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Olubukola Olayemi Olusola-Makinde

ooolusola-makinde@futa.edu.ng

Federal University of Technology

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BLOOD-DEGRADING *PLCH*-POSITIVE HAEMOLYTIC *PSEUDOMONAS AERUGINOSA* STRAINS DRIVE BIODEGRADATION OF RAW SLAUGHTERHOUSE WASTEWATER: PHYSICOCHEMICAL MARKERS OF TREATMENT PERFORMANCE

Olubukola Olayemi Olusola-Makinde

Department of Microbiology, Federal University of Technology, P.M.B. 704, Akure 340110, Nigeria;

Corresponding author Email: ooolusola-makinde@futa.edu.ng

ORCID: https://orcid.org/0000-0002-5012-2458

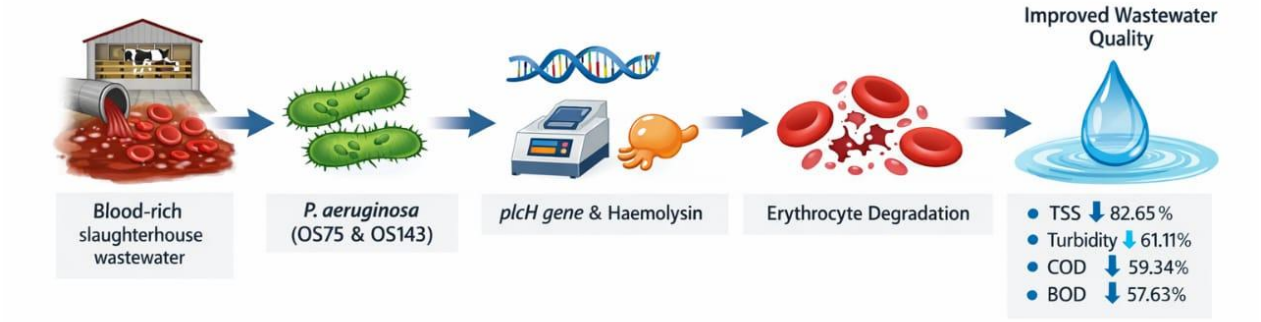
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Abstract

Microorganisms acclimate to their environmental conditions for survival by employing available substrates, whereas blood is a major substrate of slaughterhouse wastewater. This study engaged activated blood-degrading *Pseudomonas aeruginosa* to evaluate biodegradation markers in slaughterhouse wastewater. Haemolysin genes were amplified via PCR. Crude haemolysins were subjected to various treatments and animal erythrocytes. The autochthonous bacteria used as consortium identified as *P. aeruginosa* strains OS75 and OS143 presented 82.65%, 61.11%, 59.34%, and 57.63% reductions in total suspended solids, turbidity, chemical oxygen demand (COD) and biochemical oxygen demand (BOD), respectively, after achieving a 4-hour retention time. The haemolytic phospholipase C (*plcH*) gene encoding haemolysin in *P. aeruginosa* strains was amplified at 507 bp. Effects of 10 µg/mL cholesterol, 0.1 mM EDTA, and metals (5 mM AlCl, 5 mM MgCl, 5 mM NaCl, 5 mM CaCl, and 5 mM $(NH_4)_2SO_4$) on crude haemolysin revealed no increase in haemolytic activity. Isolates had the highest haemolytic activity (72%) against chicken erythrocytes. This study revealed that the *plcH* gene encoding haemolysin in *P. aeruginosa* strains OS75 and OS143 significantly improved raw slaughterhouse wastewater quality. This indicates promising autochthonous biodegradability of raw slaughterhouse wastewater, thereby reducing its associated environmental and public health risks.

Keywords: Biodegradation markers; erythrocytes; haemolysin; *Pseudomonas aeruginosa*; slaughterhouse wastewater



1 Introduction

The slaughterhouse industry is an important component of the livestock industry in Nigeria, providing meat supplies to more than 150 million people and employment opportunities for the teeming population (Abubakar et al., 2023). A slaughterhouse is a facility where apparently healthy animals are killed for human consumption (Adesola et al., 2024). Although animal slaughtering yields significant meat supplies an important protein source together with useful by-products such as leather, skin, and bones, the associated processing activities generate environmental pollution and health hazards (Alibakov et al., 2024). The meat processing industry continuously produces large volumes of slaughterhouse wastewater owing to the sustained demand for meat and other animal products, resulting in heavy slaughtering and intensive cleaning of facilities (Bustillo-Lecompte and Mehrvar, 2015).

Biological treatment of wastewater can remove more than 90% of pollutants and is cost-effective and environmentally friendly (Kanaujiya et al., 2019). Specific microorganisms such as *Streptococcus* spp. produce haemolysin, which is capable of degrading red blood cells a major component of slaughterhouse wastewater. Haemolysins are proteins and lipids that lyse red blood cells by damaging the cell membrane (Barnett et al., 2025). Although haemolysin production is commonly attributed to virulence factors, Dulermo et al. (2023) reported haemolytic activity in non-pathogenic *Streptomyces coelicolor* M145. Haemolysins can also target white blood cells (Vandenesch et al., 2012).

Blood is one of the major dissolved pollutants in slaughterhouse wastewaters and has the highest chemical oxygen demand (COD) among slaughterhouse effluent components. Blood with an ultimate BOD_5 of 405,000 mg/L far exceeds the Federal Environmental Protection Act (FEPA, 1992) guideline of 50 mg/L for slaughterhouse effluent (Muallim,

2019). Raw blood is the most concentrated of the effluent, comprising almost 100% protein (94-96% of the volatile solids), with a biochemical oxygen demand of 140-200 g/L and a COD of 375-400 g/L (Nazifa et al., 2021; Selormey et al., 2021). The readily biodegradable characteristic of the stream, which is usually presented in slaughterhouse effluent as BOD_5/COD ratio of above 0.4, makes it suitable for a biological treatment method instead of chemical treatment method (Musa and Idrus, 2021). The study site Onyearugbulem slaughterhouse located in Akure, Ondo State, Nigeria is situated along the federal expressway linking Southern Nigeria to Northern Nigeria and discharges wastewater into open drainage without conventional treatment, generating a pungent odour that causes discomfort to community residents and commuters (NiMET, 2016).

