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Life as Basic Science: an Overview and Prospects for the Future

Dr. Somnath Das; Dr. Jayanta K.Das; Dr. Mayur Doke; Dr. Vincent Avecilla

Life as Basic Science

an overview and prospects for the future

Vol. 3

Editors

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Life as Basic Science: An Overview and Prospects for Future
[Volume: 3]

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Acknowledgement

This book explores a wide range of scientific study areas, combining several disciplines. The main goal of this book is to assist academics, teachers, and other professionals by means of an in-depth investigation of several areas within the biological sciences. Written by specialists from many different branches of science, every chapter provides evidence-based analysis and stresses the interdependence of biological processes across disciplines.

This set of eight chapters written by diverse scholars has turned their ideas into accessible knowledge for readers. The book aims to close gaps across specialised study fields and promote a whole awareness of life sciences via this means.

Though the writers and editors work tirelessly, the intricacy of the content guarantees some mistakes will remain. Readers are politely asked to report any mistakes or oversights since their comments will be quite helpful in improving next editions.

We truly want that this book motivates readers to understand and use fundamental ideas of life sciences in their particular fields of interest and practice, hence stimulating more research and creativity.

Dr. Somnath Das,
Dr. Jayanta Kumar Das,
Dr. Mayur Doke,
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PREFACE

Life as Basic Science: An Overview and Prospect for the Future [Volume 3] offers a thorough and easily understandable concept of the complex interaction between human life and the continuous need to grasp fundamental science. Examining prospects and possibilities from several angles that emphasise the changing intersections of life, health and scientific progress, this book adopts a forward-looking approach.

Driven by an unyielding will to increase biological knowledge and enhance the human condition, mankind has always confronted and surmounted many obstacles throughout history. The protection of life has become a top priority in today's globalised society, where the blending of science, technology and other cultural perspectives impacts our everyday life. The interaction between life sciences and technology has become more complicated, requiring creative solutions to guarantee the well-being and sustainability of future generations.

Practitioners, teachers, students and researchers committed to crafting a sustainable future for our world will find in this book necessary knowledge and insights. The material is the outcome of a group effort combining the knowledge, experience and enthusiasm of people dedicated to pushing the frontiers of knowledge in this crucial field.

Our aim is not just to record the present state of knowledge but also to encourage fresh conversations and support inventions that will help to create a more just future for everybody.

The authors & editors of this book understand that the future of life sciences has to include multidisciplinary approaches combining knowledge from biology, we recognise the great interdependence between human health, environmental sustainability and technological progress—an interdependence that should direct our work going forward.

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Chapter- 1

Phycotoxins produced by Harmful Algal Blooms (HABs) and their role in human poisoning: A review

Debkumar Sahoo[1], Santosh Kumar Bera[1], Prabad Pratim Pal[1], Dipak Kumar Tamili[2], Nithar Ranjan Madhu[3] and Sudipta Kumar Ghorai[1,4]*

Keywords: Phycotoxin, HABs, Domoic acid, Red tides or Green tides

Abstract:

Phycotoxins are highly potent natural toxins produced by specific marine algae and cyanobacteria during Harmful Algal Blooms (HABs), which often appear as water discolorations known as "Red Tides" or "Green Tides." These toxins are classified based on their chemical structure, mode of action, target tissues, and biological effects on human health. They pose an ongoing threat to public health, marine ecosystems, and the economy, particularly through seafood contamination and water pollution. Managing their impact requires a multidisciplinary approach at both local and global levels. Historical cases highlight the severity of phycotoxin contamination. For instance, in 2015, a bloom of the toxigenic Pseudo-nitzschia species along the West Coast of North America led to domoic acid contamination in crabs and clams, prompting harvesting closures and consumer advisories from public health authorities. Similarly, in September 2016, elevated toxin levels resulted in the closu-

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re of razor clam and mussel harvesting along the Oregon coast. In another incident, massive cyanobacteria blooms in Florida led to drinking water bans in some areas due to contamination concerns. These events underscore the need for ongoing public health surveillance, environmental monitoring, and scientific research to mitigate risks associated with phycotoxins. Despite advancements in marine science, research on human exposure and long-term health consequences remains limited, even as toxigenic species blooms increase globally. Currently, diagnosis and management of phycotoxin poisoning rely heavily on clinical symptom interpretation, exposure history assessment, and identification of contamination sources. Several phycotoxins are neurotoxic, potentially fatal, or linked to chronic health effects. However, human intoxications often go misdiagnosed, underreported, or unrecognized by public health authorities, creating challenges for effective management and epidemiological tracking. To reduce risks, stronger regulatory frameworks, public health vigilance, and awareness among healthcare providers—especially in regions with frequent HAB occurrences—are crucial. However, certain populations face a higher risk of exposure, including recreational shellfish harvesters, anglers, children, and Indigenous coastal communities. Additionally, human poisoning incidents can arise globally due to the consumption of contaminated seafood, whether through travel or the importation of products from regions with insufficient food safety regulations and limited analytical testing. To address these challenges, continued research, improved diagnostic tools, and enhanced monitoring systems are essential for the early detection, prevention, and management of phycotoxin-related health risks.

Introduction:

Phycotoxins by Hurtful Algal Blooms (HABs) are an open well-being concern around the world, flare-ups persistently occur, geographic distribution changes and grows, and modern poisons are recognized, expanding the chance of human exposure and harmful events (Anderson et al., 2012; Tang et al., 2024; Stoner et al., 2024). Climate alters and natural contamination is variables embroiled in the appearance, geographic dispersion and recurrence of HABs and phycotoxins (Pulido, 2016). Topographical extensions of HABs, such as Pseudo-nitzschia species, known to synthesize domoic acid, continue to occur. HABs unfavorably affect the economy, nourishment, and water accessibility, locally and/or through trade, sports, amusement and tourism. A few later occasions depict the extent of HAB's natural contamination of aquatic biological systems and nourishment, as well as its effect on the economy and populace hazard in a few North American locales. HABs and phycotoxins are common natural contaminants of fresh, brackish and seawater, and include: i) Cyanobacteria blooms (CyanoHABs) toxins "cyanotoxins", specially contaminants of soft water stores and drinking water, with direct risk to human wellbeing (Pírez et al., 2013; Raja et al., 2015; Grattan et al., 2016). ii)Marine biotoxins/marine algal toxins by dinoflagellates and diatoms can gather at high concentrations in different tissues of aquatic living beings such as bivalve molluscs and fish, entering the nourishment chain, and threatening consumer's wellbeing. Public health warnings are issued for particular blooms and poisons based on data collected on distinguished toxins, contamination levels of water or specific seafood items, depuration times, and regulatory levels for each product and toxin group. A viable case is the domoic acid contamination of crab and clams that occurred in California November 2015, which lead to a Consumer Caution by California Department of Public Health (CDPH), showing levels of domoic acid in crabs that exceed US federal safety limits of 20 parts per million (ppm) within the meat and 30 ppm within the viscera, with the most elevated level recorded of 190 ppm in a yellow rock crab within the Monterey locale.

The event provoked the closing of the year-round rock crab fishery and postponing the recreational and commercial Dungeness crab seasons (Pulido, 2016). Built-up regulatory parameters, monitoring, and framework capabilities permitted local preventive activities to minimize the risk of intense human exposure. Determination and treatment of intoxication are based on the history of exposure, identification of the contamination event, separation of the toxic compound and creation of organism at the source, displaying side effects related to each group of toxins. Emergency clinical management may help to anticipate serious complications, including death. Long-term disabilities may follow the intense event, e.g., amnesia and epilepsy seen during the domoic acid human harming event in Canada in 1987. Even though less is known about the harmful impacts induced by chronic, repeated exposure, some mycotoxins are carcinogenic or are connected to persistent degenerative neurologic disorders such as Amyotrophic Lateral Sclerosis (ALS). Human health impacts associated with the most common phycotoxins from HABs, cyanobacteria, and marine algae are summarized. Although not talked about here, information from household animals and wildlife intoxication has been key in the identification and follow-up of health impacts, intense and constant exposure in mammals, acting as sentinels for human health hazards, e.g. ocean lions and domoic acid. Experimental information helps to understand mechanisms of activity, distribution, target tissues, and biological effects and to set up rules, policies, and regulations. Anthropogenic activities, counting nutrient contamination, huge utilize of coastal regions, alteration in the dynamics of water streams, leakage of species through ships ballast waters, take part to the worldwide spread of HABs. The availability of detergents also may be a significant anthropogenic movement, and in the last decades, there has been an exponential increment in their utilization. Climate alter incorporates sea acidification, changes in temperature, stratification, and sections in nutrients induced by precipitation and light. Temperature is one of the most natural variables that influences the structure and composition of phytoplankton community (Ianora et al., 2011), global warming in truth acts on a few stages of development and improvement of the blooms, influencing germination, photosynthesis, supplement take-up and other physiological activities, supporting toxin production in HAB species. Poisoning through the ingestion of phycotoxin-contaminated seafood is the leading reported impact that HABs have on people (Figure 1). The poisoning preparation includes the bio-concentration of the phycotoxins by filter-feeding fauna

(generally bivalve molluscs, e.g., *Mytilus* spp.) which themselves are, for the most part, unaffected by these compounds. Other vectors incorporate certain marine gastropods (e.g. whelks and moon

snails), a few shellfish (e.g. crabs), echinoderms and fish (e.g., a few planktivorous fishes or belonging to the Tetraodontidae family) that obtain biotoxins through the nourishment web (Schroeder and Bates, 2015). Phycotoxins gathered in seafood tissues can stay for significant lengths of time after the bloom has declined in the seawater. Further, these biotoxins are not devastated by cooking or by the preparation of seafood products, and because they don't have particular scents or tastes, they can be identified only through specialized research facility testing.

A phycotoxin-producing organism, such as the dinoflagellates *Dinophysis acuta* or *Alexandrium catenella*, is bioaccumulated by shellfish, which are apparently not affected by saxitoxin or lipophilic biotoxins (Anderson et al., 2014; Bragg et al., 2015). Consumption of contaminated shellfish is a traditional way of diarrhetic or paralytic poisoning (DSP, PSP). Alternatively, some toxicogenic species attach to surfaces (macrophytes, corals) by an endogenous mucus (e.g., *Gambierdiscus, Ostreopsis, Prorocentrum lima*). Fragments of corals or macrophytes covered by the microalgae enter the food web through ingestion by herbivorous fish. This is the transmission mechanism of ciguatera fish poisoning (CFP). Certain fish can also experience some sort of poisoning (Mattei Mattei et al., 2014).

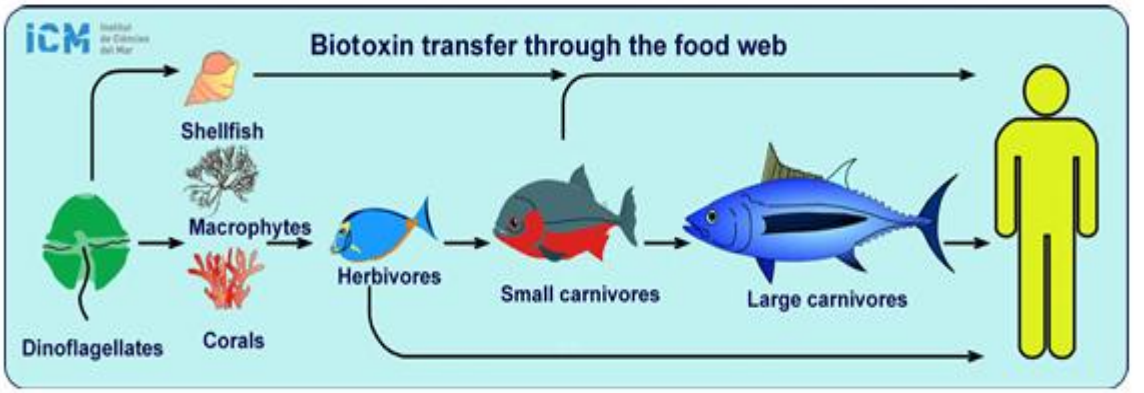

Figure 1: Phycotoxin transfer pathways through the marine food web to humans (Berdalet et al., 2015) [Source: https://doi.org/10.1017/S0025315415001733].

Effects of Phycotoxin on human health:

In mammals, antagonistic health impacts associated with phycotoxins and HABs can happen through verbal, respiratory or dermal exposure to the toxins, their metabolites, or their creating organisms in aquatic or earthbound environments. For people, the most elevated dangers are: (1) Ingestion of seafood contaminated with toxins created by diatoms and dinoflagellates and respiratory exposure through mist concentrates. (2) Exposure to soft water contaminated with cyanotoxins (Ferrão-Filho & Kozlowsky-Suzuki, 2011; Drobac et al., 2013; Farrer et al., 2015) through drinking water, freshwater fish, dermal exposure, e.g., washing in contaminated lakes, or through contaminated equipment or liquids. The poisonous effects of cyanotoxins and marine algal toxins are depicted in people, the mechanism of activity, displaying side effects, clinical disorders and forecast (Koreivienė et al., 2014; Hardy et al., 2015).

Most of the data available deals with acute exposure and effects. A few toxins are neurotoxic and can be deadly, but with appropriate clinical administration, some may completely recover. Marine algal toxins are recognized as contaminants of aquatic environments, with destructive natural impacts but without recognized unfavorable health impacts in humans.

Table 1: Cyanotoxins Biological and Human Health Effects.

Cytotoxins	Mechanism	Symptoms	Source	Prognosis
Hepatotoxins				
Cylindrosper-mopsin (CYN) (Kinnear, 2010; Weirich & Miller, 2013; Méjean & Ploux, 2021)	Inhibition of Protein Synthesis, ribosomal protein synthesis by interfering with RNA and DNA transcription. The inhibition is irreversible, causing delayed cytotoxic effects.	Symptoms up to several days' after exposure or later. Gastroenteritis abdominal pain, vomiting, bloody diarrhea, acute liver inflammation. Liver and kidney failure, hay fever, asthma	Cyanobacteria species such as *Cylindrospermopsis raciborskii*, *Aphanizomenon ovalisporum*, and *Raphidiopsis curvata*.	Chronic exposure linked to cancer e.g., colon
Microcystin MCs	Inhibition of Protein Phosphatases (PP1 & PP2A) Microcystins trigger ROS production	Nausea, vomiting, diarrhea, jaundice, liver failure. Hepatic fibrosis, cirrhosis, increased risk of liver cancer.	cyanobacteria (blue-green algae), primarily *Microcystis*, *Anabaena*, *Planktothrix*, and *Nostoc* species.	Can be lethal. Exposure: drinking water, contaminated dialysis fluid, soft water recreational environments (Azevedo et al., 2002; Banack et al., 2015)
Nodularin (Chen et al., 2013; Brezeștean et al., 2022)	Inhibition of Protein Phosphatases (PP1 & PP2A), Cytoskeletal Disruption & Hepatocyte damage	Diarrhea, vomiting, goose bumps, weakness, liver hemorrhage	Cyanobacteria, primarily *Nodularia spumigena*	–
Neurotoxins				
Anatoxin-a /Homoanatoxin-a (Méjean et al., 2014; Colas et al., 2021)	Agonist at Nicotinic Acetylcholine Receptors (nAChRs).	Muscle twitching, cramping staggering, paralysis, convulsions,	Cyanobacteria species such as *Anabaena*, *Planktothrix*, and *Aphanizomenon*.	Can be lethal

	Persistent Nerve Stimulation (Depolarizing Blockade)	gasping, respiratory failure, death by suffocation		
Cytotoxins	**Mechanism**	**Symptoms**	**Source**	**Prognosis**
beta-Methylamino-L-alanine (BMAA) (Yan et al., 2020)	Excitotoxicity via Glutamate Receptors Incorporation into Proteins (Misfolding & Aggregation). Oxidative Stress & Mitochondrial Damage	Amyotrophic Lateral Sclerosis (ALS), Parkinson's Disease (PD), and Alzheimer's Disease (AD).	cyanobacteria such as *Nostoc*, *Anabaena*, and *Microcystis*.	Chronic exposure linked to chronic neurodegenerative conditions:Amyotrophic Lateral Sclerosis
Saxitoxins (STXs) (Gad & Gad, 2004; Jeon et al., 2024; Pinto et al., 2024)	Voltage-Gated Sodium Channel (NaV) Blockade. Reversible and Highly Potent Blocker	Paralytic Shellfish Poison: Nausea, vomiting. peri-oral burning ataxia, drowsiness, paraesthesia, fever, tachycardia, muscular paralysis, respiratory failure, death	freshwater cyanobacteria *Aphanizomenon*, *Dolichospermu*, *Lyngbya*.	Death can occur within 2-12 hours after exposure. Good prognosis after 24hr, requiring good medical support system.
Irritants and Dermatotoxins				
Aplysiatoxins (Cho, 2006; Nagai et al., 2019)	Uncontrolled cell proliferation. Tumor Promotion & Carcinogenesis	Skin irritation, asthma like symptoms	marine cyanobacteria such as *Lyngbya* and *Trichodesmium*	_
Lyngbyatoxin (Weirich & Miller, 2013; Biessy et al., 2024)	Excessive cell growth (hyperplasia). Increased Intracellular Calcium (Ca^{2+}) Levels	Smooth muscle contraction. Skin irritation	marine cyanobacterium *Lyngbya majuscula*	_

Table 2a: Marine Algal Toxins Diatom and Dinoflagellates: Biological and Human Health Effects.

Toxin group	Vector	Mechanism	Symptom	Syndrome	source
Azaspiracids (AZAs) (Twiner et al., 2012; Yang et al., 2024)	Shellfish Bivalves Mollusks	Cytoskeletal Disruption & Cell Death. Alteration of Ion Channels & Cellular Signaling	Food: Nausea, vomiting diarrhea, abdominal pain. Similar to DSP	Azaspiracids Poisoning (AZP)	Dinoflagellate *Azadinium spinosum*
Brevetoxins (BTX / PbTX) (Katwa & Brown, 2014)	Shellfish Bivalves Mollusks	Persistent Activation of Voltage-Gated Sodium Channels (NaV). Cardiovascular & Gastrointestinal Toxicity	Food: nausea, vomiting, diarrhea, paresthesia, cramps, bronchoconstriction, paralysis, seizures and coma Aerosol inhalation: Rhinorrhea Asthma-like symptoms	Neurotoxic Shellfish Poisoning (NSP) (Hurley et al., 2014)	Marine dinoflagellate *Karenia brevis*
Ciguatoxins (CTXs): Pacific (P-CTX), Caribbean (C-CTX), and Indian Ocean (I-CTX)	Tropical and sub-tropical fish e.g.:eels, snappers, groupers, mackerels, jacks or barracudas	Neurotoxic: Opens sodium channels by binding site. Influx of Na, provoke action potentials, cell swell, and blebs on cell's surface	Food: Vomiting, diarrhea, nausea, tingling, itching hypotension bradycardia arthralgia, myalgia hyporeflexia, dysphagia, ataxia paralysis	Ciguatera Fish Poisoning (CFP) Severity and type of symptoms varies with the type of CTX (Friedman et al., 2008; Brett & Murnion, 2015)	Marine dinoflagellate *Gambierdiscus spp.*

Toxin group	Vector	Mechanism	Symptom	Syndrome	source
Domoic acid group (Bates, 2016)	Shellfish Mollusks Crustaceans Fish (Mazzillo et al., 2010)	Cardiotoxic: Excitatory neurotoxin, analogue of glutamate – acts through glutamate receptors.	Vomiting, diarrhea arrhythmia cardiovascular collapse. Confusion, memory loss, seizure, coma, death	Amnesic Shellfish Poisoning (ASP)	marine diatoms of the genus *Pseudo-nitzschia*
Okadaic acid (OA) and dynophysistoxins (DTXs) (Corriere et al., 2021)	Shellfish Bivalve Mollusks	Inhibit protein phosphatases 1 (PP1) and 2A (PP2A). Increased intestinal fluid secretion (diarrhea)	Severe diarrhea, nausea, and abdominal pain.	Diarrheic Shellfish Poisoning (DSP)	dinoflagellates *Dinophysis spp.* and *Prorocentrum spp.*.
Palytoxins (PlTXs) and PITX like compounds (Wieringa et al., 2014)	Shellfish Bivalve Mollusks, Crab, Fish e.g. Sardines Anchovies	Disrupt cellular ion homeostasis, leading to severe cardiovascular, muscular, and neurological effects	Nausea, vomiting fever rhabdomyolysis, vasoconstriction heart and renal failure, delayed haemolysis Rhinorrhea, cough bronchoconstriction	Haff Disease	Dinoflagellates *Ostreopsis spp.*

Table 2b: Marine Algal Toxins by Diatoms and Dinoflagellates without Known Human Health Effects.

Toxin Group	Vector	Mechanism	Source
Cyclic imines Spirolides(SPXs), gymnodimines (GYMs), pinnatoxins (PnTXs) pteriatoxins (PtTXs)	Shellfish Bivalves Mollusks	Experimental data: Neurotoxic. Fast acting Experimental in rodents parenteral toxicity more potent than oral	Dinoflagellate *like Alexandrium ostenfeldii, Karenia selliformis*

(Otero et al., 2011)			
Pectenotoxins (PTXs)	Shellfish Bivalves Mollusks	Disruption of Actin Cytoskeleton. Inhibition of Phosphorylation Pathways	*Dinophysis* species (dinoflagellates)
Yessotoxins (YTXs) (Tubaro et al., 2014; Alfonso et al., 2016)	Shellfish	Disruption of Calcium Homeostasis. Decrease cyclic AMP, activate cellular phosphodiesterases (PDEs)	Dinoflagellates

Cyanotoxin impacts on human health:

Cyanotoxins are secondary metabolites produced by cyanobacteria that pose toxic threats to various living organisms, including humans (Ibelings et al., 2014; Paerl, 2014). These toxins are classified into distinct chemical groups based on their structure and composition, including: Cyclic peptides: Microcystins and nodularins (Chen et al., 2013), Alkaloids: Anatoxin-a, anatoxin-a(s), saxitoxins, cylindrospermopsin, aplysiatoxin, lyngbiatoxin-a, Lipopolysaccharides (LPSs). Cyanotoxins can also be categorized based on their physiological effects and target tissues: Hepatotoxins: Affect the liver, Neurotoxins: Target the nervous system, Cytotoxins: Cause cellular damage, Dermatoxins: Affect the skin, Irritant toxins: Cause inflammatory responses. Among the neurotoxic cyanotoxins, key compounds include anatoxin-a, homoanatoxin-a, and β-Methylamino-L-alanine (BMAA). Chronic and repeated exposure to BMAA has been linked to neurodegenerative disorders such as Amyotrophic Lateral Sclerosis (ALS). The harmful effects of BMAA have been extensively studied in relation to dietary exposure from the seeds of *Cycas circinalis*, a plant traditionally consumed by the indigenous populations of Guam Island. More recently, the presence of BMAA has also been identified in certain species of diatoms (Jiang et al., 2014), broadening concerns regarding its potential environmental and health impacts. Harmful cyanobacteria, particularly those capable of forming dense blooms, pose a growing global threat to aquatic ecosystems. Their proliferation is primarily driven by anthropogenic nutrient enrichment—such as agricultural runoff, wastewater discharge, and industrial pollution—alongside the accelerating effects of climate change. These environmental stressors have led to the frequent occurrence of toxic cyanobacterial blooms in freshwater bodies, including lakes, reservoirs, and soft-water systems worldwide (Glibert et al., 2014; Brooks et al., 2016). The toxins released by these microorganisms can severely disrupt biodiversity, destabilizing aquatic ecosystems and posing significant risks to both wildlife and human health. Despite their harmful effects, certain cyanotoxins exhibit bioactive properties that hold promise for pharmaceutical and biotechnological applications (Zanchett and Oliveira-Filho, 2013). Some of these compounds have demonstrated potential as:

- Antimicrobial agents – Compounds that can inhibit or destroy harmful bacteria and other pathogens.

- Algaecides – Substances that suppress the growth of undesirable algal species.
- Cytotoxic agents – Compounds capable of targeting and eliminating malignant cells.
- Immunosuppressive agents – Substances that can modulate immune responses, which may be beneficial in conditions such as autoimmune disorders and organ transplant procedures.
- Enzyme inhibitors – Molecules that interfere with enzymatic processes, which can be exploited for therapeutic interventions.

Certain cyanotoxins have shown potential for anticancer applications, particularly those that act as protease inhibitors and cell cycle regulators. One notable example is Curacin A, a compound derived from marine cyanobacteria that has been investigated for suppressing tumour growth by interfering with microtubule dynamics, a key mechanism in cancer cell proliferation (Carmichael & Boyer, 2016).

Marine Algal Toxins cause Seafood Poisoning Syndromes:

Marine algal toxins, primarily produced by dinoflagellates and diatoms, are potent compounds that contaminate seawater ecosystems. These toxins accumulate in the tissues of aquatic organisms, particularly bivalve mollusks (e.g., mussels, oysters, clams, cockles, and scallops), as well as shellfish and fish, ultimately entering the food chain. As filter feeders, bivalve mollusks can concentrate both chemical and microbiological contaminants, making them a significant vector for seafood poisoning. The consumption of contaminated fish and shellfish remains one of the leading causes of marine toxin-related illnesses worldwide. Marine algal toxins pose serious threats to human health, marine wildlife, economies, and ecosystems. To mitigate these risks, international regulations and safety standards have been established to ensure seafood quality, particularly given its role as a major globally traded commodity. However, limited resources and analytical capabilities in developing nations hinder the effective detection of mycotoxins, potentially masking severe environmental health concerns and seafood safety issues.

Table 2 outlines the major clinical conditions associated with marine algal toxins produced by dinoflagellates and diatoms. These toxins can cause illness through ingestion of contaminated seafood, inhalation of aerosolized toxins, or direct skin and eye contact. The most well-documented marine toxin-related seafood poisoning syndromes include: Diarrheic Shellfish Poisoning (DSP), Paralytic Shellfish Poisoning (PSP), Amnesic Shellfish Poisoning (ASP), Neurotoxic Shellfish Poisoning (NSP), Azaspir acid Shellfish Poisoning (AZP), Ciguatera Fish Poisoning (CFP). Additionally, Palytoxin (PITX)-related disorders include: Palytoxicosis, Clupeotoxism, PITX-like Myotoxic Syndrome (Haff Disease) – Characterized by severe muscle pain (myalgia), muscle breakdown (rhabdomyolysis), and dark urine (myoglobinuria) following the consumption of cooked freshwater or brackish water fish. Unlike other PITX toxicities, Haff Disease is primarily myotoxic rather than neurotoxic. There is growing public health concern regarding PITX poisonings from inhalation, skin contact, and eye exposure, particularly during the handling of contaminated soft corals in aquariums (Pelin et al., 2016).

Medical Management of Phycotoxins:

Currently, the diagnosis of phycotoxin poisoning primarily relies on a combination of clinical symptomatology and exposure history. Medical professionals assess whether an individual has consumed contaminated food or water, and they often attempt to identify the presence of toxins in these sources. Additionally, an estimation of the quantity of contaminated food or water ingested is conducted to determine the potential toxin exposure level. From an epidemiological perspective, phycotoxin poisoning is commonly documented through reports of acute poisoning incidents, outbreaks related to the consumption of contaminated seafood, and cases linked to exposure in affected oceanic environments. However, mild cases may go undiagnosed as individuals might not seek medical attention, either due to the transient nature of their symptoms or the lack of awareness regarding phycotoxin-related illnesses. Furthermore, respiratory and skin reactions caused by phycotoxins can often be misinterpreted as allergic responses, leading to potential underreporting or misdiagnosis.

Poisoning symptoms resulting from the consumption of phycotoxin-contaminated seafood can manifest within minutes to several days after ingestion (Picot et al., 2012). These toxic effects can arise from the consumption of various seafood products, including fish and shellfish—whether freshwater or marine, cooked or raw, locally sourced or imported. The risk of exposure is not limited to any specific geographic region, as poisoning may occur both during travel to endemic areas and after returning home. Public health surveillance plays a crucial role in monitoring and managing phycotoxin-related illnesses. The assessment of the population's dietary habits, recreational activities, and cultural seafood consumption practices is essential for identifying at-risk groups (Diaz, 2015). Individuals at heightened risk of exposure due to social and occupational practices include recreational shellfish harvesters, fishermen, and Indigenous communities residing in coastal regions, where seafood forms a significant part of their diet. Multiple factors, including age, pre-existing medical conditions such as renal insufficiency, and the quantity and type of contaminated seafood consumed, influence the severity of intoxication. Public health management of outbreaks requires a thorough investigation into the source of contamination. This involves examining food samples to identify specific toxins, which is critical in determining the extent of an outbreak and implementing necessary control measures. Despite the diversity of phycotoxins, each possessing distinct mechanisms of action, clinical manifestations, and prognostic implications, acute poisoning due to neurotoxic phycotoxins commonly affects multiple organ systems. These include the gastrointestinal (GI), cardiovascular, respiratory, and nervous systems. Gastrointestinal symptoms generally appear first, while neurological manifestations may be delayed, emerging minutes to days after exposure. The severity and frequency of symptoms often escalate over time, particularly in the hours following the consumption of contaminated seafood. Timely reporting of suspected poisoning cases to public health authorities is vital for tracking outbreak events, facilitating epidemiological investigations, and maintaining a comprehensive database for future reference. Even mild cases should be documented to ensure a more accurate assessment of the public health burden. Currently, there are no specific biomarkers available to confirm the diagnosis of phycotoxin

poisoning or to definitively detect exposure. However, some laboratory tests may provide supportive evidence. For instance, in cases of Haff Disease, which is associated with a PITX-like myotoxic compound, urine tests measuring myoglobin levels can assist in diagnosis. Similarly, certain experimental assays for detecting saxitoxins (STXs) in urine exist, though they require further validation and standardization. In cases of acute poisoning, emergency medical management should be well-coordinated and tailored to the patient's clinical presentation. While mycotoxins are not explicitly included in standard toxicological guidelines, general supportive care principles, as outlined by Thompson et al. (2014), remain applicable. Treatment for phycotoxin poisoning is largely symptomatic and supportive, as no specific antidotes are currently available. Management strategies vary depending on the toxin group and its mechanism of action. For example, in cases of suspected domoic acid intoxication **(**Kirkley et al., 2014), antiepileptic medications and drugs that modulate glutamate receptors may be used to manage seizures and neurotoxicity (Boushey et al., 2016). In saxitoxin (STX) poisoning**,** respiratory support is often required due to its paralytic effects. Given the serious and potentially fatal nature of phycotoxin poisoning, early recognition and prompt intervention are essential to improving patient outcomes. Ongoing research and improvements in diagnostic methodologies are necessary to enhance the detection, treatment, and prevention of these toxin-related illnesses.

Limited information is currently available regarding the long-term effects of repeated exposure to neurotoxic phycotoxins or their combinations. However, the potential developmental toxicity of domoic acid and its association with epilepsy warrant significant attention due to their implications for public health. Emerging evidence suggests that domoic acid exposure may be a preventable risk factor for epilepsy and neurobehavioral disorders. Rapidly accumulating data from experimental research, wildlife poisoning incidents, and studies on chronic exposure support a causal relationship between prolonged domoic acid exposure and neurological consequences. Furthermore, laboratory studies have demonstrated that domoic acid can cross the placenta during pregnancy and may also be present in breast milk, posing potential risks to developing fetuses and nursing infants. Raising consumer awareness is crucial, particularly for pregnant and lactating women, who may be more vulnerable to the effects of phycotoxin exposure. This is especially important during contamination outbreaks, as seen in recently reported cases. To better understand and mitigate these risks, dietary assessments should be conducted to estimate exposure levels and guide public health recommendations.

Prevention of Human Phycotoxins Exposure:

The primary strategies for managing the risks associated with mycotoxins include prevention, achieved through regulatory policies and monitoring systems and early warning mechanisms issued by public health organizations to minimize the occurrence of human poisoning incidents. The most effective way to prevent poisoning is to avoid consuming non-commercially harvested shellfish that have not been tested for phycotoxins (Knaack et al., 2016). However, cultural and traditional dietary practices, such as recreational shellfish picking, may increase the likelihood of exposure. Travellers visiting regions with limited food safety regulations are also at risk and may

experience symptoms of intoxication upon returning home. Raising awareness about environmental conditions and systematically documenting HAB events are essential for preventive management and the development of predictive models. Detailed clinical records, including dietary history, symptom progression, and timely reporting to public health authorities, are critical for accurate diagnosis, outbreak tracking, and effective risk management.

Conclusion:

Phycotoxins and their associated health effects represent a significant global public health concern, necessitating increased awareness and proactive management strategies. This summary highlights the most common poisoning syndromes caused by these marine biotoxins and underscores the importance of enhanced awareness among healthcare professionals and consumers (Lawrence et al., 2011; Paredes et al., 2011). Improved knowledge and vigilance will lead to more effective clinical management, better case reporting, and the expansion of epidemiological databases, all of which are crucial for tracking and mitigating the impact of these toxins. However, addressing the full scope of human health risks posed by phycotoxins requires a multidisciplinary, international effort. A comprehensive understanding of their effects involves detailed exposure assessments, including data on seafood consumption patterns and contamination levels in marine and freshwater environments. Each phycotoxin group presents distinct characteristics that must be studied in-depth, such as the specific organisms responsible for toxin production, the vectors through which they enter the food chain, their toxicological properties (including toxicokinetics), biological effects, associated clinical symptoms, and overall prognosis following exposure. While acute poisoning syndromes have been well documented, the long-term effects of chronic, low-dose exposure remain poorly understood. Identifying and validating biomarkers for phycotoxin exposure is critical for advancing the diagnosis, detection, and monitoring of human health risks. The development of such biomarkers would significantly enhance disease surveillance, improve epidemiological tracking, and allow for more precise risk assessments. Certain vulnerable populations require special attention due to their heightened risk of exposure and potential long-term health consequences. These include Indigenous Arctic communities and Native American populations along the American West Coast, whose traditional diets heavily rely on seafood, as well as pregnant women, nursing infants, and individuals with pre-existing health conditions. Emerging research suggests that chronic exposure to certain phycotoxins may be linked to serious health conditions such as cancer, neurodegenerative diseases, and developmental disorders, further emphasizing the need for targeted surveillance and risk reduction strategies. In addition to preventive measures, there is an urgent need to develop effective antidotes for phycotoxins with potentially fatal effects. This remains a major scientific and medical challenge, requiring further investment in research and innovation. Until specific treatments are available, public health efforts must focus on prevention, early detection, and symptomatic management to minimize the impact of phycotoxin-related illnesses. Moving forward, international collaboration, advancements in diagnostic tools, and continuous public health monitoring will be essential in protecting populations from the risks

associated with phycotoxin exposure. Through a coordinated and evidence-based approach, the global community can work toward reducing the burden of these marine toxins and safeguarding human health.

Conflict of interest:

The authors state that they do not have any competing interests.

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Chapter- 2

A Review of Allelopathic Potential of Some of the Economically Important Members of the Family Poaceae with Special Reference to Rice for Weed Control and Sustainable Agriculture

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Abstract:

The present study is focused on reviewing the allelopathic potentiality of the family Poaceae concerning the nature of action (stimulation or inhibition) and their mechanism of action. The plant family Poaceae has been the topic of inquiry in a number of studies due to the fact that it demonstrates a significant amount of allelopathic potential. A number of secondary metabolites like phenolics, flavonoids, and alkaloids are frequently found in the family Poaceae, both cultured as well as in wild species. Growth and development of plants sometimes get encouraged below the threshold levels of allelochemicals but the harsh reduction of growth may be detected with allelochemicals exceeding the threshold concentration, which may be sensitivity-dependent for the receiving species. Some researchers illustrated that soil collected from the donor plant's base prominently reduced or, to some extent, encouraged the plants' development under experimentation. Allelochemicals have the potential effect on genetic and physiological parameters of plants of other plants and plants of the Poaceae family. Bio-standardization experiments using petri plates with methanol or aqueous extracts or fractions, along with contributory allelochemicals of phenolic nature, confirmed the considerable phytotoxicity in a concentration-dependent manner. This article makes an effort to examine and summarise previous and more recent data about the allelopathic activity of this family along with their potential for use in the development of natural product-based, environmentally friendly herbicides for sustainable agriculture, and to stimulate future discussion on this topic.

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Introduction:

The term allelopathy is a blending of the Greek words 'allelon' denoting 'each other' as well as 'pathos' standing for 'suffering', given by Hans Molisch, a plant physiologist. Pathos also refers to 'feeling' or 'sensitivity,' and thus can be applied to illustrate both optimistic (sympathetic) and pessimistic (pathetic) interactions (Gross, 1999). Allelopathy is the mechanism by which plants release secondary metabolites or allelochemicals that influence the growth and development of other plants by involving secondary metabolites produced by plants, microbes, and other organisms, thereby affecting the growth and development of agricultural or biological systems in either a beneficial or detrimental manner (Cheng, F., & Cheng, Z., 2015). Allelopathic chemical substances are present in almost all plants and most plant tissues and can positively or negatively influence vegetational development parameters (Rice, 1984; Reigosa et al., 1999; Alam et al., 2001). The majority of allelochemicals are classified as secondary metabolites in plants and are typically believed to be those that do not participate in any function related to primary metabolic pathways crucial for their continued existence (Niemeyer, 1988; Corcuera, 1993). Such secondary metabolites may be a variety of chemicals, from pigments to gases of toxic nature, and reveal none of the intrinsic effects on the plant systems. As an exceptional occasion, the above said pigments sometimes show a functional effect by attracting pollinating agents. Some also trigger plant defence mechanisms after pathogen attack revealing their structural or physiological role in plants (Einhellig, 1995; Kruse, 2000). Allelochemicals may be insecticidal, herbicidal, and antimicrobial or may possess some other biological activities (Tsao et al., 2002). Therefore, allelopathic studies may be beneficial for weed management in the organic farming system (Rey and Monalie, 2012; Sar et al., 2024). Allelochemicals released from the plant may not prevent plant growth and development in a certain amount of concentration but may reveal vital ecological involvement about the impact on soil microbes along with chemical factors (Weidenhamer et al., 2009; Ghosh et. al., 2011). Therefore, in the near future, allelopathy may be useful for protecting crops in agriculture to preserve soil efficiency and a pollution free environment. According to some researchers, Barley is cultivated with other crop plants to prevent weed growth by allelopathic interactions (Robert and Moncef, 2009). Allelochemicals in Barley can be used as a natural herbicide to improve sustainable or ecological-based weed management. The evolution of humans, in addition to their domesticated grazing mammalians viz., Cow, Buffalo, and Horse etc., the indispensable role of the Poaceae grasses can't be ignored. Skilful domestication, as well as storage of grains, permitted human civilizations to develop years ago. The family Poaceae is huge, having approximately 8000-9000 species, with those of supreme importance economically. According to FAO, rice, wheat as well as corn are considered as three most vital crops in the world (Ghosh et al., 2023; Paul et al., 2024; Das et al., 2024; Mishra et al., 2024); which belong to the said big family, along with different fodder species like Lolium and further Bamboos, Canes and a few other species with ornamental value (FAO, 1991, 1992, 1993, 1995). The plants of the Poaceae family are categorized as the most dominant from

an ecological viewpoint and undoubtedly the most significant family from an economic perspective globally (Favaretto et al., 2018).

Rice, wheat, barley, rye, and sorghum are the most researched allelopathic crops exhibiting notable allelochemical differences in connection to the cultivar (Bhowmik and Inderjit,2003; Jabran et al., 2015). The scientific community has worked on this problem quite a bit in recent years. Disparities in metabolomic and phytotoxic properties have been noted recently between two rye (*S. cereale*) and six canola (*B. napus*) cultivars (Asaduzzaman 2015; Ghimire et al., 2019). Maver et al. (2020) screened twelve barley accessions for the presence of the alkaloids gramine, hordenine, and its direct precursor N-methyltyramine. They discovered notable variations between modern genotypes and wild relatives, in addition to differences based on plant parts, which marked a significant advancement in the breeding of this crop.

It is recognised that certain biotic (diseases, infections, and plant density) and abiotic (light, drought, temperature, salinity, and mineral availability) stress factors can boost the synthesis of allelochemicals. For instance, Oueslati et al. (2005) showed that when there is a drought, barley autotoxicity increases. Another study indicated that the quantity of sesquiterpene lactones and their allelopathic activity in farmed cardoon leaf extracts increased by 60% of plant shade (Scavo et al. 2020). According to Xuan et al. (2016), as a defence mechanism, rice increases the synthesis of momilactones A and B, two recognized allelochemicals present in rice, in response to salinity and drought.

In view of this background, an attempt has been made to review the allelopathic potentiality of the family Poaceae along with the herbicidal perspective of the members.

Range of allelochemicals from the family Poaceae:

Allelochemicals are biomolecules which are released from the plant by exudation and biomass decay (Miller, 1996). Abundant allelochemicals are present in plant species. As one of the most economically significant plant families, allelopathic potentialities of the members of the family Poaceae were studied extensively, along with the identification of responsible allelochemicals. The prominent allelopathic compounds identified can be split into the following groups.

The type and composition of their allelochemicals range from phenolics to quinones, and there is a significant amount of variety present across this spectrum (Nath et al., 2024). The substances that have been isolated and characterised to the greatest extent can be classified into one of four categories: phenolic acids, hydroxamic acids, alkaloids, or quinones.

Phenolic Acid Group:

p-hydroxybenzoic acid, syringic acid, vanillic acid, ferulic acid, *p*-coumaric acid, caffeic acid, *p*-hydroxybenzaldehyde, gallic acid, protocatechuic acid, etc., are encountered in various species like Couch grass (Schulz et al., 1994), Avena spp. (Guenzi et al., 1967; Schumaeher et al., 1983; Wardle et al., 1994), Rice (Chou et al., 1981; Alsaadawiet al., 1998), Millet species (Nicollier et al., 1983; Weston et al., 1989), Wheat (Blum et al., 1992; Alsaadawi et al., 1998; Wu et al., 2001;

Wu et al., 2002), Maize (Chou & Patrick, 1976) and *Eragrostis plana* (Favaretto et al., 2015) (Table 1 and Figure 1).

Table 1: Phenolic acid compounds found in different Poaceae species.

Poaceae Species	Phenolic acid compounds	Effects
Oryza sativa (Rice)	Ferulic acid, *p*-Coumaric acid, Caffeic acid	Inhibit weed seed germination and exhibits antimicrobial properties
Zea mays (Maize)	Ferulic acid, Caffeic acid, Vanillic acid	Inhibit seed germination and root elongation
Saccharum officinarum (Sugarcane)	Ferulic acid, Syringic acid, *p*-Coumaric acid	Suppress weed growth and exhibits antimicrobial properties
Cenchrus ciliaris (Buffel Grass)	Caffeic acid, *p*-Coumaric acid	Allelopathic inhibition of neighboring plants
Echinochloa crusgalli (Barnyard Grass)	Ferulic acid, *p*-Coumaric acid	Competes with rice, affects soil microbiome
Pennisetum glaucum (Pearl Millet)	Ferulic acid, Syringic acid, Caffeic acid	Inhibit growth of legumes
Dactyloctenium aegyptium (Crowfoot Grass)	Ferulic acid, *p*-Coumaric acid	Reduces seed germination in nearby species
Chloris barbata (Swollen Fingergrass)	Vanillic acid, Caffeic acid	Suppress weed growth

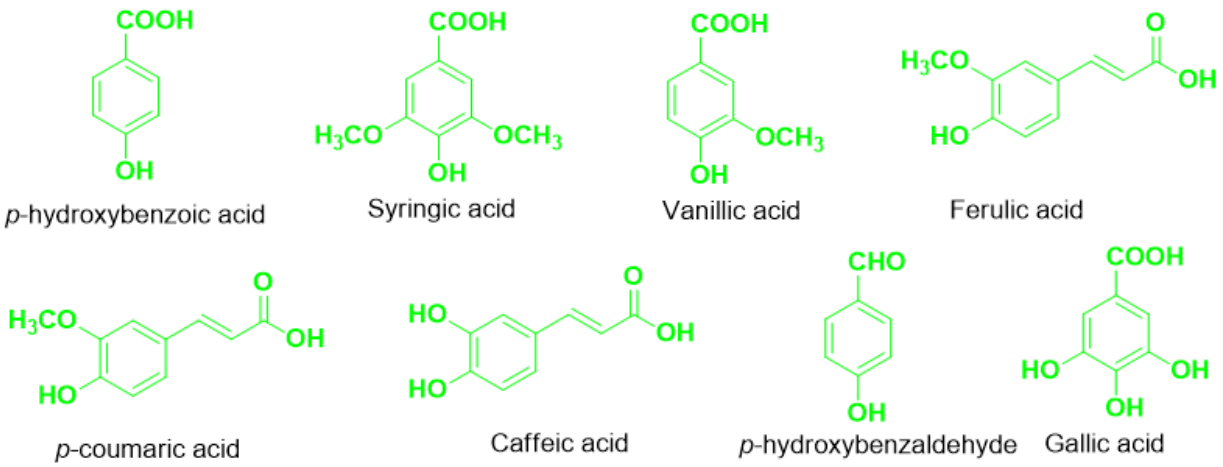

Figure 1: Molecular structure of the various types of phenolic acids found in various Poaceae species.

Hydroxamic Acid Group:

Benzoxazolin-2-one, 2,4-dihydroxy-1,4-benzoxazin-3-one, 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3(4*H*)-one, AZOB, DIMBOA-glucoside, 6-methoxybenzoxazolin-3-one, CI-

MBOA, etc. in different plants like Maize (Hedin et al., 1993; Friebe et al., 1997), Couch grass (Schulz et al., 1994), *Triticum* spp (Kato-Noguchi et al., 1998; Quader et al., 2001, Vieites-Álvarez et al., 2023) and Rye (Pérez and Ormefio-Nǜñez, 1991; Pérez and Ormefio-Nǜñez, 1993; Mwaja, 1995) but not found in rice, sorghum and cultivated barley (Niemeyer, 2009) (Table 2 and Figure 2).

Table 2: Hydroxamic acid compounds found in different Poaceae species.

Poaceae Species	Hydroxamic Acids	Effects
Zea mays (Maize)	DIMBOA (2,4-Dihydroxy-7-methoxy-1,4-benzoxazin-3-one), MBOA (6-Methoxybenzoxazolin-2-one)	Inhibits seed germination and root growth of competing plants and demonstrates antifungal properties
Triticum aestivum (Wheat)	DIMBOA, MBOA	Suppresses weed growth and reduces insect herbivory
Secale cereale (Rye)	BOA (Benzoxazolin-2-one), DIBOA (2,4-Dihydroxy-1,4-benzoxazin-3-one)	Strong allelopathic effects on weeds like Amaranthus and Lolium
Echinochloa crusgalli (Barnyard Grass)	DIBOA, BOA	Competes with rice, inhibits germination of neighboring plants

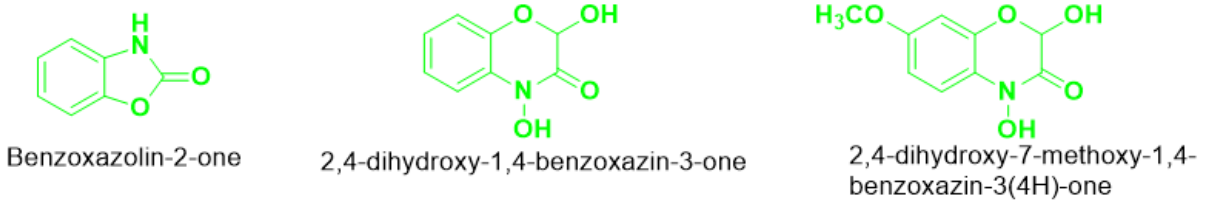

Figure 2: Molecular structure of the various types of hydroxamic acids found in different Poaceae species.

Alkaloids:

Hordenine, gramine, found in Barley (Coreuera et al., 1992; Liu DL and Lovett, 1993) (Table 3 and Figure 3).

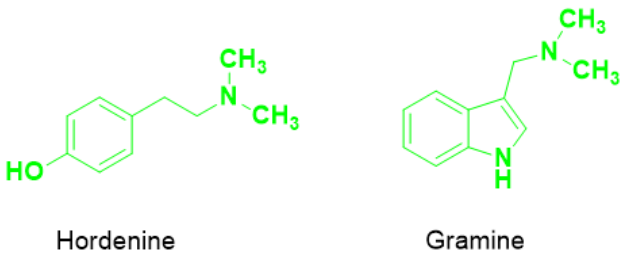

Figure 3: Molecular structure of the various types of alkaloids found in different Poaceae species.

Table 3: Alkaloids found in different Poaceae species.

Poaceae Species	Alkaloids	Effects
Zea mays (Maize)	Gramine	Inhibit seed germination
Triticum aestivum (Wheat)	Gramine, Hordenine	Affects microbial communities, reduces pest attacks
Hordeum vulgare (Barley)	Hordenine	Inhibits root growth of competing plants
Avena sativa (Oat)	Avenacins, Gramine	Antifungal activity, defence against pathogens
Echinochloa crusgalli (Barnyard Grass)	Gramine	Competes with rice, inhibits plant growth

Quinones:

Quinine, sorgoleone, *p*-benzoquinones found in Millet (Netzly et al., 1988; Einheling et al., 1993; Nimbal et al., 1996; Gonzàlez et al., 1997; Weston and Czarnota, 2001) (Table 4 and Figure 4).

Table 4: Quinones found in different Poaceae species.

Poaceae Species	Quinones	Effects
Sorghum bicolor (Sorghum)	Sorgoleone	Allelopathic effect on weeds, inhibits root growth
Zea mays (Maize)	Benzoquinones	Inhibit seed germination and root elongation
Triticum aestivum (Wheat)	Tocochromanols	Antioxidant properties, defencee mechanism
Hordeum vulgare (Barley)	Plastoquinone, Phylloquinone	Photosynthetic electron transport
Oryza sativa (Rice)	Phylloquinone	Antioxidant, possible defence against pathogens
Saccharum officinarum (Sugarcane)	Plastoquinones	Involved in oxidative stress response

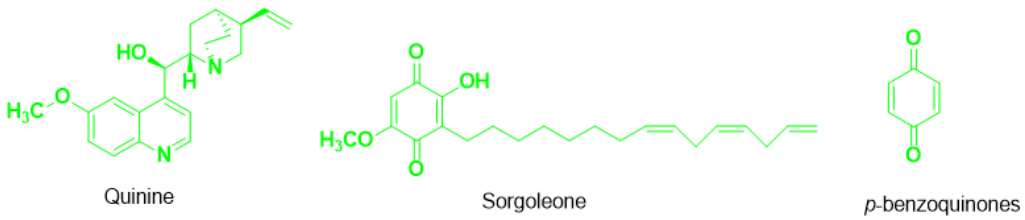

Figure 4: Molecular structure of the various types of quinones found in different Poaceae species.

Others:

Some other important allelochemicals include Momilactone-B from rice (Kong et al., 2004; Kato-Noguchi et al., 2010) linoleic acid, oleic acid, stearic acid, 9-octadecenoic in addition to 7-octadecenoic acids grouped under fatty acids from rice cultivars showing an effect on growth parameters of duck salad and barnyard grass (Ko et al., 2005), Flavones as well as O- or C-glycosides of flavones, cyclohexenone, playing vital roles in allelopathy induced through the rice (Kong et al., 2004). The most effective substances against the big brome were salicylic acid and

saponarin (a flavonoid recently discovered in barley). These were followed by "Manel" and "Ardhaoui," and they may be used in biological control against weeds (Bouhaouel et al., 2020).

The way that it is let out:

There is no one unique way that allelopathic substances are released into the environment, nor are there any particular plant sections that contain them. Therefore, phenolic acids can be found in the grains, the pollen, the root exudates, the residues, the straw that is degrading, extracts from various plant sections, soil extracts, and other places. The same can be said for the hydroxamic acids, the quinones, and any other allelopathic substances found in the Poaceae family.

Mechanism of action of allelochemicals:

The outlined allelochemicals and their secretion are not found throughout the body, root exudates or decomposing litter or even in the soil where they grow and decompose. To study their mechanism of action concerning germination parameters decline in radical and plumule length, dry and wet mass, germination index, percentage of germination and finding their site of action has also been studied by several researchers (Javaid et al., 2006; Kato-Noguchi et al., 2010; Suwitchayanon et al., 2013). To mention some of the allelopathic impact of *Sorghum bicolour* in *Pisum sativum* and *Glycine max* on the process of photosynthesis and oxygen evolution (Einheling et al., 1993), on cellular respirational process in *Zea mays (*Rasmussen et al., 1992), the effect of abundant biomass of rye that physically obstructs weed seedling appearance and interference with a light interception was studied (Teasdale and Mohler, 2000). Autotoxicity is a type of allelopathy that takes place at the intraspecific level where one plant species liberates chemicals that restrain or else cause disturbances with relation to the germination as well as growth parameters of the plant species of the same type as observed in the case of many Poaceae members like the roots exudates of wheat, oats (*Avena sativa* L.) and some other crops that release chemical substances inhibitory to their own seedlings (Schreiner and Reed, 1907; Putnam, 1985). Wheat straw residue on the field soil exerts adverse influences of autotoxicity on wheat farming procedures and has been observed in many other instances (Wuet al., 2001; Wu et al., 2008). Barley autotoxicity was reported and often reveals vulnerability in barley-barley agro-successions (Ben-Hammouda, 2002; Oueslati et al., 2005).

Pest Control in Agricultural and Natural Ecosystems:

The Poaceae family contains some of the most vital crops in the world. Allelopathic effects allow these large crops to biologically control weeds and pests is a goal that has a lot of potential and is highly interesting. In the field of pest control, cases of various species were described (Zwain et al., 1999; Chattopadhyay et al., 2010; Madhu et al., 2015; Madhu, 2017, 2019). Several compounds have been isolated from the exudates or residues of *Triticum aestivum*, *Zea mays*, *Imperata cylindrica*, *Secale cereale*, or *Hordeum vulgare*. The literature contains references to the potential for controlling fungi, bacteria, algae, and aphids with these compounds (Zwain et al., 1999; Wiseman et al., 1992). However, the allelopathic capabilities of Poaceae have garnered

the most scientific attention because of their role in weed management by crops and the benefits of crop rotation for Gramineae species, which include increases in production, vigour, and emergence.

Genetic basis of allelopathy in Poaceae:

The genetic foundation of allelopathic potential in rice through the use of F_2 generation obtained from crossing experimentation among rice varieties *PI312777*illustrated that allelopathy demonstrates quantitative inheritance (Dildayet al., 1998), while some of the researchers stated that allelopathic behaviour of rice is a quantitative as well as a polygenic characteristic which rests on several physiological and morphological features (Kong et al., 2011). Six numbers of genes, such as DEG-1, DEG-4, DEG-5, DEG-7, DEG-9 and DEG-8, were studied to understand the genetical regulation of allelopathy in rice with elevated expressive nature compared to the genes DEG-2, DEG-3 and DEG-6 which revealed a lower level of expression recognized employing molecular genetic tools while rice variety 'Sathi' were growing with cockspur grass (Junaedi et al., 2008). The locus of genes on the chromosomes that control the synthesis of wheat hydroxamic acid was examined, resulting in a multigenic control of the trait in favour of the build-up of hydroxamic acids (Niemeyer and Jerez, 1997). They also proposed that the Genes for the conversion of DIBOA to DIMBOA may be positioned in Chromosome numbers 4A and 4B. In contrast, Chromosome number 5B might include a gene controlling the conversion of methoxylated lactam into DIMBOA. Moreover, a gene positioned in chromosome 4D might hinder the accumulation of hydroxamic acids. Meanwhile, chromosome 2B was spotted with two key quantitative trait loci or QTLs linked to wheat allelopathy using RFLP and microsatellite markers on ryegrass (Wu et al., 2003). Genetic markers linked to wheat allelopathic perspective and the cytochrome P450 that codes for the production of allelopathic chemicals in wheat were also evaluated (Wu et al., 2007). It was substantiated by an assessment of allelochemicals made by the same genotypes that, like rice in the case of wheat also allelopathy is a quantitative character. In allohexaploid wheat, benzoxazinoid biosynthesis is regulated through TxBx1 to TxBx5 genes, which reveal Orthology to HlBx1 to HlBx5 genes of barley, along with Bx1 to Bx5denes of maize (Nomuraet al., 2005). In the case of maize, indole-3-glycerol phosphate is converted to indole, which is controlled by the BX1 branch enzyme, whereas production of DIBOA from indole is under the control of the rest of genes i.e., Bx2 to Bx5 (Frey et al., 1997). Homology among HlBx1to HlBx5 barley genes and the maize genes Bx1 to Bx5 reveal 72%, 80%, 76%, 81% and 78%, respectively (Grünet al., 2005). A variety of secondary metabolites considered as allelopathic potential are produced through the phenylpropanoid metabolic pathway. Few plants protective proteinaceous substances and the phenylalanine ammonia-lyase or PAL, which is related to the metabolism of phenylpropanoid in addition to the defence mechanism of plants, were established to be in an up-regulation mode in the case of rice varieties with allelopathic potential (Fang et al., 2009). Proteomics studies, in addition to bioinformatics research related to the molecular machinery associated with allelopathy when cultured along with

barnyard grass, revealed that an augmented reduction consequence of rice allelopathic potential of rice takes place on target weeds is because of the up-regulated gene expression that codes for PAL (He et al., 2005).

Physiological basis of allelopathy:

Allelochemicals demonstrate prominent physiological effects on different plant species. Different allelochemicals from the Poaceae plants reveal clear manipulations of different target species through various mechanisms. Hordenine and gramine encountered with barley allelopathy causes cell wall damage of root tips of white mustard, amplification of size as well as quantity of vacuoles, cellular autophagy, disorganization of cell organelle chloroplasts as well as mitochondrial deformation at a considerable level was induced through the action of cinnamic acid in *Cucumber* or roots cells irregularity and cell organelle disturbances were triggered by benzoic acid (Liu and Lovett, 1993). Significant reduction in the germination rate, radical as well as plumule lengths, and dry weight along with the length of the seedling as a whole with augmented cover crops residue of a few Poaceae crops was also reported (Shekoofa et al., 2020). Elongation of tap root system *Raphanus sativus* L., as well as *Gossypium hirsutum* L. grown in greenhouse conditions, revealed inhibition by residues of *Avena trigose* Schreb, *Secale cereal* L. and *Trifolium incarnatum* L. (Bauer and Reeves, 1991). The higher inhibition of root growth in barnyard grass by extract of barley in comparison to oat extract might be correlated to the variations in entire quantity as well as physicochemical features of allelochemicals synthesized by the mentioned cereal crops (Burgos et al., 1999; Burgos and Talbert, 2000; Chon and Kim, 2004). Wheat and rye have been well acknowledged for their suppressive effect on parameters like germination as well as radicle elongation of numerous plant species using liberating benzoxazinoid (Geddes et al., 2015). Hydroxamic acids are considered a well-known defensive chemical substance of Poaceae and are distinguished for their diversity of tasks in pest as well as disease resistance in addition to their display of allelopathic potential against a variety of plant species (Niemeyer, 1988; Chiapusio et al., 1997). Besides the trimming down growth parameters, yield, and nodulation percentage was also considerably hindered by the presence of allelochemicals in the aqueous shoot extracts of *Imperata cylindrical* (Afzal et al., 2000). Alteration in the plant growth regulators level or even bringing on disproportions in different phytohormones bring about inhibition of the growth as well as developmental parameters of plants by many allelochemicals like extensive stimulation of IAA oxidase activity in barnyard grass along with subsequent reduction of IAA contents and thus inhibition of the growth regulation system and thereby reducing seedling growth was monitored using aqueous rice extract (Lin et al., 2001). The germination, as well as growth parameters of barnyard grass (*Echinochloa crusgalli* L.), bristly foxtail (*Setaria verticillata* L.) and corn (*Zea mays* L.), was studied under the influence of three rye populations, six triticale and two barley cultivars which were used as cover crops. The study revealed that all the extracts of cereal crops resulted in a reduction of seed germination as well as growth parameters of barnyard grass and bristly foxtail,

but, without showing any impact on corn. The outcome of the research also advocated that certain winter cereals like barley cultivar Athinaida might be utilized as cover crops for weed suppression in case of corn cultivation. Moreover, minimization of herbicide applications can also be achieved as a consequence (Dhima et al., 2006).

