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Production of Pyocins of *Pseudomonas aeruginosa*: A Structure and Methodology Review

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Abstract

The main objective of this review is to clarify the definition, types and structures of pyocins. It also highlights the various methods and their procedures that have been employed to screen, extract, isolate and purify pyocins from *Pseudomonas aeruginosa*. Furthermore, this study provides an analysis of the advantages and disadvantages associated with each approach. This may aid future research in combating antibiotic resistance that has become increasingly prevalent in recent years creating a burden on both the medical and financial fronts. In recent years, the resistance to antibiotics has increased rapidly due to the misuse and the presence of inhibitory and sub-inhibitory concentrations of antibiotics in various terrestrial and aquatic environments. The misuse of these antibiotics has led to the emergence and spread of antibiotic resistant microbes, posing challenges in treating common infections. The understanding of these findings is crucial for devising strategies to explore alternatives such as pyocins to replace conventional antibiotics capable of efficiently combating bacterial illnesses. The study concluded that some techniques may not be very effective at differentiating between types of pyocins though they are cheap which makes them beneficial for screening. On the other hand other techniques accurately identify the pyocins, nevertheless, they are costly, complicated, and need specific tools though some are more expensive than others. Some techniques lack specificity, and left out centrifuge specifics that are essential to maintaining consistency

between experiments. The initial ammonium sulphate saturation has not been mentioned and a range with ammonium sulphate precipitation with the highest yield should be measured. Numerous strains of indicators have been employed, indicating the necessity for more extensive research to identify trustworthy indicators for consistent outcomes.

Keywords: *Pseudomonas aeruginosa* bacteriocin, pyocin, structure, induction, production, extraction.

Introduction

The demand for novel and alternative treatments is on the rise as the healthcare system worldwide grapples with the challenge of antibiotic resistance which has been increasing over the years due to misuse and increased selection pressure which forces these microbes to adapt rendering the used antibiotics nonfunctional [1, 2]. Over 1.27 million deaths were related to antimicrobial resistance in 2019 alone, while 5 million deaths were attributed to antimicrobial resistance according to a study published in 2022, this number is predicted to increase to 10 million deaths a year by 2050 [3]. Bacteriocins are peptides that are produced by some microorganisms mostly gram-positive bacteria which possess antimicrobial activity especially against closely related bacteria. They are tiny molecules that typically consist of 30-60 amino acids. They have the ability to withstand high temperatures and differ in their molecular weight, mechanism of action and antimicrobial spectrum [4]. Bacteriocins are classified to three groups based on shared characteristics such as: structure, molecular weight, mode of action, heat stability and genetic properties. Class I bacteriocins: known as lantibiotics are peptides that have a 2 - 5 kDa molecular weight and are produced by gram-positive bacteria. They are unique because they contain an amino acid called lanthionine. Class II bacteriocins: are below 10 kDa while Class III bacteriocins, have a molecular weight of more than 10 kDa [5, 6]. Each bacteriocin possesses distinct mechanisms of action and targets specific sites, For example, Bacteriocins recognize and attach to target cells, certain chemicals or surface receptors in these cells allow bacteriocins to recognize them [7, 8]. Bacteriocins can also cause pore formation and inhibit cell wall synthesis [9, 10]. Bacteriocins can halt nucleic acid and protein synthesis which may lead to cell death [11], bacteriocins can also inhibit important enzymatic actions like ATP synthetase complex, DNA gyrase and DNA polymerase [10], Anti-biofilm ability of some

bacteriocins increases their susceptibility to antimicrobials by dispersing them [12], Bacteriocins can also have synergistic antimicrobial effect when combined with other antimicrobials [13]. Though bacteriocins can be used as an alternative for conventional antimicrobial drugs, they also face the same problem of resistance that antibiotics do. Different mechanisms have been employed by microbes to defend themselves from the antimicrobial effect of bacteriocins, some of these include: Antagonistic bacteriocin receptors that bind to the bacteriocins and efflux pumps which rid the cell of them to prevent these bacteriocins from exerting their antimicrobial activity on the cells [14]. Studies have also shown that resistance to bacteriocins can be spontaneous or induced due to the exposure of bacteria to the bacteriocin which leads to alteration of the cell membrane for example rendering them less effective [15]. The presence of the mannose sugar phosphoenolpyruvate phosphotransferase system (PTS) operon plays an important role in regulating gene expression potentially leading to resistance against bacteriocins [16]. Changes in the composition of cell membranes can contribute to resistance against bacteriocins, for instance resistant cells may exhibit higher levels of unsaturated and short acyl chain phosphatidyl-glycerols, which can impact the interaction, between bacteriocins and the cell membrane itself [15]. Bacteria also have the ability to change the structure of bacteriocins rendering them ineffective either by breaking them down with enzymes or adding a chemical group to them [17]. Some types of bacteria like Pseudomonas for example have developed some mechanisms that can allow them to survive the effect of bacteriocins without developing resistance to them [18]. Some bacteria can also create biofilms which serve as a barrier that keeps antimicrobials out and therefore increases their resistance to them [14]. And last but not least the bacteria that produce bacteriocins also have specific proteins that shields them from their effect [19]. This research delves into the possibilities of pyocins produced by *Pseudomonas aeruginosa* as an alternative to conventional antibiotics. Moreover this research seeks to uncover the complexities of pyocins by examining methods of extraction, understanding their structure and investigating how they work, by gaining insights into their properties this study aims to make a contribution by highlighting different methods that can be used to induce, extract, and purify pyocins for treatments in light of the urgent concern regarding antibiotic resistance.

The method used for this review was a comprehensive search of literature that is available online. The search strategy incorporated the use of relevant keywords that are related to Bacteriocins, *Pseudomonas aeruginosa*, Pyocins, Production and Structure; the search was not limited by any time period due to scarcity of studies and to include relevant studies. Only articles that used the English language were included.

Pyocins

Pyocins are categorized into 3 types: R-pyocins, F-pyocins and S-pyocins, pyocins possess the capability to eliminate various types of bacteria. S pyocins specifically target *P. aeruginosa*, it's important to note that pyocin S2 has demonstrated results in combating *P. aeruginosa* biofilms indicating its potential as an option for antibacterial treatment. R-pyocins exhibit effectiveness against gram-negative bacteria. The structures and protein sequences of pyocins belonging to the F and R types resemble those found in bacteriophage tails. Moreover the GC content of pyocin genes is lower than those of the *P. aeruginosa* genome suggesting that their origin is from a phage [20].

