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Effect of Incubation Temperature and pH on *Lactococcus lactis* ssp *Diacetylactis* and Their Virulent Phages

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Abstract: pH and temperature conditions do effect bacterial growth and phage infectivity and development. Bacteria grew better in media at pH 7.0 as compared to other tested pH. Most of these phages showed earlier lysis of host bacteria in cultures at pH 6.5 and maximum phage titre was recorded at pH 7.0. In case of temperature bacteria showed better growth rate at 37°C as compared to 32 and 42°C respectively.

Key words: Bacteriophage, pH, Temperature, *Lactococcus lactis* ssp *diacetylactis*

Introduction

Lactic acid bacteria are widely used for the preparation of different dairy products in which they are responsible for the development of acidity and characteristic flavor. For successful fermentation, these microorganisms must produce acid rapidly. Bacteriophage infections of these cultures commonly prevent fermentation, resulting in great economic loss to the industry. Different studies on various aspects of phages including morphological and structural characterization, host phage interaction, effect on lactic fermentation and product quality and genetic studies have been carried out in order to combat phage attacks in the dairy industry (Lawrance *et al.*, 1976; Relano *et al.*, 1987; Defabrizio *et al.*, 1991). In Pakistan no detailed study on phages isolated from dahi is available despite its significance. The present study was undertaken to compare the isolated phages HTK1 and HTK2 at different pH and temperatures with respect to their host.

Materials and Methods

The bacteriophages used in this study were originally isolated from indigenous dahi samples. The genetic homogeneity of each phage stock was ensured by isolation twice of single plaques. *Lactococcus lactis* ssp *diacetylactis* was used as propagating strain for these phages at 37°C in M 17 broth (Difco). M 17 broth was further supplemented with 20m M $CaCl_2$ for phage preparation. Phage propagation, concentration and purification were carried out as described by Terzaghi and Sandine, 1981. The M 17 medium was adjusted to different pH values namely: 6.5, 6.7 and 7.0 in separate flask (50 ml). Each flask was inoculated with 1 ml of overnight grown bacterial culture (*Lactococcus lactis* ssp *diacetylactis*) at 37°C inoculated on a shaking incubator for two hours. The growth was recorded by using SHIMADZU spectrophotometer. After two hours 500 ml 1M $CaCl_2$ was added in all flask except those flask which are used for studying bacterial growth at different pH. Then after adding 500 ml 1M $CaCl_2$, 1ml of each phage used in this study was added in the flask for each pH value. Reading from spectrophotometer, at 650 nm, for six hours were taken. Reading for bacterial growth at different pH values 6.5, 6.7 and 7.0 were also recorded at 650 nm by using same spectrophotometer. Same procedure was applied in order to study temperature effect on bacterial growth and phage infectivity. Different temperature values were 32, 37 and 42 °C. pH value was taken 6.5 as it is recommended for M 17 media (Metolan and Sandine, 1986).

Results and Discussion

The starter culture for production of dahi is a heterogeneous mixture of lactic acid bacteria. However, there is a large variation in the quality of the final product because crucial parameters such as milk quality, starter composition and the quality and quantity of inoculum and incubation temperature during the preparation are not appropriately monitored. This may add to the chances of starter culture failure due to phage contamination. However, the effects of phage contamination may not necessarily results in complete batch failure because of the practice of using multiple strain starters. Bacterial strain *L. lactis* ssp *diacetylactis was* grown in M 17 adjusted to different pH values i.e. pH 6.5, 6.7 and 7.0. It was observed that bacteria showed maximum growth at pH 7.0 as compared to growth of bacteria at pH 6.7 and 6.5 as indicated by OD values in Table 1. Culture at pH 7.0 became turbid after three hours, while at pH 6.7 low turbidity was observed after four hours. In culture at pH 6.5 no turbidity was observed although OD value increased. It was also observed that bacterial multiplication after two hours gave an OD value of 0.32 in culture at pH 6.5 while, OD value of 0.044 was observed in culture at pH 6.7 and OD value of 0.051 in culture at pH 7.0 (Table 1). These results showed that bacteria grow well in medium adjusted to pH 7.0. So we could say that pH affects bacterial growth. These results are in line with the findings of Catherine *et al.* (1994). They reported that *Lactobacillus delbrueckii* ssp *thermophilus* grew well at pH 7.0 at 37°C than at pH 4.6. Similarly Fredeique and Novel (1994) described that *S. salivarius* ssp *thermophillus* showed maximum growth rate at pH 7.0 at 42°C than at pH 5.5 at 42°C. This study however, was based on monitoring the effects of pH and temperature conditions. In the present study effect of pH on phage lytic patterns were monitored using two phages HTK1 and HTK2. Phage HTK1 caused lysis of host strain after two hours of phage inoculation. In culture at pH 7.0 lysis started after three hours of phage inoculation but lysis was slow and culture was not cleared even after four hours of phage inoculation (Table 2). On the other hand phage HTK2 showed no lysis at pH 6.5, however, lysis occurred in culture at pH 6.7 and pH 7.0. In culture at pH 6.7 lysis was compared after three hours of phage inoculation. In culture at pH 7.0 turbidity of culture was lost due to lysis after four hours of phage inoculation (Table 3). With the help of above information, it could be assumed that maximum phage lysis was observed in culture at pH 7.0. Rizwan (1994) expressed similar views, who observed that phages showed maximum lytic activity at different pH and complete lysis of host strain, occurred at different pH. It may be suggested that pH values affect the adsorption and lysis by delaying such process. These results are also in accordance with the finding of Rhimani and Freids, (1993). They reported that the host interactions of Lactococcal phages FRC1, FRC2, FRC3 and FRC4 were maximum at pH

