Proceedings of the Pakistan Academy of Sciences: B
Life and Environmental Sciences 63(1): 67-76 (2026)
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ISSN (Print): 2518-4261; ISSN (Online): 2518-427X
http://doi.org/10.53560/PPASB(63-1)1129

Pakistan Academy of Sciences

Research Article

Antibacterial and Antibiofilm Activity of Pullulanase from *Paenibacillus macerans* Against Urinary Catheter-Associated Pathogens

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Abstract: Urinary tract infections and urinary catheters are frequently related. Clinicians in underdeveloped nations often encounter urinary tract infections (UTIs), which are among the most prevalent bacterial illnesses. Selecting the appropriate empirical treatment for a patient may be aided by the results of area-specific surveillance studies that seek to identify the bacteria causing UTIs and their patterns of resistance. Pullulinase, an enzyme that hydrolyzes pullulin into maltotriose, panose, and maltooligosaccharides, is capable of inhibiting bacterial colonization and biofilm formation on urinary tract catheters. The goal of this work is to detect *Paenibacillus macerans* isolated from rhizosphere soil as pullulanase producer and to use the purified pullulanase as prebiotic, antibacterial, and antibiofilm agent. Using of modified pullulin-agar for screening pullulanase producers. Purification of pullulanase by DEAE-Cellulose A-50 column followed by sephadex G-150 column. Isolation of urinary catheters bacteria and using of pullulanase and detection biofilm formation on the catheters then applied of pullulanase as antibacterial and antibiofilm agent against urinary catheters bacteria in addition to enhance the growth of lactic acid bacteria. Eleven isolates of *Paenibacillus macerans* (41%) had showed an ability to produce pullulanase with different levels. Serial steps were used Pullulanase purification which included 70% ammonium sulfate saturation, DEAE-Cellulose A-50 ion exchange chromatography and sephadex G-100 gel filtration chromatography with a recovery yield of 22.9%, 8.11 fold of purification and specific activity 15.90 U/mg. *Acinetobacter baumannii* and *Pseudomonas aeruginosa* were the most predominant isolates in the urinary catheters. The purified pullulanase has a potential prebiotic characteristic to enhance lactic acid bacteria growth while allegedly impeding the development of pathogens and inhibiting their biofilm formation. Prebiotic properties are typically intended not only to reduce pathogen growth and biofilm formation, but to promote the growth of desirable and beneficial bacteria like lactic acid bacteria. In conclusion, pullulanase can be added to animal feed to increase digestibility and gastrointestinal health. It can also be used to cover catheters to assist avoid infections and bacterial colonization.

Keywords: Pullulanase, Urinary Catheters, Prebiotic, Biofilm Inhibition, *Paenibacillus Macerans,* Enzyme Purification, Lactic Acid Bacteria.

1. INTRODUCTION

Paenibacillus macerans is considered as a facultative anaerobe and a member in paenibacillaceae family [1] that is found in different environments like soil and plants and can perform nitrogen fixation and fermentation, besides their presence in blood cultures of infants with infection [2]. A linear polymer of maltotriose, which is also known as pullulan, and linked with α-saccharoyl linkages, is utilized in various industries [3]. Pullulanases (extra cellular hydrolases that breaks-1, glycosidic linkages in pullulan, starch, and oligosaccharins) are examples of an extracellular debranching enzymes which debride these oligosaccharides from a subtype of glycans that is derived from pullulan [4, 5]. Pullulanase, a significant debranching enzyme widely used for α-1,6 glucosidic hydrolysis in starch, amylopectin, pullulan, and related oligosaccharides, is an industrial enzyme candidate [4]. Due to its special actions on the α-1,6 connections in pullulan a linear α-glucan

Received: December 2025; Revised: February 2026; Accepted: March 2026
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consisting essentially of maltotriosyl units linked by 1,6-α-bonds is preferred for microbial pullulanase in industry. Pullulanases are also hydrolysed in pullulan and amylopectin by the α-1,6 glucoside bond. Different microorganisms like *Klebsiella planticola*, *B. acidopullulyticus*, *B. deramificans*, *B. cereus* and *Geobacillus stearothermophilus* produce pullulanases [6].

