IOSR Journal of Pharmacy
e-ISSN: 2250-3013, **p**-ISSN: 2319-4219, www.iosrphr.org
Vol. 2, Issue 6, Nov-Dec. 2012, PP.49-59

Optimization of temperature and pH forthe growth and bacteriocin production of *Enterococcus faecium. B3L3*

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Abstract :-**The knowledge of the optimum condition for growth of *Ent. Faecium*strain*B3L31 and* its bacteriocin production are important when deciding on mass production of both the organism and its bacteriocin for probiotic use and as an antimicrobial agent respectively.The optimum condition viz: temperature, pH and incubation period were used to determined parameters most suitable for growthandbacteriocin production from*Ent. faecium B3L3*. These three variables of optimization for growth and bacteriocin production were determined based on the growth curves. The bacteriocin production was done by growing strain of LAB onMRS broth the bacteriocin harvest was done following the chloroform precipitation. Resultobtained showed that maximum bacteriocin production occur at the tail end of the exponential phase. The most optimum pH and temperaturefor growth and bacteriocin production were found to be pH8 and37^{O}Crespectively. The partially purified protein when resolved on SDS-PAGE was shown to be of molecular weight 10kD. The bacteriocin also produced an inhibitory action against *methicillin resistance Staphylococcus aureus*.**

Keyword :-*Ent. faecium, temperature, pH, LAB, optimization*

I. INTRODUCTION

Bacteriocins are proteins or complexes proteins biologically active with antimicrobial action against other bacteria, principally closely related species.
They are produced by bacteria and are normally not termed antibiotics in order to avoid confusion and concern with therapeutic antibiotics, which can potentially illicit allergic reactions in humans and other medical problems [1].

Bacteriocins differ from most therapeutic antibiotics in being proteinaceous agents that are rapidly digested by proteases in the human digestive tract. They are ribosomally synthesized peptides, and this fact creates the possibility of improving their characteristics to enhance their activity and spectra of action [2].Antibiotics are generally considered to be secondary metabolites that are inhibitory substances in small concentration, excluding the inhibition caused by metabolic by-products like ammonia, organic acids, and hydrogen peroxide.

Bacteriocins have been reported to be inhibitory against several other bacteria [3; 4; 5 and 6]. The production of bacteriocinsdepend largely on the pH, source of nutrients and temperature[7]. Various physicochemical factors seemed to affect bacteriocin production as well as its activity. Maximum activity is normally noted at pH 6.0, temperature 30°C and 1.5% NaCl.There are contradictory statements as to the location of the encoding genes of enterococcus bacteriocins, while some authors believed that it is located in chromosomal DNA [8 and 9].[10]in their finding reported that enter, entB and entP genes are located on the genome DNA of *Enterococcus faecium* and *Enterococcus faecalis* isolates [11]; likewise, discovered a bacteriocin gene analogous to enterocin P from the total genomic DNA of *Ent. faecium* GM-1 by PCR and direct sequencing approaches. On the other hand, the structural genes for enterocin P (entP) through hybridization studies were located on the plasmid [12]. Moreover, [13] found from PCR-amplified fragments containing the structural genes for F-58 A and B were located in a 22-kb plasmid harbored by that strain.

Bacteriocins produced by enterococcus are known to be of the class II types of peptide and are plasmid encoded. The prevalence of enterococcus bacteriocinencodingtraits cannot be ascertain due to the limited data available since only one enterococcus genus was successfully sequenced completely and only one reputed bacteriocin gene was identified. It is assumptionally true that the frequency of occurrence of bacteriocinin Enterococcus and Streptococcus is more as compared to many other lactic acid bacteria, e.g.Lactococcus and Lactobacillus [14].

The presence of hydrophobic regions in bacteriocin molecules is essential for their activity against sensitive bacteria, since in theinactivationofmicroorganisms,bacteriocins depend on the hydrophobic interaction between the bacterial cells and bacteriocinmolecules [15].Somebacteriocins appear in their native state as

aggregates with high molecular mass (ca. 30–300kDa).These aggregates may mask partially or completely the antimicrobial activity of the bacteriocins during their purificationand alsoinduceerrors in the determination of their molecular weight. This is especially true with highly non-polar low-molecular-weight bacteriocins, which easily interact with extracellular materialoflysatecells (e.g., cell wall debris and micelles of lipotheicoic acids) and other non-polar compounds from the culture medium [16].In all these cases, macromolecular complexes may be disaggregated by using dissociating agents such as urea or SDS [17], or by eliminating lipid material by extractionswith methanol-chloroform or ethanol-diethyl-ether [18].Once the bacteriocins are recovered from the cell-free supernatants, they can beconcentrated by techniques permitting separationofthe fractions according to their size and/or physicochemical properties [16].

