CHAPTER 5: MATERIALS AND METHODS

This research study followed standard bacterial growth protocol for culturing E. coli in batch culture. Compared to other microbiological growth experiments, here intervention is based on *Jyotisha Lagna Muhurta*.

5.1 MATERIALS

5.1.1 Bacterial strain: *Escherichia Coli* (*E. Coli)* strain, K-12 MG 1655, a non-pathogenic strain stored at -80^{0}C in glycerol stock, was obtained from the National Centre for Biological Sciences (NCBS), Bengaluru.

5.1.2 Bacterial Glycerol stock and Growth Media:

Glycerol stock: Initially **50%** glycerol solution was prepared by diluting 100% glycerol (v/v) in dd-water (double distilled water). Further, it was autoclaved and stored for further use. Glycerol stock of the bacterial strain was prepared by diluting (in 1:1 ratio) the overnight culture of bacteria in **50%** autoclaved glycerol solution and stored at **-80^{0}C (-112^0 F)** until further use.

Growth Media: The compositions of the different media used are given below.

Table 5.1 Compositions of the different growth media used in grams

Constituents ↓	2x YT medium - 1 L	LB medium – 1L	YT agar plates
Tryptone	16	10	8
Yeast extract	10	5	5
NaCl	5	10	5
Agar	--	--	15

Caption: Table 5.1 gives the compositions of the growth media

All the media were procured as ready mix from HiMedia Laboratories Pvt. Ltd., Mumbai, India (Luria Broth: Catalog no. M575-500G; Y.T. Agar: Catalog no. G032-

500G). Two days before conducting the GC experiment, 50 ml of Broth in conical flasks (250 ml) was prepared following manufacturer's instruction and autoclaved at 121^0C for 20 minutes. Growth agar was also prepared following manufacturers' instruction and autoclaved as above and poured into Petri plates, allowed to cool and stored at 4^0C in fridge until further use.

5.1.3 Glassware:

Pipettes: Adjustable Volume single channel of volume 0.5-10µl, 20-200µl and 200-1000µl.

Flasks: 250 ml flat bottomed conical flask (Catalogue no. 4980021, Borosil Glass Works Ltd, Mumbai)

Petri Plates: Catalog no. G032-500G, HiMedia Laboratories Pvt. Ltd., Mumbai, India.

Falcon Tubes: 15 ml and 50 ml Falcon tubes (Catalogue no. 546021, Tarsons Products Pvt. Ltd, Kolkata)

Miro-cuvettes: 3.5ml (Quartz Cuvette 190nm-2500nm, Imported from France)

5.1.4 Equipment:

Incubator-cum-shaker: 180 rpm Incubator-cum-shaker (Model:116736, GeNei™, Mumbai, India)

Spectrophotometer: Nanodrop™ 2000c spectrophotometer (Thermo Scientific™ NanoDrop 2000c, Wilimgton, USA)

5.1.5 Inclusion Criteria: Non-toxic strain of ***E. Coli*.**

5.1.6 Exclusion Criteria: Toxic strains of bacteria.

5.1.7 Ethical Criteria: S-VYASA's Institutional Ethical Committee, IEC, waived necessity for approval as the study did not involve any human or animal subjects and the organism used, bacteria, was to be grown according to well-established standard protocols without any changes.

5.2 METHODS:

The steps followed for generating each growth curve, in the order in which it was done, are given below. The overall scheme for the bacterial growth experiments is presented in Table 5.2.

Table 5.2: Timeline for a Growth Curve Experiment

Day No.	Activity
Day 1	❖ Preparation of Media & Autoclaving & Preparation of Petri plates ❖ Finding the potential Time Of Flask Inoculation / *Lagna Muhurta*
Day 2	Making YT agar Petri plates for streaking
Day 3	Streaking of Petri plate with *E. coli* & Incubation overnight at 37°C
Day 4	Pre-Culture Preparation & Incubation
Day 5	❖ Main cultures inoculation (T.O.F.I.) & 6-hour Growth study ❖ Calculating *Graha Shadbalas* for T.O.F.I. *Lagna Muhurta*

Caption: Table 5.2 gives Timeline describing each individual day's activities

5.2.1 Making a Streak plate:

Pre-prepared YT agar plates (as explained in the previous section) was used for making streak plate from the *E. Coli* stored as glycerol stock at -80^0C. One vial of glycerol stock of *E. Coli* was taken out on ice and streaked on YT agar plate (brought to room

temperature after taking out from 4^0C) using sterile inoculation loop. The streaked plate was incubated overnight (in incubator) at 37^0C along with control (without bacteria, but just streaked with sterile inoculation loop). Further, individual colonies on the plate were used for making pre-culture.

