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Optimization of hemolysin formation in *Alcaligenes* species isolated from abattoir wastewater samples in Akure, Ondo State, Nigeria

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Abstract

Hemolysin is significantly toxic and is used as a molecular marker for pathogenicity. This study evaluates the conditions for optimal hemolysin production by *Alcaligenes faecalis* strains isolated from a city abattoir wastewater. The parameters investigated for hemolysin formation were the size of the inoculum, initial pH of the production medium, bacterial incubation temperature, agitation speed, and growth media. After that, the effect of various parameters on the hemolytic activity for the formation of hemolysin was assessed. The genus *Alcaligenes* was assigned to the test organisms after analyzing their 16S rRNA gene sequence with accession numbers: MF498824, MF498825, and MF498827. Optimum conditions for hemolysin formation in *Alcaligenes faecalis* strain OS42 were inoculum size of 0.5% (v/v), pH 9, 20 ^{o}C, 0 rpm, and brain heart infusion broth. 77% and 79% of Hemolytic activities were achieved at 20 h for strains OS42 and OS61. Cholesterol and ethylenediaminetetraacetic acid did not affect hemolysin formation. This work revealed that the hemolysin formation in *Alcaligenes* strains was sourced from abattoir wastewater effluent. The effluent was contaminated with pathogenic *Alcaligenes* strains, a public health hazard to their prospective infection of humans and animals.

Keywords: Abattoir wastewater; *Alcaligenes* spp.*;* hemolysin; optimization; pathogenicity

1. Introduction

Alcaligenes faecalis is a well-reported environmental bacterium associated with freshwaters (Nduka, 2011), soil (Jiajun *et al.,* 2010), wastewaters (Rajeshkumar & Jayachandran, 2004; Abo-Amer *et al.,* 2015), and mangrove sediments (Wongwongsee *et al.,* 2003; Behera *et al.,* 2017). *Alcaligenes faecalis* synthesizes various ranges of extracellular proteins such as biosurfactant, enzymes, R-(-) mandelic acid, and the enterococcal surface protein (Wongwongsee *et al.,* 2003; Tapi *et al.,* 2010; Bharali *et al.,* 2011; Xia, 2013 and Ju *et al.,* 2016). The growth of *Alcaligenes* species is easily stimulated in water with the addition of proteins (Nduka, 2011). More so, the abattoir wastewater consists of a high number of organic matters such as blood, animal fats, aborted fetuses, condemned organs, bile, urine, and undigested feed that can stimulate the growth of most bacteria, including *Alcaligenes* species (Chukwu *et al.,* 2008 and Ogbonna, & Ideriah, 2014). Blood is the primary organic matter in the abattoir wastewater comprising hemoglobin, an

iron-containing compound that can be lysed by hemolysin produced by hemolytic microorganisms (Coker *et al.,* 2001).

Hemolysins are exotoxins that lyse erythrocytes *in vitro* by disintegrating their cell membrane (Stipcevic *et al.,* 2005; Das *et al.,* 2016). Their production is attributed to the microorganisms' virulence factor that produces them. Nevertheless, Rajesh *et al.* (2013) has reported hemolytic activity in non-pathogenic *Streptomyces coelicolor* M145. Besides red blood cells, hemolysins can target other cells, including white blood cells (Stipcevic *et al.,* 2016). Some hemolytic organisms also show the ability to utilize nicotinamide adenine dinucleotide (NAD) (factor V) and heme (factor X) released when blood is lysed (Public Health England, 2015). Hemolysin's production is attributed to various functions, such as the growth of the respective microbe (Zhang *et al.,* 2005 and Singh *et al.,* 2010) and heme uptake in mycobacteria (Jones & Neiderweis, 2011).

The abattoir generates a large amount of wastewater that constitutes 32.5% of blood (Aniebo *et al.,* 2011). In addition, the primary wastewater treatment, which entails mainly segregation, is applied at the Onyearugbulem abattoir; thus, a high blood concentration is associated with its final effluent (Akinro *et al.,* 2009). Furthermore, there is little documentation on hemolysin production by environmental bacterial strains, specifically from the abattoir.

This paper, therefore, describes hemolysin production by *Alcaligenes* strains isolated from Onyearugbulem abattoir effluent and the optimal culture conditions for hemolysin production by *Alcaligenes faecalis* strains isolated from abattoir wastewater. In addition, this study further provides information on the influence of different treatments on hemolysin production. This is the first report on optimizing hemolysin produced by *Alcaligenes* strains to our knowledge.

2. Materials and methods

2.1 Source and isolation of bacterial isolates

Final wastewater effluent samples from the Onyearugbulem abattoir (7°16'58.5"N 5°11'15.9"E) were collected over a 12-month sampling regime and analyzed within 4 h of collection. For the bacterial isolation, serial dilution of the samples was prepared, and 1 ml was pipetted into sterile prepared nutrient agar (Biolab) plates using the spread-plate method and cultured aerobically at 37 °C for 24 h. Distinct colonies were sub-cultured on sterile nutrient agar (Biolab) plates at 37 °C for 24 h. The pure cultures were aseptically transferred into well-labeled nutrient agar (Biolab) slants and preserved at 4 °C until further studies.

