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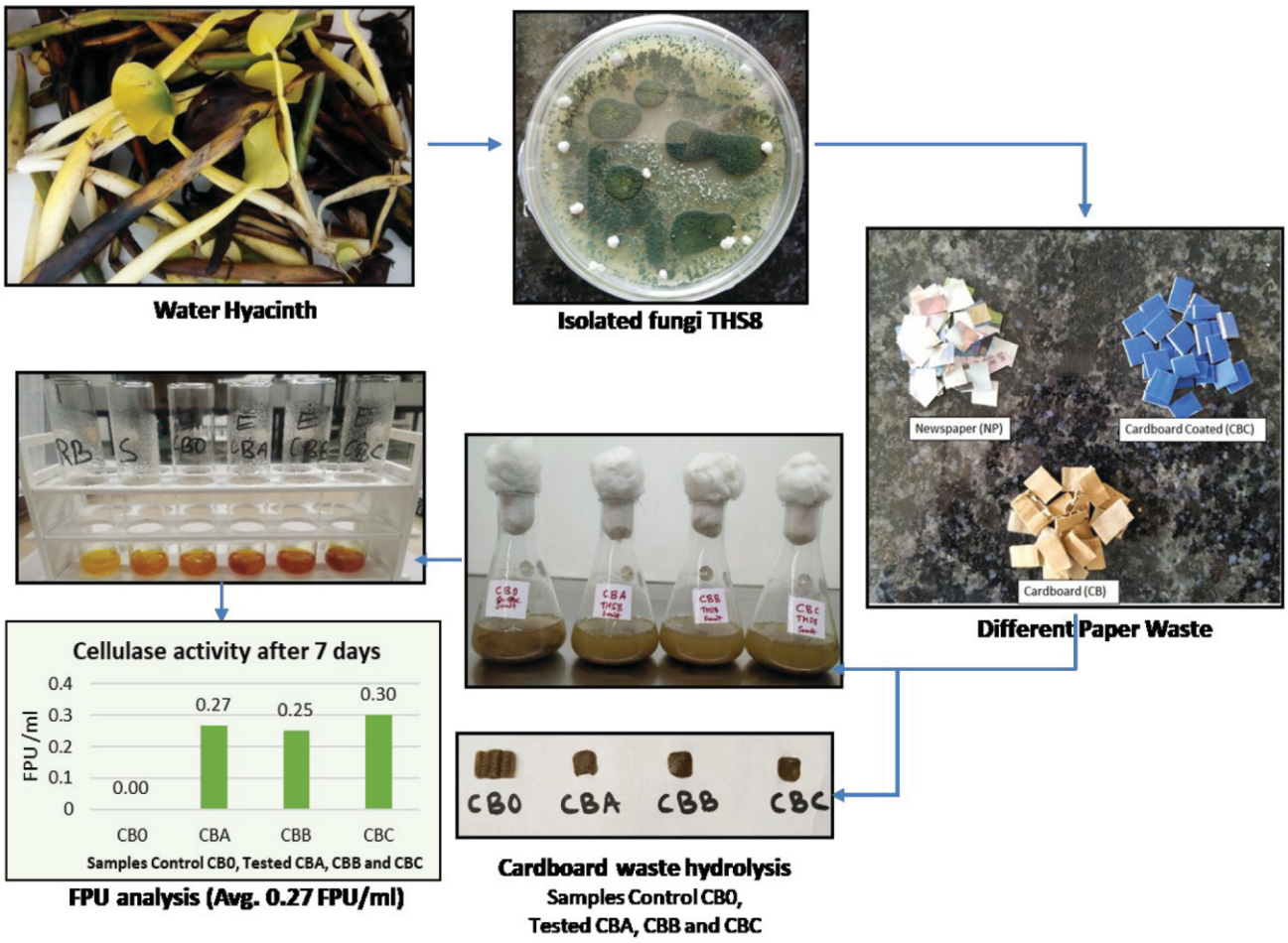

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The IJEHS is an official publication of Save The Environment (STE). It publishes peer reviewed quarterly, original articles (Research paper, Review articles, Short Communication, Case studies, etc.) related to all fields of Environment and Health Sciences. It disseminates the scientific research and recent innovations.

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International Journal of Environment and Health Sciences

From The Editor's Desk…

Advent of modern technologies is leading to persistent urbanization and capitalism which although favorable to mankind, have a plethora of environmental hazards accompany them. Air pollution, water contamination, greenhouse gas emissions, etc. are manmade tribulations that are challenging the availability of clean air and pure water. Consequently, health perils linked to environmental risk factors are snowballing at an alarming rate. In this outlook, the necessity for formation of regulatory bodies to propagate awareness for environmental sustainability is more now than ever before.

With this perspective, the International Journal of Environment and Health Sciences (IJEHS) proposes to provide a reliable platform to discuss technologies and strategies for management of aforesaid environmental matters. IJEHS has been launched as a peer-reviewed quarterly journal that will be quintessential to academicians, industry professionals and researchers who are actively engaged in the areas of environmental issues and related health effects. We are pleased to inform that ISSN for IJEHS is now available as 2582-5283. Also, IJEHS is now indexed in Crossref (DOI 10.47062).

We invite original research articles, short communications and critical reviews directed towards an academic, clinical and industrial audience. The first section of the journal focuses on burning environmental issues like pollutants and their fate, waste management, resource conservation, remediation technologies, etc. The second section includes all topics relevant to physiological impact of environmental risk factors and application of alternative medicinal approaches as remedial measures. Detailed scope can be found in the home page of the journal (www.stenvironment.org). Notes on development of any novel and validated strategy or tool to address environmental challenges are welcome. Discussion on proceedings of conferences conducted around an environmental theme will also be considered. All submissions will be meticulously scrutinized by pioneers in the field to ensure publication of only articles of high quality and relevance. Authors are requested to take special precautions to avert plagiarism and redundancy.

It is high time that we realize the gravity of circumstances and take potent steps to undo the adversities already triggered. The time is now and the place is here. I, on behalf of STE team pray for your good health and urge all of you to stay safe amid ongoing Covid-19 crisis and I hope our audience and patrons shall come together in this effort to promulgate their part in resurrecting our valuable environment.

Dr. Kshipra Misra
Editor-in-Chief, IJEHS

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BIO-SUCCINIC ACID: AN ENVIRONMENT-FRIENDLY PLATFORM CHEMICAL

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Abstract
Global research for biomass-based products is swelling gradually due to depletion of fossil-based raw material as well as their negative effects on the environment. The hazardous chemical processes used for production of valuable industrial chemicals are directly responsible for environmental pollution. Therefore, use of alternative renewable resources as feedstock to produce such chemicals is gaining popularity. Succinic acid (SA) is platform chemical which can be used to produce bulk industrial chemicals with huge global demand such as adipic acid, 1,4-butandiol, maleic anhydride. Non-hazardous biological fermentation process can be used to produce succinic acid, which can be further converted to these chemicals. The bio-based approach uses CO_2 as supplement during the process and it replaces fossil-based raw materials; therefore, it is environment friendly. Mostly, pure sugars or sugars derived from crop residues or lignocellulosic materials are used to produce succinic acid. However, utilization of agricultural waste aromatic compounds to produce succinic acid has not been investigated in detail and not being implemented anywhere. Lignin waste management is a problem for the cellulosic bio-refineries and finding the way for its biological utilization is still in the stage of research and development. Unlike sugar metabolism, bioconversion of aromatic compounds is relatively complicated. Interestingly, bioconversion of agricultural waste aromatic compounds could be targeted towards production of succinic acid. The purpose of the present review is to highlight this possibility.

Keywords
Succinic acid, Platform chemical, Market potential, Lignin, Aromatic substrates.

1. Introduction

Succinic Acid (SA) is a biochemical compound produced in almost all organisms, aerobic as well as anaerobic either as an intermediate or end product of their metabolic pathway. Knowing the positive impact of succinic acid on human health, Nobel Prize winner, Robert Koch popularized succinate by means of an industrial organic compound in food industries and generated interest intended for its high scale industrial production. Due to its impressive utilization to produce various industrial chemicals like 1, 4-butanediol, 2-pyrrolidinone, tetrahydrofuran, and various other conventional products like pigments, additives, detergents, pharmaceuticals intermediate, etc., succinic acid becomes a valuable chemical [1,2]. The production of SA was initiated by Agricola in 1546. Initially, it was extracted from Amber through dry distillation which releases acidic anhydride. At that time, the succinic acid used to recognize by odor only. Later, numerous chemical approaches of succinic acid extraction from Amber were developed such as extraction by solvents like petroleum ether, methanol, and ammonium carbonate. At the end of 18th-century, chemical-based industries started using fossils fuels (petroleum, coal and natural gases) or catalytic hydrogenation of maleic anhydride to produce succinic acid. However, due to the concern of their limited natural feedstock, and negative environmental impacts, biotechnology is now playing a vital role to develop environment friendly processes to produce succinic acid [3].

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Color versions of one or more of the figures in this article can be found online at www.ste.org

Bio-based succinic acid has various benefits over its chemically synthesized counterpart as it utilizes CO_2 during the process, renewable raw materials are used and produce fewer toxic byproducts. Moreover, succinic acid fermentation not only utilizes CO_2 but also produces three important products together i.e. ethanol, succinic acid, and diethyl succinate. Michigan biotechnology institute (USA) had isolated the first commercial micro-organism "*Anaerobiospirillum succiniciproducens*" as a natural succinic acid producer. Some other micro-organisms isolated for this purpose are *Actinobacillus succinogenes*, *Mannheimia succiniciproducens* and *Basfia succiniciproducens* [4]. Low product yield and low productivity are the major limitations of these natural producers. Metabolic engineering can resolve these problems by modifying the biosynthetic pathway as desired. Metabolic engineering can be applied to natural producers and or any other microorganism whose metabolic pathways are well studied such as *Escherichia coli*, *Corynebacterium glutamicum*, and *Saccharomyces cerevisiae*. However, *E. coli* is usually selected as the model organism for metabolic engineering to produce succinic acid as its genome, proteome, metabolome and culture conditions are well established. Thus, significant interest has been observed to develop metabolically engineered strain for succinic acid production and various technologies to transform succinic acid into industrially important derivatives [5, 6].

As the applications of succinic acid are very vast, so its demand for at commercial level is high. In the 1990s, total succinic acid was manufactured around 18,000 MT (metric tons) and estimated to increase with 27.4% CAGR to 768 million MT by the year 2025. Major markets spread over for succinic acid use are surfactants/detergents, anti-corrosion agents, flavoring agents or antimicrobial agents in foods, health industry including vitamins, antibiotics, etc. In 2009, *Bioamber* and *Biohub* are two major French corporations have begun pilot-plant for succinic acid production. They used genetically engineered *E. coli* strains for this. Later in 2010, Myriant and MBI International started pilot plant production using *Escherichia coli* and *Actinobacillus succinogenes*, respectively in the United States. As the value of bio-based succinic acid production increases, at the end of 2010, German-based BASF and Dutch CSM firms also started bio-succinic acid production in Spain. BASF isolated their own novel rumen bacteria *Basfia succiniproducens* for succinic acid production. Later on, Japanese agencies (Mitsubishi and Ajinomoto) came up with scaling-up of succinic acid production by using the *Corynebacterium* strain [7, 8]. All of these companies use direct sugar sources like corn starch, sugarcane molasses even though there are other low-cost and abundant cellulosic biomass such as wood hydrolysate [9] also available for succinic acid production. Although these materials need to be pretreated to release carbohydrate molecules available, still they are less expensive than refined carbohydrates. Apart from cellulosic source, aromatic compounds source (lignin) have the potential for succinic acid production. This review signifies the susceptibility of the succinic acid as an important building block chemical.

2. Succinic acid: Production approaches

Succinic acid production methods through biological routes are well studied along with genetic level modifications. Bio-based succinic acid production is non-hazardous and environmentally friendly while the petroleum-based method uses harmful chemicals for succinic acid and their derivatives development. Vaswani and Sudeep [10] defined both the chemical and biological processes for succinic acid synthesis. The chemical method starts with the conversion of maleic anhydride to succinic anhydride by hydrogenation with the aid of catalyst like Ni/Si alloy. After that, hydrolysis converts it into succinic acid. But hydrogenation causes toxicity and increases the cost of the whole process. However, the non-toxic biochemical approach uses glucose and CO_2 as substrates to produce succinic acid (Figure 1).

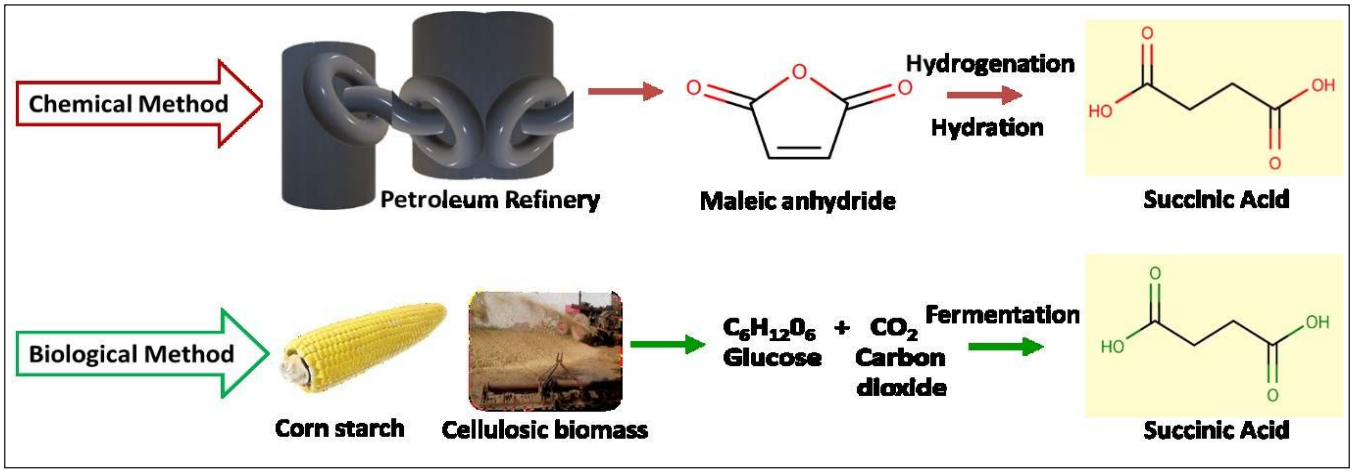

Figure 1: Succinic acid production via a chemical and biological method.

There are various applications of succinic acid in the field of food and chemical industry. It is used to produce adipic acid, 1,4 butanediol, tetrahydrofuran, succinonitrile, succinimide, polyesters, resins, acidulants, etc. Figure 2 describes various applications of succinic acid. Moreover, it is utilized in detergent and surfactant formations, as chain extender, ion-chelator, and foaming agent preparations. It is also used for making resins, inks, coating materials, plasticizers, liquors, plant growth stimulants, electrolyte bath additives, biodegradable plastics, anti-microbial agents, pH modifiers and flavoring agents.

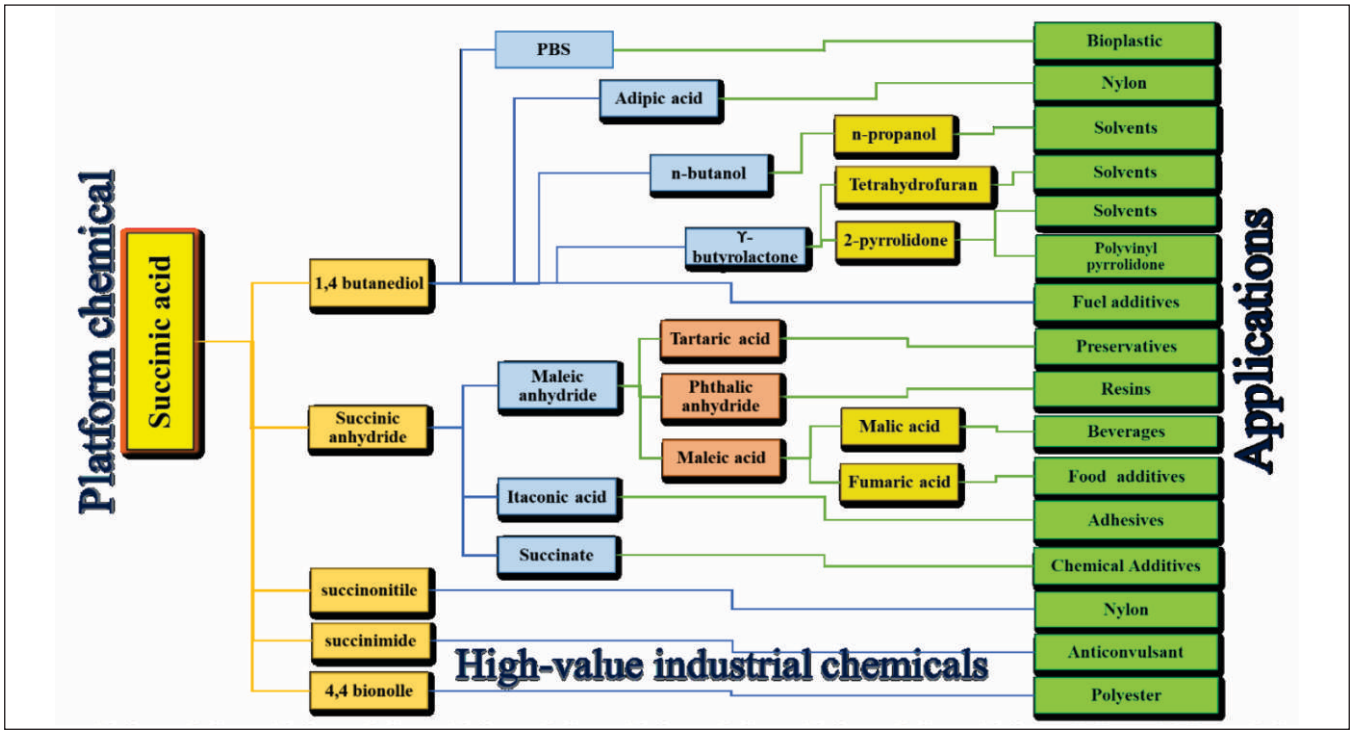

Figure 2: Applications of succinic acid as a platform chemical.

3. Global market potential of succinic acid

The global market of succinic acid is expected to reach at around 701 million USD by 2021. The global demand for succinic acid is around 40,000 tons/year-45,000 tons/year. A comparative analysis of the global market potential of succinic acid against other valuable industrial chemicals are presented in Figure 3. The major succinic acid manufacturers using petrochemical process are Kawasaki Kasei Chemicals, Linyi Lixing Chemical, Anhui Sunsing Chemicals, Mitsubishi Chemical, Nippon Shokubai, Gadiv Petrochemical, Astatech, R-Biopharm, Evonik, Thyssenkrupp [11]. On the other hand, major bio-based succinic acid manufacturers are BioAmber, Reverdia, and Succinity (Table 1). In India, Thirumalai Chemicals Ltd., A. B. Enterprises, Chemicals Chamber and others (shown in Table 2) are chemical-based manufacturers of succinic acid [12,13]. As per the authors knowledge, till now there is no bio-based succinic acid producing company in India.

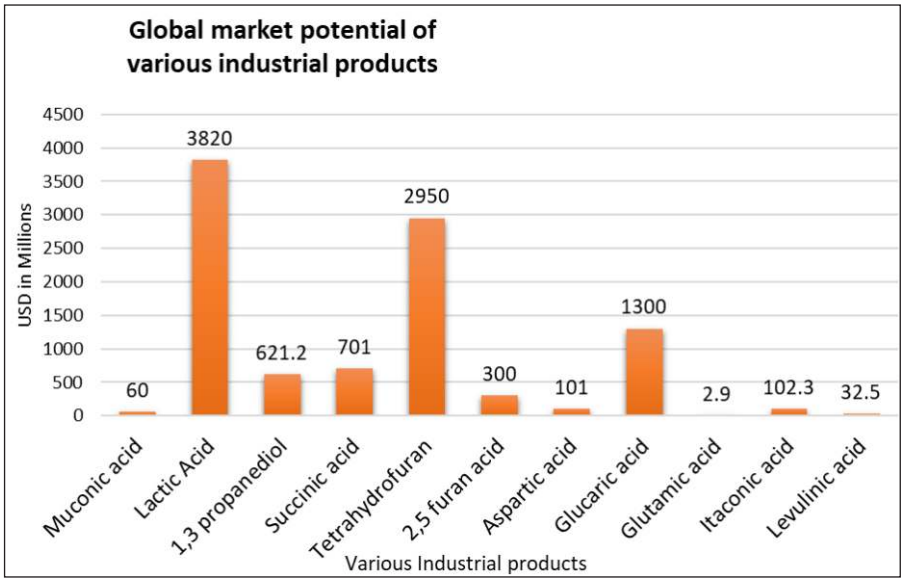

Figure 3: The global market potential of various industrial products.

Table 1: Succinic acid key player in world.

Company Name	Location
Shanghai Runkey Biotech Co., Ltd	China
Linyi Lixing Chemical Co., Ltd.	China
HeBei Oukai Biotechnology Co.,Ltd	China
A & Z Food Additives Co., Ltd.	China
ZSIKI KFT	Hungary
BioAmber	United States
Myriant	United States
Succinity	Germany
Reverdia	Italy
Gadiv Petrochemical Industries Ltd.	Israel
Nippon Shokubai	Japan
Mitsubishi Chemical Holdings	Japan
Kawasaki Kasei Chemicals Ltd.,	Japan
BASF	Germany
DSM	Netherlands

Table 2: Succinic acid key player in India.

