CHAPTER 4 RESULTS AND DISCUSSION

The research focuses on the optimization of the non-selective and selective enrichment steps. In this research, the cell cultivation was performed using a developed technique containing the reaction in 96 deep well plates to facilitate high-throughput application. The cell response to the alteration of non-selective media and inhibitory effect of common selective agent was explored. Cell multiplication and survival were monitored and studied. Several key growth characteristics, for example, maximum specific growth rate (μ_{max}), were evaluated using Sigmodal-type mathematical models and compared each cultivation conditions to find the optimal conditions for *Listeria* spp. detection and selectivity. The main goal was to understand intrinsic growth-related parameters to help fasten the growth and better select *Listeria* spp. in the liquid enrichment step. The results from this finding could lead to more efficient enrichment step reducing the overall detection time and lower the cost of analysis.

4.1 Investigation of Non Selective Enrichment Protocol

In general, the numbers of *Listeria* spp. present in food samples tend to be in low quantity (Sheridan et al., 1994a; Jay, 1996; Walsh et al., 1998). Many literatures have reported that *Liasteria monocytogenes* was frequently sublethally-stressed in food processing facilities and by environmental challenges. Therefore, an effective enrichment addition can be useful to increase the likelihood to find the target organism in those situations. This supplemental enrichment serves to escalate the cell numbers to exceed the minimal detectable levels with many detection protocols. Several research groups experimented the use of non selective enrichment media to improve *Listeria* recovering and detection (Kosonpisit et al., 2011; Sheridan et al., 1994b; Budu-Amoako et al, 1992; Beumer et al., 1996; Walsh et al., 1998; Vaz-Velho et al, 2001 and Nancy

and Herman, 2004). The purpose of this experiment was to investigate the optimum condition for non-selective enrichment step that was suitable to optimize the growth of *Listeria spp.*

4.1.1 Experimental Description

In this experiment, *Listeria innocua* culture was incubated to reach 9 log CFU/mL. The initial cell concentration of *L. innocua* was prepared at 2-3 log CFU/mL by using serial dilution technique. One hundred micro liters of *L. innocua* was inoculated into 1.5 mL eppendorf tubes containing 900 µL of 0.85% normal saline. Non selective enrichment media were assembled together in Duran bottles at 20 mL for each condition. The desired initial cell concentration of *L. innocua* at 2-3 log CFU/mL was inoculated into the bottles containing the prepared non selective enrichment broth. The mixture was homogenized using a vortex mixer. The total colony forming units were observed and counted from the lid of 96 micro-well surface (Figure 4.1).

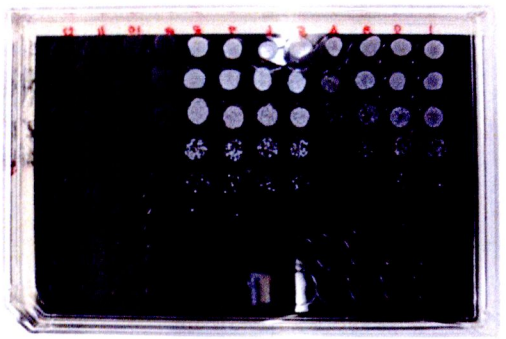


Figure 4.1 An example of photograph of *L. innocua* colonies grown on TSA

4.1.2 Results and Discussion

Non-selective media that used in this study can be divided into two groups: conventional non-selective media (i.e., Nutrient Broth: NB, Lactose Broth: LB and Tryptic Soy Broth: TSB) and *Listeria*-specific broth base (i.e., Buffered Listeria Enrichment Broth base: BLEB, Fraser Broth base: FB and Palcam Broth base: PB). The Listeria-specific broth was modified from the standard selective enrichment media that

theirs selective inhibitors were removed from the standard composition. Figure 4.2 and 4.3 show the growth profiles of *L. innocua* grown on different non-selective media and incubation temperatures.

