A PROJECT REPORT ON

MITIGATION OF ENTERIC METHANE EMISSIONS IN CATTLE THROUGH INDIGENOUS ALGAE

Submitted by

HIMANSHI BENDRE

MTech. Biotechnology 602104003

Under the guidance of

Dr. SADANAND SONTAKKE
Senior Principal Scientist
CSIR- National Environmental Engineering Research Institute, Nagpur
2022-2023

CERTIFICATE

This is to certify that **Ms. Himanshi C Bendre** has prepared this project titled **"MITIGATION OF ENTERIC METHANE EMISSIONS IN CATTLE THROUGH INDIGENOUS ALGAE"** under my guidance and to my satisfaction, in fulfilment of the requirement for two years M-TECH. (Biotechnology) Degree programmed.

Dr. Sadanand Sontakke
Senior Principal Scientist,
CSIR-NEERI, Nagpur

Debajyoti Dutta

Dr. Debajyoti Dutta
Assistant Professor
Department of Biotechnology
TIET, Patiala

DECLARATION

I hereby declare that this project titled **“MITIGATION OF ENTERIC METHANE EMISSIONS IN CATTLE THROUGH INDIGENOUS ALGAE”** is submitted to **Thapar Institute of Engineering & Technology** (TIET), Patiala. This is my individual work carried out at **Council of Scientific and Industrial Research – National Environmental Engineering Research Institute, Nagpur** under the guidance of **Dr. SADANAND SONTAKKE,** for the fulfilment of the degree of **Master in Biotechnology** (M-TECH) and this project report or part of this thereof has not been submitted elsewhere for any other degree. I also undertake that the material I reproduced in this thesis from other sources has been duly acknowledged.

Place: Patiala

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Bendre

HIMANSHI BENDRE

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ABBREVIATIONS

1.	GHGs	Greenhouse gases
2.	CFCs	Chlorofluorocarbon
3.	Co2	Carbon dioxide
4.	CH_4	Methane
5.	N_2O	Nitrous oxide
6.	NASA	National Aeronautics and Space Administration
7.	O_3	Ozone
8.	IPCC	**Intergovernmental Panel on Climate Change**
9.	TG	Tera-gram
10.	DNA	Deoxyribonucleic acid
11.	PPB	**Parts per billion**
12.	PPM	Parts per million
13.	F2 medium	Guillard’s f2 medium
14.	BG-11 medium	Blue-Green 11 Medium
15.	Nacl	Sodium chloride
16.	Kcl	Potassium chloride
17.	$CaCl_2$	Calcium Chloride
18.	MgCl2	Magnesium chloride
19.	Na_2SO_4	Sodium sulphate
20.	H2SO4	**Sulphuric Acid**
21.	C-TAB	Cetyltrimethylammonium bromide
22.	EDTA	Ethylenediaminetetraacetic acid
23.	KH2PO4	Monobasic potassium phosphate
24.	GC-MS	Gas Chromatography Mass Spectrometry
25.	VFA	Volatile fatty acid

ABSTRACT

Climate change and global warming has major negative impacts on human health and a cause of concern to the environment and biodiversity as a whole. Greenhouse gases (GHGs), in particular, methane (CH_4), are the major contributors of global warming. Livestock and farm animals alone produce more than half of total GHG emissions in the world contributing significantly to GHGs emissions through enteric fermentation and its manure. From nutritional perspective, enteric methane energy accounts for 2 – 12% of gross energy intake, and by any reduction in this energy can be diverted to production purposes. Given India's largest population of cattle and buffalo in the world, country needs strategy in place to make animal production more efficient, environmental-friendly and more sustainable and at par or above the global standards.

In an effort to mitigate methane emissions from ruminants, dietary modifications have been explored as a viable strategy, offering both environmental and nutritional benefits. Recently, Australian researchers proposed use of red seaweed in ruminants to tackle enteric methane problem. In vitro experiments have demonstrated that altering ruminant feed intake can substantially reduce methane emissions, with algal-derived feed additives showing the most promising anti-methanogenic effects with as much as 80% reduction in methane production in livestock. However, so far, there are no studies on Indian algae. The present study attempts to screen three different indigenous algae for their anti-methanogenic compounds and their potential to mitigate methane production in vitro using cow rumen fluid. Further, we determined the nutritive value of these seaweeds and attempts were also made for cultivation of algae and extraction of genomic DNA from these seaweeds. Such endeavours hold great potential for significantly reducing GHG emissions, thereby improving air quality, and combating climate change.

Chapter 1

INTRODUCTION

Global warming and climate change has significantly impacted human health and at the same time environment and biodiversity are also at risk. One of the main causes of climate change are greenhouse gases (GHGs). The average surface temperature during the 20th century was 57.0° Fahrenheit (13.9° Celsius), and in 2022 it was 1.55° Fahrenheit (0.86 °Celsius) higher than the average and 1.90° Fahrenheit (1.06° Celsius) warmer than it was during the pre-industrial era (1880–1990).

According to (Myhre et al., 2013), emissions of GHGs from livestock, such as methane, carbon dioxide and nitrous oxide, among others, both directly and indirectly contribute to global warming. About 51% of all global GHG emissions come from livestock in developing nations. India is responsible for around 15% of the world's methane emissions, 12% of its carbon dioxide emissions, and 4% of its nitrogen oxide emissions. Livestock manure makes up the remaining 7–8% of GHG emissions, while enteric fermentation is responsible for 15% of them. Due to their size and concentrated diet, cattle and buffalo are the largest greenhouse gas emissions (Caro et al., 2014). Methane production by cattle ranges from 32 to 98.6 kilogrammes per head per year.

Dairy and nursing beef cows are the main contributors (Sejian et al., 2011). The amount of dry matter consumed, the breed, the microbial population of each animal, and the nutritional content affect the enteric fermentation of ruminants. In addition to contributing to climate change, ruminant methane also causes host animals to consume less energy (2–12% gross energy intake) (Johnson et al.,1995). The energy saved from a reduction in CH_4 generation may be used to boost animal performance and increase the productivity of products like meat, milk, and wool (Choi et al., 2022).

Therefore, there is growing interest in reducing methane emissions for both environmental and nutritional reasons. especially if an animal is powered by the energy saved through methane reduction. Genetic selection, the forage-to-concentrate ratio (F:C), tannin-rich feedstuffs, pasture quality, and the use of microalgae and macroalgae in diet are all strategies to minimise ruminant methane emissions. Therefore, altering the food may be one of the approved and applied strategies to lower methane levels while also increasing the animal output. Several feed additives have been used to decrease methane production.

By controlling rumen microbial methanogenesis, CH_4 synthesis can be achieved. The most promising additions for this purpose have been found to be seaweeds, also referred to as red, green, or brown macroalgae. Seaweeds are being harvested in almost 50 different nations, with the worldwide seaweed output in 2018 reaching 32.4 million metric tonnes (Choi et al., 2022).

Numerous *in vitro* experiments that were published elsewhere suggested that altering ruminant feed intake might significantly reduce cattle methane emissions. Algal-derived feed additives have been shown to have the best anti-methanogenic effects in ruminants, which increased animal production and caused an intestinal methane output decrease as much as 50%. There are several strategies under investigation for biologically reducing bovine intestinal methane. Algae was used as a feed modulator *in vivo* trial on dairy calves, which considerably decreased methane emissions by 60% (Roque et al., 2019). Red algae exhibit strong anti-methanogenic action when added to the diet. Red seaweed contains active substances such as bromoform, dibromochloromethane, dibromo acetate, and bromo chloroacetate with anti-methanogenic properties that can be employed in enteric methane mitigation, (Machado et al., 2016). Algal biomass is well known to be a fascinating choice for useful nutritional supplements in humans, and it has the potential to be extended to cattle. It has been established that microalgae boost the DHA/EPA content of the product while having no effect on total production when used as a feed additive for breastfeeding cows (Altomonte et al., 2018). By increasing daily weight increase averages, fatty acid content, and other growth performance parameters, previous research have shown that introducing microalgae improves the quality of meat. On the other hand, the quantity of microalgae that should be added to feed varies depending on the microalgae species and the intended animal type (Madeira et al., 2017).

Even though similar research is being conducted all around the world, this one is probably unique. Australian experts have suggested using red seaweed in ruminants to address the methane problem. The suggested initiative makes an effort to examine local algae for potential anti-methanogenic properties and use them to reduce methane generation in animals, helping to combat climate change and global warming. Important dietary control issues need to be researched before commercialising algae as a common feed ingredient. Two of the most important dietary management factors that may be used to optimise ruminal fermentation without reducing animal output or intake are the practical feeding range and dose. When more than 15% of the diet is fat, it affects dietary intake and palatability. a unique feed supplement that carefully weighs the quantity of supplemental feed vs. the amount of feed the animal can tolerate. Only a small number of the more than 200,000 species of algae have been examined as future feed additions for ruminants with relation to algae as a cutting-edge feed supplement. Less than ten microalgal species and roughly 40 macroalgal taxa have thus far had their capacity to lessen CH_4 examined. Additional research is needed to ascertain the long-term viability of CH_4 reduction using algal additions.

Results to be expected include the identification of anti-methanogenic substances and biochemical tests (carbohydrate, lipid, and protein) in various algae. Algal biomass' anti-methanogenic effectiveness will be determined by *in vitro* tests, and their incorporation in feed may be standardised in terms of quantity. The polymerase chain reaction test, which used in majority of DNA molecular procedures, requires the separation of genomic DNA with the necessary degree of purity. NGS data will offer fresh perspectives on microbial ecology and ruminant metabolic potential. This will be crucial for researching biological methods to lower cattle methane emissions. Algae mix microbial cell biotechnological traits, making them capable of fast multiplication in a liquid medium with minimal nutrition requirements (unlike fungus and bacteria, which require an organic carbon source to develop) and the accumulation of certain metabolites. By implementing this innovative feeding strategy, farmers may substantially decrease methane emissions while also improving animal output. Such research can significantly lower GHGs emissions, enhance air quality, and combat climate change.

Chapter2.

REVIEW OF LITERATURE

2.1 Overview of climate change and global warming's impacts

Climatic variations are variations in the average temperature, rainfall, and other atmospheric variables brought on by persistent human involvement over a long period of time. The global temperature has increased by 1.9°F since 1880, according to the different NASA examinations of global climate change. The increase in emissions of greenhouse gases (GHG) is the primary reason for the rise in global temperature. The greenhouse effect, which is caused by certain GHGs in the atmosphere, allows heat energy from the earth's surface to escape. methane (CH_4), nitrous oxides, Carbon dioxide (CO_2), and chlorofluorocarbons (CFCs) are examples of GHGs. the Arctic Sea is receding, ice sheets are melting, snow cover is diminishing, Ocean temperatures are rising, and ocean temperatures are rising.

Increased levels of GHGs are the root cause of ocean acidification (Six et al., 2013). One of the main GHGs is methane (CH_4). The principal (GHG) that has grown up to several folds and prevents the heat contained in the atmosphere from escaping is this transparent, odour-free gas (Jones, 1984). Human actions, such as the burning petroleum and coal, the manufacture of hydrocarbons, clearing forests, the industrial revolution, etc., are to blame for the release of CH_4 into the environment.

The biggest threat to life on Earth is the climate change. It is the repercussions of both artificially created and natural emissions of air pollutants, particularly (GHGs), that have a significant impact on the climate (IPCC., 2013). In other words, GHGs are principally responsible for the complex link between air pollution and climate change (Von Schneidemesser et al., 2015). Among the GHGs contributing to global warming are (CO_2), water vapour, ozone (O_3), (CH_4) and (N_2O) (Hughes et al., 2020).

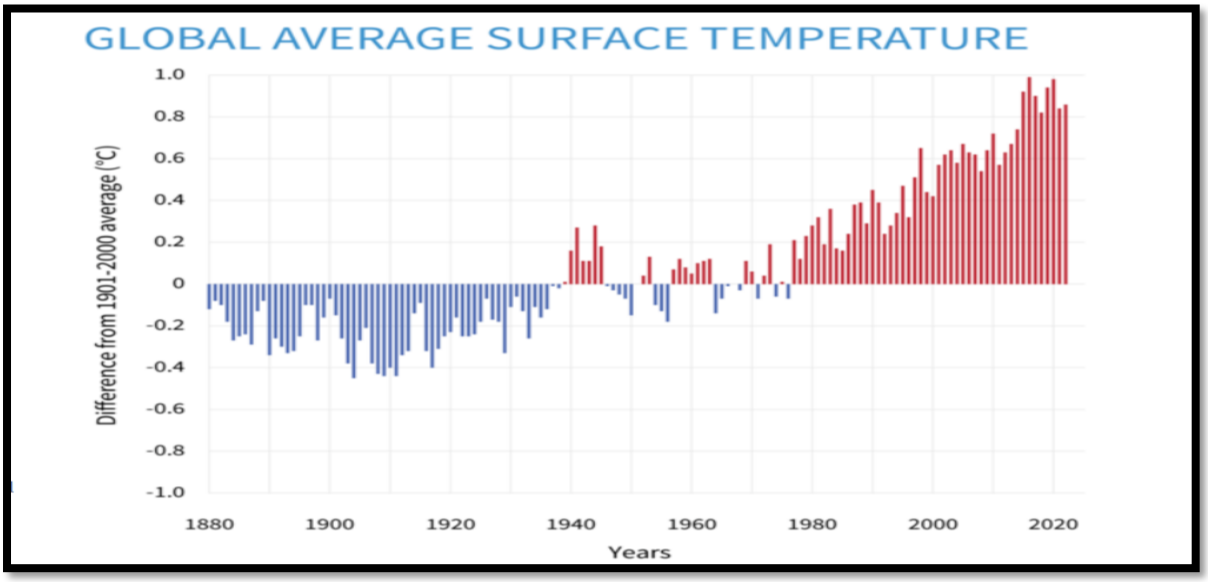


Fig1: Global average surface temperature (NOAA)

Annual surface temperature as compared to the average for the 20th century (1880–2022). Red bars reflect years that were warmer than normal, and blue bars show years that were colder than usual. Based on data from the National Centres for Environmental Information, the NOAA Climate.gov graph According to NOAA's temperature data, The sixth-warmest year on record was 2022. In the 20th century, the average surface temperature was 57.0 ° Fahrenheit (13.9 °Celsius); in 2022, that average was 1.55 ° Fahrenheit (0.86 °Celsius) hotter, and the pre-industrial era (1880–190) was 1.90 ° Fahrenheit (1.06 °Celsius) warmer.

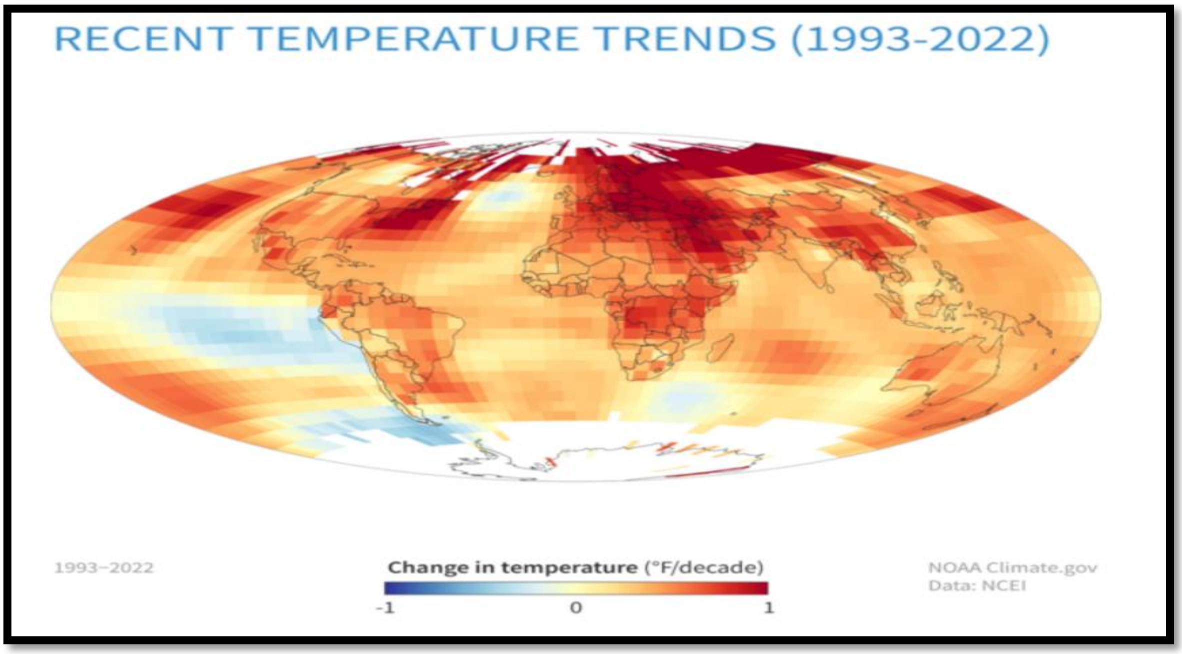


Fig2: Recent temperature trends (NOAA 2022)

Statistics in degrees Fahrenheit each decade for the worldwide average surface temperature between 1993 and 2022 The majority of the world (yellow, orange, and red) is warming. Few places, mostly in the waters of the Southern Hemisphere, experienced cooling during this time.

2.2 Greenhouse gases

Global surface temperatures have increased per result of climate change brought on by the release of GHG from diverse sources (Calabro PS 2009). (Vijaya et al., 2012) Because it is continuing to have a large and detrimental influence on people, natural resources, and economic situations globally, climate change—a consequence of greenhouse gas emissions—is a global issue (Abbasi et al., 2010). According to Russell R. 2007's research, the main greenhouse gases (GHGs) include H2O (g) (36-70%), water vapour, nitrous oxide (3–7%), carbon dioxide (9-26%), and methane (4–9%), along with other gases.

Among these GHG, CO_2, as well as CH_4, has been held responsible for increasing global surface temperatures (Hansen et al. 2009). These gases are released as a result of both entirely natural and human processes. CH_4 is the second-largest cause of global warming, behind CO_2, according to (Xiaoli et al., 2010). It has a bond angle of 109.51, is the most fundamental alkane, and accounts for the bulk of natural gas. It is created when organic compounds in the environment break down anaerobically. With a high global warming potential (GWP) of 21–25 times greater than carbon dioxide (IPCC 2006), methane is a far more powerful greenhouse gas than carbon dioxide (Todd RW et al. 2011).

Methane now makes up 20% of anthropogenic radiation, second only to carbon dioxide (60%) (Lassey 2007). However, because it is around 25 times more effective than CO_2 at trapping atmospheric heat, it has been under increased scrutiny in recent years. However, compared to the thousands of years that CO_2 has been present in the atmosphere, methane has a comparatively short persistence lifetime of just approximately 10 years. there is now a greater worldwide focus on the implementation of initiatives to reduce methane emissions. Agriculture and the use of fossil fuels are the human-related activities that are most strongly linked to the rising trend in methane concentration (IPCC 2007).