The Onyearugbulem slaughterhouse slaughters approximately ten cattle per day; the head of one cow releases approximately 22 kg (49 lbs) of blood. As population grows, slaughtering rates and thus wastewater volumes will increase. The primary wastewater treatment facility at the site is currently non-functional. Therefore, optimised microbial degradation of blood may significantly contribute to slaughterhouse effluent treatment. In practice, it is hard to maintain conventional aeration and chemical dosing at small Nigerian abattoirs, which are often lacking in power, reagents and manpower to provide adequate supervision, making a low-input, self-sustaining biological alternative appealing. The obvious starting point is bacteria that are already present in the effluent, since they are already acclimatised to the effluent's composition and don't require expensive acclimatisation. Directing the attack at the blood fraction, via the hemolytic phospholipase C (PLCH) of the autochthonous *P. aeruginosa*, is a way therefore to attack the most significant organic load at source, which is the effluent. From this basis, the study aimed to (i) confirm the presence of hemolytic activity of *P. aeruginosa* strains recovered from the effluent, (ii) optimize the physicochemical conditions for the production of hemolysin by *P. aeruginosa*, (iii) profile the hemolytic activity of the strains against erythrocytes of several livestock species and their sensitivity to the membrane-active treatments, and (iv) evaluate the resulting consortium for reducing the typical physicochemical parameters (COD, BOD, TSS, and turbidity) within a short cyclic retention regime of raw slaughterhouse wastewater.

2 Materials and Methods

Study Site

Akure, the capital of Ondo State in south-western Nigeria, is located between latitudes 7°12′ N and 7°58′ N and longitudes 5°15′ E and 5°17′ E. The climate is subtropical, with rainy and dry seasons; relative humidity ranges from 60% in January to 80% in July (Adamu et al., 2021; Dimitrakopoulou et al., 2020) (**Plate 1**). The Onyearugbulem slaughterhouse is situated along the Owo–Ilesa expressway in Akure, a city with a population of approximately 420,000 inhabitants. The facility was selected as the study area because of its large-scale operations and central location within built-up areas comprising residences, industries, and marketplaces. It lies approximately 50 m from the Ilesa–Akure–Owo expressway and covers a land area of approximately 10,000 m².

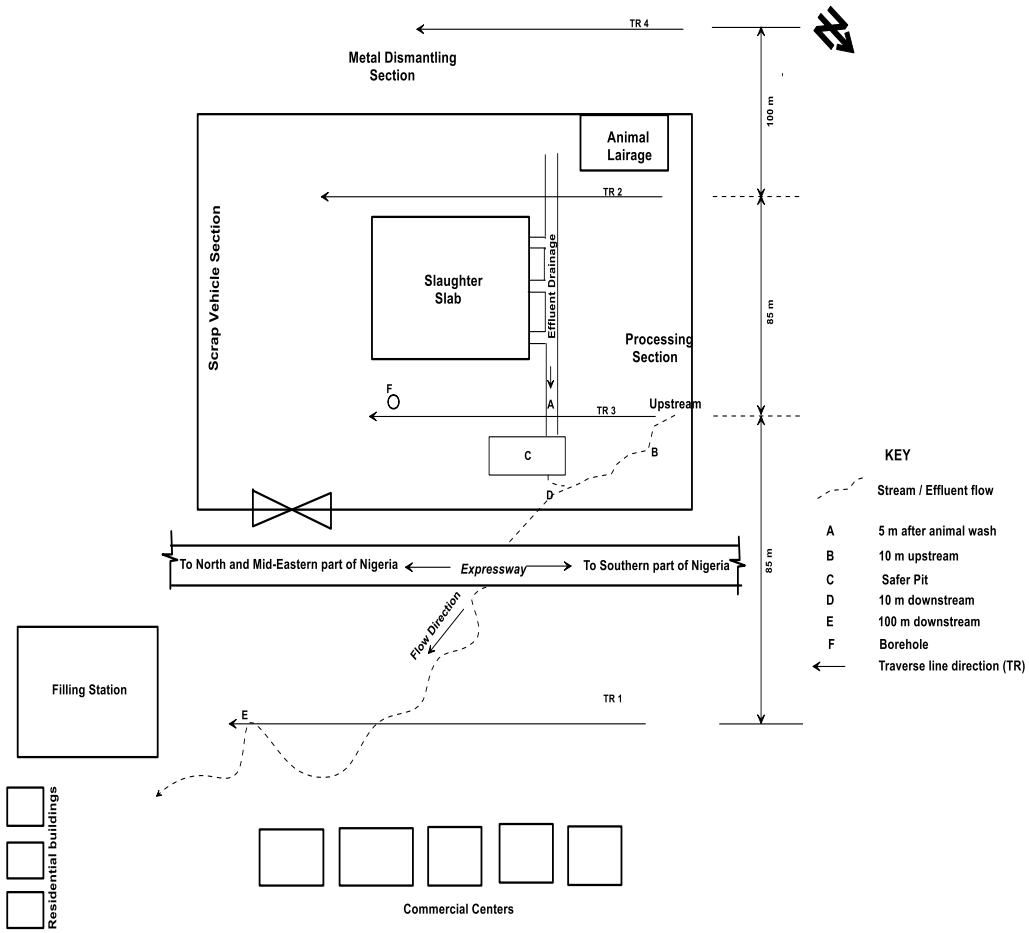


Plate 1. A schematic map of the Onyearugbulem slaughterhouse in Akure, Ondo State

Identification of Haemolysin-Coding Genes

Bacterial Purification and Genomic DNA Extraction

Bacterial isolates from slaughterhouse wastewater that demonstrated *in vitro* β-haemolysis were retrieved from the Microbiology Laboratory, Federal University of Technology, Akure, and assayed for haemolysin gene identification. An inoculum was streaked on sterile nutrient agar and grown at 37 °C overnight. Bacterial DNA was extracted from 24-h cultures using the boiling method described by Dimitrakopoulou et al. (2020). Two to three colonies were reconstituted in 200 µL of purified nuclease-free water, vortexed, and lysed by boiling (100 °C) for 10 min using an AccuBlock digital dry bath (LabNet, Woodbridge, NJ, USA). The lysate was recovered by centrifugation at 14,000 rpm for 4 min in a MiniSpin microcentrifuge (LASEC, Johannesburg, South Africa). Cell lysates (5 µL) served as template DNA in subsequent PCR assays (Costa et al., 2022). *Pseudomonas aeruginosa* strains OS75 and OS143, previously identified by conventional biochemical characterisation and 16S rRNA gene sequencing, were kept as β-hemolytic isolates, and only these confirmed β-hemolytic isolates were forwarded for the detection of hemolysin genes.

Polymerase Chain Reaction Amplification

PCR for confirmation of the haemolysin-producing gene was performed according to Das et al. (2013) using the primer pairs listed in **Table 1a** and the thermal regimens in **Table 1b.** Briefly, the PCR mixture consisted of 10× reaction buffer (5 µL), dNTPs (2 µL, 2.5 mM), $MgCl_2$ (2.5 µL, 50 mM), primers (1.2 µL each, 10 µM), Taq polymerase (0.2 µL, 5 U/µL), 5 µL of template DNA, and 32.9 µL of sterile Milli-Q water in a 50 µL total reaction volume. The negative control consisted of PCR mixtures devoid of template DNA. The plcH primer pair of Das et al. (2013) amplifies an expected product size of ~507 bp; a single band at 507 bp against the molecular-weight ladder was scored as plcH-positive.

