

Detection, Optimization, Characterization, and Cytotoxic Effect of Vaginolysin Extracted from *Gardnerella vaginalis* Isolated From Iraqi Women Who had Abortions

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Abstract

Objective: The goal of this study is the detection of the gene responsible for production of the vaginolysin toxin, discovery of the optimal condition for production of vaginolysin, purification and characterization of the toxin and showing the cytotoxic effect of vaginolysin on VK2 cell and HeLa cell.

Methods: Among 592 specimens isolated from pregnant women suffering from vaginal inflammation and threatened with abortion (these women are suffering from strong pain in the cervical region), only 62 isolates were identified as *G. vaginalis*. From Baghdad teaching hospital, detection of the gene responsible for production of the vaginolysin toxin. Study of the optimal condition to produce vaginolysin. The purification of the toxin by using ammonium sulfate, ion exchange chromatography and gel filtration, toxin characterization of pH and temperature activity and stability, the study of cytotoxic effect of vaginolysin on VK2 and HeLa cells.

Result: The optimal condition to produce the toxin is pH level 4, best temperature is 37 °C, the best media is TSB broth, and the best hours is 24 hr. The best range for precipitation saturation ratio is 50%. Ion exchanges have specific activity 4 mg. gel filtrations show an increase in the specific activity of purified toxin (10 U/mg). The characterization done by pH and temperature, Vaginolysin was active in 0.5 U/mL at pH 7, and stable in pH 7 activity, the best temperature for vaginolysin activity is 0.5 U/mL at 37°C and maximum stability was recorded at 37°C. The cytotoxic effect of the toxin on VK2 cell observed the viability ratio 69.98%, at higher toxin concentration, while the result for HeLa cell viability ratio 38.77%, at higher toxin concentration.

Conclusion: Vaginolysin causes lysis of vaginal cell and cervical cell, which may lead to abortion, the killing ratio increases by increasing the concentration of the toxin and the effect of the toxin on HeLa cell is more than on VK2 cell.

Keywords: *G. vaginalis*, abortion, vaginolysin toxin, CDCs toxin, gram positive

Introduction

A variety of other mostly anaerobic bacteria, including *G. vaginalis*, a facultatively anaerobic Gram-variable rod, are linked to bacterial vaginosis in some women. The facultative anaerobic Lactobacillus colony in the vagina is in charge of producing the acidic environment. Prescription antibiotics with anaerobic coverage may be necessary to maintain the ecosystem's equilibrium once the anaerobes have supplanted the common vaginal bacteria. Rather than being the source of bacterial vaginosis, *G. vaginalis* is believed to be a signal organism of the altered microbial ecology connected to the overgrowth of many bacterial species.¹

G. vaginalis has an extremely complex metabolism. *Gardnerella vaginalis* can metabolize glucose and other simple carbohydrates in both aerobic and anaerobic settings because of their facultative anaerobic nature. In both anaerobic and aerobic glucose metabolism scenarios.²

G. vaginalis yields Vaginolysin, a cholesterol-dependent hemolysin (CDC) that lyses susceptible cells when it interacts with the CD59 molecule. This causes the epithelial cells in the vagina and cervical regions to undergo rapid structural changes, or "blebbing," by creating pores in the cells, which may result in abortion.³ Human erythrocyte hemolysis is induced by vaginolysin; nevertheless, Gardnerella-caused bacteremia is an uncommon occurrence and is not a characteristic of bacterial vaginosis.⁴

Compared to non-BV isolates, BV-associated Gardnerella species caused more cytotoxicity in cervical HeLa cells, which was coupled with higher production of vaginolysin.⁵ Even though BV-Gardnerella isolates also generally had lower levels of vaginolysin expression, this difference was not statistically significant. In people with BV, specific immune responses to Gardnerella vaginolysin have been seen.⁶

After adhering to the host cell initially, the invasive bacteria grow and might create a biofilm community to help them survive in the future.⁷ *G. vaginalis* must produce biofilm in order to survive in the vagina. Some strains of *G. vaginalis* produce sialidase, which may increase biofilm development due to its mucinase activity.⁸ In vitro biofilms of *G. vaginalis* have been shown to be more resistant to lactic acid and hydrogen peroxide generated by lactobacilli than planktonic forms of the bacteria.⁹