Company Name	Location
B Joshi Agrochem Pharma	Mumbai
Thirumalai Chemicals Ltd.	Mumbai
Alpha Chemika	Mumbai
A. B. Enterprises	Mumbai
Axiom Chemicals Pvt. Ltd.	Vadodra
Meru Chem Pvt. Ltd.	Mumbai
Antares Chem Private Limited	Mumbai
Labdhi Chemicals	Mumbai
Arnish Laborates Private Limited.	Mumbai
Alpha Chem	Ambala
Natural Organics India	Delhi
Kapchem International	Mumbai
Chemicals Chamber	Delhi

3.1 Economic Value of Succinic Acid

The current bio-based production of compounds and polymers (excluding-fuel) is estimated to be around 50 million tons [14]. But still, chemical production from fossil-fuels was significantly higher, which was accounted to be 330 MT in 2009 [15]. The market of petrol-based production of succinic acid was estimated to decline by 2019 because of its negative influence on the environment. Due to this, bio-based production processes are preferred for use in the pharmaceutical and cosmetics industries. Its non-toxicity and high purity help in taking care of the personal health of consumers [16]. Global market of succinic acid can be divided into different sectors such as, pharmaceuticals (15%), industrial (57.1%), food (13.07%) and others (13.92%).

Among various applications of succinic acid, the largest application is BDO (1, 4-butanediol) that boosted the international bio-succinic acid marketplace by about 17% in the year 2013. BDO is used as a solvent in many industries. It can be further converted to γ-butyrolactone (GBL) and tetrahydrofuran (THF). So, OMR industry analyst and Grand view research estimated the demand for succinic acid increases with CAGR of 35% till 2020.

On an industrial scale, butyrolactone is mainly manufactured from maleic anhydride. So, the replacement of maleic anhydride (MAN) by bio-succinic acid will take over this market, which is less expensive and environment-friendly. Polyurethane, a thermosetting polymer is another example that shows increases in the market possibility of bio-succinic acid. A new polyurethane class Non-isocyanate based polythenes (NIPUs) has recently been developed, which will also increase the demand for bio-succinic acid [17]. About 25,000 to 30,000 MT (metric tons) succinic acid produces annually and expected to rise 10% every year. The addressable market of succinic acid will replace mainly the maleic anhydride, adipic acid, phthalic anhydride market. Because it can be used as a substitute for these chemicals. The succinic acid price in the market relies upon its purity, productivity, yield, recovery process, and raw material cost. The succinic acid produced from the organic process is usually sold at a price of USD 5.9 to 9 per kg.

3.2 Expansion of bio-based succinic acid productions

There are various companies that are using bio-based production routes succinic commercial production of acid. Major companies producing bio-succinic acid are BASF, BioAmber, Mitsubishi, Myriant, DSM, Mitsui & Co, Succinity, Roquette Freres S.A, Purac, and Reverdia. Most of them are joint ventures (JV) except Myriant. The main bio-based succinic acid production market was covered by BioAmber, Succinity, and Reverdia with the production capacity of 350 MT, 500 MT, and 300 MT respectively [18]. BioAmber, Reverdia, and Succinity were able to produce 3,800 MT in the year 2011. Further, the Myriant corporation also enhanced the production volume with a dramatic increase in their production units. The world's first largest bio-based succinic acid industry BioAmber is located in France and now able to produce succinic acid nearly 3,000 MT/year. All the bio-based succinic acid-producing companies are using sugars extracted from different crops or biomasses. Table 3 shows the foremost bio-based succinic acid-producing player in the succinic acid market along with the details of major production capacity, kind of substrates, and class of microbes used in production [19].

Table 3: Bio-Based succinic acid-producing companies

Company name	Location	Substrate	Microbe	Commercial Operation	Production (tons/year)
Bioamber (in JV with Mitsui)	Sarnia, Ontario, Canada	Corn, wheat, cassava, rice, sugarcane, sugar beets	Bacteria	August 2015	30,000
Myriant	Lake Providence, LA, USA	Sorghum grain, Ligno cellulosic sugar from corn stover	Bacteria	June 2013	14,000
Reverdia (Roquette-DSM JV)	Cassano Spinola, Italy	starch from corn	Yeast	December 2012	10,000
Succinity (BASF-Corbion Purac JV)	Barcelona, Spain	sucrose, glucose	Bacteria	March 2014	10,000

4. Substrate and micro-organisms for succinic acid: Natural and Engineered

Corn waste, sugarcane molasses, wheat grains, whey, lignocellulosic biomass or pure sugars are commonly used substrates for fermentation. Glucose is the main carbon source used to study the metabolic rate of microbes. Natural succinic acid-producing microbes such as *Actinobacillus carcinogens, Anaerobicspirullum succiniproducens, Mannheimia succinicproducens* are restricted to glucose only, and cannot utilize a variety of substrates [20]. Genetically engineered micro-organisms such as *Corynebacterium glutamicum*, *E. coli*, and a new specific isolate *Basfia succinoproducens* were also investigated for their role in succinic acid production [21]. *Basfia succinoproducens* isolated from the rumen gut is a natural succinic acid-producing microbe. Different kinds of bacteria,

natural and genetically engineered, were being used for getting a higher titer of succinic acid. Lignocellulosic sugar and direct pure sugar derivatives are the mostly used substrates to produce succinic acid. Some researchers suggested that corn cob, cane molasses, starch, and lignocellulosic biomass are the substrates useful for succinic acid production [22-41].

A comparative analysis of succinic acid yield and titer on different carbon sources and hydrolysates is depicted in Figures 4 and 5.

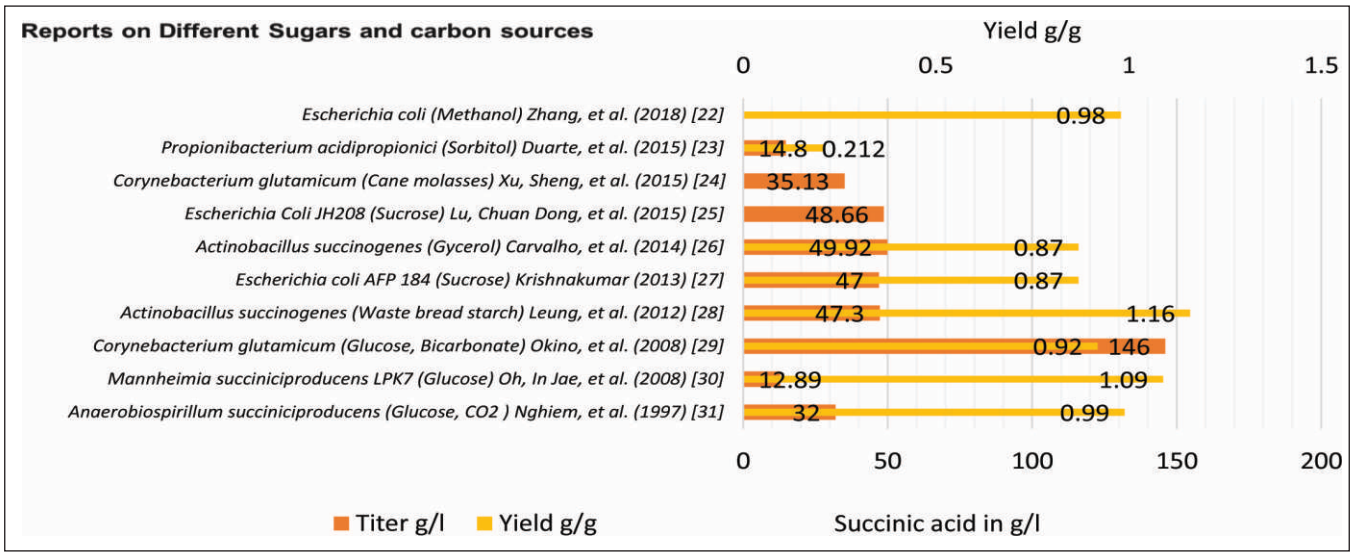

Figure 4: Comparative study of the bacterial fermentation of different sugar derivatives and other carbon sources for succinic acid production.

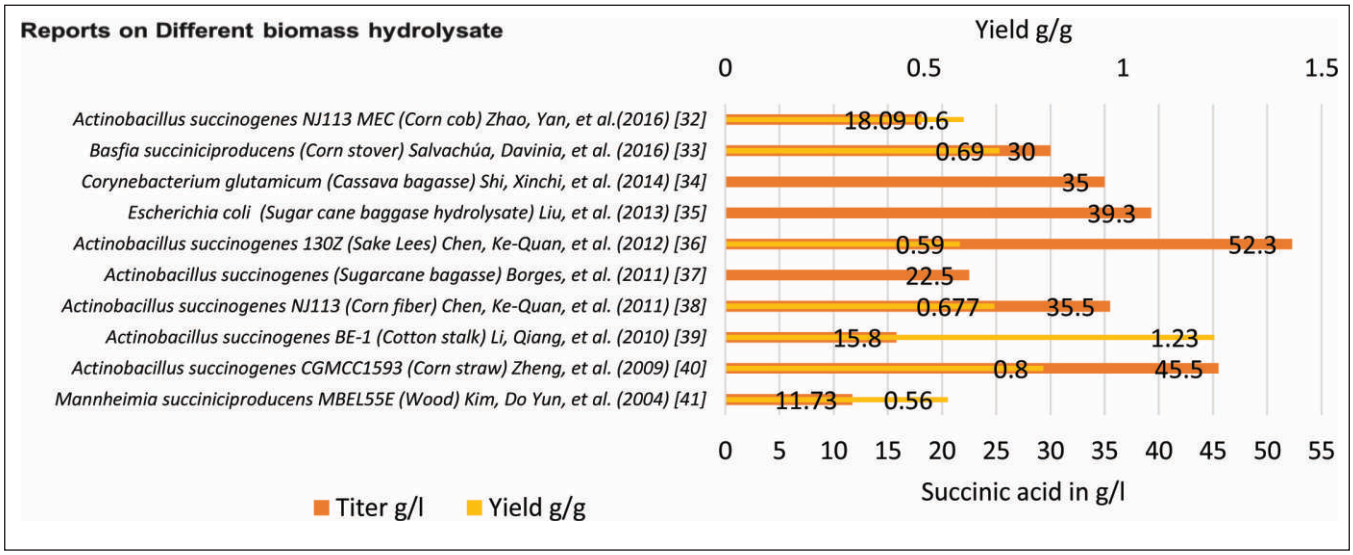

Figure 5: Comparative study of the bacterial fermentation of different biomass hydrolysates for succinic acid production.

5. Metabolic engineering strategies for succinic acid production

Glucose is the starting substrate for biosynthesis of succinic acid in microbial cells. Initially, glycolysis occurs till phosphoenolpyruvate (PEP) and pyruvate formation. When CO_2 enters the system under anaerobic condition, it activates enzymes like phosphoenolpyruvate carboxylase (ppc), phosphoenolpyruvate carboxylase kinase (ppck), and pyruvate carboxylase (pyc). Their carboxylases catalyze PEP

and pyruvate to produces oxaloacetate. At this stage, the citric acid cycle appears as a reverse second half citric acid cycle to produce succinic acid (Figure 6). Other byproducts such as, acetate, ethanol, lactate formate also start producing from pyruvate. Apart from that, keto-glutarate and malate form amino acid such as aspartate and glutamate. Figures 6 (A and B) described all probable products formed along with succinic acid-producing under anaerobic conditions. The theoretical yield measured for the succinic acid from glucose is 0.66g/g. One mole of CO_2 is used per mole of succinic acid obtained.

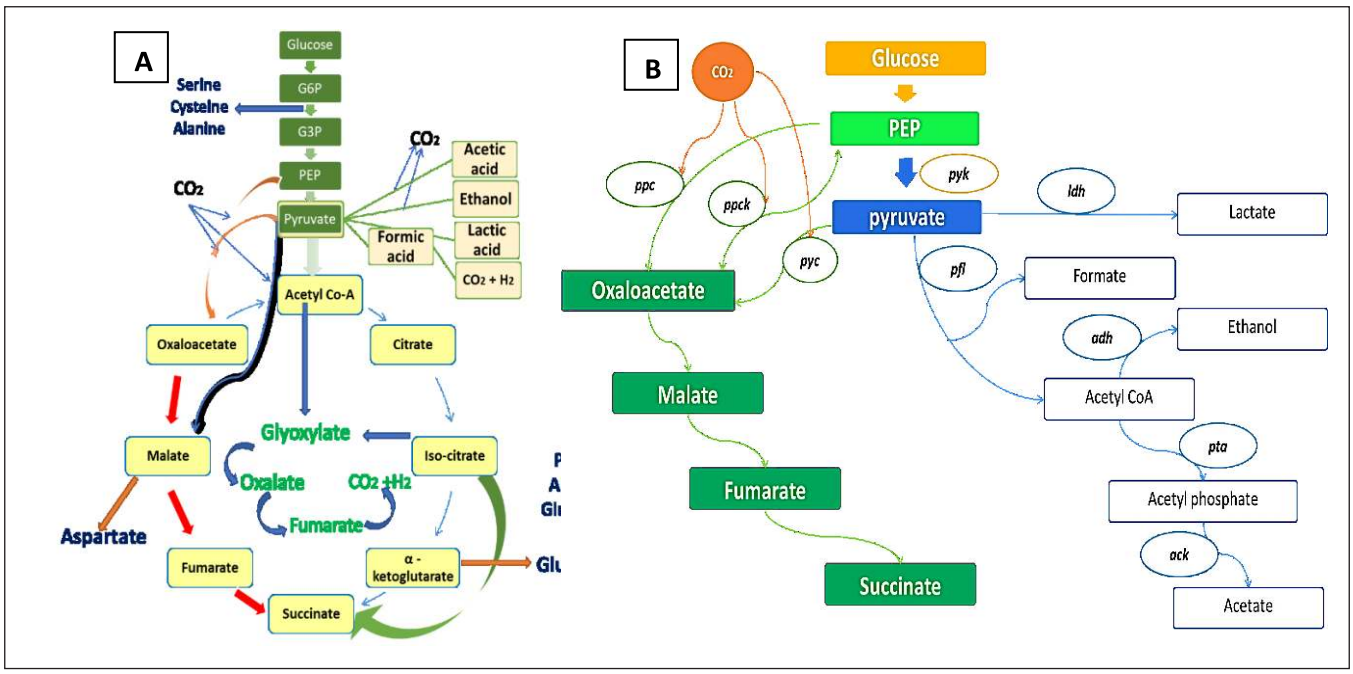

Figure 6: (A) Major metabolic routes to succinic acid production, (B) Bio-synthetic pathway of the succinic acid from glucose: Green branch of the pathway is representing succinic acid and white is for the by-product.

6. Potential to use aromatic substrates

Mostly, sugar derivatives were used to produce succinic acid, but aromatic compounds and phenol derivatives have not been studied to produce succinic acid. However, the phenolic derivatives were studied for other industrial chemicals such as adipic acid, muconic acid, but not for succinic acid. The phenol degradation potential by different micro-organisms is shown in Figure 7 [42-50]. A higher degradation rate for aromatics like catechol, phenol, benzoic acid, p-coumarate, 4-hydroxy benzoate was achieved by the implementation of a genetic engineering approach. Researchers have studied the degradation of the aromatic into products like adipic acid, pyruvate, lactate, muconic acid by *Pseudomonas putida.* Vanillin, pyridine and di-carboxylic acid were produced from *Rhodococcus josti* [51-57]. This has been done by deleting gene of *ldhA, pflB, pta and ackA* and overexpression of *pyc* in *E. coli* and in other naturally succinic acid-producing micro-organisms. Table 4 clearly differentiates the strategies used for the metabolic change in micro-organism for utilizing diverse categories of phenolics. For muconic acid production, *PcaHG*, *CatBC* and *vdh* genes suppression and overexpression of the *AroY, AsbF, CatA* genes were accomplished in *P. putida.* So, there are possibilities for enhancing the succinic acid production from phenol derivatives by appropriate metabolic engineering strategies.

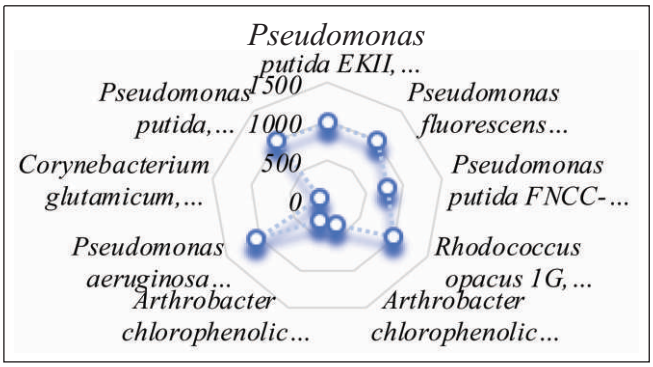

Figure 7: Comparative study of phenol degradation (mg/l) in bacteria for succinic acid production.

Table 4: Phenol derivatives used to produce various valued products.

Sr. No.	Substrate	Micro-organism	Bio-Product	Gene deletion	Gene addition	Approach	Product/ yield	Refs
1.	Catechol, phenol, and benzoic acid using glucose as additional	Pseudomonas putida Kt2440	Muconic acid adipic acid, Nylon	catB	benAB CD, catA	Homologous recombination and plasmid insertion	13 g/L	[56]
2.	Catechol, phenol, and benzoic acid using glucose as additional	Corynebacterium glutamicum MA-2	Muconic acid	muconate cyclo isomerase (catB)	Catechol-1,2-dioxy genase (catA)	Homologous recombination and plasmid insertion	85 g/L	[54]
3.	Benzoate, p-coumarate, phenol,4-hydroxy benzoate,	Pseudomonas putida Kt2440	Muconic acid, Adipic acid	Pca HG	AroY, Dmp KLMONP	Homologous recombination	13.5 g/L	[52]
4.	Benzoate, p-coumarate	Pseudomonas putida Kt2440	Pyruvate Lactate	aceEF	catBCA	Homologous recombination and plasmid insertion	41.17 g/g	[51]
5.	Wheat straw	Rhodococcus jostii RHA1	Vanillin	vdh	-	Homologous recombination	96 mg/L	[57]

7. Downstream processing techniques for succinic acid purification

The succinic acid comes in the market as a white crystalline powder form. When succinic acid is coming from the microbial broth, it should be clear and without any impurity especially, from hydrolysates which are having color, minerals and metal impurities in excess amount. Therefore, purification of the compound is very crucial for any kind of product that comes in the market. There are various methods established for succinic acid purification, some of them are patented processes developed by succinic acid-producing companies and some are research-based methods. Table 5 describes the purification process developed and currently used by bio-based succinic acid manufacturing companies.

Cost-effective downstream processes and clarification methods are always desirable to cut the purification cost. Three major categories of the purification are electrodialysis, salt precipitation and ammonia-based neutralization (Figure 8). These are mostly used due to their recyclability and low cost. Electrodialysis is done by ion-exchange membranes with electrolytic conductivity to transfer ions faster for separation of product. It can be reused several times and very effective in getting high yield [58]. In the salt precipitation process, calcium hydroxide is used for neutralization and precipitate formation. Then, the solid is dissolved by adding sulfuric acid and finally passed through ion-exchangers to get clarified product [59]. Third, ammonia neutralization is used to produce salts of succinic acid as di-ammonium succinate after the addition of liquor ammonia. It can be transformed into mono form through heating at 135°C with pressure 50 psi. Final crystals of acid are dissolved in water and purified again with ion exchangers [60]. In all the three processes, ion exchangers are used for extracting the final highly purified product. So that only the purified product will come without any other salt or metal ion impurities.

Table 5: Succinic acid purification process developed by bio-succinic acid companies.

Company	*Purification Process*
BASF	Succinic acid is salted out first as a precipitate, then, passed through an ion-change column with a mild temperature near 50 to 60°C.
BioAmber	Ammonia treatment to form di-ammonium succinate salt then heated to convert it into mono-ammonium succinate. Further dissolving in reverse osmosis water system.
Mitsubishi	Precipitation of succinic acid by reducing the overhead pressure below the atm. pressure.
Myriant	Succinic acid is purified from the vacuum dried concentrate of the broth, then acidic treatment separated it from ammonium sulfate.

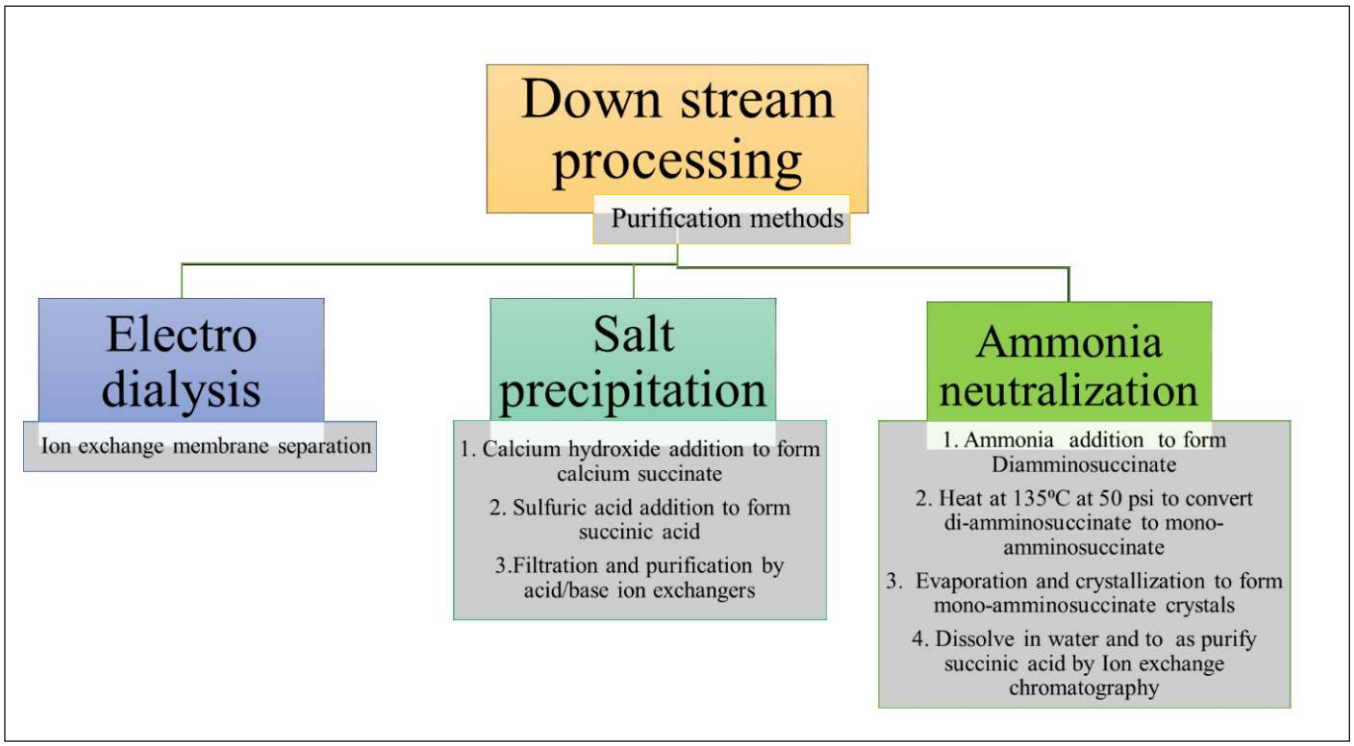

Figure 8: Various downstream processing methods for succinic acid.