In recent years bacterial antibiotic resistance has been considered a problem due to the extensive use of classical antibiotics in treatmentof human and animal diseases [19,20 and21]. As a consequence, multiple resistant strains appeared and spread causing difficulties and the restricted use of antibiotics as growth promoters. So, the continue development of new classes of antimicrobial agents has become of increasing importance for medicine[22 and23].Compared to antibiotics, most bacteriocins are relatively specific and can only affect a limited number of bacterial species makingthem particularly advantageous for applications in which a single bacterial strain or species is targeted without disrupting other microbial populations.Since bacteriocin production is linked to cell growth, it may also depend on factors affecting this parameter (such as inhibitory substances like salt or nitrite) or the lack of available nutrients (such as manganese in the case of many LAB). The optimization of bacteriocin production and enhancement of its activity are economically important to reduce the production cost. The aim of this work is to determine the optimum temperature, incubation period and pH for the growth and bacteriocinproduction of *Ent. faecium.*

II. MATERIALSAND METHODS

The isolate *Ent. faecium used* in this study was isolated from Malaysian non-broiler chicken. The bacteriocin production was done by growing the *Ent. faecium* on the MRS broth medium using a 500 ml Erlenmeyer flask with a working volume of 400 ml. One percent (1%, v/v) inoculum was used in bacteriocin production according to [24; 10 and13]. The bacteriocin harvest was donefollowing the method of [(15].Three important variables were used in the study of the optimization of bacteriocin production: temperature, pH, and incubation period. The variables of optimization of bacteriocin production were determined based on the growth curves of the three variables.The*Ent. faecium* strain was grown anaerobically at selected temperatures (4, 10, 28, 37 and 45°C) and at different pH of MRS broth adjusted with 1N HCL or 1NNaOH to pH values of 5, 6, 7, 7.5, 8, 9 and 10. Growth was determined spectrophotometrically using UV-Visible spectrophotometer by measuring theODat 600nm.ThepH was monitored throughout the incubation period.

2.1 Antibacterial spectrum of culture supernatantof*Ent. faecium*.

An aliquot (4ml) of culture broth was withdrawn from flask after time interval of 2, 4, 6, 8, 10, 12, 14, 16, and 18hrs and centrifuged at 10,000rpm for 15 min. The supernatant was filtered through 0.22mm Millipore membrane filter (Sartorius, Germany). An aliquot of 20µl of a 2-fold dilution of the culture supernatant was introduced onto a 6mm sterile filter paper and then placed onto Mueller Hilton Agar (MHA) plate inoculated with overnight growth of indicator strains (*Staphylococcus aureus* and *Pseudomonas aerogenosa*). After incubation at 37^{O} C for 18-24h, the plates were observed for inhibition of indicator strains and the zones of inhibition measured [25].

2.2 Bacteriocin extraction.

The antimicrobial compound was extracted by one step solvent extraction procedure [15] with slight modification. The cell free supernatant was mixed with an equal volume of chloroform and agitated vigorously for 20min, after which it was centrifuge at 12000rpm for 20min at a temperature of 12^{O} C. The precipitate detected at the interfacial region was collected by first gently removing the upper solvent and then the chloroform without disturbing the interfacial components. The interfacial components remaining in the tube is then centrifuge at the highest speed for 3mins to sediment the protein of interest. The residual chloroform was then removed by evaporation. The residue was termed as crude antimicrobial compound. The residue was dissolved in distilled water and its activity was checked by disc diffusion method against methicillinresistance*Staphylococcus aureus and Pseudomonas aerogenosa.* Partial purification of extracted antimicrobial substance was done by chloroform methanol protocol.

2.3 Characterization of antimicrobial compound

2.3.1 Effects of temperature, pH, enzymes and organic solvent on the antimicrobial agent.

2.3.1.1 Heat and pH sensitivity:

To test the heat sensitivity, extractedbacteriocin was heated for 15 min. at 100C, 121^{o} C, -12^{o} C, after which the bacteriocin activity was tested against methicillin resistance *Staphylococcus aureus*. The pH sensitivity of bacteriocinswere also determined at different pH by adjusting the pH of the extracted bacteriocins dissolved in buffer to 5, 6, 7, 8, 9, and 10. The inhibitory activity was detected by disc diffusion method [2].