Materials and Methods

Identification of *G. vaginalis*

One solitary colony was transferred, well-fixed, and stained with Gram stain on a microscope slide. After observing the Gram reaction, the cell's form, and organization, bacterial colonies were moved to blood agar and Colombian agar in an aerobic environment (anaerobic jar) for

routine microbiological culture studies. The shape, color, size, opacity, and border of colonies on solid media were examined.¹⁰ Additionally, the VITEK system performs the biochemical test (oxidase, catalase, and whiff test) at the final conference identification.¹¹

Identification of Vaginolysin by Real Time PCR

The VLY genomic region from *G. vaginalis* was amplified from the *G. vaginalis* 14018 strain using PCR. The amplification was done with Pfx proofreading polymerase (Invitrogen) and the primers R (5' ACA TAA GCT TGG CCA CGG TC 3') and F (5' GCC CTT GAA GAA AGA CAG CC 3'), as shown in reference.¹² The predicted VLY open reading frame was then amplified by PCR using the cloned genomic region as a template.

DNA was extracted using 1 mL aliquots of liquids following the removal of the transport swabs. Endometrial curettings were suspended in 1.5 milliliters of sterile physiological saline, and 1 milliliter was removed for DNA analysis. The remaining samples were frozen and stored at -80°C . DNA was extracted using the QiAMP Mini DNA extraction kit (Qiagen, Australia) in accordance with the manufacturer's instructions. An enzymatic lysis step was added beforehand, and the product was eluted in 50 μL of sterile water.¹³

Optimum Conditions for Vaginolysin Toxin Production

For vaginolysin produced by selected isolate were determined by inoculating 100 ml of medium with 1.0 ml of fresh culture (OD = 0.6) of the bacterial isolate and incubated for 24 h at 37°C . Then, toxin activity and protein concentration in crude extract were estimated.¹⁴

Optimum cultural medium

Read the OD at 595 nm after adding 0.1 ml of new culture at 0.6 optical density to 100 ml of medium that has been produced, such as pepton water broth, TSB broth, neutrinant broth, and brain heart broth. The medium is then incubated for 24 hours at 37°C .

Optimum pH

The production medium's pH was adjusted to several values (3, 4, 5, 6 and 7) in order to establish the ideal pH for vaginolysin production. The medium was then incubated for 24 hours at 37°C . It was established what the toxin activity, protein concentration, and specific activity were.

Optimum temperature

The chosen *G vaginalis* was used to inoculate the production medium, which was then incubated at various temperatures (25, 30, 35, 37, 40, and 45°C).

Optimum incubation period

By incubating the production medium for varying lengths of time (16, 24, 48, and 72 hours), the impact of incubation period on vaginolysin production by *G vaginalis* was investigated in order to identify the ideal time for toxin synthesis.

Vaginolysin Purification

Three steps were used in this study for the purification of vaginolysin produced from *G vaginalis*:

Crude Extraction of Vaginolysin Toxin

The chosen isolate of *G. vaginalis* was cultured for 36 hours at 37°C in an anaerobic jar fitted with an aeroculture reagent (tween 80) in tryptot-casein-soya broth (TSB broth) supplemented with 0.4% *G. vaginalis* selective supplement and 2% human plasma. The culture was routinely subcultured every 36 hours. To generate the toxin, 150 ml flasks containing 125 ml of TSB/HP, 0.1% starch, and 0.3% nonionic detergent (Tween 80) were inoculated with 107 bacteria. before harvesting, the cells were cultured anaerobically for 36 hours at 37°C . The CFU was then counted on Columbia blood agar base, which contained 0.4% *G. vaginalis* selective supplement and 5% human defibrinated blood. Anaerobic cultures were cultured at 37°C . Crude filtrate was recovered by centrifuging the cultures for 20 minutes at 4°C to obtain cell-free supernatant was used as a crude toxin activity.¹⁵

Hemolytic Assay for Measurement the Toxin Activity

The assay combination included the necessary volume of sample, 50 ul of a 1% human blood solution, and 1 ml final volume of potassium phosphate buffer (pH 7). For 30 minutes, the tubes were incubated at 37°C . An A415 of 0.90 upon full hemolysis was used to measure the A415 of the supernatant fluid containing the hemoglobin released from lysed erythrocytes following centrifugation (1,200 x g for 3 min).¹⁵

