DOI: 10.24996/ijs.2019.60.10.2

ISSN: 0067-2904

Evaluation of Secondary Metabolites of Some Fungi Isolated From Beach Soils of Lagos, Nigeria Against Some Pathogens

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Abstract

Three beach soils in Lagos, Nigeria were screened for the presence of antibiotic producing fungi against 8 test pathogenic bacteria & fungi. The physiochemical parameters of the soils were determined following standard procedures. Soil plate dilution method was employed for isolation of marine fungi and they were identified based on cultural and microscopic characteristics. Primary screening of isolated fungi for antibiotic potential was determined by perpendicular streak method against known pathogenic test organisms *(Escherichia coli, Saphylococcus aureus, Klebsiella pnuemoniae, Pseudomonas aeruginosa, Candida albicans, Saccharomyces cerevisiae,Aspergillus niger and Aspergillus fumigatus*). Further sreening of the product of secondary metabolism [antibiotic] of the active fungi was done by agar disc diffusion assay. The fungus with the highest secondary activity was subjected to molecular analysis for further identification. pH of beach soils was alkaline ranging from 7.6-8.0, while temperature and moisture were normal ranging from 26.333 ± 1.155^{a}-$30.333 \pm 0.577^{a}$and 7.142 ± 1.497^{a}-10.030 ± 3.130^{a} respectively. A total of four fungal species of 2 different genera were isolated from the beaches ;*Aspergillus niger*,*Aspergillus flavus*,*Penicillium purpurogenum* and *Penicillium islandicum*. Both primary and secondary assays revealed only antibacterial activities against *Staphylococcus aureus* and *Psedomonas aeruginosa* with no effect on all test fungi. Highest antibacterial activity[28 ± 0.2^{a}] was exhibited by the fungus identified as *Penicillium purpurogenum*. Fungi from beaches in Lagos, Nigeria may be a promising source of antibacterial agent useful in the treatment of infections caused by *S. aureus* and *P. aeruginosa*.

Keywords; Antibacterial, Beach Soil, Agar diffusion, *Penicillium purpurogenum*, Lagos

Introduction

Soils are traditionally the main source of fungi [1] and other numerous microorganisms producing structurally complex bioactive products of pharmaceutical importance. Soil microorganisms have continually been screened to harness beneficial biologically active metabolites. Out of over 23,000 identified secondary metabolites42% are from fungi especially *Penicillium* spp. [2].

The marine and fresh water environments are known to contain taxonomically diverse fungi groups which exhibit unique physiological and structural characteristics that enable them to survive in extreme environmental conditions, with the potential production of novel secondary metabolites not observed in terrestrial microorganisms. Antibiotic producing microorganisms can be isolated from aquatic habitats such as streams, rivers, lake mud, river sediments, beach sands and marine sediments [3]. Of all biologically active substances produced by microorganisms, 45% are derived from actinomycetes, 38% from fungi and 17% from other bacteria [4]. It has been claimed that marine environment is unique in terms of its specific composition in both organic and inorganic substances, as well as temperature ranges, and pressure conditions [5].Several compounds isolated from marine ecosystem have shown promising antibacterial, antifungal and antiviral activities [5].

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Fungi are found primarily in the top 10 cm of the soil and are rarely found below 30 cm. They are most abundant in well-aerated and acidic soils. Marine fungi are an important target group for useful products of industrial importance such as enzymes, sugar, alcohol, beverages, and food products [6]. The constant emergence of multidrug resistant pathogens has led to substantial morbidity and mortality and to overcome this, it is necessary to discover novel antibiotics by continuous screening of secondary metabolites from microbial sources.

Therefore, this present study is designed with a view to isolate, screen and characterize antibiotic producing fungi from marine and fresh water soils in the cities of Lagos and Ilorin, Nigeria.

2. Materials and Methods

2.1Sampling area

9 soil samples were collected from three beaches in the city of Lagos in Nigeria. They were; Alpha, Elegushi, and Oniru. Alpha beach is located in Lekki, east of Lagos mainland (Latitude-6025’23.2248’’N and Longitude – 3031’25.7664’’E). Elegushi beach is located about 8km from Alpha beach (latitude – 6025’19.6212N and longitude – 3029’11.8716’’E) while Oniru beach is situated at Victoria Island (Latitude – 6025’20.5356’’N and longitude 3026’39.138’’E).