2.3.1.2 Sensitivity of bacteriocin to enzymes.

The sensitivity of the bacteriocins extract to proteolytic enzymes was determined by treatment with proteinase k, trypsin and chloroform. 1mL of crude extract was treated with1mg proteinase-k.0.5mg trypsin [26].The proteinaceous nature of the antimicrobial compound was confirmed by the Biuret test Bacteriocin quantification. Quantification of the protein was determined by Bradford method using BSA as a standard. The molecular weight of the proteins wasanalyzed in 12% SDS-PAGE by [27]. An aliquot (10µl) of partially purified antimicrobial compound was treated with an equal volume of SDS-PAGE sample buffer and electrophoresed at 200 V /30mA for 90 min. The gel was stained with 0.25% Coomassie brilliant blue in methanol-acetic acid-water (40:10:50), and destained in the same solvent excluding the Coomassie blue to observe the protein bands. The result was then compared with SDS-PAGE standards [26].

III. RESULTS AND DISCUSSION

3.1 Growth curve of the isolate.

The isolate was grown in MRS broth (after several purification steps) at 37^{O}C for 18h with constant shaking at 150rpm anaerobically, and aliquot of the culture medium turbidity was measured spectrophotometrically at different time intervals. The pH and antimicrobial activity were also determined at these time intervals. The result obtained was then used to plot a growth curve.In another experiment the growth medium pH was adjusted to 5, 6, 7, 8, 9, and 10 with a working temperature of 37^{O}C. Furthermore, the temperature of the growth medium was also varied to obtain the optimum temperatures for growth and bacteriocin production.

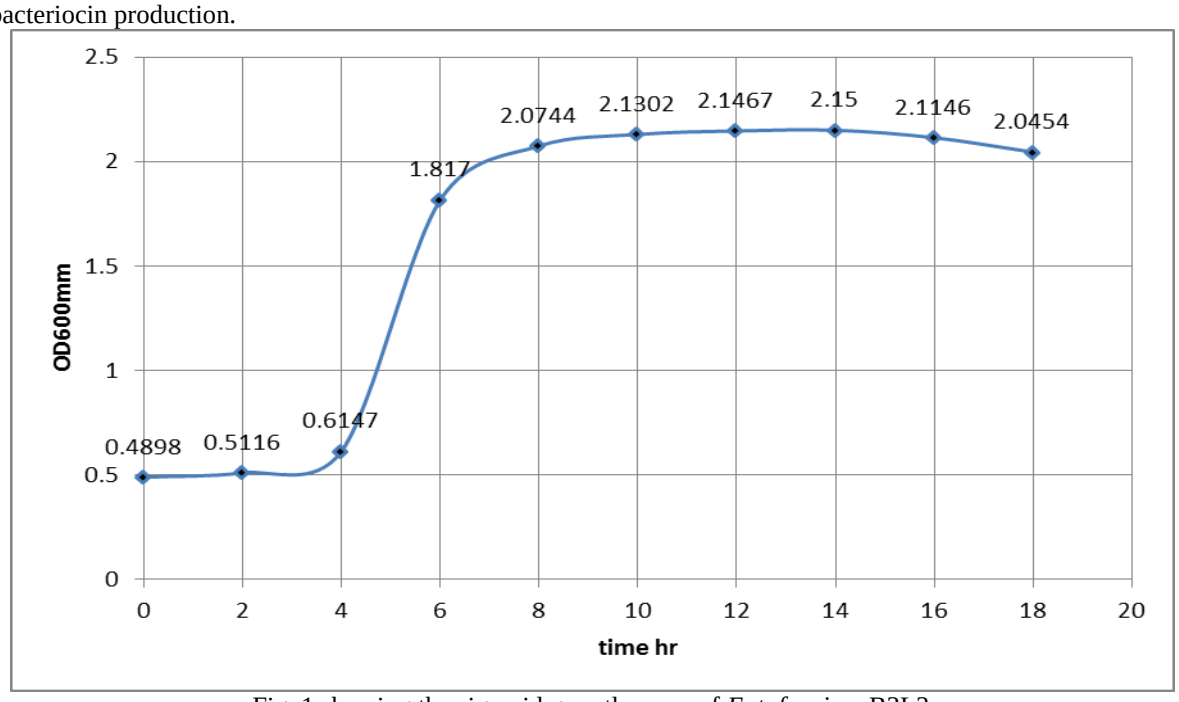

Fig. 1 showing the sigmoid growth curve of *Ent. faecium* B3L3

Fig. 1 shows an increase in the optical density of the culture medium with time. The exponential phase of growth started at the 4thh from the time of incubation and these was maintained up to the 9thh of growth.