Precipitation of Toxin by Ammonium Sulfate

The crude toxin was mixed with varying weights of ammonium sulfate at graduated (%) saturation ratios of 50, 60, 70, and 80%. The mixture was then gently swirled on a magnetic stirrer while cooling for a duration of 20 minutes. Following each stage of ammonium precipitation, the protein precipitate was dissolved in an appropriate volume of 0.5M Tris-HCl buffer at pH 9, prepared in toxin activity, protein concentration, and specific activity of toxin solution. It was then centrifuged at 10,000 rpm for 10 min. at 4°C , while the supernatant was separated and mixed with ammonium sulfate to obtain the next saturation ratio.¹⁶

Dialysis is carried out by A dialysis clip was used to seal one end of the dialysis tubing after a 10-cm section of the bag (3KDa) was cut off and washed with distilled water. Because the volume expanded during dialysis, some space was left in the dialysis tube after the purified vaginolysin was poured in.¹⁷

Purification of Vaginolysin by Ion Exchange Chromatography¹⁸

Twenty-five grams of DEAE-cellulose were suspended in one thousand milliliters of distilled water and allowed to precipitate for a while before the exchanger DEAE cellulose was

produced and packed onto a column according to Whitaker's (1972) procedure. After removing the floating flakes from the solution until the top layer was clear, a vacuum pump was used to filter the mixture. The precipitate was placed in a 250 ml solution (0.25 M NaCl, 0.25 M NaOH) that had been filtered, repeatedly rinsed with distilled water, and then rinsed again with 250 ml of 0.25 M HCl. After the column was mobilized in an ionic exchanger DEAE cellulose to a size of 2.5 by 20 cm, phosphate buffer solution was used to balance it. After that, the column was cleaned using the same amount of buffer, while the attached proteins were stepwise eluted with gradual concentrations of sodium chloride (0.1-1 M). A UV-VIS spectrophotometer was used to measure each fraction's absorbance at 280 nm while maintaining a 30 ml/h flow rate throughout the column. Each fraction's enzyme activity was measured.

Purification of Vaginolysin by Gel Filtration Chromatography

The Pharmacia Fine Chemicals Company's recommendation was followed for preparing Sephadex G-100. After a certain amount of Sephadex G-100 was suspended in 0.05 M phosphate buffer pH 7, it was heated to 90°C for five hours to guarantee the beads swelled, then it was degassed, packed, and allowed to equilibrate using the same buffer. Using the same buffer for equilibration, the concentrated sample from the preceding stage was added to the column to accomplish elution at a flow rate of 30 mL/hr. At 280 nm, the absorbance of every fraction was calculated. Each fraction's toxin activity was also ascertained.¹²

Determination of Toxin Concentration

Protein concentration was determined according to the method of Bradford (1979) using bovine serum albumin standard curve.¹⁹

Vaginolysin Characterization

Effect of pH on Vaginolysin Activity

The assay was performed by pre-incubating 1ml of purified toxin on 0.05 M phosphate buffer (pH 5, 6, 7, 8 and 9) at 37°C for 30 min the toxin activity

Effect of pH on Vaginolysin Stability

By adding equal volumes of purified toxin to test tubes containing buffer at different pH levels (5, 6, 7, 8, and 9) prepared as and incubating for 20 minutes at 37°C in a water bath, the effect of pH on vaginolysin stability was investigated. The activity with different buffers was measured according to the remaining activity (%) for vaginolysin.

Effect of Temperature on Vaginolysin Activity

measuring the activity of toxin at different temperatures (30, 35, 40 and 45)°C. for 30 min and the activity was determined.

Effect of Temperature on Toxin Stability

For thermal stability, equal volumes of purified vaginolysin were incubated in the water bath at 32, 35, 40 and 45 for 20 min and the activity was determined according to the remaining activity (%).¹⁵

The Cytotoxic Effect of vaginolysin on VK2 cell and HeLa cell

Preparation of Culture Media use in Cytotoxicity of Vaginolysin

RPML-1640 medium

A 1.6 gram powder of Media RPMI-1640 was added. 10 milliliters of 10% fetal bovine serum (FBS) was mixed with 100 micrograms of penicillin and 100 micrograms of streptomycin, as well as powdered sodium bicarbonate. 2.2 g in 1 ml/L, the volume was increased to 1 L using TDW, and the medium was sterilised according to the manufacturing company's recommendation using a Nalgene filter with a 0.22 filter unit.²⁰

MTT Protocol

The cytotoxic effect of Cuprizone 50mM with presence different concentrations from levetiracetam was performed by using MTT ready to use kit.²⁰

