"*CHLORELLA MINUTISSIMA* AS A FUNCTIONAL FOOD AND BIO-NANO-FACTORIES FOR SILVER NANOPARTICLE SYNTHESIS, CHARACTERIZATION AND APPLICATIONS"

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Submitted by

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CANDIDATE'S DECLARATION

I hereby certify that the work which is presented in the research work entitled "*Chlorella minutissima* as a functional food and bio-nano-factories for silver nanoparticle synthesis, characterization and applications" in fulfilment of the requirement for the award of Degree of Masters of Sciences in Biotechnology and submitted to the Department of Biotechnology, Delhi Technological University, Delhi is an authentic record of my own work, carried during a period from 2- Jan-2021 to 3-May-2021, under the supervision of Dr. Navneeta Bharadvaja. The matter presented in this thesis has not been submitted by me for the award of any other degree of this or any other University. The work has been published and communicated in various journal under my name with the guide.

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This is to certify that the Project dissertation titled "*Chlorella minutissima* as a functional food and bio-nano-factories for silver nanoparticle synthesis, characterization and applications" which is submitted by Lalit Mohan, 2K20/MSCBIO/11, Department of Biotechnology, Delhi Technological University, Delhi in partial fulfilment of the requirement for the award of the degree of Master of Sciences, is a record for the project work carried out by the student under my supervision. To the best of my knowledge this work has not been submitted in part or full for any Degree or Diploma to this University or elsewhere.

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"*CHLORELLA MINUTISSIMA* AS A FUNCTIONAL FOOD AND BIO-NANO-FACTORIES FOR SILVER NANOPARTICLE SYNTHESIS, CHARACTERIZATION AND APPLICATIONS"

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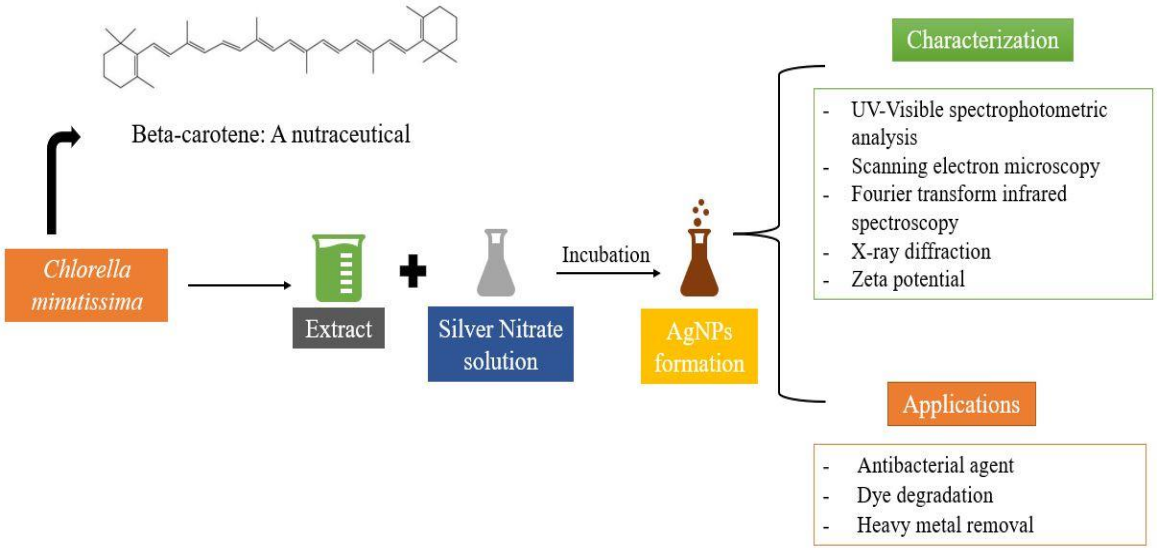
ABSTRACT

Chlorella minutissima acts as a functional food due to the presence of various nutraceuticals such as beta-carotene. Beta-carotene extracted from algal sources are sustainable and economical for use and large-scale production. Beta-carotene helps in prevention of various diseases by acting as an antioxidant. Apart from acting as a functional food, *Chlorella minutissima* also acts as bio-nano-factories for the synthesis of silver nanoparticles. This approach of synthesis of silver nanoparticle is green, ecofriendly, low-priced biotechnological approach that gives advancement over both chemical and physical methods. In the current study, an aqueous extract of *C. minutissima* fresh biomass was used for the green synthesis of Ag-NPs, since *C. minutissima* extract plays a dual part in both reducing and stabilizing Chlorella-silver nanoparticles (C-AgNPs). The UV-Visible absorption spectrum, fourier transforms infrared, X-ray diffraction, zeta-potential and field emission-scanning electron microscope were performed for confirming and characterizing the biosynthesis of C-AgNPs. FE-SEM images depicted the spherical Ag-NPs shape. FT-IR analysis demonstrated the presence of free amino groups in addition to sulfur containing amino acid derivatives acting as stabilizing agents as well as the presence of either sulfur or phosphorus functional groups which possibly attaches silver. XRD pattern depicted that the biogenic Ag-NPs were crystalline in nature with FCC structure. Zeta potential analysis relieved that the C-AgNPs are highly stable with an average size of 78.28 nm. In this study, synthesized Ag-NPs exhibited strong antibacterial activity against *Escherichia coli*, *Bacillus cereus*, *Staphylococcus aureus*, *Salmonella* sp., and *Klebsiella* sp. C-AgNPs have demonstrated a removal efficiency of 76.07% for Chromium and 99.71% for Nickel at pH 3. C-AgNPs have been demonstrated to show 82.34% photocatalytic dye degradation efficiency for methylene blue. Thus, it can be concluded that *Chlorella minutissima* is a potential microalgae agent for synthesis of silver nanoparticles having antibacterial potential, adsorption of heavy metals, and photocatalytic dye degradation. It will be sustainable approach to deal with environmental pollution and combat antimicrobial resistance.

GRAPHICAL ABSTRACT



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LIST OF ABBREVIATIONS

Ag-NPs: Silver nanoparticles

NPs: Nanoparticles

DNA: Deoxyribonucleic acid

RNA: Ribonucleic acid

C. minutissima: *Chlorella minutissima*

BG11: Blue Green 11

BBM: Bold's Basal Medium

$AgNO_3$- Silver nitrate

RO- Reverse osmosis

UV-Vis: UV-visible spectroscopy

FE-SEM: Field emission scanning electron microscopy

FTIR: Fourier transform infrared spectroscopy

XRD: X-ray diffraction

NADPH: Nicotinamide adenine dinucleotide phosphate- hydrogen

SEM- Scanning electron microscopy

AFM: Atomic force microscopy

TEM: Transmission electron microscopy

HR-TEM: High-resolution TEM

DLS: Dynamic light scattering

XPS: X-ray photo-electron spectroscopy

EDS: Energy dispersive spectroscopy

SAED: Selected area electron diffraction

MIC: Minimum inhibitory concentration

CuO: Copper oxide

ROS: Reactive oxygen species

MRSA: Methicillin-resistant *Staphylococcus aureus*

Cr: Chromium

Pb: Lead

As: Arsenic

Hg: Mercury

Cu: Copper

Zn: Zinc

Au-NP: Gold nanoparticles

CdS NPs: Cadmium sulfide nanoparticles

Fe-NPs: Iron nanoparticles

Fe_3O_4-NP: Ferric oxide nanoparticles

KI: Potassium Iodide

HCl: Hydrochloric acid

PPM: Particles per million

Rpm: rotations per minute

OD: Optical density

MB: Methylene Blue

INTRODUCTION

Algae can either be micro- or macro-algae depending upon their size and morphology. Micro and macroalgae find applications in a wide range of field starting from feedstock to medical and pharma industry, nutraceutical and cosmeceutical industry as well as modernly in bio-nanotechnology. These have been identified to be good sources of fuel in the form of biodiesel as well as high value-low yield bio-products like beta-carotene, xanthophylls and so on. Being easy to handle, algae are preferred over other green organisms for such applications. There are so many essential phyco-compounds targeted for recovery from algal species. Some of these are beta-carotene, xanthophyll, astaxanthin and so on. They are medically and industrially essential compounds which can be sustainably produced from algae. Chemical synthesis of such compounds produces compounds with similar efficacy however, its cost and treatment are not user-friendly. *Dunaliella* and *Chlorella* species are the most widely cultivated strains for the algae-based production of beta-carotene.

The science of nanotechnology includes materials in nano-scale range usually between 1-100 nm. Nanotechnology works at nano-scale range and show distinctive properties. Hence, it has been employed for various applications in the fields like bioengineering, dentistry and medical, pharmaceuticals and others [1]. There are various physical, mechanical and chemical methods that are being employed for the synthesis of nanomaterials such as microwave mediated, electrochemical method, photochemical methods, laser evaporation methods, thermolysis, co-precipitation, sol-gel method, and sonochemical method [2]. These methods involve high input of energy, expensive and toxic chemicals such as sodium borohydride or hydrazine as reducing agents for synthesis and consequently produces harmful by-products which poses negative impact on the environment thus, these methods are non-sustainable and cannot be employed for large-scale production basis [3], [4].

To overcome the limitations of physical and chemical methods, “green nano-synthesis” approach has gained momentum in the past decades. Green synthesis or the biological methods of synthesis has gained attention owing to their economical and environment friendly nature and high thermal stability of the produced nanoparticles [3]. Depending on the solvents and reducing agents employed for the synthesis of nanoparticles (NPs), the morphology (size and shape), its physicochemical properties and the use of synthesized NPs vary [5]. For green synthesis, microorganisms ranging from bacteria, to algae and higher organisms such as plants have been involved in the synthesis of metal NPs. Employing these organisms reduces the

overall production cost, leaves no harmful by-products, and makes the overall synthesis environment friendly and sustainable [6].

Plants and their parts including leaves, stems, roots as well as flowers and fruits have been used for green synthesis of NPs. Plant based production is highly rapid, non-pathogenic, ecofriendly, economical and a single step process. The various phytocompounds involved in reduction, capping and stabilization of nanoparticles are amino acids, alkaloids, alcohols, carbohydrates, vitamins, polyphenols, glycosides and flavonoids [6]. For NPs synthesis, plant extract obtained from different plant parts is incubated with precursor metal salt solution for a defined amount of time. The time duration of incubation and the morphology of NPs depends upon nature of plant extract (phytocompounds), metal salt extract and salt concentration, its pH, contact time, exposure to light and intensity of stirring [3], [6]. Pirtarighat et al., reported the synthesis of Ag-NPs of particle size range 19-125 nm using *Salvia spinosa* extract [7]. Leaf extract of *Ocimum sanctum* have been used by Jain and Mehta for the synthesis of spherical Ag-NPs of size about 12- 20 nm [8].

Bacterial cells have several enzymes, redox proteins, and nucleic acids which act as stabilizing, reducing and capping agents, essential for the synthesis of NPs. Bacterial cells have inherent potency to reduce heavy metals due to the presence of various functional groups attached to their cell wall. For the synthesis of NPs, bacterial cultures are first grown as cell suspension and then precursor metal salt solution is added into the culture medium followed by either stirring or incubation at still conditions. The progress in formation of NPs can be monitored by UV-Vis spectrophotometer [9]. *Pseudomonas aeruginosa*, *Escherichia coli*, *Bacillus cereus*, *Klebsiella* sp., *Arthrobacter gangotriensis* and *Lactobacillus* sp. are some of the most commonly employed bacterial species for the synthesis of Ag-NPs.

Fungi harbors various proteins due to which they are commonly used for reduction and stabilization during the synthesis of NPs. Fungal agents are easy to handle, have high yields and produces very low toxic residues. Fungal biomolecules enhance the biological activity of nanoparticles and also improves the stability of nanoparticles. Fungi and yeasts have high metal tolerance and are easy to manipulate and handle. Fungi harbors more than 6,400 bioactive compounds [10]. Fungi secretes large number of extracellular proteins which provides stability of nanoparticles [11]. Fungi mediated NP synthesis can take place either intracellularly or extracellularly. During intracellular synthesis, the metal precursor salt solution added along

the cultures gets internalized by mycelia. In case of intracellular synthesis, extraction of NPs is required employing chemical cell lysis, centrifugation followed by filtration.

Algae are the most abundant and easily available organism, this property of algae makes them the best candidates for nanoparticles' synthesis [12]. Algal mediated synthesis of silver nanoparticles involves three major steps, (i) algal extract preparation in distilled water or in organic solvents by boiling at a particular temperature for a predetermined duration, (ii) preparation of precursor metal molar salt solution, and (iii) incubation of algal extract with salt solution under controlled conditions [12], [13]. Various algal biomolecules such as polysaccharides, proteins, cytochromes and other pigments are responsible for the reduction and stabilization of metal ions [14]. Algal based NP synthesis takes short time as compared to any other biogenic synthesis method [15]. Algal NP synthesis takes place both extracellularly and intracellularly [1]. Intracellular synthesis of NPs require additional step of recovery of synthesized NPs from within the cells [16]. Edison et al., synthesized spherical Ag-NPs of size ~25 nm employing *Caulerpa racemose* [17]. Ag-NPs of size range 8- 12 nm have been synthesized using algal extract of *Chlorella pyrenoidosa* [18].

Amongst all the biological candidates, algae are the best source for the synthesis of nanoparticles due to its ease of cultivation, minimal requirement of nutrients, presence of various bioactive compounds. Algal strains are the best choice because it can be cultivated even employing wastewater. Employing wastewater for algal cultivation will result in the treatment of wastewater and production of algal feed stock for various purposes such as nanoparticle synthesis, lipid extraction, carotenoid extraction, etc.

Objectives of this study:

1. Synthesis parameter optimization and characterization of silver nanoparticles (Ag-NPs) using *Chlorella minutissima* extract
2. Qualitative and quantitative analysis of β-carotene from *C. minutissima*
3. Ag-NPs synthesized from *C. minutissima* extract as remediating agent for dyes, heavy metals and as an antibacterial agent.

REVIEW OF LITERATURE

Amongst all the groups that comprise of microalgae, Cyanophyceae- blue green algae, Bacillariophyceae- including diatoms, Chlorophyceae- green algae and Chrysophyceae- including golden algae are the most commonly reported ones for harbouring one or more desirable characters for carrying out economical and efficient combinations of lipid synthesis for biodiesel production, CO_2 fixation and wastewater treatment [19], [20]. Various bioprocess industries and processes rely on the microorganism's metabolism to convert carbohydrates to various value-added products such as vitamins, amino acids and organic acids such as acetic acid. In such bioprocess industries, the cost of carbohydrate feedstock plays a significant role in the total cost of production [21]. Photosynthetic organisms such as microalgae provides a production alternative and can entirely eliminate the cost associated with carbohydrate feedstock. These photosynthetic organisms employ sunlight as a source of energy which is inexpensive and readily available.

β- Carotene

Carotenoids (commonly abbreviated as Crts) are the class of natural pigments with hefty scientific attention due to their substantial properties. Out of around 600 structurally and functionally diverse natural types; three major provitamin A are the alpha, beta and gamma isomers. The beta isomers get actively cleaved at the centre in an O_2-dependent manner to produce retinal [22] Plants and algae are considered the best sources of these bright orange-red coloured pigments, the β-carotene. These oxygen lacking pigments are fat-soluble and highly hydrophobic due to the conjugated double bonds and central symmetry [23]. These have been reported to be safe for consumption, as nutritional supplements as well as food additives [24]. β-carotenes ($C_{40}H_{56}$) are 40-C isoprene containing compounds, the characteristic colour imparted due to the presence of double bonds [25]. Both the ends of the molecule have cyclic rings. During isolation, *cis*-isomers of carotenoids are most common, however, they readily undergo *cis*↔trans isomerisation in polar environments [26]. Due to the number of therapeutic and preventive effects that β-carotene offers, it has been recognised as the functional component of food. Even being categorised as a micronutrient, due to its requirement in minute amounts as compared to other requirements of the body, β-carotene is considered to be very essential. β-carotene conversion to Vitamin A has moved from 6:1 to the current ratio of 28:1. The reason is the single nucleotide polymorphisms in the β-carotene-*15',15*-monooxygenase (BCMO) enzyme, responsible for converting provitamin A into retinol. This means that, in the

affected individuals, 28 mg of β-carotene is converted into 1 mg of retinol. This exposes the population to a greater risk of developing Vitamin A deficiency and associated disorders, as well as the inability to utilise provitamin A from the diet [27].

To date, no potential adverse effects of β-carotene consumption have been reported other than 'carotenodermia' which is skin discolouration due to elevated concentrations of carotenoids. β-carotene didn't appear to be mutagenic; carcinogenic or teratogenic in any of the assays carried out [28]. In fact, no increase in serum retinol level was found even after long-term β-carotene supplementation in people with already adequate levels of Vitamin A [29]. For prolonged serum and tissue accumulation of β-carotene, its administration must be with dietary fats [30]. However, people who smoke are better not subjected to high supplementations of β-carotene as this might put them at higher risk of lung cancer and an enhanced chance of mortality [31]. Most of the absorbed β-carotene is converted by the gut microbes into unknown complex compounds, and the remainder gets excreted.

Nanotechnology for environmental remediation

The science of nanotechnology includes materials in nano-scale range usually between 1-100 nm. Nanotechnology works at nano-scale range and thus, plays a major role in various fields like bioengineering, dentistry and medical, pharmaceuticals and others [1]. There are various physical and chemical methods that are being employed for the synthesis of nanomaterials such as microwave mediated, electrochemical method, photochemical methods, laser evaporation methods, thermolysis, co-precipitation, sol-gel method and sonochemical method [2]. These methods involve high input of energy, expensive and toxic chemicals such as sodium borohydride or hydrazine as reducing agents for synthesis and consequently produces harmful by-products which poses negative impact on the environment thus, these methods are non-sustainable and cannot be employed for large-scale production basis [3], [4]. Thus, to overcome the drawbacks of physical and chemical methods, green synthesis approach has gained momentum in the past decades. Green synthesis or the biological methods of synthesis has gained attention owing to their economical and environment friendly nature and high thermal stability of the produced nanoparticles [3]. The various solvents and reducing agents employed for the synthesis of nanoparticles (NPs) affects the morphology (size, shape), physicochemical properties and the use of synthesized NPs [5]. For green synthesis, microorganisms such as bacteria, fungi, yeast and algae and higher organisms such as plants have been used for the synthesis of metal NPs. Employing these organisms reduces the overall production cost, leaves

no harmful by-products and makes the overall synthesis environment friendly and sustainable [6].

Silver (Ag) possesses many fascinating properties due to which it has been extensively used in many fields such as medical, pharmacy, remediation. Green synthesis of Ag-NPs has been highly studied over the past decade due to its wide variety of applications. The morphology and stability of Ag-NPs can be regulated according to their application by regulating the biogenic synthesis parameters such as pH, light intensity, incubation temperature, precursor metal salt ($AgNO_3$) concentration, concentration of biogenic extract employed for synthesis [32]. For example, Ag-NPs employed for drug delivery are greater than 100 nm in size whereas those used as antimicrobial agents have a size ranging between 15- 80 nm [33]. These unique properties of Ag-NPs enable them to be used in the fields of biosensing, nanomedicines, pharmacy and for enhancing fuel efficiency. Some of the practical applications of Ag-NPs includes their use as antimicrobial agents, anti-cancerous agents, bioremediation candidates, optoelectronics and as a water purifying agent [34]–[37]. Furthermore, Ag-NPs are cost effective, highly abundant in nature, and possess high potential as compared to gold NPs or any other metallic NPs [6], [38]. The current review aims to provide a brief account and advancements in the field of green synthesis of Ag-NPs employing plants and microbes (bacteria, fungi and algae) and their various applications in diverse fields such as healthcare and environmental remediation.