Cellular basis of allelopathy:

Cellular shape and structure reveal visible effects of the action of allelochemicals. A study on pollen extract from corn causes more than 50% reduction in mitotic activity, abnormalities in nuclear features and pyknosis in watermelon (*Citrullus lanatus* var. *lanatus*) besides growth inhibition in radicle as well as hypocotyl part (Cruzet al., 1998). Interactions with Hordenine and Gramine of barley (*Hordeum vulgare*) root system with white mustard (*Sinapis alba* L.) results in the development of signs of injured cell wall of radicle apex along with the augmented size as well as quantity of vacuoles, triggering organelle disorganization as well as cellular autophagy (Liu and Lovett, 1993). Citral is considered an essential oil from lemongrass (Cymbopogon citrates) and other aromatic plant species proposed to possess allelopathic behaviour (Dudai et al., 1999). Citral can result in microtubular disruption in wheat and *Arabidopsis thaliana* L. Roots with a stronger influence on the mitotic microtubule compared to cortical microtubules (Chaimovitsh et al., 2012). Additionally, it illustrates a prominent and extensively prevalent consequence of disorganization on cellular ultra-structure of plantlets of *Arabidopsis thaliana*, thickening of cell wall other than a drop of cell-to-cell communication as well as root hair development (Grana et al., 2013). Mitotic cell cycle progression is hampered by BOA, mainly the cell cycle checkpoint G2-M in lettuce (Sánchez-Moreiras et al., 200). Furthermore, sorgoleone decreased cell numbers in every cell division period, tubulin damage and the development of polyploidy in the nucleus (Hallak et al., 1999). Allelopathic substances of rye BOA and DIBOA revealed notable inhibition regarding cellular regeneration in the root cap of cucumber with the subsequent hindrance of growth (Burgos et al., 2004).

Biochemical basis of allelopathy:

Much research revealed that allelochemicals substantially obstruct the actions of antioxidant enzymes along with amplified free radical levels, determining superior membrane lipid peroxidation and altered membrane potential. This causes a diminished scavenging activity on activated oxygen with a damaged membrane system (Lin et al., 2000; Zeng et al., 2001; Harun et al., 2014; Sunmonu and Vanstaden, 2014). The growth and development of wild barley, wild oat, and wild mustard plantlets were restricted by aqueous barley extract from aerial components by means of enhancement of peroxidation of lipids (Farhoudi et al., 2012; Farhoudi and Lee, 2013; Sinha et al., 2024). Elevated essential oil concentration of lemongrass leaf extensively reduces chlorophylls as well as carotenoid contents of barnyard grass along with an influence on the action of α-amylase enzyme of seeds, denoting interference of essential oil in the process of photosynthesis (Poonpaiboonpipat et al., 2013). Sorgoleone was capable of inhibiting decline in variable fluorescence, blocking the oxidation process and inhibiting photochemical effects

(Gonzalez et al., 1997). According to rye and wheat, crop rotation lessened cotton's growth and developmental parameters, and the actual cause of this inhibition was rye allelopathy (Allen et al., 2012). Therefore, it should be of prime importance for a producer to opt for a cover crop with a thorough understanding of the harmful allelopathic effects that it might exert on the crops of interest to the farmer. *Sorghum* germplasm screening revealed a significant variation in synthesising important allelochemical sorgoleone among 25 *Sorghum* genotypes with different concentration ranges (Nimbal et al., 1996). Further research reported variations in the volume of root exudates from seven *Sorghum* accessions from fresh root weight with lower most and maximum intensities were seen in the two weed types and five cultured types, respectively (Czarnota et al., 2003). These outcomes support the fact that variation in allelopathic potential is a noticeable phenomenon and decreased in cultured genome attributable to various agronomic traits selection, for instance, production or output (Bertholdsson, 2004). Monitoring of 65 barley accessions established the presence of a significant difference in allelopathic action in lettuce (Lin et al., 2005). The knowledge about the genetic basis of allelochemical generation is vital since biosynthesis, as well as the phytotoxic action of allelopathic chemical substances is the foundation for the allelopathic potential of the plant species. Enumerations of allelochemicals can indicate the suppressive ability of the test plant species, but the best directions for study are chemical analysis, the synchronized research of germplasm by bioassays, and experiments (Wu et al., 2005; Adhikari et al., 2024).

Effects on Agroecosystems:

As a result of issues with soil sickness and the fact that certain plant species are incompatible with one another, agriculturalists have been forced to implement crop rotation practices that involve a variety of plant species and legumes. Self-incompatibility is typically brought on by a number of different reasons, including weeds, pests, nutrients, water supplies, and poisonous compounds, among others (Bhattacharya et al., 2020). It appears to be challenging to measure all of these aspects.

Conclusion and Future Perspective:

The present study on the allelopathic potential of the Poaceae family members revealed a high allelopathic potential characterized by a wide range of allelochemicals. The family Poaceae has been studied extensively by various authors, who have identified a range of allelochemicals, including phenolic compounds, hydroxamic acids, alkaloids, and other secondary metabolites. Allelochemicals have both inhibitory and stimulatory effects on plants following various physiological processes. They also act as a natural weed killer. Therefore, the allelopathic mechanism among plants could be used as an alternative to herbicides and pesticides for a pollution-free environment.

The allelopathic potential of the Poaceae family holds significant future perspectives, particularly in sustainable agriculture and weed management. Although the number of studies on allelopathy has increased recently, relatively few of these studies have examined the biological

characteristics and chemical composition of the Poaceae family. Allelopathy plays a significant role in explaining the interactions between invasive and cultivated plants, as well as the fluctuations in natural ecosystem composition. Developing bioherbicides from Poaceae extracts could offer a more environmentally friendly approach to weed control. Therefore, in addition to bioprospecting for allelochemicals with potential herbicidal usefulness, it is vital to understand the natural behavior of plants and manage pastures to comprehend this phenomenon. Poaceae species can enhance soil health through their root systems and the release of organic compounds. Allelopathic compounds can also affect soil microbial communities. Future research should examine how allelopathic Poaceae species influence soil biodiversity and nutrient cycling. Additionally, studies should explore the optimal cover crop species and management practices to maximize allelopathic effects while minimizing potential negative impacts on subsequent crops.

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Chapter- 3

Discovery of Rim Region Between Core and Surface of Proteins

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Keywords: Rim-region, topological pattern, interior cavity, non-bonded interaction, protein compaction

Abstract:

The crystal structure reveals the complexities of protein component organization from the core to the surface. In general, the existence of core and surface in protein structure is well known. Here, we raise the question of how this core and surface, respectively, end and begin. To further understand the concerns, we did a comprehensive investigation on high-resolution structures of three protein families [ADH (Alcohol Dehydrogenase), Glyceraldehyde 3-Phosphate Dehydrogenase (GDH), and Malate Dehydrogenase (MDH)] from various domains of life utilizing authentic methods. The results demonstrate the presence of a zone (designated as the rim), which has separate properties from the core and the surface. The Kyte Doolittle grand average hydrophobicity (KD) of the core, surface, and rim are positive, negative, and neutral, respectively. Compared to the rim-zone, the core and surface have more hydrophobic residues and beta-strand structure, and charged residues and coil structure, respectively. In terms of polar residues and helix structure, compared to the rim, the core and surface have essentially similar but lower contents. The core's long β-sheets and shell-water-filled cavities may restrict residue compaction in this location. These analyses, thus, demonstrate that the archaea employed a distinct approach to fine-tune the compaction of their core compared to the bacteria and eukaryotes. Simultaneously, the dominance of the coil at the surface appears to produce a similar result. Overall, our study provides evidence that the rim-zone has a very distinct structure from the core and surface. The study that applies to other proteins finds application in protein structure bioinformatics.

Introduction:

The crystal structure reveals how the codes in a protein's primary sequence are expressed in the functional state of the structure. It is really interesting to know the pattern that may exist in such a complex structure (Kendrew et al., 1958; Dill and MacCallum, 2012; Rovers& Schapira, 2022). Orthologous proteins from Archaea (ar), Bacteria (ba), and Eukaryotes (eu) have comparable topologies and biological functions despite differing in basic sequences (Bandyopadhyay et al., 2007; Bottini et al., 2013; Bandyopadhyay et al., 2019; Flower et al., 2000; Petitjean et al., 2015). In true solvent conditions, the primary sequence generates the tertiary structure through weak forces and intermediate secondary structures (Anfinsen, 1973; Dill, 1990; Bandyopadhyay et al., 2020). In terms of protein structure, amino acid residues are preferentially arranged from the core to the surface. The core and surface of a globular protein are typically enriched with hydrophobic (A, V, F, I, L, M, C) and hydrophilic residues,

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respectively (Lim, 1974; Rose et al., 1985; Stollar& Smith, 2020). Hydrophilic amino acids are classified into two types: ionizable (D, E, H, R, K) and non-ionizable (S, T, N, Q, Y, W) (Betts & Russell, 2003). Ser and charged residues are particularly important in determining protein solubility (Trevino et al., 2007). These hydrophobic and charged residues, in particular, tend to form helix and strand configurations (Williams et al., 1987). Aside from amino acid residues, SW plays an essential role in globular protein stability and specificity (Finney, 1987). Although there is no SW in the globular protein's core, SWs can be found in the inner cavity, which is generated by the interaction of several protein atoms (Ernst et al., 1995). These SWs are found inside and on the cavity's entry face, but the first type has a significantly larger interaction multiplicity (Bandyopadhyay et al., 2019). It would be interesting to learn more about the atomic composition, SW content, and region-specific distribution of these cavities. SW and protein atoms interact by non-covalent interactions such as hydrogen bonding, electrostatic (ion-pair, cation-π, anion-π), and hydrophobic (alkyl-alkyl, π-π, π-σ, etc.) to contribute to stability (Baker & Hubbard, 1984; Mecozzi et al., 1996; Martinez & Iverson, 2012; Islam et al., 2019; Surti et al., 2020). There were structural differences between the protein's core and surface. This region of accessibility is known as the in-between or rim (Lins et al., 2003; Sen Gupta et al., 2017). Alcohol dehydrogenase (ADH), glyceraldehyde-3-phosphate dehydrogenase (GDH), and malate dehydrogenase (MDH) are oxydoreductases that use NAD+ as a cofactor to transform an alcoholic substrate into an oxidized product (Williamson & Paquin, 1987; Soukri et al., 1989; Song et al., 1990; Gutheil et al., 1992; Madern, 2002; Ceccarelli et al., 2004; Xiao et al., 2023). All of these enzyme types create multimeric structural complexes to enhance NAD+ and substrate binding at the interface (Song et al., 1999; Dalhus et al., 2002; Moon et al., 2011). In the case of these three protein families, the structural database is extensive. There is little known about the rim region's structure and interactions. A comparative analysis of the distributions and non-bonded interactions of amino acid residues, residue classes, secondary structures, cavities, and SWs in these three accessible zones appears to be useful.

In the current study, we addressed and provided evidence on the aforementioned concerns using representative crystal structures of three protein families from each of the three domains of life. We have done this analysis by using our and others' authentic automated procedures. Our study, based on extensive studies of three protein structures, demonstrates that the rim region exists between the core and the surface. We hypothesize that this rim region acts as a container and carrier of protein structure and function. We believe that this rim effect is present in nearly all structures. Tour study finds application in the field of protein bioinformatics.

Materials and Methods

Retrieval of Dataset

The 3D structures (Table 1) of ADH, GDH, and MDH of different domains of life (ar, ba, and eu) were procured from the Research Collaboratory for Structural Bioinformatics (RCSB) protein data bank (PDB) (Berman et al., 2000). Each of the structures was minimized using Autominv1.0 (Islam et al., 2018) in the presence of SW interacting with protein atoms within

4.0Å distance as in our earlier studies (Nayek et al., 2014; Biswas et al., 2020; Banerjee et al., 2021; Roy et al., 2024).

Table 1. Details on protein structures used in the study.

ADH			GDH			MDH		
ar	**ba**	**eu**	**ar**	**ba**	**eu**	**ar**	**ba**	**eu**
1h2b [16]	1rjw [15]	1adb [17]	1b7g [16]	1euh [28]	1dss [23]	1o6z [13]	1b8p [22]	1civ [30]
1jvb [15]	3ox4 [23]	1b15 [17]	1cf2 [9]	1gad [19]	1j0x [21]	1v9n [20]	1bdm [21]	1mld [18]
1rhc [22]	3uog [16]	1cdo [18]	1uxt [39]	1gd1 [19]	1k3t [22]	2d4a [17]	1emd [26]	1sev [18]
2eer [13]	4cpd [23]	1d1t [16]	2czc [12]	1obf [17]	1vsu [26]	2x0i [16]	1guz [6]	2dfd [18]
2h6e [16]	4eez [15]	1ee2 [17]	2yyy [28]	2d2i [14]	1ywg [18]	4bgu [18]	1gv1 [15]	2fn7 [21]
4jbg [15]	4gkv [10]	1ht0 [21]		2g82 [11]	2i5p [22]	4bgv [8]	1ur5 [17]	2g76 [9]
6c75 [32]	4j6f [18]	1mc5 [14]		3gnq [19]	2vyn [20]	6ihd [23]	1z2i [24]	2hjr [11]
	4z6k [13]	1mg5 [17]		4dib [17]	3cps [23]		3d5t [21]	2i6t [17]
	5yln [11]	1u3t [14]		5ld5 [19]	3pym [24]		3fi9 [16]	3i0p [20]
		3wle [26]		5utm [18]	3qv1 [18]		3flk [18]	4h7p [15]
		4jji [19]		6fzh [18]	3sth [24]		3gvh [19]	4mdh [25]
		4rqt [16]			4k9d [19]		3nep [7]	4plh [14]
		4w6z [20]			4lsm [14]		3p7m [12]	4plt [20]
		5ilg [12]			5c7i [18]		3tl2 [16]	4uuo [19]
					5tso [15]		4e0b [19]	5nue [19]
							4ror [13]	5zi2 [17]
							4tvo [15]	6um4 [11]
							5ujk [20]	7mdh [21]
							6aoo [19]	
							6bal	

							[20]	
							6itk [19]	
							6itl [14]	

Protein data bank files of Alcohol Dehydrogenase (ADH), Glyceraldehyde 3-Phosphate Dehydrogenase (GDH), and Malate Dehydrogenase (MDH) from archaea (ar), bacteria (ba) and eukaryote (eu) domains. Only the first chain was processed in each case. Each of these structures was minimized in the presence of shell-water interacting with protein atoms within 4.0Å distance. The value of the third bracket indicates the number of cavities in this protein.

Protein residue accessibility (ASA) and accessibility regions

We used the novel Surface Racer v5.0 program (Tsodikov et al., 2002; Banerjee et al., 2018) to extract the accessibility and cavity details of the protein atom. Absolute accessibility of protein atoms has been converted into relative accessibility of residue. In this case, N, CA, C, and O are considered as the main-chain and the rest of the atoms of a residue are taken as side-chain.The relative accessibility of the residue's main-chain, side-chain, and all-atoms was determined by the absolute value of their folded (Tsodikov et al., 2002) and unfolded (Zielenkiewicz & Saenger, 1992) states as earlier (Bandyopadhyay et al., 2019). Based on overall KD value, the accessibility of proteins was divided into three regions namely core (ASA≤15Å^2), rim (ASA>15 Å^2 and ≤35 Å^2), and surface (ASA>35 Å^2) (Sen Gupta et al., 2017; Banerjee et al., 2018).

Details on the residue, cavity, shell-water, and non-covalent interactions

Relative to these three regions (core, rim, and surface), domains of life (ar, ba, eu) specific, protein-family (ADH, GDH, MDH) specific structures were compared to the average properties of protein residue composition, residue-class composition, Kyte-Doolittle hydrophobicity (Kyte & Doolittle, 1982; Islam et al., 2018), secondary structure type (Helix/Strand/Coil) (Islam et al., 2018), cavity details (Sen Gupta et al., 2017), non-bonded interactions (Islam et al., 2019), SW and its interactions, etc. The composition, secondary-structure details, accessibility-details of cavity residues, and their association and interaction with SWs were also extracted for comparison. Residues, A, V, L, I, F, M, C, P, G, residues, S, T, N, Q, Y, W and residues, H, R, K, E, D were taken as hydrophobic, hydrophilic, and charged types, respectively (Betts & Russell, 2012). The secondary structure details of the amino acid residue were taken from the crystal structure.

Protein residue and shell-water interaction

Biovia Discovery Studio 2020 was used to determine all atomic level non-bonded interactions that include hydrogen bond, ion-pair, π-π, π-cation, π-anion, π-amide, π-sigma, π-alkyl, and alkyl-alkyl, etc. for the above-mentioned accessibility regions of a protein. These

interactions were processed using an automated AWK script to obtain different inter-residue interactions (Islam et al., 2018; Mitra et al., 2019).

Automated extraction of accessibility (ASA) region-specific details

Our database has 108 protein PDB structures in it. Thus, the total number of proteins to analyze is 324, as each protein has three accessibility (ASA) regions. We used a fully automated AWK script (that makes use of the Surface Racer program to determine accessibility regions and cavities) to properly and accurately extract the properties of all the items mentioned above (Nayek et al., 2014; Gupta et al., 2014a; Gupta et al., 2014b; Nayek et al., 2015a; Nayek et al., 2015b; Banerjee et al., 2015). The script runs in a Cygwin environment in a user-friendly and error-free manner. It takes all PDB structures as an input that is present in the working directory. Outputs are redirected in Excel format for the ease of post-run analyses. The binary version of the script can be obtained upon request.

Statistical Analysis

All statistical tests were performed using paired t-test if not stated otherwise. P-value, thus obtained, was used to judge against the null hypothesis at the 5% level using the standard two-tailed procedure. The *p*-value is included with the relevant data in the results (Banerjee et al., 2021; Roy et al., 2023).

Results

Residue, residue-class, and secondary structure distribution in co, rm, and su zones

The primary goal of our research is to understand the topological characteristics and patterns of enzymes as they work in various earths' conditions (Bandyopadhyay & Sonawat, 2000; Gupta et al., 2015). We investigated crystal structures from ADH, GDH, and MDH enzymes (Table 1). A typical crystal structure shows how the co, rm, and su residues and a typical cavity are arranged (Figure 1a). The properties of archaea's primary sequence differ from those of ba and eu (Figure 1b and Figure 2a-h). The overall KD (GRAVY) of co, rm and su are positive, neutral and negative, respectively (Figure 1c for MDH's ba and Figure 3a-c for ADH's ar, ba, and eu; d-f for GDH's ar, ba, and eu; g-h for MDH's ar and eu, respectively). Further, co and su regions are significantly abundant in hb and cr residue classes, respectively. Again, these two regions have in common the abundance of po class (Figure 1d, e). These observations of ba MDH's are also true in other domains of life specific enzyme classes (Figure 4a-c for ADH's ar, ba and eu; d-f for GDH's ar, ba and eu; and g-h for MDH'a ar and eu; and Figure 5a-c for ar ADH's hb, hl, and cr; d-f for ba ADH's hb, hl, and cr; g-i for eu ADH's hb, hl, and cr; j-l for archaeal GDH's hb, hl, and cr; Figure **6**a-c for ba GDH's hb, hl, and cr; d-f for eu GDH's hb, hl, and cr; g-i for archaeal MDH's hb, hl and cr and j-k for eu MDH's hb, hl, and cr, respectively). Notably, for rm-region, which is between the co and su, these classes of amino acids are negligible. Relative to this region, these features of the amino acid compositions and classes of the co and the su regions are statistically significant.

The secondary structure, which is between the primary and tertiary structures, determines the topology and conformation of enzyme classes (Bandyopadhyay et al., 2001). Here, we raise the question as to how these secondary structures are distributed in these regions (core, rim and surface). Here, we see that when normalized, and average helix-content is almost equivalent to co and su, and much higher than that of rm. The strand and coil, in the core and surface are uniquely, and significantly dominating over the rim region, respectively (Figure 1f for ba MDH's helix, strand and coil; and Figure 7a-c for archaeal ADH's helix, strand, and coil; d-f for ADH's ba helix, strand, and coil; g-i for ADH's eu helix, strand and coil; j-l for GDH's archaeal helix, strand, and coil; Figure 8a-c for GDH's ba helix, strand and coil; d-f for GDH's eu helix, strand, and coil; g-i for archaeal helix, strand and coil and j-l for MDH's eu helix, strand, and coil, respectively). Here, it is important to mention that although the accessibility region-specific differential pattern in amino acid compositions, residue classes, and secondary structures described above are true and statistically significant in all domains of life and enzyme classes, their normalized scales show domains of life specific and also enzyme class specific variation (Table 2 and 3). It should be noted here that although the rm's preference for the protein structure components is low, this region has a strong preference towards shell-water (see below).

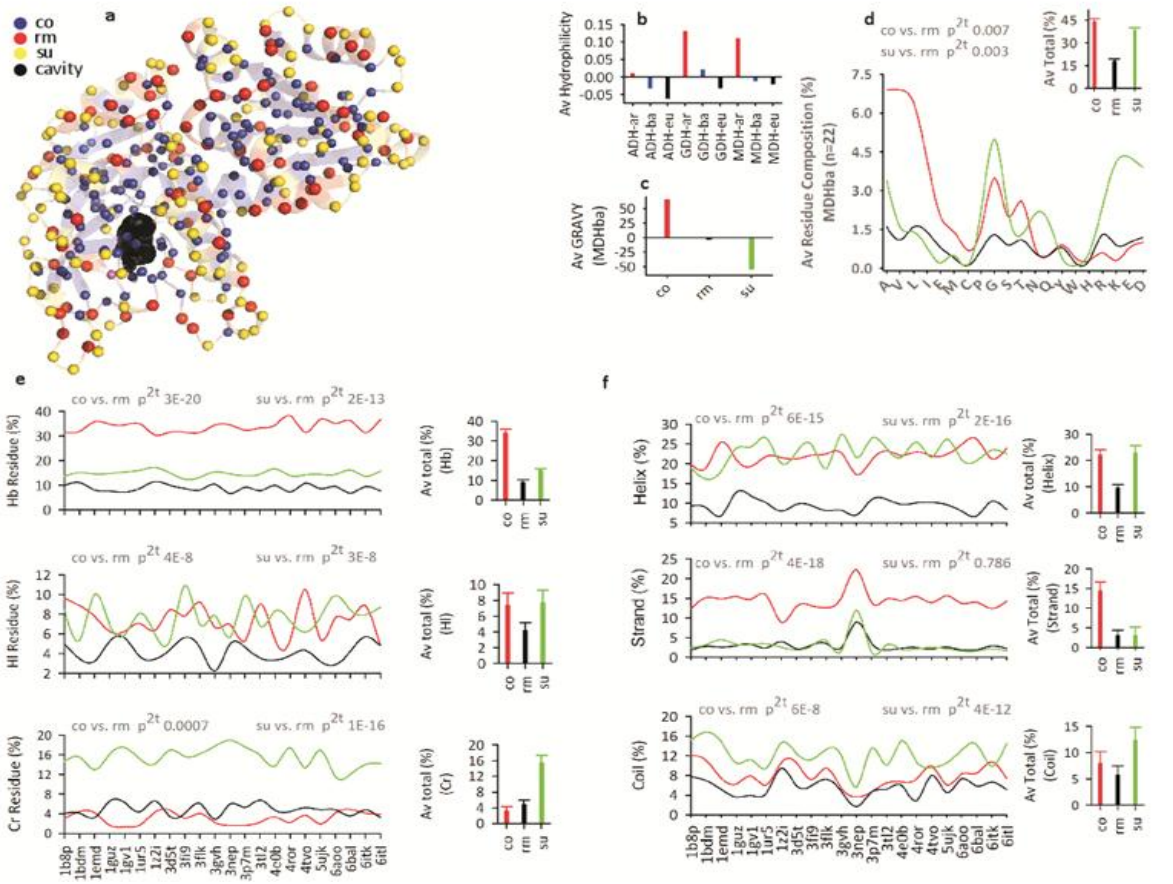


Figure 1. Details on residue composition, residue-class composition and secondary structure types.

Variation of amino acid composition, class and secondary structure in different accessibility regions along with sequence hydrophobicity relative to domains of life. **a**, A general presentation of a crystal structure where the residues (Cα) in co (blue), rm (red) and su (yellow) are shown with a typical cavity (black). **b**, Av KD of sequences in ar, ba, and eu. **c**, Av KD (n=22 PDBs) of ba MDH's co, rm and su regions. **d**, The average residue composition in the co (red), rm (black) and su (green) regions are shown with *p*-values (p^{2t}) for test of significance in co vs. rm, and su vs. rm format. **e**, The average residue class (hb, hl and cr) composition in the co (red), rm (black) and su (green) regions with *p*-values. **f**, The average secondary structure type in the co (red), rm (black) and su (green) regions with *p*-values. In each case, the total value of each of the three regions is shown as inset.

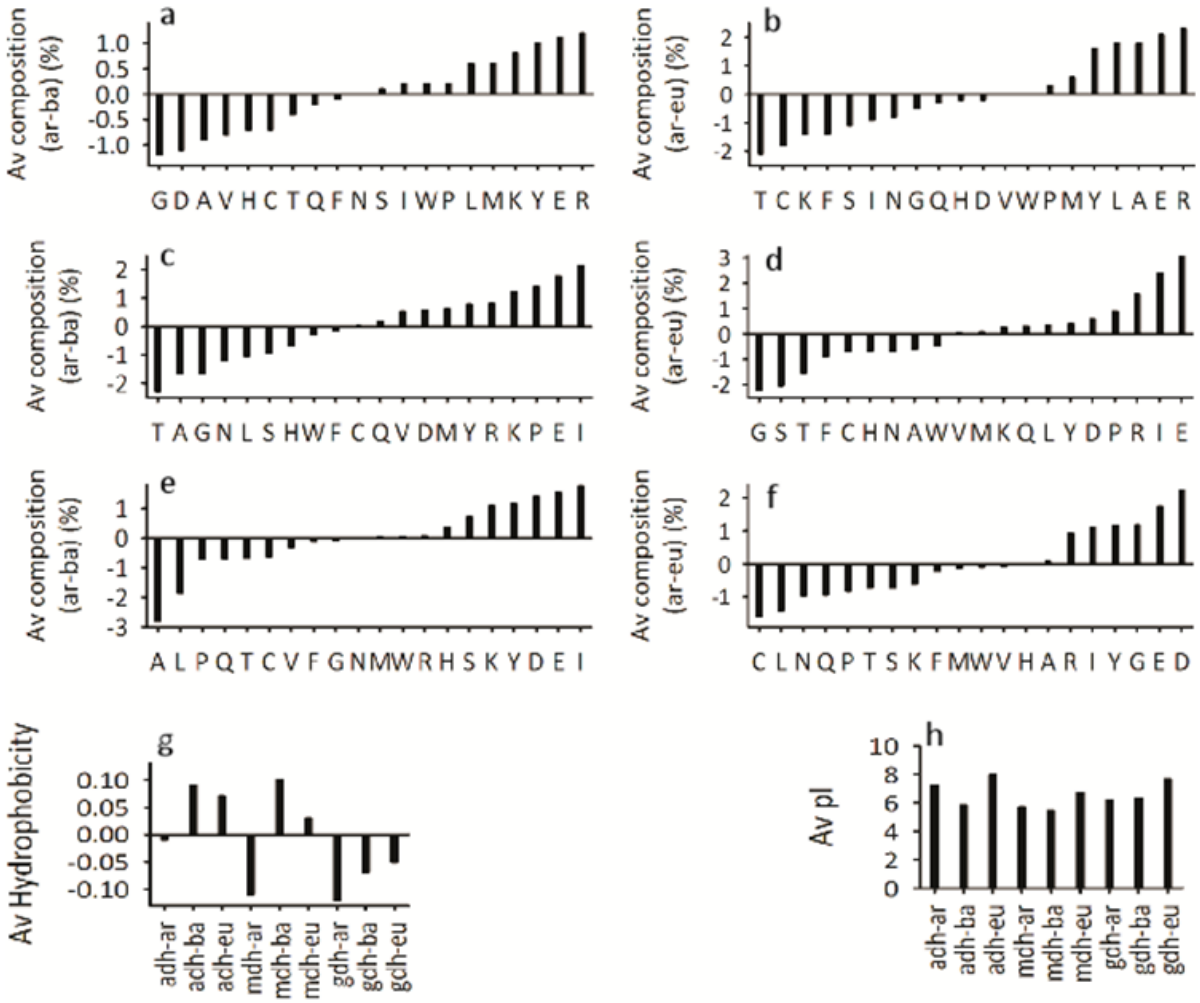


Figure 2. Relative residue compositions of archaeal proteins.

Average (av) normalized (%) relative composition of ar in reference to ba, and eu for ADH (**a** and **b**), GDH (**c** and **d**), and MDH (**e** and **f**), respectively. Grand average Kyte-Doolittle hydrophobicity of AD, GDH and MDH for ar ba and eu (**g**). Average pI value ADH, GDH and MDH for ar ba and eu (**h**).

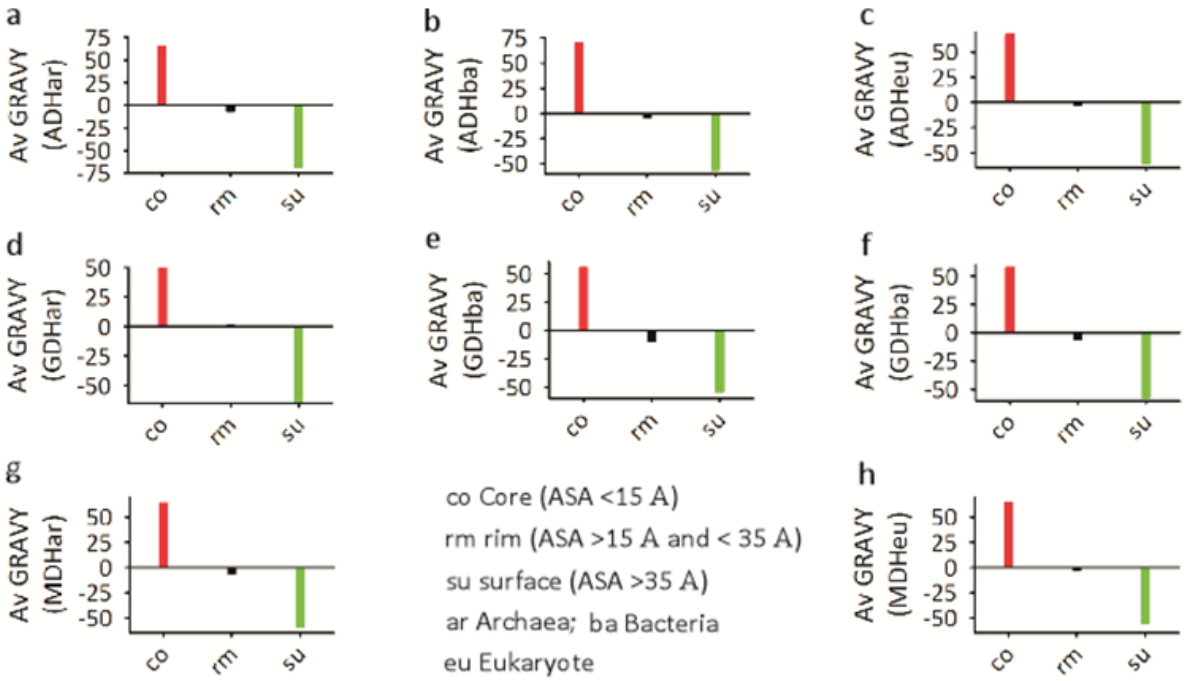


Figure 3. Details on ASA-region-specific GRAVY of proteins in our dataset.

Kyte-Doolittle overall hydrophobilicy (GRAVY) for the co, rm and su regions of ar (**a**), ba (**b**) and eu (**c**) enzyme, ADH. Similarly the GRAVY of co, rm and su regions of ar (**d**), ba (**e**) and eu (**f**) enzyme, GDH and ar (**g**), and eu (**h**) enzyme, MDH.

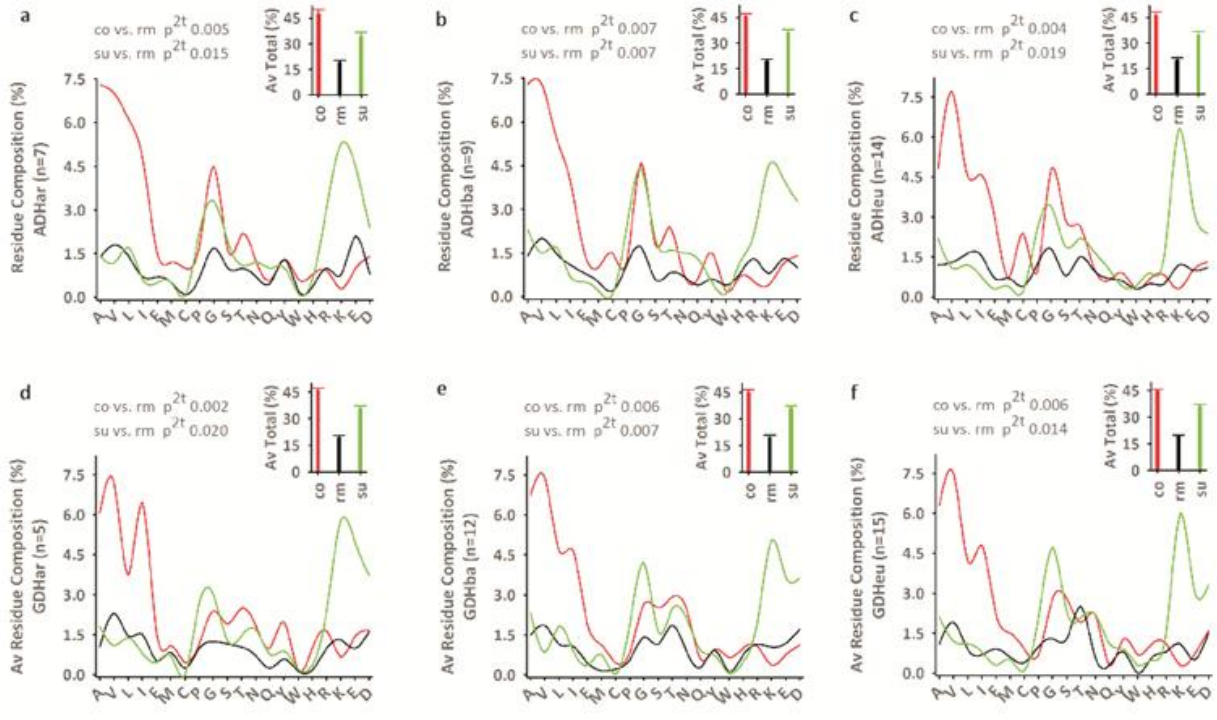
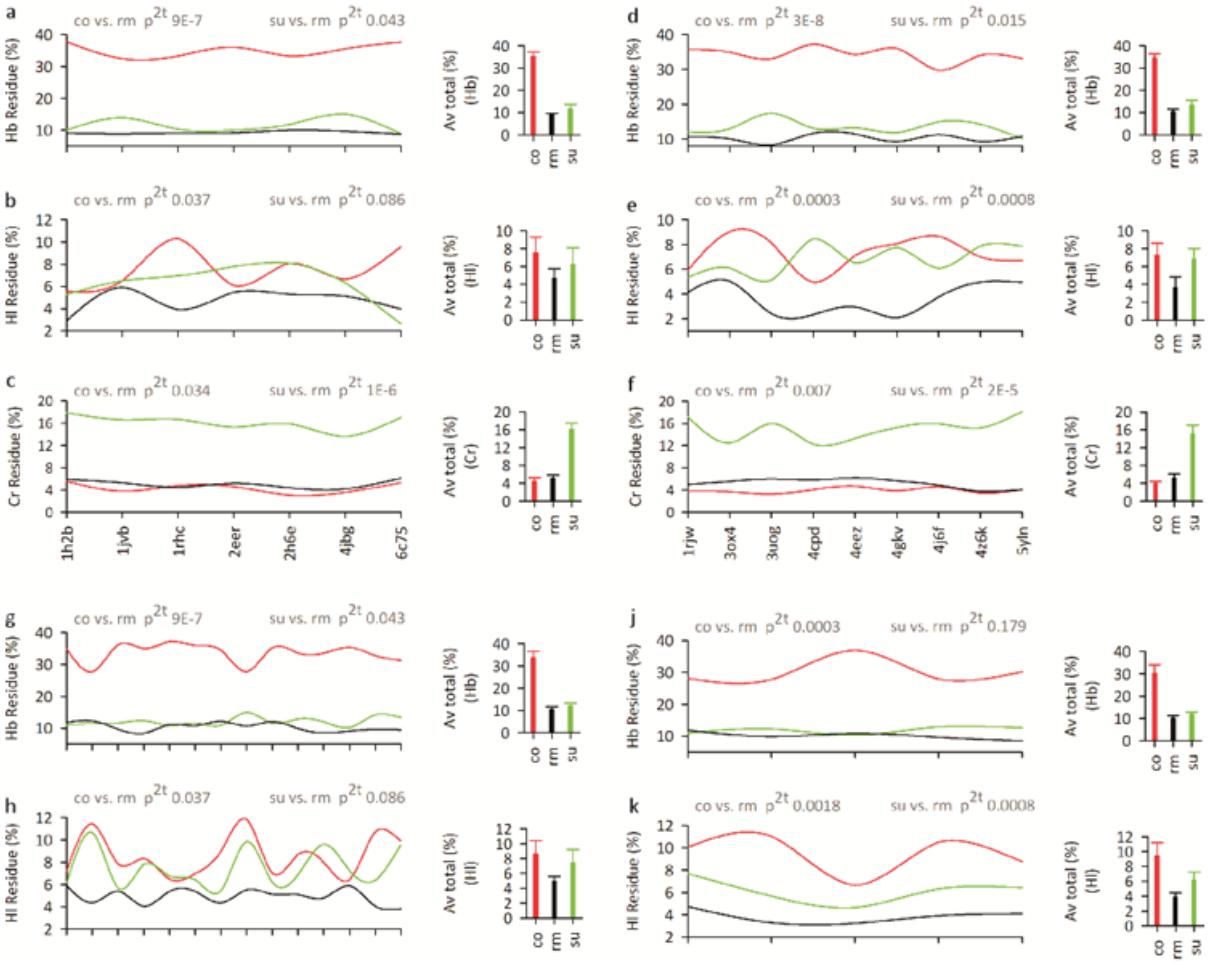


Figure 4. ASA-region-specific normalized average residue compositions of proteins of our dataset.

Average (av) normalized (%) amino acid residue composition of core (co, red), rim (rm, black) and surface (su, green) for ADH (**a** for ar, **b** for ba and **c** for eu), GDH (**d** for ar, **e** for ba and **f** for eu) and MDH (**g** for ar, **h** for eu). Individual and total values of these three regions are shown in each case. Significance of the difference of mean observation is tested using paired t-test using two tailed procedures.



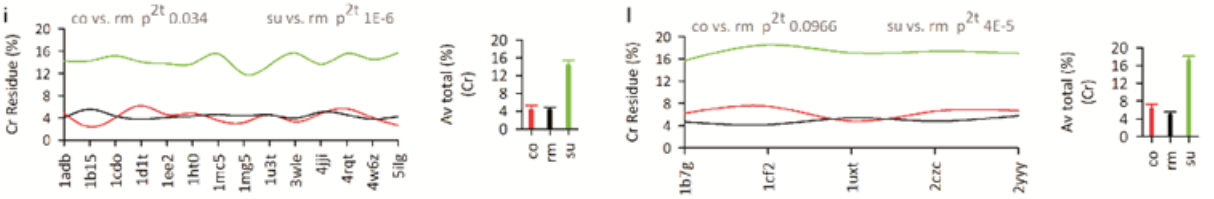


Figure 5. ASA-region-specific normalized average residue class compositions of proteins of our dataset.

Average (av) normalized (%) amino acid classes composition of core (co, red), rim (rm, black) and surface (su, green) regions of ar's ADH (**a,** hb; **b,** hl; **c,** cr), ba's ADH (**d,** hb; **e,** hl; **f,** cr), eu's ADH (**g,** hb; **h,** hl; **i,** cr) and ar's GDH (**j,** hb; **k,** hl; **l,** cr). The region-specific average plots of all the proteins are shown as bars on the side of each protein-specific profile plot. The significance of the difference of mean observation is tested using a paired t-test using a tailed procedure.

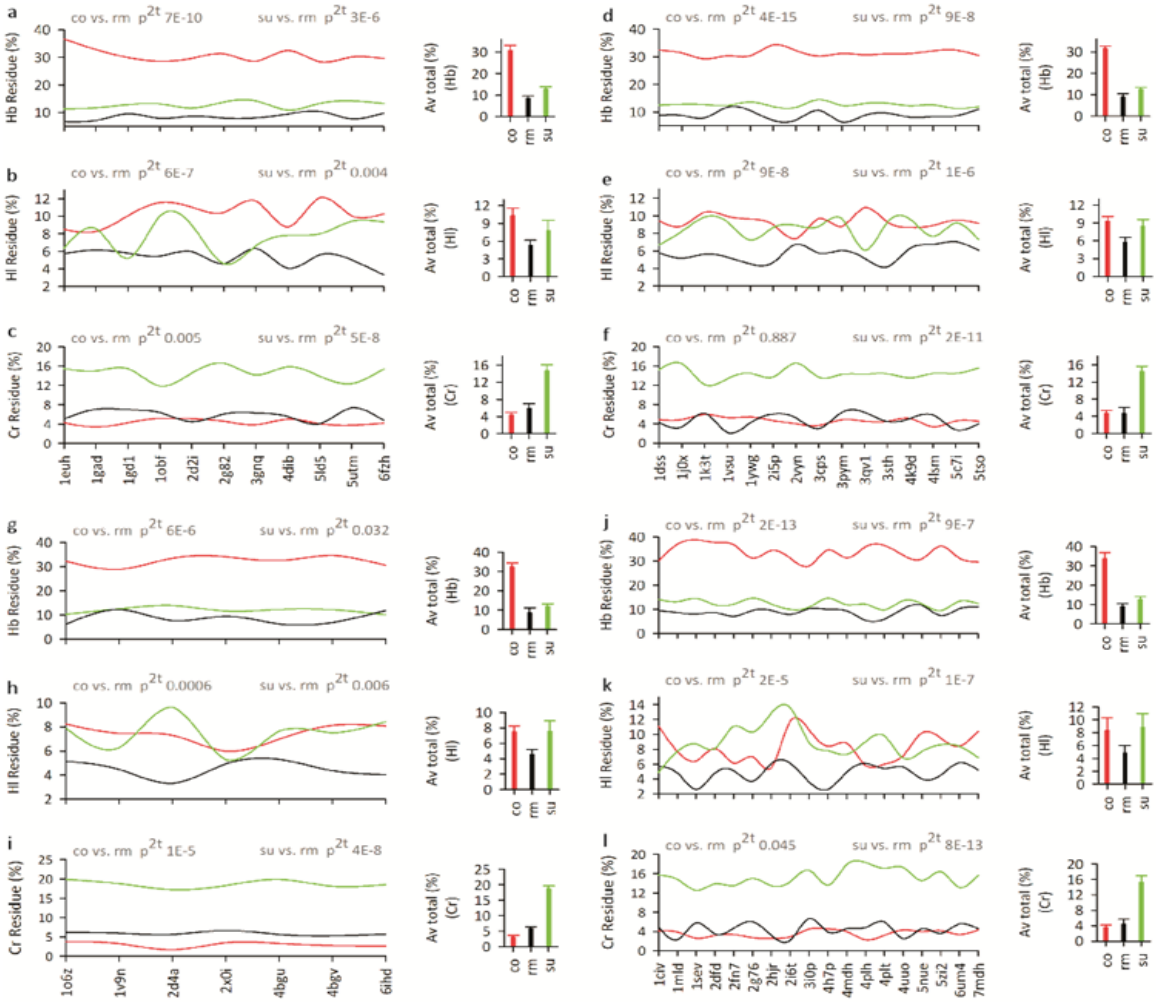


Figure 6. ASA-region-specific normalized average residue class compositions of proteins of our dataset (continued).

Average (av) normalized (%) amino acid classes composition of core (co, red), rim (rm, black) and surface (su, green) regions of ba's GDH (**a,** hb; **b,** hl; **c,** cr), eu's GDH (**d,** hb; **e,** hl; **f,**

cr), ar's MDH (**g,** hb; **h,** hl; **i,** cr) and eu's MDH (**j,** hb; **k,** hl; **l,** cr). The region-specific average plots of all the proteins are shown as bars on the side of each protein-specific profile plot. The significance of the difference of mean observation is tested using a paired t-test using a tailed procedure.

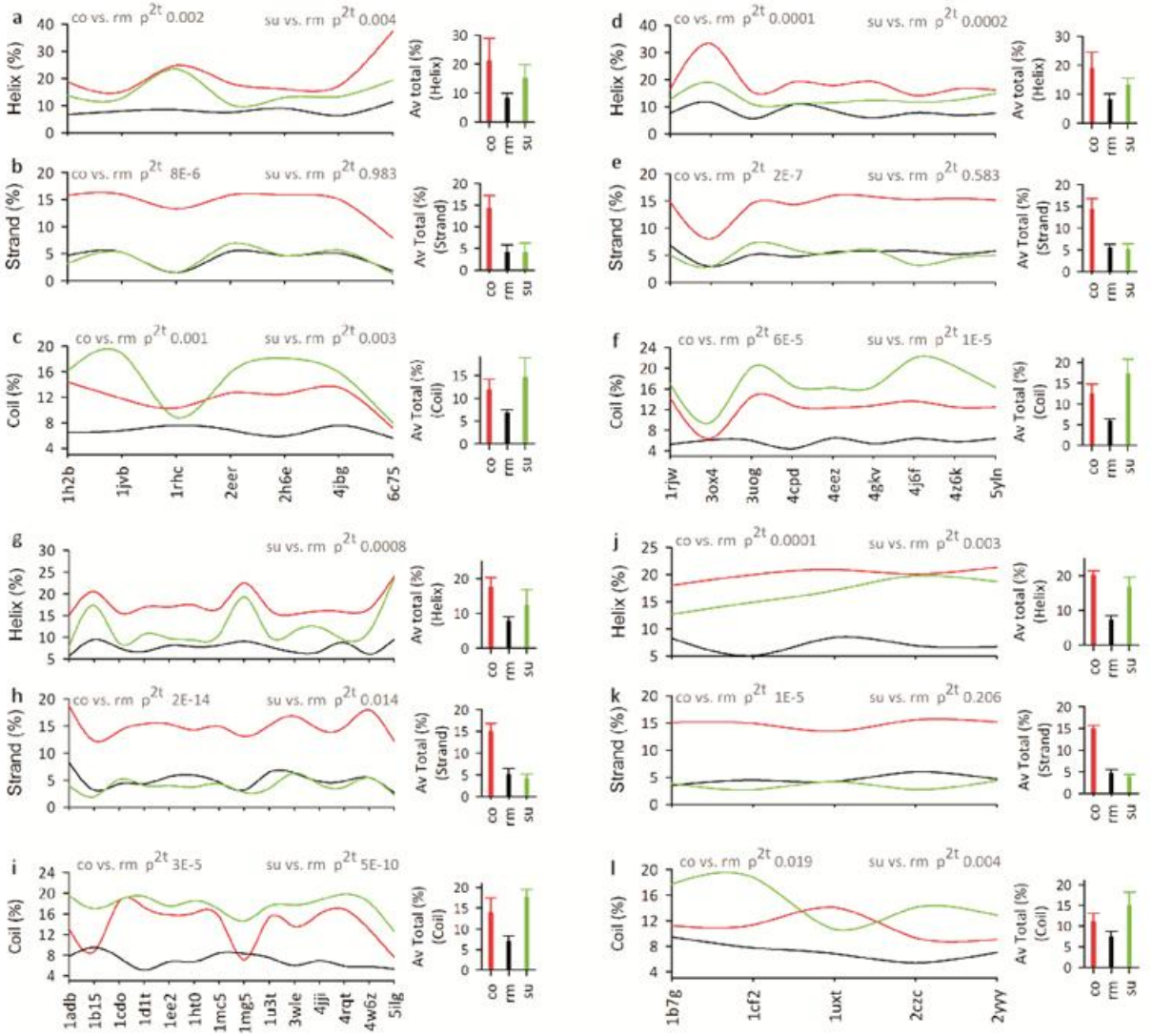


Figure 7. ASA-region-specific normalized average secondary structure types of proteins of our dataset.

Average (av) normalized (%) secondary structure types of core (co, red), rim (rm, black) and surface (su, green) regions of ar's ADH (**a,** hb; **b,** hl; **c,** cr), ba's ADH (**d,** hb; **e,** hl; **f,** cr), eu's ADH (**g,** hb; **h,** hl; **i,** cr) and ar's GDH (**j,** hb; **k,** hl; **l,** cr). The region-specific average plots of all the proteins are shown as bars on the side of each protein-specific profile plot. The significance of the difference of mean observation is tested using a paired t-test using a tailed procedure.

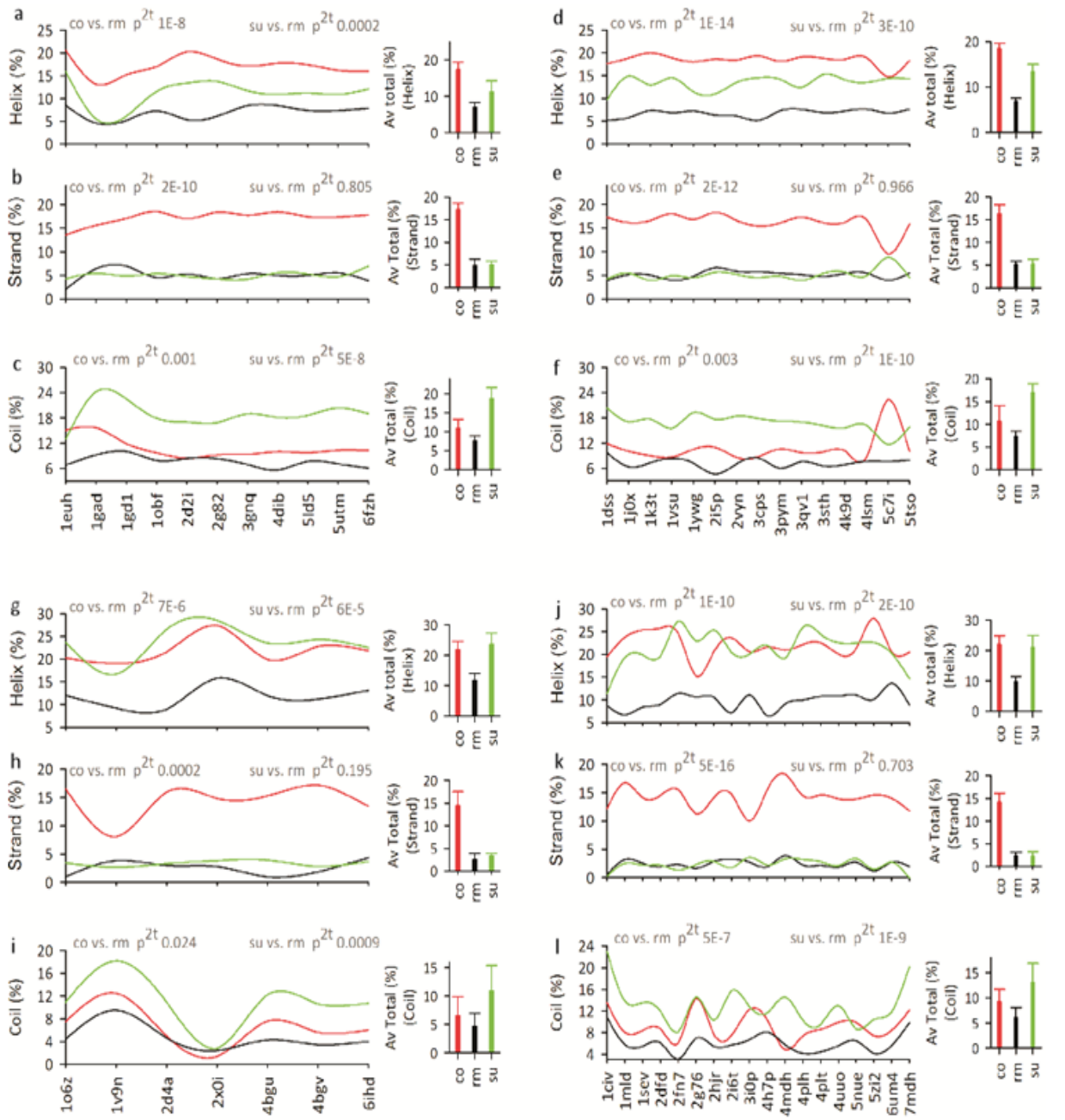


Figure 8. ASA-region-specific normalized average secondary structure types of proteins of our dataset (continued).

Average (av) normalized (%) secondary structure types of cores (co, red), rim (rm, black) and surface (su, green) regions of ba's GDH (**a,** hb; **b,** hl; **c,** cr), eu's GDH (**d,** hb; **e,** hl; **f,** cr), ar's MDH (**g,** hb; **h,** hl; **i,** cr) and eu's MDH (**j,** hb; **k,** hl; **l,** cr). The region specific average plots of all the proteins are shown as bars on the side of each protein-specific profile plot. The significance of the difference of mean observation is tested using a paired t-test using a tailed procedure.

Table 2. Domain-specific, enzyme-class-specific and ASA-specific Normalized (%) residue class.

	cohb	rmhb	suhb	cohl	rmhl	suhl	cocr	rmcr	sucr
MDH-ar	32.4	8.6	11.9	7.5	4.5	7.5	3.0	5.9	18.7
MDH-ba	33.9	8.9	14.8	7.3	4.2	7.7	3.1	4.8	15.4
MDH-eu	33.6	8.9	12.4	8.3	4.8	8.8	3.5	4.4	15.2
ADH-ar	35.2	9.2	11.5	7.5	4.7	6.2	4.4	5.1	16.1
ADH-ba	34.3	10.3	13.4	7.3	3.6	6.8	4.0	5.2	15.1
ADH-eu	33.7	10.4	12.1	8.6	4.9	7.5	4.2	4.4	14.3
GDH-ar	30.2	10.1	11.8	9.4	3.8	6.1	6.4	5.0	17.1
GDH-ba	30.7	8.4	12.7	10.3	5.3	7.7	4.4	5.9	14.6
GDH-eu	31.4	8.9	12.6	9.3	5.7	8.4	4.7	4.6	14.4

Domains of life specific, enzyme class specific and accessibility region (co, rm and su) specific percentage of residue classes such as hydrophobic (hb), hydrophilic (hl) and charged (cr).

Table 3. Domain-specific, enzyme-class-specific and ASA-specific Normalized (%) secondary structure types.

	co-h%	rm-h%	su-h%	co-s%	rm-s%	su-s%	co-c%	rm-c%	su-c%
MDH-ar	21.9	11.8	23.7	14.5	2.6	3.4	6.5	4.7	11.0
MDH-ba	22.0	9.3	22.6	14.4	2.9	3.0	7.9	5.6	12.2
MDH-eu	22.1	9.7	21.2	14.1	2.3	2.3	9.2	6.1	13.0
ADH-ar	21.1	8.2	15.2	14.3	4.1	4.1	11.8	6.7	14.5
ADH-ba	18.8	8.1	13.0	14.4	5.3	5.0	12.4	5.8	17.2
ADH-eu	17.6	7.7	12.3	14.9	5.0	4.0	13.9	7.0	17.6
GDH-ar	20.1	7.1	16.7	14.9	4.6	3.6	11.0	7.3	14.8
GDH-ba	17.3	7.0	11.2	17.2	5.0	5.1	10.9	7.6	18.8
GDH-eu	18.5	6.7	13.4	16.2	5.1	5.1	10.7	7.3	16.9

Domains of life specific, enzyme class specific and accessibility region (co, rm and su) specific percentage of secondary structure types such as helix (h), strand (s) and coil (c)

Table 4. Protein-specific shell-water-specific cavity details.

