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The purpose of the journal is to provide a platform for disseminating novel and innovative research and development to help address the global grand challenges that we confront.

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Foreword

Research in universities and research institutions has traditionally been discipline based. Therefore, research in institutions is organized to promote research in respective disciplines. To address the challenges we face today, such as climate change, long-term health, and frequent common infectious diseases, we need to cross-fertilize ideas from many disciplines such as science, engineering, social sciences, and humanities.

Multidisciplinary approach is traditionally directed at problem solving, for example in engineering. However, knowledge enhancement at the interfaces of disciplines is critically important in order to find novel solutions to increasingly complex problems. Multidisciplinary research is very much in vogue. Multidisciplinary research requires inputs from a variety of individual disciplines operating in a culture of collaborative exploration across the discipline boundaries. More and more institutions recognize the need for facilitating multi-disciplinary research and development and promoting multidisciplinary teams and research.

Recent decades have seen exciting multidisciplinary research and novel solutions being found based on new knowledge.

I am pleased to introduce the first issue of Natural Sciences, a new journal dedicated to provide a venue for dissemination of multidisciplinary research and development. While there are several other platforms for multidisciplinary and interdisciplinary research, this publication is intended for research with direct impact on emerging challenges.

Professor Rao Bhamidimarri
Editor-in-Chief

NATURAL SCIENCES

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Review Article

Recent Development in Antibacterial Activities of Chalcones

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Abstract

Chalcone is a central core for many important biological compounds. Chalcones, containing α, β-unsaturated ketone fragment are pharmacologically important active agents because of their diverse mechanisms. This review provides an update on the recent developments in the antibacterial activities of natural and synthetic chalcones. This concise and critical review will be helpful for medicinal chemists to develop more antibacterial agents.

Keywords: chalcone, antibacterial, structure-activity relationships, mechanism

1. Introduction

Over the past few years, many people have suffered from infections and diseases. [1] As a cure against the deadliest diseases in the 20thcentury, antibiotics were considered to be “miracle drugs”. Microbial infections are the world's most important cause of death after a heart attack. However, bacteria have counteracted the antibiotic pressure and developed resistance to existing antibiotic drugs, rendering them nearly inactive.Due to these current circumstances, available antibiotics may not be sustainable in the future. [2-4] These trends highlight the urgent need and challenge to develop more efficient, potent, and broad-spectrum antibacterial new drugs with good bioavailability and no or fewer side effects to cure microbial infections. [5] In this context, drug discovery has attracted considerable interest in the synthesis of novel antibacterial agents based on microbial targets.

New drugs approved by the U.S. Food and Drug Administration (FDA) between 1981 and 2018 revealed that most of the clinical drugs were derived from natural products or their synthetic derivatives. Furthermore, natural products are a major source of medicines for the treatment of human diseases. [6] In addition, natural antibiotics (derived from natural products) are directly used as medicines [7]. Studies have shown that natural antibiotics readily interact with cellular targets with high efficiency and selectivity, avoiding the development of drug resistance.

Chalcone is one of the important components of natural flavors named by Kostanecki and Tambor [8]. Chalcone contains a 1, 3-diaryl-1-one backbone which is biologically important. The most extensive method used for the preparation of chalcones is the claisen-schmidt condensation which is carried out with the reaction of equimolar ratios of aldehyde and ketone in the presence of alkali.[9] Chalcones comprise a family of the flavonoids which are widely distributed in vegetables, fruits, tea, and soy [10, 11]. Chalcones are a class of flavonoids that are generally found in vegetables, fruits, tea, and soybeans. The use of plants and herbs for thousands of years to treat different health conditions may be related to the original medicinal uses of chalcone [12]. Modern reports on chalcone show several pharmacological activities, viz. antioxidant, antiproliferative, anti-inflammatory, and anticancer effects [11, 13, 14].

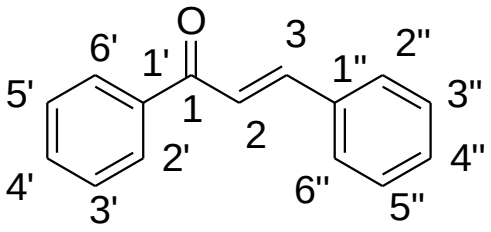


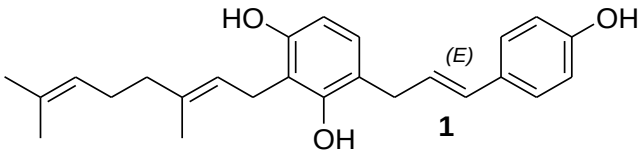
Figure 1: General structure of chalcone

Chalcones are α, β-unsaturated ketones composed of two aromatic rings (A ring and B ring) with different substituents (Fig.1). The two aromatic rings are bonded to each other through a three-carbon α, β-unsaturated carbonyl electrophilic system resulting in a linear or near-planar structure [15, 16]. Chalcones maintain conjugated double bonds and a delocalized π-electron system over the benzene rings. Chalcone compounds are usually colored compounds due to the presence of α, β-unsaturated carbonyl pharmacophore. Notably, the presence of a keto-alkenyl group in chalcone has been found to be responsible for the remarkable biological activity of chalcone. Chalcone can exist in cis and trans isomers. Amongst them, trans-chalcone is thermodynamically stable. A large number of chalcone derivatives exhibit various biological activities, such as antimalarial, [15-17], anticancer [18, 19], anti-inflammatory, [20-22], antibacterial [23] antifungal [24], anticonvulsant [25], antioxidants, [26, 27], etc. Chalcone moieties are utilised as templates in the synthesis of pyrimidines, pyrazolines, benzofurans, thiadiazines, isoxazoles, quinolinones, benzodiazepines, and other biologically important heterocyclic compounds. Some of these synthesized compounds show significant therapeutic activity [28-33].

Another advantage is a variety of chemical reactions carried out to prepare heterocyclic compounds or intermediates to design new drugs with therapeutic value. Therefore, the chalcone family has received more attention due to its wide range of interesting biological activities such as antitumor, anti-inflammatory, and antibacterial activities. However, their antibacterial activity is still not well understood.

2. Natural Chalcone

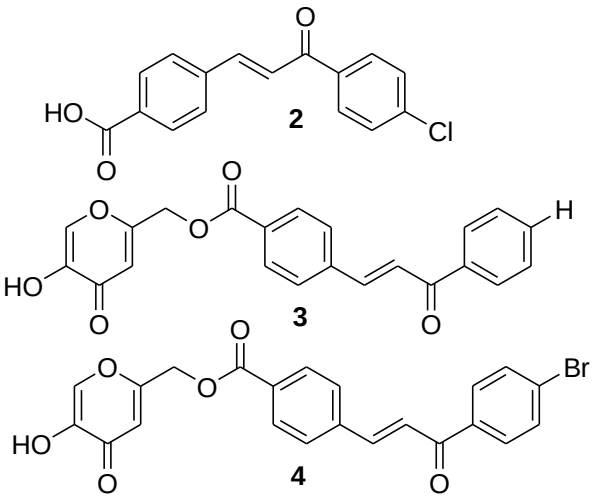
Naturally occurring, chalcones exhibit different biological activities. [10-34] During the last three decades, there are a number of chalcone-based derivatives showing their antibacterial activity. Here, we represent the latest developments in natural chalcone-based antimicrobials. Muharini et al. isolated new natural products from the fruits of Amorphafruticosa L. (Fabaceae). Amongst them, 1 (amorphastilbol) has antibacterial effect on Gram-positive bacteria *Staphylococcus aureus, Enterococcus faecalis* and *Enterococcus faecalis*, and the minimum inhibitory concentration (MIC) values were 3.1-6.3 µg/ml (Fig. 2). Further studies of these natural products revealed that a large number of novel and known phenolic metabolites in the fruit were significantly cytotoxic to the L5178Y mouse lymphoma cell line. [35]



Bacterial strains	MIC
S. aureus	3.1 µg/mL
E. faecalis	6.3 µg/mL
E. faecium	6.3 µg/mL

Figure 2: Natural occurring chalcone (amorphastilbol) as an antibacterial agent

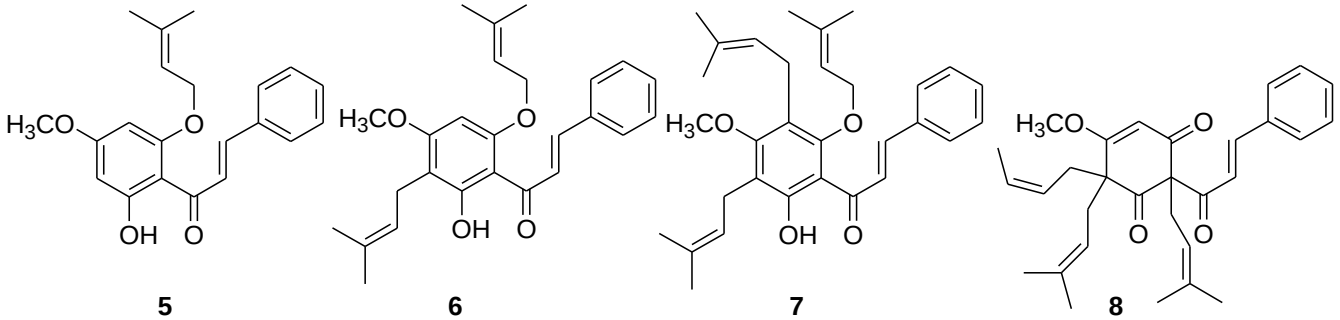
Uvaria change, also known as finger root or bush bananais commonly used in southern Benin to treat infections against Gram-positive *Staphylococcus aureus* (ATCC 25923), *E. faecalis* Van A (clinical isolate) and *E. faecalis* Van B (clinical isolate). Surprisingly, in water (at neutral pH) the ethanolic extract (25-27 mm) had a higher inhibition diameter than the water-ethanol (9-15 mm) extract. [36] Using kojic acid as a raw material, a series of kojic acid ester derivatives 2-4 were prepared using sago waste, chalcone, and azobenzene (Fig.3). The results showed that with the decrease of the electro negativity of the halogen substituent of chalcone, the antibacterial activity was enhanced and the inhibitory effect on *Staphylococcus aureus* was the strongest which was better than that of the ampicillin reference substance. The presence of C=C and N=N reactive moieties in both chalcone and azo molecules contributed to the potential biological activities of the kojic acid ester. [37]



Bacterial strains	MIC
S. aureus	8.0 µg/mL
S. aureus	11.0 µg/mL

Figure 3: Kojicacid ester derivatives and their antibacterial properties against gram-positive

K. pandurate is locally known as "Temu Kunci" in Indonesia. Chemical transformation of pinostrobin, forming K. pandurate, rhizomes (5-8) *In vitro*, it has moderate antibacterial activity against clinical bacteria *Bacillus subtilis, Staphylococcus aureus, Escherichia coli* and *Pseudomonas aeruginosa* with a MIC value of 25-50 µg/mL based on chloramphenicol. [38]. Phloretin is a natural chalcone with antibacterial activity against *P. acnes* strains (KCTC3220), (KCTC5527) and (KCTC5933) with an MIC of 16 µM. Furthermore, the results suggest that phloretin is a less toxic and more efficacious alternative to triclosan and benzoyl peroxide for the treatment of skin infections caused by *P. acnes* (Fig. 4) [39]

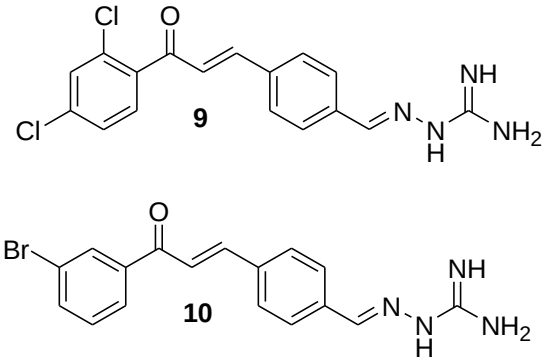


Entry	Bacterial strains	MIC
5	B. subtilis	50 µg/mL
6	S. aureus	25 µg/mL
7	E. coli	25 µg/mL
8	P. aeruginosa	25 µg/mL

Figure 4: Antibacterial activities of Temu Kunci derived chalcones

3. Synthetic Antibacterial Chalcones

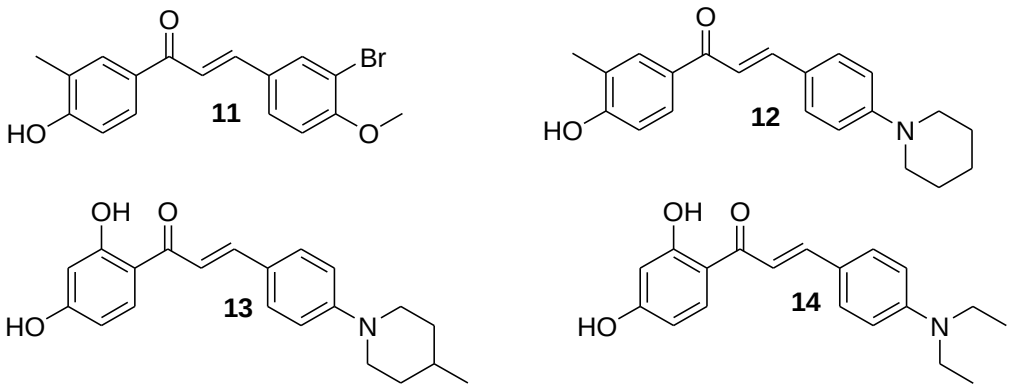
It is well known that fusion or molecular hybridization of two or more different pharmacophore is an important strategy for drug design. In addition, hybrid molecules can provide more biological targets, which can also improve bioavailability. Depending on the application, different methods such as Claisen-Schmidt condensation, Julia-Kocienski olefination, acylation, rearrangement, Witting reaction, one-pot synthesis and multiple coupling reactions can be used to prepare chalcone derivatives (Fig. 5)[40].



Bacterial strains	MIC (9)	MIC (10)
S. aureus 4220	2 µg/mL	2 µg/mL
S. aureus 503	2 µg/mL	2 µg/mL
S. aureus 209	2 µg/mL	2 µg/mL
S. mutans 3065	2 µg/mL	2 µg/mL
E. coli 1924	2 µg/mL	2 µg/mL
E. coli 1356	8 µg/mL	8 µg/mL
S. typhimurium 1926	1 µg/mL	1 µg/mL
P. aeruginosa 2742	2 µg/mL	2 µg/mL
C. albicans	1 µg/mL	2 µg/mL

Figure 5: Guanidine derived chalcone derivatives and their antibacterial activity against gram-negative strain

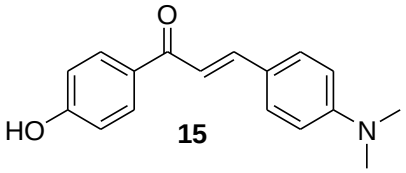
The aminoguanidine/acylhydrazone moiety-synthesized chalcone derivatives 9-10 were used to evaluate their antibacterial activity against the Gram-negative strain *S. typhimurium* 1926 and the fungus *Candida albicans* 7535 with the MIC value of around 1-8 µg/mL and making them 1 to 2-fold higher than the standard drugs. [41] M. Zhang, et al. synthesized thiol-/amine-Michael addition analogs of chalcone 11-14. Compound 11-12 had selective antibacterial activity against *Bacillus anthracis*, and the MIC value was 1.56 µg/mL, while derivative 13-14 indicated activities against *B. anthracis*, *S. aureus* (MSSA and MRSA), and *B. subtilis* with MICs ranging from 1.56 to 6.25 µg/mL. SAR analysis suggested that α, β-unsaturated linker between rings was found to be important for antibacterial activity (Fig. 6). [42]



Entry	Bacterial strains	MIC (11)	MIC (12)	MIC (13)	MIC (14)
11	B. anthracis.	1.56 µg/mL	-	1.56 µg/mL	3.12 µg/mL
12	E. faecalis	-	1.56 µg/mL	-	6.25 µg/mL
13	S. aureus (MSSA)	-	-	3.12 µg/mL	6.25 µg/mL
14	S. aureus (MRSA)	-	-	3.12 µg/mL	3.12 µg/mL
15	B. subtilis	-	-	6.25 µg/mL	3.12 µg/mL

Figure 6: Thiol-/amine-Michael addition derived chalcone analogs as gram-positive antibacterial agent

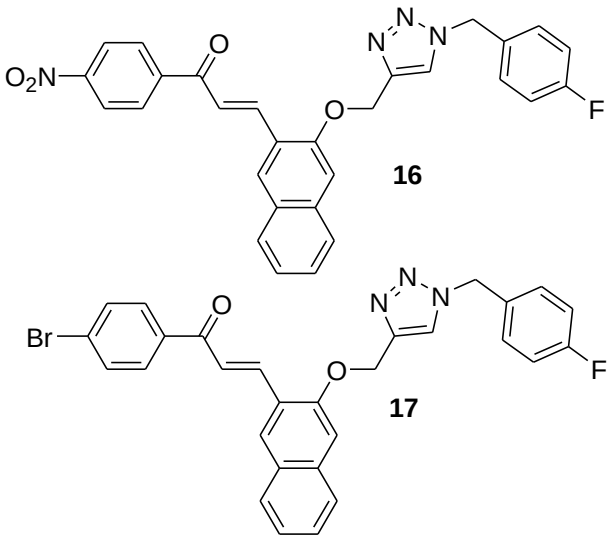
Chalcone derivative 15 was synthesised by Claisen-Schmidt condensation reaction and its antibacterial activity was evaluated against Salmonella typhimurium. Furthermore, computational studies of 15 show anti-bacterial efficacy in silico along with Lipinski's parameters (Fig. 7). [43]



Bacterial strains	MIC
K. pneumoniae	0.51 mg/mL
V. cholera	0.48 mg/mL
S. typhimurium	0.39 mg/mL

Figure 7: Antibacteiral properties of N, N'-dimethyl chalcone derivative

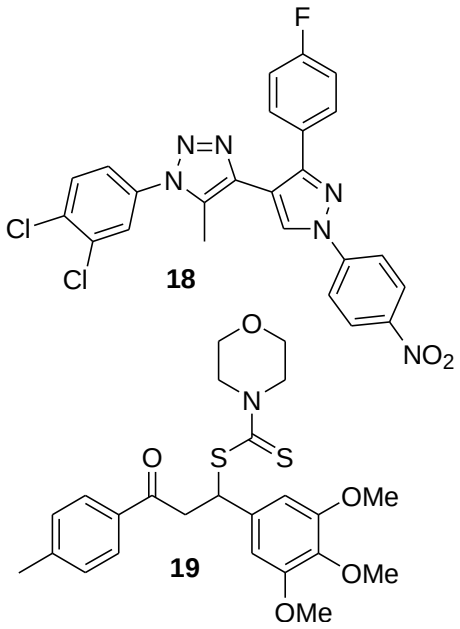
Yadav et al. synthesized some fluorinated chalcone triazole derivatives and demonstrated their antibacterial activity. The SAR results showed that the biological activity depends on the conjugation of two pharmacophore units - chalcone and triazole. Derivative 16 with 4-Nitro group has excellent antibacterial activity against *Escherichia coli, Staphylococcus epidermidis,* and *Bacillus subtilis* with MIC values of 0.0032 µmol/mL, 0.0032 µM/mL and 0.0063 µmol/mL respectively which is more potent than fluconazole while, derivative 17 with a 4-bromo group has excellent potency against *Escherichia coli, Bacillus subtilis, Staphylococcus epidermidis,* and *Candida albicans* with a MIC value of 0.0058 µmol/mL (Fig. 8). [44]



Bacterial strains	MIC (16)	MIC (17)
E. coli	0.0032 µmol/mL	0.0058 µmol/mL
B. subtilis	0.0063 µmol/mL	-
S. epidermidis	0.0032 µmol/mL	-
P. aeruginosa	0.0063 µmol/mL	-
C. albicans	0.0032 µmol/mL	-

Figure 8: Antibacteiral properties of Chalcone-triazole derivatives

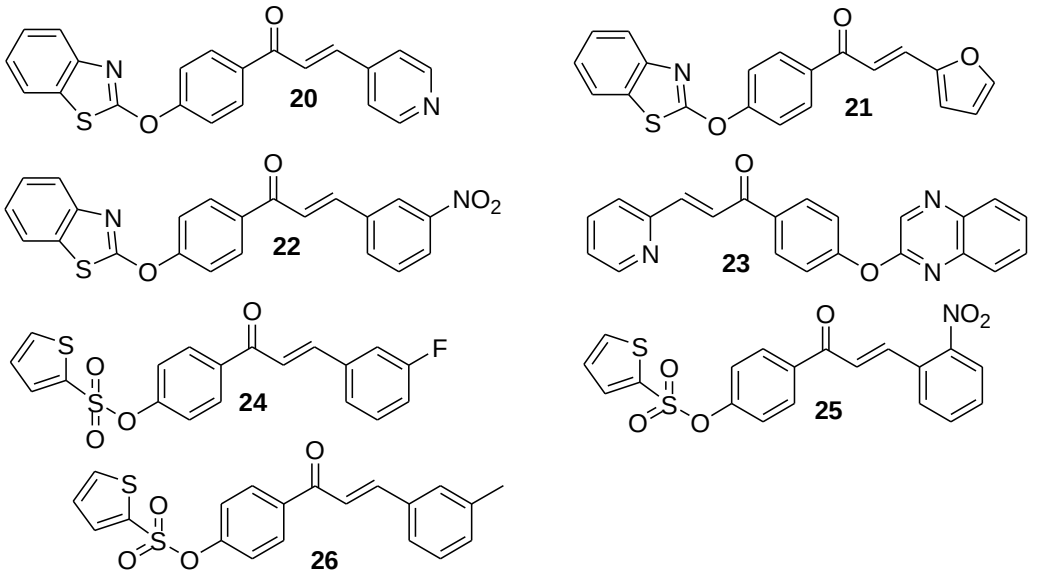
The triazole-containing chalcone synthesized by Santosh et al. was effective against *Pseudomonas aeruginosa, Escherichia coli, Staphylococcus aureus,* and *Bacillus subtilis*. The results showed that α, β-unsaturated carbonyl segment was further modified to pyrazole18 (nitro-containing pyrazole) which exhibited the highest MIC values of 83.92, 63.85, 76.63 and 92.46%, respectively [45]. Dithiocarbamate chalcone-based derivative 19 was synthesized by Ayman et al., showed activity against *Pseudomonas aeruginosa* (Ps12) and *Klebsiella pneumoniae* (K4) with the MIC value of 8 µg/mL. SAR and Pharmacokinetic properties indicated better bioavailability characters of synthesized compounds than colistin (Fig. 9). In addition, studies have shown that bacterial resistance is targeted through phosphoethanolamine transferase so bacteria become highly sensitive to the test compounds. [46]



Bacterial strains	MIC (18)	MIC (19)
P. aeruginosa	83.92 µg/mL	8 µg/mL
E. coli	63.85 µg/mL	-
S. aureus	76.63 µg/mL	-
B. subtilis	92.46 µg/mL	-
K. pneumoniae (K4)	-	8 µg/mL

Figure 9: Pyrazoles/Dithiocarbamate-based chalcone derivatives and their antibacterial properties

Synthesized benzothiazole derivatives 20-22 exhibited the strongest antibacterial activity compared with the reference drug bimerthiazol with EC_{50} values of 72.75, 54.58 and 97.18 μg/cm3 respectively. The highest activity against *Xan-thomonasoryzaepv, Oryzae (Xoo), Xanthomonas axonopodispv. Citri (Xac),* and *Ralstonia solanacearum* (Rs) was reported with EC_{50} values of 38.97, 13.42, and 36.49 μg/cm3, respectively [47]. The EC_{50} values for Xac, Xoo and Rs of analogue 23 were 6.72, 15.17 and 9.29 μg/cm3, respectively, compared with the 50% concentration of bismethylthiazole. In addition, SAM studies showed that 23 caused cell membrane wrinkling and damage, and it increased with increasing 23 concentrations (Fig. 10). [48]



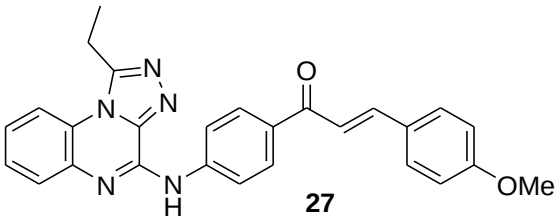
Bacterial strains	MIC (20)	MIC (21)	MIC (22)	MIC (23)	MIC (24)	MIC (24)	MIC (24)
X. oryzae pv. Oryzae (Xoo)	38.97 μg/mL	-	-	15.17 μg/mL	19.1 μg/mL	-	-
X. axonopodis pv. Citri (Xac)	-	13.42 μg/mL	-	6.72 μg/mL	-	11.4 μg/mL	-
Ralstonia solanacearum (Rs)	-	-	36.49 μg/mL	9.29 μg/mL	-	-	11.6 μg/mL

Figure 10: Benzothiazole/Thiophene sulfonate-based chalcones derivatives and their antibacterial properties

A series of thiophene sulfonate group-based chalcones were designed and the in vitro antibacterial activities of the synthetic derivatives against three phytopathogenic bacteria (Xac, Xoo and Rs) were evaluated. Compounds 24-26 exhibited significant antibacterial activity against Xac, Xoo and Rs with EC_{50} values of 11.4, 19.1 and 11.6 μg/mL, respectively (Fig. 10) [49].

A series of heteroarylations based on [1, 2, 4] triazolo [4, 3-a] quinoxaline derivatives with chalcone were designed. Compared with reference drugs such as ampicillin, gentamicin, and amphotericin B, its*in*

vitro antibacterial MIC values ranged from 0.49-15.63 µg/mL, the results of SAR analysis showed that the linker and terminal aromatic moieties play a key role in the antibacterial activity and the derivative 27 with a methoxy substituent increased the antibacterial activity against most bacterial and fungal strains (Fig. 11). [50]



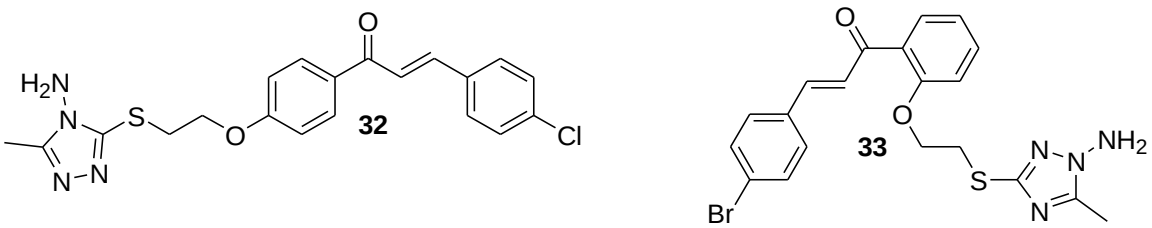
27

Bacterial strains	MIC (20)
S. Pneumoniae	0.98 µg/mL
B. Subtilis	0.98 µg/mL
E. Coli	0.49 µg/mL
A.Fumigatus	0.49 µg/mL
S.Racemosum	0.39 µg/mL
G. Candidum	0.98 µg/mL
C. Albicans	1.95 µg/mL

Figure 11: Quinoxaline-based chalcones derivative as an antibacterial agent

Twinkle et al., synthesized ferrocene based chalcone analogues-28 (E)-3-(2-methylpyrimidin-5-yl)-1-ferroceynlprop-2-en-1-one via the classic Claisen-Schmidt condensation. The synthesized compound was evaluated for its antibacterial activity against Gram-positive (*S. aureus*) and Gram-negative (*P. aeruginosa*) bacteria. Among them, *P. aeruginosa* had the highest antibacterial activity within the concentration of 75 µg. SAR analysis showed that the geometry of ferrocene and the polarity of the α-β-unsaturated carbonyl bond enhanced the antibacterial activity [51]. Ru, Rh, and Ir metal complexes 29-31 with pyridyl chalcone derivatives were prepared by Dkhar et al. and evaluated for antibacterial activities against *S. aureus* (Gram-positive) and Gram-negative (*Klebsiella pneumoniae* and *Escherichia coli*). Compounds 30 and 31 were most active against *E. coli* compared to the control drug ciprofloxacin. The MIC and minimum bactericidal concentration (MBC) values of compounds 29 and 31 against *Staphylococcus aureus, Escherichia coli,* and *Klebsiella pneumoniae* ranged from 0.12 to 1.0 mg/mL. [52]

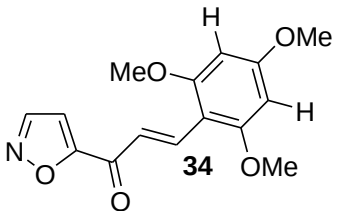
The synthesized thioethertriazole-linked chalcone derivatives 32-33 have been reported to possess antibacterial activity against *Xanthomonas oryzaepv, Oryzae* (*Xoo*), and *Xanthomonasaxonopodispv. Citri* (*Xac*). The EC_{50} value of compound-32 against Xac strain was 9.1 µg/mL while, the EC_{50} value of 33 against Xoo strain was 10.8 µg/mL which was much better than the EC_{50} value of about 54.9 to 69.3 µg/mL of the commercially available agent bismethizole-1 (Fig.12). SAR analysis showed that the chlorine substituents exhibited good inhibitory activity against Xac. In addition, when the substituent is a heterocyclic group/halogen atom, the position of the oxygen bond makes the biological activity better [53]. The SAR of isoxazole-based chalcone derivatives indicated that the nature and position of substituents on the benzene ring play an important role in antibacterial activity (Fig. 12).



Bacterial strains	MIC (32)	MIC (33)
X. axonopodis pv. Citri	9.1 µg/mL	-
X. oryzae pv. Oryzae	-	10.8 µg/mL

Figure 12: Quinoxaline-based chalcones derivative as an antibacterial agent

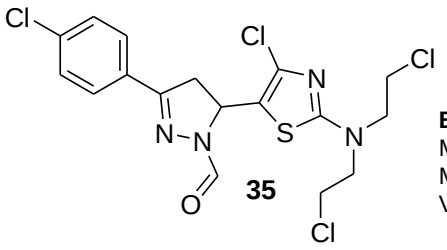
Compounds 34 had an electron-donating group (-OCH_3) substituted at positions 2,4, and 6 on the aromatic ring indicated the highest antibacterial activity against Gram-positive *S. aureus* and Gram-negative *P. aeruginosa* with MIC value 1 µg/mL which was greater than ciprofloxacin with MIC value 2 µg/mL and antioxidant activity (IC50 = 5 ± 1 µg/mL) [54]. Novel N-substituted pyrazoline derivatives were synthesised by reacting hydrazine derivatives with chalcone thiazole, and these derivatives were active against both methicillin-susceptible *Staphylococcus aureus* (MSSA), methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin intermediates have antibacterial activity (Fig. 13).