Biogenic Ag-NPs synthesis

Synthesis of Ag-NPs can take place employing a number of methods including physical, chemical and biological methods as depicted in **figure 1**. Amongst all, biological synthesis methods are the most utilized ones for NPs synthesis due to enhanced stability and safety of NPs. Biogenic Ag-NPs are highly stable, environment friendly, easy to scale up and sustainable as compared to Ag-NPs produced from any other method [5]. In the recent times, NPs have been synthesized using microorganisms, extract of plant parts (leaves, seeds, bark), enzymes and metabolites of arthropods and agricultural wastes. Biogenic Ag-NPs due to their low production cost, stability and safety find its uses in various fields such as medicine, pharmaceuticals, environment remediation and in energy enhancement. The compounds like alkaloids, flavonoids, phenolic acids, proteins, enzymes, RNA and DNA present in extract of plants and microbes acts as capping and reducing agent responsible for the conversion of Ag^+ ions into Ag-NPs [39]. Production of biogenic Ag-NPs involves some basic steps: preparation

of extract of biological material; mixing and incubation of extract with $AgNO_3$ solution of desired or optimized concentration; and finally confirming synthesis via visual color change of the mixture to brown color or via UV-spectrophotometer. The produced Ag-NPs are then characterized using various methods such as scanning electron microscopy (SEM), transmission electron microscopy (TEM), X-ray diffraction (XRD), Fourier-transform infrared spectroscopy (FTIR) [40]–[44]. Various other methods used for characterization are enlisted in **table 1**.

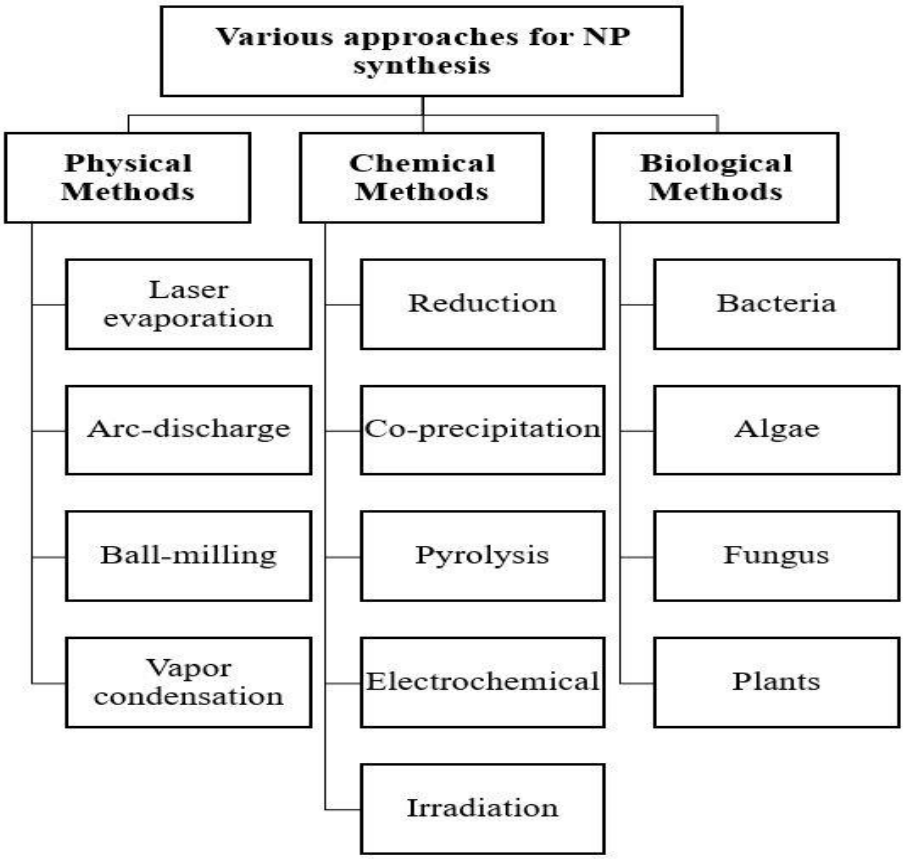


Figure 1: Various approaches for the synthesis of nanoparticles

Table 1: Common techniques used for characterization of nanoparticles

Techniques	Characteristics identified	References
SEM (Scanning electron microscopy)	Elucidate morphology and size	[45]
AFM (Atomic force microscopy)		[46]
TEM (Transmission electron microscopy)		[47]
HR-TEM (High-resolution TEM)		[48]
UV- visible spectrophotometric analysis	Analyze structure, crystallinity and composition.	[49][50]
FTIR (Fourier transform infrared spectroscopy)		[51]
XDR (X-ray diffraction)		[52]
DLS (Dynamic light scattering)		[53]
XPS (X-ray photo-electron spectroscopy)		[54]
EDS (Energy dispersive spectroscopy)		[55]
SAED (Selected area electron diffraction)		[56]

Plant mediated Ag-NPs synthesis

Plants and their parts such as leaves, stems, fruits, roots and flowers have been used for green synthesis of Ag-NPs. Plant based production is highly rapid, non-pathogenic, ecofriendly, economical and a one step process. The various phytocompounds involved in reduction, capping and stabilization of nanoparticles are amino acids, alkaloids, alcohols, carbohydrates, vitamins, polyphenols, glycosides and flavonoids [6]. For Ag-NPs synthesis, plant extract obtained from different plant parts is incubated with precursor metal salt solution ($AgNO_3$) for a defined amount of time. The time duration of incubation depends upon nature of plant extract (phytocompounds), metal salt concentration, extract concentration, pH of extract, contact time, exposure to light and stirring [3], [6]. Pirtarighat et al., reported the synthesis of Ag-NPs using *Salvia spinosa* extract with particle size ranging from 19- 125 nm [7]. Leaf extract of *Ocimum sanctum* have been used by Jain and Mehta for the synthesis of spherical Ag-NPs whose size ranged from about 12- 20 nm [8]. Studies conducted by Banerjee et al., reported the synthesis of Ag-NPs (size up to 200 nm) using 1mM $AgNO_3$ solution and leaf extract of *Musa balbisiana*, *Azadirachta indica* and *Ocimum tenuiflorum*. They also reported synthesis of Ag-NPs using seeds of *Vigan radiata* and *Cicer arietinum* [36]. Garibo et al., reported green synthesis of quasi spherical AgNPs of average size 5 nm using extracts obtained from *Lysiloma acapulcensis* [45]. *Momoridica charantia* have been reported to synthesize spherical AgNPs

of size range 11- 16 nm [9]. Pahal et al., reported the synthesis of Ag-NPs employing leaflet extracts of *Triticum aestivum* and *Oryza sativa* [46].

Bacterial mediated Ag-NPs synthesis

Bacterial cells have been used for the synthesis of Ag-NPs due to the presence of several enzymes, redox proteins, DNA and RNA which act as reducing and capping/ stabilizing agents. Bacterial cells have inherent potency to reduce heavy metals due to the presence of various functional groups in the cell wall. For the synthesis of Ag-NPs, bacterial cultures are first grown as cell suspension and then precursor metal salt solution is added into the culture medium followed by either stirring or incubation at still conditions. The progress in formation of Ag-NPs can be monitored by UV-Vis spectrophotometer [9]. *Pseudomonas aeruginosa*, *Escherichia coli, Bacillus cereus, Klebsiella* sp., *Arthrobacter gangotriensis* and *Lactobacillus* sp. Are some of the most commonly employed bacterial species for the synthesis of Ag-NPs. Ag-NPs have also been synthesized using actinomycetes. Various enzymes secreted by cell wall and membrane aids in reduction of Ag^{+} to Ag^{0} [3], [5], [21]. Yusof et al., synthesized spherical Ag-NPs of size range 14 ± 4.7 nm employing *Lactobacillus plantarum* TA4 and 2mM solution of $AgNO_3$ [47]. *Paenarthrobacter nicotinovorans* has been used for the synthesis of spherical Ag-NPs within the size range of 13- 27 nm [48]. Saleh and Alwan synthesized spherical Ag-NPs using *Klebsiella pneumoniae* culture supernatant of the size range between 26.48 to 44.42 nm [49]. Ibrahim et al., reported the synthesis of Ag-NPs (size range 5 to 7.06 nm) by using *Bacillus cereus* and 1mM $AgNO_3$ [50]. Saeed et al., reported the synthesis of Ag-NPs of size range 5-50 nm using the secondary metabolites of *Brevundimonas diminuta*, *Escherichia coli* and *Exiguobacterium aurantiacumm* [51]. Kathwate et al., demonstrated the use of *Pseudomonas aeruginosa* for the the production of Ag-NPs [52].

Fungal mediated Ag-NPs synthesis

Fungi harbors various proteins due to which they are commonly used as reducing and stabilizing agents for the synthesis of Ag-NPs. Fungal agents are easy to handle, have high yields and produces very low toxic residues. Fungal biomolecules enhance the biological activity of nanoparticles and also improves the stability of nanoparticles. Fungi and yeasts have high metal tolerance and are easy to manipulate and handle. Fungi harbors more than 6,400 bioactive compounds [10]. Fungi secretes large number of extracellular proteins which provides stability of nanoparticles [11]. Fungi mediated NP synthesis can take place either intracellularly or extracellularly. For intracellular synthesis, metal precursor salt solution is

added along the mycelial cultures which is then internalized by mycelia. In case of intracellular synthesis, extraction of NPs is required employing chemical cell lysis, centrifugation followed by filtration. For extracellular synthesis, precursor metal solution is incubated with aqueous fungal filtrate containing biomolecules. Extracellular synthesis results in free NPs formation [6]. *Fusarium* sp have been reported to be one of the best candidates for the production of Ag-NPs [53]. Feroze et al., reported the synthesis of spherical Ag-NPs from *Penicillium oxalicum* of size ranging from 60 to 80 nm [54]. Ag-NPs have been synthesized extracellularly in size range of 1- 24 nm by using *Aspergillus sydowii* [37]. Velhal et al., synthesized Ag-NPs ranging in size from 2- 13-80 nm employing *Aspergillus terrerus* [55]. Liu et al., reported the synthesis of Ag-NPs falling in size range of 50- 500 nm using *Metschnikowia* [33].

Algal mediated Ag-NPs synthesis

Algae are the most abundant and easily available organism, this property of algae makes them the best candidates for the synthesis of nanoparticles [12]. Algal mediated Ag-NPs synthesis involves three major steps, (i) algal extract preparation in distilled water or in organic solvents by boiling for predetermined duration, (ii) preparation of molar solution of precursor metal salt and (iii) incubation of algal extract along with molar solution of precursor metal solution under controlled conditions [12], [13]. Various biomolecules present in algae such as polysaccharides, proteins, cytochromes and other pigments causes the reduction and stabilization of metal ions [14]. Algal based NP synthesis takes short time as compared to any other biogenic synthesis method [15]. Algal NP synthesis takes place both extracellularly and intracellularly [1]. Intracellular synthesis of NPs require additional step of recovery of synthesized NPs from within the cells [16]. Edison et al., synthesized spherical Ag-NPs of size ~25 nm employing *Caulerpa racemose* [17]. Ag-NPs of size range 8- 12 nm have been synthesized using algal extract of *Chlorella pyrenoidosa* [18]. Spherical NPs have been reported to be synthesized from *Jania rubins* [56]. Azizi et al., reported the synthesis of spherical Ag-NPs in size range 5-15 nm using the algal extract of *Sargassum muticum* [57], [58]. *Cystophora moniliformis* extract has been reported to synthesize Ag-NPs in the size range of 75 nm [59].

Algal cells possesses the capacity to hyperaccumulate heavy metals and to reduce them to nanoparticles [59]. The presence of various bioactive compounds aids in reducing, capping and stabilizing agents for nanoparticles synthesis [1], [60]–[63]. Algal based nanoparticle synthesis occurs in three phases: (a) Activation phase- reduction of metal ions by algal bioactive compounds, (b) growth phase- nanoparticles formation takes place due to the nucleation of elements and, (c) termination phase- final shape of nanoparticles are achieved [1], [61], [64]. Algal nanoparticles are synthesized either extracellularly or intracellularly [61], [65].

APPLICATIONS OF AG-NPS

Ag-NPs as antimicrobial agents

Biogenic Ag-NPs are emerging as new age antimicrobials and the research in this area has accelerated over the period of time due to the emergence of antimicrobial resistance strains [66]. Nanoparticles are considered as an effective antibacterial agents because they interact directly with bacterial cells and consequently overcome the antibiotic resistance mechanisms adopted by bacterial species [13]. Biogenic Ag-NPs are advantageous over conventional antibacterial agents as they prevent the emergence of antimicrobial resistance strains, they are cheap to synthesize, rapid synthesis occurs and environment friendly [67]. These NPs are reported to use one of the three mechanisms or a combination of mechanisms for causing bactericidal effect (i) increased production of reactive oxygen species, (ii) penetration within membrane, and (iii) interaction with cellular components like DNA, RNA and cell organelles as depicted in **figure 2**. [68]. Dhavale et al., demonstrated the synthesis of Ag-NPs using *Amphiroa fragilissima* and its application as an antibacterial agent targeting *Bacillus subtilis*, *Escherichia coli* and *Staphylococcus sp* [69]. *Chlorella vulgaris* extract was used for the biosynthesis of Ag-NPs and was used as antibacterial agents against *E. coli* and *Pseudomonas aeruginosa* [70]. *Ocimum sanctum* was used for the synthesis of Ag-NPs and was found effective against *E. coli* [71]. Yusuf et al., synthesized Ag-NPs employing *Lactobacillus plantarum* and the synthesized particles were found to be effective against both gram positive (*S. aureus* and *S. epidermidis*) and negative (*E. coli* and *Salmonella* sp) [47]. *Aspergillus sydowii* was used for synthesis of Ag-NPs and was found effective against various fungal strains such as *Candida albicans*, *C. glabrata*, *Fusarium solani* [37]. Various algal nanoparticles used as antimicrobial agents are enlisted in **table 2**.

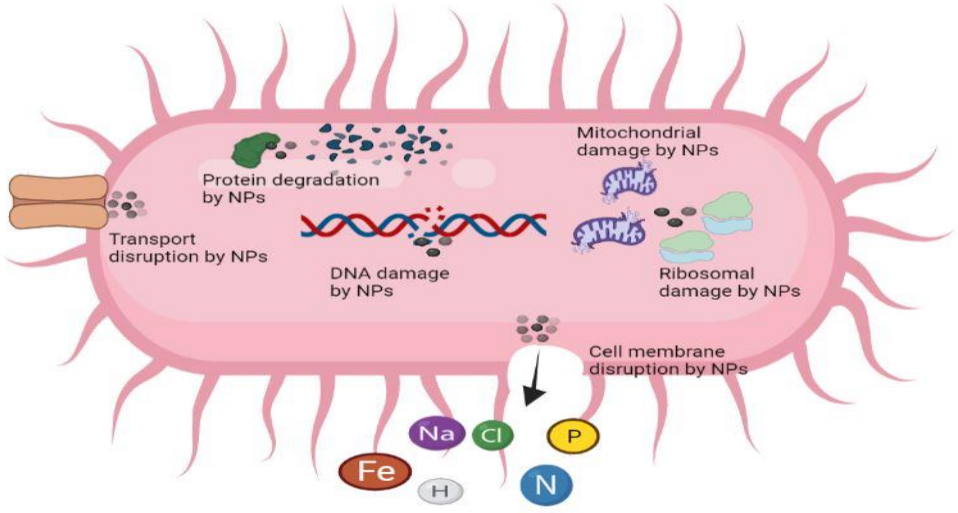


Figure 2: General killing mechanism of micro-organism by nanoparticles (NPs). The NPs may act on microbial cells by damaging DNA, RNA, ribosomes or mitochondria. The NPs can also degrade proteins present in the

cells or disturbs the ion transportation or disrupts the cell membrane and thus, eventually causing the cellular damage and cell death

Table 2: Algal synthesized nanoparticles as antimicrobial agents against various pathogens.

Algal species	Synthesized NPs	Target organism	Reference
Acanthophora specifera	Silver NPs	*Bacillus* sp., *S. aureus, E. coli, Candida* sp.	[72]
Acanthophora specifera	Silver NPs	Biofilm forming *S. typhi* and *S. flexneri*	[73]
Amphiroa fragilissima	Silver NPs	*S. aureus*	[74]
Bifurcaria bifurcata	Copper oxide NPs	*E. aerogenes* and *S. aureus*	[75]
Botryococccus braunii	Copper NPs Silver NPs	*K. pneumoniae* and *S. aureus*	[76]
Caulerpa racemosa	Silver NPs	*S. aureus* and *P. mirabilis*	[77]
Chlorella pyrenoidosa	Silver NPs	*K. pneumonia, A. hydrophila* and *S. aureus*	[18]
Chlorella vulgaris	Silver NPs	*S. aureus* and *K. pneumoniae*	[78]
Chlorochoccum humicola	Silver NPs	*E. coli*	[79]
Cladophora vagabunda	Fluorescent carbon NPs	*S. aureus* and *E. coli*	[80]
Colpmenia sinusa	Silver NPs	*E. coli and S. aureus*	[56]
Ecklonia cava	Silver NPs	*E. coli* and *S. aureus*	[81]
Euglina gracilis	Silver NPs	*E. coli*	[82]
Galaxaura elongata	Gold NPs	MRSA, *S. aureus* and *P. aeruginosa*	[83]
Gelidium amansii	Gold NPs	*E. coli* and *S. aureus*	[84]

Ag-NPs mediated Heavy metal remediation

Recently, bio-nanobiotechnology has gained a lot of interest in research especially in the area of environment. Nanoparticles are considered to be the best material for heavy metal remediation as they have high surface activity, large surface-to-volume ratio and unique physical as well as chemical characteristics. Type of nanoparticles and their physical, chemical and magnetic properties plays a major role in the abatement of heavy metal [85]. Metal-based nanoparticles like Ag, Au, Fe and metal-oxide nanoparticle are widely used for remediation of heavy metals like Cd, Cu, Cr, Zn, Pb and Hg, etc. Ag-NPs can remediate mercury, cadmium, chromium, cobalt, lead, etc. [86]. It is observed that remediation ability of Ag-NPs is dependent on the reduction potential of heavy metals [87]. Thus, it can be concluded that for every different heavy metal, a different type of nanomaterial is required. Attasi and Nsiah reported that 20 nm Ag-NPs is able to remediate 92.92% lead and 53.34% cobalt within 14 days [88]. On the other hand, El-Tawil et al observed that Ag-quartz nanocomposite enhances the

removal efficiency of mercury to 96% within 1 hour [89]. Many algal species like *Sargassum muticum, Turbinaria ornate, Sargassum polycystum, Turbinaria conoides, Gilidiella acerosa , Sargassum wightiigrevilli, Padina pavonica, Colpmenia sinusa* are able to synthesize different size of Ag-NPs [90]. *Ficus benjamina* leaf extract has been utilized for the synthesis of Ag-NPs which further has been employed for the removal of Cd^{2+} from the contaminated sources [86]. Various heavy metals that have been remediated using nanotechnology are listed in **table 3**.

