JMBFS
Journal of Microbiology, Biotechnology and Food Sciences
International peer-reviewed scientific online journal

Published by Faculty of Biotechnology and Food Sciences

ANTIBACTERIAL ACTIVITY AND IDENTIFICATION OF PRODUCED REUTERIN FROM LOCAL *Lactobacillus reuteri* LBIQ1 ISOLATE

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https://doi.org/10.55251/jmbfs.4701

ARTICLE INFO

Received 24. 4. 2021
Revised 2. 2. 2023
Accepted 20. 2. 2023
Published 3. 4. 2023

Regular article

OPEN ACCESS

ABSTRACT

Reuterin was anaerobically manufactured by developing *Lactobacillus reuteri* LBIQ1 (a local isolate) at 37 °C in the MRS broth medium. Cell biomass was harvested after 18 hours of incubation and used to ferment 278 mMol glycerol for 3 hours in a water-glycerol solution at 37 °C. Cells were then extracted from the suspension solution, which remained at 4 °C until colorimetric, UV, and HPLC assays observed the presence of reuterin in the solution. The reuterin compound has inhibitory activity against Gram-positive and Gram-negative bacteria. The highest inhibition zones measured were 23mm and 21mm against *Staphylococcus aureus* and *Escherichia coli*, respectively. The UV chromatogram of produced reuterin showed one peak at 280 nm, while HPLC analysis were appeared three peaks at 6.48, 15.6 min, and 15.49 min for the injection peak, residual glycerol, and produced reuterin, respectively. After incubation for 3 hours, a total yield of 167.382 L/ml reuterin was estimated as the productivity of *L. reuteri* LBIQ1in the glycerol-water solution. The highest inhibition zones of pure reuterin against *S. aureus* and *E. coli* were 44 and 39 mm, respectively. Reuterin is an effective compound against microorganisms that can be produced from glycerol media and used in food preservation.

Keywords: *Lactobacillus reuteri*, Reuterin, 3-hydroxypropanal, antibacterial, HPLC

INTRODUCTION

In recent years, many biologists have shown a wide interest in studying antibiotics produced by Lactobacilli. This interesting was due to many reasons such as their wide inhibition spectrum for growth of pathogens and food damaging bacteria. In addition, Lactobacilli antibiotics represent a natural inhibitor used in food preservation **(Duan *et al*., 2020)**, especially acidic and neutral foods. This, antibiotics and bacteriocins of Lactobacilli used commercially as a replacement natural material against chemical preservatives which mostly causes a great harm at the level of food safety and human health **(Naimah *et al*., 2018; Niamah, 2018)**. One of the Lactobacilli species is *Lactobacillus reuteri*, which is naturally found in the gastrointestinal tracts of humans, chickens, and other animals. *L. reuteri* can produce Reuterin, which is an intermediate metabolic compound produced from glycerol fermentation under anaerobic conditions and has a wide inhibition effect against other Gram positive and negative bacteria, fungi, yeasts, and also some protozoa **(Greppi *et al*., 2020)**. Reuterin is a non-protein compound that is neutral water soluble, and has a low molecular weight (< 200g/mol) and it derivative from glycerol **(Lindlbauer *et al*., 2017)**. Reuterin is produced from *L. reuteri* as an intermediate compound during the anaerobic hetero-fermentative metabolic pathway by removal of the water molecule from the glycerol **(Talarico *et al*., 1988)**.
Chemically, reuterin is identified as β-hydroxypropionaldehyde or 3-hydroxypropanal (3-HPA) and it is formed during the anaerobic condition growth of *L. reuteri* by the work of glycerol dehydratase which catalyzes the diversion of glycerol into reuterin compound. This bacterium uses the phoshoketolase pathway for fermentation of carbohydrates to lactate, acetic acid, ethanol, and carbon dioxide. 3-hydroxypropanal (3-HPA) is produced as an intermediate step in the conversion of glycerol to 1,3-propanediol, a pathway proposed to regenerate NAD^+ from NADH and to contribute to improved growth yield **(Rodriguez *et al*., 2003)**. Many studies were explained to the technique of activity by which the reuterin compound spends its antimicrobial impacts has been so hard to be known. The cause is the aldehyde group of the reuterin, which is highly reactive; thus, the reuterin compound can be converted to different compounds in the hydrous solutions. The reuterin can dimerized to form hydroxypropanal (HPA) dimer (this normally shown in very high concentration of 3-hydroxypropanal); or can be hydrated to form the HPA hydrate; or can also be dehydrated to form the toxic compound 'acrolein'. Depending on the two main hypotheses that have been found to explain its method, The first hypothesis was proposed that aldehyde group of the reuterin is highly reactive with the thiol groups and the primary amines, therefore, reuterin compound could inactivate or inhibited the proteins and the small molecules that containing such groups **(Katryniok *et al*., 2013)**.
Reuterin is effective against a wide range of microorganisms, including *Candida* species (*C. albicans*, *C. dubliniensis*, *C. glabrata*, *C. krusei*, *C. parapsilosis*, and *C. tropicalis*) **(Jørgensen *et al*., 2017)**, *Bacillus subtilis*, *E. coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* **(Mohammed *et al*., 2020)**, *Clostridioides difficile* **(Engevik *et al*., 2020)**, *Campylobacter jejuni* and *Campylobacter coli* **(Asare *et al*., 2020)**.
The present study was aimed at the production and extraction of reuterin from a local isolate of *Lactobacillus reuteri*, followed by a study of the antibacterial activity of extracellular metabolites offered in cell-free supernatant and the extraction and characterization of the reuterin product.