ADH_ar	Prot res	*fCv*	*fCv woW*	ADH_ba	Prot res	*fCv*	*fCv woW*	ADH_eu	Prot res	*fCv*	*fCv woW*
1h2b	341	16	1	1rjw	338	15	7	1adb	373	17	7
1jvb	338	15	6	3ox4	377	23	8	1b15	253	17	10
1rhc	330	22	4	3uog	332	16	4	1cdo	370	18	5
2eer	346	13	4	4cpd	343	23	8	1d1t	371	16	7
2h6e	321	16	6	4eez	338	15	2	1ee2	371	17	2

4jbg	331	15	2	4gkv	335	10	1	1ht0	372	21	0
6c75	377	32	19	4j6f	346	18	5	1mc5	368	14	2
				4z6k	343	13	1	1mg5	253	17	2
				5yln	343	11	8	1u3t	371	14	3
								3wle	333	26	3
								4jji	375	19	0
								4rqt	373	16	3
								4w6z	345	20	7
								5ilg	262	12	3

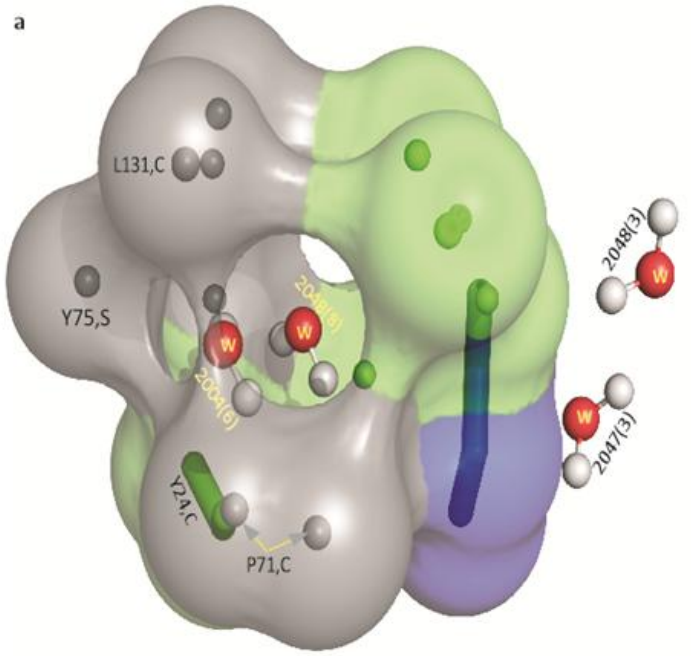
GDH_ar	Prot res	*fCv*	*fCv woW*	GDH_ba	Prot res	*fCv*	*fCv woW*	GDH_eu	Prot res	*fCv*	*fCv woW*
1b7g	338	16	6	1euh	470	28	6	1dss	329	23	6
1cf2	335	9	1	1gad	326	19	6	1j0x	328	21	12
1uxt	497	39	10	1gd1	328	19	1	1k3t	355	22	9
2czc	333	12	3	1obf	329	17	5	1vsu	322	26	10
2yyy	342	28	12	2d2i	335	14	3	1ywg	332	18	8
				2g82	326	11	3	2i5p	300	22	9
				3gnq	332	19	5	2vyn	325	20	6
				4dib	320	17	14	3cps	330	23	7
				5ld5	338	19	7	3pym	330	24	10
				5utm	339	18	3	3qv1	329	18	8
				6fzh	331	15	2	3sth	334	24	5
								4k9d	333	19	6
								4lsm	326	14	3
								5c7i	326	18	7
								5tso	328	15	4
MDH_ar	Prot res	*fCv*	*fCv woW*	MDH_ba	Prot res	*fCv*	*fCv woW*	MDH_eu	Prot res	*fCv*	*fCv woW*
1o6z	291	13	2	1b8p	322	22	5	1civ	369	30	7
1v9n	335	20	5	1bdm	316	21	5	1mld	311	18	5
2d4a	301	17	11	1emd	309	26	10	1sev	311	18	6
2x0i	284	16	12	1guz	304	6	1	2dfd	310	18	3
4bgu	301	18	4	1gv1	304	15	8	2fn7	296	21	9

4bgv	320	8	2	1ur5	297	17	2	2g76	301	9	4
6ihd	297	23	21	1z2i	348	24	7	2hjr	311	11	5
				3d5t	319	21	7	2i6t	279	17	8
				3fi9	320	16	4	3i0p	359	20	3
				3flk	346	18	6	4h7p	309	15	1
				3gvh	316	19	8	4mdh	329	25	5
				3nep	301	7	2	4plh	311	14	6
				3p7m	302	12	4	4plt	315	20	5
				3tl2	309	16	2	4uuo	323	19	16
				4e0b	299	19	5	5nue	324	19	3
				4ror	316	13	4	5zi2	336	17	3
				4tvo	323	15	0	6um4	321	11	0
				5ujk	312	20	7	7mdh	347	21	11
				6aoo	313	19	5				
				6bal	308	20	2				
				6itk	315	19	7				
				6itl	310	14	1				

Frequency of total water, total cavity and number of water-unfilled cavities in each representative protein in each of the three domains (ar, ba and eu) of each of the three classes of enzyme (ADH, GDH and MDH). Cv, cavity; *f*, frequency; wo, without; W, shell-water; freq, frequency.

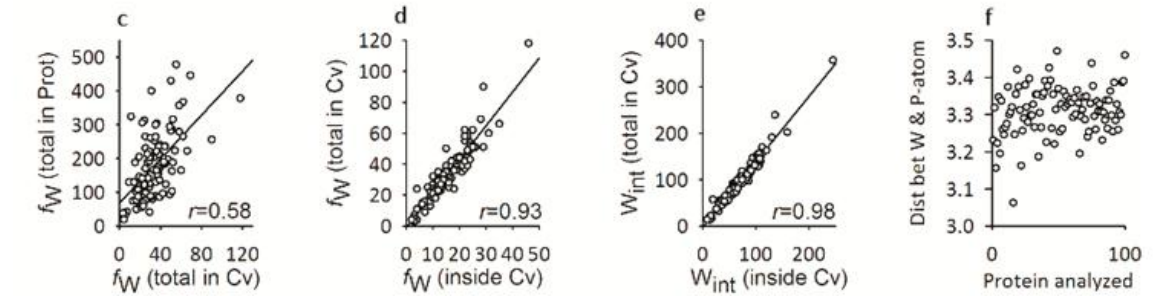
The interior cavity of the protein and its properties. (**a)** The typical cyrysy type cavity is constituted by atoms of co (grey), rm (green) and su (blue). It also has four moles of SWs. Cavity atoms in coil (C) and strand (S) are labelled and helix atoms are left unlabelled. (**b)** The grand average propensity of amino acid residues in the cavity. (**c**) Correlation between the total SW of the protein and the total SW of the cavity. (**d)** Correlation between the total SW and inside SW of the cavity. (**e)** Correlation between the total and inside interactions of the cavity atom. (**f**) Average distance of interaction of SW inside the cavity with its atoms. (**g)** Types of cavity and its frequency. (**h)** Cavity type specific water content. (**i**) Correlation between the cavity type specific frequency and their SW frequency. (**j)** Correlation between the cavity type specific frequency and their SW interaction frequency.

TW, total water in protein; Cv TW, cavity's total water; Cv Wins, cavity's inside water; Wint ins, water interaction with cavity atoms from inside; Wint T, water (inside and outside) interaction with cavity atoms; CvW:TW, total cavity water : total protein water in %; Cv_atom, protein specific total unique atoms that are present in all cavity; T_rs, total residue in protein; Cv_hT, helix% in cavity, Cv_sT, Strand% in cavity; Cv_cT, coil% in cavity; Cv_coT, cavity% in core region; Cv_rmT, cavity% in rim region; Cv_suT, cavity % in surface region; Dist, distance in Angstrom between the water and cavity atom interaction.



b

	ADH			GDH			MDH		
	ar	ba	eu	ar	ba	eu	ar	ba	eu
A	1.0	0.9	0.7	0.9	0.7	0.9	0.8	0.8	0.7
V	1.2	1.2	1.0	1.2	1.1	1.2	1.4	1.3	1.3
L	1.5	1.5	1.6	1.6	1.7	1.8	1.5	1.3	1.5
I	1.3	1.2	1.4	1.8	1.4	1.6	2.0	1.8	1.6
F	1.6	1.9	1.8	1.1	1.4	1.3	1.3	1.5	1.5
M	0.8	1.2	1.7	0.6	1.1	1.3	1.7	1.2	1.3
C	0.4	1.7	1.1	0.7	2.2	1.8	1.9	1.4	1.2
P	1.1	0.7	0.6	0.7	0.7	0.3	0.8	0.8	0.8
G	0.6	0.8	0.7	0.4	0.7	0.6	0.9	0.7	0.7
S	1.1	0.8	1.1	0.7	1.0	0.6	0.7	0.8	0.7
T	1.0	1.1	0.9	0.8	0.5	0.7	0.5	1.2	1.0
N	1.0	0.8	1.2	1.2	1.0	1.0	0.7	0.6	1.0
Q	1.4	0.9	1.1	0.8	1.2	1.4	0.5	0.7	0.6
Y	1.4	1.6	1.2	1.5	2.2	1.4	1.2	1.6	1.3
W	2.5	1.5	1.9	2.6	1.7	1.0	1.1	1.1	1.6
H	1.3	1.0	1.0	1.9	0.6	0.8	0.8	1.5	1.5
R	0.5	1.1	0.9	1.6	1.5	1.9	1.1	0.8	1.2
K	0.5	0.6	0.6	0.5	0.6	0.5	0.4	0.5	0.5
E	0.6	0.5	0.6	0.6	0.5	0.4	0.4	0.6	0.6
D	0.7	0.5	0.5	0.7	0.7	0.8	0.6	0.7	0.7



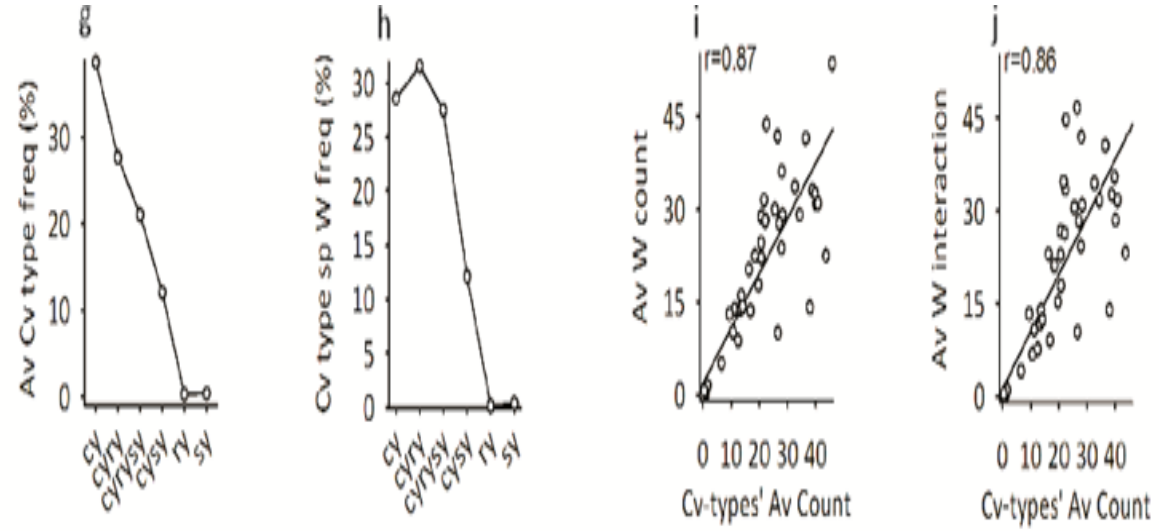


Figure 9. Cavity, its interaction and types.

Table 5. Domain-specific, enzyme-specific, cavity details.

Domain	protein	TW	Cv TW	Cv Wins	Wint ins	Wint T	CvW:TW	Cv_atom	T_rs	Cv_hT	Cv_sT	Cv_cT	Cv_coT	Cv_rmT	Cv_suT	Dist
ADH-ar	1h2b	286	51	26	108	147	17.8	131	341	44	22	65	92	19	20	3.23
	1jvb	187	27	10	33	56	14.4	154	338	50	62	42	108	27	19	3.32
	1rhc	143	37	19	90	115	25.9	155	330	74	31	50	102	33	20	3.16
	2eer	205	19	10	41	54	9.3	102	346	45	28	29	82	11	9	3.22
	2h6e	205	30	11	59	85	14.6	136	321	55	35	46	91	32	13	3.35
	4jbg	141	27	16	62	75	19.1	82	331	23	28	31	65	11	6	3.20
	6c75	98	25	16	86	97	25.5	221	377	168	20	33	174	24	23	3.34
GDH-ar	1b7g	265	25	8	34	53	9.4	108	338	36	36	36	79	11	18	3.26
	1cf2	231	25	12	46	65	10.8	81	335	44	14	23	70	4	7	3.25
	1uxt	256	90	29	136	239	35.2	314	497	109	79	126	263	37	14	3.27
	2czc	139	19	9	27	38	13.7	69	333	31	23	15	51	11	7	3.28
	2yyy	191	29	15	50	71	15.2	149	342	70	41	38	113	21	15	3.37
MDH-ar	1o6z	157	38	16	75	109	24.2	114	291	59	33	22	100	13	1	3.30
	1v9n	183	44	19	90	120	24	152	335	42	10	100	98	21	33	3.31
	2d4a	42	7	3	13	19	16.7	109	301	44	44	21	82	21	6	3.32
	2x0i	38	4	4	17	17	10.5	109	284	66	39	4	87	15	7	3.06
	4bgu	478	55	22	90	135	11.5	132	301	75	31	26	111	15	6	3.25
	4bgv	313	24	10	30	46	7.7	54	320	23	28	3	52	2		3.36
ADH-ba	1rjw	74	13	9	37	45	17.6	104	338	27	30	47	85	15	4	3.42
	3ox4	121	29	13	71	95	24	196	377	147	18	31	142	27	27	3.38
	3uog	85	33	16	83	111	38.8	162	332	55	55	52	141	10	11	3.31
	4cpd	60	20	10	37	55	33.3	159	343	76	34	49	132	14	13	3.16
	4eez	223	54	24	94	147	24.2	130	338	64	30	36	110	16	4	3.26
	4j6f	79	32	10	56	91	40.5	200	346	69	52	79	159	28	13	3.38
	4z6k	253	34	15	57	88	13.4	113	343	33	34	46	85	13	15	3.32
	5yln	32	5	3	15	17	15.6	77	343	17	34	26	54	17	6	3.28
GDH-ba	1euh	378	118	46	245	357	31.2	270	470	89	63	118	203	37	30	3.40
	1gad	132	27	12	62	81	20.5	149	326	42	57	50	104	27	18	3.30
	1gd1	170	37	15	72	110	21.8	156	328	51	50	55	98	38	20	3.30
	1obf	241	39	15	62	92	16.2	135	329	44	61	30	99	26	10	3.33
	2d2i	101	16	8	42	51	15.8	118	335	47	38	33	99	13	6	3.27
	2g82	170	25	13	62	76	14.7	82	326	46	26	10	55	11	16	3.35
	3gnq	130	35	16	70	105	26.9	146	332	53	47	46	98	30	18	3.35
	4dib	38	3	3	13	13	7.9	134	320	49	50	35	100	29	5	3.19
	5ld5	85	22	9	44	61	25.9	163	338	89	39	35	119	22	22	3.31
	5utm	235	38	20	87	109	16.2	138	339	66	32	40	95	26	17	3.27

	6fzh	430	50	15	74	118	11.6	111	331	51	28	32	76	20	15	3.35
MDH-ba	1b8p	197	47	23	102	132	23.9	150	322	59	31	60	103	29	18	3.36
	1bdm	89	35	21	81	99	39.3	156	316	64	49	43	117	22	17	3.39
	1emd	86	39	17	65	99	45.3	195	309	85	66	44	157	18	20	3.23
	1guz	188	15	6	25	35	8	51	304	26	21	4	46	4	1	3.38
	1gv1	130	9	7	30	32	6.9	90	304	40	38	12	72	4	14	3.43
	1ur5	265	62	25	112	170	23.4	172	297	106	49	17	140	25	7	3.39
	1z2i	172	39	21	84	106	22.7	182	348	59	43	80	127	40	15	3.26
	3d5t	76	25	13	55	74	32.9	173	319	75	49	49	125	30	18	3.35
	3fi9	204	34	13	56	86	16.7	117	320	54	30	33	85	13	19	3.22
	3flk	221	29	13	74	102	13.1	191	346	98	67	26	170	18	3	3.31
	3gvh	150	37	22	72	100	24.7	135	316	72	46	17	115	15	5	3.47
	3p7m	128	24	4	19	57	18.8	126	302	68	40	18	99	16	11	3.25
	3tl2	367	62	22	102	152	16.9	146	309	92	34	20	114	26	6	3.33
	4e0b	202	42	17	74	107	20.8	147	299	66	51	30	113	23	11	3.26
	4ror	304	36	18	82	111	11.8	98	316	44	37	17	81	8	9	3.36
	4tvo	237	44	22	112	157	18.6	139	323	73	39	27	122	11	6	3.32
	5ujk	282	50	24	91	125	17.7	128	312	69	43	16	105	11	12	3.35
	6aoo	163	51	29	109	139	31.3	132	313	50	47	35	99	17	16	3.33
	6bal	165	60	31	120	163	36.4	150	308	63	43	44	115	21	14	3.33
	6itk	115	29	15	74	92	25.2	134	315	72	21	41	100	24	10	3.34
	6itl	136	39	21	84	106	28.7	105	310	54	38	13	74	20	11	3.33
ADH-eu	1adb	49	16	7	45	57	32.7	167	373	25	44	98	128	26	13	3.29
	1b15	69	23	11	61	80	33.3	136	253	57	47	32	105	21	10	3.34
	1cdo	300	35	15	61	85	11.7	122	370	43	12	67	104	11	7	3.30
	1d1t	76	16	9	61	74	21.1	141	371	47	29	65	110	18	13	3.37
	1ee2	446	69	28	130	191	15.5	149	371	45	32	72	121	16	12	3.33
	1ht0	279	58	23	105	156	20.8	169	372	65	27	77	118	34	17	3.19
	1mc5	166	33	14	66	97	19.9	114	368	42	20	52	98	10	6	3.30
	1mg5	357	58	22	104	155	16.2	137	253	61	44	32	109	19	9	3.31
	1u3t	203	30	14	64	89	14.8	118	371	46	26	46	94	16	8	3.35
	3wle	222	66	35	159	202	29.7	192	333	60	72	60	131	42	19	3.29
	4jji	212	39	23	107	132	18.4	142	375	53	14	75	99	23	20	3.35
	4rqt	173	37	18	88	118	21.4	134	373	44	16	74	92	29	13	3.24
	4w6z	41	29	13	55	73	70.7	140	345	43	44	53	110	19	11	3.25
	5ilg	146	19	10	51	66	13	82	262	35	25	22	64	9	9	3.33
GDH-eu	1dss	212	38	19	55	81	17.9	148	329	49	54	44	103	17	27	3.44
	1j0x	117	23	13	53	70	19.7	153	328	59	55	39	122	22	9	3.38
	1k3t	261	30	13	64	85	11.5	164	355	76	61	27	136	19	9	3.30
	1vsu	87	31	16	68	92	35.6	178	322	65	77	36	115	35	28	3.26

	1ywg	87	18	10	56	67	20.7	152	332	67	51	34	110	22	20	3.34
	2i5p	126	29	12	50	77	23	174	300	55	67	52	136	18	20	3.31
	2vyn	206	30	15	61	82	14.6	132	325	52	47	33	96	21	15	3.33
	3cps	165	36	19	84	112	21.8	163	330	67	59	37	115	32	16	3.31
	3pym	221	36	18	92	120	16.3	187	330	79	70	38	150	20	17	3.23
	3qv1	55	21	10	47	65	38.2	137	329	54	47	36	106	21	10	3.31
	3sth	188	52	24	100	137	27.7	171	334	92	37	42	110	25	36	3.29
	4k9d	121	25	13	58	71	20.7	137	333	64	38	34	96	24	16	3.34
	5c7i	203	29	14	42	65	14.3	124	326	46	16	62	96	19	9	3.34
	5tso	264	35	12	65	99	13.3	122	328	51	39	31	86	28	7	3.36
MDH-eu	1civ	93	49	24	106	155	52.7	249	369	105	45	99	182	38	29	3.30
	1mld	123	45	20	88	123	36.6	145	311	76	41	28	120	22	3	3.25
	1sev	142	28	16	58	71	19.7	132	311	55	47	30	105	19	8	3.39
	2dfd	188	44	23	105	133	23.4	136	310	77	36	23	109	18	9	3.28
	2fn7	89	30	14	60	81	33.7	147	296	79	44	24	113	24	10	3.33
	2g76	130	14	6	26	37	10.8	86	301	37	18	31	58	18	10	3.26
	2hjr	108	18	11	48	55	16.7	89	311	40	18	31	66	11	12	3.31
	2i6t	141	22	12	41	53	15.6	113	279	37	33	43	75	20	18	3.30
	3i0p	96	42	22	101	130	43.8	150	359	69	18	63	110	32	8	3.39
	4h7p	316	52	25	105	146	16.5	117	309	53	37	25	97	7	11	3.39
	4mdh	190	43	24	104	128	22.6	199	329	97	73	29	150	26	23	3.46
	4plh	122	24	18	63	73	19.7	102	311	47	37	18	88	13	1	
	4plt	235	44	20	91	120	18.7	139	315	78	29	32	106	20	13	3.34
	4uuo	19	4	2	8	14	21.1	130	323	94	19	17	99	20	11	3.24
	5nue	294	49	23	98	131	16.7	129	324	64	37	28	106	13	10	3.36
	5zi2	103	51	25	107	145	49.5	134	336	72	37	25	101	14	19	3.29
	6um4	213	27	13	74	100	12.7	111	321	48	24	39	89	10	12	3.31
	7mdh	59	23	12	66	81	39	173	347	88	38	47	118	30	25	3.38

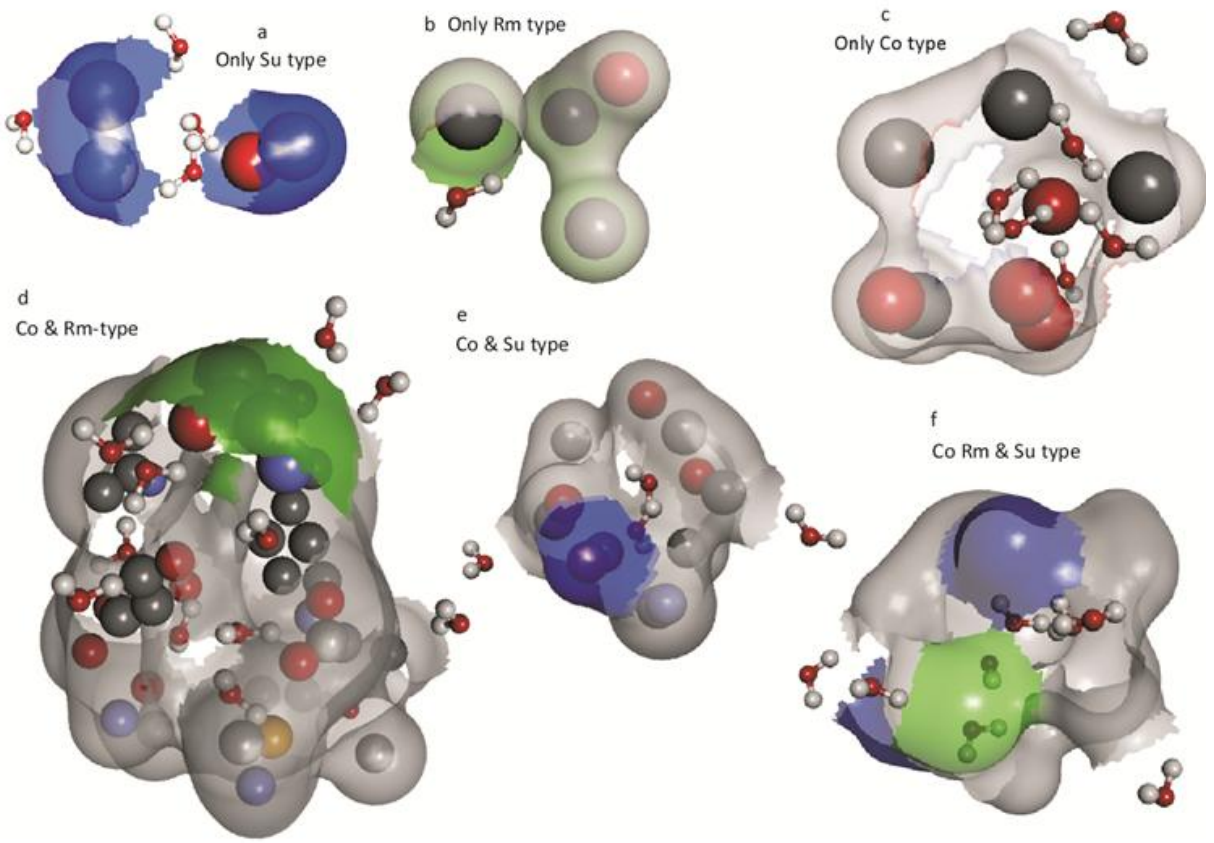


Figure 10. Shell-water and atoms in and out of cavity.

Different types of cavities formed by protein atoms from only the su type (**a**), only the rm (**b**), only the co (**c**), co and rm (**d**), co and su (**e**), and co, rm and su (**f**) regions. Atoms from su, rm, and co regions are shown by blue, green, and grey colors, respectively.

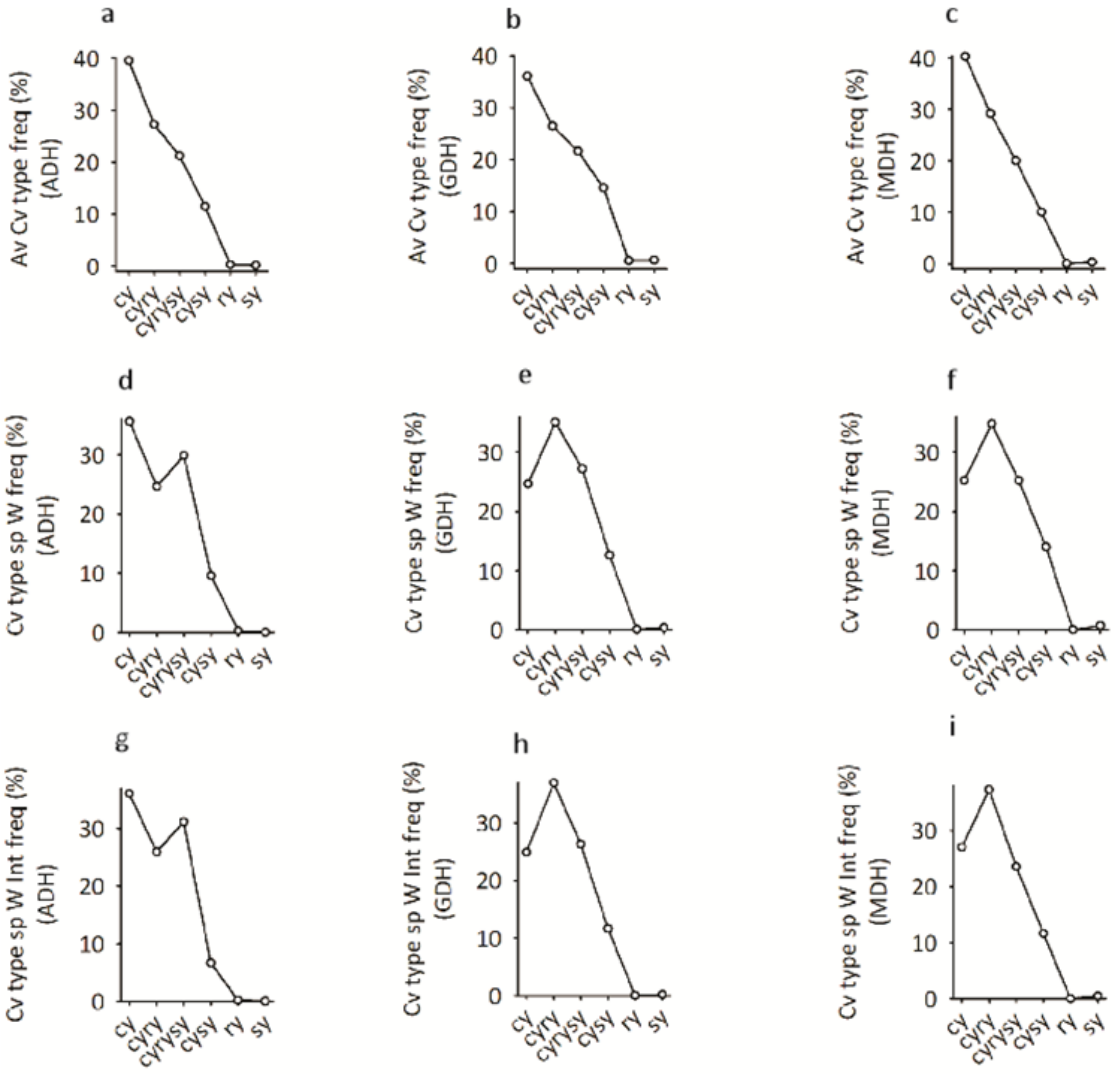


Figure 11. Enzyme-specific percent frequency of cavity

Average cavity types normalized frequency for ADH (**a**), GDH (**b**), and MDH (**c**). Similarly, cavity type specific average (%) heteroatom content for ADH (**d**), GDH (**e**) and MDH (**f**). Again, cavity type specific cavity-water and cavity-atom interaction normalized frequency.

Table 6. Domain-specific and enzyme-specific residue propensity to be part of cavity.

adh_ar	A	V	L	I	F	M	C	P	G	S	T	N	Q	Y	W	H	R	K	E	D
1h2b	0.7	0.6	1.5	0.8	1.5	1.3	1.3	1.7	1.2	1.9	0.7	1.5	0.8	1.4	5.1	1.7	0.7	0.0	0.5	0.8
1jvb	1.3	1.8	1.7	1.1	2.3	0.0	0.0	0.9	0.1	0.6	1.0	0.8	1.4	2.1	4.5	1.3	0.3	0.2	0.5	0.3
1rhc	1.3	1.1	1.5	2.1	1.3	0.6	1.2	0.4	0.2	0.8	1.1	1.2	1.4	1.3	2.2	0.9	1.0	0.3	1.1	0.3
2eer	1.0	1.1	1.5	0.7	1.1	2.1	0.0	2.0	0.8	0.6	1.4	0.9	1.8	1.5	2.7	1.8	0.3	0.5	0.4	0.7
2h6e	1.1	1.4	1.1	1.6	1.7	0.0	0.0	1.3	0.7	0.7	0.6	0.3	1.7	1.7	0.0	2.3	0.7	0.9	0.8	0.7
4jbg	0.5	1.4	1.7	1.3	1.8	0.0	0.0	0.6	0.8	1.8	1.4	1.4	2.5	1.0	2.0	0.0	0.6	0.4	0.3	1.1
6c75	0.8	1.3	1.7	1.7	1.7	1.4	0.0	0.8	0.7	1.1	1.1	1.1	0.0	1.0	1.0	0.8	0.2	0.9	0.5	0.7

av	1.0	1.2	1.5	1.3	1.6	0.8	0.4	1.1	0.6	1.1	1.0	1.0	1.4	1.4	2.5	1.3	0.5	0.5	0.6	0.7
sd	0.3	0.4	0.2	0.5	0.4	0.8	0.6	0.6	0.4	0.6	0.3	0.4	0.8	0.4	1.8	0.8	0.3	0.3	0.3	0.3
adh_ba	**A**	**V**	**L**	**I**	**F**	**M**	**C**	**P**	**G**	**S**	**T**	**N**	**Q**	**Y**	**W**	**H**	**R**	**K**	**E**	**D**
1rjw	0.7	0.9	1.7	1.0	1.4	1.1	0.6	1.0	1.3	0.0	1.9	0.0	0.0	2.1	3.8	0.6	1.4	0.6	1.1	0.4
3ox4	1.1	1.2	1.6	0.2	1.8	1.9	0.6	0.2	0.5	1.0	1.0	1.0	0.0	2.6	0.0	1.3	1.1	0.3	1.4	0.5
3uog	1.0	1.3	1.3	2.0	1.8	0.0	4.4	0.9	0.6	1.2	1.1	2.2	2.6	0.0	1.5	0.5	0.8	0.7	0.0	0.7
4cpd	0.5	0.9	0.8	2.4	3.3	2.4	2.9	1.0	1.0	0.6	0.0	0.4	0.8	2.1	0.0	1.1	1.0	0.6	0.2	0.7
4eez	0.9	1.2	1.5	1.2	1.7	1.2	1.8	1.0	0.9	0.7	1.7	0.7	0.0	0.7	2.4	1.0	1.2	0.6	0.6	0.6
4gkv	0.6	1.7	1.0	0.7	2.4	1.8	1.4	0.7	1.1	0.8	0.7	0.6	1.1	2.4	3.2	0.7	1.6	0.6	0.3	0.3
4j6f	0.7	1.3	1.6	1.4	1.8	0.0	2.4	0.2	0.7	1.6	1.0	0.5	1.4	1.7	0.0	1.6	0.9	0.4	0.2	0.8
4z6k	1.5	1.9	1.1	0.0	0.7	0.6	0.8	1.1	0.7	0.7	0.9	1.0	1.1	2.1	2.9	0.9	0.7	1.0	0.3	0.6
5yln	1.2	0.7	2.5	2.0	2.2	1.5	0.0	0.5	0.2	0.4	1.7	0.8	1.5	0.6	0.0	1.4	1.2	0.5	0.3	0.3
av	0.9	1.2	1.5	1.2	1.9	1.2	1.7	0.7	0.8	0.8	1.1	0.8	0.9	1.6	1.5	1.0	1.1	0.6	0.5	0.5
sd	0.3	0.4	0.5	0.8	0.7	0.8	1.4	0.4	0.3	0.5	0.6	0.6	0.9	0.9	1.6	0.4	0.3	0.2	0.5	0.2
adh_eu	**A**	**V**	**L**	**I**	**F**	**M**	**C**	**P**	**G**	**S**	**T**	**N**	**Q**	**Y**	**W**	**H**	**R**	**K**	**E**	**D**
1adb	0.7	0.7	1.4	1.4	2.7	1.1	0.7	0.5	0.8	1.5	1.0	1.2	1.2	0.0	2.4	0.7	0.8	0.7	0.7	0.9
1b15	0.9	1.6	1.2	2.3	0.8	4.1	2.1	0.4	0.8	0.7	0.5	1.6	0.0	2.8	0.8	0.0	0.0	0.5	0.8	0.3
1cdo	0.6	1.0	1.9	0.8	3.3	2.1	1.6	0.3	0.9	1.5	0.0	1.3	1.2	0.0	0.0	1.1	0.0	0.6	0.6	0.6
1d1t	0.5	1.0	2.2	1.0	1.2	0.6	1.1	0.5	0.7	1.1	0.7	1.1	3.1	1.0	5.2	1.5	0.9	0.8	1.0	0.6
1ee2	0.3	0.8	1.8	1.5	2.2	1.2	1.1	0.8	0.7	1.6	0.5	1.3	1.2	0.0	2.6	1.5	1.9	0.5	0.3	0.3
1ht0	0.3	0.7	1.8	1.5	2.2	1.4	0.6	1.0	0.6	1.7	1.1	1.2	0.7	1.2	2.4	0.8	1.0	0.7	0.7	0.3
1mc5	0.6	1.0	1.5	0.9	0.9	3.7	0.4	1.6	0.8	1.6	1.1	0.7	2.2	1.8	1.6	1.8	0.7	0.4	0.5	0.5
1mg5	0.6	2.0	2.0	1.3	0.8	0.0	1.8	0.0	0.6	0.5	0.8	1.8	1.0	1.2	0.7	0.0	0.7	0.6	0.4	0.0
1u3t	0.5	0.9	1.8	1.6	1.7	1.6	1.0	1.0	0.6	2.1	0.7	1.4	0.7	2.6	2.6	0.7	0.7	0.5	0.3	0.0
3wle	1.0	0.7	1.3	1.4	1.7	2.3	0.8	0.9	1.0	0.4	1.4	0.8	1.9	0.8	3.4	2.1	0.9	0.1	0.9	1.2
4jji	0.9	0.8	1.9	0.7	2.0	2.1	1.4	0.3	0.4	0.5	0.8	0.4	0.5	1.6	3.1	1.7	1.4	0.9	1.6	0.9
4rqt	1.4	0.5	1.9	1.3	1.8	0.6	2.1	0.6	0.5	0.5	1.2	1.3	0.9	0.9	0.0	1.6	2.0	0.6	0.5	0.3
4w6z	1.0	0.7	1.6	2.3	1.2	1.6	1.2	0.0	0.5	1.4	1.0	1.3	1.1	1.4	1.9	1.0	1.2	0.4	0.5	0.9
5ilg	1.0	2.2	0.6	1.6	2.1	1.5	0.0	0.0	0.6	0.5	1.2	1.0	0.0	2.0	0.0	0.0	0.6	1.0	0.0	0.4
av	0.7	1.0	1.6	1.4	1.8	1.7	1.1	0.6	0.7	1.1	0.9	1.2	1.1	1.2	1.9	1.0	0.9	0.6	0.6	0.5
sd	0.3	0.5	0.4	0.5	0.7	1.1	0.6	0.5	0.2	0.6	0.4	0.4	0.8	0.9	1.5	0.7	0.6	0.2	0.4	0.4
gdh_ar	**A**	**V**	**L**	**I**	**F**	**M**	**C**	**P**	**G**	**S**	**T**	**N**	**Q**	**Y**	**W**	**H**	**R**	**K**	**E**	**D**
1b7g	0.9	1.5	1.4	2.3	0.0	0.5	0.0	0.4	0.0	1.0	0.8	1.0	0.0	1.4	0.0	0.9	1.5	0.4	0.8	0.7
1cf2	0.9	0.9	1.2	1.5	2.2	0.6	0.0	1.0	0.4	1.1	0.8	0.5	1.1	1.4	7.6	1.1	2.7	0.6	0.6	0.8
1uxt	1.1	1.0	1.3	1.5	1.1	0.9	3.5	1.4	0.9	1.0	0.5	2.3	1.4	1.8	0.9	3.5	0.6	0.5	0.4	0.5
2czc	0.8	1.4	2.0	1.6	1.0	0.0	0.0	0.0	0.3	0.6	1.3	1.2	0.9	0.0	4.4	2.2	1.9	0.5	0.5	1.0
2yyy	1.0	1.0	2.1	2.2	1.0	0.9	0.0	0.7	0.4	0.0	0.7	1.1	0.6	2.7	0.0	2.0	1.2	0.5	0.5	0.4
av	0.9	1.2	1.6	1.8	1.1	0.6	0.7	0.7	0.4	0.7	0.8	1.2	0.8	1.5	2.6	1.9	1.6	0.5	0.6	0.7
sd	0.1	0.3	0.4	0.4	0.8	0.4	1.6	0.5	0.3	0.5	0.3	0.7	0.5	1.0	3.3	1.0	0.8	0.1	0.2	0.2
gdh_ba	**A**	**V**	**L**	**I**	**F**	**M**	**C**	**P**	**G**	**S**	**T**	**N**	**Q**	**Y**	**W**	**H**	**R**	**K**	**E**	**D**

1euh	0.4	1.2	1.6	1.3	1.7	1.9	3.4	0.9	1.0	1.3	0.8	1.2	0.8	0.9	0.0	0.0	1.3	0.7	0.6	0.7
1gad	0.7	0.9	1.9	1.9	1.1	1.2	1.4	0.9	0.6	0.0	0.6	1.4	3.3	1.0	2.8	0.0	1.7	0.8	0.6	0.8
1gd1	0.6	1.2	1.5	1.4	0.7	1.5	1.8	1.1	0.3	0.6	0.4	1.4	1.2	2.2	3.6	0.8	1.7	0.5	0.9	0.7
1obf	0.4	0.9	1.5	1.9	1.8	0.7	2.2	0.7	0.3	1.3	0.6	0.8	1.5	2.4	1.5	1.0	2.2	0.2	0.4	1.3
2d2i	0.7	1.3	1.7	1.3	0.6	0.0	2.6	0.5	0.5	0.7	0.0	2.0	0.6	3.9	1.0	0.9	1.4	0.6	0.3	0.9
2g82	0.5	1.3	2.0	1.4	1.0	1.0	0.0	0.6	0.3	1.0	0.0	0.6	1.7	2.0	2.2	0.7	1.7	1.2	0.7	0.9
3gnq	0.5	1.0	1.4	0.8	2.0	2.0	2.7	0.7	0.7	1.1	1.2	1.3	1.3	0.8	1.3	0.8	1.4	0.9	0.5	0.7
4dib	1.1	1.1	1.8	1.9	1.8	0.7	4.4	0.0	0.6	1.3	0.2	0.5	0.0	2.6	1.5	1.5	1.6	0.2	0.2	0.7
5ld5	1.0	1.2	1.7	1.6	2.3	1.3	1.4	0.3	0.7	0.8	0.4	0.6	0.8	1.5	0.0	0.0	1.7	1.0	0.6	0.6
5utm	1.1	1.0	2.1	0.7	1.2	1.3	2.3	0.8	1.5	1.2	0.8	0.5	0.4	2.9	2.3	0.0	1.1	0.5	0.2	0.4
6fzh	0.7	0.8	1.8	1.2	1.2	0.7	2.4	1.0	1.5	1.3	1.0	0.5	1.2	3.5	2.4	0.7	0.8	0.2	0.5	0.4
av	0.7	1.1	1.7	1.4	1.4	1.1	2.2	0.7	0.7	1.0	0.5	1.0	1.2	2.2	1.7	0.6	1.5	0.6	0.5	0.7
sd	0.3	0.2	0.2	0.4	0.6	0.6	1.1	0.3	0.4	0.4	0.4	0.5	0.9	1.0	1.1	0.5	0.4	0.3	0.2	0.3
gdh_eu	**A**	**V**	**L**	**I**	**F**	**M**	**C**	**P**	**G**	**S**	**T**	**N**	**Q**	**Y**	**W**	**H**	**R**	**K**	**E**	**D**
1dss	0.9	1.4	1.7	2.0	0.9	0.8	2.1	0.3	0.7	0.8	0.9	1.0	2.4	0.5	0.0	0.0	2.3	0.3	0.2	1.5
1j0x	1.3	1.2	1.9	0.9	1.1	2.5	0.0	0.7	0.2	1.3	1.3	1.4	0.6	1.3	1.3	0.3	1.9	0.3	0.3	0.7
1k3t	0.7	1.4	2.1	2.0	2.6	1.2	2.0	0.3	0.4	0.6	0.4	1.4	1.0	1.7	2.0	0.4	1.1	0.2	0.3	0.6
1vsu	1.0	1.4	1.9	1.1	0.3	1.1	1.5	0.0	0.7	0.8	1.2	0.8	0.0	0.9	0.0	2.3	2.3	0.9	0.3	0.9
1ywg	1.0	1.0	1.4	1.8	1.4	0.0	1.4	0.3	0.7	0.9	0.7	0.9	1.4	1.9	0.0	1.3	1.8	0.6	0.5	0.7
2i5p	1.0	1.4	1.1	1.4	1.9	1.2	1.5	0.3	0.9	0.5	0.6	0.8	2.3	0.9	1.5	1.5	1.0	0.8	0.6	0.6
2vyn	1.2	1.2	1.6	1.8	2.6	1.2	0.0	0.0	0.7	0.0	0.6	0.9	2.4	1.0	1.4	0.0	1.7	0.6	0.6	0.7
3cps	1.3	0.8	1.8	1.8	0.4	1.2	1.6	0.2	0.9	0.8	0.8	0.5	0.9	2.0	0.0	0.0	2.0	0.7	0.3	0.5
3pym	1.0	1.6	2.3	1.6	1.0	1.5	3.4	0.0	0.7	0.5	0.4	1.0	1.4	1.2	1.1	0.4	1.5	0.7	0.2	0.7
3qv1	0.7	1.4	1.5	1.4	2.0	0.0	3.2	0.6	0.5	0.4	0.8	0.8	2.0	2.0	2.0	1.6	1.3	0.5	0.7	0.4
3sth	0.7	0.8	2.1	1.3	1.5	1.1	2.5	0.5	0.9	0.5	1.2	1.2	0.7	1.3	0.9	0.8	2.5	0.4	0.3	0.7
4k9d	0.7	1.4	1.8	1.5	1.1	1.9	1.9	0.8	0.6	0.6	0.7	1.1	1.0	1.5	0.0	1.3	1.9	0.5	0.4	0.8
4lsm	0.6	1.3	1.9	1.8	0.6	2.3	1.7	0.0	0.3	0.7	0.6	0.9	1.4	1.0	1.7	0.0	1.3	0.8	0.9	1.1
5c7i	1.2	0.8	1.5	1.8	1.5	1.6	1.0	0.5	0.0	0.2	0.0	1.5	1.9	2.6	1.6	1.4	2.1	0.3	0.3	1.2
5tso	0.7	0.8	2.6	1.5	1.1	2.3	2.6	0.0	0.7	0.3	0.7	0.7	1.7	1.7	1.7	0.9	3.1	0.4	0.0	1.0
av	0.9	1.2	1.8	1.6	1.3	1.3	1.8	0.3	0.6	0.6	0.7	1.0	1.4	1.4	1.0	0.8	1.9	0.5	0.4	0.8
sd	0.2	0.3	0.4	0.3	0.7	0.7	1.0	0.3	0.3	0.3	0.3	0.3	0.7	0.6	0.8	0.7	0.6	0.2	0.2	0.3
mdh_ar	**A**	**V**	**L**	**I**	**F**	**M**	**C**	**P**	**G**	**S**	**T**	**N**	**Q**	**Y**	**W**	**H**	**R**	**K**	**E**	**D**
1o6z	0.6	0.7	2.5	2.6	1.4	2.7	0.0	1.1	0.9	0.6	0.4	0.6	0.5	1.6	0.0	0.8	2.1	0.7	0.2	0.7
1v9n	0.5	1.9	1.1	0.8	0.4	0.0	0.0	1.3	0.8	1.0	1.4	1.8	0.0	1.3	1.5	0.6	1.1	0.5	0.9	1.1
2d4a	0.8	1.5	2.2	1.7	1.0	1.5	0.0	1.2	0.2	0.8	0.7	0.6	0.0	1.4	0.0	1.7	1.1	0.0	0.4	0.7
2x0i	1.3	1.8	1.5	1.5	1.9	1.8	4.5	0.6	0.5	0.8	1.0	0.4	0.0	0.0	3.0	0.0	0.6	0.6	0.4	0.3
4bgu	0.7	1.0	1.8	2.5	1.9	1.7	4.2	1.2	1.3	1.2	0.2	0.8	1.4	0.0	2.1	1.4	1.3	0.0	0.2	0.3
4bgv	0.7	1.4	0.0	3.0	0.8	3.1	4.7	0.0	1.8	0.4	0.0	0.0	0.0	1.4	0.0	0.0	0.7	0.4	0.0	0.9
6ihd	1.1	1.8	1.6	2.0	1.8	0.8	0.0	0.3	0.7	0.4	0.0	0.9	1.3	2.5	1.3	0.8	0.5	0.8	0.4	0.0
av	0.8	1.4	1.5	2.0	1.3	1.7	1.9	0.8	0.9	0.7	0.5	0.7	0.5	1.2	1.1	0.8	1.1	0.4	0.4	0.6
sd	0.3	0.5	0.8	0.8	0.6	1.1	2.4	0.5	0.5	0.3	0.5	0.6	0.6	0.9	1.2	0.6	0.5	0.3	0.3	0.4

mdh-ba	A	V	L	I	F	M	C	P	G	S	T	N	Q	Y	W	H	R	K	E	D
1b8p	0.7	1.3	1.6	1.4	1.2	1.0	0.0	0.3	0.8	0.8	1.2	0.7	0.6	2.9	3.0	2.0	0.9	0.9	0.6	0.4
1bdm	0.7	1.1	1.0	1.7	0.8	1.5	0.0	0.0	0.4	1.7	1.4	2.1	0.9	0.5	0.9	3.8	1.2	0.7	0.8	1.1
1emd	0.7	1.1	2.2	1.6	1.8	1.5	2.0	0.7	0.5	0.7	0.8	0.3	0.6	2.2	0.0	1.5	0.3	0.6	0.9	0.8
1guz	1.2	1.2	1.5	2.6	0.0	0.9	3.5	0.8	0.4	0.7	2.1	0.0	0.0	0.0	0.0	2.6	0.8	0.0	1.2	0.0
1gv1	0.4	1.5	1.0	3.0	0.9	0.9	1.8	0.4	0.8	0.4	2.4	1.0	0.0	0.0	0.0	1.4	0.4	0.3	0.7	0.3
1ur5	1.1	1.3	0.8	1.4	0.6	1.5	2.2	1.3	0.8	1.3	1.6	0.0	0.0	2.5	0.0	2.2	0.0	0.3	0.9	0.6
1z2i	0.9	1.0	1.2	0.5	1.4	1.9	2.5	1.2	0.7	0.6	1.0	1.1	0.7	2.3	3.7	2.1	0.9	0.5	0.2	0.5
3d5t	0.3	1.5	1.3	1.4	1.1	1.9	1.6	0.2	0.3	0.8	1.1	2.0	1.4	1.6	3.3	2.4	1.6	0.5	0.4	0.9
3fi9	0.9	1.5	1.5	2.2	2.2	0.9	0.0	0.5	0.2	0.8	1.2	0.4	0.5	1.7	1.7	0.7	2.1	0.0	0.7	0.4
3flk	1.0	1.5	1.4	1.4	1.7	0.0	0.9	1.2	0.9	0.7	0.9	0.9	0.0	1.5	1.1	0.4	1.1	0.3	0.5	0.7
3gvh	0.7	1.5	1.6	1.8	1.6	1.0	0.0	0.6	1.1	0.5	1.3	0.0	1.0	1.1	2.2	1.5	0.0	0.4	0.6	0.8
3nep	0.9	1.3	1.2	3.5	4.3	2.3	2.1	1.4	0.0	0.8	1.3	0.0	0.0	0.0	0.0	2.1	0.6	0.0	0.4	0.3
3p7m	1.2	1.6	1.8	1.7	1.1	0.0	1.1	0.4	1.0	0.0	1.7	0.0	0.0	0.9	0.0	2.9	1.2	0.5	0.3	0.4
3tl2	0.9	1.5	0.6	1.3	1.5	2.3	0.0	0.9	1.0	1.7	1.3	0.0	1.3	0.4	0.0	2.0	1.1	0.4	0.4	0.8
4e0b	0.9	0.9	1.7	1.8	2.1	0.0	2.5	0.8	0.8	0.5	0.8	0.8	0.5	1.8	0.0	0.0	0.0	1.2	0.5	0.8
4ror	0.7	0.9	1.1	2.2	1.0	1.3	1.8	1.8	1.2	0.3	0.8	0.0	1.1	2.0	2.7	0.0	0.5	0.8	0.3	1.1
4tvo	0.3	0.9	1.7	1.4	1.8	1.7	0.0	0.6	1.2	1.0	1.4	1.1	1.2	2.8	1.7	1.4	1.7	0.4	0.2	0.4
5ujk	1.2	1.0	1.2	2.2	1.3	0.6	1.6	0.8	0.9	1.2	1.2	0.0	0.9	1.7	2.3	0.0	0.4	0.5	0.5	0.8
6aoo	1.1	1.9	1.4	0.8	1.5	2.3	3.1	0.3	0.9	0.5	0.5	0.0	1.4	1.8	0.0	0.0	0.8	0.5	0.5	1.5
6bal	0.8	1.2	1.0	1.8	1.6	0.9	2.4	0.7	0.9	0.7	0.6	0.3	1.2	2.9	0.0	1.8	1.5	0.6	0.6	1.2
6itk	0.8	1.3	1.3	2.1	1.5	1.0	0.0	1.0	0.6	0.2	1.6	0.9	0.5	3.1	2.1	1.0	1.0	0.9	0.4	0.4
6itl	0.6	1.6	1.4	1.1	2.4	0.0	1.8	1.3	0.2	0.7	0.9	0.8	1.1	2.1	0.0	1.8	0.5	0.8	1.0	1.3
av	0.8	1.3	1.3	1.8	1.5	1.2	1.4	0.8	0.7	0.8	1.2	0.6	0.7	1.6	1.1	1.5	0.8	0.5	0.6	0.7
sd	0.3	0.3	0.4	0.7	0.8	0.7	1.1	0.4	0.3	0.4	0.5	0.6	0.5	1.0	1.3	1.0	0.6	0.3	0.3	0.4
mdh-eu	**A**	**V**	**L**	**I**	**F**	**M**	**C**	**P**	**G**	**S**	**T**	**N**	**Q**	**Y**	**W**	**H**	**R**	**K**	**E**	**D**
1civ	0.2	0.8	1.6	0.8	1.1	1.3	0.0	1.0	0.7	0.8	1.2	0.9	1.5	1.3	1.1	1.4	1.8	1.3	1.1	0.9
1mld	0.7	1.7	0.9	2.6	1.9	0.7	1.1	0.8	0.6	0.5	1.2	1.1	0.5	1.7	0.0	0.8	1.1	0.4	0.6	0.3
1sev	1.1	1.3	1.5	1.0	1.3	0.6	2.8	0.8	0.7	0.9	0.6	0.9	1.2	0.8	0.0	1.6	1.0	0.6	0.3	0.7
2dfd	0.6	1.2	1.1	2.5	2.4	1.5	0.9	1.0	0.6	0.9	0.6	0.9	0.9	1.5	0.0	0.7	1.6	0.3	0.7	0.6
2fn7	0.6	1.9	0.8	1.7	1.1	1.6	1.9	0.9	0.6	1.1	0.6	0.3	0.0	1.1	3.7	2.1	1.6	0.2	0.0	1.0
2g76	0.7	1.9	1.7	1.0	3.4	2.2	1.6	0.0	1.1	0.4	0.7	0.5	1.2	0.0	0.0	2.0	0.7	0.0	0.6	0.4
2hjr	0.5	1.6	1.3	1.5	1.8	0.8	1.0	1.1	1.0	0.2	1.5	0.7	0.0	1.8	0.0	2.0	2.0	0.0	0.9	1.1
2i6t	0.3	1.3	2.1	1.3	1.8	1.8	1.5	0.0	1.1	0.3	1.4	0.4	0.9	1.5	1.1	0.0	0.0	0.9	0.4	1.1
3i0p	1.3	0.9	1.4	1.9	1.2	1.1	0.0	0.6	0.8	1.1	1.2	1.3	0.0	1.3	0.0	1.6	0.8	0.4	0.8	0.4
4h7p	0.4	0.9	1.7	2.0	0.0	0.8	2.0	1.2	0.6	0.9	1.0	2.7	0.0	2.5	3.3	2.1	1.4	0.0	0.3	0.7
4mdh	0.2	1.3	1.5	1.7	1.8	0.4	0.7	0.8	0.5	0.6	1.1	1.1	1.2	1.4	2.5	2.5	0.7	1.3	0.8	0.4
4plh	1.0	1.6	2.1	1.6	1.9	1.1	2.5	0.8	0.7	0.3	1.1	1.0	0.0	1.5	5.0	1.0	0.0	0.0	0.2	0.6
4plt	1.2	0.9	1.5	1.7	1.4	1.2	0.9	0.3	0.8	0.8	1.1	0.5	0.0	1.9	1.9	0.7	1.9	0.6	0.6	0.9
4uuo	1.0	0.6	2.2	1.6	0.7	1.1	1.9	0.6	0.2	0.4	1.3	1.3	0.6	1.8	2.2	0.0	2.0	0.9	0.5	0.6

5nue	0.6	1.3	1.5	1.7	1.4	1.0	1.4	1.0	0.6	0.3	0.7	1.4	1.4	1.2	3.2	2.2	1.3	0.4	0.0	1.0
5zi2	0.5	2.1	1.1	1.5	0.9	0.8	0.0	1.1	0.4	1.3	0.7	1.4	0.0	0.8	0.0	0.9	1.0	0.6	1.1	0.4
6um4	0.7	1.5	1.3	1.2	1.2	2.8	1.4	1.4	0.4	1.0	0.9	1.3	0.5	0.0	2.8	2.8	0.8	0.3	0.3	0.7
7mdh	0.8	0.8	1.6	2.3	1.9	2.3	0.0	0.5	0.7	0.8	0.8	0.9	1.7	1.1	1.6	1.9	1.0	0.6	0.9	0.2
av	0.7	1.3	1.5	1.6	1.5	1.3	1.2	0.8	0.7	0.7	1.0	1.0	0.6	1.3	1.6	1.5	1.2	0.5	0.6	0.7
sd	0.3	0.4	0.4	0.5	0.7	0.6	0.9	0.4	0.2	0.3	0.3	0.5	0.6	0.6	1.6	0.8	0.6	0.4	0.3	0.3

Details of residue propensity to be part of cavity for representative proteins of three enzyme classes of the domains of life. Adh _ar, archaeal ADH; adh_ba, bacterial ADH; adh_eu, eukaryote ADH; gdh_ar, archaeal GDH; gdh_ba, bacterial GDH; gdh_eu, eukaryotic GDH; mdh_ar, archaeal MDH; mdh_ba, bacterial MDH, and MDH-eu, eukaryotic MDH.

Table 7: Protein-specific frequency of types of cavity

Proteins	cy	cyry	cyrysy	cysy	ry	sy
1h2b	43.8	12.5	18.8	18.8	6.3	0.0
1jvb	46.7	13.3	33.3	6.7	0.0	0.0
1rhc	18.2	45.5	27.3	9.1	0.0	0.0
2eer	38.5	30.8	23.1	7.7	0.0	0.0
2h6e	43.8	25.0	31.3	0.0	0.0	0.0
4jbg	40.0	33.3	13.3	13.3	0.0	0.0
6c75	50.0	18.8	9.4	18.8	0.0	3.1
AD_ar_av	40.1	25.6	22.3	10.6	0.9	0.4
1rjw	53.3	20.0	26.7	0.0	0.0	0.0
3ox4	21.7	39.1	17.4	21.7	0.0	0.0
3uog	62.5	12.5	18.8	6.3	0.0	0.0
4cpd	52.2	17.4	13.0	17.4	0.0	0.0
4eez	46.7	40.0	0.0	13.3	0.0	0.0
4gkv	40.0	30.0	20.0	10.0	0.0	0.0
4j6f	38.9	33.3	22.2	5.6	0.0	0.0
4z6k	15.4	23.1	23.1	38.5	0.0	0.0
5yln	18.2	36.4	45.5	0.0	0.0	0.0
AD_ba_av	38.8	28.0	20.7	12.5	0.0	0.0
1adb	41.2	29.4	23.5	5.9	0.0	0.0
1b15	35.3	41.2	0.0	23.5	0.0	0.0
1cdo	50.0	22.2	11.1	16.7	0.0	0.0
1d1t	37.5	25.0	31.3	6.3	0.0	0.0
1ee2	41.2	29.4	17.6	11.8	0.0	0.0
1ht0	38.1	28.6	28.6	4.8	0.0	0.0
1mc5	57.1	21.4	14.3	7.1	0.0	0.0
1mg5	29.4	29.4	23.5	17.6	0.0	0.0
1u3t	35.7	28.6	28.6	7.1	0.0	0.0
3wle	30.8	30.8	30.8	7.7	0.0	0.0

4jji	26.3	21.1	26.3	26.3	0.0	0.0
4rqt	43.8	37.5	18.8	0.0	0.0	0.0
4w6z	40.0	35.0	10.0	15.0	0.0	0.0
5ilg	50.0	16.7	25.0	8.3	0.0	0.0
AD_eu_Av	39.7	28.3	20.7	11.3	0.0	0.0
1b7g	56.3	0.0	31.3	12.5	0.0	0.0
1cf2	44.4	11.1	11.1	33.3	0.0	0.0
1uxt	41.0	35.9	15.4	7.7	0.0	0.0
2czc	33.3	16.7	33.3	16.7	0.0	0.0
2yyy	42.9	17.9	21.4	14.3	3.6	0.0
GD_ar_av	43.6	16.3	22.5	16.9	0.7	0.0
1euh	35.7	28.6	28.6	7.1	0.0	0.0
1gad	36.8	31.6	21.1	10.5	0.0	0.0
1gd1	15.8	31.6	26.3	21.1	5.3	0.0
1obf	29.4	29.4	23.5	11.8	5.9	0.0
2d2i	42.9	35.7	7.1	14.3	0.0	0.0
2g82	9.1	45.5	27.3	18.2	0.0	0.0
3gnq	26.3	42.1	15.8	15.8	0.0	0.0
4dib	35.3	47.1	11.8	5.9	0.0	0.0
5ld5	21.1	31.6	15.8	26.3	0.0	5.3
5utm	27.8	38.9	27.8	5.6	0.0	0.0
6fzh	13.3	40.0	33.3	13.3	0.0	0.0
GD_ba_Av	26.7	36.5	21.7	13.6	1.0	0.5
1dss	26.1	34.8	13.0	21.7	0.0	4.3
1j0x	52.4	19.0	19.0	9.5	0.0	0.0
1k3t	50.0	22.7	13.6	13.6	0.0	0.0
1vsu	26.9	23.1	30.8	19.2	0.0	0.0
1ywg	27.8	22.2	33.3	11.1	0.0	5.6
2i5p	50.0	9.1	27.3	13.6	0.0	0.0
2vyn	40.0	20.0	25.0	15.0	0.0	0.0
3cps	39.1	21.7	26.1	13.0	0.0	0.0
3pym	45.8	29.2	12.5	12.5	0.0	0.0
3qv1	38.9	33.3	16.7	11.1	0.0	0.0
3sth	25.0	20.8	25.0	20.8	0.0	8.3
4k9d	38.9	22.2	27.8	11.1	0.0	0.0
4lsm	35.7	35.7	21.4	7.1	0.0	0.0
5c7i	38.9	50.0	5.6	0.0	0.0	5.6
5tso	33.3	33.3	13.3	20.0	0.0	0.0
GD_eu_Av	37.9	26.5	20.7	13.3	0.0	1.6
1o6z	46.2	46.2	7.7	0.0	0.0	0.0

1v9n	20.0	15.0	30.0	35.0	0.0	0.0
2d4a	41.2	29.4	29.4	0.0	0.0	0.0
2x0i	50.0	25.0	25.0	0.0	0.0	0.0
4bgu	50.0	27.8	11.1	11.1	0.0	0.0
4bgv	87.5	12.5	0.0	0.0	0.0	0.0
6ihd	26.1	39.1	34.8	0.0	0.0	0.0
MD_ar_av	45.8	27.9	19.7	6.6	0.0	0.0
1b8p	18.2	31.8	40.9	9.1	0.0	0.0
1bdm	28.6	38.1	19.0	14.3	0.0	0.0
1emd	50.0	15.4	19.2	11.5	0.0	3.8
1guz	50.0	33.3	0.0	16.7	0.0	0.0
1gv1	53.3	13.3	13.3	20.0	0.0	0.0
1ur5	52.9	23.5	17.6	5.9	0.0	0.0
1z2i	29.2	37.5	25.0	8.3	0.0	0.0
3d5t	38.1	14.3	33.3	14.3	0.0	0.0
3fi9	31.3	18.8	31.3	18.8	0.0	0.0
3flk	44.4	44.4	5.6	5.6	0.0	0.0
3gvh	52.6	31.6	10.5	5.3	0.0	0.0
3nep	57.1	14.3	14.3	14.3	0.0	0.0
3p7m	25.0	33.3	41.7	0.0	0.0	0.0
3tl2	43.8	43.8	6.3	0.0	0.0	6.3
4e0b	42.1	26.3	21.1	10.5	0.0	0.0
4ror	46.2	15.4	30.8	7.7	0.0	0.0
4tvo	40.0	40.0	13.3	6.7	0.0	0.0
5ujk	55.0	10.0	30.0	5.0	0.0	0.0
6aoo	42.1	15.8	26.3	15.8	0.0	0.0
6bal	45.0	25.0	25.0	5.0	0.0	0.0
6itk	31.6	42.1	26.3	0.0	0.0	0.0
6itl	21.4	28.6	35.7	14.3	0.0	0.0
MD_ba_Av	40.8	27.1	22.1	9.5	0.0	0.5
1civ	36.7	20.0	23.3	20.0	0.0	0.0
1mld	55.6	33.3	11.1	0.0	0.0	0.0
1sev	44.4	27.8	16.7	11.1	0.0	0.0
2dfd	44.4	33.3	16.7	5.6	0.0	0.0
2fn7	38.1	28.6	28.6	4.8	0.0	0.0
2g76	22.2	44.4	33.3	0.0	0.0	0.0
2hjr	27.3	36.4	0.0	27.3	0.0	9.1
2i6t	23.5	17.6	41.2	17.6	0.0	0.0
3i0p	30.0	45.0	20.0	5.0	0.0	0.0
4h7p	33.3	33.3	0.0	33.3	0.0	0.0
4mdh	28.0	36.0	16.0	20.0	0.0	0.0

4plh	50.0	42.9	7.1	0.0	0.0	0.0
4plt	45.0	30.0	15.0	10.0	0.0	0.0
4uuo	15.8	47.4	15.8	21.1	0.0	0.0
5nue	42.1	26.3	26.3	5.3	0.0	0.0
5zi2	35.3	11.8	17.6	35.3	0.0	0.0
6um4	27.3	36.4	18.2	18.2	0.0	0.0
7mdh	19.0	38.1	23.8	19.0	0.0	0.0
MD_eu_AV	34.3	32.7	18.4	14.1	0.0	0.5

Domains of life and enzyme class specific representative protein's normalized frequency of different types of cavity and their average (grey row) value.