Bacterial strains	MIC (34)
S. aureus	1 µg/mL
P. aeruginosa	1 µg/mL

Figure 13: Donating group containing chalcone derivative and their antibacterial properties

The results on antibacterial activity showed that N-formyl pyrazoline 35 was most active against MSSA as well as two multidrug-resistant MRSA and VISA, with MIC values of 62.5, 125, and 31.25 µg/ml respectively. (Fig.14) [55]

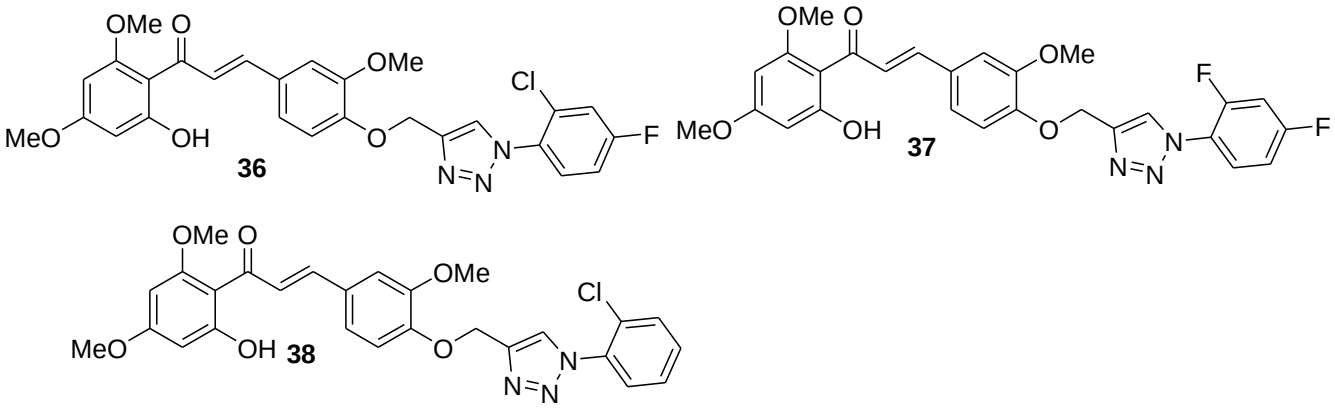


Bacterial strains	MIC (34)
MSSA	62.5 µg/mL
MRSA	125 µg/mL
VISA	31.25 µg/mL

Figure 14: Pyrazoline-based chalcone derivative as antibacterial agent

The antibacterial activity of the 1,2,3-Triazole linked chalcone and flavonoid hybrid compounds 36-38 was evaluated against Gram-positive and Gram-negative bacteria with equal or higher MIC compared to

the control drug, ciprofloxacin. Compound 36 (2-chloro-4-fluoro) had MICs of 6.25 µg/mL against *Staphylococcus aureus, Enterococcus faecalis* and 12.5 µg/mL against *Escherichia coli, Pseudomonas aeruginosa, S. boydii*, while compound 37- 38 (2,4-difluoro and 2- chloro) displayed activity against E. coli and S. boydii at MIC 6.25 µg/mL and against *S. aureus, P. aeruginosa* with MIC 12.5 µg/mL. This is very similar to ciprofloxacin MIC 6.25 µg/ml. Studies have shown that the position of these groups in the benzene ring also plays a key role in activity. [56-57] (Fig.15)



Bacterial strains	MIC (36)	MIC (37)	MIC (38)
E. coli	12.5 µg/mL	6.25 µg/mL	-
S. boydii	12.5 µg/mL	6.25 µg/mL	6.25 µg/mL
S. aureus	6.25 µg/mL	12.5 µg/mL	12.5 µg/mL
P. aeruginosa	12.5 µg/mL	12.5 µg/mL	12.5 µg/mL
E. faecalis	6.25 µg/mL	-	-

Figure 15: Triazole and flavonoid-based chalcone derivatives and their antibacterial properties against gram-positive and gram-negative bacteria

4. Conclusions

The antibacterial activities of chalcone and its derivatives are discussed in this review. It is clear from the above discussion that chalcones are precursors to various heterocyclic moieties of valuable pharmaceutical compounds. Chalcone derivatives have antibacterial activity against Gram-negative and Gram-positive bacteria. Chalcone can be used as an excellent scaffold for synthetic manipulations with a variety of biological activities. Although chalcone exhibits many interesting biological effects, its mechanism of action is still not fully understood. Due to the poor solubility of most chalcone compounds in vivo, efficacy has not yet reached the level expected from preclinical evaluations. Thus, the optimization of the physicochemical properties will be one of the most significant research directions of chalcone-based compounds.

5. Acknowledgement

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Research Article

Thermal and Structural Investigation of Gum Karaya (*Sterculia Urens*) & Gum Kondagogu (*Cochlospermum Gossypium*) Natural Acid Polysaccharide as a Potential Material for Biomedical Applications

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Abstract

In the present work, we have evaluated the thermal and structural behavior of tree gum exudates of natural anionic polysaccharides i.e. gum karaya (*sterculia urens*) and gum kondagogu (*Cochlospermum gossypium)* by Differential Scanning Calorimetry (DSC), Thermogravimetric Analysis (TGA) and Fourier Transform Infrared Spectroscopy (FTIR) respectively. The heat of melting, thermal conversion, reaction, thermal stability, and structural characteristics were determined, compared, and correlated with each other. DSC analysis reveals that both the gums majorly undergo the endothermic and exothermic reaction. In DSC analysis, the exothermic peak of gum kondagogu is higher and broader along with an overlapping of another peak suggesting a complex degradation reaction in case of gum kondagogu. This may be due to the higher acetyl group in kondagogu than karaya gum. Both the gums are prone to water which is very clear from the broad enothermic peak at lower temperatures. The thermal stability of gum karaya is higher than gum kondagogu.

Keywords: Gum karaya, gum kondagogu, thermal properties, DSC

1. Introduction

Tree exudates gum of natural acid polysaccharide has attracted huge attention among researchers because of their immense potential application in food industry, biomedical, as well as material science [1–12]. Natural acid polysaccharides are inexpensive, easily available, non toxic, biodegradable materials which exhibit peculiar physicochemical properties and applications [13–15]. Gum karaya is a partially acetylated natural polysaccharide having branched structure with high molecular mass of 16×10^6 Da and grouped under

substituted rhamnogalacturonoglycan (pectic) type tree gums. Gum karaya contains 13-26% of galactose and 15-30% of rhamnose which is higher compared to other tree gum exudates. However, protein content of karaya is lesser than other exudates gum. Gum karaya is commonly used for food and non-food applications due to its acid stability, high viscosity, and suspension properties [13]. Gum kondagogu (*C. gossypium*) belongs to the *Cochlospermum spp*. of family Bixaceae and gum karaya belongs to the *Sterculia spp*. and family of Sterculiaceae. Gum kondagogu contains ~50% of uronic acid [15] of average molecular weight of $7.23x10^6$ to $8.25x10^5$ g/mol determined by static light scattering method and Berry plots [15], [16].

Although gum karaya and gum kondagogu have both been grouped as rhamnogalacturonan types of gum, there are significant differences with respect to protein, tannin, fibre content, pH, intrinsic viscosity, molecular weight, water-binding capacity, sugar compositions, and rheological properties [16]. Gum kondagogu have lower solubility and higher viscosity compared to gum karaya [14–17]. Both the gums swell in water absorbing a large amount of water and produce gel which show thixotropic behavior. To exploit the commercial application of gum kondagogu in biomedical science, we investigated in detail the thermal behavior of gum kondagogu and compared it with gum karaya. An understanding of the thermal and structural behavior of gum kondagogu is essential in exploiting its potential in biomedical applications. In the present paper, we report and compare the thermal and structural properties of gum kondagogu with gum karaya.

2. Experimental

Materials

Gum kondagogu of grade-I, clean handpicked with no foreign material, was supplied by Girijan Cooperative, Hyderabad, India. The average molecular weight (Mw) of native pristine gum was determined to be $8.5x10^6$ g/mol. The pristine gum kondagogu was grinded in a high speed mechanical grinder and then sieved to obtain fine powder samples. Gum karaya was obtained from the Sigma-Aldrich Company (Sigma-Aldrich, USA).

Characterizations

Differential Scanning Calorimetry

DSC experiments of gum karaya and gum kondagogu was carried out on a Pyris Diamond S6 DSC (Perkin-Elmer, USA). The sample was heated from 3 to 400 ^oC at a heating ramping of 10 ^oC in N_2 atmosphere at a flow rate of 20 mL min^{-1}. 7 mg of each sample was crimped in an aluminum DSC pan. Calibration of the DSC instrument was done based on the melt onset and heat of melting of two metals namely, indium at 156.6 ^oC with 28.4 J g^{-1} and zinc at 419.6 ^oC with 108 J g^{-1}.

Thermogravimetric Analysis

Thermal stability and chemical composition of the material was determined by Mettler Toledo TGA/SDTA851e apparatus in N_2 atmosphere at a flow rate of 20 mL/min. The sample was heated from 30 to 800°C with the heating rate of 10 °C min^{-1}. From the TGA traces, differential thermogravimetric (DTG) plots were calculated as the first derivative of the TGA curve. The amount of sample was 5 mg in all the TGA experiments.

ATR-FTIR Analysis

The FTIR analysis of karaya and kondagogu samples was done in a NICOLET IZ10 instrument from Thermo scientific FTIR spectrometer Co. USA. The spectrometer is equipped with a multireflection variable angle horizontal ATR accessory. Zinc Selenide crystal was used in an attenuated total reflection (ATR) platform to analyze the sample. The angle of incidence on the crystal is set to 45 °C. The measuring signal passed the optical way with an aperture diameter of 3 mm with spectral resolution of 4 cm^{-1}. For optimal signal-to-noise ratio, 8 scans were averaged per sample spectrum and apodized by applying atmospheric suppression correction functions to avoid interference for the Fourier transformation. All the spectra were baseline corrected by automatic software control and were normalized thereafter with the highest peak.

3. Results and Discussion

3.1 Differential Scanning Calorimetric Analysis

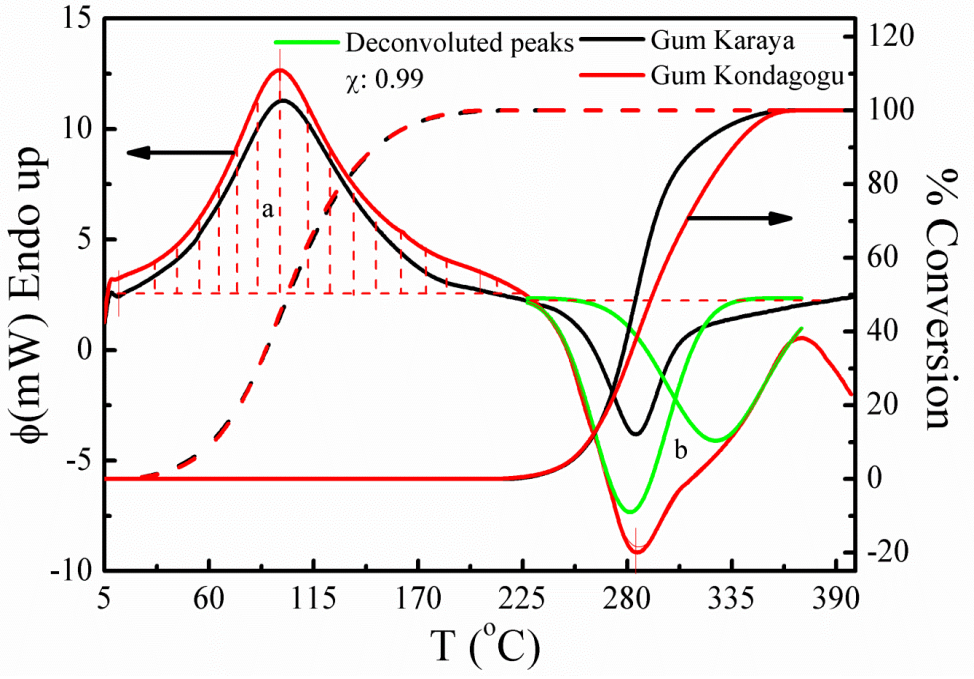

Figure 1: Differential scanning calorimetry heat flow curve of gum karaya (black) and gum kondagogu

(red) in N_2 atmosphere along with the percentage conversion curve for both endothermic (dashed line) and exothermic peak (solid line) and the deconvoluted exothermic peak (Green) for gum kondagogu

Table 1: DSC results of gum karaya and gum kondagogu

Gum Karaya (*Sterculia Urens*)					
Peak	**Peak (ºC)**	**Area under peak (mJ)**	**ΔH (J/g)**	**Peak height (mW)**	**End Temp (ºC)**
Endothermic	99	4191	599	8.88	163
Exothermic	285	-510	-216	-5.97	311
Gum Kondagogu (*Cochlospermumgossypium*)					
Endothermic	97	4111	587	9.34	151
Exothermic	284	-4728	-675	-11.19	364

Fig. 1 shows the DSC heating scans of gum karaya and gum kondagogu along with the conversion curve for both endothermic and exothermic peak respectively. For both the gum samples, the mass of the DSC sample is always the same. In order to see the consistency and reproducibility, 3 measurements for each gum polysaccharides were performed. From the DSC, the broad endothermic peak for both the gum is due to the breaking of the long nucleotides and removal of the hydrogen bound water. This peak is broader which signifies a bigger crystallite size distribution. Again after the broad endothermic peak, there is a start of an exothermic peak which shows the scissions of the chains leading to degradation of the polysaccharide. The exothermic peak of gum karaya is smaller than the gum kondagogu, indicating that degradation of gum kondagogu is more complex and needs higher energy for breaking the bonds. The exothermic degradation peak of gum kondagogu is deconvoluted in order to see two particular reactions. The acetyl group content in kondagogu is higher than the karaya. Again, the uronic acid content in kondagogu is more than karaya gum. The area under the peak is quite big in case of gum kondagogu.

3.1.1 Discussion of the Conversion Curves

In DSC, the heat of melting (enthalpy), peak temperature, and the peak area are the most important parameters in order to study the thermal behavior of the material. This thermal behavior is also to be analyzed by conversion curve of DSC of the respective peak area. The conversion curve mainly indicated the % of crystals that has melted or converted. The percent conversion α (T) is calculated from the heat flow curve:

$$\alpha(T) = \frac{\Delta H_{part}}{\Delta H_{tot}} . 100\% = \frac{\int_{T_0}^{T} \emptyset(T')dT'}{\int_{T_o}^{T_i} \emptyset(T')dT'}$$

where T_o is the lower temperature limit of the peak evaluation and T_1 is the upper temperature limit. The partial area of the peak from the temperature T_0 to the actual temperature T_1 is ΔH_{part}. The total area of the peak is $\Delta H_{tot.}$

From the conversion curve one can see that the for the endothermic conversion curve the reaction is slower till about 60 ^oC but once it reached to about 70 ^oC, the reaction becomes significantly faster. This again proves that the reaction is due to the removal of hydrogen bounded water in the gum. The exothermic conversion curve however shows some differences between the gums due to broader spectrum of gum kondagogu.

3.1.2 Thermogravimetric Analysis (TGA)

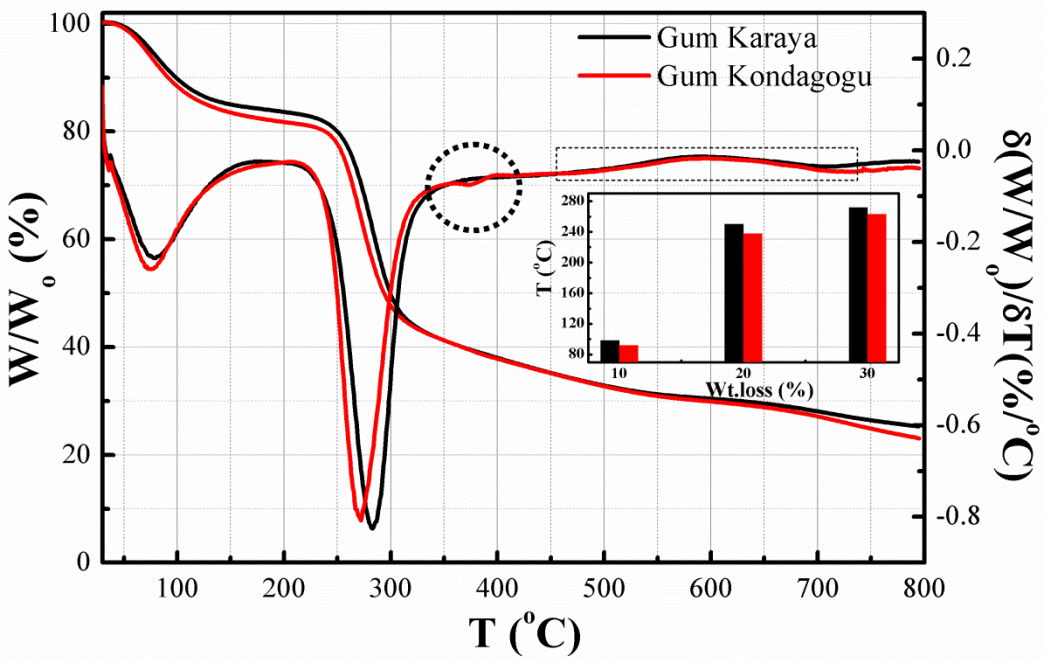

Figure 2: Thermogravimetric Analysis (TGA) and Differential Thermogravimetric Analysis (DTG) of gum karaya (black) and gum kondagogu (red)
(Insets box: temperature recorded for both the gum at 10, 20 and 30% wt loss)

Fig. 2 shows the TGA and DTG plots along with ~~in~~ the insets of temperature Vs 10, 20 and 30% mass loss respectively. TGA analysis is performed in nitrogen atmosphere. From the TGA curve, it is clear that both gum karaya and gum kondagogu decomposed in two major mass loss steps. However, it is clear that gum karaya is more stable than kondagogu although both the gums show same nature of degradation.

On the DTG curve, we can see that the black color associated with gum karaya occurs at higher temperatures in the major mass loss curve.The first degradation step is associated with the adsorbed water which is upto a temperature of 150 ^{o}C. We know that natural carbohydrate polymers are hygroscopic in nature due to the huge number of (O-H) group associated with the structure. That is the reason why carbohydrate natural polymer swells in water. The second major degradation step started at a temperature of ~240 ^{o}C. The major mass loss occur upto a temperature of 350 ^{o}C. Maximum mass loss of around ~60 % occurs at the end of this temperature. The major mass loss is due to the degradation of uronic acid. However, in the case of gum kondagogu, a small and immediate mass loss occurs after the big mass loss, as highlighted in the graph with a circle. The interesting fact we found in the TGA analysis is that after the major degradation, the mass is nearly constant, and there is a little improvement in the mass loss curve, which starts at around 450 ^{o}C and ends at around 700 ^{o}C, which could be due to oxidation of some particular acid or sugar composition of both the gums.Gum kondagogu shows a residual char of around 23% whereas the karaya gum shows residual char of 25%. Carbohydrates polymer are highly carbon containing. The first FTIR spectra of karaya gum residue obtained after the first mass loss step ends at 175 ^{o}C. From the FTIR spectra, it's clear that the received FTIR spectra of both the gums overlap with the spectra at 175 ^{o}C except the intensity of (O-H) group which is decreased for the heated residue at 175 ^{o}C. This proves the evidence that the first mass loss step is due to the adsorbed water in the gum sample. In the second degradation step, the FTIR spectra show major changes. The intensity of the (O-H) again decreases but is not completely varnished.

3.1.3 Fourier Transform Infrared Spectroscopy

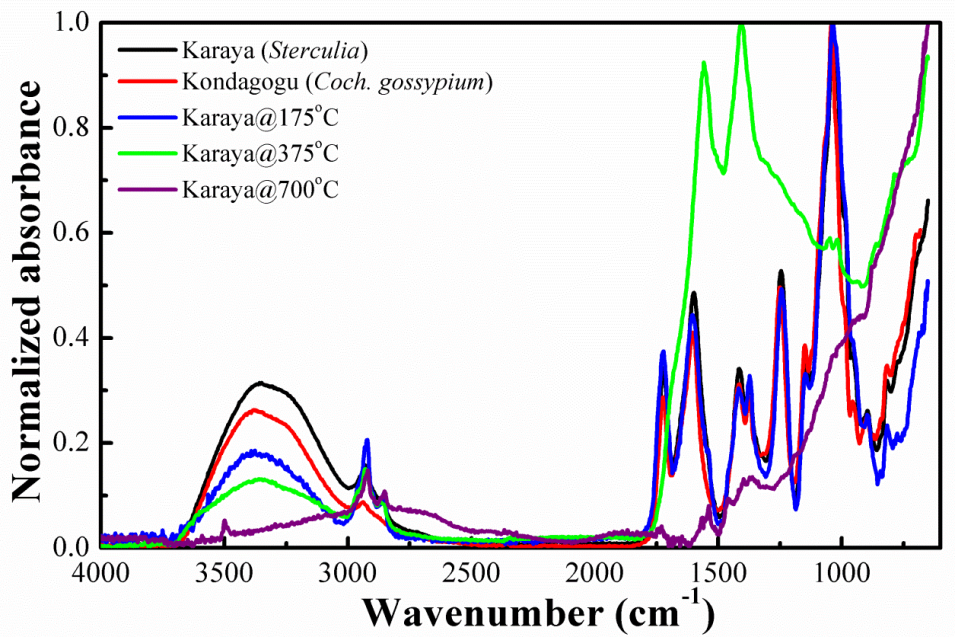

Figure 3: FTIR analysis of pristine gum karaya and gum kondagogu along with the residue of gum karaya after heating it at 175, 375 and 700 ^{o}C

Fig. 3 shows the normalized FTIR absorption spectra of gum karaya (black) and gum kondagogu (red) along with the spectra of residue of gum karaya powder after heating at different degradation temperature i.e. 175 (blue), 375 (green) and 700 ^{o}C (purple) respectively. Six major bands were determined in the spectra, corresponding to vibrations of characteristic groups. The broad band at 3338 cm^{-1} corresponds to O-H stretching band of hydroxyl group and N-H stretching band of amide group; 1725 cm^{-1} C=O due to the stretching vibration; 1595 cm^{-1} to the C=C band; 1417 to the deformation band at and 1371 cm^{-1} represents the C-H deformation. The band at 1244 cm^{-1} is due to the C-O stretching. We can clearly see that after heating the gum upto a temperature of 175, there is no change in the FTIR spectra except the hydroxyl group which proves the removal of the bounded water in gum. The FTIR spectra after heating at 375, shows ~~a~~broad changes and the carbonyl band disappears. So, the degradation occurs by breaking the carbonyl band majorly. Finally at 700 ^{o}C, the FTIR spectra disappear, showing only an exponential increase in the base line which is only due to the residual carbon.

4. Conclusions

We compared the thermal and structural behavior of gum kondagogu and gum karaya. Both the gums show the same nature of degradation, except a few low intensity peaks while the karaya gum is more stable compared to the kondagogu gum. Gum kondagogu requires more energy than karaya for degradation. The exothermic peak in DSCsuggest the area under the exothermic peak in kondagogu is more than the gum karaya. The enthalpy of fusion for the degradation step is 675 J/g whereas for karaya it is 587 J/g.

5. Acknowledgement

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Review Article

Current Status and Future Prospects of Biodiesel in India: A Review

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Abstract

Biodiesel have potential to bridge the gap between the depleting conventional energy sources and increasing energy demands. It is produced from renewable vegetable oil or animal fat via transesterification. Transesterification reactions are being classified based on the catalyst used. The various factors that affect the transesterification reaction are FFA and water content of vegetable oil, type and concentration of catalyst, molar ratio between triglyceride and alcohol, temperature and mixing intensity. New alternatives like waste oil from food processing industries and restaurants, non-edible oils such as Jatropha (Jatropha Curcas), Karanja (PungamiaPinnata), and Neem (Azadirachtin Indica), Castor (Ricinus Communis), and Algal oil have been studied for the biodiesel production. The biodiesel from algae is relatively a new technology. Biodiesel can be proved useful only by proper cultivation and management of feedstock in sustainable way with biodiversity in mind. In this research paper, an attempt has been made to review biodiesel feedstock, biodiesel production using transesterification reaction, and variable affecting transesterification, biodiesel standards, advantages and challenges associated with it.

Keywords: Renewable sources, biodiesel, transestrification, catalyst

1. Introduction

As our energy needs are increasing day by day due to industrialization and population growth, energy sources are becoming less and less. The main source of energy is fossil fuels which are limited and cause pollution by emitting greenhouse gases and contributing to global warming. With growing emissions of combustion-generated pollutants, scarcity of fossil fuels and their increasing cost creates an urgency to find an alternative for the petroleum-based fuels. Keeping these aspects in mind, researchers all over the world are working for development of renewable energy sources that have the potential to solve many of the current problems. An alternative fuel to petro-diesel must be technically feasible, economically competitive, environmentally acceptable, and easily available [1]. Nowadays, biodiesel fuels are gaining more and more attention worldwide as an eco-friendly petro-diesel equivalent. Biodiesel is a mixture of mono alkyl esters of fatty acids derived from naturally occurring renewable vegetable oils or animal fats by the process of

transesterification in the presence of catalyst. Major advantages of biodiesel fuels are that they are biodegradable and have lesser emission of potential pollutants like green house gases. Combustion of neat biodiesel decreases carbon monoxide (CO) emissions by 46.7%, particulated matter emissions by 66.7% and unburned hydrocarbons by 45.2% [2].

2. Biodiesel Feed Stocks

a) Virgin Vegetable Oil

Vegetable oils are renewable and widely available from a variety of sources. Various vegetable oils used as fuel in diesel in engine are oil from rapeseed, canola, soybean, palm, coconut, almond, olive, and sunflower [3]. They contain triglyceride molecules of three long chain fatty acids that are ester bonded to a single glycerol molecule. Vegetable oils have different fatty acid chains that differ by the number of carbon atoms, double bonds, and their orientations in these chains. Some of the common fatty acids present in different vegetable oil are listed in Table 1[4]. Vegetable oil as an alternative to the engine fuel is not a new concept. Rudolph Diesel inventor of diesel engine run his diesel engine with peanut oil in 1900 Paris exposition [1]. In 19^{th} century, he foresaw the applications of vegetable oils as a diesel engine fuel. Vegetable oils are good alternative fuel for diesel engine but they have some drawbacks on engine in the long run. The unmodified direct application of vegetable oils chokes the diesel engine [5] due to the high kinematic viscosity of vegetable oil [2]. High viscosity is due to the large molecular mass of oil. In addition, high cloud and pour point of vegetable oil causes problem in cold weather conditions [4]. The inefficient mixing of oil with air contributes to incomplete combustion, leading to high smoke emission. The higher flash point attributes to lower volatility characteristics. The other drawbacks include high Free Fatty Acid (FFA) content, lube oil dilution, ring sticking, high carbon deposits, and scuffing of the engine liner [6]. To overcome these problems, chemical modification of vegetable oils is required for successful application in diesel engine rather than its direct use.

Table 1: Chemical structure of common fatty acids [2, 6]

Name	**Chemical Name**	**Structure(xx:y)**	**Chemical formula**
Lauric	Dodecanoic	12:0	$C_{12}H_{24}O_2$
Myristic	Tetradecanoic	14:0	$C_{14}H_{28}O_2$
Palmitic	Hexadecanoic	16:0	$C1_6H_{32}O_2$
Stearic	Octadecanoic	18:0	$C_{18}H_{36}O_2$
Arachidic	Eicosanoic	20:0	$C_{20}H_{40}O_2$
Behenic	Docosanoic	22:0	$C_{22}H_{44}O_2$

Lignoceric	Tetracosanoic	24:0	$C_{24}H_{48}O_2$
Oleic	Cis-9-Octadecenoic	18:1	$C1_8H_{34}O_2$
Linoleic	Cis-9,cis-12-Octadecadienoic	18:2	$C_{18}H_{32}O_2$
Linolenic	Cis-9,cis-12,cis-15-Octadecatrienic	18:3	$C_{18}H_{30}O_2$
Erucle	Cis-13-Docosenoic	22:1	$C_{32}H_{42}O_2$

xx represent the number of the carbon and y the number of double bonds in the fatty acid chain.

b) Waste Cooking Oil

Due to higher production cost of biodiesel, biodiesel manufactures are focusing on using low cost feed stock such as waste cooking oil. Waste cooking oil is way less cheap than vegetable oils, and therefore is a promising alternative for biodiesel production [106]. The food processing industries and restaurants all over the world have large quantity of waste oil left after frying potato chips and other food items. This left over oil is not suitable for further use and should be safely disposed as improper disposal could lead to water contamination. This problem of disposal can be easily solved by using waste cooking oil for biodiesel production. Used vegetable oil can be employed for the biodiesel production but the quality of oil affect the quality of biodiesel obtained [107]. Nigam P, [111] used the waste cooking oil obtained from the canteen of the University of Ulster, UK for biodiesel production and results of the study were presented in the International ECI-USA Biofuel Conference [109].The presence of free fatty acids and water in waste cooking oil cause problem in the biodiesel conversion. Escobar et al., reported that the production cost of biodiesel derived from waste cooking oil reduces more than half compared to virgin vegetable oil [112].

c) Non-Edible Oil

The increasing use of edible oil for the biodiesel production arise dispute relating to the use of edible oil for food or fuel purpose. All over the world, more than 95% biodiesel is produced from easily available edible oil. As the agricultural land and production of edible oil crop are limited, the non-edible oil can be a promising solution to the problem. In the last few years, biodiesel has been produced from different non-edible oil [28, 35, 111, and 112]. As a green alternative, non-edible oils have been considered a promising feedstock for biodiesel production. The problem in conversion of these non-edible oils to biodiesel is that they often contain large amount of free fatty acids which cannot be transesterified by alkali catalyst. Sharma et al., [113] studied non-edible Karanja (*P.Pinnata*) oil having high amount of FFA for biodiesel production and reported optimized yield of 90-95% and high conversion of 96-100% while El. Diwani G et al., [114] studied jatropha oil for biodiesel conversion.

d) Animal Fat

Animal fat such as beef tallow, chicken fat, lamb meat, and pork lard are used as a biodiesel feed stock. Animal fats are triacylglycerols which are generally solidbut liquid at room temperature thus, cannot be use directly as biodiesel. In animal fat, saturated fatty acid component accounts for almost 50% of the total fatty acids. Chakraworti et al, [129] used animal fat and concluded that slaughter house animal fat and poultry fat can be used as biodiesel after transesterication process. However, direct transesterification may lead to formation of soap due to high acid number. Thus, a pretreatment with alcohol could reduce the FFA content of feed stock. Further, higher values of iodine number and kinematic viscosity offered a measure resistance to flow and shear. According to Tang et al., [131] and Mata et al., [130] biodiesel from poultry and slaughter animal fat possesses inferior quality of biodiesel as compared to those of ultra low sulfur diesel. Table 2 shows various properties of animal fat feed stock.

Table 2: Characteristics of slaughter house animal feed stocks viz. pork lard, tallow, and fleshing oil [129]

Parameter	**Pork lard**	**Beef tallow**	**Fleshing oil**
Acid number (mg KOH/g of fat)	0.63	1.07	24.30
Iodine number (g/100g of fat)	77.9	46.37	75
Kinematic viscosity at 40^0 C(mm^2/s)	39.53	46.37	43.33
Higher heating point (Mj/kg of fat)	39.5	38.9	39.572

e) Algae

All algae contain protein, carbohydrate, lipids and nucleic acids in different proposition. The fatty acid content of algae varies with the type of algae. There are algae which contain up to 40% fatty acid of their overall mass [116]. Algae can grow anywhere as well as in saline water in the presence of enough sunlight. The high yield of algal oil is the most significant characteristic of algae. According to National Renewable Energy laboratory report, estimated yield (per acre) of oil from algae is over 200 times the yield from the best performing plant/vegetable oils [117].Microalgae are the fastest growing algae having a growing cycle of every few days. Other advantages of microalgae are easier cultivation, higher photosynthetic efficiency, and higher oil yield compared to vegetable oils. Different algae produce different amount of oil but some algae can produce 50% oil by weight. Brain JK et al., [118] studied the production of biodiesel from *Dunaliella Tertiolecta and Nannochloropsisoculata* wild fresh water microalgae via heterogeneous catalyzed transesterification and reported 85% efficiency while conversion of triacylglycerides and free fatty acids into alkyl esters (biodiesel). Algae biodiesel is relatively new technology and research is going on for its improvement. Commercialization of algae production for biodiesel has not been undertaken. Researchers

across the world have been investigating micro algaes like *Botryococcus Braunii, Clorella, Dunaliella Tertiolecta, Gracilaria, Pleurochrysis Carterae (CCMP647), Sargasuum,* etc. for their suitability as mass oil-producers [119-123]. The lipid content each strain of microalgae produces varies. Table 3 shows the various microalgae which could be used for biodiesel production along with their corresponding lipid content.

Table 3: Micro-algae along with its lipid content [6-63]

Micro-algae	**Lipid Content (% dry weight)**
Botryococcusraunii	29–75
Chlorella sp	29
Cyclotella DI-35	42
Ankistrodesmus TR-87	28–40
Chlorella protothecoides(autotrophic/ heterothrophic)	15–55
Schiochytrium	50–77
Neochlorisoleoabundans	35–54
Crypthecodiniumcohnii	20-56
Thalassiosirapseudonana	21–31
Tetraselmissuecica	15–32
Stichococcus: 33	9–59
Scenedesmus TR-84	45
Phaeodactylumtricornutum	31
Nitzschia TR-114	28–50
Nannochloropsis	46(31–68)
Dunaliellatertiolecta	36–42
Hantzschia DI-160:	66
Nannochloris	31

Biodiesel

According to ASTM D 6751, biodiesel is defined as a diesel engine fuel comprised of monoalkyl esters of long chain fatty acids derived from vegetable oils or animal fats, designated B100 [ASTM D 6751]. B100 refers to the pure fuel before blending with diesel fuel. Biodiesel blends are denoted as, "BXX" with "XX" representing the percentage of biodiesel contained in the blend (i.e.: B20 is 20% biodiesel, 80% petroleum diesel). High viscosity of vegetable oil is reduced through various methods such as dilution,

microemulsification, pyrolysis, catalytic cracking, and transesterification. These methods bring a minor change in chemical composition of vegetable oils which in turn produce biodiesel.