2.2 Phenotypic characterization of isolates

The method described by Hussain *et al*. (2013) was applied for the phenotypic characterization of the isolates with a slight modification. Bergey's manual of systematic bacteriology was employed to suggest the phenotypic characteristics of the bacterial isolates. The assessed cultural features included the colonial color, shape, edge, elevation, and surface, while catalase, coagulase, oxidase, indole, motility, and sugars' fermentation tests were carried out for the biochemical characterization. In addition, gram-stain reactions were assayed to ascertain morphological characteristics.

2.3 Genetic characterization of bacterial isolates by 16S rRNA

WITH MINOR REVISIONS, the DNA extraction and 16S rRNA gene amplification were implemented, as reported by El Samak *et al.* (2018). Briefly, two colonies of an overnight bacterial culture were aseptically reconstituted in 200 µl sterile nuclease-free water. The cells were vortexed and lysed by boiling (100 °C) for 10 min using AccuBlock (Digital dry bath, Labnet). The lysate (supernatant) containing the DNA was recovered at 14,000 rpm for 4 min in a Mini Spin microcentrifuge (LASEC, RSA). Cell lysates were preserved at 4°C and functioned as DNA stencils in polymerase chain reaction (PCR) assays. Two universal primers; 27F (Forward primer 5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (Reverse primer 5'-GGTTACCTTGTTACGACTT-3'), were used for the amplification of isolates' 16S rRNA gene with the aid of PCR. The conditions employed for PCR amplification included: initial denaturation for 15 min at 95 °C, 35 cycles at 95 °C for 30 sec, 61 °C for 1 min, and at 72 °C for 1 min 30 sec. Lastly, an extension step at 72 °C for 10 min. For verification, amplicons were resolved in a 1.5% agarose gel electrophoresis at 120 Volts for 35 min in 0.5% TBE buffer stained with ethidium bromide solution. Sequencing the resolved PCR outcomes was done at the University of Kwa Zulu Natal, Durban, South Africa. The deoxyribonucleic acid (DNA) sequences were analyzed by the algorithm system of the Basic Local Alignment Search Tool (BLAST) from the National Centre for Biotechnology (NCBI) (http://www.ncbi.nlm.nih.gov/Blast.cgi). The sequences from the GenBank database with more than 98% similarity and the resultant sequences were copied in FASTA format and used to determine the phylogeny of the bacterial strains. The software used for the phylogeny studies was Molecular Evolutionary Genetics Analysis version 7.0 (MEGA7).

2.4 Hemolysin tube assay

2.4.1 Collection of hemolysins

The hemolysin collection from the bacterial cultures was carried out using the descriptions reported by Sato *et al.* (2012), with minor revisions. Single colony bacterial isolates were cultured at 37 °C using a blood agar base (Biolab, Hungary) with 5% defibrinated sheep blood for 24 h. They are used to inoculate brain heart infusion broth (Biolab, Hungary). The inoculated agar plates were cultured at 37 °C for 18 h and centrifuged at 12000 rpm for 10 min at 5 °C. A 0.22 µm pore size nylon filter (Micron Separations, Westboro, USA) was used to filter the supernatant. The supernatant (cell-free) was used to evaluate the hemolytic activity of the hemolysin synthesized by the bacterial isolates.

2.4.2 Determination of hemolytic activity

With minor revisions, the tube assay for hemolytic activity was adopted as described by Ktari *et al.* (2017). Freshly collected sheep blood was obtained into sterilized screw cap bottles containing anticoagulant solution (EDTA solution). Five milliliters (5 ml) of heparinized sheep blood was pipetted into 15 ml conical bottle and spun down at 4000 rpm at 4 °C for 10 min, and the serum was gently aspirated. The bacterial cells were twice washed with normal saline followed by phosphate-buffered saline (PBS) formulated by 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4 and pH adjusted to 6.8. Before aspiration, the resultant solution (4 ml) was

diluted with 10 ml of PBS. Subsequently, 1% v/v erythrocytes were prepared with PBS at a ratio of 0.15:14.85. For the preparation of bacterial samples, 18 hold bacterial cultures in brain heart infusion broth were standardized ($A_{600\ nm}$ = 0.1), whereas 1% v/v of the culture was cultured in nutrient broth (Biolab) at 37 °C for 24 h. The bacterial culture solution was centrifuged (12000 rpm, 10 min), and the supernatant was gently aspirated. After that, the hemolysin tube assay was carried out by incubating 500 µl of 1% v/v erythrocytes and 500 µl of bacterial supernatant at 37 °C for 90 min. Positive control was Triton X-100 (1%), while negative control was PBS buffer. The mixture was centrifuged (4000 rpm, 4 °C, 5 min), 100 µl of aspirated supernatant was pipetted into microtitre plates, and the absorbance$_{400\ nm}$ was taken using a microtitre reader (BioTek Instruments, USA). One hemolytic unit (HU) was equalized as the percentage of lysed cells, which was determined by Equation 1.

$$HU\ (\%) = \frac{X\ -\ X_0}{X_{100}\ -\ X_0} \times 100 \quad (1)$$

where X = sample absorbance, X_0 = negative control absorbance, and X_{100} = positive control absorbance.

2.5 Determination of optimal parameters for hemolysin production

Optimization of hemolysin production by the selected bacterial isolates was carried out by a one-variable-per-time optimization method described by Fatokun *et al.* (2016).