The amino acid sequence of **R-pyocins** lead into the classification of R-pyocins into five groups (R1-R5), with R2, R3, and R4 sharing a nearly identical sequence, while R1 and R5 differ in the C-terminal region. R-pyocins exhibit a structure resembling nonflexible contractile tail structures found in bacteriophages of the Myoviridae family. The structure of these pyocins resembles double hollow cylinders with specific components including a rigid core, sheath, baseplate, and tail fibers. The core is encased by a contractible outer sheath and the tail fibers facilitate attachment to target cells, refer to Fig. 1. The sheath contracts as it binds to the target membrane, forcing the pyocin into the cell membrane. This causes the cell membrane to depolarize, which stops the synthesis of proteins and nucleic acids and causes ions to flow out of the cell. R-pyocins are highly efficient; a bacterial cell can be killed with merely a single R-pyocin particle [21].

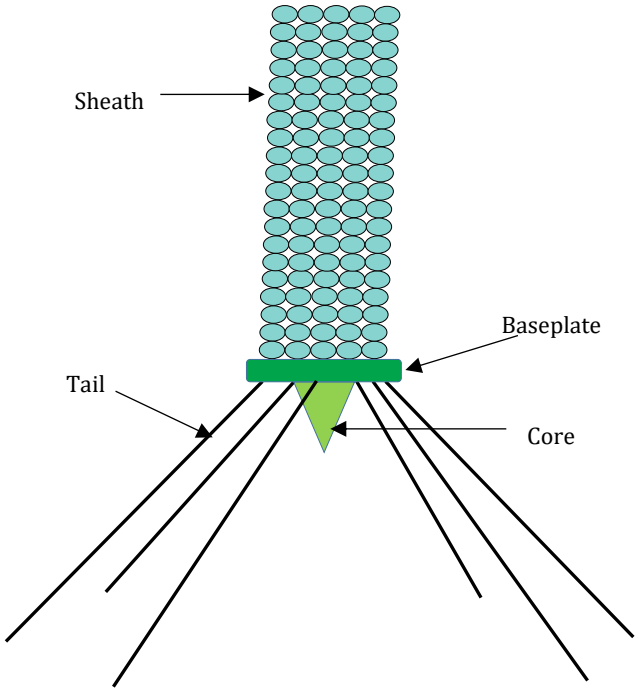

Figure 1: R-type pyocin Diagram

F-Pyocins resemble the tail of bacteriophages and are distinguished by their flexible rod shaped particles that consist of a baseplate, a core, and tail fibers with both long and short filaments. They are shorter than R-pyocins. At one end of the rods they have a square structure that tapers into fine fibers. There are three recognized F-subtypes (F1, F2, and F3) that resemble R-pyocins in both structure and action. The filaments play a crucial role in binding F-pyocins to target cells and their compositions can be altered through variations in short and long filaments. A single F-pyocin particle can eliminate the bacteria though mechanism is not clear [21, 22].

S-pyocins are colicin-like bacteriocins they are sensitive to heat and proteases and are water soluble. They consist of small and large protein complexes; the large segment carries the DNA degradation activity, while the small segment provides immunity from the host antibacterial activity. Four domains make up the large protein, arranged from the N-terminus to the C-terminus: the killing domain, the translocation domain, the receptor-binding domain, and a domain that serves an unidentified purpose. Conversely, S1 and S5 pyocins exhibit a simplified structure with only three domains, which include the translocation, the receptor-binding, and the killing domains. These pyocins work in several ways to eliminate bacteria: Pyocins S4 and

S6 suppress protein synthesis, while S1, S2, and S3 pyocins exhibit DNase activity; and S5 pyocin inhibit bacteria through its pore forming capability [21]. A summary of these pyocins has been provided in Table 1.

Table 1: Characteristics, structures and actions of pyocins

Pyocin Type	Characteristics	Structure	Action
R-pyocin	Classified into five groups (R1-R5); Highly efficient, can kill bacterial cell with a single particle	Resemble double hollow cylinders with core, sheath, baseplate and tail fibers	Kills bacteria by depolarizing cell membrane and inhibiting protein and nucleic acid synthesis
F-pyocin	Three recognized subtypes (F1, F2 and F3); Highly efficient, can kill bacterial cell with a single particle	Flexible rod-shaped particles with baseplate, core, and tail fibers	Mechanism is not clear
S-Pyocin	Colicin like Bacteriocins, classified into six groups (S1-S6)	Consist of small and large protein complexes, with various domains	Eliminates bacteria through multiple mechanisms including: suppressing protein synthesis, DNase activity, pore-forming capability

Factors Affecting the Induction of Pyocin

Pyocin production can be induced by different methods like promoting DNA damage with different factors like Mitomycin C, ciprofloxacin, oxygen radicals, and ultraviolet radiation; pyocin production can also be controlled by other factors like temperature and available nutrients.

1. Ultraviolet Radiation: Ultraviolet Radiation at different wavelengths can lead to the promotion of pyocin production through the induction of SOS response [23, 24, 25, 26].
2. Mitomycin C: Mitomycin C is an antibiotic and anti-cancer drug that has shown to upregulate the expression of pyocin genes by inducing cross-linking and inducing the SOS response in *P. aeruginosa* as reported by [27, 25].
3. Temperature: Pyocins production is also influenced by temperature, pyocins production increases as temperature rises to a maximum at 37°C where increasing temperature beyond will result in lower pyocins production. A culture medium with 500 µM of $MgCl_2$, 100 µM of $CaCl_2$, and 700 µM of methionine, and iron or glucose were not included [26].
4. Oxidative Stress: Oxidative stress has long been recognized as a catalyst for mutations, it is characterized by an upsurge in reactive oxygen species (ROS) within cells. The presence of hydrogen peroxide can trigger an elevation in the production of F, R and S type

pyocins due to the impact of stress on iron metabolism. Hydrogen peroxide reacts with iron in the cells generating hydroxyl radicals that inflict damage on DNA, proteins and lipids [28].

5. Sub inhibitory Antibiotic Concentrations: The exposure of bacterial populations to minimal antibiotic concentrations carries significant role in the promotion of mutagenesis; such as ciprofloxacin which can lead to mutagenesis through the same pathways as UV, Gamma, or X rays [23].

Pyocins are produced in response to genotoxic agents including ciprofloxacin, and the release of pyocins results in lysis of the producer cell [20].

