Study of qualitative and quantitative changes in metabolic profile of *Escherichia coli* cells in response to Glucose deprivation.

Krishi Tata [a], Abhinav Dubey [b,c] and Prof. Hanudatta S. Atreya [c]

[a]Department of Biotechnology, Indian Institute of Technology Madras, Chennai, krishitata045@gmail.com

[b]IISc Mathematics Initiative, Indian Institute of Science, Bangalore, abhinavdubey29@gmail.com

[c]NMR Research Center, Indian Institute of Science, Bangalore, hsatreya@gmail.com

ABSTRACT

E.coli is one of the most studied bacterial species. There are a number of ways in which these cells respond to external stimuli which can be in the form of nutrient starvation, temperature (heat shock or cold shock), pH etc. The question of how the metabolite profile of *E.coli* cells vary quantitatively and qualitatively in response to Glucose deprivation, a form of nutrient starvation is addressed here. The cells might respond either by change in the metabolite concentration by keeping their cell numbers constant with varying amount of glucose or they might reduce their cell numbers by producing the required amount of metabolites. The metabolic profile is measured using Nuclear Magnetic Resonance (NMR) technique. Specifically, the 2D HSQC (Heteronuclear Single Quantum Coherence) experiment is used to analyze spectrum, in order to find out the varying concentrations of the metabolites.

1. INTRODUCTION

1.1. Metabolomics

Metabolites are the intermediates of biochemical reactions. They are synthesized by the cell for the purpose of performing a useful function in cell maintenance and survival. These metabolites undergo chemical reactions regulated by enzymes which modify the chemical characteristics of the metabolites. These chemical reactions in series are called a pathway and sum of all pathways is called metabolism. Hence, the metabolites play a crucial role in bridging different pathways in the living cell. Metabolism can be either central (or primary) metabolism which is concerned with energy and core structures of the cell such as proteins and structural components which are central across species. These metabolites are influenced by nutritional environment. Secondary metabolism is the production of far more specialized metabolites which may be specific to single species.

Metabolome is a complete set of all metabolites used or formed by the cells in association with its metabolism. Analysis of the level of metabolites gives an important insight on the cellular pathways and cell functioning with regard to different environmental conditions and stimuli. The various kinds of ways to analyze the metabolome or a fraction of it can be described as metabolomics. It involves the qualitative and quantitative study of all intracellular and extracellular metabolites with a molecular mass less than 1000 Daltons using various analytical techniques. Mass spectrometry (MS) and Nuclear magnetic Resonance (NMR) are the most frequently used methods of detection in metabolomics.

1.2. NMR Spectroscopy

NMR Spectroscopy is one of the most efficient techniques of measuring very specific molecular properties that can be used to elucidate the structure of molecules. NMR measures the spin and magnetic moment properties of nuclei in a molecule. All nuclei have an intrinsic property of spin due to which they obtain angular momentum.

They exist either in +1/2 or -1/2 spin states. This spin creates a magnetic field which orients itself with the spinning axis of the nucleus.

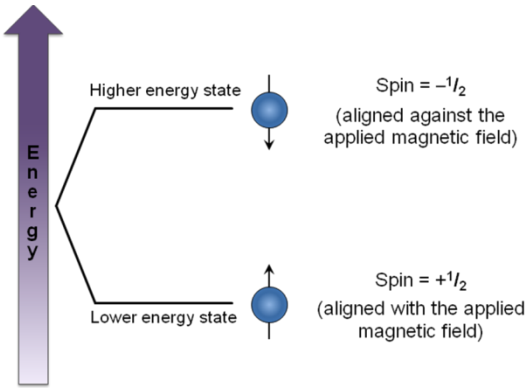

Figure 1. Zeeman splitting in presence of magnetic field

If a nucleus is placed in external magnetic field, it aligns itself with the field in one of the two directions depending on the magnetic moment of the nucleus. The potential energy in a quantum state of +1/2 is lower than that in a quantum state of -1/2, thus most nuclei reside in +1/2 quantum state. An NMR spectrum is normally created by radiating the sample with a short pulse of high energy radio frequencies (typically in the range of 100-1000 MHz depending on the field strength) that excite all the nuclei. Rather than measuring the absorption at each frequency, the energy emitted when the nuclei return to lower energy state is measured as a Free Induction Decay (FID) signal. By Fourier transformation of the FID signal, the decay can be converted into a pattern of signals. The scale is then calibrated to the frequency of a reference compound and the frequencies are converted to parts per million (ppm) of the radiation frequency to ease the task of comparing between instruments. Thus, an NMR spectrum is generally plotted as ppm-value (called chemical shifts) versus intensity. The required compound is dissolved in solvents such as Deuterium oxide or chloroforms which do not interfere with NMR signals. The sample is placed in an NMR tube and kept in the magnet.

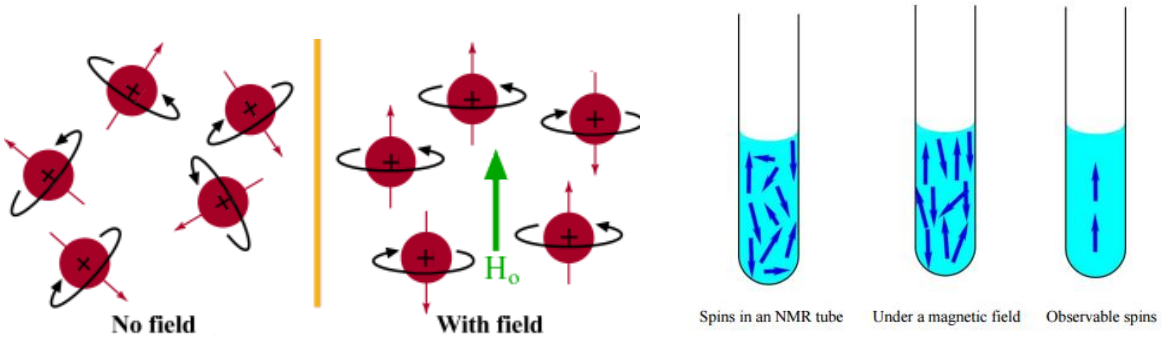

Figure 2. Nuclei aligned when placed in an external magnetic field according to their spin state.

NMR spectrum basically shows at which chemical shift, the nuclei will absorb energy. The more shielded a nucleus is, the higher is the chemical shift; thus a proton will be found at lower ppm if it is in a simple hydrocarbon and at much higher ppm if it is on a benzene ring.

