ability to lyse lipids and Voges proskauer test. *B. megaterium* has large spore while *B. subtilus* has small spore. On the other hand, *B. subtilus* is positive to lipolysis test, while *B. megaterium* is negative. Also *B. subtilis* is positive to VP test, while *B. megaterium* is negative to the test. In general, both species of bacillus are Gram positive, spore containing, catalase positive, non-lactose fermenters with ability to ferment mannitol and glucose, hydrolyse both gelatin and starch, and can't utilize urea.

Consequently, both *Pseudomonas aeruginosa* and *Proteus vulgaris* were found to be Gram negative rod, catalase positive, non-lactose fermenters, non-spore forming, negative to starch hydrolysis, but positive to gelatin hydrolysis and

negative to VP test. But they are differentiated by mannitol test, lipid hydrolysis test, glucose fermentation test, and ability to utilize urea as shown in (Table 3).

Table 3 Gram reaction and the biochemical characteristics of the other isolated organisms.

Gr	Cm	La	Sp	Ma	Li	Ca	Ge	St	Gl	Ur	Vp	Probable Organism
+	Rod	-	+ (small)	+	+	+	+	+	+	-	+	*Bacillus. subtilis*
+	Rod	-	+ (large)	+	-	+	+	+	+	-	-	*Bacillus. megaterium*
-	Rod	-	-	+	-	+	+	-	-	-	-	*P. aeruginosa*
-	Rod	-	-	-	+	+	+	-	+	+	-	*Proteus. Vulgaris*

Key;
Gr = Gram Reaction, Ge = Gelatin Hydrolysis,Cm = Cell Morphology, La = Lactose Fermentation, Sp = Spore Staining, Ma = Mannitol Fermentation,Li = Lipid Hydrolysis, Ca = Catalase, St = Starch Hydrolysis, Gl = Glucose Fermentation, Ur = Urea Utilization,VP = Voges Proskauer.

Pseudomonas. aeruginosa is a mannitol fermenter, while *Proteus vulgaris* can't ferment mannitol. *P. vulgaris* can hydrolyse lipid, while *P .aeruginosa* is negative to the test. Also *P. aeruginosa* is negative to both urea and glucose test, while *P. vulgaris* is positive to both.

The antibacterial activity of the biomolecule from the antibiotic producing bacteria. The biomolecule extracted from each of the antibiotic producing organisms were challenged against seven different bacteria pathogens viz; *Klebsiella pneumonia*, *Proteus vulgaris*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Serratia marscens*, *E. coli*, and *Enterococcus faecalis* as displayed in (Table 4).

Table 4 Antibacterial activity of the biomolecule extracted from the antibiotic producing organisms.

	Zone of inhibition (mm)			
Organisms	**SS**	**SA**	**BM**	**BS**
K. pneumonia	18.00	19.00	9.50	11.50
Proteus vulgaris	21.00	0.00	20.50	8.00
S. marscens	16.50	18.50	20.00	10.50
P .aeruginosa	3.00	6.50	14.50	2.00
E. coli	22.50	18.00	16.50	2.50
S. aureus	12.50	0.00	25.00	19.00
E. faecalis	23.50	25.00	20.00	0.00
Average total of zone of inhibition	**16.60**	**12.40**	**17.90**	**7.60**

Key:
SS= *Streptomyces somaliensis*, SA = *Streptomyces anulatus*, BS= *Bacillus subtilis*, BM = *Bacillus megaterium.*

The biomolecule extracted from *B. megaterium* was found to be the most efficacious of all the tested biomolecules and it was closely followed by *S. somaliensis*. The biomolecule from *S. anulatus* was also effective against some organisms except that it has no effect on *S. aureus* and *Proteus vulgaris*. The extract from *B. subtilis* also tends to be effective, but it has no activity against *Enterococcus faecalis*.

DISCUSSION

With the increasing demand for new antibiotics and novel bioactive compounds, it is necessary to find new strategies that can increase the effectiveness of the search. This present study was carried out to study the production of antibiotic from isolated soil organisms. Different bacteria were isolated from soil and identified as *Bacillus* and *streptomyces*. All showed antibacterial activity against various pathogens.