2.5.1 Influence of bacterial inoculum size on hemolysin production

Brain heart infusion broth was inoculated with bacterial culture (18 hold) at varying percentages (0.5 %, 1 %, 2 %, 3 % and 4 %) standardized to 0.10 at 600 nm and incubated at 37 ℃ for 24 h. Using the hemolysin tube assay, the gently aspirated supernatant, representing the crude enzyme, was assayed for hemolytic activity.

2.5.2 Influence of initial pH of the production medium on the hemolysin production

The growth media's initial pH was tuned to different pH values of 3, 5, 7, 9, and 11 using 1 M NaOH or 1 M HCl as necessary before the sterilization. The sterilized media were inoculated with a standardized ($A_{600nm} \approx 0.01$) bacterial culture. The medium was cultured for 24 h at 37 ℃, and the supernatant served as the crude enzyme fraction and was tested for hemolytic activity using the hemolysin tube assay.

2.5.3 Influence of temperature of bacterial incubation on hemolysin production

To evaluate the optimal incubation temperature required for hemolysin synthesis, the growth medium was inoculated with a standardized ($A_{600nm} \approx 0.01$) bacterial culture and incubated at the following temperatures: 20, 25, 30, 35, and 40 ℃ for 24 h. After incubation, the supernatant served as the crude enzyme and was tested for hemolytic activity using the hemolysin tube assay.

2.5.4 Influence of agitation speed on hemolysin production

To determine the optimal agitation rate, brain heart Infusion broth (Biolab, Hungary) was inoculated with the standardized ($A_{600nm} \approx 0.01$) bacterial culture and incubated under variable

agitation speed at 37 ℃ for 24 h. The agitation speeds included static (0 rpm), slow (50 rpm), standard (100 and 150 rpm), and fast agitation (200 rpm). After incubation, the supernatant served as the crude enzyme and was tested for hemolytic activity using the hemolysin tube assay.

2.5.5 Influence of growth media on hemolysin production

For the optimal growth media required for hemolysin production, standardized ($A_{600nm} \approx 0.01$) bacterial culture was inoculated in different growth media, [nutrient broth (Biolab, Hungary), blood agar base (Biolab, Hungary) broth, and brain heart infusion broth (Biolab, Hungary) after standardization ($A_{600nm} \approx 0.01$) and incubated for 24h at 37 ℃. The supernatant was tested for hemolytic activity using the hemolysin tube assay.

2.6 Time and growth kinetics of hemolysin production

The relationship between bacterial growth and hemolysin production over a while was investigated as reported by Sato *et al*. (2012) with minor alterations. Briefly, 1 ml of 18 h pre-culture cell solution at 0.10 OD_{600} was transferred to 100 ml brain heart infusion broth and cultured for 56 h, under optimal conditions for hemolysin production. The growth medium was withdrawn at 4 h intervals to determine bacterial growth and hemolytic activity. Culture turbidity at 600 nm was taken to measure bacterial growth, and hemolytic activity was determined by hemolysin tube assay, as mentioned earlier.

2.7 The effect of various treatments on the hemolytic activity

The stimulating effect of cholesterol, EDTA, $AlCl_3$, $MgCl_2$, NaCl, $CaCl_2$, and $(NH_4)_2SO_4$ on the hemolytic activity of hemolysin produced by *Alcaligenes* strains was evaluated as described by Sato *et al*. (2012), with minor modifications. Briefly, 10 µg/ml cholesterol, EDTA (0.1 mM), $AlCl_3$ (5 mM), $MgCl_2$ (5 mM), NaCl (5 mM), $CaCl_2$ (5 mM) and $(NH_4)_2SO_4$ (5 mM) were prepared and incubated individually with 5 ml aliquots of freshly collected crude hemolysin for 10 min and set at 37 ℃. Five milliliters of freshly collected crude hemolysin, without any treatment, served as a positive control.

2.8 Statistical analysis

The data were obtained in triplicates, and analysis of variance (ANOVA) was determined. Means of the treatment were obtained using Duncan's New Multiple Range Test at a 95% confidence level using SPSS (version 23).

3. Results

3.1 Molecular confirmation of bacterial isolates

Analyses of the 16S rRNA gene sequences of bacterial isolates using the BLAST program ascribed the organisms to the *Alcaligenes* genus. After depositing the 16S rRNA gene sequences at the GenBank, the assigned nomenclatures were identified as *Alcaligenes feacalis* strains OS42, OS61, and OS117 with accession numbers MF498824, MF498825, and MF498827, respectively. The

unrooted phylogenetic tree indicating the evolutionary relationships of *Alcaligenes* strains in this study with other *Alcaligenes* species available in the NCBI database is shown in Figure 1. *Alcaligenes feacalis* OS61 had 99% similarity to *Alcaligenes* sp. strain SCU-M2.