8. Conclusion

Succinic acid is well studied for its potential as a platform chemical, which has vast applications such as the formation of polymers. The process development for enhancement of yield and titer has been done by metabolic engineering. Due to the depletion of petroleum-based resources, alternate bio-based approaches are being explored. Easy sugar-based process for succinic acid production is developed and marketed; however, aromatic substrates are not evaluated yet for succinic acid production. Therefore, to compete with the increasing demand for polymers, this basic platform chemical needs to be produced in bulk quantity and preferably from biomass. Thus, succinic acid production from low-cost agro-industrial wastes has become a major research area.

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ISOLATION, IDENTIFICATION AND MOLECULAR CHARACTERIZATION OF *SERRATIA MARCESCENS* STRAIN NMRL65 AN OPPORTUNISTIC HUMAN PATHOGEN FROM THE SPOILED COCONUT

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Abstract
Microbes are the common cause of food spoilage and are widely distributed in the environment. Fruits and vegetables provide ideal nutrient conditions for the survival and growth of many types of bacteria. Bacteria responsible for food spoilage produce extracellular hydrolytic enzymes which bring about the deterioration of the food products. Coconut flesh is highly nutritious and rich in saturated fat, sugar and potassium that have wide range of health benefits. Fresh coconut flesh is often spoiled by red pigment producing bacterium *Serratia.* The present study was therefore undertaken and a red pigment producing opportunistic human pathogen *Serratia marcescens* strain NMRL 65 from spoiled coconut was isolated, identified and characterized for specific genes. Also its ability to produce multiple hydrolytic extracellular enzymes like chitinase, lipase and protease was investigated and reported.

Keywords
Food spoilage bacteria, *Serratia marcescens*, opportunistic human pathogen, lipase, protease, coconut,

1. Introduction

Many countries around the world are facing food insecurity due to increasing global human population. The second goal of United Nations Sustainable Development is to end hunger in all its forms by 2030 and to achieve food security. Large proportions of food products are spoiled during the food supply chain by food spoilage microorganisms. One third of the food products produced for human population are either spoiled or wasted according to Food and Agriculture Organization of the United Nations 2011 report.Food spoilage is broadly defined as a change in the quality of food items that makes it undesirable for human consumption, due to spoilage indicators such as bad odour, texture and appearance[1]. The cause of food spoilage is a complex process broadly grouped as microbiological, chemical and physical [2]. Microorganisms responsible for food spoilage are bacteria, fungi and yeast. Microbes are able to survive in different environmental conditions like high salt, sugar, acidic and alkaline. Fruits with high water and sugar content are easily spoiled by microbes. Fresh coconut flesh is easily spoiled by bacteria, fungi and yeast due to its sugar, fat and water content.

The *Serratia* genus are Gram negative, facultative anaerobic bacteria, which grow in a broad range of temperatures and substrates, including plant surfaces, soil, water, and food products such as fruit juices, coconut and fish.*Serratia marcescens* and *S. rubidaea*is commonly found as a contaminant in wet coconut as a pink or red colour colonies. The red pigment produced by *S. marcescens* is prodigiosin. Prodigiosin is produced in the later stages of bacterial growth, although it doesn't have any physiological role in producing organisms, it's reported to have antibacterial, antifungal, antiprotozoal and antitumor properties[3].

Serratia marcescens produces wide range of extracellular hydrolytic enzymes such as chitinase, protease and lipase, due to this it is able to degrade and spoil edible food items rich in protein and fat. The presence of *S. marcescens* in an edible food items like coconut should be considered as a cause of concern because it's classified as an opportunistic human pathogen.About 2% of nosocomial infections of the bloodstream, lower respiratory tract, urinary tract, surgical wounds and skin and soft tissues of adult persons are caused by *Serratia*[4].

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Quorum sensing allows bacterial populations to communicate and coordinate group behaviour and commonly is used by pathogens in disease and infection processes. Quorum sensing systems controls a diverse range of cell density dependent factors such as antibiotic, biosurfactant production, extracellular polysaccharide, biofilm differentiation, cell division and pathogenecity. *Serratia* utilizes two types of quorum sensing signalling molecules: N-acyl homoserine lactones (AHLs, AI-1) and AI-2 type signalling molecules. The two key enzymes in AI-2 signalling molecules in *Serratia* is S-ribosylhomocysteinase (*LuxS*) and S-adenosylhomocysteine nucleosidase (*Pfs*) are responsiblefor biosynthesis of signalling molecules, which contribute to virulence factors of the human opportunistic pathogen *S. marcescens*[5].

To detect and identify the presence of *S. marcescens*-an opportunistic pathogen in edible food products like coconut, using reliable, rapid and internationally accepted test methods is of great importance. In this study a red pigment producing bacterium was isolated from spoiled coconut and identified using *S. marcescens* species specific polymerase chain reaction as *S. marcescens* strain NMRL65. The genes coding for serrawettin biosurfactant production, chitinase, serrapeptidase, S-ribosylhomocysteinase (*LuxS*) and S-adenosylhomocysteine nucleosidase (*Pfs*) were also detected in *S. marcesccens* strain NMRL65 by polymerase chain reaction.

2. MATERIALS AND METHODS

All the microbiological media used were procured from Hi-Media Laboratory Pvt., Ltd. (Mumbai, India). Molecular biology reagents were procured from Thermo Fisher Scientific (USA). Water used throughout was of Milli-Q grade.

2.1. Sample Collection

Spoiled wet coconut with pinkish red colour discoloration was collected from D2/6, Residential Quarters of Naval Materials Research Laboratory, Ambernath.

2.2 Isolation of bacteria from pinkish red discoloured spoiled coconut

The pinkish red discoloured spoiled coconut was swabbed using sterile wooden cotton swab (Hi-Media Labs Pvt., Ltd. Mumbai) and inoculated in 50 ml of sterile Nutrient Broth in 250 ml conical flask and incubated in an rotary shaker (Orbitek Shaker, Scigenics Pvt. Ltd., Chennai, India) at 28°C for 48 hour. After 48 hours 10µl of reddish nutrient broth was inoculated in prepared sterile Nutrient Agar (NA) petri-plates and quadrilateral streaking was done and incubated in microbiological incubator at 28°C for 48 hours to isolate food spoilage bacteria. The red pigmented colony was selected and detailed morphological, biochemical and molecular biology characterization was done. The red pigmented bacterial isolate was labelled asstrain NMRL65 and stored in glycerol stock at - 80°C for further characterization [6].

2.3 Identification of red pigment producing bacterium *S. marscecens*strain NMRL65 from spoiled coconut using *S.marscecens* species specific polymerase chain reaction

Total DNA extraction of bacterial strain NMRL65 was performed using DNeasy ultraclean microbial kit (Qiagen, Germany). The strain NMRL65 was further identified till the species level by using primers specially designed for the identification of*S. marcescens* based on the 16S rRNA sequence alignment of different environmental isolates [7].The nucleotides sequence of *S. marcescens* species specific forward primer are Smar16SV89-108 –5'-GGGAGCTTGCTCACTGGGTG-3'and reverse primer Smar16SWR 499-471–5'- GCGAGTAACGTCA GTTGATGAGCGTATTA-3'. The PCR amplification was performed in the total volume of 25µl in200µl capacity thin wall PCR tube consisting of 7.5 µl of PCR grade water, 12.5 µl of 2X PCR master mix (Thermo Scientific), 1 µl (10 picomoles) of each primer, and 3 µl of template DNA (50 picomoles). Amplification was performed in a thermal cycler Mastercycler epgradient PCR machine (Eppendorf, Hamburg,Germany). The temperature profile for PCR was kept, 95° C for 3 min, 94° C for 0.5 min, 69° C for 1min, 72° C for 0.45 min, 35cycles and final extension at 72° C for 10 min. The PCR product was then electrophoresed in 1.2% agarose and documented using G:Box gel document system (Syngene, USA).

2.4 Morphological and Biochemical identification of *S. marcescens* strain NMRL65

The morphological characterizations of the strain NMRL65 was carried out by Gram Staining and hanging drop method for motility. To determine the lipase activity the strain NMRL 65 was streaked on lipase medium (peptone 10g, sodium chloride 5g, calcium chloride 0.1g, Tween 80 10ml, agar 20 g dissolved in 1000 ml of distilled water). After 3 days of incubation at 28ºC, the lipase activity was indicated by the powdery deposit zone surrounding the colonies. Protease enzyme was determined using skimmed milk agar (pancreatic digest of casein 5g, yeast extract 2.5g, glucose 1g, 7% skimmed milk solution 100 ml, agar 15g dissolved in 1000 ml distilled water). After 2 days of incubation at 30ºC, a clear zone around the cells indicated positive proteolytic activity [8].

Phosphate solubilizing enzyme was determined by the development of a clear zone in Pikovskaya's agar medium (yeast extract 0.5g, dextrose 10 g, calcium phosphate 5g, ammonium sulphate 0.5g, potassium chloride 0.2g, magnesium chloride 0.1g, manganous sulphate 0.0001g, ferrous sulphate 0.0001g, agar 15g dissolved in 1000ml distilled water) after 2-5 days incubation of assay plates at 30ºC [9].

2.5 Detection of *chiA* gene coding for chitinase A enzyme in *S. marcescens* strain NMRL 65 by PCR

The *ChiA* gene coding for chintinase A enzyme in strain NMRL 65 was detected using reported *chiA* specific primers : 5'-GATATCGACTGGGAGTTCCC-3' (forward; corresponding to nucleotide positions 931-950 of S. marcescens X03657) and 5'-CATAGA AGTCGTA GGTCATC-3' (reverse: positions 1180-1161) flanked a 225 bp fragment[10]. The PCR amplification was performed in the total volume of 25µl in200µl capacity thin wall PCR tube consisting of 7.5 µl of PCR grade water, 12.5 µl of 2X PCR master mix (Thermo Scientific), 1 µl (10 picomoles) of each primer, and 3 µl of template DNA (50 picomoles). Amplification was performed in a thermal cycler Mastercycler epgradient PCR machine (Eppendorf, Hamburg, Germany). The temperature profile for PCR was kept, 95° C for 3 min, 95° C for 0.25 min, 58° C for 1min, 72° C for 0.25 min, 35cycles and final extension at 72° C for 10 min. PCR products were visualized by agarose gel electrophoresis and documented using G:Box gel document system (Syngene, USA).

2.6 Detection of Serrapeptidase gene in *S. marcescens* strain NMRL 65 by PCR

The serrapeptidase gene was amplified in strain NMRL 65 using specific forward Ser pep (L): 5'-GGAAGCTTCATATG CAATCTACTAAAAAGGCAAT-3' and reverse [Ser pep (R): 5'- GCGAAGCTTAC ACGATAAAGTAGT GGCGACGT-3'[11]. The PCR amplification was performed in the total volume of 25µl in200µl capacity thin wall PCR tube consisting of 7.5 µl of PCR grade water, 12.5 µl of 2X PCR master mix (Thermo Scientific), 1 µl (10 picomoles) of each primer, and 3 µl of template DNA (50 picomoles). Amplification was performed in a thermal cycler Mastercycler epgradient PCR machine (Eppendorf, Hamburg, Germany). The temperature profile for PCR was kept, 95° C for 3 min, 95° C for 1.5 min, 60° C for 1min, 72° C for 1.5 min, 35cycles and final extension at 72° C for 10 min. PCR products were visualized by agarose gel electrophoresis and documented using G:Box gel document system (Syngene, USA).

2.7 Detection of *pfs and luxS* genes involved in AI-2 Dependent quorum sensing in *S. marcescens* strain NMRL 65 by PCR

The S-ribosylhomocysteinase (*LuxS)* and S-adenosylhomocysteine nucleosidase (*Pfs*) enzymes are responsible for synthesis of signalling molecules, which contribute to virulence factors of the human opportunistic pathogen *Serratia* and other genes of unknown function via quorum sensing. The luxS and pfs gene was detected in strain NMRL 65 using primers specific for *SerratialuxS* and *pfs* gene. The primers used for luxS are FluxS1- 5' GCTGGAACACCTGTTCGC-3' and RluxS2-5' ATGTAGAAACCGGTGCGG-3', the primers used for pfs are Fpfs1-5' CCGGCATCGGCAAAGTCT-3' and Rpfs2 -5'-ATCTGGCCCGGCTCGTAGCC-3'[12]. The PCR amplification was performed in the total volume of 25µl in200µl capacity thin wall PCR tube consisting of 7.5 µl of PCR grade water, 12.5 µl of 2X PCR master mix (Thermo Scientific), 1 µl (10 picomoles) of each primer, and 3 µl of template DNA (50 picomoles). Amplification was performed in a thermal cycler Mastercycler epgradient PCR machine (Eppendorf, Hamburg, Germany). The temperature profile for PCR was kept, 95° C for 3 min, 95° C for 45 s, 55° C for 0.5min, 72° C for 15 s, 30cycles and final extension at 72° C for 10 min. PCR products were visualized by agarose gel electrophoresis and documented using G:Box gel document system (Syngene, USA).

2.8 Detection of putative *SwrW*gene required for biosurfactant serrawettin W1 production in *S. marcescens* strain NMRL65

The *SwrW* gne which encodes putative serrawettin W1 synthase was detected in strain NMRL65 using primers SW2-F3 5′-GCG ACA AAA GCA ATG ACA AA-3') and SW2-R3 5′-GTC GGC GTA TTG TTC CAA CT-3'[13]. The PCR amplification was performed in the total volume of 25µl in200µl capacity thin wall PCR tube consisting of 7.5 µl of PCR grade water, 12.5 µl of 2X PCR master mix (Thermo Scientific), 1 µl (10 picomoles) of each primer, and 3 µl of template DNA (50 picomoles). Amplification was performed in a thermal cycler Mastercycler epgradient PCR machine (Eppendorf, Hamburg,Germany). The temperature profile for PCR initial denaturation at 95°C for 3 min, 95°C for 30s and extension 72°C for 50 sec, 30 cycles of denaturation at 94°C, 0.45 mins, annealing 55°C, 0.45 min, and extension at 72°C, 3 min; 1 cycle of final extension at 72°C, 10 min. Polymerase chain reaction (PCR) products were stored at 4°C. PCR products were visualized by agarose gel electrophoresis and documented using G:Box gel document system (Syngene, USA).

3. RESULTS AND DISCUSSION

3.1 Morphological and Biochemical characterization of *S. marcescens* strain NMRL65

The *S. marcescens* strain NMRL 65 isolated from pinkish reddish discoloured spoiled coconut (Figure 1a) was characterized by Gram staining as Gram negative rods, and hanging drop method revealed motile rods. The strain NMRL65 produced non diffusible red pigment prodigiosin in nutrient agar after 48 hours of incubation at 28°C as shown in Figure 1b. The strain NMRL 65 hydrolyzed casein in skimmed milk agar producing clear zone around colonies after 48 hours which indicated the protease enzyme production as shown in Figure 2a. This enzyme plays a role in spoilage of protein rich food items like milk [14].

Lipase assay using Tween 80 as substrate revealed the hydrolysis which produced powdery deposit around the strain

NMRL 65 colonies as shown in Figure 2b. Due to the production of lipase enzyme *S. marcescens* strain NMRL 65 was able to spoil food products which are rich in fats like coconut and milk[15].

The strain NMRL 65 was able to solubilise inorganic calcium phosphate by producing phosphate solubilising enzyme as shown in Figure 2c. Many researchers have reported phosphate solubilising capabilities of *S. marcescens*isolated from agricultural soils[16 – 18].

Figure 1: (a) A pinkish red discoloured spoiled fresh wet coconut (b) Isolated a red pigmented producing *S. marcescens* strain NMRL 65 in nutrient agar incubated at 28°C for 48 hours.

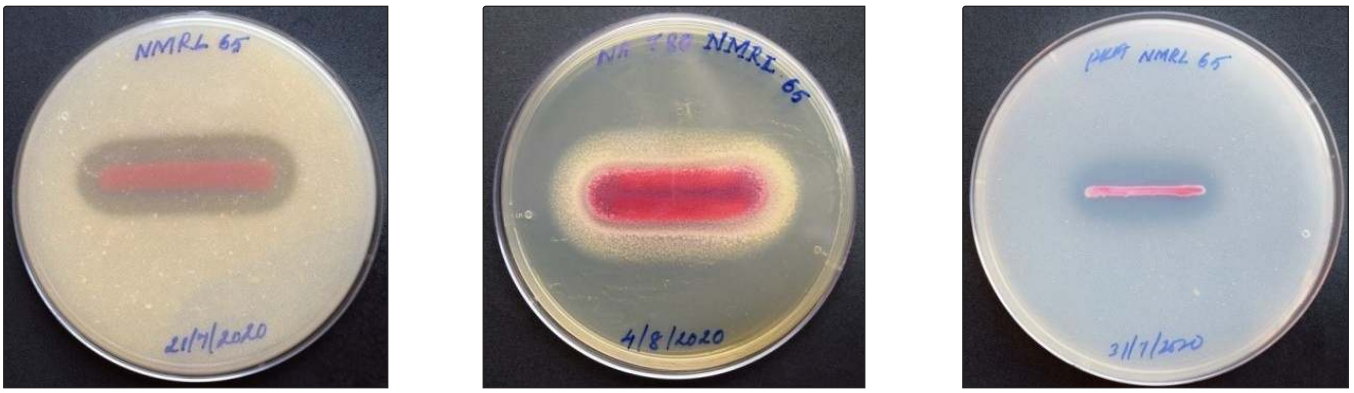
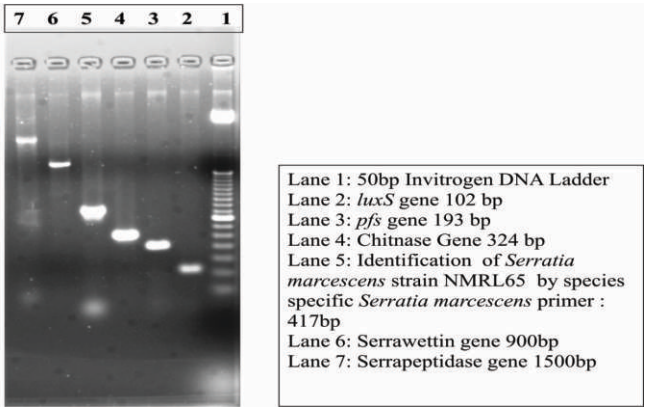


Figure 2:(a) Protease plate assay for *S. marcescens* strain NMRL65 in skimmed milk agar. (b) Lipase plate assay for *S. marcescens* strain NMRL65 in nutrient agar with 0.1% calcium chloride and 1% Tween 80. (c) Phosphate solubilising plate assay in Pikovskaya's agar.

3.2 Molecular Identification of *S. marcescens* strain NMRL65 by *S. marcescens* species specific PCR

The amplification of PCR product of 417 bp (Figure 3 Lane 5) from the genomic DNA of strain NMRL25 using *S. marcescens* species specific primer confirmed the strain NMRL65 as *S. marcescens*. A prodigiosin producing *S. marcescens* isolated from soil was identified by species level by using *S. marcescens* species specific primer[19].



3.3. Molecular characterization of *S. marcescens* strain NMRL65

The amplification of 225 bp PCR products in *S. marcescens* strain NMRL 65 (Figure 3. Lane 4) using reported *chiA* gene primer designed based on *S. marcescens* sequence confirmed the presence of chitinase A enzyme producing capabilities of the strain NMRL65. Many researchers have reported the presence of *chiA* gene in *S. marcescens* and its role in spoilage of chitin rich sea foods like shrimps[20,21].

The amplification of 1500 bp PCR products in *S. marcescens* strain NMRL65(Figure 3. Lane 7) using primer designed based on *S. marcescens* sequence confirmed the presence of Serrapeptidase enzyme capacity of this stain. Serrapeptidase is also known as miracle enzyme which can be used to dissolve blood clots and shrink varicose veins. Many researchers worldwide reported the production of serrapeptidase from *S. marcescens*[22-24].

The amplification of 900bp PCR (Figure 3, Lane 6) products in *S. marcescens* strain NMRL 65 using primer specific for serrawettin W1 synthase confirmed the biosurfactant producing property of this strain. Due to the biosurfactant producing ability *S. marcesens* is able to colonize wide range of substrates in the environment[25].

The amplification of 102bp and 193bp PCR products (Figure 3, Lane 2&3) in *S. marcescens* strain NMRl 65 using primer specific for S-ribosylhomocysteinase (*LuxS)* S-adenosylhomocysteine nucleosidase (*Pfs*) sequence of *Serratia* confirmed the presence of this two key enzymes in AI-2 dependent quorum sensing signal molecules in this strain. The quorum sensing based PCR test method can be used to detect *S. marcescens* from various environments like spoiled food items and animal host. Zhu *et al.*, 2008 [12] reported detection of *luxS* and *pfs* in 27 different *Serratia* strains.

4. Conclusion

S. marcescens is widely distributed in air and cause deterioration of food products. Wet coconut rich in sugar, fat and water content is easily colonized and spoiled by this food spoilage bacterium. The *S. marcescens* species specific PCR method can be employed for rapid detection and identification of *S. marcesens* in spoiled food items.