2.2 Sample Collection

Sterile hand trowel was used to collect sand from a depth of 10cm into sterile plastic containers and transferred aseptically to the laboratory for further analysis. These were air dried for a period of 7-10 days at 25 ^{o}C [7].

2.3 Physicochemical Characteristics of Beach Soil Samples

The following parameters were determined in the soil samples collected.

2.3.1 Determination of Soil pH

Twenty grams(20g) of soil was mixed with 20ml of water in a beaker to obtain slurry. The electrode of the pH was calibrated with distilled water and inserted into slurry to take readings [8].

2.3.2 Determination of Soil Moisture Content

The moisture content of soil samples was determined using a moisture analyzer, model type (RADWAG pmc). Soil sample (2g) was dispensed on to the crucible and the analyzer was operated at 120 ^{o}C over a period of four minutes [8].

2.3.3 Determination of Soil Temperature

The temperature of the soil was taken using a thermometer which was inserted into the freshly collected soil sample up to 5cm and allowed to stay for 10 minutes, after which reading was obtained. The average of three consecutive readings was recorded [8].

2.4 Isolation and Identification of Fungal Isolates from Beach Soils

Sabouraud dextrose agar (SDA) was used in order to encourage the growth of fungal species only. Isolation was carried out by Soil dilution technique using pour plate method and plates were incubated at 25 ^{o}C for 5 days [8].

Pure cultures of isolated fungi were identified based on cultural and microscopic methods with reference to Barnett and Hunter [9]. The percentage frequency of occurrence for each fungus was determined.

2.5 Test microorganisms

The test organisms used for this study were; *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Klebsiella pnuemoniae, Candida albicans, Aspergillus fumigatus, Aspergillus niger* and *Saccharomyces cerevisiae* obtained from the culture collection unit of Department of Microbiology, University of Ilorin, Nigeria and University of Ilorin Teaching Hospital, Ilorin, Nigeria. They were confirmed using standard methods [9] and routinely subcultured for purity.

2.6 Primary Antimicrobial Screening

Primary screening of isolates for antimicrobial activity was carried out using cross streak method; each fungal isolate was streaked as a straight line on Muller-Hinton agar plates and incubated at 25 ^{o}C. After 2days test organisms were streaked perpendicular to the growing fungus and incubated at 37 ^{o}C for 24 hours following which growth of inhibition of each test organism was measured [10].

2.7 Production of crude antibiotics from Fungal Isolates

Isolates which produced zones of inhibition in the primary screening assay were grown in 100ml malt extract and kept on a shaker incubator at 25°C for 7 days. Cultures were centrifuged at 250 rpm for 10-15 minutes. Aliquot of supernatant was filtered into sterile screw capped glass vials. Filtration was carried out twice to obtain cell-free supernatant antibiotic agent for antibacterial activity [11].

2.8 Secondary Antimicrobial Assay of Antibiotic

Agar disk diffusion assay was employed for the secondary screening. Aliquot of antibiotic (50µl) produced was impregnated onto filter paper disks and placed on appropriate agar plates inoculated with each test organisms and plates were incubated at appropriate temperatures for test fungi and bacteria and inhibition zones measured [10].

2.9 Molecular Isolation of Fungi Isolate

The fungus producing the highest activity was subjected to molecular characterization. Isolation of fungal DNA was done using CTAB method as described by Mohammed *et al* [12]. Polymerase chain reaction amplification was carried out using specific primers (ITS4) to amplify the small subunit rRNA genes. Initially, denaturation at 94 °C for 4 min was done which was then followed by 35 cycles consisting of denaturation at 94 °C for 1 min. Annealing at 50°C for 1 min and extension at 72 °C for 2 min and final extension was achieved at 72 °C for 7 min. The PCR product was checked on an 1.5% agarose containing 0.05 µg mL^{-1} ethidium bromide photographs were taken after 30 min [12].