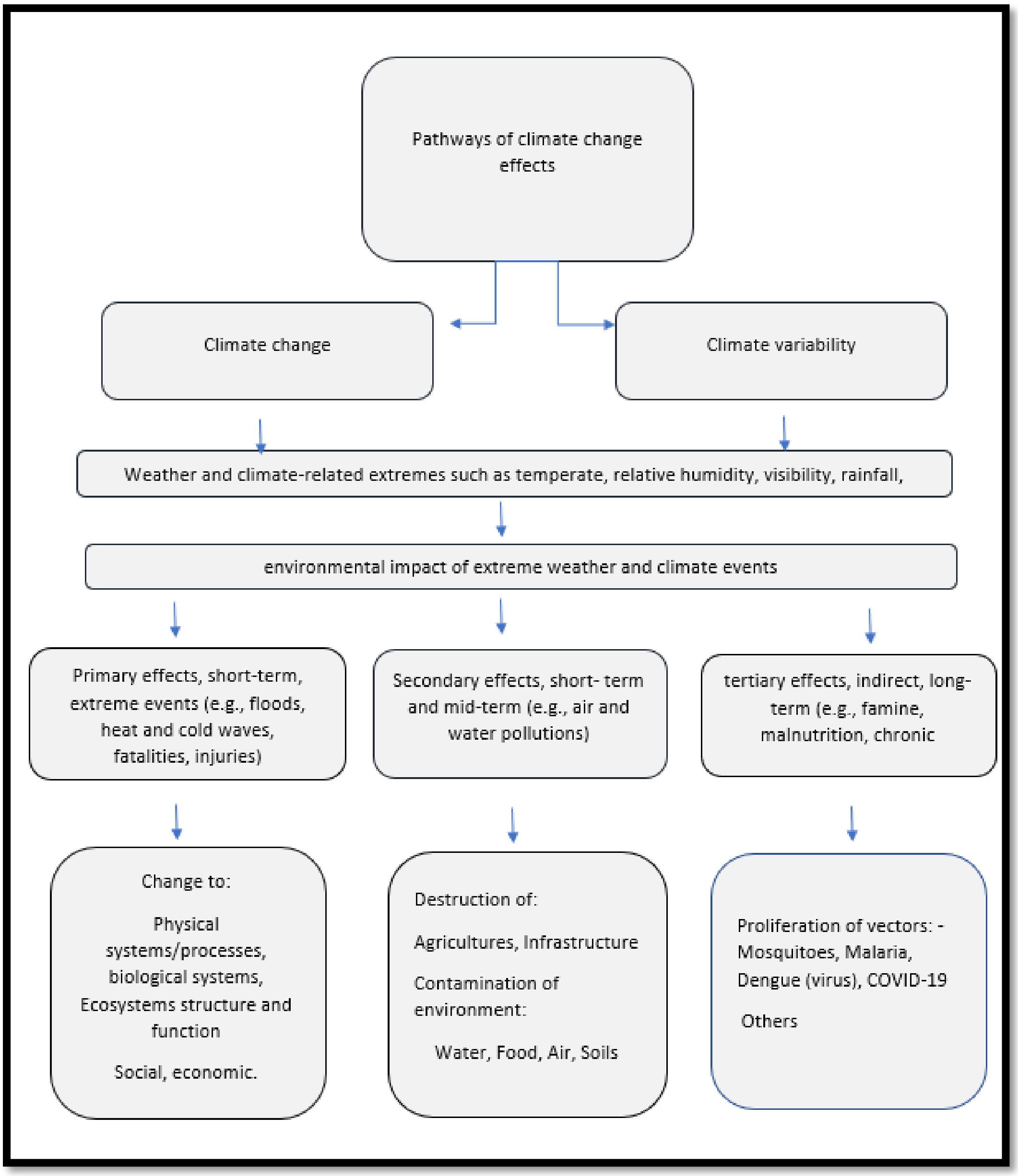


Fig 3: Climate change is the most significant threat to life on Earth (Kumar 2021)

While wetlands, termites, and seas are among the natural sources that are thought to produce roughly 40% of the world's methane emissions, manmade activities account for 64% of the total (World Meteorological Organisation 20019). The main sources of emissions from agriculture have been crop production, animal raising, the breakdown of biomass, and the application of soil inputs, both organic and inorganic. Enteric fermentation and manure management are the two processes that contribute the most to methane emissions from the livestock business in the agricultural sector. The type of animal, the type and quantity of feed consumed, and the technique used to handle waste all have an effect on how much methane is produced. (Biswal et al.,2020)

2.3 Environmental protection protocols

These GHG levels are constantly rising, which can alter the course of global climate change. According to the IPCC, the world's temperature might climb by 2.6 to 10 degrees Celsius over the course of the next century as a result of the ongoing growth in CO_2 and CH_4 concentrations. The timing of the seasons will also be affected by how much the climate is changing globally. Even more frequent droughts, high-intensity heat waves, and low-intensity cold waves would occur.

We typically estimate farm animal-related gas emissions throughout the period of 1961–2010 in 237 countries and 11 farm animal categories in accordance with IPCC criteria (IPCC 2006). We find that in 2014, farm animal emissions of methane and nitrous oxide made for around 9% of all global greenhouse gas emissions. In comparison to the quantity that was looked at, global GHG emissions from agriculture were exaggerated by 51%, mostly because of an increase in emissions in developing countries (+117%). Emissions from developed nations, however, reduced by roughly 23%. Beef and milk cows are the two primary sources of farm animal emissions, accounting for 74% of all farm animal emissions worldwide. (Caro et al. 2014).

2.4 Emissions of CH_4

Methane, along with (CO_2) and (N_2O), is one of the 3 primary GHG. In comparison to CO_2, its potential to cause global warming is 23 times higher, Methane is a flavourless, transparent, odourless, explosive gas. Methane, or CH_4, is a naturally occurring gas with a specific gravity of 0.554 and is found in the atmosphere. The melting point of methane gas is -187 °C, whereas the boiling point is -161 °C. The density of methane gas is 0.717 m3/kg. Only water is insoluble in this gas; nonetheless, organic solvents are soluble in it. Methanogenesis is the primary mechanism responsible for the creation of naturally occurring CH_4. The equation for the reaction is CO_2 + 4H2 → CH_4 + 2H2 O (Hook et al., 2010). Methane is a greenhouse gas, and during the past 250 years, increases in atmospheric methane content have resulted in more radiative forcing of the atmosphere. Since 1750, increases in atmospheric methane concentration have contributed to about 17% of increases in the atmosphere's radiative forcing. If the effects of the greenhouse gases produced by methane's atmospheric reactions are taken into account, that percentage rises by about a factor of 2.

Methane emission sources are being identified and quantified with greater scientific interest because of their contribution to the radiative forcing of the atmosphere. Biogenic, geogenic, and anthropogenic sources all contribute to methane emissions; the biggest human-made sources include natural gas and petroleum infrastructure, enteric fermentation in cattle, landfills, coal mining, and manure management (Allen 2016). It is crucial to expand the group of nations committed to aggressive methane reductions. The five nations with the biggest worldwide methane emissions (from all sources) are China, Brazil, the US, Russia, and India. They represent more than half of the world's total methane emissions. (IEA 2022).

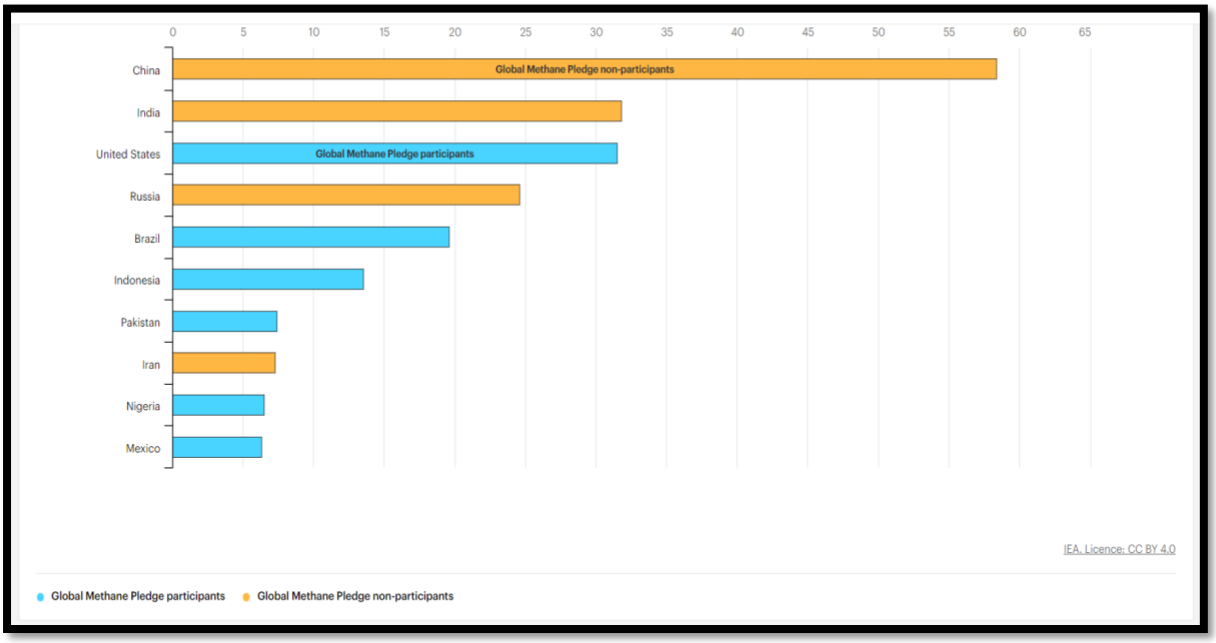


Fig 4: Top 10 methane emitting countries (IEA (2022)

Methane is a result of oxygen-deprived microbial activity in the gastrointestinal tracts of several "domestic ruminants" across the world, including, cattle, sheep, goats, buffalo, and camels. (World Economic Forum, 2019; article number 22)

2.5 Shows the trend of rising atmospheric CH_4 levels.

Methane levels in the atmosphere are about 1.9 ppm, but those in cows' breathing air are about 1000 ppm (Palangi et al., 2022). According to recent research by (NOAA) (Madsen et al., 2010), methane levels in the atmosphere increased by 17 parts per billion (ppb) in 2021. Since the start of systematic tracking, this rate represents the greatest yearly increase that has been seen. Last year, the average atmospheric methane concentration was 1.898 parts per million (ppm), This is around 162% greater than levels before the Industrial Revolution. The rise in atmospheric CH_4 levels as witnessed in the past three centuries is shown by the 2.5-fold increase in the atmospheric methane burden, which has reached levels unheard of in at least 650,000 years.

As a result, Effective and efficient generation of methane mitigation is required. strategies for ruminants throughout the world in order to help combat climate change (Palangi et al., 2022).

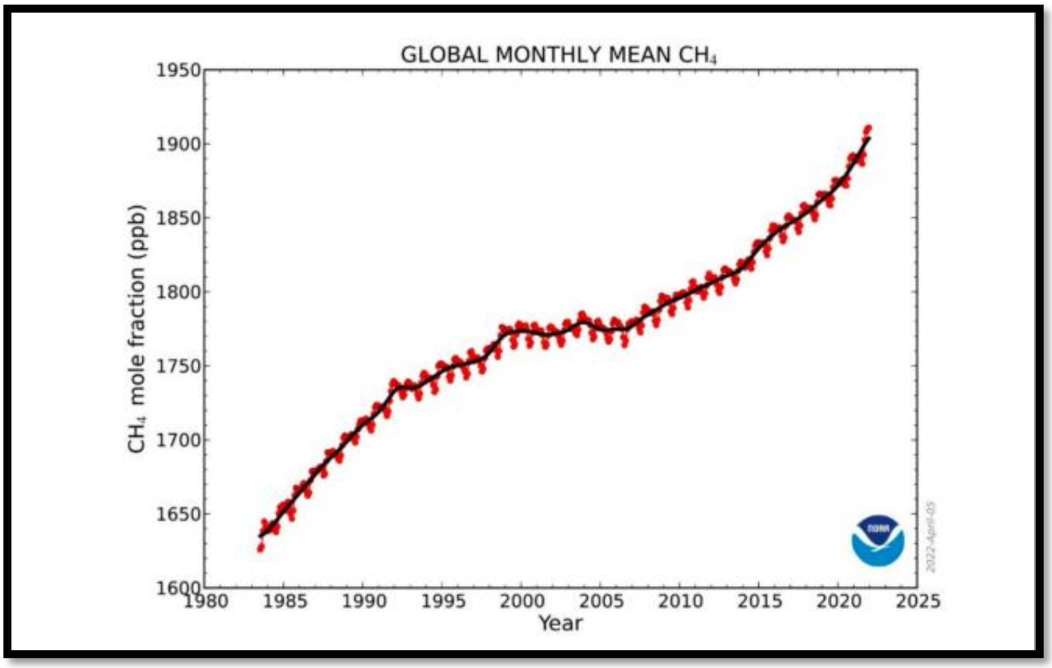


Fig 5: Increasing atmospheric CH_4 levels. (NOAA 2022)

2.6 Distinct GHG production sectors.

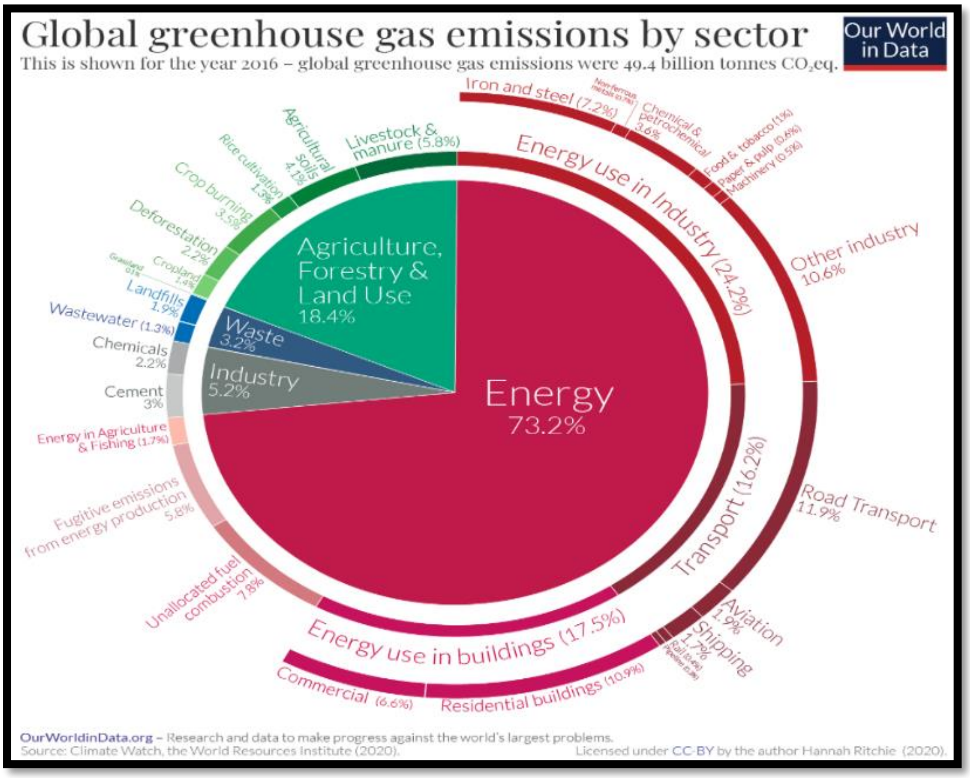


Fig 6: GHG emission by different sector (world data 2016)

Negative climatic changes are a result of the worldwide hazards posed by rising surface temperatures. Methane is one of the GHG. It is released both naturally and artificially from many sources, and the level of concentration in the environment is frightening. It is the subject of intensive examination and research due to its catastrophic effects on climate change and atmospheric chemistry. The three industries of agriculture, energy, and garbage make up the majority of the anthropogenic sources of its emissions. Methane emissions have been shown to be highest in the agricultural, energy, and waste sectors, in that order.

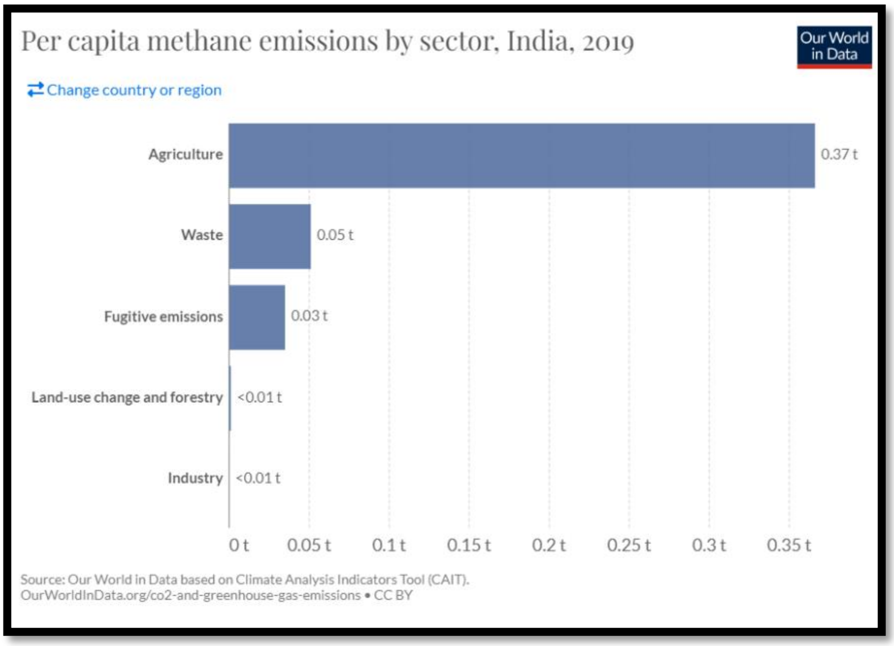


Fig 7: Methane emission by different sector in India (https://ourworldindata.org/)

This analysis looks at methane emissions from 1990 to 2010 and explains the trends that were seen. shows the trajectory of human-caused CH_4 emissions from sources in 2010. The three main sectors that make up the largest known sources of CH_4 emissions are waste electricity. Agriculture, and management.