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Conflict of Interest
The authors declare that there is no conflict of interest.

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ISOLATION OF FUNGUS FROM ROOTS OF WATER *HYACINTH* AND *CELLULASE* PRODUCTION BY THE MICROORGANISM FROM CARDBOARD WASTE

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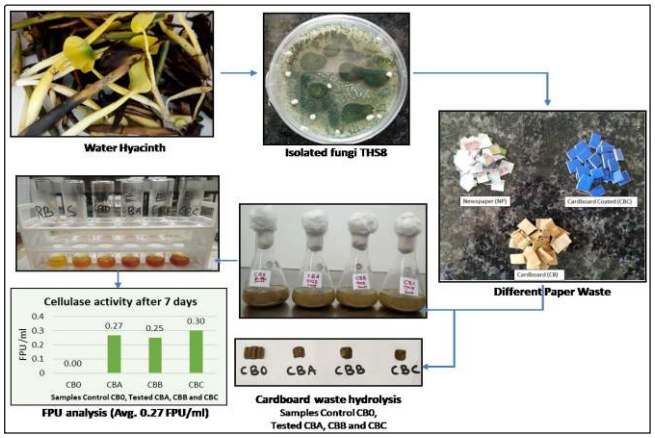
Abstract
Bioethanol production from agro-industrial waste is becoming a major research area for alternative fuel. The cellulase enzyme is the key ingredient used for the cellulosic bio-ethanol fermentation process. India is abundant in cellulosic resources that come from paper and pulp industries. Single-use newspaper and packaging cardboards constitute a significant portion of cellulosic wastes. Here in this study, an industrial enzyme cellulase was produced from single-use newspaper and cardboard wastes. A fungus was isolated (THS8) from the root of the water hyacinth collected from an abundant pond located near Badoli village, Faridabad (India). The fungus was used to do a comparative study for cellulase production by using different substrates such as pure carboxymethyl cellulose (CMC), cardboard (CB),coated cardboard (CBC) and newspaper (NP). The maximum cellulase activity 0.149 FPU (Filter paper unit)/ml was estimated in cardboard (20g/l) grown in MW (Mandel Weber) medium. Likewise, a total of 60% degradation of the cardboard was observed after 7 days of incubation at 30°C with 0.27 FPU/ml optimized at CBC 20g/l.This demonstrates the feasibility of using cardboard as a less expensive feedstock, which is usually dumped after unpacking items in households and industries, to produce cellulase enzyme by the isolated microorganism.

Keywords
Cardboard, Water hyacinth, *Trichoderma*, Cellulase, FPU.

'Graphical Abstract'



1. Introduction

Lignocellulosic biomass is a kind of renewable resource present in abundant quantities in India. Rice straw, wheat straw, sugar cane bagasse, etc. are different types of lignocellulosic biomasses. The burning of such biomass is a major concern due to resulting air pollution. Bioethanol production from lignocellulosic biomass is a suitable way to process it into a useful product. Bioethanol can be blended with fuel and have advantages of less greenhouse gas emission. The whole process of bioethanol production includes pretreatment, hydrolysis, and fermentation. Improvements in the enzymatic hydrolysis can also make it more feasible for commercialization. As the cellulosic biorefineries are setting up in India, the demand of cellulase enzyme is increasing day-by-day. So, a cost-effective process can reduce the production cost using cheaper or waste cellulosic sources for cellulase production. Cellulase is a well-studied enzyme which converts cellulose to glucose by using a cocktail CBHs (Cello-bio-hydrolases) and glucosidase. Therefore, this study is focused on finding a way

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to use the cheapest cellulosic waste for cellulase production. A waste biomasscan be applied as raw material to reduce waste pollution as well as production cost, which can boost the bioeconomy of the country.

Lignocellulosic biomass is a renewable, abundant and cost-effectivebioresource to produce bioproducts [1]. It comes from the non-edible part of the feedstock (rice straw, wheat straw, sugarcane bagasse, cotton stalk) so its application does not affect the food supply [2]. There are many challenges of this process like poor sugar recovery, high enzyme cost, long hydrolysis time, less inhibitor tolerant microbes [3]. Lignocellulosic biomass is composed of cellulose, hemicellulose and lignin, and ash in a variable amount in different kinds of biomasses. Cellulose is a major part of biomass, which contains long chains of glucose sugar that are further utilized for fermentation into ethanol [4]. Hemicellulose is made up of a combination of sugars like xylose, arabinose, and mannose [5]. Lignin is a complex polymer that gives strength to the biomass and highly cross-linked phenols are present in it [6].

Enzymatic hydrolysis is the process of conversion of cellulose to glucose by the action of cellulase enzyme. Cellulase is made up of three main enzymes exo-β-glucanase, endo-β-glucanase and β-glucosidase [7]. Similarly, hemicellulase is composed of mostly xylanase. *Trichoderma reseei* and *Aspergillus niger*are the major micro-organisms studied and used to produce cellulase and xylanase enzyme [7]. Meanwhile, the cost of the enzyme is too high so that the improvement in the enzyme activity and in-house production strategies are used to find a better solution for a reduction in the cost of the enzyme. There are various methodologies used for enzyme activity improvement, such as optimization of pH and temperature [8], immobilization methods [9], the addition of surfactants [10], chemical or radiation mutation [11,12], mild sonication [13], and enzyme cocktail from different species [14] to reduce the time and cost of the process.

Fermentation of cellulosic biomass can be done with and after enzymatic hydrolysis process. Separate hydrolysis and fermentation (SHF), simultaneous saccharification and fermentation (SHF), simultaneous saccharification and co-fermentation are three approaches [2] used with hydrolysis. Hydrolysis and fermentation are separately carried out in the case of SHF. SSF includes hydrolysis along with fermentation of only hexose sugar and SSCF includes the hydrolysis and fermentation of both hexose and pentose sugars [15]. Consolidated bioprocessing (CBP) is the new method in which one single micro-organism like C*lostridium* processes the cellulose directly into ethanol by the release of enzyme [16]. *Saccharomyces cerevisiae* is the primary source of microbe used to produce ethanol due to its robustness [17]. Some other thermo-tolerant yeast *Kluveromyces marxianus* is also used (mostly for the SSF) to carry out the process at high temperatures (nearly 45^{o}) [18]. *Zymomonas mobilis* is a bacterium that can metabolize sugars into ethanol [19]. *Clostridium themocellum* is the best thermophilic bacterium for the CBP process [16] but the yield in both the bacterium is lower than the yeast. Pentose fermenting micro-organismslike *Pichia stipitis*, *Pachysolentennophilus*, and *Candida shehata*are also used for xylose to ethanol production. *Pichia stipitis* is preferred because it does not produce any byproduct, rather has lower ethanol and pH tolerance [20].

Co-fermentation of hexose and pentose is carried out by metabolically engineering the strain. Metabolic engineering of the *Saccharomyces cerevisiae* for xylose to ethanol production can be achieved by the transfer of xylose reductase enzyme-encoding(XR)gene and xylose dehydrogenase enzyme-encoding(XDH) genes into *Saccharomyces cerevisiae* [21]. There is a co-factor imbalance issue due to which xylitol is produced instead of ethanol. While in the case of xylose isomerase enzyme coding (XI) gene does not have any cofactor imbalance issue and only ethanol is produced [22]. So, SSCF is the advanced technology focused on the bioethanol production from lignocellulosic biomass. The purpose of the present study was to find a novel isolate from a non-agricultural area filled with water hyacinth and to produce cellulase enzyme from different kinds of wastes.

2. Material and Methods

2.1 Sample collection

The water hyacinth sample was selected for isolation of micro-organism because of softwood biomass and high-water content. It was suggested as the degrading stem and root can be a source of cellulose-degrading fungi or other micro-organisms. The water hyacinth sample was collected from Badoli village, Faridabad (India).

2.2 Isolation of cellulasee nzyme-producing microbes

Water hyacinth wet root liquid and the solid portion samples were plated on the PDA (potato dextrose agar) and CMC agar (1% carboxymethyl cellulose agar medium) respectively and incubated at 37°C for 4 days. The screening was done by Congo-red zone test [23].

2.3 Cellulase enzyme production and activity assay

The cellulase enzyme production medium was MW (Mandel Weber) medium [24]. Initially, isolate S8 was tested for cellulase enzyme activity on the MW medium with CMC 1.5 % as agar plate and as broth. After that comparative study was done between all isolates for cellulase enzyme activity. All the strains SL1 to SL6 (from a liquid sample) and S8 (from the solid root) were grown in the MW agar medium at pH 5 and kept as the slant. 1ml of the autoclave distilled water was

poured into each slant sample, mixed well and 1ml of the spore solution was inoculated in 50 ml of the MW production medium containing 1% CMC. After 72 hours,the sample was centrifuged at 8000 rpm for 5 minutes and the supernatant was filtered by a 0.22-micron filter. Then, filtrate was used for the filter paper assay. FPU assay was done by the Gosh FPA assay protocol[25].

2.4. Saccharification of cellulose by cellulase crude enzyme extract

Five gm CMC was added to two 50 ml flasks and autoclaved. After autoclaving and cooling of dried cellulose, 20 ml of the crude enzyme filtrate from the S8 culture was added in the sample (CMCase). Control samples contained20 ml autoclaved water to maintain the moisture up to 80%. Components were mixed well and incubated at 50°C for 48 hours. After 48 hours, the CMCase sample became liquefied as compared to control.

2.5 Cellulase enzyme production in different paper waste

Four kinds of substrates were taken such as newspaper (NP), cardboard (CB), dye coated cardboard (CBC), carboxymethylcellulose (CMC) for the cellulase enzyme production. 20 gm/l of each substrate in 50 ml fermentation medium was inoculated with spore suspension(1ml) of the isolate THS8 with the spore count of 5.32×10^6. The samples were incubated at 30ºC at 120 rpm for 120 hours. After 120 hours, samples were extracted, centrifuged and the supernatant was taken as a crude enzyme sample. FPU assay was done by standard Gosh FPA assay protocol for cellulase enzyme activity.

2.6 Cardboard substrate utilization

2% of cardboard was mixed with 50 ml MW medium. Sterilization was done by autoclaving and 1ml of the spore suspension was inoculated and incubatedat 30°C for 7 days. After 7 days, the treated biomass is centrifuged, and the crude enzyme extract was taken out. Then, the crude enzyme was tested for FPU analysis per ml of the enzyme sample. The remaining solid substrate was also tested for the degradation and compared with the control sample.

2.7 Substrate concentration optimization for cellulase production from cardboard

The cardboard waste substrate was used from 5g/l to 50g/l concentration (5g/l, 10 g/l, 20 g/l, 30 g/l, 40 g/l and 50 g/l) in MW medium prepared in duplicates for test against control. The samples were sterilized by autoclaving and spore suspension was prepared for the inoculation. The 5.40×10^6 spores per ml were inoculated as 1 ml suspension in each test sample and1ml water in control. Then, it was kept for incubation at 30°C for 7 days. After incubation, the crude enzyme was tested for the highest cellulase activity and maximum substrate utilization. Cellulase kinetic activity with respect to enzyme to substrate concentration was applied similar to that used for the growth and consumption of substrate by microbes. Enzyme-specific activity depicted as μ^E(similar to the specific activity of microbes), a maximum enzyme-specific activity depicted as μ_{max}^E(similar to μ_{max} of microbes) and substrate saturation constant depicted as K_s^E (similar to Ks for microbes) was calculated by using Michaelis Menten equation [26], where superscript E indicates enzyme.

2.8 Characterization of isolate TSH8

THS8 isolate was grown on PDA plate for 4-5 days at 30°C. After the development of full myceliummorphological characterization was conducted. Then, a spore suspension was prepared from the plate containing nearly 5.5×10^9 spores per ml of the suspension. Then, conidial spores were examined underan inverted microscope (EVOS XL imaging system, Thermo Fisher).

3. Result and Discussion

3.1 Isolation of cellulolytic microorganism

New isolates with the ability to convert cellulose were screened.7 different fungal isolates viz., THSL1,2,3,4,5,6 and THS8 were obtained by plating the collected sample on 1% CMC agar plate. THS8 was isolated from the solid root area sample. Out of which, the S8 isolate was morphologically similar to the fungus *Trichoderma sp*. A total of 6 liquid isolates SL1 to SL6 and solid sample isolate S8 were selected, which showed cellulose degradation as presented in Figure 1. The potential cellulase producing 7 microbes were screened on Congo red medium containing carboxymethyl cellulose as a substrate with the MW medium. Hendricks *et al*. also used a similar method for identifying cellulase producing bacterial strains from the soil through Congo-red dye depletion screening [27].After screening with Congo red dye and substrate hydrolysis (Figure 2), the S8 was selected as a potential cellulase producing fungal candidate. As per morphology and growth of the isolate,S8 showed similar characteristics with cellulose-degrading fungus *Trichoderma sp.* and was selected for further analysis [28]. Congo red test also showed zone on the S8 plate as shown in Figure 2B.

3.2 Screening based on cellulase activity

Hydrolysis of cellulose can be visualized on the plate as yellow rings and glucose release from THS8 broth in Figure 3B was determined by DNS test. The filter paper unit (FPU) was also calculated as shown in Table 1 [26,29]. It was observed that sample THS8 showed fungal growth in clumps formation in which these were grown as fungal pellets and did not fully mix with the medium. When allowed to settle, these easily settled as shown in Figure 4. The isolate S8 has an advantage for purification of cellulase which may omit the centrifugation step. The FPU assay results are shown in Table 2 and THS8 showed maximum activity.

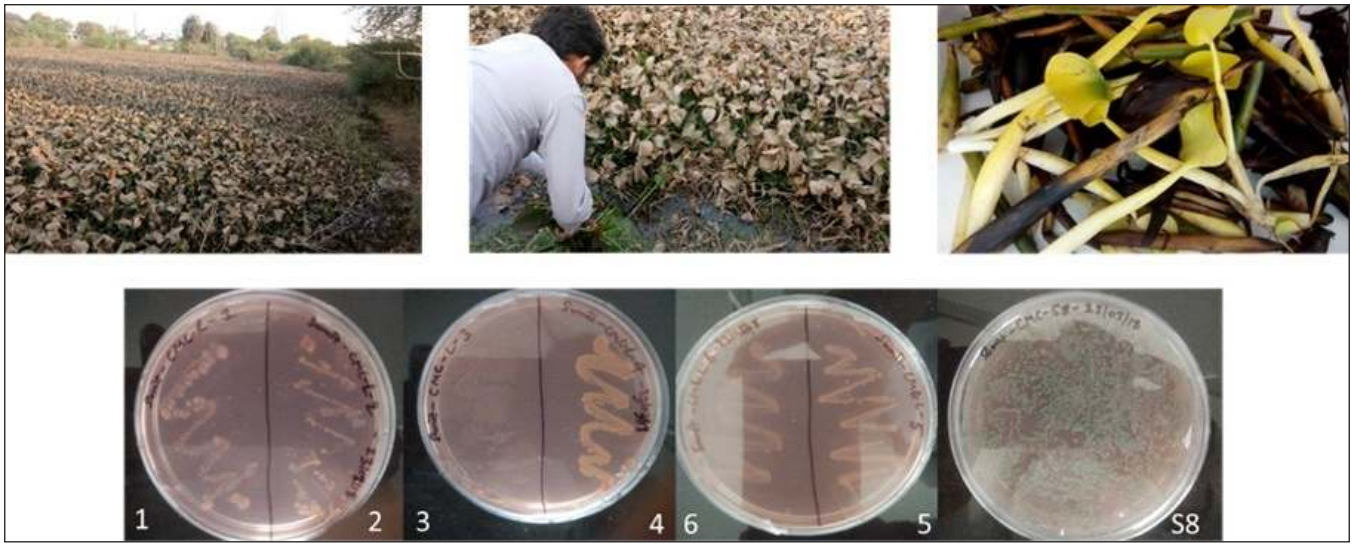

Figure 1: Sample collection of water hyacinth and isolated samples SL1 to SL6 and S8 grown of CMC agar plate with Congo red dye.

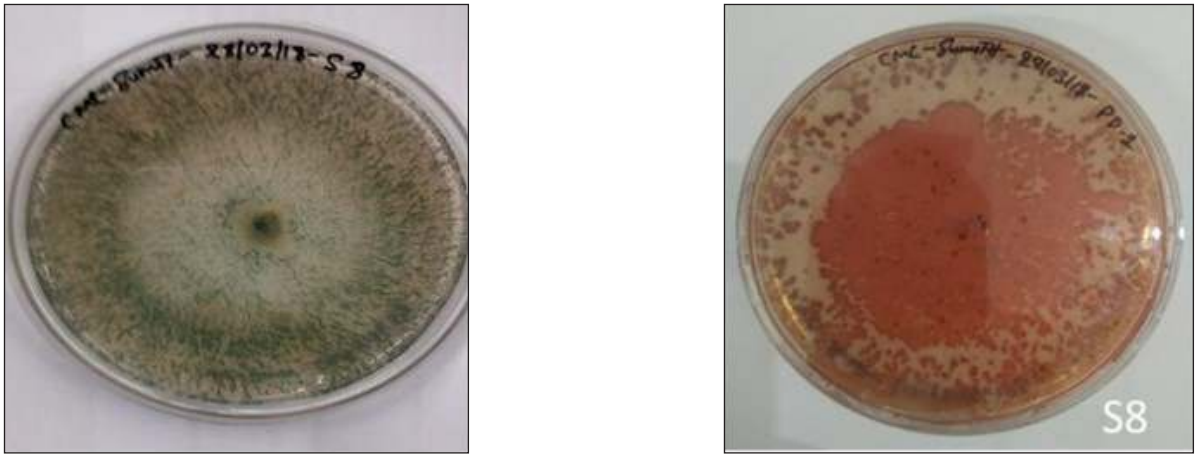

Figure 2: Congo red test showed a clear zone of degraded cellulose by isolate S8.

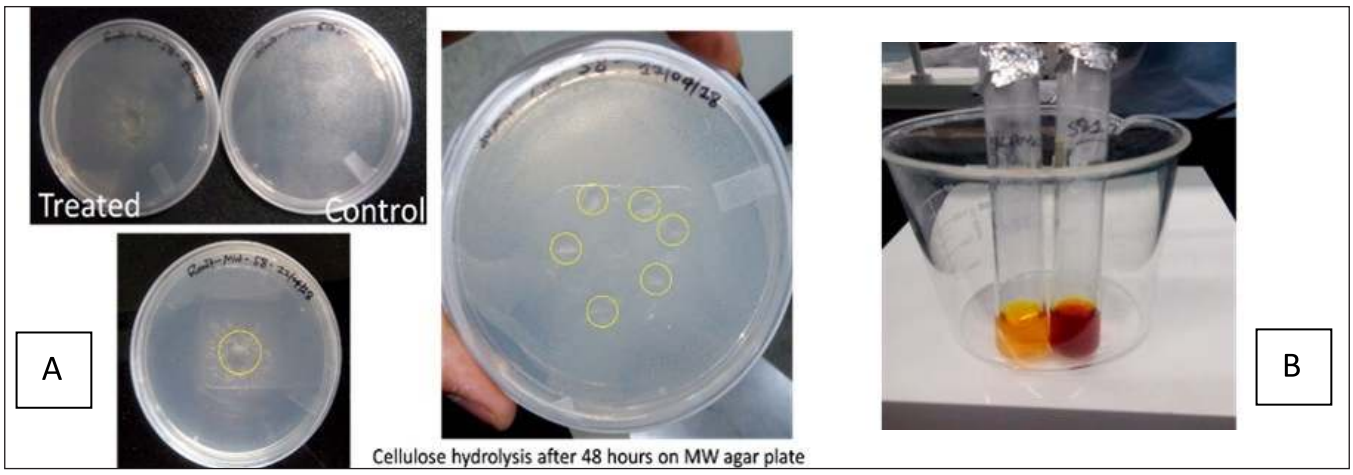

Figure 3: (A) Clear zones as a yellow ring of hydrolyzed cellulose1% with MW agar plates (B) release of sugar from cellulose observed by DNS method.

Table 1: FPU and glucose to cellulose conversion.

Parameters	Values
CMC Broth cellulose at Zero-day	15 g/l
Glucose produced after 4 days	3.16 g/l
Cellulose consumed	2.84 g/l
FPU/ml	0.1670 unit/ml

3.3. Cellulose saccharification

The saccharification study was done on pure carboxymethyl cellulose (CMC). This was done to verify hydrolysis of pure cellulose by the cellulase enzyme produced by the isolate. 5g CMC was treated with 20ml crude enzyme against control as water. After 48 hours of incubation at 50°C, sample CMCase was liquefied as compared to control (shown in Figure 5). 23.42% hydrolysis of pure cellulose was observed with THS8 crude enzyme broth. FPU of the crude enzyme was 0.0534 U/g of cellulose. Herr and Dieter used 10% cellulose suspension for hydrolysis by crude enzyme and resulted in a 40% hydrolysis. However, in this study, the suspension was 20% and hydrolysis is nearly similar to 10% cellulose suspension used by Herr and Dieter [30].

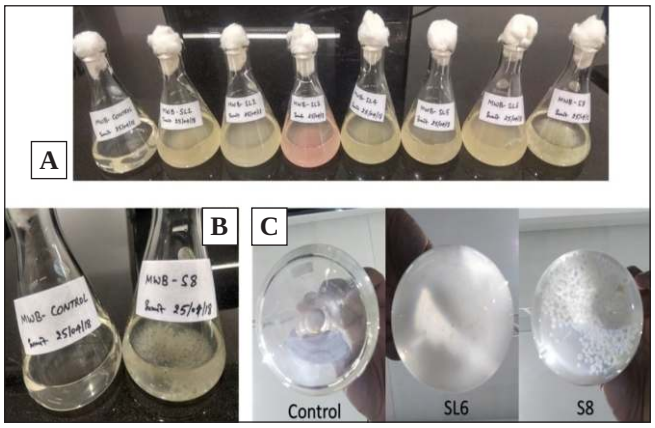

Figure 4:(A) Fungal isolates in 1%CMC broth, (B) S8 culture against control, (C) Observation of fungal pellets in S8 as compared to SL6.