MATERIAL AND METHODS

Bacteria strains

In the European Nucleotide Archive (ENA), the National Centre for Biotechnology Information (NCBI) and Gene Bank, *Lactobacillus reuteri* isolate has been checked and registered as a local Iraqi isolate. Under the name "LBIQ1" this isolate was reported (MT259030). The Food Science Department/College of Agriculture/University of Basrah, Iraq supplied *E. coli* ATCC 25922, *Listeria monocytogenes* ATCC 15313, *Pseudomonas aeruginosa* ATCC 15442 and *Staphylococcus aureus* ATCC 25923 and they were used as indicator strains.

Antibacterial activity of culture supernatant

The *L. reuteri* isolate was screened for their antibacterial activity spectra, using the well diffusion agar method **(Niamah, 2010)** and filter paper disc diffusion method **(Mulyani *et al*., 2019)**, against *E. coli*, *Listeria monocytogenes*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*. First, the cell-free culture supernatants (CFS) were prepared by growing the isolates in tubes of MRS broth (Hi Media, India), adjusting the pH to 6.2±0.2 for 16–18 h at 37 ºC, then centrifuging the tubes at 8,000 rpm for 10 min and transforming the cells into new MRS broth (pH 7.2±0.2). After completing the incubation for 24 h at 37 ºC, tubes were centrifuged again, and the culture supernatant obtained was sterilised through 0.45 Millipore filters (Millipore S.A.S.- Molsheim, France) to remove all live cells and determine their activity. At the same time, the indicator bacterial cultures were grown for 24 h in nutrient broth (Hi media, India) tubes, and then 0.1 ml (1×10^7 CFU/ml) of each were inoculated by pouring into or spreading on Muller Hinton agar (Hi media,

India) plates independently. In the spread method, 6-millimeter wells were formed onto agar plates and then filled with 0.05 ml of the CFS. The plates were kept undisturbed for 2 h and subsequently incubated at 37ºC. for 24 h. After that, the diameters (mm) of the growth inhibition zones were measured **(Al-Fekaiki *et al*., 2017)**.

