Characterization and bioassay of penicillin (Penicillium Chrysogenum) produced from oranges (Citrus Sinensis) collected from Na'ibawa orange market, Kano State, Nigeria

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ABSTRACT

This study aimed at producing penicillin from rotten and fresh oranges. The samples were prepared by extracting the orange juice, and their pHs were adjusted to 6.5 using 1% calcium hydroxide; then sterilized for fifteen minutes using an autoclave at 121°C. The samples were then inoculated with ***Penicillium chrysogenum*** and kept for fifteen days. The inoculated samples were characterized using Thin Layer Chromatography (TLC), Fourier Transform Infrared Spectroscopy (FTIR), and Gas Chromatography Mass Spectrometry (GC-MS). Bioassay test was also carried out using some clinical pathogens such as; ***staphylococcus aureus, Klebsella spp***, wild strain of ***Escherichia coli*** (E. coli), ***Methycillin resistant Staphylococcus aureus*** (MRSA) and ***Methicillin Susceptible Staphylococcus Aureus*** (MSSA) to measure the zone of inhibition of the produced penicillin. The penicillin was extracted by the liquid-liquid extraction method. The broth and the solvent were mixed in a separating funnel at 1:½ of the broth volume. The highest yield (400 mg/cm^3) of the produced penicillin was recorded for the rotten orange before optimization. The same methodology was used to optimize the penicillin yield, but the yield remained the same after optimization. The study demonstrated that fresh and rotten oranges are viable substrates for the fermentation media to be used in cultivating fungi for the production of natural penicillin antibiotics.

Introduction

Global trash production amounts to over 38 billion metric tons annually. It is commonly accepted that population growth, consumption rates, and human behavior are to blame for this sharp rise. Due to the constant disposal or burning of waste items, there are several environmental issues. While land disposal of organic waste products may directly change the soil's heavy metal status by influencing metal solubility or dissociation kinetics, burning pollutes the environment. Various therapeutic approaches and techniques have been developed and implemented by nations worldwide to address this difficult topic [39].

The conversion of nutrient-rich organic waste materials into beneficial products for sustainable farming operations has received a lot of attention. One practical method of getting rid of garbage is to use organic resources that come from plants and animals. Penicillin belongs to a class of antibiotics that are commonly used to treat bacterial infections and illnesses include rheumatoid arthritis, pneumonia, TB, and gonorrhea. Penicillin usage is under strain, so there's a need to increase supply—not only in Nigeria but worldwide. Not only will the beneficial penicillin product be more readily available, but the environment will also be of higher quality when decaying oranges are converted into it.

Solid organic wastes are referred to as organic-biodegradable wastes that have a moisture content of 85–90%. Various decomposer microorganisms, including fungi, bacteria, and invertebrates that feed on detritus, recycle these organic materials [39]. Rotten oranges are not an exception, since they can be processed to produce valuable products like penicillin. According to the Environmental Protection Agency

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(EPA) in 2017 [85], recycling is crucial since it boosts the economy of a country and creates jobs.

Similarly, EPA released significant findings on the economic benefits of the recycling industry with an update to the national Recycling Economic Information (REI) study in 2016. This study analysed the number of jobs, wages and tax revenues attributed to recycling. The study found that in a single year, recycling and reuse activities in the United States accounted for: 757,000 jobs, $36.6 billion in wages; and $6.7 billion in tax revenues. This equates to 1.57 jobs, $76,000 in wages; and $14,101 in tax revenues for every 1,000 tons of materials recycled [85].

Based on an estimated 3 million hectares of land, Nigeria produces approximately 3.4 million metric tons of citrus fruits annually (2013). The nation is the largest growing region in Africa, trailed only by Egypt, Morocco, and South Africa, and ranks ninth in the world in terms of citrus fruit production, right behind Italy. Benue, Nassarawa, Kogi, Ogun, Osun, Ebonyi, Taraba, Ekiti, Imo, Edo, and Delta are the principal citrus-producing states in Nigeria, in that order. Oranges account for the majority of citrus output, while grape fruits, lemons, and limes are also grown in considerable quantities [23].

Orange juice includes sugars like fructose, glucose, and sucrose, which can be bioprocessed to produce expensive, value-added products like antibiotics, including penicillin. About 25–30 million of the 10.8 million oranges produced in India are wasted. Pest attacks, microbiological contamination, incorrect storage, and transformation are the causes of this waste buildup in the nation. Penicillin and other value-added products can be produced effectively from this enormous amount of rotten orange trash [10].

In the process of making orange juice, it is believed that half the weight of fresh oranges is converted into juice, with the remaining weight being considered waste. Hence, by applying appropriate fermentation procedures, this enormous amount of trash can be transformed into numerous value-added goods.

Experimental Methodology, Materials and Chemicals

Sample Location

The Na'ibawa Orange Market in Kumbotso Local Government Area, Kano State, Nigeria, served as the sample location. With a population of 409,500 according to 2016 population projections, Kumbotso local government is located on latitude 11.890 and longitude 8.503 of the equator, 11°N and 8°E. Its area is 158 km³.

