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RESEARCH ARTICLE

CHARACTERIZATION OF LACTOCIN LC-09 PRODUCED BY *LACTOBACILLUS ACIDOPHILUS*

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ABSTRACT

The kinetics of lactocin LC-09 synthesis and the effect on the growth of LC -09 was determined at different temperatures i.e. 37 ˚C, 40 ˚C, 45 ˚C and 50 ˚C. Of all the temperatures tested, 37 ˚C, 40 ˚C and 45 ˚C displayed bacteriocin production in the early exponential phase. However, no lactocin production was observed at 50 ˚C. Cell dry mass and μ_{max} was also determined at such temperatures. Maximum cell dry mass obtained at 37˚C and 45 ˚C and μ_{max} increased as the dry cell mass increased. Heat stability of lactocin LC-09 indicates that lactocin LC- 09 is heat stable up to 100 ˚C even heating for 50 minutes but high temperature has marked effect on lactocin production during growth. Effect of pH on lactocin LC-09 indicates that the lactocin is active at acidic pH.

INTRODUCTION

Basically lactic acid bacteria may be characterized as gram positive, catalase negative, non spore forming bacteria (Lee *et al*., 2000). The lactic acid bacteria (LAB) including the genera *Lactobacillus, Lactococcus, Leuconostoc* and *Pediococcus* have long been used in fermentations to preserve the nutritive qualities of various foods (O'Keeffe and Hill, 1999). Lactic acid bacteria also inhibits the growth of harmful putrefactive microorganisms through other metabolic products such as hydrogen peroxide, carbon dioxide, and diacetyl, lactic acid and bacteriocins (Wood., 1992). Bacteriocins are antimicrobial peptides, proteins or protein complexes produced by many bacterial species that particularly inhibit closely related strains. Of particular interest to food industry are those produced by members of lactic acid bacteria. Many bacteriocins produced by lactic acid bacteria are also active towards food spoilers and/or food borne pathogens such as *Bacilli, Clostridia, Staphylococci and Listeriae* (Klaenhammer., 1998). The best studied bacteriocin is nisin, a bacteriocin produced by certain strains of *Lactococcus lactis* subsp. *lactis,* is inhibitory to a wide range of Gram positive bacteria. Many bacteriocins have been observed to be more stable and effective at acidic pH, higher temperatures (important in the case of temperature abuse) or lower temperatures (important for refrigerated foods). Bacteriocins are considered to play a beneficial role in the development of aroma and flavor of many cheeses, probably due to their proteolytic and lipolytic activity, as well as to their activity to catabolize citrate. They are defined as starter cultures because, when added to raw material, they initiate a rapid acidification through the production of organic acids, in particular lactic acid and sometimes acetic acid. In this way they contribute to a prolong shelf - life and a pleasant profile of the end- produce (Hurst and Dring, 1968).

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MATERIALS AND METHODS

Bacterial strains and media

Lactobacilli strain LC-09, isolated from clinical sample, was used as bacteriocin producing strain. *Lactobacillus acidophilus* LC-09 used in this study was grown in MRS broth at 37 ˚C for 24 hours and other non-lactic acid bacteria were grown in nutrient broth.

Inhibitory activity of bacteriocin produced by LC-09

The antagonistic activity of LC-09 was checked against strains of *Bacillus* such as *Bacillus subtilis* and *Bacillus cereus*, by agar well diffusion assay. Overnight broth culture of producing strain was centrifuged at 3000 rpm for 20 minutes. The supernatant containing the bacterionin was collected. The lawn of indicator strain was made by inoculating the indicator strain in 4-5 ml of soft agar (0.85% agar) and then over laid on agar plates. The soft agar was allowed to solidify, thus generating a potential mat of indicator bacteria. With the help of sterile borer, wells were cut in the agar. The resulting agar buttons were removed and the wells were filled with culture

supernatant. Plates were kept in refrigerator for 2-4 hr to let the bacteriocin diffuse into the medium. The plates were then incubated at 37 ˚C for 24 hr. At the end of incubation period the plates were checked for zone of inhibition and diameter of zone were measured.