Catheters for the urinary system are silicone or latex-filled tubes. Biofilms can easily form on the inside or outside of inserted catheters. The longer the catheter is left in place without changing, the more likely it is that microorganisms will create catheter biofilms [7]. The process of bacteria growing and irreversibly attaching to a surface to create biofilms involves the production of extracellular polymers that help in matrix development and adhesion [8, 9]. In hospital and community care settings, urethral catheters are the most frequently used medical devices. However, their usage puts patients at risk for infection and creates an environment that is perfect for the growth of bacterial biofilms [10, 11]. Individuals receiving long-term urethral catheterization are more susceptible to infections related to biofilms; urease-producing species' crystalline biofilm production frequently results in catheter occlusion and other severe clinical consequences [12]. In critical care units, urinary tract infections are mostly caused by bacterial biofilms that grow on urine catheters. Because of the development of these biofilms, cytobacteriological analysis of patient urine is frequently deceptive. Determining the type of bacteria living on the catheter surface and characterizing these biofilms are therefore crucial [13]. The aim of this research was to investigate pullulanase production by a new strain of *Paenibacillus macerans* isolated from rhizosphere soil, purification of pullulanase and prebiotic, antibacterial, and antibiofilm characteristics are being investigated.

2. MATERIALS AND METHODS

2.1. Detection of Producers of Pullulanase

Samples of soils of rhizosphere at different sites in Mustansiryiah University Garden, Baghdad, Iraq were collected at 27 locations. For ten minutes, one gram of each sample was suspended in ten milliliters of sterile distilled water and forcefully shaken. The surface of the modified pullulin-agar according to [4] that consisted of (g/l) soluble pullulan [10] was then distributed with 0.1 of the suspension fluid, NaCl (2), $MgSO_4.7H_2O$ (0.1), K_2HPO_4 (0.17) and $KH_2PO_4.7H_2O$ (0.12) and Agar (0.15), pH 7.0 [14]. Till the colonies appeared, the plates were then incubated at 30 °C. Pullulanase producers were considered colonies around which a clear hollow zone was observed.

2.2. Biological Diagnosis

Morphological features were observed in the increasing bacterial isolates and identified by Berge's Systemic Bacteriology Manual and identified, for example, size, colour, shape, and colony outline as well as gauze and biochemical tests [15]. By using Vitek 2 with Vitek GPI cards this diagnosis has been confirmed.

2.3. Submerged Fermentation for Pullulanase Production

A modified pullulan agar media has been inoculated on the selected isolate but not agar. The incubation took place for 48 hrs at 30 °C. At 8000 rpm to 10 mins, the cultivation was centrifuged and the supernatant used for crude enzymes.

2.4. Pullulanase Activity

The measurement of reduced pullulan release of sugar tested the activity a mixture of reaction (3 ml) of 0.5 ml pullulan and 2 ml sodium phosphate buffer (0.5 ml) raw enzyme sources (0.1 M, pH 6.5). 0.5 ml $CaCl_2$ (0.02 % w/v) was added to reaction mixture. The reaction was stopped by the ice bath, after incubation was carried out at 40 °C for twenty minutes, then 1 mL of 3-5-dinitrosalicyclic acid reagent was added and the sugar released by enzyme hydrolysis was decreased, followed by incubation in boiling bath for five minutes, measuring enzyme activity at 540 nm. after incubation at 20 mins [16]. The amount of the enzyme, that released one mole of sugar reduction as glucose per minute under standard conditions, is defined as one unit of pullulanase.

2.5. Estimate of Protein Determination

The concentration of protein was determined by the Bradford [17] method and the standard use of bovine serum albumin.