Table 1a. Primers for identification of haemolysin-producing genes.

Primer Name		Primer Sequence (5′–3′)	Amplicon Size (bp)	Reference
plcH	F	GCACGTGGTCATCCTGATGC	607	Das et al. [16]
	R	TCCGTAGGCGTCGACGTAC		

Table 1b. PCR thermal programme for identification of haemolysin-producing genes.

Primer Set	Initiation	Denaturation	Annealing	Extension	Final Extension
plcH F & R	1 cycle, 94 °C, 3 min	30 cycles, 94 °C, 30 s	55 °C, 1 min	72 °C, 1 min 30 s	72 °C, 5 min

Gel Electrophoresis and Staining

PCR amplicons (8 µL) were electrophoresed on 1.5% agarose gels in TBE buffer (pH 8.0; 0.089 M Tris, 0.089 M boric acid, 0.002 M EDTA) for 1 h at 80 V. A 50–2000 bp DNA ladder served as the molecular weight standard. The gel was subsequently stained with ethidium bromide (0.2 µg/mL) for 35 min, and DNA bands were visualised under ultraviolet transillumination and photographed (Costa et al., 2020).

Optimisation of Haemolysin Production

Collection of Haemolysin

Single-colony isolates were grown at 37 °C on blood agar (Biolab, Midrand, South Africa) supplemented with 5% defibrinated sheep blood and used to inoculate brain heart infusion (BHI) broth (Biolab). After 18 h at 37 °C, cultures were centrifuged at 12,000 rpm for 10 min at 5 °C, and the supernatant was filtered through a 0.22 µm nylon filter (Micron Separations, Westboro, MA, USA) (Olusola-Makinde et al., 2022).

Haemolysin Tube Assay

The tube assay was performed according to Ktari et al. (2022) with modifications. Fresh sheep blood was collected into sterile screw-cap bottles containing anticoagulant (EDTA solution). Five millilitres of heparinised blood was centrifuged (4000 rpm, 10 min, 4 °C), and the serum was aspirated. Erythrocytes were washed twice with normal saline

and once with phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 6.8), then diluted to 1% v/v with PBS. Bacterial cultures (18 h old, standardised to $A_{600} \approx 0.1$ OD) were centrifuged at 12,000 rpm for 10 min, and 500 µL of supernatant was incubated with 500 µL of 1% erythrocytes at 37 °C for 90 min. Triton X-100 (1%) and PBS served as positive and negative controls, respectively. After centrifugation (4000 rpm, 5 min), 100 µL of supernatant was transferred to microtiter plates and absorbance was measured at 400 nm. Haemolytic units were calculated as:

$$HU\ (\%) = [(A - A_0) / (A_{100} - A_0)] \times 100$$

where A, A_0, and A_{100} are the absorbances of the sample, negative control, and positive control, respectively.

Determination of Optimum Production Conditions

Inoculum size (0.5%, 1%, 2%, 3%, 4%), initial pH (3, 5, 7.5, 9, 11; adjusted with 1 M NaOH or 1 M HCl), incubation temperature (20, 25, 30, 35, 40 °C), agitation speed (0, 50, 100, 150, 200 rpm), and growth medium (nutrient broth, blood agar base broth, BHI broth; all Biolab) were varied independently (Fatokun et al., 2016). After 24 h incubation, haemolytic activity of the supernatant was determined by the tube assay. Standardised inocula were used throughout ($A_{600} \approx 0.01$ OD).

Bacterial Growth Kinetics and Haemolysin Production

Standardised 18-h broth cultures ($A_{600} \approx 0.1$ OD) were inoculated into sterile BHI broth and incubated under optimised conditions. A 1 mL aliquot of culture supernatant was harvested every 4 h over 56 h. Growth was monitored turbidimetrically at 600 nm. Aliquots were centrifuged (12,000 rpm, 10 min) and haemolytic activity of the supernatant was determined by the tube assay (Olusola-Makinde et al., 2022).

Haemolytic Activity against Erythrocytes of Various Animal Species

Following incubation at 37 °C for 24 h, cultures were centrifuged (12,000 rpm, 10 min, 4 °C) and 100 µL of filtered (0.22 µm) supernatant was mixed with 100 µL of 1% washed erythrocytes from cattle, sheep, pig, chicken, and calf suspended in PBS (Daly, 2021). Blood was drawn fresh from each species, washed three times in phosphate-buffered saline and standardised to 1% (v/v) before use to ensure the difference in lysis was not due to the number of cells per unit volume. The mixture was incubated at 37 °C for 1 h and haemolytic activity was determined as described in Section 2.3.2.

Effects of Various Treatments on Crude Haemolysin

Freshly collected supernatant (5 mL aliquots) was individually incubated with 10 µg/mL cholesterol, 0.1 mM EDTA, 5 mM AlCl, 5 mM MgCl, 5 mM NaCl, 5 mM CaCl, or 5 mM $(NH_4)_2SO_4$ for 10 min at 37 °C. Haemolytic activity was then determined by the tube assay. Untreated supernatant served as the control (100% activity) (Olusola-Makinde et al., 2022).

Biodegradation of Slaughterhouse Wastewater

Inoculum Preparation

Bacterial cultures were inoculated in 25 mL nutrient broth and incubated overnight at 35 °C and 200 rpm. Subcultures were inoculated (100 µL) into 100 mL BHI broth (pH 7.0) and grown for 16–18 h. Actively growing cultures were washed three times with sterile deionised water and centrifuged (12,000 rpm, 10 min) to obtain a wet pellet. Pellets were resuspended in sterile deionised water until turbidity reached McFarland 0.5 standard ($A_{600} \approx 0.1$ OD) (Dhall et al., 2012).

Bioremediation Under Optimised Conditions

One millilitre of standardised resuspended pellets of each selected haemolytic bacterium was inoculated into 50 mL of freshly collected slaughterhouse wastewater. Flasks were incubated in a shaking incubator at 200 rpm and 35 °C for 36 h. After incubation, COD, BOD, total suspended solids (TSS), and turbidity were estimated and compared with pre-inoculation values according to standard methods (Ajao and Yusuf-Salihu, 2022).

Cyclic Treatment to Achieve Short Hydraulic Retention Times

The cyclic treatment method of Dhall et al. (2012) was adapted to reduce retention times. After an initial 36-h treatment, wastewater quality parameters were measured. Following 30 min settling, 80% of the sample was replaced with fresh slaughterhouse wastewater and re-incubated at 200 rpm and 35 °C for 8 h; parameters were re-measured. This cycle was repeated, reducing the incubation period to 4 h in the final cycle.

