Final Project Report

Metagenomic Analysis of Ruminal Microbes

Niche Area of Excellence

(From 15.11.2011 to 31.03.2016)

SUBMITTED TO

EDUCATION DIVISION

INDIAN COUNCIL OF AGRICULTURAL RESEARCH

KRISHI ANUSANDHAN BHAWAN II

NEW DELHI

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College of Veterinary Science and Animal Husbandry
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Summary of the project

The metagenomic sequence data of the two cattle breeds viz., Kankrej, Gir and three buffalo breeds viz., Mehsani, Jaffarabadi and Surti rumen samples was analyzed using MGRASTfor its microbial diversity and functional content. The role of methanogens in the rumen of Indian cattle and its comparative context with exotic cattle was performed. Rumen Microbiome analysis performed from animals raised at different locations i.e. SK Nagar and Anand revealed that the microbiome profile of the Mehsani buffalo and Kankrej Cows raised at SK Nagar was found to have higher similarity than Gir cows raised at Anand suggesting the higher influence of geographical location than the effect of species in shaping the microbial community. Preliminary data on the comparative microbiome profile suggested predominance of Bacteoidetes in Mehsani buffalo and Kankrej Cows whereas of Firmicutes in Gir Cows. The study also generated basic information for the understanding of complexity of the rumen microbial ecology with special attention towards the resistome, Phages, Prophages, Transposable elements, Plasmids and stress responses.

Metatranscriptome sequencing runs were performed for all the 48 samples of each breed with the aim to characterize the active rumen microbiome of cattle and buffaloes adapted to different diet treatments. 16S amplicon sequencing of all the breeds were completed and primer-wise as well as sample-wise comparative analysis was performed. All the data of metagenome, and metatranscriptome sequencing were quality filtered at uniform parameters and uploaded on MGRAST for taxonomic and functional assessment. As per the proposed objective, collection of all the samples for calf to adult stages were completed, DNA isolated and amplicons generated from the same. Overall, the objectives of the programme were completed with salient achievements under each technical programme.The present study generated the basic information which delivers for the understanding of complexity of the microbial ecology of the cattle rumen with special attention towards the resistome, Phages, Prophages, Transposable elements, Plasmids and stress responses.

Salient achievements of the project

- A total of 240 (48 samples per breed, 3 breeds of buffalo and 2 breeds of cattle) metagenome, 240 metatranscriptome and 240 amplicon sequence data set generated which will serve as reference point.
- Shotgun sequencing of all the metagenomes completed and analysed for its microbial content.
- Characterization of active rumen microbiome of cattle and buffaloes adapted to different diet treatments performed through metatranscriptome sequencing of 3 breeds of buffalo and 2 breeds of cattle.
- Comparative analysis performed from 16s amplicons - both primer wise and sample wise for microbial diversity with samples of all the breeds and with different diet treatments.
- Amylolytic, anaerobic, spore forming novel strain of Bacillus nealsonii i.e. Bacillus nealsonii AAU1 isolated and reported from rumen of surti buffalo.
- A novel strain of clostridium sp. i.e. Clostridium sartagoforme AAU1 having cellulolytic and chitinolytic activity isolated and pheno-genotypically analysed.
- Role of methanogens in rumen of Indian cattle explored and its comparative context with exotic cattle studied.
- More influence of geographical location rather than the species in shaping of microbial diversity exhibited by experimental evidences.
- Special attention given to previously underexplored resistome, phages, prophages, transposable elements, plasmids and stress responses for understanding the complexity of rumen.
- Significant differences in Environmental Gene tags (EGT's) involved in metabolic processes for production of Volatile Fatty acids in response to dietary treatment variations investigated and revealed.
- Experimental evidences provided for the fact that the microbial diversity modulates itself in response to change in diet and geographical location.
- Importance of diet proportions, fraction of rumen, and type of forage affecting the rumen microbiome at taxonomic and functional level revealed.
- As evident from 16s based diversity and clustering analysis, Kankrej, Gir and Jafarabadi share microbial community with each other whereas Mehsani buffalo microbiome is quite distinct from former three.
- 16s amplicon analysis emphasizes the fact that microbial community differs in their presence and abundance in solid and liquid fractions.
- Rumen Virome analysis performed with enriched surti buffalo rumen sample to explore their taxonomy, probable hosts and metabolic profile.
- Patent application filed for Recombinant cellulaseCel-PRI (identified from Mehsani rumen metagenome) showing 4-5 times higher activity than commercially available Sigma cellulase.
- A novel multifunctional enzyme showing activity against Locust Bean Gum (LBG), beech wood xylan, CMC and pectin identified, cloned and over expressed in bacterial expression system.

- Acidic recombinant cellulases Cel PRII and P4 with optimum pH 4 & 6 and stability ranging from pH 4 to 10 cloned and expressed from Mehsani buffalo and Gir cattle rumen metagenome respectively.
- RPHY1 (Ruminal Phytase) identified, cloned and expressed from Mehsani buffalo rumen metagenome can be used as an exogenous supplement for animal feeds thus increasing the utilization of phytate, major supply of phosphorous.
- Cellulolytic clones identified by functional screening of fosmidmetagenomic library having average insert size of 40 kb DNA constructed from buffalo rumen metagenome
- Total 22 articles published in peer reviewed journal.
- A book entitled “Metagenomics- Role of Next Generation sequencing and Bioinformatics” published under the programme.
- Five short courses each of ten days organized as part of training program and training imparted to more than 100 participants across the india.
- Two chapters in conference proceedings and one review paper published under this programme.
- Six awards/recognition achieved through this program for poster and oral presentation.
- Linkages with various research institutes at national and international level generated.

Report of the project

1. Name of the University: Anand Agricultural University, Anand, Gujarat

2. Title of the Niche Area programme: Metagenomic Analysis of Ruminal Microbes

3. Date of start: 15th November 2011 **End:** 31st March 2016

4. Final accepted project proposal: Annexure I

5. Year-wise Budgetary details (Rs. in lakh):

Item	Year-1 (2011-12)	Year-2 (2012-13)	Year-3 (2013-14)	Year-4 (2014-15)	Year-5 (2015-16)	Total
Allocation	110.28	117.28	120.00	115.00	43.58800	506.148
Release by the Council	110.28	117.28	120.00	115.00	21.79400	484.354
Expenditure	96.32486	113.75849	119.72222	113.02177	8.05012	450.87746

6. Name of the PI/Co-PI with designation, and date of joining/leaving the project activities:

PI:

Dr. C. G. Joshi, Professor & Head, Dept. of Animal Biotechnology, College of Veterinary Science & AH, Anand Agricultural University, Gujarat (India).

Co-PI:

a) Dr. P. G. Koringa, Assistant Professor, Dept. of Animal Biotechnology, College of Veterinary Science & AH, Anand Agricultural University, Gujarat (India).

b) Dr. S. J. Jakhesara, Assistant Professor, Dept. of Animal Biotechnology, College of Veterinary Science & AH, Anand Agricultural University, Gujarat (India).

c) Dr. S. R. Dave, Professor & Head, Dept. of Microbiology, Gujarat University, Gujarat (India).

d) Dr. Srinivas Murthy Duggirala, Professor, Dept. of Microbiology, Gujarat Vidyapith - Ahmedabad, Gujarat (India).

e) Dr. D. N. Rank, Professor & Head, Dept. of Animal Genetics & Breeding, College of Veterinary Science & AH, Anand Agricultural University, Gujarat (India).

f) Dr. S. Parnerkar, Research Scientist & Head, Department of Animal Nutrition, College of Veterinary Science & AH, Anand Agricultural University, Gujarat (India).

g) Dr. P. R. Pandya, Associate Research Scientist, Department of Animal Nutrition, College of Veterinary Science & AH, Anand Agricultural University, Gujarat (India).

h) Sardarkrushinagar Dantiwada Agricultural University, SK Nagar

i) Navasari Agricultural University, Navasari

j) Junagadh Agricultural University, Junagadh

7. Goal:

S. No.	Approved goal	Status	Justification (if any)
1	Temporo-spatial study of buffalo and cattle rumen microbiome	Completed	--

8. Objectives:

S. No.	Approved objectives	Objective fulfilled	Justification (if any)
1	Molecular characterization and identification of rumen microbiome of cattle and buffalo.	Completed	--
2	To study effect of diet containing different proportions of nutrient ingredients on rumen microbial diversity.	Completed	--
3	To study effect of different geographical conditions on rumen microbial diversity.	Completed	--
4	Comparative study of rumen microbiome of cattle and buffalo	Completed	--
5	Metagenomic changes from calf to adult in rumen	Completed	--
6	Study of the active microbiome of cattle and buffalo by metatranscriptome sequencing	Completed	--
7	Functional metagenomics for discovery of enzymes with agricultural applications from rumen microbiome	Completed	--
8	Cloning and characterization of novel enzymes from rumen inferred using metagenome shotgun sequencing data	Completed	Research articles under communication

9. Introduction:

Biotechnology is a highly multidisciplinary and fastest growing discipline; however its application in animal health and production has been a recent phenomenon. The complex microbiome of the gastrointestinal system of herbivores plays an important role in nutrient utilization, growth and well-being of these animals as well as methane emission in the environment. With the introduction and growth of

molecular tools in microbial ecology, many culture-independent methods have been developed to overcome the cultivation biases and allow detailed information on microbial community diversity, structure, and function. New high throughput sequencing platforms based on pyrosequencing are now available hence it is possible to sequence all the genes of all species of the rumen ecosystem to produce a metagenome as well as identify genotypes of host.

10. Technical programme:

S. No.	Activities			Remarks (if any)
	Overall Approved	So far achieved	Achieved in 2015-16	
1	Hiring of animals and planning for their accommodation in different geographical conditions	Completed	--	--
2	Maintaining animals in good health and simultaneously conducting changes in diet provision	Completed	--	--
3	Collection of rumen fluid from each animal after each diet change	Completed	--	--
4	Extraction of DNA and mRNA from collected rumen fluid at each diet change	Completed	--	--
5	Metagenome, metatranscriptome sequencing and data analysis	Completed	--	--
6	Functional metagenomics for discovery of enzymes with agricultural applications from rumen microbiome	Completed	--	--
7	Discovery of enzymes with agricultural applications from rumen microbiome through functional metagenomics	Completed	--	Manuscripts under communication
8	Metagenomic changes from calf to adult in	Samples were collected, DNA	DNA isolation from all samples	Further technical

	rumen	isolated and amplicons generated	and amplicon generation	programme is under-progress

11. Monitorable targets:

S. No.	Monitorable targets	So far completed	Remarks (if any)
1	Extraction of DNA from collected rumen fluid at each diet change.	Completed	Created the DNA repository at NAE, Dept. of Animal Biotechnology, CVSc, Anand Agricultural University.
2	Metagenome, metatranscriptome and Amplicon sequencing and data analysis.	Completed	Submitted data to MG-RAST and EBI servers
3	Discovery of enzymes with agricultural applications from rumen microbiome through functional metagenomics	Completed	Identification and characterization of 2 cellulase, 1multifunctional and 1 phytase recombinant clone were completed
4	Shotgun and amplicon sequencing of calf to adult rumen samples	Completed	--
5	Screening of fosmid library on additional substrates	Completed	Screening of xylanase positive clones from fosmid library

12. Major equipments/facilities generated under the programme:

Name of the major equipment/facilities	Number	Approved Budget (Rs. in lakh)	Unit cost (Rs. in lakh)	Year of procurement	Further use plan	Present status
Fluorimeter	1	10.0	10.71274	FY 2011-12	Will be used in upcoming programmes	Working
Ultrasonic sonicator	1	9.0	8.5	FY 2011-12		Working
Cell counter	1	4.5	3.77043	FY 2011-12		Working

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- Five short courses each of ten days organized as part of training program and training imparted to more than 100 participants across the india.
- Two chapters in conference proceedings and one review paper published under this programme.
- Six awards/recognition achieved through this program for poster and oral presentation.
- Linkages with various research institutes at national and international level generated.

14. Other achievements under the programme:

i. Publications

a. Refereed Research Papers:

1. M. Chandra Shekar, Neelam M Nathani, Amrutlal K. Patel, Subhash J. Jakhesara, Chaitanya G. Joshi. 2016. Mining of ruminant microbial phytase (RPHY1) from metagenomic data of Mehsani buffalo breed: Identification, gene cloning and characterization. *Journal of Molecular Microbiology and Biotechnology*. (Accepted) (NAAS – 8.1)
2. Ravi K Shah, Amrut K Patel, Tejas Shah, Krishna M Singh, Neelam M. Nathani, Chaitanya G. Joshi. 2015. Community structure and species richness of protozoa enriched rumen metagenome from Indian Surti buffalo by shrot-gun sequencing. *Current Science*. (Accepted) (NAAS – 6.93)
3. Neelam M Nathani, Amrutlal K. Patel, Mootapally Chandra Shekar, Bhaskar Reddy Shailesh V shah, Pravin M Lunagaria, Ramesh K Kothari, Chaitanya Joshi. 2015. Effect of roughage on rumen microbiota composition in the efficient feed converter and sturdy Indian Jaffarabadi buffalo (*Bubalus bubalis*). *BMC Genomics* 16:1116. DOI 10.1186/s12864-015-2340-4. (NAAS - 9.99)
4. Vimal Prajapati, Hemant Purohit, D.V. Raje, Nidhi Parmar, Anand Patel, Oliver Jones, Chaitanya Joshi. 2015. The effect of high roughage diet on the metabolism of aromatic compunds by rumen microbes: A metagenomic study using Mehsani buffalo (*Bubalus bubalis*). *Applied*

Microbiology and Biotechnology. DOI: 10.1007/s00253-015-7239-0. (NAAS – 9.34)

5. Neelam M Nathani, Srinivas Duggirala, Chandra Shekar M, Ramesh Kothari and Chaitanya G Joshi. Isolation of chitinolytic Clostridium sp NCR from Mehsani buffalo rumen, its genomic analysis and potential role in rumen. *Genomics Data.* DOI: 10.1016/j.gdata.2015.05.017
6. Neelam M. Nathani, Ramesh K. Kothari, Amrutlal K. Patel, Chaitanya G. Joshi. Functional Characterization Reveals Novel Putative Coding Sequences in *Prevotella ruminicola* Genome Extracted from Rumen Metagenomic Studies. *Journal of Molecular Microbiology and Biotechnology*. DOI: 10.1159/000437265 (NAAS – 8.1)
7. Neelam M. Nathani, Srinivas M. Duggirala, Vaibhav D. Bhatt, Amrutlal K Patel, Ramesh K. Kothari, Chaitanya G. Joshi. Correlation between Genomic analyses with Metatranscriptomic study reveals various functional pathways of *Clostridium sartagoforme* AAU1, a buffalo rumen isolate. *Journal of Applied Animal Research.* DOI: 10.1080/09712119.2015.1091346(NAAS – 6.44)
8. N.R. Parmar, J.I. Nirmal Kumar & C.G. Joshi. 2015. Deep insights into carbohydrate metabolism in the rumen of Mehsani buffalo at different diet treatments. *Genomics Data*. DOI: 10.1016/j.gdata.2015.08007
9. N.R. Parmar, J.I. Nirmal Kumar & C.G. Joshi. 2015. Exploring diet-dependent shifts in methanogen and methanotroph diversity in the rumen of Mehsanibuffalo by a metagenomics approach. *Frontiers in Life Science.* DOI: 10.1080/21553769.2015.1063550
10. AB Patel, AK Patel, MP Shah, IK Parikh, CG Joshi**.** 2015.Isolation and characterization of novel multifunctional recombinant family 26 glycoside hydrolase from mehsani buffalo rumen metagenome. *Biotechnology and Applied Biochemistry*. DOI: 10.1002/bab.1358 (NAAS – 7.36)
11. Krishna M Singh, Amrutlal K Patel, Ravi K Shah, Bhaskar Reddy, Chaitanya G Joshi. 2015. Potential functional gene diversity involved in methanogenesis and methanogenic community structure in Indian buffalo (*Bubalus bubalis*) rumen. *Journal of Applied Genetics* 02/2015; DOI:10.1007/s13353-015-0270-0. (NAAS – 7.48)

12. K M Singh, T K Jisha, Bhaskar Reddy, Nidhi Parmar, Anand Patel, A K Patel, C G Joshi. 2015. Microbial profiles of liquid and solid fraction associated biomaterial in buffalo rumen fed green and dry roughage diets by tagged 16S rRNA gene pyrosequencing. *Molecular Biology Reports*10.1007/s11033-014-3746-9 (NAAS – 8.02)
13. Nidhi R. Parmar, Jitendra V. Solanki, Anand B. Patel, Tejas M. Shah, Amrutlal K. Patel, Subhash Parnerkar, Nirmal Kumar J.I., Chaitanya G. Joshi. 2014. Metagenome of Mehsani Buffalo Rumen Microbiota: An Assessment of Variation in Feed-Dependent Phylogenetic and Functional Classification. *J Mol Microbiol Biotechnol*. DOI: 10.1159/000365054. (NAAS – 8.1)
14. Vilas Patel, Amrutlal K Patel, Nidhi R Parmar, Anand B Patel, Bhaskar Reddy, Chaitanya G Joshi. 2014. Characterization of the rumen microbiome of Indian Kankrej cattle (*Bos indicus*) adapted to different forage diet. *Applied Microbiology and Biotechnology* 10.1007/s00253-014-6153-1 (NAAS – 9.34)
15. Dipti W Pitta, Nidhi Parmar, Amrut K Patel, Nagaraju Indugu, Sanjay Kumar, Karsanbhai B Prajapathi, Anand B Patel, Bhaskar Reddy, Chaitanya Joshi. 2014. Bacterial Diversity Dynamics Associated with Different Diets and Different Primer Pairs in the Rumen of Kankrej Cattle. *PLoS ONE* 9(11):e111710 (NAAS – 9.23)
16. KM Singh, Bhaskar Reddy, Dishita Patel, AK Patel, Nidhi Parmar, Anand Patel, JB Patel, CG Joshi. 2014. High Potential Source for Biomass Degradation Enzyme Discovery and Environmental Aspects Revealed through Metagenomics of Indian Buffalo Rumen. *BioMed Research International* (NAAS – 9.17)
17. Pitta DW, Kumar S, Veiccharelli B, Parmar N, Reddy B, Joshi CG. 2014. Bacterial diversity associated with feeding dry forage at different dietary concentrations in the rumen contents of Mehshana buffalo (*Bubalus bubalis*) using 16S pyrotags. *Anaerobe* 25:31-41 (NAAS – 8.48)
18. Dishita D. Patel, Amrutlal K. Patel, Nidhi R. Parmar, Tejas M. Shah, Jethabhai B. Patel, Paresh R. Pandya, Chaitanya G. Joshi. 2014. Microbial and Carbohydrate Active Enzyme profile of buffalo rumen

metagenome and their alteration in response to variation in the diet. *Gene* 545(1):88–9 (NAAS – 8.14)

19. Bhaskar Reddy, Krishna M Singh, Amrutlal K Patel, Ancy Antony, Harshad J Panchasara, Chaitanya G Joshi. 2014. Insights into resistome and stress responses genes in *Bubalus bubalis* rumen through metagenomic analysis. *Molecular Biology Reports* 10.1007/s11033-014-3521-y (NAAS – 8.02)
20. NM Nathani, SM Duggirala, VD Bhatt, J Ka Patel, CG Joshi. 2014. Genomic analysis of a novel strain of *Bacillus nealsonii* isolated from Surti buffalo rumen. *Advances in Bioscience and Biotechnology* 5, 235.
21. KM Singh, B Reddy, AK Patel, H Panchasara, N Parmar, AB Patel, TM Shah, VD Bhatt, CG Joshi. 2014.Metagenomic analysis of buffalo rumen microbiome: Effect of roughage diet on Dormancy and Sporulation genes. *Meta gene*. 2, 252-268
22. Nathani NM, Patel AK, Dhamannapatil PS, Kothari RK, Singh KM, Joshi CG. 2013. Comparative evaluation of rumen metagenome community using qPCR and MG-RAST. *AMB Express.* Sep 11;3(1):55. doi: 10.1186/2191-0855-3-55.

b. Review papers:

1. N.R. Parmar, J.I. Nirmal Kumar and C.G. Joshi. 2015. Advancements in Bovine Rumen Microbial Ecology: A Review. *Int.J.Curr.Microbiol.App.Sci* 4(7): 105-121.

c. Chapters in Conference proceedings:

1. Neelam M Nathani, M Chandra Shekar, Amrutlal K Patel, Bhaskar Reddy, Avani Patel, Ramesh K Kothari, Chaitanya G Joshi. 2015. Rumen Microbiome Characterization of Jaffarabadi Buffalo Adapted to Different Forage Diet using Metagenomic Approach. UGC-CAS National Conference on Biodiversity and Bioresource Utilization organized by Dept. of Biosciences, Saurashtra University, Rajkot, 17th - 18th March'2015. pp 72-73.
2. Dishita D. Patel, Senior Research Fellow of Department of Animal Biotechnology presented paper entitled "Carbohydrate Active Enzymes in Buffalo Rumen Metagenome and their Profile in Response to Variation in the Diet" at IVRI and won Women Scientist Award, 2012.

d. Book Published:

1. Joshi, C.G., Koringa, P.G., Jakhesra, S.J., Nathani, N.M. and Thakkar, J.R. (Eds.). 2015. Metagenomics- Role of Next Generation sequencing and Bioinformatics. Ome Research Facility, Department of Animal Biotechnology, College of Veterinary Science & Animal Husbandry, Anand Agricultural University, Anand, Gujarat, India, pp 1-108. ISBN 9789352360451

e. Any other:

1. Poster presentation: Ankit T. Hinsu, Nidhi R. Parmar, Neelam M. Nathani, Deepti Davla, Bhaskar Reddy, Anand B. Patel, Chaitanya G. Joshi on "Comparative Insights into the Rumen Microbiome of Gir and Kankrej Cattle (*Bos indicus*) revealed through Meta-RNAseq approach" at 2015 NextGen Genomics, Biology, Bioinformatics and Technologies (NGBT) Conference held from 1st - 3rd October, 2015, HICC, Hyderabad, India.
2. Poster presentation: Ravi K. Shah, Amrutlal K. Patel, Kamlesh C. Patel, Chaitanya G. Joshi on " Functional Screening of buffalo rumen metagenomic library for biomining novel glycosyl hydrolases of industrial importance" at PEGS : The essential Protein engineering summit held from 4th-8th May 2015, Seaport world trade convention centre, Boston, Massachusetts, USA.
3. Poster presentation: Nidhi R. Parmar on “An assessment of variation in feed-dependent phylogenetic and functional classification of microbial communities in rumen of Mehsani buffalo” at International Union of Microbiological Societies Congress (IUMS 2014), Conference held from July 27- August 1, 2014, *Montréal*, Canada

ii. New innovations commercialized (if any, give details): -Nil-

iii. Patents Applications/Granted or submitted (if any):

Title	Application /publication number	Date of application /publication	Present status	Indicate your role (1st/2nd/3rd/… inventor /others)
Buffalo rumen originated recombinant Cel_PR1 cellulase with potent CMCase activity	3928/MUM/ 2014	December 2011	Filed	1st inventor

iv. Success stories in one/two page (if any): -Nil-

v. Technologies generated/transferred or new products developed :-Nil-

vi. Resources generated (if any): - Nil-

vii. Radio/TV talks (if any): - Nil-

viii. Students completed M.Sc. /M. V. Sc. etc /Ph. D thesis work under the programme:

S. No.	Number of Student	Course Title	Whether completed thesis under the programme (Yes/No)	Role of the PI/Co-PI (Guide/Co-Guide)
1	Neelam M Nathani	Individual Genome Reconstruction of Ruminant Anaerobic Microbes from Metagenomic Studies	Yes, Submitted thesis	Evaluation of the experimental findings
2	Nidhi R Parmar	Exploring microbiota and their molecular characterization from rumen metagenome of buffalo and cattle	Yes, Submitted synopsis	Evaluation of the experimental findings

ix. Employment record of the fellows and the employment profile of the alumni:

S. No.	Number of Student	Designation & Duration	Completed thesis under the programme (Yes/No)	Placement records (if any)	Role of the PI/Co-PI in students employment
1	Amrut K Patel	RA, 16.01.2012–31.03.2014	No	Hester Bioscience	Recommendation
2	Ajai K Tripathi	RA, 01.02.2012 - 31.10.2012	No	Case Western University	Recommendation
3	Tejas M Shah	SRF, 10.02.2012–31.03.2014	No	--	--
4	Mansi Aparnathi	SRF, 10.01.2012 - 04.08.2012	No	Pursuing PhD, IIT Chennai	--
5	Ancy Antony	SRF, 10.10.2012 - 30.04.2013	No	Pursuing PhD, Messay Uni. New Zealand	--
6	Vaibhav D. Bhatt	RA, 09.11.2012 - 30.08.2013	No	Inspire Faculty, DST	--
7	Ravi K. Shah	SRF, 25.05.2013–31.03.2014	No	UGC-NET, JRF	--
8	Akhilesh S. Dhanani	RA, 28.11.2013 - 11.02.2014	No	--	--

9	Sandip G. Patil	RA, 03.07.2014 - 19.09.2014	No	--	--
10	Bhaskar Reddy	SRF, 27.06.2014- till to date	No	--	--
11	Ramesh J Pandit	SRF, 01.07.2014– 30.07.2015	No	Research Associate - DBT	--
12	M. Chandra Shekar	RA, 05.01.2015 - till to date	No	--	--
13	Brijesh Dabhi	RA, 05.02.2015 - 05.04.2015	No	Working as lecturer	--
14	Piyush Chudasama	RA, 05.05.2015 - 07.12.2015	No	Research Associate, Akanksha Infertility Clinic, India	--
15	Gajendra Joshi	RA, 06.02.2016 - till to date	No	--	--

x. Entrepreneurship developed under the programme: -Nil-

xi. Awards/recognition received by the PI/Co-PIs/other Staff/Students under the Programme:

Name/Designation of the person	Award name	Date of award	Host institution	Purpose of the Award
Ravi K Shah, UGC Junior Research Fellow	Best Poster Award PEGS-The essential Protein engineering Summit	May 2015	Cambridge Health-tech Institute, United States of America	Poster Presentation
Neelam M. Nathani, DST INSPIRE Fellow	Best oral presentation Award	Feb, 2014	Christ College, Rajkot	Oral Presentation
Dr. Vilas Patel	Best Poster Award	Dec, 2014	Scigenome Research Foundation, NIMHANS, Bangalore	Poster Presentation
Ankit T Hinsu, Research Fellow	Best Poster Award	Dec, 2014	Scigenome Research Foundation, NIMHANS, Bangalore	Poster Presentation
Dishita D. Patel, Senior Research Fellow	ISVIB-GADVASU Women Scientist Award	April, 2013	IVRI, Izatnagar	Oral Presentation
Nidhi R Parmar, Senior Research	Best Poster Award	Dec, 2012	NIAB and AIMSCS,	Poster Presentation

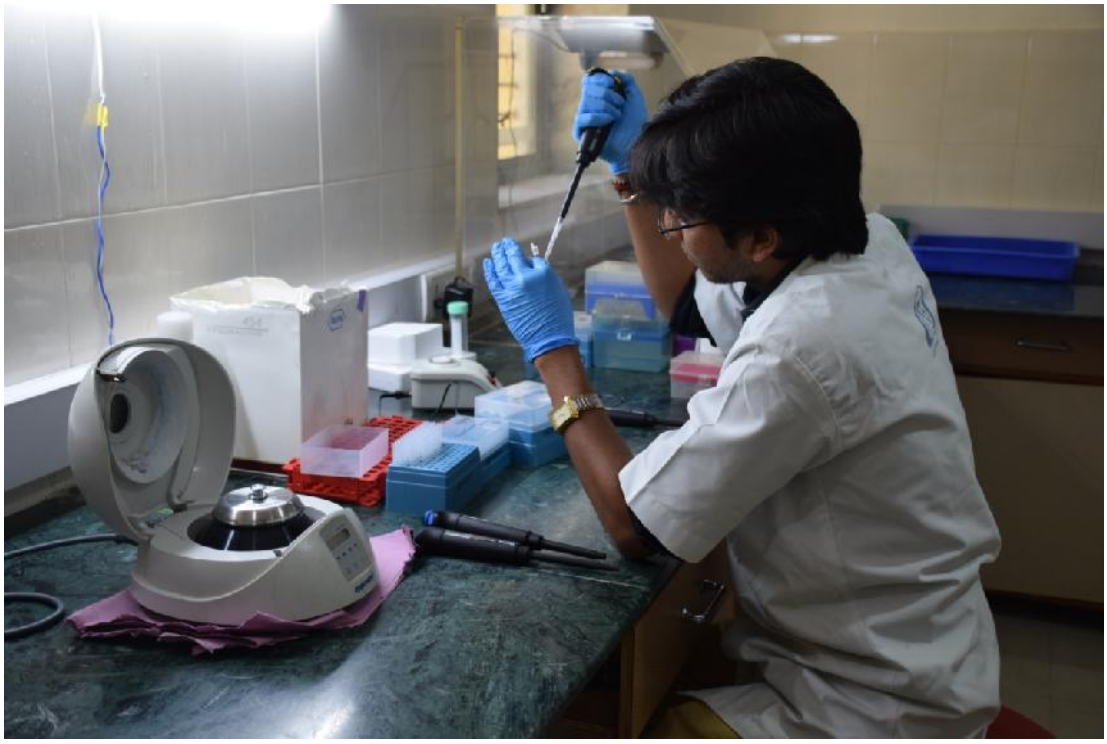
Fellow			Hyderabad	

xii. Linkages established within the country and abroad with various agencies:

S. No.	Linkages established with	Date of Linkage Establishment	Nature/Purpose of Linkages	Number of Beneficiary	Present Status
1	Dr. Oliver A.H. Jones, Senior Lecturer, School of Applied Sciences, RMIT University, Melbourne, Australia	2015	Technical interaction and interpretation of data	2	Exist
2	Dr. Hemant J Purohit Chief Scientist & Head, Environmental Genomics Division, National Environmental Engineering Research Institute, NEERI, CSIR, Nagpur 440020, India	2014	Technical interaction and interpretation of data	6	Exist
3	Dr. Rajesh Patel, Lecturer in Microbiology, Dept. of Life Sciences, HNGU, Patan- 384265, Gujarat, India.	2013	Technical interaction and interpretation of data	2	Exist
4	Dr. Dipti Pitta, Assistant Professor in Ruminant Nutrition, University of Pennsylvania, School of Veterinary Medicine, Dept. of Clinical Studies, New Bolton Center382 W. Street Road Kennett Square, PA 19348 USA	2012	Technical interaction and interpretation of data	6	Exist
5	Dr. P.U. Krishnaraj, Professor and Head, IABT University of Agricultural Sciences, Dharwad-580005 , Karnataka, India	2012	Technical interaction and interpretation of data	4	Exist
6	Dr. N.V. Patil, Director, NRC on Camel, Biknaer-334001, Rajasthan, India.	2012	Technical interaction and interpretation of data	3	Exist

7	Dr. D.N. Kamra, ICAR National Professorial Chair, Animal Nutritional Division, Indian Veterinary Research Institute, IzatNagar-243122, Bareilly, India.	2011	Technical interaction and interpretation of data	4	Exist
8	Dr. Datta Madamwar, Professor, BRD school of Biosciences, Sardar Patel University, Vallabh Vidyanagar-388120, Gujarat, India.	2011	Technical interaction and interpretation of data	4	Exist

xiii. Two-Four related photographs (with date/captions) showing the important activities in lab, field as applicable (also provide separately in jpg/jpeg format).



Photograph1_13-04-2012: Extraction of Metagenomic DNA from buffalo rumen samples.

Photograph2_ 22-10-2013: Lecture deliver by Dr. Yogesh Shouche during short course organized by Department of Animal Biotechnology, C.V.Sc. & A.H., AAU under the Niche Area of Excellence on Metagenomic Analysis of Ruminal Microbes held from 15th-24th October 2013.

Photograph3_ 24-03-2014: Sequencing run setup on *GS-Flx 454*

Photograph4_ 02-11-2015: Group photograph of Resource Persons, Participants and Course Director - Dr. C.G. Joshi, Course Coordinators - Dr. P.G. Koringa and S.J. Jakhesra, Core Faculty - Dr. D.N. Rank including Dean/Principal: Dr. A.M. Thaker participated in short course organized by Department of Animal Biotechnology, C.V.Sc. & A.H., AAU under the Niche Area of Excellence on Metagenomic Analysis of Ruminal Microbes held from 26th October to 4th November 2015.

xiv. PPP developed, if any: -Nil-

Xv. Trainings organized: Detail of the participant given separately as Annexure II

Sr. No.	Title of the training programme	Duration & dates of Organization	Number of participants				Justification for post training follow-up (if any)
			Faculty	Students	Farmers	Others	
1	Metagenomics: Role of Next generation sequencing and bioinformatics	15th-24th October 2012	22	-	-	-	-
2	Metagenomics: Role of Next generation sequencing and bioinformatics	15th-24th October 2013	18	-	-	-	-
3	Metagenomics: Role of Next Generation	6th-15th October 2014	20	-	-	1	-

	Sequencing and Bioinformatics						
4	Metagenomics: Role of Next generation sequencing and bioinformatics	26th Oct-04th Nov 2015	20	-	-	-	-
5	Metagenomics: Role of Next generation sequencing and bioinformatics	9th Feb-18th Feb 2016	4	21	-	-	-

Xvi.ATR of the Internal Review Meeting (please specify the date of the meeting): (Annexure III)

S. No.	Date of the meeting	Observations/suggestions during International Review Meeting	Action taken by the PI
1	21st - 22nd March, 2014	Metagenomics needs to be translated into functional genomics	Mining of novel enzymes from metagenomic data is completed.
		Metagenomic studies to be correlated with feeding practices.	Comparative analysis in between different roughages was done.
		The committee agreed to extend the programme for two years.	--
2	25th March, 2015	The committee found the progress of the project satisfactory in totality.	--
		The metatranscriptome sequencing runs of Jaffarabadi and Surti buffalo are in progress which are likely to be completed in short time.	Completed
		The analysis work will continue thereafter including metagenome, metatranscriptome and amplicon data and comparative analysis.	Analysis completed and manuscripts were communicated to journals
		Several enzymes screened from metagenome are being cloned and characterized for application as feed in the diet for agricultural importance.	Cloning and characterization of two GH and one phytase clones completed

		The fosmid library screening of bacterial and protozoa community will be pursued as per the proposed objectives.	Screening of bacterial fosmid library was completed
		Dr.D.N.Kamra may be requested to visit the Centre and discuss with you and other scientists and provide some technical guidance as he is also working in similar research.	Due to ill health could not able visit.

Xvii. Annual Review Meetings of Niche Area of Excellence (NAE Programme): (Annexure IV)

Xviii. Copies of AUCs (year wise form 2011 to 2015)

ANAND AGRICULTURAL UNIVERSITY
ANAND - 388 110

Proforma of AUC in respect of Niche Area of Excellence

Reference No . Grant Sanctioned vide letter No (1) F.No.10(2)/2011 EPD dated : 15.07.2014
(2) F.No.10(2)/2011-EP&HS dated : 09.03.2015

Audit Utilization certificate in respect of the Scheme entitled Metagenomic Analysis of Ruminal Microbes

For the Period **01.04.2014 TO 31.03.2015**

Name of the University **ANAND AGRICUTTURAL UNIVERSITY, ANAND**

Year	Particular	Opening balance for the year brought forwarded from the previous 01.04.2014	Remittance by the council during the year 2014-15	Council's share of receipts released from the scheme during the year 2014-15	Actual expenditure for the year 2014-15	Council's share of sanctioned grant for the year 2014-15	Concil's share of expenditure actually incurred & audited during the year 2014-15	Closing balance at the end of the year 31.03.2015
1	2	3	4	5	6	7	8	9
2014-15	Metagenomic Analysis of Ruminal Microbes							
	Works	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	Equipments	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	Recurring Contingencies	56012.00	0.00	11443988.00	11297209.00	11443988.00	11297209.00	202791.00
	Total	**56012.00**	**0.00**	**11443988.00**	**11297209.00**	**11443988.00**	**11297209.00**	**202791.00**

Certified that the expenditure under various heads had been audited & the grant has been spent for the purpose it was granted.

INANI
Chartered Accountants

Partner

COMPTROLLER
Anand Agricultural University
Anand 388 110

D:\BAKUL\2014-15\Grant Register 2014-15

Page 1

ANAND AGRICULTURAL UNIVERSITY
ANAND - 388 110

Proforma of AUC in respect of Niche Area of Excellence

Reference No . Grant Sanctioned vide letter No (1) F.No.2-(2)/F&AO/2013--F&A Unit dated : 28.06.2013
(2) F.No.10(2)2011 -EPD dated : 28.12.2013

Audit Utilization certificate in respect of the Scheme entitled Metagenomic Analysis of Ruminal Microbes

For the Period 01.04.2013 TO 31.03.2014

Name of the University ANAND AGRICUTTURAL UNIVERSITY, ANAND

Year	Particular	Opening balance for the year brought forwarded from the previous 01.04.2013	Remittance by the council during the year 2013-14	Council's share of receipts released from the scheme during the year 2013-14	Actual expenditure for the year 2013-14	Council's share of sanctioned grant for the year 2013-14	Concil's share of expenditure actually incurred & audited during the year 2013-14	Closing balance at the end of the year 31.03.2014
1	2	3	4	5	6	7	8	9
2013-14	Metagenomic Analysis of Ruminal Microbes							
	Works	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	Equipments	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	Recurring Contingencies	370150.70	28233.00	11629850.00	11972221.70	11972221.70	11972221.70	56012.00
	Total	**370150.70**	**28233.00**	**11629850.00**	**11972221.70**	**11972221.70**	**11972221.70**	**56012.00**

Certified that the expenditure under various heads had been audited & the grant has been spent for the purpose it was granted.

For, Sandip Desai & Co.
CHARTERED ACCOUNTANTS

PARTNER

SANDIP DESAI & CO. AHMEDABAD MEMBERSHIP NO. 44872 CHARTERED ACCOUNTANTS

Accounts Officer-cum-Comptroller
Anand Agricultural University
Anand 388 110

ANAND AGRICULTURAL UNIVERSITY
ANAND - 388 110
AUDIT UTILIZATION CERTIFICATE

Budget Head	2030-20

A Statement Showing the grant & expenditure incurred for the scheme shown at Sr.No 2 of the statement at the
Anand Agricultural University
for the period from 01.04.2012 to 31.03.2013

Sr No	Name of the Scheme	Opening Balance as on 01.04.12	Grant received during the year 2012-13	Other Receipt during the Year 2012-13	Total No (3+4+5)	Expenditure during the Year 2012-13	Closing Balance as on 31.03.2013	Remarks
1	2	3	4	5	6	7	8	9
1	**Metagenomic Analysis of Ruminal Microbes at Vet.Sci.College, AAU, Anand**	1395514.00	10332486.00	18000.00	11746000.00	11375849.30	**370150.70**	

Certified that the grant has utilized for the purposes for which it was sanctioned & all the conditions attached the grant were fulfilled

For, Sandip Desai & Co.
CHARTERED ACCOUNTANTS

PARTNER

SANDIP DESAI & CO. AHMEDABAD CHARTERED ACCOUNTANTS

Accounts Officer-cum-Comptroller
Anand Agricultural University
Anand 388 110

ANAND AGRICULTURAL UNIVERSITY , ANAND 380110
AUDIT UTILIZATION CERTIFICATE FOR THE YEAR 2011-12

Utilization Certification for the Scheme **Metagenomic Analysis of Ruminal Microbes, Vet.Sci.College, AAU, Anand**

For the Period from 01.04.2011 to 31.03.2012 **Budget Head 2030-20**

Opening Balance for the year brought over from the previous year 2010-11	Remittance by the Council during the year 2011-12	Council's share of Receipts released from the scheme during the year 2011-12	Actual Expenditure for the Year 2011-12	Council's Share sanctioned grant for the year 2011-12	Council's Share of Expenditure Actually incurred & audited during the year 2011-12	Closing Balance at the end of the Year 2011-12
₹ Ps	₹ Ps	₹	₹ Ps	₹ Ps	₹ Ps	₹ Ps
1	2	3	4	5	6	7
[+] 0.00	11028000.00	0.00	9632486.00	11028000.00	9632486.00	[+] 1395514.00

Certified that

1 The grant has been utilized for the purpose for which it was made by the Council
2 The Excess expenditure incurred incurred over the above the sanctioned ceiling of one or more sanctioned heads of expenditure has been met by re-appropriation of saving under the remaining "Heads"
3 The explanation for the excess Expenditure not covered by reappropriation has been furnished in the attached Performa for issue of Governing sanction by the Council

Accounts Officer-cum-Comptroller
Anand Agricultural University
Anand 388 110

SANDIP DESAI & CO. AHMEDABAD CHARTERED ACCOUNTANTS

For, SANDIP DESAI & CO.
CHARTERED ACCOUNTANTS

PARTNER

Results of the project

Shotgun Sequencing:

The sequencing of 48 Mehsani rumen metagenome led to generation of 3.9Gb total data comprising of 17.75 million reads. The sequencing of 48 Kankrej rumen metagenome led to the generation of 6 GB total data and shotgun sequencing of 48 samples of Gir cow rumen metagenomes yielded 6.94 Gbp data. Metagenome sequencing of 48 Jaffrabadi rumen samples resulted into a total data of 38 million raw reads, followed by 27 million reads post quality filtering. Metagenome sequencing of 48 Surti rumen samples resulted into a total data of 41 million raw reads, followed by 34 million reads post quality filtering. The sequencing data of individual samples obtained after quality filtering ranged from 85 Mb to 339 Mb. The sequencing data of individual samples obtained after quality filtering were further analyzed using MGRAST/EBI pipelines for its microbial diversity and functional content.

Rumen Microbiome analysis was performed from animals raised at different locations i.e. SK Nagar and Anand. The microbiome profile of the Mehsani buffalo and Kankrej Cows which were raised at SK Nagar was found to have higher similarity than Gir cows raised at Anand suggesting the higher influence of geographical location than the effect of breed in shaping the microbial community. Preliminary data on the comparative microbiome profile suggests predominance of Bacteroidetes in Mehsani buffalo and Kankrej Cows whereas of Firmicutes in Gir Cows.

Analysis of Mehsani rumen metagenome

This study recovered 3.970 GB of data from 48 metagenomes comprised by 17.75 million reads. Total data of 48 metagenomes were also uploaded to European Bioinformatics. The analysis revealed the abundance of OTUs which were found to be increased with the increase in roughage concentration (Fig. 1a). Phylogenetic assignment of the OTUs revealed significant change in the diversity of microbes at the phyla level in response to change in the diet as well as in the fiber adherent and liquid fraction of the rumen digesta (Fig. 1b).Diversity of Firmicutes and Tenericutes increased in liquid fraction with the increase ingreen or dry roughages in diet with concomitant decrease in the solid fraction of digesta. On the other hand, diversityof Bacteroidetes followed the opposite trend with anincrease in the roughages in the diet. However, diversity of Proteobacteria increased in both fractions with increase in the roughage proportion. The diversity of Bacteroidetes, Fibrobacter, Proteobacteria and

Verrucomicrobia were found higher in the liquid fraction whereas Firmicutes in the solid fraction of the rumen digesta.

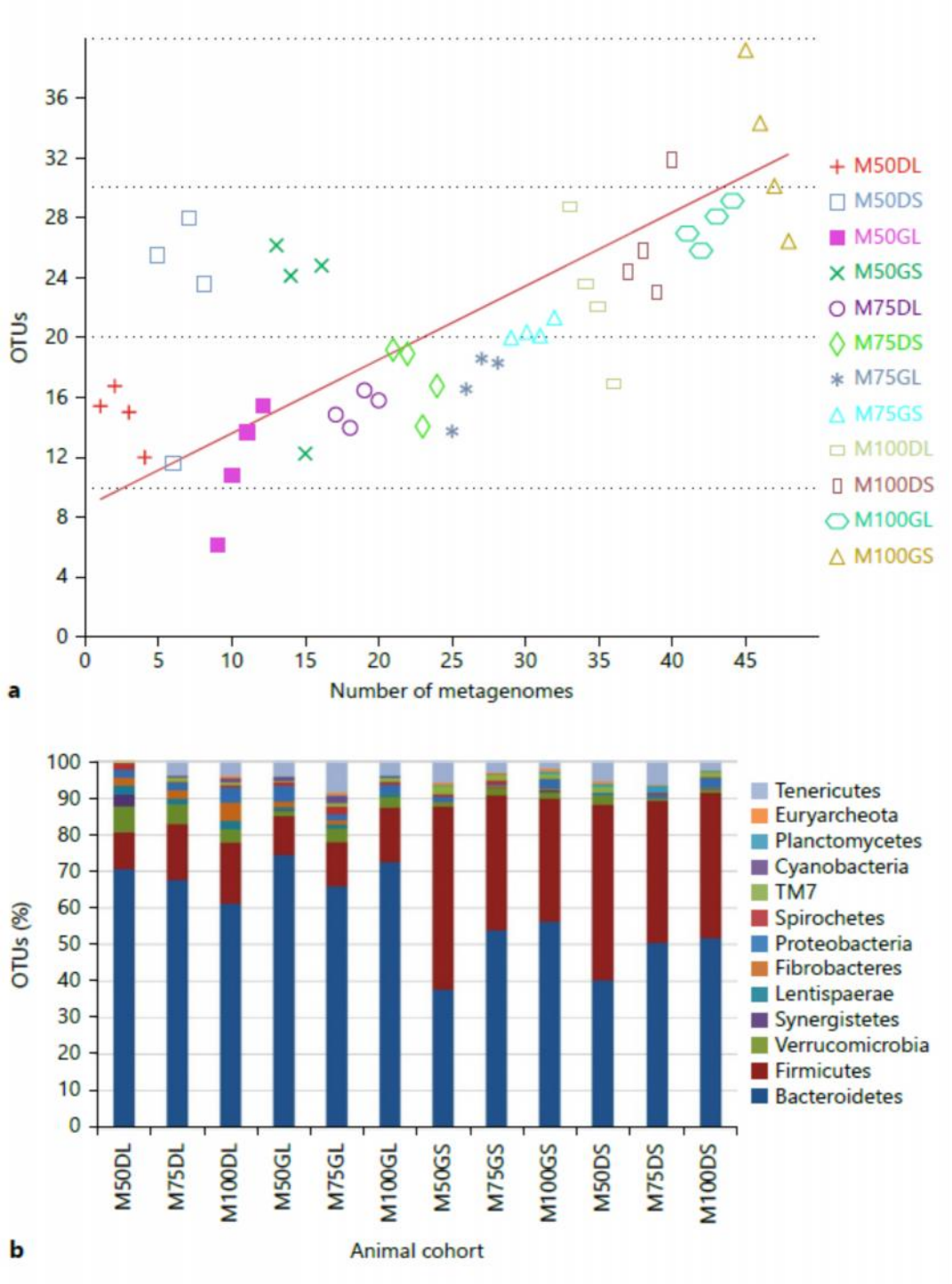


Fig. 1 a) Abundance of OTUs for all 48 metagenomes using reduced major axis method. X-axis: total number of metagenomes involved in the study; y-axis: number of OTUs of respective samples. **b)** Phyla level diversity of OTUs among rumen fractions across feeding regimes. X-axis: animal cohort; y-axis: percentage of OTUs assigned. X-axis: groups (all Mehsani breed).

Phylogenetic Classification

Assignment of sequences against the M5NR database in MG-RAST revealed a complex diversity of microbiota in rumen, which was classified from domain to species level. Instead of comparing all the metagenome individually, they were grouped into clusters based on the principle component analysis (PCA) profile (Fig. 2). PCA is a data reduction technique in which metagenomes are clustered with respect to components of variation extracted from their normalized abundance profiles. In the present study, the clustering for taxonomic content was performed using PCA.

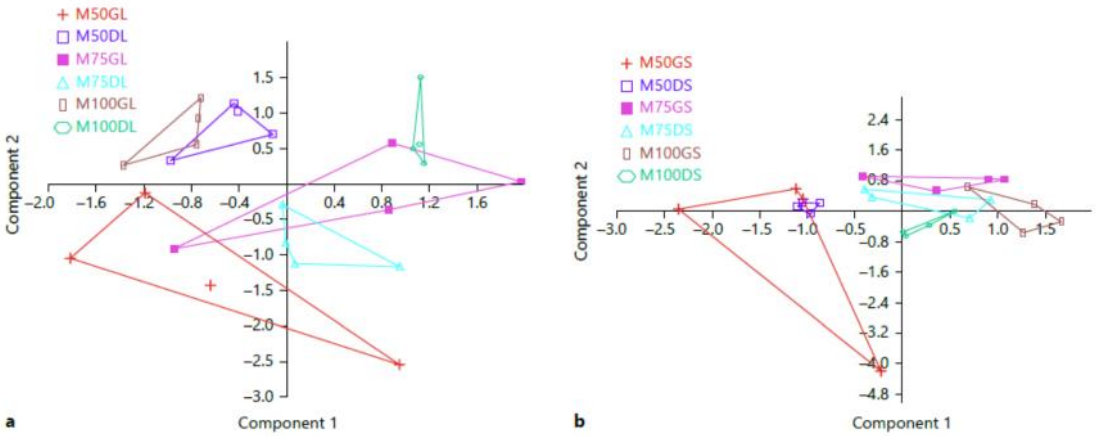


Fig. 2 PCA of metagenomes using co-relation matrix of PAST software. **a)** PCA of liquid fraction of metagenomes divided into 6 groups, i.e. 4 replicates in each groups. **b)** PCA of solid fraction of metagenomes divided into 6 groups, i.e. 4 replicates in each groups.

The phyla level taxonomy comparison of these groups revealed that Bacteroidetes are the highly predominant phyla in all the groups (Fig. 3a). The Bacteroidetes: Firmicutes ratio was found to be 3.13: 1.0 for the liquid fraction of all regimes. But, the proportion of Firmicutes in the liquid and solid fractions was around 1.0: 1.91. Moreover, the presence of *Fibrobacter* in greater proportion was observed in the liquid fraction (1.58%) as compared to the solid fraction(0.41%), whereas, the Proteobacteria were equally represented in all the samples averaging 8.5% of abundance. At the subdominant level, Actinobacteria were also found to be present in all regimes (Fig. 3a).The major observation we made at the phyla level was that the Bacteroidetes decreased significantly with the increment in regimes. On the other hand, the Firmicutes increased significantly with the increment in regimes. The phyla level diversity between the green and dry roughage groups demonstrated that the

Fibrobacter were significantly higher in the dry roughage group compared to the green roughage group.

Genus level classification unwounded the bacterial diversity and indicated predominance of *Prevotella* followed by *Bacteroides* in all the groups (Fig. 3b).The overall genus level diversity demonstrated that, in the liquid fraction, with the increment in roughage, the abundance of *Clostridium* and *Ruminococcus* genus increased significantly. In the solid fraction, *Clostridium* abundance decreased and *Bacteroidetes* increased with the increment in roughage concentration.

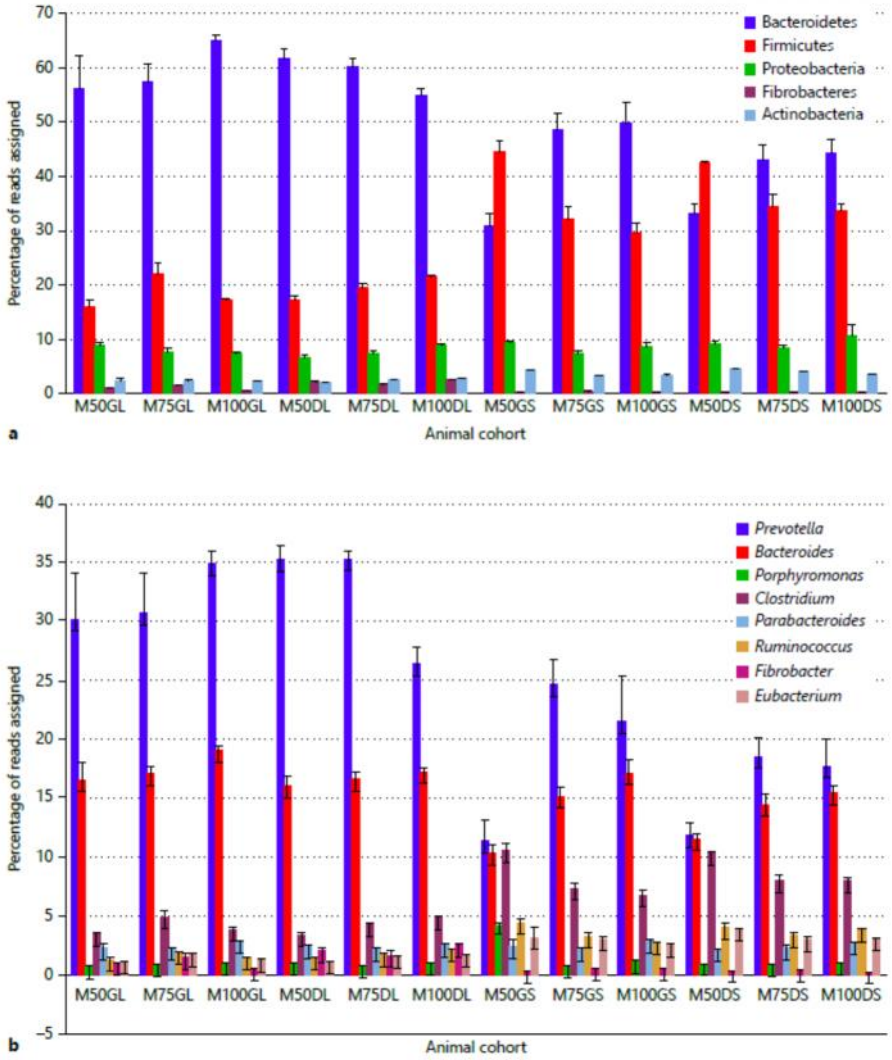


Fig. 3 Taxonomical classification of metagenomes. **a)** Abundance of each phylum in the green and dry roughage groups for M50, M75and M100 regimes for both the liquid and solid fractions. X-axis: animal cohort; y-axis: mean of percent reads assigned to particular phyla with error values. **b** Abundance of each genus in the green and dry roughage groups for all regimes. X-axis: animal cohort; y-axis: mean of percent reads assigned to particular phyla with error values.

Functional Classification

Subsystems-based annotations (SEED database) keeping 80% of the threshold were used to gain a better understanding of how this phylogenetic drift could be used to predict the metabolic potential of the microbiome. All the subsystems were divided into different categories based on their functions. Among all the subsystems mentioned, carbohydrate metabolism was the most dominant one, accounting for an average of 11.4% in all the samples (Fig. 4a). In the ion metabolism subsystem, nitrogen and sulfur metabolism had a higher proportion (Fig. 4b). In the solid fraction, with the increment in dry roughage concentration, the percentage of nitrogen metabolism also increased (Fig. 4b).

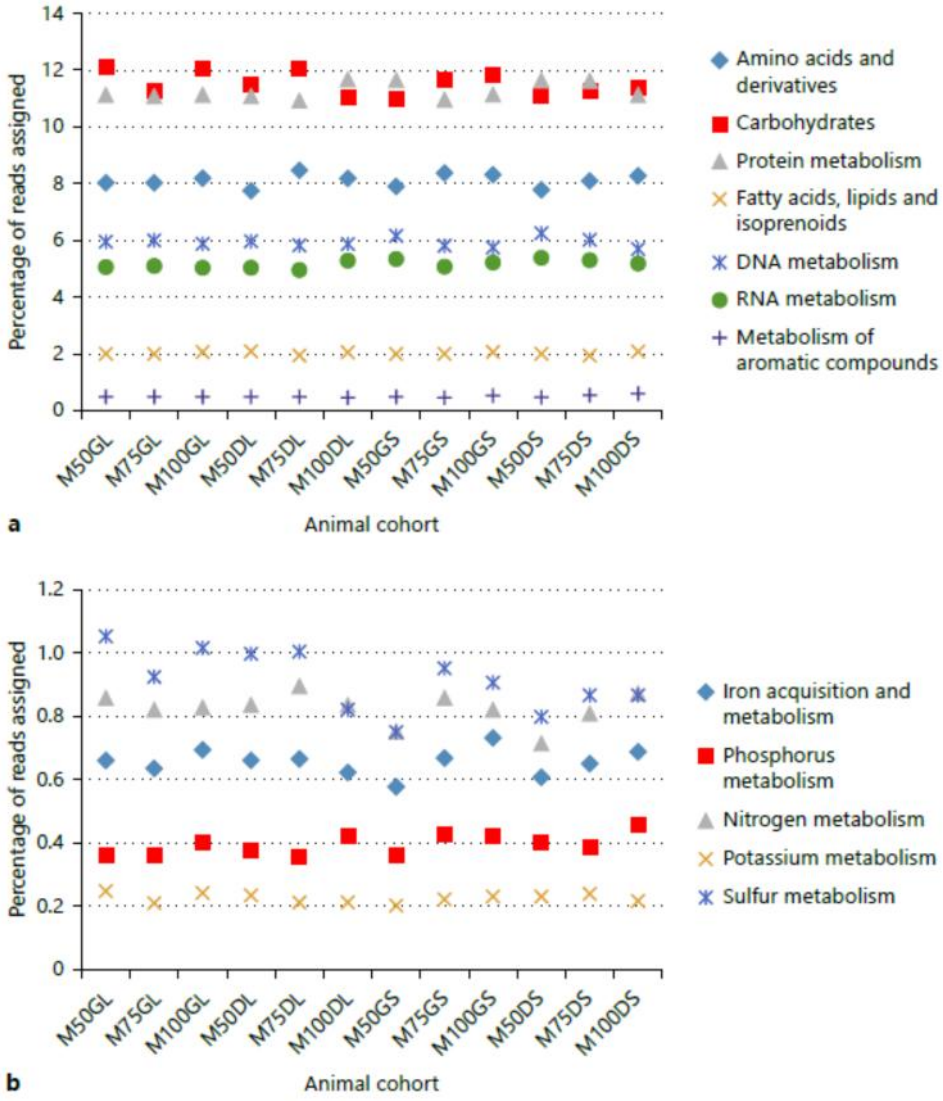


Fig. 4 Subsystem classification of the metagenome groups using MG-RAST. Xaxis: animal cohort; y-axis: percentage of reads assigned to particular metabolism category. **a)** Organic metabolism. **b)** Ion metabolism.

Carbohydrate-Active Enzymes

In present study, the carbohydrate-degrading enzymes were identified by BLAST-based approach against the CAZy database. Of identified CAZy families, most abundant families are shown in Figure 5. The glycoside hydrolase 3 (GH3) family was found to be abundant in both fractions of the dry roughage group. GH13 count decreased with the increase in the roughage: concentrate ratio in the liquid fraction of the green roughage group (Fig. 5a). In M50 regime, GH43 count decreased in both fractions of the dry roughage group with the increment in regimes (Fig. 5b).

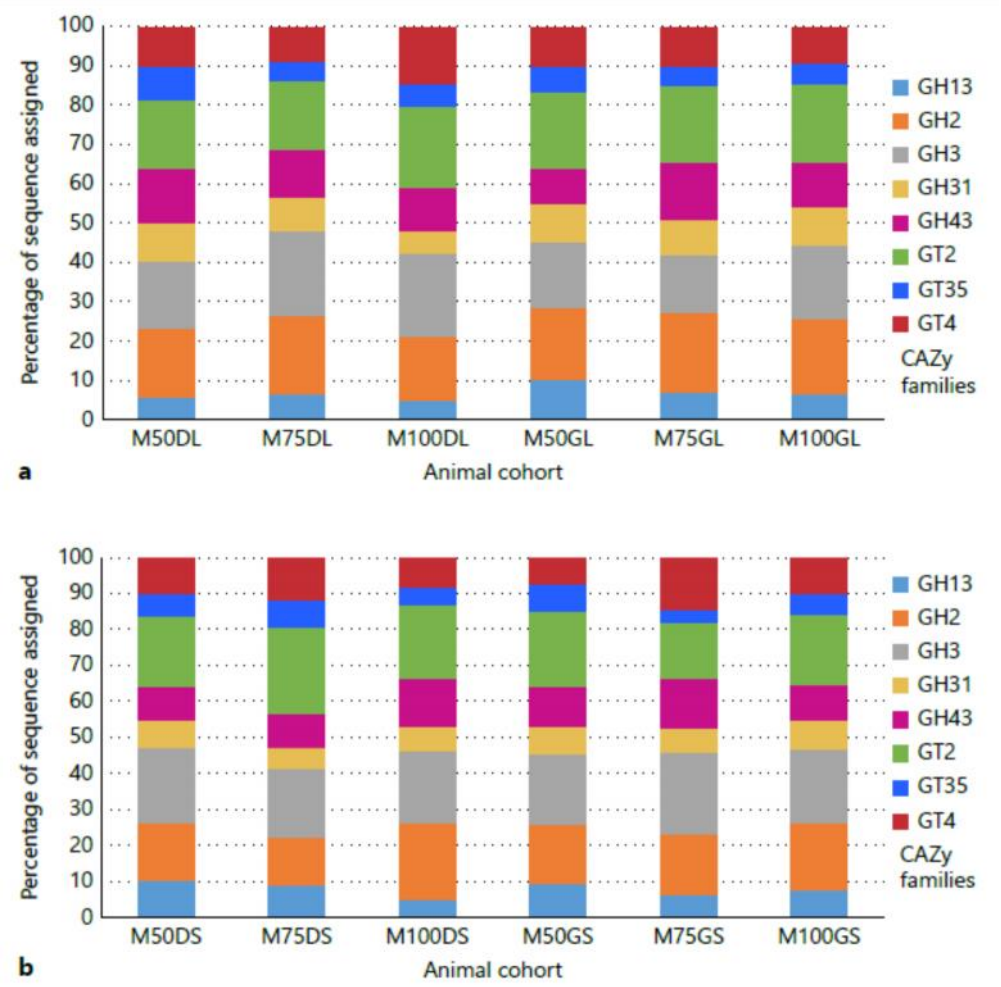


Fig. 5 CAZy classification in different groups. Bars indicate the different groups. X-axis: animal cohort; y-axis: percentage of sequences assigned to CAZy families. **a)** CAZy classification for the liquid fraction. **b)** CAZy classification for the solid fraction.

The similarities between the different groups were statistically evaluated using ANOSIM at $p<0.05$ (Fig. 6). As regards CAZy families, significant difference($p< 0.05$) was observed in the liquid fraction of the dry roughage group for all the

regimes, i.e. M50, M75 andM100 (Fig. 6a). The liquid fraction of the green roughage and dry roughage groups of M50 and M100 differed significantly ($p < 0.05$). In the M75 regime, a non-significant difference was observed for the liquid fraction. The M50GS group was significantly different ($p<0.05$) than the M100GS group (Fig. 6b).

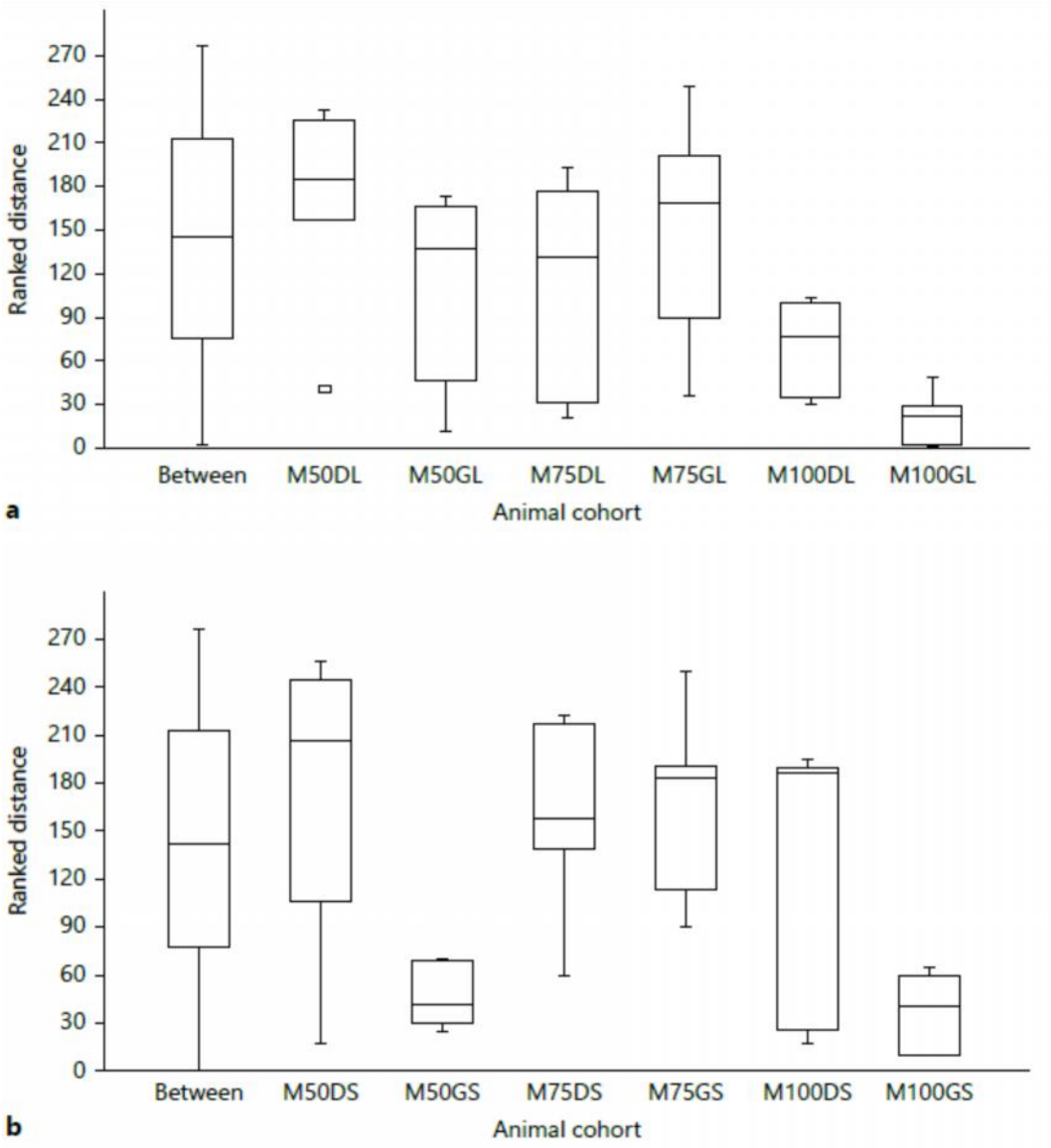


Fig. 6 ANOSIM of CAZy families for all regimes with $p < 0.05$. **a)** ANOSIM for the liquid fraction for CAZy families of all regimes($p < 0.05$). **b)** ANOSIM for the solid fraction for CAZy families of all regimes($p < 0.05$). Boxes represent the median and interquartile range (IQR); whiskers extend to the most extreme data points up to 1.5 times the IQR.

Analysis of Kankrej rumen metagenome based on EBI pipeline

In the rumen microbial community of liquid fraction of dry roughage (DL), Bacteroidetes was the most abundant phylum followed by Firmicutes, Verrucomicrobia, Fibrobacter and Proteobacteria. Whereas, in case of solid fraction of dry roughage (DS), Bacteroidetes was the most abundant phylum followed by Firmicutes, Fibrobacter, Verrucomicrobia, and Proteobacteria and other phyla. In the rumen microbial community of liquid fraction of green roughage (GL), Bacteroidetes was the most abundant phylum followed by Firmicutes, Verrucomicrobia, Fibrobacter, Proteobacteria and other phyla.In the rumen microbial community of solid fraction of green roughage (GS), Bacteroidetes is the most abundant phylum followed by Firmicutes, Fibrobacter,Proteobacteria and other phyla (Fig. 7).