During this stage there was an increase in the optical density600nm(from 0.6617nm to2.0477nm). The stationary phase begins at the 8thh of growth. A decline in optical density was observed at the 16th from the time of incubation.

3.2 Effects of temperature and pH on growth and bacteriocin production by*Ent. faecium* B3L3

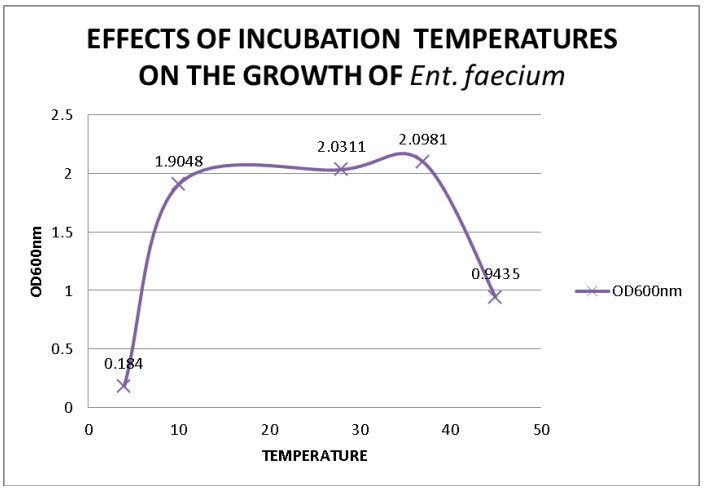

Fig. 2 Effects of incubation temperature on the growth of *Ent. Faecium B3L3*.

Temperatures of 4, 10, 28, 37, and 45^{o} C were used to determine the optimum for the growth and bacteriocin production. The highest growth 2.0981nm optical density was observed at 37^{o} C while the lowest growth 0.184nm optical density was observed at 4^{o} C Fig. 2.

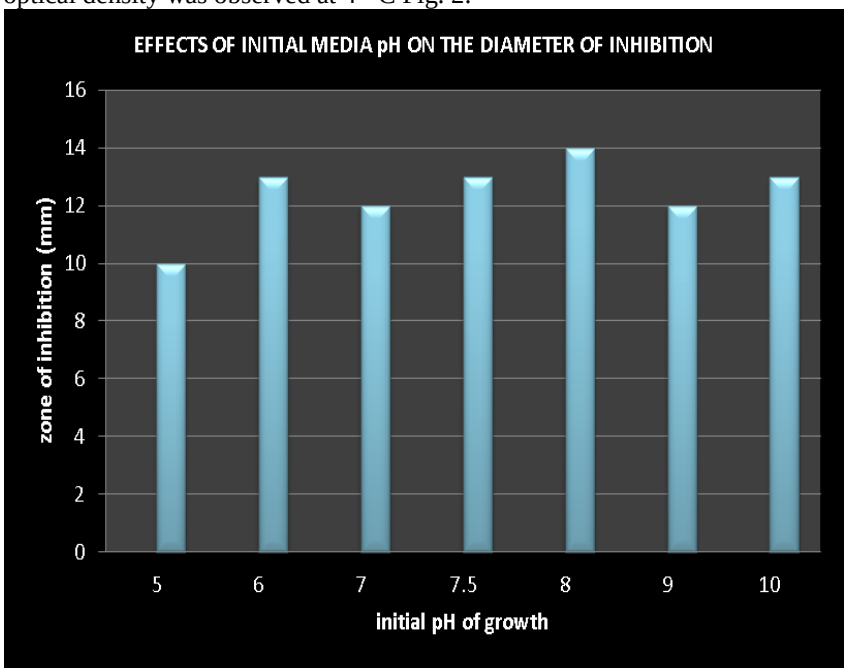

Fig. 3Effects of initial pH on the inhibition activity of the antimicrobial compound.

Different pH range was used to determine the optimum pH needed for bacteriocin production Fig.3. The highest inhibition was recorded at pH 8. residual inhibition was also produced at the highest and lowest pH10 and 5 respectively..