Table 8: Details on unique shell-water in different types of cavities.

	cyUw	cyryUw	cyrysyUw	cysyUw	ryUw	syUw
1h2b	41.2	9.8	25.5	17.6	5.9	0.0
1jvb	29.6	22.2	40.7	7.4	0.0	0.0
1rhc	5.4	43.2	43.2	8.1	0.0	0.0
2eer	31.6	42.1	15.8	10.5	0.0	0.0
2h6e	20.0	26.7	53.3	0.0	0.0	0.0
4jbg	48.1	29.6	11.1	11.1	0.0	0.0
6c75	40.0	36.0	8.0	16.0	0.0	0.0
AD_ar_av	30.8	30.0	28.2	10.1	0.8	0.0
1rjw	46.2	30.8	23.1	0.0	0.0	0.0
3ox4	31.0	34.5	20.7	13.8	0.0	0.0
3uog	42.4	21.2	33.3	3.0	0.0	0.0
4cpd	65.0	20.0	5.0	10.0	0.0	0.0
4eez	27.8	59.3	0.0	13.0	0.0	0.0
4gkv	29.0	35.5	29.0	6.5	0.0	0.0
4j6f	53.1	31.3	15.6	0.0	0.0	0.0
4z6k	2.9	32.4	32.4	32.4	0.0	0.0
5yln	0.0	60.0	40.0	0.0	0.0	0.0
AD_ba_av	33.1	36.1	22.1	8.7	0.0	0.0
1adb	50.0	31.3	18.8	0.0	0.0	0.0
1b15	0.0	78.3	0.0	21.7	0.0	0.0
1cdo	22.9	11.4	31.4	34.3	0.0	0.0
1d1t	62.5	18.8	0.0	18.8	0.0	0.0
1ee2	34.8	34.8	21.7	8.7	0.0	0.0
1ht0	31.0	27.6	36.2	5.2	0.0	0.0
1mc5	45.5	27.3	27.3	0.0	0.0	0.0
1mg5	8.6	37.9	29.3	24.1	0.0	0.0
1u3t	30.0	30.0	30.0	10.0	0.0	0.0
3wle	24.2	24.2	43.9	7.6	0.0	0.0

4jji	30.8	15.4	28.2	25.6	0.0	0.0
4rqt	21.6	35.1	43.2	0.0	0.0	0.0
4w6z	51.7	24.1	6.9	17.2	0.0	0.0
5ilg	42.1	10.5	26.3	21.1	0.0	0.0
AD_eu_Av	32.6	29.0	24.5	13.9	0.0	0.0
1b7g	28.0	0.0	60.0	12.0	0.0	0.0
1cf2	24.0	16.0	32.0	28.0	0.0	0.0
1uxt	22.2	48.9	16.7	12.2	0.0	0.0
2czc	10.5	15.8	68.4	5.3	0.0	0.0
2yyy	27.6	20.7	41.4	10.3	0.0	0.0
GD_ar_av	22.5	20.3	43.7	13.6	0.0	0.0
1euh	12.7	36.4	44.1	6.8	0.0	0.0
1gad	3.7	48.1	33.3	14.8	0.0	0.0
1gd1	8.1	48.6	24.3	16.2	2.7	0.0
1obf	2.6	48.7	28.2	20.5	0.0	0.0
2d2i	43.8	37.5	12.5	6.3	0.0	0.0
2g82	0.0	28.0	44.0	28.0	0.0	0.0
3gnq	2.9	60.0	22.9	14.3	0.0	0.0
4dib	0.0	33.3	33.3	33.3	0.0	0.0
5ld5	4.5	50.0	18.2	18.2	0.0	9.1
5utm	15.8	39.5	36.8	7.9	0.0	0.0
6fzh	16.0	26.0	48.0	10.0	0.0	0.0
GD_ba_Av	10.0	41.5	31.4	16.0	0.2	0.8
1dss	15.8	34.2	10.5	31.6	0.0	7.9
1j0x	21.7	47.8	26.1	4.3	0.0	0.0
1k3t	13.3	40.0	20.0	26.7	0.0	0.0
1vsu	6.5	29.0	54.8	9.7	0.0	0.0
1ywg	11.1	38.9	38.9	11.1	0.0	0.0
2i5p	20.7	24.1	34.5	20.7	0.0	0.0
2vyn	23.3	33.3	33.3	10.0	0.0	0.0
3cps	5.6	36.1	47.2	11.1	0.0	0.0
3pym	13.9	38.9	25.0	22.2	0.0	0.0
3qv1	19.0	52.4	28.6	0.0	0.0	0.0
3sth	1.9	28.8	34.6	30.8	0.0	3.8
4k9d	12.5	45.8	25.0	16.7	0.0	0.0
4lsm	13.6	54.5	27.3	4.5	0.0	0.0
5c7i	24.1	48.3	17.2	0.0	0.0	10.3
5tso	8.6	74.3	8.6	8.6	0.0	0.0
GD_eu_Av	14.1	41.8	28.8	13.9	0.0	1.5
1o6z	31.6	60.5	7.9	0.0	0.0	0.0
1v9n	13.6	31.8	31.8	22.7	0.0	0.0

2d4a	28.6	57.1	14.3	0.0	0.0	0.0
2x0i	50.0	0.0	50.0	0.0	0.0	0.0
4bgu	50.9	16.4	20.0	12.7	0.0	0.0
4bgv	100.0	0.0	0.0	0.0	0.0	0.0
6ihd	100.0	0.0	0.0	0.0	0.0	0.0
MD_ar_av	53.5	23.7	17.7	5.1	0.0	0.0
1b8p	19.1	21.3	51.1	8.5	0.0	0.0
1bdm	25.7	54.3	11.4	8.6	0.0	0.0
1emd	35.9	15.4	28.2	20.5	0.0	0.0
1guz	33.3	60.0	0.0	6.7	0.0	0.0
1gv1	11.1	11.1	33.3	44.4	0.0	0.0
1ur5	53.2	25.8	17.7	3.2	0.0	0.0
1z2i	28.2	41.0	20.5	10.3	0.0	0.0
3d5t	28.0	12.0	48.0	12.0	0.0	0.0
3fi9	11.8	5.9	55.9	26.5	0.0	0.0
3flk	24.1	72.4	0.0	3.4	0.0	0.0
3gvh	48.6	8.1	24.3	18.9	0.0	0.0
3nep	27.3	9.1	36.4	27.3	0.0	0.0
3p7m	4.2	29.2	66.7	0.0	0.0	0.0
3tl2	30.6	61.3	1.6	0.0	0.0	6.5
4e0b	26.2	26.2	19.0	28.6	0.0	0.0
4ror	44.4	5.6	38.9	11.1	0.0	0.0
4tvo	56.8	25.0	11.4	6.8	0.0	0.0
5ujk	64.0	4.0	26.0	6.0	0.0	0.0
6aoo	15.7	29.4	27.5	27.5	0.0	0.0
6bal	33.3	30.0	30.0	6.7	0.0	0.0
6itk	37.9	31.0	31.0	0.0	0.0	0.0
6itl	20.5	30.8	38.5	10.3	0.0	0.0
MD_ba_Av	30.9	27.7	28.1	13.1	0.0	0.3
1civ	38.8	24.5	28.6	8.2	0.0	0.0
1mld	46.7	28.9	24.4	0.0	0.0	0.0
1sev	14.3	53.6	28.6	3.6	0.0	0.0
2dfd	31.8	43.2	20.5	4.5	0.0	0.0
2fn7	56.7	16.7	23.3	3.3	0.0	0.0
2g76	0.0	71.4	28.6	0.0	0.0	0.0
2hjr	38.9	22.2	0.0	27.8	0.0	11.1
2i6t	9.1	18.2	45.5	27.3	0.0	0.0
3i0p	19.0	69.0	9.5	2.4	0.0	0.0
4h7p	17.3	32.7	0.0	50.0	0.0	0.0
4mdh	9.3	51.2	23.3	16.3	0.0	0.0

4plh	62.5	29.2	8.3	0.0	0.0	0.0
4plt	43.2	22.7	27.3	6.8	0.0	0.0
4uuo	25.0	50.0	0.0	25.0	0.0	0.0
5nue	36.7	8.2	53.1	2.0	0.0	0.0
5zi2	25.5	2.0	17.6	54.9	0.0	0.0
6um4	22.2	44.4	22.2	11.1	0.0	0.0
7mdh	26.1	17.4	43.5	13.0	0.0	0.0
MD_eu_AV	29.1	33.6	22.5	14.2	0.0	0.6

Domains of life and enzyme class specific representative protein's normalized frequency of unique water in different types of cavities and their average (grey row) value.

Table 9: Details of interaction frequency of shell-water in different types of cavities.

	cyTw	cyryTw	cyrysyTw	cysyTw	ryTw	syTw
1h2b	43.5	7.5	30.6	14.3	4.1	0.0
1jvb	23.2	23.2	50.0	3.6	0.0	0.0
1rhc	1.7	46.1	46.1	6.1	0.0	0.0
2eer	31.5	40.7	22.2	5.6	0.0	0.0
2h6e	12.9	23.5	63.5	0.0	0.0	0.0
4jbg	46.7	25.3	20.0	8.0	0.0	0.0
6c75	40.2	47.4	2.1	10.3	0.0	0.0
AD_ar_av	28.5	30.5	33.5	6.8	0.6	0.0
1rjw	42.2	42.2	15.6	0.0	0.0	0.0
3ox4	32.6	42.1	9.5	15.8	0.0	0.0
3uog	43.2	30.6	22.5	3.6	0.0	0.0
4cpd	72.7	14.5	1.8	10.9	0.0	0.0
4eez	23.8	66.0	0.0	10.2	0.0	0.0
4gkv	23.5	47.0	25.2	4.3	0.0	0.0
4j6f	52.7	36.3	11.0	0.0	0.0	0.0
4z6k	2.3	34.1	39.8	23.9	0.0	0.0
5yln	0.0	64.7	35.3	0.0	0.0	0.0
AD_ba_av	32.6	41.9	17.8	7.6	0.0	0.0
1adb	59.6	24.6	15.8	0.0	0.0	0.0
1b15	0.0	83.8	0.0	16.3	0.0	0.0
1cdo	29.4	14.1	35.3	21.2	0.0	0.0
1d1t	66.2	23.0	0.0	10.8	0.0	0.0
1ee2	35.6	36.6	20.9	6.8	0.0	0.0
1ht0	38.5	28.2	31.4	1.9	0.0	0.0
1mc5	47.4	29.9	22.7	0.0	0.0	0.0
1mg5	4.5	47.7	31.0	16.8	0.0	0.0
1u3t	43.8	30.3	16.9	9.0	0.0	0.0
3wle	26.2	23.3	44.1	6.4	0.0	0.0
4jji	27.3	19.7	32.6	20.5	0.0	0.0

4rqt	19.5	39.0	41.5	0.0	0.0	0.0
4w6z	56.2	24.7	6.8	12.3	0.0	0.0
5ilg	42.4	7.6	21.2	28.8	0.0	0.0
AD_eu_Av	35.5	30.9	22.9	10.8	0.0	0.0
1b7g	28.3	0.0	64.2	7.5	0.0	0.0
1cf2	21.5	24.6	35.4	18.5	0.0	0.0
1uxt	21.3	50.6	18.4	9.6	0.0	0.0
2czc	21.1	15.8	60.5	2.6	0.0	0.0
2yyy	23.9	23.9	45.1	7.0	0.0	0.0
GD_ar_av	23.2	23.0	44.7	9.1	0.0	0.0
1euh	12.6	34.5	47.3	5.6	0.0	0.0
1gad	2.5	51.9	32.1	13.6	0.0	0.0
1gd1	5.5	58.2	14.5	20.9	0.9	0.0
1obf	1.1	43.5	37.0	18.5	0.0	0.0
2d2i	39.2	37.3	13.7	9.8	0.0	0.0
2g82	0.0	31.6	46.1	22.4	0.0	0.0
3gnq	1.0	62.9	25.7	10.5	0.0	0.0
4dib	0.0	7.7	69.2	23.1	0.0	0.0
5ld5	11.5	55.7	13.1	16.4	0.0	3.3
5utm	20.2	38.5	33.9	7.3	0.0	0.0
6fzh	21.2	24.6	49.2	5.1	0.0	0.0
GD_ba_Av	10.4	40.6	34.7	13.9	0.1	0.3
1dss	14.8	33.3	11.1	35.8	0.0	4.9
1j0x	24.3	55.7	17.1	2.9	0.0	0.0
1k3t	11.8	51.8	22.4	14.1	0.0	0.0
1vsu	6.5	31.5	53.3	8.7	0.0	0.0
1ywg	16.4	40.3	32.8	10.4	0.0	0.0
2i5p	22.1	28.6	24.7	24.7	0.0	0.0
2vyn	19.5	32.9	37.8	9.8	0.0	0.0
3cps	5.4	41.1	45.5	8.0	0.0	0.0
3pym	10.8	44.2	27.5	17.5	0.0	0.0
3qv1	27.7	44.6	27.7	0.0	0.0	0.0
3sth	0.7	45.3	28.5	23.4	0.0	2.2
4k9d	10.0	55.7	28.6	5.7	0.0	0.0
4lsm	9.2	63.2	19.7	7.9	0.0	0.0
5c7i	21.5	53.8	16.9	0.0	0.0	7.7
5tso	8.1	77.8	7.1	7.1	0.0	0.0
GD_eu_Av	13.9	46.6	26.7	11.7	0.0	1.0
1o6z	33.9	59.6	6.4	0.0	0.0	0.0
1v9n	10.0	36.7	30.8	22.5	0.0	0.0
2d4a	31.6	57.9	10.5	0.0	0.0	0.0

2x0i	58.8	0.0	41.2	0.0	0.0	0.0
4bgu	60.7	15.6	17.8	5.9	0.0	0.0
4bgv	100.0	0.0	0.0	0.0	0.0	0.0
6ihd	100.0	0.0	0.0	0.0	0.0	0.0
MD_ar_av	56.4	24.2	15.2	4.1	0.0	0.0
1b8p	25.8	13.6	55.3	5.3	0.0	0.0
1bdm	27.3	50.5	10.1	12.1	0.0	0.0
1emd	33.3	16.2	30.3	20.2	0.0	0.0
1guz	25.7	71.4	0.0	2.9	0.0	0.0
1gv1	9.4	12.5	21.9	56.3	0.0	0.0
1ur5	50.0	28.2	19.4	2.4	0.0	0.0
1z2i	35.8	39.6	18.9	5.7	0.0	0.0
3d5t	28.4	21.6	40.5	9.5	0.0	0.0
3fi9	23.3	8.1	47.7	20.9	0.0	0.0
3flk	22.5	70.6	0.0	6.9	0.0	0.0
3gvh	48.0	10.0	22.0	20.0	0.0	0.0
3nep	27.3	4.5	31.8	36.4	0.0	0.0
3p7m	8.8	28.1	63.2	0.0	0.0	0.0
3tl2	29.6	63.2	0.7	0.0	0.0	6.6
4e0b	20.6	20.6	20.6	38.3	0.0	0.0
4ror	51.4	9.9	30.6	8.1	0.0	0.0
4tvo	66.9	21.7	9.6	1.9	0.0	0.0
5ujk	66.4	6.4	20.8	6.4	0.0	0.0
6aoo	12.9	26.6	34.5	25.9	0.0	0.0
6bal	31.3	27.6	36.8	4.3	0.0	0.0
6itk	30.4	29.3	40.2	0.0	0.0	0.0
6itl	22.6	42.5	24.5	10.4	0.0	0.0
MD_ba_Av	31.7	28.3	26.3	13.3	0.0	0.3
1civ	41.3	16.1	32.3	10.3	0.0	0.0
1mld	39.8	31.7	28.5	0.0	0.0	0.0
1sev	15.5	52.1	31.0	1.4	0.0	0.0
2dfd	32.3	37.6	28.6	1.5	0.0	0.0
2fn7	59.3	21.0	16.0	3.7	0.0	0.0
2g76	0.0	83.8	16.2	0.0	0.0	0.0
2hjr	52.7	21.8	0.0	18.2	0.0	7.3
2i6t	11.3	15.1	39.6	34.0	0.0	0.0
3i0p	23.1	63.8	9.2	3.8	0.0	0.0
4h7p	21.2	31.5	0.0	47.3	0.0	0.0
4mdh	9.4	56.3	25.8	8.6	0.0	0.0
4plh	54.8	42.5	2.7	0.0	0.0	0.0
4plt	41.7	24.2	30.8	3.3	0.0	0.0

4uuo	28.6	57.1	0.0	14.3	0.0	0.0
5nue	45.8	7.6	45.8	0.8	0.0	0.0
5zi2	29.7	2.1	17.2	51.0	0.0	0.0
6um4	23.0	44.0	21.0	12.0	0.0	0.0
7mdh	40.7	11.1	35.8	12.3	0.0	0.0
MD_eu_AV	31.7	34.4	21.1	12.4	0.0	0.4

Domains of life and enzyme class specific representative protein's normalized frequency of interacting water in different types of cavities and their average (grey row) value.

Table 10 shows the normalized quantitative details on cavity SW and residue for candidate proteins of the domain-specific protein family of our database. Several points are noteworthy from the table. First, a cavity is a homogenous or heterogeneous structural unit formed by region-specific protein atoms. In addition to these, SW can also be a component. Irrespective of domains of life and enzyme families, $1/4^{th}$ to $1/5^{th}$ of total residues of a protein can participate in cavity. More than half of which are of the hydrophobic type (Table 10; Table 11). Second, at the same time, on average, ~20% of the SW of protein also participates in cavity formation. Notably, in all enzyme classes, the SW of cavity of archaea is much less than that of ba and eu. However, about 70% of the total cavities of a protein are filled with SW and the rests are empty. Although lower in the case of archaea, the average interaction multiplicity of SW and protein atoms is ~3. Third, in cavity, the predominance of atoms in the helix is greater (~43%) than that of the strand (28%) and coil (28%). Similarly, the atoms in the co are much higher (~76%) in the cavity than that in the rm and su. Forth, a typical cavity is shown in figure 9a. It is formed by atoms of residues from the co, rm, and su regions (Table 12). The majority of the atoms are present in the helix. Cavity SWs that are present inside the cavity have much higher multiplicity than outside (Figure 9a and Table 5). Since more cavities (~76%) are present in the co, and since more are filled with SW (~70%), their role in the overall property and stability of the co is immense. Finally, it can be said that the characteristics of this cavity in a class of three enzymes regardless of the domains of life follow a certain pattern.

Table 10: Details of average cavity compositions along with standard deviation.

	ADH (average %)			MDH (average %)			GDH (average %)		
Items	**ar (n=7)**	**ba (n=9)**	**eu (n=14)**	**ar (n=7)**	**ba (n=22)**	**eu (n=18)**	**ar (n=5)**	**ba (n=11)**	**eu (n=15)**
R‡	20.7±5.9	20.3±4.6	20.9±3.7	20.2±4.8	20.8±5.4	23.0±4.6	20.3±6.5	22.7±3.9	25.2±3.7
R_hb	14.2±4.0	13.1±2.9	12.8±2.6	13.9±3.5	14.8±3.4	15.1±2.4	12.1+_5.2	13.0±2.8	15.2±2.7
R_po	4.9±1.4	3.9±1.3	4.8±1.3	3.1±1.3	4.3±1.7	4.3±1.4	3.9±1.0	5.3±1.4	5.3±0.9
R_cr	3.6±1.3	3.3±0.9	3.2±1.3	3.2±1.2	3.8±1.5	3.6±2.2	4.2±0.4	4.4±1.3	4.6±0.8
Fr(W)#	18.1±6.1	23.9±11.5	24.2±15.0	15.0±6.8	22.1±10.6	26.1±13.0	16.9±10.5	19.0±7.0	20.2±8.5
FillW#	70.7±18.2	69.7±20.5	77.1±17.0	54.5±30.6	73.8±12.3	68.9±20.1	61.8±20.9	71.6±20.0	64.4±10.0
Mul#	2.9±0.4	3.1±0.5	3.1±0.6	2.8±0.7	2.8±0.4	3.0±0.4	2.4±0.3	3.0±0.5	2.9±0.4
H*	43.2±16.0	39.8±16.1	34.9±7.3	48.3±11.9	47.4±7.8	48.9±8.4	42.9±8.8	40.3±9.4	40.7±5.6
S*	24.8±10.6	27.6±9.6	23.0±9.3	31.7±14.2	32.1±8.3	25.6±7.3	27.3±6.7	31.3±7.1	33.3±7.5

C*	32.0±10.6	32.7±8.7	42.1±12.0	20.0±21.4	20.5±10.1	25.5±9.4	29.8±7.2	28.3±8.2	26.1±7.5
co*	73.1±6.2	79.3±5.7	76.9±5.6	79.6±11.0	78.6±6.1	76.6±5.7	78.6±6.1	71.3±5.6	73.2±5.8
rm*	16.0±5.0	12.8±4.4	14.6±4.1	13.6±5.6	12.9±4.6	14.2±3.9	11.4±4.2	17.5±4.1	15.7±3.8
su*	10.9±2.7	7.9±3.6	8.5±2.3	6.9±7.3	8.5±3.9	9.3±4.2	10.0±4.4	11.2±4.4	11.1±4.7

The normalization is done by total cavity atoms of a protein. ‡Unique residues in cavities in reference to total residue of a protein. #Unique waters in reference to total shell-water. R, amino acid residue; W, Shell-water; fr(W), % fraction of shell-water in cavity (i.e. cavW*100/totW); Mul, Multiplicity; H, Helix; S, strand; C, Coil; co, core; rm, rim; su, surface

The average measurement of items of the cavity in the enzyme class and domains of life-specific manner. Items include residue (R) class (hydrophobic, R_hb; polar, R_po; charged, R-cr), SW (fractional frequency fr(W) in %; frequency of the SW-filled cavity, fillW; interaction multiplicity of SW, mul), secondary structure (helix, H; strand, S; coil, C), and accessibility regions (co, rm and su).

Table 11: Details of normalized residue content in the cavity.

Residue	ADHar		ADHba		ADHeu		mdhar		mdhba		mdheu		GDH-ar		GDH-ba		GDH-eu	
	av%	sd	%av	sd	av%	sd	av	sd	av	sd	av	sd	av	sd	av	sd	av	sd
A	2.3	1.0	2.0	1.0	1.3	0.7	1.6	0.9	2.2	0.8	1.4	0.6	1.8	1.1	1.7	0.6	2.3	0.8
V	2.8	0.9	2.7	1.0	2.2	1.1	2.7	1.1	2.8	0.9	2.8	1.0	2.5	0.9	2.5	0.6	3.3	1.2
L	3.2	1.2	2.5	1.0	2.6	1.2	2.5	1.2	3.0	1.5	3.1	1.1	2.2	1.0	3.0	0.4	2.9	0.7
I	1.9	1.0	1.4	0.9	2.1	1.1	3.0	1.0	2.5	0.9	2.7	1.2	3.3	1.6	2.2	0.8	2.6	0.6
F	1.1	0.8	1.1	0.5	1.5	0.7	0.8	0.6	1.1	0.6	1.1	0.6	0.5	0.3	0.9	0.5	1.2	0.6
M	0.5	0.5	0.5	0.6	0.6	0.3	0.8	0.5	0.6	0.5	0.7	0.3	0.3	0.2	0.5	0.3	0.9	0.5
C	0.1	0.2	0.5	0.3	0.7	0.4	0.1	0.2	0.3	0.2	0.5	0.3	0.0	0.1	0.4	0.2	0.6	0.4
P	1.1	0.4	0.7	0.4	0.6	0.4	0.7	0.4	0.8	0.5	0.8	0.5	0.8	0.9	0.5	0.3	0.3	0.3
G	1.3	0.8	1.7	0.7	1.4	0.6	1.6	0.8	1.6	0.9	1.3	0.4	0.7	1.0	1.4	1.0	1.3	0.6
S	1.0	0.5	0.8	0.6	1.3	0.7	0.8	0.4	0.9	0.5	1.0	0.6	0.6	0.5	1.2	0.5	1.0	0.5
T	1.0	0.4	1.1	0.5	1.1	0.5	0.5	0.4	1.3	0.5	1.2	0.5	0.8	0.3	1.0	0.7	1.2	0.5
N	0.6	0.2	0.4	0.4	1.0	0.9	0.6	0.5	0.5	0.7	1.0	0.4	1.0	0.4	1.3	0.7	1.2	0.4
Q	0.7	0.4	0.4	0.4	0.5	0.4	0.3	0.4	0.4	0.4	0.5	0.4	0.3	0.2	0.4	0.3	0.6	0.3
Y	1.1	0.4	0.9	0.6	0.5	0.4	0.7	0.6	0.8	0.5	0.7	0.4	1.1	0.9	1.2	0.4	1.1	0.5
W	0.5	0.5	0.3	0.3	0.3	0.2	0.2	0.3	0.3	0.4	0.4	0.4	0.2	0.2	0.3	0.2	0.2	0.2
H	0.5	0.4	0.5	0.2	0.5	0.3	0.3	0.2	0.4	0.4	0.5	0.3	0.5	0.3	0.3	0.3	0.4	0.4
R	0.6	0.3	0.9	0.6	0.5	0.4	0.9	0.5	0.9	0.8	0.9	0.5	1.4	0.2	1.4	0.4	1.5	0.3
K	0.8	0.8	0.6	0.2	0.9	0.4	0.7	0.7	0.7	0.4	0.9	0.9	0.7	0.2	0.9	0.5	1.0	0.5
E	1.1	0.6	0.6	0.6	0.7	0.4	0.6	0.6	0.8	0.4	0.8	0.6	0.8	0.1	0.7	0.5	0.4	0.3
D	0.6	0.2	0.7	0.4	0.6	0.6	0.7	0.4	1.0	0.5	0.8	0.4	0.9	0.1	1.1	0.4	1.3	0.3

Domains of life and enzyme class-specific normalized residue content (%) in the cavity along with standard deviation.

Table 12: Details of region (reg) specific atoms of a cavity.

	ATOM		RES	CH	ID	X	Y	Z	ASA	SS	Reg	
ATOM	1	CZ	TYR	A	24	77.200	-11.273	-16.829	23.5	C	rm	C
ATOM	2	OH	TYR	A	24	77.356	-12.108	-15.712	23.5	C	rm	O
ATOM	3	CE2	TYR	A	24	76.793	-9.948	-16.667	23.5	C	rm	C
ATOM	4	CA	LEU	A	60	78.264	-6.035	-13.663	0.0	H	co	C
ATOM	5	CD2	LEU	A	60	78.671	-7.963	-11.132	0.0	H	co	C
ATOM	6	O	LEU	A	60	77.763	-7.028	-15.778	0.0	H	co	O
ATOM	7	CA	GLY	A	63	80.567	-7.435	-18.164	31.5	H	rm	C
ATOM	8	N	TRP	A	65	82.969	-8.397	-14.317	19.3	H	rm	N
ATOM	9	CB	TRP	A	65	82.252	-9.140	-12.098	19.3	H	rm	C
ATOM	10	C	TRP	A	65	82.935	-10.842	-13.793	19.3	H	rm	C
ATOM	11	O	TRP	A	65	82.948	-11.793	-13.012	19.3	H	rm	O
ATOM	12	N	HIS	A	66	82.670	-11.033	-15.100	39.8	H	su	N
ATOM	13	CA	HIS	A	66	82.287	-12.325	-15.659	39.8	H	su	C
ATOM	14	CB	HIS	A	66	81.866	-12.148	-17.141	39.8	H	su	C
ATOM	15	CB	LEU	A	69	82.273	-13.356	-10.014	31.6	H	rm	C
ATOM	16	CD	PRO	A	71	79.096	-14.930	-13.625	11.3	C	co	C
ATOM	17	CG	PRO	A	71	77.727	-14.363	-13.269	11.3	C	co	C
ATOM	18	OH	TYR	A	75	75.691	-9.963	-12.033	1.2	S	co	O
ATOM	19	O	LEU	A	131	78.065	-11.628	-8.947	9.5	C	co	O
TER	20		LEU	A	131							
HETATM	3	O	HOH	A	2004	77.948	-10.686	-13.444	3.3	6	O	
HETATM	3	O	HOH	A	2045	79.476	-9.082	-15.125	3.4	8	O	
HETATM	3	O	HOH	A	2047	84.703	-10.446	-17.620	3.4	3	O	
HETATM	3	O	HOH	A	2048	86.349	-10.838	-14.422	3.6	3	O	
END												

A typical cavity in PDB format (auto generated by the AWK script) along with residue specific accessibility (ASA), secondary structure (SS) and region (co, rm and su) specific values. There are about 2000 such PDB for all proteins (Table S1). These files were further analyzed for cavity types, inside and outside water frequency, region specificity using fully-automated script.

Region-specific non-bonded interaction

The three-dimensional structure of proteins is formed by weak force or non-bonded interactions such as hydrogen bonds, electrostatic (salt-bridge; ion-pair; π-cation; π-anion, etc.), hydrophobic (π-π; π-amide; π-σ; π--alkyl; alkyl-alkyl, etc.), etc. [8, 18-24]. Hydrogen bonds are formed with different types of donor and acceptor atoms (main-chain, side-chain polar, carbon, π-systems, and SW).

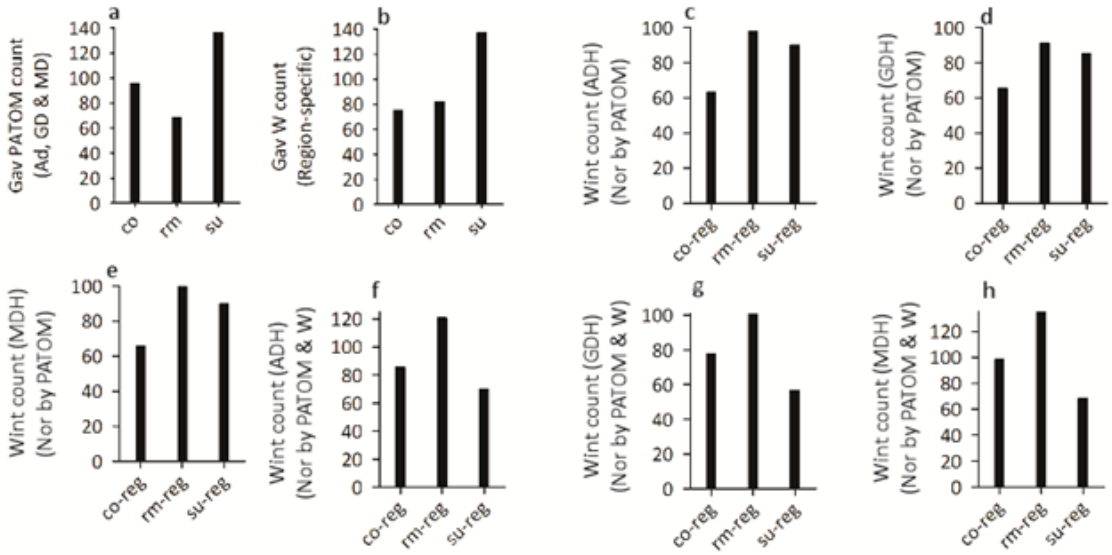


Figure 12. Protein region-specific overall frequencies of polar atoms.

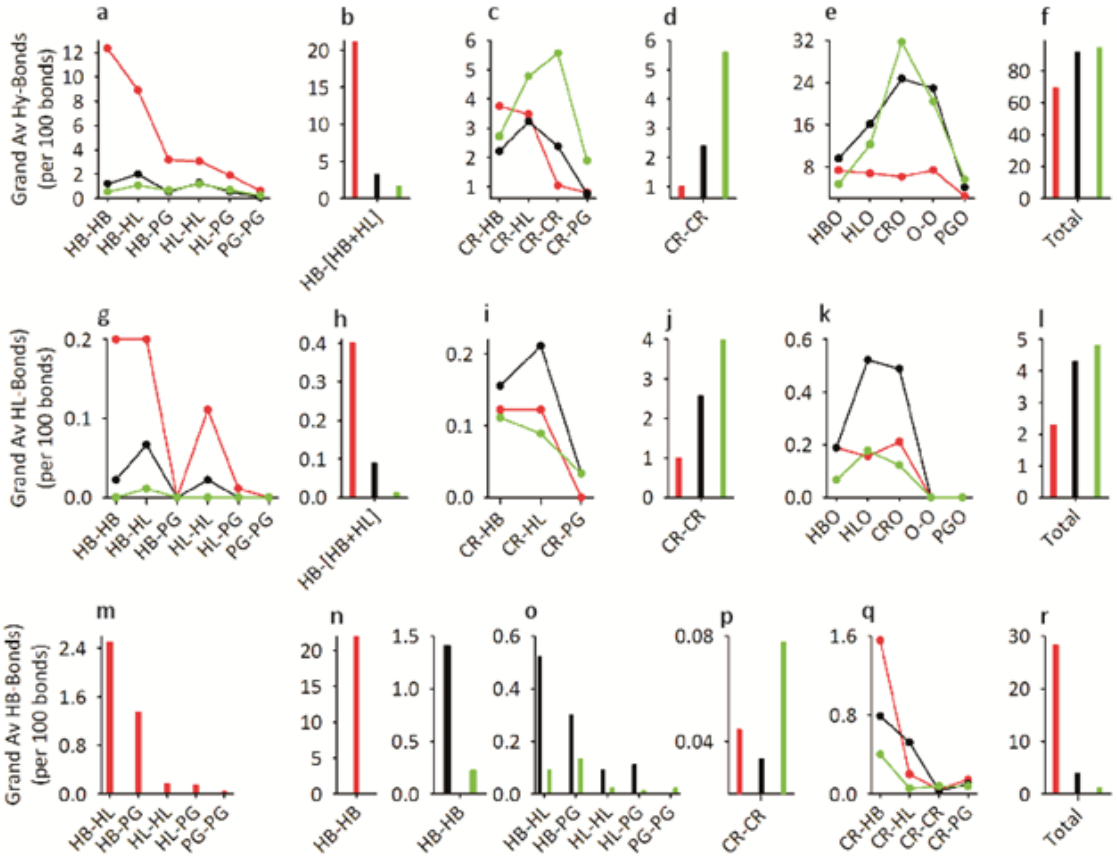


Figure 13. Normalized average nNon-bonded interactions.

Region specific grand average (irrespective of domains of life and enzyme classes) counts of protein polar atoms (PATOM) (**a**). Region specific grand average (irrespective of domains of life and enzyme classes) counts of total shell-waters (W) (**b**). PATOM normalized PATOM-

interacting Wint counts in ADH (**c**), GDH (**d**) and MDH (**e**). Both PATOM and W normalized Wint counts in ADH (**f**), GDH (**g**) and MDH (**h**).

Grand average frequency of hydrogen-bond (**a-f**), other hydrophilic (**g-l**) and hydrophobic (**m-s**) interactions regardless of enzyme classes and domains of life in different types of inter-residue connections (hydrophobic residue with hydrophobic residue i.e. HB-HB; hydrophobic residue with charged residue i.e. HB-CR, and, etc.). The interaction of each type is broadly divided into four groups namely hydrophobic and hydrophilic classes; charged class, and SW mediated, and total for each group. Some region-specific plots have been plotted together and some are plotted separately for the convenience of comparison.

Of the three regions of the protein (co, rm, and su), the grand average absolute frequency of protein atoms and SW is the highest in the su-region (Figure 12a-b). Notably, although rm is significantly less dominant in protein residues, residue-classes, and secondary structures than co and su, normalized (either by only protein atoms or both by protein atoms and total SW of protein) average SW is higher than even the su (Figure 12c-e and h-j). Here, we have compared the normalized grand average of different types of inter-residue interaction for the three regions. In the case of hydrogen bonds, in co, HB-HB, HB-HL, HB-PG, HL-HL, HL-PG, and PG-PG types dominate (Figure 13a and Table 13-14) over other regions. rm maintains an intermediate level, especially for HB-HB and HB-HL types (Figure 13b). On the other hand, except for CR-HB type, which is the highest in the co (Figure 13c), other CR types hydrogen bond (CR-HL, CR-CR, and CR-PG) are the highest in the su-region (Figure 13c, d, and Table 12-13). Here too, the rm-region is at an intermediate level (Figure 13d). Interestingly, in the case of SW-mediated hydrogen bonds, in the case of other types except for CRO, the rm again exceeds su's level so that the hydrogen bond levels of these two regions are equivalent (Figure 3e, f). In this case, it is clear that while co and su dominate in HB- and CR-mediated hydrogen bonds respectively, rm, in turn, acts as an intermediate. As far as electrostatic interactions (salt-bridge, ion-pair, cation-π, anion-π pi-sulfur, π-HB, etc.) are concerned, except for su dominating CR-CR type (Figure 3j), while HB-HB, HB-HL, and HL-HL types dominate in co (Figure 13g, h), CR-HB, CR-HL types are higher in the rm region (Figure 13i). In the case of SW-mediated electrostatic interactions, rm, as before, is dominant (Figure 13k). In this case, too, although rm is of intermediate level, the total interaction is almost equivalent to that of su (Figure 13l). Similarly, in hydrophobic interactions (π-π, π-σ, π-amide, π-alkyl, and alkyl-alkyl) especially for the HB-HB, HB-HL, HB-CR types co is highest, which is followed by the rm-region (Figure 13m-p). Notably, su and rm regions maintain the highest level of interaction for CR-CR and CR-HB types, respectively (Figure 13q, r). All in all, this rm region maintains an intermediate level here as well (Figure 13s). It should be noted here that the types that have low frequencies show a much lower level due to normalization based on the total interactions. We see that rm dominates in many cases in SW-mediated and mixed types (HB-CR and HB-HL etc) inter-residue interactions (Figure 13 and Table 13-14).

Table 13: ASA-region-specific interactions.

Total Int→	**2744**			**883**			**1631**		
ADH_ar_reg	**co**	**rm**	**su**	**co**	**rm**	**su**	**co**	**rm**	**su**
Types	Hydrogen bond category			Hydrophobic category			Hydrophilic Category		
HB-HB	354	7	6	589	8	5	6	0	0
PG-PG	17	2	1	2	0	1	0	0	0
HL-HL	68	16	9	10	1	1	2	0	0
CR-CR	22	21	100	3	0	1	24	21	77
HB-PG	100	2	6	40	4	2	0	0	0
HB-HL	204	11	21	86	6	4	11	2	0
HB-CR	109	23	32	35	5	5	6	2	2
HL-PG	60	10	8	8	2	0	0	0	0
CR-PG	33	7	30	8	2	3	0	2	2
CR-HL	104	23	63	6	10	2	5	2	1
HBO	189	107	76	0	0	0	3	1	1
PGO	73	37	102	0	0	0	0	0	0
HLO	168	133	179	0	0	0	7	12	4
CRO	175	206	552	0	0	0	7	2	4
O-O	210	196	331	0	0	0	0	0	0
Total Int→	**1886**	**801**	**1516**	**787**	**38**	**24**	**71**	**44**	**91**
Total Int	**2152**			**697**			**1488**		
GDH_ar_reg	co	rm	su	co	rm	su	co	rm	su
Types	Hydrogen bond category			Hydrophobic category			Hydrophilic Category		
HB-HB	216	11	6	424	19	5	1	0	0
PG-PG	2	1	1	5	0	1	0	0	0
HL-HL	81	6	11	0	2	1	1	0	0
CR-CR	62	24	105	2	0	2	65	19	77
HB-PG	54	4	8	29	2	6	0	0	0
HB-HL	154	30	11	62	2	3	1	1	0
HB-CR	101	19	42	50	6	9	3	1	2
HL-PG	26	4	6	4	0	0	0	0	0
CR-PG	22	4	32	0	0	0	0	0	1
CR-HL	129	14	53	8	1	0	4	0	1
HBO	149	71	60	0	0	0	2	2	1
PGO	41	33	72	0	0	0	0	0	0
HLO	138	107	149	0	0	0	1	0	5
CRO	168	176	551	0	0	0	1	1	1
O-O	146	137	266	0	0	0	0	0	0
Total Int→	**1489**	**641**	**1373**	**584**	**32**	**27**	**79**	**24**	**88**

Total Int	**1875**			**703**			**1900**		
MDH_ar_reg	co	rm	su	co	rm	su	co	rm	su
Types	Hydrogen bond category			Hydrophobic category			Hydrophilic Category		
HB-HB	267	12	12	462	4	5	4	0	0
PG-PG	30	0	7	0	0	0	0	0	0
HL-HL	47	6	19	2	0	0	1	0	0
CR-CR	10	11	126	0	0	2	7	18	61
HB-PG	69	6	10	32	1	0	0	0	0
HB-HL	194	14	12	29	6	0	4	0	0
HB-CR	51	19	50	19	7	7	3	2	0
HL-PG	42	2	16	3	1	0	0	0	0
CR-PG	12	3	46	0	0	2	0	0	0
CR-HL	33	30	109	2	5	4	2	5	7
HBO	126	40	63	0	0	0	1	0	2
PGO	66	22	73	0	0	0	0	0	0
HLO	104	99	185	0	0	0	1	3	0
CRO	97	205	637	0	0	0	3	8	0
O-O	152	174	445	0	0	0	0	0	0
Total Int→	**1300**	**643**	**1810**	**549**	**24**	**20**	**26**	**36**	**70**
Total Int	**3472**			**994**			**1379**		
ADH_ba_reg	co	rm	su	co	rm	su	co	rm	su
Types	Hydrogen bond category			Hydrophobic category			Hydrophilic Category		
HB-HB	455	9	3	866	11	3	7	0	0
PG-PG	27	3	4	0	0	0	0	0	0
HL-HL	84	10	18	10	0	0	4	0	0
CR-CR	30	30	103	1	2	2	33	33	77
HB-PG	144	3	14	51	10	3	0	0	0
HB-HL	282	14	7	80	5	0	11	0	0
HB-CR	134	27	30	40	12	6	10	1	2
HL-PG	64	4	10	3	3	1	3	0	0
CR-PG	40	15	32	7	4	2	0	0	0
CR-HL	120	25	77	5	2	0	3	1	0
HBO	211	89	86	0	0	0	8	1	1
PGO	93	53	65	0	0	0	0	0	0
HLO	249	106	162	0	0	0	12	0	5
CRO	175	285	400	0	0	0	10	2	3
O-O	200	234	263	0	0	0	0	0	0
Total	**2308**	**907**	**1274**	**1063**	**49**	**17**	**101**	**38**	**88**

Total Int→	**4008**			**1456**			**3032**		
GDH_ba_reg	co	rm	su	co	rm	su	co	rm	su
Types	Hydrogen bond category			Hydrophobic category			Hydrophilic Category		
HB-HB	440	17	23	795	20	2	8	0	0
PG-PG	7	0	3	0	0	0	0	0	0
HL-HL	196	13	30	3	0	0	7	1	1
CR-CR	50	45	133	3	5	2	23	41	112
HB-PG	94	10	19	33	4	0	0	0	0
HB-HL	379	19	31	114	6	2	8	0	0
HB-CR	163	23	76	81	12	11	6	3	0
HL-PG	55	4	15	1	1	0	0	0	0
CR-PG	19	11	42	10	0	0	0	0	2
CR-HL	202	65	143	13	7	8	8	1	3
HBO	286	122	168	0	0	0	9	3	4
PGO	72	52	209	0	0	0	0	0	0
HLO	350	266	405	0	0	0	6	9	1
CRO	238	339	928	0	0	0	12	14	3
O-O	317	343	656	0	0	0	0	0	0
Total	**2868**	**1329**	**2881**	**1053**	**55**	**25**	**87**	**72**	**126**
Total Int→Int	**7218**			**2594**			**6138**		
MDH_ba_reg	co	rm	su	co	rm	su	co	rm	su
Types	Hydrogen bond category			Hydrophobic category			Hydrophilic Category		
HB-HB	983	26	51	1669	33	24	9	0	0
PG-PG	64	0	29	1	0	0	0	0	0
HL-HL	131	14	63	10	2	0	2	0	0
CR-CR	25	53	230	1	0	7	48	62	183
HB-PG	234	9	81	136	9	11	0	0	0
HB-HL	686	38	121	124	8	1	14	0	0
HB-CR	220	39	249	89	25	11	6	8	5
HL-PG	148	6	58	17	3	1	0	0	0
CR-PG	33	21	113	6	1	2	0	0	1
CR-HL	123	73	278	12	6	0	2	6	3
HBO	603	243	297	0	0	0	12	9	7
PGO	224	109	335	0	0	0	0	0	0
HLO	451	407	768	0	0	0	17	19	7
CRO	466	698	1847	0	0	0	6	4	3
O-O	646	663	1352	0	0	0	0	0	0
Total	**5037**	**2399**	**5872**	**2065**	**87**	**57**	**116**	**108**	**209**

Total Int→	**7559**			**2629**			**4300**		
ADH_eu_reg	co	rm	su	co	rm	su	co	rm	su
Types	Hydrogen bond category			Hydrophobic category			Hydrophilic Category		
HB-HB	885	31	18	1626	30	4	29	0	0
PG-PG	54	2	11	2	1	1	0	0	0
HL-HL	225	52	27	15	0	1	6	1	0
CR-CR	56	45	208	8	0	3	75	52	173
HB-PG	285	19	12	75	3	2	0	0	0
HB-HL	642	38	33	182	10	6	16	2	0
HB-CR	273	49	73	121	18	10	16	8	3
HL-PG	168	17	23	13	5	1	0	0	0
CR-PG	105	22	62	16	8	7	1	0	2
CR-HL	189	79	169	10	6	2	5	4	2
HBO	618	312	198	0	0	0	23	4	1
PGO	236	114	243	0	0	0	0	0	0
HLO	513	436	502	0	0	0	13	17	4
CRO	441	569	1495	0	0	0	29	5	8
O-O	588	670	996	0	0	0	0	0	0
Total	**5278**	**2455**	**4070**	**2068**	**81**	**37**	**213**	**93**	**193**
Total Int→	**5211**			**1842**			**3480**		
GDH_eu_reg	co	rm	su	co	rm	su	co	rm	su
Types	Hydrogen bond category			Hydrophobic category			Hydrophilic Category		
HB-HB	610	26	7	1085	37	4	12	3	0
PG-PG	8	0	5	0	0	0	0	0	0
HL-HL	222	28	71	3	0	1	12	1	0
CR-CR	75	42	147	0	2	0	36	55	133
HB-PG	113	8	15	36	14	4	0	0	1
HB-HL	500	44	29	186	4	1	8	2	1
HB-CR	231	34	87	104	12	16	3	1	2
HL-PG	67	12	27	0	2	0	1	0	0
CR-PG	27	10	54	15	0	2	0	0	1
CR-HL	233	86	151	18	4	3	12	2	0
HBO	423	203	187	0	0	0	16	5	0
PGO	73	67	260	0	0	0	0	0	0
HLO	375	366	550	0	0	0	4	15	12
CRO	347	354	1060	0	0	0	8	14	6
O-O	348	389	643	0	0	0	0	0	0
Total	**3652**	**1669**	**3293**	**1447**	**75**	**31**	**112**	**98**	**156**

Total Int→	6160			1798			3801		
MDH_eu_reg	co	rm	su	co	rm	su	co	rm	su
Types	Hydrogen bond category			Hydrophobic category			Hydrophilic Category		
HB-HB	799	20	43	1463	30	9	15	0	0
PG-PG	38	3	16	0	0	0	0	0	0
HL-HL	139	37	96	6	0	1	11	0	0
CR-CR	46	35	215	2	1	2	57	36	160
HB-PG	197	13	37	114	4	8	0	0	0
HB-HL	618	45	76	113	9	2	17	1	2
HB-CR	210	44	155	94	17	23	9	2	8
HL-PG	171	12	45	21	1	0	0	0	0
CR-PG	21	11	81	4	4	1	0	1	0
CR-HL	156	48	260	12	8	0	7	3	4
HBO	467	155	144	0	0	0	12	4	0
PGO	127	74	189	0	0	0	0	0	0
HLO	418	344	546	0	0	0	8	4	8
CRO	372	452	988	0	0	0	8	11	5
O-O	408	369	677	0	0	0	0	0	0
Total	**4187**	**1662**	**3568**	**1829**	**74**	**46**	**144**	**62**	**187**

Non-bonded interactions for the co (core), rm (rim) and su (surface) accessibility regions of ADH, GDH and MDH of ar, ba and eu. These interactions are subcategorized as hydrogen bond (HyB), hydrophobic (hb) such as π-sigma (PS), π-π (PP), amide-π (AP), alkyl-alkyl (AL), π-alkyl (PA), and hydrophilic (hl) such as π-sulfur, π-cation (PC), π-anion (PA), π-hydrogen bond (PH), salt-bridge (SB), ion-pair (IP). Each of these interaction category is classified based on the protein's residue group such as hydrophobic-hydrophobic (HB-HB), Pro and Gly (PG-PG), Hydrophilic-Hydrophilic (HL-HL), Charged-charged (CR-CR), and other inter class residues interactions (HB-PG, HB-HL, HB-CR, HL-PG, CR-PG, CR-H). Hydrophobic-shell-water (HBO), PG-shell-water (PGO), Hydrophilic-shell-water (HLO), Charged-Shell-water (CRO), and shell-water-shell-water (O-O) types of interactions are also assessed. For each accessibility region specific PDB file (co-PDB, rm-PDB and su-PDB), all these distance dependent interactions were initially determined in the Biovia Discovery Studio Visualizer v20.1.0.19295. The interaction-detailed file thus obtained was formatted to analyze automatically using home-built AWK-script. The absolute interaction values (frequency of interaction) are placed in the table.

Table 14: domain-specific ASA-specific weak interactions.