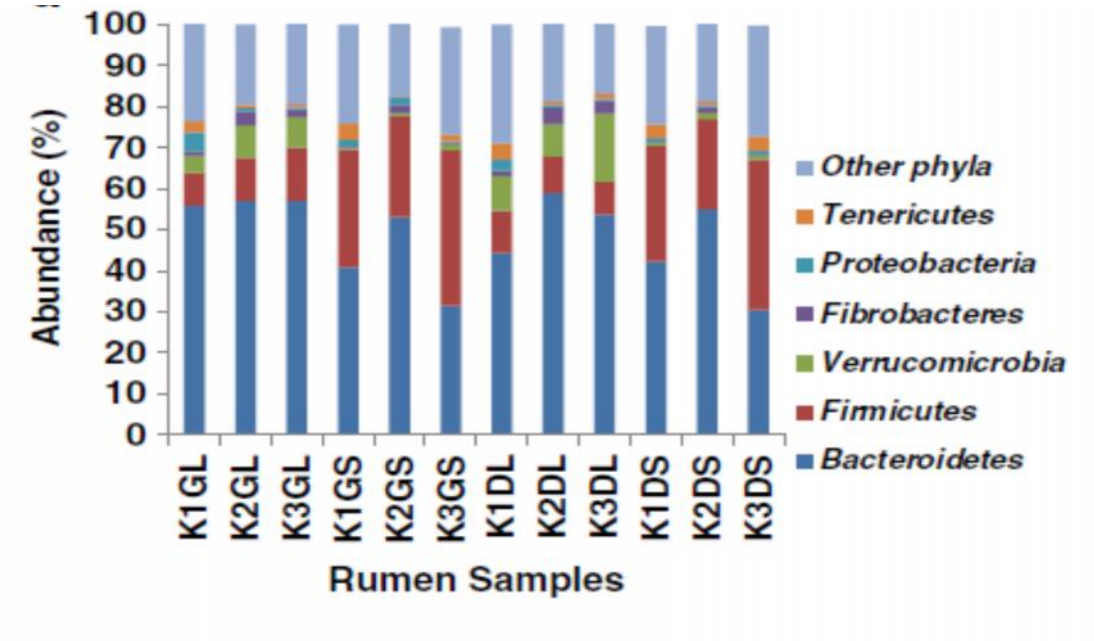


Fig.7 The taxonomic distribution of bacterial phylum level based on OTUs distribution in each sample of Kankrej cattle.

Studying the correlation between phyla using PAST, revealed that the abundance of the Bacteroidetes was positively correlated with abundance of bacterial phylum Verrucomicrobiae, Proteobacteria, and Fibrobacter, whereas negatively correlated with Firmicutes and Tenericutes during green and dry roughage treatment (Table 1 & 2).

For functional assignment, out of total functional categories, 65 were found to be significantly different between solid and liquid fraction and 58 categories were found to be significantly different between different treatments, whereas only 17

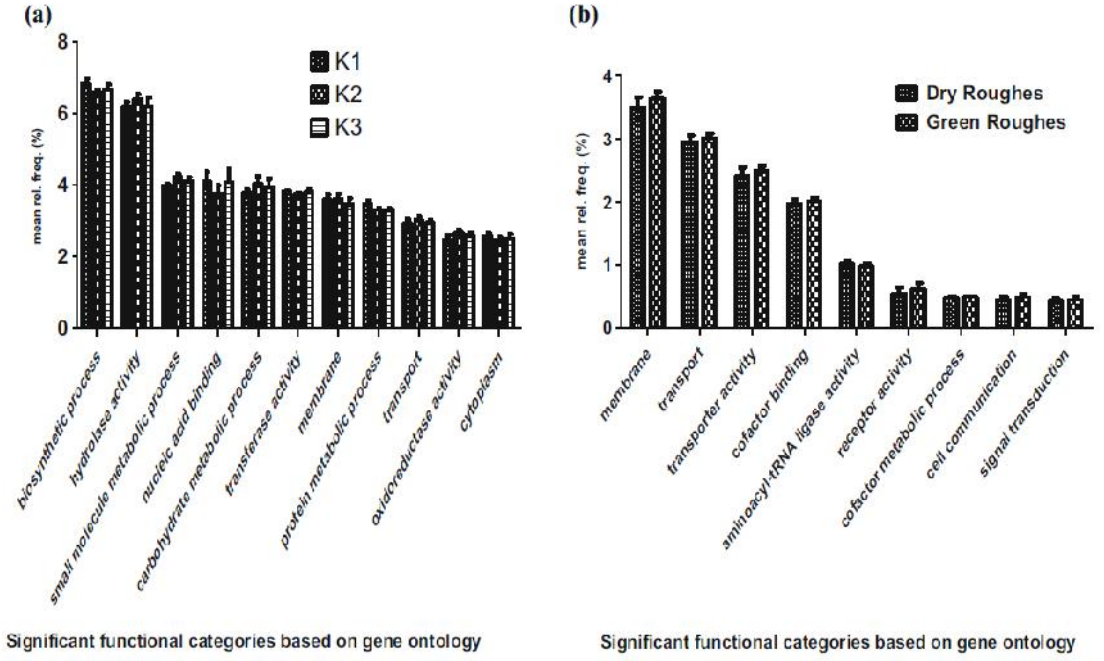
categories were found to be significantly different between green and dry roughage. Nitrogen compound metabolic process subcategory was found dominant in all the samples (Fig.8).

Table 1 Cross-phylum interaction using paleontological statistical analysis under dry roughage treatment

0	Bacteroidetes	Firmicutes	Verruco-microbia	Proteobacteria	Tenericutes	Fibrobacter
Bacteroidetes	0	0.138	0.466	0.563	0.028	0.088
Firmicutes	−0.679	0	0.018	0.594	0.479	0.035
Verrucomicrobia	0.374	−0.890	0	0.653	0.732	0.049
Proteobacteria	−0.300	−0.278	0.236	0	0.137	0.645
Tenericutes	−0.860	0.363	−0.181	0.681	0	0.148
Fibrobacter	0.747	−0.843	0.814	−0.242	−0.667	0

Table 2 Cross-phylum interaction using paleontological statistical analysis under green roughage treatment

0	Bacteroidetes	Firmicutes	Verruco-microbia	Proteobacteria	Tenericutes	Fibrobacter
Bacteroidetes	0	0.011	0.146	0.635	0.112	0.023
Firmicutes	−0.912	0	0.083	0.377	0.408	0.138
Verrucomicrobia	0.67	−0.754	0	0.805	0.317	0.113
Proteobacteria	0.249	−0.444	−0.131	0	0.603	0.686
Tenericutes	−0.712	0.42	−0.496	0.271	0	0.024
Fibrobacter	0.875	−0.679	0.711	−0.213	−0.871	0



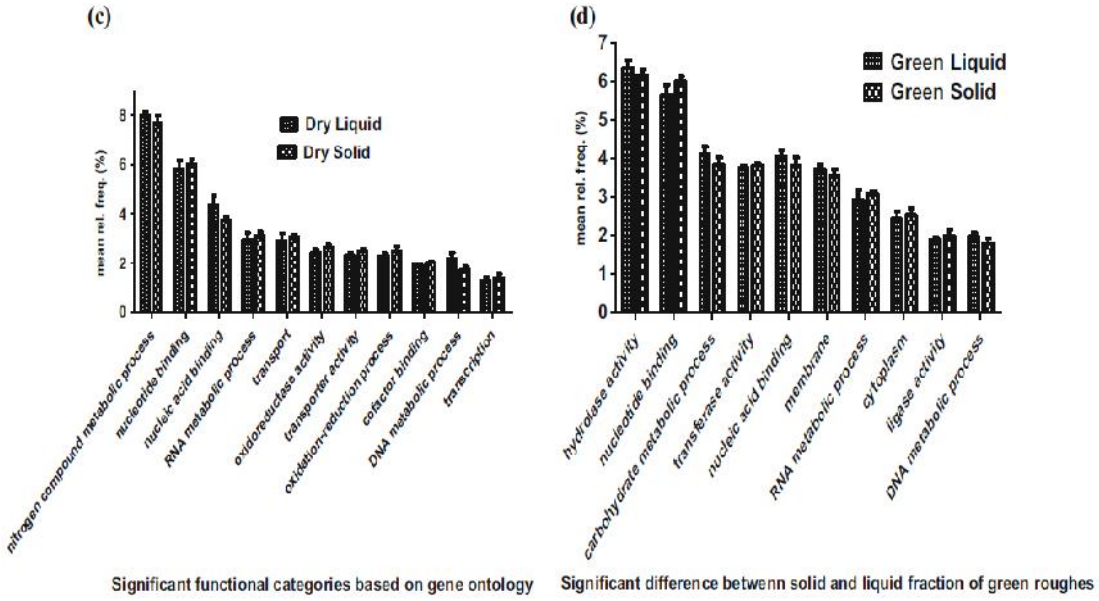


Fig.8 Statistical analysis based on functional analysis using STAMP a) Between all three treatments, b) Between dry and green roughage, c) Between liquid and solid fraction of dry roughage and d) Between liquid and solid fraction of green roughage.

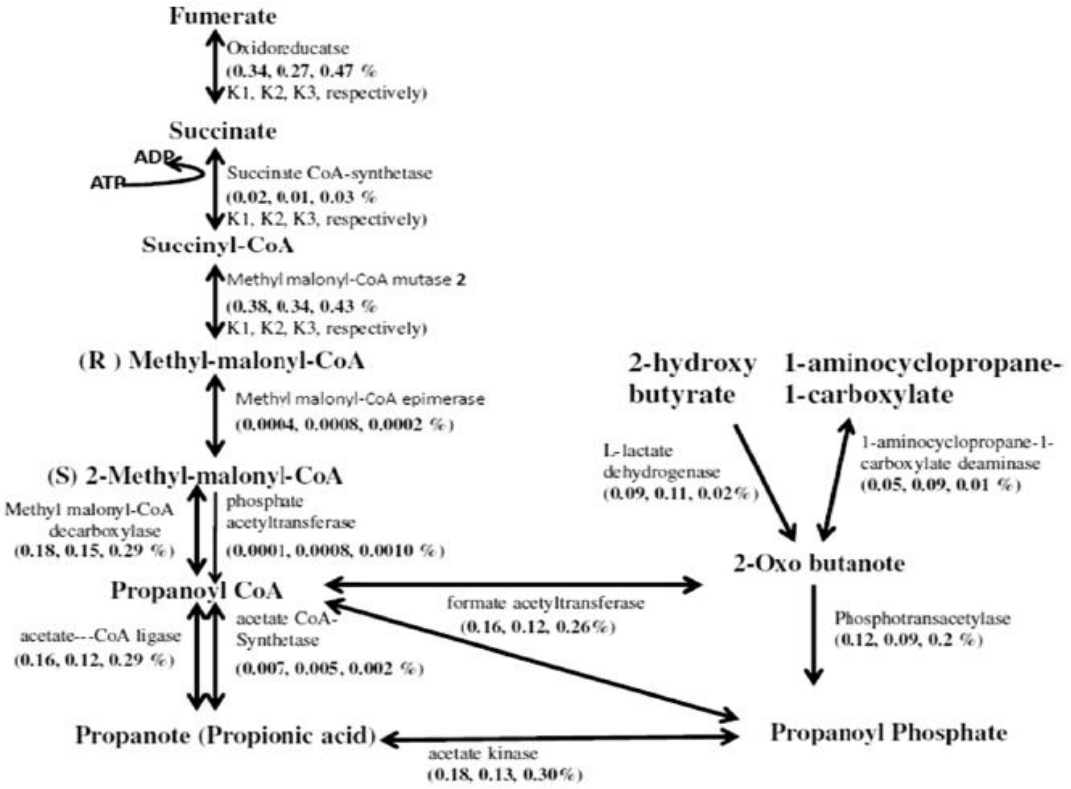


Fig.9 Schematics of propionate production pathway (abundance of enzymes during three different treatments shown in parentheses).

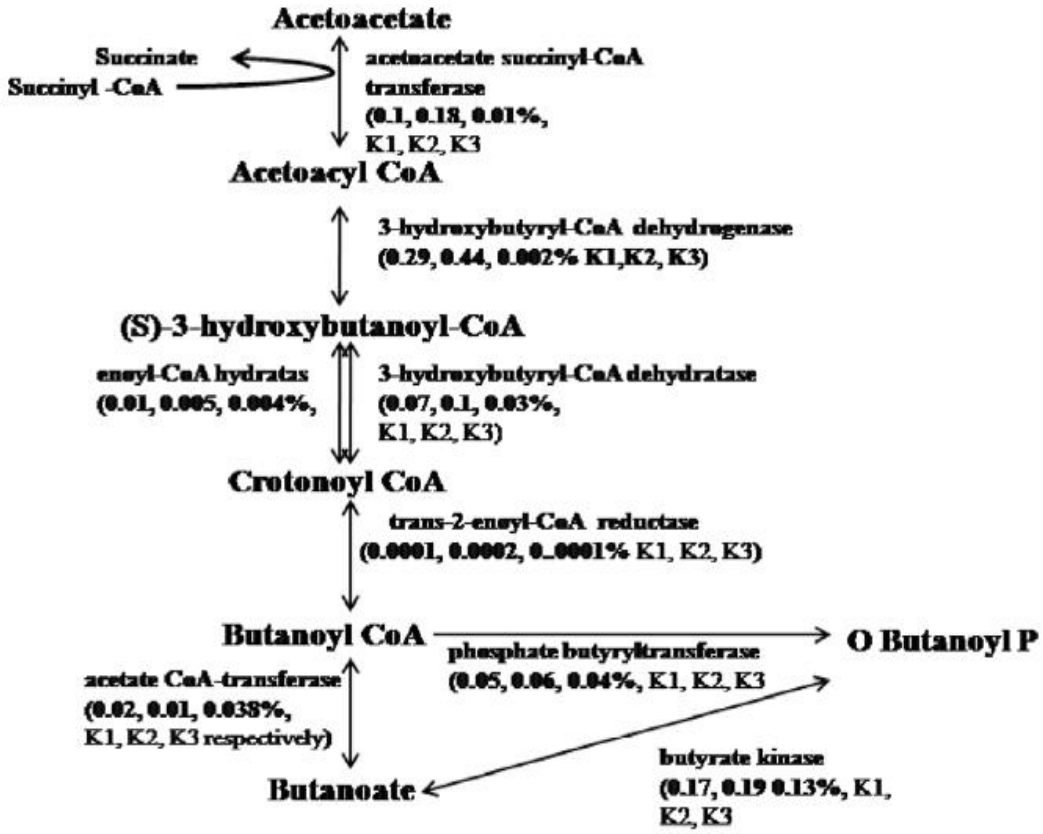


Fig.10 Schematics of butyrate production pathway (abundance of enzymes during three different treatments shown in parentheses).

EGTs involved in VFAs production such as propionate and butyrate production were also studied during three different diets. Third treatment (K3) showed consistently higher abundance of genes required for propionate production followed by first (K1) and second treatment (Fig. 9). Enzymes involved in butyrate production were found to be abundant consistently during second treatment followed by first and third treatment (Fig. 10).

Role of methanogens in the rumen of Kankrej cattle

The taxonomic annotation of the sequences based on M5nr database revealed that the percent abundance of *Methanobacteriales* genus decreased from K1 to K2 treatment, and then increased in K3 treatment. If we correlate the enzymes for methanogenesis contributed by this genus, we observed that the abundance of the enzymes involved in Formate to methane production pathway, decreased from K1D to K2D treatment, which further increased in K3 treatment (Fig. 11A and 5B). Thus, the functional profile shifts with the diet for *Methanobacteriales* corroborate with its taxonomic profile shift at each treatment.

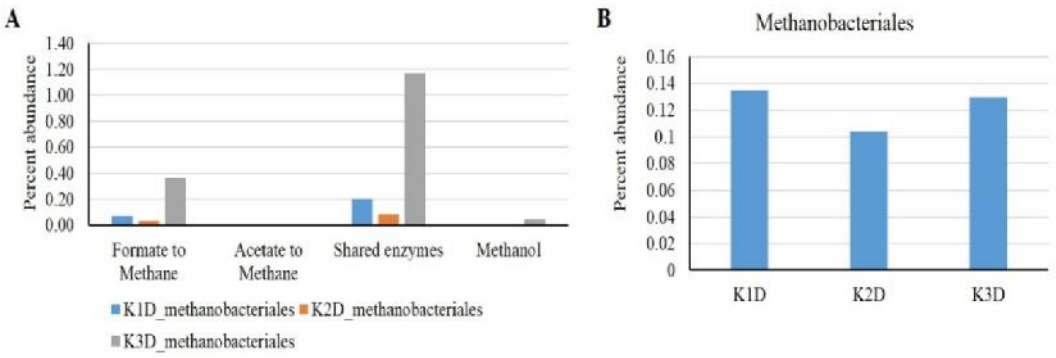


Fig.11 A. The methanogenic enzymes contributed by *Methanobacteriales* at each treatment of dry roughage. B. Percent abundance of *Methanobacteriales* order among all observed taxa at each treatment.

The taxonomic annotation of the sequences based on M5nr database revealed that the percent abundance of *Methanobacteriales* genus was found to be more in 1st treatment as compared to 2nd and 3rd treatments of the dry roughage diet. But, upon correlating the enzymes for methanogenesis contributed by this genus, we observed that the abundance of these enzymes were more in 1st and 3rd treatments as compared to 2nd treatment (Fig. 12A and 12B).

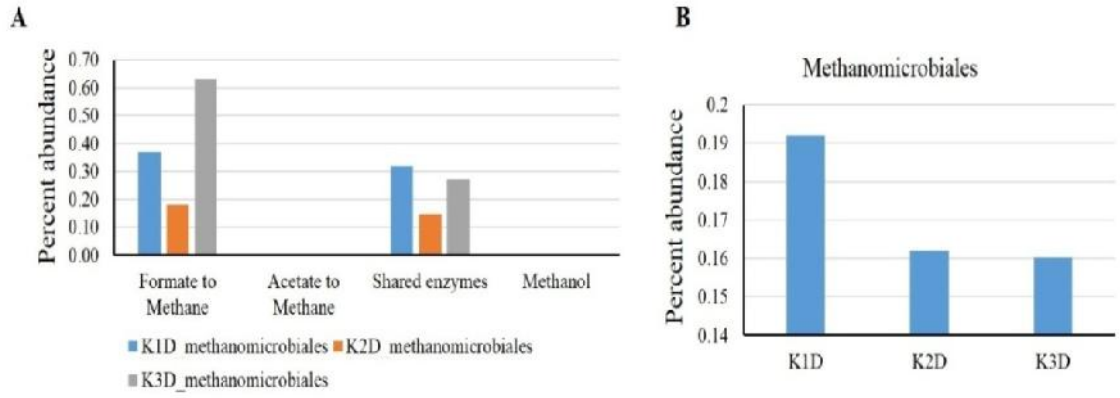


Fig.12 A. The methanogenic enzymes contributed by *Methanomicrobiales* at each treatment of dry roughage. B. Percent abundance of *Methanomicrobiales* order among all observed taxa at each treatment.

The order *Methanosarcinales* is composed of two families i.e., *Methanosaetaceae* and *Methanosarcinaceae*. The taxonomic annotation of the sequences based on M5nr database revealed that the percent abundance of *Methanosaetaceae* family was increased with the increment in the dry roughage proportion whereas, the abundance of *Methanosarcinaceae* decreased from K1D to K2D and then increased in K3D treatment. If we correlate the enzymes for methanogenesis with that of the taxonomy profile, we observed that the abundance of

the enzymes involved in acetate to methane production pathway showed quite similar profile with *Methanosaetaceae* family (Fig. 13A and 7B). *Methanosaetaceae* is a strictly acetate utilizing group of organism and *Methanosarcinaceae* can utilize all three substrates (CO_2, Acetate and Methanol). Here, more abundance of methanol to methane producing enzymes is thought to be contributed by *Methanosarcinaceae* family.

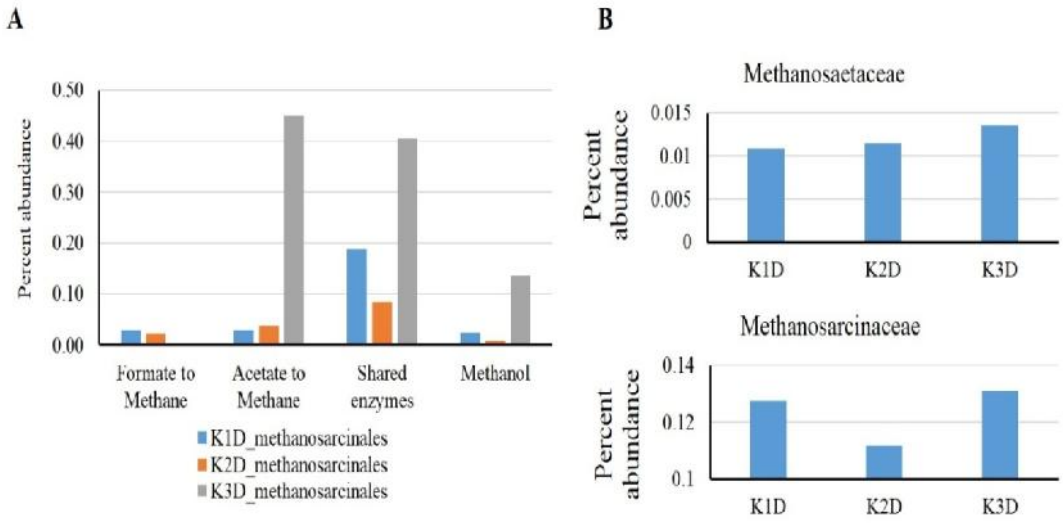


Fig.13 A. The methanogenic enzymes contributed by *Methanosarcinales* at each treatment of dry roughage. B. Percent abundance of *Methanosaetaceae* and *Methanosarcinaceae* families among all observed taxa at each treatment

Gir metagenome data analysis using MG-RAST server

The rumen bacterial population of Gir cattle at phylum level was classified in to 28 bacterial phyla from all samples. Amongst 28 bacterial phyla, *Bacteroidetes* were highly abundant, ranging from 26 to 65% with predominance in G1GL (65%), followed by *Firmicutes*, ranging from 14-43% with predominance in G1DS (Fig.14).

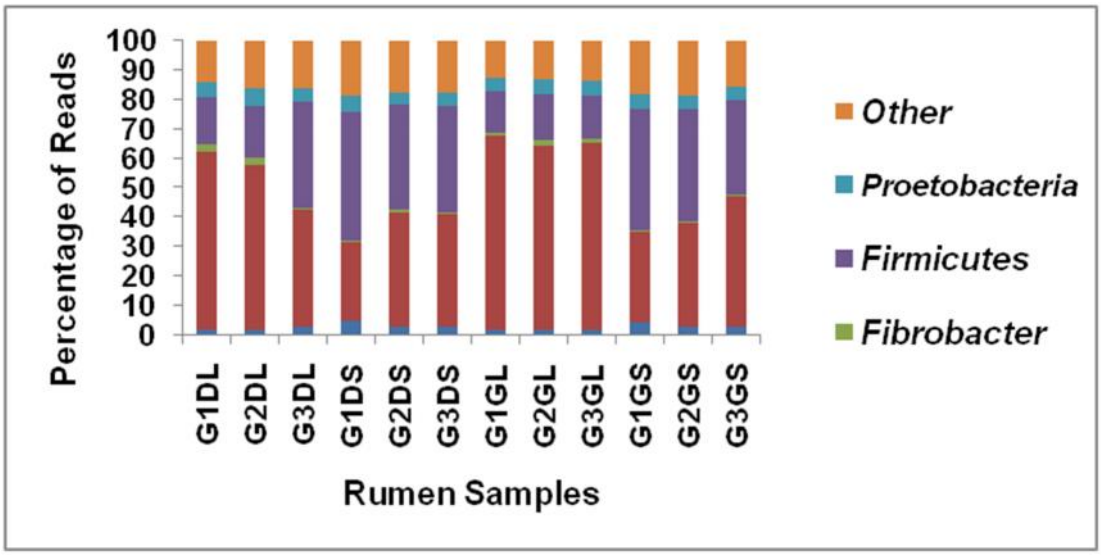
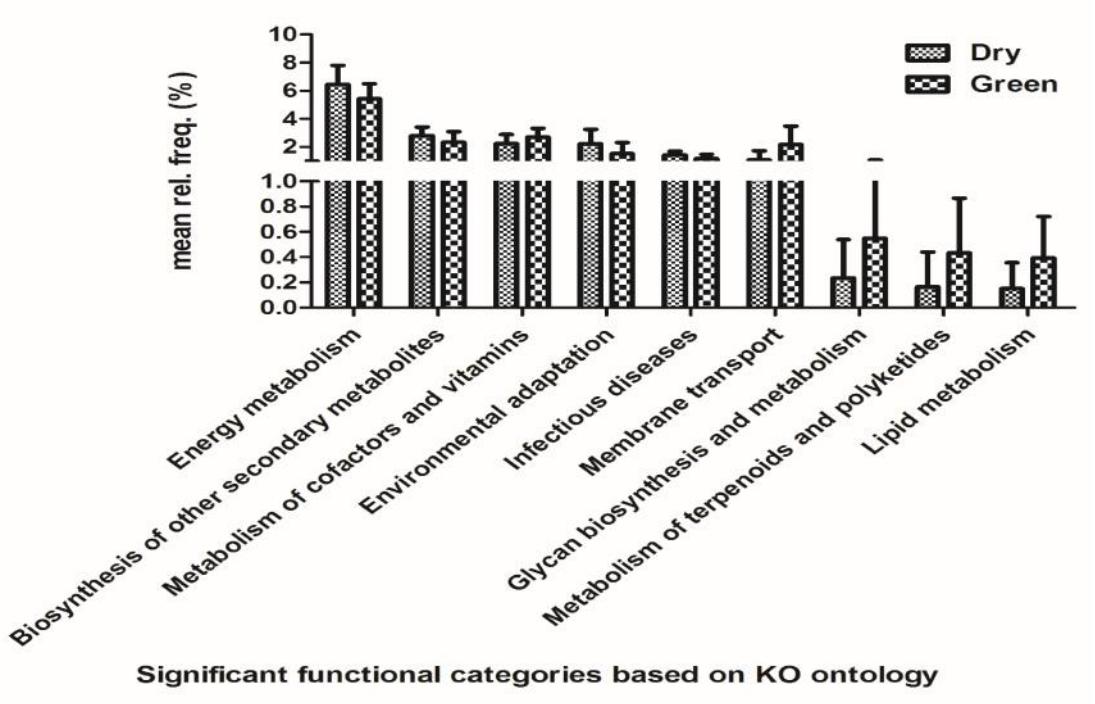
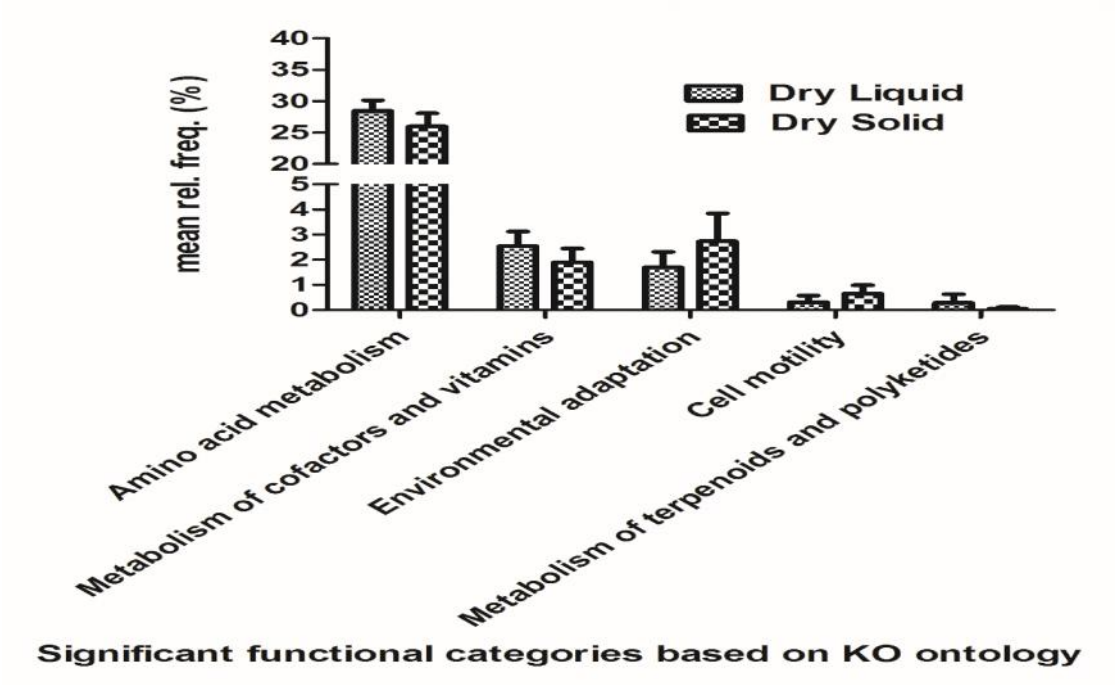


Fig.14 Taxonomic composition of all rumen microbiome samples at phylum level using M5NR database at e-value cutoff $1e^{-05}$and with a minimum alignment length of 15 bp.

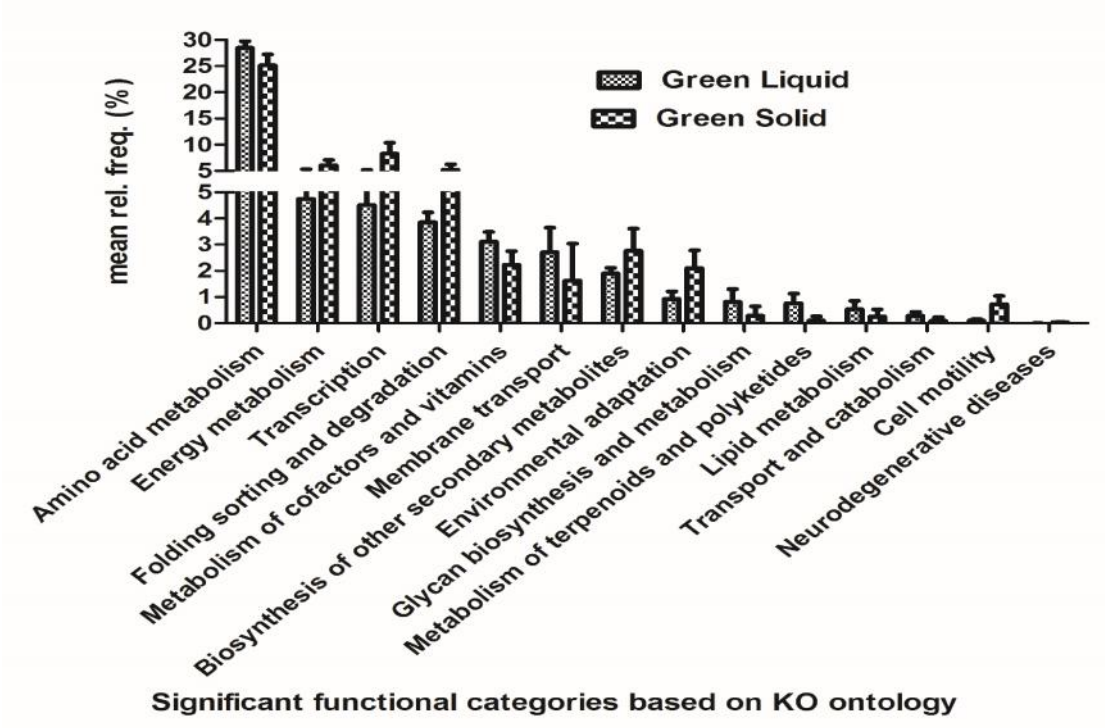
(a)



(b)


mean rel. freq. (%)
40
35
30
25
20
5
4
3
2
1
0
Dry Liquid
Dry Solid
Amino acid metabolism
Metabolism of cofactors and vitamins
Environmental adaptation
Cell motility
Metabolism of terpenoids and polyketides
Significant functional categories based on KO ontology

(c)


mean rel. freq. (%)
30
25
20
15
10
5
5
4
3
2
1
0
Green Liquid
Green Solid
Amino acid metabolism
Energy metabolism
Transcription
Folding sorting and degradation
Metabolism of cofactors and vitamins
Membrane transport
Biosynthesis of other secondary metabolites
Environmental adaptation
Glycan biosynthesis and metabolism
Metabolism of terpenoids and polyketides
Lipid metabolism
Transport and catabolism
Cell motility
Neurodegenerative diseases
Significant functional categories based on KO ontology

(d)

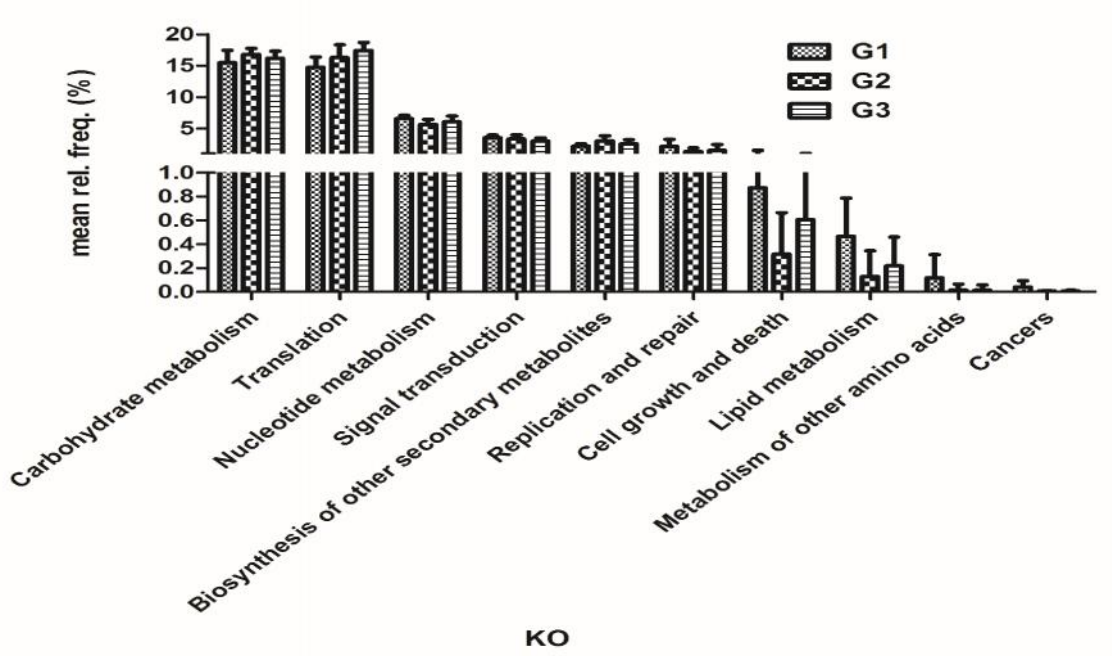


Fig.15 Statistical analysis at P<0.05 of functional abundance using STAMP (a) Between dry and green roughage (b) Between dry solid and liquid (c) Between green solid and liquid (d) Between all three treatment.

In functional metabolic profile, as expected, majority of reads were affiliated with genes involved in carbohydrate and protein metabolisms. Other categories such as, cell motility category is generally under-represented in all samples, as motility is not required by intestinal bacteria to persist in the gut due to the constant peristaltic movements (Fig.15).

A total of 20,000 to 30,000 reads with corresponding enzyme commission (EC) numbers were assigned to the metabolic pathways from all three treatments. Sequences related to the metabolism of VFA synthesis have direct relation to milk production and sequences related to methanogenesis are directly related to green house effect, hence we focused our attention on metabolic pathway for VFA synthesis and methanogenesis (Fig. 16, 17&18). Results stated that roughage concentration also exert significant effect on EGT involved in methanogenesis; significantly affecting the abundance of *mcr* gene.

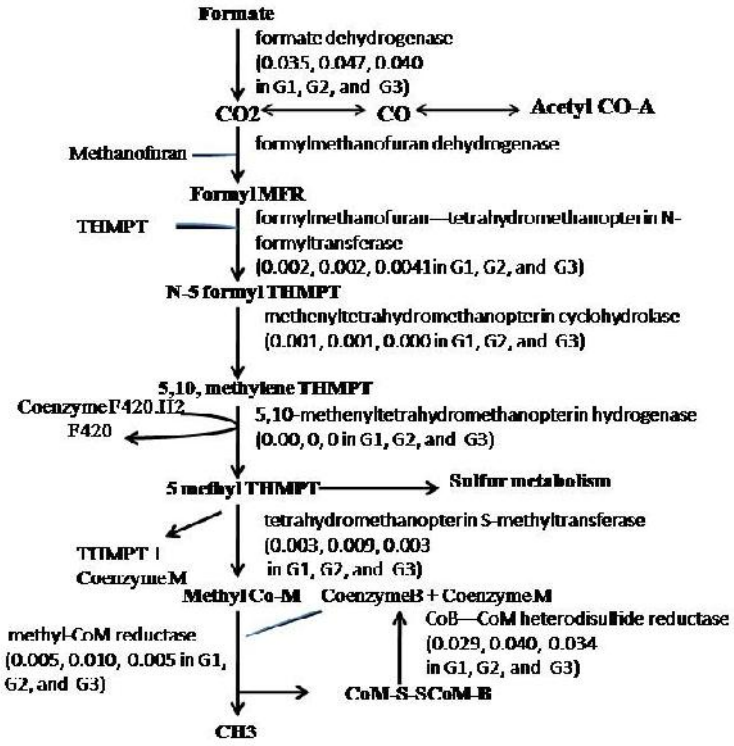


Fig.16 Schematics of methanogenesis pathway (abundance of enzymes during three different treatments shown in parentheses)

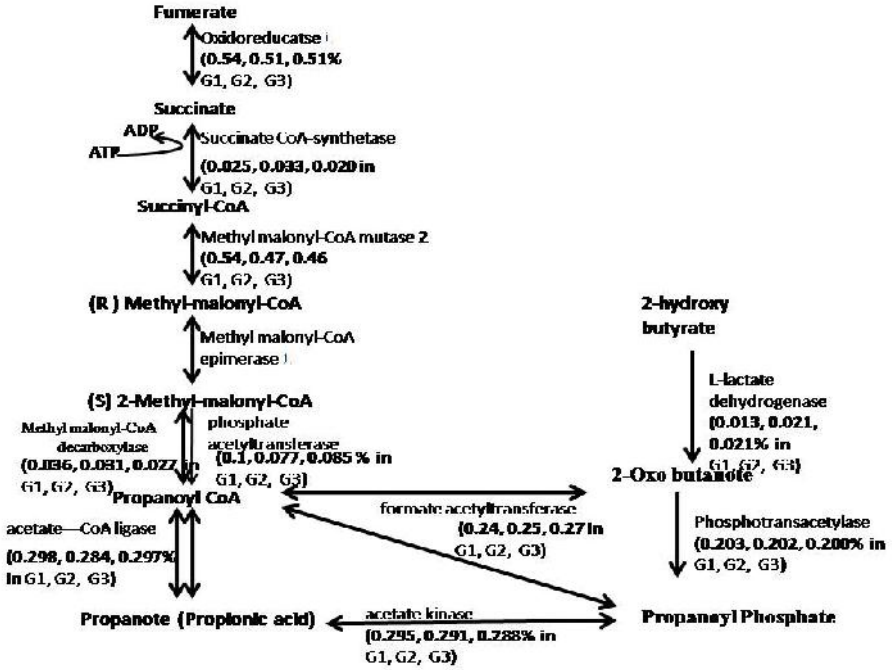


Fig.17 Schematics of propionate production pathway (abundance of enzymes during three different treatments shown in parentheses).

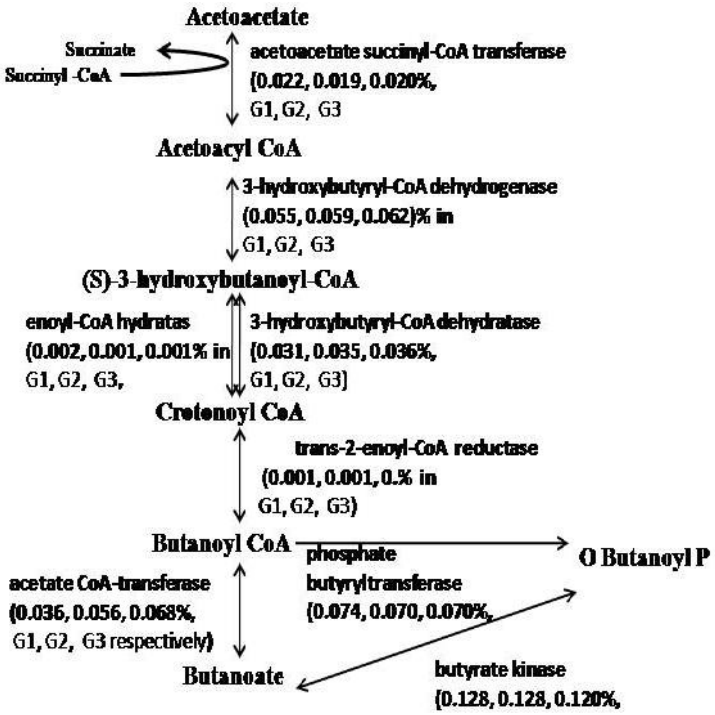


Fig.18 Schematics of butyrate production pathway (abundance of enzymes during three different treatments shown in parentheses).

Role of methanogens in the rumen of Gir cattle

The taxonomic annotation of the sequences based on M5nr database revealed that the percent abundance of *Methanobacteriales* genus increased with the increment in the dry roughage. If we correlate the enzymes for methanogenesis contributed by this genus, we observed that the abundance of these enzymes increased with the increment in the roughage proportion (Fig. 19A). Thus, the functional profile shifts with the diet for *Methanobacteriales* corroborate with its taxonomic profile shift at each treatment (Fig. 19B).

A B

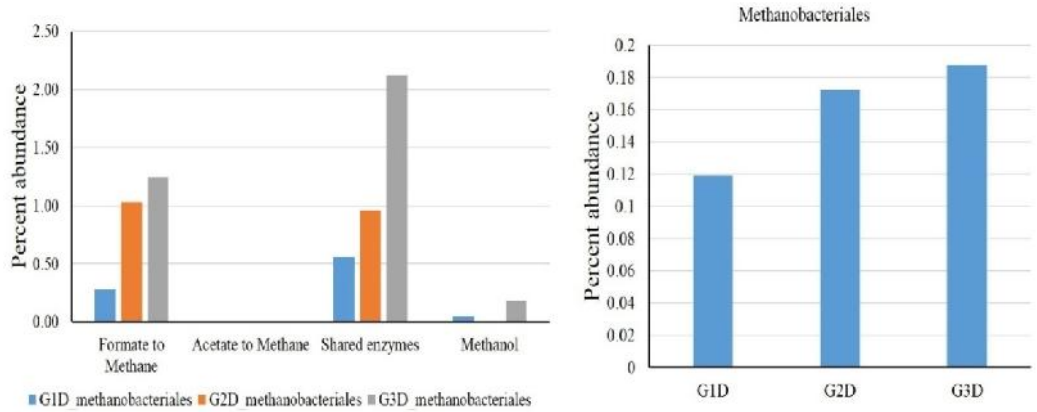


Fig.19 A. Methanogenic enzymes contributed by *Methanobacteriales* at each treatment of dry roughage. B. Percent abundance of *Methanobacteriales* order at each treatment.

The taxonomic annotation of the sequences based on M5nr database revealed that the percent abundance of *Methanobacteriales* genus was found to be more in 2nd treatment as compared to 1st and 3rd treatment of the dry roughage diet. If we correlate the enzymes for methanogenesis contributed by this genus, we observed that the abundance of these enzymes were more in 2nd treatment as compared to 1st and 3rd treatment (Fig. 20A and 20B). Thus, the functional profile shifts with the diet for *Methanomicrobiales* corroborate with its taxonomic profile shift at each treatment.

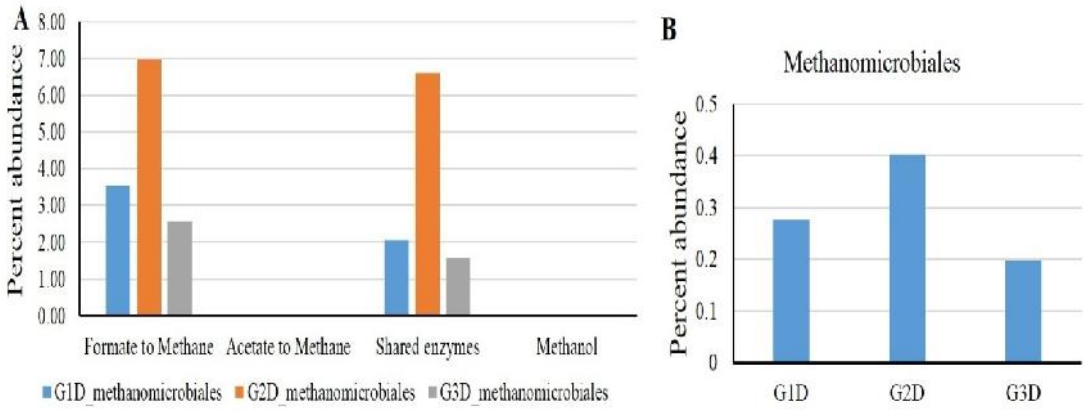
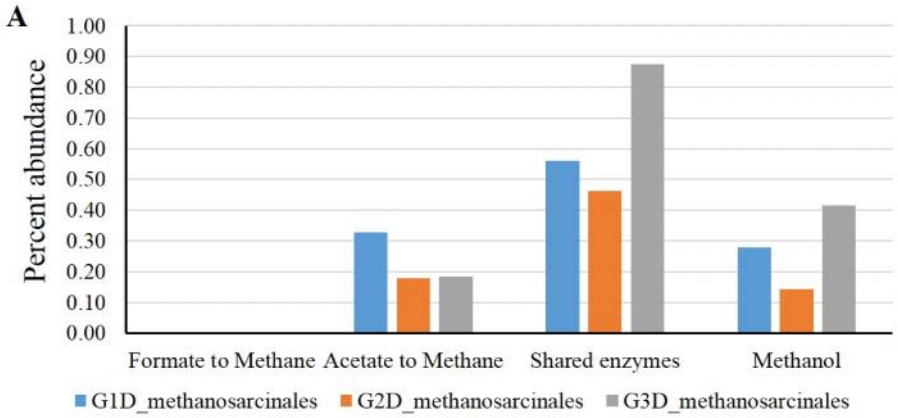


Fig.20 A. The methanogenic enzymes contributed by *Methanomicrobiales* at each treatment of dry roughage. B. Percent abundance of *Methanomicrobiales* order among all observed taxa at each treatment.

The order *Methanosarcinales* is composed of two families i.e., *Methanosaetaceae* and *Methanosarcinaceae*. The taxonomic annotation of the sequences based on M5nr database revealed that the percent abundance of *Methanosaetaceae* family was found to decrease with the increment in the dry roughage proportion whereas, the abundance of *Methanosarcinaceae* increased with the increment in the dry roughage proportion. If we correlate the enzymes for methanogenesis with that of the taxonomy profile, we observed that the abundance of the enzymes involved in acetate to methane production pathway showed quite similar profile with *Methanosaetaceae* family (Fig. 21A and 21B). *Methanosaetaceae* is a strictly acetate utilizing group of organism and *Methanosarcinaceae* can utilize all three substrates (CO_2, Acetate and Methanol). Here, more abundance of methanol to methane producing enzymes is thought to be contributed by *Methanosarcinaceae* family.



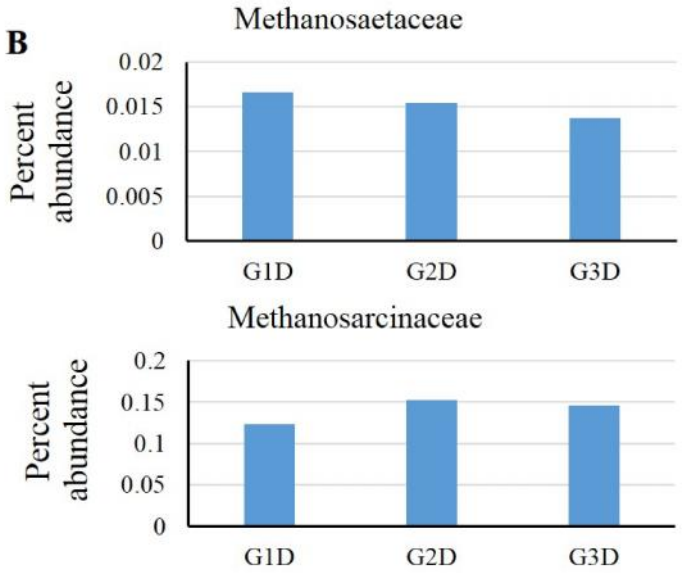


Fig.21 A. The methanogenic enzymes contributed by *Methanosarcinales* at each treatment of dry roughage. B. Percent abundance of *Methanosaetaceae* and *Methanosarcinaceae* families among all observed taxa at each treatment.

Comparative analysis of Indian cattle breed (*Bos indicus*) with foreign cattle breed (*Bos taurus*) for methanogenesis pathway and their enzymes

Upon comparing the methanogenic enzymes contributed by Indian cattle breeds with foreign cattle breeds, we found that, formate to methane producing enzymes along with shared enzymes contributed by Methanobacteriales were more in Australian Holstein cattle and Jersey cattle as compared to Indian Gir and Kankrej cattle (Fig. 22).

Whereas, the formate to methane producing enzymes along with shared enzymes contributed by Methanomicrobiales were more in Indian origin cattle breeds as compared to Holstein and Jersey breeds (Fig. 23). Acetate to methane producing enzymes contributed by Methanosarcinales group of organisms showed more representation in Indian cattle and Jersey cattle. To further evaluate the differences in the methanogenic enzyme abundance between Indian cattle and two exotic cattle were significant or not, the ANOSIM (Analysis of Similarity) was performed. The results suggested the significant difference in abundance of methanogenic enzymes between Indian cattle and Holstein cattle (Table 3).

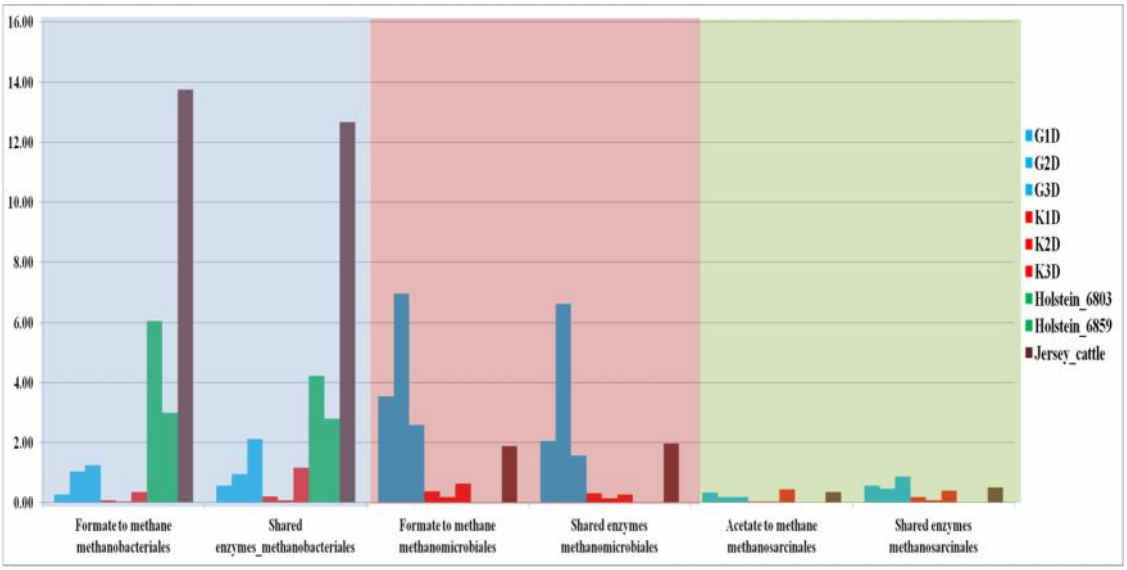


Fig.22 Comparative analysis showing the percent abundance of the enzymes involved in methane production pathway from two Indian cattle breeds (Gir and Kankrej), Australian Holstein cattle and Jersey cattle. Blue background: Enzymes contributed by Methanobacteriales, Red background: Enzymes contributed by Methanomicrobiales and Green background: Enzymes contributed by Methanosarcinales.

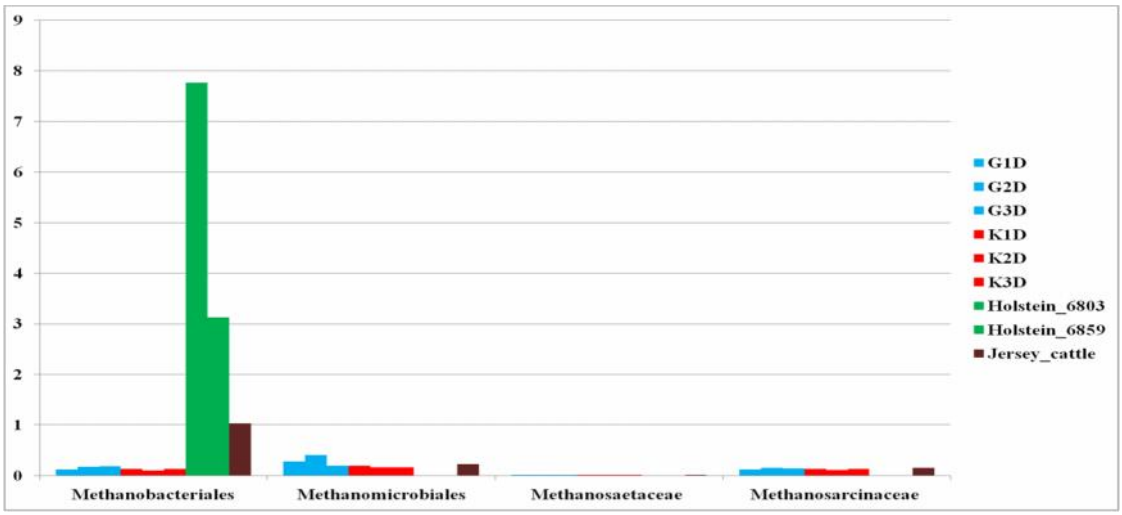


Fig.23 Comparative analysis showing the percent abundance of the Methanogens in Indian cattle, Holstein cattle and Jersey cattle.

Table 3 ANOSIM output inferring significant difference in methanogenesis enzymes contributed by Indian and Exotic cattle

	Indian cattle	Holstein cattle	Jersey cattle
Indian cattle	0.000	0.0352	0.7191
Holsteincattle	0.0352	0	0.3382
Jersey cattle	0.719	0.3382	0

PCA analysis also corroborated with the results obtained from ANOSIM analysis, where, more distinct clusters of Holstein and Indian cattle were observed (Fig. 24).

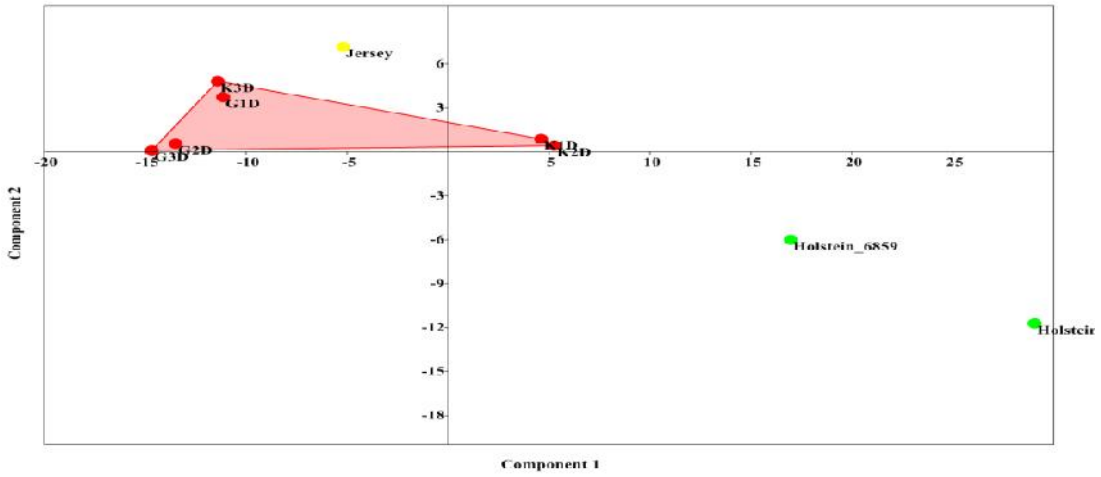


Fig.24 Principal component analysis (PCA) based visualization of the cluster formation based on the difference in methanogenesis enzymes abundance for Indian and exotic cattle

Further comparison of the total methanogenesis related enzymes abundance among Indian , Holstein and Jersey cattle, more abundance of it was observed in Holstein cattle as compared to rest of the cattle breeds. Similarly, when we compared the abundance of methanogens among these three cattle, the more abundance was observed in Holstein cattle as compared to other cattle breeds (Fig. 25).

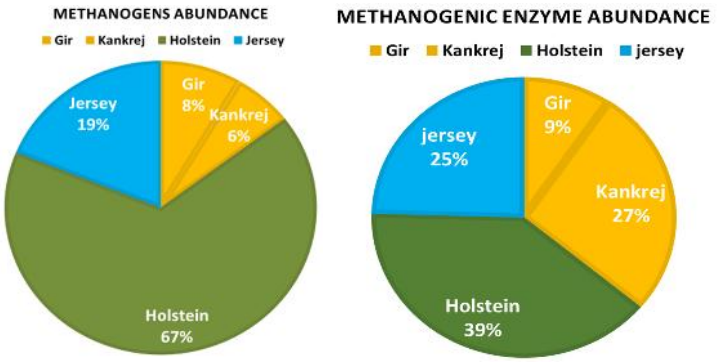


Fig.25 Total methanogens and methanogenesis related enzyme abundance (irrespective of specific pathways) and the abundance of methanogens in Indian and exotic cattle

Jaffarabadi metagenome data analysis using MG-RAST server

In the rumen microbial community of liquid fraction of Dry Roughage (DL) at phylum level using M5NR database at 60% minimum percent identity, Bacteroidetes was observed to be the most abundant followed by Firmicutes, Proteobacteria, across all the three J1, J2 and J3 treatments.

Similarly, in the solid fraction of Dry Roughage (DS), Bacteroidetes was the most abundant phylum followed by Firmicutes, Fibrobacter and Proteobacteria in J1, J2 and J3, followed by other phyla. Similarly, in the liquid fraction of green roughage (GL) as well as the solid fraction of Green Roughage (GS), Bacteroidetes was the most abundant phylum followed by Firmicutes, Proteobacteria and other phyla across all the three J1, J2 and J3 treatments (Fig.26).

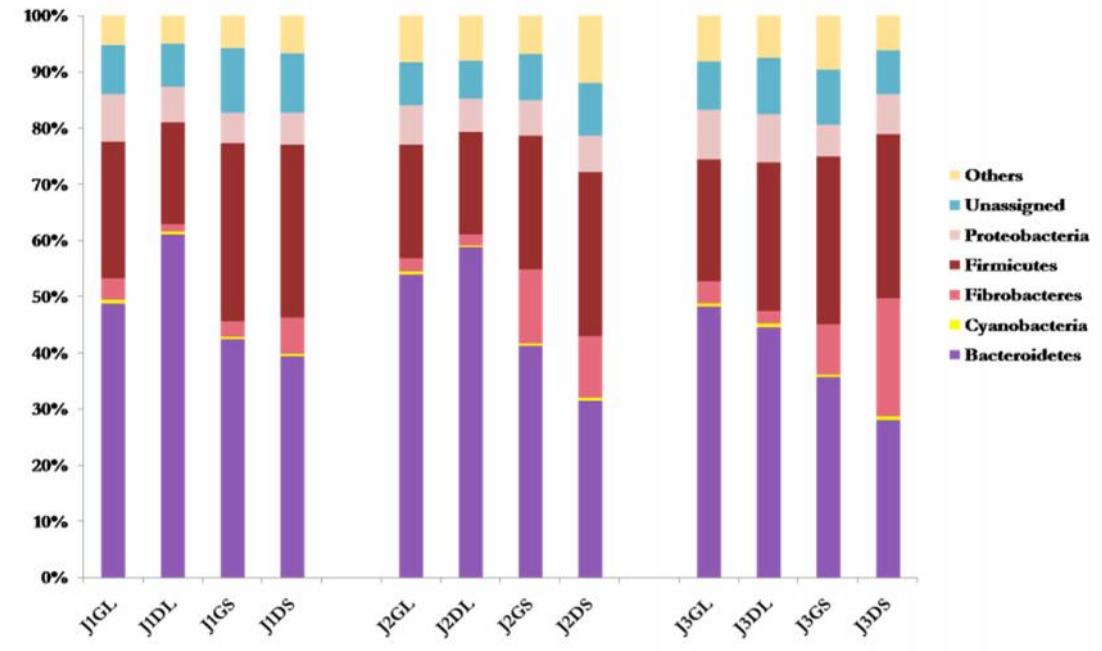


Fig.26 Taxonomic distribution of Jaffarabadi metagenome at phyla level

Functional analysis based on MG-RAST output against the SEED database at 80% identity cutoff revealed that the highest coding sequences were present for the Protein Metabolism followed by Carbohydrate metabolism category at level 1. Statistical test using STAMP revealed significant differences (p value <0.05) between solid and liquid fraction as well as across the dietary treatments. About ten categories including Secondary Metabolite; Nitrogen metabolism; DNA metabolism; protein metabolism; carbohydrates; amino acids and derivatives; sulfur metabolism; cofactors, motility and Chemotaxis; and miscellaneous were observed to be significantly different between the liquid and solid fractions (Fig.27).

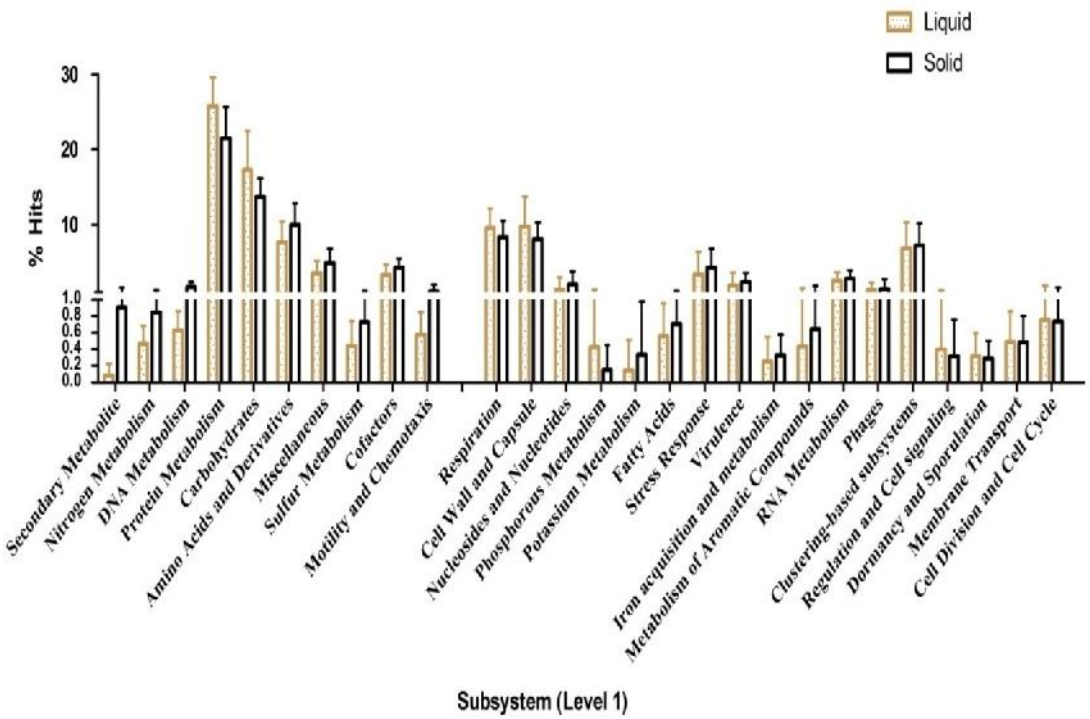


Fig.27 Functional Profile of Liquid and Solid fraction (Level 1 abundance) form Jaffarabadi metagenome

Whereas three of the categories viz. carbohydrates; amino acids and its derivatives; and miscellaneous differed significantly across the J1, J2 and J3 dietary treatments, the coding sequences related to the Carbohydrate subsystem increased with the increase in roughage concentration (Fig. 28).

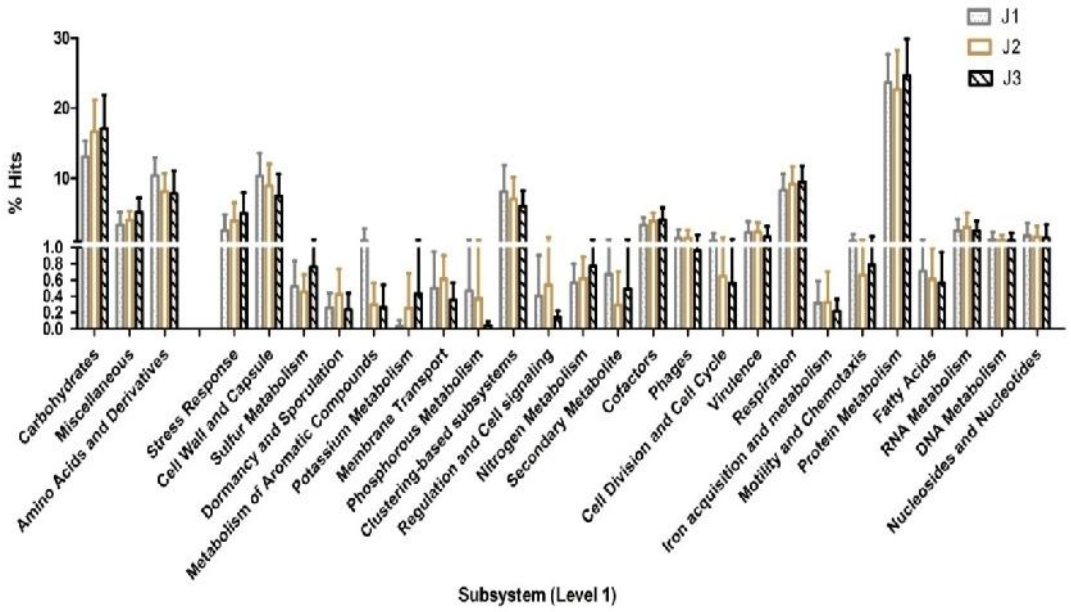


Fig.28 Functional Profile across the dietary treatments (Level 1 abundance) form Jaffarabadi metagenome

Further, coding sequences involved in VFAs production such as propionate and butyrate production were also studied during three different diets (Fig.29&30). Enzymes involved in butyrate production were found to be abundant during second treatment followed by first treatment and third treatment.

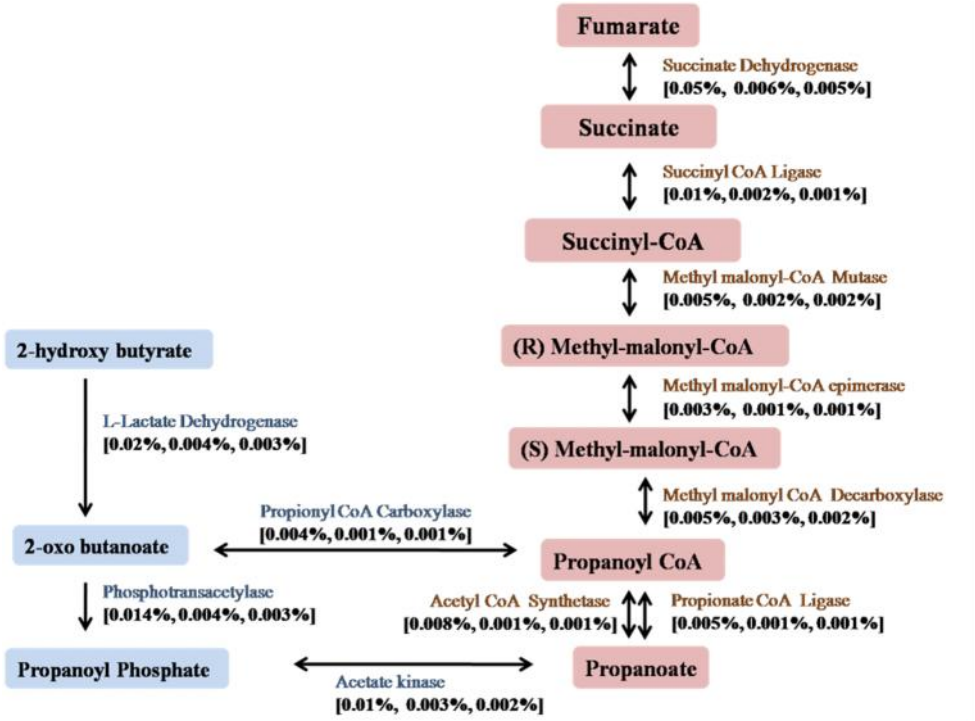


Fig.29 Schematics of Propanoate production pathway(abundance of enzymes during three different treatments shown in parentheses).