Statistical analysis and data representation

Each assay was performed three times, and the results are presented as mean ± standard error of the mean (SEM). One-way analysis of variance (ANOVA) was performed for each parameter of optimization (inoculum size, initial pH, incubation temperature, agitation speed, and growth medium) followed by Duncan's multiple range test for means separation of data; significant difference $p < 0.05$ was accepted. Time courses of growth and hemolysin production were plotted as optical density (A_{600}) and hemolytic units (HU) against time, and percentage reductions in COD, BOD, TSS, and turbidity were calculated based on the pre-inoculation value for each treatment cycle. All analyses have been carried out in SPSS (v 25; IBM Corp., Armonk, NY, USA) and bar charts were then plotted with error bars showing ± 1 SEM, grouped panels and line plots were plotted without error bars.

3 Results

Identification of Haemolysin-Producing Genes

Gel electrophoresis confirmed the presence of the haemolytic phospholipase C (*plcH*) gene in *Pseudomonas aeruginosa* strains OS75 and OS143 at an amplicon size of 507 bp (Plate 2). Lanes 2 and 3 yielded clear amplification bands consistent with positive *plcH* detection.

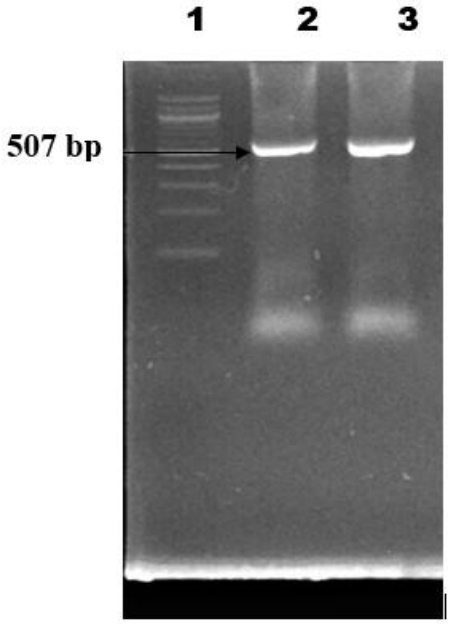


Plate 2: Gel electrophoresis; Lane 1: 100 bp DNA ladder; Lanes 2–3: *P. aeruginosa* OS75 and OS143 showing plcH amplicon at 507 bp]

Optimum Conditions for Haemolysin Production

Optimal haemolysin production was attained with a 2% inoculum size of *Pseudomonas aeruginosa* OS75 during incubation. Although there was haemolysin production by *Pseudomonas aeruginosa* OS143, there was no significant difference between the various inoculum sizes (0.5%, 1%, 2%, 3% and 4%) during haemolysin production (Figure 1). *Pseudomonas aeruginosa* OS75 and *Pseudomonas aeruginosa* OS143 displayed optimum haemolysin production at pH 7. Though, there was a significant difference in pH ($p < 0.05$), no significant difference was detected between pH 3 and 5 (**Figure 2**). Although optimal haemolysin production was recorded at 30 ℃ for *Pseudomonas aeruginosa* OS75, production was also recorded at all the incubation temperatures tested (**Figure 3**). *Pseudomonas aeruginosa* OS75 and *Pseudomonas aeruginosa* OS143 attained optimal haemolysin production at an agitation speed of 200 rpm (**Figure 4**). Brain heart infusion broth supported the highest haemolysin production by approximately 80% (0S143) and 100% (0S75) of strains (**Figure 5**).

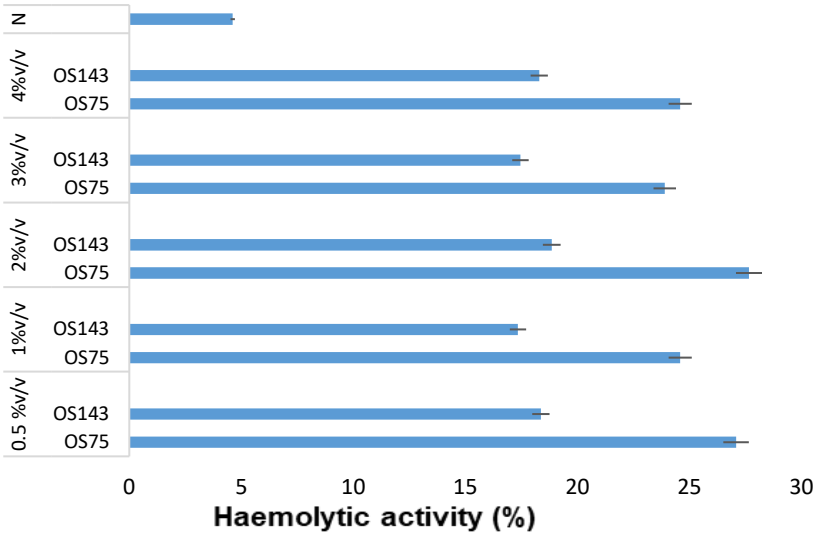
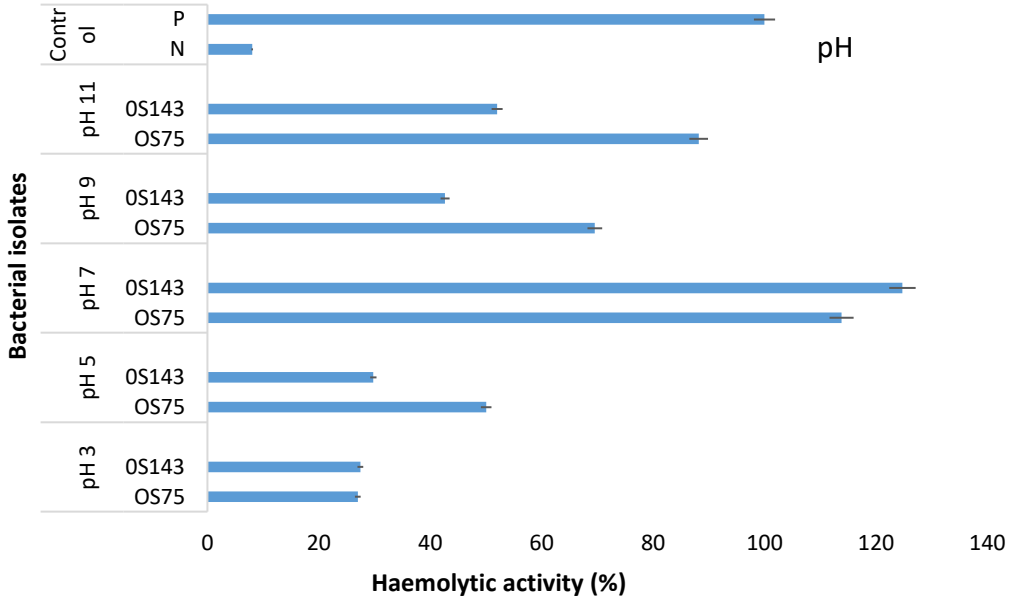