Reuterin production and extraction

Reuterin was produced from *L. reuteri* cultures using the methods described previously by **Mohammed *et al*. 2020** with some modifications. Briefly, the activated cells of the *L. reuteri* isolate were grown in 20 tubes of 50 ml MRS broth culture, harvested after 18 h of incubation by centrifugation (7000 rpm for 10 min), and washed twice with 50 mMol sodium phosphate buffer (pH 7.2±0.2). The washed cells were then resuspended in 100 ml of 278 mMol water-glycerol solution (pH 7.4±0.2). Then, water-glycerol suspension was distributed on 10 ml screw-capped tubes and incubated under anaerobic conditions at 37°C for 3 h. Reuterin was collected by tube centrifugation (8000 rpm for 15 min) and filtering the supernatant through 0.45 mm Millipore filter paper. The resulting cell-free supernatant was then stored in refrigerator at -4°C before it undergoes the analyses tests for identifying of reuterin containing.

Identification of extracted reuterin

The colour test for detection of reuterin

A colour test, based on the method of **Vimont *et al.* (2019)**, was used for the detection of 3-HPA content in the glycerol-water solution depending on the 3-HPA dehydration to acrolein, which in turn reacts with the tryptophan reagent (Sigma-Aldrich, Germany) to form a purple complex when compared with standard acrolein. Three millilitres of properly diluted samples and standard acrolein were combined with 6 ml of concentrated HCL and 1.5 ml of tryptophan reagent in a test tube. The mixtures were then incubated for 20 min at 37°C as the purple colour developed, became stable for approximately 15 min, and could be maintained for at least 1 h by cooling the tubes in the refrigerator.

UV chromatogram of sample and standard acrolein

To ensure the detection of 3-HPA in the extract solution of *L. reuteri,* the UV chromatograms of the sample and standard acrolein were measured to determine the identity between them. The method of **Chen *et al*. (2020)** was also used without adding the DL-tryptophan reagent. After Millipore filtration, the 3-HPA sample solution was subjected to dehydration under acidic conditions by the combination of 3 ml of samples, as well as standard acrolein, with 6 ml of concentrated 37% HCL and incubated for 20 min at 37°C then kept in the refrigerator until UV detection was done at the range of wave length between 200 and 800 nm to be compared between peaks obtained as an indicator of 3-HPA detection.

HPLC chromatogram of sample and standard reuterin

After centrifugation and Millipore filtration of glycerol-water solution, the extracted reuterin product was sent to characterized by HPLC technique. Samples from both extracted and standard reuterin were sent to Gahan Shemi Kuster Company laboratories /Tehran, Iran, where the analysis of the HPLC test was done by using a Shimadzu binary pump with an automatic injector and the acetone used as eluent **(Mauro and Garcia, 2019)**.

Quantification of reuterin by standard curve of acrolein

Depending on the method of **Burge *et al.* (2015)**, a standard calibration curve of acrolein, as an analytical standard, was performed for different concentrations (0.1, 0.5,0.5 ,1, 1.5, 2, 2.5, and 3 mg) prepared in water mixed with 0.75 ml of DL-tryptophan reagent and 3 ml of HCL 37% in a test tube. At the same time, a sample of 3-HPA extract was filtered through 0.22-mm Millipore filter paper, and then 1 ml of the sample was mixed with 0.75 ml of DL-tryptophan reagent and 3 ml of HCL 37%. Tube acrolein samples and standards were then incubated for 20 min at 37°C, and the optical density was measured immediately at 560 nm. If necessary, samples were diluted with distilled water to ensure an OD_{560} within the range of the OD_{560} of standard acrolein..

Pure reuterin activity against bacteria

The bioactivity of the pure reuterin were assessed by using the wells diffusion method. Both of the pure reuterin were produced in a test tube of glycerol-water solution with the pH neutralised to 7.4 for the prevention of organic acid production. Tubes were then subjected to centrifugation at 8000 rpm for 10 minutes and filtered through 0.45-micron Millipore filter papers. Fifty microliters of pure reuterin were transferred onto the wells of Muller-Hinton agar that had been previously inoculated with *E. coli, Listeria monocytogenes, Pseudomonas aeruginosa,* and *Staphylococcus aureus* and left for 60 min at room temperature until they diffused, then incubated at 37°C for 24 h. After incubation, the zone of growth inhibition was measured in millimetres (mm).

Statistical analysis

The obtained data has been treated with statistical analysis by mean ± standard deviation. One-way ANOVA table was used to analyze the using chi square to count of the significant values at ($P<0.05$) level using the statistical analysis program SPSS version 16.