Chemical Composition of Biodiesel

The elemental composition (carbon – C, hydrogen – H and oxygen – O), the C/H ratio and the chemical formula of diesel and biodiesel produced from different feedstocks is shown in Table 4[125,126]. The elemental composition of biodiesel varies slightly depending on the feedstock it is produced from. The most significant difference between biodiesel and diesel fuel composition is their oxygen content which is between 10 and 13%. The table shows elemental composition of biodiesel derived from different vegetable oils.

Table 4: Elemental composition of diesel fuel and biodiesel, % (m/m) [125,126]

Fuel	Carbon	Hydrogen	Oxygen	C/H	Empirical Formula
Diesel	86.5	13.50	0	6.24	$C_{15.05}H_{27.94}$
RME	77.2	12.0	10.8	6.50	$C_{19}H_{35.17}O_2$
SME	77.2	11.9	10.8	6.60	$C_{19.05}H_{34.98}O_2$
PME	76.35	11.26	12.39	6.16	$C_{18.07}H_{34.93}O_2$

PME – palm oil methyl ester, RME – rapeseed oil methyl ester, SME – soybean oil methyl ester

3. Methods of Biodiesel Production

3.1 Dilution

Biodiesel is most often blended with petrodiesel in ratios of 2% (abbreviated as B2 where 2% of vegetable oil is blended with 98% petrodiesel), 5% (abbreviated as B5 where 5% of vegetable oil is blended with 95% petrodiesel) or 20% (abbreviated as B20 where 20% of vegetable oil is blended with 80% petrodiesel). Fuel blending improves the performance of vegetable oil. It lowers the viscosity of vegetable oil along with solving engine problems like injector choking, oil ring stickening, and gelling of the engine lubricant. First of such blending was reported by Caterpillar Brazil in 1980 where they used pre-combustion chamber engines with a mixture of 10% sunflower oil to maintain total power without any alterations and adjustment to the engine [7].

3.2 Microemulsification

Microemulsification is the process in which vegetable oils are mixed with simple alcohols like methanol, ethanol or 1- butanol which reduces its viscosity and makes it suitable for diesel engine [8]. Ma and Hanna

reported that microemulsion biodiesel was successfully tested in lab but its performance in engines was not tested [7]. Microemulsion shows improved spray characteristics and lower viscosities [9, 10].

3.3 Pyrolysis

Pyrolysis is simple cracking of vegetable oil either by heat or heat plus a catalyst. Pyrolysis produces a large number of compounds and requires additional steps for separating these compounds [11]. Pyrolysis results in production of alkanes, alkenes, alkadienes, cycloalkanes, alkylbezenes, carboxylic acids, aromatics, water and small amount of gases compounds like CO2, CO [8, 11]. Therefore, biodiesel obtained by this method is less environmental friendly. The mechanism of the thermal decomposition of triglycerides is shown in Fig. 1

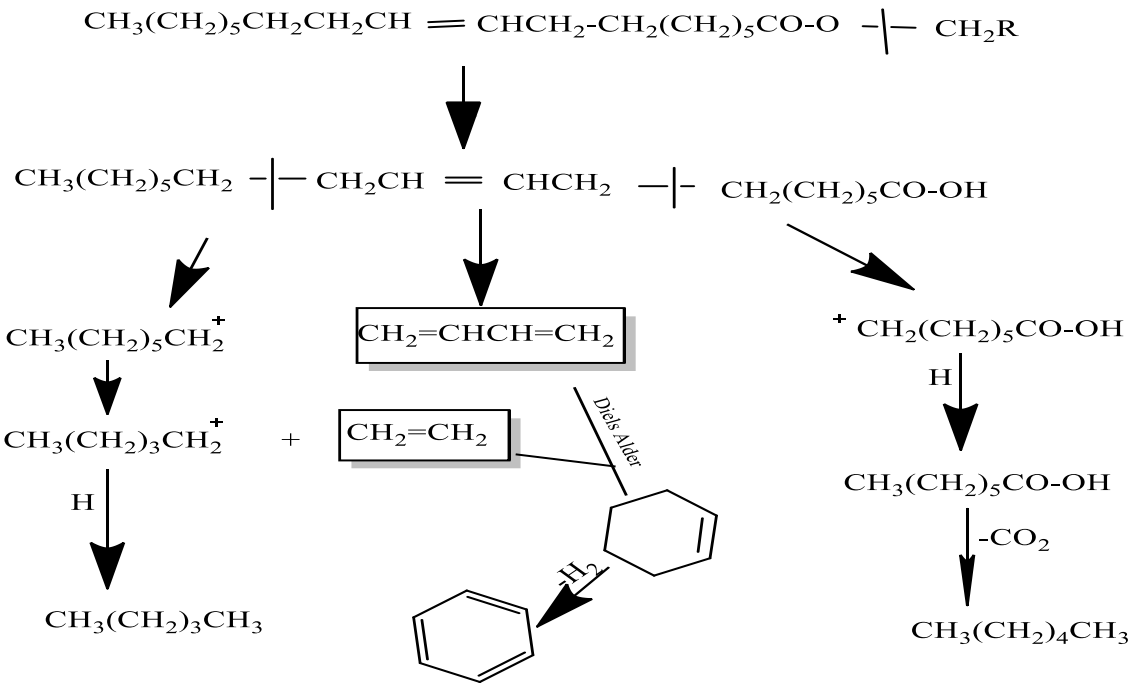
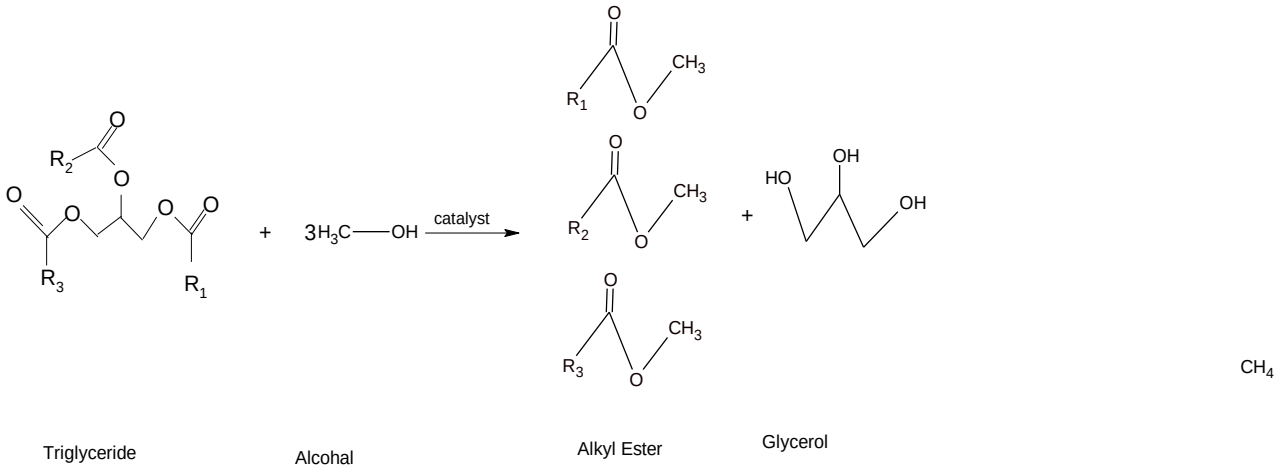


Fig -1Mechanism of the thermal decompsition of Triglycerides

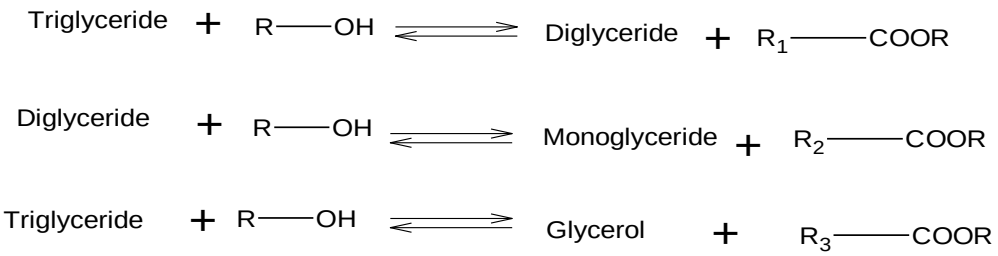
3.4 Transesterification

Transesterification is the most successful among all these method because of high yield and low cost. All over the world, approximately 90% of the biodiesel is produced by this method. Transesterification is the reversible chemical reaction between oil (triglycerides) and alcohol (methanol) which produce biodiesel (fatty acid methyl esters, FAME) and glycerol (by-product) in presence of catalyst. Production of biodiesel depends on two factors: feedstock and catalyst. The selection of catalysts depends on the amount of free fatty acid content (FFA) present in the feedstock. Catalysts are bases, acids, and enzymes. Base-catalyzed

reaction gives a better yield for triglycerides having lower content of FFA whereas for triglycerides containing high content of FFA, acid catalysts are used. The overall transesterification reaction is represented by Equation 1. Transesterification is not a single step reversible but it is a sequence of three consecutive reactions represented in Equation 2 [13]. Firstly, triglyceride is converted into diglyceride and this diglyceride is further converted into monoglyceride in the second step and this monoglyceride is finally converted into glycerol giving one molecule of alkyl ester at every step [117]. The final step, i.e. the conversion of monoglyceride into glycerol is the rate determining step as monoglyceride are the most stable intermediate [7].



Equation 1: Transesterification Process



Equation 2: Stepwise Transesterification Reaction

4. Classification of Transesterification Reaction on Basis of Catalysts Used

a) Homogeneous Acid-base Catalyzed Transesterification Reaction

Alkali/Base catalyzed transesterification is preferably done by alkaline metal hydroxide such as sodium hydroxide (NaOH), potassium hydroxide (KOH) [14,15,16,17,18,19,20] or alkoxides like sodium methoxide (CH_3ONa), potassium methoxide (CH_3OK) [21, 22] as well as by sodium or potassium carbonates[23]. Alkaline metal alkoxides give very high yield in less reaction time as they are highly reactive in comparison to hydroxide which are less reactive but cheaper than metal alkoxides[11]. Typical

mechanism of base catalysed transesterification is given in Fig. 2. As shown in Figure 2, the reaction mechanism for alkali catalyzed transesterification was formulated in three steps [24, 25, 26, 27, 28, 29, 30]. The first step is an attack on the carbonyl carbon atom of the triglycerides molecule by the anion of the alcohol (methoxide ion) to form a tetrahedral intermediate which reacts with an alcohol (methanol) to regenerate the anion of alcohol (methoxide ion). In the last step, rearrangement of tetrahedral intermediate results in the formation of a fatty acid ester and a diglyceride.Use of basic catalyst is limited only for vegetable oils which contain less than 0.5 wt. % free fatty acids (FFA) or acid value less than 1 mg KOH/g [31]. It is the most extensively used process because it is widely available, economical, takes less time, moderate temperature, low pressure, and without any intermediate step, it gives high conversion efficiency which is about 98%. However, it becomes less effective when FFA level exceeds 1% as the FFA reacts with base catalysts and form soaps (Fig 3) which in turn inhibit the separation of ester and glycerine which results in reduced conversion rate [32, 33]. Potassium carbonate used in a concentration 2 or 3% mol gives high yield of fatty acid alkyl esters and reduces saponification (Fig 4).

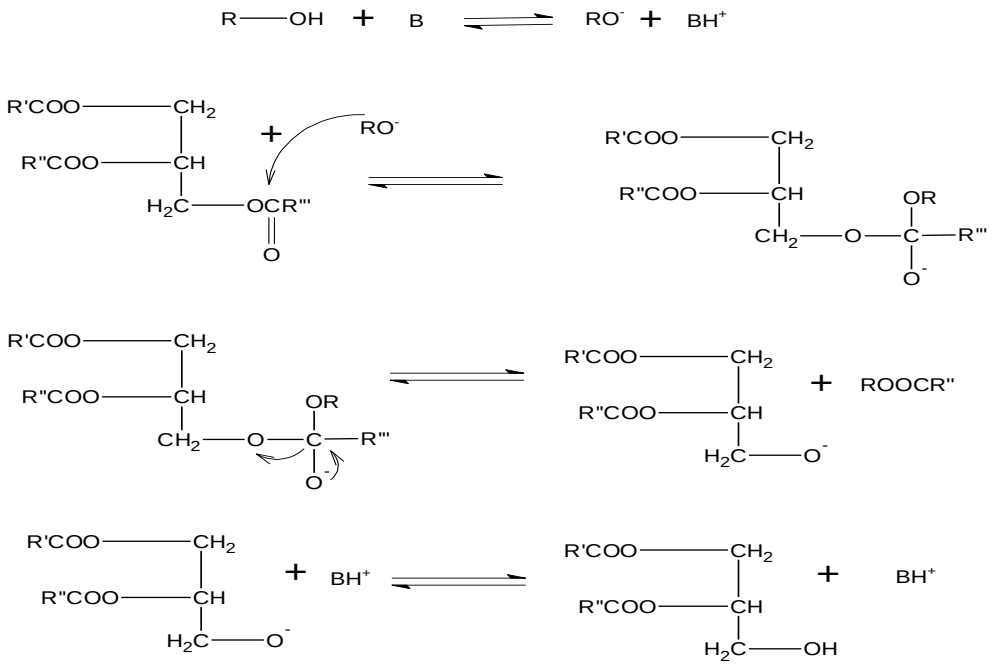
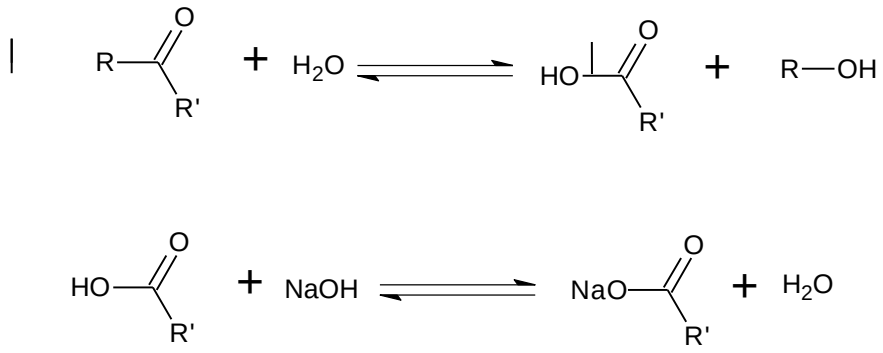
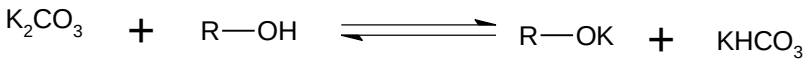


Figure 2: Mechanism of base catalysed transesterification [7]



R' = Chain of the fatty acid ; R = Alkyl group of alcohal

Figure 3: Saponification reaction of the produced FFA [7]



R = Alkyl group of alcohol

Figure 4: Reaction of Potassium Carbonate with alcohol [32-33]

Due to the drawback of base catalysts against high FFA content, acid catalysts are being used as they show low susceptibility to FFA content. The acid catalyzed transesterification is preferably done by sulfonic acid [34], sulfuric acid [35, 36, 37, 38, 39, and 40], ferric sulphate [41], methanolic boron trifluoride [42] and hydrochloric acid [11] [43, 44]. The mechanism of acid catalyzed transesterification of vegetable oil is shown in Fig. 5 [45, 46]. The protonation of carbonyl group of the ester leads to the carbocation which after a nucleophilic attack of the alcohol produces a tetrahedral intermediate. This intermediate eliminates glycerol to form a new ester and to regenerate the catalyst.

Generally acid-catalysed reactions are performed at high alcohol-to-oil molar ratios, low-to-moderate temperatures, and pressures and high acid catalyst concentrations that is the problem associated with acid catalysts [11, 47]. Acid catalyzed transesterification is sensitive to water content of vegetable oil. Water content should be kept under 0.5 wt. % to achieve high conversion efficiency. The transesterification of small esters under acid catalyzed conditions can be retarded by the presence of spectator polar compounds. Usually in industrial applications, base catalysts are preferred over acid catalysts due to their capability to complete reaction at a higher speed, at a lower reaction temperature, and with high conversion efficiency [48]. Cheaper homogenous base and acid catalysts provide high yield conversion of biodiesel.

However, they need thorough washing by water and neutralization by respective acid or base, resulting in the need for extra water and generation of excess wastewater. The biodiesel produced then must be dried to the moisture content. These limitations can be avoided by using heterogeneous solid catalyst [49].

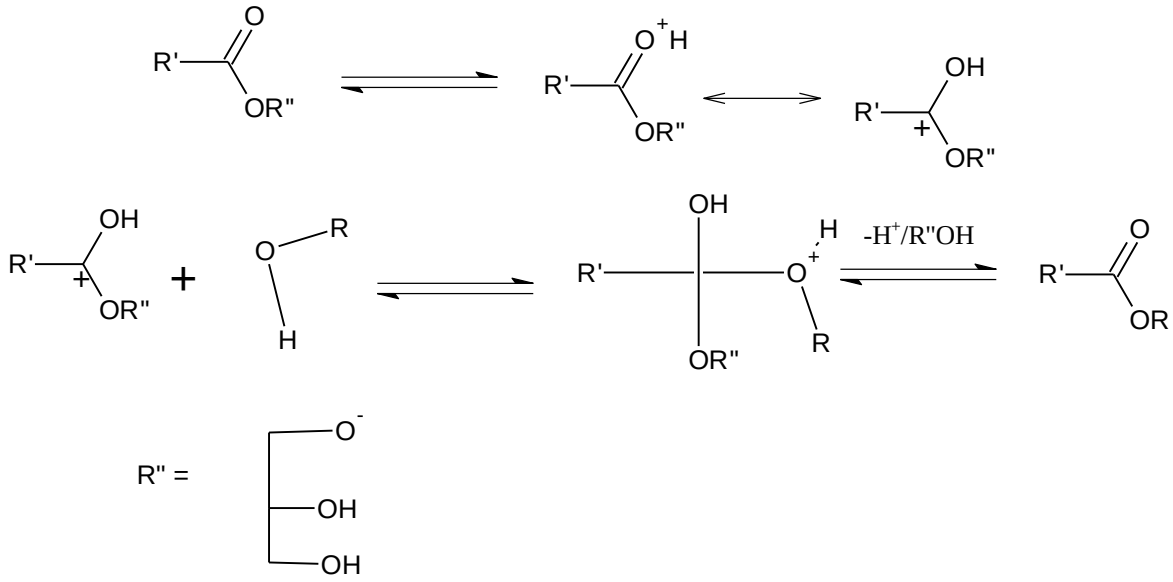


Figure 5: Mechanism of acid catalysed transesterification [45, 46]

b) Heterogeneous Solid Catalyzed Transesterification Reaction

Heterogeneous base catalysis is the most viable process for the transesterification of triglyceride into biodiesel. The heterogeneous catalysis features lower corrosiveness, environmental friendliness, easy catalyst recovery and high process integrity all at levels superior to those of homogeneous catalysis [50]. Like homogenous catalysts, heterogenous catalysts are also classified as solid base and solid acid catalyst. Examples of various heterogenous base catalysts are metal oxides: MgO [51], CaO [52], SrO [53], mixed metal oxides: $Ca_2Fe_2O_3$, $CaTiO_3$, $CaZrO_3$, $CaCeO_3$, $CaMnO_3$ [54], CaO-ZnO [55]. The reaction mechanism of the heterogenous catalysts is similar to homogenous catalysts. The use of heterogeneous solid catalyst is a simpler and more efficient process as it avoids the neutralization and washing steps. Various researches have been done by using zeolite, hydrotalcites, oxides, alumina, etc. as catalystsfor the production of biodiesel from different vegetable oils. The solid basic catalyst is more active than solid acid catalyst similarly, as base catalyzed is more active than acid catalyzed in homogeneous catalyzed transesterification [56, 57]. X.Liu et al., [58] reported production of biodiesel using CaO as a solid base catalyst from soybean oil and found long lifetime of catalyst and higher activity but reaction rate was slow. The FFAs present in

vegetable oil react with base catalyst forming soap which result in production loss. It was reported that FFA could be successfully converted into FAME (Fatty Acid Methyl Ester) prior to biodiesel production by using sulfated zirconia (SO_4^{-2}/ ZrO_2) and tungstated zirconia (WO_3/ZrO_2) as heterogeneous acid catalysts [59]. Reuse of the heterogeneous catalyst is anotherimportant aspect which makes it economic and preferableover the homogeneous one. However, most of the catalysts utilized for transesterification got deactivated and had to be reactivated by calcination or dosing with compounds. Even after reactivation, there is a limited run for which a catalyst worked and had to be discarded. Many of the heterogeneous catalysts suffered from some limitations such as low activity and leaching [60].

c) *Enzyme Catalyzed Transesterification Reaction*

Biodiesel can also be produced from biocatalytic enzyme – lipase through transesterification [61]. Lipases used in bidiesel production are generally obtained from bacteria, yeast, and filamentous fungi. Most commonly used bacterial lipases are *Burkholderiacepacialipase* (BCL) [62, 63], *Pseudomonas fluorescenslipase* [64], *Photobacterium lipolyticumlipase* [65], and *Chromobacteriumviscosumlipase* [66]. Examples of yeast lipase are *Candida antarctica lipaseB* (CALB) [67], *Candida sp*. 99–125 (also *Yarrowialipolyticalipase 2*) [68] and *Candida rugosalipase* [69]. Examples of lipases obtained from filamentous fungi include *Thermomyceslanuginosuslipase* (TLL) [70], *Rhizopus oryzaelipase* (ROL) [71], *Penicillium expansumlipase* (PEL) [72] and *Geotrichumsp.lipase* (GSL) [73]. Shah.S et al., [74] produce biodiesel from jatropha oil in a solvent free system using three different lipases – *Chromobacteriumviscosum, Candida rugosa, Porcine pancreas* and found that only *Chromobacteriumviscosum* give appreciable yield. The main purpose of enzyme catalyzed biodiesel production is to overcome the issue of byproducts recovery and treatment which requires complex processing equipment [75]. In comparison to conventional catalysts, lipases show lower energy consumption and can convert both triacylglycerols and free fatty acids into biodiesel which means they are able to catalyze oils from varied resources, including waste oils with high content of fatty acids. Biodiesel is easily seprated from the reaction mixture by filtration when catalyzed by lipases. The purification of biodiesel produced by transesterification by lipases is also much easier in comparison to other chemical catalysts. The main drawbacks of enzymatic process are high cost of lipase [76], lower yield and long reaction time [77].

(A) Variables Affecting Transesterification Reaction

1. Raw Material

The amount of free fatty acid and water content are key parameters which govern the yield of biodiesel through transesterification reaction. The raw material i.e., vegetable oil should have acid value less than 1. If it is greater than 1, more catalyst (example NaOH or KOH) is needed to neutralize these free fatty acids.

Higher amount of free fatty acids and water also cause soap formation. The resulting soap can increase viscosity by formation of gels and cause complications in glycerol separation [78]. In homogenous catalytic transesterification, presence of FFA and moisture leads to soap formation which complicates the product purification and generates lots of wastewaters. Kusdiana and Saka [79] reported that water have greater negative affect than free fatty acids on yield of biodiesel therefore, raw material should be water free. The presence of water had negligible effect on lipase catalyzed transesterification reaction [80].

2. Catalyst

Catalysts commonly employed for transesterification reaction are alkali, acid, enzyme or heterogeneous catalysts among which alkali catalysts like NaOH, KOH, NaOMe and KOMe are more effective [6]. The type of catalyst and its concentration is also an important parameter in biodiesel production which depends on the quality of the feedstock and method applied for tranesterification process. If the feedstock oil contains higher fatty acid, acid catalyzed transesterification is suitable. For the conversion of vegetable oil into ester, the catalyst concentration in the range 0.5-1.0% (w/w) gives 94-99% yield. If there is further increase in catalyst concentration, conversion is not affected but it adds extra cost for removal of catalyst after completion of reaction [81, 82]. The industrial process prefers alkali catalysts as they are less corrosive to industrial equipments.

3. Co-Solvent

Co-solvents are used to enhance the mixing between alcohols and triglyceriods. Commonly used co-solvents are tert. butanol, tert. pentanol, and isooctane. These solvents allow contact between two polar feed stocks as they act as a medium, stabilizer, and binders. Hence, they improve solubility of alcohol. Some co-solvents also act as an agent to reduce the operating temperature and pressure required for transesterification process. Encinar et al., [132] used Diethyl ether as a co-solvent and achieved high biodiesel conversion in a short interval of time.

(B) Reaction Conditions

1. Molar Ratio

Demirbas reported increase in the yield of alkyl ester when oil to alcohol molar ratio was increased [83]. According to stoichiometry, transesterification reaction requires 3 mol of alcohol per mol of triglyceride to yield 3 mol of fatty ester and 1 mol of glycerol. Transesterification is an equilibrium reaction in which excess amount of alcohol shift the equilibrium to the right that is towards the product. Ramadhas et al., [84] have taken 6:1 molar ratio during acid esterification of high FFA rubber seed oil. Sahoo et al., [35] have taken 9:1 molar ratio during alkaline esterification of polonga seed oil for biodiesel production. Various researches

have been done by taking different ratios. Veljkovic et al., [85] have taken 18:1 molar ratio for acid transesterification while 6:1 molar ratio for alkaline one. Meher et al., [86] have taken 6:1 molar ratio for acid and 12:1 for alkaline transesterification. The molar ratio between 9:1 and 12:1 shows the best result. For the molar ratio 15:1, separation of glycerin is difficult which leads to yield loss [87]. The reaction was incomplete for the molar ratio less than 6:1. However, if ratio of oil to alcohol is too large, it could give an adverse effect on the yield of fatty acid alkyl esters. Some researchers reported that addition of large quantity of methanol i.e., at ratio of 1:70, 1:84 could slow down the separation of esters and glycerol phases during the transesterification process therefore affecting the final yield [133].

2. Temperature

The rate of reaction is strongly influenced by the reaction temperature. Transesterification reaction can occur at different temperatures depending on the oil used. Freedman et al, [88] reported that trans-esterification reaction is strongly influenced by the temperature. In base catalyst transesterfication reaction, the temperature maintained between 318 and 338 K during different steps. The temperature higher than this will burn methanol as boiling point of methanol is 337.9 K and will result in yield loss. A study by Leung and Guo, [89] showed that temperature higher than 323K had a positive impact or yield for used frying oil with higher viscosities but had a negative impact for neat oil. Marchetti et al., [90] reported that increasing the temperature resulted in a rise in final conversion.

3. Mixing Intensity

Mixing is important to initiate the transesterification reaction as oils or fats are immiscible with NaOH or MeOH solution. Vigorous mixing can be used to homogenize the reaction mixture and to increase the rate of collision between the reactants [7]. Mass transfer rate can be increased by vigorous mixing of alcohol in triglyceride as fine droplets which results in increased surface area between two immiscible reactants [91]. Vicente et al. [92] reported that methyl ester formation increased as stirring rate was increased from 300 to 600 rpm. Methanolysis was conducted by Maf F et al., [93] at different stirring rate such as 180, 360 and 600 rpm and found that at 180 rpm, reaction was incomplete and rate of mixing was not sufficient.

4. Reaction Time

Reaction time is also an important factor in transesterification process as larger reaction time could also permit reversible transesterification reaction which eventually could reduce the yield of fatty acid alkyl esters. Thus optimization of reaction time is also necessary [123].

(C) Biodiesel Standards

Technically biodiesel (B100 i.e. 100% biodiesel) is monoalkyl esters of long chain fatty acids derived from vegetable oils or animal fats meeting the ASTM D 6751 specifications. Biodiesel have a number of promising advantages including reduction of exhaust emissions [94]. The ASTM specifications for biodiesel and petrodiesel fuel are shown in Table 7 [5]. Biodiesel have similar viscosity as petrodiesel. Biodiesel has higher flash point and therefore it is non explosive in comparison to petrodiesel. The standards for biodiesel in India (Table 8) are used to describe a product that represents a blending component in conventional hydrocarbon based diesel fuel, while thestandard ASTM D975 allows mixing commercial diesel oil with 5% biodiesel that meets the requirements of ASTM D6751 and ASTM D7467 specifies the quality requirements of mixtures with 5-20% of biodiesel. [127,128]

Table 7: ASTM standards of biodiesel and petro diesel fuels [135-138]

Property	**ASTMD975 (Petro diesel)**	**ASTMD6751(Biodiesel,B 100)**
Composition	Hydrocarbans (C10-C21)	FAME(C12-C22)
Specific Gravity(g/ml)	0.85	0.88
Flash point(K min)	325	403
Water and sediment(max %vol)	0.05	0.05
Kinematic viscosity (at 313K) (mm^2/s)	1.3-4.1	1.9-6.0
Sulfated ash(max %wt)	-	0.02
Ash(max %wt)	0.01 max %wt	-
Sulfur (max %wt)	0.05	-
Sulfur(max %wt)	-	0.05
Copper strip corrosion(max)	No.3	No. 3
Cetane number(min)	40	47
Aromaticity(max %vol)	35	-
Total Glycerin (%mass)		0.240
Free Glycerin(%mass)		0.020
Carbon residue(max %mass)	0.35	0.050
Distillation temp(90% volume recycle)	555 K min-611 K max	360max
Pour Point (^0C)	-35-15	-15-16
Hydrogen (wt%)	13	12

Cloud Point (^{0}C)	-15-5	-3-12
HFRR(µm)	685	384
BOCLE scuff (g)	3600	>7000

FAME- Fatty acid methyl ester, HFRR- High frequency reciprocating, BOCLE- Ball-on-cylinder lubricity evaluator

Table 8: Biodiesel standards in India [100-115]

Property	**Test Method**	**Limit**	
		Min	**Max**
Density at 15^{O}C (Kg/m^3)	ISO 3675/P32	860	900
Kinematic viscosity at 40ºC (mm^2/s)	ISO3104/P25	2.5	6.0
Flash point (closed cup) ºC	P21	120	-
Sulphur mg/kg	D5443/P83	-	50
Carbon resiue (Ramsbottom) (m/m)	D4530	-	0.05%
Sulfated ash (m/m)	ISO6245/P4	-	0.02%
Water content (mg/kg)	D2709/P40	-	500
Total contamination (mg/kg)	EN12662	-	24
Copper corrosion 3 hr at 50ºC	ISO 2160/P15	-	1
Cetane number	ISO5156/P9	51	-
Acid value (mg KOH/g)	P1	-	0.50
Methanol EN 14110 – 0.20 % (m/m)	EN14110	-	20%
Ethanol (m/m)		-	0.20%
Ester content (m/m)	EN141.03	-	96.5%
Free glycerol, max (m/m)	D6584	-	0.02%
Total glycerol, max (m/m)m	D6584	-	0.25%
Phosphorous(mg/kg)	D4951	-	10.0
Sodium and potassium mg/kg	EN14108	To Report	
Iodine value	EN14104	To Report	
Oxidation stability at 110ºC hours	EN14112	6	-

(D) Biodiesel Advantages

1. Renewable and Sustainable Fuel

Biodiesel is renewable and sustainable fuel as it is produced from renewable oil seed crops such as soybean, rapeseed, and sunflower. The raw material for biodiesel production i.e. oil seed crops can be cultivated and easily processed. Biodiesel can be used neat or mixed with petrodiesel in any ratio. B20 which is 20% biodiesel and 80% petrodiesel is the most common blend [95].