Kinetics of Lactocin LC-09 synthesis at different temperatures

The effect of temperature on growth and bacteriocin production of LC -09 was observed at 37 ˚C, 40 ˚C, 45 ˚C and 50 ˚C. LC-09 was grown in 250 ml MRS broth at 37 ˚C, 40 ˚C, 45 ˚C and 50 ˚C. At different time intervals samples were removed for demonstration of inhibitory activity and absorbance were measured at 600 nm by using spectrophotometer (Shinadzer mini 240). The inhibitory activity was measured by agar well diffusion method.

Determination of gram dry weight of cells of LC-09

For gram dry weight cell culture was centrifuged, washed and transfered into fore weighed aluminium foil and cells were dried at constant temperature in an oven. Difference between foil containing cells and empty foil was considered as Gram dry weight.

Effect of heat on Lactocin LC-09

The effect of heat on lactocin LC -09 was observed at 50 ˚C, 60 ˚C, 80 ˚C and 100 ˚C. Producing strain was grown in 150 ml MRS broth at 37 ˚C for 24 hr. Culture was then centrifuged and the supernatent was divided to study the effect of above mentioned temperatures on lactocin LC-09.

Effect of pH on Lactocin LC-09

The effect of pH on lactocin LC-09 was observed at pH 3.0, 3.5, 4.0, 4.5, 5.0, 5.5. Supernatent was divided into aliquots and pH was adjusted. Samples were removed after different intervals and activity was determined by agar well diffusion method.

Determination of LC-09 titre

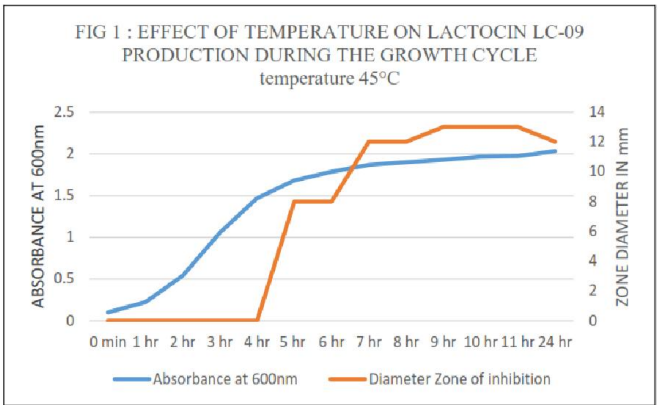
For the determination of titre of bacteriocin produced by LC-09 twofold dilution of lactocin was made (1: 2 – 1: 64) in distilled water, and agar well diffusion was performed in order to determine the highest dilution showing inhibitory activity (titre).

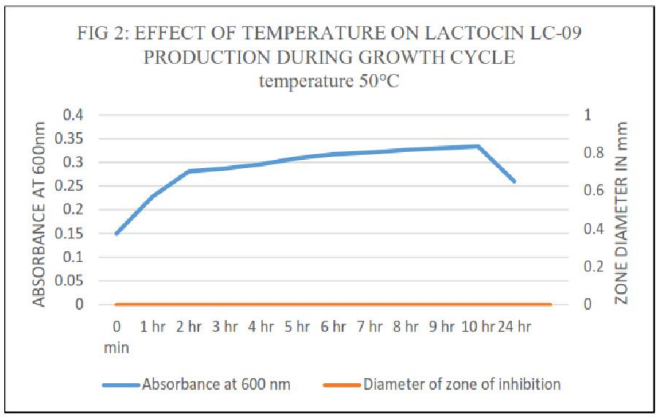
RESULTS

Lactobacillus acidophilus LC-09 was previously isolated in our lab from clinical sample capable of producing an inhibitory substance which was designated as lactocin LC-09.

Kinetics of LC-09 synthesis at different temperatures

The lactocin LC-09 production during growth cycle in MRS broth was studied at different temperatures. At 37 ˚C, 40 ˚C, 45 ˚C lactocin production was started during early exponential phase in all three temperatures. Increased volumetric production of lactocin LC- 09 was observed at 45 ˚C (Fig. 1), while absorbance was comparable at each temperature. As depicted in (Fig. 2) slow rate in absorbance and low cell turbidity was observed at 50 ˚C and consequently production of lactocin LC-09 was not observed. Cells dry mass of LC- 09 was measured during growth cycle at different temperatures i.e. at 37 ˚C, 40 ˚C, 45˚C and 50 ˚C.