HyB	arCOad	arCOgd	arCOmd	raRMad	arRMgd	arRMmd	arSUad	arSUgd	arSUmd
HB-HB	12.9	10	14.2	0.8	1.6	1.7	0.4	0.4	0.6
PG-PG	0.6	0.1	1.6	0.2	0.1	0	0.1	0.1	0.4
HL-HL	2.5	3.8	2.5	1.8	0.9	0.9	0.6	0.7	1
CR-CR	0.8	2.9	0.5	2.4	3.4	1.6	6.1	7.1	6.6

HB-PG	3.6	2.5	3.7	0.2	0.6	0.9	0.4	0.5	0.5
HB-HL	7.4	7.2	10.3	1.2	4.3	2	1.3	0.7	0.6
HB-CR	4	4.7	2.7	2.6	2.7	2.7	2	2.8	2.6
HL-PG	2.2	1.2	2.2	1.1	0.6	0.3	0.5	0.4	0.8
CR-PG	1.2	1	0.6	0.8	0.6	0.4	1.8	2.2	2.4
CR-HL	3.8	6	1.8	2.6	2	4.3	3.9	3.6	5.7
HBO	6.9	6.9	6.7	12.1	10.2	5.7	4.7	4	3.3
PGO	2.7	1.9	3.5	4.2	4.7	3.1	6.3	4.8	3.8
HLO	6.1	6.4	5.5	15.1	15.4	14.1	11	10	9.7
CRO	6.4	7.8	5.2	23.3	25.3	29.2	33.8	37	33.5
O-O	7.7	6.8	8.1	22.2	19.7	24.8	20.3	17.9	23.4

HyB	baCOad	baCOgd	baCOmd	baRMad	baRMgd	baRMmd	baSUad	baSUgd	baSUmd
HB-HB	13.1	11	13.6	0.9	1.2	1	0.2	0.8	0.8
PG-PG	0.8	0.2	0.9	0.3	0	0	0.3	0.1	0.5
HL-HL	2.4	4.9	1.8	1	0.9	0.5	1.3	1	1
CR-CR	0.9	1.2	0.3	3	3.1	2	7.5	4.4	3.7
HB-PG	4.1	2.3	3.2	0.3	0.7	0.3	1	0.6	1.3
HB-HL	8.1	9.5	9.5	1.4	1.3	1.5	0.5	1	2
HB-CR	3.9	4.1	3	2.7	1.6	1.5	2.2	2.5	4.1
HL-PG	1.8	1.4	2.1	0.4	0.3	0.2	0.7	0.5	0.9
CR-PG	1.2	0.5	0.5	1.5	0.8	0.8	2.3	1.4	1.8
CR-HL	3.5	5	1.7	2.5	4.5	2.8	5.6	4.7	4.5
HBO	6.1	7.1	8.4	9	8.4	9.4	6.2	5.5	4.8
PGO	2.7	1.8	3.1	5.3	3.6	4.2	4.7	6.9	5.5
HLO	7.2	8.7	6.2	10.7	18.3	15.7	11.7	13.3	12.5
CRO	5	5.9	6.5	28.7	23.3	26.9	29	30.6	30.1
O-O	5.8	7.9	8.9	23.5	23.6	25.6	19.1	21.6	22

HyB	euCOad	euCOgd	euCOmd	euRMad	euRMgd	euRMmd	euSUad	euSUgd	euSUmd
HB-HB	11.7	11.7	13	1.2	1.4	1.1	0.4	0.2	1.1
PG-PG	0.7	0.2	0.6	0.1	0	0.2	0.3	0.1	0.4
HL-HL	3	4.3	2.3	2	1.5	2.1	0.6	2	2.5
CR-CR	0.7	1.4	0.7	1.7	2.3	1.9	4.8	4.2	5.7
HB-PG	3.8	2.2	3.2	0.7	0.4	0.7	0.3	0.4	1
HB-HL	8.5	9.6	10	1.4	2.4	2.5	0.8	0.8	2
HB-CR	3.6	4.4	3.4	1.9	1.8	2.4	1.7	2.5	4.1
HL-PG	2.2	1.3	2.8	0.6	0.7	0.7	0.5	0.8	1.2
CR-PG	1.4	0.5	0.3	0.8	0.5	0.6	1.4	1.6	2.1

CR-HL	2.5	4.5	2.5	3	4.7	2.7	3.9	4.3	6.8
HBO	8.2	8.1	7.6	11.9	11	8.6	4.6	5.4	3.8
PGO	3.1	1.4	2.1	4.3	3.6	4.1	5.7	7.5	5
HLO	6.8	7.2	6.8	16.6	19.9	19.1	11.7	15.8	14.4
CRO	5.8	6.7	6	21.6	19.2	25.1	34.8	30.5	26
O-O	7.8	6.7	6.6	25.5	21.1	20.5	23.2	18.5	17.8

HL	arCOad	arCOgd	arCOmd	arRMad	arRMgd	arRMmd	arSUad	arSUgd	arSUmd
HB-HB	0.2	0	0.2	0	0	0	0	0	0
PG-PG	0	0	0	0	0	0	0	0	0
HL-HL	0.1	0	0.1	0	0	0	0	0	0
CR-CR	0.9	3	0.4	2.4	2.7	2.5	4.8	5.2	3.2
HB-PG	0	0	0	0	0	0	0	0	0
HB-HL	0.4	0	0.2	0.2	0.1	0	0	0	0
HB-CR	0.1	0.1	0.3	0.2	0.1	0.2	0.2	0.2	0
HL-PG	0	0	0	0	0	0	0	0	0
CR-PG	0	0	0	0.2	0	0	0.1	0.1	0
CR-HL	0.1	0.1	0.2	0.2	0	0.7	0.1	0.1	0.4
HBO	0.1	0.1	0.1	0.1	0.3	0	0.1	0.1	0.1
PGO	0	0	0	0	0	0	0	0	0
HLO	0.3	0	0.1	1.4	0	0.4	0.2	0.3	0
CRO	0.3	0	0.2	0.2	0.1	1.1	0.2	0.1	0
O-O	0	0	0	0	0	0	0	0	0

HL	baCOad	baCogd	baCOmd	baRMad	baRMgd	baRMmd	baSUad	baSUgd	baSUmd
HB-HB	0.2	0.2	0.1	0	0	0	0	0	0
PG-PG	0	0	0	0	0	0	0	0	0
HL-HL	0.1	0.2	0	0	0.1	0	0	0	0
CR-CR	1	0.6	0.6	3.3	2.8	2.3	5.6	3.6	2.9
HB-PG	0	0	0	0	0	0	0	0	0
HB-HL	0.3	0.1	0.2	0	0	0	0	0	0
HB-CR	0.3	0.1	0	0.1	0.2	0.3	0.2	0	0.1
HL-PG	0.1	0	0	0	0	0	0	0	0
CR-PG	0	0	0	0	0	0	0	0.1	0
CR-HL	0.1	0.2	0	0.1	0.1	0.3	0	0.1	0
HBO	0.2	0.2	0.2	0.1	0.2	0.3	0.1	0.1	0.1
PGO	0	0	0	0	0	0	0	0	0
HLO	0.3	0.1	0.2	0	0.6	0.7	0.4	0	0.1
CRO	0.3	0.3	0.1	0.2	1	0.2	0.2	0.1	0
O-O	0	0	0	0	0	0	0	0	0

HL	euCOad	euCOgd	euCOmd	euRMad	euRMgd	euRMmd	euSUad	euSUgd	euSUmd
HB-HB	0.4	0.3	0.2	0	0.2	0	0	0	0
PG-PG	0	0	0	0	0	0	0	0	0
HL-HL	0.1	0.2	0.2	0	0.1	0	0	0	0
CR-CR	1	0.6	0.9	2	3.1	2	4.1	3.8	4.2
HB-PG	0	0	0	0	0	0	0	0	0
HB-HL	0.2	0.2	0.2	0	0.2	0.1	0	0	0.1
HB-CR	0.1	0	0.1	0.1	0.1	0.1	0	0	0.3
HL-PG	0	0	0	0	0	0	0	0	0
CR-PG	0	0	0	0	0	0.1	0	0	0
CR-HL	0.1	0.2	0.1	0.1	0.2	0.2	0	0	0.1
HBO	0.3	0.3	0.2	0.2	0.3	0.2	0	0	0
PGO	0	0	0	0	0	0	0	0	0
HLO	0.2	0.1	0.1	0.6	0.8	0.2	0.1	0.3	0.2
CRO	0.4	0.2	0.1	0.2	0.8	0.6	0.2	0.2	0.1
O-O	0	0	0	0	0	0	0	0	0

HB	arCOad	arCOgd	arCOmd	arRMad	arRMgd	arRMmd	arSUad	arSUgd	arSUmd
HB-HB	21.5	19.6	24.7	0.9	2.6	0.5	0.4	0.4	0.3
PG-PG	0.1	0.2	0	0	0	0	0.1	0.1	0
HL-HL	0.4	0	0.2	0.1	0.3	0	0.1	0.1	0
CR-CR	0.1	0.1	0	0	0	0	0.1	0.1	0.1
HB-PG	1.4	1.3	1.7	0.4	0.3	0.1	0.1	0.4	0
HB-HL	3.1	2.9	1.7	0.7	0.3	0.8	0.3	0.2	0
HB-CR	1.3	2.2	1	0.5	0.9	1	0.3	0.6	0.4
HL-PG	0.3	0.1	0.2	0.2	0	0.1	0	0	0
CR-PG	0.3	0	0	0.2	0	0	0.2	0	0.2
CR-HL	0.2	0.3	0.2	1.1	0.1	0.7	0.1	0	0.2
HBO	0	0	0	0	0	0	0	0	0
PGO	0	0	0	0	0	0	0	0	0
HLO	0	0	0	0	0	0	0	0	0
CRO	0	0	0	0	0	0	0	0	0
O-O	0	0	0	0	0	0	0	0	0

HB	baCOad	baCOgd	baCOmd	baRMad	baRMgd	baRMmd	baSUad	baSUgd	baSUmd
HB-HB	25	19.7	23.2	1.1	1.4	1.3	0.2	0.1	0.3
PG-PG	0	0	0	0	0	0	0	0	0
HL-HL	0.3	0.1	0.2	0	0	0.1	0	0	0

CR-CR	0	0.1	0	0.2	0.4	0	0.2	0.1	0.1
HB-PG	1.5	0.8	1.9	1	0.3	0.3	0.2	0	0.2
HB-HL	2.3	2.9	1.7	0.5	0.4	0.3	0	0.1	0
HB-CR	1.2	1.9	1.2	1.2	0.9	0.9	0.5	0.4	0.2
HL-PG	0.1	0	0.2	0.3	0.1	0.1	0.1	0	0
CR-PG	0.2	0.2	0	0.4	0	0	0.2	0	0
CR-HL	0.1	0.3	0.1	0.2	0.5	0.2	0	0.2	0
HBO	0	0	0	0	0	0	0	0	0
PGO	0	0	0	0	0	0	0	0	0
HLO	0	0	0	0	0	0	0	0	0
CRO	0	0	0	0	0	0	0	0	0
O-O	0	0	0	0	0	0	0	0	0

HB	**euCOad**	**euCOgd**	**euCOmd**	**euRMad**	**euRMgd**	**euRMmd**	**euSUad**	**euSUgd**	**euSUmd**
HB-HB	21.6	20.8	23.8	1.1	2	1.6	0	0.1	0.2
PG-PG	0	0	0	0	0	0	0	0	0
HL-HL	0.2	0.1	0	0	0	0	0	0	0
CR-CR	0.1	0	0	0	0.2	0.1	0	0	0
HB-PG	1	0.7	1.8	0.1	0.8	0.2	0	0.1	0.2
HB-HL	2.4	3.6	1.9	0.4	0.2	0.5	0.1	0	0.1
HB-CR	1.7	2	1.5	0.7	0.6	1	0.2	0.4	0.6
HL-PG	0.1	0	0.3	0.2	0.1	0.1	0	0	0
CR-PG	0.2	0.3	0.1	0.3	0	0.2	0.1	0	0
CR-HL	0.1	0.3	0.2	0.2	0.2	0.5	0	0	0
HBO	0	0	0	0	0	0	0	0	0
PGO	0	0	0	0	0	0	0	0	0
HLO	0	0	0	0	0	0	0	0	0
CRO	0	0	0	0	0	0	0	0	0
O-O	0	0		0	0	0	0	0	0

Non-bonded interactions for the co (core), rm (rim) and su (surface) accessibility regions of ADH (ad), GDH (gd) and MDH (md) of ar, ba and eu. These interactions are subcategorized as hydrogen bond (HyB), hydrophobic (hb) such as π-sigma (PS), π-π (PP), amide-π (AP), alkyl-alkyl (AL), π-alkyl (PA), and hydrophilic (hl) such as π-sulfur, π-cation (PC), π-anion (PA), π-hydrogen bond (PH), salt-bridge (SB), ion-pair (IP). Each of these interaction categories is classified based on the protein's residue group such as hydrophobic-hydrophobic (HB-HB), Pro and Gly (PG-PG), Hydrophilic-Hydrophilic (HL-HL), Charged-charged (CR-CR), and other inter class residues interactions (HB-PG, HB-HL, HB-CR, HL-PG, CR-PG, CR-H). Hydrophobic-shell-water (HBO), PG-shell-water (PGO), Hydrophilic-shell-water (HLO), Charged-Shell-water (CRO), and shell-water-shell-water (O-O) types of interactions are also assessed. For each accessibility region specific PDB file (co-PDB, rm-PDB and su-PDB), all

these distance dependent interactions were initially determined in the Biovia Discovery Studio Visualizer v20.1.0.19295. The interaction-detailed file thus obtained was formatted to analyze automatically using home-built AWK-script. The absolute interaction values (frequency of interaction) are normalized using the total frequency of interaction. For different domains of life (ar, ba, and eu) the number of PDB file are different and thus, the Ti value is different. Such a variation is normalized using Ti value for comparison purpose.

Discussion

Rim has a distinct composition from the core and surface regions of the protein structure

To gain insights into the pattern of globular protein structures and interactions (Rose et al., 1985; Gutheil et al. 1992; Lins et al., 2003; Sen et al., 2017; Bandyopadhyay et al., 2019; Islam et al., 2019; Bandyopadhyay et al., 2020; Biswas et al., 2020; Banerjee et al., 2021; Roy et al., 2023), we have done the current study of three protein families of three domains of life. Our observation implies that the core and surface of the globular proteins have strong preferences towards hydrophobic and charged classes, respectively. Moreover, the polar class appears to be equally likely for these two regions. We know that the polar, like hydrophobic residues, are equally important in the core's organization (Bolon & Mayo, 2001). At the same time, some polar residues (e.g., Ser) play an important role in protein solubility, like the charged class (Trevino et al., 2007). However, the rim area's KD-neutral (Fleming et al., 2006) nature may indicate that it is a non-preferential region with an equal number of hydrophobic and hydrophilic residues. The existence of such a region was speculated earlier (Lins et al., 2003; Sen Gupta et al., 2017). Although compared to the rim, these differences for the core and the surface are significant in all enzyme cases, the observed variation in its level seems to be originating from the divergence in domain-specific orthologous sequences. Taken together, the existence of the rim between the core and the surface of these three enzyme structures appears to be a general characteristic of globular proteins.

Rim is topologically and conformationally distinct from the core and the surface

The topology of the tertiary structure of the protein is largely determined by the secondary structure (Islam et al., 2018; Mitra et al., 2019). Furthermore, because the strand is extended and the helix is compact (Bolon & Mayo, 2001), these two types of secondary structure may influence protein residue packing. The significant preference for the strand and helix in the core may indicate that they contribute to the core's balance of rigidity and flexibility. At the same time, the preference for coil and helix on the surface could play a comparable role. The preference for secondary structures in the core and on the surface appears to be general phenomena. In the context of protein function, it appears that ar, ba, and eu enzymes preserve the topological pattern of their structures for these accessible regions (core and surface) via creating combinational variations in secondary structures. Notably, the predominance of such distinctive secondary structural preference strategies is less apparent in the rim region. This

could imply that the rim zone serves as a balance between these two regions (co and su) with alternate and similar preferences (for helix) for secondary structures.

Compared to the surface, the interior cavities are more distinctive of the core and rim

The internal cavity, like residue classes and secondary structures, appears to play a crucial role in the region-specific structural organization of globular proteins. To illustrate their numerical abundance, a 100-residue protein may have six to eight cavities. Our findings suggest that the cavity is closely related to the stability of the core in particular. This is most likely why charged residue, which has a surface propensity, is significantly less concentrated in the cavity. These cavities, like the secondary structure, appear to be higher-level, confined structures in the protein folding path. These, on the one hand, limit the movement of secondary structure elements (e.g., helixes) and SW by incorporating them into their structure; while on the other hand, these stability units appear to operate as a limiting step in spontaneous residue packing during the folding process (Eisenberg, 2003; Sadqi et al., 2003). In other words, without these structural units, the protein's core, in particular, could have been significantly more compact, as it contains two-thirds of the total cavities. Most cavities (~70%) trap shell-water to reinforce their structure from inside and yet retain space, particularly in compact cores. This approach is most likely used by Archaea to compact their core structure, as the water-filled cavity is substantially lower than in other domains. Archaea's protein folding environment (such as high temperature) appears to improve the mobility of water and protein components, as opposed to a shell-water trap in the cavity. Because the rim region contains the most shell-water, the water content of the cavity increases when an atom from this region is present. This propensity most likely causes heterogeneity in the cavity at the residue, residue-class, and secondary structure levels. The helix specifies the compact structure, while the cavity includes helix residues in it. Again, hydrophobic residue, which plays an important role in hydrophobic collapse, has been the primary constituent for cavity. According to these events, just as an extended beta-structure with a strong tendency for core provides flexibility at the core, the cavity, with space within itself and the inclusion of compact structure units i.e., helix and hydrophobic residues into its structure, brings a balance of flexibility and rigidity to the core. Regardless of domains of life and enzyme classes, the correlations of shell-water and its interactions may imply that the interior cavity is a generalized and pattern-wise structural feature in globular proteins.

The rim is an interaction bridge between the core and the surface

Hydrogen bonding creates a complex interaction network while preserving the distinct properties of these three accessible zones. While the core and surface have peaks in hydrophobic (also hydrophilic) and charged residue-mediated hydrogen bonding, respectively, the rim has an intermediate level in both situations, indicating that the latter region contains both types of residue. In the case of other hydrophilic and hydrophobic interactions (Nayek et al., 2015), the presence of identical events in these three zones lends credence to the theory that

the rim exists as a distinct mixed entity between the core and surface. Furthermore, in the case of shell-water interactions, the fact that the rim is often superior to the core and surface demonstrates the abundance of SW here. Taken together, it seems that the position of the residue light but mixed and SW abundant rim between the strong residue-class trend core and the surface, on the one hand, supports their structures and, on the other hand, makes the protein dynamic.

Conclusion

The core and surface of globular proteins, which are KD-positive and KD-negative, respectively, have a strong tendency towards residue types, its classes, and secondary structure elements. The rim, on the other hand, which lies between these two regions, is KD-neutral; residue-wise light, mixed, and abundance in SW. SW filled interior cavities, which are abundant in the core and rim regions in particular, act as a sub-structural entities to limit residue compaction. The predominance of cavities with space within itself and extended β-structures in the core, and the predominance of the flexible coil on the surface seem to lower the rigidity in these regions. In non-bonded interactions, the core and surface are superior to hydrophobic and charged-mediated interactions, respectively, but the rim is at the intermediate level in both cases. The non-bonded interactions between these two types of residues are highest in their frequency in the rim region. This distinct entity of the rim seems to help maintain the structure of the core and the surface, on the one hand, and the dynamism of the protein, on the other. Our study finds applications in protein folding and protein bioinformatics.

Although our study demonstrates the existence of RIM, we believe that it is necessary to analyze it for many more proteins in the database.

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Competing interests:

The authors declare no competing interests.

Abbreviation: hb, hydrophobic residue; cr, charged residue; po, polar residue; ar, Archaea; ba, Bacteria; eu, Eukaryote; SW, shell-water; co, core; rm, rim; su, surface; ss, secondary structure

Data availability:

While the coordinate files are available in the Protein Data Bank, these structure files were minimized for their use in the study. Minimized files are available upon request. There are also approximately 2000 cavity files in PDB format along with residue atoms' accessibility, secondary structure, and interaction multiplicity details. These PDB files can also be obtained upon request.

Code availability:

The binary version of the fully automated AWK scripts was used for the analysis of accessibility region-specific residue composition, residue class, and secondary structure and, KD value, cavity properties. Scripts are also used for the analysis of cavity PDB format files for their composition, residue propensity, types, and shell-water properties. While we have tested the program to be fully functional, error-free, and user-friendly for the proteins used here, we are presently continuing our verification works. The binary version of these could be obtained by experts working in the field upon request.

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Chapter- 4

Environmental Hazards Associated with the Disposal of Municipal Solid Waste

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Keywords: Anaerobic Digestion, Microplastics, Municipal Solid Waste (MSW), Sustainable Practices, Waste Management

Abstract:

Increasing urbanization, industrialization, and population growth result in increasing amounts of municipal solid waste (MSW), which proved to be one of the major threats to the environment and public health. This type of waste mainly comprises plastics, metals, organics, electronic waste, etc. As MSW contains various components such as microplastics, heavy metals, inorganic salts, and volatile organic compounds (VOCs), it is regarded as a mixed source of various contaminants. The mismanagement of these wastes subsequently causes increased pollution around an area, degrading air, water, and land. The heavy metals that accumulate in the ecosystem, which endanger humans and biota, are lead, cadmium, and mercury. These are often derived from industrial and electronic waste. Such nutrients above cause eutrophication and disrupt ecosystems with plastics and microplastic carriers of pathogenic bacteria and antibiotic resistance genes. MSW is well known for having VOCs and POPs about air pollution and public health, bioaccumulating along food chains. It is crucial to sink waste and to rehabilitate waste management. Recent approaches like recycling, energy recovery, and circular economy models emphasize cutting waste, recovering resources, and pollution prevention. Waste can also be tackled with energy production by incineration and anaerobic digestion methods. The ideals of sustainable development, which are concerned with environmental integrity, health risk reduction, and responsible consumption of resources, cohere with international efforts to shift sustainable practice. This synthesis stresses the urgent need for integrated approaches in the regulation-technical innovation-community combination to address the multifaceted challenges of municipal solid waste management and the welfare of people and the environment.

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Introduction:

The global production of municipal solid waste is increasing with industrialization, economics, and better living. The production of MSW is estimated at 2.1 billion tonnes per year, of which about 33% remain uncollected. This is causing environmental and health problems (Peng et al., 2023; Khan et al., 2022; Lino et al., 2023). Forecasts indicate that it will reach 3.4 billion tonnes by 2050 owing to accelerating urbanization as well as increasing population growth, especially in developing countries, where urban residency is expected to rise from 54% to 68% by the middle of the century (Statista, 2023; Kaza et al., 2018; UN DESA, 2018). The three major actors among countries that produce waste in large quantities are the USA, China, and India, illustrating that the more economic activities there are, the larger the waste volume (Gour and Singh, 2023). Waste characteristics and management differ greatly between income levels. Organic waste is highest in low- and middle-income regions, while high-income countries produce more recyclable materials such as metals and glassware (Kumar and Samadder, 2017). Poor waste management aggravates greenhouse gas emissions, water pollution, and plastic pollution, adding to the global environmental challenges (Vinti et al., 2023). Environmental hazards resulting from traditional methods of waste disposal such as landfilling, which accounts for more than half of waste disposal in countries such as the USA (52.6%) and Brazil (59.1%), including methane emissions, groundwater pollution, and disease spreading by vectors (Sun et al., 2019; Costa et al., 2019). The pathogenic and harmful gases CO_2 and CH_4 that leak from these organic fractions in landfills remain even after the landfill is closed (Chavan et al., 2019; Han et al., 2022). Moreover, the leachate from landfills, including chemicals from heavy metals, inorganic salts, and other toxicants, poses a future risk to water quality and public health for a long time (Mor and Ravindra, 2023; Abdel-Shafy et al., 2023). It included, for instance, changing from traditional disposal facilities to advanced waste management technologies such as incinerators, pyrolysis, and anaerobic treatment facilities that reduce the waste footprint, provide energy, and offer possible job growth (Singh et al., 2020; Waqas et al., 2023). Nowadays, NASA recycling and energy recovery are key components as global priorities shift toward reducing greenhouse gases toward desired resource utilization. This transformation echoes a greater, finer purview of sustainable development goals such as responsible consumption and production, sustainable cities, and environment conservation (Tamboli et al., 2024). The historical context of waste production discusses the linkage of population growth and urbanization with economic development since low-income countries are responsible for only 5% of the world's waste, unlike upper-middle and high-income countries contributing more than 65% to this figure (Maalouf and Mavropoulos, 2022). The two other major contributors were the Asia-Pacific region and Europe, as the dual forces of urbanization and rising GDP will come into play in waste generation (Hoornweg et al., 2013). In Asia, urban residents generate an estimated 760,000 tonnes of MSW a day and are print-listed to see this double Bwillhattopadhyay et al. (2009). Waste management is among those aspects of urban planning that need serious valiance because it has been shaped through policies like the Waste Framework Directive of the EU and CE models, meant to engender innovative initiatives aimed at waste reduction, reuse, and recycling (Kirchherr et al.,

2017). On the legal frameworks prevalent in the European Union, waste prevention and recycling are emphasized, thus setting targets to recycle at least 55% of MSW by 2025, reducing landfill use to less than 10% by 2035 (European Commission, 2015). It gives a better insight into the gradual movement of resource extraction and disposal of wastes to a circular economy, with the capacity to prolong the lifetime of materials through these directives (Hobson et al., 2021). Some countries like Sweden have proven to be the best shift from this, with almost 47% recycling and composting rates and eliminating landfill waste by 2017 (EP, 2021). The US appears very poor in this aspect; its recycling rate for MSW is a paltry 23.6%. Challenges of waste globally demand technological innovation, the reform of laws, and people's participation. Further waste management strategies such as biogas production, value-added product development, and digitized systems could substantially make waste management effective in cities (Kurniawan et al., 2022; Norouzi and Dutta, 2022). The concept of a circular economy, building on sustainability and resource efficiency, is increasingly heralded as a strong pathway for relieving environmental and economic pressures from waste generation (Korhonen et al., 2018b). In waste management systems that include recycling, composting, and energy recovery, a nation can reduce its dependence on virgin materials, environmental degradation, and sustainable development (Erfani et al., 2023).

This article aims to consolidate all the emerging trends, challenges, and opportunities in municipal solid waste management worldwide in sustainable practice. It is meant to bring practical recommendations for policymakers, urban planners, and researchers to develop strategies to address one of the critical issues of the waste management problem in a way that would also consider the environment and people within it.

Classification and Composition of MSW:

The objectives of MSW categorization are increasing waste diversion, minimizing landfill volume, and lowering costs for waste disposal. Thus, the influence of MSW classification on the practical result of waste treatment, economic effectiveness, and environmental defense is important to identify. MSW is categorized by the source because the composition varies depending on the source. For example, kitchen waste may encompass perishable foodstuffs of animal or vegetable origin, as well as other kitchen residues such as tea leaves and coffee grounds (Zhang et al., 2021).

Branches, leaves, and charred wood are examples of natural yard waste. Waste paper and cardboard come from advertising fliers, office paper, and packing supplies. Bottles, packaging, and other types of plastic are examples of waste composed of rubber and plastic (Yang et al., 2018). Metal waste includes things like cans, cutlery, and food packaging. Bottles, lightbulbs, and food storage containers are examples of glass waste. Cell phones and PCs that are not working properly are considered electronic trash. Miscellaneous rubbish includes syringes, discarded clothing, pharmaceuticals, and pottery. Lastly, inert materials include inorganic waste, including furniture, ash, and construction waste. Each category highlights a different waste

management challenge, from the breakdown of organic materials to the recycling and disposal of non-biodegradable materials (Nanda and Berruti, 2021).

Furthermore, waste is categorized based on income level. According to Hoornweg and Bhada-Tata (2012), waste is categorized into four groups, namely Lower Income (LI), Lower Middle Income (LMI), Upper Middle Income (UMI), and High Income (HIC). Figure 1 presents the waste composition by income group, showing how the types vary between low, lower-middle, upper-middle, and high-income countries.

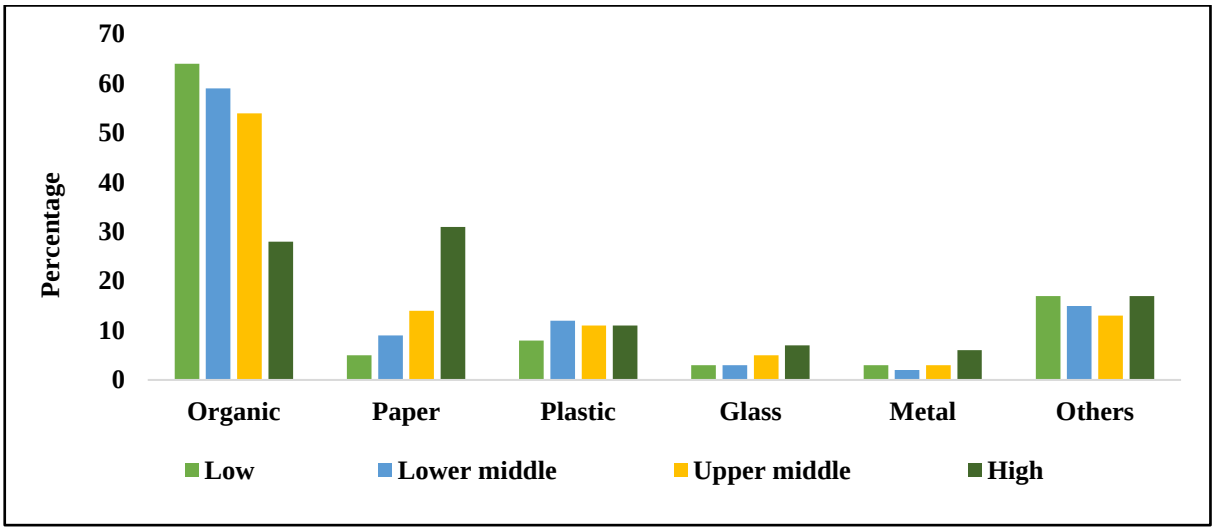

Figure 1: Composition of waste in percentage according to income (Hoornweg and Bhada-Tata, 2012).

It has been demonstrated that organic waste is lowest in high-income nations and most in low-income ones. Additionally, waste from paper and plastic grows as income rises. As income levels improve, the share of organic waste decreases, but the share of paper waste also increases considerably. The percentage of plastic waste is relatively consistent across all socioeconomic brackets. According to Feng et al. (2017) and Zhang and Yuan (2019), municipal solid waste (MSW) is frequently considered a porous media material since it exhibits the coexistence of solid, liquid, and gas phases, just like natural geotechnical materials. However, unlike natural geotechnical materials, which usually do not consider degradation-related changes in geotechnical properties, MSW shows notable time-dependent modifications (Ren et al., 2022). MSW's composition changes over time since most organic components undergo considerable biodegradation. According to Chen et al. (2016), this degradation is a complicated process that involves chemical, biological, and physical processes. The degrading process consequently significantly changes MSW's pore structure and composition, forming gases, leachate, and heat release (Zhou et al., 2024).

Environmental Contaminants present in MSW:

Many materials make up Municipal Solid Waste (MSW), such as plastics, metals, glass, and kitchen waste. Components carrying harmful contaminants can significantly affect the

environment. Various pollutants, such as organic contaminants, plastics, synthetic materials, heavy metals, electronic waste, and several volatile organic compounds (VOCs), are common in municipal solid waste (MSW). MSW poses a primary ecological concern as it contains various contaminants, and these pollutants significantly affect ecosystems and human health.

Heavy metals are highly significant. In MSW, some common examples of heavy metals are arsenic (As), lead (Pb), mercury (Hg), and cadmium (Cd). A widely recognized ubiquitous heavy metal present in MSW is Pb which generally originates from the waste of electric products (e-wastes) and components of its, including cathode ray tubes, batteries, and circuit boards, etc. (Silvetti et al., 2017; Tucker et al., 2020; Madhu et al., 2022). Contamination in MSW is influenced remarkably by the disposal of waste-bearing As from drinking water treatment plants (Clancy et al., 2013). Another notable heavy metal is Cd found in MSW, often sourced from plastics and pigments (Ashfaq et al., 2022; Aendo et al., 2022). Improper disposal of items from the residential sector includes some cleaning agents, paints, and dyes containing Cr compounds, which contribute an efficient amount of Cr in MSW (Tumolo et al., 2020). Also, wastes from some industries and agricultural fields have a significant amount of Cr in them, adding to Cr in MSW. Jalali et al. (2011) studied the common source of another frequently detected contaminant, nickel (Ni), which is waste from stainless-steel utensils, batteries, and residues from electroplating.

Inorganic salts like sulfates (e.g., Na_2SO_4, $CaSO_4$), magnesium salts (e.g., $MgCl_2$), calcium chloride ($CaCl_2$), potassium chloride (KCl), and sodium chloride (NaCl) are another significant class of pollutants which are present in MSW (Meena et al., 2019). Food scraps, agricultural residues, industrial effluents, construction debris, and household litter are some of the primary sources from where the inorganic salts originate and get mixed with MSW. The presence of these slats in a high amount In MSW creates notable environmental challenges (Kim et al., 2022).

The existence of macro-nutrients in excessive quantity, especially nitrogen (N) and phosphorus in MSW, poses serious environmental risks. Organic materials, namely food wastes, paper and cardboards, sewage sludge agricultural wastes, are the major sources of these nutrients (Sultana et al., 2021; Kaur and Kaur, 2024). Accumulating these contaminants in MSW comes with nutrient pollution, which can harm the ecosystems (Meena et al., 2019; Das et al., 2022).

MSW is also known to contain various organic pollutants from numerous sources and processes. Due to the improper disposal of unused or expired medications, residual pharmaceutical compounds have been discovered in MSW and have been found in MSW (Bu et al., 2020). Volatile organic compounds (VOCs) are commonly found in MSW, often produced by the decomposition of organic matter and industrial activities (Wu et al., 2020; Majumdar et al., 2012). Another class of organic pollutants, aromatic compounds, frequently originate from aromatic detergents, chemical solvents, food additives, and paint coatings (Nie et al., 2018).

Plastics and microplastics (MPs) in MSW are an emerging environmental concern. Plastics and MPs enter MSW through various commercial, industrial, and construction activities. Various personal care products, plastic pellets utilized in manufacturing, and microfibers shed from

synthetic textiles contribute to primary MPs, while the degradation of larger plastic items generates secondary MPs (Upadhyay et al., 2021).

Overall, the composition and contamination of MSW highlight the importance of managing waste effectively to minimize its environmental impact.

Potential Impact from the Contaminants on Environment and Biota:

The contaminants present in MSW have a profound impact on the environment and its living organisms. These pollutants can negatively impact the abiotic components, air, water, and soil, hamper human health and the ecosystems.

Contaminants from municipal solid waste, including heavy metals, inorganic salts, nutrients such as nitrogen and phosphorus, plastics, microplastics, volatile organic compounds (VOCs), and pharmaceutical residues, can leach into water bodies. By altering the physicochemical properties, such as pH, hardness, and nutrient concentrations, which can result in eutrophication and increased toxicity levels of the water bodies, these pollutants could significantly impact the quality of the water (Bhat et al., 2022).

Contaminants, such as pharmaceuticals, personal care products, and microplastics, are recognized for exacerbating risks by altering microbial communities, facilitating resistance transfer, and contaminating soil, water, and air (Anand et al.,2021). Microbes present in municipal solid waste (MSW), including bacteria, fungi, and algae, interact with heavy metals and play a crucial role in their uptake, accumulation, transformation, mobility, and bioavailability within landfills and contaminated soils (Sharma et al., 2022). MSW emits harmful air pollutants, such as particulate matter (PM), volatile organic compounds (VOCs), greenhouse gases (GHGs), and odorous compounds, which degrade air quality and pose health risks, including respiratory problems and headaches (Pekdogan et al., 2024). Various organic pollutants, such as polycyclic aromatic hydrocarbons (PAHs), phthalates, and persistent organic pollutants (POPs), present in composted municipal solid waste (MSW), can bioaccumulate in food chains and pose potential health risks to humans, including endocrine disruption, carcinogenicity, and toxicity, mainly when contaminated compost is applied to food production (Langdon et al., 2019). Microplastics in MSW landfill leachate serve as vectors, offering surfaces for the adsorption and proliferation of antibiotic resistance genes (ARGs) and pathogenic microorganisms, leading to contamination of soil, water, and potentially food chains (Jaafarzadeh et al., 2024).

Contaminants from MSW accumulate in animal bodies, causing toxic effects that disrupt reproduction, growth, and survival (de Titto et al., 2024). Additionally, open dumpsites serve as breeding grounds for disease vectors like mosquitoes and rodents, which can transmit pathogens to humans and increase the prevalence of vector-borne diseases. Pollutant introduction into different ecosystems can also alter species composition, disrupt the ecological balance, affect predator-prey dynamics, and potentially lead to the extinction of sensitive species due to habitat degradation (Abubakar et al., 2022).

Existing MSW Management Practices:

Effective Municipal Solid Waste (MSW) management is essential for maintaining environmental health and urban sustainability. Rapid urbanization and population growth have significantly increased waste generation, creating challenges for municipalities worldwide (Khan et al., 2022). This report outlines existing MSW management practices, highlights common challenges, and explores potential solutions to enhance efficiency and sustainability.

Screening of MSW:

A detailed review of studies was done to find examples of successful recycling or significant improvements through specific waste management methods. Many examples came from groups and projects that share best practices, like Zero Waste Europe, the Global Alliance for Incinerator Alternatives (GAIA), Regions for Recycling, and Pre-Waste. The Eurostat database was also used to find countries with high recycling rates, and towns or cities in those countries were studied further (Nanda and Berruti, 2021). In most cities in India, the collection is mainly done by community bins placed along highways, which might result in unlawful open collection stations; house-to-house collecting efforts are gaining momentum in megacities like Delhi, Mumbai, Bangalore, Madras, and Hyderabad, with the support of NGOs (Sharholy et al., 2008).

Identifying the Assessing Criteria:

Several criteria were applied to assess the chosen case studies. The case studies included in this paper were selected based on key factors aligning with current waste policies, laws, and the tools and strategies to encourage sustainable waste management. Different tools and methods can be used when creating a recycling program. These tools are divided into four main types: technical, economic, legal, and informational tools (R4R).

- Technical Tools
- Economic Tools
- Legal Tools
- Informational Tools

Technical Tools:

Household waste can be collected either through kerbside pickup near the property or by using designated drop-off locations; in property-close systems involve the use of various storage solutions such as bins, racks, sacks, and bags for effective collection (Dahlén and Lagerkvist, 2010).

Kerbside collection

Each household is given a waste container. Residents are responsible for storing waste in these containers, either inside or outside their homes. Some sources differentiate between door-to-door collection and yard collection. In door-to-door collection, the waste collector enters the property to pick up waste stored inside the house. In yard collection, the waste collector collects waste stored outside, usually in the yard (Kogler: Waste Collection., 2007).

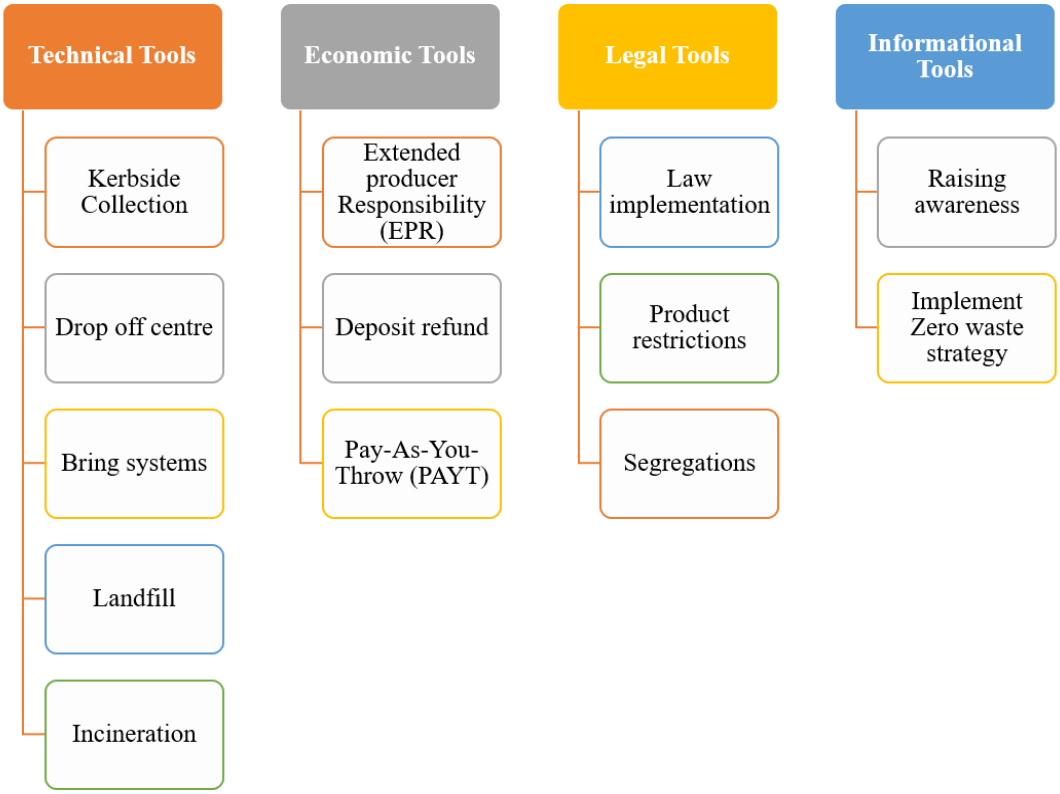

Figure 2: Methods used to improve MSW management (Xevgenos et al., 2015).

Drop off systems

Households are responsible for sorting their waste into different categories and taking it to nearby neighborhood containers. These containers come in various sizes and shapes. Families drop off their sorted waste at designated recycling or green centers (Gallardo et al., 2018).

Landfill Method

The most popular waste management technique worldwide is landfill disposal. Interest in landfill system innovation and progress has increased since these landfills operate as final waste receivers for municipal trash, industrial or agricultural leftovers, wastewater sludge, incinerator ash, recycling discards, and/or treated hazardous wastes. By choosing the best landfill location, residents' concerns may be addressed, and environmental harm can be minimized, leading to a more sustainable garbage lifecycle. Landfills are often built to contain or limit garbage to reduce exposure to the environment and people. Landfills generate methane gas in the atmosphere (Ayub and Khan, 2011).

Incineration

Incineration is one of the earliest techniques for handling and getting rid of solid waste, which has improved significantly in the past 20 years. At first, it only entailed burning garbage, seriously polluting the air. The primary benefits were illness management and waste volume reduction. Mass-burn incinerators are the most prevalent kind of incinerator. Before being transferred into the feeding chute, waste is kept in a pit and stirred using a crane arm to guarantee consistency.

Inside, the waste burns on grates, with oxygen levels controlled for efficient combustion. Extra fuel is added if needed based on the type of waste. The temperature in the incinerators varies between 980℃ and 2000℃. Once filtering to remove metal particles, the remaining bottom ash is collected. A turbine attached to a generator generates electricity using steam created by hot gasses and particles from the combustion process that go through a boiler (Karim et al., 2019).

Economic Tools:

Extended Producer Responsibility (EPR)

General Extended Producer Responsibility (EPR) is a policy aimed at reducing the environmental impact of products by making them more eco-friendly. It requires producers and related businesses to pay fees that fund the recycling of materials they introduce into the market. These fees, which vary by country, can be based on the weight or type of material and reflect the industry's responsibility to manage waste (Hickle, 2014).

Deposit–refund systems

It is also called “Bottle Bills” or container deposit laws, which are economic tools designed to collect and recycle used packaging, like beverage bottles and cans. These systems have been used worldwide for decades, starting in the mid-1900s when the packaging industry shifted from reusable containers to single-use ones. The main goals of these systems are to reduce litter, cut waste disposal costs, recycle valuable materials, conserve energy and natural resources, and create new businesses and jobs. This is applied to glass bottles, plastic containers, cans, batteries, fluorescent lamps, and tires (US EPA, 2017).

Pay-As-You-Throw (PAYT)

A major reason for promoting sustainable waste management is to ensure that the costs are shared fairly among those generating the waste, based on the impact they create. The 'pay-as-you-throw' (PAYT) system, called variable-rate or unit pricing, applies the "polluter-pays" principle clearly and equitably. PAYT is an economic tool widely used worldwide that encourages better recycling practices. By linking waste disposal costs directly to the amount of waste generated, it provides a financial incentive to recycle more and produce less waste, while addressing social, environmental, and legal concerns (Morlok et al., 2016).

Legal Tools:

Law Implementation

Bans and restrictions on waste disposal help manage waste effectively. Landfill bans aim to reduce reliance on landfills and promote better waste management methods that follow the waste hierarchy. As highlighted in case studies, several countries have introduced statewide bans or restrictions on municipal waste. These rules differ based on waste types, including (i) untreated or unsorted waste, (ii) separately collected waste, and (iii) residual waste, considering factors like combustibility, biodegradability, and organic carbon content. In India, the Ministry of Environment, Forest, and Climate Change (MoEFCC) updated the Solid Waste Management (SWM) Rules in 2016, replacing the MSW Rules 2000. These updates aim to improve waste

collection, sorting, recycling, treatment, and disposal in an eco-friendly way. Initiatives like the Hazardous Waste Management Rules (first issued in 1989 and revised in 2003) and the Swachhaa Bharat Mission (SBM) launched in 2014 also focus on promoting sustainable waste management (Sharma and Jain, 2019).

Separation of waste

One prescriptive policy tool that can support economical and effective recycling is separating waste based on its source. It can be sent directly to households or municipalities; they must be fined if they do not obey the regulations (Walls, 2011).

Informational Tool:

Informational tools are important in creating and implementing an integrated waste management plan. The goal of any informational tool about installing waste management systems should be to increase awareness among the public.

Zero Waste Strategy

Recently, more municipal, regional, and international governments started to include a "Zero Waste" goal in their waste management plans. Zero Waste is an ambitious idea rooted in the circular economy, focusing on more than just recycling. It involves redesigning products to save natural resources, reducing waste, encouraging reuse, and prioritizing resource recovery over incineration and landfill disposal (Zaman and Lehmann, 2013).

Conclusion and Recommendations:

Strategic approaches, technologies, and policies should be implemented to successfully separate and manage contaminants generated from Municipal Solid Waste (MSW). Handling those contaminants should be efficient. There are some recommendations given below:

Segregation at Sources: Encourage people to separate waste into categories like hazardous, residual, organic, and recyclable. Raise awareness through campaigns and introduce policies that motivate public participation in proper waste segregation.

Advanced Sorting and Processing Facilities: Invest in automated sorting systems, including screens, metals, and optical scanners to separate biodegradable and non-biodegradable garbage. Separate paper, glass, organic, and inert elements from combined garbage. There is much more emphasis on AI-powered sorting devices and recycling.

Containment of Hazardous Materials: Implement a separate collection system for hazardous waste (such as batteries, chemicals, and electronic waste). To avoid leakage and contamination, hazardous waste must be handled properly and securely.

Leachate Management: To prevent contamination of groundwater, landfills should be designed with suitable impermeable liners and leachate collecting systems. Reserve osmosis, activated carbon filtration, or biological treatment may be applied to treat leachate.

Air pollution control: Install landfill devices to capture methane and other harmful gases for energy use or safe disposal. Use scrubbers and advanced oxidation methods in waste-to-energy plants to lower pollutant emissions.

Biological Treatment for Organics: The biological treatment of municipal solid waste (MSW) can reduce its volume and turn organic waste into compost or biogas through processes like composting or anaerobic digestion. Ensure any byproducts meet environmental standards before disposal.

Recycling: Implement robust recycling programs for metals, plastics, paper, and glass to minimize waste contamination. Promote the reuse of recycled products through the reselling market, emphasizing the 3R (Reduce, Reuse, and Recycle) approach.

Community Engagement and Education: Organize workshops and awareness campaigns to teach communities about the harmful effects of improper waste disposal on the environment. Implement strict waste management regulations with penalties for illegal dumping.

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Chapter- 5

Epigenomic and other important functions of diet and nutrition in Mesenchymal Stem Cells: A brief review

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Keywords: Diet, Energy, Fatty acid, Mesenchymal, Nutrient, Stem cell

Abstract:

Adult stem cells stand for the regenerative ability of organisms during their lifespan. One characteristic feature of healthy aging is the sustainment of healthy SC populations capable of replenishing organs and physiological systems. The native environment of stem cells is known as the niche. It comprises the nutritional surroundings and is crucial to sustain the quality and quantity of stem cells available for renewal and regeneration. It is considered mainly that stem cells have unique metabolism and restricted nutrient requirements compared to completely differentiated cells. Nutrients play a significant role in stem cell physiology because many metabolites derived from nutrients discharged during the catabolic process can affect chromatin remodelling, epigenetic changes, and modulation of gene expression. Nutrient requirements differ throughout the lifespan and are altered by factors like individual health, physiological states including pregnancy, disease, sex, age, and during healing from injury. Even if present nutrition guidance mainly focuses on healthy populations and averting nutritional insufficiency diseases, there are growing efforts to demonstrate food-based and nutrient-based suggestions depending on decreasing chronic disease. Understanding the dynamics of stem cell nutritional needs throughout the life span, including the role of nutrition in extending biological age by blunting biological systems decay, is fundamental to establishing food and nutrient guidance for chronic disease reduction and health maintenance.

Introduction:

Nutrients, which include carbohydrates, proteins, lipids, minerals, and vitamins, are substances in food and are necessary for biological activity in organisms. During metabolism, the nutrients, after conversion into smaller molecules inside the body, are exploited in several life-sustaining chemical reactions. Metabolism constitutes catabolism and anabolism. In catabolism, the breakdown of food or fuel to obtain energy occurs, whereas the reactions in which larger molecules are produced from smaller ones are known as anabolism. Anabolic reactions utilize the energy generated in catabolic reactions. Hence, the cooperative control of both processes is essential to sustain life (Tadokoro and Hirao, 2022).

Stem cells (SCs) are undifferentiated cells with the ability by cell division to generate various cell types in an organism. They possess unique metabolic features in contrast to differentiated cells (Cerletti et al., 2012; Moussaieff et al., 2015; Baksh et al., 2020), and they can exclusively

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sustain their undifferentiated state throughout their entire life while making offspring cells devoted to differentiation in response to specific requirements to maintain tissue homeostasis. Several evidences support the idea that stem cells (SCs) are important in coordinating our body's response to nutrients, mainly because of their key role in tissue homeostasis. To accomplish this, tissue SCs, besides utilizing nutrients for their metabolic requirements, also adjust their functions, such as self-renewal, autophagy, or differentiation, to the metabolic environment and availability of nutrients (Cerletti et al., 2012; Yilmaz et al., 2012; Rafalski et al., 2012). Conversely, their relatively long lifespan, which is crucial to carry out their function in tissue turnover, holds the back of the coin of continually being exposed to important environmental factors like diet and gradually accumulating cell damage at the genetic and epigenetic level, with considerable effects on gene and protein expression as well as on molecular pathways (Blokzijl et al., 2016; Novak et al., 2021; Mondal et al., 2024).

Nutrients are usually essential in SC physiology because of the ability of various nutrient-derived metabolites, produced during the catabolic process, to trigger chromatin reshaping, epigenetic modifications, and modulation of gene expression (Lu et al., 2018). Nutrients also act as donors for moieties engaged in post-translational modifications. For example, in the hexosamine biosynthetic pathway (HBP), uridine diphosphate GlcNAc (UDP-GlcNAc) results in the O-GlcNAcylation of serine and threonine residues embedded in cytoplasmic, mitochondrial and nuclear proteins. In SCs, these post-translational changes have been found to cause linkages between the availability of glucose and nutrients with the epigenetic control of cell fate determination and differentiation (Sun et al., 2016).

Both embryonic and adult SCs have the potential to provide tissues with new lineage of cells throughout their entire life. This new lineage of cells may divide symmetrically or asymmetrically, resulting in either SC self-renewal or differentiation. Besides several other factors, nutrients play a vital role in SC specification, differentiation, and performance, thus extending their effects on aging and disease. Nutrients act directly on SCs or indirectly by controlling the SC niche (non-autonomously). In addition, nutrients can regulate the production of hormones, which can manipulate the nature of SCs and their niche. These direct and indirect stimuli result in the activation of signalling pathways, modification in metabolism, and changes in gene expression in SCs. In this way, the dietary input is converted into fate decisions in SCs (Puca et al., 2022).

For SCs, the primary molecular mechanism linking diet and function is mediated by the AMPK-mTOR-SIRT1 pathway. Fasting or exercise-induced low cellular ATP levels trigger the phosphorylation of AMP-activated protein kinase (AMPK) by the serine-threonine kinase liver kinase B1 (LKB1). This results in direct or indirect modulation of enzymes involved in glucose (Theret et al., 2017) and lipid metabolism (Wang et al., 2018). It also modulates the mTOR pathway, which regulates proteostasis and cell growth (Shackelford et al., 2009). The target proteins of AMPK include the proteins controlling cell polarity, apoptosis (through direct phosphorylation of p53), cell proliferation (cyclin D1) (Shackelford et al., 2009), differentiation (Sarikhani et al., 2020), response to hypoxia (HIF1) and autophagy (Mihaylova et al., 2011).

These proteins have also been found to modify SC fate (Shackelford et al., 2009; Chung et al., 2019). In addition, AMPK enhances cellular NAD^+, which triggers the activation of the NAD-dependent histone deacetylase SIRT1, influencing gene expression (Dai et al., 2020), protein synthesis, and SC self-renewal (Igarashi and Guarente, 2016).

Nutrition has appeared as a chief regulator of the epigenome and gene expression. As a result, nutrition and diet can affect cell metabolism and health (Hahn et al., 2017). This capacity of nutrition and diet is explained by the fact that many metabolites either directly bind to chromatin or indirectly modulate chromatin-modifying enzymes. In SCs, epigenetic changes of DNA and DNA-associated histones determine their function and fate decisions. Hence, inputs from the diet cause modification in chromatin structure and expression of genes (Van Winkle and Ryznar, 2019; Bar-El Dadon and Reifen, 2017; Afarideh et al., 2021; Shyamasundar et al., 2013) in embryonic and adult SCs. This modification, in turn, affects several processes in humans, including embryonic development, cell differentiation, determination of cell fate, aging, immune function, and oncogenic transformation (Chen, 2019; Hernández-Saavedra et al., 2017). These play important roles in closely correlating SC functions, nutrition, metabolism, and epigenetics to each other (Reid et al., 2017).

Nutrients derived from diet, after digestion, produce simple metabolites and can be uptaken by SCs. These biomolecules can act as precursors of substrates or cofactors required by chromatin-modifying enzymes. Sometimes, these enzymes can move to the nucleus and, in association with specific cofactors, bring about chromatin modifications (Boukouriset al., 2016). Epigenetic modifications induced by nutrients result in modifications of both histones (acylation, acetylation, ADP-ribosylation, glycosylation, glycation, methylation, phosphorylation, hydroxylation, and ubiquitylation) and DNA (glycation and methylation). These modifications may be achieved through enzymatic or non-enzymatic reactions (Dai et al., 2020).

Mesenchymal Stem Cells (MSCs) and their Characteristics:

MSCs undergo mitotic divisions. One of its daughter cells remains as a stem cell while the other one differentiates into a mature cell, and only small numbers of these mature cells can be seen in mature organs and tissues in the stem cell niche (Aliborzi et al., 2016). In 1966, MSCs were discovered as fibroblast-like cells within the bone marrow (Aliborzi et al., 2015). Since then, the presence of MSCs has been confirmed in various adult tissues like endometrium (Ghobadi et al., 2018), adipose tissue (Kamali-Sarvestani et al., 2018), intestine (Mani et al., 2023), dental pulp (Zare et al., 2019), and Wharton's jelly (Nazempour et al., 2020). Among various types of MSCs, intestinal stem cells (ISCs) possess a crucial role in the nutritional milieu. They are present in the crypts and do not come in direct contact with intestinal content. On the other hand, differentiated gut cells are present at the villi and come in direct contact with the intestinal lumen. They provide mature cell types of the intestinal epithelium throughout adult life (Barker et al., 2007). Inside intestine, adjacent to ISCs reside a collection of functionally differentiated cells including enterocytes, Paneth and goblet cells. These cells inhabit the intestinal epithelium and play a critical role in the nutritional environment.

The principal function of ISCs is to act as gut regenerative machinery. They undergo continuous division to reinstate their own population and create subtypes of differentiated epithelial cells. The nutritional conditions can modulate the production of secretory lineages such as Paneth cells, enterocytes, and ISCs (Alonso et al., 2018). Specific dietary exposure and fasting have been established to reduce the population of ISCs and their function (Alonso et al., 2018). Two populations of ISCs have been identified, namely Lgr5+ and Lgr4+. Lgr5+ is associated with regular cell renewal, while Lgr4+ is responsible for tissue regeneration. The quiescent Lgr4+ ISCs have the potential to be stimulated in response to injury (Wang et al., 2021). Optimal food intake can control and trigger symmetric divisions of ISCs (O'Brien et al., 2011).

The MSCs can differentiate into one or more types of full-grown cells. This property of stem cells is called "developmental plasticity," and different stem cells have distinct potency levels (Mehrabani et al., 2019). Under both pathologic and physiologic conditions, MSCs can sustain tissue regeneration. In a specialized and dynamic microenvironment along with a separate design as stem cells niche SCs play a major role in tissue homeostasis. These cells have immune-modulating properties due to the low expression of class I MHC, CD40, CD80, and CD86 and the absence of class II MHC expression (Hashemi et al., 2019).

The immune modulating activity of MSCs is attributed to their interaction with immune cells like neutrophils, T and B cells, natural killer cells (NKs), dendritic cells (DCs), and macrophages (Mohammadzadeh et al., 2022). They can be used as drug carriers and can be tracked by MRI (Mehrabani et al., 2022). Their application has also been traced to tissue engineering (Fard et al., 2018). Exosomes or extracellular vesicles (EVs) are the active constituents of paracrine secretion of MSCs. These exosomes are utilized in the management of various diseases (Khajehahmadi et al., 2016). Exosomes are used in the treatment of brain diseases as they can cross the blood-brain barrier (BBB) and enter the CNS (Payehdar et al., 2017). MicroRNAs (miRNAs) are naturally packaged into exosomes in MSCs. This feature of MSCs is successfully applied in the packaging of exogenous therapeutic miRNAs (Jahromi et al., 2017).

Mesenchymal Stem Cells and Nutrition:

Lifestyle and diet are key factors that influence health and vulnerability to diseases. In the stem cell niche, these two factors affect the quality and quantity of stem cells available for renewal, regeneration, and physiological reinstatement as a trademark of health (Stover et al., 2022). Deficiencies in the nutritional environment can modify the niche of stem cells and/or interaction between stem cells and niche, leading to age-associated modulations of the proliferation of stem cells and their functions. Stem cells have distinctive metabolism. Hence, their nutrient requirements are of immense importance. So, consideration of the nutritional requirements of stem cells throughout the life span, together with the involvement of nutrition in expanding biological age by minimizing biological systems degeneration, is key to determining food and nutrient guidance to reduce the occurrence of diseases and to retain the general health (Stover et al., 2022).

MSCs as the tissue precursor were demonstrated to be incredibly relevant for obesity during childhood and metabolic disease risk of skeletal muscle and adipose tissues (Gyllenhammer et al., 2023). In this circumstance, by controlling the stem cell niche, nutrients may directly or indirectly impact stem cells. Nutrients also control hormone production, which can modify the behaviour of the stem cells and their niche. These stimuli trigger the activation of signalling pathways in stem cells, modify their metabolism and gene expression, and transform the dietary input into fate decisions. Many stem cell features are controlled by nutrients, including balanced asymmetric/symmetric divisions, genome and epigenome integrity, gene expression, metabolism, autophagy, oxidative status, differentiation, self-renewal, and exhaustion. When there are adequate nutrients and growth factors, stem cells undergo proliferation. This tight regulation is achieved by “master regulators" like mTORC1, which can monitor nutrients and control stem cells' metabolism and fate (Rafalski et al., 2012).

Alternatively, intracellular metabolites like acetyl-CoA regulate epigenetic processes and metabolic pathways and link stem cell functions with diet and metabolism (Ghosh-Choudhary et al., 2020). For the fate determination of different stem cells, this link is very crucial, and self-renewal of stem cells can be brought about by modification of nutrients or calories (Novak et al., 2021). So, stem cells play a significant role in coordinating the body's response to nutrients because of their essential role in tissue homeostasis and health maintenance (Alvina et al., 2021). This feature of stem cells is achieved by using nutrients for their metabolic requirements and accomplishing many functions, like self-renewal, differentiation, or autophagy. Nutrient availability, metabolic environment, and diet-induced metabolic changes affect the fate of stem cells, lineage specification, and differentiation (Puca et al., 2022).

In this scenario, nutrients are vital in stem cell physiology because of the ability of many metabolites derived from nutrients released during catabolic processes to trigger chromatin reorganization, epigenetic alterations, and modulation of gene expression (Lu et al., 2021). At the same time, molecular mechanisms that sense nutrient availability regulate important self-renewal functions, protein synthesis, autophagy, and differentiation (Bjerkvig et al., 2005). The effect of diet on stem cells becomes more spectacular as stem cells have exceptional metabolic requirements which are changed depending on their developmental stages (Baksh et al., 2020). For precise activities of stem cells, stimulation of metabolic pathways is essential, making stem cells more explicitly dependent on nutrients compared to differentiated cells (Yilmaz et al., 2012). Stem cells have been reported to possess fewer reactive oxygen species (ROS) than differentiated cells. The total intracellular oxidation state and accumulation of ROS have been reported to be primarily influenced by nutrients and diet. They are believed to be key monitors of balance between differentiation and self-renewal (Smith et al., 2000).

Mesenchymal Stem Cells and Amino Acids:

Amino acids (AAs) are engaged in self-renewal, preservation of pluripotency, and differentiation capability of stem cells (Liu et al., 2019). Several essential AAs (EAAs) have been revealed to be crucial for the maintenance of MSCs (Taya et al., 2016), and their affluence was

demonstrated to enhance proliferation without disturbing the stemness (Nikolits et al., 2021). In the intestine, The Mammalian Target of Rapamycin Complex 1 (mTORC1) was reported to be a principal nutrient sensor, functioning as an essential controller of protein synthesis and growth, influencing the proliferation of stem cells and autophagy (Wang et al., 2021).

Restriction of amino acids and proteins in the diet has been described to alter stem cell fate. For instance, methionine deficiency has been shown to reduce the proliferation of ISCs (Saito et al., 2017). In *Drosophila*, in response to a reduction in methionine and the methionine-derived S-adenosyl methionine, the midgut mitosis in ISCs was shown to diminish. This inhibition of mitosis in ISCs was accomplished by regulating the protein synthesis and stimulating the Jak/STAT ligand Unpaired 3 (Upd3) (Obata et al., 2018). Stimulation of the JNK pathway enhances ISC differentiation, while ISC proliferation remains unaffected despite the attenuation of the Jak/STAT pathway (Zhang et al., 2017). Hence, methionine was revealed to regulate cell proliferation (Walvekar et al., 2018).

The function of leucine in carrying out the proliferation and differentiation of myoblasts through an mTORC1-MyoD cascade was reported (Dai et al., 2015). The mTOR has a vital function in various cellular processes, including cell growth, differentiation, and protein synthesis, via its role in regulating specific gene expression (Zhang et al., 2015). Arginine was shown to have a crucial function in the proliferation and renewal of ISCs and tissue regeneration (Hou et al., 2020). During the proliferation stage of myoblasts, glutamine has been revealed to be the second most used nutrient after glucose (Hosios et al., 2016), establishing their significant role in cell proliferation (Gaglio et al., 2009). The conditional EAA glutamine in diet supplementation was found to cause activation of ISCs, which includes an increase in total intestinal cell numbers (Viitanen, 2019). Dietary glutamate activates ISC proliferation and growth via calcium signaling (Deng et al., 2015).

Mesenchymal Stem Cells and Fatty Acids:

Fatty acids (FAs) are another class of molecules derived from nutrients and are crucial for stem cell physiology. It is confirmed by the presence of a particular lipidome signature in MSCs, performing a significant function in self-renewal and quiescence, asymmetric-symmetric division, differentiation, determination of cell fate of MSCs, and cell-to-niche interaction (Clémot et al., 2020). A high-fat diet (HFD) can trigger modifications in intestinal structure and function (Obniski et al., 2018) by modifying the regulation of ISC activity. It was described that some particular fatty acids, including oleic acid and palmitic acid, interact directly with the ISCs and stimulate peroxisome proliferator-activated receptor delta (PPAR-δ) exclusively in ISCs and progenitor cells to increase their stemness (Beyaz et al., 2016).