Table 3: Application of algal biomass-based nanomaterials in remediation of heavy metals

Heavy metal(s) and its source	**Nanotechnology enabled algae-based remediation agent**	**Removal or degradation or reduction efficiency**	**Reference**
Cr (VI) from effluent stream of paint and steel industry.	Nanoparticle synthesized by mechanical agitation of *Spirulina platensis*	99.1% removal efficiency	[40]
Pb ion solution (1000 mg/L)	Fe_3O_4 nanoparticle alginate bead synthesized from *Padina pavonica* (Linnaeus)	91% bio removal	[91]
Pb ion solution (1000 mg/L)	Fe_3O_4 nanoparticle alginate bead synthesized from *Sargassum acinarium*	78% bioremoval	[91]
As contaminated ground water	Fe_3O_4 nanoparticles from *Sargassum muticum*	High arsenic adsorbance	[60], [92]
Hg ions from wastewater reservoirs	Ag nanoparticles from *Chlorococcum humicola*	Highly efficient	[93]
Cu ions from solution with initial ion concentration of 100 mg/L	Reduced graphene oxides (rGO) from *Scenedesmus vacuolatus* (211-11n),	91% removal within 30 mins	[94]
Cu ions from solution with initial ion concentration of 100 mg/L	Reduced graphene oxides (rGO) from *Chloroidium saccharophilum* (211-9a)	74% removal with 30 mins of contact time	[94]
Cu ions from solution with	reduced graphene oxides (rGO) from *Leptolyngbya* JSC-1	93% removal with 30 mins of exposure time	[94]

initial ion concentration of 100 mg/L			
Pb ions from solution with initial ion concentration of 100 mg/L	Reduced graphene oxides (rGO) from *Scenedesmus vacuolatus* (211-11n),	95% removal with 30 mins of exposure time	[94]
Pb ions from solution with initial ion concentration of 100 mg/L	Reduced graphene oxides (rGO) from *Chloroidium saccharophilum* (211-9a)	89% removal with 30 mins of exposure time	[94]
Pb ions from solution with initial ion concentration of 100 mg/L	Reduced graphene oxides (rGO) from *Leptolyngbya* JSC-1	82% removal with 30 mins of exposure time	[94]
Cr (VI) to Cr (III) reduction	Fe nanoparticles from *Chlorococcum* sp. MM11	92% reduction of Cr (VI) to Cr (III)	[95]

Ag-NPs mediated dye degradation

Biogenic NPs have been studied for their potential use in dyes degradation present in contaminated water bodies. NPs help in effective adsorption of these dyes owing to large surface area of NPs [93]. NPs are also known to degrade dyes into simpler non-toxic forms. Ag-NPs have been used for the degradation of dyes such as Congo Red, Coomassie blue, Malachite green and methyl orange [96]. Ag-NPs derived from *Microchaete* sp have been reported to demonstrate 84.6% removal efficiency of Methyl red in contaminated water sources [96]. Aziz et al., reported the synthesis of Ag-NPs using extracts of *Chlorella pyrenoidosa* and demonstrated the degradation of methylene blue from waste water [18]. Ag-NPs have been synthesized using algal extracts of *Ulva lactuca* and was subsequently used for the degradation of methyl orange from contaminated water sources [35]. *Viburnum opulus* fruit extract has been used for the synthesis of Ag-NPs which was further used for the degradation of tartrazine, brilliant green and carmoisine [97]. Raj et al., reported the removal or degradation efficiency of 86.68% for methyl orange, 93.60% for methylene blue, 88.80% for 4- nitrophenol and 92.20% in case of congo red by Ag-NPs synthesized using *Terminalia arjuna* [98]. Ag-NPs synthesized using *Phaseolus vulgaris* extract was used for removal of reactive red-141 dye with 97% efficiency [99]. *Pestalotipsis versicolor* has been used for the synthesis of Ag-NPs and has been used for the degradation

of azo dyes such as Rhodamine B, Congo red and Orange G [100]. Various dyes that have been degraded using nanoparticles have been listed in **table 4**.

Table 4: Application of algal biomass-based nanomaterials in remediation of dyes

Dyes and its source	**Nanotechnology enabled algae-based remediation agent (Algae, Nanomaterials)**	**Removal or degradation or reduction efficiency**	**Reference**
Methylene blue from textile and paper industry	*Chlamydomonas reinhardtii*, CdS-NP	90% removal in 90 minutes	[101]
Malachite green from industrial waste	*Spirulina platensis, CdS-NP*	35-40% removal in 1 hour	[102]
Reactive black 5, effluent of dyeing industry	*Macrocystis pyrifera*, Zerovalent Fe-NP	69-80% removal in 1 hour	[103]
Rhodamine B, Chemical solution	*Turbina riaconoides* and *Sargassum tenerrimum*, Au-NP	Increased removal efficiency	[42]
Sulphorhodamine 101, Chemical solution	*Turbina riaconoides* and *Sargassum tenerrimum*, Au-NP	Increased efficiency	[42]
Methyl red, Chemical solution	*MicrochaeteNCCU-342*, Ag-NP	84.60% removal in 2 hours	[96]
Methylene blue, Chemical solution	*Chlorella pyrenoidosa*, Ag-NP	Increased efficiency	[18]
Methyl Orange, Chemical solution	*Ulva lactuca*, Ag-NP	Slow but high efficiency after 12 hours	[104]
Congo Red, Chemical solution	*Caulerpa serrulata*, Ag-NP	Decolouration in 5 minutes	[105]
Methyl Orange, Chemical solution	*Hypnea musciformis*, Ag-NP	Efficiency increases with time	[44]
Reactive navy-blue dye, Chemical solution	*Turbin ariadecurrens*, Super paramagnetic Fe_3O_4-NP	Initially fast but slows down with time. Saturation after 2 hours.	[41]

Methylene blue, Chemical solution	*Caulerpa racemosa,* Ag-NP	Complete removal in 30 minutes	[17]

MATERIAL AND METHODS

1. Algal cultivation

Growth medium optimization for *C. minutissima*

- Different algal growth medium (BG11, Bold 3N, Zarrouk synthetic medium, BBM and SE (Bristol's solution) medium) were inoculated with 5% inoculum of *Chlorella minutissima* under aseptic conditions.
- The culture flasks were incubated at 28^{o}C under 16 hours light and 8 hours dark cycle. 2 mL aliquots were withdrawn from each flask after an interval of every three day and absorbance was measured at 690 nm.
- Semi-log graph was plotted taking X-axis as number of days and Y-axis as absorbance value.

Growth analysis of *C. minutissima* using RO spent water

- Different proportions of RO spent water (0%, 25%, 50%, 75% and 100%) was supplemented with BG11 medium and was inoculated with 5% inoculum of *Chlorella minutissima* under aseptic conditions.
- The culture flasks were incubated at 28^{o}C under 16 hours light and 8 hours dark cycle. 2 mL aliquots were withdrawn from each flask after an interval of every three day and absorbance was measured at 690 nm.
- Semi-log graph was plotted taking X-axis as number of days and Y-axis as absorbance value.

Biomass measurement: Weight of empty 1.5 ml centrifuge tube was measured and were labelled accordingly. 5 ml culture of *C. minutissima* was withdrawn aseptically from different medium and were placed in prelabelled tubes. Centrifuged the cultures at 10000 rpm for 4 minutes. The supernatant was discarded and the pellet was collected. The collected pellet was washed twice with distilled water. The tube was placed in oven at 40^{0}C for drying. The dry weight was measured using weighing balance. Biomass productivity and biomass concentration was calculated using the formula mentioned below:

-1

$$Biomass\ productivity = \frac{Weight\ (mg)}{Volume\ of\ culture\ (L) \times No.of\ cultivation\ days}$$

$$Biomass\ concentration = \frac{Weight\ (mg)}{Volume\ of\ culture\ (L)} \quad -2$$

2. **Biochemical test for qualitative estimation of Bioactive compounds in *C. minutissima***

Qualitative test for Alkaloids

- Preparation of Mayer's reagent:

Mayer's reagent was prepared by mixing 1.36 gm of mercuric chloride ($HgCl_2$) and 5 gm of potassium iodide (KI) in 100 mL of distilled water.

- Sample Preparation:

Sample was prepared by dissolving 1 gm of algal powder in 10 mL solution of 70% methanol and 70% acetone. The mixture was macerated at 50 rpm for 24 hours.

- **Mayer's Test**

The prepared sample was centrifuged at 10,000 rpm for 10 minutes. 1N HCl was added to dissolve the sample and the dissolved content was filtered using Whatman filter paper number 1. Few drops of Mayer's reagent were added to the obtained filtrate and incubated at room temperature for 1 hour.

Qualitative test for Carotenoids

1 gm of dried algal powder was mixed with 10 mL solution of 4 Acetone: 1 Water. The obtained mixture was centrifuged at 12,000 rpm for 2 minutes. The supernatant was collected and the absorbance of supernatant was measured at 470 nm for confirming the presence of carotenoids.

Qualitative test for Flavonoids

- Sample preparation

1 gm of dried algal powder was dissolved in 10 mL solution of 70% methanol and 70% acetone. The mixture was macerated at 50 rpm for 24 hours.

- $AlCl_3$ Colorimetric test

0.6 mL of the prepared sample extract was mixed with 0.6 mL of 2% $AlCl_3$ and the mixture was incubated for 60 minutes at room temperature and the absorbance was recorded at 420 nm.

Qualitative test for Phenols

- Neutral Ferric chloride ($FeCl_3$) preparation

1N NaOH was added dropwise to 1% $FeCl_3$ solution to form a brown colored precipitate. The precipitate was filtered using filter paper. The collected supernatant was Neutral $FeCl_3$.

- Neutral $FeCl_3$ test

Neutral $FeCl_3$ solution was added dropwise to the wet algal sample and the development of color was noted.

3. β-Carotene: Quantitative assay

The quantitative estimation of beta-carotene from *C. minutissima* extract prepared in tetrahydrofuran (THF) using sonication (for 10 minutes), heat treatment, bead milling and sonication of wet *C. minutissima* biomass in water as a cell disruption method was performed using UV-Vis spectrophotometry. Beta-carotene, as per literature, gives peak at 450 nm.

Quantification of beta-carotene from *C. minutissima* sample was done using the following formula.

$$\text{Total beta-carotene } (\mu g/ml) = 25.2 * A_{450}$$

For extraction of beta-carotene, *C. minutissima* was cultivated on BG11 and BG11 along with glucose supplemented medium in order to compare the productivity of beta-carotene and best cell disruption technique for liberation of beta-carotene.

4. Synthesis of algal based silver nanoparticles

Extract preparation

- 100 ml of 24 days old culture was centrifuged at 10000 rpm for 5 minutes.
- Supernatant was discarded and the pellet was washed 2-3 times with distilled water.
- The washed pellet was dissolved in 100 mL of distilled water and kept at 80^oC for 30 mins.
- After cooling down of the mixture, supernatant (algal extract) was collected using centrifugation.
- The prepared extract was stored at 4^oC for further use.

Method of preparation

- Ag-NPs were prepared by the addition of a proportion of 10 mM solution of $AgNO_3$ in algal extract and incubating the obtained mixture at a particular pH and temperature.

Optimization of synthesis condition

pH optimization

- The algal extract was divided into parts and pH was varied from 4 to 10.
- 10mM fresh $AgNO_3$ solution was prepared and mixed with the algal extract kept at different pH range in the ratio of 1:10 (Precursor metal salt: algal extract).
- 0 min absorbance was scanned (200 nm- 700nm) using UV-Visible spectrophotometer.
- The mixture was incubated at 28°C, still in light conditions for 24 hours.
- Color change was observed and absorbance scan was run for all the samples.

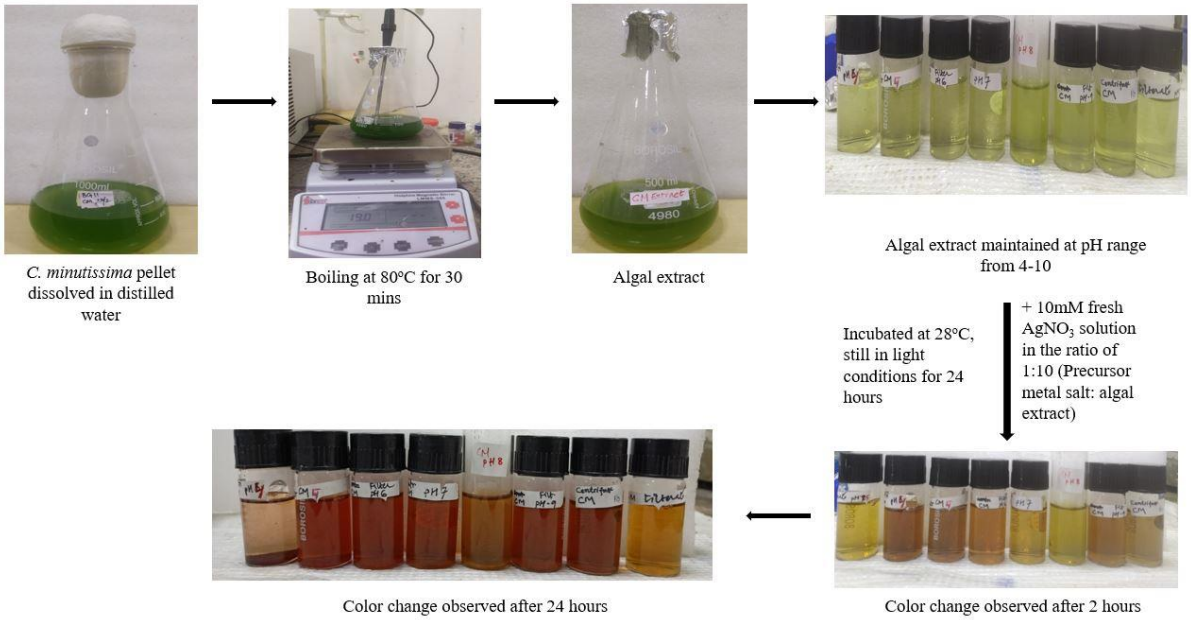


Figure 3: Experimental set up for optimization of pH for the synthesis of Ag-NPs employing *C. minutissima*

Algal extract and $AgNO_3$ solution ratio optimization

- The algal extract was maintained at pH 9.
- 10mM fresh $AgNO_3$ solution was prepared and mixed with the algal extract kept in different ratios of 1:10, 1:1 and 10:1 (Precursor metal salt: algal extract).
- 0 min absorbance was scanned (200 nm- 700nm) using UV-Visible spectrophotometer.
- The mixture was incubated at 28°C, still in light conditions.
- Color change was observed and absorbance scan was run for all the samples after 120 to check the stability of nanoparticles.

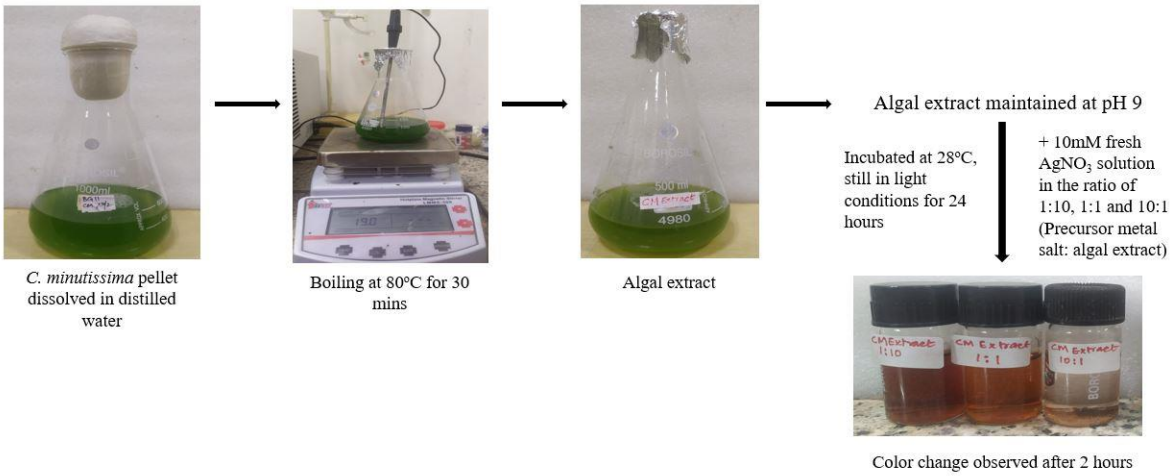


Figure 4: Experimental set up for optimization of ratio of algal extract and metal precursor salt

Ag-NP synthesis using cell free extract

- 100 ml of 24 days old culture was centrifuged at 10000 rpm for 5 minutes and the supernatant was collected in a separate flask
- The cell free extract was maintained at pH 5-10.
- 10mM fresh $AgNO_3$ solution was prepared and mixed with the cell free extract maintained at pH 5-10 in the ratio of 1:10 (Precursor metal salt: algal extract).
- 0 min absorbance was scanned (200 nm- 700nm) using UV-Visible spectrophotometer.
- The mixture was incubated at 28°C, still in light conditions for 24 hours.
- Color change was observed and absorbance scan was run for all the samples at 0 hr, 24 hrs, 48 hrs and 96 hrs for checking the stability of produced Ag-NPs.
- BG11 medium and 1g/L glucose solutions were also mixed with 10 mM $AgNO_3$ in the ratio of 1:10 (Precursor metal salt: solution) to check their ability to synthesize Ag-NPs.

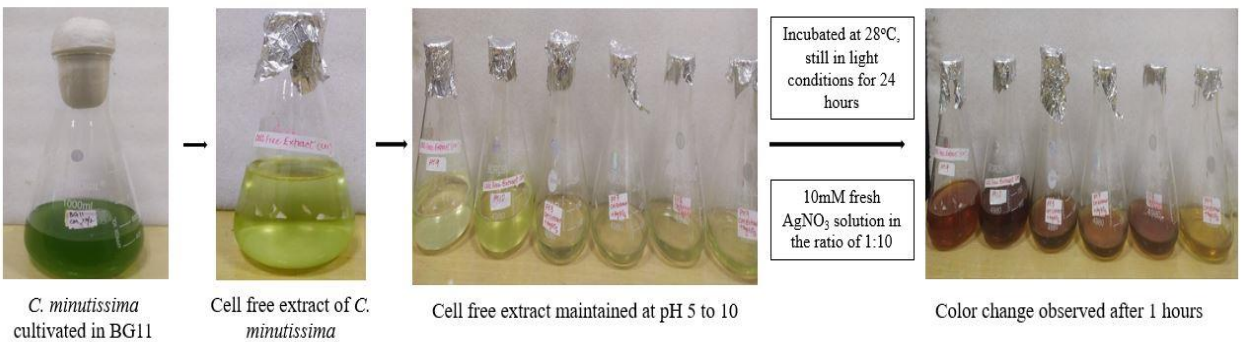


Figure 5: Experimental set up for optimization of pH for the synthesis of Ag-NPs employing *C. minutissima* using cell free extract

5. Characterization of Ag-NPs

The *C. minutissima* mediated synthesized Ag-NPs were characterized using UV-Visible Spectrophotometer (for confirmation of formation of Ag-NPs), FE-SEM (for shape determination), XRD analysis (for crystallinity determination), Zeta-potential (for size determination) and FTIR (for identification of bioactive compounds acting as stabilizing and capping agents for Ag-NPs).