Methodologies Used in Detection, Extraction and Purification of Pyocins

Different methods have been used in the pyocin production, which are generally comprised of the following: isolate collection, identification, screening for pyocin production, top producer selection, induction of pyocin production, purification, differentiation of pyocins, identify

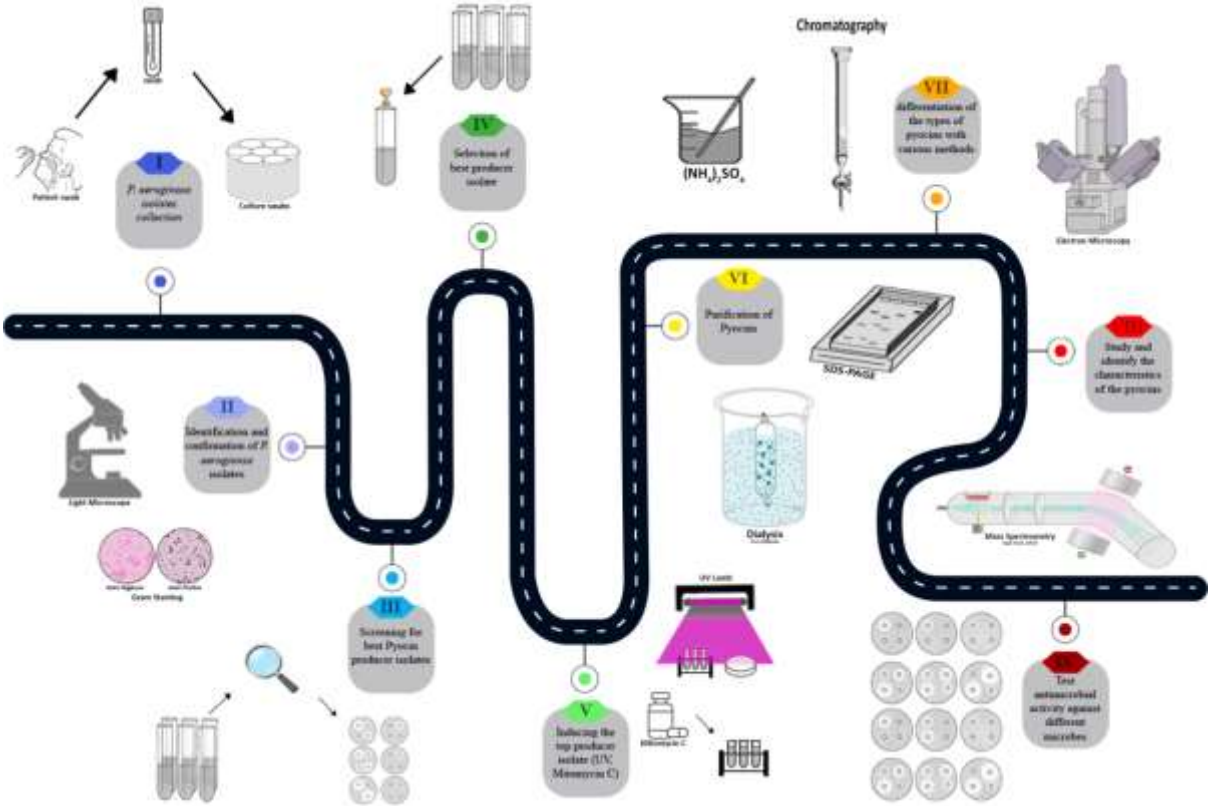

Figure 2: The general steps that are followed in pyocin

pyocin characteristics, and test their antimicrobial activity as shown in Fig. 2. Some of these methods may skip some of the general steps mentioned in the figure; on the other hand other methods may only be used for screening, although most of them should follow these general steps.

1. Well diffusion method: This can be a good method used of screening for pyocin production. In this method, wells are made in the agar, and the bacteriocin solution is added directly into the wells. The bacteriocin diffuses from the wells into the agar, creating a concentration gradient. A clear zone of inhibition around the wells is an indicator of the antimicrobial activity of the substance, which in this case can be the supernatant containing the pyocin [29].

The following steps were followed:

Culture producer strain overnight in an enrichment media of choice; Centrifuge at 6000 rpm for 15 mins to obtain the supernatant, prepare agar media that will be used for susceptibility test and inoculate it with the indicator strain of choice, make wells in the agar media used with a cork borer or another tool, add the supernatant to the wells and incubate the petri dishes at the desired conditions, measure the diameter of inhibition around the wells which can be an indicator of pyocin production. See [29] for more details about this experiment.

2. The disk diffusion method: This method can be used for screening of pyocin production. It is also used to determine the susceptibility of different indicator strains to antimicrobials which in this case is the pyocin [30]. The method consists of the following steps:

Prepare the antimicrobial agent that will be tested usually by acquiring the supernatant and purifying it in different ways, then prepare disks or filter papers (5 mm) that are soaked in the antimicrobial agent, in this case it's pyocins, culture your indicator strains on agar media of choice that will be used for susceptibility test with a swab, place the disks or filter papers on agar, observe and measure the inhibition zones. Refer to the source [30] for more details about this experiment.

3. Pyocin typing by spotting method was used by [31]. It is a viable method for screening for pyocin production by multiple strains simultaneously. The following steps were followed:

P. aeruginosa strains for single colonies should be streaked onto nutritional agar "(Columbia agar base; Oxoid Ltd., Basingstoke, London, England)" and incubated overnight at 37°C, to

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make a bacterial suspension of 10^8 to 10^9 organisms per milliliter of sterile physiological saline (absorbance at 550 nm, -0.5), use single colonies from each test strain, apply 1 µl quantities of bacterial suspensions onto 13, 90 mm-diameter plates that hold 10 milliliters of tryptone soy agar using a multipoint inoculator with 21 stainless steel pins. This makes it possible to type 20 test strains against each indication strain at once, Once the plates have dried, which normally takes a few minutes, incubate them for 4 hours at 30°C, chloroform should be impregnated into 5 cm Whatman filter paper disks. For 15 minutes, place the plates over the disks so that the vapor of chloroform can kill the bacteria, allow the plates to air dry for a further fifteen minutes in order to remove any remaining chloroform vapor, in order to grow indicator strains to a population size of about 10^7 organisms per milliliter, culture them for 4 hours at 37°C in nutrient broth without stirring, Apply indicator strains to the plates by mixing "2.5 ml of molten, semisolid agar (1% peptone, in 0.5% agar)" at 45°C, adding 0.1 ml of each bacterial indicator culture, and pouring the mixture onto each plate as an overlay (note: each plate receives a different indicator strain), determine the types of pyocins by incubating the plates at 37°C for 18 hours after the overlays have set. For a more thorough strain comparison and assessment of S-pyocin activity integrated into the typing results, consider the dimensions of the inhibition zones. For more detailed information about the experiment, see [31].

