Al-Kufa University Journal For Biology: 2023. Vol. 15 No. 3 P: 187 -195
ISSN (Print)2073-8854 ISSN (Online): 2311-6544

Original Research Paper: *Journal homepage : https://journal.uokufa.edu.iq/index.php/ajb/index*

Effect of Some culture conditions and medium composition on bacteriocin Production from *Lactobacillus helveticus* DF

Doaa Mahmood Mahdy[1*]
Wala'a Shawkat Ali[2]
Nibras Nazar Mahmood[3]

[1,3] Department of Biology, College of Science, Almustansiriyah University, Baghdad, Iraq
[2] Department of Biology, College of Science, University of Baghdad, Baghdad, Iraq

Article history
Received: 11 / 6 /2023
Revised: 12 / 9 /2023
Accepted: 18 / 9 /2023
DOI: 10.36320/ajb/v15.i3.12961

**Corresponding Author:*

Emails: dalala.alesawi@uokufa.edu.iq

Abstract: *The objective of this study was to evaluate the effects of some culture conditions and components of the culture medium on the production of bacteriocin from Lactobacillus helveticus DF to get maximum bacteriocin yield. The results showed that the best culture conditions for maximum bacteriocin production were; pH 6, incubation temperature 37°C, incubation period 48 hours and the best culture medium MRS at anaerobic conditions. Optimization of the medium ingredients was also investigated. The production of active antimicrobial peptide bacteriocin, active against Gram-positive and Gram-negative microorganisms including P.aeruginosa (B) and E. Feacalis (A), by L. helveticus DF isolated from feces of infants is influenced by complex nitrogen sources and carbon sources in the production medium. Medium components, especially peptone, beef extract and yeast extract, and their concentration contributed to an increase in the production during the stationary phase. The Optimal nitrogen sources for their production were 1.5%, 2%, 2% (w/v) of peptone, beef extract and yeast extract respectively, accordingly, the present result evidenced that the increment in bacteriocin production was attributed to nitrogen source. Bacteriocin production by L. helveticus DF emphasized that the higher bacteriocin yield was attained in the medium supplied with Tween 80 compared to the medium without the addition of tween 80, the optimal concentration for their production 0.75%. Carbon source supplementation in culture media favored the maximum bacteriocin yield by L. helveticus DF, maximum bacteriocin yield when cultured in MRS supplemented with glucose and mannose (2%w/v). Bacteriocin production was affected differently by the presence of different concentrations of K2HPO4 or KH2PO4. Optimal bacteriocin production was recorded in the presence of 0.25% K2HPO4.*

***Keywords*:** *Lactic acid bacteria (LAB), L. helveticus DF, Bacteriocin, optimization*

1. Introduction

Lactic acid bacteria (LAB) are a kind of microorganisms that have great economic importance to the food biotechnology sector as well as in the production of other health supplements [1].. *Lactobacilli* are one of the frequently used commercial probiotic groups within LAB and are considered to play a beneficial role in the human and animal intestinal tract [2]. Many species of *Lactobacillus*, used in the manufacture of fermented dairy products, which inhibit the growth of other bacteria including intestinal pathogens and spoilage organisms could be attributed to the production of antimicrobial compounds including organic acids, hydrogen peroxide, antibiotics and bacteriocins [3]. Bacteriocin is a small proteinaceous compound with low- molecular mass, consisting of 30 to 60 amino acids synthesized by bacterial ribosomes, and extracellularly excreted to inhibit or kill other bacterial strains, Bacteriocin production is a desirable trait among LAB from the perspective of controlling microbial population in fermented foods to extend product shelf –life and safety [4,5]. Optimum growth and bacteriocin production depend on various factors: culture conditions such as pH, temperature, agitation and type of medium in addition to other factors including carbon and nitrogen sources [6,7,8]. These parameters are generally specific to producer strains [6]. Optimization of bacteriocin production and improvement of its activity may have great economic significance. The production of bacteriocin is growth associated because production occurs during the mid-exponential phase and increases to reach a maximal level at the end of the exponential phase or the beginning of the early-stationary phase [9]. Moreover, several reports on bacteriocin have been published, but only limited information is available for bacteriocins produced by *Lactobacillus helveticus.* Therefore, the study aimed to evaluate the influence of certain growth conditions and medium composition on the bacteriocin production by *L. helveticus* to formulate an appropriate medium for bacteriocin production by this isolate.