2. Less Polluting Fuel

The emission from automobiles is the main cause of pollution all around the world. Many studies have been done and reported on emissions and performance of compression ignition engines fuelled with pure biodiesel and its blends with petrodiesel [95, 96]. The emission of sulphur content decreased but NO_X exhaust emission increased slightly while using biodiesel compared to petrodiesel [97]. Dorado et al., [98] studied the exhaust emissions of biodiesel from waste olive oil using Perkins engine. Schumacher et al., [99] studied and measured the emissions of CO, HC, NO_X, particular matter (PM) and polycyclic aromatic hydrocarbons (PAHs) and showed that B20 can reduce cancer causing PAH emission. The use of biodiesel reduces the emission of carbon dioxide, carbon monoxide, sulfates, PAHs, NPAHs (nitrated polycyclic aromatic hydrocarbons), unburned hydrocarbons and PM. Table 9 below summarizes how biodiesel reduces emissions compared topetrodiesel even when used as the minor component of fuel blend.

Table 9: Average B100 and B20 emissions (in %) compared to petro diesel [33]

Emission	B100	B10
Carbon monoxide	-48	-12
Total unburned hydrocarbons	-67	-20
Particulate matter	-47	-12
Nitrogen Oxides	+10	+2
Sulphates	-100	-20
Air Toxics	-60to -90	-12 to -20
Mutagenicity	-80to -90	-20.0

3. Safer Fuel

The higher flash point of biodiesel makes it a safer fuel in terms of handling, storing, and transporting as compared to conventional petrodiesel. The flash point is minimum temperature at which the vapors of liquid catches fire with a spark. Biodiesel (B100) has a flash point of 423K, whereas petrodiesel has a

flash point of 337K [122].

4. Biodegradable Fuel

The increasing environmental pollution problems have drawn interest towards the biodegradable fuel which degrades more rapidly than conventional fuels. Biodiesel has an additional advantage of being an environmental friendly biodegradable fuel. Biodiesel is oxygenated fuel which makes it degradable four times faster than petrodiesel. Heavy fuel oil is 11% biodegradable [96] due to presence of high molecular weight aromatic compounds while gasoline is 28% biodegradable [101] after 28 days laboratory studies. Zhang et al., [102] studied the biodegradation of refined rapeseed oil, refined sunflower oil, and their methylesters (biodiesel) and found the biodegradation as refined rapeseed oil (78%), refined sunflower oil (76%), rapeseed oil methyl ester (88%) and sunflower methyl ester (90%). This study concluded that biodiesel is more biodegradable than its feedstock vegetable oil.

(E) Biodiesel Challenges

1. High Cost

The high cost of bio-diesel is related to the high cost of feedstock vegetable oil or animal fats that in turn makes biodiesel costly. The cost of biodiesel varies as per the availability of feedstock crop, geographic condition, and variability in crop production from season to season. Various researches all over the world are going on for low cost feedstock to reduce the cost of biodiesel [117-120].

2. Cold Flow Properties

Most of the properties of biodiesel are comparable with that of diesel except cloud and pour point which shows that the cold flow properties of a fuel are very poor. The cloud point is the temperature at which liquid fatty material becomes cloudy due to formation of crystals. The pour point is the lowest temperature at which liquid solidify and cease to flow. The cloud point usually occurs at a higher temperature than the pour point. Various researches have been done to improve cold flow properties of biodiesel by taking additives like methanol, ethanol, kerosene, and Mg additives [103]. Bhale et al., [106] studied the effect of ethanol, kerosene, and commercial additive Lubrizol on cold flow behavior of biodiesel (B100) obtained from *Madhucaindica* and found considerable reduction in pour point by using these cold flow improvers. Caynak et al., [105] studied the improvement in the cold flow properties of biodiesel obtained from pomace oil with synthetic manganese additive.

(F) Recent Advances

Direct transesrtification of microalgae is an effective way to make biodiesel cost effective. Jesus et al., [109],

extracted biodiesel from microalgae Chlorella pyrenoidosaby direct transesterification using 2-methyltetrahydrofuran or cyclopentyl methyl ether co-solvents as an alternative to chloroform. Moreover, cost reducing can be done by mass production of biomass. According to Ranganathan and Savithri [110], hydrothermal liquefaction and hydro processing may prove as alternative tools to make biodiesl cost effective. Consequently, researchers all around the world are attempting to develop a procedure that uses no catalyst or as little catalyst as possible.

(G) Biodiesel Economy

Global consumption of petroleum is increasing day by day. It has been expected that energy demands are projected to grow by more than 50% by 2025, with much of this increasing demand emerging from several rapidly developing nations. Shifting society's dependence away from petroleum to renewable biomass is viewed as an important contributor to the development of a sustainable industrial society and effective management of GHGs emission [118]. In October 2020, the cost of B100 was $3.23l / gallon whereas cost of petrodiesel was $3.41/gallon [134]. The biodiesel industry could contribute significantly to the domestic economy and along with reducing the amount of non-renewable resources. The biodiesel industry contributes to regional economic growth by supporting local agriculture producers and industries by using vegetable oils and residual fats as its raw material [121].

5. Conclusion

The direct use of vegetable oil causes sticking and choking in diesel engine because of its high viscosity. Therefore, modification must be needed to make it suitable for diesel engine that is, conversion to biodiesel. These modifications can be brought by micro emulsion, pyrolysis, and transesterification method. Among them, transesterification is the best one because of high yield and low cost. The main advantages of biodiesel are that it is renewable, less polluting, safer, and biodegradable fuel. However, biodiesel also have certain limitations as high cost and cold flow behavior which is not up to the mark. Mostly the raw material used in biodiesel production is edible vegetable oil due to which food versus fuel condition arises. To overcome this problem, researchers all over the world are working in the direction of cost effective and non-edible raw material for biodiesel production. In this way, the new alternatives like waste oil, non-edible oils, and algal oil have been found. The biodiesel from algae is relatively a new technology. Biodiesel can be proved useful only by proper cultivation and management of feedstock in a sustainable way with biodiversity in mind.

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Review Article

Low–Temperature Plasma Technology: Impact on Indian Rural Life

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Abstract

The low temperature plasma technology has been emerged as an alternative environmental friendly technology that has a potential to replace the traditional technologies in various sectors such as textile industries, agriculture, food industries, and waste water treatment. The future prospective of the low temperature plasma technology on the life of rural Indians has been discussed in this brief report.

Keywords: Low temperature plasma, plasma agriculture, dielectric barrier discharge, plasma water treatment

1. Introduction

What are people's most common thoughts about the states of matter existing in the universe? Are these solid, liquid, and gas only? The answer is no, but why? Does any other state of matter exist? Yes, but which state? The fourth state of matter is named 'PLASMA'. It is an ionized gas which consists of negatively charged electrons and positively charged ions in which these charged particles interact via long range coulomb interaction and is capable to exhibit the collective response to an external field force [1]. Depending on the averaged energy of the electrons and ions in the mixture of ionized gas (plasma), it can be named as hot plasma (or thermal plasma) and cold plasma (or non-thermal plasma). Some examples of the hot plasma are thermonuclear fusion plasma (e.g. sun), atmospheric arcs, flames, and sparks. In a plasma, the average energy of ions is negligible than the electrons. Examples of cold plasmas include the discharge in a fluorescent tube, different glow discharges (dielectric barrier discharge, surface micro discharge) in laboratory, and earth's ionosphere [2-4]. In laboratory, non-thermal plasma is produced by the electric breakdown of gases at atmospheric pressure. A high voltage difference across the electrodes is required to break down the gases at atmospheric pressure. In the non-thermal plasma, majority of the discharged energy goes into the production of energetic electrons rather than in ion and neutron heating. These energetic electrons are the main source for production of radicals or reactive species of nitrogen and oxygen (RONS) through the electron impact dissociation and ionization processes [5, 6]. The role of the various reactive species, UV radiation, energetic electrons, and ions of non-thermal plasmas has been studied in multidisciplinary scientific fields which are discussed in subsequent sections.

2. Textile Industries

Non-thermal plasma (NTP) is widely used in the textile industries around the world. The textile materials in the form of sheets, fabrics, and polymers are treated with the non-thermal atmospheric pressure plasma to modify the surface properties and make surface anti-bacterial. In treating with the plasma, the modified surface wettability and surface textures may increase dye or finishing agents' absorptions, surface chemical and morphological modifications help to improve the hydrophilicity of fabrics, and reduction of bacterial species on the fabric surface [7–9]. There are chemical processes to treat wool and cotton fabrics to modify the surface properties of raw materials to turn them into consumable products. The use of NTP technology to treat wool and cotton fabrics has some advantages over the other processes. It is possible to treat all the organic compounds by NTP because it only modifies the surface properties without altering the bulk properties. It is also environmentally favourable because it uses less water [7, 8].

In rural India, sheep and goats play an important role in the livehood of a large proportion of small farmers and landless labourers. The production of wool from sheep and goats is a source of income. The wool quality of most Indian sheep and goats is unsatisfactory, which is one of the key reasons for low demand and limited output. Because of the low market for Indian sheep wool due to its poor quality, it does not contribute significantly to the sheep farming sector's net income. The improvement of wool quality is one of the factors that help to enhance the net profit of sheep farmers. The non-thermal plasma technology has a potential to improve the quality of the wool fibres at low input costs [7, 9].

Apart from wool, cotton is also a major crop in India. Cotton fibres are the most important and comfortable textile fibres. In some parts of the country (rural region), it is the main crop for the source of income for farmers. Cotton fibres of improved quality bring the highest money in the commodity markets. Plasma can modify the surface of cotton fibres instead of using the traditional method to improve their quality. Hence, plasma technology is considered as an advanced tool to improve the quality of wool and cotton fibres [7–9]. In this view, there is a need for installation of small scale industries of textiles in the rural region for converting raw material (wool and cotton) to finished/usable products. The low energy consumable plasma technology can be a part of the surface modification (or quality improvement) unit in the textile industries. There is a possibility to use different types of plasma sources such as dielectric barrier discharge plasma, energetic electrons beam, and ions beam to modify the surface properties of fibres. For reducing the input cost, it is also possible to operate these systems with the help of solar powers. Hence, the low energy cost, less water usage, and environment friendly non-thermal plasma technology is necessary to increase the cost of raw materials which definitely will help to increase the net income of small farmers, marginal farmers, and landless labourers who are involved in sheep farming sector.

3. Agriculture Sector

Agriculture is a back bone of the Indian economy. The majority of agriculture land in India is utilised to cultivate a variety of crops as well as vegetables and fruits. However, yields per hectare of crops in India are generally low compared to international standards. There are many factors such as shortage of water, fewer nutrients in soils, lack of suitable fertilizers, harmful insects and bugs, etc. which strongly affect the yield of the crops. The low yield of crops/vegetables/fruits is the main cause of the low income of small and marginal farmers in rural regions. It is true that India's population growth rate is positive which clearly indicates the increase in food demand for coming years. Therefore, obtaining high yield in agriculture production is necessary to compensate the increased food demand.

In recent years, low temperature plasma or non-thermal plasma is considered as an advanced green technology for enhancing productivity in agriculture sectors. The low temperature plasma has a potential to boost yield in a robust way without demanding more water and fertilizers. In this technology, crops, seeds, and soil are treated to see the combined effects on productivity [10, 11]. The low temperature plasma or plasma treated water helps in enhancing seeds germination, increasing the rooting speed, stimulating plant growth, preventing the pests and bugs, inactivating of the micro-organisms and fungal spores, etc. The presence of reactive species of nitrogen in plasma along with water can form the nitrogen rich fertilizer which is an essential nutrient for growth of the crops and keeps them healthy. The presence of ozone, oxygen reactive species, and UV radiation in non-thermal plasma can help to inactivate the microorganism, pests, and fungal spores to keep the crops healthy during the entire life cycle. The productivity in agriculture sectors can be improved by keeping the crops/plants healthy which is possible by using the non-thermal atmospheric-pressure plasma sources such as DBD, plasma jets, and surface micro discharges [10–12].

Indian government is more focused on doubling the income of farmers in the coming years. It is only possible by increasing yield of crops per hectare with low input costs. Providing high quality seeds, low cost pesticides, and low cost fertilizers to farmers are essential to reduce the input cost of farming and increase the productivity. Day by day use of chemicals (fertilizers and pesticides) in the agriculture sectors is responsible for the poor health of human beings. Alternate advanced eco-friendly technologies for increasing productivity of food grains, vegetables, and fruits are needed in the future to keep society or people healthy.It has been experimentally proven in many research labs worldwide that low temperature plasmas (or NTP) are an alternate green technology to boost the agriculture sector [10,11]. It is necessary to establish a broad multidisciplinary research network (plasma, agriculture, biotechnology, and chemistry) among different research institutes/universities of a country. Apart from the research activity, applied research projects with industrial partners are necessary to scale up the small laboratory projects at a large scale for direct applications of non-thermal plasma technology to crops/plants in the rural region. It is expected to increase the productivity up to 20 % by using the low temperature plasma technology throughout the entire life cycle (from seed germination to inactivation of micro-organism) of crops/plants. The higher productivity will

increase the net income of small and marginal farmers which is essential to survive a better life in the future.

4. Food Sector

In India, the storage of vegetables/fruits for a long time is one of the big challenges. Due to the lack of cold storage chain between farmers and consumers, small and marginal farmers are unable to sell the agriculture products such as vegetables and fruits at a reasonable price. A used amount of vegetables and fruits is wasted due to the growth of microbes under the suitable environmental conditions. However, there are some commonly used chemical agents (organics acids) and radiations for inactivation of microbes on the vegetables and fruits. But, the non-thermal plasma technology could be an alternative to currently used methods without any side effects. It has been reported in many studies that non-thermal atmospheric pressure plasma consists of different reactive oxygen and nitrogen species (RONS), energetic electrons, UV radiation, and ions. Therefore, it has a potential to inactive different contaminating microbes (or surface disinfect) on vegetables and fruits [12–14]. The life or freshness of vegetables/fruits is increased after treating them with the non-thermal atmospheric pressure plasma.

To decontaminate vegetables and fruits, they can be treated directly with gas plasma or plasma activated water [13, 15]. Hence, plasma treatment could be used to preserve the vegetables/fruits freshness and all other properties for a few days (2 to 5 days) without cold storage. This small time period (2 to 5 days) is sufficient to transport the vegetable/fruits from the local region or farm houses to cold storage or consumers in metro-city areas. The sterilization of packing materials (plastics bags/container) using atmospheric pressure plasmas also increases the life of vegetables/fruits [12–14]. In India, solar power is assumed to be a future energy source. Similar to solar water pump at farm houses in rural regions, solar power driven low temperature plasma sources could be established at the village level for treating the vegetables/fruits and packing materials before transporting to other cities. Thus, the low cost and environment friendly plasma technology can help to increase the net income of small and marginal farmers of the rural India.

5. Water Purification

In rural India, hand pumps, tube wells, and normal wells (ground water) are the main sources of drinking water. Sometimes, the quality of ground water is not good. The bacterial contamination of pumping out water continues to be a widespread problem in the rural region of India. The poor quality of drinking water due to bacterial and viral species is a major cause of illness and death affected by water-borne diseases. Such water related diseases put a financial burden on low income sections in villages. Providing safe drinking water to all homes in rural India is a very challenging task. There are many technologies to make water drinkable but we also require some advanced technologies. There is an emerging technology based on

plasma (plasma-based water purification) having the potential to inactivate/remove the micropollutantsm, bacterial species, and viruses from the pumping out water [16]. The interaction of plasma with water generates various reactive species (short and long lived) that inactivate/kill the bacterial contamination of water [17, 18]. For providing safe water to all the villagers of rural regions in India, we should use the low cost advanced water purification techniques. However, installing plasma-based water purification reactors at the local level poses numerous technical hurdles, yet this mission could be a success in the coming years.

6. Conclusion

In this report, a brief introduction about plasma and types of plasmas based on average energy of charged species (electrons and ions) are presented. The plasma with lower ions temperature than the electrons temperature is named as low temperature or non-thermal plasma. Applications of non-thermal plasmas in various fields such as textiles industries, agriculture, food sector, and waste water treatment are discussed. How an environment friendly plasma technology could transform the life of rural regions of India in coming years and challenges in implementing the technology at local level have been thoughtfully discussed.

7. Conflict of Interest: No

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Review Article

CRISPR-Cas System: Molecular Scissors Revolutionizing Genetic Engineering

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Abstract

The CRISPR-Cas system is an elegant mechanism that acts as a shield and safeguards prokaryotes from various invading genetic elements by RNA interference. Interestingly, the ability of these genetic scissors to precisely nick specific nucleic acid has restructured genetic engineering ranging from fundamental research to translational medicine. The simplicity and robustness of this technique has been used to carry out gene modifications in a single cell as well as whole organism. This review sheds light on biology and molecular mechanism of CRISPR-Cas system, its classification, and its implication as a versatile platform in transcriptional modulation creating transgenic models, genomic imaging, and translational medicine.

Keywords: CRISPR-Cas, genome editing, RNA interference, gene therapy, and translational medicine

1. Introduction

The persistent threat encountered by bacteria and archaea against invading viruses, plasmids, and transposons has empowered them with strategies to withstand infection (Gomaa et al., 2014). Recently, the defense mechanism mediated by a novel family of DNA repeats known as Clustered Regularly Interspaced Short Palindromic repeats abbreviated as CRISPR along with CRISPR-associated systems (Cas) has gained a lot of importance. These unusual DNA repeats were first discovered in 1987 in an intergenic region of *the Escherichia coli* (*E. coli*) genome but due to lack of high throughput sequencing tools, its existence in other microbes initially remained unknown (Barrangou 2013). Notably, all the repetitive DNA sequences often termed as 'repeats or CRISPR repeats' are ~ 23 – 55 base pairs (bps) in length and are separated by unique non-repetitive sequences called 'spacers' that are derivatives of invading genetic elements (Ratner, Sampson, and Weiss 2016). A study carried out in 2002 by Jansen and colleagues employed an *in silico* approach in defining the role of CRISPR. Similarity analysis of CRISPR repeats revealed lack of sequence conservation between two distinct species that is, *E. coli* and *M. tuberculosis* while significant similarity was observed between closely related species - *M. tuberculosis* and *M. bovis.* Further, analysis of genes edging both side of CRISPR loci in unrelated prokaryotic species showed significant presence of congruity between four genes which were designated as CRISPR- associated genes, *cas1* to *cas4* (Jansen et al.,

2002).Later in 2005, three independent group of researchers showed that the sequence of spacers is analogous to that of invading genetic elements including bacteriophages and that the spacer carrier strains were protected from the subsequent infection by those viruses (Bolotin et al., 2005; Pourcel et al., 2005; Soria, 2005). This observation advocated the role of CRISPR in providing immunity in bacteria. The first evidence suggesting the role of *Cas* in providing phage resistance was provided by Barrangou and colleagues wherein they observed bacteriophage challenge induced viral spacer integration in the genome of *Streptococcus thermophilus*. Subsequently, removal or addition of spacer altered this resistance phenotype (Barrangou et al., 2007). Over time, the presence of these repetitive arrays in about 45% bacteria and 90% archaea were discovered using computational algorithms (Horvath and Barrangou, 2010). Later, structural analysis of genes associated with CRISPR showed similarity to that of endonuclease and helicase which can associate with nucleic acids. This analysis further confirmed that CRISPR-Cas mediated defence system relies on targeting and silencing of invading elements by RNA interference (Brouns et al., 2008; Wiedenheft et al., 2009). The function and basis of CRISPR-Cas remained a mystery until a series of acute observations opened the way for an emerging area of biology of this prokaryotic adaptive immune system as well as the investigation of how it can be used for guided genome alteration (Ratner, Sampson, and Weiss, 2016).

2. Crispr-Cas Complex

Despite high abundance in prokaryotes, the CRISPR-Cas system shows vast variation in distribution, number, size, and components across organisms (Horvath and Barrangou, 2010). The core elements of the system include:

2.1 Crispr Loci

The most distinctive feature of this loci is the presence of repetitive sequence varying from 20-55 bps in length. Generally, these CRISPR repeats are conserved in closely related species and appear in cluster (Barrangou, 2013). Another key feature of these repeats is the presence of palindromic sequence which gives the word ‘palindromic’ in the CRISPR acronym. The dyad symmetry of these repeats enables the formation of a stable secondary structure in the form of hairpin loop. Along with the direct repeats, some CRISPR arrays often include inverted repeats (Jansen et al., 2002). In general, bacterial genome consists of three CRISPR arrays on an average while up to five CRISPR arrays are present in archaea. The CRISPR repeats are spaced by a stretch of novel sequences with uniform length known as **“spacers”**. Spacers are peculiar derivatives of foreign genetic elements such as bacteriophage, plasmids or transposons. Sequence specific immunity in bacteria is conferred by these spacers who act as a memory of previous infection (Charpentier et al., 2015; Karimi et al., 2018; Richter, Chang, and Fineran, 2012). After integration of a spacer, the architecture of the loci displays repeat-spacer-repeat pattern. Along with this, two identical spacers are not

present in the same array. Besides this, **'Leader Sequence (LS)'** is made up of long repeats rich in AT which flanks the CRISPR loci. This sequence is frequently found to be present on the 5'- end of CRISPR locus and harbors promoter element which regulates transcription of the CRISPR array (Jansen et al., 2002; Sorek, Kunin, and Hugenholtz, 2008; Wiedenheft, Sternberg, and Doudna, 2012).

*2.2 CRISPR - Associated Genes (*Cas*)*

CRISPR mediates its action in association with a core set of genes called CRISPR associated genes (*cas*) which are exclusively present in the genomes consisting of CRISPR. The positioning of these genes is invariably near CRISPR array. In 2005, Haft and his co-workers reported six core genes of CAS family named as *cas1* to *cas6*. Among these six genes, the two genes *cas1* and *cas2* are ubiquitously present in all the types of CRISPR system and are known to play a crucial role in acquisition of novel spacer (Haft et al., 2005). The abundance, existence, and distribution of these genes are variable. The Cas proteins encoded by these genes are genetically polymorphic and functionally diverse in providing adaptive immunity. One of the most commonly observed domains in Cas protein is RNA Recognition Motif (RRM) which is crucial for RNA mediated interference. Some other peculiar features of Cas proteins is the presence of functional domains for nuclease, helicase, and polynucleotide binding domain, etc. that enables the cleavage of specific strands of target DNA complementary to the CRISPR sequence using CRISPR as a guide. Recently, based on the phylogeny and the mechanism used by *cas* genes, CRISPR-Cas systems are classified into 3 main types that includes Type-I system which is marked by the presence of *cas3* gene, Type-II system is signified by the presence of *cas9*, and Type-III by the presence of *cas10* gene (Barrangou, 2013; Burmistrz, Krakowski, and Krawczyk-balska, 2020; Haft et al., 2005; Jinek et al., 2012; Karimi et al., 2018).

3. Basis of Acquiring Immunity via Crispr-Cas System in Bacteria

CRISPR-Cas functions in three discrete steps: Adaptation, Expression, and Interference.

3.1 Adaptation

To build immune memory, CRISPR-Cas systems incorporate small fragments of foreign nucleic acids called spacers via spacer acquisition (Ivančić-Bace et al., 2015). This sampling of genetic material will help in building immunity and immune memory in bacteria (Barrangou, 2013). Theoretically, acquisition process can be divided into two phases: during the preliminary phase, a small fragment of foreign sequence termed as 'protospacers' integrate proximal to the CRISPR locus, while in the later phase spacers are integrated into the CRISPR locus (McGinn and Marraffini, 2019). The two core proteins involved in this process are Cas1 and Cas2. The complex formed by the integration of Cas1 and Cas2 functions as spacer integrase and performs cleavage as well as ligation reaction (Xiao et al., 2017). Further, the integration of spacer is mediated by a nucleophilic attack through 3'- OH of protospacer DNA on the end of the CRISPR repeat,

generating structure with double stranded protospacer DNA bound to ssDNA of the repeat sequence. Later, this spacer is ligated and insertion is achieved by DNA polymerase. Apart from this, RecBCD plays a key role in preventing the acquisition of self-targeting spacers by detecting the existence of *chi* sites in bacteria (McGinn and Marraffini, 2019; Yosef, Goren, and Qimron, 2012).

3.2 Expression

The spacers acquired during adaptation phase are processed to form CRISPR RNAs (crRNA), the functional elements of defense in CRISPR-Cas system. These crRNAs are synthesized by highly specific processing events. Maturation of crRNA is a two-step event where initially a long primary transcript or precursor crRNA (pre-crRNA) is synthesized from the leader sequence harboring promoter. Further, the pre-crRNA is cleaved at specific sites such that the mature crRNA comprises of entire spacer flanked by repeat sequence partially. This crRNA serves as a guide for target cleavage thus, entitled as guide RNA (gRNA). Diversification of CRISPR-Cas systems into various subtypes and a large number of Cas variants have also evolved the biogenesis mechanisms for crRNA (Hille et al., 2018; Ratner, Sampson, and Weiss, 2016).

3.3 Interference

In the concluding phase, mature crRNA associates with Cas proteins to form a ribonucleoprotein complex. This complex is guided by crRNA to precisely restrict the invading genetic elements (Barrangou, 2013). Once the invading genetic elements are recognized based on the sequence homology, the nuclease activity of Cas cleaves the target dsDNA (Hille and Charpentier, 2016). Further, autoimmunity is prevented by the presence of proto-spacer adjacent motif (PAM) in bacteria (Ratner, Sampson, and Weiss, 2016). It is a conserved stretch of 2-5 nucleotides that serves as a tag of foreign genetic elements and is indispensable for Cas nuclease to nick DNA. Apart from recognition, PAM promotes DNA melting, RNA: DNA heteroduplex formation, and binding of Cas interference complex (Jinek et al., 2012).

4. Classification of Crispr-Cas Systems

Cas proteins are genetically and functionally diverse group of proteins which makes them difficult to classify. A new classification system was proposed by Emmanuelle Charpentier and colleagues in 2011 based on which CRISPR-Cas system is divided into 3 major groups (Makarova et al., 2015). The *cas1* and *cas2* genes are a fundamental element of all the three systems and due to their ubiquitous presence in all CRISPR-Cas systems; they are often referred as universal *Cas* genes. Cas1 and Cas2 are metal dependent nucleases (Wiedenheft et al., 2009). They play a key role in spacer acquisition process where Cas1 cleaves the DNA independent of the sequence specificity and mediates spacer acquisition along with Cas2. Apart from this, the signature genes which define Type I, II, and III systems are *cas3*, *cas9*, and *cas10* respectively

(Barrangou, 2013; Makarova et al., 2015).

4.1 Type II System

One of the best characterized CRISPR-Cas systems frequently used for genome editing purpose is Type-II system guided by Cas9 endonuclease. The Type II system is marked by the presence of **'Cas9'**which codes for proteins involved in the biogenesis of crRNA and cleavage of target DNA. Cas9 harbors the HNH nuclease domain and RuvC like nuclease domain which has endonuclease activity attributing to double-strand cleavage of the target. A remarkable feature of this system is **'trans-activating RNA'** (tracrRNA) which is complementary to CRISPR repeat and plays a key role in maturation and processing by endoribonuclease RNAaseIII. TracrRNA-Cas9-RNAaseIII forms 3 element system and mediates interference by cleaving dsDNA where HNH and RuvC domains of Cas9 cut complementary and non-complementary strands respectively (as shown in figure 1) (Barrangou, 2013; Deltcheva et al., 2011; Jakubauskas et al., 2007).However, the presence of *cas1* and *cas2* genes nearby of *cas9* is a hallmark of the Type-II system (Makarova et al., 2015). Type-II system is further classified into 3 subtypes namely Type-II A to II-C. Impressively, this system is solely observed in bacteria and is generally used in genome editing tool kit (Barrangou, 2013).

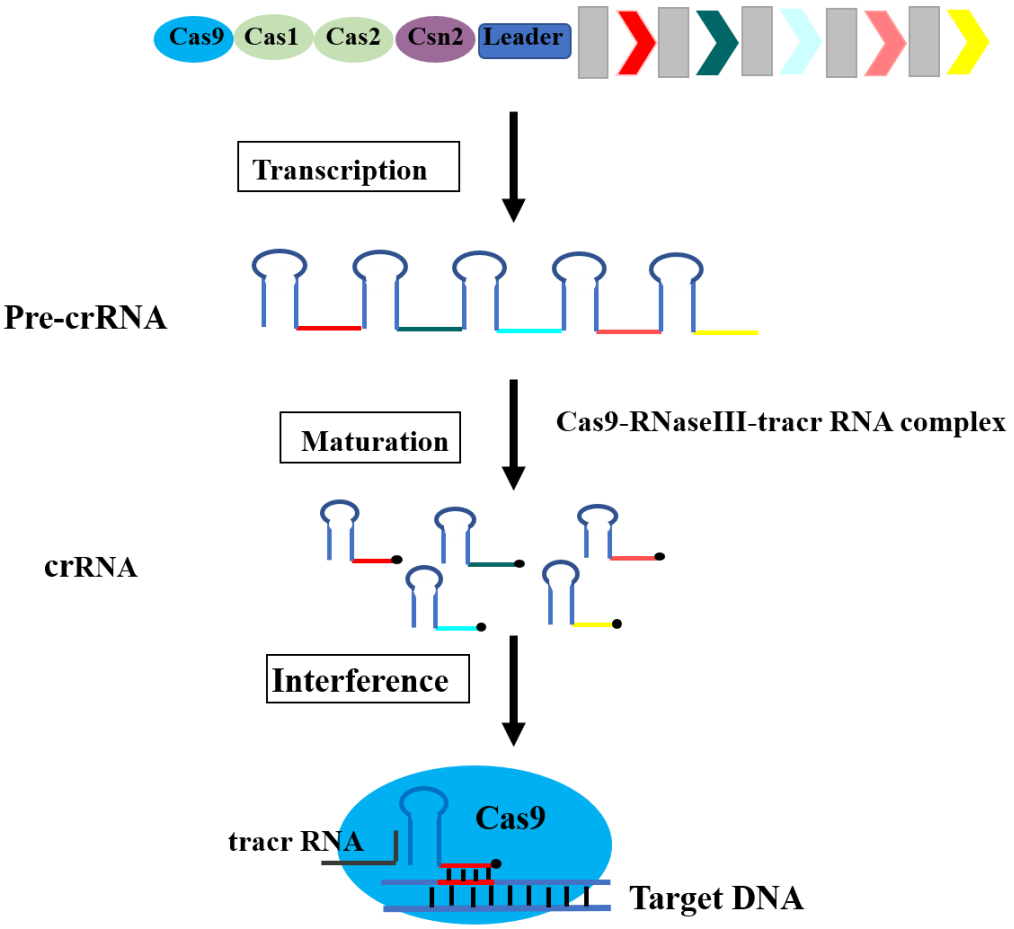

Figure 1: Mechanism of Type II CRISPR-Cas system

In this figure, universal cas1 and cas2 genes are shown in green. Cas9, the Type II signature gene is shown in blue, repeats are shown in grey, and colored arrows represent spacers. The three-component system involved in mediating interference is shown in light blue. (Image was created using Microsoft Power Point, 2016)

5. Genome Editing Using Crispr-Cas System Relies on Endogenous DNA Repair Pathway

Genome editing using CRISPR-Cas provides a robust approach for precise alteration. Type-II system is predominantly used for genome engineering. Once the cleavage of target DNA is mediated by Cas9, the double stranded break generated activates endogenous DNA repair pathway. The cell can mediate DNA damage repair by either activating non-homologous end joining which has low fidelity and often results into insertions and deletions (indels) or homology directed pathway that repairs the break by using sequence homologous to the break (Ran et al., 2013; Sander and Joung, 2014). Both of these pathways are important for editing genome as per the choice. Generally, NHEJ is the commanding pathway independent of the cell cycle stage and its ability to create insertion and deletion is harnessed when the aim of editing is to disrupt the gene function or create a knockout (Sansbury, Hewes, and Kmiec, 2019; Xue and Greene, 2021). For example, the genetic disease DMD is the result of premature stop codon in the dystrophin gene targeting of Cas9 to nearby locus introduces frameshift mutation due to indels thereby returning the function of DMD. Further, to achieve the deletion of large target sequence or gene from the genomic DNA, a pair of gRNA is used. Both the gRNAs are furnished with the sequence complementary to the target that flanks the desired region to be deleted (Ran et al., 2013; Xue and Greene, 2021). Once the Cas9 mediates its action on the target DNA, double stranded breaks are created simultaneously resulting into the loss of sequence between the two gRNA. This approach was used to delete human CDC42 gene in HEK293 cells and removal of expanding CTG repeat in myotonic dystrophy kinase (DMPK) (Dastidar et al. 2018; Zheng et al. 2014). Additionally, the gene recovery by insertion of functional gene is the most appreciated application of CRISPR-Cas system. This can be achieved by directing DNA repair pathway towards HDR. For gene recovery, the exact match of the donor DNA is provided as a template that leverages HDR. The template DNA donor can be in the form of single stranded oligonucleotides (ssODNs), DNA plasmids, or viral vectors (Ran et al., 2013). This approach was used in *ex vivo* gene correction of β-globin gene in patient derived hematopoietic stem cells and showed 90% efficiency in target gene insertion (Dever et al., 2016). One of the shortcomings of HDR activation is that it generally gets activated during S phase while induction of this pathway is less during other phases of cell cycle. To overcome this, mutant Cas9 (Cas9n) is used which has Aspartate to Alanine mutation in RuvC catalytic domain of Cas9. This Cas9n nicks the target DNA resulting

in single-stranded breaks rather than cleaving DNA, preferentially activating HDR and thus, increases the specificity of target recognition (Ran et al., 2013, 2014). This way sequence specific endonuclease facilitates precise genome editing.