4- The cross streak technique is an efficient approach for screening and evaluating the effectiveness of bacteriocins. It enables the observation of zones of inhibition which indicate the presence of bacteriocin activity [32]. The experiment consists of the following steps: Get your agar plates ready with the growth media for the bacteria you're testing; Inoculate the bacteriocin producing strain by taking your producer strain and streaking it on the agar plate in a perpendicular manner starting from the edge, give some time for the producer strain to grow and produce bacteriocins by incubating the plate, take your indicator strains and streak it perpendicular to the producer strain crossing the initial streak as seen in Fig .3. Put your plate in an incubator allowing time for the bacteriocin to diffuse from the producer strain onto the target strain, after incubation carefully examine your plate to look for any inhibition zones where both streaks intersect. These zones indicate that there is bacteriocin activity, against your target strain. See [32] for more detailed information.

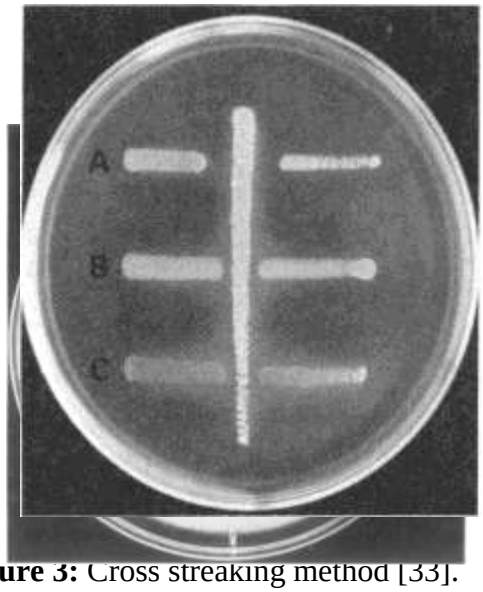

Figure 3: Cross streaking method [33].

5. Reverse Side Method: this a method was used by [34]. The method is mainly used for the screening for pyocin producers, as seen in Fig .4. The following steps were followed:

Spot inoculate, use single colonies, or use a strip to inoculate the producer strains onto the nutrient agar surface, incubate the Petri dish containing the producer strains, using a sterile spatula, separate the agar from the Petri dish edges, to ensure that the agar disc falls into the lid, invert the Petri dish and give it a firm tap on the bench, the sterile surface is now at the top of the lid, when it was formerly at the bottom of the Petri dish. Inoculate the indicator strain onto this sterile surface, incubate the plate; after incubation, observe and measure clear zones of inhibition around the producer isolates. See [34] for more detailed information about the experiment.

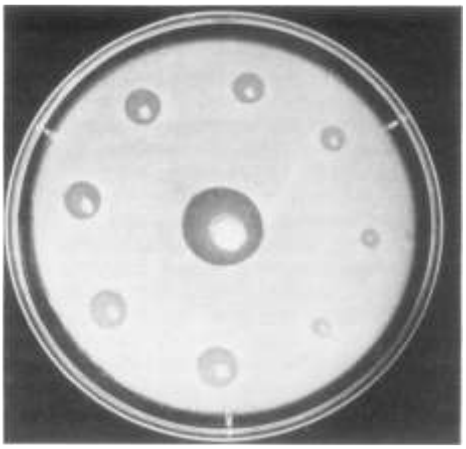

Figure 4: Reverse side method [34].

6. Ultraviolet radiation screening method as described by [25], was used for the screening of pyocin producing strains of *P. aeruginosa.* The following steps outline the steps of the experiment:

Use needles to spot test strains on nutrient agar plates, then incubate the samples until small colonies form, to induce the production of pyocin, expose the plates to a 15-W germicidal lamp at a distance of 70 cm for 1 minute, use chloroform vapor to kill any colonies that remain after a few hours of incubation, then remove them by impressing them onto sterile velvet, cover the resulting plates with 2 milliliters of 0.5% soft agar solutions that contain roughly 5×10^7 cells of the appropriate indicator strain, examine distinct inhibitory zones surrounding pyocinogenic colonies following an overnight incubation period. For more detailed information check [25].

7. Genetic approaches: Genetic techniques can be used to identify the genes responsible for bacteriocin production. These methods may include amplifying and sequencing genes or regions of interest using PCR (polymerase chain reaction) techniques [35]. Additionally tools, like BAGEL "(Bayesian Analysis of Gene EssentiaLity)" can be employed to explore genomes and identify bacteriocin genes of producer isolates [36].

8. Pyocin Production Medium 81 method was used by [37] for *P. aeruginosa* typing in hospitals and clinical labs with an advantage of being simple for not requiring the use of expensive and unavailable devices like shaking incubators and centrifuges. The experiment is carried out through these steps:

Prepare the ingredients for Pyocin Production Medium 81. (27.5 g of trypticase soy broth without glucose; 10 g of potassium nitrate; and 1,000 ml of distilled water), fill 125 by 16 mm screw-cap tubes with 10-ml aliquots, then autoclave; Inoculate one colony of the *P. aeruginosa* strain into Pyocin Production Medium 81, derived from a 24-hour culture (one colony should provide slight turbidity; optical density at 650 nm of about 0.01), to produce pyocin, incubate the cultures for 24 hours at 32°C in a water bath; Fill each tube with 1.0 ml of chloroform, shake hard for 10 seconds, allow to rest at room temperature for 10 minutes, and then shake once more, to evaporate any remaining chloroform, leave the lids off and let the products sit at room temperature for a few hours or overnight in the refrigerator, though it can be used to get rid of cellular debris, centrifugation is optional and not required for typing, drop the obtained pyocins

onto indicator strains for typing, and observe the results. For more detailed information about the experiment check [37].

9. Ciprofloxacin method: by [20] Utilized ciprofloxacin antibiotic for the induction of pyocin production. The method includes the following steps:

Grow bacteria in LB (Luria-Bertani) with or without 0.025 µg/ml ciprofloxacin to an OD600 of 1.0; Treat an aliquot of bacteria grown in LB with 0.3 µg/ml ciprofloxacin for 1 hour, Collect supernatant from the same amount of bacteria by centrifugation and filter sterilize it, Concentrate the supernatant 10-fold using an Amicon Ultra 0.5-ml, 10-kDa centrifugal filter unit; Spot 20 µl of the concentrated supernatant on a filter paper, lay the filter paper on a culture of wild type PAK indicator strain, incubate the plate at 37°C for 24 hours, measure the size of the inhibition zone after incubation. See [20] for more detailed information.

10. Hydrogen peroxide method by [28], which used hydrogen peroxide to cause oxidative stress and promote the production of pyocin. The procedure is carried out through these steps:

Initiate *P. aeruginosa* cultures at 37°C with shaking at 250 rpm using sterilized Luria-Bertani (LB) broth (10 g of tryptone, 5 g of yeast extract, and 10 g of sodium chloride per liter), after 17 hours, dilute the overnight cultures 1:100 in pre-warmed LB broth and incubate at 37°C with shaking at 250 rpm until the optical density at 600 nm (OD600) reaches the early logarithmic phase (~0.8); Re-dilute the cells 1:10 in pre-warmed LB broth and incubate at 37°C with shaking at 250 rpm, add 1 mM hydrogen peroxide immediately after OD600 reaches 0.8, ensure that culture volumes for all growth conditions are adjusted to be less than 1/10 the total flask volume to maximize aeration. Check [28] for more detailed information about this experiment.