Figure 6: Hydrolysis of pure cellulose by using crude enzyme.

Table 2. FPU/ml of different isolates on MW medium with 1% CMC.

Sample Name	FPU/ml
SL1	0.1150
SL2	0.1336
SL3	0.1460
SL4	0.1596
SL5	0.1534
SL6	0.1348
S8	0.1658

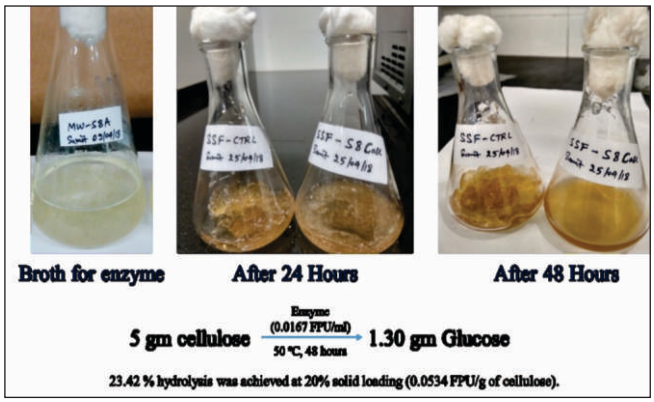

Figure 5:Hydrolysis of pure cellulose by using crude enzyme.

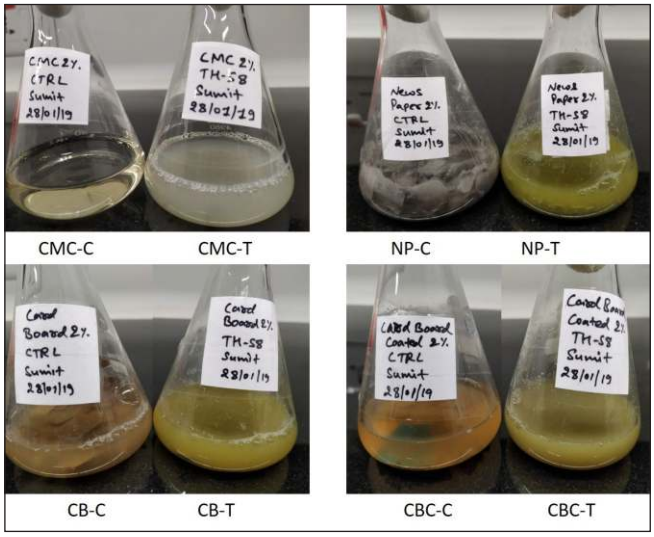

Figure 7: Comparative growth difference between control (C) and treated (T) and cellulase production in MW medium.

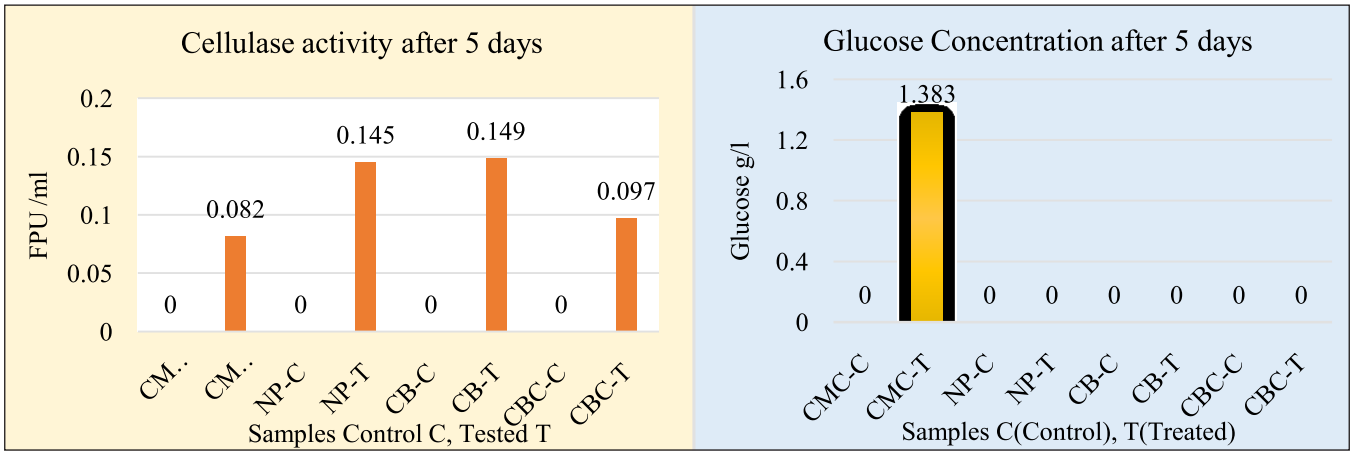

Figure 8: Left: Cellulase activity in FPU/ml in different substrates, Right: Glucose concentration after 5 days of incubation in the different samples.

3.4. Paper waste utilization

The use of the cheapest cellulose source was a major objective of this study. Therefore, 3 kinds of paper waste (Figure 6) as NP, CB, and CBC along with CMC was tested for cellulase production. Figure 7 depicts comparative growth differences in different substrates provided. The enzyme activity was found higher in NP, CB and CBC (0.145, 0.149and 0.097 FPU/ml) as compared to the CMC (0.082 FPU/ml)as shown in Figure 8(left side). The newspaper and cardboard waste samples showed nearly similar cellulase activity. Cardboard is preferable because it is free from ink and dye and mostly dumped as waste while newspapers are used for other purposes too. The consumption of sugar and its release was also estimated. It was found that sugar was left in CMC-T (Treated) as 1.383 g/l, which can cause the feedback inhibition for the production of the enzyme in the CMC-T sample (Figure 8 right side). Ahlam *et al.*, also showed the utilization of cardboard waste nearly 25 gm/l of the substrate with 2.95 FPU/ml, which is quite higher than the observation done in this study. Here, direct cardboard was used as a substrate, while Ahlam *et al.*, used 1% H_2SO_4 for pre-treatment first, then further for enzymatic hydrolysis [31]. Similarly, newspaper, filter paper and other substrates were also tested for cellulose hydrolysis [32]. Here in this study, maximum enzyme activity was found with cardboard substrate i.e. 0.149 FPU/ml.

3.5. Cardboard utilization for cellulase production

It was found that the cardboard is a good substrate for enzyme production, as it showed maximum enzyme activity. So, the experiment was done in triplicates (CBA, CBB, and CBC) with cardboard used as substrate (Figure 9 left side). Here, 2% of cardboard was suspended in MW medium treated with THS8 for 7 days at 30ºC measured as the average maximum of 0.27 FPU/ml(Figure 9 right side). There was around 60% degradation of substrate observed with respect to remaining solid wet weight as shown in Figure 10.

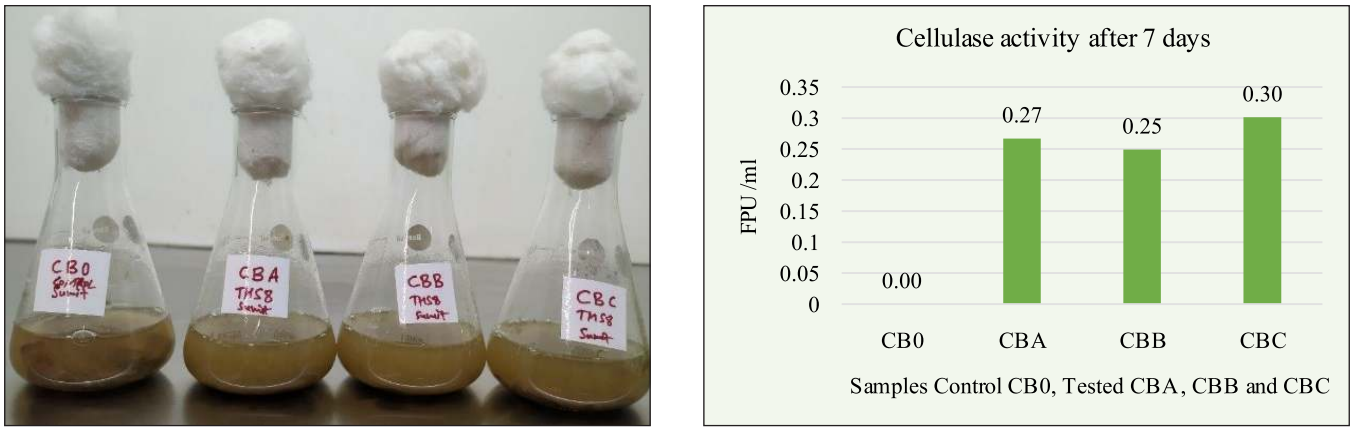

Figure 9: Left: Growth of the S8 in cardboard medium after 7 days, Right: Cellulase enzyme activity measured in cardboard medium cultures in triplicate: CB0 is controlled, CBA, CBB, and CBC are the grown cultures.

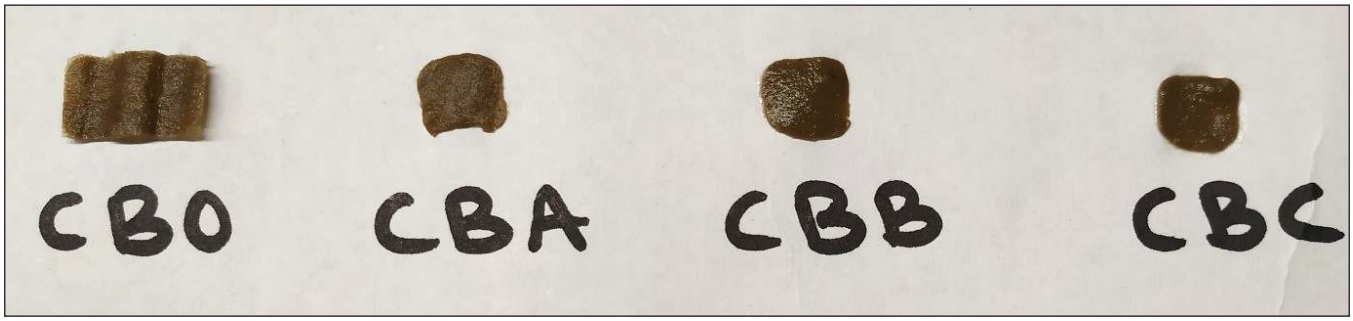

Figure 10: Degradation of the pure cardboard substrate: First left control (CB0) and the other three are samples in triplicate treated with the enzyme.

3.6. Solid-state fermentation with cardboard

Growth of mycelium on the cardboard waste as solid support was observed with nearly 85% moisture developed by the spore suspension on the substrate. Solid-state fermentation was previously used for cellulase production by using *Trichoderma reesei* QMY-1 with FPU as 17 U/ml [33]. The purpose of this study was to determine whether it is feasible to grow the fungus on solid cardboard or not. After 7 days of static incubation, the growth was observed on the medium and visually compared against control. Figure 11 clearly showed very high growth of isolate THS8 as green-colored patches on the wet cardboard.

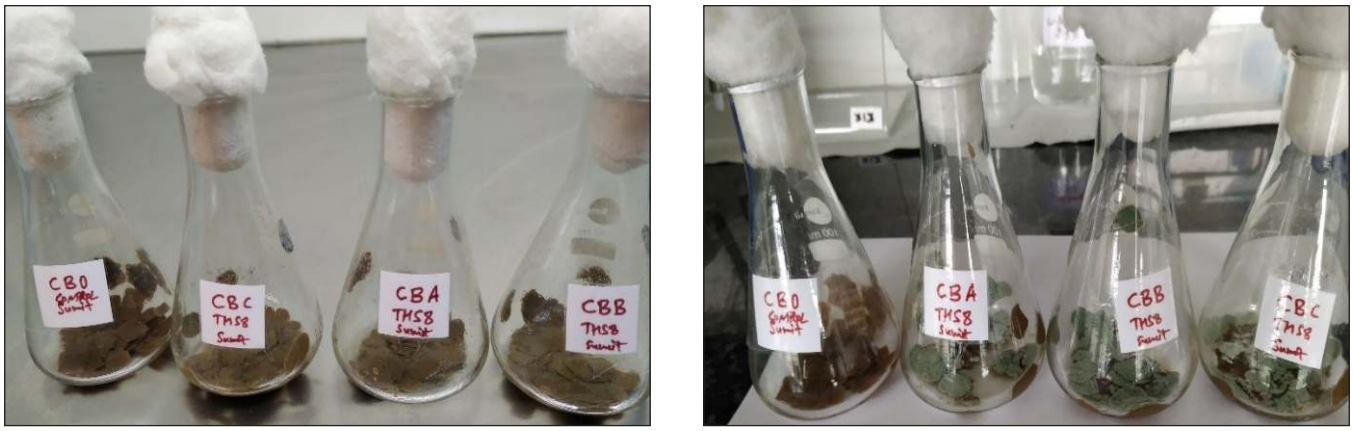

Figure 11: The solid-state fermentation of the isolate S8 on waste cardboard (Left: at zero-day Right: after 7 days).

3.7. Substrate concentration optimization for cellulase and kinetic modeling

Usually, with the increase in the substrate, there should be an increase in product yield. However, in the case of cellulases, feedback inhibition due to the product was observed. When the product is produced in a higher amount, the feedback inhibition occurs. A similar case was found in this study during substrate optimization of cardboard concentration. However, an increase in the activity was observed from day 2 to day 6. While enzyme production was not increased much due to feedback inhibition by glucose concentration, it was found higher in more concentrated substrate samples. There was a substantial increase in glucose concentration after 6 days as 0.10, 0.10, 0.11, 0.12, 0.19, 0.56 g/l in 5, 10, 20, 30,40 and 50 g/l samples respectively. Maximum enzyme activity was found to be 0.117 FPU/ml in 50g/l samples, but it was reduced due to higher glucose feedback inhibition (Figure 12). Meanwhile, inhibition was lower and nearly similar up to 30g/l sample. The μ_{max}^{E} and K_s^{E} were calculated as 14.93 min^{-1} and 0.022 respectively by using slope and intercept values of Figure 13. Similarly, other kinetic parameters like K_m and Vmax were measured by Shafique et. al. by using *Trichoderma viride* FCBP-142 with 0.6 and 8.33 values respectively [34].

3.8. Morphological identification

During the initial 2 days of inoculation, the mycelium grown as white clouds then, after maturity, the color change to dark green (Figure 14A), which showed the similarity with *Trichoderma hazaricum* [35]. However, spores shown under the microscope are oval (Figure 14B), nearly 6.250 µm in length and 3.125 µm in width as measured with the scale bar after zooming at 8×from an image captured at40x objective. Conidial spores count was ~5.42×10^6 per ml.

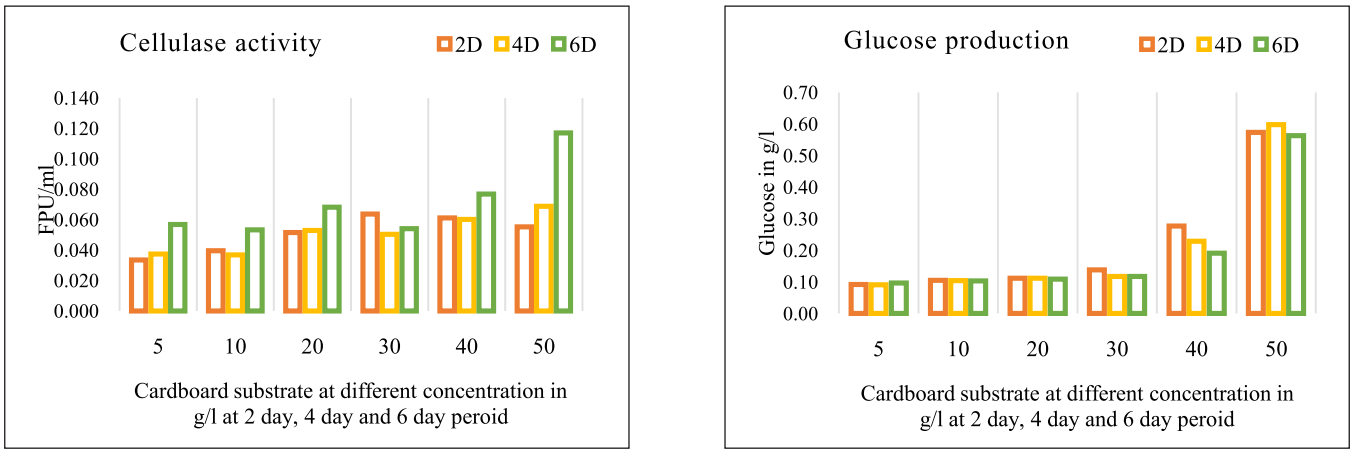

Figure 12: Left: Cellulase activity in FPU/ml in different concentrations of cardboard substrate Right: The glucose concentration of samples grown till 6 days of incubation with different concentration of substrate in g/l

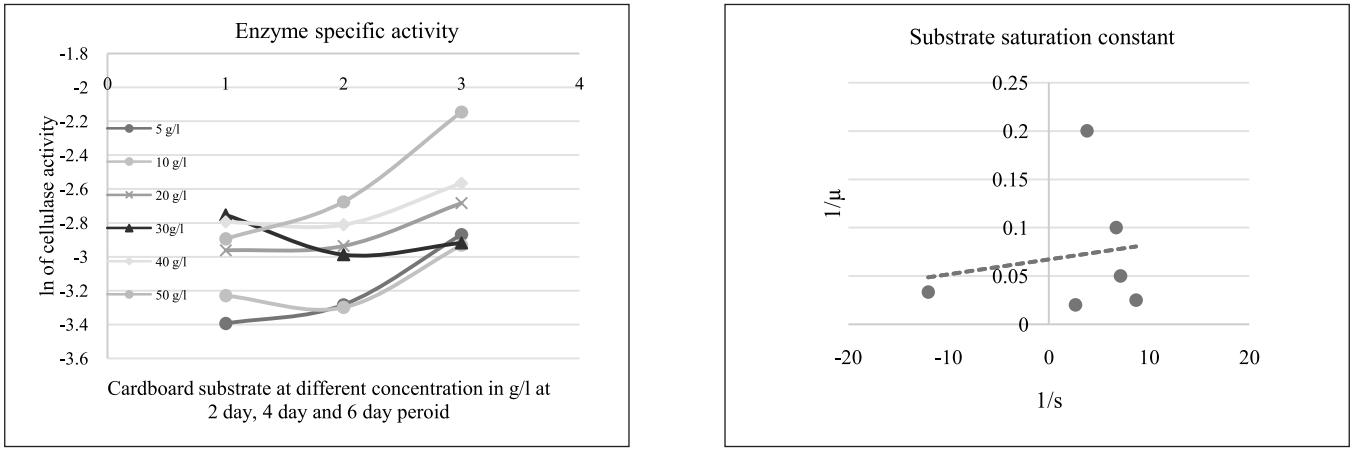

Figure 13: Left:Cellulase enzyme-specific activity measured as µ against each substrate over the time 2-day, 4 days and 6 days. Right: Substrate saturation constant KsE value determination by plotting 1/µ against 1/s values.

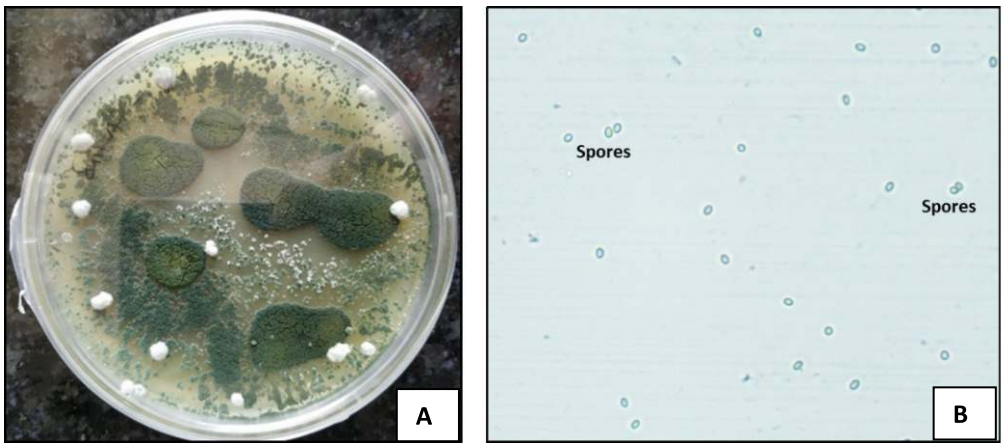

Figure 14: (A) Morphological identification of cellulase producing isolated fungi THS8. (B) microscopic image of conidial spores.

4. Conclusion

Application of paper waste for cellulase production at an industrial scale can be promising for reducing the cost of the enzyme as well as for waste management. This study signifies the use of cardboard waste as pure cellulosic feedstock replacement, which can contribute a cost-effective approach

with higher cellulase enzyme production as compared to pure carboxymethyl cellulose. According to the results, isolated THS8 fungal strain was the best cellulase producing strain. The strain was capable of degrading nearly 60% of the cardboard waste during the study period and showed maximum cellulase activity of 0.27 FPU/ml, which is a good start for its large-scale production after further process optimization. In conclusion, the cardboard waste is a very good potential source of cellulose substrate for cellulases production, irrespective of the dyes or ink it contains. It is more valuable because it can reduce the whole production cost and the process becomes simpler.