The antibiotic producing microorganisms isolated from the soil samples taken from various farm lands in Oyo and Ogun state were *Bacillus* spp and *Streptomyces* spp of all the bacterial species originally isolated. The results conform to many reviews of literature that *Bacillus* spp and *streptomyces* spp are known to produce bioactive secondary metabolites (antibiotic). **Al-Ajlani and Hasnain** (2010) in a research discovered the production of bacitracin and subtilin by *Bacillus* spp. **Ayitso and Onyango** (2016) reported that bacitracin produced by *Bacillus* spp inhibits *Escherichia coli* and *Staphylococcus aureus* which corroborated the result in this present study.

Many of the strains evaluated in the present study showed bioactivity against one or more of pathogenic microorganisms. It is becoming increasingly evident that the taxonomic and metabolic diversity encompassed by *Streptomycetes* and *Bacillus* are remarkable, as new and putatively novel *Streptomyces* species are being continuously isolated from under-researched habitats.

Of all the pathogenic organisms tested with the extract, both *Pseudomonas* and *Proteus* spp have lower zones of inhibition compared to their Gram positive counterpart. This observation corroborate with that of **(Zgurskaya and Nikaido**, 2000).

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REGULAR ARTICLE

CHARACTERIZATION AND OPTIMIZATION OF KERATINOLYTIC BACTERIA *Pseudomonas stutzeri* C3 ISOLATED FROM POULTRY WASTE SOIL SAMPLE

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ABSTRACT

Microbial degradation of keratinous wastes is preferred over physicochemical methods, because it's a cost effective and eco-friendly. Novel habitats are promising for discovery of new microbial strains. Towards isolate a keratinolytic bacteria, screening of bacterial strains from a poultry farm soil in Namakal district, Tamil Nadu, India was done and a promising the isolate C3 was found to degrade native chicken feather efficiently. It could grow over a pH range 7 at 40°C and in presence of potassium nitrate as a nitrogen source and citrate as a carbon source. Based on phenotypic characterization and 16S rRNA gene sequence analysis, the keratinolytic isolate was identified as *Pseudomonas stutzeri* C3. It produced 30.86±1.08 U/ml keratinase in raw feather medium and complete feather degradation at 5 days incubation periods.

Keywords: Microbial degradation, keratinase, poultry farm, *Pseudomonas stutzeri*

INTRODUCTION

Keratin is the most abundant protein in epithelial cells and forms major components of skin, hair, nail, feather and wool. Keratins are grouped into hard keratins, found in appendages such as feather, hair, hoof and nail and soft keratins found in callus and skin. The former have high disulfide bond content and are tough and inextensible whereas soft keratins such as skin and callus have low content of disulfide bridges and are more pliable (**Gupta and Ramnani, 2006**). Structurally, keratins are classified as α-keratins (hair, hooves, nails, etc.) and β-keratins (feather, silk fibrion, β-amyloid) **(Voet and Voet, 1995; Akthar and Edwards, 1997)**.