2- Methodology

Bacterial isolates

Bacteriocin producer

The bacterial isolate *Lactobacillus helveticus* DF which was previously isolated from feces was used for bacteriocin production. Producer isolate was preserved in MRS broth plus 20% of glycerol and inoculated with fresh bacteria, incubated at 37°C for 48 hours with anaerobic conditions. Then kept at -20°C and subcultured every 6 months.

Indicator bacteria

A total of two bacterial isolates; one isolate of *Pseudomonas aeruginosa* and one isolate of *Enterococcus feacalis* were used as indicator bacteria. They were preserved in brain heart infusion agar slants, inoculated with an 18-hours old culture of bacterial isolates and incubated at 37°C for 24 hours. Then kept at 4°C and sub-cultured monthly.

Effect of some conditions on bacteriocin production

- **Effect of culture medium**

The four-culture media included: DeMan Rogosa Sharpe broth (MRSB), brain heart infusion broth (BHIB) and acetate medium of Rogosa were inoculated with fresh broth culture of *L. helveticus* DF and incubated anaerobically at 37°C for 48 hours. [10]

- **Effect of medium composition**

The fresh broth culture of *L. helveticus* DF was used to inoculate the following media and incubated anaerobically at 37°C for 48 hours:

A- The best culture medium without tween 80 was supplemented with (0.1, 0.2, 0.25, 0.5, 0.75 and 1) % of tween 80, respectively.

B- The best culture medium without organic nitrogen sources was supplemented with (0.25 0.5, 1, 1.5, 2 and 2.5) % of peptone, beef extract and yeast extract, respectively.

C- The best culture medium without potassium phosphate was supplemented with (0.1, 0.2, 0.25 and 0.5) % of KH_2PO_4 and K_2HPO_4, respectively.

D- The best culture medium without glucose was supplemented with 2% of glucose, lactose, maltose, mannitol, sucrose, fructose and mannose, respectively.

- **Effect of medium pH**

The best culture medium was adjusted to pH 4, 4.5, 5, 5.5, 6, 6.5, 7, 8 and 9 respectively, and inoculated with fresh broth culture of *L. helveticus* DF and incubated anaerobically at 37°C for 48 hours.

- **Effect of Inoculum Size**

The best culture medium was inoculated with (10^4, 10^5, 10^6, 10^7, 10^8, 10^9 and 10^{10}) cell/ml of fresh broth culture of *L. helveticus* DF respectively and incubated anaerobically at 37°C for 48 hours.

- **Effect of incubation temperature**

The best culture medium was inoculated with fresh broth culture of *L. helveticus* DF and incubated anaerobically at (30, 35, 37 and 40) °C respectively for 48 hours.

- **Effect of the incubation period**

The best culture medium was inoculated with fresh broth culture of *L. helveticus* DF and incubated anaerobically at 37°C for 18, 24, 48 and 72 hours.

- **Effect of incubation conditions**

The best culture medium was inoculated with fresh broth culture of *L. helveticus* DF and incubated anaerobically and aerobically at 37°C for 48 hours.

Bacteriocin activity assay

Bacteriocin activity was detected by serial twofold dilutions of cell-free supernatant (crude bacteriocin) and determined against indicator bacteria using agar well diffusion assay. The arbitrary unit (AU) was calculated according to [11], and bacteriocin was concentrated according to [12].

3. Results and Discussion

L. helveticus DF was subjected to different optimization experiments to determine the best condition for bacteriocin production, also the influence of culture medium components on the production of bacteriocin was investigated using more sensitive pathogenic bacteria to bacteriocin include E. *feacalis* (A) & *p.aeruginosa* (B) as an indicator organism.

