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Comparative analysis of growth phases of lactic acid bacteria grown on different media for fermentation

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Abstract
Fermentation is a safe, acceptable and efficient food preservation technology especially using lactic acid bacteria (LAB). In addition to their enhanced fermentative ability and food safety, they improvise organoleptic properties, enrich nutrients and increase health benefits. A typical LAB growth curve shows five distinct phases of growth: lag phase, exponential phase, stationary phase and death phase when conditions become unfavourable for growth and cells lack viability. Among different phases, lag phase is the earliest and most inexplicit stage of the bacterial growth cycle. Lag phase/exponential phase provides the adaptation necessary for bacterial cells to exploit new environment. This process could include the repair of macromolecular damage that accumulated during stationary phase and the synthesis of cellular components necessary for growth. The available physiological data simply shows that lag-phase bacteria are metabolically active. However, there is currently no physiological or biochemical criteria to define lag phase and thus the physiology of bacterial lag phase remains incoherent. The aim of this study was to determine growth curve of two lactobacillus species, *Lactobacillus acidophilus* and *Lactobacillus brewis*, in order to investigate the duration of their lag phase along with the understanding of biomass in this phase of bacterial growth. The study displayed that the duration, OD_{600} and maximum biomass of lag phase of *L. acidophilus* and *L .brewis* was 3 to 8h and 2 to 8.5h, 1.235 and 1.127, 38.3 x 10^{10}CFU/ml and 38 x 10^{10} CFU/ml, respectively.

Keywords: Fermentation, LAB, growth media, lag phase, CFU, biomass

Introduction
Lactic acid bacteria (LAB) are enumerated as very important microorganisms which play a critical role in food, agricultural, and clinical applications. They possess probiotic benefits and have the capability to produce lactic acid as the end product of carbohydrate breakdown. LABs are group of gram-positive, catalase-negative bacteria, with more than 231 species and 29 subspecies and *Lactobacillus* genus being its most diverse group (Felis and Dellaglio, 2007) [2],(George *et al.*, 2018) [3]. *Lactobacillus*are present in the oral cavities, vaginas of humans and are considered major genus of gastrointestinal microbiome with health-promoting benefits. The rapid growing properties of lactic acid bacteria and their metabolic activity have been the key in its applications including food production, agricultural industry and probiotics. *Lactobacillusspp*. Is one of the most widely used genera for fermentation of protein-rich resources to release bioactive peptides? *Lactobacilli* bacteria are used for fermentation of foods, food biopreservation, or probiotic applications (Stefanovic *et al.*, 2017) [9].Their auxotrophicnature for numerous amino acids which are essential for their growth, makes them hydrolyze proteins in their environment. They hydrolyze proteins through their proteolytic system mainly by action of enzymes called cell envelope proteinases (CEPs) which leads to the release of peptides and free amino acids in the fermentation media (Savijoki *et al.*, 2006) [7]. The peptides released display biological activities alongwith their contribution in the organoleptic properties of the fermented product (Hafeez *et al*., 2014) [5].The biochemical and biophysical environments have remarkable effect on the growth and metabolic activity of LAB. Thus, much of studies were conducted to understand the impact of available nutrients on the growth and metabolic activities of LAB. In order to establish the best fermentation conditions, we described the *Lactobacillus acidophilus* (MTCC 10307) *and L. brewis* (MTCC 4463) growth curve.

Materials and methods
Pure cultures of *L. acidophilus* (MTCC 10307) *and L. brewis* (MTCC 4463) were obtained

from Microbial Type Culture Collection and Gene Bank (MTCC, India) in freeze dried state and maintained 4 °C in molecular biology laboratory, Division of Biochemistry, Sher-e-Kashmir University of Agricultural sciences and technology of Jammu.

Growth media

In this study both MRS agar and nutrient media were used in this study. The MRS agar medium was prepared by dissolving 70g Lactobacilli MRS Agar (Himedia) per litre distilled water by boiling, then autoclaved at 121 °C. The broth medium was prepared by dissolving 55 g Lactobacilli MRS Broth (Himedia) per litre distilled water, then autoclaved at 121 °C. For nutrient agar (Himedia-HiEncap) 1 capsule was suspended in 100ml distilled water and heated to dissolve the medium completely. Media was sterlized by autoclaving at 121 °C for 15 minutes.

Culturing of LAB

Freeze dried cultures were revived by using as single tube of broth (5-6ml), 0.5-1ml was withdrawn and was used to hydrate the entire pellet. Entire suspension was transferred back into the broth tube and was mixed well (MTCC). The cultures of each LAB were prepared using standard streak plate method provided by Gerhardt *et al*., 1981[4].