RESULTS AND DISCUSSION

Antibacterial spectrum of *L. reuteri* culture supernatants

Table 1 shows the inhibition effect of the culture supernatant of the *L. reuteri* isolate against four indicator strains (*E. coli, Listeria monocytogenes, Pseudomonas aeruginosa,* and *Staphylococcus aureus*). The inhibition zones were measured at (23, 22) mm against Gram-positive *S. aureus* and *L. monocytogenes,* while they were 21 mm and 16 mm for Gram-negative *E. coli* and *P. aeruginosa,* respectively. This result gives a sign that the isolates under study have the ability to produce an antibacterial substance against pathogenic bacteria. We then discovered that the antimicrobial bioactive compounds are found in the culture supernatant rather than within the bacterial cells. Our results were in agreement with many past studies that indicated the antibacterial activity of reuterin produced by *L. reuteri*. **Langa *et al*. (2018)** reported that *L. reuteri* reacts to the invasion of pathogens with the production of the antimicrobial substance reuterin. **Mohammed *et al*. (2020)** mentioned that the reuterin compound is produced in anaerobic conditions with the presence of glycerol and that it causes oxidative stress in pathogenic bacterial cells while lactobacilli, streptococci, and other commensals are more resistant to reuterin. Numerous experiments have tested the antimicrobial effects of biologically derived agents against these bacteria to date, with a new review disclosing the antimicrobial activities of *L. reuteri* supernatant against *Porphyromonas gingivalis*, *Fusobacterium nucleatum*, and *Streptococcus mutans* **(Yang *et al*., 2021).**

Table 1 Antibacterial activity spectrum of bacteria *L. reuteri* culture supernatants

Indicator strains	Inhibition Zone (mm)*
E. coli	21±0.5
Listeria monocytogenes	22±0.9
Pseudomonas aeruginosa	16±0.6
Staphylococcus aureus	23±0.3

* The mean values with standard deviation (SD), n = 3 for samples.

Identification of extracted reuterin

The colour test for detection of reuterin

After a short period of incubation at 37°C, the colour of both sample and standard acrolein solutions began to develop to red, then converted to red-purple and finally to deep purple which was left at the end of the incubation period until the purple color became stable of both tubes (Figure 1). The test gave a colour indicator for the chemical conversion of 3-HPA to acrolein as it subjected it to the dehydration process under the effect of concentrated HCL. This result was in agreement with **Sun *et al*. (2018)**, who mentioned that this method is specific for acrolein detection as a replacement for 3-HPA, which in turn reacts with tryptophan to form a purple complex..

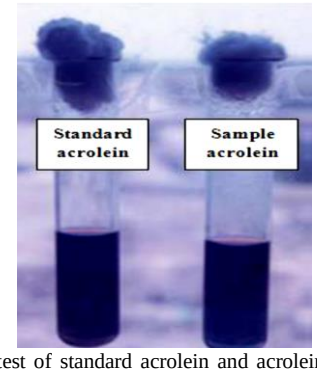


Figure 1 The color test of standard acrolein and acrolein yielded from 3-HPA dehydration.

UV chromatogram of sample and standard acrolein

The UV chromatogram result (Figure 2) showed one peak identity between the sample and standard acrolein solutions at 280 nm, indicating that acrolein was yielded by dehydration of 3-HPA content in the extraction solution under acidic conditions. The asymmetric length of the sample and standard peaks is probably due to the differences in purity and concentration between sample and standard,

despite many attempts we made to obtain further identity in the peaks of UV detection. This method was derived from the work of **Burge *et al*. (2015),** who used both acrolein (an analytical standard) in the colorimetric method and the UV chromatogram of citric acid (an internal analytical standard) in the separation and analysis of synthetic 3-HPA by HPLC assay. Therefore, this analytical method is appropriate for the detection of 3-HPA production capability efficiently and effectively based on the use of a UV chromatogram for acrolein as a diagnostic standard.