6. Implications of Crispr-Cas System in Translational Research

Potential to precisely manipulate a sophisticated network of genes and its regulatory elements by CRISPR-Cas9 is exploited globally for innovative applications in biology. The strength of the technology to unravel the elaborate network of genes and dynamic cellular process, explore genetic rearrangements, and progression of diseases as well as its ability to rectify genetic mutations responsible for hereditary disorders is revolutionizing basic research (Doudna and Charpentier, 2014). The major breakthrough in the development of the CRISPR-Cas system has been contributed by Emmanuelle Charpentier andJennifer Doudnawho were conferred with the Nobel Prize in Chemistry in 2020. Repurposing the CRISPR-Cas system in genetic engineering relies on the ability of a eukaryotic cell to repair DNA damage. To restore the nick, cells can operate either through non-homologous end joining (NHEJ), an error-prone process that often gives rise to mutations or homology-directed repair (HDR) which utilizes sequence homologous to target site as a template and reconstruct the DNA by homologous recombination (Ratner, Sampson and Weiss, 2016).

6.1 Crispr as an Antimicrobial Tool

The blooming and expansion of drug resistant pathogens continue to threaten our potentiality to treat infections exploiting its nuclease activity. The use of CRISPR as an antimicrobial tool has been recently studied (Bikard et al., 2014; Citorik, Mimee and Lu, 2014). Antimicrobial activity is achieved by designing CRISPR arrays aiming at antibiotic resistance or virulence genes which facilitate the killing of drug resistant pathogenic bacteria. To evaluate the ability of this technology in destructing virulent and antibiotic resistant strains, a study was carried out in 2014 by Bikard and colleagues where staphylococci, a predominant member of human skin microbiota as well as the leading cause of nosocomial infections were taken into consideration. In this study, sequence-specific removal of virulent and drug resistant *S. aureus* was achieved by reprogramming the CRISPR-Cas9 system against signature sequences responsible for Kanamycin resistance keeping the avirulent *S. aureus* population unharmed. Destruction of plasmid with antibiotic resistance genes re-sensitized resistant bacteria to the drug as well as prevented the transfer of plasmid induced resistance (Bikard et al., 2014). Along with this, fine control over the microbial population is a critical aspect in industrial fermentation, microbial consortium studies, medical biotechnology, etc., where general antimicrobial strategies including antibiotics fail to discriminate between closely related strains of bacteria. To explore the ability of the CRISPR-Cas system in removal of individual strain from a mixed

culture, Gomma et al., designed a CRISPR-Cas9 system targeting two *E. coli* sub-strains namely *E. coli* K-12 (BW25113-T7) and *E. coli* B [BL21 (DE3)] and observed that the sequence specific removal of individual strain was extremely high. This potency of CRISPR may open a new course of action in dealing with multidrug resistance and the development of smart antibiotics that can distinguish favorable bacteria from pathogenic bacteria (Gomaa et al., 2014).

6.2 Mammalian Genome Editing Using Crispr-Cas System and its Therapeutic Implications

Following the antimicrobial application, CRISPR-Cas9 is a coherent tool that permits editing of the mammalian genome. In 2013, Cong et al. used a CRISPR-Cas system from *Streptococcus pyogenes* including Spcas9, SpRNaseIII, trans-activating RNA (tracrRNA), and precursor RNA (pre-crRNA) to target the mammalian genome of 293 FT cells (Wyman et al., 2013). The desired alterations were observed in the target mammalian genome demonstrating activation of NHEJ or homology-directed repair after the action of Cas9 (Doudna and Charpentier, 2014). Cancer, a leading cause of mortality globally is evolving creating hurdles in the treatments available. To overcome this, programmable nuclease has also paved the way in cancer immunotherapy where chimeric antigen receptor T (CAR-T) cells are produced using CRISPR. In this approach, T-cells from the patients are collected and genetically modified to kill antigens present in cancer cells *ex vivo* and are retransferred to the patient (Cheng et al. 2020). In line with this, Novartis a leading global healthcare company has also secured exclusive rights for using Intellia's CRISPR technology in developing CAR-T cell treatment for dealing with leukemia, and lymphoma (Mullard, 2015). On the other side, tumor suppression was also achieved by rectifying genetic abnormality in tumor-associated genes including E-cadherin, Bax, and p21 in bladder carcinoma (Cheng et al., 2020). Moreover, the CRISPR-Cas system has also been able to resensitize drug-resistant tumors by targeting drug-resistant genes (Saber et al., 2020). These studies suggest an emerging role of the CRISPR-Cas system in cancer therapeutics.

6.3 Crispr in Resolving Genetic Defects

The emergence of CRISPR-Cas system as a genome editing toolkit provides an opportunity to manage monogenic disorders. Monogenic disorders are associated with a defect in single gene and include cystic fibrosis, duchene muscular dystrophy, cardiovasculardisease, beta-thalassemia, sickle cell anemia, etc.

Cystic Fibrosis (CF), a genetic disease associated with severe respiratory problems is caused by a mutation in cystic fibrosis transmembrane protein (CFTR). In one study published in 2013, intestinal stem cells of patients with CF were altered using CRISPR-Cas resulting in a gain of function of CFTR locus curing CF. Organoids with repaired CFTR protein were able to express fully functional CFTR and gave evidence for the development of treatments for CF using CRISPR technology (Schwank et al., 2013).Additionally, Cas9 directed repair has also shown preliminary success in removing HIV proviruses from infected cells via Cas9

directed cleavage and targeting hepatitis viruses putting it forward in antiviral therapeutics. This technique has also been employed in correcting the *crygc* gene responsible for cataract in mouse zygotes, Fah mutation causing tyrosinemia, interfering *Nrl*functioning for treatment of blindness, restoring functional globin gene as a remedy for patients with β-Thalassemia, etc. (Antony et al., 2018; Hu et al., 2014; Kennedy et al., 2015; Ratner, Sampson and Weiss, 2016; Yu et al., 2017). These unconventional studies bring CRISPR to the spotlight and signify its therapeutic promise.

6.4 Transcriptional Regulation Using Crispr

Beyond the permanent alteration of DNA, the CRISPR-Cas9 system has been redirected for transcriptional modulation using deactivated Cas9 (dCas9). dCas9 is the result of mutations induced in the nuclease domain of Cas9 which allows it to bind DNA specified by sgRNA but prevents it from cleaving it. This feature of dCas9 is employed in transcriptional regulation without genome modification. CRISPR based interference (CRISPRi) employs gene repression by directly targeting sgRNA to the promoter or by integrating repressor domain such as KRAB with dCas9 leading to repression of endogenous genes by inhibiting RNA polymerase (as shown in figure 2A). Suppression up to 80% was attained by targeting p53, CXCR4, and transferrin receptor with CRISPRi (Dominguez, Lim and Qi, 2016; Lawhorn, Ferreira, and Wang, 2014). Likewise, dCas9 when fused with transcription activators such as ω-subunit of *E. coli* polymerase promoted holoenzyme assembly in *E. coli* and fusion with VP4 or p65 showed activation of endogenous genes including interleukin-1 receptor antagonist, vascular endothelial growth factor A (VEGF-A), etc. in mammalian cells (as represented in figure 2B). Gene activation accomplished by this process is termed as CRISPR associated activation (CRISPRa) (Dominguez, Lim and Qi, 2016; Maeder et al., 2013).

6.5 Live Cell Imaging Using Crispr

Visualization using dCas9 tagged with GFP provides an indispensable approach to envision specific DNA sequences in live cell (as illustrated in figure 2D). This technique was first demonstrated in 2013 by Chen *et al*., where dCas9 tagged with EGFP enabled visualization of telomere shortening and elongation, positioning and dynamics of mucin producing MUC4 gene as well as chromosomal dynamics during mitosis (Chen et al., 2013). Further, chromatin ultrastructure was revealed by labeling pericentric, centric and telomeric sequences in mouse embryonic stem cell (Anton et al., 2014). Additionally, a new approach known as “track first and identify later” has been developed by combining CRISPR imaging with FISH enabling tracking of multiple loci and study chromosomal dynamics (Takei et al., 2017).

6.6 Epigenetic Regulation via Crispr

Epigenetic modifications of DNA and histone proteins play a critical role in biological processes by

regulating gene expression and CRISPR-Cas system enables us to achieve manipulation of epigenetic tags to study functional genomics (as shown in 2C). To accomplish this, dCas9 was fused with the functional domain of human acetyltransferase (p300) which permitted histone acetylation (H3K27) (Hilton et al., 2015), while the fusion of dCas9 with histone demethylase i.e. lysine demethylase (LSD1) showed reduction in acetylation at H3K27 (Kearns et al., 2015). In line with this, increased CpG methylation was also achieved by using dCas9-DNA methyltransferase fusion protein (Vojta et al., 2016). Loss of pluripotency in mouse embryonic stem cells was observed when enhancers of pluripotency factors Oct4 and Tbx3 were targeted by dCas9-LSD1 fusion. Observations from these studies provide evidence for employing CRISPR in epigenetic modulation. However, the fate of these synthetic marks and their inheritance in proliferating cells need to be addressed (Kearns et al., 2014).

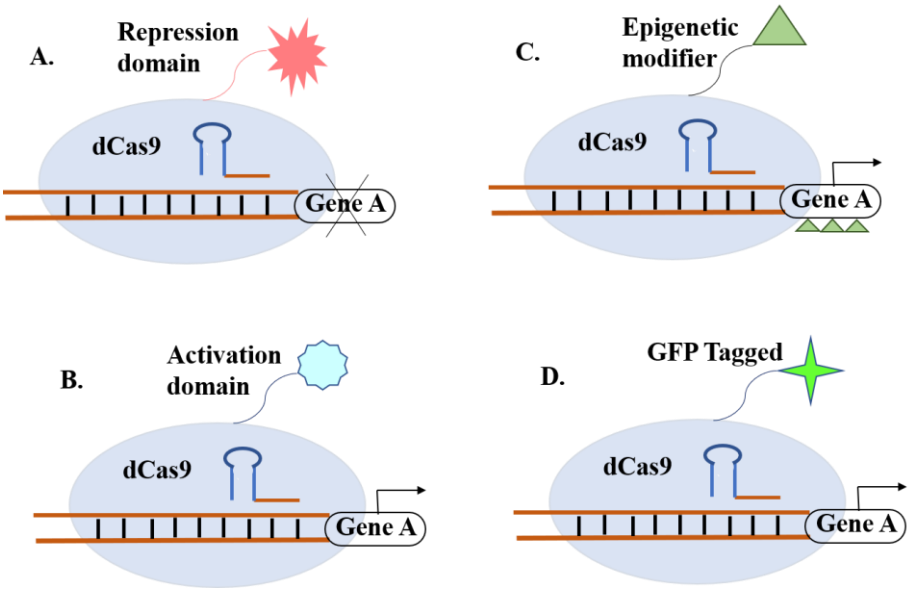

Figure 2: Transcriptional regulation using dCas9

The illustration demonstrates dCas9 mediated (a) transcription repression, (b) transcriptional activation, (c) epigenetic modification, and (d) tagging of DNA sequences. (Created using Microsoft Power Point, 2016)

7. Conclusion

Starting from the discovery of distinctive genetic components to its emerging role in biotechnology, CRISPR-Cas systems have been an electrifying field in biological sciences. This technology furnishes researchers with a remarkable tool to unravel the mystery encoded by the genome. Further, this technology has been proved versatile and has implications in all the fields including food and industrial biotechnology, agricultural science, cell therapy, anti-microbial and anti-viral therapies, biological imaging, etc. Along with the striking advantages, CRISPR-Cas technology has its flaws which must be resolved for prudent and coherent translation. One of the major concerns associated with the use of CRISPR-Cas technology in

gene therapy is the high prevalence of off-target effects. Further, the absence of PAM sequence in eukaryotes makes targeting of Cas9 difficult. In line with this, double-stranded breaks generated by CRISPR frequently triggers apoptosis instead of desired gene editing. Besides technical drawbacks, the application of this technology also raises immunological concerns in human subjects due to the use of bacterial proteins and viral vectors, while on the other side using this technology has also raised ethical concerns to be addressed. Besides this, CRISPR-Cas system has indeed reconstructed genetic engineering.

8. Conflict of Interest

The authors declare that they do not have any competing interest.

9. Acknowledgment

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Review Article

The Role of Mitochondria in the Regulation of Neuropsychological Integrity

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Highlights

- Different aspects of mitochondria-related brain disorders are discussed in this review.
- The review is quite important to comprehend organelle functions on a molecular basis.
- The importance of current research describing genetic predispositions related to mitochondrial dysfunction is analysed.
- The review emphasizes the impact of deregulated mitochondria on the neuroendocrine axis and related neurological disorders.

Abstract

From the evolutionary point of view, mitochondria originated on earth around two billion years ago by the process of ‘endosymbiosis’ in the primitive form of eukaryotic cells. Being a double-membrane-bound cellular organelle, mitochondria are mostly responsible for ATP production. The overall structure and composition of mitochondria have been significantly altered through the process of evolution and the ‘cellular powerhouse’ has acquired several additional functions inside the cell. The process of oxidative phosphorylation in mitochondria is considered the major ATP-generating pathway which fulfils the demand for maximum energy required for cellular activities. Due to genetic and environmental influences, the mitochondrial electron transport chain may be disrupted which is indicated to be an important reason for the pathogenesis of a wide range of psychiatric disorders. Some tissues which require high energy including the brain, skeletal, and cardiac muscles contain a large number of mitochondria. Interestingly, dysregulation of normal mitochondrial functions lead to malfunction of the mitochondrial electron transport system which has pathogenic implications for a wide range of neuropsychiatric, neurodevelopmental, and neurodegenerative disorders including depression, schizophrenia, bipolar disorder, childhood autism, Parkinson's disease, etc. In this review, the important role of mitochondria-related brain disorders and their

molecular mechanisms are discussed. In addition, research describing genetic predispositions and the impact of deregulated mitochondria on the neuroendocrine axis which plays a crucial role in developing such pathogenic conditions is also examined.

Keywords: Mitochondria, gene expression, neuro-psychology, mental health, neuro-hormonal regulation

Abbreviations:

ATP: Adenosine Triphosphate

EEA: Environment of Evolutionary Adaptedness

NR3C1: Nuclear Receptor Subfamily-3

mtDNA: Mitochondrial DNA

NADH: Nicotinamide Adenine Dinucleotide Hydrogen

PD: Parkinson's Disease

PINK1: PTEN-induced Kinase 1

LRRK2: Leucine-rich Repeat Kinase 2

ROS: Reactive Oxygen Species

DJ-1: Protein Deglycase DJ-1 /Parkinson Disease Protein 7

SAM: Sympathetic-Adrenal-Medullary

GABA: Gamma-Aminobutyric Acid

1. Introduction

Mitochondria have an immense contribution to energy production as well as the regulation of energy metabolism in eukaryotic cells (Piomboni et al., 2012). From the neurobiological point of view, the role of mitochondria in psychiatric diseases has been investigated thoroughly by researchers worldwide (Scaglia, 2010). In this review, we will elaborate the concepts of pathogenesis associated with dysregulated mitochondria in neurons which are eventually responsible for disruption in energy metabolism and such abnormal metabolic signalling is often implicated in stress-related neuropsychological disorders.

2. Comprehending the 'Diseases of Modernity'

Our modern life is becoming very fast, competitive, and hectic. We are facing more stress and competition in day-to-day life. As a consequence, extreme changes in daily lifestyle over a long time are responsible for accelerating the disease burden including atherosclerosis, osteoporosis, hormone-liked disorders, gastrointestinal malignancies, and type 2 diabetes mellitus (Smets et al., 2018). Scientific studies revealed that these diseases exert higher co-morbidity along with behavioural abnormality even when they are associated with metabolic and inflammatory disorders in the human body (Song et al., 2018). Earlier researchers proposed the theory that humans are mostly adapted from pre-historic 'hunter-gatherer

lifestyles' which are communally considered like the "Environment of Evolutionary Adaptedness (EEA)" (Hidaka, 2012).Interestingly, the inconsistency between the modern lifestyle and the ancient human EEA ultimately presents the scenario for the ultimate etiology of the chronic diseases related to stress (Eaton et al., 2002). These diseases are collectively mentioned as "Diseases of Modernity." According to a research, diseases of modernity are becoming the greatest risk and concerns for public health in several countries of the developed world (Yach et al., 2006).

3. Mitochondrial Inheritance and Episodes of Depression

Several studies concerning statistics demonstrated that women suffer from depression more than men (Salk et al., 2017). In addition, research revealed the fact the stress level in women is quite high (Chaplin et al., 2008). It adversely affects their social and reproductive life along with symptoms of irregular menstrual cycles (Nagma et al., 2015). According to recent studies, maternal depression during pregnancy can cause severe damage to the physical and mental health of the foetus (Wu et al., 2020). Studies have shown that during pregnancy, women who have long-term anxiety and depression symptoms, experience a modification of DNA methylation in the 'promoter sequence' of the NR3C1 (nuclear receptor subfamily 3- a human glucocorticoid receptor) gene on the foetus' chromosome (Oberlander et al., 2008). As a result, the child's brain structure, mental development, normal immunity, etc. are severely disrupted. However, it is surprising that mitochondria were identified as the first cause of stress and mental depression among men and women (Picard & McEwen, 2018b). The mitochondria in our body come from our mother. As a result, mutations in mitochondrial DNA or mitochondrial dysfunction are significantly associated with child's mental health.

Mitochondria were an ancient bacteria-like organism capable of producing its energy through oxidative phosphorylation. During evolution, such an organism entered into the body of the precursor of prokaryotic cells and established a symbiotic relationship (Martin et al., 2015). After a long time, this organism loses its unique properties and begins to live inside the cell only as a cellular organelle. Evolutionists explain this phenomenon with the help of 'endosymbiosis theory' (Zimorski et al., 2014). All eukaryotic cells supply energy to the body of animals and plants by producing mitochondria 'ATP'- or Adenosine Triphosphate. Almost all of the metabolic reactions that take place in all the cells of our body depend on the effectiveness of ATP. With the slight exception of this, the biochemical reaction will be disrupted causing disease in the human body. Recent studies suggested the potential connection between stress, depression, and mitochondria (Bansal & Kuhad, 2016). According to scientific studies, in comparison with healthy individuals, the levels of ATP were found lower in the neural tissue of the patients with chronic depression (Moretti et al., 2003). Interestingly, some reports indicated the pharmacodynamics of different antidepressant drugs which have a selective effect on mitochondrial energy metabolism. Consequently, these drugs may play important roles specifically in pre-synaptic and post-synaptic compartments (Villa et al.,

2017). Additionally, in recent days, researchers are focusing on the deregulation of glucocorticoid hormone signalling which is associated with the concept of 'Mitochondrial Allostatic Load' (Picard et al., 2014). The concept demonstrates that events of mitochondrial damage, reactive oxygen species (ROS) generation, and the release of mtDNA outside the cell established an association between chronic stress-inducing factors (catecholamine, cortisol, and blood glucose level) and episodes of major depression (Caruso et al., 2019).

4. Mitochondrial Dysfunction and Neurodevelopmental Disorders: Important Connections

The mitochondria in the nerve cells produce adequate energy and help in the proper exchange of neural signals between different neurons. Interestingly, scientists have found that the size and shape of the mitochondria in cells do not always remain the same, but they do change (A. Li et al., 2020).Inside the cell, where more energy is required for metabolism, the mitochondria proceed with the reaction in the shape of two or three spherical or elongated sac-like structures (there is a membrane link between the sacs) to supply more ATP (Campello & Scorrano, 2010). It was observed that during the time course, the sac-shaped mitochondria are joined together to form integral and single mitochondria (Bulthuis et al., 2019). Studies have also shown that a mutation in the DNA of mitochondria in neurons in the brain or an anomaly in its physiological function interferes with the normal functioning of neurons. This can lead to a variety of diseases such as vision and hearing problems, memory loss and mental retardation, imbalance of feelings of joy and sorrow, and even mental exhaustion. Significantly, due to abnormal mitochondrial function, diabetes, high blood pressure, etc. can also occur (Sivitz & Yorek, 2010). According to these scientific sources, it can be speculated that the stress in the mother's body during pregnancy can cause abnormalities in the mitochondrial functions and dysregulated mitochondria can enter the body of the child and lead to various pathogenic complications in the nerve cells of the brain. Several clinical studies indicated that mitochondrial dysfunction is highly associated with neurodevelopmental disorders in children including syndromic autism, intellectual disability, and epileptic encephalopathies (Ortiz-González, 2021). Interestingly, scientists also observed the link between deregulated mitochondria with late stages of many neurodegenerative diseases. For example, researchers observed that inhibition of mitochondrial respiratory complex-I (NADH dehydrogenase-ubiquinone oxidoreductase) can develop Parkinson's disease (PD)-like syndrome (Giachin et al., 2016). Additionally, autopsy samples from PD patients clearly showed impeded respiratory complex I activity in the brain tissue (H. Li et al., 2018). Surprisingly, rare early childhood onset of PD was observed due to mutations in proteins PINK1 (which is crucial for mitochondrial quality control) (Quinn et al., 2020), LRRK2, (Whiffin et al., 2020) and the reactive oxygen species (ROS)-sensitive chaperone Protein deglycase DJ-1 (Saito, 2017). Of note, patients with Alzheimer's disease were found with deformed mitochondria with irregular diameters and surface areas (Granat et al., 2020).

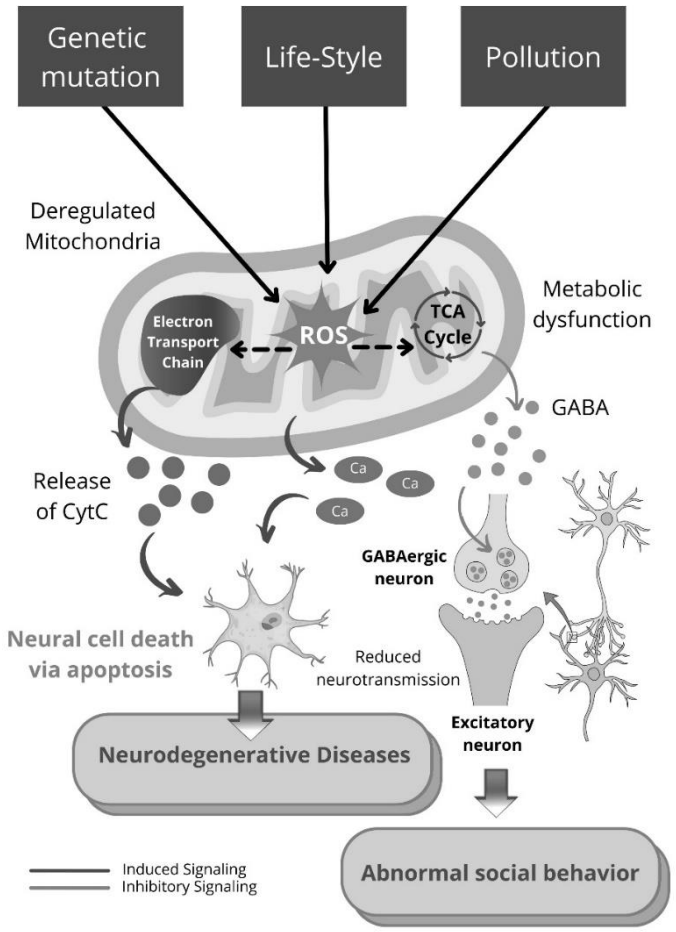

Figure 1: Mitochondrial dysfunction and associated pathogenesis

Genotoxic insults, lifestyle, and high pollution levels are causative factors for mitochondrial dysfunction. Above mentioned factors are potential inducers of Reactive Oxygen Species (ROS) which influence aberrant Ca^{+2} signaling and increase Cytochrome C (CytC) release from the mitochondria leading to apoptosis of neural cells. Moreover, ROS deregulates the mitochondrial energy metabolism pathway which reduces the rate of GABA-mediated neurotransmission. Collectively, abnormal mitochondrial functions give rise to neurodegenerative and neuropsychological disorders.

5. Impact of Mitochondria on the Social Behaviour

From the anatomical point of view, the cortico-limbic networks in our brain are specialized for perceiving and adapting to environmental and genotoxic insults through the stress response mechanism (McEwen & Gianaros, 2010). The exogenous stimuli selectively activate specific regions of the brain which contribute to bio-behavioural response in a coordinated manner. The prefrontal cortex of the brain is solely responsible

for learning, memory, cognition, and control of impulse (Friedman & Robbins, 2022). Moreover, apart from the prefrontal cortex, the amygdala was known as the emotional regulator and important contributor to the 'Sympathetic-Adrenal-Medullary (SAM)' and neuroendocrine regulatory systems (Godoy et al., 2018). Now, the basic question arose in the scientific communities that how can mitochondria cause disturbances in normal human behaviour and emotional exhaustion? Dr. Martin Picard, a researcher at Columbia University and Dr. Bruce McEwen, a well-known scientist in stress biology discussed in a 2016 study in "*Psychosomatic Medicine*" that stress causes several changes in the size and function of mitochondria. They also studied the neurons which are surrounding the synapses (the junction of two neurons) have a much larger number of mitochondria compared to other sites (Picard & McEwen, 2018a). The reason discovered by them is to provide extra energy and help to transmit neural signals. In addition, studies revealed that damaged mitochondria of a neuron come out from it and are absorbed by the oligodendrocytes and astrocytes around the nerve cells (Jeon et al., 2020). Eventually, with the help of these cells, healthy and normal mitochondria re-enter the neurons. Any disruption of this cellular phenomenon can lead to neurological disorders. Mitochondrial DNA fragments have been found in the serum of many mentally retarded patients. Studies claim that it came from damaged mitochondria (Varga et al., 2018).

It was observed that different people behave differently under the pressure of the same situation. What could be the explanation? People when faced with a difficult or uncomfortable situation, they are prepared to deal with it or withdraw from it. Such a condition is considered as the 'fight or flight' which solves the problem (Adamo, 2014). The report suggested that the adrenaline hormone secreted by adrenal glands help to cope with stressful conditions (Ranabir & Reetu, 2011). One of the components of adrenaline is the cortisol steroid, and mitochondria were known to play a key role in the formation of cortisol (Midzak & Papadopoulos, 2016). Once cholesterol enters the mitochondria, it is converted to steroid precursor molecules. The steroid precursor then exits the mitochondria and enters the surrounding endoplasmic reticulum. Thereafter, biochemical modifications or 'molecular processing', the modified steroid precursor re-enters the mitochondria. After the conversion of cortisol steroids, they get released from the cells and enter the circulatory system. Thus, if the mitochondria inside the adrenal gland cells are unable to function normally, they secrete much lower adrenaline hormones under stressful conditions and are responsible for behavioural abnormalities (Yaribeygi et al., 2017). Apart from neuroendocrine regulation, the exact role of mitochondria and energy metabolism in relation to social behaviours has yet to be determined, despite the fact that mitochondrial dysfunction has been studied in social disorders. Recently, Kanellopoulos et al. demonstrated that hyperactive mitochondria in GABAergic neurons are responsible for social behavioural impairments (Zhang et al., 2020). The study indicated that mitochondrial homeostasis is crucial for normal social behaviours through GABA signal transduction. Such important findings provide anew insight into neuropsychological disorders concerning mitochondrial dysfunction.

6. Discussion

Deregulation in normal mitochondrial functions was observed in several neurobiological pathogeneses including, aberrant synaptic signalling, a higher rate of neural cell death via apoptosis along long-term psychiatric disorders (Fricker et al., 2018). Experimental research already established the fact that deregulation in energy metabolism pathways is related to behavioural disorders and strongly associated with mitochondrial dysfunction (E Silva et al., 2019). Interestingly, the scientific communities proposed the "*Mitochondrial Hypothesis*" which suggests that behavioural abnormalities and emotional disorders are partially triggered by the deregulation of mitochondrial functions. Importantly, mitochondria-associated disease pathogenesis could be closely connected to a varied range of therapeutic outcomes, progression of the disease, and severity (Saito, 2017). In-depth research in mitochondrial functions from a bio-chemical point of view could immensely aid in the process of identifying neurological disorders and specific therapeutic biomarkers. Extensive research is a prerequisite to comprehend underlying mechanisms of action of the drugs that treat neuro-psychological disorders related to specific mitochondrial functions. Henceforth, future neuroscience research is paying special attention to the genes, structure, and physiological functions of mitochondria. At the same time, scientists have been working tirelessly to find effective treatments and drugs for various mental and neurological diseases specific to mitochondrial functions.

7. Conclusion

Mitochondria stand for one of the most important cellular organelles for generating ATP and play a major role in the energy metabolism process. Any discrepancies in mitochondrial functions may lead to neuropsychological disorders through deregulation of the neuroendocrine axis. Extensive research is going on to discover new treatment regimens and therapeutic drugs for different neurological diseases related to deregulated mitochondria.

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9. Conflict of Interest

The author has no conflict of interest in this study.

10. Author's Contribution

SB: Conceptualized the review and has made a direct and intellectual contribution to this work.

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Review Article

Quantifying Tannins: Extraction and Recovery

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Abstract

Tannins are polyphenolic chemicals found in leaves and wood, and are most commonly associated with tea. Tea is the world's second most popular drink, after only water. Historically, tea was consumed as an herbal concoction to soothe various ailments. The role of tannins in regulating hunger and metabolism is overshadowed by their anticarcinogenic, antimutagenic, and wound healing enhancing properties. Towards the negative end of the spectrum, tannins are excellent iron chelators which may lead to anaemia. The extraction of tannins is the first step in investigating its medicinal potential or determining its antagonistic traits. This review article presents the techniques which can be employed to extract tannin from tea/coffee samples along with the methods which can help in determining the concentration of tannin in the extract.

Keywords: Tannins, polyphenolic compounds, tea, extraction

1. Introduction

Tannins are polyphenolic, water soluble compounds majorly obtained from plants. They have a molecular weight ranging from 500-3000 Daltons (gallic acid esters) to 20,000 Daltons (proanthocyanidins) (Nair et al., 2015). It exhibits antibacterial, antiviral, and antioxidant properties (Anburaj, 2019). Major features of tannin are derived from its phenolic nature itself. For example, the antioxidant property of tannin is associated with the number of phenol rings present in their structure. The phenol ring acts as an electron scavenger to trap ions and radicals. Besides, tannins stand as the second largest source of phenol compounds after lignin (Silva et al., 2021). Tannins are amorphous and astringent substances. They may exhibit anti nutritional properties when in higher amounts (Khasnabis et al., 2015).It is commonly found in coffee, tea, wine, grapes, strawberries, cranberries, blueberries, apples, pomegranates, etc. The main focus of this review lies in tea as it is the most famous and favourite beverage in all parts of India.

Tea is a non-alcoholic beverage and is loved worldwide. Now tea is known to have antioxidant and artificial colouring benefits and this is due to the presence of tannin in it. Approximately, one gram of dried tea leaves has 12.66 mg of tannin (Rahman et al., 2020). Out of the three main types of tea, i.e. green tea, black tea, and oolong tea, green tea was found to contain the lowest amount of tannin while black tea was

found to contain the highest amount of tannin (Khasnabis et al., 2015). Tea tannin is amorphous in nature and is easily soluble in water. Its aqueous solution has an acid reaction and gives the feeling of dryness to the tea. Pure form of tannin is nearly colourless which when oxidized in air gives a reddish brown colour (Michiyo).Taste, colour, and aroma of tea is influenced by tannin. Tannin also gives a distinguished bitterness to tea (Rahman et al., 2020).