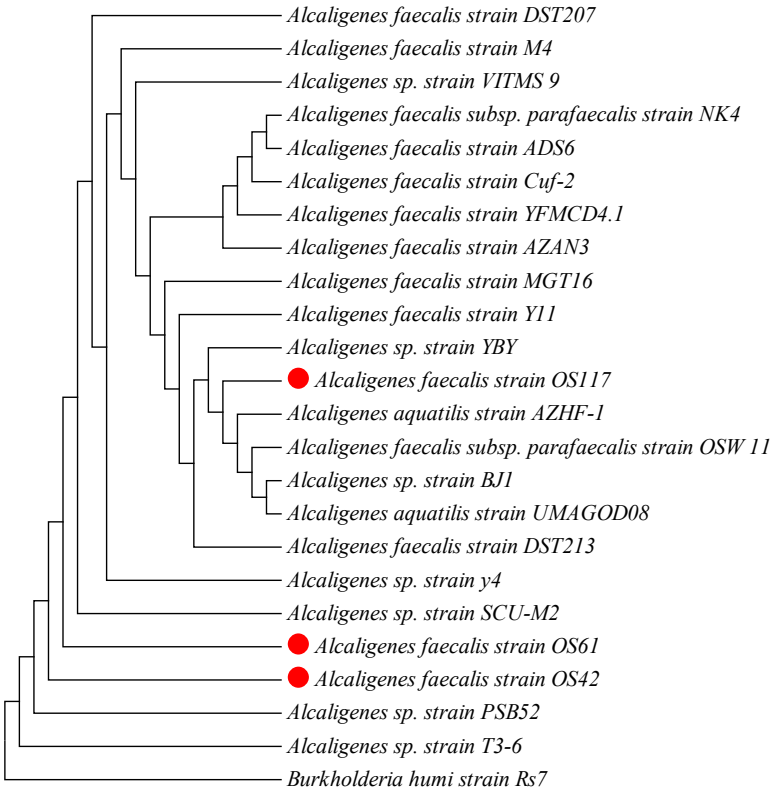

Fig. 1. Phylogeny of the relationship of *Alcaligenes feacalis* strains OS 42, 61, and 117 (bulleted) with certain Burkholderiales in GenBank database.

3.2 Influence of bacterial inoculum size on hemolysin production

The impact of bacterial inoculum size on hemolysin synthesis was investigated, and the results are shown in Figure 2. Optimal hemolysin production (35.96%) compared to 100% hemolysis by Triton-X 100 was attained with 2% v/v inoculum size of *Alcaligenes faecalis* OS61. *Alcaligenes faecalis* strains OS42 and OS117 had the highest hemolysin formation with 0.5% v/v and 1% v/v inoculum sizes. Triton-X 100 functioned as the positive control. It was observed that hemolysin formation depended on the inoculum sizes (0.5%, 1%, 3%, and 4%).

3.3 Influence of initial pH on hemolysin formation

The influence of initial pH on hemolysin synthesis by *Alcaligenes* strains was assessed, and the results are shown in Figure 3. Hemolysin synthesis was studied at variable pH, and the optimal hemolysin formation was obtained at pH 9 for *Alcaligenes faecalis* OS42, pH 7 for *Alcaligenes faecalis* OS61 and *Alcaligenes faecalis* OS117. There was a significant difference among tested pH ranges ($p < 0.05$) for hemolysin formation in *Alcaligenes faecalis* OS42. Furthermore, a steep decline of up to 50% in hemolysin formation was observed at pH 11 from *Alcaligenes faecalis* OS42 and *Alcaligenes faecalis* OS61.

3.4 Effect of bacterial incubation temperature on hemolysin formation

The impact of incubation temperature on the hemolytic activity of the bacterial isolates was studied, and the results are shown in Figure 4. The optimal hemolysin formation was recorded at 25 °C for *Alcaligenes faecalis* strains OS61 and OS117, but the appearance was also observed at all other studied incubation temperatures (20 °C, 30 °C, 35 °C as well as 40 °C). No significant difference in hemolysin formation at variable incubation temperatures was observed for *Alcaligenes feacalis* OS42.

3.5 Influence of agitation speed on hemolysin formation

The influence of agitation speed on hemolysin formation in *Alcaligenes* strains was assessed, and the results are presented in Figure 5. The hemolysin formation was little affected at variable agitation speeds, and its optimal formation was recorded at 0 rpm (static) for *Alcaligenes faecalis* strains OS41 and OS117. 200 rpm was recorded to be the most suitable for *Alcaligenes faecalis* strains OS61.

3.6 Influence of growth media on hemolysin formation

The impact of growth media composition on the synthesis of hemolysin by *Alcaligenes faecalis* strains was studied, and the results are presented in Figure 6. *Alcaligenes faecalis* strains OS42 and OS117 preferred brain heart infusion broth more than other substrates for hemolysin formation with 19.13% and 99.89%, respectively. On the other hand, the nutrient broth was most suitable for hemolysin for *Alcaligenes faecalis* strains OS61 (112.38%).

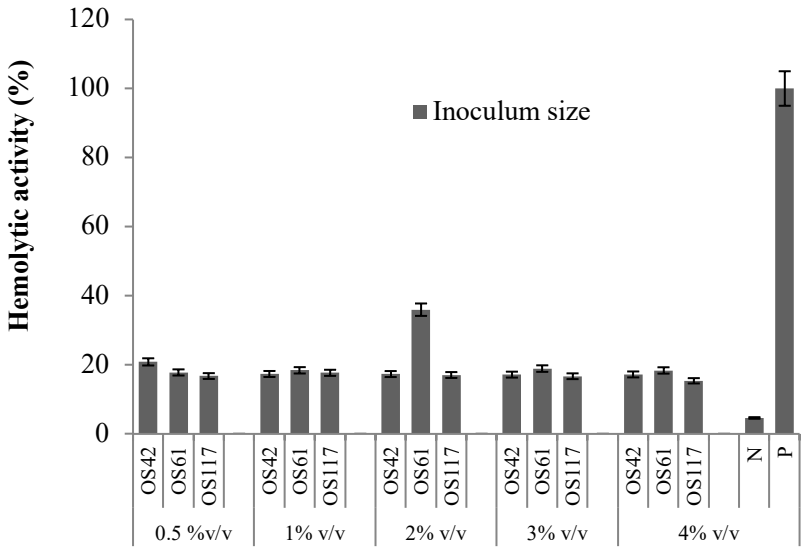

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Fig. 2. Influence of bacterial inoculum size on hemolysin formation; N = negative control, P = positive control