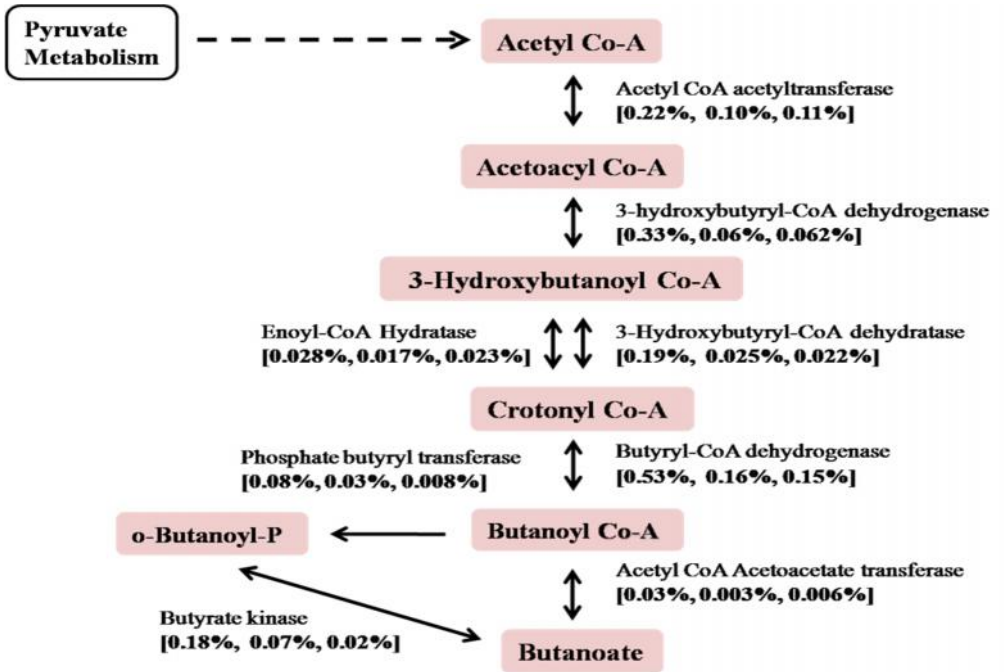


Fig.30 Schematics of butanoate production pathway(abundance of enzymes during three different treatments shown in parentheses).

The rumen metagenomic study revealed the significant variation in the microbial community composition and its functional gene profile in response to change in diet. It also depicted that individual host genotype did not significantly affect bacterial community structure. The study enhances overall understanding of the rumen microbial ecology in buffalo at taxonomic and functional level under different diets. This modulating behavior of microbiota can be manipulated to improve livestock nutrient utilization efficiency.

The taxonomic analysis that revealed that *Bacteroidetes* was the most abundant phylum followed by *Firmicutes*, *Fibrobacter* and *Proteobacteria* and the functional analysis of Jaffarabadi rumen metagenome data that revealed protein (25-30%) and carbohydrate (15-20%) metabolism as the dominant categories were detailed. Similarly, at the genus level the two predominant genus of *Bacteroidetes* family, viz. *Prevotella* and *Bacteroides* were found decreasing with the increase in roughage proportion (Fig. 31).

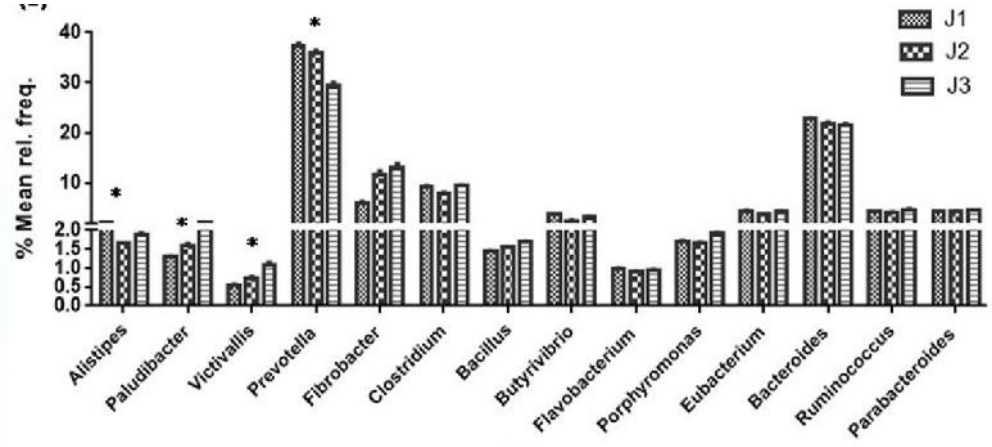


Fig.31 Statistical analysis using STAMP based on genus level taxonomic assignments between treatments (* indicates p<0.05, ** indicate p<0.01, *** indicate p<0.001)

Both were significantly (p-value < 0.05) higher in the liquid as compared to solid portion (Fig. 32). Out of the fourteen most abundant genera comprising about 70% of total genera, four were significantly varying across treatments and thirteen were varying between the solid and liquid fractions. *Paludibacter* and *Victivallis* were found increasing with the increase in roughage content. *Allistepes* was highest in J1, followed by J3 and then J2. Further at species level, *Prevotella ruminicola* was the predominant species in all the liquid samples throughout different treatments followed by the two important fibrolytic species of rumen ecosystem viz., *Fibrobacter*

succinogenes and *Ruminococcus albus.* In the solid fraction, *Fibrobacter succinogenes* and *Ruminococcus albus* were predominant followed by *Prevotella ruminicola*.

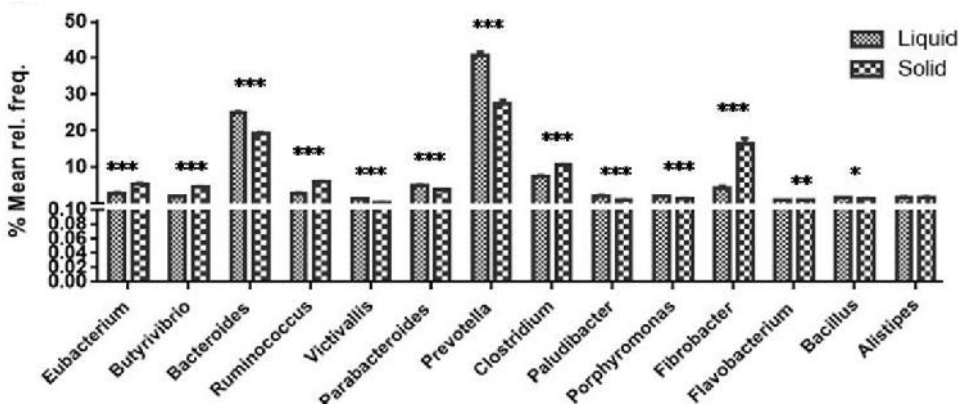


Fig.32 Statistical analysis using STAMP based on genus level taxonomic assignments between liquid and solid fraction(* indicates $p<0.05$, ** indicate $p<0.01$, *** indicate $p<0.001$)

Roughage vs. Firmicutes/Bacteroidetes ratio

Bacteroidetes and *Firmicutes* were the most prevalent phyla throughout the treatments. For the *Firmicutes*/*Bacteroidetes* ratio, significant differences between liquid (0.3 to 0.59) and solid (0.6 to 1.1); as well as between the three treatments were observed for both green and dry roughage fed animals (Fig. 33).

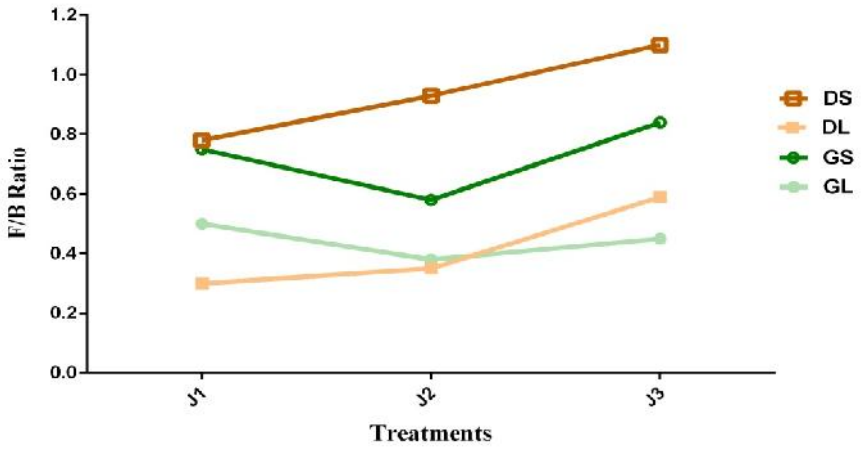


Fig. 33 *Firmicutes/Bacteroidetes* ratio during the three treatments

Notably specific trend was observed for green and dry diets, whereby in the green roughage fed animals the ratio was found to be higher in J1, reduced in J2 and again increased in the J3 treatment, whereas in dry roughage fed animals the ratio was found increasing with increase in roughage proportion (J1 > J2 > J3).

Diet based variation in CAZymes distribution

Results of the CAZyme Analysis Tool (CAT) revealed that the most encoded CAZyme domain included the Glycoside Hydrolase (GH) family (~50% of total assignment), followed by the Carbohydrate Binding Modules (CBM) (~30%), Glycosyl Transferases (GT) (~15%) and a rest of the proportion (~5%) comprising Pectate Lyases (PL) and Auxiliary Activities (AA). The proportion of GH family was found to increase marginally with increase in the roughage proportion for the dry fed individuals. GT family was represented more in the liquid samples as compared to the solid samples.

		J1				J2				J3			
		Green		Dry		Green		Dry		Green		Dry	
		Liquid	Solid	Liquid	Solid	Liquid	Solid	Liquid	Solid	Liquid	Solid	Liquid	Solid
Cellulases	GH5	6.02	3.02	2.74	6.37	2.23	4.62	2.20	7.17	4.64	6.82	3.29	9.90
	GH9	3.01	3.52	1.37	7.16	1.20	6.79	1.33	10.00	2.61	9.69	1.32	12.66
	GH45	0.52	1.01	0.18	1.38	0.00	1.64	0.00	2.83	0.18	1.88	0.15	2.57
	GH72	1.57	1.01	0.82	0.98	2.23	0.30	0.93	0.87	1.04	0.56	2.27	0.85
	GH88	0.92	0.50	0.18	0.13	0.86	0.10	0.40	0.22	0.27	0.13	0.15	0.06
	GH95	0.92	1.51	1.46	0.53	1.03	0.10	0.93	0.65	1.08	0.56	2.12	0.73
Endohemicellulases	GH8	1.70	2.01	0.09	2.89	0.51	3.31	0.87	4.02	1.31	4.75	1.02	6.32
	GH10	2.09	2.18	1.28	2.36	0.86	1.87	1.27	2.39	1.85	3.69	0.59	2.37
	GH11	0.79	1.01	0.00	1.58	0.00	0.85	0.13	2.93	0.32	2.19	0.00	2.26
	GH12	0.26	0.17	0.18	0.13	0.34	0.03	0.33	0.00	0.36	0.13	1.02	0.08
	GH26	1.05	0.50	1.28	2.23	1.03	1.77	0.60	1.96	1.04	2.31	1.17	2.06
	GH28	1.05	1.51	1.92	0.20	2.74	0	2.07	0.00	2.25	0.06	1.98	0.03
	GH53	0.79	0.34	1.74	4.60	0.51	1.70	2.20	2.93	1.31	2.38	0.88	5.56
Cell Wall Elongation	GH16	0.79	0.67	0.64	1.64	1.54	1.41	1.53	3.04	1.08	1.81	1.46	2.85
	GH17	0.13	0.34	0.55	0.13	0.86	0	0.33	0.11	0.23	0.13	0.29	0.14
	GH94	2.75	5.86	1.55	2.89	1.71	0.66	1.33	3.59	1.44	2.88	1.10	1.49
Debranching	GH23	3.66	1.17	2.19	3.09	5.31	1.08	4.54	2.83	5.09	2.50	8.71	3.75
	GH33	0.13	0.50	0.64	0.79	0.68	0.36	1.27	0.98	1.13	0.50	1.24	1.83
	GH51	4.97	5.53	2.47	5.98	4.45	1.57	2.87	5.00	3.02	5.50	2.34	2.50
	GH77	3.01	0.84	2.47	1.44	2.40	0.52	2.13	2.83	1.89	4.82	1.76	1.86
	GH78	1.44	1.34	2.01	0.39	2.23	0.36	1.33	0.00	2.48	0.75	0.95	0.08
	GH84	0.26	0	0.09	0.07	0.51	0.03	0.13	0.00	0	0.19	0.37	0.25
	GH103	0.00	0	0.00	0.00	0.00	0.10	0.00	0.00	0.36	0.13	0.22	0.00
	GH127	0.26	0.84	1.19	0.66	0.51	0.26	0.53	0.00	1.13	0.13	0.44	0.34
Oligosaccharide degrading	GH1	0.92	0.34	1.00	0.39	0.68	0.30	1.07	0.22	0.86	0.19	1.98	0.62
	GH2	10.73	8.71	10.87	4.73	9.25	2.92	10.27	5.54	10.60	7.07	9.74	4.65
	GH3	6.68	11.89	7.76	7.03	9.08	2.23	7.27	5.87	6.40	5.69	7.39	4.65
	GH13	3.14	5.70	4.47	3.61	3.42	1.21	5.47	2.83	4.73	3.81	6.66	2.74
	GH18	6.81	3.18	7.58	3.68	4.11	0.72	5.47	8.37	0.77	3.44	1.76	3.27
	GH20	0.52	0.50	1.00	0.39	0.68	0.10	0.87	0.33	1.04	0.31	1.83	0.28
	GH27	0.92	0.34	0.82	0.53	0.51	0	0.40	0.33	0.41	0.25	0.22	0.37
	GH29	0.79	1.01	2.37	1.12	1.03	0.16	2.94	0.11	0.81	0.38	1.90	0.39
	GH31	4.06	3.02	5.02	1.18	3.25	0.43	3.57	2.17	2.71	1.81	1.54	1.24
	GH32	0.65	0.67	0.91	0.72	0.51	0.03	0.93	0.76	0.90	0.19	1.61	0.48
	GH35	1.57	1.51	1.19	0.66	2.40	0.33	1.40	0.54	1.44	1.00	1.39	0.37
	GH38	0.39	2.35	0.00	0.00	0.00	1.31	0.27	0.33	0.36	3.56	0.00	0.06
	GH39	0.52	1.34	0.82	2.76	0.86	0.23	0.87	0.65	1.67	0.81	0.66	0.79
	GH43	4.45	6.53	7.95	6.89	8.22	2.89	8.14	5.33	8.97	6.82	4.32	5.78
	GH57	1.57	1.17	0.91	3.22	1.20	1.84	1.27	2.83	1.13	3.06	1.02	2.40
	GH92	2.49	2.68	2.47	2.23	1.20	0.43	1.67	0.54	2.21	0.69	2.42	1.30
	GH97	2.49	3.02	3.93	1.38	5.99	0.56	3.60	0.33	2.84	1.38	7.10	0.85
	GH130	0.26	0.34	1.00	0.46	0.86	0.13	1.60	0.00	0.45	0.25	0.37	0.08

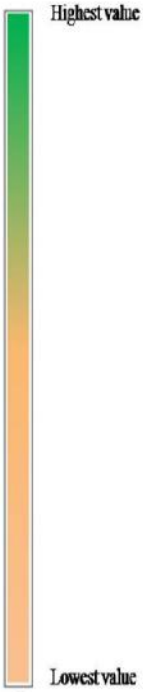


Fig.34 Glycosyl Hydrolase family distribution

GH family can be further classified based on the functional role of the enzymes such as degradation of oligosaccharide, cellulose, hemicelluloses, pectin and

other plant polymers. The contigs were found to comprise sequences related to a total of 52 different GH families amongst which majority included those playing role as oligosaccharide degrading enzymes (18 families) followed by debranching enzymes (8 families), cellulose degrading endo-hemicellulases (7 families) and cellulases (5 families), and cell wall elongation related enzymes (3 families) (Fig. 34). Out of these, oligosaccharide degrading GH2, GH3, GH18, and cellulase families GH5 and GH9 were highly represented. Majority of the GH families in oligosaccharide degradation were more abundant in the liquid and well represented in the J1 and J3 treatments as compared to the J2 treatment except GH3, GH43, GH35, GH39, GH97 and GH130 which had slightly higher proportion in J2GL. The results thus reflect that diet has an impact on the microbial diversity further resulting in variability of the CAZyme families present in the studied samples.

The findings revealed that dietary changes have a significant impact on rumen microbiome at taxonomic as well as functional levels. The information generated may allow us to modulate the rumen microbiome which would ultimately improve livestock nutrient utilization efficiency for better agricultural yield and have a global impact on environment by controlling the emission of methane gas.

Analysis of Surti rumen metagenome based on MG-RAST

Metagenomes of Surti shotgun for 48 samples were uploaded on MG RAST server. The Ion torrent shotgun sequencing of the all rumen metagenome samples together generated 11.319 GB data (Table 4).

Table 4 Metagenomic sequence details for 48 samples of Surti

Sample	Pre QC bp Count	Pre QC Read Count	Post QC bp Count	Post QC Read Count	rRNA genes	Predicted proteins	α-diversity - species
S1GL1	317,814,204	958,364	177,823,707	751,606	2,509	333,307	200.51
S1GL2	180,218,530	526,041	108,678,494	439,261	715	162,207	169.874
S1GL3	278,572,755	830,870	167,189,500	694,882	1,419	280,405	171.212
S1GL4	376,710,387	1,154,365	235,560,707	967,341	1,826	387,979	176.475
S1GS1	365,226,620	1,121,429	199,543,460	918,736	3,699	390,725	215.349
S1GS2	208,926,447	613,748	121,904,905	505,464	1,017	216,551	238.578
S1GS3	700,465,609	2,181,578	393,226,624	1,779,808	8,392	742,390	243.178
S1GS4	198,711,756	593,591	121,121,441	487,748	956	210,472	252.932
S1DL1	163,527,484	474,957	94,650,852	395,713	829	165,642	145.79
S1DL2	216,084,834	693,834	129,450,513	569,586	956	215,933	168.763
S1DL3	212,425,040	790,948	105,081,676	553,722	847	184,272	191.452
S1DL4	273,636,102	999,242	141,006,204	723,127	1,166	245,392	188.392
S1DS1	269,910,374	846,239	146,126,855	685,853	1,679	273,733	294.057
S1DS2	155,370,359	492,905	86,710,686	400,252	926	166,249	229.239
S1DS3	288,476,149	942,139	154,102,587	746,677	3,022	303,393	257.992
S1DS4	151,741,936	448,997	79,824,071	349,536	772	143,144	242.523
S2GL1	398,711,790	1,206,795	245,387,732	1,021,418	1,881	443,222	134.991
S2GL2	388,958,234	1,158,240	240,181,614	993,253	1,747	414,534	104.284
S2GL3	417,227,782	1,247,367	249,087,297	1,072,270	1,908	430,602	124.088
S2GL4	385,009,641	1,154,088	235,174,227	990,609	1,650	425,163	112.427
S2GS1	357,251,640	1,169,163	217,606,851	967,193	4,160	444,924	234.073
S2GS2	287,065,234	883,489	198,641,576	768,006	1,707	370,418	240.170
S2GS3	288,890,039	906,656	197,092,338	782,931	1,711	370,970	244.749
S2GS4	286,907,090	889,747	185,922,842	770,347	1,672	319,721	253.325
S2DL1	158,835,247	535,852	75,828,610	350,106	1,823	160,439	250.258
S2DL2	169,598,834	560,027	92,705,078	408,979	872	165,674	203.549
S2DL3	172,032,083	559,770	96,451,424	422,365	870	179,047	173.579
S2DL4	187,870,179	550,288	116,056,326	451,272	1,015	206,641	168.583
S2DS1	476,387,389	1,446,992	294,717,355	1,180,263	2,886	545,467	241.667
S2DS2	341,306,260	1,036,033	212,391,421	849,286	2,229	382,092	286.808
S2DS3	239,291,393	744,628	139,286,959	565,425	1,223	253,334	275.167
S2DS4	239,338,906	721,113	141,310,463	576,812	1,420	264,257	262.754
S3GL1	212,945,552	644,244	129,372,689	537,168	855	193,756	240.75
S3GL2	310,540,187	912,640	191,721,569	761,342	1,369	292,048	196.454
S3GL3	224,292,470	674,581	145,591,647	566,600	1,099	226,108	223.485

S3GL4	163,927,823	487,883	103,774,666	413,326	667	154,340	205.607
S3GS1	159,984,307	485,613	88,734,873	392,551	729	143,774	296.481
S3GS2	140,674,749	431,413	69,453,722	295,783	1,002	109,784	291.047
S3GS3	194,824,140	596,637	114,077,904	496,357	1,189	183,513	322.038
S3GS4	203,224,932	639,082	120,858,949	526,452	2,015	205,971	291.108
S3DL1	355,403,049	1,124,257	227,438,123	912,679	3,429	393,505	235.564
S3DL2	407,820,712	1,224,909	252,235,375	1,005,161	1,830	387,221	281.908
S3DL3	345,863,980	1,019,406	209,770,871	836,878	1,583	335,817	249.557
S3DL4	482,357,093	1,432,228	296,571,918	1,181,640	2,265	453,261	284.41
S3DS1	556,121,706	1,681,311	341,809,842	1,377,385	5,682	599,485	321.768
S3DS2	207,969,342	618,518	123,822,230	490,477	1,187	203,501	343.884
S3DS3	179,647,586	546,778	111,249,360	447,289	918	193,275	303.315
S3DS4	165,871,631	582,139	98,978,479	433,973	880	182,574	293.443

Taxonomic analysis

Abundant bacterial phyla were Bacteroidetes, Firmicutes, Proteobacteria, Fibrobacteres, Actinobacteria, respectively and among the archaea, the phylum Euryarchaeota was abundant in all the treatments. Other phyla Chlorobi, Cyanobacteria, Fusobacteria, Lentisphaerae, Spirochaetes and Verrucomicrobia were also higher.

In liquid fraction, Bacteroidetes were dominant as compare to solid fraction, and in solid fraction, Firmicutes were dominant. In statistical analysis total 46 different phyla were showing P<=0.05 significance difference in Solid/ Liquid fraction. Actinobacteria, Firmicutes, Fusobacteria, Spirochaetes were significantly higher in solid fraction of rumen samples. Bacteroidetes, Chlorobi, Cyanobacteria, Euryarchaeota were significantly higher in liquid fraction of rumen samples. In statistical analysis total 41 different phyla were showing P<=0.05 significance difference in S1/S2/S3 treatment. Bacteroidetes were significantly high in S1 treatment than S3 treatment, while Tenericutes were significantly high in S3 treatment than S1 treatment. Chordata and Fibrobacteres were significantly lower in S1 treatment. Proteobacteria and Spirochaetes were significantly high in S3 treatment.

The ratios of the phyla Firmicutes/Bacteroidetes were found to be higher in the solid fraction samples compared to the liquid fraction samples. The ratio was found to be increasing from S1 to S3 treatment in liquid fraction of dry roughage diet, and in solid fraction of dry and green diet. Whereas, in the liquid fraction of green roughage diet, ratio was found to be lower during S2 treatment, followed by S1 and S3.

At the genus level classification, *Prevotella*, *Bacteroides*, *Parabacteroides*, *Paludibacter*, *Porphyromonas*, *Alistipes* of Bacteroidetes phylum; *Clostridium*, *Ruminococcus*, *Eubacterium*, *Butyrivibrio*, *Bacillus*, *Blautia*, *Roseburia*, *Streptococcus*, *Lactobacillus* of Firmicutes phylum; *Fibrobacter* of Fibrobacteres phylum; *Slackia* of Actinobacteria phylum; *Treponema* of Spirochaetes phylum; and *Victivallis* of Lentisphaerae were found abundant.

Functional analysis

In S3 treatment, pathways related to protein metabolism; carbohydrates; RNA metabolism; respiration; and photosynthesis were higher whereas pathways related to DNA metabolism; nucleosides and nucleotides; stress response; virulence, disease and defense; phages, prophages, transposable elements, plasmids; motility and chemotaxis were lower as compared to S1 and S2 treatments. In S2 treatment, pathways related to amino acids and derivatives; cofactors, vitamins, prosthetic groups, pigments; cell division and cell cycle; fatty acids, lipids, and Isoprenoids; dormancy and sporulation; nitrogen metabolism; metabolism of aromatic compounds; potassium metabolism; phosphorus metabolism; and secondary metabolism were higher as compared to S1 and S3 treatments. In S1 treatment, pathways related to cell wall and capsule; and clustering- based subsystems were higher whereas pathways related to membrane transport; sulfur metabolism; iron acquisition and metabolism; and regulation and cell signaling were lower as compared to S2 and S3 treatments.

Taxonomic profile was differing significantly between different treatments and fraction of rumen, whereas type of roughage did not reflect major differences in rumen microbiome. Functional profile was differing significantly between different treatments, whereas type of roughage and fraction of rumen did not reflect major differences in rumen metagenomes. Present study indicates that treatment S1 (50% roughage 50% concentrate) was unbalanced diet whereas treatment S2 (75% roughage 25% concentrate) and S3 (100% roughage) diets provided a less selective environment for bacteria.

CAZymes analysis

We identified total 70033 contigs, encoding carbohydrate active enzymes including glycoside hydrolases (GH: 35639 contigs), glycosyl transferases (GT: 14173 contigs), carbohydrate binding molecules (CBM: 10591 contigs), carbohydrate esterase (CE: 7782 contigs), polysaccharide lyase (PL: 1209 contigs) and auxiliary activities (AA: 639 contigs).

Comparative analysis of Gir and Kankrej cattle and Mehsani buffalo metagenomes

M5NR database under MG-RAST pipeline was used to compare the metagenomes of Gir and Kankrej cattle and Mehsani buffalo. At Phylum level, *Bacteroidetes* (22.62%-42.61%) was found to be highly abundant followed by *Firmicutes* (21.83%-39.95%), *Proteobacteria* (9.96%-16.96%), *Actinobacteria* (2.11%-5.94%) and others (Fig.35). At species level, *Prevotellaruminicola* (1.76%-9.49%) was most abundant followed by *Bacteroides sp*. (1.98%-3.92%), *Bacteroides fragilis* (0.95%-1.76%), *Fibrobacter succinogenes* (0.42%-1.35%) and others (Fig.36).

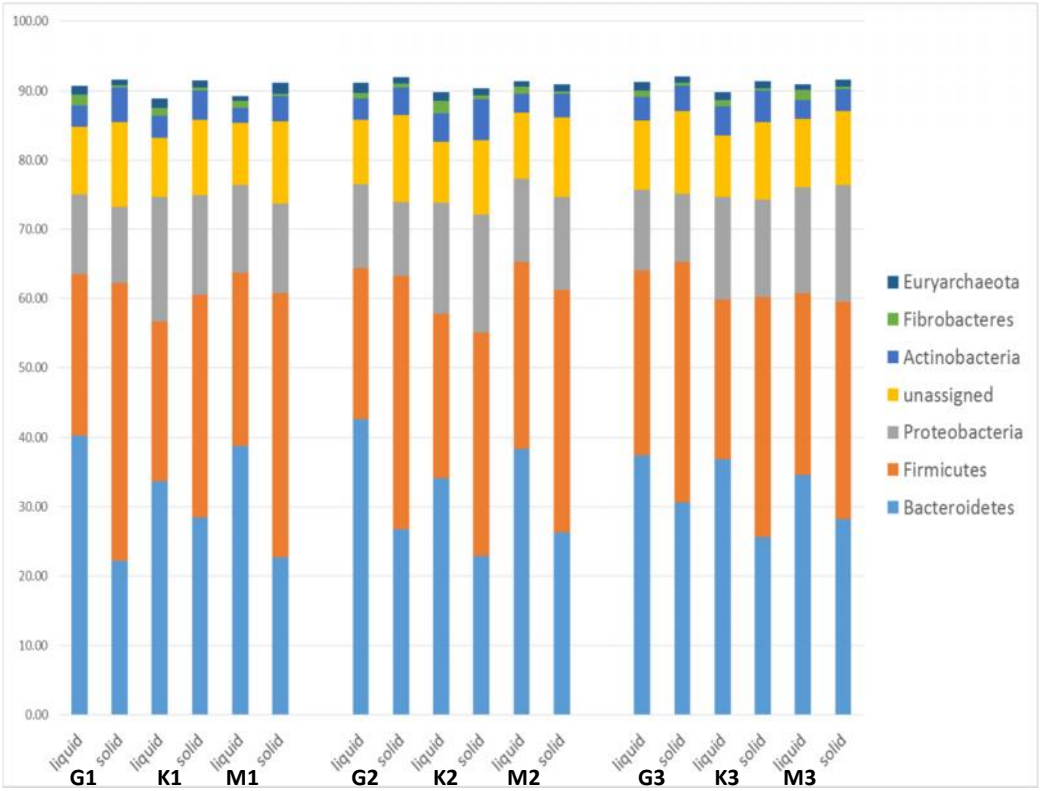


Fig.35 Phylum level taxonomic classification of Mehsani, Gir and Kankrej metagenomes

Effect of breed on metabolic pathways for methanogenesis

Methane production in rumen is a matter of great concern worldwide for its role as a greenhouse gas in the atmosphere, as well as the wastage of energy fed to the animals. EGTs involved in methane production were also studied among three different breeds (Fig.37). We found that, enzymes involved in methane production were showing more similar profiles between Kankrej cattle and Mehsani buffaloes compared to Gir cattle.

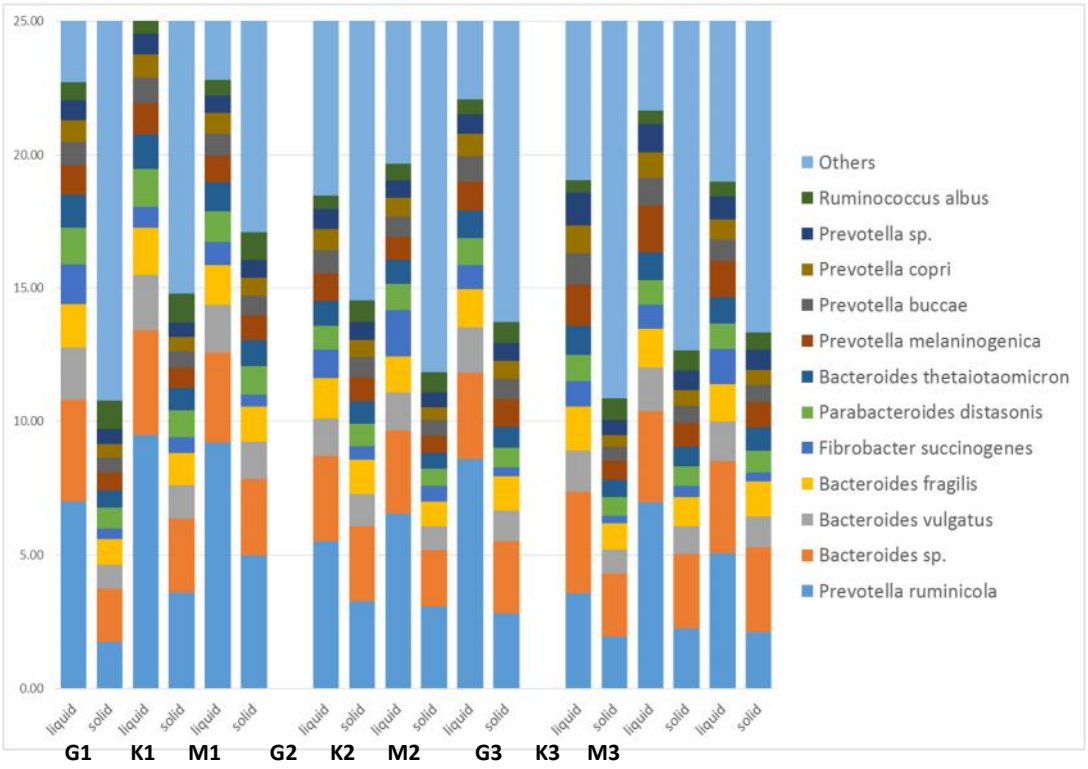


Fig.36 Species level taxonomic classification of Mehsani, Gir and Kankrej metagenomes

Effect of breeds on metabolic pathways for production of VFAs

Volatile fatty acids (VFA) such as propionic acid and butyric acid are produced in the rumen by microbial fermentation, which play a major role in milk and meat production as well as in maintaining the health of the animal. Environmental Gene Tag (EGTs) involved in VFAs production such as propionate and butyrate productions were studied during three different treatments (Fig. 38).

Presence of succinate pathway for propionate production was evident from experimental results. However, we were unable to retrieve any EGTs for methylmalonyl Co-A carboxytransferase that play a role in conversion of (S) methyl-malonyl-CoA into propanoyl CoA in Gir cattle and Mehsani buffalo. In butyrate synthesis pathway, conversion of crotonyl Co-A to butanoyl CoA was a rate limiting step for butyrate production.

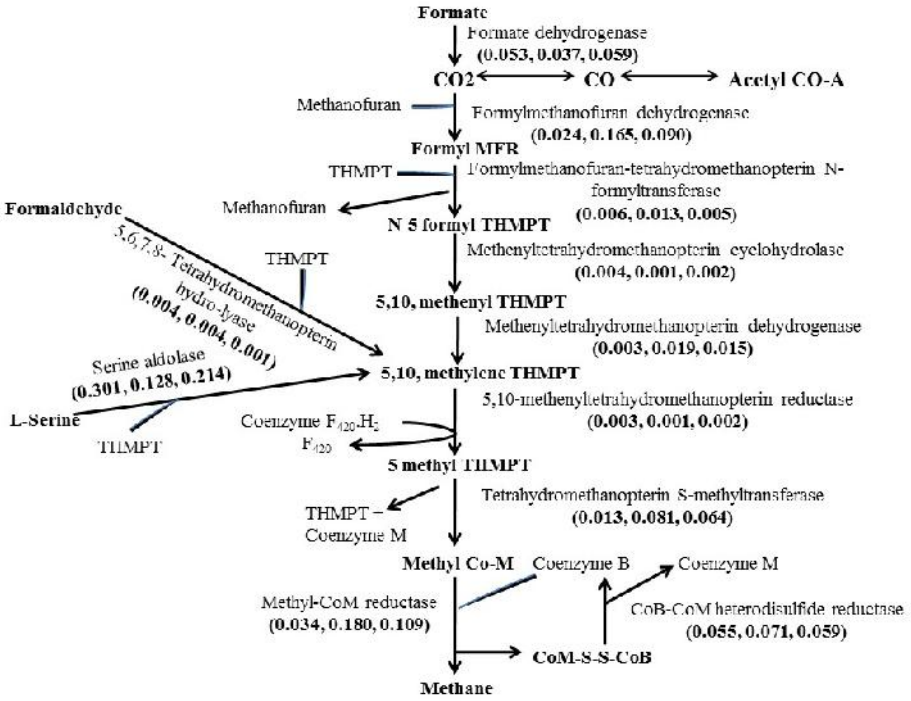


Fig.37 Schematics of Methanogenesis pathway. Numbers in bracket represent relative proportion of gene in Gir, Kankrej and Mehsani metagenome respectively.

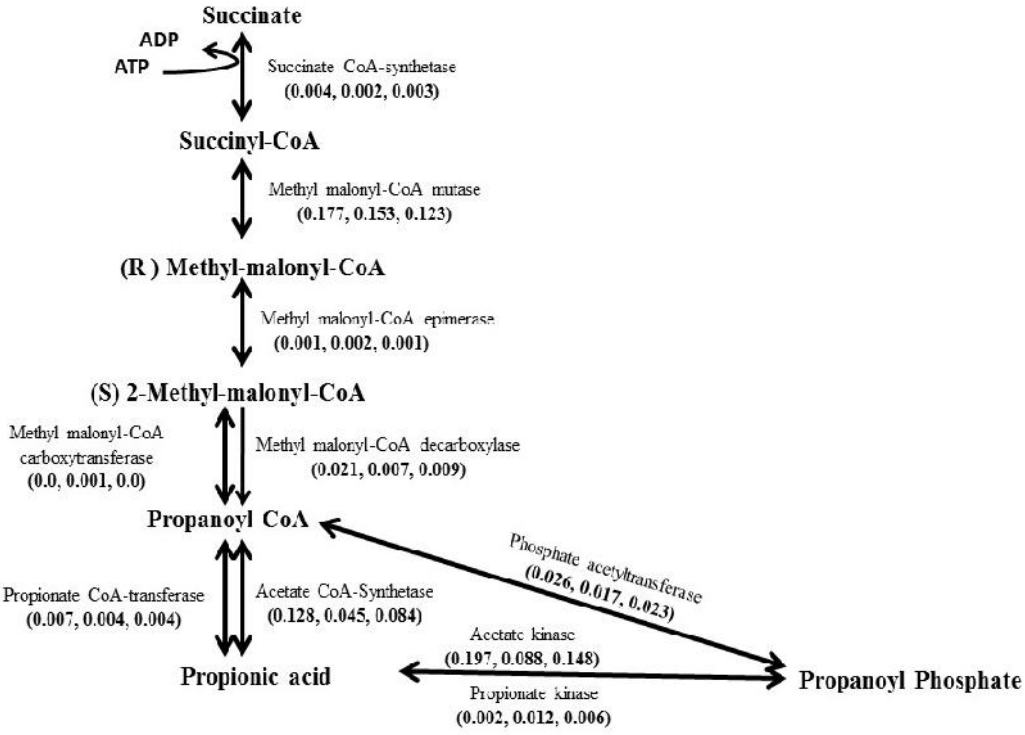


Fig. 38 Schematics of Propanoate synthesis pathway. Numbers in bracket represent relative proportion of gene in Gir, Kankrej and Mehsani metagenome respectively.

Analysis of resistome, stress and phages and prophages in Kankrej cattle to mine the relative abundance profile with respect to increasing fiber diet

The subsystems-based annotations (SEED) database (MG-RAST) was utilized to gain a better understanding of metabolic potential (content of EGTs) of these microbiomes. The subsystems are annotated based on biochemical pathways, fragments of pathways, gene clusters that function together, and any group of genes considered to be related. Looking to specific metabolic pathways, reads assignment with virulence, disease and defense were 4.14, 4.12 and 2.18 percent in K1 dry, K2 dry, K3 dry treatment, respectively. In the K1 green, K2 green and K3 green were 2.23, 4.12 and 2.24 percent, respectively. In metabolic pathway of stress response, reads assignments were 3.58, 3.67 and 1.84 percent in K1 dry, K2 dry, K3 dry treatment, respectively whereas in K1 green, K2 green and K3 green were 1.93, 3.70 and 1.81 percent, respectively. In metabolic pathway of Phages, Prophages, Transposable elements, Plasmids, reads assignments were 4.09, 4.10 and 2.73 per cent in K1dry, K2dry, K3dry treatment, respectively whereas in K1green, K2 green and K3 green were 1.75, 3.91 and 1.94 percent, respectively.

Assignment of virulence, disease and defense

Figure 39 and 40 shows the SEED subsystem assignment of virulence, disease and defense of Kankrej cattle dry and green roughage treated rumen microbiome (EGTs) profiles.

Table 5 SEED subsystem assignment of Virulence, Disease and Defense of Kankrej cattle rumen microbiome.

Virulence, Disease and Defense	K1D	K2D	K3D	K1G	K2G	K3G
-	10.15	8.06	16.26	21.77	8.43	17.24
Adhesion	5.55	4.73	2.60	3.09	4.79	2.84
Bacteriocins, ribosomally synthesized antibacterial peptides	0.60	0.38	0.12	0.23	0.38	0.13
Detection	3.57	4.04	12.46	10.48	3.68	11.50
Invasion and intracellular resistance	8.07	7.65	0.96	0.82	7.98	0.80
Resistance to antibiotics and toxic compounds	67.63	70.03	67.56	63.44	69.5	67.44
Toxins and super antigens	4.42	5.10	0.10	0.16	5.18	0.05
Grand Total	100	100	100	100	100	100

The distribution of resistance to antibiotic and toxic compounds (RATC) category was predominant with similar proportion in all roughage treatment. The statistical analysis of metagenomic profile (STAMP) analysis is described in table 5 and 6.

Table 6 SEED subsystem assignment of Stress response of Kankrej cattle rumen microbiome.

Stress response	K1Dry	K2Dry	K3Dry	K1Green	K2Green	K3Green
-	13.81	14.88	14.76	16.84	14.67	16.00
Acid stress	1.38	1.35	3.76	3.53	1.31	3.42
Cold shock	2.03	1.93	0.13	0.21	1.97	0.10
Dessication stress	1.49	1.32	0.10	0.10	1.23	0.12
Detoxification	6.29	5.84	1.50	1.96	5.66	1.60
Heat shock	13.02	12.66	41.47	37.47	11.92	40.33
Osmotic stress	11.46	10.74	4.00	4.39	11.23	4.05
Oxidative stress	47.20	47.97	30.92	31.69	48.63	31.00
Periplasmic Stress	3.33	3.30	3.36	3.81	3.38	3.38
Grand Total	100.00	100.00	100.00	100.00	100.00	100.00

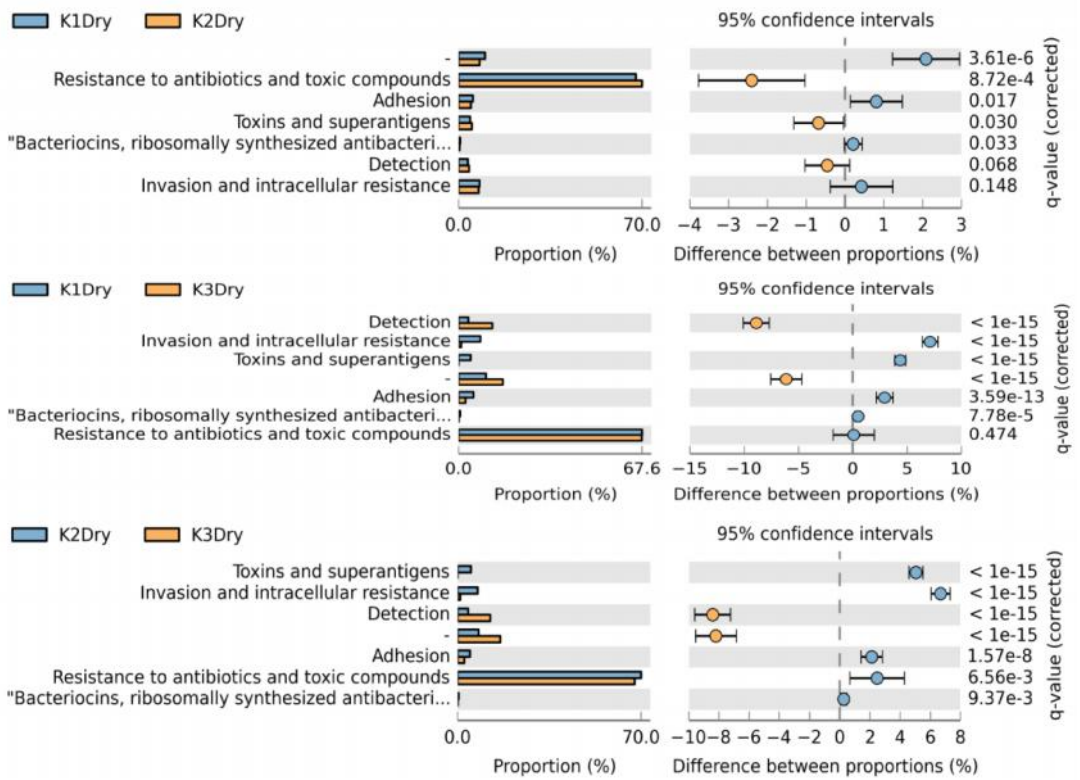


Fig.39 Statistical analysis of Virulence disease and defence with respect to the dry roughage (K1:K3 dry) treatment in Kankrej cattle

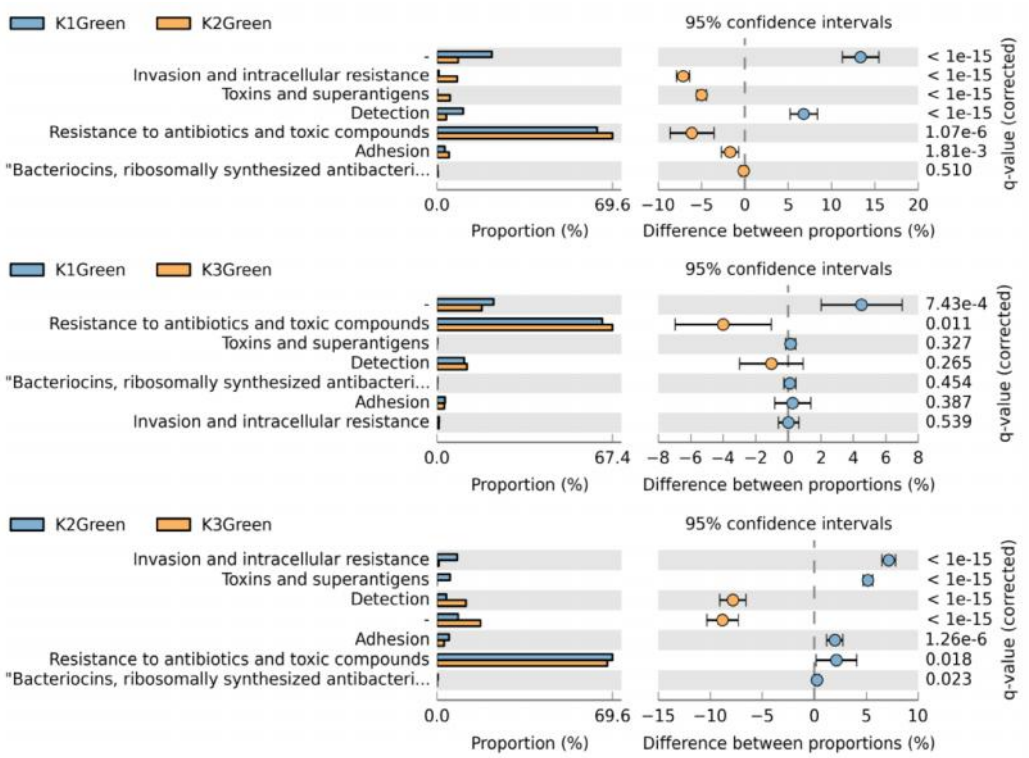


Fig.40 Statistical analysis of Virulence disease and defence with respect to the green roughage (K1:K3 green) treatment in Kankrej cattle

Assignment of Stress response

In the category of stress responses; heat shock, oxidative stress and detoxification were predominant in all the samples (K1 to K3) (Fig. 41&42).

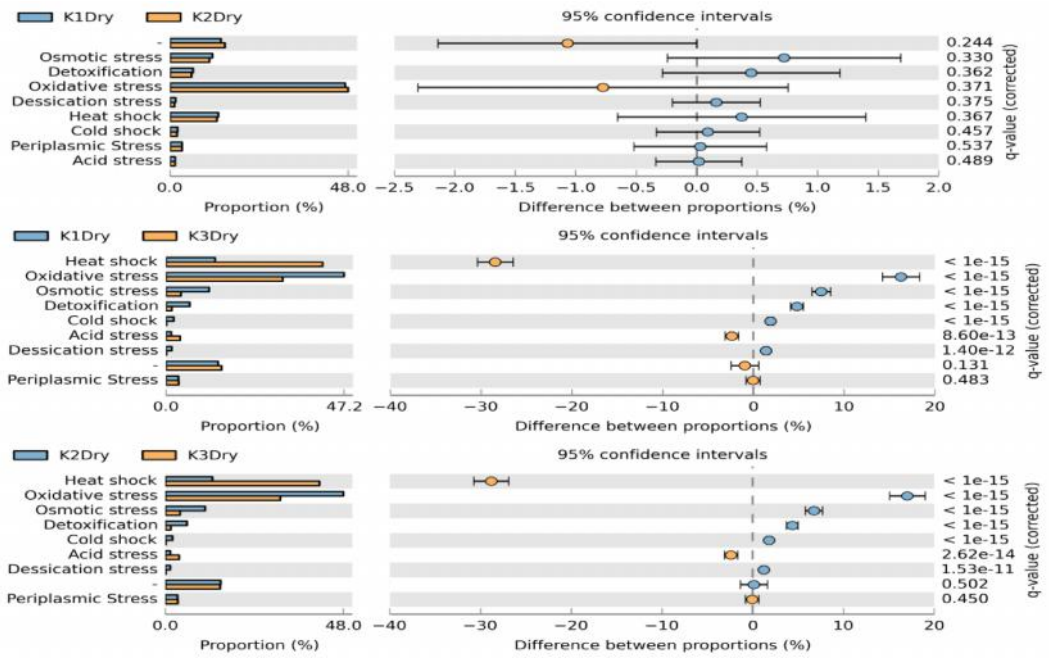


Fig. 41 Statistical analysis of stress response with respect to the dry roughage (K1:K3 dry) treatment in Kankrej cattle

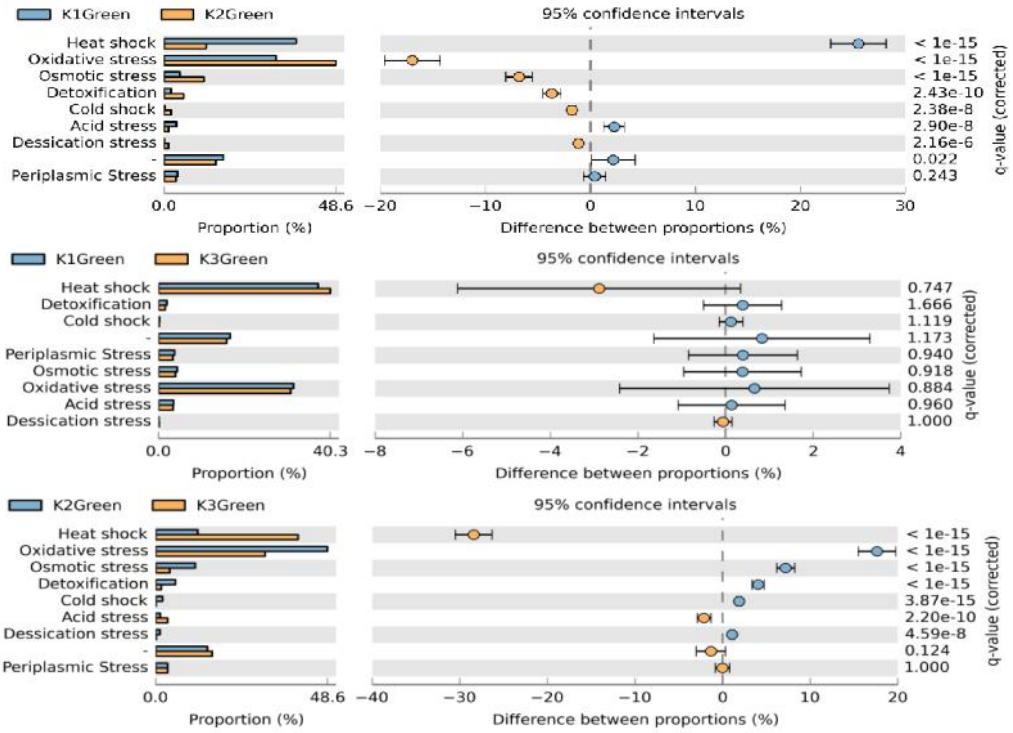


Fig. 42 Statistical analysis of stress response with respect to the green roughage (K1:K3 green) treatment in Kankrej cattle

Assignment of Phages, Prophages, Transposable elements, Plasmids

In the category of K1 vs K2 dry, gene transfer agents (GTA) (7.59%) dominated in the K1 dry while phages prophages (63.19%), pathogenicity islands (22.67%) in the K2 dry treatment. In the category of K1 vs K3, phages and prophages (78.36%) were predominant in the K3 dry while pathogenecity islands (21.20%) in the K1 dry. In the category of K2 vs K3 green, GTA (6.69%), pathogenicity islands (22.67%) in K2 dry while phages and prophages (78.36%) in K3 dry treatment were dominant with the statistical evidence (Fig. 43). In the category of K1 vs K2 green, phages and prophages (69.22%) in the K1 green while pathogenicity islands (22.10%) dominated in the K2 green treatment. In K1 vs K3 green, transposable elements (10.98%) in K1 green while phages and prophages (72.22%), transposable elements (7.96%) dominated in the K3 green treatment. In K1 vs K3 green, pathogenicity islands (22.10%), GTA (6.84%) in K2 green while phages and prophages (72.22%) and transposable elements (7.96%) were dominated in K3 green treatment with the statistical evidence ($p < 0.05$) (Fig. 44).

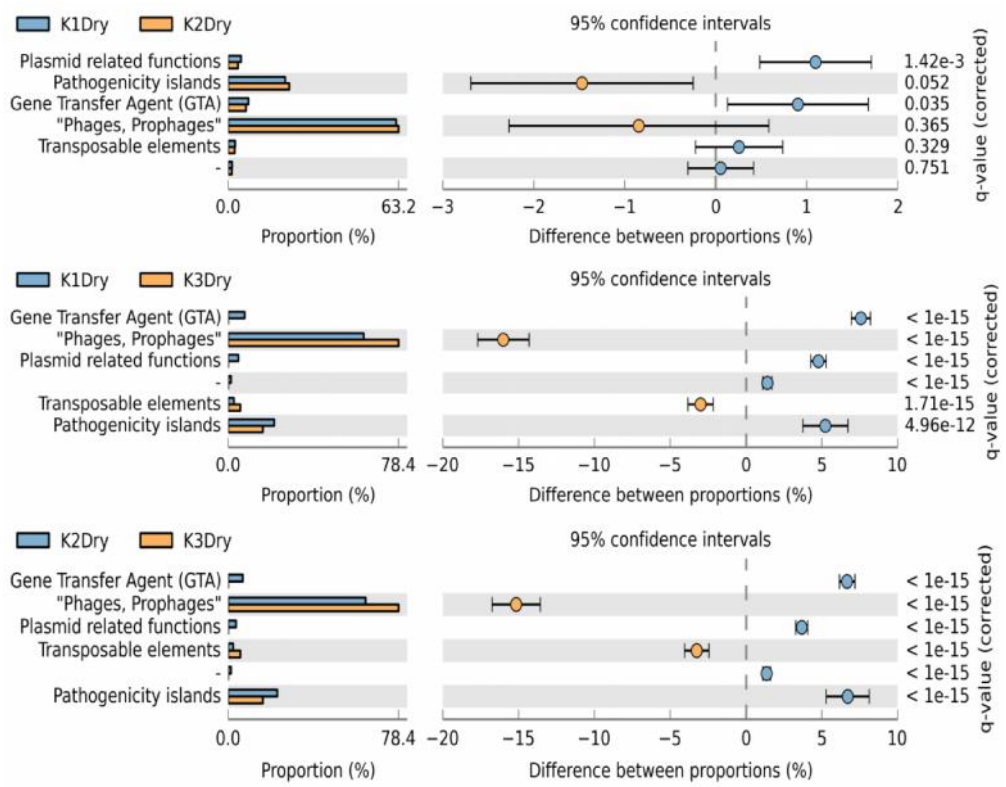


Fig. 43 Statistical analysis of Phages, Prophages, Transposable elements, Plasmids with respect to the dry roughage (K1:K3 dry) treatment in Kankrej cattle

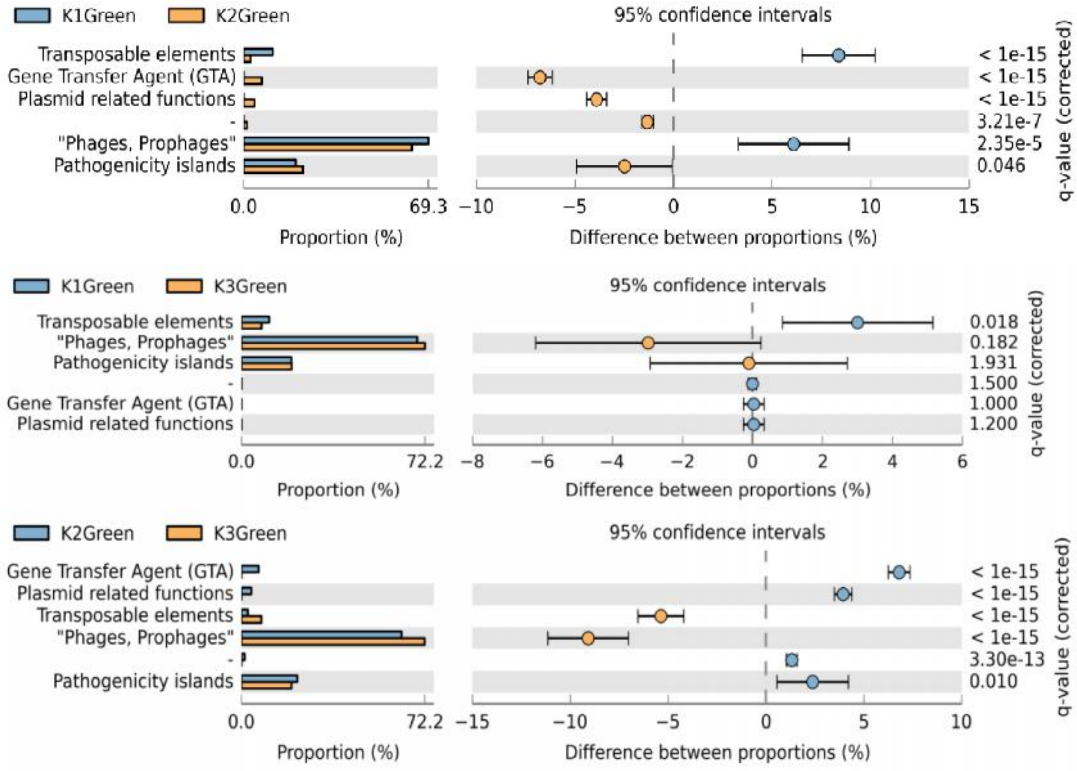


Fig.44 Statistical analysis of Phages, Prophages, Transposable elements, Plasmids with respect to the dry roughage (K1:K3 green) treatment in Kankrej cattle.

Metagenomic Analysis of Resistome, Stress Response and Phages and Prophages of the Gir cattle fed with different fiber diet content

Further into specific metabolic pathways, reads assignment with virulence, disease and defense were 2.19, 2.25 in G1 liquid and solid, 2.34, 2.34 in G2 liquid and solid, 2.31, 2.34 in G3 liquid and solid fraction, respectively. In the metabolic pathway of stress response, reads assignments were 1.81, 1.86 in G1 liquid and solid, 1.92, 1.93 in G2 liquid and solid, 1.93, 1.94 percent in G3 liquid and solid fraction, respectively. In metabolic pathway of Phages, Prophages, Transposable elements, Plasmids, reads assignments were 2.11, 1.97 in G1 liquid and solid, 1.68, 1.56 in G2 liquid and solid, 1.63, 1.67 in G3 liquid and solid fraction, respectively.

Assignment of virulence, disease and defense

The distribution of resistance to antibiotic and toxic compounds (RATC) category was predominant in the liquid over solid fractions whereas detection and adhesion was predominant in the solid fractions of all treatment with statistical evidence ($p<0.05$). In G2 liquid and solid fractions, RATC was predominant (68.55%) in G2 liquid and detection (11.61%), adhesion (3.61%) in G2 solid fraction. In the G3 liquid and Solid, RATC (67.14%) in G3 liquid and detection (11.81%) and adhesion (3.34%) in G3 solid fraction were predominant (Table 7).

Table 7 SEED subsystem assignment of Virulence, Disease and Defense of Gir cattle rumen microbiome

Virulence, Disease and Defense	G1L	G1S	G2L	G2S	G3L	G3S
-	17.52	14.37	18.94	18.09	18.44	19.41
Adhesion	2.53	3.55	2.53	3.61	2.95	3.34
Bacteriocins, ribosomally synthesized antibacterial peptides	0.13	0.22	0.14	0.27	0.18	0.22
Detection	9.43	13.21	9.31	11.61	10.37	11.81
Invasion and intracellular resistance	0.50	1.32	0.42	1.19	0.80	0.94
Resistance to antibiotics and toxic compounds	69.77	67.27	68.55	65.14	67.14	64.11
Toxins and super antigens	0.12	0.05	0.10	0.09	0.12	0.17
Grand Total	100	100	100	100	100	100

Assignment of stress response

In the category of stress responses; heat shock, oxidative stress, osmotic stress were predominant in solid fraction whereas acid stress and periplasmic stress were

predominant with statistical evidence ($p < 0.05$) in the liquid fraction of all the samples (G1 to G3) (Table 8).

Table 8 SEED subsystem assignment of Stress response of Gir cattle rumen microbiome

Stress response	G1Liquid	G1Solid	G2Liquid	G2Solid	G3Liquid	G3Solid
-	18.21	16.08	17.41	15.17	15.87	15.33
Acid stress	4.46	2.29	4.51	2.98	3.49	3.11
Cold shock	0.07	0.18	0.06	0.15	0.14	0.16
Dessication stress	0.05	0.20	0.02	0.10	0.06	0.11
Detoxification	1.50	1.86	1.66	1.87	1.72	1.68
Heat shock	39.15	40.64	39.62	39.82	39.96	40.42
Osmotic stress	3.47	4.86	3.43	5.15	4.15	4.55
Oxidative stress	28.62	30.83	28.58	31.31	30.73	31.66
Periplasmic Stress	4.46	3.05	4.70	3.45	3.89	2.98
Grand Total	100.00	100.00	100.00	100.00	100.00	100.00

Assignment of Phages, Prophages, Transposable elements, Plasmids

Pathogenicity island was predominant in the solid fraction (Fig.45, 46& 47) whereas phages prophages in liquid fraction of all treatments with statistical evidence ($p<0.05$) (Table 9).

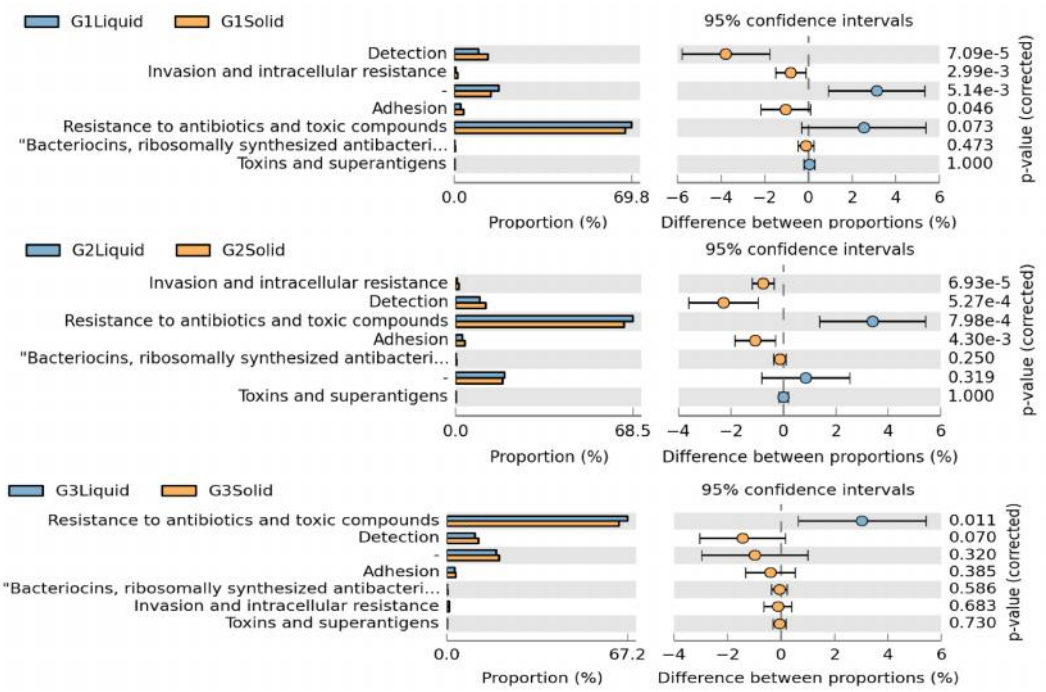


Fig.45 Statistical analysis of Virulence disease and defence with respect to the fraction of liquid and solid states (G1:G3 liquid and solid) samples in Gir cattle.

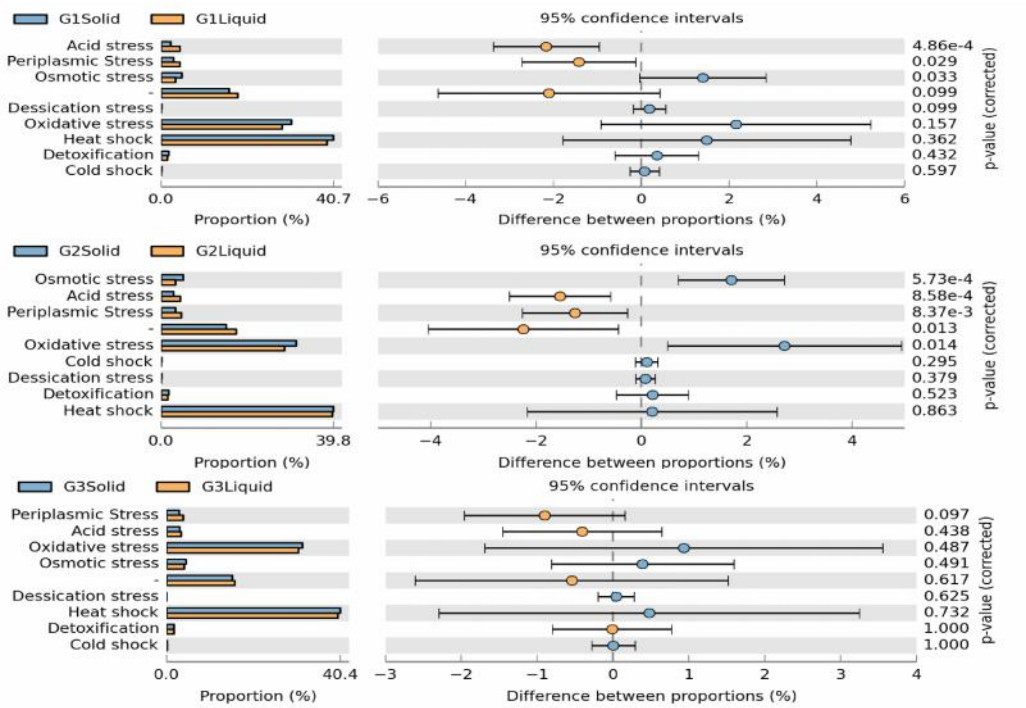


Fig. 46 Statistical analysis of Stress responsewith respect to the fraction of liquid and solid states (G1:G3 liquid and solid) samples in Gir cattle.