2.9.4 Nucleotide Sequencing

The PCR product was used for another PCR reaction that was now sequencing reaction. The program, process and quantities used were according to BigDye® Terminator v3.1Cycle Sequencing Kit. The sequencing reaction was also purified using the protocol of BigDye® Terminator v3.1Cycle Sequencing Kit. Sequencing was then performed using an automated sequencer (ABI genetic analyzer model 3500, Applied Biosystems, USA). The product from the purification process was loaded on the 3130xl genetic analyzer from Applied Biosystems to give the sequences. The PCR product was purified from contaminating products by adding 2vol (20µl) of absolute ethanol to the PCR product followed by incubation at room temperature for 15minutes. It was then spun down at 10000rpm for 15minutes, and the supernatant was decanted. Two volumes (40µl) of 70% ethanol were added after spinning at 10000rpm for 15minutes and supernatant was decanted, the product was air dried and about 10µlof ultrapure water was added. Finally, the amplicon was checked on 1.5% agarose. Sequencing was performed using an automated sequencer (ABI genetic analyzer model 3500, Applied Biosystems, USA). Sequences of each isolate were subjected to similarity check with previously identified species by using BLASTn tool on the NCBI database (www.ncbi.nlm.nih.gov:80/BLASTN/) to identify the isolates up to strain level.

3 Results

3.1 Physicochemical Parameters of Beach Soils

Table -1 shows the result of the physico-chemical properties of soil from 3 beaches in Lagos State, Nigeria with the temperature ranging from $26.333 \pm 1.155^{a}$to 30.333 ± 0.577^{a}. Temperature plays an important role in the growth and development of fungi. Earlier studies have also demonstrated that the optimal growth temperature for marine fungi was between 20 ^{o}C – 30 ^{o}C [13]. According to Das *et al.* [14] pH often interferes with the growth of antibiotic producers in the soil. Fungi grow best within the pH range of 5.0-9.0 and this is in agreement with the result of this study [7.667 ± 0.058^{b}- 8.000 ± 0.100^{a}]. Thus the capability of these fungi to grow at higher pH values is uncommon and may be a reflection of adaptation to factors such alkalinity [15]. Soil moisture depends on the availability and retention levels of water in soils. Beach soils are usually sandy hence retaining little water due to large pore spaces in between the soil particles [15]. Thus the moisture content of soil from beaches is lower to other types of soil. This finding is in line with the moisture content from beaches studied in the present study which ranged from $7.142 \pm 1.497^{a}$to 10.030 ± 3.130^{a}.

Table1-Physico-chemical Properties of beach Soils in Lagos Metropolis

Sample sites	Temperature	pH	Moisture content
Elegushi	29.667 ± 1.155^{a}	$7.767 \pm 0.152^{a,b}$	8.857 ± 0.754^{a}
Oniru	30.333 ± 0.577^{a}	7.667 ± 0.058^{b}	10.030 ± 3.130^{a}
Alpha	26.333 ± 1.155^{a}	8.000 ± 0.100^{a}	7.142 ± 1.497^{a}

3.2 Identification of Fungal Isolates and their Distribution in Beach Soils

From the present study, a total of four fungal species from 2 different genera were isolated from the three beaches and these were; *Aspergillus niger*,*Aspergillus flavus*,*Penicillium purpurogenum* and *Penicillium islandicum*. The genus *Aspergillus* has been previously reported as the most commonly isolated microfungi from Mediterranean coast beaches [16]. According to another report, *Penicillium* was the most occurring fungal genus isolated from Ipanema beach sand in Brazil [17]. However, the

diversity of microfungi in the three beaches in Lagos was not as much as those isolated from sandy beach soil in Teluk amounting to 7 different genera with several species in a study carried out by Zakaria *et al* [18]. This differences may be owing to variations in sampling techniques, method of isolation, level of salinity of the beaches as well as nutrient status [19]. Table-2 shows their cultural and microscopic characteristics.