Environmental wastes are found in large quantities in many countries. Although some of them contain a considerable amount of protein and various carbon compounds, little attention is given to utilizing or recycling these wastes in a technological way. Additionally, the accumulation of some of these wastes in nature is considered to be a serious source of pollution and health hazards. Therefore, their proper disposal may be considered as a means of avoiding environmental pollution. Recently, we have focused on the utilization of some polymeric wastes, mainly feather waste (**Park and Son 2009**). It is estimated that nearly 24 billion chickens are killed yearly (which is increasing per year) and leads to production of nearly 4 billion pounds of feathers as a waste from commercial (large and small scale) poultry industries around the world. Naturally a feather takes 3 to 4 years to get degraded due to solid structure of keratin protein. Disposal of feather waste is a major problem because simple dumping in the ground leads to the soil pollution and burning it adds to the SO_2 and CO_2 content in the environment and causes air pollution. This mammoth size of discarded feather, apart from polluting the soil or air, also causes various human ailments including chlorsis, mycoplasma and fowl cholera (**Revathi *et al.*, 2013**). Traditional ways to feather converted to feather meal through steam pressure and chemical treatment (**Poole *et al.*, 2009** and **Freeman *et al.*, 2009**). Though making keratin waste more digestible, this chemical treatment process is both costly and destructive to certain amino acids (**Xie *et al.*, 2010, Syed *et al.*, 2009** and **Tatineni *et al.*, 2008**). The nutritional upgrading of feather meal with the treatment of microbial keratinase might lead to a significant increase in the availability of amino acids in feather keratin. Keratinase is an enzyme which was secreted by many microorganisms that hydrolysis keratin in to smaller molecule (**Bin *et al.*, 2009**). Despite the recalcitrance keratin waste can be efficiently by degraded by a myroid of bacteria (**Cheng *et al.*, 2008; Bin *et al.*, 2009**); actinomycets (**Amany *et al.*, 2009**) and fungi (**Scott *et al.*, 2004**) due to the elaboration of keratinolytic proteases and keratinase (**Onifade *et al.*, 1998**). In fact, keratinases have been successfully applied in several biotechnological processes, including the introduction of enzymatic dehairing in the leather industry (**Marimuthu *et al.*, 2016**), production of slow-release nitrogen fertilizers in the agricultural industry (**Siddharthan *et al.*, 2017**), and synthesis of biodegradable films and coatings in the biomedical industry (**Anbu *et al.*, 2007, Freeman *et al.*, 2009** and **Brandelli *et al.*, 2010**). The present study aims at the degradation of feather by keratinase produced by *Pseudomonas stutzeri* C3, a keratinolytic bacteria isolated from poultry farm waste site and the optimization of keratinase production.

MATERIALS AND METHODS

Collection of Sample

The poultry waste soil sample was collected from Poultry Farm, Namakkal district, Tamil Nadu, India. The soil sample was collected in sterile polythene bag and transported to the laboratory maintaining temperature around 4°C as early as possible for analysis.

Isolation of Keratinolytic Bacteria

The soil sample was serially diluted with sterile distilled water upto $10^{-7}$and the bacteria were isolated on the feather meal agar plates by the spread plate technique. Feather meal agar (FMA) plates containing: feather meal (0.5g), NH_3Cl_2 (0.05g), NaCl (0.05g), $MgCl_2$ (0.01g), K2HPO4 (0.03g), KH_2PO_4 (0.04g), yeast extract (0.1g), agar (1.7g) and distilled water (100 ml) with a pH of 7.5 (**Saraniya and Baskaran, 2016**). Plates were then incubated at 37°C for 24-36 h. Colonies forming transparent zones around the bacterial colony due to hydrolysis of keratin. The keratin hydrolyzed bacteria were screen to further process.

Screening of Keratinase Production

A raw feather basal medium containing: $MgSO_4.7H_2O$ (0.2 g), K_2HPO_4 (0.3 g), KH_2PO_4 (0.4 g), $CaCl_2$ (0.22g), yeast extract (0.1g) and chopped raw feather (0.1g) in 100 ml distilled water. The medium was sterilized at 121ºC for 15 minutes. The isolates which showed positivity in feather meal agar medium were inoculated and the flasks were incubated at 37ºC for 5 days on a rotary shaker at 180 rpm. 5 ml of samples were, centrifuged at 5000 rpm for 30 minutes and used for keratinase determination. (**Saraniya and Baskaran, 2016**)

The keratinolytic activity was assayed as follows: 1.0 ml of crude enzyme properly diluted in phosphate buffer (0.05 mol/L, pH 8.0), was incubated with 1 ml keratin solution at 50°C in a water bath for 10 min, and the reaction was stopped by adding 2.0 ml of 0.4 mol/L trichloroacetic acid. After centrifugation at 4000 rpm for 30 min, the absorbance of the supernatant was determined at 280 nm against a control. The control was prepared by incubating the enzyme solution with 2.0 ml TCA without the addition of keratin solution. One unit (U/ml) of keratinolytic activity was defined as an increase of corrected absorbance of 280 nm (**Gradisar *et al.*, 2000**) with the control for 0.01 per

minute under the conditions described above and calculated by the following equation:

$U=4\times n\times A280/(0.01\times 10)$

Where *n* is the dilution rate; 4 is the final reaction volume (ml); 10 is the incubation time (min) according to **Cai *et al*., (2008)**.