Around (1x10⁴ to 1x10⁶ cells/ml) of HeLa or VK2 cells were grown in 96 wells culture plates containing 200 ml/well growth culture medium. The plate was covered by sterile parafilm and Shaked gently, then incubated at optimum cell line culture conditions (37°C & 5% CO₂) for 24 hr. On the next day, the culture medium was removed and two folds serial dilutions of the Levetiracetam (200, 100, 50, 10, 2.5 & 0.5 M/ml) were added to the wells. Triplicate wells were used per each concentration as well as the controls (serum free medium treated cells). Plates were incubated at the optimum mammalian cell lines culture conditions at 37°C and 5%CO₂ for 4hr. At the end of the incubation period, 50 mM/ml of cuprizone was add to each well for 24 hr, after which, 10ml of the MTT solution was added and the mixtures were further incubated at optimum culture conditions for another 4 hr. Medium from each well was carefully removed and 100 ml/well of solubilization solution was added and incubated at room temperature for 5 min till complete solubilization. Optical densities of different wells were measured at 575 nm and statistically analyzed to calculate the concentration of toxin that causes 50% reduction in VK2 or HeLa cells viabilities, using the following equation: $Y = D + A - D / 1 + 10^{(x - \log C)} B$.

Results

Isolation and Identification of G vaginalis from Pregnant Women Have Abortion

In the current study, five hundred ninety two specimen were collected from pregnant women suffering from vaginal inflammation and threatened with abortion (these women is suffering from strong pain in the cervical region) in Baghdad teaching Hospital, al. Only 62 confirmed isolates

belong to *G vaginalis*. The cotton swabs were cultured on Columbia agar with 5% blood, blood agar medium, under aerobic conditions at 37 °C and incubated for 24–48 hours.

Cultural Characteristic

whereas on Columbia agar light grey -transparent colonies small, round, with regular edges as show on figure, the bacteria cultured on Columbia agar with 5% blood, 15% nalidixic acid and 10% collistin.²¹

Detection the Ability of *G vaginalis* to Produce Vaginolysin Toxin²²

In our study, 90% of *G vaginalis* isolate have vly gene detected by real time PCR. DNA Extraction from pure cultures was performed using the Qiagen DNA easy tissue kit (Qiagen, Valencia, CA, USA) according to the manufacturer’s instructions, using logarithmic growth phase cells in Todd Hewitt broth (THB, Difco).

Optimization of Vaginolysin Toxin Production

The optimal conditions for vaginolysin toxin production, including pH, temperature, medium, and incubation period, are detailed in reference.²³ These factors determine the best conditions for Gardnerella vaginalis to produce the vaginolysin toxin. The result show that the perfect pH level is 4 with specific activity 0.6 U/mg as shown in (Figure 1) while the lower specific activity pH 7 is 0.3 U/mg.

The results in Figure 2 showed that the optimal temperature for producing the toxin is 37°C, with a specific activity of 0.65 U/mg, while the specific activity is lower at 45°C.

The results show that the optimal medium for producing the toxin is TSB broth, with a specific activity of 0.51 U/mg. In comparison, the specific activity is lower in nutrient broth and brain heart infusion broth, with values of 0.2 U/mg. These findings are illustrated in Figure 3.

The results in Figure 4 indicate that the optimal time to produce the toxin is 24 hours, achieving a specific activity of 0.73 U/mg. In contrast, the lower specific activity is 0.5 U/mg.

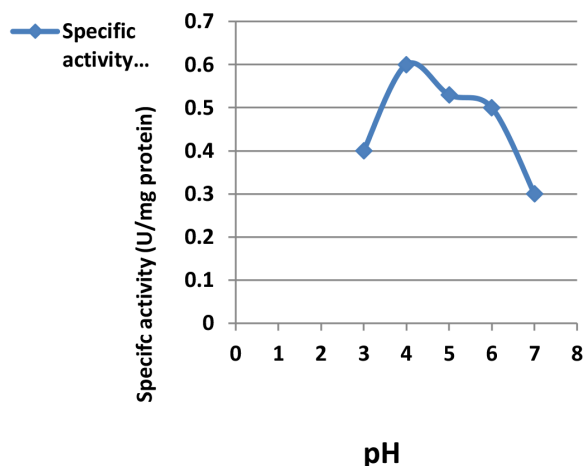


Fig. 1 The optimal pH to produced vaginolysin from *G vaginalis*.