The maximum cell dry mass were obtained at 37 ˚C and 45 ˚C, while absorbance found to be the same at each temperature. But low turbidity and low cell dry mass was observed at 50 ˚C. The maximal specific growth rate (μ_{max}) obtained by testing the effect of temperature (37˚ C, 40 ˚C, 45 ˚C and 50 ˚C) without pH regulation on growth of LC-09 cells (Table 1).

Table 1. Kinetic constant constant of growth

Fermentation conditions	X_{max} (g l^{-1})	μ_{max} (h^{-1})	(AU ml^{-1})	q_p (AU h^{-1})
37˚C	1.4	0.058	2000	8.3 x 10^4
40˚C	1.0	0.0416	ND	ND
45˚C	1.4	0.058	ND	ND
50˚C	0.6	0.025	ND	ND

Effect of temperature on Lactocin LC-09

Effect of temperature on lactocin LC-09 was studied against two sensitive strains *Bacillus subtilis* and *Bacillus cereus* at 50 ˚C, 60 ˚C, 80 ˚C and 100˚C. Such temperatures did not exhibit any effect on lactocin LC-09 and a consistent zone of inhibition was observed at each temperature even after heating of lactocin LC-09 for 50 minutes at 100 ˚C (Fig. 3, 4, 5, 6&10).

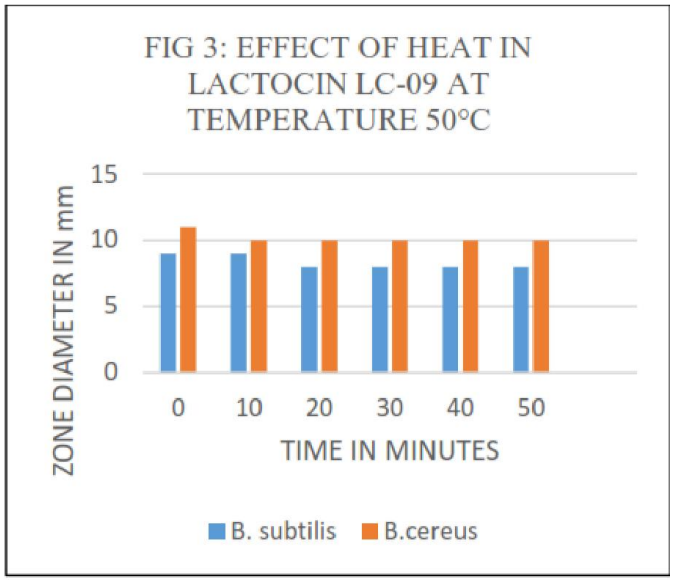


FIG 3: EFFECT OF HEAT IN LACTOCIN LC-09 AT TEMPERATURE 50°C
ZONE DIAMETER IN mm
15
10
5
0
0 10 20 30 40 50
TIME IN MINUTES
B. subtilis
B.cereus

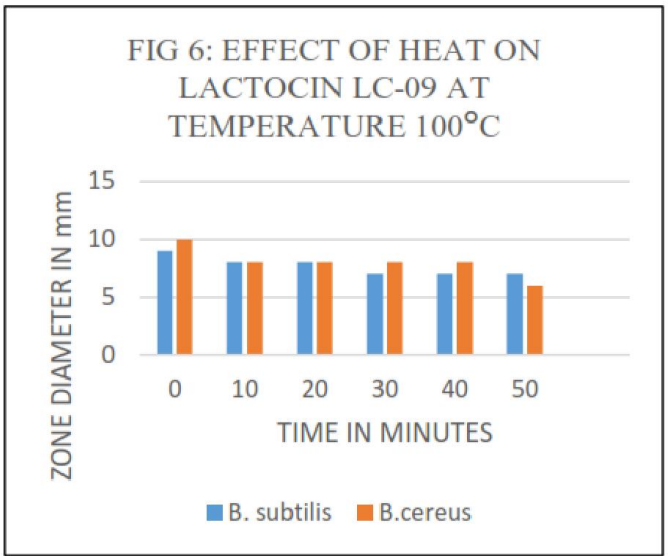


FIG 6: EFFECT OF HEAT ON LACTOCIN LC-09 AT TEMPERATURE 100°C
ZONE DIAMETER IN mm
15
10
5
0
0 10 20 30 40 50
TIME IN MINUTES
B. subtilis
B.cereus