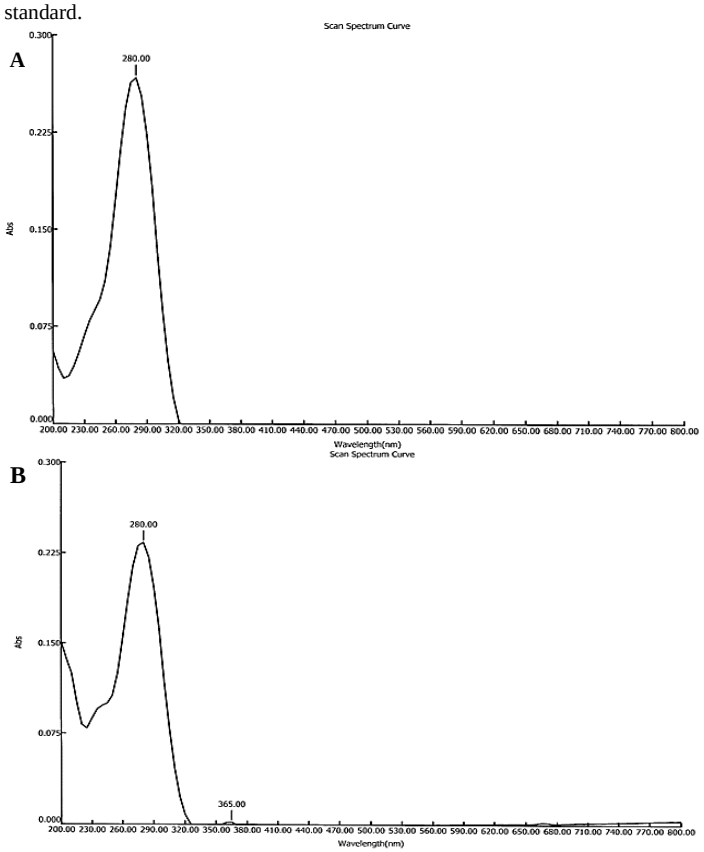
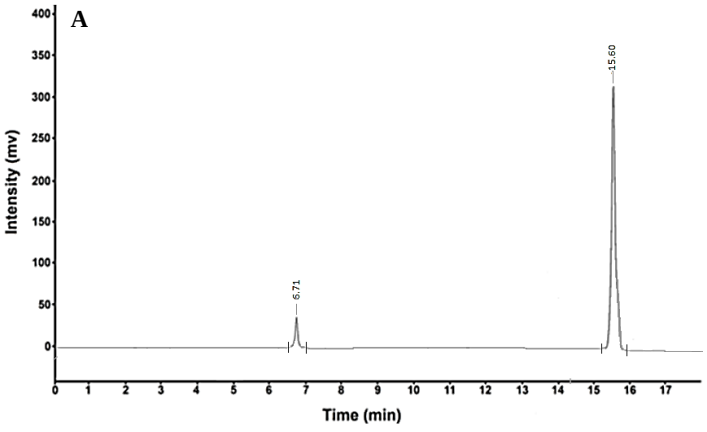


Figure 2 UV chromatogram detection at 280nm for (A) acrolein standard solution (B) acrolein sample solution.

HPLC analysis of extracted and standard acrolein

Results showed the high purity of reuterin, which was represented by the sharp reuterin peak in the purified, extracted sample in comparison with the standard (Figure 3). The first small peaks in both (A) and (B) represent the injection peak, which appeared at 6.71 and 6.48 of standard and sample reuterin, respectively, while the reuterin peaks appeared at 15.6 min and 15.49 min, respectively. The third peak that shown in figure (B) the sample was represented the residual glycerol in the production solution.