Collection of Samples

Clean polythene bags were used to collect both fresh and rotting orange samples from the Na'ibawa orange market in Kumbotso L.G., Kano State. The samples were then transported immediately to the Department of Microbiology at Bayero University, Kano, for additional processing. The samples were gathered simultaneously.

Preparation of Samples

To extract the juice, the raw orange samples—both rotten and fresh—were cleaned with tap water and squeezed by hand. Each sample's juice extract was filtered using a muslin cloth. Orange juices in the 120 cm3-200 cm3 range for every sample were measured into thoroughly cleaned Bama bottles, autoclaved for 15 minutes at 121°C, and allowed to cool at room temperature. Before inoculating the orange samples with 1% calcium hydroxide, the pH was first adjusted to 6.5 using a digital pH meter [10].

Isolation and Identification of *Penicillium Chrysogenum*

Penicillium Chrysogenum was isolated from soil, rotten tomatoes, and rotten pineapple. The samples were chopped to a 5µm size in order to isolate the decaying pineapple and tomato. Following surface sterilization with 1% hypo chloride solution, the samples were cleaned with distilled water. After that, five duplicates of the samples were put in a Petri dish with Potato Dextrose Agar (PDA) media and allowed to incubate for a full day at room temperature.

The soil sample was then isolated using the serial dilution method, in which 10^{-1} dilution of the soil sample was obtained by adding 1 g of the soil sample to 9 cm^3 of sterile distilled water. Nevertheless, in order to produce a 10^{-2} dilution, 1 cm^3 of the soil suspension was moved to a second tube that had 9 cm^3 of sterile distilled water.

Similarly, 1 cm^3 was transferred to a third tube that held 9 cm^3 of sterile distilled water in order to obtain a 10^{-3} soil sample. The soil sample was diluted one more time until it reached a 10^{-5} dilution [5]. PDA was added after plating the final 1 ml of the soil sample dilutions (10^{-4} and 10^{-5}), then completely combined to ensure that the soil suspension was properly mixed. and the medium (PDA). The set up was incubated at room temperature for 5-7 days for fungi to mature.

The sparkling blue-green mold with white mycelium around it allowed Penicillium Chrysogenum to be distinguished from the other colonies in the Petri plates. Lactophenol Cotton Blue was used to visualize the colonies under a light microscope in order to confirm the presence of the fungi's mycelium and conidia [5].

Test Organisms

The test organisms used were; *staphylococcus aureus, Klebsella spp*, wild strain of *Escherichia coli* (E. coli), *Methycillin resistant Staphylococcus aureus* (MRSA)

and *Methicillin Susceptible Staphylococcus Aureus* (MSSA).

Media Preparation

Potato Dextrose Agar (PDA) was the media utilized for Penicillium Chrysogenum isolation. In order to prepare it, 10 g of PDA were measured and diluted in 250 cm3 of distilled water. After giving the mixture a thorough shake, it was sterilized for 15 minutes at 121 degrees Celsius in an autoclave. After letting the liquid cool, it was put into the Petri dishes and left to solidify.

Fungal Inoculum Preparation and Standardization

Each isolate was subcultured twice to guarantee its viability after it was grown from the stock water suspensions on potato dextrose agar slants at room temperature. Penicillium chrysogenum isolate inoculum suspensions were generated by gently probing the colonies with the tip of a Pasteur pipette after covering them with 10 milliliters of sterile 1% normal saline. Conodia and hyphal particles made up the resultant combination, which was taken out and placed in a sterile tube. For standardization, the upper homogeneous suspension was utilized after the suspension's heavier particles were given three to five minutes to settle [1]. The mixture was calibrated using a spectrophotometer set to 530 nm to yield a 75% transmittance percentage [1].

Thin Layer Chromatography (TLC)

Thin Layer Chromatography (TLC) was used to evaluate the penicillin content of the broth made from both fresh and rotten oranges. The solvent used in TLC was ethyl acetate: methanol: acetic acid (45:50:5), and the samples were seen using iodine crystal, which gives penicillin its yellow color [32]. The analysis was done both rights after production and immediately after recovery [44].

The mobile phase, which consisted of acetic acid, methanol, and ethyl acetate, was saturated in the chromatographic chamber for 30 minutes. After that, the chromatographic chamber was filled with spots representing various components found in the samples. After 20 minutes, the plates were developed and the spots eluted. The Rf values were computed and the distances were measured [31].

Using an iodine crystal, all of the spots were visible as demonstrated. The dots and phase gave off a yellow color, supporting the findings of [31], [60], and [76].

Filtration and Purification of Penicillin from Production Media

The most adaptable technique for extracting insoluble materials from the broth is filtration. The penicillin-rich aqueous broth was treated with activated charcoal to remove colors and contaminants, therefore the first step in purifying penicillin from the production media was filtration using Whatman No. 1 filter paper, where big particles and microbial cells (mat culture) were separated [22].