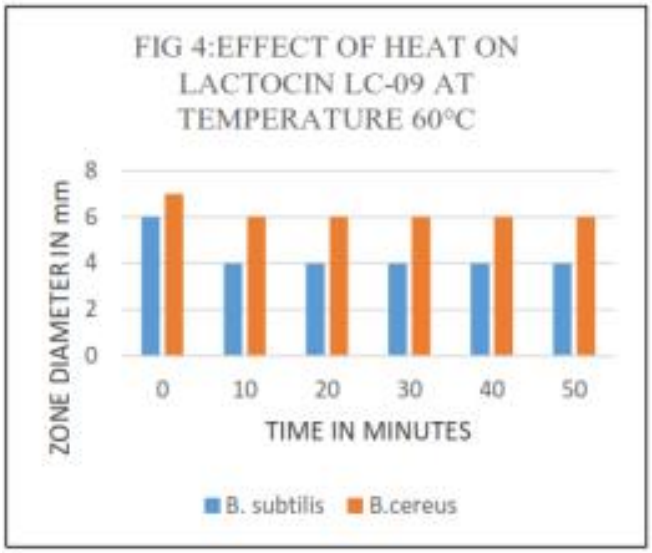


FIG 4:EFFECT OF HEAT ON LACTOCIN LC-09 AT TEMPERATURE 60°C
ZONE DIAMETER IN mm
8
6
4
2
0
0 10 20 30 40 50
TIME IN MINUTES
B. subtilis
B.cereus

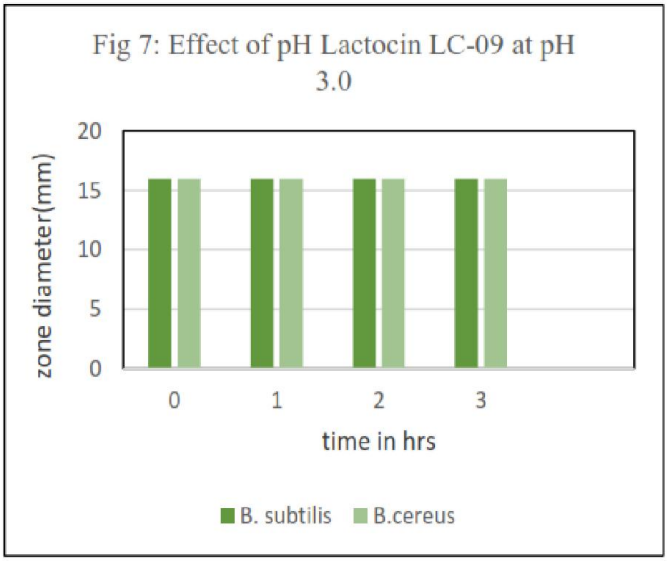


Fig 7: Effect of pH Lactocin LC-09 at pH 3.0
zone diameter(mm)
20
15
10
5
0
0 1 2 3
time in hrs
B. subtilis
B.cereus

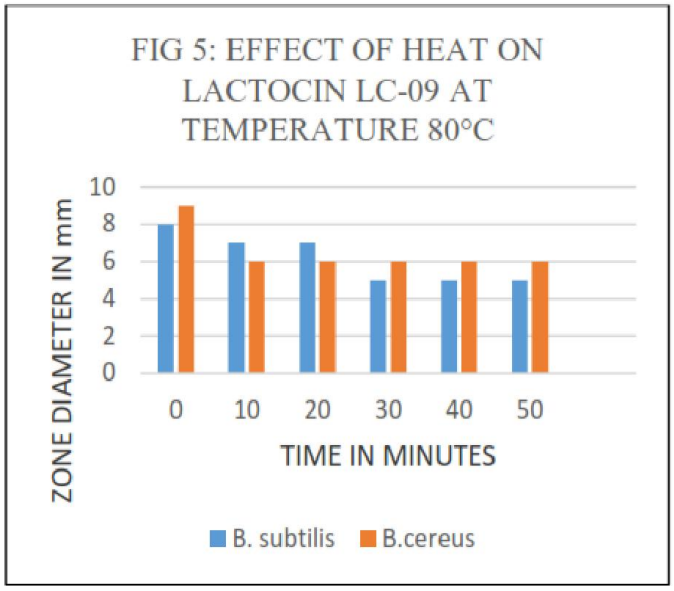


FIG 5: EFFECT OF HEAT ON LACTOCIN LC-09 AT TEMPERATURE 80°C
ZONE DIAMETER IN mm
10
8
6
4
2
0
0 10 20 30 40 50
TIME IN MINUTES
B. subtilis
B.cereus