Table 2- Macroscopic and Microscopic characteristics of isolated fungi

Colour of mycelia	Reverse colour	Margin colour	Hyphae	Branching Pattern	Conidia shape	Identification
Black	Light yellow	White	Aseptate	_	Globulose	*Aspergillus niger*
Bluish-grey	Yellow	White	Septate	Branch, biverticillate	Flask	*Penicillium purpurogenum strain* A27
Yellowish-green	Light yellow	_	Aseptate	_	Globulose	*Aspergillusflavus*
Green	Red	Orange	Septate	Two-stage branched, Terverticillate	Flask	*Penicillium islandicum*

The frequency of occurrence of the isolates from the sampling sites is presented in Table-3.

Table 3-Distribution of Fungi isolated from Different Beaches in Lagos

Fungal isolates	Locations			No	Frequency(%)
	Elegushi	Oniru	Alpha		
Aspergillus niger	+	+	+	3	37.5
Penicillium purpurogenum	+	_	_	1	12.5
Aspergillus flavus	+	+	+	3	37.5
Penicillium islandicum	_	+	_	1	12.5

Their colony forming units were not indicated as a result of the low number of colonies formed on the soil dilution plates which were less than 30 and therefore considered insignificant as reported by Foght and Aislabie [20].

3.4 Primary Antimicrobial Activity of Fungi Isolated from Beach Soils

The result of the primary screening revealed that *S. aureus* and *P. aeruginosa* were the only susceptible test bacteria to three of the aquatic fungi isolated (Figure -1).

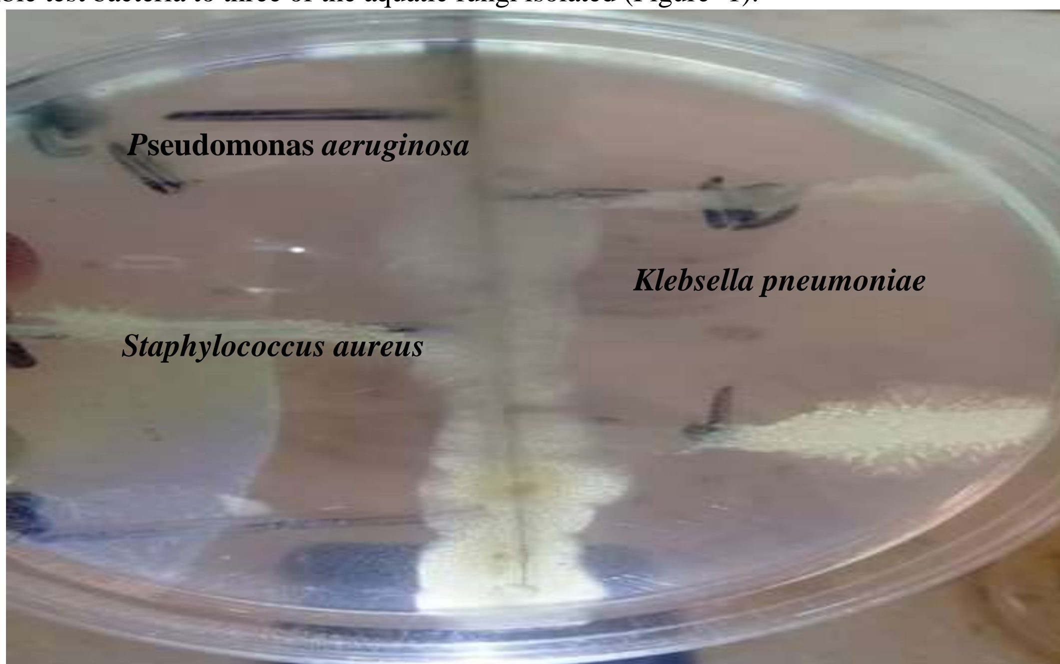

Figure 1- Sensitivity plate showing inhibition of some test bacteria by Penicillium purpurogenum

Aspergillus niger did not inhibit any of the test organisms. This observation is similar to that obtained by Trinh *et al*. [21] on the antimicrobial activity of marine fungi isolated from the son tra peninsula, Da Nang,Vietnam noting that *S. aureus* and *P. aeruginosa* among other test bacteria were inhibited by some marine fungi . The highest inhibition was observed with *P. purpurogenum* against *S. aureus* (Table-4).