1.2.1 One Dimensional NMR spectroscopy

One dimensional NMR spectra are plots of intensity versus frequency. In one dimensional pulsed Fourier transform NMR, the signal is recorded as a spectrum which is a function of one frequency variable. The spectrum consists of signals which are split up into several lines (the split signals are called multiplets). This arises from the interaction of different spin states through the chemical bonds of a molecule and result in splitting of NMR signal.

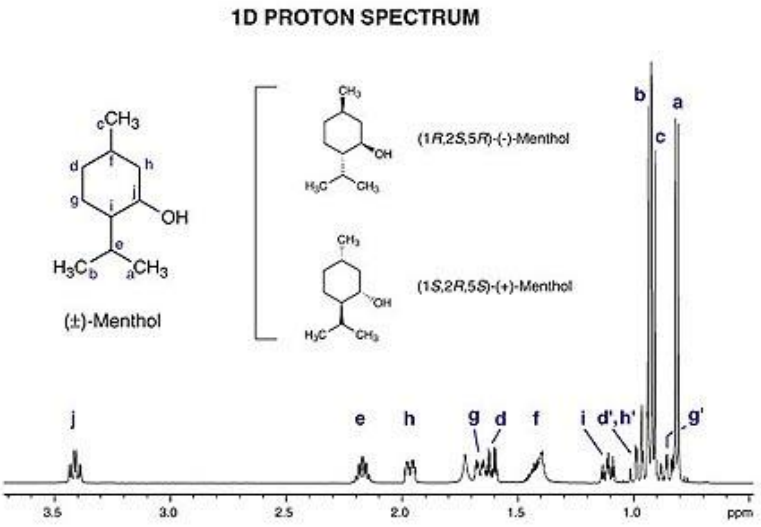

Figure 3. ^{1}H spectrum of menthol with chemical shift in ppm on the horizontal axis. Each proton has a characteristic shift, and coupling to other protons appears as splitting of peaks into multiplets.

One dimensional spectrum is far too complex for interpretations as most of the signals overlap heavily. By introduction of additional spectral dimensions, these spectra are simplified and some extra information is also obtained.

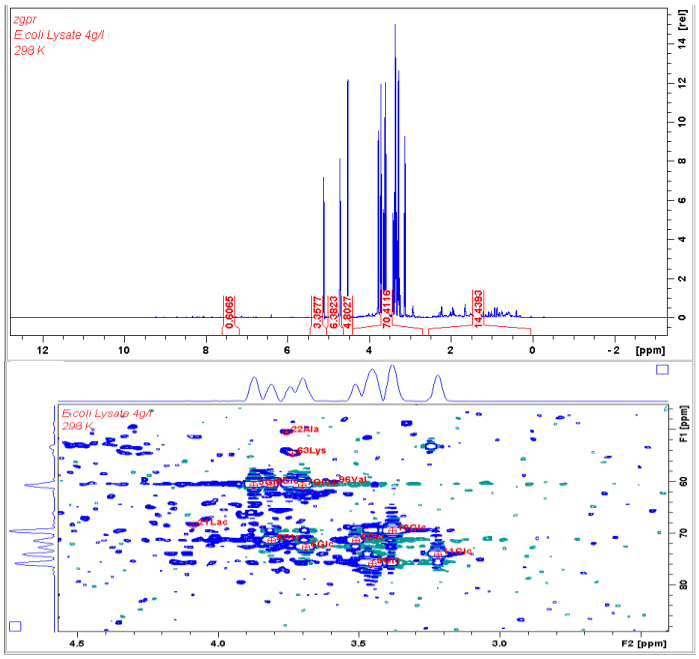

Figure 4. Comparison of one dimensional and two dimensional spectra.

1.2.2. Two Dimensional NMR spectroscopy

In two dimensional NMR, the signal is recorded as a function of two time variables and the resulting data is Fourier transformed twice to yield a spectrum which is a function of two frequency variables.

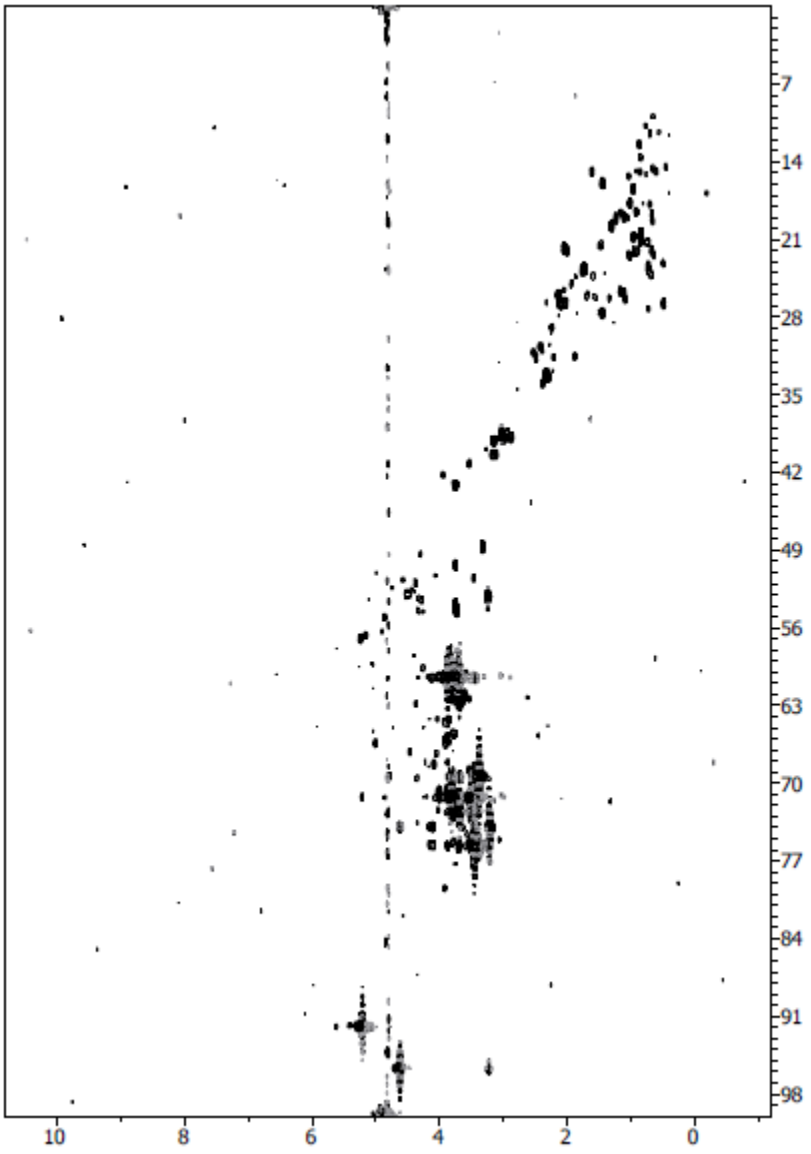

Figure 5. ^{13}C HSQC spectrum.