5.2.2 Finding the potential TOFI a.k.a. *Lagna Muhurta*: *Jyotisha* Intervention

TOFIs for bacterial growth experiments were decided using a standard and widely used Indian Telugu Panchangam, 'Andhra Patrika Panchangam'. TOFIs were selected when a *Rasi* containing *Guru* and/or *Rahu* was in *Lagna* for the length of time available to start the main experiment. Only such of those TOFIs are selected which are devoid of '*Rahukala*', '*Durmuhurta*' and '*Varjyam*', which are readily made available in any standard Panchangam, as per the traditional approach. For eclipse TOFIs, the lagna muhurta was the starting time of the various phases of the eclipse.

***Jyotisha* Intervention:** Time of Flask Inoculation of the main flask was taken as *Jyotisha Muhurta*, analogous to an intervention. Also TOFIs were used to compute *graha Shadbalas*.

5.2.3 Culturing bacteria for generating growth curves: One hundred & fifty growth curves were generated. The following preparations were done before performing the growth experiments.

First, 50 ml of Luria Broth was prepared in as many 250 ml conical flasks as required for the day's experiment (along with one additional control flask for every individual growth curve experiment) following standard procedure as explained above. Separately, for every growth curve experiment, the same LB broth in 3 ml portions were prepared and placed in 15 ml Falcon tubes (one for one growth curve experiment

along with one for control). All containers thus prepared, were sterilized by autoclaving at 120^0C and 15 bar pressure for 30 mins (Widdel, 2007).

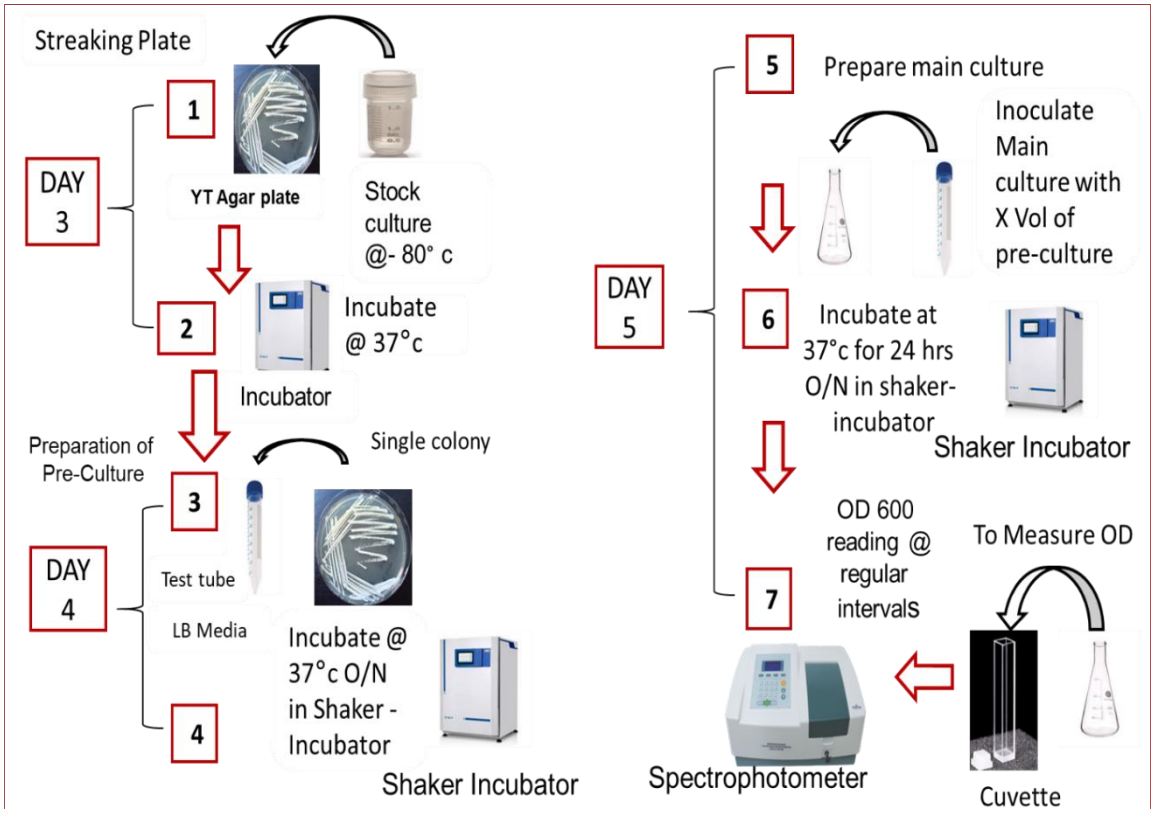
The next day, a pre-culture was made by inoculating a autoclaved 3ml LB broth in Falcon tube with a single colony from the streaked YT agar plate, and incubating it overnight at 37°C and 180 rpm in a Shaker-cum-Incubator. In all individual experiments care was taken to uniformly maintain the pre-culture culture duration to be between 12 to 14 hours before using the pre-culture to inoculate the main culture at the intended *muhurta* (TOFI). This pre-culture was used to inoculate a pre-autoclaved 250 ml conical flask with its 50 ml LB broth as the main culture for that growth experiment. The starting OD of the main culture was fixed to be **0.002.** The volume of pre-culture required to get a starting OD of 0.002 for the main GC experiment was arrived at by dividing **overnight OD of pre-culture / starting OD required** in main culture (0.002). For example: when overnight OD of pre-culture is 4.5; dilution required was calculated as **4.5/0.002=** 2,250 times of dilution is required.