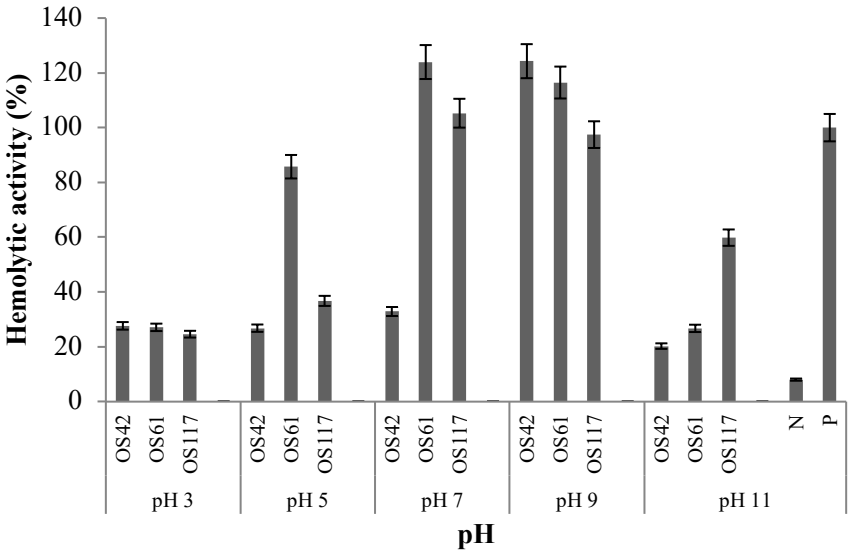

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Fig. 3. Influence of pH on hemolysin formation; N = negative control, P = positive control

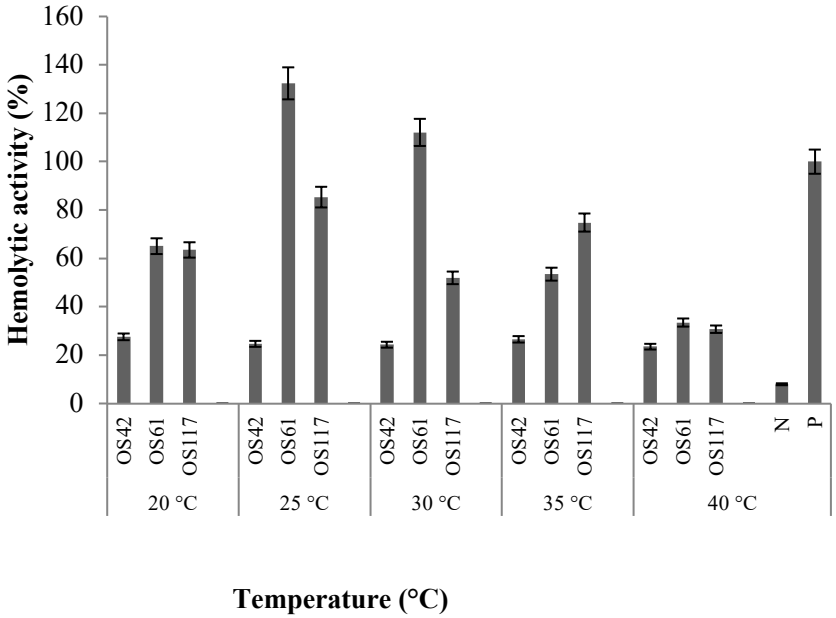

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Fig. 4. Influence of temperature (°C) on hemolysin formation; N = negative control, P = positive control

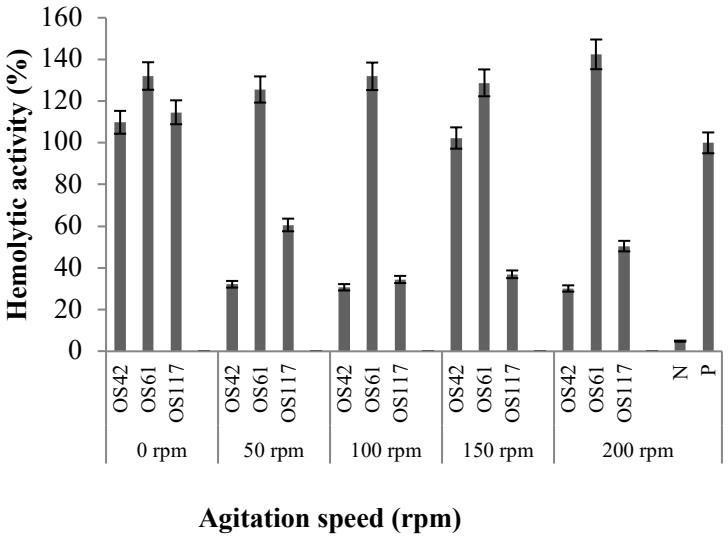

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Fig. 5. Influence of agitation speed (rpm) on hemolysin formation; N = negative control, P = positive control