Figure 1. Effect of inoculum size on haemolysin production by Onyearugbulem slaughterhouse bacterial isolates

Key: OS75 = *Pseudomonas aeruginosa* strain OS75, OS143 = *P. aeruginosa* strain OS143



Error bars: ± 1 SE

Figure 2. Effects of pH on haemolysin production by Onyearugbulem slaughterhouse wastewater bacterial isolates

Key: OS75 = *Pseudomonas aeruginosa* strain OS75, OS143 = *P. aeruginosa* strain OS143

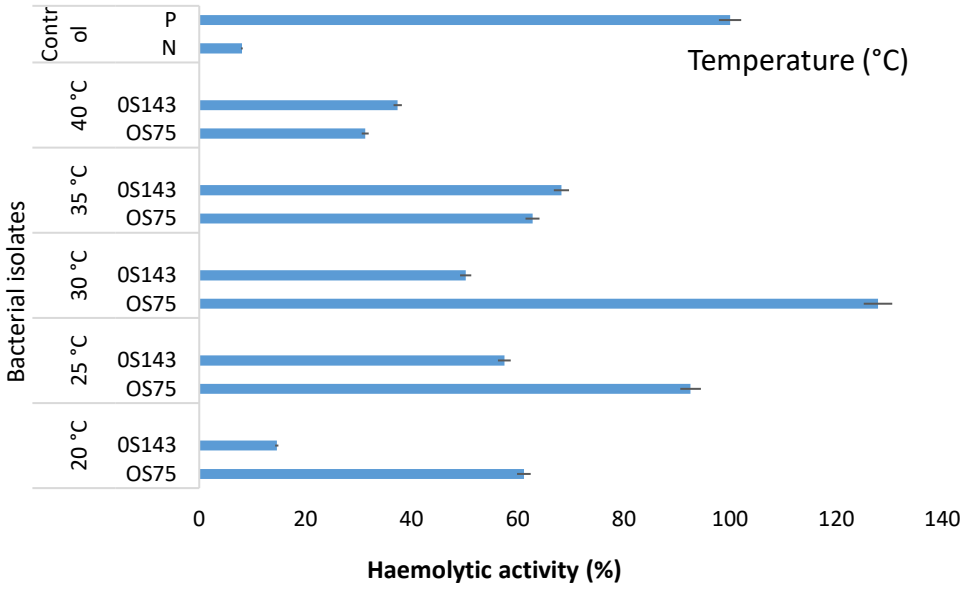


Figure 3. Effects of temperature (°C) on haemolysin production by Onyearugbulem slaughterhouse wastewater bacterial isolates

Key: OS75 = *Pseudomonas aeruginosa* strain OS75, OS143 = *P. aeruginosa* strain OS143

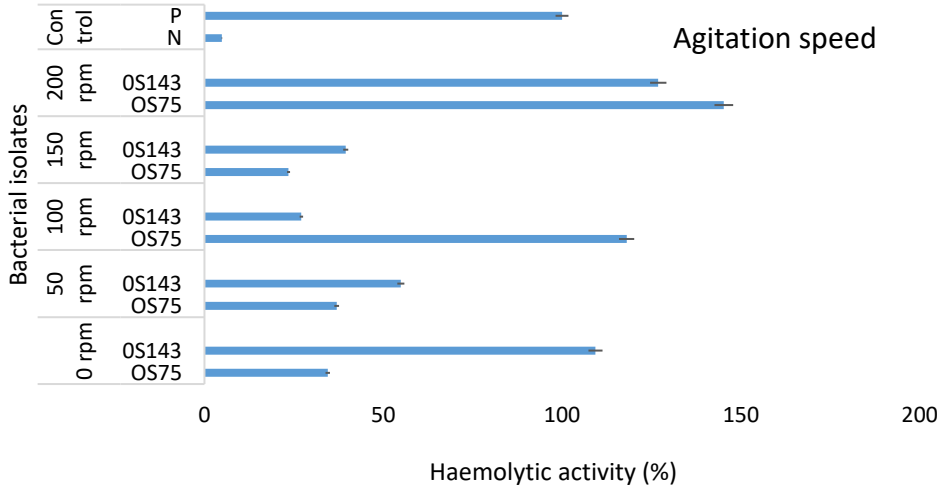
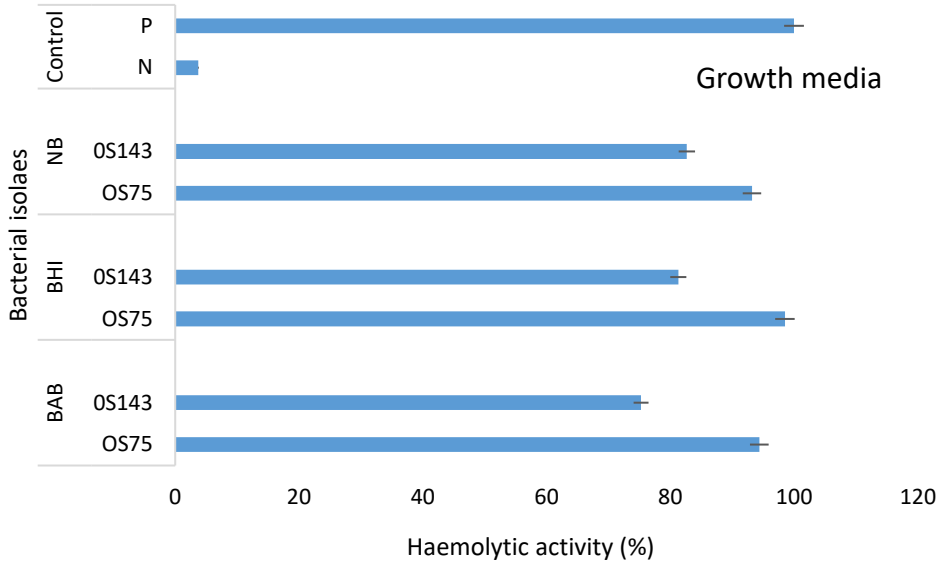


Figure 4: Effects of agitation speed (rpm) on haemolysin production by Onyearugbulem slaughterhouse wastewater bacterial isolates

Key: OS75 = *Pseudomonas aeruginosa* strain OS75, OS143 = *P. aeruginosa* strain OS143