Table 4-Preliminary Antimicrobial Activity of Isolated Fungi from Beach Soils

Antibiotic Producing Isolates	Zones Of inhibition (mm)							
	S.A	**E.C**	**P.A**	**K.P**	**C.A**	**A.F**	**S.C**	**A.N**
F1	8±1.5[a]	0±0.0	12±1.0[a]	0±0.0	0±0.0	0±0.0	0±0.0	0±0.0
F2	0±0.0	0±0.0	0±0.0	0±0.0	0±0.0	0±0.0	0±0.0	0±0.0
F3	7.8±0.2[a]	0±0.0	0±0.0	0±0.0	0±0.0	0±0.0	0±0.0	0±0.0
F4	4.6±2.3[a]	0±0.0	0±0.0	0±0.0	0±0.0	0±0.0	0±0.0	0±0.0

Keys

F1- Penicillium purpurogenum strain , F2 –Aspergillus niger, F3 –Aspergillus flavus, F4 –Penicillium islandicum, S.A– Staphylococcus aureus, E.C – Escherichia coli, P.A – Pseudomonas aeruginosa K.P – Klebsiella pneumonia, C.A – Candida albican, A.F – Aspergillus fumigatus, S.C - Saccharomyces cerevisiae, A.N – Aspergillus niger

Interestingly, however, the fungi did not produce antifungal activity, hence all test fungi were resistant to the isolates. This observation is similar to [22] reporting fungal metabolites to be active against only bacterial pathogens with no activity on the test fungus studied. One possible reason for lack of antifungal activity may be as a result of similarity in genes coding for antimicrobial substances in both the isolates and the test fungi, especially since *A. niger* and *A. flavus* isolated were screened against other sources of the same species.

3.5 Secondary Antibacterial Activity of Active Fungi Isolated from Beach Soils

The secondary antibacterial activity of active fungi isolated from beach soils against *Pseudomonas aeruginosa* is shown in Figure-2.

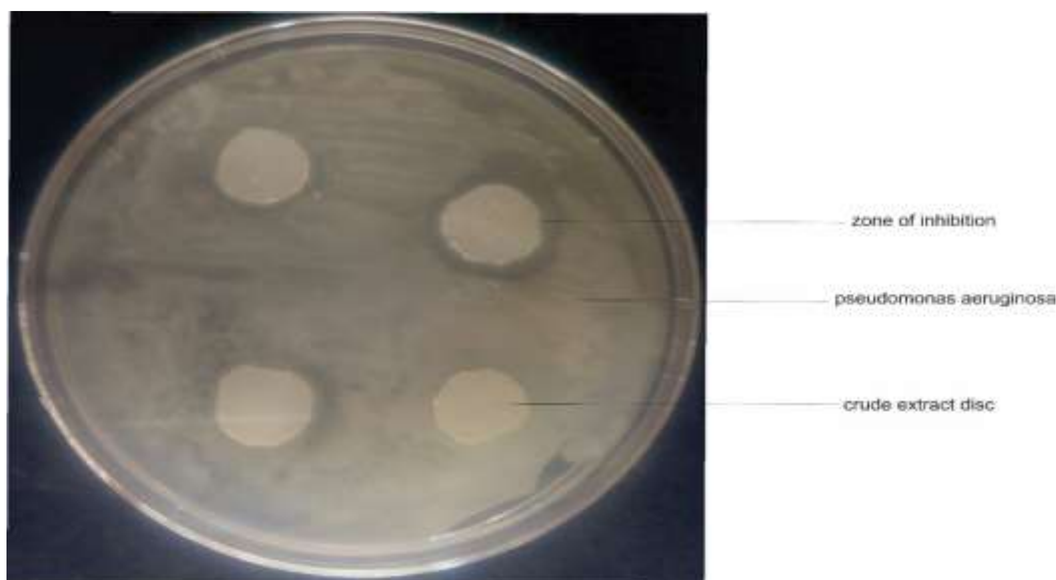

Figure 2-Sensitivity plate showing zone of inhibition of *Pseudomonas aeruginosa* when assayed with secondary metabolite from *Penicillium purpurogenum*.

The result of the three active fungi from beach soils are presented in Table-5.