6. Application of algal Ag-NPs

Antibacterial effect of algal Ag-NPs

The prepared Ag-NPs were tested for their antimicrobial activity against *E. coli*, *Salmonella* sp, *Bacillus cereus*, *Staphylococcus aureus* and *Klebsiella* sp in triplicates.

- 0.01 ml of overnight grown bacterial culture was spread using a spreader on the nutrient agar plate.
- Circular disks were cut from filter paper under aseptic conditions.
- The filter-paper disks were absorbed with different volumes of (10, 20, 30, 40 and 50 µL) synthesized Ag-NP solution and placed carefully on the nutrient agar plates containing cultures.
- The plates were incubated in upright manner for 24 hrs.
- The plates were checked for zone of inhibition around the disks and was measured carefully using a millimeter scale.

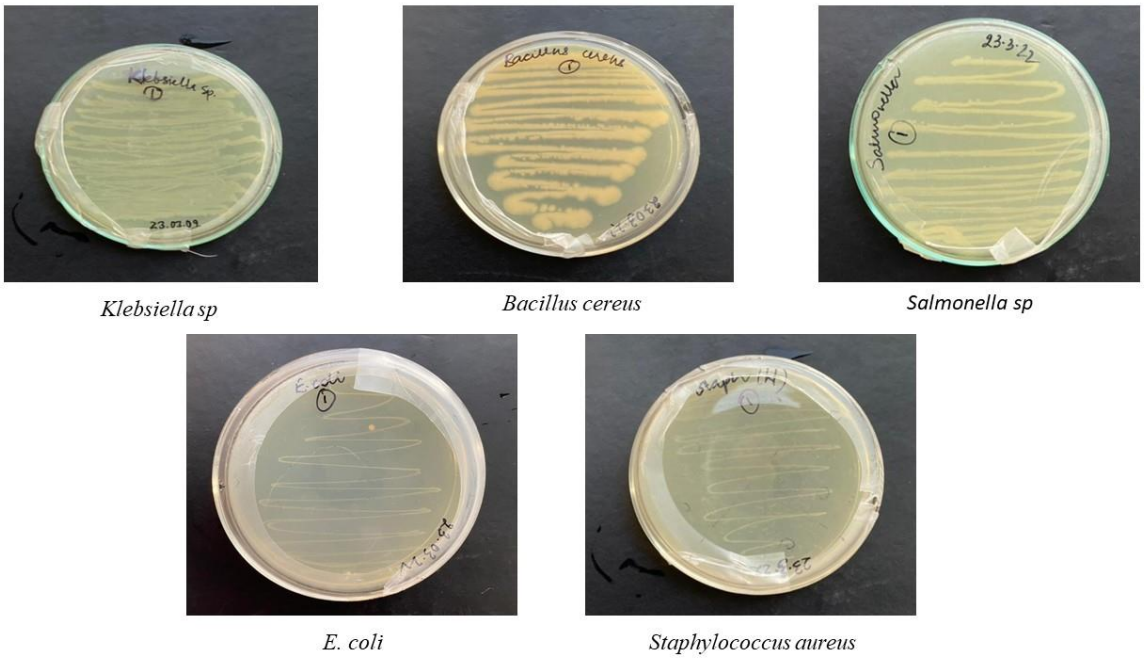


Figure 6: Nutrient agar plates containing different bacterial species

Photocatalytic dye degradation using algal Ag-NPs

The biosynthesized Ag-NPs were tested for their photocatalytic degradation of Methylene blue.

- Standard curve of different concentration of Methylene blue (MB) dye was prepared and absorbance at 663 nm was noted for each dilution.
- 10 mL of 10 PPM solution of MB was taken and 1 mL of biogenic Ag-NPs was mixed.
- Absorbance was noted after mixing Ag-NPs with the MB solution.
- The solutions were incubated in the sunlight and absorbance was noted after every hour.
- Removal efficiency was calculated using the standard curve.

Heavy metal remediation using algal Ag-NPs

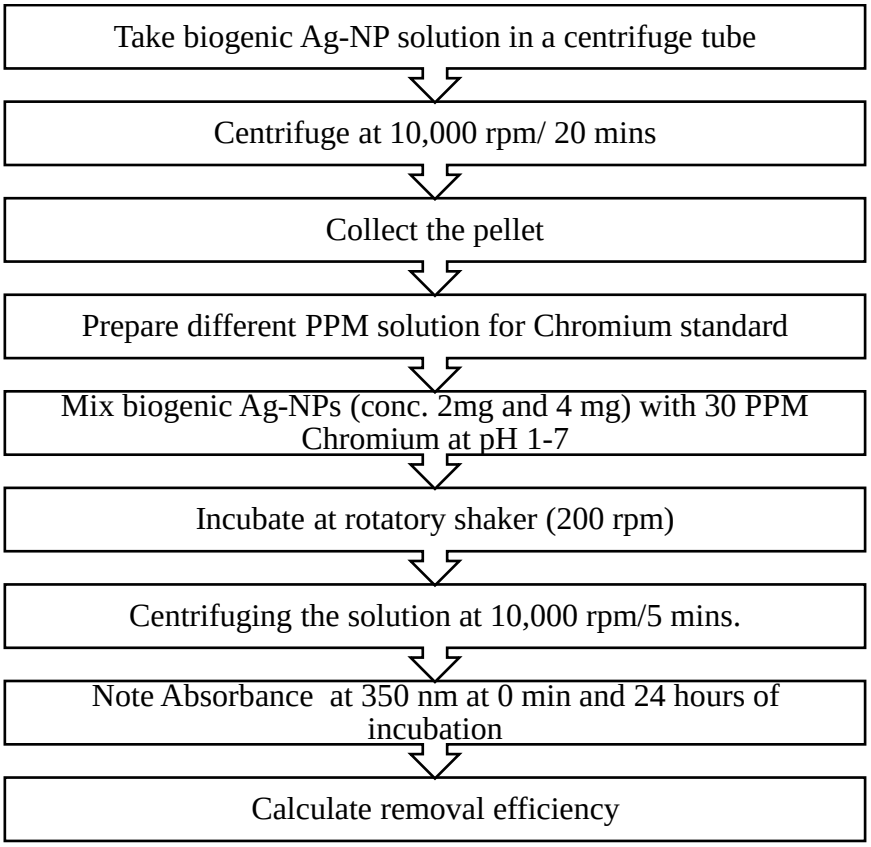
Chromium removal:

The biosynthesized Ag-NPs were tested for their Chromium removal efficiency from synthetic wastewater.

- Standard curve of different concentration of Chromium was prepared and absorbance was noted for each dilution.
- 30 PPM Chromium solution was taken and pH was varied from 1 to 7 and biogenic Ag-NPs was mixed at concentration 2 mg and 4 mg.
- Absorbance was noted after mixing Ag-NPs with the chromium solution.

- The solutions were incubated in rotatory shaker (200 rpm) at room temperature for 24 hours.
- The solution was centrifuged at 10,000 rpm for 5 minutes after 24 hours of incubation and absorbance was taken.
- Removal efficiency was calculated using the standard curve.

Figure 7: Steps for the removal of Chromium using biosynthesized Ag-NPs



Removal efficiency (%) = [(Initial Concentration – Final concentration)/ Initial concentration] * 100

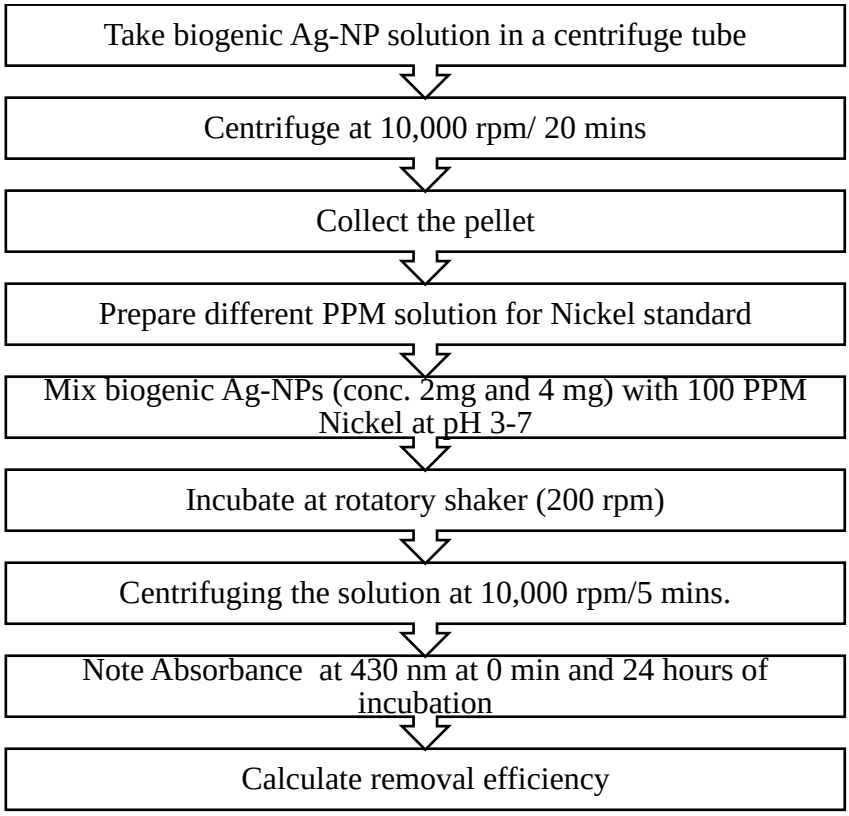
Nickel removal:

The biosynthesized Ag-NPs were tested for their Nickel removal efficiency from synthetic wastewater.

- Standard curve of different concentration of Nickel was prepared and absorbance was noted for each dilution.

- 100 PPM Nickel solution was taken and pH was varied from 3 to 7 and biogenic Ag-NPs was mixed at concentration 2 mg and 4 mg.
- Absorbance was noted after mixing Ag-NPs with the chromium solution.
- The solutions were incubated in rotatory shaker (200 rpm) at room temperature for 24 hours.
- The solution was centrifuged at 10,000 rpm for 5 minutes after 24 hours of incubation and absorbance was taken.
- Removal efficiency was calculated using the standard curve.

Figure 8: Steps for the removal of Nickel using biosynthesized Ag-NPs



Removal efficiency (%) = [(Initial Concentration – Final concentration)/ Initial concentration] * 100

RESULT AND DISCUSSION

1. Algal cultivation and Biomass production for Ag-NPs synthesis

Cultivation of *C. minutissima* on different medium

Absorbance at 690 nm of *Chlorella minutissima* grown on different medium (BG11, Bold 3N, Zarrouk synthetic medium, BBM and SE (Bristol's solution) medium) were taken and noted in order to plot growth curve. The plotted growth curve was used for the selection of best growth medium for cultivating *Chlorella minutissima*.

Table 5: Optical density of *C. minutissima* cultivated on different medium noted after every 3 days at 690 nm.

Media/ Days	Day1	Day 3	Day 6	Day 9	Day 12	Day 18	Day 24
BG11	0.0041	0.1237	0.2652	0.4906	0.6699	0.8274	1.2334
B3N	0.0052	0.0861	0.1267	0.3318	0.6812	0.8752	1.331
BBM	0.0051	0.1335	0.2426	0.4056	0.6468	0.7289	0.9818
ZARROUK	0.0041	0.0437	0.0543	0.2213	0.4025	0.492	0.8775
SEM	0.0050	0.1413	0.2173	0.39	0.8002	1.1019	1.543

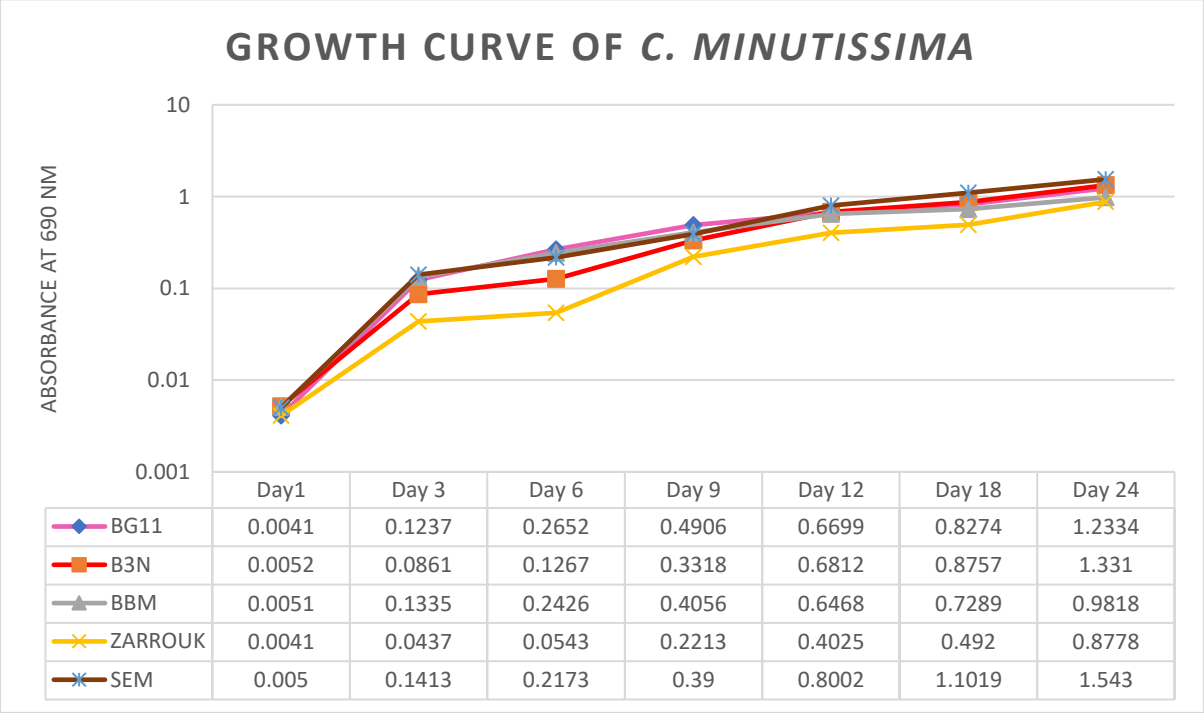


Figure 9: Growth curve of *C. minutissima* cultivated on BG11, Bold 3N medium, BBM, Zarrouk medium and SE (Bristol's solution) medium.

The growth of *C. minutissima* was tested on five different media (BG11, Bold 3N medium, BBM, Zarrouk medium and SE (Bristol's solution) Medium) maintained at pH-8. It was found that SE (Bristol's solution) and BG11 medium (pH-8) was found to best suited for cultivation of *C. minutissima* amongst all the five tested medium. The lowest growth rate was found to be on Zarrouk medium. *C. minutissima* exhibited moderate growth rate while being cultivated on Bold 3N and BBM medium. This difference in growth rates can be attributed to the difference in the amount of micro and macro-nutrients present in the medium and to the chemical complexes which provide these nutrients.

Cultivation of C. minutissima on different proportions of RO Spent water

Absorbance at 690 nm of *C. minutissima* grown on different proportions of RO spent water and BG11 were taken and noted in order to plot growth curve. The plotted growth curve was used for the selection of best proportions of RO spent water medium for cultivating *C. minutissima*.

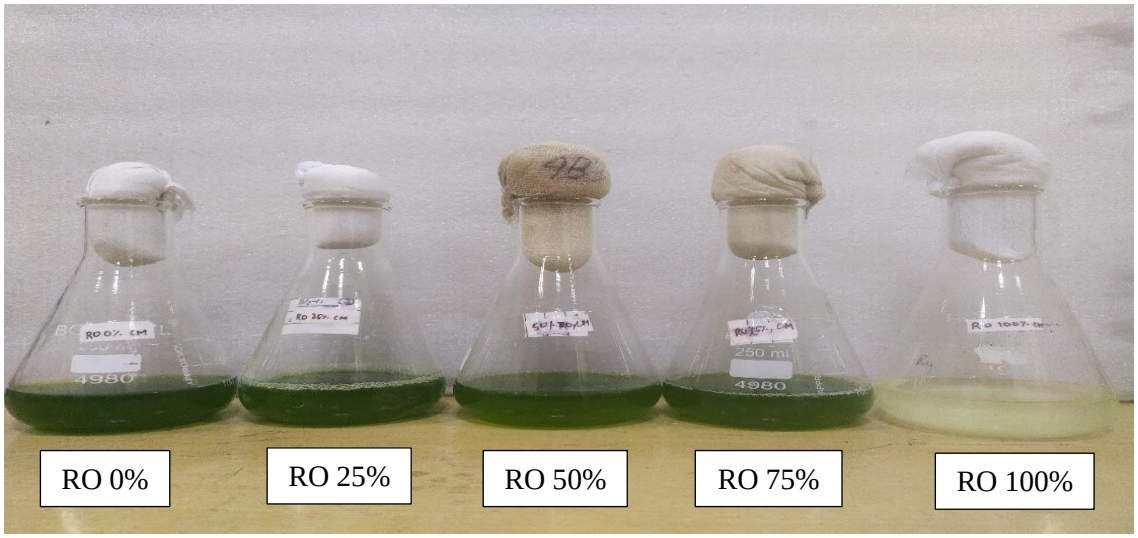


Figure 10: *C. minutissima* cultivated on different proportions of RO spent water and BG11

Table 6: Optical density, biomass productivity and biomass concentration calculated for C. minutissima cultivated on different proportions of BG11 and RO spent water.