2. Types of Tannin

Tannins are mainly classified into two groups: hydrolysable tannins and condensed tannins. Figure 1(a) shows structure of condensed tannin while figure 1(b) shows structure of hydrolyzable tannin.

- ***a. Hydrolyzable tannins*** are polymers of simple phenolic acids like gallic acid or ellagic acid esterified to a core molecule, usually glucose which is highly soluble in water. On heating with hydrochloric or sulphuric acid, it yields Gallic or ellagic acids (Nair et al., 2015).
- ***b. Condensed tannins*** do not split easily and are flavonoid dyes formed by biosynthesis of flavins and catechins. It is usually formed by condensation of two or more molecules of flavan-3-ols, specifically catechins (Nair et al., 2015).

Chemical structure of tannin is very diverse and different. They are often referred to as tannic acids. Its chemical formula is $C_{76}H_{52}O_{46}$. Its molecular mass is 1701.19 gm/mol and density is 2.12 gm/cm^3. It decomposes above200℃ and is soluble in water, ethanol, glycerol, acetone but insoluble in benzene, chloroform, diethyl ether, carbon disulfide, petroleum, and carbon tetrachloride.

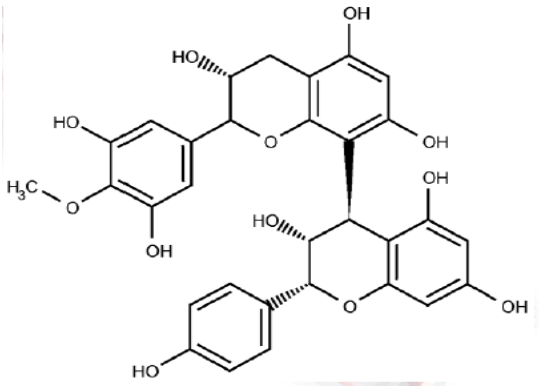


Figure 1(a)

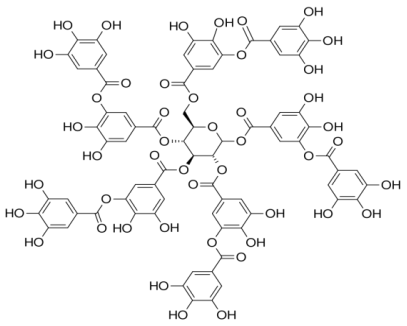


Figure 1(b)

Tannins are found in both free and bound states in plants. Now, tannins found in terrestrial plants are divided into four major groups: gallotannins, ellagitannins, proanthocyanidins, and complex tannins. Tannins from sea plants have been named as phlorotannin. (Cuong et al., 2016) structures are shown in

figure 1(c), 1(d), 1(e), 1(f), and 1(g) respectively.

Tannins are found to have very high biological potential. Owing to their number of hydroxyl radicals and aromatic rings, they are proven to exhibit antimicrobial, anti-fungal, anti-tumor, and antioxidant activities. Also, the phenolic groups play an important role in tannin. The phenolic groups form strong hydrogen bonds with -NH groups in protein which cannot be broken down by digestive enzymes or any other microorganism. Thus, protein precipitates. Tannins are also found to have the feature of chelating metal ions which is because of its ortho-hydroxyl groups. (Cuong et al., 2016)

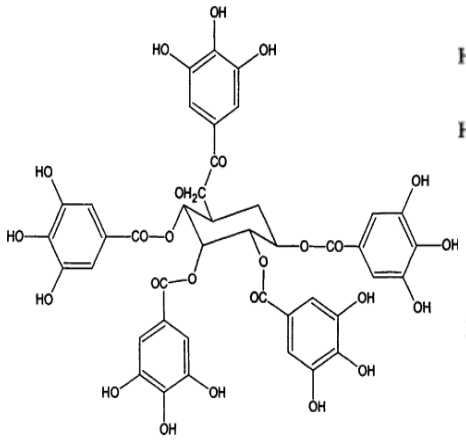


Figure 1(c)
Ellagitannin

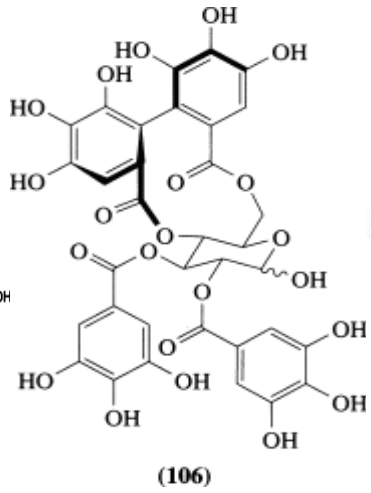


Figure 1(d)
Proanthocyanidins

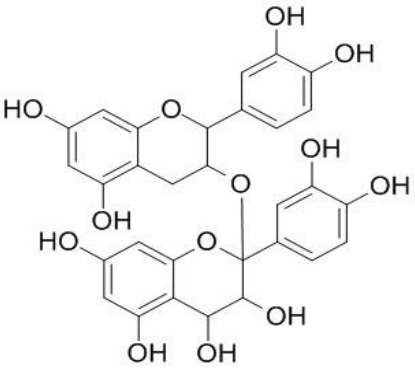


Figure 1(e)
Gallotannin

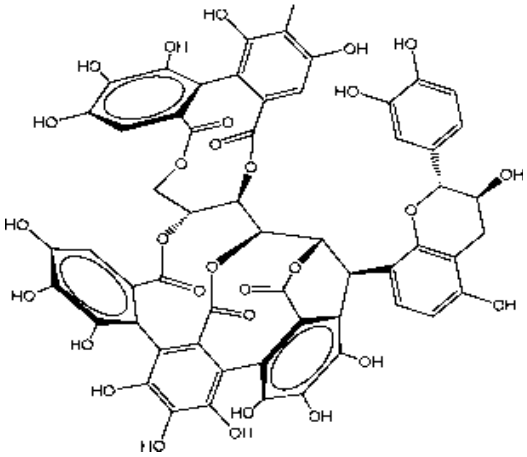


Figure 1(f)
Complex tannin

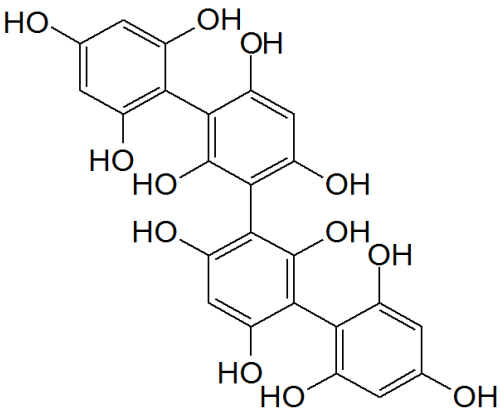


Figure 1(g)
Phlorotannin

3. Extraction of Tannin

Tannins are found commonly in the bark, wood, leaves, buds, stems, fruits, seeds, and roots of the plant. Tea and coffee are also one of the richest sources of tannin. For the production of tannin, one has to extract it and then purify it. Amount of tannin present in the plant depends on their geological and biological origin, age, species, population, and position (Das et al., 2020).

3.1 Methods to Extract Tannin

There are a number of methods available to extract tannin but due to its heterogeneous nature, it becomes difficult to enhance its yield (Das et al., 2020). The extraction procedure of tannin is widely variable and requires more than one protocol (Das et al., 2020). Extracted tannin contains different impurities like sugars and minerals (Das et al., 2020). Parameters like particle size, temperature, pressure, time, solvent type, and solute to solvent ratio affects the amount of impurities (Bello et al., 2020). Therefore, it is necessary to extract tannin under controlled operating parameters (Das et al., 2020). Below are a few commonly used methods to extract tannin.

3.1.1 Decoction

It is a simple and affordable method. For the condensed type of tannin, decoction is the best method. Only a few equipments like gas stove, funnel, flask, and whatman paper to collect filtrate are required here in this method. The workflow of the method goes like-

1. Take the powdered starting material and add it to the water.
2. Let this mixture/solution containing tannin, boil. Make sure the temperature reaches 100°C at least.
3. Filter the boiled solution through whatman filter paper.
4. Collect the filtrate.

Boiling water has high solvent action and therefore this method is used to extract oils, volatile organic compounds, and other various chemical substances including woody, hard, and thermo stable compounds. This is the best method for extracting plant material on a large scale; however it is not suitable for heat-sensitive materials (Cuong et al., 2016).

3.1.2 Pressurized Water Extraction

Pressurized water extraction method is simple and somewhat similar to the decoction method. Here, in this method, water is used as solvent. This method is faster than any other method in use. High pressure i.e. 100 to 150 bars and high temperature i.e. 60℃ to 70℃ plays a vital role in this technique (Teo et al., 2010) (Cuong et al., 2016). By adjusting pressure and temperature of the solvent, polarity can be modulated and tannin in significant amounts can be extracted (de Hoyos-Martínez et al., 2019).

There are two more types in this extraction method (Teo et al., 2010).

1. Dynamic extraction method: Here, extraction time and flow are important parameters for optimization.
2. Static extraction method: In this type, extraction efficiency strongly depends on the partition-equilibrium constant and solubility of compounds at elevated temperatures.

3.1.3 Microwave Assisted Extraction

Microwave-assisted extraction is a good alternative to the other traditional extraction methods for polyphenols (Jovanovic et al., 2017). The main advantage of using this method is that it takes less time for extraction, less solvent consumption, higher extraction rate with higher yield, and better quality product (with higher antioxidant activity) at lower cost (Suwal & Marciniak, 2019). For the extraction of tannins, this method is a good choice because it uses microwave (electromagnetic radiation in the frequency range from 300 MHz to 300 GHz) energy (Cuong et al., 2016).

Steps involved in microwave assisted extraction:

1. Extraction of polyphenolic compounds is performed by using a commercial microwave oven.
2. Mixtures of ethanol in water are used as solvent for recovery of phenolic contents.
3. Set the microwave power to 170, 340, 900 W, ethanol concentration to 30, 60, 90%, and solvent-to-sample ratio as 10, 20, 30 ml/g.
4. There is no temperature monitoring system in the oven so treat it till the boiling point of the solvent.
5. After that, filter it through Whatman filter paper 1.
6. Collect the filtrate and keep it at 4℃ for further use.

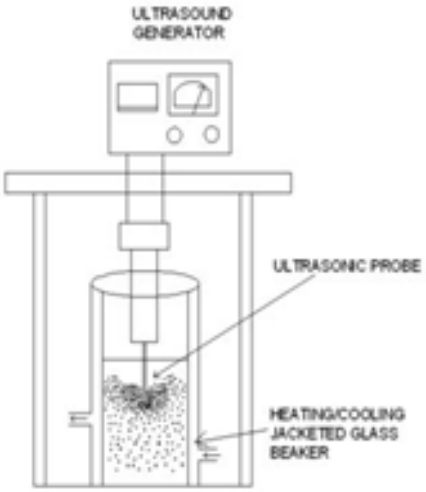
The setup for MAE is shown in the pictures below.

Figure 2 (a) Industrial Scale

Figure 2(b) Lab Scale

3.1.4 Ultrasound Assisted Extraction (UAE)

In this method, the use of ultrasound (wave frequencies of more than 20 kHz to 10MHz) is made for the extraction of bioactive molecules (Suwal & Marciniak, 2019). For the extraction of condensed tannins, hydrolysable tannins, and valonea tannins from plants and seaweeds, ultrasonic-assisted maceration method is commonly used (Cuong et al., 2016). The use of UAE has been found to improve the extraction efficiency by 35 % during the extraction of phenolic compounds from various plant sources. Also, tannins by ultrasonic-assisted maceration method are 17.6% higher in concentration than traditional methods (Suwal & Marciniak, 2019) (Cuong et al., 2016). Here, factors like ultrasonic power, temperature, and time of ultrasound affects the yield of tannin extraction. The following figures show a typical ultrasound extractor. Figure 3(a) shows the hardware of a typical ultrasound extractor and figure 3(b) shows an actual image of the ultrasound extractor.



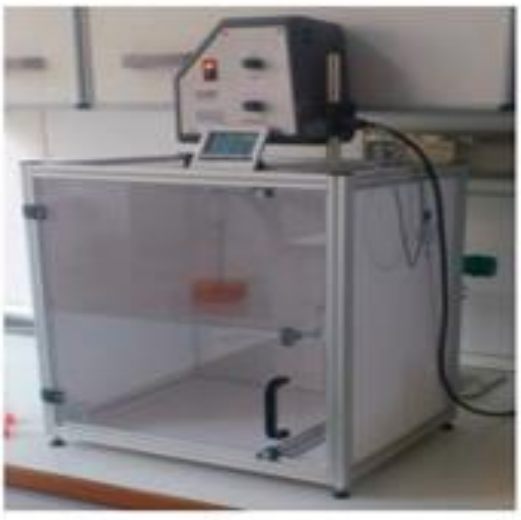


Figure 3 (a) **Figure 3(b)**

In the UAE, pulse intensity (10%-100%) controls the process.

1. Take 1 gm of the powdered starting material.
2. Mix it with deionized water (7.9 to 48.8 ml) at room temperature for 2-40 minutes.
3. Then, the mixture is kept in the ultrasound extractor with temperature ranging from 15℃ to 75℃ and time from 2 to 40 minutes with 10% pulse intensity of ultrasound power during the extraction process.
4. The extract is then filtered through whatman filter paper 1.
5. Collect the filtrate and store it at -4℃ for further use.

Advantages of ultrasound-assisted extraction: (Jovanovic et al., 2017)

1. Increased extraction yield
2. Improved quality of the extract
3. Fast kinetics – an important property for industrial processes
4. Easy to work
5. Affordable instruments when compared to other techniques
6. Wide range of solvents can be used here

Disadvantages of ultrasound-assisted extraction: (Jovanovic et al., 2017)

1. Polyphenol compounds can degrade easily
2. Ultrasound waves sometimes affect the yield in an adverse manner
3. Extraction kinetics depends on the characteristics of plant material used
4. Presence of large quantities of plant particles contributes to the ultrasound wave's attenuation which results in the restriction of the active part of ultrasound inside the zone located in the vicinity of the ultrasonic emitter

3.1.5 Supercritical Fluid Extraction

Supercritical fluid extraction method is one of the best methods because it is modern and environment friendly. It is used to separate substances using a supercritical fluid as a solvent. Solvents like carbon dioxide, hexane, pentane, butane, nitrous oxide, sulfur hexafluoride, and fluorinated hydrocarbons can be used. Supercritical solvents are important in extraction for efficient tannin yield (Cuong et al., 2016). An advantage of using carbon dioxide is that it is non toxic, non-flammable, and non-corrosive in nature. Also, it is easily available and has a low critical temperature i.e. 31°C and pressure 7.28 MPa. Therefore, it can easily be removed by avoiding further oxidation by creating an oxygen free environment (de Hoyos-Martínez et al., 2019). Along with CO2, co solvents like methanol, ethanol, acetone or water are used to get better yield of extraction (de Hoyos-Martínez et al., 2019) (Hassim et al., 2021). Extraction efficiency may increase due to the influence of pressure on solubility of desired compound (Týskiewicz et al., 2018). Extraction was conducted for one hour followed by four hours of dynamic extraction wherethe extracts were collected every 30 mins. Then, collected extracts were dried in an air oven at 40 °C to remove the remaining co-solvent. All extracts were cooled at room temperature.

Steps involved in supercritical fluid extraction method: (Pansera et al., 2004)

1. Take 50 g of starting material and extract it with 500 ml of solvent for 24 h.
2. The extracted mixture is concentrated in a Lyophilizer.

3. Pansera used Hewlett Packard 7680 extraction module. It consists of nozzle/tap assembly (it acts as a controllable variable restrictor allowing an instantaneous depressurization of the supercritical fluid as well as the decoupling of flow and pressure).
4. The material is extracted before loading to a self-sealing extraction cell of 7.0 ml thick-walled stainless steel thimble.
5. Supercritical fluid extracts are deposited into an internal trap which runs off into a vial containing 1 ml of hexane and methanol.

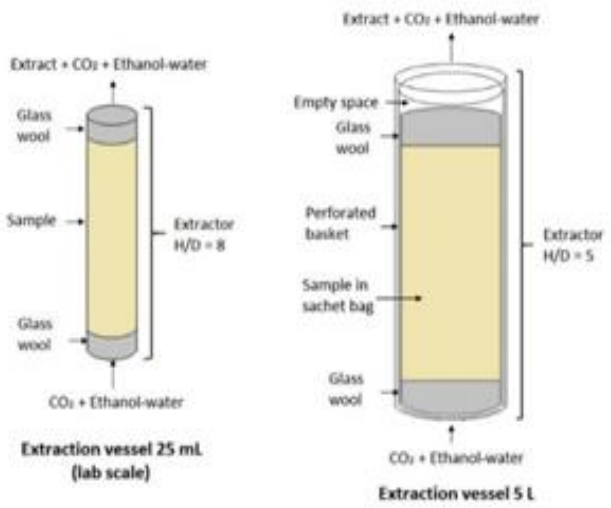


Figure 4

6. 0.4 g extract is obtained with supercritical carbon dioxide as per the described procedure.
7. The operation temperature was kept 40℃ to 80ºC and pressure ranged from 150 to 200 bars. Remaining variables were kept constant i.e. CO_2 flow (2.0 ml/min) and extraction time (30 min).
8. Analysis of tannin is done by TLC and concentration is measured by spectrophotometer.

3.1.6 Soxhlet Extraction

Both method and solvent play important roles in extraction of tannins. Soxhlet extraction is also one of the best methods for extracting tannins (Cuong et al., 2016). This type of extraction is performed with different solvent mixtures such as hexane-dichloromethane, dichloromethane-light petroleum, cyclohexane-acetone, hexane-acetone, ethanol-water or even methanol depending on the nature of the sample. For tannins, a mixture of ethanol and water is mainly used (Abilleira et al., 2021).

Steps involved in the Soxhlet extraction: (Abilleira et al., 2021)

1. 10 gm of sample is taken and 200 ml of solvent is used for each extraction.
2. Then, the mixture is introduced into the solvent extractor for 3 hours which runs six extraction cycles.
3. Measure weight of the extract and remaining solid material. Keep it in an oven for 24 hours at temperature 100 ± 5℃. Measure the weight again and obtain the data of yield.
4. Instead of mixture, if one takes only distilled water as a solvent then after extraction, directly the extract can be kept in an oven at temperature 100 ± 5℃. The water will therefore, evaporate out and concentrated mixture will remain containing tannin.

3.1.7 Magnetic Stirrer Based Technique: (Khasnabis et al., 2015)

1. Take 5 grams of the starting material and add it into the flask which contains 50 ml of water. Keep the system on a magnetic stirrer.
2. Start the magnetic stirrer and set the temperature at around 70-80℃ at 400-500rpm for 30-40 mins.
3. Filter it through whatman filter paper 1. Collect the filtrate and centrifuge it at 10,000 rpm for 15 mins.
4. Discard the pellet and transfer the supernatant to collection tubes. Perform qualitative analysis to see the presence of tannin.

4. Estimation of Tannin Content

We have a number of qualitative and quantitative estimations to detect tannin in a solution. Few of them are mentioned here.

4.1 Qualitative Estimation: Prepare 5 % w/v aqueous solution of Ferric chloride. Take 1 ml of extract and add 2-3 drops of Ferric chloride. Formation of greenish or black precipitates shows the presence of tannin in the given solution (jyotismitakhasnabis).

4.2 Quantitative Estimation: Different quantitative methods are used to identify the amount of tannin present in any given sample. Some commonly used ones are listed below.

4.2.1 Folin-denis Method: In an alkaline solution, phosphorus molybdic acid gets reduced by tannin (polyphenolic in nature) which gives a highly coloured compound. The more the intensity, the more tannin present in the sample.

Materials: Folin-denis reagent, sodium carbonate solution, standard tannic acid solution, working standard solution

Method: Take 0.5 g of starting material containing tannin. Add 75 ml water and transfer it into a flask. Boil it for 30 mins. After that, Centrifuge at 2,000 rpm for 20-25 mins. Collect the supernatant into the 100 ml

volumetric flask and make up the volume. Discard the pellet. Take 1 ml of the sample extract to a 100-ml volumetric flask which contains 75 ml water. Add 5 ml of Folin-Denis reagent into it with 10 ml of sodium carbonate solution and dilute to 100 ml with water. Mix well and check the absorbance at 700 nm using a UV-visible spectrophotometer after half an hour. Blank is prepared only by water. Make proper dilution if the absorbance goes more than 0.7. Draw standard graphs.

4.2.2 Folin-ciocalteu Method: This is a very simple and quick method for estimation of tannin present in any given sample.

Materials: Folinciocalteu reagent, sodium carbonate, sample, distilled water

Method: Take 10 ml volumetric flask and add 7.5 ml distilled water in it. After that, add 0.5 ml of Folin Ciocalteu reagent. Add 0.1 ml of sample and make up the volume to 10ml. Mix it well and put it in the dark for 30 minutes. Take readings at 700 nm with the help of UV-visible spectrophotometer. Note down the reading and compare it with the standard graph for the same.

4.2.3 Titrimetric Method: (Khasnabis et al., 2015) $KMnO_4$ solution is used to titrate against the extract. During the titration, the blue color of the indigo carmine passes through the various shades and gives a faint pink color as the end point.

Materials: Potassium permanganate solution, distilled water, gelatin solution, indigo-carmine, acidic NaCl solution, 5 g powdered kaolin, NaCl solution

Method: Take 5 ml aliquot of the extract, mix it with 12.5 ml of indigo- carmine solution and add 375 ml of distilled water. Now, this is titrated against the $KMnO_4$ solution. Blue color of the indigo carmine converts into a faint pink colored solution which marks the end point.

For determination of non tannin compounds, take another aliquot of 50 ml extract and mix it with 25ml of gelatin solution, 50 ml of the acidic NaCl solution, and 5gms powdered kaolin. Shake the mixture well for 10-15 minutes and filter it through whatman paper 1.Collect the filtrate. Take 12.5 ml of this filtrate and mix it with 12.5 ml indigo-carmine and add 375 ml distilled water. Titrate it against the $KMnO_4$ solution. Conversion of blue colour to faint pink marks the end point.

Calculate the concentration of the tannin present in the sample by given relation:

1ml of standard KMnO4 solution = 0.595 ml of 0.1N Oxalic acid

1ml of 0.1 N Oxalic acid = 0.0042 grams of tannin

5. Applications

1. In the food industry, tannins are used in food preservatives, packaging materials, and food enhancements owing to their antimicrobial and antioxidant properties (P. Singh & Kumar, 2020).

2. In the leather industry, to prevent the disintegration of collagen fibres of leather (P. Singh & Kumar, 2020).
3. In dyeing industry, it is used as a common mordant (Ammayappan & Jeyakodi Moses, 2009).
4. In preparation of wine and beer to provide colour, aroma, flavor, and to prevent over oxidation of wine. Also, precipitates out proteins thus clarifying the wine (P. Singh & Kumar, 2020).
5. In medicine and pharmaceuticals, used as preventive therapeutic agents and as astringents (P. Singh & Kumar, 2020).
6. Also, has a diverse application in the paper industry for sizing and as a mordant of coloured paper (P. Singh & Kumar, 2020).
7. It is also a significant ingredient in cosmetics. It has anti-inflammatory properties that help reduce redness and inflammation (P. Singh & Kumar, 2020).
8. It is also used as a drug to prevent diseases like cancer, cardiovascular diseases, and kidney diseases (Huang et al., 2018).
9. Tannins are proven to show significant activity against viruses like human immunodeficiency virus, norovirus, etc (Huang et al., 2018).
10. In textile industries, tannins are used for tanning cotton, wool, linen, and silk. Tanning is a process where textile fibres are treated with tannic acid (Ammayappan & Jeyakodi Moses, 2009).
11. Tannic acid can be used for removal of gumming substance from mulberry silk yarn (Ammayappan & Jeyakodi Moses, 2009).
12. Tannic acid has been in use for electrostatic flocking for the past several years (Ammayappan & Jeyakodi Moses, 2009).
13. It is also used in preparation of galvano plastics and in electroplating (Ammayappan & Jeyakodi Moses, 2009).
14. It is used in deodorization of crude oils and for preparation of inks (Ammayappan & Jeyakodi Moses, 2009).
15. Tannic acid is also used in local treatment of minor burn wounds (Halkes et al., 2001).

6. Future Prospects

The antioxidant property of tannin can be used to the proper extent thus giving rise to new drugs for betterment of human health. Also, use of tannins in fertilizers can help plants grow in an efficient manner and help prevent various diseases caused by microorganisms. As tannins have metal chelating properties, their ability to take up heavy metals can be studied thus, giving a new scope in bioremediation.

7. Acknowledgment

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Research Article

Altitude-Phytochemicals Correlation on Regulating Ag/Ag_2O Nanoparticles Formation and their Colloidal Stability in Surfactants

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Abstract

Plants growing at different altitude differ in their phytochemical concentration. This paper investigates the impact of altitude variation on the formation of silver nanoparticles (AgNPs) using the extract of tea leaves cultivated at different altitudes in eastern Nepal. Multidisciplinary approaches including UV-visible absorbance spectroscopy, XRD, FTIR, XPSand DLS are conducted to systematically investigate how altitude influences the contents of active substances in tea leaves which eventually diversify the AgNPsphysicochemical properties. AgNPs synthesized using the extract of tea leaves cultivated at the hilly region were observed to be homogeneously dispersed and smallest sized, while those synthesized using the extract of tea leaves cultivated at the mountainousregion were heterogeneously dispersed and large sized. Since surfactants can severely affect the stability of AgNPs aqueous dispersion, the stability of the AgNPs dispersed in aqueous surfactants (cationic, anionic and nonionic) were also studied. Cationic surfactant having a smaller alkyl chain length acted as the best stabilizing agent. With the possibility of production of other metal nanoparticles using plant extracts, this study may hold great promise wherein altitude based variable physicochemical properties may be developed for better and enhanced applications of metallic nanomaterials.

Keywords: Tea leaves, silver nanoparticles, cultivation altitude, surfactants, colloidal stability, phytochemicals

1. Introduction

As the domain of plant based green synthesis of AgNPs is getting saturated, studies focusing on the impact of ecological element like cultivation altitude on the concentration of the active component in plant extracts could open a new scientific window for the synthesis of highly stable metallic nanoparticles. Among the

most common food and drinks we eat today, tea leaves (*Camellia Sinensis*) have one of the highest amounts of flavonoids. Secondary metabolites (active components) like phytochemicals (catechins, flavonols, flavones, tannin acids, flavonoids, polyphenols) and amino acid: theanine, are present in green tea.[1] Among these active components, the most abundant main bioactive compounds in tea are catechins, a group of flavonoids which include: (-)-epigallocatechin-3-O-gallate (EGCG), (-)-epicatechin-3-gallate (ECG), (-)-epigallocatechin (EGC), and (-)-epicatechin (EC).[2] EGCG is the primary bio-active tea polyphenol found in tea leaves, demonstrating strong antioxidant activity. The main constituents of fresh tea leaves are polyphenols which comprise up to 30% of the dry weight of young shoots. The main flavonols in tea are conjugates of kaempferol and quercetin with conjugated glycosides moiety associated with glucose. Phenolic acids are largely composed of quinic acid esters of caffeic acid and gallic acid, while >60% of the total amino acid is accounted to theanine.[3] Since altitude is a global reflection of ecological elements like sunlight, humidity, and temperature; numerous studies have correlated the content of active ingredients in the tea leaves at the altitude of the place of cultivation.[4]Based on the previous works on the silver nanoparticles synthesis using the tea leaf extract as a reducing and stabilizing agent,[5-11] we in this work have therefore, tried to conceptualize the mechanism over the influence of altitude variation in tea leaves on the AgNPs formation and size control. The green tea (*Camellia sinensis*) leaves used in this work were collected from eastern Nepal, and cultivated at three different altitudes of 506 m, 1828 m, and 3643 m. Acting as reducing and capping agent, the variation in the amount of tea leaves secondary metabolites due to variation in the cultivation altitude were related to the AgNPsphysicochemical properties through various structural characterization techniques.

Despite the ability of silver to interact with structural component of the tea leaf phytochemicals and the concentration dependent relationship of the tea leaves active components with their cultivation altitude, the literature reports no such research work on their collective effect on AgNPs formation. This is the first such study wherein the effect of altitude variation in tea leaves phytochemicals on formation and size control of AgNPs have been investigated and thereby, establishing an functioning linkage between the plant extract mediated AgNPs physicochemical properties and the plant resources (fruit, seed, leaves, flowers, etc) growing altitude.

Our study have revealed that un-aggregated and smallest sized AgNPsmay be prepared using tea leaves extract prepared from tea leaves cultivated at the hilly region. Moreover, we have shown that the colloidal stability of the AgNPs aqueous dispersion may be greatly enhanced using cationic surfactant as a stabilizing agent. Such excellent colloidal stability of the AgNPs aqueous dispersion obtained with cationic surfactant may in future be implemented to increase the sensitivity and selectivity of AgNPs as colorimetric sensors. Our work thus have given a new dimension to the plant mediated nanoparticles synthesis, wherein the altitude at which the plant resources used for their synthesis are grown plays a key factor towards

modifying the physicochemical properties of metallic nanomaterials.

2. Experimental Section

2.1 Materials and Methods

Green tea leaves were received from three cultivation locations in eastern Nepal: (a) Jhapa (flat land region at 506 m) (b) Ilam (hilly region at 1828 m), and (c) Taplejung (mountainous region at 3643 m). The following chemicals were procured from Sigma-Aldrich and were used as received: $AgNO_3$, Sodium bis (2-ethylhexyl) sulfosuccinate, Sodium octyl sulphate, Trimethyloctylammonium bromide, Decyltrimethylammonium bromide, Dodecyltrimethylammonium bromide, Tetradecyltrimethylammonium bromide, Hexadecyltrimethylammonium bromide, n-octyl-β-D-glucoside

2.2 Tea Extract Mediated Synthesis of Silver Nanoparticles (AgNPs)

All the tea extracts (TE) were individually prepared by mixing 350 mg of the dried tea leaves samples with 20 mL of Milli-Q water in a 100 mL Erlenmeyer flask and then boiling the mixture for 15 min before finally decanting it. Centrifugation at 8000 rpm for 15 minutes removed any colloidal content and the final supernatant volume was made to 50 mL using Milli-Q water. The aqueous TE prepared from tea leaves collected from the mountainous region, hilly region, and flat land region were named as T1, T2, and T3, respectively. For synthesizing the AgNPs, toa known volume of TE (1 mL, 2 mL and 4 mL), 100 μL of $AgNO_3$ solution (1.75 M) was added and the overall volume was made 20 mL using Milli-Q water. The resulting reaction mixture was incubated for 45 min at 373.15 K and the AgNPs dispersion obtained thereafter was purified by centrifugation at 16000 rpm for 20 mins and re-dispersed in 100 mL Milli-Q water. The reaction parameters such as volume of aqueous TE, $AgNO_3$ concentration and reaction time were optimized using absorption spectroscopy.

2.3 Structural Characterization of AgNPs

A UV-visible spectrophotometer (Spectro 2060 plus) was used to measure the absorbance and Ag/Ag2O content of the AgNPs. Particle size and zeta-potential were measured using Malvern Zetasizer 3000 DLS. The XRD analysis were performed using Bruker, D8 Focus instrument of Cu-Kα wavelength (λ = 1.54 A°), with scanning over a 2θ range of 20–90° with 0.017° step size. The FTIR spectra of the AgNPs were recorded using PerkinElmer Spectrum 65 series FTIR Spectrophotometer. OriginPro 8 software was used for the Gaussian curve fitting analysis.