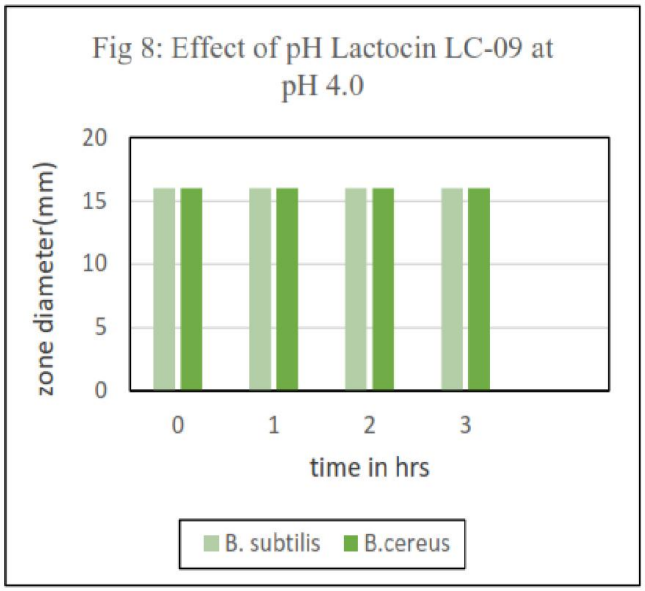
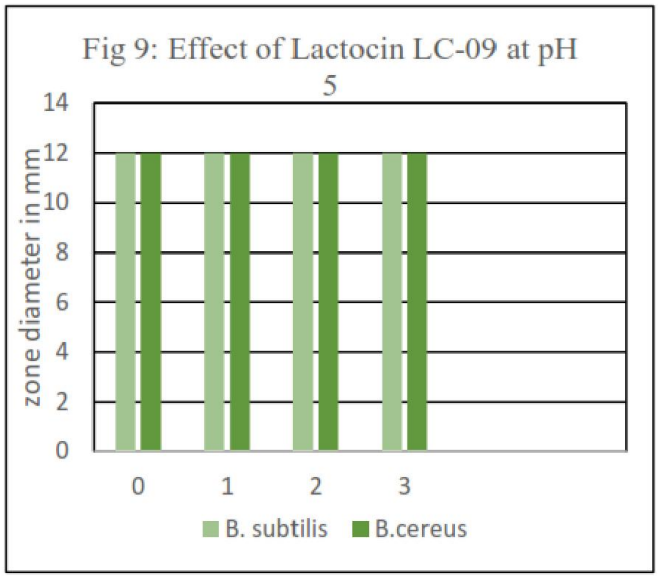


Fig 8: Effect of pH Lactocin LC-09 at pH 4.0
zone diameter(mm)
20
15
10
5
0
0 1 2 3
time in hrs
B. subtilis
B.cereus



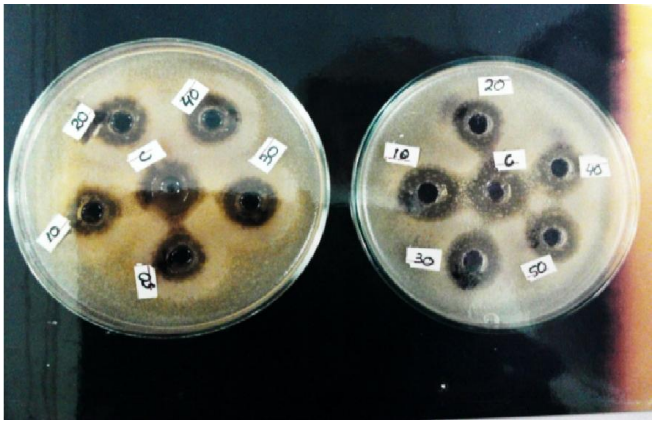


Fig. 10. Effect of heat on lactocin LC-09(50°C)