Identification by 16S rRNA Sequencing

The maximum keratinase sproducing potential bacterial isolate was studied for its morphological, cultural and biochemical characteristics according to Bergey's Manual of Systemic Bacteriology. The strain was also identified by PCR using 16S rRNA as a molecular marker. The primers used were as follows: forward primer (5′ CAGCCGCGGTAATAC 3′), and reverse primer (5′ ACGGGCGGTGTGTAC 3′). PCR was run for 30 cycles using the DNA thermal cycler. The PCR products were analyzed in a 1% (w/v) agarose gel with ethidium bromide before being sent for sequencing analysis. The DNA sequences were aligned and compared using the BLAST algorithm to find homologous sequences in the GenBank database of NCBI. The data were submitted to the GenBank database (**Younes Ghasemi *et al*., 2012**). A phylogenetic study of the selected strain was performed using the ClustalW program within MEGA6 software (**Tamura *et al*., 2013**). The branching pattern was designed based on the neighbor-joining method.

Optimization of Cultural conditions

Feather degradation was carried out in raw feather medium. The medium was inoculated with 5 % (v/v) inoculum. The inoculated flasks were kept incubated on a rotary shaker in150 rpm at 30 °C. Time course of feather degradation was monitored by taking the fermentation broth at various time intervals and monitoring bacterial growth rate and keratinase production. Effect of feather concentration was monitored by adding feather at different concentrations. Effects of incubation temperature were also studied by growing C3 in raw feather medium at different temperatures. For evaluating effects of carbon and nitrogen supplements on feather degradation, various carbon sources viz. glucose, sucrose, citrate, corn starch and lactose were added in raw feather medium at 1 % (w/v) concentration and nitrogen sources viz. peptone, yeast extract, soybean meal, beef extract, KNO_3 and NH_4Cl were added at 0.5 % (w/v) concentration.

Microscopic Observation of Feathers

The potential bacterial strain was grown in FM and the fermentation broth was filtered with Whatman No.3 filter paper after incubation. The filtered feather was fixed with 2.5 % (v/v) glutaraldehyde and 2 % (v/v) formalaldehyde for 24 h. The specimens were dehydrated several times with 70–80 % acetone and dried at 50 °C for 10 min. The specimens were observed using FEI Quanta 250 scanning electron microscope and bright field microscope (**Rahayu *et al*., 2012**).

Statistical Analysis of Data

All the above experiments were repeated in triplicate and the final values have been presented as mean ± S.D.

RESULTS AND DISCUSSION

Isolation of Keratinolytic Bacteria

In the preliminary screening, 8 isolates showing keratinolytic activity by producing clear zones on feather meal agar plates (Fig.1) were selected for transfer to basal feather medium. However, 8 colonies growing well on this medium were chosen for further studies. Initial morphological identification showed that 5 isolates were Gram-positive, spore-forming bacilli and 3 were Gram-negative and rod-shaped.

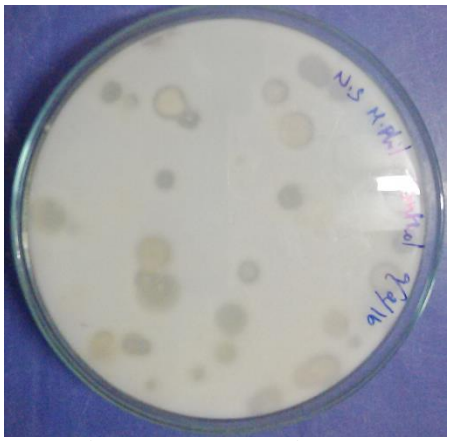

Figure 1 Isolation of Keratinolytic Bacteria from Feather meal agar Plate

Screening of Keratinase Production

The promising feather degrading isolates were selected for quantitative determination of enzyme activity using raw feather medium. The measurement of the keratinolytic activity revealed a range from 15.80±0.32 to 30.86±1.08 U/ml (Table.1). One isolate exhibited the highest keratinolytic activity (30.86±1.08 U/ml) in its culture supernatant. Moreover, it presented pronounced growth and complete hydrolysis of native chicken feathers. This isolate was designated C3 and was selected for identification, characterization and optimization studies. *Bacillus* sp FK 46 was maximum keratinase produced at 0.9 U/ml from soil sample (**Suntornsuk and Suntornsuk, 2003**). *Arthrobacter* sp 108 showed 5 U/ml keratinase activity on feather meal agar at 7 days of incubation periods (**Kate and Archana, 2014**).