Sub-culturing of mother cultures

Pure culture of *L. acidophilus* (MTCC 10307) *and L. brewis* (MTCC 4463) were subcultured on nutrient media or MRS media twice a month Sharma *et al.* (2013) [8]. Microbial culture activation was done by transferring single colony using inoculation loop from mother culture plate to a fresh petriplate containing media (nutrient agar or MRS).The petriplates were then incubated for 24h at 37 °C and were stored in refrigerator (4 °C) for further use (Fig 1,2).

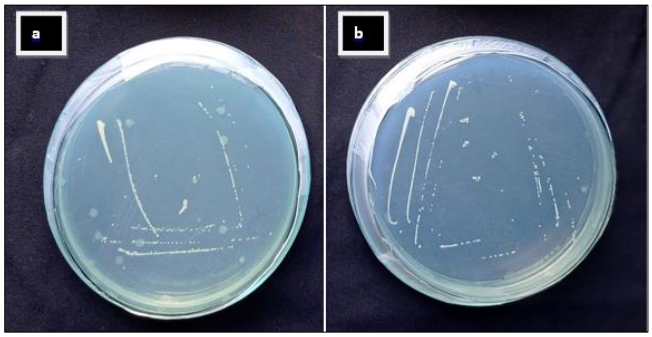

Fig 1: *Lactobacillus acidophilus* (a) and *Lactobacillus brewis* (b) cultured on nutrient agar

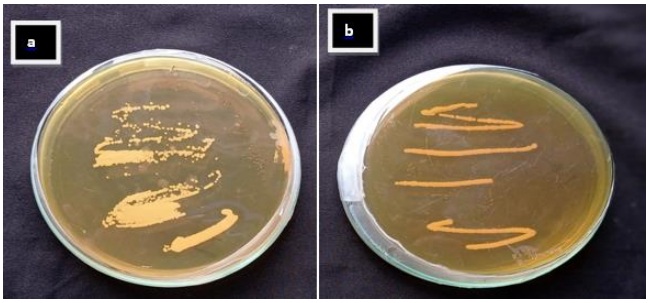

Fig 2: *Lactobacillus acidophilus* (a) and *Lactobacillus brewis* (b) cultured on MRS media

Gram staining test

The bacteria were examined using gram staining kit (Himedia) according to method given by Becker*et al.* (2004) [1] and was observed under light microscope (Magnus CH2O*i*LED) with a magnification of 100X.

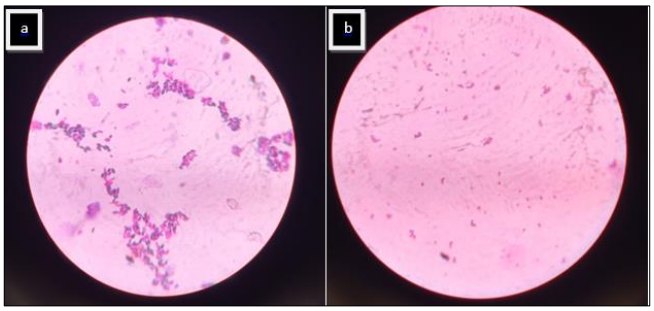

Fig 3: *Lactobacillus acidophilus* (a) and *Lactobacillus brewis* (b) as viewed under light microscope (100X)

Determination of growth curves of bacteria

Growth curves of each bacteria, *Lactobacillus acidophilus* and *Lactobacillus brewis*were determined using spectrophotometric analysis. Inoculating loop full of bacteria was transferred from sub-culturedpetriplate to Erlenmeyer flask which contained MRS broth (50 ml). The broth was incubated in BO.D incubator (Temp star) followed by continuous shaking at 130rpm and 37° C for 24h. Growth curves were determined by recording absorbance at 600nm using UV-Vis spectrophotometer (Shimdzu) after every 30 minutes interval upto 12h. Sterile broth was used as blank for spectrophotometer.

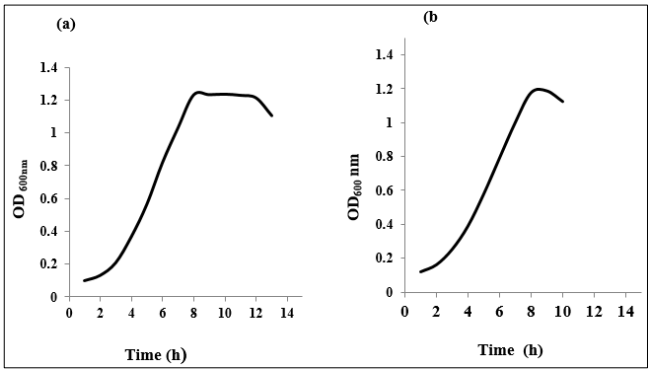

Fig 4: Growth curves of *Lactobacillus brewis* (a) and *Lactobacillus brewis* (b)