There are various ways of representing such a spectrum, but the one most usually used is to make a contour plot in which the intensity of the peaks is represented by contour lines drawn at suitable intervals, in the same way as a topographical map. The position of each peak is specified by two frequency coordinates F_1 and F_2 .

The Heteronuclear Single Quantum Coherence (HSQC) spectra are two dimensional NMR experiment with one axis for proton (^1H) and other for a hetero nucleus (an atomic nucleus other than proton which may be ^{13}C or ^{15}N). The spectrum contains a peak for each unique proton attached to the hetero nucleus considered. The 1H signal is detected in the directly measured dimension in each experiment, while the chemical shift of ^{15}N or ^{13}C is recorded in the indirect dimension which is formed from these experiments.

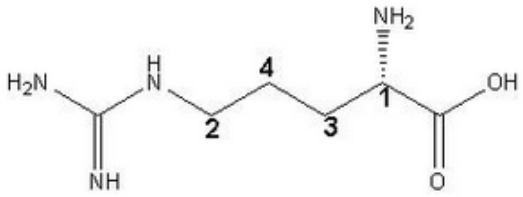

Figure 6. Chemical shifts of carbons and hydrogen marked on the structure of L-Arginine from the ^{13}C HSQC spectra

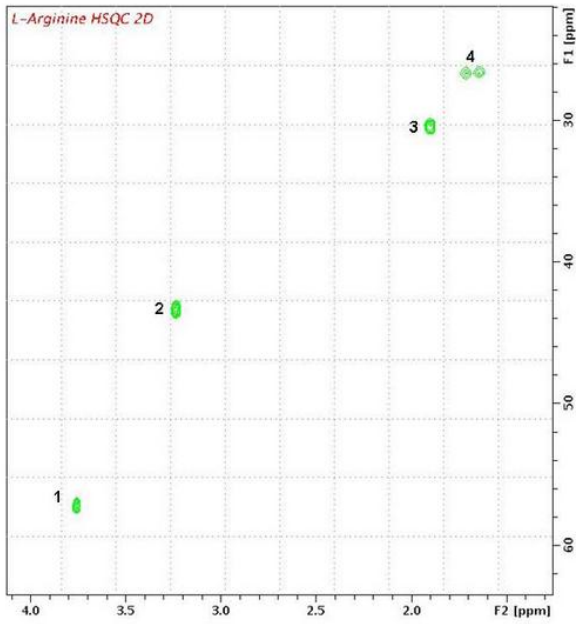

Figure 7. The 13C HSQC spectra of L-Arginine and Figure 6 correspond to assignment of chemical shift to corresponding carbon.

1.3. *Escherichia coli* cells

Escherichia coli is a facultative (aerobic and anaerobic growth) , gram negative, rod shaped bacteria that can be mainly found in animal feces, lower intestine of mammals and even on the edge of hot springs. They grow best at 37 degree centigrade. *E.coli* has only one circular chromosome, some along with a circular plasmid. It is a rod shaped bacteria which possesses adhesive fimbriae and a cell wall that contains an outer membrane made of lipopolysaccharides, a periplasmic space with a peptidoglycan layer and an inner cytoplasmic membrane. Even though it has extremely simple cell structure, with only one chromosomal DNA and plasmid, it can perform complicated metabolism to maintain its cell growth and cell division.

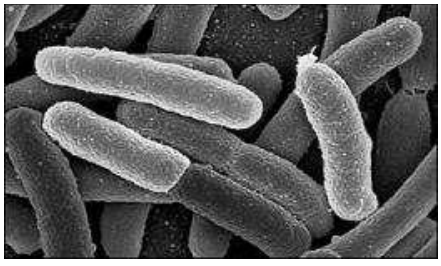

Figure 8. Cells of *E.coli*

E.coli cells that were used are required to have antibiotic resistance genes in order to avoid undesirable growth of other microorganisms. For this, the cells are required to be transformed using Ampicillin resistance gene. *E.coli* cells are more likely to incorporate foreign DNA if their cell walls are altered so that DNA can pass more easily. Such cells are said to be competent. Cells are made competent and transformed by a process that uses calcium chloride and heat shock. Competence results from the alterations in the cell wall that makes it permeable to large DNA molecules. Cells that are undergoing very rapid growth are made competent more easily than cells in other stages of growth.

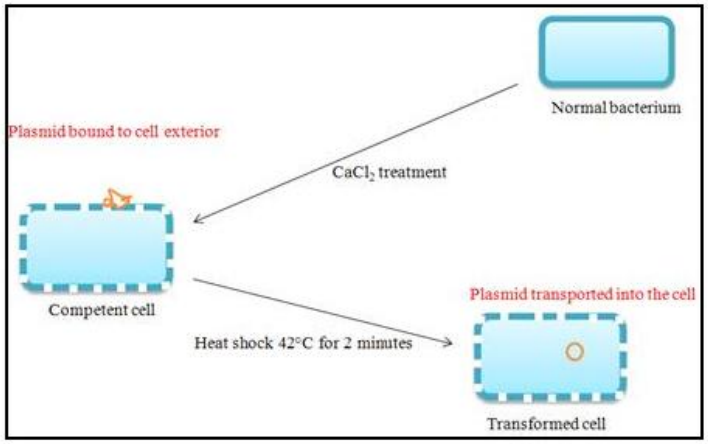

Figure 9. Overview of competence and heat shock.

Transformation is the genetic alteration of the cell resulting from the direct uptake and incorporation of exogeneous genetic material from its surroundings and taken up through the cell wall. Because transformation usually produce a mixture of a relatively few transformed cells and more of non transformed cells, the plasmid therefore requires a selectable marker such that those cells without plasmid maybe killed or have their growth arrested. Antibiotic resistance is most commonly used marker for bacteria.