Characterization of Penicillin Using FTIR Analysis

Fourier transformed infrared spectroscopy (FIRCary 630) was used at Bayero University in Kano at the Department of Pure and Industrial Chemistry. The FTIR was calibrated with an $8cm^{-1}$ resolution and a wavelength range of 650–4000 (cm^{-1}). Samples were examined in liquid form, and a computer was used to record and display the spectra. The rationale behind the selection of FTIR analysis was its ability to provide an accurate picture of a sample's entire makeup. FTIR spectra of different organisms will vary because of differences in their overall chemical composition. The spectra also serve as spectroscopic fingerprints that enable highly accurate identification of a compound [73].

Penicillin Recovery Using Liquid-Liquid Extraction

Using the liquid-liquid extraction (solvent extraction) method with amyl acetate, the penicillin generated from both fresh and rotten oranges was recovered. In a separating funnel, the solvent and broth were combined at a ratio of 1:½ of the broth volume. After giving the combinations a thorough 20-minute shake, they were allowed to separate. Following their separation, the broth was disposed of, and the solvent was left to evaporate, allowing the penicillin to survive [44].

Gas Chromatography Mass Spectrometry (GC-MS) Analyses

Gas chromatography mass spectrometry (GC-MS) was carried out at Multi user laboratory in Ahmadu Bello University (ABU), Zaria.

The GC-MS analysis was performed using GC system (Agilent 19091S) coupled with a HP-5MS column (HP-5MS 30 m x 250 µm x 0.25 µm). GC grade helium was used as a carrier gas at a flow rate of 9 cm^3/min. A 2 µl of penicillin samples were injected in split flow of 5 cm^3/min at a split ratio of 5:1, at 250 °C and pressure of 11.089 psi. Septum purge flow was 3cm^3/min at total flow of 9 cm^3/min. Oven temperature was 110 °C, ramp at 10 °C/min to 200 °C, then ramp at 5 °C/min to 280 °C and hold for 9 min.

The compounds eluted from the column as they were separated and went into a detector, which was able to provide an electronic signal each time a chemical was found. The signal that was acquired and then analyzed by a computer increased with the concentration of the sample. Retention time (RT) is the interval of time between the injection time (starting time) and the elution time. While the instrument was run, the computer generated a graph from the signal called

chromatogram. Each of the peaks in the chromatogram represented the signal created when a compound eluted from the gas chromatography column into the detector [36]. Before analysing the extract using GC-MS, the temperature of the oven, the flow rate of the gas used, and the electron gun were programmed. The temperature of the oven was maintained at 110 °C. The compounds were identified by comparing their spectra to those of National Institute of Standard and Technology (NIST) [36].

Optimization of Penicillin Production

To increase penicillin yield, optimization was done. The optimization approach involved the use of five parameters: the size of the inoculums (1%, 2%, 3%, and 5%) was determined by introducing the isolated Penicillium at varying proportions into the extracted orange samples. Additionally, several temperatures (30 °C, 32 °C, 37 °C, 40 °C, and 45 °C) were altered using incubators that were set at varying temperatures. Each gram weighed in the inoculated samples of the orange extracts was dissolved, and glucose (1g, 2g, and 3g) was added as a carbon source. Peptone (1g, 2g, and 3g) was dissolved for each gram of peptone to be added as a source of nitrogen to the infected orange extracts. The orange extracts' pH (4.0, 6.5, 7.0, 8.0, and 10.0) were adjusted at various pHs using 1 % Calcium Hydroxide. The results were then recorded accordingly.

Penicillin Bioassay Test

Using the agar-well diffusion method, the penicillin's potency against the pathogens *Streptococcus (STR), Methycillin-resistant Staphylococcus aureus (MRSA), Methycillin-sensitive Staphylococcus aureus (MSSA), and Klebsella spp. (Klep.)* was tested both before and after recovery. After spinning the samples for five minutes at 4000 rpm in a centrifuging machine, the supernatant was collected using sterile pipettes to test for sensitivity. [10].

To guarantee that the test organisms were evenly distributed across the surface of a solidified Nutrient Agar (N/A), the test organisms were swabbed three times with a sterile swab stick while the plate was rotated by about 60°. The agar was punched on the surface using a sterile cork borer to create the agar wells. Two to three drops of the samples were then added to the wells, which were then incubated for twenty-four hours at 37 °C and allowed to absorb the injected inoculated orange samples for fifteen minutes before diffusion. Using digital vernier calipers, the zones of inhibition were evaluated following incubation [10].

Statistical Analysis

Microsoft Excel was used to analyze the data. There were two elements Replication in Analysis of Variance (ANOVA) was found at a statistically significant level of $P \leq 0.05$.