Table 1 Keratinase Activity of Isolated Strains

S.No	Isolated Strains	Keratinase Activity (KU/ml)
1	C1	27.95±1.25
2	C2	15.81±1.31
3	C3	30.86±1.08
4	C4	15.80±0.32
5	C5	13.45±0.99
6	C6	25.13±0.34
7	C7	21.18±0.75
8	C8	18.93±0.50

Identification by 16S rRNA sequencing

The cultural, morphological and physiological characteristics of strain C3 was summarized in Table 2. Briefly, the isolate *Pseudomonas stutzeri* C3, proved to be Gram negative straight rod-shaped cells with non-spore forming. It formed opaque creamy circular colonies on feather-agar plates whereas the colonies were irregular in shape and mucoid on nutrient agar plates. Based on morphological characterization and 16S rDNA sequence analysis, C3 was identified as a *Pseudomonas* sp. and designated as *Pseudomonas stutzeri C3* (NCBI GenBank Accession No. **MF092721**). It showed closest 16S rRNA gene sequence similarities with *Pseudomonas stutzeri* (93 %) (Fig.2). However, earlier study rachises were completely degraded by strain *Pseudomonas stutzeri* at 5 days incubation, indicating complete degradation of chicken feathers (**Venkatesh *et al*., 2013**).

Table 2 Morphological and Biochemical Characteristics of Potential Strain

S.No	Characteristics	Result
1	Gram staining	Negative
2	Shape	Rod
3	Motility test	Motile
4	Capsule staining	Non capsulated
5	Spore forming	Non spore forming
6	Pigment	Positive
7	Catalase	Positive
8	Oxidase	Positive
9	Indole	Negative
10	Methyl red	Negative
11	Voges proskauer	Negative
12	Citrate test	Positive
13	Urease test	Negative
14	H_2S production	Negative
15	Gas production	Positive
16	Nitrate reduction test	Positive
17	Starch hydrolysis	Positive
18	Coagulase test	Negative
19	Cetrimide agar	Positive

The evolutionary history was inferred using the Neighbor-Joining method (**Saitou and Nei, 1987**). The optimal tree with the sum of branch length = 12.52928651 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (**Tamura *et al.*, 2004**) and are in the units of the number of base substitutions per site. The analysis involved 9 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 911 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 **Tamura *et al.*, 2013**).

Optimization of Keratinase Production

Feather concentration was affected to bacterial growth and feather degradation. Bacterial growth also increased as the feather concentration increased, while the percentage of feather degradation was inversely proportional to the feather concentration. Keratinase was, produced at a similar level at different feather concentrations. It was demonstrated that high feather concentrations may cause substrate inhibition /repression of keratinase production, resulting in a low percentage of feather degradation. The results were similar to a previous report of **Cheng *et al.* (1995)** which showed that the minimum amount of feather powder (1%) yielded the highest keratinase activity from *B. licheniformis* PWD-1. The initial pH of the medium also greatly affected bacterial growth, percentage of feather degradation and keratinase production. The optimum pH for bacterial growth, feather degradation and keratinase production was 7. The growth, feather degradation and keratinase production of *Pseudomonas stutzeri C3* were optimal at an incubation temperature of 40ºC (Fig. 3). Similarly maximum keratinase production was observed at pH 7 and 40°C, inoculum size of 5% (v/v) and inoculum age of 7 d (**Ningthoujam *et al.*, 2016**). Citrate as a carbon source and a potassium nitrate was a nitrogen sources to obtained maximum enzyme production. Among the carbon sources tested, sodium citrate was found to be the best carbon supplement for keratinase production followed by glucose, corn starch and sucrose (**Kshetri and Ningthoujam, 2016**).