2.6. Purification of Pullulanase

Culture supernatant obtained after incubation in the pullulan broth medium (8,000 g, 30-minute, 4 °C) and subjected to ammonium sulphate prescription with 20-80% saturation, through centrifugation (8,000 g, 30-minute, 4 °C). The sample has been removed overnight at 4 °C (2000 rpm) and the precipitate has been collected at 20 mM phosphate-buffered saline (PBS) with a pH of 7.2, and then dialyzed overnight in a centrifugation buffer at pH 7.0. The dialyzed protein was loaded on a column (2cm x 25cm) of the DEAE-Cellulose A-50 and eluted with same buffer at a flow rate of 0.1-0.4 m NaCl. In the sephadex G-150 column (2 x 70 cm), which was previously balanced with that buffer, the fractions with highest activity of the pullulanase were collected. For further studies after protein concentrations of 280 nm and Pullulanase activity was measured.

2.7. Isolation Urinary Catheters Bacteria

Urinary catheters that hospitalized patients had been using for longer than a week were meticulously removed in an aseptic manner. Each catheter was carefully packed in a sterile glass bottle before being sent straight to the lab for examination. These urinary catheter discs were sliced, and the discs were cultured on Blood agar and MacConkey. Macroscopical and microscopic analysis of cultures carried out in compliance with guidelines provided by the Clinical and Laboratory Standards Institute (CLSI) [18]. To authenticate the isolates, the Vitek-2 system was utilized.

2.8. Quantitative Detection of Biofilm Formation by Urinary Catheters Bacteria

The method of quantitative microtiter plates, as detailed by Castilla-Sedano *et al*. [19], was employed. First, 3 mL of brain heart infusion broth was used as the inoculation medium for each uropathogenic bacterial colony that was recovered from a new agar plate. The culture was incubated for 18 to 24 hours at 37 °C with agitation (150 rpm). Following the incubation period, 20µL of the bacterial inoculum was combined with 180µL of brain heart infusion broth and injected into separate sterile, disposable, 96-well polystyrene tissue culture microplates with a U-bottom and an untreated surface. As a negative control, brain heart infusion broth devoid of cells was used to confirm the sterility of the media. For 48 hours, the TCP was incubated at 37 °C. After the incubation period, the contents of each well were carefully removed, and the free-floating planktonic bacterial cells were removed by air-drying them after four washes in 200 µL of sterile phosphate-buffered saline (PBS) with a pH of 7.2. After carefully rinsing the wells three times with deionized water, the wells were stained for 15 minutes with 1% crystal violet. The dye was then dissolved in 96% ethanol for another 15 minutes. An automated micro-ELISA reader was used to measure the optical density (OD) at 590 nm. for every isolation, three separate trials were run. By using the OD values of adhering bacterial cells, biofilm production was classified as negative, weak, or strong, in accordance with the guidelines given by Stepanović *et al.* [20]; the experiment was performed in triplicate.

2.9. Enhancement of Lactic Acid Bacteria by Pullulanase

The effect of pullulanase on the growth of two *Lactobacillus reuteri* isolates, *Lactobacillus plantarum*, *Leuconostoc mesenteroides* and *Pediococcus sp*., was tested. These bacterial isolates were cultivated 24-hour at 37 °C in 50 ml MRS broth. 1% of inoculums adjusted to 0.5 absorption in 600 nm were subsequently moved to 5 ml of MRS broth with 1% purified pullulanase or without then for 4 hours was incubated at 37 °C. The cell number of MRS agar plating and cultivated overnight at 37 °C was determined following 4 hours of incubation. The experiment was performed in triplicate. Enhanced (percent) activity was identified as [21]:

$$\textit{Enhancement Activity}\ (\%) = \frac{SB-CB}{CB} \times 100$$

where SB is the quantity of MRS colonies with purified pullulanase (cfu/ml) and CB, there are no purified pullulanase (cfu/ml) cells within MRS.