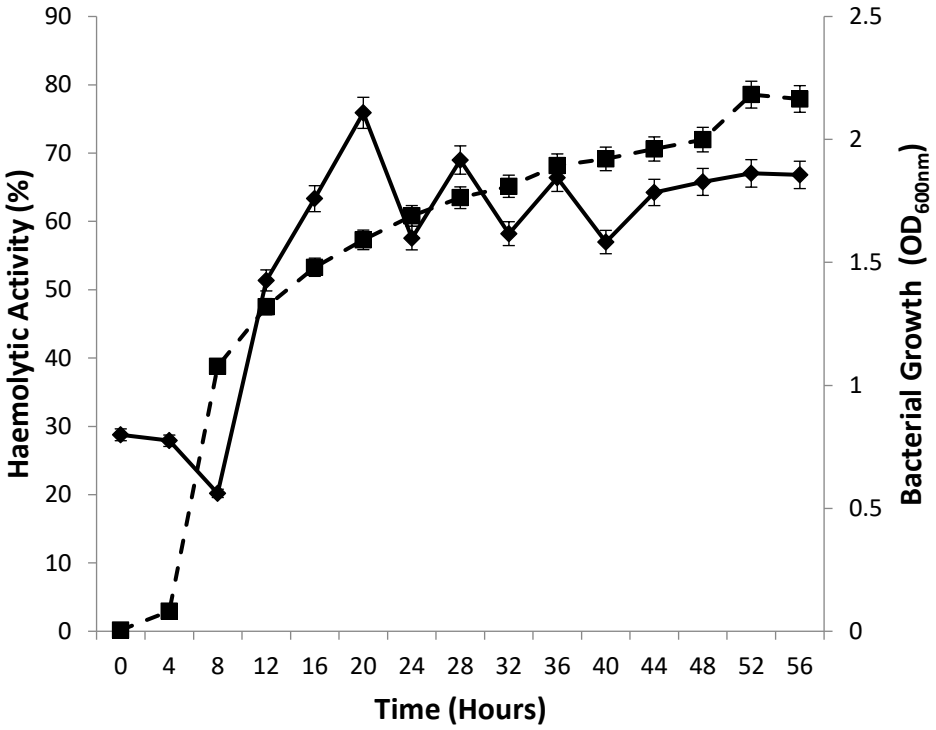
Error bars: ± 1 SE

Figure 5: Effects of growth media on haemolysin production by one-year bacterial isolates; BAB = blood agar broth, BHI = brain heart infusion broth, NB = nutrient broth

Key: OS75 = *Pseudomonas aeruginosa* strain OS75, OS143 = *P. aeruginosa* strain OS143

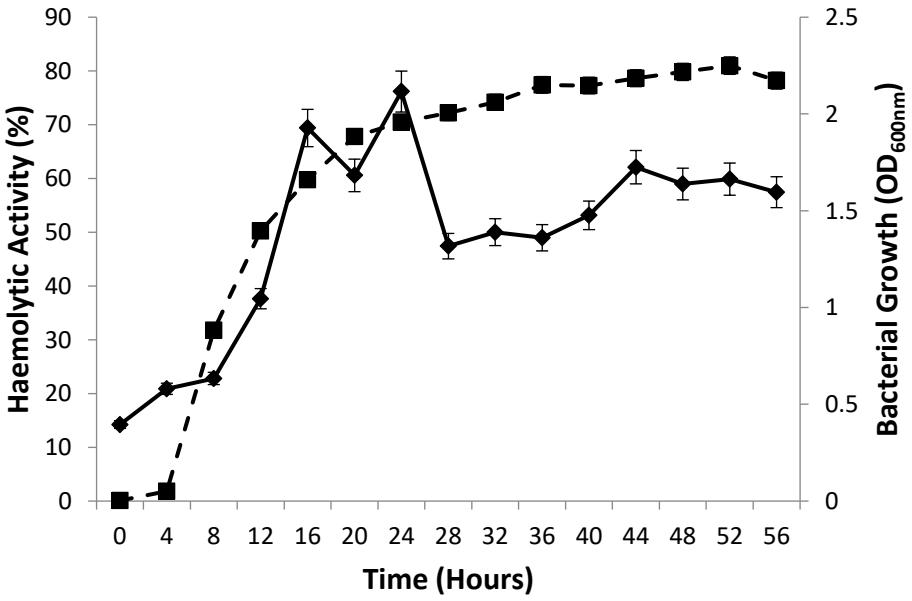
Growth Kinetics and Haemolysin Production

All the isolates were cultured under optimised conditions and monitored for growth and haemolysin production for a period of 56 hours. The *Pseudomonas aeruginosa* strain OS75 attained optimum haemolysin production at 20 h, which also corresponded with the early logarithmic growth of the strains. Growth and haemolysin production occurred in tandem until 44 h, after which there was a decline in production. **Figure 6** illustrates *Pseudomonas aeruginosa* strain OS75 growth and haemolytic patterns. This strain showed robust growth (OD range approximately 0.2-1.8) with remarkably high haemolytic activity peaking at approximately 85-90% after 36-48 hours. The lowest haemolytic activity was recorded during early growth at approximately 10-15%. **Figure 7** illustrates *Pseudomonas aeruginosa* strain OS143's growth kinetics and haemolytic activity over time. The strain demonstrated robust growth (OD range approximately 0.2-1.7) with peak haemolytic activity reaching about 80-85% at approximately 36-48 hours. The lowest haemolytic activity was observed during early growth phases (0-12 hours) at approximately 15-20%.



Error bars: ± 1 SE

Figure 6. Growth kinetics and haemolysin production of *Pseudomonas aeruginosa* strain OS75; time versus biomass (dotted line), time versus haemolytic activity (straight)

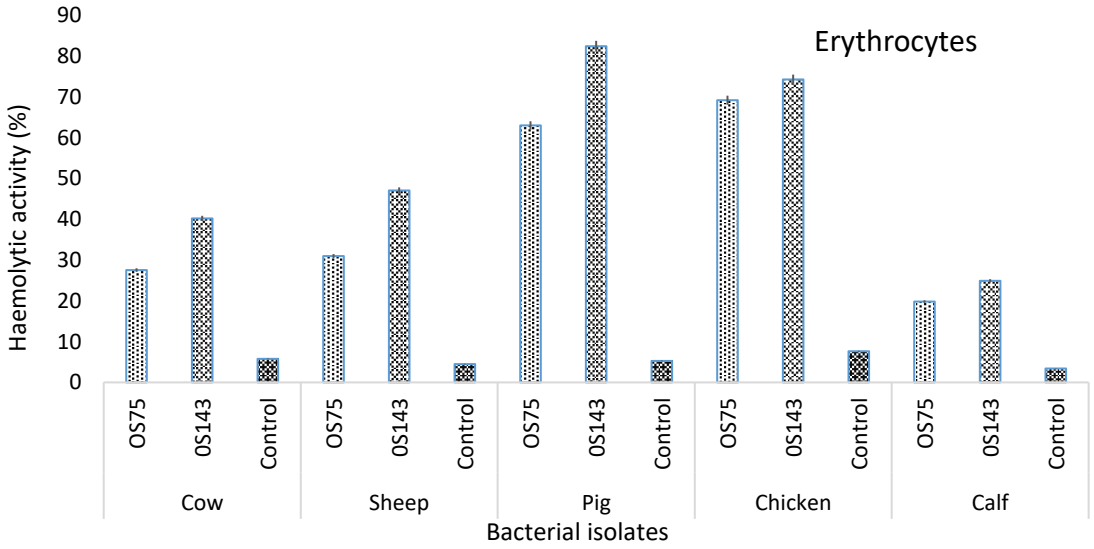


Error bars: ± 1 SE

Figure 7. Growth kinetics and haemolysin production of *Pseudomonas aeruginosa* strain OS143; time versus biomass (dotted line), time versus haemolytic activity (straight)