In stem cells, the presence of excellent coordination between the synthesis of fatty acids and oxidation of fatty acids is necessary, and damage or removal of one or the other can lead to stem cell retardation (Clémot et al., 2020). High-fat diets have been shown to enhance ISC proliferation and self-renewal while reducing Paneth cell number and resulting in an increased risk of intestinal hyperplasia (Wang et al., 2021). It was found that a high-fat western-style diet

in mice resulted in transcriptional reprogramming in both Lgr4+ and Lgr5+ ISCs populations, mutations in stem cells, and nutrient-triggered modifications in stem cell populations, which are in line with a carcinogenesis event (Li et al., 2019). HFD-induced stress causes activation of the JNK pathway, and this pathway leads to Upd3 ligand secretion and activation of ISC proliferation (Richards et al., 2016).

In *Drosophila*, short-chain fatty acids derived from microbiota were reported to control carbohydrate and lipid metabolism to maintain ISCs (Koh et al., 2016). In *Drosophila*, it was demonstrated that high-cholesterol diets, by changing the δ-ligand and Notch stability in the endoplasmic reticulum, can alter ISC cell differentiation (Obniski et al., 2018).

Mesenchymal Stem Cells and Minerals:

Impaired dietary intake of calcium at early stages of life might alter the adipogenic differentiation capability of MSCs from male offspring, with considerable expressions on the Wnt/β-catenin signalling pathway to exacerbate high-fat diet-induced obesity in adulthood (Li et al., 2022). This adipogenic differentiation is controlled by coordinating a complex network of several signalling pathways, which include SIRT1/SIRT2, JAK2/STAT3, TGF-β/BMP, Wnt/β-catenin, ERK1/ERK2, and RHO family GTPase (Porro et al., 2021). Stimulation of Wnt/β-catenin signaling can additionally prevent adipogenic differentiation and trigger osteogenic differentiation with the help of endogenous regulatory genes including Wnt1, Wnt10a, Wnt10b, Wnt5a, CTNNB1, Axin2, Gsk3β, and TGF7L2 (Matsushita., and Dzau, 2017). This differentiation capability was considerably decreased with age (Matsushita and Dzau, 2017). Hence, the nutritional posture and exposure to unfavorable factors during pregnancy and during lactation have a significant function in the differentiation ability of MSCs to influence later metabolic troubles in adulthood (Zhang et al., 2020). The Ca^{2+} produced in the culture medium was reported to have osteo-inductive features to support the osteogenic differentiation of MSCs (Chen et al., 2015).

Mesenchymal Stem Cells and Energy:

It was demonstrated that changes in energy sources can affect stem cell differentiation through glycolysis, the TCA cycle, as well as alterations in the generation of ROS (Burgess et al., 2014). ISCs were reported to have vigorous responses to intake of energy, including caloric constraint, fasting, and a variety of energy sources resulting from ketogenic, high carbohydrate, or high-fat diets (Wang et al., 2021). Under this circumstance, energy has been illustrated as the Lkb1/AMPK triggered kinase pathway to operate as a metabolic checkpoint and principal regulator of stem cell proliferation and fate. This pathway is activated when mTORC1 signalling is suppressed in response to reduced level of ATP and ceased cell growth. So, it can be said that the complex relationship between LKB1-AMPK activity and mTORC1 can affect stem cell proliferation, self-renewal, and apoptosis (Wang et al., 2021; Das et al., 2023) since LKB1-AMPK signalling has an impact on Sirt1 and is triggered by fasting, caloric restriction and exercise which can influence the development of the ISCs and enhance the ability for tissue repair and regeneration (Igarashi., and Guarente, 2016).

Sirt1 functions as a NAD-dependent histone and nonhistone protein deacetylase and controls gene expression, metabolism, cell proliferation and differentiation. As level of Sirt1 declines with age and restored by dietary NAD, it can control the stem cell quantity (Igarashi et al., 2019). Ketogenic diets mimic low caloric states by increasing stem cell self renewal and tissue regeneration and minimizing the gradual loss of tissue functions during aging. However, diets with high fat and carbohydrate levels have opposing effects (Cheng et al., 2019). The intracrine ketone bodies can delineate the fate of ISCs and act as moderators of the pro-regenerative results of fasting. Diets containing high carbohydrates were reported to inhibit the formation of ketone bodies and diminish function, stemness, self-renewal, regenerative power, and epithelial homeostasis of ISCs by activating the formation of goblet cells and Paneth cells at the expense of enterocytes formation (Cheng et al., 2019).

ISCs were shown to monitor and respond differently to macronutrients and dietary energy sources. Ketogenic diets can enrich intestinal health since the increased production of ketone bodies influences the functions of Lgr5+ stem cells and intestinal epithelial homeostasis. Hindrance in the production of ketone bodies in Lgr5+ cells can hamper stemness by increasing the formation of Paneth and goblet cells. It is now established that the release of stem cell growth factors and Wnt ligands by Paneth cells can protect epithelial homeostasis (Cheng et al., 2019). Dietary supplementation with N-acetyl-Dglucosamine (GlcNAc) was sufficient to sustain ISC proliferation amid caloric prohibition independent of food intake (Igarashi et al., 2019). Diets with high sugar can cause modifications in intestinal structure and function and ISCs (Kapinova et al., 2018) through alterations in the control of ISC activity.

Conclusion:

Lifestyle and diet have significant effects on health and vulnerability to diseases. The nutritional requirements of stem cells and their function in quality and quantity are of immense significance for the replenishment of cells and the curative process in wounded tissues, as nutrients play a vital role in stem cell physiology because many nutrient-derived metabolites have genetic and epigenetic roles. Preserving stem cell populations for tissue renewal, regeneration, and restoration is one of the features of health posture. Depending on the participation of stem cells in tissue renewal and regeneration, demonstrating the nutritional needs in diseases, during recovery from trauma, and in the aging process must come into discussion for determining nutrient endorsements to reduce the occurrence of diseases and to progress the interpreting of the biological pathways and mechanisms that link nutritional requirements of stem cells with diseases and aging.

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Chapter- 6

From Trash to Treasure: Innovations in Waste Management for a Sustainable India

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Keywords: Waste management, Valorization, Agro-industrial waste, Solid waste, Pyrolysis, Gasification

Abstract:

Waste management in India is at a critical juncture, with rapid urbanization and population growth generating massive amounts of municipal, industrial, and agricultural waste. Conventional disposal methods, such as landfilling and incineration, pose significant environmental and health risks, necessitating innovative and sustainable solutions. This chapter explores cutting-edge waste management strategies that transform waste into valuable resources, promoting a circular economy. Key advancements include biomethanation for energy recovery, pyrolysis and gasification for biofuel production, and refuse-derived fuel (RDF) as an alternative to conventional incineration. Additionally, the role of biotechnology in bioremediation, enzyme-based degradation, and microbial conversion of organic waste into bio-compounds is examined. Policy frameworks, technological interventions, and community-driven initiatives are also discussed to highlight the multi-faceted approach required for an efficient waste-to-wealth model. By leveraging scientific innovations and integrated waste management practices, India can transition toward a more sustainable and resource-efficient future.

Introduction:

"Someone's waste is someone else's treasure". In a common man's eye, anything that is unwanted or useless is called garbage or waste. Waste is often perceived as an environmental burden, yet with the right approach, it can be transformed into a valuable resource. As a developing nation amidst rapid economic growth and urbanization, India Is now facing a rapidly growing waste management challenge also. With rapid economic growth and urbanization and due to the everchanging consumption pattern continuously shifting to more disposable goods, a

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higher amount of waste is generated every year. India is generating 62 million metric Tonnes of waste annually, with projections reaching 165 million metric Tonnes by 2030 [Annual Report on Solid Waste Management (2020-21), CPCB, Delhi]. People are continuously exposed to a variety of risks in their daily lives as a result of the ongoing growth in waste generation and its constantly shifting composition, including air pollution, methane gas emissions, ground water pollution, the search for new landfill sites, and numerous other health issues. Unfortunately, the ever-increasing disposal issues cannot be resolved by straightforward, quick fixes as society advances. The ongoing development of an Integrated Waste Management (IWM) strategy, which includes the recovery of valuable materials, is the answer to these issues (Kathiravale et al., 2008). The process of turning waste materials into useful goods or energy is known as the "Waste-to-Wealth" idea. Recycling, upcycling, composting, and energy recovery (including waste-to-energy facilities, biogas production, and biofuel production) are ways to do this. The goal is to turn waste into revenue, advancing a circular economy and sustainability (Kalkanis et al., 2022). Thus, the possibility of converting waste to usable products and using them for energy production opens a new era of waste management and job opportunities.

The current waste landscape of India:

India is a fast-developing nation. As of 2024, India is 5th largest economy in the world in terms of Gross Domestic Product (GDP) and the 3rd largest economy in the world in terms of Purchasing Power Parity (PPP) (Bhattacharyya & Dastidar, 2024). It is witnessing a boom in industrialization, urbanization, population growth. Being the most populous nation in the world with a population of 1.43 billion, India is now facing a strong declining thrust on nation resources and generation of burgeoning amount of waste. India produces more than 100,000 metric Tonnes of solid waste per day, according to recent studies (Joseph and Rattan, 2018). Kalyanasundaram et al. have confirmed this statistic, stating that 147.6 million metric Tonnes of solid waste are generated annually. This results in a waste generation per capita that ranges from 170 g to 620 g per day, with an annual rise of 1.3% (Kalyanasundaram et al., 2021). Effective management techniques are required due to the enormous amount of waste generated.

Despite these alarming statistics, the effectiveness of waste management systems in India is inadequate. Collection rates hover around 70%, while only about 12.45% of the collected waste is processed scientifically. The majority, unfortunately, end up in open dumps, contributing to environmental degradation (Pratap et al., 2021). Singh and Singh's review highlights that waste generation rates vary across different regions, with estimates indicating production as low as 0.12 kg to as high as 0.60 kg per capita per day across various states (Singh & Singh, 2021). These discrepancies reflect the need for tailored strategies that align with local demographics and consumption patterns. In India, the main types of waste include municipal solid waste (MSW), industrial waste, agricultural waste, and e-waste, each containing different portions of biodegradable, non-biodegradable and recyclable wastes.

Big cities in India collect about 70-90 percent of MSW generated while for small town and urban areas this value reduces to less than 50 percent. More than 91% of the initially collected

MWS is dumped and landfilled on open lands. Nearly 22000 Tonnes of pollutants are released annually due to Open burning of MSW and landfill fires (Bharti et al., 2017).

Sources of organic wastes in India:

Most of the organic waste comes from MSW and agricultural wastes. It includes agricultural residues, yard wastes, leftover food, and other types of biodegradable sources. With increasing population and urbanization, the organic waste generation in India is on the rise.

Agricultural waste:

As an agrarian nation, India generates a substantial quantity of agricultural waste, encompassing crop residues such as straw, husk, and stems, as well as animal manure and various farming by-products. According to the Central Pollution Control Board, approximately 620 million Tonnes of organic agricultural waste are produced annually in India. The prevalent practice of burning these residues in fields not only exacerbates air pollution but also significantly contributes to greenhouse gas emissions. Nevertheless, there exists considerable potential for the conversion of this waste into valuable resources. Techniques such as bio-composting, vermicomposting, and the generation of biofuels present promising avenues for the utilization of agricultural waste. By transforming these residues into sustainable energy sources or organic fertilizers, it is possible to not only alleviate environmental challenges but also to create alternative revenue streams for the agricultural community, thereby fostering a circular economy within the sector (Jain and Naik, 2022).

Household waste:

In India, household activities are a predominant source of organic waste, primarily comprising kitchen refuse such as leftover food, vegetable peels, fruit skins, and other biodegradable materials (Haldar et al., 2022). Studies have shown that organic waste constitutes a significant portion of household solid waste. For instance, research conducted in the city of Tulsipur revealed that approximately 46% of household solid waste is organic in nature (Haldar et al., 2022). Furthermore, the emphasis on fresh produce in Indian cuisine, coupled with daily cooking routines shaped by cultural and regional preferences, contributes to the substantial generation of organic waste (Priya et al., 2023). A study estimated that Indian households generate about 50 kilograms of food waste per capita annually, totaling approximately 68.76 million tonnes each year (Priya et al., 2023). Implementing sustainable waste management strategies is imperative for both urban and rural communities, as improper disposal can lead to environmental degradation and public health challenges, particularly in densely populated regions. Challenges such as inadequate waste collection systems and improper disposal practices, including open burning and dumping in fields or water bodies, exacerbate these issues (Kapoor & Chakma, 2024).

Green waste:

Green waste, encompassing garden debris such as grass clippings, leaves, branches, and other plant materials, constitutes a significant portion of India's organic waste. Rapid urbanization and

the expansion of green spaces within urban areas have led to a notable increase in the generation of green waste. Although these materials are biodegradable, improper disposal methods-such as incineration or indiscriminate dumping—can result in severe environmental consequences, including air and water pollution, land degradation, and the emission of greenhouse gases like methane, contributing to climate change (Abubakar et al., 2022). To mitigate these effects, implementing effective green waste management strategies, such as mulching, composting, and biomass conversion, is essential. These approaches not only reduce the burden on landfills but also promote sustainable practices in urban landscaping (Kumar et al., 2017).

Market waste:

Local markets in India, particularly those specializing in fruits, vegetables, and meats, play a crucial role in the nation's economy. However, these markets generate substantial amounts of organic waste daily, primarily consisting of unsold or spoiled produce, packaging materials, and other by-products. For instance, in Lucknow, municipal solid waste generation in 2022 was approximately 2,000 tons per day, with organic matter representing about 45% of the waste stream (TERI, 2023). Unfortunately, a significant portion of this waste ends up in landfills, exacerbating waste management challenges. In 2020-21, India generated approximately 160,038.9 tons per day of solid waste, with only 50% being treated and 18.4% landfilled, leaving about 31.7% unaccounted for [Central Pollution Control Board (CPCB), 2021]. To mitigate environmental impacts and reduce the strain on waste management systems, it is essential to implement waste segregation practices at the source and promote responsible disposal methods within these markets. Effective waste segregation can significantly decrease landfill waste, promote composting and recycling, and conserve resources (Grow Purpose, n.d.).

Food industry waste:

The food and beverage industry-including restaurants, hotels, and food processing units—significantly contributes to India's organic waste challenge. This waste predominantly consists of food scraps, cooking oils, and residues from food production processes. With the growing demand for processed and fast food, the volume of organic waste generated by this sector is on the rise. In 2021, India generated approximately 68.7 million tons of food waste, with 11.9 million tons attributed to the food service sector, which includes restaurants, hotels, caterers, and canteens (HPG Consulting, n.d.). Effective waste reduction strategies, such as food donation programs, recycling used cooking oil into biofuel, and implementing sustainable food processing practices, can minimize waste and lead to cost savings for businesses while promoting environmental sustainability. The Food Safety and Standards Authority of India (FSSAI) has launched the "Repurpose Used Cooking Oil" (RUCO) initiative to enable the collection and conversion of used cooking oil into biodiesel, creating a circular economy model that benefits both the environment and the economy (FSSAI, n.d.).

Industrial waste generation:

Industrial waste in India encompasses by-products, residues, sludge, and other materials resulting from production processes. While the environmental impact of organic wastes is often immediate and visible, dissolved chemical wastes from industries can remain undetected for extended periods, eventually emerging as significant chronic pollution issues affecting large populations. For instance, the village of Bichhri in Rajasthan has faced water contamination for over 35 years due to pollution from fertilizer and acid manufacturing factories, leading to severe water scarcity and health problems among residents (Mongabay, 2023). The composition and characteristics of industrial wastes in India vary across sectors, necessitating diverse approaches for their effective management and utilization. According to the Ministry of Environment, Forest and Climate Change (MoEFCC), approximately 10 to 15 percent of industrial waste in India is considered hazardous, highlighting the need for stringent waste management practices (Wastech India, 2020).

Table 1: Industrial waste generation and utilization (Balaganesh et al., 2023).

Industry	Waste Generation	Utilization
Textile	Excess fabric, dye wastewater, fiber waste	Recycling for textile production, biofuel production
Chemical	Chemical residues, solvents, by-products	Co-processing, recycling, chemical recovery
Electronics	E-waste, electronic components, soldering residues	E-waste recycling, component recovery, precious metal extraction
Food Processing	Organic waste, food by-products, packaging waste	Composting, anaerobic digestion, animal feed production
Pharmaceutical	Pharmaceutical waste, packaging waste, chemical residues	Incineration, co-processing, recycling
Refineries	Sludge, oil residue, wastewater	Energy recovery, co-processing, re-refining
Thermal Power Plants	Fly ash, bottom ash, flue gas desulfurization residue	Cement manufacturing, construction materials
Mining	Tailings, waste rock, overburden	Land reclamation, material recovery, backfilling
Paper	Paper sludge, wastepaper	Paper recycling, pulp production, composting
Metal	Metal scraps, metal sludge, metal plating waste	Metal recycling, metal recovery, manufacturing feedstock
Construction	Construction debris, demolition waste	Recycling for aggregate production, building materials

Waste to wealth – An Indian perspective:

Waste to Wealth refers to the process of converting waste materials into valuable products, resources, or economic benefits. Instead of treating waste as a burden, this approach focuses on recycling, reusing, and repurposing waste into useful goods, raw materials, or sustainable

solutions. The Government of India's Waste to Wealth Mission aims to identify, develop, and deploy technologies to treat waste, generate energy, recycle materials, and extract valuable resources, thereby reducing landfill waste, pollution, and greenhouse gas emissions (Office of the Principal Scientific Adviser to the Government of India, n.d.). This strategy also maximizes the use of materials, reducing the need for virgin resources, creating jobs, supporting circular economy businesses, and promoting eco-friendly innovations. For instance, the Pune Municipal Corporation, in collaboration with the SWaCH cooperative, has implemented waste management practices that minimize landfill use and create employment opportunities for waste pickers (Centre for Science and Environment, n.d.).

Current waste wealth opportunities India:

Conversion of Agro-industrial waste into value-added products:

Agricultural residues:

Agricultural waste mainly includes Crop residue which refers to the remaining plant material, such as leaves, stalks, and roots, after the economic part of the plant has been separated for various uses (Bhattacharyya et al., 2021). According to the Indian Ministry of New and Renewable Energy (MNRE), India generates an average of 500 million Tonnes (Mt hereafter) of crop residue per year. The same report shows that a majority of this crop residue is, in fact, used as fodder and fuel for other domestic and industrial purposes. However, there is still a surplus of 140 Mt out of which 92 Mt is burned each year. The composition of crop residue differs across various crops and is identified by different terms. For instance, the parts of the plant that remain in the soil after harvesting are called stubble, while in the case of rice, the harvested stalk is referred to as straw, the unfilled grains are known as chaff, and the leftover part after milling is called husk. In India, the primary crop residues produced include rice straw, wheat straw, millet straw, sorghum straw, pulses, oilseed crop residues, maize stalks, maize cobs, cotton stalks, jute sticks, sugarcane trash, and mustard stalks (Bhattacharyya et al., 2021).

Food Industrial wastes:

A significant volume of organic residues and effluents is generated annually by India's food processing industries, including sectors such as juice, chips, meat, confectionery, and fruit production. Notably, approximately 22% of the country's foodgrain output, equating to around 74 million tonnes, is lost each year, accounting for roughly 8% of global food waste (Times of India, 2023). India is a major producer of crops like apples, cotton, soybeans, and wheat; as production increases, the amount of waste generated from these crops also rises. Waste from food processing industries often contains high levels of Biological Oxygen Demand (BOD), Chemical Oxygen Demand (COD), and suspended solids. A significant portion of this waste remains unutilized or untreated, leading to adverse environmental effects and posing risks to human and animal health. However, these wastes are rich in organic compounds with the potential to be converted into various value-added products.

In the oil industry, a substantial amount of processed residues, known as oil cakes, is generated after oil extraction from seeds. These oilseed cakes are categorized into edible and non-edible types. Edible oilseed cakes, such as those from soybean and groundnut, are commonly used as poultry and animal feed due to their high protein and mineral content. Non-edible oilseed cakes, like castor oil cakes, are typically utilized as concentrated manure or biopesticides (Popović et al., 2020). The composition of oil cakes varies based on their source, including types such as canola oil cake, sunflower oil cake, coconut oil cake, sesame oil cake, mustard oil cake, palm kernel cake, soybean cake, groundnut oil cake, cottonseed cake, olive oil cake, and rapeseed cake. These agro-industrial residues are relatively inexpensive and contain high levels of valuable constituents, making them promising alternative substrates for fermentation and other value-added applications (Vichare and Morya, 2024).

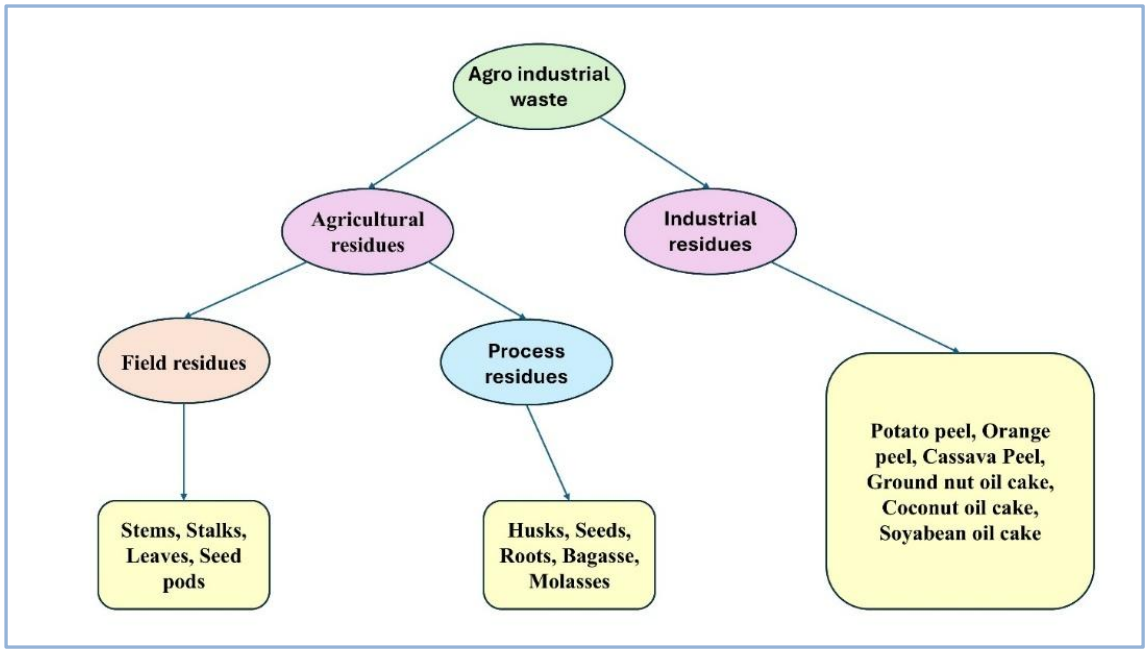

Figure 1: Agro-industrial waste and their types.

Table 2: Composition of agro-industrial wastes (Source: Agro-industrial wastes and their utilization using solid-state fermentation: a review) (Sadh et al., 2018).

Agro-industrial wastes	Cellulose (%)	Hemicellulose (%)	Lignin (%)	Ash (%)	Total solids (%)	Moisture (%)
Sugarcane bagasse	30.2	56.7	13.4	1.9	91.66	4.8
Rice straw	39.2	23.5	36.1	12.4	98.62	6.58
Corn stalks	61.2	19.3	6.9	10.8	97.78	6.40
Sawdust	45.1	28.1	24.2	1.2	98.54	1.12
Sugar beet waste	26.3	18.5	2.5	4.8	87.5	12.4
Barley straw	33.8	21.9	13.8	11.1	–	–
Cotton stalks	58.5	14.4	21.5	9.98	–	7.45
Oat straw	39.4	27.1	17.5	8	–	–

Soya stalks	34.5	24.8	19.8	11.84	–	–
Sunflower stalks	42.1	29.7	13.4	11.17	–	–
Wheat straw	32.9	24.0	8.9	6.7	95.6	7

Composition of Agro-industrial waste:

Agro-industrial waste varies in type and availability depending on its source. It primarily consists of cellulose, hemicellulose, and lignin, making it structurally complex and difficult to degrade. Table 2 summarizes the biochemical composition of Agro-industrial waste. Table 3 summarizes the composition of oil cakes.

Table 3: Composition of oil cakes (Source: Agro-industrial wastes and their utilization using solid-state fermentation: a review) (Sadh et al. 2018).

Oil Cakes	Dry Matter	Crude Protein	Crude Fiber	Ash	Calcium	Phosphorus
CaOC	90	33.9	9.7	6.2	0.79	1.06
COC	88.8	25.2	10.8	6.0	0.08	0.67
CSC	94.3	40.3	15.7	6.8	0.31	0.11
GOC	92.6	49.5	5.3	4.5	0.11	0.74
MOC	89.8	38.5	3.5	9.9	0.05	1.11
OOC	85.2	6.3	40.0	4.2	–	–
PKC	90.8	18.6	37	4.5	0.31	0.85
SuOC	91	34.1	13.2	6.6	0.30	1.30

Valorization techniques:

The valorization of agricultural residues into upcycled products like bioactive compounds, antioxidants, nutraceuticals, and fine chemicals offers a sustainable and economically viable alternative to traditional waste management methods. Utilizing agro-waste as a substrate in Biotechnological and chemical processes enables the recovery of valuable compounds essential to the pharmaceutical industry, including antibiotics, industrial enzymes, and bioactive peptides. These processes, which integrate physical, chemical, and biochemical stages to prevent microbiological hazards, enhance the extraction and utility of bioactive molecules crucial for drug development and other pharmaceutical applications. Various valorization methods, such as cascade or on-site processing of seasonal leftovers, industrial symbiosis, and green chemical or biotechnological conversion, help reduce dependence on fossil resources. By supporting sustainable resource management, these approaches play a key role in advancing pharmaceutical, cosmetic, and nutraceutical innovations (Bala et al., 2023). Table 4 summarizes the modern valorization techniques, including physical, chemical, biological and green solvent techniques.

Table 4: Pretreatments of Agricultural waste and its related pros and cons (Bala et al., 2023).

Application Sector(s)	Pretreatment Method	Pros	Cons	Recovery of Biocompounds
Agriculture, Food	Grinding (Physical)	Produces fine biomass powder (≤0.2 mm).	High energy demand, low	Increases surface area for enzymatic hydrolysis and

			long-term viability.	bioactive compound extraction.
Agriculture, Food	Ultrasonic (Physical)	Enhances lignocellulosic breakdown.	Prolonged sonication may cause degradation.	Improves efficiency of bioactive compound extraction from plant materials.
Agriculture, Food	Steaming Explosion (Physical)	Low energy requirement.	Incomplete lignin-carbohydrate cleavage, inhibitor formation.	Enhances accessibility of cellulose for fermentation processes.
Agriculture, Food	Microwave (Physical)	Efficient, handles large agro-waste.	High electricity consumption, temperature rise.	Facilitates hydrolysis of lignocellulose for bioethanol and bioactive compound extraction.
Agriculture, Food	Pyrolysis (Physical)	High cellulose-to-sugar conversion.	Expensive.	Produces bio-oil, biochar, ethanol, and high-value biochemical products.
Pharma, Agriculture, Biorefinery, Food	Irradiation (Physical)	Increases surface area, hydrolyzes hemicellulose.	Costly.	Improves enzymatic digestibility for biofuel and pharmaceutical applications.
Agriculture, Biorefinery	Acid Hydrolysis (Chemical)	Converts lignin, hydrolyzes hemicellulose.	Corrosive, produces harmful byproducts.	Converts lignocellulosic biomass into fermentable sugars for biofuel and pharmaceutical synthesis.
Agriculture, Biorefinery	Alkaline Hydrolysis (Chemical)	Removes lignin, increases surface area.	Requires high alkalinity and long processing time.	Enhances sugar recovery and bioactive compound extraction from agricultural waste.
Agriculture, Biorefinery	Ozonolysis (Chemical)	Reduces lignin without hazardous byproducts.	High ozone demand, expensive.	Facilitates the breakdown of lignin for improved cellulose utilization in pharmaceuticals.

Agriculture, Biorefinery	Organosolv (Chemical)	Hydrolyzes lignin and hemicellulose.	Volatile, costly solvents.	Extracts lignin-based phenolics and organic acids for pharmaceutical and biorefinery applications.
Agriculture, Biorefinery	Wet Oxidation (Chemical)	Removes lignin effectively, low inhibitors.	High cost (oxygen, acid catalyst use).	Improves enzymatic digestibility of cellulose for bioethanol and pharmaceutical precursor production.
Agriculture, Biorefinery, Pharma, Food	Enzyme (Biological)	Mild conditions, minimal effort.	Low hydrolysis rate, requires sterile conditions.	Converts polysaccharides into fermentable sugars for biofuels and pharmaceutical intermediates.
Agriculture, Biorefinery, Pharma, Food	Bacteria (Biological)	Cost-effective, mild conditions.	Slow processing time.	Produces organic acids and enzymes useful for biofuel and pharmaceutical industries.
Agriculture, Biorefinery, Pharma, Food	Fungi (Biological)	Cheap, destroys lignin, low energy demand.	Long processing duration.	Facilitates enzymatic degradation of lignocellulose for bioactive compound extraction.
Food, Pharma	Ionic Liquids (Green Solvents)	Efficient cellulose dissolution, recovery.	Toxicity, high cost, impractical for mass use.	Used for dissolving and extracting pharmaceutical-grade biomolecules.
Food, Pharma	Deep Eutectic Solvents (Green Solvents)	Eco-friendly, safe.	Can create contaminants, high viscosity.	Enables the extraction of antioxidants, flavonoids, and alkaloids from agro-waste.
Food, Pharma	Natural Deep Eutectic Solvents (Green Solvents)	Low-cost, readily available, safe.	Limited industrial-scale application.	Extracts bioactive compounds such as polyphenols and essential oils for pharmaceutical use.

Applications of valorization process for Agro-industrial waste:

Agro-industrial waste can be transformed into valuable products through various valorization methods, including physical, chemical, and biochemical processes. These methods facilitate the production of biodegradable products, biofuels, biochar, fibers, bio-bricks, sustainable construction materials, and the extraction of bioactive compounds for pharmaceutical and cosmetic applications. Several applications of valorization of Agro-industrial waste are summarized in Table 5.

Table 5: Applications of valorization process for Agro-industrial waste.

Agro-Industrial Waste	Valorization Method	Process Description	Application	Reference
Mango Peels and Seeds	Solvent Extraction	Utilizing solvents to extract polyphenols and carotenoids from mango by-products.	Pharmaceutical and Cosmetic Industries.	Berardini et al. (2005)
Grape Pomace	Supercritical Fluid Extraction	Employing supercritical CO_2 to extract phenolic compounds from grape residues.	Pharmaceutical and Cosmetic Industries.	Dikmetas et al. (2024)
Tomato Pomace	Ultrasound-Assisted Extraction	Applying ultrasonic waves to enhance the extraction of lycopene and carotenoids.	Pharmaceutical and Cosmetic Industries.	Topal et al. (2006)
Citrus Peels	Microwave-Assisted Extraction	Utilizing microwave energy to extract essential oils and flavonoids efficiently.	Pharmaceutical and Cosmetic Industries.	Khan et al. (2010)
Apple Pomace	Enzyme-Assisted Extraction	Using enzymes to break down cell walls, facilitating the release of phenolic compounds.	Pharmaceutical and Cosmetic Industries.	Bhushan et al. (2008)
Pineapple Leaves	Mechanical Processing	Extracting fibers from pineapple leaves for textile applications.	Fiber Production.	Asim et al. (2019)
Banana Peels	Anaerobic Digestion	Microbial decomposition of banana peels in the absence of oxygen to produce biogas.	Biofuel Production.	Achinas et al. (2019)

Olive Pits	Torrefaction	Mild pyrolysis process producing biochar.	Biochar Production.	Eder et al. (2021)
Coffee Grounds	Pyrolysis	Heating spent coffee grounds at 350°C to convert them into biochar.	Construction Material.	Roychand et al. (2023)
Rice Husk Ash (RHA)	Chemical Activation	Enhancing cementitious composites with rice husk biochar and rice husk ash.	Construction Materials.	Muthukrishnan et al. (2019)
Sugarcane Bagasse Ash (SCBA)	Thermochemical Conversion	Burning sugarcane bagasse to obtain silica-rich ash for cementitious material.	Bio-Bricks and Concrete.	de Sande et al. (2021)
Wheat Straw	Biochemical Conversion	Fermentation of wheat straw to produce bioethanol.	Biofuel Production.	Chen et al. (2021)
Corn Stover	Thermochemical Conversion (Pyrolysis)	Heating corn stover in an oxygen-limited environment to produce bio-oil and biochar.	Biofuel and Biochar Production.	Fodah et al. (2021)
Rice Straw	Mechanical Processing	Incorporating rice straw fibers into cementitious materials.	Bio-Brick Production.	Rautray et al. (2021)

Municipal waste management and harnessing value-added products from it:

Municipal solid waste (MSW) is a heterogeneous solid waste stream generated as a result of human activities (Yan et al., 2020). Unlike industrial solid waste, hazardous waste (including medical waste), and construction waste, MSW is characterized by non-point source pollution. Extensive research has been conducted to examine the generation and characteristics of MSW across various countries and regions, focusing on aspects such as waste quantity, composition, moisture content, and calorific value (Abylkhani et al., 2019; Mmereki et al., 2016). The generation and composition of MSW vary significantly based on geographic location (Shi et al., 2021), making accurate forecasting an essential step in the planning and implementation of an effective waste sorting management system (Abbasi and El Hanandeh, 2016; Gu et al., 2015). This process is crucial in determining appropriate treatment methods and assessing the potential for resource recovery.

Generation of Municipal solid waste:

According to data from the World Bank in 2018 the global average municipal solid waste (MSW) generation rate was 0.74 kilograms per capita per day (PCPD) (Yan et al., 2020). The total generation of MSW is primarily influenced by factors such as gross domestic product (GDP) (Lu et al., 2017) and population size (Oribe-Garcia et al., 2015). In general, higher GDP growth rates lead to a significant increase in both per capita and total waste generation. In India, an estimated 143,449 metric tonnes of municipal solid waste (MSW) is generated daily, with approximately 111,000 metric tonnes being collected and around 35,602 metric tonnes undergoing treatment (Kumar et al., 2017). The per capita waste generation rate varies significantly across cities, exhibiting an exponential increase ranging from 0.24 to 0.85 kg per capita per day between 2001 and 2018, as reported by the Central Pollution Control Board (CPCB) in its 2018 annual report. Major metropolitan cities such as Mumbai, Delhi, Kolkata, Chennai, Hyderabad, and Bangalore contribute significantly to India's total solid waste generation. These densely populated regions produce a heterogeneous mix of waste, accounting for approximately 70–80% of the country's daily waste output (MNRE, 2018). According to the MNRE 2018 report, highly populated states, including Maharashtra, Tamil Nadu, Uttar Pradesh, the National Capital Region, Gujarat, Karnataka, and West Bengal, generate a substantial share of India's total waste.

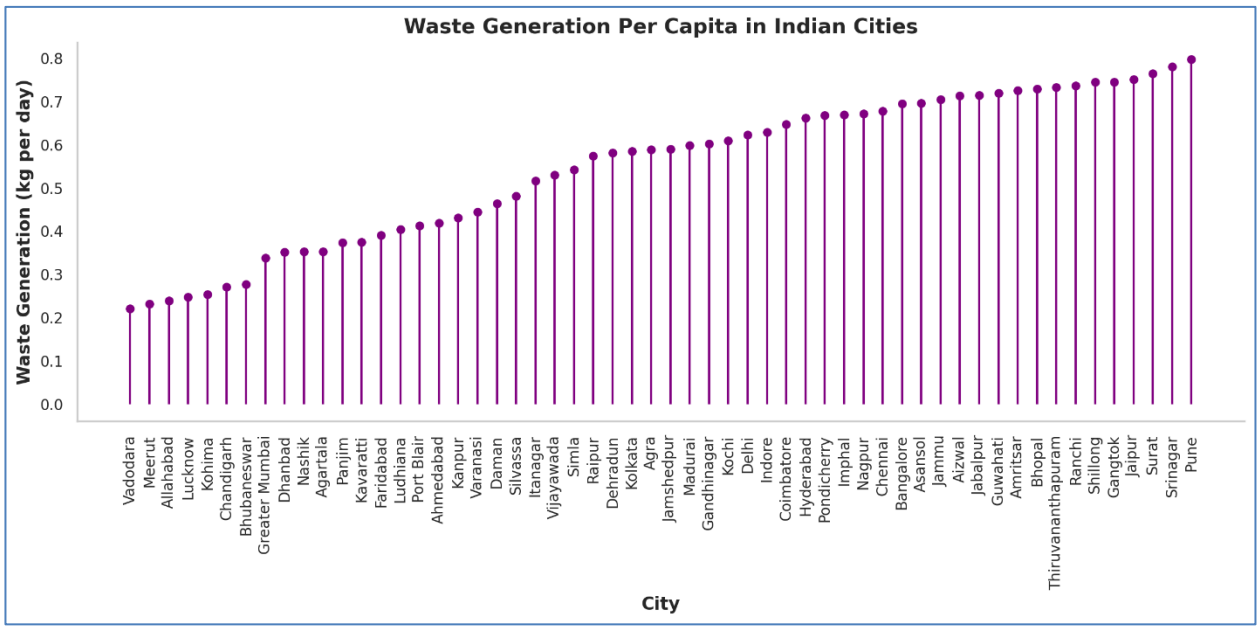

Figure 2: Indian cities waste generation per capita (CPCB India, 2018b).

Composition of MSW in India:

Waste composition significantly impacts waste management practices. High-income groups generate more paper, glass, metals, plastics, and textiles due to increased packaged product consumption (Sridevi et al., 2012). Municipal solid waste (MSW) may also contain hazardous materials like paints, medicines, pesticides, e-waste, and batteries. The informal sector relies on

waste composition for economic activities. Studies show that MSW comprises 40-50% organic waste, 30% inert and construction waste, and the rest recyclable materials. In India, the average calorific value of solid waste ranges from 1500-2200 Kcal/kg, lower than developed countries due to less paper and plastic waste. Additionally, moisture content in Indian solid waste is higher than in developing nations. Figure 4 shows the physical composition of waste in a typical Indian city whereas Table 5 shows the chemical composition of MSW in India as per the population range.

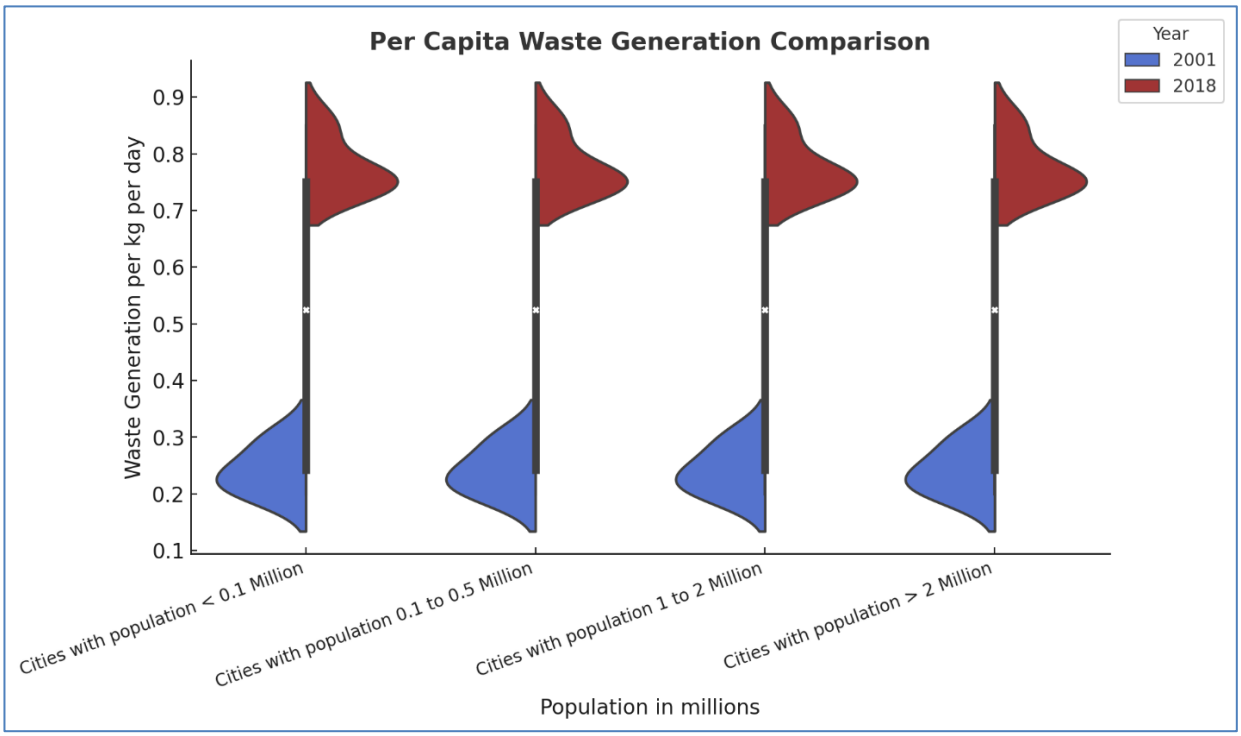

Figure 3: Per capita waste generation comparison in Indian cities (Kumar et al., 2017) (CPCB India, 2018a).

Management of MSW and Possible strategies and options for valorizing this waste:

The management of waste in India is done in two stages 1. Collection and sorting 2. Treatment and final disposal.

Collection and segregation:

Source separation [involves separating waste into different categories (like biodegradable, dry, and recyclable) at the point of generation (e.g., households, businesses) to facilitate recycling, reuse, and proper disposal)] is rare in India, so unsorted waste is typically collected by municipalities daily with the help of the inadequate number of staff; Collection of waste is done door to door from highly congested and narrow streets on the manual bases (CPHEEO India, 2016). As per the report of State Pollution Control Boards/ Pollution Control Committees (in between the year 2009–12),127,486 Tonnes per day MSW was generated in the country during

the year 2011–12, out of which, 89,334 Tonnes per day (70%) of MSW was collected (CPCB, 2013).

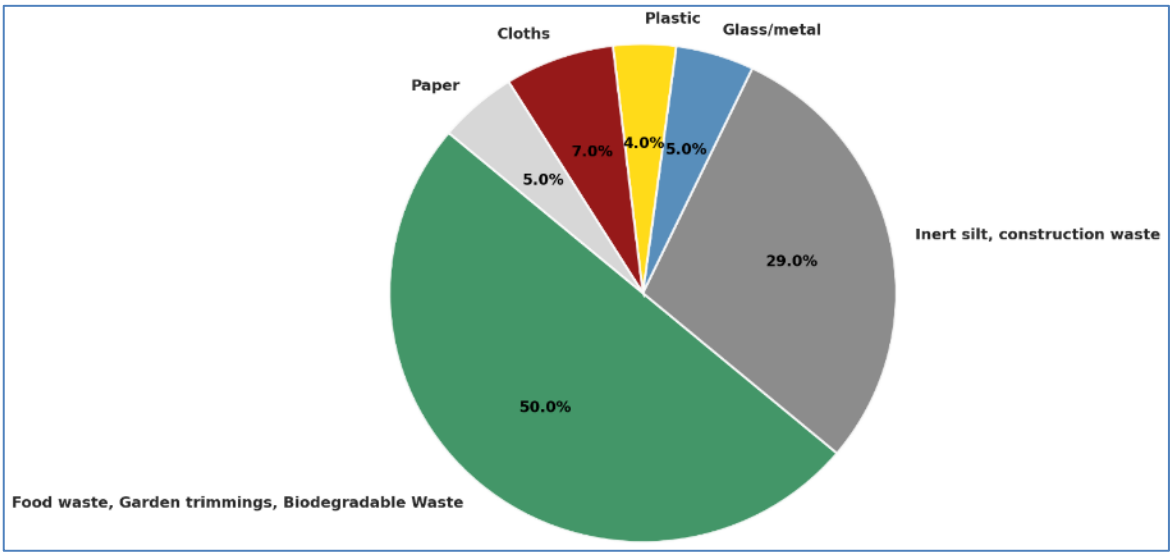

Figure 4: Physical composition of Waste in typical Indian cities.

Table 6: Chemical composition of MSW in India as per the population range (in million) (Sharma and Jain 2019). Entire values, excluding moisture and calorific value, are on a dry weight basis. (Source: NEERI, 1995).

Range of Population (in million)	No. of Cities Surveyed	Moisture %	Organic Matter %	Nitrogen as Total Nitrogen %	Phosphorous as P_2S_5 %	Potassium as K_2O %	C/N Ratio	Calorific Value (kcal/kg)
0.1 to 0.5	12	25.81	37.09	0.71	0.63	0.83	30.94	1009.89
0.5 to 1.0	15	19.52	25.14	0.66	0.56	0.69	21.13	900.61
1.0 to 2.0	9	26.98	26.98	0.64	0.82	0.72	23.68	980.05
2.0 to 5.0	3	21.03	25.60	0.56	0.69	0.78	22.45	907.18
5.0 and above	4	38.72	39.07	0.56	0.52	0.52	30.11	800.70

Storage & Transportation of Solid Waste:

Solid waste is primarily stored in community bins and individual containers. However, residents often use a single bin for mixed waste, leading to improper waste management. During the rainy season, these bins frequently overflow and accumulate leachate. Waste collection vehicles operate on a weekly basis, but they are often uncovered, have insufficient capacity, and

rely on traditional, manual collection methods in many cities. Additionally, the limited availability of land for waste disposal presents a significant challenge (Annepu, 2012).

Figure 5: Pictures showing the current situation of waste collection by ragpicker and waste transporter [Source: Wikimedia, India Today].

Treatment and final disposal:

The processing pathways for municipal solid waste (MSW) can be categorized into mechanical recycling, thermal conversion, and biological treatment methods.

Recycling of Non-Biodegradable Waste:

Recycling involves the recovery and reprocessing of materials that would otherwise become waste, allowing them to be transformed into new products. This process reduces the reliance on virgin resources in manufacturing and significantly lowers energy consumption compared to producing goods from raw materials. Additionally, recycling helps mitigate greenhouse gas emissions across various stages of a product's lifecycle, including extraction, manufacturing, and decomposition. Commonly recycled materials include paper, cardboard, glass, plastics, and metals.

India's rapid economic growth and urbanization have led to a significant increase in plastic consumption and waste generation. While India's per capita plastic consumption stands at 11 kg—considerably lower than that of the United States and China—it is expected to rise sharply due to continued urban expansion and economic development (Plast India, 2015).

Using plastic waste in formulation of plastic road:

An innovative approach to addressing plastic waste while improving infrastructure is the incorporation of soiled and torn thin plastics into road construction. When integrated into bitumen (tar) roads, these plastics significantly enhance the durability and longevity of the pavement. Research indicates that plastic-infused roads exhibit increased resistance to deformation, improved stability, and enhanced fatigue life compared to conventional roads (Government of India, Ministry of Road Transport and Highways, n.d.).

The process involves introducing finely shredded plastic films into hot-mix plants, where asphalt/bitumen mixes are prepared for road construction. The shredded plastic is added to heated aggregates, creating a polymer coating on the aggregate as the plastic softens under heat. This polymer-coated aggregate exhibits superior binding properties with bitumen, resulting in roads

that are more resistant to wear, tear, and water damage, particularly during heavy rains (Dhruv Consultancy Limited, n.d.).

India has been a pioneer in adopting this technology. Tamil Nadu, for instance, has utilized waste plastic in the construction of approximately 1,000 km of various road stretches (Central Pollution Control Board, n.d.). More recently, urban centers such as Chennai, Pune, and Indore have integrated plastic waste into their road-laying projects (Indian Express, 2024). India has already constructed around 40,000 km of rural roads using plastic waste, and efforts are underway to expand this sustainable initiative to highways as well (Swarajya, 2024).

Studies by the Central Road Research Institute (CRRI) and various municipal bodies have shown that plastic roads exhibit enhanced resistance to pothole formation, reduced maintenance costs, and improved load-bearing capacity (Swarajya, 2024). Additionally, this method provides an eco-friendly solution to the growing problem of plastic waste disposal, effectively diverting non-recyclable plastic from landfills and incineration (World Bank Blogs, 2023).

Governments and private contractors are increasingly recognizing the benefits of plastic roads, and policies are being formulated to encourage their widespread adoption. In 2015, the Indian government mandated the use of waste plastic in road construction to address the growing problem of plastic waste disposal in urban centers (Government of India, Ministry of Road Transport and Highways, n.d.). As a result, plastic waste management and sustainable road construction are being seamlessly integrated, paving the way for greener and more resilient infrastructure.

The recycling rate of paper and paper-based products in India stands at 27%, significantly lower than that of industrialized countries such as Germany (73%), Sweden (69%), Japan (60%), and the United States (49%) (CPPRI, 2013). In these countries, a substantial portion of wastepaper is exported to recycling facilities. Industry assessments indicate that over 50% of India's paper demand is met through imported wastepaper, with one-third sourced from the U.S. Recycling wastepaper offers substantial environmental benefits, leading to up to 70% savings in energy and water consumption compared to paper production from virgin wood pulp. However, inadequate segregation and the absence of a dedicated primary collection system for recyclables hinder effective paper recycling. Paper is particularly susceptible to contamination, which further reduces its recyclability. Implementing proper wastepaper management practices could not only reduce dependence on imports and conserve critical resources such as water and trees but also mitigate greenhouse gas emissions associated with virgin paper production. According to a study by the Central Pulp and Paper Research Institute (2013), a 1% increase in wastepaper recovery could prevent approximately 20 kilotonnes of greenhouse gas emissions annually, highlighting the significant environmental impact of improved recycling efforts.

Biodegradable Waste Processing:

Composting of Organic Waste (Wet Waste):

The segregated organic fraction of municipal solid waste can be directly processed through composting. This natural decomposition process, facilitated by microbial activity, results in the production of organic fertilizer and energy while emitting gases such as CO_2, CH_4, SO_2, and H_2S.

The six main types of composting materials are food processing residues, manure and agricultural by-products, forestry and wood industry residues, organic waste and sludge, yard and garden waste, and separated organic waste from municipal sorting (Krstic et al., 2019).

There are two primary composting methods: aerobic and anaerobic. Common aerobic composting techniques include pit/pot composting, vermicomposting, windrow composting, and aerator drum composting. Among these, aerobic composting is particularly advantageous as it produces compost with minimal odor. Vermicomposting, which involves the use of earthworms under controlled conditions, has been promoted by the Ministry of Fertilizers and Chemicals, Government of India (Aalok et al., 2008). With a scientific approach, high-quality compost can be produced and utilized for gardening or sold to local farmers at subsidized rates. Vermicomposting has already gained commercial traction in countries such as the United States, Canada, Italy, and Japan. Given its benefits, India should consider adopting vermicompost technology on a larger commercial scale to enhance sustainable waste management practices.

Biomethanation:

Biomethanation is an advanced biochemical process for converting biodegradable waste through anaerobic decomposition. In this process, organic matter is broken down by microbial action in the absence of oxygen, producing biogas, which primarily consists of methane. This biogas can serve as a substitute for conventional fuels such as LPG or CNG. Additionally, it can be purified and compressed into Compressed Biogas (CBG), which can be used for electricity generation through generators with a conversion efficiency of approximately 30%. However, nearly 70% of the energy is lost as heat during this process. A significant byproduct of biomethanation is slurry, which serves as a highly effective liquid manure for agriculture. This makes biomethanation not only an energy-generating solution but also a contributor to soil nutrient enrichment (Pathak et al., 2020).

Like composting, biomethanation can be implemented at both small-scale decentralized units and large centralized facilities. Several small-scale biomethanation plants, ranging from 0.5 to 10 tonnes per day (TPD) capacity, have been established in various Indian cities, including Pune, Bengaluru, Mumbai, Delhi, Coimbatore, Matheran, Vadodara, and Nasik. These plants generate electricity from biogas, which is often used to power streetlights in nearby areas (Vaish et a., 2016).

Pune has taken the lead in implementing decentralized biomethanation, with 25 plants of 5 TPD capacity (except for two plants with 3 TPD capacity) processing 121 TPD of biodegradable waste at 80% capacity utilization. Collectively, these plants manage approximately 10% of the city's biodegradable waste as of 2017. The Pune Municipal Corporation supports this initiative by providing 600 square meters of land per plant, along with 5,000 liters of water per day and free on-site electricity. Bengaluru adopted a similar decentralized biomethanation model, establishing 15 plants across the city. However, most of these facilities are non-operational, primarily due to inadequate waste segregation. For efficient operation, feedstock waste must be meticulously segregated and delivered in time to biomethanation plants. Poor segregation levels

and inferior quality of feedstock keeps the plants from running at their full capacity. Frequent breakdowns result from the presence of non-biodegradable materials in feedstock waste. The processing therefore takes much longer as secondary sorting is carried out at the plant. (Ahluwalia et al., 2018).

Table 7: Medium and Large-scale Biomethanation Plants in India (Ahluwalia et al., 2018).

City	Developer	Installed Capacity (TPD)	Output
Pune	Nobel Exchange	300*	Bio-CNG: 4 TPD, Manure: 7.5 TPD
Bengaluru	Nobel Exchange	250#	Bio-CNG: -, Manure: 25 TPD
Solapur	Organic Recyclers	400#	Electricity: 3 MW, Manure: 60 TPD
Chennai	Ramky	30	Electricity: 0.26 MW, Manure: 3 TPD
*Operational capacity as of 2017 is 25%; #Currently operational capacity is not available			

Dry waste processing by waste to energy conversion:

Dry waste can be processed for energy generation through three primary methods: refuse-derived fuel (RDF), incineration, and gasification. However, it is important to recognize that solid waste is neither the most efficient nor the most cost-effective source of energy. Waste-to-energy (WTE) plants should be considered as components of an integrated solid waste management system, ensuring compliance with emission standards and environmental regulations. Proper implementation and monitoring are essential to minimize environmental impact and enhance the sustainability of waste-to-energy initiatives.

Incineration:

Mass incineration, the burning of mixed waste with minimal pre-processing, is a common practice in India. While it reduces waste volume and generates heat energy, it poses significant environmental risks. The process releases toxic gases, especially when waste contains heavy metals, PVC, or halogenated compounds. Additionally, wet waste and inert materials lower the calorific value, reducing furnace temperatures below the required 1000°C. Using auxiliary fuel to compensate decreases energy efficiency and increases costs. Since December 2016, the National Green Tribunal (NGT) has banned the mass incineration of unsegregated municipal solid waste, though enforcement remains weak. Refuse-Derived Fuel (RDF) is a superior alternative as it involves pre-processing waste to remove non-combustible materials, ensuring higher calorific value, reduced toxic emissions, and improved energy recovery. This makes RDF-based combustion more efficient, environmentally safer, and economically viable (Tihin et al., 2023).

Refuse Derived Fuel (RDF):

Non-biodegradable, non-recyclable, and non-hazardous waste with a high calorific value can be utilized for energy recovery through combustion. To enhance energy output, this waste is shredded, dried, and compressed into pellets or briquettes, known as Refuse Derived Fuel (RDF),

which can serve as a coal substitute for heat generation in various industries. Thus, Refuse-derived fuel (RDF) is a highly combustible fraction of municipal solid waste. It has a high content of biomass, ranging from 11 to 82 wt.% with a plastic content ranging from 13 to 45 wt%. However, it is crucial to maintain furnace temperatures at 1000°C or higher to prevent the release of toxic air pollutants such as dioxins and furans during RDF combustion (Khatibi et al., 2024).

Under the Solid Waste Management Rules (2016), industries within 100 km of an RDF plant are required to replace at least 5% of their fuel consumption with RDF. However, compliance with this mandate has been limited, and RDF utilization has not gained significant traction since the introduction of the regulation. Similar to compost, RDF manufacturers face challenges in marketing their products due to low industrial demand. Additionally, the cost of segregating mixed waste, along with production, storage, and transportation expenses, often makes RDF as expensive as, or even more costly than, conventional fuels in India. Further challenges, such as high volume and excessive residual ash, reduce its desirability among consumers, posing significant barriers to widespread RDF adoption.

Gasification:

Conversion technologies such as pyrolysis, gasification, and plasma gasification have emerged as effective methods for transforming high-calorie dry waste into valuable products like syngas, ethanol, and biochar. Gasification is a thermochemical process that converts carbonaceous materials into syngas—a mixture primarily composed of carbon monoxide (CO) and hydrogen (H_2) by heating the feedstock in a controlled oxygen environment. Operating temperatures typically range from 480°C to 1,650°C. The resulting syngas can be utilized for electricity generation, chemical production, and as a fuel source. Plasma gasification is an advanced form of gasification that employs plasma torches to achieve extremely high temperatures, often exceeding 2,760°C. This process effectively breaks down waste materials into their basic molecular components, producing cleaner syngas with reduced tar content. The high temperatures also facilitate the vitrification of inorganic components into a stable, glass-like slag, minimizing environmental impact. While plasma gasification shows promise for waste-to-energy applications, its economic viability and scalability for municipal solid waste management are still under investigation (Arena, 2012).

Pyrolysis:

Pyrolysis involves the thermal decomposition of organic materials in the absence of oxygen, occurring at temperatures between 300°C and 760°C. There are two primary pyrolysis techniques: fast pyrolysis, which is optimized for bio-oil and syngas [a mixture of CO, H_2, and methane (CH_4)] production, and slow pyrolysis, which facilitates prolonged heat exchange to primarily produce charcoal (Shukla et al., 2000). Pyrolysis offers a versatile approach to waste management, with the produced bio-oil serving as a potential renewable energy source and biochar being utilized to improve soil health. Several small-scale pyrolysis projects are currently operational, contributing to sustainable waste-to-energy initiatives. Collectively, these

technologies present promising avenues for converting waste into valuable energy resources, aligning with sustainable development goals and circular economy principles.

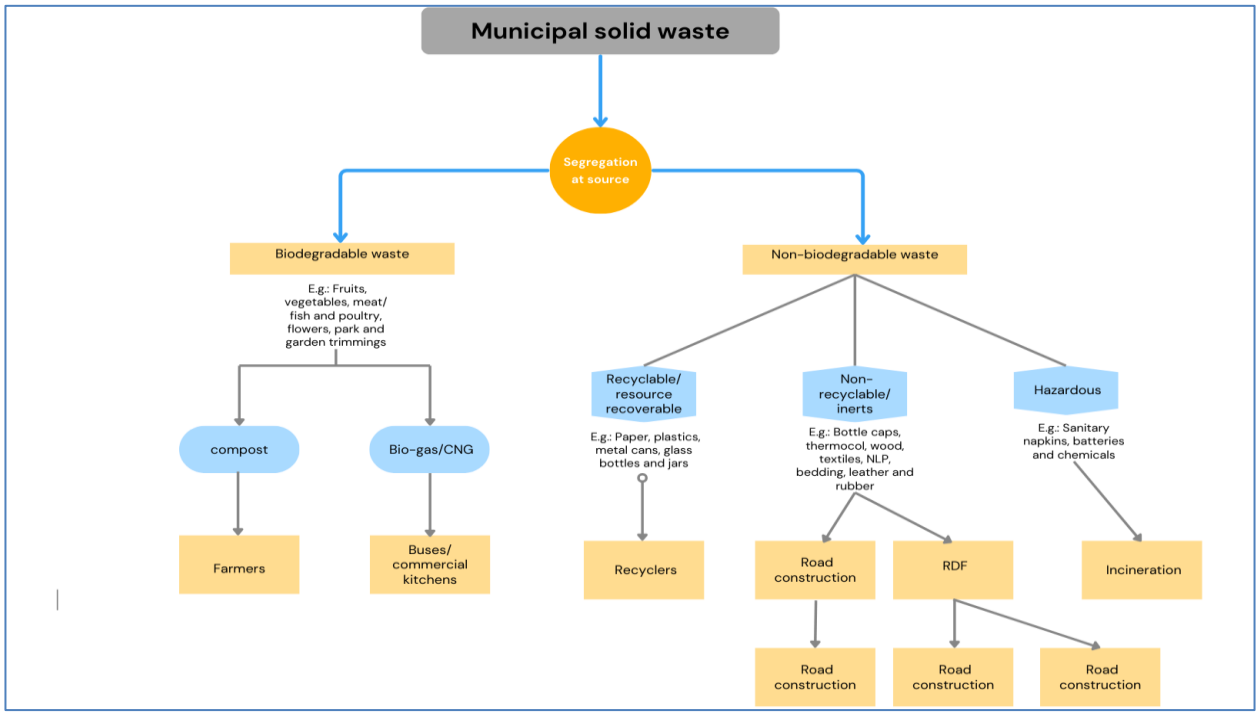

Figure 6: Components of MSW management.