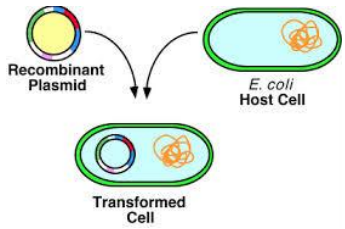

Figure 10. Transformation of *E.coli* cells.

2. PROBLEM STATEMENT

Many responses have been recorded in *E.coli* to nutrient starvation and metabolic regulation. Seceral global receptors such as RpoD, SoxRS, Cra, FadR and IclR may help *E.coli* cope with different kinds of stress. In the following experiment, the glucose is provided as sole carbon source and it is decreased by half in each culture grown in minimal media. The response of the cells is studied to understand the qualitative and quantitative changes in the metabolic profile by targeting a few known metabolites and understanding how they vary with respect to change in glucose levels. The metabolites were analyzed with the help of ^{13}C HSQC NMR experiment.

3. MATERIALS AND METHODS

3.1. Transformation of *E.coli* cells

3.1.1. Materials and Instruments

- BL21 Competent *E.*coli cells
- Plasmid with Ampicillin resistance gene
- Luria- Bertani broth
- Agar plates with Ampicillin (100µl of Ampicillin for 100ml of rich media)
- Incubator
- Ice bath
- Centrifuge machine

3.1.2. Method

- 50µl of calcium chloride treated competent cells are added with 1µl of plasmid with Ampicillin resistance gene.
- The eppendorf with the above mixture is kept in ice for 30 minutes.
- Heat shock is given to the cells at a temperature of 42º C for 90 seconds.
- The cells are then immediately kept in ice bath and allowed to chill for 2 minutes.
- 700µl of LB media is added to the cells and incubated at 37ºC for 30-45 minutes.
- The cells are then pelleted down and about 400µl of media are removed.
- The cells are resuspended in the remaining media and plated on Agar plates with Ampicillin using an L-rod.
- The plates are incubated at 37ºC for 12 to 16 hours.

3.2. Sample preparation

3.2.1. Materials and Instruments

- Four 10ml LB media
- Transformed cells of Ampicillin resistance gene
- Minimal media (for 100ml media)

Glucose	0.4 g
Ammonium chloride	0.2g
Calcium chloride (0.5M)	100µl
Magnesium Sulphate (0.1M)	1ml
M9 Salts (Na_2HPO_4,KH_2PO_4 and NaCl)	10ml
Vitamin mix	100µl
Ampicillin	100µl

- Agar plates with Ampicillin resistance gene
- Cuvette and UV spectrophotometer
- Incubator
- 80% methanol solution in water
- Sonicator

- Rota vapor and vacuum pump
- Lyophilizer
- NMR machine

3.2.2. Method

- The colonies of transformed cells from the Agar plate are picked up and inoculated in four 10ml LB media tubes and incubated at 37ºC for about 12 hours.
- The cells are then pelleted down at 8000rpm for about 5 minutes and the supernatant is thrown away.
- Minimal media with varying amounts of glucose that is 4 g/l, 2g/l, 1g/l and 0.5 g/l is made already. 1ml of minimal media from each 100ml media is taken and added to the pellet.
- The cells are slowly resuspended in the media and pooled.
- 1ml of this mixture is equally added to each of the four 100 ml minimal media flasks.
- The conical flasks are kept in incubator at 37ºC and at 200 rpm.
- The cells are grown for about 6-7 hours until the Optical Density (OD) in one of the flask reached 0.6.
- 10µl of the cell culture is diluted in 990µl of water.
- Agar plates with Ampicillin resistance gene are made and the above diluted cell culture is plated with an L-rod.
- These plates are incubated overnight at 37ºC and the number of colonies is counted to keep a track of cell number.
- The cell culture is then taken out from the incubator and each 100ml culture is added to four different centrifuge tubes.
- The cultures are pelleted down at 4000 rpm, 4ºC for about 10 minutes.
- 2ml of the supernatant is stored in separate eppendorfs from the four cultures and rest is discarded.
- The pellet is added with 1.5ml of 80% methanol solution and resuspended.
- The resuspended pellet is taken into a 2ml eopendorf and immediately kept in ice.
- This pellet is then sonicated for about 12 minutes in ice bath.
- These cells are now lysed. This is centrifuged at 12,000 rpm at 4ºC for about 20 minutes.
- The pellet containing the cell debris is discarded and the supernatant is collected.
- Four such eppendorfs with supernatants from the sonication procedure are kept in the rota vapor for about 2hours to remove the methanol.
- High vacuum pump is used to remove any remaining traces of methanol.
- The water in the lysate is removed by keeping it in the lyophilizer overnight.
- The dried sample is then added with 500µl of Deuterium oxide.
- This sample in taken in the NMR tube and placed in the machine to record the spectrum.

3.3. Analysis of 2D ^{13}C HSQC spectra

3.3.1. Method

- A peaklist of 10 metabolites was made and calibrated on the 13C HSQC spectrum of 4g/l glucose sample which is the control.
- The peaks were integrated on Bruker Topspin NMR software and the integral values are saved for each metabolite. A total of 29 peaks are observed for these 10 metabolites.
- The same peaklist was used to integrate the peaks in other three samples which are 2g/l, 1g/l and 0.5g/l glucose concentrations.
- The relative ratios of individual peaks of 2g/l, 1g/l and 0.5g/l glucose concentrations with the peaks of 4g/l glucose concentration sample for individual metabolite is calculated.

- A matrix is constructed using these 29 peaks.

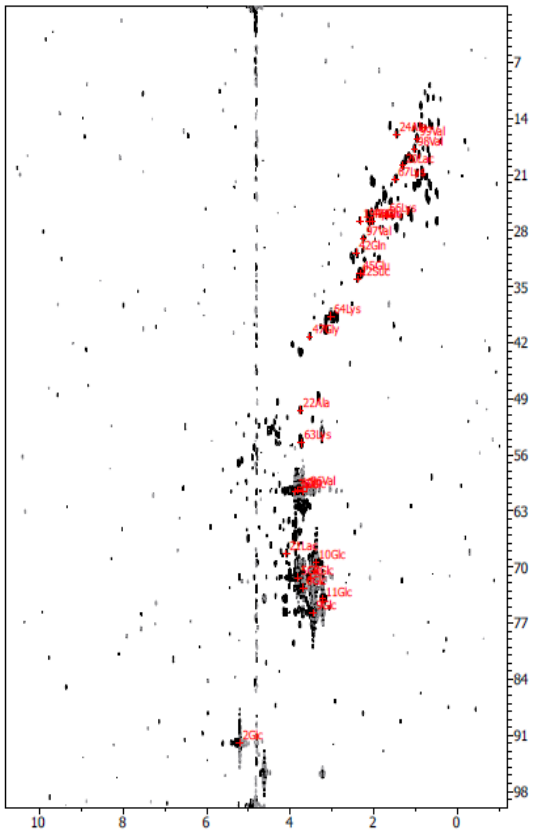

Figure 11. Calibrated peaklist of 10 metabolites on 4g/l glucose concentration sample made on CARA.