2.10. Effect of Pullulanase on Pathogenic Bacteria Growth

In aerobial conditions 50 ml of the nutrient broth medium was grown in patenous bacterial isolates by shaking at 150 rpm for 24 hours at 37 °C. 5 ml of nutritional broth medium, 5 ml of nutrient broth plus 1% of purified pullulanase, and 1% of the inoculum adjusted to absorb at 600 nm were combined, and

the mixture was shaken at 150 rpm for 4 hours at 37 °C to generate the desired result. The number of cells was determined by nutrient agar tapering and incubation at 37 °C overnight. The experiment was performed in triplicate. Activity of inhibition (%) has been determined as [21]:

$$Inhibition\ Activity\ (\%) = \frac{CB-SB}{CB} \times 100$$

where SB is cell volume in nutrients pullulanase (cfu/ml) and CB is quantity of cells in nutrient pullulanase (cfu/ml) without purification in nutrient pumps.

2.11. Effect of Pullulanase on Biofilm Formation by Pathogenic Bacteria

In a microtiter plate approach, 125 µl of pure pullulanase in 20 mM phosphate-buffered saline (PBS) with a pH of 7.2 was combined with 125 µl of a chosen bacterial culture to measure pullulanase activity against biofilm formation. Following a 24-hours and 48-hours incubation period at 37 °C, the biofilm experiment was conducted as previously indicated. As previously said, the biofilm activity was duplicated. The percentage of biofilm inhibition was computed as follows [22]:

$$Percentage\ of\ biofilm\ inhibition\ (\%) = \frac{OD\ treatment}{OD\ control} \times 100$$

3. RESULTS

3.1. Detection of Producers of Pullulanase

Following pullulan agar plate incubation, the bacterial isolates from soil and agricultural wastes exhibited a clear hollow zone surrounding their colonies, indicating that they were pullulanase producers. These 11(41%) isolates were identified as *Paenibacillus macerans*, based on microscopic and biochemical tests. The pullulan hydrolysis zone ratio of 0.9 to 3.3 (Figure 1) and *Paenibacillus maceramic* SR8, isolated from rhizospheric soil, showed the highest level of pullulanase production.

3.2. Purification of Pullulanase

In *Paenibacillus macerans* SR8 cultivation broth, pullulanase was obtained as an extracellular enzyme. The pullulanase purification results are summarized in Table 1. Precipitation was performed using ammonium sulphate at 70 percent saturation, precipitation of protein from the cell-free surnatant in the specific activity reaching a level of 2.795 U/mg. To extract this salt from the sample, use ammonium sulfate precipitation followed by dialysis. First, the sample was loaded to a column of A-50 DEAE-Cellulose. The elution of the 0.1 - 0.5 M gradient NaCl led to the appearance of three protein peaks and the activity of pullulanase was at the second protein peak (Figure 2). Pullulanase was purified in this step 2.98 times with 26.3% yield. The fractions collected were loaded to G-100 column of sephadex. The active fractions revealed a protein maximum of 22.9 % with a purification fold of 8.11 and a specific activity of 15.90 U/mg, which included the activity of pullulanase in the second peak, as shown in Figure 3.

3.3. Distribution of Gram-Negative Bacterial Species Isolated from Urinary Catheters

Nineteen Gram negative bacterial isolates were found among the twenty-one urine catheter discs that were taken from eleven urinary catheters. These isolates included seven isolates of *A. baumannii,* four isolates of *E. coli,* six isolates of *P. aeruginosa*, and two isolates of *E. aerogenus*.

3.4. Quantitative Detection of Biofilm Formation by Urinary Catheters Bacteria

All bacterial isolates showed signs of being able to produce biofilm at different intensities, according to the results of the TCP technique. Of these isolates, 53%, 21%, and 29% formed strong, moderate, and weak biofilms, respectively (Table 2).