As shown in Figure (1) different culture media (MRSB, SL broth and BHIB) were tested to select the one that can support the maximum production of bacteriocin. The results showed that the MRS was the best medium for the bacteriocin production which gave the highest antagonistic activity against *P.aeruginosa* B and *E. feacalis* A with diameters (17.5 and12.5) mm respectively, followed by SL broth medium, which recorded (14 and 8) mm respectively against the same indicator bacteria above. On the other hand, no production was occurred in BHIB, which is a nutrient medium suitable for the growth of a variety of microorganisms but not suitable for the growth and production of bacteriocin from *Lactobacillus* spp. However, the MRS medium is a better one for cell growth and bacteriocin production.

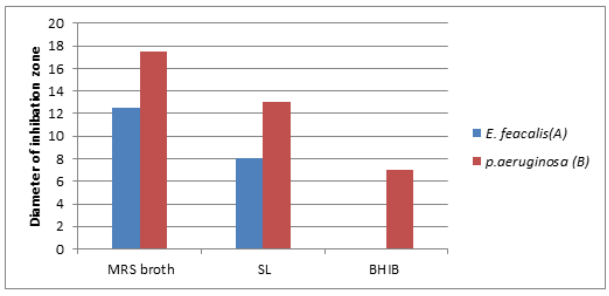

Figure (1): Antagonistic activity of *L. helveticus* DF against indicator bacteria in different culture media (MRSB: De Man Rogosa Sharp, SL: Acetate medium of Rogosa, BHIB: Brain Heart Infusion Broth)

As shown in Figure (2) there was an increase in bacteriocin production as a result of the addition in different concentrations (0.5 to 1) %, Tween 80 strongly affected the production of bacteriocin, the highest agoniantagonistic activity was recorded at 0.75% concentration, the diameter of inhibition zone at this concentration aswas (31 and 19) mm against *p.aeruginosa* (B) and *E. Feacalis* (A) respectively.

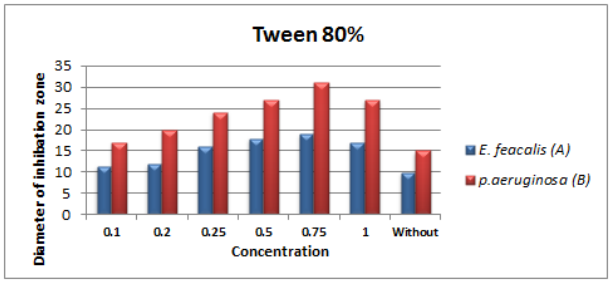

Figure (2): Effect of tween 80 on production of bacteriocin produced by *L. helveticus* DF.

In this study, different nitrogen sources such as Pepton, yeast extract and meat extract were used as ingredients to increase the production of bacteriocin in the media against indicator bacteria (*E. Feacalis* (A) and *p.aeruginosa* (B).

As shown in Figure (3), the addition of peptone in concentration 1.5% has a great impact on bacteriocin production during the growth of *L. helveticus* DF, the result showed from this experiment noticeable increase in antimicrobial activity to (24 and 15.5) mm against *p.aeruginosa* (B) and *E. Feacalis* (A), in comparison with the medium without the addition of peptone.

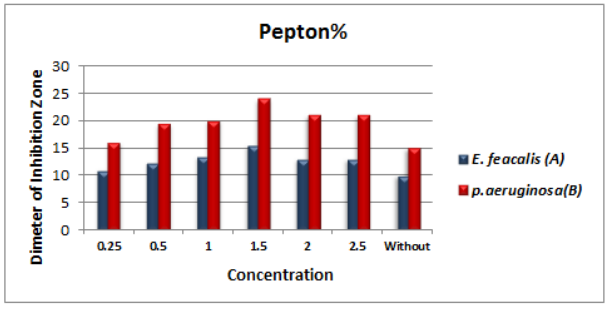

Figure (3): Effect of peptone on the production of bacteriocin produced from *L. helveticus* DF

As shown in Figure (4) the effect of different concentrations of beef extract on producing of bacteriocin from *L. helveticus* DF which gives the highest antimicrobial activity at 2% concentration with a diameter of inhibition zone (22 and 15) mm against P.*aeruginosa* (B) and *E.feacalis* (A) respectively. The optimum concentration of beef extract for producing bacteriocin from L. *helveticus* DF was 2%.