3. Results and Discussion

3.1 Synthesis of AgNPs

The simultaneous colour change of the reaction mixture demonstrated the creation of the AgNPs by reduction of Ag^+ ions with aqueous TE. Within 2 minutes of starting the synthesis, the colour changed from colourless to yellowish but after 20 minutes, a brown colour appeared. Owing to the surface plasmon resonance (SPR) band, the AgNPs displayed characteristic absorption maxima at 420-455 nm in the dispersion spectra (Figure 1). UV–visible spectroscopy was used to determine the optimal TE (T1, T2, and T3) volume, reaction time, and $AgNO_3$ concentration for the synthesis of AgNPs. The absorption spectra for the optimised (a) TE volume, (b) $AgNO_3$ concentration, and (c) reaction time are shown in Figure 1. After 45 minutes of incubation at 373.15 K, absorption spectra of the AgNPs made with 100 μL (1.75 M) $AgNO_3$ and various TE volumes (1, 2, 4, and 5 mL) are shown in Figure 1a-1d. The concentration of AgNPs grew as the amount of TE (T1, T2, and T3) rose, as shown by increased absorbance.[12] The maximum absorbance with 4 mL TE followed by a minor change in absorbance with > 4 mL TE shows that all Ag^+ ions are converted to Ag (0). Variations in $AgNO_3$ concentration were used to study the formation of AgNPs with 4 mL TE, as shown in Figure 1e-1g. With increasing $AgNO_3$ concentration, the increased absorbance resulted from increased oxidation of hydroxyl groups found in TE phytochemicals (catechins, flavonols, flavones, tannic acids, flavonoids, and polyphenols) by Ag^+ ions. With > 100 μL, there was no discernible change in absorbance (1.75 M). At this $AgNO_3$ concentration, all of the reducing agents in the TE have been exhausted and no further reduction of the Ag+ ion is conceivable. Variation of the reaction time at a given TE volume (4 mL) and $AgNO_3$ concentrations of 100 L (1.75 M) was used to assess the progress of AgNPs production (Figure 1h). With increasing incubation time, the reducing ability of TE rises, possibly because more and more hydroxyl groups in the TE phytochemicals were oxidized to carbonyl and or carboxylic groups which in-turn reduced the Ag^+ ions to Ag. Meanwhile, no change in absorbance after 45 minutes of incubation indicates that the precursors have been completely consumed. Therefore, 4 ml TE (T1, T2, and T3), 100 μL (1.75 M) $AgNO_3$ solution and 45 mins reaction time were the optimized values thus used for the synthesis of AgNPs namely: T1A4 (synthesized using TE from mountainous region), T2A4 (synthesized using TE from hilly region), and T3A4 (synthesized using TE from flat land region).

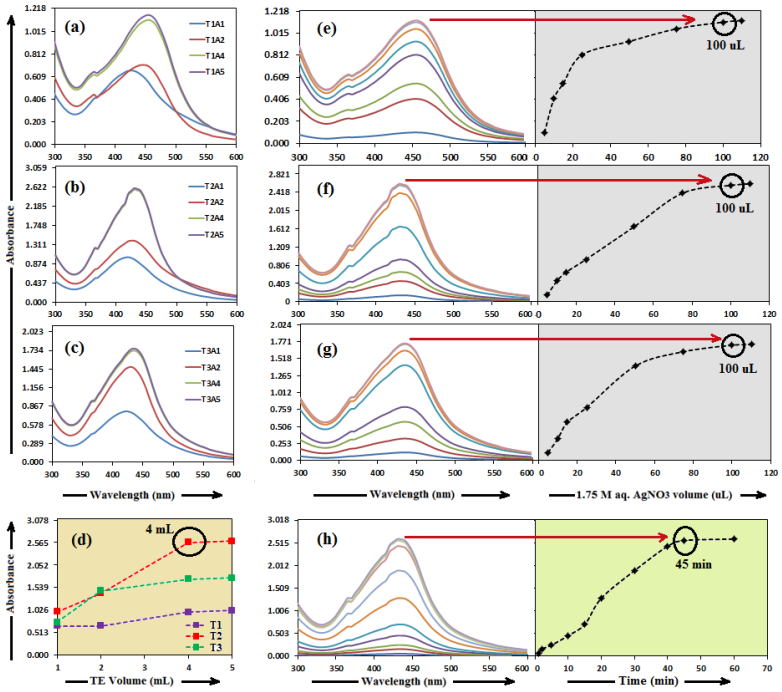

Figure 1: (a-d) Optimization plot for Tea Extract (TE) concentration, (e-g) optimization plot for $AgNO_3$ concentration, and (h) optimization plot for reaction time for the synthesis of T2A4.

3.2 Characterization

The AgNPs hydrodynamic diameter obtained using DLS depicted a narrow size distribution for T2A4, while for T1A4 and T3A4, the size distribution was wider (Figure 2). It appears that since altitude is the collective reflection of multiple ecological elements like: temperature, sunshine and sun radiation; the concentration of the phytochemicals present in the TE (catechins, flavonols, flavones, tannins, flavonoids, polyphenols) may thus vary with altitude. Therefore, with such possibilities of high complexation ability of the phytochemicals towards Ag^+ ions, and the influence of altitude on the concentration of the phytochemicals present in tea leaves may have caused nucleation of Ag nuclei. This may have provided a narrow to wider size distribution for the AgNPs. It seems that in case of T2A4, the narrow size distributionresulted from ordered nucleation. As a result, phytochemicals in the TE play a directional regulatory role during the AgNPs nucleation and development processes. The AgNPs size distribution varies as well depending on the altitude dependent phytochemical content.

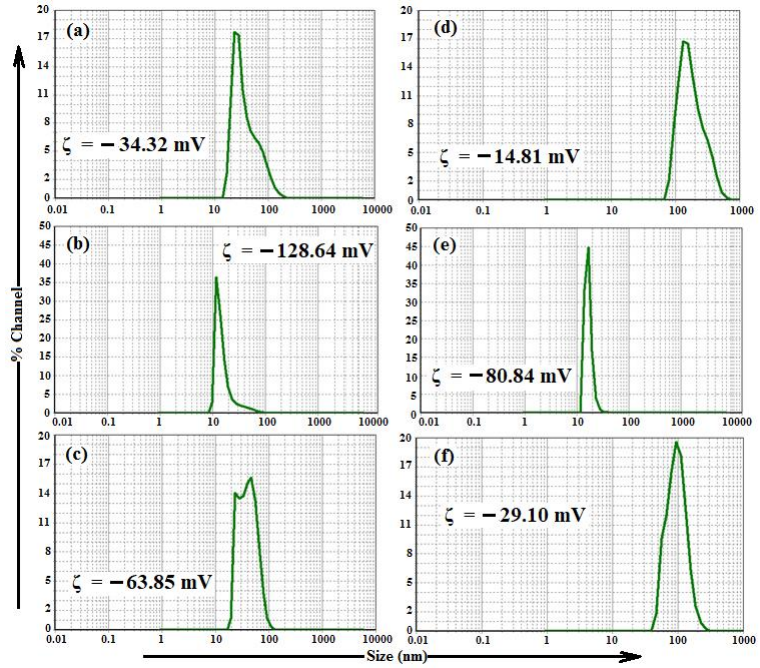

Figure 2: Particle size distribution and zeta potential (ζ) of aqueous dispersed AgNPs: (a) T1A4 (b) T2A4 (c) T3A4; and 0.02 M aqueous C8 dispersed AgNPs: (d) T1A4 (e) T2A4 (f) T3A4

The XRD patterns reflect high crystallinity of the AgNPs (Figure 3), while the d-spacing values confirm presence of standard cubic phases of Ag_2O and Ag.[13,14] T2A4 has the largest concentration of Ag crystallites among the AgNPs, as seen by the 100% intensity peak of Ag (111) planes. Similarly, T1A4 have the highest concentration of Ag_2O crystallites, depicted by the 100% intensity peak of Ag_2O (111) planes. Moreover, the broadest XRD peaks observed for T2A4 hints towards their size being the smallest, while the narrowest XRD peaks observed for T1A4 hints towards their size being the largest among the AgNPs, i.e., their size follows the order: T1A4>T3A4>T2A4. The percentage peak intensities of the Ag_2O and Ag crystallites were used to calculate the Ag and Ag_2O contents in the AgNPs (Table 1), wherein T2A4 has the highest while T1A4 has the least Ag^0 content. Therefore, this investigation establishes that tea leaves cultivated at (a) high altitude contributes more towards the formation of Ag_2O (110) and (111) planes but inhibits the growth of Ag (311) planes and (b) at hilly altitude contributes more towards the formation of Ag (111) planes. Hence, during the nucleation and growth stages of the TE mediated AgNPs, the concentration of the phytochemicals present in the tea leaf used for preparing TE (T1, T2 and T3) must have been altitude dependent and may have introduced peculiar features in the AgNPs crystalline structure, thereby regulating their physicochemical properties like stability and dispersibility. As observed in this work, the tea extract mediated synthesis of Ag/Ag_2O nanocomposite establishes an efficient relationship between their chemical composition and crystallinity of the AgNPs and the altitude dependent concentration of phytochemicals in tea leaves and adds to an otherwise inadequate literature.

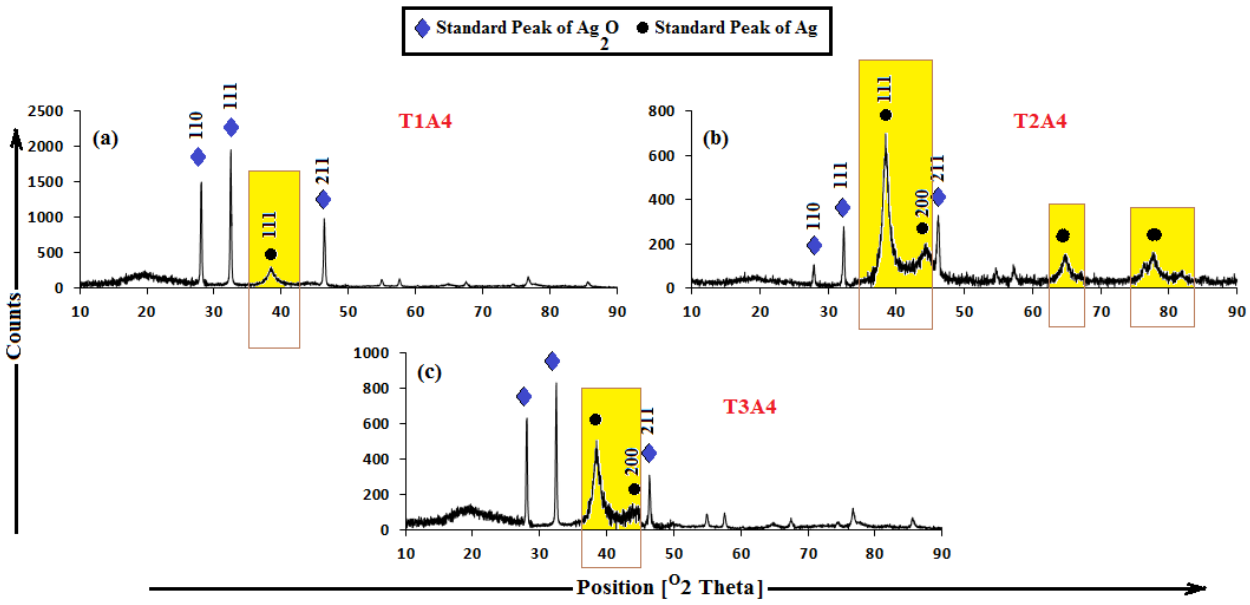

Figure 3: XRD patterns of AgNPs: T1A4, T2A4 and T3A4

Proteins and polysaccharides from TE as well as oxidised metabolites of TE phytochemicals introduces electron rich sites on the AgNPs surfaces.[15] When AgNPs are aqueous dispersed, these electron-rich sites form a negatively charged electrostatic double-layer around them. The overall negative charge carried by these electrostatic double-layers determines the dispersion stability of AgNPs. As a result, zeta potential of the AgNPs may provide a clear picture of their stability. Aqueous tea extract contains phytochemicals which are majorly: catechins, flavonols, flavones, tannin acids, flavonoids, polyphenols); amino acid: theanine; and polysaccharides which are mainly composed of arabinose, galactose and glucose.[3,16] The negative zeta potential values of AgNPs may have been caused by the negatively charged carboxyl groups adsorbing on their surfaces. Phytochemicals in TE operate as a reducing agent by converting Ag^{+} ions to Ag^{0}. The substantial difference in AgNPs zeta potential can thus be linked to the phytochemical content which varies with altitude. For higher concentrations of photochemical in TE, the average size of the silver nanoparticles may increase with increased aggregation as also reported by Oliveira.[17] An increased concentration of the phytochemicals in TE can induce partial removal of carboxylic groups weakly associated on the AgNPs surface, consequently making their surface less negatively charged, promoting partial AgNPs aggregation with decreased zeta potential. With a larger zeta potential, the nanoparticles create a strong electrostatic repulsive force. Consequently, the AgNPs agglomeration is preventing, leading to increased stability of the dispersion. Therefore, it appears that especially high altitude is responsible for high content of phytochemicals in the TE collect from mountainous region (T1) which may have resulted in large sized T1A4 with the lowest dispersion stability. The DLS results thus support the findings from XRD results.

FTIR spectroscopy was employed to understand the involvement of TE phytochemicals in the formation of AgNPs and its surface chemistry. Figure 4 shows a comparison between the FTIR spectra of T2 and T2A4.

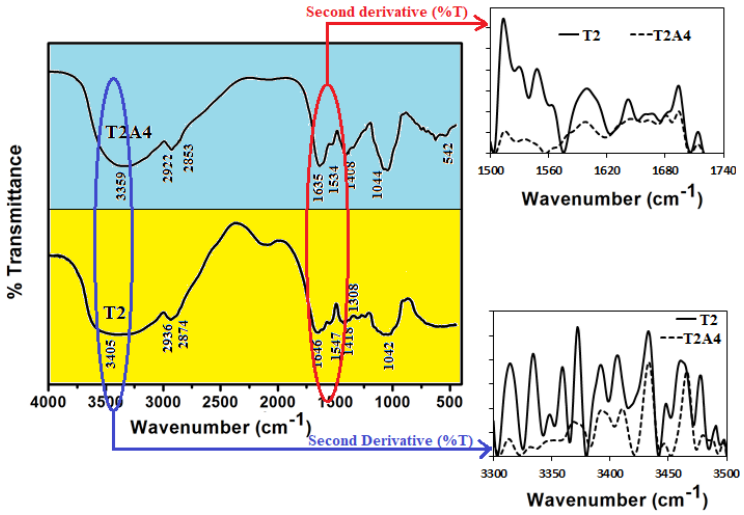

Figure 4: FTIR spectra of (a) tea extract (T1, T2, T3), (b) AgNPs: (T1A4, T2A4 and T3A4) (c) comparative FTIR spectra of T2 and T2A4 (d) comparative second derivative FTIR spectra of T2 and T2A4

The peaks obtained for T2 can be assigned to -OH stretching at 3405 cm^{-1} in phenols and carboxylic acids; -CH stretching at 2936 and 2874 cm^{-1} in aliphatic groups; >C=O stretching at 1646 cm^{-1} in tannins, aldehydes, ketones, esters and carboxylic acids; aromatic skeletal vibration of the tea polysaccharides at 1547 cm^{-1}; O-H in plane bending at 1418 cm^{-1} in carboxylic acids; C-N stretching at 1308 cm^{-1}in aromatic amines; and C-O-C stretching at 1042 cm^{-1} in polysaccharides and ethers.[14] In the FTIR spectra of T2A4, the peaks at 3405, 2936, 2874, 1646, 1547, 1418, 1308 and 1042 cm^{-1} shifted, indicating that functional groups contained in T2 were involved in the reduction of Ag^{+} ions into Ag^{0}. Furthermore, the peak at 542 cm^{-1} due to Ag-O bond stretching validates the presence of Ag_2O in T2A4 as shown by XRD analysis. On comparing the deconvoluted FTIR spectra of T2 and T2A4 (Figure 4) in the regions (1720-1500 cm^{-1}) and (3500-3300 cm^{-1}), it was found that intensity of many of the deconvoluted peaks for T2A4 decreased, while some of the peaks either shifted or disappeared. This clearly shows that structural and quantitative changes in the phytochemical content of TE occurred during the formation of AgNPs through phytochemical induced reduction of Ag^{+} ions into Ag^{0}. Thus, during the formation of AgNPs using TE, majority of the phytochemicals were mostly consumed and oxidized to carboxylic acids and eventually gets adsorbed over the AgNPs. As shown in Figure 5, the decreased pH and conductance after the formation of AgNPs further supports the FTIR findings.

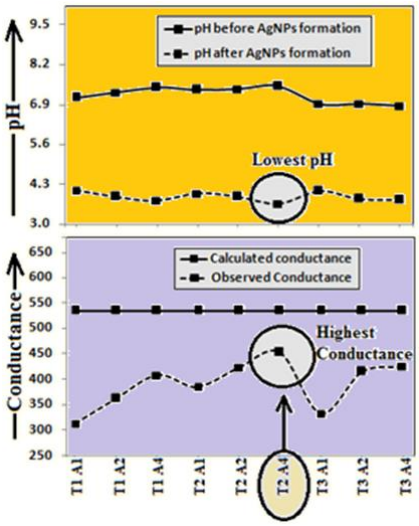

Figure 5: The pH and conductance of the reaction mixture before and after AgNPs synthesis

For the experimental validation of Ag^0 and Ag_2O percentage in the AgNPs as obtained from XRD analysis, an earlier wet lab protocol developed by Chandra et al was used.[15] Briefly, an absorbance based experiment was carried out based on the reaction given below:

$$Ag_2O\ (s) + 4\ NH_4OH\ (aq) \rightarrow 2[Ag\ (NH_3)_2]\ OH\ (aq)$$

Figure 6 describes the impact of ammonia on the absorbance of AgNPs. After ammonia addition, the AgNPs λ_{max} undergoes a blue shift with decreased absorbance indicating a decrease in AgNPs size. The dissolution of water insoluble Ag_2O (s) crystallites into water soluble $[Ag(NH_3)_2]OH$ (aq) is the cause of the decreased size. From the experiment, it was calculated that T2A4 is made up of >72% Ag^0, while T1A4 and T3A4 is made up of <38% Ag^0. The calculated values from the wet lab experiment agree well with the XRD data and are compared in Table 1.

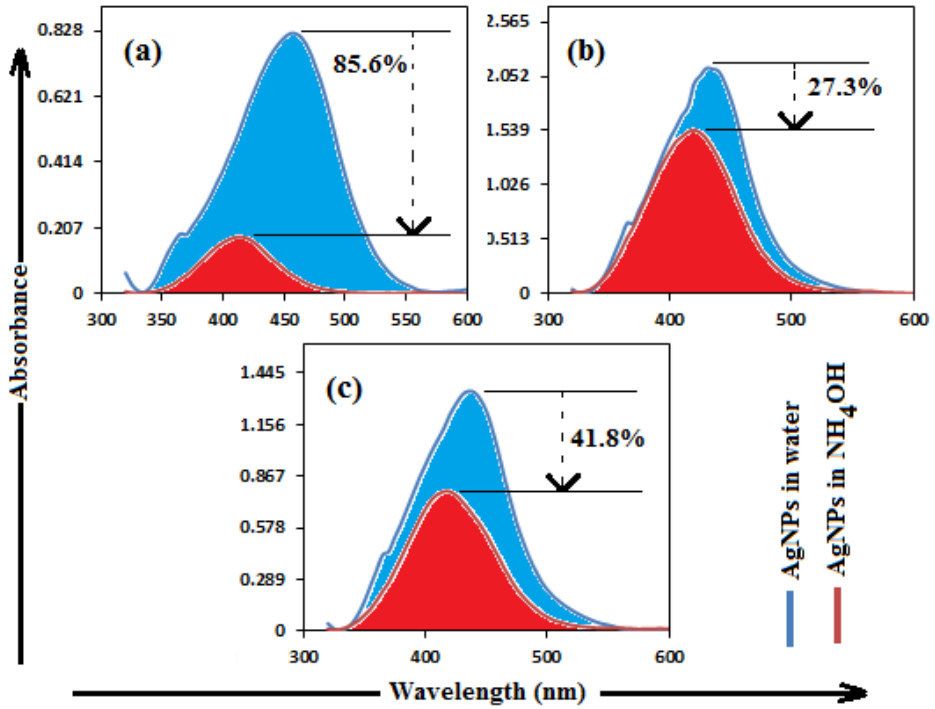

Figure 6: Ag_2O and Ag^0 contents in the AgNPs obtained from the UV-Visible spectra of (a) T1A4 (b) T2A4 (c) T3A4, dispersed in water and 30% aq. NH_4OH

Table 1: Percentage of Ag_2O and Ag^0 in AgNPs, as obtained from XRD and absorbance spectroscopy

AgNPs	$\%Ag^0$	$\%Ag_2O$	$\%Ag^0$	$\%Ag_2O$
	XRD		Abs. Spectroscopy	
T1A4	15	85	14.4	85.6
T2A4	83	17	72.7	27.3
T3A4	37	63	41.8	58.2

3.3 Mechanism of AgNPs Formation

Keeping in mind that all the reaction parameters were kept constant during the tea leaves mediated synthesis of AgNPs: (T1A4, T2A4, T3A4), the findings from AgNPs characterization strongly suggest that the nucleation, growth, and extent of oxidation of Ag to Ag_2O depends on the concentration of the phytochemicals present in the tea leaf used for preparing the TE (T1, T2, T3). The phytochemicals concentration must have been therefore dependent on the altitude from where the tea leaves were collected. The synthesis of the AgNPs can thus be divided into two parts (a) TE phytochemicals induced reduction of Ag^+ ions to Ag^0 (b) variation of the AgNPs physicochemical properties due to altitude dependent phytochemicals concentration in the collected tea leaves.

The presence of hydroxyl groups in TE phytochemicals appears to aid in the complexation of Ag^+ ions. Complexation allows the hydroxyl groups to reduce the Ag^+ ions into $Ag^o$and itself being oxidized

into carboxylic groups. The polysaccharides and proteins present in the TE initially trap the Ag^+ ions through electrostatic interactions. The TE's catechins, flavonols, flavones, tannins, flavonoids, polyphenols, and flavonoid glycosides function as a reducing agent, thereby lowering the Ag^+ ions and initiating the formation of Ag^0 nuclei. The reaction mixture's pH dropped after AgNPs were synthesized indicating that the oxidation products were mostly acidic.[17]Moreover, although having the maximum conductivity, the pH of the reaction mixture created following T2A4 synthesis was the lowest of all the AgNPs.This infers that the highest concentration of carboxylic acids was present after the T2A4 synthesis, depicting controlled nucleation of the Ag^0 nuclei obtained using TE prepared from tea leaves collected from hilly region (T2). Oliveira et al., have earlier demonstrated that higher reducing agent concentration leads to the formation of large sized silver nanoparticles with increased aggregation.[17] Based on this, the AgNPs particle size obtained through DLS and XRD analysis gives us a clue that T1 must have the highest concentration of phytochemicals, while T2, having the least, among the TE. The T2 with lowest phytochemicals concentration must have then assisted in the formation of smallest size AgNPs (T2A4) with the highest surface area. This may have resulted in the highest concentration of carboxylic acids as the oxidized byproduct in the reaction mixtures obtained after T2A4 synthesis. As a result, the reaction mixture obtained after T2A4 synthesis has the lowest pH and maximum conductance (due to increased H_3O^+ ions population) (Figure 5).

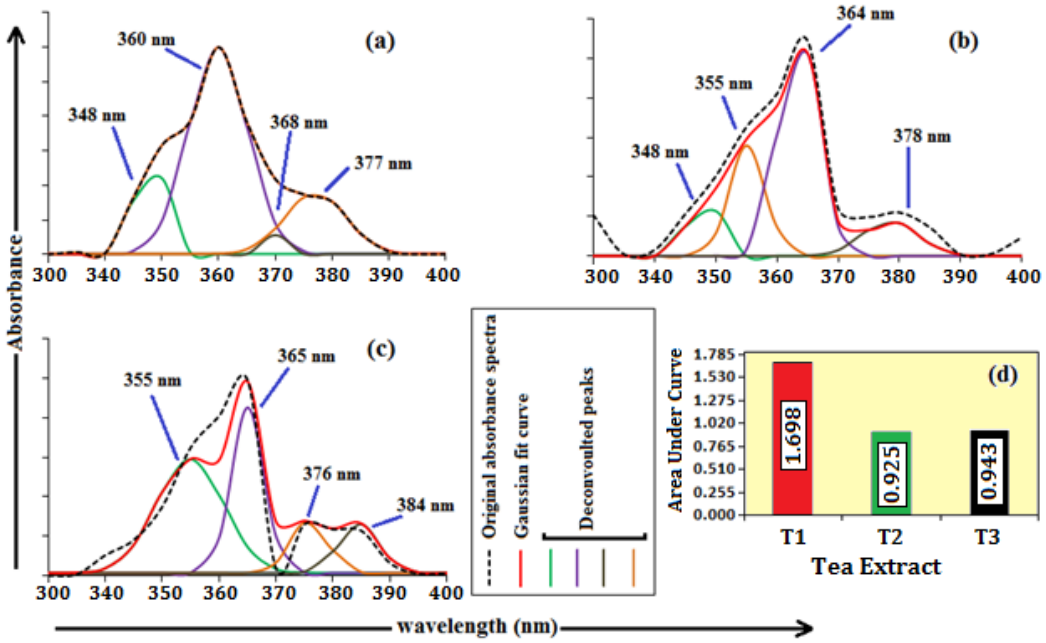

Figure 7: Deconvoluted absorbance spectra of Tea Extract: (a) T1 (b) T2 (c) T3, obtained by UV-Vis spectroscopy (d) Area under Gaussian fitted curves

Figure 7 is the deconvoluted UV-Visible absorbance spectra of TE: (T1, T2, T3). It is evident from the area under the Gaussian fitted curves (Figure 7) that the content of phytochemicals is the highest in T1,

while lowest in T2, as the peaks observed in the region 348-384 nm are due to the flavonoid content in the TE.[18]To support this finding,the antioxidant potential of the TE were correlated with their phytochemical content through scavenging of free radicals because phytochemicals are known for their antioxidant potential.

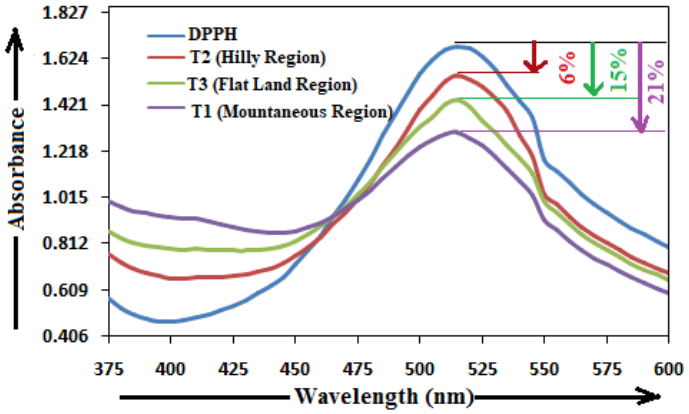

Figure 8: DPPH free radical scavenging activity of TE: (T1, T2 and T3)

The DPPH free radical scavenging activity of the TE (Figure 8) revealed that the TE prepared from tea leaves from mountainous regions (T1) has the highest DPPH free radical scavenging activity, while the TE prepared from tea leaves from hilly regions (T2) has the lowest. This analysis also reveals that in the TE, phytochemical concentrations are in the following order: T1 > T3 > T2.

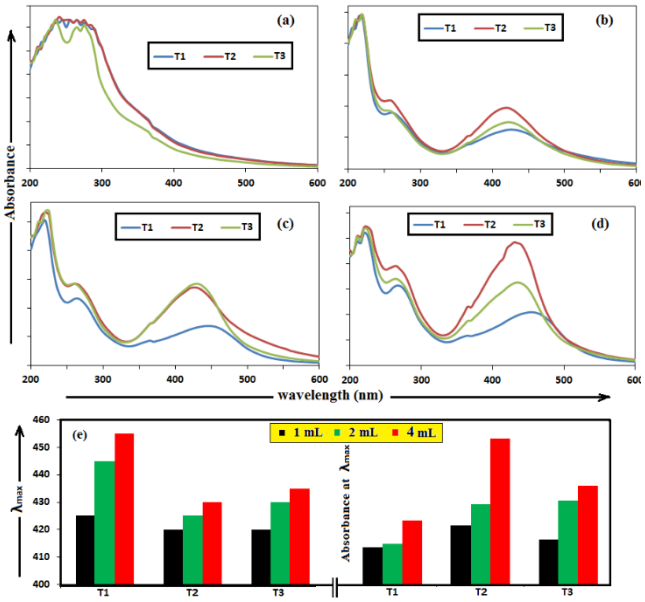

Figure 9: UV-Visible absorbance spectra of Tea Extract (TE) and AgNPs: (a) Tea Extract (b) AgNPs synthesized using 1 mL TE (c) AgNPs synthesized using 2 mL TE (d) AgNPs synthesized using 4 mL TE (e) SPR peak maxima (λ_{max}) and absorbance at λ_{max} of AgNPs synthesized using 1 mL, 2 mL, and 4 mL TE (T1, T2, and T3)

The absorbance spectra of TE and AgNPs produced using varied amounts of TE (1, 2, and 4 mL) are shown

in Figure 9. It is evident from the figure that the $\lambda_{max}$and absorbance of the AgNPs increases with the increasing TE volume, and also the surface resonance maxima (λ_{max}) of T2A4 is the lowest, while of T1A4 is the highest among all the AgNPs synthesized using 4 mL TE. This valuable information strengthens our claim that with increasing TE volume, the phytochemical concentration increases, resulting in large sized AgNPs, as well as that T2 contains the lowest, while T1 contains the highest phytochemical concentration. Because a single Gaussian peak function could not properly fit all of the spectra, the absorbance spectra for AgNPssynthesised at varied TE volumes were deconvoluted (Figure 10) and the band parameters of the deconvolution are reported in Table 2.