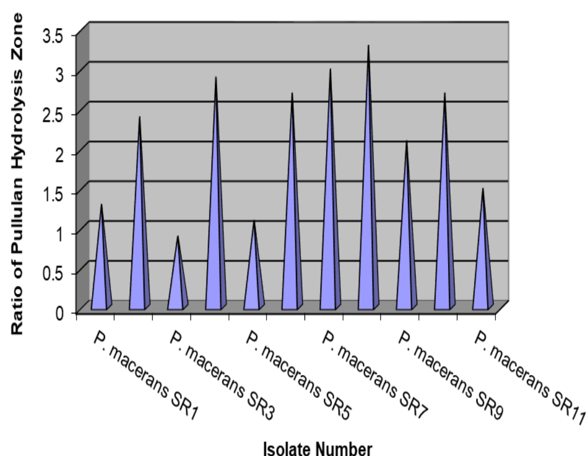

Fig. 1. *Paenibacillus macerans* isolates of rhizosphere soils in pullulan hydrolysis zones.

Table 1. Steps of pullulanase purification from *Paenibacillus macerans* SR_8.

Purification Step	Size (ml)	Pullulanase Activity (U/ml)	Protein Conc. (mg/ml)	Specific Activity (U/ mg)	Total Activity	Purification Fold	Yield (%)
Crude extract	150	65.8	33.6	1.96	9870	1	100
$(NH_4)_2SO_4$ precipitation	70	84.7	30.3	2.795	5929	1.43	60.0
DEAE-Cellulose A-50	23	112.8	19.9	5.668	2594.4	2.98	26.3
Sephadex G-100	19	119.3	7.5	15.90	2266.7	8.11	22.9

Table 2. Biofilm formation by different negative bacilli isolated from urinary catheters.

Biofilm Formation Level	*E. coli*	*A. baumannii*	*P. aeruginosa*	*E. aerogenus*	Biofilm Formation (%)
Weak	1	2	2	-	29
Moderate	1	1	1	1	21
Strong	2	4	3	1	53
Total	4	7	6	2	

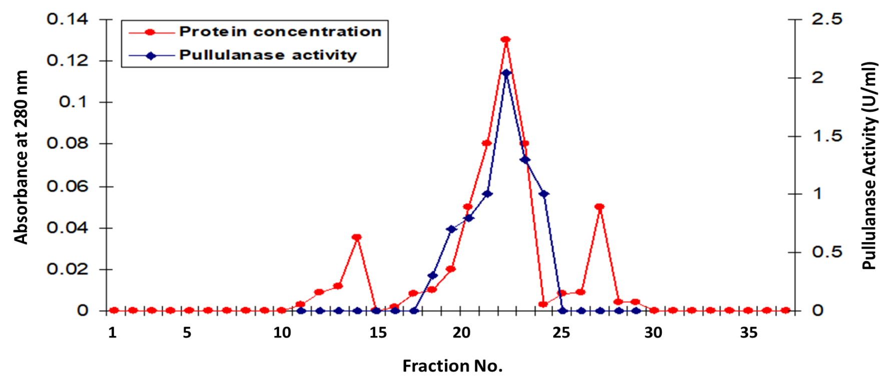

Fig. 2. DEAE-Cellulose A-50 column as ion exchanger was used to purify pullulanase from *Paenibacillus macerans* SR8.

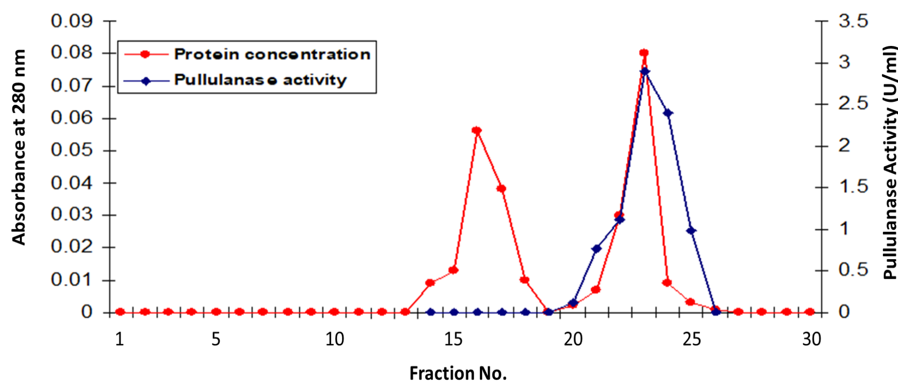

Fig. 3. Gel filtration on a sephadex G-100 column was used to purify pullulase from *Paenibacillus macerans* SR8.