RO spent water (ml)	BG11 medium (ml)	Total volume (ml)	OD- 28 days	Biomass (mg)	Biomass Productivity (mg L^{-1} day^{-1})	Biomass Concentration (mg/L)

0	100	100	2.7914	4.2	55.5	1160
25	75	100	2.8572	5.33	44.5	1066
50	50	100	2.5006	5.58	44.57142857	1166
75	25	100	1.0287	2.9	7.928571429	580
100	0	100	0.2045	1.69	4.35714286	338

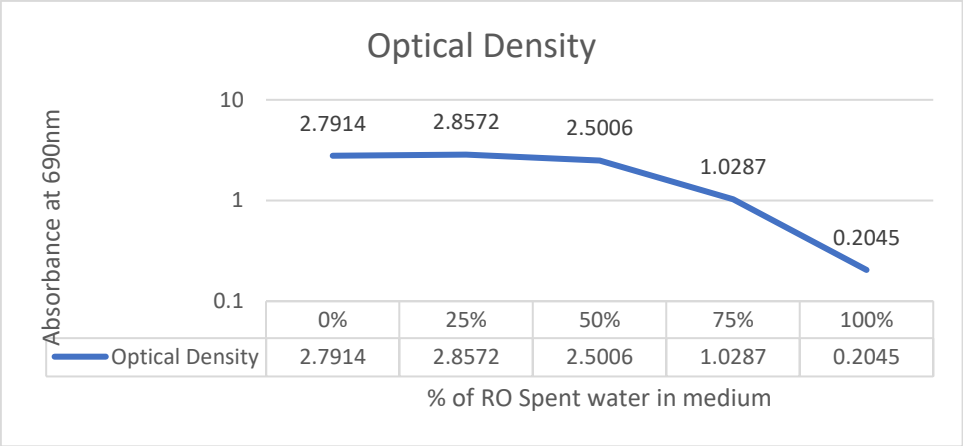


Figure 11: Growth pattern of *C. minutissima* over 28 days on different proportions of BG11 and RO spent water

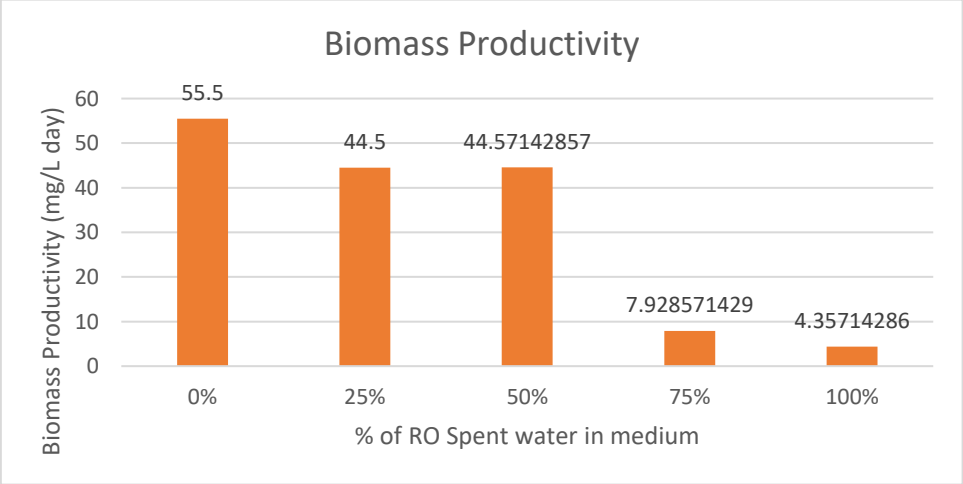


Figure 12: Biomass productivity of *C. minutissima* over 28 days on different proportions of BG11 and RO spent water

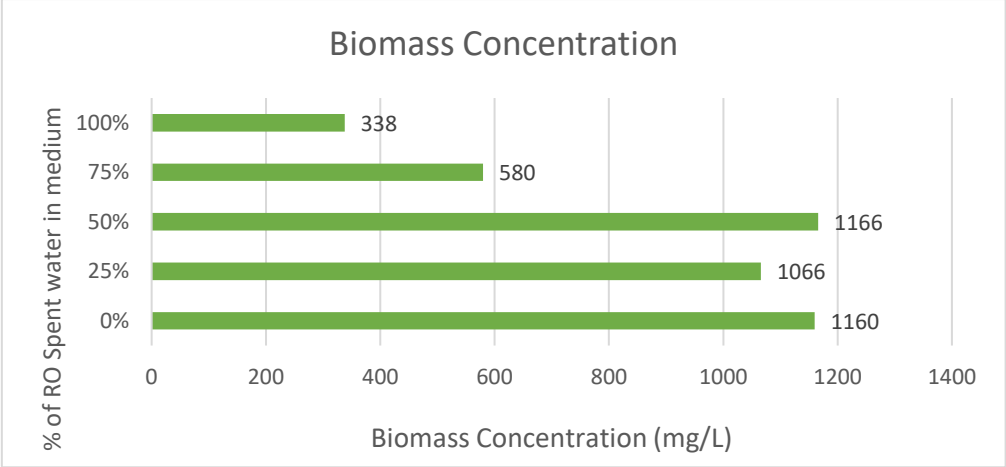


Figure 13: Biomass concentration of *C. minutissima* over 28 days on different proportions of BG11 and RO spent water

RO spent water 25% and 50% with BG11 were found out to be best for growth of *C. minutissima* as an increased biomass productivity and biomass concentration was obtained at these mixture proportions. Thus, RO spent water 25% and 50% with BG11 can also be used for the cultivation and biomass production of *C. minutissima* for biogenic Ag-NPs synthesis.

2. Qualitative assessment of bioactive compounds in Algal extract

On performing various qualitative tests on *C. minutissima*, it was confirmed that the algal species under consideration contains various biochemical compounds such as alkaloids, phenols, flavonoids, and carotenoids.

Table 7: Results of presence and absence of various biochemical compounds in *Chlorella minutissima*.

Bioactive Compound	**Result**	**Inference**
Alkaloids	Formation of white precipitate	Presence of Alkaloids was confirmed in the algal sample
Carotenoids	OD_{470}- 0.8178	Presence of Carotenoid was confirmed in the algal sample
Flavonoids	OD_{420}- 0.2887	Presence of Flavonoids was confirmed in the algal sample
Phenols	Green colored solution was observed	Presence of phenol was confirmed in the algal sample

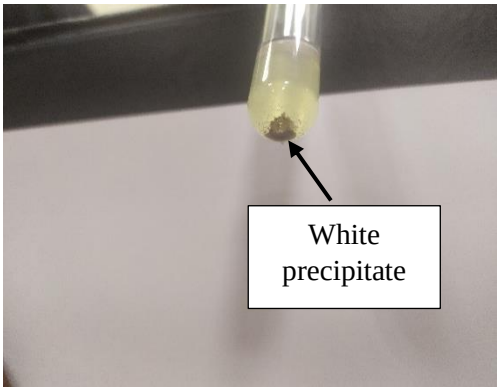


Figure 14: White ppt formation in test for Alkaloids

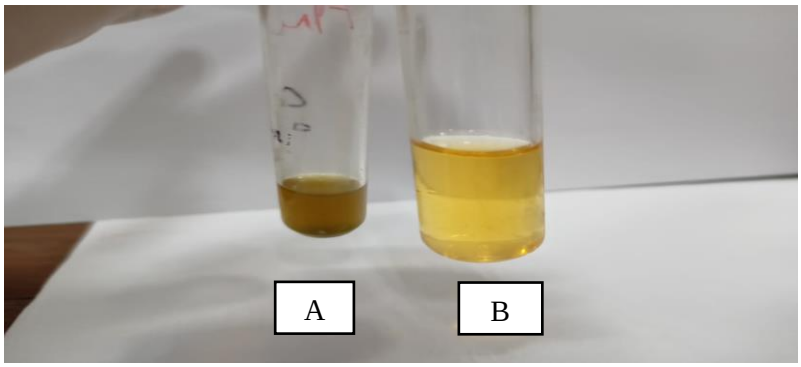


Figure 15: A- Formation of green coloration – confirmation for the presence of ethanol. B- Neutral $FeCl_3$ solution

3. β-Carotene: Quantitative assay

Beta-carotene yield was found to be much higher in case of C. minutissima when cultivated in BG11 alone as compared to when cultivated in glucose supplemented BG11. The highest amount of beta-carotene was extracted using THF as a solvent and bead milling as a cell disruption technique.

Table 8: Amount of beta-carotene extracted using various cell disruption methods and C. minutissima cultivated on different medium.

Samples	Amount of Beta-carotene (µg/mL)
C. minutissima cultivated in BG11 (THF + Sonicated)	5.292
C. minutissima cultivated in BG11 (THF + Heat treatment)	5.932

C. minutissima cultivated in BG11 (THF + Bead milling)	6.630
C. minutissima cultivated in BG11 (Water + Sonicated)	4.029
C. minutissima cultivated in BG11 + Glucose (THF + Sonicated)	1.432
C. minutissima cultivated in BG11 + Glucose (THF + Heat treatment)	0.8643
C. minutissima cultivated in BG11 + Glucose (THF + Bead milling)	1.9429
C. minutissima cultivated in BG11 + Glucose (Water + Sonicated)	0.68544

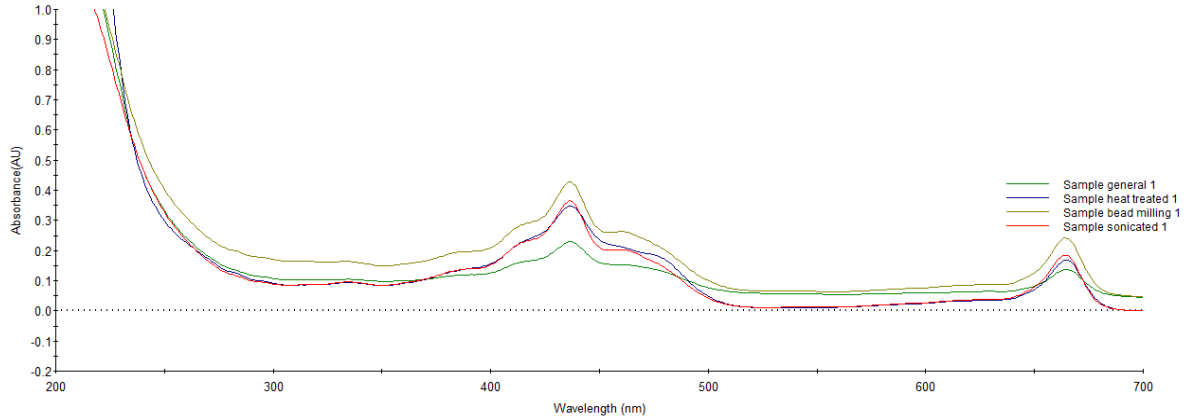


Figure 16: Absorbance plot of beta-carotene extracted from *C. minutissima* cultivated in BG11

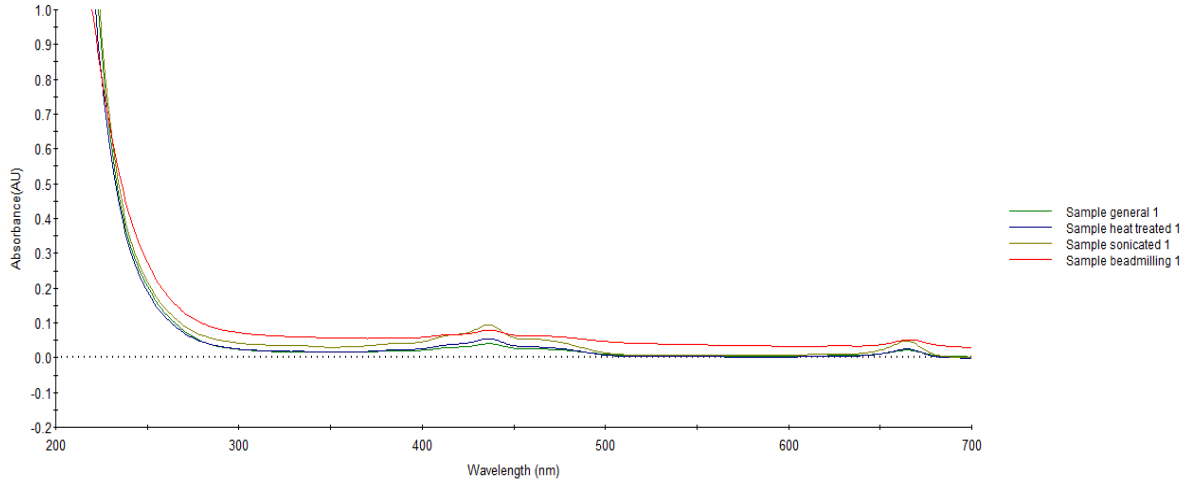


Figure 17: Absorbance plot of beta-carotene extracted from C. minutissima cultivated in BG11 supplemented with glucose

4. Ag-NPs synthesis

These bio-compounds are responsible for various activities performed by these algal species such as capping and stabilizing of nanoparticles, etc.

Ag-NPs were prepared using 100 mL of the wet biomass. 10 mM freshly prepared silver nitrate solution was added to the *C. minutissima* extract. Bio-reduction of silver ions started the moment algal extract was mixed with silver nitrate solution. The process of reduction can be easily be observed as a color change from light green or colorless to light brown in color. The intensity of brown coloration increased with an increase in incubation time. This color change indicated the reduction of silver ions and the formation of Ag-NPs as shown in **figure 18**. The synthesized Ag-NPs were found to be stable when prepared at pH 9 and extract to precursor metal salt solution 10:1. The Ag-NPs produced using cell free extract were found to be stable when prepared at pH 10 and extract to precursor metal salt solution 10:1. Thus, it can be concluded that both the algal extract and cell free extract can be used for the synthesis of Ag-NPs. Using cell free extract can enable us to achieve the concept of zero waste. In BG11 and 1g/L glucose solution there was no color change and absorption scan also indicated that there is no synthesis of Ag-NPs using these solutions as depicted in **figure 19**. The stability of Ag-NPs was checked using UV-Visible spectrophotometer over a duration of period.

$$Ag^{+} \xrightarrow{\text{Bioreduction}} Ag^{0} \xrightarrow{\text{Stability}} Ag^{0}$$

Figure 18: Steps in the bio-reduction and stabilization of silver ions to form Ag-NPs

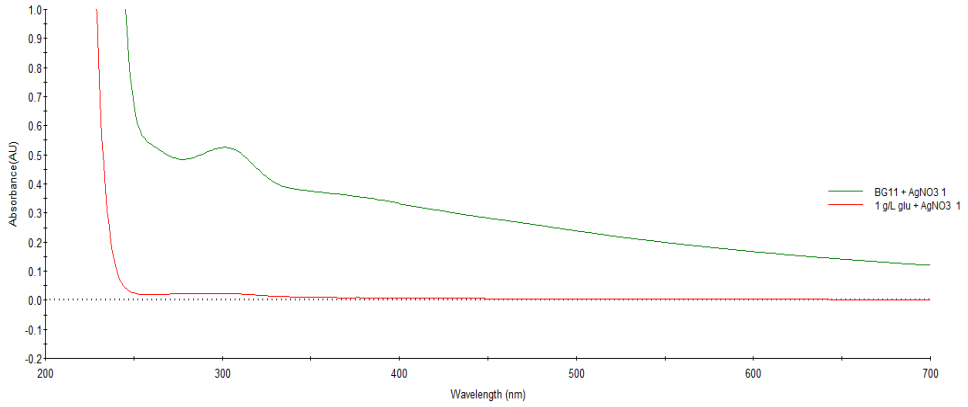


Figure 19: Absorbance scan of mixture of BG11 + 10 mM $AgNO_3$ and 1g/L glucose + 10 mM $AgNO_3$ solution incubation in light condition after 48 hours.

5. Characterization

The initial identification was for the synthesis of Ag-NPs were done through observing the visual changes in color of the mixture of extract and silver nitrate solution. The intensity of color depends upon the concentration of precursor metal salt solution, pH, concentration of nanoparticles. It was observed that higher concentrations of Ag-NPs lead to a dark brown coloration. UV- Visible spectroscopy was used for the confirmation of synthesis of Ag-NPs. It was observed that formation of Ag-NPs was observed around 400-450 nm, while the peaks of silver nitrate was observed at around 300 nm and that of *C. minutissima* extract at 300-350 nm as depicted in **figure 20 (a & b)**. FE-SEM analysis of the nanoparticles synthesized at the optimized conditions depicted that the Ag-NPs were spherical in shape as depicted in **figure 21**. FTIR was performed in order to identify the probable biochemical compounds responsible for the reduction of silver anion and capping of the reduced silver nanoparticles synthesized using microalgae *Chlorella minutissima* extract. The **figure 22** represents the FTIR spectrum of biogenic silver nanoparticles synthesized using *C. minutissima* extract. The spectrum exhibits the peak at 3350 cm^{-1} (H–bonded hydroxyl groups), 1600 cm^{-1} (C O stretching band of the carboxylic acid group), 1150 cm^{-1} (C–O or C–O–C stretching vibrations), 1100 cm^{-1} (C–O stretching of alcoholic groups), 1000 cm^{-1} (C-N amine maybe the nitro groups) and 950 cm^{-1} (vinyl bond trisubstituted alkanes). XRD pattern of the algal mediated synthesized Ag-NPs is illustrated in **figure 23**. Distinct XRD patterns were observed at 38.31°, 44.61°, 64.5° and 76.5°. The distinct peaks at 2θ values of 38.31°, 44.61°, 64.5° and 76.5° corresponds to (1 1 1), (2 0 0), (2 2 0), and (3 1 1) planes of FCC (face-centered cubic) structure of silver lattice [106]. XRD pattern reveals that the bio-synthesized Ag-NPs are crystalline in nature. Zeta-potential analysis of Ag-NPs revealed that the bio-synthesized Ag-NPs were highly stable with a mean size of 78.28 nm. The size distribution graph obtained from zeta-potential analysis is shown in **figure 24**.

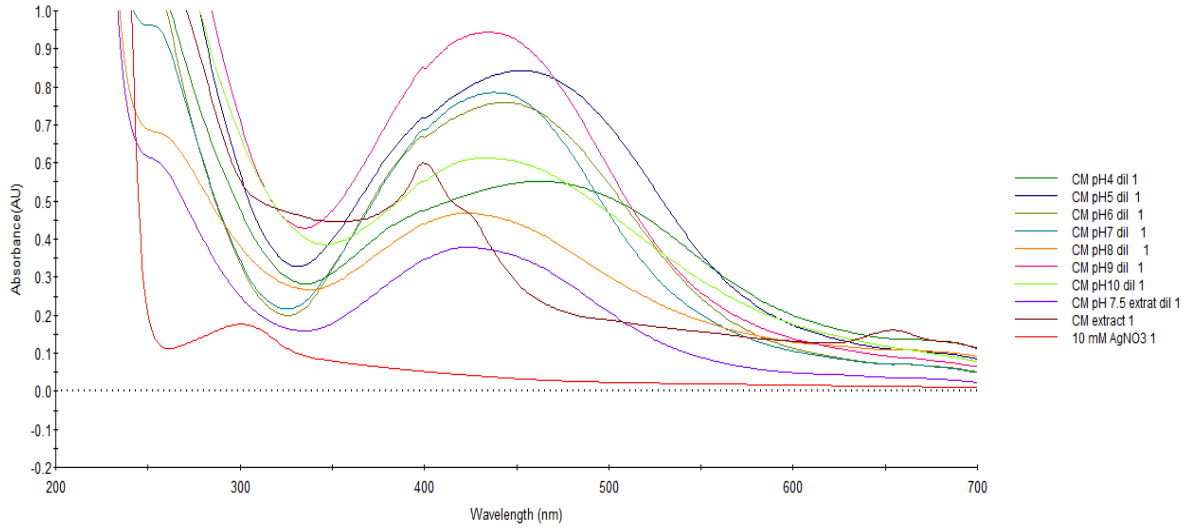


Figure 20 (a): Absorbance scan plot of Ag-NPs synthesized using *C. minutissima* extract after 24 hours of incubation.

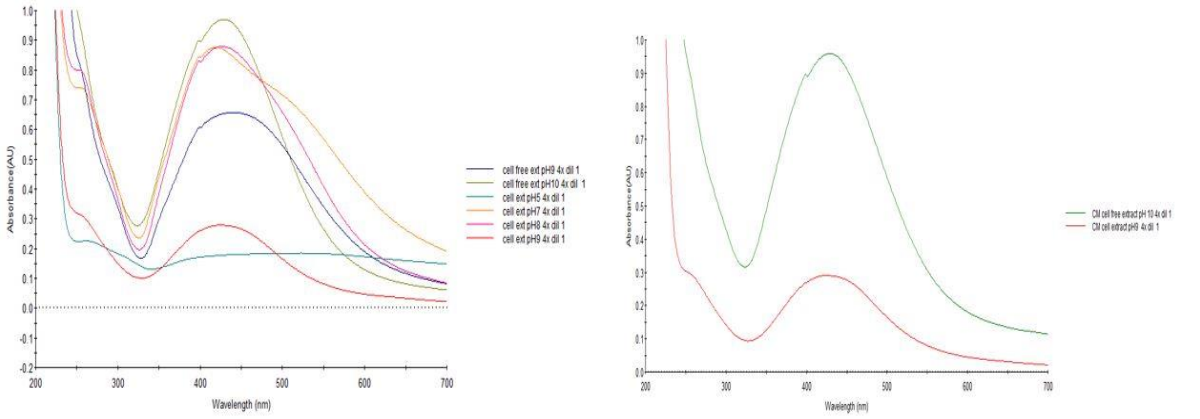


Figure 20 (b): Absorbance scan of the synthesized AgNPs from cell free extract, pH 9 and 10 after 24 and 120 hours of incubation in light condition.

