Antimicrobial activity of pectin against bacterial strains isolated from marine habitats

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Abstract: Pectin is a polysaccharide found in fruits and vegetables. Pectins are added to several types of foods because of their nutritional benefits to humans due to the presence of dietary fiber. It is also considered a natural antimicrobial material. In this study, antibacterial activity of pectin was investigated against unknown marine bacteria (isolated from marine sand). The samples were identified using PCR. 5% pectin solution was used in well diffusion assay. The result showed that pectin 5% was able to exhibit antimicrobial activity against *Bacillus pumilus*, *Bacillus areophilus* and *Bacillus* sp. However, the minimum activity of pectin was found in *Bacillus altitudinis* (has no clear zone). The most obvious finding to emerge from this study, pectin is capable of reducing activity of some marine bacteria.

Keywords: Pectin, antimicrobial, clear zone, *Bacillus pumilus, Bacillus areophilus* , *Bacillus sp*.

Introduction

There is a wide range of plant species on Earth (400,000–500,000). A considerable number of drugs in use today come from nature, either the microbial world, plants, or animals. The discovery and development of new compounds with pharmacological activity is largely based on the natural world. Approximately 75% of compounds with antibacterial activity introduced into clinical practice in the last 40 years derive from natural products (Alibi *et al*.,2021). Antibiotics have many problems such as undesirable side effects on humans and resistance issues among microorganisms. After a while they will be no longer effective on microorganisms due to significant expansion of resistance and shortage of new sources of antibiotics. The replacement with natural and effective products is very important. Natural products have been used in traditional medicine all over the world. Medical plants produce variety of compound have therapeutic properties. There are several reports on antimicrobial activity of different herbal substrate. The publications could contribute to a rational basis for finding compounds or extracts from plants that may address the problem of anti-microbial resistance (Eloff, 2019). Using chemical preservatives have negative impacts including: human health hazards of the chemical applications, chemical residues in food and feed chains and acquisition of microbial resistance to the used chemicals. Because of such concerns, the necessity to find a potentially effective, healthy safer and natural alternative preservatives is increased. Within these texts, Plant extracts have been used (Mostafa *et al*., 2018). Pectin is a complex polysaccharide found in plants (cell wall and middle lamella); they consist of homogalacturon (HG) which might be methyl esterified or deesterified to generate co-polymer structure. Also, the HG backbone may be attached with neutral sugars in the side of chains generating the rhamnogalacturon I (RGI) region, or rhamnogalacturon II (RGII) (Wicker *et al*., 2014). Pectins are found in fruits and vegetables such as the peel of orange and lemons and the pulp of tomato and pineapple. In these substances, D-galacturonic acid units are combined via α-1, 4- glycosidic bonds

and the carboxyl groups are esterified with methanol. (Gummadi and Panda, 2003). The amount of pectin present and its molecular weight differs between fruits and vegetables (citrus > apple > beet). The components of pectin include protopectin, pectin polysaccharides and associated galactans, arabinans and arabinogalactans. Protopectin is considered to be an insoluble complex molecule form which is found with cellulose and hemicelluloses in the cell walls of plants (Ovodov, 2009):

Importance of pectin substances

Pectin substances can be found in all higher plants. Pectin allows plant growth and cell wall extension. Pectins give plants resistance against drought and low temperatures and contribute in human nutrition as a food and fiber source (Ovodov, 2009). It has effects on human health, including reducing the level of cholesterol in the blood and deceleration of the absorption of glucose in obese and diabetic patients. Furthermore, citrus pectin is very active as anti-cancer agents in at all stages of cancer (Benoit *et al*., 2012). This research will focus on pectin and its antimicrobial activity on marine bacteria.

Material and Methods

Five isolates of unknown marine bacteria were tested for pectin activity.

Isolation of unknown marine bacteria

Marine bacteria were isolated from marine sand using APY agar medium.