3.5. Enhancement of Lactic Acid Bacteria by Pullulanase

Lactic acid bacteria, including *Lactobacillus reuteri*, *Lactobacillus plantarum, Leuconostoc mesenteroides,* and *Pediococcus* sp., were used to assess the prebiotic qualities of pure pullulanase. Pullulanase stimulated isolates of *Lactobacillus reuteri* isolates with 82–85% followed by *Leuconostoc mesenteroides* with 75-80% while little stimulation for *Pediococcus* sp. isolates with 45-50% as reported in Figure 4.

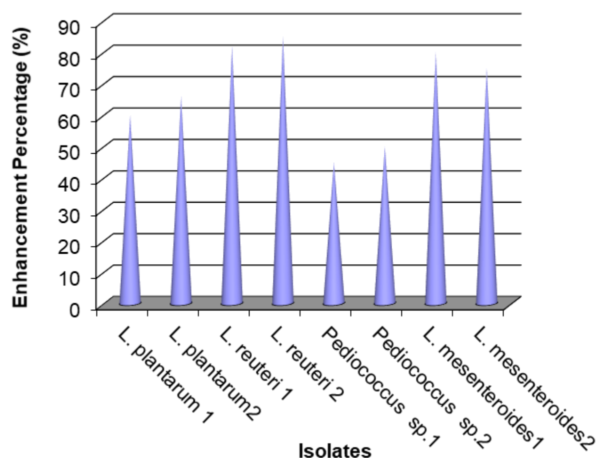

Fig. 4. Purified pullulanase from *Paenibacillus macerans* SR8 enhances lactic acid bacteria.

3.6. Effect of Pullulanase on Pathogenic Bacterial Growth

The pathogenic bacteria such as *A. baumannii*, *E. coli*, *P. aeruginosa* and *E. aerogenus* when combined with purified pullulanase led to inhibition of the bacterial growth with an inhibition percentages ranged between 72 to 83% for *P. aeruginosa* followed by 62 to 68% for *E. coli.* In contrast, lower inhibition rate appeared with *A. baumannii* at 23-44% as shown in Figure 5.

These findings suggest that pullulanase has the potential prebiotic property of enhancing lactic acid bacteria growth while inhibiting pathogenic bacteria growth. Prebiotic properties, in general, do not only inhibit pathogen growth but additionally promote the development of advantageous microorganisms like *Lactobacillus*. This is the first study to look at the purification of pullulanase from *Paenibacillus macerans* and its prebiotic properties.

3.7. Effect of Pullulanase on Biofilm Formation by Pathogenic Bacteria

Using the strongest producers, pullulanase activity against uropathogeinic bacteria's biofilm formation on urinary catheters was examined. It was shown that pullulanase that had been isolated from *Paenibacillus macerans* had antibiofilm action against many genera. Figure 6 shows that the percentage of biofilm inhibition was 44–76% after 24 hours and rose to 58–84% after 48 hours. *A. baumannii* isolates had the highest percentage of inhibition, followed by *P. aeruginosa* isolates with 68–75%, and *E. aerogenus* isolates with 58%.