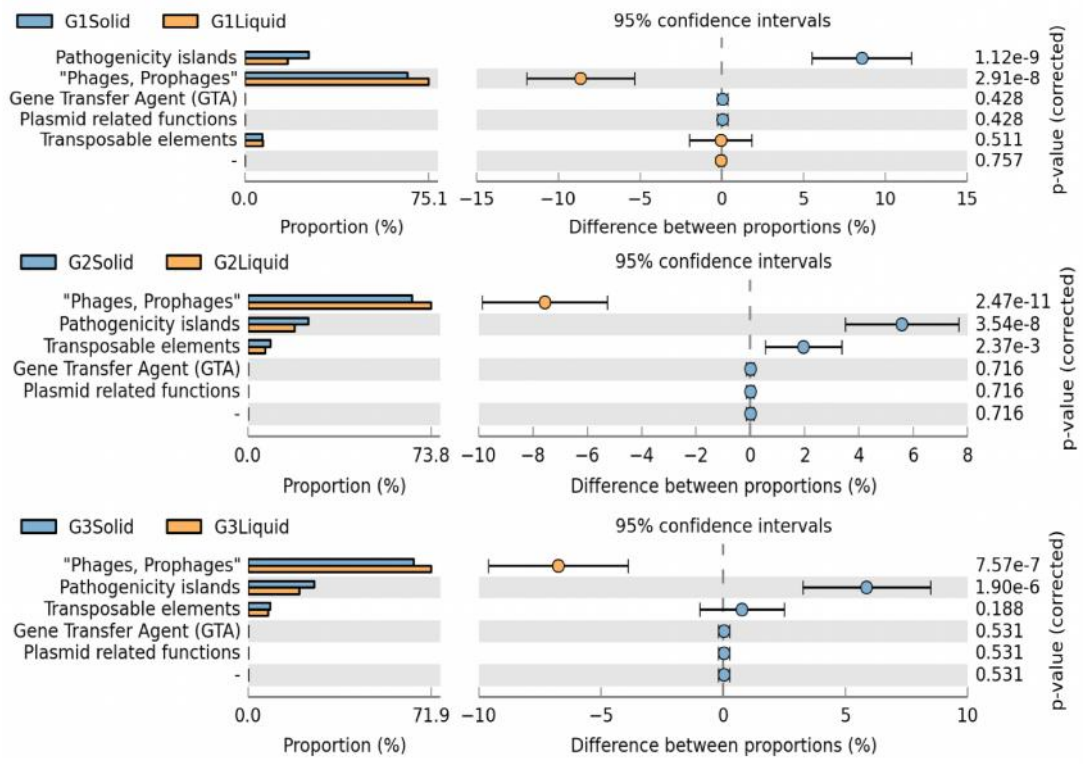


Fig.47 Statistical analysis of Phages, Prophages, Transposable elements, Plasmids with respect to the fraction of liquid and solid states (G1:G3 liquid and solid) samples in Gir cattle.

Table 9 SEED subsystem assignment of Phages, Prophages, Transposable elements, Plasmids of Gir cattle rumen microbiome

Phages, Prophages, Transposable elements, Plasmids	G1L	G1S	G2L	G2S	G3L	G3S
-	0.03	0.00	0.03	0.03	0.01	0.06
Gene Transfer Agent (GTA)	0.03	0.09	0.03	0.03	0.03	0.06
Pathogenicity islands	17.48	26.04	18.91	24.49	20.15	26.03
Phages, Prophages	75.15	66.52	73.86	66.30	71.91	65.14
Plasmid related functions	0.02	0.09	0.02	0.03	0.01	0.06
Transposable elements	7.29	7.25	7.16	9.11	7.89	8.64
Grand Total	100	100	100	100	100	100

The rumen metagenomic study revealed the presence of resistome and stress response genes in Kankrej and Gir cattle rumen. Among the virulence disease and defense, distribution of resistance to antibiotic and toxic compounds (RATC) category was predominant in all category of Kankrej and Gir cattle with statistical evidence ($p<0.05$). Among the stress responses category, heat shock, oxidative stress, osmotic stress predominant with statistical evidence ($p<0.05$). Among the phages, prophages, transposable elements, plasmids: Pathogenicity island was predominant with statistical evidence ($p<0.05$).

The present study generated the basic information for the understanding of complexity of the microbial ecology of the cattle rumen with special attention towards the resistome, Phages, Prophages, Transposable elements, Plasmids and stress responses.

Metatranscriptomics Sequencing:

Metatranscriptome analysis of Surti samples

Metatranscriptome sequencing of 48 Surti rumen samples resulted into a total data of 31 million raw reads (Table 10). The sequencing data of individual samples obtained after quality filtering ranged from 85 Mb to 339 Mb.

Table 10 Sequencing output details of 48 Surti samples

Sample	Sequence count	bp count
S1DL1	549315	109242479
S1DL2	598426	126306158
S1DL3	547725	104896936
S1DL4	618451	122475666
S1DS1	497866	90632475
S1DS2	511878	97160829
S1DS3	511934	87402885
S1DS4	628329	123512909
S1GL1	534512	109073094
S1GL2	617850	126830357
S1GL3	509914	104173281
S1GL4	1806034	338908719
S1GS1	609853	112507557
S1GS2	561525	100483456
S1GS3	888994	162961427
S1GS4	509782	84890446
S2DL1	600909	129922751
S2DL2	572247	120900452
S2DL3	538114	113040665
S2DL4	695057	146369556
S2DS1	618428	115738741
S2DS2	663970	125688514
S2DS3	661773	124124779
S2DS4	578859	110773927
S2GL1	537594	117196862
S2GL2	512703	115062407
S2GL3	508850	110405708
S2GL4	502695	108789661
S2GS1	499851	96300154
S2GS2	663066	130620085
S2GS3	621879	119747086
S2GS4	503198	96831067
S3DL1	939520	206657225
S3DL2	696496	163131023
S3DL3	936497	208044001
S3DL4	932832	211080374
S3DS1	505444	108028873

S3DS2	498450	92968924
S3DS3	500580	96707521
S3DS4	606045	110147856
S3GL1	853060	188273815
S3GL2	586225	117697912
S3GL3	494861	111740103
S3GL4	977065	215201194
S3GS1	664566	112988803
S3GS2	610644	106611460
S3GS3	834622	150130448
S3GS4	573530	97960506

Analysis of Mehsani Metatranscriptomic data

Raw reads from all the samples were uploaded to the MG-RAST (Metagenomic Rapid Annotations using Subsystems Technology v3.4) server (http://*metagenomics.anl.gov/*) for annotation. All the reads were passed through quality filters and filtered for host sequences (*Bos taurus*) within MG-RAST and reads classified under bacterial domain were further analyzed using the workbench feature in MG-RAST. Taxonomic classification was carried out using M5NR database with maximum e-value of 1e-5 and minimum identity of 60%. The KEGG Orthology database was used with maximum e-value of 1e-5 and a minimum identity of 60% for functional classification. Statistical Analysis of Metagenomic Profiles (STAMP) v2.0.9 software package was employed to test for significant differences between rumen microbiome samples. Statistically significant differences ($P < 0.05$) between multiple groups were assessed using the ANOVA test with Benjamini-Hochberg FDR multiple test correction within STAMP software.

Taxonomic annotation

Principle Component Analysis plot (PCA plot) revealed clear cut demarcation between samples of liquid and solid fractions at both phylum level and genus level (Fig. 48). However, samples were not segregating differently when grouped on the basis of type of diet (dry and green) or on the basis of treatments (proportion of roughage in ration).

Further, *Bacteroidetes* was found to be the most dominating phyla across all the samples (38.51% - 74.80%) (Fig. 49 a & b). *Bacteroidetes* along with *Firmicutes* (11.70% - 34.52%), *Proteobacteria* (4.86% - 13.60%), *Actinobacteria* (1.10% - 5.33%) and *Spirochaetes* (0.23% - 1.34%) represented the top five abundant phylum. *Bacteroidetes* was found comparatively higher ($P<6.44e-14$) in liquid fraction than solid samples whereas, reverse was observed for *Firmicutes* ($P<8.85e-14$), *Fibrobacteres* ($P<0.336$), *Proteobacteria* ($P<0.027$) and *Actinobacteria* ($P<0.0017$).

To further resolve the microbial abundance, classification was done at genus level wherein different profiles for liquid and solid fraction was observed. Amongst liquid fractions, *Prevotella* (22.14% - 34.53%) and *Bacteroides* (25.49% - 32.56%) were the most abundant genus followed by *Parabacteroides* (3.02%-3.78%), *Clostridium* (1.81% - 2.79%), *Poryphyromonas* (0.65% - 1.70%) and others.

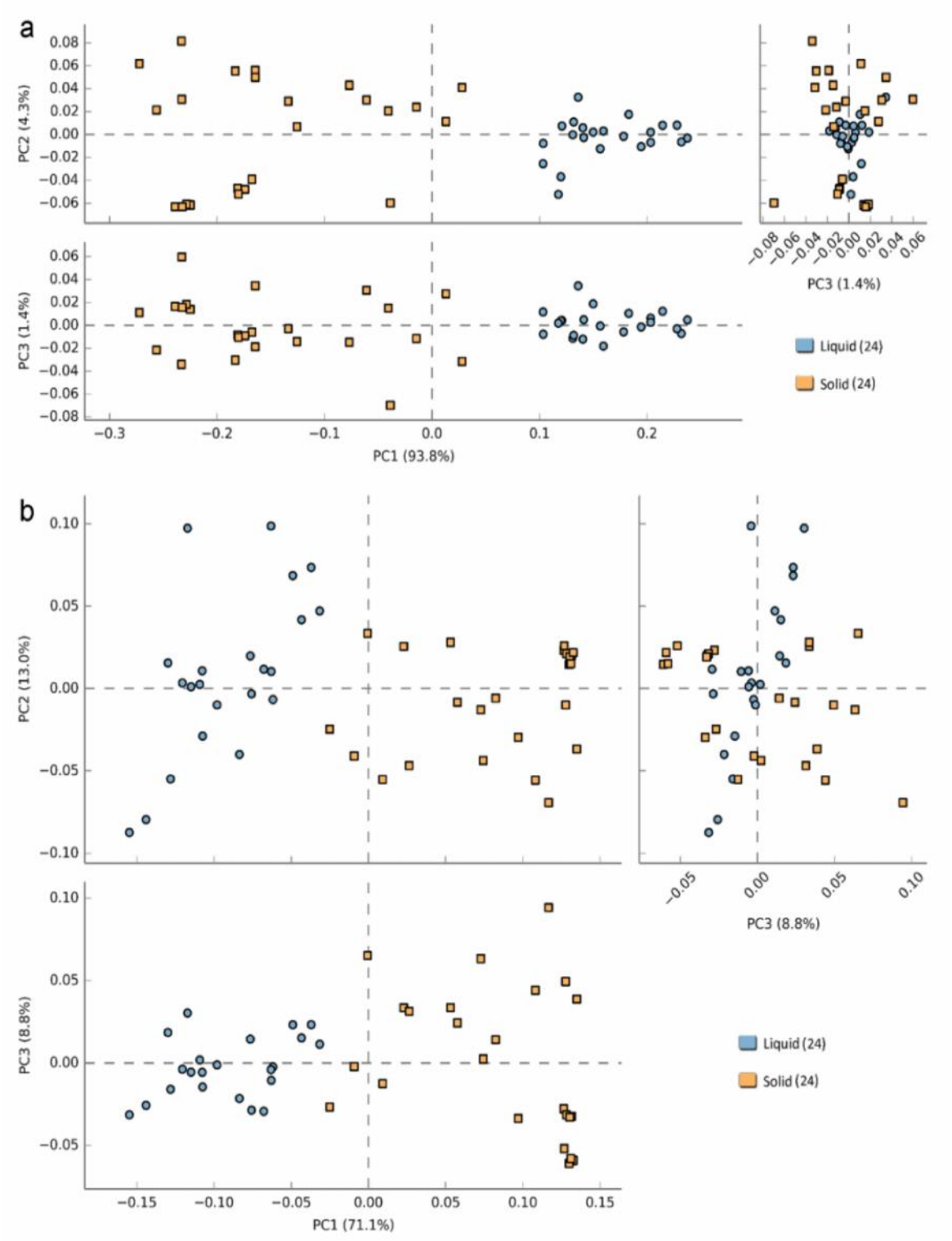


Fig. 48 Principle Component Analysis showing clear demarcation between liquid and solid fraction samples at a) Phylum level and b) Genus level

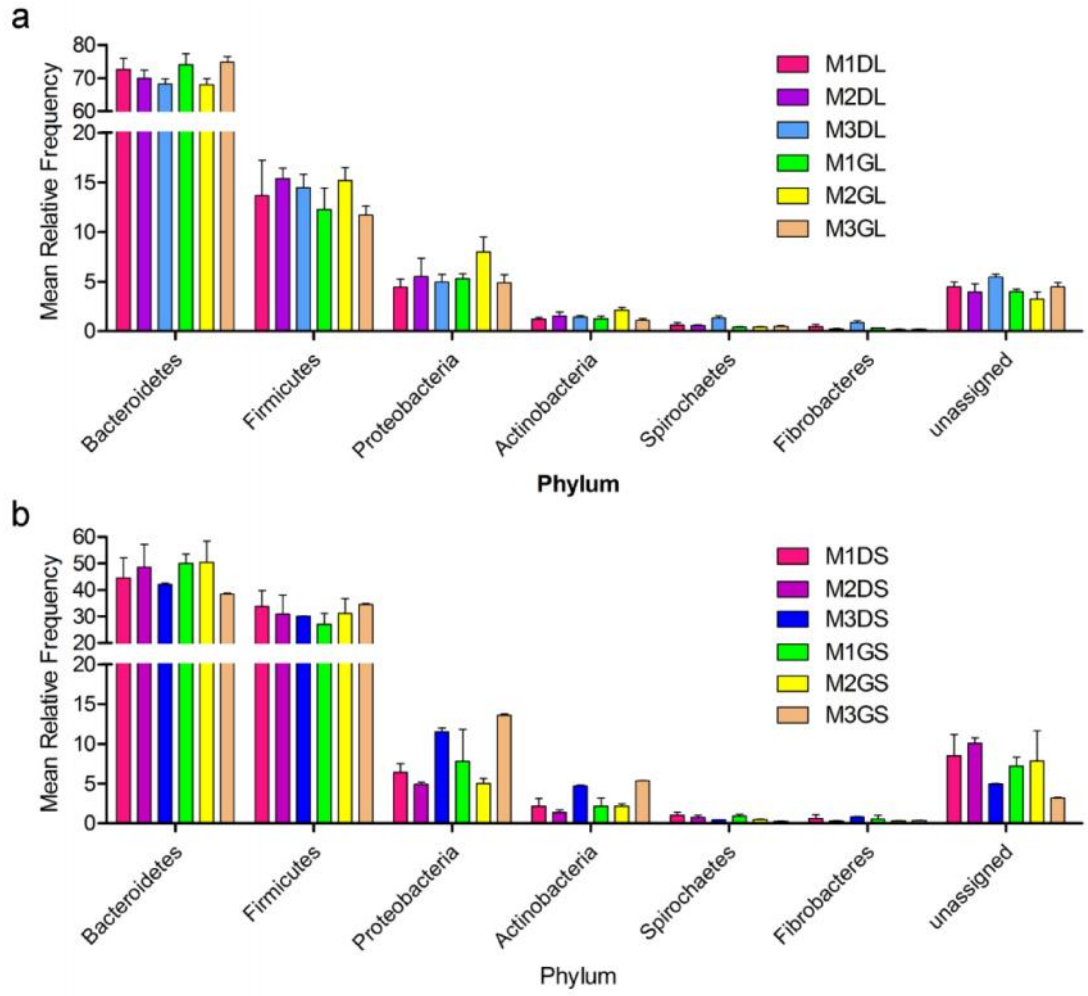


Fig. 49 Taxonomic classification at Phylum level using M5NR database in MG-RAST among samples of a) Liquid fraction and b) Solid fraction

While in solid fraction, highly abundant genus includes *Prevotella* (12.63% - 23.90%), *Bacteroides* (15.99% - 23.39%), *Clostridium* (4.80% - 6.22%), *Butyrivibrio* (0.56% - 4.59%), *Ruminococcus* (1.36% - 4.30%) and others (Fig.50).

Bacteroides was abundant in liquid and solid fractions of M3D, M3G and M2D while *Prevotella* was abundant in liquid and solid fractions of M1D, M1G and M2G. *Prevotella* (P<4.45e-7), *Bacteroides* (P<3.2e-8), *Parabacteroides* (P<0.113) and *Poryphyromonas* (P<0.055), all belonging to *Bacteroidetes* phylum, had higher abundance in liquid fraction as compared to solid fraction and vice versa in case of *Clostridium* (P<2.78e-8), *Ruminococcus* (P<6.87e-5) and *Butyrivibrio* (P<0.006), all belonging to *Firmicutes* phylum. These results clearly showed the comparatively higher abundance of genera from *Firmicutes* and *Bacteroidetes* phyla in solid and

liquid fractions, respectively. Still, up to 5% and 10% reads remained unassigned in liquid and solid fractions respectively.

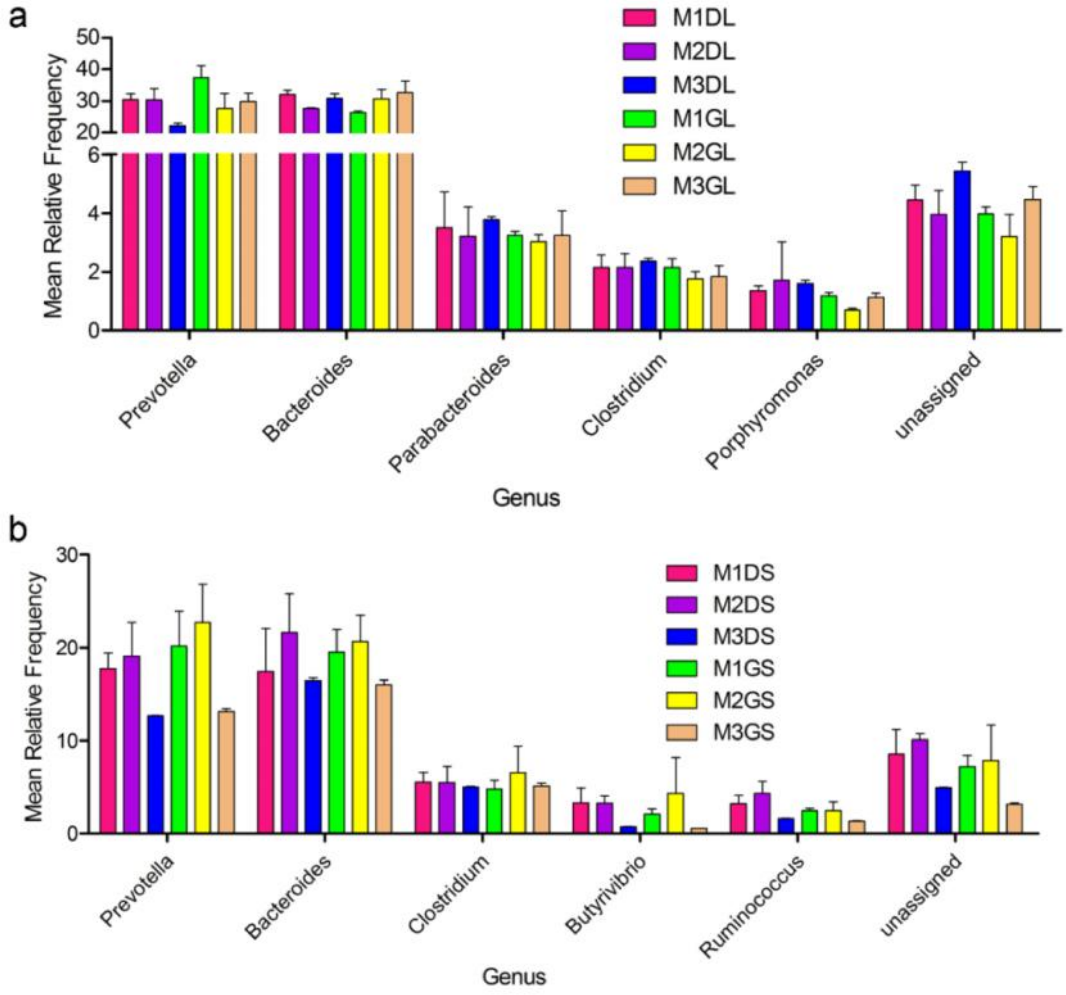


Fig. 50 Taxonomic classification at genus level using M5NR database in MG-RAST among samples of a) Liquid fraction and b) Solid fraction

Functional annotation

For functional annotation KEGG Orthology, a hierarchical database for functional annotation under the MG-RAST pipeline was used followed by analysis in STAMP. At level 1, database has six categories. All samples showed highest abundance of Metabolism category (43.05% - 51.55%) followed by Genetic information processing (30.62% - 38.81%), Environmental information processing (7.03% - 11.26%), Organismal systems (3.03% - 7.88%), Cellular processes (2.46% - 9.33%) and Human diseases (0.51% - 1.04%) (Fig. 51 a & b). When combined Metabolism and Genetic information processing categories represented about 80% of total abundance.

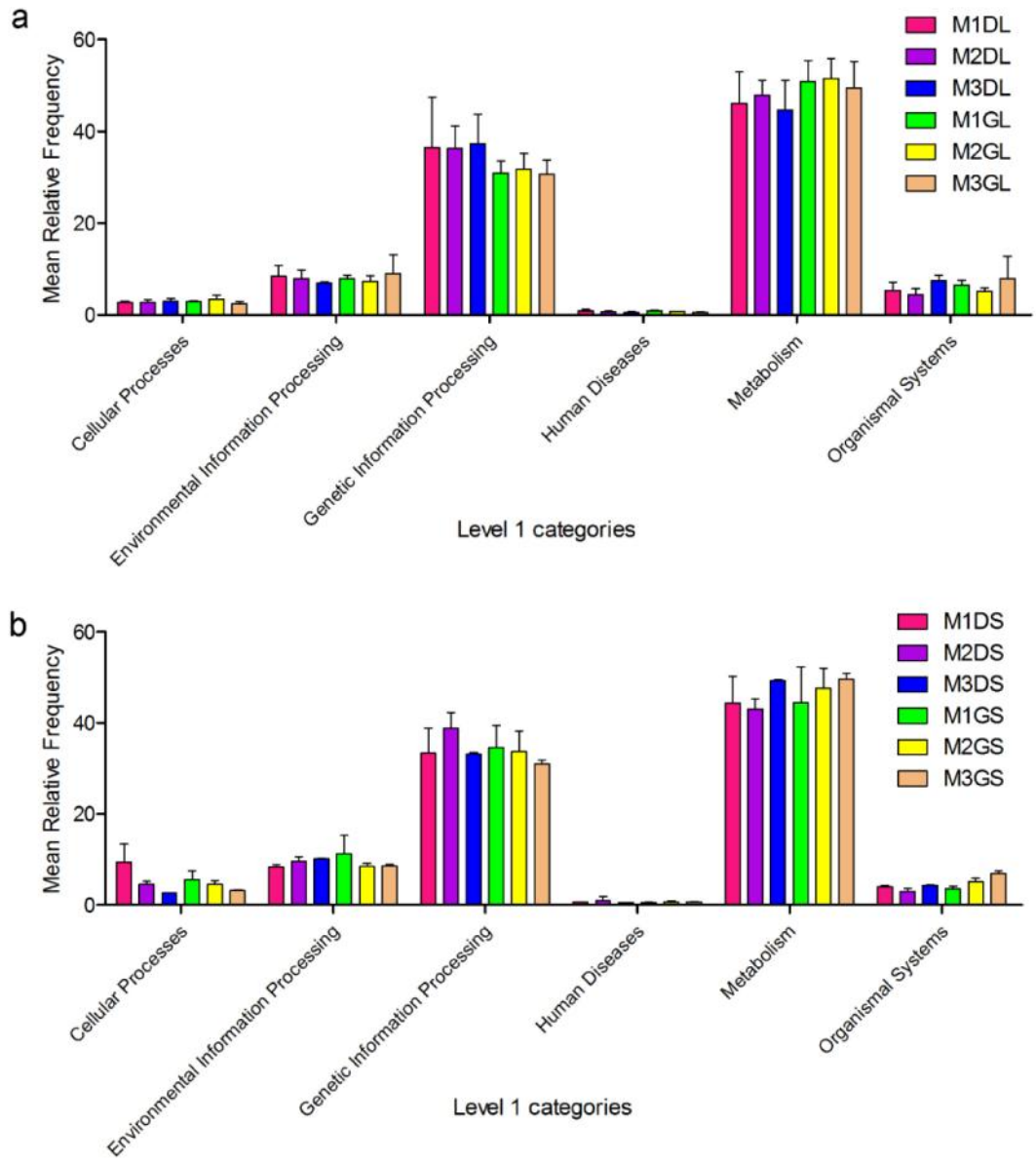


Fig. 51 Functional profile of all samples based on Level 1 categories of KO database among samples of a) Liquid fraction and b) Solid fraction

At level 2, Translation (19.36% - 33.02%) was the most abundant category across all the samples followed by Carbohydrate metabolism (13.39% – 20.82%) and Amino acid metabolism (10.17% – 16.68%) (Table 11). On an average, Metabolism category covered around 46% of all functionally annotated reads at level 1 while, 30% of all annotated reads were shared among Carbohydrate metabolism and Amino acids metabolism at level 2. Within Metabolism category, Carbohydrate metabolism was the most abundant followed by Amino acid metabolism, Energy metabolism and Nucleotide metabolism while, Xenobiotics biodegradation and metabolism category

was the least annotated preceded by Metabolism of other amino acids; Metabolism of terpenoids and polyketides; and Glycan biosynthesis and metabolism.

Among three treatments, Carbohydrate metabolism (P<0.002) showed higher abundance in first treatment followed by second and third treatments (Fig. 52). Metabolism of terpenoids and polyketides; Metabolism of cofactors and vitamins; and Lipid metabolism showed higher abundance in second treatment. While, all other categories showed highest abundance in third treatment.

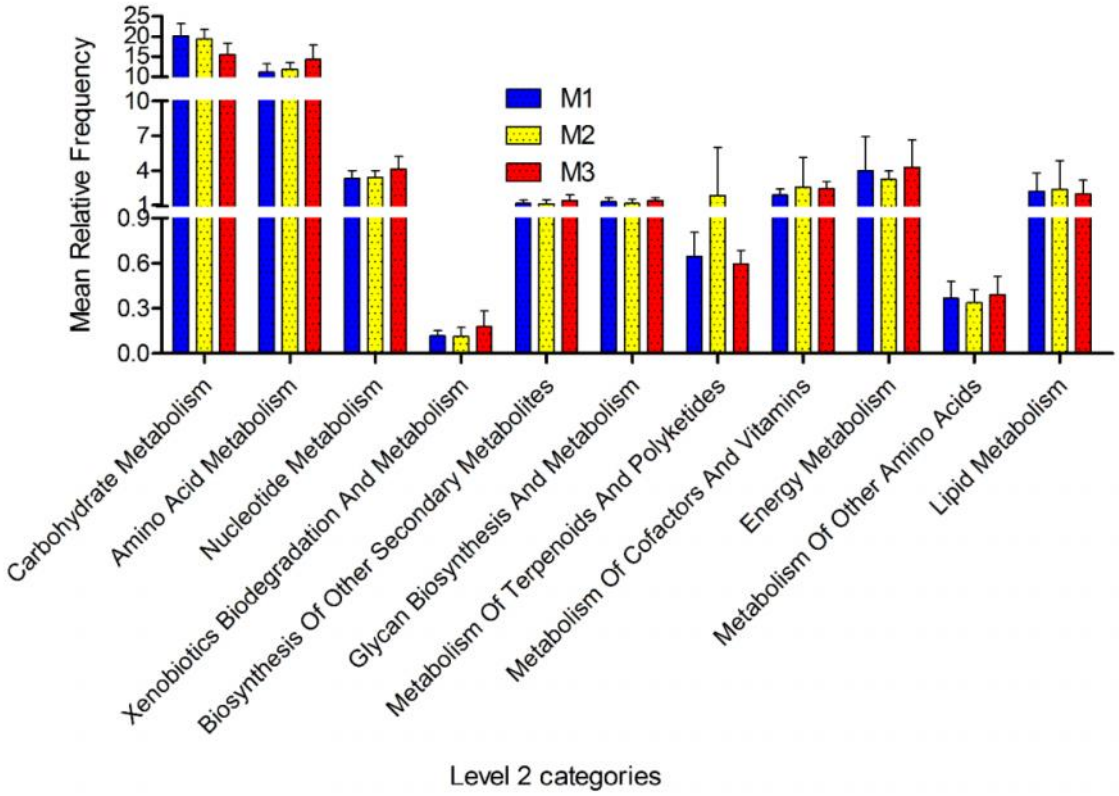


Fig. 52 Functional profile of Level 2 categories of Metabolism using KO database among three treatment

Table 11 Treatment wise distribution of Level 2 categories as annotated using KEGG Orthology database

level 1	level 2	p-values	p-values (corrected)	M1 (%)	M2 (%)	M3 (%)
Cellular Processes	Cell Communication	0.284451	0.533344878	0.002949	0.001698	0.004317
Cellular Processes	Cell Growth And Death	0.226704	0.453407077	1.229372	1.270724	1.393415
Cellular Processes	Cell Motility	0.006506	0.039038265	3.512329	2.016485	0.764511
Cellular Processes	Transport And Catabolism	0.174815	0.374602883	0.548366	0.545553	0.644639
Environmental Information Processing	Membrane Transport	0.65469	0.72743325	5.968228	5.414905	5.37128
Environmental Information Processing	Signal Transduction	0.836382	0.865223126	3.089042	2.987992	3.319516
Genetic Information Processing	Folding, Sorting And Degradation	0.002638	0.039571992	2.467632	2.612794	3.448639
Genetic Information Processing	Replication And Repair	0.004384	0.043838234	2.255348	2.334692	3.077665
Genetic Information Processing	Transcription	0.121009	0.330025521	3.838498	4.937974	5.236351
Genetic Information Processing	Translation	0.531798	0.66474727	25.45435	25.4872	23.07916
Human Diseases	Cancers	0.548046	0.65765521	0.075846	0.063879	0.062768
Human Diseases	Endocrine And Metabolic Diseases	0.363928	0.574622578	0	0.000248	0
Human Diseases	Immune Diseases	0.732585	0.784912217	0.005427	0.005557	0.007226
Human Diseases	Infectious Diseases	0.403391	0.605087179	0.644499	0.674125	0.515848
Human Diseases	Substance Dependence	0.162503	0.406256646	0	0	0.000157
Metabolism	Amino Acid Metabolism	0.005681	0.042606352	11.15052	11.81507	14.25939
Metabolism	Biosynthesis Of Other Secondary Metabolites	0.08401	0.252030244	1.140793	1.106902	1.397046
Metabolism	Carbohydrate Metabolism	6.87E-05	0.002061595	20.15326	19.37261	15.48409
Metabolism	Energy Metabolism	0.440605	0.600824831	3.967721	3.231128	4.26821
Metabolism	Glycan Biosynthesis And Metabolism	0.173081	0.399417611	1.281135	1.153815	1.376539
Metabolism	Lipid Metabolism	0.850951	0.850950539	2.190537	2.357306	1.974615
Metabolism	Metabolism Of Cofactors And Vitamins	0.458523	0.598073591	1.856979	2.544011	2.408447
Metabolism	Metabolism Of Other Amino Acids	0.427882	0.611260607	0.366006	0.338538	0.390991
Metabolism	Metabolism Of Terpenoids And Polyketides	0.314949	0.55579204	0.643846	1.814504	0.596153
Metabolism	Nucleotide Metabolism	0.025167	0.107859475	3.305628	3.414456	4.105035
Metabolism	Xenobiotics Biodegradation And Metabolism	0.03326	0.124725685	0.117011	0.110606	0.17754
Organismal Systems	Circulatory System	0.586523	0.676756777	0.000399	0.000466	0
Organismal Systems	Digestive System	0.349666	0.582777033	0.093623	0.114381	0.110832
Organismal Systems	Endocrine System	0.047898	0.159660491	0.057553	0.053556	0.083289
Organismal Systems	Environmental Adaptation	0.011332	0.056658479	4.583097	4.21882	6.442328

Organisms playing role in different functional categories were studied using the workbench feature of MG-RAST. All the reads corresponding to total functional annotation, Metabolism category and Carbohydrate metabolism category were annotated taxonomically using M5NR database in MG-RAST. In all the samples *Bacteroidetes* showed the highest activity followed by *Firmicutes*, *Proteobacteria*, *Spirochaetes* and *Actinobacteria* (Fig. 53a & b). However, difference was observed between the solid and liquid fraction samples. Trends similar to those in overall taxonomic annotation were observed in all the samples. In all the samples, higher proportion of *Actinobacteria* and *Proteobacteria* were involved in Metabolism category from all functional categories while, proportion of Carbohydrate metabolism category from Metabolism category was higher in *Proteobacteria* and *Firmicutes*.

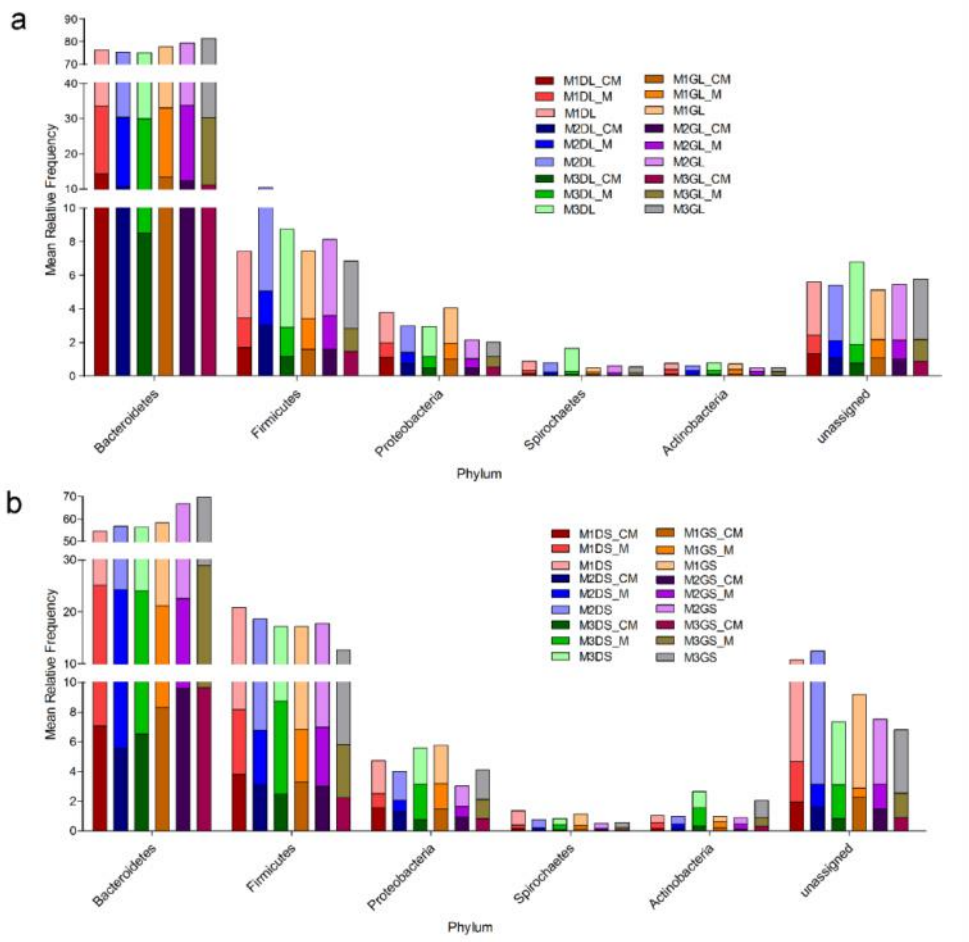
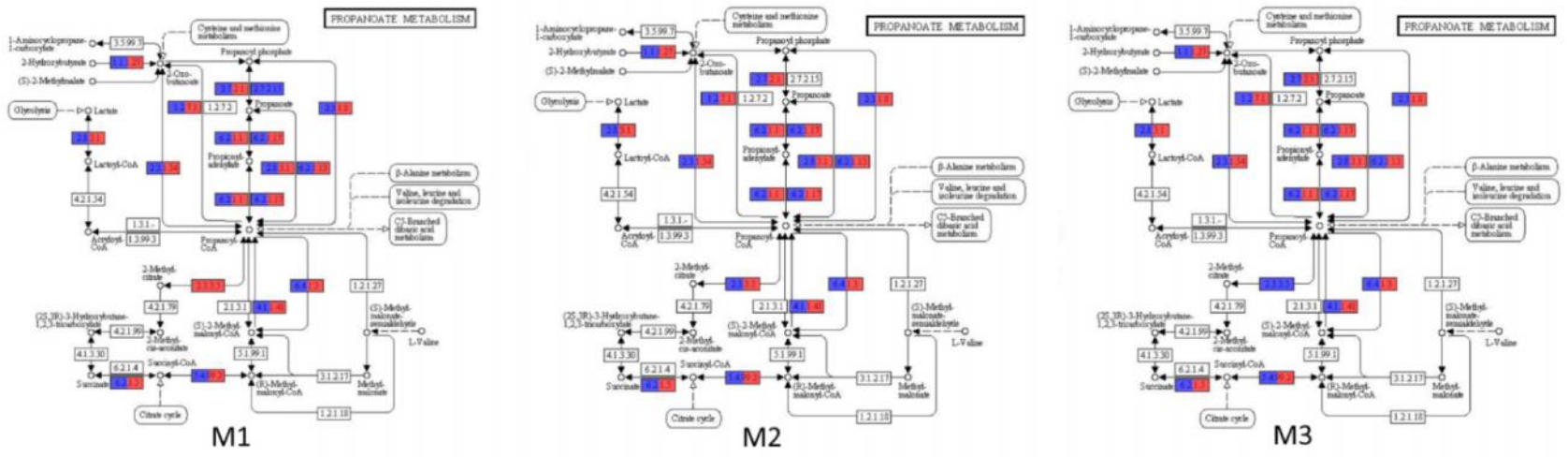


Fig. 53 Taxonomic annotations of reads annotated as all functional categories, Metabolism category and Carbohydrate metabolism category in samples of a.) Liquid fraction and b.) Solid fraction. The samples with suffix CM and M denote Carbohydrate metabolism category and Metabolism category respectively while, those without suffix denote other functional categories. The shown graphs are overlapped bar graph

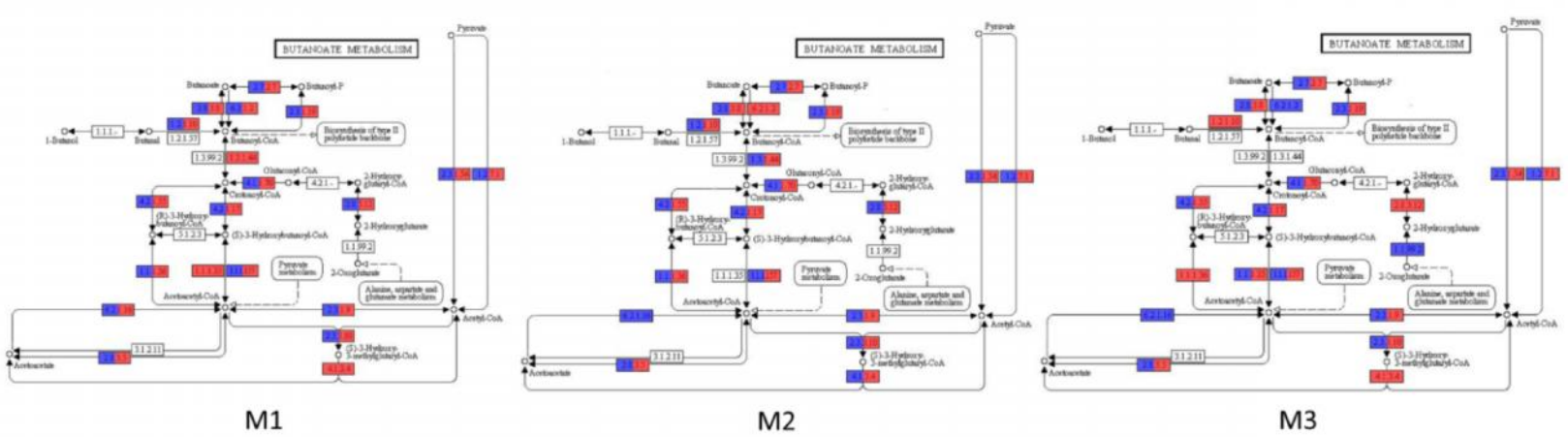
VFA production pathways

The genes involved in production of Propanoate and Butanoate were analysed using KEGG mapper option in MG-RAST as well as by using their mean relative frequency (Fig. 54 & 55). For Propanoate production, succinate pathway was highlighted very well. However, enzyme required for epimerization of (R)-Methyl malonyl CoA into (S)-Methyl malonyl CoA was absent in all the samples. In case of Butanoate production pathway, enzyme responsible for conversion of Crotonyl-CoA, a major intermediate of the pathway, into Butanoyl-CoA was absent in M3 treatment and M1 liquid sample. Apart from that, enzyme for epimerization of (R)-3-Hydroxybutanoyl-CoA to (S)-3-Hydrobutanoyl-CoA was also absent in all the samples.



Function	EC number	M1L	M2L	M3L		M1S	M2S	M3S		M1	M2	M3
L-lactate dehydrogenase	1.1.1.27	0.014968	0.013621	0.005199		0.026716	0.016848	0.002042		0.020842	0.015234	0.003621
Pyruvate ferredoxin oxidoreductase, alpha subunit	1.2.7.1	0.002401	0.006492	0.005817		0.036385	0.011822	0.018566		0.019393	0.009157	0.012192
Pyruvate ferredoxin oxidoreductase, beta subunit	1.2.7.1	0.001206	0.898815	0.013535		0.111071	0.02946	0.039877		0.056139	0.464138	0.026706
Pyruvate ferredoxin oxidoreductase, delta subunit	1.2.7.1		0.000603	0.000645		0.006732	0.0018			0.003366	0.001202	0.000322
Pyruvate ferredoxin oxidoreductase, gamma subunit	1.2.7.1	0.000929	0.009374	0.007289		0.088328	0.024483			0.044629	0.016928	0.003644
Phosphotransacylase	2.3.1.8	0.000371	0.000258	0.000482						0.000186	0.000129	0.000241
Formate C-acetyltransferase	2.3.1.54	0.188916	0.171272	0.125993		0.443636	0.56925	0.300757		0.316276	0.370261	0.213375
Acetate kinase	2.7.2.1	0.324402	0.234929	0.302858		0.24581	0.163159	0.1919		0.285106	0.199044	0.247379
Propionate kinase	2.7.2.15	0.000464								0.000232		
Propionate CoA-transferase	2.8.3.1	0.000521	0.000413	0.001529		0.003226	0.003379			0.001873	0.001896	0.000764
Methylmalonyl-CoA decarboxylase	4.1.1.41	0.001073	0.000672	0.00113		0.000399	0.000895			0.000736	0.000783	0.000565
Methylmalonyl-CoA/ethylmalonyl-CoA epimerase	5.1.99.1											
Methylmalonyl-CoA mutase	5.4.99.2	1.350376	1.364726	0.900949		0.626161	0.674925	0.540213		0.988269	1.019826	0.720581
Acetyl-CoA synthetase	6.2.1.1	0.086861	0.078568	0.071833		0.074353	0.081757	0.081841		0.080607	0.080162	0.076837
Acetyl-CoA synthetase (ADP-forming)	6.2.1.13	0.000464	0.002083	0.000347		0.000748				0.000606	0.001041	0.000174
Propionyl-CoA synthetase	6.2.1.17	0.000852	0.001233	0.000122		0.000955	0.002201	0.003384		0.000903	0.001717	0.001753
Succinyl-CoA synthetase alpha subunit	6.2.1.5	0.006476	0.005615	0.003979		0.007652	0.007119	0.026003		0.007064	0.006367	0.014991
Succinyl-CoA synthetase beta subunit	6.2.1.5	0.007105	0.006172	0.002995		0.011123	0.010761	0.003384		0.009114	0.008466	0.003189

Fig. 54 Schematics of proposed pathway for Propanoate production. The KEGG pathways shows presence of enzymes in liquid (blue color) and solid (red color) fractions in each treatment. The images represent the presence of enzyme from all domains while the table shows the relative frequency of enzymes in bacterial domain



Function	EC number	M1L	M2L	M3L		M1S	M2S	M3S		M1	M2	M3
Acetoacetyl-CoA reductase	1.1.1.36	0.000475	0.00019	0.00076			0.000453			0.000237	0.000321	0.00038
3-hydroxybutyryl-CoA dehydrogenase	1.1.1.157	0.028622	0.030732	0.031087		0.079234	0.096992	0.065544		0.053928	0.063862	0.048316
Pyruvate ferredoxin oxidoreductase, alpha subunit	1.2.7.1	0.002401	0.006492	0.005817		0.036385	0.011822	0.018566		0.019393	0.009157	0.012192
Pyruvate ferredoxin oxidoreductase, beta subunit	1.2.7.1	0.001206	0.898815	0.013535		0.111071	0.02946	0.039877		0.056139	0.464138	0.026706
Pyruvate ferredoxin oxidoreductase, delta subunit	1.2.7.1		0.000603	0.000645		0.006732	0.0018			0.003366	0.001202	0.000322
Pyruvate ferredoxin oxidoreductase, gamma subunit	1.2.7.1	0.000929	0.009374	0.007289		0.088328	0.024483			0.044629	0.016928	0.003644
Acetyl-CoA C-acetyltransferase	2.3.1.9	0.01971	0.03111	0.017834		0.165069	0.081353	0.035493		0.09239	0.056231	0.026663
Phosphate butyryltransferase	2.3.1.19	0.055275	0.027563	0.028545		0.070599	0.056448	0.028918		0.062937	0.042005	0.028732
Formate C-acetyltransferase	2.3.1.54	0.188916	0.171272	0.125993		0.443636	0.56925	0.300757		0.316276	0.370261	0.213375
Butyrate kinase	2.7.2.7	0.053567	0.03277	0.060835		0.044615	0.041946	0.064528		0.049091	0.037358	0.062681
3-oxoacid CoA-transferase subunit A	2.8.3.5			0.000174			0.000336				0.000168	8.68E-05
3-oxoacid CoA-transferase subunit B	2.8.3.5	0.032773	0.005248	0.015904		0.010512	0.005381	0.02607		0.021642	0.005315	0.020987
Acetate CoA-transferase alpha subunit	2.8.3.8	0.007797	0.004046	0.01457		0.014741	0.014431	0.036042		0.011269	0.009239	0.025306
Acetate CoA-transferase beta subunit	2.8.3.8	0.011862	0.006191	0.015186		0.015472	0.007798	0.021736		0.013667	0.006995	0.018461
Enoyl-CoA hydratase	4.2.1.17	0.001502	0.000382	0.000489		0.001561	0.000694			0.001531	0.000538	0.000244
3-hydroxybutyryl-CoA dehydratase	4.2.1.55	0.043244	0.023667	0.027331		0.104711	0.078389	0.020018		0.073977	0.051028	0.023675
Acetoacetyl-CoA synthetase	6.2.1.16	0.001037	0.000468			0.001123		0.000955		0.00108	0.000234	0.000477
3-hydroxyacyl-CoA dehydrogenase	1.1.1.35			0.000523		0.000239				0.000119		0.000262
Trans-2-enoyl-CoA reductase (NAD+)	1.3.1.44		0.000413			0.001147	0.000347			0.000573	0.00038	

Fig. 55 Schematics of proposed pathway for Butanoate production. The KEGG pathways shows presence of enzymes in liquid (blue color) and solid (red color) fractions in each treatment. The images represent the presence of enzyme from all domains while the table shows the relative frequency of enzymes in bacterial domain

Amplicon Sequencing:

The experiments were carried out using about 50ng DNA for generating 16S rRNA gene amplicons from each sample using three composite pairs of primers. The first set of primer consisted of 5′-**CCATCTCATCCCTGCGTGTCTCCGAC**GACTNNNNNNNNNNNN*GAGTTTGATC CTGGCTCAG*-3′ wherein the bold sequence is the GS FLX Titanium Primer A, the italicized sequence are the universal broadly conserved bacterial primer 8F, the sequence NNN, in the forward primer of each pair, designates the unique 11-base barcode used to tag each PCR product and under line 4 mer sequence is the key sequence. The reverse primer was 5′-**CCTATCCCCTGTGTGTGCCT TGGCAGTC**GACT*ATTACCGCGGCTGCTGGC*-3′: the bold sequences are GS FLX Titanium Primer B, and the italicized sequence is the broad-range bacterial primer 534R. Similarly second and third set of primer consists of forward primer 517F 5′-GCCAGCAGCCGCGGTAA-3′ and 917F GAATTGACGGGGRCCC and reverse primer 1114R GGGTTGCGCTCGTTRC and 1541 R AAGGAGGTGATCCAGCCGCA respectively.

The PCR amplification mix contained 1.25 units of DNA polymerase (Roche, USA), 2.5 µl reaction buffer, 0.5ul 10mm dNTPs (Roche, USA), and 1 µM of each primer in a total volume of 25 µl. Gel-purified genomic DNA (50ng) was then added to each amplification mix. Cycling condition was an initial denaturation at 95°C for 5 min, 30 cycles of 95°C for 30 s, 60°C for 45 s and 72°C for 60 s, followed by 10 min final extension at 72°C and 4°C hold. All PCR products were confirmed by agarose gel electrophoresis on 1.5% gel.DNA amplicons were gel-purified by gel extraction kit (QIAGEN) and pooled in equimolar before sequencing. Samples were further proceed for Roche 454 GS FLX Titanium chemistry pyrosequencing. Samples were analyzed using the QIIME (Quantitative Insights into Microbial Ecology) version 1.7.0-dev pipeline. Raw sequences were de-multiplexed and then quality-filtered using the default parameters in QIIME. Sequences were then clustered into operational taxonomic units (OTUs, 97% similarity) with UCLUST.

The resulting representative sequence set were aligned using PyNAST and given a taxonomic classification using RDP. Comparative data analysis was done in QIIME using 4 breed and 3 primer pair.

Comparative analysis in Jaffarabadi and Mehsani buffalo breeds microbial diversity

Principle component analysis was done using unifrac distance matrix in QIIME. Clustering was done on the basis of taxonomic presence or absence of organism in a particular samples. PCoA of two buffalo breeds i.e. Jaffarabadi and Mehsani was done using 3 composite pair of primer sets (Fig. 56). Here, we observed separate clustering of samples according to the primer pairs used except that Mehsani treatment 1 (M1) with primer pair 1 falls clustering with primer pair 2.

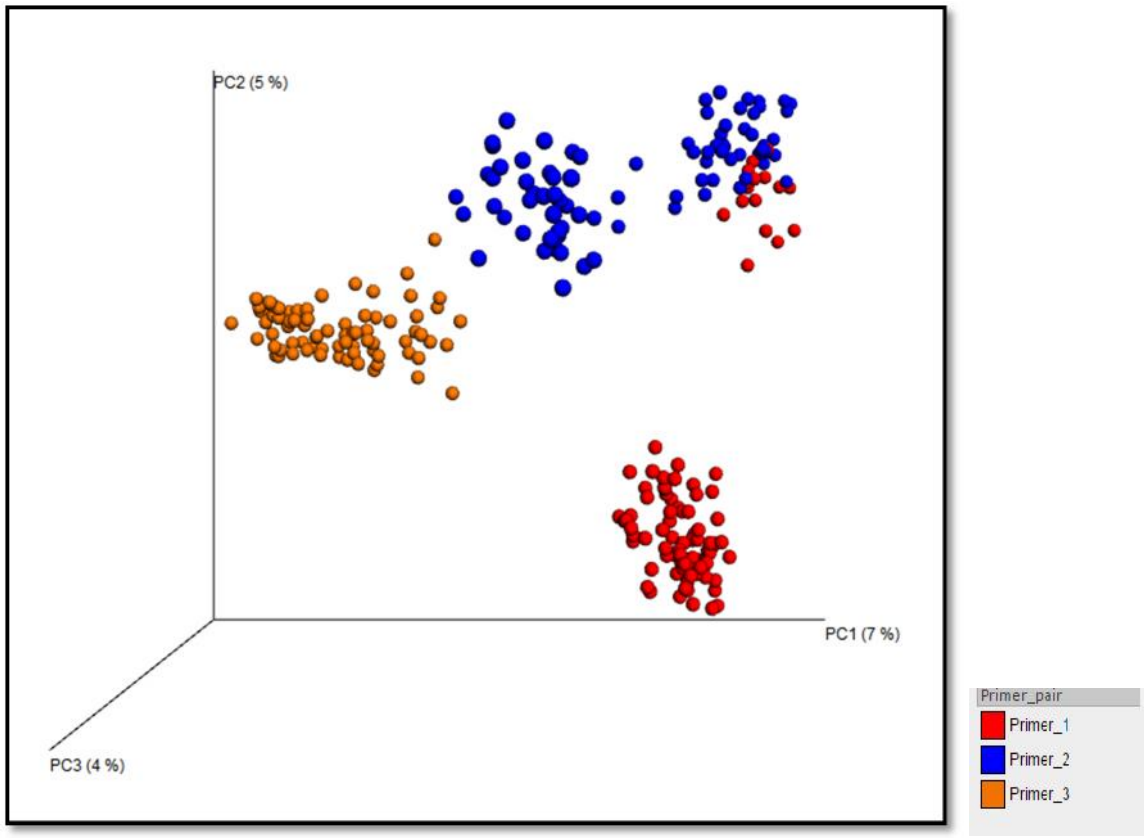


Fig. 56 Principal component analysis in two different breeds of buffalo using all three set of primers.

However, when on separation of all the samples of primer pair 1 (Fig. 57), it was observed that all the samples of Mehsani treatment 1 were falling under separate clusters while rest all samples clustered together.

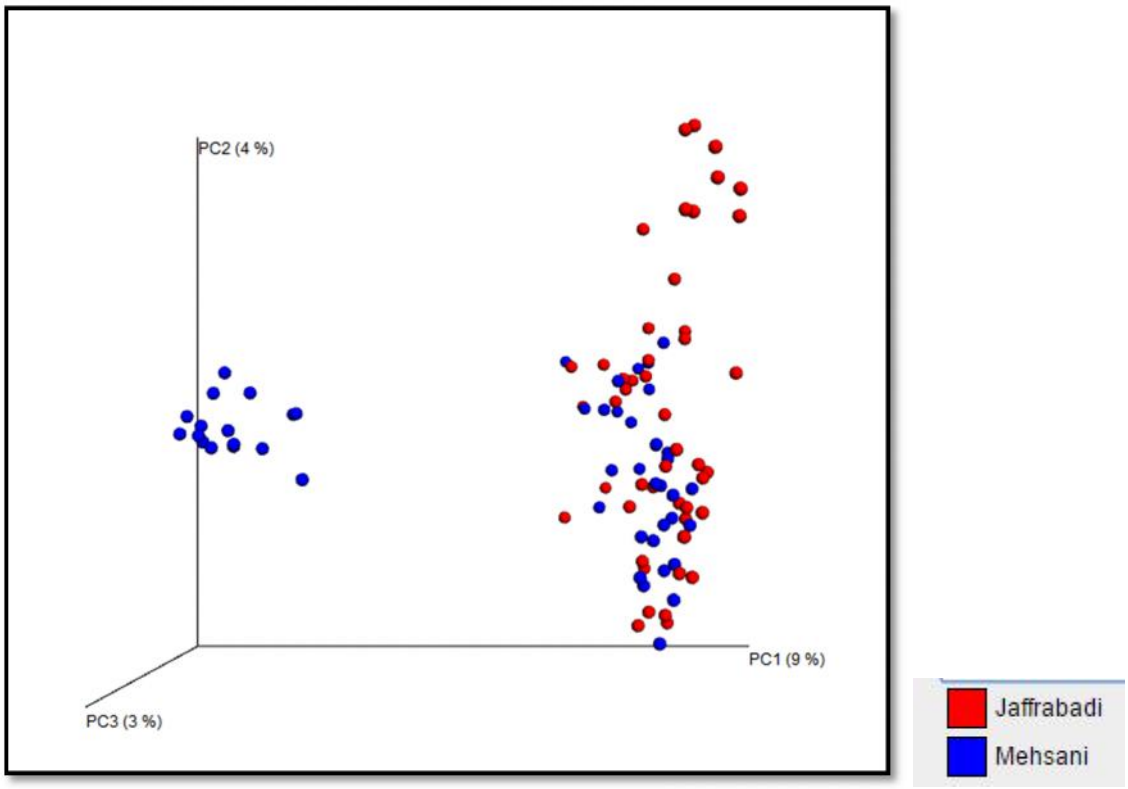


Fig. 57 Principal component analysis in two different breeds of buffalo using primer pair 1.

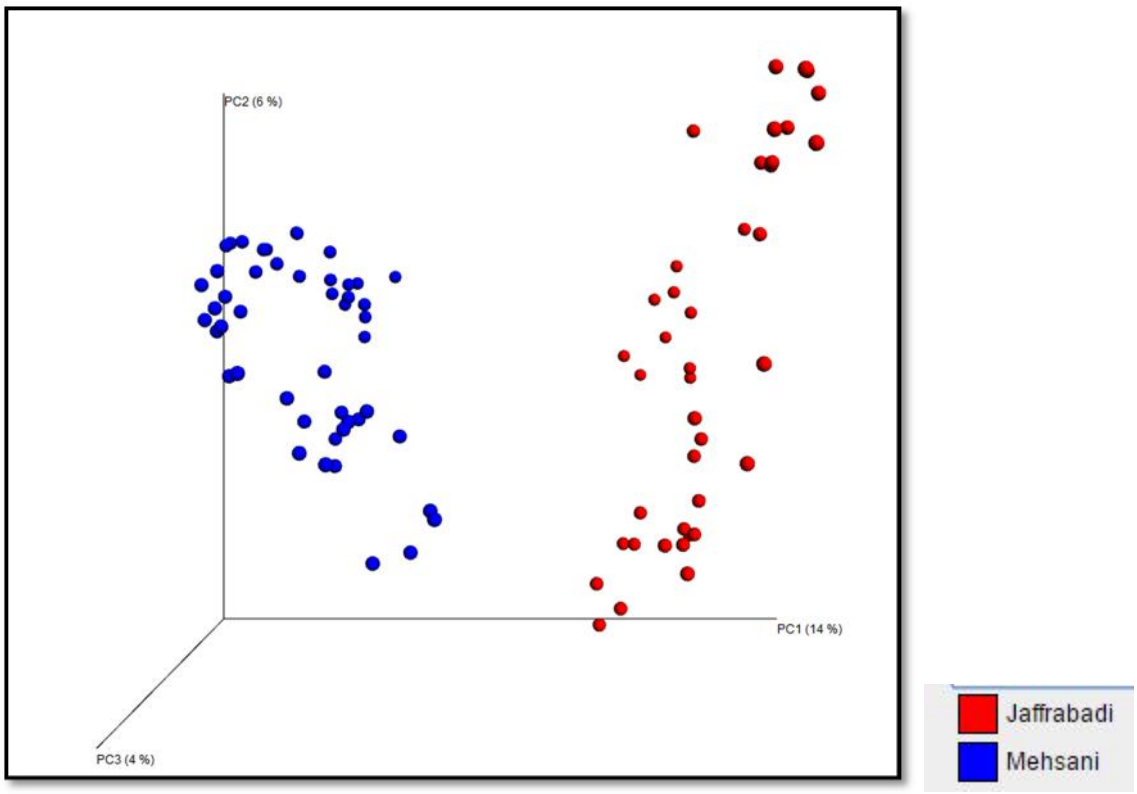


Fig. 58 Principal component analysis for all samples of two buffalo breeds using primer pair 2.

Similarly when cluster analysis of only primer pair 2 samples was performed, a clear cut clustering was found between both the breeds i.e Jaffarabadi and Mehsani (Fig. 58). However, in these samples solid liquid fraction also clustered separately (Fig. 59). Distinct differences in species richness and diversity were evident by it.

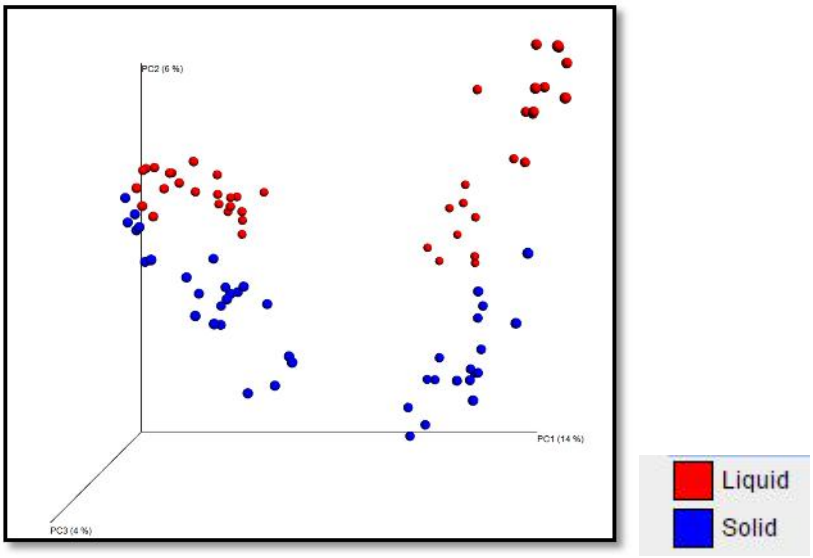


Fig. 59 Principal component analysis in two different breeds of buffalo using primer pair 2 based on solid and liquid fractions.

Similarly with primer pair 3 also similar type of results were obtained. Separate clustering between the breeds and here in this case also solid and liquid samples are clustered together showing the distinct diversity among the samples (Fig. 60).

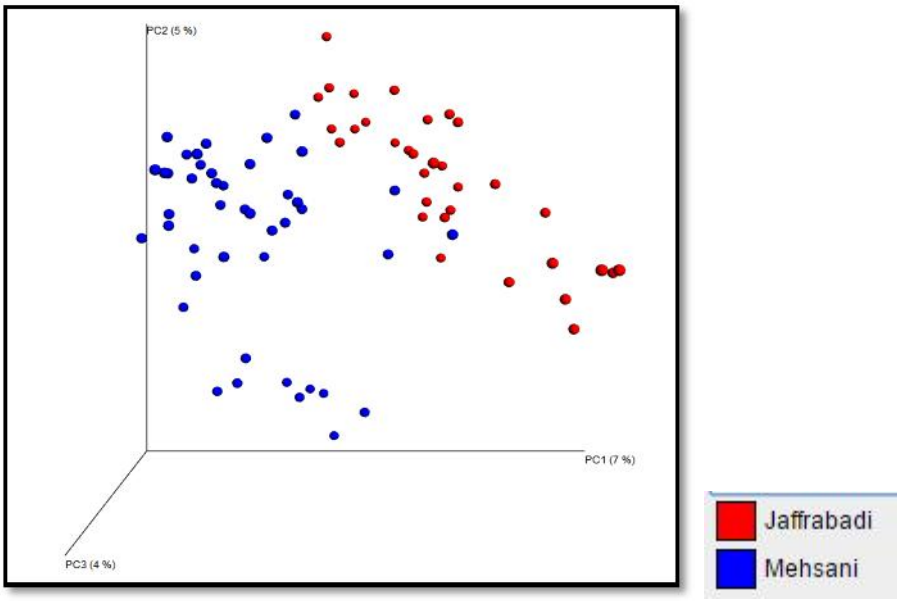


Fig. 60 Principal component analysis in two different breeds of buffalo using primer pair 3

Comparative analysis of cattle breeds microbial diversity using 16S amplicon

All samples of Gir and Kankrej cattle were analyzed together using all three composite pairs of primers. However all the samples of Gir treatment 2 and 3 with primer pair 1 were not falling in the same clusters of primer 1 and were clustering somewhere in clusters of primer pair 3 clusters. Also samples from Kankrej treatment 1 with primer pair 2 and primer pair 3 were also not falling in their respective clusters. Apart from these, all the samples showed separate clusters indicating different diversity among the samples (Fig. 61).

In both the breeds of cattle, all samples were also analyzed separately with different primer pairs. By comparison of the bacterial communities, it is apparent that both liquid (Red) and solid (Blue) fractions have distinct community compositions in all the three primer sets. Similarly by comparing different breeds of cattle, different clusters were observed except few as also seen in comparative analysis that Gir treatment 1 with primer pair 1 clustered with Kankrej samples, and Kankrej treatment 1 with primer pair 2 and 3 formed separate clusters. However, solid and liquid samples were forming distinct clusters, indicating distinct microbial communities in these breed as different and separate clusters were being formed in PCoA analysis (Fig. 62, 63 and 64).

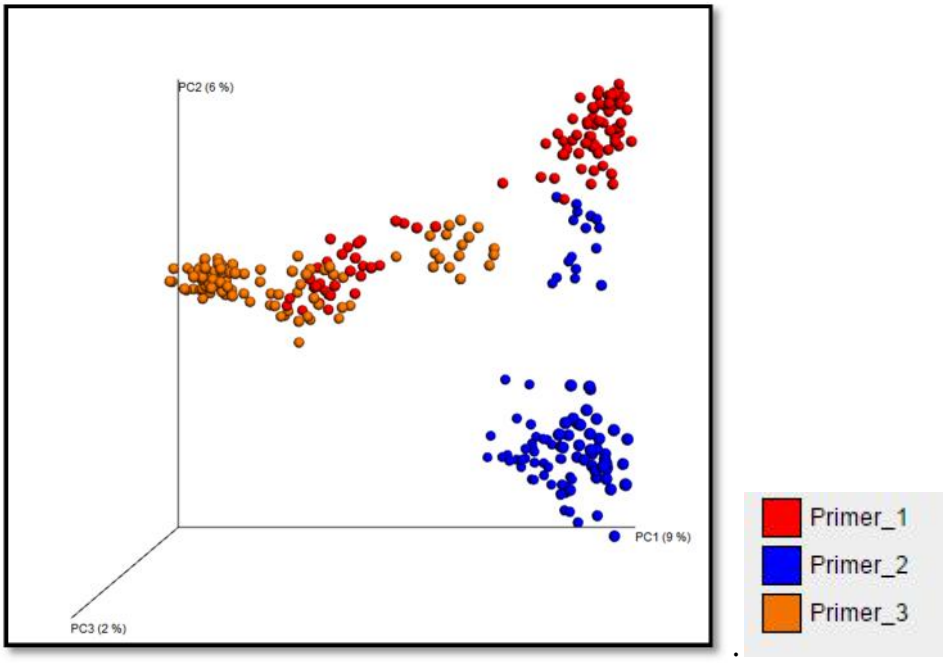


Fig. 61 Principal component analysis in two different breeds of cattle using all three set of primers.

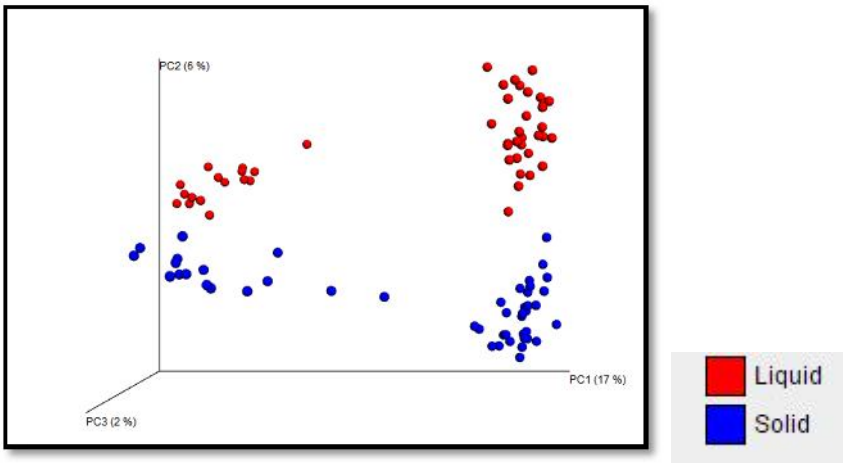


Fig. 62 Principal component analysis in two different breeds of cattle using primer pair 1.

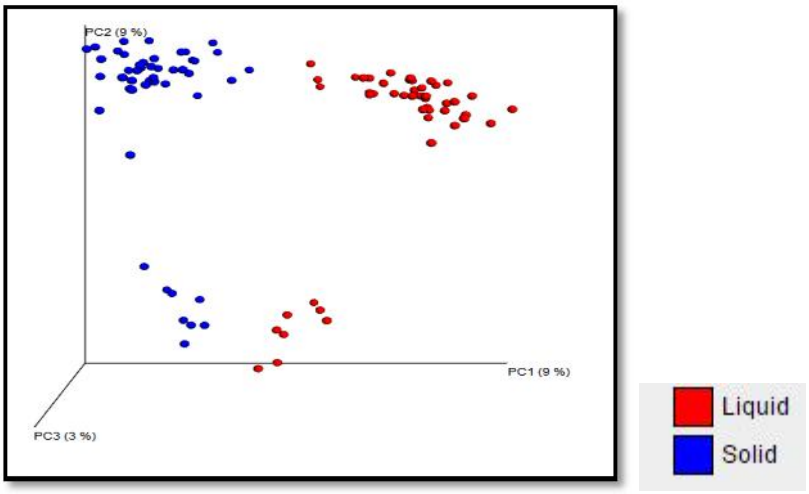


Fig. 63 Principal component analysis in two different breeds of cattle using primer pair 2.