11. Iwalokun's method was used by [26]. It involves the following steps:

Strains that produce pyocins were grown aerobically for one night at 37°C and 120 rpm of shaking in a Tris-minimal succinate solution that is devoid of iron and glucose but contains 500 µM of $MgCl_2$, 100 µM of $CaCl_2$, and 700 µM of methionine, Centrifuge the resultant cultures for ten minutes at 4000 rpm, Use ethyl acetate to acidify the supernatant at a volume ratio of 5:2, Using a rotary evaporator set to 50°C, concentrate the acidified pyocin fraction while operating at low pressure, In 400 µL of sterile water, dissolve the crude pyocin preparation, use a 0.45 µM filtration unit to sterilize the preparation; Calculate the pyocin preparation's yield, as

a control, employ *Pseudomonas putida* PUC 34, a non-pyocin producing strain. Check [26] for more information about the experiment.

12. A revised method was developed by [24], for pyocin production and purification. It included the following steps:

Grow the producer strain on cetrimide agar overnight at 37°C; Inoculate 10 ml^{-1} G-medium "(20 g sodium glutamate, 5 g glucose, 0.50 g yeast extract, 0.10 g $MgSO_4.7H_2O$, 0.25 g KH_2PO_4, and 5.63 g Na_2HPO_4 l^{-1})" with a suspension made from the overnight culture, for 24 hours, incubate the culture at 37°C with agitation, centrifuge the cells at 9184 × g for 20 minutes at 4°C to pellet them. Then, wash the cells once with 0.85% (wt vol^{-1}) NaCl, pellet them again, and resuspend them in 10 ml of 0.85% (wt vol^{-1}) NaCl, in a class II biosafety hood, expose a 30 cm distance to 365 nm of UV radiation for 1 minute to induce the production of pyocin, transfer 10 ml of the bacterial suspension that was induced to a sterile petri dish with a diameter of 10 cm, to achieve a final volume of 100 ml^{-1}, add the induced cells to 90 ml^{-1} of G-medium in a sterile flask, then, incubate for 18 hours at 37°C in a shaker incubator at 150 rpm, shake the culture for an extra half-hour at 37°C after adding DNase I (2 µg ml^{-1}), centrifuge at 9184 × g for 20 minutes to remove cell debris, use a Millipore filter with 0.45µm pore size to filter the supernatant, use ammonium sulfate to precipitate crude pyocin at 70% (w v^{-1}) saturation to partially purify it. Then, let the mixture sit at 4°C overnight, the pyocin precipitate should be centrifuged for 20 minutes at 15521 × g and 4°C, resuspend the pellets in a pH 6.8 Tris-HCl buffer (0.1M), dialyze the pyocin against the same buffer for 20 hours. See [24], for more detailed information. Ammonium sulfate precipitation is an important technique that can and has been used to precipitate different compounds like extracellular proteins and enzymes like urease and tannase [38, 39, 40] respectively.

13. Mitomycin C by [27] which uses Mitomycin C to induce pyocin production and multiple techniques to purify it, as follows:

For 10 minutes, centrifuge a *P. aeruginosa* culture that was cultured overnight at 2,100 × g, resuspend the pellet back into 1/10th of its original volume in a pH 6.5 solution that contains 0.1% cysteine hydrochloride and 0.85% NaCl, grow the cultures in a gyratory shaker at 37°C using an inoculum of 1% (vol/vol), add Mitomycin C to the culture at a concentration of 1 µg/ml

once the turbidity reaches about 150 Klett units, after adding Mitomycin C, usually within three hours, keep the culture incubated until significant lysis occurs, centrifuge the culture at 2,400 × g for 30 minutes to remove cellular debris, supernatant is then treated with chloroform (5%, vol/vol), designating this fraction as "crude pyocin", add 1 M $MnCl_2$ while slowly stirring to further purify crude pyocin, use 1 M NaOH to bring the pH down to 7.5, then centrifuge the precipitate (2,400 × g) for 15 minute. The supernatant is referred to as "partially purified pyocin". To achieve additional purification, add $(NH_4)2SO_4$ to 70% saturation and incubate it at 4°C overnight, dissolve the pellet containing pyocin activity in 50 ml of 0.01 M Tris (hydroxymethyl) aminomethane (Tris)-hydrochloride (pH 7.5) containing 0.01 M $MgSO_4$ and 0.01 M $MgCl_2$ following centrifugation (2,400 × g for 30 minutes) at 4°C, perform a dialysis against two liters of the same buffer over night at 4°C, centrifuge the preparation (2,400 × g for 15 minutes at 4°C) to clear it if needed, centrifuge the pyocin preparation for 90 minutes at 100,000 × g; Chromatograph the gelatinous pellet on diethylaminoethyl (DEAE)-cellulose after gently dissolving it in 20 milliliters of buffer, apply to a 1.5 by 28 cm column with an 8 ml the pyocin sample, and let it adsorb for an hour, Using 200 milliliters of buffer, wash the column to get rid of anything that isn't adhering to the DEAE-cellulose, use 800 milliliters of a NaCl gradient (0 to 1.0 M) in 0.01 M Tris buffer that contains 0.01 M $MgCl_2$ and $MgSO_4$ to elute the pyocins, gather 5 ml portions and measure pyocin activity and absorbance at 280 nm. Use ultracentrifugation to increase concentration (100,000 × g for 90 minutes) after dialyzing pool fractions displaying pyocin activity against 0.01 M Tris buffer containing 0.01 M $MgCl_2$ and 0.01 M $MgSO_4$ to remove NaCl. The operating temperature for all chromatographic processes is 4°C. See [27] for more detailed information about the experiment.

14. The Naz & Rasool method [41] for the isolation and partial purification of pyocin. The method comprises of the following steps:

Grow *P. aeruginosa* in Brain Heart Infusion (BHI) medium at 29°C for 18 hours; centrifuge at 6000 × g for 30 minutes at 4°C, adjust the cell-free supernatant (CFS) to pH 7.0, filter sterilize the CFS using a 0.45 µm pore size membrane filter, partially purify the crude bacteriocin preparation by 70% ammonium sulfate precipitation at 4°C, sediment the precipitate by centrifugation at 6000 × g for 45 minutes at 4°C, suspend the resulting pellet in 50mM Sodium

Phosphate Buffer of pH 7.0, referring to it as a partially purified bacteriocin preparation, determine the activity units (AU) of both crude and partially purified bacteriocin preparations using the agar well diffusion method, express the bacteriocin titer as activity units, or arbitrary units per milliliter (AU/ml); Check [41] for more information.