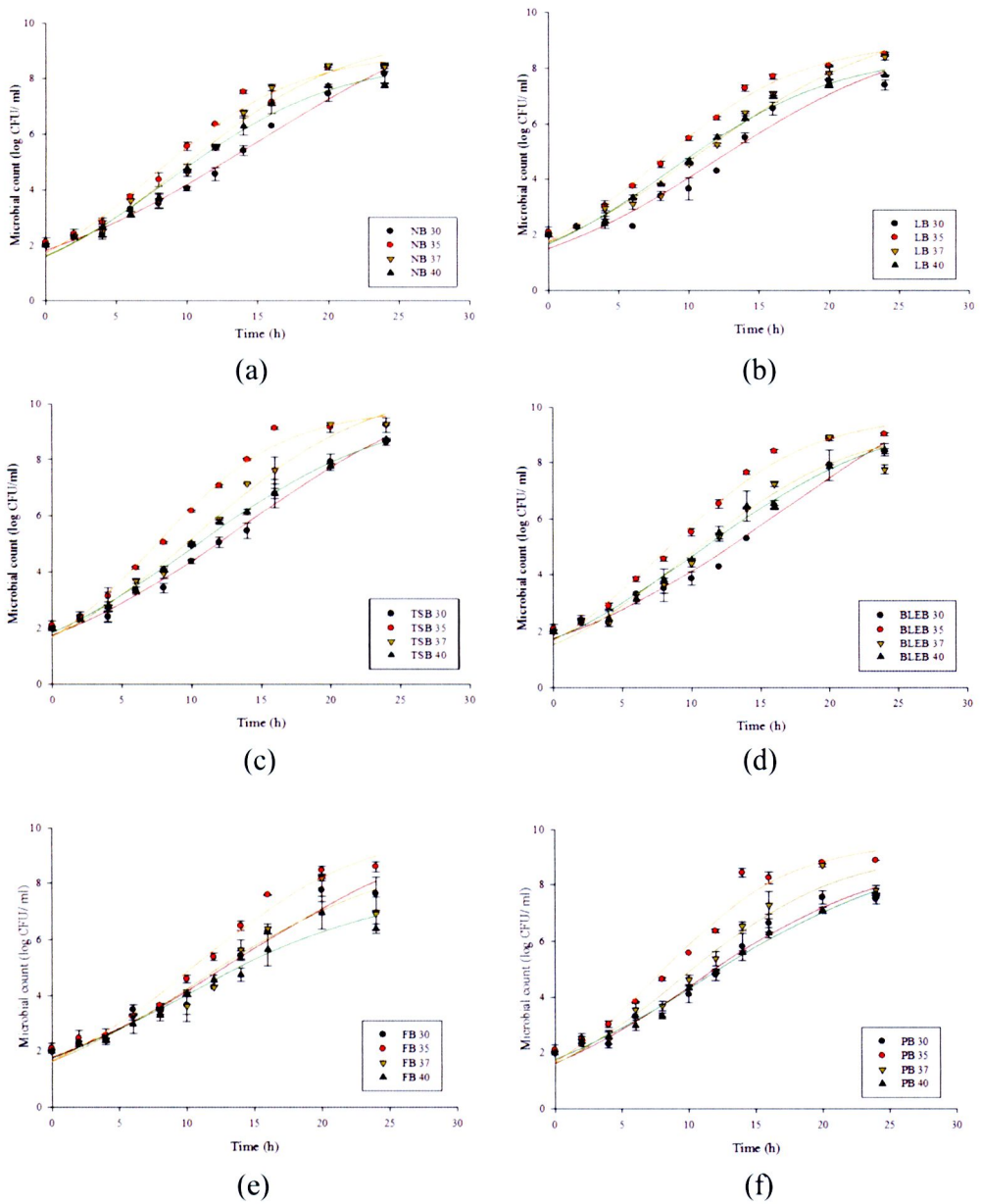


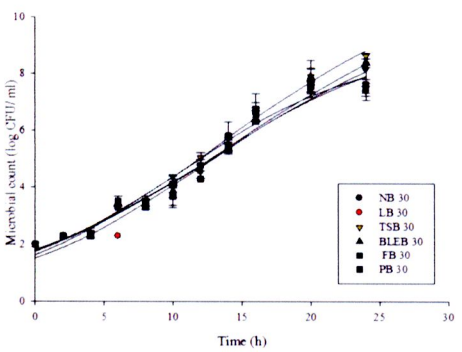
Figure 4.2 Effect of different incubation temperatures on growth profiles of *L. innocua* in different non-selective enrichment broths; a) Nutrient Broth (NB), b) Lactose Broth (LB), c) Tryptic Soy Broth (TSB), d) Buffered Listeria Enrichment Broth (BLEB), e) Fraser Broth (FB) and f) Palcam Broth (PB)

The growth profiles clearly displayed the effect of incubation temperatures on the growth of *L. innocua.* At the temperature 35°C, the growth curves in all substrate media returned the steepest slope during the exponential growth phase. The increase of cell density initiated sooner than that in other conditions. This growth condition reflected the best cell expansion kinetic. At the lower and upper experimental boundaries (i.e., 30 and 40°C, respectively), the *L. innocua* growth was apparently suboptimal. The incubation at 37°C showed slower growth kinetics; however, it still can be very useful as an additional selective strategy to discourage other psychrophiles if need be. The compromise of *L. innocua* growth kinetics was only marginally; the range of incubation temperatures between 35 and 37°C was recommended to culture *L. innocua.*

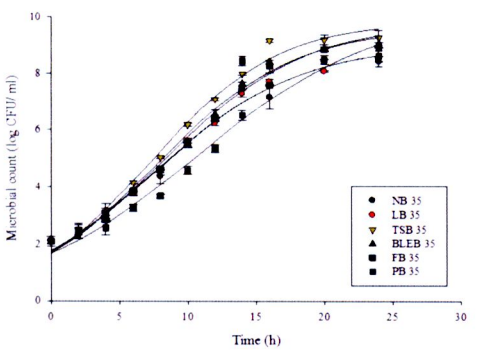
Figure 4.3 alters the *L. innocua* growth profiles as a function of medium types. These plots provided different insight into the growth in the non-selective enrichment step. At very low incubation temperature (i.e. 30°C), it was irrelevant what types of non-selective media used to grow *L. innocua.* When the incubation temperature was increased to 35°C, the convergence of growth profiles started to disappear. The TSB showed stronger *L. innocua* preference than other media. These plots helped emphasize the effect of temperatures on the final cell density at the end of the batch growth curve. In the 30°C treatment, it was hypothesized that the temperature was too low such that it slowdowns all cell metabolisms and the surplus and the variation of carbon and nitrogen sources in each medium recipe didn't not affect *L. innocua* growth profiles. The cell grew to significantly lower final cell density. When the incubation temperature was at the optimum, different medium compositions started to make distinctive growth characteristics (Figure 4.3b).

From these plots, there was an interesting inclination that the nutrient compositions in the selective broth base performed poorly to sustain growth of *L. innocua* in comparison

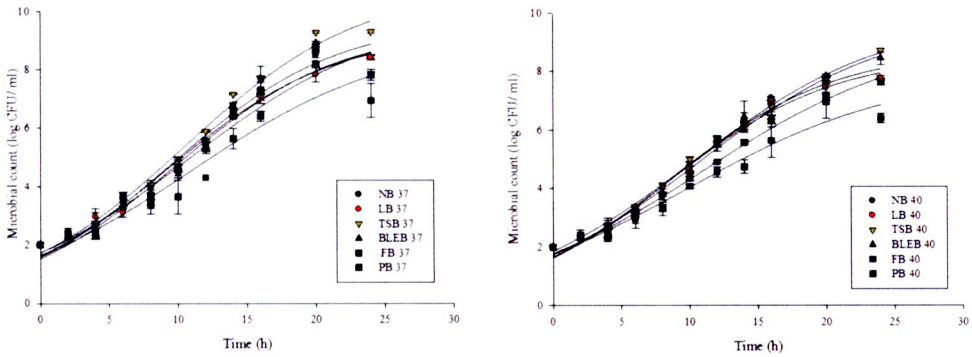
to the performance of the conventional non-selective media. Together with high incubation temperatures (e.g., 35-40°C), this combined effect differentiated the growth of *L. innocua* grew on those treatments. Perhaps, the heightened cell metabolism due to temperature played some role on exacerbating the inappropriateness of the non-selective medium composition. At temperature rise, chemical and enzymatic reactions proceed at a faster rate, and the growth rate increases. Above a certain temperature proteins are irreversibly damaged. As temperature is increased within a certain range therefore, growth and metabolic activity increases up to a point where inactivation reactions set in. Evidently, the high temperature exceeding the optimum inflicted a substantial negative effect on the growth kinetics. Even for the most all around medium, like TSB, the 40°C incubation brought down the final cell density from approximately 9 log CFU/mL (incubated at 35-37°C) to 8 log CFU/mL and delayed the exponential growth phase significantly.