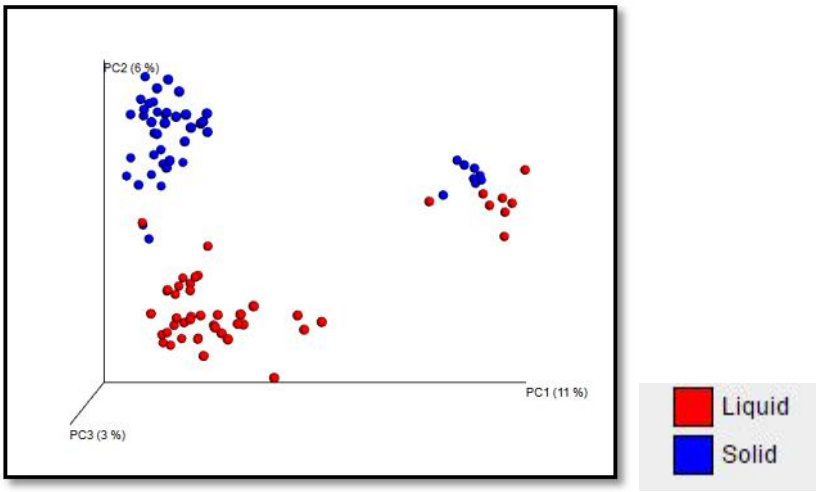


Fig. 64 Principal component analysis in two different breeds of cattle using primer pair 3.

Comparative analysis of buffalo and cattle rumen microbial diversity using amplicon data

Distinct differences in species richness and diversity were evident by primer pair 2. In figure 65, samples from Gir, Jaffarabadi and Kankrej formed long clusters. Mehsani breed is forming separate clusters with Kankrej treatment 1 in it. However in this also liquid and solid samples are forming separate clusters (Fig. 65).

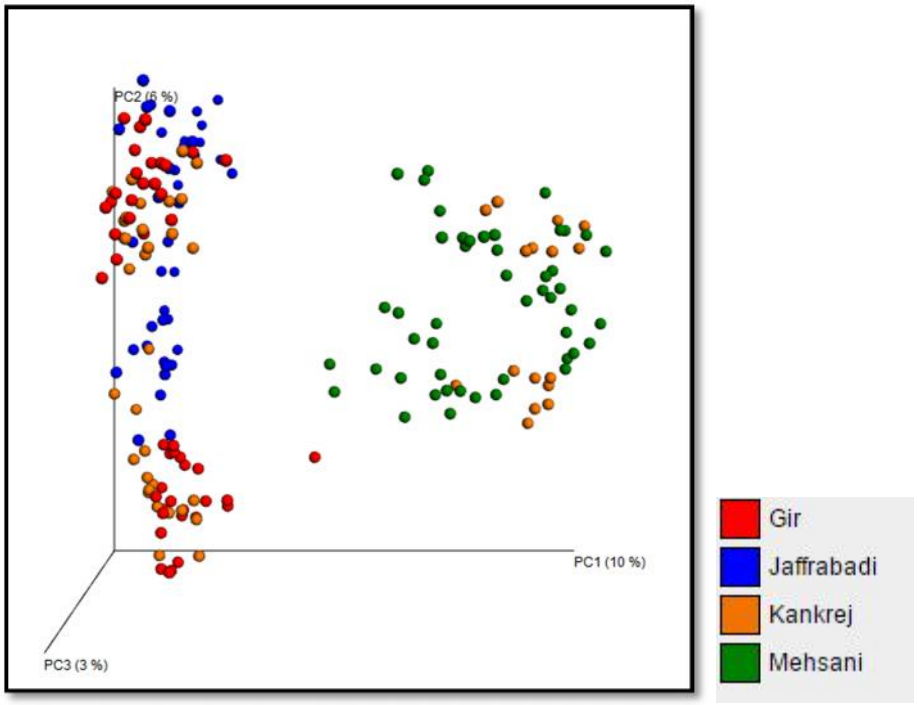


Fig. 65 Principal component analysis in two breeds of cattle and two breed of buffalo using primer set 2.

Jaffarabadi amplicon data analysis

Amplicon analysis of rumen samples highlighted the predominance of Bacteroidetes, Firmicutes and Fibrobacters (Fig. 66). Results indicated a striking abundance of bacterial lineages in microbiomes for all groups of buffalo. The relative abundance of *Bacteriodetes* were higher in treatment group 2 as compared to other treatment (Fig. 67).

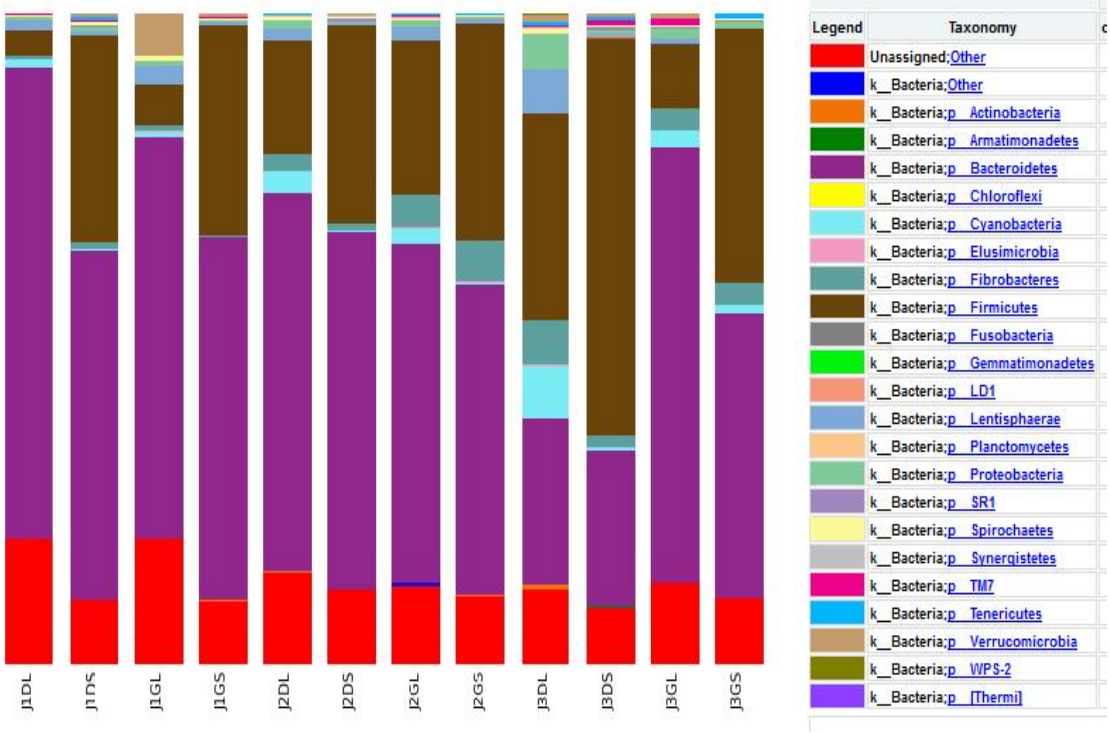


Fig. 66 Bacterial taxonomic composition at Phylum level

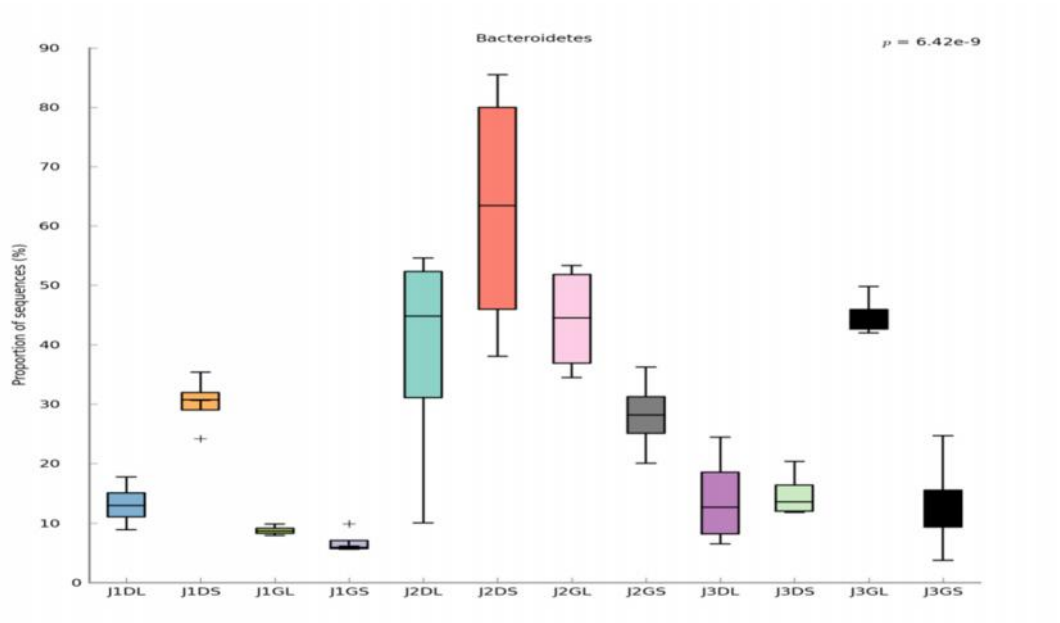


Fig. 67 Differences in the relative abundance of the bacterial Phyla *Bacteroidetes.*

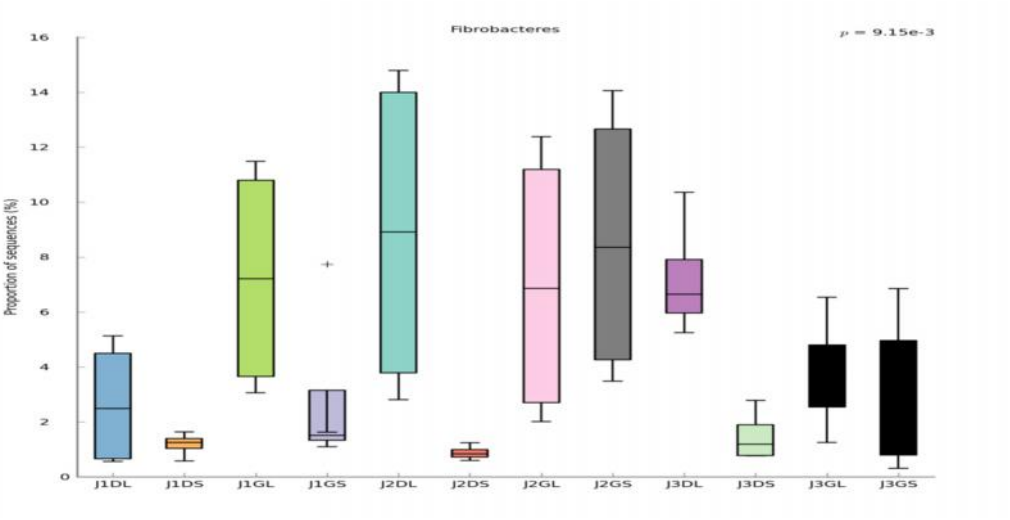


Fig.68 Differences in the relative abundance of the bacterial Phyla *Fibrobacter*.

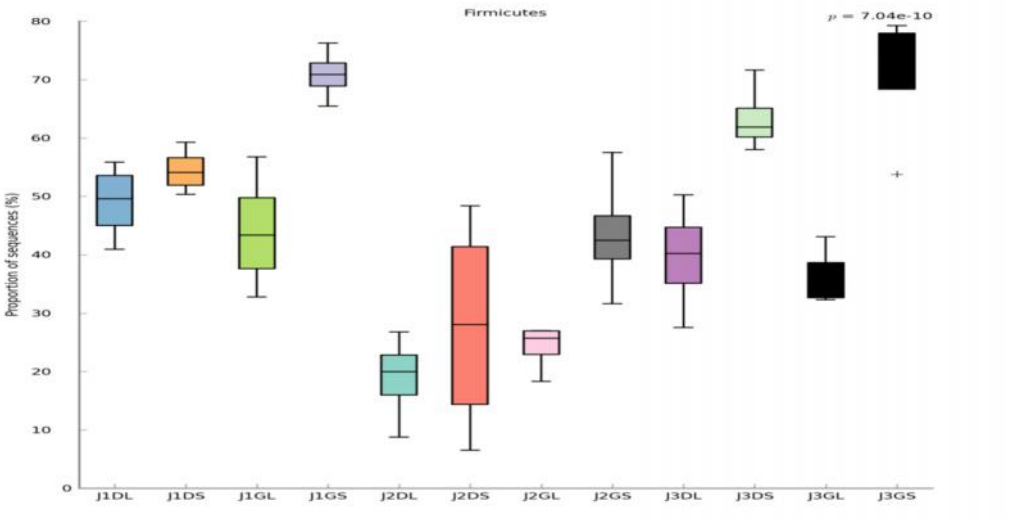


Fig.69 Differences in the relative abundance of the bacterial Phyla *Firmicut*es.

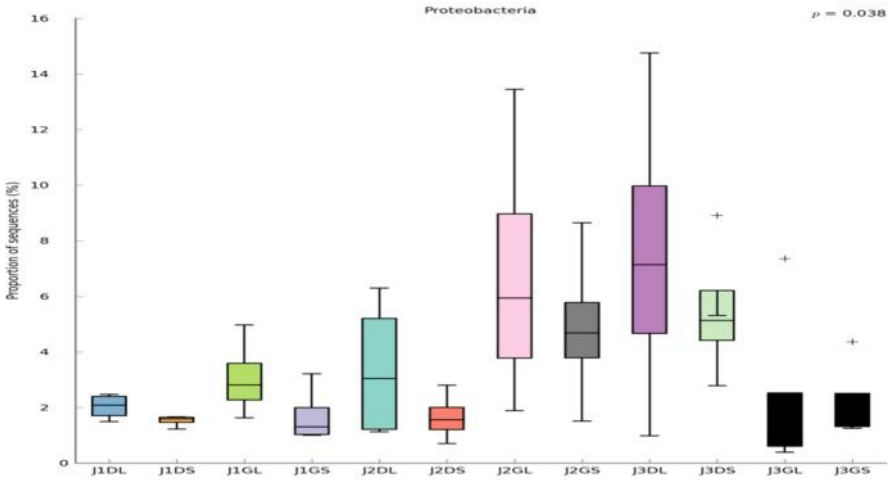


Fig.70 Differences in the relative abundance of the bacterial Phyla *Prot*eobacteria.

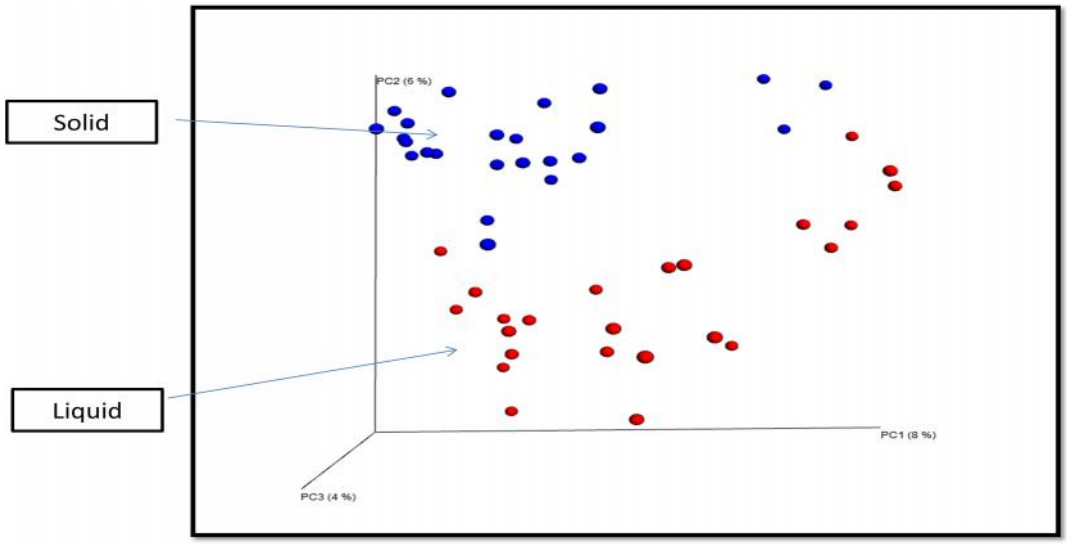


Fig. 71 Principal component analysis among different fraction in primer1 i.e. Liquid and Solid

Clustering of communities was influenced by the interactions between primer pair, treatment, and fraction. Distinct differences in species richness and diversity were evident by primer pair (Fig.72).

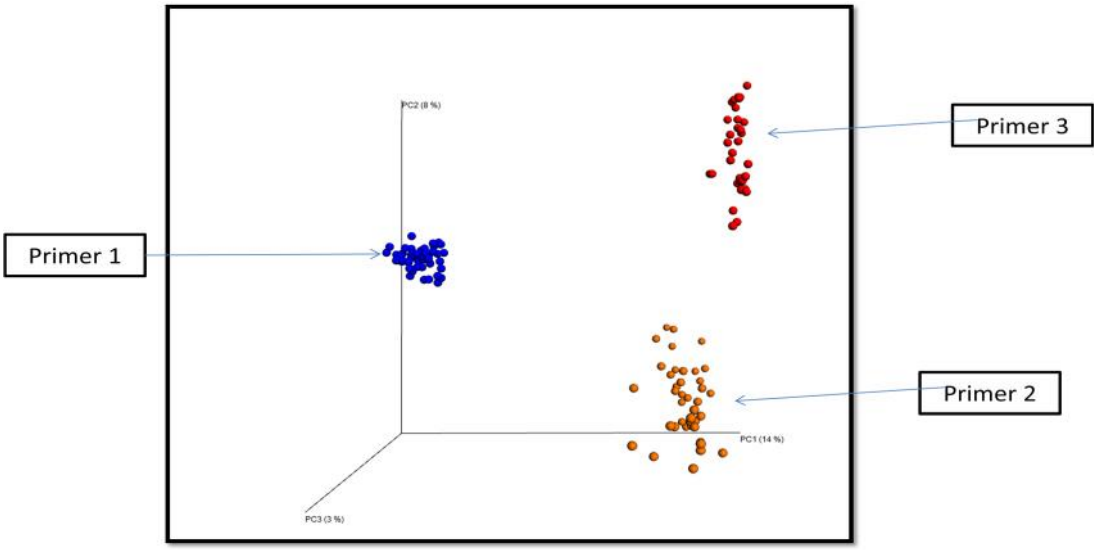


Fig.72 Principal component analysis among different fraction with primer pair1, 2 and 3.

Surti Buffalo amplicon data analysis

A total of 18.64297 lakh reads were obtained out of which 15.97034 lakh passed the quality parameters from 48 samples. These are the total number of reads obtained in each sample and treatment 1, 2, and 3 . A total of 4.41 lakh in J1, 3.61 lakh J2 and 7.93 lakh in J3 were obtained. A total 1,29,971 OTU were observed.

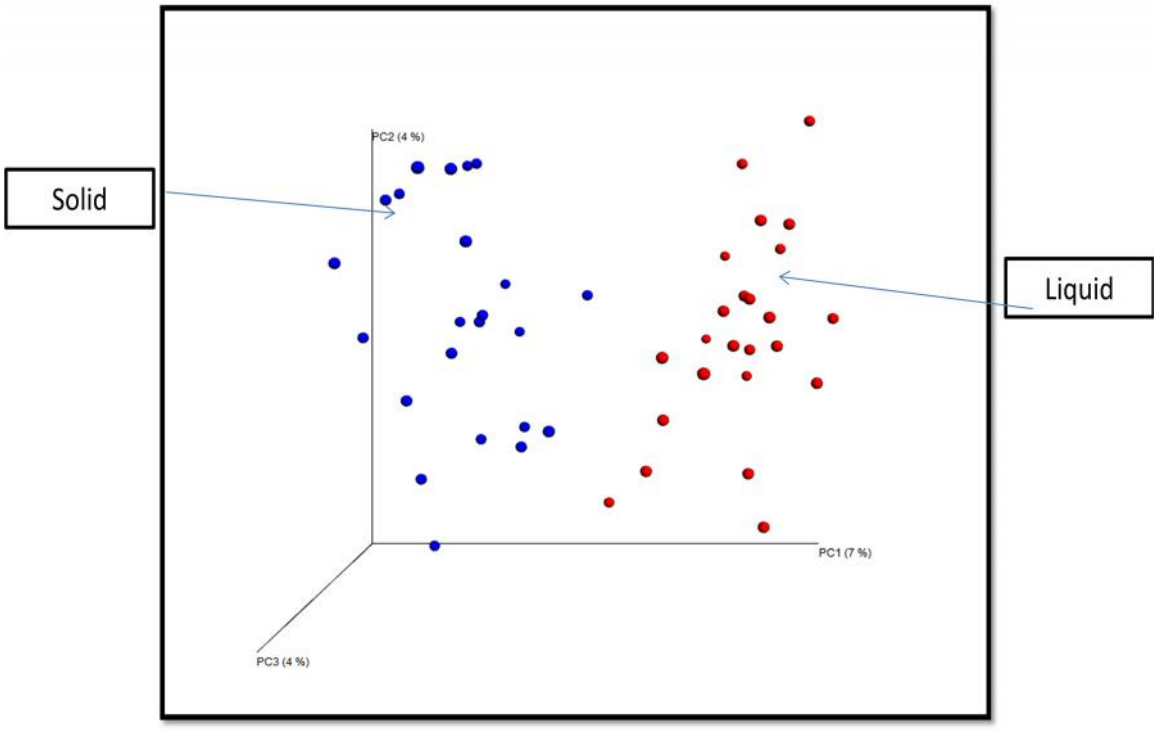


Fig.73 Principal component analysis among different fraction in primer pair1 i.e Liquid and Solid of Surti buffalo

Amplicon analysis of rumen samples highlighted the predominance of *Bacteroidetes, Firmicutes, Proteobacteria* and *Fibrobacters*. Results indicated a striking abundance of bacterial lineages in microbiomes for all groups of surti buffalo. The relative abundance of *Bacteriodetes* was higher in liquid fraction as compared to solid fraction (Fig.74).Whereas abundance of *Firmicutes* was high in solid as compared to liquid (Fig.75). Also evident by principle component analysis that liquid and solid fraction is forming different clusters so having different microbial diversity (Fig. 73).

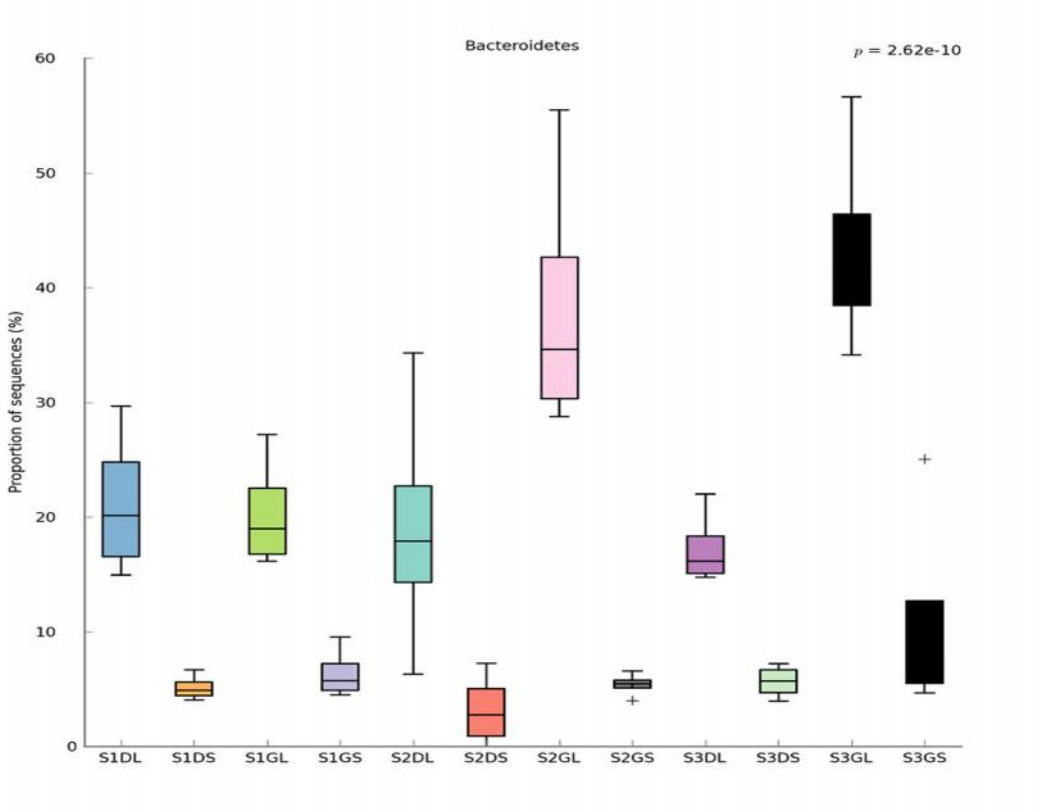


Fig.74 Differences in the relative abundance of the bacterial Phyla *Bacteroidetes.*

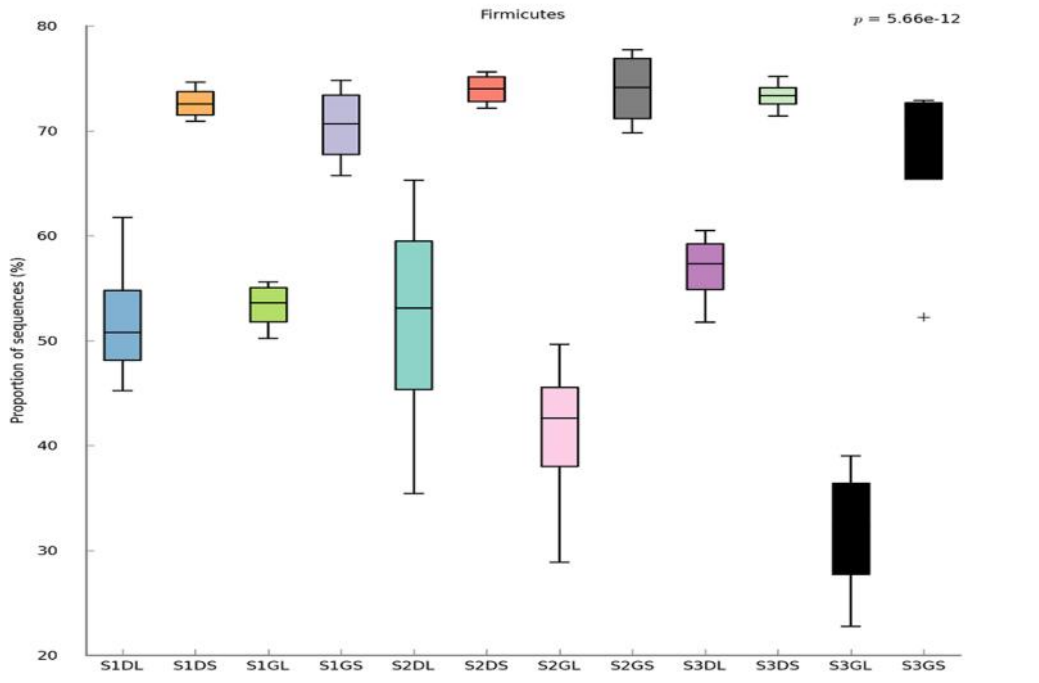


Fig.75 Differences in the relative abundance of the bacterial Phyla *Firmicut*es.

Comparative analysis of Jaffarabadi and Surti buffalo amplicon data

Shifts in the abundance of bacterial populations were apparent from phylum in two different breeds of buffalo i.e. Jaffarabadi and Surti. Bacteroides abundance was higher in Jaffarabadi buffalo as compared to Surti buffalo. Whereas Firmicutes abundance is higher in Surti buffalo compared to Jaffarabadi buffalo. Proteobacteria was also found to be abundant in Surti buffalo (Fig.76). The abundance of individual bacterial populations was different in two breeds. So here it is demonstrated that the bacterial diversity is host specific.

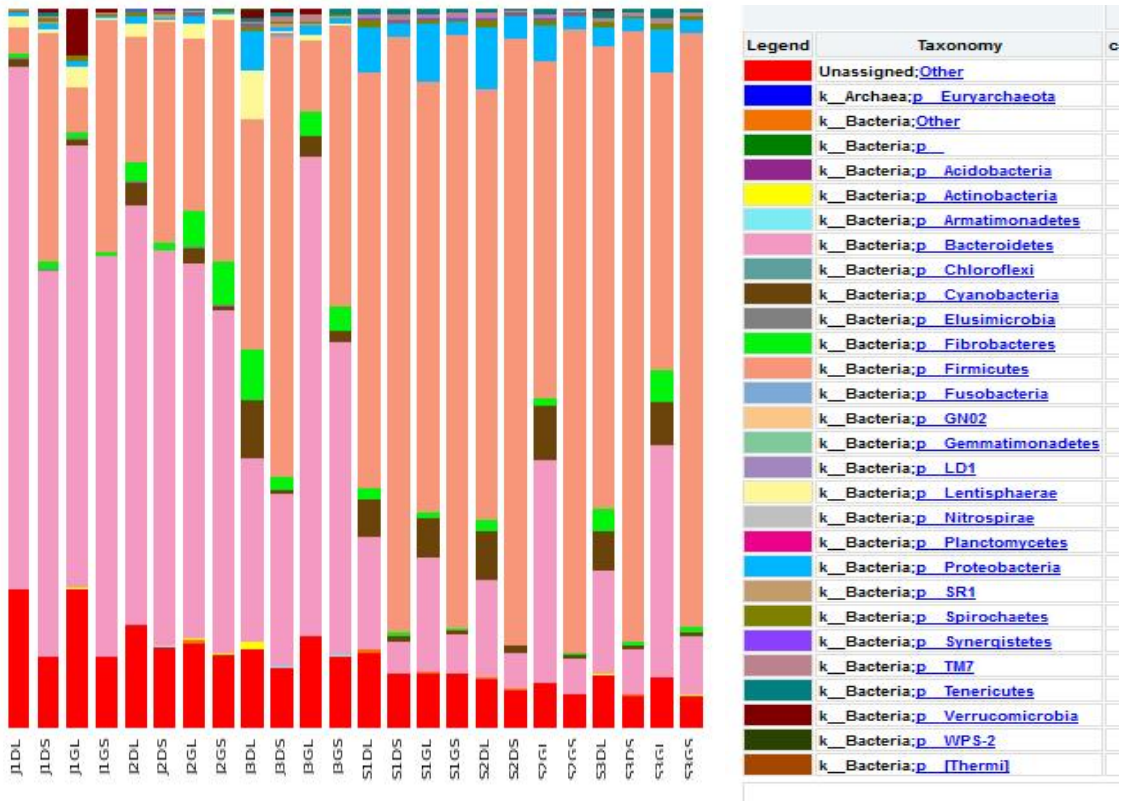


Fig.76 Bacterial taxonomic composition at Phylum level

From comparisons between bacterial communities it was apparent that both liquid (Red) and solid (Blue) fractions had distinct community compositions. Similarly by comparing different breed of buffalo we also observed different clusters indicating distinct microbial communities in these breed as different and separate clusters are being formed in PCoA analysis (Fig. 77 and 78).

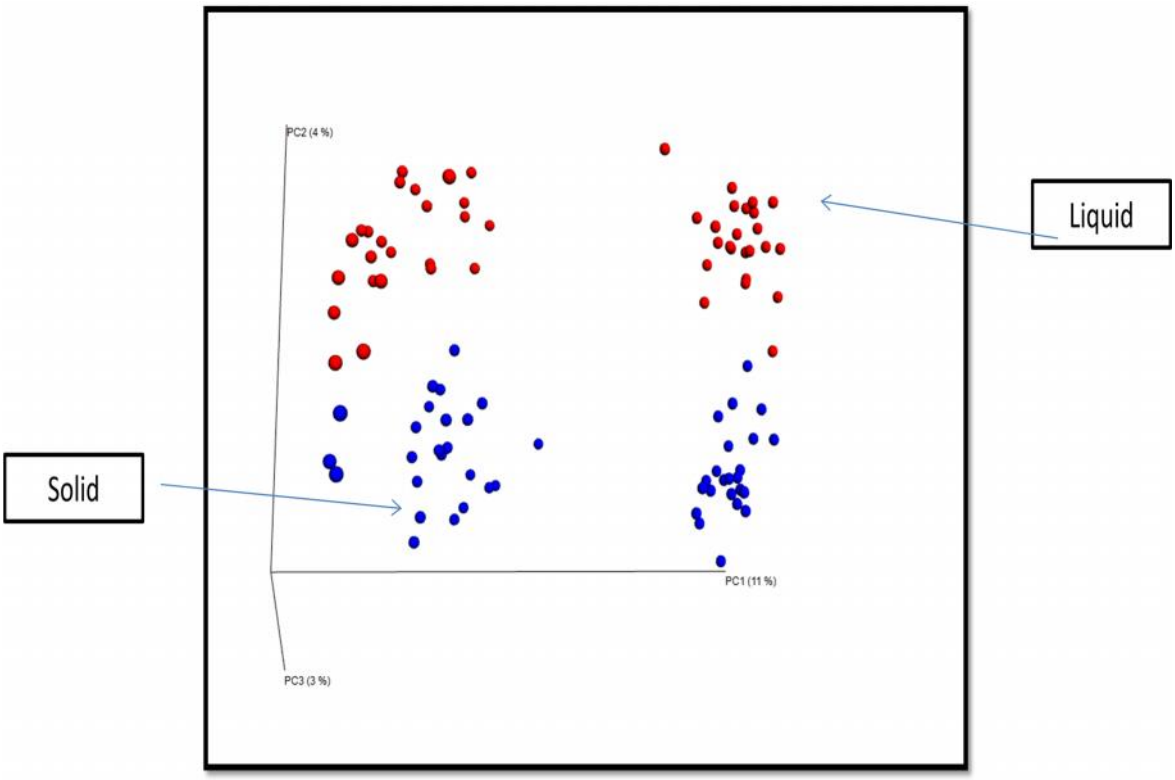


Fig. 77 Comparative principal component analysis among different fraction in primer pair1 i.e. Liquid and Solid of Jaffarabadi and Surti buffalo

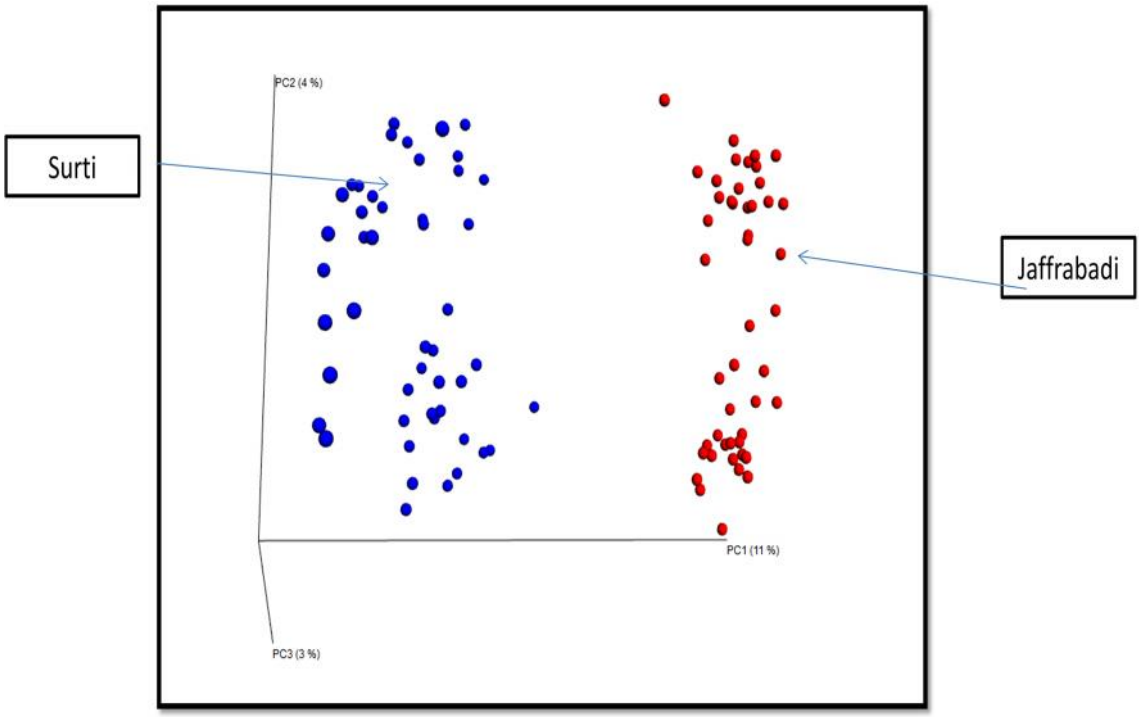


Fig.78 Comparative principal component analysis among different fraction in primer pair1 of Jaffarabadi and Surti buffalo

Isolation and characterization of enzymes from metagenomic data:

Cel PRI enzyme from Mehsani rumen metagenome:

Cloning and sequencing of PRU_2516 cellulase

The full length coding sequence of PRU_2516 of *Prevotella ruminicola* originated from buffalo rumen was amplified from metagenomic DNA using the specific primers (Forward: CGCGGATCCATGGCTGCGCCTGCTAAGG; Reverse: GCGCTCGAGTTATTTCTTGCCATCCAAAATACCTTG) designed based on the NCBI reference sequence (GenBank Accession number: NC_014033.1). The PCR amplified product was analyzed by agarose gel electrophoresis for the amplification of product and it was purified by gel extraction. The gel purified fragment was digested with *BamH*I and *Xho*I restriction enzymes and cloned in respective sites of pET32a (+) vector. The cloned fragment was sequenced using vector specific primers (pET32Fwd: AACGCCAGCACATGGACAG and pET32Rev: CAGCTTCCTTTCGGGCTTTG). Expected 1152 bp product was observed on to the agarose gel.

SDS-PAGE analysis of the induced crude extract of GH5_PR1 showed protein expression (His tagged), as observed by the appearance of protein band at about 55 kDa. The size of expressed GH5_PR1 was similar to the molecular mass calculated from the amino acid sequence. After Purification with the Ni-NTA column and desalting with Spin-X UF column single band was observed on the SDS-PAGE gel consistent with the size of the enzyme, suggesting that the enzyme was purified to homogeneity. In the western blot single band of 55 kDa was observed that confirmed the full length protein.

Standard Enzyme Assay

For semi quantitative measurement of enzyme activity, different dilutions of enzyme were spotted onto the L-agar + CMC plate along with the standard sigma cellulase (positive control). It was observed that the zone of clearance for enzyme GH5_PR1 was higher compared to the standard sigma cellulase at the same concentration of 1mg/ml. Apart from the diameter of the zone, intensity of the zone was also higher for GH5_PR1. On performing DNS assay for the quantification of enzyme activity, linearity was not followed for our enzyme compared to that of standard sigma cellulase, in terms of glucose generation. The activity of commercial grade sigma cellulase is 1.3 U/mg. Upon calculating the activity of our enzyme (based on the equation of graph) it showed an activity of 4.11U/mg which is 4-5 times higher activity as compared to that of the sigma cellulase.

Enzyme Characterization

Activity of our enzyme against CMC was observed by the formation of clear zone. GH5_PR1 did not show any activity towards LBG, Avicel, Xylan, and Pectin; that was confirmed by absence of cleared zone into respective plates. Optimum pH determination was done by spotting of enzyme incubated onto plates having different pH and the enzyme showed maximum activity at pH 5.This can be confirmed by intensity of the zone of clearance at pH 5. As the pH of media increased towards alkalinity, activity of enzyme got reduced as observed through the intensity of zone of clearance at each pH, ranging from 6 to 10. At pH 4 media turns acidic and zone of clearance turned dark bluish color. Enzyme is stable across the broad range of pH and shows activity onto the plate after pre-incubation in different buffers having pH range of 4-10 at 4˚C for 48 Hrs. Enzyme loses its activity completely at pH 3. Temperature optima for enzyme determined by incubating suitably diluted enzyme on to plate at various temperature showed that enzyme has maximum activity between the temperature range of 35-45˚C. Enzyme activity gets reduced at 50˚C and; at even higher temperatures it loses its activity completely. Enzyme was pre-incubated at temperature range of 5˚C-70˚C for 1 hr in pH-5 buffer to determine thermo stability and it shows stability in the range of 5˚C-50˚C. Activity of enzyme gets abolished completely in the temperature range from 55-70˚C. Effect of additives on activity of enzyme showed that NaCl enhances the activity of enzyme at the concentration of 10mM.$MgCl_2$ and $CaCl_2$ did not have any effect on the enzyme activity. Activity of enzyme gets reduced when we add SDS into medium but activity was not abolished completely. In the presence of KCl and EDTA into medium, zone of clearance turned blackish due to high activity of enzyme that might have produced some acidic compound.

Identification of unknown compound after reaction of Carboxymethyl cellulose (CMC) with PR-1

PR-1 enzyme was allowed to react with the substrate glucose for 30 min and then the reaction was filtered and analyzed using HPLC. Amino column was used with RI detector. Mobile phase was set with Acetonitrile and water with different rations for standardizing the method. Run time was kept 10 min for all the mobile phase rations (Fig. 79 to 81).

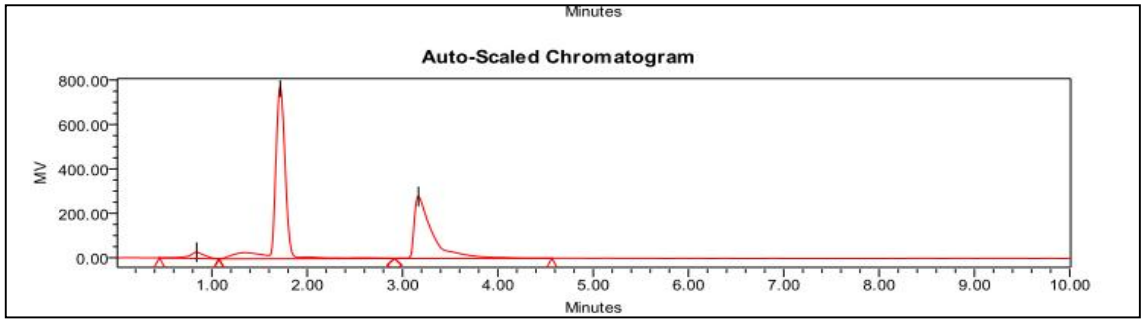


Fig. 79 Negative control: (Buffer, Phosphate buffer saline)

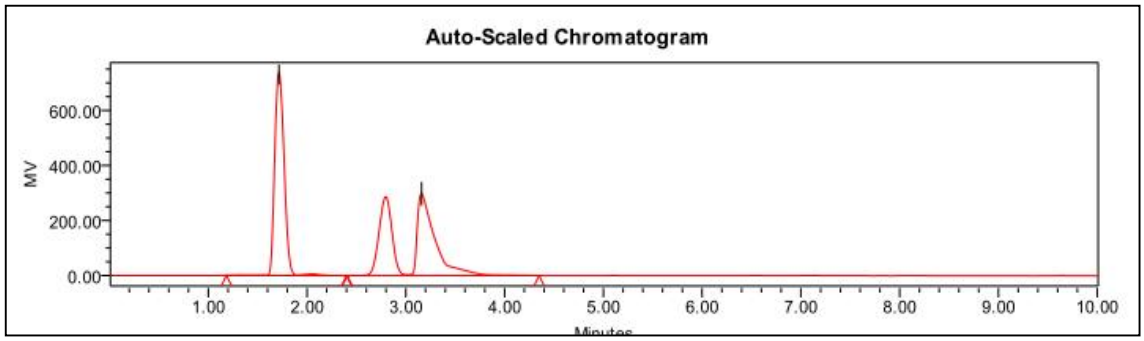


Fig. 80 Control: (Glucose in Phosphate buffer)

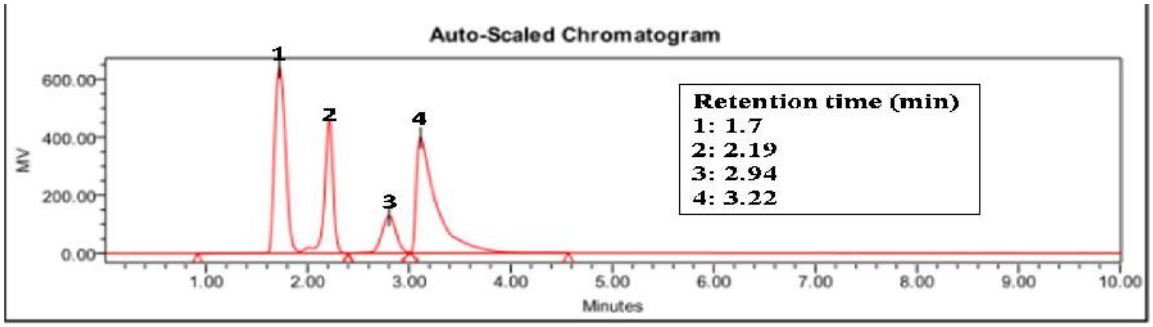


Fig. 81 Sample: (Glucose reacted with Enzyme PR-1)

It can be seen from the chromatogram that after reaction with PR-1 enzyme, new distinct peak was observed which can be degraded or modified product of glucose or enzyme (Fig. 81).

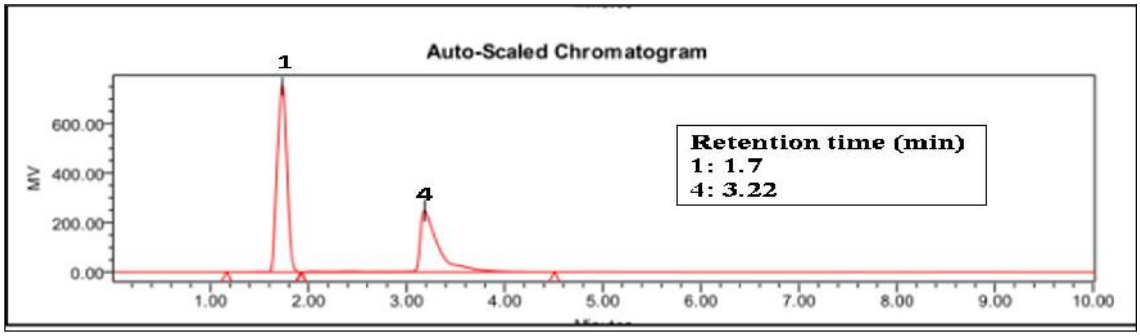


Fig. 82 Only Enzyme in PBS

LC-MS analysis of the PR-1: Glucose reacted with PR-1 enzyme was subjected to LC-MS analysis using amino column**.** It showed two distinct peaks with a mass of 475.2 and 701.3 (Fig. 83 & 84).

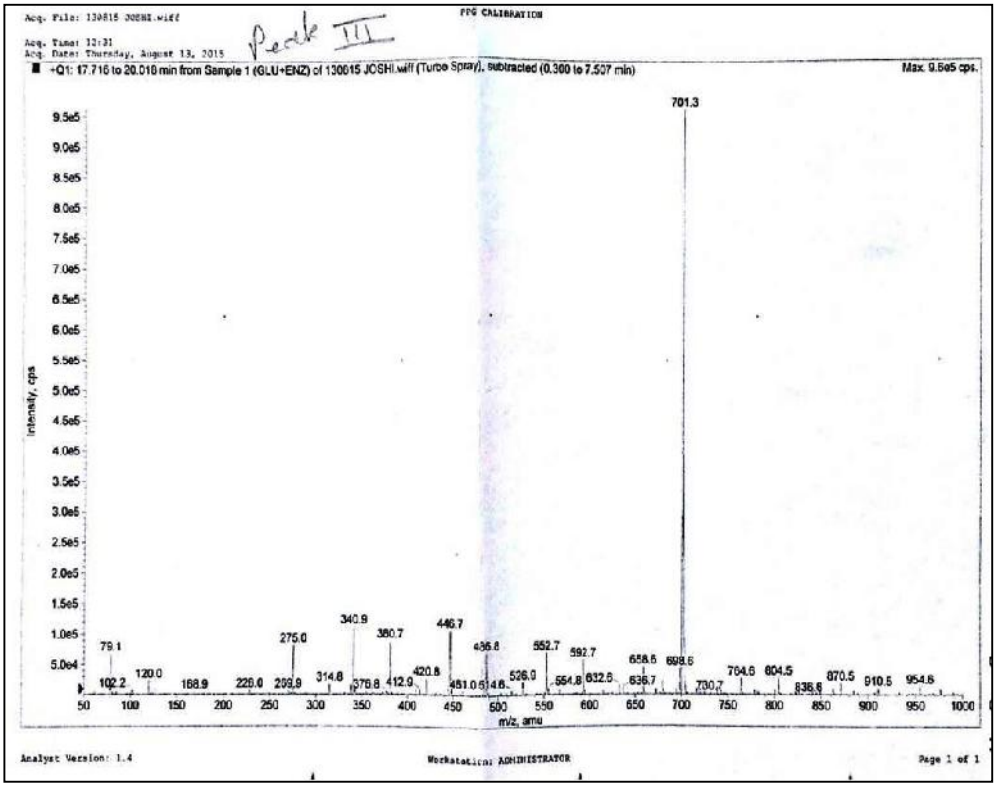


Fig. 83 LC-MS analysis

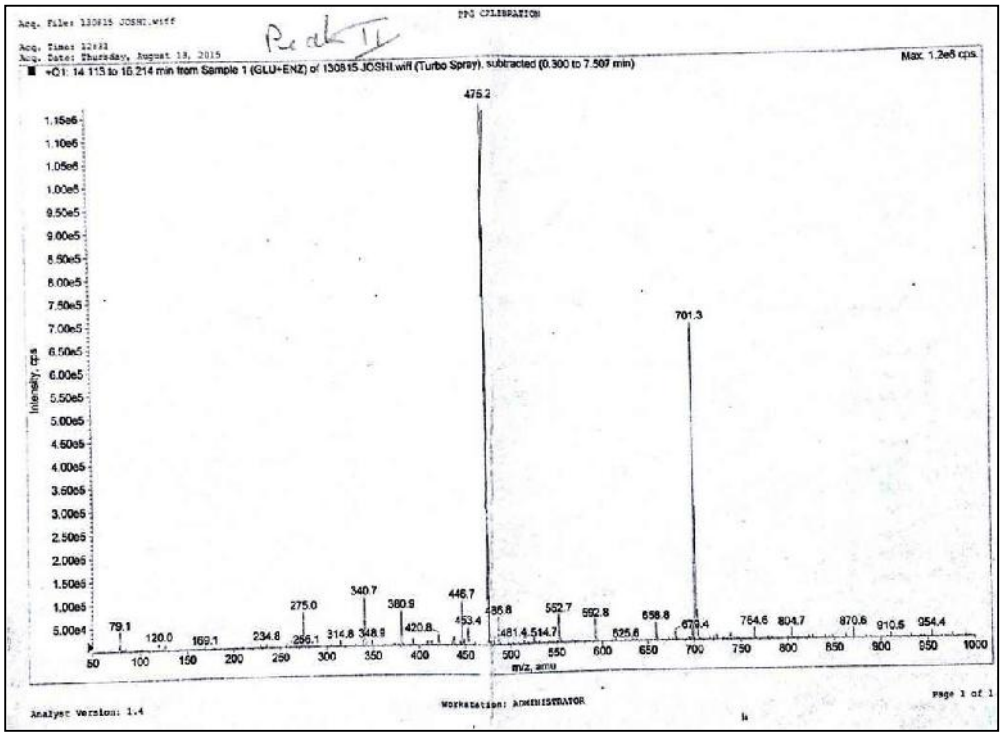


Fig. 84 LC-MS analysis

Determination of rate of Glucose degradation by PR-1: To determine the actual rate of glucose degradation by PR-1 with respect to time, experiments were set with 100 µg/ml of glucose solution incubated with 3 mg/ml of PR-1 (Final concentration in the reaction system). Samples were collected at different time interval and subjected for detection of glucose using chemical and analytical method using HPLC (Fig. 85 & 86.A). Degradation of glucose was monitored using DNSA method at different time intervals mentioned below:

Enzyme concentration (3mg/ml)	Actual glucose concentration	Final glucose concentration	Degraded or depleted glucose
PR-1 (15min)	100 µg/ml	43.8 µg/ml	56.2 µg/ml
PR-1 (30min)	100 µg/ml	44.01 µg/ml	55.99 µg/ml
PR-1 (60min)	100 µg/ml	46.58 µg/ml	53.42 µg/ml

The same reactions were also subjected for HPLC analysis to monitor the depletion of glucose in the reaction. To achieve this, Standards of different glucose concentrations were run on HPLC and their area of curve (AOC) were taken into account to measure the depletion of glucose.

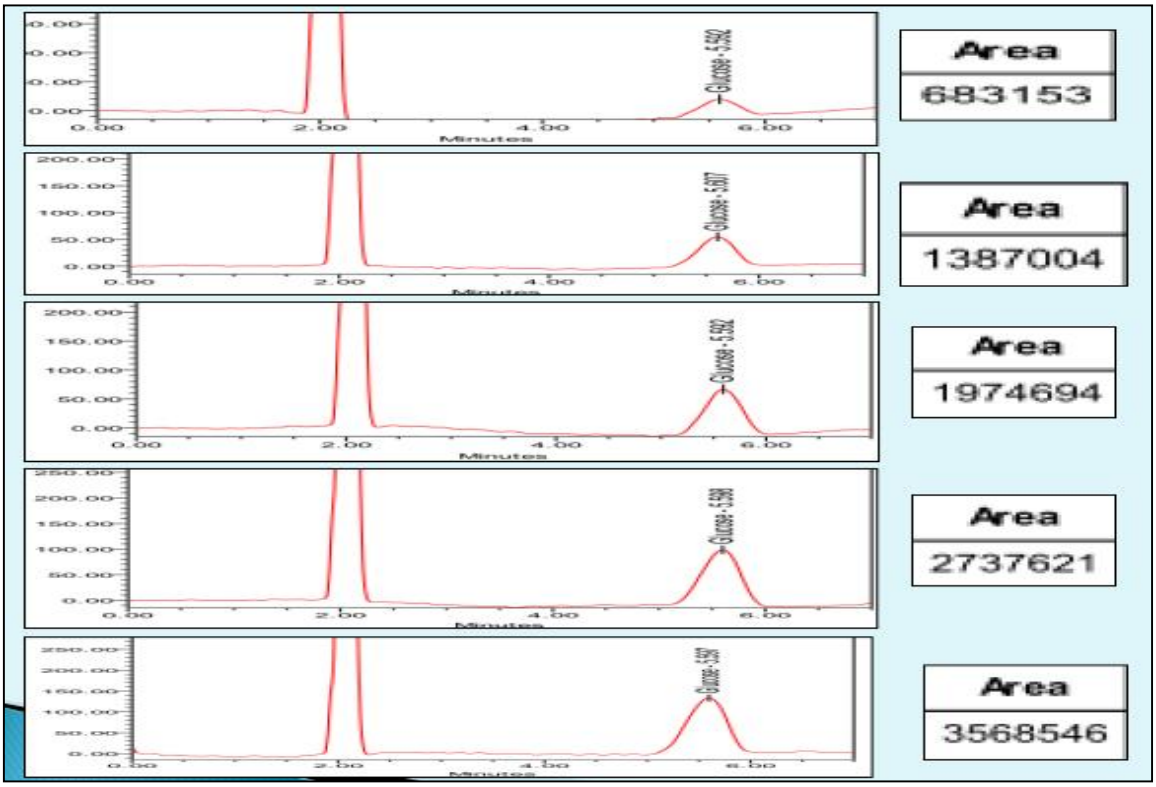


Fig. 85 Standard Curve for glucose

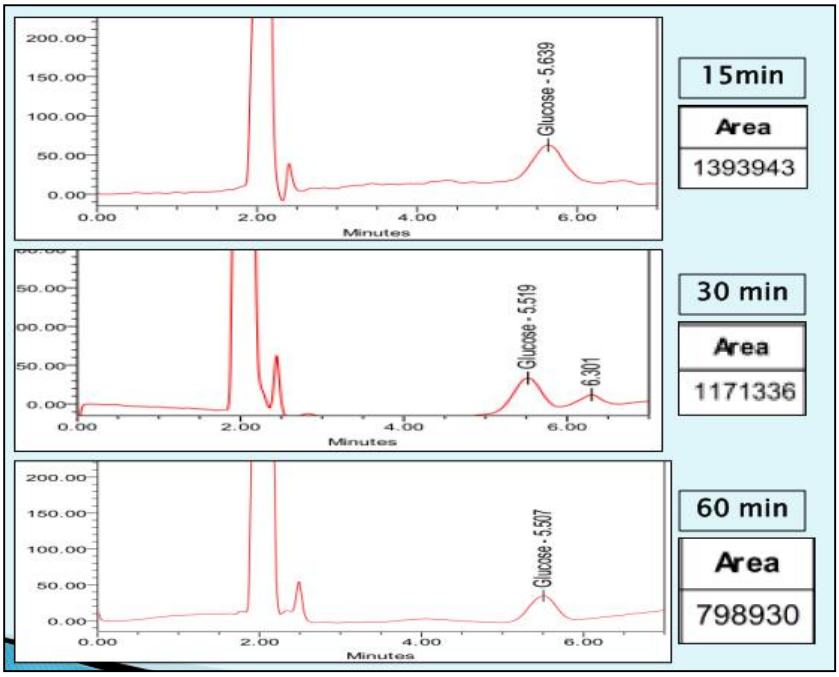
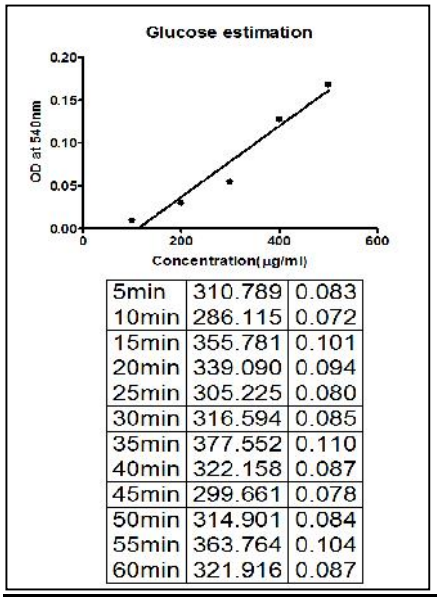
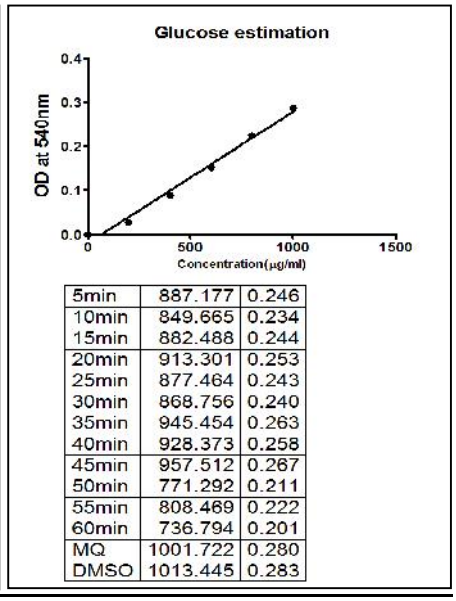


Fig. 86 A. HPLC analysis of Samples

Glucose depletion was measured at different time interval (5 min to 60 min) using DNSA method. Reaction was set according to previously mentioned concentrations of glucose and PR-1 and incubated for varied time period. To check the variability in the results, the assay was performed for intra and inter-day variations. PR-1 (3mg/ml) was incubated with two different glucose concentrations (500 µg/ml and 1000 µg/ml) and glucose depletion was measured (Fig 86. B). The depletion can be observed in both the cases at the end of incubation period (60 min). The PR-1 substantially reduces the level of free glucose in the system as evident by the results shown below.



5min	310.789	0.083
10min	286.115	0.072
15min	355.781	0.101
20min	339.090	0.094
25min	305.225	0.080
30min	316.594	0.085
35min	377.552	0.110
40min	322.158	0.087
45min	299.661	0.078
50min	314.901	0.084
55min	363.764	0.104
60min	321.916	0.087



5min	887.177	0.246
10min	849.665	0.234
15min	882.488	0.244
20min	913.301	0.253
25min	877.464	0.243
30min	868.756	0.240
35min	945.454	0.263
40min	928.373	0.258
45min	957.512	0.267
50min	771.292	0.211
55min	808.469	0.222
60min	736.794	0.201
MQ	1001.722	0.280
DMSO	1013.445	0.283

PR-1 + Glucose (500 µg/ml) **PR-1 + Glucose (1000 µg/ml)**

Fig.86 B. Measurement of glucose depletion after incubation with PRI

Evaluation of the enzyme kinetic parameters (Km and Vmax) based on its cellulase activity (For PR-1): For determining the Km and Vmax values for PR-1, Reactions were set from 100 µg/ml to 2000 µg/ml of glucose solution and it were incubated with 1mg/ml of PR-1 (Final concentration in reaction system). After the incubation for 60 min, glucose was monitored using DNSA method and activity was measured using the standard formula (Fig. 87).

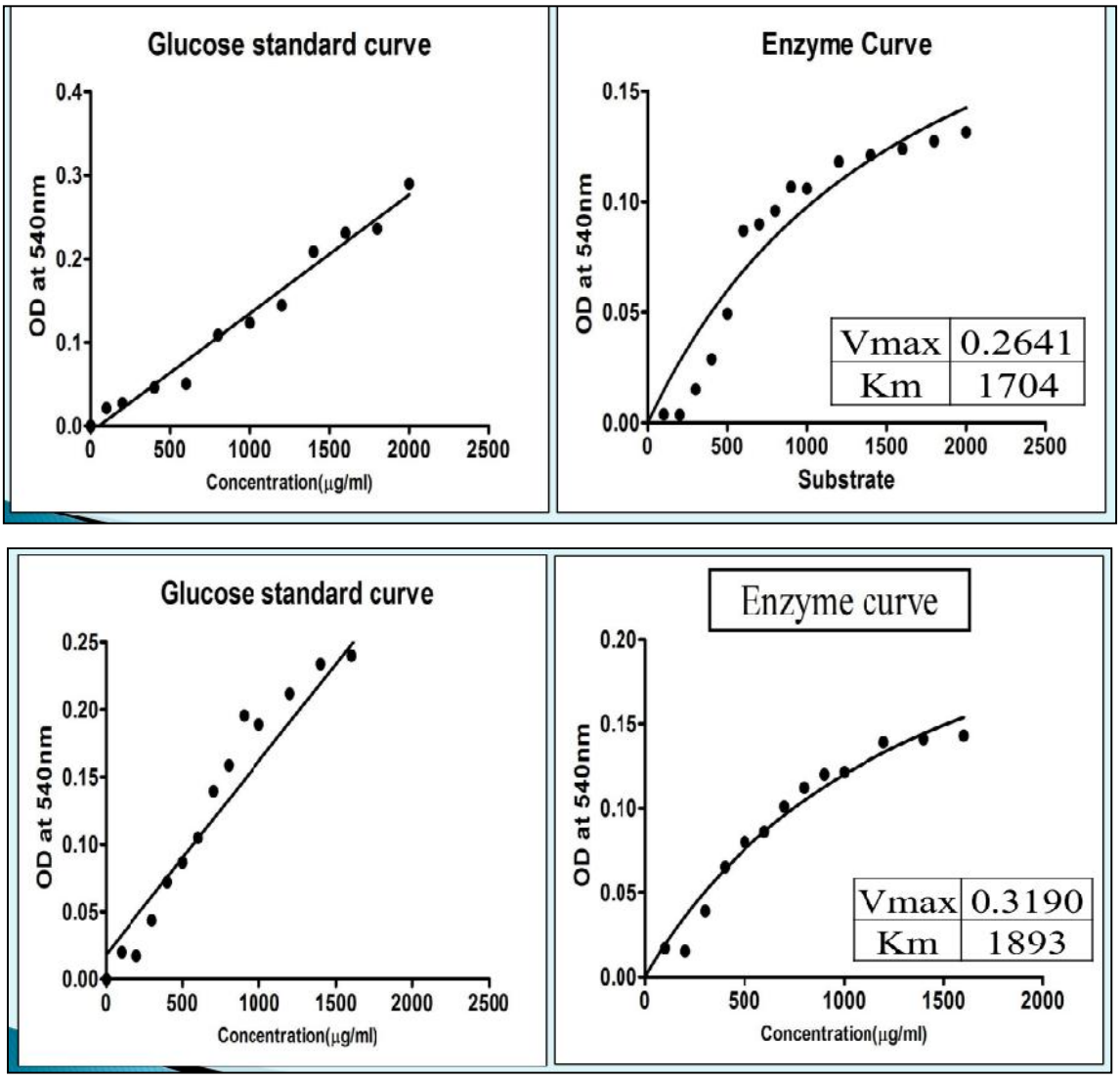


Fig. 87 Enzyme kinetics of PR-I

Comparison between Sigma cellulase and PR-1 based on their cellulase activity

The Cellulase activity of the commercially available cellulase from Sigma (USA) was compared with in house produced cloned enzyme PR-1. The activity was compared at two different concentrations (1 mg/ml and 10 mg/ml) (Fig. 88 to 91).

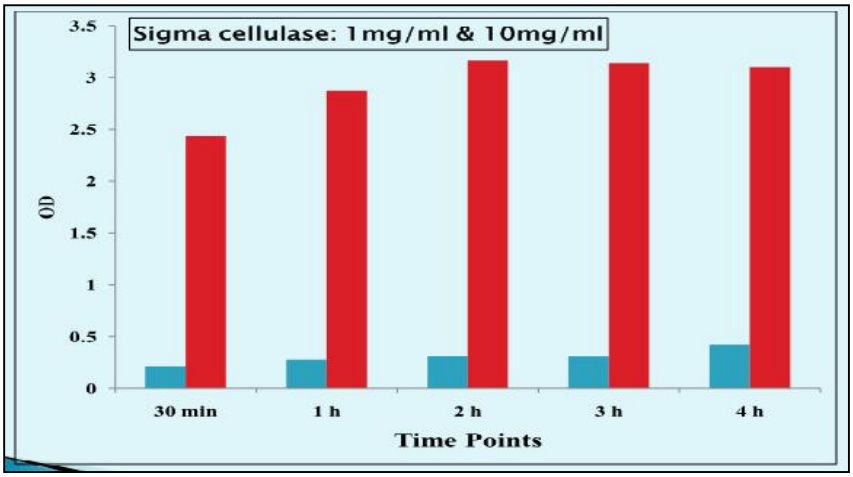


Fig. 88 Activity of sigma cellulase at two different concentrations (Blue bar: 1 mg/ml and Red bar: 10 mg/ml)

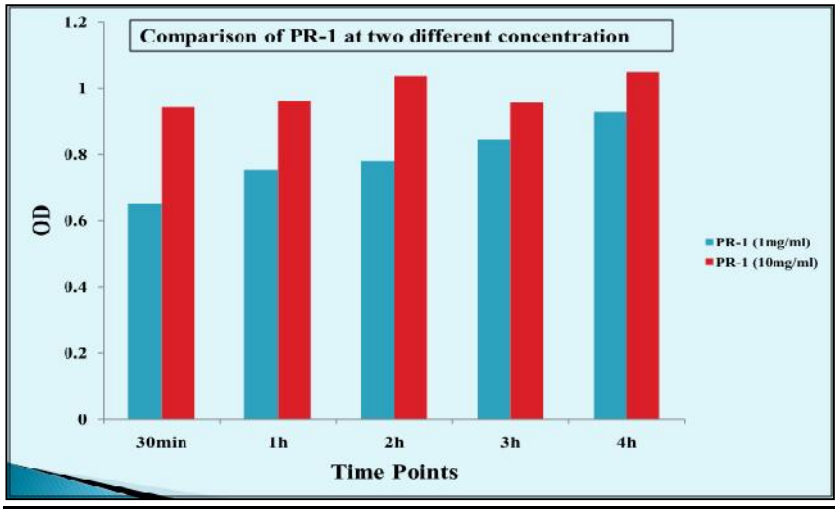


Fig. 89 Activity of PR-1 at two different concentrations

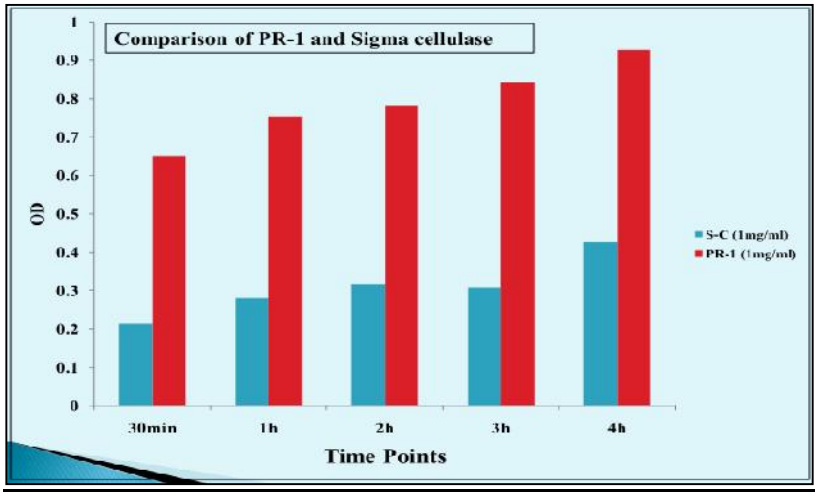


Fig. 90 Comparison of PR-1 and Sigma cellulase at 1 mg /ml concentration

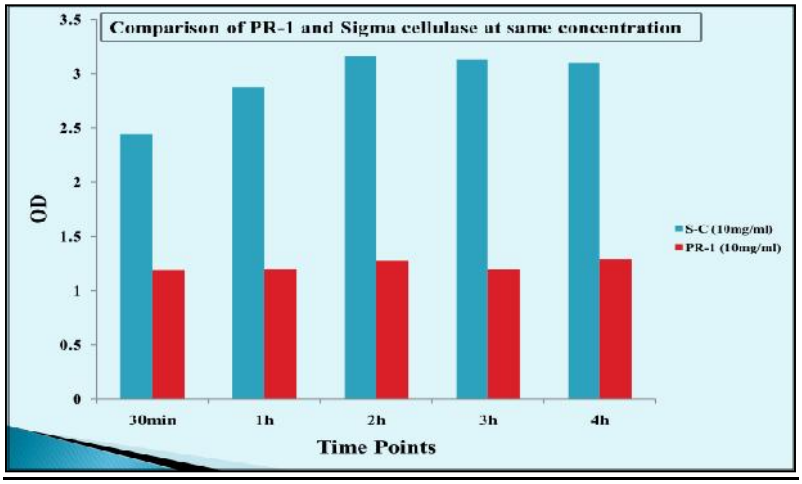


Fig. 91 Comparison of PR-1 and Sigma cellulase at 10 mg /ml concentration

Estimation of true cellulase activity for PR-1 using Viscosity measurement: CMC solution (1%) was incubated with PR-1 enzyme (1mg /ml) for different time intervals and then the viscosity of the solution was measured using Rheometer (Anton Parr, USA). PR-1 treatment showed reduction in viscosity more efficiently than sigma cellulase (Fig. 92 to 94).