7.0, 7.2 and 7.6 after 10 minutes. On the other hand Sijtsma *et al.* (1991) observed that when *Lactococcus lactis* ssp *cremoris* cell wall was subjected to low pH and high temperature, the strain lost its ability to resist phage attack.

Table 1: Spectrophotometric analysis of bacteria *Lactococcus lactis* ssp *diacetylactis* at different pH

Time(min)	pH 6.5 OD Values	pH 6.7 OD Values	pH 7.0 OD Values
0	0.026	0.029	0.032
60	0.027	0.033	0.033
120	0.032	0.044	0.051
180	0.054	0.038	0.070
210	0.060	0.047	0.090
240	0.072	0.056	0.117
270	0.096	0.070	0.131
300	0.113	0.092	0.164
330	0.131	0.119	0.172
360	0.158	0.170	0.186

Culture conditions
Host bacteria

Lactococcus lactis ssp *diacetylactis* was grown from over night culture inoculum on one ml in 50 ml M 17 media.
Absorbance = 650 nm Temperature = 37°C Duration = Six hours

Table 2: Spectrophotometric analysis of bacteriophage HTK 1 at different pH

Time(min)	pH 6.5 OD Values	pH 6.7 OD Values	pH 7.0 OD Values
0	0.033	0.103	0.084
60	0.031	0.151	0.090
120	0.019	0.151	0.147
	1 ml HTK 1 + 500μ 1M $CaCl_2$		
180	0.011	0.150	0.619
210	0.029	0.351	0.647
240	0.057	0.254	0.669
270	0.013	0.239	0.697
300	0.032	0.191	0.691
330	0.015	0.168	0.665
360	0.012	0.309	0.650

Culture conditions
Host bacteria

Lactococcus lactis ssp *diacetylactis* was grown from over night culture inoculum of one ml in 50 ml M 17 media.
Absorbance = 650 nm Temperature = 37°C Duration = Six hours

While Furtado *et al.* (1990) observed that lactic acid bacteria at low pH 5.0, 5.5 and 5.8 showed low sensitivity to phages. Wu *et al.* (1990) four types of *Bacillus thuringensis* and their four types of phages were studied and it was observed that these phages showed stability at pH 5 to pH 8 and were inactive at pH 3 and pH 11 to 12.The tested bacterial strain *L lactis* ssp *deiacetylactis* was grown at three different temperature values i.e 32, 37 and 42°C. It was observed that bacteria grow better at 37°C as compared to growth at 32 and 42°C as indicated by OD values (Table 4). Autolysis of *L lactis* ssp *diacetylactis* was observed at 32°C after one hour of bacterial inoculation. Autolysis of host strain was not observed at 37 and 42°C. After one hour of bacterial inoculation OD value of 0.087 and 0.050 was observed in culture at 32 and 42°C, respectively. While, at 37°C OD values increased rapidly as compared to OD values of cultures at 32 and 42°C. At the end of six hours of bacterial inoculation OD value 0.144 and 0.145 was observed in culture at 32 and 42°C, respectively. While, in culture at 37°C OD value of 0.568 was observed. Autolysis of *L lactis* ssp *diacetylactis* was observed at 32°C after three hours of phage inoculation. This is indicated by decrease in OD value from 0.087-0.063 (Table 4).