Isolation of bacteria from marine habitats

Isolation of bacteria was carried out from marine sand using APY agar medium described by Sawabe *et al*., (1995). The medium consists of 0.5g sodium alginate (Sigma), 0.1g tryptone, 0.1 g yeast extract, 15g agar (Oxoid No1), in 1000 ml natural sea water at pH 8.0. All components were dissolved and sterilised by autoclaving at 121°C for 15 minutes. Nystatin (Sigma) was added as solution to avoid fungal growth. The isolation of bacteria was carried out as follows: 25g of marine sand were added to a flask containing 250 ml of sterile seawater. From 10^{-1} to 10^{-5} dilutions, 0.1 ml was inoculated on an APY agar medium and incubated at 37°C for 24 hours. The colonies on the APY agar plates were purified on the same medium. Developed colonies were then, streaked onto new APY plates and incubated in order to obtain pure cultures. To ensure strain purity, the isolations were conducted in triplicate. The colonies were maintained on Tryptic soy agar (Fluka) consisted of; 17g casein, 3g soya peptone, 5g sodium chloride, 2.5g glucose, 2.5g dipotassium hydrogen phosphate and 15g agar in 1000 ml deionised water.

Identification of unknown bacterial isolates using 16s rRNA technique

A bacterial suspension in nutrient broth was prepared and incubated overnight at 37°C. After the incubation period, 1-3 ml of media was transferred in a sterile Appendorf tube and centrifuged at 6000 x g for 2 min at room temperature and the supernatant was decanted completely. A KeyPrep bacterial DNA extraction kit supplied by ANACHEM® was used and all steps were done as described in the instructions provided by the company. 100µl of buffer R1 was added to the pellet and the cells were resuspended completely by pipetting up and down. After full cell homogenising, 20 µl of lysosyme was added and mixed thoroughly and incubated at 37°C for 20 min. The mixture was centrifuged at 10,000xg for 3 min and the supernatant was completely decanted. The pellet was resuspended in 180 µl of buffer R2 and 20 µl of proteinase K was added and incubated at 65°C for 20 min in a water bath with occasional mixing every 5 min. 400 µl of buffer BG was added and mixed thoroughly by inverting the tube several times until a homogeneous solution was obtained and then incubated

for 10 min at 65°C. After the incubation period 200 µl of absolute ethanol was added and mixed thoroughly. The sample was transferred into a column which was assembled in a clean collection tube and centrifuged at 10,000xg for 1 min while the flow was discarded. The column was washed by addition of 750 µl of wash buffer and centrifuged at 10,000xg for 1 min while the flow was discarded. Finally, the column was placed in a clean microcentrifuge tube and 70µl of elution buffer was added and centrifuged at 10,000xg for 2 min to elute DNA. DNA was stored at -20°C until the next step.

Gel Electrophoresis

Gel electrophoresis was done to make sure that the bacterial DNA was well extracted and purified. The following steps were taken:

Agarose gel Preparation

0.5g of molecular biology grade agarose was dissolved in 50 ml of 1x TAE (Tris Acetate EDTA) buffer and 40ml distilled water by heating in a microwave oven. The solution was mixed gently and allowed to cool to 55°C, and 2.5 µl of ethidium bromide was added. After mixing, the solution was poured into a sealed gel rack and a comb was inserted at one side of the gel vertically and left at room temperature for 20 min to solidify. Then the comb was removed and gel was placed into an electrophoresis tank and submerged in 1x TAE buffer.

Sample Loading

10 µl of the DNA sample was mixed with 2 µl of Blue/Orange 6x loading dye with glycerine and loaded into the wells. 6 µl of hyper ladder was added into an adjacent well as a reference. The set was adjusted for 40 minutes at 80V to allow the DNA to migrate toward the anode. After the supposed period the DNA fragments were visualized under a UV transilluminator and the images were captured using a connected digital camera.