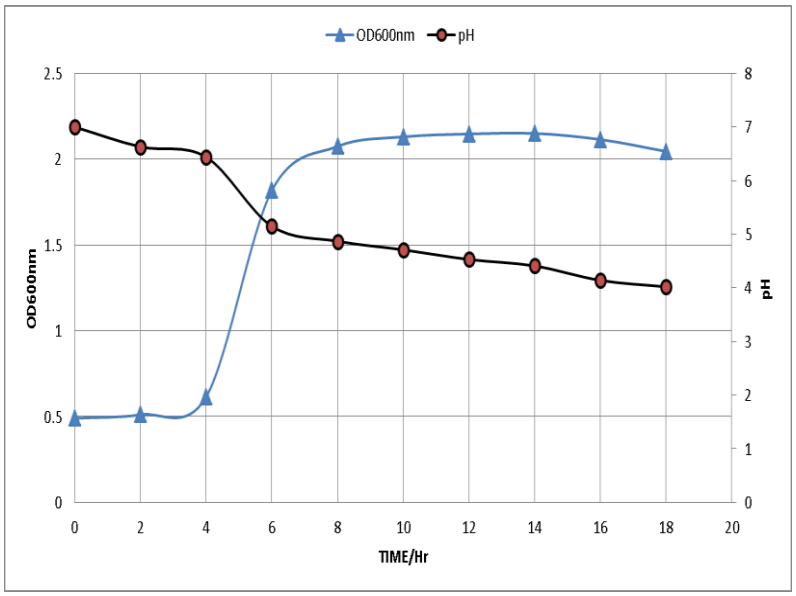

Fig. 4pH and turbidity changes with duration of incubation of*Ent.faecium B3L3*

A gradual decrease in the pH was noticed as the cell biomass increase with time of incubation Fig.4.at the end of the incubation period, pH of 4.02 was recorded showing the production of acid by the isolate which happen to be the main characteristic of LAB.

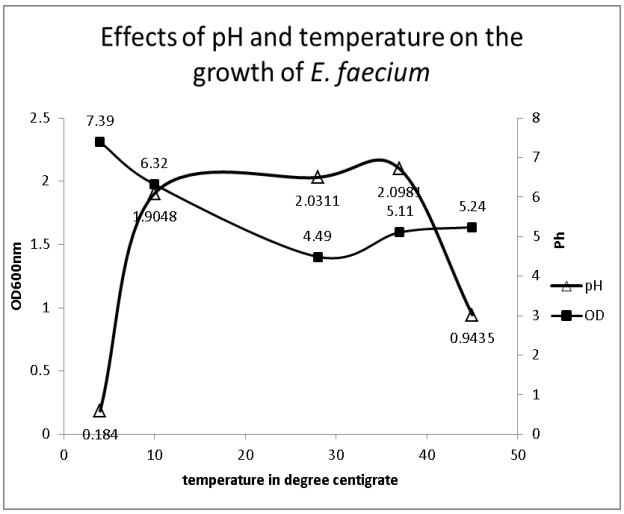

Fig. 5 Effects of different temperatures on the growth and final pH of the growth medium

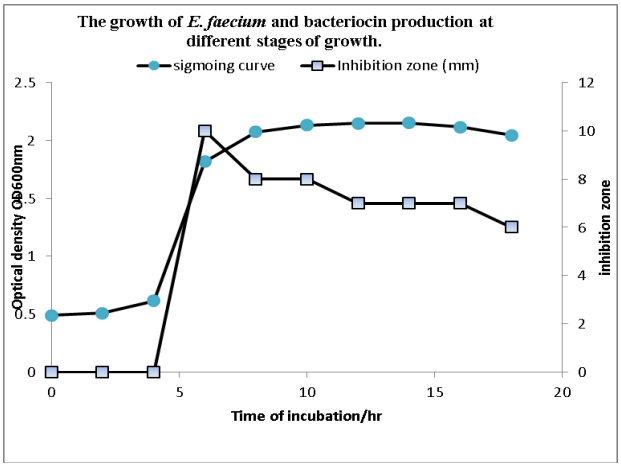

Fig.6*Ent.faecium* B3L3 growth curve indicating critical phaseof bacteriocin production:

Bacteriocin production started early during logarithmic phase and the activity reached to maximum in early stationary phase and then remained constant for certain period during this stage ofgrowth. This is in partial agreement with the results obtained by Leroy and De Vuyst[28] in which production of enterocin RZC5 (from *E. faecium*) occur in early growth phase.An increase in turbidity with time and decrease in the pH of the medium were also observed. The exponential phase of growth started from the 4th hour after incubation and the production of antimicrobial peptides starts at 6thhour which happens to be the peak of the exponential phase. It was also noticed that the stationary phase started at the 8th hour after incubation, residual inhibitory activity was also noticed here. The decline in growth was seen at the 16th hour from the onset of incubation. All experimentswere conducted in a shaker incubator at150rpmincubation temperature of 37^{o} C for18h under anaerobic condition. This result is in partial agreement with the findings of [29] in the batch fermentation of *Ent. faecium* HJ35, enterocin HJ35, that the production of bacteriocin was produced at the mid-log growth phase, reaching a maximum production of up to 2,300 AU/mL during the late stationary phase. [30; 31], in their findings discovered that highest bacteriocin production occurs at the end of the exponential and early stationary phase. According to [32 and 31] degradation of the bacteriocin by proteolytic enzymes is the main cause of bacteriocin reduction. .

Table 1 :pH changes, turbidity and AU/ml at the end of the incubation period of 24hr.

Initial pH	Final pH	Turbidity 600nm	Inhibition in AU/ml
5	4.98	1.6315	200AU/ml
6	5.0	1.8814	300AU/ml
7	4.93	1.9565	300AU/ml
7.5	4.96	2.0644	300AU/ml
8	4.99	2.0771	300AU/ml
9	5.00	2.1654	300AU/ml
10	5.19	2.2037	300AU/ml

Table 1 shows gradual increase in the turbidity with a decrease in pH and almost stable individual residual activities. The initial pH of growth medium plays an important role in the growth and bacteriocin production as shown in table 1. The result shows production of the highest turbid culture is at an initial pH10 followed by pH9. The lowest growth was observed at pH5. Also the highest final pH was recorded from the initial pH 10 (5.19) while lowest was recorded from the initial pH 7 (4.93). Most of the bacteriocin activity recorded was almost identical (200-300AU/ml).

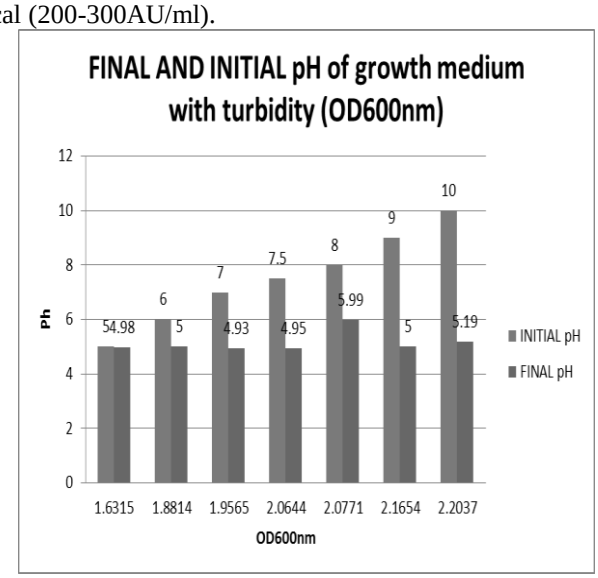

Fig.7The initial and final pH of the growth medium with increase in optical density of the growth medium.

From Fig. 7, there appear to be a drop in the pH of the medium as the optical density increases or increase in growth biomass. At the end of the incubation (24hr at 37^{o} C), the pH range obtained was between 4.93 for pH 7 to 5.99 for pH 8. This report is in agreement with the findings of [33] who suggested that the increase of biomass cells and organic acids production are the main reasons for pH reduction in fermented food. [33; 34] reported that lactic acid and acetic acids produced by L. plantarum strains during

itsmetabolite production augment the growth of the producer cells. The maintenance of the acidic pH of the metabolites is supplied by the lactic and acetic acid which are the major contributor to the acidic environment. [16; 35 and36].