4. DISCUSSION

Differences in hydrolytic zone diameter in the isolates of *Bacillus* lead to differences in pullulanase genetic expression [3]. In previous reports, pullulanases were mainly extracellular enzymes produced by various kinds of bacteria, especially by *Bacillus* spp. [3, 4]. According to Muslim *et al*. [6], *Paenibacillus macerans*, isolated from agricultural waste, was capable of producing pullulanase using salmon plants as a screening medium. An ammonium sulfate was the most common used salt,

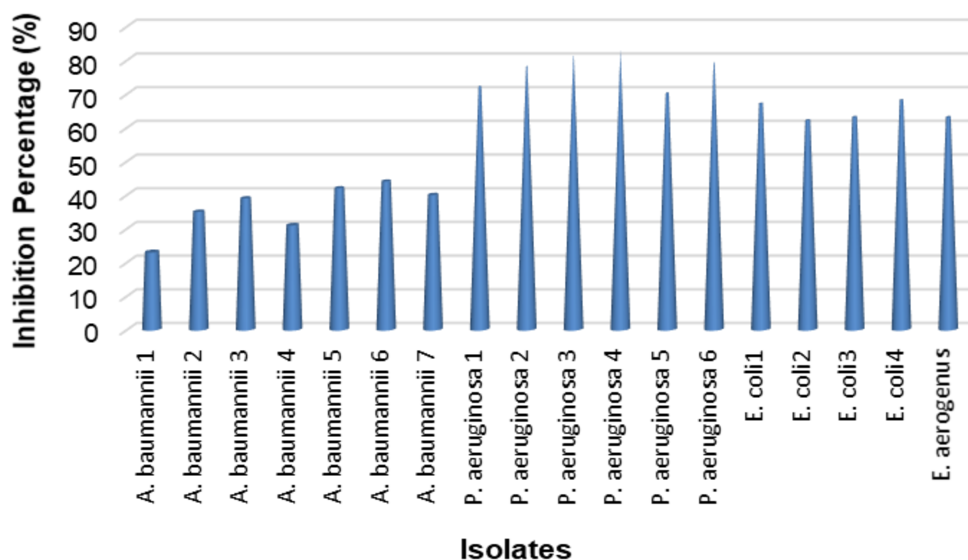

Fig. 5. An inhibition of urinary catheters pathogenic bacteria by purified pullulanase from *Paenibacillus macerans* SR_8.

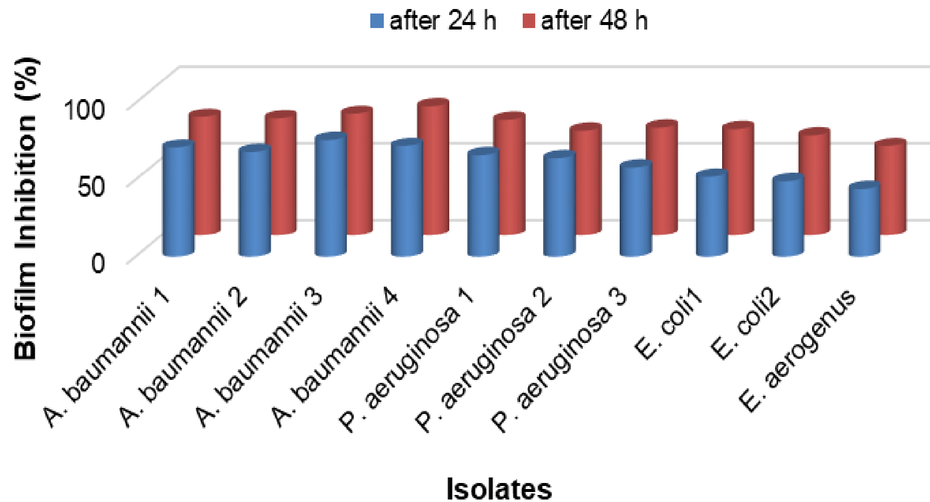

Fig. 6. Inhibition biofilm formation by urinary catheters pathogenic bacteria by purified pullulanase from *Paenibacillus macerans* SR_8.