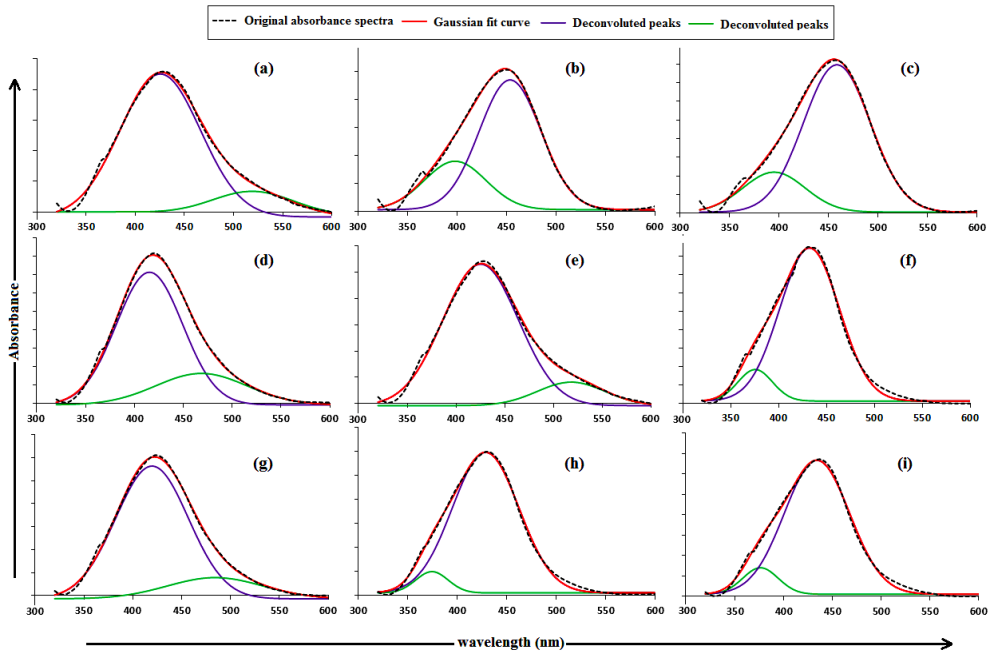

Figure 10: Deconvolution of UV-Visible absorbance spectra of AgNPs dispersion synthesized using TE, obtained by UV-Vis spectroscopy. The absorbance spectra of colloidal dispersion, deconvoluted mathematically (Gaussian curve fit) to two bands: (a)–(c) AgNPs synthesized using 1 mL, 2 mL, and 3 mL of T1 (d)–(f) AgNPs synthesized using 1 mL, 2 mL, and 3 mL T2 (g)–(i) AgNPs synthesized using 1 mL, 2 mL, and 3 mL of T3

Here again, it is marked that when the AgNPs (T1A1, T2A1, T3A1) are synthesized using 1 mL TE (T1, T2, T3), no deconvoluted peak is observed at λ_{max}< 400 nm (absorption region for phytochemicals), whereas when the AgNPs (T1A2 and T3A2) are synthesized using 2 mL TE (T1 and T3), deconvoluted peak appears at λ_{max}< 400 nm but not in case of T2A2, which is synthesized using 2 mL T2. Moreover, for the AgNPs: (T1A4, T2A4 and T3A4), the ratio of area under curve observed for the silver nanoparticles to that of the adsorbed phytochemicals over the AgNPs was found to be 4:9:6. These valuable spectroscopic inputs illustrates that the amount of phytochemicals adsorbed over the AgNPs surface is much higher when they are synthesized from TE prepared from tea leaves collected from mountainous and flat land region.This confirms that the phytochemical content of tea leaves from mountainous and flat land regions is significantly

higher than that of tea leaves from hilly areas. Because of the differing cultivation altitudes, the phytochemical content of green tea leaves differed significantly. The highest phytochemical content were found in leaves from the mountainous region (Taplejung, 3643 m above sea level), whereas the lowest phytochemical content were observed in leaves from the hilly region (Ilam, 1828 m above sea level). These rich differences may be due to the changing altitudes where they are grown because altitude reflects several ecological elements such as: temperature, solar radiation, and humidity. The statistical significance demonstrated through this study demonstrated that tea extract samples from the hilly region (T2) is verified to be the most desired region for synthesizing AgNPs because of the lowest phytochemical concentration. The rich variation in the phytochemical concentration may lead to significant differences in effectiveness of AgNPs formation, stability, and application. Multiple studies have confirmed that tea leaves flavonoids concentration is proportional to the cultivation altitude.[4] Because the altitude of Taplejung (3643 m) is higher than that of other cultivation altitudes, the total flavonoids content is likewise higher. Flavonoids absorb UV radiation due to their ortho-dihydroxylated structure. As a result of the strong UV radiation, there is a considerable positive connection between total flavonoids concentration and altitude among tea species dispersed at higher altitudes. Furthermore, UV-absorbing flavonoids and phenolic acids have been a vital feature of such plants' vegetative organs to protect them from harmful UV radiation.[19] Earlier reports have validated that plants growing at a low temperature region have an increased production of phytochemicals, majorly phenolics, for self-defense.[20] Because the mean temperature drops by 0.55°C with every 100 meters gained in altitude, the content and concentration of phenolic compounds must have increased due to the lower temperature at higher elevations.[21] Second, the synthesis and accumulation of phytochemicals in plants is influenced by sunlight irradiation. According to Zheng and group,[22] exposure to UV-B radiation stimulates the formation of tea catechins however, excessive UV-B irradiation suppresses their formation. The concentration of phytochemicals grows as illumination time increases [4] and as sunlight illumination time is the highest in the mountainous region and the lowest in the hilly region, the lowest phytochemical concentration must be noticed in tea leaves grown in the hilly region (Ilam, 1828 m). In addition to variations in phytochemical concentration due to altitude, the temperature at which the phytochemicals are extracted has a significant impact on the efficiency and stability of catechin extraction. The EGCG content of catechins extracted at temperatures above 80°C is significantly higher, accounting for up to 43% of total catechins.[23] Therefore, it may be concluded that the EGCG content was the highest in T1, while the least in T2, which could have resulted in large sized T1A4, while the smallest sized T2A4 with the least aggregation. Based on all these findings, T2A4 must be selected as the AgNPs of interest for the colorimetric sensing.

Table 2: Band parameters of deconvolution of UV-Visible absorbance spectra of AgNPs colloidal

dispersion synthesized using TE

Peak	FWHM	Area
	T1A1	
425 nm	82 nm	48
520 nm	83 nm	8
	T1A2	
398 nm	62 nm	14
454 nm	62 nm	36
	T1A4	
395 nm	63 nm	17
459 nm	68 nm	67
	T2A1	
415 nm	68 nm	62
469 nm	94 nm	20
	T2A2	
425 nm	78 nm	105
518 nm	70 nm	17
	T2A4	
376 nm	36 nm	19
432 nm	62 nm	162
	T3A1	
420 nm	75 nm	54
484 nm	98 nm	11
	T3A2	
375 nm	33 nm	7
429 nm	67 nm	99
	T3A4	
376 nm	36 nm	17
434 nm	67 nm	110

3.4 Stability of AgNPsin Aqueous Surfactants

The fundamental features of TE mediated silver nanoparticles (AgNPs) were aimed to be used for their possible application as colorimetric sensors. Thus, it is of utmost importance to study the stability of these AgNPs in different surfactants to determine the most suitable stabilizing agent. Thus as stabilizing agent; cationic, anionic, nonionic surfactants were used in the present study for improving colorimetric sensing ability of these AgNPs by creating an appropriate electrostatic atmosphereto improve the colorimetric sensing ability of the AgNPs by creating an appropriate electrostatic atmosphere.[24,25] Figure 11 shows absorbance spectra of the AgNPs (T1A4, T2A4, T3A4) dispersed in aqueous and 0.2 mM aqueous surfactants. For clarity, abbreviation have been used for the surfactants used in this study which are: (0)C8 for n-octyl-β-D-glucoside, C8 for Trimethyloctylammonium bromide, C10 for Decyltrimethylammonium bromide, C12 for Dodecyltrimethylammonium bromide, C14 for Tetradecyltrimethylammonium bromide, C16 for Hexadecyltrimethylammonium bromide, and (-)C8 for Sodium octyl sulphate.

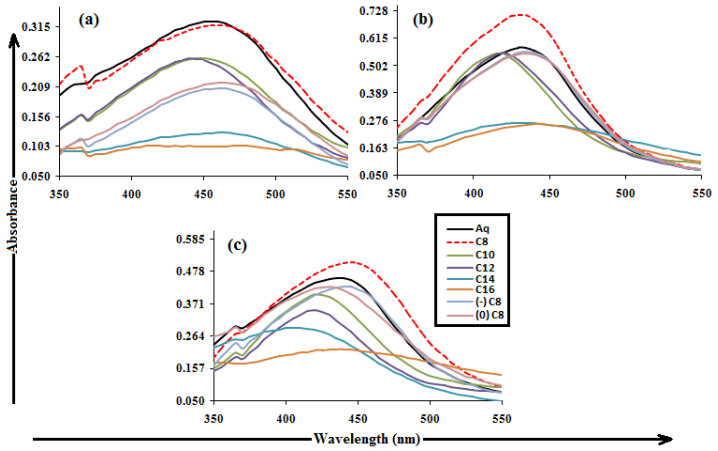

Figure 11: UV–Visible absorption spectra of aqueous and aqueous surfactant dispersed (a) T1A4 (b) T2A4 (c) T3A4

In all the aqueous surfactants, the SPR peak intensity of T1A4 was lower than in water, wherein the least decrease (by 2.5%) was observed with C8 (Figure 11). In all the aqueous surfactants, the SPR peak intensity of T2A4 and T3A4 was lower than in water except with C8, wherein the SPR peak intensity increased by 23.5 and 10.1%, respectively, and T2A4 having the highest absorbance. If we compare the structures of the cationic surfactant: C8, anionic surfactant: (-) C8 and nonionic surfactant: (0)C8; we will observe that all of them have different head groups but have one thing in common, the octyl chain. The C8, (-)C8 and (0)C8 are having trimethylammonium, sulfate and β-D-glucoside head groups, respectively. Accounting to the presence of four hydroxyl groups in the β-D-glucoside head of (0)C8 and four oxygen atoms (−2 oxidation state) in the sulfate head of (-)C8, there is a development of strong solute-solvent interaction in aqueous environment due to the formation of multiple hydrogen bonding between these head groups and water. With C8, in comparison to (-)C8 and (0)C8, the strength of the solute-solvent interaction is much weaker due to formation of lesser number of hydrogen bonding between the trimethylammonium head group and water. Thus, in the aqueous surfactant – AgNPs systems, due to the prevalence of stronger solute-solvent interaction between surfactants [anionic: (-)C8 or nonionic:(0)C8] and water which is greater than those existing between the AgNPs and water, the surfactant molecules traps water molecules in its hydration sphere thereby causing the AgNPs to form aggregates with decreased absorbance (Figure 11). Moreover, the impact of decrease in absorbance due to aggregation should be the highest for T1A4, while the least for T2A4 due to their respective zeta potential values (Figure 2). This aggregation mechanism is supported by Figure 12 wherein, for the AgNPs, the highest decrease in the SPR peak absorbance in presence of (-)C8 and (0)C8 is observed for T1A4 (37% and 36.4%), while the least decrease is observed for T2A4 (3.5% and 4.7%). The extent of decreased absorbance of the AgNPs with (-)C8 and (0)C8 are of similar magnitude: T1A4 (37% and 36.4%), T2A4 (3.5% and 4.7%), T3A4 (6.8% and 7%); demonstrating that the degree of hydrogen bonding between the (-)C8 and (0)C8 head groups and water is the same. Among the aqueous

surfactant – AgNPs systems, (a) prevalence of weakest interaction among cationic surfactant (C8) and water, (b) order of aqueous AgNPs dispersion zeta potential as T2A4>>T3A4>>T1A4, and (c) the favorable interaction of the trimethylammonium head of C8 over the AgNPs surfaces, may explain the highest increase (23.5%) in the SPR peak intensity for T2A4 in presence of C8.[24,25]In case of T2A4 and T3A4, it seems that the increase in their SPR peak intensity by 23.5 and 10.1%, respectively, in presence of 0.2 mM aqueous C8 is associated to their very high zeta potential values in water (-128.64 and -63.85 mV, respectively). Among the aqueous AgNPs dispersions, T2A4 with the highest zeta potential value may have adsorbed more C8 molecules, whereas T1A4, adsorbing less. Thus, in comparison to T2A4 dispersed in water and in aqueous cationic surfactant (C8), the number density of T2A4 will be lower in aqueous C8. Self-assembled single layers of C8 are formed over the T2A4 surface due to the phenomenon of physisorption. Thus, T2A4 surface will be passivated and its surface energy will be reduced, thereby reducing the particle-particle interactions. This will add dispersion stability to the T2A4 in aqueous cationic surfactant (C8) with the highest increase (23.5%) in its SPR peak absorbance. Since in case of nanoparticles, their increased SPR peak intensity on addition of surfactant infers decreased coalescence, thus in the present study, stability (in terms of decreased coalescence) of the AgNPs was found to be the highest for T2A4, followed by T3A4, dispersed in aqueous C8.

Based on the good stability of T2A4 in aqueous C8 in terms of least coalescence, it became necessary to study the effect of C8 concentration on T2A4 dispersion stability and consequently, the concentration of C8 was increased from 0.1 mM to 0.4 mM. The absorbance vs wavelength plot (Figure 12) shows that when the C8 concentration increases from 0.1 mM to 0.2 mM, the SPR peak absorbance at 430 nm for T2A4 increases; however, as the ionic strength increases, the absorbance reduces, leading to aggregation. As the ionic strength of C8 increases, there is an increase in the solute-solvent interaction between the trimethylammonium head of C8 and water and as a result, the C8 molecules traps water molecules in its hydration sphere thereby causing the T2A4 to form aggregates with decreased absorbance.[26]Thus, [C8] = 0.2 mM was considered the most effective concentration with T2A4 being the most stable AgNPs without aggregation.

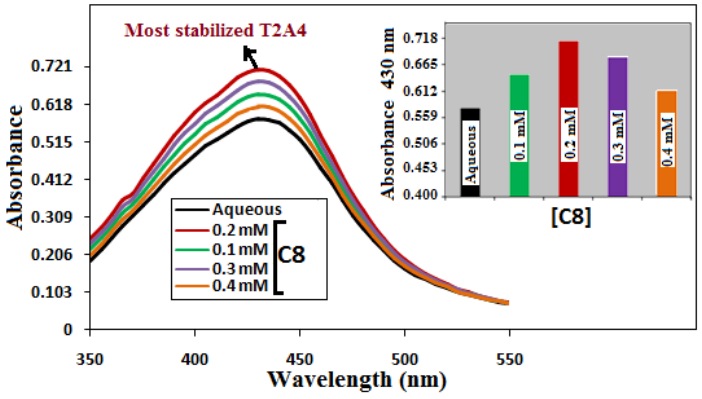

Figure 12: Absorbance versus wavelength scan of T2A4 dispersed in water and aqueous C8. Inset: Absorbance of T2A4 at 430 nm dispersed in 0-0.4 mM aqueous C8

Since zeta potential is the reflection of AgNPs dispersion stability wherein, a higher zeta potential (> ±30 mV) infers excellent dispersion stability. The zeta potentials shown in Figure 2illustrates T2A4 having the highest, while T1A4 having the least aqueous stability. Interestingly, in presence of 0.2 mM aqueous C8, the zeta potential of T1A4, T2A4 and T3A4 decreases by 56.8, 37.2, and 54.4 %, respectively. Moreover, in presence of 0.2 mM aqueous C8, the width of T1A4 particle size distribution (Figure 2) increases drastically by 121.60 nm, while the least increase by 2.14 nm is observed in case of T2A4. These numbers signify that 0.2 mM aqueous C8 containing T1A4 is a highly heterogeneous dispersion, while containing T2A4 is highly homogeneous. Through DFT study, Moudgil *et al* have earlier shown that the head end of the cationic surfactant interacts positively with the Ag clusters.[27] Thus, in the present scenario, there is a high probability that when the AgNPs are dispersed in aqueous C8, the positively charged trimethyloctylammonium head group of C8 molecules comes in the vicinity of the AgNPs, and decreasing their overall negative charge density due to inductive effect (+I) of the trimethyloctyl chain of C8. For T1A4 having the lowest zeta potential among the aqueous dispersed AgNPs, the highest decrease in the zeta potential with an increased particle size distribution in presence of C8 is may be due to the increased neutralization of the charge of the negatively charged double electrostatic layer surrounding the T1A4 by the +I effect of the C8 trimethyloctyl chain. This also provides the reason behind the 2.5% decrease in the absorbance of T1A4 in presence of C8, wherein the greater extent of aggregation resulting from decreased repulsion between the T1A4 resulted in the decreased absorbance. The SPR peak intensity of AgNPs tends to decrease as the alkyl chain length of the cationic surfactants increases as seen in Figure 12 and follows the order C8>C10>C12>C14>C16. This could be attributed to the growing +I effect owing to the increasing length of the cationic surfactant alkyl tail neutralizing the negatively charged double electrostatic layer around the AgNPs. Thus, the AgNPs-cationic surfactant-water system's dispersion stability is destabilized as the alkyl chain length of the cationic surfactant increases.

4. Conclusions

A detailed study on the effect of altitude variation in tea leaves over the physicochemical properties of tea leaf extract mediated AgNPs has been carried out. It was discovered that AgNPs synthesized using tea leaves grown in mountainous areas will be of large size, have a wider particle size distribution, and poor dispersion stability. Tea leaves grown in mountainous areas on the other hand, will be of small size, have a narrow particle size distribution, and excellent dispersion stability. Reaction of the AgNPs with ammonia solution along with their XRD, FTIR, and UV-Visible absorption spectrophotometric characterization revealed them

of being made of Ag/Ag_2O nanocrystallites. When compared to other surfactants, the AgNPs synthesized using hilly region tea leaves have the maximum dispersion stability when dispersed in 0.2 mM aqueous Trimethyloctylammonium bromide. This research also demonstrates a strong link between the altitude at which tea leaves are grown and the surface adsorption of surfactants by AgNPs, indicating a combination that will be the most successful for AgNPs in colorimetric sensing applications.

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Review Article

Use of Sand as Alternative to Low-Cost Thermal Storage Material: A Short Review

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Abstract

With rising energy demand, one of the most promising technologies for storing energy from renewable sources is thermal energy storage. However, there are limitations in the availability and efficiency of energy storage materials. Sand is one of the low cost energy storage materials used in various studies to enhance the performance and energy storage capacity of various storage devices. This study reviews the use of sand and its composites in various forms to increase the performance and efficiency of energy storage devices. It was found that sand with various compositions around the globe is proven to be an alternate to the conventional energy storage materials in terms of energy efficiency.

Keywords: Sand, thermal energy, energy from renewable sources, alternate to the conventional energy, energy efficiency

1. Introduction

Rise in the human population across the world puts a huge demand on energy and resources which is followed by rapid urbanization and industrialization. To accomplish the same, our conventional sources of energy are depleting very fast and the focus of the world's scientists is now to harness and store the energy from renewable sources of energy such as solar energy, wind energy, hydro energy, etc. [1–3]. However, due to intermittent supply of energy, it is very important to develop efficient energy storage systems. Some of them are specifically needed to store the thermal energy which is available from sun, exhaust gases, wastewater from industry, and geothermal energy [4–6]. Thermal storage systems are categorized into 1) Sensible heat storage systems in which the thermal energy is stored in the form of sensible heat. Some of the sensible heat storage systems are solar air heaters, solar water heaters, solar lake etc.[7–9] 2) A latent heat storage system in which heat is stored by the materials in the form of latent heat of phase change. Examples of latent heat storage systems are concentrated solar plants [10–12].There are various researches going around the world to improve the efficiency and charging-discharging time of such systems. The same could be achieved by improving the geometrical design, developing new thermal storage material, and improving the property of existing thermal storage materials. However, scientists are also focusing on

various naturally available materials which are cheap, abundant, and can be used as an efficient thermal storage material such as beeswax, carnauba wax, natural oils, and sand.

Sand has been used for both latent heat storage and sensible heat storage systems from a few decades due to its low cost and abundance. However, its application into thermal storage system varies with the type and composition of the sand.

2. Sand and its Composite as Heat Storage Material

Sand is ~~a~~perfect to store sensible heat storage material and can be used as support material to enhance the thermal storage performance of various solar based energy storage systems. Diago et al., 2018[13] conducted the investigation on desert sand dunes as potential thermal storage material and its characterization. The study revealed that desert sand contains a variable mass of calcium carbonate and calcium hydroxide which decomposed at average temperature of 408°C and 635°C, respectively. However, the reaction can be reversed during the discharging process. Therefore, this scenario should be discussed during the designing and development of sand based thermal energy storage system. The study also revealed that sand can be used as sensible storage material as it remains stable up to the temperature of 1000°C and could be used as an alternative to salt in concentrated solar plants. Lv et el., [14] studied the yellow sand in south east of Tenggri desert's thermal storage medium. They found that thermal conductivity, density, and heat storage capacity of unscreened sand is more than the screened sand which separates on the mesh size of 40-60 mesh, 60-80 mesh, and 80-100 mesh. It was also found that on giving thermal shock and grinding screened sand, it was more resistant to breakage into small particle size and also offers better heat storage performance. Deshmukh & Thombre, 2017 [15] conducted study on a solar still using sand as sensible heat storage medium. They found that in comparison to conventional solar stills, the solar stills with sand have higher thermal storage performance. Quninones et al., 2021 conducted experiments with two sand samples i.e. lime stone and beach sand originated from Mexico. The test was carried out using a solar dryer, and it was found that Schlipf et al 2015[16] investigated the effect of grain size on the charging and discharging behaviour of the packed bed latent heat thermal storage system, and it was discovered that small grain sized materials are best suited for thermal energy storage. Quinones et al. 2021[17] conducted a study with two types of sensible heat storage materials i.e., beach sand and limestone originated from Mexico using indirect type solar dryer with thermal storage system and concluded that absorber plate reached 79.3°C for beach sand and 86.8 °C with lime stone. It was also found that the drying efficiency for limestone is 1.55% higher than that of beach sand which may be due to small particle size of limestone as compared to the beach sand.

Table 1 details the review of sand and its composite with various other materials as sensible heat storage materials. It can be seen from the table that sand has been used as thermal storage material in both pure form as well as composite form in different applications. The efficiency of thermal solar stills was

shown to rise as the thermal energy storage material sand was used. The reason for this was the long-term availability of thermal energy. In some of the applications, it was found that sand when mixed with phase change materials provides better thermal efficiency in terms of storage as well.

Sr. No.	References	Sensible Storage Material	Application of Material	Conclusion
1.	[18]	Sand and mixture of sand with basalt	Moving bed heat exchanger for CSP	It was found that efficiency of moving bed heat exchanger was improved by 30% with mixture of sand and basalt (50:50), as compared to sand only
2.	[19]	Black sand and yellow sand	Single basin solar still	It was found that by using black sand bed and yellow sand bed, the thermal efficiency of solar still increases by 42% and 17% respectively.
3	[20]	Sand	Solar air collector	Solar air collector with sand as thermal storage material provided 39% and 42% more thermal efficiency than black paint coated aluminium absorber.
4.	[21]	Sand with iron grits, brick powder, and charcoal powder	Solar cooker	It was found that thermal storage efficiency of solar cooker was raised by 16.1%

5.	[22]	Pure sand	Single basin solar still	It was found that the daily productivity and efficiency of solar still is 31.44% and 23.12% more with sand than without sand.
6.	[23]	Sand and paraffin mixture	Flat panel ground heat exchanger	It was concluded that PCM mixed with sand has enhanced thermal storage efficiency.
7.	[24]	Sand with acetamide	Scheffler reflector solar cooking system	Composite with Sand-acetamide composite was found to be having 3-3.5 more heat as compared to iron balls-acetamide composite.
8.	[25]	Sand	Solar greenhouse drier	Study concluded that drying with sand saves 62% of drying time as compared to drying in open sun.
9.	[26]	Sand	Solar still	Results showed that exergy efficiency of the system with heat storage improves by 30% as compared to the conventional solar still.
10.	[27]	Jute cloth knitted with sand	Solar still	The yield of fresh water under the least water mass of 20 kg with sensible heat storage material was found to be 5.9 kg/m^2 as compared to

				the 5.0 Kg/m^2 without sensible heat storage material
11.	[28]	Portland cement and alluvial sand	Solar distiller	Solar distillers with layers of alluvial sand and Portland cement produces distilled water of very high quality and low cost as compared to other methods.
12.	[29]	Crushed gravel sand	Biomass evaporator assisted solar still	Use of crushed gravel sand as thermal storage material improved the efficiency of biomass assisted solar still by 34.4% as compared to the conventional biomass assisted solar still.

3. Conclusion

As the cost of efficient heat storage materials are high due to the cost involved in their production, there is a need to identify the materials which are cheap and low cost. Sand and its composites are one of the best options available which are natural in sources and have very low cost. Desert, the vast source of sand, can provide unlimited supply for the development of various solar based applications such as solar still, solar dryer and solar cooker. It was evident from various studies that the use of sand and its composites with various other materials increases the thermal storage efficiency of the material. Due to its sensible heat storage capacity, sand could also be use as heat transfer fluid in various applications and therefore, further studies need to be carried out in this direction.

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Research Article

Changing Relation between Life Expectancy and Per Capita Income - India

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Highlights

- The relation between life expectancy and per head income has changed over time from 1962-2019 in India.
- Low level income has strongly influenced life expectancy and assisted the growth of life expectancy.
- Surpassing low level income, individual weekly income has influenced life expectancy. The growth of life expectancy is slow and minimal in India.

Abstract

The thought that average income may be an influential parameter driving the economic development process is intriguing. Economic development is a multifaceted process driven by a multitude of factors. One important parameter to judge economic development is life expectancy. This paper explores the relationship between life expectancy and per capita income for India where it finds that relationship exhibits the Preston curve.

Keywords: Preston curve, development economics, income, health

1. Introduction

For the 21st century, economic development is the primary objective for the majority of the nations. This truth can be accepted without any controversy. To increase standard of living (per capita income), well-being (life expectancy) and economic capabilities (education) of people is the most crucial social task that we are facing today. Economic development is a multifaceted process driven by multitude of factors. The thought that average income may be an important influential parameter driving the economic development process is intriguing. As quoted by R.E. Lucas in his famous paper - On the mechanics of economic development,

"By the problem of economic development I mean simply the problem of accounting for the observed pattern, across countries and across time, in levels and rates of growth per capita income. This may seem

too narrow a definition, and perhaps it is, but thinking about income patterns will necessarily involve us in thinking about many other aspects of societies too, so I would suggest that we withhold judgment on the scope of this definition until we have a clearer idea of where it leads us." [1]

The quote by Lucas proposes a simplified approach for economic development with the belief that the universal features of economic development (health, life expectancy, literacy, and so on) follow in some natural way from the growth of average income (per capita income), perhaps with the passage of time [2]. This approach strives towards the world view about the possibility of finding a smaller set of variables that correlates well with the multifaceted process of economic development.

Life expectancy at birth is one of the crucial parameters for development which shows the expected years an individual may expect to live when subjected to age-specific mortality rates prevailing during the given time period. Life expectancy is a complex statistical measure, calculated by developing life tables from mortality rates.

There are strong reasons to focus on income rather than other socio-economic variables. Firstly, the income per capita is probably the best single indicator of living standards in a country, since it comprises the value of all final products (goods and services) produced in a certain period. From this, a wide range of products can be expected to influence mortality, and expenditures in all of them are represented by varying weights in income per capita. Secondly, as the leading index of the level of economic development, income per capita is the focus of growth models from which policy measures are derived. For example, Leibenstien and Nelson argued that a small gain in income per head in low-income countries will tend to produce a decline in mortality, causing a more rapid rate of population growth that will bring the population back to its initial level of income [3].

In his classic paper titled "The changing relation between Mortality and Level of Economic Development" published in 1975 [4], Samuel H Preston boldly researched the association between average income and life expectancy. Preston found that life expectancy was highly sensitive to income variation for low-income countries. However, the correlation became weak as one move from low income to high income. The paper was the cornerstone for both global public health policy and academic discussion of public health, strongly showing the influential role income had on improving life expectancy. The paper suggested studies at the individual country level. However, there are a few studies conducted for India.This study strives to explore the relationship between life expectancy and income per head for India.

2. Methodology

To explore the relation between life expectancy and gross national income per capita, stastical tool Pearson correlation is utilized. Further, the relationship is visualized using scattered diagram and logistic curve.

3. Result and Discussion

The secondary data on life expectancy at birth (average, male and female combined) and gross national income per capita (GNI per capita) are sourced from the World Bank. The criterion for starting time series analysis from the year 1962 was simply the availability of reliable data. Hence, the time interval spans from 1962 to 2019 (58 years).

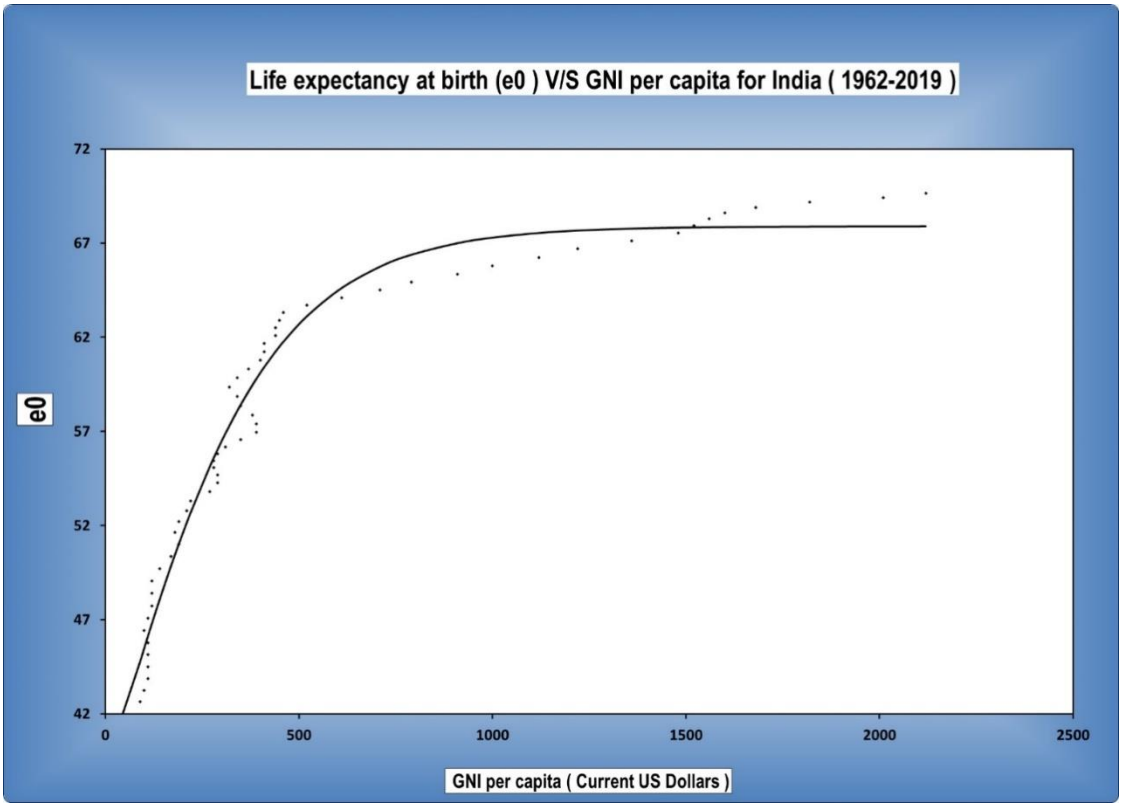

Figure 1: Scatter-diagram of relation between life expectancy at birth (e0) and GNI per capita for India for time period (1962-2019)

As seen from the scatter diagram, the relationship closely resembles the logistical growth rate. Therefore, the logistic curve has been fitted having equation as below.

$$e0 = \frac{70}{1 + EXP^{0.003(42.637 - x)}}\ ,\quad where\ e0 = Life\ expectancy\ at\ birth$$

$$x = GNI\ per\ capita$$

The relationship has a strong Pearson correlation coefficient of 0.827 (computed using SPSS). However, the correlation is weak as income increases as reflected by the flat curve. At income below 910$,

life expectancy is associated strongly with variation in income. A given increment in income tends to be associated with significant gains in life expectancy whereas, after 910 $ income level, the gain in life expectancy was small with increments in income. This phenomenon is known as the Preston curve named after Samuel.H.Preston who published it in 1975. There are several assertions for the phenomenon by using factors endogenous and exogenous to a country's level of income.

Firstly, at low income, the mortality resulting from diseases is closely associated with standard of living like diarrhoeal disease resulting from nutritional adequacy and personal sanitation level, tuberculosis resulting from living space conditions, and so on decreases as endogenous factors such as personal care spending, hygiene, living space are improved with increment in income. It generally reduces mortality in children, adolescents, and adults.

Secondly, as the income increases over time, the role of endogenous factors ceases. The reason is that the population has surpassed the low-income level, while mortality sensitivity to endogenous factors has by far reduced. However, the mortality arising from diseases like cardiovascular disease, cancer, diabetes, chronic respiratory, prevails. Mortality from this disease is more sensitive to factors exogenous to income like technological advancement, importation of health care technology and personnel, and government health care expenditure. The flat curve reflects the weakness of these exogenous factors to improve life expectancy and why life expectancy has hit slow in increments over the period 2000-19.

Lastly, the non-linearity of the mortality-income relationship reflects diminishing returns to an increase in income. At the macro level, it shows that a wide variety of dose-response relationships at the individual level exhibits diminishing returns to income. If the relationship was linear where individuals have the same income, there would be no flat curve. Ergo, income inequality must have an influence on life expectancy.

In India, the Preston curve shows that the link between per capita income and life expectancy has diminished over time. It is mainly due to the weakness of factors exogenous to income to stimulate life expectancy.

4. Conclusion

The relationship between income and life expectancy was highly correlated at low level income in India. The relationship weakened as the level of income incresead, a general characteristic of the Preston curve. As the level of income increased, mortality arising from diseases closely related with living standards decreased as people's standard of living improved. As the population suprasses low level income, the income is not able to stimulate life expectancy with mortality arising from diseases whose treatment depends on factors exogenous to income in general. The relation between income and life expectancy is disassociating in India and the growth of life expectancy is minimal.

5. Limitations

The study is limited to specific variables, mortality and income. Health in itself is a broad topic where mortality is influenced by a multitude of variables. Ceteris paribus, the study here, considers only the effect of income on mortality as per the objective.

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