Effect of pH on lactocin LC-09

Effect of pH on lactocin LC – 09 was studied by using different pH values such as 3.0, 3.5, 4.0, 4.5, 5.0 and 5.5. It was observed that the zone of inhibition was decreased as the pH values of lactocin LC-09 was increased. The minimum zone of inhibition was observed at pH value 5.5 whereas the maximum zone of inhibition was observed at pH 3.0 (Fig. 7, 8, 9&11).

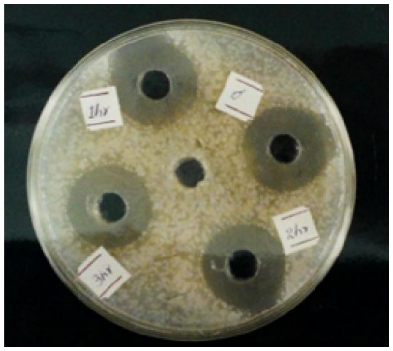


Fig. 11. Effect of ph-4.5 on lactocin against *B.subtilis*

Determination of titer of lactocin LC-09

The titer of lactocin LC-09 was determined by using critical dilution method, a commonly used method for determining the titer of bacteriocin. The titer of lactocin LC-09 was determined in terms of AU / ml (arbitrary units / ml) against the indicator strains *Bacillus subtilis* and *Bacillus cereus* and was found to be 4000 AU / ml. The specific production rate q_p was also determined, i.e. 8.3 x 10^4 (AU $h^{-1)}$ (Table 2).

Table 2. Titre of lactocin lc-09 by critical dilution method

Dilutions	Zone diameter (mm)	
	B. subtilis	*B. cereus*
1:2	6	6
1:4	0	0
1:8	0	0
1:16	0	0
1:32	0	0
1:64	0	0
control	10	10

DISCUSSION

Predictive microbiology generally focuses on potential outgrowth of spoilage bacteria and food-borne pathogens in foods. Little attention has been paid to be bio kinetics of beneficial food- grade microorganisms, such as lactic acid bacteria. It is commonly used in the food fermentation industry, mainly for the in situ production of the antimicrobial lactic acid to extend the shelf life of the food (Leroy *et al*., 2002). Bacteriocin from lactic acid bacteria are potent antimicrobial agents that enables the producer cells to selectively and efficiently inhibit a part of the competing background flora, including several spoilage bacteria and food borne pathogens (Leroy *et al*., 2002). *Lactobacillus acidophilus* LC- 09 a previously isolated strain of lactic acid bacteria in our lab capable of producing an inhibitory substance designated as Lactocin LC-09 was used in the present study. Main focus during the present research was on the kinetics of lactocin LC- 09 production during the growth cycle of *Lactobacillus* LC- 09 and to elaborate the preserving effect of lactocin LC- 09 against *B.subtilis, B.cereus.* As *Bacillus* is a ubiquitous organism contaminating food raw materials. Endospore of this organism can be found in virtually all foods that have not been subjected to spore inactivating process for example, autoclaving, UHT (Ultra High Temperature) treatments. *B. subtilis* is also found commonly in pasteurized milk and dairy products (Hladikova *et al*., 2012). When lactocin LC -09 production was studied at different temperature that is at 37 ˚C, 40 ˚C, 45 ˚C and 50 ˚C, it was observed that at 37 ˚C, 40 ˚C and 45 ˚C, the production started at early growth phase and reached at peak level after 24 hours. However there was no lactocin production at 50 ˚C and also slow increase in absorbance during whole growth cycle. This indicates that 50 ˚C is not suitable for growth and lactocin LC-09 production. The production of bacteriocin at a high temperature is important for the industrial purpose. In previous reports, bacteriocin activity increased rapidly while cells were growing exponentially, which supposes that the production rate of bacteriocin is related to production of biomass, and

indicated clearly that the production of bacteriocin follows primary metabolite kinetics.