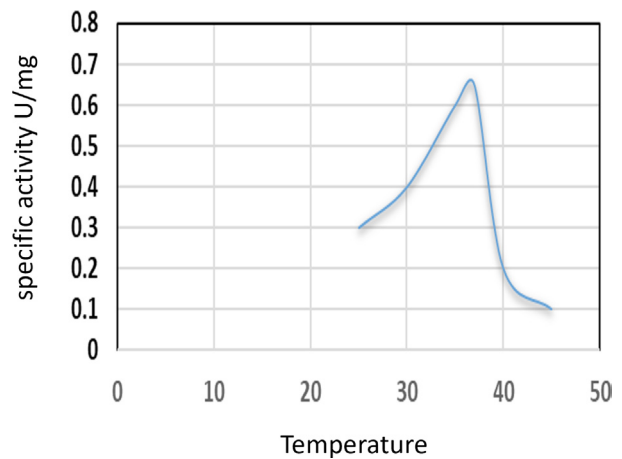


Fig. 2 The optimal temperature to produced vaginolysin from *G vaginalis*.

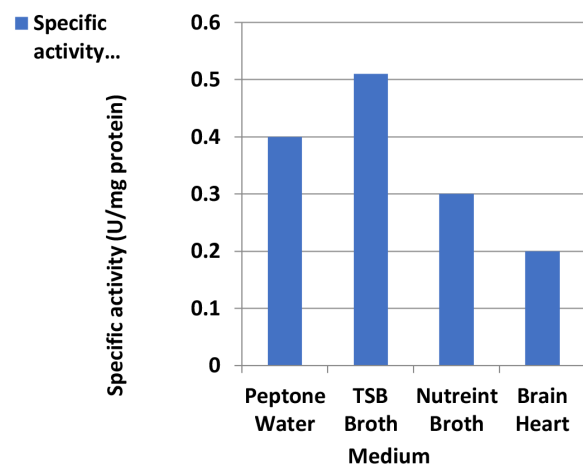


Fig. 3 The optimal cultural media to produced vaginolysin from *G vaginalis*.

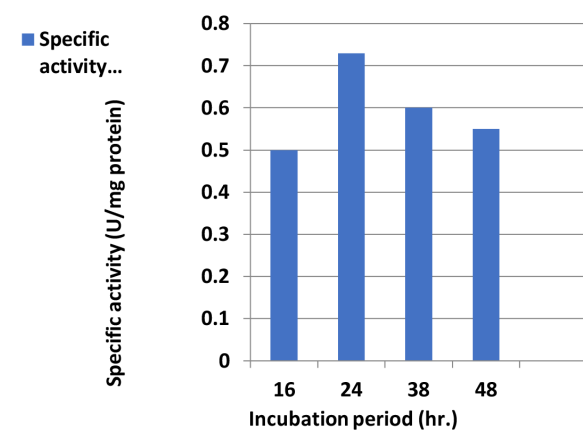


Fig. 4 Optimum incubation period to produce vaginolysin.

Purification of Vaginolysin From *G vaginalis* Isolate

Preparation of Vaginolysin Crude

The vaginolysin toxin was extracted from *G vaginalis* after incubating it in 125 ml tryptot -casein-soya broth (TSB broth)

Table 1. Purification steps for vaginolysin produced by *G vaginalis*

Purification step	Volume (ml)	Toxin activity (U/ml)	Protein concentration (mg/ml)	Specific activity (U/mg)	Total activity (U)	Purification (folds)	Yield (%)
Crude enzyme	75	0.3	0.4	0.75	22.5	1	100
Ammonium sulphate precipitation 50 %	13	0.9	0.5	1.8	11.7	2.4	52
DEAE-cellulose	24	0.4	0.1	4	9.6	5.3	42.6
Sephadex- G150	18	0.5	0.05	10	9	13.3	40

with 2% human plasma and 0.4%, 0.1% starch, and 0.3% non-ionic detergent (Tween 80). The inoculated medium was then incubated anaerobically for 36 h at 37°C and washed with (0.05 M) sodium phosphate buffer (pH 7.0) broke the cell wall of bacteria after Centrifugation was used to separate the cells for 15 minutes at 10000 rpm and, the toxin activity in crude supernatant was estimated. The toxin activity was 0.3 U/ml, and specific activity 0.75 U/mg toxin, as shown in (Table 1).¹⁵

Ammonium Sulfate Precipitation

The findings demonstrated that when ammonium sulfate was utilized at different saturation rates (40, 50, 60, 70, 80, and 90%), the optimal range for toxin precipitation was at the saturation ratio of 50%. The toxin specific activity was found to be 1.8 IU/mg protein, and its purification fold reached 2.4 with a 52% overall yield, as per the results shown in Table 1.²⁴

Ion Exchange Chromatography (DEAE-Cellulose)

Tubs 43–55 exhibited the highest vaginolysin activity, with only one peak among the gradient elution peaks corresponding to 0.5 M of NaCl. As demonstrated in Figure 5, this finding suggests that the vaginolysin generated by *G. vaginalis* has a negative net charge because it bound with the anionic ion exchange DEAE-cellulose.