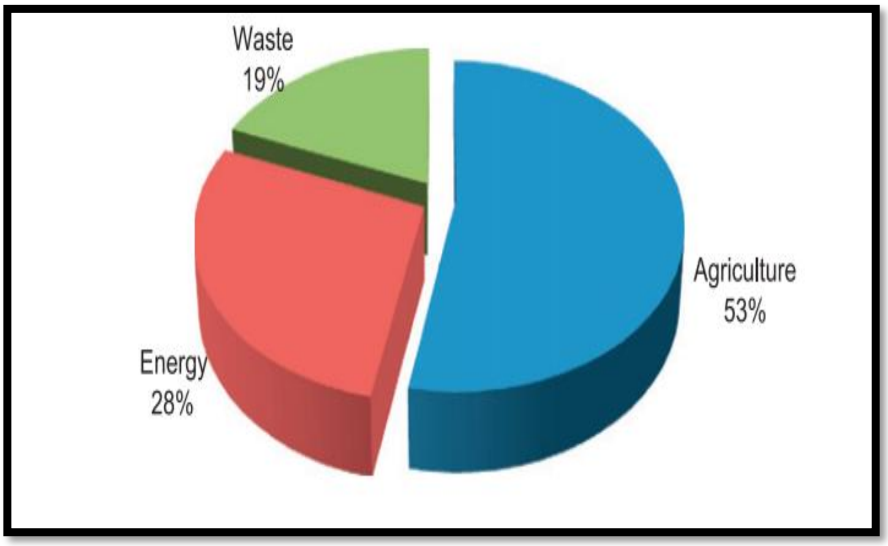
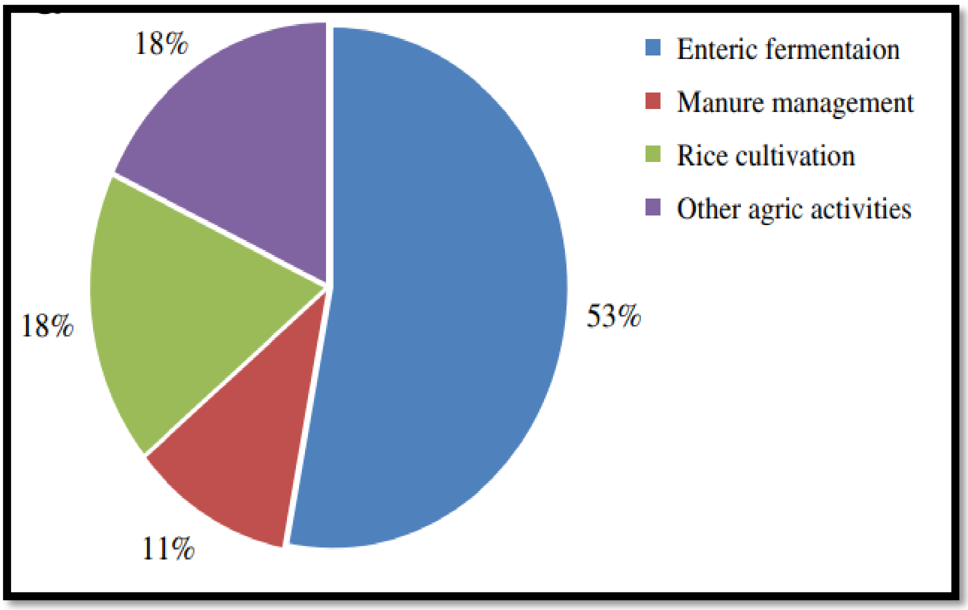


Fig 8: Anthropogenic methane emission by sectors (Sejian V. et al. 2011)

In contrast to the growing of rice, additional agricultural operations, and managing manure, which each contributed 18%, 18%, and 11% of agricultural emissions, respectively (UPEPA 2020), enteric fermentation (livestock rearing) alone was responsible for 53% of agricultural emissions (Naqvi et al., 2011). 85 Tg of the 550 Tg of gases containing methane produced globally each year are produced by ruminant cattle.

Fig9:



Methane emissions from agriculture

The primary cause of this higher trend the increased agricultural production is responsible for the rise output necessary to fulfil the demands of the continuously expanding populations in many nations. Due to increases in population and affluence, agricultural product consumption is rapidly rising in many of these areas.

2.7 For animals, we are concentrating on the agricultural sector.

About 25.6% of all global (GHG) emissions come from the agriculture sector. While greenhouse gases garner the majority of attention as a factor in heating, CH_4, N_2O, and CFCs also significantly contribute to radiative forcing. This paper discusses enteric CH_4 emission forecast models, estimation techniques, and mitigation strategies. The amount of CH_4 that ruminants emit varies between developed and developing nations and is influenced by things like animal type, breed, stomach fluid pH, the ratio of propionate to acetate, the number of archaebacteria, meal composition, amount of concentrate provided. Bos taurus, a ruminant species, is the one that contributes the most to atmospheric phenomena through gas emission, followed by sheep, goats, and buffalos, in that order. In industrialised nations, the calculated rates of CH_4 emission per Bos taurus, buffalo, sheep, and goat are much higher in developing nations.

To identify mitigation strategies for lowering CH_4 emissions, there is a great deal of interest in creating new and improved CH_4 projection models. The most recent method designed to more precisely quantify CH_4 is the micrometeorological mass distinction approach. because there are significant regional differences in the conditions in which animals are kept. Producers are more inclined to use economical tactics, boost production, and have minimal unanticipated negative effects on the yield of cattle. It is crucial to assess CH_4 mitigation options in relation to the entire GHG budget and to take different techniques' economics into account. Although reducing GHG emissions from the cattle sector is of utmost importance, the economic viability of firms cannot be jeopardized in the process. (Sejian et al.,2011)

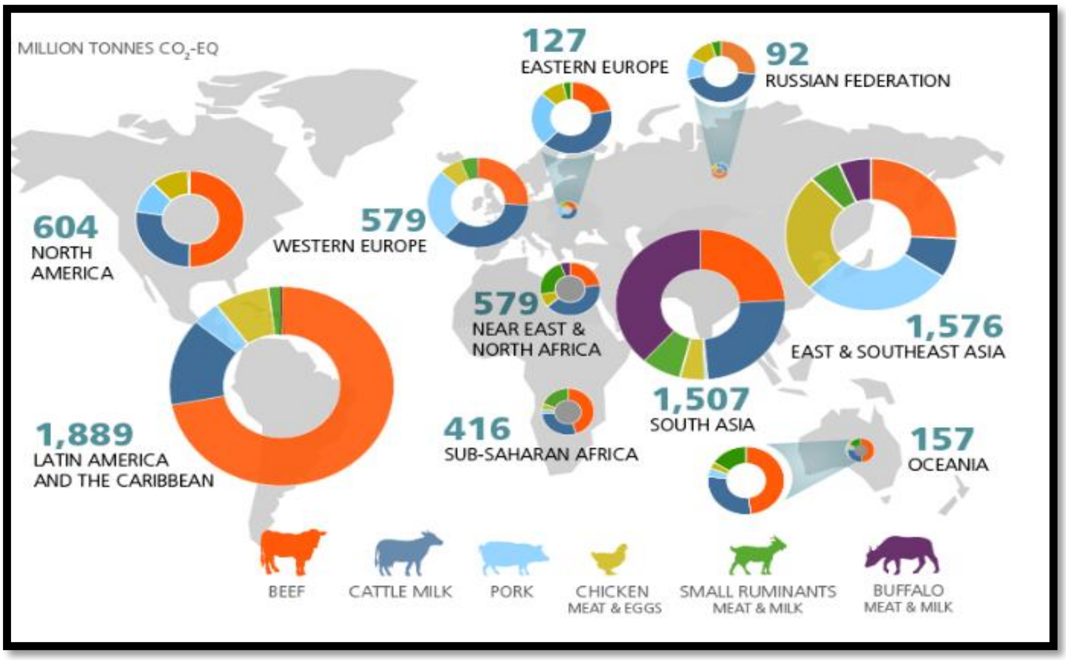


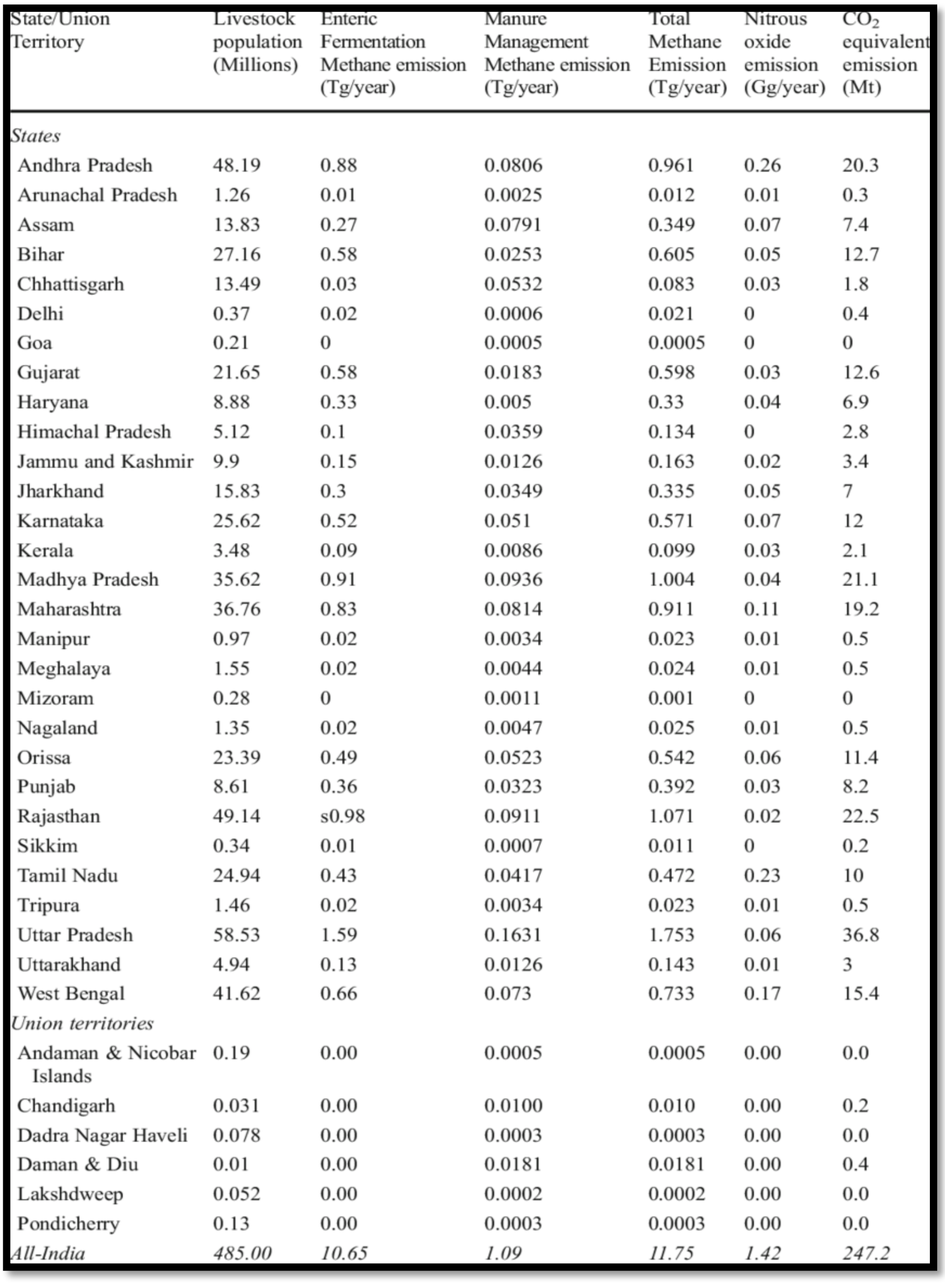
Fig10: Methane emission by different countries through various livestock. (World Economic Forum articles 22, 2019)

2.8 Methane outflow from animals in India

Strong GHG methane contributes to global warming. Livestock is a substantial human contributor to methane emissions from agriculture. India has the largest and most diversified population of livestock in the world, numbering 485 million animals. When it comes to livestock, cattle make up 38.2% of the total, followed by goats (25.7%), buffalo (20.2%), sheep (12.7%), and other species (3.3%). The most recent livestock census from 2013 and the country-specific and Indian feed standard-based emission coefficients were used to estimate the comprehensive state-wise methane emission11.75 TG (tera gram) of methane, including emissions from manure management and enteric fermentation, were emitted by Indian animals in 2003. Enteric fermentation accounts for 10.65 Tg (91%), whereas manure management only provides 1.09 Tg (9%). 60% of all methane emissions come from indigenous dairy cattle and dairy buffalo combined. The three states with the greatest methane emissions are Uttar Pradesh (14.9%). Rajasthan (9.1%), and Madhya Pradesh (8.5%) The livestock available feed and fodder area collected from remote sensing was used to determine the overall methane flow from Indian cattle, which was estimated to be 74.4 kg/ha. (Chhabra et al., 2009) Although production of meat and eggs accelerated after 1980, milk production in India had a rapid increase after 1970. In the past, India's trade balance for items derived from animals and cattle was negative. (Sonavale et al., 2020)

The population of India generated 23% of the nation's greenhouse gas emissions and 17% of the GDP. 77% of the country's Two-thirds of methane emissions from the agricultural sector were attributed to enteric fermentation, manure use, and rice production, according to estimates for the year 2008; methane emissions from urban solid waste have been steadily rising over the past 20 years, reaching 8% of all methane emissions. Using the aggregated emissions estimates (Garg et al., 2011).

Table 1: comprehensive estimates of methane emissions at the state/regional level (Tg) (Chhabra et al.,2013)



State/Union Territory	Livestock population (Millions)	Enteric Fermentation Methane emission (Tg/year)	Manure Management Methane emission (Tg/year)	Total Methane Emission (Tg/year)	Nitrous oxide emission (Gg/year)	CO_2 equivalent emission (Mt)
States						
Andhra Pradesh	48.19	0.88	0.0806	0.961	0.26	20.3
Arunachal Pradesh	1.26	0.01	0.0025	0.012	0.01	0.3
Assam	13.83	0.27	0.0791	0.349	0.07	7.4
Bihar	27.16	0.58	0.0253	0.605	0.05	12.7
Chhattisgarh	13.49	0.03	0.0532	0.083	0.03	1.8
Delhi	0.37	0.02	0.0006	0.021	0	0.4
Goa	0.21	0	0.0005	0.0005	0	0
Gujarat	21.65	0.58	0.0183	0.598	0.03	12.6
Haryana	8.88	0.33	0.005	0.33	0.04	6.9
Himachal Pradesh	5.12	0.1	0.0359	0.134	0	2.8
Jammu and Kashmir	9.9	0.15	0.0126	0.163	0.02	3.4
Jharkhand	15.83	0.3	0.0349	0.335	0.05	7
Karnataka	25.62	0.52	0.051	0.571	0.07	12
Kerala	3.48	0.09	0.0086	0.099	0.03	2.1
Madhya Pradesh	35.62	0.91	0.0936	1.004	0.04	21.1
Maharashtra	36.76	0.83	0.0814	0.911	0.11	19.2
Manipur	0.97	0.02	0.0034	0.023	0.01	0.5
Meghalaya	1.55	0.02	0.0044	0.024	0.01	0.5
Mizoram	0.28	0	0.0011	0.001	0	0
Nagaland	1.35	0.02	0.0047	0.025	0.01	0.5
Orissa	23.39	0.49	0.0523	0.542	0.06	11.4
Punjab	8.61	0.36	0.0323	0.392	0.03	8.2
Rajasthan	49.14	s0.98	0.0911	1.071	0.02	22.5
Sikkim	0.34	0.01	0.0007	0.011	0	0.2
Tamil Nadu	24.94	0.43	0.0417	0.472	0.23	10
Tripura	1.46	0.02	0.0034	0.023	0.01	0.5
Uttar Pradesh	58.53	1.59	0.1631	1.753	0.06	36.8
Uttarakhand	4.94	0.13	0.0126	0.143	0.01	3
West Bengal	41.62	0.66	0.073	0.733	0.17	15.4
Union territories						
Andaman & Nicobar Islands	0.19	0.00	0.0005	0.0005	0.00	0.0
Chandigarh	0.031	0.00	0.0100	0.010	0.00	0.2
Dadra Nagar Haveli	0.078	0.00	0.0003	0.0003	0.00	0.0
Daman & Diu	0.01	0.00	0.0181	0.0181	0.00	0.4
Lakshdweep	0.052	0.00	0.0002	0.0002	0.00	0.0
Pondicherry	0.13	0.00	0.0003	0.0003	0.00	0.0
All-India	*485.00*	*10.65*	*1.09*	*11.75*	*1.42*	*247.2*

2.9 Ruminant methane production alleyway

According to (Sejian et al., 2012), ruminant digestion involves fermenting the plant matter that has been ingested in the animal's forestomach (rumen). Ruminants lack the hydrolytic enzymes required to glean energy from the structural sugars in plants. Instead, they depend on a diverse consortium of microorganisms living in their digestive systems, such as bacteria, archaea, protozoa, and fungi (Choudhury et al.,2015). The first step in digestion is when microbial enzymes break down carbs into monomers, which are subsequently fermented to become VFAs. According to the research of (Boadi et al.,2004), These volatile fatty acids, are converted to CH_4 by butyrate in the rumen wall or are absorbed via the ruminal epithelium and utilized as the ruminant's energy source.

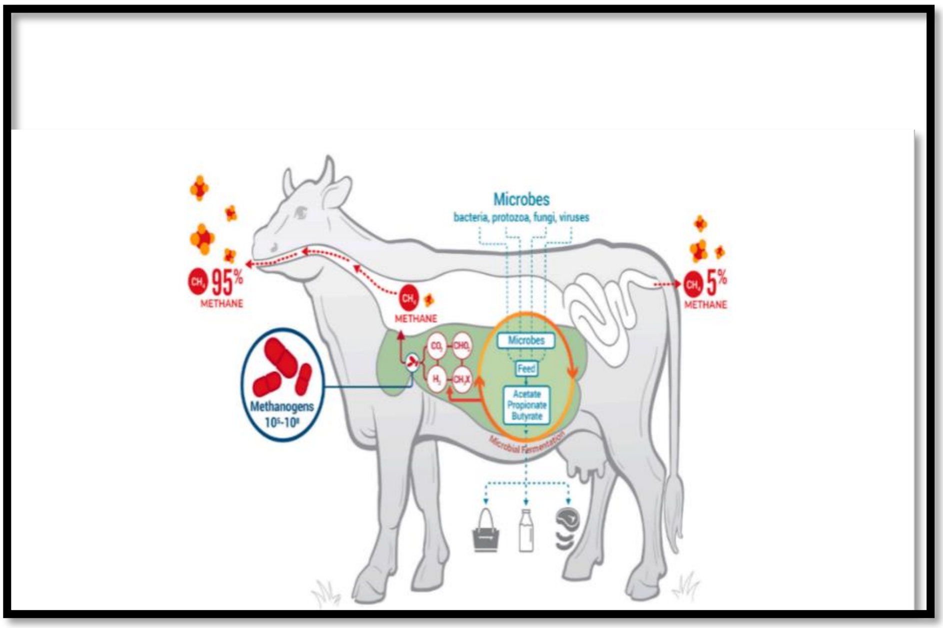


FIG11: Ruminant fermentation processes, microbial contribution and product (Bélanger et al.,2019)

2.10 There are several methods for measuring intestinal CH_4 emissions.

There are now measurement techniques for ruminant enteric CH_4 emissions. The respiration chamber is one of them and is recognized as the reference technique, despite neither being portable nor suitable for capturing many animals (Goopy et al.,2016). In actual agricultural circumstances, (SF6) the sulphur hexafluoride tracer technique (Grainger et al.,2007) provides a high level of accuracy. However, both CH_4 recording techniques are expensive and could impact animal behaviour (Garnsworthy et al.,2019). While labour-intensive and portable, sniper systems like Greenfeed also require some animal training. The laser methane detector (LMD) technique was initially advised for massive trait recording in the field by (Chagunda et al.,2009). Since then, the strategy has been applied in several ruminant research investigations (Sorg et al., 2016). The LMD is currently the most adaptable and straightforward method for on-farm estimation of enteric CH_4 emissions due to its mobility, low cost, ease of handling, non-invasiveness, and not to interfere with animal behaviours (Pinto et al., 2020). However, concerns about the consistency and precision of the measurements do exist (Pickering et al., 2015).

2.11 Methane from a Ruminant Source

2.11(A) Enteric fermentation-produced methane

Enteric CH_4 is a waste product of ruminant digestion created by the bacteria Archaea through a process known as fermentation or methanogenesis, according to (Dana et al.,2017). Animal characteristics including chewing, salivation, and digestive kinetics have an impact on the rate and type of fermentation (Varga et al.,1997). Sheep and goats create around 7 and 9 times as much CH_4 as cattle do. The most typical way that animals release CH_4 into the environment is through their mouths and noses. Only 11% of the CH_4 generated in the hindgut was found to be released through the anus, compared to 95% of the CH_4 created by intestinal fermentation in the foregut that was ejected by eructation (Murray et al.,1999). As seen in the image above, the anus was responsible for 5% of the overall CH_4 gastrointestinal gases released by the oral cavity, nose, and rectum. Even though absorbed CH_4 is also present in the air from the lungs and is inhaled with air, the concentration in the breath fluctuates, being lower when the breath originates from the lungs and greater when the "breath" is gases belched from the front abdomen (Madsen et al.,2010). The focal point will be considerably more intense in a barn or bigger area.