Microscopic Observation of Feathers

Scanning electron microscopy was applied to visualize and confirm the keratinolytic degradation of feather. As illustrated on Fig.4, the degree of feather degradation increased when feather was incubated with *Pseudomonas stutzeri C3*. The pictures show the degradation of feather barbules along with disintegration of the feather rachis structure after incubation. Similarly **Pedersen *et al.*** (2015) reported the protease from *B. subtilis* highly feather degrading compared with 3 commercial available proteases.

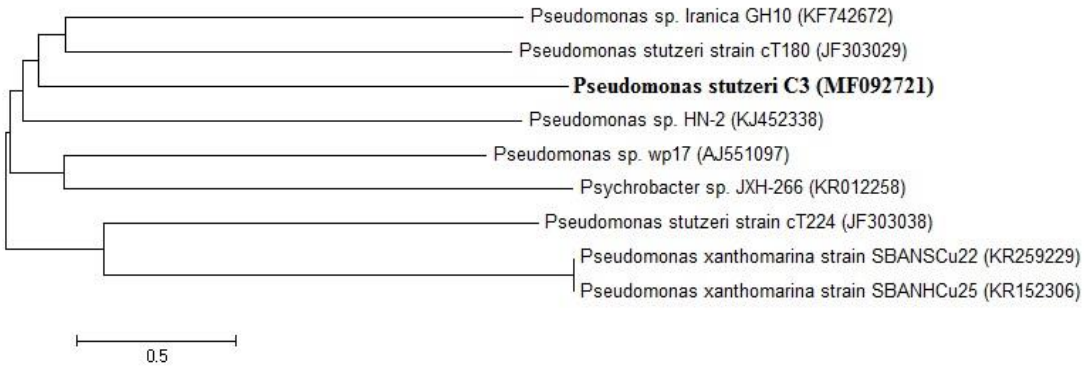

Figure 2 Phylogenetic Analysis of Potential Strain

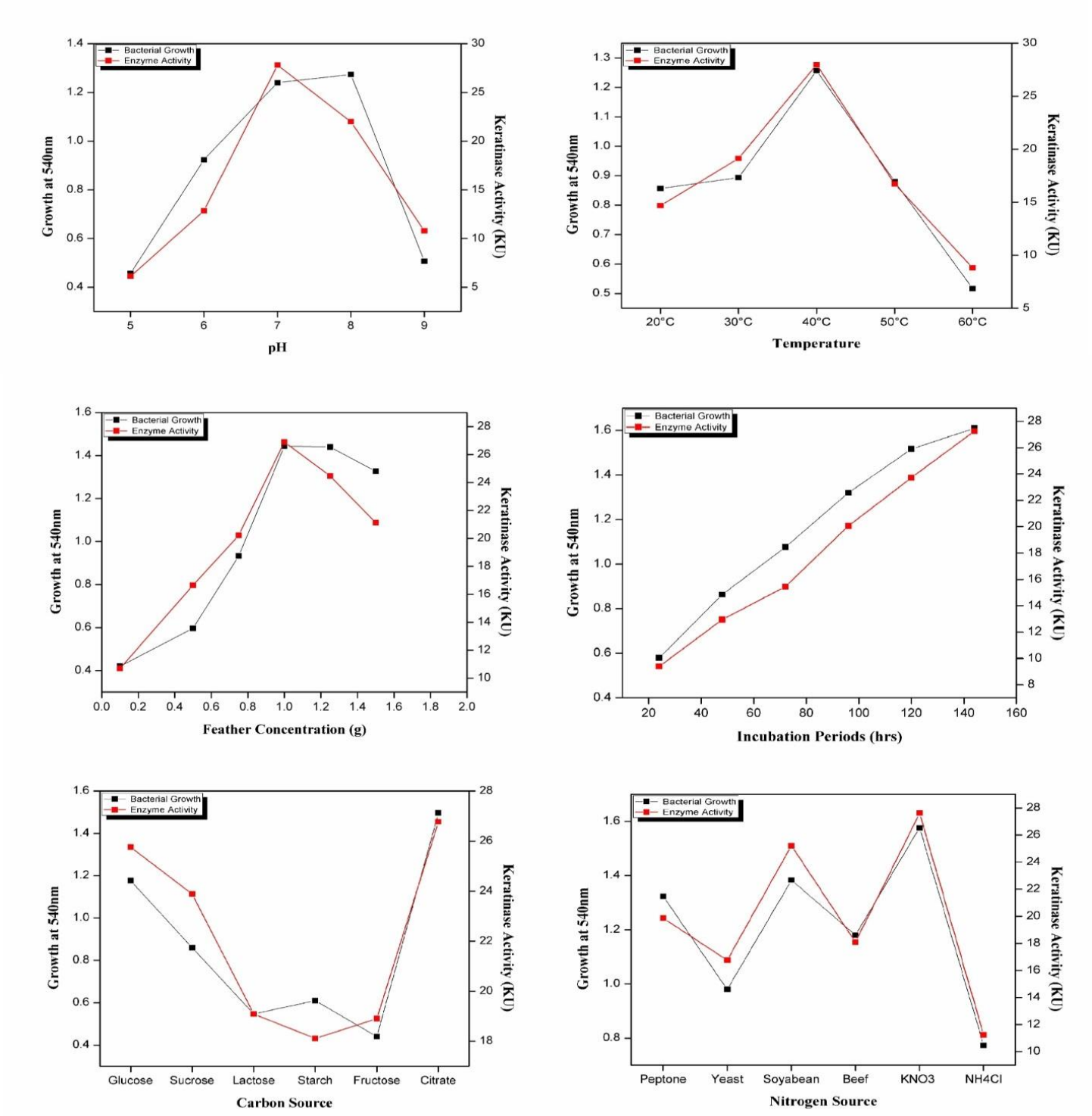