Samples amplification

Samples were amplified using Poly Chain Reaction (PCR) technique. A mixture in a sterile Appendorf tube was prepared as follows: 12 µl of Master mix, 1 µl of forward primer, 1 µl of reverse primer, 1 µl of the DNA sample, and 35 µl of sterile distilled water. The mixture was inserted in a PCR machine and the programme was adjusted as follows:

Serail	Temperature	Time(Min)	Passes total
1	94°C	3	1
2	94°C	1	35
3	60°C	1	35
4	72°C	1	35
5	72°C	5	1
6	4°C	∞	1

16S rRNA sequencing and phylogenetic analysis

After PCR, aliquots of 10 µl of each sample were allowed with 1µl of forward primer (16SUN1.FOR) and 1µl of reverse primer(16SUN1. REV) in a sterile small size tube and sent to the DNA sequencing (Weisburg *et al.*, 1991) . 16S rRNA gene sequences were adapted using the Finch TV software and then exported into the Basic Local Alignment Search Tool (BLAST), available from the website of the National Centre for Biotechnology Information (NCBI), to identify matches with existing characterized reference sequences.

Antimicrobial assay for unknown bacteria

One approach was done to assess pectin activity in the agar medium. For antimicrobial activety of pectin bacterial suspension was prepared by measuring the turbidity.

Preparation of turbidity standard suspension

Sensitivity tests were adjusted to $1.5x10^6$ colony forming units (CFU) which equals 0.5 McFarland. Two isolated colonies of the same

morphological type were taken with a sterile loop and inoculated into a tube containing 3 ml of buffer phosphate and incubated in 37°C for 2 hrs. After incubation the turbidity was measured in spectrophotometer at 600 nm and the measurement was recorded. The tube was diluted by taking 1 ml of the broth and transferred to another tube containing 9 ml sterile buffer phosphate; this was the first dilution (10^{-1}), the same step was then repeated six times to achieve the dilution of (10^{-5}); 0.1 ml was taken from the last dilution and poured and spread on sterile nutrient agar plate. The plate was incubated for 48 hrs then the total colony count was counted and timed 10^5 and 10 was added to the total to get the actual concentration of 10^6 which is the suspension density. If the colony number was too high then more dilution will be needed to the original inoculation tube with nutrient broth and same steps then should be repeated to obtain 1-2 colonies forming units in dilution 10^{-5}.

Antimicrobial activity of pectin using Well diffusion assay method

This experiment was designed to determine the effect of pectin on unknown bacteria. The following bacteria were used: S1, S2, Alg2 and Alg3. Pectin, esterified potassium salt, from citrus fruit (Sigma) was dissolved in sterile distilled water. The concentration of the solution of pectin was 5% (w/v). The solution was stored at 4°C as stock. Phosphate buffer suspension of all strains, equivalent to 0.5 McFarland standard containing 1.5×10^6 colony forming units (CFU). Five wells were made in each nutrient agar plate. A suspension (0.1 ml) of pectin was added to each well in nutrient agar, and the cultures were incubated at 37°C for 48 hours. The clear zones were then measured (Daoud *et al.*, 2013).

Results and Discussion

Growth of marine bacteria isolated from marine sand

Under the experimental conditions, the bacteria isolated from marine sand was able to grow on APY agar medium.

Phylogenetic identification of unknown fungi

Polymerase chain reaction (PCR) with the help of primers, used to amplify bacterial genes. Agarose 1% gel was used to check the expected size of the amplified product. Figure 1 illustrates the successful PCR products.

The amplified product was sequenced and searched against bacterial sequences accessible in the BLAST database. The results were used to identify species with high and lower similarity. Table 1 shows the species; *Bacillus pumilus, Bacillus areophilus, Bacillus altitudinis* and *Bacillus sp* designed as S1, S2 , Alg 2, and Alg 3 respectively.