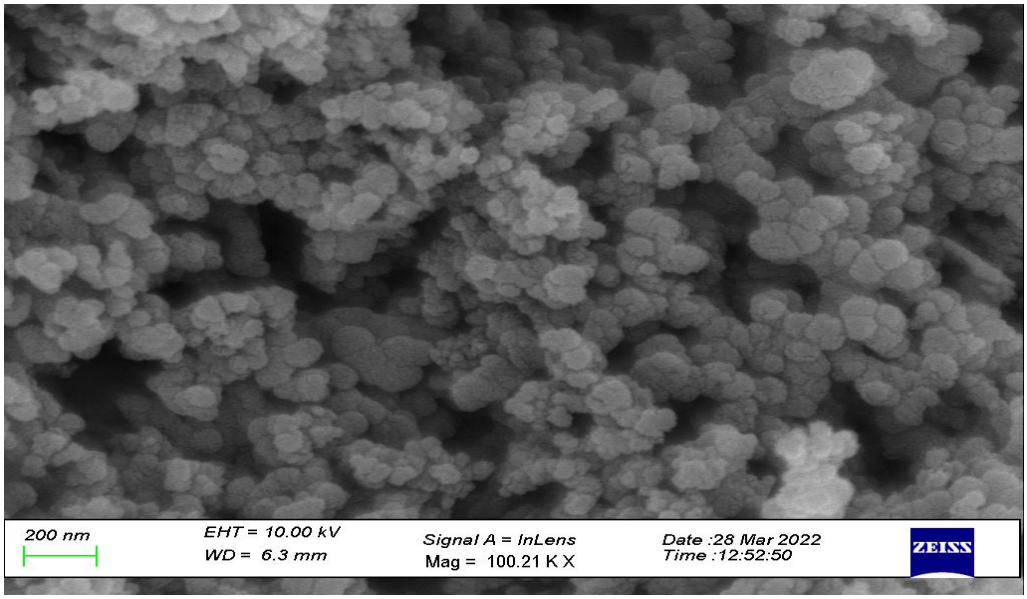


Figure 21: Morphological analysis of Ag-NPs synthesized using *C. minutissima* by FE-SEM.

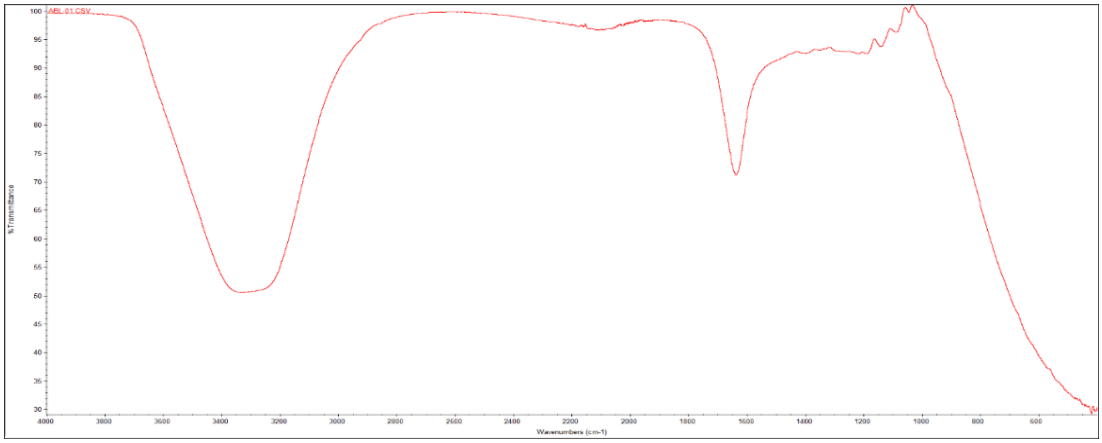


Figure 22: FTIR spectrum of Ag-NPs synthesized using *C. minutissima* extract.

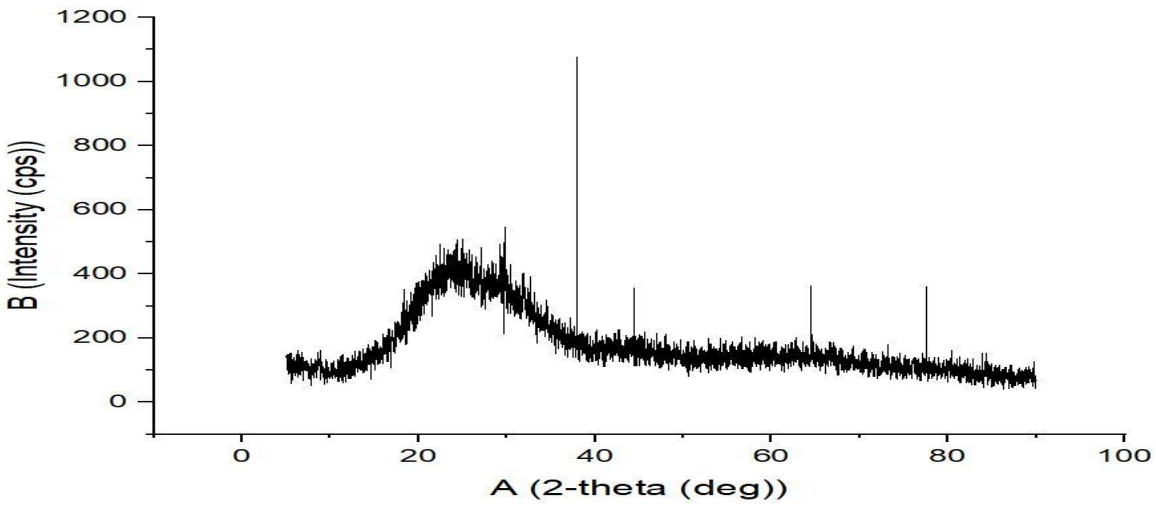


Figure 23: XRD plot of Ag-NPs synthesized using C. minutissima extract

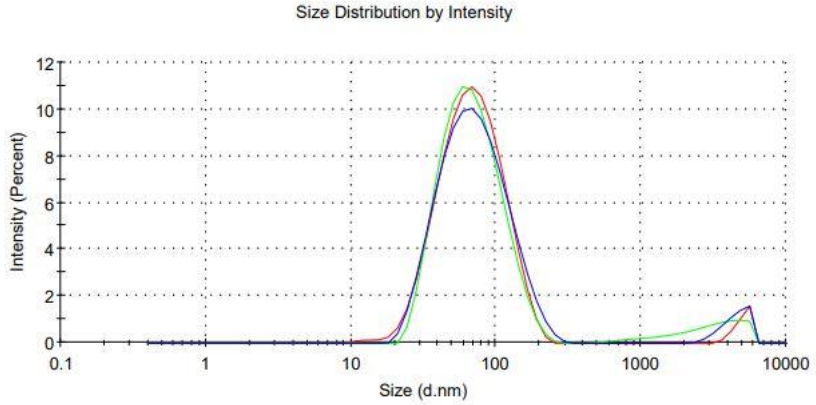


Figure 24: Size distribution of Ag-NPs prepared using *C. minutissima* extract analyzed using Zeta- potential

6. Applications

Antibacterial activity of Ag-NPs

The effectiveness of synthesized Ag-NPs as antibacterial agent was evaluated using zone of inhibition produced by different volumes of Ag-NPs and for control algal extract and $AgNO_3$ solution was also placed on the disk and zone of inhibition was checked for the test solutions as well. The synthesized Ag-NPs were found to be highly effective antibacterial agents at higher concentrations as compared to lower concentrations. Amongst the five test bacterial species, Ag-NPs produced maximum zone of inhibition against *B. cereus* (21 ± 1 mm), followed by *S. aureus* (20 mm) and *E. coli* (20 mm). Ag-NPs were found to be moderately effective against *Salmonella* sp. *Klebsiella* sp. was found to be least susceptible towards Ag-NPs as depicted in **figure 25**. This least susceptibility can be attributed to the presence of acidic capsule around the bacterial cell wall which might have prevented the diffusion of Ag-NPs inside the cell and thus preventing the death of bacterium.

Table 8: Zone of inhibition (mm) depicted by various amounts of Ag-NPs on 5 different bacterial species

Test Compound	**Average zone of inhibition (mm)**				
	E. coli (G-ve)	*Salmonella* sp (G-ve)	*Klebsiella* sp (G-ve)	*Staphylococcus aureus* (G+ve)	*Bacillus cereus* (G+ve)
Algal extract	0	0	0	0	0

$AgNO_3$ solution	7	6	7	8	7 ± 1
Ag-NPs (10 µL)	10	6 ± 1	7 ± 1	12 ± 1	12
Ag-NPs (20 µL)	14 ± 1	10	10	14 ± 1	15 ± 1
Ag-NPs (30 µL)	17 ± 1	12 ± 1	11 ± 1	16	17 ± 1
Ag-NPs (40 µL)	18 ± 1	14	13 ± 1	18	18
Ag-NPs (50 µL)	20	17 ± 1	15	20	21 ± 1

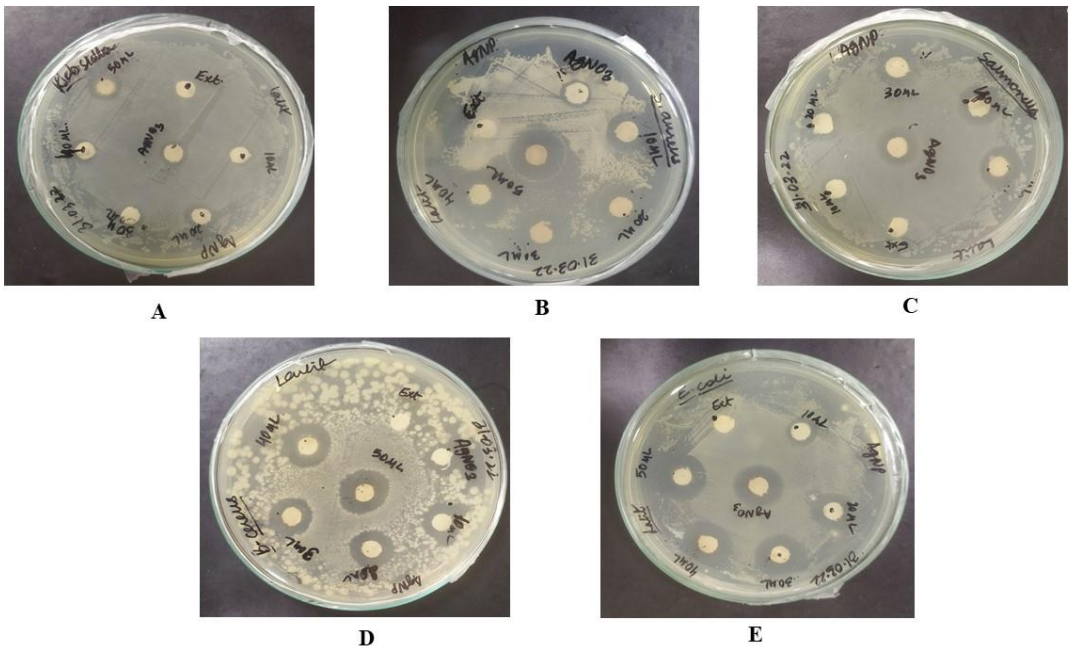


Figure 25: Nutrient agar plate showing zone of inhibition due to the activity of Ag-NPs produced by extract of *Chlorella minutissima*. (A) Ag-NPs tested against *Klebsiella* sp., (B) Ag-NPs tested against *Staphylococcus aureus*, (C) Ag-NPs tested against *Salmonella* sp., (D) Ag-NPs tested against *Bacillus cereus*, and (E) Ag-NPs tested against *E. coli*.

Heavy metal remediation using Ag-NPs synthesized from *C. minutissima*

Chromium removal

The removal efficiency of Chromium (30 PPM) was found to be best employing 4 mg/ 5mL of biosynthesized AgNPs as compared to 2 mg/ 5mL concentration of AgNPs. Removal efficiency was found to be pH dependent and the best removal was carried out at pH 3 and slightly comparable at pH 4 also as shown in **figure 26 and 27**.

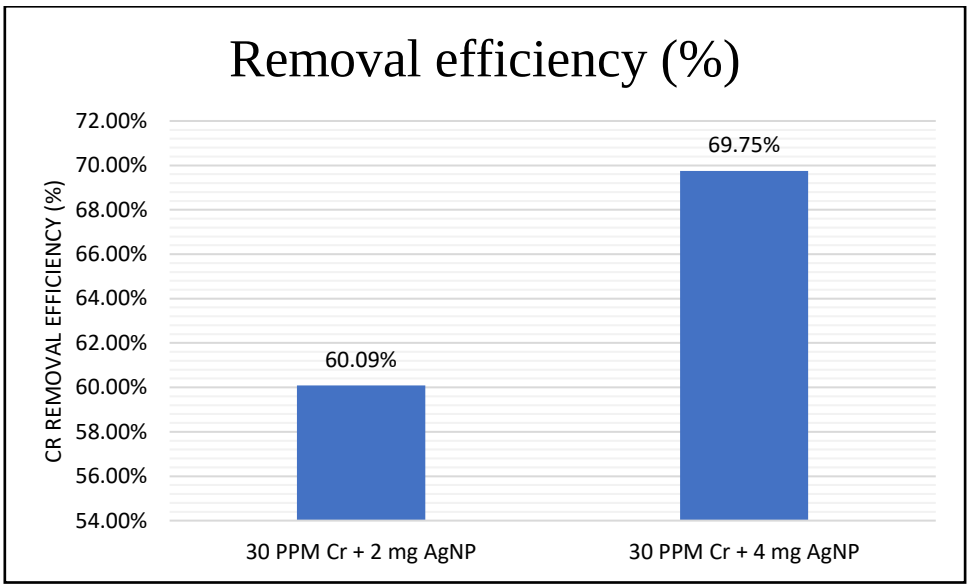


Figure 26: Removal efficiency of Chromium at different concentrations of Ag-NPs

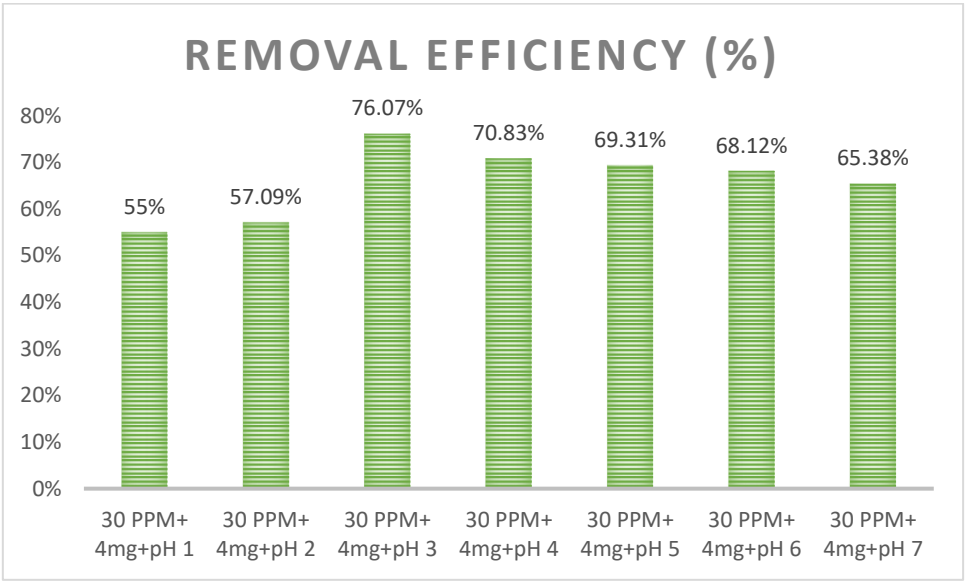


Figure 27: Removal efficiency of Chromium maintained at different pH.

Nickel removal

The removal efficiency of Nickel (100 PPM) was found to be best employing 4 mg/ 5mL of biosynthesized AgNPs as compared to 2 mg/ 5mL concentration of AgNPs. Removal efficiency

was found to be pH dependent and the best removal was carried out at pH 3 and slightly comparable at pH 4 also as represented in **figure 28 and 29**.

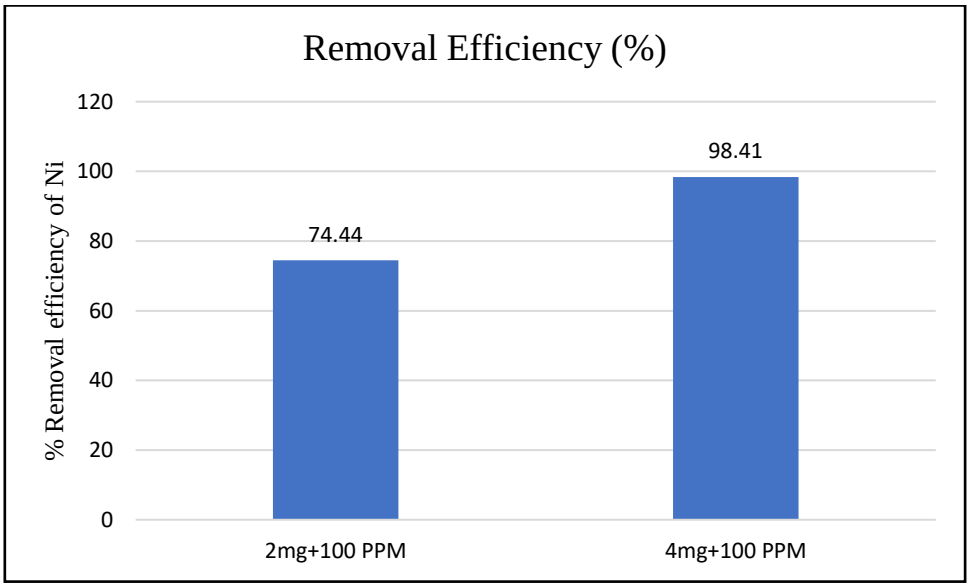


Figure 28: Removal efficiency of Nickel at different concentrations of Ag-NPs

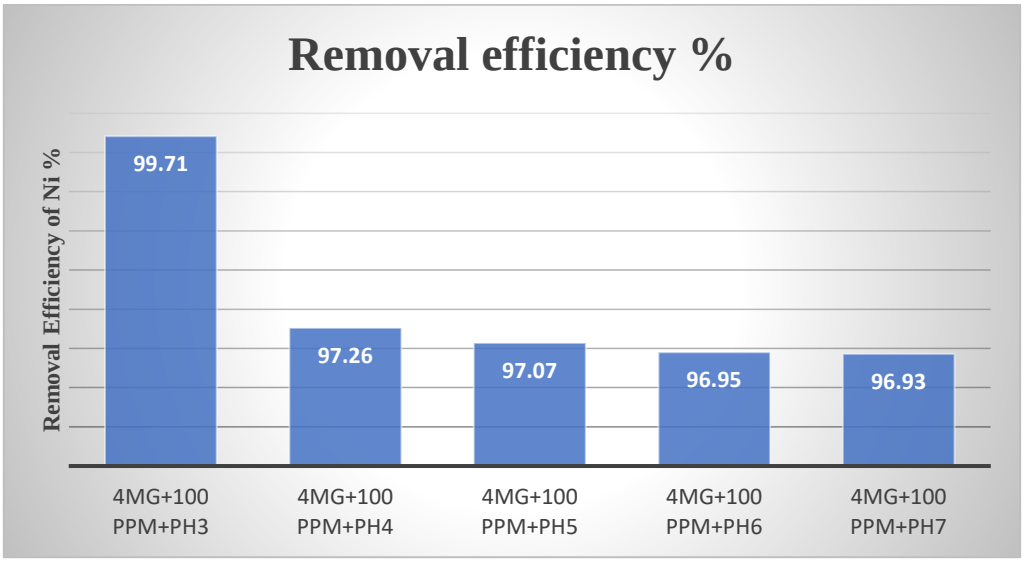


Figure 29: Removal efficiency of Nickel maintained at different pH.