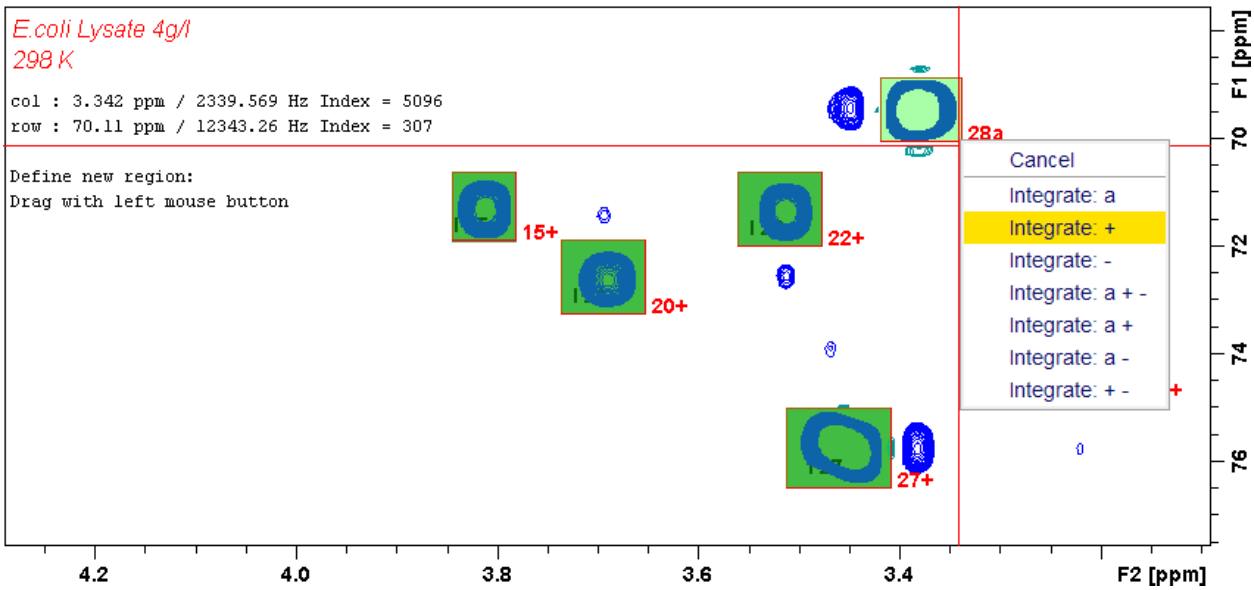

Figure 12. Integartion of peaks done in Bruker topspin NMR software.

4. RESULTS AND DISCUSSION

4.1. One dimensional NMR experiment

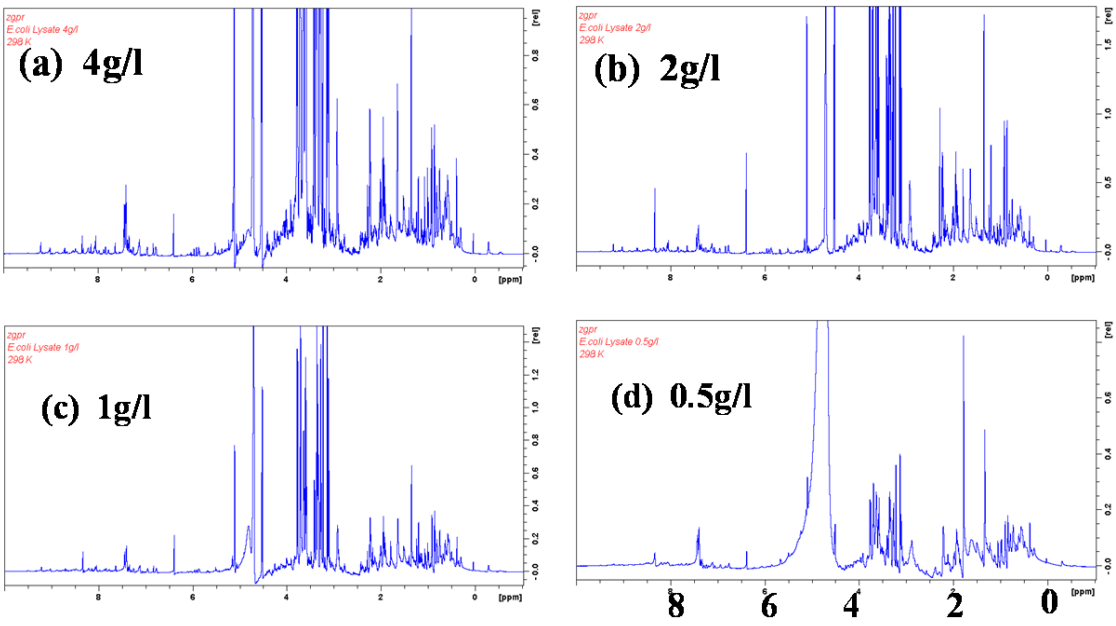

Figure 13. The 1D zgpr spectra for all four samples.

The above figure represents the comparison between the four samples in 1D experiments. We can see the intensity of peaks reducing from 4 g/l to 0.5 g/l sample.