Determination of CFU/ml

The colony forming units were determined according to method described by Yang *et al.* (2019) [10] alongwith some modifications. A serial dilution of the concentrated *Lactobacillus acidophilus and Lactobacillus brewis* broth was performed upto 9th dilution. Meanwhile, 200 ml MRS agar solution was prepared, sterilized, and poured into 8-9 petriplates for solidifying. We took nine 20ml test tubes and prefilled one with 10ml concentrated culture broth (*Lactobacillus acidophilus or Lactobacillus brewis)* and the remaining eight with 10ml sterile broth. We transferred 1ml of the well suspended broth from the first tube to the second one and thoroughly mixed, and transferred 1ml of broth from second tube to third. We repeated the same dilution procedure up to the ninth tube. Then from each of the test tubes containing the final diluted samples, we loaded 100µL of the

broth onto the surface of the agar plate, and spread the sample evenly over the surface. For each dilution rate, we prepared two agar plates as duplicates. All agar plates were then put upside down (agar up) into BOD incubator at 37°C for 24h till distinct colony forming units (CFU) became available. Based on the number of CFU (N) on each agar plate, the volumetric CFU can be calculated using the equation:

$$\frac{\text{CFU (N)}}{\text{ml}} = \frac{\text{Numberofcolonies xdillutionfactor}}{\text{Volumeofcultureplaced (ml)}}$$

All the dilutions were saved for optical density measurement at 600 nm (OD600) using an UV-Vis spectrophotometer. The sterile MRS broth was used to set blank and as the diluent to keep the direct OD readings below 2.0. After data collection, OD600 was correlated with volumetric CFU to create a standard curve for this specific *Lactobacillus acidophilus* and *Lactobacillus brewis* strain.

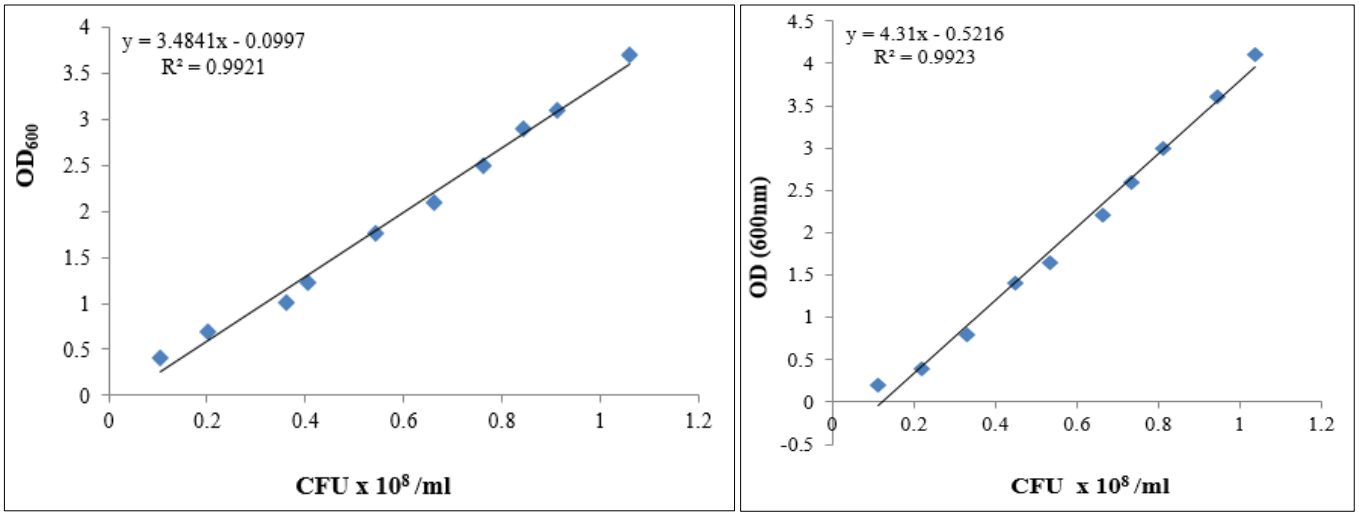

Fig 5a,b: Standard curve correlating OD_{600} and CFU/ml of *Lactobacillus acidophilus* (a) and *Lactobacillus brewis* (b)