Results and Discussion

Characterization of the Produced Penicillin Using Thin Layer Chromatography (TLC)

When separating components of mixtures, one of the most crucial analytical methods is thin-layer chromatography (TLC). TLC is frequently employed as a quick, simple, and straightforward procedure. Consequently, the presence of penicillin that was produced in the orange samples was verified using the TLC technique [61]. For the fresh and rotten oranges, the mean Rf values (Table 1) were 0.88 and 0.82, respectively, which were within the standard Rf value (0.82) given by [77]. The preserved rotten oranges' mean Rf value (0.94) was higher than the standard, nevertheless. There were no appreciable differences in the penicillin Rf values across the orange samples ($P \leq 0.05$).

In a similar vein, TLC was also performed using normal benzathine penicillin after penicillin recovery. According to Table 2, the Rf values of SB2, SB4, and standard were all the same (0.84), which was in line with the finding (0.83) that was published by [77]. The Rf value of 0.89 was observed in the triplicate rotten orange samples (SA1, SA3, and SA5), surpassing the threshold reported by Thangadurai et al. (2002). However, some more replicate samples (SA2, SA4, SB1, and SB5) from both fresh and rotten oranges did not exhibit any elution.

Table 1: R_f values of the produced penicillin after fermentation process

Sample	DMS (cm)	DMP (cm)	Rf
SA1	5.1	4.6	0.9
SA2	5.1	4.5	0.88
SA3	5.8	5	0.86
SA4	5.8	4.9	0.85
SA5	5	4.5	0.9
SB1	6	4.9	0.82
SB2	6	4.7	0.78
SB3	5.8	4.8	0.83
SB4	5.8	4.6	0.79
SB5	5	4.5	0.9

SA_{1-5}: Five replicate rotten orange samples, SB_{1-5}: Five replicate fresh orange samples

Table 2: R_f values of the produced penicillin after recovery

Sample	DMS(cm)	POI (cm)	Rf
SA1	4.4	3.9	0.89

SA2	4.4	3.9	0.89
SA3	4.4	3.9	0.89
SB1	4.4	3.7	0.84
SB2	4.4	3.7	0.84
Standard	4.4	3.7	0.84

DMS: Distance moved by the solvent, POI: Point of interest

SA_{1-3}: Five replicate rotten orange samples, SB_{1-2}: Five replicate fresh orange

Characterization of the Produced Penicillin Using Fourier Transformed Infrared Spectroscopy (FTIR)

The penicillin made from rotten, and fresh oranges had an FTIR spectrum (Figures 1-3) that resembled the commercial penicillin (Benzathine penicillin) (Figure 1). Those from rotten orange samples (SA_1, SA_3, and SA_5) and those from fresh orange samples (SB_1, SB_3, and SB_5) were found to be identical to the commercial penicillin. SA_2 and SA_4 from rotting oranges, as well as SB_2 and SB_4 from fresh oranges also showed minor variations from the standard.

The FTIR spectra of the penicillin produced from the orange samples and the standard, and the ones that were produced from orange samples were obtained within the range of 650 to 4000 cm^{-1} as presented in Figures 1-3. The similar spectra indicated a broad band at 3342 cm^{-1}, representing bonded –OH stretching vibration and the –NH stretching of the protein, as well as the acetamido group of the chitin fraction. The peaks appearing in the 2977 cm^{-1} and 2836 cm^{-1} region can be attributed to the asymmetric and symmetric stretching vibration of CH_2, respectively. The strong adsorption band at 1659 cm^{-1} represents C=O stretching vibration and NH deformation (amide I). The peak at 1566 cm^{-1} was assigned to a motion combining both –NH bending (amide II) and –CN stretching vibration of the protein. The typical amide III band, which represents COO^- anions, appeared at 1380 cm^{-1}. The weak peak at 1398 cm^{-1} was induced by the C-N stretching vibration of amine groups. The strong adsorption peaks 1249 cm^{-1} and 1026 cm^{-1} are indicators of $–SO_3$ groups and the C-N stretching vibration of amine groups, respectively. The moderate peak at 888 cm^{-1} may be attributed to aromatic –CH stretching vibration. The fingerprint zone of the spectra, ranging from 650cm^{-1} to 800 cm^{-1}, represents phosphate or sulphur functional groups.

A significant shift in FTIR spectra adsorption peaks was observed in the some of the samples (SA2 and SA4, SB2 and SB4), however within the expected range. The FTIR spectra observed from this study were similar to the results reported by [85].

Characterization of the Produced Penicillin Using Gas Chromatography Mass Spectrometry (GC-MS)

In addition, GC-MS analyses were performed on the crude extract of penicillin obtained from both fresh and rotten orange substrates. The compounds that were identified as prevalent are listed in Tables 3 and 4, respectively, based on their peak number, retention time, compound, reference number, Chemical Abstract Service (CAS) number, and quality match. The samples (SA1, 3, 5, and SB2, 4) that shown the greatest activity in the bioassay test and those that exhibited elution during TLC examination following recovery were the ones that underwent GC-MS studies.