Table 8: Waste to energy plants in India (CPCB India, 2018).

States	Plant Location	Power Generation (MW)	Remarks and Waste Intake
Andhra Pradesh	10 Numbers (7 numbers received CFE from Board)	63	-
	Elikkta (Nonoperational)	6.6	200 Mt./day (RDF)
	Vijayawada (Nonoperational)	6	225 Mt./day (incineration)
	Rajahmundry (Nonoperational)	13	1075 Mt./day (incineration)
Chandigarh	01 (M/s Jaiprakash Associates Ltd. Green-tech Fuel Processing plant, open dumping ground, Dadumajra, Sector-25 West Chandigarh)	RDF generated utilized in their hot air generator, rest is supplied to nearby industries	Operational RDF production: 175 MT/day (Optimum), Approx 60 MT/day (present production)
Delhi	Okhla (Operational)	16	2000 Mt./day (RDF)

	Ghazipur (Operational)	12	1300 Mt./day (RDF)
	Narela-Bawana (Operational)	24	1300 Mt./day (RDF)
Goa	01 (Hindustan Waste Treatment Plant at Saligao, Bardez Goa)	0.4	In Operation
Himachal Pradesh	01 (Shimla - Under Construction)	2.5	In this plant, heterogeneous SW 70-100 t converted to bio-briquettes in the drum drier and further producer gas used for power generation through gasification.
Madhya Pradesh	01 (Jabalpur MSW Pvt. Ltd., Village Kathonda)	11.5	Waste utilized 300-320 TPD
Maharashtra	Pune (Partially Operational)	11	700 Mt./day (Gasification/Pyrolysis)
	Solapur (Operational)	3	400 Mt./day (Anaerobic Digestion) NA
	Kolahapur (Proposed)	2	-
Orissa	01 (Bhubaneswar MC)	11.5	Yet to be commissioned
Punjab	08 (Bathinda, Ludhiana, GMADA, Patiala, Ferozepur, Amritsar, Jalandhar, Pathankot)	-	1 plant at Nakodar for Ludhiana Cluster is already installed but yet not operational
Pondicherry	Kurumbapet Waste Processing Facility	120 KVA	Under Construction: Based on Nisargruna Technology (by BARC)
Tamil Nadu	Greater Chennai Corporation	-	Under Operation
	Pulianthope	12 KWH	Biomethanation
	Velankadu	4.8 KWH	Biomethanation
	Otteri	7.5 KWH	Biomethanation
	Trichy Corporation	0.45 MW	Biomethanation/Power Plant
	Erode Municipal Corporation (Vendipalayam)	500 M^3/day	Biomethanization Plant

	Parambalpur Municipality	300 units/day	Biomethanation Plant
	Nagapattinum Municipality	0.5 MW/day	Biomethanation Plant
	Namakkal Municipality SWM site	200 units/day	-
	Tiruchengode Municipality	117 units/day	-
	Pallipalayam Municipality	98 units/day	-
	Karur Municipality	400 units/day	Biogas Plant
Telangana	Karimnagar (Operational)	12	1100 Mt./day (RDF)
Uttar Pradesh	4	42	NA
	Kanpur (Nonoperational)	15	1500 Mt./day (RDF)
West Bengal	01 (Barasat Municipality)	NA	Ongoing
Total	29 plants in operation/partial operation, under construction, and proposed	-	These Waste-to-Energy plants include RDF-based and Biomethanation plants

Disposal of Solid Waste:

Once the principles of reduction, recycling, and resource recovery have been effectively implemented in solid waste management, and with the assurance that wet waste is not mixed with dry waste, the remaining residual waste must be securely disposed of. This ensures that non-recoverable and hazardous waste is isolated and prevented from negatively impacting the environment.

A sanitary landfill is a specially designed pit with a protective bottom and side liners, where unrecoverable and stabilized waste is disposed of in layers. To maximize space, the waste is compacted and covered with an inert layer. The landfill is equipped with gas vents to allow for the safe release of gases and a bottom drainage system to collect leachate, which is then treated. The Solid Waste Management Rules (2016) clearly outline the types of waste that are allowed to be landfilled. However, unlike countries like Germany, the implementation of landfill regulations in India is often inconsistent and inadequately enforced.

Policy Framework and Support for Waste-to-Energy (WtE) Initiatives in India:

India's policy framework for Waste-to-Energy (WtE) initiatives is designed to promote sustainable waste management practices and renewable energy production. This framework is supported by various national policies and programs, which collectively aim to address the

growing waste management challenges while fostering clean energy solutions. The key policies and initiatives include:

1. **Swachh Bharat Mission (SBM), 2014:** The Swachh Bharat Mission, launched in 2014, is a national initiative aimed at improving cleanliness and sanitation across India's cities and rural areas. This mission promotes waste segregation at source, recycling, and the establishment of waste treatment facilities, including Waste-to-Energy (WtE) plants. The SBM has played a significant role in encouraging municipal corporations to adopt WtE technologies, thereby contributing to sustainable waste management practices (Ministry of Housing and Urban Affairs, 2017).
2. **Solid Waste Management Rules, 2016:** The Solid Waste Management Rules, 2016, provide a comprehensive regulatory framework for managing municipal solid waste (MSW) in India. These rules mandate municipal authorities to establish WtE plants wherever feasible and promote energy recovery from non-recyclable dry waste. The regulations emphasize waste reduction at source and the importance of minimizing waste sent to landfills. They also highlight the need for effective waste segregation and treatment to support WtE technologies (Ministry of Environment, Forest and Climate Change, 2016).
3. **National Policy on Biofuels, 2018:** The National Policy on Biofuels, introduced in 2018, aims to promote the production and use of biofuels derived from diverse sources, including municipal solid waste. The policy encourages investment in biofuel technologies and provides financial incentives for the establishment of biofuel production facilities. By supporting WtE projects, the policy helps in reducing dependence on conventional fuels and encourages sustainable energy solutions (Ministry of New and Renewable Energy, 2018).
4. **National Clean Energy Fund (NCEF):** The National Clean Energy Fund was established to finance innovative clean energy initiatives that enhance energy security and reduce the environmental impact of traditional waste disposal methods. The NCEF provides financial support for WtE projects, facilitating the development and deployment of clean energy technologies that contribute to India’s sustainability goals (Press Information Bureau, 2011).
5. **Atal Mission for Rejuvenation and Urban Transformation (AMRUT), 2015:** The AMRUT mission aims to ensure basic infrastructure services in urban areas, focusing on water supply, sewage, urban transport, and green spaces. The mission also encourages the integration of WtE technologies within urban waste management systems to promote environmental sustainability and improve urban cleanliness (Ministry of Urban Development, 2015).
6. **Clean Energy Fund under the Pradhan Mantri JI-VAN Yojana, 2019:** The Pradhan Mantri JI-VAN (Jaiv Indhan- Vatavaran Anukool Fasal Awashesh Nivaran) Yojana aims to promote the generation of second-generation biofuels. This policy supports the development of technologies for converting agricultural waste and other non-food waste into biofuels, thus supporting WtE efforts through energy recovery from organic waste. The program aligns with India’s efforts to reduce its carbon footprint while enhancing energy security (Ministry of New and Renewable Energy, 2019).

7. **National Electric Mobility Mission Plan (NEMMP), 2013:** While focused on the promotion of electric vehicles, the NEMMP also aligns with waste-to-energy goals by encouraging the development of energy infrastructure that can integrate renewable energy sources, including energy generated from waste. The policy indirectly supports WtE projects by promoting the adoption of cleaner energy technologies and enhancing the overall sustainability of urban transportation (Department of Heavy Industries, 2013).

8. **The Bioenergy Policy:** The Bioenergy Policy supports the development of bioenergy from a variety of organic waste streams, including agricultural and municipal solid waste. This policy encourages the establishment of biogas and other bioenergy plants that utilize waste for energy production, offering financial incentives and facilitating research and development in bioenergy technologies. It aims to reduce reliance on fossil fuels and promote energy production from waste (Ministry of New and Renewable Energy, 2020).

These policies, along with the regulatory frameworks and financial support mechanisms, create a conducive environment for the growth of WtE technologies in India. Together, they aim to improve waste management practices, promote renewable energy production, and contribute to the achievement of India's sustainability and clean energy goals.

Table 9: Waste-to-Wealth Challenges in India and their probable mitigation (Reference: Kumar et al., 2017).

Problem	Explanation	Probable Mitigation
1. Lack of Segregation at Source	Many households, industries, and institutions mix biodegradable and non-biodegradable waste, making it difficult to efficiently process waste for recycling and waste-to-energy (WtE).	- Promote public awareness campaigns about the importance of waste segregation.
		- Implement stricter regulations requiring waste segregation at the source.
		- Provide incentives to households and industries for segregating waste.
2. Insufficient Waste Management Infrastructure	India lacks adequate waste processing plants, recycling units, and waste-to-energy (WtE) facilities to handle the growing volume of waste.	- Increase investment in building more waste processing, recycling, and WtE facilities.
		- Encourage public-private partnerships (PPPs) to develop necessary infrastructure.
		- Utilize existing resources, such as landfills, for waste-to-energy technologies.

3. High Initial Investment Costs	Establishing waste-to-energy plants and recycling units requires significant capital investment, which deters private sector involvement.	- Provide financial incentives, grants, or subsidies for private sector investments in waste-to-energy and recycling plants.
		- Facilitate access to low-interest loans or venture capital for waste management startups.
		- Offer tax incentives and reduced tariffs on technology and equipment imports.
4. Technological Limitations	Many waste-to-energy technologies are not suited to India's waste composition, which has high moisture content and low calorific value, making energy conversion inefficient.	- Invest in research and development (R&D) to develop waste-to-energy technologies suitable for India’s waste composition.
		- Adapt and improve existing technologies to increase energy efficiency in handling high-moisture and low-calorific waste.
		- Encourage collaborations between international and local experts to transfer relevant technologies.
5. Policy and Regulatory Gaps	Despite having policies like the Solid Waste Management Rules (2016), enforcement remains weak, reducing the effectiveness of waste-to-wealth initiatives.	- Strengthen the enforcement of waste management policies and regulations, with clear penalties for non-compliance.
		- Regularly monitor and review the effectiveness of waste management policies and make necessary updates.
		- Ensure better coordination between local governments and central authorities for smoother policy implementation.
6. Informal Waste Sector Challenges	A large portion of waste collection and segregation is carried out by informal waste pickers who lack proper training, financial support, and social security, limiting their efficiency.	- Provide training programs for informal waste pickers on safe and efficient waste segregation and collection.
		- Offer financial support and social security benefits to waste pickers.

		- Integrate informal waste pickers into formal waste management systems through partnerships and legal recognition.
7. Low Public Awareness and Participation	Many people are unaware of the benefits of waste segregation and recycling, leading to low participation in waste management programs.	- Launch nationwide awareness campaigns to educate the public on waste segregation, recycling, and the benefits of waste-to-wealth.
		- Engage local communities through workshops, seminars, and school programs to raise awareness.
		- Encourage the adoption of community-based waste management systems.
8. Market Challenges for Recycled Products	The demand for recycled products in India remains low, affecting the economic viability of recycling businesses and discouraging investment in waste conversion industries.	- Develop and promote a stronger market for recycled products by creating demand through government procurement policies.
		- Educate consumers and businesses on the quality and benefits of using recycled products.
		- Create incentives for industries to use recycled materials in their manufacturing processes.
9. Logistical and Collection Issues	Waste collection and transportation are challenging, especially in urban areas with narrow roads and dense populations. Inefficient logistics lead to delays and improper waste handling.	- Improve waste collection infrastructure with better vehicles and technologies suited to urban environments.
		- Implement optimized waste collection routes and schedules to increase efficiency.
		- Promote decentralized waste collection points and community-driven waste management practices.
10. Environmental Concerns	Some waste-to-energy technologies, like incineration, can contribute to air pollution if not managed properly,	- Ensure proper management and pollution control measures for waste-to-energy technologies,

	raising concerns about their environmental impact.	such as advanced filtration systems for incinerators.
		- Adopt cleaner and more sustainable waste-to-energy technologies, such as gasification or anaerobic digestion.
		- Regularly monitor emissions and environmental impacts to ensure compliance with environmental standards.
11. Land Acquisition Difficulties	Setting up waste processing plants requires large areas of land, which can be difficult to acquire due to high costs, regulatory restrictions, and public opposition.	- Simplify the land acquisition process for waste management facilities, including waste-to-energy plants.
		- Explore alternative land-use models, such as utilizing unused or underutilized public land for waste processing plants.
		- Provide compensation or incentives to local communities to mitigate opposition to land acquisition for waste management projects.

Conclusion:

The Indian government has acknowledged the significance of organic and industrial waste management through initiatives like the *Swachh Bharat Abhiyan* and the *Solid Waste Management Rules, 2016*. Efforts to promote waste segregation, composting, recycling, and waste-to-energy projects highlight a commitment to sustainable waste utilization. However, to fully realize the potential of industrial waste management, several key areas require attention. Investment in research and development (R&D) is essential for advancing innovative waste processing technologies and identifying sustainable solutions for emerging waste streams. Stakeholder collaboration among industries, academia, policymakers, and waste management experts can foster knowledge-sharing, pilot projects, and industrial symbiosis networks. Additionally, policy interventions that offer incentives for sustainable practices and enforce compliance with waste management regulations can create an enabling environment for industries to integrate circular economy principles. By addressing these areas, India can transition towards a more sustainable and resource-efficient waste management system, ensuring environmental and economic benefits while reducing industrial waste's ecological footprint.

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Chapter- 7

The role of Folk medicine in achieving the traditional goals through IKS: A Review

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Keywords: Folk medicine, Indigenous Knowledge Systems (IKS), traditional healing, herbal remedies, cultural identity, healthcare integration

Abstract:

Folk medicine, deeply embedded within Indigenous Knowledge Systems (IKS), plays a crucial role in preserving traditional healing practices, fostering cultural identity, and enhancing healthcare accessibility. This review explores the significance of folk medicine in achieving traditional health goals by examining its therapeutic efficacy, psychological benefits, and socio-cultural contributions. Herbal remedies, such as *Azadirachta indica* (Neem), *Allium sativum* (Garlic), *Zingiber officinale* (Ginger) and *Curcuma longa* (Turmeric), have been widely used for their medicinal properties, with some transitioning into modern pharmaceuticals. Additionally, indigenous healing rituals, such as Ayahuasca ceremonies and sweat lodge practices, provide psychological and emotional well-being, reinforcing community cohesion and resilience. Despite its relevance, folk medicine faces several challenges, including the need for scientific validation, ethical concerns related to intellectual property rights, and difficulties in integrating traditional healing with modern healthcare. The lack of empirical research limits its acceptance within biomedical frameworks, while the commercialization of indigenous remedies raises issues of biopiracy. Regulatory barriers hinder the full incorporation of traditional practices into formal healthcare systems. Addressing these challenges requires interdisciplinary collaboration, legal protections, and standardized protocols to bridge traditional knowledge with scientific research. As global interest in holistic and integrative medicine increases, recognizing the value of folk medicine can contribute to a more inclusive and sustainable healthcare system. This review underscores the importance of preserving indigenous healing traditions while fostering ethical and scientific advancements to ensure their continued relevance in modern healthcare.

Introduction:

Folk medicine, as a key component of Indigenous Knowledge Systems (IKS), has been instrumental in achieving traditional health and well-being goals across cultures (Maiti et al., 2013; Acharya et al., 2022, 2023; Biswas et al., 2023; Islam & Karmakar, 2023; Madhu et al., 2023; Rai & Sharma, 2024). Rooted in centuries-old practices, folk medicine encompasses herbal remedies, spiritual healing, and holistic approaches that integrate cultural beliefs with medicinal applications (Sarkar et al., 2016; Das, 2022; Sanyal, 2023; Pawar et al., 2023). The effectiveness of these practices is often validated through experiential learning, oral traditions, and intergenerational transmission of knowledge (Hlatshwayo & Phasha, 2021). From a theoretical

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perspective, folk medicine is closely linked to ethnomedicine and cultural epistemology, which emphasize the value of non-Western health paradigms in addressing community health needs (Sarkar & Madhu, 2016; Sarkar, 2017; Hobsbawm, 2019; Maity, 2023; Nath et al., 2024; Kumar et al., 2024). Theoretical frameworks such as the constructivist approach highlight how Indigenous communities develop knowledge based on lived experiences and environmental interactions (Smith & Wane, 2022). These frameworks underscore the significance of traditional healing methods in maintaining societal balance and resilience.

Textual analysis of historical manuscripts, folklore, and ethnographic records reveals that folk medicine has consistently played a crucial role in traditional societies (Sarkar et al., 2016; Mazrui, 2020; Sarkar et al., 2021; Sarkar et al., 2023). Many indigenous texts document the use of plant-based remedies and spiritual healing, illustrating the deep connection between cultural beliefs and health practices. Studies also highlight the way oral literature and indigenous scripts serve as repositories of medical knowledge, ensuring its preservation across generations (Chavunduka, 2018).

Empirical research further validates the role of folk medicine in achieving traditional health goals. Several studies demonstrate the efficacy of indigenous remedies in treating various ailments, complementing biomedical approaches (Oyebode et al., 2016). Additionally, research on indigenous healthcare models illustrates the socio-cultural significance of traditional healing, showing how these practices contribute to holistic well-being, community cohesion, and identity preservation (Mutwa, 2017). By integrating theoretical insights, textual evidence, and empirical research, this review explores the critical role of folk medicine in achieving traditional health and well-being objectives within Indigenous Knowledge Systems. It highlights the need for further research to bridge the gap between traditional and modern medical paradigms, ensuring the continued relevance of IKS in contemporary health discourse (Das, S. 2022).

1) To examine the role of folk medicine within Indigenous Knowledge Systems (IKS) in preserving and transmitting traditional healing practices and cultural values.
2) To analyze how folk medicine contributes to achieving traditional health and well-being goals by integrating indigenous beliefs, rituals, and natural remedies.

Methods and Materials:

This study employs a qualitative systematic review to explore the role of folk medicine in achieving traditional health goals within Indigenous Knowledge Systems (IKS). By synthesizing peer-reviewed journal articles, books, and institutional reports from sources like the World Health Organization (WHO), the research aims to highlight folk medicine's contributions to healthcare accessibility, cultural preservation, psychological well-being, and integration with modern medical systems. Data was collected through academic databases such as Google Scholar, PubMed, ScienceDirect, and JSTOR using search terms related to folk medicine, traditional healing, indigenous knowledge, and herbal remedies. Studies published within the last two decades were prioritized, though historically significant works were included for contextual depth. Thematic content analysis identified key aspects, including the medicinal efficacy of

herbal treatments, psychological and social benefits of healing rituals, and challenges in integrating traditional medicine with modern healthcare. Empirical research validating the pharmacological properties of plants like *Artemisia annua* for malaria and *Azadirachta indica* for antimicrobial use was examined alongside anthropological perspectives on healing practices. Ethical concerns such as biopiracy and intellectual property rights were also assessed. To ensure credibility, only peer-reviewed and scientifically recognized sources were used, incorporating multidisciplinary perspectives from ethnopharmacology, anthropology, and public health. Cross-referencing findings helped maintain consistency and reduce bias. However, the study is limited by the lack of empirical data on certain orally transmitted indigenous practices and the diversity of folk medicine traditions, which may affect generalizability. Future research should include ethnographic field studies and clinical trials to further validate traditional healing methods. This review underscores the enduring significance of folk medicine within IKS and provides a basis for future research and policy initiatives aimed at integrating traditional and modern healthcare practices.

The role of folk medicine within Indigenous Knowledge Systems (IKS) in preserving and transmitting traditional healing practices and cultural values:

Folk medicine, as a vital component of Indigenous Knowledge Systems (IKS), plays a crucial role in preserving and transmitting traditional healing practices and cultural values. This objective seeks to explore how folk medicine maintains indigenous health traditions and supports cultural continuity. Through a content analysis approach, this review categorizes key aspects of folk medicine's role in IKS, highlighting its functions, significance, and impact.

Table 1: Key Themes in the Role of Folk Medicine in IKS.

Theme	Description	Relevant Studies
Preservation of Traditional Healing Practices	Folk medicine ensures the survival of ancient healing techniques, such as herbal remedies, spiritual healing, and holistic treatments, passed down through generations.	(Sharma & Patel, 2021; Smith, 2019)
Transmission of Indigenous Knowledge	Knowledge transfer occurs through oral traditions, apprenticeship, and community-based practices, ensuring that younger generations inherit medicinal wisdom.	(Oyebode et al., 2016; Ahmed et al., 2020)
Cultural and Spiritual Significance	Many indigenous healing methods are deeply connected to spiritual beliefs, rituals, and ancestral heritage, reinforcing cultural identity.	(Warren et al., 2018; Kimmerer, 2013)

Sustainability and Biodiversity Conservation	The use of local plants and natural resources in folk medicine promotes environmental sustainability and biodiversity conservation.	(Gómez-Beloz, 2002; Berkes, 2018)
Integration with Modern Healthcare	Traditional medicine is increasingly being integrated into modern healthcare systems, offering complementary approaches to health and wellness.	(World Health Organization, 2013; Bannerman, 2022)

Preservation of Traditional Healing Practices:

Folk medicine remains a cornerstone of indigenous communities, where healing practices such as herbal therapy, massage, and spiritual healing have been safeguarded through generations. These traditions, rooted in centuries of experiential knowledge, play a crucial role in addressing physical, emotional, and spiritual well-being. Sharma and Patel (2021) emphasize that these practices are not only therapeutic but also serve as a means of cultural preservation, reinforcing indigenous identity (Das, 2022). The reliance on natural remedies, including medicinal plants, minerals, and animal-derived substances, demonstrates a deep understanding of local biodiversity and ecological balance. Indigenous healers, often regarded as custodians of ancestral wisdom, utilize inherited knowledge to diagnose and treat ailments, ensuring that these traditional methodologies persist despite modernization pressures (Smith, 2019). This accumulated wisdom is transmitted orally or through apprenticeship, fostering a lineage of healing expertise that remains integral to indigenous healthcare systems. By maintaining these practices, communities safeguard their medical heritage while promoting sustainable resource use and environmental stewardship.

Transmission of Indigenous Knowledge:

The transmission of indigenous medicinal knowledge is primarily oral, rooted in the cultural practices of indigenous communities where elders, healers, and practitioners pass down their expertise through verbal communication, apprenticeships, and community-based learning. Oyebode et al. (2016) highlight that this method of knowledge transfer ensures the continuity of traditional healing practices within families and communities, where younger generations learn directly from experienced healers. These practices are often taught through hands-on experience and guidance, allowing for deep, contextual understanding that goes beyond mere information transfer. Ahmed et al. (2020) further emphasize the role of storytelling and ritual in this process, explaining that narratives and ceremonies are not only used to teach specific medical knowledge but also serve to preserve cultural identity and spiritual beliefs. These oral traditions, which include the recounting of past healings, mythological stories, and sacred practices, reinforce both the spiritual and medicinal significance of the practices, ensuring that indigenous health knowledge remains embedded in the cultural fabric of the community (Das, 2022). Through this

intricate web of oral transmission, indigenous medical knowledge thrives across generations, adapting to changing circumstances while preserving its foundational wisdom.

Cultural and Spiritual Significance:

Many folk medicine practices are deeply rooted in spiritual beliefs and cultural traditions, serving as more than just methods for treating physical ailments. Warren et al. (2018) emphasize that indigenous healing approaches focus on restoring spiritual balance and harmony, which are considered essential for overall well-being. This perspective aligns with the holistic nature of Indigenous Knowledge Systems, where illness is often viewed as a disruption in the interconnected relationship between the body, mind, spirit, and environment. Kimmerer (2013) further illustrates that ceremonies and rituals play a vital role in traditional healing, as they not only activate medicinal properties but also reinforce a sense of belonging and cultural continuity within communities. These rituals, which may involve prayers, chants, or symbolic offerings, are designed to invoke ancestral wisdom and connect individuals with the spiritual realm, thereby enhancing the healing process. Such culturally embedded practices contribute to the preservation of indigenous identity and strengthen communal bonds, ensuring the continuity of folk medicine traditions across generations.

Sustainability and Biodiversity Conservation:

The use of natural resources in folk medicine fosters ecological awareness by encouraging sustainable harvesting and conservation efforts among indigenous communities. Gómez-Beloz (2002) highlights that traditional healers follow ethical guidelines when collecting medicinal plants, such as seasonal harvesting, selective picking, and replanting to ensure long-term availability. These practices help maintain biodiversity and prevent overexploitation of critical flora. Berkes (2018) further elaborates that indigenous medicinal traditions are deeply rooted in environmental stewardship, emphasizing the interconnectedness of humans and nature. Rituals and cultural taboos often regulate the use of medicinal plants, ensuring that ecosystems remain balanced and regenerative. Additionally, knowledge transmission within communities reinforces the importance of biodiversity conservation as younger generations are taught to respect and protect their natural environment. By integrating ecological principles into their healing systems, indigenous groups contribute to the sustainability of both medicinal resources and broader ecosystems.

Integration with Modern Healthcare:

The integration of folk medicine into modern healthcare systems has gained increasing recognition, as it offers valuable insights into holistic healing practices. The World Health Organization (2013) underscores the importance of incorporating traditional medicine into national healthcare policies, highlighting its potential to complement biomedical approaches and address diverse health needs. This inclusion fosters culturally appropriate healthcare, particularly in regions where indigenous healing practices remain prevalent. Bannerman (2022) further

elaborates on the benefits of collaboration between indigenous healers and biomedical professionals, emphasizing that such partnerships enhance healthcare accessibility, especially in remote areas where conventional medical services are limited. By combining indigenous knowledge with scientific advancements, healthcare systems can develop more inclusive and patient-centered treatment strategies. These collaborations also contribute to validating and documenting traditional healing methods, ensuring their preservation while promoting evidence-based practices. However, successful integration requires mutual respect, regulatory frameworks, and ongoing dialogue to bridge the gap between indigenous wisdom and modern medical standards, ultimately improving overall healthcare outcomes.

The Contribution of Folk Medicine to Traditional Health and Well-being Goals:

Folk medicine is a critical component of Indigenous Knowledge Systems (IKS), deeply rooted in centuries of empirical knowledge and cultural heritage. Unlike modern biomedicine, which focuses primarily on physical symptoms, folk medicine integrates indigenous beliefs, rituals, and natural remedies to promote holistic health and well-being (Bodeker, 2019). This review analyzes the role of folk medicine in achieving traditional health goals by examining its foundational principles, key practices, and societal impact.

Table 2: Key Components of Folk Medicine in Traditional Health and Well-being.

Component	Definition and Role	Examples and Case Studies	Impact on Health & Well-being
Indigenous Beliefs in Healing	Traditional medicine is based on spiritual and cosmological beliefs, where illness is often seen as an imbalance in life forces. Healers diagnose not just physical symptoms but also emotional and spiritual disturbances (Gupta & Sharma, 2020).	- **African Traditional Medicine**: Beliefs in ancestral spirits guiding healing (Mwenda & Chitindingu, 2022). - **Native American Healing**: The concept of harmony between humans and nature, where illnesses are treated with sacred herbs and spiritual ceremonies (Cohen, 2019).	Promotes a holistic understanding of health that includes mental, emotional, and physical well-being.
Rituals and Ceremonies	Healing rituals often include prayers, offerings, and chants to invoke divine or ancestral assistance in curing diseases (Laderman & Roseman, 2021). These ceremonies help strengthen the	- **Ayahuasca Healing (South America)**: A shaman-led ritual using plant-based hallucinogens for emotional and mental healing (Dos Santos et al., 2018). - **Balinese Usada Medicine (Indonesia)**:	Rituals create a strong placebo effect and reinforce communal support in healing processes.

	patient's psychological and emotional state.	Involves mantras, meditation, and herbal baths to cleanse the body and spirit (Hobart, 2020).	
Use of Natural Remedies	Folk medicine relies on plant-based, mineral, and sometimes animal-derived remedies that are prepared and administered according to ancestral wisdom (Kumar et al., 2018).	- **Traditional Chinese Medicine (TCM)**: Ginseng for boosting energy, turmeric for inflammation (Zhang et al., 2021). - **Indian Ayurveda**: Ashwagandha for stress relief, Neem for skin infections (Das, S. 2022).	Natural remedies offer accessible and cost-effective treatments with fewer side effects compared to synthetic drugs.
Community-based Healing and Knowledge Transmission	Healing knowledge is preserved and transmitted orally by elders and traditional healers (Oyebode et al., 2016). Community participation ensures cultural continuity and trust in traditional practices.	- **Zulu Traditional Healing (South Africa)**: Sangomas (healers) pass knowledge to apprentices through initiation (Ngubane, 2021). - **Mayan Medicine (Mexico and Guatemala)**: Midwives and herbalists train younger generations through experiential learning (Barrett, 2019).	Strengthens intergenerational knowledge transfer, preserving cultural identity and local health sovereignty.
Adaptation and Integration with Modern Medicine	Folk medicine is increasingly being integrated into modern healthcare systems, with governments recognizing the value of indigenous healing methods (Van der Kooi & Theobald, 2018).	- **China**: Traditional Chinese Medicine (TCM) clinics operate alongside Western hospitals (Zhang et al., 2021). - **India**: The AYUSH Ministry promotes Ayurveda, Yoga, and Unani medicine as complementary therapies (Das, 2022).	Enhances medical pluralism, allowing patients access to diverse healing approaches.

Enhancing Physical Health through Herbal Medicine:

The use of herbs in folk medicine has been integral to traditional healing practices, offering natural remedies for various ailments. Neem (*Azadirachta indica*), widely used in Indian Ayurveda, possesses antibacterial, antifungal, and anti-inflammatory properties, making it effective for treating skin infections, diabetes, and digestive disorders (Mukherjee, 2022; Kaur et al., 2021). Similarly, Garlic (*Allium sativum*), a staple in Mediterranean folk medicine, has been scientifically validated for its cardiovascular benefits, including reducing blood pressure and cholesterol levels and enhancing immune function (Kumar et al., 2018; Rahman & Lowe, 2019). Other notable examples include Ginger (*Zingiber officinale*), known for its anti-nausea and anti-inflammatory effects, and Turmeric (*Curcuma longa*), which has been extensively researched for its curcumin content, offering antioxidant and anticancer properties (Gupta et al., 2013; Prasad & Aggarwal, 2011). Many of these herbal treatments have transitioned into modern pharmaceuticals, with active compounds extracted and synthesized for wider medical applications, demonstrating the scientific credibility of traditional herbal medicine (Das & Bandyopadhyay, 2023). Despite their efficacy, challenges such as standardization, dosage regulation, and potential side effects remain concerns, necessitating further research and integration into evidence-based healthcare practices (World Health Organization, 2013).

Psychological and Emotional Well-being through Rituals:

Rituals in folk medicine play a dual role, blending spiritual healing with psychological therapy, thereby contributing significantly to traditional health goals. Research indicates that ceremonial practices, such as Ayahuasca rituals among Amazonian tribes, not only facilitate spiritual transcendence but also serve as therapeutic interventions for mental health disorders, including PTSD and depression (Dos Santos et al., 2018). The psychoactive properties of Ayahuasca, combined with the structured ceremonial setting, promote introspection, emotional processing, and neural modulation, which are linked to improved mental well-being (Palhano-Fontes et al., 2019). The communal aspect of these rituals fosters social cohesion and reduces psychological distress, as shared experiences within a supportive group setting enhance emotional resilience and decrease isolation (Laderman & Roseman, 2021). Similar effects are observed in other indigenous healing ceremonies, such as sweat lodge rituals among Native American communities, which are reported to alleviate stress and trauma through symbolic purification and collective participation (Gone, 2016). The rhythmic elements of rituals, including drumming and chanting, have been shown to induce altered states of consciousness, activating brain regions associated with emotional regulation and stress reduction (Winkelman, 2010). The interplay between symbolic meaning, sensory stimulation, and social support within folk medicine rituals underscores their psychological efficacy, aligning them with both traditional and contemporary therapeutic frameworks (Katz & Csordas, 2020). As modern psychological research continues to validate these practices, integrating indigenous healing rituals into broader mental health discussions may offer valuable insights into holistic well-being (Tupper, 2022).

Strengthening Community Cohesion and Cultural Identity:

Folk medicine plays a crucial role in preserving cultural identity by maintaining indigenous healing traditions and fostering community cohesion. Healers, such as Sangomas in South Africa and Curanderos in Latin America, serve not only as medical practitioners but also as spiritual and cultural custodians, ensuring the intergenerational transmission of traditional knowledge (Mwenda & Chitindingu, 2022). These practitioners employ a holistic approach that integrates physical healing with spiritual guidance, counseling, and communal rituals, reinforcing social bonds within their communities (Tshuma & Moyo, 2020). Through ceremonies, oral traditions, and apprenticeship systems, indigenous healing knowledge is preserved and adapted to contemporary health challenges (Das, 2022). These practices contribute to emotional and psychological well-being, as patients receive care within a familiar cultural framework that validates their beliefs and traditions (Langwick, 2011). The recognition of folk medicine within indigenous communities strengthens cultural resilience, affirming identities that have historically been marginalized by colonial and biomedical frameworks (Wreford, 2008). Furthermore, the growing interest in integrating traditional medicine with modern healthcare highlights its enduring relevance, ensuring that these cultural practices continue to evolve while maintaining their foundational values (World Health Organization, 2013).

Accessibility and Cost-effectiveness in Healthcare:

Folk medicine plays a crucial role in improving healthcare accessibility, particularly for marginalized communities in remote areas with limited access to modern medical facilities. Traditional healers serve as primary healthcare providers, offering treatments based on centuries-old indigenous knowledge. The use of *Artemisia annua* (Sweet Wormwood) in African traditional medicine is a notable example, as its active compound led to the development of Artemisinin-based Combination Therapies (ACTs), now a global standard for malaria treatment (Gupta & Sharma, 2020). Similarly, indigenous Amazonian communities rely on *Cinchona* bark, which contains quinine, historically used for malaria management before modern pharmaceuticals (Pérez et al., 2019). Ayurvedic medicine in India provides cost-effective alternatives for chronic diseases through herbal formulations like *Withania somnifera* (Ashwagandha) for stress and immune support (Sharma & Patel, 2021). The affordability of these remedies makes them vital in low-income regions, where Western medicine may be financially out of reach (Das & Bandyopadhyay, 2023). Traditional healing practices emphasize holistic care, integrating physical, spiritual, and emotional well-being, which aligns with the World Health Organization's (WHO) recognition of traditional medicine as an essential component of primary healthcare (WHO, 2021). The resilience of folk medicine highlights its relevance in global health discussions, reinforcing the need for further research and integration into formal healthcare systems while preserving indigenous knowledge (Das, 2022).

Challenges and Future Considerations:

Despite the significant contributions of folk medicine, several challenges hinder its broader acceptance and integration into modern healthcare. One major issue is the lack of scientific validation for many traditional healing practices, as empirical research remains limited (Van der Kooi & Theobald, 2018). Without rigorous clinical trials, concerns about efficacy and safety persist, restricting the formal adoption of indigenous remedies. For instance, while traditional Chinese medicine (TCM) has been widely used for centuries, only a fraction of its treatments, such as *Artemisia annua* for malaria, have undergone extensive scientific evaluation (Liu et al., 2020). Another critical challenge is the ethical and intellectual property rights (IPR) issues surrounding indigenous knowledge (Das & Bandyopadhyay, 2023). Biopiracy, where multinational corporations patent traditional remedies without compensating the indigenous communities that developed them, remains a pressing concern (Oyebode et al., 2016). The case of the neem tree (*Azadirachta indica*), whose antifungal properties were patented by Western companies before legal disputes led to revocations, highlights the need for stronger IPR protections (Kumar & Gupta, 2019). Additionally, integrating traditional medicine with modern healthcare remains a slow process despite growing recognition from organizations like the World Health Organization (WHO). While some countries, such as China and India, have successfully incorporated traditional medicine into national healthcare policies, others struggle with regulatory barriers and limited collaboration between traditional healers and biomedical professionals (Zhang et al., 2021). Strengthening interdisciplinary partnerships, establishing standardized protocols, and fostering mutual respect between traditional and modern healthcare practitioners could enhance patient outcomes, particularly in low-resource settings where folk medicine is a primary healthcare source. Addressing these challenges through scientific validation, ethical regulations, and systemic integration is crucial for the sustainable and equitable use of traditional medicine in global healthcare (Das & Bandyopadhyay, 2023).

Conclusions:

Folk medicine remains a vital component of indigenous communities, preserving healing traditions that address physical, emotional, and spiritual well-being. Rooted in experiential knowledge, these practices not only provide therapeutic benefits but also reinforce cultural identity and environmental sustainability. The oral transmission of indigenous medicinal knowledge through storytelling, apprenticeship, and rituals ensures the continuity of these traditions across generations. Additionally, the spiritual and cultural significance of folk medicine highlights its holistic approach to health, where healing is deeply connected to harmony between individuals, their communities, and nature. Sustainable harvesting practices further demonstrate indigenous communities' commitment to biodiversity conservation, ensuring the long-term availability of medicinal resources.

Despite its enduring relevance, integrating folk medicine into modern healthcare systems presents both opportunities and challenges. Collaboration between traditional healers and biomedical professionals can enhance healthcare accessibility, particularly in remote areas, while

also fostering culturally sensitive medical approaches. However, successful integration requires scientific validation, ethical protections, and regulatory frameworks to preserve indigenous knowledge while promoting evidence-based practices. Recognizing the value of folk medicine alongside modern advancements can contribute to a more inclusive, holistic, and sustainable healthcare system that respects and utilizes the wisdom of indigenous traditions.

Folk medicine remains an essential aspect of indigenous healing traditions, offering holistic healthcare solutions that address physical, psychological, and spiritual well-being. Rooted in centuries of experiential knowledge, these practices continue to serve as accessible and cost-effective alternatives, particularly in marginalized communities with limited access to modern healthcare. Herbal remedies, such as *Azadirachta indica* (Neem) and *Allium sativum* (Garlic), have demonstrated significant medicinal properties, contributing to both traditional healing and modern pharmacological advancements. Additionally, indigenous rituals provide psychological and emotional benefits, fostering social cohesion and cultural resilience.

Despite its widespread use, folk medicine faces challenges, including the need for scientific validation, ethical concerns over intellectual property rights, and difficulties in integrating traditional practices into modern healthcare systems. While organizations like the World Health Organization recognize the importance of traditional medicine, further efforts are required to bridge the gap between indigenous knowledge and biomedical research. Strengthening interdisciplinary collaboration, developing standardized protocols, and implementing protective legal frameworks can enhance the credibility and sustainability of folk medicine. As global interest in holistic and integrative medicine grows, acknowledging and preserving indigenous healing traditions is crucial for a more inclusive healthcare system. By respecting and incorporating traditional knowledge alongside scientific advancements, healthcare systems can become more comprehensive, culturally sensitive, and sustainable, ensuring that these valuable healing practices continue to benefit future generations.

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Chapter- 8

Pyrococcus abyssi's Methionine-tRNA Synthetase Exhibits Hyperthermophilic Signatures in Its Weak Forces and Cavities

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Keywords: Salt-bridge and microenvironment, energetics, weak forces, interior cavity

Abstract: Weak forces, including the salt-bridge and the inner cavity, are of particular importance in protein folding and functioning in extreme environments. A comparative study on these may reveal insights into the intrinsic protein thermostability. Here, we study salt-bridge energetics and its microenvironment, other weak interactions, and interior cavity properties of Methionine-tRNA synthetase from hyperthermophilic, *Pyrococcus abyssi* (PMRS) and mesophilic, *E. coli* (EMRS). Results show that PMRS, which is more hydrophilic, is uniquely distinct from EMRS. PMRS's complete and domain-specific sequences are favorable for more salt-bridges and other weak interactions than EMRS's. In the former, the recruitment of excess networked, long-ranged, and inter-domain salt bridges with energetically advantageous pairs and ME around them suggests that these properties originate from the underlying sequence. The fact that the net stability (ΔΔGnet) per salt bridge of PMRS exceeds that of EMRS denotes a novel design in its salt bridge. Furthermore, compared to EMRS, an excess of hydrogen bonds (HyB), hydrophobic, and other electrostatic interactions in PMRS's core and surface demonstrate that these also contribute to its thermostability. Notably, PMRS has a much lower level of water-mediated HyB than EMRS, pointing to an altered strategy. In addition, compared to EMRS, a lower and higher mostly empty interior cavities in PMRS's core and surface, respectively, indicate that surface engineering is more prominent in PMRS. We think that these differences are indeed related to the thermostability of the PMRS, which would apply to other similar systems.

Introduction:

The crystal structure of protein reveals the arrangements and interactions of the atoms, secondary structures, cavities, and shell-waters of the protein. A general insight into protein thermostability was found from a comparative study of mesophilic and thermophilic proteins (Vogt et al., 1997; Menéndez-Arias and Argosf, 1989; Szilágyi and Závodszky, 2000; Banerjee et al., 2021). Such knowledge helps to understand the basic mechanisms of protein function at high temperatures on the one hand and the practical applications on the other (Vogt et al., 1997). Although the topologies of orthologous proteins of normal and stress environments are similar, considerable differences exist in their homologous position in their sequence (Vogt et al., 1997;

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Menéndez-Arias and Argosf, 1989; Banerjee et al., 2021). The same topology is formed by the interplay of weak forces and secondary structures arising from the intrinsic sequence properties (Anfinsen, 1973; Dill, 1990). Protein-thermostability is due to various mechanisms such as an increase of ion-pair, HyB, polar surface, and helical structure, a decrease of flexible loops, and packing of amino acid residues (Vogt et al., 1997; Menéndez-Arias and Argosf, 1989; Hurley et al., 1992; Russell et al., 1997; Yip et al., 1998; Kumar et al., 2000; Banerjee et al., 2021; Sumida et al., 2024). In the case of thermophilic proteins, the number of low-volume cavities also increases (Vogt et al., 1997; Sternke et al., 2019; Biswas et al., 2020), whose importance in protein thermostability is not so clear (Vogt et al., 1997; Dubey and Jagannadham, 2008). Using the Poisson-Boltzmann Equation based method, it was demonstrated that compared to the mesophilic counterpart, the average electrostatic strength of thermophilic glutamate dehydrogenase and prolyl oligopeptidase increases as the number of salt bridges increases (Kumar et al., 2000; Banerjee et al., 2021). The net-stability of salt-bridge is derived from the sum of three-component terms, such as energetically costly desolvation-term ($\Delta\Delta G_{dslv}$), contributing bridge-term ($\Delta\Delta G_{brd}$), and background-term ($\Delta\Delta G_{bac}$) (Nayek et al., 2014; Nayek et al., 2015; Bandyopadhyay et al., 2019; Bandyopadhyay, 2020; Banerjee et al., 2021). The latter is formed by a handful of residues around the salt-bridge (i.e., the ME) that interact with the positive and negative partners of the salt-bridge (Bandyopadhyay, 2020; Banerjee et al., 2021). Although, in principle, it can be costly or contributing, it has been demonstrated that compared to the mesophiles, thermophile's ME plays a crucial role in protein thermostability (Banerjee et al., 2021; Banerjee et al., 2021). Although aromatic and bulky aliphatic residues' side-chain mediated interactions such as π-π, π-σ, π-amide, π-alkyl, alkyl\alkyl, are important in protein thermostability (Puchkaev et al., 2003; Martinez and Iverson, 2012), these are less studied. Similarly, like the above-mentioned salt bridges and HyBs, it is particularly interesting to understand the importance of other electrostatic interactions, such as π-cation, π-anion, π-HyB, etc., in thermostability (Mecozzi et al., 1996).

Methionine-tRNA ligase (EC:6.1.1.10) from *Pyrcoccus abyssi* (PMRS) and *E. coli* (EMRS) are cytosolic and class-I type enzymes that participate in elongation and initiation of protein synthesis (Crepin et al., 2003; Crepin et al., 2004). The enzyme, in structure, has five distinct domains such as Rossmann, Connective peptide (CP), KMSKS, anticodon, and C-terminal (Crepin et al., 2004). In this context, it is pertinent to understand hyperthermophilic features of PMRS by the use of only available crystal structure, 1RQG relative to its most high-resolution mesophilic counterpart i.e., 1PG2 from *E coli*.

Here, we have done a comparative study of PMRS and EMRS for the above-mentioned concerns. We apply various in silico methods to extract the sequence and structural properties of these proteins to understand their importance in the thermostability of PMRS in the background of EMRS. We believe that our work reveals heitherto unknown insights, which will find application in other similar systems.

Materials and methods:

Sequence and salt-bridge:

Complete and domain-specific sequences of PMRS (722 residues) and EMRS (677 residues) were analyzed using PHYSICO2 (Banerjee et al., 2015). The normalized relative composition of PMRS was plotted using Sigma Plot v12.0. Hoop-Woods hydrophilicity and Kyte-Doolittle hydrophobicity (Banerjee et al., 2015) were computed using an aligned sequence. The absolute frequency of SBFRs was determined from the sequence and salt-bridge (IP and NU) of PMRS and EMRS's structure. From these two, SBFR's normalized frequency used at the salt-bridge was determined. Six pairs, such as HD, HE, RD, RE, KD, and KE can take part in the salt-bridge. The frequencies of these pairs were compared for an equal length of 1RQG (606 residues) and 1PG2 (488 residues).

Salt-bridge energetics:

There are two types of salt-bridge, namely IP and NU. The binary properties of these salt-bridges (such as long-ranged vs. short-ranged, core vs. surface, etc.) were extracted using SBION2 (Gupta et al., 2015; Banerjee et al., 2021). Inter-domain salt-bridge was determined manually. IPM method was followed as earlier for the extraction of IP salt-bridge's energy terms using the PDB2PQR (Dolinsky et al., 2004) and APBS (Baker et al., 2001) programs (Nayek et al., 2014; Banerjee et al., 2021). The energy terms for NU salt-bridge were extracted using the NUM method, since, unlike IP, NU is made up of more than two salt-bridge partners (Bandyopadhyay et al., 2019; Banerjee et al., 2021). The net energy of a salt-bridge is the sum of the component energy terms. Average accessibility was determined using NACCESS program (Banerjee et al., 2021; Banerjee et al., 2021).

Microenvironment of salt-bridge:

Practically only a few residues of protein (~ 1-2%) contribute most of the background energy of a salt-bridge (Bandyopadhyay, 2014; Banerjee et al., 2021). These residues (mostly charged and polar types) orient around the positive and negative partners of the salt-bridge to interact with them. These residues are called ME-residues and their interaction energy is ME-energy (Bandyopadhyay, 2014; Banerjee et al., 2021). After obtaining the background energy by the above-mentioned method, a low energy cut-off is set on it to get the ME-residues and their energy. ME-residues' accessibility was computed using NACCESS program. ME-residues' secondary structure details are extracted from the PDB file itself. Other attributes are manually or programmatically configured if not mentioned otherwise (Bandyopadhyay, 2014; Banerjee et al., 2021).

Hydrogen bonds and other weak interactions:

Depending on the accessibility of the residue, the core and surface residues of the protein were separated and saved as different PDB files. Thus, during such a division of the residue, the shell waters that are within 3.9Å of the residues' atoms were also included in the PDB. It was used as

an input to BIOVIA Discovery Studio 2020 and HyBs, hydrophobic, and other electrostatic interactions were extracted using default parameters. The total number of interactions in the output file and their nature are huge, for example, the 1PG2_Core fraction has a total of 887 interactions (535 HyBs, 316 hydrophobic, and 36 electrostatic). In this inter-residue interaction, each type was normalized using the total Interactions. The same types of PMRS and EMRS subcategories were then compared.

Interior cavity:

The inner cavity of the protein was computed using the default parameters of the Surface_Racer program (Tsodikov et al., 2002). If shell-waters are within 3.9 Å of an atom of a cavity, those are included in the cavity. Surface_Racer determines the relative accessibility of the cavity atoms. The secondary structure details of the cavity atoms were taken from the PDB file. All this information is included together to create a PDB file. In this way, PDB files of all the cavities obtained from the protein were made. These PDB files were analyzed for shell-water content, secondary structure type, and residue class. The average and normalized values of each item were compared between PMRS and EMRS.

Results and discussion:

The sequence and the salt-bridge properties are interrelated:

The properties of the protein structure are derived from the underlying properties in its sequence (Anfinsen, 1973; Dill, 1990). The different forms of this property, therefore, seem to be the main source of deliberate living of hyperthermophiles in their extreme environments. Here, we see that hyperthermophilic, PMRS's sequence is much more hydrophilic than that of EMRS (Fig. 1a). The primary reason for this seems to be in the charged residues (Glu, Arg, Lys), as PMRS is relatively less in most polar residues (Ser, Thr, Asn, Gln) than in EMRS's (Fig. 1b) of which Asn, Gln are thermolabile (Russell et al., 1997).

(a) Hopp-Woods average hydrophilicity of PMRS (red) and EMRS (green). (b) Relative residue composition of PMRS. (c) Kyte-Doolittle hydrophilicity of PMRS (red) and EMRD (green). (d) SBFR's ratio in salt-bridge to the sequence. (e) PMRS and EMRS's salt-bridge pairs' frequency in 606 residue protein. (f) Details of a typical highly stable inter-domain networked unit in the core of PMRS. (g) Correlation between desolvation-term and ASAav for PMRS and EMRS. (h) Total desolvation-energy. (i) Total bridge-energy. (j) Correlation between bridge-term and ASAav for PMRS and EMRS. (k) Correlation between background-term and ASAav for PMRS and EMRS. (l) Total background-energy (m) Net-energy. (n) per salt-bridge stability. (o) Overall ME-energy.

In addition, the relative abundance of bulky hydrophobic residues (Ile, Val, Leu) in PMRS's sequence is noticeable in abundance. Although relative compositions of PMRS's domains (Fig. 2) are almost similar, some observations are very characteristic (Fig. 3a, b, c, d, e, f). The predominance of only acidic (Glu) and basic (Arg, Lys) residues in PMRS's KMSKS and CT (C-terminal) domains (Fig. 3c, e), respectively, may point towards the enhancement of inter-domain interaction specificity. The relative abundance of Cyseine that coordinates Zn^{++} (Crepin et al.,

2003) is only present in the CP domain (Fig. 2 and Fig. 3b) might be for its thermolabile nature (Russell et al., 1997). The decline in PMRS's higher level of hydrophobicity compared to EMRS's after the KMSKS domain (Fig. 1c) may indicate that the hydrophilicity of the charged residues of anticodon and CT domains supersedes the hydrophobic effect (Fig. 3d, e).

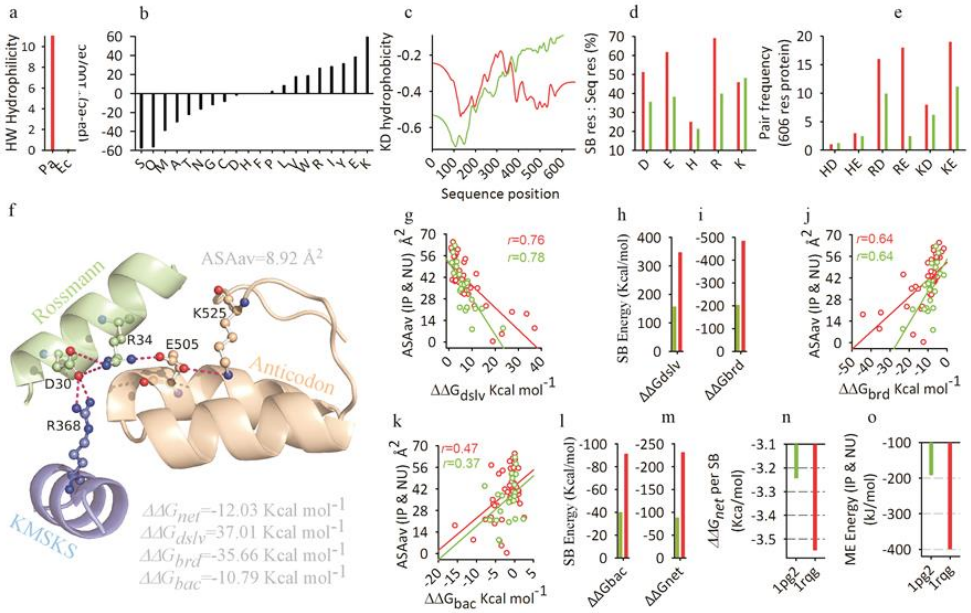

Figure 1: Sequence and salt-bridge properties of PMRD (Pa) and EMRS (Ec).

Figure 2: Crystal structure portion (488 for *E. coli* PDB, 1PG2 and 601 for *Pyrococcus abyssi* PDB, 1RQG) with different domains of Methionyl tRNA synthetase from bacteria (ec, *E. coli*) and *Pyrococcus abyssi* (ab).

Named domains are Rossmann domain (have *HIGH* region; sequence range: 15-25 for ec, and 10-21 for ab), CP domain (Zn binding region; in between Rossmann regions; here, 126-185 residues are absent in the crystal structure of ec), MNKS domain (for ATP binding; 333-337 for ec and 344-348 for ab), Anticodon (in between MNKS regions), and C terminal extreme region (CeX). The tRNA binding region at the C-terminal end is not shown (575-677 for ec and 622-722 for ab).

Table 1: Inter-domain salt-bridges for hyperthermophilic, *Pyrococcus abyssi* (structure: 1RQG). ASAav, average accessibility; ASA-ac, accessibility of acid partner; accessibility of base partner; Dist, distance.

ROSSMANN's partner	CP's partner	Dist (Å)	ASAav (Å^2)	ASA-ac (Å^2)	ASA-bs (Å^2)
E256	H100	3.8	0.4	0.6	0.2
D298	R161	3.3	57.4	57.1	75.8
E325	R198	3.2	52.1	44.8	59.4
ROSSMANN's partner	MNKS's partner				
D30	R368	2.7	10.6	0	21.2
R84	D360	2.9	40.6	39.3	42
K297	D380	3.7	35.9	27.5	44.2
ROSSMANN's partner	ANTICODON's partner				
D34	E505	3.5	15.1	16.3	13.9
K86	D510	2.7	51.5	39.7	63.3
MNKS's partner	ANTICODON's partner				
E377	K453	4.1*	20.5	11.3	29.7
E377	R451	4.8*	32.5	11.3	53.7
MNKS's partner	CT's partner				
E341	K575	3.2	22.2	1.8	42.7

32.5% and 26.8% residues of PMRS and EMRS are salt-bridge forming residue (SBFR) types, respectively, of which 17.7% and 10.3% are forming salt-bridge. In the case of 1RQG, this fraction for the Asp, Glu, and Arg is much higher than 1PG2 (Fig. 1d). Interestingly, of the six possible salt-bridge pairs (Asp-Arg, Asp-Lys, Asp-His, Glu-Arg, Glu-Lys, and Glu-His), the frequency of Asp-Arg, Glu-Arg, and Glu-Lys, which are known to be energetically advantageous (Meuzelaar et al., 2016) and helix promoter (Williams, et al., 1987), is much higher in 1RQG (Fig 1e).

(a) 1PG2's crystal structure and its salt-bridge. Here, the salt-bridges of the inter-domain (R, Rossman i.e., greenish; CP, connective peptide i.e., blueish; AC, anticodon; M, KMSKS i.e., cyan and CT, C-terminal, i.e., brownish) are visible. (b) 1RQG's crystal structure and its salt-bridge. Here, the salt-bridges of the inter-domain. (c) A typical, long-ranged IP-type salt-bridge

of 1RQG, which is absent in 1PG2. (d) Salt-bridge's intervening-distance specific frequency. Here, the frequency in salt-bridge for the range ≤10 is shown differently as it has much higher frequency. (e) Comparison of the frequencies of 1RQG (red) and 1PG2's (green) residue-classes. (CR, charged-class; PO, polar-class; HB, hydrophobic class).

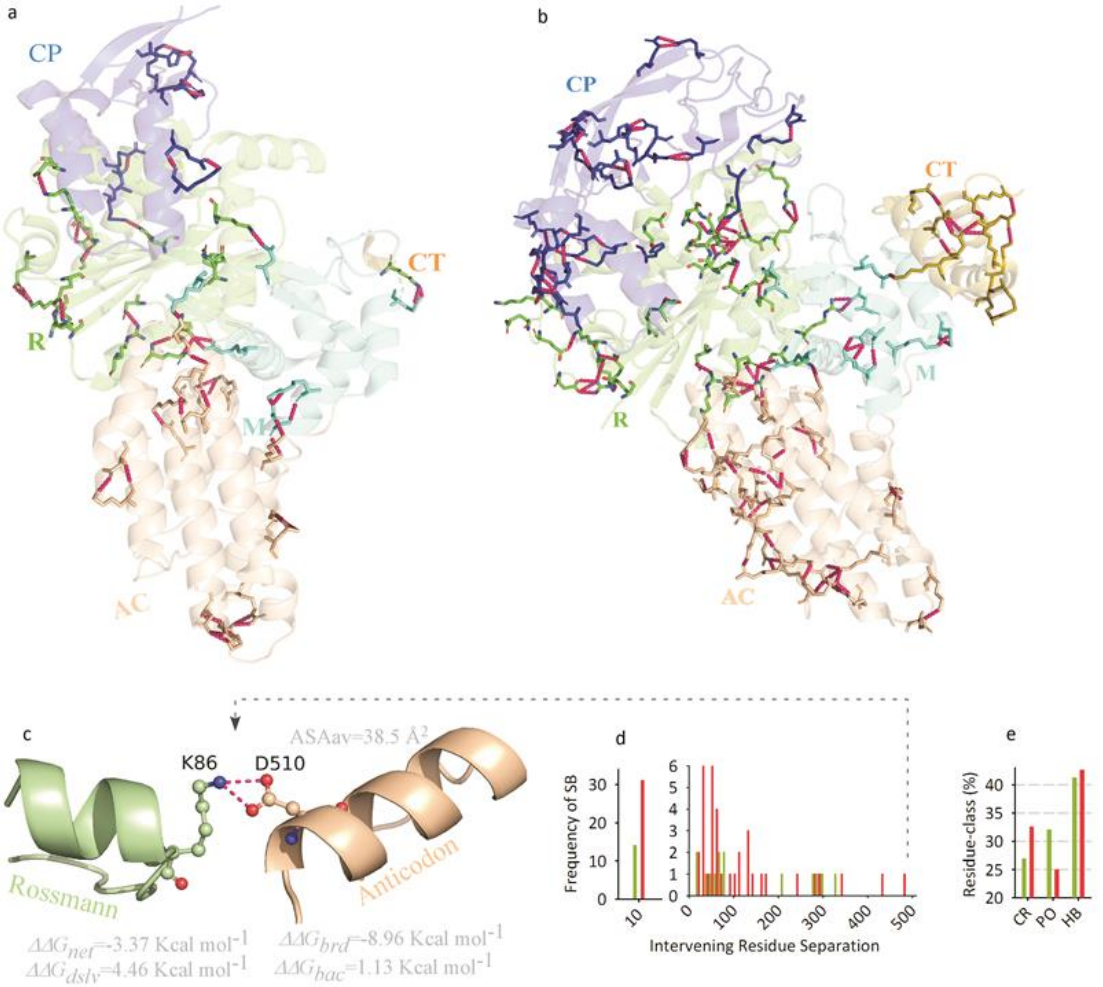

Figure 3: Unique features of salt-bridge in the structure of hyperthermophilic, *Pyrococcus abyssi* (structure: 1RQG) and mesophilic, *E. coli* (structure: 1PG2).