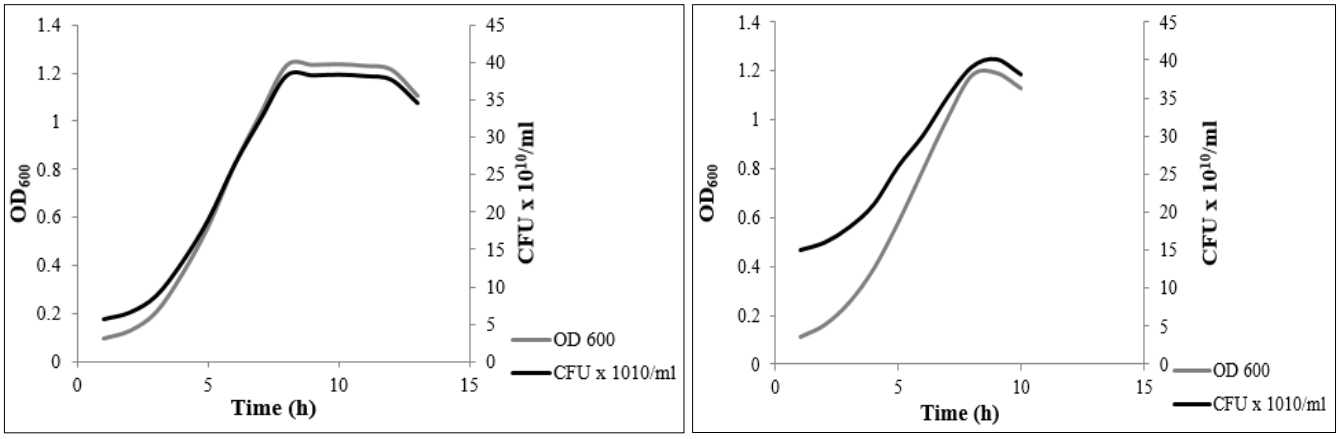

Fig 6: Growth curves of *Lactobacillus acidophilus* (a) and *Lactobacillus brewis* (b) and CFU/ml

Results and Discussion

The *Lactobacillus acidophilus* and *Lactobacillus brewis*were observed under light microscope (100X). It was clear that the bacteria was gram positive, rod shaped coccobacilli, occurring singly or in chains (Fig 3). The gram staining results indicated that the isolated bacteria could be identified as lactobacilli (Holt *et al*., 1994) [6]. Growth curves of *L.acidophilus* and *L. brewis* were prepared by plotting absorbance measured at 600nm against time (hours). For *L. acidophilus* late log phase was at 8h and in case of *L. brewis*, late log phase was at 8h 30min (Fig 4a, 4b). During serial dilution, after 24hr of incubation in BOD incubator, the distinct colony forming units (CFU) were present on petriplates for each dilution. Based on which, the number of CFU/ml (N) on each agar plate were calculated and correlated with the respective OD600 readings to create a standard curve (Fig. 5). The correlation observed was: CFU/ml = OD_{600}x 10^8 + 0.099/3.484 (*L.acidophilus*) (Fig 5a) and CFU/ml = OD_{600}x 10^8 + 0.256/4.585 (*L. brewis*) (Fig 5b), respectively. Based on the equation, conversion between optical density and volumetric CFU and growth curvesof each bacteria were drawn (Fig 6). From the figure 6a, it is evident that a distinct exponential growth phase was observed between 3 and 8 hours and the maximum OD600 and CFU occurred at 9h for *L.acidophilus*. For *L. brewis*, the maximum exponential phase lied between 2 and 8.5 hours and the maximum OD600 and CFU occurred at 10h (Fig 6b). The maximum OD600 reached 1.235 and 1.127 and maximum biomass was 38.3 x 10^{10}CFU/ml and 38 x 10^{10}CFU/ml for *L.acidophilus* and *L. brewis*, respectively. This could be explained as the log/exponential phase is the quickest phase of growth and shows highest cell multiplication. Under optimum nutritional and physical conditions, the physiologically robust bacterial cells reproduce at a uniform and rapid rate by binary fission.

Thus there is a rapid exponential increase in population, which doubles regularly until a maximum number of cells is reached. The length of the log phase varies, depending on the organisms and the composition of the medium, although the average may be estimated to last 6 to 12 hours. Yang *et al*., 2019[10] performed microaerobic fermentation of *Lactobacillus acidophilus* within gut microbiome physiological conditions. The results showed robust growth of *Lactobacillus acidophilus*, which reached biomass concentrations of 3.13 x 10^9 CFU/ml and 3.73 x 10^9 CFU/ml respectively.

Conclusion

This study displayed growth curves of *L.acidophilus* and *L. brewis*, which depicted different phases of their growth (Log, lag, stationary and death phase) along with the biomass produced at specific stages. This will be very helpful for peers in the field of microbiome research and microbial fermentation based bioprocesses.

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