(a)



(b)



(c) (d)

Figure 4.3 Effect of different non-selective on growth profiles of *L. innocua* at the same temperature; a) 30°C b) 35°C c) 37°C and d) 40°C

The predicted lines in Figure 4.2 and 4.3 demonstrated the application of the sigmoid mathematical model in representing batch growth curves of *L. innocua* using various substrates and incubation conditions. The good agreement of the data and prediction resonate the usefulness and appropriateness of the model to extract key kinetic parameters. Figure 4.4 show the maximum specific growth rate of *L. innocua* in non-selective broths at different incubation temperatures. The highest maximum specific growth rate shown in the condition that *L. innocua* was cultured in TSB at 35°C. And also, at this temperature give the very good maximum specific growth rate only Fraser Broth gave rather inferior maximum specific growth rate value. Followed by 37°C, that reveals the lower maximum specific growth rate value. Although at 30 and 40 °C the maximum specific growth rate values are very low, that mean *L. innocua* grow very slow at these temperatures

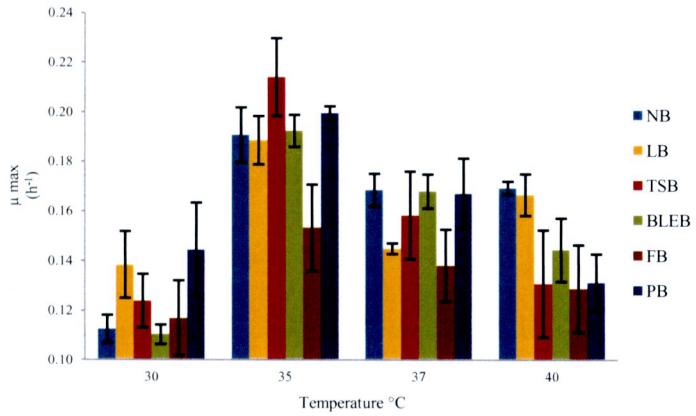


Figure 4.4 Effect of non-selective enrichment media and incubation temperature on growth of *L. innocua*.

Also, when considering final cell density (Figure 4.5) of *L. innocua* in different non-selective broths at different incubation temperatures. *L. innocua* that cultured in TSB at 35 and 37 °C returned the very good final cell count. This optimal temperature range was consistent with Farber and Peterkin. (1991), which reported that the optimum temperature for growth of *Listeria* spp. lied between 30 and 37°C. It can grow between 1 and 45°C (Smith et al., 1991).

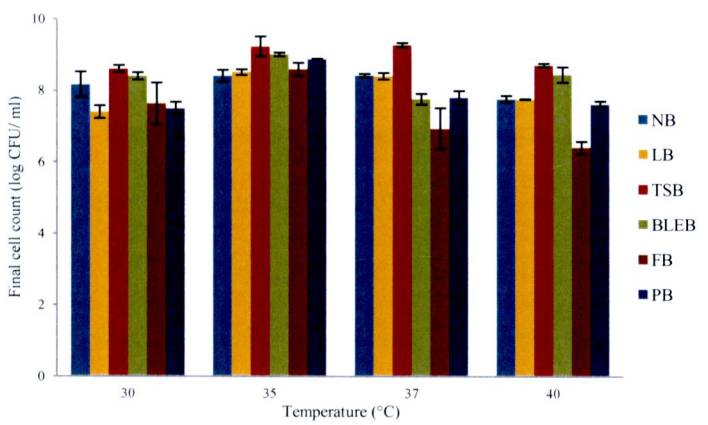


Figure 4.5 Effect of non-selective enrichment media and incubation temperature on growth of *L. innocua*.

4.2 Examination of Inhibitory Effect of the Conventional Selective Agents on Growth and Selectivity of *L. innocua*

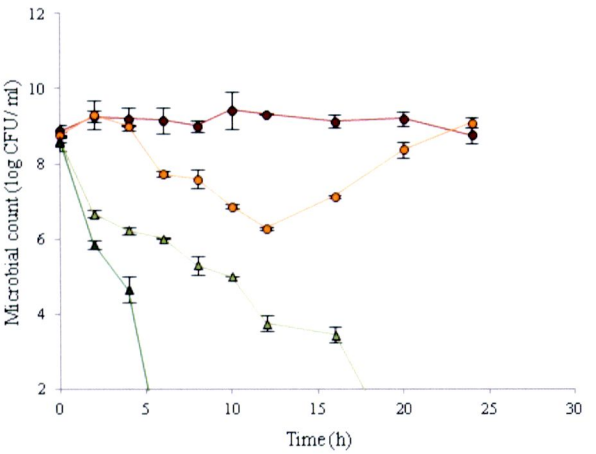
4.2.1 Experimental Description

In this experiment, *L. innocua, E. coli* and *S. aureus* cultures were prepared in TSB to reach 9 log CFU/mL in shake flasks. One hundred twenty μL. of each culture was inoculated into 1.2 mL of tested media that contained different types and concentrations of selective agents in a 96 deep well plate. Selective agents included acriflavine, polymyxin B, nalidixic acid and lithium chloride. The cultivation temperature was controlled at 35°C for 24 h to study the growth kinetics. The total colony forming units were detected on the agar surface of 96-micro well lid.

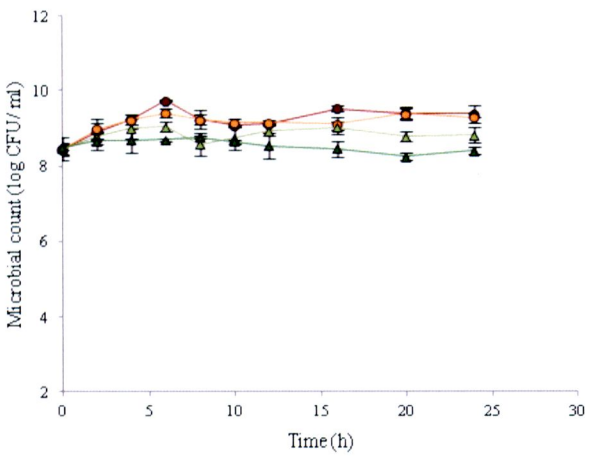
4.2.2 Results and Discussion

4.2.2.1 Acriflavine

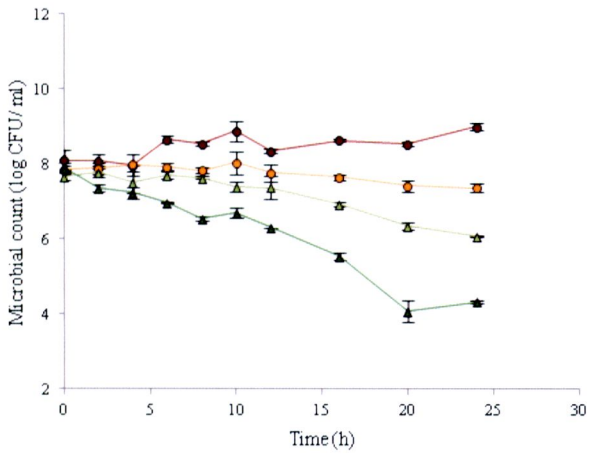
The effect of acriflavine as selective agents on growth of *L. innocua* is shown in Figure 4.6, which depicts the kinetic comparison among growth profiles of *L. innocua, E. coli* and *S. aureus* on TSB containing various concentration of acriflavine. Figure 4.6 (a) represents the growth profile of *L. innocua* using different concentrations of acriflavine. The lowest concentration treatment (5 mg/L) didn't affect the growth of *L. innocua.* At higher concentration, there was the evidence of *L. innocua* lethality. The 10 mg/L acriflavine treatment showed the decline of cell number to the 10^{th} hour but later on the growth was recurred. The cell number was increased until the end of cultivation. Perhaps due to *L. innocua* was able to adapt itself to tolerate the toxicity of acriflavine. Intrinsic resistance to acriflavine of *L. innocua* was performed by efflux transporters that occurred when it was induced (Mata et al., 2000).