Table 3 indicates that of the 31 chemicals present in SA1, 3, 5 were found using GC-MS analysis. Consisting of cis-13-octadecenoic acid, methyl ester, pentyl ester, 2,3-dihydroxypropylester, 9-octadecenoic acid (Z)-, 2-hydroxy-1-(hydroxymethyl)ethyl ester, and 4-Oxatricyclo[20.8.0.0(7,16)]triaconta-1(20),7(16)-diene were the main common compounds found in the penicillin made from rotten oranges. These compounds had respective peak numbers of 4, 8, 22, 23, and 29 and retention times of 13.6 min, 16.05 min, 21.90 min, 21.94 min, and 30.86 min. Each compound's quality match was determined to be 99, 98, 97, 95, and 70, in that order. Others were noted in little quantities, with peak values between 0.49 and 4.99%.

As seen in Table 4, the penicillin derived from fresh oranges (SB2, 4) contains fifteen components. Hexadecanoic acid, methyl ester, methyl 10-trans,12-cis-octadecadienoate, 11-octadecenoic acid, methyl ester, 1-(phenylmethyl)-, 1H-Indole-3-carboxylic acid, and cis,cis-7,10,-hexadecadienal were the chemicals. The compounds had the following peak numbers: 2, 3, 4, 5, and 7 in that order; retention times: 11.50, 13.60, 13.67, 14.00, and 18.78 minutes; and quality match: 98, 99, 99, 64, and 89 in that order.

Every detected compound was matched to the National Institute of Standards and Technology (NIST) library's collection. It was discovered that the most common bioactive substances in the penicillin that was produced were physiologically active [75]; [73].

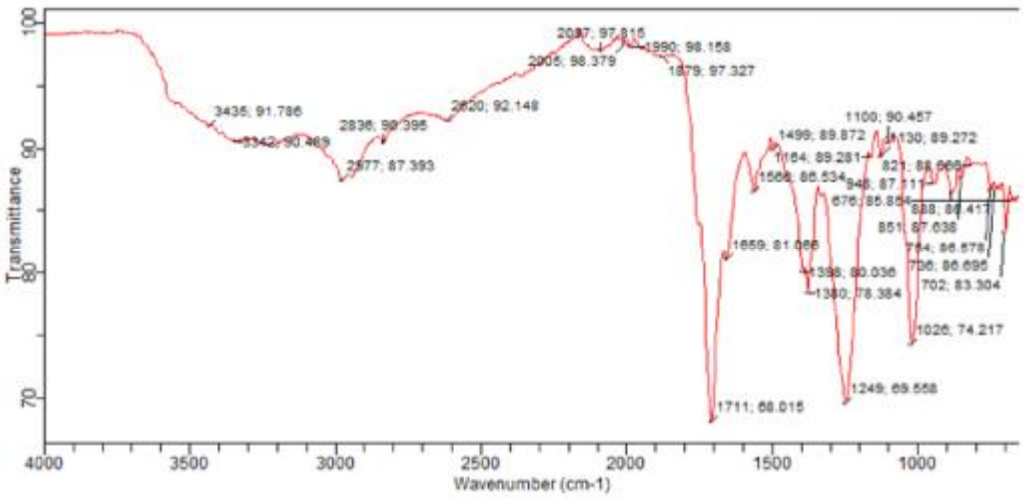


Fig.1. FTIR spectrum for the standard (Benzathine Penicillin Injection)