The report of (De Vuyst *et al* 1996) about *L. sake* CTC 494 is in agreement with our findings, that in our study also it was noticed that cell dry mass and bacteriocin activity increased as the cells were growing exponentially at 37 ˚C and 45 ˚C, but further increase in temperature to 50 ˚C, low cell dry mass and complete inhibition of lactocin production was observed. In a previous finding instead of complete inhibition of bacteriocin, decrease in bacteriocin activity was observed, once the cell mass production began to level off. This decrease was more pronounced at the high temperatures and pH values, indicating greater proteolytic degradation or adsorption (Loubiere *et al*., 1997). Gram dry weight of *Lactobacillus* LC-09 was also observed at the same temperatures i.e. at 37 ˚C, 40 ˚C, 45 ˚C, 50 ˚C. Results indicate that the maximum cell dry mass obtained at 37 ˚C and 45 ˚C because absorbance was also maximum at that temperatures. But further increase in temperature decrease the cell absorbance as well as cell biomass. So by these results it was concluded that 37 ˚C and 45 ˚C are suitable temperatures for maximum cell turbidity.

Equations for cell growth are generally based on the specific growth μ (per hour), which dictates evolution of biomass concentration [X] (in gram of CDM per liter) over time (t) (in hours):

$$d [X] / dt = \mu [X]$$

In the early phase of growth, reported previously that specific growth rate μ is at its maximal value (μ_{max}), but it decreases as biomass concentration increases (Wijtzes *et al*., 1995). In our study the maximal specific growth rate (μ_{max}) obtained at different temperatures (37 ˚C, 40 ˚C, 45 ˚C and 50 ˚C), was dependent on cell dry mass. As the temperature has a pronounced effect on growth and volumic production of lactocin LC-09, further work carried out on effect of heat on lactocin LC- 09 to determine heat stability of lactocin. As it was observed that high temperature affected the production of lactocin LC- 09 during growth and complete inhibition was observed at 50 ˚C but lactocin was heat stable showed inhibitory activity even after heating at 100 ˚C for 50 minutes, Heat stability of antibacterial substances produced by *Lactobacillus* sp has been well established (Nettles and Barefoot, 1993). The previous report of heat stability of *L. brevis* OG1 at 121 ˚C for 60 minutes is novel, and heat resistant pattern of lactocin LC-09 seems closely related to *L. brevis* bacteriocin. Temperature stability is important if the bacteriocins are to be used as food preservative, because many procedures of food preparation involve a heating step. The effect of pH on the activity of lactocin LC- 09 indicates that the lactocin was not so stable in the wide range of pH. The activity of lactocin elaborated by the *Lactobacillus acidophilus* LC- 09 was also pH dependent. The highest antibacterial activity was exhibited in an acidic pH range 3 to 5.5, while activity was lost at pH value above 5.5. The results show that maximum activity found to be at pH value 3.0 and less activity found at pH value 5.5. So it was concluded that the lactocin LC- 09 is active at low pH value. Bacteriocin of lactic acid bacteria active at low pH values have been described by (Reddy *et al*., 1984; Abdel-Bar *et al*., 1987), where two bacteriocins namely bulgarican and lactobulgarican, isolated from *L. bulgaricus* were shown to have the highest activity and stability at pH 2.2 and 4.0 respectively, against a range of pathogenic and spoilage bacteria, comparing with our lactocin, active between a wide pH range i.e. 3-5.5, and thus can be used for relatively food samples having broader pH range.

The titre of lactocin LC- 09 against *Bacillus subtilis* and *Bacillus cereus* in terms of AU / ml were found to be 2000 AU / ml. This finding led us to categorize lactocin LC-09 as a moderately active bacteriocin as compared to more active bacteriocins of lactobacillus (Aly and Abo-Amer, 2011). In conclusion, main focus of our study was based on the effect of temperature on growth and bacteriocin production during growth cycle and maximal specific growth rate (μ_{max}). So it was found out that temperature had a pronounced effect on growth and lactocin production during growth cycle, while (μ_{max}) was determined at different temperatures dependent on the cell dry biomass. It was also concluded that this lactocin is heat stable and active in acidic pH value. The effectiveness of bacteriocins as food preservative is well demonstrated and now a days instead of chemical preservative, bacteriocins, the new biological preservatives will be appreciated as they are considered “safe”.

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