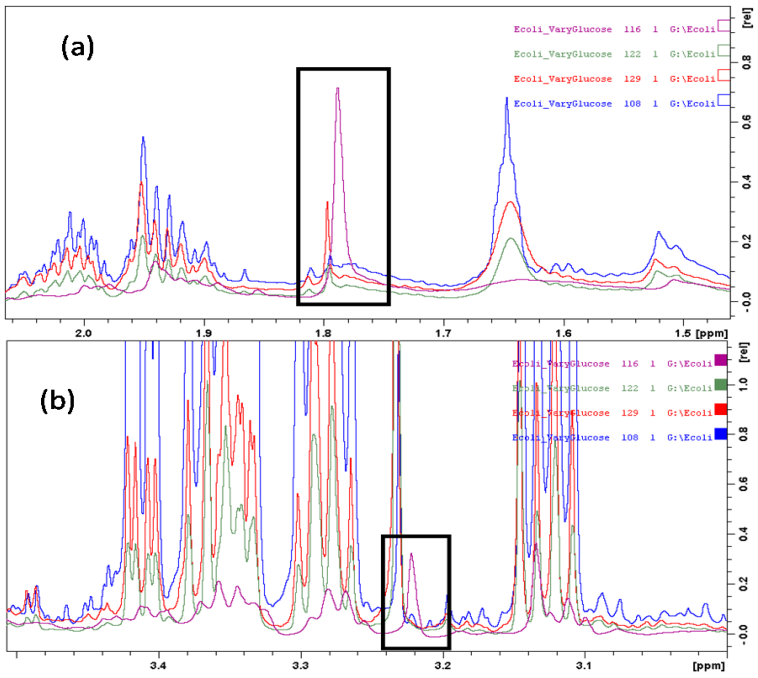

Figure 14. Overlay of four zgpr spectra. Blue color for 4g/l, red color for 2g/l, green color for 1g/l and purple for 0.5 g/l.

From figure 14, we can say that for most of the peaks there is a gradual decrease in the concentration of the peak when the concentration of glucose is halved by 2 that is 4g/l, 2g/l,1g/l and 0.5 g/l. The same trend is seen in most other peaks on the zgpr spectrum. But in box, we can see a peak which shows high concentration in 0.5 g/l whereas its concentration is not significant in other samples as in 4g/l, 2g/l and 1 g/l. So, we can conclude that this might be one peak which is shown in conditions of glucose starvation.

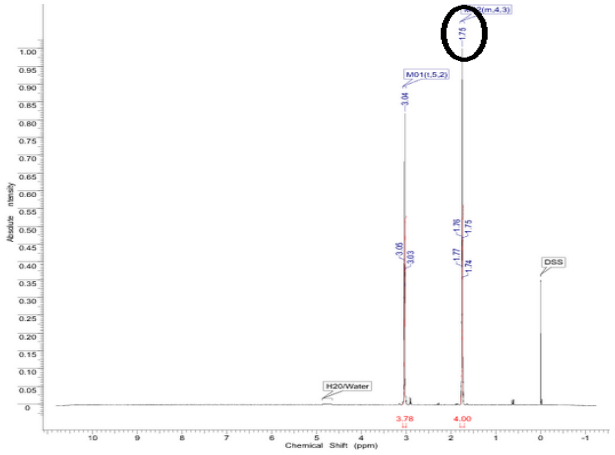

Figure 15. Spectrum of Putrecine from HMDB website. The circled peak might correspond to the peak shown in Figure 13, box 2 as it has almost the same peak value of 1.78 ppm.

From the HMDB database, the compound might be Putrecine with peak at 1.78 ppm from the above figure 15. There are two peaks seen in the spectrum mostly due to the low concentration of it being produced and the other peaks might not have appeared.

4.2. Two Dimensional ^{13}C HSQC NMR experiment

4.2.1. List of metabolites used in the analysis

Name	Chemical shift values F1 (ppm) F2(ppm)		Intensity 4g/l	Intensity 2g/l	Intensity 1g/l	Intensity 0.5g/l
Glucose	3.382	69.457	43244000000	22837000000	13702000000	2716100000
	3.221	73.991	27165000000	14080000000	8758300000	1731800000
	5.211	91.932	11256000000	5881900000	3424700000	613430000
	3.874	60.583	26394000000	14320000000	8168300000	1580700000
	3.814	60.424	15485000000	9544600000	5380600000	1263700000
	3.812	71.317	16614000000	9570900000	5421200000	1431100000
	3.69	72.574	14610000000	7560100000	4269100000	816770000
	3.702	60.594	26558000000	14254000000	8455000000	2061200000
	3.513	71.338	16792000000	8828800000	5287600000	1201600000
	3.453	75.717	49443000000	26469000000	16257000000	3269000000
Succinic acid	2.386	34.054	222640000	1965300000	457680000	41122000
Pyruvate	2.331	26.747	117740000	941510000	391800000	123510000
Lactic acid	1.306	19.876	509420000	2055800000	1328800000	130410000
	4.09	68.329	324920000	1359300000	727090000	8688800

Alanine	3.759	50.386	409920000	2206100000	1066700000	69119000
	1.455	16.002	555110000	2998600000	1526800000	1199100000
Glutamine	2.425	30.712	354970000	359910000	380590000	123140000
	2.103	26.827	1251200000	3835600000	2078700000	891940000
Glutamic Acid	2.327	33.343	2626100000	6131400000	3834400000	1824000000
	2.041	26.795	1002300000	2571100000	1735200000	929950000
Glycine	3.535	41.264	229090000	209570000	101990000	80468000
Lysine	3.736	54.482	1936400000	4278000000	2659800000	465860000
	3.026	38.66	3061300000	7608700000	4689500000	1617200000
	1.694	26.098	303250000	677240000	479270000	372150000
	1.485	21.599	452000000	879360000	197860000	106540000
Valine	3.588	60.102	158590000	1263200000	652290000	198330000
	2.252	29.037	488060000	1028200000	841580000	356660000
	1.024	17.813	401430000	1802300000	1045500000	487180000
	0.97	16.533	402810000	2012400000	1071400000	694960000

The above table gives a list of the metabolites which have been used in the analysis of ^{13}C HSQC spectra of the four samples. Glucose has a total of ten peaks; Succinic acid,Pyruvate and Glycine has each one peak ; Lactic acid, Alanine, Glutamine, Glutamic acid each have two peaks and Lysineand valine have four peaks each.

We can see that the integral values of Glucose are decreasing in a pattern from 4g/l to 0.5 g/l but we cannot conclude anything from this because we are not sure of the cell numbers.