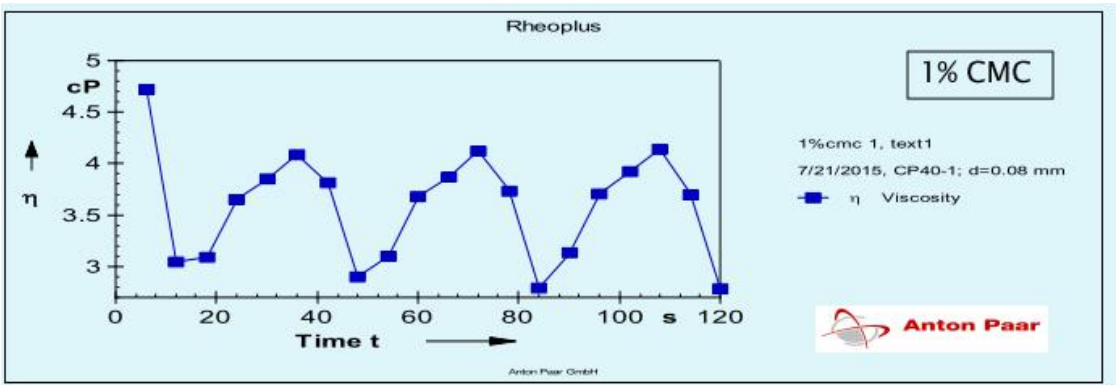


Fig. 92 Viscosity of 1% CMC solution (Approx 4.5 cP value, cP: Centipoise)

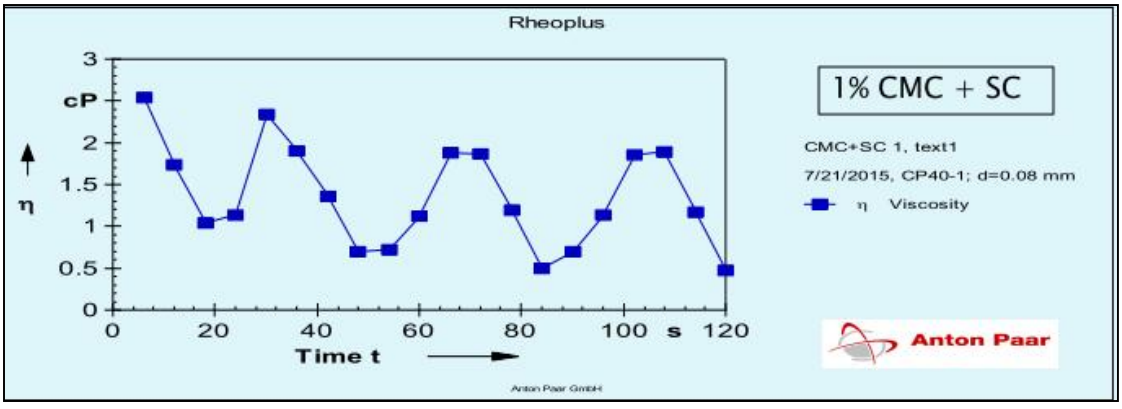


Fig. 93 Viscosity of 1% CMC after treatment with Sigma Cellulase (Approx. 2.5 cP value)

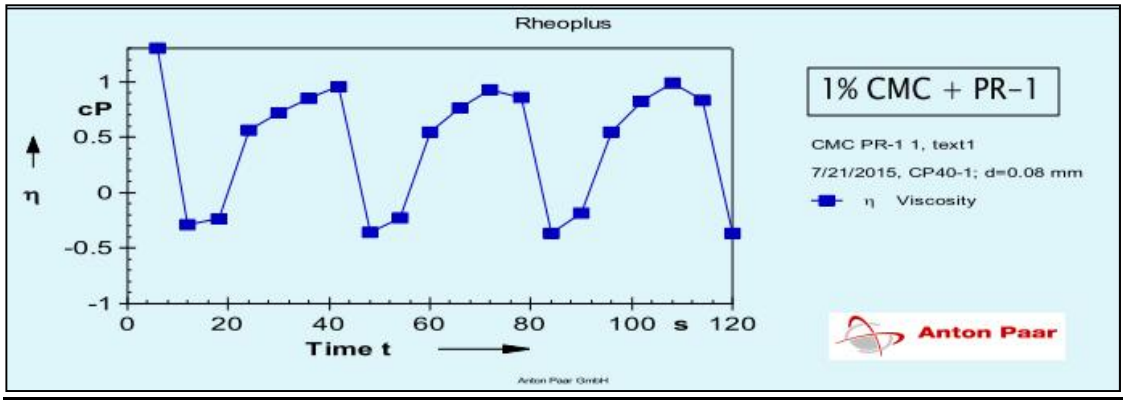
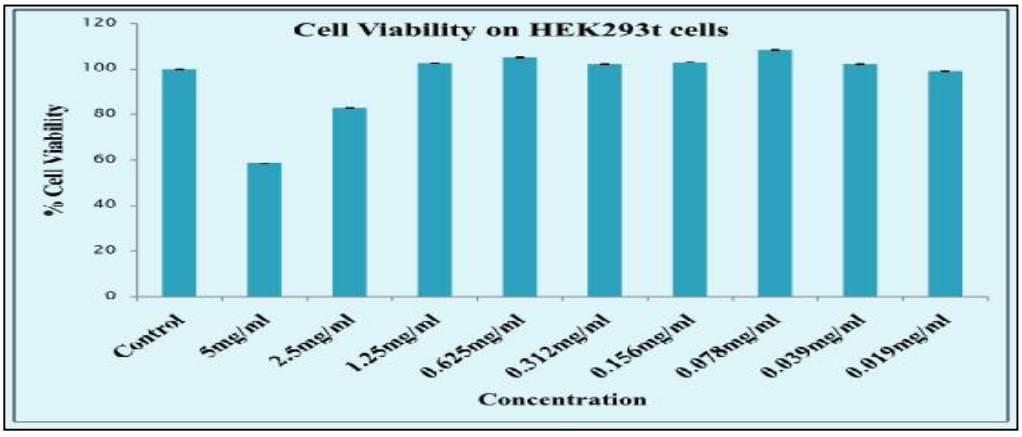
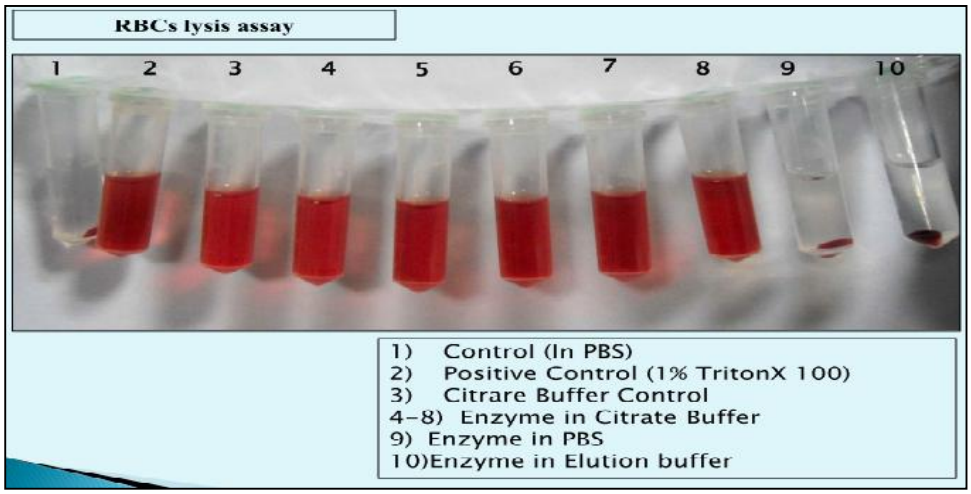


Fig. 94 Viscosity of 1% CMC after treatment with PR-1 (Approx 1.2 cP value)

In vitro toxicity evaluation of PR-1 on cultured cells as well on RBCs: The objectives included toxicity evaluation using MTT assay on HEK293t cells. Toxicity evaluation using RBCs lysis assay was performed for understanding its blood compatibility. Results showed the toxicity determination for PR-1 at different concentrations on HEK293t cells using MTT assay. Result demonstrated low toxicity induction on cells due to treatment with PR-1 in the range of 2.5 mg/ml to 19 µg/ml concentration. The results can be utilized to plan animal experiments for the efficacy study of PR-1 on glucose lowering (Fig. 95).





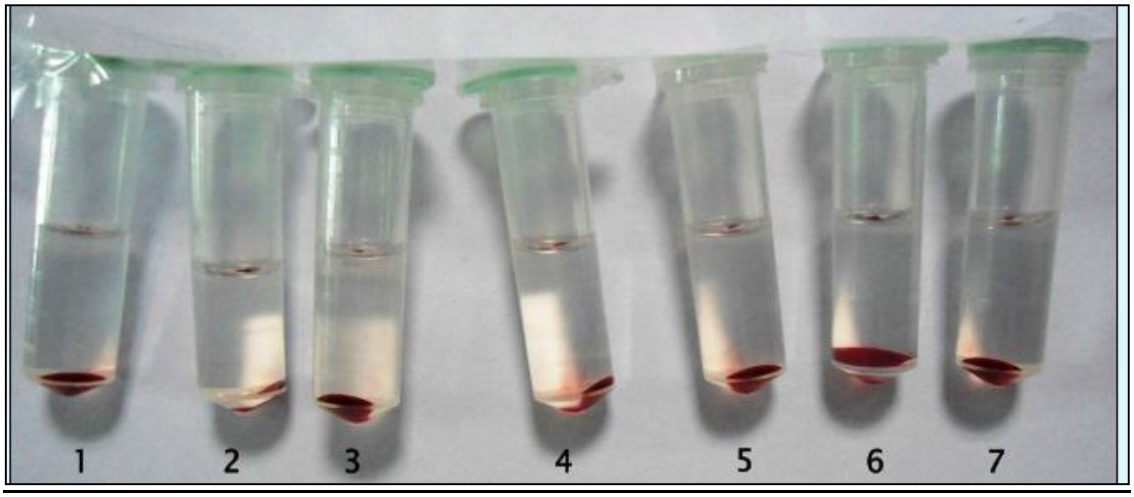


Fig. 95 *In vitro* RBCs lysis assay

Results demonstrated that the PR-1 has no RBC lysis activity when compared with positive control (Triton X100). Lower image showed no toxicity of PR-1 at different concentrations (100 µg/ml to 10 mg/ml).

In vivo **experiments for understanding glucose lowering action of PR-1 (Using different dose routes: IP- intraperitoneal, IM- intramuscular and IV-intravenous):** The objectives included developing Normal OGTT curve in Rats. And evaluating the effect of PR-1 on OGTT pattern after injecting at 1mg dose via various routes. Results showed the effects of PR-1 on OGTT pattern in normal rats. Results demonstrated that PR-1 shifts the normal OGTT curve significantly. It showed the potential glucose lowering effects of PR-1 in rats (Fig. 96 to 99).

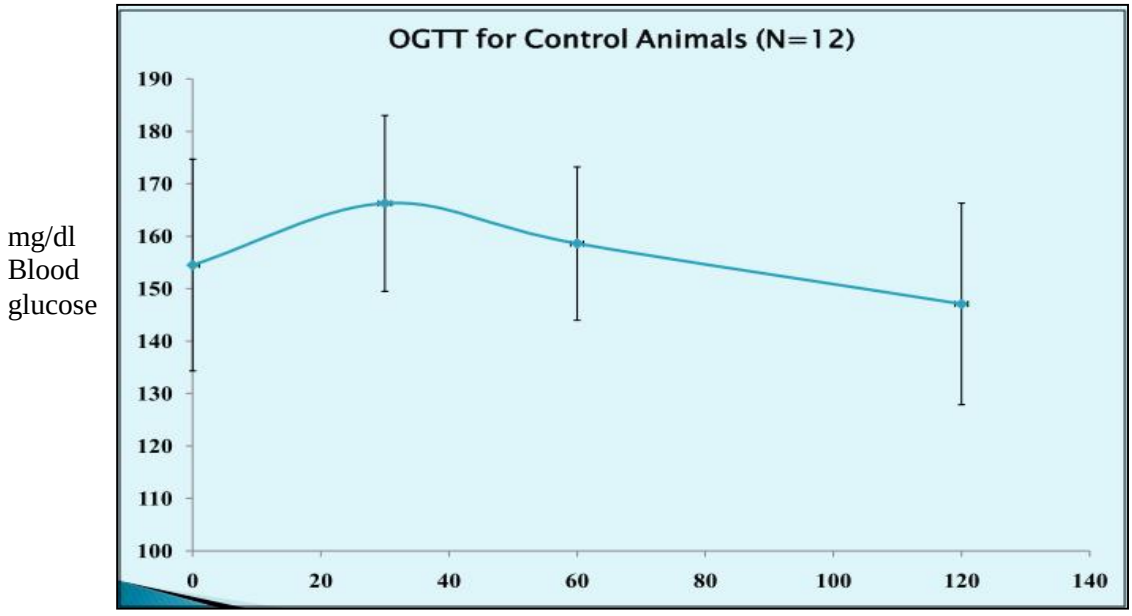


Fig. 96 OGTT curve in Normal Rats

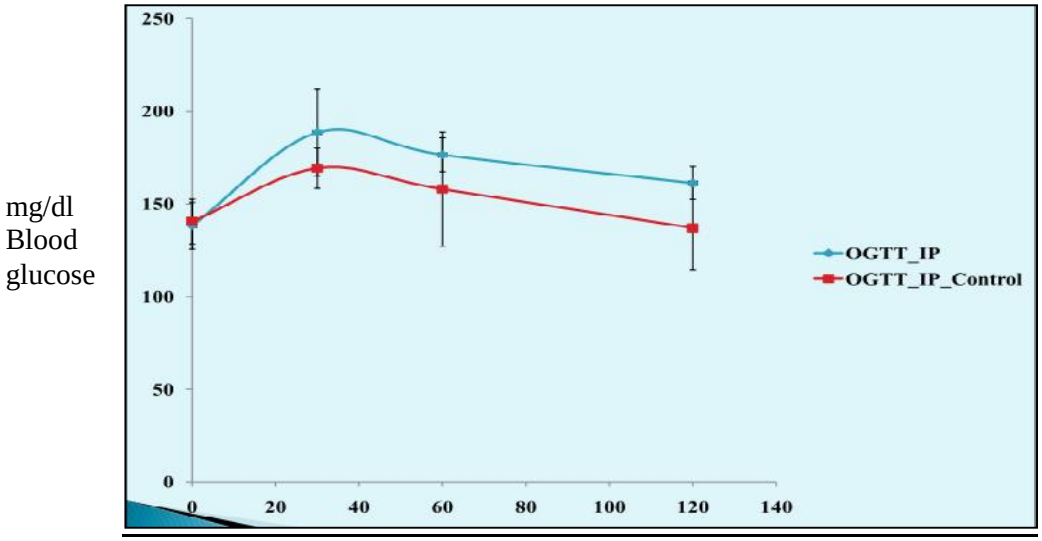


Fig. 97 OGTT pattern after PR-1 injection via IP route

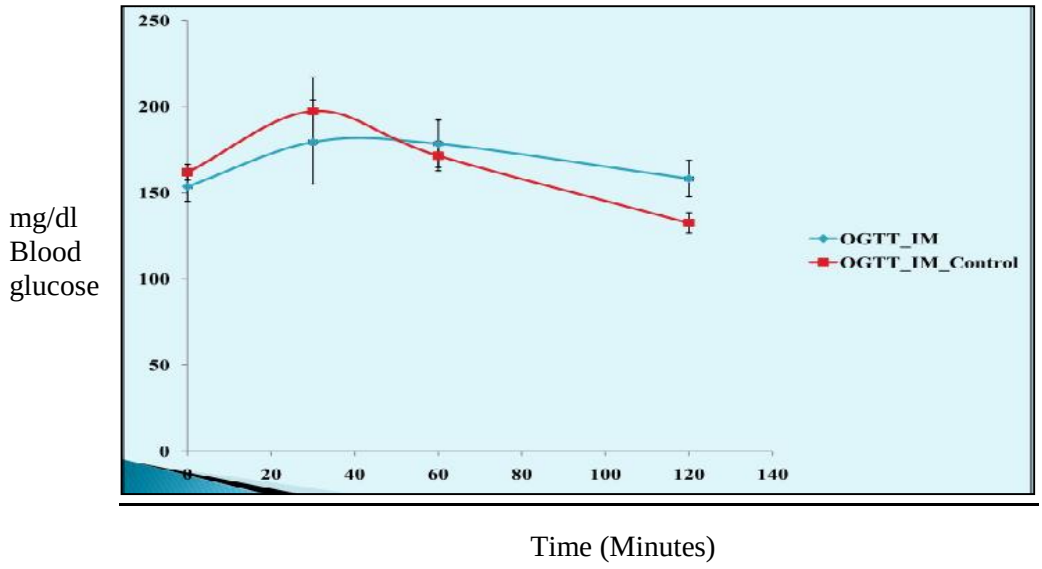


Fig. 98 OGTT pattern after PR-1 injection via IM route

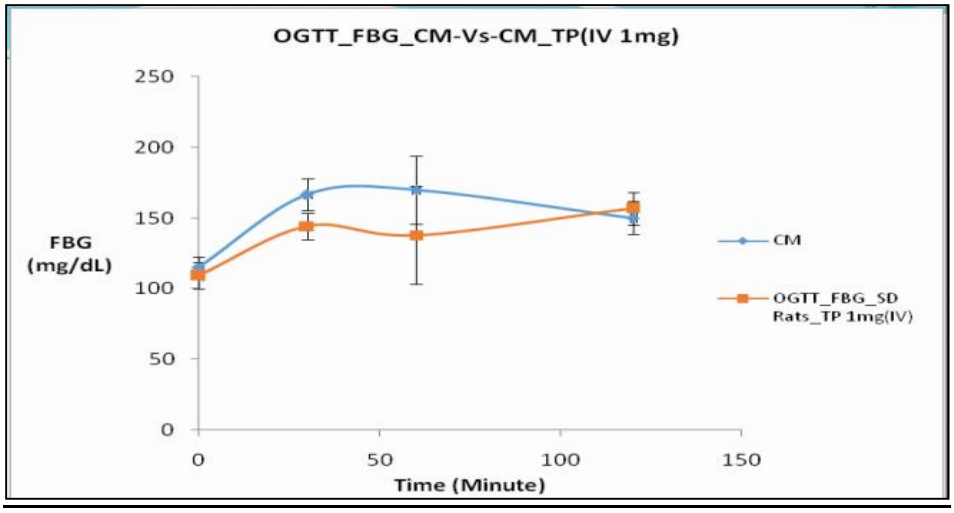


Fig. 99 OGTT pattern after PR-1 injection via IV route

Single Pass Perfusion of rat Intestine to understand the effects of PR-1 in glucose absorption: To check the effects of PR-1 in glucose absorption (Fig. 100 to 103).

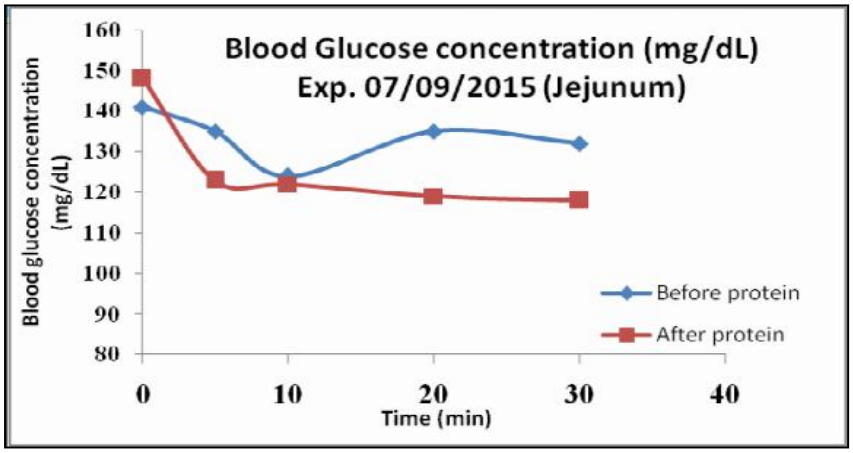


Fig. 100 Blood Glucose concentration (mg/dL) before and after PR-1 treatment (Jejunum Section)

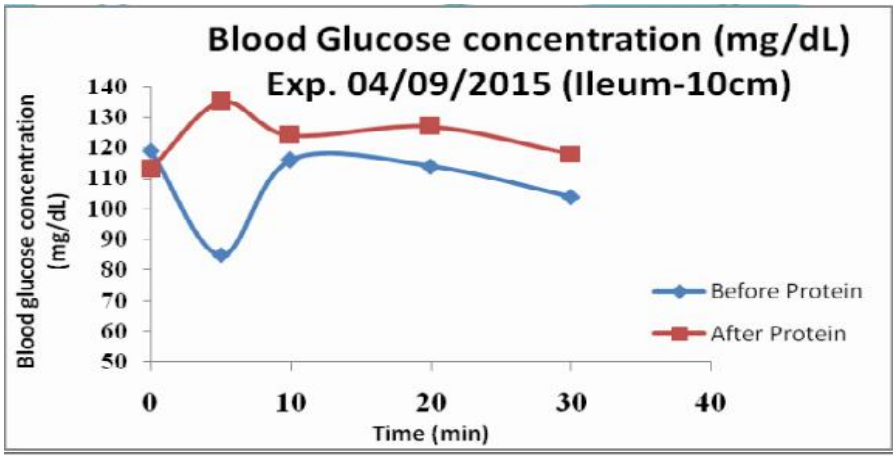


Fig. 101 Blood Glucose concentration (mg/dL) before and after PR-1 treatment (Ileum Section)

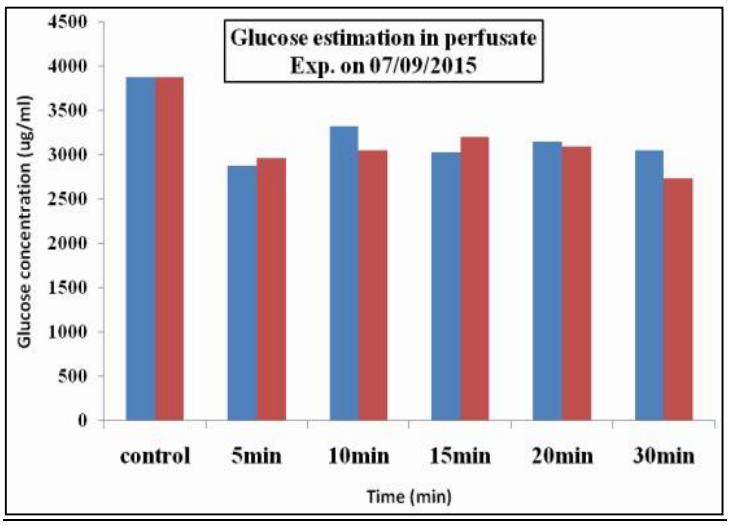


Fig. 102 Estimation of glucose in perfusate before and after PR-1 (Jejunum Section)

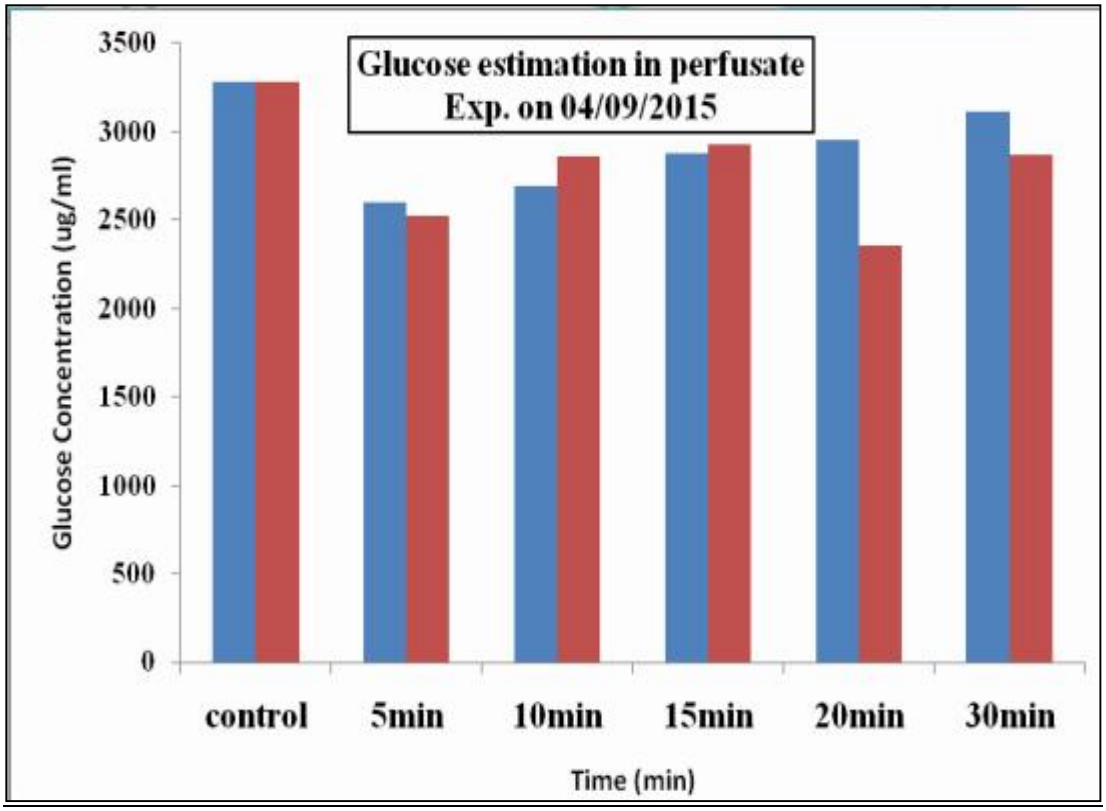
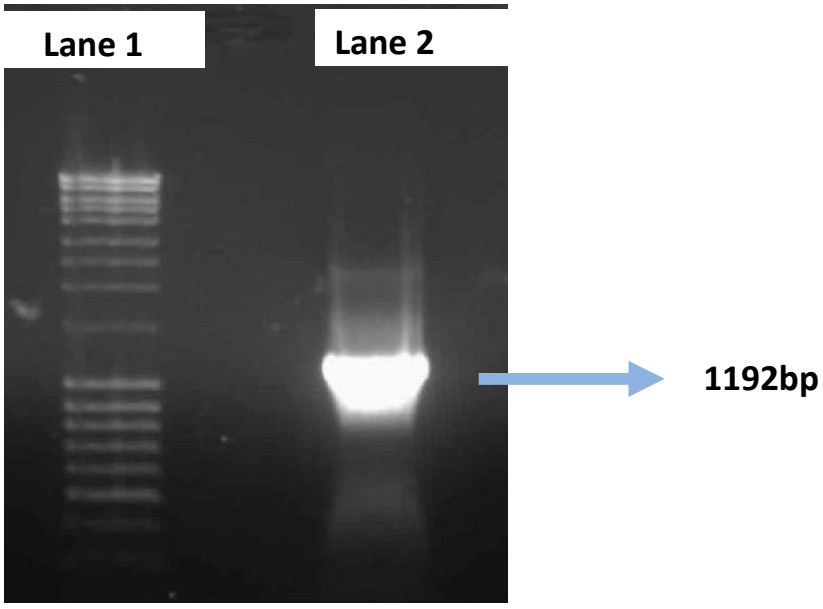


Fig. 103 Estimation of glucose in perfusate before and after PR-1 (Ileum Section)

Cel PRII enzyme from Mehsani rumen metagenome

A total of 2597 contigs, encoding putative CAZymes were identified by CAT analysis which showed hits corresponding to 87 different CAZy families. The 4 prime Carbohydrate Active Enzyme families: Glycoside Hydrolases (GH), Glycoside Transferases (GT), Carboxyl Esterase (CE) and Polysaccharide Lyase (PL) were represented by 1929, 373, 259, and 17 contigs, respectively. The full length ORF encoding D5EU37 was further cloned in pET32a, an *E. coli* expression vector. The expression of recombinant protein in BL21 (DE3)pLysS *E.coli* host revealed expression of insoluble protein with expected molecular weight of 60 kDa fusion protein of His-Tag and D5EU37. The *E.coli* transformants for D5EU37 showed cellulolytic activity on carboxymethyl cellulose. The purified protein showed activity on carboxy methyl cellulose. The enzyme was stable till 60°C and gave an optimal enzyme activity at 40°C. The maximum enzyme activity was observed at pH 7.0 and was stable in a pH range of 2.0-10.0. The enzyme also showed efficient activity in presence of K^{+} and divalent metal ions viz. Ca^{+2}, Mg^{+2}. In presence of chelating agent EDTA, comparatively less activity was observed and no activity was observed in presence of detergent SDS.

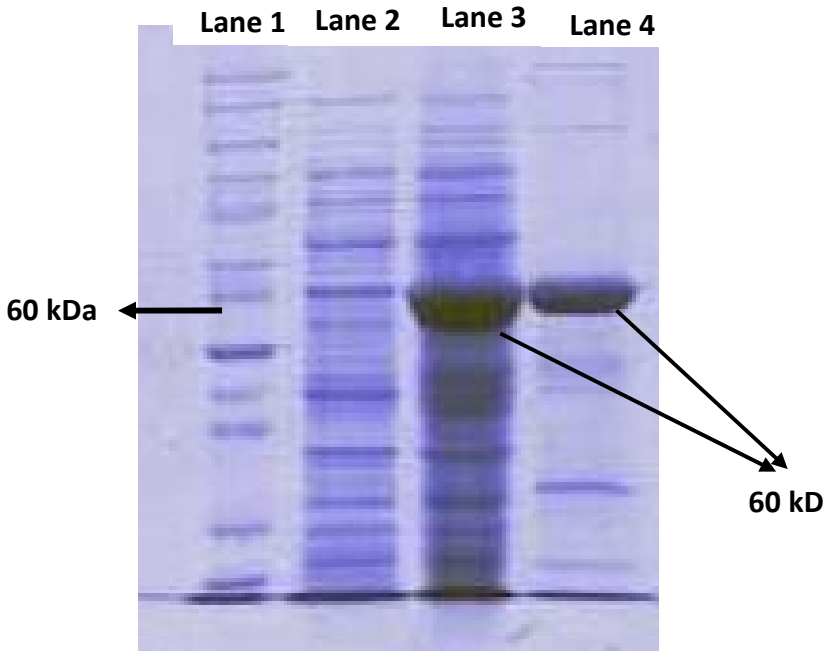


PCR amplification of Cel PRII encoding gene from Buffalo rumen metagenome.

Lane 1: Fermentas high range + low range DNA ladder.

Lane 2: Amplified product of 1192bp.

SDS-PAGE analysis for Cel PRII enzyme:



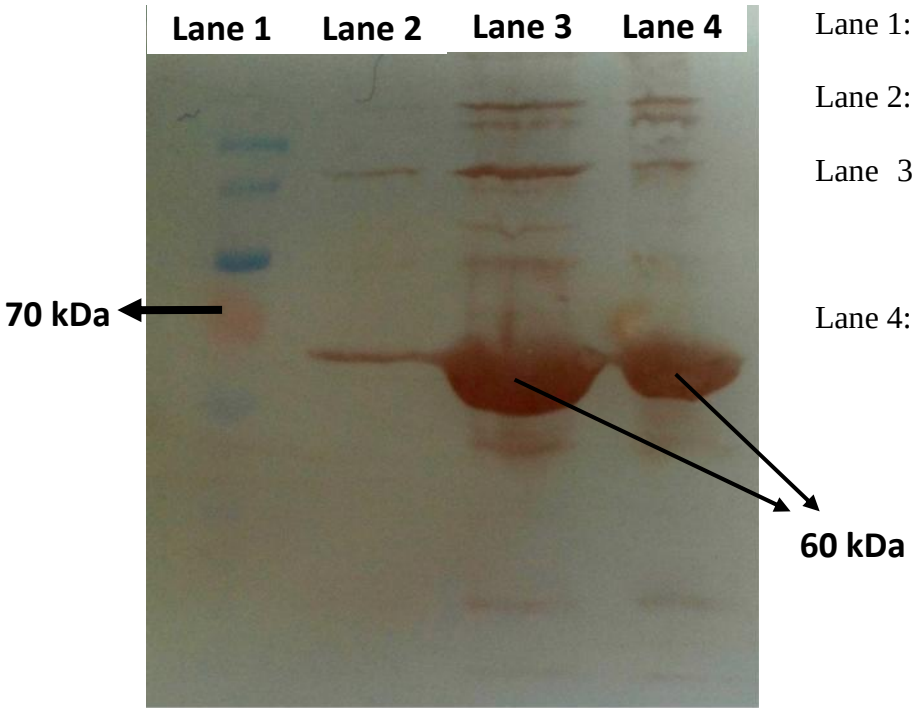
Lane 1:EZ run pre-stained protein ladder

Lane 2: Non-induced Cel PRII.

Lane 3: Induced Cel PRII crude lysate showing 60kDa band

Lane 4: Purified Cel PRII.

Western blotting for Cel PRII enzyme:



Lane 1:EZ run pre-stained protein ladder

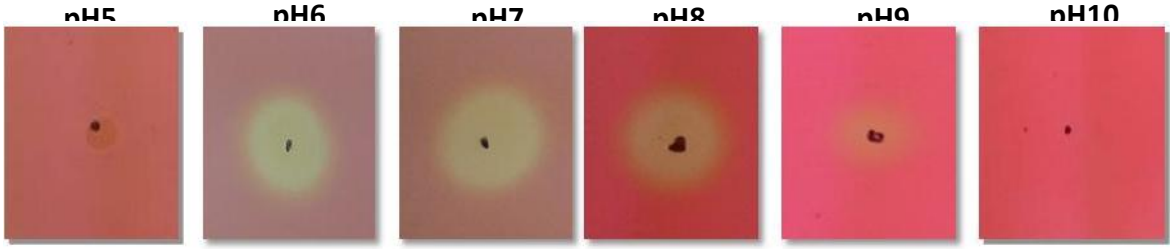
Lane 2: Non-induced Cel PRII.

Lane 3: Induced Cel PRII crude lysate showing 60kDa band

Lane 4: Purified Cel PRII.

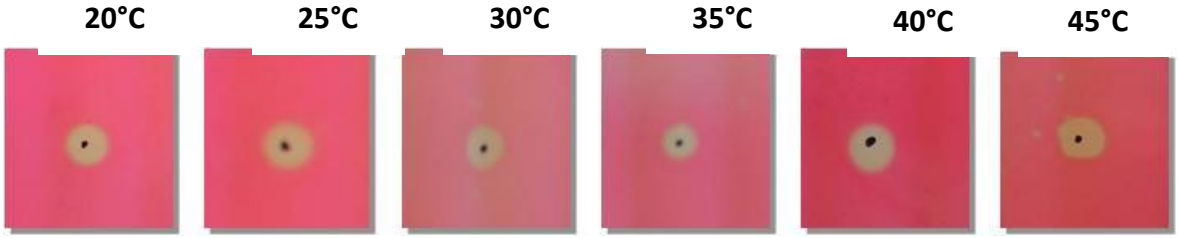
Celluloytic activity of Cel PRII enzyme on CMC plate:

pH optimization for Cel PRII enzyme:Enzyme gives best activity at pH 7.



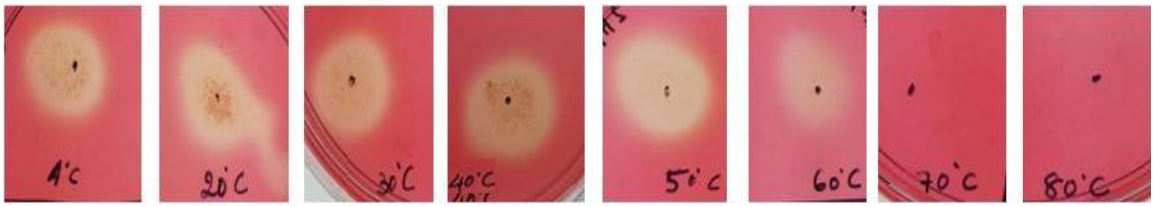
Temperature optimization for Cel PRII enzyme:

Purified enzyme was pre-incubated at a temperature range of 20°C- 45°C for 1 hour and the residual activity was measured by spotting on L.Agar+CMC plate. Enzyme gives best activity at 40°C.



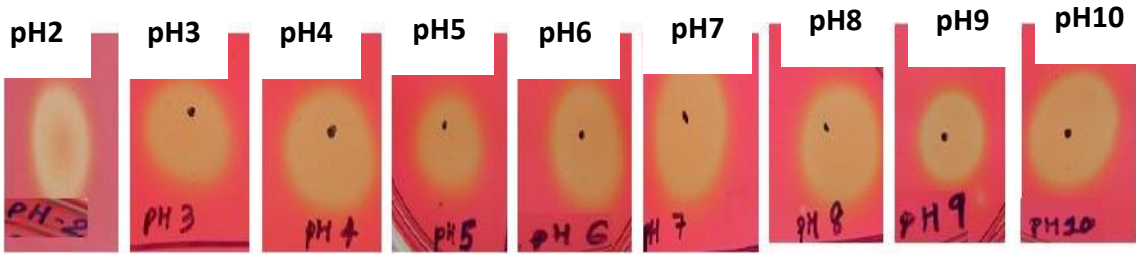
Temperature stability for Cel PRII enzyme:

Purified enzyme was pre-incubated at a temperature range of 4°C-80°C and the residual activity was observed by spotting on L.Agar+CMC plate. Enzyme is stable till 60°C.



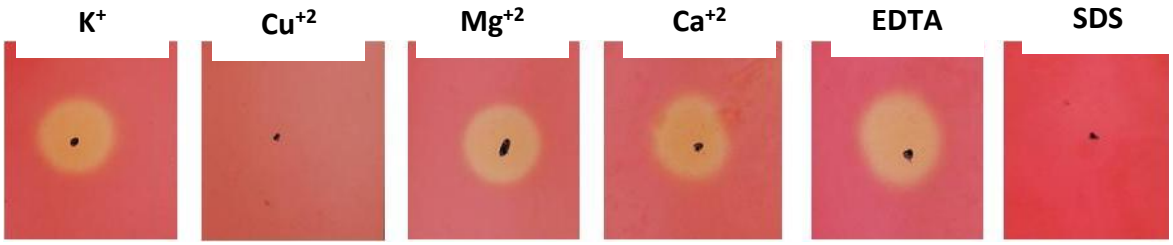
pH stability for Cel PRII enzyme:

Purified enzyme was incubated for 48 hrs in 50mM phosphate buffer ranging from pH 2.0- 10.0 and spotted on L.Agar + CMC plate. Enzyme is stable from pH 2.0 till pH 10.0



Effect of metal ions (K^{+}, Cu^{+2}, Mg^{+2}, Ca^{+2}), EDTA and SDS on Cel PRII enzyme:

Purified enzyme was spotted on L.Agar+CMC plate consisting of metal ions having 10mM final concentration in media. Enzyme activity is influenced in the presence of chelating agent EDTA and detergent SDS.

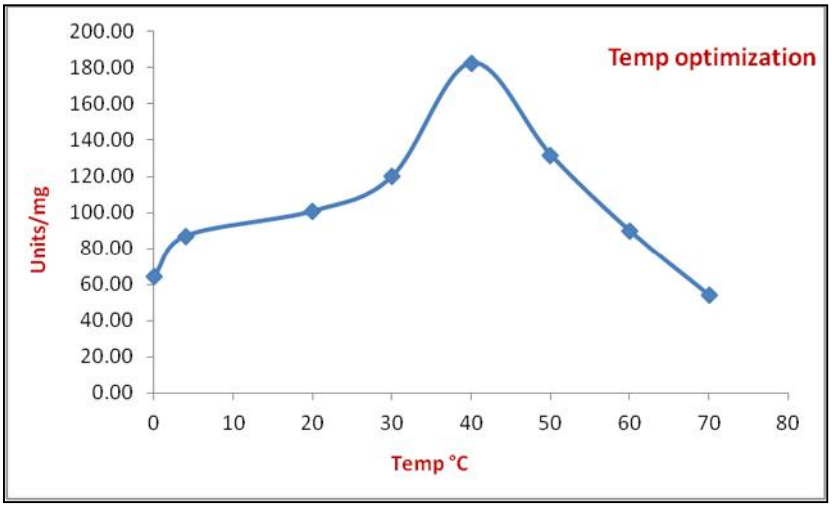


To determine the enzymatic activity DNS-based enzyme assays for Cel PRII was performed by using 5% carboxymethyl cellulose as substrate and enzyme concentration of 100µg. The enzyme-substrate reaction was incubated for 1h and reaction was stopped by adding DNS (3, 5- dinitrosalicylic acid) reagent and heating in boiling water bath for 10 min. The absorbance was read at 540nm using Tecan plate reader. Glucose with

different concentrations (50µg-800µg) was used as a standard. One unit of enzyme activity is defined as one micromole of glucose released per minute.

Temperature optimization for Cel PRII

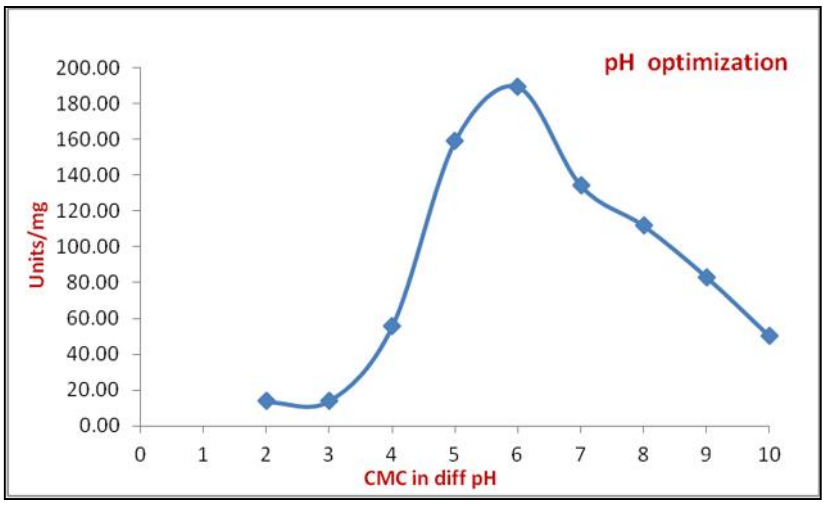
Optimum temperature for Cel PRII was determined by incubating the enzyme-substrate reaction at a temperature range of 0°C -80°C and further activity was determined as described above. The enzyme shows best activity at 40°C.



Temperature optimization

pH optimization for Cel PRII

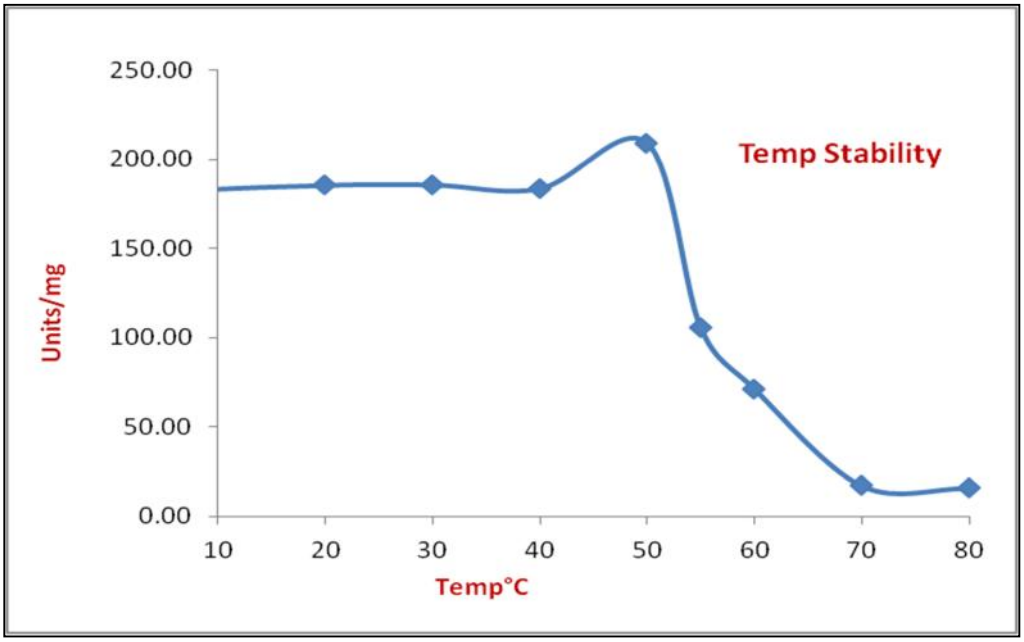
For determining optimum pH of enzyme, substrate was prepared in 50mM citrate buffer of various pH ranging from 2 to 10.The reaction was carried out as mentioned above. The enzyme showed best activity at pH 6.



pH optimization

Temperature stability for Cel PRII

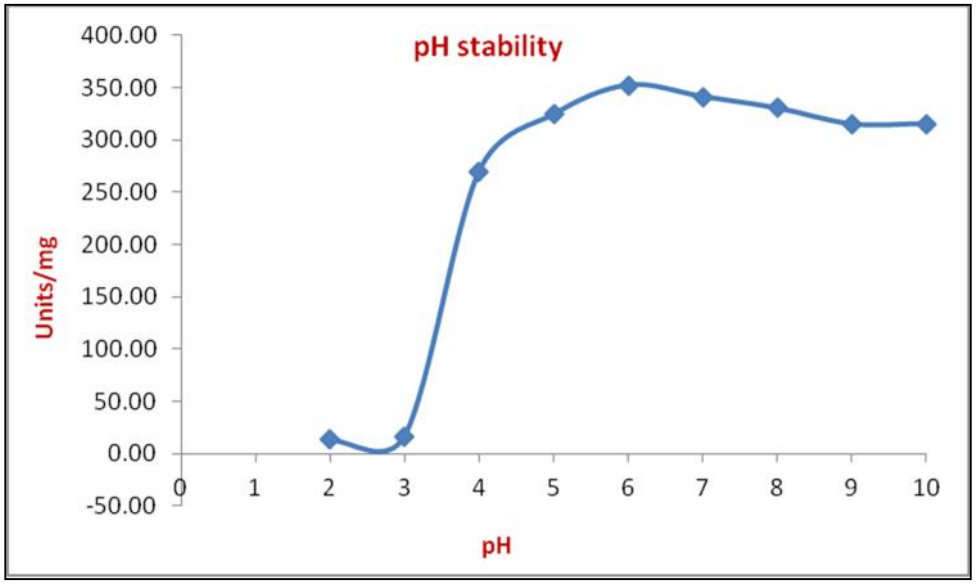
Purified enzyme was pre-incubated at a temperature range of 10°C - 80°C for 1h and further it was incubated with substrate and activity was determined as described above. The enzyme shows stability till 55°C.



Temperature stability

pH stability for Cel PRII

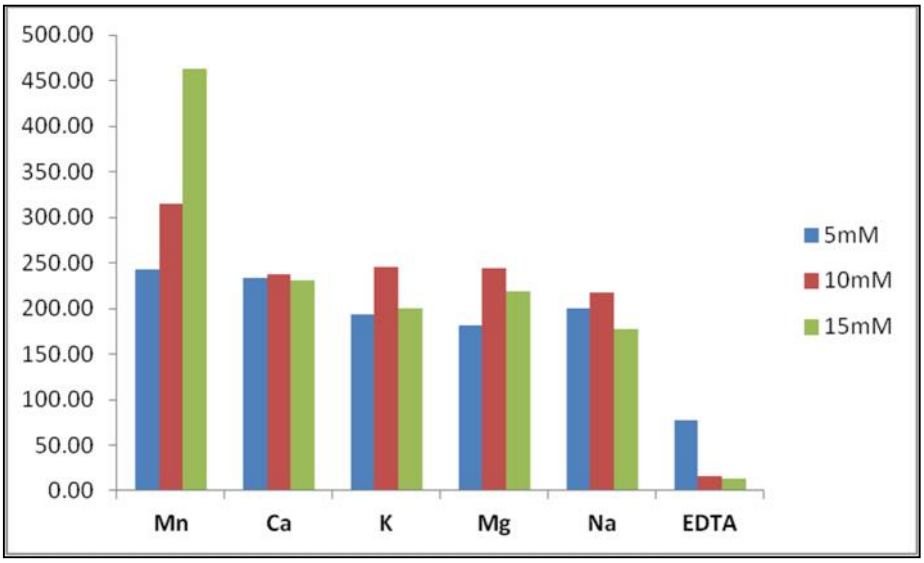
For determining the pH stability of Cel PRII, enzyme was incubated in citrate buffer of pH 2.0 – pH 10.0 for 48 hrs and the residual activity was measured using DNS method as described above. The enzyme is not much stable under highly acidic conditions (pH 2.0 and pH 3.0), but stable in range of pH 4.0 -pH 10.0.



pH stability

Effect of metal ions and chelating agent on Cel PRII activity

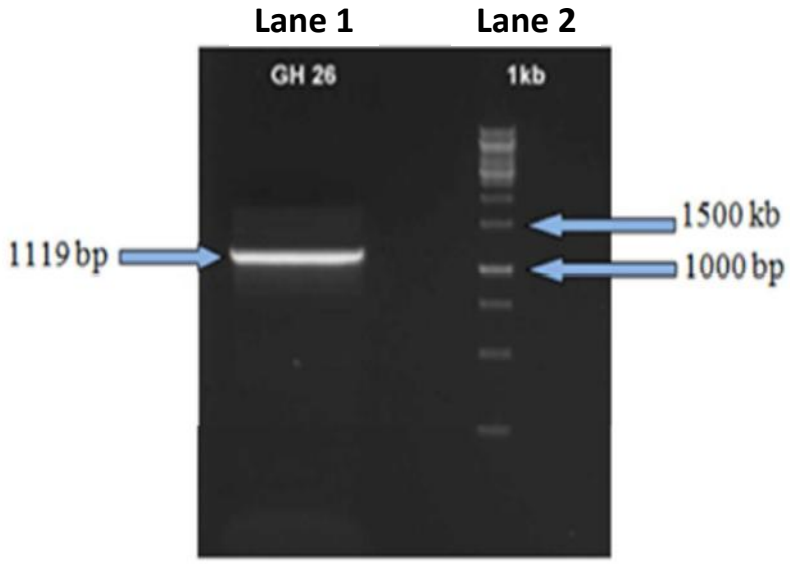
Metal ions viz. Mn^{+2}, Ca^{+2}, K^{+}, Mg^{+2}, Na^{+} and EDTA was supplied to the enzyme-substrate reaction in a range of 5mM, 10mM and 15mM. The activity was determined as described above. In case of metal ions, in presence of 15mM Mn^{+2}, highest activity was recorded, while in case of other ions, no significant effect of activity was observed. In presence of EDTA, enzyme activity was hindered and less activity was recorded.



Effect of metal ions and chelating agents

Novel Multifunctional Recombinant Family 26 Glycoside Hydrolase from Mehsani Buffalo Rumen Metagenome

A novel GH26 gene comprised of 1119 base pairs was successfully amplified using the gene specific primers inferred based on the contig generated from metagenome sequence assembly and cloned in pET32a (+) expression vector as N-terminal histidine tag fusion protein. A novel GH26 protein from unknown rumen microorganism shared a maximum of 68% identity with the *Prevotella ruminicola* 23 encoded carbohydrate esterase family 7 and 46% with *Bacteroides* sp. 2_1_33Bl encoded mannan endo-1, 4-beta-mannosidase. The purified enzyme displayed multifunctional activities against various carbohydrate substrates including locust bean gum, beech wood xylan, pectin and carboxymethyl cellulose. Clear zone of hydrolysis was observed on LBG, beech wood xylan, CMC and pectin substrate plates. Optimum activity of the enzyme was observed at pH 5.0 and 6.0 and optimum activity at temperature ranging from 4ºC to 45ºC, with slightly higher activity at 35ºC. The enzyme was found to be stable at pH ranging from 5.0 to 9.0.

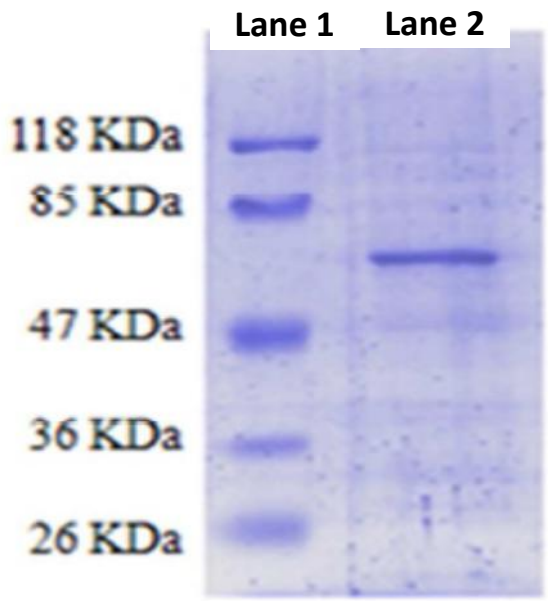


PCR Amplification:

Lane 1: 1119 bp amplicon of GH26 gene

Lane 2: O' Gene Ruler 1Kb

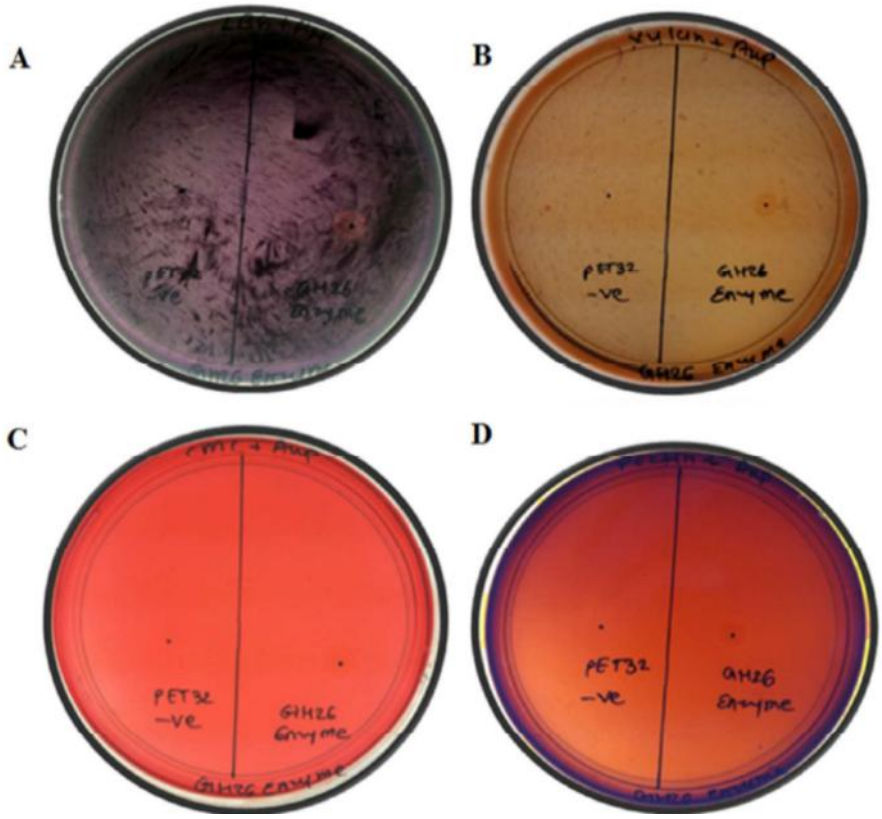
SDS-PAGE analysis of GH26 protein:



Lane1: EZ run prestained protein Ladder

Lane2: Purified 59.5 kDa GH26 protein with N terminal His tag

Activity profile of GH26 protein:



Clear zone of hydrolysis observed for the following substrates:

(A) LBG

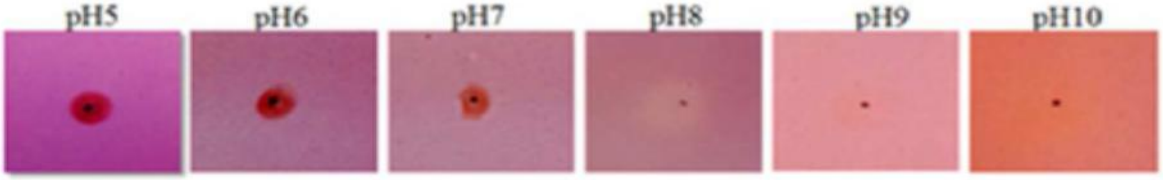
(B) Beechwood Xylan

(C) CMC

(D) Pectin

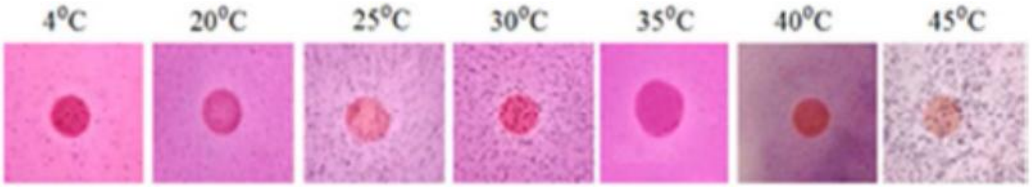
pH optimization for GH26 enzyme :

Purified enzyme was spotted on L. Agar + LBG plate having pH 5.0-10. Enzyme gives best activity at pH 5.0 and 6.0.



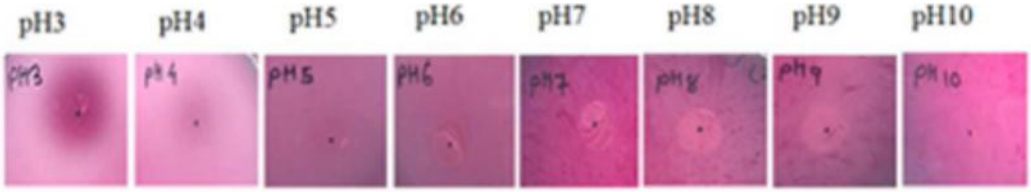
Temperature optimization for GH26 enzyme:

Purified enzyme was spotted on L.Agar+ LBG plate and incubated at a temperature range of 4°C- 45°C . Enzyme gives best activity at 35°C.



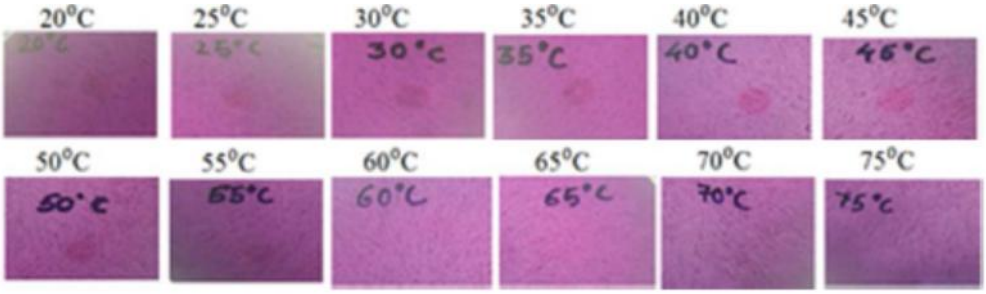
pH stability for GH26 enzyme:

Purified enzyme was incubated for 48 hrs in 50mM citrate buffer ranging from pH 3.0- 10.0 and spotted on L. Agar + LBG plate. Enzyme is stable from pH 5.0 till pH 9.0.



Temperature stability for GH26 enzyme:

Purified enzyme was pre-incubated at a temperature range of 20°C-75°C and the residual activity was measured by spotting on L.Agar + LBG plate. Enzyme is stable till 45°C.



Potent mannanase C_4373 enzyme from Mehsani rumen metagenome:

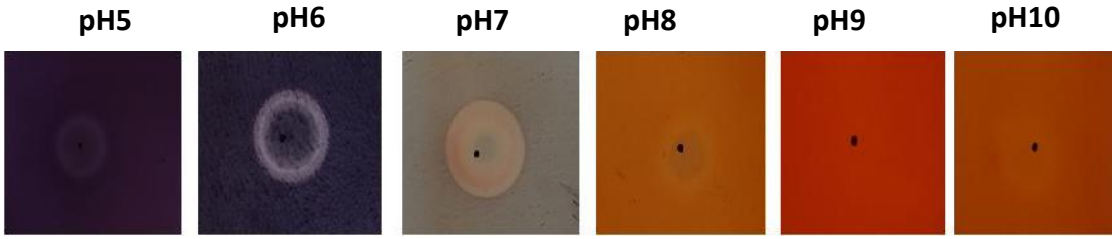
A potent mannanase showing high activity on locust bean gum (LBG) substrate was cloned from metagenome of Mehsani breed of buffalo. Shotgun sequencing of rumen microbiota, followed by CAZyme analysis, cloning in *E. coli* and characterization yielded insoluble protein of 50kDa showing potent mannanase activity against Locust bean gum (LBG)substrate. The enzyme was purified under denaturing conditions and recovered by column refolding. Optimum activity of enzyme was observed at pH 7.0

Mannanase activity of C_4373 clone on LBG plate:

Stability and Optimization assays for C4373 enzyme:

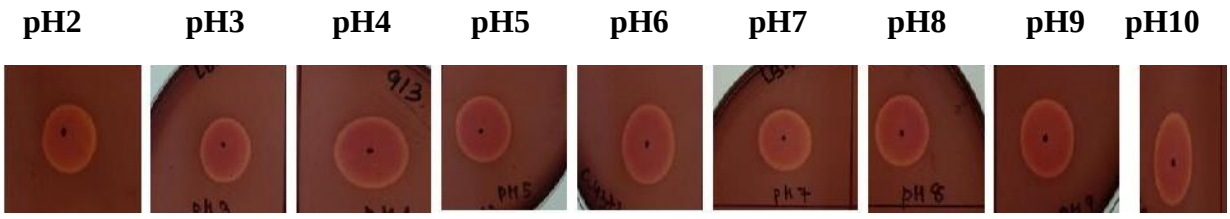
pH optimization for C_4373 clone:

Purified enzyme was spotted on L.Agar + LBG plate of pH 5-10. Enzyme gives best activity at pH 7.

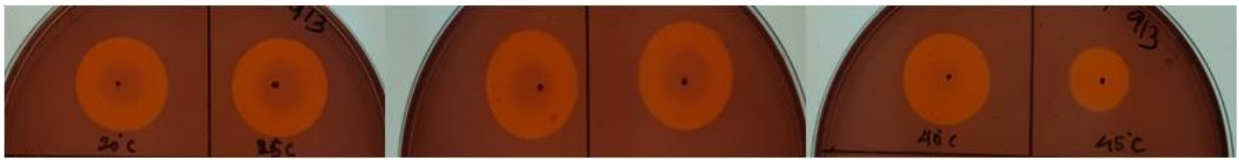


pH stability for C_4373 clone:

Purified enzyme was incubated for 48 hrs in 50mM citrate buffer ranging from pH 2- 10 and spotted on L.Agar + LBG plate. Enzyme is stable from pH 2 till pH 10.

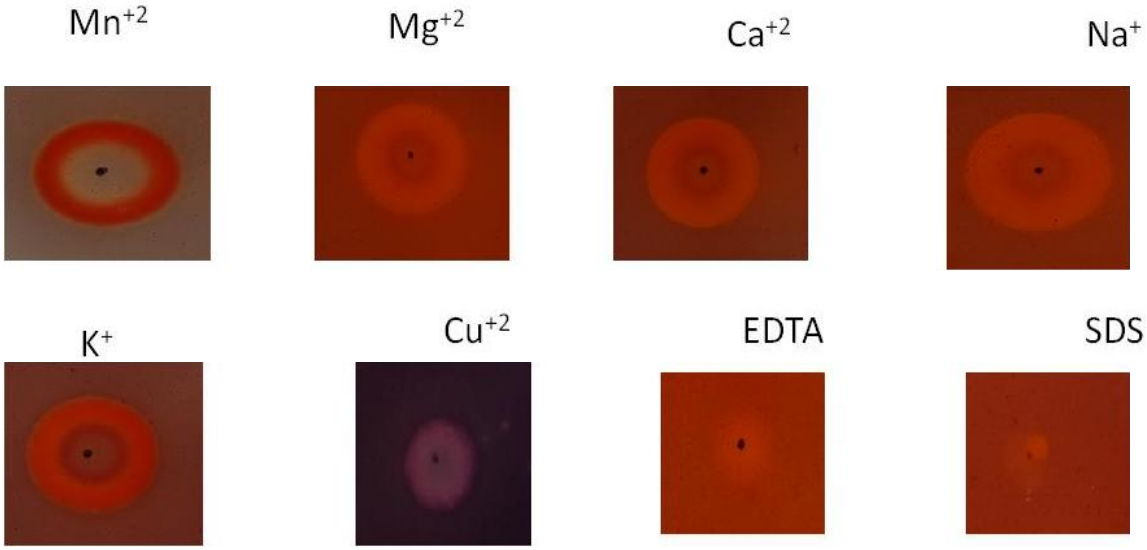


Temperature stability for C_4373 clone:



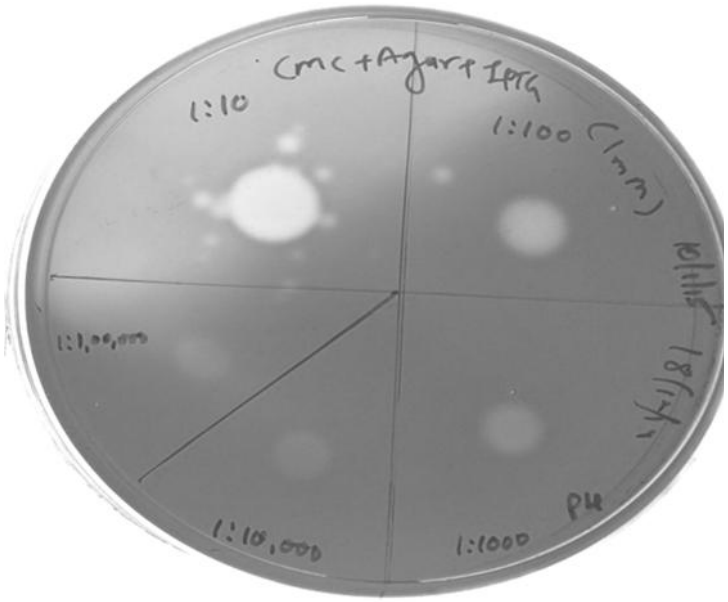
Effect of metal ions on enzymatic activity:

Purified enzyme was spotted on L. Agar + LBG plate supplemented with metal ions having 10mM final concentration in media. Enzyme activity is influenced by presence of chelating agent EDTA and SDS.