(a) *L. innocua*



(b) *E. coli*



(c) *S. aureus*

Figure 4.6 Comparison of growth profiles of *L. innocua, E. coli* and *S. aureus* on acriflavine; ( ) acriflavine 5 mg/L, ( ) acriflavine 10 mg/L, ( ) acriflavine 20 mg/L and ( ) acriflavine 40 mg/L

Nevertheless, at the concentrations higher than 20 mg/L, *L. innocua* density decreased rapidly as a function of incubation time and showed no indication of cell resuscitation. Figure 4.6 (a) demonstrated the strong toxicity of acriflavine in the media on *L. innocua.* When the concentration of acriflavine exceeded 40 mg/L. it was able to bring down the *L. innocua* density from 9 log CFU/mL to less than 2 log CFU/mL in less than 5 h However, in the 20 mg/L treatment, the lethality of *L. innocua* was mitigated and it took more than 18 h to reduce the cell density to less than 2 log CFU/mL. The application of acriflavine in the selective media from 5 to 40 mg/L was very critical to isolate L. innocua since it returned significant impact to *L. innocua* growth from no kill to drastic reduction of cell numbers.

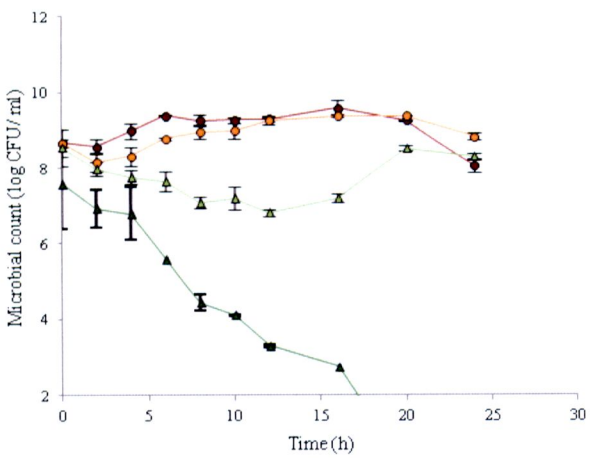
The effect of acriflavine on *E. coli* considerably differed from that on *L. innocua* (Figure 4.6 (b)). While *L. innocua* was rapidly inactivated at the concentration higher than 20 mg/L, E. coli density was stabilized in all acriflavine treatments. Some researcher also

reported the strong resiliency of *E. coli* strains on acriflavine. The wild-type strains of *E. coli* K-12 was able to resist to different concentrations of acriflavine (Nakamura, 1968). It was later identified that the Gene acrA+, which was located near the lac region of the chromosome, was responsible to the resistance of *E. coli* to acriflavine. As Gram-negative bacteria, *E. coli* was in general more resistant to a wide array of antibiotics and chemotherapeutic agents than most Gram-positive bacteria (Nikaido, 2011). It was hypothesized that the narrow porin channels of outer membrane limited the penetration of hydrophilic and lipophilic solutes (Nikaido, 1985; Plésiat, 1992). In addition, Phalanisong (2008) revealed that the complexity of outer membrane of Gram-negative was effective in protecting Gram-negative cell.

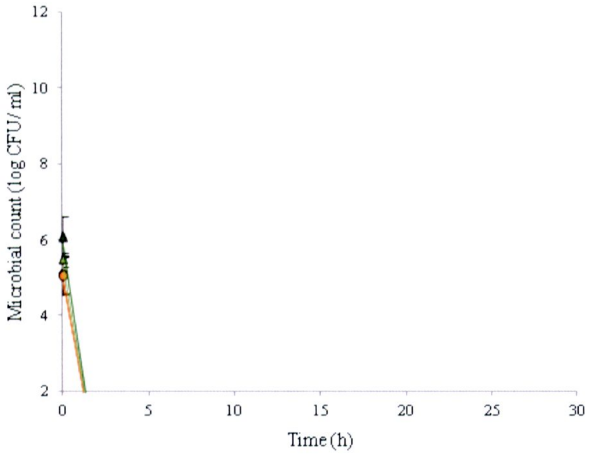
The effect of acriflavine on the growth of *S. aureus* was shown in Figure 4.6 (c). Acriflavine affected *S. aureus* in the same way as it did on *L. innocua* but to a lesser extent. *S. aureus* was, however, more resistant to the toxicity of acriflavine than *L. innocua.* At lower concentrations of acriflavine ranging from 5 to 10 mg/L, its impact on growth profile was minimal; the *L. innocua* growth was stagnant or slightly decreased. Starting from 20 mg/L, the effect on acriflavine on growth curve was noticeable. Log reductions of 2 and 4 orders of magnitude were observed when the acriflavine concentration was raised to 20 and 40 mg/L after 24-hour incubation, respectively. Maybe *S. aureus* strains were naturally equipped with peptidoglycan and was able to thicken the already impermeable cell wall in respond to the presence of hostile selective inhibitors (like, acriflavine). The development of its own natural defend mechanism serving as permeability barrier resulted in higher acriflavine resistance in *S. aureus* than other Gram-positive and -negative strains (Kawai et al., 2009).