2.11(B)Methane from manure

Excreta is a source of CH_4 in addition to intestinal synthesis, particularly when CH_4 is stored during respiration (Klevenhusen et al.,2011). Enteric fermentation is unquestionably the primary source of methane emissions in regions with minimal inputs, accounting for 2% and

0.4% of worldwide CH_4 and GHG emissions from ruminant and non-ruminant animal species, respectively. However, large sources of emissions in industrialized areas come from high manufacturing and food processing (Gerber et al.,2013). Because grazing is generally done under aerobic conditions, manure emissions from Compared to vast or grazing operations, intensively managed dairy farms with manure storage systems contribute more to overall farm CH_4 emissions. A part of the organic substances in livestock manure, including proteins, carbohydrates, and lipids, are accessible to anaerobic bacteria as food and energy. Of course, the energy value of the gas itself might be a gain from methane generation. But the effectiveness of the operating system is what mostly determines how much gas can be produced from manure. According to (Song et al.,2011), gas yield may be defined as the volume of gas generated for every unit of solid matter broken down by anaerobic bacteria. The natural process of anaerobic digestion involves bacteria consuming organic material in an atmosphere devoid of oxygen. GHG (CO_2 and CH_4) as well as microbial biomass are produced as a result. The volatile substances in manure have an impact on the formation of CH_4 and the anaerobic breakdown of organic matter. According to (Getabalew et al.,2019), the volatile solids in manure are mostly made up of proteins, carbohydrates and fatty acids all are readily biodegradable.

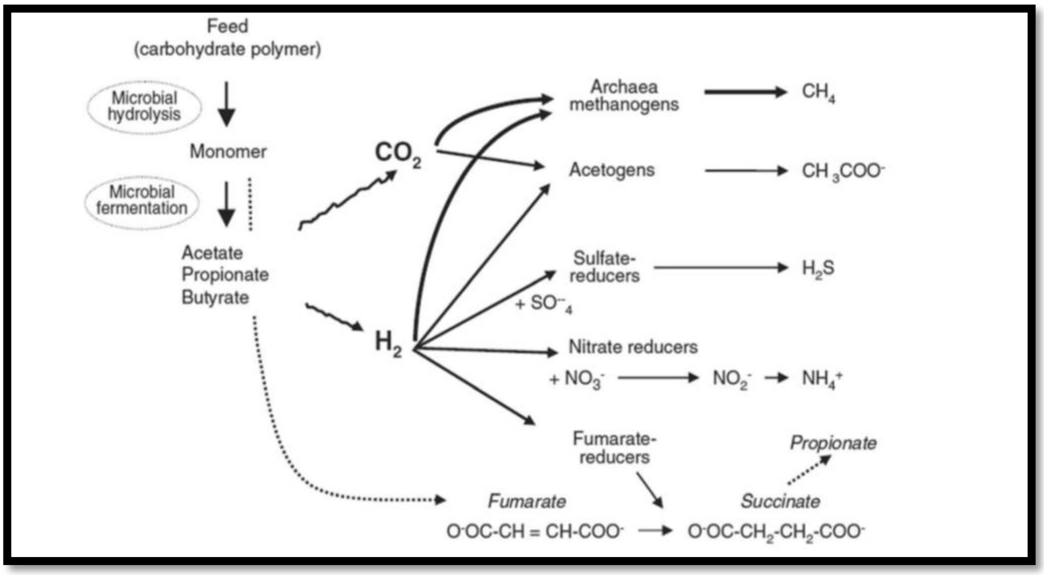


Fig 12: Schematic microbial fermentation and h2 reduction pathway in the rumen (Getabalew et al.,2019)

2.12 Methods to Reduce Ruminant Enteric Methane Emissions

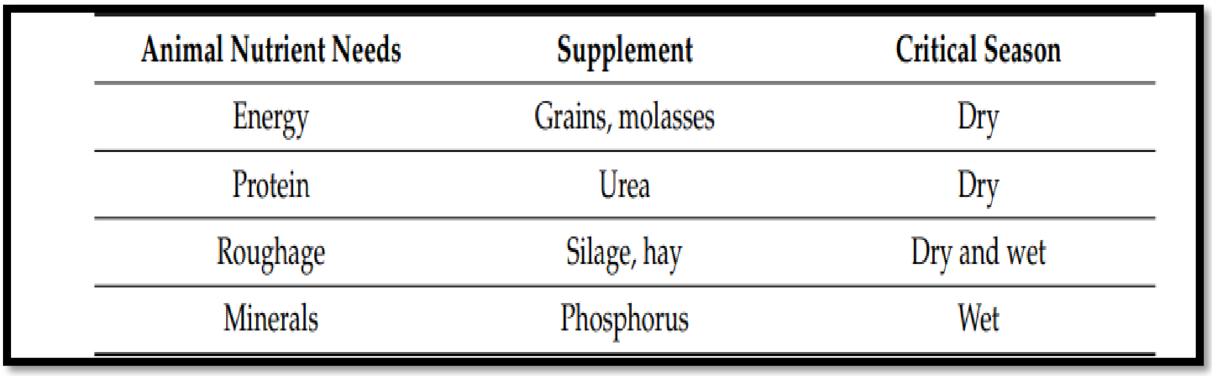
2.12(A) Genetic Selection

According to growing evidence (Lvendahl et al. 2018), dairy cows' feed conversion efficiency (FCE) and methane emissions are heritable features. In order to determine these qualities, it is important to take the rumen microbiota into account. Therefore, individuals with lower CH_4 emissions must be chosen in order to take advantage of the hereditary element (Lassen et al. 2016).

2.12(B) Forage to Concentrate Ratio (F: C)

2.12(C) Compared to pasture diets, high-concentrate diets create less methane.

Table2: Standard tropical animal nutrition during certain times of year (Suybeng et al.,2019)



Animal Nutrient Needs	Supplement	Critical Season
Energy	Grains, molasses	Dry
Protein	Urea	Dry
Roughage	Silage, hay	Dry and wet
Minerals	Phosphorus	Wet

2.12 (D)Adding dietary oils or lipids to the meal

The efficiency of the rumen's microbial flora would be significantly altered by the inclusion of fat in the diet, which would also result in a decrease in methane generation (Beauchemin et al., 2006). It may be considered that lipids and oils may be employed efficiently for enteric methane reduction, either alone or in conjunction with other methods. By including more fat in the diet, the generation of methane may be reduced in five different ways: by reducing the digestion of fibre, the intake of feed, the number of methanogens, the population of rumen protozoa, and by boosting biohydrogenation reactions. They also investigated how fish oil supplements may reduce methane emissions. (McGinn et al.,2004)

2.12 (E) Tannin-rich feedstuffs

The epidermis, roots and leaves of most plants contain tannins (derivatives of tannic acid), a category of soluble in water phenolics with a wide range of chemical properties that interact with proteins to create insoluble or soluble complexes (Hagerman et al.,1992). Tannins are known to affect rumen bacteria indirectly and directly reducing the methanogenesis and biohydrogenation of unsaturated fatty acids as well as ruminal protein degradation.

2.12(F) Quality of a Pasture

Many things affect the fodder's quality. As previously mentioned, the quantity and kind of meal ingested are closely associated with gastrointestinal methane emissions, and several studies have shown that including legumes in feed has a favourable impact. (Dini et al.,2017) investigated how varying pasture quality levels affected CH_4 emissions. In comparison to the control group, the releases of CH_4 from the excellent pasture-grazing animals were 11% lower when expressed as g CH_4/kg dry matter intake and 14% lower when represented as a percentage of total energy intake.

When taken as a dietary supplement, (Vázquez et al., 2020) looked at the inclusion of herbs in feed. The antimethanogenic effects of Mexican aster (*Cosmos bipinnatus*), chamomile (*Matricaria chamomilla*), and lemon grass (*Cymbopogon citrat*us) on beef cattle given a high-concentrate diet were studied.

2.13 List of substances that reduce methane

Medium- or long-chain fatty acids, such as omega-3 (DHA), bromoform/bromochloro-methane, ethylene, aliphatic molecules, and nitro compounds Halogenated acyclic hydrocarbons, hydroxymethylglutaryl-SCoA,

However, once directly consumed by people and animals in high quantities, these chemicals will produce severe responses and have semipermanent toxic effects (Risher et al., 2005). Therefore, administering these substances directly to ruminants could be problematic. However, meal replacements with organically generated, exceedingly low amounts of these chemicals yet are nonetheless efficient in increasing CH_4 synthesis might provide a different option. It is estimated that up to 70% of the volatile, transitory ozone-depleting chemical tribromomethane is produced by algae (Chipperfield et al., 2020). Whenever it is produced, it acts as a bacterial defence mechanism. Red algae are a somewhat unique and accessible source of the chemical for ruminants because of its volatility and the rarity with which it accumulates inside the algal cell itself (Paul et al., 2006).

2.14 Determining and examining a bioactive compound's impact

Tribromomethane, dibromochloromethane, bromo chloroacetic acid, and dibromo carboxylic acid were the main natural products (Machado et al., 2016). Red algae are a source of these chemicals.

In addition to having over 100 low relative molecular mass natural products identified, red algae also contain halo acids, haloalkanes, halomethanes and haloketones (Burreson et al., 1975).

With lesser concentrations of bromo chloroacetic acid and dibromochloromethane present, tribromomethane ($CHBr3$, tri-brominated halomethane) bromoform is consistently the most prevalent of those natural products (Burreson et al.,1975). These immune systems from herbivores and bacteria are produced and kept in certain organ cells from wherever they are discharged onto the surface (Paul et al., 2006a, 2006b).

In *in vitro* ruminal fermentation experiments, algae have shown antagonistic methanogenic activity (Machado et al.,2015). These natural compounds are effective against the bacteria that produce CH_4 through microbial production because they strongly impede the assembly of CH_4 once adscititious at a concentration of 1.5-5% of organic matter incubated. Notably, synthetic halomethanes and tribromomethanes are intended to control methanogenesis and breadbasket fermentation, is concentrating on bromochloromethane, a synthetic halomethane that blocks the creation of CH_4 both in vivo and *in vitro*. These low relative molecular mass halomethanes operate by interacting with reduced vitamin B12 to prevent the carbamide-dependent methyl group enzyme phase, which is essential for methanogenesis.

Various haloalkanes, such as 2-chloroethanesulfonate and 2-bromoethanesulfonate, its structurally comparable to coenzyme-M and inhibits the methyl group transfer procedures that are essential for the production of CH_4 via competition. The efficiency of synthetic halomethanes as anti-methanogens suggests that the natural substance's method of action is quite similar. Red algae can control the production of enteric CH_4 by using all of its biomass, extract, or refined parts. For this to be employed, the production of VFA, which are the animals' primary source of energy, must be effective with no negative effects on the fermentation properties or the breaking down of organic feedstock.

Instead of artificial inhibitors like chemical-based and halomethanes BCM, which are prohibited in several nations because of their possible impacts on the degradation of ozone and malignant neoplasms, the public is more inclined to embrace natural goods or complete biomass. (Hristov et al.,2013).

To get around all of this, methane emissions should be constrained ("mitigated") or eliminated in an environmentally responsible way. Different reductions optimized to lower enteric greenhouse gases haven't been very effective. Despite *in vitro* trials showing a considerable reduction in methane emissions, no study has been done to investigate the effects of red algae on dairy cattle. One of our objectives was to impartially assess the effect of intense algal feeding on cattle on methane production. In terms of feeding, changing the food has been recognised as one of the most practical strategies that farmers may adopt and use to reduce CH_4 (Maccarana et al.,2014). This method of strategically managing CH_4 production from enteric fermentation must take into account a variety of factors.

2.15 Algal biomass as a methane reducer

Golden algae, eubacteria, red algae, diatoms, and euglenoids are some of the several types of algae that may be found in different bodies of water across the world. They range in size from microscopic to enormous and may be found in marine water, stagnant water, the sea, oceans, rivers, streams, lakes, ponds, etc. (Diatoms of North America 2019). The sea is the greatest surviving repository of bioactive chemicals and the habitat for half of the world's variety. Usually, marine red algae are distinguished as huge benthal sea plants that are alive or cellular. The three pigmentation groups of Chlorophyceae (green algae), Rhodophyceae (red algae) and Phaeophycean (brown algae) are frequently used to classify marine red algae. Pigments like chlorophyl for green, and fucoxanthin for yellow and phycobilin’s for red are thought to be responsible for marine algae's colour.

The phylogenetically extremely old creatures known as red seaweeds, or Rhodophyta (from the phyla "plant and the Greek rhodo- "rose”), have numerous unique characteristics in their shape and manner of reproduction. They are grouped with around 6100 other species with a wide range of sizes and forms within the Primo plantae clade of non-vascular plants.

Rhodophyta are photosynthetic, do not have flagella, and have phycobiliproteins and chlorophylls a and d. (phycoerythrin, phycocyanin, and allophycocyanin), carotenoids, and accessory pigments. Red seaweeds, as previously stated, have a distinctive polymer makeup and do not include chloroplasts for glucose; as a fallback supply, they utilize floridean glucose from the cytoplasm. Red seaweeds are present at all latitudes; however, they are particularly common in tropical areas. Polar and subpolar areas have few species and are dominated by brown and green algae. Red algae are mostly short filamentous plants in tropical oceans, but larger species with huge thalli can be found in colder and more moderate climates. Rhodophyta is better able to survive at extreme levels than other algae groups and may grow up to 210 m below the surface because of the presence of optional pigments. (Cian et al.,2015).

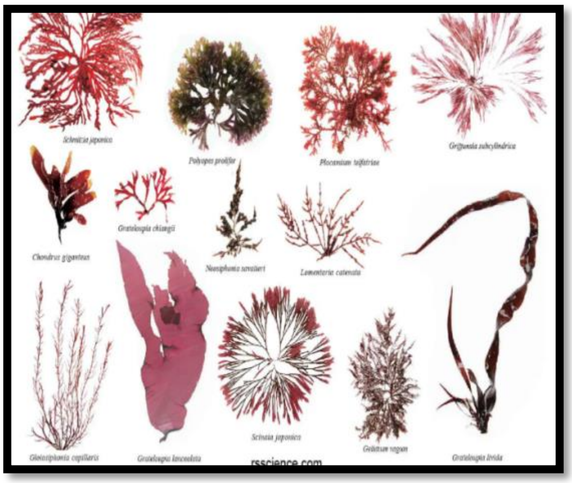


Fig13: Red algae (Rhodophyceae) (Serisawa et al., 2015)

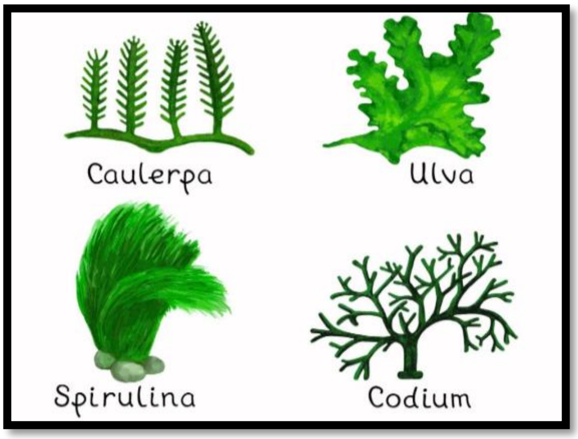


Fig14: green algae (Chlorophyceae)

(www.vectorstock.com%2Froyalty-free-vector%2Fgreen-algae-vector-)

Fig15: Brown algae (Phaeophycean) (https://qsstudy.com)

The least number of findings was found to be in fresh algae. An infinite amount was shown to be necessary since higher levels of algae in eating habits tend to lower substrate edibleness milk output and HDMI. Since it was observed that the red marine algae were dreadfully poor at decreasing CH_4 (80%) at a sporadic level of feed inclusion, with a detrimental effect on overall fermentation, it has attracted a lot of attention. (McCauley et al., 2020).

Some brown and red macroalgae will reduce microbic methanogenesis and are utilized as nourishment to minimize methanogenesis during intestinal fermentation, according to *in vitro* studies (Machado et al., 2014; Roque et al., 2019).

2.16 Using algae biomass as a feed additive

Multicellular macroalgae may be found in a wide range of settings and have intricate life cycles. They can range in size from a few millimetres to up to fifty metres. Macroalgae, also known Historically, seaweeds have been fed to pregnant mammals as food. (Makkar et al., 2016) noted several benefits of macroalgae, including their capacity to meet the macromolecule and energy requirements of the animals as well as their presence of bioactive compounds, which may improve the animal's assembly and state of health.

Cellular photoautotrophs with a size of under 400 m, microalgae can be utilized as a source of feed for animals. Ruminant species appear to be suitable candidates for this unique diet since they can both use and digest the non-protein nitrogen present in red algae. There are just a few articles that have been published that explore the effects of phytoplankton on milk supply and quality, even though microalgae have the potential to be used in ruminant nutrition.

The main effects of microalgae on milk production are changes in the carboxylic acid profile of the milk, particularly those linked to long-chain fatty acids and the omega-3 family, including DHA and EPA, which are good for human health. Given that efforts to boost the omega-3 content of milk through feeding have thus far had very moderate benefits, these findings are noteworthy. However, excessive algal supplementation might harm feed intake, ruminal metabolism, and palatability, as well as lower milk output and fat content. (Altomonte et al. 2018)

Fig 16: Macroalgae (Planted Reef Red Bamboo Marine Macro Algae Solieria spp.)

The natural marine resource known as microalgae is being studied for a variety of commercially feasible uses, including animal feed. This article discusses the impact of nutritional microalgae on the production and meat quality of placental animal species, including pigs, ruminants, rabbits, and poultry. The four subgroups of microalgae include diatoms, chlorophytes, golden algae, and blue-green eubacteria. The most significant phototrophic species are found within the genera Arthrosporic, Chlorella, Dunaliella, and Haematocussus. Additionally, it is possible to effectively cultivate multifaceted marine organisms that produce n-3 long-chain unsaturated fatty acids. The bulk of microalgae's nutrients include carbohydrates, lipids, proteins, minerals, vitamins, and bioactive elements like carotenoids. This varied nutrient content is dependent on the species, strain, and algal growth circumstances. Evidence has shown that including microalgae in animal, diets can improve the growth and meat nutritional value of ruminant, pig, poultry, and rabbit animals.