Characterization of P4 cellulase enzyme from the metagenomic DNA of Gir cattle:

P4 cellulase belonging to GH5 family showed potent activity against carboxy methyl cellulose (CMC).

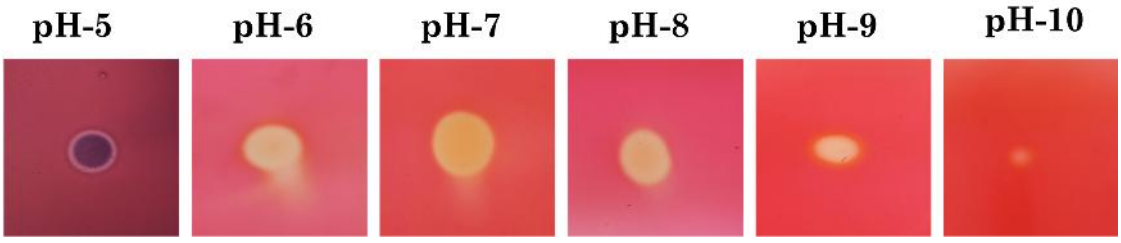


Activity of P4 cellulase on CMC plate at different dilutions.

Enzyme P4 cellulase shows activity up to 1, 00,000 dilutions on CMC plate.

pH optimization

For optimum pH determination enzyme was spotted onto CMC plate having different pH (5-10)

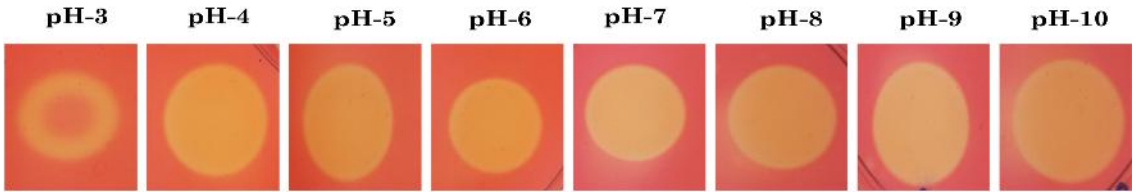


pH optimization for P4 cellulase enzyme

Enzyme P4 cellulase shows best activity at pH 7.

pH stability

To determine the pH stability for P4 cellulase, enzyme was incubated into buffer of pH-3 to pH-10 for 48 hrs at 4ºC and then spotted onto CMC plate.

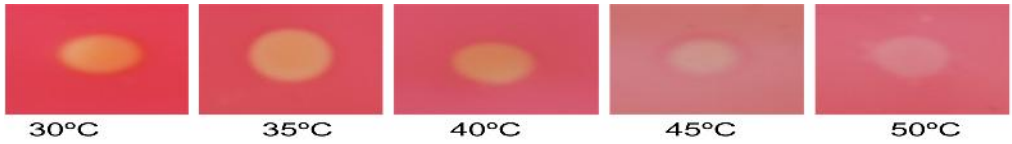


pH stability for P4 cellulase enzyme

Enzyme P4 cellulase is stable in the pH range of 3-10

Temperature optimization

Enzyme was spotted onto CMC plate having pH-7 and incubated at different temperature. (30-50ºC)

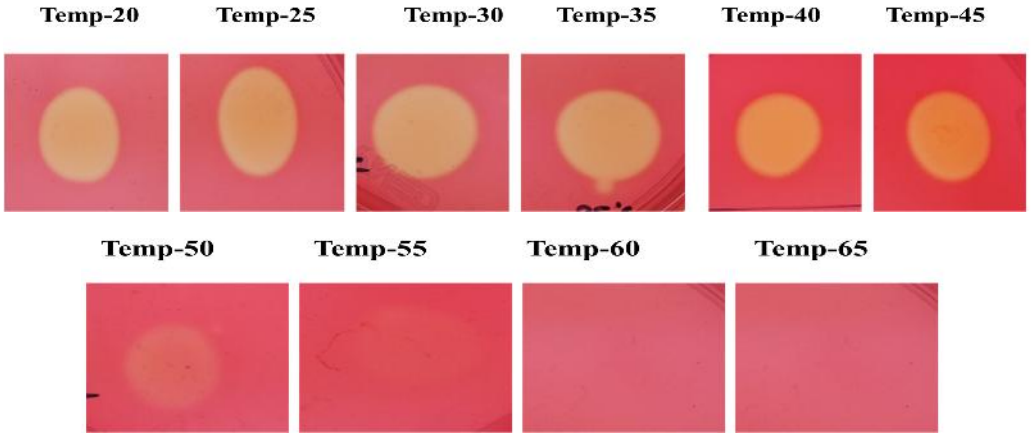


Temperature optimization for P4 cellulase

Enzyme shows good activity at 35°C

Thermo stability of P4

Enzyme was pre-incubated at different temperature (20-65ºC) for 1 Hr and then spotted onto CMC plate of pH-7.

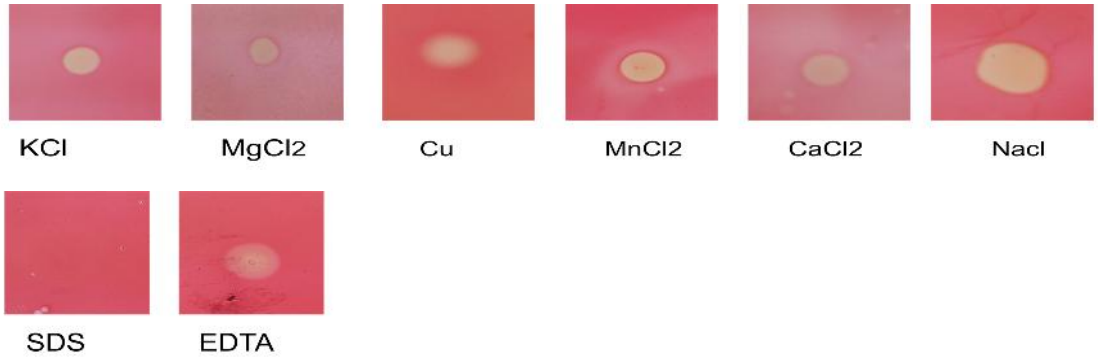


Thermostability for P4 cellulase enzyme

P4 cellulase is stable up to 45ºC. At higher temperatures like 50 ºC and 55 ºC enzyme starts to lose its activity and above 55 it loses its activity completely.

Effect of metal chlorides, EDTA and detergent (SDS)

Different metal chlorides, EDTA and SDS were added into medium at the concentration of 10mM to determine the effect of additives on activity of P4 cellulase



Enzyme shows good activity in presence of NaCl, KCl and $MnCl_2$, while in presence of Cu, $CaCl_2$ and EDTA activity of enzyme gets reduced. In the presence of SDS enzyme loses its activity.

P4 cellulase was purified using His60 column according to the manufacturer's protocol and subsequently used for characterization. The enzyme characterization (pH and Temperature) was done using 100ug of enzyme (Final concentration in the reaction volume). 2.5% Carboxymethyl cellulase was used as substrate in the reaction (Fig. 104 to 106). For pH optimization, enzyme was reacted with substrate made in a citrate buffer with different pH (2 to 12). Stability was checked after incubating the enzyme in a citrate buffer with different pH for 24 hr and one week. Temperature optimization and stability was done using temperature range from 0 to 60 at an interval of 5^0C.

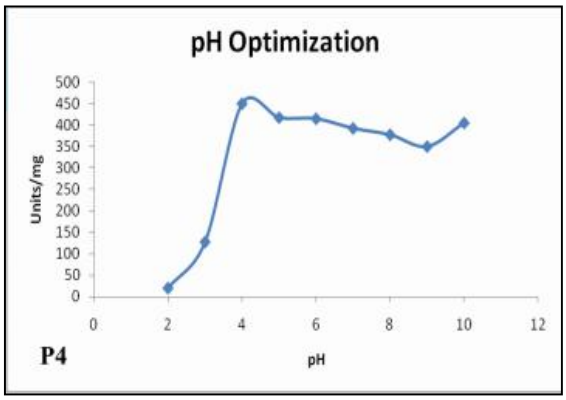


Fig. 104 pH Optimization

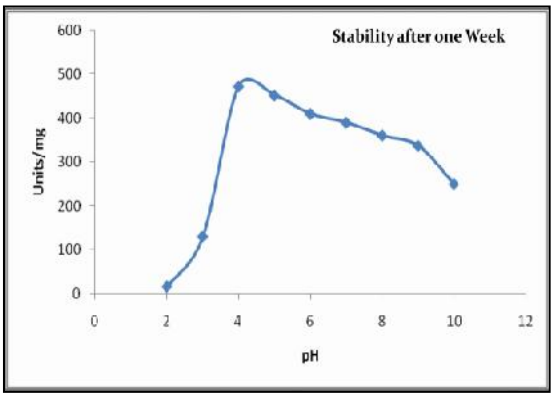


Fig. 105 pH stability

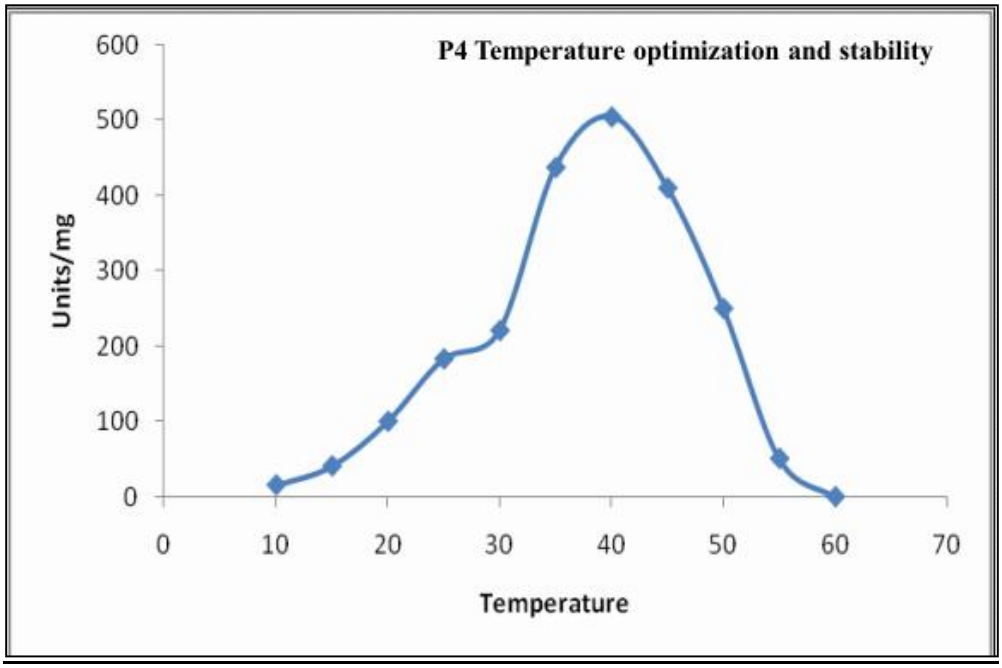


Fig. 106 Temperature optimization and Stability

Immobilization of P4 enzyme on chitosan coated iron oxide nano particles: For recycling the same enzyme for more number of reactions, immobilization was done using chitosan coated nano-particles made of iron oxide (Fe_3O_4). Synthesized chitosan coated particles were characterized using modern tools like FTIR, TGA and XRD. After successful coating with chitosan, P4 enzyme was immobilized on to the coated particles and used in a reaction volume for several times (in this case, 17 cycles). After the end of reaction, free sugars were detected using DNSA method and activity was determined (Fig. 107 to 113).

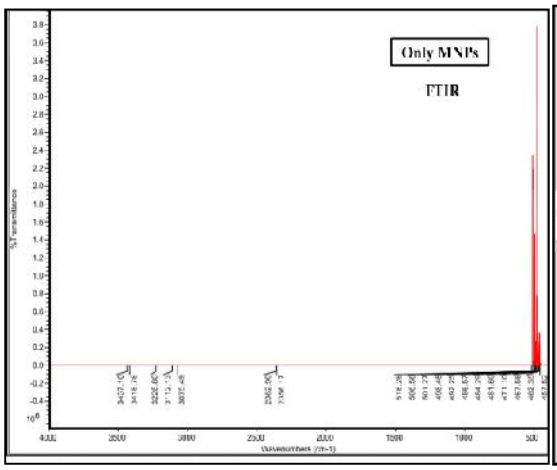


Fig. 107 FTIR of synthesized iron oxide nano particles

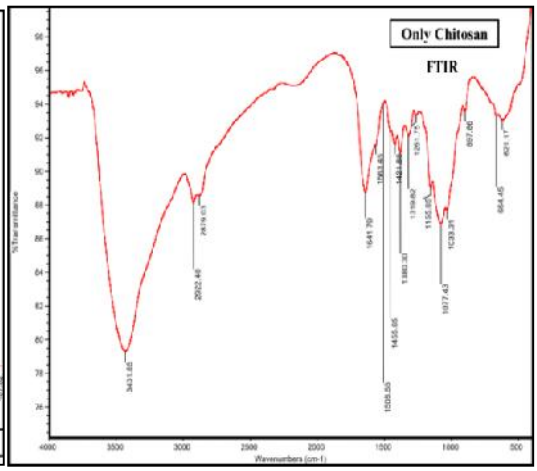


Fig. 108 FTIR of Chitosan

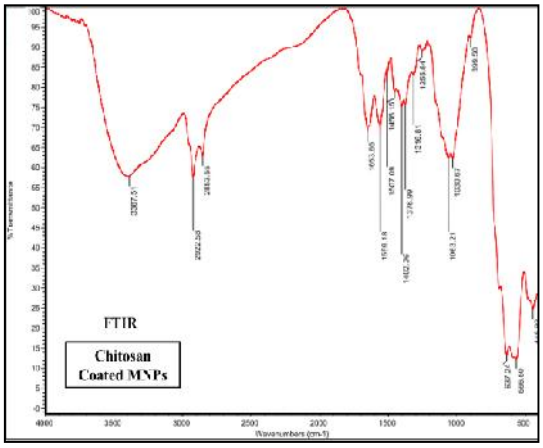


Fig. 109 FTIR of Chitosan coated nano particles

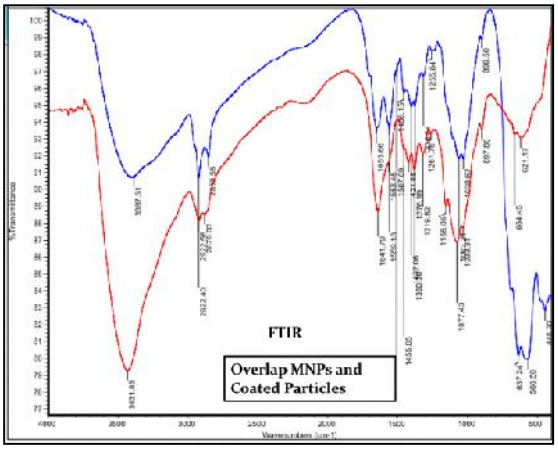


Fig. 110 Overlay of Chitosan and coated particles

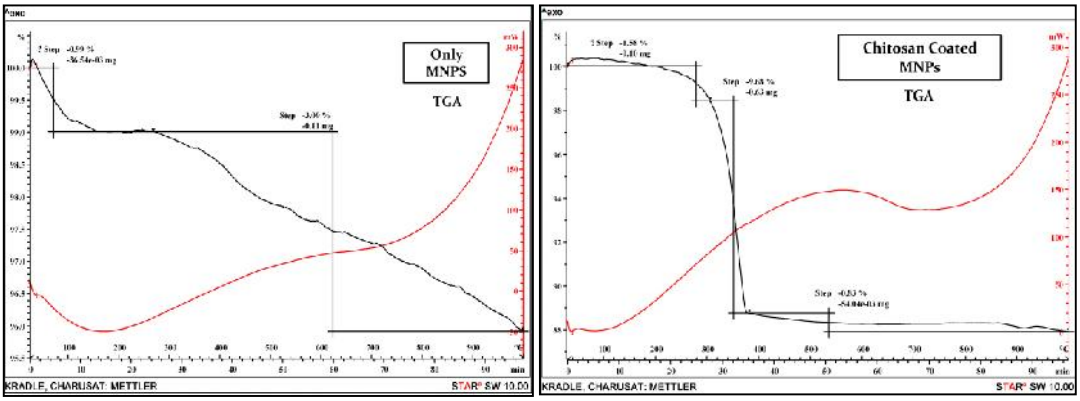


Fig. 111 Thermogravimetry Analysis (TGA) of coated and non coated particles

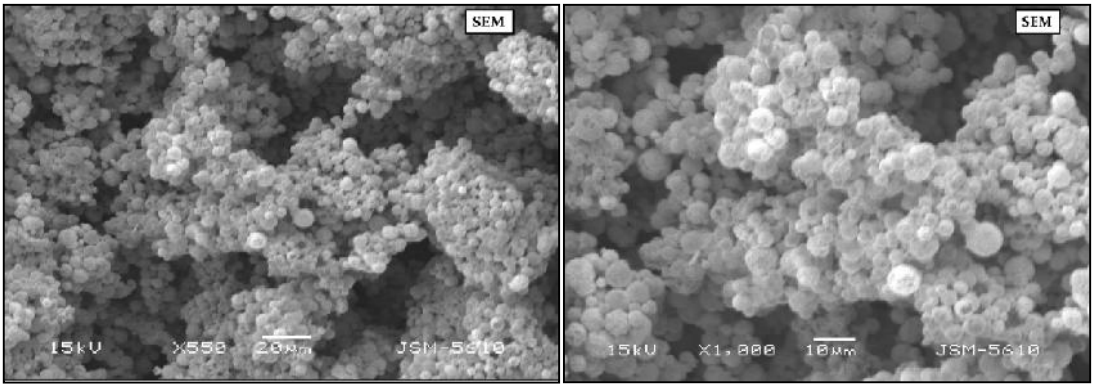


Fig. 112 Scanning electron microscope (SEM) images of coated particles

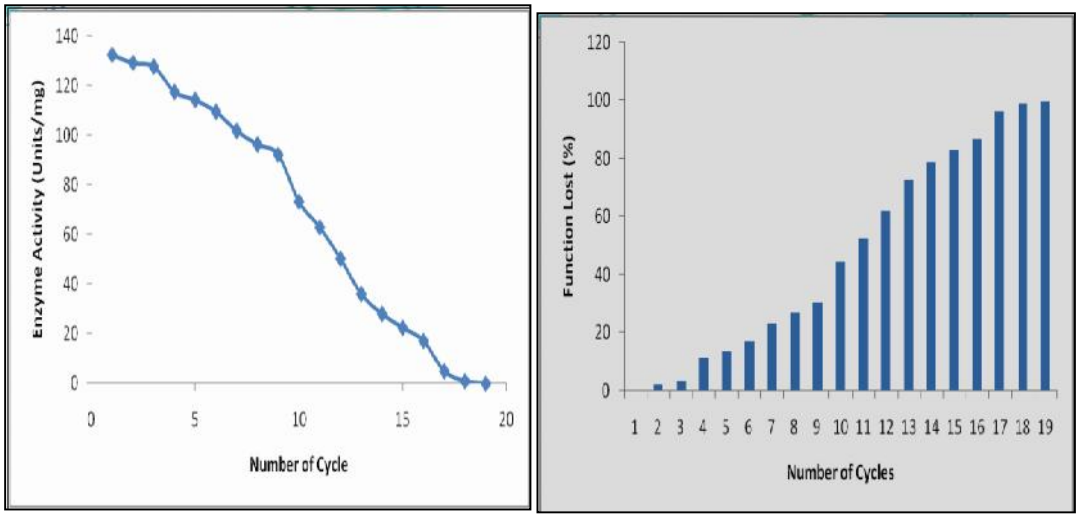


Fig. 113 Enzyme activity after immobilization with coated particles and its recycling

Ruminant microbial phytase (RPHY1) from metagenomic data of Mehsani buffalo breed:

A well diffusion assay was performed on agar plate containing 2 mM calcium phytate by 10 µl of purified enzyme (1 $mgml^{-1}$) and sodium acetate buffer as negative control in individual plates to confirm the restore of protein biological activity. Plates were incubated at 37°C for overnight and enzyme activity was confirmed based on the zone of hydrolysis. Enzyme activity was determined using 0.1 ml of enzyme solution with 0.9 ml of 2 mM calcium phytate in 0.1 M Tris–HCl buffer for 10 min. One unit of phytase activity (U) was defined as 1 µmol of phosphate liberated per minute under the above said assay conditions. Parameters such as optimum temperature and pH, kinetic parameters, stable temperature and pH, effect of cations and effect of potential inhibitors were determined. The effect of cations, EDTA and SDS on enzyme activity was investigated by pre-incubating the compounds, individually as well as in possible combinations, with the purified phytase (RPHY1) for 12 hours. The following metal ions were used in 0.005 M, 0.01 M and 0.015 M concentrations: KCl, $MgCl_2$, $CaCl_2$, $MnCl_2$ and NaCl. Effect of 0.01 M EDTA and 1% SDS on the phytase activity was also checked individually and in combination of both.

Amplification and DNA sequence analysis of RPHY1

The homology based search for phytase encoding gene sequences in rumen metagenomic sequence assembly of Mehsani breed of buffalo and subsequent protein blast confirmation against the non redundant protein database revealed a near full length open reading frame (ORF) corresponding to a conserved hypothetical protein sequence with predicted putative domain for histidine acid phosphatases (HAPS) from *Prevotella ruminicola* strain 23 (GenBank accession number CP002006.1). The contig encoding a 1251 bp ORF of HAPS was selected and amplified using specific PCR primers designed against the predicted functional phytase encoding fragment from the total rumen metagenomic DNA of Mehsani buffalo. Amplified target (RPHY1) was sequenced by Sanger sequencing method using ABI 3500 genetic analyzer™ (Applied Biosystem®, USA) with gene specific primers and assembled in SeqScape v2.5 sequence alignment tool (Applied Biosystems) to generate the full length sequence of phytase encoding gene and was submitted to GenBank under the following accession number: KT601171. Further, homology based search of the full length RPHY1 nucleotide and protein sequences against the NCBI non redundant database was done by performing BLASTn

and BLASTp, respectively (http://blast.ncbi.nlm.nih.gov/Blast.cgi), for structure and protein binding site prediction RaptorX web server was used (http://raptorx.uchicago.edu/), and for molecular weight and isoelectric point calculation was done by Compute MW/pI tool.

The amplified (Fig. 114) and sequenced full-length ORF of RPHY1 gene from rumen metagenome of Mehsani buffalo consisted of 1251 nucleotides, encoding for 417 amino acid residues. The BLASTn homology search revealed 100% query coverage and 95% of sequence identity with *P. ruminicola* 23 followed by *Prevotella sp.* Sc00033 with 99% query coverage and 80% of sequence identity.

BLASTp analysis of amino acid sequence of RPHY1 gene against the non-redundant protein sequences (nr) revealed 92% identity with histidine acid phosphatase encoded by *Prevotella sp.* Sc00033. BLASTp analysis also revealed two conserved domains spanning 52 to 326 amino-acids as Histidine acid phosphatases and phytases and 1 to 277 amino-acids as Histidine phosphatase superfamily_branch 2 with the 1.65e-05 and 3.87e-05, respectively. The protein structure predicted by RaptorX revealed a single domain and 42, 11 and 45 % of α-helix, β-strands and loops, respectively. The structure possessed the phosphate binding pocket with a multiplicity of 84 and the pocket comprised of R54 H55 R58 I61 R146 W272 H315 E316 V317 residues involved in the ligand binding (Fig. 115A and 115B). Compute MW/pI predicted the theoretical isoelectric point of the RPHY1 to be 7.15 and molecular weight to be 47.75 KDa

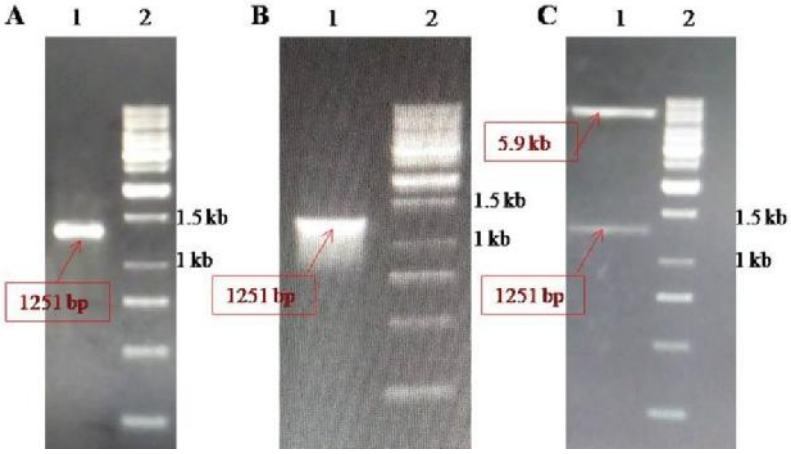


Fig. 114 PCR amplification of RPHY1 (A), colony PCR confirmation of positive recombination (B) and restriction digestion confirmation of purified recombinant plasmid using *BamHI* and *HindIII*.

Colony PCR followed by plasmid restriction digestion confirmed positive clone and further its sequencing revealed phytase gene (RPHY1) in-frame expression within

the thioredoxin and poly histidine tag. The N-terminal His-tagged fusion protein expression was confirmed by the band size of 63 kDa (46 kDa of the insert and 17 kDa of the fusion protein) in the SDS-PAGE and western blot (Fig. 116A, 116B and 116C) after an optimum protein induction at 1.5 mM IPTG concentration. The desired protein bands were observed in the insoluble fraction of the induced culture.

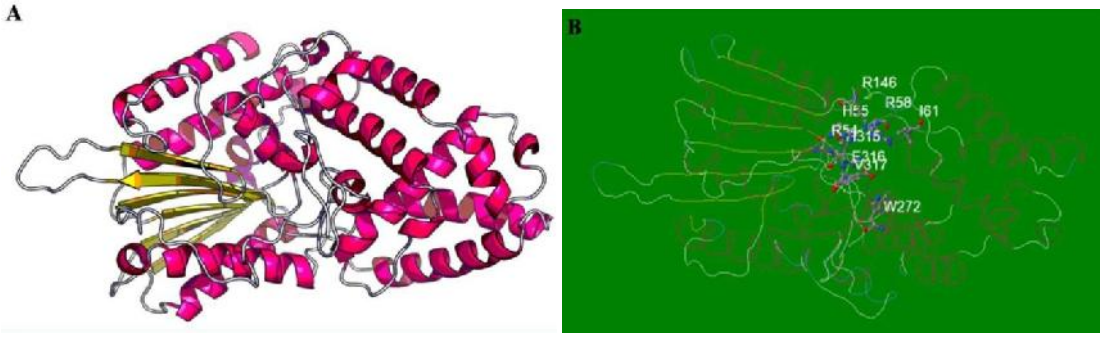


Fig. 115 RPHY1 protein structure prediction (A) and ligand binding pocket of PO_4 (B).

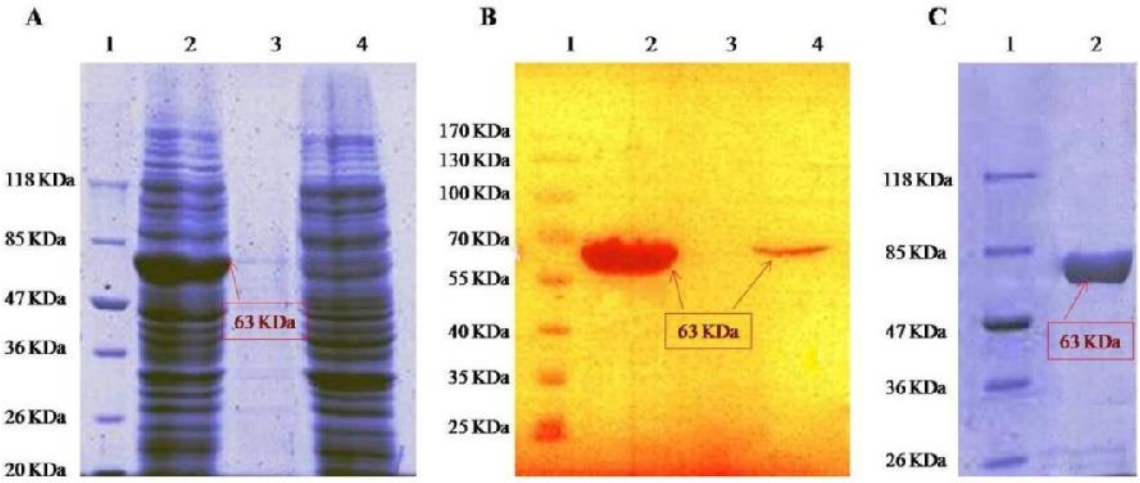


Fig. 116 Analysis of expression of RPHY1 protein by (A) SDS PAGE, (B) Western Blot (Lane1: Prestained protein Ladder, Lane2: IPTG induced insoluble crude lysate, Lane3: IPTG induced soluble crude lysate, Lane4: Non induced crude lysate) and (C) SDS-PAGE of RPHY1 (Lanes1: EZ run prestained protein Ladder and Lane2: Purified RPHY1 protein with N terminal His tag).

Further, the purified and confirmed recombinant plasmid was transformed into expression host and the protein expression level and solubility was confirmed on 10% SDS-PAGE and western blot analysis. Purified and acetone precipitated protein obtained using denaturing and refolding on Ni–NTA affinity column was also confirmed by SDS-PAGE and it showed a homogeneous single band of approximately 63 kDa indicating the

desired purity (Fig. 116C). In plate based bioassay, a clear zone of hydrolysis was observed, which indicated retain of protein biological activity (Fig. 117).

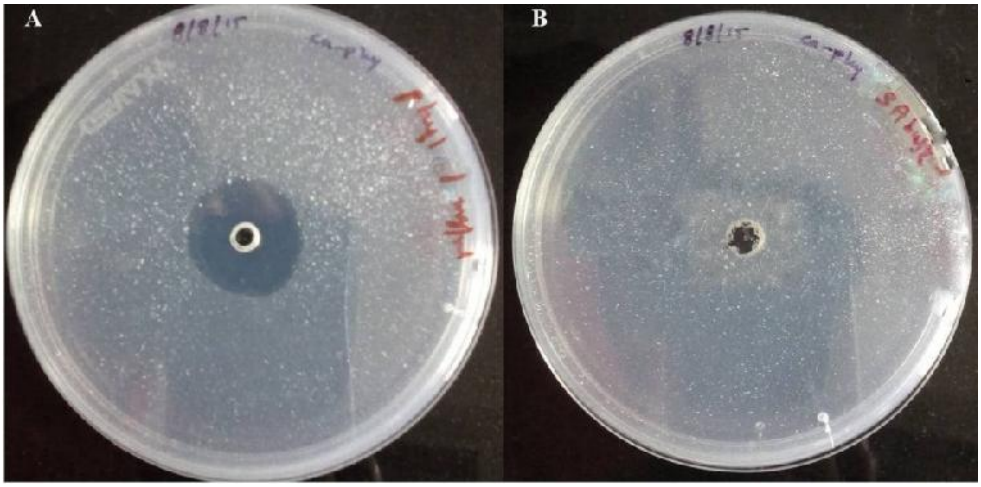


Fig. 117 Determination of RPHY1 enzyme activity through well diffusion assay on agar plate. (A) Clear zone of hydrolysis indicated retain of protein biological activity against calcium phytate (B) sodium acetate buffer as negative control.

Optimum temperature and pH for enzyme activity

The RPHY1 enzyme activity was determined for the optimal temperature; the purified phytase was screened at a temperature range from 20°C to 80°C. Results revealed that the enzyme possesses broad range of activity with optimal activity of 1951 IU at 55°C (Fig. 118A). Similarly, determination for optimal pH within range of 1.0 to 10.0 at the optimum temperature of 55°C revealed that the enzyme gave highest activity of 2084 IU at pH 5.0 (Fig. 118B).

The RPHY1 enzyme activity profile in terms of temperature stability determined by preincubation of enzyme at different temperatures ranging from 0 to 30°C for 12 h at observed optimum pH revealed the maximum stability at around 5°C (Fig. 118C). The enzyme stability was found to be reduced as temperature increases above 5°C. Similarly, the activity profile in terms of pH stability, when preincubated at different pH ranging from 0 to 10 for 12 h at observed stable temperature revealed the enzyme stability through a wide range of pH 1.0 to 10.0 and maximum stability was observed at pH 5.0 (Fig. 118D).

Effect of metal ions, EDTA and SDS on enzyme activity

The effect of metal ions on enzyme activity determined by allowing the ions (0.005 M, 0.01M, 0.015 M) to react with the RPHY1 enzyme by pre-incubating them for 12 hours at 4°C revealed that the metal ions modulated the phytase activity. Among them, Mg^{2+} and K^{+} individually inhibited the enzyme activity by ≥50% as compared to the control irrespective of the three metal ions concentration, while in combination with each other and the rest of the metal ions under study it was found to retain the enzyme activity of approximately 50-55%. Apart from these, Ca^{2+}, Mn^{2+}, and Na^{+} were found to have very low inhibitory effect on the enzyme activity with more than 15% activity loss when compared to the control (Fig. 119A). Moreover, SDS also slightly inhibited the phytase activity (10.7%), while EDTA, individually as well as in combination with SDS was found to increase the RPHY1 enzyme activity by almost two fold compared to the control (Fig. 119B).

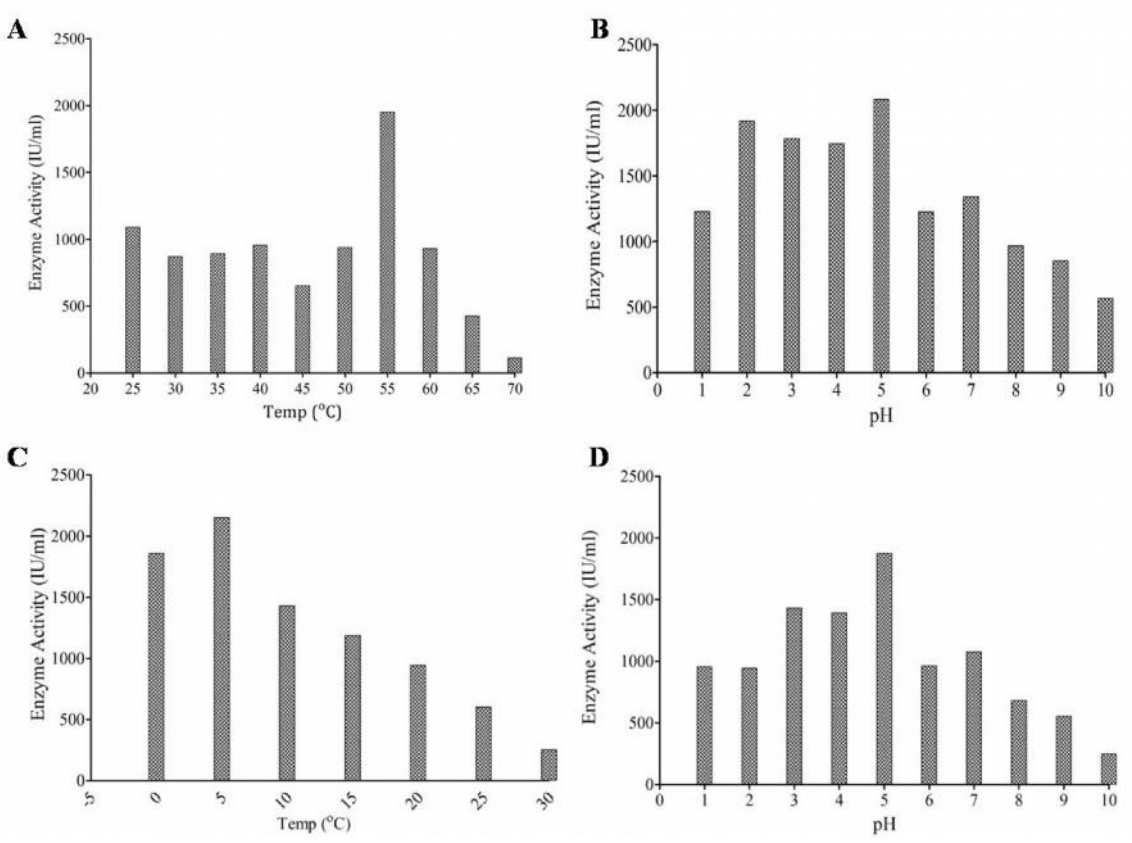


Fig. 118 Determination of optimum temperature and pH for RPHY1 enzyme activity and stability. Assessment of enzyme activity for optimum (A) temperature and (B) pH and enzyme stability at range of (C) temperatures and (D) pH.

Phytate, the major supply of phosphorus in animal feeds is poorly utilized by monogastric animals and hence exogenous phytase is supplemented in feeds. In context to the same, the present study showed that the full length phytase gene (RPHY1) of 1251 bp encoding 417 amino acids from the anaerobic rumen bacterium *P. ruminicola* was cloned, expressed and characterized. The results revealed that the RPHY1 enzyme had much higher activity compared to the commercial available phytase (SIGMA # P1259) and also higher compared to other microbial phytases. The specific parameters like optimum temperature and pH proved its rumen origin and the efficacy of rumen environment for exploration of highly active variety of industrially important enzymes. Further, the temperature and pH stability of RPHY1 reveal its potency for application as feed supplemented enzyme.

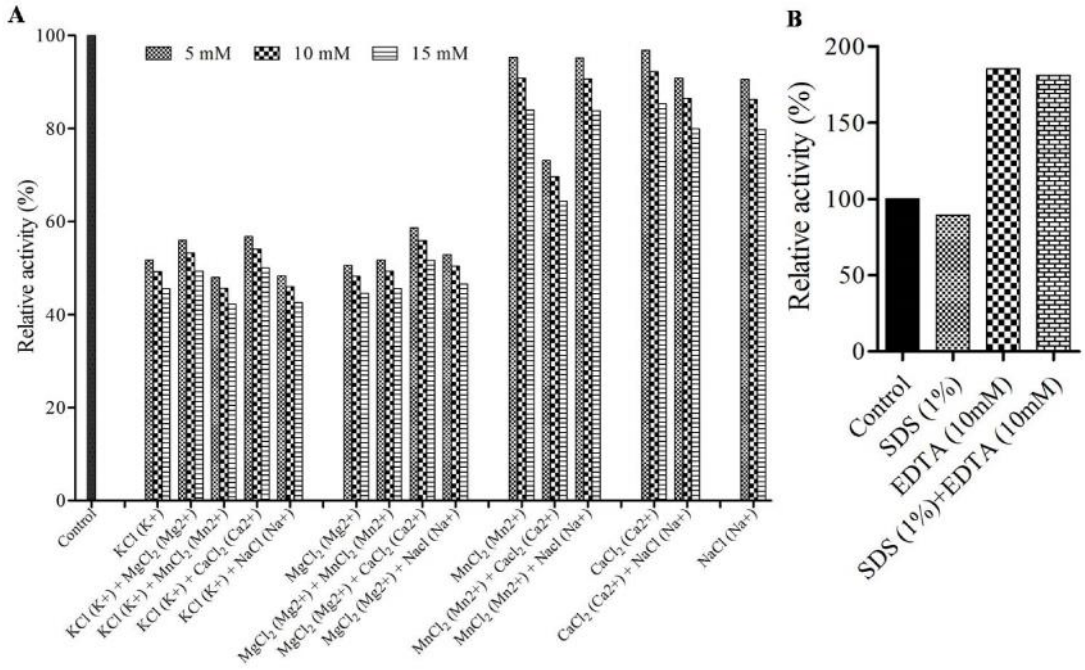
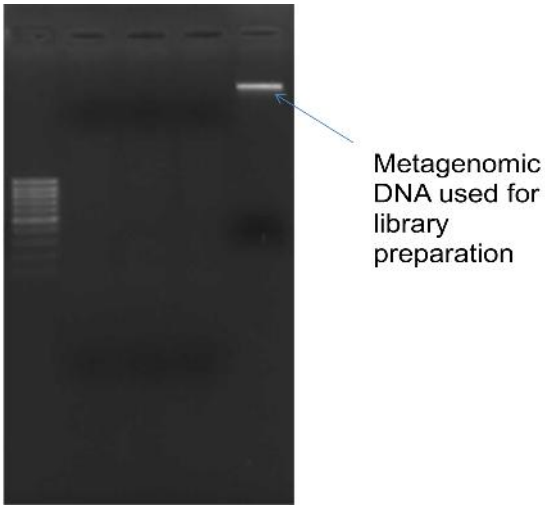


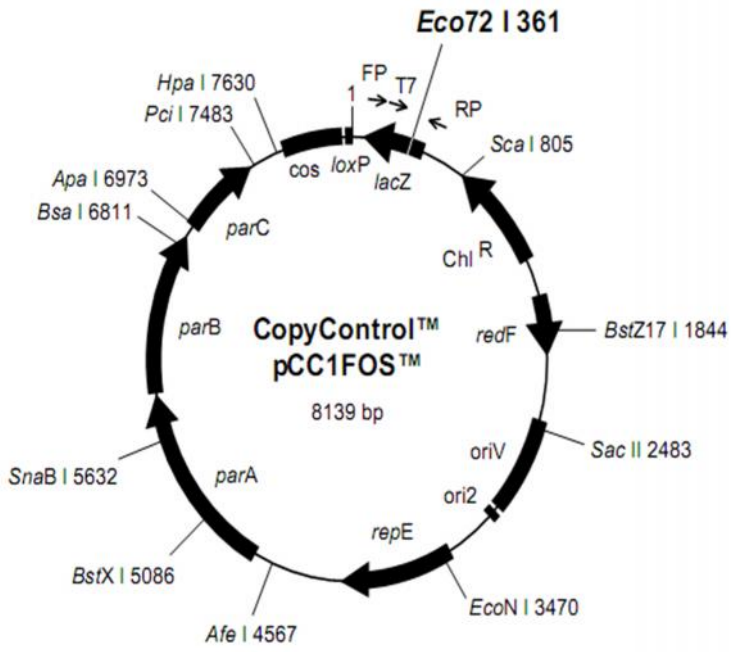
Fig. 119 Effect of (A) metal ions, (B) EDTA and SDS on RPHY1 enzyme activity.

Functional screening of buffalo rumen metagenomic library for biomining novel enzymes of industrial importance

The natural diet of ruminants is mainly plant material, and most of the digestion of plant mass takes place under anaerobic conditions within the rumen. Many enzymatic genes have been isolated and characterized from cultivable ruminal microbes. However, it is generally accepted that a large proportion of the microorganisms in many complex natural environments remain uncultured. Even for the relatively intensively studied rumen microbial community it is estimated that more than 85% of its members have still not been cultivated. This unexplored microbial diversity represents an untapped source of potentially novel and unique enzymatic activities and metabolic pathways that can be applied to industrial biomass conversion. Activity-based metagenomics enables direct identification of genes and enzymes of interest by screening metagenomic libraries for desired heterologous phenotypic traits expressed in a surrogate bacterial host.

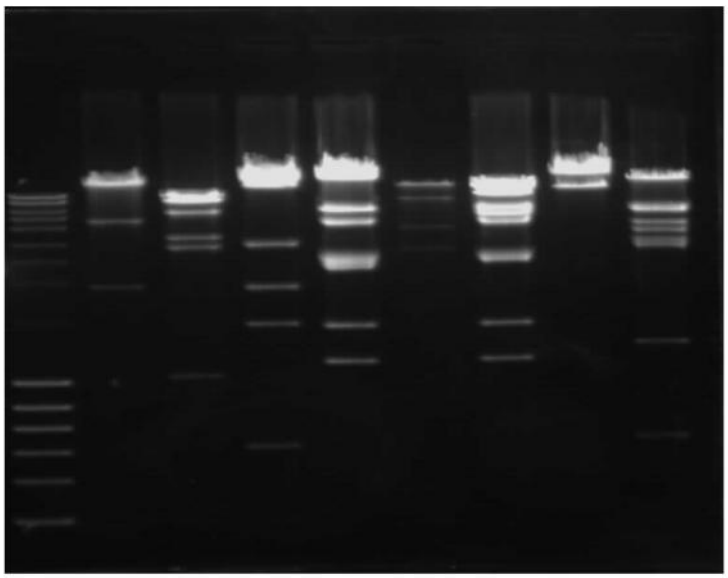
A Fosmid metagenomic library of approximately 1.5 million clones with an average size of 40 kb insert DNA was constructed from buffalo rumen metagenomic DNA. Figure 120 shows the HMW metagenomic DNA and PCCFOS1 vector used for the construction of fosmid library. Figure 121 shows the digestion of random clones from fosmid library to check the average insert size of library and similarity between clones. A small fraction of clones were screened on Agar plates supplemented with xylan as sole carbon source to screen for Xylanase activity. Apart from recombinant *E. coli* showing xylanase activity, a natural microbe exhibiting good xylanase activity was observed on the plate. To deduce the taxonomic information of the microbe, 16s rRNA gene sequencing was performed using Sanger sequencing.





Vector map of PCC1FOS fosmid vector

Fig. 120 (A) High Molecular metagenomic DNA isolated from rumen microbes and (B) pCC1FOS vector used for metagenomic library construction.



RE digestion of random fosmid clones to check the average insert size and similarity between clones

Fig. 121RE digestion of random fosmid clones to check the average insert size and similarity between clones

When compared against the NCBI database, blast results showed top match with *Bacillus Sonorensis* (Fig. 122). By literature survey, it was identified that the genome for this microbe has already been sequenced. Gene mining was performed on the available genome of *B. Sonorensis* to identify the gene encoding the xylanase enzyme. Primers were designed based on available sequences and gene was amplified from the lab culture. A PCR product of expected size (~646 bp) was obtained from the B. *Sonorensis* DNA. (Fig. 123). These gene (BSXYL) was then cloned into pET32a vector (Expression vector) and expressed into the appropriate host cells (BL21 cells). Good Zone of hydrolysis is observed on Agar plates supplemented with Xylan (Fig. 124). Further Characterization of this clone is in progress. As Majority of the fraction of these enzyme is expressed in insoluble form, efforts are made to express these enzyme in soluble form. Cloning of this gene along with a secretory peptide is under process for expression of major part of protein in soluble form. Regarding the Fos3 enzyme, still majority of the fraction is expressed as insoluble portion and studies are being performed for conversion of insoluble enzyme to the soluble form. Regarding the optimization for host competent cells, BL21 cells are showing good activity compared to Lemo cells (Fig. 125).

Apart from this screening of metagenomic library for other targets like pectinase, amylase and pullulanase is in progress. Our findings indicate that BSXYL can be a potential candidate for the industrial applications. Further functional screening of metagenomic library from ruminal microbes is still in progress to identify the effective and novel biocatalysts for potential industrial applications.

Description	Max score	Total score	Query cover	E value	Ident	Accession
Bacillus sp. C9-E3 16S ribosomal RNA gene, partial sequence	1917	1917	99%	0.0	100%	KC920591.1
Bacillus sonorensis strain ZN19 16S ribosomal RNA gene, partial sequence	1917	1917	99%	0.0	100%	KJ542770.1
Bacillus sonorensis strain N5 16S ribosomal RNA gene, partial sequence	1917	1917	99%	0.0	100%	KC441815.1
Bacillus licheniformis strain Jy01 16S ribosomal RNA gene, partial sequence	1917	1917	99%	0.0	100%	KC132795.1
Bacillus sonorensis strain KSI 1339 16S ribosomal RNA gene, partial sequence	1917	1917	99%	0.0	100%	KC113131.1

Fig. 122 Results obtained after comparing 16s rRNA gene sequence obtained from the available micro-organism with the available database

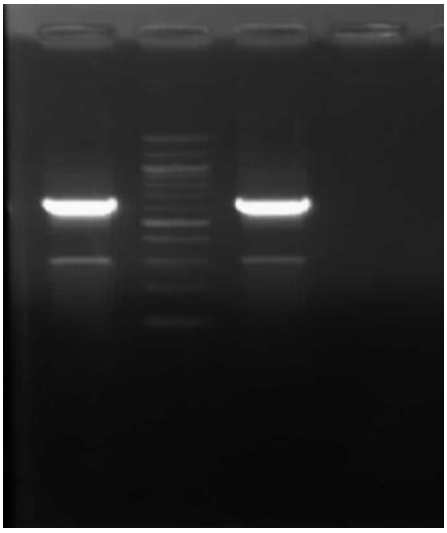


Fig. 123 PCR amplification of BSXYL gene from the Bacillus sonorensis available in lab using the PCR primers designed based on the available sequence in database

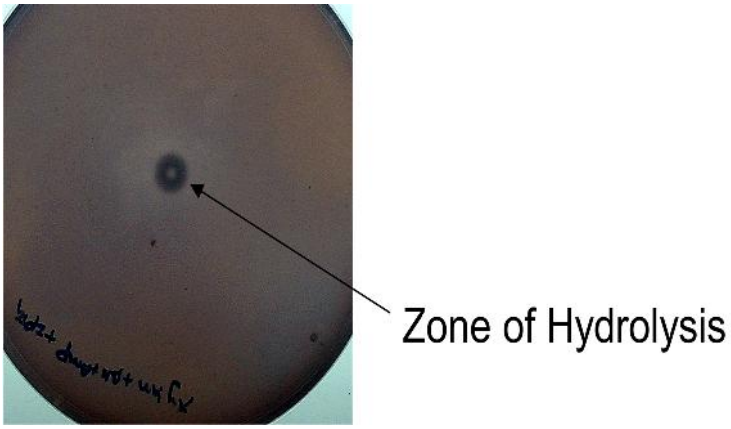


Fig. 124 Zone of hydrolysis obtained on Xylan-Agar plates after hydrolysis of xylan by recombinant xylanase enzyme

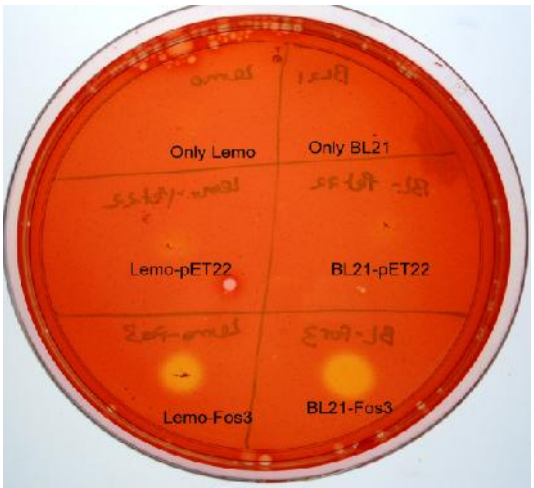


Fig. 125 Optimization of host cells for Fos-3. BL21 cells transformed with recombinant clone is exhibiting good activity compared to Lemo cells transformed with Fos3.

Shotgun and amplicon sequencing of calf to adult rumen samples

Sampling from zero day to 6th months (with interval of 15 days) and from 6th to 9th month (with interval of 30 days) is completed. Total 19 samples were collected and preserved at -80 C. DNA isolation from all the samples were completed, amplicons and shotgun libraries generated. Sequencing runs under progress.

Authentication:

(Name & Signature of the Principal Investigator)
Date:
Place:

(Name & Signature of the Head of the University)
Date:
Place:

Annexure I

4. Final accepted project proposal

Final - accepted proposal

Niche Area of Excellence (NAE) Programme

Under Scheme- Strengthening and Development of Higher Agricultural Education in India

Revised project proposal based on suggestions of reviewer and Expert committee headed by DG, ICAR

Submitted by

Department of Animal Biotechnology
College of Veterinary Science and A.H.
Anand Agricultural University
Anand-388001
Gujarat

To

Education Division
Indian Council of Agricultural Research
KrishiAnusandhanBhawan II, Pusa
New Delhi-110 012

Summary

1. Name of the University : Anand Agricultural University
2. Name of the Niche Area : Metagenomic analysis of Ruminal Microbes
3. If area is Multidisciplinary : Yes.
4. Reasons for Selecting Niche Area :

Biotechnology is a highly multidisciplinary and fastest growing discipline, although its application in animal health and production has been a recent phenomenon.

The complex micro biome of the gastrointestinal system of herbivores plays an important role in nutrient utilization, growth and well-being of these animals as well as methane emission in the environment. With the introduction and growth of molecular tools in microbial ecology, many culture-independent methods have developed to overcome the cultivation biases and allow detailed information on microbial community diversity, structure, and function. New high throughput sequencing platforms based on pyrosequencing are now available hence it is now possible to sequence all the genes of all the species of the rumen ecosystem to produce a metagenome as well as identify genotypes of host.

5. Existing strength in the Niche Area :

5.1 Past Achievements

(a) Research Programmes completed: 6 (six); in operation: 6 (six)
(b) Publications : 52
(c) Patents: Patent Application no. 2272/DEL/2008
(d) Students completed M.Sc. /M. V. Sc. /Ph. D in Animal Biotechnology: 29
(e) Employment profile of alumni: All employed
(f) Awards/recognition: 2

5.2 Faculty available in the Niche Area (with one page bio-data for each)

(a) Within the Department: 2; (b) Outside the Department: 3; (c) Outside the University: 2

5.3 Supporting staff available in the Niche Area: Nil

5.4 Infrastructure, equipment and other facilities available: Available

1. Proposal in support of Niche area

4.1 Goal: Temporo-spatial study of buffalo rumen microbiome

4.2 Objectives

- Molecular characterization and identification of rumen microbiome of buffalo.
- To study effect of diet containing different proportions of nutrient ingredients on rumen microbial diversity.
- To study effect of different geographical condition on rumen microbial diversity.
- **Mapping of ruminal metagenome for single buffalo animal through calf to adult**

4.3 Budgetary requirements

A) Manpower (Rs. In lakhs): Rs.33.264; B) Equipments (Rs. In lakhs): Rs.23.5

C) Consumable and contingencies (Rs. In lakhs) : Rs.221.292

Total Rs. In lakhs: Rs.**278.056**

2. In-charge of Niche Area (attach two page bio-data) :

(a) Name	:	Dr. Chaitanya G. Joshi
(b) Designation	:	Professor (Animal Biotechnology)
(c) Address	:	Department of Animal Biotechnology College of Veterinary Science & Animal Husbandry Anand Agricultural University Anand, Gujarat – 388001 India
(d) Telephone	:	02692-261201 / 9227131075
(e) Fax	:	02691-261201
(f) E-Mail	:	cgjoshi@aau.in cgjoshi@rediffmail.com

PROFORMA FOR SUBMITTING THE PROPOSAL FOR NICHE AREA OF EXCELLENCE

(Give separate one page summary at the beginning of proposal)

1. Name of the University : Anand Agricultural University
2. Name of the Niche Area : Metagenomic analysis of Ruminal Microbes
3. If area is Multidisciplinary : Yes.
4. Reasons for Selecting Niche Area :

Biotechnology is a highly multidisciplinary and fastest growing discipline, although its application in animal health and production has been a recent phenomenon. It is must to have timely and accurate diagnosis and treatment of animal diseases for profitable business of animal husbandry. The area in which biotechnology can play role in animal science are includes production of antibiotics, recombinant and synthetic vaccines, monoclonal antibodies, recombinant hormones, valuable transgenic and cloned animals. Other areas includes molecular diagnosis of animal disease, micropropagation, transformation, germplasm maintenance and storage, production of commercially useful chemicals by transgenesis (drug secrete from milk) and animal genetic engineering as well as in-vitro fertilization and embryo transfer technology. Animal biotechnology has also scope for molecular markers, and QTL mapping of different quantitative traits, functional genomics (proteiomics, transcriptomics) resistances improvement of bacterial strains by recombinant DNA technology, fermentation and metabolic engineering. These techniques are generally used to study basic physiology, improvement in animal health, production as well as production of disease resistance animal and propagation of highly produced animal through embryo transfer and artificial insemination.

The complex micro biome of the gastrointestinal system of herbivores plays an important role in nutrient utilization, growth and well-being of these animals. The gastrointestinal tract of animals harbors a large, complex, and dynamic microbial community, and the composition of this community ultimately reflects the co-evolution or selection of microorganisms with their animal host and the diet adopted by the host. The majority of these microbial species cannot be cultured under traditional culturing techniques, and therefore, we have a very limited understanding of the capabilities of most species. More recently, with the introduction and growth of molecular tools in microbial ecology, many culture-independent methods have developed to overcome the cultivation biases and allow detailed information on microbial community diversity, structure, and function. In the late 1990's and early 2000 sequencing technology had advanced to the point where it was possible to obtain a catalogue of all the genes in a single species of bacteria. Sequencing technology has since advanced even further, and new platforms based on pyrosequencing are now available. It is now possible to sequence all the genes of all the species of the rumen ecosystem to produce a metagenome.

Each ruminant hosts ten times more microbial cells in their gut than the total number of cells in their body. These microbes are highly evolved to survive in our gastro-intestinal tract in a symbiotic relationship, where both bacteria and animal host benefit. Proportionally, ruminants are highly evolved to protect symbiotic gut bacteria while destroying potentially pathogenic bacteria. Massive depth metagenomic sequencing is an invaluable complement to what has already been learned about lignocelluloses degradation in the rumen. The ease with which bovine ruminants can be fistulated allows for simple and rapid sampling strategies, and changes in diet and

management can be easily implemented for metagenomic investigations on the microbial community and metabolic potential. We are rapidly increasing our knowledge on the genetic mechanisms that symbiotic bacteria use to survive in our gut, however we know very little about the balance of the microflora in the gut. Disruption of this natural microbial flora is linked to infection and metabolic diseases but detailed knowledge about microbial component is very scarce. Recent technological advances permit, for the first time, comparative metagenome analysis of a gastrointestinal microbiome from Buffalo by using the inexpensive, massively parallel, and rapid method of pyrosequencing.

5. Existing strength in the Niche Area :

5.1 Past Achievements

(a) Research Programmes completed/in operation

Sr. No.	Name of Scheme	Funding Agency	Budget (in Lakh)
1	Identification of differentially expressed gene in lactogenic pathways of buffalo - Completed	ICAR	25.26
2	Somatotropine mediated gene expression in lactogenic pathways of buffalo– Completed	GSBTM	21.41
3	Differential gene expression in buffalo udder during lactogenic pathways in induced lactation model - Completed	DBT	40.84
4	Density, Diversity and Dynamics of rumen microbes in buffaloes– Completed	DBT	40.50
5	Genetic Engineering and Biotechnology – Ongoing	AAU	
6	Centre of excellence in Animal Biotechnology - Ongoing	AAU	
7	Genetic characterization of four indigenous cattle breeds found in MP using molecular markers - Completed	DBT	39.0
8	Development of goat having knocked down myostatin gene through RNA interference technology to enhance the meat production– Ongoing	NAIP	420
9	BIT^{virtual} Virtual Learning of Bioinformatics - Ongoing	GSBTM	5.0
10	PCR based identification and genotyping of prokaryotes and eukaryotes– Ongoing	MPUH	28.00
11	Biotechnological approaches for containment of animal diseases	RKVY	420
12	Increasing milk production through recent biotechnological approach	RKVY	190

(b) Publications (10 recent publications; remaining publications as Annexure)

1. Jhala, N. B.; Rank, D. N. ; Vataliya, P. H.; Joshi, C.G.; Mehta H.H. and Patil, A.V. (2009). Cloning and Sequencing of TheLeptin Gene in Gir Cattle and Mehsana Buffalo. *Buffalo Bulletin* Vol.28 No.1.
2. Chaudhari, M.V.; Parmar S.N.S.; Joshi, C.G.; Bhong, C.D.; Fatima,S.; Thakur, M.S. and Thakur, S.S. (2009). Molecular characterization of Kenkatha and Gaolao*(Bosndicus)* cattle breeds using microsatellite markers.*Animal Biodiversity and Conservation* 32.2
3. Mehta TY, Prajapati LM, Mittal B, Joshi CG, Sheth JJ, Patel DB, Dave DM, Goyal RK. (2009) Association of HLA-B*1502 allele and carbamazepine-induced Stevens-Johnson syndrome among Indians.Indian J DermatolVenereolLeprol. 75(6):579-82.

4. Pandya P.R.; Singh, K.M.; Parnerkar, S.; Tripathi, A.K.; Mehta, H.H.; Rank, D.N.; Kothari, R.K.; Joshi, C.G. (2010) Bacterial diversity in the rumen of Indian Surti buffalo (Bubalusbubalis), assessed by 16s rDNA analysis. J Appl. Genet 51 (3): 395-402.

5. Kalc, D S, Rank, D N, Joshi, C G, Yadav, B R, Koringa, P G, Thakkar, K M, Tolenkhomba, T C, Solanki, J V (2010) Genetic diversity among Indian Gir, Deoni and Kankrej cattle breeds based on microsatellite markers.Indian Journal of Biotechnology, 9, pp 126-130.
6. Singh KM, Pandya PR, Parnerkar S, Tripathi AK, Ramani U, Koringa PG, Rank DN, Joshi CG, Kothari RK. (2010) Methanogenic diversity studies within the rumen of Surti buffaloes based on methyl coenzyme M reductaseA (mcrA) genes point to Methanobacteriales.Polish J Microbiol. 2010;59(3):175-8.
7. Tripathi AK, Ramani UV, Ahir VB, Rank DN, Joshi CG. (2010) A modified enrichment protocol for adult caprine skeletal muscle stem cell. Cytotechnology;62(6):483-8.
8. S.J. Jakhesara, P.G. Koringa, U.V. Ramani, V.B. Ahir, A.K. Tripathi, P.S. Soni, K.M. Singh, V.D. Bhatt, J.S. Patel, M.M. Patel, M.R. Sajnani and C.G. Joshi (2010) Comparative Study of Tannin Challenged Rumen Microbiome in Goat Using High Throughput Sequencing Technology. Developmental Microbiology and Molecular Biology (1): 95-106
9. Sharma, R., Parmar, SNS, Joshi, C. G.; Thakur, M S.; Bhong C. D.; Chaudari, M.V. (2010) Molecular characterisation of Nimari cattle using fluroscently labelled microsatellite markers. The Indian Journal of Animal Sciences, 80(7)
10. Ahir V.B.; Koringa, P.G.; Bhatt, V.D.; Ramani, U.V.; Tripathi, A.K.; Singh K.M.; Dhagat, U.M.; Patel, J.S.; Patel, M.M.; Katudiya, K.H.; Sajanani, M.R.; Jakhesara, S.J.; Joshi, C.G. (2010) Metagenomic analysis of poultry gut microbes. Indian J of poultry Science (August)

(c) Patents

Patent Application no. 2272/DEL/2008

(d) Technologies generated

(e) Resources generated:

Sixty Five students have completed master's degree research work in animal biotechnology and more than 100 students of associated departments took advantage of animal biotechnology department during their postgraduate study. Department had completed several research schemes and presently running several training programmes and also imparting summer training to a students from other universities.

(f) Students complcted M.Sc. /M. V. Sc. /Ph. D

M.V.Sc.	:	29
Ph.D.	:	00

(g) Employment profile of alumni

Pursuing study abroad: 8
Pursuing study India: 10
Private Industry job : 9
Government job : 1
Own business :1

(h) Awards/recognition:

P.K.Pani award
Ayurvet award

5.2 Faculty available in the Niche Area(with one page bio-data for each)

(a) Within the Department

1. Dr. Prakash G. Koringa
   Assistant Professor (Animal Biotechnology)
   [Biodata attached separately as Annexure-I]

2. Dr. Subhash J. Jakhesara
   Assistant Professor (Animal Biotechnology)
   [Biodata attached separately as Annexure-I]

(b) Outside the Department

1. Dr. S. Parnerkar, Professor & Head, (Animal Nutrition)
2. Dr. D.N.Rank, Professor & Head, (Animal Genetics & Breeding)
3. Dr.P.R.Pandya, Associate Professor, (Animal Nutrition)

(c) Outside the University

1. Dr.Shailesh R. Dave, Professor (Microbiology), Gujarat University
2. Dr.SrinivasMurthyDuggirala, Professor (Microbiology), Gujarat Vidhyapith.
3. Sardarkrushinagar Dantiwada Agricultural University, SKNagar
4. Navsari Agricultural University, Navasari and
5. Junagadh Agricultural University, Junagadh

5.3 Supporting staff available in the Niche Area

Nil

5.4 Infrastructure, equipments and other facilities available

Well buildup separate high-tech molecular biology laboratory with following research facilities are available with us.

Sr. No.	Name of Equipment	No.
1	GS-FLX Titanium Whole Genome Sequencer (Roche)	1
2	Automated DNA sequencer (ABI 310)	1
3	Bioanalyzer 2100	1
4	Real Time PCR	1

5	Gel Documentation system	2
6	Advanced Research Microscope with Camera (Fluorescent, Phase contrast & Inverted)	5
7	Thermal cycler	5
8	Electrophoresis Units-various types	8
9	Stereo Zoom Microscope	1
10	Multichannel Nanodrop Spectrophotometer	1
11	Nanodrop Spectrophotometer	1
12	2-D GEL Electrophoresis	1
13	Eliza Reader	1
14	Refrigerated Centrifuge, table top	5
15	Bio-safetyCabinet Class III	1
16	Bio-safety Cabinet Class II B2	1
17	Laminar Air Flow	3
18	Ultra low deep freeze	3
19	Other miscellaneous equipments used in molecular biology experiments	

3. Proposal in support of Niche area

6.1 Goal: Temporo-spatial study of buffalo rumen microbiome

6.2 Objectives

- Molecular characterization and identification of rumen microbiome of buffalo.
- To study effect of diet containing different proportions of nutrient ingredients on rumen microbial diversity.
- To study effect of different geographical condition on rumen microbial diversity.
- **Mapping of ruminal metagenome for single buffalo animal through calf to adult.**

6.3 Technical Programme

Selection of experimental animals of buffalo (heifers and Adults)

Rearing under (four) different geographical locations containing different climate of Gujarat

Different proportions of roughage and concentrate i) 100% roughage ii) 50% roughage & 50 % concentrate iii) 25 % roughage and 75% concentrate

Collection of rumen fluid sample and metagenomic DNA extraction

Pyrosequencing with whole genome sequencer

Comparative analysis between different groups using bioinformatics tools

6.4 Activity milestones (yearly)

Period of study	Achievable Targets
6 Months	• Hiring of animals and planning for their accommodation in different geographical conditions
12 Months	• Maintaining animals in good health and simultaneously conducting changes in diet provision • Collection of rumen fluid from each animal after each diet change • Extraction of DNA from collected rumen fluid at each diet change
12-24 Months	• Sequencing of extracted DNA on GS-FLX genome analyzer • bioinformatics analysis of obtained data • Training
24-30 months	• Analysis of obtained data
30-36 months	• Documentation of obtained data and findings • Preparation of publication based on research findings • Conducting training cum workshop

6.5 Monitorable targets

1. Animals located (24 animals in each location) in different geographical locations (4 locations) of Gujarat
2. Starting feeding regimen (three regimen) lasting for six weeks. Collection of rumen samples from each animal.
3. Metagenomic analysis of samples completed
4. Mapping of ruminal metagenome for single buffalo animal through calf to adult

6.6 Major equipments/facilities required

A. Equipments: Ultrasonic fragmentar, Fluorimeter

B. Chemicals:

- Kit for sequencing run on GS-FLX genome analyzer
  35 Kits for metagenomic sequencing

C. Biological material:

- 96 animals for feed/fodder trial and metagenomic study
- Fodder – concentrate & roughage for above animals

6.7 Budgetary requirements

A) Manpower (Rs. In lakhs):

Sr. No.	No. of position	Consolidated amount	Ist Year	IIndyear	IIIrd year	Total
1.	RA– TWO	24000/-+HRA	6.336	6.336	6.336	19.008
2.	SRF - TWO	18000/-+ HRA	4.752	4.752	4.752	14.256
Total			**11.088**	**11.088**	**11.088**	**33.264**

(B) Equipments (Rupees in Lakh)

Sr. No.	Name of equipment	Ist Year	IIndyear	IIIrd year	Total
1.	Fluorimeter	10.0	00	00	10.0
2.	Ultrasonic sonicator	9.0	00	00	9.0
3.	Cell counter	4.5	00	00	4.5
		23.5	**00**	**00**	**23.5**

(C) Consumable and contingencies (Rs. In lakhs)

Sr. No.	Items	Ist Year	IIndyear	IIIrd year	Total
1	Purchase (lease) of animals	5.0	00	00	5.0
2	Feed and Fodder	4.0	00	00	4.0
3	kit for genome sequencer GS-FLX	60.0	100.0	15.0	175.0
4	Ampure beads	0.6	00	00	0.6
5	Bioanalyzer kits	1.5	1.5	00	3.0
6	Travel and transport including training to scientists from different parts of the country	2.9	2.9	2.6	8.4
7	Contractual support staff	1.44	1.44	1.44	4.32
8	Miscellencous (reagents for DNA extraction, plasticware etc.)	0.5	0.5	0.4	1.4
9	Overhead charges	7.394	10.434	1.744	19.572
Total		83.334	116.774	21.184	221.292
Total (A + B + C)		**117.922**	**127.862**	**32.272**	**278.056**

Justification:

- Total 3 animals can be included in one pyrosequencing run.