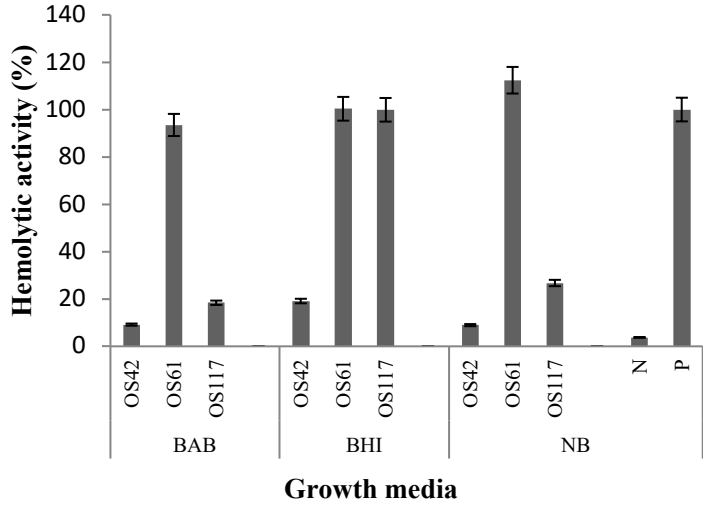

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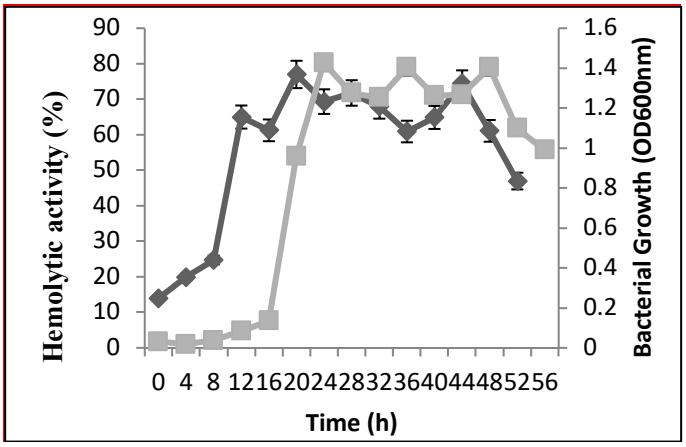
Fig. 6. Effect of growth media on hemolysin production; N = negative control; P = positive control; BAB = Blood agar broth; BHI = Brain heart infusion broth; NB = Nutrient broth

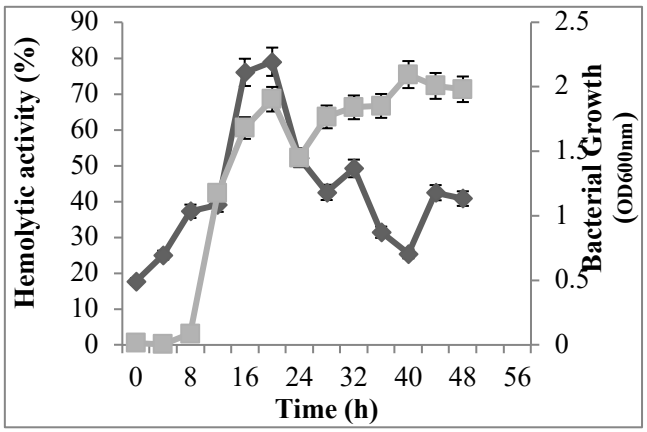
3.7 Examination of the relationship between bacterial growth and hemolysin production

The time progression of hemolysin synthesis and growing line of tested *Alcaligenes* strains was observed in Figure 7. All the bacterial isolates were cultured at optimum conditions and monitored for growth and hemolysin synthesis for 56 h. *Alcaligenes faecalis* strain OS42 and *Alcaligenes faecalis* strain OS61 reached optimum hemolysin production at 20 h with 77% and 79% hemolytic activity, respectively, which corresponded with the early logarithmic growth of the strains. Meanwhile, optimal hemolysin production was achieved at 36 h by *Alcaligenes faecalis* strain OS117 with 85.3% hemolytic activity. Double peaks of hemolytic activity were observed in *Alcaligenes faecalis* OS42 at 20 h and 44 h of incubation and in *Alcaligenes faecalis* OS117 at 28 h and 36 h. Growth and hemolysin production by *Alcaligenes faecalis* OS42 increased until 44 h,

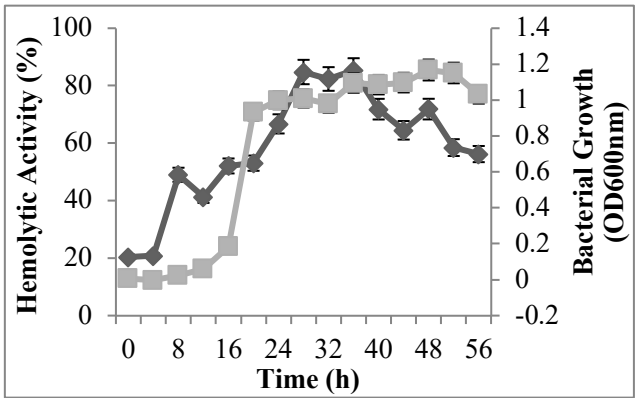
when a reduction in hemolysin synthesis was observed. Contrarily, there was a sharp decline in hemolysin production by *Alcaligenes faecalis* OS61 after it reached its peak at 20 h, although the bacterial growth continued. Hemolysin production by *Alcaligenes faecalis* OS117 increased as the incubation time increased until 48 h, and after that, the production rate decreased with time.