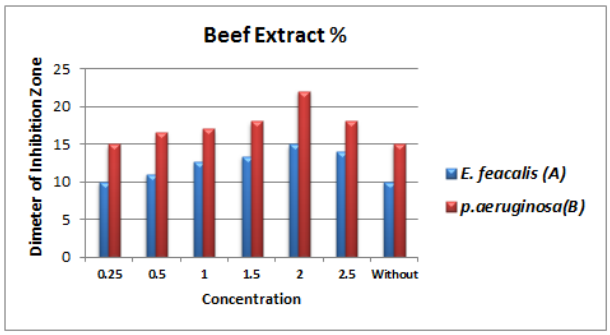

Figure (4) Effect of beef extract on the production of bacteriocin produced by *L. helveticus* DF

As shown in Figure (5), bacteriocin production from *L. helveticus* DF was tested with different concentrations of yeast extract, and a pronounced increase in the antimicrobial activity of bacteriocin and bacterial growth in the media supplemented with different concentrations of yeast extract was observed in comparison with the medium without the addition of yeast extract. An increase in antimicrobial activity to (26 &16.5) mm against P.*aeruginosa* (B) and *E. Feacalis* (A) respectively at 2% concentration. The optimum concentration of yeast extract for producing bacteriocin from L. *helveticus* DF was 2%.

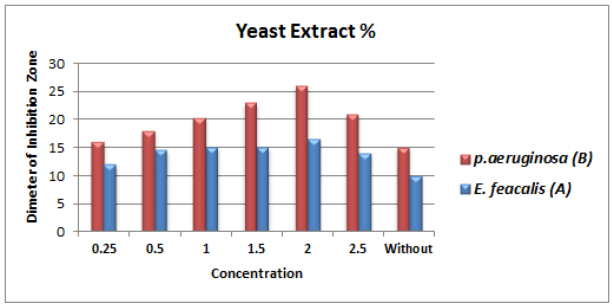

Figure (5): Effect of yeast extract on the production of bacteriocin produced by *L. helveticus* DF

Bacteriocin production was affected by the addition of different concentrations of K2HPO4 or KH2PO4. As shown in figure (6), the addition of K2HPO4 in different concentrations to the medium due to an increase the antimicrobial activity of bacteriocin to (22 & 15) mm against P.*aeruginosa* (B) and *E. feacalis* (A) respectively at 0.25 concentration. K2HPO4 contains a phosphorus source which is very important for cell growth and thus bacteriocin production.

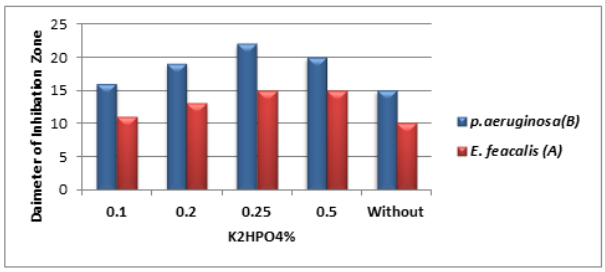

Figure (6) Effect of K2HPO4 on production of bacteriocin produced by *L. helveticus* DF

The highest antimicrobial activity was (26 and 17) mm against P.*aeruginosa* (B) and *E. Feacalis* (A) respectively at 2% glucose concentration whereas maltose, mannitol and fructose did not affect the production of bacteriocin from producer isolate. At a 2% concentration of mannose, the diameter of the inhibition zone was (23and 15) against the same indicator above.

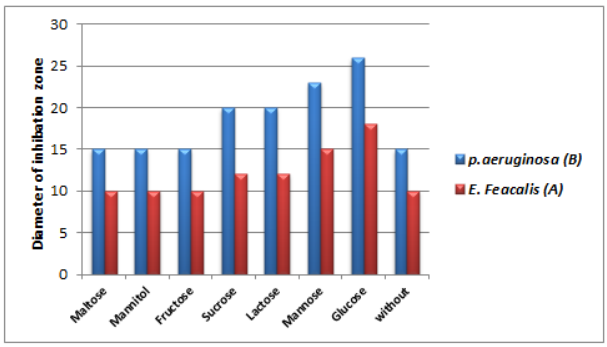

Figure (7) Effect of different carbon sources on production of of bacteriocin produced by *L. helveticus* DF.