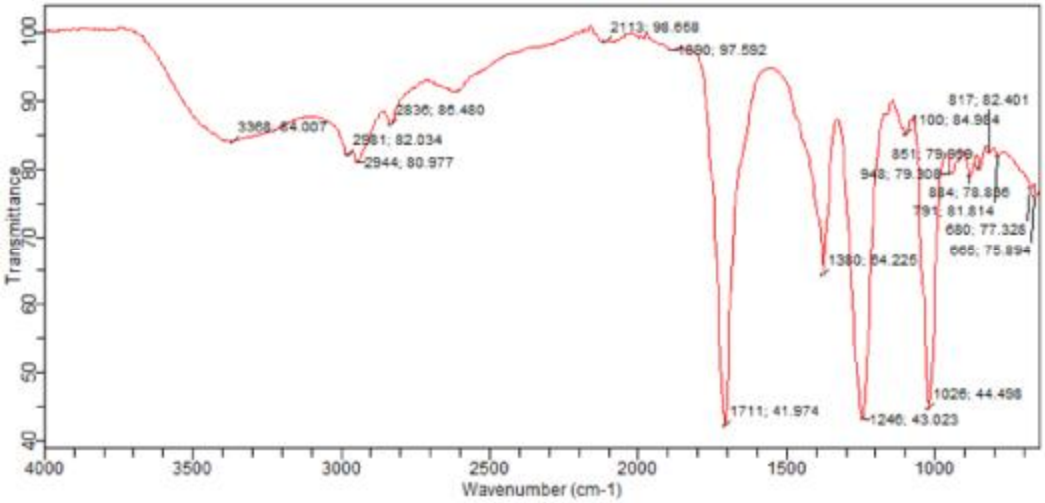


Fig. 2. FTIR spectrum for penicillin in rotten orange

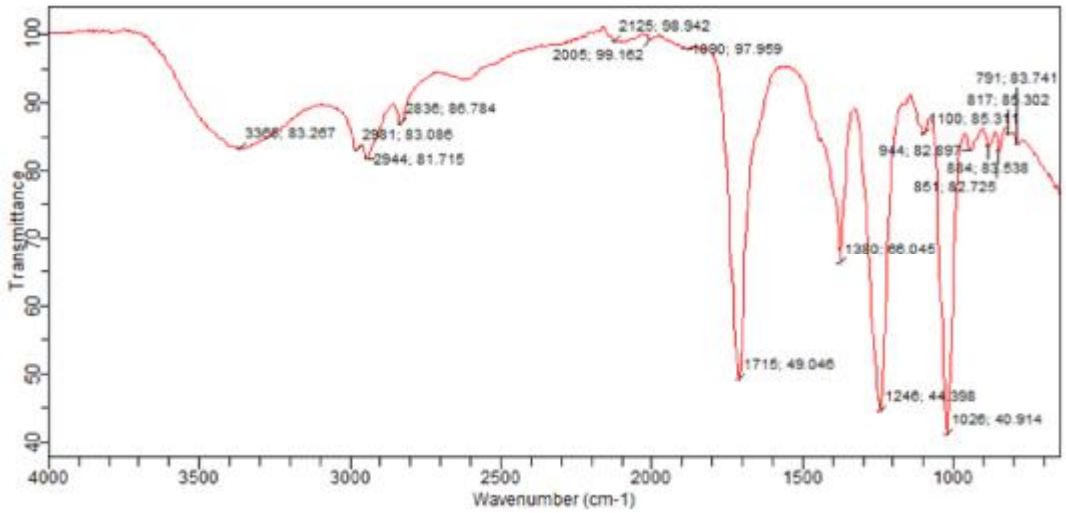


Fig. 3. FTIR spectrum for penicillin in replicate fresh orange (SB3)

Table 3: GC-MS result of most prevalent bioactive compounds in replicate of rotten orange (SA 1, 3, 5)

PK	RT	Area Pct	Library/ID	Qual
4	13.6752	12.5345	cis-13-Octadecenoic acid, methyl ester	99
8	16.05	9.5847	Hexadecanoic acid, pentyl ester	98
22	21.9035	6.7118	9-Octadecenoic acid (Z)-, 2,3-dihydroxypropyl ester	97
23	21.9395	7.4222	9-Octadecenoic acid (Z)-, 2-hydroxy-1-(hydroxymethyl)ethyl ester	95
29	30.8618	6.3339	4-Oxatricyclo[20.8.0.0(7,16)]triaconta-1(20),7(16)-diene	70

PK: Peak, RT: Retention Time, Area Pct: Percentage, Qual: Quality match
SA: Sample A

Table 4: GC-MS result of most prevalent bioactive compounds in replicate of fresh orange (SB 2, 4)

PK	RT	Area Pct	Library/ID	Qual
2	11.5966	8.0229	Hexadecanoic acid, methyl ester	98
3	13.6045	5.8326	Methyl 10-trans,12-cis-octadecadienoate	99
4	13.6738	30.7787	11-Octadecenoic acid, methyl ester	99
5	13.9928	18.2713	1H-Indole-3-carboxylic acid, 1-(phenylmethyl)-	64
7	18.78	16.321	cis,cis-7,10,-Hexadecadienal	89

PK: Peak, RT: Retention Time, Area Pct: Percentage, Qual: Quality match
SB: Sample B

Optimization and Yield of Penicillin

Table 5 displays the penicillin production from both fresh and rotting oranges. Penicillin has been obtained from just two substrates: fresh and rotten oranges. From the rotten orange samples, the yields (Table 5) were 400 mg/cm3 for SA2, 4 and 77 mg/cm3 for SA1, 3, 5. Fresh orange samples yielded yields of 100 mg/cm3 for SB1, 3, 5, and 100 (mg/cm3) for SB2, 4. It was discovered that the amount of penicillin produced exceeded the amount stated by [22].

However, in order to increase the yield of penicillin production, optimization was done based on changing a few factors. The size of the inoculums, temperature, carbon source (glucose), nitrogen source (peptone), and pH were the variables that changed [10]; [81]. Table 6 shows that the largest yields (114.3 mg/cm3) were obtained from 1g of nitrogen, followed by 84 mg/cm3 from pH 8 and 33.9 mg/cm3 from temperature (37°C).