which supports centrifugation separation because of its low solution density in comparison to other salts, high solubility, lack of buffer capacity, and low cost [23]. Extra-cellular pullulanase Type I has been cleansed with CM Sepharose FF ion exchange chromatography, followed by Sephadex G-150 gel filtration chromatography as the last purification step, folding from 14,367 with the final purification step of *Lactococcus lactis*, type I, by using ammonium-sulfate fractionation and dialysis (rather than ultrafiltration) [24]. A 5-step purification method was used to purify a 150-kDa monomeric pullulanase: (1) an original enzyme was purified 50.0 times for the ultimate specific activity of 3.0 U/mg, (2) Ammonium sulphate salts, (3) dialysis and anion exchange, (4) permeation of the gel, and (5) hydrophobial chromatography. Urinary tract pathogens are commonly isolated and counted using CHROM agar Orientation media, a chromogenic culture medium. It has several benefits, including improved gram-negative bacilli differentiation and easier detection and presumed identification of gram-negative bacilli [25]. It has been demonstrated in a number of earlier investigations that urinary tract pathogens, such as gram-negative bacilli, enterococci, and staphylococci, may be effectively isolated and differentiated using this medium. The majority of strains linked with *A. baumannii, Klebsiella ornithinolytica*, and *Pseudomonas aeruginosa* are responsible for catheter-related urinary tract infections [26]. Urinary catheter biofilm growth and biomineralization can result in serious side effects like infection and blockage. *Proteus mirabilis* and *Pseudomonas aeruginosa*, two uropathogens, were used to assess biofilm development and biomineralization in vitro on catheters retrieved from hospitalized patients [27]. Recurrent, complex UTIs are frequently caused by biofilm-forming bacteria, which are typically linked to MDR bacteria [28]. The creation of novel treatments depends on our ability to comprehend the pathophysiology and variables connected to biofilm formation [29]. The isolated *E. coli* were exposed to the biofilm-forming medium, 125 (62.5%) of the isolates formed biofilms on Congo Red Agar [30]. Because biofilm-forming bacteria have a strong polymeric matrix that prevents antibiotic penetration, they often show better resistance than planktonic cells [31]. Prebiotics are indigestible dietary additives that benefit the host by encouraging the colon's few resident microorganisms to grow and/or function. Pullulan is a non-digestible carbohydrate that is fermented by the microbiota and can change the composition of the intestinal microbiota. It is a -1,6 connected polymer of maltotriose subunits [32]. The natural microflora of the infants'colon was selectively affected by pullulan and its derivatives. While pullulan had no impact on the growth of *Bifidobacterium* and *Lactobacillus*, it did increase their acidifying activity, which was likely the cause of the decrease in the number of *E. coli* bacteria [33]. As a result, animal feed containing distilled pullulanase may be used to increase digestibility and promote gastrointestinal health. In the future, more investigation will be done to ascertain the kind of synbiotic impact that might arise from combining it with a suitable probiotic.

5. CONCLUSIONS

Certain isolates of Gram-negative bacteria, including *A. baumannii, E. coli, P. aeruginosa*, and *E. aerogenus*, were obtained from urinary catheters. Pullulanase may have a prebiotic property that promotes the growth of lactic acid bacteria while ostensibly hindering the growth of pathogens and preventing the creation of their biofilms. Consequently, pullulanase can be added to animal feed to increase digestibility and gastrointestinal health. It can also be used to cover catheters to assist in preventing bacterial colonization, infections, and the care of patients with biofilm-associated infections.

6. ACKNOWLEDGEMENTS

The Authors would like to thank Mustansiriyiah University (http://uomustansiriyah.edu.iq/) Baghdad, Iraq for its support in the present work.

7. CONFLICT OF INTEREST

The authors declare that there is no competing interest.

8. AUTHORS' CONTRIBUTIONS

Sahira Nsayef Muslim: Conceptualization, Methodology, Supervision. Nehad A. Taher: Formal analysis, Investigation. Wafaa Hassan Muslem: Data curation, Writing - original draft (Corresponding Author). Saba Riad Khudhaier: Validation, Writing - review and editing.

9. FUNDING

This work was self-funded by the authors and no external grant was received.

10. ETHICAL STATEMENT

This study does not involve human participants or animal subjects. Therefore, ethical approval was not required.

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