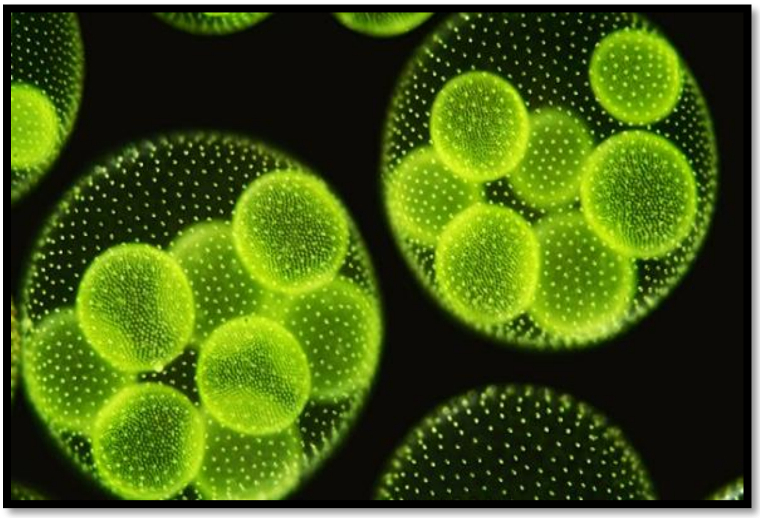
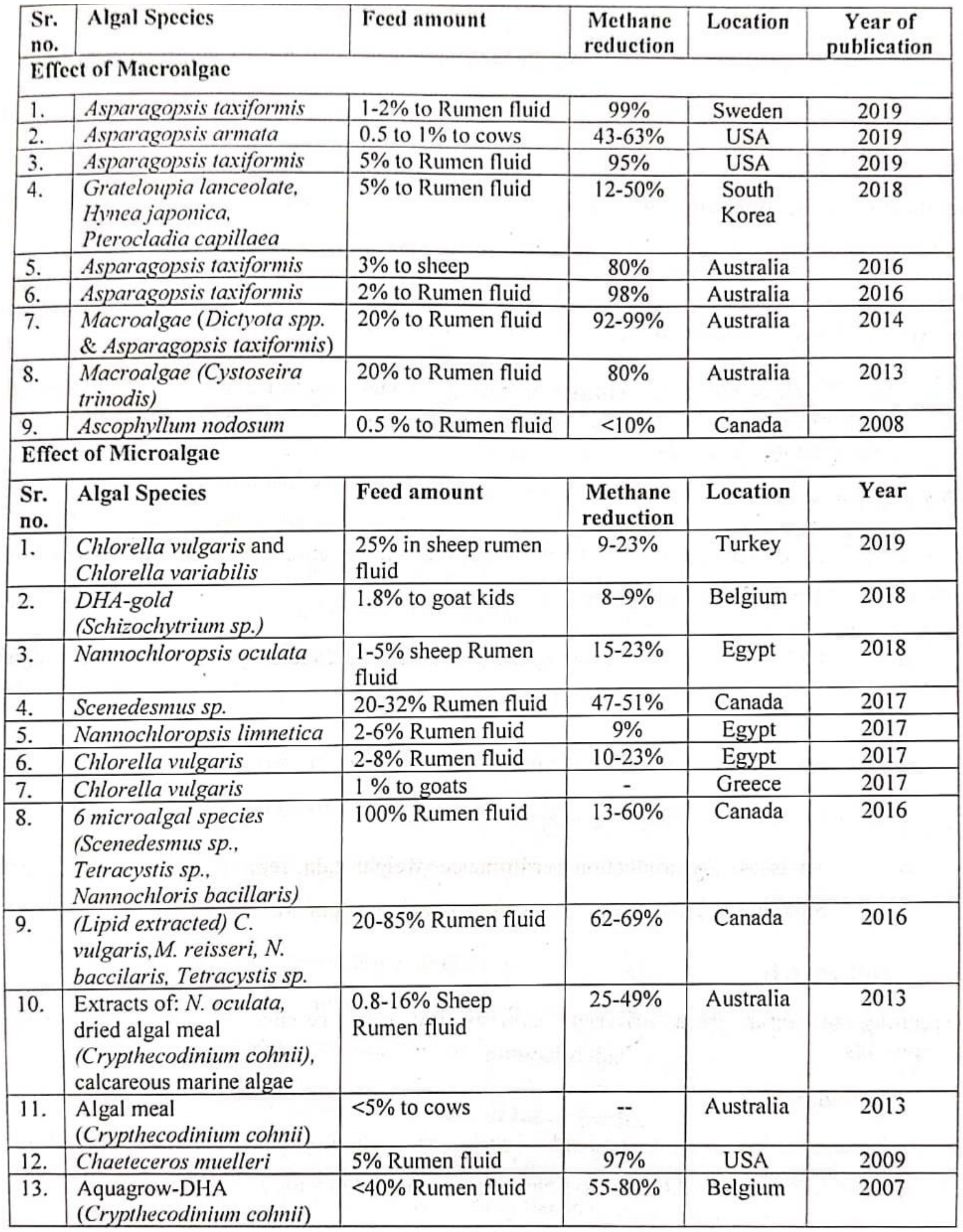


Fig17: Microalgae (biorefineries-algaepro4u-extraction-and-fractionation-of-functional-proteins-from-microalgae.htm)

These data reveal a great deal about the makeup of microalgae and how much of it is consumed. In place of maize and soybeans, the use of algae as feed components is highly resulting, reducing rivalry between the food, feed, and biofuel industries. Additionally, algae help safeguard the environment and natural resources, notably red algae have potent opposing methanogenic activity In in-vitro biological tests, 2% of natural material was added as a feed supplement. This study used cow stomach contents to identify the most beneficial natural compounds and their effects on decomposition. We evaluated polar and non-polar extracts made from water, methanol, etc. The most effective extract, which reduced gas generation by 80%, Bromoform, or haloform ($CHBr_3$), was the most abundant organic compound in the red algal biomass (1723 μg g−1 DW), followed by, bromo chloroacetic acid (9 μg g−1 DW), dibromochloromethane (15.8 μg g−1 DW) and dibromo acetic acid (0.9 μg g−1 DW). With concentrations of ≥1 μm, haloform and dibromochloromethane exhibited the best effectiveness in preventing gas production against water scarcity and land degradation. Additionally, microalgae have a special ability to access n-3 LCPUFA, protecting the world's stocks of fatty fish. However, there may be a big issue with affordable cultivation and use of microalgae in the coming years. This algae-growing method has to be improved to minimize the cost of production. (Madeira et al.,2017). However, only bromoform was administered in significant concentrations inside the biomass at a 2-5% organic content to have this impact. Significantly, at practical amounts, neither organic matter nor volatile fatty acids were altered in their capacity to degrade (Machado et al.,2016). Algae have been used in several experiments to decrease methane output.

Table 3: Effect of different algae in methane reduction (McCauley et al., 2020)



Sr. no.	Algal Species	Feed amount	Methane reduction	Location	Year of publication
Effect of Macroalgae					
1.	*Asparagopsis taxiformis*	1-2% to Rumen fluid	99%	Sweden	2019
2.	*Asparagopsis armata*	0.5 to 1% to cows	43-63%	USA	2019
3.	*Asparagopsis taxiformis*	5% to Rumen fluid	95%	USA	2019
4.	*Grateloupia lanceolate, Hynea japonica, Pterocladia capillaea*	5% to Rumen fluid	12-50%	South Korea	2018
5.	*Asparagopsis taxiformis*	3% to sheep	80%	Australia	2016
6.	*Asparagopsis taxiformis*	2% to Rumen fluid	98%	Australia	2016
7.	*Macroalgae (Dictyota spp. & Asparagopsis taxiformis)*	20% to Rumen fluid	92-99%	Australia	2014
8.	*Macroalgae (Cystoseira trinodis)*	20% to Rumen fluid	80%	Australia	2013
9.	*Ascophyllum nodosum*	0.5 % to Rumen fluid	<10%	Canada	2008
Effect of Microalgae					
Sr. no.	**Algal Species**	**Feed amount**	**Methane reduction**	**Location**	**Year**
1.	*Chlorella vulgaris* and *Chlorella variabilis*	25% in sheep rumen fluid	9-23%	Turkey	2019
2.	*DHA-gold (Schizochytrium sp.)*	1.8% to goat kids	8–9%	Belgium	2018
3.	*Nannochloropsis oculata*	1-5% sheep Rumen fluid	15-23%	Egypt	2018
4.	*Scenedesmus sp.*	20-32% Rumen fluid	47-51%	Canada	2017
5.	*Nannochloropsis limnetica*	2-6% Rumen fluid	9%	Egypt	2017
6.	*Chlorella vulgaris*	2-8% Rumen fluid	10-23%	Egypt	2017
7.	*Chlorella vulgaris*	1 % to goats	-	Greece	2017
8.	*6 microalgal species (Scenedesmus sp., Tetracystis sp., Nannochloris bacillaris)*	100% Rumen fluid	13-60%	Canada	2016
9.	*(Lipid extracted) C. vulgaris,M. reisseri, N. baccilaris, Tetracystis sp.*	20-85% Rumen fluid	62-69%	Canada	2016
10.	Extracts of: *N. oculata,* dried algal meal *(Crypthecodinium cohnii),* calcareous marine algae	0.8-16% Sheep Rumen fluid	25-49%	Australia	2013
11.	Algal meal (*Crypthecodinium cohnii*)	<5% to cows	--	Australia	2013
12.	*Chaeteceros muelleri*	5% Rumen fluid	97%	USA	2009
13.	Aquagrow-DHA (*Crypthecodinium cohnii*)	<40% Rumen fluid	55-80%	Belgium	2007

In order to identify and characterise anti-methanogenic compounds in seaweeds, anti-methanogenic properties and effectiveness of representative algae *in vitro* using cow rumen fluid, we used three species of algae viz. S*pirulina spp. Gigartina spp.*, and *Gracilaria spp.*

Isolation of genomic DNA from seaweeds also growing marine algae

Chemical procedures, including solvent extraction and steam distillation, are used to identify anti-methanogenic chemicals. A serum bottle setup is created to study the effectiveness and anti-methanogenic qualities of representative algae *in vitro* using cow rumen fluid. The Bradford Method, Bligh and Dyer Method, and Phenol Sulphuric Acid Method are used in biochemical tests to measure total carbohydrates, protein, and lipid, respectively. The C-TAB technique is used for total genomic DNA extraction. Microalgae cultivation is also simpler than macroalgae cultivation, which calls for ideal lab conditions. It needed a medium for development, which consists of

A) MACRONUTRIENTS – carbon, nitrogen, phosphate, silicon

B) TRACE METALS – Chloride, zinc, cobalt, manganese, selenium, nickel, iron, boron, keep in solution of EDTA

C) VITAMINS - B12, thiamine, biotin

D) PH BUFFER – Tris, glycylglycine

E) CHELATORS – EDTA, NTA, citric acid

The batch culture involves a single injection of cells into a container of fertilized saltwater, a growth phase that lasts for several days, and then harvesting when the algal population achieves its maximum or almost maximum density. Before entering the stationary phase, algae are often transplanted to bigger culture volumes, which are subsequently maximally densely grown and collected. The following sequential steps might be used.

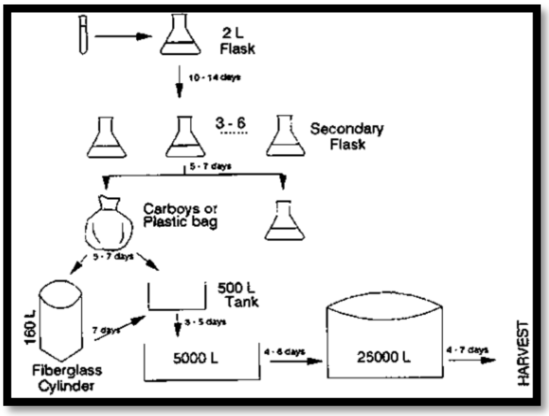


Fig18: Production scheme for batch culture of algae (Lee and Tamaru., 1993).

Fig19: ***Spirulina*** **spp. (https://www.sahasrara-enterprises.co.in/pure-*spirulina*-powder.htm) commercially available**

Fig20: Gigartina spp. (https://www.pronovex.com/gigartina commercially available)

Fig21: ***Gracilaria*** **spp. (Aquagri processing pvt. ltd Manamadurai, Tamilnadu)**

Chapter3

OBJECTIVES

1. **To isolate and characterize anti-methanogenic compounds in seaweeds.**

2. **To determine anti-methanogenic properties of red algae *in vitro* using cow rumen fluid.**

3. **To determine the nutritive value of seaweeds and cultivation of seaweed *in vitro***

4. **Isolation of total genomic DNA of seaweeds for molecular identification.**

Chapter4.

MATERIALS AND METHODS

4.1 Algae**:** Three different algal species (*spirulina spp, Gigartina* spp, *Gracilaria)* were obtained from different sources. *Gracilaria* spp was obtained from Aquagri processing pvt ltd Plot.F1&85, Sipes industrial state Manamadurai-630606
whereas *Gigartina* spp. powder was purchased commercially. *Spirulina* spp. was taken from the culture of our laboratory.

4.2 Quantitation of anti-methanogenic bromoform compound

Equipment-Centrifuge tube, Waring-type blender, dry ice, centrifuge machine, sonicate machine, GC glass vials, rotary shaker

Sample - *spirulina* powder*, Gigartina* powder, *Gracilaria* powder

Reagents- acetone, methanol, ethyl acetate

Method - Solvent extraction (Donato et al.,2021)

protocol

The analysis of freeze-dried seaweed was made possible by quantification technique. To establish a representative sample, a large amount of biomass composed of many different weighing around 100 g was employed for fresh-frozen seaweed. The biomass was combined with a similar amount of dry ice pieces in a blender, uniformed into a powder, and then stored at 20 °C until it was required again. The dry ice was allowed to melt during storage, resulting in a frozen algal powder that was prepared for extraction.

Three batches of homogenized biomass for freeze-dried. The dried algae powders were extracted and analysed using the following procedures:

Ten ml of ethyl acetate were placed in 50 ml screw-cap polypropylene centrifuge tubes. One gram of freeze-dried powder of algae biomass in addition to the ethyl acetate. The tubes were shaken constantly for two to three days at room temperature. After that, algae were filtered through the Whatman filter paper then centrifuging the tubes for 10 minutes at 6500 rpm.

Then, sample is used for methanol extraction or kept chilled at 4º C. A twofold extraction was carried out using 10 ml of methanol and in water bath equipped with ultrasonic technology set at 40 kHz and 0 °C later. The two processed extracts were combined together to give the extracts with varying levels of activity at various times, (Donato et al.,2021).

Sample purification process - For GC quantification, the extracted sample should be colourless; however, because algae include colour, cleaning is necessary. Cleaning with a silica gel. Heat sodium sulphate to 200 degrees C for 4 to 6 hours and activated silica to 100 degrees C for 5 to 6 hours in a hot air oven.

Constructing a silica column - A column is filled with 1 g of sodium sulphate, 5 g of silica, and a glass wool plug at the bottom.

Elution - Pre-elute with 20 ml of hexane, then discard. Transfer the 1 ml sample of algae right away, then discard it. 30 ml of methanol are used to elute once again, followed by 30 ml of hexane: DCM (60:40) collet it. Focus on the rotating evaporator until it is completely dry. Transfer 1.5 ml of the hexane mixture into GC vials. Take a sample of the mixture that has been diluted to 1.4 ml (1400 ul) in 0.1 ml (100 ul) of acetone. (Poly aromatic hydrocarbons).

GC-MS was used to evaluate these diluted extract samples (Paul et al., 2006).
Conditions for GC-MS analysis
Standards of bromoform were prepared for analysis.Commercial bromoform was diluted at 1 ppm, 10 ppm, 100 ppm, and 1000 ppm because it dissolves in methanol at a rate of 5000 ug/ml (5000 ppm). For the standardisation of bromoform in GC-MS, five standards—1000ppb, 500 ppb, 250 ppb, 100 ppb, and 50 ppb—were prepared with10 ppm.
After adding the solvent acetone to the GC, five standards are run, ranging in value from lower to higher. Then, a fragment of bromoform with the bromoform masses of 173, 171, 175, 91, 93, and 252 is obtained. After that, the GC is configured for SIM mode. The column is then always cleaned with the solvent before a new sample is inserted. Determine the amount of bromoform present in the algae sample in this manner.

GC-MS circumstance.
The use of a triple quadrupole gas chromatograph, model 7000D GC/TQ from Agilent Technologies.

The most successful GC/TQ in history, the Agilent 7000D triple quadrupole GC/MS, is currently available in a new model. The 7000D, a fourth-generation GC/TQ used by organisations all over the world to develop and validate a large number of now-standard GC/MS methods, is the ideal option for the majority of applications.

Bromoform compound quantification is carried out in this manner. One can then determine the bromoform content of the algae according to the methodology. It is necessary to add 5% of the organic weight of algal biomass. The least effective concentration needed to reduce methanogencity is assessed using analytical standards in an *in vitro* assay (Machado et al., 2016).

4.3 *In Vitro* study on methane production using algae

Material/ Equipment-

- Waring Blender
- Cheesecloth
- Glass wool and enclosed funnel assembly (serum bottles)
- Automatic syringe, 10 ml.
- Glassware refluxing apparatus as used for detergent preparations, for collection of ruminal fluid.
- Rumen -content source from an animal
- Shaking incubator
- Rudder sealer, aluminium cover
- SBR Super Basic Ration (concentrated feed, mineral mixed, green Napier grass)/fodder.
- Pvp syringe

Sample- *Spirulina* powder, *Gigartina* powder, *Gracilaria* powder

Reagent- sodium bicarbonate, ammonium bicarbonate, distilled water

Protocol

Measure 4 grammes of ammonium bicarbonate and 35 grammes of sodium bicarbonate, then combine them with 1000 millilitres of distilled water to make a saliva buffer.

The rumen mixture is gathered from the abattoir and placed in a preheated, warm container that is tightly sealed to prevent gas leaks. Before use, serum vials are thoroughly cleaned and autoclaved. Powder the algae sample to a fine consistency. The correct proportions of the fodder (Super Basic Ration) should be mixed properly (for preparation, 100 g of cereal feed, 800 g of grass, and 160 g of dry matter). Making the ruminal mixture by diluting it three times with saliva buffer. Considering that we needed 900 ml of ruminal combination, 300 ml of ruminal should be combined with 600 ml of saliva buffer.

Fig22: set up of *In Vitro* Experimental Design of Ruminant stomachs

Take 8 serum bottles and divide them into 2 control bottles where no algae are added and 2 bottles each of *Spirulina, Gigartina*, and *Gracilaria* where algae are added. Add 100 ml of the ruminal mixture after adding 1 g of the 5% organic matter algae to the 1 g of feed.

Following this, quickly close the serum container to preserve pressure, then thoroughly shake the bottles. After 24 hours, use a syringe to read the total gas generation and maintain the bottles in the shaking incubator at 85 rpm at 38 °C for about 15 days or one month. GC-MS analysis can be done to calculate the concentration of CH_4. (Charles et al.,2020)

Fig23: Gas measurement using disposable syringe

4.4 Estimation of nutrient content in seaweeds.

Biochemical Analysis

Quantification of Carbohydrates, Protein and Lipids

4.4(A) Estimation of carbohydrates content.

Reagents

- 5% phenol
- Sulphuric acid (H_2SO4)

Equipment

- UV Visible spectrophotometer
- Weighing balance
- Centrifuge

Method – Phenol Sulphuric Acid Method for the estimation of total carbohydrates.

protocol

One gram of finely ground algae sample has to be measured, added, well mixed, and subsequently centrifuged at 8000 rpm for ten minutes.After discarding the upper layer, add 1.5 ml of the buffer involving 50 mM kh2p04 and 0.1 mM EDTA. Now sonicate algal samples at a fixed amplitude of 50um at 100W for 1 to 5 pluses separated by 30 seconds. A ten-minute centrifugation at 8000 rpm is performed.

Take 200 µl of the algae sample from the supernatant, 200 µl of the 5% phenol, and 1 ml of H_2SO_4. Incubate in a water bath for 15–20 min at 30ºC. At 490 nm, obtain the final reading on the UV-visible spectrophotometer. (Mecozzi et al., 2005)

4.4(B) Estimation of Protein contents in algae powder

Reagents

- 50 mM (KH_2PO4) Potassium dihydrogen phosphate (PH- 6.8)
- 1.1 mM (EDTA) Ethylene diacetate tetra acetic acid
- Bradford reagent

Equipment:

- Centrifuge
- Ultrasonication
- UV Visible spectrophotometer.