Using a DEAE-cellulose exchanger purification fold, the fractions were pooled and evaluated for vaginolysin activity, resulting in a specific activity of 4 mg and a purification fold of 5.3 with an overall yield of 42.6%, as seen in Table 1.

Gel Filtration Chromatography

In this investigation, the specific activity of vaginolysin in peak fractionation tubes 13–18 was examined. The findings in Table 1 demonstrate an increase in the specific activity of the purified toxin (10 U/mg), with a purification fold of 13.3 and a vaginolysin yield of 40%.

The vaginolysin obtained from *G. vaginalis* was further purified and characterized using the Sephadex G-100 chromatography system. This was the third step in the process, following the precipitation of ammonium sulfate and the DEAE-cellulose, to produce a purified toxin with a specific activity of 1.457 U/mg and a fold 22%, as shown in Figure 6.¹⁵

Characterization of Purified vaginolysin

The Characterization of Purified vaginolysin includes study the effect of pH, temperature, on vaginolysin activity.

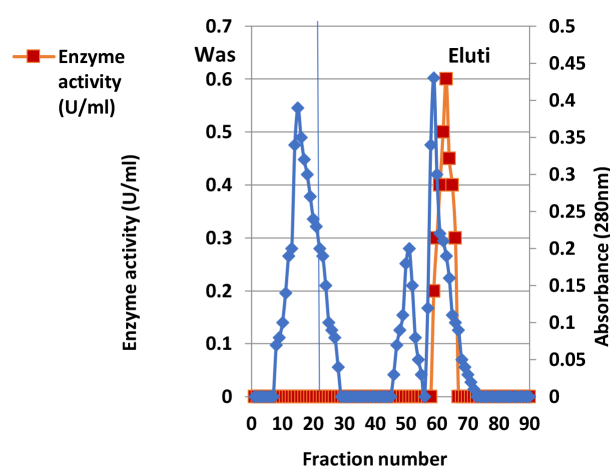


Fig. 5 Ion exchange chromatography of vaginolysin produced by local isolate *G vaginalis*.

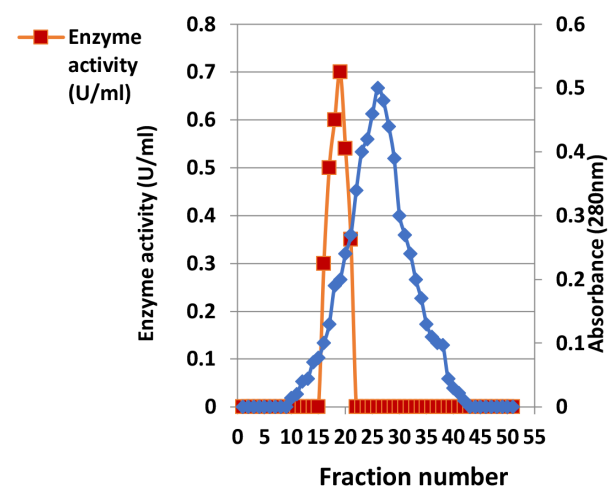


Fig. 6 Gel filtration chromatography of vaginolysin produced by locally isolated *G vaginalis*.

Effect of pH on Vaginolysin Activity

The purified vaginolysin was active over a wide pH range of 5 to 9, with an optimum of 0.5 U/mL at pH 7. At higher pH values, the toxin's activity was reduced, but it still retained 0.1 U/mL activity even at pH 9. This is shown in Figure 7.