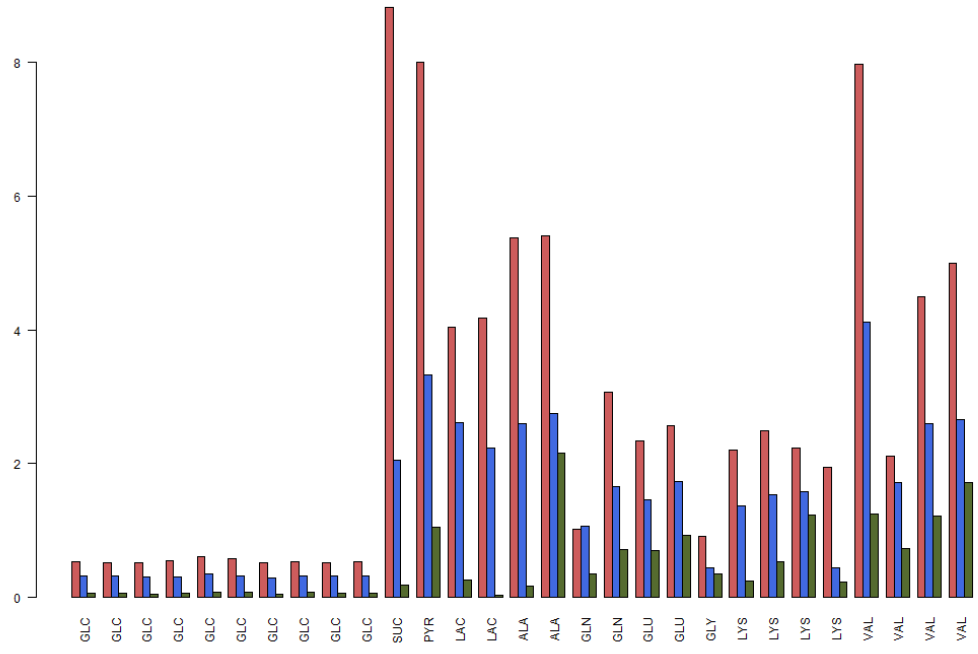

Figure 16. A bar plot showing the relative ratios of peak volume of the 29 peaks with respect to 4 g/l sample. Red color represents 2g/l, blue color represents 1 g/l and green color represents 0.5 g/l glucose concentration.

From figure 16, we have the bar plot of ratio of relative peaks with respect to 4g/l sample. We have done this keeping in mind that the OD of all the four samples lies between 0.5 to 0.6, so the cell numbers are almost constant. The glucose peaks in 2g/l, 1g/l and 0.5 g/l samples decrease as expected. But when we take the case of 2g/l with respect to 4g/l sample, we can say that almost all the metabolites have increased. The same is with 1 g/l sample. But the metabolites of 0.5 g/l sample have shown decrease with some exceptions of Alanine and Valine. Nothing seem to have significantly increased only in the case of 0.5 g/l sample.

4.2.2. Assignment of Metabolites

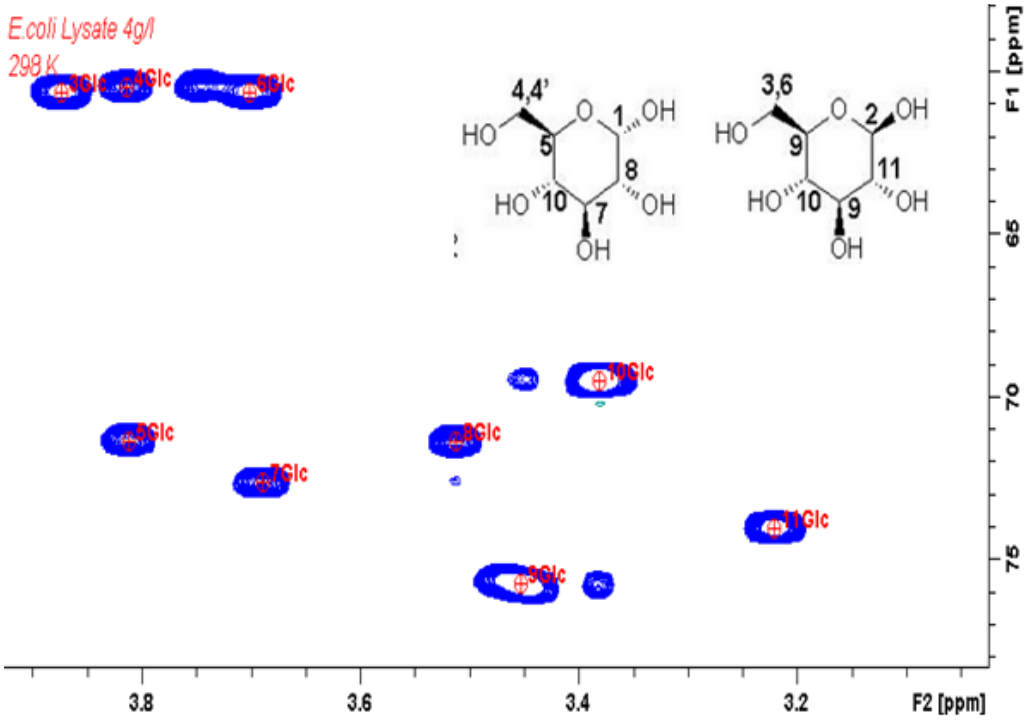

Figure 17. Assignment of Glucose peaks in the ^{13}C HSQC spectrum of 4g/l glucose sample.

Figure 17 shows the assignment of glucose peaks in the spectrum and how the peaks in the spectrum correspond to the carbon atoms in the structure of glucose. Other peaks are also assigned in a similar way. The number of peaks shown by a particular metabolite in the spectrum depends on its concentration in the sample.

4.2.3. Heat Maps of metabolites

Heat maps of the metabolites in samples of glucose concentration 2g/l, 1 g/l and 0.5 g/l with respect to the metabolite concentrations in 4g/l glucose sample gives an idea of how the corresponding metabolites are varying in the different concentrations of glucose as compared to the control.

In Figure 16 below, we can see how many folds the particular metabolites are increasing or decreasing with respect to the control sample that is 4g/l glucose sample. It is the heat map of 2g/l glucose sample with respect to control. We can see that metabolites such as succinic acid vary four fold, pyruvate vary 16 times, Lysine vary four times etc as seen from the heat map. Lactic acid seems to decrease by four folds from 4g/l glucose sample to 2 g/l glucose sample.

A similar trend is seen in the heat maps of 1g/l and 0.5 g/l glucose concentration sample as compared to 4g/l sample. Glutamate and Glutamine have seem to be increased by four folds and Valine has increased by 16 folds. Therefore,

from these maps we can see that the OD being almost same for the four samples, Glucose concentration has decreased in a trend but the concentration of most other metabolites seem to have increased.