The maximum production of bacteriocin and bacterial growth of *L. helveticus* DF was found at pH values 5.5 and 6 with high antagonistic activity against more sensitive bacterial isolate (*E. Feacalis* (A) and *P.aeruginosa* (B). The diameters of inhibition zones were (16&13) mm at pH 5.5 against *P.aeruginosa (B), and E. feacalis* (A)respectively, (19&14.6) mm at pH 6 against the same indicator bacteria above. At alkaline pH *L. helveticus* DF showed a sharp decline in the production of bacteriocin.

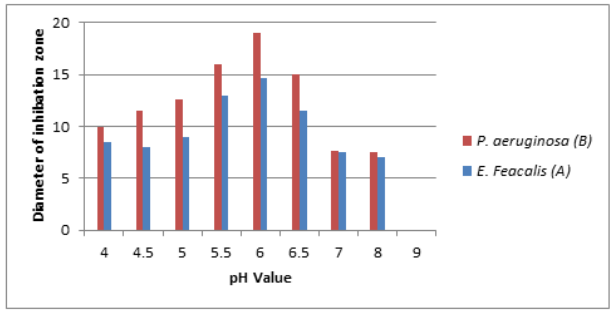

Figure (8) Influence of different pH values on bacteriocin production *L. helveticus* DF.

Bacteriocin production from *L. helveticus* DF increase by inoculation of culture medium with large numbers of bacterial cells, as shown in figure (9) it was observed from this experiment that optimum production when the number of the bacterial cells was 1×10^9 cell/ml with diameter of inhibition zone (21&15) mm against *P.aeruginosa* (B)& *E. Feacalis (A)* respectively.

The low number of bacterial cells recorded a reduction in bacteriocin production with a diameter of inhibition zone (12& 8) mm against the same indicator bacteria above at 1×10^6 cell/ml. The results demonstrate a strong relationship of inoculum size with the production of bacteriocin, inoculum size of 4% of cells number 1×10^9 cell/ml fostered the best production of bacteriocin.

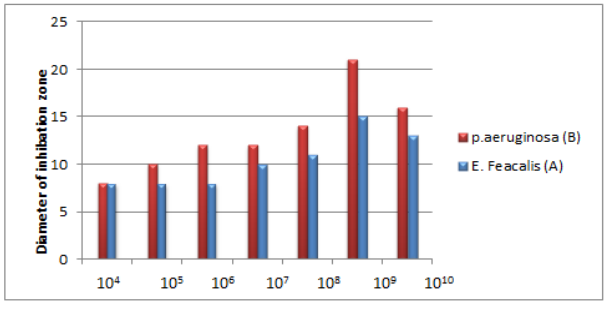

Figure (9): Influence of the number of cells (10^4 -10^{10}) cell/ml on bacteriocin production *L. helveticus* DF

Figure (10) showed the impact of different temperatures on the production of bacteriocin by *L. helveticus* DF, the result showed from the experiment that the highest production of bacteriocin at 37 °C with a diameter of inhibition zones (19&14) against *P.aeruginosa (B)* and *E. Feacalis (A)* respectively**.** At 35°C, showed a noticeable decrease in antimicrobial activity giving a (15&11,) mm against *E. Feacalis* (A) and *P.aeruginosa* (B) respectively**.**

At 40 °C the diameter of inhibition zones (14&10) mm against *P.aeruginosa (B)and E. feacalis (A)* respectively**,** is evidence that *L. helveticus* DF is capable of producing bacteriocin at high temperatures. The lowest production of bacteriocin was observed at 30 °C giving a (10.5&8) mm against the same indicator bacteria above**.** A decrease in temperature led to a decrease in the bacteriocin antimicrobial activity of L. *helveticus* DF. The optimum temperature for the growth and bacteriocin production is 37 °C.