Acknowledgement
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B.
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VASCULAR DISEASES AND ROLE OF NITRIC OXIDE1

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Abstract
Endothelium, the inner-most layers of blood vessels is an active monolayer of cells which has been evolved to become specialized barrier between blood and other structures of the vessel wall. Endothelial cell layer is known to communicate between blood and extravascular tissues and is actively involved in cardiovascular homeostasis. Intact endothelium regulates vascular tone, permeability and maintains non-inflammatory, anti-thrombotic surface. Through its ability to express pro-coagulants, anticoagulants, vasoconstrictors, vasodilators, cell adhesion molecules and cytokines, the endothelium has emerged as one of the pivotal regulators of haemostasis. Under normal conditions, endothelial cell sustains a vasodilatory, anticoagulant and fibrinolytic state in which coagulation, platelet adhesion as well as leukocyte activation and inflammation are suppressed by continuous release of nitric oxide (NO). Endothelium plays a major role in development of cardiovascular diseases (CVDs). Endothelial cell injuries and resultant endothelial dysfunction (ED) plays a key role in the deployment of CVDs. Therefore, the imbalance of endothelial function due to suppression of anticoagulant molecules like nitric oxide (NO), tissue factor pathway inhibition (TFPI), thrombomodulin etc, and over expression of pro-coagulant molecules like tissue actor (TF), endothelin-1, von Willebrand factor (vWF), plasminogen activator inhibitor (PAI)-1 secreted by endothelial surface is seen during stress. Several factors like infection, hyperglycaemia, hyperlipidaemia, malignancy, oxidative stress, and aging can interfere with endothelial function. It is widely believed, that ED plays a crucial role in the development of cardiovascular diseases. Also it has been reported to be involved in atherosclerosis, thrombosis, hypertension, diabetes and other vascular conditions. In this article we will specifically highlight and review the role of ED in different vascular conditions.

Keywords
Nitric Oxide, CVDs, hypertension, thrombosis, inflammation, stress..

1. Introduction

Vascular endothelium is considered as the largest endocrinal organ in the body which has been shown to have a role in homeostasis in the body by exerting various functions [1]. It is made up of simple squamous epithelial cells that line blood vessels, lymphatic vessels and the heart. The vascular endothelium has a total weight of about 1. 5kg. The endothelium has been recognized as a smart barrier and a key regulator of blood flow in micro and macro vascular circulation [2]. Endothelial function is very important, as it interacts with nearly every system in the body, and selectively supplies nutrients and growth factors to every organ. On the other hand, endothelium is also the recipient of active metabolites and delivers them back to the circulation. Previously, it was believed that, endothelium is an inactive barrier between blood and extravascular tissues. However, recent studies have shown that the vascular endothelium is an active paracrine, endocrine, and autocrine organ, responsible

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for the regulation of vascular tone and the maintenance of vascular homeostasis.

2. Physiological functions of endothelium

When immediate surrounding tissues are at basal conditions, the endothelium maintains the vessel homeostasis which favors vessel dilation over vasoconstriction [3]. The endothelium being a dynamic reactive tissue, responds to various intrinsic and/or external stimuli (e.g. shear stress, temperature, transmural pressure, temperature, mental stress, neurohumoral responses, immune response and medications [2,4].

Under physiological condition, endothelial cells maintain basal perfusion which is determined by cardiac output, systemic and local vascular resistance. Endothelial metabolism, which is a key regulator of perfusion, is impaired during several diseases like infection, injury, aging, and inflammation, local, blood flow is the result of vascular relaxation and contraction that is balanced by endothelium derived vasodilatative and vasoconstrictive factors [5]. Among these factors, nitric oxide (NO) stands out as hub and target of many pathways and mechanisms [6]. It is important to understand the biochemical foundations of NO for endothelial functions. NO, a potent vasodilator, is released from the endothelium due to shear stress. This NO is released by endothelial nitric oxide synthase (eNOS) by utilizing L-arginine as substrate which leads to the production of intracellular cyclic GMP (cGMP) [7]. However, in an event when the NO-dependent vasodilation is compromised, the cytochrome-derived factors, natriuretic peptide [8], and prostacyclin [9] dependent vasodilator mechanism comes into action. During diseased state, there is impaired endothelial function and this results in the balance shift towards prevailing constrictive factors and/or down-regulation of vasodilatative factors. An important counterweight in the vascular balance is cyclooxygenase (COX). This mostly induces COX-1 which is endogenous, and may involve COX-2 if it is induced. The COXs have a key role in generating vasoconstrictive factors.

The COX enzymes transform arachidonic acid into endoperoxides and further into thromboxane A_2 (TXA_2) [10], prostaglandins and prostacyclin [11]. Local presence of thrombin evokes inducible NO release. Release of serotonin and ADP from platelets in turn increases the NO synthesis and release in healthy endothelium to induce dilatation [12]. When vasodilatory function of endothelium is impaired, then the thrombus formation is mechanically promoted by vasoconstriction via TXA_2 and by the direct effect of serotonin on smooth muscle cells [13].

3. Endothelial Dysfunction

3.1 Nitric oxide – generation and significance

In last 20 years, world has witnessed a gripping surge in the field of NO biology. NO is a free radical, an endogenous product that was first reported as endothelium-derived relaxing factor (EDRF) by Furchgott and Ignarro in 1986 [14,15]. Nitric Oxide (NO) plays a significant role in several pathophysiological conditions such as atherosclerosis, hypertension, angiogenesis-associated disorders, nervous and immune systems, defense mechanisms against infectious diseases and tumors [16-25]. NO is mainly produced from l-arginine by eNOS [26-29]. L-arginine was first discovered and characterized as substrate for NOS for the production of NO [30-32]. Three distinct genes catalyze the production of NO from L-arginine: neuronal NOS (nNOS), inducible NOS (iNOS) and endothelial NOS (eNOS). However, endothelium-derived NO is a potent vasodilator [33,34]. In the vasculature, NO stimulates sGC to produce cGMP, decreases the intracellular concentration of calcium and causes relaxation of vascular smooth muscle. NO also mediates hypoxic augmentation of contraction in coronary arteries, a response which depends on sGC but independent of cGMP production (Figure 1) [35].

Traditionally, ED has been associated with pathological conditions that might have altered anticoagulant function, impaired anti-inflammatory properties of the endothelium, impaired modulation of vascular growth, and dysregulation of vascular remodeling. For instances, a plethora of studies has confirmed that the impairment of endothelium-dependent vasorelaxation is caused by a loss of NO bioactivity/availability in the vessel wall [4]. The loss of NO bioavailability is the salient feature of a dysfunctional endothelium, which in turn is the sentinel of systemic or focal vascular disease.

Numerous studies have shown that most of the cardiovascular diseases were initiated from ED. The decline in NO bioavailability may be caused by decreased expression in endothelial cells [36], a lack of substrate or cofactors for eNOS [37], the presence of inhibitor of NOS [38], and alterations of cellular signaling and finally, accelerated NO degradation by reactive oxygen species (ROS) (Figure 1) [39]. Another aspect of ED is impaired endothelial barrier function. Depending on the mode of pathophysiological changes, barrier function may be impaired locally or systemically (Figure 2). Localized loss of the selective barrier function (manifested as edema), coupled with the mobilization of leukocytes, have been recognized as cardinal signs of inflammation [40]. From an immunological point of view, reaction to tissue injury or infection leads to cross-talk between leukocyte and endothelium. However, from the perspective of hemostasis and thrombosis, ED is characterized by activation of pro-inflammatory and pro-coagulant molecules, as well as the suppression of anti-inflammatory, and anti-coagulant molecules. The intact and normal functioning endothelial lining provides a stable

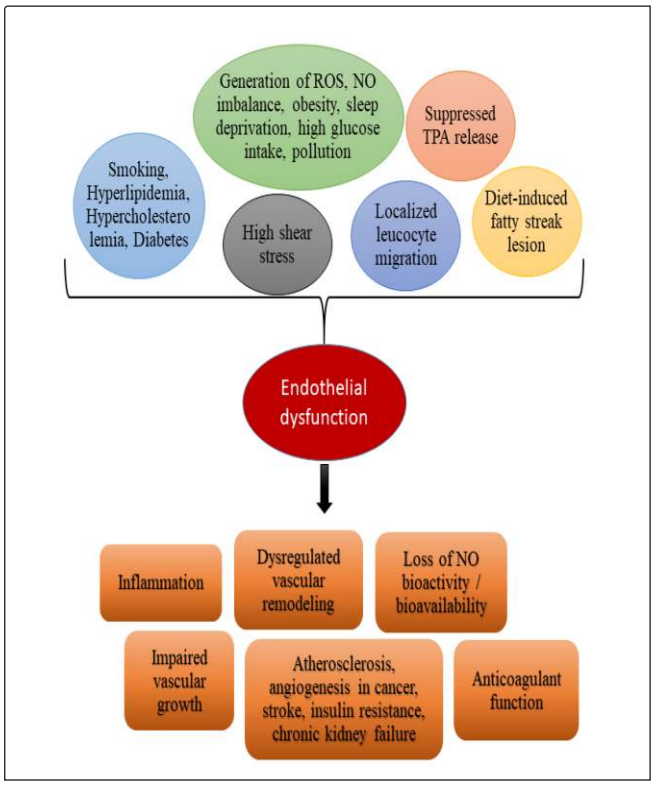

Figure 1: Ischemia-reperfusion induces generation of reactive oxygen species; its role in generation of nitric oxide, coronary vasoconstriction/dilation, causing decreased NO production, followed by impaired endothelium-dependent vasodilation.

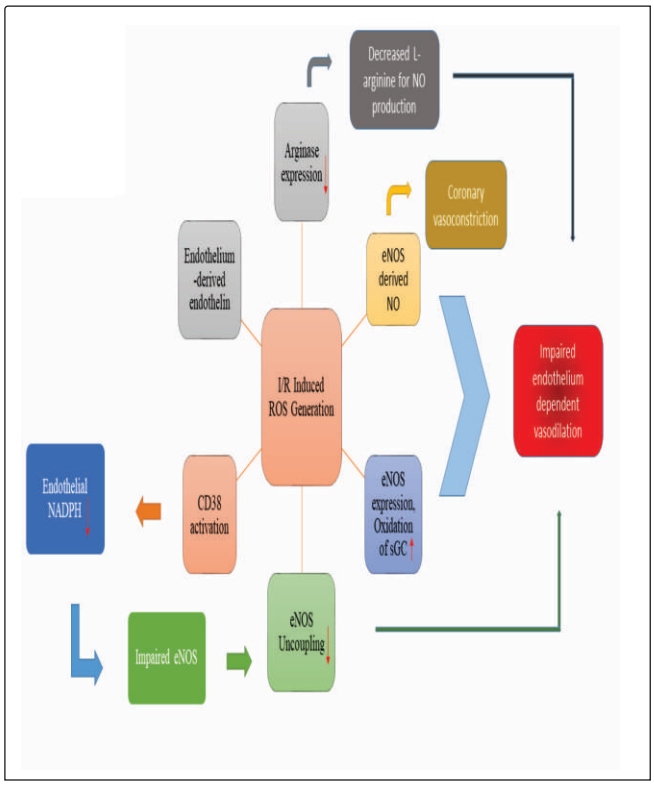

Figure 2: Differential role of endothelial dysfunction cause and effects.

reservoir for blood as its luminal surface does not activate the coagulation cascade or promote leukocyte-platelet adhesion, and it also exhibits anticoagulant and fibrinolytic properties (Figure 2) [41]. Systemic endothelial dysfunction may lead to wide spread inflammation, vascular leakage, thrombocytopenia and disseminated intravascular coagulation (DIC). Therefore, localized ED and leukocyte adhesion may lead to venous thrombosis. Other than altered endothelial barrier function, localized ED also leads to tissue factor (TF) induction and increased von Willebrand factor (vWF) release that shifts the homeostatic balance towards the pro-coagulant-pro-inflammatory phenotype (Figure 2) [42]. Intact endothelium releases pro-fibrinolytic molecules like tissue plasminogen activator (TPA) [43], whereas dysfunctional endothelium suppresses TPA release thereby impairing fibrinolytic function of the endothelium [44]. In contrast to venous endothelial cells and microvascular endothelial cells, arterial endothelial cells are surrounded by vascular smooth muscle layer and adventitial layer. Arterial endothelial cells physiologically experience high sheer stress and synthesize ample amount of NO that facilitate vascular relaxation. In the context of atherogenesis, dysfunction of endothelium is mainly characterized by, a loss of anatomical integrity of the intima, as described by the seminal *"Response-to-Injury Hypothesis"*. Endothelial cell injury and subsequent sub-endothelial matrix exposure lead to platelet adhesion and activation through sub-endothelial collagen layer [45]. The initiating event in the atherogenic process is some form of overt injury to the intimal endothelial lining, that is induced by noxious substances (e.g., oxidized cholesterol, cigarette smoke, hyperlipidemia, hypercholesterolemia, hyperglycemia, etc.) or altered hemodynamic sheer stress (e.g., abnormal blood flow caused by hypertension) (Figure 2) [46]. In particular, mechanical tearing of local endothelial is seen as the inciting stimulus for platelets adhesion, activation and the localized release of platelet-derived growth factors (PDGFs). This might then elicit the migration, proliferation and phenotypic modulation of medial smooth muscle cells and thus generate a fibromuscular plaque [47]. It is of great importance to establish the sequential event that lead to the atherogenesis from endothelial injury. But, the direct link between endothelial injury and the genesis of atherosclerotic lesion is still unclear. However, the detailed morphologic examination in diet-induced fatty streak lesions in animal models failed to demonstrate unconcealed intimal injury or platelet adhesion. In this context, it is highly relevant that several molecules including high mobility group protein (HMGB-1) [48], heat

shock proteins (HSPs) [49] are released from injured endothelium, facilitate monocyte adhesion, a crucial step for plaque formation.

3.2 Endothelial dysfunction in atherosclerosis

ED of lesion-prone areas of the arterial vasculature lead to atherosclerotic plaque formation [50]. Sequential deterioration of arterial vasculature along with increased sheer stress contribute in lesion formation. ED is one of the early events that are responsible for the deterioration of arterial vasculature [51]. Recent insight into the cellular mechanisms involved in atherogenesis shows that deleterious modifications of endothelial physiology or metabolism is the initial event of vascular remodeling that represents a crucial step in the development of atherosclerosis and are also involved in development of plaque and the occurrence of atherosclerosis [2]. The sequential event including focal permeation, trapping and physicochemical modification of circulating lipoprotein particles in the sub-endothelial space construct an inflammatory lesion [52]. This initiates a coordinated cellular signaling, followed by complex pathogenic sequence and endothelial activation. Activated endothelial cells express several cell adhesion molecules, which facilitate selective recruitment of circulating monocytes from the blood, and invade the tunica intima, where they differentiate into macrophages. These macrophages also abnormally take up modified lipoproteins to become foam cells (the hallmark of early fatty streak lesions [53, 54]. The activated endothelium and macrophages release multiple chemokine, and growth factors which act on neighboring smooth muscle cells (or precursors cell) to induce their proliferation and synthesis of extracellular matrix components within the intimal compartment, thus generating a fibromuscular plaque [55, 56]. This progressive structural remodeling of developing lesions results in the formation of a fibrous cap, overlying a lipid-rich necrotic core that consists of oxidized lipoproteins, cholesterol crystals and cellular debris. This is also accompanied by varying degrees of matrix remodeling and calcification [57, 58]. The lateral edges of these complicated plaques also contain a rich population of inflammatory cells i.e. activated macrophages, T-lymphocyte, dendritic cells, which secrete several cytokines and chemokines that further activate endothelial pro-inflammatory phenotype, and contribute to structural instability of the plaque through release of proteolytic enzymes (matrix metalloproteases) further leading to modification of sub-endothelial matrix components [59, 60]. Another aspect of atherogenesis is also governed by lipoproteins, mainly through low-density lipoproteins (LDL). This initial arterial remodeling through accumulation of lipids is known as fatty streak formation. The first changes in the arterial wall occur at the branch points of arteries, where adaptive intimal thickening occurs in response to normal hemodynamic stresses [61].

During the early stage of atherogenesis, LDL particles leave the blood and enter the arterial intima, composed of endothelial cells. Accumulation of fat droplets i.e LDL may also occur in the cytoplasm of vascular smooth muscle cells (VSMCs) [58]. LDL particles are then modified by enzymes and are oxidized into highly reactive pro-inflammatory molecule (oxidized LDL), that are recognized by pattern recognition receptors i.e. toll like receptors (TLRs) present in endothelial cells as well as pro-inflammatory macrophages [62]. Oxidized LDL incite the reaction of the innate inflammatory system within the intima and contributes in vascular remodeling. Inflammation begins when activated endothelial cells (through TLRs) express cell adhesion molecules and VSMCs secrete chemokines and chemoattractant, which together draw monocytes, lymphocytes, mast cells, and neutrophils into the arterial wall [63]. Once monocytes enter into the arterial wall through the intima, they become activated into macrophages. These macrophages take up lipids as multiple small inclusions and become transferred into foam cells [56]. The degree of lipid accumulation is critical for early-stage diagnosis of atherosclerosis. Atherosclerosis is believed to start when the lipid accumulation appears as confluent extracellular lipid pools and extracellular lipid cores with decreased cellularity [64]. ED is also responsible for VSMC proliferation and differentiation to myofibroblast. In an intact vessel, VSMCs never come in contact with plasma proteins and therefore devoid of growth factor present in plasma. In physiological condition VSMCs are always maintained in quiescent states. But through early inflammation and endothelial cell activation, VSMCs receive signal from dying cells or growth factors that modify VSMCs to myofibroblast (more proliferative counterpart). Altered VSMCs (myofibroblast) also secrete proteoglycans, collagen and elastic fibers into the sub-endothelial matrix [65]. This transformation of VSMCs further worsens the histological structure and leads to formation of thin-cap fibroatheroma formation [66]. Fibroatheroma can be of two different types depending on the content and stability of the plaque. Stability of the plaque also determines the fate of the fibroatheroma. Unstable fibroatheroma lead to thrombotic plaque formation whereas, stable fibroatheroma accumulate calcium, become stiff and eventually lead to occlusion [66, 67]. Possibility of ruptured plaques may lead to a catastrophic transition into atherosclerotic lesion plaque rupture, with luminal release of the highly thrombogenic contents [68, 69]. Else, some significant clinical sequelae can be seen from superficial intimal erosions, without any indication of plaque rupture [70]. Therefore, an acute transition appears leading to endothelial cell apoptosis, with localized endothelial denudation and thrombus formation leading to obstruction in regional blood flow in later stage [71, 72]. Whereas, the stable lesions, having thick fibrous cap and less lipid as well as inflammatory cell content, can gradually invade the lumen of

the vessel causing ischemic symptoms [73,74]. Ruptures of many thin fibrous caps remained clinically silent and are subsequently healed by forming fibrous tissue matrices of cells, collagen fibers, and extracellular space but may rupture again with thrombus formation of the necrotic core, triggering an atherothrombotic occlusion. These cyclic occurrences of rupture, thrombosis, and healing as many as four times at a single site in the arterial wall, results in multiple layers of healed tissue. In these conditions calcium deposition in the wall of the vessels forms micro-aggregates of thrombus, which in turn forms large nodules at later stage. Later on these plaques ruptures and exposes the nodules and becomes sites for thrombus formation [69]. Therefore, the increasing number of plaques itself might be adequate to form significant stenosis which may cause acute ischemic event [75].

Another area of great importance is the role of ROS producing enzymes in atherosclerosis. A number of enzyme systems can produce ROS in the vascular wall. Among them four have gained major attentions in recent years, namely, NADPH oxidase, xanthine oxidase, enzymes of the mitochondrial respiratory chain and especially a dysfunctional endothelial NO synthase (eNOS) [76]. Here in this section we will focus mainly to NADPH oxidase and eNOS. NADPH oxidases are major sources of ROS in the vasculature, producing superoxide from molecular oxygen using NADPH as the electron donor. The oxidases are multi-subunit enzyme complexes incorporating one of several homologs of the membrane-bound Nox catalytic subunits [77, 78]. In the vascular wall, Nox1 and Nox4 are expressed in vascular smooth muscle cells whereas Nox2 and Nox4 are predominantly found in endothelial cells [79-82]. It is reported that, although an activation of endothelial Nox2 in other cell types makes an indispensable contribution to progression of atherosclerosis, under physiological conditions, eNOS produces NO, which represents a key element in the vasoprotective function of the endothelium [83-86]. Under pathological conditions associated with oxidative stress, however, eNOS may become dysfunctional [76]. eNOS- derived NO can diffuse from endothelial cells into the underlying smooth muscle cells and induce vasodilation by stimulating NO-sensitive guanylyl cyclase. Endothelial NO can also diffuse into the blood and inhibit platelet aggregation and adhesion [76]. eNOS- derived NO also possesses multiple anti-atherosclerotic properties, including prevention of leucocyte adhesion, migration and vascular smooth muscle cell proliferation through the inhibition of oxidation of LDL [84-86]. Consistent with the anti-atherosclerotic role of eNOS-derived NO, genetic depletion of eNOS leads to accelerated and enhanced atherosclerotic in ApoE-KO mice [87,88]. Uncoupling of eNOS is a crucial mechanism contributing significantly to atherogenesis. It not only reduces NO production, but also potentiates the pre-existing oxidative stress [89]. It has been reported that, the damaging effects of superoxide is produced by uncoupled eNOS-derived NO in atherosclerosis [90].

Based on its multi-regulatory roles throughout this complex series of events, it is evident that ED constitutes a well-coordinated multicellular pathogenic sequence that lead to atherosclerosis.

3.3 Endothelial dysfunction in hypertension

Millions of people get affected by hypertension leading to worldwide cardiovascular morbidity and mortality and considered as a crucial factor for cardiovascular disease. Hypertension appears to have a complex association with ED, a phenotypical alteration of the vascular endothelium that precedes the development of adverse cardiovascular events. Endothelial cells along with the vascular smooth muscle cells of resistance vessels (arteries and arterioles) regulate hypertension as they continuously constrict and dilate according to the rhythm of cardiac cycle. In response to the blood flow (perfusion), the quiescent healthy endothelium continuously releases potent vasodilators, which have the potential to lower vascular resistance, thereby lowering the blood pressure [91, 92]. In normal condition, basal perfusion is determined by cardiac output, systemic and local resistance. Endothelial cell always maintains a vasodilatory rather than a vasoconstrictive phenotype in an intact healthy vessel. ED is a condition comprising not only of attenuated endothelium-dependent vasodilatation but also an augmented inflammation-induced endothelial activation that leads to vasoconstriction. ED contributes significantly in the development of hypertension, whereas hypertension also leads to endothelial dysfunction. In healthy endothelial tissues, a balance between endothelium-derived relaxing factors (EDRFs) and endothelium-derived contracting factors (EDCFs) is maintained. Endothelium secretes a number of vasodilating factors including NO, PGI_2, ET and adenosine. Generation of NO can activate the guanylate cyclase (cGMP) which causes vasodilation through relaxation of vascular smooth muscle cells [93]. Another vasodilatory factor is PGI_2, secreted by the endothelium which inhibits platelet aggregation and proliferation of vascular smooth muscle cells [94]. Several vascular contracting factors including: angiotensin-II (Ang-II), endothelin-I (ET-I), dinucleotide uridine adenosine tetraphosphate (UP4A), COX derived TXA_2 are also secreted by endothelial cells [95]. Endothelins (ETs) are potent vasoconstrictor molecules having a key role in vascular homeostasis. Although there are three types of ET, vascular ECs mainly produce only ET-1 which has a prominent role in vasoconstriction. Active ET molecule is generated by the actions of an ET converting enzyme (ECE) found on the endothelial cell membranes.