Table 5-Secondary Antibacterial Activity of Active Fungi

Antibiotic Producing Isolate	Zone of inhibition (mm)of test isolates			
	Staphylococcus aureus	***Escherichia coli***	***Pseudomonas Aeruginosa***	***Klebsiella pneumoniae***
***Penicillium purpurogenum strain* A27**	18±2.0[a]	0±0.0	28±0.2[a]	0±0.0
Aspergillus Flavus	10±0.7[a]	0±0.0	0±0.0	0±0.0
Penicillium Islandicum	6±0.3[a]	0±0.0	0±0.0	0±0.0

The cell-free supernatant antibiotic produced from fungal isolates showed a similar pattern of activity with the primary assay. The same test bacteria were inhibited in both primary and secondary assays but activity was higher in the secondary screening assay with *P. aeruginosa* exhibiting the highest zone of inhibition (28±0.2[a]) to *Penicillium purpurogenum*. According to an earlier report, screening techniques based on cultivation of live organisms on solid agar may be often affected by certain extraneous factors such as other cell lysates thereby giving false positive results [23] however, fermentation to obtain secondary end product is a veritable tool in industrial microbiology. During the process, the producer organism multiplies rapidly in a free flowing medium with optimized nutrients and exponential growth to induce the secretion of copious quantities of several bioactive components. This process also allows for the separation of the desired metabolite from the cell biomass as well as other unwanted substances which may militate against the activity of the antimicrobial agent. This suggests fermentation as a better means of obtaining antibacterial extract from microorganisms.

3.7 Molecular characterization of fungal isolate

DNA extraction ladder by gel electrophoresis is shown in Figure-3. On the basis of position sequence of the fungal isolate on phylogenetic tree, the fungal isolate producing the highest antibacterial activity showed 98% similarity to *Penicillium purpurogenum.* The gene sequences of the isolates have been submitted to GenBank to obtain accession number; while respective pure cultures of these strains have been deposited at the culture collection center of the Department of Microbiology, University of Ilorin under same name as identified. Further analysis is ongoing to establish the novelty of the isolate (Figure-4).