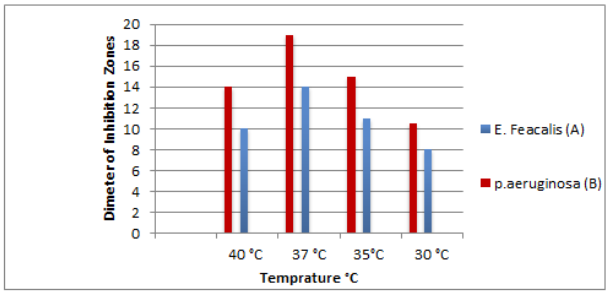

Figure (10): Effect of different temperatures on the production of bacteriocin from *L. helveticus* DF

The evolution of the bacteriocin production from this isolate was followed up at different incubation periods, as shown in Figure (10) it was observed that optimum production at the end of 48 hours with a diameter of inhibition zone range between (18&12) mm against *p.aeruginosa (B) and E. Feacalis (A)* respectively, while no inhibition zones were recorded at 18h. At 24h diameter of the inhibition zone ranged between (11&9) mm against *P.aeruginosa (B)* and *E. feacalis (A)*respectively, and at 72h a noticeable decrease in antimicrobial activity giving (14.5&10) mm against the same indicator bacteria above.

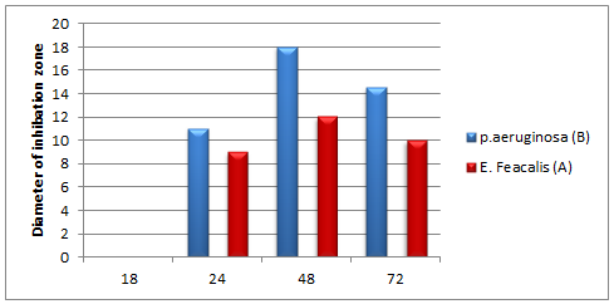

Figure (12): Effect of the incubation period (hours) on bacteriocin production from L. *helveticus* DF.

The medium composed of 1.5 % peptone + 2% yeast extract + 2% beef extract + 0.25% K2HPO4, 0.75% tween 80, 2% glucose, 2% mannose, prepared in this study referred to as Modified MRS was used in all subsequent experiments in this study. with pH 6, at 37°C incubation temperature for 48 hours under anaerobic conditions and inoculum size of 4% of cells number 1× 10^9 cell/ml fostered the best production of bacteriocin by *L. helveticus* DF.

Antibacterial activity of bacteriocin

After optimization and the use of Modified MRS for the production of bacteriocin from producer isolate, Bacteriocin activity was increased to 80 and 160 AU\ML against *E. feacalis* (A) and *P.aeruginosa (B)* respectively, also the specific activity of bacteriocin recoded 390.24 U/ mg.as in figure (13).

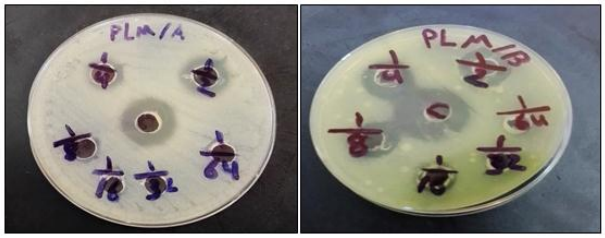

Figure (13) Bacteriocin activity 160 AU/ML against P.*aeruginosa* (B) Bacteriocin activity 80 AU/ML against *E. feacalis (A)*

Under optimized conditions, the probiotic *L.helveticus DF* produced a higher amount of bacteriocin that effectively inhibited the growth of Gram-negative and Gram-positive bacteria. The production of bacteriocin in MRS due to the presence of Tween 80, dipotassium phosphate and the mixture of different carbon and nitrogen sources that induce *Lactobacillus* for the production of bacteriocin,Simliar result was observed by [13,14] who found that MRS broth gave the highest bacteriocin production by *Lactobacillus*. Tween 80 had positive effects on the production of bacteriocin, which is consider as an emulsifier that possibly decreases the surface tension of cell membranes. Therefore, some of the metabolites, such as bacteriocins, can be released more smoothly in vitro [15].