4.2.2.2 Polymyxin B

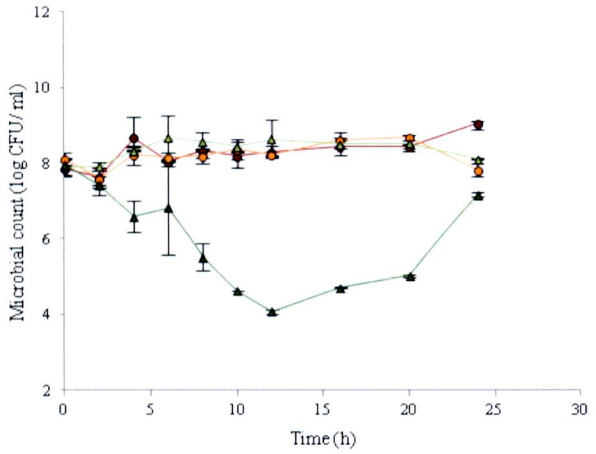
The effect of polymyxin B on growth of *L.innocua* and its selectivity on *E. coli* and *S.aureus* were explored (Figure 4.7). Polymyxin B was the most potent selective agent affecting the survival of both Gram-positive (*L. innocua* and *S. aureus*) and Gram-negative (*E. coli*) bacteria. It was obvious that it inflicted high level of toxicity to Gram-negative than Gram-positive bacteria. Polymyxin B was able to produce rapid reduction of *L. innocua* cell density when the concentration was increasd to 100 mg/L. It brought down the number of the viable cells of *L. innocua* from approximately 8 log CFU/mL to less than 2 log CFU/mL within 16 h. Again, L. innocua recovery was observed at the 50 mg/L treatment as seen earilier on the effect of acriflavine at the 20 mg/L treatment.



(a) *L. innocua*



(b) *E. coli*



(c) *S. aureus*

Figure 4.7 Comparison of growth profiles of (a) *L. innocua,* (b) *E. coli* and (c) *S. aureus* on polymyxin B; (—●—) polymyxin B 10 mg/ L, (—●—) polymyxin B 20 mg/ L, (—▲—) polymyxin B 50 mg/ L and (—▲—) polymyxin B 100 mg/ L.

The levels of Polymyxin B applied were very effective in inhibiting on *E. coli* growth. Even at the lowest concentrations of 10 mg/L, E. coli was disappeared to lower than 2 log CFU/mL within 2 h of incubation period. Substantial difference of polymyxin B

potency on *L. innocua* and *E. coli* can be very useful to select Gram-positive from – negative. The rather benign effect of polymyxin B on other Gram-positive bacteria was observed on the *S. aureus* treatment. The application of polymyxin B marginally disturbed the growth of *S.aureus* when its concentration was lower than 50 mg/L; hence, the growth profile of *S. aureus* was fairly constant throughout the incubation period. Only at the highest concentration (100 mg/L of polymyxin B) did the *S. aureus* growth profile show the constant decrease of cell viability from 8 log CFU/mL to 4 log CFU/mL at 12 h of incubation. However, the number of cell density was later increased until finish the incubation. The event showed rather mild toxicity of polymyxin B on Gram-positive displaying cell recovery when the polymyxin B concentration didn't exceed the threshold of cell toxicity.

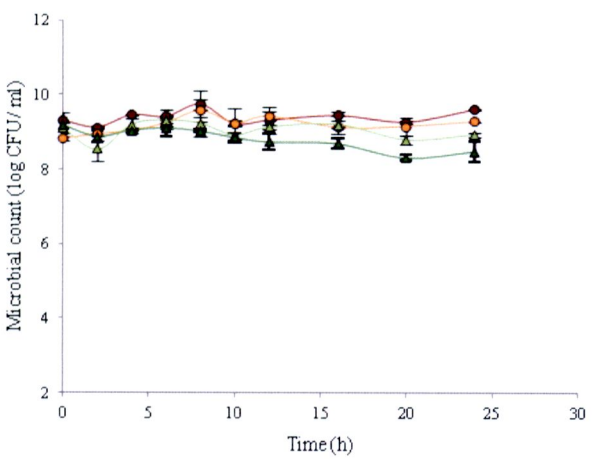
The role of polymyxin B as selective agent was well-defined. Polymyxin B is a polypeptide that has the positively-charged amino groups in the form of cyclic peptides and fatty acid attachment. This polypeptide binds specially on to negatively charged sites of cell membrane, like phospholipids and lipopolysaccharides of bacteria (Hsu Chen and Feingold, 1973; Newton, 1956; Teuber, 1973; Teuber and Bader, 1971). These reactions made cells more permeable to extracellular elements resulting in cell death. It appeared capable in damage Gram-negative bacteria than Gram-positive bacteria. Because Gram-negative bacteria have thinner cell wall than Gram-positive bacteria. Other than thin cell wall, Gram-negative bacteria have more lipopolysaccharides than Gram-negative bacteria, so it easier to damage.

4.2.2.3 Nalidixic acid

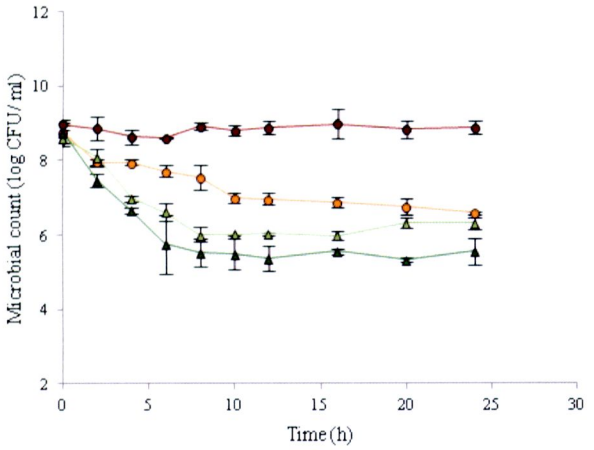
The selective capability of nalidixic acid for *L.innocua* was shown in Figure 4.8. The growth of *L.innocua* on media contained various concentration of nalidixic acid was different from *E. coli* but similar to *S. aureus*. Gram positive bacteria, *L.innocua* and *S.*

aureus that used in this experiment weren't inhibited by nalidixic acid. Appling *L. innocua* onto nalidixic acid stabilized viable cell of *L. innocua*, while *S. aureus* slightly decreased when cultured on TSB contained different concentration of nalidixic acid. Gram negative bacterium was interrupted by nalidixic acid but lesser efficient than polymyxin B. The lowest concentration treatment, at 10 mg/L cell number of *E. coli* was stabilized throughout the incubation period. The higher concentration treatment the viable cell decreased with the concentration increased. At the highest concentration treatment the viable cell continuously decreased till 8th hour after that the viable cell stabilized at 5 log CFU/mL until the end of incubation.