15. Another method which also used Mitomycin C described by [27]. This method partially purifies Pyocin S and also Purifies Pyocin R, it comprises of the following steps:

To prepare the lysate rich in pyocin, dilute an overnight culture 50–100 times with fresh medium, and then incubate the culture for two to three hours at 37°C while shaking continuously, when the culture reaches the logarithmic phase of development, introduce 2 µg/ml of Mitomycin C. The culture becomes more turbid for approximately ninety minutes prior to the onset of cell lysis. About three hours after adding Mitomycin C, obtain the clear lysate, centrifuge the Mitomycin-induced lysate to remove any remaining bacterial debris after treating it for an hour at 37°C with DNase (1 µg/ml), to a lysate that has been treated with DNase, add ammonium sulfate (80% saturation). Gather the precipitate, dissolve it in and dialyze it against 0.01 M Tris solution that contains 0.05 M NaCl (pH 7.5), use the same buffered saline to prewash a DEAE-cellulose column before charging it with the protein solution. While pyocin R4 activity is maintained and eluted by the gradient increase in NaCl concentration, pyocin S activity flows through the column. Notice that while pyocin S activity is not retained by the column, R-type pyocins are adsorbed by DEAE-cellulose chromatography. The recovery of pyocin S activity at this point is around 20–30% of the lysate, this makes it easy to distinguish between the two activities. Check [27] for more details. Take note that chromatography using CM-cellulose causes a considerable loss of activity and therefore additional purification of pyocin S is challenging.

Applications

Pyocins as an alternative to antibiotics: These antimicrobial agents have shown effectiveness against *P. aeruginosa* infections in immunocompromised patients like those with Cystic fibrosis. The rise of resistance in these bacteria has made treatment challenging. Pyocins offer a potential solution from this aspect. Recent studies have demonstrated the efficacy of pyocins in treating lung diseases caused by gram-negative bacteria, such as *P. aeruginosa*. In fact the

S5 pyocin has shown effectiveness at a 100 fold lower concentration compared to inhaled tobramycin antibiotic therapy. An added advantage is that pyocins have lower immunogenicity making them an attractive option [42, 23]; Pyocins have also been shown to have an anti-biofilm activity against clinical *P. aeruginosa* isolates [43]; Pyocins have great potential for the treatment of traumatic burns, wounds and injuries that are exposed to *P. aeruginosa* infections [44]; Pyocins have been suggested for use in epidemiological fingerprinting of *P. aeruginosa*, indicating their potential application in identifying and characterizing bacterial strains [22, 45]. A simplified method has also been proposed called pyocin typing of *P. aeruginosa* [31]; Pyocins have been suggested for use in bioremediation and bio sensing [44]; Pyocins have also been used in food preservation, skin and oral care, and peptic ulcer treatment [46]. Applications of pyocins have been summarized and presented in Table 2.

Table 2: Applications of pyocins

Applications	Description	Reference
Treatment of *P. aeruginosa* infections in immunocompromised patients (e.g., Cystic fibrosis)	Effective against *P. aeruginosa* infections, offering a potential solution to antibiotic resistance challenges.	[42], [23]
Anti-biofilm activity	Demonstrated activity against clinical *P. aeruginosa* isolates, aiding in combating biofilm-associated infections.	[43]
Treatment of traumatic burns, wounds, and injuries	Potential for treating infections caused by *P. aeruginosa* in these contexts.	[44]
Epidemiological fingerprinting of *P. aeruginosa*	Used for identifying and characterizing bacterial strains, aiding in epidemiological studies.	[45]
Bioremediation and biosensing	Potential applications in environmental cleanup and sensing technologies.	[44]
Food preservation, skin and oral care, and peptic ulcer treatment	Versatile applications beyond healthcare, including in food safety and personal care products.	[46]

Conclusion

Some methods may not differentiate between pyocin types effectively, for example, screening with well diffusion method, disk diffusion method, spotting method, cross streak method, reverse side method, *P. aeruginosa* typing method, the Long method, and Chang method but can be viable for screening. On the other hand, the genetic method, Ito methods, Morse method, and Mohameds method can identify the exact types of pyocins and differentiate between them,

though the Genetic, Ito, Long, Chang, Morse, and Mohameds methods require more specialized equipment, expertise, are more complex and higher cost. Ito method is a lot more complex and expensive and requires specialized equipment and unavailable materials compared to Jones method which is inexpensive, requires no specialized equipment and isn't complex but requires the use of a special media. Some methods that have been reviewed throughout this study like used a centrifuge without referring to the type of the centrifuge, the radius, or the RCF (G force), which is problematic if consistency in the experiment is being pursued. The studies provided that used ammonium sulphate precipitation provided a specific percentage (70% or 80%) instead of a range like (50-70%). The initial saturation with ammonium sulphate is not specified which should be required to maintain the consistency of the experiment. Also the methods that utilized the use of Mitomycin C did not mention what happens to it after it induces the production of pyocin and cell lysis, and whether they are involved in the antimicrobial activity against the indicator strains. Further studies and clarifications have to be done to resolve this issue. Different indicator strains of bacteria have been used in different studies for example, *P. aeruginosa, Stphylococcus aureus, Klebsiella, Escherichia coli* and *Neisseria gonorrhea* have been used, a broader study should include these and more indicator bacteria to determine a reliable indicator to have consistent results. To summarize, the goal of this review is to be an invaluable tool for scientists, physicians, and decision-makers who are engaged in the fight against antibiotic resistance. Through a summary of current knowledge on pyocins, clarification of their mechanisms of action, and investigation of production procedures, this review hopes to add to the continuous endeavors to devise alternative antibacterial tactics capable of efficiently combating bacterial illnesses. The efficacy of pyocin types screening techniques varies. Some techniques may not be very effective at differentiating between types, such as well diffusion, disk diffusion, spotting, cross streak, reverse side, *P. aeruginosa* typing (Jones method), Long method, and Chang methods but can be really effective and cheap. The techniques of Jong, Jones, Ito, Morse, and Mohameds provide accurate identification; nevertheless, they are costly, complicated, and need specific tools. Notably, compared to the easier, equipment-free Jones approach, the Ito method is more expensive and complex. Some techniques lack specificity, for example, Iwalokun's method and Mohsin's, left out centrifuge specifics that are essential to

maintaining consistency between experiments. The initial ammonium sulphate saturation has not been mentioned and a range with ammonium sulphate precipitation with the highest yield should be measured. The use of Mitomycin C, what happens to it after induction and whether it is involved in the antimicrobial activity require more explanation. Numerous strains of indicators have been employed, indicating the necessity for more extensive research to identify trustworthy indicators for consistent outcomes.