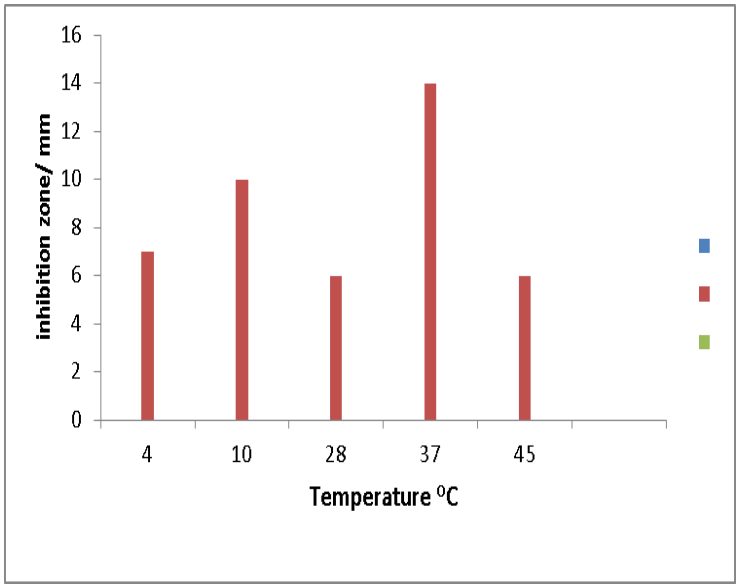

Fig.8 The effects of incubation temperatures on the inhibition zone.

The highest inhibition zone was recorded at the optimum growth temperature of 37^{O} C with a reduction at 10, 4, 28 and 45^{O} C. In several studies, the production of Bacteriocin have been known to be temperature regulated especially in enterocin 1146 [37], enterocin AS-48 [38], a bacteriocin formed by E. faecium RZS C5 [28] and enterocin P [39], which is generated by Enterococcus spp. In obvious cases, greater level production of bacteriocin has been detected at sub- optimal growth situations [40; 37; 41; 42; 43]. A reduction of the bacteriocin production at 4, 10, 28, and 45^{O} C for 48 hours was observed when compared to l incubation at 37^{O} C (Fig. 8). [28] studied the production of bacteriocin by *Enterococcusfaecium* RZS C5 under different temperatures and obtained similar results with less bacteriocin production below 35°C; probably due to cellular environment regulation and growth related processes.

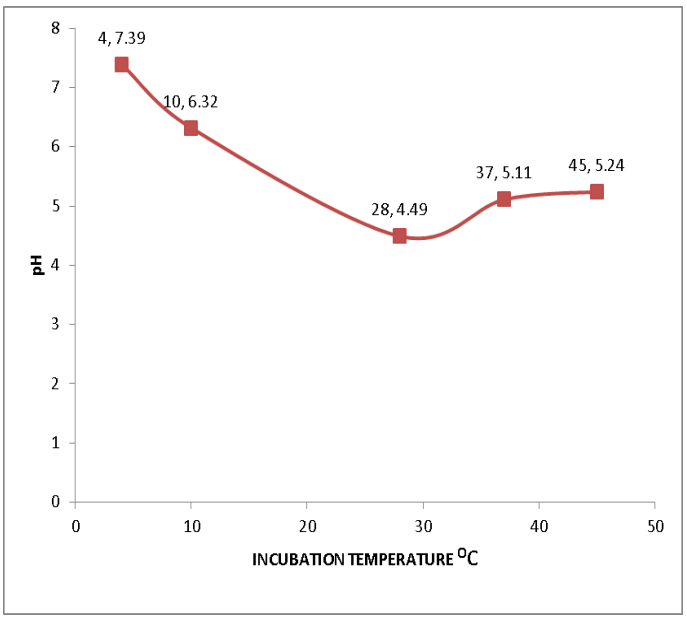

Fig 9 Changes in the initial culture medium pH (7.5) at the end of the incubation period 24h at different temperatures of incubation.

FromFig. 9, at the end of the incubation period (24h), the lowest final pH was recorded at the incubation temperature of 28°C while the highest recorded final pH was noticed at the temperature of 4°C. The high final pH recorded at 4°C showed that lesser biomass was obtained.

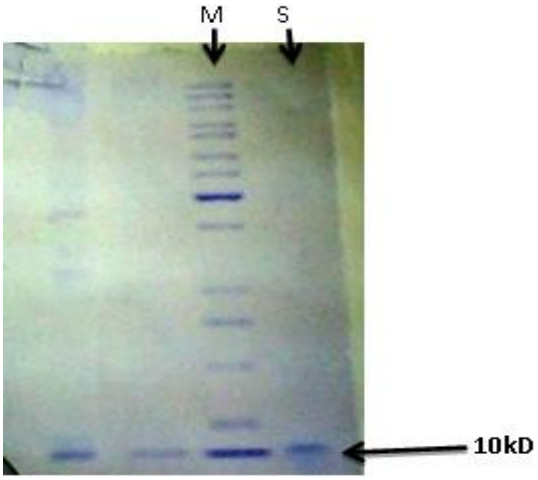

Fig. 10.SDS-PAGE showing single band of bacteriocin.Key M=marker (Fermentas) S=sample kD=kilo Dalton