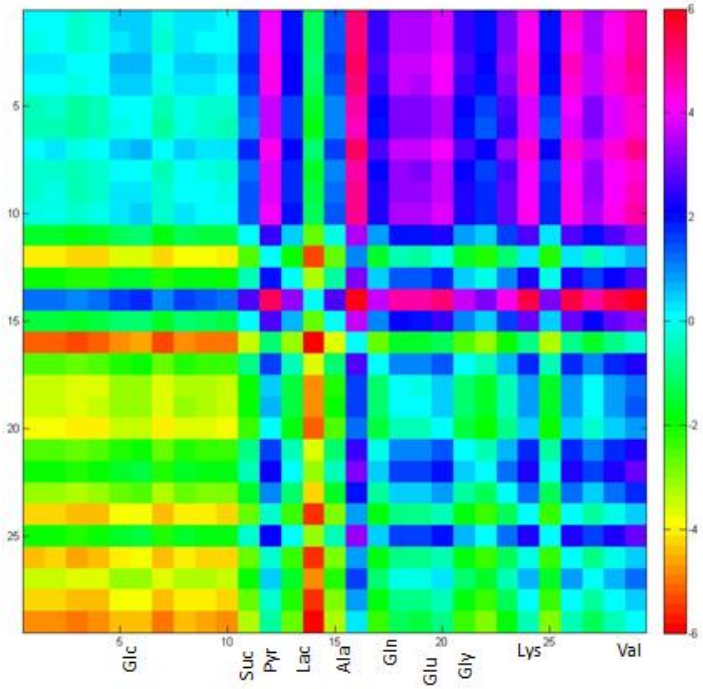

Figure 18. Heat map of metabolites of 2g/l glucose sample varying with the control sample. Different metabolites are marked according to their peaks.

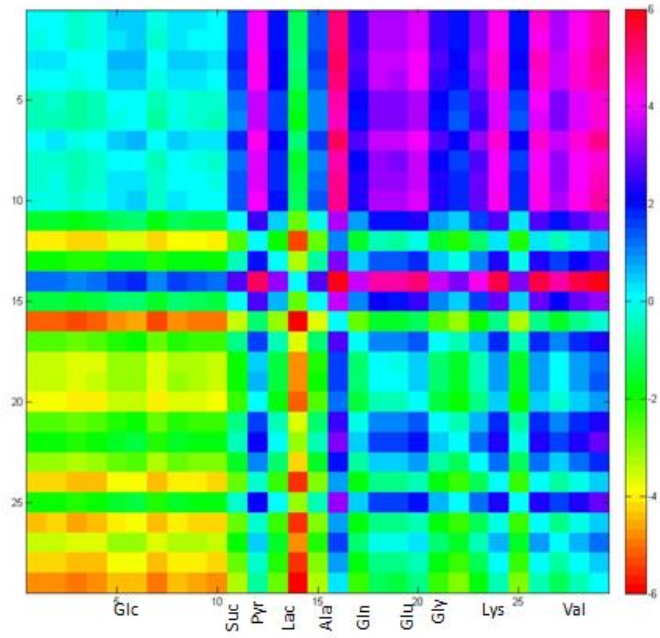

Figure 19. Heat map of metabolites of 1g/l glucose sample varying with the control sample. Different metabolites are marked according to their peaks.

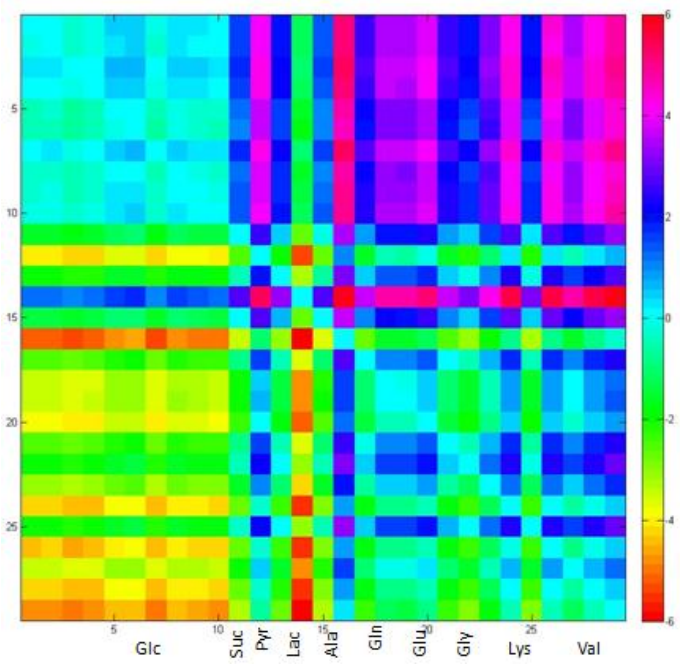

Figure 20. Heat map of metabolites of 0.5g/l glucose sample varying with the control sample. Different metabolites are marked according to their peaks.

5. CONCLUSION

From the experiment and the data, we can say that the *E.coli* cells prefer to keep the cell numbers constant and vary the concentrations of metabolites under starvation, in this case glucose deprivation. But the variation in the concentration of metabolites cannot be quantified as we are not quite sure of the cell numbers and the experiment needs to be repeated at least two times more to have confidence in the findings. Many of the metabolites have not been assigned. This I'm intending to do in future. A similar strategy of quantifying changes in metabolic profile can be applied to many other studies on other organisms also.

ACKNOWLEDGEMENTS

I would like to thank my guide, Prof. Hanudatta S. Atreya for introducing me to NMR and providing me with knowledge, resources and all the help that was required to carry out the experiment.

I would like to extend my gratitude to my co-guide, Abhinav Dubey who has been a constant help all through my project, who has taken the pain in making me understand the basics of NMR and taught me different ways of approaching a given problem. I could have not successfully carried out this project without him.

Iam grateful to all other members of the lab, Sujeesh, Gitanjali, Shahid, Indrani, Varsha and Somnath for helping me at various stages in the lab work and have shared their experience in doing experiment and verifying my mistakes in lab.

I would also like to thank Bhavesh, Sunil and Karunakaran from Organic Chemistry department for taking time off their work and helping me with my experiment.

I would also like to thank BEST program organizers for the lectures and seminars they have conducted which have greatly enhanced my knowledge in field of Bioengineering.

I am also thankful to my co interns, Jugal, Meena and Sai without whom the stay at IISc would not have been as amazing as it was.

Finally, I would like to thank my parents for their constant help and support in letting me explore new avenues and motivating me to achieve higher goals in life.

Krishi Tata

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