Haemolytic Activity on Erythrocytes from Different Animal Species

All the bacteria tested had the highest haemolytic activity on chicken erythrocytes, with the exception of *Pseudomonas aeruginosa* strain OS143, which had the highest haemolytic activity on pig erythrocytes. (**Figure 8**). A significant difference was observed between haemolytic activity on all animal erythrocytes and the control ($p < 0.05$).



Error bars: ± 1 SE

Figure 8. Haemolytic activity of bacterial isolates from Onyearugbulem slaughterhouse wastewater on erythrocytes from different animals

Key: OS75 = *Pseudomonas aeruginosa* strain OS75, OS143 = *P. aeruginosa* strain OS143

Effects of Treatments on Crude Haemolysin

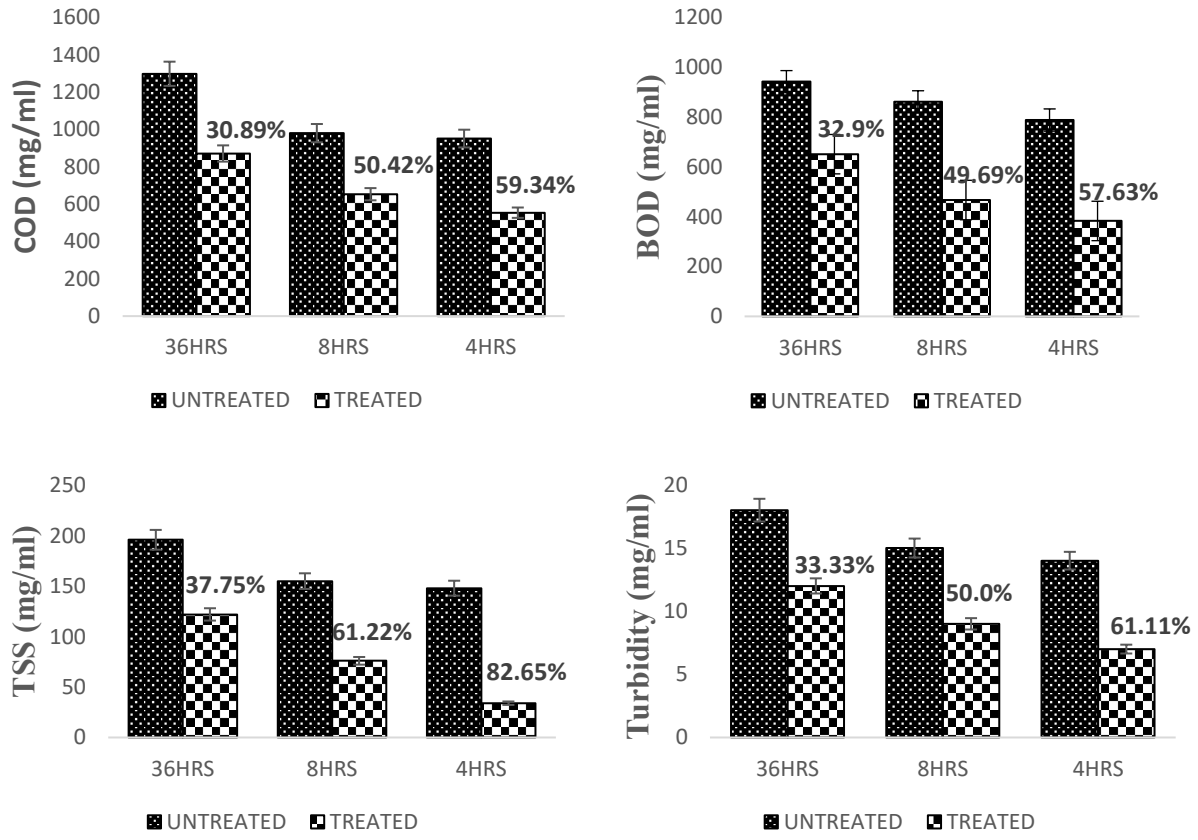
None of the treatments tested increased haemolytic activity relative to untreated crude haemolysin (100% activity). Results are presented in **Table 2.**

Table 2. Effects of various treatments on crude haemolysin (%)

Treatment	Haemolytic Activity (%)
10 µg/mL Cholesterol	22.22
5 mM AlCl	23.11
0.1 mM EDTA	38.82
5 mM MgCl	20.23
5 mM NaCl	26.48
5 mM CaCl	24.07
5 mM $(NH_4)_2SO_4$	30.04
Untreated (Control)	100.00

Biodegradation of Slaughterhouse Wastewater

The treatment was measured on the basis of several physicochemical parameters, such as the chemical oxygen demand (COD), biochemical oxygen demand (BOD), total suspended solids (TSS) and turbidity. The results revealed a significant decrease in all the parameters measured after the third round, which spanned 4 hours. The treatment had the greatest impact on the TSS, with an 82.65% decrease from the initial value. There was a final 59.34%, 57.63% and 61.11% decrease in the COD, BOD and turbidity, respectively (**Figure 9**). The decreases were gradual through the three treatment cycles, and each of the four parameters was significantly different from the pre-inoculation level ($p < 0.05$). Notably, the consortium was able to maintain this performance while reducing the hydraulic retention time from 36 to 4 h; the removals in the last cycle are a near-order-of-magnitude increase in throughput at a comparable removal efficiency, arguably the most practically important outcome of the study.