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References

[1] CDC, How do germs become resistant?, Centers for Disease Control and Prevention, 24-Oct-2022. https://www.cdc.gov/drugresistance/about/how-resistance-happens.html.

[2] M. A. Salam, M. Y. Al-Amin, M. T. Salam, J. S. Pawar, N. Akhter, A. A. Rabaan, M. A. A. Alqumber, Antimicrobial Resistance: A Growing Serious Threat for Global Public Health, Healthcare, 11, 1946(2023), DOI(https://doi.org/10.3390/healthcare11131946)

[3] J. Oneill, Antimicrobial resistance: Tackling a crisis for the health and wealth of nations (The Review on Antimicrobial Resistance), United Kingdom Rev. Antimicrob. Resist, 2014.

[4] M. P. Mokoena, Lactic acid bacteria and their bacteriocins: Classification, biosynthesis and applications against uropathogens: A mini-review, Molecules, 22, 1255(2017), DOI(https://doi.org/10.3390/molecules22081255)

[5] C. K. Anumudu, O. Omoregbe, A. Hart, T. Miri, U. A. Eze, H. Onyeaka, Applications of bacteriocins of lactic acid bacteria in biotechnology and food preservation: A bibliometric review, Open Microbiol. J., 16, (2022), DOI(http://dx.doi.org/10.2174/18742858-v16-e2206300)

[6] A. Ben Lagha, B. Haas, M. Gottschalk, D. Grenier, Antimicrobial potential of bacteriocins in poultry and swine production, Vet. Res., 48, 22(2017), DOI(https://doi.org/10.1186/s13567-017-0425-6)

[7] L. García-Bayona, M. S. Guo, M. T. Laub, Contact-dependent killing by Caulobacter crescentus via cell surface-associated, glycine zipper proteins, Elife, 6, (2017), DOI(https://doi.org/10.7554/eLife.24869)

[8] D. B. Diep, M. Skaugen, Z. Salehian, H. Holo, I. F. Nes, Common mechanisms of target cell recognition and immunity for class II bacteriocins, Proc. Natl. Acad. Sci. U. S. A., 104, 2384-2389(2007), DOI(https://doi.org/10.1073/pnas.0608775104)

[9] F. W. J. Collins, P. M. O'Connor, O. O'Sullivan, B. Gómez-Sala, M. C. Rea, C. Hill, R. P. Ross, Bacteriocin Gene-Trait matching across the complete Lactobacillus Pan-genome, Sci. Rep., 7, 3481(2017), DOI(https://doi.org/10.1038/s41598-017-03339-y)

[10] A. Simons, K. Alhanout, R. E. Duval, Bacteriocins, antimicrobial peptides from bacterial origin: Overview of their biology and their impact against multidrug-resistant bacteria, Microorganisms, 8, 639(2020), DOI(https://doi.org/10.3390/microorganisms8050639)

[11] M. G. K. Ghequire, R. De Mot, Ribosomally encoded antibacterial proteins and peptides from Pseudomonas, FEMS Microbiol. Rev., 38, 523–568(2014), DOI(https://doi.org/10.1111/1574-6976.12079)

[12] K. Kirtonia, et al., Bacteriocin: A new strategic antibiofilm agent in food industries, Biocatal. Agric. Biotechnol., 36, 102141(2021), DOI(https://doi.org/10.1016/j.bcab.2021.102141)

[13] K. I. Wolska, K. Grześ, A. Kurek, Synergy between novel antimicrobials and conventional antibiotics or bacteriocins, Pol. J. Microbiol., 61, 95–104(2012)

[14] L. L. Newstead, K. Varjonen, T. Nuttall, G. K. Paterson, Staphylococcal-produced bacteriocins and antimicrobial peptides: Their potential as alternative treatments for Staphylococcus aureus infections, Antibiotics, 9, 40 (2020), DOI(https://doi.org/10.3390/antibiotics9020040)

[15] V. Vadyvaloo, J. W. Hastings, M. J. van der Merwe, M. Rautenbach, Membranes of class IIa bacteriocin-resistant Listeria monocytogenes cells contain increased levels of

desaturated and short-acyl-chain phosphatidylglycerols, Appl. Environ. Microbiol., 68, 5223–5230(2002), DOI(https://doi.org/10.1128/AEM.68.11.5223-5230.2002)

[16] M. Opsata, I. F. Nes, H. Holo, Class IIa bacteriocin resistance in Enterococcus faecalis V583: the mannose PTS operon mediates global transcriptional responses, BMC Microbiol., 10, 224 (2010), DOI(https://doi.org/10.1186/1471-2180-10-224)

[17] H. Etayash, S. Azmi, R. Dangeti, K. Kaur, Peptide Bacteriocins - Structure Activity Relationships, Curr. Top. Med. Chem., 16, 220–241(2015), DOI(https://doi.org/10.2174/1568026615666150812121103)

[18] P. P. Kandel, D. A. Baltrus, K. L. Hockett, Pseudomonas can survive tailocin killing via persistence-like and heterogenous resistance mechanisms, J. Bacteriol., 202, (2020), DOI(https://doi.org/10.1128/jb.00142-20)

[19] M. Kjos, L. Snipen, Z. Salehian, I. F. Nes, D. B. Diep, The Abi proteins and their involvement in bacteriocin self-immunity, J. Bacteriol., 192, 2068–2076(2010), DOI(https://doi.org/10.1128/JB.01553-09)

[20] Y. Long, et al., Fis contributes to resistance of Pseudomonas aeruginosa to ciprofloxacin by regulating pyocin synthesis, J. Bacteriol., 202, (2020), DOI(https://doi.org/10.1128/JB.00064-20)

[21] A. Alqahtani, J. Kopel, A. Hamood, The in vivo and in vitro assessment of pyocins in treating Pseudomonas aeruginosa infections, Antibiotics, 11, 1366 (2022), DOI(https://doi.org/10.3390/antibiotics11101366)

[22] L. Blasco, et al., Study of 32 new phage tail-like bacteriocins (pyocins) from a clinical collection of Pseudomonas aeruginosa and of their potential use as typing markers and antimicrobial agents, Sci. Rep., 13, 117 (2023), DOI(https://doi.org/10.1038/s41598-022-27341-1)

[23] L. Laureti, I. Matic, A. Gutierrez, Bacterial responses and genome instability induced by subinhibitory concentrations of antibiotics, Antibiotics, 2, 100–114 (2013), DOI(https://doi.org/10.3390/antibiotics2010100)