Table 5. Penicillin yield (mg/cm^3) of each sample after production

Sample	Volume (cm^3)	Yield (mg/cm^3)
SA1,3,5	200	77
SA2,4	200	400
SB1,3,5	120	100
SB2,4	120	100
SC1,3,5	180	0
SC2,4	180	0

SA_{1-5}= Replicate rotten orange samples
SB_{1-5}= Replicate Fresh orange samples
SC_{1-5}= Replicate Preserved rotten orange samples

Table 6. Penicillin yield (mg/cm^3) of each sample after optimization

Parameter	Volume (ml)	Yield (mg/cm^3)
Inoculum size		
I2%	100	60.27
I3%	100	33.9
I5%	100	22.3
Temperature		
T30°C	100	3
T37°C	100	77
T40°C	100	11.4
T45°C	100	1.7
Carbon source		
C1g	160	7.8
C2g	140	7.3
C3g	200	7.3
Nitrogen source		
N1g	120	114.3

N2g	140	9.6
N3g	200	3
pH		
P4	170	1.2
P7	200	6.3
P8	200	84
P10	200	3

Bioassay of Penicillin produced before and after recovery

The zone of inhibition shows whether or not penicillin is effective against a particular strain of bacteria. *Escherichia coli (E. coli), Streptococcus (Strep.), Methycillin-resistant Staphylococcus aureus (MRSA), Methycillin-sensitive Staphylococcus aureus (MSSA), and Klebseilla spp. (Klep.)* were the microorganisms employed in this bioassay both before and after recovery.

To find out how sensitive penicillin was to the test organisms listed above, a bioassay (sensitivity) test was performed. Table 7 demonstrated how the penicillin made prior to recuperation inhibited the growth of E. Coli and Strep. From the replicate rotten orange samples (SA1-SA5), the highest zone of inhibitions was measured for SA2 and SA4 (22.4 mm), while the lowest zone was SA1 (10.56 mm) for both E. coli and Strep. The highest zones were measured for SA2 and SA4 (12.10 mm and 12.14 mm, respectively), while the least zone was SA1 (10.2 mm). SA2 and SA4 had the largest zone of inhibitions (22.4 mm) in E. coli, followed by SA3 (10.58 mm), SA1 (10.56 mm), and SA5 (10.57 mm), as shown in Figure 4. SA4 had the highest zone (12.14 mm) for Strep, followed by SA2 (12.1 mm), and SA1 had the lowest zone (10.2 mm).

As E. coli did not exhibit any action against the penicillin made from fresh orange samples (Table 7), the penicillin made from fresh oranges was ineffective against gram negative bacteria. Based on the gram positive bacterium Strep (Table 7), penicillin derived from fresh orange samples (SB2 and SB4) showed the maximum zones of inhibition, measuring 18.24 mm and 18.22 mm, respectively. With 13.76 mm, SB1 had the smallest zones of inhibition.

The bioassay test results are displayed in Table 8, Figure 4, following recovery. A few samples exhibit comparable behavior: SA1, 3, 5, and SA2, 4 are replicated rotten oranges; SB1, 3, 5, and SB2, 4 are replicated fresh oranges. With a zone of inhibition of 26 mm, the standard (benzathine penicillin) was obviously the most inhibited, followed by SA2, 4 and SB2, 4 (20 mm each). The SB1, 3, and 5 samples exhibit no activity. Penicillin made from the orange samples was inactive against MRSA, including the standard, with the exception of SB1, 3, and 5, which displayed an 8 mm zone of inhibition. This is consistent with the outcome that [6] reported. The rotten orange replicate (SB1, 3, 5) in MSSA showed the largest zone of inhibition (32 mm), then the fresh orange replicate (SB2, 4) with a 26 mm zone of inhibition. There was no inhibition seen in the sample SA2, 4, or standard. The results that Klebsiella produced were comparable to those that [22] reported. The samples SA2, 4 had the largest zone of inhibition (34 mm), as indicated in Table 8, Figure 4. Samples SB2, 4 had zones of 30 mm. The standard's zone of 16 mm was the smallest.

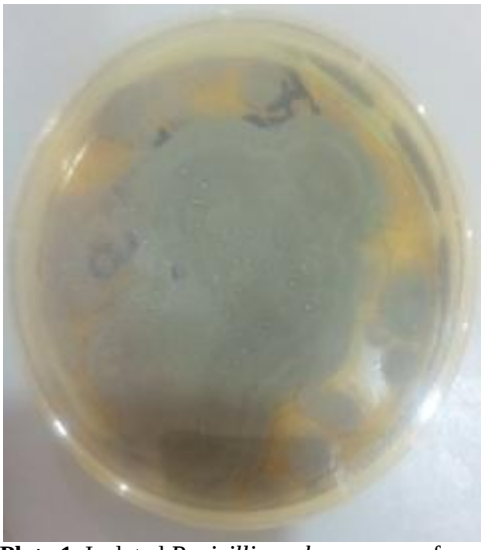


Plate 1. Isolated *Penicillium chrysogenum* from soil

Table 7. The zones of penicillin inhibition before recovery

Sample	E.coli (mm)	STR(mm)
SA1	10.56	10.2
SA2	22.4	12.1
SA3	10.58	10.3
SA4	22.4	12.14
SA5	10.57	10.23
SB1	0	13.76
SB2	0	18.24
SB3	0	13.78
SB4	0	18.22
SB5	0	13.77