Notably, these have increased almost evenly in every domain as compared to 1PG2 (Fig. 4a-f). Again, the inter-domain salt-bridges are more exhaustive and high in frequency in 1RQG than 1PG2 (Table 1 and 2). Here, the question of whether the additional non-salt-bridge forming residues (nSBFRs) of the sequence (as isolated charged residue) are of any importance is relevant. The answer to this question may come from the energetics of salt-bridge (see below).

Relative composition of Rossmann (**a**), CP (**b**), MNKS (**c**), and anticodon (**d**) domains. pI of complete and domain-specific sequence of MetG tRNA synthetase (**e**). Comparison of normalized total charge (at physicological pH) of *E. coli* (circle) and *Pyrococcus abyssi* (triangle) for complete and region-specific sequences (f). Here, CeX is plotted separately for better visibility of other regions. Comparison of normalized net charge (at physicological pH) of *E. coli*

(circle) and *Pyrococcus abyssi* (triangle) for complete and region-specific sequences (g). Here, CeX is plotted separately for better visibility of other regions. Comparison of normalized GRAVY of *E. coli* (circle) and *Pyrococcus abyssi* (triangle) for complete and region-specific sequences (h). Here, CeX is plotted separately for better visibility of other regions.

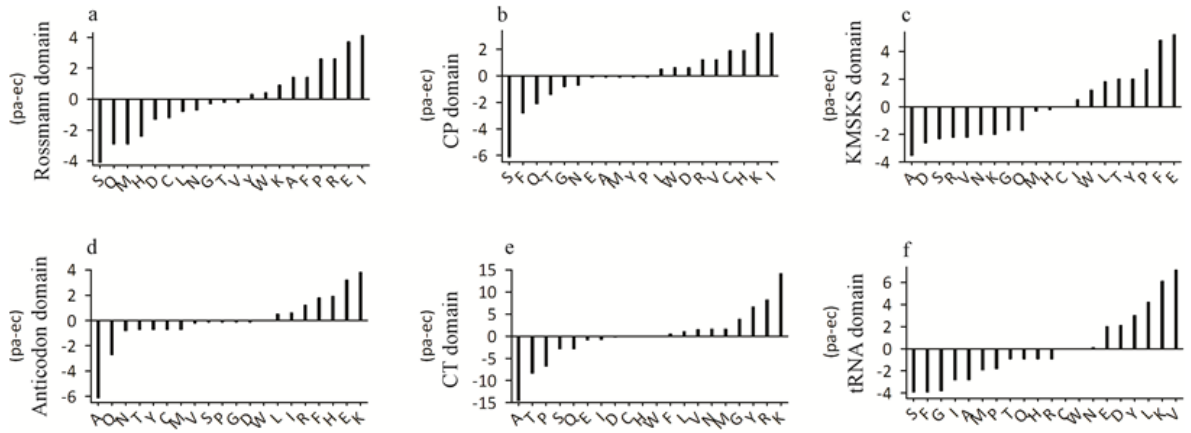

Figure 4: Relative (with reference to *E. coli*) composition and physicochemical properties of *Pyrococcus abyssi* MetG tRNA synthetase.

Table 2: Inter-domain salt-bridges for mesophilic, *E. coli* (structure: 1PG2). ASAav, average accessibility; ASA-ac, accessibility of acid partner; accessibility of base partner; Dist, distance.

ROSSMANN's partner	CP's partner	Dist (Å)	ASAav (Å^2)	ASA-ac (Å^2)	ASA-bs (Å^2)
H95	E101	2.7	22.4	21	23.9
ROSSMANN's partner	MNKS's partner				
D32	R356	2.7	7.2	0	14.3
K295	D369	3.1	37.5	31.1	44
MNKS's partner	ANTICODON's partner				
R366	E436	3.4	50.3	75	25.6
R384	K388	2.6	10.9	2.9	19

Per salt-bridge electrostatic strength is higher in PMRS:

In the former, on the one hand, as the long-ranged, inter-domain salt-bridge has increased (Fig. 4c and Fig. 1f), so has the number of complex networked, intra-domain, and core salt-bridge with more partners (Fig. 5a, b, Table 3-6).

(a) A typical intra-domain (Rossmann) and inter-helix complex networked core salt-bridge. As the salt-bridge is in the core, the desolvation cost is very high. At the same time the bridge energy term is also high. Further, the contribution of the microenvironment is also high. Sum of these three terms, i.e., $\Delta\Delta G_{net}$ is highest among all salt-bridges of the protein. (b) A typical intra-domain (Anticodon) and inter-helix complex networked core salt-bridge. Although, the

desolvation and bridge energy terms are similar as (a), the background term is weak. Here, the $\Delta\Delta G_{net}$ is moderately high, i.e., -12.9 Kcal/mol. This is a cyclic salt-bridge where all acidic and basic partners are interconnected to each-other.

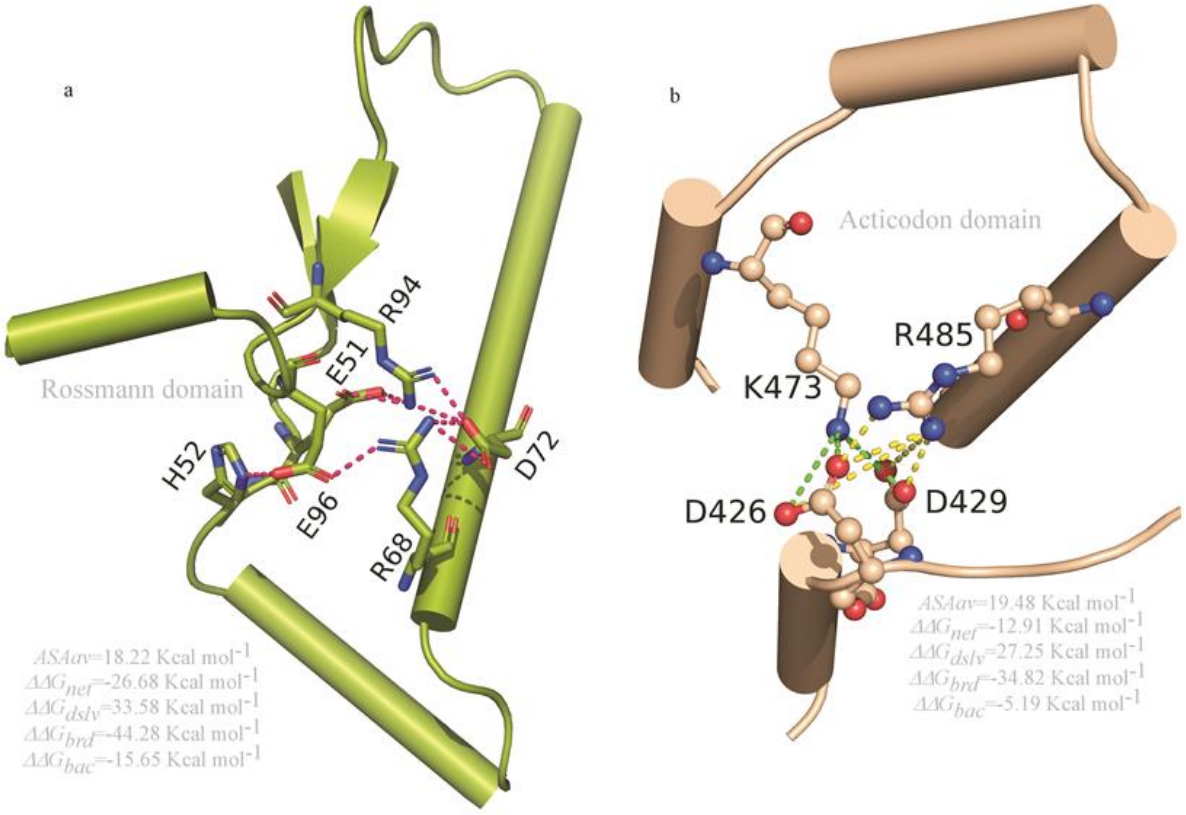

Figure 5: Intra-domain complex networked, core salt-bridge of hyperthermophilic, *Pyrococcus abyssi* (structure: 1RQG).

Table 3: Component and net energy terms, average-distance, and accessibility of isolated pair type of salt-bridge of 1PG2. Energy terms (in Kcal/mol) are extracted following the isolated pair method (IPM). The net energy of the salt-bridge is the sum of the component (desolvation i.e., $\Delta\Delta G_{dslv}$, bridge i.e., $\Delta\Delta G_{brd}$, and background i.e., $\Delta\Delta G_{bac}$) energy terms. Accessibility of acidic (A) and basic (B) residues was extracted by the ACCESS program, whose average is the ASA_{av}.

Salt-Bridge	$\Delta\Delta G_{dslv}$	$\Delta\Delta G_{brd}$	$\Delta\Delta G_{bac}$	$\Delta\Delta G_{net}$	AvDist	ASAav
LYS5_GLU288	4.8	-6.4	-1.5	-3.1	3.2	33.2
LYS316_GLU310	7.2	-10.1	-1.5	-4.3	3.2	20.4
LYS217_GLU220	1.9	-4.5	0.4	-2.3	3.3	58.2
LYS497_GLU500	2.1	-6.0	-0.8	-4.7	3.0	51.5
ARG469_ASP472	3.3	-6.0	-0.5	-3.3	2.7	48.9
LYS439_GLU443	2.1	-3.6	0.0	-1.5	3.1	44.7
HIS95_GLU101	4.8	-7.3	2.4	-0.2	2.7	22.4

LYS419_ASP423	2.7	-5.9	0.2	-3.1	3.2	62.5
ARG366_GLU436	4.6	-1.7	-7.8	-5.0	3.4	43.8
LYS295_ASP369	5.4	-8.0	-0.4	-2.9	3.1	37.5
LYS265_ASP269	5.3	-9.2	-0.2	-4.1	2.8	34.4
ARG103_GLU100	3.5	-5.2	-0.7	-2.4	3.7	52.1
HIS323_GLU27	10.0	-7.4	-3.8	-1.2	3.1	8.2
ARG403_ASP470	8.2	-9.5	0.1	-1.2	2.8	39.5
ARG395_ASP456	6.7	-9.4	-3.2	-5.9	2.9	37.6
LYS547_GLU544	1.4	-8.1	0.4	-6.3	2.8	51.5
ARG233_ASP230	6.7	-11.7	0.0	-5.0	3.0	37.2
LYS492_GLU503	7.9	-10.8	0.2	-2.8	2.6	22.4
ARG39_ASP92	9.9	-8.9	-3.7	-2.7	3.2	27.4

Table 4: Component and net energy terms of network unit type of salt-bridge of 1PG2 by network unit method (NUM). The net energy of the salt-bridge is the sum of the component (desolvation i.e., $\Delta\Delta G_{dslv}$, bridge i.e., $\Delta\Delta G_{brd}$, and background i.e., $\Delta\Delta G_{bac}$) energy terms. Accessibility of partners of NU residues was extracted by NACCESS program whose average is the ASA_{av}.

Salt-Bridge	$\Delta\Delta G_{dslv}$	$\Delta\Delta G_{brd}$	$\Delta\Delta G_{bac}$	$\Delta\Delta G_{net}$	AvDist	ASAav
R356-D32:R36-D32	23.4	-23.3	-4.1	-3.9	3.3	8.9
H189-D234:K248-D234	14.3	-12.0	-6.5	-4.1	2.9	21.7
K6-E44:K6-E279	7.8	-9.9	-4.3	-6.4	3.3	38.2
K388-D384:R380-D384	11.4	-18.1	-4.7	-11.4	2.9	21.6

Table 5: Component and net energy terms, average-distance, and accessibility of isolated pair type of salt-bridge of 1RQG. Energy terms (in Kcal/mol) are extracted following the isolated pair method (IPM). The net energy of the salt-bridge is the sum of the component (desolvation i.e., $\Delta\Delta G_{dslv}$, bridge i.e., $\Delta\Delta G_{brd}$, and background i.e., $\Delta\Delta G_{bac}$) energy terms. Accessibility of acidic (A) and basic (B) residues was extracted by the ACCESS program, whose average is the ASA_{av}.

Salt-Bridge	$\Delta\Delta G_{dslv}$	$\Delta\Delta G_{brd}$	$\Delta\Delta G_{bac}$	$\Delta\Delta G_{net}$	AvDist	ASAav
LYS575_GLU341	11.45	-11.12	-7.67	-7.34	3.21	22.2
LYS441_GLU445	2.73	-7.12	-0.45	-4.84	2.73	61.8
ARG231_ASP136	7.78	-13.61	-3.31	-9.14	3.02	21.9
ARG406_ASP471	6.34	-8.03	-0.87	-2.56	2.71	43.2
ARG3_ASP286	11.08	-13.06	-6.16	-8.14	2.82	23.5
LYS279_GLU321	5.67	-7.53	1.47	-0.39	2.61	32.4
ARG422_GLU531	4.42	-6.86	-2.54	-4.98	2.69	41.8
ARG198_GLU325	2.67	-5.48	-0.08	-2.89	2.87	52.1

ARG37_ASP90	8.47	-8.72	-4.58	-4.83	3.08	31.1
LYS477_GLU481	1.85	-4.17	0.09	-2.23	3.17	64.9
LYS280_GLU277	1.58	-6.25	-0.38	-5.05	3.21	40.3
ARG451_ASP454	2.86	-7.04	-0.03	-4.21	2.92	45.6
ARG183_GLU128	6.56	-10.1	-4.09	-7.63	3.13	51.8
LYS86_ASP510	4.46	-8.96	1.13	-3.37	2.7	38.7
LYS200_GLU221	5.76	-8.57	-0.75	-3.56	2.73	34
ARG84_ASP360	5.73	-7.09	1.14	-0.22	2.88	40.6
LYS79_GLU76	2.41	-7.06	0.01	-4.64	3.11	52.1
ARG391_ASP387	5.69	-9.24	-5.57	-9.12	2.9	32.1
ARG584_GLU588	3.89	-7.07	-1.24	-4.42	2.78	60
LYS389_ASP550	2.61	-6.72	0.26	-3.85	3.17	39.4
LYS152_ASP150	2.94	-6.79	1.02	-2.83	3.09	41
ARG161_ASP298	3.24	-5.88	-6.08	-8.72	2.96	57.4
HIS517_GLU513	2.25	-3.76	-1.01	-2.52	2.76	55.3
HIS100_GLU256	18.98	-12.45	-2.01	4.52	3.43	0.4
LYS120_GLU226	2.65	-5.11	0.14	-2.32	2.63	42.8
ARG430_GLU434	1.38	-2.84	-0.06	-1.52	2.63	59.1
ARG65_GLU73	4.6	-8.48	-0.8	-4.68	3	46.2
ARG526_GLU528	2.77	-4.97	-0.21	-2.41	2.68	55.8
ARG227_GLU225	2.23	-5.57	1.07	-2.27	2.76	55.5
LYS318_GLU323	1.01	-1.73	0.18	-0.54	3.73	61.1
LYS270_GLU267	3.08	-7.26	-1.79	-5.97	2.92	40

Table 6: Component and net energy terms of network unit type of salt-bridge of 1RQG by network unit method (NUM). The net energy of the salt-bridge is the sum of the component (desolvation i.e., $\Delta\Delta G_{dslv}$, bridge i.e., $\Delta\Delta G_{brd}$, and background i.e., $\Delta\Delta G_{bac}$) energy terms. Accessibility of partners of NU residues was extracted by NACCESS program whose average is the ASA_{av}.

Salt-Bridge	$\Delta\Delta G_{dslv}$	$\Delta\Delta G_{brd}$	$\Delta\Delta G_{bac}$	$\Delta\Delta G_{net}$	AvDist	ASAav
K249-D232:H187-D232:	22.9	-17.9	-3.4	1.6	3.1	5.2
R201-E197:R201-E204:	7.1	-8.2	-3.1	-4.2	2.9	53.9
K119-E243:K119-D245:	12.3	-15.3	-5.7	-8.7	3.2	35.0
K297-E336:K297-D380:	6.7	-8.2	-5.2	-6.8	3.1	31.1
R530-E523:K498-E523:	16.1	-21.2	2.6	-2.5	3.1	23.4
R599-E595:K587-E595:	4.5	-10.1	0.5	-5.0	2.6	43.2

R458-E435:K438-E435:R458-E442:	9.4	-18.7	0.6	-8.8	3.2	35.1
H52-E96:R94-E51:R94-D72:R68-D72:R68-E96:	33.6	-44.3	-15.7	-26.4	2.8	18.2
K110-E113:K110-E106:K102-E106:	8.0	-16.5	-1.1	-9.7	2.8	36.4
R368-D30:R34-D30:K525-E505:R34-E505:	37.0	-35.6	-10.8	-9.3	3.2	8.9
R570-E567:R570-D574:R579-D574:	11.9	-23.0	-1.2	-12.4	2.6	44.2
R485-D426:K473-D426:R485-D429:K473-D429:	27.3	-34.8	-5.2	-12.8	2.9	19.5

In some cases, these salt-bridges are of the self-neutralized (cyclic) (Fig. 5b) and secondary structures locking type (Fig. 4c, Fig. 1f and Fig. 5a, b). The abundance of such complexly designed salt-bridges in PMRS is largely unseen in EMRS. How does such a design of salt bridge affect its energetics? Several points are noteworthy in this aspect. First, PMRS has more core and intricate NU-type salt-bridges than EMRS (Table 4 vs. 6), and thus, the costly desolvation-term ($\Delta\Delta G_{dslv}$) is somewhat higher in PMRS (Fig. 1g, h). However, due to the recruitment of energetically advantageous and helix promoting pairs (Williams et al., 1987; Meuzelaar et al., 2016), the bridge-term ($\Delta\Delta G_{brd}$) has also been more contributing in PMRS than EMRS (Fig 1i, j) such that it causes an overall energy gain even after neutralizing the cost of $\Delta\Delta G_{dslv}$ (Table 3-6). These two terms, which rely solely on the salt-bridge partners (Bandyopadhyay et al., 2019; Bandyopadhyay, 2020; Banerjee et al., 2021), show an apparent linear relation to the protein's location-specific dielectric constant related parameter, ASAav (Fig. 1g, j). Second, unlike these two terms, the background term ($\Delta\Delta G_{bac}$) that relies on other residues of protein other than salt-bridge partners, and, which is equally likely to be costly or contributing, has been more contributing in PMRS and is almost unrelated to ASAav (Fig. 1k, l) indicating the involvement of other factors (Banerjee et al., 2021), which appears to be related with the intrinsic ME (Banerjee et al., 2021). Notably, the net ($\Delta\Delta G_{net}$) (Fig. 1m) and per salt-bridge (Fig. 1n) stability in PMRS is more favorable than that of the EMRS indicates a novel strategy. Third, since NU reduces desolvation cost more efficiently than IP (Bandyopadhyay et al., 2019; Banerjee et al., 2021), its higher level in PMRS seems to be a strategy (Table S4 vs. S6). ME, which is much more in PMRS due to an increase in sequence hydrophilicity than EMRS (Fig. 10), seems to be an intrinsic strategy. Overall, the change in the intrinsic property of the sequence seems to be the reason for the more favorable salt-bridges in PMRS.

Significance of ME in salt-bridge mediated thermostability:

ME-residues, which are mostly composed of charged and polar residue classes and derive from the underlying sequence, positioned themselves around the positive and negative partners

of salt-bridge and interacts with them (Banerjee et al., 2021). Here, several points are noteworthy. First, there are three ME-classes in charged-class. These are nSBME, IPME, and NUME (Table 7-10). Second, relative to EMRS, in PMRS, more nSBME act as ME-residues (Table 7 and Table 8), which explains why some of these do not participate in the salt-bridge, even though there is an excess of charged residues in the sequence. Third, due to the increase of salt-bridge, a higher proportion of ME-residues are also present in PMRS than EMRS. Along with the location at the core and surface of the salt-bridge, these MEs also orient themselves in those locations (Table 7-10). Fourth, the overall ME-energy is seen to be more contributing to PMRS than EMRS's, which appears to be largely due to energetically advantageous substitution at homologous positions in PMRS's sequence (Table 7-10). This is probably why its sequence hydrophilicity is much higher than that of its mesophilic counterpart (Nayek et al., 2015; Bandyopadhyay, 2020; Banerjee et al., 2021). Notably, although less polar class in PMRS than in EMRS, ME-energy is far greater in the former, which may indicate the proper use of selective polar residues. Surprisingly, the ME energy of the charged class has been less than the polar class might be for repulsive interactions between the salt-bridge partners and the charged ME residues. In the balance of protein rigidity and flexibility, this observation may have a beneficial role to play. Fourth, PMRS's ME-population is enriched in helix and also coil than EMRS's (Table 9). Finally, as ME-residue may interact with partners of more than one salt-bridge, the TU-value is greater than the actual counts of ME-residue (Table 7-10). This type of use seems to be particularly helpful in the structural integrity of the multi-domain protein. However, such exhaustive and overlapping usage of ME-residue is much more prominent in PMRS than EMRS and appears to play a crucial role in PMRS thermostability.

Table 7: Classes, categories, binary details, and interaction energies of microenvironment residues of 1RQG (606 residues). The energy cut-off=±0.75 kJ/mol. Co, core; su, surface; H, helix; S, strand; C, coil; TU, times used.

Res class	ME-class	nSBME	IPME	NUME	Total	co	su	Enz kJ/mol	H	S	C	TU
Charged	IP	20	6	14	40	11	29	-32.31	21	3	16	49
	NU	17	21	4	42	6	36	-10.59	16	5	21	49
	IP&NU	17	8	10	35	21	14	-55.07	18	4	13	92
Polar	IP	23	-	-	-	14	9	-129.73	17	4	2	23
	NU	17	-	-	-	12	5	-118.25	8	7	2	18
	IP&NU	6	-	-	-	3	3	-65.28	2	0	4	12
PG	IP	1	-	-	-	1	0	2.03	1	0	0	1

	NU	7	-	-	-	6	1	15.18	2	0	5	8
	IP&NU	2	-	-	-	2	0	-0.11	1	0	1	4
Hydrophobic	IP	0	-	-	-	0	0	0.00	0	0	0	0
	NU	3	-	-	-	3	0	-2.65	0	1	2	4
	IP&NU	1	-	-	-	1	0	-0.37	0	1	0	2
	Grand Total	**114**	**35**	**28**	**-**	**80**	**97**	**-397.14**	**86**	**25**	**66**	**262**

Table 8: Classes, categories, binary details, and interaction energies of microenvironment residues of 1PG2. The energy cut-off=±0.75 kJ/mol. H, helix; S, strand; C, coil; TU, times used.

	1pgm class of 1pg2 (488 Res)	nSBME	IPME	NUME	Total	co	su	Enz kj/mol	H	S	C	TU
CR	IP	13	7	4	24	5	19	-30.16	11	4	9	29
	NU	15	8	2	25	6	19	-9.37	19	5	1	26
	IP&NU	14	5	0	19	12	7	-48.75	10	2	7	44
PO	IP	20	-	-	-	13	7	-43.62	12	2	6	20
	NU	5	-	-	-	4	1	-35.90	2	3	0	5
	IP&NU	3	-	-	-	3	0	-30.18	0	1	2	6
PG	IP	3	-	-	-	3	0	-0.26	1	0	2	4
	NU	2	-	-	-	2	0	10.88	1	0	1	2
	IP&NU	0	-	-	-	0	0	0.00	0	0	0	0
	IP	1	-	-	-	1	0	-1.88	0	1	0	1
HB	NU	0	-	-	-	0	0	0.00	0	0	0	0
	IP&NU	1	-	-	-	1	0	-0.24	0	1	0	2
	Grand Total	**77**	**20**	**6**	**-**	**50**	**53**	**-189.48**	**56**	**19**	**28**	**139**

Table 9: Residue specific details of the microenvironment for isolated and network unit types of salt-bridge of 1PG2. Microenvironment (ME) residue can participate in isolated (IP) or network (NU) or both(IPNU) types of salt-bridge (SB). Thus, types of ME are of three types: IP_ME (ME for IP type of SB), NU_ME (ME for NU type of SB), and IPNU_ME ME for both IP and NU types of SB). Each residue (row-wise) is presented with residue name, residue ID as per the PDB file. ME_Energy is the interaction energy between the ME residue with the positive and negative partners of the concerned salt-bridge. An ME that can be either an IP partner (IP_ME) or an NU partner (NU_ME) or a non-salt-

bridge ME partner (nSBME) by itself can participate in the IP or NU or both types salt-bridge's microenvironment as a ME candidate. Residue-specific total interaction energy (ME_Energy) with the partners of salt-bridge is expressed in kJ/mol. Residue side-chain accessibility (ASA), type of secondary structure (Coil C, Helix H, and strand S) are also shown. Again, an ME residue can be used as an ME candidate for multiple salt-bridges. It has been denoted by the Times Used (TU) parameter. Only those residues were considered as ME-residue whose interaction energy was either greater than 0.75 kJ/mol (unstable) or less than -0.75 kJ/mol (stable). If a residue is ME for multiple times, the sum of the energy was not used as screening criteria.

Type of ME	Residue name	Residue ID	ME-Energy (kJ/mol)	ASA Å^2	SECONDARY STRUCTURE	Times Used	Type of partner SB (IP or NU) nSB
NU_ME	ARG	122	1.3228	55.9	S	1	nSBME
NU_ME	ARG	41	0.7853	42.4	H	1	nSBME
NU_ME	ARG	453	0.9133	66.7	H	1	nSBME
NU_ME	ARG	485	1.0376	6.2	H	1	nSBME
NU_ME	ARG	501	2.2538	44.8	H	1	nSBME
NU_ME	ASN	121	-0.9986	44.1	S	1	nSBME
NU_ME	ASP	278	0.7864	76.2	H	1	nSBME
NU_ME	ASP	351	-6.4147	34.9	H	1	nSBME
NU_ME	ASP	376	-3.6467	52.7	H	1	nSBME
NU_ME	ASP	449	-7.5481	49.5	H	1	nSBME
NU_ME	ASP	83	-1.2096	7	H	1	nSBME
NU_ME	GLU	188	-4.5967	55.9	S	1	nSBME
NU_ME	HIS	21	0.9332	26.5	H	1	nSBME
NU_ME	LYS	114	0.9407	56.7	H	1	nSBME
NU_ME	LYS	282	-2.2694	58.6	H	1	nSBME
NU_ME	LYS	362	2.5006	38.5	H	2	nSBME
NU_ME	PRO	493	2.136	0	C	1	nSBME
NU_ME	PRO	496	8.7403	3.9	H	1	nSBME
NU_ME	SER	187	-18.3885	19.5	S	1	nSBME
NU_ME	SER	232	-4.7789	0.1	S	1	nSBME
NU_ME	TYR	280	1.7722	0.6	H	1	nSBME
NU_ME	TYR	357	-13.51	0.1	H	1	nSBME
NU_ME	ARG	233	8.3635	36.1	S	1	IP
NU_ME	ARG	380	1.1995	42.8	H	1	NU
NU_ME	ARG	395	0.8532	21.5	H	1	IP
NU_ME	ASP	230	-8.6499	38.2	S	1	IP
NU_ME	ASP	384	-1.8952	2.9	H	1	NU
NU_ME	ASP	456	-0.8221	53.8	H	1	IP
NU_ME	ASP	92	2.5489	21.3	C	1	IP

NU_ME	GLU	27	-0.8644	9.3	H	1	IP
NU_ME	GLU	288	1.3147	27.1	S	1	IP
NU_ME	LYS	497	2.791	44.5	H	1	IP
IP_ME	ASN	102	-1.5138	0.4	H	1	nSBME
IP_ME	ASN	266	3.5294	20.5	H	1	nSBME
IP_ME	ASN	391	-1.4173	45.6	H	1	nSBME
IP_ME	ASN	396	-2.8365	0	H	1	nSBME
IP_ME	ASN	452	-12.1257	15.9	H	1	nSBME
IP_ME	ASN	93	-0.8118	14.1	S	1	nSBME
IP_ME	ASP	296	1.005	61.9	C	1	nSBME
IP_ME	ASP	368	-1.3897	64.8	C	1	nSBME
IP_ME	ASP	51	-1.9834	1.3	S	2	nSBME
IP_ME	CYS	11	-1.881	0	S	1	nSBME
IP_ME	GLN	104	-1.1144	65.8	H	1	nSBME
IP_ME	GLN	213	-1.2185	49.2	H	1	nSBME
IP_ME	GLN	30	2.5311	5.7	H	1	nSBME
IP_ME	GLN	466	-3.2526	49.1	C	1	nSBME
IP_ME	GLU	107	-4.8439	44.9	H	1	nSBME
IP_ME	GLU	212	-1.2791	78.1	H	1	nSBME
IP_ME	GLU	241	-2.5921	61.4	C	1	nSBME
IP_ME	GLU	411	0.9272	81.5	C	1	nSBME
IP_ME	GLU	433	-2.2742	40.1	C	1	nSBME
IP_ME	GLY	262	0.8528	0	H	1	nSBME
IP_ME	GLY	324	0.2795	0	C	2	nSBME
IP_ME	HIS	301	-4.3184	4	H	1	nSBME
IP_ME	HIS	80	-1.8035	0.6	H	1	nSBME
IP_ME	HIS	98	1.9319	35.6	C	1	nSBME
IP_ME	LYS	270	-0.5939	52	H	3	nSBME
IP_ME	LYS	283	-7.6117	46.3	C	1	nSBME
IP_ME	PRO	14	-1.3966	3.1	C	1	nSBME
IP_ME	SER	263	-1.5621	0.1	H	1	nSBME
IP_ME	SER	318	0.8864	22.5	C	1	nSBME
IP_ME	SER	364	-3.5692	31.9	C	1	nSBME
IP_ME	SER	365	-20.0916	12.4	C	1	nSBME
IP_ME	SER	394	0.8162	45.1	H	1	nSBME
IP_ME	SER	99	3.8422	8.9	H	1	nSBME
IP_ME	TYR	237	-0.8812	7.5	C	1	nSBME
IP_ME	TYR	260	-1.0041	1.1	H	1	nSBME
IP_ME	TYR	290	-2.7545	16.9	S	1	nSBME
IP_ME	TYR	325	-1.0673	59.1	C	1	nSBME
IP_ME	ARG	36	3.9893	26.6	H	3	NU

IP_ME	ASP	234	-0.9192	42.1	S	1	NU
IP_ME	ASP	269	2.3121	36.4	H	1	IP
IP_ME	ASP	32	-8.0664	0	H	1	NU
IP_ME	ASP	369	-2.9	44	C	1	IP
IP_ME	GLU	100	0.7595	72	H	1	IP
IP_ME	GLU	436	-0.7644	25.6	H	1	IP
IP_ME	GLU	44	1.0752	35.4	S	1	NU
IP_ME	HIS	95	-0.8721	21	S	1	IP
IP_ME	LYS	265	-2.6437	32.4	H	1	IP
IP_ME	LYS	295	2.6967	31.1	C	1	IP
IPNU_ME	ARG	271	-12.116	39	H	2	nSBME
IPNU_ME	ARG	315	-3.7326	8.8	C	3	nSBME
IPNU_ME	ARG	435	3.9613	10.7	C	3	nSBME
IPNU_ME	ARG	442	0.646	73.7	H	2	nSBME
IPNU_ME	ASN	46	-7.1105	0.1	S	2	nSBME
IPNU_ME	ASN	88	3.3187	18.9	C	2	nSBME
IPNU_ME	ASP	255	-6.3145	0.5	H	3	nSBME
IPNU_ME	ASP	273	5.245	15.3	C	2	nSBME
IPNU_ME	ASP	353	-21.7569	1.3	H	2	nSBME
IPNU_ME	ASP	52	2.3307	3.3	C	2	nSBME
IPNU_ME	GLU	509	-2.1962	75.6	C	2	nSBME
IPNU_ME	HIS	24	-2.1175	39.2	H	2	nSBME
IPNU_ME	HIS	28	-9.1918	1.8	H	2	nSBME
IPNU_ME	HIS	291	-5.1287	0	S	3	nSBME
IPNU_ME	HIS	43	0.1077	14.3	C	2	nSBME
IPNU_ME	HIS	54	5.1465	0.2	C	3	nSBME
IPNU_ME	PHE	47	-0.2388	0	S	2	nSBME
IPNU_ME	SER	90	-26.3875	16.4	C	2	nSBME
IPNU_ME	ARG	39	-2.7158	33.4	H	3	IP
IPNU_ME	GLU	500	-2.6301	58.5	H	2	IP
IPNU_ME	GLU	503	-1.6814	20.9	H	2	IP
IPNU_ME	HIS	323	0.1196	7	S	2	IP
IPNU_ME	LYS	492	3.2775	24	H	2	IP

Table 10: Residue specific details of the microenvironment for isolated and network unit types of salt-bridge of 1RQG. Microenvironment (ME) residue can participate in isolated (IP) or network (NU) or both (IPNU) types of salt-bridge (SB). Thus, types of ME are of three types: IP_ME (ME for IP type of SB), NU_ME (ME for NU type of SB), and IPNU_ME ME for both IP and NU types of SB). Each residue (row-wise) is presented with residue name, residue ID as per the PDB file. ME_Energy is the interaction energy between the ME residue with the positive and negative partners of the concerned salt-bridge. An ME that can be either an IP partner (IP_ME) or an NU partner (NU_ME) or a non-salt -bridge ME partner (nSBME) by itself can participate in the IP or NU or both types salt-bridge's microenvironment as a ME candidate. Residue-specific total interaction energy (ME_Energy) with the partners of salt-bridge is expressed in kJ/mol. Residue side-chain accessibility (ASA), type of secondary structure (Coil C, Helix H, and strand S) are also shown. Again, an ME residue can be used as an ME candidate for multiple salt-bridges. It has been denoted by the Times Used (TU) parameter. Only those residues were considered as ME-residue whose interaction energy was either greater than 0.75 kJ/mol (unstable) or less than -0.75 kJ/mol (stable). If a residue is ME for multiple times, the sum of the energy was not used as screening criteria.

Type of ME	Residue name	Residue ID	ME-Energy (kJ/mol)	ASA Å^2	SECONDARY STRUCTURE TYPE	Times Used	Type of partner SB (IP or NU) nSB (non SB)
NU_ME	ALA	186	-1.0743	14.1	S	1	nSBME
NU_ME	ARG	379	-2.108	78.3	C	1	nSBME
NU_ME	ASN	114	-1.1267	26.9	H	1	nSBME
NU_ME	ASN	335	-1.1178	8.7	S	1	nSBME
NU_ME	ASP	242	1.036	102.8	C	1	nSBME
NU_ME	ASP	331	1.2415	22.4	C	1	nSBME
NU_ME	ASP	482	-3.0462	44	H	1	nSBME
NU_ME	GLN	124	-9.1322	37.5	S	1	nSBME
NU_ME	GLU	166	-2.1892	77.2	C	1	nSBME
NU_ME	GLU	244	1.7658	83.3	C	1	nSBME
NU_ME	GLU	427	1.5903	98.1	H	1	nSBME
NU_ME	GLU	431	1.0963	57.2	H	1	nSBME
NU_ME	GLY	501	4.1481	0	H	2	nSBME
NU_ME	HIS	116	0.1216	21.7	C	2	nSBME
NU_ME	HIS	293	-1.0113	0.5	S	1	nSBME
NU_ME	HIS	472	1.1845	46.2	H	1	nSBME
NU_ME	HIS	536	8.3675	20	C	2	nSBME
NU_ME	LYS	191	0.9639	32.4	C	1	nSBME
NU_ME	LYS	209	-5.5604	53.5	H	1	nSBME
NU_ME	LYS	247	-0.347	62.2	C	2	nSBME
NU_ME	LYS	344	-0.9828	88.2	S	1	nSBME
NU_ME	LYS	606	2.0919	108.3	C	1	nSBME

NU_ME	MET	246	-2.5007	0.1	C	2	nSBME
NU_ME	PHE	133	0.9274	18.8	C	1	nSBME
NU_ME	PRO	240	0.9272	5.2	C	1	nSBME
NU_ME	PRO	28	1.1074	0	C	1	nSBME
NU_ME	PRO	376	-1.6134	1	C	1	nSBME
NU_ME	PRO	506	2.1667	0	C	1	nSBME
NU_ME	PRO	509	6.5422	8.1	H	1	nSBME
NU_ME	PRO	533	1.9015	55.2	C	1	nSBME
NU_ME	SER	185	-17.9638	9.5	S	1	nSBME
NU_ME	SER	461	2.1193	46.2	H	1	nSBME
NU_ME	SER	512	-30.7314	0	H	1	nSBME
NU_ME	THR	122	1.2366	29	S	1	nSBME
NU_ME	THR	230	4.3965	0	S	1	nSBME
NU_ME	THR	489	-1.2946	2.6	H	1	nSBME
NU_ME	THR	49	-13.8669	0	S	1	nSBME
NU_ME	TRP	235	-2.8618	16.9	C	1	nSBME
NU_ME	TRP	516	-5.0178	6.4	H	2	nSBME
NU_ME	TYR	112	-1.2563	50	H	1	nSBME
NU_ME	TYR	188	-17.2889	4.7	S	1	nSBME
NU_ME	TYR	314	-2.4072	5.9	C	1	nSBME
NU_ME	TYR	469	-19.1123	0.3	H	1	nSBME
NU_ME	TYR	602	-2.8232	4.5	H	1	nSBME
NU_ME	ARG	65	-0.8191	39.1	C	1	IP
NU_ME	GLU	73	0.7663	53.4	H	1	IP
NU_ME	ARG	84	1.2178	39.3	H	1	IP
NU_ME	LYS	86	4.5995	37.4	H	1	IP
NU_ME	ASP	90	0.9376	27.7	C	1	IP
NU_ME	LYS	119	5.3804	30.1	S	1	NU
NU_ME	LYS	120	2.8316	41.2	S	1	IP
NU_ME	ARG	161	-0.7991	68.7	C	1	IP
NU_ME	LYS	200	-13.8854	10	H	1	IP
NU_ME	GLU	221	8.9047	57.9	H	1	IP
NU_ME	GLU	225	-1.1613	68.9	C	1	IP
NU_ME	GLU	226	-1.2253	44.3	C	1	IP
NU_ME	ARG	227	1.9857	42.1	S	1	IP
NU_ME	GLU	243	-3.7	31.6	C	1	NU
NU_ME	ASP	245	-4.9659	43.2	C	1	NU
NU_ME	ARG	391	0.8623	34.5	H	1	IP
NU_ME	ARG	422	-0.1494	24.7	C	2	IP
NU_ME	ARG	430	-0.935	70	H	1	IP

NU_ME	ARG	458	1.2504	19.4	H	1	NU
NU_ME	ASP	510	-6.0814	40	H	1	IP
NU_ME	GLU	513	-11.1232	57.4	H	2	IP
NU_ME	HIS	517	3.476	53.2	H	1	IP
NU_ME	ARG	526	2.8417	52.1	C	2	IP
NU_ME	GLU	528	-2.8484	59.5	C	2	IP
NU_ME	GLU	531	-2.1616	58.9	C	1	IP
IP_ME	ARG	137	1.908	27.1	H	2	nSBME
IP_ME	ARG	271	-3.2062	63.7	H	1	nSBME
IP_ME	ARG	59	1.7813	41.4	H	2	nSBME
IP_ME	ASN	276	8.1087	61.8	C	1	nSBME
IP_ME	ASN	399	-0.796	26.8	H	1	nSBME
IP_ME	ASN	402	5.636	47.6	H	1	nSBME
IP_ME	ASN	467	-13.0092	14.4	H	1	nSBME
IP_ME	ASN	491	-14.057	2.8	H	1	nSBME
IP_ME	ASP	130	0.8901	21.7	C	1	nSBME
IP_ME	ASP	319	-1.0115	81	C	1	nSBME
IP_ME	ASP	42	-10.1651	47.6	S	2	nSBME
IP_ME	ASP	551	0.7996	56.6	H	2	nSBME
IP_ME	ASP	581	-2.033	47	H	2	nSBME
IP_ME	GLN	288	-9.698	38.4	C	1	nSBME
IP_ME	GLN	77	-1.3277	37.7	H	1	nSBME
IP_ME	GLU	149	0.8351	95.3	C	1	nSBME
IP_ME	GLU	157	-2.7898	51.1	C	1	nSBME
IP_ME	GLU	320	-2.5453	78.7	C	1	nSBME
IP_ME	GLU	357	0.8039	28.8	H	1	nSBME
IP_ME	GLU	41	0.7703	13.2	C	1	nSBME
IP_ME	GLU	63	-1.4641	47.5	H	1	nSBME
IP_ME	GLU	69	-2.5449	62.4	H	1	nSBME
IP_ME	GLY	260	2.0305	0	H	1	nSBME
IP_ME	HIS	268	-6.0303	8.1	H	2	nSBME
IP_ME	HIS	405	1.8099	55.1	H	1	nSBME
IP_ME	LYS	316	1.0981	72.4	C	1	nSBME
IP_ME	LYS	39	-2.5337	45.4	C	2	nSBME
IP_ME	LYS	457	-0.8622	77.3	H	2	nSBME
IP_ME	SER	104	-5.9616	0	H	1	nSBME
IP_ME	SER	181	-12.9052	27.3	S	1	nSBME
IP_ME	SER	263	-1.7311	0.3	H	1	nSBME
IP_ME	SER	384	-17.2879	23.5	H	1	nSBME
IP_ME	THR	289	-11.9535	1.7	S	1	nSBME
IP_ME	THR	54	-16.053	1.5	H	1	nSBME

IP_ME	THR	81	-1.8627	1.2	H	1	nSBME
IP_ME	TRP	278	-1.0982	11.3	H	1	nSBME
IP_ME	TYR	126	-2.536	18.8	S	1	nSBME
IP_ME	TYR	189	-1.6057	14.9	S	1	nSBME
IP_ME	TYR	252	-0.8824	29.3	H	1	nSBME
IP_ME	TYR	281	-1.0112	8.2	H	1	nSBME
IP_ME	TYR	370	-15.6696	0.5	H	1	nSBME
IP_ME	TYR	560	1.9456	20.8	H	1	nSBME
IP_ME	TYR	576	-14.8017	23.6	H	1	nSBME
IP_ME	TYR	577	-1.1728	12.8	H	1	nSBME
IP_ME	ARG	3	2.8145	15.8	S	1	IP
IP_ME	ASP	30	-8.2999	0	C	1	NU
IP_ME	ARG	34	2.3936	16.3	C	2	NU
IP_ME	GLU	51	-2.5145	0	C	1	NU
IP_ME	ASP	72	0.8184	41.3	H	1	NU
IP_ME	ARG	94	1.423	13.2	S	1	NU
IP_ME	LYS	102	1.0106	60.6	H	1	NU
IP_ME	GLU	197	-1.8345	61.8	H	1	NU
IP_ME	ARG	201	4.6109	61	H	1	NU
IP_ME	GLU	204	-7.7596	38.9	H	1	NU
IP_ME	GLU	277	0.8048	43.3	C	1	IP
IP_ME	LYS	280	2.5558	37.4	H	1	IP
IP_ME	ASP	286	-0.8984	31.1	C	1	IP
IP_ME	LYS	297	-2.6693	27.5	C	1	NU
IP_ME	GLU	321	1.1885	53.3	C	1	IP
IP_ME	GLU	336	1.3549	21.6	C	1	NU
IP_ME	ARG	368	-1.5757	1	H	1	NU
IP_ME	LYS	389	-0.8271	30.7	H	1	IP
IP_ME	GLU	435	0.8136	19.9	H	1	NU
IP_ME	LYS	438	-1.2326	56.1	H	1	NU
IPNU_ME	ARG	349	-1.9963	34.9	C	2	nSBME
IPNU_ME	ARG	539	3.6259	57	C	2	nSBME
IPNU_ME	ASP	154	-0.3218	72.1	C	3	nSBME
IPNU_ME	ASP	184	-2.4382	79.2	S	2	nSBME
IPNU_ME	ASP	365	-5.4679	5.9	H	2	nSBME
IPNU_ME	ASP	382	6.2772	41	S	3	nSBME
IPNU_ME	ASP	50	0.1984	15.8	C	3	nSBME
IPNU_ME	GLN	495	0.6853	0.2	H	2	nSBME
IPNU_ME	GLU	377	2.8516	10.1	C	3	nSBME
IPNU_ME	GLU	395	-11.795	37.4	H	2	nSBME

IPNU_ME	GLY	236	0.2928	0	C	2	nSBME
IPNU_ME	GLY	53	-0.3979	0	H	2	nSBME
IPNU_ME	HIS	18	-1.2938	19.4	H	3	nSBME
IPNU_ME	HIS	21	-1.4011	48.3	H	3	nSBME
IPNU_ME	HIS	303	-2.3218	3.2	H	2	nSBME
IPNU_ME	HIS	356	3.7468	21.8	H	2	nSBME
IPNU_ME	HIS	75	-13.6707	17.1	H	4	nSBME
IPNU_ME	LYS	213	-4.4662	26.3	H	2	nSBME
IPNU_ME	LYS	453	-2.2062	29.7	H	3	nSBME
IPNU_ME	LYS	514	1.4098	39.8	H	2	nSBME
IPNU_ME	PHE	45	-0.3661	0	S	2	nSBME
IPNU_ME	SER	88	-25.7323	17.5	C	2	nSBME
IPNU_ME	THR	95	-3.7044	0	C	2	nSBME
IPNU_ME	TYR	101	-15.1646	35.3	H	2	nSBME
IPNU_ME	TYR	12	0.2771	29.9	C	2	nSBME
IPNU_ME	TYR	337	-21.6394	47.4	C	2	nSBME
IPNU_ME	ARG	37	-4.7152	34.6	C	2	IP
IPNU_ME	HIS	52	-9.598	0.2	C	2	NU
IPNU_ME	HIS	100	18.8738	0.2	H	4	IP
IPNU_ME	ASP	136	-5.6937	25	H	3	IP
IPNU_ME	HIS	187	6.6996	0.4	S	5	NU
IPNU_ME	ARG	231	8.0118	18.7	S	4	IP
IPNU_ME	ASP	232	-2.268	10.7	C	2	NU
IPNU_ME	LYS	249	-1.4446	4.4	C	3	NU
IPNU_ME	GLU	256	-34.4997	0.6	H	3	IP
IPNU_ME	LYS	279	0.0894	11.5	H	2	IP
IPNU_ME	GLU	341	2.2417	1.8	C	2	IP
IPNU_ME	ASP	387	0.6113	29.7	H	2	IP
IPNU_ME	ASP	429	-5.1754	0	H	2	NU
IPNU_ME	LYS	498	8.0995	0.8	H	2	NU
IPNU_ME	GLU	505	0.8737	13.9	H	4	NU
IPNU_ME	GLU	523	-6.9789	22	C	2	NU
IPNU_ME	LYS	525	-4.4687	13.4	C	3	NU
IPNU_ME	ARG	530	3.5406	47.3	C	2	NU

Importance of non-salt-bridge weak interactions in thermostability:

(a) Comparison of inter-residue HyBs in core (co). (b) Comparison of inter-residue HyBs in surface (su). (d) A typical presentation of HOH-HOH and HOH-residue types of HyBs. (e) A typical presentation of different types of hydrophobic interactions. (g) Comparison of inter-residue hydrophobic interactions in the core. (h) Comparison of inter-residue hydrophobic

interactions in the surface. (i) Comparison of inter-residue electrostatic interactions in the core. (j) Comparison of inter-residue hydrogen bonds in the surface

To understand the role of hydrogen bonds, hydrophobic and other electrostatic interactions (Mecozzi et al., 1996; Puchkaev et al., 2003; Martinez and Iverson, 2012), their normalized frequency of the core and surface of the PMRS and EMRS have been compared. The frequency of HyBs at PMRS's core and the surface is much higher than that of EMRS (Fig. 6a, b, Table 11). Oppositely, the shell-water mediated HyBs dominate in EMRS (Fig. 6c, d, e). Aromatic π-systems and bulky alkyl groups participate in a variety of hydrophobic interactions (Fig. 6f, Table 11). These hydrophobic forces in PMRS's core and the surface are much higher in PMRS than EMRS (Fig. 6g, h). Furthermore, the normalized frequency of other electrostatic interactions other than the salt-bridge also dominated in PMRS.

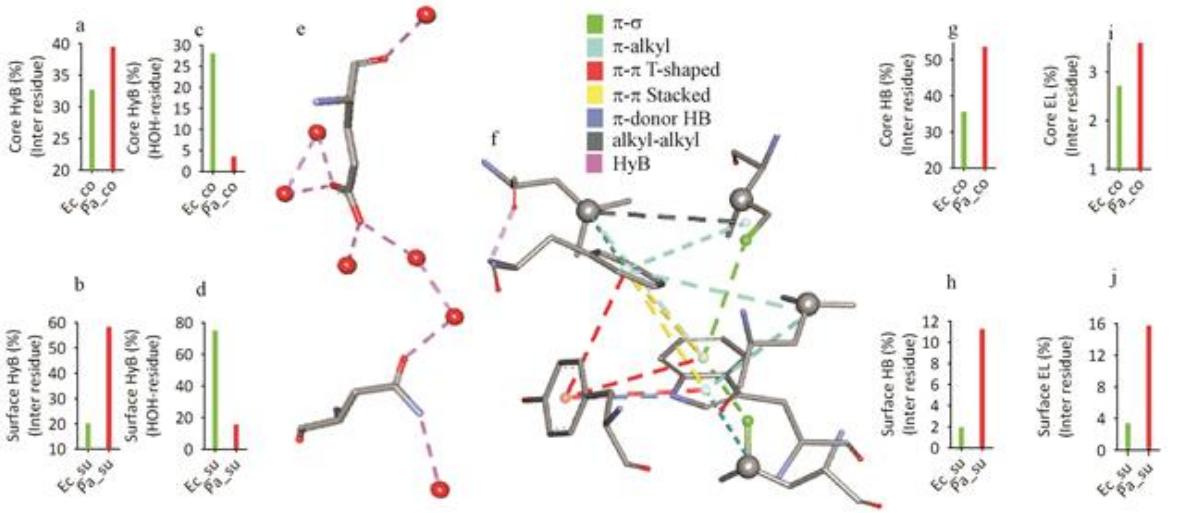

Figure 6: Details on weak interactions other than salt-bridge of PMRS (Pa, red) and EMRS (Ec, green).

Hydrophobic interactions, the most dominant of all weak interactions (Dill, 2005), are more prevalent in PMRS than in EMRS. Just as salt-bridges are important in PMRS's thermostability, so are the higher levels of HyBs and other electrostatic interactions. The latter seems to have some specific roles as their counts are much less (after global normalization) than others. The amount of shell-water in PMRS is much less than in EMRS and so, the HOH-mediated interactions are less. While this may seem counterintuitive in the thermostability of PMRS, the low shell-water, in turn, may enhance collapse-mediated folding and residue packing (Hurley et al., 1992; Russell et al., 1997). Taken together, it can be said that not only the salt-bridge but also other weak interactions are actively involved in PMRS's thermostability.

Cavity at thermostability of PMRS:

The cavity in a folded protein is a highly heterogeneous sub-structure, where, along with the atoms of the protein, shell-waters also participate in its structure. Further, these atoms belong to

the different residue and secondary structure classes. In these aspects, is there any discriminatory feature of the cavity in the thermostability of PMRS compare to EMRS?

Table 11: Normalized frequency of weak forces (except the salt-bridge) in the core and the surface of 1PG2 and 1RQG. Weak forces are divided into hydrogen bond (HyB), hydrophobic and hydrophilic categories. In hydrogen bond there are three sub-categories such as HB/HL, charged and water mediated. In HB/HL, HyB formed by hydrophobic-hydrophobic, hydrophobic-hydrophilic, hydrophilic-hydrophilic residues are included. In charged-mediated HyB, charged-charged, charged-hydrophilic, charged-hydrophobic are included. In water mediated HyB, water-water, water-hydrophobic, water-hydrophilic, water-charged mediated interactions are included. Ion-pair interactions are like salt-bridge where the distance of interaction is greater than 4.0Å. All these interactions were computed using the BIOVIA Discovery Studio 2020 using default parameters for the distance and defined angles. Normalization was done using the total interactions (HyB, hydrophobic, and hydrophilic).

	1PG2_core	1RQG_core	1PG2_surface	1RQG_surface
Hydrogen bond				
HB/HL residue mediated	23.7	28.4	6	8.6
Charged residue mediated	8.9	11	13.9	49.5
Water-mediated	27.9	3.4	74.8	15.3
Hydrophobic				
π-σ	5	8.6	0.2	1.8
π-π	1.9	3.1	0.1	0.8
Amide-π	0.2	0.4	0	0
Alkyl-alkyl	15.8	24.6	0.5	5
π-alkyl	12.5	16.7	1.1	3.6
Hydrophilic				
Ion-pair	0.1	0.8	2.1	12.1
π-cation	0.2	0.2	0.5	0.7
π-anion	0.2	0.1	0.1	0
π-donor HyB	1.4	2.1	0.6	2.5
π-sulfur	0.8	0.4	0	0.4

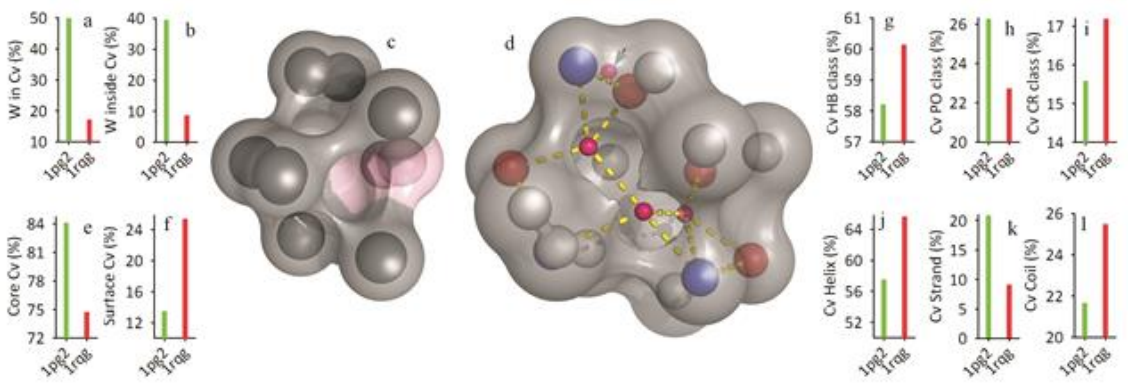

Figure 7: Details on structural components of interior cavities. Here, the red and green bars are of PMRS (1rqg) and EMRS (1pg2), respectively.

(a) Comparison of water (W) containing cavity (Cv). (b) Comparison of inside water-containing cavity. (c) A typical empty cavity. (d) a typical water-filled cavity with water interaction from inside. Arrow indicates an outside W. (e) Comparison of cavity atoms in the core. (f) Comparison of cavity atoms in the surface. (g) Comparison of hydrophobic-class of cavity residues. (h) Comparison of polar-class of cavity residues. (i) Comparison of charged-class of cavity residues. (j) Comparison of cavity atoms in helix. (k) Comparison of cavity atoms in strand. (l) Comparison of cavity atoms in coil.

To check this, we have presented Figure 3. Several points are noteworthy. First, the cavity in PMRS (7.8%) is more than EMRS (5.7%). However, the shell-water-filled total and just inside-filled cavities are almost negligible in PMRS compared to EMRS (Fig. 7a,b) may imply that these types of the cavity are less important in the former (Bandyopadhyay et al., 2019) (Fig. 7c). In EMRA, in inside-filled cavities, the shell-waters form HyBs to themselves and with polar constituents of the cavity to stabilize (Fig. 7d), and thus, make them voluminous. In turn, largely in PMRS, the empty cavity is usually smaller (Vogt et al., 1997), which seems to be stabilized by self-assembly. Second, although the number of cavities in the core is relatively less in PMRS than that of the EMRS, they are highly abundant in its surface regions (Fig. 7e, f). Third, on a residue-class basis, hydrophobic and charged-classes are more in PMRS's cavity than that of EMRS, but polar-class like salt-bridge is less here (Fig. 7g, h, i). Fourth, there are more helix and coil-prone residues in PMRS's cavity than in EMRS.

What is the significance of the above observations in PMRS's thermostability? Since PMRS has more cavities, and since those cavities also show selective preference towards residue-class and secondary structure type, it seems that these cavities are more active in restricting (i.e., packing) these structural elements. Very little shell water inside the cavity of PMRS seems to be another distinct strategy. The abundance of cavities on the PMRS's surface compared to the core demonstrates the importance of surface engineering in thermostability. Taken together, the cavity appears to be an assembler or container of different structural components of protein and, thus, maintains its conformation. In other words, the different elements (especially the secondary structure elements such as helis, strand and coil) would not have come together without these

interior cavities. Since the cavity has a space within itself, it may also have a role in the balance of rigidity and flexibility, which is required in the case of effective proteins in different environments (Dubey and Jagannadham, 2008).

Our study shows that in PMRS, compared to EMRS, salt-bridge and other weak forces and cavities are involved in its thermophilic properties. The importance of the microenvironment is immense as the stability of the salt-bridge towards PMRS is higher than that of EMRS. Regarding PMRS's thermostability in relation to EMRS, its inter-domain and surface engineering are worth noting. These discriminatory structural features seem to stem from its inherent properties in the sequence as about 2/3rd of the homologous position of the former differs from that of the latter. We believe that our study will be applied to other similar systems.

Declaration of competing interest:

There are no competing interests to declare.

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Abbreviations used:

PMRS, Methionine-tRNA synthetase from hyperthermophilic, *Pyrococcus abyssi*; EMRS, Methionine-tRNA synthetase from mesophilic, *E. coli*; ME, microenvironment of salt-bridge; APBS, Adaptive Poisson Boltzmann Solver; SBFR, salt-bridge forming residue; nBSFR, non-salt-bridge forming residue; IP, Isolated pair; NU, Network Unit; IPM, Isolated Pair Method; NUM, Network Unit Method; IPME, isolated pair's partner as ME-candidate; NUME, network unit's partner as ME-candidate; nSBME, ME-candidate but not salt-bridge's partner; HyB, Hydrogen bond

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