Figure 3 Optimization of Potential Strain and Enzyme Production

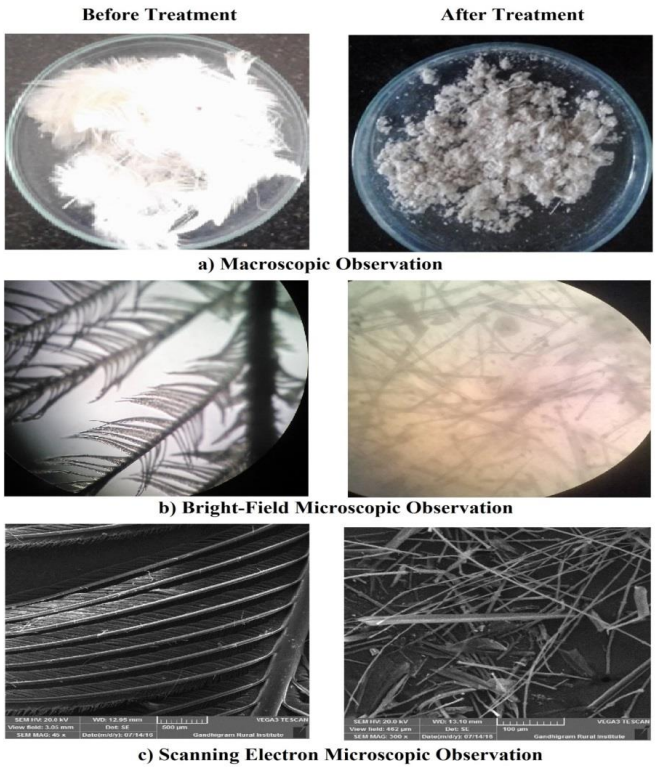

Figure 4 Macroscopic and Microscopic Observations of Feather Degradation

CONCLUSION

In conclusion, this present study was isolated *Pseudomonas stutzeri* C3 possesses high keratinolytic activity and is effective in feather degradation in a short period of 5 days at 40°C and pH 7, potential uses for biotechnological processes. This study also demonstrate the potential useful of feather as a cheap source fermentation substrate for the production of industrial enzyme and as a source of valuable amino acids and soluble proteins that can be used as a digestible feed for animal.

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