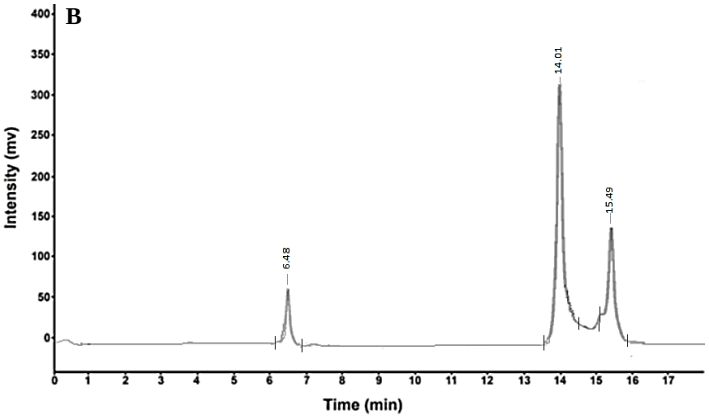


Figure 3 HPLC chromatogram of reuterin compound. (A)standard solution, (B)sample solution.

Quantification of reuterin by the standard curve of acrolein

Depending on **Burge *et al*. (2015)**, who referred to 1 mol of 3-HPA dehydrating to 1 mol of acrolein, the quantification of 3-HPA content in the extracted samples was assessed by the analytical calibration curve of the standard acrolein in a range of concentrations tested. The linear equation was obtained between the prepared concentrations of standard acrolein and its optical density (OD) at 560 nm (Figure 4).

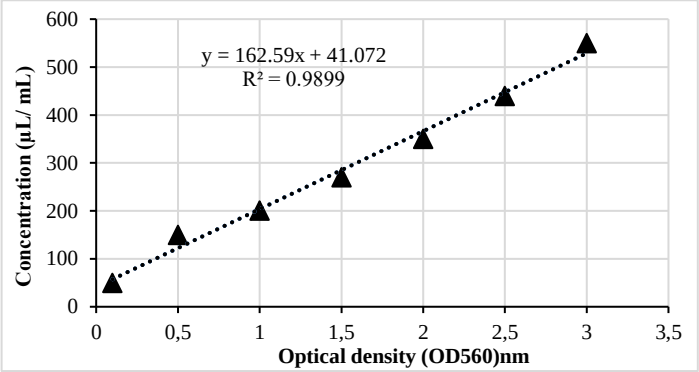


Figure 4 Standard curve of standard acrolein concentrations at optical density (OD) at wavelength 560 nm.

The incorporeal treatments of the regression equation for the prediction of the reuterin (3-HPA) concentrations that are produced in the supernatant of *L. reuteri* suspension were significant ($P < 0.05$) depending on optical density (OD_{560}) for any sample. So, by compensating for the absorption value of the sample supernatant in the equation, we can predict its concentration. For obtaining the actual concentration of sample supernatant, the obtained value from the equation has been multiplied by the sample dilution factor. According to this result, the predicted concentrations of the produced 3-HPA were calculated in the glycerol-water supernatant of the *L. reuteri* suspension. As clarified in table (2), the regression equation above was applied in the calculation and prediction of 3-HPA concentrations (column Y) using the optical density OD_{560} (column X) obtained for *L. reuteri* supernatant. The OD_{560} data has been recorded after each 30 minutes for the production period (total period = 3 hours), which started beyond 18 hours of growth in the MRS broth and the transformation of cells into a glycerol-water solution. Figure 4 shows the slope and the straight increasing in concentration gradually with incubation time, depending on the absorption indicator of the supernatant.

Table 2 Concentrations of produced reuterin (3-HPA) per each 30min of incubation period, predicted according to acrolein OD_{560} absorption.

Time (min)	Absorption nm. (X)	Concentration µL/ml (Y)
30	0.083	165.99
60	0.135	168.13
90	0.145	168.54
120	0.157	169.03
150	0.168	169. 49
180	0.199	170.76