Photocatalytic degradation of Methylene blue dye using Ag-NPs synthesized from *C. minutissima*

The photocatalytic dye degradation efficiency of Ag-NPs synthesized using *C. minutissima* was found to be 82.34% after incubation of 10 hours in the presence of sun light represented in **figure 30**.

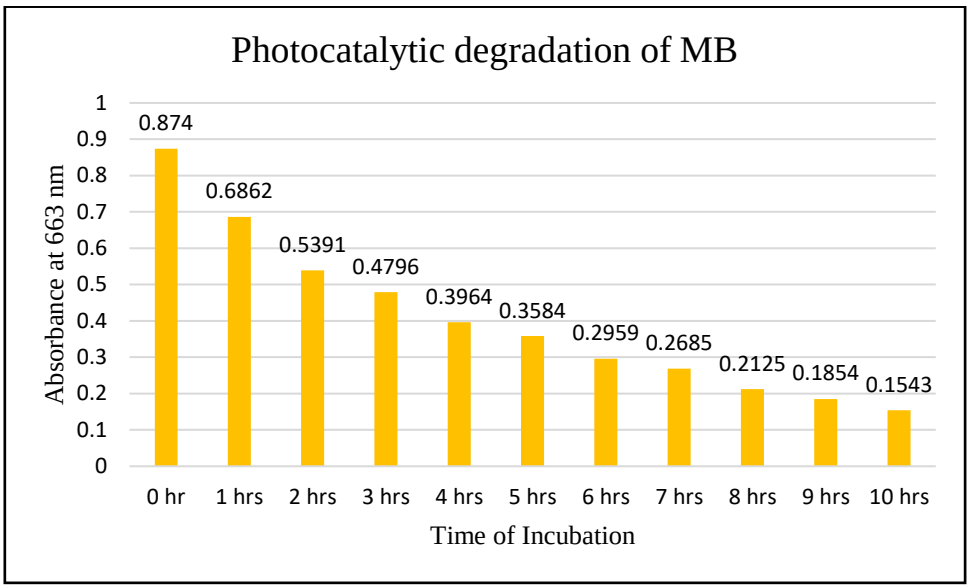


Figure 30: Photocatalytic degradation efficiency of Ag-NPs on Methylene blue dye

Degradation efficiency (%) = [(Initial Concentration – Final concentration)/ Initial concentration] * 100

CONCLUSION

Chlorella minutissima acts a good source of beta-carotene- a nutraceutical. Beta-carotene possesses various health benefits such as it acts as an antioxidant. Extraction of beta-carotene can be done using various organic solvents and cell disruption techniques. Indiscriminate utilization of antimicrobial agents has culminated into antimicrobial resistance among a number of pathogenic microbial strains. Infections caused by antimicrobial resistant strains are difficult to treat and is highly expensive, making it more harmful to the lower income population. Consequently, the search for newer compounds exhibiting antimicrobial effect started. Nanoparticles have inherent antimicrobial properties and can potentially replace the traditionally used antimicrobials. Environmental pollution is a prominent threat in the current times. The pollution led irreparable and irreversible loss in public and environmental health. Several physicochemical methods have been devised to tackle the pollution led problems. But these methods are either economically inefficient or ineffective or not sustainable as a whole. Nanotechnology based interventions are recently gained attraction for remediation of pollution. To make nanoremediation more meaningful, biogenic synthesis has been suggested. In this direction, algae-based nanoparticles synthesis and their use as agent for remediating environmental pollutants. In this study, beta-carotene was best extracted using THF as a solvent and bead milling as a cell disruption technique. C*hlorella minutissima* is used for synthesis of the silver nanoparticles. The synthesis of the silver nanoparticles was confirmed initially using spectrophotometer. As time progress, the silver nanoparticles formation takes place upon mixing of silver metal precursors and *Chlorella* extract in a certain proportion. Further characterization was confirmed via FE-SEM, FTIR, XRD, and Zeta potential analyzer. Synthesized silver nanoparticles exhibited antibacterial activity against five different bacteria namely in order *Bacillus cereus*, followed by *Staphylococcus aureus* and *E. coli* and were moderately effective against *Salmonella* sp. and *Klebsiella* sp. Thus, it can be a potential alternative for antibiotics and can aid into fight against antimicrobial resistance led public health loss. In addition to this, these silver nanoparticles were used as adsorbent and catalyst for removal of chromium and nickel from synthetic wastewater, and photocatalytic degradation of methylene blue dye respectively. Thus, it can be concluded that C*hlorella minutissima* is a potential alga for synthesis of silver nanoparticles to be used as antimicrobial agent, adsorbent for heavy metals, and a catalyst for dye degradation.

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APPENDIX

Media Composition:

Media 1- BG11 Medium (pH-8)

Stock 1

Chemical Compound	Concentration (g/L)
Na-MGEDTA	0.1
Ferric ammonium citrate	0.6
Citric acid.H_2O	0.6
$CaCl_2.2\ H_2O$	3.6

Stock 2

Chemical Compound	Concentration (g/L)
$MgSO_4$	7.5

Stock 3

Chemical Compound	Concentration (g/L)
$K_2HPO_4.3H_2O$	4
Or K_2HPO_4	3.05

Stock 4

Chemical Compound	Concentration (g/L)
H_3BO_3	2.86
$MnCl_2.4H_2O$	1.81
$ZnSO_4.5H_2O$	0.222
$CuSO_4.5H_2O$	0.079
$CoCl_2.6H_2O$	0.050
$NaMoO_4.2H_2O$	0.018

Final Composition

Stock solution	Amount (per Liter)
Stock 1	10 mL
Stock 2	10 mL
Stock 3	10 mL
Stock 4	1 mL
Na_2CO_3	0.02
$NaNO_3$	1.5

Medium 2- Zarrouk Synthetic Medium (pH-8)

Compound	Concentration (gm/L)
$NaHCO_3$	16.8
$NaNO_3$	2.5
K_2HPO_4	0.5
K_2SO_4	1.0
NaCl	1.0
$MgSO_4.7H_2O$	0.2
$CaCl_2$	0.04
$FeSO_4.7H_2O$	0.01
EDTA	0.08
Micronutrients	

Medium 3- BBM medium (pH-8)

Compound	Stock (gm/L)	Amount (mL per L of medium)
$NaNO_3$	25	10
$CaCl_2.2H_2O$	2.5	10
$MgSO_4.7H_2O$	7.5	10

$10K_2HPO_4$	7.5	10
KH_2PO_4	17.5	10
NaCl	2.5	10
Alkaline EDTA solution EDTA (Triplex III) KOH	 50- 1 mL 31- 1 mL	1
Acidified Fe Solution $FeSO_4.7H_2O$ H_2SO_4 (Conc)	 4.98 1 mL	1
H_3BO_3	11.42	1
Trace Elements		1

Medium 4- SE (Bristol's solution) Medium (pH-8)

Compound	**Stock (gm/100 mL)**	**Amount (mL per L of medium)**
$NaNO_3$	25	1
K_2HPO_4	7.5	1
$MgSO_4.7H_2O$	7.5	1
$CaCl_2.2H_2O$	2.5	1
KH_2PO_4	17.5	1
NaCl	2.5	1
$FeCl_3.6H_2O$	0.5	1
EDTA- Fe-Monosodium	1 mg in 1 mL	1
Trace elements		1
Soil Extract		40

Trace elements	**Concentration (mg/mL)**
H_3BO_3	2.86

$MnCl_2.4H_2O$	1.86
$ZnSO_4.7H_2O$	0.22
$Na_2MoO_4.2H_2O$	0.39
$CuSO_4.5H_2O$	0.08
$Co(NO_3)_2.6H_2O$	0.05

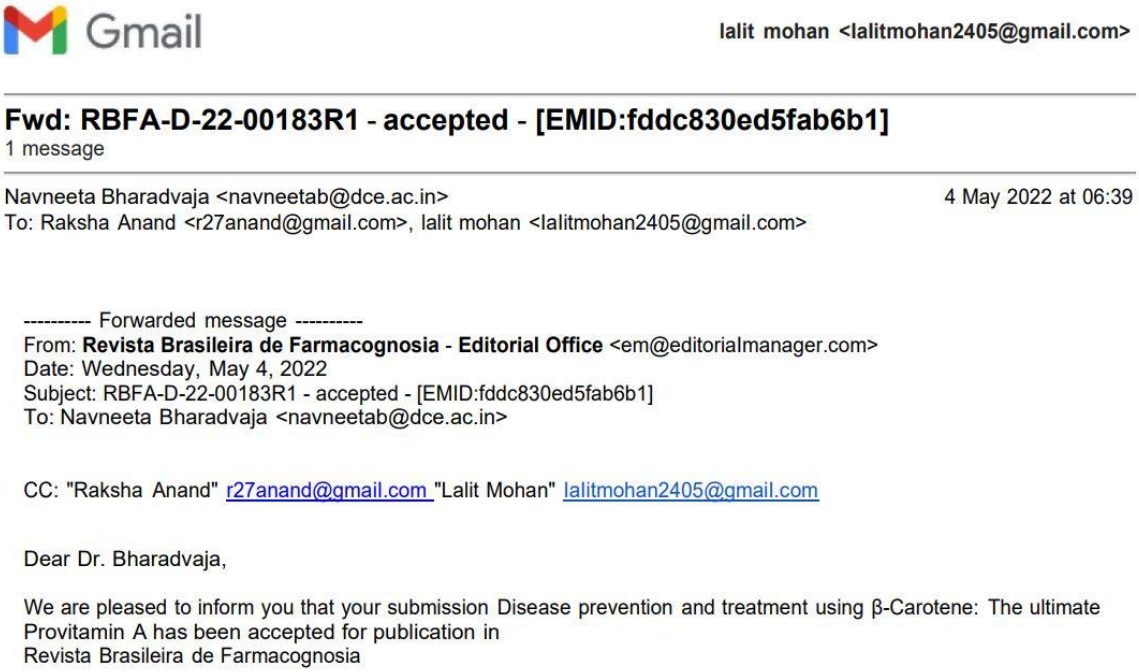
Medium 5- Bold 3N medium (pH-8)

Compounds	**Stock (gm/ 400 mL)**	**Amount (mL/L)**
$NaNO_3$	10	30
$CaCl_2.2H_2O$	1	10
$MgSO_4.7H_2O$	3	10
K_2HPO_4	3	10
KH_2PO_4	7	10
NaCl	1	10
Soil Extract		1
P IV		6

P IV	**Concentration (g/L)**
Na_2 EDTA	0.75
$MnCl_2.4H_2O$	0.041
$ZnSO_4$	0.005
$Na_2MoO_4.2H_2O$	0.004
$FeCl_3.6H_2O$	0.097
$CoCl_2.6H_2O$	0.002

LIST OF PUBLICATIONS

1. Anand Raksha, Mohan Lalit, and Bharadvaja Navneeta (2022). Disease prevention and treatment using β-carotene: the ultimate pro-vitamin A. Revista Brasileira de farmacognosia. (Accepted)- SCIE
2. Anand Shaubhik, Mohan Lalit and Bharadvaja Navneeta (2022). A Review on Ayurvedic Non-Carbohydrate Prebiotics. ECS Transactions, 107 (1) 13505-13514 doi: 10.1149/10701.13505ecst



Gmail lalit mohan <lalitmohan2405@gmail.com>

Fwd: RBFA-D-22-00183R1 - accepted - [EMID:fddc830ed5fab6b1]
1 message

Navneeta Bharadvaja <navneetab@dce.ac.in> 4 May 2022 at 06:39
To: Raksha Anand <r27anand@gmail.com>, lalit mohan <lalitmohan2405@gmail.com>

---------- Forwarded message ----------
From: **Revista Brasileira de Farmacognosia - Editorial Office** <em@editorialmanager.com>
Date: Wednesday, May 4, 2022
Subject: RBFA-D-22-00183R1 - accepted - [EMID:fddc830ed5fab6b1]
To: Navneeta Bharadvaja <navneetab@dce.ac.in>

CC: "Raksha Anand" r27anand@gmail.com "Lalit Mohan" lalitmohan2405@gmail.com

Dear Dr. Bharadvaja,

We are pleased to inform you that your submission Disease prevention and treatment using β-Carotene: The ultimate Provitamin A has been accepted for publication in
Revista Brasileira de Farmacognosia

Gmail lalit mohan <lalitmohan2405@gmail.com>

Fwd: ECS Transactions: Manuscript #ICTSGS-1990 Decision Letter

1 message

Navneeta Bharadvaja <navneetab@dce.ac.in> 28 January 2022 at 15:51
To: lalit mohan <lalitmohan2405@gmail.com>

---------- Forwarded message ----------
From: <ecs@confex.com>
Date: Friday, January 28, 2022
Subject: ECS Transactions: Manuscript #ICTSGS-1990 Decision Letter
To: navneetab@dce.ac.in

Dear Dr. Navneeta Bharadvaja,

I am pleased to inform you that your manuscript, "A Review on Ayurvedic Non-Carbohydrate Prebiotics", has been reviewed and accepted for publication in the issue of "ECS Transactions" (ECST) from the First International Conference on Technologies for Smart Green Connected Society 2021. This issue is scheduled to be published in March 2022.

Authors whose papers will be published in ECST are also urged to submit their papers to one of the Society's peer-reviewed journals: the Journal of The Electrochemical Society (JES) or the ECS Journal of Solid State Science and Technology (JSS). While the expectation is that six months is sufficient time to revise an ECST paper to meet the stricter standards of the journals, there is no deadline for submission. Submissions to the journals must be made using the online submission system. Click here for author instructions: http://ecsdl.org/site/ecs/manuscript_submissions.xhtml.

Thank you for contributing your work to ECST. If you have any questions or comments, please feel free to contact the ECST staff at ecst@electrochem.org.

Sincerely,

Srinesh Thakur
Editor, First International Conference on Technologies for Smart Green Connected Society 2021
"ECS Transactions", Volume 106

INTERNATIONAL CONFERENCE ON TECHNOLOGIES FOR SMART GREEN CONNECTED SOCIETIES 2021

NOVEMBER 29-30, 2021|ONLINE| WORLDWIDE

LALIT MOHAN

DELHI TECHNOLOGICAL UNIVERSITY

Presented a paper titled **A Review on Ayurvedic Non-Carbohydrate Prebiotics**
at the ICTSGS-1 conference led by Yamagata University Japan.
ICTSGS-1 IS ORGANIZED BY SPAST FOUNDATION AND ASSOCIATED PARTNER INSTITUTIONS.

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Hidemitsu Furukawa,
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Ajit Khosla,
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A Review on Ayurvedic Non-Carbohydrate Prebiotics

ECS Transactions, 107 (1) 13505-13514 (2022)
10.1149/10701.13505ecst ©The Electrochemical Society

A Review on Ayurvedic Non-Carbohydrate Prebiotics

Shaubhik Anand[a], Lalit Mohan[a], and Navneeta Bharadvaja[a*]

[a] Plant Biotechnology Laboratory, Department of Biotechnology, Delhi Technological University, Shahbad Daulatpur, Main Bawana Road, Delhi, 110042

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Prebiotics facilitates the growth of microorganisms which makes a healthy gut microbiota hence known as food for probiotics. They also lower the risk of inflammation and oxidative damage in the gut. Gut enzymes cannot digest prebiotics so gut microbiota uses their own enzymes to ferment them. Upon their fermentation, SCFAs are produced which helps to lower the level of pathogenic species. Primary prebiotic sources are carbohydrate-based like inulin which are present in fiber-based foods. Prebiotics are an essential part of a healthy diet but, their primary sources have a limitation to their consumption. Maintaining a healthy lifestyle through a healthy diet is the key focus in Ayurveda. It highlights the use of non-carbohydrate sources of prebiotics like curcumin, *triphala*, anthocyanin, licorice, and other such phytochemicals, spices, and supplements. It also describes the health benefits of all the individual components of a balanced meal and their prebiotic potential.

Introduction

In 1995, the first definition of prebiotics came out as "a non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and activity of one or limited number of colon microflora and thus improving host health" (1). In the year 2010, International Association for Prebiotics and Probiotics defined prebiotics on functionality basis as "a selectively fermented ingredient that results in specific changes in the composition and activity of gut microbiota and thus, conferring health benefits to the host". FAO defined prebiotics as "non-viable food components that confers a health benefit on the host associated with the modulation of the microbiota" (2). A compound should possess the following characteristics to be classified as prebiotics: 1. compound should be resistant towards the acidic pH found in the human stomach, 2. Compound should be resistant to the hydrolysing enzymes found in the human gut and should not get absorbed by the gastrointestinal tract of humans, 3. Compound should easily get fermented by the gut microbiome and stimulate their growth which ultimately confers health benefit to the consumers (3). Compound such as inulin, which is non-digestible, bifidogenic compound qualify the criteria of being a prebiotic and is naturally found in *Allium cepa* (onions), *Triticum* (wheat), *Allium porrum* (leeks), *Jerusalem artichoke* (sunroot) and *Allium sativum* (garlic). These compounds act as a source of carbon for the gut microbiota and thus stimulates growth by supplying carbon. It also enhances host immune status as fermentation of these compounds synthesize butyric acid, lactic acid, and other such organic acids that decreases the overall pH in the colon thus competitively excludes the pathogens. Prebiotics reduces the intestinal inflammation, helps to prevent diarrhoea, reduces colon cancer

risk, and enhances uptake of minerals. Prebiotics also possesses antioxidant and anti-inflammatory properties which blocks the progression of various diseases (4).

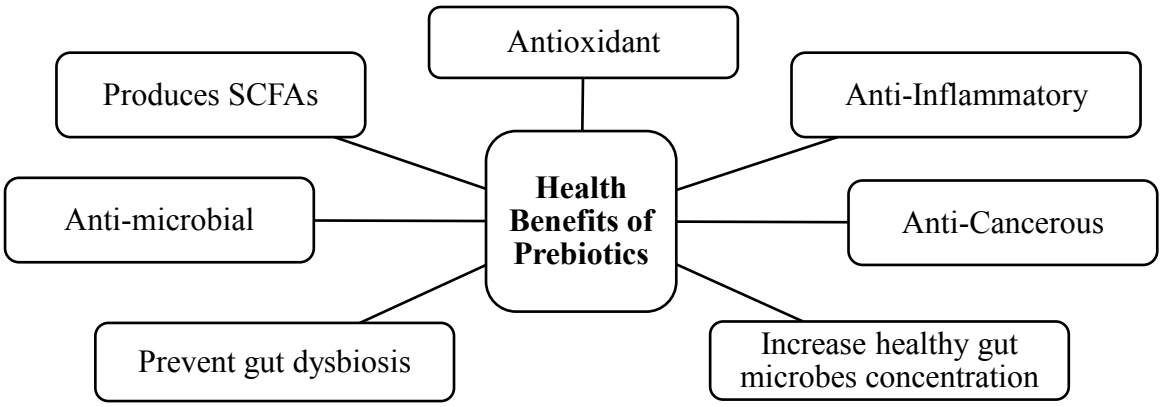

Figure 1: Various health benefits associated with the consumption of prebiotics.