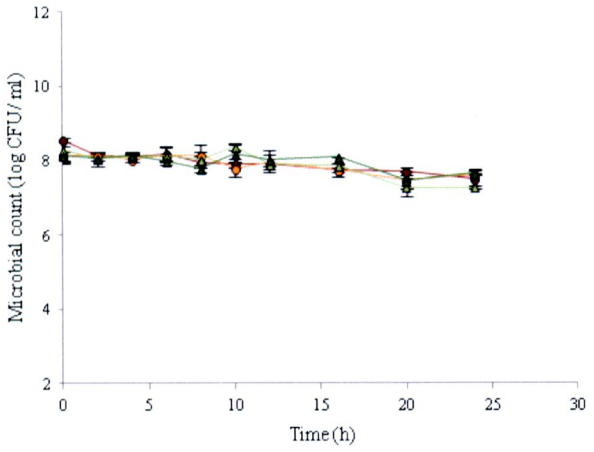
Different mechanisms of inhibition of polymyxin B and nalidixic acid caused the variation of this selective capability. Polymyxin reacted with the components of cell wall resulted in more permeable that lead to cell death rapidly, whereas nalidixic acid inhibited DNA synthesis by suppressing the activity of DNA gylase system. The DNA gylase system participated in the DNA synthesis that is slower reaction when compared to the reaction of polymyxin B (Bhanot et al., 2001; Andriole, 1998). And also at the low concentration, nalidixic acid was only the bacteriostatic that mean it only inhibited the growth and reproduction but not killed the microorganisms (Goss et al., 1964).



(a) *L. innocua*



(b) *E. coli*



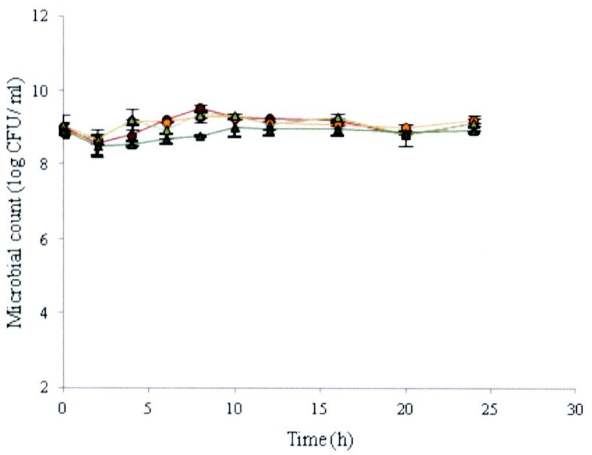
(c) *S. aureus*

Figure 4.8 Comparison of growth profiles of (a) *L. innocua,* (b) *E. coli* and (c) *S. aureus* on nalidixic; ( ) nalidixic 10 mg/L, ( ) nalidixic 20 mg/L, ( ) nalidixic 50 mg/L and ( ) acriflavine 100 mg/L

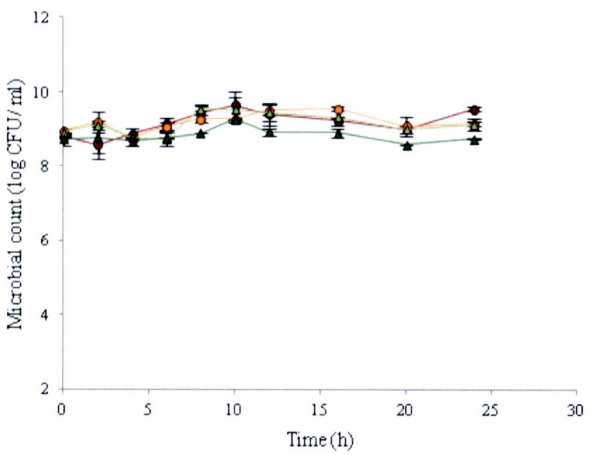
4.2.2.4 Lithium chloride

The effect of different concentration of lithium chloride on growth of *L. innocua, E. coli* and *S. aureus* was shown in Figure 4.9. The results showed that lithium chloride had no effect on inhibiting the growth of used bacteria that grown in liquid media. Lithium

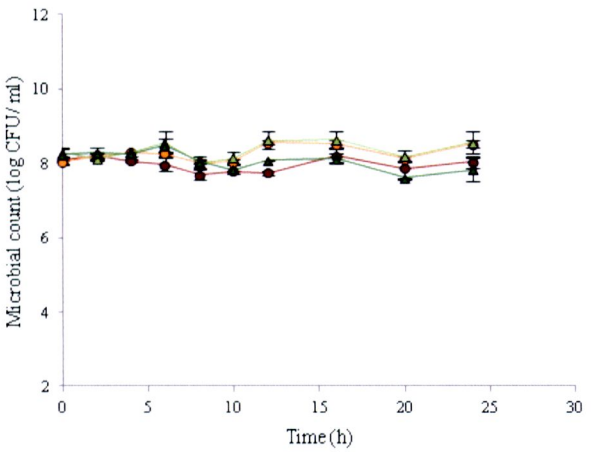
chloride provides hypertonic growth condition forcing water to diffuse from cell and rendering cell death. However *L. innocua,* and *S. aureus* is salt tolerant bacteria, they were able to tolerate salt concentration up to 10% w/v (Seeliger and Jonesy, 1986; Shahamat and Woodbine, 1981; Carr and Hageman, 2005), the experiment carry on liquid media that the highest concentration 20 g/L or 2% w/v which is low concentration. Thus, the destruction of used microorganisms didn't appear.



(a) *L. innocua*



(b) *E. coli*



(c) *S.aureus*

Figure 4.9 Comparison of growth profiles of (a) *L. innocua,* (b) *E. coli* and (c) *S. aureus* on lithium chloride. (—●—) lithium chloride 5 g/ L, (—●—) lithium chloride 10 g/ L, (—▲—) lithium chloride 15 g/ L and (—▲—) lithium chloride 20 g/ L.

4.3 Optimize Combination of the Effective Inhibitors

4.3.1 Experimental Description

L. innocua, E. coli and *S. aureus* cultures were used to investigate the optimum combination of inhibitors. They were prepared in TSB to reach 9 log CFU/mL in shake flasks. One hundred twenty µL. of each culture was inoculated into 1.2 mL of media that contained different formulas according to Table 4.1 in 96 deep well plate. Selective agents that were chosen from the previous experiment consisted acriflavine (5 and 10 mg/L), polymyxin B (10 and 25 mg/L) and lithium chloride (20 and 40 g/L). The experimental design and analysis of obtained data which was the initial and the final cell count of microorganisms were performed using Minitab software. The cultivation condition was to incubate 96 deep well plate at 35°C for 24 h to study the growth

kinetics. Total colony forming units were detected in the media surface of 96-micro well lids.