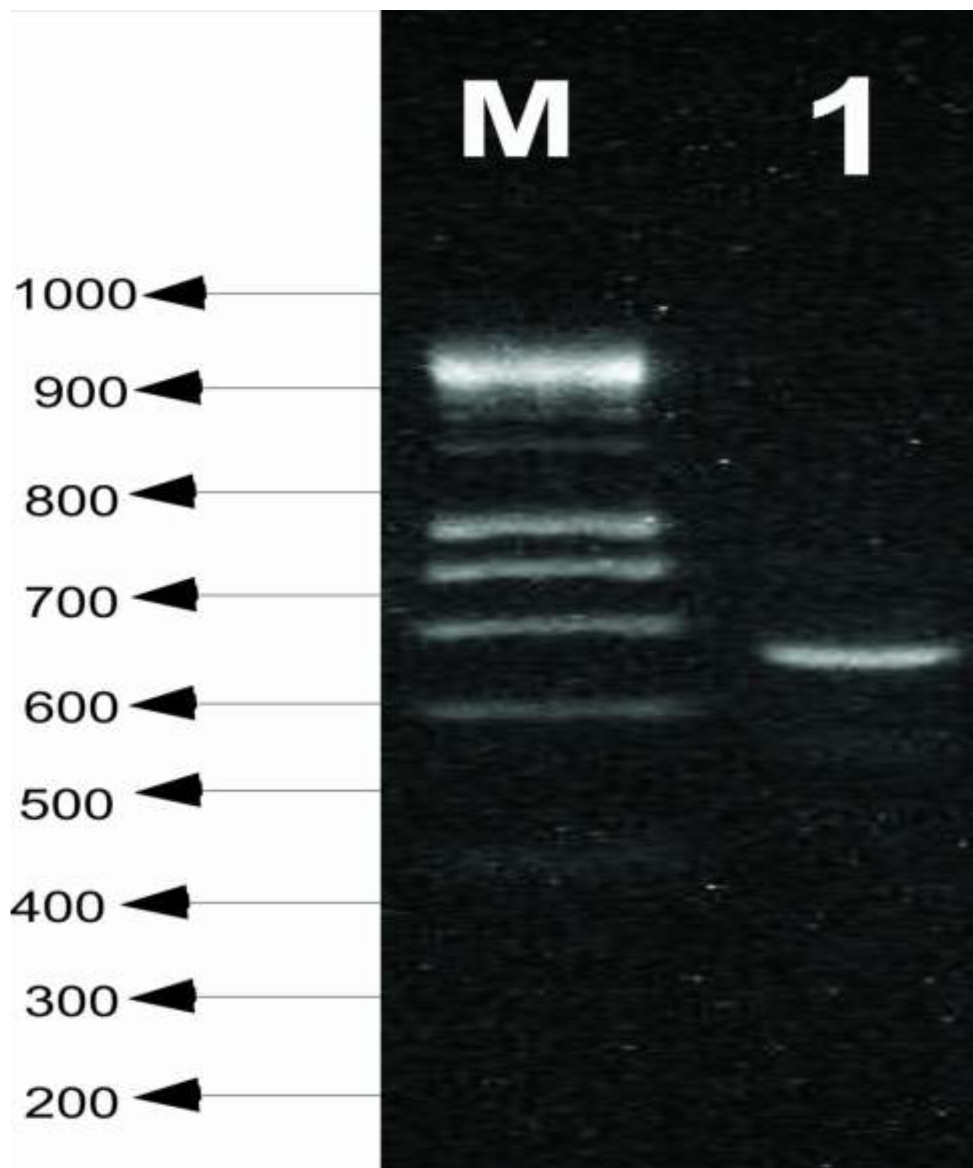

Figure 3-DNA Gel Electrophoresis of *Penicillium purpurogenum strain* A27

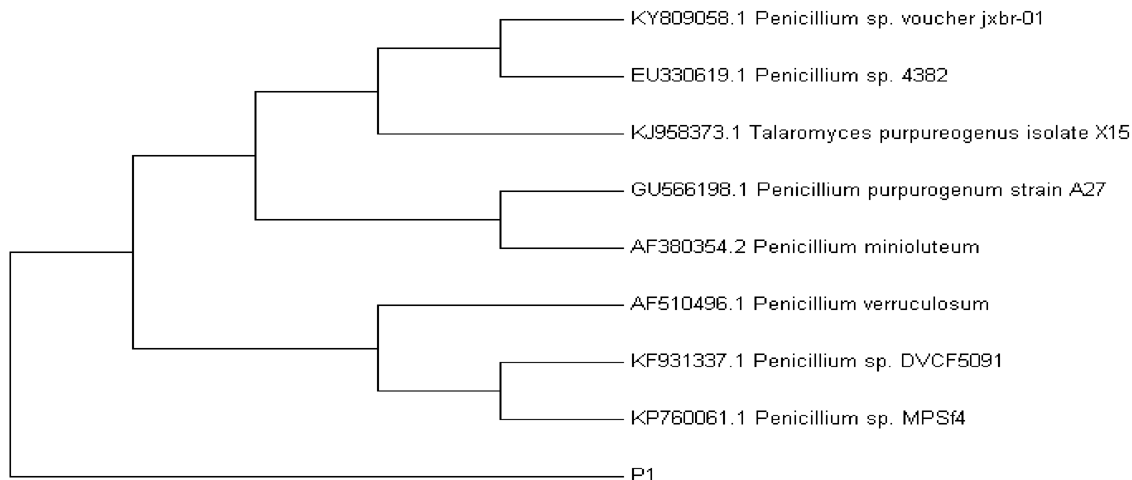

Figure 4-Maximum likelihood tree of the sequenced 16S rRNA gene of isolated *Penicillium purpurogenum strain* A27

4. Conclusion

Marine fungi have become an important source of bioactive metabolites and constant screening of their metabolites is on the increase. It was evident from this study that microfungi isolated from the three beaches in Lagos, Nigeria have potentials as antibacterial agents especially *P. purpurogenum* isolated from Elegushi beach which produced the highest activity against *S. aureus* and *P. aeruginosa*.Further investigations on metabolites from this fungus are necessary in order to determine the mechanisms of action and the nature of the active components.

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