There are two types of ET-1 receptors: ET-A and ET-B. Under normal conditions the ET-A receptor is dominant in blood vessels [96]. ET-1 exerts vasoconstriction through activation of dihydropyridine channel (DHP channel) or long lasting Ca^{++} channels (L-type) by binding to ET-A receptors on vascular smooth muscle cells. Smooth muscle cells express both ET-A and ET-B receptors. However, endothelial cells express only ET-B receptors which negatively regulate NO release. Another vasorelaxing factor adenosine, released from endothelial cells acts through purinergic receptor, helps maintain vascular perfusion [97]. Other than these factors several cytokines and chemokines also play important role in hypertension. Inflammatory cytokine induces generation of reactive oxygen species (ROS), one of the critical factors that link ED and hypertension [98]. It is well established that Ang-II induces NADPH oxidases (NOX). Recent finding indicates additional source of ROS generation. In small subcutaneous arteries, a significant portion of Ang-II induced ROS is produced by COX-2. In mouse aorta, the mitochondrial monoamine oxidase is another mediator of ROS generation and Ang-II or inflammation induced ED [99]. Therefore, mitochondrial monoamine oxidase-A and B are also induced due to ED in the vessels and generate a significant amount of H_2O_2, sufficient to quench endothelial NO. Apart from above, another mitochondrial ROS generating system i.e. p66Shc, also contribute to hypertension-induced ROS production. ROS production is also regulated by several intracellular signaling which further attenuate endothelial dysfunction and hypertension.

3.4 Endothelial dysfunction in Heart Failure

Heart failure (HF) is the most common cause of hospitalization in cardiovascular disease with a high mortality rate. Despite novel treatment options for patients suffering from HF, morbidity and mortality rates are still high. With the advancement of medical management, survival of acute coronary disease and cardiac ischemia has been improved. However, in myocardial infarction, prognosis is still poor, as HF with preserved ejection fraction (HFpEF) has a 65% mortality rate at 5 years. While the heart was an initial focus as the failing "pumping" organ in research and treatment, neurohumoral activation and subsequently the role of a failing endothelium was recognized and investigated in the recent years. Traditionally, HF was recognized as impairment of cardiac muscle activity, known as cardiomyopathy. It has been reported that, altered perfusion in cardiac arteries, due to atherogenesis also contributes to cardiac ischemia and cardiomyopathy.

Reduction in myocardial perfusion due to impaired ventricular function are at least in part, a consequence of reduced endothelium dependent vasodilator capacity of coronary arteries. The prominent regulatory activity of the vascular endothelium in HF was discovered about two decades ago, and its assessment in different cardiovascular disorders, including HF, has been the focus of intense research [100]. On the other hand, declined peripheral vasodilation causes higher systemic and pulmonary vascular resistance, and together with stiffness of conductance arteries, leads to increased afterload. Elevated afterload further increases cardiac workload and therefore worsens the myocardial function. Altered endothelial metabolism further contributes in increasing cardiac afterload [13]. Indeed, various aspects of endothelial function are affected in heart failure, including vasomotor, hemostatic, antioxidant, and anti-inflammatory activities [85, 86]. Differences also exist in the pattern of ED depending on etiology, severity, and stability of HF in individual patients. ED also plays a central role in HF. Heart failure is also characterized by an altered redox state with overproduction of ROS. The increasing evidence suggest that the abnormal cardiac and vascular phenotypes characterizing the failing heart are caused in large part by imbalances between NO bioavailability and oxidative stress [87]. During initial stages of HF, inflammatory mediators from the myocardium, and altered local shear forces modulate gene expression, leukocyte infiltration, increased cytokine production, increased ROS generation and diminished NO bioavailability. Many diverse and often contradictory effects of NO or NO donors on myocardial function have been reported which, until relatively recently, have been difficult to make sense of. However, there is now emerging consensus that NO generally acts to fine tune and optimize cardiac pump function. Studies have shown that, suboptimal doses of NO exert small positive inotropic effects, which may serve to enhance basal cardiac function [101-104]. Augmented data suggests that NO derived both from eNOS from sarcolemmal caveolae and nNOS in the sarcoplasmic reticulum (SR) of the cardiac myocyte may modulate events like calcium influx through sarcolemmal L type channels and the release and re-uptake of calcium by SR [105,106]. At physiological doses, NO myocyte relaxation and diastolic function are observed [107-109]. The effects have been confirmed in normal human subjects studied invasively in the catheterization laboratory with intracoronary infusions of the NO donor, sodium nitroprusside, an agonist that releases NO from endothelial cells [110,111]. However, there is no direct evidence for deleterious role of NO in human heart failure. The initial speculative suggestions that excessive NO production by iNOS has acute negative ionotropic effects are almost certainly too simplistic. Treatment with NOS inhibitors had no effect on basal function either in myocardial strip preparations or isolated myocytes from end stage failing hearts [112,113]. The functional consequences of altered NOS expression and NO bioactivity in the failing human heart are only just beginning to be explored.

Clinical studies showed significant up-regulation of plasma markers of endothelial activation (e.g. E-selectin) and

endothelial damage (e.g. vWF) in HF [44,85]. However, it is difficult to determine if ED is the cause or effect of the HF. Therefore, HF is regarded as thrombotic complication. As mentioned earlier, during atherogenesis, decreased lumen of cardiac arteries leads to reduced perfusion to the heart muscle. This phenomenon is coupled with increased sheer stress and impaired blood flow. This reduced perfusion either led to ischemia-reperfusion injury or coronary artery thrombosis [85]. Studies showed that ED is one of the principle mediators of ischemia-reperfusion injury and thrombosis. This explains the increased ED markers in coronary artery disease, HF and thrombosis.

3.5 Endothelial dysfunction in stroke

The global burden of neurological diseases including cerebro-vascular stroke has significantly increased, and development of new treatment modalities for cerebro-vascular diseases is an urgent need. Cerebrovascular stroke can be broadly subdivided into acute ischemic stroke and hemorrhagic stroke [114]. Acute ischemic stroke is among the leading causes of death and long-term disability. Cerebro-vascular stroke in small vessel has functional (lacunar stroke, cognitive impairment, gait and movement disorders) and structural (small subcortical infarct, lacunar infarct, lacunas, white matter lesions, micro bleeds) consequences. In the past few decades the immense development of neuro-radiological methods enabled better imaging of cerebral blood vessels. From the clinical point of view, it is very important to identify the location of vascular lesion. However, the treatment strategies do not depend on the location of vascular impairment. It is now well recognized that ED represents a systemic syndrome involving multiple vascular beds, including the cerebral vasculature [115]. Endothelial function is not uniform throughout the arterial system. It differs between organs and potentially also between different vascular beds within the same organ. Cerebral endothelium is probably one of the most specific types since it is the crucial element of the well-known blood-brain barrier (BBB). The BBB is a term used to describe the unique properties of the microvasculature of the central nervous system that protects the brain from harmful agents and pathogens [116]. CNS vessels are continuous non-fenestrated vessels, but also contain a series of additional properties that allow them to tightly regulate the movement of molecules, ions, and cells between the blood and the CNS. This heavily restricting barrier capacity allows BBB to tightly regulate CNS homeostasis, which is critical to allow for proper neuronal function, as well as protect the CNS from toxins, pathogens, inflammation, injury, and disease. The cell-to-cell interaction with astrocytes, microglia and neurons mainly play an important role for maintenance of BBB controlled by endothelial cells and pericytes [117].

However, the integrity of BBB is primarily disrupted due to decrease in endothelial cell –cell junction proteins and the detachment of pericytes from the endothelial membrane in homorganic condition [118]. Cerebral autoregulation maintains constant blood flow (CBF) through the brain in spite of changing mean arterial pressure. Autoregulation of cerebral blood flow consists of mechano-and chemo-regulation. The serum level of carbon dioxide (CO_2) is directly controlled by the chemo-regulation independent of changes in mean arterial pressure [119,120]. However, mechano-regulation depends on transmural pressure gradient and endothelial vasodilatation.

As mentioned in previous section, strokes could be divided in two types: ischemic and hemorrhagic. In both, nitric oxide (NO) plays an important role where inducible NOS (iNOS) and neuronal NOS (nNOS) plays the role of neurotoxic agent and endothelial NOS (eNOS) plays the neuroprotective role in acute ischemic stroke [121]. NO thus produced by iNOS and nNOS exerts its neurotoxic effects by producing nitrites and releasing free radicals which eventually damage the mitochondria and genetic materials [122-125]. On the other hand, NO produced by eNOS exerts the neuroprotective effects through the regulation of vascular bed and peripheral nerve tissue [126,127]. It has been reported that, the concentration and distribution of NO in brain tissue is altered significantly after cerebral ischemia [121]. The neuroprotective role of NO in middle cerebral artery occlusion (MCAO) model shows that NO mediates the neurovascular protection through the inhibition of serine racemases [128]. The integrity of BBB could also be achieved by regulating NO/caveolin1/MMP pathway, while reduction in mRNA and protein level of iNOS and nNOS would also provide neuroprotection [129,130]. The neurotoxic effects of NO in MCAO exhibits its role by increasing infarct size and cerebral vascular injury [131] and activation of iNOS induced cell apoptosis in a rat model of cerebral ischemia-reperfusion injury [132,133]. NO and peroxides cause microvascular dysfunction and poor prognosis [134]. Hence, NO plays a dual role in hemorrhagic and acute ischemic stroke.

4. Role of Inflammasomes in endothelial dysfunction

Ample research has showed reactive species-mediated activation of inflammasomes (NLRP3), in sterile inflammatory conditions. Inflammasomes are multi-protein platforms, with a molecular mass of at least 700 kD [135] controlling the activation of caspase-1 and the cleavage of pro-IL-1β, enabling the release of the active mature 17 kD cytokine [136,137]. Caspases are responsible for crucial aspects of inflammation and cell death and can be broadly divided into two classes based on their substrate specificity pro-apoptotic/inflammatory (Figure 3) [138]. Inflammasome complexes assemble upon activation by an appropriate stimulus, leading to the multimerization of the adaptor molecule Adaptor Protein apoptosis-associated speck-like protein containing CARD (ASC) (Figure III). In

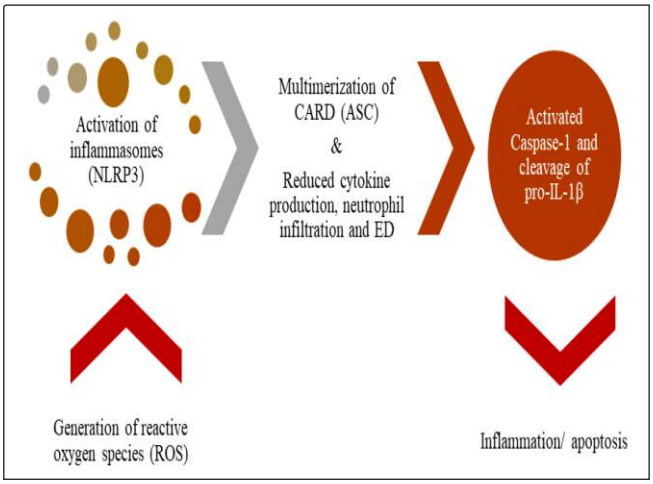

Figure 3: Role of reactive oxygen species on the activation of inflammasome, activation caspase-I, leading to leading to inflammation/apoptosis.

ischemia/reperfusion (I/R), release of ATP and/or mitochondrial DNA following mitochondrial permeability transition pore opening and/or rupture of mitochondrial membranes serve as strong danger signals that initiate sterile inflammation [139,140]. Reactive species production by mitochondria also induces detachment of thioredoxin from the potent NLRP3 activator TXNIP in microvascular endothelial cells [141,142]. Postischemic RS production, NLRP3 activation, TXNIP/NLRP3 signaling reduce postischemic cytokine production, neutrophil infiltration, dysfunctional endothelial barrier and cell death [143,144]. In postischemic tissues, NLRP3 forms an inflammasome composed of apoptosis-associated Spec-like protein containing a caspase activation and recruitment (ASC), which recruits and activates caspase-1(Figure 3) [145]. \

Mounting evidence indicates that inflammation and immune responses play an important role in the overall pathogenesis of ischemic stroke by activating various cascades of damage. Several reports show that ischemic stroke increases the expression and activation of the NLRP3 inflammasome in the neurons and glial cells [146-148]. Several mechanisms trigger NLRP3 inflammasome during cerebral ischemia, acidosis, increased ROS formation, cathepsin release, oxidized mitochondrial DNA, intracellular Ca^{2+} accumulation, cell swelling, and protein kinase R (PKR) activation [149-155]. Recent studies have indicated that NO enhances the removal of the dysfunctional mitochondria and prevents assembly of the inflammasome, which leads to downregulation of the NLRP3 inflammasome and NO in myeloid cells of the mice and humans; inhibits the activation of the NLRP3 inflammasome; and consequently prevents ASC pyroptosome formation, caspase-1 activation and IL-1β secretion (Figure 3) [156-158]. In conclusion, physiological functions of NO encompass reduction of inflammatory responses and hence plays an important role in neuroprotection after stroke.

5. Endothelial dysfunction – role in preeclampsia

Preeclampsia, which is a hypertensive pregnancy disorder affects around 1-5% pregnant women and is characterized by hypertension, proteinuria, maternal organ dysfunction and uteroplacental dysfunction (Figure 4) [159,160]. Preeclampsia is a major cause of maternal and fetal morbidity and mortality, affects the health of the mother in the years directly following preeclampsia [161]. Women with a history of preeclampsia have a 2.2 times higher risk of developing ischemic heart disease [161]. In preeclampsia, ED is characterized by oxidative stress, angiogenic and vasodilatory imbalance which could be paired with endoplasmic reticulum stress and endothelial cell apoptosis [162] and reactive oxygen species raises the risk for CVD for example hypertension, hypercholesterolemia and diabetes [163,164]. In case of endothelial dysfunction, reactive oxygen species stimulate inflammation via the NF-kB pathway and activation of the macrophages in the plaque [164]. Reactive oxygen species induces the activation of proteases and matrix metalloproteinases (MMP), degrades basement membrane which in turn get involved in plaque

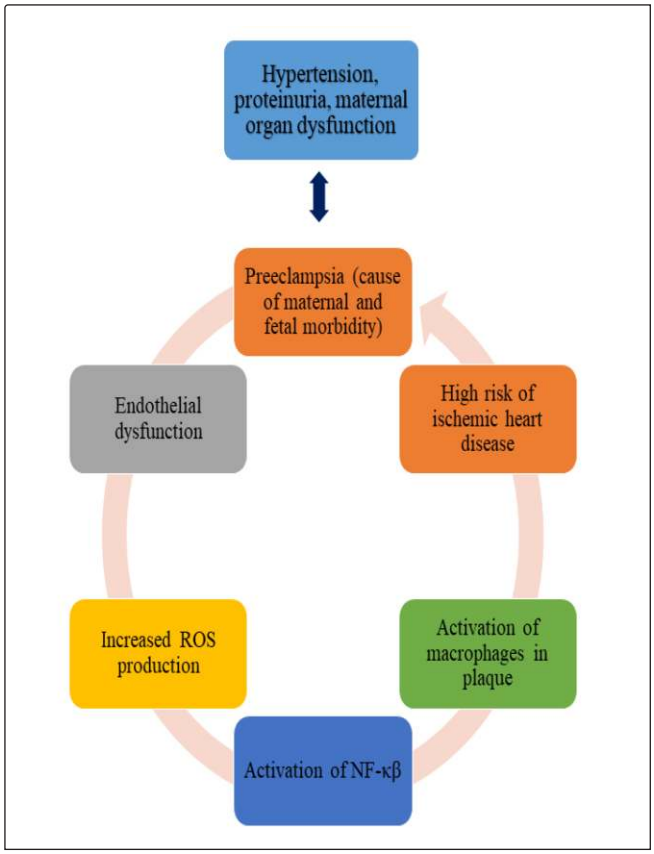

Figure 4: Role of endothelial dysfunction and hypertension in maternal and fetal morbidity during preeclampsia.

erosion [165,166]. Nitric oxide concentration has been shown to have variable results ranging from decreased or increased or even unchanged levels in terms of NO metabolites in preeclampsia, [167-172]. Although the whole body NO may not change in PE, a reduction in endothelial NO signaling, vascular relaxation in PE and NO bioavailability could be expected [173]. Attempts to assess eNOS activity in PE led to the conclusion that it is unknown whether eNOS deficiency plays a casual role there. In the murine model, chronic NOS inhibition reversed systemic vasodilation and glomerular hyperfiltration in pregnancy, which suggested its role for endothelial damage and decreased NO in the pathogenesis of preeclampsia (Figure 4) [174]. Data from PE women is quite limited and without consensus on eNOS expression, as higher, lower and unchanged levels of mRNA or enzyme have been reported [175,176].

6. Conclusion

In this review we have tried to focus on the role of ED in CVDs and cardiovascular morbidity where sterile inflammatory responses pose a credible threat, owing to considerable attention.

Patients with chronic inflammatory and / or sterile inflammatory diseases are at high risk for cardiovascular morbidity and mortality. In many inflammatory diseases, this heightened risk of CVDs are reflected in early ED, even in the absence of any other detectable diseases. Several others mechanisms i.e. auto-antibodies, oxidative stress and interactions with traditional risk factors like dyslipidemia and insulin resistance might also be involved. Current literature search provides an insight into the cross-talks between oxidative stress, ED and inflammasomes. Therefore, further research is required to delineate the importance of these processes. The current approaches to diminish cardiovascular morbidity and mortality are focused on controlling traditional modifiable cardiovascular risk factors and reduction of disease risk. Therefore, the precise mechanisms leading to development of CVDs due to inflammation/or sterile inflammation need to be explored. These studies might help to identify unique therapeutic targets to combat these diseases.

The endothelium therefore represents an integrator of vascular risk and the study of its dysfunction may help elucidate mechanisms driving accelerated CVDs in future which could help to develop therapeutic targets for control of CVDs.

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EXPLORING INDIAN TRADITIONAL MEDICINAL SOURCES AS PROPHYLACTIC STRATEGY AGAINST COVID-19

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Abstract
Coronaviruses have engendered many instances of widespread epidemics across the globe in the past couple of decades. The severity of diseases caused by coronaviruses is complicated manifold due to their characteristic hypermutations which pose the biggest obstacle in delineating any specific vaccine or drug against these viral strains. The world at present is battling the ongoing novel coronavirus disease 2019 (Covid-19), caused by SARS-CoV-2 virus. Covid-19 has claimed about 4, 00, 000 lives as of 15 June 2019 since early reports of the outbreak in December 2019. The unavailability of a particular treatment strategy has resulted in a rising trend of active Covid-19 cases and casualties. Such a scenario has motivated the healthcare professionals and general public to adopt a preemptive approach by accentuating overall immunity as well as maintaining personal hygiene and social distancing. The past few months have witnessed increased awareness and acceptance of the age old convention of Ayurveda and traditional medicines for augmentation of immunity and/or subsiding the symptoms against SARS-CoV-2 infection. In line of this, the present review focuses on the importance of various traditional medicinal sources as preventive leads against the pandemic. The sources discussed here include plants namely, Curcuma longa and Hippophae rhamnoides, and medicinal mushrooms i.e., Cordyceps sinensis and Ganoderma lucidum. Evidence-based antiviral properties of extracts and fractions prepared from these traditional medicines, along with their prospective use for immunity enhancement and supportive healthcare in novel coronavirus disease have also been elucidated. Also, the interplay between Covid-19 and hypoxia-induced ailments of lungs have been elaborated for exploring the usage of aforesaid traditional medicinal sources, as prophylactics against the current pandemic.

Keywords
Covid-19; preventive medicine; prophylactic; SARS-CoV-2; traditional medicinal system.

1. Introduction

The ongoing 'Covid-19' pandemic also known as 'novel coronavirus disease 2019' is a form of respiratory ailment, accompanied by symptoms typical to flu, for instance cough, fever, shortness of breath, and as recently added, a loss in perception of smell and taste [1]. In extreme severities, acute respiratory stress syndrome, associated multiple organ failure and blood coagulation are also observed [2]. Covid-19 spreads chiefly by respiratory droplets released by disease-ridden individuals while coughing and sneezing. The pandemic has already claimed 4, 40, 000 lives across the world, with 8 million active cases as of 15 June, 2020 [3].Similar to all the main epidemic and pandemic outbreaks recorded so far in history, namely, West Nile fever, severe acute respiratory syndrome (SARS), avian influenza/bird flu, Middle East respiratory syndrome (MERS), Ebola hemorrhagic fever and Zika fever, the Covid-19 disease is also caused by a virus, i.e., severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). The simple structure of viruses comprising a protein coat and genetic material (DNA or RNA), permits easy assembly and rapid multiplication of viruses in the host cells, thus accounting for their high contagion rates. Inside the host cells, virions cause several cytopathological effects like cell lysis, apoptosis and instigate host cell machinery to produce multiple copies of virus particles [4].