(a)



(b)



(c)

Error bars: ± 1SE

Fig. 7. Relationship between bacterial growth and hemolysin formation by (a) *Alcaligenes faecalis* strain OS42, (b) *Alcaligenes faecalis* strain OS61, and (c) *Alcaligenes faecalis* strain OS117; time versus biomass (dotted line), time versus hemolytic activity (straight).

3.8 Effects of various treatments on crude hemolysin

The effects of different treatments on hemolysin activity by *Alcaligenes* strains were investigated, and the results are shown in Table 1. The treatment substances used were 10 µg/ml cholesterol, 5 mM $AlCl_3$, 0.1 mM of EDTA, 5 mM of $MgCl_2$, 5 mM of NaCl, 5 mM of $CaCl_2$and 5 mM of $(NH_4)_2SO_4$. These treatments had variable effects on the hemolytic activity; 5 mM. $AlCl_3$ had the highest inhibitory effect (65.3%) on the hemolytic activity of the crude hemolysin. The hemolytic activity of the untreated hemolysin served as the positive control.

Table 1. Effect of different treatments on crude hemolysin

Treatment reagents	**Hemolytic activity (% ± S.D)**
10 µg/ml Cholesterol	71.23 ± 0.12
5 mM AlC_3	65.30 ± 0.08
0.1 mM EDTA	81.28 ± 0.16
5 mM $MgCl_2$	81.96 ± 0.55
5 mM NaCl	80.37 ± 0.18
5 mM $CaCl_2$	77.85 ± 0.34
5 mM $(NH_4)_2SO_4$	79.91 ± 0.42
Untreated	100 ± 0.26

Key: SD = standard deviation; AlC_3= aluminium chloride, EDTA = ethylene diamine tetraacetic acid, $MgCl_2$= magnesium chloride, NaCl = sodium chloride, $CaCl_2$ = calcium chloride, $(NH_4)_2SO_4$ = ammonium sulphate.

4. Discussion

For survival, microorganisms tend to adapt to environmental conditions, and for their growth and viability, they develop mechanisms to utilize available substrates. One of the mechanisms is the production of hemolysin by the microorganisms, which is associated with untreated abattoir wastewater, containing 32.5% of blood (Aniebo *et al.,* 2011). Hemolysin plays a significant role in controlling infectious diseases (Zhang *et al.,* 2005; Rahman *et al.,* 2010), and it functions as a toxin (Sato *et al.,* 2012). Therefore, it is used as a molecular marker for pathogenicity, primarily in clinical isolates. The hemolytic activities in the environmental microorganisms are steadily being documented (Rajesh *et al.,* 2013). The present study, using PCR, demonstrates the optimization of hemolysin formation by *A. faecalis* strains isolated from a significant city abattoir wastewater plant in Akure, Nigeria. The evolutionary analyses (Figure 1) showed that the strains OS61 and OS117 were more closely related compared with *A. faecalis* strain OS42.

The genus *Alcaligenes* has long been known as a contrivance of several metabolites as well as hydrolytic enzymes (Bharali *et al.,* 2011; Naeem *et al.,* 2017), but its hemolysin synthesis has only been connected to the biosurfactant formation, but petite reports have appeared on the optimization and characterization of this enzyme. Furthermore, it is known that the appearance of enzymes by microorganisms is controlled by multiple variables (Fatokun *et al.,* 2016). Therefore,

to obtain a prime enzymatic activity, it is essential to validate and measure the impact of such variables.

The size of the inoculum is a vital variable that determines microbial growth. We observed optimal hemolysin formation with the most petite sizes of the inoculum among an array of inoculum percentage volumes; *A. feacalis* strains OS42, OS61, and OS117 produced hemolysin optimally at 0.5 %v/v, 2 %v/v, and 1 %v/v inoculum sizes. This is supported by Jaapar *et al.* (2011), who have shown that a high substrate to cell ratio could lead to a prolonged growth phase, extending the period of metabolite production. The long period of metabolite production indicates a higher yield of the metabolite. In addition, more culture densities may reduce extracellular substance synthesis because the cell replication may have used up most of the nutrients leaving behind a minuscule amount of nutrients for cellular demands (Nwodo *et al.,* 2013).

pH is a part of the critical variables that determine the best enzymatic activities. Most enzyme activities and movements through the cell membrane are subjective to the original pH of the growth media (Gupta & Kar, 2008; Ahmad *et al.,* 2017; Yusuf *et al.,* 2019). Olson (1993) has reported that the metabolic activities of microorganisms, the production of genes, and their expression are pH-dependent as a reaction to their outer milieu. In addition, the bacterial protein syntheses and their processes are tuned in line with both the inner and outer pH of a system. The original pH of the production medium regulates the oxidation-reduction potential in the cells affecting nutrient absorption and enzyme production. In this study, optimal hemolysin synthesis was observed at pH 7.0 in *A. faecalis* strains OS61 and OS117, as well as at pH 9.0 for *A. faecalis* OS42. In contrast, Sato *et al.* (Sato *et al.,* 2012) reported pH 6.0 was suitable for hemolysin formation in *Prevotella oris*. Funk *et al.* (1996) concurred with our results in which maximum hemolytic activity was found within the pH range of 5 - 9. Considerable differences in the optimal pH of hemolysin synthesized by different bacterial isolates have been reported (Takada *et al.,* 2003). This variable optimal initial pH for hemolysin formation in other bacterial species may be because of the variations in media composition and microbial and sample differences (Shu and Lung, 2004).