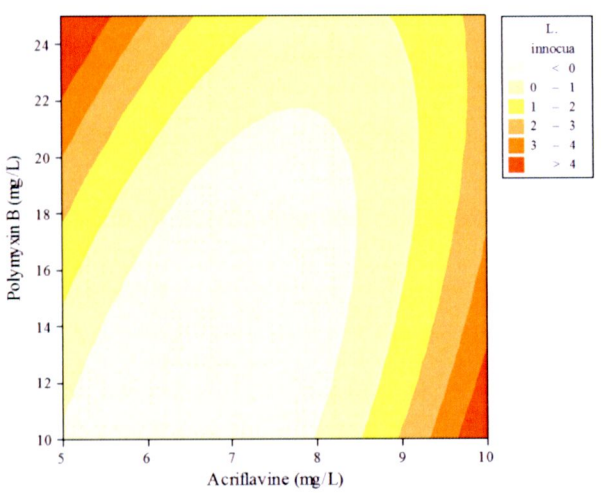
The experiments were carried out according to Box-behnken design. Box-behnken design was used to design the experiment to reduce the number of experiment from 20 experiment of Central composite design to 15 experiment of Box-behnken design. The innitial and final cell of *L. innocua, E. coli* and *S. aureus* which incubated in TSB that contained inhibitos acording to formulas from experimental design was compared (Equation 3.1) inhibitory effect and used to be resposne variable to analyse in the design of experiment using Minitab software. Table 4.1 showed inhibitory effect of *L. innocua, E. coli* and *S. aureus* from experimental design. 100 percent Inhibitory effect means that microorganism was totally inhibited.

Table 4.1 Inhibitory effect of *L. innocua, E. coli* and *S. aureus* from Box-behnken design

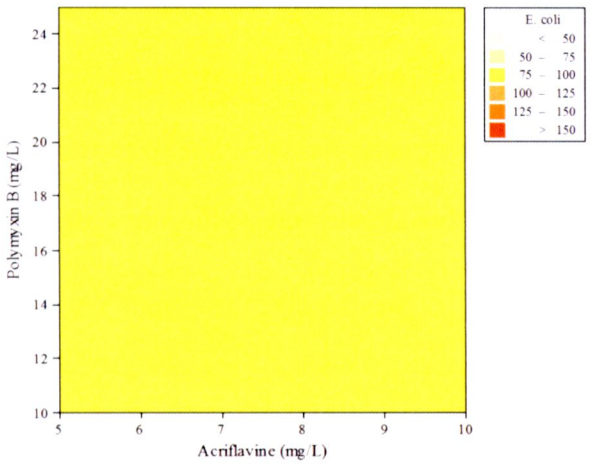
RunOrder	Acriflavine (mg/L)	Polymyxin B (mg/L)	Lithium chloride (g/L)	Percent Inhibitor Effect		
				L. innocua	*E. coli*	*S. aureus*
1	7.5	17.5	30	-3.03	100	-2.48
2	7.5	17.5	30	1.44	100	-1.48
3	5	25	30	4.32	100	-0.56
4	5	17.5	20	1.71	100	3.17
5	5	17.5	40	1.13	100	-2.39
6	10	25	30	-0.23	100	-5.70
7	7.5	10	20	-1.47	100	-0.09
8	10	10	30	4.40	100	4.46
9	7.5	25	20	0.98	100	1.97
10	7.5	10	40	0.64	100	3.70
11	5	10	30	0.43	100	2.11
12	7.5	25	40	1.06	100	-0.84
13	7.5	17.5	30	-3.62	100	-6.40
14	10	17.5	40	3.35	100	1.78
15	10	17.5	20	4.01	100	-12.38

4.3.2 Results and Discussion

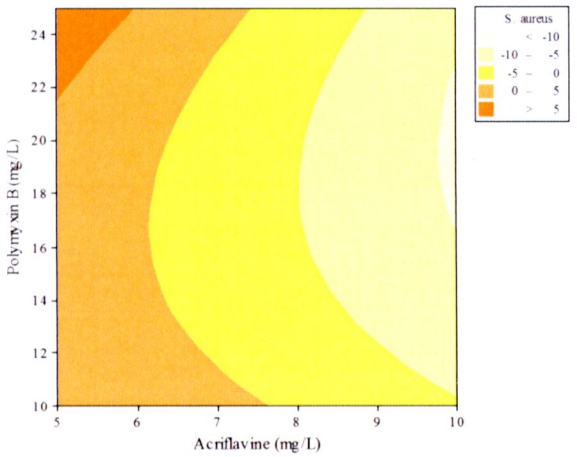
The results from experiment then were plotted in a contour format as shown in Figure 4.10 to 4.12. Figure 4.10 a) showed inhibitory effect of acriflavine and polymyxin B on *L. innicua* when the concentration of lithium chloride was stabilized. *L. innocua* was able to survive at the concentration of 8 mg/L acriflavine and 21 mg/L polymyxin B. Interestingly *L. innocua* was inhibited when the concentration of each selective agent was at the high level. *E. coli* was totally suppressed in all concentrations at the 24th hour. For *S. aureus*, the high inhibitory effect occurred at the high level of polymyxin B and low level of acriflavine. These results indicated that the use of acriflavine together with polymyxin B resulted in the decrease of inhibitory efficiency. In the sample used, acriflavine was able to bind to protein and polymyxin B, which is a highly-active polypeptide. Thus, part of acriflavine binds to polymyxin B anddecreases the inhibitory activity as a result (Beumer et al, 1996).



(a) *L. innocua*



(b) *E. coli*

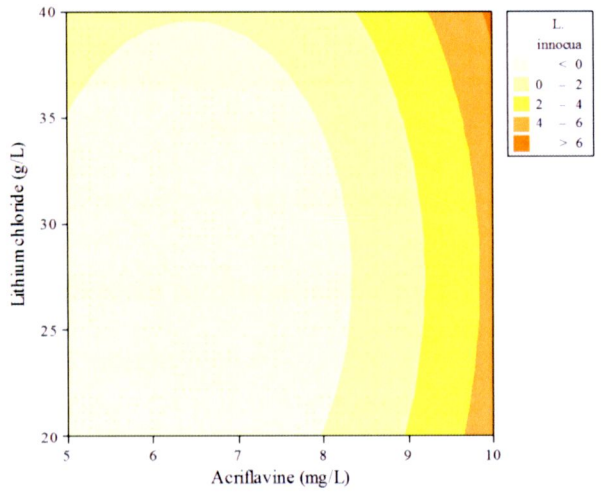


(c) *S. aureus*

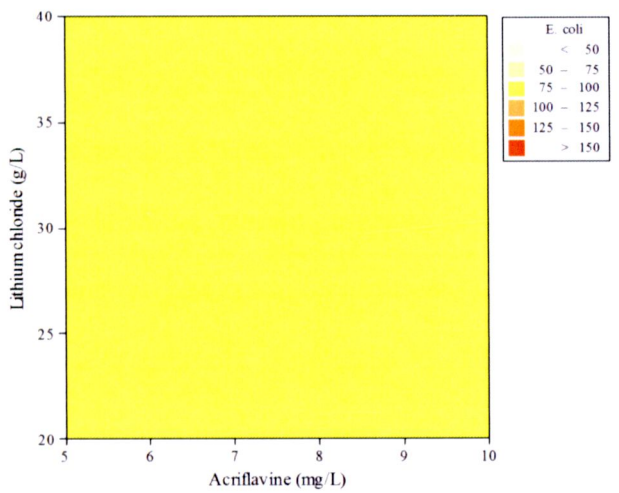
Figure 4.10 Inhibitory effects of acriflavine and polymyxin B on a) *L. innocua*, b) *E. coli* and c) *S. aureus*

At the lowest polymyxin B treatment, the effect of combination using different levels of acriflavine and lithium chloride on the growth of target microorganisms was studied. *L. innocua* was not affected by the increase of lithium chloride quantity. The inhibitory effect of *L. innocua* increased with the concentration of acriflavine. Although *E. coli* was cultured in the lowest polymyxin B, *E. coli* was absolutely repressed at 24th hour.