Error bars: ± 1 SE

Figure 9. Measurement of the biodegradation rate of slaughterhouse wastewater by an acclimatised haemolytic bacteria consortium from Onyearugbulem slaughterhouse wastewater via physico-chemical parameters. (a) Chemical oxygen demand (COD), (b) biochemical oxygen demand (BOD), (c) total suspended solids (TSS) and (d) turbidity

4 Discussion

The harsh consequences of untreated slaughterhouse wastewaters, coupled with the challenges of sustaining conventional wastewater treatment in the Nigerian economic context, represent a major environmental and public health concern (Bustillo-Lecompte and Mehrvar, 2015; Olusola-Makinde et al., 2022; Alibakov et al., 2024). This study focused on blood-degrading bacteria for the removal of blood, the principal organic contributor to slaughterhouse effluent from wastewater (Nazifa et al., 2021). Haemolysins produced by *Pseudomonas aeruginosa*, as demonstrated in the present study, include a heat-stable glycolipid and a heat-labile haemolytic phospholipase C (plcH) (Singh et al., 2023; Mackinder et al., 2024). The blood fraction of the effluent is degraded by the hydrolytic attack of PlcH on the membrane phospholipids, not by simple pore formation, which causes the lysis of blood cells, as the very phospholipids of the outer leaflet of the erythrocyte membrane are those most preferred for hydrolysis by PlcH (Mackinder et al., 2024). *P. aeruginosa* is nutritionally undemanding, enriched with enzymes, toxins, and glycocalyx, and forms coherent layers on moist surfaces (Iseppi et al., 2020).

Haemolysin production is markedly influenced by pH. The initial pH of the cultivation medium significantly affects enzyme transport and microbial metabolic activities (Bendary et al., 2024). The optimal pH values of 7.0 and 9.0 observed for OS75 in the present study align with the range reported by Khayat et al. ((2023) (pH 5–9) and contrast with the optimal pH of 6.0 for haemolysin production by *Prevotella oris* (Diaz et al., 2024). Variations in optimal pH reflect differences in medium composition, microbial characteristics, and sample origins (Mirbag et al., 2024).

Temperature is another critical determinant of haemolysin production. Deviations from optimal temperature reduce metabolic activities and inhibit both growth and enzyme synthesis (Rocha et al., 2022). The optimal temperature of 30 °C observed for OS75 contrasts with the high haemolysin production of *Clostridium chauvoei* at 10 °C (Al-humndu and Farj, 2025), reflecting strain-specific adaptations to temperature fluctuations (Dougnon et al., 2021).

High agitation (200 rpm) enhanced haemolysin production, likely by improving oxygen transfer in the growth medium (Xu et al., 2024; Ma et al., 2022). BHI broth supported maximum haemolysin production, consistent with the carrier protein requirements for haemolysin activity and release (Stachurova et al., 2020; Ali, 2024)).

Haemolysin production was coupled with the exponential growth phase, consistent with reports for multiple species including *Aggregatibacter actinomycetemcomitans*, *Prevotella intermedia*, *Porphyromonas gingivalis*, and *Prevotella oris* (Sato et al., 2012; Mohammed and Mahdi, 2022; Liwarska-Bizukoic, 2022; Morni et al., 2023). The post-peak decline at 44–56 h likely reflects nutrient depletion, proteolysis, or autolysis of aging cells (Shak et al., 2013; Yossapol et al., 2023).

The differential susceptibility of erythrocytes from various species to *P. aeruginosa* haemolysin parallels results for *Prevotella intermedia* and *Actinobacillus actinomycetemcomitans* (Chen et al., 2020). Chicken erythrocytes were the most susceptible in this study, reaching about 72% haemolytic activity. Species differences of this type have been documented in bacterial hemolysins; they are typically linked to the composition of the target membrane, which is more likely to contain phosphatidylcholine and sphingomyelin, the preferred substrates of PlcH, and are normally associated with the ability to lyse erythrocytes (Mackinder et al., 2024; Reynolds et al., 2025). Susceptibility is not therefore rigidly defined throughout the literature; the susceptibility depends on the substrate and on the specific membrane to which the enzyme is directed, hence the different rank orderings for the same species as different

structurally different hemolysins are used. The extensive lysis of chicken and pig erythrocytes is seen in the present plcH-driven system and is indicative of a mechanism of phospholipid hydrolysis on membranes exposed to abundant phosphatidylcholine and sphingomyelin.

The insensitivity of *P. aeruginosa* OS75 haemolysin to cholesterol, EDTA, and metals contrasts with the behaviour of listeriolysin O and streptococcal haemolysins (Zhu et al., 2021), but is consistent with haemolysins from *Actinomyces pyogenes* (Morton et al., 2019). This suggests a membrane-damage mechanism distinct from cholesterol-dependent cytolysin pathways, such as that of streptolysin O (Popoff, 2018).

The significant reductions in TSS (82.65%), turbidity (61.11%), COD (59.34%), and BOD (57.63%) achieved by the acclimatised consortium The COD removals, typically range between 55–95%, are the typical values used in biological slaughterhouse-wastewater treatment, and are mostly achieved with engineered aeration or anaerobic reactors over retention times that can be hours to days long (Musa and Idrus, 2021; Musa & Idrus, 2021). The uniqueness of the present result is that similar reductions were achieved in a 4 h cycle with only the resident bacteria and shake-flask culture conditions. These outcomes reflect the capacity of autochthonous bacteria from slaughterhouse environments to develop catabolic enzyme systems tailored to the specific organic composition of the wastewater (Dhall et al., 2021). The COD reduction is attributable to the availability of dissolved organic matter as a microbial nutrient source (Ojobor and Egbuchua, 2020). BOD reduction reflects microbial consumption of organic substrates and may concurrently diminish coliform populations (Olalemi et al., 2020). The turbidity decrease parallels similar observations in dairy wastewater treatment using *Oceanibackillus* and *Halobacillus* species (88.3% reduction) (Cosa and Okoh, 2014).

5 Conclusions

This study demonstrated that *P. aeruginosa* strains OS75 and OS143 isolated from slaughterhouse wastewater and characterised as *plcH*-positive haemolytic isolates effectively degraded blood constituents and reduced key physicochemical pollutants in raw slaughterhouse wastewater. The haemolytic activity of the strains was active across erythrocytes of multiple animal species, confirming broad-spectrum blood-degrading capacity. Optimised production conditions (pH 7.0, 30 °C, 200 rpm, BHI broth, 2% inoculum) and consortium-based cyclic treatment substantially reduced TSS, turbidity, COD, and BOD within 4 h of hydraulic retention time, representing a cost-effective and sustainable biotreatment strategy. These findings provide a foundation for scaling up autochthonous microbial consortia for the management of slaughterhouse wastewater in resource-limited settings, thereby mitigating associated environmental and public health risks. The study demonstrates that a molecular determinant (the phospholipase C/sphingomyelinase encoded by plcH) can be linked to a measurable improvement in effluent quality, from a simple observation to a mechanistically-based and therefore more easily optimised treatment option for low-resource abattoirs.

Declarations

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Data Availability Statement

Data supporting the reported results are available upon reasonable request from the corresponding author.

Conflicts of Interest

The author declares no conflicts of interest.

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