According to this example, 22µl of pre-culture was inoculated into 50 ml of main culture to get a starting OD of 0.002 (50/2250=0.022ml). Finally, the required amount from 3 ml pre-culture grown overnight in the 15 ml Falcon Tube was transferred to the pre-autoclaved 50 ml Luria Broth medium in its 250 ml conical flask (Widdel, 2007).

The main culture was then incubated in the Shaker-cum-Incubator maintained at 37°C and 180 rpm. Growth experiments were conducted in single flask or in replicates (for each TOFI). This was purely decided based on the logistic resources. On each day selected to run experiments, two or three growth curves were generated with different main culture inoculation-time (TOFI) *muhurta* (Shriram, 1996). A pre-autoclaved

blank control, treated in the same way as culture flask/falcon tube/petri plate except inoculum, was used at each step: for step one, a blank YT Agar plate; for step two, a blank 15 ml Falcon Tube with its 3 ml Luria Broth; and for step three, a blank 50 ml Luria Broth in its 250 ml flat bottomed conical flask.

Figure 5.1: Sequence of important steps in a growth curve experiments



Caption: Figure shows sequence of steps for three days of a growth curve experiment

5.2.4 Steps in Measuring OD-600 and Data Extraction:

1. OD600 (optical density at 600nm) measurements (Widdel, 2007) were made using Nanodrop™ 2000c spectrophotometer.

2. TOFI forms the 0 minute reading; subsequently periodical OD measurements

were also done.

3. OD measurements were done against blank LB broth from the control flask (see above) maintained.

4. In all, the OD was measured at nine time points 0, 60, 120, 150, 180, 210, 240, 300 and 360 minutes.

5. For this purpose, 1 ml aliquots from the flask growing the main culture in the incubator-cum-shaker were taken at the above time points in sterile condition (under flame, using sterile micropipette tips). After measurement, the 1 ml aliquot was discarded.

6. Every measurement was immediately recorded in the logbook.

5.2.5 Generating growth curves:

After conducting each experiment, the OD 600 data were entered in Libre Office Calc spreadsheets (versions 5.1 to 7.2.2) under Ubuntu (14.04 to 20.04) O.S., a popular Linux distribution. To generate each growth curve, Measurement Times on Abscissae, and OD values on Ordinates were plotted. Of the three typical phases of growth exhibited by bacteria grown in batch culture (lag phase, log/exponential phase and stationary phase), it was decided to look for differences in growth due to TOFI at the exponential phase. For the strain of *E. Coli,* we were using, the exponential phase of growth was identified to span 180 minutes between 180^{th} and 360^{th} minute from the TOFI through pilot growth experiments. And so the steepest slope, observed between these 180 and 360 mins, was computed by calculating the gradients of best-fit lines to (spanning) those five time points in the exponential phase. See Fig.1 for a general

understanding of the concept of exponential growth phase.