The crude bacteriocin of *Ent. faecium* B3L3 was subjected to SDS-PAGE. The electrophoretogram of the gel stained with Coomassie Brilliant Blue R-250 showed a single band of protein corresponding to a molecular mass of ~10.0 kDa (Fig.10). When the gel was overlaid with the indicator bacteria a large inhibitory zone corresponding to this band could be observed data not shown.This is in agreement with the work of Simonová M, Lauková A. 2007 who showed that *Enterococcusfaecium* (EF2019, EF1819, EF2119, EF1839, EF529, EF24/10) isolated from rabbits faeces produce bacteriocin of molecular mass ranging from 3 to 10 kDa. The molecular size of *Ent. faecium*bacteriocin is close to that produced by *E. avium*[44]. On the other hand, enterocin ON-157 from *E. faecium* NIAI 157 is one of the lowest molecular weight bacteriocin [45].

3.3 Characteristics of extracted bacteriocin of *Ent. faecium* B3L3..

Effect of different treatments on bacteriocin extracts on the indicator organism and relative activity was measured by a disc diffusion test.

Table 2 : Effect of different treatments on bacteriocin extracts on the indicator organism and relative activity.

Treatments	**Relative activity**
Enzymatic treatments	
Proteinase K	-
Trypsin	-
Catalase	++
Control	++
pH treatments	
4	+
5	+
6	++
7	++
7.5	++
8	++
9	+
10	+
Control	++
Heat treatments (°C/min)	
112/15min	-
100/15min	+
112/15min	+
100/30min	+

Legend: (−): no inhibition; (+): low inhibition; (++): high inhibition.

The bacteriocin of *Ent. faeciumB3L3* retained at least 80% of its activity in MRS broth in the pH range of 4 to10 and when stored at 10 °C for 24 hours (Table 2). It remained stable after 15 minutes at 100°C and 112°C; but lost 50% of activity after treatment at 100°C for 30 minutes (Table 2).This results are also supported by findings of [10; 46; 12; 47; 48].

IV. DISCUSSION

4.1Temperature and bacteriocin activity

The activity of bacteriocins produced by *Ent.faecium B3L3* was thermo-stable and retained their activity even afterexposure at 100°C for 15min and long storage period at 4, and -20°C.(Table 2). These results may be in accordance with the stability of bacteriocins by the other findings.[8] reported that the activity of enterocin BFE 900 retained its activity after heating at 100 °C and 121°C. [46]hadalso reported a stable enterocin MR99 from *Ent. faecium*..

4.2 pH and bacteriocin activity

The crude bacteriocin of strain*Ent.faecium B3L3* could withstand a wide pH range (4-10). The maximum activity was obtained between pH 6-8[49] showed that bacteriocins isolated from Enterococcus, A5-11 was found to be active over a wide range of pH from 2.0 to 10.0 [50]. Also based on the findings of [10; 46; 12; 47; 48], showed that Partially purified bacteriocin appeared stable to adjustment of pH range of 4-10.

4.3 Enzyme and bacteriocin activity

Antibacterial activity of the partially purified bacteriocin of *Ent. faecium B3L3* studied was completely destroyed upon treatment with trypsin (residual activity of 0 AU/ml). Bacteriocinactivities were not affected by lysozyme, and catalase. These results are consistent with other findings of [51; 12; 52 and 48] which identified and characterized enterocin produced by *Ent. Faecium.*They reported on their inactivation by proteinase K, trypsin, α-chymotrypsin and papain, but not by lysozyme, lipase, catalase or β-glucosidaseSensibility to proteolytic enzymes showing the proteinaceous characteristic of bacteriocins.

V. CONCLUSION

In this research three parameters were used in assessing the optimum for the growth of *Ent. faecium* B3L3 and the bacteriocin it produces, from the results obtained, we discovered that 37°C, pH 8 were the most conducive condition for production of bacteriocin while growth was noticed to be maximum at 37°C, at a pH 9 to 10. This characteristic of *Ent. faecium*B3L3 to grow and produce bacteriocins that can inhibit pathogenic strain (Methicillin resistance *Staphylococcus aureus*) is a welcome idea in the fight against human pathogens. From different findings, Enterococci species is known to be the highest producer of bacteriocin. The flexibility of the growth and production of its bacteriocinsand its stability to a wide range of physiochemical agents is another added advantage.

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