Effect of Temperature on Vaginolysin Activity

The purified vaginolysin activity (Figure 8) was active over a wide range of temperature range of 32 to 45°C with an

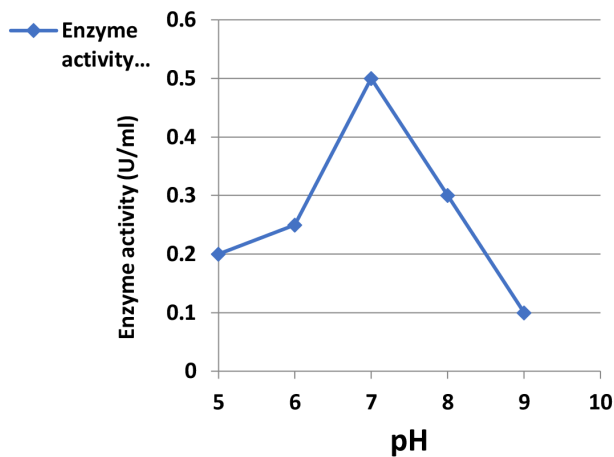


Fig. 7 Effect of pH on vaginolysin activity.

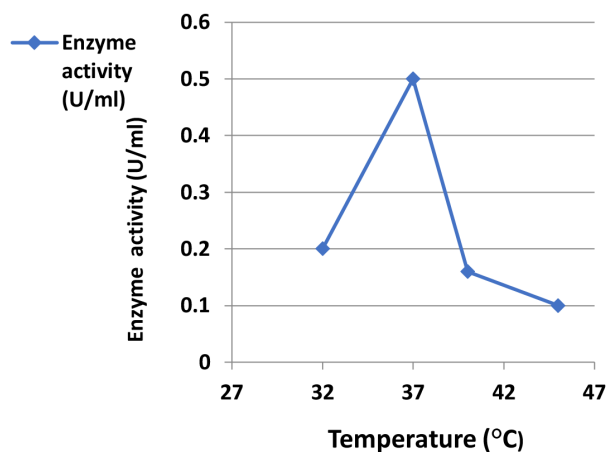


Fig. 8 Effect of Temperature on vaginolysin activity.

optimum vaginolysin activity of 0.5 U/mL at 37°C and lower vaginolysin activity observed at higher temperatures (45°C).

Effect of pH of Vaginolysin Stability

Results in Figure 9 displayed that purified vaginolysin was steady at pH 7 Because the toxin has its highest remaining activity (100%) after incubation for 6 h

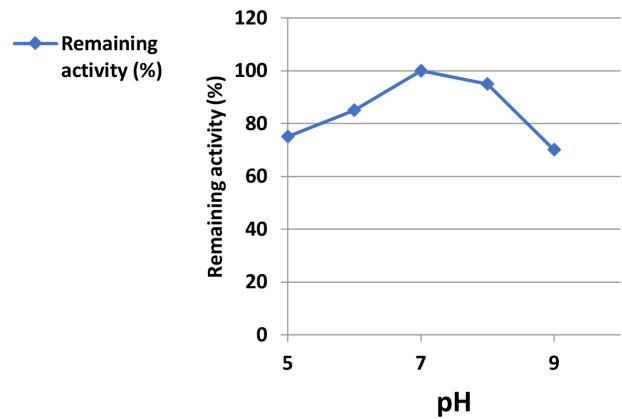


Fig. 9 Effect of pH on purified vaginolysin stability.

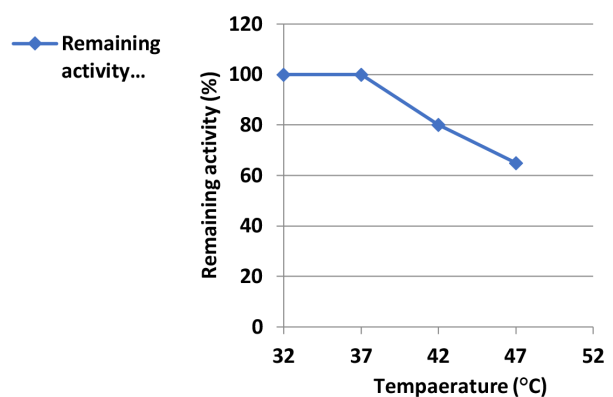


Fig. 10 Residual activity of purified vaginolysin at different temperatures.

Effect of Temperature of Vaginolysin Stability

After studying the effect of temperature on stability, the maximum vaginolysin stability was seen at 37°C (Figure 10), with 100% of the initial toxin activity being retained over a 20-minute incubation period. Toxin activity, however, was shown to rapidly diminish with residual activity of 80% and 65% after exposure to higher temperatures and longer incubation times of 42°C and 45°C for 90 minutes, respectively.