The observation from this experiment was that yeast extract resulted in a significant increase in bacteriocin production, due to it providing a larger proportion of free amino acid and more growth factors than other protein hydrolysates [16]. The result of this study was in agreement with [17], which found from their studies that yeast extract has great impact on the production of bacteriocin from *L.curvatus*. *Lactobacillus* strains are nutritionally fastidious bacteria their growth and bacteriocin production are influenced by organic nitrogen sources. However, any additional increase in the concentration of nitrogen sources resulted in significant changes in their growth and bacteriocin production [18]. Sometimes, the type of nitrogen source does not affect the bacteriocin production as shown by the study [19]. K2HPO4 contains a phosphorus source which is very important for cell growth and thus bacteriocin production. Similar results have been recorded by [20]. The observation from this experiment indicated that the production of bacteriocin from producer isolate was affected by the type of carbon source and the best carbon source was glucose and mannose. Glucose is considered the main carbon source by all microorganisms due to its molecular weight, rapid uptake, utilization and cellular energy conversion.

Also, growth culture condition has a great impact on bacteriocin production, Similar observations were made by [21] who reported that bacteriocin produced by *L. helveticus* had maximum activity at acidic pH. The pH of the medium appears to be an important factor that affects bacteriocin production possibly indicating that it has a regulatory effect driven by the ionic conditions of the medium or that it may have a general effect on the cell envelope [22].

Number of the bacterial cells plays an important role in bacteriocin production where the optimum inoculate size is favorable for the highest productivity. The optimal size of inoculate may vary from strain producers due to their cell proliferation rate, ability to metabolize medium, medium size and nutrient composition [15]. The optimum temperature for the growth and bacteriocin production is 37 °C. A similar result was observed by [23]

Conclusion

In the present study, there is a clear correlation between bacteriocin production and *L. helveticus* DF inoculum size. Variation in bacteriocin production due to indicator strain. Optimization of bacteriocin production by modification of medium composition and culture conditions will greatly benefit commercial and clinical applications for subsequent steps in this study.

References

1. 1 Riaz Rajoka, M. S., Shi, J., Zhu, J., Shao, D., Huang, Q., Yang, H., & Jin, M. (2017). Capacity of lactic acid bacteria in immunity enhancement and cancer prevention. *Applied microbiology and biotechnology*, *101*, 35-45.
2. Lin, X., Chen, X., Tu, Y., Wang, S., & Chen, H. (2017). Effect of probiotic lactobacilli on the growth of Streptococcus mutans and multispecies biofilms isolated from children with active caries. *Medical science monitor: international medical*

journal of experimental and clinical research, *23*, 4175.