Prebiotics compounds are broadly classified in two categories based on their composition: 1. Carbohydrate-based and 2. non-carbohydrate based. Majority of the prebiotics are carbohydrate based. Inulin and fructo-oligosaccharides (FOS) belong to the category of Fructans as they contain linear chain of β (2→1) linked fructose and a terminal β (2→1) linked glucose. These fructans selectively stimulates the growth of lactic acid bacteria. Galacto-oligosaccharides (GOS) are a product formed due to extension of lactose. GOS are known to stimulate the population of *Bifidobacterium*, *Lactobacillus*, *Bacteroidetes* and *Firmicutes* (5). Resistant starch is known to promote the production of butyrate. They show resistance to the upper gut digestive enzymes and are thus named as resistant starch. It is known to promote the growth of *Firmicutes*, *Ruminicoccus bromii* and *Bifidobacterium* (6,7). β-glucans are non-digestible carbohydrates homopolymer of β-D-glucose which are joined together via glycosidic bond. β-glucans pose many potential health benefits and thus are one of the best candidates for prebiotics (8).

Non-carbohydrate-based prebiotics includes polyphenols, flavanols and anthocyanin stimulates the growth of lactic acid bacteria. Flavanols obtained from cocoa can stimulate the growth of lactic acid producing bacteria (4).

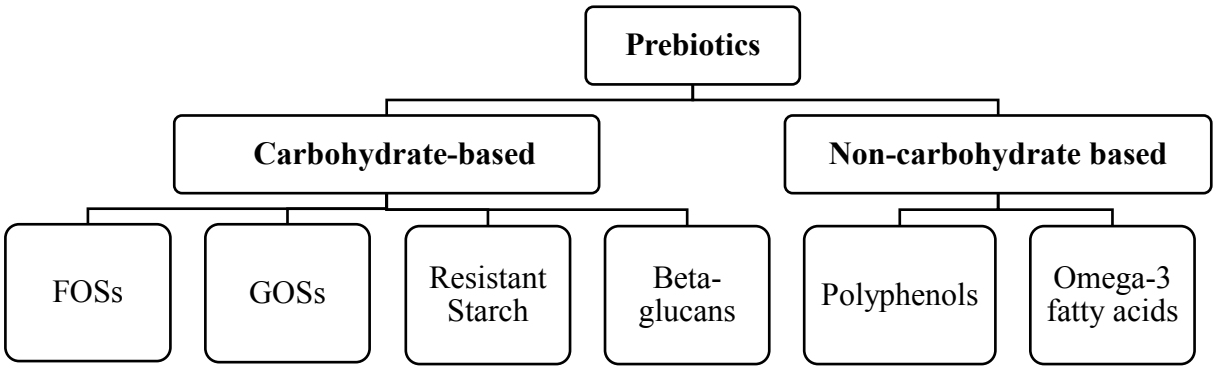

Figure 2: Classification of prebiotics (FOS- Fructo-oligosaccharides, GOS- Galacto-oligosaccharides)

Ayurveda is the ancient science which prevailed in the Indian sub-continent since centuries and is facts mentioned in it is still found to be valid and thus people are orienting back to the traditional knowledge. Ayurveda provides insight about the treatment of diseases and, also provides a way to lead a healthy lifestyle. It contains information about various herbs and associated formulations which is useful for the treatment of various diseases including cancer and other life threating diseases and, also if incorporated in daily routine prevents the development of many lifestyle disorders. Certain formulations present in Ayurveda poses anti-inflammatory and antioxidant properties and can also serve as a source of carbon for the gut microbiota thus, can suitably be used as a prebiotic. This review emphasizes on various such ayurvedic non-carbohydrate prebiotics and, also enlightens the need to incorporate these prebiotics in our daily routine.

Importance of Healthy Gut Microbiota

The population of certain bacterial species like *Lactobacillus* and *Bifidobacterium* should be maintained in the gut because these bacterial species provide numerous health benefits by enhancing the activities of the gut (9). *Bifidobacterium* species inhabit the gut naturally and ferments oligosaccharides and produces butyrate, propionate, and such short-chain fatty acids, SCFAs. Butyrate provides energy to colonocytes found in human colon where it causes apoptosis in the cancer cells. Propionate regulates gluconeogenesis after it gets transferred to the liver. Acetate is utilized in the metabolism of glucose and lipogenesis. It is also hypothesized that acetate plays a key role in appetite control system (10,11). Production of

SCFAs acts an identification for gut friendly bacterial species. Decreased CFU of *Bifidobacterium* inside the gut may also lead to inflammation in the gut. Optimum concentration of *Bifidobacterium* keeps a check on weight gain and obesity (12). While on the other hand, *Lactobacillus* species downregulates the inflammation of the gastrointestinal (GI) tract. *Lactobacillus* species are also known to diminish the symptoms of IBS (irritable bowel syndrome), tackle the problem of lactose intolerance and also helps in relieving constipation. Inflammation of gut is majorly associated with the disturbance in the gut microflora (13,14). Decreased levels of both *Bifidobacterium* and *Lactobacillus* results in gut inflammation. Consuming prebiotics enhances their growth as a result reduces inflammation. A stable and healthy gut microflora prevents the invasion and colonization of the gut mucosa by pathogenic species by competing for the receptors for attachment, competition for nutrients. The normal gut microflora also lowers the gut pH with their secretions which exempts the growth of pathogenic species (12). Probiotic organisms also produce certain bacteriocins and other inhibitory compounds that inhibits the growth and further colonization of gut by pathogenic bacteria. Gut microflora increases the production of antioxidants inside the body which prevents the onset of many gut related disorders (15). Healthy gut microflora helps to lower the concentration of indoxyl sulphate which is a potent kidney toxin (16).

Healthy gut microbiota further improves the lipid metabolism. Any disturbance or dysbiosis in the gut microbiota leads to “diet induced obesity” and several metabolism associated complications which may lead to dysregulation of immune system, dysregulation in the energy regulation, alterations in the level of gut associated hormones and inflammatory responses (10,17). Individuals suffering from diseases like diabetes both type I and type II, inflammatory bowel disease, psoriatic arthritis, and arterial stiffness have been reported with decreased levels of gut microbiota as compared to their healthy counterparts. Thus, reduced diversity of gut microbiota can be associated with several diseases (17). The important function of gut microbiota can be demonstrated or established by the transplantation of faecal microbiota for several pathogenic infections such as during the infection caused by drug resistant *Clostridium difficile* (18). Also, the dependence of gut microbiota is very high on dietary intake of an individual. Microbial consortium in the gut can alter within few days of change in diet. Along with diet several other factors responsible for alterations in gut microbiota are age of the individual and consumption of antibiotics (19).

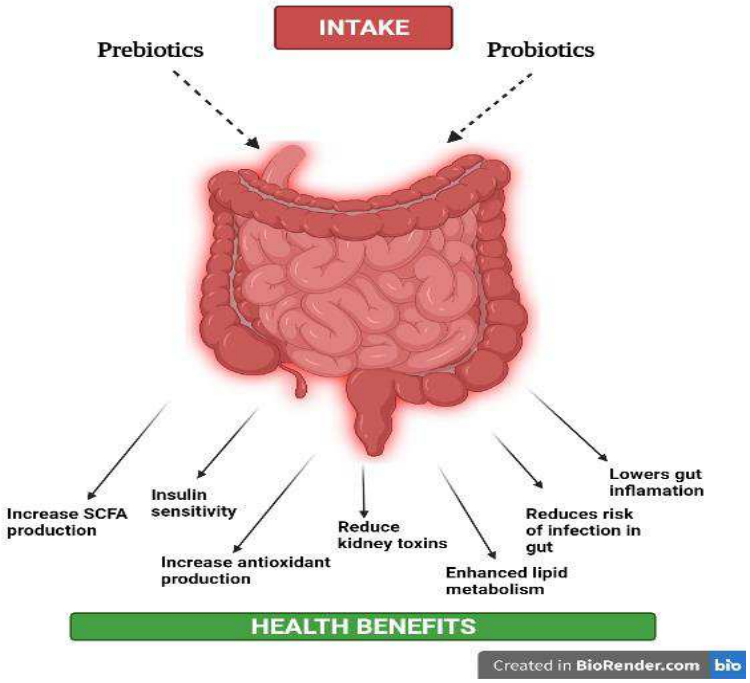

Figure 3: Schematic representation of the various health benefits associated with the intake of probiotics and prebiotics which enables to maintain a healthy gut microflora (created using biorender.com).

Ayurveda and Prebiotics

In Ayurveda, food is considered as source of nutrition as well as a medicine. A proper diet can serve as a preventive as well as curative measure against several diseases. A proper diet which contains the right amount of balance of all six *rasas* is an essential part of a healthy lifestyle. Not only that, but a proper diet should also contain micronutrients, macronutrients, and fibers. It also should have several bioactive compounds like flavonoids, tannins, isoflavones, and lignans. These bioactive compounds provide several health benefits to our body like scavenging reactive species, reducing inflammation, increases the population of good gut bacteria while killing the pathogenic ones. These bioactive compounds have been a part of our diet or has been taken after the meal. They are non-digestible by the gut enzymes, can be used as carbon source and provides additional health benefits. Thus, if taken as is regulatory manner they can serve as a source of non- carbohydrate prebiotics (20).

Table I. Potential non-carbohydrate prebiotic sources and their health benefits

Potential prebiotics	Bioactive compounds	Anti-inflammatory	Antioxidant	Healthy Microbiome growth	Antimicrobial	Prevent gut dysbiosis	Additional benefits	References
Ginger	Gingerol	✔	✔	✔ *Bifidobacterium, Lactobacillus*	✔ *Klebsiella pneumoniae, Escherichia coli, Bartonella, Atopostipes*	✔	Isoflavone and bile acid biotransformation	(21,22)
Pipli and Black Pepper	Piperine	✔		✔ *Bifidobacterium,* Bacteroidaceae	✔ *Salmonella enterica*	✔	Increase bioavailability of mannose, arabinose, xylose	(23)
Turmeric	Curcumin	✔ Lowers inflammatory mediators' expression	✔	✔ *Bifidobacterium, Lactobacillus,* butyrate producers	✔ *Enterobacterium*, *Enterococcus* Pervotellaceae	✔	Anticarcinogenic	(24,25)
Triphala	Quercetin, gallic acid, chebulinic acid	✔ Reduced inflammatory mediators' expression like IL-17	✔	✔ *Bifidobacterium, Lactobacillus*	✔	✔	Stimulate appetite, reduce acidity, antipyretic, anticarcinogenic	(21,26)
Liquorice	Glycyrrhizin	✔	✔	✔ *Bifidobacterium,* butyrate producers	✔		Laxative, demulcent	(27)
Brinjal Carrot Potato	Anthocyanin	✔ Lowers IL-6 expression	✔	✔	✔ *Escherichia coli*	✔	Cure chronic inflammation	(20,28,29)

Anthocyanin

Found in almost all plants specially in deeply-coloured vegetables like potato, carrot, sweet potato and brinjal, the phytochemical anthocyanin has always been a part of our diet. It is soluble in water; however, it reaches directly into colon without being absorbed by small intestine (29,30). There it undergoes fermentation through the healthy gut microbial residents as a carbon-source for their growth (31). Their fermentation in gut produces antimicrobial by-products which lowers the population of pathogenic gut species like *Escherichia coli.* Thus, maintaining a healthy gut microbiota and preventing gut dysbiosis (29). As an antioxidizing agent anthocyanin also scavenges ROS which downregulates IL-6 inflammatory pathway expression. This adds anti-inflammatory activity to anthocyanin's repertoire, thus making it a potential aid for chronic inflammations (28).

Spices

Spices have been an integrated part of Indian Cuisines. They are used to imbibe dishes with appetizing aroma and enhanced flavor. Ayurveda mentions spices as a herbal medicine which

improves digestion nutrient absorption. The phytochemicals present in spices are known maintain a healthy gut, to reduce inflammation, cellular oxidation, and shows anti-cancerous, anti-diabetic and anti-microbial effects in the gut. This makes culinary spices a potent source of prebiotics (23).

Turmeric. It is widely used spice both in culinary science and Ayurveda. In cuisines it is used as a flavouring, colouring agent, and aids in digestion. On the other hand, it possesses many therapeutic properties which can cure many diseases that has been penned down in Ayurveda (25). Curcumin facilitates the growth of *Bifidobacterium, Lactobacillus* and butyrate producing bacteria in gut thus establishing a healthy gut. The increase in butyrate-producing bacteria decreases the inflammatory mediator expression from mucosal mRNA. It also helps to limit the activation of NF-κB which all together provides anti-inflammatory activity to curcumin. This anti-inflammatory as well as immunomodulatory property of curcumin prevents the growth of pathogenic species in the gut (24).

Ginger. Apart from being a popular flavouring agent in many dishes ginger inherit many health benefits. It has a pool of bioactive compounds like β-carotene, alkaloids, ascorbic acid, polyphenols with most prominent one being gingerol provides gingerol with anti-inflammatory, anti-microbial and bio-absorptive property (22). As an anti-microbial, gingerol reduces the number of pathogenic species belonging to *Bartonella* and *Atopostipes* genera. It also kills the population of *Escherichia coli, Klebsiella pneumoniae* and other such bacterial species which causes inflammation in the gut. Ginger facilitates the growth of species belonging to Coriobacteriaceae family. These bacteria then speed up the biotransformation of bile acid and isoflavones. It also increases the population of *Bifidobacterium* and *Lactobacillus* which helps to prevent gut dysbiosis (23,27).

Pipli and Black Pepper. Just like ginger, both pipli and black pepper contains a large pool of bioactive compounds. It includes antioxidants, terpenes, alkaloids, and vitamin B. The primary alkaloid present in both pipli and black pepper is piperine which shows chemo-preventive and anti-inflammatory activity. They also improve the food absorption and bioavailability. In Ayurveda, an equal volume concoction of pipli and black pepper with ginger called *Trikatu* is used as an aid for a healthy gut (32). Piperine increases the population of bacteria belonging to Bacteroidaceae family and glycosyl hydrolase produces. These bacteria catabolize polysaccharides such as xylose, mannose and arabinose which otherwise would not have been utilized. It increases their bio-absorption, and their utilization provides additional source of carbon for the growth of *Bifidobacterium* and *Lactobacillus.* Piperine act as an antibiotic against *Salmonella enterica* thus inhibit its growth. As a result, pipli and black pepper help in establishing a healthy gut (23).

Slippery Elm. It is an endangered spice known for its mucous membrane restoration property. It acts as a mucous membrane emollient and demulcent in the gut. When mixed with water the extensive amount of mucilage present in slippery elm forms a gel like matrix which lines the gut with a protective layer. It prevents the attachment and invasion of pathogenic species through the gut. It also supports the growth of *Bifidobacterium, Lactobacillus,* and some butyrate and propionate producing bacteria (27).

Licorice

In Ayurveda, it mentioned by the name *Yashtimadhu* which is known for reducing oxidative stress, and cellular inflammation. These properties are an attribute of glycyrrhizin, a

phytochemical present in licorice. Glycyrrhizin is also known to have anti-microbial activity. Just like slippery elm it also increases the population of *Bifidobacterium* and butyrate producing bacteria and act as a demulcent (27).

Triphala

A panacea of Ayurveda for gastro-intestinal tract infection, *Triphala* is an equatorial combination of *Amalika, Bibhitaki,* and *Haritaki* by weight. When it is mixed with honey and clarified butter, *triphala rasayan* is concocted. The *triphala rasayan* have *tridoshic* effect, meaning it has the potential to balance out *vat, pitt* and *kapha* all three *doshas.* Apart from salty taste *triphala* have the rest five out of six *rasas,* sweet, pungent, sour, bitter and astringent. Potency of *triphala* and its action also known as *virya* is neutral, while its *vipaka,* post-digestive effect is sweet. Although all the three components have a dry *guna,* but individually *Amalika* has heavy and both *Bibhitaki* and *Haritaki* have light *guna* (21,33).

Quercetin and gallic acid present in *triphala* enhance the growth of healthy gut bacteria *Bifidobacterium* and *Lactobacillus* while limiting the inhibiting the growth of pathogenic species like *Escherichia coli.* The presence of chebulinic acid provides *triphala* antioxidant properties. As the number of healthy gut microbes increases, they start utilizing chebulinic acid, another compound present in *triphala.* After its degradation, chebulinic acid is transformed into urolithin, which is a potent antioxidant (26). *Triphala* also limits inflammatory mediators like IL-17 expression thus reducing chances of inflammation. The effect of *triphala* in the gut is dose dependent. At low dose it acts as a bowel tonic, at high dose it acts as a purgative and as a mild laxative at mild dose. Additional health benefits of *triphala* include appetite stimulation, hyperacidity reduction, anticancer, antipyretic, and hypoglycemic effect (21,26).

Conclusion

Prebiotics are known to be food for probiotics thus they are a crucial part of a healthy gut and lifestyle. They enhance the population and activity of gut microbiota, and aids in some way to fight several diseases. Most prebiotics that are consumed are a type of fibrous food, majorly a form of carbohydrate like inulin, FOS etc. Though these are beneficial to us but there is a limit to the amount these can be consumed. The reason being these sources are a primarily fiber they act as roughage. If they are consumed in large quantities, they may erode out the beneficial gut microorganisms along with them without being consumed as well. Thus, prebiotics sources which can be consumed on a regular basis and in different forms can be used as a potential substitute for the fiber-based prebiotics. These sources of prebiotics are together termed as non-carbohydrate prebiotics. They can form an essential component of a healthy and balanced diet. The importance of a healthy diet to prevent and cure disease has been mentioned in our *Rigvedic* text of *Ayurveda.* It underlines the various advantages of a proper diet to sustain a healthy lifestyle. Different sources of non-carbohydrate prebiotics which are a part of our daily diet have been mentioned in Ayurveda. Anthocyanin which is present in many deeply coloured foods can be utilized as a carbon source, increase population of healthy gut microbiome, and reduces inflammation. Similar attributes are also present in different culinary spices like turmeric, ginger, pipli, black pepper and slippery elm. Some of the non-carbohydrate prebiotics sources are primarily not a part of our diet but can be consumed as a supplement after a meal. It includes *triphala* which is a one stop cure for all gastro-intestinal tract disorder and *Yashtimadhu* which act as demulcent. The study of *prakriti* in *Ayurveda* is the pioneer of

personalized medicine. As the science of life, *Ayurveda* directs all its attention to maintain a harmony between mind, body, and soul. Using this approach along with the specific requirement of an individual by analyzing their *prakriti* an individual specific proper diet can be formulated which can be the first step of attaining optimum healthy lifestyle.

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