The bacterial incubation temperature is the crucial player for prime enzyme synthesis, and this may be due to the microbial protein modification influenced by differential incubation temperatures (Juturu and Wu, 2014). Ray *et al.* (2007) have reported that when incubation temperature is not optimal, metabolic turnovers are lower, resulting in growth retardation and reduced enzyme production and activity. In the present study, optimal hemolysin production was recorded at 25 ℃ for *A. faecalis* strains OS61 and OS117. This is not in agreement with Hang'ombe *et al.* (2006), who have reported the optimal temperature for hemolysin production by *Clostridium chauvoei* at 10 ℃ and a significant reduction of over 80% productivity at 30 ℃. Disparities in the incubation temperature for the enzyme production may be accredited to different strains among microbial species and compliance with the temperature range and the environmental growth conditions of the bacteria (Techapun *et al.,* 2002).

The optimal agitation speed for hemolysin synthesis varied among the studied bacterial strains. The observed high agitation speed (200 rpm) for hemolysin production by *A. faecalis* strain OS61 may result from the maximum oxygen transfer in the growth medium. It is known that the agitation speed influences the aeration level of the bacterial cells. It determines the dissolved

oxygen level required for the respiration of the bacterial cells and subsequently affects enzyme production in batch fermentation (Jang and Chang, 2005). However, other studies have highlighted that a high agitation speed may lead to solid shear stress and adversely affect growth (Balan *et al.,* 2013; Xia *et al.,* 2014). This is in line with our observation of *A. faecalis* strains OS42 and OS117.

The different growth media compositions significantly impacted hemolysin synthesis and conservation of activity. The studied bacterial strains showed optimal hemolysin production in various growth media. Hang'ombe *et al.* (2006) have reported an unclarity of variation in hemolysin activity associated with different growth media. This may be due to a specific protein carrier requirement by individual microorganisms. Marchlewicz and Duncan (1982) have shown that Group B streptococcal hemolysin needed the availability of particular proteins as carriers for conservation of its activity. An induction with streptolysin S may happen during the availability of various proteins (Zhu *et al.,* 2017). Funk *et al*. (1996) have also reported that the lack of specific proteins may result in reduced transporters of hemolysin, justifying the absence of hemolysin synthesis indefinite media.

The bacterial growth kinetics have suggested that hemolysin is synthesized during the logarithmic and primary stationary growth phase (Sato *et al.,* 2012). Higher hemolytic activity has been recorded from the crude hemolysin obtained from the early logarithmic growth phase than the crude hemolysin obtained from the stationary phase. The same pattern of hemolysin synthesis has been revealed by *Aggregatibacter actinomycetemcomitans* (Kimizuka *et al.,* 1996), *Prevotella intermedia* (Beem *et al.,* 1998), *Porphyromonas gingivalis* (Deshpande & Khan, 1999), *Prevotella nigrescens* (Silva *et al.,* 2004) and *Prevotella oris* (Sato *et al.,* 2012). In our study, the reduced hemolysin synthesis at the late logarithm phase of incubation may be due to many factors, such as nutrient exhaustion and proteolysis. (Papagianni and Moor-Young, 2002).

Crude hemolysin from the bacterial strains was treated with cholesterol and EDTA. The hemolytic activity did not enhance by any of these treatments. Funk *et al.* (1996) has also reported a non-impact of cholesterol on the hemolytic activity of hemolysins from *Actinomyces pyogenes*. This contrasts with Alouf (1980) and Jacobs *et al.* (1994), who have reported a significant effect of cholesterol on hemolysins from *Listeria monocytogenes* and *Streptococcus suis.* Former studies have revealed that hemolysin complexes with cholesterol result in a decline in cytolytic activity (Prigent and Alouf, 1976). Sato *et al*. (2012) has suggested that the variance in the effect of cholesterol on different hemolysins may be due to specific differences in the membrane damage caused by individual hemolysin produced. Additional studies are needed to study the mechanism of the formation of hemolysins in the abattoir wastewater of Nigeria.

5. Conclusion

Our results suggest the occurrence of hemolytic *Alcaligenes* species in the final wastewater effluent of Onyearugbulem abattoir in Akure, Nigeria. Our study has provided critical information on the best conditions for hemolysin synthesis in *A. feacalis* strains by assessing their hemolytic activity using variable cultivation parameters. The traditional optimization for hemolysin formation in *A. feacalis* strain OS117 resulted in 85.31% activity compared with 100% lysis with Triton-X 100. In *A. feacalis* strain, OS117 hemolysin formation is supported at neutral pH, 25 ˚C,

in stationary cultures, in the early logarithmic phase using brain heart infusion broth by 1% v/v inoculum. Further studies about purification, mechanisms of action, and the molecular structure of hemolysins formed in *Alcaligenes* species are required.

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