E.coli**:** *Escherichia coli,* STR**:** *Streptococcus,*

SA_{1-5}= Rotten orange (samples in five replicates)
SB_{1-5}= Fresh orange (sample in five replicates)
SC_{1-5}= Preserved rotten orange (samples in five replicates)

Table 8. The zones of penicillin inhibition after recovery

Sample	E.coli (mm)	MRSA (mm)	MSSA (mm)	Klep. (mm)
SA2,4	20	0	0	34
SB1,3,5	0	8	32	20
SB2,4	20	0	26	30
Standard	26	0	0	16

E.coli**:** *Escherichia coli,* MRSA: *Methycillin resistant Staphylococcus aureus,* MSSA: *Methycillin-sensitive Staphylococcus aureus,*
Klep.: *Klebsella spp*
SA_{1-5}= Rotten orange (samples in five replicates)
SB_{1-5}= Fresh orange (sample in five replicates)
SC_{1-5}= Preserved rotten orange (samples in five replicates)

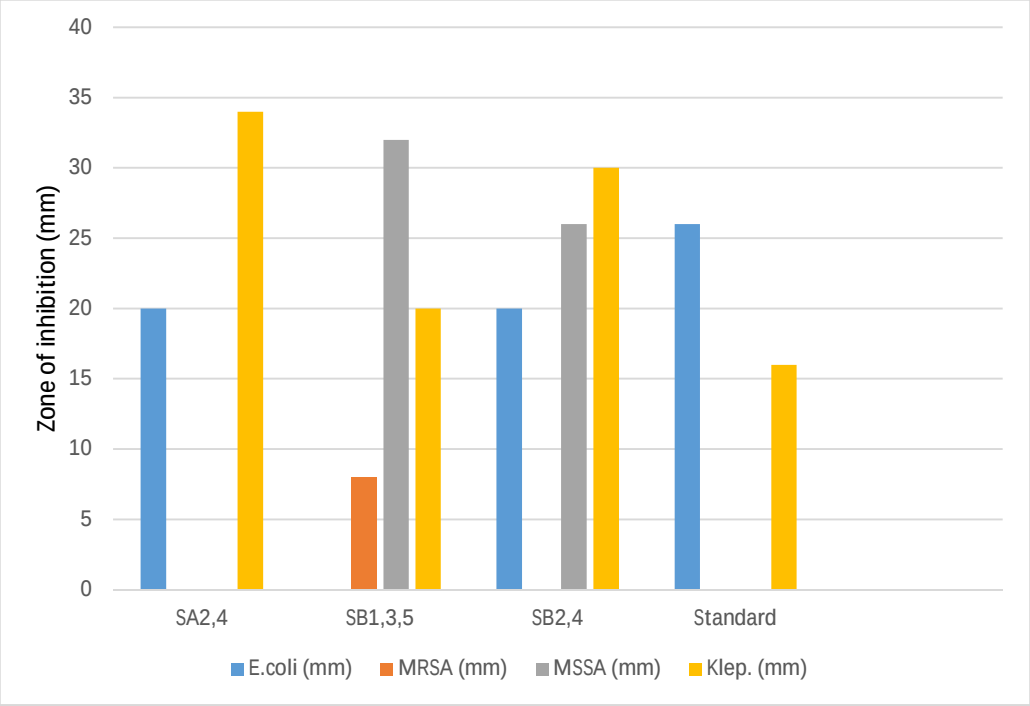


Fig. 4: The zones of inhibition (mm) of penicillin against some pathogens using rotten and fresh orange samples after recovery

E.coli**:** *Escherichia coli,* STR**:** *Streptococcus,* MRSA: *Methycillin resistant Staphylococcus aureus,* MSSA: *Methycillin-sensitive Staphylococcus aureus,*
Klep.: *Klebsella spp*
$SA_{2,4}$= Rotten orange (samples in five replicates)
SB_{1-5}= Fresh orange (sample in five replicates)
Standard= Benzylpenicillin (Penicillin G)

Conclusion

According to the study, both fresh and rotten oranges can serve as a good substrate for the fermentation media that are used to grow the fungus that produces natural medicines like penicillin. Given that both fresh and rotten oranges are produced in vast quantities in Nigeria, this is extremely significant. Therefore, oranges (fresh or rotten) can be used as the only carbon and energy source when creating inexpensive microbial medium, as opposed to synthetic media that contains glucose. Its benefits over industrial synthetic substrates include being natural, easily obtainable, economical, sustainable, and eco-friendly.

Fresh and decaying oranges may be reliable and secure sources of the natural antibiotic Penicillin G, according to bioassay and TLC, FTIR, and GC-MS characterization of the generated penicillin. The natural technique of generating penicillin by medium formulation is possible since the yield of penicillin produced before to optimization yielded more penicillin than the parameters utilized during optimization.

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