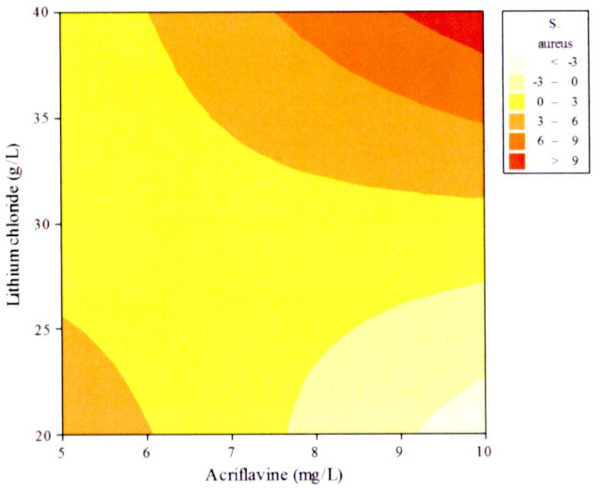
Lithium chloride affected the inhibitory effect levels of *S. aureus*. At low level of lithium chloride and high level of acriflavine, the inhibitory effect of *S. aureus* was less than 0. The inhibitory effect of *S. aureus* was higher than 9 when applied to treatments that contained both the high levels of lithium chloride and acriflavine.



(a) *L. innocua*



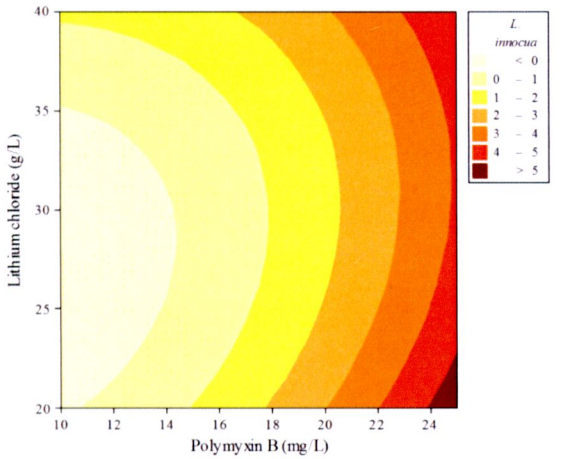
(b) *E. coli*



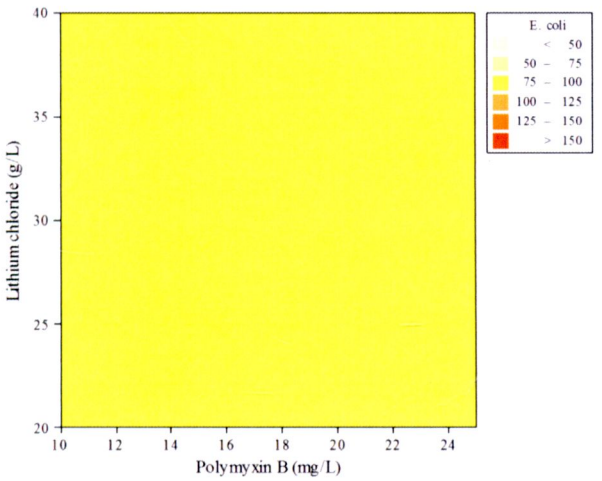
(c) *S. aureus*

Figure 4.11 Inhibitory effects of acriflavine and lithium chloride on a) *L. innocua*, b) *E. coli* and c) *S. aureus*

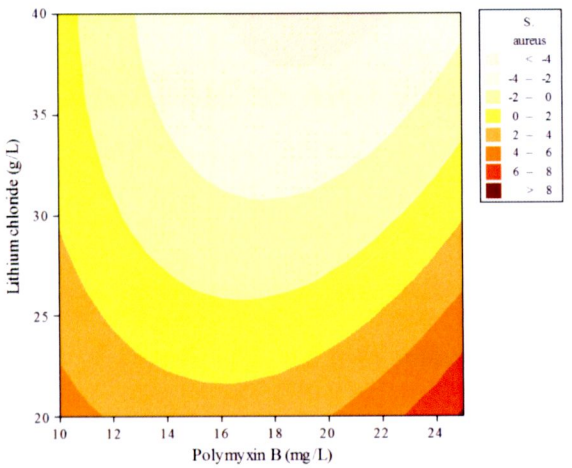
At the lowest acriflavine concentration, different levels of lithium chloride and polymyxin B acted on the growth of each microorganism in different ways. Polymyxin B was effective in restraining the expansion of *L. inoccua*, while increasing of lithium chloride hardly had any effect on *L. innocua.* Again, *E. coli* was terminated in all treatments in these experiments.. Interestingly, the quantity of lithium chloride added in the treatment was very effective when considered in conjunction with the use of polymyxin B. In increasing the concentration of lithium chloride, the inhibitory level of *S. aureus* was decreased. In addition, the highest level of inhibitory effect was at the highest concentration of polymyxin B and lowest level of lithium chloride.



(a) *L. innocua*



(b) *E. coli*



(c) *S. aureus*

Figure 4.12 Inhibitory effects of polymyxin B and lithium chloride on a) *L. innocua*, b) *E. coli* and c) *S. aureus*

The contour plot revealed that the concentration of each inhibitor in combination affected the inhibitory activity of each target microorganism. Thus, response optimizer tool in Minitab software was used to optimize the optimum inhibitors combination. The optimization was carried out by defining minimum inhibitory effect for *L. innocua*, maximum inhibitory effect for *E. coli* and *s. aureus*. The analysis revealed the optimum combination, including 5.7 mg/L acriflavine , 10.0 mg/L polymyxin B and 20.7 g/L lithium chloride. The predicted inhibitory effect of microorganisms was as follows; *L. innocua* = -1.00, *E. coli* = 100.00 and *S. aureus* = 3.504. The composite desirability was 0.517. This combination was similar to the formula of Palcam Broth that contained 5 mg/L acriflavine and 10 mg/L polymyxin B except for 10 g/L lithium chloride.