Method: Bradford Method

protocol

Take falcon tubes with different one g algae sample.. Centrifuged for 15 minutes at 4°C at 16000 rpm. 1.5 ml of the buffer involving 50 mM kh2p04 and 0.1 mM EDTA were added to the pellets. The buffered sample is transferred and sonicated using a sonication range between 1 and 5 pulses spaced 3 seconds apart at a constant amplitude of 25% (100 w).

Centrifuge for 15 minutes at 4 °C at 16000 rpm. Consider the supernatant while measuring proteins at 595 nm. 0.1ml of sample plus 1 ml of Bradford reagent, with distilled water used as a control. Through the use of a UV-visible spectrophotometer, estimate protein at 595 nm. (Kruger et al.,2009).

4.4(C)Estimation of lipid contents present in the algae powder

Chemicals

- Chloroform

- Methanol

Equipment

- Weighing balance
- Centrifuge
- Incubator, Shaker
- Hot air oven

Sample

- *Spirulina* powder
- *Gigartina* powder
- *Gracilaria* powder

Method-Bligh and Dyer method.

Protocol

In a centrifuge tube, 1g of algae crushed was dissolved in 10 ml Millipore water of and spin for 10 minutes at 8000 rpm.

Transferring the combination (2:1) into clean glass vials requires the removal of the solution and the addition of 5 ml of distilled water. 10 ml of the solvent methanol and 5 ml of chloroform should then be added.

The glass vials were fully sealed with Teflon tape and overnighted in the mixer or incubator.

The suspension was transferred to a new falcon tube the next day, 5 ml of Millipore water and 5 ml of chlorine solution were added, and the mixture had been spinning for five min at 8000 rpm.

Three layers were formed, with the lowest lipid phase being removed and transferred to pre-weighted glass vials to remove impurities.

The vials were dried in an oven, and the dried vials and lipid were weighed to gravimetrically ascertain the lipid's real concentration. (Jensen et al.,2009)

4.5 Total Genomic DNA Extraction Studies from Seaweeds.

Equipment

- Water bath
- centrifuge machine
- nanometre
- electrophoresis setup
- gel dog machine

Reagents

- 3× extraction buffer containing: 3% CTAB (w/v),
- 1.4 M NaCl,
- 0.3% 2-β-Mercaptoethanol.
- Chloroform: isoamyl alcohol (24:1 v/v).
- 0.8 M Tris-HCl pH 8.0,
- 6 M NaCl
- 3 M potassium acetate
- 0.5 M EDTA pH 8.0 (autoclaved)
- Ice cold 100% isopropyl alcohol
- 70% ethanol
- 1× TE buffer (10 mM Tris-HCl, pH 8.0; 1 mM EDTA, pH 8.0, autoclaved).
- Agarose (molecular grade)

Sample

- *Spirulina* powder
- *Gigartina* powder
- *Gracilaria* powder

Method -CTAB method.

Protocol

The extraction buffer needs to be warmed to 65 °C in a water bath. Add 0.3% 2-mercaptoethanol right before adding it to the CTAB extraction buffer.

0.05 g of algae specimens should be powdered using dry ice before being thoroughly mixed using a mortar and pestle. Now 800µl of the hot 3 CTAB extraction buffer into the crushed algae samples while using the pestle to gently mix

The sample mixture should be transferred to a 2-ml centrifuge tube, incubated for 1 hour at 65 °C in a water bath, carefully mixed by inverting the tube 20 times every 20 minutes, and then allowed to cool to atmospheric temp.

800 µl of a 24:1 v/v chloroform: isoamyl alcohol mixture should be added. Mix by minor inverted.

Spin at 13,000 rpm for 15 minutes at room temperature (RT). Gently transfer the top aqueous phase that contains the DNA to a new 2-ml Eppendorf tube.

700 µl of the aqueous phase is obtain, add 350 µl of 6 M NaCl, and vigorously mix.

mixing 500 µl of 100% isopropyl alcohol with 1/10 the amount (70 µl) of 3 M potassium acetate Invert the precipitated DNA slowly to allow DNA strings to form. At -20 °C, incubate for a full 1hour.

Spin at 13,000 rpm for 5 minutes; eliminate the upper layer.

Turn upside-down the tube containing the DNA pellet on a piece of tissue paper to fully drain away the supernatant.

The DNA pellet should be washed in 500 µl of 70 percent ethanol before being inverted once.

At 13,000 rpm, centrifuge for five minutes. Delete the 70% alcohol in the tubes. The pellet-filled tubes should air dry at ambient temperature for 10 minutes after the filter paper has been turned over.

Re-suspending the DNA pellet in 50 µl of TE buffer is necessary. The DNA should be incubated for one to two hours at 50 °C to ensure complete re-suspension.

Keep in a -20 °C storage place up until use. (Maaty et al.,2019)

Quantitative and qualitative analysis of DNA

4.5(A) Quantitative

DNA was extracted using the standard C-TAB technique. NanoDrop1000 (Thermo Scientific) was used to measure DNA content, purity, and quality using spectrophotometry at 260 nm (A260) absorption.

Reagent/equipment

- distilled water

- tissue paper
- TE-buffer
- nanodrop.

Sample

DNA samples of *spirulina, Gigartina*, *Gracilaria* spp.

Method

Nanodrop 2000c Microvolume Nucleic Acid Quantification

Protocol

Pipette 2 µl of deionized water added over the lower optical surface of the microvolume spectrophotometer's specimen retaining mechanism to clean the top and lower visual surfaces.

Make sure the uppermost pedestal reaches the deionized water by closing the lever arm. Cleanse the surfaces of both oculars while elevating the lever arm. Open the Nanodrop programme and choose the DNA option. Fill the bottom optical surface with 1 µL of TE-buffer to do a blank measurement using a small-volume, calibrated pipettor. Lower the lever arm and choose "Blank" from the menu.

Clean both optical surfaces following the completion of the blank measurement. Place a 1 µL sample of algae on the bottom optical pedestal, then secure the lever arm. Choose "Measure" from the application software's menu. The DNA concentration and purity ratios will be calculated automatically by the programme. Review the spectrum output after the sample measurement.

The ratios for purity and concentration of nucleic acids are automatically calculated by the programme. Review the spectral figure after sample measurement to judge specimen quality.

For an accurate assessment of sample quality, 260/230or260/280 ratios should be studied together with average spectrum quality. The typical 260/280 ratio of pure DNA is 1.8, respectively. (Desjardins et al.,2010)

4.5(B) Qualitative

In order to further assess the quality of the DNA acquired using both techniques, all DNA samples were electrophoresed on a 0.1% agarose gel and stained with ethidium bromide (1 ug/ml).

Sample – DNA samples of *Spirulina, Gigartina*, *Gracilaria*

Material/equipment-microwave, ice-petals, gel dog machine, electrophoresis setup

Reagent- agarose powder, distilled water, TAE-buffer, loading dye, ladder, ethidium bromide

Method-Agarose Gel Electrophoresis

Protocol

In the 1X TAE buffer, a 0.1% agarose solution was prepared. Without adding any air bubbles, the solution was poured into the gel caster along with the comb when the temperature reached 60°C. The gel-containing tray was withdrawn from the gel caster after solidification and put into the electrophoretic unit containing 1X TAE buffer and ethidium bromide at a concentration of 3 µg/ml. A 1 kb ladder is loaded first, and then 5 µl of DNA and 3 µl of loading dye are combined. A micropipette is then allowed to fill half of each well before the mixture is electrophoresed at 50 V for two hours. The gel was then examined using a UV transilluminator and captured on the recording device. (Maaty et al.2019).

4.6 Cultivation of marine algae

Materials / Equipment: 250ml flask,100ml measuring cylinder,1000 ml flask, rotary shaker incubator, laminar air flow, autoclave, vacuum filter

Reagent- Guillards f/2, marine water, sea water (Mumbai), saline water (Sodium chloride (NaCl), Magnesium chloride (MgC12-6H2O), Sodium sulphate (Na2S04), Calcium chloride (CaCl2), Potassium chloride (KCI), distilled water.BG-11 media

Sample: *Spirulina* powder*, Gigartina* powder, *Gracilaria* powder

Method: lab culturing technique.

Protocol

The process of making saline water

Take one litre of distilled water, measure the salts. After carefully combining the ingredients, saline water is autoclaved with 24g of sodium chloride (NaCl), 11.1g of magnesium chloride (MgC12-6H2O), 4.1g of sodium sulphate (Na2S04), 1.2g of calcium chloride (CaCl2), and 0.7g of potassium chloride (KCI).

Then thoroughly combine them and autoclave saline water. Take one g of algae and mix it with 50 ml of saline water, 50 ml of Guillard's f/2, in 250 ml of a flask to study green algae and red algae are studied using BG11 media.

Similar procedures should be followed for seawater, which consists of 50 ml of seawater and 50 ml of Guillard's f/2 in a 250 ml flask to study green algae and red algae are studied using BG11 media.

After that, put the flask in a rotating shaker incubator set to rotate at a speed of 90 to 100 rpm at a temperature of 24 to 30 degrees. All of this culture work is done in a laminar air flow environment. Take 10 ml of the culture and mix it with 90 ml of the medium after 7 days. Repeat these steps again after 15 days, and the sample of pure algae will have grown.

The growth can be seen with a hemacytometer or by physical modifications in algae.

Chapter 5.

RESULTS

5.1 To identify and characterise the anti-methanogenic substances in seaweeds.

Using the solvent extraction technique, bromoform was dissolved in methanol to prepare bromoform standards of different concentrations viz. 50, 250 and 500 ppb.

Table 4: Bromoform standards

Sr.no	Conc. (ppb)	Area of the peaks
1	50	2885.9
2	250	80874.65
3	500	155100.63

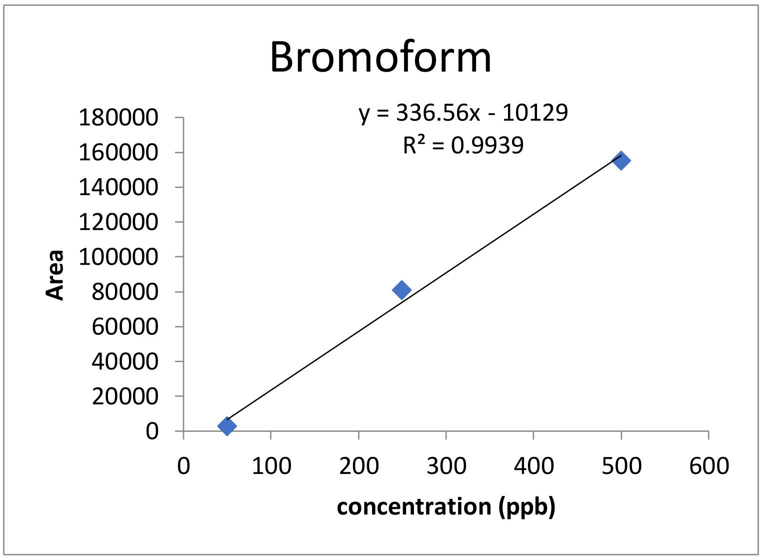
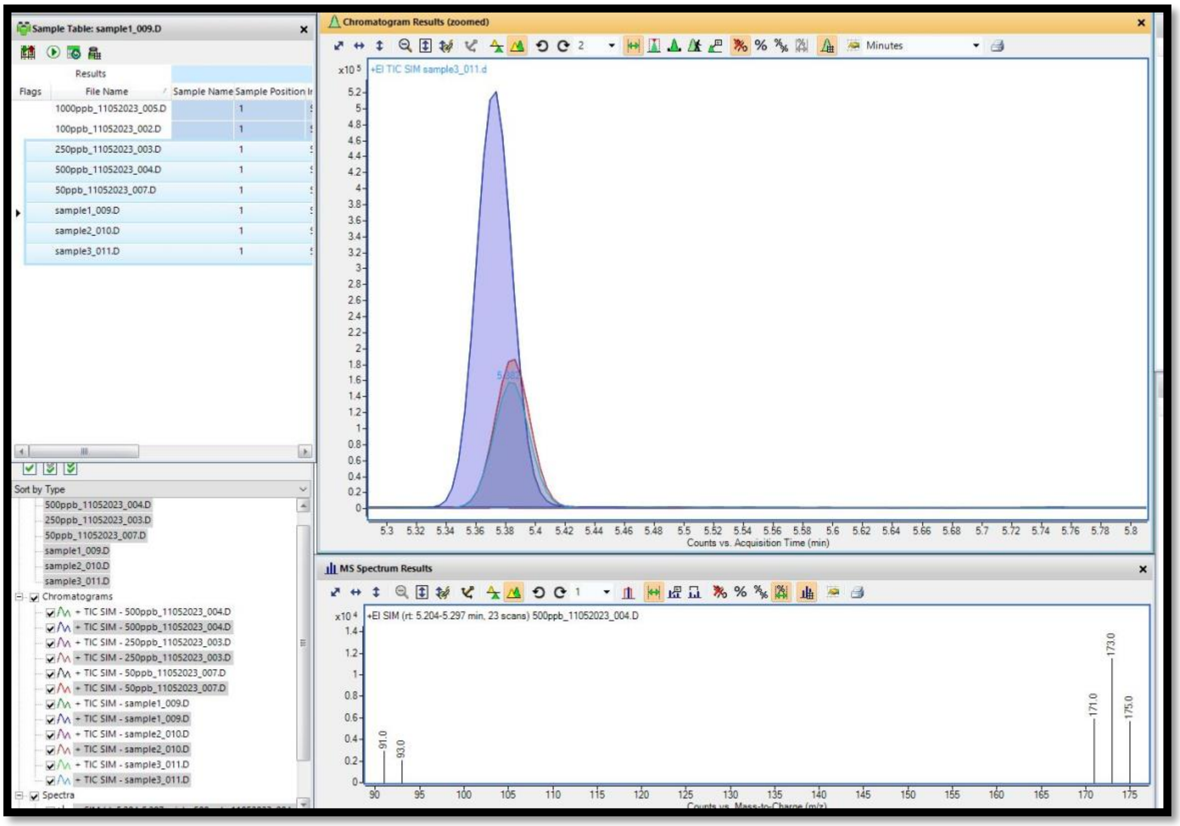


Fig24: The graphical representation of standard bromoform concentration for the estimation of bromoform from algae samples.

Now that we have completed GC-MC, we are able to determine the area of the algae sample. With this information, we can use the linear equation Y= MX + C to get the bromoform concentration in the algae sample.


Sample Table: sample1_009.D

Flags	File Name	Sample Name	Sample Position
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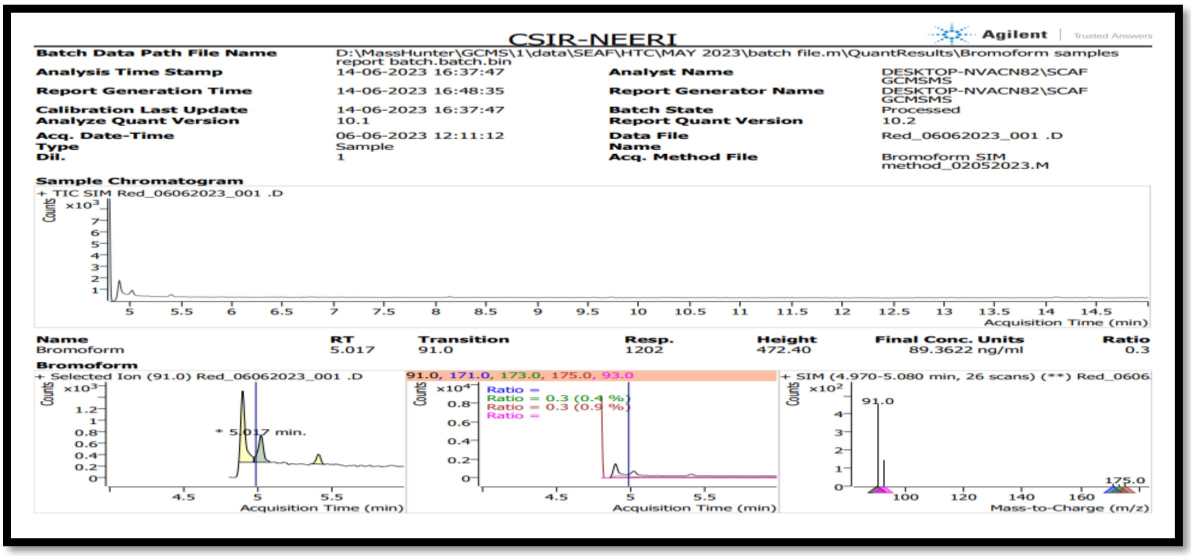
	100ppb_11052023_002.D		1
	250ppb_11052023_003.D		1
	500ppb_11052023_004.D		1
	50ppb_11052023_007.D		1
	sample1_009.D		1
	sample2_010.D		1
	sample3_011.D		1

Sort by Type
500ppb_11052023_004.D
250ppb_11052023_003.D
50ppb_11052023_007.D
sample1_009.D
sample2_010.D
sample3_011.D
☑ Chromatograms
☑ + TIC SIM - 500ppb_11052023_004.D
☑ + TIC SIM - 500ppb_11052023_004.D
☑ + TIC SIM - 250ppb_11052023_003.D
☑ + TIC SIM - 250ppb_11052023_003.D
☑ + TIC SIM - 50ppb_11052023_007.D
☑ + TIC SIM - 50ppb_11052023_007.D
☑ + TIC SIM - sample1_009.D
☑ + TIC SIM - sample1_009.D
☑ + TIC SIM - sample2_010.D
☑ + TIC SIM - sample2_010.D
☑ + TIC SIM - sample3_011.D
☑ + TIC SIM - sample3_011.D
☑ Spectra

Fig 25: Bromoform standard, quantifier and qualifier chromatograms during the present study.