The viruses having RNA as genetic material are usually much more virulent than their DNA equivalents, due tothe absence of proofreading function in viral RNA polymerases. An

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example of viral family with RNA genome is 'coronaviruses' which cause infectious diseases like common cold, hepatitis A, hepatitis C, measles, polio and the current Covid-19 pandemic. RNA viruses demonstrateunrelenting hyper mutations and reassortment, hence, the task of devising vaccines or any antiviral drug becomes a grim imposition. This necessitates the role of preventive strategies like the use of traditional medicinal sources, as deliberated in the upcoming paragraphs. In this perspective, the present review illustrates the pathophysiology of ailments inflicted by coronaviruses, and the importance of traditional medicinal sources in treatment or prevention of Covid-19, with special focus on Indian turmeric, sea buckthorn, keerajhari and reishiasputative natural alternatives against viral incidences.

2. Coronaviruses

Coronaviruses are categorized under RNA viruses having a lipid bilayer to which the envelope (E), spike (S) and membrane (M) proteins are attached (Figure 1). Entry into host cells is by attachment via spike protein. Once the virus has gained entry into target host cytoplasm, its positive-sense single-stranded RNA genome replicates arbitrated by RNA dependent RNA polymerase [5]. Coronaviruses trigger respiratory tract infections in human beings, ranging from common cold to serious illnesses such as SARS, MERS and Covid-19. The causal strain of Covid-19 -'SARS-CoV-2' is the seventh known coronavirus to wreak havoc in human population, the earlier ones being 229E, NL63, OC43, HKU1, SARS-CoV and MERS-CoV [6]. Particularly, SARS-CoV-2 is a beta coronavirus categorized under 'coronaviridae 'family. Its structure is depicted in Figure 1 [7].

3. Pathophysiology of novel coronavirus disease

SARS-CoV-2 virus primarily infects the respiratory tract in human host. Its spike protein renders accessibility to the epithelial cells in lung alveoli. The virus gets attached with the angiotensin-converting enzyme 2 (ACE2). ACE2 is plentifully present in alveolar cells, hence lungs become the predominant target in SARS-CoV-2 infection. Other organs where ACE2 is expressed are gut, heart, kidney and brain thus, Covid-19 leads to severe gastric, duodenal and rectal infections, acute myocardial risks, kidney injuries and also, complications in central nervous system [8-10]. Etiology of Covid-19 proves that all the vital organs, in more or less severities depending on the patient's immunity, are affected. Hence, individuals with already prevailing comorbidities like diabetes, hypertension, heart diseases, kidney diseases are more susceptible to Covid-19.

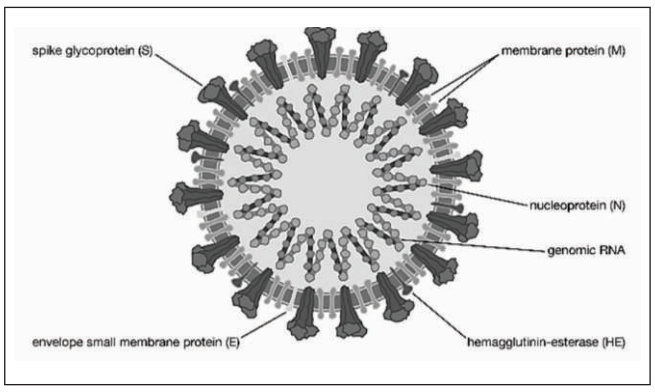

Figure 1: Structure of SARS-CoV-2

4. Ongoing medical approaches for management of Covid-19

The high contagion rate and rapid mutation tendency of SARS-CoV-2are the main roadblocks in developing a substantial vaccine or drug against the novel coronavirus disease. These factors have steered the status of Covid-19 from epidemic in December 2019 to pandemic in March, 2020.Several research groups the world over are persistently striving to delineate an anti-SARS CoV-2 vaccine; however, it is a long way before the final phase trials are completed [11]. The Union Health Ministry of the Government of India has stated that, as of May 25, 2020, four prospective vaccine candidates are being considered for clinical trial phases.

In the unavailability of a steadfast vaccine candidate, certain antiviral drugs are being administered to Covid-19 patients, to restrict the viral flare. The drugs currently in the task arelopinavir, chloroquine phosphate, arbidol, ribavirin, favipiravir, etc. The named antiviral medications follow varied mechanisms of action, for example, acting as structural analogues, reducing replication rate, deterring RNA-dependent RNA polymerase activity, etc. However, all these drugs exhibit many side effects like nausea, diarrhea, and complications in patients with already existent chronic illnesses. So far, hydroxychloroquine in conjunction with azithromycin has been conceded as the best medication combination against the SARS-CoV-2 infection [12,13]. Although hydroxychloroquine decreases IL-6 production implicated in cytokine storm in Covid-19 patients, however, this this drug is effective only during the early stages of viral entry and replication and not in the advanced stages of the disease.[14].

Amidst this, the World Health Organization (WHO) and the Indian Council for Medical Research (ICMR), since the beginning of the pandemic phase, have recommended to count on 'traditional' or 'complementary' medicinal systems and preventive approaches against contracting Covid-19 or alleviating the symptoms [15]. In this regard, the present review intends to discuss the efficacy of some traditional medicinal sources already recognized in literature as antiviral therapeutics and suggest their prospective prophylactic abilities against Covid-19. Traditional medicines have been amply proven to be resourceful preventive and alternative modes of healthcare. Therefore, this article highlights the role of some natural herbs e.g.: Indian turmeric and sea buckthorn, and medicinal mushroomsi.e., keerajhari and reishi against Covid-19 disease and its associated symptoms.

4.1 Traditional medicinal system

Traditional medicinal system is a popular strategy of alternative healthcare that has been well represented in traditional Chinese medicine (TCM), traditional Tibetan medicine and Indian systems of Ayurveda, Siddha and Unani since past many centuries. Descriptive literature is plentifully available about the use of therapeutic principles sourced from plant, animal and mineral sources in curing several illnesses or maintaining well-being. The traditional medicinal system of India is home to diverse medicinal plants and mushrooms that possess many pharmacological properties [16, 17]. The Indian traditional system of healthcare, i.e., Ayurveda ('ayus' meaning 'life' + 'veda' meaning 'knowledge') is adopted as primary healthcare by more than half of the country's population, owing to its ease of accessibility, affordability and holistic purposes. Ayurvedic or naturopathic restorative formulations provide significant respite from numerous illnesses or diseased conditions. The pharmacological effects of these traditional preparations are at par with modern allopathic drugs. In Ayurved literature, Maharishi herbal mixtures have been mentioned to be stronger antioxidants than ascorbic acid and α-tocopherol [18]. The rising popularity of traditional medicine has motivated the establishment of The Ministry of Ayurveda, Yoga & Naturopathy, Unani, Siddha and Homoeopathy (AYUSH) by Government of India where AYUSH is dedicatedly working on research and dissemination of indigenous traditional medicines.

Not only in India, but also in other countries, traditional medicinal system is an established approach for wellness management and improvement of human health. In China, 40% of primary health care consists of traditional medicines where natural sources are a central component of remedial measures. Similar trends in Asian and South American countries have also been cited [19]. Traditional medicine has gathered impetus in developed countries like Australia, Belgium, Canada, France and the United States of America [20]. The benefit of not having any side effects is the major contributing factor to the popularity of traditional medicines worldwide.

4.2 Traditional medicines as a workforce to augment immunity and restrict viral flare

Both TCM and Ayurveda describe the use of several herbs and other natural sources for enhancing individual immunity.E.g.: Panax ginseng (ginseng) herb has recuperative potential in immune-compromised cancer patients [21]. Ginseng fractions also aid in helper T cell-mediated immune response against viral infections caused by parvoviruses, rhino viruses, influenza viruses, HIV, etc. [22]. In addition to above, other herbal preparations from Radix spp., Fructusforsythiae (Lanhua), Andrographispaniculata (chirayata), etc. are known to exert inhibitory effects on influenza and hepatitis viruses [23]. Traditional drugs have also displayed efficacy against specific coronavirus strains as explained below.

4.2.1. Traditional medicines: targeting antiviral action against coronaviruses

Traditional medicines are known to be appreciable preventive agents against infections caused by coronaviruses. In the 2002-2004 SARS epidemic in China, general public was suggested to intake traditional medicines as a prophylactic. Here, 'glycyrrhizin' collected from liquorice root curbed the replication of SARS viruses [24]. Chinese herbs in adjunction with modern medication helped in recovery of SARS patients by bringing about antipyretic action, clearance of fluid accumulation in lungs and reduced dependency on steroids. Even for the ongoing Covid-19, TCM herbal remedies have been endorsed by Chinese healthcare experts to contain and eradicate theSARS-CoV-2 infliction. A TCM formulation named 'qingfeipaidu decoction (QPD)' made up of 21 herbs has been reported to be seemingly efficient in treating Covid-19 by repressing viral replication and improving overall immune response. The recipe of QPD includes medicinal herbs such asBupleuri radix, Cinnamomiramulus Ephedraeherb, Glycyrrhizaeradix, Scutellariae radix and Zingiber spp. [25].

In a manner similar to TCM, traditional Indian medicineshave been increasingly reported to be highly reliable in enhancing individual immunity and downgrading pathogenesis of SARS-CoV-2 in Covid-19 patients. An Ayurvedic formulation comprising Elettariacardamomum(cardamom), Piper nigrum(black pepper), Syzygiumaromaticum(clove), Withaniasomnifera(ashwagandha) and Zingiberofficinale(ginger) has been seen to impart immune-stimulant action in HIV positive patients by elevating levels of IFN-γ, interleukin-2 (IL-2) and tumor necrosis factor-alpha (TNF-α) [26]. Polyphenols and tannins present in Triphala, containing equal proportions ofEmblica officinalis (amla), Terminalia belerica (baheda) and Terminalia chebula(haritaki) are known to have antiviral properties and increase immunity by augmenting neutrophils action [27]. Previously,virucidal effects of Eugenia jambolana(jamun) extracts were observed on H5N1 avian influenza viral strain [28].

4.2.2. Traditional medicines as therapeutic strategy against Covid-19

Several research groups in India have insisted upon increasing an individual's overall immunity as a beneficial preventive approach against contracting Covid-19. For this, numerous traditional medicinal herbs with well-established recuperative uses have been proposed. One among them is W. somnifera commonly known as 'ashwagandha' has been elucidatedas a remedial means against COVID-19. The underlying action is said to be via 'withanones' which influence helper T cells (Th-1/Th-2) immunity [29]. Keeping in consonance with reported medical uses of ashwangandha, generation of immunity against viral by IFN-γ responses and repression of immune inflammatory parameters such as IL-1, IL-6 and TNF-α have been suggested. Yet other Ayurveda

concoctionswith ingredients like Asparagus racemosus(shatavari), Phylanthusemblica (amalaki) and Tinosporacordifolia (guduchi) have also been shown to improve immunity in individuals infected with SARS-CoV-2 [30].

The Ministry of AYUSH has advocated consumption of Chyavanprash in order to strengthen immunity. Warm decoctions ('kaadha') containingOcimum sanctum (tulsi), Cinnamomumverum (cinnamon), black pepper and dried Z. officinale (sounth) have also been advised. Golden milk, simply milk with an added portion ofCurcuma longa (Indian turmeric/haldi) powder is being administered to Covid-19 patients as a means to ensure faster recovery[31].

Patanjali foundation'sSwasariRas, a methodicalpreparation of Glycyrrhizaglabra (liquorice/mulethi), S. aromaticum (clove), cinnamon, Pistaciachinensis (kakdasingi), sunthi, Piper longum (pepper), mica ash/abhrakbhasma and kapardakbhasma plus extracts of ashwagandha, guduchi, tulsi, already recognized remedial actions against phlegm and cough, has been advised for preventive action against Covid-19. Consumption of all these compounds have been advised in addition to ICMR-prescribed allopathic treatments.

It may be especially noted that all the aforementioned traditional medicinal preparations are extremely rich in secondary metabolite content like alkaloids, flavonoids, glycosides, phenolic, proteins, sterols, sugar alcohols, etc. that in turn fortifies their concept of being utilized as alternative medicines against viral outbreaks [29].

4.2.3. Application of traditional medicinal sources as prophylactic against Covid-19

Current research outcomes mention that SARS-CoV-2 is responsible for mucus build up in lungs that further aggravates to pulmonary edema [32]. Severe hypoxemia followed by loss of perfusion regulation and hypoxic vasoconstriction in affected lungs has been reported [33]. With disease progression, elevation in host inflammatory reaction causes onset of hypoxia that worsens one's pulmonary abilities [34]. In a breakthrough study, substantial similarities in pathophysiology of Covid-19 and high altitude pulmonary edema have been derived. These similarities embody lowered partial pressure of oxygen in tissues, patchy infiltrates in computerized tomography-based imaging of lungs and bilateral diffuse alveolar damage. An increased lung capacity and higher systemic immunity is being sounded as a better way to gain faster recovery from the pandemic [35].

Keeping in accordance with the observations in preceding paragraph, this review article discusses the efficacy of four Indian traditional medicinal sources, widely recognized in traditional medicinal scriptures and contemporary research alike, for their efficacy againsthigh altitude induced hypoxia and other respiratory ailments, as promising preventive agents against Covid-19. These sources include medicinal plants namely, Curcuma longa (Indian turmeric) and Hippophaerhamnoides(sea buckthorn), and medicinal mushrooms i.e., Cordyceps sinensis (keerajhari) and Ganoderma lucidum (reishi). Their highly efficient hypoxia protective action in addition to antiviral and immune-enhancing effects have been iterated below, thus,strengthening their prospects as putative therapeutics against the ongoing pandemic.

4.2.3.1. *Curcuma longa*

C. longa or Indian turmeric is pharmacologically the most important species in Curcuma genus. Literature cites that thirteen curcuminoids isolated from *C. longa* have exhibited effective inhibition of H1N1 swine flu virus by disrupting neuraminidase activity [36]. Similarly, aqueous and ethanol extracts of *C. longa* have been reported to inhibit the replication of avian influenza virus H5N1 by augmenting levels of TNF-α and IFN-β mRNA [37]. It has also been illustrated that curcumin supplementation normalizes the quantities of hypoxia inducible factor-1α (HIF-1α) - an established biochemical marker of hypoxia conditions in tissues along with upregulating hemeoxygenase-1 (HO-1) and nuclear factor (erythroid-derived 2)-like 2 factor (Nrf2), thus, bringing about pulmonary surfactant homeostasis under hypoxic [38]. Even for the novel coronavirus disease, the immune – enhancing and defensive action of *C. longa* has clearly been projected. [39].

4.2.3.2. *Hippophae rhamnoides*

H. rhamnoides, commonly referred to as 'sea buckthorn', is known to display significant antiviral properties against specific viral strains. For example, sea buckthorn water extracts inhibit viral neuraminidase of the influenza virus; obstructs the replication of herpes virus and alsoappreciably controls the rapid replication of HIV [40-41]. *H. rhamnoides* bud dry extract was found to reduce replication of the influenza-A and H1N1 virus in vitro [42]. In yet another study, sea buckthorn leaf extract demonstrated significant antidengue activity [43]. The importance of sea buckthorn in averting hypoxia effects are well represented [44]. H. rhamnoides leaf extract significantly reduces hypoxia-induced transvascular permeability in lungs. This leaf extract imparted significant protection against hypoxia-induced transvascular permeability by stabilizing the levels of reduced glutathione and antioxidant enzymes [45]. In CHR multiple stress setting, aqueous extract of *H. rhamnoides* efficiently decremented malondialdehyde, normalized catalase and restored the amounts of reduced glutathione and superoxide dismutase [46]. Furthermore, H. rhamnoidesis considered to be a considerable repository of vitamin C. It has been recently verified that vitamin C encourages immunity enhancement by augmenting differentiation and proliferation

of B and T lymphocytes cells, and is specifically beneficial in preventing and treating respiratory and vascular infections. The role of vitamin C in providing resistance against SARS-CoV-2 infection has been established by now. Hence, H. rhamnoidescan certainly be regarded as a promising potent preventive herb against the coronavirus infection [47].

4.2.3.3. *Cordyceps sinensis*

'Cordycepin', the major marker compound of C. sinensis/keerajhari has antiviral action against Epstein-Barr virus (EBV), HIV and influenza virus [48]. It also quells the characteristic morphological changes occurring in human lymphocytes after infection with EBV [49-50]. Infection with hepatitis B virus (HBV) is contained by C. sinensis extracts by bringing about an increase in humoral and cellular immunity in murine models [51]. Also, C. sinensis aqueous extracts have shown immunomodulatory action in mice with lymphoma by improving phagocytic activity of peritoneal macrophages[52]. In the present context of Covid-19, the use of C. sinensis as an immunomodulatory agent against the2001 SARS outbreak is worth mentioning. Reports accrediting C. sinensis for its antiviral as well as bronchio-alveolar muscle relaxing effects, thus, aiding in lung decongestion are available. The effect of C. sinensis in regulating T-cell, natural killer (NK) cell and macrophagesfunction as antiviral defense in SARS patients paves its way as an anti-Covid-19 therapeutic as well [53]. Aqueous and alcoholic extracts of C. sinensisare known to mollify hypoxia induced stress in human lung epithelial cell lines (A549) via Nrf2 activation and in murine neuronal hippocampal cell lines (HT22) by reducing inflammatory cytokines [54-55]. Also, in a recent study conducted on murine model, phenolic fractions of Indian variety of C. sinensis have been observed to impart protective action against CHR stress, by moderating dysregulated levels of malondialdehyde, superoxide dismutase and glutathione in lungs, heart and muscle tissues [56]. Combined, the observations described herestrongly put forth the salubrious role of keerajhari in prophylaxis of Covid-19.

4.2.3.4 *Ganoderma lucidum*

G. lucidum, or popularly called 'reishi' mushroom, is widely recognized both in traditional medicine and modern research for its numerous pharmacological properties. Its rich content of alkaloids, flavonoids, phenolics and triterpenes elicit antiviral effects against different viruses. As instance,ganoderic acids obtained from *G. lucidum* fruiting bodies display antiviral activity against HIV and Epstein-Barr virus [57]. Ganodermadiolexhibits inhibitory effect in herpes simplex virus type [58]. Aqueous extracts and methanolic fractions of reishi mushroom have proven virucidal action against human papilloma virus and influenza A virus, respectively [59]. Besides, there are ample scientific reports vouching for the immune enhancing role of *G. lucidum*. Polysaccharides and glycoproteins present in *G. lucidum* upregulate the major histocompatibility complex (MHC) expression in melanoma cell lines; treatment with G. *lucidum* fractions lead to elevated cytokine secretion from immune cells, thus, boosting innate and adaptive immunity [61-62]. *G. lucidum* as a protective agent against hypobaric hypoxia has been authenticated in recent scientific literature. Aqueous and ethanolic extracts from reishi mushroom have been emulated to be cardioprotectant in murine cardiomyoblasts, H9c2 under simulated hypoxic environment. Here, addition of G. lucidum extracts have been shown to stabilize HIF-1α, caspase-3 and caspase-7 [63]. In a cold, hypoxia, restraint (CHR) multiple stress set up, water extract of G. lucidum mycelium has been demonstrated as an adaptogen by regulating oxidative biomarkers like malondialdehyde, reduced glutathione and superoxide dismutase [64]. The promising action of *G. lucidum* as an antiviral, immune enhancing and anti-hypoxia therapeutic bolster its applicability in supportive care of Covid-19.

An overall interplay in the levels of various oxidative stress biomarkers that are modulated after supplementation with different traditional medicinal plants or mushrooms, whilst protecting against hypoxia stress is depicted in Figure 2 [65].

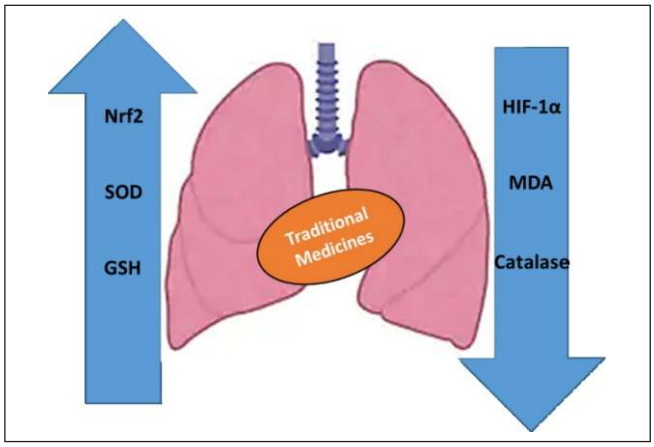

Figure 2: Modulation of oxidative stress markers by traditional plants/ mushrooms under hypoxia, indicative of their prophylactic activity against Covid-19

5. Conclusion

In the ongoing pandemic situation, WHO and ICMR have asserted the importance of improving immunity and use of sanitation measures to prevent contracting Covid-19. It is the need of the hour to explore our country's vast reserve of traditional medicines to bolster immunity. Research conducted by AYUSH, Patanjali foundation and many other scientific groups has to be coordinated with ICMR guidelines. Also, validated interdisciplinary approaches could be undertaken to translate the aforementioned traditional herbal immunity-boosters and immuno-

modulators into 'herbal adjuvants' or 'prophylactic products'.The absence of a guaranteed anti-Covid-19 vaccine/drug candidate, has made it even more essential to recommend the use of traditional medicines as preventive and alternative remedies, along with personal hygiene measures, to emerge victorious amidst these pandemic times.

Conflict of interest
The authors have no conflict of interest to declare.

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SAVE THE ENVIRONMENT (STE) was founded and registered on 19th November 1990. In 1992 with the collaboration of WWF (India), the organization started working to combat arsenic poisoning problem of water in the arsenic prone areas of West Bengal. Since then STE has been involved in various projects related to combat arsenic problem in India.

Our Vision
To protect present and future generations from various environmental hazards.

Our Mission
To create awareness and motivation among rural communities & provide cost effective, energy efficient & environment friendly technologies.

Our Activities
Conducting interactive sessions, workshops/ seminars, awareness programs, field operations through projects, science fairs, posters & quiz competitions.

Please join us and become part of our family
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