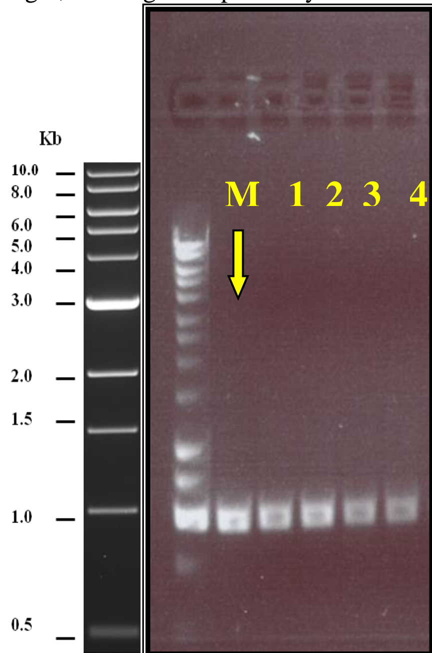


Figure 1: The polymerase chain reaction (PCR) on agarose gel (1%) electrophoresis. (M) shows the 1-Kb DNA ladder. Lane 1:S1 , Lane 2: S2, Lane 3: Alg 2, Lane 4: Alg 3. Yellow arrow (M) indicats the 1-kb DNA ladder.

Table 1: The bacterial sequence analysis of marine bacteria cultured from marine sand.

Source of Sample	Representative sequence	Closest matches identification	Sequence identity	Length of Sequence (bp)	NBCI (Accession number)
Marine sand	S 1	*Bacillus pumilus*	97%	697	GQ90342.1
	S2	*Bacillus areophilus*	98%	776	JX680140.1
	Alg2	*Bacillus altitudinis*	97%	729	KC172054.1
	Alg3	*Bacillus sp*	99%	766	KF010630.1

Blast analysis of the bacterial sequence of S2 showed similarity of 98% with *Bacillus areophilus.*

Antimicrobial activity of pectin

The results presented here show that pectin exhibits antibacterial activity against *Bacillus pumilus, Bacillus areophilus* and *Bacillus sp* (5% pectin). However, pectin had no effect against Bacillus altitudinis (Fig.2). The maximal clear zone of pectin achieved within 48 hours at 37°C These findings suggest that pectin has a narrow range of antibacterial activity and exhibits selective toxicity against *Bacillus pumilus, Bacillus areophilus* and *Bacillus sp*. it can be clearly seen that the maximum clear zone produces by *Bacillus areophilus* and *Bacillus sp* occurred after 48 hours incubatino. Over all, the results show that Some types of marine bacteria can be regulated via pectin 5%. The results are shown in table 2. *Bacillus pumilus, Bacillus areophilus* and *Bacillus sp* showed a quick response. (i.e. it releases clear zone at 48h). The results show that marine bacteria was sensitive to pectin substrate.

Table 2: Clear zone produced from marine bacteria.

Bacterial isolates	Mean of inhibition
Bacillus pumilus	11
Bacillus areophilus	12
Bacillus altitudinis	0
Bacillus sp	12

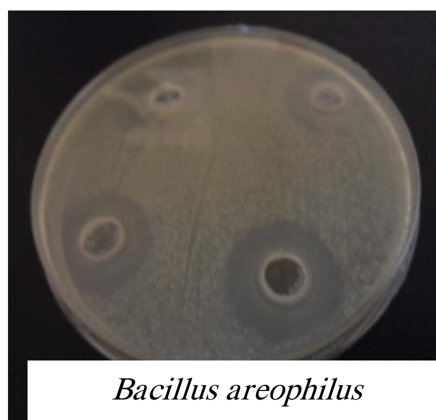
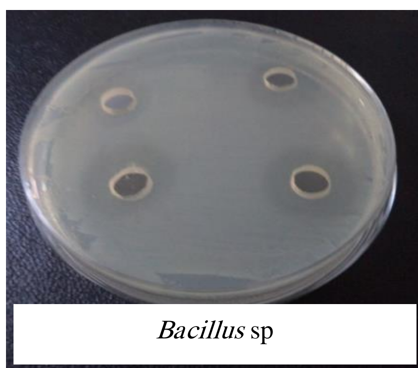

Bacillus areophilus

Figure 2 (A): Antimicrobial activity of pectin 5% after 48 hours incubation.



Bacillus sp

Figure 2 (B): Antimicrobial activity of pectin 5% after 48 hours incubation.

Bacillus pumilus

Figure 2 (C): Antimicrobial activity of pectin 5% after 48 hours incubation.

Bacillus altitudinis

Figure 2 (D): Antimicrobial activity of pectin 5% after 48 hours incubation.

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