3. Nissen, L., Chingwaru, W., Sgorbati, B., Biavati, B., & Cencic, A. (2009). Gut health promoting activity of new putative probiotic/protective Lactobacillus spp. strains: a functional study in the small intestinal cell model. *International journal of food microbiology*, *135*(3), 288-294.
4. Moradi, M., Molaei, R., & Guimarães, J. T. (2021). A review on preparation and chemical analysis of postbiotics from lactic acid bacteria. *Enzyme and Microbial Technology*, 143, 109722.
5. Raval, V., Patel, J., Raol, G., Bhavsar, N., Surati, V., Gopani, Y., & Vaidya, Y. (2020). Evaluation of bacteriocin purified from potent lysinibacillussphaericus mk788143 isolated from infant faecal. *Journal of Advanced Scientific Research*, 11.
6. Aidani, E., Delfan, P., Akbarian, M., Akbarian, A., Ghasemkhani, N., & Moayedi, F. (2014). Genomics of lactic acid bacteria: features, function, and comparative genomics: a review. *Int J Biosci*, *5*(4), 34-46
7. Kumar, M., Jain, A. K., Ghosh, M., & Ganguli, A. (2012). Statistical optimization of physical parameters for enhanced bacteriocin production by L. casei. *Biotechnology and Bioprocess Engineering*, *17*, 606-616
8. Bali, V., Panesar, P. S., Bera, M. B., & Kennedy, J. F. (2016). Bacteriocins: recent trends and potential applications. *Critical reviews in food science and nutrition*, *56*(5), 817-834.
9. Garsa AK, Kumariya R, Sood SK, Kumar A, Kapila S. (2014). Bacteriocin production and different strategies for their recovery and purification. Probiotics Antimicrob Proteins. 6(1):47–58. doi: 10.1007/s12602-013-9153- z. [PubMed: 24676767].
10. Kandler, O. and Weiss, N. 1986)"Genus Lactobacillus. In : Bergey's Manual of Systematic Bacteriology", Vol. 2. (edited by Sneath, P.H. A.; Mair, N.S. and Hold, J.G.), William and Wilkins Co., Baltimore, USA(
11. Pilasombut, K., Sakpuaram, T., Wajjwalku, W., Nitisinprasert, S., Swetwiwathana, A., Zendo, T., ... & Sonomoto, K. (2006). Purification and amino acid sequence of a bacteriocins produced by Lactobacillus salivarius K7 isolated from chicken intestine. *Songklanakarin J Sci Technol*, *28*(Suppl 1), 121-131.
12. Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical biochemistry*, *72*(1-2), 248-254.
13. Nouralhuda A. H. and K. Luti (2022). Evaluation of in vitro antimicrobial activity of Lactobacillus parabuchneri as a potential probiotic in toothpaste against some oral pathogens, University of Baghdad.
14. Ali, W.S. and Musleh, R.M.(2011). Determination of Optimum Conditions for Plantaricin VGW8 Production by Lactobacillus plantarum VGW8. *Al-Mustansiriyah J. Sci*. Vol. 22, No. 4.
15. Lajis, A. F. B. (2020). Biomanufacturing process for the production of bacteriocins from Bacillaceae family. *Bioresources and Bioprocessing*, *7*, 1-26.
16. Aasen, I. M., Møretrø, T., Katla, T., Axelsson, L., & Storrø, I. (2000). Influence of complex nutrients, temperature and pH on bacteriocin production by Lactobacillus sakei CCUG 42687. *Applied Microbiology and Biotechnology*, *53*, 159-166.
17. Foudjing, G. G. D., Sarmast, E., Allahdad, Z., Salmieri, S., & Lacroix, M. (2023). Influence of growth parameters on bacteriocin-like inhibitory substances (BLIS) production by lactic acid bacteria. *Letters in Applied Microbiology*, *76*(1), ovac013.
18. Kim, W. S., Hall, R. J., & Dunn, N. W. (1997). The effect of nisin concentration and nutrient depletion on nisin production

of Lactococcuslactis. *Applied Microbiology and Biotechnology*, *48*, 449-453.

19. Todorov, S., Gotcheva, B., Dousset, X., Onno, B., & Ivanova, I. (2000). Influence of growth medium on bacteriocin production in Lactobacillus plantarum ST31. *Biotechnology & Biotechnological Equipment*, *14*(1), 50-55.
20. Zhang, J., Bu, Y., Zhang, C., Yi, H., Liu, D., & Jiao, J. (2020). Development of a low-cost and high-efficiency culture medium for bacteriocin lac-b23 production by Lactobacillus plantarum j23. *Biology*, *9*(7), 171.
21. Joerger, M. C., & Klaenhammer, T. R. (1986). Characterization and purification of helveticin J and evidence for a chromosomally determined bacteriocin produced by Lactobacillus helveticus 481. *Journal of bacteriology*, *167*(2), 439-446.
22. Bárcena, J. M. B.; Sińeriz, F.; De Liano, D. G.; Rodriguez, A. and Suárez, J.E. (1998). Chemostate production of plantaricin C by Lactobacillus plantarum LL441. Appl. Environ. Microbiol., 64(9): 3512– 3514.
23. Yang, E., Fan, L., Yan, J., Jiang, Y., Doucette, C., Fillmore, S., & Walker, B. (2018). Influence of culture media, pH and temperature on growth and bacteriocin production of bacteriocinogenic lactic acid bacteria. *AMB express*, *8*(1), 1-14.