Table 3: Spectrophotometric analysis of bacteriophage HTK 2 at different pH

Time(min)	pH 6.5 OD Values	pH 6.7 OD Values	pH 7.0 OD Values
0	0.031	0.058	0.096
60	0.093	0.053	0.077
120	0.010	0.020	0.027
	1 ml HTK 2 +500 ml 1M $CaCl_2$		
180	0.068	0.196	0.278
210	0.167	0.288	0.464
240	0.427	0.424	0.639
270	0.010	0.084	0.491
300	0.015	0.011	0.186
330	0.018	0.016	0.127
360	0.072	0.011	0.077

Culture conditions
Host bacteria

Lactococcus lactis ssp *diacetylactis* was grown from over night culture inoculum of one ml in 50 ml M 17 media.
Absorbance = 650 nm Temperature = 37°C Duration = Six hours

Table 4: Spectrophotometric analysis of bacteria *Lactococcus actis* ssp *diacetylactis* at different temperatures

Time(min)	32°C OD Values	37°C OD Values	42°C OD Values
0	0.073	0.182	0.054
60	0.087	0.184	0.049
120	0.063	0.220	0.055
180	0.090	0282	0.091
240	0.130	0.348	0.123
300	0.126	0.431	0.155
330	0.128	0.499	0.149
360	0.144	0.568	0.145

Culture conditions
Host bacteria

Lactococcus lactis ssp *diacetylactis* was grown from over night culture inoculum on one ml in 50 ml M 17 media.
Absorbance = 650 nm pH =6.5 Duration = Six hours

Table 5: Spectrophotometric analysis of bacteriophage HTK 1 at different temperatures

Time(min)	32°C OD Values	37°C OD Values	42°C OD Values
0	0.089	0.024	0.050
60	0.085	0.049	0.059
120	0.055	0.165	0.123
	1 ml HTK 1+500 ml 1M $CaCl_2$		
180	0.066	0.223	0.174
240	0.124	0.002	0.127
300	0.123	0.009	0.120
330	0.124	0.002	0.116
360	0.081	0.035	0.092

Culture conditions
Host bacteria

Lactococcus lactis ssp *diacetylactis* was grown from over night culture inoculum of one ml in 50 ml M 17 media.
Absorbance = 650 nm pH = 6.5 Duration = Six hours

These results are in line with the observation by Lortal *et al.*, 1991; Mou *et al.*, 1976 while, Vegrud *et al.*, 1993, studied the influence of temperature and media composition on autolysis of 14 Lactococcal strains. They reported that the Lactococcal cells autolyzed spontaneously when they are transferred from M17 broth to a buffer solution. They also reported that *L lactis* ssp *lactis* showed maximum growth at 37°C as compared to 40°C. In another study it was observed that growth rate of *S.salivarius* ssp *thermophillus* increased as temperature was increased (Frederique and Novel, 1994).

Table 6: Spectrophotometric analysis of bacteriophage HTK 2 at different temperatures

Time(min)	32°C OD Values	37°C OD Values	42°C OD Values
0	0.098	0.010	0.026
60	0.093	0.068	0.016
120	0.065	0.167	0.087
	1 ml HTK 2 + 500 ml 1M $CaCl_2$		
180	0.092	0.427	0.135
240	0.134	0.010	0.135
300	0.129	0.015	0.113
330	0.133	0.186	0.082
360	0.130	0.072	0.043

Culture conditions

Host bacteria

Lactococcus lactis ssp *diacetylactis* was grown from over night culture inoculum of one ml in 50 ml M 17 media.

Absorbance = 650 nm pH = 6.5 Duration = Six hours

Goldberg *et al.* (1994) observed that *E.coli* growth was inhibited when temperature was raised from 37 to 41°C. The effect of temperature on phage lytic pattern was monitored by two phages, which included HTK1 and HTK2 Table (5 and 6). At temperature 37°C phage HTK1 showed complete lysis of host bacteria after two hours of phage inoculation and OD values decreased from 0.223 to 0.035. At 32 and 42°C lysis occurred after three hours of phage inoculation and OD value decreased from 0.124 to 0.081 and 0.127 to 0.092, respectively. Phage HTK2 also caused lysis of host strain at 37°C after two hours of phage inoculation and OD value decreased from 0.427 to 0.015. Bacterial culture at 32°C showed very slow lysis, which is evident from very slow decrease of OD value from 0.134 to 0.130 in four hours. At 42°C lysis occurred after two hours of phage inoculation and culture lost its turbidity after four hours and the OD value decreased from 0.135 to 0.043. It was observed that temperature at which complete lysis of host strain occurred by some phage is different form the temperature at which maximum phage lytic activity was observed. This effect of temperature may be due to the fact that temperature may delay phage-adsorption, restriction-modifications and development were found to be temperature sensitive, permitting full lytic development at 40°C, in contrast to greatly restricted phage development at 30°C (Chopin *et al.*, 1976). In another study on *Streptococcus cremoris* strain and it's bacteriophages. It was observed that at 38°C, the titre of phage was 101 to 104 times greater than observed at 25 to 30°C it was described that transition from reversibly adsorbed to reversibly adsorbed phage occurs more rapidly on host bacteria grown at higher temperature (Eddy and Hull, 1987). In a study of 19 phages from *L lactis* and *S cremoris* it was observed that at higher temperature greater than 37°C latent period was decreased but the effect on the burst size was variable (Keogh 1973).

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