From the monitoring of the production process by this curve, we observed that 148.173µL/ml began to yield after 30 minutes of incubation and, when the incubation time continued to 3 hours, a total yield of 167.382µL/ml reuterin was estimated as the productivity of *L. reuteri* LBIQ1, under study, in the glycerol-

water solution. The yield of reuterin produced at different glycerol concentrations in glucose and other sugars as carbon dioxide sources depends on the action of the catabolite repression element sequence existent in the DNA of *L. reuteri* bacteria, where this result was distinguished in comparison with other previous studies. **Liang and Sung (2001)** reported that the fermentation optimum conditions for reuterin production were 100mg/ml in 300mM glycerol-water suspension of *L. reuteri* incubated anaerobically at 37°C for 1 h. A notable variance in reuterin concentrations and susceptibility among bacteria strains has been reported. 4000 activity units (AU)/ml of reuterin were obtained from using *L. reuteri strain 12002* in a 2-step fermentation process for the production of the greatest amount of bacterial biomass, which was then collected and re-suspended in a glycerol-water solution and incubated anaerobically to produce reuterin. The produced reuterin was then water-diluted and used in food decontamination, where MIC values against *E. coli* and *Listeria monocytogenes* were 4 AU/ml and 8 AU/ml, respectively **(EI-Ziney *et al*., 1999)**. The reason of this reuterin activity was clarified by **Fujiwara *et al*. (2017)** who's referred to HPA system which contains three compounds, were used together in food preservation, there were HPA-hydrate and HPA-dimmer which beat equilibrium state.

Pure reuterin activity against bacteria test

The bioactivity of the pure reuterin against *E. coli*, *Listeria monocytogenes*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* test bacteria revealed a significant difference ($p<0.05$) between their frequencies. The diameter of the inhibition zones for the pure reuterin was 39, 38, 30, and 44 mm, respectively (Table 3 and Figure 5). This result clarifies that the purity of reuterin is important for its mode of action. As mentioned previously, the aldehyde group of reuterin is highly reactive, which leads to the conversion of reuterin in a glycerol-water solution to various compounds, such as HPA dimer or HPA hydrate, termed the "reuterin system," or to the toxic acrolein compound.

Table 3 The diameters inhibition zones (mm) of pure reuterin activity against bacteria test

Indicator strains	Pure reuterin*
E. coli	39±1.2
Listeria monocytogenes	38±1.0
Pseudomonas aeruginosa	30±0.5
Staphylococcus aureus	44±0.3

* Mean values with standard deviation (SD), n = 3 for samples.

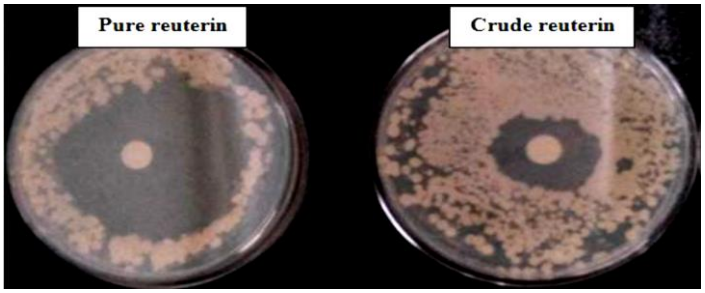


Figure 5 The activity of pure and crude reuterin (CFS) against *staphylococcus aureus* test bacteria.

L. reuteri use glycerol as the raw material for secreting high levels of reuterin when grown or incubated in the presence of excess amounts of glycerol. When the glycerol amount decreased, the amount of reuterin decreased too. **Doi (2019)** reported that glycerol is metabolised in two pathways: the oxidation by a dehydrogenase to dihydroxyacetone (DHA), which is phosphorylated by DHA kinase and enters the glycolysis pathway; or the reductive pathway, where glycerol is converted to 1,3-propanediol via 3HPA as an intermediate compound.

CONCLUSION

The results obtained in this research explained that reuterin compound is a strong natural disinfecting agent able to inhibit many species of Gram positive and negative bacteria. This opens up one of the alternate ways to tackle the antibiotic-resistance menace that is rampant around the world in this century. In addition, it uses a preservative as an alternative to chemical additives in food. In the reuterin pathway, glycerol is converted to 1,3-propanediol via 3HPA as an intermediate compound that has inhibitory activity against bacteria. The purification method of the reuterin compound increases the inhibitory activity against the bacterial species tested.

Acknowledgments: Thank you for Staff of Biotechnology Lab., Department of food science, College of Agriculture, University of Basrah, The Ministry of Higher Education and Scientific Research of Iraq.

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