Total 35 (32 for pyrosequencing of rumen microbiome from 96 animals + 10% extra) runs are required for the project. Sequencing cost is 5 lakh per run i.e. 35 x 5=175 lakhs. Pyroequencing also requires bioanalyzer kits and Ampure beads.

- Feed and fodder is required for rearing of animals under project.
- Cell counter is required to obtain accurate bead count during sequencing.
- Ultrasonic sonicator is required to generate fragments of desired size of DNA for sequencing.
- Contractual support staff is needed for management of animals under experiment and other non-technical work.
- Travel and transport during Training to the scientists from different parts of India

6.8 Expected outcome for the stake holders at the end of the project

Comparison of the gene content needed for efficient digestion and utilization for different type of diets will reveal nature of microflora needed for particular feed digestion. It will become also clear about the changes in rumen microbial population after diet changes and what those changes are, i.e. the organisms which can result in efficient digestion and more amount of energy produced can be identified. Genetical basis of

change in microflora will also be investigated and it will aid in selection of better animals.

(a) For the country : Formulation of better feed for buffalo results in to efficient utilization of feed resource, reduced methane production and thus economically and environmentally beneficial.
(b) For the state : As in Country
(c) For the region : As in Country
(d) For the university : Human Resource Development & Development of Research Capabilities
(e) For students : Training on most modern molecular biology techniques
(f) For farmers : Socio-economic development.
(g) For agribusiness management/industries :More profitable end products

4. In-charge of Niche Area(attach two page bio-data) :

(a) Name	:	Dr. Chaitanya G. Joshi
(b) Designation	:	Professor (Animal Biotechnology)
(c) Address	:	Department of Animal Biotechnology College of Veterinary Science & Animal Husbandry Anand Agricultural University Anand, Gujarat – 388001 India
(d) Telephone	:	02692-261201 / 9227131075
(e) Fax	:	02691-261201
(f) E-Mail	:	cgjoshi@aau.in cgjoshi@rediffmail.com

5. Authentication :

(Project Incharge)

CHAITANYA G. JOSHI
PROFESSOR (ANIMAL BIOTECHNOLOGY)
ANAND AGRICULTURAL UNIVERSITY
ANAND-388001. GUJARAT, INDIA.

(Dean, Faculty of Veterinary Science)

Principal & Dean
Veterinary Science College
ANAND.

(Vice-Chancellor)

Annexure-I

1. Joshi, C.G.; Rank, D.N.; Kanakraj, P.; Kumanan, K. and Krishnan, A.R. (1997) Differentiation of chromosomes in mouse X buffalo somatic cell hybrid. *Indian Veterinary Journal,* 74. pp. 762-764.
2. Joshi, C.G.; Rank, D.N.; Brahmkshtri, B.P.; Patel, A.V.; Vataliya, P.H.; Muraleedharan, P.; Khoda, V.K. and Solanki, J.V. (1998) RAPD analysis by PCR using arbitrary primers in different animal species. *Indian Veterinary Journal*, 75. pp. 1029-1031.
3. Joshi, C.G.; Rank, D.N.; Brahmkshtri, B.P.; Patel, A.V.; Vataliya, P.H. and Solanki, J.V. (1998) Comparison of DNA extraction protocols on frozen stored blood samples. *Indian Veterinary Journal,* 75. pp. 1038-1039.
4. Patel, M.K.; Patel, K.S.; Brahmkshtri, B.P.; Vataliya, P.H. and Solanki, J.V. (2000) Cytogenetic profile of kachchhi camel (Camelusdromedarius). *Journal of Camel Practiceand Research,* 7 (2). pp. 201-202.
5. Joshi, C.G.; Rank, D.N.; Jani, R.G.; Tank, P.H.; Brahmkshtri, B.P.; Vataliya, P.H. and Solanki, J.V. (2001) A case of E. asinus X E. heminouskhur hybrid. *Indian Veterinary Journal,* 78. pp. 549-550.
6. Patel, R.M.; Joshi, C.G.; Solanki, J.V. and Patel, P.R. (2001) PCR based detection of tuberculosis from blood using oligoprimers in cattle. *Indian Veterinary Journal,* 78. pp. 480-482.
7. Acharya, C.P.; Pipalia, D.L.; Rank, D.N.; Joshi, C.G.; Brahmkshtri, B.P.; Solanki, J.V. and Shah, R.R. (2002) BOLA DRB3 gene polymorphism in Jaffrabadi and Mehsani buffaloes as revealed b PCR-RFLP. *Indian Veterinary Journal*, 79. pp. 652-656.
8. Acharya, C.P.; Pipalia, D.L.; Rank, D.N.; Joshi, C.G.; Solanki, J.V. and Shah, R.R. (2002) Detection of BoLA-DRB3 gene polymorphism in Gir and Kankrej cattle using PCR-RFLP. *Indian Journal of Animal Sciences,* 72 (8). pp. 680-683.
9. Ladani, D.D.; Pipalia, D.L.; Brahmkshtri, B.P.; Rank, D.N.; Joshi, C.G.; Vataliya, P.H. and Solanki, J.V. (2003) Prolactin genotyping of Indian buffalo breeds using PCR-RFLP. *Buffalo Journal*, 2. pp. 203-208.
10. Rank, D.N.; Joshi, C.G.; Tank, P.H.; Brahmkshtri, B.P.; Vataliya, P.H. and Solanki, J.V. (2003) Chromosomal aberration in a donkey (E. asinus). *Indian Veterinary Journal,* 80. pp. 83-84.
11. Ladani, D.D.; Pipalia, D.L.; Brahmkshtri, B.P.; Rank, D.N.; Joshi, C.G.; Vataliya, P.H. and Solanki, J.V. (2003) PCR-RFLP polymorphism at prolactin locus in buffaloes. *Buffalo Journal*, 2. pp. 237-242.
12. Kadam, Megha; Joshi, C.G. and Jhala, M.K. (2003) Detection of infectious bursal disease virus from bursal samples. *Cheiron,* 32 (5 & 6). pp. 142-143.
13. Rank, D.N.; Joshi, C.G.; Thiagrajan, V.; Kanakraj, P. and Krishnan, A.R. (2004) Development of somatic cell hybrid using buffalo leukocytes and mouse cell line. *Indian Veterinary Journal,* 81. pp. 156-159.
14. Pipalia, D.L.; Joshi, C.G.; Rank, D.N.; Brahmshktri, B.P. and Solanki, J.V. (2004) PCR-SSCP typing of MHC in cattle and buffaloes. *Indian Journal of Animal Sciences*, 74 (6). pp. 637-639.
15. Tank, P.H.; Joshi, C.G.; Archibald David, W.P. and Balasubrmanian, N.N. (2004) A modified method for photographic documentation of tissue fluorescence. *Indian Journal of Animal Sciences,* 74 (5). pp. 504-505.

16. Pipalia, D.L.; Joshi, C.G.; Rank, D.N.; Brahmshktri, B.P. and Solanki, J.V. (2004) PCR-SSCP typing of MHC in cattle and buffaloes. *Indian Journal of Animal Sciences,* 74 (6). pp. 637-639.
17. Goriya, H.V.; Kalia, A.; Bhavsar, S.K.; Joshi, C.G.; Rank, D.N. and Thaker, A.M. (2005) Comparative evaluation of phenobarbital-induced CYP3A and CYP2H1 gene expression by quantitative RT-PCR in Bantam, Bantamized white leghorn and white leghorn chicks. *Journal of Veterinary Science*, 6 (4). pp. 279-285.
18. Shanaz, S.S.; Joshi, C.G.; Jhala, M.K.; Rank, D.N.; Khanna, K.; Barot, V.N.; Brahmkshtri, B.P. and Solanki, J.V. (2005) Molecular characterization of B-Lβ II family (class II MHC) alleles in three strains of poultry and its association with immune response. *Indian Journal of Poultry Science*, 40 (1). pp. 1-8.
19. Pandya, G.M.; Singh, Satinder; Joshi, C.G.; Rank, D.N.; Khanna, Kuldeep; Barot, V.N. and Solanki, J.V. (2005) Association of LEI-146 and MCW-43 microsatellites with body weights in bantam, bantamised white leghorn and white leghorn chicken. *Indian Journal of Poultry Science,* 40 (1). pp. 9-15.
20. Jain, Anshu; Roy, A.; Rank, D.N.; Joshi, C.G. and Purohit, J.H. (2005) Characterization of the Pasteurellamultocida isolates by their outer membrane protein profile. *Indian J. Compo Microbiol. Immunol. Infect. Dis.,* 26 (1). pp. 63-65.
21. Makadiya, N.R.; Jhala, M.K.; Gaba, Amit; Joshi, C.G. and Rank, D.N. (2006) Detection of infectious bursal disease virus (IBDV) from bursal tissue by RTPCR and its comparative efficacy with conventional precipitation assays. *Indian Journal of Poultry Science*, 41 (1). pp. 82-84.
22. Anand, S.K.; Shadma, Fatima; Joshi, C.G. and Purohit, J.H. (2006) Molecular characterization of Escherichia coli using PCR single strand conformation polymorphism analysis of 16S rRNA gene. *Indian Journal of Poultry Science*, 41 (1). pp. 1-5.
23. Pipalia, D.L.; Joshi, C.G.; Khanna, K.; Rank, D.N.; Thakkar, K.M.; Brahmkshtri, B.P. and Solanki, J.V. (2006) RAPD profiling of Bantam, white leghorn and Bantamised white leghorn birds. *Indian Journal of Poultry Science,* 41 (2). pp. 111-114.
24. Thakur, M.S.; Parmar, S.N.S.; Tolenkhomba, T.C.; Srivastava, P.N.; Joshi, C.G.; Rank, D.N.; Solanki, J.V. and Pillai, P.V.A. (2006) Growth hormone gene polymorphisms in Kadaknath breed of poultry. *Indian Journal of Biotechnology*, 5. pp. 189-194.
25. Joshi, Jarina; Patel, R.K.; Singh, K.N.; Soni, K.J.; Chauhan, J.B.; Rank, D.N.; Joshi, C.G. and SambasivaRao, K.R.S. (2007) Genome identity and diversity in Gir and Kankrej (Bosindicus) cattle breeds using RAPD fingerprints. *Biotechnology,* 6 (3). pp. 322-327. ISSN 1682-296X
26. Jain, Shally; Brahmbhatt, M.N.; Rank, D.N.; Joshi, C.G. and Solanki, J.V. (2007) Use of cytochrome b gene variability in detecting meat species by multiplex PCR assay. *Indian Journal of Animal Sciences,* 77 (9). pp. 880-881.
27. Kerur, N.; Jhala, M.K. and Joshi, C.G. (2007) Genetic characterization of Indian peste des petits ruminants virus (PPRV) by sequencing and phylogenetic analysis of fusion protein and nucleoprotein gene segments. *Research in Veterinary Science*, 85 (1). pp. 176-183.
28. Patel, A. K.; Koringa, P. G.; Nandasana, K. N.; Ramani, U. V.; Goriya, H. V. and Panchal, K. M. (2007) Comparative histology of mammary gland in Heifer, Pregnant, Lactation and Dry buffaloes. *Indian Journal of Veterinary Anatomy,* 19 (1). pp. 71-78. ISSN 0971-1937

29. Gaba, Amit; Makadiya, N.R.; Joshi, C.G. and Jhala, M.K. (2007) Isolation of infectious bursal disease virus in cell culture, detection and characterization by RT-PCRSSCP analysis. *Indian Journal of Poultry Science*, 3 (42). pp. 339-342.
30. Pawar, R.S.; Tajane, K.R.; Joshi, C.G.; Rank, D.N. and Brahmkshtri, B.P. (2007) Growth hormone gene polymorphism and its association with lactation yield in dairy cattle. *Indian Journal of Animal Sciences*, 77 (9). pp. 884-888.
31. Arya, Gitanjali; Roy, Ashish; Chaudhary, Vandana; Yadav, Mahendra M. and Joshi, C.G. (2008) Serogroups, atypical biochemical characters, colicinogeny and antibiotic resistance pattern of shiga toxin-producing Escherichia coli isolated from diarrhoeic calves in Gujarat, India. *Zoonoses Public Health*, 55 (2008). pp. 89-98.
32. Fatima, Shadma; Bhong, C.D.; Rank, D.N. and Joshi, C.G. (2008) Genetic variability and bottleneck studies in Zalawadi, Gohilwadi and Surti goat breeds of Gujarat (India) using microsatellites. *Small Ruminant Research*, 77. pp. 58-64.
33. Singh, Y.; Brahmbhatt, M.N.; Bhong, C.D.; Jain, Shally and Joshi, C.G. (2008) Detection of meat species by polymerase chain reaction of actin gene family. *Haryana Veterinarians* (46). pp. 25-27.
34. Jain, Lata; Kanani, A.N.; Purohit, J.H.; Joshi, C.G.; Rank, D.N.; Kumar, Vinay and Jain, V.K. (2009). Detection of bovine herpes virus 1 (BHV-1) infection in semen of Indian breeding bulls by polymerase chain reaction and its characterization by DNA sequencing. *Buffalo Bulletin*, 28 (2). pp. 76-84.
35. Verma, Amit Kumar; Joshi, C.G.; Koringa, P.G. and Nauriyal, D.S. (2009). Defensin gene polymorphism analysis in bovine intramammary infections in cattle and buffaloes of Gujarat. The *Indian Journal of Animal Sciences*, 79 (3). pp. 300-302.
36. Mathakiya, R.A.; Roy, A.; Nandasana, K.N.; Koringa, P.G. and Joshi, C.G. (2009). Evaluation of a rapid molecular method for detection of Listeria monocytogenes directly from broth culture. *Veterinary World*, 2 (5). pp. 177-178.
37. Mehta, Timir Y.; Prajapati, Laxman M.; Mittal, Bharti; Joshi, C.G.; Sheth, Jayesh J.; Patel, Dinesh B.; Dave, Dinkar M. and Goyal, Ramesh K. (2009) Association of HLA B1502 allele and carbamazepine-induced Stevens–Johnson syndrome among Indians.*Indian Journal of Demarmatology, Venereology &Laprology*, 75 (6). pp. 579-582.
38. Koringa, P.G.; Joshi, C.G.; Solanki, J.V. and Rank, D.N. (2008). Genetic characterization and bottleneck studies in Kathiawari horse breed of India. *Haryana Veterinarians*, 47. pp. 77-83.
39. Sacravarty, G.; Vadodaria, V.P.; Joshi, C.G.; Brahmkshtri, B.P.; Shah, R.R. and Solanki, J.V. (2008). Prolactin gene polymorphism and its association with economic traits in Kankrej cattle.*Indian Journal of Dairy Science*, 61 (4). pp. 273-276.
40. Bhong, C.D.; Brahmbhatt, M.N.; Joshi, C.G. and Rank, D.N. (2008). Detection of virulence determinant by real time PCR in E.coli isolated from mutton.*Meat Science*, 80 (4). pp. 1129-1131.
41. Kalyani, I.H.; Joshi, C.G.; Jhala, M.K.; Bhanderi, B.B. and Purohit J.H. (2008). Detection of pathogenic Marek's disease virus in layer birds by PCR. *Indian Journal of Poultry Science*43(3): 325-328.
42. Patel, T.J.; Kanani, Amit; Jain, Lata; Joshi, C.G. and Purohit, J.H. (2008). Evaluation of PCR and Indirect enzyme linked immunosorbent essay on milk samples for diagnosis of brucellosis in bovines.*Buffalo Bulletin* Vol.27 No.2.

Annexure II

Xv. Details of the participants in the trainings organized under NAE

1. Training organized during 9th - 18th Febraruy 2016:

Sr. No	Name	Designation	Institute	Address	Place	Phone
1	Dr. Jaynudin Khorajiya	PhD Scholar	IVRI-ICAR	Izatnagar, Bareilly, Uttar Pradesh 243122	Uttar Pradesh	9662072286
2	Miss Nilambari Pawar	Research Associate	Central Inst. Of Fisheries Edu.	Panch Marg,Off Yari Road, Versova, Andheri West, Mumbai, Maharashtra 400061	Maharashtra	9867667317
3	Dr. Varsha Sharma	Professor & Head	NDVSU, Jabalpur	Indira Gandhi Marg, Panagar, South Civil Lines, Jabalpur, Madhya Pradesh 482001	Madhya Pradesh	9425311329
4	Miss Sweta Tripathi	Research Scholar	Gujarat Univeristy, Ahmedabad	Navrangpura, Near L D Engineering college, Ahmedabad, Gujarat 380009	Gujarat	9722641135
5	Miss Visha Rathod	Research Associate	Junagadh Agricultral University	Junagadh Road, Junagadh, Gujarat 362001	Gujarat	9428840025
6	Dr. Govind Kumar	Post Doc. Fellow- UGC	GBPUA & T, Pantnagar	District - Udham Singh Nagar, Pantnagar, Uttarakhand 263145	Uttarakhand	9456193092
7	Dr. S. Vishnuvardhan Reddy	DST-SERB, Young Scientist	Dept. of Biochemistry, Mahatma Gandhi Univeristy	Hyderabad Road, Aneparti, Dist Nalgonda, Yellareddy Gudem, Telangana 508254	Telangana	9247350187
8	Dr. Ravindra	DSK Kothari Post DOC Fellow	Saurastra University	Saurashtra University Campus, Rajkot, Gujarat 360005	Gujarat	7742761306

	Pal Singh					
9	Mrs. Kavitha S	SRF	Manipal University	Madhav Nagar, Near Tiger circle, Manipal, Karnataka 576104	Karnataka	9620401934
10	Mr. Ninian Prem Prashanth P	Research Scholar	NIT-Warangal	Hyderabad Highway, National Institute of Technology Campus, Warangal, Telangana 506004	Telangana	7673996655
11	Miss Disha Patel	Ph.D Scholar	st. Xeviers College, Ahmedabad	Gulabai Tekra, Ahmedabad, Gujarat 380009	Gujarat	9925074807
12	Miss Krupa Parmar	Project Asssistant II	Environmental Genomics Division, NEERI	Nehru Marg, Nagpur - 440 020.	Maharashtra	9712001735
13	Mr. Abhishek Saxena	Ph.D Scholar	TERI University	Plot No. 10, Institutional Area, Vasant Kunj, New Delhi, Delhi 110070	Delhi	8130713327
14	Mr. Ranjeet Verma	Research Scholar	Central Lab, CIRB, Hisar	7, Sector 14, Hisar, Haryana 125001	Haryana	9450933755
15	Mr. Khomdram Niren Singh	Ph.D. Scholar	Microbiology Laboratory, Gauhati Univeristy	Gopinath Bordoloi Nagar, Guwahati, Assam 781014	Assam	8486402564
16	Mr. Jacky Bhagat	Ph.D. Scholar cum SRF	CSIR-National Inst. Of Oceanography	Raj Bhavan Rd, Dona Paula, Goa, 403004	Goa	8698663259
17	Mr. Prabhakar Pandit	Ph.D. Scholar	Environmental Genomics Division, NEERI	Nehru Marg, Nagpur - 440 020.	Maharashtra	9145252709, 9970860389
18	Dr. Pritesh Sabara	SRF	Dept. of Biotechnology, JAU	Junagadh Road, Junagadh, Gujarat 362001	Gujarat	9427216099

19	Miss Madhusmita Dehingia	SRF	IASST, Vigyan Path	IASST, Vigyan Path, Paschim Boragaon, Guwahati, Assam 781035	Assam	8486355493
20	Mr. Mounil Mankad	Research Associate	Dept. of Agricultural Biotechnology, AAU	Anand Agricultural University, Hadgood Road, Hadgood, Anand, Gujarat 388110	Gujarat	9825962997
21	Mrs. Armi Patel	Research Associate	Dept. of Agricultural Biotechnology, AAU	Anand Agricultural University, Hadgood Road, Hadgood, Anand, Gujarat 388110	Gujarat	9429160671
22	Mr. Sandipkumar Patel	Research Associate	Dept. of Agricultural Biotechnology, AAU	Anand Agricultural University, Hadgood Road, Hadgood, Anand, Gujarat 388110	Gujarat	9925711003
23	Mr. Pratik Jaisani	Ph.D. Scholar, JRF	Anand Agricultural University	Anand Agricultural University, Hadgood Road, Hadgood, Anand, Gujarat 388110	Gujarat	9737249737
24	Dr. Abid Ali Bhagat	Assistant Professor	Dept. of Microbiology, College of Basic Sciences & Humanities	sardarkrushinagar dantiwada agricultural university, Dantiwada, Banaskantha, Gujarat 385506	Gujarat	9426249501
24	Shri Karen P. Pachchigar	Assistant Professor	Agri. Biotech. Department, Sardarkrushinagar Dantiwada Agricultural University	Sardarkrushinagar, Dantiwada, Banaskantha, Gujarat 385506	Gujarat	9099262684

2. Training organized during 24th October 2015 -4th November 2015:

Sr. No	Name	Designation	Institute	Address	Place	Phone
1	Dr. Arjun	Scientist	ICAR-National	ICAR-National	Uttar	9455936139

	Singh		Bureau of Agriculturally Important Microorganisms (ICAR-NBAIM)	Bureau of Agriculturally Important Microorganisms Kushmaur, Mau, Uttar Pradesh-275103.	Pradesh	
2	Dr. Ramesh K Kothari	Professor	UGC-CAS Department of Biosciences, Saurashtra University	Saurashtra University, Rajkot-5	Gujarat	9428598941
3	Dr. Arun C Patel	Assistant Professor	Dept. of Microbiology, SDAU	S.K.Nagar, Dantiwada Gujarat- 385 506	Gujarat	8866805005
4	Dr. Lata Jain	Scientist	ICAR-National Institute of Biotic Stress Management	ICAR-National Institute of Biotic Stress Management, Baronda, Raipur, Chhattisgarh 493225	Chhattisgarh	9479038712
5	Dr. Vikrant M Bhor	Scientist-C	Division of Molecular Immunology, National Institiute of Research in Reproductive Health (NIRRH-ICMR)	National Institute of Research in Reproductive Health, Jehangir Merwanji Street, Parel, Mumbai-400 012	Maharashtra	9987036993
6	Dr. Ashok Bhonsle	Assistant Professor	Department of Veterinary Microbiology, College of Veterinary & Animal Sciences	Department of Veterinary Microbiology, College of Veterinary & Animal Sciences.Udgir-413517, Dist. Latur, Maharashtra	Maharashtra	7721025073
7	Dr. Ashish C Patel	Assistant Professor	Animal Genetics &Breeding dept. College of Veterinary Science & Animal Husbandry.	College of Veterinary Science & Animal Husbandry, AAU, 388 110	Gujarat	9429407557
8	Dr. Jasmi Patel	Assistant Professor	Sardarkrushinagar, Dantiwada,Agricultural University	Sardarkrushinagar, Dantiwada, Banaskantha, Gujarat 385506	Gujarat	9769777922

9	Dr. Shweta Yadav	Associate Professor	Department of Zoology, School of Biological Science, Dr H S Gour Central University	Sagar, Madhy Pradesh 47000	Madhya Pradesh	9479983812
10	Dr. Vishal Kothari	Research Associate	Dept of Biotechnology, Junagadh Agricultural University,	Junagadh Agricultural University Junagadh-362001	Gujarat	9904226016
11	Dr. Vipul Parekh	Assistant Professor	ASPEE College of Horticulture & Forestry	Navsari Agricultural University	Gujarat	
12	Dr. Pradeep M.A.	Scientist	Marine Biotechnology Division, Central Marine Fisheries Research Institute	Central Marine Fisheries Research Institute, Cochin-682018. Ernakulam, Kerala	Kerala	08547118155
13	Dr. Amar A. Sakure	Assistant Professor	Agril. Biotechnolgy, Anand Agricultural University	Anand Agricultural University 388 110	Gujarat	7600053668
14	Dr. Supriya Kshirsagar	Deputy General Manager,	Poultry Diagnostic & Research Centre	Venkateshwara Hatcheries, Poultry Diagnostic & Research Centre, Loni Kalbhor, Sholapur road Haveli, Pune	Pune	
15	Dr. Sunita Varjani	Assistant Professor	Dept. Of Environmental Sciences, University of IAR	University of IAR, PDPU Rd, Koba Institutional Area, Gandhinagar 382 007	Gujarat	9909828684
16	Dr. Geeta Gaudar	Assistant Professor	Department of Agril. Microbiology, College of Agriculture	Department of Agril. Microbiology, College of Agriculture, Hitnalli farm, Vijaypur-586101, Karnataka	Karnataka	9902670206
17	Dr Ponnanna	Scientist II	NDDB R&D Laboratory, Indian	NDDB R&D Laboratory,	Telangana	9290264259

	N.M.		Immunologicals Ltd. Campus	Indian Immunologicals Ltd. Campus, Gachibowli P.O., Hyderabad-32		
18	Dr. Himani Gandhi	Teaching Associate	Hemchandracharya North Gujarat University	Hemchandracharya North Gujarat University,Patan, 384265	Gujarat	
19	Dr. P.C.Latha	Scientist	Soil Science Section, Indian Institute of Rice Research	Indian Institute of Rice Research, Rajendranagar, Hyderabad-500030. Telangana State	Telanagana	040-24591263
20	Dr. S.R. Krupesh Sharma	Principal Scientist	Central Marine Fisheries Research Institute	Central Marine Fisheries Research Institute, Karwar Research Centre, PB. No. 5, Karwar-581301 Karnataka	Karnataka	9449810746

3. Training organized during 6th-15thOctober 2014:

Sr. No.	Name	Designation	Institute	Address	Place	Phone
1	Dr.Jitendra Singh	Scientist-1	NDDB, Anand	Scientist-1, PPD Group, CALF Building, NDDB, Anand - 388001	Gujarat	--
2	Dr.Digant Narzary	Assistant Professor	Guwahati University	Dept. of Botany, Guwahati University, Guwahati -781 014	Assam	--
3	Dr.Padma V. Putta	Scientist-III	Asian Institute of Gastroenterology	6-3-661, Somajiguda, Hyderabad-500082	Aandhra Pradesh	--
4	Dr. Anuradha S. Nerurkar	Professor	M. S. University	Department of Microbiology and Biotechnology Centre, Feculty of Science, The M. S. Uni.of Baroda, Vadodara - 390 002	Gujarat	--
5	Dr.Subhash Taraphder	Assistant Professor	West Bengal. Uni. Of Animal & Fishery Sci.	Dept. of Animal Genetics & Breeding, W. B. Uni. of Animal & Fishery Sci., K. B.	Kolkata	--

				Sarani - 700 037		
6	Dr.Trupti Navinchandra Patel	Associate Professor	School of Bioscience & Technology	School of Bioscience & Technology, Vellore - 632014	Tamilnadu	--
7	Dr.A. Kathirvelpandian	Scientist	NBFGR, Kochi Unit, CMFRI Campus,	NBFGR Kochi Unit, CMFRI Campus, Ernakulam-682018	Kerala	--
8	Dr.Rakesh Ranjan	Senior Scientist	National Research Center on Camel	National Research Center on Camel, Jorbeer, Bikaner - 334001	Rajasthan	--
9	Dr.Prashant Ramchandra Suryawanshi	Assistant Professor(Veterinary Microbiology)	Department of Veterinary Microbiology, College of Veterinary Science & A.H.	Department of Veterinary Microbiology, College of Veterinary Science & A.H., R.K.Nagar, Agartala - 799008	Tripura	--
10	Dr.Madhu Chaudhari	Scientist	Central Soil Salinity Research Institute	Central Soil Salinity Research Institute, Kachhawa road, Karnal - 132001	Haryana	--
11	Dr.Mani Chellappan	Associate Professor	AINP on Agricultural Ornithology	AINP on Agricultural Ornithology, College of Horticulture, KAU, Thrissur - 680656	Kerala	--
12	Dr.Padma Ambalam	Assistant Professor	Dept. of Biotechnology	Christ College,Vidya Niketan, Saurashtra University, P. O., Rajkot - 360005	Gujarat	--
13	Dr.Basdeo Kushwala	Principal Scientist	National Beauro of Fish Genetic Resources	National Beauro of Fish Genetic Resources, Canal Ring Road, P.O.- Dilkusha, Lukhnow-226025	Uttar Pradesh	--
14	Dr.Sreeja V.	Assistant Professor	Department of Dairy Microbiology, AAU	Department of Dairy Microbiology, SMC College of Dairy Science, AAU, Anand	Gujarat	--
15	Dr.Prasenjit Dhar	Assistant Professor	Dept.of vetrinary microbiology, DGCN, COVAS,CSKHPKV	Dept.of vetrinary microbiology, DGCN, COVAS,CSKHPKV, Palampur, Kangra - 176062	Himachal Pradesh	--
16	Dr.K. Vijayarani	Professor	Madras veterinary College	Dept. of Animal Biotechnology, Madras veterinary College, Chennai - 600007	Tamilnadu	--

17	Dr.Aarti Ozarkar	Scientist	University of Pune	Dept. of Zoology, University of Pune, Pune - 411007	Maharashtra	--
18	Dr.R. A. Mathakiya	Assistant professor	Anand Agricultural University	Dept. of Veterinary Microbology, Veterinary Collge, AAU, Anand - 388001	Gujarat	--
19	Dr.D. Girija	Professor & Head	Kerala Agricultural University	Dept. of Agricultural microbology, Collge of Horticulture, Kerala Agricultural University, Thrissur - 680656	Kerala	--
20	Mr.Harish E.R.	Scientist	AINP on Agricultural Ornithology	AINP on Agricultural Ornithology, College of Horticulture, KAU, Thrissur -680656	Kerala	--
21	Mr.Pinal Chandarana	NGS - Analyst and Researcher	iBIO Analysis Pvt.Ltd	C/O iBIO Analysis Pvt.Ltd, 202, Campus Corner, Nr. St. Xaviers College, Navarangpura, Ahmedabad - 380009	Gujarat	--

4. Training organized during 15th -24th October 2013:

Sr. No	Name	Designation	Institute	Address	Place	Phone
1	Dr. M. Bagath	Scientist	National Institute of Animal Nutrition and Physiology,	Dr. Bagat M., Gut Microbiology Lab. Animal Nutrision Lab. NIANP, Bangalore-560030	Karnataka	9035865536
2	Dr.Megha K. Purohit	Research Associate	Veer Narmad South Gujarat University	Dept. Bio Science, Veer Narmad South Gujarat University, Surat - 395 007	Gujarat	8530303010
3	Dr.Avijit Dey	Sr. Scientist	Central Institute for Research on Buffaloes (CIRB)	Buffalo Nutrition Division, Central Institute for Research on Buffaloes Sirsa Road, Hissar(Haryana)	Haryana	9728334810
4	Dr.Jerome A.	Sr. Scientist	Central Institute for Research on Buffaloes (CIRB)	Buffalo Nutrition Division, Central Institute for Research on Buffaloes Sirsa Road, Hissar(Haryana)	Haryana	8053954239

5	Dr.Prashant Devjibhai Kunjadia	FTYS Fellow	B. N. Patel Institute of Paramedical & Science (Science Division)	B. N. Patel Institute of Paramedical & Science (Science Division), Bhalej Road, Anand-388001	Gujarat	9824252544
6	Dr.Sarita G. Bhat,	Associate professor	Cochin University of Science and Technology	Dept. of Biotechnology, Cochin University of Science and Technology, Cochin, Kerala, India-682022	Kerala	0484-2576267
7	Mis.Jani Vishakha Ramakrishna	Adhoc Lecturer	Municipal arts and urban bank science college	Dept. of Biotechnology, Municipal arts and urban bank science college, Mehsana-384022	Gujarat	9624724975
8	Mr.Rashmin M. Dhingani	Assistant professor	AAU, Anand	Dept. of Food Quality Assurance College of Food Processing Technology and BioEnergy, AAU,	Gujarat	9998471983
9	Mr.J.K. Momin	Assistant professor	AAU, Anand	Dept. of Food Quality Assurance College of Food Processing Technology and BioEnergy, AAU,	Gujarat	9998606042
10	Dr. Bijay Kumar Behera,	Sr. Scientist	Central Inland Fisheries Research Institute	Central Inland Fisheries Research Institute, Barrackpore, Kolkata-700120	West Bengal	9163209580/82
11	Mr.Solly Solomon	JRF	Cochin University of Science and Technology	Dept. of Marine Biology, Microbiology and Biochemistry, School of Marine Sciences, Fine Arts Avenue, Cochin University of Science and Technology, Cochin 16, Kerala, -682016	Kerala	9895529824
12	Dr.S. Rajkumar	Scientist	Iladevi Cataract and IOL Research Centre	Dept. of Molecular Genetics, Iladevi Cataract and IOL Research Centre, Gurukul Road, Memnagar, Ahmedabad, Gujarat-380052	Gujarat	8511262859

13	Dr.Alpesh Patel	Scientist	Iladevi Cataract and IOL Research Centre	Dept. of Molecular Genetics, Iladevi Cataract and IOL Research Centre, Gurukul Road, Memnagar, Ahmedabad, Gujarat-380052	Gujarat	9824999084
14	Dr.Arvindaxan	Professor	KVASU	Centre for advanced studies in Animal Genetics & Breeding, Kerala Veterinary and Animal Sciences University, Mannuthy, Thrissur, Kerala-680 651	Kerala	9895268573
15	Dr.Murugadas Vaiyapuri	Scientist	CIFT	MFB division, Central Institute of Fisheries Technology, CIFT Junction, Matsyapuri, Cochin-682029	Kerala	9567973533
16	Dr.Rani Singh	Scientist	Project Directorate on Cattle.	Project Directorate on Cattle.Meerut Cant, India	Uttar Pradesh	9412173380
17	Dr.Gayatri Dave	Assistant professor	Charotor University of Science & Technology,	P. D. Patel Institute of Applied Sciences, Charotor University of Science & Technology,Charusat Campus, Changa-388 421	Gujarat	7600414303
18	Dr.Sumesh K.M.	Assistant professor	College of Agriculture, DharwadUAS.	Dept. of Biotechnology, Institiute of Agri. Biotechnology, College of Agriculture, DharwadUAS	Karnataka	9035333774

5. Training organized during 15th -24th October 2012:

Sr. No	Name	Designation	Institute	Address	Place	Phone
1	Dr. Chandra Sekhar Mukhopadhyay	Assistant Scientist	GADVASU	School of Animal Biotechnology, GADVASU	Punjab	9779541452
2	Dr. Aparna Chaudhari	Senior Scientist	CIFE	Fish Genetics and Biotechnology Division, CIFE, Versova	Mumbai	9821687228

3	Dr.Rashmi H. M.	Scientist	NDRI	Molecular Biology Unit. Dairy Microbiology Division, NDRI	Harayana	9996441256
5	Dr.Radhakrishnan E. K	Assistant Professor	M.G. University	School of Biosciences	Kerala	9847901149
6	Dr.C.A. Jayprakash	Principal Scientist & Head	Central Tuber Crops Research Institute	Thiruvanthampuram	Kerala	9447138123
7	Dr.Janardan Jani	Associate Research Scientist	Anand Agricultural University	Biological Control Research lab	Gujarat	--
8	Dr.Jigar Mistri	Assisstant Research Scientist	Anand Agricultural University	Department of Agri. Biotechnology	Gujarat	9898796597
9	Dr.Jigar Kansara	Scientist-I	NDDB	-	Gujarat	9427326732
10	Dr.Sunil S. Trivedi	Professor & Principal	Pramukhswami Medical College	Microbiology Dept.	Gujarat	9825629204
11	Dr.Chandrakant Dadaso Bhong	Assistant Professor	KNP College of Veterinary Science	Shirawa	Maharashtra	8600236300
12	Dr.Bharat Bhanderi	Assistant Professor	Dept. of Vet. Microbiology, Anand Agricultural University	Anand	Gujarat	--
13	Dr.Jitendra Dhruve	Associate Professor	Dept. of Agricultural Biochemistry, BACA, AAU	Anand	Gujarat	--
14	Dr. Birbal Singh	Senior Scientist	IVRI	Palampur	Himachal Pradesh	--
15	Dr.Chhabi lal Patel	Scientist	IVRI, Biological Product Division	Izatnagar	Uttarpradesh	9760821407
16	Dr. Akarsh Parihar	Associate Professor	Dept. of Genetics & Plant Breeding	Saradarkrushinagar	Gujarat	9879442267
17	Dr.R Anandham	Assistant Professor	Dept. of Agricultural Microbiology	Madurai	Tamilnadu	9159029745
18	Dr. O.P. Ahlawat	Principal Scientist	Directorate of Mashroom Research, ICAR	Solan	Himachal pradesh	9418022025

19	Dr.Prahlad Balakrishnan	Scientist	Gangaram Hospital	Rajendranagar		9899311406
20	Dr.Lal Chandra Chaudhary	Principal Scientist	Animal Nutrition Division, IVRI	Izatnagar	Uttar pradesh	9412585551
21	Dr. Prasanta K Dash	Scientist(Senior Scale)	NRC on Plant Biotechnology, IARI	Pusa	New Delhi	9654785655

Annexure III

Internal Review meeting of the Niche Area of Excellence Program on "Metagenomic Analysis of Ruminal Microbes" held in the Department of Animal Biotechnology, College of Veterinary Science & A.H., Anand Agricultural University on **March 21-22, 2014**.

Following members were present:

Review Committee

1. Dr. K. Pradhan, Chairman
2. Dr. D. N. Kamra, Member
3.Dr.C.G.Joshi PI and Member Secretary

Invited Scientists of the College

Dr. J. V. Solanki (Invited Guest)
Dr. Dave (Co-PI)
Dr. Murthy (Co-PI)
Dr. D. N. Rank (Co-PI)
Dr. N.V. Patil, Director, NRC on camel, Bikaner (invited Guest)
Research Fellows

Meeting commenced with the welcome address by Dr. C.G. Joshi,the Principal Investigator of the Project and then he introduced the Committee members appointed by the ICAR for review of the project.

Dr. K. Pradhan, the chairman while welcoming all scientists present in the meeting briefly described about the high standard of research attached to Nichea Area of Excellence Projects and mode of selecting a discipline and scientist by the ICAR to execute the programme. He congratulated Dr. Joshi and his Team of scientists for recipient of such a research project.

Dr. C. G. Joshi (PI) presented overview of the project including the objectives, date of initiation, and overall achievements. The details project execution were presented by concerned scientists.

Dr. Amrut Patel presented the research progress on metagenomic analysis by shotgun sequencing of rumen metagenome of Mehsani buffalo, Gir and Kankrej cows followed by presentation by Miss Nidhi Parmar on 16S amplicon sequencing of rumen metagenome of Mehsani buffalo and Gir and Kankrej cows. Further, Dr. Amrut Patel, Mr. Ishan, Miss Neelam and Mr. Ravi presented the additional work carried out related to cloning and expression of recombinant glycoside hydrolases, genome reconstruction from metagenome, culture of rumen originated microbes and their genome analysis and analysis of protozoa enriched metagenome.

Objective wise progress is given below:

1. Molecular characterization and identification of rumen microbiome of cattle and buffalo.

Analyzed rumen metagenome of Mehsani buffalo and Kankrej and Gir cows which revealed predominance of Bacteroidetes followed by Firmicutes, Proteobacteria, Actinobacteria and Fibrobacter in the Mehsani buffalo and Kankrej cow metagenome. The Gir cow metagenome revealed predominance of the Firmicutes and Fibrobacter over Bacteroidetes.

2. To study effect of diet containing different proportions of nutrient ingredients on rumen microbial diversity.

Studied effect of three different proportions of roughages (Green and Dry) to concentrate ratio (50% roughage + 50% concentrate, 75% roughage + 25% concentrate, and 100% roughage) on rumen microbial diversity. Increasing the roughage proportion in the diet caused reduction in the Bacteroidetes in liquid fraction with simultaneously increasing the solid fraction of the rumen digesta. The proportion of Firmicutes was seen increased in the liquid fraction with simultaneous decrease in solid fraction with increasing proportion of roughage diet. The metagenome profile in the Kankarej cows showed increased proportion of Firmicutes and decreased proportion of Bacteroidetes with respect to increasing roughage proportion in the diet. In Gir cows, proportion of Firmicutes was found to be significantly higher than Bacteroidetes.

3. To study effect of different geographical conditions on rumen microbial diversity.

Rumen Microbiome analysis was performed from animals raised at two different locations i.e. SK Nagar and Anand. The microbiome profile of the Mehsani buffalo and Kankrej cows which were raised at SK Nagar was found to have higher similarity than Gir cows raised at Anand suggesting the higher influence of geographical location than the effect of species in shaping the microbial community.

4. Comparative study of rumen microbiome of cattle and buffalo

The comparative microbiome profiles in cows and buffalo fed on different diet regime at various geographical locations is currently being analyzed. Preliminary data on the comparative microbiome profile suggests predominance of Bacteoidetes in Mehsani buffalo and Kankrej Cows whereas of Firmicutes in Gir Cows.

5. Metagenomic changes from calf to adult in rumen

This objective could not be started for want of precise protocol for collecting stomach content from day old to 90 days.

Other notable achievements:

- 4 research papers published and 6 others are under publication
- 1 Best poster presentation award, 1 young woman scientist award, and 1 best oral presentation award.
- The programme has developed collaborations, with Gujarat University, Gujarat Vidhyapith, University of Pennsilvenia, IVRI, NRC on Camel and UAS, Dharvad on metagenomics

Internal Review meeting of the Niche Area of Excellence Program on "Metagenomic Analysis of Ruminal Microbes" held in the Department of Animal Biotechnology, College of Veterinary Science & A.H., Anand Agricultural University on **25th March, 2015**.

Following members were present:

Review Committee

1. Dr. K. Pradhan, Chairman
2. Dr.C.G. Joshi PI and Member Secretary

Dr. D.N. Kamra could not attend, because of ill health of his mother.

Invited Scientists of the College

1. Dr. Dave (Co-PI)
2. Dr. Murthy (Co-PI)
3. Dr. D. N. Rank (Co-PI)
4. Dr. Subhash Parnerkar (Co-PI)
5. Dr. P. R. Pandya (Co-PI)
6. Dr. P. G. Koringa (Co-PI)
7. Dr. S. J. Jakhesara (Co-PI)
8. Research Fellows

Meeting commenced with the welcome address by Dr. C.G. Joshi, the Principal Investigator of the Project followed by introduction of the Committee members appointed by ICAR for review of the project and handed over the floor to the Chairman.

Dr. K. Pradhan, the chairman while welcoming all scientists present in the meeting briefly described about the high standard of research attached to Niche Area of Excellence Projects and mode of selecting a discipline and scientist by the ICAR to execute the programme. He congratulated Dr. C.G. Joshi and his Team of scientists for recipient of such a research project.

Dr. C. G. Joshi (PI) presented overview of the project including the objectives, date of initiation, and overall achievements. The progress of project execution was presented by concerned scientists.

Miss. Nidhi Parmar presented the research progress on metagenomic analysis by shotgun sequencing of rumen metagenome of Gir and Kankrej cows followed by presentation by Mr. Ankit Hinsu on comparative metagenomic analysis of Mehsani buffalo with Kankrej and Gir cows. Miss. Neelam Nathani presented analysis of Jaffarabadi shotgun metagenomic analysis and Mr. Bhaskar Reddy discussed the resistome and stress responses genes analysis in Kankrej and Gir rumen. Miss. Neelam Nathani presented work of genome reconstruction from metagenome, culture of rumen originated microbes and their genome analysis.16S amplicon sequencing of rumen metagenome of Jaffarabadi and Surti buffalo was presented by Miss. Reena Patel followed by presentation of Metatranscriptome analysis of Mehsani buffalo by Mr. Ankit Hinsu and Dr. Ramesh Pandit. Further, Mr. Ishan and Miss. Deepti presented the work carried out related to cloning and expression of recombinant enzymes including cellulases, esterases and mannanases. Mr. Ravi presented work on Fosmid Metagenomic library for enzyme mining. Dr. M. Chandra Shekar presented the research progress of protozoa cDNA fosmid metagenomic library and details about work initiated on cloning and expression of phytases.

Objective wise progress is given below:

1. Molecular characterization and identification of rumen microbiome of cattle and buffalo. Analyzed rumen metagenome of Kankrejcattle and Jaffarabadi buffalo revealed predominance of Bacteroidetes phyla followed by Firmicutes, Proteobacteria, Actinobacteria and Fibrobacter. The Gir cow metagenome revealed predominance of the Firmicutes and Fibrobacter over Bacteroidetes.

2. To study effect of diet containing different proportions of nutrient ingredients on rumen microbial diversity.

   In all the studied breeds the increase in proportion of roughages (Green and Dry) (50% roughage + 50% concentrate, 75% roughage + 25% concentrate, and 100% roughage) had an impact on the rumen microbial diversity. Increasing the roughage proportion resulted in the reduction of theBacteroidetesphyla in liquid fraction with simultaneous increase in the solid fraction of the rumen digesta. While, the proportion of Firmicutes was observed to be increasing in the liquid fraction with simultaneous decrease in solid fraction. The metagenomic profile of the Jaffarabadi buffalo showed increase in Firmicutes and decrease of Bacteroidetes with respect to increasing roughage proportion in the diet.

3. Study of the active microbiome of cattle and buffalo by metatranscriptome sequencing.

   Metatranscriptome analysis of Mehsani breed revealed predominance of Bacteroidetes and Firmicutes in the liquid and solid fraction, respectively. Roughage proportion had an impact on the active rumen microbiota as revealed by various functional pathways and gene abundance analysis.

4. Functional metagenomics for discovery of enzymes with agricultural applications from rumen microbiome.

   a) Metagenomic Fosmid library and screening for potent enzymes. Fosmid metagenomic library of approximately 1.5 million clones with an average size of 40 kb insert DNA was constructed from buffalo rumen metagenomic DNA.A small fraction of clones from the amplified library when plated onto the Agar plates supplemented with Azo labelled CMC substrate, three clones exhibited enhanced capability to hydrolyze the carboxy Methyl Cellulose (CMC) substrate. These clones were also able to hydrolyzexylan.

   b) Study of active protozoa biota by cDNA library construction and screening for enzymes. Protozoa cDNA library of approximately 7.6 million clones was constructed from buffalo rumen protozoa metagenomic DNA.Further clones from the amplified library will be screenedfor enzyme activity on Agar plates supplemented with various Azo labelled substrates.

5. Cloning and characterization of novel enzymes from rumen inferred using metagenome shotgun sequencing data. Three clones including cellulase, mannanase and multifunctional GH26 were cloned and characterized and showed good activity on

their respective substrates, were stable at various pH and temperature range, and showed higher zone of hydrolysis compared to the available commercial enzymes.

6. To understand metagenomic dynamics of rumen microbiome from birth to adulthood, samples from one animal are collected.

Other notable achievements:

- 10 research papers published and 8 others are under publication.
- 1 patent filed.
- 2 Best poster presentation awards.
- The programme has developed collaborations, with Gujarat University, Gujarat Vidhyapith, University of Pennsylvania, IVRI, NRC on Camel and UAS, Dharvad on metagenomics

The committee found the progress of the project satisfactory in totality. The metatranscriptome sequencing runs of Jaffarabadi and Surti buffalo are in progress which are likely to be completed in short time. The analysis work will continue thereafter including metagenome, metatranscriptome and amplicon data and comparative analysis. Several enzymes screened from metagenome are being cloned and characterized for application as feed in the diet for agricultural importance. The fosmid library screening of bacterial and protozoa community will be pursued as per the proposed objectives. The project was sanctioned in November, 2011 hence in March, 2015 the project is completing three and a half years.

Dr.D.N.Kamra may be requested to visit the Centre and discuss with you and other scientists and provide some technical guidance as he is also working in similar research.

Considering the present outcome of the project, and its importance for the discipline, there is a need to work further for generating basic data on metagenomics of different animal species (domesticated and wild ruminants), under different geographical locations, feeding schedules and by different advanced techniques of metagenomics.

Annexure IV

Xvii. Annual Review Meetings of Niche Area of Excellence (NAE Programme)

Proceedings of the IX Annual Review Meeting of Niche Area of Excellence (NAE) Programme

The IX Annual Review Meeting of Niche Area of Excellence Programme was held on 25th May, 2015 at Conference Facility, NAS Complex, New Delhi from 9.00 AM onwards under the chairmanship of Dr. S. Ayyappan, Secy. DARE & DG, ICAR. The following experts participated:

- Dr. Arvind Kumar, DDG (Agricultural Education)
- Dr. B Meenakumari, DDG (Fishery Science)
- Dr. NK Krishna Kumar, DDG (Horticultural Science)
- Dr. AK Sikka, DDG (Natural Resources Management)
- Dr. K Alagusundaram, DDG (Agricultural Engineering)
- Dr. AK Singh, DDG (Agriculture Extension)
- Dr. Jeet Singh Sandhu, DDG (Crop Science)
- Dr. M. L. Madan, Former DDG (Animal Science)
- Dr. M. P. Yadav, Former VC, SVBPUAT, Meerut
- Dr. K. K. Vass, Former Director, CIFRI, Kolkata
- Dr. B. Gangwar, Former Director, IIFSR, Modipuram
- Dr. BangaliBaboo, Former National Director, NAIP
- Dr. T. R. Sharma, Director, NRCPB
- Dr. B. N. Tripathi, Director, NRCE, Hisar
- Dr. D. K. Benbi, National Prof., PAU, Ludhiana
- Dr. V. Upadhyay, Professor & Co-ordinator, NPTEL, IIT-Delhi
- Dr. V. S. Reddy, Group Leader, ICGEB, New Delhi
- Dr. M. S. Saini, Professor, Dept. of Zoology, Punjabi University, Patiala
- Dr. S. R. Bhat, Principal Scientist, NRCPB, New Delhi
- Dr. Dhyan Singh, Former Principal Scientist, Div. of SSAC, IARI, New Delhi
- Dr. Gaya Prasad, ADG (Animal Science)
- Dr Madan Mohan, ADG (Marine Fisheries)
- Dr S D Singh, ADG (Inland Fisheries)
- Dr. MB Chetti, ADG (HRD)
- Dr. S Mauria, ADG (IPTM & PME)
- Dr. PS Pandey, ADG (EP & HS)
- Dr K.L Khurana, Pri. Sci., Education Division
- Dr Vanita Jain, Pri. Sci., Education Division

Dr. Arvind Kumar, DDG (Agricultural Edn), ICAR, welcomed the Chairman, invited experts, DDGs, other colleagues from Education Division, the PIs and Co-PIs, who had come from various States of the country. He elaborated the concept and genesis of Niche Area of Excellence, which was launched in the X Plan for the first time with the objective to achieve educational excellence in teaching, research, consultancy and other services. He briefed the house about the achievements made under the programme by the various centre still date and raised the issues pertaining to Niche Area of Excellence and underlined the continuing importance of agricultural research, education and extension for sustaining agricultural production against the odds of reducing profitability, increasing global competition, and adverse impact of biotic and abiotic stresses. He reiterated the criticality of capacity building as the core

- Developed SNP markers of Cytochrome b gene in wild herbivores (13 species) of Madhya Pradesh. PCR-RFLP profile was also developed for the same species using Cytochrome b gene.
- Developed species specific primers for identification of Indian Wild Pig (*Susscrofacristatus*) and differentiated it from the (n=17) pig races of world and filled patent for the primer.
- Techniques developed for species identification from biological samples such as dried blood stained materials like clothes and weapons used for poaching..
- Decollaring and treatment of deep maggotised wound of a tiger at Sanjay Tiger Reserve, Madhya Pradesh.
- Played a major role in planning and execution of reintroduction program of Hard Ground Swamp Deer (*Cervusduaceallibranderi*) from Kanha Tiger Reserve to Satpura Tiger Reserve.
- Initiated quality assessment of drinking water in water holes in remote areas of different Protected Areas in Madhya Pradesh.

Specific Comments:

i. The progress was considered satisfactory.
ii. The PI, was advised not to deviate from objectives while presenting the results and focus on forensics.
iii. The annual report may be resubmitted again after restructuring.
iv. Approach of achieving the technical programme must be systematic and focussed.

7. **Metagenomic analysis of ruminal microbes (AAU, Anand).** The achievements of the programme were presented by the PI, Dr. C. G. Joshi.

Salient Achievements:

- Metagenome sequencing of 48 samples of Jaffarabadi and Surti breed each.
- Metatranscriptome sequencing runs of 48 samples for Kankarej, Gir, Jaffarabadi breeds.

Specific Comments:

i. The progress of the programme was appreciated.
ii. The centre was advised to submit the schedule for the capacity building programmes for the year 2015-16.

8. **Nutrition and gut health; probiotics, prebiotics and phytogenic as functional foods to augment gut health of dogs (IVRI, Izatnagar).** The presentation of the achievements was by Dr. A.K. Patnaik, PI.

Salient Achievements:

- A canine-origin probiotic *Lactobacillus johnsonii* CPN23 developed following a series of *in vitro* evaluation procedures, partially sequenced (Gen Bank Accession No. KP065494)
- *In vivo* evaluation of the developed probiotic (in dogs followed by rats) revealed its superiority over the dairy-origin probiotics when used in dogs

Proceedings of the VIII Annual Review Meeting of Niche Area of Excellence (NAE) Programme

The VIII Annual Review Meeting of Niche Area of Excellence Programme was held on 3rd June, 2014 at Conference Facility, NAS Complex, New Delhi from 9.30 AM onwards. The following is the list of the experts:

- Dr. Swapan Kumar Datta, DDG (CS)
- Dr KML Pathak, DDG (Animal Sci.)
- Dr. Meenakumari DDG (Fy.Sci)
- Dr NK Krishna Kumar, DDG (Hort. Sci)
- Dr AK Sikka, DDG (NRM)
- Dr. R. P. Sharma, Former Dir, NRCPB
- Dr H. S. Gupta, Director, IARI
- Dr. K K Vass. Former Dir, CIFRI, Kolkata
- Dr. GautamKalloo, Former VC, JNKVV, Jabalpur
- Dr. M. P. Yadav, Former VC, SVBPUAT, Meerut
- Dr. Gajender Singh, Former VC, Doon University, Dehradun
- Dr. T. R. Sharma, Dir, NRCPB
- Dr. BhushanLalJalali, Ex Dir of Res., HAU
- Dr. Opender Koul, Dir, IBRC, Punjab
- Dr. N. N. Goswami, Former Dean, IARI
- Dr. D. K. Benbi, National Prof. PAU, Ludhiana
- Dr. S. Rajan, Head Crop Improvement, CISH, Lucknow
- Dr. Gaya Prasad, ADG (AH)
- Dr KK Singh, ADG (PE)
- Dr. R P Dua, ADG (FC)
- Dr. B Mohan Kumar, ADG(Agro & AF)
- Dr PK Chakrabarty, ADG(PP)

Dr K. Sharma, ADG (EPD), welcomed the Chairman, invited experts, DDGs, other colleagues from education Division, the PIs and Co-PIs, who had come from various States of the country. He elaborated the concept and genesis of Niche Area of Excellence, which was launched in the X Plan for the first time with the objective to achieve educational excellence in teaching, research, consultancy and other services. He briefed the house about the achievements made under the programme by the various centres till date.

Dr. Arvind Kumar, DDG (Edn), ICAR, underlined the continuing importance of agricultural research, education and extension for sustaining agricultural production against the odds of reducing profitability, increasing global competition, and adverse impact of biotic and abiotic stresses. He reiterated the criticality of capacity building as the core mandate for this programme. He gave a brief overview of the thrust areas piloted in the XII Plan by the Education Division in enhancing the quality and capacity building in the agricultural universities and highlighted the new initiatives under consideration. He stressed that the purpose of the review meeting will not be based only for asking the deliverables but also to get feedback where we faltered and why. The subject matter experts may look into these issues and clearly suggest action points for continuing, strengthening or changes in the NAE programme.

Salient Achievements:

- ❖ Standardized uniplex PCR for 2 different species isolated.
- ❖ Studied the effect of low level Mycotoxicosis in broiler birds to study immunotoxicity: CD4 & CD8 estimation by FACS analysis, Interleukins & TNFα estimation by Sandwich ELISA, DNA fragmentation Assay.

Specific Comments:

- Reconstitution of the Internal Review Committee was recommended.
- The technical programme does not focus on innovative approaches and need to be revisted.
- A committee constituted under the chairmanship of DDG (AS) and Dr. A. C. Varshney, VC, DUVASU, Mathura, Dr Amresh Kumar, Former Dean, Veterinary College, GBPUAT, Pantnagar as members may carry out the critical review of the technical programme and align it with the objectives of the programmes.

XVII. Metagenomic Analysis of Ruminal Microbes (PI- Dr. C. G. Joshi; AAU, Anand)

Salient Achievements:

- ❖ Shotgun sequencing of rumen metagenome of 48 samples.
- ❖ Amplicon sequencing of rumen metagenome of 48 DNA and 48 RNA samples targeting 4 regions of 16S rRNA(total of 384 amplicons).
- ❖ Amplicon sequencing of fecal metagenome of 24 samples targeting 4 regions of 16S rRNA (Total of 96 amplicons).
- ❖ sequenced 144 metagenome samples by ion torrent PGM and sequenced 1184 amplicons by 454 GS-FLX titanium platforms.

Comments:

- Metagenomics needs to be translated into functional genomics.
- Metagenomic studies to be correlated with feeding practices.
- The committee agreed to extend the programme for two years and centre may submit the technical programme and activity milestones along with the relevant budget.

XVIII. Development of Bio-sensors for Diagnosis of *Peste des petits ruminants* (PPR) and Brucellosis (PI- Dr B. Mishra; IVRI, Izatnagar)

The report was presented by Dr Sameer Srivastava (Co-PI).

Salient Achievements:

- ❖ Purified and checked the reactivity of recombinant OMP-25, OMP-28 and OMP-31 protein genes by ELISA and Western Blot.
- ❖ Raised hyper-immune sera against purified recombinant OMP-25, OMP-28 and OMP-31 proteins and determined the end-point titre and purified IgGs.
- ❖ Collected cattle serum samples from UP, Orissa and Tripura for *Brucella* antibodies and used these serum samples to develop a panel of positive and negative sera samples.
- ❖ Monoclonal antibody 4G6 against PPR virus was affinity purified and immobilized on gold sensor surface.

Proceedings of Seventh Annual Review Meeting of Niche Area of Excellence

The VII Annual Review Meeting of NAE programme was organized under the chairmanship of Dr. S. Ayyappan, Secretary, DARE & DG, ICAR on 24th May, 2013 at 10:00 AM in the Mahatma Jyoti Phule Hall, Krishi Bhawan, New Delhi.

In addition to the PIs and Co-PIs of the various NAE centres, the following ICAR officials and resource persons participated in the meeting:

1. Dr. S. Ayyappan, Secy. DARE & DG, ICAR
2. Dr. Arvind Kumar, DDG (Edn)
3. Dr Meenakumari, DDG (Fy)
4. Dr. A. K. Sikka, DDG (NRM)
5. Dr D. K. Benbi, ICAR, National Professor, ICAR
6. Dr Ashok Bhatia, Ex Advisor, CSIR
7. Dr G Kalloo, Former DDG (Hort)
8. Dr R. P. Sharma, Ex-Dir., NRCPB
9. Dr Lalkrishna, Former ADG (AS) & Animal Commissioner
10. Dr O. P. Dubey, Former ADG (PP)
11. Dr. Madan Mohan, ADG (Fy)
12. Dr. R. P. Dua, ADG (F&FC)
13. Dr C. Devakumar, ADG (EPD)
14. Dr Suresh Honnappagol, ADG (EQR)
15. Dr BS Parakash, ADG (A&P)
16. Dr S. K. Malhotra, ADG (Hort-II)
17. Dr. Jyoti Misri, Principal Scientist (AS)
18. Dr Devendar Dhingra, Principal scientist (Agril. Engg.)
19. Dr. Vanita Jain, Principal scientist (EPD)

Dr. Arvind Kumar, DDG (Edn.) welcomed the chairman, experts, DDGs, ADGs, PIs/Co-PIs and other participants for the review meeting. He elaborated the concept and genesis of Niche Area of Excellence, launched in the X Plan with the objective to achieve educational excellence in teaching, research, and capacity building as core mandate. He underlined the continuing importance of agricultural research, education and extension for sustaining agricultural production against the adverse impact of biotic and abiotic stresses and reiterated the importance of capacity building as one of the core mandates of this programme. He gave a brief overview of the NAE centres continuing in the XII Plan and their achievements and informed that the annual review meeting has become one of the regular features of NAE and it has helped in refining the objectives and technical programmes of the centres of the NAE.

Dr. S. Ayyappan, Secretary, DARE & DG, ICAR welcomed all experts and other participants. He reiterated that this concept of NAE is to build a school around an idea of local relevance with global standards. He reiterated that it is not a project but a support for the chosen centre to grow

in an interdisciplinary mode creating a brand value for the university. The centres must undertake to elevate their outlook, outputs and outcomes in an excellent manner and noting that some centres have created a website webpage dedicated to the programme, urged all the centres to emulate this feature this year for enhancing the visibility. He emphasized that it was important that the vision and scope of the NAE as understood by most, needs to be reviewed, so that envisaged objectives are fully achieved and the deficiencies in the programme are rectified. He emphasized on economic feasibility of the technologies developed and PIs should take care to publish the findings in high impact journals. He also emphasized that the universities should give the PIs financial/administrative powers to run the programme smoothly. He suggested formulation of the advisory committees for each centre.

The programme was divided into three technical sessions viz. Animal and Fishery sciences, NRM and Agricultural Engineering and Plant Sciences, and the PIs made brief presentations covering *inter alia* the objectives, technical programme (2012-13), the achievements made and the technical programme (2013-14). The PI/Co-PI of each centre made a brief presentation and thereafter experts offered their suggestions and comments.

1. Dr. C.J. Joshi, AAU, Anand made a presentation of the centre "*Metagenomic Analysis of Ruminal Microbes* ".

Salient achievements

- Metagenomic analysis of Mehsani buffalo and Kankrej cow rumen total microbiota and fecal samples by shotgun and amplicon sequencing under different feeding regimes
- A shortcourse on Metagenomics: Role of Next generation sequencing and bioinformatics" was organized from 15th Oct. to 24th Oct., 2012
- Publications: Scientific bulletins: A practical manual for shotgun sequencing on Ion Torrent PGM platform and also for amplicon sequencing on 454 Roche GS-FLX Titanium platforms.

Expert comments

- The progress made under the programme was appreciated.
- Microbial libraries of both culturable and non-culturable categories may be created and maintained.
- The work should also give more importance to metagenomic part .
- It was suggested to increase the number of training programmes for capacity building in the area.
- The application part of the metagenome biome may be kept in view.

2. Dr. B. P. Mishra, IVRI made a presentation of the salient achievement the centre *"Development of Bio-sensors for Diagnosis of Peste des petits ruminants (PPR) and Brucellosis"*.

Proceedings of Sixth Annual Review Meeting of Niche Area of Excellence

The VI annual review meeting was organized under the chairmanship of Dr. S. Ayyappan Secretary, DARE & DG, ICAR on 21st June, 2012 at 9:00 AM in the DG's committee room, Krishi Bhawan.

In addition to the PIs and Co-PIs of the various NAE centres, the following ICAR officials and resource persons participated in the meeting:

1. Dr. P. L. Gautam, Chairperson, PPVFRA, New Delhi
2. Dr. Arvind Kumar, DDG (Edn.)
3. Dr. M. M. Pandey, DDG (Engg.)
4. Dr. Meenakumari, DDG (Fy.)
5. Dr. K.M.L. Pathak, DDG (AS)
6. Dr A. P. Singh, VC, DUVASU, Mathura
7. Dr. R. P. Sharma, Former Director, NRCPB
8. Dr. H. S. Gupta, Director, IARI
9. Dr. Ashok Bhatia, Ex-Advisor, CSIR
10. Dr. C. Devakumar, ADG (EPD)
11. Dr. T.P.Rajendran, ADG (PP)
12. Dr. Dr K.K. Singh, ADG (PE)
13. Dr. Madan Mohan, ADG (Fy.)
14. Dr. B.S. Prakash, ADG (ANP)
15. Dr. J. N. Kataria, Jt. Director (Research), IVRI and
16. Dr. (Mrs) M. Dadlani, Jt. Director (Research), IARI

Dr. Arvind Kumar, DDG (Edn.) welcomed the chairman, experts, DDGs, ADGs, PIs/Co-PIs and other participants for the review meeting. He informed that the annual review meeting has become one of the regular programmes of Education Division and it has helped in refining the objectives and technical programmes of the centres of the NAE. In today's meeting' eight centres would present their progress and seek technical and administrative guidance in pursuing the path of niche with excellence in the chosen area.

Dr. S. Ayyappan, Secretary, DARE & DG, ICAR welcomed all experts and other participants. He reiterated that this concept of NAE is to build a school around an idea of local relevance with global standards. It is not a project but a support for the chosen centre to grow in an interdisciplinary mode creating a brand value for the university. Centres must undertake to carryout training programmes for the targeted students and faculty so that a pool of trained manpower in the area is created and sustained within NARS.

The PIs made brief presentations covering the objectives, technical programme (2011-12), the achievements made and the technical programme (2012-13) with concomitant demand of the budget.

1. Dr. Anna Mercy, PI of the center ***"Integrated Centre for Ornamental fish Research, Training and Management"***, KUFOS explained her

difficulties in not making a headway in the programme and reluctantly requested closure of the centre. It was resolved that the centre should be closed and the university may be asked to refund the grant released. The VC who joined later sought the permission to change the PI and continue the centre. This was not agreed to, as the NAE is based on the expertise and competence of the PIs/Co-PIs collectively, in the chosen field and two Co-PIs have purportedly left the scheme.

2. Dr. C. G. Joshi, PI of the centre ***"Metagenomic Analysis of Ruminal Microbes"***, AAU, Anand made an excellent presentation highlighting the following significant progress made.

Salient achievements:

- The first stage of the experiment which consisted of adaption of animals (Mehsani buffaloes) on high concentrate diet, reared at S.K. Nagar campus, and collection of rumen sample at the end of six week treatment has been successfully completed.
- A pilot study to standardize the methodology for metagenomic analysis of active and total rumen microbial communities was performed using 16 amplicon pyrosequencing from fiber adherent and liquid fraction of buffalo rumen and significant variations were observed in the active and total microbial communities present in the rumen liquid and solid fractions as well as variation between the fiber adherent communities compared to the ones enriched in the liquid fraction.
- Analysis of effect of different feed regimens on rumen microbial communities has been undertaken.

Expert comments:

- The experts appreciated the good work done by the centre. In order to minimize physiological variabilities, the centre was advised to use dry animals as treatments and not to include lactating cattles.

3. Dr. B. P. Mishra, IVRI made a presentation on the work carried out under the centre ***"Development of Bio-sensors for Diagnosis of Peste des petits ruminants (PPR) and Brucellosis"***.

Salient achievements:

- Full length N gene (1578bp) of PPR virus was amplified from vaccine strain using specifically designed primers. The amplified PPR-N gene was cloned in pET 32b vector and recombinant clone was confirmed by RE digestion and nested PCR. Sub-cloning of PPR-N gene was further done in prokaryotic expression vector pQE-30 to produce recombinant N protein.