Cytotoxic Effect of Purified Vaginolysin

The results indicated that the cytotoxicity of the toxin with VK2 cells increased with concentration as well as with HeLa

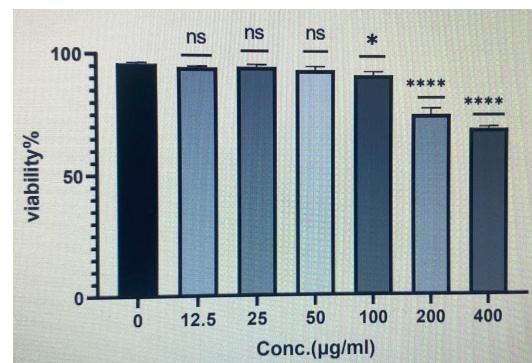


Fig. 11 Cytotoxic activity of purified vaginolysin produced by *G vaginalis* on VK2 cell line.

cells, the result for VK2 cell we observed that 69.98%, 75.96%, 92.12%, 94.25%, 95.44% and 95.02% of cell death occurred when 400, 200, 100, 50, 25 and, 12.5 µg/ml respectively show Figure 11, while the result for HeLa cell is 38.77% , 46.21%, 52.31%, 62.53%, 87.5 % and 95.17% of cell death occurred when 400, 200, 100, 50, 25 and ,12.5 µg/ml , as shown in Figure 12 and Table 2.

Discussion

Under a microscope, *Gardnella vaginalis* might seem to be either Gram-positive or Gram-negative despite having a

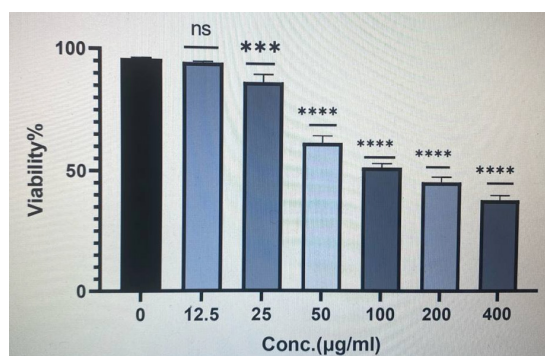


Fig. 12 Cytotoxic activity of purified vaginolysin produced by *G vaginalis* on HeLa cell line.

Table 2. Cytotoxic activity of purified vaginolysin on VK2 and HeLa cell line

Conc.(µg/ml)	VK2		HeLa	
	Mean	SD	Mean	SD
400	69.98	0.85	38.77	2.05
200	75.96	2.55	46.21	2.190
100	92.17	1.55	52.31	1.62
50	94.25	1.51	62.53	2.93
25	95.44	1.075	87.5	3.22
12.5	95.023	0.53	95.17	0.40
0	96.18	0.53	96.29	0.34

Gram-positive cell wall due to its thinness. Microscopically, clue cells—epithelial cells coated in bacteria—are linked to it.²⁵

G. vaginalis is thought to be a signal organism of the changed microbial ecology linked to the overgrowth of numerous bacterial species rather than the cause of bacterial vaginosis.²⁶

There has never been a study that shows the ideal conditions for producing vaginolysin. In contrast, my research shows that the ideal pH level for toxin production is 4, with a specific activity of 0.6 U/mg; the ideal temperature is 37°C, with a specific activity of 0.65 U/mg; the ideal media is TSB broth, with a specific activity of 0.51 U/mg; and the ideal hours are 24 hours, with a specific activity of 0.73 U/mg. In this study the best ratio for precipitating vaginolysin from *G vaginalis* was 50% saturation of ammonium sulfate Ammonium sulphate as well as in study done by G Rottini (1990).¹⁵

Study done by Ragaliauskas et al., (2019)²⁷ demonstrate that the VLY activity is marginal at acidic pH and peaks at neutral pH. This data agrees with mine, which showed that the toxin's action was highest at pH 7.

The fractions were pooled and tested for vaginolysin activity a specific activity of 4 mg, and that its purification fold was 5.3 with a 42.6% overall yield. While in study done by Giandomenico et al (1990)¹⁵ discovered on an ion exchange chromatography technique to purify negatively charged vaginolysin extracted from *G vaginalis* gave highly purified vaginolysin specific activity of 186 U/mg protein with a 33% over yield.

In this study I discovered that when the concentration of the toxin increase there is increase in killing the VK2 cell and HeLa cell. by the decrease of concentration of toxin will be decrease the killing of the available cell.

Conclusion

vaginolysin cause lysis of vaginal cell may lead to abortion, the killing ratio increase by increasing the concentration of the toxin and the effect of the toxin on HeLa cell is more than on VK2 cell.

Conflict of Interest

None. ■

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