[24] A. A. Mohamed, A. M. Elshawadfy, G. Amin, A. Askora, Characterization of R-pyocin activity against Gram-positive pathogens for the first time with special focus on

Staphylococcus aureus, J. Appl. Microbiol., 131, 2780–2792 (2021), DOI(https://doi.org/10.1111/jam.15134)

[25] S. Ito, M. Kageyama, F. Egami, Isolation and characterization of pyocins from several strains of Pseudomonas aeruginosa, J. Gen. Appl. Microbiol., 16, 205–214 (1970), DOI(https://doi.org/10.2323/jgam.16.3_205)

[26] B. A. Iwalokun, K. A. Akinsinde, O. Lanlenhin, C. C. Onubogu, Bacteriocinogenicity and production of pyocins from Pseudomonas species isolated in Lagos, Nigeria, African J. Biotechnol., 5, 1072–1077 (2006), DOI(https://doi.org/10.5897/AJB06.094)

[27] S. A. Morse, P. Vaughan, D. Johnson, B. H. Iglewski, Inhibition of Neisseria gonorrhoeae by a bacteriocin from Pseudomonas aeruginosa, Antimicrob. Agents Chemother., 10, 354–362 (1976), DOI(https://doi.org/10.1128/AAC.10.2.354)

[28] W. Chang, D. A. Small, F. Toghrol, W. E. Bentley, Microarray analysis of Pseudomonas aeruginosa reveals induction of pyocin genes in response to hydrogen peroxide, BMC Genomics, 6, 115 (2005), DOI(https://doi.org/10.1186/1471-2164-6-115)

[29] Z. A. Mohsin, W. S. Ali, Antagonistic Activity of Bacteriocin-producing Lactobacillus Against Candida spp, Iraqi J. Sci., 2153–2162 (2021), DOI(https://doi.org/10.24996/ijs.2021.62.7.4)

[30] E. Ünal Turhan, Z. Erginkaya, A. Bayram Türemiş, M. Terkuran, Antibacterial effect of bacteriocinogenic enterococci from different sources on Listeria monocytogenes, Tarım bilim. derg., (2020), DOI(https://doi.org/10.15832/ankutbd.448475)

[31] J. A. Fyfe, G. Harris, J. R. Govan, Revised pyocin typing method for Pseudomonas aeruginosa, J. Clin. Microbiol., 20, 47–50 (1984), DOI(https://doi.org/10.1128/jcm.20.1.47-50.1984)

[32] G. Zheng, M. F. Slavik, Detection and demonstration of inhibitory activities of bacteriocins by isoelectric focusing, J. Rapid Methods Automat. Micro., 6, 219–224 (1998), DOI(https://doi.org/10.1111/j.1745-4581.1998.tb00201.x)

[33] R. Harasawa, K. Suzuki, T. Mitsuoka, Streptococcus faecium-derived antibacterial substance antagonistic to Bifidobacteria, Antimicrob. Agents Chemother., 18, 58–62 (1980), DOI(https://doi.org/10.1128/AAC.18.1.58)

[34] D. A. Kékessy, J. D. Piguet, New method for detecting bacteriocin production, Appl. Microbiol., 20, 282–283 (1970), DOI(https://doi.org/10.1128/am.20.2.282-283.1970)

[35] H. Tomita, S. Fujimoto, K. Tanimoto, Y. Ike, Cloning and genetic organization of the bacteriocin 31 determinant encoded on the Enterococcus faecalis pheromone-responsive conjugative plasmid pYI17, J. Bacteriol., 178, 3585–3593 (1996), DOI(https://doi.org/10.1128/jb.178.12.3585-3593.1996)

[36] A. de Jong, S. A. F. T. van Hijum, J. J. E. Bijlsma, J. Kok, O. P. Kuipers, BAGEL: a web-based bacteriocin genome mining tool, Nucleic Acids Res., 34, (2006), DOI(https://doi.org/10.1093/nar/gkl237)

[37] L. F. Jones, B. V. Pinto, E. T. Thomas, J. J. Farmer 3rd, Simplified method for producing pyocins from Pseudomonas aeruginosa, Appl. Microbiol., 26, 120–121 (1973), DOI(https://doi.org/10.1128/am.26.1.120-121.1973)

[38] H. A. Ibrahim, N. H. Zaki, The biological activity of some extracted proteins from Bacillus spp. Isolated from soil against pathogenic bacteria, Al-Mustansiriyah J. Sci., 30, 29–38 (2020), DOI(https://doi.org/10.23851/mjs.v30i4.685)

[39] D. S. Bedan, Extraction, precipitation and characterization of urease from Vicia faba L. Al-Mustansiriyah, J. Sci., 31, 9–14 (2020), DOI(https://doi.org/10.23851/mjs.v31i1.555)

[40] S. N. Muslim, Production and partial purification of tannase from Serratia marcescens isolated from different sources, Al-Mustansiriyah J. Sci., 28, 109–121 (2018), DOI(https://doi.org/10.23851/mjs.v28i3.550)

[41] S. A. Naz, S. A. Rasool, Isolation, production and characterization of bacteriocins produced by strains from indigenous environments, Pakistan J. Bot., 45, 261–267(2013)

[42] L. C. McCaughey, N. D. Ritchie, G. R. Douce, T. J. Evans, D. Walker, Efficacy of species-specific protein antibiotics in a murine model of acute Pseudomonas aeruginosa lung infection, Sci. Rep., 6, 30201(2016), DOI(https://doi.org/10.1038/srep30201)

[43] Š. Paškevičius, U. Starkevič, A. Misiūnas, A. Vitkauskienė, Y. Gleba, A. Ražanskienė, Plant-expressed pyocins for control of Pseudomonas aeruginosa, PLoS One, 12, e0185782 (2017), DOI(https://doi.org/10.1371/journal.pone.0185782)

[44] D. K. Karig, S. Bessling, P. Thielen, S. Zhang, J. Wolfe, Preservation of protein expression systems at elevated temperatures for portable therapeutic production, J. R. Soc. Interface, 14, 20161039 (2017), DOI(https://doi.org/10.1098/rsif.2016)

[45] J. J. Farmer III, L. G. Herman, Epidemiological fingerprinting of Pseudomonas aeruginosa by the production of and sensitivity to pyocin and Bacteriophage1, Appl. Microbiol., 18, 760–765 (1969), DOI(https://doi.org/10.1128/am.18.5.760-765.1969)

[46] A. W. Negash, B. A. Tsehai, Current applications of bacteriocin, Int. J. Microbiol., 2020, 4374891 (2020), DOI(https://doi.org/10.1155/2020/4374891)