Table5: Algae samples concentration and area obtained by GC-MS analysis

Sr.no	Algae sample	Conc. (ppb)	Area of peaks
1	*Gracilaria* spp.	Sample1-89.3622 Sample2-87.4245 Sample 3-80.3967	1202 59907 1016
2	*Gigartina* spp.	Sample1-104.8631 Sample2-96.4757 Sample3-86.8788	1349 1150 1523
3	*Spirulina* spp.	Sample1-103.8432 Sample2-80.4718 Sample3-63.5858	1502 1017 667



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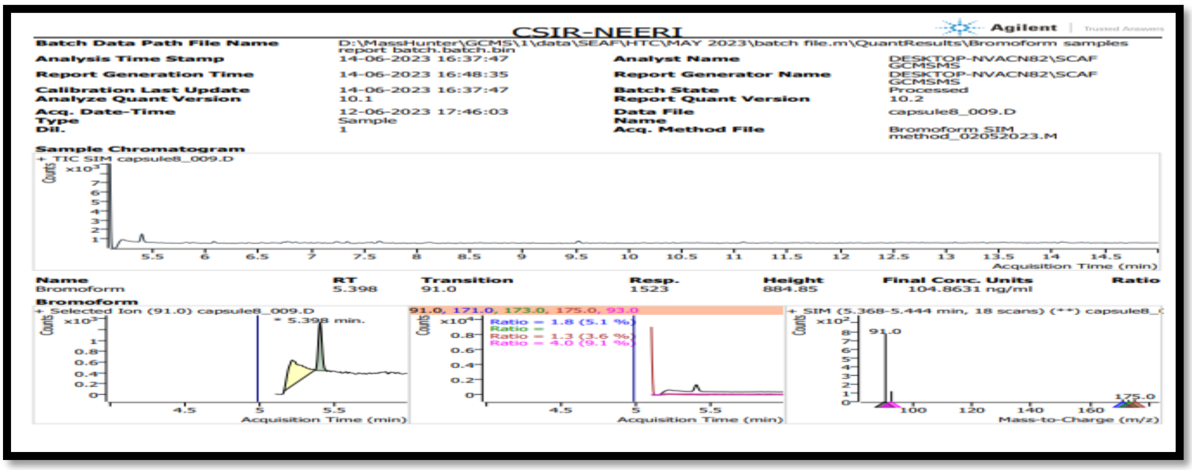
Batch Data Path File Name D:\MassHunter\GCMS\1\data\SEAF\HTC\MAY 2023\batch file.m\QuantResults\Bromoform samples report batch.batch.bin
Analysis Time Stamp 14-06-2023 16:37:47 **Analyst Name** DESKTOP-NVACN82\SCAF GCMSMS
Report Generation Time 14-06-2023 16:48:35 **Report Generator Name** DESKTOP-NVACN82\SCAF GCMSMS
Calibration Last Update 14-06-2023 16:37:47 **Batch State** Processed
Analyze Quant Version 10.1 **Report Quant Version** 10.2
Acq. Date-Time 06-06-2023 12:11:12 **Data File** Red_06062023_001 .D
Type Sample **Name**
Dil. 1 **Acq. Method File** Bromoform SIM method_02052023.M

Sample Chromatogram

Name	**RT**	**Transition**	**Resp.**	**Height**	**Final Conc. Units**	**Ratio**
Bromoform	5.017	91.0	1202	472.40	89.3622 ng/ml	0.3

Bromoform

FIG26: GC-MC report of *Gracilaria* algae sample



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Report Generation Time 14-06-2023 16:48:35 **Report Generator Name** DESKTOP-NVACN82\SCAF GCMSMS
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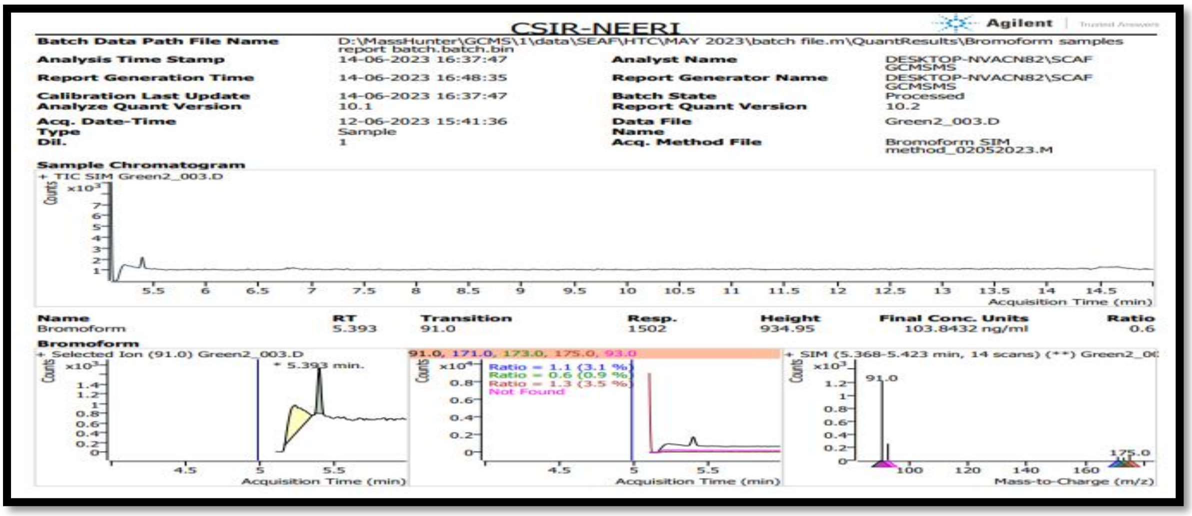
Dil. 1 **Acq. Method File** Bromoform SIM method_02052023.M

Sample Chromatogram

Name	**RT**	**Transition**	**Resp.**	**Height**	**Final Conc. Units**	**Ratio**
Bromoform	5.398	91.0	1523	884.85	104.8631 ng/ml	

Bromoform

FIG27: GC-MC report of *Gigartina* algae sample



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Batch Data Path File Name D:\MassHunter\GCMS\1\data\SEAF\HTC\MAY 2023\batch file.m\QuantResults\Bromoform samples report batch.batch.bin
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Report Generation Time 14-06-2023 16:48:35 **Report Generator Name** DESKTOP-NVACN82\SCAF GCMSMS
Calibration Last Update 14-06-2023 16:37:47 **Batch State** Processed
Analyze Quant Version 10.1 **Report Quant Version** 10.2
Acq. Date-Time 12-06-2023 15:41:36 **Data File** Green2_003.D
Type Sample **Name**
Dil. 1 **Acq. Method File** Bromoform SIM method_02052023.M

Sample Chromatogram

Name	**RT**	**Transition**	**Resp.**	**Height**	**Final Conc. Units**	**Ratio**
Bromoform	5.393	91.0	1502	934.95	103.8432 ng/ml	0.6

Bromoform

FIG28: GC-MC report of *spirulina* algae sample

Table 6: Concentration of bromoform present in algae samples as analysed on GC-MS

Sr.no	Algae sample	Average (µg/ml)	Standard deviation (µg/ml)
1	*Gracilaria* spp.	85.728	4.717429
2	*Gigartina* spp.	96.0725	8.998926
3	*Spirulina* spp.	82.6336	20.21558

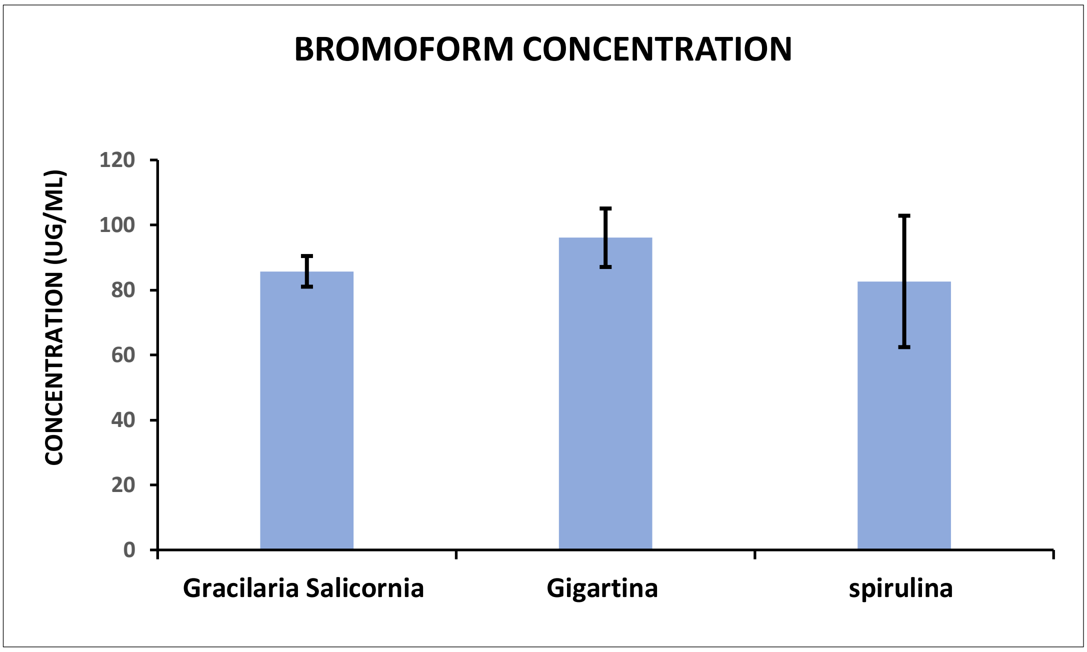

Fig29: Estimation of bromoform in *Spirulina, Gigartina*, *Gracilaria* algae. Bromoform concentration was considerably higher in *Gigartina* spp. algae followed by *Gracilaria* and *spirulina* spp.

Now after performing GC-MS we were able to know the area of algae sample with which we could find the concentration of bromoform in algae sample using linear equation Y=MX+C. It was observed that there were no significant differences in bromoform content between algae tested, although. *Gigartina* algae contained higher (non-significant) concentration of bromoform than *Gracilaria* and *Spirulina* algae, indicating that this Indian algal species may be considered as feed additive for mitigation of enteric methane emissions from cattle.

2 To investigate anti-methanogenic properties and efficiency of representative algae *in vitro* using cow rumen fluid.

Table7: During the first batch's in-vitro fermentation, total gas, CH_4, CO_2[ML/G] was produced in vessels with and without 5% of algae as an addition. The reading was taken everyday

DAYS	control	*Gracilaria*	*Gigartina*	*Spirulina*
1	0	0	0	0
2	7.2	9.5	12	12.5
3	7.5	8.5	10.75	11
4	10	10.5	11	10.5
5	10.75	9.5	11.25	10.25
6	10.5	11	11	11
7	11.25	12	11	13.5
8	11.5	12	13	12
9	17	17.5	15	14.5
10	19.5	22.5	22.5	20.5
11	21	19.5	18	17.5
12	16.5	16.5	17	15
13	16.75	14.5	13.75	13.65
14	22.5	17	20.1	22.5
15	14.8	16.75	14.25	15
16	13.1	15.35	14.75	16
17	13.35	12	13.85	13
18	18.75	12.5	15.5	10.5
19	11	11.8	13.5	10
20	12.5	9	12	11
21	12.5	9.5	12.5	10
22	12	10.3	11	9.8
23	10	9.6	11.25	9.5
24	12.05	8.5	10	7.5
25	10.25	6.8	9.3	6
26	8	6	8.65	6.8
27	7.65	4.5	7	5.6
28	5	4.3	6.5	4
29	4.5	4	4	4
30	3.5	2.5	4	4

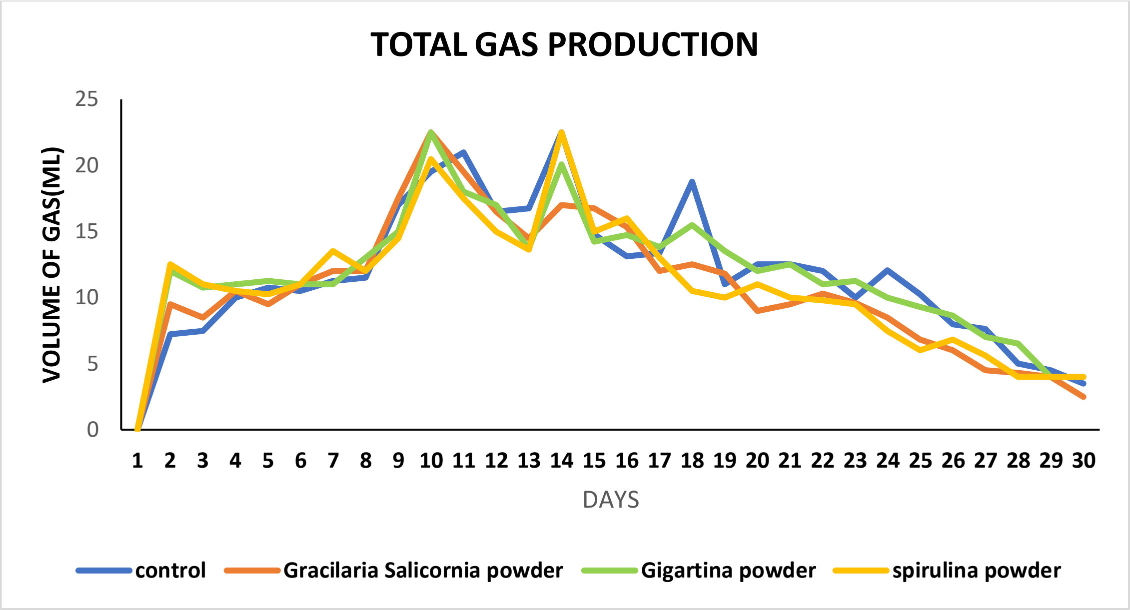

Fig30: Effects of Algae on gas properties.

It is well known that in fermentation bottles, total gas is produced in which usually CO_2 gets dissolved with rumen fluid as its solubility is higher (3.48 g gas/kg of water) than methane gas which is 0.04 g gas/kg of water, therefore, it is considered that CH_4 is the major gas present in the headspace of the bottle. Therefore, it is considered that volume of gas we are measuring in a syringe mostly contains methane (Engineering Toolbox, 2008)

An invitro experiment was repeated thrice. In the first batch, when vessels examining the impacts of algae on ruminal fermentation properties in-vitro at a 5% organic matter (OM) inclusion rate, a substantial decrease in CH_4 generation was observed. The total experiment's findings indicate about -50% reduction in total gas production, which is consistent with earlier in-vitro research studies demonstrating the benefits of red algae in reducing the methane emission in the rumen.

When vessels were used in the second batch to evaluate the effects of algae on the properties of ruminal fermentation in-vitro at a 5% OM inclusion rate, a substantial decrease in CH_4 generation was also observed. The total experiment's findings indicate a about 40-50% reduction in total gas production, which is consistent with several in-vitro research on the benefits of algae. Consistent results were obtained in subsequent series of experiments.

According to Paul et al., (2006) and Salvador et al .(2007), algae species are very active against both gram-negative and gram-positive bacteria. Additionally, *in vitro* ruminal fermentation tests using algae showed anti-methanogenic activity (Machado et al. 2015). When given at a dosage of 5% of the OM incubated, it severely inhibits the formation of CH_4, demonstrating that these natural compounds are also active against the archaea responsible for the microbial generation of CH_4.

5.3Estimating the nutrient content in seaweeds

5.3(A)Estimation of Carbohydrate content**:**

The standard protocol for carbohydrates concentration was followed by using D-glucose as standard with Phenol-Sulphuric Acid method.

Table 8: Given above shows absorbance of glucose at 490 nm.

Conc. of glucose (µg/ml)	Absorbance at 490 nm
s0	0
40	0.786
80	1.643
120	2.489
160	3.398
200	3.983

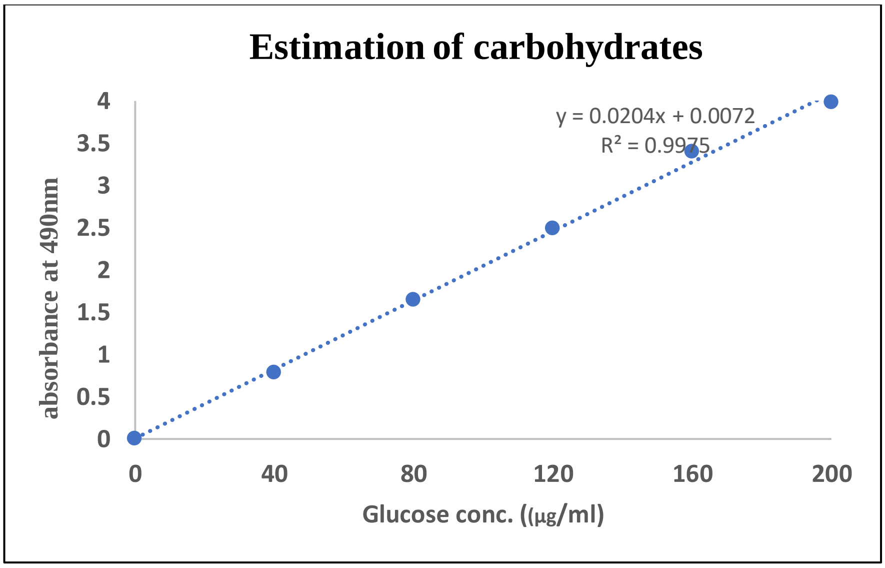

Fig31: The graphical representation of standard glucose concentration for the estimation of carbohydrates at 490 nm.

Table9: Average and standard deviation of algae sample

Sr.no	Algae sample	AVREAGE(µg/ml)	Standard deviation (µg/ml)
1	*Gracilaria* spp.	138.360	17.51285
2	*Gigartina* spp.	175.043	12.42223
3	*spirulina* spp.	190.582	2.744141

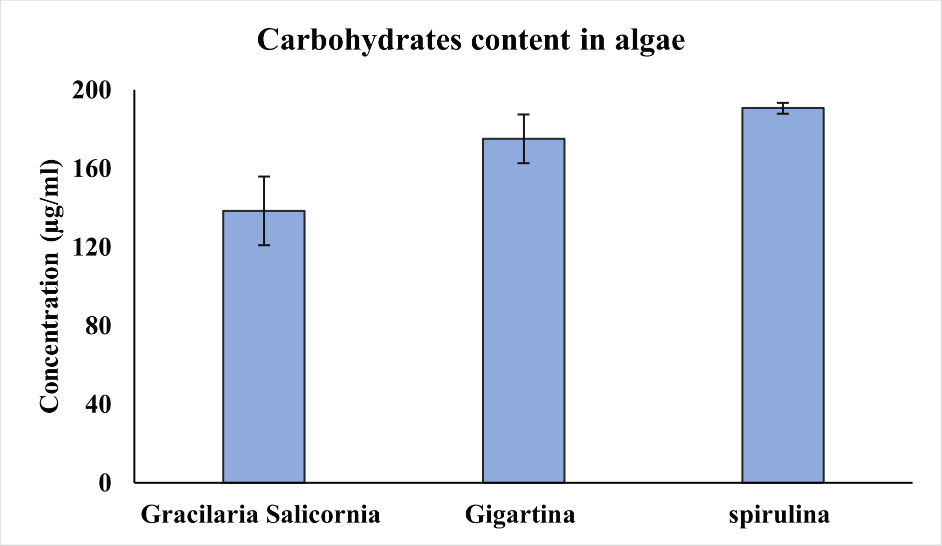

Fig32: Estimation of carbohydrates in *Spirulina, Gigartina*, *Gracilaria* algae. carbohydrates concentration is considerably higher in *spirulina* algal then *Gigartina* from the *Gracilaria*.

5.3(B)Estimation of Protein content:

The unknown protein concentration of samples was compared using BSA as standard. The standard curve of BSA was plotted using 0.2 µg/ml BSA stock and diluting it to respective concentrations

Table10: Standard BSA Absorbance for protein estimation

BSA concentration (µg/ml)	Absorbance at 595 nm
0	0
100	0.096
200	0.224
300	0.338
400	0.436
500	0.541
600	0.655
700	0.754
800	0.834
900	0.954
1000	1.059
1100	1.165
1200	1.270
1300	1.378
1400	1.480
1500	1.585

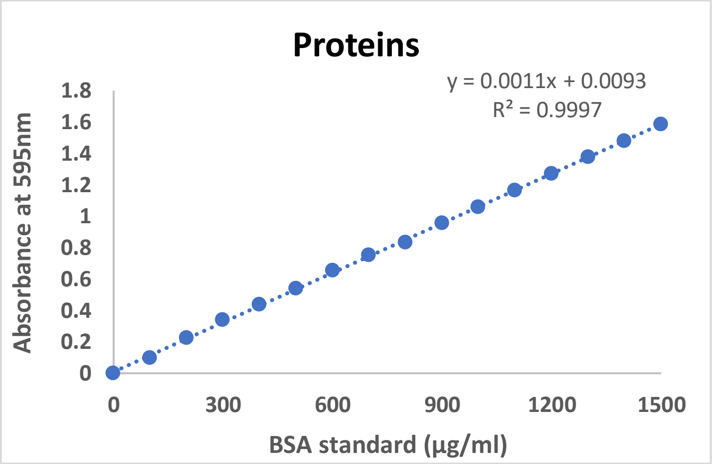

Fig33: The graphical representation of BSA standard for the estimation of protein at 595 nm.