5.2.6 Calculating *Graha Shadbalas*:

Applying principles of *Jyotisha* (Santhanam, 1984), *Saptagraha Shadbala* strengths were calculated for each TOFI *muhurta* using Vedic Astrology Software, Jagannatha Hora, Version 8.0 (www.VedicAstrologer.org). Correlations between exponential phase growth rates and the seven *Graha Shadbalas* were computed. Detailed method for the calculation of Graha Shadbalas of seven grahas is given at the end of this chapter. All further analysis was done using **IBM SPSS Statistics for Windows, version 21.0 (IBM Corp. Armonk, N.Y. USA)**

5.3 ECLIPSE DAY EXPERIMENTS:

Thirty-three growth curves were generated for 30 TOFIs on the six eclipse days between August 2017 and July 2018, with the TOFIs coinciding *with **distinctly different identifiable 'phases of eclipses'** as commonly named in astronomical parlance*. Three days were those of partial or total solar eclipses, while three were days of lunar eclipses, either partial or total. Rates of exponential phase growth on these six eclipse days are reported. There were used to empirically test hypotheses based on earlier results which indicated retarded bacterial growth during eclipse due to its malefic effect

5.4 STUDY VARIABLES

1.0 *Shadbala* strengths: Applying principles of *Jyotisha* (Santhanam, 1984), *Shadbala* strengths of seven planets were computed for each *muhurta*, i.e. the TOFI from the six sources of constituent parts of six-fold Shadbala. Total points in

Shashtiamsas obtained make the 'Net or Total' Shadbala of a graha for given place at a specified time. Shashtiamsas are also known as units of **Virupas**. As per tradition, 60 units of Virupas or Shashtiamsas make One unit of Rupa of Shadbala. Usually Shadbala value is mentioned either in 'Rupas' or 'Virupas'. **A more detailed account on Shadbala calculation procedures is attached as Appendix-3.**

2.0 Growth rate*:* Exponential phase growth rate was calculated as the slope of the line of regression **spanning five data points for times 180, 210, 240, 300 and 360 minutes**. The experiment also studied growth variables connected to bacterial growth, like cell numbers, doubling time which are **computed from OD at the five data points**. Growth rate or Slope used in analysis, is **taken as representative of growth parameters derived from OD by formula**. Then compared them to *Jyotisha* properties of the TOFI *muhurta*. These data were used for further data analysis, e.g. **to disprove null hypotheses**. A Model Bacterial Growth Curve is given below showing Main Phases.

5.5 DATA ANALYSIS

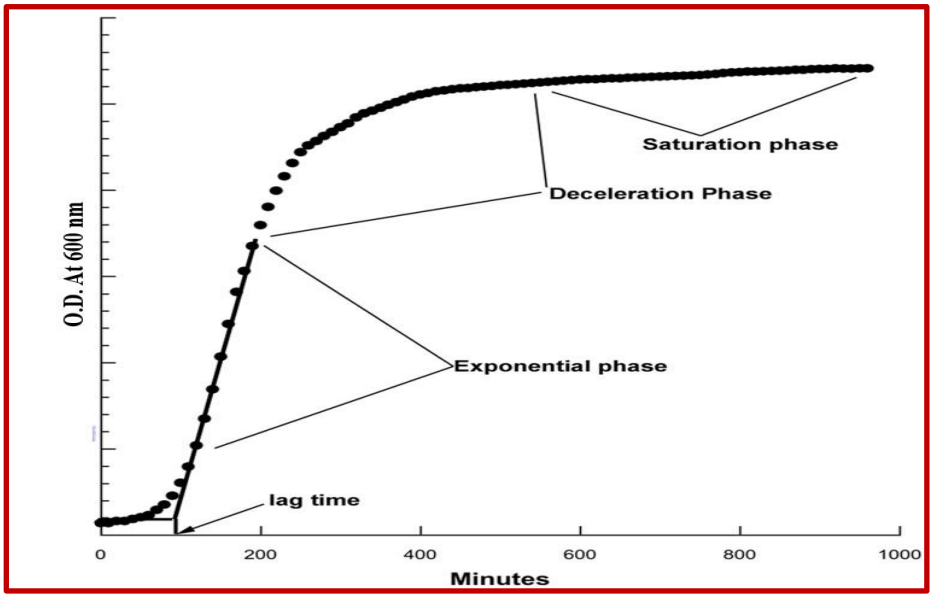
5.5.1 F-Tests and Sub-Group Variance Analysis: F tests were conducted comparing within-column variances of Table 6.4's group of 91 growth curves. Verified the sequence of increasing within column variances from Table 6.4, columns 2 to 5, particularly Row 6 in bold; and also conducted F tests in the last 3 rows. Values of column 3 of Table 6.5are graphically displayed (Vegaraju et al., 2020a**).**