Protein concentration in Samples:

Table11: Average and standard deviation of algae sample

Sr.no	Algae sample	AVREAGE(µg/ml)	Standard deviation (µg/ml)
1	*Gracilaria* spp.	192.727	21.18182
2	*Gigartina* spp.	420.97	20.33121
3	*Spirulina* spp.	1056.48	90.34033

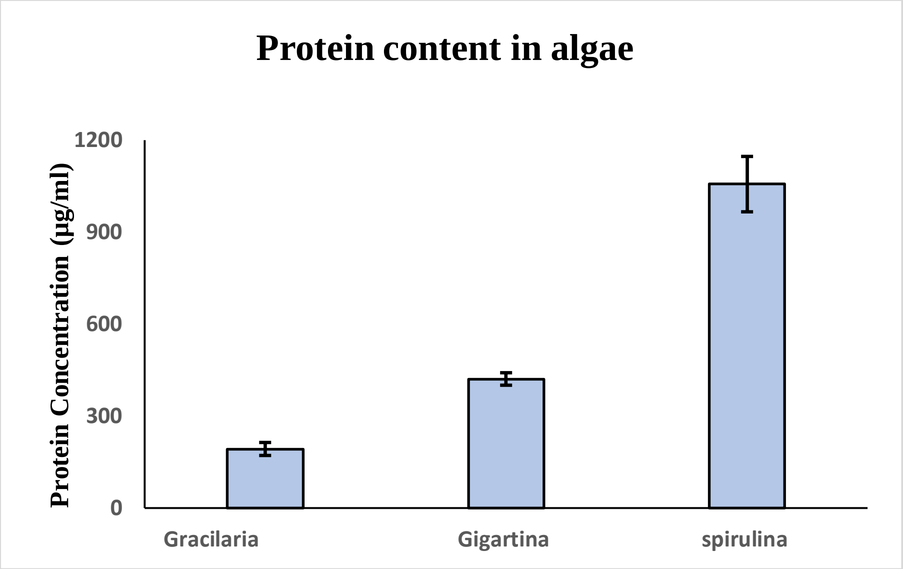

Fig34: Protein contents of different algae is estimated by Bradford reagent method. Protein concentration is considerably higher in *Spirulina* algae followed by *Gigartina* and *Gracilaria spp.*

5.3(C)Estimation of Lipid content**:**

Lipid analysis of *Spirulina, Gigartina*, *Gracilaria* algae respectively was conducted by Bligh and Dyer method. The lipid content was measured gravimetrically and expressed with respect to dry weight. (g/ml)

Table12: Average and standard deviation of algae sample

Sr.no	Algae sample	Average (g/g)	Standard deviation (g/g)
1	*Gracilaria* spp.	0.047867	0.039426
2	*Gigartina* spp.	0.033000	0.017642
3	*Spirulina* spp.	0.367067	0.149976

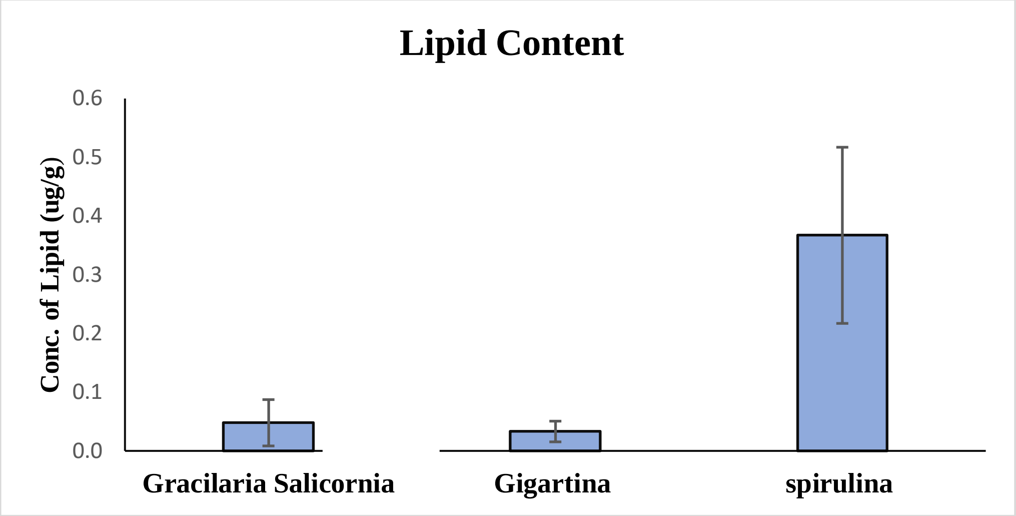

Fig35: Lipid is estimated by Bligh and Dyer Method Gravimetrically. Lipid concentration is considerably higher in *spirulina* as compared to *Gracilaria* and *Gigartina* algae.

5.4 Total Genomic DNA Extraction from Seaweeds.

A fundamental prerequisite for molecular analysis is DNA. Consequently, an appropriate technique for extracting DNA from seaweed is required. The purpose of this study was to examine several seaweed species' DNA extraction techniques and evaluate the DNA quality using PCR-RAPD.DNA was isolated from seaweed samples using the modified C-TAB technique.

Most molecular genetics research needs accurate determination of DNA concentration and purity. Polysaccharides, polyphenols, and other secondary metabolites are found in varied amounts in different plant species. These compounds react with nucleic acids during DNA isolation to influence the extracted DNA's integrity. With further changes for the extraction of DNA from varied plant seeds and crops belonging to seven distinct orders, the present extraction methodology is based on the traditional Cetyl trimethylammonium bromide (CTAB) approach. The two main adjustments currently being used for DNA extraction are a higher CTAB concentration and a higher amount of 2-mercaptoethanol. Additionally, for the precipitation of DNA devoid of polysaccharides, larger quantities of sodium chloride and potassium acetate were added concurrently with absolutely ice-cold isopropanol.

The best DNA extraction result was obtained after an overnight incubation at 60°C in water bath, according to the results. Additional DNA extraction optimisation is required to acquire high quality DNA for genetic analysis.

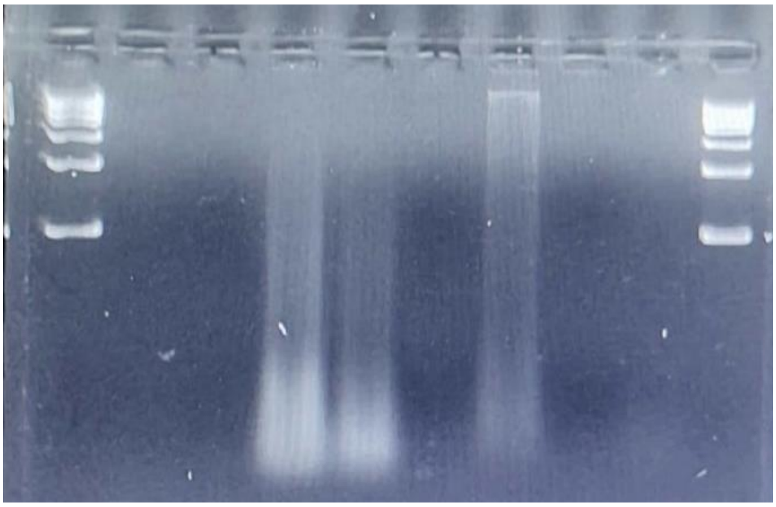

Fig36: *Spirulina, Gigartina*, and *Gracilaria* seaweed species' DNA was extracted after one hour of incubation in CTAB buffer. On a 1.0% agarose gel matrix stained with ethidium bromide in a 1 X TAE solution, electrophoresis separation was carried out.

Several samples, including those, did not have effective DNA extraction after an hour of incubation. *Spirulina, Gigartina*, *Gracilaria* and This may be because there wasn't enough extraction buffer volume or incubation time for this sample, making the buffer effective in lysing cell walls and membranes. asserts that a longer incubation time in lysis buffer enhances the cells' exposure to the solution, resulting in more thorough cell lysis according to (Rogers et.al 2019)

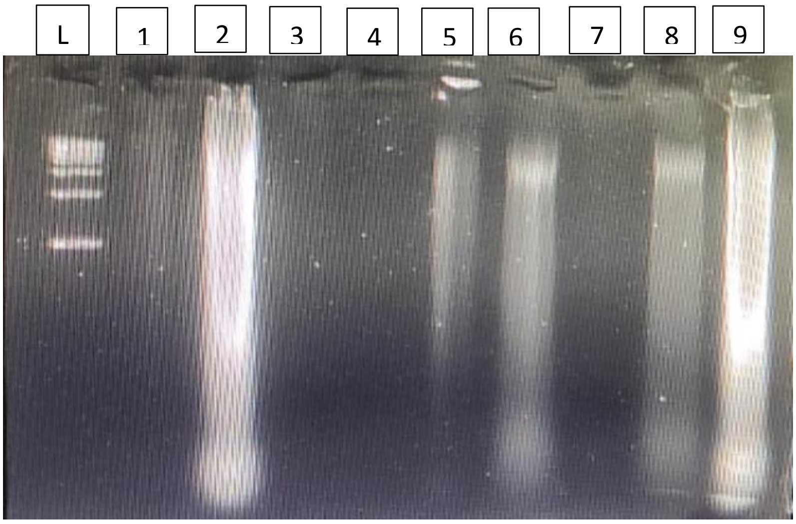

Fig37: Seaweed species with overnight incubation in CTAB buffer cause the development of smear bands. L-ladder, 1, 2, 3, spirulina, 4,5, 6, Gigartina, 7, 8, and 9-Gracilaria is indicated.

DNA that is readily visible in numerous kinds of seaweed was created by extraction by altering the overnight incubation duration. Therefore, 10 µl of DNA from each species was loaded and electrophoresis was repeated. The electrophoresis result was displayed in gel-documentation instrument and Nanodrop 2000c instrument used for Quantification as its result is not that good as the ratio 260/280 is not coming in the range of 1.8 for DNA.

Table13: Concentration and absorbance of algae sample showing DNA bands

Sr no.	Algae Sample	Concentration(ng/ul)	260/280(nm)
2	*Spirulina* spp.	50.970	2.18
5	*Gigartina* spp.	937.822	2.07
6	*Gigartina* spp.	533.434	2.04
8	*Gracilaria* spp.	160.535	1.16
9	*Gracilaria* spp.	509.812	1.04

5.5 Cultivation of marine algae

Both micro and macro algae needed the right conditions for development in the lab. As we utilised B11 medium to culture the green algae and F2 Galliard's media to culture the red algae, it is simpler to cultivate micro algae than macro algae.

As the conditions necessary for micro algae development are met, their growth rate rises and they can be resurrected for continued cultivation. Macro algae first exhibits growth, but since it lacks space to expand, it transitions to a stationary phase before dying off as a result of nutritional deficiency. In recent years, synthetic salt combinations have tended to take the role of real sea water as the mixed mineral salt foundation for culture medium meant for marine unicellular algae. Although other nutritional investigations do not often need quickly reproducing populations of high cell concentration, they must be taken into account jointly since they require these synthetic combinations.

The synthetic salt combinations that have thus far been detailed would be predicted to behave quite similarly to real sea water in terms of their carbon dioxide concentration, buffering capabilities, and propensity to produce precipitate thus calcium carbonate, according to theoretical considerations.

Fig38: Culture of green algae (*Spirulina* spp.)

It is plausible that using a greater ionic strength might minimise the risk of calcium carbonate precipitating if medium with lower calcium contents cannot be tolerated. While improving pH control during autoclaving and culture growth, the addition of artificial buffer to natural sea water or synthetic salt mixtures is bound to have a negative impact on the total amount of carbon dioxide in solution if the buffering capacity of the artificial buffer is to be fully utilised. The method also demands that starting pH values for media be much below the range of naturally occurring sea water before they can be considered acceptable. Because of this, artificial synthetic media exhibit more pronounced growth than natural marine media.

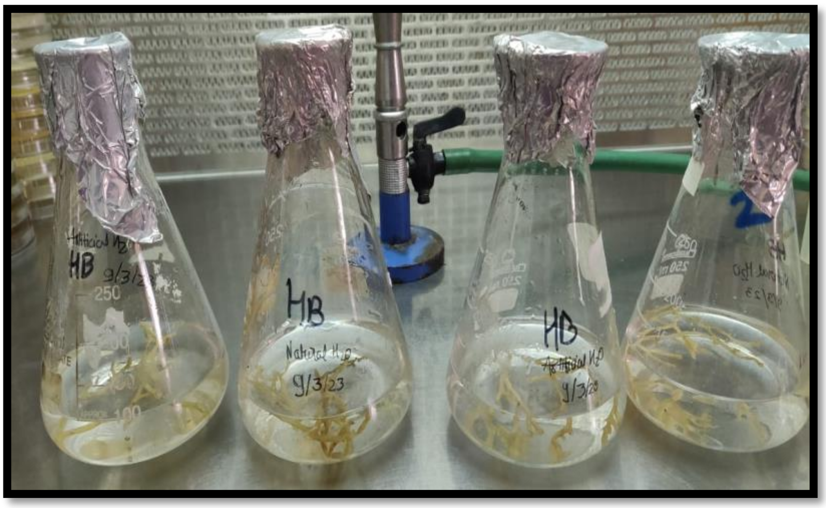

Fig39: Culture of Red algae (*Gracilaria spp.*)

Chapter 6

DISCUSSION

Cattle enteric methane emissions along a rural-urban gradient is increasing in a growing megacity. The surface temperature profile throughout time has served to emphasise the risks faced by prolonged global warming. Through the growing reliance on fossil fuels and the destruction of forests, anthropogenic activities have been linked to this global warming. It has been shown that methane is responsible for 20% of global warming. These emission levels will continue to rise in the future due to population growth and rising living standards, particularly in developing nations.

Because these algae contain anti-methanogenic compounds such as bromoform, dichloromethane, dibromochloromethane, Bromo chloroacetic acid, and dibromo acetic acid, which can be used to reduce methane emissions from the agriculture sector. However, we concentrated on the bromoform compound, which is primarily dominant in algae, so for that extraction of algae sample has been done. Then, for detection, GC-MC was carried out, and we successfully could extract good amount of bromoform in all three candidate algae used. Of which, the highest concentration was found in *Gigartina*, followed by *Gracilaria* and lowest in *Spirulina spp*. Additionally, earlier studies on ruminal fermentation patterns revealed that the group that consumed algae had higher concentrations of propionate and lower concentrations of acetate. These Volatile fatty acids profile alteration raises the possibility that algae might cause hydrogen to be diverted to propionate, reducing the amount of hydrogen available for methanogenesis (Li et al., 2019). The presence of numerous secondary metabolites may be the cause of benefits of algae. Bromoform (CHBr3), which is chemically related to bromochloromethane (CH2BrCl) and has recognised antibacterial effects, has been found to decrease CH_4 in *Asparagopsis* (Kinleyet et al., 2016). According to (Li et al.,2019), *Asparagopsis taxiformis* was added at different doses, and this resulted in a linear reduction in the production of CH_4 and CH_4/DMI of up to 81.3% for CH_4 and 62.6% for CH_4/DMI.

After confirming presence of an anti-methanogenic compound in algae, we created an *in vitro* environment using cow rumen fluid and added 5% of the appropriate amount of algae to a serum bottle to monitor daily total gas production to see if it was significantly less than the control bottle. We discovered that including algae in cow feed can reduce methane output as a result, the addition of algae to cattle feed to maintain VFA levels while restricting the production of methane and the effectiveness of microbial biomass in the rumen by lowering the digestibility and fermentation of OM. Adding algae to ruminant diets, in particular, offers a feed alternative since these diets increased the amounts of propionic and butyric acids, lowered the ratio of acetic acid to propionic acid, and reduced enteric CH_4 synthesis by 15.6 to 31.6%. (Albores-Moreno et al.,2009). According to research, it won't have an impact on milk production or growth capacity (Palangie et al., 2022). Similar to the outcomes shown in dairy cattle, *Asparagopsis taxiformis* at 0.05, 0.1, and 0.2% (organic matter basis) in the feed of beef cattle for 90 days decreased CH_4/DMI by 9, 38, and 98%, respectively (Kinley et al. 2016).

Then, biochemical studies were carried out to determine the nutritional components in algae—namely, carbohydrate, protein, and lipid—that would be employed as feed. *Spirulina* and *Gigartina* spp. were discovered to have equal amounts of protein and carbohydrates but higher amounts of lipids. *Spirulina*, *Gracilaria* spp. is reported to have lower amounts of lipids overall. Given their high levels of protein and dietary fibre, red algae are low-calorie meals, according to earlier studies. Additionally, they contain more protein than brown and green algae, with typical amounts up to 18.8 g per 100 g. Despite having a low-fat content, they have exceptionally high-quality fatty acids.

They include EPA, DHA, oleic acid, arachidonic acid, and essential fatty acids, all of which are thought to be good for health. in contrast to green algae. According to (Gamero et al., 2020), the omega-6/omega-3 ratio is quite low (median = 0.8), which is excellent for human health. The red macroalgae in this study had the greatest total carbohydrate content; the value was noticeably greater than that of the other algae. In contrast to green algae, macroalgae had an adequate total carbohydrate content. The potential of macroalgae as a useful source for development is therefore concluded. (Ilhami et al.,2002)

Then, DNA was extracted from the algal sample using the C-TAB technique, which is quite challenging to execute since after 1 hour of incubation, in water bath we did not see any bands; however, after modifying the procedure, we kept it overnight for water bath incubation and saw smears. The algae sample is then subjected to nanodrop, and the resultant smear indicates that the DNA quantity may not be as excellent because it may be in degraded form. According to previous study, the greatest results were obtained with DNA extraction when it was incubated for 3 hours in 2 ml of extraction buffer. As seen by the thin DNA bands and smeared DNA in numerous species, the quality of the seaweed DNA collected was poor. The inability of PCR-RAPD to amplify seaweed DNA was another sign of poor DNA quality. (Basyar et al.,2020)

Microalgae are now cultured in BG11 medium, while macroalgae are cultured in F2 Guillard's medium. Microalgae can successfully grow in lab settings, while macroalgae cannot. Microalgae grew more quickly when grown in the BG11 culture medium, with a marked exponential phase. Although the culture conducted in the Guillard medium took less time to reduce nitrate, the maximum amount of algal biomass generated in the BG11 medium was larger. Differences in the growth rate were found between the two treatments (Da Cruz Coelho et al., 2013)

Chapter7

CONCLUSIONS

Overall, this study highlights the potential of algae as a feed additive as well as for reducing enteric methane emissions from cattle. The use of algae in cattle diets has shown promising results in terms of methane reduction without negatively affecting milk production or growth capacity. Additionally, algae offer nutritional benefits and could serve as a sustainable and environmentally friendly alternative to conventional feed sources. Implementing such strategies could contribute to mitigating greenhouse gas emissions from the agriculture sector and addressing the challenges of global warming and climate change. Further research and development in this area are essential to optimize algae cultivation, improve DNA extraction methods, and evaluate long-term effects on methane emissions and animal performance.

Chapter 8

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MITIGATION OF ENTERIC METHANE EMISSIONS IN CATTLE THROUGH INDIGENOUS ALGAE

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