5.5.2 Pearson's Correlation: A Pearson's product-moment correlation coefficient was run to assess the relationship between Mean Slope of exponential phase segment of growth curve and the Shadbala of Saptagrahas 72 growth curves were generated and evaluated for data analysis (Vegaraju et al., 2020b**).**

5.5.3 Eclipse Days experiments: The mean slopes of exponential phase segment for all growth curves done on days of eclipses were analyzed taking into account ***Gamma*** ***(γ)*** of an eclipse values (Vegaraju et al., 2019a**).**

5.5.4. Compared effects of *Guru* versus *Rahu* in *Lagna* over many weeks of slope **data of ten days' growth experiments**, with a pairing of *Kanya* and *Simha Lagnas*

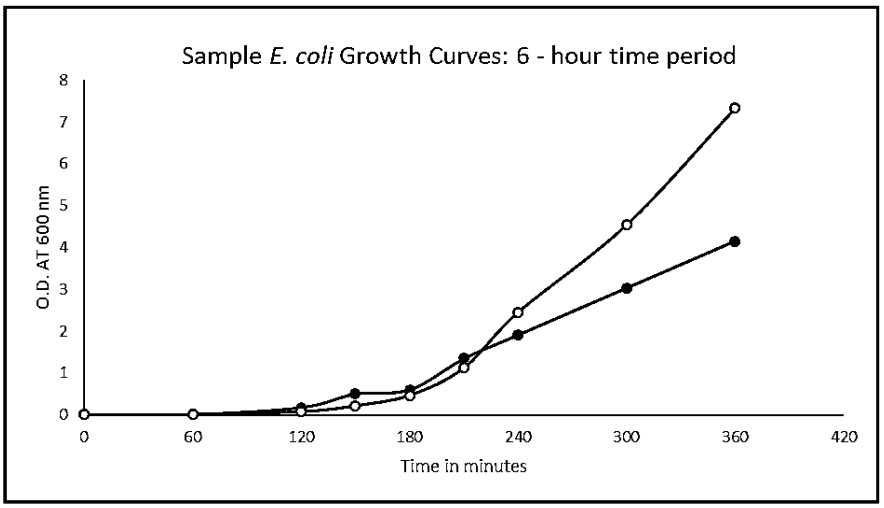
Figure 5.2: Model Bacterial GC showing Exponential, Deceleration, and Saturation Phases (Numbers on Time Axis Arbitrary)



The above Figure 5.2 Model Bacterial GC depicts Exponential, Deceleration, and Saturation Phases with Numbers on Time Axis considered arbitrary in our case and part of this graph portion is adopted from "Growth Rates Made Easy" (Hall et al, 2013) for illustration purposes only.

An example for anticipated difference in GC started at two different TOFIs is illustrated in Figure 5.3. below are given typical *E. Coli* bacterial growth curves.

Figure 5.3: Typical *E. Coli* Bacterial Growth Curves started at two different TOFIs illustrating differences in exponential phase of growth.



The following Table 5.3 gives constituent components of Graha Shadbalas.

Table 5.3: Graha Shadbalas of Sapta Grahas as per Parashara Rishi (Santhanam R., 1984)	
No.	Six Constituent Components of Graha Shadbala
1	Sthanabala (Positional): Strength according to Rasi & Amsas
2	Kaalabala (Temporal): Strength due to Time of Day
3	Digbala (Directional): strength due to Direction in Sky
4	Cheshtabala (Motional): Prograde, Stationary, Retrograde
5	Drigbala: strength due to Aspects by other Grahas
6	Naisargikabala: Fixed Natural Strength; unchangeable.
Caption: Table 5.3 details Six Constituent parts of a Graha Shadbala	

Table 5.4 gives Sapta Graha Shadbalas in ‘Virupas’, units of Shadbala as per *Jyotisha,* for 3 typical T.O.F.I.s Jyotisha *Muhurtas*

Table 5.4: Graha Shadbalas in ‘Virupas’, for 3 typical T.O.F.I.s			
	TOFI 1	TOFI 2	TOFI 3
Surya	486	661.4	487.47
Chandra	412	429.45	484
Kuja	374	359.1	320
Budha	402.09	465.06	460
Guru	428	584.88	504
Sukra	541	458.79	526
Sani	358	279.06	326